NONO couples the circadian clock to the cell cycle

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Mammalian circadian clocks restrict cell proliferation to defined time windows, but the mechanism and consequences of this interrelationship are not fully understood. Previously we identified the multifunctional nuclear protein NONO as a partner of circadian PERIOD (PER) proteins. Here we show that it also conveys circadian gating to the cell cycle, a connection surprisingly important for wound healing in mice. Specifically, although fibroblasts from NONO-deficient mice showed approximately normal circadian cycles, they displayed elevated cell doubling and lower cellular senescence. At a molecular level, NONO bound to the p16-Ink4A cell cycle checkpoint gene and potentiated its circadian activation in a PER-dependent fashion. Loss of either NONO or PER abolished this activation and circadian expression of p16-Ink4A and eliminated circadian cell cycle gating. In vivo, lack of NONO resulted in defective wound repair. Because wound healing defects were also seen in NONO-deficient mice, we show that coupling of the cell cycle to the circadian clock is necessary for normal wound healing in mice. Specifically, although fibroblasts from NONO-deficient mice showed approximately normal circadian cycles, they displayed elevated cell doubling and lower cellular senescence.

The mechanism of the cell cycle has been reviewed extensively elsewhere (13, 14). Rather than having a circadian phenotype of slight period shortening in animal behavior and cellular gene expression that was expected from our previous studies (22), we noticed that the primary circadian feedback loops of transcription of core oscillator genes (Per) and cryptochromes (Cry) are activated by BMAL1:CLOCK heterodimers at cis-acting elements called E-boxes, and their protein products form complexes that repress their own transcription (11). We originally identified the RNA-binding protein NONO (also called p54nrb) biochemically as a new member of this circadian transcriptional repressor complex in mice, and mutation of its ortholog NonA in flies resulted in severe attenuation of circadian rhythmicity (12). However, apart from its interaction with this circadian repressor complex, NONO’s mechanism of action within the cell remains unknown.

The mechanism of the cell cycle has been reviewed extensively elsewhere (13, 14). Rather than having a fixed duration, its period is tightly regulated by cellular processes via checkpoint proteins that gate cell cycle progression. The circadian clock is one such regulatory process, and it has been shown that the circadian clock can directly time cell division (3, 4), although not necessarily in all cell types (15, 16). The exact mechanism of this regulation is at the moment only partially understood and may involve circadian regulation of cell cycle checkpoint genes like wee1 (3) and p21-Waf1 (17), or interaction of clock factors with CSK-homologous kinase 1/2 (CHK1/2) proteins (18, 19). In addition, a limited number of accessory factors have been assigned roles in both processes, including the mammalian Timeless protein (TIM), which interacts with both the circadian CRY2 protein and the cell cycle CHK1 protein (18), and p54nrb/NONO, which interacts with proteins involved in DNA damage repair (20, 21).

To understand the role of the NONO protein in the circadian clock and its relationship to the cell cycle, we created NONO-deficient mice. Although these mice showed only minor circadian defects (22), circadian gating by the cell cycle was lost in fibroblasts from these mice, allowing us to probe both the mechanism and the physiological relevance of the connection between the two processes. Our investigations not only uncover a unique role for NONO in the circadian transcriptional regulation of the p16-Ink4A cell cycle checkpoint gene, but also suggest that circadian gating of the cell cycle by NONO plays an unsuspected role in tissue regeneration.

Results

Fibroblasts from NONO-/- Mice Show Increased Proliferation and Decreased Senescence. To better understand the function of NONO, we used a “genetrapped” embryonic stem cell line (23) to generate NONO-/- mice that completely lack coding NONO transcript and NONO protein in all tissues tested (Fig. L4). In addition to a circadian phenotype of slight period shortening in animal behavior and cellular gene expression that was expected from our previous studies (22), we noticed that the primary fibroblasts taken to characterize these mice showed more robust division than their WT counterparts. To quantify this effect, we serially split cells from WT and NONO-/- littermates at a predetermined frequency so that confluence was not reached and counted cells at each passage. NONO-/- cells indeed showed a markedly increased rate of population doubling relative to WT cells (Fig. 1B). In principle, such an effect could have arisen either because cells divided faster or because they reached senescence—the normal postproliferative arrest of cell division in adult tissues—at a lower rate. To examine senescence, we stained the same cells for senescence-associated β-galactosidase (SA-βgal) activity (24) at each passage from their

