# Supporting Information

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### SI Text

**Animals.** Naked mole-rats were from the colony of K.C.C. at Vanderbilt University. Mice used for isolation of cell lines were C57BL6.

**Cell Isolation and Culture.** Primary mouse and naked mole-rat cells were isolated from lung and under arm skin. Cells were obtained from five animals of each species. The initial growth characteristics did not differ between the cell lines from different animals therefore we performed most of the experiments on two lung and two skin cell lines from two animals. All cell lines were used at early passage (<12–15 PDs).

We used cell isolation protocol adapted from S. Austad (University of Texas Health Sciences Center, San Antonio). Skin was shaved and cleaned with 70% ethanol. Tissue was minced and incubated in DMEM F-12 media (Gibco) with 0.14 Wunsch U/mL collagenase (Liberase Blendzyme 3, Roche) at 37 °C on a stirrer for 30–90 min. Dissociated cells were then washed, and placed in tissue culture dishes with DMEM F-12 media (Gibco), 15% FBS, and antibiotics/antimycotic (Gibco). Mouse cells were cultured at 37 °C, 5% $CO_2$, 3% $O_2$; naked mole-rat cells were cultured at 32 °C, 5% $CO_2$, 3% $O_2$ on treated polystyrene culture dishes (Corning). After the plates became confluent, cells were re-plated, and alliquotes were frozen in liquid nitrogen. All of the subsequent culture was performed in EMEM media (ATCC) supplemented with 15% FBS (Gibco), nonessential amino acids, sodium pyruvate, 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin (Gibco). All of the experiments were performed using the same batch of FBS.

**Cell Growth Analysis.** To measure cell proliferation and the confluent density cells were seeded on 60-mm grided plates (Corning). Three 2 × 2 mm squares were marked on each plate and the number of cells in those squares was counted each day for 20 days. Images of the squares were taken using SPOT Advanced (Diagnostic Instruments) and analyzed using the colony counting program on ImageQuant TL (GE). The average count of the three squares for each day was multiplied by 458.33 to give the total cell count per 10-cm plate. Cell count data were analyzed using Microsoft Excel.

**Thymidine Incorporation.** Thymidine incorporation assay was performed as described (62). Briefly, cells were grown on BD BioCoat Fibronectin pretreated Culture Slides (BD Biosciences), for 2 (sparse) and 10 (confluent) days for mouse fibroblasts, and for 4 (sparse) and 20 (confluent) days for naked mole-rat fibroblasts, and [$^3$H]thymidine was added to the cells. Cells were then incubated for an additional 2 days for mice and 4 days for naked mole-rat fibroblasts, fixed, and subjected to autoradiography. Percentage of cells incorporating tritiated thymidine was determined by counting at least 200 labeled and unlabeled nuclei.

**Transfections.** Naked mole-rat skin fibroblasts were seeded at 2 × $10^5$ cells/100-mm plate 7 days before transfection. Mouse skin fibroblasts were seeded at 5 × $10^5$ cells/100-mm plate 3 days before transfection. For transfection, cells were harvested, counted and $10^6$ cells were transfected with 5 $\mu$g plasmid DNA using Amaxa Nucleofector II on program U-020 and solution NHDF (Amaxa). After transfection, cells were seeded at 2 × $10^5$ live cells per 10-cm plate for apoptosis analysis and 7 × $10^4$ live cells per 6-cm grid plates (Corning) for cell growth analysis in the same media as stated above. Media was replaced 24 h post-transfection to remove dead cells due to electroporation. Transfections included a mock with no DNA, pEGFP-N1 (Clontech), pSG5 Large T (Addgene 9053), pSG5 Large T K1 (Addgene 9055), pSG5 Large TΔ434–444 (Addgene 9054), and a no electroporation control.

