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Kinase Suppressor of Ras (KSR1) modulates multiple Kit-ligand-dependent mast cell functions

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Abstract

The intricately regulated Ras pathway coordinates multiple kit-ligand induced mast cell functions, including chemotaxis, proliferation and degranulation. However, the intracellular proteins that modulate the intensity and duration of SCF-induced signals and the consequent cellular response are incompletely understood. Scaffolding proteins coordinate the spatial organization of MAPK proteins that may potentiate and/or inhibit cell functions. The Kinase Suppressor of Ras (KSR1) protein is known to function as a molecular scaffold and coordinates the organization of Raf/Mek/Erk in response to receptor tyrosine kinases. However, the impact of *KSR1* in myeloid mast cell functions and in response to stem cell factor remains unknown. In the present study, we investigated the role of *KSR1* in regulating cellular functions of bone marrow derived mast cells (BMMCs) of *KSR1* deficient ($^{-/-}$) mice. Genetic disruption of KSR1 resulted in both striking reductions in kit-ligand mediated proliferation and degranulation that are commonly attributed to MAPK signals. Surprisingly, disruption of the KSR1 scaffold also resulted in a decline in migration that is generally not linked to Raf-Erk signals. We found that loss of KSR1 does impact the biochemical activation of Pak kinase, a kinase that is known to modulate Raf-Erk signals and also F-actin polymerization key to mast cell migration. Collectively, these studies demonstrate that the scaffolding protein KSR1 has an important role in multiple kit-ligand mediated mast cell functions. This study elucidates varied mast cell physiological functions for KSR1, including those related to cytoskeletal organization, and it suggests a novel molecular target for attenuating mast cell-mediated inflammation.

Introduction

Mast cells have a key role in initiating innate host defense in sepsis, are key instigators in acute and chronic allergic asthma [1–5], and are increasingly recognized for their potential to promote inflammation and even alter the microenvironment of cancers. Upon stimulation,

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mast cells elaborate histamine, heparin, over 30 cytokines, growth factors, and proteases integral to host defense, asthma, and inflammation [1, 6, 7]. Stem cell factor (SCF), the ligand for the c-kit receptor tyrosine kinase, coordinates multiple mast cell functions, including differentiation, proliferation, survival, migration, and degranulation [8–10]. SCF/c-kit signaling initiates both the Ras-Raf-MEK-extracellular regulated kinase (ERK) and Ras-phosphoinositol-3-kinase (PI3K) cascades [11–15]. Accordingly, *K-ras* deficient mast cells demonstrate impaired proliferation, survival, migration, and degranulation [16], and both genetic and chemical inhibition studies have demonstrated various roles for effector molecules within SCF-stimulated Ras pathways [11, 13–15]. SCF-induced MEK-ERK signaling generally is associated with mast cell proliferation, protein synthesis, and degranulation while PI3K-Rho-GTPase signals are associated with mast cell migration. However, recent studies have implicated the close interconnection of these pathways [15].

Despite the known data regarding SCF signaling in the mast cell, novel molecular effectors continue to emerge. Kinase Suppressor of Ras (KSR1), a scaffolding protein which binds Raf-MEK-ERK at the plasma membrane, enhances ERK signaling and permits coordinated feedback mechanisms [17, 18]. KSR1 scaffolding potentiates Ras-dependent signal transduction and cellular functions, including proliferation and oncogenic potential [19, 20] and the survival of multiple cell types, including TNF-stimulated intestinal epithelium [21] and BDNF-stimulated neurons [22]. KSR1 additionally interacts with 14-3-3 proteins and heat shock proteins, among others, and MEK-KSR1 interactions can modulate tropomyosin expression and actin cytoskeletal architecture in Ras-transformed 3T3 cells [23]. Thus, KSR1 may coordinate cellular physiology within and beyond known aspects of the Raf-MEK-ERK pathway.

