

Suppression of p53-dependent senescence by the JNK signal transduction pathway

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The JNK signaling pathway is implicated in the regulation of the AP1 transcription factor and cell proliferation. Here, we examine the role of JNK by using conditional and chemical genetic alleles of the ubiquitously expressed murine genes that encode the isoforms JNK1 and JNK2. Our analysis demonstrates that JNK is not essential for proliferation. However, JNK is required for expression of the cJun and JunD components of the AP1 transcription factor, and JNK-deficient cells exhibit early p53-dependent senescence. These data demonstrate that JNK can act as a negative regulator of the p53 tumor suppressor.

AP1 | cJun | cell cycle

It is established that the JNK signaling pathway is a critical regulator of AP1-dependent gene expression (1). JNK phosphorylation of a number of AP1 and AP1-related transcription factors (e.g., cJun, JunB, JunD, and ATF2) causes increased AP1 transcription activity. In addition, JNK can regulate AP1 activity by increasing the expression of *cJun* mRNA (by acting at AP1-like sites in the *cJun* promoter) and regulating the half-life of the cJun protein (1). This role of JNK to increase AP1-dependent gene expression suggests that JNK may play an important role in cellular responses to mitogens (2). Indeed, primary murine fibroblasts (MEF) isolated from compound mutant *Jnk1*^{-/-} *Jnk2*^{-/-} embryos proliferate slowly (3).

The observation that MEF that lack JNK expression grow slowly suggests that JNK may function to regulate cell-cycle progression. However, the target of JNK signaling that mediates this effect of JNK on proliferation has not been defined. In addition, it is unclear whether the requirement of JNK for proliferation reflects a role for JNK-mediated phosphorylation of a substrate that is critically required for cell-cycle progression or whether it is the prolonged absence of JNK that causes slow growth.

To test whether JNK is required for proliferation, we examined the effect of acute loss of JNK function on cellular proliferation. A drug with validated specificity for JNK is not yet available (4). We therefore used genetic approaches to examine the effect of acute loss of JNK function on proliferation.

Results and Discussion

Construction of a Conditional *Jnk1* Allele. Mice with a *floxed* allele of *Jnk1* were constructed by using standard techniques (Fig. 1 A–C). These mice were crossed with JNK2-deficient mice to create *Jnk1*^{LoxP/LoxP} *Jnk2*^{-/-} mice. Primary MEF prepared from these animals expressed JNK1. Alternative splicing of *Jnk1* mRNA results in the expression of 46-kDa isoforms of JNK1 with a small amount of 54-kDa JNK1 isoforms (5). *Cre*-mediated excision of the *floxed Jnk1* alleles resulted in primary MEF that lack detectable JNK expression (Fig. 1D). We have previously reported that JNK-deficient primary MEF display reduced UV-stimulated apoptosis and increased TNF-stimulated apoptosis (3, 6). The compound knockout MEF derived from conditional JNK mice similarly exhibited reduced UV-

stimulated apoptosis and increased TNF-stimulated apoptosis [supporting information (SI) Figs. 7 and 8]. These data indicate that primary MEF isolated from conditional JNK-deficient mice are suitable for the analysis of the acute effects of compound JNK deficiency.

PCR analysis of genomic DNA demonstrated that the *Cre*-mediated deletion of *floxed Jnk1* alleles was rapid. However, immunoblot analysis indicated that JNK1 expression slowly decreased after *Jnk1* gene ablation (Fig. 2A). Indeed, low levels of JNK1 protein were detected at 3 days after *Cre*-mediated deletion. This slow loss of JNK1 protein expression is consistent with the half-life of the JNK1 protein (≈ 8 h) (1). Nevertheless, MEF without JNK (*Jnk-null* MEF) were obtained within 6 days after exposure to *Cre*-recombinase (Fig. 2A).

