

NIR is a novel INHAT repressor that modulates the transcriptional activity of p53

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Most transcriptional repression pathways depend on the targeted deacetylation of histone tails. In this report, we characterize NIR, a novel transcriptional corepressor with inhibitor of histone acetyltransferase (INHAT) activity. NIR (Novel INHAT Repressor) is ubiquitously expressed throughout embryonic development and adulthood. NIR is a potent transcriptional corepressor that is not blocked by histone deacetylase inhibitors and is capable of silencing both basal and activator-driven transcription. NIR directly binds to nucleosomes and core histones and prevents acetylation by histone acetyltransferases, thus acting as a bona fide INHAT. Using a tandem affinity purification approach, we identified the tumor suppressor p53 as a NIR-interacting partner. Association of p53 and NIR was verified in vitro and in vivo. Upon recruitment by p53, NIR represses transcription of both p53-dependent reporters and endogenous target genes. Knock-down of NIR by RNA interference significantly enhances histone acetylation at p53-regulated promoters. Moreover, p53-dependent apoptosis is robustly increased upon depletion of NIR. In summary, our findings describe NIR as a novel INHAT that plays an important role in the control of p53 function.

[*Keywords:* INHAT; transcription; HDAC-independent repression; p53]

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There is growing evidence that major aspects of transcriptional regulation can be explained by the histone code model (Strahl and Allis 2000). The covalent modification of the N termini of core histones by transfer and removal of acetyl, methyl, or phospho groups determines whether a candidate gene is expressed or remains in a silenced state. A major mechanistic principle in transcriptional activation has been unraveled by the discovery of basal and recruited transcriptional coactivators that contain histone acetyltransferase (HAT) activity (Mizzen et al. 1996; Ogryzko et al. 1996). Transfer of acetyl groups to lysine residues in histone tails weakens the tight contact between DNA and nucleosomes, and

decompacted chromatin is thus rendered accessible to the action of the RNA-polymerase II holoenzyme complex (Glass and Rosenfeld 2000). Consequently, the return to silenced chromatin or the specific repression of target genes requires enzymatic activities that counteract the action of coactivators. Therefore, a commonly used mechanism for short-term repression involves the enzymatic action of histone deacetylating enzymes (HDACs). Most repressors have been reported to recruit diverse HDAC-containing complexes (Jepsen and Rosenfeld 2002). HDACs are grouped into three classes based on their similarity to known yeast factors, structural features, and their subcellular localization (Verdin et al. 2003). Class I and class II HDACs are sensitive to inhibitors such as Trichostatin A (TSA) or Na-butyrate, whereas class III HDACs are blocked by nicotinamide. In contrast to the wealth of information on HDAC-dependent repression pathways, there are only a few studies that describe HDAC-independent repression (Deltour et

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al. 1999; Sun and Taneja 2000; Koipally and Georgopoulos 2002; Christian et al. 2004).

A multiprotein complex has been described that inhibits the HAT activity of the major histone acetyltransferases p300/CBP and p/CAF on histone substrates (Seo et al. 2001). The active moiety of this complex, the Set/TAF1 β oncoprotein, binds to histone tails and blocks the access of HATs to their histone substrates. This complex and its novel activity were referred to as INHAT, inhibitor of histone acetyltransferases. Subsequently, another protein of the Set INHAT complex named pp32 (Seo et al. 2002), the polyglutamine-tract-containing protein Ataxin-3 (Li et al. 2002), and the corepressor PELP1 (Choi et al. 2004) were shown to contain INHAT activity. Set/TAF1 β and pp32 preferably bind to hypoacetylated histones, while acetylation of histones H3 and H4 abrogates binding of INHAT subunits (Kutney et al. 2004; Schneider et al. 2004). The INHAT subunits Set/TAF1 β and pp32 associate with HDAC activity, suggesting a more global function of the previously characterized INHATs by linking targeted deacetylation and maintenance of the unmodified status (Kutney et al. 2004).

p53 is a tumor suppressor that is activated in response to various stress signals. p53 function is mediated largely through the action as a DNA-binding transcription factor that recruits transcriptional cofactors to regulate genes that control cell cycle, cell growth, or apoptosis (Vogelstein et al. 2000). Recent studies demonstrated p53-dependent recruitment of p300 and targeted acetylation of histones at p53-responsive genes (Espinosa and Emerson 2001; Liu et al. 2003; Espinosa et al. 2003). Nevertheless, to date little is known about transcriptional corepressors that counteract p53-mediated transcriptional activation (Allison and Milner 2004).

In this work we characterize the novel corepressor NIR (Novel INHAT Repressor). NIR binds to nucleosomes and histones and blocks their acetylation by histone acetyltransferases, thus acting as a bona fide INHAT. We identify the tumor suppressor p53 as a NIR-interacting partner and demonstrate that, upon recruitment, NIR inhibits p53-activated gene expression and regulates p53-mediated apoptosis. Thus, NIR is the first INHAT repressor that plays an important role in the control of p53 function.

Results

Cloning of NIR

In order to define new pathways in transcriptional regulation, we searched for novel INHAT repressors. We applied low-stringency iterative BLAST analyses to screen databases for proteins with putative INHAT regions. By this *in silico* approach, we identified a cDNA coding for an unknown protein that we named NIR. The human full-length NIR cDNA was obtained from a prostate cDNA library by PCR. Sequence analysis confirmed that the NIR cDNA corresponds to the deposited sequence with the accession number BC003555. The human NIR locus codes for a 749-amino-acid protein (Fig. 1) with orthologs present from worms to mammals (Supplemen-

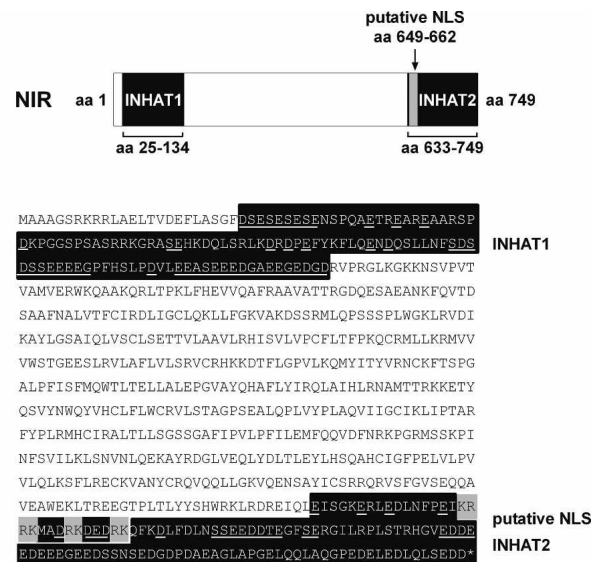


Figure 1. Schematic representation and sequence of human NIR. (Top) Schematic representation of the human NIR protein. (Bottom) Amino acid sequence and functional motifs in NIR. INHAT regions are shaded in black. Set/TAF1 β homology regions are underlined white. The putative bipartite nuclear localization signal (NLS) is in gray.

tary Fig. 1). The cDNA of the murine ortholog was also cloned, and sequence analyses verified the deposited partial sequence (accession no. AF155546) (Supplementary Fig. 2). Bioinformatic analyses suggested that NIR harbors two INHAT regions, one at its N terminus and one at its C terminus. A homology alignment of NIR with the INHAT domains of published human INHAT proteins is displayed in Supplementary Figure 3. Additionally, NIR contains a putative bipartite nuclear localization signal (NLS).

NIR is ubiquitously expressed

As shown by RT-PCR analyses, *Nir* mRNA was present at all analyzed stages of mouse embryogenesis (Fig. 2A). Consistent with the mRNA expression profile, Nir protein was detected at all murine embryonic stages analyzed. A representative immunohistochemical analysis at embryonic day 9.5 (E9.5) shows ubiquitous Nir protein expression (Fig. 2B). RT-PCR analyses detected mouse *Nir* mRNA to varying extents in all adult tissues examined (Fig. 2C), and in accordance with this finding, mRNA dot blot assays showed expression of human NIR mRNA in all tissues tested (Fig. 2D). Northern blot analyses revealed the presence of a single NIR transcript of 3.3 kb (Supplementary Fig. 4). In summary, NIR is ubiquitously expressed during embryonic development and in adult tissues.

