Supporting Information

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SI Experimental Procedures

Plasmid Construction. pCMV6 plasmid was digested with NotI and SalI (New England Biolabs). Adapter primers were used to add appropriate restriction sites to clones of naked mole rat $p15^{INK4b}$, $p16^{INK4a}$, and $pALT^{INK4a/b}$ cDNA. Plasmids and inserts were ligated, subcloned, and sequenced to confirm correct construction. All plasmids used were prepared using an EndoFree Maxi kit (Qiagen).

qRT-PCR. RNA extraction from cell lines and tissues (brain, heart, muscle, liver, lung, spleen, kidney, and testes from a 1-y-old naked mole rat) was performed using the Qiagen RNeasy kit (cat. 74104) according to the manufacturer's instructions. RNA samples were treated with DNase I followed by heat inactivation. cDNA was synthesized by SuperScript III (Invitrogen) unless otherwise noted. RNA was heated to 80 °C for 5 min and immediately cooled on ice before RT reaction. RT reactions were all performed at 55 °C for 1 h.

qRT-PCR was performed using the FastStart SYBR Green (Rox) mix from Roche in a total volume of 25 μL. Primers used are summarized in Table S2. Fold-differences in expression were calculated using the ΔΔCt method, with beta-actin as the reference gene.

Cell Culture. Naked mole rat fibroblasts were grown at 32 °C (in vivo body temperature of naked mole rat), 5% CO_2, 3% O_2 on treated polystyrene culture dishes (Corning) in EMEM media (ATCC) supplemented with 15% (vol/vol) FBS (Gibco), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco). Unless otherwise indicated, all cell-culture experiments were performed in at least three primary naked mole rat skin fibroblast lines at low passage (NSF8, NSF13, NSF14, and/or NSFJ2). All of the cell lines were obtained from different naked mole rat individuals. The experiment measuring the reduction of irradiation-induced apoptosis was performed in NSF2 mut cells that spontaneously lost INK4 locus expression, the senescence response, and the ECI phenotype (1).

Analysis of INK4 Expression in Different Growth States. Four independent naked mole rat skin fibroblast cell lines were passaged twice at low cell density before being grown for 48 h and harvested for RNA extraction; these samples are referred to as "growing." ECI cells were achieved by passaging twice and allowing to grow on the same plate for 10–14 d until they stopped growing and exhibited the ECI phenotype. Hyaluronidase-treated cells were obtained by passaging and growing with 3 U/mL *Streptomyces hyalurolyticus* hyaluronidase (Sigma-Aldrich H1136) for 48 h. Confluent cells were cells grown with 3U/mL hyaluronidase until reaching confluence and maintained for another 48 h.

UV Irradiation. Naked mole rat skin fibroblasts were passaged twice after recovery from liquid nitrogen and grown for an additional 48 h. Cells were then subjected with UV-C radiation of 0, 200, 400, and 1,200 J/m^2, respectively. Cells were then grown for 24 h and harvested for RNA extraction and qRT-PCR analysis.

γ-Irradiation–Induced Senescence. Growing naked mole rat skin fibroblasts were treated with 20 Gy γ-irradiation and allowed to grow for 30 d to fully develop the senescent phenotype. Media were changed every 7 d. Senescent cells were harvested, and RNA was extracted for qRT-PCR analysis.

Anoikis. Growing naked mole rat skin fibroblasts were harvested, and 2×10^6 cells were put into the plates precoated with 1% agarose for 48 h. Cells were then collected, and RNA was extracted for qRT-PCR analysis.

Oncogene Overexpression. Naked mole rat skin fibroblasts were transfected with pEGFP-N1 (Clontech), pHRAS V12 (Clontech), or pBRAF E600, respectively. Transfections were performed 48 h after splitting using Amaxa Nucleofector II on program U20 and solution NHDF (Lonza). Media were replaced 24 h after transfection to remove dead cells. Cells were then maintained for 10 d before harvesting for qRT-PCR analysis of INK4 locus.

Cell-Cycle Analysis. Naked mole rat skin fibroblasts were transfected with *INK4* plasmids and were plated at a low density (1×10^5 cells per 10-cm plate) to avoid ECI-induced cell-cycle arrest. The overexpression level was examined by RT-PCR using a primer pair on the vector flanking the INK4 cDNA. The cells were harvested at different time points and then fixed in 70% (vol/vol) ethanol at −20 °C for 16 h. The cells were centrifuged, ethanol was decanted, and the pellet was resuspended in 1 mg/mL RNase A in PBS followed by incubating for 30 min. The cells were centrifuged again, resuspended in 20 μg/mL propidium iodide in PBS, and stained for 10 min. Cell-cycle status was determined by flow cytometry using a FACS Canto machine with previously published parameters (2).

