INK4 locus of the tumor-resistant rodent, the naked mole rat, expresses a functional p15/p16 hybrid isoform

Xiao Tian[a,1], Jorge Azpurua[a,1,2], Zhonghe Ke[a], Adeline Augereau[b], Zhengdong D. Zhang[c], Jan Vijg[c], Vadim N. Gladyshev[b], Vera Gorbunova[a,3], and Andrei Seluanov[a,3]

[a]Department of Biology, University of Rochester, Rochester, NY 14627; [b]Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115; and [c]Albert Einstein College of Medicine, Bronx, NY 10461

Edited* by Eviatar Nevo, Institute of Evolution, Haifa, Israel, and approved December 1, 2014 (received for review September 21, 2014)

The naked mole rat (*Heterocephalus glaber*) is a long-lived and tumor-resistant rodent. Tumor resistance in the naked mole rat is mediated by the extracellular matrix component hyaluronan of very high molecular weight (HMW-HA). HMW-HA triggers hypersensitivity of naked mole rat cells to contact inhibition, which is associated with induction of the *INK4* (inhibitors of cyclin dependent kinase 4) locus leading to cell-cycle arrest. The *INK4a/b* locus is among the most frequently mutated in human cancer. This locus encodes three distinct tumor suppressors: $p15^{INK4b}$, $p16^{INK4a}$, and ARF (alternate reading frame). Although $p15^{INK4b}$ has its own ORF, $p16^{INK4a}$ and ARF share common second and third exons with alternative reading frames. Here, we show that, in the naked mole rat, the *INK4a/b* locus encodes an additional product that consists of $p15^{INK4b}$ exon 1 joined to $p16^{INK4a}$ exons 2 and 3. We have named this isoform $pALT^{INK4a/b}$ (for alternative splicing). We show that $pALT^{INK4a/b}$ is present in both cultured cells and naked mole rat tissues but is absent in human and mouse cells. Additionally, we demonstrate that $pALT^{INK4a/b}$ expression is induced during early contact inhibition and upon a variety of stresses such as UV, gamma irradiation-induced senescence, loss of substrate attachment, and expression of oncogenes. When overexpressed in naked mole rat or human cells, $pALT^{INK4a/b}$ has stronger ability to induce cell-cycle arrest than either $p15^{INK4b}$ or $p16^{INK4a}$. We hypothesize that the presence of the fourth product, $pALT^{INK4a/b}$ of the *INK4a/b* locus in the naked mole rat, contributes to the increased resistance to tumorigenesis of this species.

naked mole rat | INK4 | p16 | p15

The naked mole rat (*Heterocephalus glaber*) is notable for being the longest-lived rodent, with an observed maximum lifespan of over 30 y (1). Additionally, despite being kept in captivity since 1974, there has been no reported incidence of neoplasia in these animals (2, 3). Naked mole rats remain healthy until almost the end of their lives, showing resistance to multiple age-related diseases (4). Understanding the mechanisms responsible for tumor resistance of this unusual rodent may lead to the discovery of novel targets to cancer therapy that cannot be found in cancer-prone laboratory species.

In tissue culture, naked mole rat fibroblasts display hypersensitivity to contact inhibition, termed early contact inhibition (ECI) (5). ECI is directly linked to the cancer resistance of naked mole rats. The signal triggering ECI comes from an extracellular matrix component, hyaluronan, which, in the naked mole rat, is characterized by an extremely high molecular weight (6). Removal of high molecular weight hyaluronan abrogates ECI and makes naked mole rat cells susceptible to malignant transformation (6). ECI is associated with an increase in $p16^{INK4a}$ expression, and cell lines that spontaneously lost expression from the *INK4a/b* (inhibitors of cyclin dependent kinase 4) locus no longer display ECI (5).

Deletion or silencing of the *INK4a/b* locus is the hallmark of human cancer (7, 8). This small (<50 kb) locus is remarkable because, in mammals, it encodes three distinct tumor suppressors: $p16^{INK4a}$, $p15^{INK4b}$, and $p19^{ARF}$ ($p14^{ARF}$ in human) (9). These three proteins coordinate a signaling network that depends on the activities of the retinoblastoma protein (RB) and the p53 tumor suppressor protein. The $p16^{INK4a}$ and $p15^{INK4b}$ proteins are cyclin-dependent kinase inhibitors that directly inhibit the binding of cyclins to their target cyclin-dependent kinases (10). $p16^{INK4a}$ is involved in establishing replicative senescence, oncogene-induced senescence, and stress-induced premature senescence (11, 12), plays a role in stem-cell aging (9, 13), and acts as a barrier to induced pluripotency (14). Many genes involved in senescence are also associated with age-related disease (15, 16). Indeed, evidence has accumulated to support the role of the locus in atherosclerosis and other aging-related pathologies (17–20). Differences between $p15^{INK4b}$ and $p16^{INK4a}$ include expression profile and stability, with $p15^{INK4b}$ having a lower overall half-life (21, 22). ARF (alternate reading frame) protein $p19^{ARF}$ (in mice) also has a tumor-suppressing function (23): it acts to repress the oncogene MDM2, which in turn binds p53, repressing its activation (24). ARF has other functions in regulating the cell cycle, independently of p53 (25).

