Stem cell function and stress response are controlled by protein synthesis

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Whether protein synthesis and cellular stress response pathways interact to control stem cell function is currently unknown. Here we show that mouse skin stem cells synthesize less protein than their immediate progenitors in vivo, even when forced to proliferate. Our analyses reveal that activation of stress response pathways drives both a global reduction of protein synthesis and altered translational programmes that together promote stem cell functions and tumorigenesis. Mechanistically, we show that inhibition of post-transcriptional cytosine-5 methylation locks tumour–initiating cells in this distinct translational inhibition programme. Paradoxically, this inhibition renders stem cells hypersensitive to cytotoxic stress, as tumour regeneration after treatment with 5-fluorouracil is blocked. Thus, stem cells must revoke translation inhibition pathways to regenerate a tissue or tumour.

Protein synthesis is a fundamental process for all cells, but its precise regulatory roles in development, stem cells and cancer are not well understood. We recently identified post-transcriptional methylation of transfer RNA (tRNA) at cytosine-5 (m5C) by NSUN2 as a novel mechanism to repress global protein synthesis1,2. Loss of Nsun2 causes hypomethylation of tRNAs, allowing endonucleolytic cleavage by angiogenin and accumulation of 5′ tRNA fragments3,4. These fragments repress cap-dependent protein translation4,5.

Correct RNA methylation is essential for development and tissue homeostasis. Loss-of-function mutations in human NSUN2 cause growth retardation and neurodevelopmental defects including microcephaly6,8–10. In mouse, Nsun2-associated microcephaly can be rescued by inhibiting angiogenin-mediated tRNA cleavage1. In adult tissues (testis and skin), NSUN2 is only expressed in a subpopulation of committed progenitors, in which its activity balances self-renewal and differentiation11,12.

Here, we reveal that the interplay between RNA methylation and translation shapes stem cell fate. Using skin as a model, we demonstrate that stem cells have lower protein synthesis than committed cells in both homeostasis and tumorigenesis. Low translation functionally contributes to maintaining stem cells, and is not merely a consequence of quiescence or cell cycle state. By genetically deleting Nsun2 in a tumour mouse model, we find that protein synthesis is globally repressed; however, distinct transcripts escape this repression and establish a translational programme crucial to stimulate stem cell functions. Unexpectedly, the selective alteration of translation is remarkably effective in rendering stem cells sensitive to cytotoxic stress.

Protein synthesis is low in stem cells

In skin, the best-characterized stem cell populations reside in the hair follicle13. Hair follicle stem cells (HFSGs) are periodically activated at the onset of hair growth (anagen), which is followed by phases of regression (catagen) and rest (telogen) (Extended Data Fig. 1a)14,15. HFSGs located in the bulge express the stem cell markers CD34, K19 (also known as KRT19) and LGR5 (Fig. 1a)14,16,17. To visualize HFSGs and their progeny, we genetically labelled K19− and LGR5− expressing bulge stem cells with a tdTomato (tdTom) reporter (Fig. 1a, b and Extended Data Fig. 1a)16,18. To measure global protein synthesis we quantified incorporation of O-propargyl-puromycin (OP-puro) into nascent proteins (Fig. 1b)19. Protein synthesis was uniformly low in the interfollicular epidermis, but highly dynamic in hair follicles throughout the hair cycle (Extended Data Fig. 1b). In telogen, highly translating cells at the follicle base were not stem cells, as they were negative for tdTom (Fig. 1c, d and Extended Data Fig. 1c). In late anagen, OP-puro co-localized with tdTom in committed progenitors located in the hair bulb (Fig. 1e, f and Extended Data Fig. 1d, arrows). The highest translation was displayed above the hair matrix, which contains committed progenitors that divide a finite number of times before differentiating (Fig. 1e, f and Extended Data Fig. 1d, arrowheads)20.

Co-labelling of OP-puro with markers for all hair lineages identified the Henle's and Huxley's layers of the inner root sheath (IRS) as the lineages with highest translation (Fig. 1g–k and Extended Data Fig. 1e, f)21,22. Both IRS layers exclusively contain committed and differentiated cells22.

To quantify protein synthesis fully in distinct epidermal populations, we flow-sorted bulge stem cells (CD34+/ITGA6+), non-bulge cells (CD34−/ITGA6−), and differentiated cells (CD34−/ITGA6−) (Fig. 2a–c)17. To capture epidermal cells giving rise to the highly translating IRS, we enriched for OP-purohigh cells (top 2.5% in rate of translation) (Fig. 2b). The selection for high translation did not perturb the proportion of cell populations found in the epidermis (Extended Data Fig. 2a–d). Quantification of OP-puro incorporation confirmed that protein synthesis was highest in differentiated populations in late anagen (Fig. 2d). Translation in bulge stem cells significantly increased from telogen to anagen (Fig. 2d), suggesting a correlation between translation rate and stem cell activation.

Next, we focused on HFSCs and their progeny and quantified protein translation in tdTom− cells that were sorted into bulge stem cells, non-bulge cells, and differentiating cells (Fig. 2e, f). Translation rates significantly increased in bulge HFSGs from telogen to anagen (Fig. 2e, f). In addition, the average translation rate increased in differentiating cells in late anagen, and was around twofold higher compared to the background cells (tdTom−) (Fig. 2d–f and Extended Data Fig. 2e, f). These results were robust to the specific threshold used to identify cells as highly translating (top 2.5–50%) (Extended Data Fig. 3a–c).
Thus, as stem cells proceed into a fully committed progenitor state, protein translation steadily increases.

Proliferation does not dictate translation

Protein synthesis was highest in growing hair follicles. However, cellular division alone did not explain translation rates as the greatest protein synthesis was found in differentiating but non-dividing (Ki67−) cells (Fig. 2g). Although the percentage of cycling (S/G2/M) cells correlated with increasing translation rates (Extended Data Fig. 3d, e), differentiating (CD34+/ITGA6−) and non-dividing (G1/G0) cells represented the population with the highest translation (Extended Data Fig. 3f, g).

To test directly whether protein synthesis was determined by lineage commitment instead, we measured the translation rate in bulge HFSC and their offspring (tdTom+) along the cell cycle. In late anagen, non-cycling (G1/G0) tdTom+ cells synthesized significantly more protein than their cycling (S/G2/M) counterparts (Fig. 2h and Extended Data Fig. 3h). Thus, increasing translation rates correlated with stem cell commitment and differentiation rather than proliferation (Extended Data Fig. 4p).

Low translation in tumour-initiating cells

To test whether low protein synthesis simply reflected a quiescent state, we investigated translation rates in cancer-initiating cells, which exhibit both high self-renewal and proliferation capacity. We used K5-Sos mice, which constitutively activate RAS in basal epidermal cells and develop well-differentiated tumours resembling human squamous tumours23,24.

Undifferentiated progenitors expressed markers for tumorigenesis and tumour-initiating cells (ITGB1, ITGA6, CD44, CD34, PDPPN)25–29 and exhibited lower protein synthesis than committed progenitors (Fig. 3a, d and Extended Data Fig. 4a–c, f–i). Translation was highest in suprabasal and differentiating committed progenitors (Ki10+), but absent in terminally differentiated, non-tumorigenic cells (Fig. 3a, b). In cancer, elevated translation has been associated with increased proliferation30. However, in our data, high translation was uncoupled from proliferation because both OP-purohigh and OP-purolow cells expressed Ki67 (Fig. 3c), and protein synthesis did not correlate with cycling cells (Fig. 3e).

