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Normal p53 Function in Primary Cells Deficient for *Siah* Genes

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Overexpression studies have suggested that Siah1 proteins may act as effectors of p53-mediated cellular responses and as regulators of mitotic progression. We have tested these hypotheses using *Siah* gene knockout mice. *Siah1a* and *Siah1b* were not induced by activation of endogenous p53 in tissues, primary murine embryonic fibroblasts (MEFs) or thymocytes. Furthermore, primary MEFs lacking *Siah1a*, *Siah1b*, *Siah2*, or both *Siah2* and *Siah1a* displayed normal cell cycle progression, proliferation, p53-mediated senescence, and G_1 phase cell cycle arrest. Primary thymocytes deficient for *Siah1a*, *Siah2*, or both *Siah2* and *Siah1a*, E1A-transformed MEFs lacking *Siah1a*, *Siah1b*, or *Siah2*, and *Siah1b*-null ES cells all underwent normal p53-mediated apoptosis. Finally, inhibition of *Siah1b* expression in *Siah2 Siah1a* double-mutant cells failed to inhibit cell division, p53-mediated induction of p21 expression, or cell cycle arrest. Our loss-of-function experiments do not support a general role for *Siah* genes in p53-mediated responses or mitosis.

p53 acts as a tumor suppressor by inducing cell cycle arrest, apoptosis, and senescence in response to cellular stresses such as DNA damage or oncogene activation. These effects are mediated largely through the function of p53 as a transcriptional activator or repressor (5, 25). A diverse range of p53 target genes have been identified, for example, *p21*, an important mediator of G_1 phase cell cycle arrest and cellular senescence (10), and several proapoptotic genes including *Noxa* and *Bax* (53). However, understanding of the full range of p53 effectors is incomplete and it is likely that multiple signaling pathways are involved in determining cellular responses to p53 activation.

Recent studies have proposed that Siah1, a member of the Siah family of E3 ubiquitin ligases, may act as a downstream effector of p53. Overexpression of p53 induces the transcriptional activation of *Siah1* family genes in a variety of mammalian cell lines (1, 20, 32, 35, 43, 44, 47), and overexpression of *Siah1* can mimic the effects of p53 activation and induce cell cycle arrest or apoptosis (37, 43, 44).

A molecular mechanism by which Siah1 proteins may mediate p53 function has been proposed. Human SIAH1 functions as a component of an E3 ubiquitin ligase complex including Siah-interacting protein, Skp1, and the F-box protein Ebi, which is proposed to target β-catenin for ubiquitin-mediated degradation in response to activation of p53 (32, 36, 42). Importantly, overexpression of either *p53* or *SIAH1* can induce β-catenin degradation, and this is blocked by coexpression of an N-terminally truncated SIAH1 protein lacking the RING domain. By this model, p53-mediated induction of SIAH1 following DNA damage induces degradation of β-catenin independently of the GSK3β-mediated degradation pathway. Since overexpression of β-catenin promotes cell cycle progression and inhibits cell cycle arrest induced by gamma irradiation (41), SIAH1-mediated β-catenin degradation may contribute to p53-dependent cell cycle arrest. Interestingly, accumulation of a β-catenin mutant protein that is resistant to degradation by the GSK3β pathway induces activation of p53 (7). Since overexpression of *SIAH1* can induce degradation of wild-type or mutant forms of β-catenin, SIAH1 may function downstream of p53 in a pathway that senses and degrades oncogenic β-catenin and thereby contributes to tumor suppression.

Mice have three unlinked *Siah* genes: *Siah1a*, *Siah1b* (collectively *Siah1*), and *Siah2*, while humans have single *SIAH1* and *SIAH2* genes (9, 20). The mammalian Siah proteins are highly homologous to one another. Siah1a and Siah1b proteins are 98% identical, while Siah1 and Siah2 proteins diverge significantly only at their N termini. Siah proteins interact via their RING domains with the E2 ubiquitin conjugating enzymes UbcH5 (36) and UbcH8 (54) and with UbcH9, which catalyzes conjugation of the ubiquitin-like protein SUMO-1 (22). Overexpression of Siah proteins induces the ubiquitination and proteasome-dependent destabilization of diverse substrate proteins. These include DCC, Kid, β-catenin, c-myb, Obf-1, Numb, and TIEG-1 for Siah1 (3, 15, 21, 22, 26, 32, 36, 47, 49, 51), and DCC, Bag-1, N-CoR and synaptophysin for Siah2 (22, 46, 54, 55). Siah1 and Siah2 proteins function equivalently to induce the degradation of some substrates (e.g., DCC), while other substrates (e.g., Bag-1) appear to be specific targets of either Siah1 or Siah2.

In addition to their suggested role as mediators of p53 function, Siah1 proteins have also been implicated in mitosis and meiosis. *Siah1a* is required for progression past metaphase during meiosis I of spermatogenesis (11), and overexpression of *SIAH1* in MCF7 cells induces multinucleation and mitotic abnormalities (4, 15). These findings are consistent with studies showing that overexpression of *SIAH1* induces the degradation of the kinesin Kid (15), a DNA-binding microtubule

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motor protein that forces chromosome arms away from mitotic spindle poles to facilitate chromosome alignment at the metaphase plate (30). Proteasome-mediated degradation of X-Kid is required for chromosome segregation in *Xenopus* oocytes (2, 13). It has therefore been hypothesized that degradation of Kid by Siah proteins may be necessary to allow chromosome segregation during mitosis and/or meiosis.

To date, the functions of the *Siah* gene family have been investigated largely through overexpression studies, often in transformed or immortalized cell lines. To begin to characterize the physiological functions of the *Siah* genes, we have generated and analyzed mice deficient for *Siah1a* (11), *Siah2*, or both *Siah2* and *Siah1a* (R. Dickins, I. Frew, V. Hammond, and D. Bowtell, unpublished results). Whereas *Siah1a* knockout mice are subviable and growth retarded and *Siah2* knockout mice are completely viable, *Siah2 Siah1a* double-mutant mice die at birth. This result supports previous biochemical findings that Siah1 and Siah2 proteins have both distinct and overlapping functions. In this report we describe the generation of embryonic stem (ES) cells and MEFs lacking *Siah1b* and have used these, and primary cells derived from other *Siah* knockout mice, to comprehensively investigate whether *Siah* genes are necessary for p53-mediated responses or for mitosis. We find that *Siah* genes are not induced by activation of endogenous p53 in mouse tissues or in primary MEFs or thymocytes and are not necessary for p53-mediated senescence, cell cycle arrest, or apoptosis. While *Siah1a* is clearly required for meiotic progression (11), cellular proliferation and cell cycle progression of MEFs are unaffected by the loss of *Siah* genes, suggesting that Siah proteins are not necessary for chromosome segregation in somatic cells.

MATERIALS AND METHODS

Targeted mutation of *Siah1b*. The *Siah1b* targeting construct was generated by using the vector pPNTloxP (gift of Paul Orban). DNA for the targeting arms was derived from a 129Sv genomic library λ phage clone encompassing the *Siah1b* locus (9). PCR was used to generate the 2.6-kb 5′ and 7.6-kb 3′ targeting arms (see Fig. 2A). Transfection of ES cells and selection of drug-resistant colonies were as previously described (11). Genomic DNA from resistant clones was digested with *Bam*HI and Southern blotted. Blots were probed with a 400-bp *Kpn*I-*Hin*dIII fragment (probe 4) located 5′ of the region of DNA used as the 5′ targeting arm. Homologous recombination introduces a new *Bam*HI site, leading to a size shift of the hybridizing band from 3.7 to 3.3 kb. A single targeted clone was isolated from 452 clones screened. Probing with a 560-bp *Ssp*I fragment (probe 8) of the *Siah1b* gene confirmed specific deletion of the *Siah1b* coding region. Chimeras were derived by injection of targeted ES cells into C57Bl/6J blastocysts. Live chimeras were born at low frequency (four were obtained from 320 blastocyst injections, compared with approximately 50% frequency for other ES cell clones injected in parallel experiments) and failed to transmit the agouti coat color to progeny when mated to C57Bl/6J mice.