To date, no study has examined KSR1's modulation of SCF-dependent pathways in the mast cell. Here, we utilized a *KSR1* knockout (*KSR1*^{-/-}) mouse to examine consequences of *KSR1* deletion in SCF-dependent mast cell physiology and biochemistry. *KSR1*^{-/-} bone marrow derived mast cells (BMMCs) demonstrated decreased SCF-dependent ERK1/2 signaling. The biochemical change is associated with reductions in SCF-induced proliferation and migration. We also found a decreased capacity for allergen-induced degranulation in both SCF-stimulated and unstimulated cells, providing functional evidence that KSR1 influences the mast cell cytoskeleton in response to multiple stimuli. Collectively, these data implicate *KSR1* in the coordination of multiple SCF-induced mast cell functions, including proliferative and cytoskeleton related events. These findings suggest that, in the mast cell, KSR1 modulates signals in the SCF-induced Raf-MEK-ERK cascade.

Design and Methods

Animals

Generation of mice bearing genetic disruption of *KSR1* has been previously described [24]. *KSR1*^{+/-} mice were crossed to generate *KSR1*^{-/-} progeny and WT (*KSR1*^{+/+}) littermate controls. Progeny from these crosses were genotyped by polymerase chain reaction (PCR) as previously described [24]. All protocols were approved by the Institutional Animal Care and Use Committee. WT and *KSR1*^{-/-} mice were six to eight weeks old at the time of tissue harvest.

Mast cell culture and phenotypic analysis

Low density bone marrow mononucleocytes (BMMNCs) were isolated by ficoll-histopaque from the tibial and femoral marrow of WT and *KSR1*^{-/-} mice as described previously [25]. Low density cells were cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented

with 10% fetal bovine serum (FBS, Biomedica), 2% penicillin/streptomycin, 1% glutamine, and 10 ng/ml murine recombinant interleukin-3 (IL-3, Peprotech) for four weeks at 37°C, 5% CO₂ in a humidified incubator, as previously described [14]. All experiments were performed with at least three independent cultures generated from independent mice of each genotype. To verify the mast cell purity, c-Kit and FcεR1 expression was examined by flow cytometry following the staining of cells with phycoerythrin (PE)-conjugated anti-FcεR1 (MAR-1) and fluorescein isothiocyanate-conjugated anti-c-Kit (CD117, 2B8) antibodies (BD Biosciences).

Proliferation, cell cycle, and survival assays

Proliferation of mast cells was evaluated using a [³H] thymidine incorporation assay with a minor modification [26]. Briefly, mast cells were deprived of growth factors overnight, plated in a 96-well plate at 5×10⁴ cells in 100 μl of RPMI 1640 with 10% FBS and 20 ng/ml recombinant SCF (Peprotech), and incubated at 37°C, 5% CO₂ for 48 hours, followed by pulsing with [³H] thymidine (PerkinElmer Life and Analytical Sciences) for 16 hours and harvested on glass fiber filters (PerkinElmer). β emission was measured with a Beckman Coulter LS 6500 Scintillation Counter. To kinetically examine mast cell maturation, the expression of c-Kit/FcεR1 was examined by flow cytometric analysis weekly upon establishment of WT and *KSR1*^{-/-} mast cell cultures. The cell numbers of mast cells were also assessed by multiplying the percentage of c-Kit/FcεR1 cells in each culture with the total cell number of the culture.

To determine if loss of KSR affects the cell cycle, mast cells were deprived of growth factors overnight, plated in a 24-well plate at 1×10⁵ cells in 1000 μl of RPMI 1640 with 10% FBS and 20 ng/ml recombinant SCF (Peprotech), and incubated at 37°C, 5% CO₂ for 48 hours. Cells were stained with equal volumes of 2mg/ml RNase A in PBS and 0.6% NP40 containing 0.1% mg/ml propidium iodide (PI; Calbiochem, La Jolla, CA) in PBS at 4°C for 30 minutes [14]. Thereafter, cell cycle distribution was analyzed by flow cytometry as previously described [14]. For survival assay, mast cells were deprived of growth factors overnight, plated in a 96-well plate at 2×10⁶ cells in one ml of RPMI 1640 with 0.5% bovine serum albumin (BSA) and 50 ng/ml SCF, and incubated at 37°C, 5% CO₂ for 48 hours [14]. Aliquots were stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) per the manufacturer's instructions (BD Pharmingen) and analyzed by flow cytometry (FACSCalibur, BD Biosciences).