Thus, similar to normal skin, stem and progenitor cells in tumours produced less protein than their committed progeny.

Low translation maintains tumour stem cells

To test whether low translation is a cause or a consequence of a stem cell state requires the ability to modulate protein synthesis. An excellent system is the genetic deletion of the RNA-methyltransferase NSUN2. NSUN2 modulates global translation by protecting tRNAs from cleavage31. In normal skin, NSUN2 is restricted to distinct hair follicle populations31 that overlap with OP-purohigh cells in early and late anagen (Extended Data Fig. 4k, l). Nsun2 deletion delayed HFSC differentiation in adult31 and developing skin (Extended Data Fig. 4m–o). NSUN2 is upregulated in epithelial tumours and homogenously expressed in mouse and human squamous cell carcinomas (Extended Data Fig. 5a)31,32, and its expression is restricted to highly translating cells in K5-Sos tumours (Fig. 3f).

We deleted Nsun2 in K5-Sos mice, and measured OP-puro incorporation into the tumours of the offspring. As expected, Nsun2 ablation reduced protein synthesis in tumours (Fig. 3g–i and Extended Data Fig. 4d, e). K5-Sos/Nsun2−/− mice developed more tumours that appeared earlier, grew larger, and reduced their life span (Fig. 4a and Extended Data Fig. 5b–d).

Nsun2−/− tumours appeared more proliferative; however, 5-ethyl-2-4-5-6-nyldeoxyuridine (EdU)/5-bromodeoxyuridine (BrdU) pulse-chase experiments revealed that high EdU incorporation reflected an increased undifferentiated population, but not a faster division rate (Fig. 4b, c and Extended Data Fig. 5e, f). Nsun2−/− tumours were poorly differentiated and in a later stage of tumorigenesis, as shown by increased expression of stem cell and tumour progression markers (Fig. 4d–i and Extended Data Fig. 5f–j).
tRNA fragments modulate translation

A likely mechanism for the translational repression in Nsun2-deficient tumours was that 5′ tRNA fragments inhibit protein synthesis. Using RNA-bisulfite sequencing, we confirmed that in tumours, NSUN2-dependent methylation occurred at most tRNAs (65%), but only at a small proportion of messenger RNA exons (2%) and introns (Fig. 5a, b).

Figure 3 | Tumour-initiating cells synthesize less protein than their progeny. a–c, Co-labelling OP-puro (OP-P) with the indicated markers. Arrows indicate marker-positive cells. Boxed areas in the left-hand panels are shown magnified in the panel to the right. d, Flow cytometry for OP-puro incorporation. Percentage of marker-positive cells. Boxed areas in the left-hand panel are shown magnified in the panel to the right. Arrows indicate marker-positive cells. Nuclei are stained with DAPI; dotted line indicates basal membrane. All analyses are in a cell-autonomous manner.

To test for the cell-intrinsic potential to initiate tumours, we injected Nsun2–/– tumour cells subcutaneously into nude mice (Extended Data Fig. 6a). Only Nsun2–/– cancer cells reconstituted the original squamous tumour with high proliferative potential and elevated levels of ITGβ1 and PDPN (Extended Data Fig. 6b–f). Thus, Nsun2 deletion enhances the self-renewal potential of tumour-initiating cells in a cell-autonomous manner.

Furthermore, in human skin cancers, NSUN2 expression was inversely correlated with malignancy when we compared protein expression levels in normal skin and cutaneous cancers of increasing tumour/node/metastasis (TNM) stages (Fig. 4j) and Extended Data Fig. 6g–m).

These results indicate that the reduction of translation rates caused by Nsun2 deletion increased the tumour-initiating population.

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In contrast, hypomethylation of tRNAs directly caused by loss of Nsun2 led to the accumulation of 5′ tRNA fragments that influenced translation in mouse tumours and patient-derived NSUN2-deficient fibroblasts (Extended Data Fig. 8a, b and Supplementary Tables 5, 6a–c). We verified the high quality of our data by testing for triplet periodicity of ribosomal footprints, increased ribosomal density near translational start sites, and correlation between RNA expression levels and translation (Extended Data Fig. 8c–j). The distinct translational programme in Nsun2–/– mouse tumours was not driven by transcriptional alteration, as the changes in protein synthesis caused by Nsun2 removal were decoupled from the corresponding changes in RNA expression levels (Fig. 5e and Extended Data Fig. 8e). The differences in translation were more likely to be caused by accumulated 5′ tRNA fragments than by changes in mRNA methylation, because translation of NSUN2-methylated mRNAs remained unaltered (Extended Data Fig. 7h).

In summary, the undifferentiated cellular phenotype of Nsun2–/– tumours was primarily driven by translational, and not transcriptional, changes.

Translational signatures in NSUN2–/– cells

Accumulation of 5′ tRNA fragments can activate a cap-independent stress-response programme; and stress stimuli can increase ribosomal
density in 5′ untranslated regions (UTRs)\textsuperscript{43,44}. Consistent with such a stress response, 5′ UTRs in NSUN2-deficient cells showed increased ribosome densities (Fig. 5f, g, Extended Data Fig. 8k, l and Supplementary Tables 7–9). The increased ribosome density in 5′ UTRs is probably due to the occurrence of upstream open reading frames (uORFs)\textsuperscript{41,45}. Functionally, uORFs repress translation by sequestering initiation events or facilitating downstream re-initiation and translation\textsuperscript{45–48}, which may explain why protein synthesis of the corresponding coding sequences (CDS) remained unaltered (Fig. 5f, g).

Although the underlying mechanisms are unclear, differential ribosome density in 5′ UTRs should alter the protein production of distinct genes. Indeed, transcripts with increased ribosome density in 5′ UTRs were linked to apoptosis, stress response, cell shape and migration (Fig. 5h–j). In tumours, transcripts with reduced ribosome density in the CDS were related to differentiation (Fig. 5h, i). Thus, the ribosome profiling data correlated well with the phenotypic reduction of epidermal differentiation of Nsun2-deficient tumours; and the cell-intrinsic NSUN2-controlled translational programme(s) related to stress responses and cell motility was conserved between species.

To identify the translational programme that directly depended on RNA methylation, we performed ribosomal profiling after rescuing Nsun2\textsuperscript{+/−} human fibroblasts with the wild-type or enzymatically dead constructs of NSUN2 (Extended Data Fig. 9a–d). Modulators of cell adhesion and motility represented a quarter of translational repressed transcripts that depended on the enzymatic activity of NSUN2 (Extended Data Fig. 9e–g and Supplementary Tables 10a–c). Consequently, motility and adhesion were reduced and differentiation increased in primary human keratinocytes when NSUN2 was repressed or enzymatic-dead versions overexpressed (Extended Data Fig. 9h–m).

Thus, the undifferentiated stem cell state in Nsun2-deficient tumours was primarily driven by differential translation of proteins regulating cell migration, adhesion and stress responses (Extended Data Fig. 10a, b and Supplementary Fig. 1).