Generation of MEFs lacking *Siah* genes. MEFs were derived from embryonic day 13.5 (E13.5) embryos according to standard protocols. *Siah1a*$^{-/-}$, *Siah2*$^{-/-}$, or *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ MEFs were derived from intercrosses of 129Sv.C3-$+^{c}+^{p}$ background *Siah1a*$^{+/-}$, *Siah2*$^{+/-}$, or *Siah2*$^{-/-}$ *Siah1a*$^{+/-}$ mice, respectively. Appropriate control cultures were derived from littermate embryos. *Siah1b*$^{-/Y}$ MEFs were obtained from chimeric embryos produced via injection of *Siah1b*-targeted ES cells into blastocysts and reimplantation into foster mothers. Mutant MEFs were selected by culturing in the presence of G418 (200 μg/ml; GibcoBRL) for 2 passages (5 to 7 days), and the elimination of wild-type cells was confirmed by Southern blotting using probe 4. Cells were frozen at passage 2 and were assayed at passage 4 or 5.

Cell culture and MEF proliferation assays. MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (500 IU/ml), streptomycin (500 μg/ml), and 200 μM β-mercaptoethanol. For proliferation assays, passage 4 MEFs were seeded at 2×10^5 cells per plate in replicate 60-mm-diameter dishes and cell numbers were determined daily for 7 days. Media were replaced every second day. For 3T3 assays, passage 4 MEFs were seeded at 3×10^5 cells per 60-mm-diameter dish, cell numbers were determined after 3 days, and cells were reseeded for the next passage at the starting density. Cell cycle synchronization experiments using HeLa cells were undertaken as described previously (29). CEM (IC3, 3H3) and erythroleukemic (DP-16, Ts5) cell lines were maintained at 37°C in DMEM supplemented with 10% FBS, 250 μM L-asparagine, 13 μM folic acid, penicillin (500 IU/ml), streptomycin (500 μg/ml), and 50 μM β-mercaptoethanol. Temperature shift was achieved by placing cells in media prewarmed to 32°C and by subsequent incubation at 32°C.

p53 activation. p53 activation was induced by gamma irradiation of mice or cultured cells (^{137}Cs source, 0.75 Gy/min) or by treatment of cells with UV-C irradiation (Stratalinker 1800; Stratagene), disodium *N*-(phosphonacetyl)-L-aspartic acid (PALA; Developmental Therapeutics Program, National Cancer Institute), doxorubicin (Pharmacia), or etoposide (Sigma). Retrovirus (pME2 SV-puro) (23) expressing E1A 12S was a gift of Dobrila Nesic. Phoenix cells were transfected (Lipofectamine Plus) with the retroviral construct. After 2 days, the retrovirus-containing media were harvested and polybrene (4 μg/ml) was added. MEFs were incubated overnight with this mixture, washed to remove polybrene, and cultured for 24 h before addition of puromycin (3 μg/ml). Transformed MEFs were cultured with the drug for 4 days before the assay.

RNA and protein analysis. Northern blots were probed with coding region DNA fragments from mouse *cyclin G*, human *p21* or *GAPDH* genes, an 850-bp *Eco*RV fragment from the 3′ untranslated region (UTR) of mouse *Siah2*, or a 680-bp *Eco*RI-*Hin*dIII fragment from the 3′ UTR of mouse *Siah1a*. Protein extracts from mouse tissues were prepared as previously described (11), and whole-cell extracts were generated from cultured cells by lysis in 1% sodium dodecyl sulfate–10 mM Tris (pH 7.4) followed by boiling for 5 min. Protein lysates were quantitated by the Bio-Rad D_c protein assay, and 20 to 40 μg of protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting using antibodies against Siah1 (11), p21 (F-5; Santa Cruz,), p53 (FL-393; Santa Cruz), α-tubulin (B-5-1-2; Sigma), hsp73 (spa-815; StressGen), E1A 12S (M58; BD PharMingen), and cyclin B1 (H-433; Santa Cruz).

Cell cycle analysis and cell death assays. S-phase cells were labeled by incubation with BrdU (30 μM; Sigma) for 30 min (or 4 h; see Fig. 5). Cells were harvested by trypsinization, fixed in 70% ethanol, and treated with 2N HCl–0.5% Triton X-100 for 30 min followed by 0.1 M sodium tetraborate (pH 8.5). Incorporated BrdU was detected by using anti-BrdU-fluorescein isothiocyanate (BD PharMingen) and DNA stained with propidium iodide (PI) in the presence of RNase A (40 μg/ml). Flow cytometry (FACSCalibur) and WinMDI analysis software were used to determine cell cycle distribution after gating to exclude cellular debris and fixation artifacts (e.g., doublets).

ES cells were grown on fibroblast feeder layers, and adherent and nonadherent cells were harvested by trypsinization 24 h after UV irradiation and fixed in 70% ethanol; apoptosis was quantitated by flow cytometry and PI staining to measure the percentage of cells with sub-2N DNA content.

Adherent and nonadherent E1A-transformed or -nontransformed MEFs were harvested by trypsinization 24 h after treatment with doxorubicin, and the percentage of viability was quantitated by flow cytometry and exclusion of PI by live cells.

Thymocytes were isolated from 6- to 10-week-old mice by crushing thymi through a wire mesh. Cells were washed twice in culture medium (DMEM supplemented with 10% FBS, 250 μM L-asparagine, 13 μM folic acid, penicillin [500 IU/ml], streptomycin [500 μg/ml], and 50 μM β-mercaptoethanol) prior to culture at 2×10^6 cells/ml. Percent viability was determined by trypan blue exclusion 24 h after gamma irradiation. *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ thymocytes were obtained 13 weeks after adoptive transfer of E14.5 *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ fetal liver cells (19). Briefly, embryos from timed matings of *Siah2*$^{-/-}$ *Siah1a*$^{+/-}$ mice were genotyped by PCR, as described previously (11), and 10^6 fetal liver cells were injected into the tail veins of B6.SJL-Ptprc^{a} (CD45.1) mice (Animal Resources Centre, Perth, Australia) that had been gamma irradiated (5 Gy followed 3 h later by 4.5 Gy). Flow cytometry using anti-CD45.2-FITC (BD PharMingen) showed that >98% of thymocytes were derived from donor fetal liver cells.

Antisense treatment. Passage 4 MEFs were seeded in 12-well plates at 4×10^4 cells per well the day before transfection with phosphothioate- and 2′-*O-methyl*-modified oligonucleotides (120 nM) by using cationic peptoid L1 (750 nM) and C (750 nM) transfection reagents (Chiron Corp.). Transfection media were removed after 16 h. Oligonucleotide sequences were as follows: Siah1b AS, 5′-GCTGTGCAATGCTGGTGTCAAACAC; Siah1b RC, 5′-CACAAACTGT GGTCGTAACGTGTCG; Siah1a AS, 5′-ACCGAGGAGTCGCTTCCCAAGT CA; Siah1a RC, 5′-ACTGAACCCTTCGCTGAGGAGCCA.

