Title
RIN3 negatively regulates SCF-mediated responses of mast cells

Author
Janson, Christine

Publication Date
2012

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

RIN3 negatively regulates SCF-mediated responses of mast cells

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Biological Chemistry

by

Christine Marie Janson

2012

ABSTRACT OF THE DISSERTATION

RIN3 negatively regulates SCF-mediated responses of mast cells

by

Christine Marie Janson

Doctor of Philosophy in Biological Chemistry

University of California, Los Angeles, 2012

Professor John Colicelli, Chair

Stimulation of the receptor tyrosine kinase KIT by Stem Cell Factor (SCF) triggers activation of RAS and its downstream effectors. Proper KIT activation is essential for the maturation, proliferation and survival of mast cells. In addition, SCF activation of KIT is critical for recruiting mast cells to sites of infection or injury, where they release a mix of pro-inflammatory substances. RIN3, a RAS effector and RAB5-directed guanine nucleotide exchange factor (GEF), is highly expressed and enriched in human mast cells. SCF treatment of mast cells increased the amount of GTP-bound RAB5, and the degree of RAB5 activation correlated with the expression level of RIN3. SCF treatment also caused the dissociation of a pre-formed complex of RIN3 with BIN2, a membrane bending protein. Silencing of RIN3 increased the rate of SCF-induced KIT internalization, while persistent RIN3 over-expression led to KIT downregulation. These observations strongly support a role for RIN3 in coordinating the early steps in KIT endocytosis. Importantly, RIN3 also functions as an inhibitor of mast cell migration toward SCF. Finally, we demonstrate that elevated RIN3 levels sensitize mastocytosis cells to treatment with a KIT tyrosine kinase inhibitor, suggesting the value of a two-pronged inhibitor approach for this difficult to treat malignancy. These findings directly connect KIT activation with a mast cell-specific RAS effector that regulates the cellular response to SCF and provide new insight for the development of more effective mastocytosis treatments.

The dissertation of Christine Marie Janson is approved.

Jonathan Braun

Gregory Payne

Leonard Rome

John Colicelli, Chair

University of California, Los Angeles

2012

I dedicate my thesis to my parents.

Thank you for teaching me the value of hard work and a good education.

I also dedicate this to Ian for all his support and encouragement.

Table of Contents

List of Figures

Acknowledgements

I'd like to acknowledge fellow Colicelli lab members Joanne Bliss, Minh Thai, Xiaoqing Cao, Chelsea Mooser, Lucia Huynh, Pamela Ting, and Kavitha Balaji for advice and discussion concerning experiments and manuscripts. Emmanuelle Faure-Kumar, Janet Treger, Makoto Sato, and Brooke Bogan of the UCLA Vector Shared Resource & Core for their assistance with Ad5/F35 vector production; Sarah Dry (UCLA) for assistance with mastocytosis samples; Ben Van Handel (UCLA) for flow cytometry assistance; Juan Rivera and Ryo Suzuki (NIH) for degranulation assay assistance; A. Kirschenbaum (NIH) and J. Butterfield (Mayo Clinic) for providing mast cell lines; and Andre Lieber (University of Washington) for providing the adenovirus vector system.

Chapter Two is a version of: Colicelli, J., Ting, P., and Janson, C. (2012) RIN family Proteins (RIN1, RIN2, RIN3). *Encyclopedia of Signaling Molecules*. In Press. John Colicelli wrote the Historical Perspective, RIN family: Evolution and General Properties, RIN2, and Summary sections. Pamela Ting wrote the section on RIN1.

Chapter Three is a version of: Janson, C., Kasahara, N., Prendergast, G.C., and Colicelli, J. (2012) RIN3 Is a Negative Regulator of Mast Cell Responses to SCF. *PLoS ONE*. In Press. John Colicelli is the prinical investigator for this work. Nori Kasahara provided the adenovirus and George C. Prendergast provided the BIN2 antibody.

I'd like to thank the Cellular and Molecular Biology Training Grant and the Dissertation Year Fellowship for supporting this work.

Vita

2002-2006	B.S., Biology Truman State University, Kirksville, Missouri
2007-2010	Cellular and Molecular Biology Training Grant University of California, Los Angeles
2011-2012	Dissertation Year Fellowship University of California, Los Angeles

Publications

Janson, C., Kasahara, N., Prendergast, G.C., and Colicelli, J. (2012) RIN3 Is a Negative Regulator of Mast Cell Responses to SCF. *PLoS ONE*. In Press.

Balaji, K., Mooser, C., Janson, C., Bliss, J., Hojjat, H., and Colicelli J. (2012) EGFR Endocytosis and Signaling is Regulated by RIN1 Though Both RAB5GTPase and ABL Tyrosine Kinase Effectors. *Journal of Cell Science.* In Press.

Colicelli, J., Ting, P., and Janson, C. (2012) RIN family Proteins (RIN1, RIN2, RIN3). *Encyclopedia of Signaling Molecules*. In Press.

Chapter One

Mast cell biology;

SCF mediated signaling and biological output

1.1 Historical perspective

Paul Ehrlich was the first to describe mast cells in his doctoral thesis in 1878. They were distinguishable from other cells based on their large, negatively staining granules. He named the cells "Mastzellen," from the German "mast," which means feeding. Ehrlich hypothesized that the cells and their granules were providing nutrients to the surrounding tissue. However, the true functions of mast cells remained a mystery to the Nobel laureate, as well as the greater scientific community for generations (1).

In the early 20th century, Portier and Richet were pursuing a seemingly separate line of research, studying the mechanisms of allergic reactions. They were the first to describe acquired hypersensitivity reactions and coined the term anaphylactic shock. They used this term to describe the set of symptoms observed in dogs after receiving a second injection of extract from *Physalia* tentacles (2). In the 1920s great strides were made to better understand allergic response. Scientists discovered that allergy was triggered by an antibody-antigen interaction, as well as the fact that histamine was a major factor released. However, none of these scientists gave much thought to the mysterious mast cell. It wasn't until a series of papers from Riley and West (3,4) published in the 1950s that mast cells were established as the predominant source of histamine during an allergic response. After 60 years scientists had finally linked mast cells with their primary function.

Discovering that mast cells are the primary mediator of allergic response was a great leap forward for understanding this cell and further investigation lead to greater understanding of this cell's mediators and receptors. However, all the data indicated that this cell had harmful effects for the organism. This posed an obvious and perplexing question: why did evolution give rise to a cell type whose only role is to mediate potentially fatal reactions to benign antigens? It wasn't until 1996 that two labs (5,6) independently demonstrated that mast cells are crucial for responding to, properly clearing, and surviving bacterial infection *in vivo*. Finally, after one hundred years of research, scientists had discovered both the benefits and drawbacks of this complex player in innate immunity.

1.2 Mast cells in allergy and immune response

Mast cells are bone marrow-derived hematopoetic cells of the immune system (7). Although mast cells are derived from bone marrow, they circulate as immature precursors before taking up residence in the tissue where they mature (8). They reside in tissues that function as a barrier to the external environment, such as the skin, lung, and gut. Mast cells reside at these locations to facilitate their role as the first line of defense against pathogens. In the early stage of infection, mast cells help alert the rest of the immune system to the presence of the pathogen. Mast cells release pro-inflammatory mediators, cytokines, and growth factors to stimulate the proximal immune cells and recruit distal cells through the lympathic system (9). Mast cells are also known to support chronic inflammatory diseases such as arthritis (10).

As previously stated, mast cells primary role is to function in allergic response. The first time the body is exposed to a foreign substance (i.e. pollen), B cells make antibodies to antigens of the substance. One type of antibody is Immunoglobulin E (IgE). The FcεRI receptors on the

surface of mast cells bind to IgE; now the cells have been primed to respond to the antigen when the person is exposed a second time. Antigens will then bind to and crosslink the IgE on the surface of the cell causing the receptors to cluster. This causes activation of FcεRI signaling pathway resulting in degranulation, the exocytosis of granule contents. This event gives rise to the hallmarks of allergic response.

Granules are membrane bound sacs filled with histamine, heparin, proteases, and other pro-inflammatory factors. Histamine causes vasodilatation and increased capillary permeability, which allows white blood cells to invade the tissue, as well as bronchial constriction. In addition, histamine is a neurotransmitter and can cause sensory-neural stimulation (sneezing). Heparin is an anticogulant, which allows greater access of white blood cells to tissue. The proteases help to break down the extracellular matrix also aiding in white blood cell recruitment. These mediators are the primary factors that cause the edema, sneezing, trouble breathing, itching, and pain associated with severe allergic reactions.

1.3 Biological importance of KIT signaling

In addition to FcεRI, another important receptor for mast cell function is KIT (aka c-KIT, CD117). It is the most abundant tyrosine kinase receptor on the mast cell's surface; its ligand is Stem Cell Factor (SCF) (reviewed in (11-14)). The gene was first discovered as a viral oncogene (v-KIT) in Hardy-Zuckerman 4 feline sarcoma virus (15). Its cellular counterpart was cloned and sequenced shortly afterward (16). Mice with loss of function mutation in the KIT locus are termed W^{sh}/W^{sh}. These mice have defects in pigmentation, are anemic and sterile (17). These defects are mostly due to KIT's role in haematopoiesis (18) and development and function of germ cells (19) and melanocytes (20). Mast cell deficiency is also seen these mice, indicating

that KIT is critical for mast cell maturation (21,22). In fact, KIT signaling is critical for development of mast cells from human bone marrow, cord blood, and peripheral blood progenitor cells from humans (23). Gain of function mutations in KIT cause hyper-proliferative pathologies originating from different cell types, including mast cell-derived mastocytosis, a family of diseases characterized by mast cell hyper-proliferation (24). These can range from asymptomatic, indolent systemic mastocytosis to malignant, aggressive mast cell leukemia (25). Currently, there is no effective treatment option for patients with the most aggressive form. Most patients harbor the KIT^{D816V} gain of function mutation which is resistant to the tyrosine kinase inhibitor imatinib. Other kinase inhibitors are being tested in clinical trials; however, the results have been disappointing especially compared to the effectiveness of TKIs in treating chronic myelogenous leukemia (26).

KIT signaling is not only necessary for proper formation of mature mast cells, but also for mast cell survival, migration, and inflammatory response. Murine mast cells undergo programmed cell death within six hours after removal of SCF from the media (27). KIT promotes survival by inactivating proapoptotic BIM through both its transcriptional regulation and by regulating its phosphorylation by MEK (28). It is also well established that SCF, which is expressed and secreted by mast cells as well as other cell types, is a chemotatic factor for mast cells (29). Migration toward SCF is a complex process which utilizes multiple signal transduction pathways including MAP kinase (30), PI3 kinase (31), and Lyn kinase (32) activation. SCF also enhances mast cell responses leading to allergic airway inflammation and hyperreactivity (33).

1.4 Signaling through KIT regulates many pathways

Signaling is induced when the SCF homodimer binds to two monomeric KIT receptors on the plasma membrane (34) resulting in autophosphorylation of several residues in the cytoplasmic domain. These residues then serve as docking sites for Src-homology2 (SH2) containing proteins which promote signal transduction (Fig 1-1).

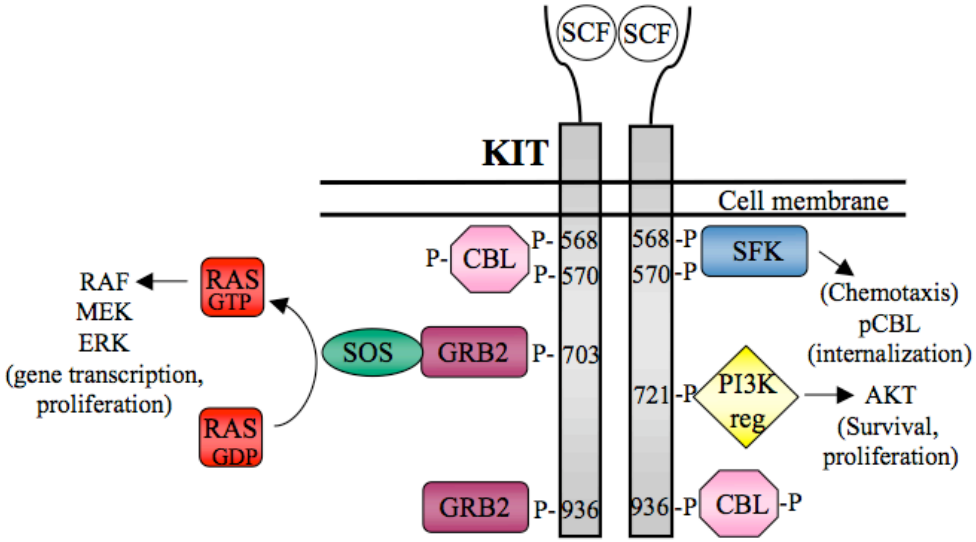


Figure 1-1. ***KIT binding partners and signal transduction.*** PI3Kreg denotes the p85 regulatory subunit of PI3-kinase.

Activation of the RAS/ERK pathway is well studied for its role in cell division and survival. RAS is a small G protein that can exist in either the active GTP bound or inactive GDP bound form. This process is regulated by SOS, a guanine exchange factor (GEF), which facilitates the exchange of GDP for GTP on RAS. In turn, SOS is regulated by its association with SH2 containing adaptor GRB2. After SCF stimulation, KIT is phosphorylated on Y703 and Y936; these sites recruit GRB2 (35) in a preformed complex with SOS to the plasma membrane. SOS is then properly placed for RAS activation resulting in activation of RAF/MEK/ERK pathway influencing gene transcription.

The lipid kinase, PI3-kinase phosphorylates the 3' hydroxyl group on phosphatidylinositol (PtdIns). The PIP_3 product recruits pleckstrin homology (PH) domain containing proteins to the plasma membrane where they are activated. PI3K contains SH2 domains and is recruited to phosphorylated Y721 of KIT (27). Signaling through this molecule leads to activation of AKT and phosphorylation and inactivation of BIM, a pro-apoptotic factor (36). Deletion of the p85a subunit of PI3K greatly reduced SCF-mediated proliferation of bone marrow derived mast cells from mice (37). Taken together, these results show that PI3K pathway is important for cell survival and proliferation downstream of KIT signaling.

The family of Src kinases (SFKs) consist of ubiquitously expressed Src, Yes, and Fyn as well as the more hematopoietic-restricted Lck, Hck, Fgr, Lyn, and Blk. These proteins contain a SH2, SH3, and kinase domain as well as a myristoylated and palmitoylated N terminus for anchorage in the membrane (reviewed in (38)). SFKs bind primarily to pY568 of KIT. Studies in Mo7e cells, a human hematopoietic cell line, show that chemotaxis toward SCF is deterred when cells are treated with the SFK inhibitor PP1 (31). Expression of dominant negative LYN in bone marrow derived mast cells causes decreased chemotaxis and proliferation (39).

In short, activation of KIT causes phosphorylation of multiple tyrosine residues on the protein. These residues then serve as docking sites for the activation of many other signaling molecules. This in turn leads to increased survival, proliferation, and migration for the cell.

1.5 Endocytosis and signaling

Endocytosis is a dynamic process by which a cell takes in molecules from its environment (reviewed in (40)). This term can be used to describe the uptake of many different types of substances through varying mechanisms. Different types of endocytosis are clathrin- mediated

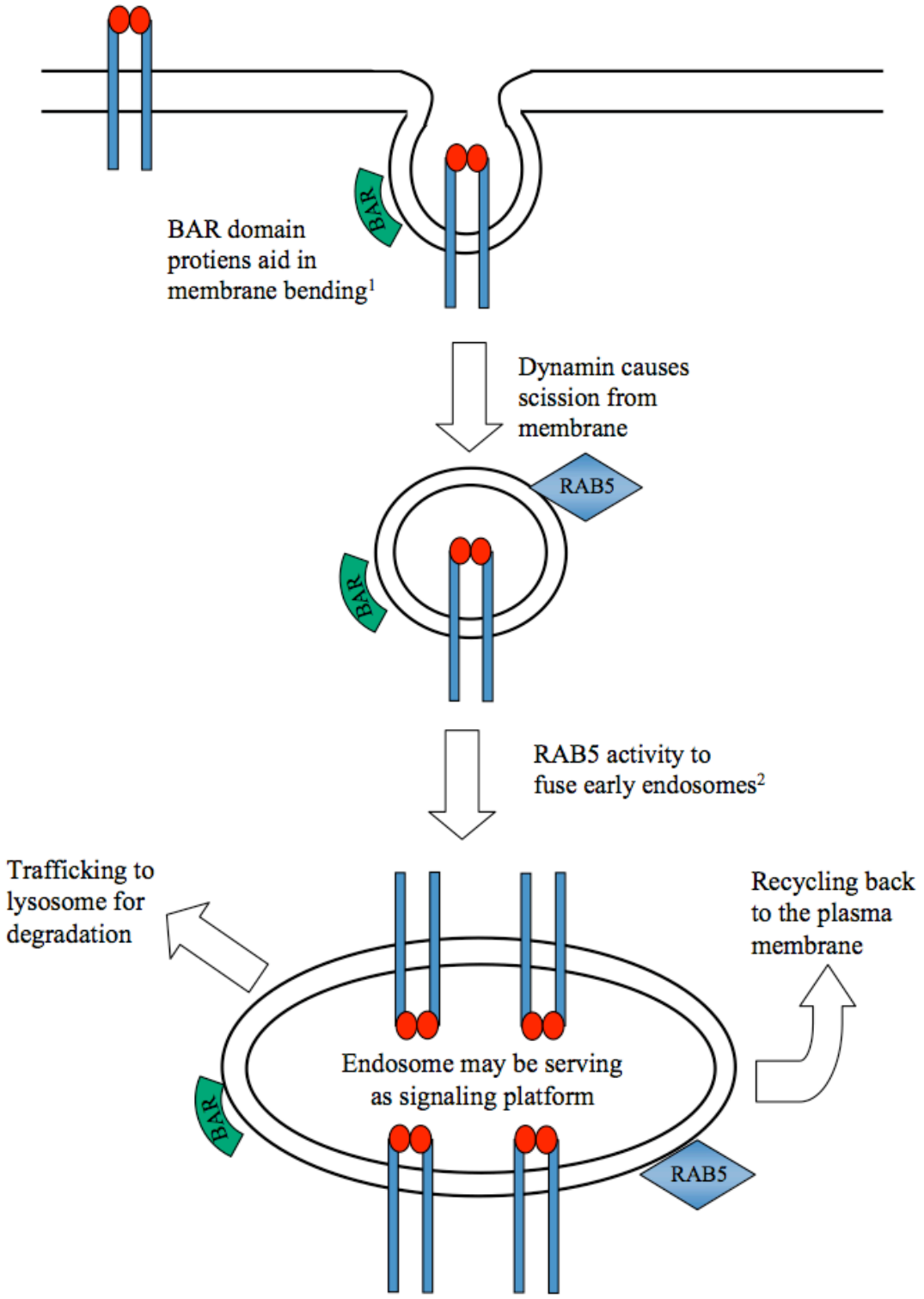


Figure 1-2. ***Schematic of RTK endocytosis and downstream trafficking.***
[1,2]Denote steps where RIN3 may aid in regulation.

endocytosis, caveolae endocytosis, macropinocytosis, and phagocytosis. Clathrin-mediated endocytosis is the canonical and best studied type of endocytosis for receptors. Following ligand binding to an RTK, epsin and BAR domain proteins are recruited to the plasma membrane to deform and invaginate the membrane. Clathrin then forms a scaffold around the invaginated membrane to build a sphere shape. Dynamin binds at the neck of this structure and mediates scission from the plasma membrane. This structure is then stripped of its clathrin coat. Through the activation of the small GTPase RAB5, this vesicle can now fuse with others to form early endosomes. From the endosome the RTK can be recycled back to the plasma membrane or it can continue down the pathway to the late endosome and finally to the lysosome for degradation (Fig 1-2).

