**SUPPLEMENTARY MATERIAL AND METHODS**

**Cells and treatments**

MCs were isolated from human foreskin tissue as described 1,2. Briefly, the skin was cut into strips and treated with dispase (26.5 mL per preparation, activity: 3.8 U/mL; Boehringer-Mannheim, Mannheim, Germany) at 4°C overnight, the epidermis was removed, and the dermis finely chopped, and digested with 2.29 mg/mL collagenase (activity: 255 U/mg; Worthington, Lakewood, NJ, USA), 0.75 mg/mL hyaluronidase (activity: 1000U/mg; Sigma, Deisenhofen, Germany), and DNase I at 10 µg/mL (Roche, Basel, Switzerland). Cells were filtered stepwise from the resulting suspension. MC purification was achieved by anti-human c-Kit microbeads and the Auto-MACS separation device (both from Miltenyi-Biotec, Bergisch Gladbach, Germany), giving rise to 98-100% pure preparations (FACS double staining of KIT/FcεRI and acidic toluidine blue staining, 0.1 % in 0.5 N HCl), as described 3,4 (Figure 1B).

MCs were cultured in the presence of SCF (100 ng/ml) and IL-4 (20 ng/ml) freshly provided twice weekly when cultures were re-adjusted to 5x105/ml. 3-4 d after the last addition of cytokines, cells were deprived of growth factors (GFs) and FCS (minimal medium) for 16 h prior to the experiments. For each experiment, a different culture, originating from 2 to 15 donors, was used. For inhibition studies, cells deprived of GFs and FCS were pre-incubated with SCH772984 (ERK1/2 inhibitor), LY3214996 (ERK1/2 inhibitor), Vx-11e (ERK2 inhibitor), Pictilisib (PI3K inhibitor) or Trametinib (MEK1/2 inhibitor) each at 10 µM for 20 min (Enzo Life Sciences, Germany).

**LC-MS/MS**

**Sample preparation for phosphosite quantification by mass spectrometry**

Cell pellets from 3 replicates per condition were resuspended in sodium deoxycholate lysis buffer containing protease and phosphatase inhibitors (1% sodium deoxycholate, 100 mM Tris (pH 8.0), 1 mM EDTA, 150 mM NaCl, Roche complete, phosphatase inhibitor cocktail 2 and 3 (1:100, Sigma), 50 mM NaF, 10 mM dithiothreitol (DTT, Sigma), incubated for 10 minutes at 95°C and sonicated 2 times for 10 seconds. After centrifugation (15 minutes, 16,000 x*g*) the soluble fraction was collected and alkylated for 30 minutes using 40 mM chloroacetamide. To each sample 4 µg endopeptidase LysC (Wako, Osaka, Japan) and 4 µg trypsin (Promega, Mannheim, Germany) was added and incubated for 16 h at 37°C. The digestion was stopped by acidifying each sample to pH < 2.5 with trifluoroacetic acid (1% final concentration). Peptides were extracted and desalted using C18 cartridges (Empore, 3M). A small aliquot (10%) of each sample was desalted and stored using stage tip protocol 5.

**Phosphopeptide enrichment**

Phosphopeptide enrichment was performed as described previously 6. Briefly, iron-chelated IMAC beads were prepared from Ni-NTA superflow agarose beads (Qiagen, Hilden, Germany) that were stripped of nickel with 100 mM EDTA and incubated in an aqueous solution of 10 mM FeCl3 (Sigma). 100 μg dried peptides were reconstituted in 50% acetonitrile/0.1% trifluoroacetic acid and then diluted 1:1 with 100% acetonitrile/0.1% trifluoroacetic acid to obtain a final 80% acetonitrile/0.1% TFA peptide solution at a concentration of 0.5 μg/μL. Peptide mixtures were enriched for phosphorylated peptides with 10 μl IMAC beads per sample for 30 min. Enriched IMAC beads were loaded on equilibrated C18 silica-packed stage tips, washed twice with a solution of 80% acetonitrile/0.1% trifluoroacetic acid and 1% formic acid. Phosphorylated peptides were eluted from IMAC beads with 500 mM dibasic sodium phosphate, pH 7.0 (Sigma, S9763) and washed twice with 1% formic acid before being eluted from stage tips with 50% acetonitrile/0.1% formic acid. Samples were lyophilized in a vacuum concentrator and stored at -80°C until analysis by LC-MS/MS.

