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Srcasm corrects Fyn-induced epidermal hyperplasia by kinase downregulation

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Summary

Src-family tyrosine kinases (SFKs) are important regulators of epithelial cell growth and differentiation. Characterization of cellular mechanisms that regulate SFK activity will provide insights into the pathogenesis of diseases associated with increased SFK activity. Keratin 14-Fyn (K14) transgenic mice were derived to characterize the effect of Fyn on epidermal growth and differentiation in vivo. The epidermis of K14-Fyn mice is thickened, manifests prominent scale, and exhibits features consistent with hyperproliferation. Increased epidermal Fyn levels correlate with activation of p44/42 MAP kinases, STAT-3, and PDK-1; key signaling molecules that promote epithelial cell growth.

The Src-activating and signaling molecule (Srcasm) is a substrate of SFKs that becomes tyrosine phosphorylated downstream of the EGF receptor. In vitro, increased Srcasm levels promote activation of endogenous Fyn and keratinocyte differentiation. To study the in vivo effect of Srcasm upon Fyn, double transgenic lines were derived. K14-Fyn/Srcasm transgenic mice did not manifest the hyperproliferative phenotype. In contrast, K14-Fyn/Srcasm-P transgenic mice, that express a non-phosphorylatable Srcasm mutant, maintain the hyperproliferative phenotype. Resolution of the hyper-proliferative phenotype correlated with reduced Fyn levels in vivo in three experimental systems: transgenic mice, primary keratinocytes, and cell lines. Biochemical studies reveal that Srcasm-dependent Fyn downregulation requires Fyn kinase activity, phosphorylation of Srcasm, and the SrcasmGAT domain. Therefore, Srcasm is a novel regulator of Fyn promoting kinase downregulation in a phosphorylation-dependent manner. Srcasm may act as a molecular ‘rheostat’ for activated SFKs, and cellular levels of Srcasm may be important for regulating epithelial hyperproliferation associated with increased SFK activity.

Activation of protein tyrosine kinases is an important mechanism for promoting epithelial cell growth.(1, 2) Increased Src-family tyrosine kinase (SFK) activity is present in many human cancers, including colonic and breast carcinomas.(3–5) Increased SFK activity in tumors could result from activating mutations and/or impairment of downregulatory mechanisms. However, activating mutations of SFKs are rare in these carcinomas raising the hypothesis that impaired down-regulation of activated SFKs could account for increased tumoral SFK activity. (6, 7) Therefore, characterization of negative regulatory mechanisms that target activated SFKs may provide insights into carcinogenesis associated with increased SFK activity.

In skin, neoplasia is associated with increased cellular proliferation, epidermal hyperplasia, and increased EGFR activity.(8) Many signaling pathways that are persistently activated in cutaneous neoplasia are also stimulated in psoriasis, a cutaneous disorder also associated with T-cell inflammation, epidermal hyperplasia and increased EGFR activity.(9–11) Given

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these observations, delineation of SFK-regulatory mechanisms in keratinocytes should provide insights into the pathogenesis of cutaneous neoplasia and psoriasis. (12–14)

In vitro studies of keratinocytes from Fyn-deficient mice demonstrate abnormalities in differentiation, suggesting an important role for Fyn in differentiation (15–17). Increased Fyn expression in primary murine keratinocyte cultures promotes differentiation and withdrawal from the cell cycle.(18) To evaluate the in vivo effects of increased Fyn expression, K14-Fyn transgenic mice were derived and characterized. K14-Fyn mice demonstrate a thickened, hyperplastic, and scaly epidermis dependent on increased Fyn expression. The K14-Fyn epidermis manifests activation of p44/42 MAP kinases, STAT-3, and PDK-1, molecules associated with keratinocyte growth.(19–22) In addition, keratin 6 expression was upregulated consistent with a hyper-proliferative phenotype.

Srcasm, Src-activating and signaling molecule, is an SFK substrate that is tyrosine phosphorylated secondary to EGF and TGF-α stimulation of primary human keratinocytes (PHKs); Srcasm modulates p44/42 MAP kinase signaling in an EGF-dependent manner.(14, 23) Increased Srcasm levels activate endogenous Fyn, promote differentiation, and decrease the S-phase fraction of PHKs, even after EGF stimulation. Srcasm levels are decreased in cutaneous SCC and associated precursor lesions.(14) These data suggest that Srcasm is an important regulator of SFKs in keratinocytes that promotes keratinocyte differentiation.

The in vivo relationship between Fyn and Srcasm was evaluated by generating double-transgenic lines co-expressing Fyn with native Srcasm or Srcasm-P, a mutant lacking Fyn phosphorylation sites. K14-Fyn/Srcasm mice did not exhibit a hyperproliferative phenotype, while the K14-Fyn/Srcasm-P mice did. Increased Srcasm, but not Srcasm-P, expression in K14 Fyn/Srcasm mice correlated with decreased Fyn levels. Biochemical studies delineate a mechanism of Srcasm-dependent Fyn downregulation that requires Fyn kinase activity, the Fyn phosphorylation sites of Srcasm, and the Srcasm GAT domain. Srcasm efficiently downregulates constitutively activated Fyn mutants but not kinase inactive mutants. High levels of Srcasm also interfere with the downregulatory process suggesting a biphasic relationship between activated SFKs and Srcasm. Therefore, Srcasm is a novel molecular ‘rheostat’ for activated SFKs that limits cellular proliferation and promotes differentiation.

METHODS

Expression vector construction and generation of transgenic mice

Murine HA-Srcasm and Fyn cDNAs were cloned into a human keratin 14 expression vector. (23, 44, 45) The Srcasm-P mutant contained Y to F mutations at tyrosines 392, 440, 441, and 457. All K14-transgene cassettes were excised from the targeting vector, and purified via TAE agarose electrophoresis. C57BL/6 x CBA fertilized oocytes were microinjected with the K14 transgene cassettes using standard protocols of the Univ. of Penn. Transgenic Core Facility.

