LETTER

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High-molecular-mass hyaluronan mediates the cancer resistance of the naked mole rat

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The naked mole rat (*Heterocephalus glaber*) displays exceptional longevity, with a maximum lifespan exceeding 30 years[1–3]. This is the longest reported lifespan for a rodent species and is especially striking considering the small body mass of the naked mole rat. In comparison, a similarly sized house mouse has a maximum lifespan of 4 years[4,5]. In addition to their longevity, naked mole rats show an unusual resistance to cancer. Multi-year observations of large naked mole-rat colonies did not detect a single incidence of cancer[2,6]. Here we identify a mechanism responsible for the naked mole rat's cancer resistance. We found that naked mole-rat fibroblasts secrete extremely high-molecular-mass hyaluronan (HA), which is over five times larger than human or mouse HA. This high-molecular-mass HA accumulates abundantly in naked mole-rat tissues owing to the decreased activity of HA-degrading enzymes and a unique sequence of hyaluronan synthase 2 (*HAS2*). Furthermore, the naked mole-rat cells are more sensitive to HA signalling, as they have a higher affinity to HA compared with mouse or human cells. Perturbation of the signalling pathways sufficient for malignant transformation of mouse fibroblasts fails to transform naked mole-rat cells. However, once high-molecular-mass HA is removed by either knocking down HAS2 or overexpressing the HA-degrading enzyme, HYAL2, naked mole-rat cells become susceptible to malignant transformation and readily form tumours in mice. We speculate that naked mole rats have evolved a higher concentration of HA in the skin to provide skin elasticity needed for life in underground tunnels. This trait may have then been co-opted to provide cancer resistance and longevity to this species.

Mice and rats are standard animal models for cancer research due in part to their short lifespan and high incidence of cancer. However, these traits indicate that mice and rats have fewer anticancer mechanisms, and novel tumour resistance mechanisms are less likely to be discovered using these models. Here we focused our research on a small rodent, the naked mole rat, which in contrast to mice and rats is long-lived and cancer resistant.

Our previous studies identified a novel anticancer mechanism in the naked mole rat, termed early contact inhibition (ECI)[7]. Contact inhibition is a process of arresting cell growth when cells come in to contact with each other or the extracellular matrix. Contact inhibition is a powerful anticancer mechanism that is lost in cancer cells[8]. Naked mole-rat cells arrest at a much lower density than mouse cells, and the loss of ECI makes cells more susceptible to malignant transformation[7]. However, the signals triggering ECI in naked mole rats remained unknown.

While culturing multiple lines of naked mole-rat fibroblasts we noticed that the culture media became very viscous after a few days. Viscosity measurements confirmed that the media conditioned by the naked mole-rat cells was more viscous than the media conditioned by human, guinea-pig or mouse cells (Fig. 1a). We included the guinea-pig because it is phylogenetically closer to the naked mole rat than the mouse. We identified the viscous 'substance' secreted by the naked mole-rat fibroblasts as high-molecular-mass HA (HMM-HA). Treatment with hyaluronidase (HAase), which specifically digests HA, reduced the media viscosity to background levels (Fig. 1a). Naked mole-rat embryonic fibroblasts, which do not display ECI, did not increase viscosity of the culture media (Fig. 1a and Supplementary Fig. 1).

HA is an unbranched disaccharide glucuronic acid/*N*-acetylglucosamine polymer and is one of the main components of the extracellular matrix[9]. Biological responses triggered by HA depend on the HA polymer length. HMM-HA represses mitogenic signalling and has anti-inflammatory properties[10], whereas low-molecular-mass HA promotes proliferation and inflammation[11].

Analysis of HA from tissue culture media using pulse-field electrophoresis showed that the HA secreted by naked mole-rat cells has a molecular mass of 6–12 MDa, whereas mouse and guinea-pig HA range from 0.5 to 3 MDa (Fig. 1b); human HA has a molecular mass of 0.5–2 MDa[12]. Naked mole-rat embryonic fibroblasts did not secrete HMM-HA (Fig. 1b).

Notably, a mutated clone NMR SF Mut (mutated naked mole-rat skin fibroblasts), which spontaneously lost the ECI phenotype and $p16^{INK4a}$ expression[7], still produced HMM-HA (Fig. 1a, b), indicating that the physical presence of HMM-HA is not sufficient for the ECI phenotype; rather, the intact signalling pathway leading from HMM-HA to induction of $p16^{INK4a}$ is required. These experiments establish HMM-HA as the extracellular signal that triggers ECI.

In vertebrate cells, HA is produced by HA synthases HAS1, HAS2 and HAS3 that differ in tissue distribution and the size of HA produced[13]. Naked mole-rat skin fibroblasts overexpress HAS2, the enzyme responsible for the synthesis of HMM-HA, in comparison with mouse and human fibroblasts (Fig. 1c). Naked mole-rat embryonic fibroblasts, which do not secrete HMM-HA, did not show increased levels of HAS2. The levels of HAS1 and HAS3 were similar between mouse, human and naked mole-rat cells (Fig. 1c). Collectively, these results show that naked mole-rat cells, which display ECI, secrete HA of exceptionally high molecular mass.

