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, which express a nonphosphorylatable Srcasm mutant, maintained the hyperproliferative phenotype. Resolution of the hyperproliferative phenotype correlated with reduced Fyn levels in vivo in three experimental systems: transgenic mice, primary keratinocytes, and cell lines. Biochemical studies revealed that Srcasm-dependent Fyn down-regulation requires Fyn kinase activity, phosphorylation of Srcasm, and the Srcasm GAT domain. Therefore, Srcasm is a novel regulator of Fyn promoting kinase down-regulation 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)2 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 down-regulatory 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 EGF receptor 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 EGF receptor activity (9–11). Given 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 up-regulated consistent with a hyperproliferative phenotype.

Srcasm, the Src-activating and signaling molecule, is an SFK substrate that is tyrosine-phosphorylated secondary to EGF and transforming growth factor-α stimulation of primary human keratinocytes. 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 primary human keratinocytes, even after EGF stimulation. Srcasm levels are decreased in cutaneous squamous cell carcinoma and associated precursor lesions (14). These data suggest that Srcasm is an...
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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, whereas 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 down-regulation that requires Fyn kinase activity, the Fyn phosphorylation sites of Srcasm, and the Srcasm GAT (GGA and Tom-1) domain. Srcasm efficiently down-regulates constitutively activated Fyn mutants but not kinase-inactive mutants. High levels of Srcasm also interfere with the down-regulatory 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.

MATERIALS AND METHODS

Expression Vector Construction and Generation of Transgenic Mice—Murine HA-Srcasm and Fyn cDNAs were cloned into a human keratin 14 expression vector (23–25). The Srcasm-P mutant contained Tyr to Phe mutations at tyrosines 392, 440, 441, and 457. All K14 transgene cassettes were excised from the targeting vector and purified via Tris acetate-EDTA-agarose electrophoresis. C57BL/6 × CBA-fertilized oocytes were microinjected with the K14 transgene cassettes using standard protocols of the University of Pennsylvania 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′-ATCTTGCAGACCTCAGGG-3′; noncoding primer (Srcasm), 5′-GTTGAGCCACAGAGGATG-3′. This strategy produced a 900-bp 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 noncoding (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 microinjected with the K14 transgene cassettes using standard protocols of the University of Pennsylvania Transgenic Core Facility.

Expression of Fyn was evaluated by Western blotting with antibodies against Fyn (51). K14-Fyn 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 down-regulation that requires Fyn kinase activity, the Fyn phosphorylation sites of Srcasm, and the Srcasm GAT (GGA and Tom-1) domain. Srcasm efficiently down-regulates constitutively activated Fyn mutants but not kinase-inactive mutants. High levels of Srcasm also interfere with the down-regulatory 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.

In Vivo BrdUrd Labeling of Transgenic Mice—Mice were injected intraperitoneally with 100 µl (1 mg) of BrdUrd solution and sacrificed 1 h later. Skin was cut, formalin-fixed, and processed for BrdUrd immunohistochemistry. Proliferation indices were determined by counting positive nuclei. p values were calculated using a Pearson Chi-squared test. BrdUrd indices (% positive nuclear staining) were: control, 12.0%, n = 382; K14-Fyn, 21.5%, n = 620. For Ki-67 staining: control, 52 positive nuclei/344 cells; K14-fyn, 43 positive nuclei/1544 cells. A Pearson Chi-squared test was applied to determine the statistical significance of positive staining percentages.

Antibodies—α-HA antibody (clone 3F10, Roche Applied Science) 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. α-phospho-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 Western blotting. Fyn (SC-16, Santa Cruz Biotechnology) was used at 1/100 for IHC and 1/1000 for Western blotting. α-Mouse keratin 6 antibody (PRB-169P, Covance) was used at 1/150 for IHC and 1/1000 for Western blotting. α-Ki-67 antigen antibody (NCL-L-Ki67-MM1, Novocastra Laboratories, Ltd.) was used at 1/100 for IHC. α-Phospho-PDK1 (Ser-241) antibody (Cell Signaling Technology) was used at 1/100 for IHC. α-BrdUrd antibody (Roche Applied Science) was used at 1/10 for IHC. α-β-Actin