NIR is an inhibitor of histone acetylation

Since NIR contains two putative INHAT regions, we assayed for a direct interaction of NIR with core histones and nucleosomes. In GST-pulldown experiments, we

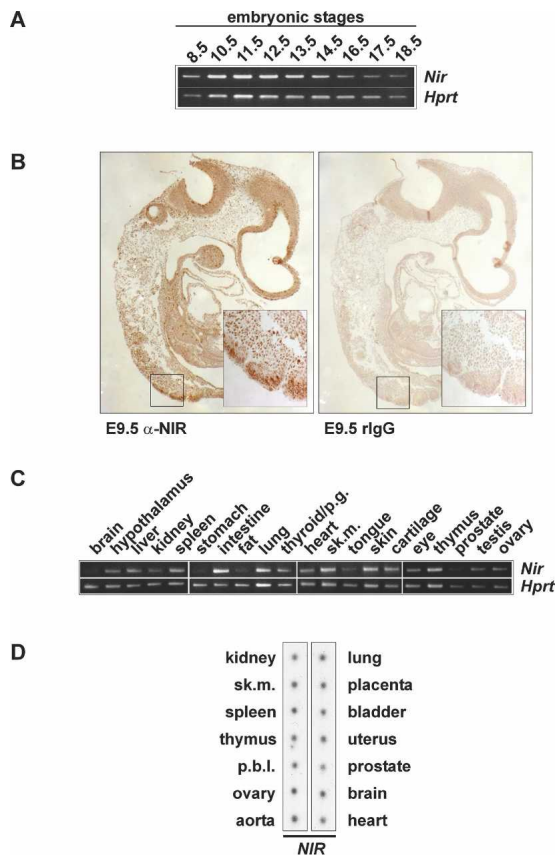


Figure 2. NIR expression analyses. (A) Expression of *Nir* during mouse embryogenesis. RT-PCR analyses were performed at the indicated stages from E8.5 to E18.5. (B, left) Expression of *Nir* in sagittal sections of E9.5 mice. *Nir* is expressed throughout the embryo. Higher magnification exemplifies *Nir* expression in the somites. The right panel displays staining with a control antibody. (C) *Nir* expression in tissues of adult mice as determined by RT-PCR. (D) Expression of *NIR* in human adult tissues. mRNA dot-blot hybridization shows that *NIR* is ubiquitously expressed in human adult tissues. (p.b.l.) Peripheral blood lymphocytes, (sk.m.) skeletal muscle, (thyroid/p.g.) thyroid and parathyroid gland.

demonstrated physical interaction of both the GST-tagged N and C termini (INHAT1 and INHAT2, respectively) with calf thymus core histones and nucleosomes isolated from HeLa cells (Fig. 3A). The unrelated control protein GST-Nix1 (Greiner et al. 2000) did not interact, thus demonstrating specific interaction of both INHAT regions. Sepharose-pulldown assays showed direct interaction between NIR and the unmodified N-terminal tail of histone H3 comprising amino acids 1–30 (Fig. 3B). Similarly to the previously described INHAT subunits (Kutney et al. 2004; Schneider et al. 2004), acetylation of the histone H3 tail (combinations of acetylated K9, K14, K18, and K23) blocked the association with NIR.

Subsequently, we tested the ability of NIR to directly block acetylation of core histones. Both putative NIR INHAT regions were expressed as His-tagged proteins and then individually preincubated with each of the four core histones. After addition of enzymatically active

p300 HAT-domain and [14 C]-labeled acetyl-CoA substrate, histone acetylation was analyzed by autoradiography (Fig. 3C, left panel). Addition of either of the putative NIR INHAT regions robustly reduced acetylation of all core histones by the histone acetyltransferase p300. The unrelated control protein Nix1 did not alter acetylation by p300, thus corroborating specific action of both INHAT domains. Coomassie staining revealed equal amounts of His-tagged proteins and excluded degradation of the core histones (Fig. 3C, right panel). In the same assay system, both NIR INHAT domains also blocked acetylation of core histones H3 and H4 by p/CAF (Supplementary Fig. 5).

Since neither full-length NIR nor NIR Δ INHAT1/2, which lacks both INHAT domains, could be recombinantly expressed in *Escherichia coli*, we affinity-purified full-length TAP-tagged NIR (NIR-TAP), NIR INHAT1-TAP, NIR INHAT2-TAP, and NIR Δ INHAT1/2-TAP from 293 cells. NIR-TAP as well as NIR INHAT1-TAP and NIR INHAT2-TAP associated efficiently with nucleosomes, whereas the central part of NIR (NIR Δ INHAT1/2-TAP) failed to do so (Fig. 3D, middle panel). Consequently, TAP-tagged NIR, NIR INHAT1, and NIR INHAT2 significantly blocked acetylation of core histones and nucleosomes by p300 in INHAT assays (Fig. 3D, bottom panel). In contrast, NIR Δ INHAT1/2-TAP did not inhibit acetylation, thus serving as an internal control. Ponceau S staining revealed equal amounts of nucleosomes in all reactions, excluding degradation of the substrate by the purified NIR proteins (Fig. 3D, bottom panel). In summary, these data demonstrate that NIR contains two independent INHAT domains and establish NIR as a novel functional INHAT.

NIR is an HDAC-independent transcriptional repressor

To analyze the effects of NIR on transcription, a Gal4 DNA-binding domain NIR fusion protein was tested in transient transfections. Gal-NIR potently repressed both minimal and complex Gal-dependent promoters in a dose-dependent manner in all cell lines tested (Fig. 4A). To verify whether NIR is able to repress activator-driven transcription, we performed experiments with a combined LexA/Gal-reporter (Lemerrier et al. 2000). LexA-VP16-mediated activation was severely inhibited by cotransfection of Gal-NIR, whereas LexA-VP16 activation was not significantly impeded by cotransfection of the unrelated cofactor Gal-Nix1 (Fig. 4B, upper panel). The potent repressive effect of NIR was also observed in the reverse set-up in which LexA-NIR suppressed activation by the transcriptional activator Gal-CREB as well as the transcriptional coactivator Gal-TIF2 (Fig. 4B, lower panels). In summary, these data demonstrate that promoter-recruited NIR is able to repress basal as well as activated transcription in a cell-line- and promoter-independent manner.

Next, we addressed a potential effect of HDACs in NIR-mediated repression. Addition of the class I and class II HDAC inhibitors TSA or Na-butyrate, or the inhibitor of NAD $^{+}$ -dependent class III deacetylases, nico-