Hyaluronan synthases are highly conserved in vertebrates. The HAS2 protein has 98.7% identity and 100% similarity between human and mouse. We cloned and sequenced HAS2 complementary DNA from the naked mole rat and compared it to other mammalian *Has2* genes (Fig. 1d). Two asparagines that are 100% conserved among mammals were replaced with serines in the naked mole-rat HAS2. This change occurs in no other mammalian *Has2* genes deposited in GenBank, including the close relative of the naked mole rat, the guinea-pig. HAS2 contains seven putative transmembrane domains and a cytoplasmic loop[14]. The conserved regions carrying asparagine to serine substitutions correspond to the cytoplasmic loop containing the enzyme's active site. These unique amino acid changes may be responsible for the high processivity of the naked mole-rat HAS2. Indeed, when the cDNA for the naked mole-rat HAS2 was overexpressed in human HEK293 cells, they began secreting HMM-HA (Fig. 2a).

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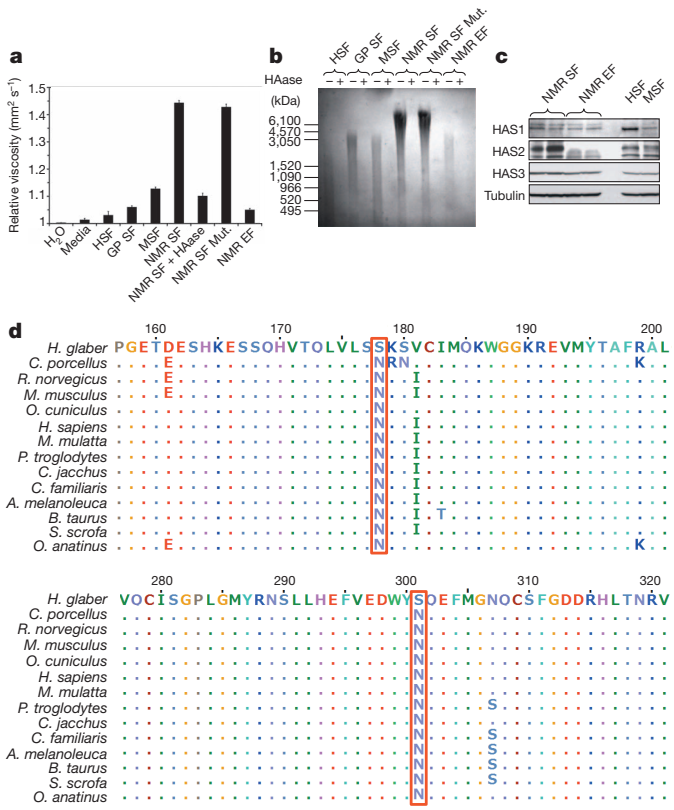

Figure 1 | Naked mole-rat cells secrete HA of exceptionally high molecular mass. a, Naked mole-rat cells make the culture media viscous. The viscosity of water, media or media conditioned with human skin (HSF), guinea-pig skin (GP SF), mouse skin (MSF) or naked mole-rat skin (NMR SF) fibroblasts for 20 days is shown. The NMR SF + HAase bar shows naked mole-rat conditioned media digested with HAase to specifically digest HA. NMR SF Mut are naked mole-rat skin fibroblasts that spontaneously lost the ECI phenotype[7]. NMR EF are naked mole-rat embryonic fibroblasts that do not show ECI. The experiment was repeated three times; error bars show s.d. **b**, Purified HA separated on pulse-field gel. Each sample was either run intact or pre-digested with HAase. The experiment was repeated five times, using both skin and lung fibroblasts (Supplementary Fig. 9), and a representative gel is shown. **c**, Western blot showing the levels of HA synthases in naked mole-rat adult skin fibroblasts, naked mole-rat embryonic fibroblasts, human skin fibroblasts or mouse skin fibroblasts. **d**, Conserved catalytic domain of mammalian HAS2 proteins. The top sequence is the naked mole-rat HAS2. Dots indicate amino acids identical to the naked mole-rat sequence. The two amino acid changes unique to the naked mole rat are indicated by red boxes.