Migration assay

Mast cells were assessed for migration using a Transwell haptotaxis assay, as previously described [16]. Cells (1×10⁵) in serum-free medium were placed in the upper chamber of a fibronectin (CH-296; Takara Bio) coated transwell filter (8-μm pore, 24-well cluster; Costar) with the lower chamber containing 600 μl RPMI 1640 with 10 ng/ml SCF or no growth factors. After a 4-hour incubation at 37°C, 5% CO₂, cells in the upper chamber were removed by cotton swab, and the migrated cells attached to the bottom surface of the transwell were stained with 0.1% crystal violet in 0.1 M borate (pH 9.0) and 2% ethanol for 10 minutes at room temperature. Migrated mast cells were quantified by averaging cell counts of at least 20 high power fields on an inverted microscope.

Degranulation assay

Degranulation of mast cells was assessed by β-hexosaminidase release as described previously [27]. Mast cells plated at 2×10⁶ cells in one mL RPMI in a 24-well plate were sensitized with 1.5 μg/ml IgE anti-dinitrophenol (DNP) (Sigma Aldrich) for 2 hours at 37°C, 5% CO₂ in a humidified incubator. Cells were washed with Tyrode's buffer (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and

0.5% BSA), followed by stimulation with either 30 ng/ml DNP-human serum albumin (Sigma Aldrich), 30 ng/ml DNP-human serum albumin and/or 10 ng/ml SCF, or no stimulation for 15 minutes at 37°C, 5% CO₂ in a humidified incubator. After stimulation, supernatants were collected and cell pellets lysed in 0.5% Triton X-100 in Tyrode's buffer. Supernatants and lysates were incubated in 1 mM *N*-acetyl-β-D-glucosaminidase (Sigma Aldrich) in citrate buffer [0.1 M citric acid and 0.1 M sodium citrate (pH 4.5)] for 1 hour at 37°C, 5% CO₂ in a humidified incubator, followed by the addition of 0.1 M sodium carbonate/sodium bicarbonate buffer. Absorbance was read at 405 nm using an Lmax microplate luminometer (Molecular Devices) and percent granule release was calculated as follows: (OD of supernatant)/(OD of supernatant + OD of pellet) × 100.

Immunoblots

To evaluate total protein and protein phosphorylation levels, mast cell cultures were deprived of growth factors overnight, standardized to 1.5×10^6 /mL, stimulated with 10 ng/mL SCF for 0, 2, or 5 minutes and western blots were performed as described previously [27]. Samples were lysed in equal volume SDS page buffer, loaded in equal volumes into 4–12 % Bis-Tris NuPAGE gels (Invitrogen), subjected to electrophoresis, and transferred to PVDF membranes. Membranes were blocked in 5% milk/TBS-Tween for 1 hour at room temperature and then incubated with antibodies overnight at 4°C. Antibodies included phospho-ERK1/2, total ERK1/2 antibodies, phospho-p38 MAP kinase (Thr180/Tyr182) antibody, total p38 antibody, anti-phospho-PAK2 (Thr423), and total PAK2 antibody. All the antibodies were purchased from Cell Signaling Technology. Secondary antibodies were either anti-rabbit or anti-mouse IgG horseradish peroxidase linked (Amersham Biosciences, Piscataway, NJ). Proteins were detected using enhanced chemiluminescence (Amersham) and densitometry determined with NIH Image software.

Statistical Analysis

Two-tailed Student's *t* test was used to evaluate statistical differences. *P* values less than 0.05 were considered significant.

