Predicting probability of tolerating discrete amounts of peanut protein in allergic children using epitope-specific IgE antibody profiling

Maria Suprun | Paul Kearney | Clive Hayward | Heather Butler | Robert Getts | Scott H. Sicherer | Paul J. Turner
Dianne E. Campbell | Hugh A. Sampson

ARTICLE SUMMARY

- Existing diagnostic testing is not predictive of severity or the threshold dose of clinical reactivity, and many patients still require an Oral Food Challenge (OFC). While OFCs are very useful for making an allergy diagnosis and determining clinical reactivity, they often cause anaphylaxis, which can increase patient anxiety. and are time and resource intensive.[1]
- An extensive validation was performed across 5 cohorts (all with confirmed oral food challenge results) across six different countries. Cohorts used: BOPI, OPIA, CAFETERIA, CoFAR6, and PEPITES with specimens from Australia, UK, US, Ireland, and Germany.
- This paper reports the first validated algorithm using two key peanut specific IgE epitopes to predict probabilities of reaction to different amounts of peanut in allergic subjects and may provide a useful clinical substitute for peanut oral food challenges.
- Using the algorithm, subjects were assigned into "high", "moderate", or "low" dose reactivity groups. On average, subjects in the "high" group were 4 times more likely to tolerate a specific dose, compared to the "low" group.[1] For example, 88% of patients in the high dose reactivity group were able to tolerate ≥ 144 mg of peanut protein whereas only 29% were able to tolerate the same amount in the low dose reactivity group.[1-2]

CLINICAL CONSIDERATIONS

- The new epitope test offers more granular information to help clinicians stratify treatment and peanut avoidance plans for their patients.
- See below for summary of clinical considerations based on threshold reactivity level.[1]

allergenis peanut diagnostic result	clinical considerations[1]
likely allergic – low dose reactor	• inform or avoid oral food challenge to reduce risk of anaphlyaxis • confirm strict avoidance of peanut • consider immunotherapy to reduce risk of reaction
likely allergic – moderate dose reactor	• consider a single oral food challenge (30 to 100 mg) to reduce anxiety and improve quality of life • less stringent avoidance of peanut regime • consider inclusions of precautionary labeled foods such as 'May contain peanut' • consider immunotherapy to reduce risk of reaction
likely allergic – high dose reactor	• consider a single oral food challenge (100 to 300 mg) to reduce anxiety and improve quality of life • less stringent avoidance of peanut regime • consider inclusions of precautionary labeled foods such as 'May contain peanut' • consider starting immunotherapy at higher doses to shorten time to maintenance dose
unlikely allergic	• oral food challenge to rule out the diagnosis of peanut allergy

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ORIGINAL ARTICLE

Basic and Translational Allergy Immunology

The SCF/KIT axis in human mast cells: Capicua acts as potent KIT repressor and ERK predominates PI3K

Kristin Franke[1,2] | Marieluise Kirchner[3] | Philipp Mertins[3] | Torsten Zuberbier[1,2] | Magda Babina[1,2]

[1]Institute of Allergology, Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany

[2]Fraunhofer Institute for Translational Medicine and Pharmacology ITMP, Immunology and Allergology IA, Berlin, Germany

[3]Core Unit Proteomics, Berlin Institute of Health at Charité- Universitätsmedizin Berlin and Max Delbrück Center for Molecular Medicine (MDC), Berlin, Germany

Correspondence
Magda Babina, Institute of Allergology, Corporate Member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Fraunhofer Institute for Translational Medicine and Pharmacology ITMP, Immunology and Allergology IA, Hindenburgdamm 27, 12203 Berlin, Germany.
Email: magda.babina@charite.de

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Abstract

Background: The SCF/KIT axis regulates nearly all aspects of mast cell (MC) biology. A comprehensive view of SCF-triggered phosphorylation dynamics is lacking. The relationship between signaling modules and SCF-supported functions likewise remains ill-defined.

Methods: Mast cells were isolated from human skin; upon stimulation by SCF, global phosphoproteomic changes were analyzed by LC–MS/MS and selectively validated by immunoblotting. MC survival was inspected by YoPro; BrdU incorporation served to monitor proliferation. Gene expression was quantified by RT-qPCR and cytokines by ELISA. Pharmacological inhibitors were supplemented by ERK1 and/or ERK2 knockdown. CIC translocation and degradation were studied in nuclear and cytoplasmic fractions. CIC's impact on KIT signaling and function was assessed following RNA interference.

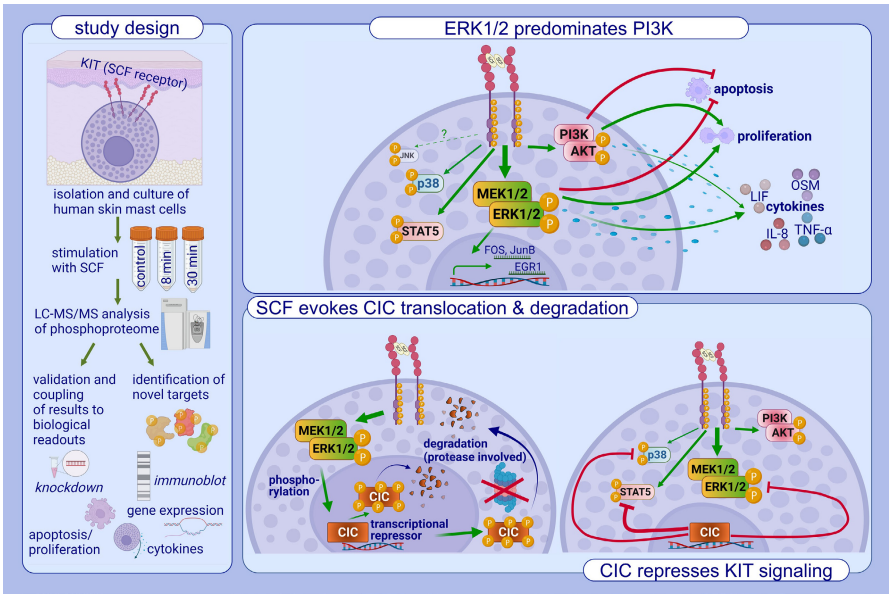
Results: ≈5400 out of ≈10,500 phosphosites experienced regulation by SCF. The MEK/ERK cascade was strongly induced surpassing STAT5 > PI3K/Akt > p38 > JNK. Comparison between MEK/ERK's and PI3K's support of basic programs (apoptosis, proliferation) revealed equipotency between modules. In functional outputs (gene expression, cytokines), ERK was the most influential kinase. OSM and LIF production was identified in skin MCs. Strikingly, SCF triggered massive phosphorylation of a protein not associated with KIT previously: CIC. Phosphorylation was followed by CIC's cytoplasmic appearance and degradation, the latter sensitive to protease but not preoteasome inhibition. Both shuttling and degradation were ERK-dependent. Conversely, CIC-siRNA facilitated KIT signaling, functional outputs, and survival.

Conclusion: The SCF/KIT axis shows notable strength in MCs, and MEK/ERK as most prominent module. An inhibitory circuit exists between KIT and CIC. CIC stabilization in MCs may turn out as a therapeutic option to interfere with allergic and MC-driven diseases.

Abbreviations: Akt, Protein kinase B; CIC, Capicua; EGR1, Early growth response 1; ERK, Extracellular signal-regulated kinase; FOS, Fos proto-oncogene; IL-8, Interleukin 8; JNK, c-Jun N-terminal kinase; JunB, JunB proto-oncogene; KIT, SCF-receptor; LC–MS/MS, Liquid chromatography tandem mass spectrometry; LIF, Leukemia inhibitory factor; MAPK, Mitogen-activated protein kinase; MEK, MAPK kinase; OSM, Oncostatin; PI3K, Phosphoinositide-3-kinase; SCF, Stem cell factor; STAT5, Signal transducer and activator of transcription 5; TNF-α, Tumor necrosis factor alpha.