JNK Deficiency Causes a Delayed Decrease in Proliferation. We have previously reported that MEF isolated from *Jnk1*^{-/-} *Jnk2*^{-/-} embryos grow slowly (3). In contrast, flow cytometry analysis of BrdU incorporation into DNA demonstrated that JNK deficiency caused no decrease in DNA synthesis at passage 3 (9 days) after *Cre*-mediated ablation of JNK expression (Fig. 2B). This observation indicates that there is no essential requirement of JNK for proliferation. The slow growth of MEF isolated from *Jnk1*^{-/-} *Jnk2*^{-/-} embryos (3) contrasts with the rapid proliferation of *Jnk-null* MEF created by using *Cre*-recombinase. One possible explanation for the different phenotypes of these MEF is that slow growth is a delayed consequence of JNK deficiency. To test this hypothesis, we passaged MEF by using a 3T3 protocol (Fig. 2 C and D). Studies of *Jnk-null* MEF demonstrated a progressive decrease in growth with increasing passage number. Indeed, at passage 7 (21 days after *Cre*-mediated JNK ablation) the *Jnk-null* MEF exhibited a flattened morphology (Fig. 2C) and proliferated very slowly (Fig. 2D). Together, these data indicate that JNK is not essential for proliferation, but long-term JNK deficiency causes a severe decrease in proliferation.

The mechanism that accounts for the delayed requirement of JNK for proliferation is unclear. One possible explanation is that JNK deficiency causes changes in gene expression. It is established that JNK is a critical regulator of the AP1 transcription factor (1) and that AP1 is important for proliferation (2). Decreased AP1 in *Jnk-null* MEF may therefore contribute to the

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Abbreviations: MEF, murine fibroblasts; 1NM-PP1, 1-naphthylmethyl-4-amino-1-*tert*-butyl-3-(*p*-methylphenyl)pyrazolo[3,4-*d*]pyrimidine; HPV, human papilloma virus.

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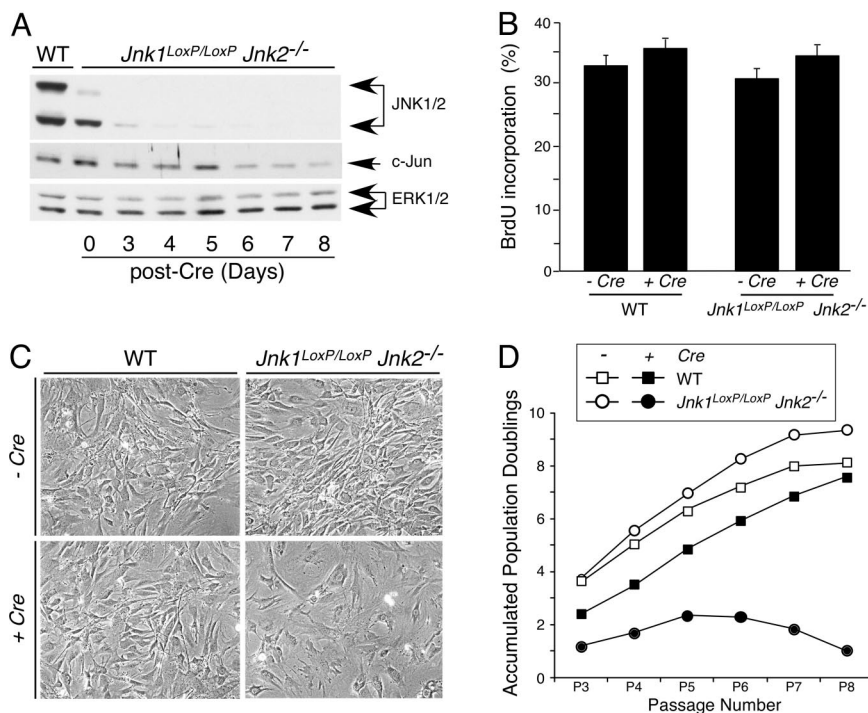