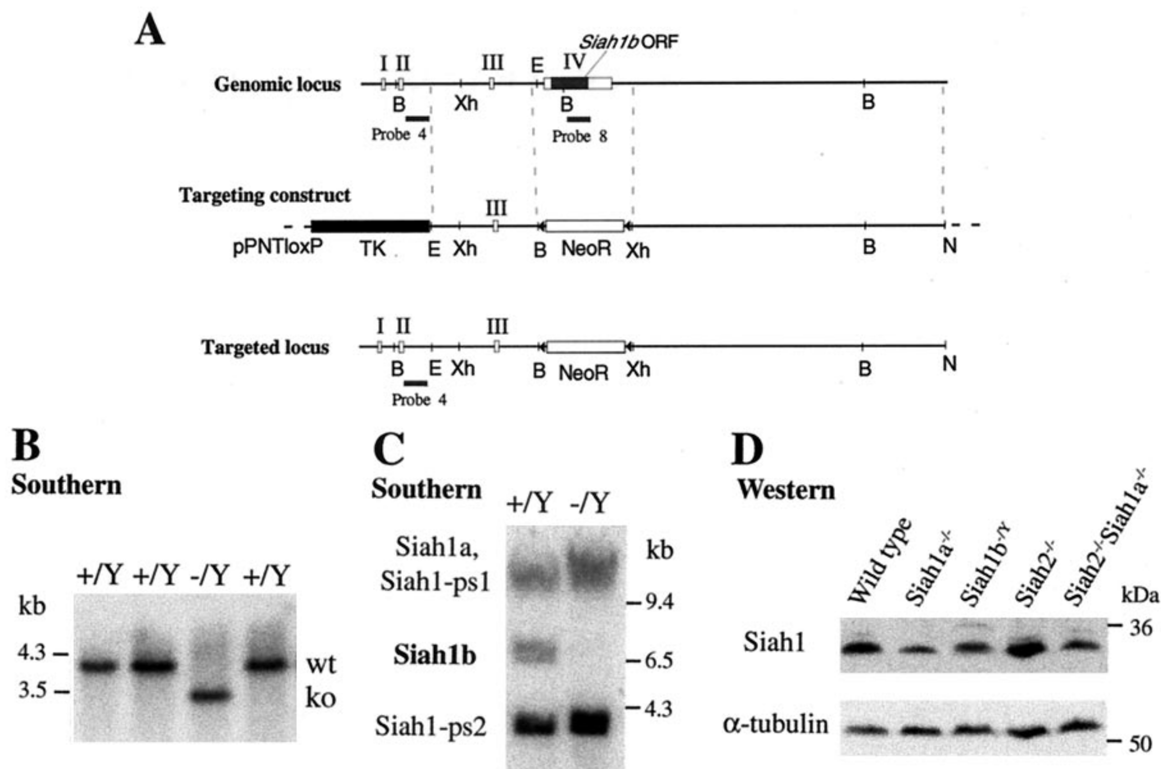

FIG. 1. Disruption of *Siah1b* in ES cells by gene targeting. (A) Exons I, II, III, and IV of *Siah1b* are depicted as open rectangles; the coding region of *Siah1b* is a filled rectangle. The targeting construct in the vector pPNTloxP is depicted. DNA fragments used as the left and right arms of homology are represented by dotted lines. Homologous recombination yields the targeted locus in which the entire coding region is replaced by a loxP-flanked neomycin resistance gene cassette. B, *Bam*HI; Xh, *Xho*I; E, *Eco*RI; triangle, loxP element. (B) Southern analysis of *Bam*HI-digested genomic DNA from wild-type (+/Y) or targeted (−/Y) ES cells, probed with probe 4 (located 5′ of the 5′ targeting arm) to identify the targeted locus. (C) Southern analysis (as for panel B) with coding region probe 8 that hybridizes to the four murine *Siah1* genes (including the two pseudogenes *Siah1-ps1* and *Siah1-ps2*), to confirm the loss of the *Siah1b* gene. (D) Western blot analysis of total protein lysates derived from wild-type, *Siah1a*$^{-/-}$, *Siah1b*$^{-/Y}$, *Siah2*$^{-/-}$, or *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ MEFs, using a monoclonal anti-Siah1 antibody that recognizes both Siah1a and Siah1b.

RESULTS

Generation of *Siah1b*-null ES cells and of MEFs lacking *Siah* genes. To complete the disruption of the *Siah* gene family, we attempted to generate *Siah1b* knockout mice. We designed a targeting vector to completely remove the single coding exon of the *Siah1b* gene (Fig. 1A) and screened ES cells by using a positive (neomycin resistance) and negative (thymidine kinase) selection strategy to enrich for homologous recombinant ES cell clones. Since the W9.5 ES cell clone used in these experiments is genotypically male (48) and the *Siah1b* gene resides on the X chromosome (18), homologous recombinant clones are hemizygous (null) for *Siah1b*. Southern blotting identified a single targeted clone (designated −/Y) from 452 drug-resistant colonies (Fig. 1B). The low targeting frequency likely reflects difficulty in targeting the locus rather than selection against ES cells that had lost *Siah1b* expression through gene targeting, since we failed to generate targeted clones with a loxP targeting vector designed to retain the *Siah1b* gene after homologous recombination (0 of 254 clones screened; data not shown). Specific deletion of the *Siah1b* coding region was confirmed by Southern blotting using coding region probe 8 that also hybridizes to bands corresponding to *Siah1a* and the two *Siah1* pseudogenes (Fig. 1C) (9). The targeted clone was karyotypically normal (data not shown) but failed to generate chimeras that could transmit the mutation to progeny. Live chimeras were born at low frequency (four were obtained from 320 blastocyst injections), and many embryos exhibited developmental abnormalities or were resorbed during pregnancy (data not shown). These data suggest that *Siah1b* may be necessary for the viability of certain tissues during embryonic development.

While we were unable to produce *Siah1b* knockout mice, we successfully generated MEFs lacking *Siah1b* from chimeric embryos (see Materials and Methods). To investigate the proposed roles of *Siah* genes in p53-mediated responses and in chromosome segregation, we also generated a panel of isogenic primary MEFs deficient for *Siah1a* or *Siah2* or both *Siah2* and *Siah1a*. Western blotting, using a monoclonal antibody that recognizes both Siah1a and Siah1b (11), demonstrated that total Siah1 protein levels are decreased by the loss of either *Siah1a* or *Siah1b* and that Siah1b protein expression in *Siah1a*$^{-/-}$ or *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ cells represents approximately one-half the level of total Siah1 expression seen in wild-type cells (Fig. 1D). Antibodies that detect endogenous Siah2 are not available.