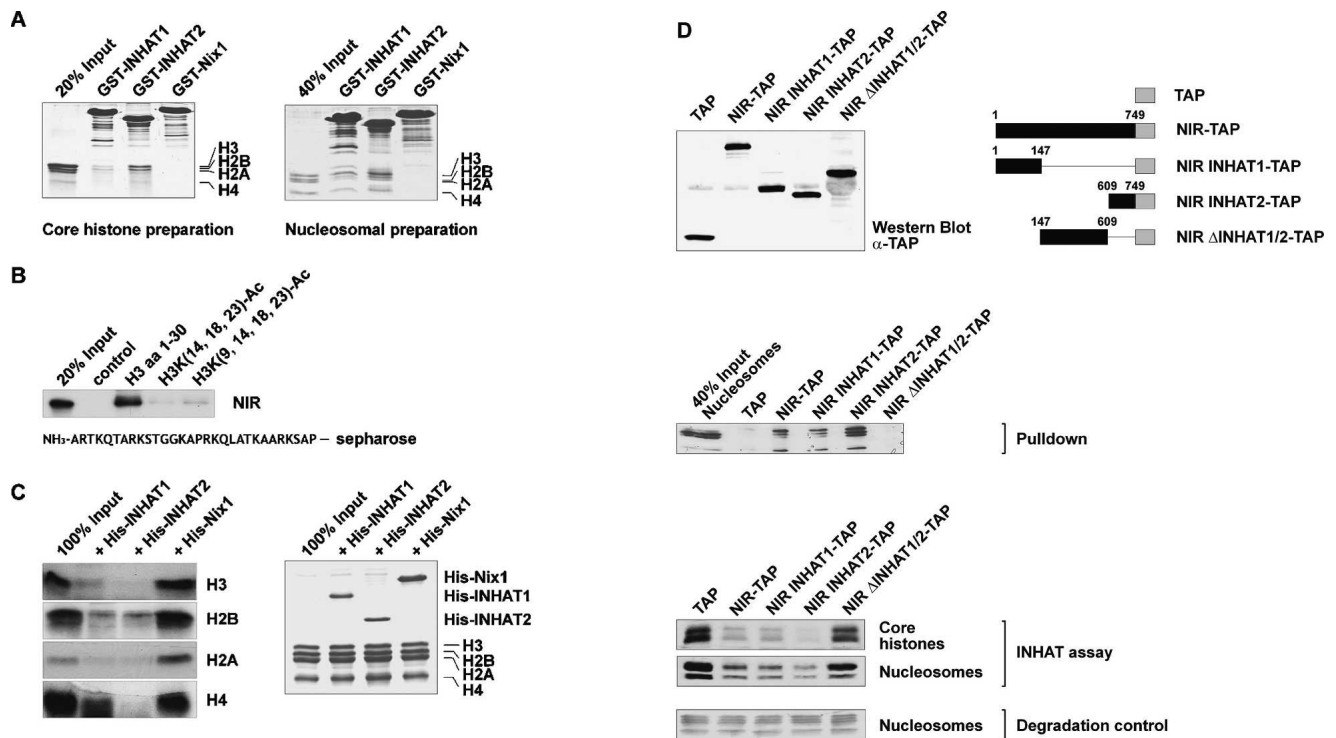


Figure 3. NIR is an inhibitor of histone acetylation. (A) NIR interacts with core histones and nucleosomes in vitro. Bacterially expressed GST-NIR INHAT domains interact with core histones (*left panel*) and nucleosomes (*right panel*). The unrelated protein Nix1 serves as a control. Proteins are visualized by Coomassie staining. (B) [³⁵S]methionine-labeled in vitro translated NIR interacts with nonacetylated Sepharose-coupled peptides corresponding to the N-terminal tail of histone H3 (amino acids 1–30). Acetylation at K9, K14, K18, or K23 no longer permits binding of NIR. Interaction of radioactively labeled NIR with histone tails is visualized by autoradiography. (C) NIR is a functional INHAT. (*Left panel*) Bacterially expressed NIR INHAT regions prevent acetylation of all core histones by the p300 histone acetyltransferase in vitro. To demonstrate specificity, the unrelated protein Nix1 is used as a control. INHAT activity is visualized by autoradiography. (*Right panel*) The Coomassie stain verifies the use of equal amounts of His-tagged proteins and absence of protease contaminations that could potentially degrade histones. (D) Full-length NIR-TAP and the indicated deletion mutants were tested for association with nucleosomes and INHAT activity. (*Upper panel*) Equal amounts of TAP fusion proteins are visualized by Western blotting. (*Middle panel*) Pulldown of NIR-TAP proteins with nucleosomes. The *lower panel* displays the INHAT assays using either core histones or nucleosomes as substrates and full-length NIR-TAP or the indicated mutants. Full-length NIR and both INHAT-domains block acetylation by the p300. INHAT activity is visualized by autoradiography. Ponceau S staining verifies the presence of equal amounts of nucleosomes and excludes protease contamination of affinity-purified NIR proteins.

tinamide, did not block NIR-mediated repression (Fig. 4C). The increase in fold repression by Gal-NIR upon addition of TSA or Na-butyrate results from the fact that both inhibitors significantly increase basal transcription levels of Gal-DBD (that harbors a weak minimal transactivation function), whereas repression by Gal-NIR was not influenced. The functionality of the HDAC inhibitors in our assay system was verified (Supplementary Fig. 6). Importantly, immunopurified NIR did not display detectable activity in HDAC assays (Fig. 4D). Moreover, NIR does not associate with HDACs or diverse members of different corepressor families (Supplementary Fig. 7). These results reveal that the novel INHAT NIR, in contrast to PELP1 and Set/TAF1 β , functions independently of HDAC activity.

NIR is a novel interaction partner of p53

To understand the biological function of NIR, we isolated NIR-associated proteins by tandem affinity purifi-

cation from 293 cells. NIR-associated proteins were analyzed by MALDI-TOF/TOF. Among other proteins, we identified the tumor suppressor p53 as a NIR-interacting transcription factor (Supplementary Fig. 9). To visualize the cellular localization of both proteins, we performed immunofluorescence analyses in cells that were treated with the DNA-damaging agent doxorubicin to increase endogenous p53 protein levels. It is important to note that under these conditions NIR protein levels were not detectably altered (Supplementary Fig. 10). Confocal laser scanning microscopy revealed colocalization of endogenous p53 and NIR in the nucleus (Fig. 5A). NIR and p53 directly interacted in GST-pulldown assays in vitro (Fig. 5B). The p53-interaction domain localizes to the central part of NIR, spanning amino acids 147–609. The NIR-interaction domain in p53 comprises the DNA-binding domain (amino acids 102–292) and the C-terminal tetramerization domain (amino acids 293–359). To demonstrate association between p53 and NIR in vivo,

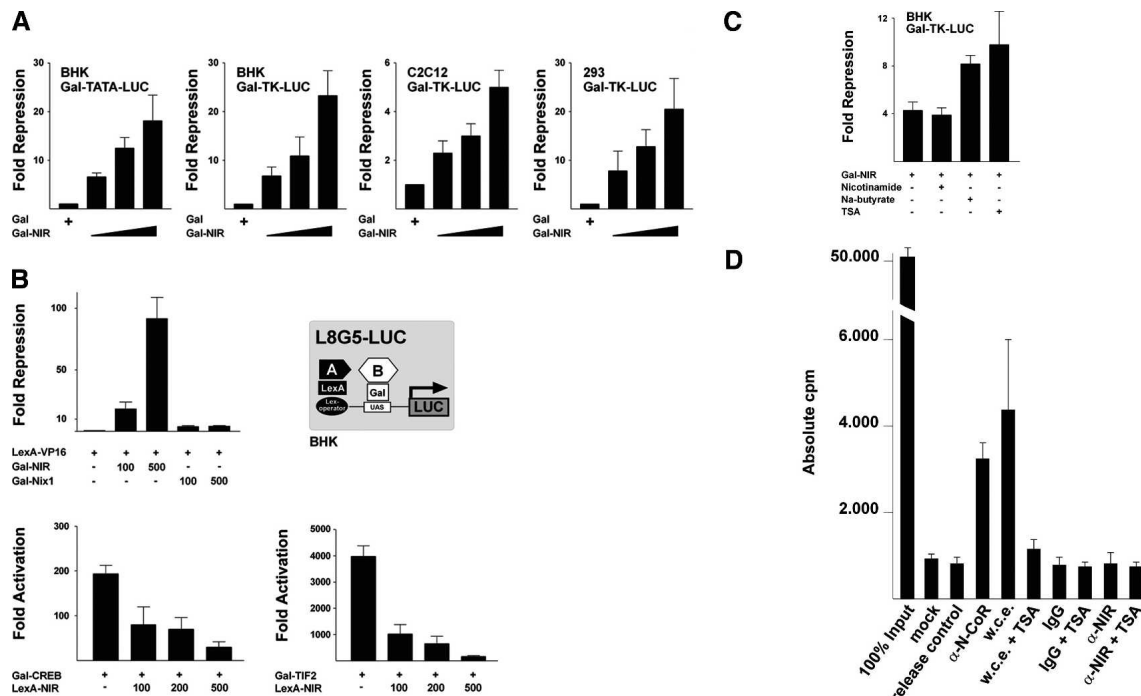


Figure 4. NIR is a potent HDAC-independent transcriptional repressor. (A) Gal-NIR represses transcription of synthetic minimal or complex Gal-dependent reporters in a dose-dependent manner in various cell lines. In transient transfection assays, 50, 100, or 500 ng of Gal-NIR expression plasmid were used. As a control, the corresponding amounts of Gal4-DBD (Gal) were transfected. (B) NIR represses activated transcription. BHK cells were cotransfected with 500 ng of L8G5-LUC reporter and 100 ng of LexA-VP16, Gal-CREB, or Gal-TIF2 expression plasmids. Fold activation is calculated in relation to the corresponding amounts of Gal4-DBD. (C) NIR repression is not influenced by HDAC inhibitors. BHK cells were cotransfected with 500 ng of Gal-TK-LUC reporter and 50 ng of Gal-NIR expression plasmid. Na-butyrate (5 mM), nicotinamide (10 mM), and TSA (330 nM) were applied for 24 h post-transfection. Fold repression is calculated in relation to the corresponding amounts of Gal4-DBD. (D) NIR is not associated with HDAC activity. NIR and associated complexes were immunopurified from 293 cells with an α -NIR (2719) antibody. Release of radiolabeled acetyl groups from H3 peptides is observed only when using whole-cell extract (w.c.e.) or purified N-CoR-associated complexes. The amount of released radiolabeled acetyl groups (measured by β -counting) in NIR immunoprecipitates corresponds to the release control. Specificity of the reactions is demonstrated by using the HDAC-inhibitor TSA. Bars represent mean \pm SD ($n \geq 6$).

extracts from BHK cells transfected with expression plasmids for myc-p53 and NIR-TAP were immunoprecipitated using an α -TAP antibody. Western blot analyses showed that p53 is specifically coprecipitated in the presence of NIR-TAP (Fig. 5C). Identical results were obtained using another cell line and performing the immunoprecipitations with α -p53 and α -myc antibodies (Supplementary Fig. 11). Importantly, we verified association of p53 and NIR endogenously expressed in 293 cells or wild-type p53-containing HCT116/p53^{+/+} cells (untreated or treated with doxorubicin for 6 h) (Fig. 5D), thus demonstrating interaction *in vivo*. In summary, the data identify NIR as the first p53-interacting INHAT.