**Anchorage-Independent Soft Agar Growth Assay.** $10^6$ mouse, naked mole-rat wild type (SF4) and naked mole-rat mutant (SF2) exponentially growing skin fibroblast cells were transfected by Amaxa with the following plasmid DNA mixtures: 5 $\mu$g pEGFP-N1 (Clontech) and 5 $\mu$g pSG5 Large T, 5 $\mu$g pRas-V12 (Clontech) and 5 $\mu$g pSG5 Large T, 5 $\mu$g pRas-V12 and 5 $\mu$g pSG5 Large T K1, or 5 $\mu$g pRas-V12 and 5 $\mu$g pSG5 Large T Δ434–444. After transfection, cells were seeded and allowed to recover for 24 h on 10-cm treated polystyrene plates (Corning) in 1× Minimum Essential Medium, Eagle with Earle's Balances salt Solution supplemented with 15% FBS and antibiotics Gibco). The following day, a 2-mL final solution of 0.5% Difco Agar Noble (BD Biosciences) and 1× media mixture was poured into 6-cm treated polystyrene plates (Corning) and allowed to solidify in incubators at 37 °C. After harvesting and counting cells transfected 24 h previous, 50 to 50,000 cells were serially diluted and resuspended in 1 mL of 2× media. This cell suspension was then quickly mixed with 1 mL 0.7% liquid Difco Agar Noble, making a final 0.35% agar/1× media solution, and seeded on top of the solidified 0.5%/1× media. After 1 week of incubation at 37 °C, 5% $CO_2$, and 3% $O_2$, an additional 1 mL of 1× liquid media was added to the soft agar plates to supplement lost nutrients and to keep cells hydrated. Cells and colonies were incubated for 21 days post-seeding in soft agar and then photographed (Diagnostic Instruments) at 10× and 20× under a phase contrast microscope (Nikon TS100).

**Luciferase Assays.** Cells were harvested and transfected using Amaxa Nucleofector II. To test for p53 response to selected oncoproteins, cells were transfected with 0.1 $\mu$g *Renilla* luciferase expressing phRL-CMV Vector (Promega) and either 5 $\mu$g pEGFP-N1 (Clontech), pSG5 Large T K1 (Addgene plasmid 9055), or pSG5 Large T (Addgene plasmid 9053) with 0.5 $\mu$g firefly luciferase expressing p53 response plasmid pp53-TA-Luc Vector (Clontech). To test for Rb response to selected oncoproteins, cells were transfected with 5 $\mu$g of pEGFP-N1, pSG5 Large T Δ434–444 (Addgene plasmid 9054), or pSG5 Large T with 0.5 $\mu$g firefly luciferase expressing Rb response plasmid pRb-TA-Luc Vector (Clontech). Mouse cells were harvested 3 days post-transfection and naked mole-rat cells were harvested 7 days post-transfection and counted using a Beckman Coulter Z2 Particle Count and Size Analyzer. Cells were lysed using 100 $\mu$L Passive Lysis Buffer (Promega) per $10^5$ cells and flash frozen/thawed three times in liquid nitrogen and a 37 °C water bath. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) and program DLR-2-INJ on a Glomax 20/20 Luminometer (Promega) with 25 $\mu$L cell extract as the input. Although both firefly luciferase and *Renilla* luciferase relative luminescence units (RLU) were obtained, only firefly luciferase RLU was used to analyze Rb and p53 activity in these cells upon transfection with oncoproteins. This was due to the oncoproteins increasing the transcriptional activity of the CMV enhancer and early promoter elements controlling *Renilla* luciferase expression.

**Analysis of Apoptosis.** Apoptosis was examined using TUNEL and Annexin-V-FLUOS assay kits (Roche). Floating and adherent cells were harvested, stained according to manufacutrer's instructions and analyzed on a BD Biosciences FACSCanto flow cytometer.