Apoptosis Analysis. NSF2 mut cells that spontaneously lost INK4 locus expression and the ECI phenotype (1) were transfected with plasmids encoding NMR INK4 proteins or a control plasmid expressing HPRT. Forty-eight hours after transfection, cells were subjected to 20 Gy γ-irradiation. Media were changed immediately after radiation. Four or 10 d after irradiation, cells (including the dead cells) were collected, and Annexin-V (Roche) staining was conducted to measure the cell death on a FACS Canto machine.

RNA-seq Analysis. Total RNA was from brain, kidney, and liver of newborn, 4-y-old, and 20-y-old NMRs, or from a pooled sample of 7 organs of a 4-y-old NMR (3). Abundance of various expressed forms derived from the *INK4* locus was analyzed by BlastN using 60 nucleotide fragments corresponding to segments of $p15^{INK4b}$ and $p16^{INK4a}$ genes mapped to various RNA-seq libraries. To assess relative expression of transcripts derived from the *INK4* locus, only uniquely mapped fragments corresponding to the exon 1/exon 2 junction were considered.

1. Seluanov A, et al. (2009) Hypersensitivity to contact inhibition provides a clue to cancer resistance of naked mole-rat. *Proc Natl Acad Sci USA* 106(46):19352–19357.
2. Mao Z, Ke Z, Gorbunova V, Seluanov A (2012) Replicatively senescent cells are arrested in G1 and G2 phases. *Aging (Albany, NY Online)* 4(6):431–435.
3. Kim EB, et al. (2011) Genome sequencing reveals insights into physiology and longevity of the naked mole rat. *Nature* 479(7372):223–227.

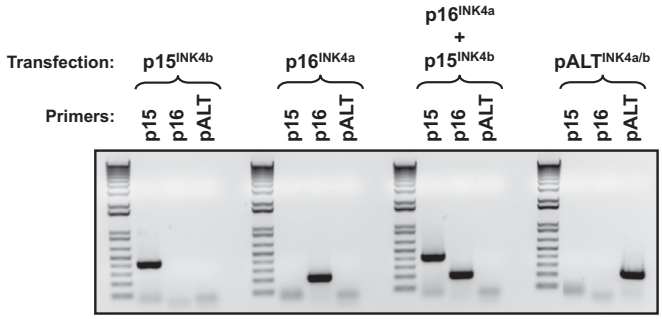

Fig. S1. The pALT$^{INK4a/b}$ product is not the result of a template switching by reverse transcriptase. Human skin fibroblasts not expressing any *INK4a/b* products were transfected with pCMV6 constructs encoding naked mole rat p15^{INK4b}, p16^{INK4a}, or pALT$^{INK4a/b}$ cDNAs. One cell line was simultaneously transfected with the INK4a and INK4b plasmids. RNA was extracted 24 h posttransfection and reverse transcribed with SuperScript III RT. Each cDNA was amplified with isoform-specific primers.