Activation of endogenous p53 does not induce *Siah* expres-

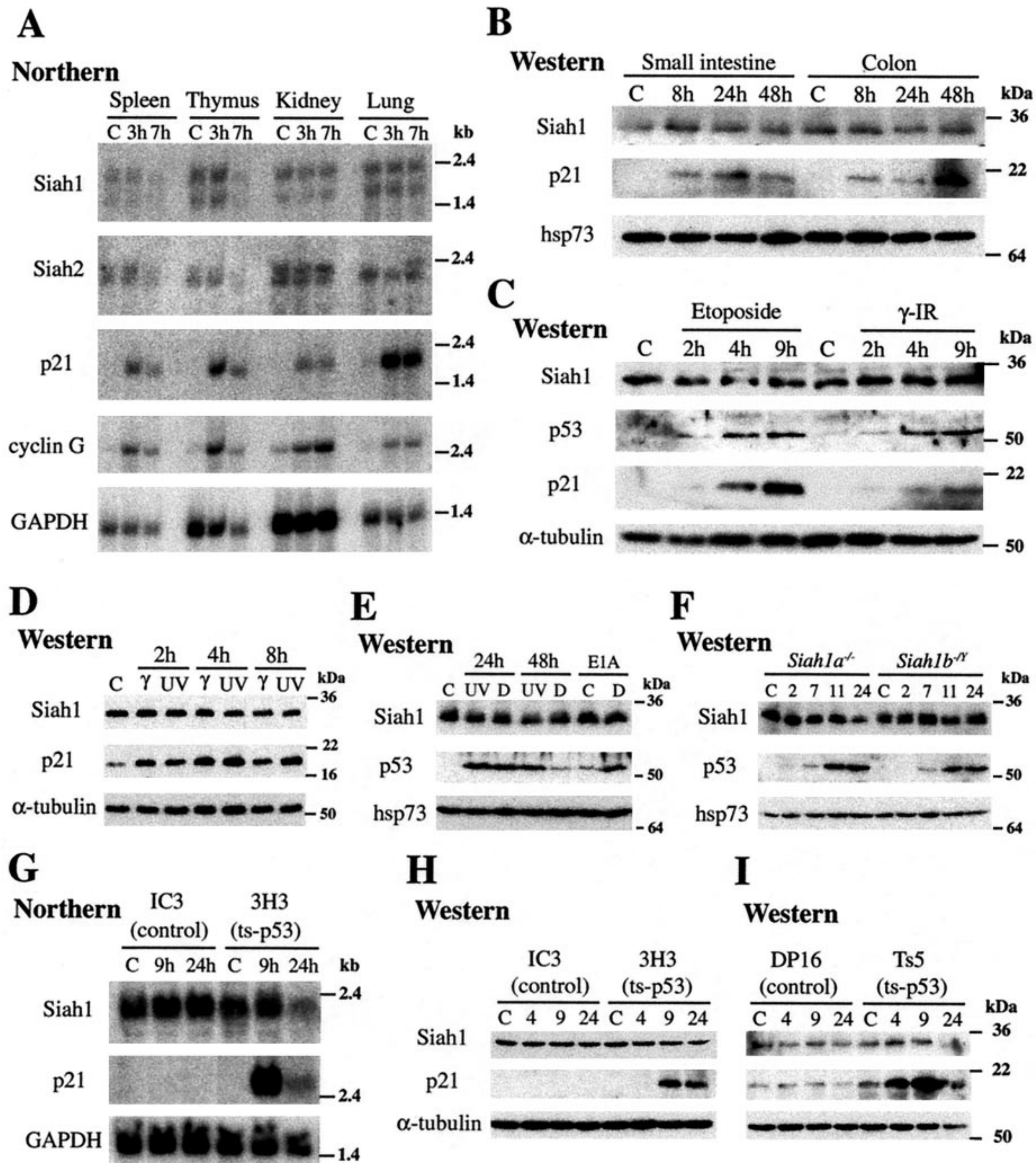

FIG. 2. Activation of endogenous p53 does not induce *Siah1* expression. In all panels, C refers to untreated control mice or cells. (A) Northern blot analysis of mRNA (10 μg) isolated from spleen, thymus, kidney, or lung from mice 3 or 7 h after treatment with gamma irradiation (10 Gy). Blots were probed with DNA fragments from the 3′ UTR of mouse *Siah1a* or *Siah2* genes or coding region fragments of mouse *cyclin G*, human *p21*, or *GAPDH* genes. The Siah1 probe detects *Siah1a* and *Siah1b*. The upper band includes both *Siah1a* and *Siah1b* transcripts. The lower band corresponds to a shorter *Siah1a* transcript. (B to F, H, and I) Western blot analyses of total protein lysates prepared from the indicated tissues or cell types. Siah1, p53, and p21 protein levels were determined by use of specific antibodies. The anti-Siah1 monoclonal antibody recognizes both Siah1a and Siah1b. Probing with antibodies against α-tubulin or hsp73 verified equal loading and transfer. (B) Small intestine and colon from mice 8, 24, or 48 h after gamma irradiation (8 Gy). (C) Mouse thymocytes 2, 4, and 9 h after etoposide (2 μg/ml) addition or gamma irradiation (5 Gy). (D) Primary MEFs 2, 4, and 8 h after gamma (10 Gy) or UV-C (50 J/m^2) irradiation. (E) Primary MEFs 24 and 48 h after UV-C irradiation (50 J/m^2) or doxorubicin (D) treatment (1 μg/ml). The final two lanes represent lysates from MEFs transformed with E1A 12S, either untreated or treated with doxorubicin (D) (0.1 μg/ml) for 24 h. (F) *Siah1a*- and *Siah1b*-null MEFs 2, 7, 11, or 24 h after treatment with UV irradiation (50 J/m^2). (G) CEM cells lacking functional p53 (IC3) or expressing a ts-p53 mutant (3H3) were cultured either at 37°C (control) or at 32°C for 9 or 24 h to induce p53 activity. Total RNA (10 μg) was probed with the coding regions of mouse *Siah1b* or human *p21* or *GAPDH*. (H) Western analysis of total protein lysates from CEM cells (as in panel G) at times after shifting to 32°C. (I) Control erythroleukemic cells lacking functional p53 (DP-16) or expressing a ts-p53 mutant (Ts5) were cultured and analyzed as described for panel H.

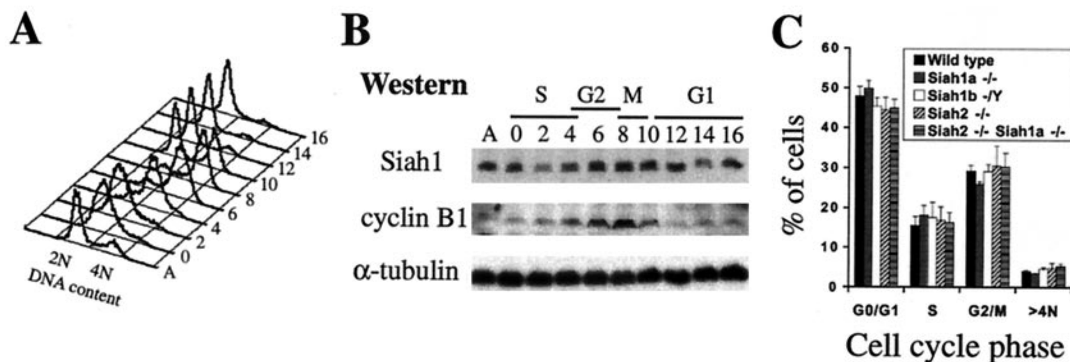

FIG. 3. *Siah* genes are not required for cell cycle progression (A) Flow cytometry analysis (DNA content) of HeLa cells after release from double thymidine block. A, asynchronous culture. Cells were released from early S phase at time zero and collected every 2 h for 16 h. Progression through mitosis occurred after 8 to 10 h. (B) Western blot analysis of total protein extracts prepared from the HeLa cells described for panel A. The accumulation and degradation of cyclin B1 (high in G_2, degraded at the end of mitosis) provided an independent marker of progression through the cell cycle. (C) Asynchronous cell cycle distribution of passage 4 wild-type, *Siah1a*$^{-/-}$, *Siah1b*$^{-/Y}$, *Siah2*$^{-/-}$, or *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ MEFs. Data represent means ± standard deviations of duplicate assays of MEF preparations from three independent embryos of each genotype.

sion. Based on overexpression evidence, current models propose that *Siah1* genes are transcriptionally activated by p53. These studies have not investigated *Siah* gene induction in response to activation of endogenous p53. To address this question, we analyzed the levels of *Siah1a*, *Siah1b*, and *Siah2* mRNA in tissues harvested from wild-type mice in which p53 activation was induced by whole-body gamma irradiation (Fig. 2A). While *Siah* gene expression was not increased, established p53 target genes *p21* (34) and *cyclin G* (40) were strongly activated in these tissues. As Siah1 proteins are proposed to mediate p53-induced β-catenin degradation in the gut (32, 36, 42), we analyzed Siah1 protein expression in mouse small intestine and colon for up to 2 days following p53 activation by gamma irradiation. Siah1 protein levels were unaltered by this treatment, yet sustained accumulation of p21 confirmed that p53 was transcriptionally active (Fig. 2B). Thus, expression of *Siah* genes is not induced in various mouse tissues, including gut, following activation of endogenous p53 by gamma irradiation.