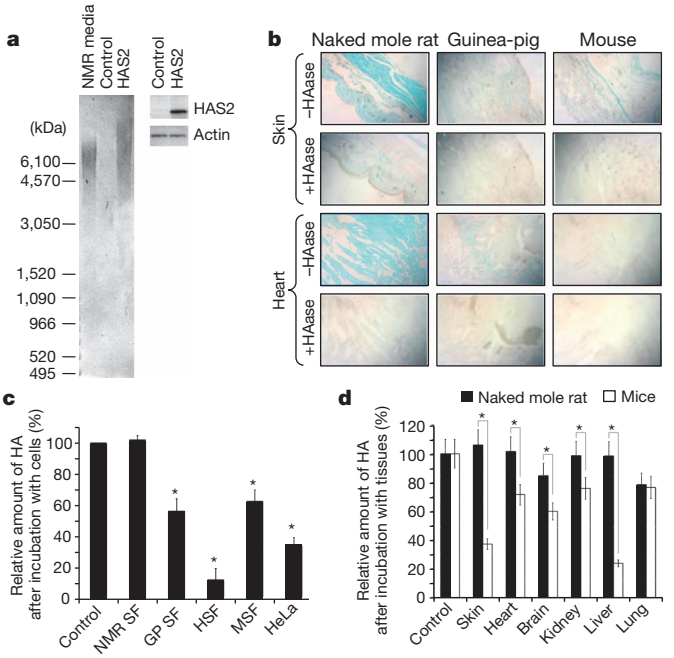

Figure 2 | Naked mole-rat tissues contain high levels of HA. a, Naked mole-rat HAS2 overexpressed in human HEK293 cells secretes HMM-HA. The small panel on the right shows immunoblot with anti HAS2 antibodies on whole-cell extracts from the control and HAS2-transfected cells. **b**, Tissues from the naked mole rat, mouse and guinea-pig stained with alcian blue. The control samples treated with HAase do not show blue staining, demonstrating that the staining is specific to HA. Staining was performed on three different animals and representative skin and heart images are shown at ×40 magnification. Brain and kidney are shown in Supplementary Fig. 2. **c**, Naked mole-rat fibroblasts have low HAase activity. Naked mole-rat skin fibroblasts (NMR SF), guinea-pig skin fibroblasts (GP SF), human skin fibroblasts (HSF), mouse skin fibroblasts (MSF) or HeLa cells were incubated with the media containing HMM-HA for 4 days. The levels of HA were then analysed by pulse-field gel. Control samples were incubated in the absence of cells. The experiments were repeated four times (all samples except guinea-pig), and three times for guinea-pig; error bars show s.d.; asterisk indicates $P < 0.01$ by *t*-test. **d**, Naked mole-rat tissues have low HAase activity. Media containing HMM-HA was incubated with corresponding tissue fragments from naked mole rats or mice for 6 h and HA levels analysed by pulse-field gel. The experiments were repeated three times and error bars show s.d.; asterisk indicates $P < 0.05$ by *t*-test.

We then examined whether naked mole-rat tissues contain high levels of HA in comparison to mouse and guinea-pig. Tissue sections were stained with alcian blue, and control samples were treated with HAase before staining to show that the staining is specific to HA. Naked mole-rat skin, heart, brain and kidney were highly enriched for HA (Fig. 2b and Supplementary Fig. 2). Furthermore, the HA extracted from naked mole-rat tissues had a higher molecular mass than HA from mouse tissues (Supplementary Fig. 3). These results indicate that production of HMM-HA by naked mole-rat cells is not an artefact of tissue culture, but a unique *in vivo* property of this species.

HA levels are regulated by HA-degrading enzymes, HAases[15]. We measured HAase activity in naked mole-rat, mouse and human cells by quantifying HA degradation after incubation with these cells. HAase activity of the naked mole-rat cells was much lower than that of human, mouse or guinea-pig cells (Fig. 2c). Similarly, HAase activity was lower in the naked mole-rat tissues than in the mouse tissues (Fig. 2d). These results indicate that two mechanisms contribute to the accumulation of HMM-HA in the naked mole-rat: more robust synthesis and slower degradation.

We previously demonstrated that ECI contributes to cancer resistance of the naked mole rat by arresting cell cycle via the induction of $p16^{INK4a}$ (ref. 7). To determine the role of HMM-HA in ECI we cultured naked mole-rat fibroblasts in the presence of bacterial HAase. Enzymatic digestion of HMM-HA abrogated the ECI phenotype and caused naked mole-rat cells to grow to complete confluence (Fig. 3a, b). Upon subsequent removal of HAase from the culture media, a fraction of cells detached from the plate and died by apoptosis (Supplementary Fig. 4), whereas the remaining cells re-acquired the ECI phenotype (Fig. 3c).

CD44 is a major HA receptor in human and mouse cells[9,11,16]. To confirm that HA signalling triggers ECI via the CD44 receptor we cultured naked mole-rat cells in the presence of a CD44-blocking antibody. Naked mole-rat cells grown with CD44 antibodies reached a higher cell density (Fig. 3b), indicating that the ECI signal from HMM-HA is in part transmitted via the CD44 receptor.

We then used a flow cytometric assay to measure the affinity of the naked mole-rat cells to fluorescently labelled HA. Naked mole-rat cells displayed a twofold higher affinity to HA than mouse or human cells (Fig. 3d); this higher affinity can contribute to higher sensitivity of naked mole-rat cells to HA signalling.

