An inducible long noncoding RNA amplifies DNA damage signaling

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Cells are constantly subject to an array of external stimuli. Signal transduction pathways must record diverse inputs and integrate these with prior experiences of the cell to decide whether to proliferate, differentiate, or die. The DNA damage response is critical for normal cell proliferation and suppression of cancer and relies on the transcription factor p53. In mammals, the p53-dependent response to DNA damage is complex and tissue specific1. While p53-dependent, p21-mediated cell cycle arrest in response to DNA damage can protect against radiation-induced gastrointestinal, cardiac, and hematologic toxicity2-4, p53-dependent apoptosis drives radiation toxicity in hematopoietic cells in a p53 dose-dependent manner5. Indeed, Trp531−/− mice are strikingly resistant to myeloablative doses of ionizing radiation because they do not activate the intrinsic apoptosis pathway, while Trp531+/− mice have intermediate radiation sensitivity between that of Trp531+/− and Trp531−/− mice. Thus, fine-tuning the choice and amplitude of different p53 target genes is a critical aspect of the DNA damage response.

Eukaryotic genomes are pervasively transcribed to generate diverse lncRNAs, especially from highly regulated enhancers and promoters6,7. Recently, several lncRNAs have been identified that regulate specific subsets of the p53-dependent gene expression signature8,9. The DNA damage-induced lncRNA PANDA negatively regulates apoptosis by blocking the transcription factor NF-YA, while long intergenic noncoding RNA (lincRNA)-p21 recruits heterogeneous nuclear ribonucleoprotein K (hnRNPK) to regulate p21 in cis10-12. Furthermore, the APELA RNA expressed in mouse embryonic stem cells binds to heterogeneous nuclear ribonucleoprotein L (hnRNPL) to block its interaction with p53 and permit p53 accumulation in the mitochondria to elicit apoptosis13. While p53 is known to bind RNA14, the role of lncRNAs in regulating the p53 protein remains mostly unknown.

p53 signaling is modulated by the regulation of p53 protein abundance. During normal cell cycles, a low level of damage resulting from DNA replication transiently activates p53 but is insufficient to robustly induce p53-responsive genes. This is because p53 has a short half-life and exists in a conformation with limited DNA binding efficiency15,16. With sustained DNA damage, p53 is stabilized and p53-responsive genes (such as CDKN1A, DDB2 and PUMA) are activated, leading to either cell cycle arrest or apoptosis. While still poorly understood, sustained DNA damage is believed to invoke a signal amplification mechanism that requires an as-yet-unidentified coactivator of the p53 response16. Here we report that a p53-inducible lncRNA serves as a key gating mechanism in the DNA damage response.

RESULTS

DINO is a conserved, DNA damage-inducible lncRNA

We identified a DNA-damage-induced transcription unit upstream of CDKN1A, hereinafter named DINO (Damage Induced Noncoding), in a screen for transcribed regions in human cell cycle promoters10 (Fig. 1a and Supplementary Fig. 1a–d). RACE showed that DINO is a 951-base RNA transcribed divergently from CDKN1A. Codon substitution

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frequency (score = −55.7) and in vitro translation suggested that DINO does not encode a protein (Supplementary Fig. 1e). DINO was induced ~100-fold in primary human fibroblasts in response to sustained doxorubicin-induced DNA damage, peaking at 10–24 h after damage and reaching ~1,000 copies per cell (Supplementary Fig. 1f). In contrast, the known p53 target gene CDKN1A was induced 5–10 fold (Fig. 1b). DINO was also induced upon DNA damage in human cancer cell lines and by other stressors, albeit at lower levels (Supplementary Fig. 1g,h).

Because DINO is situated adjacent to a p53 binding site (Fig. 1a), we reasoned that its induction may require p53. Knockdown of p53 in human fibroblasts abrogated DINO induction (Fig. 1c). Wild-type HCT116 colon cancer cells induced DINO in response to doxorubicin, while isogenic TP53−/− cells did not (Fig. 1d). Similarly, TP53-null H1299 human lung adenocarcinoma cells only induced DINO after complementation with wild-type p53, but not with a p53 mutant derived from cancer-prone Li-Fraumeni syndrome (Supplementary Fig. 1i). DINO was not induced in p53 mutant tumor cell lines (Supplementary Fig. 1j).

