Induction of p53-Dependent Senescence by the MDM2 Antagonist Nutlin-3a in Mouse Cells of Fibroblast Origin

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Abstract

Cellular senescence is emerging as an important *in vivo* anticancer response elicited by multiple stresses, including currently used chemotherapeutic drugs. Nutlin-3a is a recently discovered small-molecule antagonist of the p53-destabilizing protein murine double minute-2 (MDM2) that induces cell cycle arrest and apoptosis in cancer cells with functional p53. Here, we report that nutlin-3a induces cellular senescence in murine primary fibroblasts, oncogenically transformed fibroblasts, and fibrosarcoma cell lines. No evidence of drug-induced apoptosis was observed in any case. Nutlin-induced senescence was strictly dependent on the presence of functional p53 as revealed by the fact that cells lacking p53 were completely insensitive to the drug, whereas cells lacking the tumor suppressor alternative reading frame product of the *CDKN2A* locus underwent irreversible cell cycle arrest. Interestingly, irreversibility was achieved in neoplastic cells faster than in their corresponding parental primary cells, suggesting that nutlin-3a and oncogenic signaling cooperate in activating p53. Our current results suggest that senescence could be a major cellular outcome of cancer therapy by antagonists of the p53-MDM2 interaction, such as nutlin-3a. [Cancer Res 2007;67(15):7350–7]

Introduction

The tumor suppressor p53 is among the most important antitumoral defenses in mammalian cells. This is reflected by the fact that the large majority of malignant tumors have acquired mutations that impair the functionality of p53. All together, approximately half of all human malignancies harbor mutations in p53 (1). The remaining tumors usually contain alterations in the two main regulators of p53 stability [i.e., amplification of the negative p53 regulator murine double minute-2 (MDM2) or loss of the MDM2 inhibitor alternative reading frame product of the *CDKN2A* locus (ARF); refs. 1–3]. In agreement with the high frequency of aberrations in the ARF/MDM2 regulatory pair, we and others have recently shown that ARF is critical in mice for p53-dependent tumor suppression (4, 5).

The importance of ARF for p53-dependent cancer protection suggests that ARF-mimicking drugs could have therapeutic activity in p53-proficient cancers. Nutlin-3a was recently discovered as a small molecule that binds MDM2 at the pocket used for interaction with p53 (6–8). Hence, nutlin-3a prevents MDM2 from recognizing p53 and, consequently, it results in stabilization of p53 and activation of the p53 pathway. p53 is thought to acquire full transcriptional activity by the concurrence of two effects: stabilization, usually achieved by inhibition of MDM2, and posttranslational modifications produced by stress signaling cascades (1). Cancer cells, and even normal cells under *in vitro* culture conditions, are subject to intrinsic and extrinsic stresses, generally presenting high constitutive levels of DNA damage signaling; therefore, it is not surprising that stabilization of p53 by nutlin-3a suffices to trigger p53 transcriptional activity. Moreover, it is also possible that simple stabilization of p53, in the absence of stress signaling, could be sufficient to activate a p53 transcriptional response (9, 10). The rationale for using MDM2 antagonists in cancer treatment is twofold (8). First, malignant cells have constitutive stress signals, particularly DNA damage signaling (11, 12), which presumably will contribute to enhance the p53 activation upon treatment with a p53-stabilizing drug. In this regard, additional infliction of damage to tumor cells by standard genotoxic chemotherapy is known to enhance the effects of nutlin (13–17). Second, normal cells, by having intact checkpoints and low stress signaling, are thought to undergo a mild and transitory p53-dependent cell cycle arrest after treatment with nutlin, and this transient arrest, in fact, may protect normal cells from the toxicity of standard chemotherapy (18, 19). The latter approach implies that nutlin, by protecting normal tissues, could increase the therapeutic window to standard chemotherapy even in the treatment of p53-deficient tumors.

The study of the cellular responses elicited by nutlin has been restricted mainly to cell cycle arrest and apoptosis; however, the ability of nutlin to induce cellular senescence has remained unexplored. Cellular senescence is emerging as a particular type of cell cycle arrest of high relevance for tumor suppression and chemotherapy response (20–23). Indeed, there are indications that senescence may play an important role in tumor regression induced by standard genotoxic chemotherapy (24, 25). Cellular senescence is triggered by a multitude of stresses and, in general, it requires the engagement of the Rb and p53 tumor suppressor pathways (26). A main feature that distinguishes cellular senescence from other forms of cell cycle arrest is the irreversibility of this phenomenon. Full engagement of the Rb and p53 pathways results in chromatin remodeling that includes the heterochromatinization of genes important for proliferation (27–29). In this manner, cellular senescence is a terminal stage that prevents further proliferation of the cell, even if the initial causative stress is eliminated or transient. As we have discussed elsewhere (22), the fact that senescent cells are viable *in vitro* for long periods of time does not necessarily imply a long-term residence time in the context of the organism. In this regard, it has recently been shown that the induction of

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tumor senescence by p53 is followed by clearance of the senescent cells by the innate immune system, which results in tumor regression (30, 31). Here, we have taken advantage of genetically defined mouse cells, either primary or neoplastically transformed, to examine the ability of nutlin-3a to induce senescence.