On the cytoplasmic face, the CD44 receptor interacts with NF2 (merlin), which mediates contact inhibition[17]. The phosphorylated,

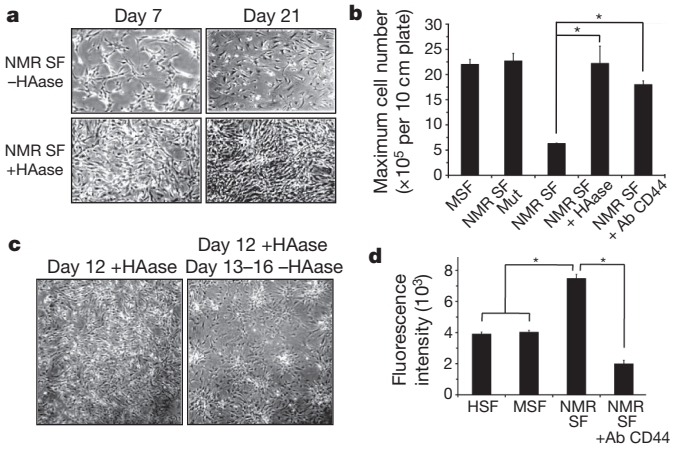

Figure 3 | HMM-HA is required for ECI. **a**, Naked mole-rat cells (NMR SF) grown in the presence of HAase do not display ECI and proliferate to high cell density. **b**, Quantification of cell growth, showing the maximum cell number per plate achieved under the indicated growth conditions. The last bar shows naked mole-rat cells grown in the presence of CD44-blocking antibody. The experiments were repeated four times (except the last bar, which was repeated three times) and error bars show s.d.; asterisk indicates $P < 0.001$ by *t*-test. **c**, Naked mole-rat cells were grown in the presence of HAase for 12 days, then HAase was removed. **d**, Naked mole-rat cells show the highest affinity to HA. Cells were incubated with fluorescein-labelled HA, and the average fluorescence was plotted. Experiment was repeated four times; error bars are s.e.m.; asterisk indicates $P < 0.001$ by *t*-test. Cells were photographed at ×40 magnification.

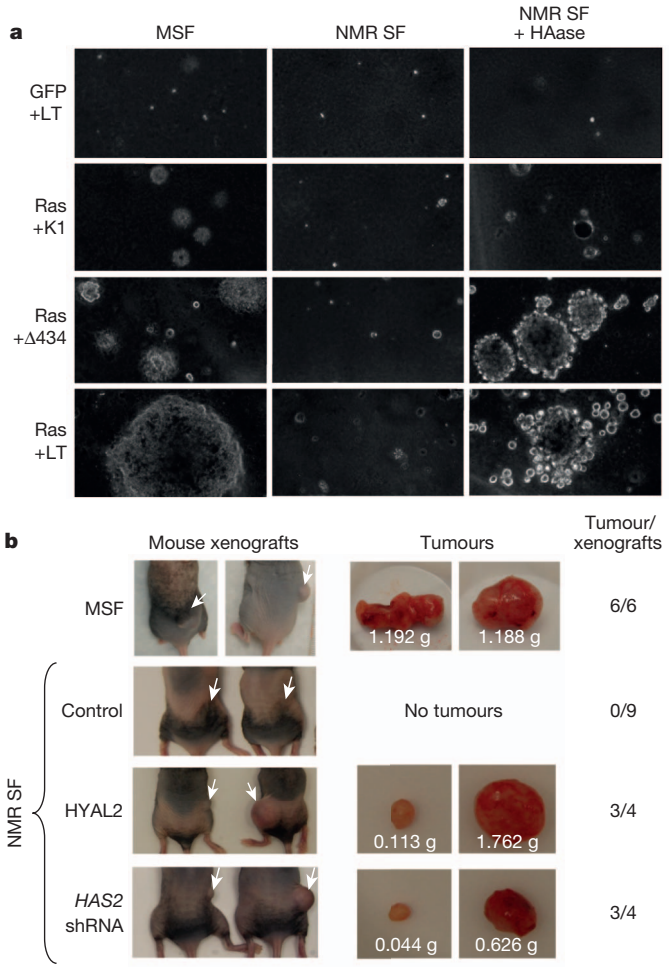

Figure 4 | Removal of HMM-HA makes naked mole-rat cells susceptible to malignant transformation. **a**, Soft agar assays of anchorage-independent growth. Mouse (MSF) or naked mole-rat (NMR SF) cells were transfected with vectors encoding SV40 LT (LT) or its mutant derivatives K1 or Δ434, and H-Ras V12 (Ras), and plated in soft agar. Cells were cultured with or without HAase. The image shows colonies after 3 weeks of growth at ×200 original magnification. The experiment was repeated three times. **b**, Mouse xenograft experiment with naked mole-rat cells in which HMM-HA was abolished. NIH-III immunodeficient mice were injected with mouse (MSF) cells expressing SV40LT and H-Ras V12 as a positive control, or naked mole rat (NMR SF) cells expressing SV40LT and H-Ras V12 and either control shRNA, HYAL2 cDNA, or shRNA targeting *HAS2*. All xenografts with mouse cells formed large tumours. Xenografts with naked mole-rat cells expressing control shRNA did not form tumours, whereas naked mole-rat cells overexpressing HYAL2 or *HAS2* shRNA formed tumours in mice. The images show xenograft sites (arrows) and representative tumours. The number of xenografts resulting in tumour formation per the total number of xenografts with each cell type is shown on the right.