The structure of the *INK4a/b* locus is complex and has undergone several modifications during its evolutionary history (26).

Significance

The naked mole rat is a longest lived and cancer-resistant rodent. Tumor resistance in the naked mole rat is mediated by signals from the extracellular matrix component hyaluronan triggering the induction of *INK4* (inhibitors of cyclin dependent kinase 4) locus expression. The human and mouse *INK4* locus encodes three critical tumor-suppressor proteins, $p15^{INK4b}$, ARF (alternate reading frame), and $p16^{INK4a}$, which are among the most frequently mutated in cancer. Furthermore, $p16^{INK4a}$ is implicated in aging and senescence. Here, we show that the naked mole rat *INK4* locus encodes an additional product, a hybrid between $p15^{INK4b}$ and $p16^{INK4a}$. The novel product, named $pALT^{INK4a/b}$, may contribute to tumor resistance and longevity of the naked mole rat. Understanding the regulation of the *INK4* locus is critical for cancer and aging research.

Author contributions: X.T., J.A., V.N.G., V.G., and A.S. designed research; X.T., J.A., Z.K., and A.A. performed research; Z.D.Z., J.V., and V.N.G. contributed new reagents/analytic tools; X.T., J.A., Z.K., A.A., Z.D.Z., J.V., V.N.G., V.G., and A.S. analyzed data; and X.T., J.A., A.A., V.N.G., V.G., and A.S. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

[1]X.T. and J.A. contributed equally to this work.

[2]Present address: University of Texas Health Science Center at San Antonio, San Antonio, TX.

[3]To whom correspondence may be addressed. Email: vera.gorbunova@rochester.edu or andrei.seluanov@rochester.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1418203112/-/DCSupplemental.

CELL BIOLOGY

The emergence of separate $p16^{INK4a}$ and $p15^{INK4b}$ genes is the result of an ancient duplication event (27). They are structurally very similar, featuring several ankyrin repeats. The ARF is a completely different protein produced by using an alternative first exon that splices to the second exon of $p16^{INK4b}$ in a different frame (28). The first exon of ARF was acquired before the *INK4a/b* duplication event (29). The whole-genome sequencing of the naked mole rat revealed that the *INK4a/b* locus was altered by early translation termination events (30), suggesting that it may have undergone specific evolutionary changes to increase cancer resistance in this species. This genomic finding complemented our earlier results that *INK4a/b* was required for ECI and cancer resistance.

Because of the importance of *INK4a/b* in cancer resistance, we decided to characterize the expression of its products. Unexpectedly, we found that, in the naked mole rat, the *INK4a/b* locus produced a novel hybrid protein isoform from the *INK4b* promoter that splices $p15^{INK4b}$ exon 1 to $p16^{INK4a}$ exon 2. This isoform, which we named $pALT^{INK4a/b}$, is unique to the naked mole rat and is not observed in human or mouse. $pALT^{INK4a/b}$ is present in cultured cells and in naked mole rat tissues in vivo. $pALT^{INK4a/b}$ is activated by a variety of stresses, similar to $p15^{INK4b}$ and $p16^{INK4a}$, but has higher capacity to induce cell-cycle arrest. We hypothesize that the presence of the additional, more potent, cyclin-dependent kinase inhibitor $pALT^{INK4a/b}$ contributes to the cancer resistance of the naked mole rat.

Results

Identification of the Novel INK4 Isoform in the Naked Mole Rat. Previous work in naked mole rat fibroblasts had indicated that the *INK4* locus was an important mediator of the tumor resistance of this species (5). To examine the expression of INK4 products, we designed two pairs of primers (Fig. 1*A*) to amplify $p15^{INK4b}$ and $p16^{INK4a}$ cDNAs from three independent lines of naked mole rat fibroblasts. We observed the expected $p16^{INK4a}$, $p15^{INK4b}$ products in primary skin fibroblasts (NSF8) and embryonic fibroblasts (NEF9). The cell line NSF2 mut, which spontaneously lost the early contact inhibition phenotype, did not express any INK4 products (Fig. 1*B*), consistent with our previous observations (5). Unexpectedly, we observed a previously unidentified transcript comprised of exon 1 of $p15^{INK4b}$ and exons 2 and 3 followed by the 3′ UTR from $p16^{INK4a}$ (Fig. 1*B*). The junction between the exon 1 of $p15^{INK4b}$ and exon 2 of $p16^{INK4a}$ corresponded to the canonical splice junction (Fig. 1*C*). We named this isoform $pALT^{INK4a/b}$. In the fibroblasts, the three isoforms were expressed at roughly similar levels, but the direct comparison was difficult because the primer sequences were similar but not identical. We next examined the expression of the three INK4 products in a panel of naked mole rat tissues obtained from a 1-y-old animal. We observed that $pALT^{INK4a/b}$ was expressed in several tissues, with the highest levels present in lung and testes, indicating that $pALT^{INK4a/b}$ is produced in vivo. The expression of various INK4 products appeared to be tissue-specific (Fig. 1*D*), suggesting that different tissues rely on various combinations of INK4 products for cell-cycle control. It is important to note that the testes were collected from a nonbreeding male, which was characterized by suppressed spermatogenesis, explaining the high levels of INK4 products in the testes. Consistent with the PCR data, the $pALT^{INK4a/b}$ product was also detected in the RNA-seq dataset that we previously reported (30). In that dataset, which combined seven tissues, $pALT^{INK4a/b}$ appeared to be less abundant than p15INK^{4b} but more abundant than p16INK^{4a} (Table S1).

