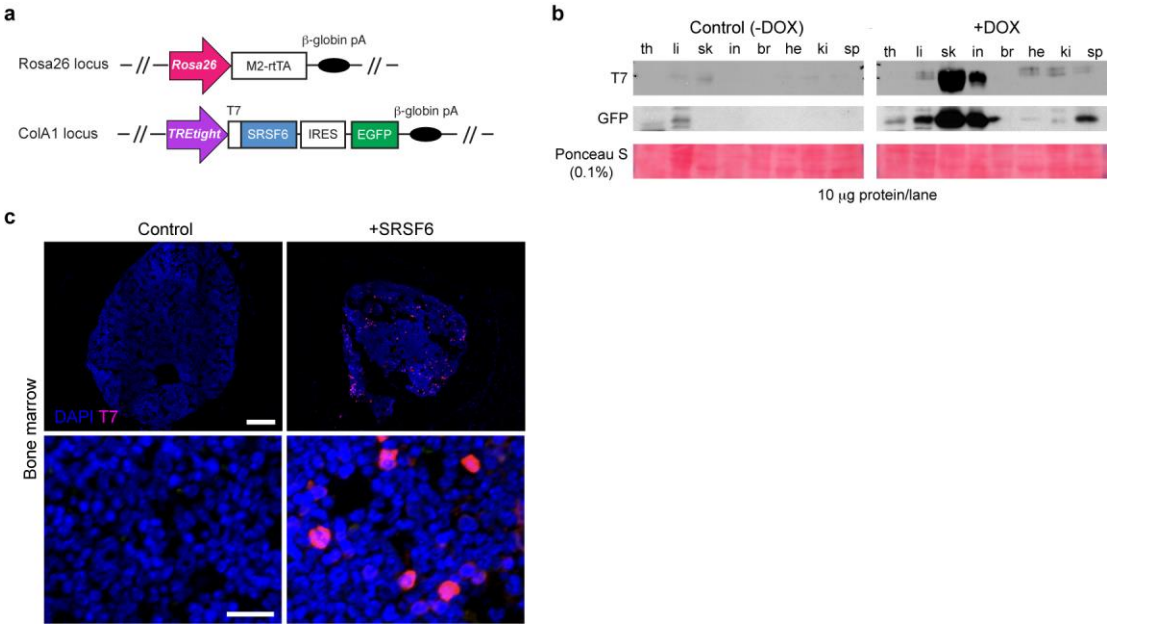
Supplementary Information

Splicing factor SRSF6 promotes hyperplasia of sensitized skin

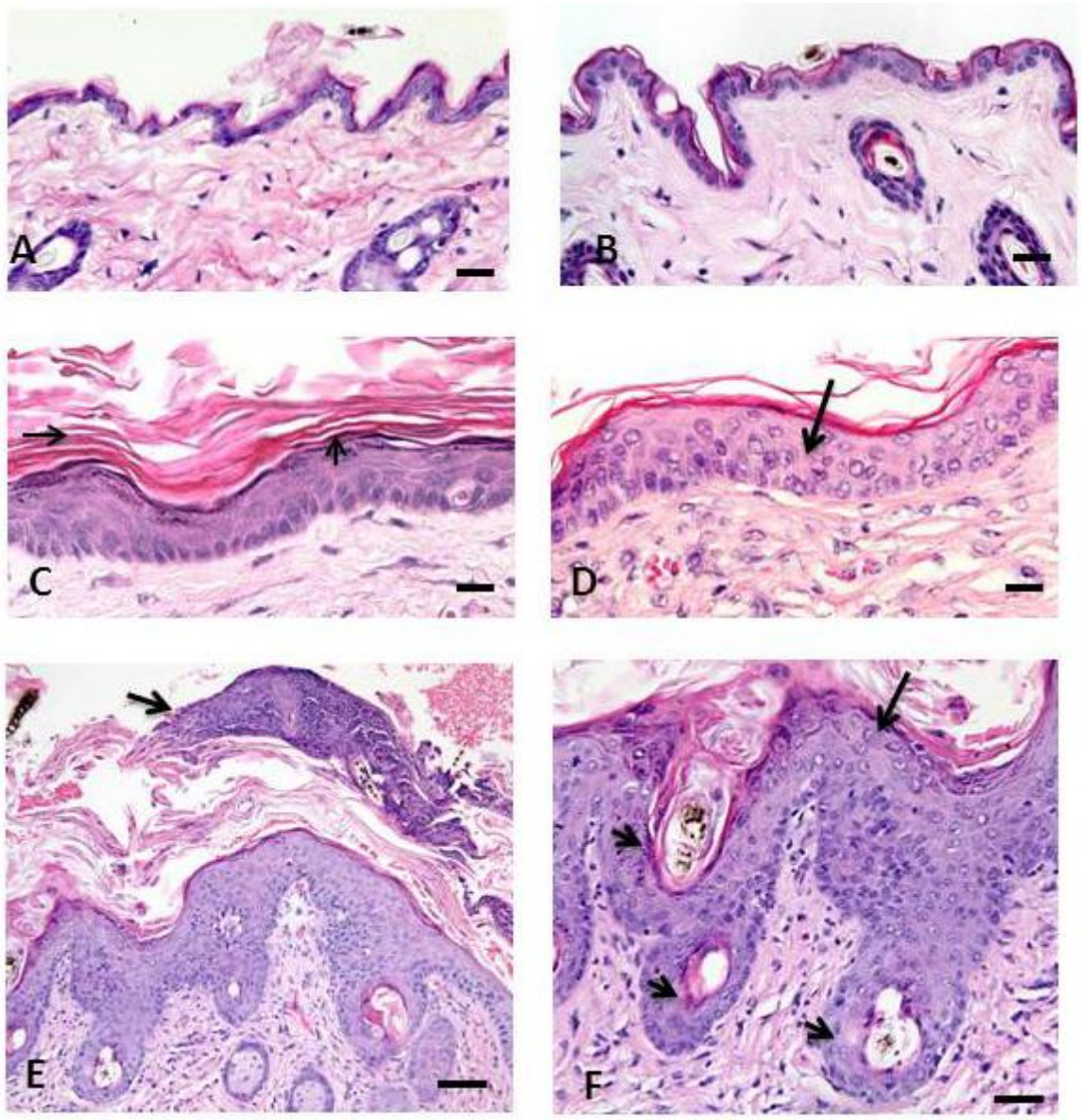
Inventory of Supplementary Information:

Supplementary Figure 1: Generation and testing of transgenic *SRSF6* conditional mice.



