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Supplementary information

Transformation of naked mole-rat cells

In the format provided by the authors and unedited

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Animals

All experimental animal work was performed in accordance to the UK Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 and approved by the Animal Welfare and Ethical Review Bodies at the University of Cambridge and the Wellcome Trust Sanger Institute. Various tissues (see below) from adult male and female NMRs aged between 37 and 244 weeks were used in this study. Mouse cell lines were derived from two C57BL/6N female mice (age: 23 weeks). NMRs were bred in-house and maintained in an inter-connected network of cages in a humidified (~55 %) temperature-controlled room (28 °C) with red lighting (08:00-16:00) and had access to food ad libitum. In addition, a heat cable provided extra warmth under 2-3 cages/colony. NMRs used in this study came from two different colonies. All animal experiments were conducted under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 under Project Licenses (70/7705 & P7EBFC1B1) and P1958D980 granted to E. St. J. Smith and Walid T. Khaled respectively by the Home Office and approved by the University of Cambridge Animal Welfare Ethical Review Body.

NMR Primary Cell Isolation

Following CO_2 exposure and decapitation, five different tissues namely skin, pancreas, lung, kidney and intestine were collected from each animal on ice-cold NMR Cell Isolation Medium (DMEM high glucose (Gibco # 11965092) supplemented with 100 units ml^{-1} Penicillin, 100µg ml^{-1} Streptomycin (Gibco # 15140122)). Skin came from either underarm or underbelly area and was cleared of any fat or muscle tissue and generously sprayed with 70% ethanol. Intestines were collected from the jejunum to the ileum and cleared of any faeces by flushing with phosphate-buffered saline (PBS) before removing any fat tissue. Similarly, fat tissue and adrenal glands were removed from kidneys. Once cleared each tissue was washed twice with PBS before finely mincing with sterile scalpels. The minced lung tissue was then washed with 4 ml NH_4Cl solution (containing 0.8% NH_4Cl in H_2O (w/v) and 1 mM EDTA, buffered with $KHCO_3$ (final concentration 10mM), final pH 7.2 - 7.6) to lyse any red blood cells. Minced skin, pancreas, kidney, intestine and lungs cleared of blood cells were then mixed with 5 ml of NMR Cell Isolation Medium containing 500 µl of Cell Dissociation Enzyme Mix (10 mg ml^{-1} Collagenase (Roche # 11088793001), 1000 units ml^{-1} Hyaluronidase (Sigma # H3506) in DMEM high glucose (Gibco # 11965092)) and incubated at 37 °C for 3 – 5 hours. Each tissue was briefly vortexed every 30 minutes to aid cell dissociation and inspected for cell dissociation. In order to reduce cell death, intestinal tissue was incubated

with NMR Cell Isolation Medium containing Cell Dissociation Enzyme Mix for only 1 hour and then vigorously pipetted to dissociate cells. After complete dissociation, cells were pelleted by centrifuging at 500 g for 5 minutes and resuspended in NMR Cell Culture Medium (EMEM (ATCC® # 30-2003) / DMEM high glucose (Gibco # 11965092) supplemented with 15% fetal bovine serum (Gibco), non-essential amino acids (Gibco # 11140050), 1 mM sodium pyruvate (Gibco # 11360039), 100 units ml^{-1} Penicillin, 100 µg ml^{-1} Streptomycin (Gibco # 15140122) and 100 µg ml^{-1} Primocin (InvivoGen # ant-pm-2)). This cell suspension was passed through 70 µm filter (Corning # 352350) and seeded on treated cell culture dishes (Greiner Bio-One). Each cell suspension was seeded on 1x 75 cm^2 dish and 7x 25 cm^2 dishes and incubated in a humidified 32 °C incubator with 5% CO_2 and 3% O_2. The cell cultures on 25 cm^2 dishes were used for cell line generation (see below) whereas those on the 75 cm^2 dish were expanded and frozen as primary cells within four passages.