Fig. 2. JNK deficiency causes a delayed reduction in cellular proliferation. (A) Time-course analysis of JNK expression after gene ablation. *Jnk1^{LoxP/LoxP} Jnk2^{-/-}* MEF were transduced by using a retroviral Cre expression vector. Cell extracts were prepared on different days after infection and examined by immunoblot analysis using antibodies to JNK, c-Jun, and ERK. Extracts prepared from WT MEF were also examined. (B) The effect of retroviral transduction of Cre in WT and *Jnk1^{LoxP/LoxP} Jnk2^{-/-}* MEF was examined 10 days after infection by measurement of the incorporation of BrdU by flow cytometry. (C) The morphology of WT and *Jnk1^{LoxP/LoxP} Jnk2^{-/-}* MEF at passage 7 after retroviral transduction of Cre was examined by phase-contrast microscopy. (Magnification: $\times 100$.) (D) The growth of WT and *Jnk1^{LoxP/LoxP} Jnk2^{-/-}* MEF was examined by using a 3T3 assay. The data are presented as the accumulated population doublings during eight passages in culture. The effect of retroviral transduction of Cre was investigated.

Role of JNK in Cell Proliferation and Senescence. The conclusion that JNK is not required for proliferation markedly contrasts with conclusions drawn from previous studies (3). Acute loss of JNK function does not inhibit proliferation (Fig. 2B). However, JNK deficiency does cause p53-dependent senescence (Fig. 4). The slow proliferation of JNK-deficient MEF isolated from *Jnk1^{-/-} Jnk2^{-/-}* embryos that was reported previously (3) is most likely a consequence of chronic JNK deficiency and p53-dependent senescence rather than a direct role for JNK in the cell cycle. Interestingly, the effect of JNK deficiency to cause early p53-dependent senescence is similar to that caused by deficiency of the AP1 proteins cJun (10, 16) and JunD (9). The large decrease in cJun and JunD expression caused by JNK deficiency (Fig. 3) may therefore contribute to the engagement of the p53 senescence pathway in *Jnk*-null MEF. Indeed, ectopic expression of JunD in *Jnk*-null MEF partially suppressed p53-mediated senescence (SI Fig. 9).

The progressive decrease in AP1 protein expression after loss of JNK over several days (Figs. 2A and 3B) may explain the finding that loss of JNK does not immediately inhibit proliferation (Fig. 2B), but p53-induced senescence is observed later (Fig. 4). Collectively, these data indicate that JNK can act as a negative regulator of the p53 tumor suppressor. This conclusion is consistent with recent reports that have implicated a role for the JNK signaling pathway in some human tumors (17).

Materials and Methods

Animal Studies. Mice with disruptions of the *Jnk1* or *Jnk2* genes or a knock-in mutation in the *Jnk2* gene (*Jnk2^{MG}*) have been described (13, 18, 19). Mice expressing a 4-hydroxytamoxifen-inducible Cre recombinase from the endogenous

Gt(ROSA)26Sor promoter (20) were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice with a germ-line mutation in the *Jnk1* gene with *LoxP* elements inserted into two different introns (*Jnk1^{LoxP}*) were constructed by using homologous recombination in ES cells. A mouse strain 129/Svev genomic BAC clone containing the *Jnk1* gene was used to create a targeting vector with a floxed *Neo^R* cassette and a thymidine kinase cassette (Fig. 1A). ES cells were electroporated with this vector and selected with 200 μ g/ml G418 and 2 μ M gangcyclovir. Two correctly targeted ES cell clones were identified by Southern blot analysis and PCR analysis. These ES cell clones were injected into C57BL/6J blastocysts to create chimeric mice that transmitted the mutated *Jnk1* allele through the germ line. The floxed *Neo^R* cassette was excised by using Cre recombinase. The mice were backcrossed to the C57BL/6J strain (Jackson Laboratory) and housed in a facility accredited by the American Association for Laboratory Animal Care. These studies were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Genotype Analysis. The genotype at the *Jnk1* locus was examined by Southern blot analysis of BamHI-restricted genomic DNA by probing with a random-primed ³²P-labeled probe (320 bp) that was isolated by PCR using a *Jnk1* genomic clone as the template and the primers 5'-GGTACTGTGTCAGCCTGGTCAAG-3' and 5'-GGGGGAACCAACCAAAAAAC-3'. The WT and conditional alleles of *Jnk1* were also detected by PCR amplification of genomic DNA using the primers 5'-GGATTTATGCCCTCTGCTTGTC-3' and 5'-GAACCACTGTTCCAATTTCATCC-3'; agarose gel electrophoresis indicates the presence of a 520-bp DNA fragment (WT *Jnk1* allele) or a 330-bp DNA