Since gene expression changes mediated by p53 depend on the nature of the inducing stimulus, the level of p53 expression, and the cell type (56), we treated primary thymocytes and MEFs with a range of p53-activating stimuli. Siah1 protein expression in thymocytes was unaltered during p53-mediated apoptosis induced by gamma irradiation or etoposide treatment (Fig. 2C) (6). Activation of p53 in MEFs by treatment with gamma or UV irradiation did not alter Siah1 protein levels at early time points (2, 4, or 8 h) after treatment (Fig. 2D). Similarly, sustained (24 and 48 h) activation of p53 by treatment of MEFs with UV irradiation (Fig. 2E), DNA-damaging drugs including doxorubicin (Fig. 2E) and cisplatin (data not shown), or the disruption of microtubules with nocodazole (data not shown) also failed to increase Siah1 protein levels. Furthermore, Siah1 abundance was not increased in MEFs in which p53 was stabilized as a result of transformation with E1A 12S oncogene (45) or when p53 was further activated in these cells by doxorubicin treatment, leading to induction of p53-mediated apoptosis (Fig. 2E) (12). Since the anti-Siah1 monoclonal antibody detects both Siah1a and Siah1b, we treated *Siah1a*- or *Siah1b*-null MEFs with UV irradiation to examine the effect of p53 activation on the expression of each gene individually. Neither Siah1b nor Siah1a expression increased in *Siah1a*- and *Siah1b*-null MEFs, respectively, in response to p53 activation (Fig. 2F). Stabilization of p53 and/or induction of p21 confirmed p53 activation in these assays. Thus, we conclude that activation of endogenous p53 in primary MEFs and thymocytes does not induce *Siah1* gene expression.

As we observed no induction of *Siah1* genes in response to activation of endogenous p53, we sought to determine whether p53 overexpression is sufficient to induce *Siah1* expression. Activation of temperature-sensitive p53 (ts-p53) by temperature shift has been reported to induce *Siah1* expression in MEF and myeloid leukemia cell lines (1, 20, 43, 44, 47). Here we show that p53-mutant human T-cell lymphoma CEM cells expressing ts-p53 (14) do not induce *SIAH1* mRNA or protein expression upon shifting to the permissive temperature (Fig. 2G and H). Similarly, activation of ts-p53 in a p53-null mouse erythroleukemic cell line (27) also failed to increase SIAH1 protein levels (Fig. 2I). Induction of p21 mRNA and/or protein in these cell lines verified that p53 was functionally activated. Finally, in contrast to previous reports (32, 35, 37), we found that transfection of *p53* in 293 or 293T cell lines had no effect on SIAH1 expression (data not shown). We conclude from these findings that induction of *Siah1* expression is not a general feature of p53 activation and may be restricted to specific cell types or stimuli or dependent on the level of p53 overexpression.

Loss of *Siah* genes does not alter mitotic progression. Since *Siah1* genes have been linked to negative regulation of the cell cycle and also to mitotic and meiotic progression, we investigated whether SIAH1 protein expression is regulated during the cell cycle. HeLa cells synchronized in early S phase by a double thymidine block were released into the cell cycle, and cultures were harvested at various time points for flow cytometric analysis of DNA content (Fig. 3A) and Western blotting of total protein lysates (Fig. 3B). SIAH1 protein levels initially decreased as cells entered S phase and then increased during the G_2 and M phases before again decreasing as the cells exited into G_1. Upregulation of SIAH1 expression during the G_2 and M phases may reflect an important role in mitosis.

We have previously shown that Siah1a is necessary for

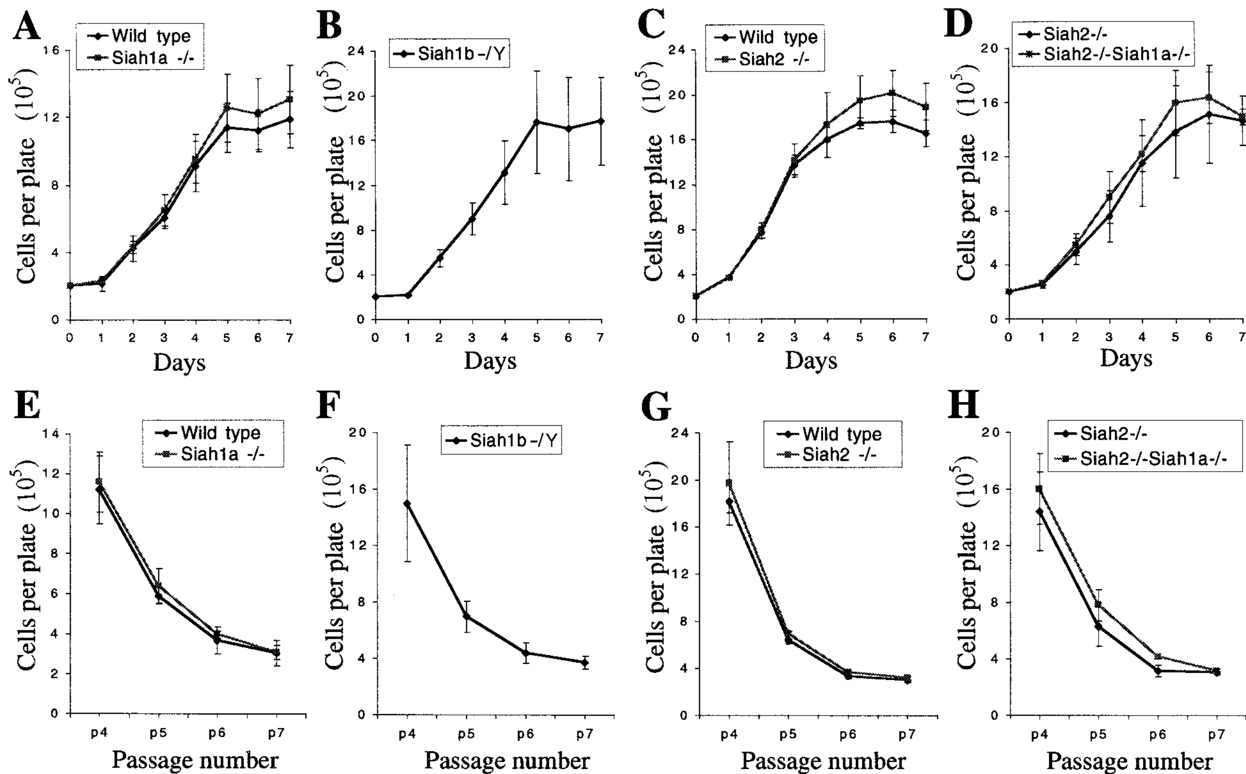

FIG. 4. Normal proliferation, saturation density, and senescence in MEFs lacking *Siah* genes. (A to D) Proliferation and saturation density of passage 4 MEFs; (E to H) 3T3 analysis of MEF proliferation and senescence. All genotypes entered senescence after passage 7. Due to variations in proliferation rates among MEFs prepared from different pregnancies, comparisons were made only between groups of MEFs derived from littermate embryos, with the exception of *Siah1b*$^{-/-}$ MEFs, which were derived from chimeric blastocysts by G418 selection and therefore do not have littermate controls. (A and E) Wild type ($n = 2$) versus *Siah1a*$^{-/-}$ ($n = 3$); (B and F) *Siah1b*$^{-/Y}$ ($n = 3$); (C and G) wild type ($n = 2$) versus *Siah2*$^{-/-}$ ($n = 2$); (D and H) *Siah2*$^{-/-}$ ($n = 3$) versus *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ ($n = 3$). Data points depict the means ± standard deviations of duplicate assays of MEF preparations from n independent embryos.