Materials and Methods

Cells and reagents. Mouse embryonic fibroblasts (MEF) from E13.5 embryos of wild-type (wt), ARF-null (32), p53-null (33), or p21-null (34) genotype were obtained as previously described (35) and grown in the presence of atmospheric oxygen and in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone). In all experiments, low-passage (<3 passages) MEFs were used. Oncogenic Ras (H-Ras^{V12}) was ectopically expressed from vector pLPC-puro and cells were retrovirally transduced following standard procedures (36). Infected cells were selected with 1.5 μg/mL puromycin for 2 to 3 days as described (37). Murine fibrosarcoma cell lines were obtained from tumors induced with 3-methyl-cholanthrene as previously described (38). On observation of overt tumors, mice were sacrificed and tumors were excised and processed as follows. The tumor mass was immersed briefly in PBS with antibiotics. Small pieces of the tumor mass were minced in DMEM plus 10% FBS with scissors and allowed to attach to 60-mm-diameter dishes. Clonal cell lines were established from tumor outgrowths after four to six passages. Nutlin-3a and nutlin-3b were provided by Hoffman-La Roche, Inc..

Colony formation assays. Cells (10^4) were seeded in 10-cm-diameter plates and treatment was initiated the following day by addition of nutlin-3a or nutlin-3b (5 μmol/L). The drug was added only at the beginning of the treatment. One week later, cells were fixed in paraformaldehyde and stained with Giemsa. For reversibility studies, cells were exposed to the drug for 1 week, followed by another week in the absence of the drug.

Proliferation assays. Cells (5×10^4) were plated in 3.5-cm-diameter plates and, 24 h later, 5 or 10 μmol/L of nutlin-3a or nutlin-3b was added. For direct cell counting, attached cells were trypsinized and counted using a Neubauer chamber slide. Reversibility was evaluated by exposing the cells to the drug for variable periods of time, as indicated, followed by a recovery period of 3 days in the absence of the drug. For S-phase and sub-G_0-G_1 quantification, both floating and attached cells were fixed in 70% ethanol, resuspended in PBS, and treated with RNase A. Propidium iodide was added to the cells and cell cycle profiles were analyzed by flow cytometry in a FACScalibur instrument (BD Biosciences) and using the ModFit software (Verity Software House).

Senescence-associated β-galactosidase staining. Cells (5×10^4) were plated in 3.5-cm-diameter plates and treated for 1 week with nutlin-3a or nutlin-3b (10 μmol/L). For senescence-activated β-galactosidase (SA-βGal) staining, we used Senescence-βGal Staining Kit (Cell Signaling Technology) following the manufacturer's instructions.

Immunoblots. Whole-cell protein extracts were obtained using RIPA buffer. For immunoblotting, we used the following primary antibodies: anti-p53 (NCL-p53-CM-5p, Novocastra), anti–phospho-Ser^{18} p53 (Cell Signaling Technology), anti-p21 (p21-C-19-G, Santa Cruz Biotechnology), anti-MDM2 (2A10, Abcam), and anti–β-actin (clone AC-15, Sigma). Protein levels were visualized after incubation with the appropriate secondary antibodies conjugated with horseradish peroxidase followed by detection with enhanced chemiluminescence plus (Amersham).

Results

Nutlin-3a induces p53-dependent cell cycle arrest in primary MEFs. Normal primary MEFs have a limited proliferation potential under standard culture conditions (i.e., 20% oxygen and 10% serum) due to the accumulation of oxidative and mitogenic stress (39, 40). The main molecular barrier activated by "*in vitro* culture" stress in murine fibroblasts is the ARF/p53 pathway and, accordingly, ARF-null or p53-null MEFs have an indefinite proliferative potential in culture (32, 41). Culture stress can be evaluated by plating cells at low density and scoring their ability to form colonies. We began our studies on nutlin by evaluating its effect on colony formation. In the presence of nutlin-3a, primary ARF-null MEFs lost their capacity to form colonies whereas p53-null MEFs were completely insensitive (Fig. 1*A*). Wild-type MEFs, as expected, could not form colonies either in the absence or presence of nutlin-3a. To better define the antiproliferative effect of nutlin-3a, we carried out short-term proliferation assays. Cell proliferation was measured directly by cell counting (Fig. 1*B*) and by cytometry to measure the proportion of cells in S phase (Fig. 1*C*). Nutlin-3a was a potent proliferation inhibitor for wt and ARF-null MEFs, whereas it had no effect on the proliferation of p53-null MEFs. As a control, the enantiomer nutlin-3b, which has 150-fold less activity compared with nutlin-3a (7), did not affect the proliferation of MEFs (Fig. 1*B* and *C*). Noteworthy, cell numbers for wt and ARF-null MEFs remained essentially constant in the presence of nutlin-3a, suggesting that the drug has a cytostatic effect on these cells (see Fig. 1*B*). In this regard, cytometric analyses did not show evidence of apoptosis in any of the genotypes on prolonged exposure to nutlin-3a (data not shown). The activation of p53 by nutlin-3a was confirmed by immunoblot analysis that showed stabilization of p53, increased p53 phosphorylation at Ser^{18}, and increased levels of the p53 downstream targets MDM2 and p21 (Fig. 1*D*). It is known that p21 is an important, although not essential, mediator of p53-dependent cell cycle arrest (42). In line with this, we observed that p21-null MEFs arrest efficiently on treatment with nutlin (Supplementary Fig. S1*A*), although the irreversibility of the arrest has slower kinetics compared with wt MEFs (see below). Together, these results indicate that nutlin-3a inhibits the proliferation of primary MEFs in a p53-dependent manner.