were observed. 15 of 34 K14-Fyn/Srcasm-P and 6 of 17 K14-Fyn mice maintained the phenotype. 35 K14-Srcasm-P and 20 littermate controls demonstrated no epidermal hyperplasia. All mice were handled in accord with University of Pennsylvania Institutional Animal Care and Use Committee Protocol 705452. A two-sided Fisher’s exact test was used to determine the statistical significance of phenotype expression.
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Phenotype of transgenic mice. a, K14-Fyn transgenic founder mouse at 4 weeks demonstrates scaly skin on the head. b, K14-Fyn progeny demonstrate thickened skin with scale on the back and head. Affected pups also exhibit running (Con, littermate controls). c, localized hyperkeratotic plaques lacking hair were seen at 2 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.

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, cultured in MCDB-153 medium with supplements (14), and maintained at 34 °C in an atmosphere of 8% CO2. Keratinocytes were used at passage 3 or 4; cells at 60–70% confluency were infected with Ad-Fyn (murine Fyn) or control virus at the indicated m.o.i., and were analyzed 16 h 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 (26). Kinase-weak Fyn is a K296R mutation (2). As a transfection control, some cells were transfected with 0.25 μg of E-GFP Living Colors plasmid (BD Biosciences). Cells at ~60–70% confluency were transfected for 5 h with the indicated expression vectors using Lipofectamine and recovered in medium plus serum for 16 h before lysis. Transfected cells were subjected to the following treatments before lysis: 10 mM ammonium chloride (for 1 h); proteasomal inhibitors (lactacystin at 10 μM for 1, 3, and 4 h; MG132 at 0.1 mM for 1, 3, and 4 h; ALLN at 0.1 mM for 1, 3, and 4 h; ALLN, MG132, and lactacystin for 1 h); and lysosomal protease inhibitors (E64 at 0.1 mM for 1, 3, and 4 h; leupeptin at 0.1 mM for 1, 3, and 4 h; E64 and leupeptin for 1 h).

For doxycycline-induced Srcasm expression, 0.5 μg of pcDNA 3.1 Fyn plasmid was co-transfected with empty vectors or pCMV-rtTA (reverse tetracycline-controlled transactivator) + pTRE-Srcasm (1.0 μg each). Some cells were incubated with 10, 50, or 100 ng/ml doxycycline-containing medium for 6 h prior to lysis.

Immunoblotting—Cell and tissue lysates were prepared using a radio-immune precipitation assay buffer with protease and phosphatase inhibitors (14). Lysates were incubated on ice for 15 min and then cleared by centrifugation at 14,000 × g for 10 min 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 (PerkinElmer Life Sciences). Western blotting was conducted in a standard manner with the indicated antibodies and developed using an enhanced chemiluminescence kit as described by the manufacturer (Lumilight Plus, Roche Applied Science). 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 TRIzol reagent, and total RNA was isolated. 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 an 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, which was prominent by 7 days (Fig. 1b). Approximately, 10% of the affected pups died by 1 week of age; such pups were runted and exhibited decreased feeding activity. Over a period of 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 with 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 upon staining with hematoxylin and eosin (H&E, low). K14-Fyn epidermal keratinocytes are larger and exhibit nuclear atypia, and the K14-Fyn dermis contains an inflammatory infiltrate comprised of lymphocytes and neutrophils (H&E, high \( (×630) \)). The K14-Fyn epidermis is hyperproliferative as indicated by increased Ki-67 staining and BrdUrd labeling. Formalin-fixed sections of back skin from 1-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. Sections of dorsal tongue from control and K14-Fyn 1-week-old mice stained with hematoxylin and eosin and for activated SFKs (pY416). Proliferation index for BrdUrd and Ki-67 staining. Epidermal sections of K14-Fyn and control mice were stained for BrdUrd and Ki-67 as in panel a. BrdUrd indices (percent positive nuclear staining): control, 12.0%; K14-Fyn, 20.3%; \( p = 0.001 \). Ki-67 indices: control, 15.1%; K14-Fyn, 27.9%; \( p < 0.001 \).