proper progression past metaphase during meiosis I of spermatogenesis (11). While *Siah1a*$^{-/-}$ MEFs do not display cell cycle or proliferative defects (11), it is possible that *Siah1b* and/or *Siah2* may compensate for loss of *Siah1a* in somatic cells and allow mitotic progression. It is significant that chromosome segregation defects are seen only during spermatogenesis in *Siah1a*$^{-/-}$ mice. As *Siah2* expression is detected only in postmeiotic spermatocytes (8) and the *Siah1b* gene resides on the X chromosome and is thus likely to be silenced by X inactivation during spermatogenesis (17), it is possible that this setting reflects a true *Siah* gene loss-of-function phenotype. To begin to examine this possibility, we have analyzed the cell cycle properties of MEFs lacking *Siah1a*, *Siah1b*, or *Siah2* or lacking both *Siah2* and *Siah1a*. MEFs of all genotypes displayed unaltered asynchronous cell cycle distribution profiles and did not accumulate cells with greater than 4N DNA content (Fig. 3C), suggesting that mitotic progression was unimpaired.

Normal proliferation, contact inhibition and senescence in MEFs lacking *Siah* genes. To further assess the integrity of the cell cycle of MEFs lacking *Siah* genes, we undertook short-term and long-term (3T3) proliferation assays (52). Since Siah1 proteins have been proposed to mediate p53 function and p53 is required for senescence and contact inhibition in MEFs (16), these experiments also tested p53 function in the absence of *Siah* genes. The rate of proliferation and saturation density of passage 4 MEFs were not altered by the loss of *Siah* genes (Fig. 4A through D). All genotypes reached cellular densities similar to those of their littermate controls at all passages in 3T3 assays and underwent senescence by passage 7 (Fig. 4E through H). The rates of proliferation, saturation densities, and entry into senescence of *Siah1b*$^{-/Y}$ MEFs were similar to those of MEFs of the other genotypes. Thus, *Siah1a*, *Siah1b*, or *Siah2* alone or both *Siah2* and *Siah1a* in combination are not required for p53-mediated contact inhibition or senescence.

***Siah* genes are not necessary for p53-mediated cell cycle arrest.** As well as mediating senescence and contact inhibition, p53 is required in MEFs for G_1 phase cell cycle arrest in response to DNA damage or cellular stress (10). To investigate whether *Siah* genes are necessary for p53-mediated G_1 arrest, we treated MEFs lacking *Siah1a*, *Siah1b*, or *Siah2* or both *Siah2* and *Siah1a* with gamma irradiation (0 or 15 Gy) and analyzed cell cycle distribution after 24 h (Fig. 5A). MEF cultures of all genotypes exhibited a decreased percentage of cells in S phase following irradiation, indicating that the p53-dependent G_1 checkpoint was unaffected. Like wild-type cells, MEFs lacking *Siah* genes also accumulated in the G_2/M phase after DNA damage, indicating that the p53-independent G_2 checkpoint (28) does not require *Siah* genes. MEFs of all genotypes also displayed a p53-dependent G_1 arrest in re-

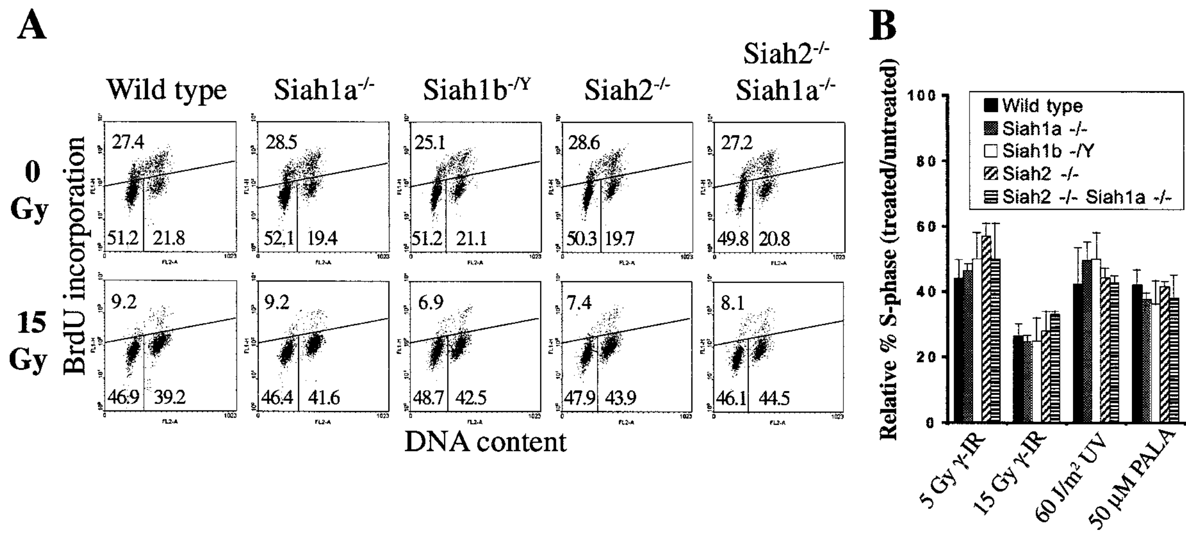

FIG. 5. p53-dependent cell cycle arrest does not require *Siah* genes. (A) Asynchronously growing wild-type, *Siah1a*$^{-/-}$, *Siah1b*$^{-/Y}$, *Siah2*$^{-/-}$, or *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ MEFs were subjected to gamma irradiation (0 or 15 Gy). Cell cycle distributions were assessed 24 h after treatment by BrdU labeling and flow cytometry. The percentage of cells in each phase of the cell cycle (lower left, G_1; upper, S; lower right, G_2/M) is shown. Distributions shown are representative of duplicate determinations of each of two or more independent MEF preparations. (B) MEFs were harvested 24 h after gamma irradiation (5 or 15 Gy) or UV-C irradiation (60 J/m^2) or after 48 h of culture in the presence of PALA (50 μM) and analyzed as described for panel A. The extent of G_1 cell cycle arrest is expressed as relative percent S phase, calculated by the following formula: (% BrdU positive in untreated cultures/% BrdU positive in treated cultures) × 100. The means ± standard deviations are derived from duplicate determinations of each of three independent MEF preparations of each genotype. Similar results were obtained in an independent experiment.

sponse to submaximal doses of gamma irradiation (5 Gy), UV irradiation, or ribonucleotide depletion (PALA treatment) (Fig. 5B) (10). In summary, we find that p53-mediated cell cycle arrest induced by a variety of stimuli is unaffected by mutation of *Siah1a*, *Siah1b*, or *Siah2* alone or by combined mutation of *Siah2* and *Siah1a*.