NIR modulates p53 function

To address the relevance of the interaction between p53 and NIR, we analyzed the consequence of NIR expression on p53 target genes. In transient transfection experiments performed in the p53-negative cell lines Saos-2 and HCT116/p53^{-/-}, NIR suppressed, in a dose-depen-

dent manner, the activation of the synthetic minimal PG13-reporter mediated by exogenous p53 (Fig. 6A). The control reporter MG15 that contains mutated p53-binding sites was unaffected by transfection of either p53 or NIR, thus demonstrating specificity. Moreover, p53-mediated activation of the *p21*, *PIG3*, and *Noxa* promoters was inhibited by cotransfection of NIR. The equivalent promoters containing mutated p53-binding sites did not respond significantly to p53 or NIR (Supplementary Fig. 12). In addition, NIR failed to block activity of other transcription factors such as CBF1, androgen receptor (AR), or thyroid hormone receptor (TR), further confirming specificity (Supplementary Fig. 13). RT-PCR analyses showed that exogenous NIR significantly down-regulated doxorubicin-induced expression of endogenous p53 target genes such as *NOXA*, *p21*, and *PIG3* in HCT116/p53^{+/+} cells (Fig. 6B). In contrast, transfection of NIR in HCT116/p53^{-/-} cells yielded no detectable change in p53 target gene expression.

To determine whether p53 recruits NIR to chromatin *in vivo*, HCT116/p53^{-/-} cells, which express NIR endog-

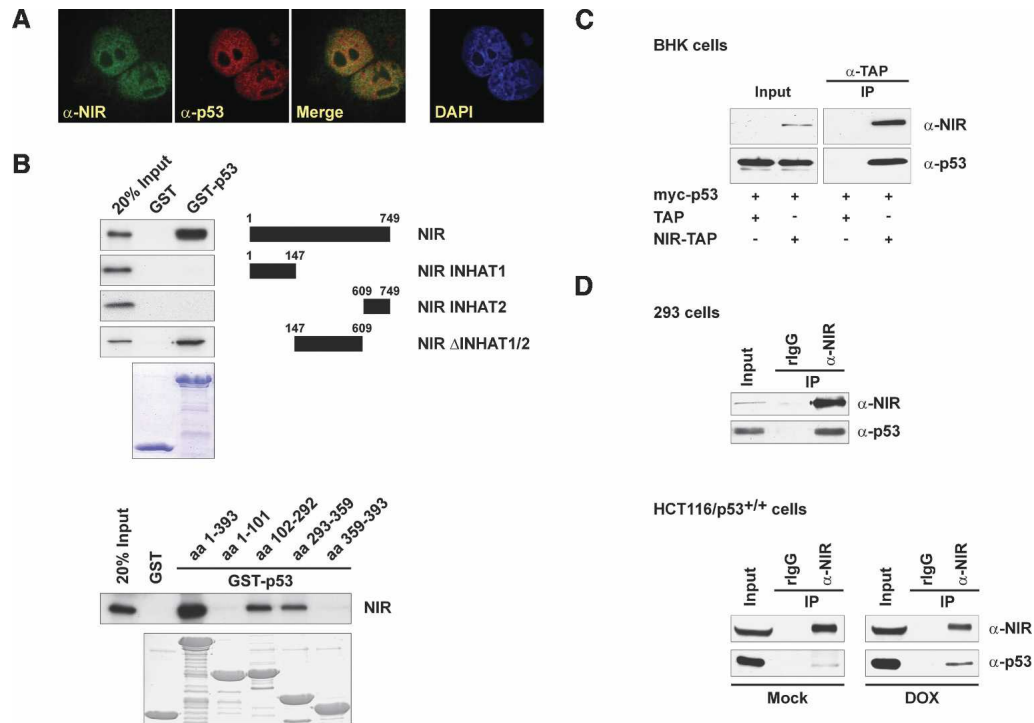


Figure 5. NIR interacts with p53 in vitro and in vivo. (A) NIR is a nuclear protein and colocalizes with p53 in vivo in BHK cells. NIR immunoreactivity is displayed in green, p53 in red. Nuclei were stained with DAPI (blue). For clarity, the merge image displays only NIR and p53 immunoreactivity. (B) p53 and NIR interact in vitro. Interaction domains were mapped in GST-pull-down assays using GST-p53, GST-p53 deletion mutants, and [³⁵S]methionine-labeled in vitro translated NIR or mutants thereof. The use of equal amounts of GST fusion proteins is demonstrated by Coomassie staining. (C) Coimmunoprecipitation of TAP-tagged NIR and myc-tagged p53 in BHK cells. Membranes were decorated with the α -NIR 2910 or the α -p53 DO-1 antibody. Five percent of whole-cell extract was used as input. (D) Endogenous p53 coimmunoprecipitates with NIR. Whole-cell extracts from 293 cells and nuclear extracts from HCT116/p53^{+/+} cells mock-treated or treated with doxorubicin (DOX) were immunoprecipitated with the α -NIR 2910 antibody. Membranes were decorated with α -NIR (2910) or α -p53 (DO-1) antibody. We used 2.5% and 25% of whole-cell extract as inputs for p53 and NIR, respectively.

enously, were transfected with a p53 expression construct and subjected to chromatin immunoprecipitation (ChIP). Genomic DNA corresponding to the p53 response elements located in the promoters of the *p21* and *PIG3* genes was immunoprecipitated with either α -NIR or α -p53 antibodies only in the cells that expressed exogenous p53 (Fig. 6C). Association of NIR and p53 with the *p21* and *PIG3* promoters is specific, since genomic DNA from the promoters of the *U6* and *GAPDH* genes was not enriched. Next, we verified that NIR and p53 endogenously expressed in HCT116/p53^{+/+} cells specifically associate with the *p21* promoter (Fig. 6D, left panel). In contrast, NIR failed to do so in HCT116/p53^{-/-} cells (Fig. 6D, right panel), further demonstrating that NIR associates with the *p21* promoter in a p53-dependent manner. Indeed, a sequential ChIP (Re-ChIP), first with an α -NIR antibody followed by either α -p53 antibody or control mouse IgG, demonstrated that NIR and p53 form a complex on chromatin (Fig. 6E, upper panel). Cellular stress, induced by treatment with doxorubicin, increased the occupancy of the *p21* promoter by p53, but did not alter its occupancy by NIR (Fig. 6E, bottom panel).

To study the effects of endogenous NIR on p53 func-

tion, HCT116/p53^{+/+} cells with an intact p53 response pathway were transiently transfected with siRNAs directed against NIR. In the absence of cellular stress, NIR knock-down resulted in a significant increase in endogenous p21 protein and mRNA expression in p53-proficient but not in p53-deficient HCT116 cells, thus demonstrating that the observed enhancement of p21 expression is indeed p53 dependent (Fig. 6F). The p53 protein level was unaffected, indicating increased transcriptional activity of p53 in response to down-regulation of NIR in the absence of cellular stress. Doxorubicin treatment led to maximal stimulation of p21 expression, which was not further elevated by NIR knock-down (Fig. 6F). Similar results were obtained using a second, independent NIR siRNA (Supplementary Fig. 14). Expression of the proapoptotic p53 target genes *BAX*, *PIG3*, and *NOXA* is not affected by depletion of NIR (Supplementary Fig. 15).

Since NIR is a transcriptional INHAT repressor, we analyzed the acetylation status in the vicinity of p53-binding sites following siRNA-mediated depletion of NIR in HCT116/p53^{+/+} and HCT116/p53^{-/-} cells. In agreement with NIR functioning as an INHAT, ChIP experiments revealed a significant increase in H3/H4

