

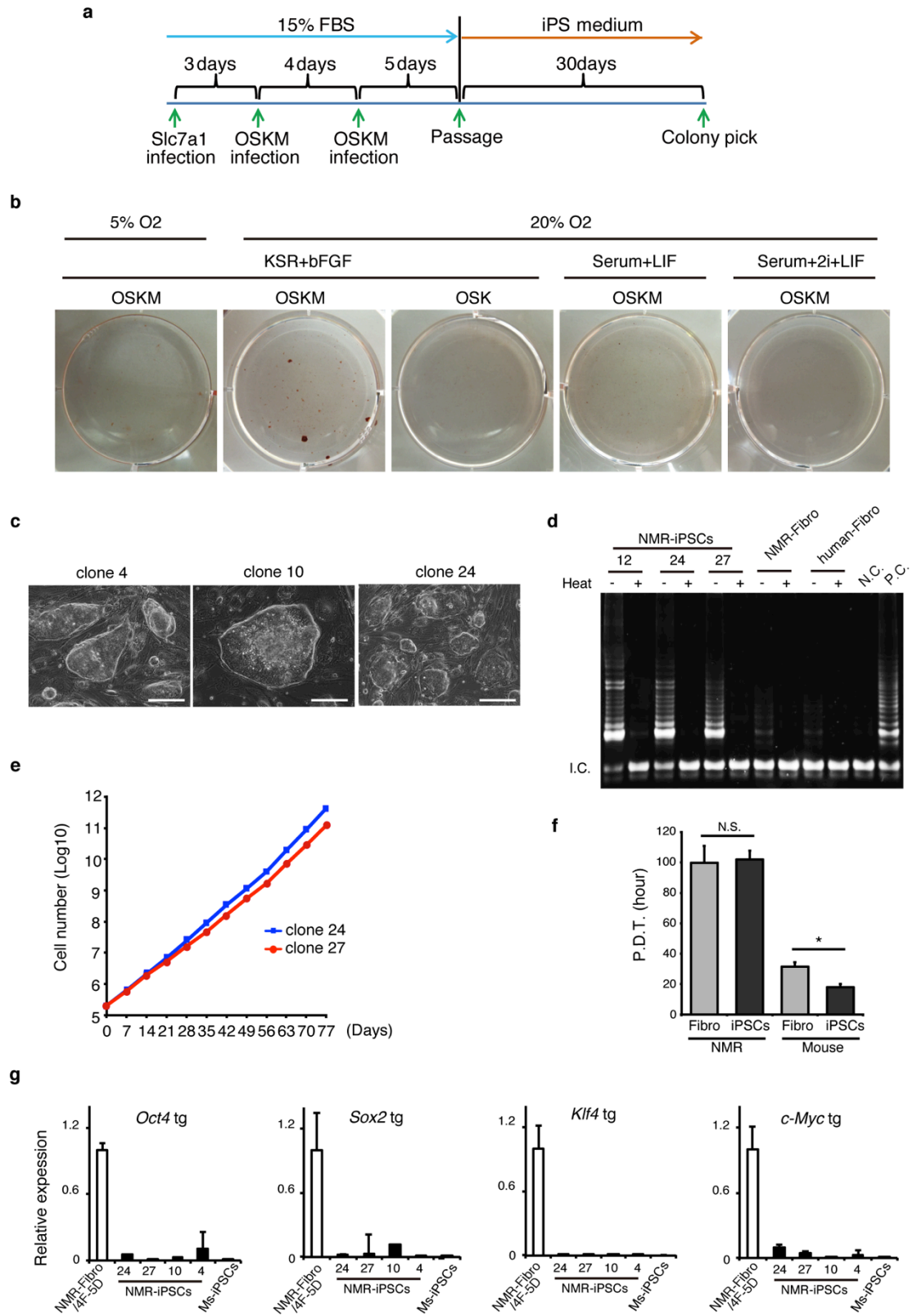


|                        |   |
|------------------------|---|
| Title                  | Tumour resistance in induced pluripotent stem cells derived from naked mole-rats  |
| Author(s)              | Miyawaki, Shingo; Kawamura, Yoshimi; Oiwa, Yuki; Shimizu, Atsushi; Hachiya, Tsuyoshi; Bono, Hidemasa; Koya, Ikuko; Okada, Yohei; Kimura, Tokuhiko; Tsuchiya, Yoshihiro; Suzuki, Sadafumi; Onishi, Nobuyuki; Kuzumaki, Naoko; Matsuzaki, Yumi; Narita, Minoru; Ikeda, Eiji; Okanoya, Kazuo; Seino, Ken-ichiro; Saya, Hideyuki; Okano, Hideyuki; Miura, Kyoko |
| Citation               | Nature communications, 7, 11471<br><a href="https://doi.org/10.1038/ncomms11471">https://doi.org/10.1038/ncomms11471</a>  |
| Issue Date             | 2016-05-10  |
| Doc URL                | <a href="http://hdl.handle.net/2115/62277">http://hdl.handle.net/2115/62277</a>   |
| Rights(URL)            | <a href="https://creativecommons.org/licenses/by/4.0/">https://creativecommons.org/licenses/by/4.0/</a>   |
| Type                   | article   |
| Additional Information | There are other files related to this item in HUSCAP. Check the above URL.  |
| File Information       | ncomms11471-s1.pdf (Supplementary Information)  |

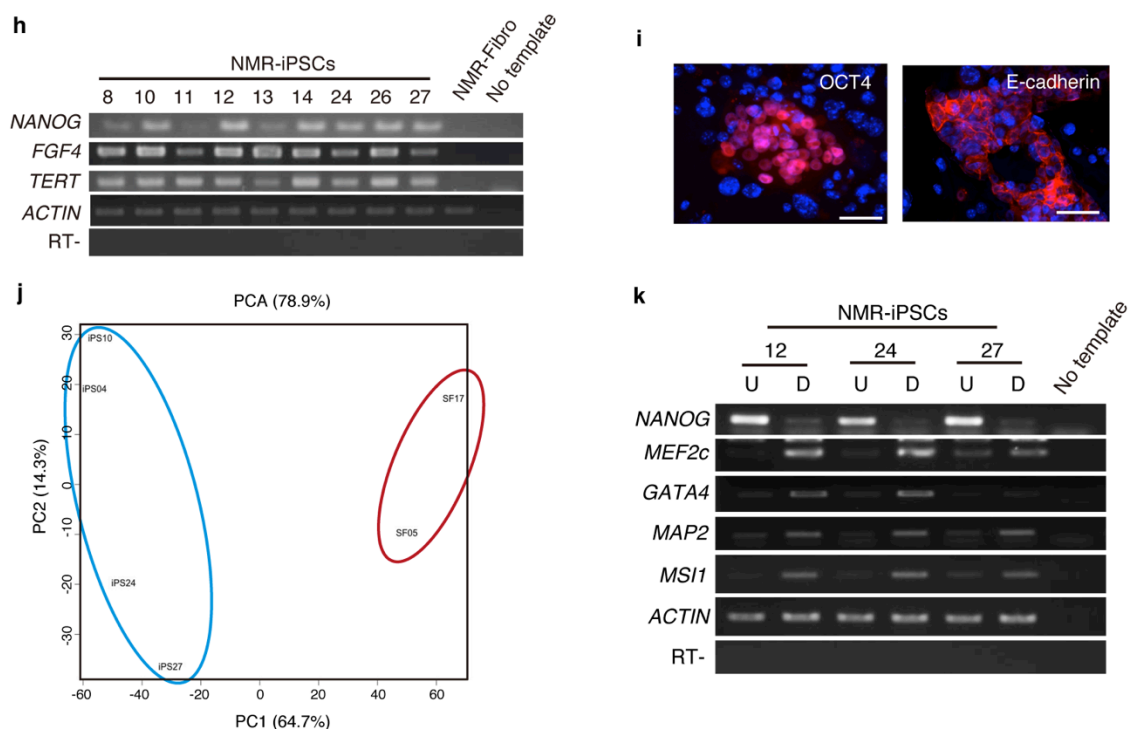


[Instructions for use](#)