growth-promoting form of NF2 was present in naked mole-rat cells grown in the presence of HAase, whereas cells cultured without HAase contained mainly the unphosphorylated growth-inhibitory form of NF2 (Supplementary Fig. 5). We previously showed that ECI is associated with induction of $p16^{INK4a}$, whereas the NMR SF Mut cells that do not display ECI have lost $p16^{INK4a}$ expression[7]. Accordingly, naked mole-rat cells grown in the presence of HAase showed reduced levels of $p16^{INK4a}$ (Supplementary Fig. 5). Collectively, these results establish that ECI is controlled by the HA–CD44–NF2 pathway.

We then tested the role of HMM-HA in the resistance of naked mole-rat cells to malignant transformation in a soft agar assay. SV40 large T antigen (SV40 LT) is a viral oncoprotein that binds and inactivates p53 and pRb. The mutant derivative LTK1 (K1) inactivates only p53, whereas LT(Δ434–444) (Δ434) inactivates only pRb and its family members[18]. A combination of H-Ras V12 and SV40 LT is sufficient to transform mouse fibroblasts[19], but, as we showed earlier, it is not sufficient to confer anchorage-independent growth to naked mole-rat cells[7]. To test the role of HMM-HA in the naked mole-rat's resistance to transformation, we transfected naked mole-rat fibroblasts with H-Ras V12 combined with SV40 LT or its mutants K1 or Δ434 and cultured them in soft agar in the presence of HAase. Under these conditions, cells transfected with H-Ras V12 and SV40 LT, or H-Ras V12 and Δ434, formed robust colonies (Fig. 4a). Similarly, naked mole-rat cells cultured in the presence of CD44-blocking antibody formed colonies in soft agar (Supplementary Fig. 6). These results demonstrate that if HMM-HA is degraded by HAase or HA signalling is blocked by a CD44 antibody, naked mole-rat cells become susceptible to anchorage-independent growth triggered by H-Ras V12 and SV40 LT. In embryonic naked mole-rat fibroblasts, which do not secrete HMM-HA, H-Ras V12 and SV40 LT or Δ434 were sufficient to trigger anchorage-independent growth (Supplementary Fig. 6).

We then generated H-Ras V12- and SV40 LT-expressing naked mole-rat cells, in which HMM-HA was abolished by either integrating short hairpin RNA (shRNA) targeting *HAS2* (Supplementary Fig. 7a) or overexpressing an HA-degrading enzyme HYAL2 (Supplementary Fig. 7b). These cells no longer increased the viscosity of their culture media (Supplementary Fig. 7c) and readily formed colonies in soft agar (Supplementary Fig. 8). To confirm that HMM-HA inhibits tumour formation *in vivo*, we performed xenograft experiments with naked mole-rat cells containing a knockdown of HAS2, or overexpressing HYAL2 (Fig. 4b). In the positive control, mouse cells expressing H-Ras V12 and SV40 LT readily formed tumours in mice. Naked mole-rat cells expressing H-Ras V12 and SV40 LT did not form tumours, consistent with an earlier report[20]. Notably, naked mole-rat cells expressing H-Ras V12 and SV40 LT and shRNA to *HAS2* or overexpressing HYAL2 formed tumours in mice. This experiment establishes HMM-HA, produced by HAS2, as a key component responsible for the elevated cancer resistance of the naked mole rat.

The HMM-HA in the naked mole rat could have evolved as an adaptation to a subterranean lifestyle to provide flexible skin needed

to squeeze through underground tunnels. Interestingly, we found that cells of a different subterranean rodent, the blind mole rat, which is phylogenetically closer to mice and rats than to the naked mole rat, also secreted HMM-HA (Supplementary Fig. 9). In summary, our results demonstrate that extremely HMM-HA, its binding to the CD44 receptor, and lower HAase activity have a key role in mediating the cancer resistance of the naked mole rat. Using naked mole-rat HMM-HA in the clinic or targeting HYAL2, or the HA–CD44 signalling pathway, opens new avenues for cancer prevention and life extension.

METHODS SUMMARY

All animal experiments were approved and performed in accordance with guidelines set up by the University of Rochester Committee on Animal Resources. Naked mole rats were from the University of Rochester colonies. C57BL/6 mice and NIH III nude mice were purchased from Charles River Labs. Non-albino guinea-pigs were obtained from Elm Hill Labs. Cells and tissues were obtained from at least three different animals. Fibroblasts were isolated from lung and underarm skin. All cell lines were used at early passage (<12–15 population doublings).

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions X.T. performed HA analysis, HAase assays, soft agar assays, and generated cells for xenograft experiments; J.A. performed immunoblots and cloning and analysis of HAS2; C.H. identified HA, performed tissue staining, and soft agar assays; A.V. performed xenografts; M.-M.R. performed HA affinity assays; J.A. purified HA; Z.M. performed experiments with HAS2 expression; E.N. provided essential materials; X.T., J.A., C.H., A.S. and V.G. designed the study and analysed data; A.S. and V.G. wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to V.G. (vera.gorbunova@rochester.edu) or A.S. (andrei.seluanov@rochester.edu).