One indication of a lncRNA’s functional significance is its evolutionary conservation. Functionally related lncRNAs between human, mouse and zebrafish can be identified by genomic synteny and conserved regions of microhomology, despite limited overall sequence identity[17,18]. Thus, we queried the mouse Cdkn1a and zebrafish CDKN1A promoters for a DNA damage-inducible lncRNA immediately upstream of the first exon. A mouse transcript sense to Cdkn1a was observed at this position in mouse embryonic fibroblasts (MEFs) after doxorubicin treatment (Supplementary Fig. 2a). RACE, RNA blotting, and reverse transcription (RT)-PCR established the identity of the putative mouse Dino (Supplementary Fig. 2a–c). DNA damage induction of mouse Dino was also attenuated in Trp53−/− MEFs (Supplementary Fig. 2a). Similarly, RT-PCR and RACE analysis of zebrafish embryos identified a UV-inducible sense-strand lncRNA at the precise syntenic location upstream of CDKN1A (Supplementary Fig. 2d–f). We observed several regions of microhomology in human, mouse, and zebrafish DINO that were also conserved in the putative DINO encoding regions of five additional mammalian species (Supplementary Fig. 2g–i). Thus, DINO represents a potentially conserved transcriptional response to DNA damage, though notably, the predicted DNA strand encoding DINO varies across the eight species examined (Supplementary Fig. 2i).

DINO regulates the p53-dependent DNA damage response

LncRNAs can regulate gene expression both in cis and in trans. Depletion of DINO by RNA interference identified a key role in the DNA damage response. DINO depletion by multiple independent short interfering RNAs (siRNAs) in primary human fibroblasts blunted CDKN1A induction upon DNA damage (Fig 2a). Microarray analysis showed that 215 of 417 genes normally regulated by DNA damage failed to respond after DINO depletion, including canonical p53-responsive genes CDKN1A, DBD2, and GADD45A (Fig. 2b). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis identified an enrichment of the p53 signaling pathway (P = 7.9 × 10−3, false discovery rate = 0.09) (Gene Ontology terms, Supplementary Table 1; gene lists, Supplementary Table 2), and a majority of the genes affected by siRNA to DINO (60%) had a canonical p53-binding site. Assay of Transposable Accessible Chromatin by sequencing (ATAC-seq)19 showed that chromatin accessibility at cognate p53 binding sites was induced by DNA damage genome-wide, but was reduced in DINO-depleted human fibroblasts (Fig. 2c and Supplementary Fig. 3b). Indeed, chromatin immunoprecipitation (ChIP) followed by quantitative PCR (qPCR) showed that DINO depletion caused a loss of p53 occupancy at its cognate target genes, such as at CDKN1A (Fig. 2d) and additional genes (Supplementary Fig. 3c,d). Finally, DINO is required for cell cycle arrest in response to DNA damage. Like cells treated with p53 short hairpin RNA (shRNA) cells, DINO-depleted, human osteosarcoma U2OS cells continued to divide following DNA damage to a greater extent than control DINO-proficient cells (Fig. 2e and Supplementary Fig. 3e). Similar results were obtained with independent siRNAs targeting DINO (Supplementary Fig. 3f).

DINO and p53 physically interact following DNA damage

LncRNAs can act as modular scaffolds for chromatin modification complexes[20], but their roles in mediating signal transduction are less understood. Therefore, we asked whether DINO may physically interact with p53. In vitro transcribed DINO RNA preferentially retrieved p53 from the lysate of DNA-damaged cells, but did not retrieve the Polycromb complex or several other control proteins (Supplementary Fig. 4a,b). DINO also had higher affinity for recombinant purified p53 than for Polycromb Repressive Complex 2 (PRC2) (Fig. 3a). In complementation experiments, immunoprecipitation of p53 from the chromatin of DNA-damaged cells retrieved endogenous DINO RNA (Fig. 3b). Analysis of RNA fragments UV-crosslinked to endogenous p53 in
DNA-damaged cells identified a major p53-binding site in DINO (Fig. 3c). Expression of wild-type p53 or a DNA-binding-incompetent R273H p53 in Trp53-null cells enabled p53 retrieval of DINO, but a C-terminal deletion truncating a RNA-binding region of p53 (ref. 14) decreased DINO interaction (Fig. 3d). Thus, the C terminus of p53, which is also known to undergo extensive post-translational modification, binds to a discrete domain of DINO. To investigate whether DINO localized to regulatory regions adjacent to DINO-dependent genes, we used chromatin isolation by RNA purification (ChIRP) to map DINO’s chromatin occupancy. DINO RNA was specifically retrieved with orthogonal ‘even’ and ‘odd’ pools of biotinylated capture probes along with associated DNA; RNase-treated chromatin served as a negative control (Supplementary Fig. 4c). We observed DINO occupancy at p53 binding elements in seven of ten p53-target genes examined, including CDKN1A, GADD45A, and DDB2; DINO occupancy was not detected at any of the five negative control loci (Supplementary Fig. 4e). These results suggest that DINO and p53 colocalize at multiple p53 target genes throughout the genome.