Low translation impairs stress responses
To test whether the stress-related programme in Nsun2\textsuperscript{+/−} tumours altered their sensitivity to external stress in vivo, we applied the cytotoxic agent 5-fluorouacil (5FU). 5FU is commonly used to treat squamous cell carcinomas\textsuperscript{49}. While wild-type tumours only showed a mild reduction in growth, 5FU-treatment blocked progression of Nsun2\textsuperscript{+/−} tumours (Fig. 6a and Extended Data Fig. 10c, d). Nsun2\textsuperscript{+/−} tumour cells were unable to re-enter the cell cycle after drug treatment, despite induction of p53 being detectable in all samples (Fig. 6b, c and Extended Data Fig. 10e, f). We obtained similar results using cisplatin (Extended Data Fig. 10g–i). 5FU-treated Nsun2\textsuperscript{+/−} tumour cell layers were reduced, and the remaining ITGA6\textsuperscript{+} basal cells unusually co-expressed the differentiation marker K10 (Fig. 6d, arrows). Thus, Nsun2-deficient tumours fail to activate survival pathways in response to stress.

**Figure 5** | Nsun2 deletion imposes distinct translational programmes. a, b, Percentage of NSUN2-methylated cytosines (dark red). c, Unmethylated RNAs are cleaved and 5′ RNA fragments accumulate. d, Relative frequencies of RNA fragments in tumours. e, No correlation between changes in protein synthesis and RNA expression in tumours. f, g, Significantly (P < 0.05) changed 5′ UTR ribosome densities in tumours (f) and human NSUN2\textsuperscript{+/−} cell lines (excluding significant (P < 0.05) changes in Nsun2\textsuperscript{+/−}) (g) and corresponding CDS. ****P < 0.0001 (two-tailed Student’s t-test). NS, not significant. RPKM, reads Per kilobase of transcript per million mapped reads. h, i, Frequency of ribosomal density values (P < 0.05 and abs(log(FC) > 0; FC, fold change) (h) and Gene Ontology (GO) categories in tumours. KO, knockout; WT, wild type. j, GO categories of significant (P < 0.05) changed 5′ UTRs in NSUN2\textsuperscript{+/−} lines versus NSUN2\textsuperscript{+/−}. Dev., development; diff., differentiation; resp., response. Data are average of 4 replicates per condition in human (g, j) and mouse (a–d), or 3 in e, f, h, i.

**Figure 6** | Nsun2 deletion sensitizes tumour-initiating cells to cytotoxic stress. a–c, Tumour size (mean ± standard error of the mean (s.e.m.)) (a) and Ki67 detection (b, c) in control (Ctrl) or 5FU-treated mice. d, K10 and ITGA6 detection in treated Nsun2\textsuperscript{+/−} tumours. Arrows indicate K10\textsuperscript{+}/ITGA6\textsuperscript{+}-positive cells. e, Tumour size in mice treated with 5FU and/or angiogenin inhibitor (AI) (horizontal line, mean). f, Quantification of tumour-initiating cells in tumours shown in e (mean ± s.d.). *P < 0.05, **P < 0.01 (two-tailed Student’s t-test). n = mice. Scale bars, 100 μm. Source Data for this figure is available in the online version of the paper.
Finally, we asked whether the increased sensitivity to 5FU depended on angiogenin-mediated cleavage of non-methylated tRNAs. We rescued tRNA cleavage by administering the angiogenin inhibitor N65828. (refs 1, 50). The high toxicity of this drug combination only allowed treatment for up to 7 days. Nevertheless, Nsun2−/− tumours failed to regress, and the survival of undifferentiated tumour-initiating cells (CD34+/ITGA6high) significantly increased when they were exposed to both drugs (Fig. 6c, f), indicating that tRNA fragments reduce the survival of Nsun2−/− tumour-initiating cells.

In conclusion, combining cytosine-5 RNA methylation inhibitors with conventional chemotherapeutic agents may provide an effective anti-cancer strategy for solid tumours (Extended Data Fig. 10).

Discussion

Similar to the haematopoietic system15, epidermal stem cells produce less protein than their immediate progenitors, and forced entry into the cell cycle is not sufficient to reverse this translation repression. Instead, global protein synthesis in normal and tumour cells is determined by lineage commitment, but not by proliferation.

We identify RNA methylation as an important pathway to modulate global protein synthesis and cell fate. Both protein synthesis and NSUN2 expression are low in epidermal stem cells, but increase upon commitment to differentiate. NSUN2-mediated methylation protects tRNA from cleavage into non-coding 5′ tRNA fragments, thereby promoting protein translation and differentiation1. External stress stimuli inhibit NSUN2 activity4, permitting cleavage into 5′ tRNA fragments, which then decrease protein synthesis in human cells4. Inhibition of post-transcriptional methylation in squamous tumours promotes stem cell function and tumorigenesis. However, re-activation of cytosine-5 RNA methylation pathways is required to exit the specific translation inhibition programme after cytotoxic stress. Thus, activation of RNA methylation or inhibition of tRNA cleavage is essential for cell survival of tumour-initiating cells in response to cytotoxic stress (see Supplementary Discussion).

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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

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**Author Contributions** M.F., S.B. and R.B. designed experiments and performed data analysis. S.B., R.B., M.P., S.H., A.S., H.T., R.C.-G. and N.G. performed experiments. P.L., J.A. and S.D. performed bioinformatics analysis. M.F., S.B. and R.B. wrote the manuscript.

**Author Information** Mouse next-generation sequencing data have been deposited in the Gene Expression Omnibus under accession number GSE72067. Human data have been deposited in dbGAP under accession number phs000645.v2.p1. 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 M.F. (mf364@cam.ac.uk).
METHODS

Transgenic mice. Rosa-CAG-LSL-tdTomato (ref. 51), K19-CreER (ref. 52) and Lgr5-
CreERT2 (ref. 53), Nsun2+/−/− (or homozygous Nsun2+G501D+D193Y)11, and K5-Sox-F
(in a w2a/w2a background) mutant mice have been described previously. Balb/C
athymic nude mice purchased from Charles River were used in transplantation
experiments. All mice were housed in the Wellcome Trust—Medical Research
Council Cambridge Stem Cell Institute Animal Unit. All mouse husbandry and
experiments were carried out according to the local ethics committee under the
terms of a UK Home Office licence 488-conjugated secondary antibodies (Thermo
Fisher Scientific) were added at a dilution of 1:1,000 for 1 h at room temperature.
Apoptotic cells were visualized staining sections with DeadEnd Fluorometric
Detection Kit (Thermo Fisher Scientific) and 5 μM of Alexa Fluor 488 or Alexa
Fluor 647 conjugated to azide (Thermo Fisher Scientific). After the 30-min
reaction, the cells were washed twice in PBS with 3% fetal bovine serum and 0.1%
saponin and then resuspended in PBS. When indicated cells were further stained
for cell surface markers and DAPI as described later. Immunofluorescence staining
to visualize protein synthesis together with antibody staining in skin or tumour
paraffin embedded or frozen sections; frozen sections were first fixed with 1%
parafomaldehyde in PBS for 15 min on ice. Next samples were washed in PBS, and then permeabilized in PBS
supplemented with 3% fetal bovine serum (Sigma-Aldrich) and 0.1% saponin
(Sigma-Aldrich) for 5 min at room temperature. To conjugate OP-puro to a fluo-
rochrome, an azide-alkyne cycloaddition was performed using the Click-IT Cell
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parafomaldehyde in PBS for 15 and paraffin sections were first de-waxed and progressive rehydration sections were then blocked and stained with primary
antibodies overnight at 4 °C. Next, the sections were washed and stained with
secondary antibodies for 1 h at room temperature. After washes sections were
stained using the Click-IT Cell Reaction Buffer Kit with Alexa Fluor-647 or -488
azide (Thermo Fisher Scientific) as described earlier.