Kristin Franke and Marieluise Kirchner contributed equally.

KEYWORDS
capicua, KIT, mast cells, RTKs, signal transduction



GRAPHICAL ABSTRACT

This study presents the first global phosphoproteome downstream of the SCF/wildtype-KIT axis in mast cells detecting over 5400 regulated sites in known and novel targets. The MEK/ERK-module is potently activated predominating PI3K/Akt; functional programs preferentially depend on ERK while in proliferation and anti-apoptosis both modules are equivalent. Being phosphorylated and degraded following SCF, CIC is uncovered as potent repressor of KIT signaling.

Abbreviations: Akt, Protein kinase B; CIC, Capicua; EGR1, Early growth response 1; ERK, Extracellular signal-regulated kinase; FOS, Fos proto-oncogene; IL-8, Interleukin 8; JNK, c-Jun N-terminal kinase; JunB, JunB proto-oncogene; KIT, SCF-receptor; LC-MS/MS, Liquid chromatography tandem mass spectrometry; LIF, Leukemia inhibitory factor; MAPK, Mitogen-activated protein kinase; MEK, MAPK kinase; OSM, Oncostatin; PI3K, Phosphoinositide-3-kinase; SCF, Stem cell factor; STAT5, Signal transducer and activator of transcription 5; TNF-α, Tumor necrosis factor alpha

1 | INTRODUCTION

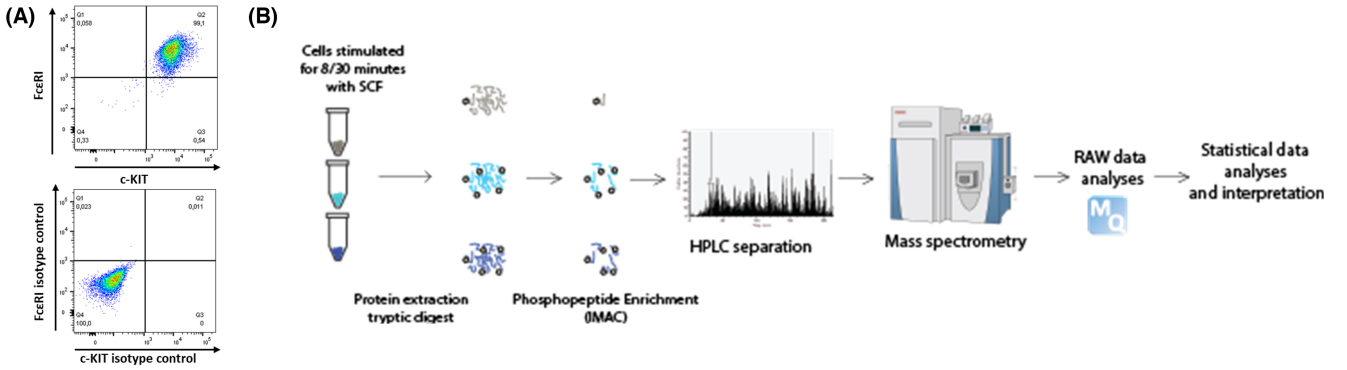
Mast cells (MCs) are principal effector cells of IgE-mediated type-I-hypersensitivity, encompassing allergic rhinitis, asthma, food allergy, and anaphylaxis.[1,2] In the skin, MCs are additionally associated with itch sensations, forming operating units with sensory neurons.[3–6] Thereby, MCs are also intimately linked to urticaria, and atopic dermatitis.[7–11] MCs either arise from the yolk sac or originate in the bone marrow.[12–15] The (c-)KIT/SCF (stem cell factor) receptor tyrosine kinase (RTK)/ligand pair is tightly linked to mast cell (MCs) development, survival, and function.[13,16,17] The binding of the specific ligand stem cell factor (SCF) activates its intrinsic tyrosine kinase activity and induces phosphorylation at key amino acid residues, thereby initiating multiple pro-survival and function-molding downstream signaling pathways.[18,19] When aberrantly (re-)activated and/or mutated, KIT is highly oncogenic and somatic gain-of-function mutations (especially the D816V mutation) are causal for systemic mastocytosis.[13,18,20]

Despite substantial research efforts in SCF-triggered signal transduction, a comprehensive view of the molecular underpinnings downstream of KIT activation in MCs is missing. Since reversible phosphorylation dictates and directs almost all cellular processes.[21] We set out to investigate phosphorylation dynamics elicited by SCF in human skin MCs (characterized by abundant expression of KIT [Figure 1A]) with the aim to estimate the importance of distinct cascades and to identity new targets to interfere with MC associated diseases.[22] The wide-scale view of cellular phosphorylation networks initiated by KIT served as basis to experimentally associate specific modules to functional programs. Dense phosphosite clustering in the nuclear protein CIC, which acts as a tumor suppressor,[23,24] allowed the disclosure of a novel repressor potently interfering with SCF signaling, MC survival, and functional outputs.

2 | MATERIAL AND METHODS

2.1 | Cells and treatments

Mast cells were isolated from human foreskin tissue as described.[25] Each mast cell preparation/culture originated from several donors, as routinely performed. Skin specimens were obtained from circumcisions with written, informed consent of the patients or legal guardians and approval by the university ethics committee (protocol code EA1/204/10, 9 March 2018). The experiments were conducted according to the Declaration of Helsinki Principles. Refer to the Appendix S1 for further details.



(C)

site	class 1	percent	SCF-regulated 8'	percent	SCF-upregulated 8'	percent	SCF-downregulated 8'	percent	SCF-regulated 30'	percent	SCF-upregulated 30'	percent	SCF-downregulated 30'	percent
all	10516	100	3446	100	2932	100	514	100	4708	100	4162	100	546	100
pSer	9282	88.27	3037	88.13	2582	88.06	455	88.52	4193	89.06	3722	89.43	471	86.26
pThr	1079	10.26	349	10.13	293	9.99	56	10.89	435	9.24	366	8.79	69	12.64
pTyr	155	1.47	60	1.74	57	1.94	3	0.58	80	1.70	74	1.78	6	1.10