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Nonogt dividing broblasts were stained with CFSE and allowed to divide for 4 additional days. Dye intensity was then measured by flow cytometry (duplicate plates of cells in green and blue). As a control, other plates of the same cells were treated with mitomycin C to inhibit cell division immediately after infection. Nonogt dividing broblasts were infected with lentivirus WPI (expressing NONO) and allowed to proliferate via serial primary plates of WT and Nonogt broblasts, we luciferase reporter for Ets1, Ets2, and Id1 loci—transcript levels in Nonogt cells were unchanged or inconsistent with the observed down-regulation of p16-Ink4A (Fig. S1 A–E), supporting our hypothesis that p16-Ink4A is a possible direct regulatory target of NONO. More broadly, we compared expression of cell cycle genes in Nonogt fibroblasts and their WT counterparts using a cell cycle gene RT-PCR array, and not surprisingly found transcriptional misregulation of multiple other cell cycle genes (Fig. 2E and Table S1), confirming the effects of NONO upon the cell cycle in general and the extensive coregulation of cell cycle processes. To examine further whether NONO might directly regulate the p16-Ink4A locus transcriptionally, we first cotransfected 3T3 fibroblast cells with a vector expressing NONO and with a luciferase reporter for p16-Ink4A promoter activity. The addition of NONO resulted in a dose-dependent increase up to 14-fold in

①) Reintroduction of NONO into primary Nono<sup>−/−</sup> fibroblasts via lentiviral transduction slowed division and increased senescence, and addition of NONO to WT cells slowed division even further (Fig. 1E), confirming the dose-dependent role of NONO in restraining cell proliferation and pointing to a probable role for this protein in the cell cycle.

**NONO is a Transcriptional Activator of the p16-Ink4A Cell Cycle Checkpoint Gene.** To identify the cell cycle stage at which NONO has a role, we fixed actively dividing fibroblasts from WT and Nono<sup>−/−</sup> animals and labeled them with propidium iodide, a fluorescent DNA-binding dye (26). Subsequent flow cytometry experiments allowed us to quantify the proportion of cells in different stages of the cell cycle in each population. Our results demonstrated a twofold increase of cells in synthesis phase (S phase) of the cell cycle when NONO was absent. NONO may thus act as a regulator of exit from the G1 phase (Fig. 2A and B). To confirm the effects of NONO upon S phase, we also pulse-labeled cells with 5-ethynyl-2′-deoxyuridine (EdU), an immunohistochemically detectable nucleotide analog incorporated during DNA replication in S phase. By comparing signals from EdU with those from propidium iodide in the same cells, we were able to verify that more cells were indeed in S phase in Nono<sup>−/−</sup> fibroblasts than in WT ones (Fig. 2C).

In most other systems, NONO has been identified as a transcriptional cofactor regulating either gene expression or RNA processing (27). Therefore, we considered it likely that NONO would exert its effects upon senescence and the cell cycle via transcriptional regulation of cell cycle genes. On the basis of the knowledge that NONO acted upon both senescence and the exit from the G1 phase checkpoint, we considered the p16-Ink4A locus as a likely target. P16-Ink4A has been implicated previously as a regulator of the mitogen-responsive retinoblastoma pathway and is one of the key cellular components regulating senescence. It is known to repress the cyclin-dependent kinases 4 and 6 (Cdk4 and Cdk6), resulting in a G1 arrest that slows cell division and promotes senescence (28, 29). To investigate whether the p16-Ink4A gene is systematically misregulated in Nono<sup>−/−</sup> fibroblasts, we serially passed cells from both WT and Nono<sup>−/−</sup> mice and examined the expression levels of p16-Ink4A at each passage. Indeed, quantitative RT-PCR confirmed that RNA abundance of p16-Ink4A was systematically down-regulated in Nono<sup>−/−</sup> cells at each passage, and this change in expression was also reflected in levels of p16-Ink4A protein (Fig. 2D).