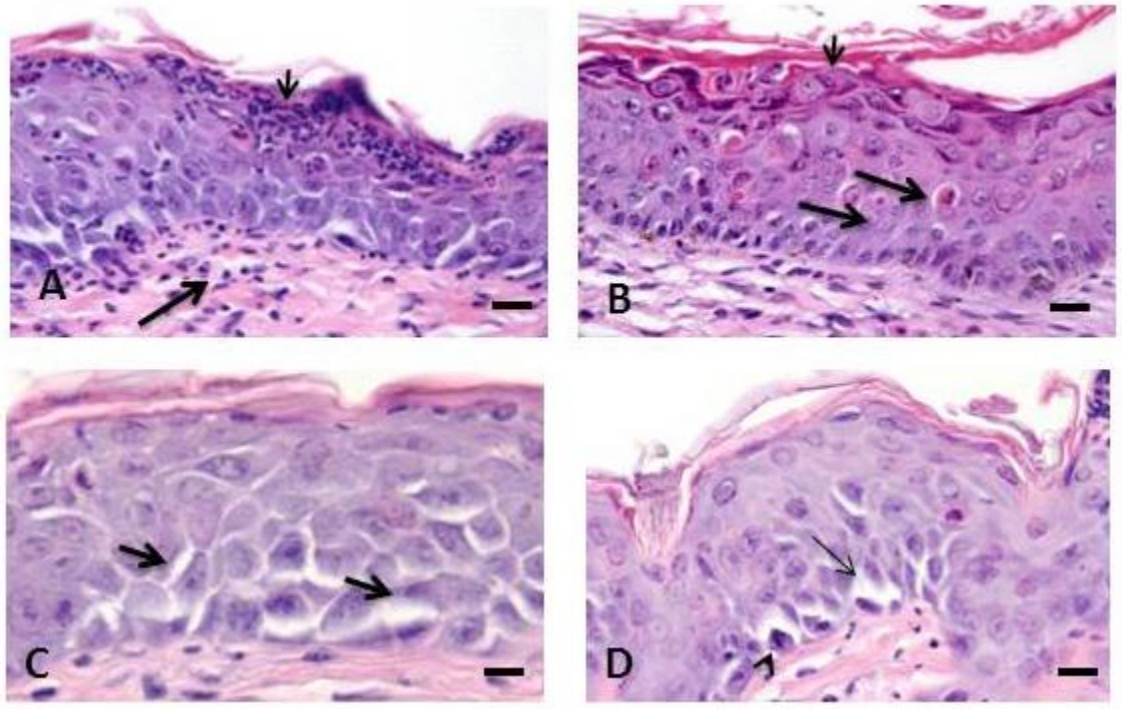
Supplementary Figure 1 Tet-on transgenic *SRSF6* mouse. (**a**) A human *SRSF6* cDNA (with an N-terminal T7-tag) was inserted into pBS31'-RBGpA-TREtight-ColA1 flp-in. Upon induction, the cDNA is expressed as a bicistronic transcript, including an IRES element followed by EGFP cDNA. The reverse tetracycline-controlled transactivator (M2-rtTA) is expressed from the Rosa26 locus in KH2 ES cells. (**b**) Expression of ectopic SRSF6 (T7) and GFP protein was measured by immunoblotting using antibodies against the T7-tag or GFP upon DOX treatment of R26-rtTA/ColA1-SRSF6-transgenic mice. Protein was extracted from thymus (th), liver (li), skin (sk), small intestine (in), brain (br), heart (he), kidney (ki), and spleen (sp). Loading: 0.1% Ponceau S staining. (**c**) Sporadic expression of ectopic SRSF6 in bone marrow. Control (left panel), +DOX (right panel). T7-tag antibody detects ectopically expressed SRSF6. Bar = 100 μm (upper panel), 20 μm (lower panel).

Supplementary Figure 2: Epidermal hyperplasia in mild to moderate lesions.