Endocytosis can contribute to the downregulation of RTK signaling. Internalization of the receptor negatively regulates the strength and duration of plasma membrane associated signaling processes. For example, PLCγ1 and PI3K signaling happens at the plasma membrane. When epidermal growth factor receptor (EGFR) is internalized signaling through this pathway is inhibited (41). Also, the reduction of surface receptors can influence the dose response of the cell to that receptor's ligand. A higher concentration of ligand is needed for biological output that would otherwise be triggered at lower concentrations.

Until recently, endocytosis was thought of as strictly negative regulation of receptor mediated signaling; however, a growing body of evidence shows that endocytosis can positively influence signaling. The endosome may be serving as a signaling platform since it can provide a location for signaling complexes that either cannot form or form with low efficiency at the plasma membrane. Several studies have shown that endocytosis of various RTKs is important for full activation of ERK (42-44). This is explained by the presence of adaptors on the

endosomal membrane that bind to MEK and ERK, thus facilitating MEK phosphorylation of ERK (45).

Work from Marino Zerial's lab has proposed that APPL (adaptor protein, phosphotyrosine interaction, PH domain, and leucine zipper-containing) positive endosomes serve as specialized signaling endsomes (46,47). APPL proteins localize to endosomes by binding to activated RAB5, BAR domain proteins, and some RTKs (47). A study using zerbafish showed that knockdown of APPL causes increased, aberrant apoptosis during development. This phenotype was due to inhibition of Akt-GSK3β signaling (48). This evidence suggests that signaling through specific endosomal compartments can greatly influence biological processes.

In summary, endocytosis is a dynamic and important process for regulating receptor mediated signaling. Whether the signaling is enhanced or inhibited depends on the location of the signaling partner. Therefore, endocytosis influences the strength and duration of signaling which, in turn, affects biological outputs such as proliferation, adhesion, and migration.

1.6 Endocytosis and downregulation of KIT

KIT is rapidly internalized following SCF binding. Phosphorylation events causing the activation of the receptor also primes the receptor for internalization and downregulation. KIT, PI3K, and SFK activity as well as Ca^{+} influx are shown to play a role in KIT internalization. Working with W^{sh}/W^{sh} BMMCs, Yee et al (49) showed that KIT^{W42}, a mutant with impaired intrinsic kinase activity, is internalized more slowly. This result is broadly accepted; however, the role of PI3K signaling in internalization is more controversial. This paper also showed that KIT^{Y719F} (which corresponds to Y721 in human KIT) internalized at the same rate as wild type. This tyrosine serves as the binding site for PI3K, implicating that PI3K signaling is not required

for internalization. Later it was shown that internalization of KIT^{Y719F} expressed in the murine lymphoma cell line DA-1 was inhibited which was also dependent on Ca^{+} influx (50). A more recent paper (38) used murine KIT fused to EGFP to study internalization. Internalization of EGFP-KIT^{Y719F} was indistinguishable from EGFP-KIT. Overall, PI3K signaling might play a role in internalization, but detecting differences may be dependent on expression levels and cell type.

Signaling through SFKs is necessary for both internalization and down regulation of the receptor. PP1 treatment blocks SCF induced KIT internalization in Mo7e cells (50). This result was supported by another group, which showed that murine $Kit^{Y567/569F}$ has decreased internalization (51). SFK signaling also leads to degradation of KIT by activating the E3 ubiquitin ligase CBL. Phosphorylation of CBL by SFKs is necessary for activation of the protein; however, CBL also directly binds to Y568 and Y936 of KIT, which also causes CBL to become active. CBL mediates the ubiquitination of KIT, which targets the receptor for degradation. Recent studies (52,53) suggest that lack of CBL activation and proper KIT ubiquitination can cause reduced internalization, thereby, causing attenuated KIT signaling resulting in mast cell deficiency.

The serine/threonine kinase, Protein Kinase C (PKC), can down regulate KIT signaling through two distinct mechanisms. After SCF stimulation, PKC is activated by diacylglycerol (DAG). PKC then phosphorylates S741 and S746 in the kinase insert region of KIT, which inhibits KIT's kinase activity (39,54). Additionally, it has been shown that treatment with phorbol myristate acetate (PMA), which activates PKC, causes proteolytic cleavage of KIT. The SCF binding domain is released causing the cell to be less responsive to SCF (55,56). KIT

signaling can also be down regulated by Suppressors of Cytokine Signaling (SOCS)-1 and -6 (57), as well as the tyrosine phosphatase SHP-1 (58).

References

1. Beaven, M. A. (2009) *European journal of immunology* **39**(1), 11-25

2. Ring, J., Behrendt, H., and de Weck, A. *Chemical immunology and allergy* **95**, 1-11

3. Riley, J. F. (1953) *The Journal of pathology and bacteriology* **65**(2), 471-479

4. Riley, J. F., and West, G. B. (1953) *The Journal of physiology* **120**(4), 528-537

5. Echtenacher, B., Mannel, D. N., and Hultner, L. (1996) *Nature* **381**(6577), 75-77

6. Malaviya, R., Ikeda, T., Ross, E., and Abraham, S. N. (1996) *Nature* **381**(6577), 77-80

7. Tertian, G., Yung, Y. P., Guy-Grand, D., and Moore, M. A. (1981) *J Immunol* **127**(2), 788-794

8. Nakano, T., Sonoda, T., Hayashi, C., Yamatodani, A., Kanayama, Y., Yamamura, T., Asai, H., Yonezawa, T., Kitamura, Y., and Galli, S. J. (1985) *The Journal of experimental medicine* **162**(3), 1025-1043

9. Abraham, S. N., and St John, A. L. *Nature reviews* **10**(6), 440-452

10. Metz, M., Grimbaldeston, M. A., Nakae, S., Piliponsky, A. M., Tsai, M., and Galli, S. J. (2007) *Immunological reviews* **217**, 304-328

11. Ronnstrand, L. (2004) *Cell Mol Life Sci* **61**(19-20), 2535-2548

12. Roskoski, R., Jr. (2005) *Biochemical and biophysical research communications* **337**(1), 1-13

13. Roskoski, R., Jr. (2005) *Biochemical and biophysical research communications* **338**(3), 1307-1315

14. Besmer, P., Murphy, J. E., George, P. C., Qiu, F. H., Bergold, P. J., Lederman, L., Snyder, H. W., Jr., Brodeur, D., Zuckerman, E. E., and Hardy, W. D. (1986) *Nature* **320**(6061), 415-421

15. Yarden, Y., Kuang, W. J., Yang-Feng, T., Coussens, L., Munemitsu, S., Dull, T. J., Chen, E., Schlessinger, J., Francke, U., and Ullrich, A. (1987) *The EMBO journal* **6**(11), 3341-3351

16. Russell, E. S. (1979) *Advances in genetics* **20**, 357-459

17. Broudy, V. C. (1997) *Blood* **90**(4), 1345-1364

18. Sette, C., Dolci, S., Geremia, R., and Rossi, P. (2000) *The International journal of developmental biology* **44**(6), 599-608

19. Yoshida, H., Kunisada, T., Grimm, T., Nishimura, E. K., Nishioka, E., and Nishikawa, S. I. (2001) *The journal of investigative dermatology. Symposium proceedings / the Society for Investigative Dermatology, Inc* **6**(1), 1-5

20. Wershil, B. K., Tsai, M., Geissler, E. N., Zsebo, K. M., and Galli, S. J. (1992) *The Journal of experimental medicine* **175**(1), 245-255

21. Kirshenbaum, A. S., Goff, J. P., Kessler, S. W., Mican, J. M., Zsebo, K. M., and Metcalfe, D. D. (1992) *J Immunol* **148**(3), 772-777

22. Valent, P., Spanblochl, E., Sperr, W. R., Sillaber, C., Zsebo, K. M., Agis, H., Strobl, H., Geissler, K., Bettelheim, P., and Lechner, K. (1992) *Blood* **80**(9), 2237-2245

23. Boissan, M., Feger, F., Guillosson, J. J., and Arock, M. (2000) *Journal of leukocyte biology* **67**(2), 135-148

24. Iemura, A., Tsai, M., Ando, A., Wershil, B. K., and Galli, S. J. (1994) *The American journal of pathology* **144**(2), 321-328

25. Metcalfe, D. D. (2008) *Blood* **112**(4), 946-956

26. Ustun, C., DeRemer, D. L., and Akin, C. *Leukemia research* **35**(9), 1143-1152

27. Moller, C., Alfredsson, J., Engstrom, M., Wootz, H., Xiang, Z., Lennartsson, J., Jonsson, J. I., and Nilsson, G. (2005) *Blood* **106**(4), 1330-1336

28. Nilsson, G., Butterfield, J. H., Nilsson, K., and Siegbahn, A. (1994) *J Immunol* **153**(8), 3717-3723

29. Sundstrom, M., Alfredsson, J., Olsson, N., and Nilsson, G. (2001) *Experimental cell research* **267**(1), 144-151

30. Ali, K., Bilancio, A., Thomas, M., Pearce, W., Gilfillan, A. M., Tkaczyk, C., Kuehn, N., Gray, A., Giddings, J., Peskett, E., Fox, R., Bruce, I., Walker, C., Sawyer, C., Okkenhaug, K., Finan, P., and Vanhaesebroeck, B. (2004) *Nature* **431**(7011), 1007-1011

31. O'Laughlin-Bunner, B., Radosevic, N., Taylor, M. L., Shivakrupa, DeBerry, C., Metcalfe, D. D., Zhou, M., Lowell, C., and Linnekin, D. (2001) *Blood* **98**(2), 343-350

32. Campbell, B. G. (1999) *Pathology* **31**(1), 17-22

33. Thommes, K., Lennartsson, J., Carlberg, M., and Ronnstrand, L. (1999) *The Biochemical journal* **341 (Pt 1)**, 211-216

34. Lemmon, M. A., Pinchasi, D., Zhou, M., Lax, I., and Schlessinger, J. (1997) *The Journal of biological chemistry* **272**(10), 6311-6317

35. Serve, H., Hsu, Y. C., and Besmer, P. (1994) *The Journal of biological chemistry* **269**(8), 6026-6030

36. Fukao, T., Yamada, T., Tanabe, M., Terauchi, Y., Ota, T., Takayama, T., Asano, T., Takeuchi, T., Kadowaki, T., Hata Ji, J., and Koyasu, S. (2002) *Nature immunology* **3**(3), 295-304

37. Abram, C. L., and Courtneidge, S. A. (2000) *Experimental cell research* **254**(1), 1-13

38. Broudy, V. C., Lin, N. L., Liles, W. C., Corey, S. J., O'Laughlin, B., Mou, S., and Linnekin, D. (1999) *Blood* **94**(6), 1979-1986

39. Yee, N. S., Hsiau, C. W., Serve, H., Vosseller, K., and Besmer, P. (1994) *The Journal of biological chemistry* **269**(50), 31991-31998

40. Doherty, G. J., and McMahon, H. T. (2009) *Annual review of biochemistry* **78**, 857-902

41. Haugh, J. M., and Meyer, T. (2002) *Journal of cell science* **115**(Pt 2), 303-310

42. Vieira, A. V., Lamaze, C., and Schmid, S. L. (1996) *Science (New York, N.Y* **274**(5295), 2086-2089

43. Lampugnani, M. G., Orsenigo, F., Gagliani, M. C., Tacchetti, C., and Dejana, E. (2006) *The Journal of cell biology* **174**(4), 593-604

44. Sigismund, S., Argenzio, E., Tosoni, D., Cavallaro, E., Polo, S., and Di Fiore, P. P. (2008) *Developmental cell* **15**(2), 209-219

45. Nada, S., Hondo, A., Kasai, A., Koike, M., Saito, K., Uchiyama, Y., and Okada, M. (2009) *The EMBO journal* **28**(5), 477-489

46. Miaczynska, M., Christoforidis, S., Giner, A., Shevchenko, A., Uttenweiler-Joseph, S., Habermann, B., Wilm, M., Parton, R. G., and Zerial, M. (2004) *Cell* **116**(3), 445-456

47. Lin, D. C., Quevedo, C., Brewer, N. E., Bell, A., Testa, J. R., Grimes, M. L., Miller, F. D., and Kaplan, D. R. (2006) *Molecular and cellular biology* **26**(23), 8928-8941

48. Schenck, A., Goto-Silva, L., Collinet, C., Rhinn, M., Giner, A., Habermann, B., Brand, M., and Zerial, M. (2008) *Cell* **133**(3), 486-497

49. Gommerman, J. L., Rottapel, R., and Berger, S. A. (1997) *The Journal of biological chemistry* **272**(48), 30519-30525

50. Jahn, T., Seipel, P., Coutinho, S., Urschel, S., Schwarz, K., Miething, C., Serve, H., Peschel, C., and Duyster, J. (2002) *Oncogene* **21**(29), 4508-4520

51. Orinska, Z., Foger, N., Huber, M., Marschall, J., Mirghomizadeh, F., Du, X., Scheller, M., Rosenstiel, P., Goldmann, T., Bollinger, A., Beutler, B. A., and Bulfone-Paus, S. *Blood* **116**(15), 2665-2675

52. Blume-Jensen, P., Ronnstrand, L., Gout, I., Waterfield, M. D., and Heldin, C. H. (1994) *The Journal of biological chemistry* **269**(34), 21793-21802

53. Blume-Jensen, P., Wernstedt, C., Heldin, C. H., and Ronnstrand, L. (1995) *The Journal of biological chemistry* **270**(23), 14192-14200

54. Yee, N. S., Langen, H., and Besmer, P. (1993) *The Journal of biological chemistry* **268**(19), 14189-14201

55. De Sepulveda, P., Okkenhaug, K., Rose, J. L., Hawley, R. G., Dubreuil, P., and Rottapel, R. (1999) *The EMBO journal* **18**(4), 904-915

56. Bayle, J., Letard, S., Frank, R., Dubreuil, P., and De Sepulveda, P. (2004) *The Journal of biological chemistry* **279**(13), 12249-12259

57. Kozlowski, M., Larose, L., Lee, F., Le, D. M., Rottapel, R., and Siminovitch, K. A. (1998) *Molecular and cellular biology* **18**(4), 2089-2099

58. Ashman, L. K. (1999) *The international journal of biochemistry & cell biology* **31**(10), 1037-1051

Chapter 2

RIN Family Proteins (RIN1, RIN2, and RIN3)

2.1 Historical Background

The RIN1 and RIN2 genes were first identified in a selection for expressed human cDNAs that suppress phenotypes associated with oncogenic RAS mutations in a yeast model system (1). Subsequent analysis demonstrated that RIN1 binds specifically to activated (GTP-bound) human HRAS (2,3), suggesting that RIN1 is a downstream effector of RAS family proteins. RIN2 and RIN3 also bind to activated HRAS (4). All three RIN proteins contain a carboxy terminal RAS association (RA) domain that mediates this interaction (5).

It was subsequently reported that RIN1 encodes a VPS9-related guanine nucleotide exchange factor (GEF) function specific for the RAB5 family of early endocytosis GTPases (6). RIN2 and RIN3 also encode VPS9-type GEF domains upstream of the RA domain. This suggests that RIN family proteins are RAS effectors that promote RAB5 function, connecting RAS activation to receptor endocytosis.

Another defining feature of RIN proteins is their amino terminal SRC homology 2 (SH2) domain. The RIN1 SH2 domain mediates binding to activated receptor tyrosine kinases (7), which likely facilitates endocytosis and down regulation of these receptors. The RIN2 and RIN3 SH2 domains show some species variation (discussed below) (Fig 2-1).

RIN1 also binds and activates ABL1 and ABL2 tyrosine kinases (8,9). This capacity requires an amino terminal fragment of RIN1, which may not be functionally conserved in RIN2 and RIN3.

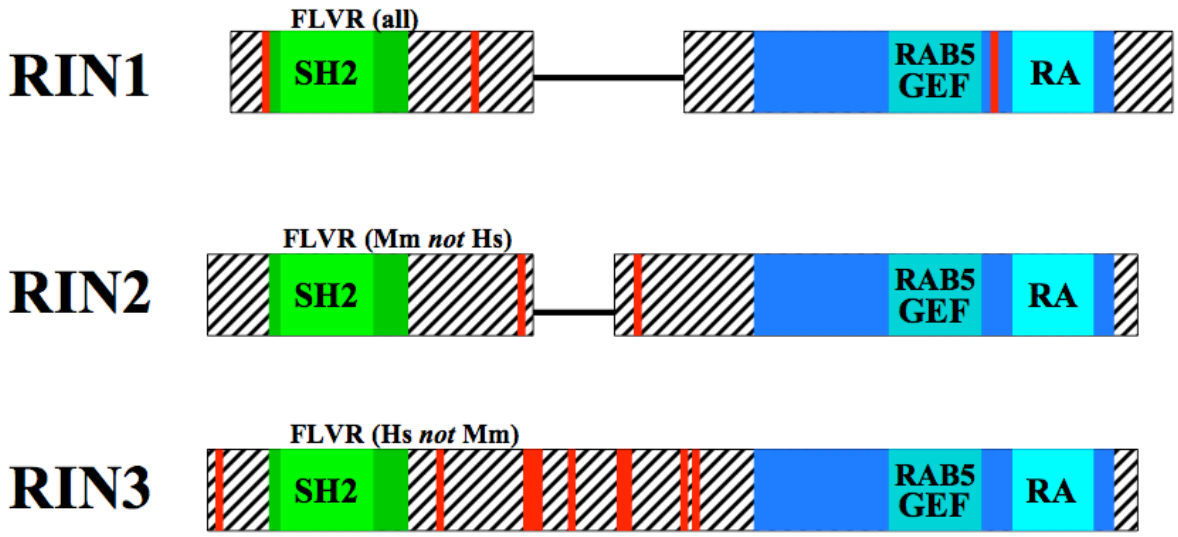


Figure 2-1. ***Domain structures for RIN family proteins.*** RA denotes the RAS Association domain; red areas are proline rich regions.

2.2 RIN Family: Evolution and General Properties

The RIN family of genes likely evolved from a progenitor represented in arthropods by the fruit fly *Drosophila melanogaster* gene Sprint (10) and in echinoderms by the sea urchin *Strongylocentrotus purpuratus* (annotated as Rin2L in www.spbase.org). The fruit fly and sea urchin gene products show strong conservation with vertebrate RIN proteins in the amino terminal SH2 domain and the carboxy terminal GEF domain. However, the fruit fly and sea urchin RIN proteins show no significant alignment with vertebrate RIN proteins in the central region or in the RA domain. This suggests that the gene expansion that gave rise to a RIN family in vertebrates was accompanied by the development of RAS effector capability. A recently annotated vertebrate RINL gene (11) encodes a protein that aligns with RIN1-3 but has no RA domain and may represent the vestige of a pre-vertebrate RIN gene.

Specific properties of each are described in the following sections.

2.3 RIN1

RIN1, the defining member of the RIN protein family, is a RAS effector that couples cell signaling with receptor trafficking and cytoskeletal remodeling. It is localized to the cytoplasm and the plasma membrane. Binding of RIN1 to RAS is GTP-dependent and mediated by the carboxy-terminal region of RIN1 (3). RIN1 overexpression suppresses fibroblast transformation by $HRAS^{G12V}$, likely by competing with RAS effectors that promote mitosis and suppress apoptosis (12). RIN1 localizes in cytoplasmic and membrane compartments, and this partition is regulated through a 14-3-3 interaction. Phosphorylation of RIN1-Ser^{351} by PKD enhances 14-3-3 binding, and mutation of this residue shifts RIN1 localization to the plasma membrane (12). This result suggests that 14-3-3 binding reduces access to RAS proteins, which are membrane tethered.