**LC-MS/MS analyses**

Peptide separation was done on a 20 cm reversed-phase column (75 µm inner diameter, packed with ReproSil-Pur C18-AQ; 1.9 µm, Dr. Maisch GmbH, Ammerbuch, Germany) using a 200 min gradient with a 250 nL/min flow rate of increasing Buffer B concentration (from 2% to 60%) on a High Performance Liquid Chromatography system (Thermo Fisher Scientific, Berlin, Germany). Peptides were measured on a Q Exactive HF-X instrument (Thermo Fisher Scientific). The mass spectrometer was operated in the data dependent mode with a 60K resolution, 3 x 106 ion count target and maximum injection time of 10 ms for the full scan, followed by TOP 20 MS2 scans with 15K resolution and 1 x 105 ion count target. Maximum injection time for phosphopeptide samples was 100 ms.

**Proteomics data analyses**

Raw data were processed using the MaxQuant software package (v1.6.2.6).7 The internal Andromeda search engine was used to search MS2 spectra against a decoy human UniProt database (HUMAN.2019-07, with isoform annotations) containing forward and reverse sequences. The search included variable modifications of oxidation (M), N-terminal acetylation, deamidation (N and Q), phosphorylation (S, T, Y) and fixed modification of carbamidomethyl cysteine. Minimal peptide length was set to seven amino acids and a maximum of three missed cleavages was allowed. The false discovery rate (FDR) was set to 1% for peptide and protein identifications. Unique and razor peptides were considered for quantification. Retention times were recalibrated based on the built-in nonlinear time-rescaling algorithm. MS2 identifications were transferred between runs with the “Match between runs” option, in which the maximal retention time window was set to 0.7 min. The integrated LFQ quantitation algorithm was applied. ProteinGroups and Phospho(STY)Site text files were used for further analyses. Entries were filtered to exclude reverse database hits, and potential contaminants. Statistical data analysis was performed using Perseus software (v1.6.2.1). Technical replicates for each condition were defined as groups. For phosphosite quantitation intensity values from Phospho(STY)Site output file were used, filtered for a minimum of 2 values in at least one group. After log2 transformation missing values were imputed with random noise simulating the detection limit of the mass spectrometer. Imputed values were taken from a log normal distribution with 0.3x the standard deviation of the measured, logarithmized values, down-shifted by 1.8 standard deviations. Differences in phosphosite abundance between groups were calculated using two-sample Student´s t test and significance was defined by a permutation-based FDR cut-off of 5%. Summaries for both replicates including novel sites are given in Excel-Files S1 and S2.

**Enrichment analyses**

KEGG pathways and GO term enrichment analyses were performed in Perseus using a hypergeometric test (Fisher´s exact test). Terms with more than 2 genes and an Benjamini Hochberg adjusted p-value < 0.02 were considered significant. The top ten enriched terms are shown in Figure S1B and complete lists are given in Excel-File 3.

