

Biological Functions of Mammalian Nit1, the Counterpart of the Invertebrate NitFhit Rosetta Stone Protein, a Possible Tumor Suppressor^{*[5]}

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The "Rosetta Stone" hypothesis proposes that the existence of a fusion protein in some organisms predicts that the separate polypeptides function in the same biochemical pathway in other organisms and may physically interact. In *Drosophila melanogaster* and *Caenorhabditis elegans*, NitFhit protein is composed of two domains, a fragile histidine triad homolog and a bacterial and plant nitrilase homolog. We assessed the biological effects of mammalian Nit1 expression in comparison with Fhit and observed that: 1) Nit1 expression was observed in most normal tissues and overlapped partially with Fhit expression; 2) Nit1-deficient mouse kidney cells exhibited accelerated proliferation, resistance to DNA damage stress, and increased cyclin D1 expression; 3) cyclin D1 was up-regulated in *Nit1* null mammary gland and skin; 4) Nit1 overexpression induced caspase-dependent apoptosis *in vitro*; and 5) *Nit1* allele deficiency led to increased incidence of *N*-nitrosomethylbenzylamine-induced murine forestomach tumors. Thus, the biological effects of Nit1 expression are similar to Fhit effects. Adenoviruses carrying recombinant *NIT1* and *FHIT* induced apoptosis in Fhit- and Nit1-deficient cells, respectively, suggesting that Nit1-Fhit interaction is not essential for function of either protein. The results suggest that Nit1 and Fhit share tumor suppressor signaling pathways, while localization of the *NIT1* gene at a stable, rather than fragile, chromosome site explains the paucity of gene alterations and infrequent loss of expression of the *NIT1* gene in human malignancies.

The "Rosetta Stone" hypothesis was proposed for proteins consisting of fused domains in one organism that are expressed as separate polypeptides in other organisms (1, 2). According to

this theory, supported by bioinformatic and experimental evidence, the existence of a fusion protein in one genome powerfully predicts that the separate polypeptides function in the same cellular or biochemical pathway in other organisms (3, 4).

In a search for the tumor suppressor *fragile histidine triad (FHIT)*² gene homolog in *Drosophila melanogaster* and *Caenorhabditis elegans*, NitFhit was cloned and identified as a fusion protein composed of a C-terminal Fhit domain and a domain related to plant and bacterial nitrilase (5). The fusion of unrelated proteins is believed to indicate involvement in the same biological pathway if the distinct proteins have similar gene expression patterns. Indeed, murine Nit1 and Fhit have overlapping patterns of tissue-specific mRNA expression and were expected to be closely associated functionally (5). *FHIT* is a tumor suppressor gene located at chromosome region 3p14.2, encompassing the most active common fragile site of the human genome, *FRA3B* (6–7); frequent deletion at the *FHIT* locus, aberrant transcripts, and promoter hypermethylation and resultant loss of Fhit expression have been associated with many types of human malignancies (6, 8–11). Fhit is a member of the histidine triad nucleotide-binding protein superfamily, encoding a diadenosine polyphosphate (Ap_nA) hydrolase that cleaves substrates such as Ap₃A to AMP plus the other nucleotide (12, 13). Fhit hydrolytic activity is lost when histidine 96 is replaced with asparagine (12), but the enzymatically dead mutant Fhit protein also induced caspase-dependent apoptosis in cancer cells, confirming that Fhit Ap₃A binding activity is necessary for the proapoptotic function of Fhit but that the hydrolytic activity is dispensable (14, 15). Overexpression of *FHIT* by adenoviral gene delivery in human cancer cells suppressed cell growth and induced caspase-dependent apoptosis *in vitro* and *in vivo* (16–20). In addition, *Fhit*^{-/-} and *Fhit*^{+/-} mice exhibited increased susceptibility to spontaneous tumors, and *Fhit* deficiency in mice enhanced sensitivity to the carcinogens *N*-nitrosomethylbenzylamine (NMBA) and dimethylnitrosamine (21–23). Fhit-deficient cells show altered sensitivity to DNA damage by mitomycin C (MMC), UVC, and

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1 and S2 and Table S1.

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² The abbreviations used are: FHIT, fragile histidine triad; Ap_nA, diadenosine polyphosphate; NMBA, *N*-nitrosomethylbenzylamine; MMC, mitomycin C; RT, reverse transcription.

ionizing radiation (24, 25). These biological functions of Fhit are consistent with its tumor suppressor activity.

In *Arabidopsis*, Nit family members (Nit1–3) are believed to convert indole-3-acetonitrile into indole-3-acetic acid, known as a plant growth hormone auxin, which plays an important role in development of the embryo, leaf formation, and root initiation (26–28). In addition, an apoptosis-inducing effect of Nit1 in plant wound and herbicide-induced cell death has been proposed (29). Structural analysis of *C. elegans* NitFhit protein, gene name *nft-1*, showed that NitFhit is a tetramer in which the Nit domains are localized at the center with Fhit dimers at the poles with Fhit nucleotide binding sites facing away from Nit and that recombinant *D. melanogaster* NitFhit was capable of cleaving Ap₃A to AMP and ADP (30, 31). The biological role of worm and fly Nit domains, as well as Nit1-interacting proteins and substrates, is unknown. In this study, we conducted diverse experiments *in vitro* and *in vivo* to elucidate the biological or physiological effects of Nit1 expression in mammalian cells and in mice. In particular, possible roles of Nit1 as a tumor suppressor were assessed on the basis of the previous data that supported Fhit-modulated cell cycle progression, apoptosis, and tumorigenesis *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Mouse Models and Genotyping Protocols—A conditional deletion construct for the murine *Nit1* gene was designed to flank exon 4 through 7 with *LoxP* sites. Briefly, *LoxP* sites were put into intron 3 and intron 7 with a phosphoglycerate kinase-neomycin cassette, and a diphtheria toxin cassette was included in the construct for negative selection. This targeting vector was transfected into 129/SVJ embryonic stem cells, and after neomycin selection, embryonic stem clones with heterozygously recombined *Nit1* alleles were identified by Southern blot. DNAs digested with BamHI and PvuII and blotted onto a nylon membrane were hybridized with ³²P-labeled probes. Correctly targeted embryonic stem cell clones were injected into blastocysts. Germ line transmitters were obtained by crossing the chimeric mice with C57BL/6 mice, which were then crossed with EIIa Cre transgenic mice (The Jackson Laboratory) to obtain mosaic mice. Segregation of the mosaic mice by crossing with C57BL/6 mice resulted in *Nit1^{fl/fl}* and *Nit1^{fl/-}* mice. The *whely acidic protein (Wap)-Cre* transgenic mice (National Cancer Institute Mouse Models to Human Cancers Consortium, Rockville, MD) were mated with the *Nit1^{fl/fl}* mice and ultimately produced *Wap-Cre Nit1^{fl/fl}* mice. All animals used in the studies were treated humanely in accordance with federal guidelines and institutional policies. Screening of founder animals and their offspring was performed by genomic PCR with the following primer sets: *Nit1* floxed/wild-type allele, 5'-GTTGGTCTAGCAATCTGTTATGA-3' and 5'-GTGCTGGATTAAAGGTGTGC-3'; *Nit1* deletion allele, 5'-GTACCGATAACCGATTACTTCGA-3' and 5'-GTGCTGGGATTAAAGGTGTGCA-3'; *Wap-Cre* transgene, 5'-TAGAGCTGTGCCAGCCTCTTC-3' and 5'-CATCACTCGTTGCATCGACC-3'. Genotypes of *Nit1^{fl/-}* and *Nit1^{-/-}* mice obtained from the offspring of *Nit1^{fl/fl}* mice were also confirmed by genomic PCR.