Supplementary Figure 1



## Supplementary Figure 1

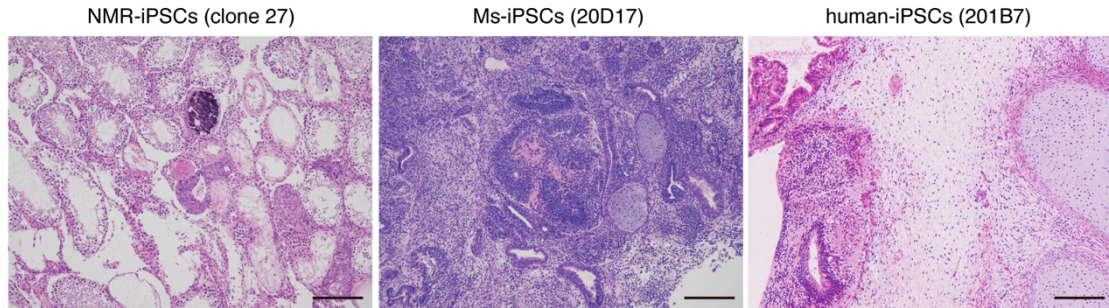


### Supplementary Figure 1 | Generation and characterization of NMR-iPSCs.

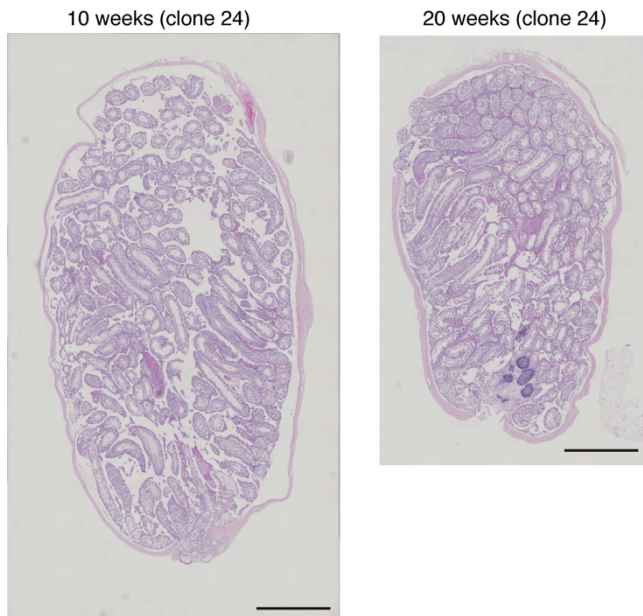
**a**, iPSC generation from NMR-fibroblasts. **b**, Culture conditions. AP activity 37 days after introduction of OSKM. **c**, Morphology of NMR-iPSCs (clones 4, 10 and 24). Scale bar, 200  $\mu$ m. **d**, Telomerase activities of NMR-iPSCs. NMR-Fibro, NMR-fibroblasts; human-Fibro, human skin fibroblasts (TIG113); N.C., Heat-inactivated (+) samples; I.C., internal control; P.C., positive control.  $n = 3$  clones. **e**, Proliferation of NMR-iPSCs (clones 24 and 27). **f**, Population doubling times (PDT).  $n = 3$  clones.  $*P < 0.05$ ; N.S., not significant ( $t$ -test). **g**, qRT-PCR analysis of transgene expression in NMR-iPSCs. NMR-Fibro/4F-5d, NMR-fibroblasts 5 days after the transduction with OSKM. Ms-iPSCs, 20D17.  $n = 4$  clones. Results are represented as mean  $\pm$  SD. **h**, RT-PCR analysis of pluripotency markers in NMR-iPSCs and NMR-fibroblasts. **i**, Immunofluorescence analysis of the expression of pluripotency markers OCT4 and E-cadherin. Hoechst dye (blue), nuclei. Scale bar, 100  $\mu$ m. **j**, Principal component analysis (PCA) of global gene expression patterns of four NMR-iPSC clones and two NMR-fibroblast lines. **k**, RT-PCR analysis of the expression of pluripotency and differentiation markers in EBs. *MEF2c*, mesoderm; *GATA4*, endoderm; *MAP2* and *MSI1*, ectoderm. U, undifferentiated; D, differentiated.  $n = 3$  clones.

Supplementary Figure 2

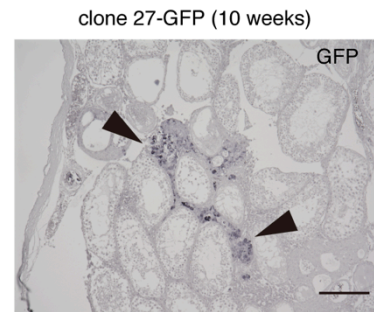
**a**



**b**



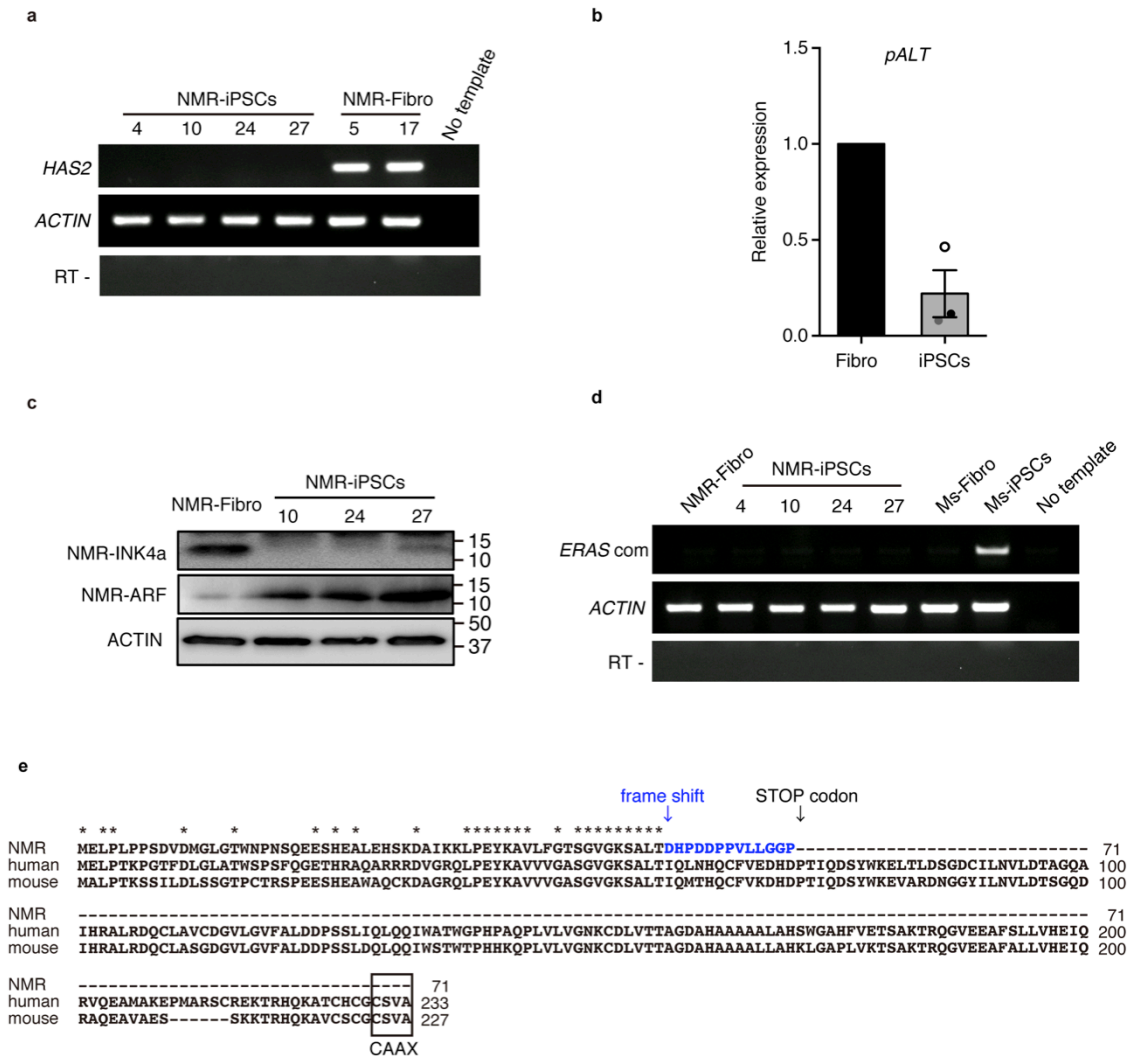
**c**



**Supplementary Figure 2 | Histopathological analysis of tumours and testes after transplantation of iPSCs. a and b, Haematoxylin and eosin staining. Sections of tumours and testes of mice transplanted with NMR-iPSCs (clone 27), Ms-iPSCs (20D17) or human-iPSCs (201B7) (a). Scale bar, 200  $\mu$ m. Testes injected with NMR-iPSCs (clone 24) 10 or 20 weeks after transplantation (b). Scale bar, 1 cm. c, Immunohistochemical analysis of GFP. Transplanted NMR-iPSCs were lentivirally labelled with GFP. Arrowhead, area of the engrafted site. Scale bar, 200  $\mu$ m.**