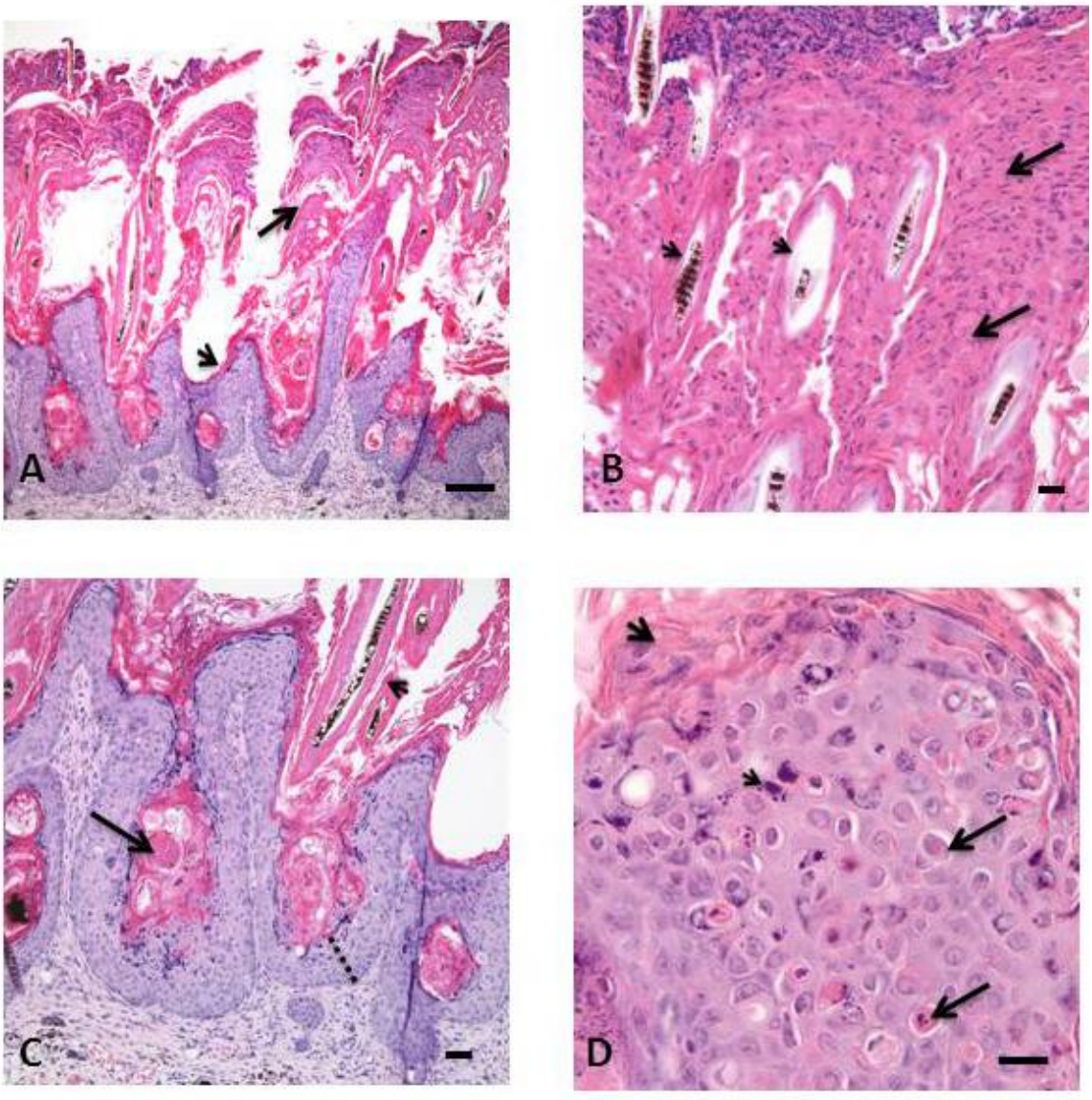
Supplementary Figure 2 Epidermal hyperplasia in mild to moderate lesions. (**a**) Epidermis from transgenic mouse not exposed to doxycycline. Note continuous basal layer, 1-2 cell thick spinous layer, and thin stratum corneum (bar = 50 µm). (**b**) Normal epidermis 200 µm from area of hyperplasia (bar = 50 µm). (**c**) Overexpression of SRSF6 for 7 days leads to mild epidermal hyperplasia. Note the orthokeratotic hyperkeratotic (arrow) stratum corneum. The granular layer is also more prominent (short arrow) (bar = 50 µm). (**d**) Prominent thickened stratum spinosum (arrow) and minimal dysplasia in mild early lesion (bar = 50 µm). (**e**) Overexpression of SRSF6 for 14 days results in severe hyperplasia. Note the severe hyperplasia in the epidermis and hair follicles and thick parakeratotic hyperkeratotic crust (arrow) (bar = 100 µm). (**f**) Higher magnification of **e**. There is severe hyperplasia of the epidermis (arrow) and hair follicles (short arrow) (bar = 100 µm).

Supplementary Figure 3: Abnormalities in the epidermis following overexpression of SRSF6 for 14-21 days.



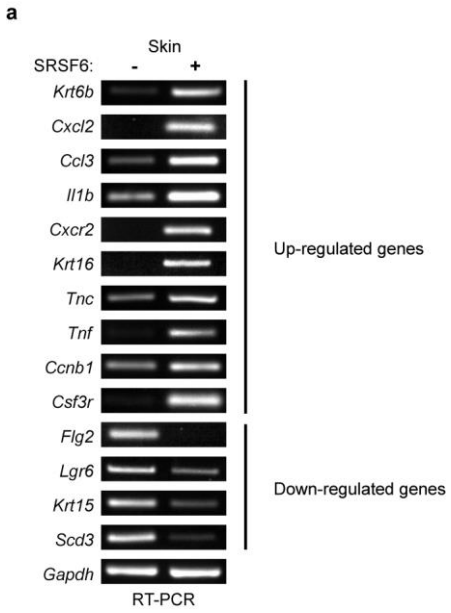
Supplementary Figure 3 Abnormalities in the epidermis following overexpression of SRSF6 for 14-21 days. (**a**) Intra-epidermal microabscess (short arrow) and mild inflammation (arrow) at the disturbed dermal-epidermal interface. (bar = 100 µm). (**b**) Multiple apoptotic bodies (arrows) and dyskeratosis (faulty development of the epidermis with abnormal keratinization) (short arrow) (bar = 100 µm). (**c**) Spongiosis (loss of adhesion and intercellular edema) in the epidermis (bar = 100 µm). (**d**) Basal cell apoptosis (Civatte body) (arrow), spongiosis, and dyskeratosis (bar = 100 µm).

Supplementary Figure 4: Verrucose hyperplasia in plucked skin of mice overexpressing SRSF6 for 21 days.