**Immunoblot analysis**

After stimulation with SCF, MCs were collected by centrifugation and immediately solubilized in SDS-PAGE sample buffer and boiled for 10 min. Samples of equal cell numbers were subjected to immunoblot analysis. The following primary antibodies were purchased from Cell Signaling Technologies (Frankfurt am Main, Germany) and are as follows: anti-p-ERK1/2 (T202/Y204, #9101), anti-pp38 (T180/Y182, #9211), anti-pAKT (S473, #9271), anti-pSAPK/JNK (T183/Y185, #9251), anti-p-STAT5 (Y694, #9359), anti-ERK1/2 (#9102), anti-p38 (#9212), anti-AKT (#9272), anti-SAPK/JNK (#9252), anti-STAT5 (#25656), anti-β-Actin (#4967), and anti-Cyclophilin B (#43603). As detection antibody a goat anti-rabbit IgG peroxidase-conjugated antibody was administered (Merck, #AP132P). Proteins were visualized by a chemiluminescence assay (Weststar Ultra 2.0, Cyanagen, Bologna, Italy) according to the manufacturer’s instructions and bands were recorded on a chemiluminescence imager (Fusion FX7 Spectra, Vilber Lourmat, Eberhardzell, Germany). Semi-quantification of recorded signals was performed using the ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018). Individual intensity values for the detected proteins were normalized to the intensity of the housekeeping protein cyclophilin B of the same membrane.

For experiments presented in Figure 2A/F, we used times 0 (unstimulated control, “w/o”), 2, 5, 15, 30, 60 and 120 minutes to cover a very broad spectrum; for experiments shown in Figure 2B-E/G, we replaced 5 by 8 minutes, since 2 and 5 minutes appeared too close to each other, resulting in better kinetic resolution.

For SCF-mediated CIC degradation experiments, nuclear and cytoplasmic fractions from accordingly treated cells were prepared using the NE-PER buffer system (Thermo Fisher Scientific) according to the manufacturer’s instructions. Protein concentration was determined by the BCA (bicinchoninic acid) method (Thermo Fisher Scientific). Equal protein amounts of cytoplasmic (~15 µg) and nuclear fractions (~10 µg) were separated through 4–12% Bis-Tris gels (Thermo Fisher Scientific), transferred to nitrocellulose membranes, and the detection of CIC (#PA5-83721, Thermo Fisher Scientific) and beta-actin was performed as described above. Beta-actin was chosen as a loading control due to its presence in the cytosol as well as nucleus 8-10.

**Apoptosis**

All MC samples were incubated with the according inhibitors (or PBS as control for SCF “only”) for 20 minutes. To half of the samples SCF (10 ng/ml) was added (in formula named “treated”) and after 24 h, all samples (with SCF/”treated” and without SCF/“untreated”) were stained using YoProTM-1/PI(Thermo Fisher Scientific) according to the manufacturer’s protocol. The % rescue effect of SCF as given in Figure 3A was calculated as follows:

The right panel of Figure 3A (also showing an example of untreated cells) highlights the YoProTM-1/PI cells (sum of Q1 and Q2, red frame); the term apoptotic cells referred to as apoptotic cells throughout this study. For each culture the rescue effect was calculated. The following serves as an example: w/o SCF (“untreated”) we find 35 % apoptotic cells and with SCF (“treated”) 13 %. Entering the values in the formula, we arrive at around 62 % reduction of apoptosis given as % rescue effect of SCF. The strategy is in accordance with previous studies: 11,12.

**BrdU incorporation**

DNA duplication was assessed with the BrdU flow Kit (BD Bioscience, Heidelberg, Germany) as described13. In brief, MCs pre-treated with inhibitors or vehicle were kept in the presence (or absence) of 10 µM BrdU for 5 days and assayed for its incorporation.

**Flow cytometry**

Intracellular staining of p-ERK followed our protocol for intracellular staining of MCs14 and cells were measured on the MACSQuant® Analyzer10 (Miltenyi Biotec, Bergisch-Gladbach, Germany). The same anti-p-ERK1/2 (T202/Y204, #9101) antibody was used as for immunoblotting. As secondary antibody, a FITC-goat anti rabbit IgG was used (Jackson, #111-095-003). Data were analyzed with the FowJo analysis software (FlowJo LLC, Ashland, OR, USA).