***Siah* genes are not necessary for p53-mediated apoptosis.** In some cell types, p53 induces apoptosis in response to DNA damage. Thymocyte apoptosis in response to gamma irradiation is abolished by the loss of p53 (6) (Fig. 6A). Thymocytes isolated from *Siah1a* or *Siah2* knockout mice or from mice in which the hematopoietic system was reconstituted with fetal liver cells from *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ embryos underwent apoptosis in a manner equivalent to that of thymocytes isolated from wild-type mice (Fig. 6A). Similarly, *Siah1b* hemizygous ES cells underwent normal p53-dependent apoptosis following UV irradiation (Fig. 6B) (5). While primary MEFs are normally resistant to apoptosis induced by DNA damage, MEFs transformed by the E1A 12S oncogene are highly sensitive to apoptosis in response to p53 activation (33). Wild-type,

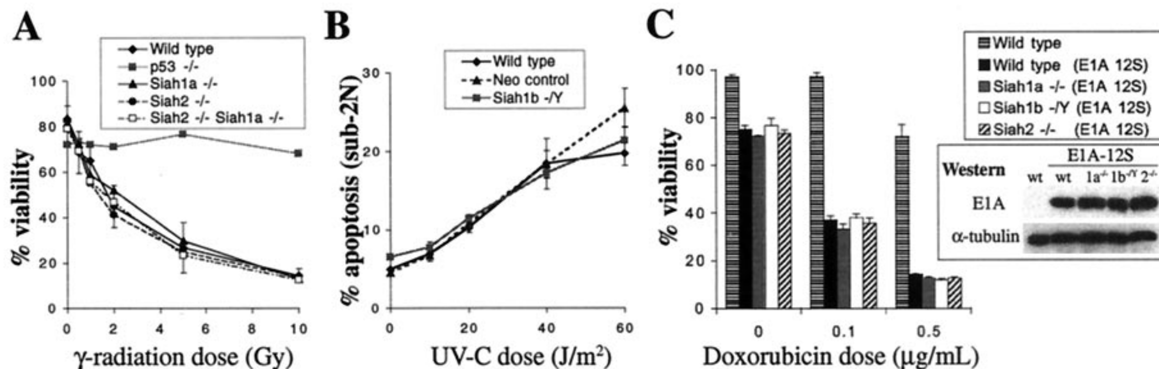

FIG. 6. *Siah* genes are not required for p53-mediated apoptosis (A) Thymocytes were isolated from wild-type (n = 3), *Siah1a*$^{-/-}$ (n = 2), *Siah2*$^{-/-}$ (n = 2), or *p53*$^{-/-}$ (n = 1) mice or from lethally irradiated mice (n = 2) that had been reconstituted with *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ fetal liver cells. Cell viability was assessed 24 h after gamma irradiation. Duplicate cultures from each of n mice of each genotype were analyzed. Means (± standard deviations [SD] where applicable) are shown. (B) Susceptibility to UV-induced apoptosis of the parental ES cell clone (wild type), a clone containing a random insertion of the *Siah1b* targeting vector (Neo control), and the *Siah1b*-targeted clone (*Siah1b*$^{-/Y}$). Apoptosis was assessed 24 h after treatment. Means ± SD of triplicate assays are shown. Data are representative of three independent experiments. (C) Wild-type, *Siah1a*$^{-/-}$, *Siah1b*$^{-/Y}$, or *Siah2*$^{-/-}$ MEFs were infected with retrovirus expressing E1A 12S. The viability of E1A-expressing MEFs or of noninfected wild-type MEFs was assessed 24 h after treatment with doxorubicin (0.1 or 0.5 μg/ml). Means ± SD of triplicate assays are shown. Western blotting (inset) confirmed equal levels of E1A 12S expression among the genotypes.

Siah1a$^{-/-}$, *Siah1b*$^{-/Y}$, and *Siah2*$^{-/-}$ MEFs transformed by retroviral infection with E1A 12S were equally sensitive to p53-mediated apoptosis induced by doxorubicin treatment (Fig. 6C) (12). Western blotting confirmed equal levels of expression of E1A 12S for these genotypes. Thus, *Siah1a*, *Siah1b*, or *Siah2* alone, or *Siah2* and *Siah1a* together, are dispensable for p53-mediated apoptosis.

Inhibition of *Siah1b* expression in *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ MEFs does not alter cell cycle progression or p53-mediated G_1 arrest. Since the Siah proteins are highly homologous to one another, and Siah1 and Siah2 can function equivalently to induce the degradation of some substrates (22), it is possible that Siah1b provides sufficient Siah activity in *Siah1a*$^{-/-}$ or *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ cells to allow normal p53 responses and mitosis. To analyze the effects of inhibition of the expression of different combinations of Siah proteins, MEFs were transfected with antisense or reverse control oligonucleotides directed against *Siah1a* or *Siah1b* 24 h prior to induction of cell cycle arrest by gamma irradiation or doxorubicin treatment. Siah1 protein levels were efficiently decreased 24 and 48 h after antisense transfection of wild-type, *Siah1a*$^{-/-}$, *Siah1b*$^{-/Y}$, or *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ MEFs, in both treated and untreated cultures (Fig. 7A and E). Densitometry revealed that, in comparison to wild-type cells, levels of Siah1 protein in antisense-treated *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ MEFs were reduced by 80 to 90%. Stabilization of p53 and induction of p21 protein expression in response to doxorubicin treatment or UV irradiation were unaffected by antisense treatment of *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ MEFs (Fig. 7E). Furthermore, the expression level of β-catenin, a putative target of degradation by Siah1 proteins, was not altered by the loss of Siah protein expression (Fig. 7E). Flow cytometry showed that antisense treatment did not alter cell cycle progression in unirradiated cells (Fig. 7B) or impair p53-mediated G_1 cell cycle arrest in response to gamma irradiation (Fig. 7C) or doxorubicin (Fig. 7D). Thus, mitotic progression, p53-mediated induction of p21 expression, and p53-mediated cell cycle arrest occur normally in *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ cells in which total Siah1 protein levels have been markedly reduced.

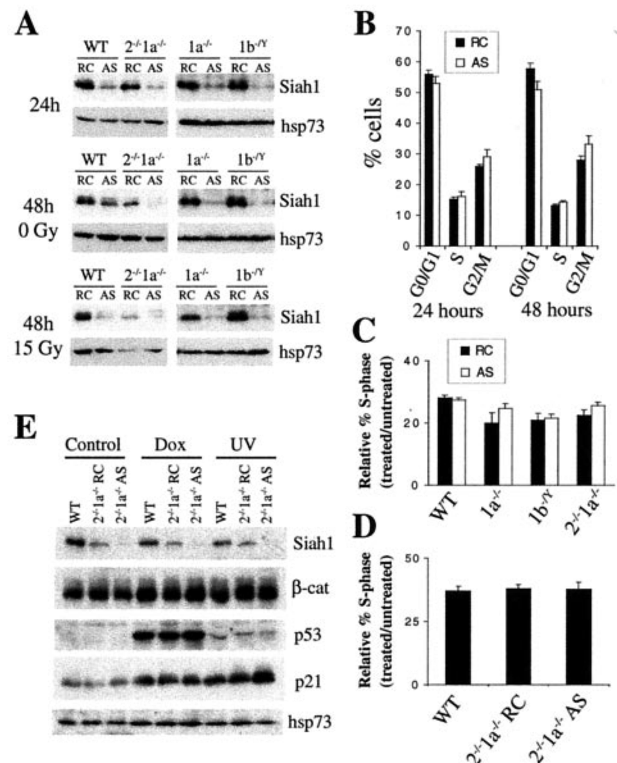

FIG. 7. Inhibition of *Siah1a* and *Siah1b* expression does not alter cell cycle progression or p53-mediated G_1 arrest. Wild-type (WT), *Siah1a*$^{-/-}$ (1a$^{-/-}$), or *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ (2$^{-/-}$1a$^{-/-}$) MEFs were transfected with antisense (AS) oligonucleotides directed against *Siah1b* or with reverse control (RC, reverse sequence of antisense) oligonucleotides. *Siah1b*$^{-/Y}$ (1b$^{-/Y}$) MEFs were transfected with AS or RC oligonucleotides directed against Siah1a. Twenty-four hours after transfection, cells were treated with gamma irradiation (15 Gy), doxorubicin (1 μg/ml), or UV-C irradiation (50 J/m^2). (A) Siah1 protein levels were assessed by Western blotting of total protein lysates prepared at the time of gamma irradiation (24 h) or 24 h after gamma irradiation (48 h). Probing with antibodies against hsp73 verified equal loading and transfer. (B) AS treatment of *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ MEFs does not alter cell cycle distribution. Cultures were analyzed by BrdU labeling (30 min) 48 h after transfection. Data represent means ± standard deviations (SD) of triplicate transfections. (C and D) G_1 phase cell cycle arrest was assayed (as for Fig. 5B) by BrdU labeling (30 min) at the 48 h time point (24 h after gamma irradiation [C] or doxorubicin treatment [D]). Data represent means ± SD of triplicate oligonucleotide transfections. (E) Siah1, β-catenin, p53, and p21 protein levels were assessed by Western blotting of total protein lysates prepared 6 h after treatment with doxorubicin or UV irradiation. Probing with antibodies against hsp73 verified equal loading and transfer.