Nutlin-3a induces p53-dependent arrest in MEFs expressing oncogenic Ras. Primary ARF-null MEFs and p53-null MEFs are permissive to Ras-driven proliferation, whereas wt MEFs undergo a permanent proliferative arrest, termed oncogene-induced senescence, which is dependent on the activation of the ARF/p53 pathway (43). We wondered whether the presence of oncogenic signaling would affect the response of cells to nutlin. Primary MEFs of different genotypes were retrovirally transduced with H-Ras^{V12} and the resulting cells were tested for their sensitivity to nutlin. The ability of ARF-null/Ras MEFs to form macroscopic colonies was completely abrogated in the presence of Nultin-3a, whereas p53-null/Ras MEFs were not affected by the presence of the drug (Fig. 2*A*). As expected, wt/Ras MEFs were unable to form colonies even in the absence of nutlin due to the activation of oncogene-induced senescence. In short-term assays, the proliferation of ARF-null/Ras MEFs was completely blocked by nutlin-3a, whereas p53-null/Ras MEFs proliferated in the presence of the drug (Fig. 2*B*). On the other hand, Ras-infected wt or p21-null MEFs had a severely impaired proliferation due to oncogenic stress, and the residual proliferation disappeared in the presence of nutlin-3a (see Fig. 2*B* and Supplementary Fig. S1*B*). As it was the case with primary MEFs, the drug seemed to have a cytostatic effect and no evidence of apoptosis was observed in any of the Ras-expressing cells regardless of the functionality of the ARF/p53/p21 pathway (data not shown). As shown in Fig. 2*C*, the stabilization and activation of p53 was confirmed by immunoblot analysis in MEFs after selection of retrovirally infected cells (i.e., before the onset of oncogene-induced senescence in wt/Ras MEFs). A strong accumulation of p53 and phospho-Ser^{18} p53 was evident in wt/Ras and ARF-null/Ras cells treated with nutlin-3a, together

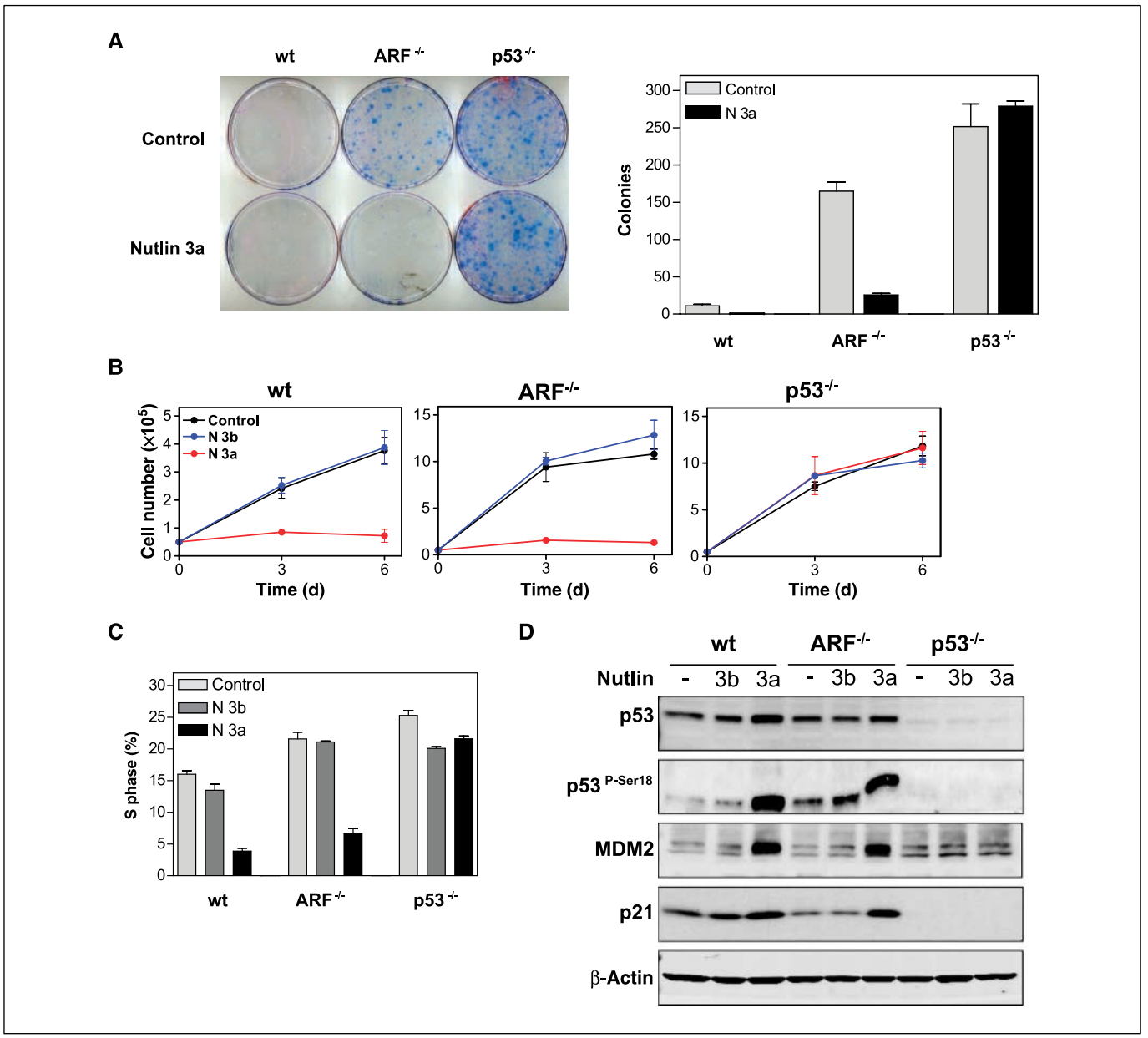

Figure 1. Nutlin-3a induces p53-dependent cell cycle arrest in primary MEFs. *A,* cells of the indicated genotypes were seeded and allowed to form macroscopic colonies during 1 wk in the absence or presence of nutlin-3a (5 μmol/L); after this time, plates were fixed and stained (*left*). The number of colonies was scored in three independently derived MEFs for each genotype (*right*). *Columns,* average; *bars,* SE. *B,* MEFs of the indicated genotypes were treated with nutlin-3a or nutlin-3b (5 μmol/L) and the number of cells was counted at the indicated days. *Points,* average of three independent MEF preparations for each genotype; *bars,* SE. Representative of a total of three assays. *C,* cells treated for 2 d with nutlin-3a or nutlin-3b (10 μmol/L) were used to quantify the proportion of cells in S phase by cytometry using propidium iodide. *D,* immunoblot analysis of cells treated for 8 h with nutlin-3a or nutlin-3b (5 μmol/L).