To further characterize the pALT product and exclude a possibility that it is the result of a PCR template switch, we transfected human fibroblasts with pCMV vectors containing naked mole rat $p15^{INK4b}$, $p16^{INK4a}$, or $pALT^{INK4a/b}$ cDNAs, or with a

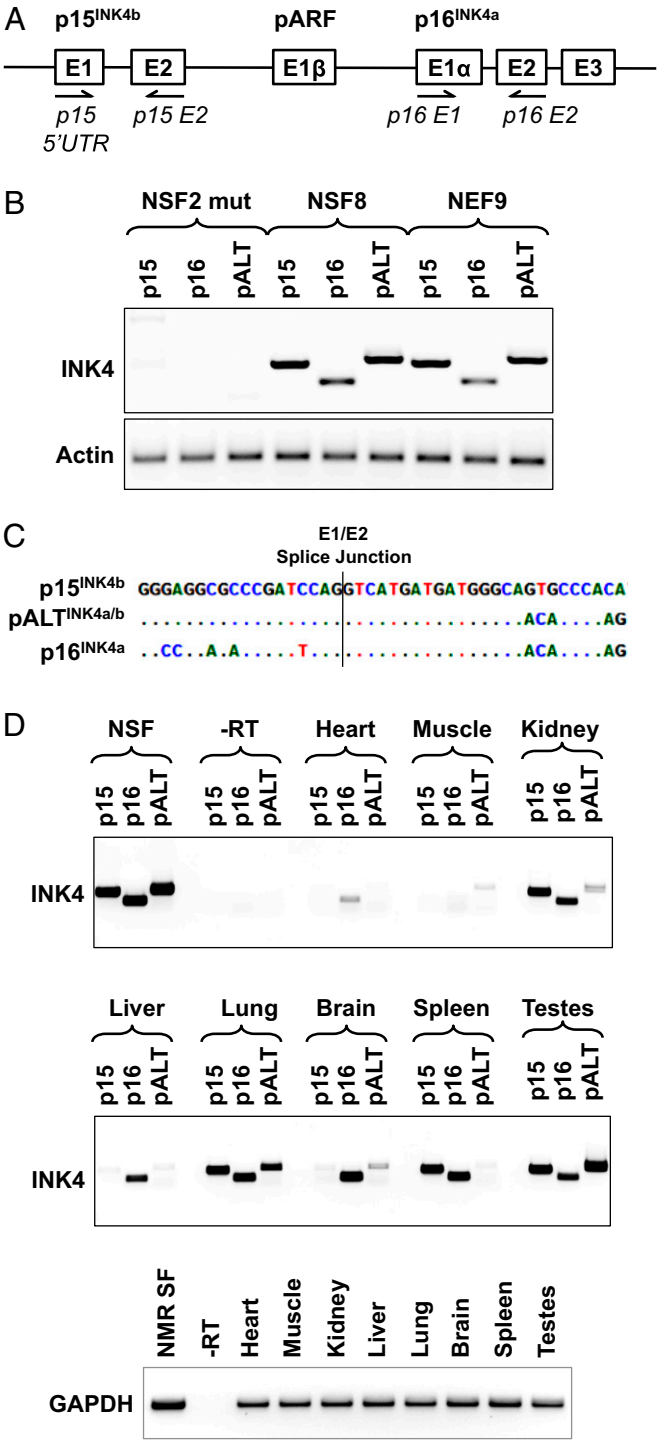

Fig. 1. The *INK4a/b* locus of the NMR produces an alternative protein isoform $pALT^{INK4a/b}$ composed of the first exon of $p15^{INK4b}$ and the second and third exons of $p16^{INK4a}$. (*A*) Schematic of the *INK4a/b* locus showing the location of the primers used to amplify the three *INK4a/b* transcripts. (*B*) RT-PCR products corresponding to the three *INK4a/b* transcripts amplified from RNA isolated from three lines of naked mole rat fibroblasts, with primers shown in *A* and Table S2; $pALT^{INK4a/b}$ was amplified with primers p15-5UTR and p16-E2. NSF2 mut is the line of spontaneously mutated naked mole rat fibroblasts that lost *INK4a/b* locus expression and do not show ECI; NSF8 are primary naked mole rat skin fibroblasts; NEF9 are naked mole rat embryonic fibroblasts. (*C*) Sequences of the Exon1/Exon2 splice junctions in p15, p16, and pALT. (*D*) RT-PCR showing *INK4a/b* products amplified from naked mole rat tissues. GAPDH was used to show that equal amounts of RNA template were used for each tissue. The reactions were repeated at least three times, and representative gels are shown.

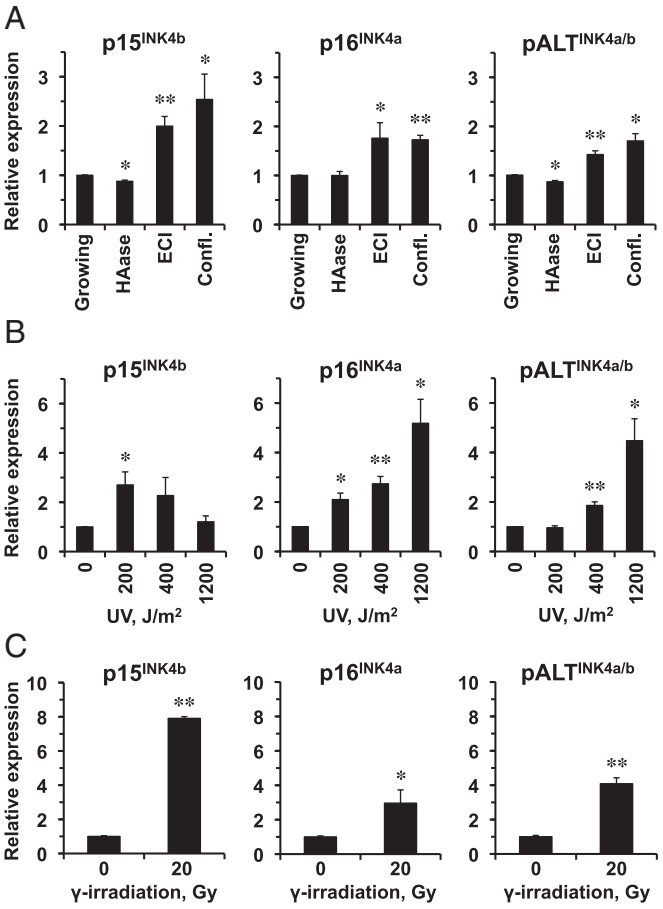