RIN1 encodes a VPS9 sub-family guanine nucleotide exchange factor (GEF) domain that mediates endosome fusion and receptor endocytosis through activation of RAB5 family proteins (6). RIN1 preferential associates with and activates the RAB5A isoform (13). The SH2 domain of RIN1 binds to activated (tyrosine phosphorylated) EGFR to promote receptor down regulation (7). Through a proline-rich domain, RIN1 may recruit the STAM2, a component of the ESCRT (endosomal sorting complex required for transport) machinery, and facilitate trafficking of ubiquitinated EGFR to lysosomes (14). RIN1 also promotes internalization of the TGF-β receptor through RAB5 activation. In this case, however, the result is increased signaling through SMAD2/3 and the transcription repressor SNAI1 (15). The contribution of RIN1 to growth factor receptor internalization and signaling may account for the observed silencing of RIN1 in breast tumor cells (16) and enhanced expression in non-small cell lung adenocarcinoma cells (17).

Other downstream effectors of RIN1 are the ABL family non-receptor tyrosine kinases. The ABL SH3 domain mediates binding to a proline-rich motif in RIN1, leading to ABL-mediated phosphorylation of RIN1-Y^{36} and subsequent association with the ABL SH2 domain (3,18). Binding of RIN1 to ABL relieves autoinhibition and stimulates ABL tyrosine kinase activity (8,9). As a positive regulator of ABL activity, RIN1 regulates actin remodeling. Mammary epithelial cells from $RIN1^{-/-}$ mice show extensive peripheral-actin networks, enhanced attachment to fibronectin, and increased cell motility (9). RIN1 potentiates the catalytic and transforming activity of the BCR-ABL1 fusion oncogene, and RIN1 over-expression increases BCR-ABL1 mediated leukemogenesis in a mouse model system (18). Importantly, the $ABL1^{T315I}$ mutant resistant to therapeutic kinase inhibitors remains responsive to positive regulation by RIN1 (8), and *Rin1*$^{-/-}$ bone marrow cells were refractory to transformation by BCR-$ABL1^{T315I}$ (19). These results suggest that the RIN1::ABL1 interaction may be a drugable vulnerability of oncogenic ABL fusion proteins.

Rin1 is most strongly expressed in mature forebrain neurons, with moderate expression in hematopoietic and epithelial cells (20). This restricted expression of Rin1 is mediated in part by SNAI1. The Rin1 promoter sequence also contains a consensus recognition site for the transcription repressor REST. Deletion of this region surprisingly led to a reduction in reporter gene expression, suggesting that elements in this region enhance expression and may be positively regulating Rin1 expression in neuronal cells (20).

Rin1$^{-/-}$ mice are viable and fertile and show no gross morphological abnormalities. However, they have elevated amygdala LTP (long term potentiation) and enhanced fear conditioning, suggesting that Rin1 normally acts as a negative regulator of synaptic plasticity in this region (21,22). In addition, *Rin1*$^{-/-}$ mice are deficient in conditioned fear extinction and latent

inhibition (22). *Rin1*$^{-/-}$ mice have normal hippocampal-dependent learning, as well as normal motor learning, anxiety and exploratory behavior, suggesting that *Rin1*$^{-/-}$ mice may be a useful model for studying neuropsychiatric conditions such as PTSD (post-traumatic stress disorder).

2.4 RIN2

The RIN2 gene is widely expressed in mouse, based on analysis of mRNA levels ((23), BioGPS.gnf.org). The SH2, RAB5-GEF and RA domains first characterized in RIN1 are well conserved in RIN2, which has demonstrable guanine nucleotide exchange activity on RAB5 (24) and RAS interaction properties (4). However, RIN2 gene products in primates (human and chimpanzee) differ from their orthologs in other vertebrates (cow, dog, mouse, opossum, chicken, frog and fish) in two notable aspects. First the amino termini of primate RIN2 proteins extend about 50 residues beyond other vertebrate RIN2 gene products. Second, an arginine residue critical for the phospho-tyrosine binding function of SH2 domains (mouse Rin2: FLVR^{122}) is instead a histidine in primate RIN2 (human RIN2: FLVH^{171}).

Loss of function mutations in RIN2 are associated with two related human connective tissue disorders referred to as MACS (25) and RIN2 Syndrome (26).

2.5 RIN3

RIN3 contains the guanine nucleotide exchange factor (GEF), Ras Association (RA), and SH2 domains conserved throughout the RIN gene family. In fact, RIN3 was first described as a novel RAB5 GEF, isolated from a human leukocyte cDNA library based on a yeast two-hybrid screen for RAB5B^{Q79L} interacting proteins (23).

RAB proteins are a small GTPases involved in vesicle fusion. GTPases cycle between active, GTP, and inactive, GDP, states (Fig 2-2). Guanine nucleotide exchange factors such as RIN3 aid in removal of GDP in order for a GTP to be added to activate the protein. RIN3 demonstrates guanine nucleotide exchange activity for RAB5 (23) and RAB31, but not RAB21 (27). RAB5 is well studied for its role in early endosome fusion downstream of receptor

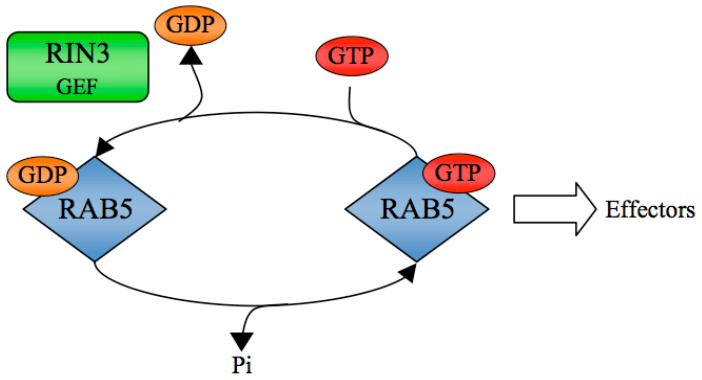


Fig 2-2. ***GEF activation of RAB5.*** RIN3 has GEF activity for RAB5, promoting its transition to the active state.

endocytosis. Kaijiho et al (23) showed that RIN3 and RAB5 co-localize when both are overexpressed in HeLa cells. However, RIN3 did not co-localize with the early endosome marker EEA-1. This suggests that RIN3 may be localized to a specific subset of endosomal vesicles. Another study (28) showed that tyrosine phosphorylation can cause RIN3 to translocate to RAB5 positive endosomes and deletion of the RA domain caused RIN3 to be constitutively located to endocytic vesicles. This suggests that the RAS Association (RA) domain has an inhibitory effect on RIN3's endosomal localization. However, this study was conducted in HeLa cells which show undetectable levels of endogenous RIN3 (see Chapter 3); therefore all experiments involved overexpression of the protein. Also, the phospho-tyrosine signal was achieved by treatment of the cell with pervanadate, a cell permeable phosphatase inhibitor,

instead of by stimulating a specific RTK or more physiological means. Although these results are interesting, it is important to take into account the artificiality of the system in which they were generated. Further studies on endogenous RIN3 in cells stimulated with a biological growth factor would better help to elucidate its relationship with RAB5.

RAB31 is a member of the RAB5 subfamily of RAB proteins; however, it is not as well characterized as RAB5. Rodrigeuz-Gabin et al (29) showed that RAB31 is important for trafficking the mannose 6-phosphate receptor from the trans-golgi network to early endosomes. When RIN3 is overexpressed along with labeled mannose 6-phosphate receptor, the receptor is enriched endosomes versus the TGN. This phenotype is similar to what is seen with expression of constitutively active RAB31 (29). These results indicate that RIN3 expression leads to higher levels of RAB31 activity.

A study from Frank McCormick's lab showed that RIN3 binds to constitutively active K-RAS and N-RAS when both RIN3 and RAS are over expressed in HEK293T cells (4). RAS is a very well studied small GTPase that is activated downstream of RTKs. The canonical RAS pathway involves RAS binding to MAPkinase, which activates MEK, which in turn activates ERK. Signaling downstream causes changes in gene transcription important in cell proliferation and survival. Activating mutations of RAS are found in approximately 30% of all human cancers (30).

Src homology 2 (SH2) domains bind to phosphorylated tyrosine residues. The SH2 domain sequence of RIN3, like that of RIN2, shows a curious divergence at the FLVR motif. The sequences of most vertebrates, including human, encode the arginine residue critical for phosphotyrosine binding. But several species (pig, cow, cat, rat and mouse) have a cysteine substitution at this key position (Fig 2-3), implying that RIN3 in these organisms may function

somewhat differently.

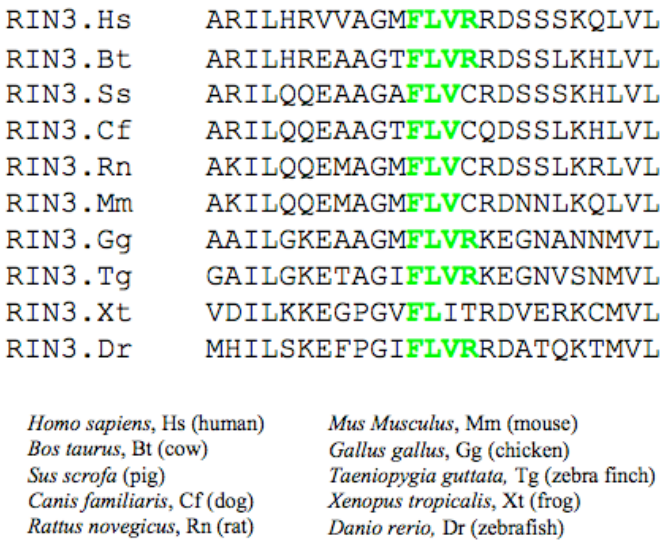


Figure 2-3. ***Comparison of RIN3 SH2 domain protein sequence across species.*** FLVR motif is highlighted in green.

RIN3 also associates with BIN1 (a.k.a. amphyphisin II), a membrane bending protein involved in endocytosis (31,32). This interaction was discovered from a yeast two-hybrid screen using full length RIN3 as bait (23). This study also confirmed that the N-terminal region of RIN3, containing multiple proline-rich motifs, interacted with BIN1; and this interaction was reduced when BIN1's SH3 domain was deleted. This study also showed that BIN1 co-localizes with RIN3 positive vesicles. BIN1 is a BAR-domain containing protein; this domain is banana-shaped and binds to membranes to cause bending (31). BIN1 is important for synaptic vesicle endocytosis in neurons (33) and T-tubule organization in muscles (34); however, it is expressed in a variety of cell types where it is involved in general endocytic processes (35). Overall, this data suggests that RIN3 may be interacting with BIN1 (and possibly other BAR family members) to aid in endocytosis.

In mice, RIN3 expression is highly enriched in mast cells with lower expression levels in

other hematopoietic tissue including lymph node, bone, and T cells (BioGPS). RIN3's restricted expression profile differs from other RIN family members (see above) as well as other known RAB5 GEF proteins. For example, RABGEF1 (aka RABEX-5) has GEF activity for RAB5 but is widely expressed in many different tissue types ((36), Chapter 3). The fact that RIN3 expression is so high in and specific to this cell type implies that RIN3 may have evolved a specific function for mast cells.

2.6 Summary

RIN1-3 proteins connect RAS signal transduction with RAB5 activation and receptor endocytosis. RIN1 has a special role in ABL tyrosine kinase regulation, with a likely contribution to cytoskeleton remodeling. A mouse knockout model suggests RIN1 involvement in stimulation-induced signal transduction in multiple cell types. Mouse models of RIN2 and RIN3 deficiencies have not yet been described.

There are several outstanding questions to be answered regarding the biochemistry of RIN proteins. Particularly curious is a species-specific divergence in the SH2 domain arginine residue required for phospho-tyrosine binding. Does this imply an alternate function for these SH2 domains? Further study is also needed to identify the full range of protein partners, and to determine the location and consequence of these interactions.

Evidence for the involvement of RIN proteins in human pathologies is just beginning to emerge. RIN1 appears to collaborate in tumorigenesis at multiple levels in ways that are complex and cell type-specific (16,19,37,38). In addition, the fear learning and extinction phenotypes of *Rin1*$^{-/-}$ mice suggest that reduced RIN1 function in forebrain neurons could contribute to post traumatic stress disorder. The correlation of RIN2 deficiency with connective

tissue disorders provides another clear indication that RIN proteins play diverse and essential roles in human physiology. A better understanding of the function of RIN family proteins should facilitate the development of new therapeutic avenues for these conditions.

2.7 Goals of this study

As this chapter describes, RIN1 is the best studied of the RIN family of RAS effectors. In comparison, little is known about the function of RIN3. Based on what is known about RIN family members and the conserved domain structures in RIN3, we hypothesized that RIN3 is involved in receptor tyrosine kinase endocytosis and biological outputs downstream of RTKs.

In order to gain a better understanding of the biological role of RIN3 we would first need to determine which cell type(s) would be best for studying the protein and what it's likely role would be. Until now, most of what is known about RIN3 has been discovered using overexpression systems in cell lines that do not have detectable levels of endogenous RIN3. Based on the mouse expression data we hypothesized that RIN3 expression would be highest in hematopoietic cells, especially mast cells. We looked at protein expression in several human cell lines, which confirmed that RIN3 expression is highly enriched and specific to human mast cells (see Chapter 3). From this information we decided to pursue the relationship between RIN3 and the most highly expressed RTK in mast cells, KIT (see Chapter 1).

We chose to use the LAD2 human mast cell line as our primary model system. LAD2 cells were isolated from a leukemia patient and can be grown in culture in the presence of SCF. Unlike most human mast cell lines, LAD2 have a wild type KIT as well as a fully functional IgE receptor (39). These qualities make this cell line more biologically relevant than the typical mastocytosis-derived, transformed cell line. Instead of an activated KIT mutation driving their

proliferation and signaling, these cells can still respond to a wide range of growth factor concentrations. However, the drawback of this more primary cell line is that it grows very slowly (with a doubling time of three weeks) and is not the best model for testing mastocytosis drug sensitivity. For these reasons, we used the HMC1.1 human mastocytosis cell line for drug sensitivity assays (see Chapter 3).

As previously discussed, KIT receptor tyrosine kinase plays an important role in mast cell maturation, immune response, and migration. However, there are still many remaining questions about how KIT signaling and response is regulated. Based on our knowledge of other RTKs, we know that internalization, endocytosis, and trafficking of receptors are all critical players in regulating growth factor response (see Chapter 1). In addition, activating mutations of KIT are typically found in mastocytosis, a group of diseases characterized by hyperproliferation of mast cells (40). Currently, clinical trials treating these patients with tyrosine kinase inhibitors targeting KIT have yielded disappointing results (41). By studying RIN3 in mast cells, we may learn more about how this important receptor is regulated as well as uncover a new druggable target for treatment of mastocytosis.

References

1. Colicelli, J., Nicolette, C., Birchmeier, C., Rodgers, L., Riggs, M., and Wigler, M. (1991) *Proceedings of the National Academy of Sciences of the United States of America* **88**(7), 2913-2917

2. Han, L., and Colicelli, J. (1995) *Molecular and cellular biology* **15**(3), 1318-1323

3. Han, L., Wong, D., Dhaka, A., Afar, D., White, M., Xie, W., Herschman, H., Witte, O., and Colicelli, J. (1997) *Proceedings of the National Academy of Sciences of the United States of America* **94**(10), 4954-4959

4. Rodriguez-Viciana, P., Sabatier, C., and McCormick, F. (2004) *Molecular and cellular biology* **24**(11), 4943-4954

5. Colicelli, J. (2004) *Sci STKE* **2004**(250), RE13

6. Tall, G. G., Barbieri, M. A., Stahl, P. D., and Horazdovsky, B. F. (2001) *Developmental cell* **1**(1), 73-82

7. Barbieri, M. A., Kong, C., Chen, P. I., Horazdovsky, B. F., and Stahl, P. D. (2003) *The Journal of biological chemistry* **278**(34), 32027-32036

8. Cao, X., Tanis, K. Q., Koleske, A. J., and Colicelli, J. (2008) *The Journal of biological chemistry* **283**(46), 31401-31407

9. Hu, H., Bliss, J. M., Wang, Y., and Colicelli, J. (2005) *Curr Biol* **15**(9), 815-823

10. Szabo, K., Jekely, G., and Rorth, P. (2001) *Mechanisms of development* **101**(1-2), 259-262

11. Kajiho, H., Fukushima, S., Kontani, K., and Katada, T. *PloS one* **7**(1), e30575

12. Wang, Y., Waldron, R. T., Dhaka, A., Patel, A., Riley, M. M., Rozengurt, E., and Colicelli, J. (2002) *Molecular and cellular biology* **22**(3), 916-926

13. Chen, P. I., Kong, C., Su, X., and Stahl, P. D. (2009) *The Journal of biological chemistry* **284**(44), 30328-30338

14. Kong, C., Su, X., Chen, P. I., and Stahl, P. D. (2007) *The Journal of biological chemistry* **282**(20), 15294-15301

15. Hu, H., Milstein, M., Bliss, J. M., Thai, M., Malhotra, G., Huynh, L. C., and Colicelli, J. (2008) *Molecular and cellular biology* **28**(5), 1573-1583

16. Milstein, M., Mooser, C. K., Hu, H., Fejzo, M., Slamon, D., Goodglick, L., Dry, S., and Colicelli, J. (2007) *Cancer research* **67**(24), 11510-11516

17. Tomshine, J. C., Severson, S. R., Wigle, D. A., Sun, Z., Beleford, D. A., Shridhar, V., and Horazdovsky, B. F. (2009) *The Journal of biological chemistry* **284**(39), 26331-26339

18. Afar, D. E., Han, L., McLaughlin, J., Wong, S., Dhaka, A., Parmar,K., Rosenberg, N., Witte, O. N., and Colicelli, J. (1997) *Immunity* **6**(6), 773-782

19. Thai, M., Ting, P. Y., McLaughlin, J., Cheng, D., Muschen, M., Witte, O. N., and Colicelli, J. *Leukemia* **25**(2), 290-300

20. Dzudzor, B., Huynh, L., Thai, M., Bliss, J. M., Nagaoka, Y., Wang, Y., Ch'ng, T. H., Jiang, M., Martin, K. C., and Colicelli, J. *Molecular and cellular neurosciences* **43**(1), 108-116

21. Dhaka, A., Costa, R. M., Hu, H., Irvin, D. K., Patel, A., Kornblum, H. I., Silva, A. J., O'Dell, T. J., and Colicelli, J. (2003) *J Neurosci* **23**(3), 748-757

22. Bliss, J. M., Gray, E. E., Dhaka, A., O'Dell, T. J., and Colicelli, J. *Journal of neuroscience research* **88**(4), 917-926

23. Kajiho, H., Saito, K., Tsujita, K., Kontani, K., Araki, Y., Kurosu, H., and Katada, T. (2003) *Journal of cell science* **116**(Pt 20), 4159-4168

24. Saito, K., Murai, J., Kajiho, H., Kontani, K., Kurosu, H., and Katada, T. (2002) *The Journal of biological chemistry* **277**(5), 3412-3418

25. Basel-Vanagaite, L., Sarig, O., Hershkovitz, D., Fuchs-Telem, D., Rapaport, D., Gat, A., Isman, G., Shirazi, I., Shohat, M., Enk, C. D., Birk, E., Kohlhase, J., Matysiak-Scholze, U., Maya, I., Knopf, C., Peffekoven, A., Hennies, H. C., Bergman, R., Horowitz, M., Ishida-Yamamoto, A., and Sprecher, E. (2009) *American journal of human genetics* **85**(2), 254-263

26. Syx, D., Malfait, F., Van Laer, L., Hellemans, J., Hermanns-Le, T., Willaert, A., Benmansour, A., De Paepe, A., and Verloes, A. *Human genetics* **128**(1), 79-88