Results

Loss of KSR1 did not affect mast cell differentiation in vitro

Raf-Erk signals have been found to significantly modulate the potential of cells to differentiate in other cell systems. To evaluate whether loss of *KSR1* influences mast cell differentiation from BMMNCs *in vitro*, we established mast cell cultures from the BMMNCs of *KSR1*^{-/-} and WT mice by culturing low density mononuclear cells in IL-3 for four weeks, and mast cell maturation was kinetically evaluated weekly by flow cytometric analysis with CD117- FITC and FcεRI- PE antibodies. As shown in Figure 1, during the culture in the presence of IL-3, WT and *KSR1*^{-/-} cells demonstrated similar expression patterns of c-Kit and FcεRI, surface receptors that serve as critical mast cell phenotypic markers. This data indicate that loss of *KSR1* does not substantially influence mast cell differentiation *in vitro*.

Genetic deletion of KSR1 reduced SCF induced mast cell proliferation

To evaluate whether deletion of *KSR1* alters SCF-induced mast cell proliferation, [³H]thymidine incorporation assays were performed. *KSR1*^{-/-} mast cells demonstrated a profound reduction in [³H]thymidine incorporation as compared to WT mast cells at multiple doses of SCF (Figure 2A), indicating a reduced proliferation in *KSR*^{-/-} mast cells. To determine whether the decreased proliferation of *KSR*^{-/-} mast cells was associated with DNA synthesis, cell cycle was examined. SCF stimulation increased the number of cells in

S/G2/M phase as compared with no growth factor condition regardless of the genotypes of mast cells (Figure 2B). There was a significant reduction in the proportion of *KSR*^{-/-} mast cells as compared to WT mast cells (30% WT versus 12% *KSR*^{-/-}). In addition, to evaluate the impact of deletion of *KSR1* in cell survival, BMMCs were deprived of FBS and cultured with or without SCF for 24 hours. The cells were then stained with Annexin V and PI and analyzed for the percent of Annexin V positive (+) and PI negative (-) by flow cytometry. *KSR1*^{-/-} BMMCs failed to demonstrate a significant change in the Annexin V⁺/PI⁻ population and early apoptotic cells as compared to WT BMMCs (Figure 2C).

To kinetically examine mast cell expansion, the cell numbers of mast cells were also assessed by multiplying the percentage of c-Kit⁺/FcεRI⁺ cell numbers with the total cell number in each culture. Compared to the mast cell numbers in WT cultures, deletion of *KSR1* significantly reduced mast cell numbers at each time point upon establishment of mast cell cultures Figure 2D. These *in vitro* data implicate a critical and specific role of *KSR1* in mast cell proliferation mediated by the changes in cell cycle but not cell survival.

KSR1 modulates SCF-mediated mast cell migration

Though *KSR* mediated functions have been principally linked to Ras-Raf-Erk signals, Raf and Mek are known to also be modulated by p21 activated kinases (Paks). In addition to their role in mediating Raf-Erk signals, Paks also modulate F-actin polymerization that is important for mast cell migration and degranulation. To test whether *KSR1* modulates SCF-induced mast cell migration, a transwell assay was used as previously described [12, 16]. In 4 independent experiments, *KSR1*^{-/-} BMMCs demonstrated substantially reduced SCF-induced migration as compared to WT BMMCs (Figure 3A). Representative photographs are shown in Figure 3B.

KSR1 positively regulates SCF/allergen-induced mast cell degranulation

Stem cell factor and cross-linking of the Fcε receptor both mediate mast cell degranulation. To evaluate the role of *KSR1* in regulating mast cell degranulation, β-hexosaminidase release assays were performed as previously described [16]. *KSR1*^{-/-} BMMCs demonstrated less β-hexosaminidase release following different dosages of SCF stimulation plus 50ng/ml of DNP (Figure 4A). β-hexosaminidase release in WT mast cells reached a peak at 25ng/ml of SCF plus DNP (50ng/ml) stimulation, while the peak of β-hexosaminidase release in *KSR1*^{-/-} mast cells was under 50ng/ml of SCF plus 50ng/ml of DNP stimulation. In addition, significantly less β-hexosaminidase release was observed with different doses of DNP stimulation plus a fix dosage of SCF (50ng/ml) as compared to WT mast cells (Figure 4B). β-hexosaminidase release reached a peak at 25ng/ml of SCF plus DNP (50ng/ml) stimulation in both WT and *KSR1*^{-/-} mast cells. These data provide initial evidence that *KSR1* regulates FcεRI-dependent pathways.