Quantification of protein synthesis rates. Protein synthesis rates in specific
cell populations were calculated by normalizing the mean of OP-puro signal of
each population of interest to the signal of the whole epidermal or tumour cell
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secondary antibodies for 1 h at room temperature. After washes sections were
stained using the Click-IT Cell Reaction Buffer Kit with Alexa Fluor-647 or -488
azide (Thermo Fisher Scientific) as described earlier.

Quantification of protein synthesis rates. Protein synthesis rates in specific
cell populations were calculated by normalizing the mean of OP-puro signal of
each population of interest to the signal of the whole epidermal or tumour cell
preparation, using the following formula: mean OP-puro signal of each population of interest/mean
OP-puro signal of whole epidermal or tumour cell population. To visualize protein synthesis together with antibody staining in skin or tumour
paraffin embedded or frozen sections; frozen sections were first fixed with 1%
parafomaldehyde in PBS for 15 and paraffin sections were first de-waxed and progressive rehydration sections were then blocked and stained with primary
antibodies overnight at 4 °C. Next, the sections were washed and stained with
secondary antibodies for 1 h at room temperature. After washes sections were
stained using the Click-IT Cell Reaction Buffer Kit with Alexa Fluor-647 or -488
azide (Thermo Fisher Scientific) as described earlier.
of the mice throughout the length of the experiment (approximately 6–8 weeks). To monitor tumour occurrence and growth mice were weighed, the number of all tumours formed all over the body were counted and the growth of each tumour monitored every other day from P16 (the earliest time at which K5-Sos-F mice start developing papillomas). Papillomas in the tail tended to fuse into one covering the whole tail, and therefore were counted as one tumour from the beginning of the experiments. Other tumours also developed in ears, mouth, back skin or feed. The growth of each tumour was monitored by measuring the diameter of the widest area of the tumour. The decision as to when the tumour size cut-off was reached (>1 mm) was made to allow discriminating size modifications >0.1 mm. If animals have to be treated with drugs, experiments started also at the third week of age. The end point of the experiments was determined by health deterioration and casualties or by the length of the treatments when mice were under a treatment regime. K5-Sos+/Nsun2+/− (referred as K5-Sos/ Nsun2+/-) survived longer than K5-Sos+/Nsun2+/+ (K5-Sos/Nsun2+/-) and K5-Sos+/Nsun2+/- (K5-Sos/Nsun2+/-). All mouse tumour experiments were carried out according to the local ethics committee under the terms of a UK Home Office license PPL/80/2231 and PPL/80/2619. Following these regulations the mean diameter of a tumour should not normally exceed 1.4 cm (PPL/80/2619, 19b/7). While K5-Sos+/Nsun2−/− and K5-Sos+/Nsun2+/- had to be killed before mice reached 2 months of age due to the size and aspect of the tumours and weight loss or general health deterioration due to excessive tumour burden, K5-Sos+/Nsun2+/- only reached the deterioration state later than 2 months of age. For the analysis in Fig. 4a we measured the percentage of mice with tumours for each indicated day. The average number of tumours in each mouse genotype is shown in Extended Data Fig. 5c. Note that data points are shorter for K5-Sos+/Nsun2−/− and K5-Sos+/Nsun2+−/− as mice survival was shorter. The diameter of the tumours was normalized to the size of each mouse (body weight) in Extended Data Fig. 5b to eliminate genotype variance because K5-Sos+/Nsun2−/− mice are significantly smaller than K5-Sos+/Nsun2−/−.

Cutaneous tumours in transgenic K5-Sos+/Nsun2+−/− and K5-Sos+/Nsun2+−/− mice were topically treated with 5-Fluorouracil (5FU) (Efudix 5% Fluorouracil Cream, Meda Pharmaceuticals) every second day for 2 weeks. 5FU inhibits thymidylate synthetase leading to the upregulation of p53 and cell death18. Tumours were also treated with 5FU in combination with an angiogenin inhibitor (AI; N65828, NCI, US)19,20 administered by i.p. injections at 2 mg kg−1 every alternative day to 5FU treatment. Owing to the high toxicity of the drug combination, we were only able to simultaneously treat with 5FU and AI for a short period of time (up to 7 days). Caspatin (CDDP) (Sigma) was dissolved in PBS and injected intra-peritoneally at 14 mg kg−1 every other day. All treatments started when the first cutaneous lesions appeared and the end point was indicated by the length of the treatment, after which all mice were killed. Control mice were administered PBS.

BrDU and EdU labelling. To measure proliferation, K5-Sos mice were injected i.p. with 50 mg of BrdU per kg of body weight, 23 h later with 20 mg kg−1 of EdU. One hour later mice were killed and tumour samples were processed for histology as described previously. BrdU was visualized as described previously21. EdU was stained with Click-IT Edu Alexa Fluor 488 Imaging Kit (Thermo Fisher Scientific). Images of random areas of the slide were collected using a confocal microscope (Leica SP5). Nuclei of BrdU- and EdU-positive cells were quantified using Velocity software (PerkinElmer).

Tumour assay. Epidermal keratinocytes from K5-Sos/Nsun2+−/− and K5-Sos+/Nsun2−/− cutaneous tumours were isolated as described earlier. GFP-expressing dermal fibroblasts were isolated from healthy skin of newborn mice. For this, skin was first incubated in a 1:1 solution of 5% dispase (BD Biosciences) at 37 °C for 1 h. The epidermis was then peeled from the dermis.

Migration assays. For migration analysis in Boyden chambers, human primary keratinocytes were transfected with siRNAs as described earlier. Transfections were carried twice every 48 h and migration assay was performed 24 h after the second transfection. Cells were treated with mitomycin C for 2 h to arrest cell cycle progression. After mitomycin C treatment cells were trypsinized and counted and seeded on Boyden chambers (transwell inserts of 8 μm, 24-well plates, BD Biosciences). 8 × 104 cells were seeded with KBM growing medium (Lonza) without human recombinant (hr)EGF. Media containing 10 ng ml−1 of hrEGF (Lonza) was placed under the transwell inserts as a chemoattractant to attract cells. Media without chemoattractant was placed under the transwell inserts in control experiments. Cells were allowed to migrate for 6 or 12 h, after which the inserts were washed once with PBS, fixed with 4% PFA for 10 min, and cells were stained with DAPI. Cells from the upper side of the membrane were scratched off with a cotton bud and washed off with PBS several times. Cells on the bottom side of the membrane were imaged with a colony scan microscope. Cells were then quantified with the software CellProfiler.