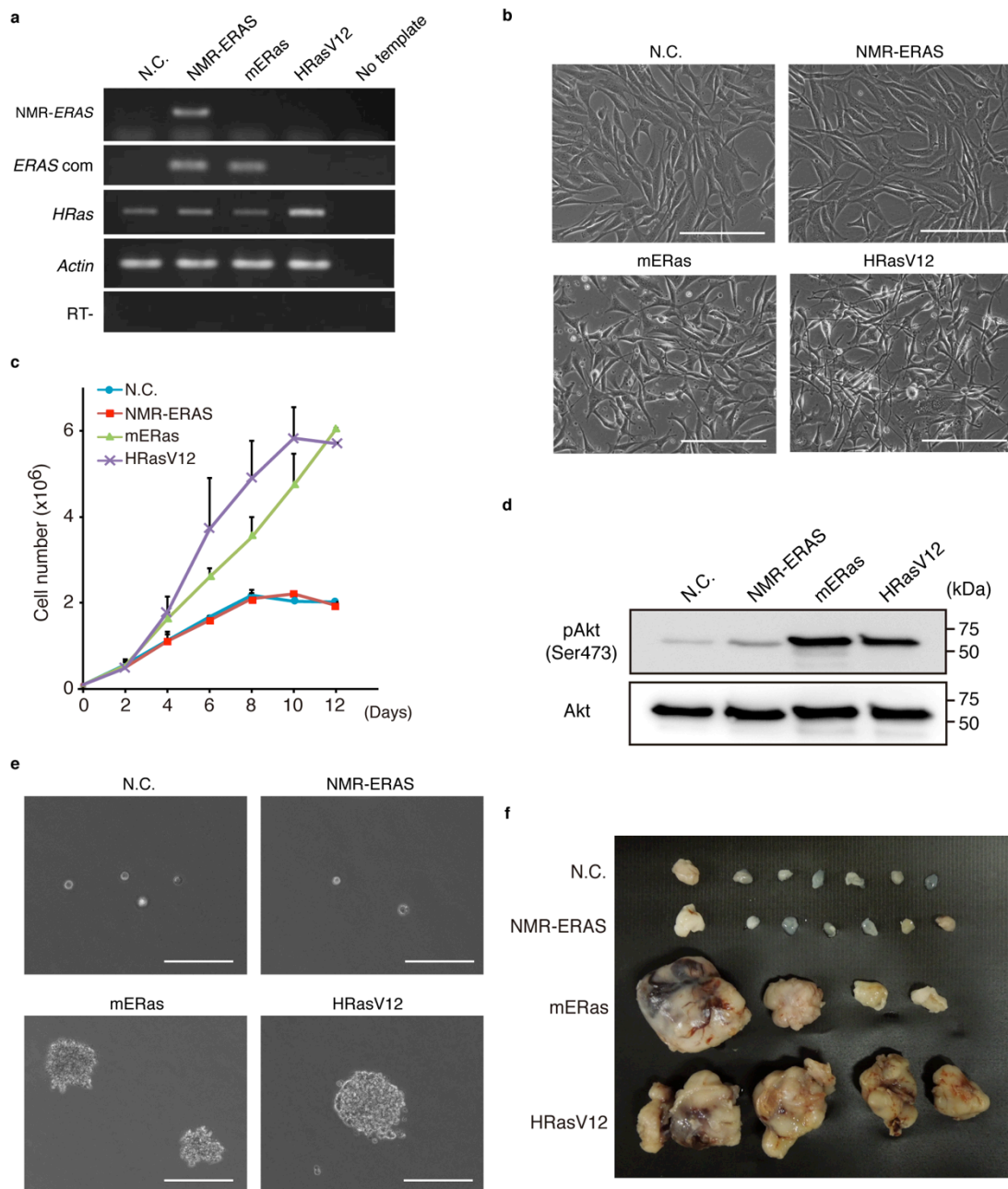


Supplementary Figure 3



**Supplementary Figure 3 | Activation of *ARF* and frameshift mutation of *ERAS* in NMR-iPSCs. a**, RT-PCR analysis of *HAS2* expression in NMR-iPSCs. **b**, Expression of *pALT*. Results are presented as mean  $\pm$  SEM.  $n = 3$  clones. **c**, Western blotting of INK4a and ARF expression in NMR-iPSCs and NMR-fibroblasts.  $n = 3$  clones. **d**, RT-PCR analysis of *ERAS* expression in NMR-iPSCs. Ms-iPSCs (20D17), positive control. *ERAS* com, primer-set designed to amplify the sequence shared by NMR-*ERAS* and *mERAs*. **e**, Amino acid sequence comparison among Ms-, human- and NMR-*ERAS* genes. Blue arrow: frameshift mutation. Box: CAAAX motif.

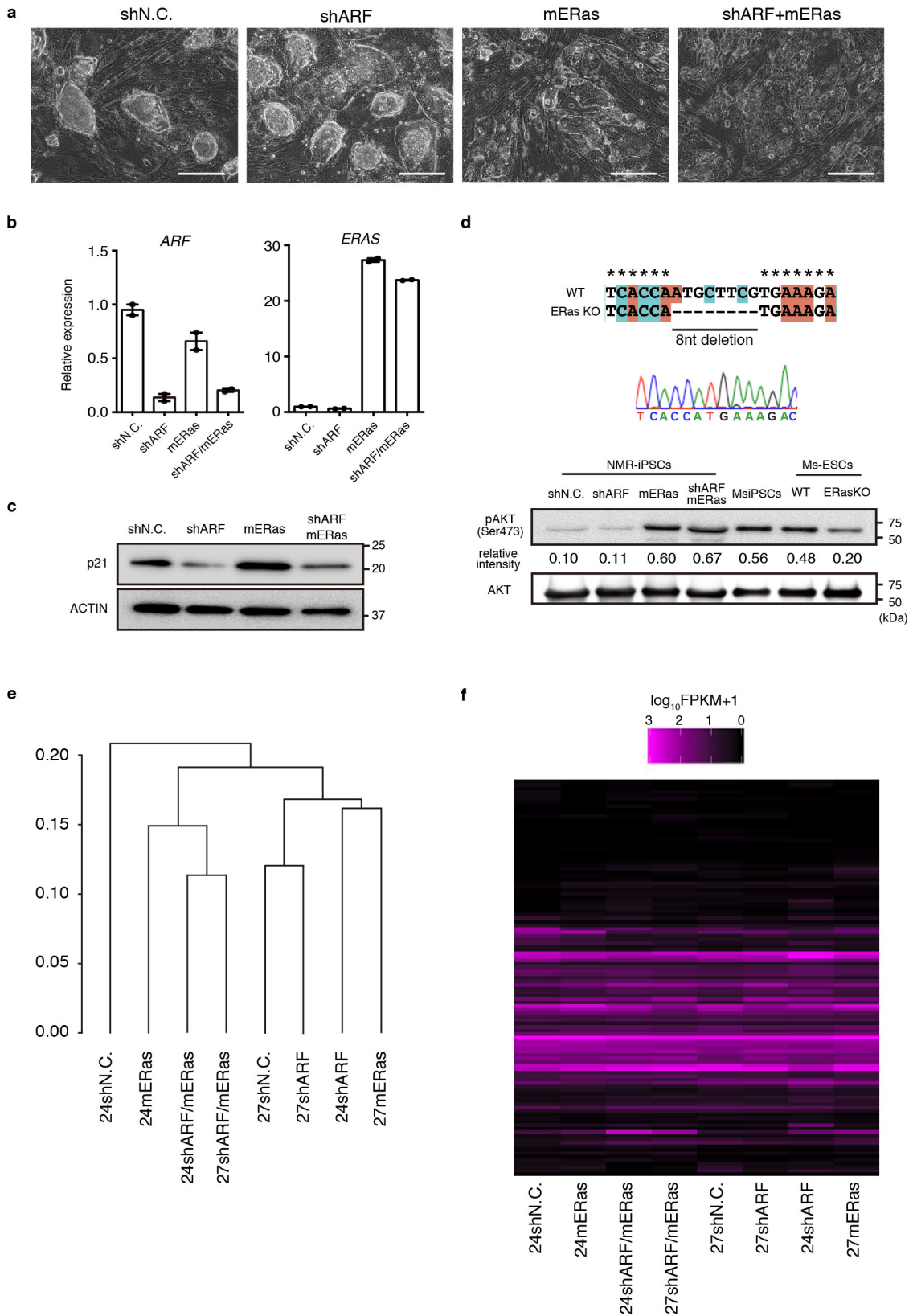
Supplementary Figure 4



**Supplementary Figure 4 | Transforming potential of NMR-ERAS in NIH-3T3 cells.**

NIH-3T3 cells were infected with lentiviral vectors expressing NMR-ERAS or mERas. HRasV12, positive oncogenic control; N.C., EGFP as negative control. **a**, RT-PCR analysis of transgene expression. **b**, Cell morphology. Scale bar, 200  $\mu\text{m}$ . **c**, Cell proliferation. Cells ( $1 \times 10^5$ ) were plated on 10 cm dish and counted every other day. Data are represented as mean  $\pm$  SD. **d**, Western blotting for AKT or phosphorylated-AKT expression. **e**, Soft agar growth assay. Scale bar, 200  $\mu\text{m}$ . **f**, Tumour formation in nude mice. Cells ( $1 \times 10^6$ ) were subcutaneously injected and tumours were dissected 25 days later.