**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 cm² 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              |

**FIGURE 2.** Histologic analysis of K14-Fyn and control mice. a, affected back skin from 1-week-old K14-Fyn mice demonstrates marked hyperplasia and hyperkeratosis upon staining with hematoxylin and eosin (H&E, low). K14-Fyn epidermal keratinocytes are larger and exhibit nuclear atypia, and the K14-Fyn dermis contains an inflammatory infiltrate comprised of lymphocytes and neutrophils (H&E, high \( (×630) \)). The K14-Fyn epidermis is hyperproliferative as indicated by increased Ki-67 staining and BrdUrd labeling. b, formalin-fixed sections of back skin from 1-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 1-week-old mice stained with hematoxylin and eosin and for activated SFKs (pY416). d, proliferation index for BrdUrd and Ki-67 staining. Epidermal sections of K14-Fyn and control mice were stained for BrdUrd and Ki-67 as in panel a. BrdUrd indices (percent positive nuclear staining): control, 12.0%; K14-Fyn, 20.3%; \( p = 0.001 \). Ki-67 indices: control, 15.1%; K14-Fyn, 27.9%; \( p < 0.001 \).
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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 down-regulation 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 SFK activation, as in the K14-Fyn mice, increased Srcasm levels promote Fyn down-regulation.

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 with 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 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 down-regulation.

 Srcasm-dependent Fyn down-regulation 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 pretreatment with 10 mM NH4Cl for 60 min 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 down-regulation.

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, 29). 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 down-regulation 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 phosphatidylinositol 3-kinase; therefore, K14-Fyn skin was assessed for phosphatidylinositol 3-kinase activation by evaluating the levels of activated PDK-1 (30, 31). K14-Fyn skin demonstrated prominent cytoplasmic staining for activated PDK-1 in a mosaic pattern (Fig. 5). K14-Fyn/Srcasm

FIGURE 3. Comparative analysis of transgenic lines. a, sections of ear skin from 1-week-old pups of the indicated genotypes were stained with hematoxylin and eosin (H&E) for the following antigens: HA epitope on Srcasm transgene, Fyn, levels of activated SFKs (pY416), and keratin 6. b, Western blot analysis of transgenic skin lysates. Skin protein lysates were derived from two randomly selected 1-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. Con, control.

FIGURE 4. Elevated Srcasm but not Srcasm-P levels promote Fyn down-regulation in murine keratinocytes. Primary keratinocytes cultures were derived from control (Con), K14-Srcasm, and K14-Srcasm-P 2–3-day-old mice. Some cultures were infected with Ad-Fyn at the specified m.o.i. at 16 h before harvesting, and a subset of K14-Srcasm cultures was treated with 10 mM NH4Cl for 1 h 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 m.o.i. 200 samples was analyzed by densitometry and standardized to β-actin. Fold differences are reported. n = 3.

[Image 53x533 to 348x714]
[Image 96x391 to 256x409]
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![Image](https://example.com/image1.png)

**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 was performed on at least 3 mice/strain.

![Image](https://example.com/image2.png)

**FIGURE 6.** Biochemical analysis of Srcasm-dependent Fyn down-regulation. a, Srcasm levels influence degree of Fyn down-regulation. COS cells were transfected with a Fyn plasmid or control vector and varying amounts HA-Srcasm plasmid (numbers indicate μg of 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 down-regulation. COS cells were transfected with Fyn plasmid and control vectors or CMV-rtTA and TRE (tetracycline-response element)-Srcasm plasmids. Some cells were incubated with doxycycline-containing (ng/ml) medium (Dox) for 6 h. Lysates were subjected to SDS-PAGE and Western blot analysis to detect Fyn, Srcasm, GFP, and β-actin. Data are representative of four experiments. c, biphasic relationship between Fyn and Srcasm levels. A densitometric analysis of the data in a, after standardization to β-actin signals, is represented graphically.