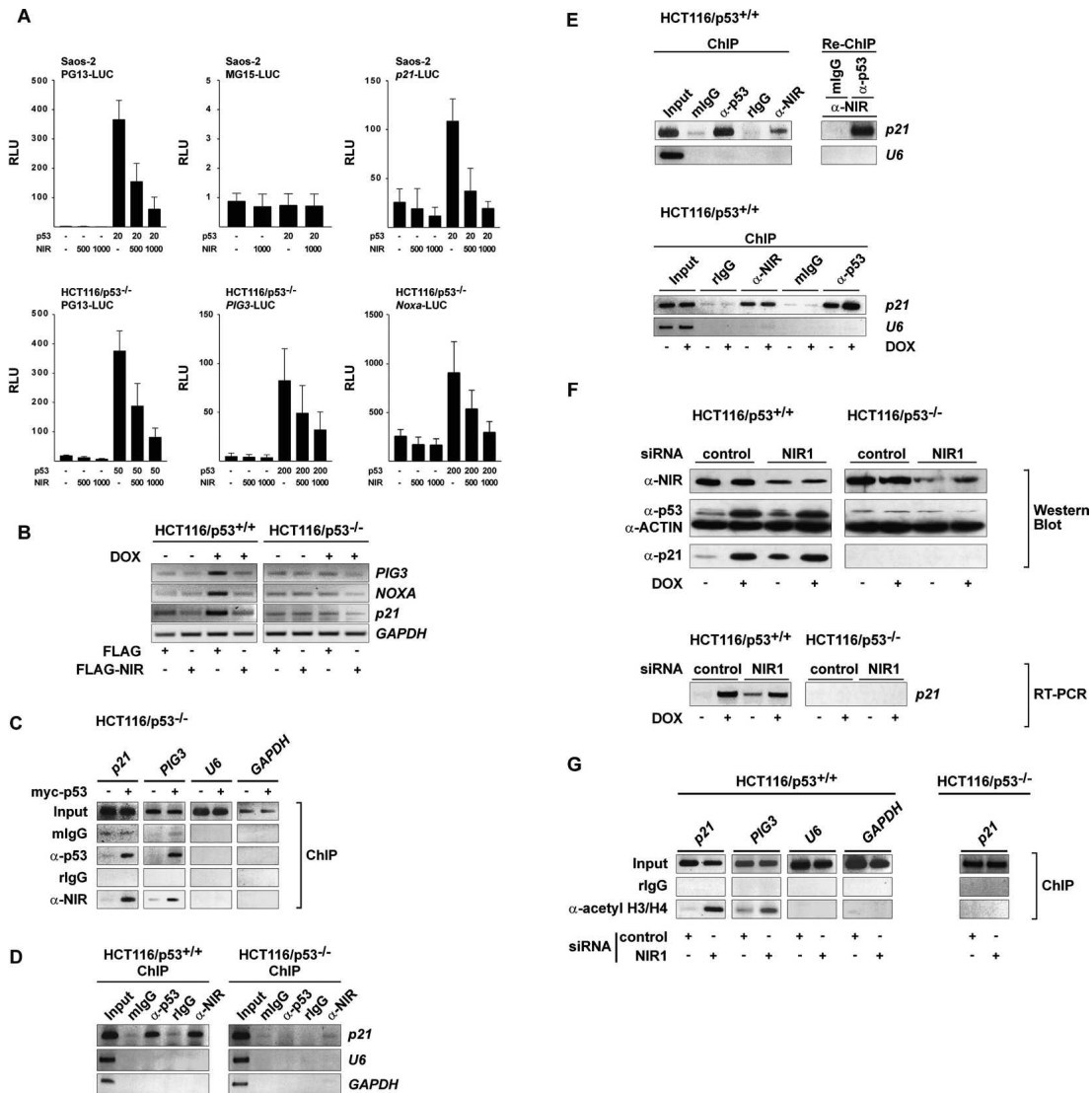


Figure 6. NIR regulates p53 function. (A) NIR represses p53 transcriptional activity. Transient transfections were carried out in the p53-negative cell lines Saos-2 and HCT116/p53^{-/-}. NIR blocks p53-induced expression of the synthetic p53-responsive reporter PG13, whereas the control reporter with mutated p53-binding sites (MG15) is not influenced by either p53 or NIR. NIR represses activation of the known p53 target genes *p21*, *PIG3*, and *Noxa*. (RLU) Relative light units. Bars represent mean \pm SD ($n \geq 6$). (B) NIR mediates repression of endogenous p53 target genes. Mock-treated or doxorubicin (DOX)-stressed HCT116/p53^{+/+} and HCT116/p53^{-/-} cells were transfected with either a Flag-control plasmid or with a plasmid coding for Flag-NIR. RT-PCR analyses were performed with primers amplifying the p53 target genes *PIG3*, *NOXA*, and *p21*. Amplification of the housekeeping gene *GAPDH* serves as a control. (C) NIR and p53 are present at endogenous p53-regulated promoters. ChIP assays show that NIR is localized at p53 target promoters in HCT116/p53^{-/-} cells only in the presence of transfected p53. The p53 target genes *p21* and *PIG3* are precipitated using α -NIR (2910) or α -p53 (DO-7) antibody. Unrelated promoters (*U6* and *GAPDH*) are not enriched. (D) Endogenously expressed NIR and p53 are present at p53-regulated promoters in HCT116/p53^{+/+} cells. In ChIP assays the *p21* promoter is precipitated using antibodies directed against either NIR (2910) or p53 (DO-7). The unrelated *U6* and *GAPDH* promoters are not enriched. (E, upper panel) Re-ChIP confirms that NIR and p53 form a complex on the endogenous *p21* promoter. First ChIP was performed using the α -NIR 2910 antibody. Re-ChIP was performed using either mIgG or the α -p53 DO-1 antibody. The unrelated *U6* promoter is not precipitated by the antibodies. (Lower panel) NIR and p53 are present at the endogenous *p21* promoter in HCT116/p53^{+/+} cells in the absence (-) or presence (+) of doxorubicin-induced cellular stress. (F) siRNA-mediated NIR knock-down correlates with increased p21 protein and RNA levels in unstressed HCT116/p53^{+/+} cells. Western blotting and RT-PCR analyses were performed at day 3 post-transfection with siRNA. ACTIN serves as a control for equal protein loading. (G) NIR acts as a p53-recruited INHAT in vivo. Knock-down of NIR by siRNA (NIR1) in HCT116/p53^{+/+} cells leads to histone hyperacetylation at p53 target promoters as shown by ChIP assays using a combination of α -acetylated H3 and H4 antibodies. Unrelated promoters (*U6* and *GAPDH*) are not enriched. The control ChIP from HCT116/p53^{-/-} cells did not reveal changes in the acetylation status of the *p21* promoter, thus demonstrating specificity.

acetylation at target promoters in HCT116/p53^{+/+} cells upon NIR knock-down (Fig. 6G). No increase in the acetylation status was observed in HCT116/p53^{-/-} cells. Similar results were obtained using the second, independent NIR siRNA (Supplementary Fig. 16). In summary, our data present NIR as a bona fide corepressor of p53 that, upon recruitment, blocks histone acetylation at p53-regulated target promoters in vivo.

Knock-down of NIR leads to increased apoptosis

Finally, we asked whether NIR controls p53-dependent apoptosis. In HCT116/p53^{+/+} cells, NIR knock-down robustly increased apoptosis, while treatment of cells with an unrelated control siRNA resulted only in a minimal increase in apoptotic cells (Fig. 7A). Apoptosis induced by NIR depletion peaked at day 3 post-transfection. In

contrast, cell survival of HCT116/p53^{-/-} cells was not influenced by knock-down of NIR (Fig. 7B), thus demonstrating that apoptosis induced by NIR depletion is p53 dependent.

In addition, we used a well-characterized siRNA directed against p53 (QIAGEN commercial siRNA #1027020) to knock down p53 in HCT116/p53^{+/+} cells. Figure 7C shows that depletion of NIR by the two independent NIR siRNAs (NIR1 and NIR2) specifically increased apoptosis in comparison to the unrelated control siRNA in unstressed HCT116/p53^{+/+} cells. Apoptosis caused by NIR depletion was significantly reduced by simultaneous p53 knock-down. These data strongly suggest that apoptosis induced by NIR depletion is p53 dependent. Finally, we show that p53-dependent apoptosis induced by 5-fluorouracil was not further increased upon depletion of NIR, indicating that a maximum response of cell death had been reached. Apoptosis induced by treatment of cells with 5-fluorouracil alone or in combination with NIR siRNA was severely impaired by p53 knock-down. In summary, these data show that NIR is critically involved in the regulation of p53-dependent apoptosis.

Discussion

Iterative BLAST analyses and degenerate, low-complexity screening with the core INHAT domain of the Set/TAF1 β oncoprotein (Seo et al. 2001) allowed us to isolate NIR, a novel INHAT repressor. Enzymatic assays demonstrate that NIR contains two functional INHAT domains. Gal-NIR is a potent repressor independent of the cell line and promoter context analyzed. In contrast to most other known repressors, NIR-mediated repression is not influenced by HDAC inhibitors. Neither TSA or Na-butyrate nor the class III HDAC inhibitor nicotinamide block NIR-mediated repression, pointing to a mechanism that differs from the behavior of previously described repressors. Concomitantly, NIR does not associate with HDACs or diverse members of different corepressor families. These data provide evidence that NIR is a novel HDAC-independent repressor.