For motility analysis of human keratinocytes, 104 cells were seeded in 24-well ImageLock plates (Essen Instruments) in growing medium and kept for 26 h at 37 °C in 5% CO2. Cell mobility was recorded with an automated IncuCyte microscope (Essen Instruments). Images were collected at 15-min intervals. Two-dimensional migration tracks were generated by manually tracing the nucleus of each cell. Migrated distance was obtained by measuring the linear distance travelled between the first and last position (after 26 h) of each tracked cell.

rRNA sequencing library preparation. Small RNA libraries were generated from snap-frozen skin papillomas from 4-week-old mice. Four independent biological replicates were used. For RNA library generation we followed the protocols described previously22. Briefly total RNA was extracted using Trizol reagents (Thermo Fisher Scientific) and treated with DNase (Turbo DNase, Thermo Fisher Scientific). Ribosomal RNA was removed with Ribo-zero (Epizentrum, Illumina). The remaining RNA fraction was size-selected using MirVana Isolation Kit (Thermo Fisher Scientific). Using MirVana RNA purification columns with two sequential filtration steps with different ethanol concentrations, an RNA fraction highly enriched in RNA species ≤200 nucleotides was obtained. The...
small RNA fraction (approximately 200 ng) was first treated with 0.1 M Tris-HCl pH 9.0 and 1 mM EDTA for 30 min at 37 °C to de-aminoacylate mature tRNAs and later T4-PNK (NEB) treated to ensure phosphorylated 5’ ends and 3’ OH ends to proceed with RNA adaptor ligation and library preparation. tRNA libraries were generated using TruSeq Small RNA Preparation Kit (Illumina). Briefly, 3’ dephosphorylated and 5’ phosphorylated adapters suitable for Illumina RNA sequencing were ligated to the small RNA fraction. RNA was reverse-transcribed at 37 °C for 1 h (SuperScript III cDNA synthesis kit, Thermo Fisher Scientific), followed by rDNA depletion by subtractive hybridization (as indicated in the original protocol) using oligonucleotides listed previously and following the protocol recommendations. Recovered complementary DNAs were PCR amplified with no more than 12 PCR cycles. All samples were multiplexed and sequenced in HiSeq platform (Illumina).

Preparation of mRNA-seq libraries. mRNA-seq libraries were generated from 4-week-old K5-Sos/Nsun2+/− and K5-Sos/Nsun2+− mice, from mouse healthy back skin from 3.5–4-week-old Nsun2+− or Nsun2+− mice (without tumours) and from human dermal fibroblasts (NSUN2+/+, NSUN2+− and NSUN2−+) growing in culture and infected when indicated. At least four replicates were performed for each sample. All samples were multiplexed and sequenced using Illumina next-generation sequencing kits (Illumina). Total RNA was extracted using Trizol (Thermo Fisher Scientific) from cells in culture or snap-frozen tissues. Total RNA was DNase (Turbo DNase, Thermo Fisher Scientific) and ribo-zero (Epicentre, Illumina) treated. RNA-depleted RNA was used to generate mRNA-seq libraries using NEXTFlex Directional RNA-seq Kit V2 (Illumina). All samples were multiplexed and sequenced in HiSeq platform (Illumina).

Next-generation sequence data analyses. For all data analyses, FastQC was used for the initial assessment of the quality and basic processing of the reads (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequencing adapters were trimmed from the 5’ and 3’ ends of the reads using cutadapt (v.1.4.2; https://pypi.org/pyppi/cutadapt/1.4.2).

RNA BS-seq analysis. To determine RNA methylation levels in mouse K5-Sos tumours, two complementary protocols for the analysis of BS-seq data were used. Alignment to the genome. BS-seq reads were aligned to mouse reference genome (GRCm38/mm10) with Bismark (http://www.bioinformatics.babraham.ac.uk/projects/bismark; v.0.13.1; options: ‘-directional −n 0 −10’). Methylation levels for all cytosines with at least coverage of ≥5 reads (5’ coverage) in both K5-Sos/Nsun2+/− and K5-Sos/Nsun2−− tumour samples were inferred with Bismark ‘methylation_extractor’. Cytosine positions displaying a difference in RNA methylation of at least 10% between K5-Sos/Nsun2+/− and K5-Sos/Nsun2−− tumour samples were extracted based on the ENSEMBL (GRCm38, Release 74; http://www.ensembl.org/info/data/ftp/) transcript annotations and tRNA gene predictions in the mouse (GRCm38/mm10) reference genome obtained from GRNAdb (http://lowelab.ucsc.edu/GRNAdb).

Alignment to representative transcripts. Sequences for ENSEMBL transcripts and tRNAs were extracted in FASTA format. All transcript isoforms were considered, and in addition the longest gene at full length including introns was retained as a representative sequence to identify RNA methylation sites in introns. Cs were converted to Ts in the reference transcript sequences, and in the processed BS-seq reads. Alignment of converted BS-seq reads against converted transcript sequences were performed using bowtie (v.1.1.1; http://bowtie-bio.sourceforge.net; options ‘-m 500 –v 2 –a –best –strata’). Following alignments, the reads that aligned in the sense direction were obtained, and the original transcript sequences and reads were used to compile RNA methylation (C(+)/T(+)) levels considering only cytosines with at least 5’ coverage. Heatmaps displaying either C+(+) or T+(+) in the aligned reads at each cytosine position were generated using custom PERL scripts and matrix2png (http://www.chibi.ubc.ca/matrix2png/) for visualization. Cytosine positions on the heatmaps were reported relative to the annotated transcriptional start sites of the transcripts.

tRNA-seq data analysis. The abundance of tRNA fragments was determined according to a previously published protocol. Adaptor-trimmed tRNA-seq reads (>20 nucleotides and <200 nucleotides in length) were mapped to the mouse reference genome (GRCm38/mm10) using bowtie (v.1.1.1; http://bowtie-bio.sourceforge.net; options ‘-m 1 –v 2 –a –best –strata’) considering only reads that aligned to the sense strand. In order to account for the polymerization of CCA 3’ ends onto mature tRNAs, the remaining unmapped reads were trimmed of CCA(CCA) ends and realigned using the same options. Annotations were conducted based on tRNA genes predicted for the mouse reference genome (GRCm38/mm10) and downloaded from GRNAdb (http://lowelab.ucsc.edu/GRNAdb). Reads that exceeded the annotated tRNA gene start or end by more than 10% were discarded. All distinct reads, which were shorter than 90% of the annotated tRNA gene length, were considered as tRNA fragments. Counts per fragments were normalized, and the differential abundances of fragments processed from the 5’ or 3’ half of the tRNAs were statistically evaluated using the R/Bioconductor DESeq2 package (https://bioconductor.org/packages/release/biocon/DESeq2/html). tRNA fragment abundances are given by log2(DESeq-normalized counts).

mRNA-seq and Ribo-seq data analyses. Ribosome profiling data was processed following established protocols. The first 5’ base of the adaptor-trimmed Ribo-seq reads was removed, as this is usually an artefact of reverse transcription.
For removing abundant contamination from digested RNA present in the libraries, the reads were aligned to a collection of rRNA sequences obtained from GenBank and UCSC using bowtie (options: ‘-n 2 –seedlen = 23’). Reads aligning to rRNA were discarded, with the average rRNA contamination per sample being around 60%. Only reads with at least 24 nucleotides and less than 30 nucleotides length were retained in accordance with the observed length distribution of ribosome footprints.

Both the Ribo-seq and mRNA-seq reads were aligned to the human (GRCh37/ hg19) and to the mouse (GRCm38/mm10) reference genomes using TopHat2 (v2.1.2; options: ‘--read-mismatch 1(2)−max-multihits 1−GTF’ guided by ENSEMBL gene models (release 76), allowing for two mismatches per read for human and one mismatch per read for mouse, and unique alignments only.

To determine mRNA abundance, mRNA-seq read counts for the full transcript were calculated using htsseq-count (http://www.huber.embl.de/HTSeq/doc/overview.html), data sets were normalized, and the statistical significance of differential expression was evaluated by using the R/Bioconductor DESeq2 package (https://bioconductor.org/packages/release/bioc/html/DESeq2.html).

To evaluate differences in translation, the following additional Ribo-seq data analyses and normalizations were performed.