***Nit1^{fl/fl}* and *Nit1^{-/-}* Mouse Kidney Cells and Cell Culture**—Whole kidneys from a *Nit1^{fl/fl}* mouse were dissected and placed

in culture, and cells were grown and subcultured in minimal essential medium (Sigma) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. To generate *Nit1*-deficient (*Nit1^{-/-}*) cells, *Nit1^{fl/fl}* cells were infected with retrovirus-expressing *Cre* recombinase, constructed using pLNCX1-IRES-EGFP (Clontech). Viral infection efficiencies were determined by inspection of enhanced green fluorescent protein expression and fluorescence-activated cell sorter-based counting. A549 (Fhit-positive) and H1299 (Fhit-negative) human lung cancer cells (15) and human embryonic kidney 293 cells were maintained in minimal essential medium and Dulbecco's modified Eagle's medium (Sigma), respectively.

Cell Viability Test, DNA Damage Induction, and Flow Cytometric Analyses—The ViCell cell counter (Beckman Coulter) was used for cell counting and viability tests (15). MMC treatment and UVC light exposure experiments were carried out as reported (24). Cells were treated with medium supplemented with 2 or 5 μM MMC (Fisher) or exposed to 30 or 60 J/m² UVC (254 nm) irradiation. Viable cell numbers were estimated using a Cell Counting Kit-8 (Dojindo). To analyze cellular DNA content and cells positive for active caspase-3 with a fluorescence-activated cell sorter Calibur cytometer (BD Biosciences), cells were stained with propidium iodide and phycoerythrin-conjugated monoclonal anti-active (cleaved) caspase-3 (BD Biosciences), respectively (15).

RT-PCR and Quantitative Real-time RT-PCR—*Nit1* mRNA expression was confirmed by RT-PCR and quantitative real-time RT-PCR. Total RNAs were amplified with OneStep RT-PCR kit (Qiagen) and QuantiTect SYBR Green RT-PCR kit (Qiagen), using primers as follows: 5'-GTACTTTGTA CTCA-GCCAG-3' (forward) and 5'-CCATAGAGGTCAGGTCT-GCG-3' (reverse). Quantitative real-time RT-PCR analyses were performed using the iCycler multicolor real-time PCR detection system (Bio-Rad). The primer set for amplification of glyceraldehyde-3-phosphate dehydrogenase mRNA served as a housekeeping control gene (15), and the levels of *Nit1* transcripts were determined by the ΔΔCt method (32).

Western Blot Analysis—Preparation of total protein and the immunoblotting procedure used are described elsewhere (15). Total protein (40 μg) was separated on a polyacrylamide gel, transferred to a membrane, and probed with antisera against Fhit (33), cyclin D1 (NeoMarkers), and other cell cycle markers, including cyclin B1, cyclin E, cdk2, cdc2, cdc25A, p21CIP1, p53, Chk1 (Santa Cruz), Cdk4, and p27KIP1 (BD Biosciences). Rabbit polyclonal *Nit1* antiserum against human *Nit1* C-terminal peptide (QHRRPDLYGSLGHPLS) was custom-made by Zymed Laboratories Inc. and shown to specifically detect both human and mouse *Nit1* protein. Specificity was shown by immunoblot and immunohistochemistry using tissues from mice with homozygous deletion of the floxed *NIT1* alleles. These tissues were shown to be negative for staining of the *Nit1*-sized band on immunoblots and negative for immunohistochemical staining relative to *Nit1*-positive controls, as seen below under "Results" and in supplemental Figs. S1 and S2. Tubulin (NeoMarkers) and green fluorescent protein (Molecular Probes) antibodies were used in detection of protein in loading controls.

Tumor Suppressor Effect of Mammalian Nit1

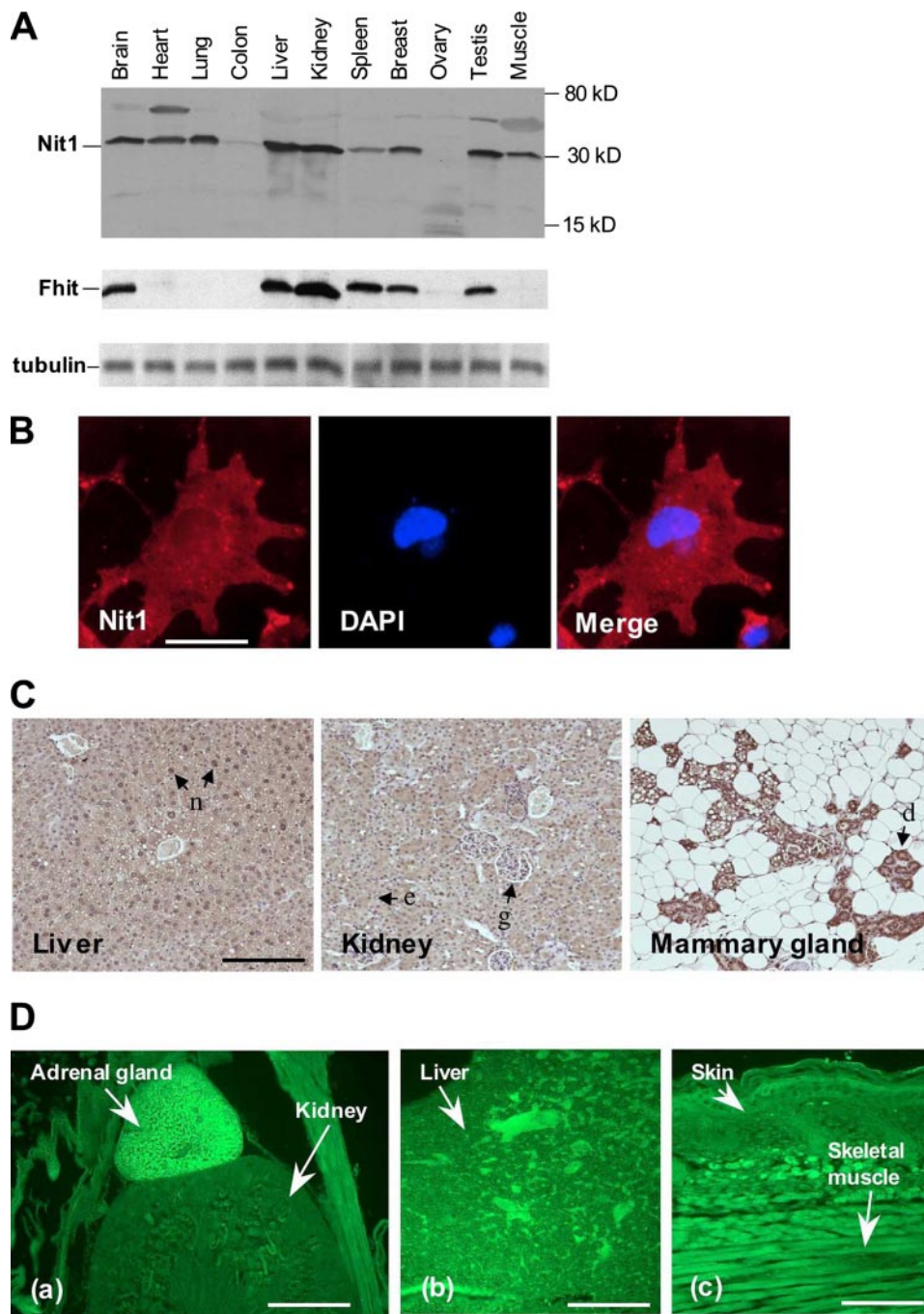


FIGURE 1. Expression of Nit1 in mouse tissues. *A*, Western blot of Nit1 (upper panel) and Fhit expression (middle panel). Antiserum against tubulin was used as a loading control (lower panel). *B*, immunofluorescence of Nit1 expression in cultured mouse kidney cells (red). The nucleus was stained with 4',6-diamidino-2-phenylindole (blue). Scale bar, 10 μm . *C*, immunohistochemical analyses of Nit1 expression in murine adult tissues. Arrowheads point out nuclei (*n*) in liver, a glomerulus (*g*) and tubule epithelial cells (*e*) in kidney, and ductal epithelial cells (*d*) in mammary gland. Nit1 knock-out tissues similarly stained for Nit1 protein were completely negative, as shown in supplemental Figs. 1D and 2B. Scale bar, 100 μm . *D*, indirect immunofluorescent detection of Nit1 expression in mouse embryo (E13). *a*, adrenal gland and kidney; *b*, liver; *c*, skeletal muscle and skin. Scale bar, 200 μm in sections *a* and *b*, 50 μm in section *c*.