Supplementary Figure 5

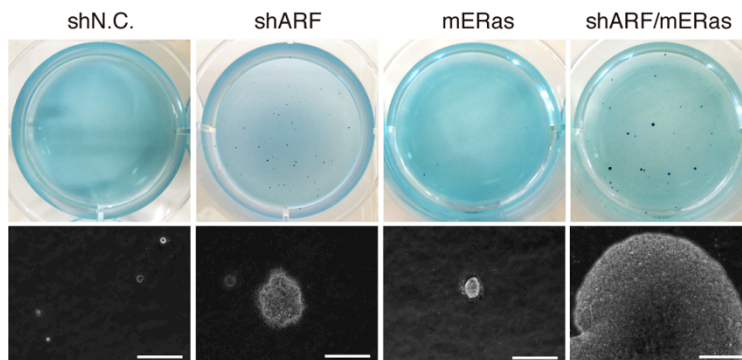


Supplementary Figure 5

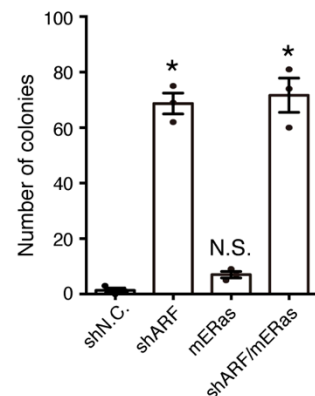
**g**

| Gene List |        | Undifferentiated | Mesoderm | Endoderm | Ectoderm |
|-----------|--------|------------------|----------|----------|----------|
| hDNMT3B   | DNMT3L | BMP2             | AFP      | OTX2     |          |
| DPPA4     | KRT17  | BMP4             | FOXA1    | SLC6A3   |          |
| ERAS      | DPPA2  | CDH1             | FOXA2    | CHAT     |          |
| FBXO15    | TERT   | EOMES            | KRT19    | LMX1B    |          |
| FGF4      | IFITM1 | HAND1            | SOX17    | GFAP     |          |
| KLF4      | NODAL  | FABP4            | TTR      | MAP2     |          |
| LIN28A    | GRB7   | VEGFA            | GATA4    | PAX6     |          |
| LIN28B    | PODXL  | MEF2C            | PDX1     | NES      |          |
| NANOG     | CD9    | NKX2-5           | KRT8     | MSI1     |          |
| NR0B1     | BRIX1  | ACTA2            | ALB      | GFAP     |          |
| POU5F1    | ESRRB  | FLT1             | PDGFRA   | REST     |          |
| SALL4     | RNF17  | CDH11            | HNF4A    | TH       |          |
| SOX2      | NR6A1  | RUNX1            | HNF1B    | SYP      |          |
| UTF1      | NUMB   | MESDC2           | SERPINA1 | MNX1     |          |
| ZFP42     | REST   | MYOD1            | CPS1     |          |          |
| ZNF296    | LIFR   | WT1              | TAT      |          |          |
| ZSCAN4    | T      | NPPA             | LAMC1    |          |          |
| TDGF1     | MYC    | HBB              | INS      |          |          |
| FGF5      | MYCN   | RUNX2            | FN1      |          |          |
|           |        | COL2A1           | PAX4     |          |          |
|           |        |                  | SST      |          |          |

**h**



**i**



**Supplementary Figure 5 | Characterization of NMR-iPSCs expressing mERas and/or**

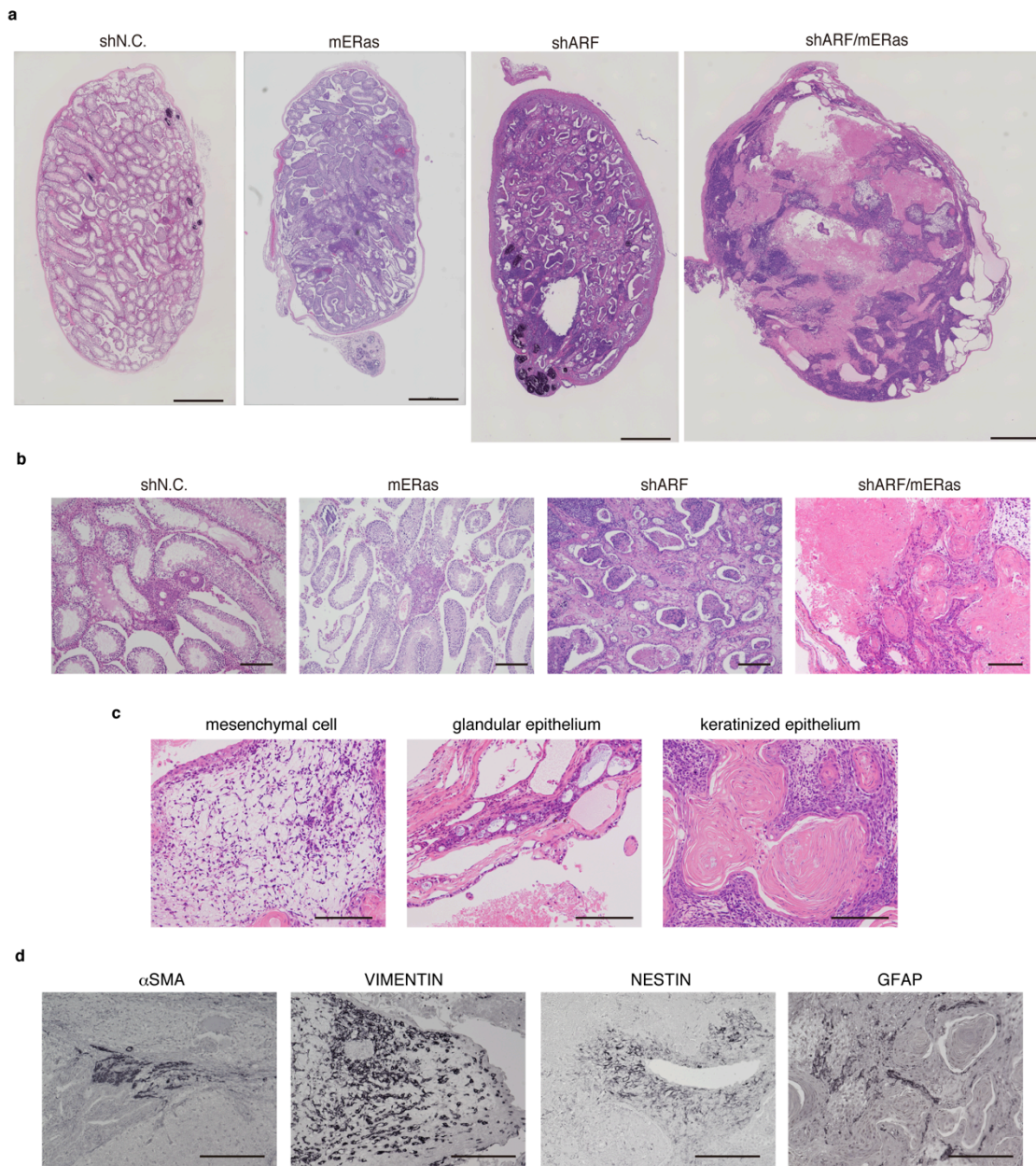
**shARF. a**, Morphology of NMR-iPSCs (clone 24) transduced with mERas and/or shARF. Scale bar, 200  $\mu$ m. **b**, qRT-PCR analysis of *ARF* and *mERas* expression in NMR-iPSCs.  $n = 2$  clones.

Data are represented as mean  $\pm$  SEM. **c**, Western blotting for p21. **d**, The genomic sequence of *ERas* in *ERas* knock-out Ms-ES cell line (EGR-G101). Western blotting for AKT or phosphorylated-AKT expression in the indicated cell lines. Values indicate the relative intensity of AKT expression and phosphorylation. **e**, Hierarchical clustering analysis. #24, NMR-iPSC clone 24; #27, NMR-iPSC clone 27. **f**, Heat map of the selected markers of undifferentiated and differentiated cells. **g**, Selected genes shown in Supplementary Fig. 5e and f. **h**, Soft agar growth assay of NMR-iPSCs expressing mERas and/or shARF. **i**, Number of colonies.  $n = 2$ .

Results are presented as mean  $\pm$  SD for three experimental replicates.  $*P < 0.05$  (one way ANOVA). N.S., not significant. N.C., negative control. Scale bar, 200  $\mu$ m (**a** and **g**).