27. Kajiho, H., Sakurai, K., Minoda, T., Yoshikawa, M., Nakagawa, S., Fukushima, S., Kontani, K., and Katada, T. *The Journal of biological chemistry* **286**(27), 24364-24373

28. Yoshikawa, M., Kajiho, H., Sakurai, K., Minoda, T., Nakagawa, S., Kontani, K., and Katada, T. (2008) *Biochemical and biophysical research communications* **372**(1), 168-172

29. Rodriguez-Gabin, A. G., Yin, X., Si, Q., and Larocca, J. N. (2009) *Experimental cell research* **315**(13), 2215-2230

30. Fernandez-Medarde, A., and Santos, E. *Genes & cancer* **2**(3), 344-358

31. Qualmann, B., Koch, D., and Kessels, M. M. *The EMBO journal* **30**(17), 3501-3515

32. Rao, Y., and Haucke, V. *Cell Mol Life Sci* **68**(24), 3983-3993

33. Wigge, P., and McMahon, H. T. (1998) *Trends in neurosciences* **21**(8), 339-344

34. Lee, E., Marcucci, M., Daniell, L., Pypaert, M., Weisz, O. A., Ochoa, G. C., Farsad, K., Wenk, M. R., and De Camilli, P. (2002) *Science (New York, N.Y* **297**(5584), 1193-1196

35. Pant, S., Sharma, M., Patel, K., Caplan, S., Carr, C. M., and Grant, B. D. (2009) *Nature cell biology* **11**(12), 1399-1410

36. Horiuchi, H., Lippe, R., McBride, H. M., Rubino, M., Woodman, P., Stenmark, H., Rybin, V., Wilm, M., Ashman, K., Mann, M., and Zerial, M. (1997) *Cell* **90**(6), 1149-1159

37. Inoue, T., Goi, T., Hirono, Y., Katayama, K., and Yamaguchi, A. *Oncology research* **19**(12), 527-534

38. Wang, Q., Gao, Y., Tang, Y., Ma, L., Zhao, M., and Wang, X. *Acta histochemica* **114**(5), 463-468

39. Kirshenbaum, A. S., Akin, C., Wu, Y., Rottem, M., Goff, J. P., Beaven, M. A., Rao, V. K., and Metcalfe, D. D. (2003) *Leukemia research* **27**(8), 677-682

40. Boissan, M., Feger, F., Guillosson, J. J., and Arock, M. (2000) *Journal of leukocyte biology* **67**(2), 135-148

41. Ustun, C., DeRemer, D. L., and Akin, C. *Leukemia research* **35**(9), 1143-1152

Chapter 3

RIN3 Is a Negative Regulator of Mast Cell Responses to SCF

3.1 Introduction

Mast cells are critical for allergic inflammatory responses, including type I hypersensitivity, anaphalxysis, asthma, and arthritis (reviewed in (1,2)). The most abundant tyrosine kinase receptor on a mast cell surface is KIT (c-KIT, CD117) (reviewed in (3-5)). Signaling is induced by the binding of its ligand, Stem Cell Factor (SCF), and is required for mast cell maturation, proliferation and migration. SCF also enhances mast cell responses leading to allergic airway inflammation and hyperreactivity (6). KIT is expressed in germ cells and hematopoietic stem/progenitor cells, but among mature somatic cells it is restricted primarily to mast cells and melanocytes (7). Gain of function mutations in KIT are causative in hyper-proliferative pathologies originating from these cells, including mast cell-derived mastocytosis, a family of diseases characterized by mast cell hyper-proliferation (8). These can range from asymptomatic, indolent systemic mastocytosis to malignant, aggressive mast cell leukemia (9). The endocytosis of receptor tyrosine kinases (RTKs), such as KIT, begins with ligand-induced receptor dimerization and transphosphorylation. This leads to engagement of downstream signal transduction pathways, most notably those mediated by RAS family GTPases, that drive the cells immediate and long term response to stimulation. Activated RTKs are typically internalized through an endocytosis mechanism mediated by clathrin and membrane deforming proteins including those in the amphiphysin family of BAR domain proteins (10-12). Internalized RTKs may continue to send downstream signals from early endosomes. Endocytosed receptors

ultimately face one of two fates: recycling and replacement on the plasma membrane or degradation via late endosome transit to the lysosome. The RAB5 family of GTPases mediate early steps in endocytosis including early endosome fusion (13-16) and play an important role in determining the fate of internalized receptors (17,18).

RIN3 is a member of the RIN family of RAS effectors (19), all of which have a guanine nucleotide exchange factor (GEF) domain with specificity for RAB5 family GTPases as well as a RAS association (RA) domain and an SH2 domain. The most extensively studied member of the RIN family is RIN1, which directly controls the signaling and stability of EGFR and other receptor tyrosine kinases (20-22) and may indirectly influence the endocytosis of other receptors (23-26). In epithelial cells, growth factor stimulation of RTKs leads to activation of RAS effectors including RIN1, which in turn activates RAB5 proteins and promotes RTK down regulation.

In this study we show that RIN3 displays a tissue-specific expression pattern, with highest levels restricted to mast cells. RIN3 was an effective promoter of endogenous RAB5 activation in human mast cells. RIN3 silencing slowed the rate of KIT internalization following SCF stimulation, while down regulation of KIT was significantly enhanced by RIN3 over-expression. The ability of mast cells to migrate toward SCF, which requires KIT recycling and prolonged signaling, was inversely correlated with RIN3 expression. Importantly, RIN3 over-expression sensitized a mastocytosis cell line to treatment with the KIT inhibitor imatinib. By regulating KIT response and stability, RIN3 may play a key role in basic mast cell functions as well as pathologies involving mast cell mediated chronic inflammation and mast cell hyperproliferation.

3.2 RIN3 is highly enriched in mast cells

The domain structure of RIN3 (Fig. 3-1A) suggests functional similarity with RIN1, a known regulator of RTK endocytosis in epithelial cells and neurons (23,24,27,28). This led us to hypothesize that RIN3 may serve as a regulator of RTK endocytosis in a restricted and perhaps distinct set of cell types. We therefore examined an expression database (BioGPS) and found that human RIN3 mRNA was most highly expressed in $CD14^{+}$ monocytes (51 fold above median for all tissues) and mouse Rin3 expression was highest in mast cells (24 fold above median for all tissues). To examine if this relatively restricted expression pattern was also true at the protein level, a panel of human cell lines derived from various lineages was immunoblotted for endogenous RIN3. Two mast cell lines, HMC and LAD2, showed by far the highest expression of RIN3 protein (Fig. 3-1B). Representative macrophage and osteoclast cells, which, like mast cells, are derived from bone marrow, showed low or undetectable RIN3 protein. This was also true for representative B cell, myeloblast, T cell, fibroblast and gliobastoma lines.
RIN3 was detected by immunohistochemistry in tissue samples from mastocytosis patients (Fig. 3-1C). While RIN3 expression was easily detected in the mast cells, bordering epidermal cells showed signal intensity about equal to tissue stained with secondary antibody only. These results confirm that RIN3 expression is characteristic of primary human mastocytosis cells, and did not result from the generation of mast cell lines.

We next compared the expression of RIN3 to other members of the RIN family of proteins (Fig. 3-1D). Expression of RIN3 was high in all three mast cell lines examined (LAD2, LUVA, and HMC1.1) with little expression in representative epithelial and glioblastoma cell lines (Fig. 3-1D). RIN1, the best characterized of the RIN paralog family, showed highest expression in glioblastoma cells and low but detectable expression in epithelial cells but was

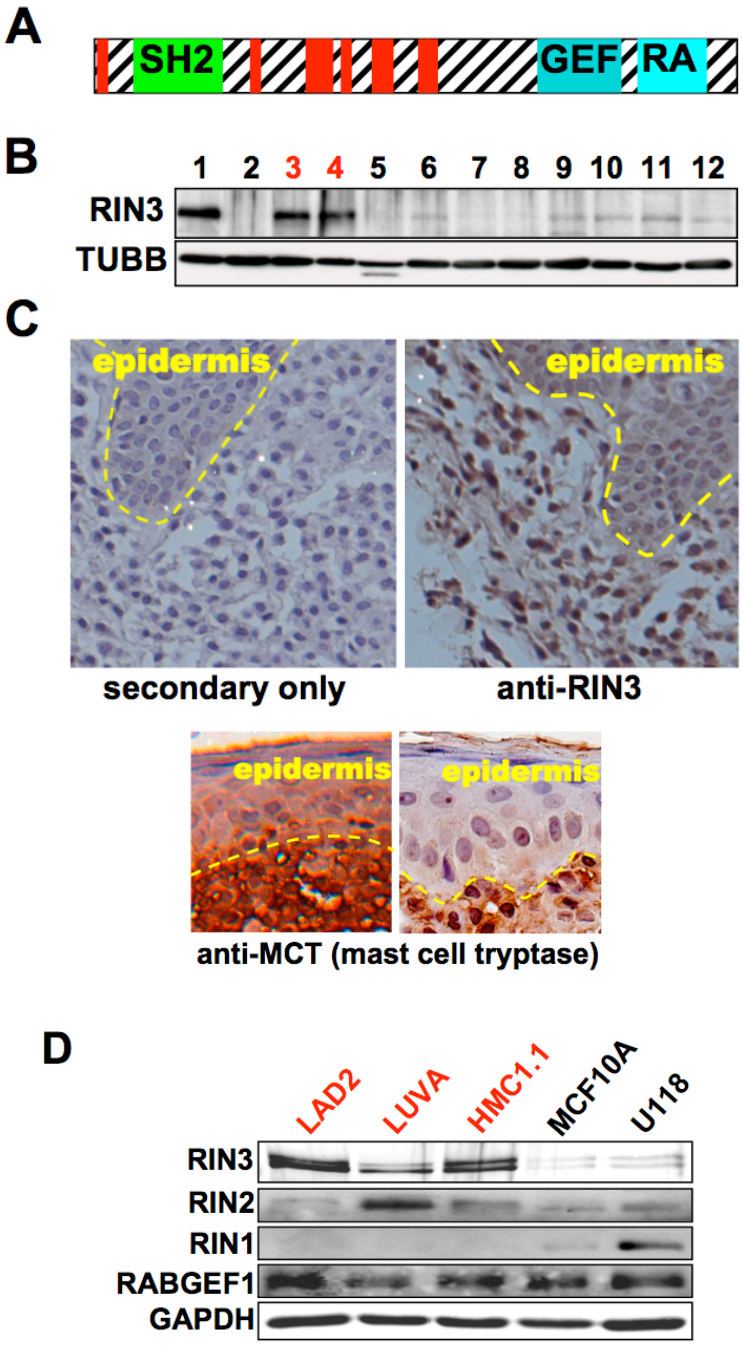


Figure 3-1. ***RIN3 is highly expressed and enriched in human mast cells.*** **A.** The domain structure of RIN3. **B.** Immunoblot analysis of RIN3 expression in human cell lines. 1:NIH3T3 w/ RIN3 (+ctr), 2:NIH3T3 (-ctr), 3:HMC1 (mast), 4:LAD2 (mast), 5:THP1 (macrophage), 6:SaOs2 (osteoclast), 7:RAMOS (B cell), 8:K562 (myoblast), 9:Jurkat (T cell), 10: MCF10A (epithelial), 11:IMR90 (fibroblast), 12:U118 (glioblastoma). RIN3 appears as a double or single band depending on exposure time of the immunoblot. **C.** Human mastocytosis sections stained using immunohistochemistry for RIN3 or secondary only as a control. Sections stained with anti-mast cell tryptase are shown to identify the infiltrating mast cells. **D.** Lysates from three human mast cell lines (LAD2, LUVA, HMC1.1), an epithelial cell line (MCF10A), and a neuronal cell line (U118) were run on an SDS-PAGE gel and immunoblotted for RIN family members (RIN1/2/3) as well as RABGEF1, another GEF for RAB5.

undetectable in the mast cell lines. RIN2 expression was detected in all cell types with relatively high expression in the LUVA mast cell line.

RIN3 is a GEF for RAB5 GTPases. We therefore compared the expression profile of RIN3 to that of RABGEF1 (Rabex-5), a RAB5-targeted GEF known to play a role in mast cell function. RABGEF1 promotes internalization and affects the downstream signaling of both FcεRI, the high affinity IgE receptor, and KIT in mast cells (29,30). RABGEF1 also influences the endocytosis of RTKs in other cell types (31). We found RABGEF1 expression in all cell lines probed (Fig. 3-1D), as expected based on its reported expression in multiple cell types (BioGPS.org and (32)).

The protein and mRNA data show that RIN3 expression is highly skewed, with notably elevated levels in mast cells. This restricted expression profile contrasts with what was seen for RIN1, RIN2 and the more distantly related RABGEF1, which show quite different tissue distribution biases or are widely expressed. Taken together, these findings suggest that RIN3 makes a unique contribution to mast cell function.

3.3 RIN3 interacts with endogenous BIN2 in mast cells

Previous studies (19) showed that RIN3 interacts with BIN1, a BAR domain protein that binds to lipid membranes to induce bending (33,34). Endogenous BIN1 protein was below the level of detection in LAD2 cells. The paralog protein BIN2 is more highly expressed in hematopoetic cells compared to BIN1 (35); therefore, we evaluated RIN3 binding to BIN2 in LAD2 cells. Again, cells were stimulated with 100 ng/mL SCF and RIN3 was immunoprecipitated from lysates. BIN2 is bound to RIN3 in unstimulated cells, but this

interaction is lost as early as 2 minutes after stimulation with SCF (Fig. 3-2). This experiment suggests that RIN3 is bound to BIN2 in resting cells, but is quickly released upon stimulation.

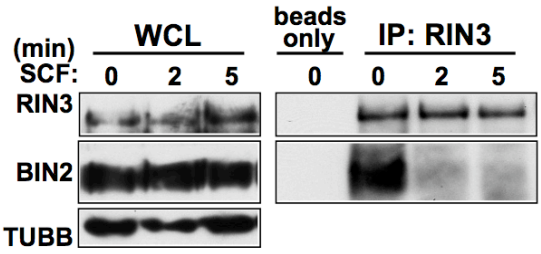
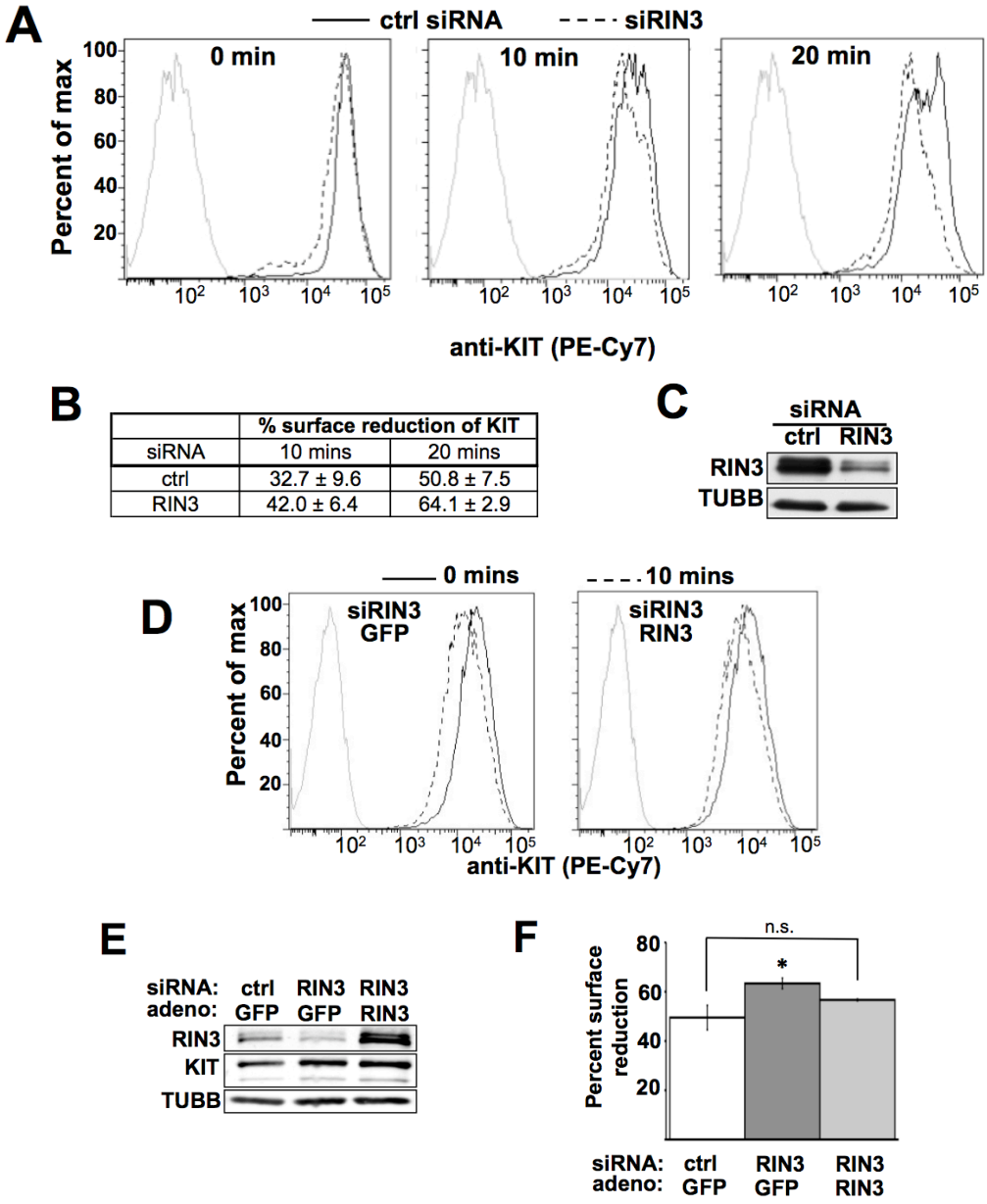


Figure 3-2. ***RIN3 interacts with endogenous BIN2.*** Cells were stimulated with 100 ng/ml SCF for indicated timepoints and lysates were immuno-precipitated with anti-RIN3 or beads alone (ctrl). WCL and IP samples were immunoblotted for RIN3 and BIN2. The immunoblots shown are representative of two independent experiments. At five minutes post-stimulation the amount of BIN2 precipitated was reduced by 81 ± 6% compared to unstimulated, $p<0.05$.