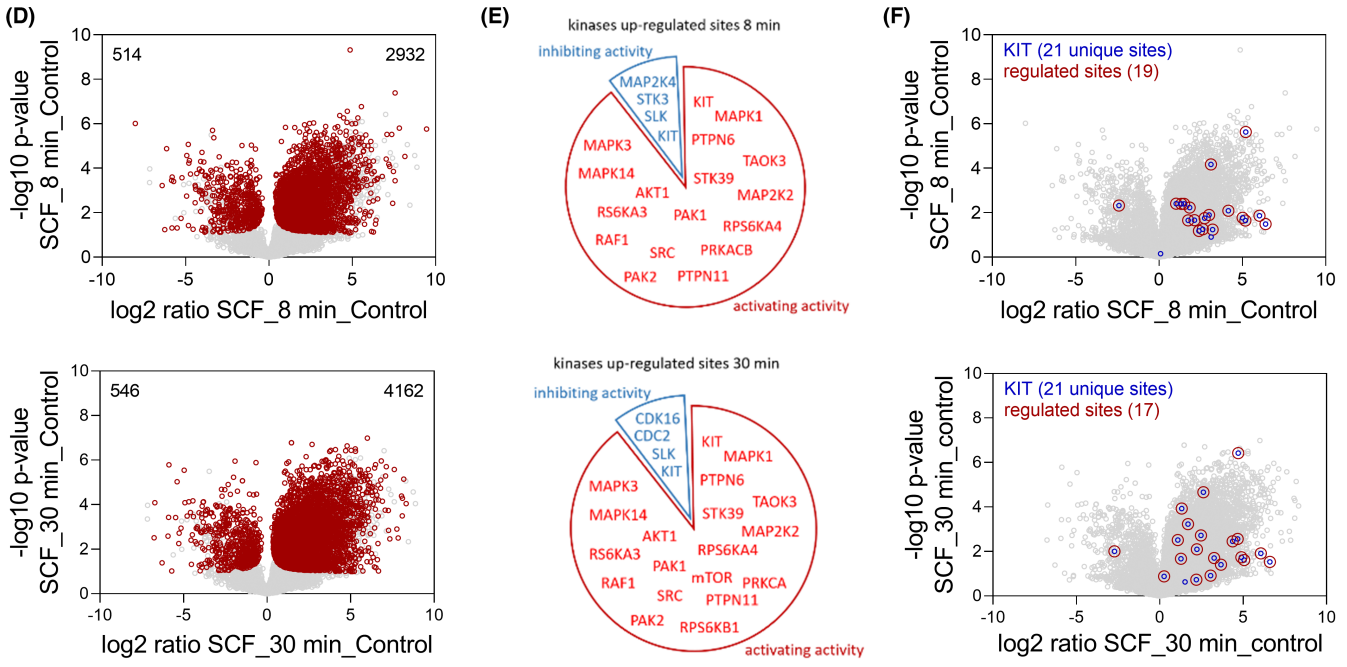


FIGURE 1 SCF regulated phosphoproteome in skin MCs (A) Double staining of KIT and FcεRI shows purity of MC cultures and a high degree of KIT expression. (B) Workflow of the phosphoproteome analysis (LC–MS/MS). (C) Summary of all identified and SCF-regulated phosphosites. (D) Volcano plots depicting the distribution of regulated sites (red circles) at 8 (upper panel) and 30 min (lower panel) after SCF stimulation, respectively, as well as all sites pertaining to the pathway (blue dots). (E) Kinases with regulated sites and known regulatory function. (F) SCF-regulated KIT sites (red circles) at 8 (upper panel) and 30 min (lower panel) after SCF stimulation, respectively. The precise events are specified in File S1

2.2 | Phosphoproteome analyses by LC–MS/MS

1×10^7 MCs per group were stimulated with SCF (10 ng/ml) (or PBS as control) for 8 or 30 min, lysed, trypsin digested, and desalted, and phosphopeptide enrichment was performed using IMAC. Peptides were separated on a 20-cm-reversed-phase column connected to a High-Performance Liquid Chromatography system (Thermo Fisher Scientific). The Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific) was operated in the data-dependent mode with a full scan in the Orbitrap followed by top 20 MS/MS scans using higher-energy collision dissociation. Data were analyzed using the MaxQuant software package with a decoy human UniProt database. For detailed description, see Appendix S1. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[26] partner repository with the dataset identifier PXD028245.

A more detailed description of the following methods can be found in the Appendix S1: Apoptosis assessment, BrdU incorporation, reverse transcription-quantitative PCR (RT-qPCR), flow cytometry, immunoblot analysis, ELISA, siRNA interference, and statistics (using PRISM 9.0 [GraphPad Software]). Basic information regarding the methods and statistical analyses are also given in the respective figure legends.

3 | RESULTS

3.1 | The SCF/KIT axis is an efficient inducer of phosphorylation

To get a glimpse into the phosphosignaling network downstream of KIT in skin MCs (Figure 1A), we first used phosphopeptide enrichment coupled to MS/MS-based quantitative proteomics (Figure 1B). Of the resulting 18,461 phosphosites (P-sites) 10,516 qualified as high confidence class-I-sites (localization probability >0.75) and were used for quantification.

As expected, the relative frequency of phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY) (Figure 1C) was 88.27:10.26:1.47 for S/T/Y sites and therefore consistent with other studies.[27–29]

3446 sites (≈1600 proteins) were significantly regulated upon 8 min, with 514 down- and 2932 upregulated (Figure 1C/D). At the 30-min-point, 4708 sites changed in abundance (546 down- and 4162 upregulated). In total, 5512 phosphosites out of 10,516 were affected by SCF at the two times combined, corresponding to 51.5%, a remarkable rate highlighting the strength of the SCF/KIT axis. Conversely, SCF had no impact on global protein expression as expected at these short intervals (Figure S2A). Data inspection revealed high reproducibility within replicates (sample clustering and Pearson correlation ≈0.80), confirming high quality of the datasets (Figure S2B). To determine biological reproducibility, results of another culture were integrated. ≈80% of the P-sites from the second replicate did overlap with replicate 1 (Figure S3A) demonstrating high reproducibility across datasets.

The activity of many kinases often requires their own phosphorylation. Of 171 kinase- and 40 phosphatase-derived phosphopeptides, 94/22 (kinases/phosphatases) were differently phosphorylated at 8 and 113/24 at 30 min (File S1). 28 and 30 sites with positive impact on activity were upregulated at 8/30 min (Figure 1E). Examples of upregulated P-sites encompass KIT itself, MAPK1/ERK2, and MAPK3/ERK1. Well-documented KIT sites[18] were reproduced in our dataset (Figure 1F, File S1, S2 and Figure S3C). Y568 and Y570, the two instant autophosphorylation sites, were highly upregulated at 8 min. Y703 (linked to Grb2/Ras/ERK activation), showed the strongest induction (fold change [FC] ~66), while Y721 (bound by PI3K-p85) was moderately phosphorylated (FC ~2.5–3.7). Phosphorylation dynamics identified 1. early initiator sites (Y568, S959, Y570, Y721, S943), 2. late effector sites (Y730, Y609, Y936), 3. continuous sites (Y703, S746), and 4. one downregulated site (S967) (Figure S1A, File S1).

Overall, the mitogen-associated protein kinase (MAPK) module was the most prominent GO term in pathway enrichment analysis of upregulated sites (Figure S2B, File S3). The results were confirmed in replicate 2 (Figure S3D).

Accordingly, the highest phosphorylation FC (≈115–220) was detected for the two activating sites (T185/Y187) of ERK2 at both times. The sites of ERK1 (T202/Y204) showed a lesser but still prominent FC (≈13–32). Conversely, p38 (MAPK14) sites T180/Y182 were only moderately modified (FC ≈ 3–5), while those in JNK1 (MAPK8) were not detected. Within PI3K/AKT, S259 of PIK3C2A was phosphorylated (FC ≈ 48/90), but of the three known AKT isoforms,[30] only AKT1 (S124) was modestly affected (File S1). The other well-described AKT1 phosphosites S473 and T308, indicative of activation, were not detected, likely due to limitations in analytical sensitivity. S473 could, however, be detected by immunoblotting (Figure 2A/C). Immunoblotting and semi-quantification confirmed all above-described results (Figure 2A–E). STAT5 phosphorylation (Y694) was likewise validated (Figure 2F/G).

Receptor tyrosine kinase stimulation can elicit profound changes in the transcriptional landscape. We inspected P-sites within the repertoire of transcription factors (TFs) (File S1). Notably, for several lineage-defining entities (MITF, MYB, TAL, GATA2),[31,32] the phosphorylation status was altered by SCF.

3.2 | ERK and PI3K pathways independently orchestrate survival and proliferation

As expected, SCF exerted a pro-survival effect on mast cells reducing apoptosis by ≈60% (Figure 3A). Both Ras–ERK and PI3K/AKT reportedly contribute to survival and proliferation.[18,19] Here, inhibition of either ERK or PI3K individually did not modulate the short-term rescue effect of SCF. However, concomitant suppression of both kinases strongly interfered with survival promotion (Figure 3A).