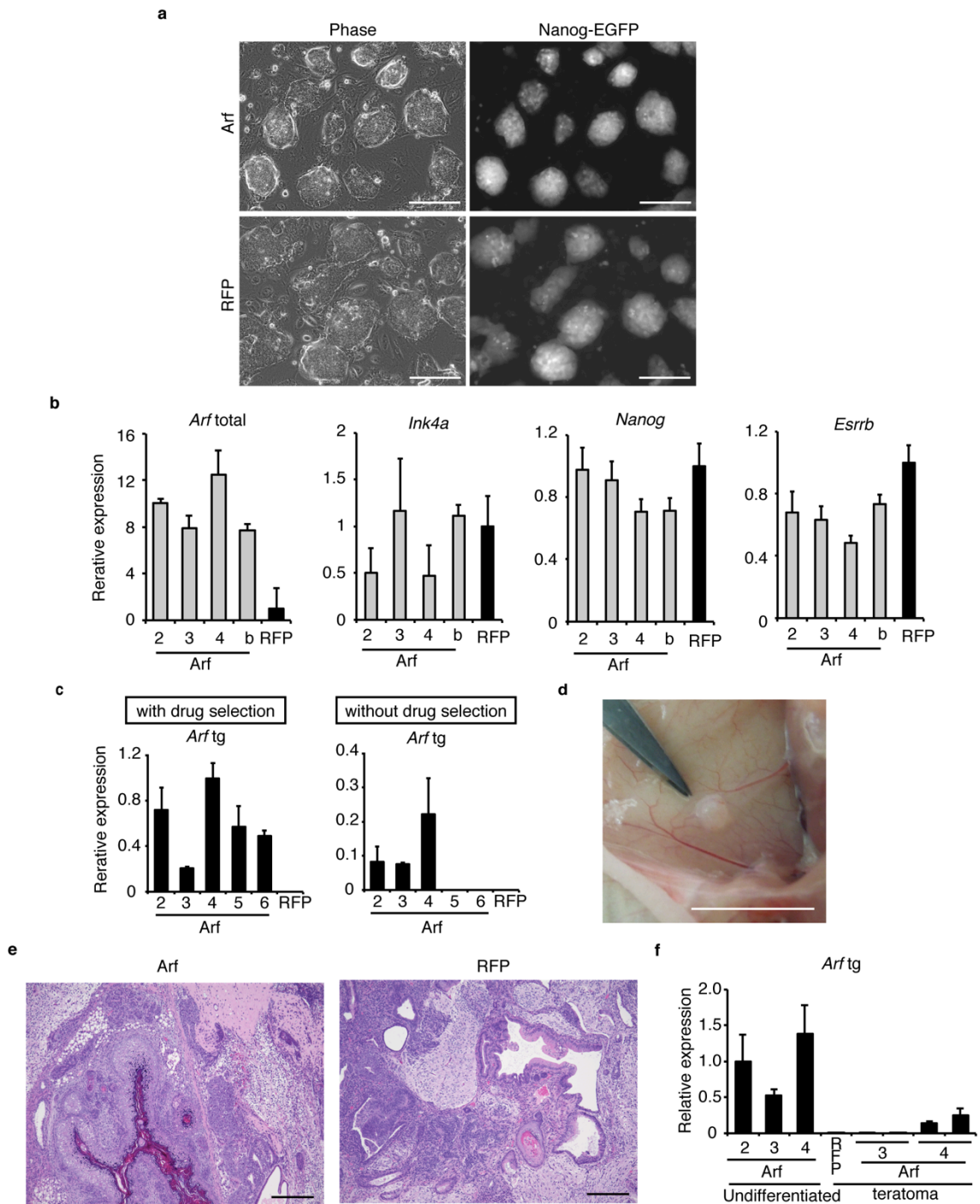
Supplementary Figure 6



**Supplementary Figure 6 | Histopathological analysis of tumours derived from NMR-iPSCs expressing mERas and/or shARF. a, b and c, Haematoxylin and eosin staining. Sections of tumours and testes 10 weeks after transplantation of NMR-iPSCs expressing mERas and/or shARF. Testes and tumours (a). Scale bar, 1 cm. High magnification (b). Scale bar, 200  $\mu$ m. Representative images of teratomas formed by mERas/shARF-NMR-iPSCs (c). Scale bar, 200  $\mu$ m. d, Immunohistochemical analysis of teratomas formed by mERas/shARF-NMR-iPSCs.  $\alpha$ SMA, mesoderm; VIMENTIN, mesoderm and parietal endoderm; NESTIN and GFAP, ectoderm. Scale bar, 200  $\mu$ m.**



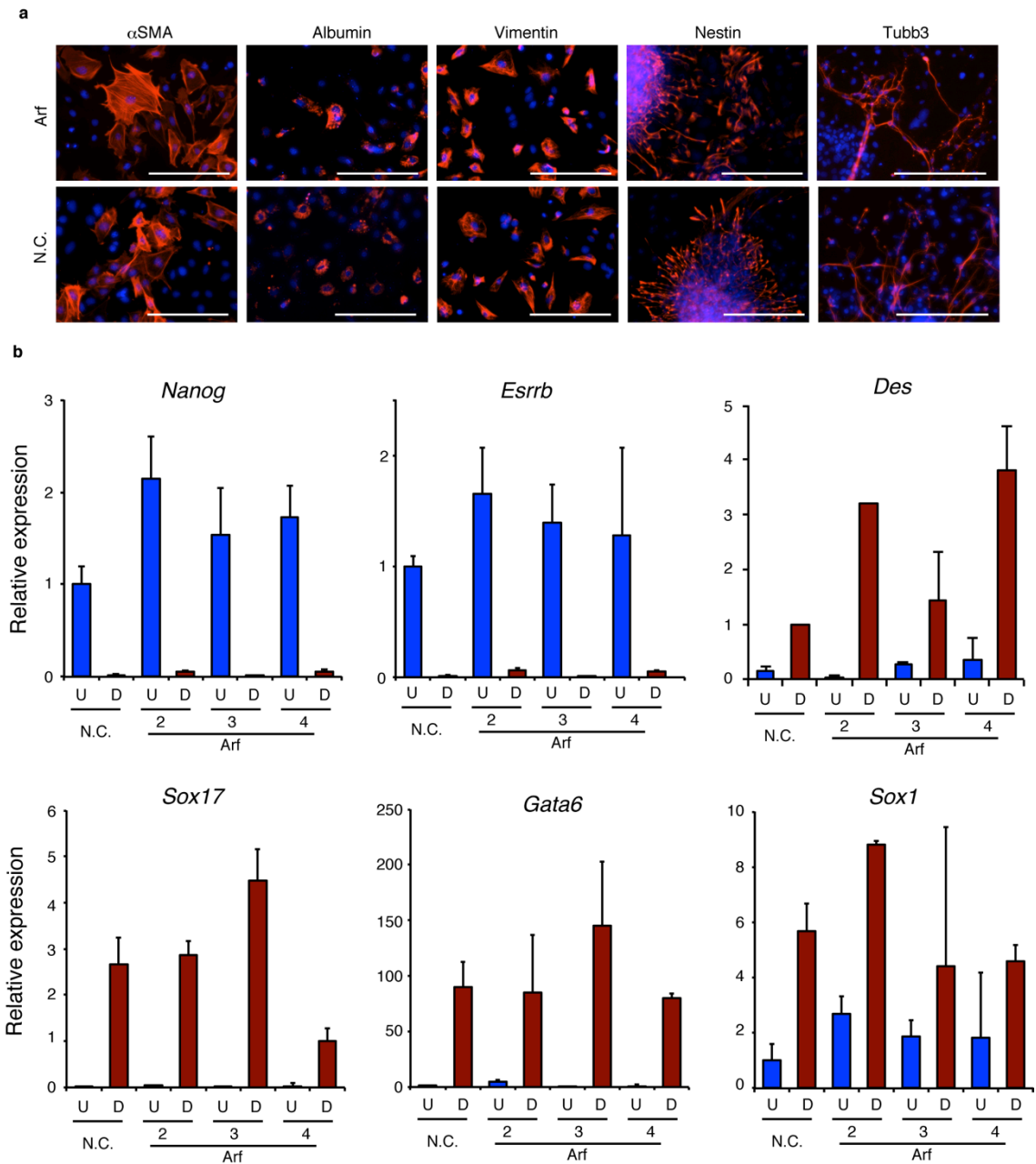
Supplementary Figure 7



**Supplementary Figure 7 | Characterization of Ms-iPSCs expressing Arf.** **a**, Morphology of Ms-iPSCs (20D17) expressing Arf. GFP: Nanog reporter (Nanog-GFP). Scale bar, 200  $\mu$ m. **b**, qRT-PCR analysis of total *Arf* (endogenous and transgenic), *Ink4a*, *Nanog* and *Esrrb* expression in Arf-Ms-iPSCs.  $n = 3$  clones (2, 3 and 4). b, bulk culture. **c**, Expression of the *Arf* transgene (*ARF* tg) in Arf-Ms-iPSC clones with (left panel) or without (right panel) hygromycin selection.