3.4 RIN3 negatively regulates KIT internalization

RIN1 is known to regulate the endocytosis and downstream signaling of receptor tyrosine kinases (RTKs) in epithelial cells (23,24). We hypothesized that RIN3 may be playing a role in regulating KIT, the Stem Cell Factor (SCF) receptor and most abundant RTK found on mast cells. To test our hypothesis, we measured the internalization of KIT following stimulation with SCF. We chose to perform these experiments in LAD2 cells, which express wild type KIT and are highly dependent on SCF for proliferation in culture (36). RIN3 was silenced using a targeted siRNA. We observed a consistent decrease in RIN3 protein levels compared to control siRNA transfected cells, which were indistinguishable from mock infected cells (data not shown). Control and RIN3 silenced LAD2 cells were stimulated with SCF at 5 ng/mL, a relatively low but still physiological concentration that facilitates measurement of early events after KIT activation. Stimulated mast cells were then analyzed for cell surface KIT by flow



	% surface reduction of KIT	
siRNA	10 mins	20 mins
ctrl	32.7 ± 9.6	50.8 ± 7.5
RIN3	42.0 ± 6.4	64.1 ± 2.9

Figure 3-3. ***RIN3 negatively regulates the internalization of KIT post SCF stimulation.*** **A.** Cells transfected with control (solid) or RIN3 siRNA (dashed) were stimulated with 5 ng/ml SCF for indicated time points. Cells were stained for surface intensity of KIT and analyzed by flow cytometry. Gray line indicates unstained control. **B.** Table shows the percent surface reduction of KIT from three independent flow cytometry experiments. Surface reduction at 20 minutes post stimulation is significantly increased in RIN3 knock down cells $p<0.05$. **C.** Immunoblot indicating the levels knockdown of RIN3. **D.** Cells were transfected with RIN3 siRNA and then infected with GFP (siRNA / GFP) or RIN3 (siRIN3 / RIN3). Surface intensity of KIT was determined by flow cytometry at 0 mins (solid) and 10 mins (dashed) post stimulation. Percent reduction: ctrl/GFP: 26%, siRIN3/GFP: 37%, siRIN3/RIN3: 29%. **E.** Immunoblot for RIN3 and KIT levels in transfected / infected cells. **F.** Graph represents percent surface reduction at 20 minutes post stimulation from two independent experiments. Percent reduction of ctrl/GFP versus siRIN3/RIN3 is not significant ($p>0.34$). Percent reduction siRIN3/GFP versus siRIN3/siRIN3 is significant ($p<0.05$).

cytometry. RIN3 silencing did not influence the basal level of detectable KIT prior to SCF treatment (Fig. 3-3A, left panel), but by 10 minutes post stimulation the cells with reduced RIN3 expression showed a marked decrease in cells exhibiting high intensity surface KIT compared to control cells (Fig. 3-3A, middle). This difference was even more pronounced at 20 minutes post stimulation, when RIN3 silenced cells had a well defined, low staining intensity peak and little overlap with the high intensity peak of control cells (Fig. 3-3A right panel). At 20 minutes post stimulation knock down cells have finished internalization of the receptor while a population within the control cells still had high levels of surface KIT. Cells with reduced RIN3 have significantly greater percent of KIT surface reduction 20 minutes post stimulation compared to control (Fig. 3-3B). Control and knock down cells reached the same low level of surface KIT by 90 minutes post stimulation (see Chapter 4). These results indicated that RIN3 plays a role in setting the rate at which activated KIT is internalized in mast cells.

To verify that accelerated KIT internalization was the result of RIN3 silencing, we restored RIN3 expression by transduction with an adenovirus expression vector. The resulting ectopic RIN3 level in transduced cells was higher than endogeneous RIN3 in control cells (Fig 3-3E) but whole cell lysate levels of KIT were unchanged (Fig 3-3E). Cells with restored RIN3 showed a KIT internalization rate close to that seen in control cells, and lower than that of RIN3 silenced cells (Fig 3-3D&F). Hence, the more rapid KIT internalization rate observed in RIN3 siRNA-transfected cells (Fig. 3-3A) can most easily be explained by a loss of RIN3 protein.

3.5 The Proline Rich (PR) region of RIN3 is involved in KIT surface expression and internalization, but is not necessary for binding to BIN2

In this study, we describe an interaction between endogenous RIN3 and BIN2 in LAD2 cells. Overexpression experiments performed by another lab described RIN3 binding to BIN1 through BIN1's SH3 domain (19). We then hypothesized that RIN3 binds BIN1 and BIN2 through its proline rich regions. In fact, RIN3 has a more extensive proline rich region compared to other RIN family members. We hypothesized that deletion of RIN3's proline rich region would alter the cell's ability to regulate KIT.

In order to study the role of the proline rich region of RIN3, we deleted residues 275 through 387, which contains ten PxxP motifs. This mutant form of RIN3, RIN3-ΔPR, was overexpressed using a lentiviral system in HMC1.1 cells. Since RIN3 is involved in KIT internalization, we tested the influence of RIN3-ΔPR over KIT internalization. As seen previously, surface levels of KIT are reduced following SCF stimulation. Cells expressing RIN3-ΔPR had reduced rate of KIT internalization compared to cells overexpressing wild type RIN3 (Fig 3-4A). This difference was significant 15 minutes post stimulation ($p<0.01$). This suggests that the proline rich region of RIN3 plays a positive regulatory role in KIT internalization.

When measuring the rate of KIT internalization, we normalized the amount of surface expression to the zero time point for each cell line. However, the raw data showed that surface expression of KIT was higher in cells expressing RIN3-ΔPR compared to vector and wild type RIN3 (Fig 3-4B). In contrast, there is no change in the total protein level of KIT as determined by western blot (Fig 3-4C). Therefore, increase in surface expression was not due to increased KIT levels overall. This suggests that RIN3-ΔPR influences the balance between internal and surface KIT to favor more surface expression of KIT.

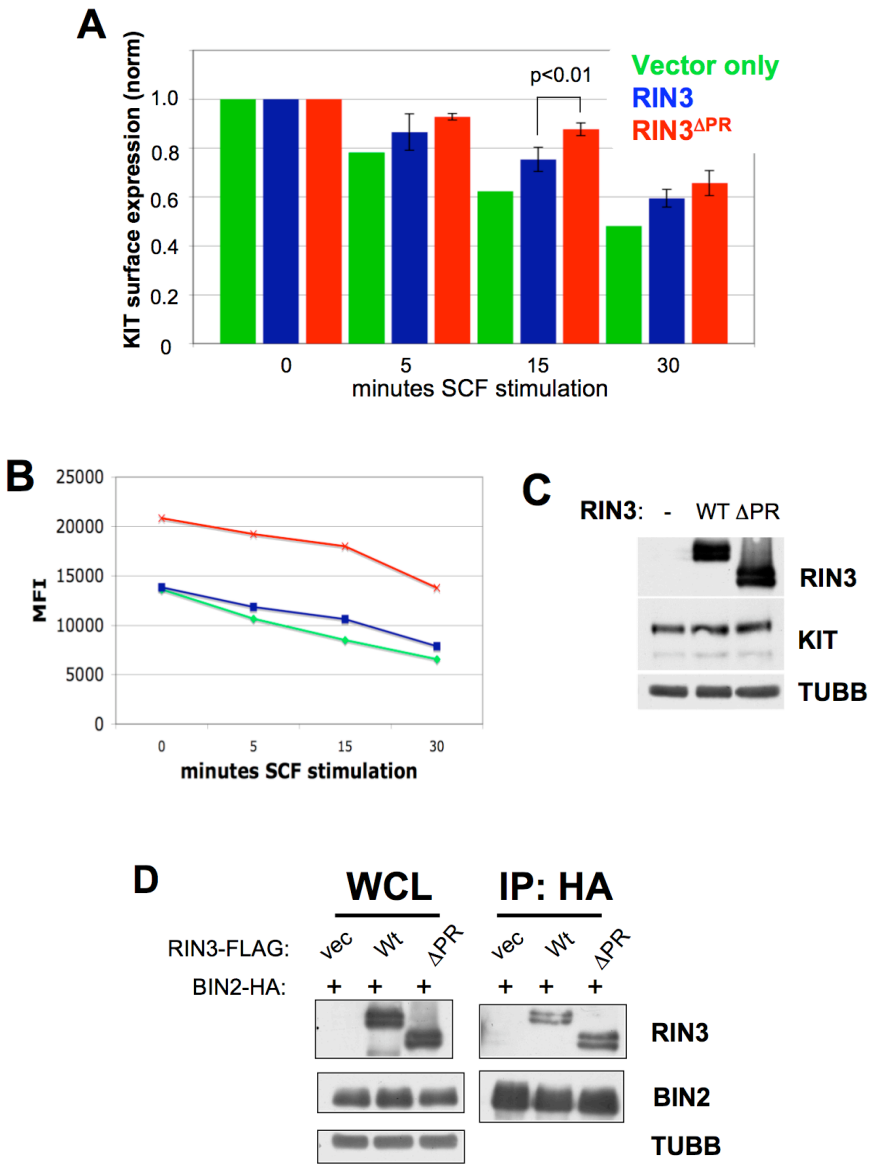


Figure 3-4. ***Expression of RIN3ΔPR causes increased surface expression of KIT and slows KIT internalization.*** **A.** HMC1.1 cells expressing vector (green), RIN3 (blue), or RIN3-ΔPR (red) were stimulated with SCF for indicated times. Percent surface expression of KIT was determined by flow cytometry. Values were normalized to the amount of surface KIT without stimulation for each cell type. **B.** Graph shows the MFI values for KIT surface staining for the same experiments shown in B, but without normalization. **C.** Western blot demonstrates the whole cell lysate levels of RIN3 and KIT. Tubulin is included as a loading control. **D.** NIH3T3 cells were transfected with vector, wild type RIN3-FLAG, or RIN3-ΔPR-FLAG as well as BIN3-HA. Lysates were immuno-precipitated for HA and blotted for BIN2 and RIN3.

Since we now have determined RIN3-ΔPR role in KIT internalization and surface expression, we wanted to test if the known binding partner BIN2 would no longer interact with mutant RIN3. RIN3-DPR-FLAG was overexpressed in NIH3T3 cells along with BIN2-HA. As expected, wild type RIN3 co-immunoprecipitates with BIN2; however, RIN3-ΔPR binds just as well to BIN2 (Fig 3-4D). Thus, the phenotypes observed with RIN3-ΔPR expression are not due to a loss of interaction between RIN3 and BIN2. It is important to remember that not every PxxP motif was deleted from RIN3 in this mutant; therefore, BIN2 may still be binding to RIN3 through an SH3::PR interaction. It is likely then that deletion of this proline rich region abolished interaction with another partner or partner(s), which is causing the phenotypes we observed. Determining this unknown partner would need to be pursued with an unbiased screen, which is beyond the scope of this study.

Overall, we show that expression of RIN3-ΔPR leads to increased surface expression of KIT as well as a reduced rate of KIT internalization post SCF stimulation. Also, these differences are not dependent on the RIN3::BIN2 interaction.

3. 6 RIN3 positively regulates RAB5 activation in mast cells

Because RIN3 is a known GEF for the RAB5 family of small GTPases (19), we examined whether the levels of activated RAB5 correlated with RIN3 expression levels. In order to measure activated endogenous RAB5 we utilized a 4xZFYVE-GST construct that contains four copies of the 40 amino acid zinc finger domain of the RAB5 effector Rabenosyn that preferentially binds to the active (GTP-bound) conformation of RAB5. There was no significant difference between the basal levels of active RAB5 in control versus RIN3 knock down cells (Fig. 3-5A). SCF stimulation, which was confirmed by elevated pERK levels, led to higher levels

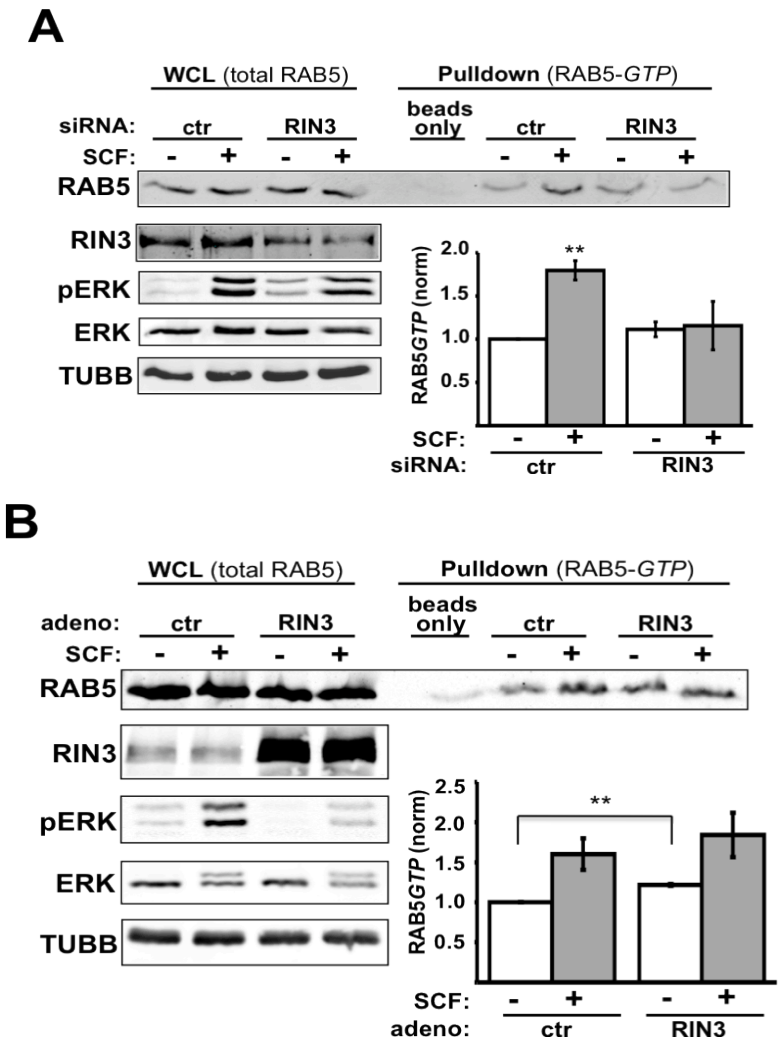


Figure 3-5. ***RIN3 promotes RAB5 activation.*** **A.** LAD2 cells transfected with control or RIN3 siRNA were stimulated with 100 ng/ml SCF for 15 minutes. Cells were lysed and activated RAB5 was pulled down using 4X-ZFYVE-GST on glutathione beads as indicated in the methods. Precipitates from the beads were immunoblotted for RAB5 (RAB5*GTP*). The whole cell lysates were immunoblotted for total RAB5, RIN3, pERK (to show stimulation), ERK, and TUBB (loading control). Graph represents data from three independent experiments, **p<0.01. **B.** Cells infected with Ad-GFP or Ad-RIN3 and treated as in (A). Graph represents data from three independent experiments, ***p<1 x 10^5.

of active RAB5 in control cells (average of 80% increase, $p<0.001$). In contrast, there was no significant change in the amount of active RAB5 in RIN3 silenced cells ($p>0.80$). As a result, following SCF treatment there was significantly less active RAB5 in the RIN3 silenced cells than in control cells ($p<0.025$). Less activated RAB5 in the cell post stimulation would be expected to reduce early endosome fusion. Under these circumstances, internalized KIT may stay in the early endosome for a prolonged period.

We next measured RAB5 activation in RIN3 over-expressing LAD2 cells. As in the previous experiments, SCF stimulation of control cells caused an increase in the level of active RAB5. Even before SCF stimulation, however, RIN3 transduced cells had higher levels of active RAB5 (Fig. 3-5B, 20% average increase, $p<1x10^{-5}$), presumably due to stimulus-independent GEF activity from over-expressed RIN3. There was no significant difference in the amount of activated RAB5 post stimulation between control and RIN3 over-expressing cells ($p>0.2$), however, which might indicate a ceiling for RAB5(GTP) levels due to negative regulation by RAB5-directed GAPs.

3.7 RIN3 over-expression promotes KIT down regulation

In contrast to the accelerated KIT internalization observed in RIN3 silenced mast cells, RIN3 over-expression in the same cells had no effect on the rate of KIT internalization following stimulation with SCF (data not shown). We noted, however, that unstimulated RIN3 over-expressing cells here and in Fig 3-3 had reduced surface KIT expression compared to control cells. The reduction of surface KIT, as determined by flow cytometry (Fig 3-6A and B), was dependent on the relative level of RIN3 over-expression (Fig. 3-6C). When RIN3 was

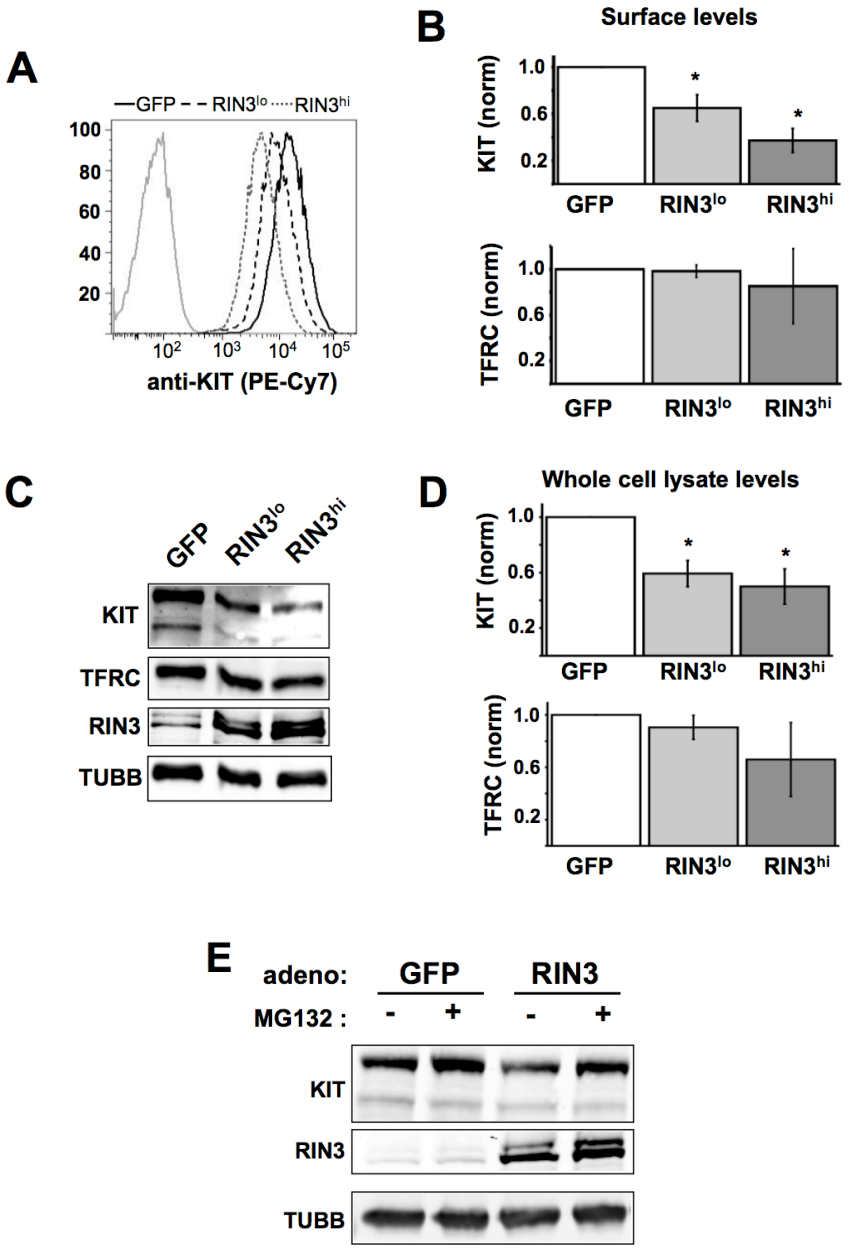


Figure 3-6. ***KIT downregulation is enhanced by RIN3 activity.*** **A.** Cells infected with Ad-GFP (solid line) or Ad-RIN3 at high (dotted line) and low (dashed line) concentrations were stained with PE/Cy7 labeled anti-KIT and analyzed by flow cytometry for basal surface expression of KIT. Gray line represents unstained control. **B.** Graphs showing surface intensity of anti-KIT and anti-TFRC as determined by flow cytometry. Surface expression of GFP transduced cells was set to one. Graphs are compilation of two independent experiments, *$p<0.05$. **C.** Lysates from cells from A and B were immunoblotted to determine the amount of RIN3 overexpression and basal KIT and TFRC levels. **D.** Graphs showing intensity of western blot signal for KIT and TFRC compiled from two separate experiments, *$p<0.05$. **E.** The proteasome inhibitor MG132 partially restores KIT expression. Immunoblot for KIT levels in cells incubated for 3 hours with vehicle (ethanol) or 10 μM MG132 after an overnight SCF starvation. Experiment was done twice, KIT levels increase 24.5 ± 12.5 % for GFP infected cells and 47.5 ± 3.5 % for RIN3 infected cells.

moderately over-expressed ($RIN3^{lo}$) surface KIT was significantly reduced compared to cells expressing GFP; this reduction was even more pronounced in cells with high RIN3 over-expression ($RIN3^{hi}$) (Fig 3-6B, $p<0.05$, $p<0.025$). As a control, the relative levels of transferrin receptor (TFRC) were measured under the same conditions. High level over-expression of RIN3 did not significantly alter TFRC surface expression (Fig 3-6B). In these same cells, we also tested to see if whole cell lysate levels of KIT and TFRC were changed with increasing RIN3 levels. We observed that total cellular KIT decreased markedly as RIN3 levels increased (Fig. 3-6C&D); however, TFRC levels remain largely unaffected, with a slight decrease at the highest level of RIN3 expression. Previous work (37) has shown that expression of constitutively active RAB5 decreases the surface and whole cell lysate levels of EGFR in HeLa cells. Like EGFR, KIT normally undergoes continuous cycles of internalization with endosome-localized receptors either returned to the cell surface or degraded. This habitual receptor turnover occurs through RAB5 mediated endocytosis, and suggests that RIN3 over-expression, which leads to higher basal levels of activated RAB5, is promoting receptor degradation over recycling. To test this we incubated control or RIN3 over-expressing LAD2 cells with the proteosome inhibitor MG132. Control cells showed a modest increase in KIT levels, while RIN3 over-expression cells showed a more dramatic increase in KIT levels (Fig 3-6E). This result suggests that RIN3 over-expression leads to decreased KIT levels due to increased degradation.

