Cullin5 destabilizes Cas to inhibit Src-dependent cell transformation

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Supplementary table

Table S1. qPCR primers used to quantify *socs* gene mRNA expression

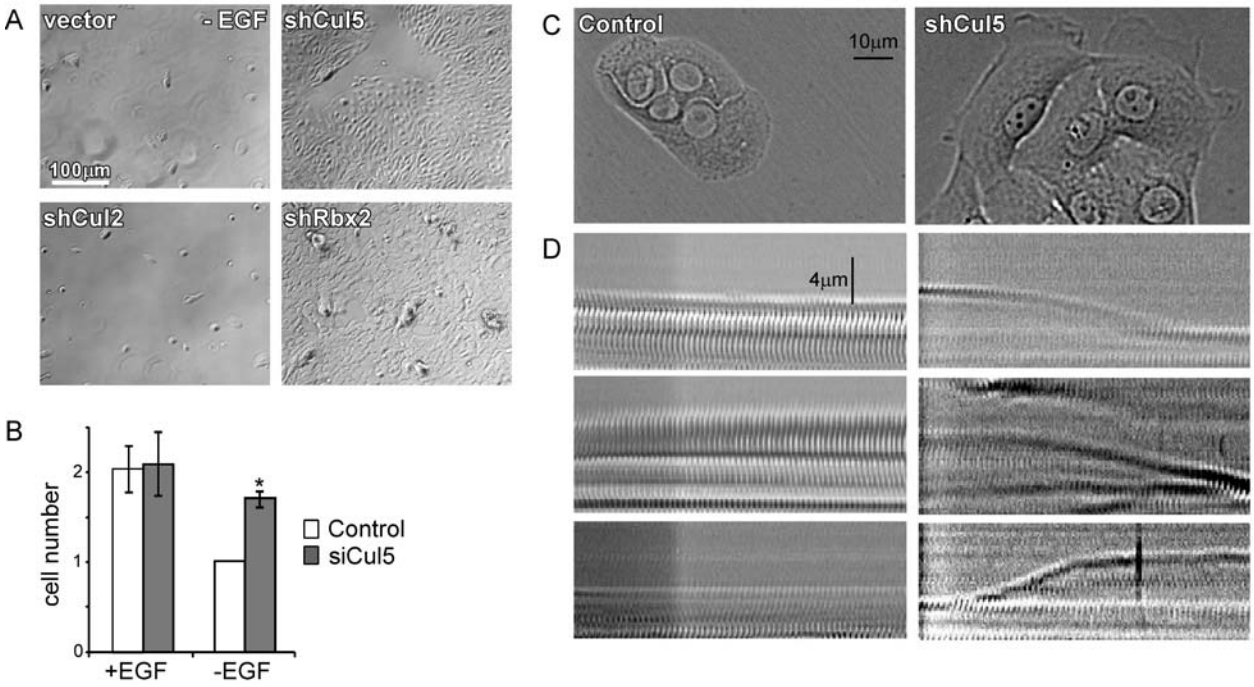


Fig S1. Cul5 regulates epithelial cell proliferation and membrane ruffling.

(A) Cul5- and Rbx2-deficient cells become EGF-independent. Phase contrast micrographs of vector, Cul5-, Cul2- and Rbx2-deficient MCF10A cells grown for 8 days in the absence of EGF. (B) Transient knockdown of Cul5 using siRNA induces EGF-independent growth. Cells were transiently transfected with control siRNA or an siRNA pool targeting the Cul5 sequences GACACGACGTCTTATATTA, CGTCTAATCTGTTAAAGAA, GATGATACGGCTTTGCTAA, GTTCAACTACGAATACTAA. *, $P < 0.05$ by *t* test. (C,D) Ruffling of shCul5 MCF10A cells plated at low density in monolayer culture. Images were recorded using cells plated on glass chamber slides and grown in media lacking EGF for 5 days. Cells were kept at 37C in a heated chamber during imaging and images captured every 15 s using Metamorph software. Kymographs were created using ImageJ.