Characterization of transgenic mice

Tail genomic DNA was isolated, and a genomic PCR was performed using the following primers: coding primer (K14 promoter)- 5′ ATCTTGAGA ACTTCAGGG 3′ and non-coding primer (Srcasm) 5′ GGTGACCC ACAGAGGTAG 3′; this strategy produced a 900bp Srcasm transgene product. To detect Fyn transgenes, the primer pair of K14 promoter coding: 5′ AAC GTG CTG GTT ATT GTG CTG 3′ and Fyn non-coding 5′ TTC CGT CCG TGC TTC ATA GT 3′ was used to amplify a 400 bp product. Transgenic founders and littermate controls were crossed with C57BL/6 mice to generate lines. F1 transgenic progeny were crossed with C57BL/6 mice to maintain lines. The skin of mice was examined at least biweekly after birth; mice exhibiting severe runting were monitored daily. The phenotypes

described were maintained through at least 7 generations. In the K14-Fyn A line, 368 mice were observed, with 74 of 203 genotype positive mice expressing a positive phenotype; epidermal hyperkeratosis less than 1 cm^2 was not considered a positive phenotype. The phenotype, as defined, exhibited incomplete penetrance, and was expressed in 36% of K14-Fyn genotype positive mice in both lines and all crosses. No epidermal hyperkeratosis was seen in 165 genotype negative mice. In the K14-Fyn/Srcasm crosses, 148 mice were observed. One of 46 K14-Fyn/Srcasm mice exhibited a phenotype while 13 of 32 K14-Fyn mice in these crosses had a phenotype. Forty-three K14-Srcasm and 27 littermate controls did not exhibit any epidermal hyperkeratosis. In the K14-Fyn/Srcasm-P crosses, 108 mice were observed. Fifteen of 34 K14-Fyn/Srcasm-P and 6 of 17 K14-Fyn mice maintained the phenotype. Thirty-five K14-Srcasm-P and 20 littermate controls demonstrated no epidermal hyperplasia. All mice were handled in accord with Univ. of Penn. IACUC protocol. #705452. A two-sided Fisher's exact test was used to determine the statistical significance of phenotype expression.

Histologic and immunohistochemical analysis

Skin samples from the back, abdomen, ear, tail, and footpad were fixed overnight in 10% buffered formalin. Tissues were subjected to standard processing followed by paraffin embedding. Five-micron-thick sections were stained with hematoxylin and eosin. For immunohistochemistry, ear or back skin sections of one-week-old mice were deparaffinized, rehydrated, blocked with 10% goat serum, and then incubated with the specified primary antibody, biotinylated secondary antibodies, streptavidin HRP or AP conjugate, and colorimetric substrates. Hematoxylin or Nuclear Fast Red were used for counter staining. Sections were incubated with 3% H_2O_2 solution to block endogenous peroxidase activity when streptavidin-HRP was used. Prior to BrdU or Ki-67 immunostaining, antigen retrieval was performed by boiling sections in 10 mM sodium citrate solution (pH 6.0) for 10 minutes. Photomicrographs were obtained using a Leica DC300 digital camera coupled to a Zeiss Axiophot microscope; all paired photos were obtained under identical conditions. The frequency of BrdU and Ki-67 staining in control and K14-Fyn mice was quantified by determining the percentage of positive nuclei. For BrdU staining: control- 12% positive nuclei, N= 382, K14-Fyn- 21% positive nuclei, N= 620. For Ki-67 staining: control- 52 positive nuclei per 344 cells, K14-fyn – 431 positive nuclei per 1544 cells. A Pearson Chi-squared test was applied to determine the statistical significance of positive staining percentages.

In vivo BrdU-labeling of transgenic mice

Mice were injected intraperitoneally with 100μl (1mg) of BrdU solution and sacrificed 1 h later. Skin was cut, formalin-fixed and processed for BrdU immunohistochemistry. Proliferation indices were determined by counting positive nuclei. P values were calculated using a Pearson Chi squared test. BrdU indices (% positive nuclear staining): control-12.0% N = 382, K14-Fyn-20.5% N = 620, P = 0.001. Ki-67 indices: control-15.1% N = 344, K14-Fyn-27.9% N = 1544, P = <0.001.

Antibodies

α-HA antibody (clone 3F10, Roche Molecular Biochemicals) was used at 1/100 for immunohistochemistry (IHC). Activated Src-family kinase antibody (phospho-Tyr-416) (Cell Signaling Technology)was used at 1/25 for IHC, and 1/1000 for Western blotting (WB). α-p44/42 MAP kinase antibody and α-phospho-p44/42 MAP kinase antibody (Cell Signaling Technology)were used at 1/100 for IHC and 1/1000 for WB. α-Fyn (SC-16, Santa Cruz Biotechnology) was used at 1/100 for IHC and 1:1000 for WB. α-mouse keratin 6 antibody (PRB-169P, COVANCE) was used at 1/150 for IHC and at 1/1000 for WB. α-Ki-67 antigen antibody (NCL-L-Ki67-MM1, Novocastra Laboratories, Ltd.) was used at

1/100 for IHC. α-phospho PDK1 (Ser241) antibody (Cell Signaling Technology) was used at 1/100 for IHC. α-BrdU antibody (Roche) was used at 1/10 for IHC. α-beta actin antibody (ab-6276, Abcam) was used at 1/5000 for WB. α-GFP (#2555, Cell Signaling Technology) antibody was used at 1/1000 for WB. α-Srcasm antibody was used at 1/1000 for WB.(23)

Primary keratinocyte culture and adenoviral infection

Primary cultures of murine keratinocytes were obtained from pups less than 3 days old. Isolated skin was subjected to trypsin digestion (0.25%, overnight at 4°C) and agitation to generate a cell suspension. Cells were plated on rat type I collagen coated dishes and cultured in MCDB-153 medium with supplements (14), and maintained at 34 °C with an atmosphere of 8% CO_2. Keratinocytes were used at passage 3 or 4; cells at approximately 60–70% confluence were infected with Ad-Fyn (murine Fyn) or control virus at the indicated MOI, and were analyzed 16 hours after infection.

Transfection studies

COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM glutamine, and antibiotic (200 units/ml ampicillin and 200 mg/ml streptomycin). For EGF stimulation, serum was decreased to 0.5%. Murine cDNAs of HA-Srcasm, Fyn, or specified mutants were cloned into pcDNA3.1 (Invitrogen). Srcasm GAT domain mutant lacks residues 199–287. Activated murine Fyn is a Y528F mutation.(46) Kinase-weak Fyn is a K296R mutation.(2) As a transfection control, some cells were transfected with 0.25 ug E-GFP Living Colors plasmid (BD Biosciences). Cells at approximately 60–70% confluency were transfected for 5 hours with the indicated expression vectors using Lipofectamine, and recovered in media plus serum for 16 hours before lysis. Transfected cells were subjected to the following treatments before lysis: 10 mM ammonium chloride for one hour; proteosomal inhibitors- lactacystin at 10 μM for 1, 3, and 4 hours; MG132 at 0.1 mM for 1, 3, and 4 hours; ALLN at 0.1 mM for 1, 3, and 4 hours; ALLN, MG132, and lactacystin for 1 hour; lysosomal protease inhibitors- E64 at 0.1 mM for 1, 3, and 4 hours; leupeptin at 0.1 mM for 1, 3, and 4 hours; E64 and leupeptin for 1 hour.