Mouse Primary Cell Isolation

Following CO_2 exposure and cervical dislocation, a section of flank was shaven and a piece of skin was collected in Cell Isolation Medium (DMEM high glucose (Gibco # 11965092) supplemented with 100 units ml^{-1} Penicillin, 100µg ml^{-1} Streptomycin (Gibco # 15140122)). Skin came from either underarm or underbelly area and was cleared of any fat or muscle tissue and generously sprayed with 70% ethanol. Once cleared the tissue was washed twice with PBS before finely mincing with sterile scalpels. Minced skin was next mixed with 5 ml of Cell Isolation Medium containing 500 µl of Cell Dissociation Enzyme Mix (10 mg ml^{-1} Collagenase (Roche # 11088793001), 1000 units ml^{-1} Hyaluronidase (Sigma # H3506) in DMEM high glucose (Gibco # 11965092)) and incubated at 37 °C for 3 – 5 hours. The sample was briefly vortexed every 30 minutes to aid cell dissociation and inspected for cell dissociation. After complete dissociation, cells were pelleted by centrifuging at 500 g for 5 minutes and resuspended in Mouse Cell Culture Medium (DMEM high glucose (Gibco # 11965092) supplemented with 15% fetal bovine serum (Gibco), non-essential amino acids (Gibco # 11140050), 1 mM sodium pyruvate (Gibco # 11360039), 100 units ml^{-1} Penicillin, 100 µg ml^{-1} Streptomycin (Gibco # 15140122)). This cell suspension was passed through 70 µm filter (Corning # 352350) and seeded on treated cell culture dishes (Greiner Bio-One) and incubated in a humidified 37 °C incubator with 5% CO_2.

Lentiviral Plasmid Construction

The *pKLV2-U6gRNA5(BbsI)-PGKpuro2ABFP-W* (Addgene # 67974)[17] was used as backbone plasmid for generating the *PGK-SV40LT-HRAS^{G12V}* vector. DNA fragments coding for the *T2A-SV40LT-T2A-HRAS^{G12V}* sequence was synthesized using GeneArt Gene Synthesis service (ThermoFisher Scientific). The synthesized DNA fragment included the sequence encoding for the last 9 amino acids of the puromycin resistance gene and SexAI restriction site at the 5' end and the NotI restriction site at the 3' end to allow for direct cloning into *pKLV2-U6gRNA5(BbsI)-PGKpuro2ABFP-W*. Since the SexAI enzyme is methylation sensitive and its restriction site (ACCWGGT) is methylated by Dcm, Dam and Dcm deficient *E. coli* (NEB # C2952I) were used for cloning. This generated the *pKLV2-U6gRNA5(BbsI)-PGKpuro2A-SV40LT-2A-HRAS^{G12V}-W* vector.

In order to generate the *pKLV2-U6gRNA5(BbsI)-PGKpuro2A-SV40LT-W* vector, the T2A-*SV40LT* sequence was PCR amplified using a primer pair (01+02, Supplementary Table 1) (final concentration 500 nM) in a 50 µl reaction. The primers carried the SexAI and STOP-NotI restriction sites in Forward and Reverse primer respectively. PCR was set up as follows. 20 µg of the *pKLV2-U6gRNA5(BbsI)-PGKpuro2A-SV40LT-2A-HRAS^{G12V}-W* vector was used as template and the Phusion Green Hot Start II High-Fidelity PCR Master Mix (ThermoFisher Scientific # F566) was used. The following PCR conditions were used:

98 °C	30 seconds	} x1
98 °C	10 seconds	)
70.7 °C	15 seconds	} x25
72 °C	60 seconds	)
72 °C	120 seconds	} x1
4 °C	hold	}

Amplification of the desired product was confirmed by running the PCR product on an 0.7% agarose gel (ThermoFisher Scientific # 16500). Next, two PCR reactions were set up under the optimised conditions and the product was cleared of polymerase and salts in the PCR reaction by running through a gel extraction kit (Qiagen # 28704) without running on agarose gel under the manufacturer's recommended protocol and eluted in 20 µl of nuclease-free water (Qiagen # 129114). The purified PCR product was next digested with SexAI and NotI enzymes and ligated to the SexAI and NotI digested backbone plasmid (Addgene # 67974).