**Reverse transcription-quantitative PCR (RT-qPCR)**

MCs (at 5 x105 cells/mL) in minimal medium were treated with inhibitors for 20 minutes prior to SCF addition for 25 minutes, after which time cells were harvested for RNA extraction. RT-qPCR was performed using optimized conditions. Briefly, RNA was isolated using the NucleoSpin RNA kit from Machery-Nagel (Düren, Germany) following the manufacturer’s instructions. cDNA synthesis (reverse transcription kit from Thermo Fisher Scientific), and qRT-PCR were performed as described elsewhere using materials from Roche (Roche Diagnostics, Mannheim, Germany)15. The primer pairs are summarized in table S1. The 2-ΔΔCT method was applied to quantify the relative expression levels of the target genes to three reference genes (appearing at the end of Table S1).

**Table S1** Primer pairs

|  |  |  |
| --- | --- | --- |
| gene | forward 5’-3’ | reverse 5’-3’ |
| ERK1 | CCATCAAGAAGATCAGCCCCT | AGGTCAGTCTCCATCAGGTCC |
| ERK2 | ACTGCGCTTCAGACATGAGA | TGCTGAGGTGTTGTGTCTTCA |
| TNF-α | TCTCGAACCCCGAGTGACAA | TCAGCCACTGGAGCTGCC |
| IL-8 | ATGACTTCCAAGCTGGCCGTGGCT | TCTCAGCCCTCTTCAAAAACTTCTC |
| OSM | GAGACTCATGACCAGGGGAC | CCCAGCTCCCACCTCTTAAA |
| LIF | GAACCTCTGAAAACTGCCGG | TTGGCTCCTGATCTGGTTCA |
| CIC | CCAATGGATCCTGCCACCTT | CACCAGTGTCTGCAGGATGT |
| Fos | AGTGACCGTGGGAATGAAGT | GCTTCAACGCAGACTACGAG |
| EGR1 | CTTCCCTTCCTCAGCTGTCA | TAGAGAGGGAGGACTTGGCT |
| JunB | GCCCGGATGTGCACTAAAAT | GACCAGAAAAGTAGCTGCCG |
| Beta-actin | CTGGAACGGTGAAGGTGACA | AAGGGACTTCCTGTAACAATGCA |
| CyclophilinB | AAGATGTCCCTGTGCCCTAC | ATGGCAAGCATGTGGTGTTT |
| GAPDH | ATCTCGCTCCTGGAAGATGG | AGGTCGGAGTCAACGGATTT |

**Accell® mediated RNA interference**

MCs were transfected twice by gene-targeting siRNA or non-targeting siRNA (at 1 µM for a total duration of 78 h in the case of ERK1 and ERK2) in Accell® medium (supplemented with Non-Essential Amino Acids and L-Glutamine). Briefly, 3 to 4 days after the last feeding, cells were transfected for the first time with 1 µM of each siRNA. The following siRNAs (“smart pool of 4”) were employed: ERK1 siRNA (E-003592-00-0050), ERK2 siRNA (E-003555-00-0050), and non-target siRNA (D-001910-10-50). After a 24-h-incubation, this process was repeated and cells were kept for a total of 78 h. After the incubation period, cells were stimulated with SCF (10 ng/mL) or PBS as control for 25 min (for RT-qPCR analysis), or 5, 8, 10 and 15 min (for immunoblot and FACS analysis). For the knockdown of CIC (“smart pool of 4”, CIC (E-015185-00-0050), the same protocol as given for ERK1 and ERK2 was used, i.e. cells were transfected twice, but they were harvested after 48 h when cell numbers were determined. The “% survival of plated” as given in Figure 5F was calculated for each culture and condition as follows:

The dotted lines in Figure 5F connect the siRNA treatments of the according culture and a paired t-test was used for analysis.

**ELISA**

MCs (at 1 x106 cells/mL) in minimal medium were treated with inhibitors for 20 minutes prior to SCF addition and supernatants were collected 24 h later. The LIF and OSM ELISAs were performed according to the manufacturer’s instructions (#BMS242 and #EHOSM, Thermo Fisher).