Fig. 2. pALT$^{INK4a/b}$ expression is induced by ECI, confluence, and senescence. (*A*) Naked mole rat fibroblasts were passaged under four different conditions. "Growing" cells were passaged at low cell density to ensure continuous cell proliferation. "HAase" cells were passaged at low cell density in the presence of hyaluronidase. "ECI" cells were kept in the same plate without passaging for 10–14 d until they stopped proliferation and entered the ECI state. "Confl." cells were cultured in the presence of hyaluronidase until they reached confluence at high cell density. RNA was extracted from the cells, and expression from *INK4a/b* locus was analyzed by qPCR. (*B*) Naked mole rat cells were treated with indicated doses of UV-C and analyzed for expression of *INK4a/b* products as described above. (*C*) Naked mole rat cells were treated with 20 Gy of γ-irradiation, kept for 30 d until they developed senescent morphology, and analyzed for expression of *INK4a/b* products as described above. Four lines of low-passage naked mole rat skin fibroblasts were used for each experiment. Error bars indicate SD; $*P < 0.05$; $**P < 0.005$; Student's *t* test.

mix of the p15^{INK4b} and p16^{INK4a} plasmids. We then performed RT-PCR analysis using primer pairs specific to p15^{INK4b}, p16^{INK4a}, and pALT$^{INK4a/b}$ (Fig. S1). As expected, the cells expressing only one gene showed one respective band whereas the mix showed a p15^{INK4b} and p16^{INK4a}, but no pALT$^{INK4a/b}$, band. If template switching had occurred, we would have expected to detect the pALT$^{INK4a/b}$ product in the mixed transfection. This experiment further verifies that the pALT$^{INK4a/b}$ transcript is a genuine gene product and not a result of a PCR artifact.