The *pKLV2-U6gRNA5(BbsI)-EF1Apuro2A-SV40LT-2A-HRAS^{G12V}-W* vector was generated by replacing the *PGK* promoter in *pKLV2-U6gRNA5(BbsI)-PGKpuro2A-SV40LT-2A-*

HRAS^{G12V}-W vector with the human *EF1A* promoter using Gibson assembly (NEBuilder HiFi DNA Assembly Cloning Kit (NEB # E5520S)). The *EF1A* promoter was PCR amplified using Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Scientific # F566) and using 20 ng of pL-CRISPR.EFS.GFP (Addgene # 57818)[18] as template. The primer pair (03+04, Supplementary Table 1) was used at a final concentration of 500nM in a 50 µl reaction with the following conditions:

98 °C	10 seconds	} x1
98 °C	10 seconds	} x25
72 °C	40 seconds	
72 °C	120 seconds	} x1
4 °C	hold	}

The PCR product was run on 1% agarose (Thermo Scientific # 16500) gel, the band (size = 300 bp) was cut out and the DNA fragment was extracted using a gel extraction kit (Qiagen # 28706) following manufacturer's instruction. The purified DNA fragment (fragment a) was eluted in nuclease-free water (Qiagen #129114). Next, the *pKLV2-U6gRNA5(BbsI)-PGKpuro2A-SV40LT-2A-HRAS^{G12V}-W* vector (without the *PGK* promoter) was PCR amplified using Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Scientific # F566) and using 20 ng of *pKLV2-U6gRNA5(BbsI)-PGKpuro2A-SV40LT-2A-HRAS^{G12V}-W* vector as template. The primer pair (05+06, Supplementary Table 1) was used at a final concentration of 500nM in a 50 µl reaction with the following conditions:

98 °C	30 seconds	} x1
98 °C	10 seconds	} x25
61.9 °C	15 seconds	
72 °C	300 seconds	
72 °C	120 seconds	} x1
4 °C	hold	}

The PCR product was passed through gel extraction column (Qiagen # 28706) to remove salts and enzymes before incubating 37 °C with DpnI for one hour to remove the template DNA. The product was next run on 1% agarose (Thermo Scientific # 16500) gel and the fragment (size = 10170 bp) was excised and the DNA fragment was purified using gel extraction kit (Qiagen # 28706) following manufacturer's instruction. The purified DNA fragment (fragment b) was eluted in nuclease-free water (Qiagen #129114).

The *EF1A* promoter (fragment a) and the *pKLV2-U6gRNA5(BbsI)-PGKpuro2A-SV40LT-2A-HRAS^{G12V}-W* vector (without the *PGK* promoter) (fragment b) were then combined in 5:1 molar ratio on ice in a 25 µl Gibson assembly reaction (NEB # E2621) before incubating at

50 °C for one hour. Next, the entire Gibson assembly reaction was used to transform the NEB 5-alph chemical competent cells (NEB #C2987). Next day, colonies were picked and grown overnight in LB (Luria-Bertani) broth. Positive colonies were identified by a diagnostic restriction digest with BamHI and confirmed through sanger sequencing.

The *pKLV2-U6gRNA5(BbsI)-CMVIE-SV40LT-HRAS^{G12V}* and *pKLV2-U6gRNA5(BbsI)-SV40E-SV40LT-HRAS^{G12V}* vectors were generated by replacing the *EF1A* promoter in the *pKLV2-U6gRNA5(BbsI)-EF1Apuro2A-SV40LT-2A-HRAS^{G12V}-W* vector with *CMVIE* and *SV40E* promoters respectively using Gibson assembly (NEBuilder HiFi DNA Assembly Cloning Kit (NEB # E5520S)). The *pCMV-RasV12* (Clontech # 631924) and *pSG5-largeT* (Addgene # 9053) were used as templates for the *CMVIE* and the *SV40E* promoter respectively. In each case 20 ng of the template vectors were used. The Q5 Hot Start High-Fidelity PCR Master Mix (NEB # M0494) was used for PCR amplification. The *SV40E* promoter (fragment c) was amplified with primer pair (07+08, Supplementary Table 1) at a final concentration of 500nM in a 50 µl reaction with the following conditions:

98 °C	30 seconds	x1
98 °C	10 seconds	x25
72 °C	10 seconds	
72 °C	35 seconds	
72 °C	120 seconds	x1
4 °C	hold	

The *CMVIE* promoter (fragment d) was amplified with primer pair (09+10, Supplementary Table 1) at a final concentration of 500nM in a 50 µl reaction with the following conditions:

98 °C	30 seconds	x1
98 °C	10 seconds	x25
72 °C	10 seconds	
72 °C	25 seconds	
72 °C	120 seconds	x1
4 °C	hold	