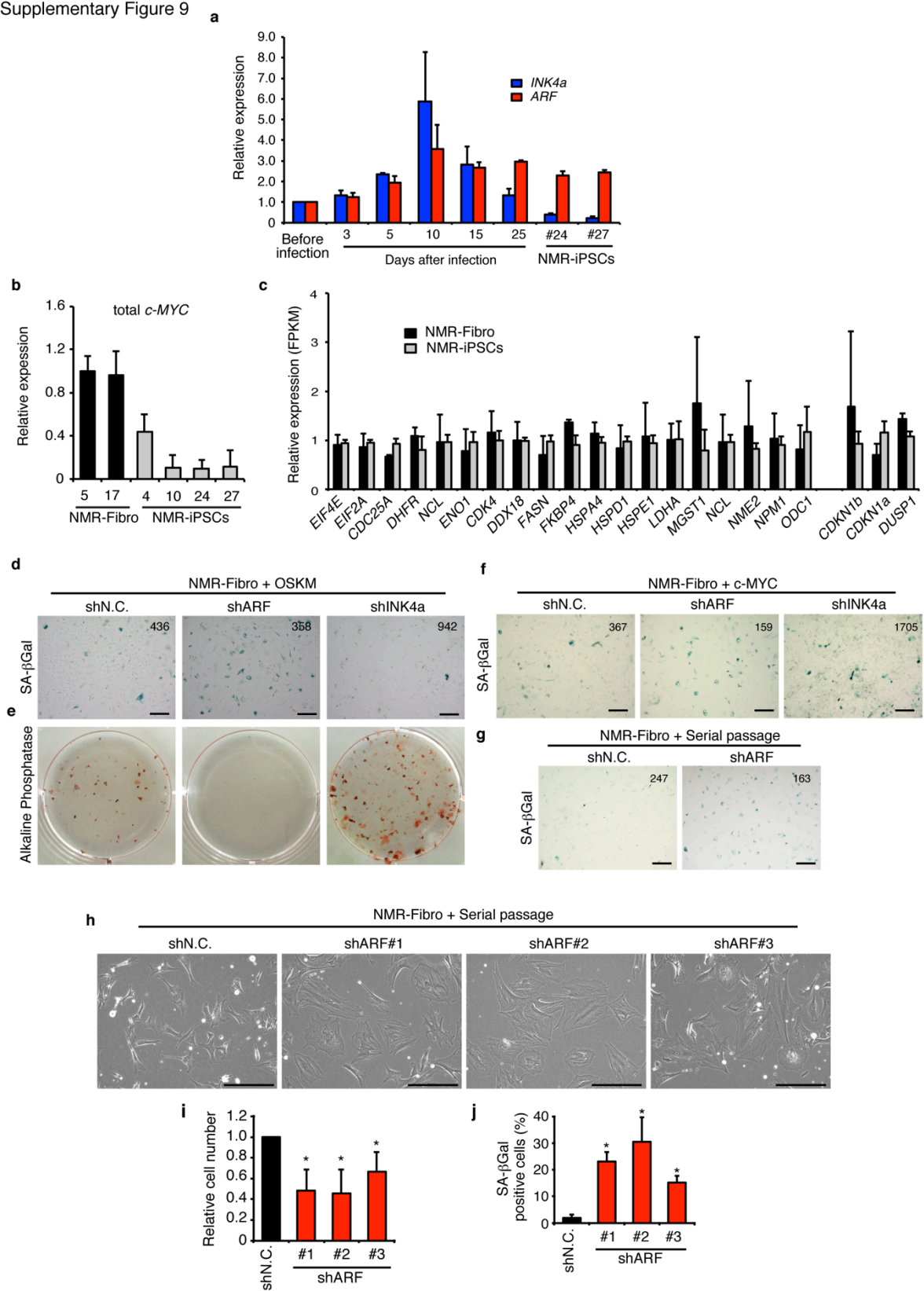
Experimental duplicate. **d**, Small teratomas observed 10 weeks after transplantation of the High-ARF-group clone 3. Scale bar, 1 cm. **e**, Haematoxylin and eosin staining of a teratoma derived from High-ARF-group clone 3 (12 weeks) or control Ms-iPSCs (3 weeks) after transplantation. **f**, qRT-PCR analysis of *Arf* transgene expression in teratomas or undifferentiated iPSCs. Scale bar, 200  $\mu$ m. Data represent the mean  $\pm$  SD (**b**, **c** and **f**).

Supplementary Figure 8



**Supplementary Figure 8 | Differentiation potential of Arf-Ms-iPSCs.** **a**, Immunocytochemical analysis of differentiated cells from the High-Arf-group clone 4. mesoderm ( $\alpha$ SMA), endoderm (Albumin and Vimentin), ectoderm (Nestin and Tubb3). Scale bar, 200  $\mu$ m. **b**, qRT-PCR analysis of differentiated cells from Arf-Ms-iPSCs (clone 2, 3, 4). pluripotent marker genes (*Nanog* and *Esrrb*) and differentiation marker genes (*Des*, *Sox17*, *Gata6* and *Sox1*). U, undifferentiated; D, differentiated. Data represent the mean  $\pm$  SD.

Supplementary Figure 9

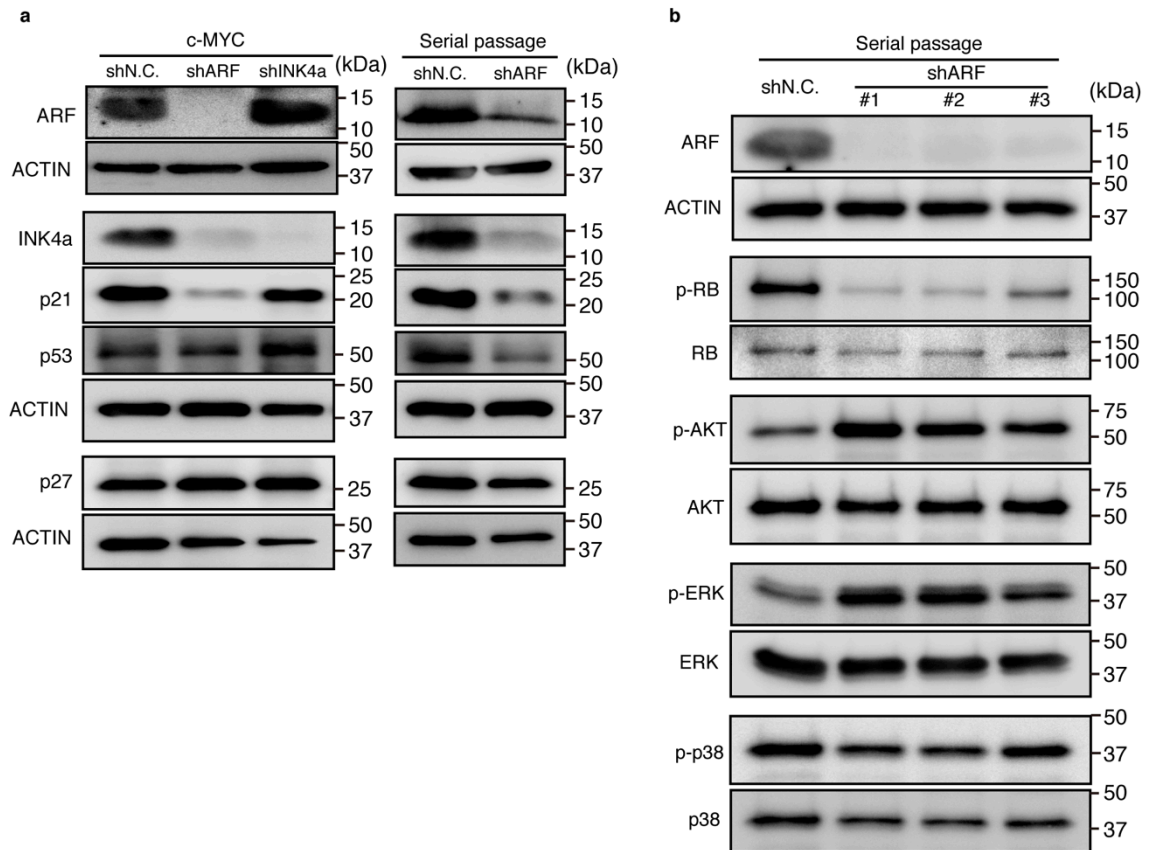


Supplementary Figure 9 | ASIS as a safeguard against reprogramming and oncogenic transformation. **a**, qRT-PCR analysis of the kinetics of *INK4a* and *ARF* expression during

reprogramming of NMR-fibroblasts. Experimental triplicate. **b**, qRT-PCR analysis of the expression of total *c-MYC* in NMR-iPSCs and parental NMR-fibroblasts. The primers amplified endogenous and transgenic *c-Myc*. *n* = 4 clones. **c**, RNA-seq of the levels of c-MYC target genes. Nineteen genes on the left, upregulated genes by c-Myc; three on the right, downregulated genes by c-Myc. Y-axis: expression level (FPKM) of NMR-fibroblasts relative to NMR-iPSCs clone 4. **d**, SA-βGal activity of NMR-fibroblasts expressing shARF and/or shINK4a and OSKM 14 days after infection. **e**, AP activity of NMR-fibroblasts expressing shARF or shINK4a and OSKM 37 days after infection. **f**, SA-βGal activity of NMR-fibroblasts expressing shARF or shINK4a and c-MYC. **g**, Transduction of shARF of fibroblasts with derepressed ARF expression induced by serial passage. SA-βGal activity 14 days after transduction. **h**, **i** and **j**, Reproducibility of the experiment of ASIS in serial passaged NMR fibroblasts using three independent short hairpins RNA against ARF. Cell morphology (**h**). Cell growth (**i**). SA-βGal-positive cells (%) (**j**). Data are represented as mean ± SD. \**P* < 0.05 (*t*-test). Scale bar, 200 μm. The number in the right upper corner indicates Hoechst-positive cells.