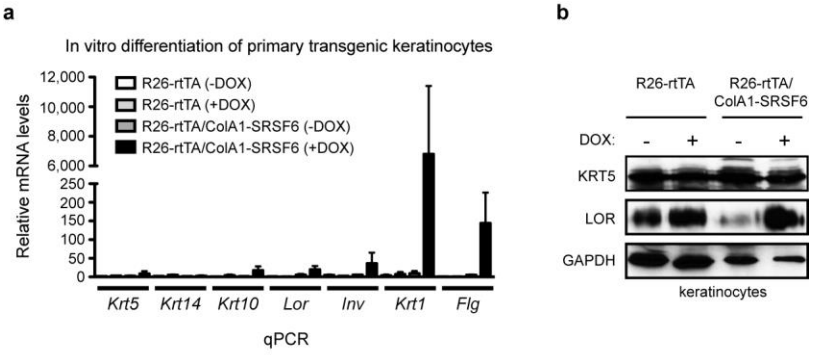
Supplementary Figure 4 Verrucose hyperplasia in plucked skin of mice overexpressing SRSF6 for 21 days. (**a**) Severe epidermal hyperplasia (short arrow) and exorbitant parakeratotic hyperkeratosis (arrow) (bar = 100 µm). (**b**) Dyskeratosis with severe parakeratosis (arrows). Hair follicles (short arrows) are trapped in the rapidly proliferating hyperplastic epidermis (bar = 50 µm). (**c**) Thickened epidermis (dotted line) with extensive hyperkeratosis and keratin pearls trapped in the hyperplastic stratum corneum (arrow) (bar = 50 µm). (**d**) Severe dysplasia with severe dyskeratosis, excessive apoptotic bodies (arrows), and dysplastic keratinocytes (short arrows) (bar = 25 µm).

Supplementary Figure 5: Gene expression analysis of SRSF6-overexpressing skin.



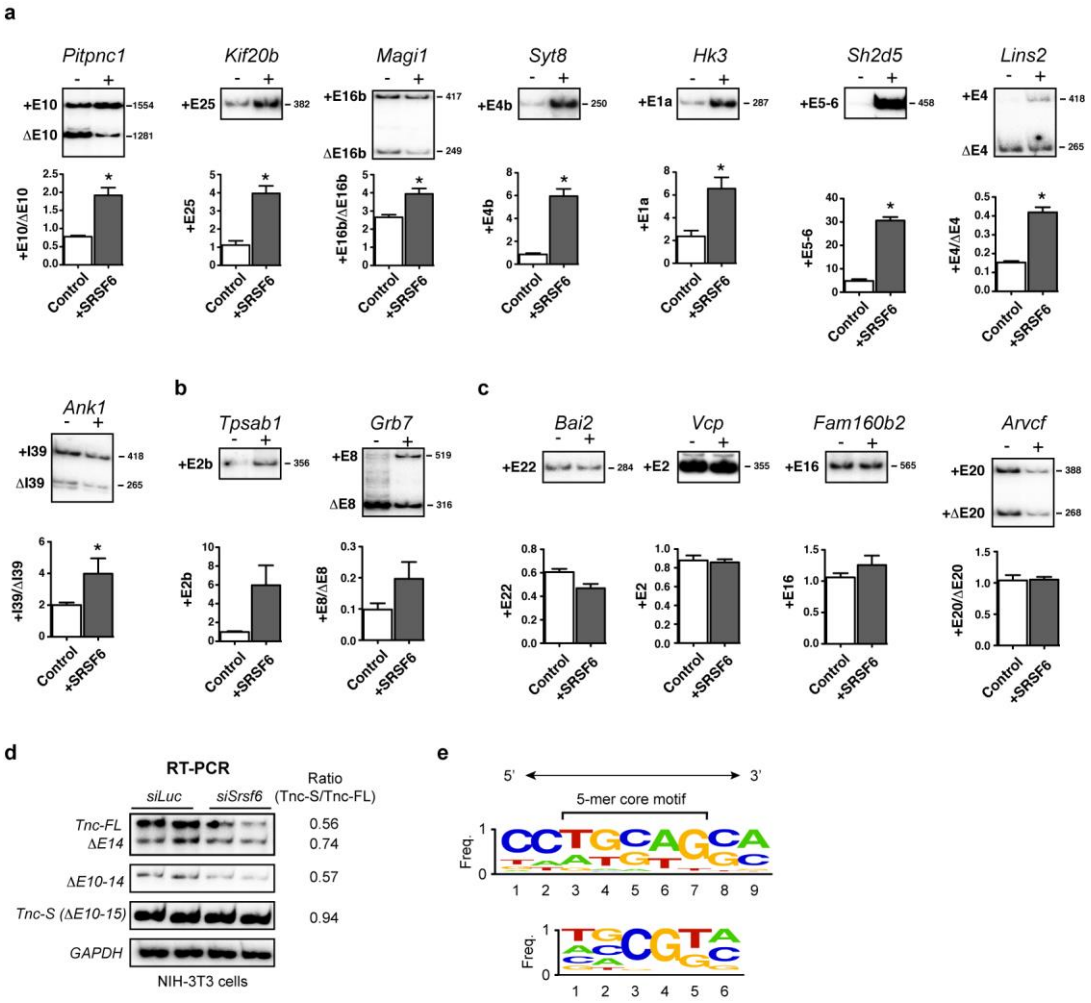
Supplementary Figure 5 RT-PCR validation of differentially expressed genes upon SRSF6 overexpression in skin. 14 out of 14 genes tested were successfully validated by RT-PCR. Confirmed upregulated genes include: genes induced upon tissue injury, such as *Keratin 6* (*Krt6*) (microarray: +154-fold), *Keratin 16* (*Krt16*) (microarray: +13-fold) and *tenascin C* (*Tnc*) (microarray: +6-fold); and pro-inflammatory cytokines important for wound healing, such as *tumor necrosis factor* (*Tnf*) (microarray: +6-fold), *Interleukin-1 b* (*Il1b*) (microarray: +24-fold), *Chemokine (C-X-C motif) ligand 2* (*Cxcl2*) (microarray: +97-fold), *Chemokine (C-C motif) ligand 3* (*Ccl3*) (microarray: +43-fold). Confirmed downregulated genes included: genes related to skin stem cells, such as *Keratin 15* (*Krt15*) (microarray: -5-fold), *Leucine-rich repeat-containing G-protein coupled receptor 6* (*Lgr6*) (microarray: -5-fold); and genes related to differentiation, such as *Filaggrin-2* (*Flg2*) (microarray: -4-fold) and *Stearoyl-coenzyme A desaturase 3* (Scd3) (microarray: -18-fold).