Recombinant Adenoviral Vector Construction—Adenoviruses carrying human recombinant *NIT1* (Ad-*NIT1*) and *FHIT* (Ad-*FHIT*) were constructed as described previously (14). The transfer vector pAdenoVator-CMV5-GFP (Qbiogene) carrying full-length human *NIT1* or *FHIT* cDNAs and the AdVator $\Delta\text{E1/E3}$ adenoviral genome construct were mixed and recombination performed in BJ5183

Escherichia coli. Resulting recombinant vectors were transfected into human embryonic kidney 293 cells to package viruses. Isolated single viral plaques were expanded and assessed for Nit1 or Fhit expression by Western blot. Ad-*GFP* virus was used as a non-specific control (Qbiogene). Viral titers were determined by absorbance measurement, multiplicity of infection test, and tissue culture infectious dose 50 methods. Cells were incubated with adenoviral aliquots at a desired multiplicity of infection (0–100) for 4 h prior to addition of culture medium ($>25 \times$ volume of virus inoculum).

Transfections—For transient expression of exogenous wild-type and mutant Nit1, *Nit1*^{-/-} cells were transfected with pIRESneo vector (Clontech) encoding wild-type (pNit1) or C203A mutant Nit1 (pNit1-C203A). Transient transfection was conducted with Lipofectamine reagent (Invitrogen). At 48 h after transformation, cells were assessed for viability, active caspase-3, and expression of specific proteins.

Mating Experiments for the Mammary Gland Development Study—Wap-Cre *Nit1*^{fl/fl} and *Nit1*^{fl/fl} matings were set up late in the afternoon and female mice checked for copulation plugs over the following few days to determine time of mating. The morning on which a copulation plug was observed was pregnancy day 0.5 and the female separated from the male. Mice were sacrificed for phenotypic analysis during pregnancy (P13, P16, P19) or lactation (L1, L4). For whole mount analysis of mammary glands, the fourth inguinal mammary glands were spread on glass, fixed in Carnoy's solution, defatted, and stained in carmine alum solution overnight. The tissue was then dehydrated and mounted on glass. The average numbers of branch points and total number of endbuds between nipple and endbuds were counted microscopically (34, 35). For histological examination, formalin-fixed, paraffin-embedded sections were stained with hematoxylin and eosin or subjected to immunohistochemical analyses.

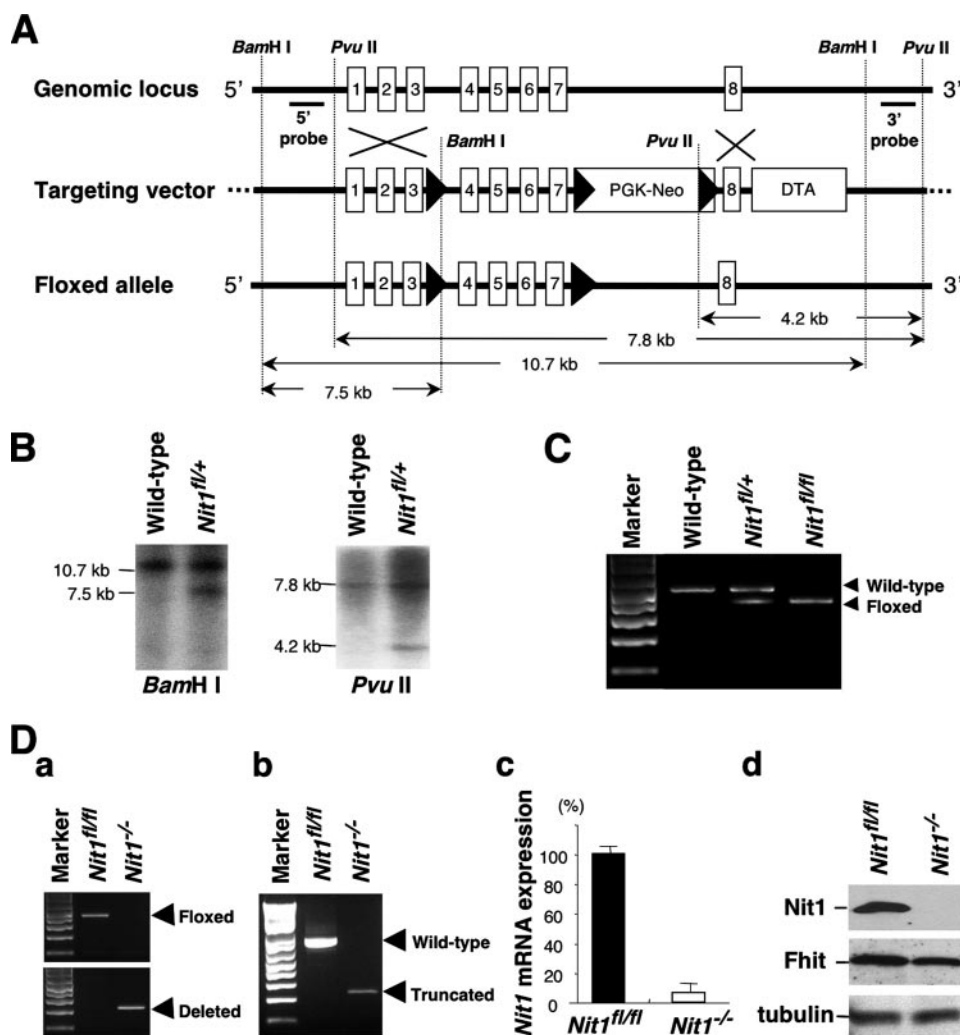


FIGURE 2. Conditional null mutation in the murine *Nit1* gene. *A*, the targeted insertion of *LoxP* sites for generation of *Nit1^{fl/fl}* mice. *B*, Southern blot analysis of the heterozygously recombined embryonic stem clones (*Nit1^{fl/+}*) and the wild-type counterpart. Genomic DNAs digested with *Bam*HI and *Pvu*II were hybridized with 5' probe (5'-CTATAACCACTGCCCGTAAGTTAC-3') and 3' probe (5'-TTGCCTTGCGAGAGCCGTCTC-3'), respectively. *C*, genomic PCR analysis to identify mouse genotype. *D*, establishment of mouse *Nit1^{fl/fl}* and *Nit1^{-/-}* mouse kidney cells. *a*, disruption of floxed *Nit1* allele was confirmed by genomic PCR. *b*, full-length and truncated *Nit1* mRNA expression detected by RT-PCR. *c*, quantitative real-time RT-PCR analysis. The levels of wild-type *Nit1* mRNA expression in *Nit1^{fl/fl}* cells were considered to be 100%. *d*, Western blot of *Nit1* expression in *Nit1^{fl/fl}* and *Nit1^{-/-}* cells. Deletion of *Nit1* did not affect *Fhit* expression. As a control, tubulin expression was also analyzed.