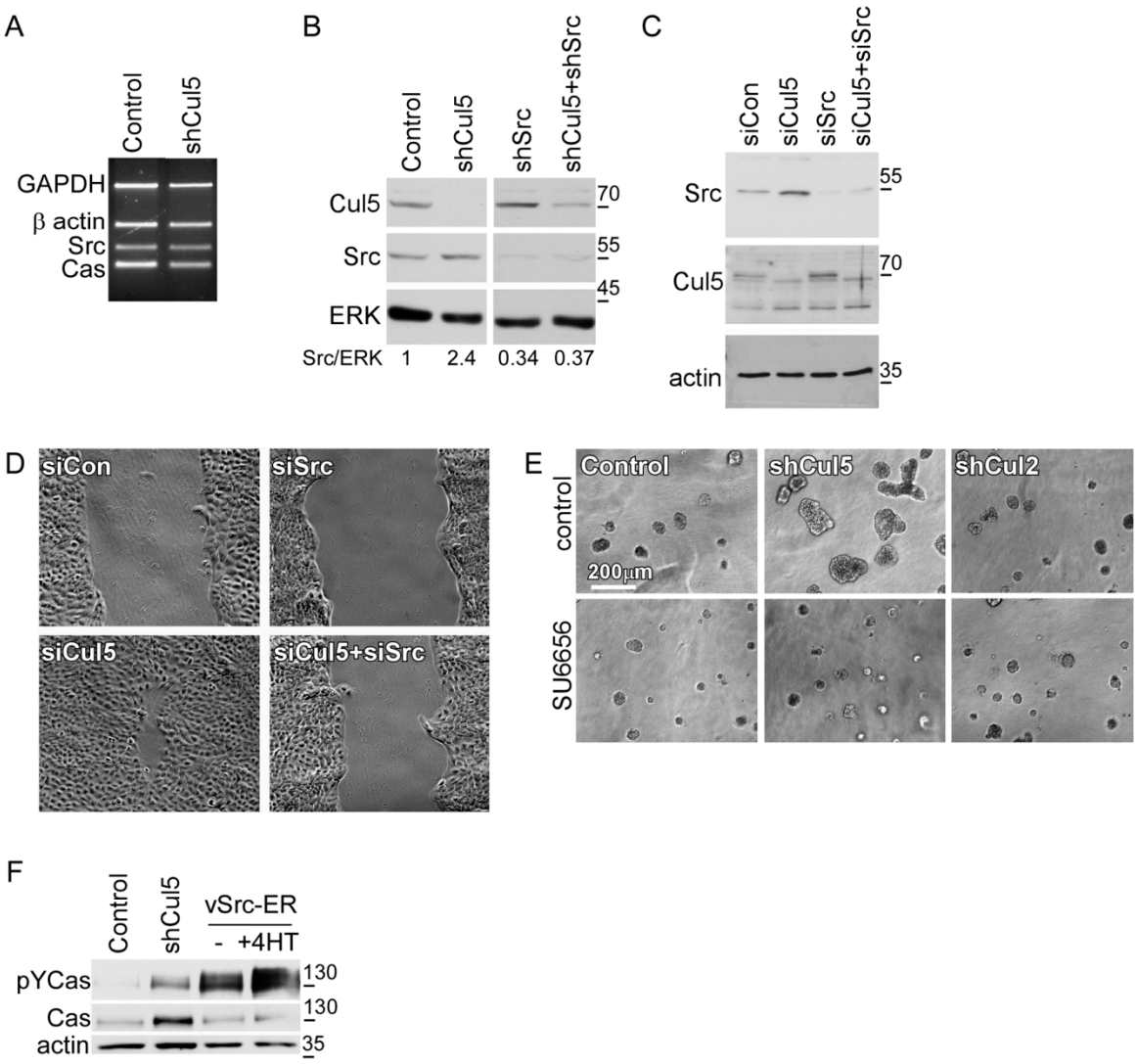
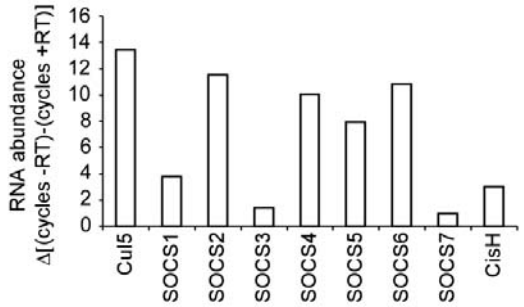


Fig S2. Src is necessary but not sufficient for EGF-independent migration and acini dysmorphogenesis of Cul5-deficient cells.

(A) RT-PCR analysis of Cas and Src mRNA levels, relative to β actin and GAPDH controls. (B) Knockdown of Src and Cul5 in stable cell lines expressing the respective shRNAs. (C) Knockdown of Src and Cul5 by a single round of transfection with their respective siRNAs. (D) Transient knockdown of Src inhibits migration of Cul5-deficient cells. Confluent monolayers of single and double knockdown MCF10A cells were transferred to EGF-deficient medium and scratched. Images were captured 24 h later. (E) Src kinase activity is required for dysmorphic acini. Phase contrast images of control, shCul5 and shCul2 acini grown for 12 days in the absence and presence of SU6656. (F) vSrc-ER induces robust Cas phosphorylation. Western blot analysis of lysates of stable lines of control and Cul5-deficient cells and cells expressing a vSrc-ER fusion protein.