Because p16-Ink4A negatively regulates cell division and positively regulates senescence, its repression is consistent with the phenotype observed in Nono<sup>−/−</sup> fibroblasts. As a control, we also looked at key regulators of other pathways known to control senescence: the DNA damage-responsive p53 locus, and tankyrase, a downstream regulator of telomere length. No differences were observed in the levels of tankyrase mRNA between WT and knockout cells, and p53 was twofold lower, a direction inconsistent with the phenotype that we observe. In addition, for all known upstream regulators of p16-Ink4A—the Ets1, Ets2, and Id1 loci—transcript levels in Nono<sup>−/−</sup> cells were unchanged or inconsistent with the observed down-regulation of p16-Ink4A (Fig. 2E), confirming the effects of NONO upon the cell cycle in general and the extensive coregulation of cell cycle processes.
we performed ChIP of endogenous NONO protein with the 
*p16-Ink4A* promoter. In murine liver NONO indeed binds to the 
*p16-Ink4A* promoter region (Fig. 3B). As a control, we verified that 
NONO does not bind to the promoter of the *Ets1* gene, an up-
stream regulator of *p16-Ink4A* (Fig. 3C). Importantly, NONO 
binds to *p16-Ink4A* in circadian fashion (Fig. 3B), implying that it 
might confer a circadian expression pattern upon this important 
cell cycle checkpoint gene.

To test this hypothesis, we examined the expression of *p16-
Ingk4A* directly in mouse liver at different times of day. As predicted, 
*p16-Ink4A* mRNA levels were indeed circadian in their abundance. In livers from NONO<sup>−/−</sup> mice, this circadian regulation 
was lost, and *p16-Ink4A* transcript levels were nonrhythmic and 
low (Fig. 3D). Therefore, circadian regulation by NONO is in-
deed responsible for the circadian gene expression of *p16-Ink4A*.

We have shown previously that overall NONO transcript and 
protein levels are constant throughout the day but that one particular NONO complex (also containing the PER proteins of 
the circadian oscillator) shows circadian variations in abundance (12). Because the *p16-Ink4A* promoter was occupied by NONO

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**NONO and PER Proteins Bind to the p16-Ink4A Promoter to Direct Its Circadian Transcription in Vivo.** To investigate whether NONO also acts directly as a transcriptional coactivator of *p16-Ink4A* in vivo,
in circadian fashion, we also tested to determine whether PER proteins bound there. ChiP analyses from mouse liver demonstrated that PER2 proteins are present at the p16-Ink4A promoter with the same kinetics as NONO. As expected, no PER2 binding was observed in a per2<sup>Brdm1/Brdm1</sup> strain lacking this protein (Fig. 4A). To determine whether PER proteins were necessary for the effects of NONO upon the p16-Ink4A promoter, we examined the levels of mRNA from this locus in per1<sup>Brdm1/Brdm1</sup>, per2<sup>Brdm1/Brdm1</sup> mutant mice (henceforth designated per1/per2<sup>mut</sup> for simplicity), which lack both proteins (30).

In livers from these mice, the binding of NONO to the p16-Ink4A promoter was noncircadian and low or absent (Fig. 4B), and p16-Ink4A transcript itself was also constantly low (Fig. 4C).

These results suggest that a PER–NONO complex is responsible for the effects of NONO upon the p16-Ink4A promoter. To test this hypothesis explicitly and determine whether PER proteins are required for NONO to activate p16-Ink4A, we compared the ability of NONO to activate transcription of a p16-Ink4A reporter in transient transfections in primary fibroblasts from WT and per1/per2<sup>mut</sup> mice. Transcriptional activation by the circadian transcriptional activator complex BMAL1/CLOCK was achieved in both WT and per1/per2<sup>mut</sup> cells to the same extent (Fig. 4D). Whereas activation by NONO was observed in WT cells, no activation was observed in mutant cells (Fig. 4D), indicating that PER proteins are required for NONO activity (or vice versa).