We also tested mouse and human cells for the presence of a similar alternative splice product. Mouse skin fibroblasts were induced by UV irradiation to produce p16^{INK4a}, and RNA was extracted from these cells. Replicatively senescent human skin fibroblasts served as human cell lines expressing p16^{INK4a}. Several primer pairs (p15 5′ UTR and p16 3′ UTR) for each species were used, and no hybrid products between the p15 and p16 were detected. The canonical p16^{INK4a} product was detected normally in these extracts (Fig. S2). Although this result does not eliminate the possibility that a pALT$^{INK4a/b}$-like transcript may occur under some conditions in mouse and human, it strongly suggests that this isoform is unique to the naked mole rat.

pALT$^{INK4a/b}$ Is Induced by High Cell Density and a Stress. To determine the biological significance of pALT$^{INK4a/b}$, we examined the expression of pALT$^{INK4a/b}$, p15^{INK4b}, and p16^{INK4a} in naked mole rat cells under various conditions that lead to the induction of the *INK4a/b* locus. We used four lines of naked mole rat fibroblasts derived from four individual animals and analyzed expression using quantitative PCR (qPCR).

First, we examined the induction of p15^{INK4b}, p16^{INK4a}, and pALT$^{INK4a/b}$ under different growth conditions: logarithmically growing cells; logarithmically growing cells in the presence of an enzyme, hyaluroinidase (HAase), that breaks down hyaluronan, leading to more rapid cell growth; cells in ECI; and cells that entered confluence in the presence of HAase. We found that all three transcripts were up-regulated in cells growth-arrested by either confluence or ECI (Fig. 2*A*). Furthermore, in growing cells, HAase treatment significantly reduced p15^{INK4b} and pALT$^{INK4a/b}$ expression. This result indicates that pALT$^{INK4a/b}$ responds to hyaluronan-induced ECI and cell density.

Next, we tested the induction of p15^{INK4b}, p16^{INK4a}, and pALT$^{INK4a/b}$ under stress. Ultraviolet-C (UV-C) exposure induces pyrimidine dimer formation in DNA and is known to induce *INK4a* expression in human melanocytes and keratinocytes (31). UV-C irradiation induced all three transcripts but with different kinetics. p15^{INK4b} was induced by lower doses of UV but declined at the highest dose whereas p16^{INK4a} and pALT$^{INK4a/b}$ showed similar patterns of induction that increased with the UV

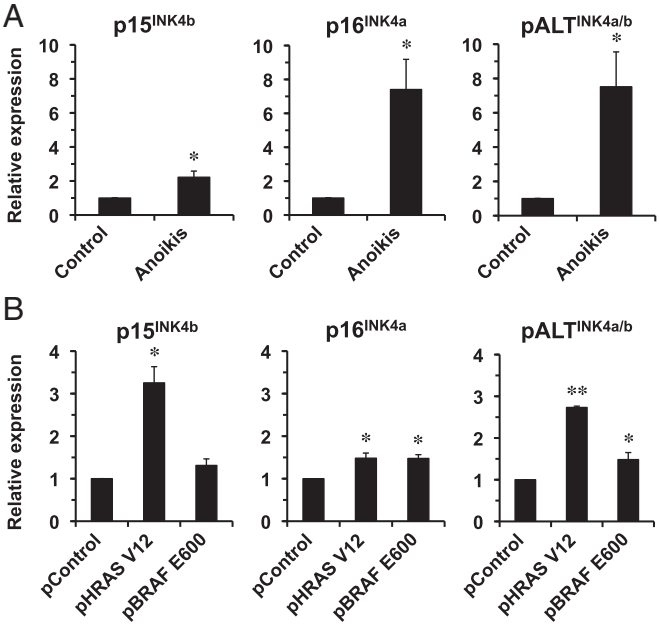

Fig. 3. pALT$^{INK4a/b}$ expression is induced by oncogenic stimuli. (*A*) Naked mole rat cells were subjected to anchorage-independent growth to trigger anoikis by placing them in plates precoated with 1% agarose for 48 h. RNA was extracted from the cells, and expression from *INK4a/b* locus was analyzed by qPCR. (*B*) Naked mole rat cells were transfected with plasmids encoding HRas V12 or BRAF E600 oncogenes or pControl plasmid encoding GFP. RNA was extracted 10 d after transfection, and expression from *INK4a/b* locus was analyzed by qPCR. Four lines of low-passage naked mole rat skin fibroblasts were used for each experiment. Error bars indicate SD; $*P < 0.05$; $**P < 0.005$; Student's *t* test.