METHODS

Animals. All animal experiments were approved and performed in accordance with guidelines set up by the University of Rochester Committee on Animal Resources. Naked mole rats were from the University of Rochester colonies. C57BL/6 mice and NIH III nude mice (NIH-Lyst bg-JFoxn1nu Btk xid) were purchased from Charles River Labs. Non-albino guinea-pigs were obtained from Elm Hill Labs. Cells and tissues were obtained from at least three different animals.

Cell culture. Primary mouse, guinea-pig, blind mole-rat and naked mole-rat cells were isolated from lung and underarm skin. Cells were obtained from five naked mole rats, three mice, three guinea-pigs, and three blind mole rats. The growth characteristics, and HA secretion, did not differ between the cell lines from different animals, therefore we performed the experiments on three skin cell lines from three animals. All cell lines were used at early passage (<12–15 PDs). Human primary skin fibroblasts HCA2 were a gift from O. Pereira-Smith. Embryonic naked mole-rat fibroblasts were isolated from eight mid-gestation embryos.

Mouse, human, guinea-pig and blind mole-rat 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) in EMEM media (ATCC) supplemented with 15% fetal bovine serum (Gibco), non-essential amino acids, sodium pyruvate, 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (Gibco).

Viscosity assay. To determine relative kinematic viscosity, 3 ml of distilled H_2O, unused complete EMEM media, or media conditioned with naked mole-rat, mouse, or human cells were run through a 0.6-mm capillary Ostwald viscometer (Barnstead International) at 22 °C and timed for the passage of the media or distilled H_2O through the capillary. For HAase control, naked mole-rat media was treated with hyaluronidase 1 U ml^{-1} HAase from *Streptomyces hyalurolyticus* (Sigma-Aldrich). The relative viscosity of unused and conditioned media was determined by comparing times required to pass through the capillary to that of distilled H_2O. Samples were run three times to determine an average relative viscosity.

Cell growth analysis. To measure cell proliferation and the confluent density, cells were seeded on 60-mm gridded 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. For cell growth in the presence of HAase, 24 h post plating the media was changed to media containing 3 U ml^{-1} HAase (Sigma). Media was then replaced with fresh media containing the enzyme every 48 h. Images of the squares were taken using SPOT Advanced (Diagnostic Instruments) and analysed 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 analysed using Microsoft Excel.

To calculate maximum cell number, cells were collected from the confluent plates and counted using Z2 Coulter counter (Beckman Coulter). Every sample was counted three times and the averages were used to calculate the maximum cell number from at least three independent experiments.

HA analysis by pulse-field gel electrophoresis. HA was purified from conditioned media (typically at day 20) by first treating 2 ml of conditioned media with 500 μg of proteinase K (Roche) at 50 °C for 45 min to remove proteins. Samples were then precipitated by adding 2 ml of 100% ethanol. The pellet was dissolved in 500 μl TE Buffer and incubated overnight at 4 °C. The following day, aliquots were removed and control samples treated with 1 U ml^{-1} of HAase from *Streptomyces hyalurolyticus* (Sigma-Aldrich). Twenty-five microlitres of each sample was mixed with 5 μl 4M sucrose loading solution and loaded to a 0.4% pulse-field SeaKem Gold agarose gel (Cambrex). Ten microlitres of HA molecular size markers—HiLadder (~500 kDa to ~1,500 kDa) and Mega-HA Ladder (1,500 kDa to 6,000 kDa) (from Hyalose)—were run to determine the size of HA from each sample. Samples were run overnight at 4 °C at 75 V with a 1–10 running ratio in TBE buffer using CHEF-DRII system (Bio-Rad). The gel was next stained in a method adapted from ref. 21. Briefly, the gel was placed in a 0.005% (w/v) Stains-All (Sigma-Aldrich) in 50% ethanol solution overnight. To de-stain, the gel was placed in distilled H_2O for 18 h in the dark and then placed under ambient light in distilled H_2O for 1 h to complete the final de-staining stages and then photographed under white light. The amount of HA was quantified by counting pixels using Image J software.

HA extraction from tissues. Tissues were excised immediately after killing the animals and weighed. Tissues were chopped and the same amount of tissues were digested at 50 °C overnight in the digestion buffer containing 10 mM Tris-Cl, 25 mM EDTA, 100 mM NaCl, 0.5% SDS and 0.1 mg ml^{-1} proteinase K (Roche). Then 27 mM $MgCl_2$ was added to chelate EDTA and Pefabloc SC was added to inhibit proteinase K. 500 units of benzonase endonuclease (Sigma-Aldrich) was added to remove nucleic acid. The complete digestion of DNA and RNA was confirmed by running the agarose gel and staining with ethidium bromide (Bio-Rad). Total polysaccharide extraction was performed by phenol/chloroform extraction followed by ethanol precipitation. Finally, corresponding volume (100 μl/100 mg) of 10 mM Tris-Cl (pH 8.5) was added to dissolve the pellet.