BrdU-incorporation revealed that inhibition of ERK1/2 or PI3K alone countered SCF-supported proliferation and the proportion of S-phase cells (Figures 3B/C and S4A/B), concurrently increasing apoptotic cells over this 5-days-period (Figure 3C). Accordingly, the proportion of cells in G1/G0 among the survivors increased (Figure S4B). Again, effects on proliferation were augmented when both pathways were inhibited simultaneously. Therefore, intact ERK or PI3K activity can maintain MC survival and proliferation to a certain degree when the other kinase is suppressed, while the concurrent inhibition of both modules leads to full-scale cell demise.

3.3 | ERK activity is essential for SCF-stimulated immediate early genes and cytokines

The immediate-early genes (IEGs) Fos, Egr1, and JunB are typically upregulated rapidly. They were vastly induced by SCF, while inhibition of ERK (but not of PI3K) completely prevented this induction (Figure 4A). Suppression was not only achieved by SCH772984

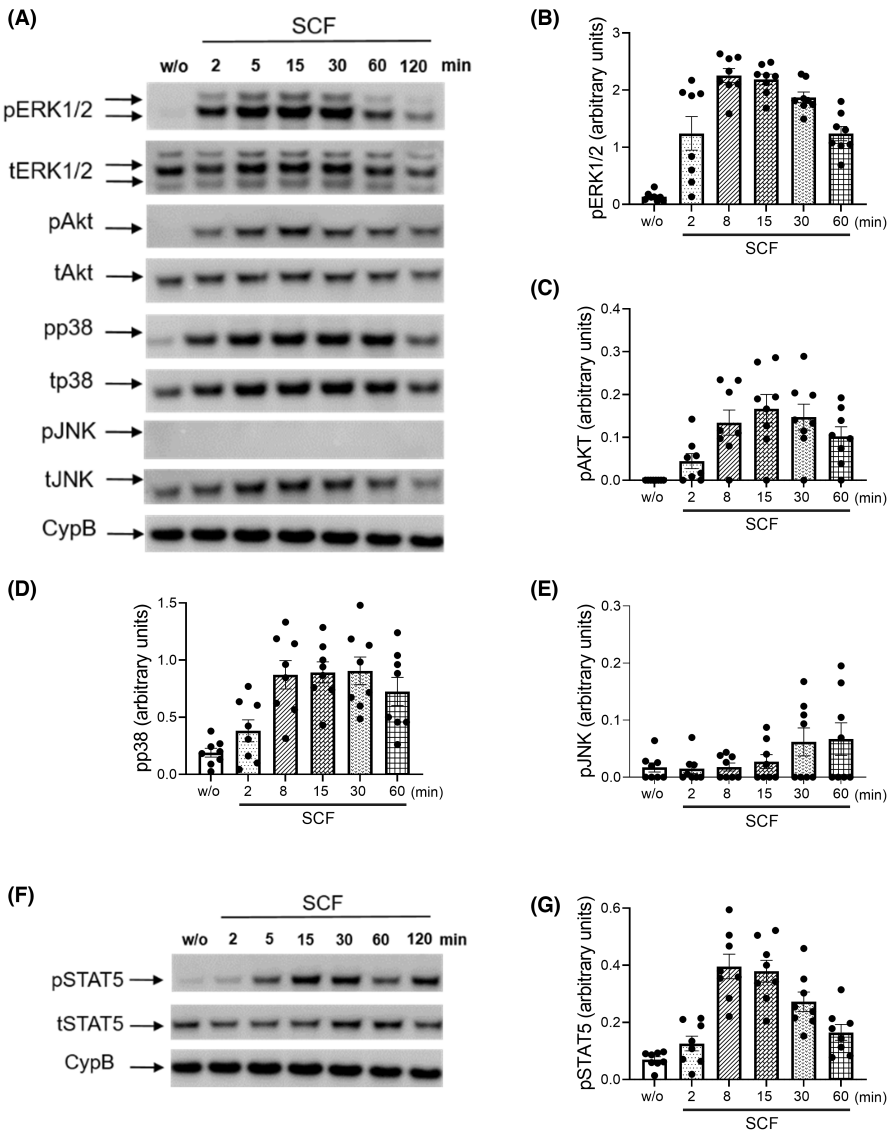


FIGURE 2 ERK1/2 is rapidly and heavily phosphorylated after SCF treatment. MCs were deprived of growth factors (16 h), and phosphorylation kinetics of signaling components upon SCF stimulation (10 ng/ml) were detected by immunoblot and analyzed using Image J. (A) One representative blot (consecutive detection of the respective proteins on the same membrane) for the according kinases is shown. (B–E) Image J based semi-quantification of the detected signals for pERK1/2 (B), pAkt (C), pp38 (D), and pJNK (E). (F) One representative blot for pSTAT5 and (G) its semi-quantification using Image J are given. Regarding the semi-quantitative analysis, each dot represents an independent MC preparation. Data are presented as mean ± SEM of 8 experiments for each time point (arbitrary units, calculated as explained in Appendix S1). CypB = cyclophilin B (loading control)

(ERK1/2 inhibitor), but similarly by Vx-11e (ERK2-selective). Further validation came from the use of additional pharmacological inhibitors (Figure S5A).

We used RNA interference to further verify the involvement of ERK (Figure S6A/B). Due to the long half-life of ERK1/ERK2 (68/53 h, respectively[33]), their knockdown required some adaptations of our original protocol.[34] Interestingly, cell recovery was compromised, especially when both kinases were simultaneously silenced (Figure S6C), indicating that ERK expression is required for the maintenance of MCs.

Due to the less complete nature of ERK, perturbation by RNAi effects were weaker than upon pharmacological inhibition. Nevertheless, silencing of ERK1 and ERK2 combined interfered with the induction of IEGs (Figure 4B).

Regarding MC secretory competence, inhibition of ERK1/2 (or ERK2) suppressed TNF-α, IL-8, Oncostatin (OSM), and Leukemia inhibitory factor (LIF) induction (Figures 4C and S7). The results were largely confirmed by RNAi (Figure 4D) and additional inhibitors (Figure S5B). Alongside ERK, the PI3K pathway contributed to cytokine transcription, although its significance tended to be lower compared with ERK (Figure 4C). We detected robust protein expression of OSM and LIF, and SCF-triggered release of both cytokines was ERK- and PI3K-dependent, matching regulation at mRNA level (Figures 4E and S5C)

3.4 | Identification of novel phosphosites

Inspection of all P-sites revealed around 400 novel robust (at least 3 MS/MS evidences) P-sites (4%–6%) not detected previously in any cell type. Their distribution was pS: 339, pT: 52, and pY: 10 with the highest proportion for pY (File S1). Between 30% (after 8 min) and 44% (after 30 min) of these novel sites were differentially phosphorylated upon SCF treatment.

Novel sites were detected in various protein categories, some of which expressed uniquely in MCs, including MC-selective proteases (TPSAB1;TPSB2;TPSD1, CMA1) and lineage markers newly uncovered in FANTOM5 (e.g., GCSAML/C1ORF150, VWA5A).