Immunofluorescent and Immunohistochemical Analyses—*Nit1^{fl/fl}* cells on coverslips and formalin-fixed, paraffin-embedded whole mount mouse embryo (E13) sections were used. Cells were incubated with the antiserum against *Nit1* and goat Texas Red or fluorescein isothiocyanate-conjugated anti-rabbit IgG (Zymed Laboratories Inc.). Mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories) was used for nuclear staining. Immunohistochemical staining by a modified version of the immunoglobulin enzyme bridge technique was performed to detect *Nit1* and cyclin D1 expression (15). Cyclin D1-positive cells in mammary ducts and lobules were counted in at least 10 high power fields ($\times 400$) for each section. For detection of apoptotic bodies, ApopTag Peroxidase kit (Chemicon) was used. Briefly, mouse mammary gland sections were pretreated with proteinase K and incubated with 2 N HCl. Terminal deoxynucleotidyl transferase enzyme was added to

the pre-equilibrated cells. Anti-digoxigenin peroxidase conjugate IgG and 3,3'-diaminobenzidine-tetrahydrochloride-dihydrate were added to the slides. Slides were counterstained with hematoxylin. The percent of ApopTag-positive cells was determined by counting cells in at least 10 high power fields.

NMBA Carcinogenicity Study—*Nit1^{fl/fl}*, *Nit1^{fl/+}*, and *Nit1^{-/-}* mice (15 each) were given intragastric doses of NMBA (Ash Stevens), 2 mg/kg body weight, a total of six times over a 3-week period. All mice were sacrificed 10 weeks after the final NMBA dose and examined for end-point tumor incidence (33). At autopsy, whole stomachs were removed and opened longitudinally. The number of animals bearing tumors in the esophagus, the forestomach, and the squamocolumnar junction and number of papillomas and dysplasias (carcinomas *in situ*) were scored by histological examination. *Fhit^{-/-}* mice, which were known to show enhanced susceptibility to NMBA-induced upper digestive tract tumors, were included in the study as controls.

Statistical Analysis—*p* values calculated by the Student's two-sided *t* test were used to determine significance of differences in measurements of mammary gland developmental stages in the *Nit1^{fl/fl}* and *Wap-Cre Nit1^{fl/fl}* mice.

RESULTS

Expression of *Nit1* Protein in Normal Tissues—

To study the function of *Nit1* in murine homeostasis and embryogenesis, we initially examined *Nit1* protein expression in adult mouse tissues and embryos. *Nit1* expression was detected in most tissues and was particularly abundant in adult liver and kidney (Fig. 1, *A* and *C*) and in embryonic adrenal gland and skeletal muscle (Fig. 1*D*). In cultured mouse kidney cells, endogenous *Nit1* was distributed mainly in the cytoplasm, weakly in nuclei, and, apparently, partially in mitochondria (Fig. 1*B*). Because the strongest case for Rosetta Stone proteins decoding real interactions can be made when the separate genes have similar gene expression patterns (1), we compared expression patterns of *Nit1* and *Fhit* in mouse tissues and found that the pattern of *Nit1* expression in adult mouse tissues partially overlapped the *Fhit* pattern (Fig. 1*A*).

***Nit1* Deficiency Results in Up-regulated Growth, Increased Cyclin D1 Expression, and Resistance to DNA-damaging Agents—**We generated conditional knock-out mice in which *Nit1* coding

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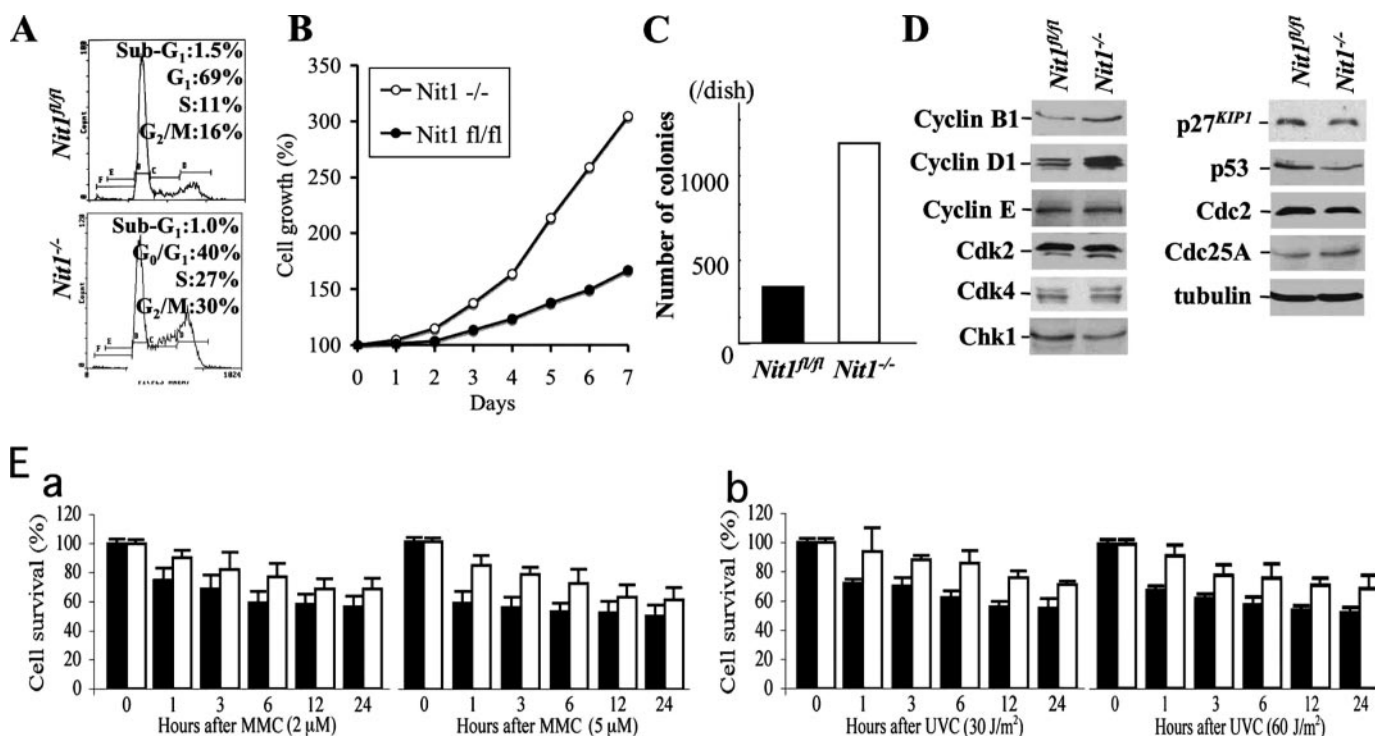


FIGURE 3. Characterization of *Nit1^{-/-}* cells in comparison with *Nit1^{fl/fl}* cells. *A*, flow cytometric analysis. *B*, cell growth assay. *C*, clonogenicity assay. *D*, expression of cell cycle regulators in *Nit1^{fl/fl}* and *Nit1^{-/-}* cells. Note the difference in cyclin D1 expression levels. Anti-tubulin antibody was used for loading control. *E*, enhanced survival of *Nit1*-deficient cells after MMC treatment and UVC light exposure. Percent surviving cells after MMC treatment (*a*) and UVC irradiation (*b*). Viability of cells without treatment was considered to be 100%. Filled column, *Nit1^{fl/fl}* cells; open column, *Nit1^{-/-}* cells. Differences in MMC and UVC sensitivity at 24 h were statistically significant (MMC 2 μ M, $p = 0.0073$; MMC 5 μ M, $p = 0.017$; UVC 30 J/m², $p < 0.0001$; UVC 60 J/m², $p = 0.0005$; Fisher's 2-tailed t test).

exons 4–7 can be deleted through Cre-mediated recombination (Fig. 2A); *Nit1^{fl/+}* mice were mated and *Nit1^{fl/fl}* mice obtained (Fig. 2, B and C). Mice with two floxed *Nit1* alleles developed normally to adulthood; males and females were fertile, without phenotypic abnormalities, suggesting that insertion of the selectable marker did not affect transcriptional regulation of the *Nit1* gene.