**Statistics**

Statistical analyses were performed with PRISM 8.0 (GraphPad Software, La Jolla, CA, USA). Unless specified otherwise in the figure legends, differences between more than two groups were calculated by RM one-way ANOVA with Dunnett’s multiple comparisons test. Comparisons between 2 groups were performed with Student’s t-test. p < 0.05 was considered statistically significant. Mass spectrometry data were normalized before further analysis. P-values were calculated by Student’s two tailed t-test. A statistically significant difference was concluded when the permutation-based FDR was below 5%. KEGG pathways and GO term enrichment analyses were performed using a hypergeometric test (Fisher´s exact test). Terms with more than 2 genes and an adjusted p-value < 0.02 were considered significant.

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**SUPPLEMENTARY FIGURE LEGENDS**

**Figure S1: The SCF regulated phosphoproteome in skin MCs (replicate 1)** A) Dynamics of SCF-regulated KIT sites. The phosphorylation dynamics of all detected KIT phosphosites (grey lines with according regulated phosphosite given on the right-hand side) were categorized into four groups: 1. early initiator sites – up-regulated/temporarily, 2. late effector sites - up-regulated/increasing, 3. continuous sites - up-regulated/stable, and 4. down-regulated site. B)Site distribution of pathway targets (Volcano plots depicting the distribution of regulated sites (red circles) on comparison to all sites in the according pathways (blue dots)) and pathway enrichment analyses/KEGG upon SCF stimulation (left panel, 8 min; right panel, 30 min; significance cut-off Benjamini-Hochberg 0.02). The precise events are specified in Excel-Files S1/S3.

**Figure S2: Sample overview of the main dataset (replicate 1)** A) Volcano plots depicting global protein expression upon SCF treatment (10 ng/ml). Upper panel 8 minutes after SCF administration; lower panel: 30 min after SCF administration. Red circles indicate proteins significantly altered in comparison to control. Note that KITLG (Kit ligand) is the gene name for SCF. B) upper panel: Clustering of samples; lower panel: a comparison of the Pearson correlation coefficients across samples in the phosphoproteome and in the global proteome shows relative stability of the latter.

**Figure S3:** **Overview of the replication dataset (replicate 2).** A) Overlap of identified P-sites with the main dataset (replicate 1) B) Volcano plots depicting the distribution of all regulated sites (red circles) and summary of all identified and SCF-regulated P-sites. C) Volcano plot depicting the distribution of regulated sites (red circles) at 8 min upon SCF on comparison to all sites in the pathway (blue dots). D) Site distribution of pathway targets (Volcano blots depicting the distribution of regulated sites (red circles) compared to all events in the pathways (blue dots)) and pathway enrichment analyses/KEGG (significance cut-off Benjamini-Hochberg 0.02). The precise events are specified in Excel-File S2.

**Figure S4: Inhibition of ERK- and PI3K-pathways counters proliferation.** BrdU-incorporation assays were performed over a 5-d-period. A) Representative dotplots showing the different phases of the cell cycle. B) Cumulative results from n=3-6 separate experiments given as mean ± S.E.M. Note that inhibition of ERK1/2 (SCH772984 10 µM) or PI3K (Pictilisib, 10 µM) increased the number of cells in G0/G1 phase, while the proportion of cells in G2/M phase was decreased or unchanged. \*\*\*\*p<0.0001, \*\*\*p<0.001, \*p<0.05 (mixed-effects analysis)

**Figure S5: SCF-triggered induction of immediate early genes and cytokines depends on the MEK/ERK cascade.** MCs were deprived of growth factors (16 h) and inhibitors (ERK1/2 LY3214996, MEK1/2 Trametinib, each at 10 µM) were applied prior to SCF-stimulation (10 ng/ml). A) IEGs and B) cytokines were quantified by RT-qPCR after 25 min and are given as fold increase relative to the unstimulated control. C) Concentrations of LIF and OSM protein in supernatants, as measured by ELISA after 24 h. Data is shown as mean ± S.E.M.; n=5-8, \*\*\*\*p<0.0001, \*\*\*p<0.001, \*\*p<0.01 (RM-ANOVA)