The *pKLV2-U6gRNA5(BbsI)-SV40E-SV40LT-HRAS^{G12V}-W* vector (without the *EF1A*) promoter (fragment e) was amplified using the Q5 Hot Start High-Fidelity PCR Master Mix (NEB # M0494) and 20 ng of *pKLV2-U6gRNA5(BbsI)-PGKpuro2A-SV40LT-2A-HRAS^{G12V}-W* vector as template. The primer pair (05+06, Supplementary Table 1) were used at a final concentration of 500nM in a 50 µl reaction with the following conditions:

98 °C	30 seconds	} x1
98 °C	10 seconds	} x25
72 °C	10 seconds	
72 °C	305 seconds	
72 °C	120 seconds	} x1
4 °C	hold	}

Amplification of the desired product in each case (fragment c, d and e) was confirmed by running the PCR product on an 1% agarose gel (ThermoFisher Scientific # 16500). Next, two PCR reactions for each of the three fragments (fragment c, d, and e) were set up under the optimised conditions and the products were cleared of polymerase and salts in the PCR reaction by running through a gel extraction kit (Qiagen # 28704) without running on an agarose gel under manufacturer's recommended protocol and eluted in 20 µl of nuclease-free water (Qiagen # 129114).

The cleared PCR products were next incubated at 37 °C with DpnI for one hour to remove the templates DNA before running through a gel extraction kit (Qiagen # 28704) without running on an agarose gel under manufacturer's recommended protocol to get rid of salts and enzymes and eluted in 20 µl of nuclease-free water (Qiagen # 129114).

Next, Gibson assembly reactions were set up. To generate the *pKLV2-U6gRNA5(BbsI)-SV40Epuro2A-SV40LT-2A-HRAS^{G12V}-W* and the *pKLV2-U6gRNA5(BbsI)-CMVIEpuro2A-SV40LT-2A-HRAS^{G12V}-W* vectors, the amplified *SV40E* promoter (fragment c) and the amplified *CMVIE* promoter (fragment d) were respectively combined with the amplified *pKLV2-U6gRNA5(BbsI)-EF1Apuro2A-SV40LT-2A-HRAS^{G12V}-W* vector (without the *EF1A* promoter) (fragment e) in 5:1 molar ratios on ice in 25 µl Gibson assembly reactions (NEB # E2621). The Gibson assembly reactions were next incubated at 50 °C for one hour before transformation of NEB 5-alph chemical competent cells (NEB #C2987). Next day, colonies were picked and grown overnight in LB (Luria-Bertani) broth. Positive colonies for the *pKLV2-U6gRNA5(BbsI)-SV40Epuro2A-SV40LT-2A-HRAS^{G12V}-W* vector were identified by a diagnostic restriction digest with NotI and BamHI whereas positive colonies for the *pKLV2-U6gRNA5(BbsI)-CMVIEpuro2A-SV40LT-2A-HRAS^{G12V}-W* vector were identified by digestion with BamHI and EcoRI restriction enzymes. In each case, vector sequences were confirmed through sanger sequencing.

Cell Culture

All NMR cells (both primary and immortalised) were cultured in NMR Cell Culture Medium (as described above) in a humidified 32 °C incubator with 5% CO_2 and 3% O_2 (Binder CB160). Media was changed twice a week and upon confluency cells were washed with PBS and detached using 1X Trypsin-PBS (Trypsin-EDTA (Sigma # T4174-100ml). After detaching cells, trypsin was inactivated by addition of NMR Cell Culture Medium and centrifuged at 450 g for 5 minutes to remove the trypsin containing medium. The cell pellet was resuspended in NMR Culture Medium and seeded on appropriate size flasks. HEK-293FT cells were cultured in DMEM high glucose (Gibco # 11965092) supplemented with 10% fetal bovine serum (Gibco) and 100 units ml^{-1} Penicillin, 100µg ml^{-1} Streptomycin (Gibco # 15140122) in a humidified 37 °C incubator with 5% CO_2. Mouse skin fibroblasts were cultured in DMEM high glucose (Gibco # 11965092) supplemented with 15% fetal bovine serum (Gibco) and 100 units ml^{-1} Penicillin, 100 µg ml^{-1} Streptomycin (Gibco # 15140122) in a humidified 37 °C incubator with 5% CO_2.