Loss of *KSR1* depresses SCF-induced ERK phosphorylation in mast cells

Proliferation and degranulation functions in mast cells are known to be mediated at least in part through activation of Raf-Mek-Erk. Given the reductions observed in these cellular functions in *KSR*^{-/-} mast cells, we next examined Erk phosphorylation. *KSR1*^{-/-} BMMCs demonstrated a markedly decreased ERK phosphorylation as compared to WT cells while there were similar protein levels of total Erk1/2 and β-actin (Figure 5A) in multiple independent experiments. In contrast, Akt phosphorylation was equivalent in both SCF stimulated WT and *KSR*^{-/-} mast cells (Figure 5B), consistent with *KSR1*^{-/-} BMMCs not having a defect in survival.

The consistent observation of a defect in mast cell migration suggests an additional role for *KSR* beyond the Raf-Mek-Erk signals. We have recently demonstrated that p21 activated

kinase functions as a key signaling kinase that links signals from Rho-GTPases to the Raf-Mek-Erk signaling pathway following SCF stimulation. Given the important role of KSR in physically positioning Raf-Mek-and Erk, we hypothesized that loss of KSR may directly interfere with the ability of Pak2 to modulate Raf-Erk signals. To test this hypothesis, we next evaluated SCF-mediated Pak2 phosphorylation. In three independent experiments, we found that Pak activation was consistently reduced in *KSR*^{-/-} mast cells while the protein loading was identical as evidenced by the total protein level of Pak2 (Figure 5C), consistent with the reduction observed in migration of *KSR*^{-/-} mast cells. Pak is known to be upstream of p38, a key mediator of migration. To assess if the loss of KSR is impacting other known Pak dependent functions, we next measured p38 phosphorylation. We found that p38 phosphorylation was consistently reduced in *KSR*^{-/-} mast cells following SCF stimulation (Figure 5D). Collectively, the data demonstrates that KSR, though having an important role for Raf-Mek-Erk signals, also has a broader role in mediating SCF dependent signals.

Discussion

The KSR1 scaffolding protein directly interacts with Raf-MEK-ERK in a Ras-dependent manner [24], critical components of SCF-induced mast cell physiology [16]. KSR1 has been shown to modulate cytoskeletal aspects in transformed 3T3 cells through ROCK-dependent mechanisms, connecting KSR1 to RhoGTPase cytoskeletal coordination [23]. However, the role of KSR1 in mast cell physiology remains unknown. Studies here elucidate three novel findings.

First, utilizing a genetic approach, we found that *KSR1* deletion induced mast cell functional defects *in vitro*. Although IL-3 cultured *KSR1*^{-/-} marrow produced viable c-kit⁺, FcεRI⁺ and granular mast cells, we found severe defects in SCF-induced proliferation, which is associated with the reduction in cell cycle, but not cell survival in *KSR1*^{-/-} mast cells. This finding reinforces the supposition of KSR1 as a Raf-MEK-ERK enhancing element, as ERK activation regulates proliferation in many cell types [28], including mast cells [15]. Importantly, loss of KSR1 depresses but does not ablate ERK activation. This activity may permit differentiation of *KSR1*^{-/-} low density mononucleocytes into mast cells, and it shows that MAPK signals in the SCF-induced mast cell do not wholly depend upon KSR1's scaffolding function.

Second, KSR1's positive regulation of SCF-induced migration suggests a role for KSR beyond commonly perceived signals in modulating Raf-MEK-ERK interactions and signals from p21Ras proteins. Indeed p21activated kinase phosphorylation, that is known to have an important role in phosphorylating Raf and Mek following SCF stimulation, is strikingly reduced in *KSR1*^{-/-} mast cells. Additionally, Pak2 is reduced in *KSR1*^{-/-} mast cells. Given the fact that Pak2 is known to modulate both F-actin and p38 phosphorylation following SCF stimulation, the reduction in Pak2 phosphorylation in *KSR1*^{-/-} mast cells may be responsible for the declined migration.