In in vitro-assays, full-length NIR and recombinantly expressed NIR INHAT domains directly associate with nucleosomes, core histones, and histone tails. As for Set/TAF1 β , association of NIR with the histone tail is sensitive to acetylation, supporting its role as an INHAT to maintain histones in the nonacetylated state. Consequently, acetylation of all core histones by p300 is blocked in the presence of NIR. ChIP analyses further corroborate the in vivo function of NIR as an INHAT, since reduction of endogenous NIR significantly increases histone acetylation at p53 target promoters. In contrast to the Set/TAF1 β and pp32 INHATs (Kutney et al. 2004) and the recently described PELP1 (Choi et al. 2004), repression mediated by NIR does not rely on HDAC activity. Accordingly, immunopurified NIR and associated complexes do not contain detectable HDAC activity. Additionally, NIR does not display interaction with known transcriptional corepressors such as N-CoR,

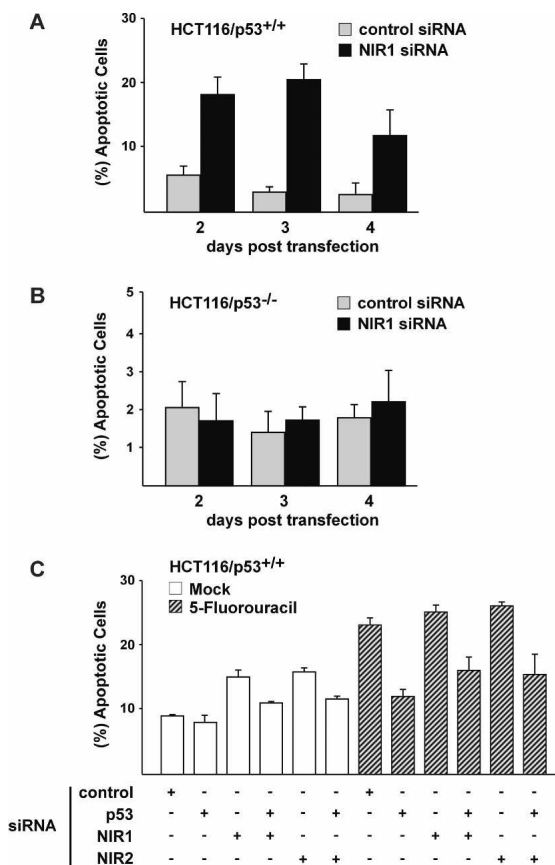


Figure 7. Knock-down of NIR increases p53-dependent apoptosis. NIR knock-down in HCT116/p53^{+/+} cells results in a strong apoptotic response. p53-containing (A) and p53-deficient (B) HCT116 cells were transfected with either a control siRNA or a siRNA directed against NIR (NIR1). The percentage of apoptotic cells was determined at the indicated time points after transfection. (C) Apoptosis induced by NIR knock-down is p53-dependent. HCT116/p53^{+/+} cells were transfected with either an unrelated siRNA (control) or the indicated combinations of siRNAs directed against p53 or NIR (NIR1 and NIR2). Apoptosis was assessed under unstressed or stress-induced (5-fluorouracil) conditions. Bars represent mean \pm SD ($n \geq 6$).

Smrt, Sin3, SuN-CoR, or HDACs 1, 4, 5, and 6 (Supplementary Fig. 7), and none of the TAP purifications revealed association with known corepressors or INHAT proteins. Furthermore, the CHIP and Re-CHIP experiments clearly demonstrate that although HDACs (such as HDAC1) occupy the endogenous p21 promoter, NIR and HDAC1 are not present in the same protein complex in vivo (Supplementary Fig. 8). These data indicate that NIR is functionally divergent from PELP1, Set/TAF1 β , and pp32.

Using a tandem affinity purification approach, we isolated p53 as a NIR-interacting partner. The C-terminal NIR-interaction domain in p53 is distinct from the Sin3-interaction site (Zilfou et al. 2001) and coincides with a described repression domain in the C terminus of p53 (Hong et al. 2001). Recent publications have shown that repression of the p53-regulated *stathmin* and *Map4* promoters requires the recruitment of HDACs by the p53-binding partner Sin3 (Murphy et al. 1999), and recruitment of the corepressor CtBP2 is acquired only in presence of the E3 ubiquitin ligase Mdm2 (Mirnezami et al. 2003). In contrast, we demonstrate NIR to be the first INHAT that is able to directly interact with p53 and to modulate p53-mediated transcriptional activation. Whether NIR is also involved in p53-mediated repression is an intriguing question that needs to be addressed in future studies.

The tumor suppressor p53 is thought to function primarily as a transcription factor, which in response to cellular stress binds to target gene promoters (Harris and Levine 2005). However, recent work has documented that p53 can occupy a subset of its target promoters even in the absence of genotoxic stress (Espinosa and Emerson 2001; Kaeser and Iggo 2002). This observation indicates that other mechanisms besides the control of DNA binding are involved in the regulation of p53 transcriptional activity in vivo, setting the requirement for negative regulatory factors. The inhibition of histone acetylation upon association of NIR with promoter-occupying p53 might be such a mechanism. Thus, it is tempting to speculate that p53 present at promoters in the absence of genotoxic stress functions as a signaling platform for negative regulators to silence transcription. In agreement with this hypothesis, NIR knock-down in unstressed cells results in stimulation of p21 RNA and protein expression. In contrast, the p53-regulated genes *PIG3*, *NOXA*, and *BAX* are significantly stimulated only in response to doxorubicin treatment but not by NIR knock-down. This difference might mirror the recent findings that *p21* constitutes a gene of high promoter occupancy by p53 while *PIG3* and *BAX* belong to a group of genes with much lower promoter occupancy (Kaeser and Iggo 2002). Overproduction of NIR robustly inhibited stimulation of the p53 target genes *p21*, *NOXA*, and *PIG3* upon genotoxic stress in p53-proficient cells, whereas basal expression of these genes in the absence of stress was unaffected. This shows that in stressed cells, p53 activation is, at least in part, counteracted by NIR.

NIR knock-down provokes apoptosis only in p53-containing HCT116 cells. In contrast, HCT116/p53^{-/-} cells

do not display any significant cell death upon addition of NIR siRNA, thus demonstrating the physiological importance of NIR in the regulation of p53-mediated apoptosis. However, NIR depletion does not stimulate expression of the proapoptotic genes *PIG3*, *NOXA*, and *BAX*, indicating that these genes do not mediate induction of apoptosis in response to NIR knock-down. It is well established that p53 provokes apoptosis not only through the transcriptional activation of proapoptotic genes. In fact, p53-mediated apoptosis is also correlated with the ability of p53 to actively repress genes (Ryan and Vousden 1998). Recent work has indicated that transcriptional repression by p53 may depend on the efficient acetylation of histone tails by HATs (Imbriano et al. 2005). Since NIR is an INHAT, NIR knock-down resulting in elevated histone acetylation thus may support apoptosis by facilitating p53-dependent gene repression.

In summary, our results establish NIR as a novel transcriptional INHAT that directly interacts with p53, inhibits p53-activated gene expression, and regulates p53-dependent apoptosis. Future research including the analyses of *Nir*-deficient mice will shed further light on the impact of NIR on p53 function in cell cycle control, development, and tumorigenesis.

Materials and methods

Plasmids

NIR cDNA was obtained by PCR from a prostate cDNA library and cloned into pcDNA6/His-C, pCMX, pCMX-Flag, and pCMX-Gal. Myc-tagged NIR deletion mutants were generated either by PCR or restriction enzyme digestion. Expression plasmids pGEX4T1-NIR-INHAT1 (amino acids 3–147), pGEX4T1-NIR-INHAT2 (amino acids 609–749), pRSET-NIR-INHAT1, and pRSET-N*/H-NIR-INHAT2 were generated by subcloning the NIR cDNA fragments into the corresponding parental vectors. For TAP analyses, the cDNAs of NIR or NIR deletion mutants were inserted into a modified pCMX expression plasmid (pURB) containing a C-terminal TAP-tag, a flexible polyalanine linker, and, for the NIR deletion mutant, a SV40 nuclear localization signal. Constructs are referred to as pURB-NLS-TAP-tag (TAP), pURB-Flag-NIR-linker_{4x}-TAP-tag (NIR-TAP), pURB-NIR-aa147–609-NLS-TAP-tag (NIR INHAT Δ 1/2-TAP), pURB-NIR-aa609–749-TAP-tag (NIR INHAT2-TAP), and pURB-NIR-aa2–147-NLS-TAP-tag (NIR INHAT1-TAP). Lienhart Schmitz (Department of Chemistry, University of Bern, Bern, Switzerland) kindly provided pGEX-human p53. All GST-p53 deletion mutants were generated by PCR and cloned into pGEX4T1. Cloning details are available upon request. All constructs were verified by sequencing. G5E1b-LUC, Gal-TK-LUC, pCMX, pCMX-Gal, and pRSET-N*/H are described (Müller et al. 2002). Other plasmids were kindly provided as follows: p21-LUC (Michael Datto, Department of Pathology, Duke University Medical Center, Durham, NC); *PIG3*-LUC (Matthias Döbelstein, Institute of Virology, University of Marburg, Marburg, Germany); pGEX-4T1-p300-HAT (Richard Eckner, Institute for Molecular Biology, University of Zurich, Zurich, Switzerland); pCMX-Gal-Nix1, pRSET-Nix1, and pGEX-Nix1 (Erich Greiner, Deutsches Krebsforschungszentrum [KDFZ], Heidelberg, Germany); pGal-TIF2.12 (Hinrich Gronemeyer, Department of Cell Biology and Signal Transduction, Institut de Génétique et de Biologie Moléculaire et Cellulaire [IGBMC], Strasbourg,

France); pcDNA3.1-myc-p53 (Makoto Hijikata, Institute for Virus Research, Kyoto University, Kyoto, Japan); L8G5-LUC and LexA-VP16 (Saadi Khochbin, Laboratoire Biologie Moléculaire et Cellulaire de la Différenciation, INSERM U309, Institut Albert Bonniot, La Tronche, France); pcDNA3.1-Gal-CREB-Z1A (Marc Montminy, Peptide Biology Laboratories, Salk Institute for Biological Studies, La Jolla, CA); mNoxa-LUC (Xinshou Ouyang, School of Medicine, University of Tokyo, Tokyo, Japan); and the p53 reporters PG13-LUC and MG15-LUC (Bert Vogelstein, Howard Hughes Medical Institute, Johns Hopkins Medical Institutions, Baltimore, MD).