Lentivirus Production

HEK-293FT cells were used as packaging cells for producing lentiviral particles. One day before transfection, HEK-293FT cells were seeded on 10 cm dishes (Greiner # 664960) coated with 0.1% gelatin (Sigma # 1393-100ml) in PBS (w/v) for 30 minutes. The next day, cells were cotransfected with one of the immortalization (or control) vectors (described above) and second-generation lentivirus packaging plasmids: pMD2.G (Addgene # 12259), psPAX2 (Addgene # 12260) as follows. For each 10 cm dish, 12 µl of Plus reagent (Gibco # 15338100) was mixed with 5.4 µg immortalization (or control) vector, 5.4 µg psPAX2 and 1.2 µg pMD2.G in 3 ml of Opti-MEM I (Gibco # 31985062). This mixture was briefly vortexed and then incubated at room temperature for 5 minutes before adding 36 µl Lipofectamine LTX (Gibco # 15338100), followed by vortexing and incubating for another 30 minutes at room temperature. Just before adding the transfection mixture, 5 ml fresh medium (DMEM high glucose (Gibco # 11965092) supplemented with 10% fetal bovine serum) was added to the cells. Next, the transfection mixture was added to the cells and incubated at 37 °C in a humidified 5% CO_2 incubator. After 5 hours, the medium containing the transfection mixture was removed and replaced with 10 ml fresh media (DMEM high glucose (Gibco # 11965092) supplemented with 10% fetal bovine serum). The cells were then incubated in a 37 °C humidified 5% CO_2 incubator for 36 – 48 hours. Following the incubation, the lentiviral particle-containing medium was collected and passed through a 0.45 µm Mixed Cellulose

Ester (MCE) filter (Millipore # SLHA033SS) to remove any cellular debris. Three volumes of this cleared lentiviral particle-containing medium was combined with one volume of Lenti-X Concentrator (Clontech # 631232) and mixed gently before incubating overnight at 4 °C. The Lenti-X Concentrator – lentiviral mixture was centrifuged at 1500 g for 45 minutes at 4 °C. The supernatant was discarded, and the viral pellet was resuspended in a ratio of 1:25 of the original volume in antibiotic free media and 200 µl aliquots were stored at –80 °C.

Lentiviral Transduction

Primary NMR cells were seeded on 25 cm^2 dishes and when the cells reach a minimum of 20% confluency, the cells were washed with PBS and 5 ml of fresh NMR cell culture media supplemented with 200-250 µl of defrosted lentiviral particles were added. 72 hours post-transduction, the cells were washed with PBS and fresh NMR cell culture media was added. The cells were allowed to recover for another 24 hours before starting selection with puromycin (Sigma # P7255-100MG) at a final concentration of 2 µg ml^{-1} for 4 days for NMR skin, pancreas, lung and intestinal cells. For the kidney cell lines 4 µg ml^{-1} of puromycin was used for 3 days. In each case, a flask of non-transduced cells was used as a control to ensure for antibiotic selection.

To achieve single transduction events in Extended Figure 2f, the *PGK-BFP*, *PGK-SV40LT* and *PGK-SV40LT-HRas*^{G12V} vectors were packaged in parallel into lentiviral particles and the same number of cells were transduced with different volumes of the *PGK-BFP* viral preparation. The BFP expression was used as surrogate of transduction and measured using flow cytometry. Transducing half a million cells with ~700 µl of this viral preparation resulted in ~25% BFP positive cells. Based on this experiment, 300 µl of the viral preparation was used to transduce half a million primary NMR skin cells.

Anchorage-Independent Growth (Soft Agar Assay)

NMR cell lines generated using the lentiviral vectors (above) and their primary counterparts were tested for anchorage-independent growth by seeding them in soft agar. One day before the assay was performed, 0.7 g and 1 g of Difco Noble Agar (BD Bioscience # 214220) was added to 100 ml of deionised water to make 0.7% and 1% agar suspensions. These were autoclaved in a benchtop autoclave (Classic Prestige Medical) before storing at 4 °C. The 1% agar was melted in a microwave and incubated at 37 °C. Melted 1% agar and Soft Agar Medium (2X MEM (Gibco # 11935046) supplemented with 15% fetal bovine serum (Gibco),