Finally, loss of *KSR1* did not affect survival or Akt phosphorylation in mast cells. These data point to an important specificity of the KSR scaffold as has been noted in other cellular contexts. This specificity, together with the reduction but not ablation of signals through the Raf-Erk pathway, points to the potential utility of KSR1 as a viable molecular target for therapeutics. Mast cells coordinate allergic asthma and, in certain contexts, the inflammatory bases of neoplastic conditions [6, 29, 30]. Recently, c-kit dependent bone marrow has been shown to critically underpin the inflammatory tumor microenvironment of a mouse plexiform neurofibroma model, where degranulating mast cells infiltrate tumor tissue [7]. Although this study has described a physiological consequence of genetic *KSR1* deletion in SCF-mediated mast cell physiology, further biochemical investigations could characterize

the exact mechanisms by which KSR1 modulates Raf-MEK-ERK-dependent and/or independent cytoskeletal coordination. Further insight into KSR1 may enhance our ability to molecularly target different features of mast cell-mediated inflammation.

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References

1. Galli SJ, Grimaldeston M, Tsai M. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. *Nat Rev Immunol.* 2008; 8:478–486. [PubMed: 18483499]
2. Piliponsky AM, Chen CC, Nishimura T, et al. Neurotensin increases mortality and mast cells reduce neurotensin levels in a mouse model of sepsis. *Nat Med.* 2008; 14:392–398. [PubMed: 18376408]
3. Metz M, Piliponsky AM, Chen CC, et al. Mast cells can enhance resistance to snake and honeybee venoms. *Science.* 2006; 313:526–530. [PubMed: 16873664]
4. Maurer M, Wedemeyer J, Metz M, et al. Mast cells promote homeostasis by limiting endothelin-1-induced toxicity. *Nature.* 2004; 432:512–516. [PubMed: 15543132]
5. Yu M, Tsai M, Tam SY, Jones C, Zehnder J, Galli SJ. Mast cells can promote the development of multiple features of chronic asthma in mice. *J Clin Invest.* 2006; 116:1633–1641. [PubMed: 16710480]
6. Coussens LM, Werb Z. Inflammatory cells and cancer: think different! *J Exp Med.* 2001; 193:F23–F26. [PubMed: 11257144]
7. Yang FC, Ingram DA, Chen S, et al. Nf1-dependent tumors require a microenvironment containing Nf1+/- and c-kit-dependent bone marrow. *Cell.* 2008; 135:437–448. [PubMed: 18984156]
8. Tsai M, Takeishi T, Thompson H, et al. Induction of mast cell proliferation, maturation, and heparin synthesis by the rat c-kit ligand, stem cell factor. *Proc Natl Acad Sci U S A.* 1991; 88:6382–6386. [PubMed: 1712491]
9. Taylor AM, Galli SJ, Coleman JW. Stem-cell factor, the kit ligand, induces direct degranulation of rat peritoneal mast cells in vitro and in vivo: dependence of the in vitro effect on period of culture and comparisons of stem-cell factor with other mast cell-activating agents. *Immunology.* 1995; 86:427–433. [PubMed: 8550081]
10. Galli SJ, Tsai M, Wershil BK. The c-kit receptor, stem cell factor, and mast cells. What each is teaching us about the others. *Am J Pathol.* 1993; 142:965–974. [PubMed: 7682764]
11. Ingram DA, Hiatt K, King AJ, et al. Hyperactivation of p21(ras) and the hematopoietic-specific Rho GTPase, Rac2, cooperate to alter the proliferation of neurofibromin-deficient mast cells in vivo and in vitro. *J Exp Med.* 2001; 194:57–69. [PubMed: 11435472]
12. Yang FC, Ingram DA, Chen S, et al. Neurofibromin-deficient Schwann cells secrete a potent migratory stimulus for Nf1+/- mast cells. *J Clin Invest.* 2003; 112:1851–1861. [PubMed: 14679180]
13. Kalesnikoff J, Rios EJ, Chen CC, et al. RabGEF1 regulates stem cell factor/c-Kit-mediated signaling events and biological responses in mast cells. *Proc Natl Acad Sci U S A.* 2006; 103:2659–2664. [PubMed: 16533754]
14. Yang FC, Kapur R, King AJ, et al. Rac2 stimulates Akt activation affecting BAD/Bcl-XL expression while mediating survival and actin function in primary mast cells. *Immunity.* 2000; 12:557–568. [PubMed: 10843388]
15. McDaniel AS, Allen JD, Park SJ, et al. Pak1 regulates multiple c-Kit mediated Ras-MAPK gain-in-function phenotypes in Nf1+/- mast cells. *Blood.* 2008; 112:4646–4654. [PubMed: 18768391]
16. Khalaf WF, Yang FC, Chen S, et al. K-ras is critical for modulating multiple c-kit-mediated cellular functions in wild-type and Nf1+/- mast cells. *J Immunol.* 2007; 178:2527–2534. [PubMed: 17277161]
17. Therrien M, Michaud NR, Rubin GM, Morrison DK. KSR modulates signal propagation within the MAPK cascade. *Genes Dev.* 1996; 10:2684–2695. [PubMed: 8946910]