For doxycycline-induced Srcasm expression, 0.5μg of pCDNA 3.1 Fyn plasmid was co-transfected with empty vectors or pCMV-rtTA + pTRE-Srcasm (1.0 μg each). Some cells were incubated with 10, 50 or 100 ng/ml doxycycline-containing media for six hours prior to lysis.

Immunoblotting

Cell and tissue lysates were prepared using a RIPA buffer with protease and phosphatase inhibitors (14); lysates were incubated on ice for 15 minutes then cleared by centrifugation at 14,000 × g for 10 minutes at 4°C. Supernatants were assayed for protein content using the MicroBCA protein assay kit (Pierce Chemical Co.). Aliquots of lysate were separated by SDS-PAGE and transferred to PolyScreen (NEN Life Sciences Products, Inc.). Western blotting was conducted in a standard manner with the indicated antibodies and developed using an enhanced chemiluminescence kit according as described by the manufacturer (Lumilight Plus, Boehringer Mannheim). Band densitometry was performed using a Canon Lide 50 flatbed scanner and Scion image analysis software. Values are standardized to actin levels.

Quantitative RT-PCR for Fyn transcript

Transfected cells were lysed in Triazol reagent and total RNA was isolated. Of total RNA was subjected to reverse transcription using poly-dT. Equivalent amounts of cDNA were

subjected to quantitative RT-PCR to detect Fyn transcript using Sybr-green technology on a MJ Research Opticon 2 system.

Results

Phenotype of Fyn and Srcasm transgenic mice

Two-independent K14-Fyn founder mice exhibited epidermal scaling with hair loss on the snouts and hindquarters (Fig. 1a). K14-Fyn progeny exhibited diffuse epidermal scale at 2–3 days post-natal that was prominent by 7 days (Fig. 1b). Approximately, ten percent of affected pups died by one week of age; such pups were runted and exhibited decreased feeding activity. Over a few weeks, diffuse scaling coalesced into prominent hyperkeratotic plaques lacking hair; these plaques usually resolved by 8 weeks (Fig. 1c). The scaling phenotype was observed through 7 generations, and was not seen in transgene-negative mice (N = 165). No spontaneous epidermal tumors were seen in 25 phenotype-positive, unstimulated mice carried to 6 months.

K14-Fyn mice were crossed with K14-Srcasm or K14-Srcasm-P mice, a mutant not phosphorylated by Fyn, to generate K14-Fyn/Srcasm and K14-Fyn/Srcasm-P double transgenic lines. K14-Fyn/Srcasm double transgenic mice lacked the scaly phenotype; this decreased phenotypic incidence was statistically significant compared to that of K14-Fyn littermates ($p<0.0001$) (Fig. 1d). In contrast, K14-Fyn/Srcasm-P double transgenic mice maintained the scaly phenotype with an incidence equivalent to K14-Fyn parent lines and littermates (Fig. 1d). The K14-Srcasm (N = 318) and K14-Srcasm-P (N = 232) strains did not exhibit a hyperproliferative phenotype (Fig. 1d). The numbers of mice characterized for each strain with their corresponding phenotype and phenotypic incidence are shown in Table 1.

Histologic analysis of K14-Fyn mice

Epidermal sections from K14-Fyn mice demonstrated marked hyperplasia and hyperkeratosis (Fig. 2a). Keratinocytes in the K14-Fyn epidermis were larger and exhibited nuclear atypia (Fig. 2a, high power). A mixed inflammatory infiltrate containing lymphocytes and neutrophils was present in the superficial dermis of K14-Fyn mice, but not in control mice (Fig. 2a). Increased Ki-67 staining and BrdU labeling of keratinocytes in the K14-Fyn epidermis was consistent with hyperproliferation (Fig. 2a). The differences in positive staining between K14-Fyn and control mice for Ki-67 ($p < 0.001$) and BrdU ($p = 0.001$) staining were statistically significant (Fig. 2d).

The epidermal hyperplasia in K14-Fyn mice correlated with elevated Fyn levels and increased SFK activity (Fig. 2b). The K14-Fyn epidermis demonstrated increased cytoplasmic and nuclear staining for activated p44/42 MAP kinase, linking increased Fyn levels with p44/42 MAP kinase activation (Fig. 2b). (13, 24, 25) Staining for keratin 6, a marker of epidermal hyperproliferation, was prominent in the hyperplastic epidermis (Fig. 2b). Runted K14-Fyn pups demonstrated hyperkeratosis of the dorsal tongue that correlated with loss of the filiform papillae and increased SFK activity (Fig. 2c).

Characterization of K14-Fyn/Srcasm and Fyn/Srcasm-P mice

The thickened epidermis of K14-Fyn transgenic mice normalizes when this line is crossed with a K14-Srcasm line (Figs. 1d and 3a). Phenotype resolution in K14-Fyn/Srcasm mice correlates with increased Srcasm expression and decreased levels of Fyn, activated SFKs, and keratin 6 (Figs. 3a and b). The K14-Fyn/Srcasm-P mice maintain a hyperkeratotic epidermis demonstrating high levels of Fyn, activated SFKs, and keratin 6 (Fig. 3a). Subtle activation of SFKs and mildly increased Fyn levels could be detected via

immunohistochemistry in the K14-Srcasm mice but not in the control or K14-Srcasm-P mice. These findings suggest that endogenous SFK levels coupled with supraphysiologic Srcasm levels may result in impaired downregulation of activated SFKs. Such findings are supported by previous experiments in primary human keratinocytes.(14) These data suggest that with endogenous Fyn levels increased native Srcasm expression may increase levels of activated SFKs; however, with supraphysiologic levels of SFKs activation, as in the K14-Fyn mice, increased Srcasm levels promote Fyn downregulation.