![Image](https://example.com/image3.png)

**FIGURE 7.** Effects of ammonium chloride, proteasomal inhibitors, and EGF upon Srcasm-dependent Fyn down-regulation. a, COS cells were transfected with plasmids containing Fyn and HA-Srcasm. Cultures were subjected to 10 mM NH₄Cl for 1 h prior to lysis; preincubation with lysosomal protease inhibitors (E64 + leupeptin at 0.1 mM) for 4 h (Lysosome); or preincubation with proteasomal inhibitors (lactacystin (10 μM) + MG132 (0.1 μM) + ALLN (0.1 μM)) for 4 h. Lysates were subjected to SDS-PAGE and Western blot analysis to detect Fyn, Srcasm, and β-actin. Data are representative of three experiments. b, effect of EGF on Srcasm-dependent Fyn down-regulation. COS cells were transfected as described for panel a and cultured in low serum medium. Cultures were subjected to no stimulus, 10 mM NH₄Cl for 1 h prior to lysis, or EGF at 100 ng/ml for 1 h. Lysates were subjected to SDS-PAGE and Western blot analysis to detect Fyn, Srcasm, and β-actin. n = 2.

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 Down-regulation**—Transfection studies in COS cells were performed to characterize Srcasm-dependent Fyn down-regulation. 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 biphase 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 down-regulation; 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 Fig. 6a. These experiments demonstrate a temporal relationship between increased Srcasm expression and Fyn down-regulation.

**Inhibition of Srcasm-dependent Fyn Down-regulation**—COS cells pretreated with 10 mM ammonium chloride for 60 min before lysis did not exhibit Srcasm-dependent Fyn down-regulation, 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 h prior to lysis did not appreciably alter Srcasm-dependent Fyn down-regulation (Fig. 7a). Incubation of transfected COS cells with proteasomal inhibitors lactacystin, MG132, and ALLN for up to 4 h before lysis mildly inhibited Srcasm-dependent Fyn down-regulation. These data suggest that Srcasm-dependent Fyn down-regulation requires an intact endosomal/lysosomal pathway and may require proteasomal function.

The ability of EGF to modulate Srcasm-dependent Fyn down-regulation was evaluated. EGF treatment alone did not
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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, 29). 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 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 down-regulation does not involve modulation of Fyn transcript levels.

The Role of Fyn Kinase Activity in Srcasm-dependent Fyn Down-regulation—Because Fyn phosphorylation of Srcasm appears necessary for Srcasm-dependent Fyn down-regulation, the role of Fyn kinase activity in Srcasm-dependent Fyn down-regulation was evaluated.

Structure-Function Analysis of Srcasm-dependent Fyn Down-regulation—A structure-function analysis of Srcasm was performed to identify domains and residues required for Srcasm-dependent Fyn down-regulation. The Srcasm GAT domain binds monoubiquitinated proteins, and this post-translational modification appears to be important for down-regulating EGF receptor and SFK signaling (32–35). A mutant Srcasm lacking the GAT domain failed to induce Fyn down-regulation; in contrast, this mutant Srcasm appeared to interfere with Fyn down-regulation (Fig. 8a). Similarly, co-transfection of Srcasm-P with Fyn did not decrease Fyn levels but increased Fyn levels relative to controls. These data demonstrate that the Srcasm GAT domain and Fyn phosphorylation sites are required for Srcasm-dependent Fyn down-regulation. 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

Structure-function analysis of Srcasm-dependent Fyn down-regulation. a, COS cells were transfected with plasmids containing Fyn (0.5 µg), vector alone, Srcasm, dGAT (Srcasm lacking GAT domain), and Srcasm-P. Srcasm plasmid doses: low (L), 1.0 µg; high (H), 2.0 µg. Lysates were subjected to SDS-PAGE and Western blot analysis to detect Fyn, Srcasm, and β-actin (n = 3). WT, wild type. b, quantitative RT-PCR for Fyn transcript. Cells were transfected as described for panel a, and total mRNA was isolated and subjected to quantitative RT-PCR to detect Fyn transcripts. Transfection conditions indicated on the x axis: F, Fyn; S, Srcasm; dGAT, Srcasm GAT domain mutant; DN, Srcasm-P. Numbers indicate micrograms of plasmid. Relative Fyn transcript amounts were assessed by quantitative RT-PCR amounts after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript amounts. This is a duplicate sample analysis of two experiments.