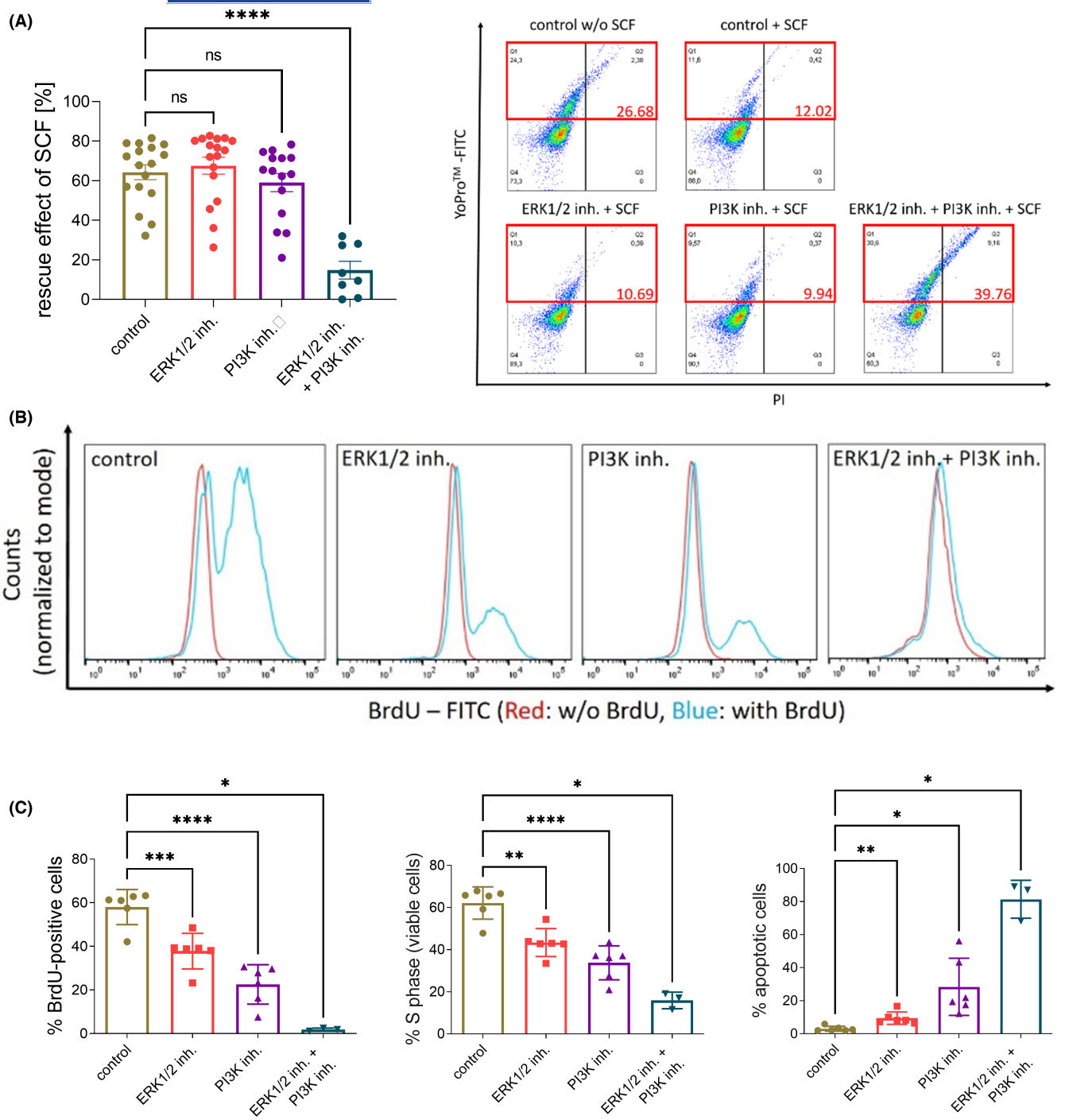


FIGURE 3 Inhibition of ERK and PI3K pathways counters SCF-mediated survival and proliferation of skin MCs. (A) MCs were kept in medium without growth factors and FCS for 16 h prior to addition of inhibitors (ERK1/2 SCH772964, PI3K Pictilisib or their combination, each at 10 μM) and stimulation by SCF (10 ng/ml) for 24 h. Membrane permeability was measured using the YoPro™-1 dye. Inhibition of either pathway by ERK1/2 and PI3K inhibitor alone did not change the percentage of apoptotic cells. Only simultaneous inhibition of both pathways increased the proportion of apoptotic cells (left panel). Representative FACS analyses are shown. Red numbers depict the sum of cells in Q1 and Q2 considered apoptotic (right panel). (B/C) MCs were pretreated with inhibitors as in A followed by SCF stimulation. BrdU-incorporation was analyzed after 5 days. (B) Representative histograms after treatment with the specified inhibitors (red: treatment without BrdU, blue: treatment with BrdU). (C) Cumulative data of $n = 3–7$ independent experiments/cultures (given as a dots), mean ± S.E.M. Refer to Figure S4B for assignment of cells to the distinct fractions/phases. ****$p < .001$, ***$p < .001$, **$p < .01$, *$p < .05$ (RM-ANOVA, mixed effects analysis)