with increased levels of p21 and MDM2. In summary, we conclude that nutlin-3a blocks Ras-driven proliferation of MEFs in a p53-dependent manner.

Nutlin-3a induces p53-dependent senescence. The activation of the p53 pathway, either by strong DNA damage or by oncogenic stress, is known to result in an irreversible cell cycle arrest termed senescence (44, 45). We asked whether the cell cycle arrest produced by nutlin in primary MEFs and Ras-infected MEFs had features of senescence. First, nutlin-3a induced a p53-dependent morphologic change in primary MEFs (not shown) and in Ras-infected cells (Fig. 3*A, left*), which is compatible with senescence (i.e., large and flat cells). Moreover, both primary and Ras-infected ARF-null MEFs treated with nutlin-3a became positive for the widely used senescence marker SA-βGal (Fig. 3*A, right*; ref. 46). As a first approach to evaluate the irreversibility of the arrest induced by nutlin, we carried out colony formation assays in which cells were exposed to the drug for 1 week and then were incubated for an additional week in the absence of the drug. Both primary and Ras-infected ARF-null MEFs were unable to form colonies under these conditions (Fig. 3*B*), suggesting that the arrest induced by nutlin is irreversible after 1 week of exposure to the drug. To estimate the minimal exposure time required to trigger an irreversible cell cycle