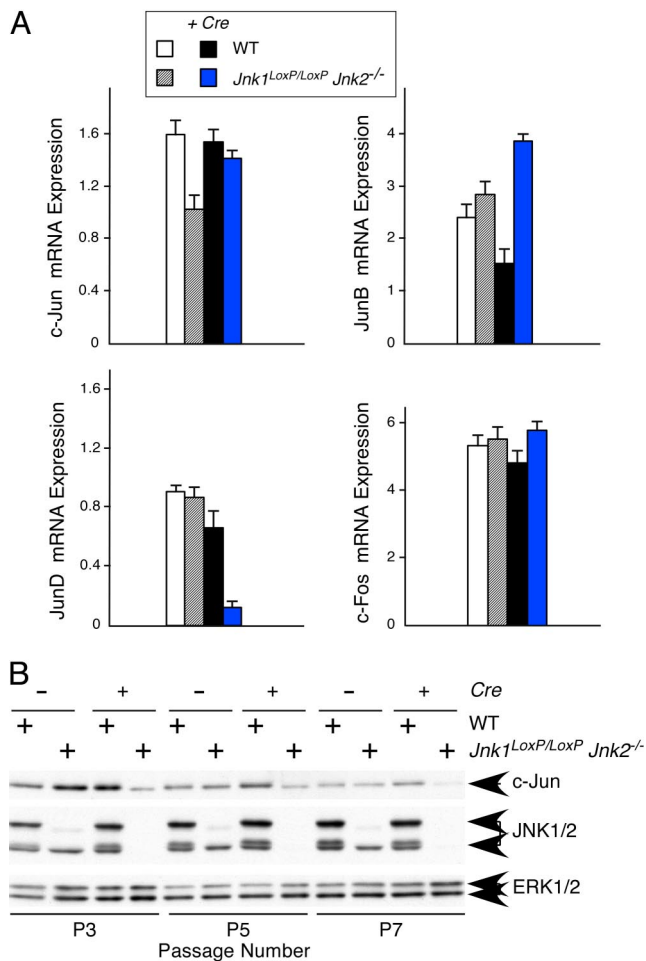


Fig. 3. JNK deficiency causes reduced expression of cJun and JunD. (A) WT and *Jnk1^{LoxP/LoxP} Jnk2^{-/-}* MEF were transduced by using a Cre retroviral expression vector. RNA was isolated from these cells at passage 7 postinfection and examined by quantitative RT-PCR (Taqman) analysis using probes for AP-1 transcription factors (cJun, JunB, JunD, and cFos) and GAPDH. The data are presented as the normalized ratio of [AP-1 mRNA]/[GAPDH mRNA]. (B) Protein extracts were prepared from MEF at 3T3 passages 3, 5, and 7 postinfection and examined by immunoblot analysis using antibodies to cJun, JNK, and ERK.

fragment (*floxed Jnk1* allele). The deleted *Jnk1* allele ($\Delta Jnk1$) was detected by PCR amplification of genomic DNA using the primers 5'-CCTCAGGAAGAAAGGGCTTATTTC-3' and 5'-GAACCACTGTTCCAATTTCCATCC-3'; agarose gel electrophoresis indicates the presence of a 1,550-bp fragment (WT *Jnk1* allele), a 1,095-bp DNA fragment (*floxed Jnk1* allele), or a 395-bp DNA fragment ($\Delta Jnk1$ allele).