Immunohistochemical studies of the K14 transgenic lines were supported by western blot analysis of epidermal lysates. Lysates from K14-Fyn mice demonstrated elevated levels of Fyn, activated SFKs, and keratin 6 compared to lysates from control, K14-Srcasm, and K14-Srcasm-P mice (Fig. 3b). Lysates from K14-Fyn/Srcasm mice contained lower levels of Fyn and activated SFKs than K14-Fyn mice; the levels of keratin 6 were reduced but remained higher than levels in control mice (Fig. 3b). In vivo, increased Srcasm expression is associated with decreased Fyn levels; however, increased expression of Srcasm-P does not promote Fyn downregulation.

Srcasm-dependent Fyn downregulation was studied in primary murine keratinocytes derived from neonatal control, K14-Srcasm, and K14-Srcasm-P transgenic mice. Infection of K14-Srcasm keratinocytes with Fyn adenovirus resulted in lower Fyn levels than those detected in parallel infections of control and K14-Srcasm-P keratinocytes (Fig. 4). Fyn levels in K14-Srcasm keratinocytes were restored to control levels by pre-treatment with 10 mM NH_4Cl for 60 minutes before lysis. Parallel experiments were attempted using primary keratinocyte cultures from K14-Fyn mice transduced with Srcasm or Srcasm-P adenoviruses. However, primary cultures from K14-Fyn mice were difficult to establish and maintain, given that increased Fyn activity in murine keratinocytes promotes cell cycle withdrawal. (18) These experimental results suggest that the functional integrity of the endosomal/lysosomal pathway may influence Srcasm-dependent Fyn downregulation.

STAT-3 and PDK-1 activation correlate with Fyn levels

STAT-3 activation is associated with keratinocyte hyperplasia, murine epidermal tumors, human squamous cell carcinomas, and psoriasis. (20, 21, 26) Therefore, the epidermis of the transgenic lines was evaluated for STAT-3 activation. Increased nuclear and plasma membrane staining for activated STAT-3 was identified in the K14-Fyn hyperplastic epidermis but not in littermate controls, linking increased Fyn levels with STAT-3 activation in keratinocytes (Fig. 5). Levels of activated STAT-3 decreased in K14-Fyn/Srcasm mice, suggesting that Srcasm-induced Fyn downregulation is associated with normalization of activated STAT-3 levels. Levels of nuclear and plasma membrane phospho-STAT-3 remained high in the skin of K14-Fyn/Srcasm-P mice.

Fyn activates PI-3 kinase; therefore, K14-Fyn skin was assessed for PI-3 kinase activation by evaluating levels of activated PDK-1.(27, 28) K14-Fyn skin demonstrated prominent cytoplasmic staining for activated PDK-1 in a mosaic pattern (Fig. 5). K14-Fyn/Srcasm epidermis exhibited little staining for activated PDK-1 and was indistinguishable from controls. The levels of PDK-1 activation remained strong in K14-Fyn/Srcasm-P mice. Together, these data link increased Fyn levels with PDK-1 activation; Srcasm, which modulates Fyn levels, also regulates PDK-1 activation.

Characterization of Srcasm-dependent Fyn downregulation

Transfection studies in COS cells were performed to characterize Srcasm-dependent Fyn downregulation. Increasing Srcasm levels leads to a decrease in Fyn levels (Fig 6a). However, as Srcasm levels continue to rise, Fyn levels rebound from a nadir, rise mildly, but

do not reach control levels (Fig. 6a). The data from these experiments suggest a biphasic relationship between Fyn levels and Srcasm levels.

Co-transfection of Fyn with plasmids constituting a doxycycline-inducible Srcasm expression system demonstrated that small increases in Srcasm levels induced significant Fyn downregulation; this expression system is ‘leaky’ yielding subtle increases in Srcasm expression in the absence of exogenous doxycycline (Fig. 6b). At relatively high Srcasm levels, Fyn levels rebounded in a manner similar to that seen in figure 6a. These experiments demonstrate a temporal relationship between increased Srcasm expression and Fyn downregulation.

Inhibition of Srcasm-dependent Fyn downregulation

COS cells pre-treated with 10 mM ammonium chloride for 60 minutes before lysis did not exhibit Srcasm-dependent Fyn downregulation confirming findings seen in primary murine keratinocytes (Fig. 7a). Treatment of transfected COS cells with the lysosomal protease inhibitors, E64 and leupeptin, for up to 4 hours prior to lysis did not appreciably alter Srcasm-dependent Fyn downregulation (Fig. 7a). Incubation of transfected COS cells with proteosomal inhibitors, lactacystin, MG132, and ALLN, for up to 4 hours before lysis did mildly inhibit Srcasm-dependent Fyn downregulation. These data suggest that Srcasm-dependent Fyn downregulation requires an intact endosomal/lysosomal pathway and may require proteosomal function.

The ability of EGF to modulate Srcasm-dependent Fyn downregulation was evaluated. EGF-treatment alone did not appreciably effect Fyn downregulation by Srcasm. However, EGF-treatment of cells appeared to lessen the inhibitory effect of ammonium chloride on Fyn downregulation (Fig. 7b). Therefore, EGF stimulation may promote Srcasm-dependent Fyn downregulation under specific conditions.

Structure-function analysis of Srcasm-dependent Fyn downregulation

A structure-function analysis of Srcasm was performed to identify domains and residues required for Srcasm-dependent Fyn downregulation. The Srcasm GAT domain binds mono-ubiquinated proteins and this post-translational modification appears to be important for down-regulating EGF receptor and SFK signaling.(29–32) A mutant Srcasm lacking the GAT domain failed to induce Fyn downregulation; in contrast, this mutant Srcasm appeared to interfere with Fyn downregulation (Fig. 8a). Similarly, co-transfection of Srcasm-P with Fyn did not decrease Fyn levels but increased them relative to controls. These data demonstrate that the Srcasm GAT domain and Fyn phosphorylation sites are required for Srcasm-dependent Fyn downregulation. To exclude a potential transcriptional effect of Srcasm upon Fyn mRNA levels, quantitative RT-PCR for Fyn transcript was performed on mRNA isolated from all transfection conditions. Fyn transcript levels did not vary consistently or significantly across transfection conditions, and mRNA levels for glyceraldehyde-3-phosphate dehydrogenase also remained constant in all samples (Fig. 8b). Fyn transcript levels did not correlate with Fyn protein levels. Therefore, Srcasm-dependent Fyn downregulation does not involve modulation of Fyn transcript levels.