To investigate the role of Nit1 in cultured cells, we established *Nit1^{-/-}* cells by infecting *Nit1^{fl/fl}* mouse kidney cells with a retrovirus expressing Cre recombinase. Homozygous genomic deletion of the targeted alleles, decreased levels of truncated *Nit1* mRNA expression, and resultant loss of Nit1 protein expression were confirmed; Fhit expression was unaffected in *Nit1* null cells (Fig. 2D). *Nit1^{-/-}* cells exhibited round and compact shapes, loss of lobular structure, higher cell density with increased S and G₂/M cell populations, and >3-fold higher clonogenicity in comparison with *Nit1^{fl/fl}* cells (Fig. 3, A–C). To clarify mechanisms of acceleration of cell growth induced by Nit1 deficiency, *Nit1^{fl/fl}* and *Nit1^{-/-}* cell lysates were immunoblotted for assessment of expression of specific proteins. Deficiency of Nit1 expression caused higher cyclin D1 expression, whereas differences in the other cell cycle-associated proteins appeared minimal (Fig. 3D). Because Fhit modulates cell survival and resistance to MMC and UVC light (24), we examined sensitivity to these DNA-damaging agents in cells with or without Nit1 expression. Enhanced survival of the *Nit1^{-/-}* cells after treatment with MMC and UVC light was observed; these DNA-damaging agents seemed to influence cell viability in a dose-dependent manner (Fig. 3E).

Mutation of the Nit1 Catalytic Site Did Not Alter Nit1-mediated Biological Effects—Previous reports have demonstrated induction of caspase-3-dependent apoptosis by Ad-*FHIT*-wild-type in human cancer cells (16, 17). However, Nit1 has not been shown to interact with Fhit nor has its biological activity been examined. Thus, we assessed the apoptosis-inducing effect of Ad-*NIT1* and Ad-*FHIT* in cells with or without Nit1 expression. Recombinant protein expression and equivalent infectivity of the two viruses were confirmed by Western blot (data not shown). Irrespective of Nit1 expression, overexpression of *NIT1* and *FHIT* by adenovirus-mediated gene delivery resulted in increased populations of active caspase-3 positive cells and cells with sub-G₁ DNA content in a multiplicity of infection-dependent manner; Ad-*FHIT*-mediated induction of caspase-3-dependent apoptosis was more effective in *Nit1^{fl/fl}* cells than in *Nit1^{-/-}* cells (Fig. 4A). Conversely, we examined Ad-*NIT1* effects in cells with or without Fhit expression. Ad-*NIT1* could induce caspase-dependent apoptosis in A549 (Fhit-positive) and H1299 (Fhit-negative) human lung carcinoma cells (Fig. 4B), as did Ad-*FHIT* as reported previously (14, 15).

Because substrates and interacting proteins for mammalian Nit1 have not been found, we wondered whether the conserved, predicted nitrilase catalytic site is necessary for Nit1 proapoptotic activity. Expression plasmids encoding human wild-type (pNit1) or predicted catalytic site mutant (pNit1-C203A) Nit1, in which the conserved cysteine residue present in nitrilases and amidases (31) is substituted with alanine, were prepared and tested for Nit1 function. Both Nit1 and Nit1-C203A proteins suppressed cell viability and caused increased caspase-3-

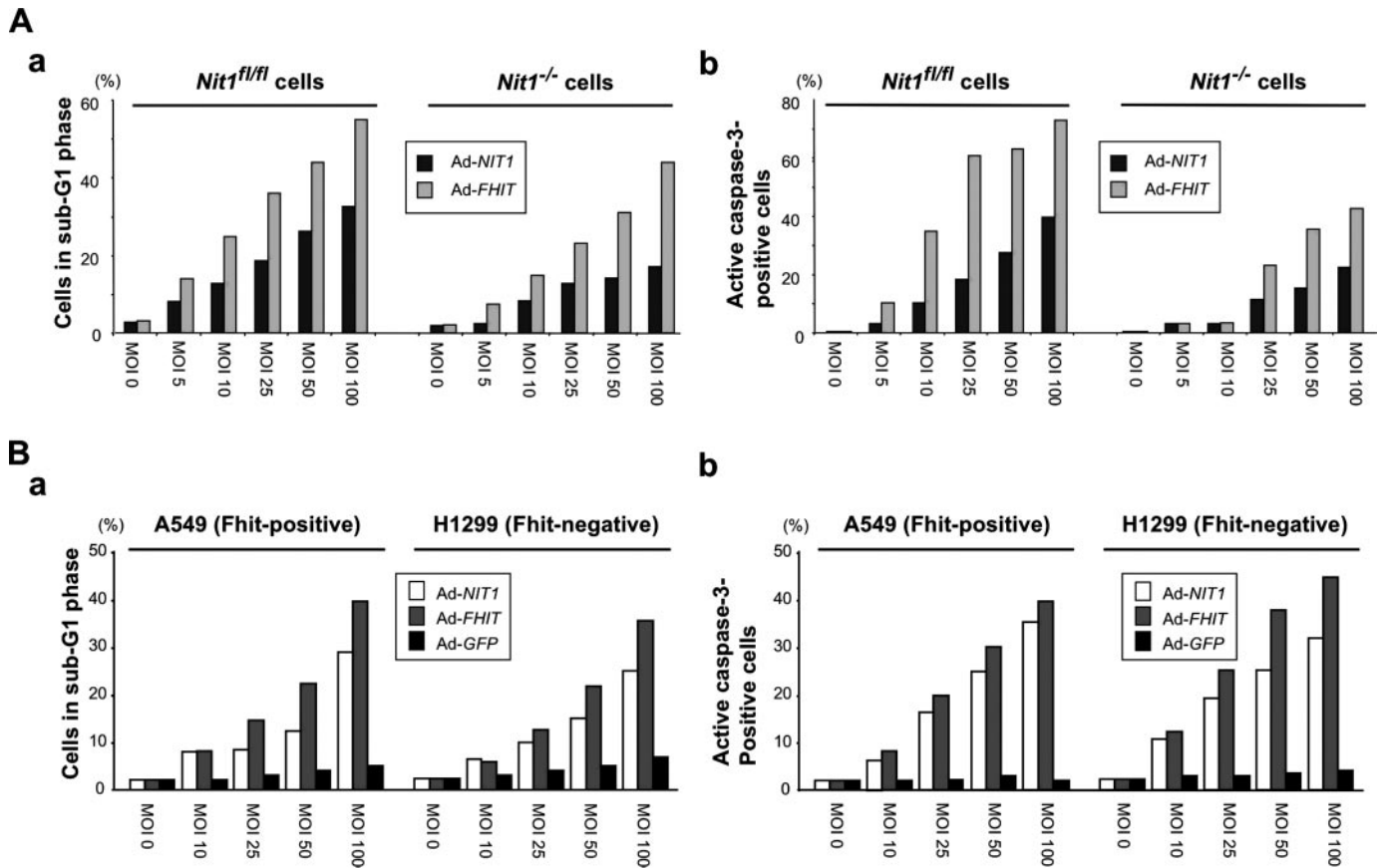


FIGURE 4. Apoptosis-inducing effect of Ad-NIT1 and Ad-FHIT infection. *A*, results of flow cytometric analysis of Nit1^{fl/fl} and Nit1^{-/-} cells infected with Ad-NIT1 and Ad-FHIT (multiplicity of infection 0–100) 5 days after infection. *a*, the percent of cells with sub-G₁ DNA content. *b*, the percent of active caspase-3-positive cells. *B*, results of flow cytometric analysis of A549 (Fhit-positive) and H1299 (Fhit-negative) human lung carcinoma cells 5 days after infection with Ad-NIT1 or Ad-FHIT. *a*, the percent of cells with sub-G₁ DNA content. *b*, active caspase-3-positive cells are shown. As a control, Ad-GFP was used.