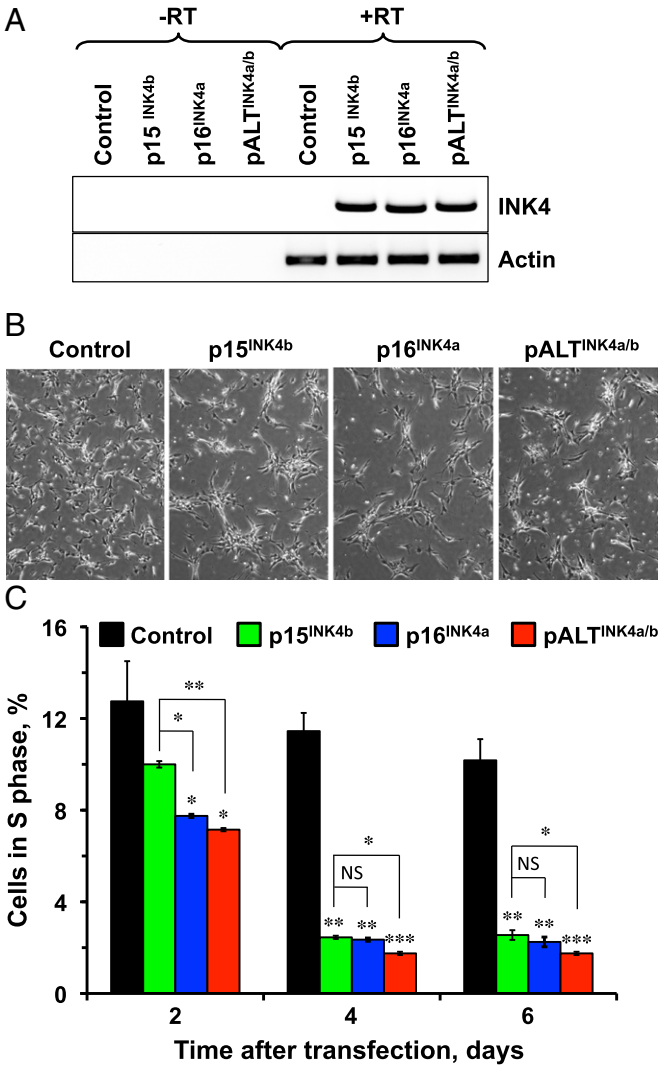

Fig. 4. pALT$^{INK4a/b}$ overexpression triggers cell-cycle arrest. Naked mole rat cells were transfected with constructs expressing HPRT (control), p15^{INK4b}, p16^{INK4a}, or pALT$^{INK4a/b}$ under the CMV promoter. (*A*) Expression of the *INK4a/b* products was verified with RT-PCR. (*B*) Microphotograph showing cells transfected with p15^{INK4b}, p16^{INK4a}, or pALT$^{INK4a/b}$ 6 d after transfection. Control cells continued to proliferate whereas cells transfected with INK4a/b products entered cell-cycle arrest. (*C*) Cell-cycle arrest induced by expression of p15^{INK4b}, p16^{INK4a}, or pALT$^{INK4a/b}$ was quantified at indicated times after transfection by staining cells with propidium iodide and quantifying the number of cells in S-phase by FACS analysis. The experiments were repeated three times, and error bars show SD; statistical significance is indicated by asterisk. Three lines of low-passage naked mole rat skin fibroblasts were used for each experiment. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$, Student's *t* test.

dose (Fig. 2*B*). Stress-induced premature senescence triggered by γ-irradiation also induced all of the three transcripts, with the highest induction observed for p15^{INK4b}, followed by pALT$^{INK4a/b}$ and weaker induction observed for p16^{INK4a} (Fig. 2*C*).

All of the three transcripts were induced to a similar extent by anoikis (Fig. 3*A*). We additionally tested whether oncogene overexpression known to activate p15^{INK4b} and p16^{INK4a} would also activate pALT$^{INK4a/b}$. The cells were transfected with either a GFP plasmid or an RASV12 plasmid, or a BRAF V600E-expressing plasmid, and RNA was extracted 10 d later. RASV12 significantly activated p15^{INK4b}, p16^{INK4a}, and pALT$^{INK4a/b}$ whereas BRAF V600E induced p16^{INK4a} and pALT$^{INK4a/b}$ (Fig. 3*B*). These results show that pALT$^{INK4a/b}$ is induced by oncogenic stimuli, suggesting that, similarly to p15^{INK4b} and p16^{INK4a}, it has a tumor-suppressor function.

pALT$^{INK4a/b}$ Is Efficient at Inducing Cell-Cycle Arrest. We next set out to test whether pALT$^{INK4a/b}$ can function to induce cell-cycle arrest. We cloned p15^{INK4b}, p16^{INK4a}, and pALT$^{INK4a/b}$ cDNAs into a pCMV6 expression vector and transiently transfected them into naked mole rat skin fibroblasts. Similar levels of expression were confirmed by semiquantitative PCR (Fig. 4*A*). We found that, compared with mock or GFP transfection, cells overexpressing p15^{INK4b}, p16^{INK4a}, or pALT$^{INK4a/b}$ had substantially lower rates of growth (Fig. 4*B*). Remarkably, pALT$^{INK4a/b}$ protein was significantly more capable of halting cell cycle than p15^{INK4b}, p16^{INK4a} (Fig. 4*C*). We speculate that this enhanced ability of pALT$^{INK4a/b}$ to mediate cell-cycle arrest may contribute to stricter cell-cycle control and ultimately increased cancer resistance in the naked mole rat.