The role of Fyn kinase activity in Srcasm-dependent Fyn downreguation

Since Fyn phosphorylation of Srcasm appears necessary for Srcasm-dependent Fyn downregulation, the role of Fyn kinase activity in Srcasm-dependent Fyn downregulation was evaluated. Co-transfection of activated Fyn Y528F with native Srcasm results in downregulation of the activated kinase similar to that seen with native Fyn (Fig. 9a). Under similar conditions, Srcasm-P did not induce downregulation of activated Fyn but promoted accumulation of the activated Fyn kinase. Native Srcasm does not efficiently downregulate

kinase-deficient Fyn K296R relative to native Fyn (Fig. 9b). These data demonstrate that efficient Srcasm-dependent Fyn downregulation requires Fyn kinase activity and Srcasm phosphorylation by Fyn.

Discussion

The K14-Fyn transgenic mouse is a novel model of epidermal hyperplasia that may provide insights into cutaneous disorders associated with keratinocyte hyperproliferation. Characterization of the dermal and epidermal inflammatory infiltrate will reveal if the K14-Fyn mouse mimics inflammatory disorders such as psoriasis. The hyperproliferative epidermis of the K14-Fyn mice demonstrates increased STAT-3 activation; a finding consistent with other models of psoriasis and cutaneous squamous cell carcinoma.(20, 21, 26) Fyn appears to be an upstream activator of STAT-3 in keratinocytes, and phospho-STAT-3 may transmit some Fyn-initiated signals to the nucleus.

The epidermal hyperplasia present in K14-Fyn transgenic mice requires elevated Fyn levels. As Fyn levels decrease with increasing Srcasm levels, the epidermal hyperplasia normalized. Spontaneous SCC formation was not seen in 25 mice followed for 6 months; this cohort was not subjected to any pro-carcinogenic stimuli. Additional studies will determine whether K14-Fyn transgenic mice represent a model for characterizing cutaneous neoplasia.

Increasing Srcasm levels decreases Fyn levels in three different experimental systems: double-transgenic mice, adenoviral infection of primary keratinocytes, and transfection of cell lines. Phenotype maintenance in K14-Fyn/Srcasm-P mice supports the hypothesis that Srcasm-dependent Fyn downregulation requires Fyn phosphorylation of Srcasm. Biochemical characterization of Srcasm-dependent Fyn downregulation in COS cells demonstrates that Srcasm levels influence the degree of downregulation. The biphasic relationship between Srcasm and Fyn levels suggests that Srcasm may associate with other molecules to promote degradation of Fyn; in such a model, high Srcasm levels may dilute the pool of these down-regulatory molecules thereby decreasing the efficiency of downregulation. It will be interesting to determine if molecules such as Tollip, TSG101, Cbl, or other mono-ubiquinated proteins play a role in downregulation of activated SFKs. (32–34)

Srcasm-dependent Fyn downregulation in COS cells not only requires phosphorylation of Srcasm by Fyn but also a functional Srcasm GAT domain. Srcasm contains VHS and a GAT domains that are found in a number of proteins associated with endosomal trafficking.(23, 32, 34, 35) The Srcasm GAT domain binds to mono-ubiquinated proteins and to Tollip (Toll-interacting protein) in a mutually exclusive manner.(32) Immunofluorescence studies in cell lines demonstrate that Fyn and Srcasm co-localize to the multi-vesicular body.(34) Therefore, Srcasm lies at a signaling nexus involving mono-ubiquinated proteins, growth factor/cytokine signaling, and the endosomal/lysosomal pathway.(14, 36, 37) Given these characterisitics, Srcasm appears to play an important role in terminating SFK-dependent signals downstream of growth factors and cytokine receptors (Fig. 10). In fact, previous data show that increased Srcasm levels promote keratinocyte differentiation and that cutaneous squamous cell carcinoma and related precursor lesions exhibit decreased Srcasm levels.(14) Characterization of the cellular mechanisms associated with Srcasm downregulation in carcinomas may provide new insights into how SFK activity is increased in carcinomas in the absence of activating mutations. Ammonium chloride treatment reliably inhibited Srcasm-dependent Fyn downregulation; this treatment will raise lysosomal pH thereby globally affecting lysosomal protease activity and promoting secretion of lysosomal proteases.(38, 39) In addition, cells exposed to ammonium chloride exhibit altered intracellular membrane trafficking and receptor complex disassembly within the endosomal-

lysosomal pathway.(40, 41) The inability of E64 and leupeptin to alter Srcasm-dependent Fyn downregulation may reflect incomplete inhibition of all lysosomal proteases. Proteosomal inhibitors mildly inhibited Srcasm-dependent Fyn down regulation, suggesting that Srcasm may, in part, promote Fyn downregulation through the proteosome. Current studies characterizing how Srcasm interacts with the intracellular membrane surfaces of the endosomal-lysosomal pathway to promote Fyn downregulation should provide novel insights into SFK signal regulation.

Srcasm, but not Srcasm-P, downregulates the activated Fyn Y528F kinase to levels similar to that seen with native Fyn. These results parallel findings seen in Cbl-dependent Fyn downregulation, where Fyn tyrosine kinase activity is critical in promoting kinase downregulation.(29, 33) The relationship between Srcasm and Cbl in regulating the levels of Src-family kinases will be important to explore.(42, 43)

The data presented support a novel mechanism of regulating activated SFKs through modulating Srcasm levels. The ratio of SFK-to-Srcasm appears important for determining whether there is increased or decreased kinase activity (Fig. 10). The ability of Srcasm to attenuate increased Fyn activity has profound effects upon keratinocyte growth and differentiation as manifested by the phenotypic variation of the various transgenic strains. Further work to delineate the molecular partners in Srcasm-dependent Fyn downregulation should provide important insights into epithelial diseases and their biology.