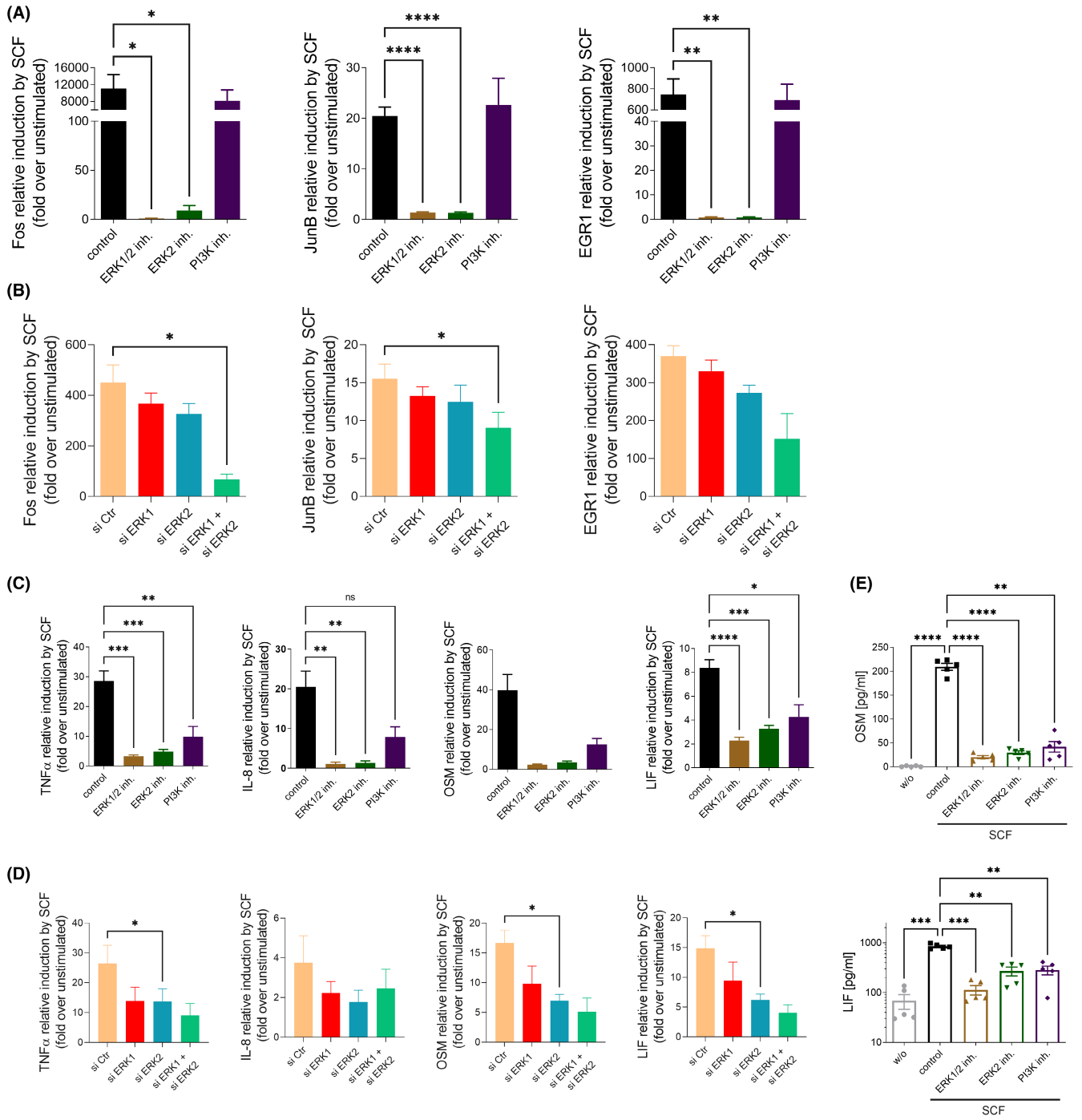


FIGURE 4 SCF-triggered induction of immediate early genes and cytokines depends on ERK1/2. (A) MCs were kept in medium without growth factors and FCS for 16 h, and inhibitors (ERK1/2 SCH772964, ERK2 Vx-11e, PI3K Pictilisib, each at 10 μM) were applied prior to SCF stimulation (10 ng/ml) or vehicle as control for 25 min. Gene expression was analyzed by RT-qPCR. The ratio of SCF-treated/untreated cells is depicted. (B) 78 h after silencing of ERK1, ERK2, or both simultaneously, cells were stimulated with SCF (or vehicle), and gene expression was quantified as in A). (C) MCs were treated, and gene expression quantified as in A. (D) Cell treatments and quantifications were performed as in B. (E) Concentrations of LIF and OSM protein in supernatants of differently treated MCs, as measured by ELISA 24 h after SCF stimulation. Each dot represents an individual culture, and the data are shown as mean ± S.E.M.; $n = 4–8$, $^{****}p < .0001$, $^{***}p < .001$, $^{**}p < .01$, $^{*}p < .05$ (RM-ANOVA; mixed-effects analysis)

In accordance with the highly efficient changes in the ERK module, new P-sites were detected in proteins related to this cascade, including SOS1 and RAPGEF2. Regarding the PI3K module, novel sites were found in phosphatases counteracting PI3K function, that is, INPP5D/SHIP, and INPPL1/SHIP2 and in PIK3R6.

A general overview of novel sites is presented in File S1. Collectively, the wealth of novel posttranslational modifications

nicely fits the uniqueness of MCs within the hematopoietic system and across the body.[22,35]

3.5 | CIC acts a as potent KIT repressor: identification of a CIC-KIT inhibitory circuit

Overall, the transcriptional repressor Capicua (CIC) attracted particular attention. With more than 20 regulated sites, it grouped within the ten most phospho-regulated proteins, similar to the major target KIT (File S1, Figure S8). CIC is highly expressed by MCs.[22] Its partners, ATXN1 and ATXN1L, were likewise phosphorylated on several sites, further supporting the significance of the CIC network downstream of KIT. Inspecting the connection between CIC and KIT, we first found that the two known isoforms, namely CIC-L and CIC-S[23,24] are expressed in skin MCs, with an apparent dominance of the shorter isoform (Figure 5A). In contrast to preliminary evidence in drosophila, the specific functions of CIC-S and CIC-L in humans have not been defined yet.[36] It is known, however, that CIC-S is the dominant form in most cells investigated (e.g., References[24,37]), as also found herein for MCs. CIC expression at baseline was almost completely restricted to the nucleus (Figure 5A). A key question was whether SCF-supported phosphorylation of CIC has consequences on its expression and/or distribution. Indeed, SCF treatment led to transient re-appearance in the cytoplasm accompanied by efficient degradation in the nucleus as well as cytoplasm, further reflected by the transient appearance of CIC degradation products in both cellular compartments (Figure 5B). Considering the dominance of the MEK/ERK module for KIT signaling, we asked whether ERK is involved in these processes. In fact, inhibition of ERK did not only completely prevent CIC degradation and cytoplasmic appearance downstream of KIT triggering, but it also seemed to stabilize CIC in the steady-state (compare the "without" lane in the SCF panel with the corresponding lane in the SCF + ERK inhibitor panel in Figure 5C). Collectively, these results demonstrate that CIC is downstream of KIT activation, by which it is phosphorylated to be subsequently degraded in an ERK-dependent manner. Further investigations revealed that CIC degradation requires the action of (a) protease(s) that is not part of the proteasome, since three distinct proteasome inhibitors did not stabilize CIC, while PMSF did (Figure S10). Together with the literature on other RTKs,[23,24] the above results indicated that a drop in CIC levels may be required for efficient KIT signaling. Using RNAi to reduce CIC expression ectopically (Figure 5D/E), we noticed that CIC attenuation indeed promotes MC survival (Figure 5F). We therefore interrogated whether KIT signaling is altered when CIC levels are reduced. In fact, repression of CIC facilitated the phosphorylation of signaling components by SCF from its already highly efficient baseline. The rank order was STAT5 > ERK ≈ p38 > AKT (Figure 6A). More efficient signal transduction was mirrored by enhanced induction of IEGs (Figure 6B) and, more strongly, cytokine genes (Figure 6C). Overall, through rational combination of datasets, we identified CIC as a novel influential repressor of KIT-mediated signaling, survival, and functional programs, whose deregulation may contribute to MC-dependent diseases.

4 | DISCUSSION

This study provides a first global view of phosphoproteomic dynamics upon SCF-mediated activation of KIT in MCs, encompassing both well-known and novel signaling events. In our study, KIT activation seemed long-lasting, as signals persisted for at least 30 min, while reported to vanish after 5–10 min.[38] The strongest event was Y703, associated with Grb2-Ras, while Y936, associated with both Grb2-Ras and PLCγ, was weak. In fact, no tyrosine phosphorylation was detected for PLCγ.

Extracellular signal-regulated kinase plays important roles in the regulation of cellular functions through cytoplasmic and nuclear pathways. SCF elicited pronounced and prolonged pERK1/2 signals with modest p38 response while pJNK was below detection. Our study also revealed STAT3 and STAT5 as crucial targets of the axis.[19] STAT3 hyperactivity appears to be involved in tumorigenesis resulting from the D816VKIT mutation,[39] while STAT5 is also an important regulator of normal mastopoeisis.[40]

Differential intensities of individual nodes not only apply to the distinct MAPKs (ERK > p38 > JNK), but also to the crucial modules PI3K/AKT and MEK/ERK, respectively. It is widely accepted that depending on the cellular context both pathways may positively and negatively influence each other's signaling.[18,41] It is even described that depending on the maturation status of a cell, the dependence of one pathway on the other may change.[42] Within our study, we find, for example, for the induction of IEGs that in skin MCs these cascades have to be separated (Figure 4A), since PI3K inhibition had no effect and thus could not be upstream of ERK.

It is widely accepted that depending on the cellular context both pathways may positively and negatively influence each other's signaling.[18,41] It is even described that depending on the maturation state of a cell, the dependence of one pathway on the other may change.[42] In our study, we find these cascades to be separated in skin MCs since, for example, IEG induction is independent of PI3K inhibition (Figure 4A).

Indeed ERK-connected signaling was the most prominent at network level (Figure S1B, File S3), but also at the level of individual proteins, including ERK2 and ERK1 themselves, the corresponding KIT residues, MEK1/2 (MAP2K1/MAP2K2) and ERK substrates (MSKs, RSKs, FOXO3, ELK1). Signals within the PI3K/Akt cascade were weaker including major participants (PTEN, AKT1, MTOR) and matching sites within KIT. The reason for the relatively poor activation of PI3K may lie in ERK-dependent Gab2 phosphorylation which can interfere with PI3K recruitment,[43] or the preponderance of ERK may potentially result from continuous signaling by internalized KIT, which apparently favors MAPK signaling.[19]

Cardinal functions of GFs like SCF are to maintain survival, support cellular growth and facilitate cell cycle entry. In fact, MCs are