3.8 RIN3 negatively regulates cell migration toward SCF

Activated mast cells release SCF, the KIT ligand, which recruits more mast cells to sites of allergen infiltration and infection. Because RIN3 silencing caused accelerated KIT

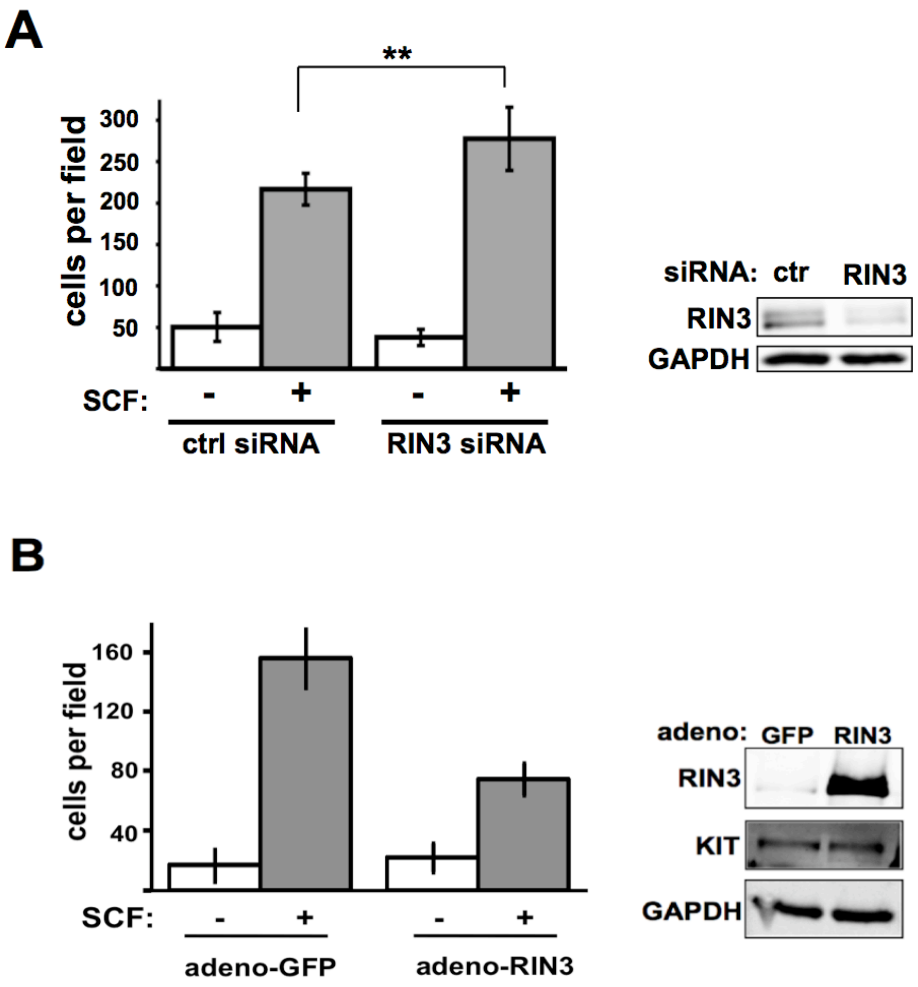


Figure 3-7. ***RIN3 inhibits mast cell migration toward SCF.*** **A.** LAD2 cells transfected with control or RIN3 siRNA were allowed to migrate toward SCF. Experiment was performed twice in triplicate, **p<0.01. **B.** Cells infected with Ad-GFP or Ad-RIN3 were allowed to migrate toward SCF. Experiment was performed in triplicate, **p<0.01. Immunoblots for RIN3 and TUBB are shown to the right of each graph (a lighter exposure was chosen to better indicate the level of RIN3 knock down). Immunoblot quantification indicated a 26% reduction in KIT (normalized to GAPDH).

internalization, we asked whether RIN3 might also influence the physiological response of mast cells to SCF. To examine this, we measured the ability of mast cells with altered RIN3 expression levels to migrate toward SCF. These experiments were performed using high (100 ng/ml) SCF to elicit a robust migratory response. Control cells showed approximately four-fold more migrated toward SCF medium compared to SCF-free medium (Fig. 3-7A). RIN3 silenced mast cells exhibited a modest, but significant increase in migration toward SCF (ctrl siRNA: 217 ± 19 cells; RIN3 siRNA: 277 ± 38 cells, $p<0.01$), but exhibited no difference from control cells in their rate of migration toward SCF-free medium (Fig 3-7A; $p>0.15$), demonstrating the KIT-dependence of this effect.

To test whether RIN3 functions as a negative regulator of mast cell migration, we established RIN3 over-expressing mast cells by adenovirus transduction. Migration of these cells toward SCF was markedly reduced when compared with mast cells infected with a control adenovirus expressing GFP (Fig. 3-7B). In these experiments RIN3 over-expression resulted in a two-fold reduction in migration (Ad-GFP: 156 ± 20 cells; Ad-RIN3: 75 ± 11 cells; $p<0.01$).

3.9 RIN3 sensitizes HMC1.1 mastocytosis cells to the KIT inhibitor imatinib

The most common genetic alterations in mastocytosis are mutations that activate KIT and confer a degree of SCF-independent growth. Imatinib, which inhibits KIT and several other tyrosine kinases, provides therapeutic benefits for some mastocytosis patients. We tested whether RIN3 over-expression, which causes KIT down regulation, might increase imatinib sensitivity. HMC1.1 is an established mastocytosis cell line with a KIT^{V560G} mutation. HMC1.1 cells show moderate sensitivity to imatinib with an EC_{50} of 50-150 nM (38). HMC1.1 cells were transduced with a RIN3 lentivirus vector to create stable over-expression cells, which showed a

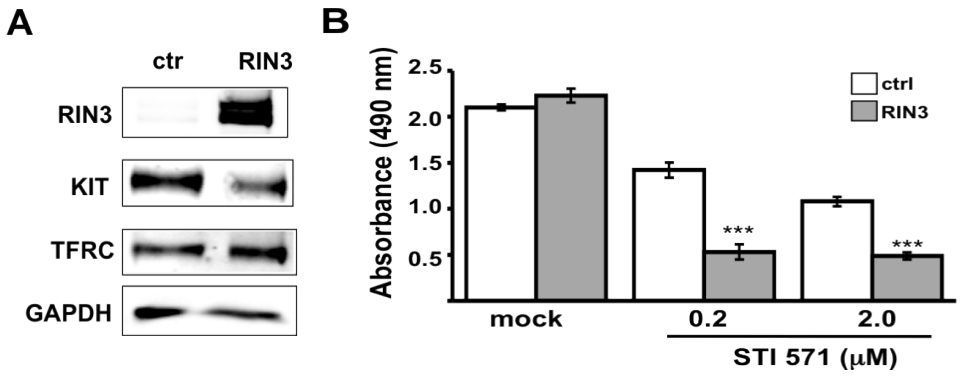


Figure 3-8. ***RIN3 over-expression sensitizes mastocytosis cells to imatinib.*** **A.** HMC1.1 cells infected empty vector (ctrl) or RIN3 were incubated with increasing amounts of STI 571 for 24 hours. **B.** The graph represents MTS assay results for cell viability. Assays were performed in quadruplicate, ***$p<1 \times 10^{-5}$.

reduction in steady state KIT levels (Fig 3-8A). Importantly, we observed no effect of RIN3 over-expression on cell proliferation in the absence of drug (Fig. 3-8B). After a 24-hour incubation in medium containing 0.2 or 2 μM imatinib, control cells showed a drop in viability as determined by MTS assay (Fig. 3-8B). The HMC1.1 cells over-expressing RIN3 showed an additional, synergistic reduction in cell viability. Hence, by facilitating KIT down regulation, RIN3 sensitizes mastocytosis cells to the therapeutic kinase inhibitor imatinib.

3.10 RIN3 silencing does not affect degranulation

Finally, we examined whether RIN3 is involved in regulating signals from FcεRI, the high affinity IgE receptor on mast cells. FcεRI activation by antigen (Ag)-clustered IgE causes rapid mast cell degranulation with the release of histamine and other inflammation mediators. Degranulation was measured as β-hexosaminidase release following treatment of LAD2 cells with biotinylated IgE and streptavidin (Fig. 3-9). We observed no significant difference in

timing or intensity when comparing RIN3 silenced cells and control cells (0 ng/ml $p>0.66$, 1 ng/ml $p>0.90$, 5 ng/ml $p>0.49$, 10 ng/ml $p>0.51$, 100 ng/ml $p>0.40$). It remains possible that RIN3 influences other events downstream of FcεRI activation, such as migration or secretion, but this determination would require extensive investigation beyond this study.

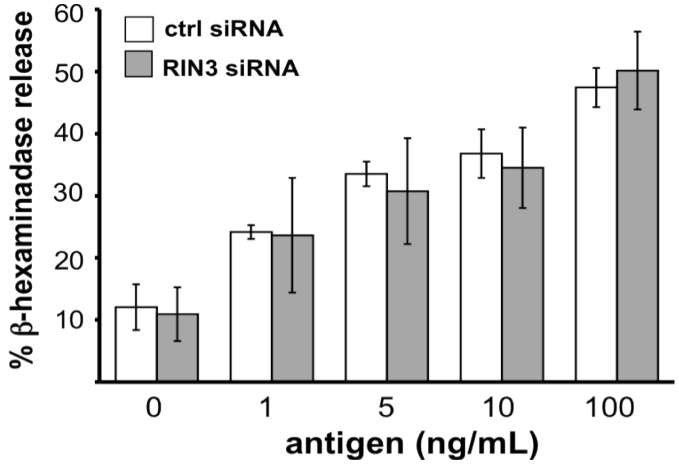


Figure 3-9. ***RIN3 silencing does not affect degranulation.*** The amount of granule release was measured in cells with control (white bars) or knock down levels of RIN3 (gray bars). All cells were primed with biotinylated IgE and then incubated with indicated concentrations of streptavidin (antigen). Percent granule release was calculated as β-hexosaminidase activity: supernatant/(supernatant + lysate). Graph is compilation of two independent experiments performed in triplicate.

3.11 Discussion

The limited expression profile of RIN3 is unique among the RIN family of RAS effectors and RAB5 activators. Our observation that RIN3 is enriched in, and largely restricted to, mast cells suggests that it evolved to provide a function specific to endocytosis of mast cell receptors. RABGEF1, which includes a RAB5 GEF domain but is otherwise unrelated to RIN proteins, is also found in mast cells ((29) and this work). In contrast, however, the *RABGEF1* gene shows a broad pattern of expression that is more consistent with a basic trafficking function utilized by a wide range of cell types.

The observation that RIN3 silencing accelerates internalization of stimulated KIT was initially surprising. Previous work had reported that mast cells from *Rabgef1*$^{-/-}$ mice show delayed KIT internalization (29). This difference highlights the importance of considering GEF activity in the context of the entire protein. In addition to its VPS9 type (RAB5-directed) GEF domain, RABGEF1 has a zinc finger domain that has been implicated in targeting HRAS for ubiquitination (39). By contrast, the much larger RIN3 protein includes an RA (Ras-Association) domain, SH2 domain and prominent PR (proline-rich) domain. Of particular relevance, we show that RIN3 interacts with BIN2, an amphiphysin related protein with a BAR domain typically used to promote membrane bending during endocytosis and vesicular trafficking. BIN2 is bound to RIN3 in resting cells but the two proteins dissociate soon after KIT stimulation by SCF. We propose that following KIT stimulation and RAS activation, RIN3 is recruited to activated RAS at the plasma membrane where it releases BIN2. Through this process, RIN3 might deliver BIN2 from the cytoplasm to the membrane where it could begin bending the membrane in a way that facilitates KIT internalization. BAR domain proteins can dimerize with other family members and can also form homodimers (40). Given the low level of BIN1 in LAD2 cells, a BIN2 homodimer might well be operating in this system. When RIN3 is silenced there may be more BIN2 protein free to dimerize and associate with the plasma membrane, leaving cells "primed" for rapid receptor internalization. Notably, BIN2 expression is restricted to hematopoietic granular cells (35) and mast cells (this work), suggesting an evolved partnership with RIN3.

Highly over-expressed RIN3 led to reduced steady state levels of total and surface resident KIT protein, likely due to elevated levels of endogenous activated (GTP-bound) RAB5. Similar reductions of receptor tyrosine kinases (RTKs) have been seen upon over-expression of

related RAB5 GEF domain proteins: RINL over-expression can reduce the basal level of EphA8 (41) and RIN1 over-expression can decrease EGFR protein levels (Balaji and Colicelli, in press). In unstimulated wild type cells, RTKs such as KIT are typically internalized and recycled back to the plasma membrane. When RAB5 activity is elevated, early endosomes with internalized KIT are more likely to undergo fusion and follow a receptor degradation pathway. This interpretation is supported by the rescue of KIT levels in RIN3 over-expressing cells treated with a proteasome inhibitor. Total KIT levels were unchanged when RIN3 was silenced, but under these conditions we observe very little RAB5 activity pre or post SCF stimulation.

RIN3 silenced cells exhibited greater migration toward SCF. These cells internalize KIT more quickly than normal and they have lower levels of active RAB5 following SCF treatment. This might lead to a prolonged stay in the early endosome, with more downstream signaling and a greater chance for KIT recycling back to the plasma membrane while migrating toward SCF. Conversely, RIN3 over-expressing mast cells demonstrated less migration toward SCF. In these cells, decreased levels of surface and total RTK, which correlate with elevated basal levels of active RAB5, would be expected to diminish downstream signaling and biological output. The ability to migrate toward SCF is fundamental to mast cell function, suggesting that a deeper understanding of RIN3 signaling may provide new avenues for intervention in mast cell pathophysiology.

KIT gain-of-function mutations are common in mastocytosis and a limited number of other hyperproliferative disorders. Imatinib is an effective tyrosine kinase inhibitor for KIT, as well as ABL and PDGF receptor (42). This drug has been used with considerable success in the treatment of BCR-ABL1 positive chronic myeloid leukemias (43). Imatinib has also shown some effectiveness in the treatment gastointestinatal tumors with mutant KIT (44). In addition,

imatinib has been shown to prevent and lessen the symptoms of rheumatoid arthritis (45), an inflammatory joint disease with mast cell involvement (46). Previous studies have established that reduced KIT expression, in conjunction with tyrosine kinase inhibitors, can decrease proliferation and increase apoptosis in the mastocytosis cell line HMC1.1, which carries the KIT^{V560G} activating mutation (47). We demonstrate here that RIN3 over-expression markedly sensitizes HMC1.1 cells to imatinib treatment. This may open new avenues for synergistically enhancing the potency of KIT tyrosine kinase inhibitors. Such combination therapies would be applicable to malignancies characterized by KIT gain-of-function mutations, as well as chronic inflammation pathologies with mast cell involvement.

3.12 Materials and Methods

Antibodies

To make human RIN3 antibodies, the full length protein was first expressed as a 6 x His fusion from a baculovirus expression vector in Sf9 cells. Metal affinity chromatography (Ni-NTA beads, Qiagen) purified protein was used to generate rabbit polyclonal antibodies (21^{st} Century Biochemicals) that were used for immunoblot staining at a 1/5,000 dilution, for immunoprecipitation at a 1/250 dilution and for immunohistochemistry at 1/5,000.
Other antibodies, their sources and the dilutions used for immunoblot probing were: rabbit anti-RIN1 (48) 1/1,000; rabbit anti-RAB5 (Abcam ab18211) 1/250; rabbit anti-RAS (Novus Bio EP1125Y) 1/5000; rabbit anti-KIT (Cell Signaling 3392) 1/500; mouse anti-KIT (Abcam Ab81) 1/500; mouse anti-α tubulin (Sigma) 1/3000; mouse anti-KIT conjugated to PE/Cy7 (Biolegend 104D2) 1/100; rabbit anti-GAPDH (Abcam) 1/3000; mouse anti-pTyr clone 4G10 (Millipore)

1/500; rabbit anti-RABGEF1 (Sigma) 1/1,000; rabbit anti-BIN2 (35) 1/3000; mouse anti-transferrin receptor (Invitrogen) 1/500; anti MCT (DakyCytomation) 1/2000.

Cell culture

The LAD2 cell line (36) was a generous gift from A. Kirsenbaum (NIH, Besthesda, MD). They were maintained in StemPro with 100 ng/ml SCF at less than 500,000 cells/ml. The HMC1.1 and HMC1.2 cell lines were generous gifts from Dr. J.H. Butterfield (Mayo Clinic, Rochester, MN) (49) maintained in Iscove's DMEM with 10% FBS, 1% glutamax, and 0.01% mono-thioglycerol. The LUVA cell line was a generous gift from J. Steinke (Asthma and Allergic Diseases Center, University of Virginia) (50) maintained in StemPro without SCF. Parental HMC cells were obtained from Dr. Chad Oh (Department of Pediatrics, Harbor-UCLA Medical Center).

Degranulation Assay

Degranulation was determined by amount of β-hexosaminidase released as previously described (36). LAD2 cells were primed with 100 ng/mL biotinylated IgE (Abbiotec) in HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.4 mM Na2HPO4, 5.6 mM glucose, 1.8 nM $CaCl_2$, 1.3 nM $MgSO_4$ pH 7.4) + 0.4% BSA for 1 hour at 37ºC. Cells were washed three times and resuspended in HEPES + BSA at 20,000 cells per well. Indicated amount of stepavidin (Sigma) was added to each well; each concentration was done in triplicate. After 30 minutes incubation, cells were pelleted and lysed in 1% Triton X-100 in PBS. Supernatants were aliquoted into a 96 well plate; β-hexosaminidase activity for both the supernatants and the lysates

were determined by incubation with p-nitrophenyl N-acetyl-β-D-glucosamide. Percent release was calculated as amount in supernatant/(supernatant + lysate).

Immunoprecipitation and immunoblotting

For immunoblotting, proteins were transferred to ECL membranes and blocked with 5% milk in PBS. Primary antibodies were incubated overnight at 4ºC in 5% milk in PBST (PBS + 0.1% Tween). This was followed by three washes in PBST and one hour incubation with secondary antibodies at room temperature. This was true for all antibodies with the exception of 4G10 antibody, which was used according to Millipore's recommendations. Membranes were developed using the ECL plus immunoblotting reagent (VWR). Immunoblots exposed on film were quantified using ImageJ software. All other immunoblots were quantified using a Li-Cor Odyssey scanner and software.