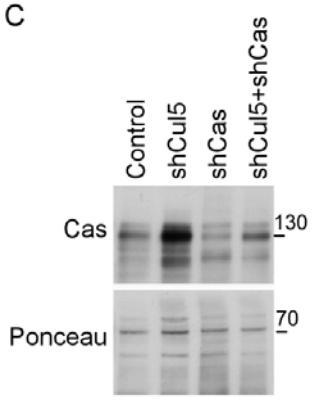
A



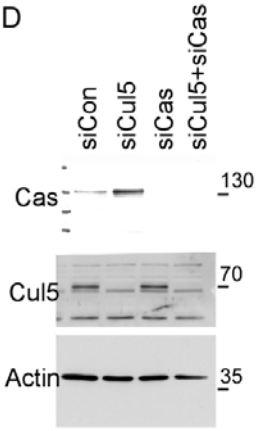
B

Protein	IP #	spectral count vector	shCul5	shCul2	peptide sequence	position
Cas	00011998	0	18	0	DIY"DVPPV; GLY"QVPGP	Y128;Y249
CDK1	00026689	0	9	0	GTY"GVVYK	Y15
ITSN2	00236575	0	7	0	LIY"LVPEK	Y552
EEF1A1	00396485	0	6	0	LAY"TLGVK	Y141
GIT1	00384861	0	4	0	AIY"SVHVP	Y545
RPS10	00008438	0	3	0	AIY"ELLFK	Y12
RDH16	00289551	0	2	0	DKY"VFITG	Y31
Transferrin R	00022462	0	2	0	LSY"TRFSL	Y20
CD40LG	00024686	0	2	0	ETY"NQTSP	Y5
Talin-1	00298994	0	2	0	LDY"YMLRN	Y70
FKBP4	00219005	0	1	0	KLY"ANMFE	Y411
CAV1	00009236	1	3	0	HLY"TVPIR	Y14
Cbl	00027269	0	1	1	AIY"DLAAR	Y674
KIAA1217	00103018	20	40	20	GFY"ADPYL	Y393
PTK2	00012885	8	16	8	STY"YKASK	Y576
paxillin	00220030	5	10	5	HVY"SFPNK	Y118
GRLF1	00334715	2	4	2	NIY"SVPHD	Y1105
EGFR	00018274	1	2	1	PDY"QQDFF	Y1172
PKP4	00021076	10	15	10	ATY"AEPYR	Y478

C



D



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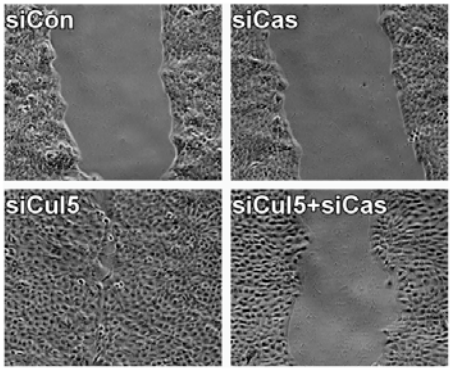

Fig S3. SOCS gene expression, phosphoprotein analysis, and the role of Cas in migration of Cul5-deficient cells.

(A) Expression of SOCS gene mRNAs in MCF10A cells. RNA was prepared from MCF10A cells, on-column treated with DNaseI, and cDNA was prepared in the presence or absence of reverse transcriptase. cDNA was quantified using SYBRgreen. Since different mRNAs amplified with different primer pairs cannot be compared one against the other, results are reported as

the difference in C_t due to the presence of reverse transcriptase. A small difference implies that the mRNA was scarcely detected above the non-specific background. By this measure, SOCS2, 4, 5 and 6 are the major SOCS genes expressed in MCF10A cells. (B) Phosphoproteins of Cul5-deficient cells. Protein names, IPI number, and spectral counts for pY peptides recovered using PY99 antibody from trypsin digests of proteins from control, Cul5- and Cul2-deficient cells. The list shows all proteins for which there were more pY peptides from Cul5-deficient cells than control or Cul2-deficient cells. Proteins are ordered according to their enrichment in Cul5-deficient cells relative to control cells (ratio [N+1]/[M+1], where N and M represent the number of peptides in Cul5-deficient and control samples, respectively). Note that this analysis does not distinguish the mechanism of regulation (protein synthesis, degradation or phosphorylation state), and the phosphopeptide sequences recovered are not necessarily sites for recognition by Cul5-CRLs. (C) Knockdown of Cas and Cul5 in stable cell lines expressing the respective shRNAs. (D) Knockdown of Cas and Cul5 by a single round of transfection with their respective siRNAs. (E) Transient knockdown of Cas inhibits migration of Cul5-deficient cells. Confluent monolayers of single and double knockdown MCF10A cells were transferred to EGF-deficient medium and scratched. Images were captured 24 h later.