Expression of naked mole-rat *HAS2* gene in human cells. HEK293 cells were transfected with an expression vector containing HAS2 under the CMV promoter and allowed to express HAS2 for 2 days, after which HA secreted into the media was analysed by pulse-field gel. Control cells were transfected with a GFP expression vector.

HAase activity assay. Culture media containing HMM-HA secreted by naked mole-rat cells was mixed 1:1 with fresh media and incubated with 2×10^5 naked mole-rat fibroblasts, human diploid fibroblasts, mouse fibroblasts, or HeLa cells for 4 days. Then the media was collected, HA was extracted and analysed on a pulse-field gel as described above. HA levels before and after incubation were compared as a measure of HAase activity. For analysis of tissues, corresponding tissues were chopped into 1-mm cubes and washed twice with PBS. HMM-HA containing media was incubated with equal amounts (by weight) of tissue fragments of each tissue type for 6 h and HA levels were analysed as above.

Antibodies. The following antibodies were used: HAS1 (ab104864 Abcam), HAS2 (sc-66916 Santa Cruz), HAS3 (sc-66917 Santa Cruz), α-tubulin (ab4074 Abcam), CD44 (monoclonal mouse IgG_{2A} clone 2C5, catalogue no. BBA10, R&D Systems), NF2/merlin (ab30329 Abcam), p16 (ab14244 Abcam), HYAL2 (ab68608b Abcam).

Tissue staining. HA detection in tissues was done as follows. Tissue samples from young animals (3-year-old naked mole rats, 3–5-month-old mice, 1-year-old guinea-pigs) were fixed in 10% buffered neutral formalin, embedded in paraffin and quadruple sections cut at 5 μm were mounted on glass slides. Slides were deparaffinized and hydrated in distilled H_2O. They were then placed in Coplin jars containing 40 ml of hyaluronidase digestion solution (40 U hyaluronidase from *Streptomyces hyalurolyticus* (Sigma) in 40 ml of PBS) for the samples to be digested or in 40 ml of PBS for the non-digested samples. The jars were microwaved for 1 min at 60 W and then transferred to a 37 °C oven for 1 h. The slides were rinsed four times in distilled H_2O, followed by three rinses with 3% acetic acid. Next, slides were placed in new Coplin jars containing 1.0% filtered alcian blue solution at pH 2.5 (alcian blue 8GX, C.I. 74240 (Leica)) in 3% acetic acid and microwaved at 60 W for 3 min followed by an additional 5 min incubation in the hot 1.0% alcian blue solution. Slides were rinsed three times in distilled H_2O, dehydrated in graded alcohols and rinsed three times in xylene. Images were taken by light microscopy.

Naked mole-rat cell growth assays with CD44 antibody. Naked mole-rat cells were seeded 50 cells per square onto cell-culture-treated 6-cm polystyrene gridded tissue culture plates (Corning). Twenty-four hours after plating, the media was changed to contain 5 μg ml^{-1} of CD44 specific antibody (monoclonal mouse IgG_{2A} clone 2C5, catalogue no. BBA10, R&D Systems) or no antibody control. Media was changed every 24 h and images were taken daily using SPOT Advanced imaging software (Diagnostic Instruments). Images from three different squares from two independent plates were counted from both the CD44 antibody treated or control groups.

HA affinity assay. Naked mole-rat and mouse cells were collected at subconfluent exponential phase. One-hundred-thousand (10^5) cells of each type were incubated for 45 min on ice in 210 μl PBS containing 1.5% fetal calf serum and 35 μg ml^{-1} fluorescein-labelled HA (fluorescein-labelled HA from bovine trachea, sc-221733, Santa Cruz Biotech). Five-thousand cells from each replicate were analysed by FACS. The experiment was repeated four times.

Transfections. Naked mole-rat skin fibroblasts were seeded at 2×10^5 cells per 100 mm plate 7 days before transfection. Mouse skin fibroblasts were seeded at 5×10^5 cells per 100-mm plate 2 days before transfection. For transfection, cells were collected, counted and 10^6 cells were transfected with 5 μg of 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 after transfection to remove dead cells due to electroporation.