**Alignment to representative regions.** Coding sequences (CDS) and 5′ UTRs were downloaded from ENSEMBL including ‘protein_coding’ and ‘nonsense-mediated decay’ types of transcripts. Intron sequences were excluded. Ribo-seq reads, which uniquely aligned to the genome in the initial alignment step using TopHat2, were aligned to 5′ UTR or CDS sequences using bowtie (options: ‘-m 1000 –v1’), allowing for multiple mappings to overlapping regions of the same gene.

**Statistical analysis of differential ribosome footprint densities and normalization.** In concordance with other studies performed in yeast or mammalian cells, we observed a characteristic 5′ ‘ramp’ of ribosome footprints at the translation start site of the CDS for our samples. It has been suggested that these excess footprints are a result of cycloheximide-inflicted accumulation of ribosomes. To prevent any artefactual bias in our analysis, we followed the instructions for normalization described previously. Read counts were extracted that aligned to either (1) all full-length CDS (see Supplementary Tables 6–10) or (2) all CDS sequences without the initial 150 nucleotides (50 codons) corresponding to the ribosomal ramp (see Supplementary Table 5 and Fig. 5e). For both data sets, statistical tests were performed with the R/Bioconductor DESeq2 package. The two sets of DESeq scaling factors were subsequently used for normalization of data sets. The DESeq-normalized counts for all regions were divided by their length in kb to define ribosome footprint densities.

**Analysis of ribosome footprint densities at 5′ UTRs.** Reads that mapped uniquely to the genome by using TopHat2 were mapped to the 5′ UTR sequences with bowtie (options: ‘-m 1000 –v1’). Differences in ribosome footprint densities on the full 5′ UTRs were evaluated by using the DESeq scaling factors obtained from the analysis of CDS (Supplementary Tables 7–9) for normalization, and DESeq to perform statistical tests for differences. DESeq-normalized counts for 5′ UTRs were divided by their length in kb to define ribosome footprint densities.

**Analysis of triplet periodicity.** Footprints of length 28 nucleotides were extracted, since they report with high precision on the position of the ribosome. The frequencies of the 5′ starts of the footprints, which were aligned close to the annotated translation initiation sites, were aggregated for all genes.

**Heatmap analysis.** For the heatmap analysis in Extended Data Fig. 8d, we specifically selected the 43,625 representative and well-annotated protein-coding transcripts from GENCODE that overlap ENSEMBL transcript structures (‘ensembl_havana’). The positions of the start codons were obtained from the ENSEMBL Gene sets’ gtf file (http://www.ensembl.org/info/data/htp/index.html). Heatmaps of ribosome footprint densities (RPKM)s were generated for regions ±1,500 nucleotides around the start codon by using ngplot (https://github.com/shenlab-sinai/ngplot). If not indicated otherwise, Gene Ontology categories represent GOTERM_BP_FAT in DAVID (http://david.ncicrf.gov).

**Protein extraction and western blot analysis.** To extract proteins from squamous tumours, samples were snap frozen in liquid nitrogen, transferred to lysis buffer (1% NP-40, 200 mM NaCl, 25 mM Tris–HCl, pH 8, 1 mM DTT) including protease inhibitor cocktail (Roche) and homogenized and cleared by centrifugation at 13,000 r.p.m. To extract proteins from cells in culture, the same lysis buffer was added to the plate and scratched the cells from the plate surface, left lysing for 20 min in ice and cleared by centrifugation. Total protein quantification was performed using BCA Protein Assay (Thermo fisher). Equal amounts of protein were run in polyacrylamide gels. Western blotting was performed as described previously. The following primary antibodies were used for western blot analyses: anti-PSAT (Protein Tech Group, 10501–1-AP), anti-THBS1 (Santa Cruz, sc-65612), anti-SESN2 (Protein Tech Group, 10795–1-AP), anti-calreticulin (Abcam, ab29070), anti-INHBA (Sigma-Aldrich, SAB140893), anti-NSUN2 (Arviva Systems Biology, ARP48811_P050), anti-K19 (Abcam, ab52652), anti-CD44 (IM7, Biolegend, 103004), anti-BCL10 (H197, Santa Cruz, sc-5611), anti-semaphorin 3A (SEMA3A) (Abcam, ab23393), anti-PSMD11 (Abcam, ab66646), anti-SHPIK1 (Cell Signaling, 3297), anti-APTX (Abcam, ab31841), anti-Slug (Cell Signaling, 9585P), anti-Snail (Abcam, ab180714), anti-SOD2 (Abcam, ab13534), anti-CLSPN (Bethyl Laboratories, A300–266A), anti-ZAG (Sigma, HPA017205), anti-CHAF1B or CAF1 p60 (Abcam, ab15246) was used as a loading control. Band intensity was quantified with Image J software.

**Statistical methods.** Group data are always represented by mean and s.d., unless otherwise indicated in figure legends. To test statistical significance between samples, unpaired two-tailed Student’s t-tests were used. To test for significance of populations (that is, stem cells versus differentiated cell populations) within one sample (mouse) we used the paired Student’s t-test. To analyse the differences among group means we used analysis of variance (ANOVA). Violin plots were created using the vioplot package (https://cran.r-project.org/package=vioplot) in R. The outline of the violin plots represents the kernel probability density of the data at different values. Violin plots include a marker for the median of the data and a box indicating the interquartile range. Boxplots were created with Prism 6 software. The box extends from the 25th to 75th percentiles and the line in the middle of the box is plotted at the median. The whiskers show minimum to maximum. Scatter plots, linear regression lines and coefficient of correlation ($r^2$) were calculated using Prism 6 software by computing non-parametric Spearman correlation and two-tailed $P$ values.

**Sample sizing and collection.** No statistical methods were used to predetermined sample size, but at least three samples were used per experimental group and condition. The number of samples used in each experiment is indicated in Figs 1–6, in the legends of Extended Data Figs 1–10 and in Source Data files.

Samples and experimental animals were randomly assigned to experimental groups. Sample collection was also assigned randomly. Experimental procedures *in vitro*, sample collection and data analysis were performed blindly whenever possible. Whenever possible automated quantifications were performed using the appropriate software. Most animal procedures (that is, mouse treatments) were performed blindly by individuals unaware of the experimental design.

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**Extended Data Figure 1 | Protein synthesis in epidermal populations.**

**a.** Hair cycle stages and genetic lineage marking using K19- and LGR5 tdTom mice. Cell surface markers to isolated bulge stem cells are CD34 and ITGA6. Telogen: stem cells (CD34+/ITGA6+) are quiescent and resting in the bulge (BG). Early anagen: stem cells divide and give rise to committed progenitors in the hair germ (HG), which then grow downwards into the bulb (BU) surrounding the dermal papilla (DP). Late anagen: cells differentiate upwards to form the hair. Catagen: intermediate phase, when the hair bulb degenerates into a new resting bulge. IFE, interfollicular epidermis; SG, sebaceous glands. Mouse transgenes label K19- (red) and LGR5- (orange) positive stem cells and their progeny.

**b.** OP-puro detection in mouse epidermis at all hair cycle stages. Dotted lines indicate hair follicle and epidermal basal layer. Arrows indicate OP-purohigh cells in the hair follicle. Arrowheads indicate OP-purolow cells in the interfollicular epidermis. Nuclei are stained with DAPI.