18. McKay MM, Ritt DA, Morrison DK. Signaling dynamics of the KSR1 scaffold complex. *Proc Natl Acad Sci U S A*. 2009; 106:11022–11027. [PubMed: 19541618]
19. Kortum RL, Lewis RE. The molecular scaffold KSR1 regulates the proliferative and oncogenic potential of cells. *Mol Cell Biol*. 2004; 24:4407–4416. [PubMed: 15121859]
20. Razidlo GL, Kortum RL, Haferbier JL, Lewis RE. Phosphorylation regulates KSR1 stability, ERK activation, and cell proliferation. *J Biol Chem*. 2004; 279:47808–47814. [PubMed: 15371409]
21. Kim M, Yan Y, Kortum RL, et al. Expression of kinase suppressor of Ras1 enhances cisplatin-induced extracellular signal-regulated kinase activation and cisplatin sensitivity. *Cancer Res*. 2005; 65:3986–3992. [PubMed: 15899786]
22. Szatmari E, Kalita KB, Kharebava G, Hetman M. Role of kinase suppressor of Ras-1 in neuronal survival signaling by extracellular signal-regulated kinase 1/2. *J Neurosci*. 2007; 27:11389–11400. [PubMed: 17942733]
23. Janssen RA, Kim PN, Mier JW, Morrison DK. Overexpression of kinase suppressor of Ras upregulates the high-molecular-weight tropomyosin isoforms in ras-transformed NIH 3T3 fibroblasts. *Mol Cell Biol*. 2003; 23:1786–1797. [PubMed: 12588996]
24. Nguyen A, Burack WR, Stock JL, et al. Kinase suppressor of Ras (KSR) is a scaffold which facilitates mitogen-activated protein kinase activation in vivo. *Mol Cell Biol*. 2002; 22:3035–3045. [PubMed: 11940661]
25. Ingram DA, Yang FC, Travers JB, et al. Genetic and biochemical evidence that haploinsufficiency of the Nf1 tumor suppressor gene modulates melanocyte and mast cell fates in vivo. *J Exp Med*. 2000; 191:181–188. [PubMed: 10620616]
26. Ingram DA, Zhang L, McCarthy J, et al. Lymphoproliferative defects in mice lacking the expression of neurofibromin: functional and biochemical consequences of Nf1 deficiency in T-cell development and function. *Blood*. 2002; 100:3656–3662. [PubMed: 12393709]
27. Chen S, Burgin S, McDaniel A, et al. Nf1^{-/-} Schwann cell-conditioned medium modulates mast cell degranulation by c-Kit-mediated hyperactivation of phosphatidylinositol 3-kinase. *Am J Pathol*. 177:3125–3132. [PubMed: 21037083]
28. Meloche S, Pouyssegur J. The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. *Oncogene*. 2007; 26:3227–3239. [PubMed: 17496918]
29. Coussens LM, Raymond W, Bergers G, et al. Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. *Genes Dev*. 1999; 13:1382–1397. [PubMed: 10364156]
30. Coussens LM, Werb Z. Inflammation and cancer. *Nature*. 2002; 420:860–867. [PubMed: 12490959]