**DINO stabilizes p53 and induces p53 target genes**

Since DINO directly binds p53, we examined whether DINO expression regulates p53 protein following DNA damage. In response to DNA damage, p53 is stabilized by a series of post-translational modifications and inhibition of ubiquitination, leading to protein accumulation and transactivation of p53 targets. DINO depletion blocked the ability of DNA damage to induce p53 stabilization and p21 induction, despite preservation of p53 phosphorylation at Ser9 (Fig. 4a), suggesting that DINO is required for p53 stability in the DNA damage response.

If DINO binding stabilizes p53 protein directly, overexpression of DINO in the absence of DNA damage may be sufficient to stabilize p53 and activate DNA damage signaling. Cyclolideine chase showed that p53 protein was rapidly degraded in control cells, whereas enforced human or mouse DINO expression, in the absence of DNA damage, caused p53 stabilization and increased p21 protein levels (Fig. 4b). DINO overexpression induced a large panel of p53 target genes, including RRM2, DDB2, and GADD45A, as measured by nCounter and RT-qPCR assays, and also caused substantial G2 cell cycle arrest, a well-known p53-dependent checkpoint (Fig. 4c). These results indicate that DINO can act in trans when expressed separately from the CDKN1A locus and highlight the functional conservation of human and mouse DINO.

Although DINO directly binds p53, it is possible that DINO regulates p53 in a manner independent of p53 binding. To examine this possibility, we generated DINO constructs with focal deletion of the p53-binding motif. This motif forms a stem loop structure in vitro (Supplementary Fig. 5a), and deletion of this motif abrogated p53 binding in vivo (Fig. 4e). Overexpression of DINO mutants failed to induce p53 target genes (Fig. 4f) or stabilize p53 protein (Supplementary Fig. 5c), confirming that DINO binding to p53 is important for activating the DNA damage response.

**Dino knockout mice are deficient in p53 pathway functions**

We generated C57BL/6 mice with genetic modification of Dino to characterize the role of the mouse lncRNA in the p53 response (Fig. 5a). The knock-in allele Dinooff was designed to achieve two purposes: (i) report the activity of the putative Dino promoter and (ii) disrupt the function of Dino by replacing the bulk of Dino sequence with GFP. We designed the targeting construct to avoid removal of endogenous p53 response elements within the Cdkn1a promoter (asterisks in Fig. 5a). Frt-flanked neomycin selection cassettes were removed by recombination in ES cells before blastocyst injection. Dinooffoff mice were born with the expected male:female Mendelian ratio and were viable, fertile, and without apparent developmental defects.

MEFs isolated from Dinooffoff E13.5 embryos induced GFP expression in response to DNA damage (Supplementary Fig. 6a), indicating that we did capture the Dino promoter. While Dinooffoff MEFs retained 90% of the basal and DNA-damage-induced Cdkn1a expression found in Dinooffoff MEFs, the expression of Cdkn1a in homozygous Dinooffoff
MEFs decreased by nearly 50% relative to Dino+/− MEFs (Fig. 5b). That Cdkn1a expression remains largely intact in Dino+/+/− MEFs, unlike in Dino+/−/− MEFs, suggests that a single wild-type allele of Dino is sufficient to maintain near wild-type levels of Cdkn1a. This supports a model of Dino regulation of gene expression in trans. We next examined whether mouse p53, like human p53, regulates p53-dependent gene expression in response to DNA damage. We cultured MEFs under low physiologic oxygen tension (2%), avoiding hyperoxic conditions that can activate p53 and induce senescence.23 Dino+/−/− MEFs exhibited substantially dampened induction of canonical p53 target genes that control cell cycle, apoptosis, and DNA repair, including Cdkn1a, Mdm2, Bax, Puma, and Gadd45a (Fig. 5c).