NONO is necessary for Circadian Gating of the Cell Cycle. Circadian transcriptional activation of p16-Ink4A by NONO has predictable consequences for cellular physiology. In numerous previous studies in a variety of contexts, increased expression of p16-Ink4A leads to a disinhibition of the G1/S transition at specific phases due to low levels of p16-INK4A. leads to a disinhibition of the G1/S transition at specific phases due to low levels of p16-INK4A. To test whether NONO is required for normal wound repair, we wounded the skin of adult WT and NONO<sup>−/−</sup> mice, closed the resulting full-thickness incisional wound with Steri-strips, and followed the wound healing process after 3, 7, 13, and 20 d by histological wound healing scores. Before wounding, skin structure appeared indistinguishable among WT and NONO<sup>−/−</sup> animals (Fig. S3A), and shortly after wounding, scab formation, inflammation, and angiogenesis were normal (Fig. 6A, Left). However, later after wounding, histological analysis revealed profound alterations in both dermal and epidermal regeneration. WT wounds exhibited good reepithelialization and granulation tissue organization. Granulation tissue consisted of oval or spindle fibroblasts (dark red) embedded in a dense fascicular system of collagen fibers (gray; Fig. 6A, Center). The epidermal layer was well structured and gave, together with the well-organized granulation tissue, rise to new skin that reestablished tissue integrity (Fig. 6A, Right). In NONO<sup>−/−</sup> mice, immature granulation tissue was characterized by continued fibroblast proliferation, occupying most of the wound area (red, Fig. 6A, Right) by day 20. These plumped and round-to-polyhedral fibroblasts were distributed within a loose matrix with hardly any collagen production. Similarly, the keratinocyte layer (stained in pink) was characterized by hyperproliferation and little epidermal organization (Fig. 6A, Center and Right). Thus, even as collagen-secreting fibroblasts hyperproliferated in wounded NONO<sup>−/−</sup> animals, collagen secretion was dramatically diminished (Fig. 6B). The dividing cells could form only an immature dysfunctional epidermal layer resting on disorganized granulation tissue, which prevented healing (Fig. 6B and S3B).

Circadian Clock-Deficient Mice Also Show Defects in Wound Healing. In principle, the defect in wound healing observed in NONO<sup>−/−</sup> mice might be linked to the circadian cell cycle phenotype described above, or arise from another unrelated function of NONO. To distinguish between these hypotheses, we also tested wound healing in two strains of mice lacking functional circadian clocks: per1/per2<sup>mut</sup> mice and bm11<sup>−/−</sup> mice. Both strains also showed defective wound healing. Consistent with the requirement of PER proteins for NONO activity at the p16-Ink4a promoter, the per1/per2<sup>mut</sup> mouse showed a thick layer of immature granulation tissue that was dominated by an excess of fibroblasts as well as polymorphonuclear cells. Bm11<sup>−/−</sup> mice also showed severe wound healing defects, although this time marked by lack of epithelial coverage and a highly disorganized granulation tissue throughout the observation period. Most wounds in bm11<sup>−/−</sup> mice consisted mainly of an inflammatory fibrin clot with hardly any fibroblast or keratinocyte proliferation (Fig. 7A and B). Taken together, our data suggest that a functional circadian
clock is important for wound healing and that its effects are likely mediated by NONO.

Discussion

In this article we show that the NONO protein—which we have identified previously as a component of the basic circadian oscillator in Drosophila and in mouse cells (12)—plays an equally important role to gate circadian cell division in fibroblasts. Elimination of NONO entirely abrogated circadian-cell cycle coupling in fibroblasts, suggesting that NONO serves as a necessary link between these two processes, at least in these cells. This uncoupling allowed us to probe possible functions of this link.