pALT$^{INK4a/b}$ Protects Cells from Radiation-Induced Apoptosis. NSF2 mut fibroblasts have lost the expression of the INK4 locus (5) and are defective in cell-cycle arrest. Unlike primary fibroblasts that undergo stress-induced senescence, NSF2 mut cells undergo apoptosis in response to γ-irradiation. To test whether pALT$^{INK4a/b}$ can rescue the apoptotic response in NSF2 mut cells, the cells were transfected with plasmids encoding NMR INK4 proteins or a control plasmid expressing the HPRT gene. Two days after transfection, cells were subjected to 20 Gy γ-irradiation. Four or 10 d after irradiation, floating and adherent cells were harvested and analyzed by Annexin-V staining and flow cytometry. Cells transfected with the control plasmid underwent massive apoptosis,

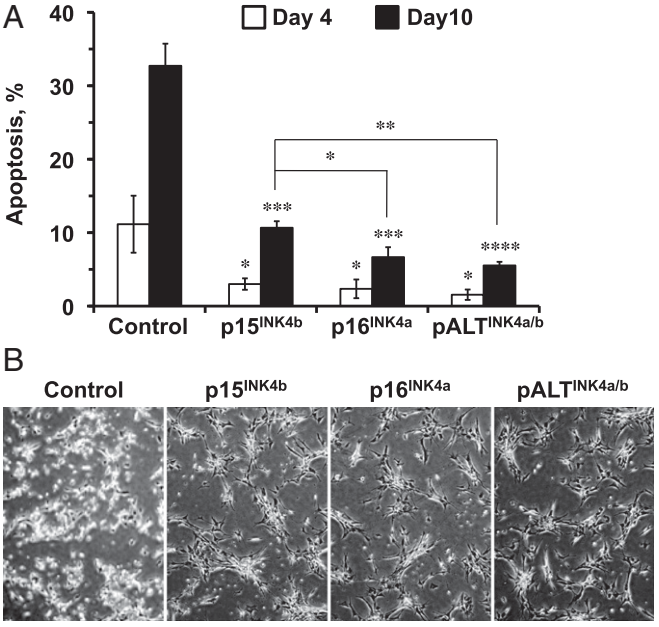

Fig. 5. pALT$^{INK4a/b}$ overexpression attenuates γ-irradiation–induced apoptosis in NSF2 mut cells. NSF2 mut cells were transfected with plasmids encoding HPRT (control), p15^{INK4b}, p16^{INK4a}, or pALT$^{INK4a/b}$ under the CMV promoter. Two days after transfection, cells were subjected to 20 Gy γ-irradiation. (*A*) At four and 10 d after γ-irradiation, the cells were harvested, and the percent of apoptotic cells was analyzed by Annexin-V staining and flow cytometry. (*B*) Cells were photographed 10 d after γ-irradiation. Control plate shows many floating apoptotic cells whereas plates transfected with *INK4a/b* products show lower numbers of apoptotic bodies and many growth-arrested cells. The experiments were repeated three times, and error bars show SD; statistical significance is indicated by asterisk. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$; ****$P < 0.00005$, Student's *t* test.

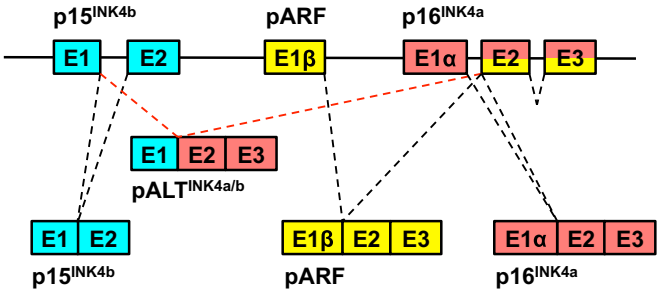

Fig. 6. Structure of the naked mole rat *INK4a/b* locus. The diagram shows the genomic locus and the canonical $p15^{INK4b}$, $p16^{INK4a}$, and pARF transcripts, as well as the $pALT^{INK4a/b}$ transcript unique to the naked mole rat.

which was significantly reduced by expression of the INK4 proteins (Fig. 5). Interestingly, $p16^{INK4a}$ and $pALT^{INK4a/b}$ were significantly more effective at reducing apoptotic cell death of NSF2 mut cells (Fig. 5). There was a trend toward even lower apoptotic response in $pALT^{INK4a/b}$-transfected cells compared with $p16^{INK4a}$-transfected cells. This result suggests that $p16^{INK4a}$ and $pALT^{INK4a/b}$ are more efficient in inducing cell-cycle arrest and/or senescence than $p15^{INK4b}$.