**p16 Quantitative RT-PCR on Growing and Early Contact-Inhibited Naked Mole-Rat Cultures.** Naked mole-rat skin fibroblasts were harvested, counted on a Beckman Coulter Z2 particle counter, and treated with RNA*later* RNA Stabilization Reagent and RNase-free DNase (Qiagen). mRNA was extracted from the cells using the RNeasy Mini kit and QIAshredder (Qiagen) and concentrations were determine by A260 nM spectrophotometry on a SMartSpec Plus (Bio-Rad). RT-PCR was done using the Titan one-tube RT-PCR kit (Roche) with 0.4 $\mu$g total mRNA as input. Primer sequences for analyzing naked mole rat p16 are 5′-CGAACTGCGCTGACCCTGTCACC-3′ and 5′-TTTGTGGGGGTGACTACACAGGCATCGCTGCC-3′ and primers used for the control RT-PCR of the 18S ribosomal subunit were from QuantumRNA 18S internal standards kit (Ambion) and used at a 1:8 18S primer:competimer ratio. The following RT-PCR program was used to amplify the transcripts: (1) heat 0.4 $\mu$g total mRNA in a 16-$\mu$L volume of dd$H_2O$ at 85 °C for 3 min followed by immediate placement onto ice; (2) add enzyme/primer/dNTP/buffer mix; (3) place reaction tubes into thermal cycler and run at the following program: 50 °C for 30 min, 94 °C for 2 min, 10 cycles of (94 °C for 1 min, 57 °C for 1 min, 68 °C 1 min), 22 cycles of (94 °C for 1 min, 57 °C for 1 min, 68 °C 1 min + 5 s/cycle) and finally 68 °C for 7 min. PCR products were run on a 2% NuSieve 3:1 agarose gel (Cambrex) and analyzed using ImagequantTL (Amersham).

**Western Blot.** Cells were harvested, washed in PBS, and boiled in 1× Laemmli sample buffer with protease inhibitors (Compleat Mini, Roche) for 10 min with vortexing every 3 min. Extracts were incubated at room temperature for 2 min, and centrifuged at 10,000 × *g* for 10 min. Protein concentrations in the extracts were measured according to modified Lowry procedure (RC DC Protein Assay, Bio-Rad) using BSA as a standard. An aliquot (40 $\mu$g proteins) from each sample was then separated on SDS/PAGE (16% for analysis of p16, 15% for analysis of p27, and 4–15% gradient gel for analysis of pRb). After electroblotting, the nitrocellulose membrane was incubated with antibodies as indicated, washed twice, and incubated with secondary antibodies conjugated to horseradish peroxidase. Identity of each protein was confirmed with two different antibodies: p16, ab14244 and ab17517 (Abcam); p27, DCS-72.F6 (Lab Vision) and 2552 (Cell Signaling); pRb, ab6075, and ab24 (Abcam). Equal loading of samples was verified by hybridizing the same membranes with pan-Actin Ab-5 antibodies (Lab Vision).

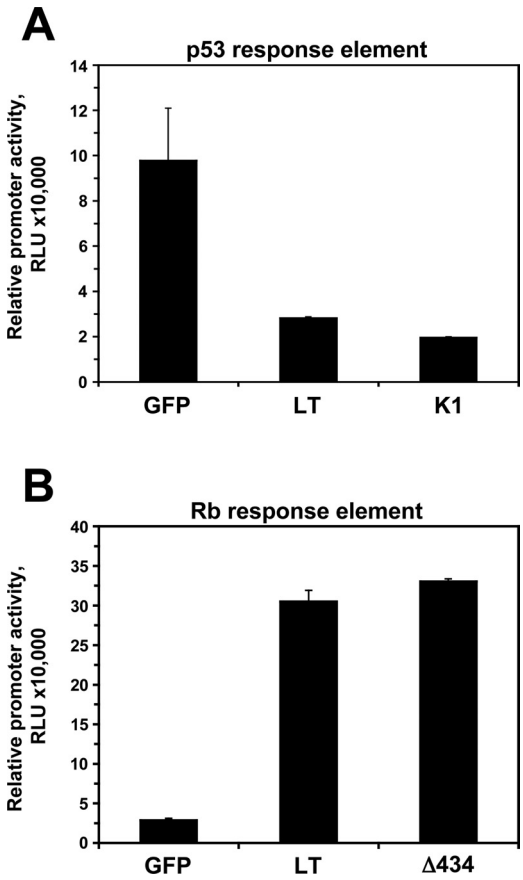


**Fig. S1.** SV40 Large T antigen is active in naked mole-rat fibroblasts. (*A*) LT and K1 inactivate p53 in naked mole-rat cells. The cells were co-transfected with the plasmids encoding GFP, LT, or K1, and a plasmid encoding firefly luciferase under control of p53-response element. (*B*) LT and Δ434 inactivate pRb in naked mole-rat cells. The cells were co-transfected with the plasmids encoding GFP, LT, or Δ434, and a plasmid encoding firefly luciferase under control of pRb-response element.