NONO as a Circadian Effector. NONO was initially identified by its homology to splicing factors (33) and as a Drosophila factor involved in courtship songs (34). Since then, diverse studies have described it as a protein with pleiotropic functions mainly involved in RNA processing and transport, as well as a transcription factor (27). It has been implicated in diverse pathways, such as nuclear receptor signaling (35), DNA repair (36, 37), and viral infection (38). Here we have shown that it can directly activate the p16-Ink4A locus, an important regulator of the G1 exit checkpoint of the cell cycle. The detailed mechanism of its coactivator function remains unclear. In the case of transducer of regulated CREB-binding protein (TORC)-mediated coactivation by NONO, Amelio et al. (39) suggested that NONO directly bridges interactions between RNA polymerase II and other activators. Other groups have shown that NONO regulates nuclear retention of RNAs (40, 41) or transcription termination (42), so it is possible that NONO couples transcription initiation and downstream RNA processing.

NONO itself is not expressed in circadian fashion. Nevertheless, it binds to PER proteins and has been shown to antagonize their repressive activity (12). Recently, a protein highly homologous to NONO called splicing factor, proline- and glutamine-rich (SFPQ) has been shown to bind to PER proteins as well, but to assist in their repressive activity by recruiting the mSin3A histone deacetylase (43). Consistent with these results, we have previously demonstrated that all three members of the DBHS protein family [Drosophila Behavior Human Splicing, of which NONO, SFPQ, and parasplice component 1 (PSCPC1) are members] likely play redundant roles via cis-acting E-box elements at circadian genes (22). Here we show that NONO plays a strong activating role, also circadian, at the p16-Ink4A promoter. However, this activation is dependent upon a particular 175-bp sequence within the promoter region of this gene that does not contain an E-box. Thus, multiple sequences likely direct activities of DBHS proteins, and these can be activating or repressing. DBHS proteins themselves seem to have no sequence-specific DNA-binding activity, although structure-specific binding (DNA triplexes, RNA loops) has been postulated (44, 45). We propose that NONO and homologous factors might act as circadian output effectors, linking circadian regulation via PER proteins to activating and repressing moieties to confer circadian expression upon diverse aspects of physiology and gene expression. The exact mechanism of this regulation will doubtless constitute a fascinating subject of future investigations.

NONO as a Link Between the Circadian Clock and the Cell Cycle. In keeping with NONO’s proposed role as a circadian output effector, we present here data that show that NONO directly activates the p16-Ink4A cell cycle checkpoint gene in circadian fashion to regulate cell cycle progression. Multiple circadian influences upon the cell cycle have already been documented, acting at multiple different checkpoints. For example, it has been suggested that transcriptional control of cell cycle genes such as p21-waf1 and wee1 might play important roles. Both Weel transcript and protein levels show circadian fluctuation probably governed by cis-acting E-box elements, and one of the targets of the WEE1 kinase, CDC2, also shows circadian phosphorylation (3). A similar story has been constructed for p21-waf1, whose circadian transcription is governed by REV-ERBα response element (RRE) elements within the promoter that respond to REV-ERBα and retinoid-related orphan receptor (ROR) proteins (17). Another class of clock factors, the PER proteins, themselves function as tumor suppressors (40, 47). Similarly, TIM—shown by some groups to play a role in the mammalian circadian clock (48)—has been shown to bind to the CHK1 and CHK2 cell cycle proteins (19). Thus, in total, five different sites of action of the circadian clock upon the cell cycle have been proposed. One of the principal difficulties in evaluating the role of these independent mechanisms in different tissues lies in the importance of cell cycle checkpoint proteins, which precludes meaningful loss-of-function experiments. What is particularly interesting about NONO is that its disruption completely abrogates circadian gating of the cell cycle in fibroblasts without severely disrupting either the circadian clock or the cell cycle. Importantly for our conclusions about circadian regulation of the cell cycle, even if the NONO homolog NON-A is essential for circadian function in flies (12), NONO-deficient mice possess essentially normal clocks in all tissues examined. This discrepancy likely arises because of functional redundancy between NONO and homologous proteins.