**Figure S6: Knockdown of ERK1/2 compromises MC survival.** Silencing of ERK1, or ERK2, or both combined was performed by an adapted protocol of our original RNAi-approach. A) ERK1 (left panel) and ERK2 (right panel) mRNA expression upon individual and combined silencing. B) Representative pERK1/2 FACS analysis for individual and combined silencing of ERK1 and ERK2. C) Knockdown of ERK1/2 compromises MC recovery. The data are presented as mean ± S.E.M., n=5-8, \*\*\* p<0.001, \*\*p<0.01 (RM-ANOVA; mixed-effects analysis)

**Figure S7: OSM and LIF, two IL-6 family cytokines, are expressed in human skin MCs**

The data are derived from the FANTOM5 atlas encompassing over 1,800 individual samples 16-18. A+B) Left panels: samples are presented using the zenbu browser19. The samples are arranged by expression level (tpm = transcripts per million) and due to space limitations only the top parts of the lists are shown. Right panels: Samples are given in alphabetical order and zoomed in on mast cells. A) OSM is manly detected in stimulated (IgER-crosslinking) samples (donor 1, 5 and 8) whereas LIF (B) is already expressed at baseline and further inducible by stimulation.

**Figure S8: CIC is under the top 20 proteins most strongly regulated by SCF regarding number of phosphosites** A) Overview of phosphorylation sites per protein (replicate 1). The distribution of phosphoproteins based on the number of phosphosites per protein is given for all Class 1 sites (upper panel) and the regulated sites (lower panel). Main figures below: absolute numbers, smaller figures above: calculated as percentage of all proteins. B) Top 20 phosphorylated proteins upon SCF. The upper panel gives the top 20 proteins with the highest number of detected phosphosites (independently of induction by SCF) and the lower panel shows the top 20 proteins with the highest number of SCF-regulated sites. Note that CIC has even slightly more regulated sites than KIT.

**Figure S9: CIC shuttles from the nucleus to the cytoplasm and is degraded upon SCF treatment** MCs were deprived of growth factors (16 h) and stimulated with SCF (10 ng/ml) with or without pre-treatment with ERK1/2 inhibitor (SCH772964, 10 µM) for the indicated times. Cytosolic and nuclear fractions were prepared using the NE-PER buffer system and resolved by SDS-PAGE side-by-side. Image J based semi-quantification of the detected signals for CIC and its degradation products was performed and data is given as mean ± SEM of 3-5 experiments resulting from individual MC cultures. A) Kinetics of SCF-provoked CIC shuttling from the nucleus to the cytoplasm. B) Inhibition of ERK1/2 prevents the SCF-mediated CIC shuttling (left panel: short isoform CIC-S; right panel: long isoform CIC-L). C) Inhibition of ERK1/2 prevents the SCF-mediated CIC degradation. CICdegrP1-3 = CIC degradation products 1-3. The quantifications correspond to Main Fig. 5.

**Figure S10: CIC degradation is independent of proteasomal function but sensitive to protease inhibition** MCs were deprived of growth factors (16 h), different proteasome inhibitors (bortezomib - borte, carfilzomib - carfil or MLN-2238 – MLN; each 10 µM) or Phenylmethylsulfonylfluorid (PMSF, a protease inhibitor; 500 µM) were added for 30 minutes before a 15-minute stimulation with SCF (10 ng/ml) was performed. Cytosolic and nuclear fractions were prepared using the NE-PER buffer system and resolved by SDS-PAGE side-by-side. A) None of the proteasome inhibitors interfered with SCF-induced cytoplasmic cycling or degradation of CIC. B) PMSF did not interfere with SCF-induced cytoplasmic cycling of CIC but did stabilize both isoforms and prevented CIC degradation (less degradation products); short isoform CIC-S, long isoform CIC-L, CICdegrP1-3 = CIC degradation products 1-3.