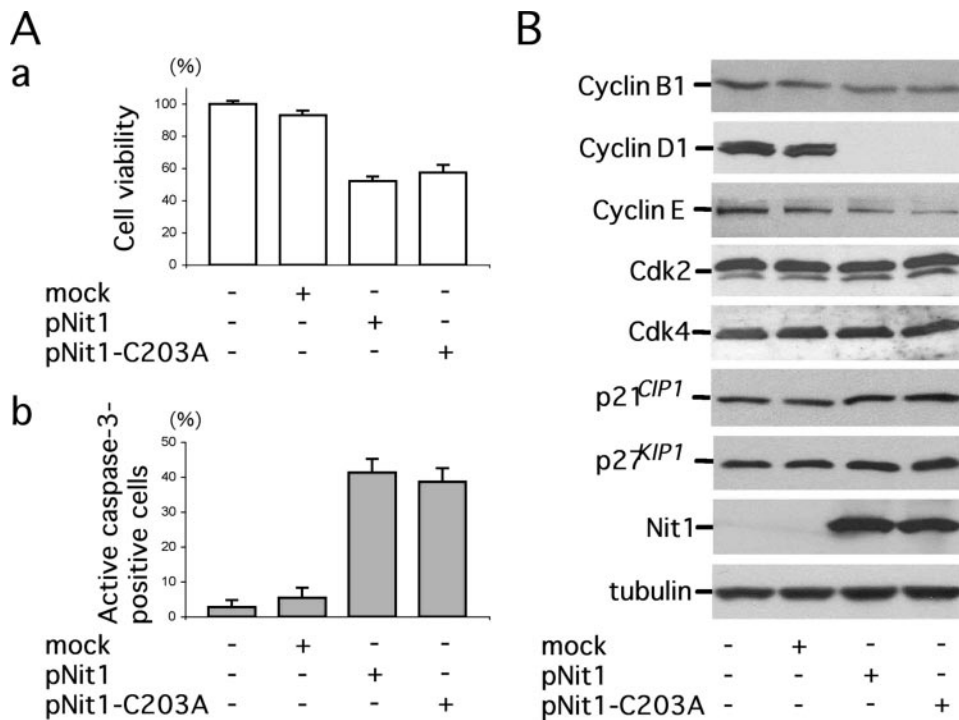


FIGURE 5. Nit1 C-terminal nitrilase catalytic site mutation does not affect Nit1-mediated biological activity. *A*, analysis of Nit1 catalytic site mutant function. *a*, cell viability test. *b*, the percentage of active caspase-3-positive cells. *B*, Western blot showing protein expression levels of the indicated cell cycle regulators in Nit1^{-/-} cells. Antiserum against tubulin was used as a loading control.

dependent apoptosis (Fig. 5A); also, expression of wild-type and C203A mutant Nit1 led to decreased cyclin D1 expression (Fig. 5B). Thus, the predicted catalytic site, C203, is apparently unnecessary for these biological effects of Nit1. Because the mutant Nit1 was overexpressed in these experiments, we cannot rule out the possibility of some loss of function due to the mutation.

Loss of Nit1 Expression Promotes Precocious Mammary Gland Development with Increase of Cyclin D1 Expression—We generated Nit1 conditional knock-out mice carrying a Wap-Cre transgene in the Nit1 homozygous floxed background (Wap-Cre Nit1^{fl/fl}) to address the physiological functions of Nit1 during mouse mammary gland development. Wap-Cre Nit1^{fl/fl} mice appeared normal at birth, their growth indistinguishable from that of their wild-type littermates. To determine the stage at which loss of

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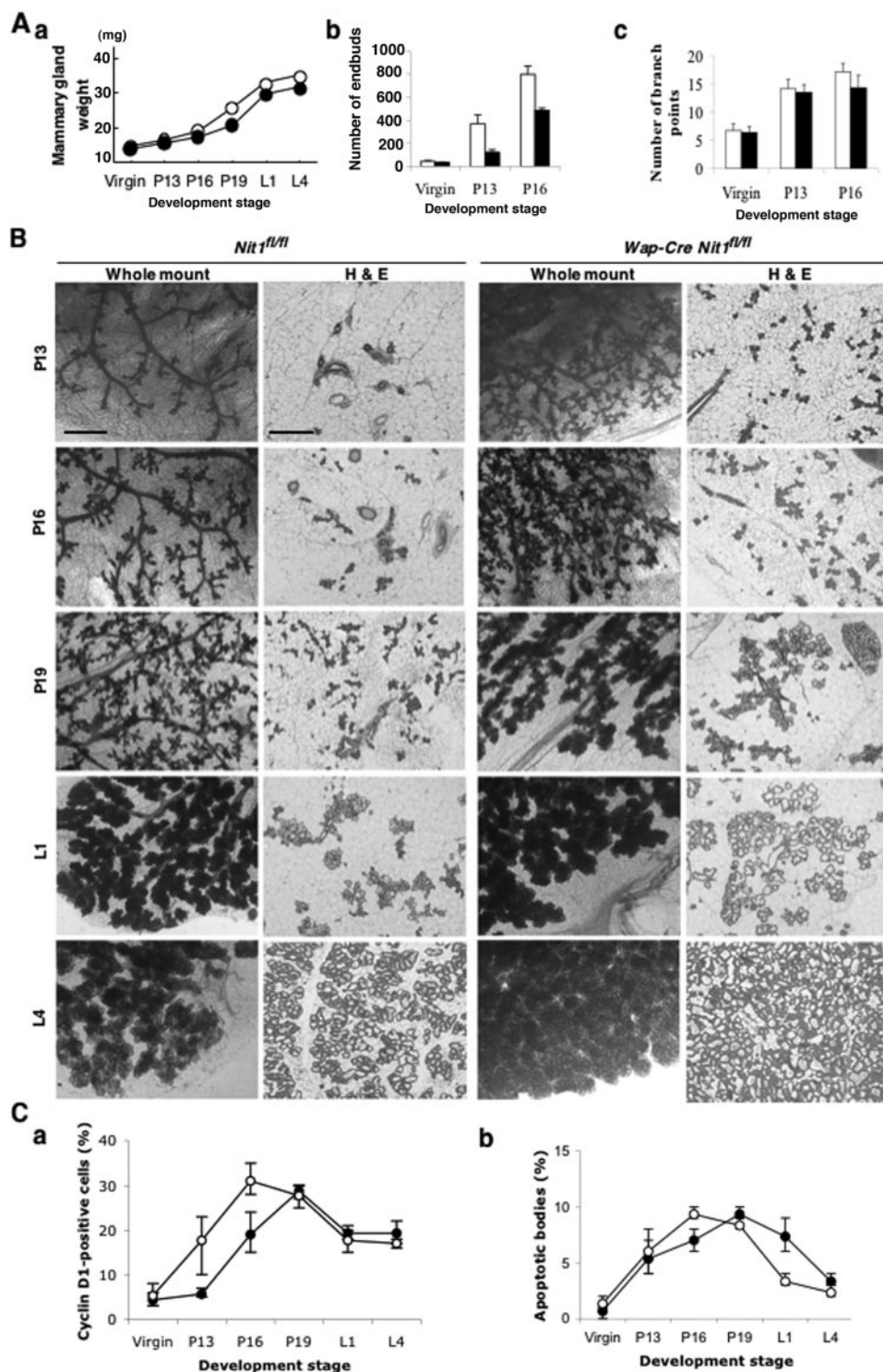


FIGURE 6. Nit1 null mutation in murine mammary glands. A, characterization of mammary glands in the *Nit1^{fl/fl}* and *Wap-Cre Nit1^{fl/fl}* mice. a, average weight of fourth inguinal mammary gland. ●, *Nit1^{fl/fl}*; ○, *Wap-Cre Nit1^{fl/fl}*. b, the number of endbuds (/duct). Filled column, *Nit1^{fl/fl}*; open column, *Wap-Cre Nit1^{fl/fl}*. c, the number of branch points between the nipple and endbuds (/duct). Filled column, *Nit1^{fl/fl}*; open column, *Wap-Cre Nit1^{fl/fl}*. B, whole mount and histological examination of mammary glands. Note the precocious development in *Wap-Cre Nit1^{fl/fl}* mammary glands. Scale bars, 400 μm in whole mount sections and 200 μm in hematoxylin and eosin sections. C, results of immunohistochemical analyses; the number of cyclin D1-positive cells (a) and apoptotic bodies (b). ●, *Nit1^{fl/fl}*; ○, *Wap-Cre Nit1^{fl/fl}*.