DISCUSSION

Many studies have proposed that *Siah1* genes are induced by p53 activation and may function as negative regulators of cell cycle progression, mediators of apoptosis, or tumor suppressors. Evidence for these hypotheses derives almost exclusively from gain-of-function studies in which *p53* or *Siah1* genes were overexpressed in transformed cell lines. In this study we have tested these models using a loss-of-function approach. We find no evidence to support a general role for *Siah* genes in a range of p53-mediated responses.

Previous reports have shown that transient overexpression of p53 in several different cell lines (32, 35) or activation of ts-p53 in immortalized MEF and mouse myeloid leukemic cell lines (1, 20, 43, 44, 47) induces *Siah1* gene expression. Additionally, stable transfection of U937 cells with *p21* increased *SIAH1* mRNA levels (31, 32, 35) and enforced expression of *SIAH1* or *p21* induced the expression of an overlapping set of genes, supporting a model whereby Siah1 proteins function downstream of p53 and p21 (44). However, it has yet to be shown that *Siah1* genes are induced by stimuli that activate endogenous p53. In this study we demonstrate that *Siah1a* and *Siah1b* mRNA and protein levels are not increased by activation of endogenous p53 in mouse tissues or in primary MEFs or thymocytes. Furthermore, we show that activation of ts-p53, in two different cell lines that lack functional endogenous p53, does not increase expression of *Siah1* genes. The discrepancy between previous reports and our findings may be reconciled by the fact that gene expression changes induced by p53 are dependent on the inducing stimulus, the level of p53 induction, and the cell type (56). It is noteworthy that in some, but not all, experimental settings, p53 overexpression induces cell cycle arrest in G_2 (50). As Siah1 protein levels are high in G_2 (Fig. 3B), previously observed induction of Siah1 by p53 overexpres-

sion may be a secondary consequence of altered cell cycle distribution. We cannot exclude that *Siah1* genes may be targets of endogenous p53 in a restricted range of cell types or in response to restricted stimuli. It is significant, however, that *Siah1* gene expression was not induced in diverse tissue and cell types (spleen, thymus, lung, kidney, gut, embryonic fibroblasts, thymocytes) under conditions where p53-mediated cell cycle arrest or apoptosis was induced by a wide range of p53-activating stimuli, including DNA damage (gamma irradiation, UV irradiation, etoposide, doxorubicin, cisplatin), oncogenic transformation (E1A 12S), disruption of microtubules (nocodazole), or depletion of ribonucleotide pools (PALA).

A number of observations have been used to support the hypothesis that Siah1 proteins may be effectors of p53-mediated responses. Siah1 proteins have been linked to negative regulation of cell cycle progression. *SIAH1* transfection into 293 cells or GM701 immortalized fibroblasts induced growth arrest and blocked S-phase entry without inducing apoptosis (37). Furthermore, stimulation of human fibroblasts with serum induced marked repression of *SIAH1* mRNA expression with kinetics similar to those of transcripts encoding proteins that inhibit the cell division cycle (24). *Siah1* genes have also been linked to p53-induced apoptosis. Coexpression of *Siah1a* and *Pw-1/Peg-3*, a p53-inducible Siah1-binding protein, induced apoptosis in immortalized fibroblasts (43). *SIAH1* overexpression also induced apoptosis in U937 cells (4, 44). Significantly, apoptosis induced by activation of ts-p53 in a myeloid leukemia cell line was reduced by expression of antisense *Siah1a* (47). Thus, overexpression of *Siah1* genes can mimic the effects of p53 activation and induce cell cycle arrest and apoptosis.

In this study we have utilized a genetic approach to assess whether *Siah* genes participate in p53-mediated responses. We show that (i) p53-mediated contact inhibition and senescence in MEFs does not require *Siah1a*, *Siah1b*, or *Siah2* alone, or *Siah2* and *Siah1a* in combination; (ii) p53-mediated apoptosis of thymocytes occurs normally in the absence of *Siah1a*, *Siah2*, or both *Siah2* and *Siah1a*; (iii) p53-mediated apoptosis of ES cells does not require *Siah1b*; (iv) p53-mediated apoptosis of E1A 12S-transformed MEFs does not require *Siah1a*, *Siah1b* or *Siah2*; and (v) p53-mediated G_1 phase cell cycle arrest in MEFs is normal in the absence of *Siah1a*, *Siah1b*, or *Siah2* or of both *Siah2* and *Siah1a*. These data clearly demonstrate that the loss of *Siah* genes individually or the loss of two of the three highly homologous *Siah* family genes (*Siah2* and *Siah1a*) does not impair p53 function in growth arrest and apoptosis. We also used antisense oligonucleotides to markedly decrease Siah1b expression in *Siah1a*$^{-/-}$ or *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ cells or to decrease Siah1a expression in *Siah1b* mutant cells. Despite attenuation of Siah1 protein levels by 80 to 90%, cell cycle progression, p53-mediated induction of p21 expression, and p53-mediated cell cycle arrest were unaffected. These findings, based on loss-of-function evidence, are inconsistent with existing hypotheses that *Siah* genes are necessary for mitotic progression or for p53 function.

SIAH1 has been reported to be the limiting factor of a novel E3 ubiquitin ligase complex that targets β-catenin for degradation (32, 36). It is hypothesized that p53 activation increases SIAH1 protein levels and thus induces the degradation of β-catenin. This process has been proposed to contribute to p53-mediated cell cycle arrest and tumor suppression. Our data does not support this model. Notably, we fail to observe induction of Siah1 protein levels following p53 activation in the gut, a tissue in which β-catenin functions as an oncogene during tumorigenesis (42), nor do we see defects in p53-mediated cell cycle arrest in cells lacking *Siah* genes. Moreover, total cellular β-catenin levels are not elevated by loss or inhibition of Siah2, Siah1a, and Siah1b protein expression. While other correlative evidence (39, 44) has been used to advance the argument that *Siah* genes function as tumor suppressors, *Siah1a*$^{-/-}$, *Siah2*$^{-/-}$, and *Siah2*$^{-/-}$ *Siah1a*$^{-/-}$ mice do not develop tumors or display tissue hyperplasia (11; also unpublished results). Previous studies have also failed to detect loss of *SIAH1* expression or *SIAH1* gene mutations in human cancers and cancer-derived cell lines (20, 38), suggesting that there is no selective pressure to lose *SIAH1* gene expression or function during tumorigenesis.

In summary, we do not observe induction of *Siah1* genes under conditions in which established p53 target genes and p53-mediated cell cycle arrest or apoptosis are clearly induced. Furthermore, the loss of *Siah* genes, singly or in combination, does not alter p53-mediated responses in primary MEFs, thymocytes, or ES cells. While we cannot exclude that Siah proteins may function to regulate p53 responses in cell types that were not examined in this study, such a requirement is clearly not a general feature of p53 signaling. We suggest that widely held models that place Siah1 proteins downstream of p53 in growth arrest, apoptosis, and tumor suppression should be reassessed. It is possible that they are based on nonspecific consequences of overexpression of Siah or p53 proteins.

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