non-essential amino acid (Gibco # 11140050), 1 mM sodium pyruvate (Gibco # 11360039) and 100 units ml^{-1} Penicillin, 100 µg ml^{-1} Streptomycin (Gibco # 15140122)) were mixed in equal volumes and 1.5 ml of which was added into each well of a 6-well plate to make a bottom layer of 0.5% agar. This layer was allowed to solidify at room temperature in a cell culture hood for 2-hours. Once solidified, NMR cells were trypsinised, counted and diluted in Soft Agar Medium. The cells were then mixed in a 1:1 ratio with 37 °C, 0.7% agar making a single cell suspension of 4000 cells per ml. 1.5 ml of the cell-agar suspension (or 6000 cells) was added on top of the solidified bottom layer prepared earlier and allowed to solidify at room temperature for 2-hours before being supplemented with 1 ml of NMR media and incubated in a humidified 32 °C incubator with 5 % CO_2 and 3 % O_2 and 6 wells were seeded per condition per line. Cells were incubated for 6 or 3 weeks to permit colony formation and the medium on top was changed twice a week to replenish nutrients. To quantify the number of colonies observed, images in a single focal plane were taken using a 1.25x objective on a EVOS FL2 (Thermo Scientific) or 1.25x / 4x objective on EVOS FL Auto2 (Thermo Scientific). This amounted to a collection of more than 83,000 images in total which were analysed and quantified using an ImageJ macro.

Protein Extraction, Antibodies and Immunoblotting

Cells were gently washed with PBS and collected by trypsinization, followed by centrifugation at 1,000 x rpm. After the centrifugation, supernatants were discarded and ice-cold 150 mM lysis buffer (20 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 1% NP-40, 5 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 50 mM NaF, 20 mM beta-glycerophosphate, 1 mM Na_3VO_4), freshly supplemented with benzamidine, DTT, leupeptin, PMSF (1:1000), were added to cells and incubated on ice for 30 minutes. After the incubation, samples were centrifuged at 15,000 x rpm and supernatants were transferred into pre-chilled Eppendorf tubes. Samples were used for immunoblotting after the addition of laemmli buffer.

For immunoblotting, samples were resolved by precast gels (Mini-PROTEAN® TGX™ Gels, Biorad #4561086), followed by transfer onto Immobilon-P membranes (Millipore). Following transfer, membranes were washed with autoclaved water twice and blocked for at least 30 minutes with 5% skim milk blocking solution (made with TBS-T, consisting of 50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Tween 20). After the blocking, membranes were incubated overnight with the corresponding primary antibodies. Next day, membranes were incubated with horseradish peroxidase-linked secondary antibodies for 2 hours, followed by chemiluminescent detection with ECL prime (Amersham). The following antibodies were

used; SV40 T ag (sc-148, Santa Cruz), Ras (G12V mutant specific antibodies, D2H12, Cell Signalling), and anti-alpha Tubulin (DM1A, Abcam). The concentration of antibodies used, the supplier and catalogue numbers are provided in Supplementary Table 2.

Xenograft Assay

Trypsinized cells were counted and 6 million cells were resuspended in 450 µl of HF (HBBS (Gibco # 24020091) + 1% fetal bovine serum (Gibco)). The cell suspension was then mixed with 150 µl ice-cold matrigel (Corning # 354230) before injection to get a final cell suspension of 6 million cells in 600 HF - 25% matrigel. 100 µl of the cell suspension (or 1 million cells) were injected subcutaneously at the flank of NSG mice using a 25-gauge needle. Animals were monitored for tumour growth and tumour volume was measured every 48-72 hours using calipers. Cells were allowed to grow in the animals for a maximum 9 weeks (63 days) post injection or until the tumour reached a maximum allowed area of 1.2 cm^2. Importantly the experiment was double blind - cells were assigned random names by one experimenter who handed them over to a second experimenter who then injected the animals and tumours were measured by an animal technician who was unaware of the identity of the lines.