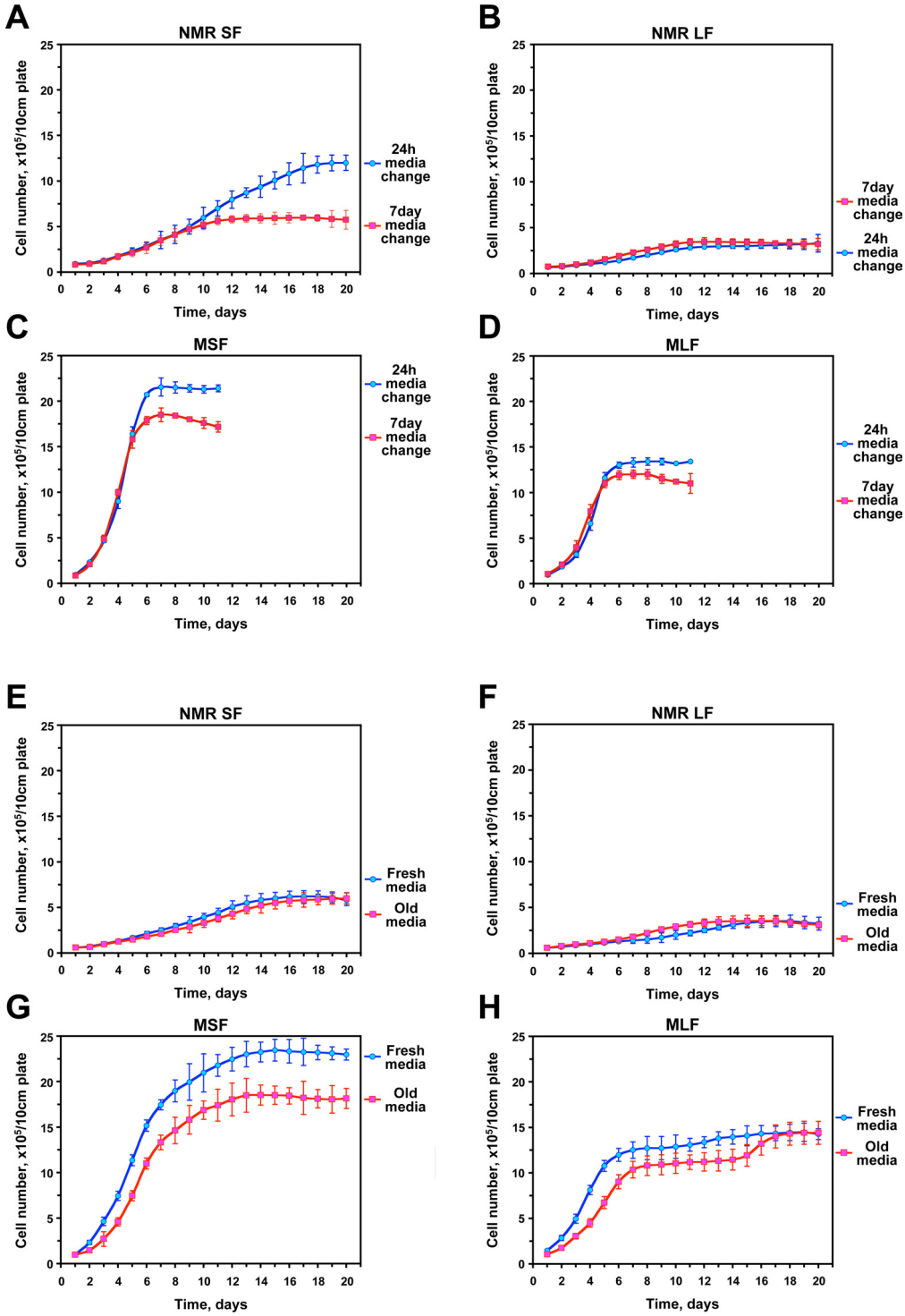


**Fig. S2.** Early contact inhibition is mediated by cell-cell contact rather than by a secreted factor. (*A–D*) Comparison of cell densities attained by fibroblast cultures grown in media which was replaced with fresh media every 24 h to cultures grown under standard conditions where the media is replaced every 7 days. Cells were seeded on grided plates, and cell numbers were counted daily for each plate. (*A*) Naked mole-rat skin fibroblasts. (*B*) Naked mole-rat lung fibroblast cultures.(*C*) Mouse skin fibroblasts cultures. (*D*) Mouse lung fibroblasts cultures. (*E–H*) Comparison of cell densities reached by fibroblast cultures grown in fresh media, and cultures grown in the media composed of 50% fresh media and 50% ''old media'' collected from a corresponding confluent cell culture plate after 7-day incubation. Both fresh and old media were