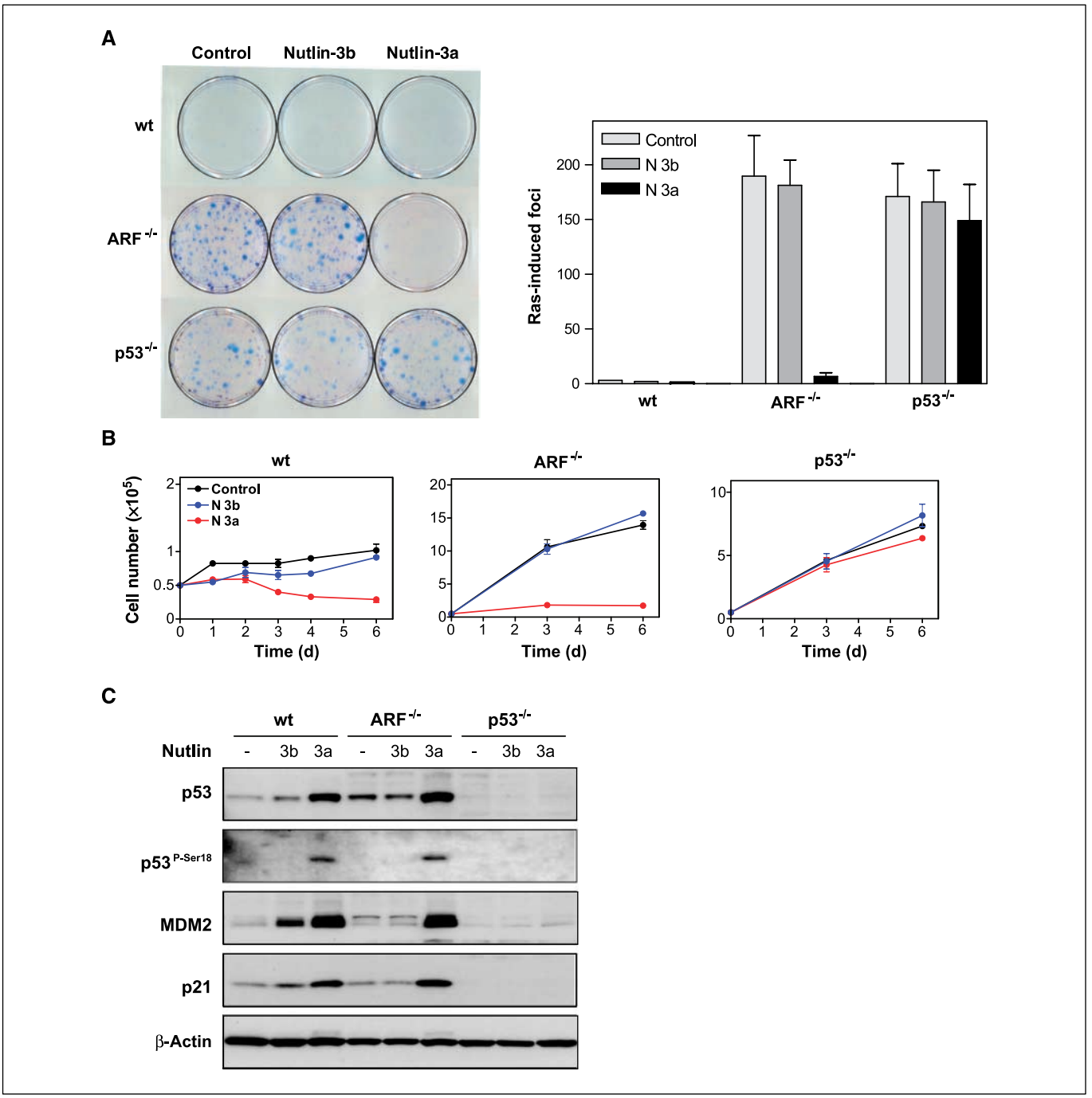

Figure 2. Nutlin-3a induces p53-dependent cell cycle arrest in Ras-infected MEFs. *A,* Ras-infected MEFs of the indicated genotypes were seeded and allowed to form macroscopic colonies during 1 wk in the absence or presence of nutlin-3a or nutlin-3b (5 μmol/L); after this time, plates were fixed and stained (*left*). The number of colonies was scored in three independently derived MEFs for each genotype (*right*). *Columns,* average; *bars,* SE. *B,* Ras-infected MEFs of the indicated genotypes were treated with nutlin-3a or nutlin-3b (5 μmol/L) and the number of cells was counted at the indicated times. *Points,* average of two or three independent MEF preparations for each genotype; *bars,* SE. Representative of a total of three assays. *C,* Ras-infected MEFs were treated for 24 h with nutlin-3a or nutlin-3b (10 μmol/L) and the status of the p53 pathway was analyzed by immunoblot. Treatment with the drug was begun 1 d after selection of Ras-infected MEFs (for 2–3 d with puromycin).

arrest, cells were treated for various time periods and then observed for their ability to resume proliferation. In the case of primary wt MEFs, 1 day of treatment was sufficient to produce an irreversible arrest (Fig. 3*C*). ARF-null MEFs also underwent an irreversible arrest but this required longer exposure time (4 days; see Fig. 3*C*). As anticipated, p53-null MEFs were insensitive to the drug. Finally, in the case of p21-null MEFs, complete irreversibility was not achieved even after 4 days (Supplementary Fig. S1*C*), which we interpret as evidence of the involvement of p21 in the establishment of p53-dependent senescence. The same type of analysis was done with Ras-expressing MEFs (Fig. 3*D*). Again, p53-null/Ras MEFs were insensitive to nutlin, whereas ARF-null/Ras MEFs turned out to be extremely sensitive. It is interesting to note the difference between primary ARF-null and ARF-null/Ras MEFs