Generation of NIR-specific antibodies

Proteins corresponding to NIR amino acids 3–147 and amino acids 609–749 were expressed as His-tagged fusion proteins in *E. coli*. Purified proteins were used for rabbit immunization using standard procedures. Antibodies directed against the N and C termini ("2719" and "2910," respectively) were affinity-purified.

Cell culture and transient transfections

For transient transfections, 293, C2C12, BHK, HCT116/p53^{+/+}, HCT116/p53^{-/-}, and Saos-2 cells were cultured in DMEM supplemented with 10% fetal calf serum, penicillin, streptomycin, and 2 mM glutamine. Transient transfection assays were carried out in 12-well plates (1 × 10⁵ cells per well) using the calcium phosphate coprecipitation technique. 293 and C2C12 cells were transfected using high molecular weight polyethyl- enimine (PEI) (Durocher et al. 2002). As indicated, media were replaced with DMEM and 330nM TSA, 5 mM Na-butyrate, or 10 mM nicotinamide (all Sigma). The total amount of transfected DNA was kept constant by addition of empty pCMX expression vector and pUC19. Luciferase activity was determined as described (Müller et al. 2002). For mRNA analyses, exponentially growing HCT116 cultures (p53^{-/-} and p53^{+/+}) were transiently transfected by Nanofectin (PAA). Twenty-four hours after transfection, subsets of the cultures were treated with 0.34 μM doxorubicin (Sigma) for another 6 h.

Immunofluorescence analyses

Tissue culture cells were treated essentially as described (Müller et al. 2002). Prior to staining, BHK cells were cultured for 6 h in the presence of 0.34 μM doxorubicin (Sigma) to increase p53 protein levels. For primary antibodies, the indicated dilutions were used: α-NIR 2910 (1:100), α-p53 DO-1 (Calbiochem; 1:250). Localization was visualized using secondary Alexa Fluor 488- and Alexa Fluor 555-labeled antibodies (Invitrogen/Molecular Probes; both 1:7000); nuclei were stained with 1 μg/mL DAPI (Roche). Images were taken with a Zeiss LSM510meta confocal laser scanning microscope.

mRNA analyses

Total RNA was extracted using the RNAwiz kit (Ambion) followed by isopropanol precipitation. RNA (6 μg) was treated with DNase I (Roche) and purified by phenol/chloroform extraction. cDNA synthesis was performed by addition of 100 ng of dT₁₈- primer using SuperScript reverse transcriptase (GIBCO-BRL). As a control, the housekeeping genes *Hprt* or *GAPDH* were amplified for 25 cycles with the following primers: fw, 5'-GTT GAGAGATCATCTCCACC-3'; rv, 5'-AGCGATGATGAACC AGGTTA-3'; and fw, 5'-TGGTATCGTGGAAGGACTCAT GAC-3'; rv, 5'-AGTCCAGTGAGCTTCCCGTTCAGC-3'; re-

spectively, at an annealing temperature (T_A) of 58°C. RT-PCR experiments used cDNA amounts according to the control PCR and the indicated primers. PCR oligonucleotides for *Nir* were fw, 5'-AGAAAGTGCAAGAGAATGCACA-3'; rv, 5'-ACTG GCAGTATGTGCACTGC-3' (T_A: 60°C, 35 cycles). For the analysis of p53 target genes, cells were stimulated with 0.34 μM doxorubicin for 6 h. The PCR oligonucleotides were *p21* (fw, 5'-CCTGGCACCTCACCTGCTCTGCTG-3'; rv, 5'-GCAGAA GATGTAGAGCGGGCCTTT-3'; T_A: 60°C, 26 cycles); *PIG3* (fw, 5'-GTGCACCTTGACAAGCCGGGAGGA-3'; rv, 5'-CAG CCTGGGTCAGGGTCAATCCCT-3'; T_A: 57°C, 29 cycles); *NOXA* (fw, 5'-AGATGCCTGGGAAGAAG-3'; rv, 5'-AGTC CCCTCATGCAAGT-3'; T_A: 58°C, 30 cycles); and *Bax* (fw, 5'-CCCCGAGAGGTCTTTTCCGAGTG-3'; rv, 5'-GAAAAAT GCCCATGTCCCCCAATC-3'; T_A: 65°C, 34 cycles). Northern blot analyses were performed with a human Multiple Tissue mRNA Dot blot (BD Biosciences Clontech) and a NIR-specific probe spanning base pairs 1447–2250.

Protein expression

Recombinant proteins were expressed in *E. coli* using either pRSET (Invitrogen) or pGEX4T1 (GE Healthcare). Expression of proteins was induced by addition of 1 mM IPTG at 37°C for 3 h for NIR and at 16°C for 24 h for GST-p53 and GST-p53 deletion mutants. Proteins were extracted by sonication in PBS containing 350 mM NaCl and solubilized by addition of 0.5% (v/v) Triton X-100.

Purification of TAP-tagged NIR proteins

293 cells were transfected with TAP, NIR-TAP, NIR INHAT1-TAP, NIR INHAT2-TAP, and NIR ΔINHAT1/2-TAP as described. Proteins were purified using IgG Sepharose (GE Healthcare). Transfections, cell lysis, and protein purification were performed essentially as described below in the section Tandem Affinity Purification. Equal amounts of the purified NIR proteins were verified by Western blotting using α-Protein A (α-TAP; Rockland; 1:5000).

GST-pulldown

Equal amounts of GST or GST fusion proteins were immobilized on GSH Sepharose (GE Healthcare) at 4°C for NIR/core histone and NIR/nucleosome interactions or at 37°C for NIR/p53-pulldown assays. Unspecific binding was removed by repeated washing with pulldown buffer (20 mM HEPES at pH 7.7; 75–600 mM KCl; 0.1 mM EDTA; 25 mM MgCl₂; 10 mM DTT; 0.075%–0.5% NP-40). Thirty micrograms of calf thymus core histones (Roche) or 5 μg of nucleosomes prepared from HeLa cells (O'Neill et al. 1992) was mixed with [³⁵S]methionine-labeled *in vitro* translated NIR proteins. Complexes were allowed to form for 1 h at 4°C (NIR/core histone and NIR/nucleosome interactions) or 37°C for NIR/p53 interactions. After extensive washing, protein complexes were separated by SDS-PAGE and visualized by Coomassie staining or autoradiography.

Protein A-pulldown

IgG Sepharose-bound NIR-TAP proteins were washed in pulldown buffer (20 mM HEPES at pH 7.7; 150 mM KCl; 0.1 mM EDTA; 25 mM MgCl₂; 10 mM DTT; 0.15% NP-40) and incubated with 5 μg of a nucleosomal preparation (O'Neill et al. 1992) for 1 h at 4°C. After extensive washing in pulldown buffer, protein complexes were separated by SDS-PAGE and visualized by Coomassie staining.

Histone tail-pulldown

Pulldowns with chemically synthesized peptides (corresponding to histone H3 amino acids 1–30) coupled to Sepharose were performed with [³⁵S]methionine-labeled in vitro translated NIR protein as described (Schneider et al. 2004). Unspecifically bound proteins were removed by washing five times in wash buffer at 4°C (20 mM Tris at pH 8.5; 150 mM NaCl; 0.5% NP-40). Protein complexes were separated by SDS-PAGE and visualized by autoradiography.

HDAC assay

Histone deacetylase activity was measured in immunoprecipitates or whole-cell extracts from 293 cells. Extracts were treated with antibodies directed against the N or C terminus of NIR or N-CoR (Santa Cruz, SC-1609). HDAC assays were performed with [³H]-labeled hyperacetylated histone H3 N-terminal tail peptides as described (Heinzel et al. 1997). As controls, immunopurified N-CoR-containing complexes or 50 µg of 293 whole-cell extracts were subjected to HDAC assays with or without 15 min of preincubation with the HDAC inhibitor TSA (330 nM; Sigma). Mock-treated reactions include histones incubated with IP buffer. The release control displays release of radiolabeled acetyl groups in storage buffer (PBS).