For immunoprecipitation experiments, LAD2 cells were starved of SCF in complete StemPro media overnight, then stimulated with SCF for indicated time points and concentrations of growth factor. Cells were centrifuged, washed once in PBS, and lysed in NP-40 buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% glycerol, 1% NP-40) on ice for 15 minutes. Lysates were spun at 16000 x g and the pellet was discarded. To enrich for RIN3 and its binding partners, RIN3 antibody was added to cell lysate with protein A and protein G beads and incubated at 4ºC with rotation for three hours to overnight. Immunoprecipitated samples were gently spun down at 800xg and the pelleted beads were washed twice in lysis buffer. Beads were boiled in 5x SDS loading buffer to release proteins from beads. Samples were then analyzed by SDS PAGE and immunoblotting.

Expression constructs, lentiviruses and adenoviruses:

RIN3 cDNA (Open Biosystems) was PCR amplified and cloned into pBluescript KS to create pKS-RIN3. A FLAG tag was fused onto the C-terminus of the protein to create RIN3-FLAG. This was subsequently inserted into an M4 vector that was used for lentiviral production as previously described (24). A 6 x Histidine tag was added to RIN3 using the following oligonucleotides 5'- TCGACCACCACCATCACCATCACCATCACTAACTAGT and 5'-GTAGACTAGTTAGTGATGGTGATGGTGATGGTGGTGG. RIN3-His was then cloned into the donor vector pFastBac (Invitrogen) in order to produce baculovirus for Sf9 cell infections following established procedures.

Adenoviral vector construction was performed by homologous recombination in *E. coli*, as previously described (51). The Ad5/F35 viral vector contains a 2,115-bp-long chimeric fiber gene consisting of the Ad5 tail and the Ad35 shaft and knob domains (52). RIN3-FLAG was cloned into the pShuttle-CMV plasmid and transformed into recombinogenic *E. coli* strain BJ5183 that had already been transformed with a plasmid containing the Ad5/F35 vector genome. Full-length recombinant adenoviral vectors genomic plasmids were amplified after re-transformation into Stabl3 cells, and transfected into Ad293 cells to generate an initial virus stock. The virus was then propagated in progressively larger scale cell cultures, and after the final amplification step, the virus was purified using an AdEasy Purification kit (Clontech). A TCID50 assay was performed to determine the titer (IU/ml) of the working virus stock. Low and high RIN3 over-expression cell lines were created by adjusting the multiplicity of infection for adenovirus and LAD2 cells (range from 2 to 20 infectious units per cell).

Flow cytometry

To measure internalization, LAD2 cells were starved of SCF overnight and stimulated with 5 ng/ml SCF at 37ºC for times indicated. Cells were spun down, washed once in cold PBS. For the KIT recovery assay LAD2 cells were starved of SCF overnight and stimulated with 5 ng/ml SCF for 90 minutes at 37ºC. Cells were spun down, washed once in room temperature PBS and resuspended in fresh StemPro without SCF. They were incubated at 30ºC for times indicated then washed in cold PBS. Staining was performed the same for both assays: cells were resuspended in PBS + 0.1% BSA with PE/CY7-anti-KIT (Biolegend 104D2) diluted 1/100 or Alexa 467-anti-CD71 (Santa Cruz) diluted 1/50 and incubated for 45 minutes at 4ºC. After washing once in PBS + 0.1% BSA, they were passed through a 2 μm filter, and run on FACS (BD FSR II) using BD FACS Diva software. Results were analyzed using FloJo. FACS graphs were gated on the viable cell population.

RIN3 knockdown

Knock down experiments were conducted using the ON-TARGETplus system from Dharmacon. The non-targeting siRNA (ON-TARGETplus Non-targeting siRNA #3) and siRNA targeted to RIN3 (GCAGCAUGUUCCACGCUUU) were transfected with Dharmafect 1 according to manufacturer's directions. The final concentration of RNA in each culture was 3 nM; LAD2 cells were at a concentration of 1.25 x 10^5 cells/ml. To restore RIN3 expression, the Ad-RIN3 adenovirus was added 24 hours post siRNA transfection.

Immunohistochemistry

Immunohistochemical analysis was performed on formalin-fixed, paraffin-embedded sections from mastocytosis patients (UCLA Translational Pathology). Sections were de-waxed

at 60ºC and rehydrated with Safeclear and ethanol, then incubated in 0.5% trypsin at 37ºC for 20 minutes for antigen retrieval. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 15 minutes. Samples were blocked in 5% goat serum for 30 minutes before being incubated overnight with anti-RIN3 or anti-MCT. Slides were washed three times in PBS and incubated with biotinylated goat anti-rabbit secondary for 40 minutes. Staining was performed using avidin-biotin complex (ABC, Vectastain) and detected using 3,diaminobezidine (DAB, Vectastain). Samples were counterstained with hematoxylin and neutralized with ammonia hydroxide. Slides were then dehydrated and coverslips were attached with Permamount.

Migration assay

Migration assays were performed using a modified Boyden Chamber (8.0 um pore size). The bottom of the chamber was coated overnight with 800 ng/mL fibronectin. LAD2 cells were starved of SCF overnight and resuspended at 5 x 10^5 cells/mL in fresh media without SCF. Chambers were place above media with or without 100 ng/mL SCF and allowed to migrate for 1 hour at 37ºC. Chambers were then washed in PBS, fixed with 4% PFA for 15 minutes, and stained with 0.1% crystal violet in 10% ethanol for 20 minutes. Cells were wiped from the top chamber that was then washed in PBS to remove excess stain. Cells were counted by light microscopy.

Activated RAB5 pulldown

BL21 cells were transformed with pGEX 4xZFYVE-GST, a tagged RAB5 effector domain (Balaji and Colicelli, in press). After induction for 3 hours at 37ºC, cells were lysed by sonication in buffer containing 20 mM Tris, 250 mM NaCl, 10% glycerol, and 0.01% Triton.

Glutothione sepharose was added to lysate and incubated for 1 hour. Beads were centrifuged and resuspended in NP-40 lysis buffer. Cells were prepared with SCF starvation overnight in then 0 or 5 minutes of 100 ng/mL SCF treatment at 37ºC. Cells were then spun down, washed once in cold PBS and lysed in NP-40 buffer with the addition of 1 mM DTT and 10 mM $MgCl_2$. Glutathione beads preloaded with effector were added to each lysate and allowed to bind for one hour followed by immunoblot detection of total RAB5.

MTS assays

HMC1.1 cells were plated at 50,000 cells per 100 µl in each well of 96 well plate. Imatinib was added at 0, 0.2, and 2 µM to each well. Cells were incubated at 37ºC, 5% CO_2 for 24 hours. Twenty microliters of MTS AQ reagent (Promega) was added to each well, mixed, and incubated for 3 hours at 37ºC, 5% CO_2. Absorbance at 490 nm was read on (Victor^3, 1420 Multilabel Counter, PerkinElmer) and normalized to media only control.

References

1. Gilfillan, A. M., and Beaven, M. A. *Critical reviews in immunology* **31**(6), 475-529
2. Amin, K. *Respiratory medicine* **106**(1), 9-14
3. Ronnstrand, L. (2004) *Cell Mol Life Sci* **61**(19-20), 2535-2548
4. Roskoski, R., Jr. (2005) *Biochemical and biophysical research communications* **338**(3), 1307-1315
5. Roskoski, R., Jr. (2005) *Biochemical and biophysical research communications* **337**(1), 1-13

6. Campbell, E., Hogaboam, C., Lincoln, P., and Lukacs, N. W. (1999) *The American journal of pathology* **154**(4), 1259-1265

7. Ashman, L. K. (1999) *The international journal of biochemistry & cell biology* **31**(10), 1037-1051

8. Boissan, M., Feger, F., Guillosson, J. J., and Arock, M. (2000) *Journal of leukocyte biology* **67**(2), 135-148

9. Metcalfe, D. D. (2008) *Blood* **112**(4), 946-956

10. Qualmann, B., Koch, D., and Kessels, M. M. *The EMBO journal* **30**(17), 3501-3515

11. Rao, Y., and Haucke, V. *Cell Mol Life Sci* **68**(24), 3983-3993

12. Pant, S., Sharma, M., Patel, K., Caplan, S., Carr, C. M., and Grant, B. D. (2009) *Nature cell biology* **11**(12), 1399-1410

13. Barbieri, M. A., Roberts, R. L., Mukhopadhyay, A., and Stahl, P. D. (1996) *Biocell* **20**(3), 331-338

14. Fukuda, M. (2008) *Cell Mol Life Sci* **65**(18), 2801-2813

15. Grosshans, B. L., Ortiz, D., and Novick, P. (2006) *Proceedings of the National Academy of Sciences of the United States of America* **103**(32), 11821-11827

16. Somsel Rodman, J., and Wandinger-Ness, A. (2000) *J Cell Sci* **113 Pt 2**, 183-192

17. Clague, M. J., and Urbe, S. (2001) *J Cell Sci* **114**(Pt 17), 3075-3081

18. Navaroli, D. M., Bellve, K. D., Standley, C., Lifshitz, L. M., Cardia, J., Lambright, D., Leonard, D., Fogarty, K. E., and Corvera, S. (2012) *Proceedings of the National Academy of Sciences of the United States of America* **109**(8), E471-480

19. Kajiho, H., Saito, K., Tsujita, K., Kontani, K., Araki, Y., Kurosu, H., and Katada, T. (2003) *J Cell Sci* **116**(Pt 20), 4159-4168

20. Hu, H., Bliss, J. M., Wang, Y., and Colicelli, J. (2005) *Curr Biol* **15**(9), 815-823

21. Kong, C., Su, X., Chen, P. I., and Stahl, P. D. (2007) *The Journal of biological chemistry* **282**, 15294-15301

22. Tomshine, J. C., Severson, S. R., Wigle, D. A., Sun, Z., Beleford, D. A., Shridhar, V., and Horazdovsky, B. F. (2009) *The Journal of biological chemistry* **284**(39), 26331-26339

23. Barbieri, M. A., Kong, C., Chen, P. I., Horazdovsky, B. F., and Stahl, P. D. (2003) *The Journal of biological chemistry* **278**(34), 32027-32036

24. Hu, H., Milstein, M., Bliss, J. M., Thai, M., Malhotra, G., and Colicelli, J. (2008) *Molecular and cellular biology* **28**, 1573-1583

25. Hunker, C. M., Galvis, A., Veisaga, M. L., and Barbieri, M. A. (2006) *Anticancer Res* **26**(2A), 905-916

26. Hunker, C. M., Giambini, H., Galvis, A., Hall, J., Kruk, I., Veisaga, M. L., and Barbieri, M. A. (2006) *Exp Cell Res* **312**(7), 1106-1118

27. Deininger, K., Eder, M., Kramer, E. R., Zieglgansberger, W., Dodt, H. U., Dornmair, K., Colicelli, J., and Klein, R. (2008) *Proceedings of the National Academy of Sciences of the United States of America* **105**(34), 12539-12544

28. Tall, G. G., Barbieri, M. A., Stahl, P. D., and Horazdovsky, B. F. (2001) *Developmental cell* **1**(1), 73-82

29. Kalesnikoff, J., Rios, E. J., Chen, C. C., Nakae, S., Zabel, B. A., Butcher, E. C., Tsai, M., Tam, S. Y., and Galli, S. J. (2006) *Proceedings of the National Academy of Sciences of the United States of America* **103**(8), 2659-2664

30. Kalesnikoff, J., Rios, E. J., Chen, C. C., Alejandro Barbieri, M., Tsai, M., Tam, S. Y., and Galli, S. J. (2007) *Blood* **109**(12), 5308-5317

31. Zhu, H., Liang, Z., and Li, G. (2009) *Molecular biology of the cell* **20**(22), 4720-4729

32. Horiuchi, H., Lippe, R., McBride, H. M., Rubino, M., Woodman, P., Stenmark, H., Rybin, V., Wilm, M., Ashman, K., Mann, M., and Zerial, M. (1997) *Cell* **90**(6), 1149-1159

33. Lee, E., Marcucci, M., Daniell, L., Pypaert, M., Weisz, O. A., Ochoa, G. C., Farsad, K., Wenk, M. R., and De Camilli, P. (2002) *Science* **297**(5584), 1193-1196

34. Leprince, C., Romero, F., Cussac, D., Vayssiere, B., Berger, R., Tavitian, A., and Camonis, J. H. (1997) *The Journal of biological chemistry* **272**(24), 15101-15105

35. Ge, K., and Prendergast, G. C. (2000) *Genomics* **67**(2), 210-220

36. Kirshenbaum, A. S., Akin, C., Wu, Y., Rottem, M., Goff, J. P., Beaven, M. A., Rao, V. K., and Metcalfe, D. D. (2003) *Leukemia research* **27**(8), 677-682

37. Dinneen, J. L., and Ceresa, B. P. (2004) *Traffic (Copenhagen, Denmark)* **5**(8), 606-615

38. Aichberger, K. J., Mayerhofer, M., Gleixner, K. V., Krauth, M. T., Gruze, A., Pickl, W. F., Wacheck, V., Selzer, E., Mullauer, L., Agis, H., Sillaber, C., and Valent, P. (2007) *Blood* **109**(7), 3031-3041

39. Xu, L., Lubkov, V., Taylor, L. J., and Bar-Sagi, D. (2010) *Curr Biol* **20**(15), 1372-1377

40. Prendergast, G. C., Muller, A. J., Ramalingam, A., and Chang, M. Y. (2009) *Biochimica et biophysica acta* **1795**(1), 25-36

41. Kajiho, H., Fukushima, S., Kontani, K., and Katada, T. (2012) *PloS one* **7**(1), e30575

42. Capdeville, R., Buchdunger, E., Zimmermann, J., and Matter, A. (2002) *Nature reviews. Drug discovery* **1**(7), 493-502

43. Druker, B. J., Tamura, S., Buchdunger, E., Ohno, S., Segal, G. M., Fanning, S., Zimmermann, J., and Lydon, N. B. (1996) *Nature medicine* **2**(5), 561-566

44. Joensuu, H., Roberts, P. J., Sarlomo-Rikala, M., Andersson, L. C., Tervahartiala, P., Tuveson, D., Silberman, S., Capdeville, R., Dimitrijevic, S., Druker, B., and Demetri, G. D. (2001) *The New England journal of medicine* **344**(14), 1052-1056

45. Miyachi, K., Ihara, A., Hankins, R. W., Murai, R., Maehiro, S., and Miyashita, H. (2003) *Clinical rheumatology* **22**(4-5), 329-332

46. Nigrovic, P. A., and Lee, D. M. (2005) *Arthritis research & therapy* **7**(1), 1-11

47. Jin, Y., Lu, Z., Cao, K., Zhu, Y., Chen, Q., Zhu, F., Qian, C., and Pan, J. (2010) *Molecular cancer therapeutics* **9**(1), 211-223

48. Han, L., Wong, D., Dhaka, A., Afar, D., White, M., Xie, W., Herschman, H., Witte, O., and Colicelli, J. (1997) *Proceedings of the National Academy of Sciences of the United States of America* **94**(10), 4954-4959

49. Butterfield, J. H., Weiler, D., Dewald, G., and Gleich, G. J. (1988) *Leukemia research* **12**(4), 345-355

50. Laidlaw, T. M., Steinke, J. W., Tinana, A. M., Feng, C., Xing, W., Lam, B. K., Paruchuri, S., Boyce, J. A., and Borish, L. (2011) *The Journal of allergy and clinical immunology* **127**(3), 815-822 e811-815

51. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kinzler, K. W., and Vogelstein, B. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95**(5), 2509-2514

52. Shayakhmetov, D. M., Papayannopoulou, T., Stamatoyannopoulos, G., and Lieber, A. (2000) *Journal of virology* **74**(6), 2567-2583

Chapter 4

RIN3 does not significantly affect KIT degradation, recycling, or AKT phosphorylation downstream of KIT activation

4.1 Introduction

We have established that RIN3 regulates KIT internalization, stability, and biological responses in mast cells. Next we wanted to determine if other processes downstream of SCF activation are influenced by RIN3. Also, we wanted to probe further into possible mechanisms behind the differences in biological output we see when RIN3 levels are changed.

First of all, it has recently begun to be appreciated that the rate and method of internalization can effect signaling downstream (reviewed in (1)). FYVE-domain containing proteins are recruited to early endosome by binding to activated RAB5. Studies have shown that these proteins can affect signaling and receptor fate. For example, in Transforming Growth Factor β signaling a FYVE-domain containing protein facilitates TGFβ-receptor interaction with SMAD proteins thereby enhancing signaling (2). In contrast, Tumor Necrosis Factor receptor 1 is marked for degradation in the endosome by a FYVE domain containing ubiquitin ligase (3). Since we have now established that RIN3 influences KIT internalization and RAB5 activity we hypothesized that it may prolong or attenuate phosphorylation events downstream of SCF. Differences in signaling events may also help to explain differences in biological responses. In particular, we know that MAP kinase and PI3 kinase pathways are activated during migration (4,5). Decreased phosphorylation in these pathways may be responsible for decreased migration.

We have shown that in cells overexpressing RIN3 KIT levels are decreased on the surface as well as in the whole cell lysate. We attribute this difference to increased degradation due to increased RAB5 activity (Chapter 3). However, these experiments only address KIT levels in the absence of stimulation. Previous studies (Balaji and Colicelli unpublished data, (6)) have shown that both RIN1 and RINL increase the rate of RTK degradation post stimulation with growth factor. Based on these data, we hypothesized that RIN3 may also influence the rate of KIT degradation post SCF stimulation.

Lastly, migration toward growth factor is a very complex process. Even in vitro where the process has been simplified, the cell needs to be able to sense the gradient of SCF, adhere to the fibronectin-coated chamber, and squeeze through the perforated bottom. Being able to sense the growth factor is dependent on the amount of receptor on the surface, stimulation of the receptor, and consequent redistribution of receptor to the plasma membrane. We already established that RIN3 influences KIT internalization; however, we also wanted to determine if RIN3 influences the rate at which KIT is recycled back to the membrane. Differences observed in rate of receptor recycling could be key to explaining the cell's ability to migrate toward SCF.

4.2 RIN3 does not significantly affect KIT phosphorylation or phosphorylation events downstream of SCF stimulation

It is well established that the rate of endocytosis and receptor trafficking can affect signaling pathways downstream of RTK (reviewed in (1)). Since we know that RIN3 affects KIT internalization and biological responses to KIT, ie migration, we wanted to see if we could detect changes in phospho-tyrosine signaling when RIN3 is silenced or overexpressed. We took three

approaches to this question. First, we looked at global phospho-tyrosine levels post stimulation. Second, we probed for KIT phosphorylation specifically. Lastly, we observed phosphorylation of ERK and AKT, which are known downstream effectors of KIT (reviewed in (7)).