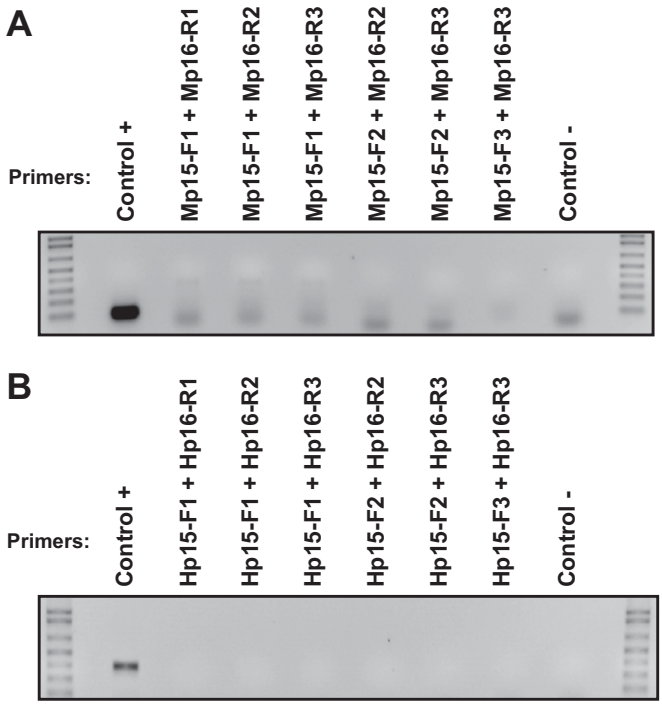

Fig. S2. Human and mouse cells induced to transcribe *INK4a/b* locus do not show the alternative splicing product. PCR using primers to p15 5′ UTR and p16 3′ UTR did not yield a similar splice isoform to that observed in the naked mole rat. Primer pairs are indicated above the gel image. (*A*) Mouse cDNA from cell lines irradiated with 1,200 J/cm^3 UV-C. The positive (+) control reaction was performed with primers to canonical mouse p16^{INK4a}; the negative (–) control is a reaction containing no template DNA. (*B*) Human cDNA from senescent skin fibroblasts (population doubling >70). The positive (+) control reaction was performed with primers to canonical human p16^{INK4a}; the negative (–) control is a reaction containing no template DNA.

Table S1. Total numbers of unique reads from RNA-seq libraries corresponding to the junction between exon 1 and exon 2 of $p15^{INK4b}$, $p16^{INK4a}$, and $pALT^{INK4a/b}$

Samples		No. of reads of the E1/E2 splice junction		
Tissues	Age	$p15^{INK4b}$	$p16^{INK4a}$	$pALT^{INK4a/b}$
Pool*	4 y	16 (66.7%)	3 (12.5%)	5 (20.8%)
Brain	Newborn	0	0	0
	4 y	0	10	0
	20 y	4	24	0
Kidney	Newborn	0	0	0
	4 y	1	4	0
	20 y	3 (27.3%)	5 (45.4%)	3 (27.3%)
Liver	Newborn	0	0	0
	4 y	0	0	0
	20 y	1	1	0

*Pooled sample of seven organs (brain, liver, kidney, spleen, heart, testis, and lung).

Table S2. PCR primers used to amplify *INK4a/b* transcripts

Name*	Sense or antisense	Sequence (5′→3′)	Specificity
NMR p15-5UTR	S	CAGGAAAAGCCCGGAACTAACTAC	Specific to NMR p15 5′ UTR.
NMR p15-E2	AS	GCAGAAGCTCAGCCATGTGGGCAC	Specific to NMR p15 Exon 2
NMR p16-E2	AS	GGTGACAGGGTCAGCGCAGTTCG	Specific to NMR p16 Exon 2
NMR p16-E1	S	GCAGCATGGACTCGTGGGGCGAAAAGCTGG	Specific to NMR p16 E1, includes start codon
H p15-5UTR-F1	S	TGGGAAAGAAGGGAAGAGTGTCGTTAAG	Human INK4b 5′ UTR
H p15-5UTR-F2	S	TTTTTCCCAGAAGCAATCCAGGCGC	Human INK4b 5′ UTR
H p15-5UTR-F3	S	CAGGAAAAGCCCGGAGCTAACG	Human INK4b 5′ UTR
H p16-3UTR-R1	AS	AGTTTCCCGAGGTTTCTCAGAGCC	Human INK4a 3′ UTR
H p16-3UTR-R2	AS	AACTCCAACACAGTGAAAAGGCAGAAGC	Human INK4a 3′ UTR
H p16-3UTR-R3	AS	TTTGAGCTTTGGTTCTGCCATTTGCTAGC	Human INK4a 3′ UTR
M p15-5UTR-F1	S	GAACGCTGCAGCTCAGTGCCTG	Mouse INK4b 5′ UTR
M p15-5UTR-F2	S	ACCGAAGCTACTGGGTCTCCAGAG	Mouse INK4b 5′ UTR
M p15-5UTR-F3	S	CGAAGGACCATTTCTGCCACAGAC	Mouse INK4b 5′ UTR
M p16-3UTR-R1	AS	GGCTGAGGCCGGATTTAGCTC	Mouse INK4a 3′ UTR
M p16-3UTR-R2	AS	CTCTGCTCCCTCCCGTGATTGC	Mouse INK4a 3′ UTR
M p16-3UTR-R3	AS	CTCATGCCATTCCTTTCCTGTCC	Mouse INK4a 3′ UTR

*Letters preceding the primer name indicate the species for which INK4 loci the corresponding primers were designed: NMR, naked mole rat; H, human; M, mouse.