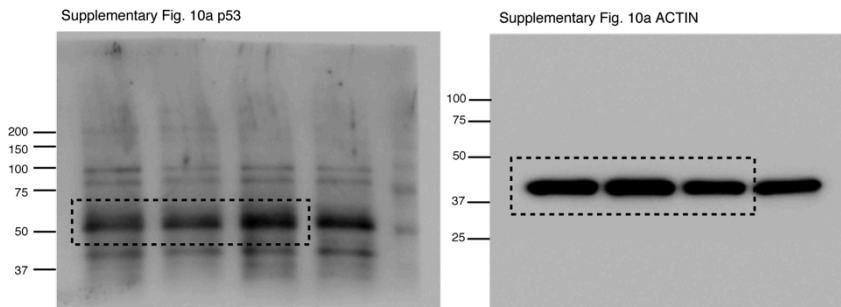
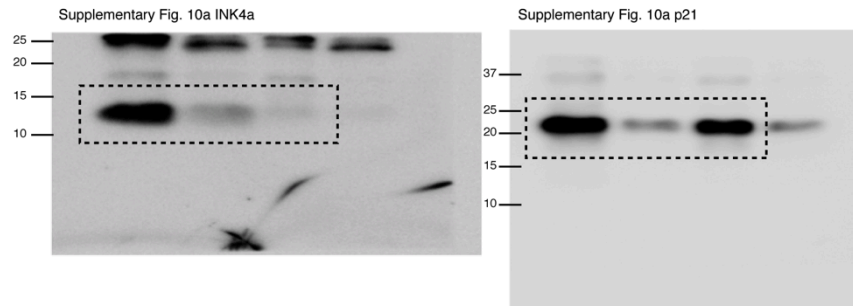
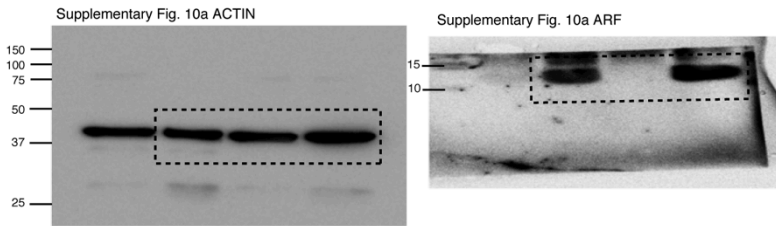
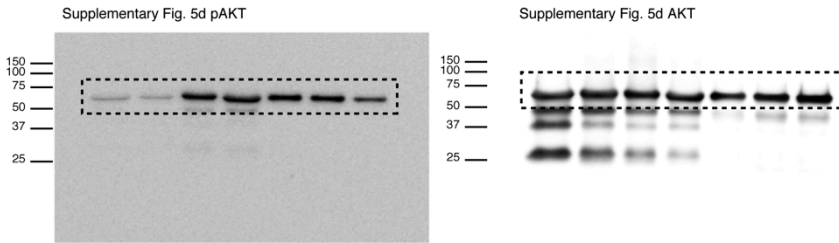
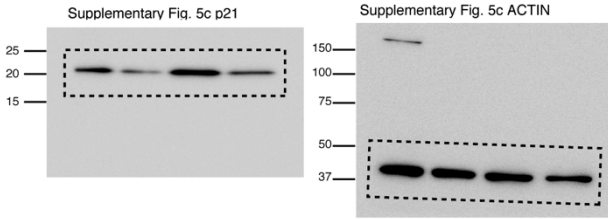
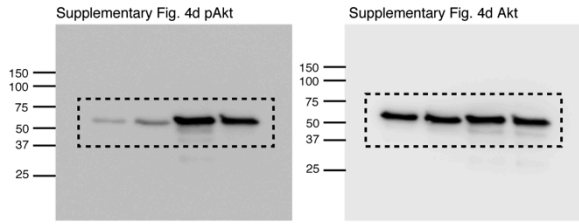
Supplementary Figure 10



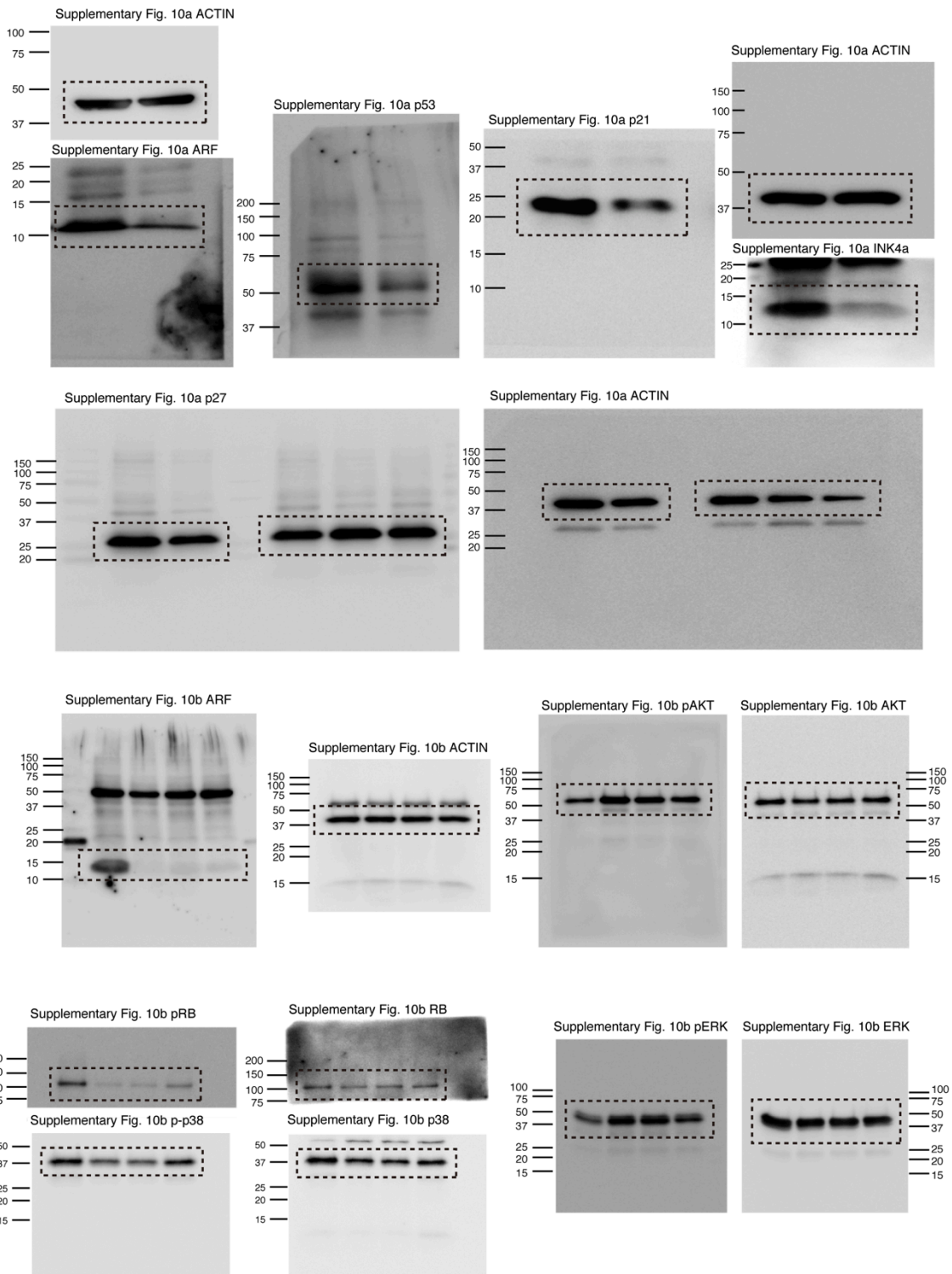
**Supplementary Figure 10 | Expression status of senescence-associated genes in NMR fibroblast undergoing ASIS.**

**a**, Western blotting of ARF, INK4a, p21 and p53 expression in stressed NMR-fibroblasts expressing shARF or shINK4a. **b**, Western blotting of RB, AKT and MAPK (ERK and p38) expression in NMR fibroblast undergoing ASIS.