Since gene expression analysis indicated that mouse Dino regulated apoptosis signaling, we next examined Dino+/−/− mice for defects in DNA-damage-induced apoptosis. Ex vivo irradiation of thymocytes produced diminished apoptosis and enhanced survival in Dino+/−/− mice.

Figure 3 p53 is a DINO-binding protein. (a) Left, summary of RNA chromatography with DINO retrieval of proteins from whole cell lysate of doxorubicin (24 h)-treated fibroblasts. Right, RNA chromatography using 6 pmol RNA and 1 μg of each recombinant protein as indicated. (b) RT-qPCR identifies the DINO region bound by p53 in human fibroblasts followed by RT-qPCR of DINO, GAPDH and U6 small nuclear RNA. *P = 0.01 compared to IgG control (Student’s t-test). Mean ± s.d. are shown, n = 3. (c) CLIP of p53-bound DINO RNA in doxorubicin (24 h)-treated human fibroblasts. RT-qPCR identifies the DINO region bound by p53 in vivo. Location of primer pairs along the DINO transcript indicated in the diagram above. Bottom shows immunoblot (IB) of p53 retrieved by CLIP. *P = 0.0001, **P = 0.002. Mean ± s.d. are shown, n = 3. Shaded box over DINO transcript maps to the identified DINO hairpin structure (Supplementary Fig. 4a,b). (d) p53 RIP of DINO with pcDNA vector or pcDNA containing the indicated p53 constructs in TP53−/− H1299 cells, p53 lacking the C-terminal 31 amino acids (p53ΔC31). A control lacking reverse transcriptase (RT) is also shown. IP, immunoprecipitation; IB, immunoblot. *P < 0.01. Mean ± s.d. are shown, n = 3. See also Supplementary Figure 4.

Figure 4 DINO stabilizes p53 and enhances regulation of its target genes. (a) Immunoblot of p53, p53 phospho-Ser9 (S9phos), and p21 in control shRNA (shSCR) and DINO shRNA (shDINO) U2OS cells. Doxorubicin (Dox; 12 h) was used at the following concentrations: 0, 0.05, 0.1, 0.2 μg/ml. (b) Human DINO or mouse Dino expression stabilizes p53 in fibroblasts, as indicated by cycloheximide (CHX) chase analysis. p53 and p21 are shown at indicated hours after CHX addition. p53 densitometry values are indicated below. (c) p53 target gene expression in human embryonic kidney (HEK 293) cells with empty vector, DINO overexpression, Dox treatment (16 h), or DINO + Dox. Left, nCounter quantification. Right, confirmation using RT-qPCR. Mean ± s.d. are shown; *P < 0.05 (Student’s t-test), n = 3. (d) Cell cycle analysis of DINO-transfected HEK 293 cells labeled with bromodeoxyuridine (BrdU). The percentage of cells remaining in G2/M phase is shown for each treatment. Mean ± s.d. are shown, n = 3. See also Supplementary Figure 5.
thymocytes relative to Dino\(^{+/+}\) thymocytes (Supplementary Fig. 6b). Since p53-dependent radiation-induced apoptosis differs among thymocyte subsets\(^24\), we specifically examined the subset most sensitive to radiation, CD4\(^+\)CD8\(^+\) cells. We observed reduced levels of apoptosis in the CD4\(^+\)CD8\(^+\) thymocytes of Dino\(^{+/+}\) mice relative to those of Dino\(^{+/−}\) mice 6 h after in vivo irradiation with 5 Gy total body irradiation (Supplementary Fig. 6c). The significant but partial defect in apoptosis in Dino\(^{+/+}\) thymocytes was similar to the defect observed in heterozygous Trp53\(^{+-}\) thymocytes (Fig. 5d)\(^24\). Such partial abrogation of DNA-damage-induced apoptosis has also been observed in mice mutant for other important mediators of p53-induced apoptosis, such as Puma\(^-/-\) and Perp\(^-/-\) mice\(^25,26\).