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Abbreviations

Ad	adenovirus
BrdU	bromodeoxyuridine
EGF	epidermal growth factor
GFP	green fluorescent protein
HA	hemagluttinin epitope
MAP kinase	mitogen activated protein kinase
MOI	multiplicity of infection
PHKs	primary human keratinocytes
PI-3 kinase	phosphoinositol-3-kinase
rtTA	reverse tetracycline-controlled transactivator
SCC	squamous cell carcinoma
SFK	Src family kinase
Srcasm	Src activating and signaling molecule
TGF-α	transforming growth factor alpha
TRE	tetracycline-response-element

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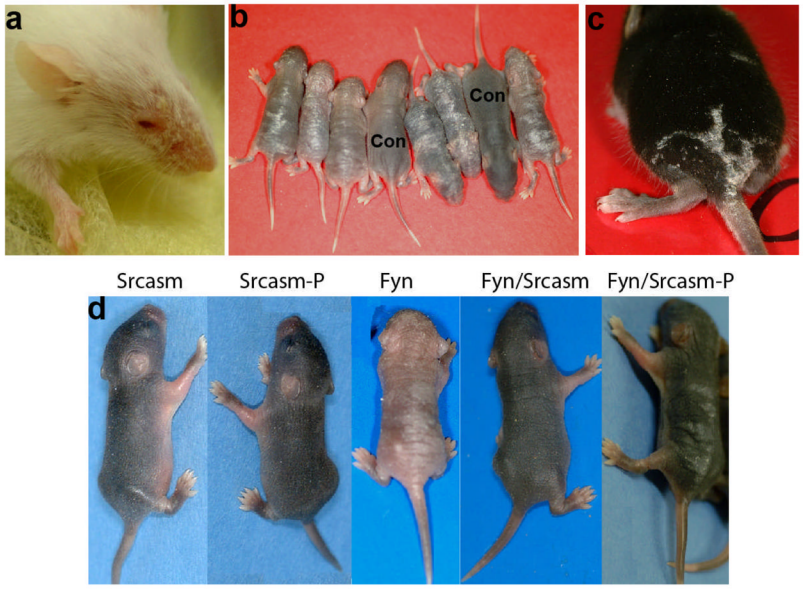

Figure 1. Phenotype of transgenic mice

a) K14-Fyn transgenic founder mouse at 4 weeks demonstrated scaly skin on the head. **b)** K14-Fyn progeny demonstrate thickened skin with scale on the back and head. Affected pups also exhibit runting. (Con-littermate controls) **c)** Localized hyperkeratotic plaques lacking hair were seen at two weeks. **d)** K14-Fyn and K14-Fyn/Srcasm-P mice maintain the thickened skin phenotype; K14-Srcasm, K14-Srcasm-P, and K14-Fyn/Srcasm mice lack the skin phenotype.

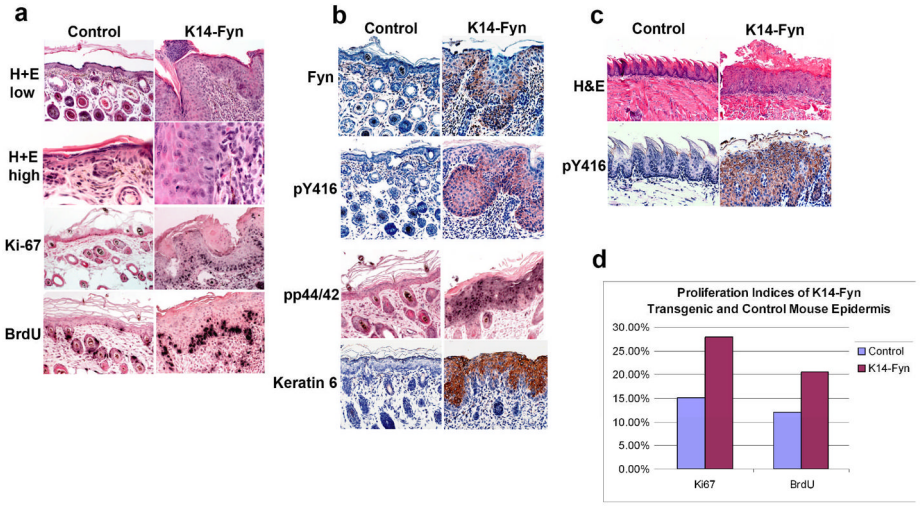

Figure 2. Histologic analysis of K14-Fyn and control mice

a) Affected back skin from one-week-old K14-Fyn mice demonstrated marked hyperplasia and hyperkeratosis (H+E, low); K14-Fyn epidermal keratinocytes are larger, exhibit nuclear atypia, and the K14-Fyn dermis contains an inflammatory infiltrate comprised of lymphocytes and neutrophils (H+E, high (630X)). The K14-Fyn epidermis is hyperproliferative as indicated by increased Ki-67 staining and BrdU labeling. **b)** Formalin-fixed sections of back skin from one-week old control and K14-Fyn mice were stained with hematoxylin and eosin or with antibodies to detect the following antigens: Fyn, activated SFKs (pY416), phospho-p44/42 MAP kinases, keratin 6. **c)** Sections of dorsal tongue from control and K14-Fyn one-week old mice stained with hematoxylin and eosin and for activated SFKs (pY416). **d)** Proliferation index for BrdU and Ki-67 staining. Epidermal sections of K14-Fyn and control mice were stained for BrdU and Ki-67 as in a). BrdU indices (% positive nuclear staining): control-12.0%, K14-Fyn-20.5%, P = 0.001. Ki-67 indices: control-15.1%, K14-Fyn-27.9%, P = <0.001.

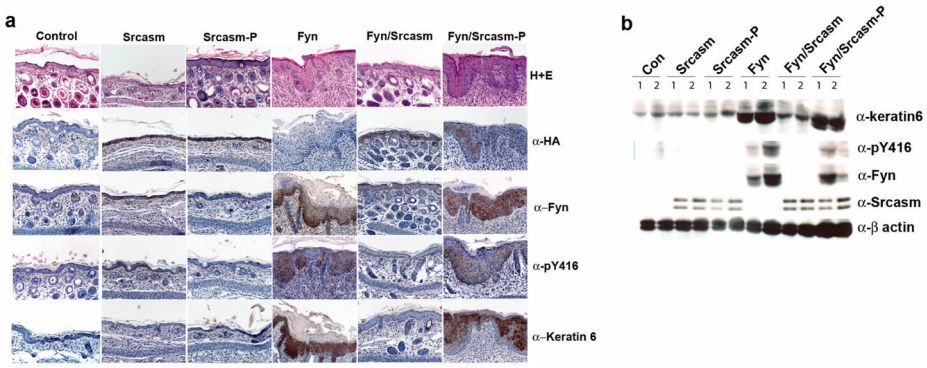

Figure 3. Comparative analysis of transgenic lines

a) Sections of ear skin from one-week old pups of the indicated genotype were stained with hematoxylin and eosin and for the following antigens: hemagluttinin epitope (HA) on Srcasm transgene, Fyn, levels of activated SFKs (pY416), and keratin 6. **b)** Western blot analysis of transgenic skin lysates. Skin protein lysates derived from two randomly selected one week-old transgenic mice of the indicated genotypes. Equivalent amounts of protein were subjected to SDS-PAGE and western blot analysis to detect: keratin 6, activated SFKs, Fyn, Srcasm, and β-actin. N=2.