**c, d.** tdTom and OP-puro detection in back skin of K19tdTom and Lgr5tdTom mice in telogen and late anagen. Arrows indicate tdTom+ cells. Arrowheads indicate Tomato+/OP-purohigh cells. Dotted line indicates lower bulge. Merged panels from c, d are shown in Fig. 1c–f. e. Hair follicle lineages and differentiation markers used in Fig. 1g–j. Ci, cuticle of inner root sheet; Ch, cuticle; Co, cortex; Cp, companion layer; He, Henle's layer; Hu, Huxley layer; IRS, inner root sheet; Me, medulla; ORS, outer root sheet. f. P-cadherin and OP-puro detection in a late anagen. Scale bars, 50μm.
Extended Data Figure 2 | Quantification of protein synthesis in epidermal populations. a, b, Top 2.5%, 10%, 25% and 50% translating epidermal cells (OP-purohigh) (a) were sorted for CD34 and ITGA6 (b). c, Protein synthesis in CD34+/ITGA6+, CD34−/ITGA6− and CD34−/ITGA6+ epidermal populations in the top 2.5%, 10%, 25%, 50% or 100% (all) translating cells at indicated hair follicle stages. d, Percentage of CD34+/ITGA6+, CD34−/ITGA6+ and CD34−/ITGA6− cells in the top 2.5%, 10%, 25%, 50% or 100% (all) of translating epidermal cells at indicated stages of the hair cycle. Error bars show mean ± s.d.

e, f, Violin plots of protein synthesis in top 2.5% OP-purohigh cells in tdTom− epidermal cells sorted for CD34 and ITGA6 from K19-tdTom (e) or Lgr5-tdTom mice (f) at all stages of the hair cycle. (n = mice). Source Data for this figure is available in the online version of the paper.
Extended Data Figure 3 | Protein synthesis and cell cycle analyses in epidermal cells. a–c, Violin plots of protein synthesis in indicated epidermal populations sorted for K19-tdTom-positive (a) and LGR5-tdTom-positive (b) and -negative (c) populations. Protein synthesis is shown for top 10%, 25% or 50% OP-purohigh cells. d, e, Cell cycle analysis (d) and percentage of cells in G1/G0 or S/G2/M in the top 2.5%, 10%, 25% or 50% OP-purohigh cells in late anagen (e). Data represent mean ± s.d. f, Scatter plots correlating protein synthesis in the 2.5% OP-purohigh population with percentage of cells in S/G2/M (top) and G1/G0 (bottom) using all samples independent of hair cycle stage. Linear regression, correlation coefficient (r²) and P value are shown. g, Box plots of protein synthesis (top) and number of cycling cells (bottom) in the top 2.5% translating cell populations (OP-purohigh). h, Box plots of protein synthesis in cycling (S/G2/M) and non-dividing (G1/G0) cells in the 2.5% OP-purohigh population isolated from Lgr5-tdTom mice. Shown are all cells (top), tdTom− (Tom−) (middle) and tdTom+ (Tom+) (bottom) cells at the indicated hair cycle stages. **P < 0.01, ***P < 0.001, ****P < 0.0001, two-tailed Student’s t-test. n = mice. Source Data for this figure is available in the online version of the paper.
Extended Data Figure 4 | Protein synthesis in squamous tumours.

a–c, Co-labelling of OP-puro with markers for undifferentiated basal cells: ITGA6 (a), CD44 (b) and PDPN (c) in mouse tumours. Nuclei are stained with DAPI. Arrows indicate low translating and marker-positive cells. Dotted line indicates invasive front of the tumour. Boxed areas are magnified on the right. d, Gating of low, medium and high OP-puro cells in Nsun2+/+ (wild type) and Nsun2−/−; K5-Sos skin tumours analysed in e–g. e, Percentage of OP-puro+ cells in tumours from Nsun2+/+ (wild type) and Nsun2−/−; K5-Sos mice. f, g, Flow cytometry for ITGA6 and CD34 in unfractonated epithelial cells from mouse tumours (all cells) or epithelial cells with high, medium and low OP-puro incorporation (f) and quantification (g) (mean ± s.d.; n = 3 mice). h, Flow cytometry for ITGA6 and CD44 in unfractonated epithelial cells from mouse tumours.

i, j, Histogram (i) and quantification (j) showing OP-puro incorporation of cells as gated and quantified in h (mean ± s.d.; n = 4 mice).

k, l, Detection of endogenous expression of NSUN2 (LacZ) in early (P23) (k) and late (P30) anagen (l) hair follicles. Sections were co-stained with eosin or markers for bulge stem cells K15 and the hair lineages Huxley’s (Hu), cuticle (Ci) (GATA3), and cortex (Co) (LEF1). m–o, Haematoxylin and eosin staining (m) and immunostaining for LEF1 (n), K72 and DLX3 (o) in wild-type (WT) and Nsun2−/− skin at P1. Nuclei are stained with DAPI. Insets: magnified boxed area (1, 2). Scale bars, 50 μm.