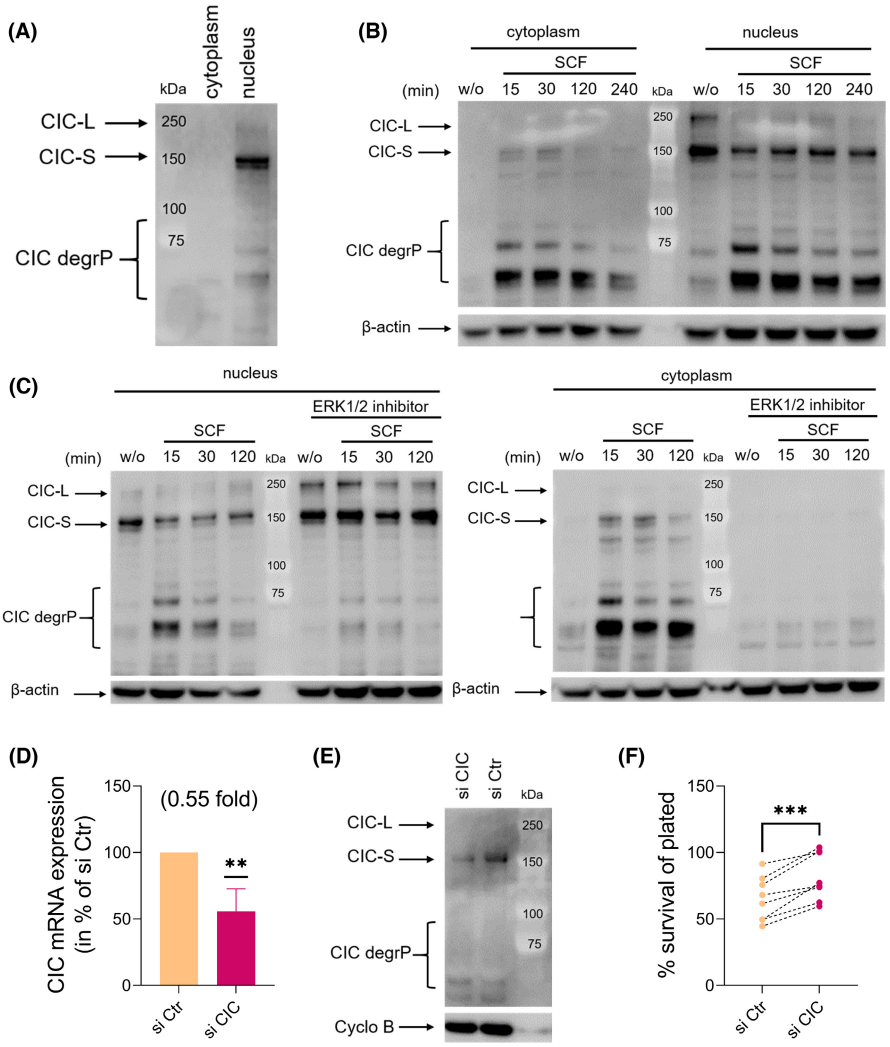


FIGURE 5 CIC is expressed in skin mast cells and ERK1/2 inhibition counters CIC relocation and degradation. (A) MCs were deprived of growth factors for 16 h, and cytosolic and nuclear fractions were prepared using the NE-PER buffer system and resolved by SDS-PAGE side-by-side. Baseline expression of CIC was detected. The immunoblot shows the (mainly) nuclear expression of CIC (both isoforms: CIC-L and CIC-S) in skin mast cell extracts. (B) Upon SCF treatment for different time points, CIC (re-)appears in the cytosol and is rapidly degraded in both fractions. CICdegrP = CIC degradation products (C) MCs were deprived of GFs, treated with ERK1/2-inhibitor and stimulated with SCF for the indicated times. Cytosolic and nuclear fractions were prepared as in A. Note that inhibition of the ERK module inhibits the SCF-provoked degradation in both compartments (right and left panels) and prevents CIC's cytoplasmic appearance (right panel); ERK inhibition also stabilizes CIC-L. CICdegrP = CIC degradation products. (D) CIC mRNA expression upon CIC silencing achieved by exposing cells to CIC-siRNA (si CIC) for 2 days; non-target siRNA served as control (si ctrl). (E) CIC protein level upon CIC silencing as in D. Note that under the conditions required for RNAi, only CIC-S remains undeniably detectable after the 48-h-incubation period. (F) CIC silencing promotes cell survival. The data are shown as mean ± S.E.M.; $n = 5–8$, $^{**}p < .01$, $^{***}p < .001$ (one-sample t-test [D]; paired t-test [F])

overabundant in and functionally associated with diseases like atopic dermatitis, psoriasis, and urticaria.[10,11,20,44,45] Important sources of SCF in the skin, that can elicit MC hyperplasia, are keratinocytes and fibroblasts.[46,47]

Attempting to dissect the relative contribution of PI3K/AKT vs. MEK/ERK in KIT-facilitated protection, we found redundancy in short-term survival, as cells remained intact upon treatment with either PI3K or ERK1/2 inhibitors given individually, while only their combination resulted in reduced protection. This finding indicates that each pathway alone is sufficient to maintain survival for a certain period. In contrast, both ERK and PI3K pathways were crucial for long-term maintenance, whereby blocking of either impaired the anti-apoptotic effect of SCF. However, survival was further reduced on combined treatment, indicating some redundancy also over longer periods. A similar outcome was observed in proliferation assays, whereby both BrdU-positive and S-phase cells were reduced by any inhibitor alone, but nearly abolished on their combination.

Besides growth and survival, the SCF/KIT axis is implicated in secretion and transcription. We studied the relative contribution of ERK vis-à-vis PI3K in cytokine generation and IEG induction. Fos, Egr1, and JunB constitute classical IEGs induced via pathways like RhoA, ERK, and p38.[48] Some IEGs encode TFs that trigger secondary response