**Fig. 5.** NONO protein is necessary for circadian gating of the cell cycle in primary fibroblasts. Dividing primary fibroblasts were clock-synchronized with dexamethasone and then harvested at different times of day, fixed, and stained with propidium iodide. The percentage of dividing cells (i.e., in S phase) from (A) WT, (B) per1/per2<sup>−/−</sup>, and (C) Nano<sup>−/−</sup> cultures was then quantified by FACS analysis. (Times indicated are relative to synchronization.)

**Fig. 6.** NONO protein is necessary for correct dermal wound healing. (A) WT and Nonogt<sup>−/−</sup> mice were incisionally wounded and then killed 3, 7, or 20 d later. Representative Masson-Goldner trichrome-stained paraffin sections from the center of these wounds are shown. (B) Wound healing subscores of normal healing WT mice compared with Nonogt<sup>−/−</sup> mice on day 3, 7, 13, and 20 after incisional wounding in the dorsal skinfold. Data represent means ± SD (n = 5).
growth (50), and a similar role in skin regeneration is not only possible, but to the authors likely. Through its role in replicative senescence, the p16-INK4A protein plays a key role in mesenchymal stem cell differentiation (51), so it is possible that circadian regulation of this protein via NONO would affect not only cell division but also stem cell populations. However, these processes are unasparable and intertwined: our observation of overproliferation of keratinocytes at wounds in *Nono* animals also implies an overrepresentation of prekeratinocyte stem cells at these times, and the overproliferation of fibroblasts equally shows direct control of cell division itself. The key to both observations—and also the key conclusion of this study—is that circadian control of the p16-INK4A checkpoint via NONO is necessary for correct dermal wound healing, and lack of this control results in loss of circadian cell cycle gating in vitro and tissue overproliferation in vivo. The exact downstream mechanism of this control and its consequences for tissue structure will provide a fascinating and medically relevant subject for future investigations. It should be noted, however, that tissue formation during development in both *Nono* and clock-disrupted mice seems normal. Therefore, it is likely that such control is either unnecessary or redundant earlier in development.

As more is known about the feedback loops of clock control circadian clock mechanism, increasing numbers of laboratories have begun to address the questions of how and why the circadian oscillator communicates timing to all of the physiological and cellular process that it governs. In these studies a pattern has begun to emerge: basic clock components can themselves directly regulate genes involved in the “output pathways” that control diurnal function (52). In this article we show that the NONO protein plays an essential intermediary role. More generally, coopting the circadian clock as a synchronizing timekeeper—completely independent of its role in diurnal adaptation—might provide an important future paradigm for clock function at a cellular level.

**Materials and Methods**

**Plasmids.** The bioluminescence reporter construct *pBmal1-Luciferase* and the NONO-targeting siRNAs have been described previously (12). For NONO overexpression, a commercially obtained pSPORT construct was used (clone ID 360A835 from Open Biosystems). p16-promoter-luciferase plasmid (D-761) and minimal p16 promoter plasmid (D-141) (53) were obtained from B. A. Mock and S. Zhang (Laboratory of Genetics, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD).

**Animal Husbandry.** Chimeric mice were obtained from *Nono* ES cells (C57Bl6 genotype) via standard blastocyst injection into SV129 mice by the University of California, Davis. Individual chimeric mice were back-crossed 4–10 generations against C57Bl6. All experiments were performed with littermates. Animal housing and experimental procedures are in agreement with veterinary law of the canton of Zurich. Genotyping was done as described at [www.mmrn.org/strains/38ctr_protocol.pdf](http://www.mmrn.org/strains/38ctr_protocol.pdf) using a NONO-specific primer set (sense 5′-TTA GGG GGC CGA ACT ACT AGT TTT T3′; antisense 5′-GGG CGG AGA TTT ACT AGT TTT T3′; Quantitative PCR (qPCR) primer sequences are listed in the quantitative real-time PCR section, below).