Supplementary Figure 6: In vitro differentiation of primary keratinocytes.



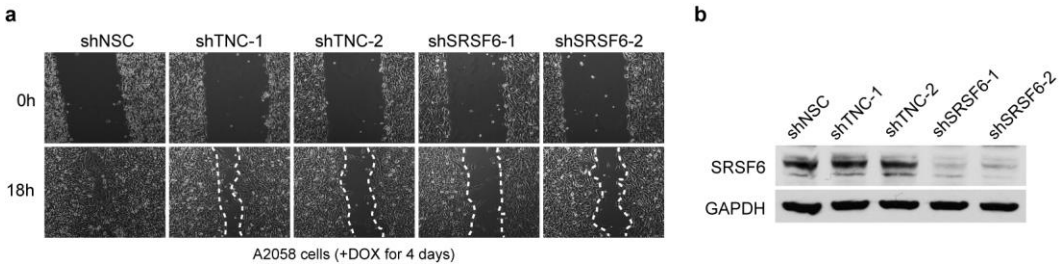
Supplementary Figure 6 *In vitro* differentiation of primary keratinocytes. Primary keratinocytes from control (R26-rtTA) or double-transgenic (R26-rtTA/ColA1-SRSF6) mice were allowed to differentiate in the presence of 1.2 mM Ca^{2+} for 5 d. (**a**) Induction of SRSF6 by DOX-treatment (+) strongly promoted differentiation of primary compared to control keratinocytes, as seen by the strong induction of genes associated with keratinocyte differentiation, measured by RT-qPCR: *loricrin* (*Lor*: +4-fold), *involucrin* (*Inv*: +7-fold), *keratin 1* (*Krt1*: +743-fold), *filaggrin* (*Flg*: +32-fold). In contrast, genes associated with undifferentiated keratinocytes, such as *keratin 5* (*Krt5*: +3-fold) and *keratin 14* (*Krt14*: +2-fold) were relatively unchanged upon SRSF6 induction. (**b**) The corresponding increase in loricrin protein levels upon SRSF6 induction was validated by immunoblotting; no change was observed in DOX-treated control keratinocytes (note: loading was different than for double-transgenic keratinocytes). Keratin 5 protein levels were unaffected by DOX treatment in both control and double-transgenic keratinocytes.

Supplementary Figure 7: Splicing-target validation.



Supplementary Figure 7 Splicing-target validation. Validation of SRSF6-responsive ASEs using ^{32}P-radioactive RT-PCR. (a) Strong ASEs with significant change; *p<0.05: *Phosphatidylinositol transfer protein, cytoplasmic 1* (*Pitpnc1*), *kinesin family member 20B* (*Kif20b*), *membrane-associated guanylate kinase, WW and PDZ domain containing 1* (*Magi1*), *synaptotagmin VIII* (*Syt8*), *hexokinase 3* (*Hk3*), *SH2-domain-containing 5* (*Sh2d5*), *lines homolog (Drosophila)*(*Lins2*), *ankyrin 1, erythrocytic* (*Ank1*), *ETS-domain protein (SRF accessory protein 1)* (*Elk4*), *Pyruvate Kinase 2* (*Pkm2*). (b) Weak ASEs that were not significant: *tryptase alpha/beta* (*Tpsab1*), *growth factor receptor-bound protein 7* (*Grb7*). (**c**) ASEs that did not show anticipated splicing changes by RT-PCR and therefore could not be validated: *brain-specific angiogenesis inhibitor 2* (*Bai2*), *valosin containing protein* (*Vcp*), *family with sequence similarity 160, member B2* (*Fam160b2*), *armadillo-repeat gene deleted in velocardiofacial syndrome* (*Arvcf*). Statistical analysis employed a Mann-Whitney test (n=10). Data are represented as mean +/- s.e.m. (**d**) *Tnc* splicing changes upon *Srsf6* knockdown in NIH-3T3 cells. ^{32}P-radioactive RT-PCR validation shows reciprocal splicing changes in *Tnc* upon *Srsf6* knockdown, using primers specific for *Tnc* exons 9 and 16. *Tnc* +E10-15 and ΔE14 isoforms decreased upon *Srsf6* depletion, whereas *Tnc-S* (ΔE10-15) (constitutive isoform) was unaffected. Lanes shown correspond to two biological replicates. (**e**) Pictogram of the predicted SRSF6 in vivo binding motif (9-mer; CCWKSWGSM, Top) which shows similarity to the reported SRSF6 functional SELEX binding motif (6-mer; YRCRKM, Bottom). The central 5-mer of the predicted SRSF6 in vivo binding motif is denoted '5-mer core motif'.

Supplementary Figure 8: SRSF6 and its target TNC is required for wound healing in vitro.

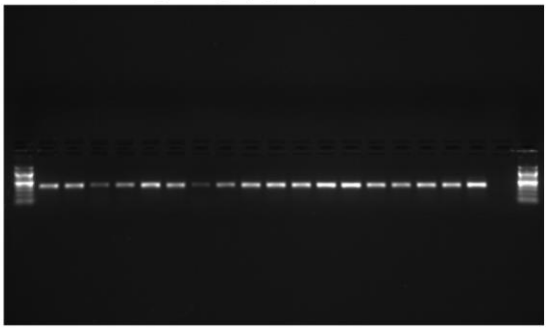


Supplementary Figure 8 *SRSF6* and its target *TNC* are required for wound healing *in vitro*. (**a**) Human A2058 melanoma cells transduced with inducible short-hairpins against either *TNC* or *SRSF6* were used for wound-healing assays. Cells were treated with DOX (10 µg/ml) for 4 d in total. Cell migration was measured over 18 h. Knockdown of either *SRSF6* or *TNC* impairs wound healing (2 shRNAs per gene). (**b**) Immunoblotting confirms efficient *SRSF6* knockdown. NSC=no-silencing control.