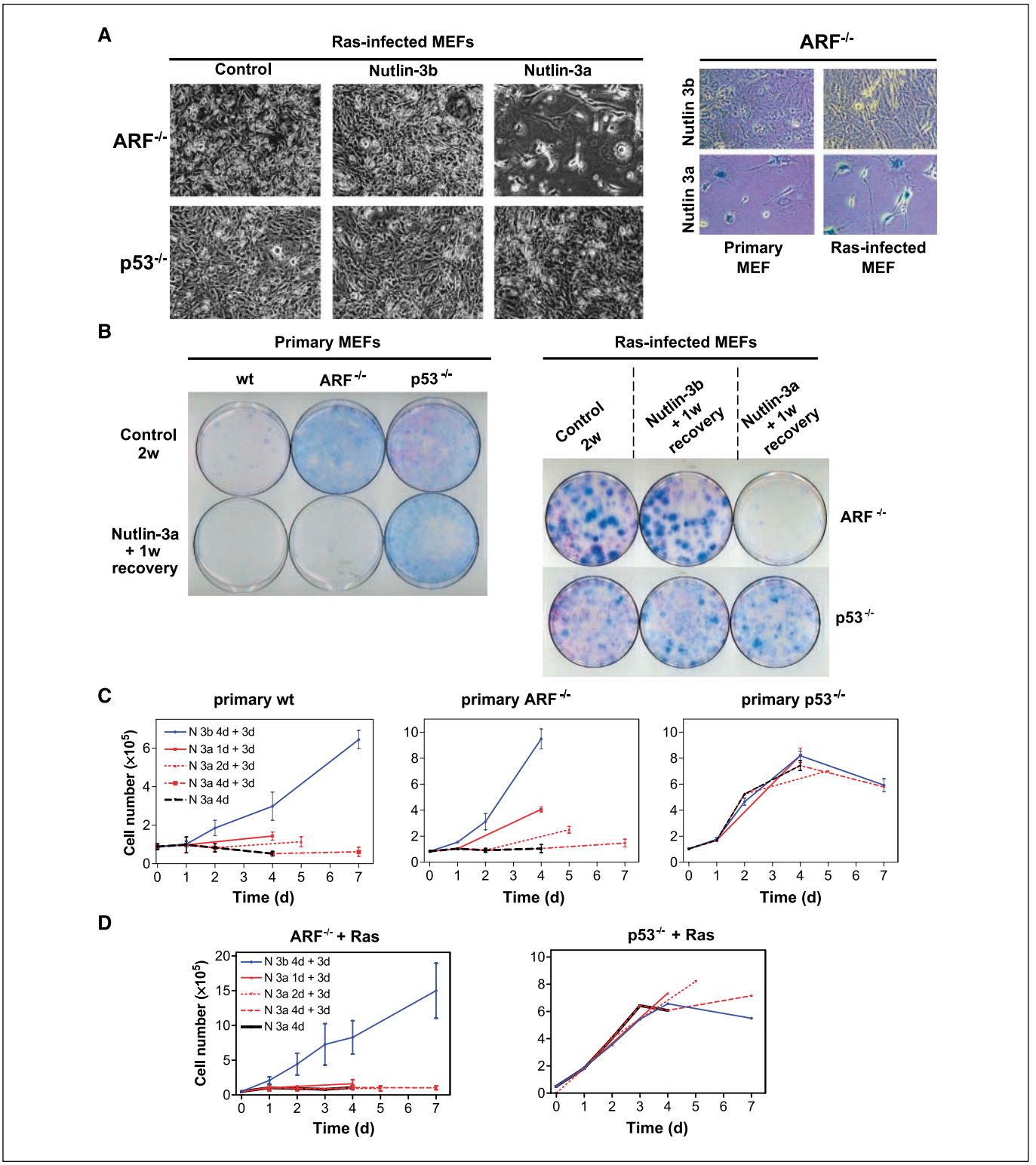

Figure 3. Nutlin-3a induces p53-dependent senescence. *A, left,* representative photomicrographs of Ras-infected MEFs of the indicated genotypes after 3 d in the presence of nutlin-3a or nutlin-3b (5 μmol/L). Note that ARF-null/Ras and p53-null/Ras cells present a typical neoplastic morphology, which in the case of ARF-null/Ras cells is dramatically converted into a senescent morphology by nutlin-3a. *Right*, representative photomicrographs of primary ARF-null MEFs and ARF-null/Ras MEFs stained with SA-βGal after treatment with nutlin-3a (10 μmol/L for 1 wk). *B,* cells of the indicated genotypes, either primary or Ras-infected, as indicated, were seeded and incubated for 2 wks in the absence of drug (*Control 2w*) or for 1 wk in the presence of the drug (5 μmol/L) followed by 1 wk in its absence (*Nutlin + 1w recovery*). After incubation, plates were fixed and stained. *C,* primary MEFs were treated with nutlin (10 μmol/L) for the indicated periods of time (1, 2, or 4 d) followed by 3 d in the absence of the drug. Cells were counted at the end of the treatment with the drug as well as at the end of the 3-d recovery period. *Points,* average of two or three independent MEF preparations for each genotype; *bars,* SE. Representative of a total of three assays. *D,* cells retrovirally transduced with Ras were seeded and incubated in the same manner as in (*C*). All the Ras-infected MEFs used in these experiments were treated with the drug the day after selection (for 3 d with puromycin). The experiment was quantified and repeated as in (*C*).

with regard to the time required to achieve irreversibility. In the case of ARF-null/Ras MEFs, the time for irreversibility was significantly shortened compared with primary ARF-null MEFs (1 day versus 4 days, respectively; see Fig. 3*C* and *D*). This differential sensitivity to nutlin likely reflects a cooperative effect between the drug and oncogenic Ras, the latter known to activate p53 by multiple pathways including the generation of DNA damage (47, 48).

Nutlin-3a induces p53-dependent senescence in murine fibrosarcoma cell lines. In the light of the previous results, we wondered whether nutlin-3a would also be able to produce p53-dependent senescence in fibrosarcoma cell lines. For this, we generated primary fibrosarcomas in wt and ARF-null mice by intramuscular injection of a well-known carcinogen, 3-methylcholanthrene. The resulting fibrosarcomas were excised and used to derive fibrosarcoma cell lines. The status of p53 was determined by DNA sequencing to identify point mutations and by Northern blot to identify those cases in which expression is completely lost (not shown); similarly, the status of ARF was determined by Northern blot to identify those fibrosarcoma cell lines that had completely lost the expression of ARF (not shown). Based on these analyses, we selected a total of seven cell lines (see Fig. 4*A*). Some of these cell lines retained functional p53 but had lost the expression of ARF ($p53^{wt}$;$ARF^{silenced}$, line 1); others were generated in ARF-null mice and retained functional p53 ($p53^{wt}$;$ARF^{-/-}$, lines 2–4); and, finally, others lacked functional p53 ($p53^{mut}$;ARF^{wt}, lines 5 and 7; $p53^{mut}$;$ARF^{silenced}$, line 6). We evaluated the effect of nutlin-3a in these malignant cells and found that the drug selectively induced cell cycle arrest in the p53-proficient cell lines (lines 1–4) but not in the p53-deficient cell lines (lines 5–7; Fig. 4*A*). As another control, we also obtained fibrosarcoma cell lines from $p53^{+/-}$ mice, which in all cases had lost p53 expression while retaining ARF (i.e., $p53^{-/-}$;ARF^{wt}), and, as anticipated, these cell lines were completely insensitive to the drug (data not shown). Once more, apoptosis was not observed in any of the cell lines evaluated (data not shown). Activation of the p53 pathway in the p53-proficient cell lines was confirmed by immunoblot analysis (Fig. 4*B*). After establishing that nutlin-3a induces p53-dependent cell cycle arrest in murine fibrosarcoma cells, we wondered whether this arrest had features of senescence. On exposure to nutlin-3a, the four p53-proficient cell lines (lines 1–4) gave positive