p53 modulates the organismal response to DNA damage and is a major determinant of tissue injury following lethal irradiation. Therefore, we examined whether loss of Dino altered the sensitivity of mice to lethal irradiation. Following exposure to a single, high dose of 12 Gy total body irradiation, Dino\(^{+/+}\) mice lived signficantly longer than Dino\(^{+/−}\) mice (P = 0.01, Fig. 5e). Heterozygous Dino\(^{+/-}\) mice had radiation resistance that was indistinguishable from that of wild-type mice. The organismal resistance to radiation toxicity in Dino\(^{+/+}\) is reminiscent of that in mice deficient in Puma, a key mediator of p53-induced apoptosis\(^25,26\). Furthermore, because Cdkn1a\(^{-/-}\) animals have increased radiation sensitivity by several regimes\(^3,4\), the opposite of the Dino\(^{+/−}\) phenotype, our findings indicate that Dino's role in DNA damage response must extend beyond cis regulation of Cdkn1a.

**Dino regulates p53 signaling independent of p21**

While the Dino\(^{+/-}\) allele created a robust gene knockout and was ideally suited to investigate the role of Dino in regulating genes in trans, we created a second mouse with a focal modification of the Dino promoter to study the activity of Dino both in cis and in trans (Fig. 6a). The Dino\(^{lox}\) allele contains ~150 bp of exogenous DNA consisting of Frt and loxP recombination sites and was intended to be used as a conditional knockout. This targeting strategy resulted in a minimal alteration of the Dino locus and did not disturb any known transcription factor binding sites or Cdkn1a exons. Unexpectedly, Dino expression was nearly completely attenuated in Dino\(^{lox}\) MEFs even in the absence of Cre-mediated recombination, and DNA-damage-induced Dino was abrogated to a level similar to that in Trp53\(^{+-}\) MEFs (Fig. 6b). The Dino\(^{lox}\) allele thus serves as a Dino promoter knockout.

Dino\(^{lox}\) MEFs exhibited significant defects in the activation of a subset of DNA damage-inducible genes, including Cdkn1a, Bax, Sfn (encoding 14-3-3σ), and Pmaip1 (encoding Noxa) (Fig. 6c, P < 0.05 for each), despite p53 mRNA being at or above wild-type levels (Supplementary Fig. 7a,b). Furthermore, Cdkn1a\(^{-/-}\) MEFs had no defect in the induction of these p53 target genes, demonstrating that Dino regulates DNA damage-inducible genes independently of p21 (Supplementary Fig. 7c). Both isoforms of Cdkn1a were impaired in Dino\(^{lox}\) MEFs (Supplementary Fig. 7d). Furthermore, DNA-damage-induced expression of Mdm2 exhibited a greater dependence on Dino when MEFs were cultured in 0.1% serum, when proliferative stress is...
diminished compared to standard growth conditions of 10% serum (Supplementary Fig. 7d). We observed less p53 protein in Dino\textsuperscript{lox/lox} MEFs than in Dino\textsuperscript{+/+} MEFs in response to increasing doses of DNA damage (Fig. 6d), indicating that Dino is required for robust accumulation of mouse p53 protein following DNA damage, especially at moderate doses of DNA damage. Similar results were observed in Dino\textsuperscript{lox/lox} MEFs (Supplementary Fig. 7e). This defect in Dino\textsuperscript{lox/lox} cells also translated to a substantial reduction in damage-induced accumulation of p21 protein, as an example of a p53 target. These results are analogous to the findings on the effect of human DINO on human p53 protein abundance was rescued by the proteasome inhibitor MG-132 (Supplementary Fig. 7e, f). Representative population doublings of serially passaged primary Dino\textsuperscript{+/+} and Dino\textsuperscript{lox/lox} MEFs, repeated three times. See also Supplementary Figure 7.