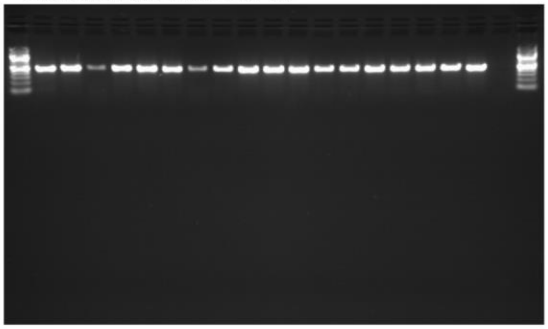
Supplementary Figure 9: Original images

a Original DNA gel image (Fig. 1a)

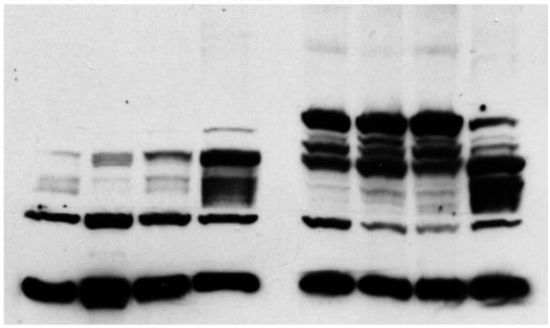
b Original DNA gel image (Fig. 1a)



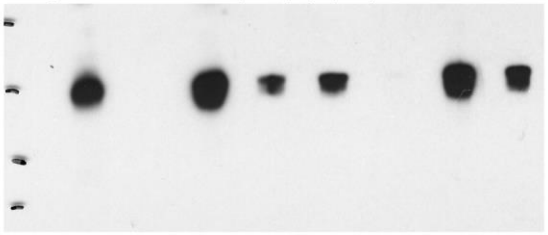
c Original DNA gel image (Fig. 1a)



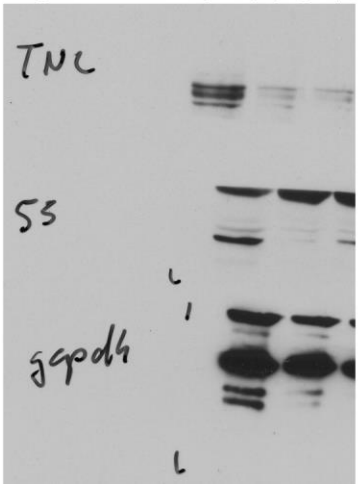
d Original western blotting image (Fig. 3d)



e Original IP western blotting image (Fig. 5d)



f Original western blotting image (Fig. 5e)



Supplementary Table 1a: Summary of transgenic mouse studies.

Genotype	DOX	Hyperplasia (Skin)	Hyperplasia (Small Intestine)
WT	Yes	0/4	0/4
R26-rtTA/ColA1-SRSF6	No	0/4	0/4
R26-rtTA/ColA1-SRSF6	Yes	8/8 (p=0.0002)	5/9 (p=0.03)

Fisher's exact test was used for statistical analysis (n=16 and n=17).

Supplementary Table 1b: Summary of allogenic skin transplantation studies.

Name	Graft genotype	Recipient genotype	Graft: GFP+ (+DOX for 1 week)	Pathology
C1	*R26-rtTA*	Wt (nude)	No	Normal skin
C2	*Wt*	Wt (nude)	No	Normal skin
C3	*Wt*	Wt (nude)	No	Normal skin
G1	*R26-rtTA/ColA1-SRSF6*	Wt (nude)	Yes	Skin hyperplasia
G2	*R26-rtTA/ColA1-SRSF6*	Wt (nude)	Yes	Skin hyperplasia
G3	*R26-rtTA/ColA1-SRSF6*	Wt (nude)	Yes	Skin hyperplasia

Supplementary Table 2: Gene expression analysis, functional gene annotation analysis, and splicing targets (separate excel-file).

Supplementary Table 3a: Summary of splicing-target analysis.

Validated	ASE (ratio)	ASE (percent)	Gene (ratio)	Gene (percent)
All	16/22	72.7%	11/17	64.7%
| SI | >2	12/14	85.7%	7/9	77.8%
| SI | <2	4/8	50.0%	4/8	50.0%

Supplementary Table 3b: Analysis of 5' splice site strength of alternatively spliced region of *Tnc*.

Tnc	**Type**	**Splice site**	**Score**	**Base pairs**	**ΔG (Free Energy)**	**Freq. Motif A**	**Freq. Motif B**	**Freq. Motif C**	**Exon size**
E9	5'	CGGgtaagt	90.84	9	-9.4	3	2	1	90
E10	**5'**	**CAGgtactt**	**77.2**	**7**	**-4.8**	**3**	**6**	**1**	**264**
E11	**5'**	**CAGgtattt**	**77.98**	**8**	**-4.9**	**2**	**8**	**0**	**273**
E12	**5'**	**CAGgtatca**	**72.27**	**7**	**-4.9**	**6**	**9**	**0**	**273**
E13	**5'**	**CAGgtacat**	**77.38**	**7**	**-4.8**	**2**	**6**	**2**	**273**
E14	**5'**	**CAGgtattt**	**77.98**	**8**	**-4.9**	**6**	**10**	**0**	**273**
E15	**5'**	**CAGgtactg**	**72.03**	**6**	**-4.8**	**1**	**6**	**0**	**273**
E16	5'	GAGgtaggg	81.68	7	-8.5	3	5	1	123

a) Summary of splicing-target analysis. Validation success rates were measured for either alternative splicing events (ASEs) or genes; weak splicing change (|SI|<2), strong splicing change (|SI|>2), or all.
b) Analysis of 5'splice site strength was performed as described in Methods. SRSF6 motif frequency analysis was done based on the presence of MEME-predicted or SELEX SRSF6 motifs, and calculated as the number of motifs per exon; Motif A (9-mer SRSF6 in vivo: CCWKSWGSM), Motif B (SRSF6 in vivo (5-mer core motif): WKSWG), or Motif C (SRSF6 SELEX: HVCGKM) were mapped using the SFmap algorithm[1]. Motifs are in IUPAC format. Settings used were: window size=50, significant p-value (0.01), suboptimal p-value (0.05) and scoring function=WR. Average number of motifs per exon was: Motif A (alternative region=3.3, constitutive region=3.0), Motif B (alternative region=7.5, constitutive region=3.5), and Motif C (alternative region=0.5, constitutive region=1.0). Alternative exons are in bold (E10-15).