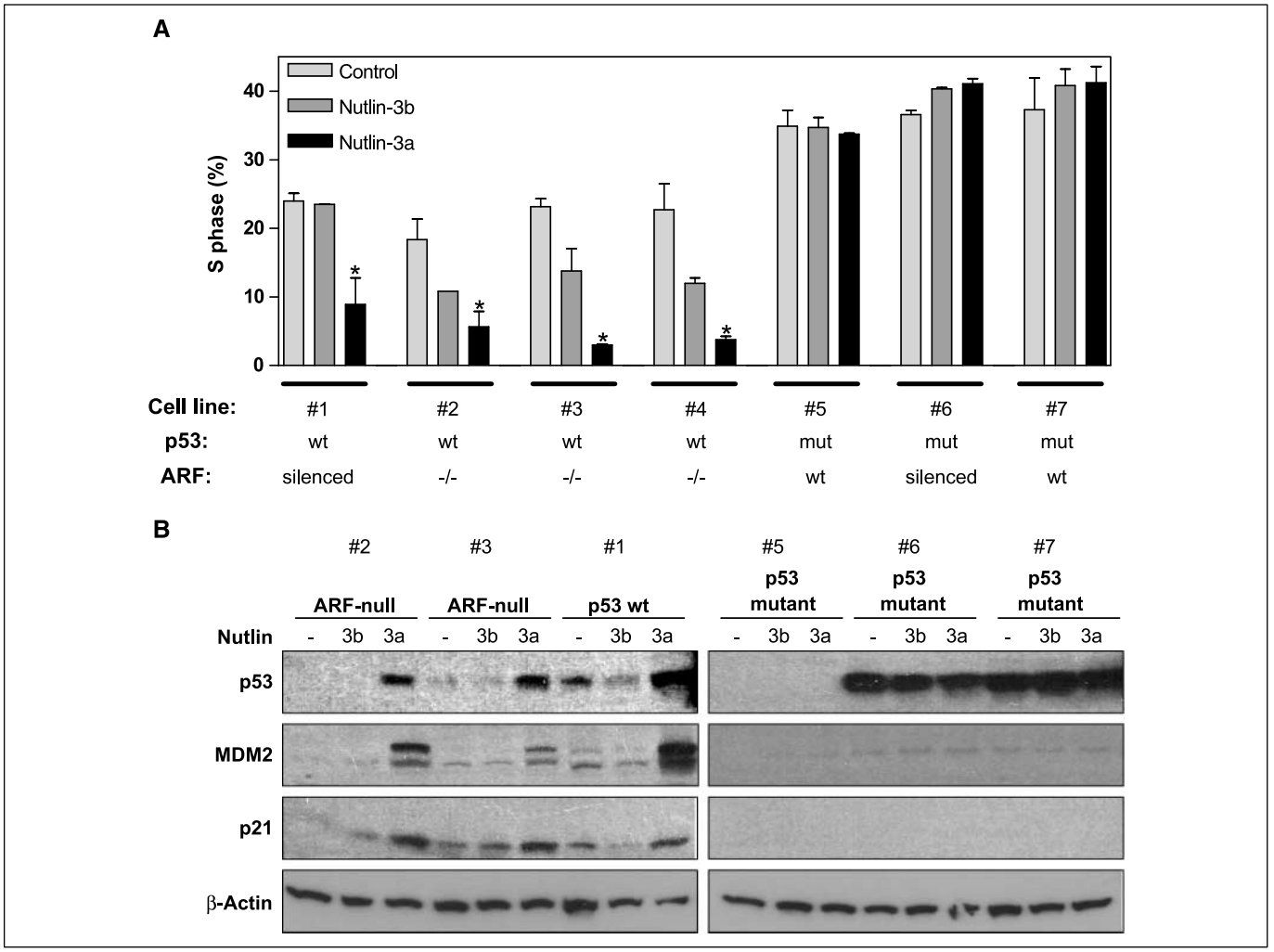

Figure 4. Nutlin-3a induces p53-dependent cell cycle arrest in murine fibrosarcoma cell lines. *A,* murine fibrosarcoma cell lines were treated with nutlin-3a or nutlin-3b (10 μmol/L) for 48 h and the percentage of cells in S phase was quantified. *Columns,* average of two independent determinations; *bars,* SE. *, statistically significant. ($P < 0.05$) of nutlin-3a–treated cells with respect to the nontreated controls (Student's *t* test). The status of p53 and ARF for each cell line is indicated at the bottom (see text and part *B* of this figure). *B,* fibrosarcoma cell lines were treated with nutlin-3a or nutlin-3b (10 μmol/L) for 24 h and the levels of p53, MDM2, and p21 were determined by immunoblot of total protein extracts.