Figure 1. Phenotypic analysis of bone marrow derived mast cells in vitro
 BMMCs cultures were established by culturing bone marrow mononuclear cells of WT and *KSR1*^{-/-} mice in the presence of IL-3, and the expression c-Kit/FcεRI was evaluated by flow cytometric analysis. The Kinetic expression of c-Kit/FcεRI on WT and *KSR1*^{-/-} mast cells is shown. Representative flow cytometric analysis data of a WT mouse and a *KSR1*^{-/-} mouse at weeks 2, 3 and 4 in the culture in the presence of IL-3 are shown. X-axis indicated the intensity of the c-Kit expression and Y-axis indicated the intensity of the FcεRI. Data represented three independent experiments of three individual mice in each genotype.

Figure 2. Impact of KSR1 on SCF mediated mast cell proliferation

(A) Mast cell proliferation was measured by [³H]thymidine incorporation assay. Black dots indicated WT mast cells and open circles stand for *KSR1*^{-/-} mast cells. Y-axis indicated [³H]thymidine incorporation and X-axis indicated doses of SCF (ng/ml). Triplicate cultures were conducted. Data represented one of three independent experiments with similar results. *p<0.01 for WT vs. *KSR1*^{-/-}. (B) Analysis of cell cycle. After 48 hour culture in the presence of SCF (20ng/ml), mast cells were stained with PI and flow cytometric analysis was conducted. Proportion of mast cells in S/G2/M phase was shown as hatched area as red arrow pointed. (C) Analysis of apoptosis and cell death by staining with PI (Y-axis) and Annexin V (X-axis). Mast cells were cultured with SCF (20ng/ml). After 48 hours, cells were stained with PI and Annexin V as described in Material and Methods. Percent cells in the lower right panels represent apoptotic cells. (D) Mast cell numbers in each culture were calculated by multiplying total cell number in each culture with the percentage of cKit⁺/FceR1⁺ cells in the culture. Mast cell numbers at each time point are shown. *P<0.01 for WT vs. *KSR1*^{-/-} mast cells. Three independent cultures of each genotype were used.

Figure 3. Genetic deletion of KSR1 reduced SCF-mediated mast cell migration

Mast cell migration was examined by transwell assays following different doses of SCF stimulation. (A) Y-axis indicated migration mast cell number/HPF and X-axis indicated dosages of SCF in the bottom chambers. Data represented mean $\pm$ SE of triplicates. * $p < 0.01$ for WT and *KSR1*^{-/-} mast cells. Three independent experiments were conducted with similar results. (B) Representative photographs are shown.

Figure 4. β -Hexosaminidase release

Following stimulation of SCF and/or DNP at different dosage, degranulation of mast cells was assessed by β -hexosaminidase release. Mast cells (2×10^6 cells) were used for each condition. **(A)** % β -hexosaminidase release is shown following stimulation with a range of concentrations of SCF and fixed concentration of DNP. * $P < 0.01$ and ** $P < 0.05$ for WT vs. *KSR1*^{-/-}. **(B)** % β -hexosaminidase release is shown following stimulation with a range of concentrations of DNP and fixed concentration of SCF (50ng/ml). * $P < 0.01$ for WT vs. *KSR1*^{-/-}, respectively. Triplicates were conducted and three independent experiments were conducted.

Figure 5. Impaired SCF-mediated Erk, p38, and Pak phosphorylation in *KSR1*^{-/-} mast cells
 WT and *KSR1*^{-/-} mast cells were stimulated with SCF for the indicated times. Whole cell lysates were separated by SDS-PAGE. Representative immunoblots probed with antiphospho-p44/42 antibody (A), anti-phospho-Akt antibody (B), anti-phospho-Pak2 (C), and anti-phospho-p38 (D). Determination of comparable loading of protein lysates was assessed (lower blots) by reprobing the blots with anti-p44/p42, anti-Akt, anti-Pak2, and anti-p38, respectively. Data represented one of three independent experiments with similar results.