**DISCUSSION**

Our studies have identified the lncRNA DINO as a component of the DNA damage response, providing a feed forward mechanism that amplifies p53 activity in response to DNA damage (Fig. 7). In the absence of DINO expression, p53 protein remains destabilized. However, induction of DINO by DNA damage or by enforced expression of DINO from a heterologous plasmid enhanced p53 protein stability and transactivation of p53 targets. Thus, the feed forward loop of DINO and p53 stabilization may serve as a filter, ensuring that the DNA damage response is activated only after surpassing a threshold of damage. Moreover, these results highlight the concept of a direct physical feedback loop between a transcription factor and its target inducible lncRNA for coordinat-
These findings reinforce the importance of p53 protein dosage in the DNA damage response. Like Trp53+/− cells, Dino knockout cells contain diminished p53 protein levels both at baseline and following DNA damage. While the remaining p53 protein is able to elicit cell cycle arrest and apoptosis following DNA damage, these responses are dampened. The moderate reduction in p53 protein abundance can alter animal survival following lethal irradiation. Furthermore, the observations that Dino knockout mice and cells are phenotypically similar to Trp53+/− mice in the acute DNA damage response raises the intriguing possibility that Dino knockout mice may be tumor prone, like Trp53+/− and epi-allelic p53 hypomorphs.

That DINO’s interaction can functionally regulate p53 protein suggests a possible broader function for RNAs in the regulation of p53 signaling. The maternally imprinted IncRNA MEG3 can also enhance p53 activity, suggesting that multiple IncRNAs can regulate p53. Additionally, the fact that IncRNAs are expressed in a highly tissue- and context-specific manner may provide cells with unique opportunities to confer specificity on otherwise global processes.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Accession codes. GEO: microarray and sequencing data sets are available under accession codes GSE42368 and GSE76420. GenBank: human DINO and mouse Dino sequences are deposited under accession codes JX993265 and JX993266, respectively.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.Y.C. conceived and supervised the study. A.M.S., T.H., and H.Y.C. designed the experiments. A.M.S., J.T.G., T.H., R.A.F., A.Y.P., A.P.-d.-S., and R.B. performed experiments. A.M.S., J.T.G., T.H., R.A.F., Y.S., and K.Q. performed statistical analyses and analyzed the data. D.K.B., S.G., J.K.C., and L.D.A. contributed materials and advice. A.M.S., T.H., and H.Y.C. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Cell culture and treatments. Human cells were obtained from ATCC free of mycoplasma and maintained under standard growth conditions with growth medium formulated according to ATCC recommendations for each cell line. Unless otherwise specified, doxorubicin was used at a concentration of 0.2 µg/mL. Unless specifically noted, the duration of doxorubicin treatment was 12 h for ChIP experiments, 16–26 h for gene expression experiments, and 26 h for cell cycle analyses. For in vitro thymocyte apoptosis analysis, freshly isolated thymocytes were treated with indicated dose of radiation from a 137Cs source and cultured for 6 h before AnnexinV/PI staining. In cycloheximide chase experiments, growth medium was supplemented with 25 µg/mL cycloheximide (Sigma) for the indicated times.

RNA interference. Human fetal lung fibroblasts were transfected with three independent 50 nM ON-TARGETplus siRNAs (Dharmacon) targeting DINO using Lipofectamine 2000 or three independent 100 nM antisense oligonucleotides targeting DINO (Isis Pharmaceuticals). Large-scale transfections were performed using the Amaxa Nucleofector NHDF kit. The siRNA and ASO sequences are provided in Supplementary Table 3.

Gene expression analyses. Total RNA was extracted using TRIZol (Invitrogen) and the RNeasy Mini Kit (Qiagen), and genomic DNA was eliminated using TURBO DNA-free (Ambion). RT-PCR using 50 ng of total RNA was performed using the One-Step RT-PCR MasterMix SYBR Green (Stratagene). RT-qPCR was performed using recombinant p53 (Active Motif) in binding buffer (50 mM Tris-Cl 7.9, 10% glycerol, 100 mM KCl, 5 mM MgCl2, 10 mM β-mercaptoethanol, 0.1% NP-40) for 1 h at 25 °C, then washed five times with binding buffer.

ChIRP and ATAC-seq. ChIRP was performed using biotinylated probes according to previously described methods33 except that cells were crosslinked in 3% formaldehyde + 1 mM EGS (ThermoFisher). See Supplementary Table 3 for probe sequences and qPCR primer pairs. Independent even and odd probe pools were used to ensure DINO-specific retrieval, and RNAses were treated with negative controls. ATAC-seq libraries were generated using 50,000 primary human fibroblasts according to a previously published protocol33 with two technical replicates and two biological replicates per condition.