**Primary Cell Isolation and Culture.** Primary adult dermal fibroblasts (ADF) were taken from a 0.5-cm piece of mouse tail that was cut into several small pieces by using a razor blade. Digestion occurred in 1.8 mL DMEM containing 20% FBS, 1% penicillin/streptomycin, and 1% amphotericin B supplemented with 0.7 U Liberase blendzyme (Roche), at 37 °C and 5% CO₂ for 8 h (all concentrations are vol/vol). After centrifugation in 1x PBS, the pellet was resuspended in DMEM containing 20% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B and kept at 37 °C and 5% CO₂. The next day, medium was exchanged, and remaining tail pieces were removed. Another medium exchange was done 3 d later. After 1 wk the medium was exchanged for medium without amphotericin B. Subsequently, ADFs were cultured at 37 °C and 5% CO₂ in DMEM supplemented with 20% FBS and 1% penicillin/streptomycin.

**cDNA Production, Quantitative Real-Time PCR, and PCR Arrays.** RNA was extracted as described in (12). Five hundred nanograms of total RNA was transcribed to cDNA with SuperScript II (Invitrogen) using oligo (dT) primers
according to the manufacturer’s instructions. For quantitative real-time PCR 20 ng of cDNA was used, and transcript levels of genes were detected by Taqman probes used with the Taqman PCR mix protocol (Roche) using the ABI7900 thermocycler. Primers used for detection of specific genes are listed in Table S2.PCR arrays to quantify cell cycle components were performed according to the manufacturer’s instructions using 500 ng of total RNA (SABiosciences; array name, PAMM-020E). RNA was extracted from duplicate plates of either WT or Nonogt primary fibroblasts 14 d after isolation.

Western Blotting and Immunohistochemistry. Western blotting was performed using standard procedures (Current Protocols in Molecular Biology, Wiley). Equal loading and size detection using protein ladder was verified by Ponceau-S staining of membranes before probing. The probing of the primary antibody and Fluoro (polyclonal antibody raised by Charles River Labs using bacterially produced NONO protein, affinity-purified against the antigen used for injection) was done at a 1:200 dilution. The probing of the secondary antibody was done at a 1:10,000 dilution for anti-rabbit IgG coupled to horseradish peroxidase (Sigma) and 1:1,000 for anti-mouse HRP (Sigma), respectively.

Cellular Senescence and Cytometric Measurements. For growth curves, primary fibroblasts from WT and Nonogt animals were split every other day, plating each time 1 × 105 cells so that confluence was never reached. Within these plates, colonies of cells that were too small to be individually scored for senescence-associated β-galactosidase staining were analyzed in the same way as described above with CFSE. In brief, cells were harvested after 60 h by washing once with 1× PBS and extracting with the supplied buffer from a luciferase assay kit (Promega). Quantification was done by scintillation counting, and normalized against total protein amount.

ChIP. Chromatin from mouse liver and tissue culture cells was obtained as described previously (55). Equal amounts of precleared chromatin were incubated overnight at 4°C with 1 μl of anti-NONO antibody or anti-Per2 antibody. The capture of the DNA:protein complexes, the washing conditions, and the purification of the DNA fragments before qPCR, as well as the control antibodies have been described previously (56). The region-specific primer/probe pairs are listed in Table S2.

Histological Wound Scoring. WT and Nonogt mice were anesthetized i.p. with a mixture of 50 μg/kg body weight (BW) ketamine hydrochloride (Ketavet; Parke Davis) and 25 μg/kg BW xyazine hydrochloride (Rompun; Bayer). The dorsal region was shaved and treated with a depilatory agent (Pilca Perfect; Stafford-Miller Continental). Three full-thickness incisions (6 mm) perpendicular to the dorsal midline were made at one anterior and two posterior dorsal sites, and the skin margins were closed with Steri-Strips (Steri-Strip Surgical Skin Closure 3M). Mice were killed on day 3, 7, 13, after wound healing and the wounds embedded according to standard procedures and stained with Masson-Goldner trichrome and hematoxylin eosin. A total of 16 animals (8 WT and 8 Nonogt, n = 2 for each time point) were wounded and killed on the corresponding time points, and two additional WT and Nonogt mice without any incisions served as controls. Subsequently, identical experiments were performed on three each of WT, Bmal1+/−, and per1/−/per2 mice, with killing on day 6 after wounding. Histological wound scoring has been described previously (57) and was based on the quality of dermal organization, epidermal regeneration, collagen deposition, cellular content, and wound vascularity. The criteria used as histological scores of wound healing are summarized in Table S3.