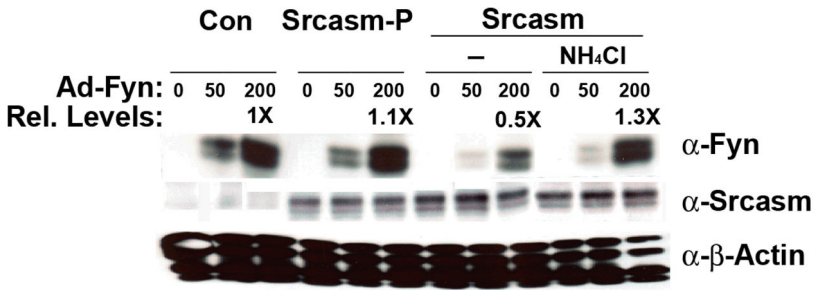

Figure 4. Elevated Srcasm but not Srcasm-P levels promote Fyn downregulation in murine keratinocytes

Primary keratinocytes cultures were derived from control, K14-Srcasm, and K14-Srcasm-P 2–3 day-old mice. Some cultures were infected with Ad-Fyn at the specified MOI 16 hours before harvesting, and a subset of K14-Srcasm cultures was treated with 10 mm NH_4Cl for one hour prior to lysis. Lysates were analyzed by western blotting to detect Fyn, Srcasm, and β-actin. The relative intensity of the Fyn bands corresponding to the MOI 200 samples was analyzed by densitometry and standardized to β-actin. Fold differences are reported. N=3

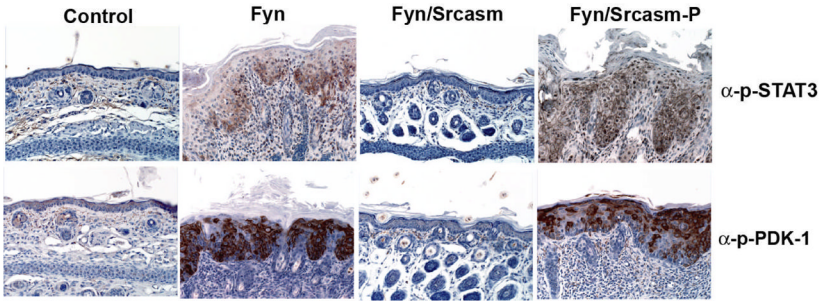

Figure 5. Immunohistochemical analysis of STAT-3 and PDK-1 signaling in transgenic strains

Formalin-fixed sections of ear skin from the specified genotypes were stained for activated STAT-3 and activated PDK-1. Immunohistochemistry performed on at least 3 mice per strain.

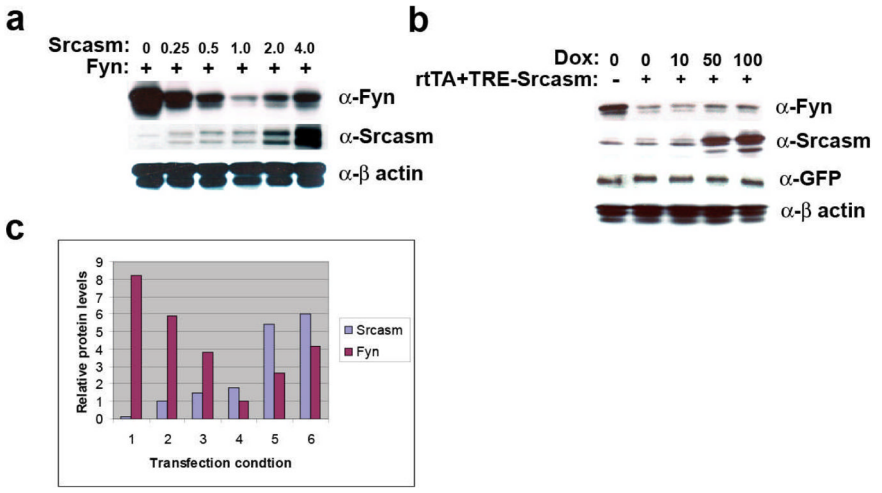

Figure 6. Biochemical Analysis of Srcasm-Dependent Fyn Downregulation

a) Srcasm levels influence degree of Fyn downregulation. COS cells were transfected with a Fyn plasmid or control vector and varying amounts HA-Srcasm plasmid (numbers indicate μg DNA). Lysates subjected to SDS-PAGE and western blot analysis to detect Fyn, Srcasm, and β-actin. N=3 **b)** Inducible Srcasm expression promotes Fyn downregulation. COS cells were transfected with Fyn plasmid and control vectors or CMV-rtTA and TRE-Srcasm plasmids. Some cells were incubated with doxycycline-containing (ng/ml) media for six hours. Lysates subjected to SDS-PAGE and western blot analysis to detect Fyn, Srcasm, GFP, and β-actin. Data representative of four experiments. **c)** Biphasic relationship between Fyn and Srcasm levels. Graphical representation of densitometric analysis of data in "a" after standardization to β-actin signals.

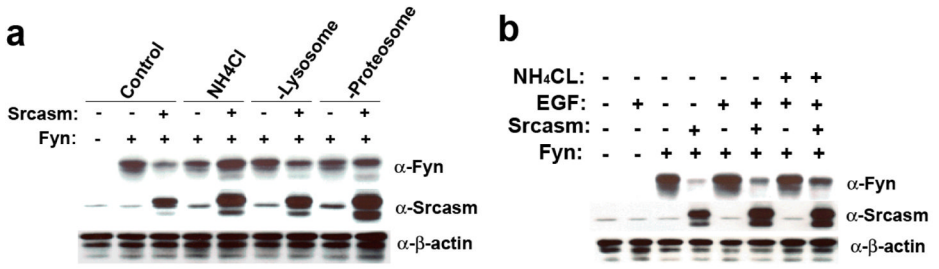

Figure 7. Effects of ammonium chloride, proteosomal inhibitors, and EGF upon Srcasm-dependent Fyn downregulation

a) COS cells were transfected with plasmids containing Fyn and HA-Srcasm. Cultures were subjected to 10 mM NH_4Cl for one hour prior to lysis, preincubation with lysosomal protease inhibitors: E64 + leupeptin at 0.1mM for 4 hours (−Lysosome), or preincubation with proteosomal inhbitors: lactacystin 10 uM + MG132 0.1 mM + ALLN 0.1 mM for 4 hours. Lysates were subjected to SDS-PAGE and western blot analysis to detect Fyn, Srcasm, and β-actin. Data representative of 3 experiments. **b)** Effect of EGF on Srcasm-dependent Fyn downregulation. COS cells were transfected as in A and cultured in low serum media. Some cultures were subjected to no stimulus, 10 mM NH_4Cl for one hour prior to lysis, or EGF at 100 ng/ml for one hours. Lysates subjected to SDS-PAGE and western blot analysis to detect Fyn, Srcasm, and β-actin. N=2