replaced every 3 days. (*E*) Naked mole-rat skin fibroblasts. (*F*) Naked mole-rat lung fibroblasts. (*G*) Mouse skin fibroblast cultures. (*H*) Mouse lung fibroblast cultures. The experiments were repeated three times, and standard deviations are shown.

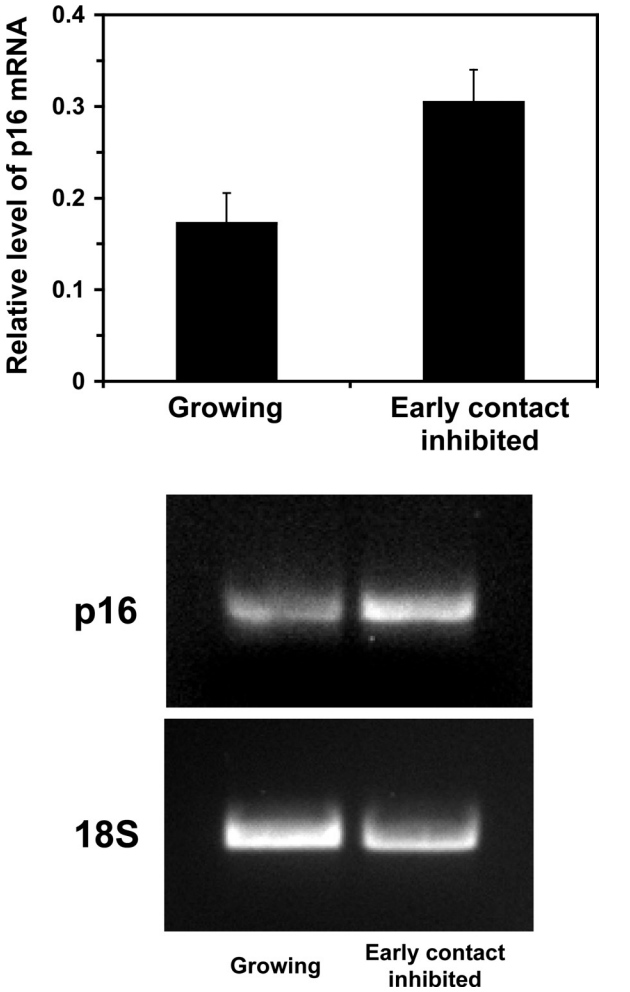


**Fig. S3.** p16 transcript is elevated in early contact-inhibited cells. Quantitative RT-PCR was performed on total RNA isolated from naked mole-rat cell plates incubated for 7 days (growing) and 20 days (early contact inhibited). The relative p16 mRNA level is the ratio between p16 transcript and 18S RNA. The primers for naked mole-rat p16 were designed based on the partial naked mole-rat p16 sequence cloned in our laboratory. The experiments were repeated three times, and the difference in p16 transcript levels between growing and early contact inhibited cells is significant with the $P < 0.01$.