Animal models. All experiments were performed in accordance with the Stanford Institutional Animal Care and Use Committee. Dino+/− and Dino−/− mice were generated by the Howard Hughes Medical Institute. C57BL/6 ES cells were selected with neomycin after introduction of the targeting construct. Following confirmation of anticipated recombination, the neomycin selection cassette was removed using Flp recombinase before blastocyst injection. E13.5 MEFs were isolated from timed pregnant mice according to standard protocols and genotypes confirmed by PCR.

Total body irradiation (TBI) experiments were performed using a Kimtron model #IC-224 small animal X-ray irradiator. Littermate mice were treated with a single fraction of 5 Gy TBI at 6–8 weeks of age for in vivo thymocyte apoptosis analysis. For survival studies, 9-week-old littermates were treated with a single fraction of 12 Gy TBI and observed twice daily until death. No animals treated with TBI were removed from analysis. Littermate offspring of two heterozygous parents were genotyped and assigned in roughly equal proportions to treatment groups in order to achieve cohorts that were nearly 50% male, 50% female for each group. Investigators were not blinded to the genotype.

DNA methylation analyses. Human and mouse cells were harvested and DNA isolated using the Zymo DNA Miniprep kit (Zymo Research) according to the manufacturer’s instructions. DNA methylation status was analyzed using the Human and Mouse MethylRChip reagent kits (ArrayMesh) on Illumina BeadArrays. The data were analyzed with the BeadChip Expression Console (BEC) software (Illumina) and normalized to the Illumina HumanMethylCel PIP file. DNA methylation status was quantified as z-scores between −3 and 3 (p < 0.05).

ATAC-seq data analysis. ATAC-seq data preprocessing, paired end reads were trimmed for Illumina adaptor sequences and transposase sequences using an in-house script and mapped to hg19 using Bowtie (v0.12.9)35 with parameters –S –x2000 –m1. Duplicate reads were discarded with Samtools (v0.1.8)36. Peak calling using ZINBA was as described37. Chromosomal regions with a posterior probability of >0.99 were identified as peaks. For analysis of the ATAC-seq signal intensity around p53 binding sites, we used p53 ChIP-seq data downloaded from GEO accession code GSE46641 and CTCF ChIP-seq signal intensity around p53 binding sites for all cell lines, the average fragment count plotted around p53 binding sites for all cell lines, the average fragment count plotted around p53 binding sites for all cell lines, the average fragment count plotted around p53 binding sites for all cell lines, the average fragment count plotted around p53 binding sites for all cell lines, the average fragment count plotted around p53 binding sites for all cell lines, the average fragment count plotted around p53 binding sites for all cell lines, the average fragment count plotted around p53 binding sites for all cell lines, the average fragment count plotted around p53 binding sites for all cell lines, the average fragment count plotted.
of ATAC-seq tags in the peak summit was compared in four different cell lines by t-test. The heat maps of ATAC-seq at all the p53 motifs were generated using Java TreeView 3.0.

nCounter assay. nCounter assays using the p53 Virtual Pathway Gene Set (Nanostring) were performed using isolated RNAs according to manufacturer's instructions. Data were normalized using the geometric mean of the six internal references.

Statistical analyses. A two-tailed Student's t-test was used for analysis of statistical significance, with a P < 0.05 considered significant. For mouse survival studies, the log-rank test was used. We determined that a sample size of at least 7 animals per genotype was required to observe a 2-d increase in time to event (death) between genotypes following lethal irradiation with a power of 0.9.

DINO cloning and sequence analysis. DINO was initially identified using 5-bp-resolution high density tiling arrays covering the promoters of CDKN1A and multiple other genes. 3' and 5' RACE was performed using the FirstChoice RLM–RACE Kit (Ambion) for human DINO and GeneRacer (Life Technologies) for mouse DINO. RNA was extracted from 0.2 µg/ml doxorubicin (Sigma)-treated human fetal lung fibroblasts or MEFs, poly(A)-selected using the Poly(A)Purist MAG kit (Ambion), and RACE was performed according to the standard manufacturer’s protocol.