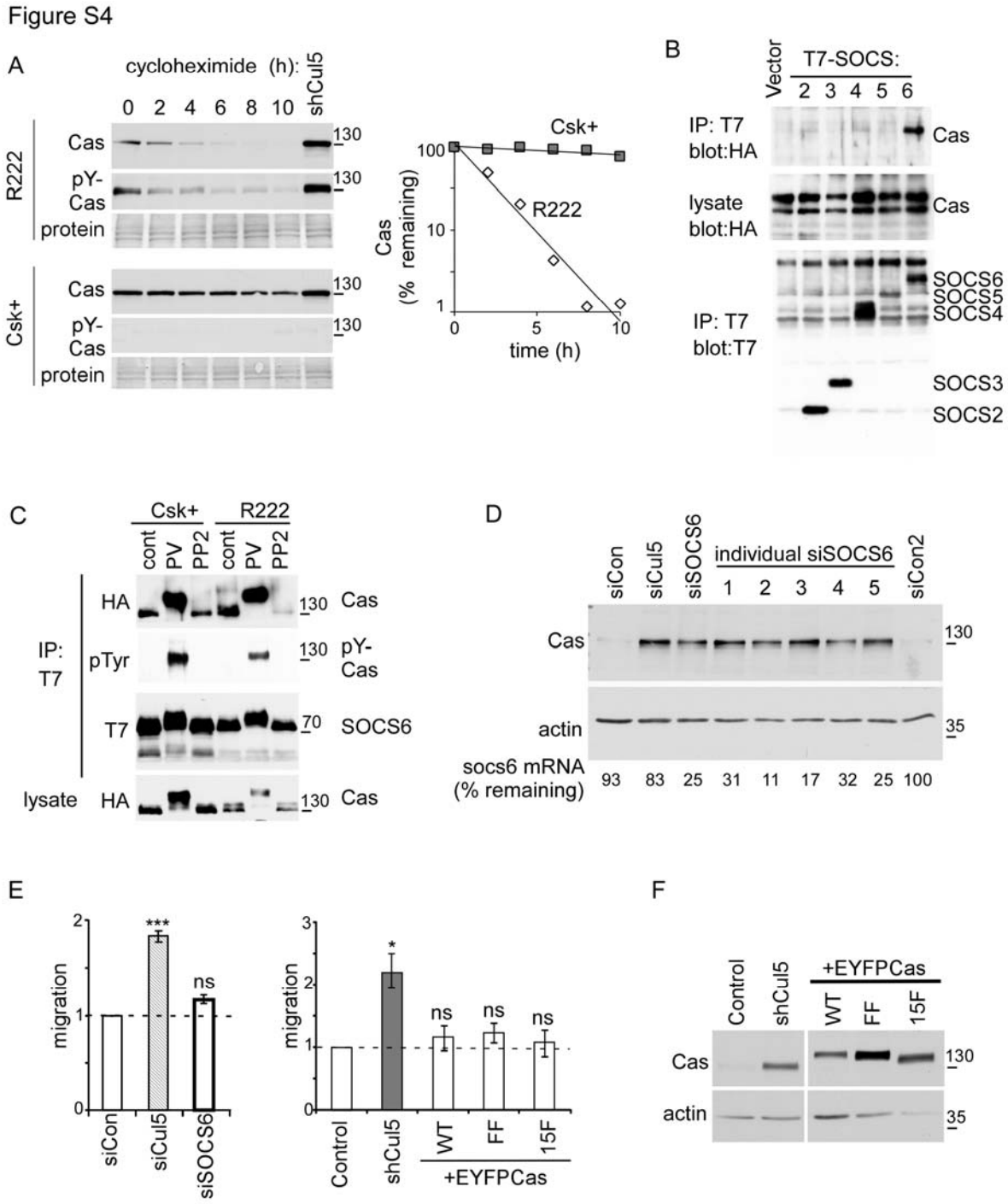

Fig S4. Cas half life, Cas binding to SOCS6 and Cul5, and controls for SOCS6 siRNA and Cas mutants.