INHAT assays

Individual core histones H3, H2B, H2A, and H4 were obtained from Roche Molecular Biochemicals. INHAT assays were performed by incubating 2 µg of the individual core histone with 1 µg of recombinantly expressed His-INHAT1 or His-INHAT2 in HAT-buffer (Eckner et al. 1996) for 15 min on ice followed by 15 min at room temperature. As a control, the unrelated cofactor protein Nix1 (Greiner et al. 2000) was incubated with the histones. Following preincubation, active GST-p300 HAT (Eckner et al. 1996) and 25 nCi of [¹⁴C]acetyl-coenzyme A (GE Healthcare) were added and incubated at 30°C for 2 h under constant shaking at 1000 rpm. Reaction products were separated by SDS-PAGE, and acetylation was visualized by autoradiography. For INHAT assays with affinity-purified NIR, 3 µg of core histones or nucleosomes were used as substrate. INHAT assays were performed as described for the recombinant NIR proteins. After separation by SDS-PAGE, proteins were transferred on PVDF membranes (ImmobilonP; Millipore) and stained with Ponceau S to control for equal loading. Dried membranes were treated with EN³HANCE spray (NEN) and exposed for autoradiography.

Coimmunoprecipitation assays and Western blot analyses

For coimmunoprecipitations, myc- and TAP-tagged proteins were coexpressed in BHK cells. Whole-cell extracts were prepared in lysis buffer (50 mM Tris/HCl at pH 8.0; 420 mM NaCl; 0.1% [v/v] NP-40; 50 mM NaF; 2 mM Na₃VO₄; 0.2 mM DTT; Complete protease inhibitor cocktail; 20% [v/v] glycerol). Whole-cell extracts (300 µg) were incubated with 3 µg of the respective antibodies (α-TAP; Rockland; α-NIR 2910) in 500 µL of lysis buffer. For immunoprecipitation of endogenously expressed proteins, 1 mg of 293 whole-cell extract was incubated with 3 µg of specific antibody α-p53 (DO-1) or α-NIR (2910) in 500 µL of RIPA buffer (1% [v/v] of 1 M Na₂HPO₄ solution, buffered to pH 7.2 with 1 M NaH₂PO₄; 150 mM NaCl; 1% NP-40; 0.5% deoxycholate; 0.1% SDS; Complete protease inhibitor cocktail). Fifty microliters of pre-equilibrated Gamma-Bind G Sepharose (GE Healthcare) was added for 1 h while ro-

tating at 4°C. Beads were washed five times with RIPA buffer, and precipitated proteins were analyzed on a 12% SDS gel. To coimmunoprecipitate endogenous NIR and p53, 293 and HCT116/p53^{+/+} cells were either mock-treated or exposed to doxorubicin for 6 h. IPs were performed as described (Müller et al. 2002) from 1 mg of nuclear extract in salt-adjusted IP buffer (150 mM) using 5 µg of α-NIR 2910 or rabbit IgG. Western blots were performed with 15 µg of whole-cell extracts. Western blots were decorated with the indicated primary antibodies: α-NIR 2910 (1:3000), α-p53 DO-1 (Calbiochem; 1:1000), α-ACTIN (Sigma; 1:3000), and α-p21 (PharMingen; 1:1000). Secondary antibody and chemiluminescence procedures were performed according to the manufacturer (Perkin Elmer).

Immunohistochemical analyses

Mouse embryos were fixed with 4% paraformaldehyde and paraffin-embedded. Antibody staining and HRP-coupled detection followed the Vecta Stain Elite kit (Vecta). Epitopes were uncovered by boiling the slides in antibody retrieval solution (Vecta). Primary α-NIR antibody (2719; 1:1000) was incubated in 5% milk powder with 2.5% horse serum overnight at 4°C. After repeated washing in PBS/0.1% Tween 20, secondary avidin-conjugated antibodies (1:200) were added for 1 h at room temperature, followed by extensive washing. Biotinylated HRP was added and specific staining was detected using NovaRed HRP-substrate (Vecta). Nuclei were stained with hematoxylin.

Tandem affinity purification

TAP purification was essentially performed as described (Rigaut et al. 1999) with the following modifications. 293 cells were transfected using PEI with either TAP, NIR-TAP, or mutants thereof. Approximately 1 × 10⁸ cells were lysed by repeated freeze-thaw cycles in buffer A (20 mM HEPES/KOH at pH 7.9; 420 mM NaCl; 1.5 mM MgCl₂; 10 mM KCl; 25% glycerol; 0.5 mM DTT; 0.1% NP-40; 0.1 mM EDTA; 50 mM NaF; 0.2 mM Na₃VO₄; and Complete protease inhibitor cocktail). TAP-tagged proteins were bound to IgG Sepharose (GE Healthcare) in salt-adjusted buffer A (150 mM NaCl) at 4°C overnight followed by repeated washing. Complexes were released with TEV-protease (100 U; Invitrogen) in TEV buffer (10 mM Tris/HCl at pH 8.0; 150 mM NaCl; 0.1% NP-40; 1 mM DTT; 1 mM EDTA). Complexes containing CBP-tagged NIR were immobilized on a calmodulin affinity resin (Stratagene) for 4 h at 16°C. Complexes were washed five times with CBP buffer (10 mM Tris/HCl at pH 8.0; 150 mM NaCl; 0.1% NP-40; 1 mM Mg-acetate; 1 mM imidazole; 10 mM β-mercaptoethanol; 2 mM CaCl₂) and finally eluted with CBP buffer containing 10 mM EGTA instead of CaCl₂. Proteins were size-separated by SDS-PAGE, visualized by Coomassie staining, and identified by MALDI-TOF/TOF.

NIR RNAi and apoptosis assays

Short interfering RNAs (NIR1, 5'-GACCTGAACTTCCCAGA GATT-3'; NIR2, 5'-GACAGGAAGGATGAAGACATT-3'; p53 siRNA, QIAGEN #1027020: 5'-GACTCCAGTGGTAATCTA CTT-3') were used to silence gene expression in human HCT116 cells. As a control, an unrelated siRNA targeting luciferase (5'-CGTACGCGGAATACTTCGA-3') was used. Exponentially growing cells were transfected with siRNA (40 nM) by RNAiFect (QIAGEN). With a routine transfection efficiency of 80%–90%, a significant knock-down of NIR was observed at days 2–3 post-transfection, but not when cells were transfected with the unrelated control siRNA. At 72 h post-transfection, cultures were mock-treated or treated with 0.34 µM doxorubi-

cin as indicated. Cells were cultured in normal growth conditions and apoptosis induced by NIR knock-down was determined at the indicated time points by flow cytometry analysis of the numbers of cells with a sub-2n DNA content (Mahyar-Roemer and Roemer 2001). To assay 5-fluorouracil-induced apoptosis, cultures were mock-treated or exposed to 375 μ M 5-fluorouracil 24 h post-transfection and incubated for another 24 h.

ChIP

ChIP experiments in HCT116 cells were performed essentially as described (Shang et al. 2002). Immunoprecipitation was performed with α -NIR (2910), α -p53 (DO-1 or DO-7), and a 1:1 mixture of α -acetyl H3/ α -acetyl H4 (Upstate) antibodies on GammaBind G Sepharose (GE Healthcare). For PCR, 1–5 μ L out of 50 μ L of DNA extractions were used. For Re-ChIP assays, reactions were immunoprecipitated with the α -NIR (2910) antibody, and sequentially washed with TSE I, TSE II, buffer III, and TE as described (Shang et al. 2002). Complexes were eluted by incubation in 10 mM DTT at 37°C for 30 min and diluted 50 times with dilution buffer, followed by a second immunoprecipitation with either the α -p53 (DO-1) antibody or mIgG. The primer sequences and PCR conditions used to amplify the corresponding promoter fragments were as follows: *p21* (fw, 5'-ACCTTTCACCATTCCTAC-3'; rv, 5'-GCCCCAAGGACAA AATAGCCA-3'; T_A: 56°C), *PIG3* (fw, 5'-CAGGACTGTCAG GAGGAGGCGAGTGATAAGG-3'; rv, 5'-GTGCGATTCTAG CTCTCACTTCAAGGAGAGG-3'; T_A: 63°C); *GAPDH* (fw, 5'-TCCTCCTGTTTCATCCAGC-3'; rv, 5'-TAGTAGCCGGGC CCTACTTT-3'; T_A: 60°C); *U6* (fw, 5'-GGCCTATTTCCCAT GATTCC-3'; rv, 5'-ATTGCGTGTCATCCTTGC-3'; T_A: 60°C).

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