Nit1 expression may alter mammary gland development, we analyzed whole mounts and histological sections of mammary glands in *Wap-Cre Nit1^{fl/fl}* mice as well as *Nit1^{fl/fl}* control mice.

Forestomach Tumors—During production and maintenance of *Nit1^{fl/fl}* mice, we also obtained mice with *Nit1^{fl/-}* and *Nit1^{-/-}* genotypes (Fig. 7A). Loss of Nit1 expression was confirmed by

Knock-out of *Nit1* alleles was confirmed by genomic PCR, RT-PCR, and Western blot (supplemental Fig. S1). There were not significant differences in the weights of mammary fat pads and only a small significant difference in the number of branch points (stage P16, $p = 0.045$); however, the number of endbuds in mid-pregnant stages (P13 and P16, $p < 0.0001$) was significantly increased in *Nit1^{fl/fl}* controls (Fig. 6A). Representative illustrations of whole mount and hematoxylin and eosin staining of these mammary glands (Fig. 6B) and *Nit1* immunohistochemistry (supplemental Fig. S1) are shown; at P13, P16, and P19, *Nit1* null mammary glands exhibited an increase in ductal structures, and, at L1 and L4 lobuloalveolar structures were clearly better developed (Fig. 6B). Because the promotion in alveolar development could arise from increased proliferation, decreased apoptosis, or both, we assessed cyclin D1 expression levels and apoptotic bodies. In mid-pregnancy (P13 and P16), the *Nit1*-deficient mammary glands exhibited more cyclin D1-positive cells (P13, $p = 0.04$; P16, $p = 0.023$), but differences in the number of apoptotic bodies were smaller (P16, $p = 0.025$) and occurred precociously (Fig. 6C). We also examined the role of *Nit1* in mouse skin development by crossing with mice carrying the *K14-Cre* transgene but did not observe differences in skin of *K14-Cre-Nit1^{fl/fl}* and control mice either in macroscopical or microscopical appearance. However, the number of cyclin D1-positive cells in the basal layer of epidermis in *K14-Cre Nit1^{fl/fl}* mice was higher than in control mice (supplemental Fig. S2). Conditional *Nit1* null mammary gland and skin did not develop spontaneous tumors during the time of observation.

Deletion of Nit1 Alleles Increases Susceptibility to NMBA-induced

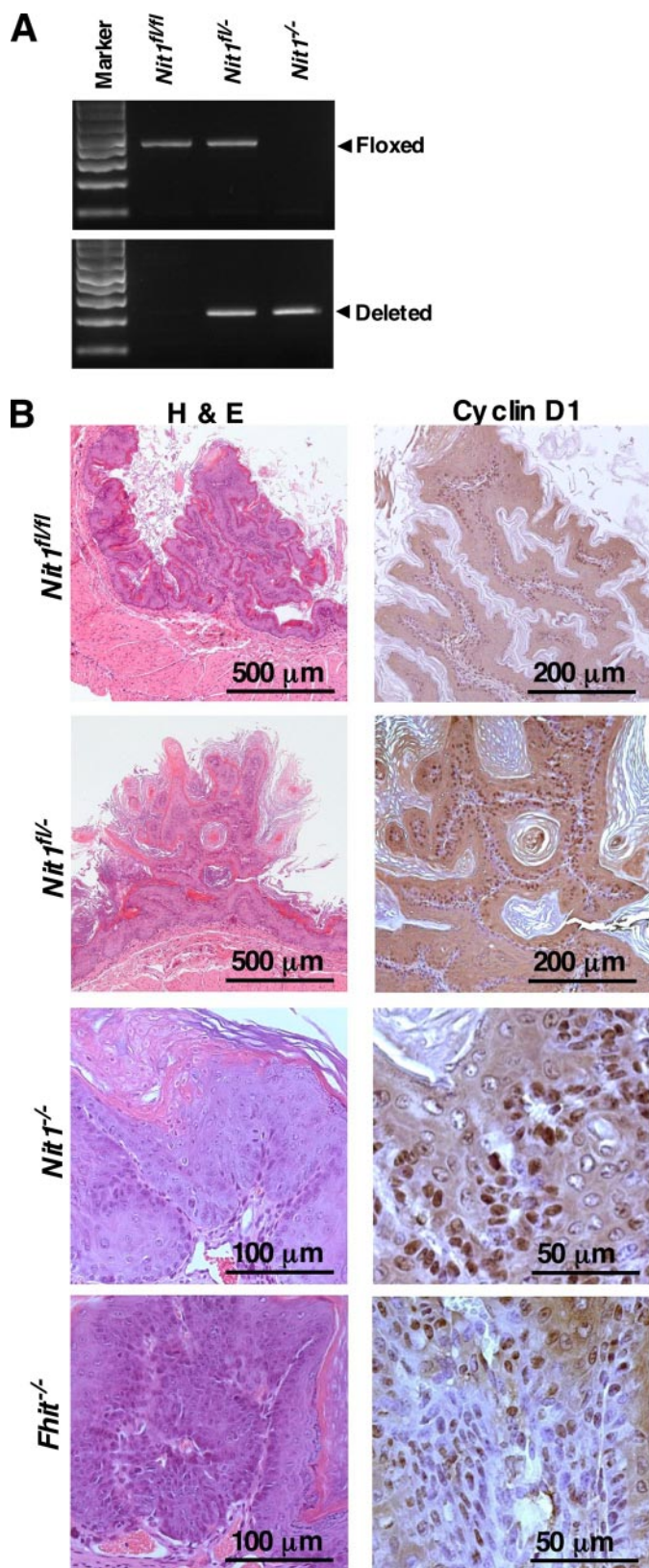


FIGURE 7. NMBA-induced murine forestomach tumors. *A*, genotyping of *Nit1*^{-/-} mouse. *B*, histological and immunohistochemical examination of papillomas developed in *Nit1*^{fl/fl} and *Nit1*^{fl/-} mice and dysplasias (carcinoma *in situ*) developed in *Nit1*^{-/-} and *Fhit*^{-/-} mice. Note that deletion of *Nit1* alleles increased cyclin D1-positive cells, as did homozygous loss of *Fhit* alleles.

RT-PCR, Western blot, and immunohistochemistry (data not shown). These mice were also normal at birth, and their growth and development were indistinguishable from that of their littermate *Nit1*^{fl/fl} mice; therefore, complete *Nit1* deficiency did not affect mouse embryogenesis, development, or fertility. In addition, these mice did not develop spontaneous tumors during a year of observation. According to previous reports, heterozygous and homozygous deletion of *Fhit* alleles increased susceptibility to development of NMBA-induced tumors in the mouse forestomach (33). We therefore assessed the possibility that loss of *Nit1* allele(s) may increase NMBA-induced tumor development. As shown in Table 1, the incidence of tumors involving papillomas and dysplasias (carcinomas *in situ*) in the *Nit1*^{fl/-} and *Nit1*^{-/-} forestomach was significantly higher than in *Nit1*^{fl/fl} forestomach. The frequency of papillomas and dysplasias (carcinoma *in situ*) in *Nit1*^{-/-} mice was almost equivalent to that in *Fhit*^{-/-} mice. The numbers of cyclin D1-positive cells in *Nit1*-deficient tumors tended to be higher than in *Nit1*^{fl/fl} mice (Fig. 7B).