Statistical Methods. In bar graphs, Student’s t test was used to determine significant differences between control and experimental groups. In all figures, n = 3–4 independent experiments, with each experiment conducted in technical duplicate. Data are plotted ±SEM. In line graphs, for each experimental data set, linear regression was conducted to determine best-fit line describing the data from each independent experiment. Overall significance of differences in doubling (e.g., Fig. 1A) or in accumulation rate (e.g., Fig. 2A) were then determined using a Student two-tailed t test of the regression lines from each data set (n = 2–4).

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Supporting Information
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Fig. S1. Transcription of senescence-implicated genes in serially passaged WT and Nonogt primary fibroblasts. WT and Nonogt primary fibroblasts were counted and passaged every 2 d and a constant number of cells plated to a new dish. Total RNA was harvested from cells in each passage, and quantitative PCR (qPCR) was used to quantify the transcript levels of senescence-implicated genes tankyrase (A) and p53 (B), as well as upstream regulators of p16-Ink4A, the genes Id1 (C), Ets1 (D), and Ets2 (E). Values are plotted in arbitrary units relative to WT levels at passage 1.
Fig. S2. NONO activates transcription of p16-Ink4A promoter reporters. (A) Relative expression levels of the diagrammed p16-luc promoter construct alone, in the presence of a NONO-targeting RNAi hairpin that reduces NONO levels 10-fold, and in the presence of a NONO-overexpressing vector. (B) Similar experiments using the diagrammed construct containing the p16-Ink4A 3’ UTR.
Fig. S3. Dermal structure and incisional wound healing in WT and Nonogt mice. (A) H&E-stained paraffin sections from uninjured dorsal skin. Normal skin morphogenesis is not affected in Nonogt mice compared with WT littermates (10–11 wk of age, 24 g body weight). (B) Full wound healing subscores of normal healing WT mice compared with Nonogt mice on day 3, 7, 13, and 20 after incisional wounding in the dorsal skinfold. Data represent means ± SD (n = 5).
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Total RNA was harvested from dividing cultures of WT and Nonogt primary fibroblasts and subjected to qPCR array analysis. All array targets are shown, with fold-regulation of Nonogt vs. WT fibroblasts. Note that the p16-Ink4A transcript itself was not probed on this commercial array. Independent qPCR analysis using the primers in Table S2 confirmed fivefold reduction in expression of p16-Ink4A in Nonogt vs. WT fibroblasts in both replicates of the cellular RNA used for this array.
### Table S2. Primer sequences

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TaqMan Gene Expression Assays from Applied Biosystems are tested, but sequences are not provided. Assay consists of primer forward, primer reverse, and probe, as usual.

### Table S3. Histological wound healing scores

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<th>Wound vascularity</th>
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<td>No epithelial closure</td>
<td>Gap without ingrowing collagen fibrils</td>
<td>Low cell proliferation, mainly inflammatory cells</td>
<td>1–3 capillaries per visual field</td>
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<td>50% thickness of granulation tissue compared with healthy tissue</td>
<td>Strong hyperproliferative epithelium</td>
<td>Gap with ingrowing collagen fibrils</td>
<td>Predominantly inflammatory cells or dysfunctional fibroblasts, hyper-proliferation</td>
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<td>75% thickness of granulation tissue compared with healthy tissue</td>
<td>Moderate hyperproliferative epithelium</td>
<td>No gap, but unstable adhesion</td>
<td>Predominantly normal fibroblasts</td>
<td>7–9 capillaries per visual field</td>
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<td>Thickness of granulation tissue equal to healthy tissue</td>
<td>Thickness and structure equal to normal epithelium</td>
<td>No gap, stable adhesion</td>
<td>Low cell proliferation, mainly fibroblasts</td>
<td>&gt;9 capillaries per visual field</td>
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