Supplementary Table 4: Summary of nude mouse tumor studies.

Genotype: MEFs p53-/-	No. cells injected	No. sites	No. tumors (after 4 weeks)	Average tumor size (mm^3)
Vector+c-myc	$2x10^6$	8	0	-
SRSF6+c-myc	$2x10^6$	8	8 (p=0.0002)	1068.5 (+/-385.5)

Fisher's exact test, n=16, mean+/-s.e.m.

Supplementary Note 1: Sequences of RT-PCR/qPCR primers and siRNA oligonucleotides.

Application*	Name	Sequence (5' to 3')
GE	Ccl3-F	ATGAAGGTCTCCACCACTGC
GE	Ccl3-R	CCCAGGTCTCTTTGGAGTCA
GE	Ccnb1-F	GGCTGACCCAAACCTCTGTA
GE	Ccnb1-R	TGCAATAAACATGGCCGTTA
GE	Csf3r-F	GCCCCAGTCTACACCCTACA
GE	CSf3r-R	AGGTGTATGTCCTGCCCTTG
GE	Cxcl2-F	AGGCTACAGGGGCTGTTGT
GE	Cxcl2-R	TTAGCCTTGCCTTTGTTCAG
GE	Cxcr2-F	GCTGCCTCACTTTCTTCCAG
GE	Cxcr2-R	GACCAGCATCACCAAGGAGT
GE	Flg2-F	TGCGTCAGGCCTTATCCTAC
GE	Flg2-R	GGCCAGAGCCAGTTTGAATA
GE	GAPDH-F	GAATGGGAAGCTTGTCATCAACGG
GE	GAPDH-R	CCGTTCAGCTCTGGGATGACCTTG
GE	Il1b-F	GGGCCTCAAAGGAAAGAATC
GE	Il1b-R	TACCAGTTGGGGAACTCTGC
GE/qPCR	Krt15-qF	TGGAGATGCAGATTGAGCAG
GE	Krt15-R	ATGCTGAGCTGAGACTGCAA
GE	Krt16-F	TGATCAGCAGTGTGGAGGAG
GE	Krt16-R	CAGCTTGAGAGGCAGTTGTG
GE	Krt6b-F	CAGGCTGCTGAAGGAGTACC
GE	Krt6b-R	CGCTGGTGGTGTATTTGATG
GE	Lgr6-F	ACCTCTGGCTGGATGACAAT
GE	Lgr6-R	GCCCATGAAGGCTTTCTCT
GE	Scd3-F	CCTCCTGCAAGCTCTACACC
GE	Scd3-F	CTTCTCGGCTTTCAGGTCAG
GE	Tnc-E16F	CCATGAAGGGATTCGAAGAA
GE	Tnc-E18R	AAACTTGGTGGCGATGGTAG
GE	Tnf-F	GAACTGGCAGAAGAGGCACT
GE	Tnf-R	CACTTGGTGGTTTGCTACGA
GE	Actb-F	AGAAAATCTGGCACCACACC
GE	Actb-R	GTCAGGCAGCTCGTAGCTCT
GE	SRSF6-F	AGCCGCAGTAGATCTCGAAG
GE	SRSF6-R	TTGAGGGTGGAGCAGGTAGA
GE	Ires-R	AGACCCCTAGGAATGCTCGT
AS	Ank1-F	GGAGGACGACACAGTGGATT
AS	Ank1-R	CCTGCTCGTATTCCTGAAGC
AS	Arvcf-F	CAGGGGACACCTCTGAGAAA
AS	Arvcf-R	GCCTCCTCTCCTACCACACA
AS	Bai2-F	CATGCTCATCGGGATTATCG
AS	Bai2-R	TGACAAAGCCTTGTGCAGAG
AS	Elk4-F	TCTTTGAGGTCCGAGTGTGA
AS	Elk4-R	CCTGCAGAAGCTTGAACTCC
AS	Fam160b2-F	CCCTTTTGCTTCCTTTTCCT
AS	Fam160b2-R	CCACCAAGATCTGCTCCTGT
AS	Grb7-F	CTGGCAGCTTCCCTGAGAT
AS	Grb7-R	GTCCGACTCTGCTCATCCTC
AS	Hk3-F1	AATGTTGAGTGTGCGAGCAG
AS	Hk3-R	TGGTGTGGACCTCACGTATG
AS	Kif20b-F	CCATGGATGACCTAGACGTG
AS	Kif20b-R	CTCTTTGCTGCAGCCTTTCT
AS	Lins2-F	GGATGTGGGAGGAACACAGT
AS	Lins2-R	TCATCTTCAAGAGTGGTTCCAA
AS	Magi1-F1	CAAGGCAGCCAGAACTCTCT
AS	Magi1-R1	GGTGGCAATCTTCTCAGCAT
AS	Pitpnc1-F	GTGGTCCGAGACATCTTGCT
AS	Pitpnc1-R	GGTCTTTGGGAATGGACAGA
AS	Pkm2-F	GTGCTGAAGGCAGTGATGTG
AS	Pkm2-R	CATGGCCAAGTTTACACGAA
AS	Sh2d5-F	TCCTGTGGATGACTTGGACA
AS	Sh2d5-R	TGAAGGGCTCTCGCACTAAT
AS	Syt8-F	CAGGGGAAAAGAACCTCTTG
AS	Syt8-R	CACCTTCAGAGTGGCTTTGG
AS/CLIP	Tnc-E9F	GAGCCAGGGCAAGAATACAC
AS	Tnc-E16R	AGTGATCCGGAAACTCTCCA
CLIP	Tnc-E10R	CTCCACATTGTTGGCTTCCT
AS	Tpsab1-F	TCCTCACTGTGTCCAAATGC
AS	Tpsab1-R	CGATGTAGAAGTCGGGGTGT
AS	Vcp-F	GTCGCCTGCCTTTCTCATAG
AS	Vcp-R	AGGCGAACTCGGAGGTTATT
AS	Wnk2-F	ACGAGTGGACAACGAAGACC
AS	Wnk2-R	GTCTGCCGCTTCTTCTCCTG
qPCR	Tnc-E8qF	GGGATTGGTGTTTCTGCTGT
qPCR	Tnc-E9qR	AGGCTGTAGTTGAGGCGGTA
qPCR/CLIP	Tnc-E15qF	CCGAACGTACCAGGGACATA