DISCUSSION

According to the Rosetta Stone hypothesis, genomic information opens new pathways to biochemical discoveries; the finding in a genome of many pairs of protein sequences A and B that are both homologs to a single sequence AB in another genome suggests the possibility that A and B are binding partners and provides robust functional information about A and B (1). This is easily applicable to prokaryotes. In eukaryotes, the evolutionary path followed from one state to the other is largely unknown. In human and mouse, the *Nit* and *Fhit* homologs are encoded by two separate genes, *Nit1* and *Fhit*, on chromosomes 1 and 3 in human and 1 and 14 in mouse, respectively. Although *Fhit* binds Ap_nA as a member of the histidine triad nucleotide-binding protein superfamily, the function of *Nit* homologs and substrates is unknown in mammals and there is no obvious connection with the ability to bind and hydrolyze Ap₃A (39). Because *Nit1* is the most highly expressed nitrilase gene among the nitrilase family (*Nit1*, *Nit2*, *Nit3*, and *Nit4*) in plants, *Nit1* has been believed to control auxin plant growth hormone levels (38). The *NitFhit* fusion protein forms a stable tetramer that displays *Fhit* dimers on opposite poles and *Nit* active sites around the equator (31, 39).

We demonstrated ubiquitous *Nit1* expression in adult human and mouse tissues and during mouse embryogenesis, overlapping the pattern of *Fhit* expression and consistent with possible complementary or redundant roles of *Nit1* and *Fhit* in controlling cell growth and apoptosis. Interestingly, the biological effects of *Nit1* under- and overexpression were consistent with a role for *Nit1* as a tumor suppressor: loss of *Nit1* expression promoted cell growth, clonogenicity, and resistance to DNA damage stress, and overexpression of *Nit1* caused caspase-3-dependent apoptosis *in vitro*. Insufficiency of *Nit1* alleles led to enhanced NMBA-induced forestomach tumorigenesis, and conditional *Nit1* null mice showed precocious mammary gland development. The results suggest participation of *Nit1* and *Fhit* in a common signaling pathway. As a result of inactivation of phosphatidylinositol 3-OH kinase-Akt-survivin signaling by *Fhit* overexpression, *Fhit* down-regulates cyclin D1 expression

Tumor Suppressor Effect of Mammalian Nit1

TABLE 1

Effect of Nit1 and Fhit insufficiency on NMBA-induced forestomach lesions

Mouse genotype	Total number of NMBA-treated mice	Dysplasia (carcinoma <i>in situ</i>) incidence	Papilloma incidence	Total tumor incidence
<i>Nit1^{fl/fl}</i>	n = 15	0 (0%)	5 (33%)	5 (33%)
<i>Nit1^{fl/-}</i>	n = 15	0 (0%)	8 (53%)	8 (53%)
<i>Nit1^{-/-}</i>	n = 15	2 (13%)	11 (73%)	13 (87%)
<i>Fhit^{-/-}</i>	n = 15	2 (13%)	12 (80%)	14 (93%)

NMBA treatment was performed as follows. Each *Nit1^{fl/fl}*, *Nit1^{fl/-}*, *Nit1^{-/-}*, and *Fhit^{-/-}* (12–20-week-old) mouse was given intragastric doses of NMBA for 3 weeks (twice a week) at 2 mg/kg body weight. All mice were killed 10 weeks after the initial NMBA dose. Five control animals did not develop any tumors. The difference in *Nit1^{fl/fl}* and *Nit1^{-/-}* total tumor incidence is statistically significant ($p = 0.008$, Fisher's two-tailed t test).

at the protein level; Fhit knock-out mouse tissues have higher levels of cyclin D1 expression, and overexpression of Fhit decreases cyclin D1 expression.³ Thus, down-modulation of cyclin D1 protein levels *in vitro* and *in vivo* is one of the common effects of Nit1 and Fhit expression, suggesting that Nit1 and Fhit may be involved in control of stability of specific proteins, directly or indirectly. The common effects on cyclin D1 expression levels may provide clues to the Nit1-, Fhit-, and Nit-Fhit-mediated tumor suppressor mechanisms. Preliminary studies suggest that p27^{KIP1} is stabilized in Ad-*FHIT*-infected cancer cells,³ supporting the notion that Fhit modulates cell functions through effects on stability of key cell growth regulators. To identify genes differentially expressed in *Nit1^{fl/fl}* and *Nit1^{-/-}* cells, microarray analysis was also performed (supplemental Table S1). We did not detect altered *Ccnd1* mRNA levels, but loss of *Nit1* expression led to up- and down-regulation of expression of various genes involved in cell cycling, checkpoint/chromatin regulation, and apoptosis. Interestingly, the microarray expression profile revealed up-regulated Survivin mRNA expression in *Nit1^{-/-}* cells (supplemental Table S1); Survivin was among genes specifically induced by Ad-*FHIT* (15), supporting the idea that Nit1 and Fhit may be involved in the same pathway.

So is Nit1 and Fhit interaction necessary for their functions? Although the overlapping patterns of Nit1 and Fhit in mouse and human tissues suggests collaboration of Nit1 and Fhit as a Rosetta Stone protein, the apoptosis-inducing effects of Ad-*FHIT* in Nit1-deficient mouse kidney cells and Ad-*NIT1* in Fhit-deficient H1299 lung cancer cells suggested that Nit1-Fhit interaction is not necessary for Nit1- and Fhit-induced apoptosis signaling. Based on lack of detection of a physical interaction between Nit1 and Fhit after co-infection of Ad-*NIT1* and Ad-*FHIT* in H1299 cells (data not shown), Nit1 and Fhit independently might control cell growth and cell death, presumably through the same pathway. Fhit Ap₃A binding activity is required for Fhit tumor-suppressing activity, but a single nucleotide substitution of tyrosine residue at codon 114 (Tyr-114), a phosphorylation target of Src tyrosine kinase family members that alters Ap₃A binding (40, 41), diminished caspase-dependent apoptosis (15). It is intriguing that overexpression of the Nit1-C203A nitrilase mutant had effects similar to wild-type Nit1 in regulation of apoptosis and cyclin D1 levels. Understanding the mechanism of Fhit-mediated signaling may lead to identification of Nit1 interactors and elucidation of the Nit1-Fhit tumor suppression pathway.

The NitFhit fusion protein may have evolved to the mammalian split polypeptides by the “copy-and-paste” process, which allows for multiplication of domains. The human *FRA3B/FHIT* locus is the most active fragile site in the human genome, and frequent deletion or cytogenetic abnormalities at the *FHIT* locus have been reported in many types of human malignancies (7, 9, 13). In *Nit1* recombinant mice, homozygous or heterozygous deletion of *Nit1* alleles increased susceptibility to NMBA-induced forestomach tumors. However, the incidence of loss of Nit1 expression in human breast cancer cases was low,⁴ whereas loss of Fhit expression is frequent (42–72%) and significantly correlated with disease progression and poor prognosis (7). Furthermore, no *NIT1* somatic mutations have been found in various human carcinoma lines.³ The difference in frequencies of gene alteration in the *NIT1* and *FHIT* loci in cancers is likely due to the fact that *FHIT*, unlike *NIT1*, encompasses a fragile locus with extreme susceptibility to DNA damage. Thus, although Nit1 can act as a tumor suppressor in experimental models, in pathways that intersect with Fhit suppressor pathways, it is rarely altered in expression in human cancers.

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Biological Functions of Mammalian Nit1, the Counterpart of the Invertebrate NitFhit Rosetta Stone Protein, a Possible Tumor Suppressor
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