Primary Cell Culture. MEF were isolated from E13.5 embryos and cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 units/ml of penicillin, and 100 μ g/ml of streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere with 5% CO₂. Similar data were obtained in experiments using independently isolated MEF cultures. The cells were treated without or with the drug 1NM-PP1 (13). Retroviral transduction experiments were performed with a self-inactivating vector (HR-MMPCreGFP) that transiently expresses Cre recombinase (21), a vector (pLXSN16E6) that expresses HPV-16 E6 (22), and a vector (pMSCV-JunD-IRES-GFP) that expresses JunD (6). MEF proliferation was monitored with a 3T3 protocol (23). Proliferation assays were performed by staining with crystal violet (3), measurement of cell number with a hemacytometer, and measurement of BrdU incorporation by

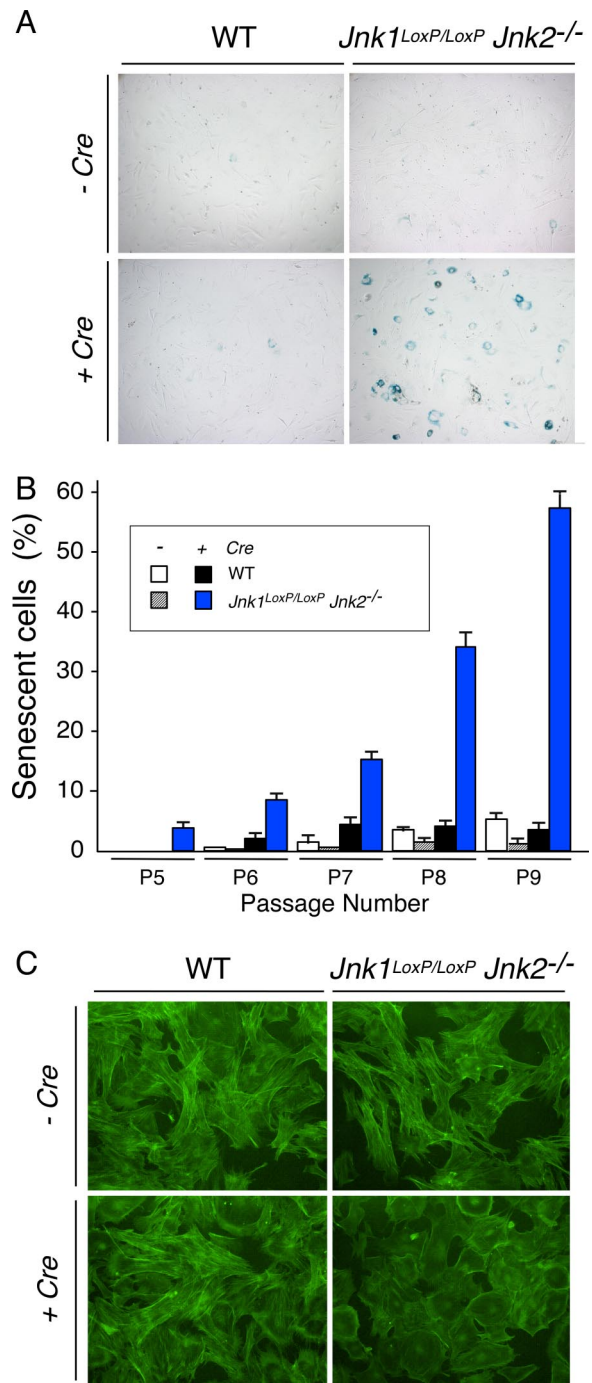


Fig. 4. JNK deficiency causes senescence. (A) WT and *Jnk1^{LoxP/LoxP} Jnk2^{-/-}* MEF were transduced by using a Cre retroviral expression vector and stained for a senescence marker (acidic β -gal) using X-Gal. A culture stained at 3T3 passage 9 postinfection is illustrated. (B) The percentage of cells that stained with X-Gal was examined at passages 5, 6, 7, 8, and 9 postinfection was quantitated. The data shown represent the mean \pm SD of three independent experiments. (C) MEF at 3T3 passage 9 postinfection were stained with phalloidin (green) and examined by fluorescence microscopy. (Magnification: $\times 100$.)

flow cytometry (24). Senescent cells were detected by staining for β -gal using X-Gal (Cell Signaling, Danvers, MA).

Fluorescence Microscopy. MEF were fixed with 2% formaldehyde (15 min), permeabilized with 0.1% Triton X-100/PBS (5 min), stained with Alexa Fluor 488-conjugated phalloidin (Invitrogen)

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