Supplementary Figure 11



Supplementary Figure 11



Supplementary Figure 11 | Uncropped scans of western blot results displayed in this study

**Supplementary Table 1 | Primers sequences**

|               |                              |   |
|---------------|------------------------------|---|
| NMR-INK4a-F   | GACCCGAAGTGCCTGACCCCT        | qRT-PCR for NMR-INK4a                       |
| NMR-INK4a-R   | CCGCGTCATGCACCGTAGTGTGA      |   |
| NMR-ARF-F     | CCATCGTGTGTCCGGCTTTCGT       | qRT-PCR for NMR-ARF                         |
| NMR-ARF-R     | CTGCTGCCCTCTCCGGTGTCT        |   |
| NMR-INK4b-F   | AACGCCGTCATCGCTTCGGGAG       | qRT-PCR for NMR-INK4b                       |
| NMR-INK4b-R   | TCCTCAGCTAGGTCCACGGGCAA      |   |
| NMR-CDKN1a-F  | ACCTGTCGCTGTCTGCACCCCTTG     | qRT-PCR for NMR-p21                         |
| NMR-CDKN1a-R  | CGTCATGCTGGTCTGCCGCCGTT      |   |
| pMXs-Oct4-F   | GACTGCCGGATCTAGCTAGTT        | qRT-PCR for Oct4 tg                         |
| pMXs-Oct4-R   | GGGGTGAGAAGGCGAAGTCTGA       |   |
| pMXs-Sox2-F   | CATGGCCCAGCACTACCAGA         | qRT-PCR for Sox2 tg                         |
| pMXs-Sox2-R   | CCTTACGCGAAATACGGGCAGA       |   |
| pMXs-Klf4-F   | GCCGACACCACTAAGAACC          | qRT-PCR for Klf4 tg                         |
| pMXs-Klf4-R   | GGAGAAGGACGGGAGCAGAG         |   |
| pMXs-cMyc-F   | GGAATTCGCCCTTACCATTGC        | qRT-PCR for c-Myc tg                        |
| pMXs-cMyc-R   | TGGGAAGCAGCTCGAATTCT         |   |
| NMR-NANOG-F   | AAGTACCTCAGCTGCAGCAGATGC     | RT-PCR for NMR-NANOG                        |
| NMR-NANOG-R   | TTTTCTGCCACCGCTTACATTTCAT    |   |
| NMR-FGF4-F    | CGTGAGCATCTTTGGAGTGCCAGC     | RT-PCR for NMR-FGF4                         |
| NMR-FGF4-R    | CAGCCTGGGAGAAAGTGGGTGAC      |   |
| NMR-TERT-F    | CCTGCTCAAGCTGGTTCACCCGTG     | RT-PCR for NMR-TERT                         |
| NMR-TERT-R    | TCAGTCCAGGATGGTCTTGAAGT      |   |
| ACTB-F        | ACAACGGCTCCGGCATGTCAA        | RT-PCR and qPCR for ACTIN common Ms and NMR |
| ACTB-R        | CATTGTAGAAGGTGTGGTGCCAGA     |   |
| NMR-MEF2c-F   | GTTTAACACAGCCAGTGCCTTC       | RT-PCR for NMR-MEF2c                        |
| NMR-MEF2c-R   | CTCTCTGTCGCTGCCGTCGTA        |   |
| NMR-GATA4-F   | CCCTCCATCCACCGTCTC           | RT-PCR for NMR-GATA4                        |
| NMR-GATA4-R   | ACGCAGTGATTATGTCCCGTGACT     |   |
| NMR-MAP2-F    | GATTCTCTATGCCAAGTCCCT        | RT-PCR for NMR-MAP2                         |
| NMR-MAP2-R    | CTGTTTCTGCCACTTTATCAGGT      |   |
| NMR-MSI1-F    | AGCTCGACTCCAAACAATTGACCC     | RT-PCR for NMR-MSI1                         |
| NMR-MSI1-R    | CATCCACCTTCCGAACTGCT         |   |
| NMR-HAS2-F    | GAAAAGGGTCTGGTGGAGACGGATGAG  | RT-PCR for NMR-HAS2 <sup>14</sup>           |
| NMR-HAS2-R    | TTCAACCTCTCCACAGATGAGGCAGG   |   |
| pALT-p15S-F   | CAGGAAAAGCCCGAACTAACTAC      | qRT-PCR for NMR-pALT <sup>16</sup>          |
| pALT-p16AS-R  | GGTGACAGGGTCAGCGCAAGTTCG     |   |
| NMR-ERAS-CI-F | CACCATGGAGCTGCCACTGCCACCTAGT | NMR-ERAS cloning                            |
| NMR-ERAS-CI-R | TCATGGTCTCCAAGAAGCACT        |   |
| ERAS-com-F    | CACAGAGCAGCCACAGCTACAC       | RT-PCR for Ms and NMR ERAS                  |
| ERAS-com-R    | GGCAAGGTGTGGAGGAAGCCTT       |   |
| NMR-ERAS-F    | TTCCACCTGCTTCTGCCAT          | RT-PCR for NMR-ERAS                         |
| NMR-ERAS-R    | GCTCCCTTCGTGAAACCTCA         |   |
| HRasV12-F     | AAGAGTGCCTGACCATCCAG         | RT-PCR for HRasV12                          |
| HRasV12-R     | TTTGATCTGCTCCCTGACTGGTG      |   |
| Ms-Ink4a-F    | GTGTGCATGACGTGCGGG           | qRT-PCR for Ms-Ink4a                        |
| Ms-Ink4a-R    | GCAGTTGGAATCTGCACCGTAG       |   |
| Ms-Arf-F      | GCTCTGGCTTTCGTGAACATG        | qRT-PCR for Ms-Arf                          |
| Ms-Arf-R      | TCGAATCTGCACCGTAGTTGAG       |   |
| Ms-Ink4b-F    | AGATCCCAACGCCCTGAAC          | qRT-PCR for Ms-Ink4b                        |
| Ms-Ink4b-R    | CCCATCATCATGACCTGGATT        |   |
| Ms-Cdkn1a-F   | TCCCGTGGACAGTGAGCAGTTG       | qRT-PCR for Ms-p21                          |
| Ms-Cdkn1a-R   | CGTCTCCGTGACGAAGTCAAAG       |   |
| Ms-Nanog-F    | TTCTTGCTTACAAGGGTCTGC        | qRT-PCR for Ms-Nanog                        |
| Ms-Nanog-R    | AGAGGAAGGGCGAGGAGA           |   |
| Ms-Esrrb-F    | CTGCCGATTTCCACCTG            | qRT-PCR for Ms-Esrrb                        |
| Ms-Esrrb-R    | TGAGGAACACAAGCTCCCGAT        |   |
| Arf-tg-F      | CCAAGAGCGGGGACATCAAGACA      | qRT-PCR for Arf tg                          |
| Arf-tg-R      | CCACACCAGCCACCCCTT           |   |
| Ms-Des-F      | AGCTCTCCCGTGTCCCTC           | qRT-PCR for Ms-Des                          |
| Ms-Des-R      | CAGCGACCCCAAGCCTCC           |   |
| Ms-Sox17-F    | GTGGACCCGACGGAATTCGAA        | qRT-PCR for Ms-Sox17 <sup>45</sup>          |
| Ms-Sox17-R    | GCAATAGTAGACCGCTGAGCTA       |   |
| Ms-Gata6-F    | ACCTTATGGCGTAGAAATGCTGAGGGTG | qRT-PCR for Ms-Gata6                        |
| Ms-Gata6-R    | CTGAATACTTGAAGTCACTGTTCTCGGG |   |
| Ms-Sox1-F     | TGAACGCCTTCATGGTGTGGTC       | qRT-PCR for Ms-Sox1 <sup>45</sup>           |
| Ms-Sox1-R     | GCGCGCCGGTACTTGTAAAT         |   |
| cMYC-com-F    | TGCTCCACCTCCAGCCTGTACCT      | pRT-PCR for Ms and NMR c-MYC                |
| cMYC-com-R    | CCTCATCCTCTTGTCTCTCTCAG      |   |

**Supplementary Table 2 | shRNA sequences**

| Target gene | Sequence                     |
|-------------|------------------------------|
| NMR-ARF #1  | 5'-GGGCTTTCGTGGTGCAGATCC -3' |
| NMR-ARF #2  | 5'-GCGGGCTTTCGTGGTGCAGAT-3'  |
| NMR-ARF #3  | 5'-GGCCCTCTTGCTGATGCTAGT-3'  |
| NMR-INK4a   | 5'-GGTCCAGGAGGTACGCGAGCT-3'  |