(A) Regulation of Cas half life by Src activity. Western blots of Cas in lysates of CskR222 and Csk+ MEFs, in which endogenous Src is activated or repressed, respectively, treated for various times with cycloheximide to inhibit new protein synthesis. Anti-pY Cas blots show

increased phosphorylation of Cas in CskR222 cells, in which Cas has short half-life. Half-life quantification. Data points are averages of two independent experiments. (B,C) Association of pY-Cas with SOCS6. (B) Cas co-immunoprecipitates with SOCS6. Cul5-deficient, Csk-mutant MEFs were co-transfected with plasmids expressing HA-Cas and T7-tagged SOCS proteins. Cells were treated with pervanadate to stimulate tyrosine phosphorylation, lysed and immunoprecipitated with T7 antibodies. Cas was only detected in complexes with SOCS6. (C) SOCS6 association with Cas is regulated by tyrosine phosphorylation. Cul5-deficient MEFs containing repressed Src (Csk+) or activated Src (CskR222) were co-transfected with plasmids expressing HA-Cas and T7-SOCS6. Cells were treated with pervanadate (PV) to stimulate tyrosine phosphorylation or PP2 to inhibit Src family kinases for 30 min prior to lysis and immunoprecipitation of T7-SOCS6. Note the mobility shift caused by hyperphosphorylation of Cas in pervanadate-treated cells, and the lower level but more efficient co-precipitation of Cas when Src is activated. (D-F) SOCS6 and Cas mutant expression. (D) Different SOCS6 siRNA sequences inhibit expression of *socs6* mRNA and increase expression of Cas protein. A pool of four SOCS6 siRNAs (Qiagen) and single siRNAs targeting different SOCS6 sequences were transfected into MCF10A cells and Cas protein levels and *socs6* mRNA levels were compared. siRNA target sequences were: 1: 5'-TTGATCTAATTGAGCATTCAA-3' (Qiagen), 2: 5'-TTTAAGCTTGAGCTTTCGCTC-3' (Life Technologies), 3: 5'-GAACATGTGCCTGTCGTTA-3' (Thermo Fisher Scientific), 4: 5'-GTCCATAGTTGATCTAATT-3' (Thermo Fisher Scientific) and 5: 5'-GAAAGTATGCGCTGTCATT-3' (Thermo Fisher Scientific). Both control siRNAs were from Qiagen. Representative blots from one of three independent experiments are shown. *socs6* mRNA levels were determined by RT-qPCR. (E) Transient knockdown of SOCS6 or over-expression of WT, FF, 15F Cas does not stimulate migration. Confluent cell monolayers were transferred to EGF-deficient medium and scratched. Images were captured 24 h later and migration distance measured. Graphs show mean and standard error from three independent experiments. The data for siCon and siCul5 are the same as those shown in Fig 4A *, $P < 0.05$ and ***, $P < 0.001$ by *t* test. (F) Expression of EYFP-Cas wildtype and mutants. Note that wildtype EYFP-Cas is expressed at similar level to endogenous Cas in shCul5 cells, while CasFF is expressed at significantly higher level.

Table S1. qPCR primers used to quantify *socs* gene mRNA expression

	Forward	Reverse
Cul5	5'-TTTTATGCGCCCGATTGTTTTG-3'	5'-TTGCTGGGCCTTTATCATCCC-3'
SOCS1	5'-TTTTCGCCCTTAGCGTGAAGA-3'	5'-GAGGCAGTCGAAGCTCTCG-3'
SOCS2	5'-CAGATGTGCAAGGATAAGCGG-3'	5'-GCGGTTTGGTCAGATAAAGGTG-3'
SOCS3	5'-CCTGCGCCTCAAGACCTTC-3'	5'-GTCACTGCGCTCCAGTAGAA-3'
SOCS4	5'-CTCAGACTGAATTGAGGGATGGT-3'	5'-CACAGGGCTGAACATTGGTAT-3'
SOCS5	5'-GTGCCACAGAAATCCCTCAAA-3'	5'-TCTCTTCGTGCAAGTCTTGTTC-3'
SOCS6	5'-ATCACGGAGCTATTGTCTGGA-3'	5'-CTGACTCTCATCCTCGGGGA-3'
SOCS7	5'-CCAGGCCCTGAATTACCTCC-3'	5'-CGACTGAGGCGGATTTTGAAG-3'
CisH	5'-GAACTGCCCAAGCCAGTCAT-3'	5'-GCTATGCACAGCAGATCCTCC-3'
GUSB	5'-AGCGTGGAGCAAGA-3'	5'-ATACAGATAGGCAG-3'