Fyn kinase activity is necessary for Srcasm-dependent Fyn down-regulation. Srcasm down-regulates activated Fyn. a, COS cells were transfected with plasmids containing Fyn or activated FynY528F (0.5 µg each). Cells were co-transfected with plasmids containing no insert, Srcasm, or Srcasm-P (low (L), 0.5 µg; high (H) 1.0 µg). Lysates were analyzed to detect Fyn, Srcasm, and β-actin. n = 2. b, kinase-inactive Fyn is not efficiently down-regulated 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 for panel a. n = 2.

appreciably effect Fyn down-regulation by Srcasm. However, EGF treatment of cells appeared to lessen the inhibitory effect of ammonium chloride on Fyn down-regulation (Fig. 7b). Therefore, EGF stimulation may promote Srcasm-dependent Fyn down-regulation under specific conditions.

Structure-Function Analysis of Srcasm-dependent Fyn Down-regulation—A structure-function analysis of Srcasm was performed to identify domains and residues required for Srcasm-dependent Fyn down-regulation. The Srcasm GAT domain binds monoubiquitinated proteins, and this post-translational modification appears to be important for down-regulating EGF receptor and SFK signaling (32–35). A mutant Srcasm lacking the GAT domain failed to induce Fyn down-regulation; in contrast, this mutant Srcasm appeared to interfere with Fyn down-regulation (Fig. 8a). Similarly, co-transfection of Srcasm-P with Fyn did not decrease Fyn levels but increased Fyn levels relative to controls. These data demonstrate that the Srcasm GAT domain and Fyn phosphorylation sites are required for Srcasm-dependent Fyn down-regulation. 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
Srcasm Corrects Fyn-induced Epidermal Hyperplasia

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 down-regulate 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 down-regulation.

mice requires elevated Fyn levels. As Fyn levels decrease with increasing Srcasm levels, the epidermal hyperplasia normalized. Spontaneous squamous cell carcinoma formation was not seen in 25 mice followed for 6 months; this cohort was not subjected to any procarcinogenic 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 down-regulation requires Fyn phosphorylation of Srcasm. Biochemical characterization of Srcasm-dependent Fyn down-regulation in COS cells demonstrates that Srcasm levels influence the degree of down-regulation. 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 down-regulation. It will be interesting to determine whether molecules such as Tollip (Toll-interacting protein), TSG101, Cbl, or other monoubiquitinated proteins play a role in the down-regulation of activated SFKs (35–37).

Srcasm-dependent Fyn down-regulation in COS cells requires not only phosphorylation of Srcasm by Fyn but also a functional Srcasm GAT domain. Srcasm contains VHS and GAT domains, which are found in a number of proteins associated with endosomal trafficking (23, 35, 37, 38). The Srcasm GAT domain binds to monoubiquitinated proteins and to Tollip in a mutually exclusive manner (35). Immunofluorescence studies in cell lines demonstrate that Fyn and Srcasm co-localize to the multivesicular body (37). Therefore, Srcasm lies at a signaling nexus involving monoubiquitinated proteins, growth factor/cytokine signaling, and the endosomal/lysosomal pathway (14, 39, 40). Given these characteristics, 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 down-regulation 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 down-regulation; this treatment will raise lysosomal pH, thereby globally affecting lysosomal protease activity and promoting secretion of lysosomal proteases (41, 42). In addition, cells exposed to ammonium chloride exhibit altered intracellular membrane trafficking and receptor complex disassembly within the endosomal-lysosomal pathway (43, 44). The inability of E64 and leupeptin to alter Srcasm-dependent Fyn down-regulation may reflect incomplete inhibition of all lysosomal proteases. Proteasomal inhibitors mildly inhibited Srcasm-dependent Fyn down-regulation, suggesting that Srcasm may, in part, promote Fyn down-regulation through the proteasome. Current studies characterizing how Srcasm interacts with the intracellular membrane surfaces of the endosomal-lysosomal pathway to promote Fyn down-regulation should provide novel insights into SFK signal regulation.

Srcasm, but not Srcasm-P, down-regulates the activated Fyn Y528F kinase to levels similar to that seen with native Fyn.
These results parallel findings seen in Cbl-dependent Fyn down-regulation, where Fyn tyrosine kinase activity is critical in promoting kinase down-regulation (32, 36). The relationship between Srcasm and Cbl in regulating the levels of Src family kinases will be important to explore (45, 46).

The data presented support a novel mechanism for 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 down-regulation should provide important insights into epithelial diseases and their biology.

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