qPCR/CLIP	Tnc-E16qR	AGTGATCCGGAAACTCTCCA
qPCR	Lgr4-qF	CACAACCTGTCTGATCTGCAT
qPCR	Lgr4-qR	TGCTGCTTATTTTTGTCCCTGT
qPCR	Lgr5-qF	CAGCCTCAAAGTGCTTATGCT
qPCR	Lgr5-qR	GTGGCACGTAACTGATGTGG
qPCR	Lgr6-qF	GATGGGGACAGAGGACTCAA
qPCR	Lgr6-qR	CCAGCTCTCAAAGAGGTGCT
qPCR	GAPDH-qF	AGGTCGGTGTGAACGGATTTG
qPCR	GAPDH-qR	TGTAGACCATGTAGTTGAGGTCA
qPCR	Inv-qF	ATGTCCCATCAACACACACTG
qPCR	Inv-qR	TGGAGTTGGTTGCTTTGCTTG
qPCR	Flg-qF	ATGTCCGCTCTCCTGGAAAG
qPCR	Flg-qR	TGGATTCTTCAAGACTGCCTGTA
qPCR	Krt1-qF	ATGACCAAAGTTGAGCTTCAGG
qPCR	Krt1-qR	CAGATCCAAACTGCGGTTGTT
qPCR	Krt5-qF	CTCTGTCGTTACAAACAGTGTCT
qPCR	Krt5-qR	CTTAGCCCGCTACCCAAACC
qPCR	Krt10-qF	CGAAGAGCTGGCCTACCTAAA
qPCR	Krt10-qR	GGGCAGCGTTCATTTCCAC
qPCR	Krt14-qF	AGCGGCAAGAGTGAGATTTCT
qPCR	Krt14-qR	CCTCCAGGTTATTCTCCAGGG
qPCR	Lor-qF	CTCCTGTGGGTTGTGGAAAGA
qPCR	Lor-qR	TGGAACCACCTCCATAGGAAC
qPCR	Krt15-qR	TACATCCCGACGGTTTTTCT
CLIP-qPCR	CP-E10F	GACTGCTCACAACTTCACAGTACC
CLIP-qPCR	CP-E10R	TCTATAAGAAGTGGCAACCTTGA
CLIP-qPCR	CP-E11F	ACTGGACTGCTCCAGAAGGA
CLIP-qPCR	CP-E11R	CGTGTCAGCCTCTAGCACCT
CLIP-qPCR	CP-E12F	TGGCCTATAAGCACTTTGTCG
CLIP-qPCR	CP-E12R	GGTACCGTGAGGTTCTGAGC
CLIP-qPCR	CP-E13F	TCATTGTCTACCTCTCTGGAATTG
CLIP-qPCR	CP-E13R	TGTGGTAGCCGTGGTACTGA
CLIP-qPCR	CP-E14F	CACTTTCAGGAACCCAGAGG
CLIP-qPCR	CP-E14R	CCTCATAGCCAATGCCAGTT
CLIP-qPCR	CP-E15F	AGCTGAACCGGAAGTTGACA
CLIP-qPCR	CP-E15R	AGTCCAGGACAGACGGAAAC
siRNA	siSrsf6	CGUUCUAGAUCUCGUUCAA
siRNA	siSrsf6-as	UUGAACGAGAUCUAGAACG
siRNA	siLuc	GCCAUUCUAUCCUCUAGAGGA
siRNA	siLuc-as	UCCUCUAGAGGAUAGAAUGGC

*Application: Validation of gene expression (GE), validation of alternative splicing (AS), quantitative RT-PCR validation (qPCR), cross-linking immunoprecipitation (CLIP), quantitative RT-PCR of exon-specific cross-linking immunoprecipitation (CLIP-qPCR) and siRNA oligo sequences (siRNA).

Supplementary Note 2: Target sequence information for short-hairpin vectors.

Target sequence (mouse)
shTNC: **CCAAACCATCTTCACAACAAT**
(pGIPZ (V2LMM_8781), Thermo Scientific)

Target sequences (human)
shTNC-1: **CGAAATTGAACTCTATGGAAT**
(pTRIPZ (V2THS_133232), Thermo Scientific)

shTNC-2: **CAGAGGTGACATGTCAAGCAA**
(pTRIPZ (V3THS_321947), Thermo Scientific)

shSRSF6-1: **CCGCAGTAGATCTCGAAGTAT**
(pTRIPZ (V2THS_20638), Thermo Scientific)

shSRSF6-2: **GAAATCTAGATCAAAGAGCAA**
(pTRIPZ(V2THS_20637), Thermo Scientific)

Supplementary References

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