Anchorage-independent soft agar growth assay. One million mouse, naked mole-rat wild-type and naked mole-rat mutant exponentially growing skin fibroblast cells were transfected by Amaxa with the following plasmid DNA mixtures: 5 μg pEGFP-N1 (Clontech) and 5 μg pSG5 large T (Addgene 9053), 5 μg pRas-V12 (Clontech) and 5 μg pSG5 large T, 5 μg pRas-V12 and 5 μg pSG5 large T K1 (Addgene 9055), or 5 μg pRas-V12 and 5 μg pSG5 large T Δ434–444 (Addgene 9054). 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% fetal calf serum and antibiotics (Gibco). The following day, a 2-ml final solution of 0.5% Difco Agar Noble (BD Bioscience) 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 previously, 50 to 50,000 cells were serially diluted and re-suspended 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. Plates were incubated at 32 °C, 5% CO_2, and 3% O_2 for 24 h before the addition of 1 ml of EMEM media with or without HAase from *Streptomyces hyalurolyticus* (Sigma) at 3 U ml^{-1}. To test the effect of CD44 antibody, 5 μg ml^{-1} of CD44 antibody (BBA10, R&D Systems) was added and changed daily. Cells were grown for 6 weeks, with the removal of old liquid EMEM media and the addition of 1 ml of new EMEM with or without 3 U ml^{-1} HAase every 48 h to ensure efficient digestion of HMM-HA. Plates were monitored every 48 h and photographed at week 3 (mouse cells) and week 6 (naked mole-rat cells) after plating at ×200 on a Nikon TS100 phase contract microscope using SPOT software (Diagnostic Instruments).

shRNA-mediated HAS knockdown and HYAL2 overexpression. shRNAs were designed by Integrated DNA Technology (IDT) with shRNA Design Tool: 5′-GATCCGCCAGCTGCCTCAGAGGAATTCAAGAGATTCCTCTGAGGCAGCTGGCTTTTTTGGAAA-3′; 5′-AGCTTTTCCAAAAAAGCCAGCTGCCTCAGAGGAATCTCTTGAATTCCTCTGAGGCAGCTGGCG-3′. The corresponding 63-bp DNA oligonucleotides harbouring the 19-mer hairpin sequence, loop sequence, polythymidine tract (U6 terminator), BamHI and HindIII restriction site overhangs were designed according to the user manual of pSilencer 2.1-U6 neo kit (Life Technologies) and chemically synthesized by IDT. The complementary oligonucleotides were annealed and ligated to the pre-cut pSilencer2.1-U6 neo vector using the rapid DNA ligation kit (Roche). Following transformation into Top10 competent cells (Life Technologies), successful ligation was confirmed using restriction digestion and DNA sequencing with M13F primer (5′-GTAAAACGACGGCCAGT-3′).

Human HYAL2 cDNA was amplified from pCMV6-HYAL2 (sc117754 OriGene) using the primers 5′-CCG*GAATTC*GCCACCATGCGGGCAGGCCCAGGCCCCACCG-3′ and 5′- ATAAGAAT*GCGGCCGC*CTACAAGGTCCAGGTAAAGGCCAGGGC-3′ (restriction enzyme sites are shown in italics) and cloned into pEGFP-N1-neo plasmid to replace the EGFP fragment using EcoR1 and NotI restriction enzymes.

Transfection grade plasmids were prepared with EndoFree plasmid maxi kit (Qiagen) and linearized with ScaI. One microgram of linearized plasmid was transfected into ~1×10^6 cells by Nucleofector (Amaxa) with U20 program, followed by G418 selection at 1 mg ml^{-1} for 2 weeks. Clones that stably expressed the shRNA were picked and expanded to characterize the knockdown efficiency. Clones with highest levels of HAS knockdown efficiency or the best HYAL2 expression were used for the *in vivo* xenograft assay.

Quantitative RT–PCR. Total RNA was extracted from cells at 80% confluence (2 days after splitting) using RNeasy mini kit (Qiagen). cDNA was generated using SuperScriptIII reverse transcriptase (Life Technologies) with Oligo(dT)18 primer. First-strand cDNA was amplified using FastStart Universal SYBR Green Master (Roche; 04913850001) with corresponding primers in which QuantumRNA β-actin internal standards (Life Technologies) were used as reference. Quantitative PCR was conducted with Applied Biosystems 7300 real-time PCR systems at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The standard curves for the quantitative PCR were set using 2, 1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 and 1/256 μl cDNA generated with RNA from NSF-LT-RAS cells. qPCR primers: HAS2-forward: 5′-GAAAAGGGTCCTGGTGAGACGGATGAG-3′; HAS2-reverse: 5′-TTCACCATCTCCACAGATGAGGCAGG-3′.

Tumour xenograft assay. NIH-III nude mice (Crl:NIH-Lyst$^{bg\text{-}J}$ Foxn1^{nu}Btk^{xid}) were purchased from Charles river Laboratories Inc. Seven-week-old female mice were used to establish xenografts. For each injection, 4×10^6 cells were collected and re-suspended in 100 μl of ice-cold 20% matrigel (BD Bioscience) in PBS (Gibco). This 100-μl solution was injected subcutaneously close to the base of the external ear or into the flank just in front of the hind legs with a 22-gauge needle. Transplantations of MSF-LT-Ras cells were allowed to grow for 2–3 weeks, whereas xenografts with NMR cells were allowed to grow for 65 days before death. Tumours were excised and size and weight were recorded. The mice were dissected and tumour metastasis was examined for each organ.

21. Lee, H. G. & Cowman, M. K. An agarose gel electrophoretic method for analysis of hyaluronan molecular weight distribution. *Anal. Biochem.* **219,** 278–287 (1994).