Transfection

Actively growing cells were harvested by trypsinizing. Cells were counted and pelleted by centrifugation at 90g for 10 minutes. 1 million cells were resuspended in 100 µl of room temperature Normal Human Dermal Fibroblasts (NHDF, Lonza # VPD-1001) or Primary Mammalian Fibroblasts (PMF, Lonza # VPI-1002) Nucleofector Solutions. 5 µg of *pSG5-largeT* (Addgene # 9053), 5 µg of *pCMV-RasV12* (Clontech # 631924) and 5 µg of *pmaxEGFP* (Lonza) were mixed and added to the cell-nucleofector suspension. Both linearized (with DraIII) and circular versions of *pSG5-largeT* (Addgene # 9053) and *pCMV-RasV12* (Clontech # 631924) were used. Cells were next transferred to cuvettes for nucleofection. U020 or U023 programs were used on Nucleofector 2b (Lonza # AAB-1001). Following nucleofection, 500 µl of cell recovery media (RPMI (Gibco # 11875093), supplemented with 15% fetal bovine serum (Gibco), non-essential amino acids (Gibco # 11140050), 1 mM sodium pyruvate (Gibco # 11360039), 100units ml^{-1} Penicillin, 100 µg ml^{-1} Streptomycin (Gibco # 15140122) was added to the cells and the mixture was transferred to sterile 1.5 ml tubes. Cells were allowed to recover at 32 ºC (for NMR cells) or 37 ºC (for mouse cells) for 15 minutes. Next, the cells were transferred to 25 cm^2 dishes containing cell

culture media pre-equilibrated at 32 ºC, 3% O_2 and 5% CO_2 (for NMR cells) or 37 ºC, 5% CO_2 (for mouse cells). Cells were allowed to recover for 24 hours prior to further experiments. NMR cells transfected with PacI-linearized lentiviral vectors (Supplementary Figure 1a) were selected with puromycin (Sigma # P7255-100MG) at a final concentration of 2 μg ml^{-1} for 4 days. In each case, a flask of non-transfected or *pmaxEGFP*-transfected cells was used as a control to ensure for antibiotic selection.

RNA Isolation, Library Preparation and Sequencing

Actively growing cells were harvested by trypsinizing and pelleted by centrifuging at 450 g for 5 minutes. Next, supernatant was aspirated, and cell pellet frozen on dry ice for processing later. Cell pellets were kept at -80 ºC and processed within a week. Total RNA was isolated using RNA Isolation Kit (Qiagen # 74106) following manufacturer's instructions. Genomic DNA was digested and removed by incubating the column with RNAse-Free DNAse (Qiagen # 79254) for 30 minutes at room temperature. RNA was eluted in RNAse-Free water and quantified using Nanophotometer N60 (Implen). RNA samples were QC'd with Qubit and Tapestation. 3 ug of total RNA was next used in the NEBNext® Poly(A) mRNA Magnetic Isolation Module (E7490). Next, 30 ng of the PolyA RNA was used with the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (E7760), with 8 cycles of PCR. QC of the finished libraries was done with Qubit and Tapestation. The samples were then pooled to 20 nM with NEBNext Multiplex Oligos 96 Unique Dual Indexes (E6440) and sequenced on one lane of a Novaseq S1 flowcell with paired end 150 bp reads.

RNA-Seq Data Analysis

FASTQ files were aligned to the reference genome of either Mus Musculus (GRCm38 with GRC38.91 annotation from ENSEMBL) or to the HetGla2.0 reference genome with the GCF_000247695.1 annotation from NCBI. The mapping was performed using the STAR aligner (2.7.1a) with default settings and –quantMode GeneCounts to derive gene level counts. The counts were then normalized with the trimmed mean of M-values (TMM) method to account for differences in sequencing depth and library composition[19]. The principle component analysis for mouse and naked mole rat cell lines was performed on log-transformed CPM (counts-per-million) values for a set of 16054 published orthologous genes[20]. Genes with high or low loading for PC2 (>0.02 or < -0.02) were characterized by gene set enrichment analysis based on gene-ontology (GO) terms using topGO with default

settings (Alexa, A. & Rahnenfuhrer, J. TopGO: Enrichment Analysis for Gene Ontology. R package version 2.28.0 (2016)). All differential expression tests were performed using edgeR[21] by fitting a negative binomial generalised log-linear model with the cell line as covariate. Genes that have a higher log fold change than 1 at an FDR of 0.01 were identified using the 'glmTreat' function.

Code Availability

The source code used for analysis of the RNA-seq data is available online at https://github.com/KaBach/NMR.

Data Availability

The RNA-seq data is available from the European Nucleotide Archive (accession number # E-MTAB-8932.). Raw western blot data is provided as Supplementary Figure 1 in the Supplementary Information. The authors declare that all remaining supporting data are available within the article and its Supplementary Information or from the authors upon reasonable request.

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