DINO evolutionary conservation analyses and statistics. DINO syntenic sites and regions of microhomology were identified as previously described. Briefly, the iterative process was as follows: (i) begin with empirically defined, strand-specific sequences for human, mouse, and zebrafish DINO, followed by (ii) a search for putative DINO orthologs in marmoset, rat, rabbit, elephant, and horse using synteny and conserved motifs identified by MEME, then (iii) trim and reorient putative DINO orthologs based on the strand of conserved motifs and rerun MEME.

MEME reports an estimate of the statistical significance of each motif it finds, the “motif E-value,” as well as an estimate of how well each occurrence, “site P-value,” matches the motif. The motif E-value is an estimate of how likely it is that the motif is not just a statistical artifact. The site P-values should only be used as relative indications of how well each site matches the motif. MEME uses an algorithm called expectation maximization to find short patterns of nucleic acids or amino acids that occur more frequently in the input sequences than would be expected by chance. Because these patterns need not be exact matches, they are described using a matrix, the position-specific score matrix (PSSM). MEME has generated the PSSM, it uses a second algorithm, MAST, to find the best matches to that PSSM in the input data. The P-value tells how well a particular site matches the PSSM found by MEME. The smaller the P-value, the more significant the match.

DINO overexpression. For overexpression assays, the Lipofectamine 2000 (Invitrogen), Fugene 6 reagent (Roche) or Cell Line Nucleofector Kit V Kit (Ambaxa) was used according to the manufacturer’s protocol. The following plasmids were used: pcDNA-human DINO, pcDNA-human DINO320–450, pcDNA-human DINO323–405, pcDNA-mouse DINO, pcDNA, pcDNA-Flag-p53 (Addgene #10838), pcDNA-Flag-p53 R273H, and C terminus truncation pcDNA-Flag-p53 1–362 (AC31).

Antibodies. The following antibodies were used for p53 analysis: DO-1 (Sigma, catalog #P6874), 1C12 (Cell Signaling Technologies, catalog #2524). Other antibodies for immunoblot analysis: anti-β-tubulin (Abcam, catalog #ab6046), anti-H3 (catalog #ab1791), anti-LSD1 (Abcam, catalog #ab17721), anti-SUZ12 (Abcam, catalog #ab12073), anti-p21 (Santa Cruz Biotech C-19, catalog #sc-397), and β-actin (Abcam, catalog #ab8227).

RNA interference. Human fetal lung fibroblasts were transfected with three independent 50 nM ON-TARGETPlus siRNAs (Dharmacon) or 100 nM ASOs targeting DINO using the Lipofectamine 2000 reagent. Large scale transfections were performed using the Amaxa Nucleofector NHDF kit. siRNAs for mRNAs (Ambion) were used as a pool of two. U2OS cells were infected with shRNAs targeting DINO using the pGIPZ shRNAmir lentiviral system.

Cycloheximide chase. Cells were reverse transfected (Lipofectamine 2000, Invitrogen) for 24 h, then medium was supplemented with 25 µg/ml cycloheximide (Sigma). At the indicated times after cycloheximide addition, cells were collected for immunoblot analysis, snap frozen overnight, lysed 30 min on ice in NET buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P40, freshly added 1 mM phenylmethylsulfonyl fluoride, protease inhibitors), sonicated with a Bioruptor (Diagenode) for 5 min (30-s ‘on' and 30-s ‘off’), then centrifuged for 15 min at 16,000g. Lysates were normalized by BCA and resolved by SDS–PAGE.

SHAPE. SHAPE analysis was performed as described. NMIA (13 mM final concentration) or DMSO was used for modification or mock modification reactions, respectively. ddGTP and ddTTP were used in two separate sequencing reactions. cDNA extensions were visualized by phosphorimaging (STORM, Molecular Dynamics). cDNA bands were integrated with SAFA. SHAPE reactivities were normalized to a scale spanning 0 to 1.5, where 1.0 is defined as the mean intensity of highly reactive nucleotides. RNA secondary structures were predicted using RNA structure software. SHAPE data and RNA structure data were reconciled using SeqFOLD.

RNA blotting. RNA blotting was performed using the NorthernMax kit (Ambion) with 2.5–5 µg of poly(A) selected RNA for each sample. Antisense RNA probes to human DINO nucleotides 203–716 and mouse DINO nucleotides 291–842 were synthesized using T7 MegaScript (Ambion) with incorporation of [α-32P]UTP.

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