p, Correlation between proliferation and protein synthesis with differentiation of quiescent (QSC) or committed stem cells (CSC), committed progenitors (CP), differentiating progenitors (DP), and terminally differentiated (TD) cells. Source Data for this figure is available in the online version of the paper.
Extended Data Figure 5 | NSUN2 in mouse skin squamous cell carcinomas. a, Immunostaining for NSUN2, ITGA6, K10 (differentiation marker), laminin 5α and K8 (tumour progression markers), and Slug (epithelial to mesenchymal transition-related gene) at different stages of DMBA-TPA-induced malignant progression to squamous cell carcinoma (SCC). b–d, Quantification of tumour diameter normalized to body weight (BW) (b), tumours per mouse (c), and mouse life span (d) in K5-Sos/NSun2+/− (K5-Sos), K5-Sos/NSun2−/− and K5-Sos/NSun2−/− littermates. Measurements start at P16. Data collection discontinued when mice died (indicated by a dagger). Data represent mean, n ≥ 5 mice of each genotype. e, f, Haematoxylin and eosin staining (e) and immunostaining for ITGB1 (f) in sections from K5-Sos (K5-Sos/NSun2+/−) and K5-Sos/NSun2−/− skin tumours. b, basal undifferentiated cells; sb, suprabasal layers. Arrows indicate ITGB1+ cells. g, Relative mRNA expression levels of the indicated transcripts in skin tumours (mean ± s.d.; n = mice). h, Flow cytometry using ITGA6 and CD44 in K5-Sos/NSun2−/− and control K5-Sos (K5-Sos/NSun2+/−) tumours. i, Percentage of cells in cell populations as gated in h (mean ± s.d.; n = mice). *P < 0.05; ***P < 0.001 (two-tailed Student’s t-test) (i). j, TdT-mediated dUTP nick end labelling (assay) (TUNEL) assay on sections of K5-Sos tumours expressing (K5-Sos/NSun2+/−) or lacking Nsun2 (K5-Sos/NSun2−/−). Arrows indicate TUNEL+ (apoptotic) cells. Nuclei are stained with DAPI. Dotted line indicates boundary of epithelia and stroma (f, j). Scale bars: 25 μm (a), 100 μm (e, f, j). Source Data for this figure is available in the online version of the paper.
Extended Data Figure 6 | Deletion of Nsun2 enhances self-renewal of tumour-initiating cells in a cell-autonomous manner and NSUN2 expression in human skin tumours. a, Tumour size after grafting of K5-Sos/ Nsun2+/− (K5-Sos) and K5-Sos/Nsun2−/− tumour cells subcutaneously into nude mice (mean ± s.d.; n = 3 mice). b–f, Histology (haematoxylin and eosin staining) (b), staining for GFP (c), Ki67 (d), ITGB1 (e) and PDPN (f) in grafted tumour sections. Dotted line indicates boundary between epithelia and stroma. Arrows indicate basal and suprabasal expression. Nuclei are stained with DAPI. g–l, Immunohistochemistry for NSUN2 in human normal skin, benign tumours, malignant basal cell carcinomas (BCC) and squamous cell carcinomas (SCC) with increased malignancy (stages classified using the TNM system). Arrows indicate NSUN2high cells. Arrowheads indicate NSUN2low cells. m, Distribution of cells shown in g–l according to NSUN2 protein levels. (n ≥ 3 samples). Scale bars, 100 μm. Source Data for this figure is available in the online version of the paper.
Extended Data Figure 7 | NSUN2-dependent RNA methylation of coding and non-coding RNA in mouse tumours. a, Percentage of NSUN2-methylated sites (>0.15 m^5C in Nsun2^+/+; <0.05 m^5C in Nsun2^-/-) out of all covered sites (left) and in non-coding RNA (ncRNA) or introns and exons (right). b, Methylation level in coding and non-coding RNAs (>0.15 m^5C in Nsun2^+/+; <0.05 m^5C in Nsun2^-/-; coverage >10 reads). c–e, Examples of NSUN2-targeted non-coding RNA (Rppl1) and mRNA (Elf1 and Dscam1) in Nsun2^+/+ (top) and Nsun2^-/- (bottom) tumours. f, Number of NSun2-methylated sites in exons 1 to 60 (top) or distance from the transcriptional start site (TSS) in introns (bottom). Number and location of lost (red) or unchanged (grey) m^5C sites in K5-Sos/Nsun2^-/- tumours. Gucleotide position in tRNA is shown on the x-axis (j). Examples of NSUN2-targeted tRNAs in Nsun2^+/+ (top) and Nsun2^-/- (bottom) K5-Sos tumours (k, l). Heatmaps show methylated (red) and unmethylated (grey) cytosines. Cytosines are shown on the x-axis, and sequence reads on the y-axis. Numbers indicate the m^5C position in the RNA (c–e, k, l). Bisulphite-seq and RNA-seq data represent average of 4 replicates per condition.
Extended Data Figure 8 | Nsun2 deletion drives translational changes independent of mRNA expression. a, Ribosome profiling and RNA-sequencing experiments (see Fig. 5) using Nsun2-expressing (Nsun2+/+) and Nsun2-deficient (Nsun2−/−) K5-Sos skin tumours, or cultured human skin fibroblasts (NSUN2+/− line1 and NSUN2−/− line2 and healthy donors; NSUN2+/−, NSUN2−/−). HTS, high-throughput sequencing. b, Correlation between protein synthesis (ribosome footprint density) in Nsun2+/+ and Nsun2−/− tumours. c, Example of triplet periodicity in ribosome footprints (K5-Sos/Nsun2+/+, replicate 1) shown as number of reads against nucleotide position relative to the translational start site for all ORFs. d, Heatmaps showing ribosome footprint reads around the translational start site (0) in Nsun2+/+ and Nsun2−/− tumours (3 replicates per condition; ribosome density >0; colour indicates RPKM values of footprints). e, Log2 fold change (FC) per transcript in normal skin (left) and tumour samples (right) of significant (P < 0.05) expression differences. Nsun2 RNA levels (red). f–j, Scatter plots, linear regression lines and coefficient of correlation (r²) of mRNA expression and protein synthesis (density of ribosome footprints per kb) in Nsun2+/+ (grey) and Nsun2−/− (red) mouse tumours (f) and human fibroblasts (g–j). k, Venn diagram of transcripts with significant (P < 0.05) different ribosome footprint density in the 5′ UTR in NSUN2+/−, NSUN2−/− line1 and NSUN2−/− line2 human fibroblasts relative to NSUN2+/+ cells. l, Box plots of ribosome footprint read counts in the 5′ UTR (left) and corresponding CDS (right) of the 192 transcripts in k. ****P < 0.0001 (two-tailed Student’s t-test).
Extended Data Figure 9 | RNA methylation–dependent changes of protein synthesis. a, Venn diagram of transcripts with differential protein synthesis in NSUN2+/+ and NSUN2−/− human fibroblasts relative to NSUN2+/+ cells. b, GO terms enriched in 424 commonly differentially translated transcripts in NSUN2−/− lines (a). c, Western blot for NSUN2 and tubulin in NSUN2−/− human fibroblasts rescued with viral constructs expressing wild-type NSUN2 (NSUN2-wt), two catalytically dead mutants (C271A and C321A) or the empty vector. d, Venn diagram of differentially translated transcripts in the indicated rescued cells relative to empty vector–infected control cells. Translation of 173 out of 746 of transcripts (23%) depended on the enzymatic activity of NSUN2.

Data represent mean ± s.d. (n = 3 assays). Western blot confirms downregulation of NSUN2 in the presence of the siRNA construct. i, j Reduced motility in keratinocytes expressing the enzymatic-dead NSUN2 construct (K190M) (K190M; n = 13; NSUN2; n = 19 cells) (i). Western blot confirms equal protein expression levels of K190M and NSUN2 (j). k, Reduced differentiation in primary human keratinocytes expressing the enzymatic-dead NSUN2 (K190M). Staining for NSUN2, ITGA6 or involucrin (IVL) and nuclei (DAPI). Control: empty vector (left); NSUN2: wild-type NSUN2 (middle); K190: enzymatic-dead NSUN2 (right). Arrows indicate NSUN2-expressing ITGA6−/IVL+ cells. Arrowheads indicate K190M-expressing ITGA6+/IVL− cells.

m, Quantification of IVL+ infected keratinocytes grown in suspension for 24 h to stimulate differentiation. *P < 0.05, **P < 0.01 (two-tailed Student’s t-test) (h–m). Scale bar, 100 μm. Source Data for this figure is available in the online version of the paper.
Extended Data Figure 10 | Protein expression differences, drug treatment of Nsun2−/− tumours and graphical summary.

**a, b,** Western blot analysis of translationally repressed (a) or induced (b) mRNAs in Nsun2−/− (−/−) compared to Nsun2+/+ (WT) skin tumours with quantification of band densitometry on the right (mean ± s.d.; n = 3 mice). *P < 0.05, ***P < 0.001 (two-tailed Student’s t-test).

**c, d,** Control and 5FU-treated tumours, before and after treatment.

**e, f,** Immunohistochemistry for p53 in tumours shown in c, d, g–i. Immunostaining for cleaved caspase 3 (Cl−CASP3) (g), Ki67 (h), ITGA6 and K10 (i) in K5-Sos tumours expressing (+/+ or lacking (−/−) Nsun2 and treated with CDDP (see Methods). Scale bars, 100 μm.

**j,** Graphical summary: (1) quiescent undifferentiated stem and progenitor cells are characterized by the absence of NSUN2 and low global protein synthesis; (2) upregulation of NSUN2 counteracts angiogenin-mediated cleavage of tRNAs through site-specific methylation of tRNAs, allowing increased translation of lineage-specific transcripts driving terminal differentiation; (3) cytotoxic stress inhibits NSUN2 and global protein synthesis in particular of lineage-specific transcripts and promotes an undifferentiated quiescent cell state. Yet cell survival after the insult requires re-methylation of tRNAs by NSUN2 (see (2)); (4) the inability to upregulate NSUN2 in response to the cytotoxic insult leads to cell death. Source Data for this figure is available in the online version of the paper.