Discussion

The dynamic regulation of the *INK4a/b* locus is of tremendous importance to tumorigenesis (32–36), stem-cell maintenance (9, 13), cellular senescence (37), and systemic aging (19, 38). Here, we have demonstrated that naked mole rat cells express a novel INK4 isoform, $pALT^{INK4a/b}$, composed of the first exon of $p15^{INK4b}$ and the second and third exons of $p16^{INK4a}$ (Fig. 6). This novel isoform was detected in multiple naked mole rat tissues and in cultured cells by RT-PCR, as well as by RNA-seq. A concern that arose during this work was the possibility that the alternative splicing we had observed was an aberrant intermolecular "template switching" product of the reverse transcriptase reaction (39). We do not believe that template switching is a likely explanation for the following five independent reasons: artifacts of this nature do not often occur at canonical splice junctions whereas the isoform we observed was reproducibly found to occur between the canonical E1 and E2 junction; we could replicate our results on multiple cell lines and even extracts from tissues from live animals; we found this product independently using different primer pairs; a similar product could not be found in human or mouse cells even when the locus was strongly induced; and we could not find the ALT transcript when NSF2 mut cells were simultaneously transfected with $p15^{INK4b}$ and $p16^{INK4a}$ overexpression plasmids.

We previously showed that ECI in naked mole rat cells is associated with the induction of $p16^{INK4a}$ (5). Our current work shows that the entire *INK4a/b* locus, including the $pALT^{INK4a/b}$ isoform, undergoes up-regulation in response to cell crowding and buildup of hyaluronan. We show that $pALT^{INK4a/b}$ is induced by other stresses, such as UV- and γ-irradiation, expression of oncogenes, and anoikis. Although both $p15^{INK4b}$ and $p16^{INK4a}$ function as repressors of the cell cycle, their relative importance in different cancers has not been fully resolved. $p15^{INK4b}$ has been proposed to serve as a backup to $p16^{INK4a}$, and mice in which both genes are mutated show higher cancer susceptibility than animals missing $p16^{INK4a}$ alone (40). However, only $p16^{INK4a}$, but not $p15^{INK4b}$, expression has been reported to be induced during the aging process (41, 42). $p15^{INK4b}$ and $p16^{INK4a}$ are induced to a different extent by partially overlapping sets of stimuli (7, 43), which likely contributes to the complex regulation of the cellular stress response. The pattern of $pALT^{INK4a/b}$ expression we observed in naked mole rat fibroblasts was intermediate between $p15^{INK4b}$ and $p16^{INK4a}$, which may be explained by $pALT^{INK4a/b}$ and $p15^{INK4b}$ sharing the same promoter and the 5′ UTR and to regulatory elements located in the 3′ UTR shared with $p16^{INK4a}$. Furthermore, we demonstrate that the novel isoform $pALT^{INK4a/b}$ is a biologically active repressor of the cell cycle. Remarkably, $pALT^{INK4a/b}$ was more efficient at arresting the cell cycle upon overexpression than either $p15^{INK4b}$ or $p16^{INK4a}$, and more efficient at mediating cell-cycle arrest after γ-irradiation than $p15^{INK4b}$. $p16^{INK4a}$ protein was found to be more stable than $p15^{INK4b}$ (the C-terminal segment is responsible for differential stability of INK4 proteins) (21, 22). Therefore, we hypothesize that $pALT^{INK4a/b}$ has similar stability to $p16^{INK4a}$, combined with the expression profile intermediate between $p15^{INK4b}$ and $p16^{INK4a}$. The presence of an additional product of the *INK4a/b* locus, with higher capacity to induce cell-cycle arrest, may confer an added protection to naked mole rat cells, contributing to cancer resistance of this species. It was recently shown that contact inhibition represses mTOR and thus suppresses the senescence program (44). Thus, it is tempting to speculate that ECI in naked mole rats mediated by the unusual structure of the *INK4a/b* locus may contribute to naked mole rat longevity, in addition to conferring cancer resistance.

Furthermore, the balance between tumor suppression and proliferation is important for longevity. For example, overly zealous tumor suppression, such as that observed in mice with an overactive p53 gene (45, 46), may limit stem-cell reserves and lead to premature aging; and very loose tumor suppression would also shorten lifespan by increasing cancer incidence. We hypothesize that an additional cyclin-dependent kinase inhibitor may provide an added layer of redundancy, allowing naked mole rats to further fine-tune their cell-cycle checkpoints for more optimal balance between growth and tumor suppression necessary to achieve longevity.

Experimental Procedures

Detailed experimental procedures including plasmid construction, qRT-PCR, cell culture, cell-growth analysis, analysis of INK4 expression, cell-cycle analysis, apoptosis analysis, and RNA-Seq are provided in *SI Experimental Procedures*.

ACKNOWLEDGMENTS. This work was supported by grants from the US National Institutes of Health (to Z.D.Z., J.V., V.N.G., V.G., and A.S.), the Life Extension Foundation (to V.G. and A.S.), the Glenn Foundation for Medical Research (to V.G. and J.V.), and the Ellison Medical Foundation (to V.G. and A.S.).

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