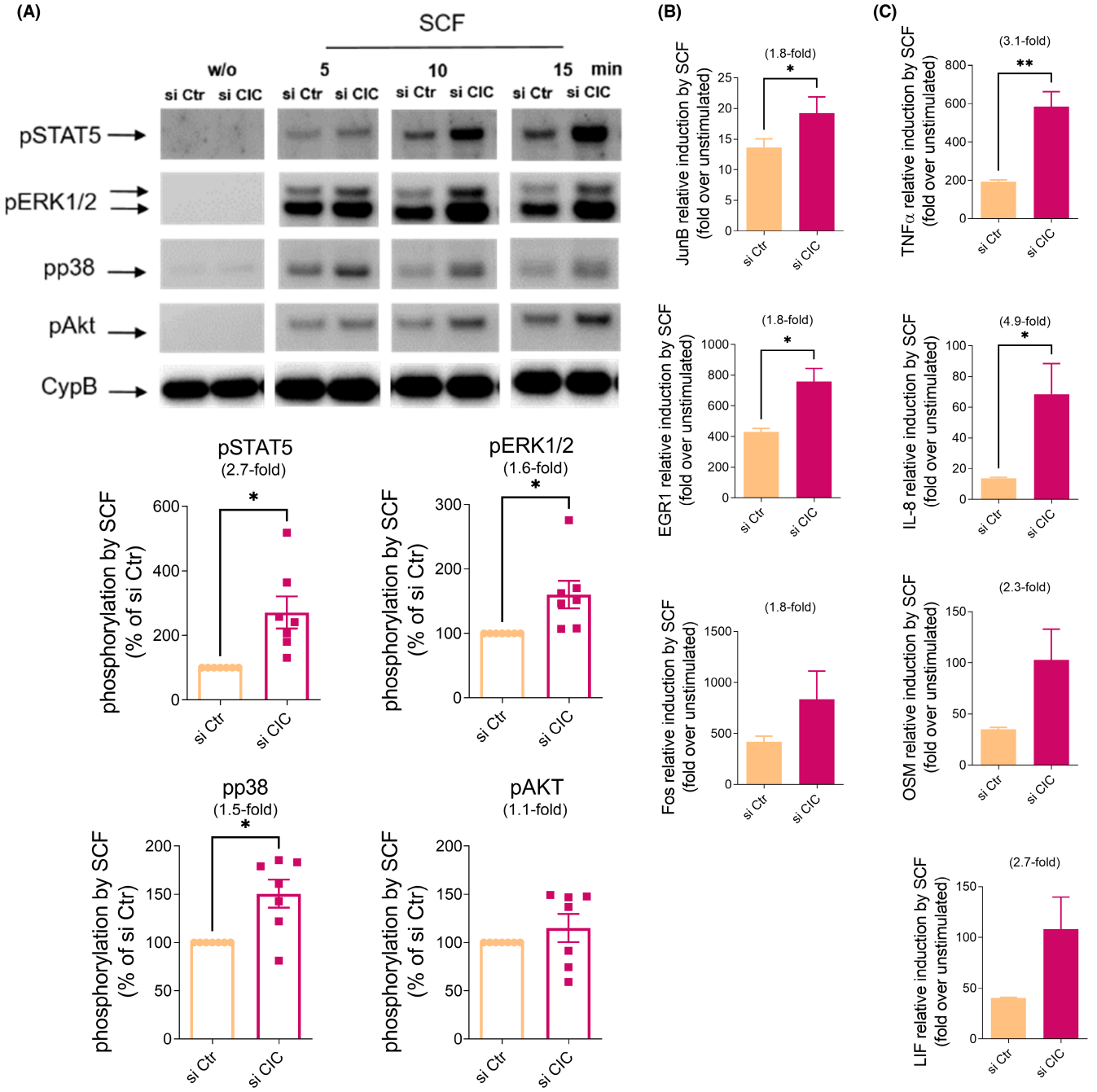


FIGURE 6 CIC is a repressor of KIT function. CIC silencing was achieved by exposing cells to CIC-siRNA (si CIC) for 2 days; non-target siRNA served as control (si ctrl). (A) Upon silencing, cells were stimulated with SCF (10 ng/ml) for different times, signaling components detected by immunoblotting and semi-quantified by ImageJ. Upper panel: representative blot (consecutive detection of the distinct proteins on the same membrane), lower panel: cumulative data of 7 independent experiments (highlighted by individual dots) for the 15-min-point. (B/C) Cells were stimulated with SCF (10 ng/ml) for 25 min and gene expression quantified by RT-qPCR. (B) IEGs, (C) cytokines. The data are shown as mean ± S.E.M.; $n = 5–7$, $^{**}p < .01$, $^{*}p < .05$ (one-sample *t*-test [A]; paired *t*-test [BC])

genes to drive downstream functions. In our study, IEG induction by SCF depended solely on ERK, whereby only the ERK2 isoform seemed to be required since the ERK2-selective inhibitor was as suppressive as compounds targeting ERK1 and ERK2 indiscriminately (Figure 4A). Apart from differentiation and proliferation,[49] IEGs can feed into cytokine generation, since both AP-1 (Fos/Jun) and EGR1 bind to crucial elements within regulatory sequences of their genes.[50–52]

Mast cell cytokines are a key component in chronic (skin) diseases[53–55]; their regulation is complex as it requires a broad range of signaling components and TFs. In our study, PI3K and ERK both contributed to SCF-triggered cytokine generation, yet ERK appeared the more relevant partaker. ERK activity is typically but not indiscriminately involved in MC cytokine responses.[56] In skin MCs, for example, ERK activity is indispensable for several FcεRI-stimulated

cytokines, while IL-33-triggered expression of overlapping entities requires p38 action instead.[25,57] PI3K can activate cytokine genes partially through the IKK-NFκB cascade.[58]

We uncover OSM and LIF as prominent (SCF-induced) skin MC cytokines (Figure S7). In contrast to IL-6 (linked to inflammation and important in mastocytotic MCs[59]), which is produced by skin MCs, but at levels that vary strongly across donors and cultures,[60] the family members LIF and OSM, far less studied in MCs compared to IL-6, are rather associated with regeneration and tissue repair of liver, bone, muscle, and nerve.[61] Therefore, MCs may contribute to such restorative activities. Collectively, the above findings emphasize the significance of the ERK cascade as the major downstream effector of KIT in normal MCs.

Of particular interest were phosphorylation events on proteins not yet described to be regulated by SCF. The massive modulation in CIC indicated important implications as nearly all detected P-sites were regulated by SCF (File S1, Figure S8). CIC can be downstream of RTK pathways as was initially discovered in drosophila and later reproduced in mammals.[23,24] However, its relevance for the SCF/KIT axis has not been studied so far. RTK triggering can lead to the degradation of CIC via the MEK/ERK pathway in other cell types to allow for activation of CIC-repressed genes. Thereby CIC, or rather its inactivation, operates as a key effector of the Ras/MEK/ERK pathway.[23,24] Here, we demonstrate for the first time that this mechanism is operative and of pivotal significance to the SCF/KIT axis, as uncovered through an unbiased phosphoproteomics approach in MCs, which (together with brain) express highest levels of CIC across the body.[22,35] Prominent expression of CIC-S and (lesser) CIC-L could be detected in the nuclear fraction of MCs. Upon SCF, CIC not only experienced massive phosphorylation on numerous sites, but it also transiently accumulated in the cytoplasm and was efficiently degraded in both compartments in an ERK-dependent manner. Investigations of the underlying mechanism revealed that CIC degradation seems to be independent of the proteasome but does require the action of (a) protease(s) (Figure S10). While SCF thus interfered with CIC function naturally through ERK-assisted degradation, we were interested in the effects of ectopic CIC inactivation. In fact, CIC perturbation by RNAi had profound consequences on the efficiency of KIT signaling and function, whereby a circular inhibitory relationship between CIC and ERK could be unearthed: While ERK (or one of its downstream effector kinases) phosphorylates CIC and inactivates its function through degradation, CIC represses ERK on its own, as established in other systems.[62,63]

Interestingly in addition to ERK, the STAT5 cascade was most strongly inhibited by CIC, an event that has not been reported so far. STAT5 is a key player of the MC lineage.[40] It directly regulates the master regulator GATA-2[64] and anti-apoptotic Mcl-1[65] in primary MCs. Importantly, overactivity of STAT5 is also associated with the initiation and progression of mastocytosis.[59,66]

Further downstream, induction of IEGs and cytokines was substantially enhanced upon CIC inactivation (Figure 6B/C). Cytokines were especially affected, which mirrors their complex regulation by several kinases and TFs, most of which attenuated by CIC. For example, STAT5 is also involved in the regulation of cytokine genes.[67] A further possibility is NF-κB, whose activity can be repressed by CIC, at least in drosophila.[68] If CIC interferes not only with MC survival, but also with MC-driven inflammation, it could turn out as an attractive target in conditions like mastocytosis, allergic inflammation, and chronic dermatoses.

Collectively, our results suggest that CIC keeps KIT (over-)activity at bay. Even though still understudied, CIC has indeed been recognized as a critical tumor suppressor.[24,69–72] It will be of great interest to delineate whether CIC expression or stabilization may interfere with aberrant KIT-driven programs. Our discovery provides the basis to comprehensive research into CIC function in MCs and its relevance for MC-associated disease.

AUTHOR CONTRIBUTION

M.B. involved in conceptualization and supervision. K.F., M.B., and M.K. involved in formal analysis and writing—original draft preparation. K.F. and M.K. involved in investigation and visualization. M.B., K.F., M.K., P.M., and T.Z. involved in writing—review and editing. M.B. and T.Z. involved in funding acquisition. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no competing conflict of interest.

ORCID

Kristin Franke https://orcid.org/0000-0002-7402-4211
Marieluise Kirchner https://orcid.org/0000-0002-7049-534X
Philipp Mertins https://orcid.org/0000-0002-2245-528X
Torsten Zuberbier https://orcid.org/0000-0002-1466-8875
Magda Babina https://orcid.org/0000-0002-4500-7615

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