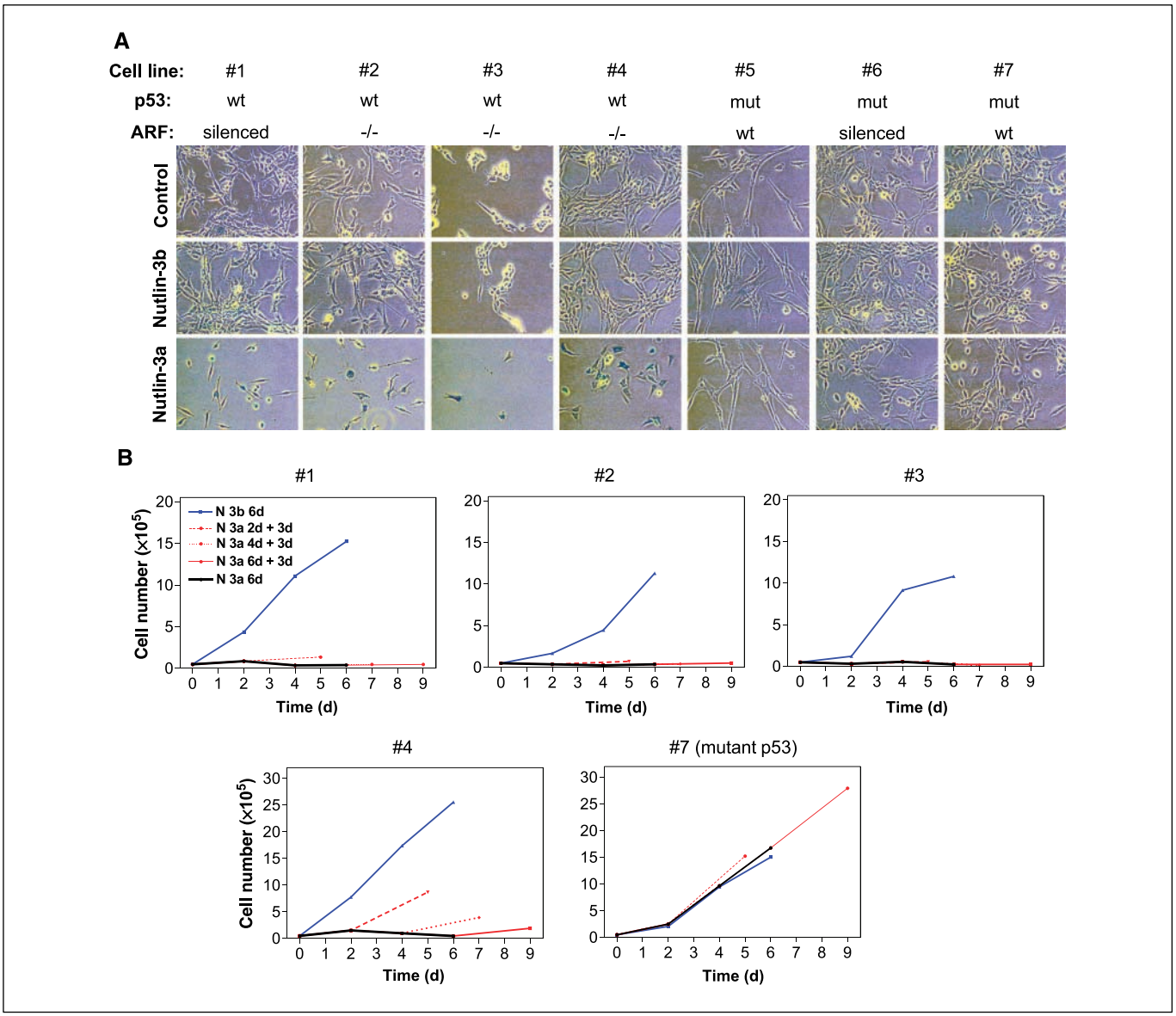

Figure 5. Nutlin-3a induces p53-dependent senescence in murine fibrosarcoma cell lines. *A,* representative photomicrographs of fibrosarcoma cells stained with SA-βGal after treatment with nutlin-3a (10 μmol/L for 1 wk). *B,* the indicated fibrosarcoma cell lines were treated with nutlin-3a or nutlin-3b (10 μmol/L) for the indicated periods of time (2, 4, or 6 d) followed by 3 d in the absence of the drug. Cells were counted at the end of the treatment with the drug as well as at the end of the 3-d recovery period.

staining for SA-βGal, whereas the others (lines 5–7) remained negative and maintained their neoplastic morphology (Fig. 5*A*). More importantly, we evaluated the irreversibility of the drug-induced arrest and we found that 2 days of treatment was sufficient to produce a complete and irreversible arrest in most of the p53-proficient cell lines (lines 1–3), with the only exception of line 4, which required 4 or 6 days of treatment before undergoing irreversible arrest (Fig. 5*B*). In summary, we conclude that, in murine fibrosarcoma cell lines, nutlin-3a induces a strong and irreversible arrest with features of senescence and in a manner that is strictly dependent on the functionality of p53.

Discussion

Here, we have examined the response to nutlin-3a in a variety of genetically defined mouse cells. Primary MEFs, oncogenically transformed fibroblasts, and murine fibrosarcoma cell lines were all shown to undergo a robust irreversible cell cycle arrest with features of senescence on exposure to nutlin-3a. In all cases, the induction of senescence was strictly dependent on the presence of functional p53. Remarkably, as previously noted (6–8), the proliferation of cells lacking p53 was essentially unaffected even after long periods of exposure (1 week; see Figs. 1*A* and 2*A*). The fact that primary p53-null fibroblasts were insensitive to the drug further reinforces the concept that p53 is the only relevant target of nutlin. In the case of oncogenically transformed ARF-null fibroblasts, the exposure time required for irreversible cell arrest (1 day; see Fig. 3*E*) was significantly shorter than the time required in nontransformed primary ARF-null cells (4 days; Fig. 3*D*). In the case of fibrosarcoma cell lines, the exposure time required for irreversibility was more variable, but in three of four cell lines, irreversibility was achieved after 2 days of drug treatment (Fig. 5*B*).

We interpret this as evidence of cooperation between nutlin and oncogenic signaling in p53 activation. These results, derived from direct comparison of primary and oncogenically transformed cells of the same origin, provide a strong support to the notion that MDM2 antagonists are more effective against cancer cells than against normal cells.

It is noteworthy that no apoptosis was observed in any of the cells of fibroblast origin used in our study. It has previously been shown that normal fibroblasts are generally resistant to p53-dependent apoptosis and usually undergo cell cycle arrest and/or senescence after p53 activation. Our results indicate that this property is retained in fibrosarcoma cell lines. Amplification of the *MDM2* gene and overexpression of the protein is particularly frequent ($\sim$30%) in soft-tissue sarcomas (1). Moreover, it has been shown that among p53-wt cancer cell lines, those that overexpress MDM2 are the most sensitive to nutlin-3a treatment (49). This observation has been interpreted as evidence that the overexpression of MDM2 is the only alteration in the p53 pathway of these cells, leaving intact other signaling cascades both upstream and downstream of p53 (49). Determination of the status of MDM2 and p53 is necessary to predict the response of tumors to nutlin-based chemotherapy. In summary, it is reasonable to speculate that tumor senescence may play an important role in the chemotherapy of soft-tissue tumors with MDM2 antagonists like nutlin-3a.

Acknowledgments

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