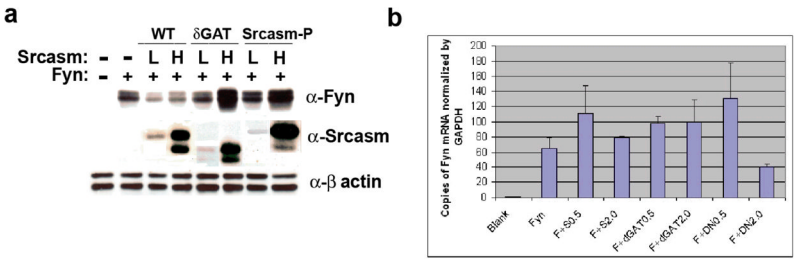

Figure 8. Structure-function analysis of Srcasm-dependent Fyn downregulation

a) COS cells were transfected with plasmids containing Fyn (0.5μg), vector alone, Srcasm, δGAT (Srcasm lacking GAT domain), and Srcasm-P. Srcasm plasmid doses: L- 1.0μg, H-2.0μg. Lysates subjected to SDS-PAGE and western blot analysis to detect Fyn, Srcasm, and β-actin. N=3 **b)** Quantitative RT-PCR for Fyn transcript. Cells were transfected as in A and total mRNA was isolated and subjected to quantitative RT-PCR to detect Fyn transcript. Transfection conditions indicated on X-axis, F-Fyn, S-Srcasm, dGAT-Srcasm GAT domain mutant, DN-Srcasm-P. Numbers indicate micrograms of plasmid. Relative Fyn transcripts amounts were assessed by quantitative RT-PCR amounts after normalization to glyceraldehyde-3-phosphate dehydrogenase transcript amounts. Duplicate sample analysis of two experiments.

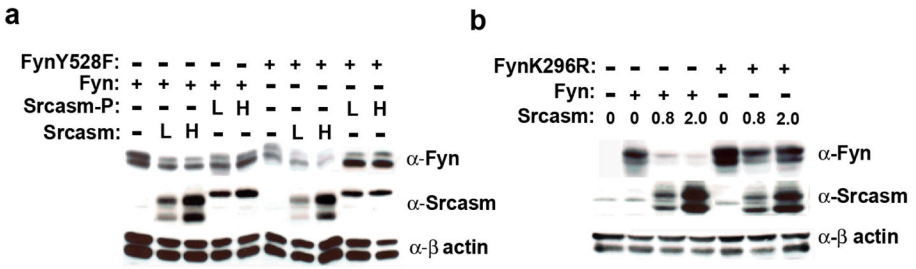

Figure 9. Fyn kinase activity is necessary for Srcasm-dependent Fyn downregulation

Srcasm downregulates activated Fyn. **a)** COS cells were transfected with plasmids containing Fyn or activated FynY528F (0.5 μg each). Cells co-transfected with plasmids containing no insert, Srcasm, or Srcasm-P. (L-0.5μg H–1.0μg). Lysates were analyzed to detect Fyn, Srcasm, and β-actin. N=2 **b)** Kinase-inactive Fyn is not efficiently downregulated by Srcasm. COS cells were transfected with plasmids containing Fyn or mutated Fyn K296R (0.5 μg each). Cells also were co-transfected with plasmids containing no insert or Srcasm. (numbers indicate μg of plasmid). Lysates were analyzed as in a. N=2

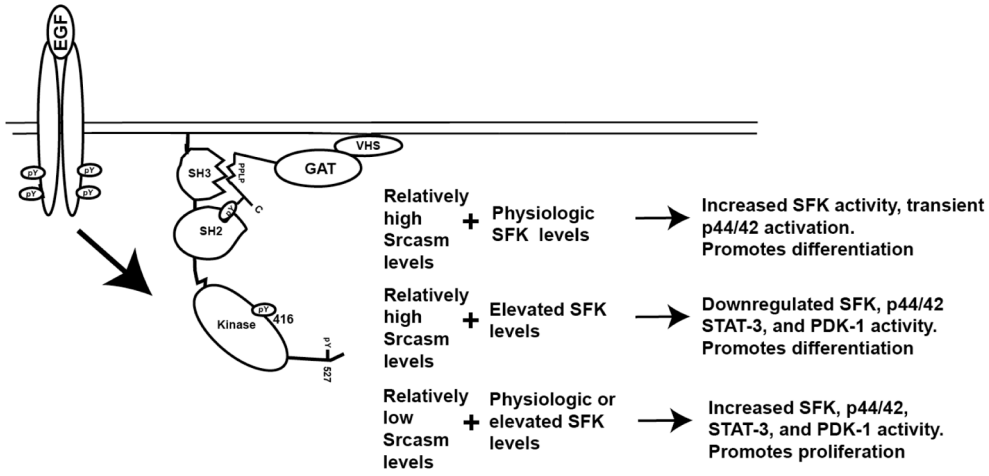

Figure 10. Srcasm modulation hypothesis

EGF receptor stimulation promotes activation of SFKs, Srcasm phosphorylation, and SFK-Srcasm association. Relatively high Srcasm levels promote activation of endogenous SFKs (14) or downregulate supraphysiologic levels of activated SFKs, thereby promoting keratinocyte differentiation. Persistent SFK signaling occurs with relatively low Srcasm levels leading to enhanced cell proliferation. The ratio of Srcasm to activated SFKs appears important for efficient kinase downregulation.

Table 1

Phenotype and Genotype of Mice Studied

The numbers of genotype positive mice from each strain are provided. The numbers of phenotype positive (> 1 cm^2 of hyperkeratosis) mice are also indicated. Controls and single transgenic strains represent the sum of genotypes from all crosses.

Strains:	Controls	K14-Srcasm	K14- Srcasm-P	K14-Fyn	K14-Fyn/Srcasm	K14-Fyn/Srcasm-P
Phenotype(+)	0	0	0	93	1	15
Genotype(+)	247	318	232	252	46	34

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