In the first approach, we took cells with silenced RIN3 and stimulated them with low

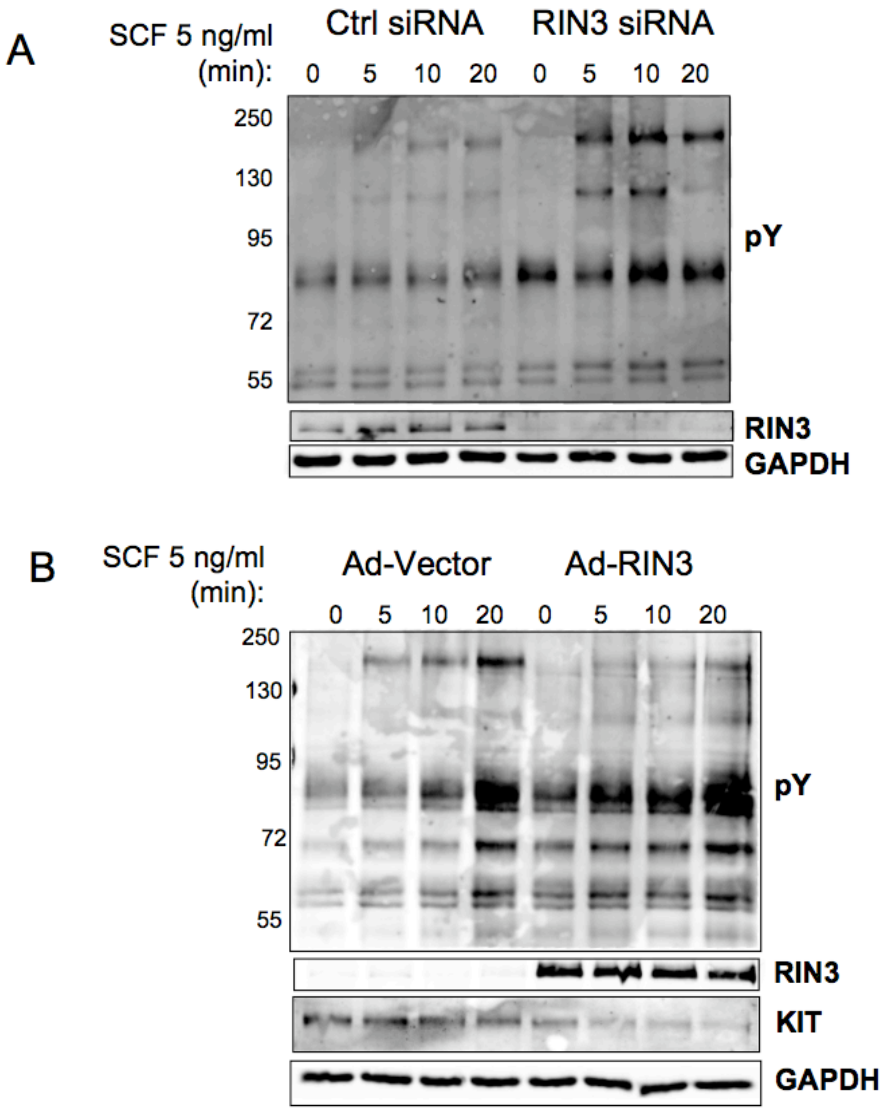


Figure 4-1. ***Manipulation of RIN3 does not greatly affect phosphotyrosine signaling downstream of KIT.*** **A**. Cells were transfected with control or RIN3 siRNA. After starvation from SCF overnight, cells were pre-treated with pervanadate inhibitor then stimulated with 5 ng/ml SCF for indicated time points. Lysates were probed for RIN3 levels, total phosphotyrosine (4G10) and GAPH (loading control). **B.** Cell were infected with GFP vector or RIN3 adenovirus, then treated the same as A. Whole cell lysate blot for KIT is included to show reduced levels.

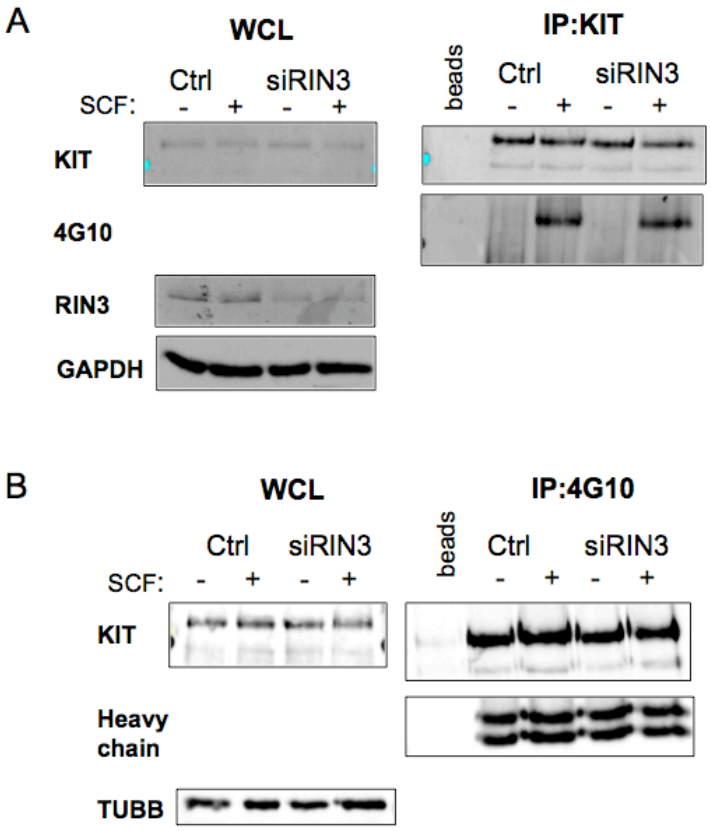


Figure 4-2. ***RIN3 silencing does not significantly effect KIT phosphorylation when measured by immunoprecipitation.*** **A.** Cells were transfected with control or RIN3 siRNA. After starvation from SCF overnight and pre-treatment with pervanadate, cells were stimulated with 5 ng/ml of SCF for 10 minutes. KIT was immunoprecipitated from solution and then blotted for phosphotyrosine (4G10). **B.** Cells were treated the same as A; however, lysates were immunoprecipitated for phosphotyrosine and blotted for KIT. Heavy chain is shown to indicate antibody added to each sample.

levels of SCF for times indicated (Fig 4-1A). Analysis of global phospho-tyrosine, as indicated by 4G10 signal, shows higher signal for bands around 250, 130, and above 72 kilodalton markers. However, this band also shows higher intensity when RIN3 is overexpressed (Fig 4-1B). Because we do not know which protein this band represents and because of the discrepancy between silencing and overexpression we have chosen not to pursue this band. However, the doublet bands at the highest molecular weight run have increased intensity when RIN3 is silenced and lower intensity when RIN3 is overexpressed. Also, these band run at a similar place

that KIT is typically seen. It is possible that the decrease in phospho-tyrosine signal in these bands with RIN3 overexpression is simply a reflection of less KIT overall (Fig 4-1B). We hypothesized that KIT undergoes more phosphorylation when RIN3 is silenced.

In order to address the KIT phosphorylation state specifically we decided to immunoprecipate KIT and blot for phospho-tyrosine. When RIN3 is silenced we do not detect a difference in phosphorylation state of KIT that is precipitated from solution (Fig 4-2A). It is possible that the immunoprecipitation with anti-KIT somehow enriches the sample for unphosphorylated or differently phosphorylated KIT protein. KIT is phosphorylated on many different sites and RIN3 may only be affecting certain sites and not overall phosphorylation. In order to address this difference we immunoprecipitated for phosphorylated proteins (with 4G10 antibody) and blotted for KIT. Again, we saw levels of KIT in the IP go up with stimulation, but did not observe a difference when RIN3 levels are silenced (Fig 4-2B). From these experiments we are not able to conclude any difference in KIT phosphorylation status due to RIN3 levels.

SCF binding oligomerizes KIT on the cell surface to activate KIT's tyrosine kinase domain, causing direct phosphorylation of multiple substrates as well as a downstream cascade of tyrosine phosphorylation by other kinases. We hypothesized that although we cannot detect changes in KIT phosphorylation there may still be changes in signaling in downstream effectors. It is well established that KIT is upstream of PI3K and RAS/RAF/ERK pathways. Therefore, we probed for phosphorylation levels of AKT and ERK post stimulation. When RIN3 is silenced basal level of pERK is elevated (Fig 4-3A). However, there is no significant difference in other time points for both ERK and AKT signaling. Overexpression of RIN3 does not cause significant changes in pAKT levels post SCF stimulation (Fig 4-3B). In contrast, there is a subtle change in pERK levels. Twenty minutes post stimulation pERK levels have started to

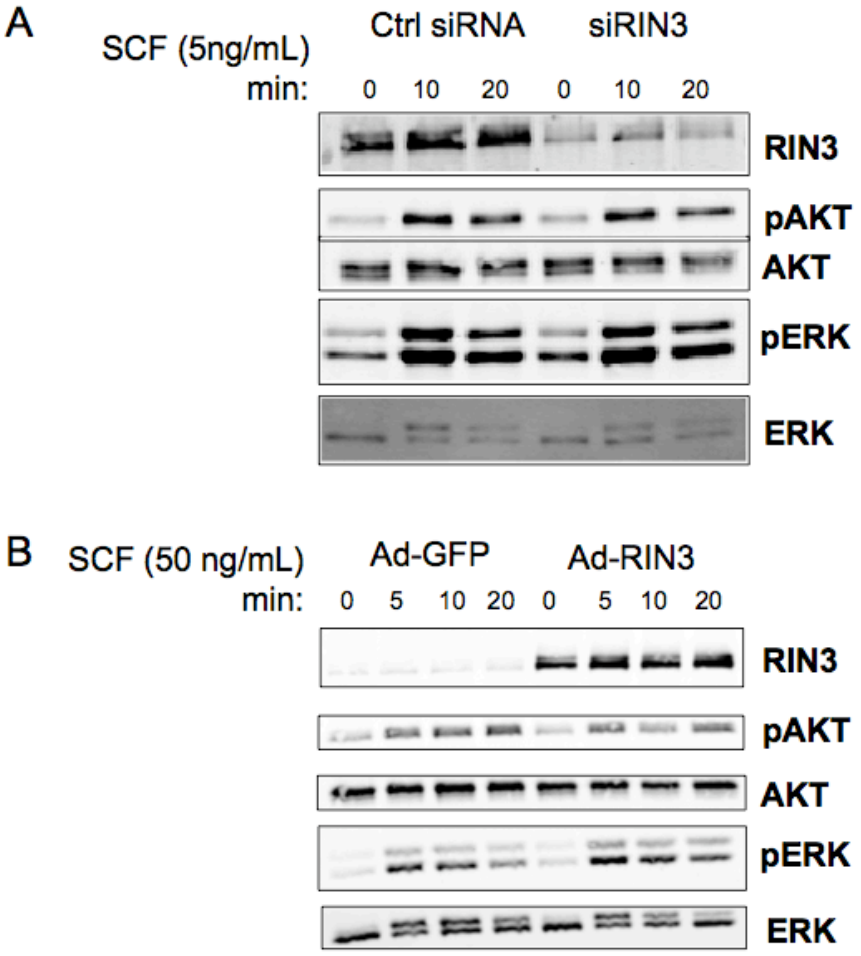


Figure 4-3. ***Manipulation of RIN3 levels does not effect AKT activation, while overexpression prolongs ERK activation.*** **A.** Cells were transfected with control or RIN3 siRNA, starved overnight and stimulated with 5 ng/ml SCF for indicated time points. **B.** Cells were infected with GFP or RIN3 adenovirus, starved overnight and stimulated with 50 ng/ml SCF for indicated time points. Lysates were probed for pAKT and pERK as well as total levels of these proteins.

decrease in control cells; however, cells with high RIN3 expression still have high levels of pERK. This indicates that RIN3 overexpression may lead to prolonged signaling through the RAS/RAF/ERK pathway. Previous studies have shown that decreased RAB5 causes attenuation of pERK signaling downstream of another RTK, Fibroblast Growth Factor Receptor (8). Increased RAB5 activity due to RIN3 overexpression may be causing prolonged signaling downstream of KIT.

4.3 RIN3 does not significantly affect KIT degradation post SCF stimulation

Overexpression of RIN1 and RINL are known to increase the rate of degradation of Receptor Tyrosine Kinases (RTKs) after stimulation with growth factor (Balaji and Colicelli unpublished data (6)). Therefore, we hypothesized that RIN3 may be able to regulate the rate of KIT degradation post stimulation. Since overexpression of RIN3 can cause decreased basal levels of KIT (see Chapter 2), we were concerned that a degradation phenotype might be masked

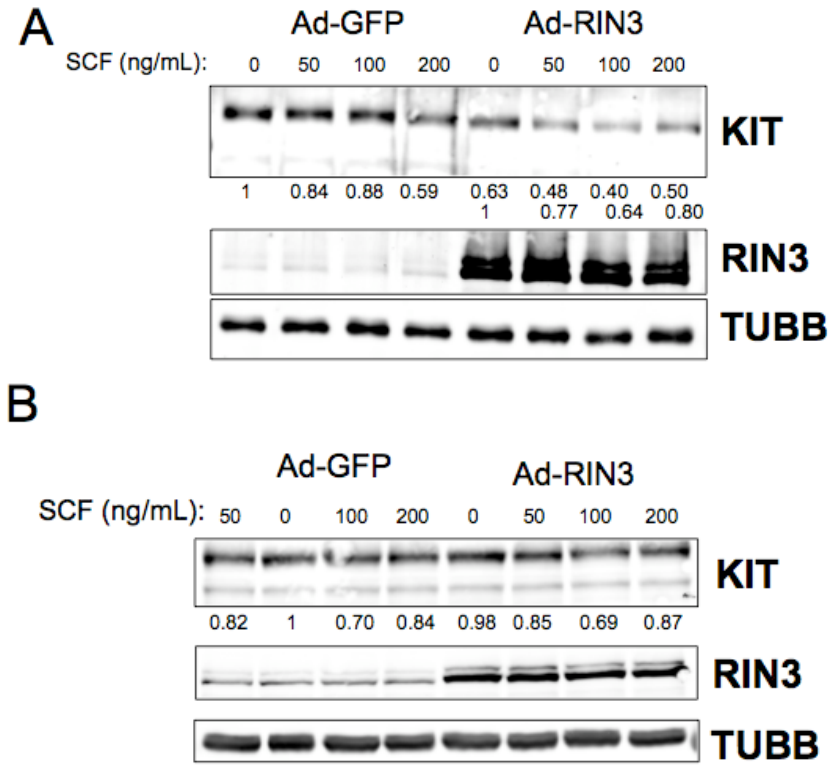


Figure 4-4. ***Overepression of RIN3 does not significantly affect KIT degradation.*** **A.** Cells were infected with high amounts of GFP or RIN3 virus (20 IU/ml). After 5 minutes of stimulation at indicated SCF concentration, KIT levels were meausured by western blot. **B.** Cells were infected with low amounts of GFP or RIN3 virus (2 IU/ml), then treated the same as A. Numbers below the KIT panel indicate the relative amounts of KIT as determined by Li-Cor.

by very robust overexpression. For this reason we conducted the experiment with both high and moderate RIN3 overexpression. High expression of RIN3 in LAD2 cells show that KIT levels are decreased approximately 36% before stimulation (Fig 4-5A). The quantification shows that if the starting level of KIT is set to one for cells with high RIN3, KIT is further reduced by 10-15% after the addition of SCF. With moderate RIN3 overexpression the basal level of KIT is the same between the control and RIN3 cells (Fig 4-5B). However, the rate of degradation post stimulation is also the same in cells with control GFP or RIN3 expression. From these experiments we conclude that very high expression of RIN3 can cause a slight increase in KIT degradation post SCF stimulation.

4.4 KIT recycling to plasma membrane is not changed with RIN3 silencing

When RIN3 is silenced in LAD2 cells we observe accelerated internalization of KIT from the plasma membrane (Chapter 3). Because of this result we also wanted to test the cell's ability to recycle the receptor back to the plasma membrane following stimulation and removal of the growth factor from the media. Control and RIN3 silenced cells have the same amount of KIT on the surface before stimulation (Fig 4-5 top left) as well as 30 minutes post stimulation, pre-recovery (Fig 4-5 top right). At both 45 and 90 minutes recovery time KIT surface levels have begun to come back up (Fig 4-5 bottom panels). These levels are the same regardless of RIN3 expression level. Although RIN3 influences the internalization of KIT, it does not significantly affect the cell's ability to recycle KIT back to the cell surface.

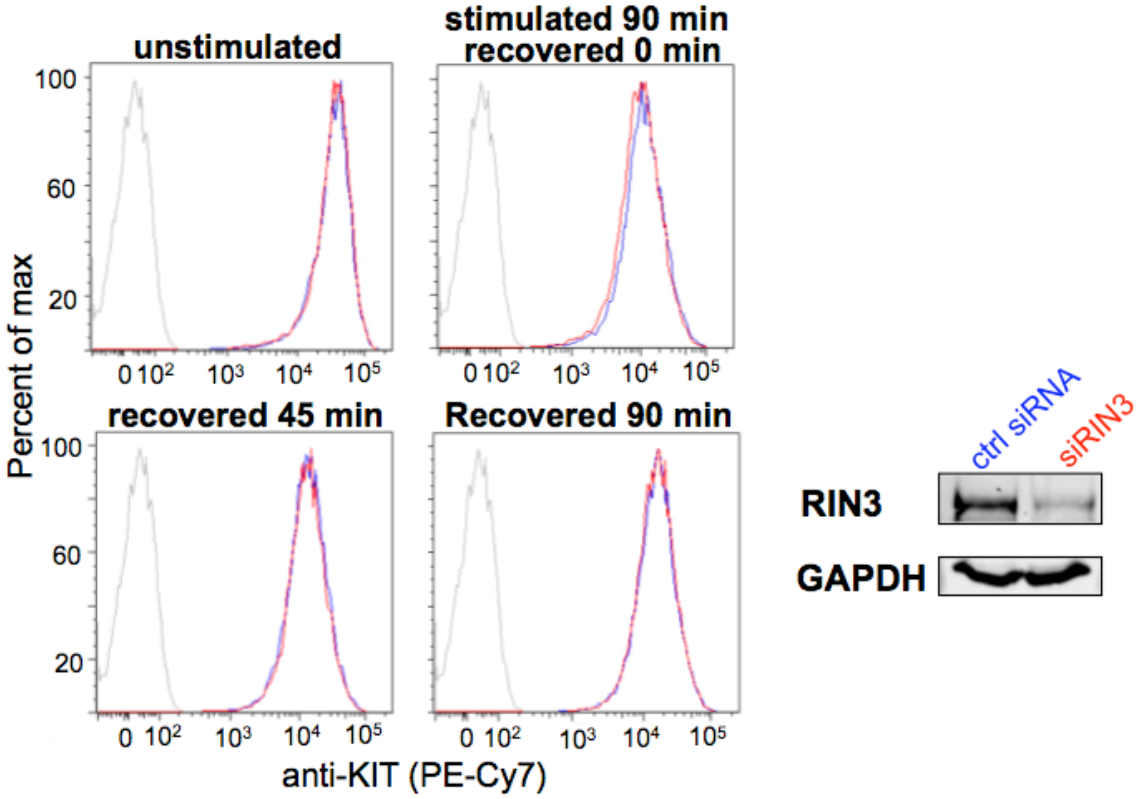


Figure 4-5. ***RIN3 knockdown does not affect KIT recycling.*** Cells were transfected with control (blue) or RIN3 siRNA (red). Surface expression of KIT was measured by flow cytometry before stimulation (top left), after stimulation with 5 ng/ml SCF (top right) and at two time points of recovery in SCF free media (bottom panels). Gray line represents unstained control. Immunoblot indicates level of RIN3 in lysates.

4.5 Discussion

Changes in RIN3 expression levels alter the internalization rate of KIT and the cell's ability to migrate toward SCF. We tested if RIN3 also influences KIT recycling, degradation, and phospho-tyrosine signaling downstream of stimulation.

When probing for changes in phospho-tyrosine signaling we see prolonged pERK with RIN3 overexpression, which is likely attributed to higher RAB5 activity. In the future, either knocking down RAB5 or expressing dominant negative $RAB5^{S34N}$ could test this hypothesis. We would expect these treatments to return pERK levels closer to control. Unfortunately, we are unable to

make a strong conclusion about how RIN3 affects the phosphorylation state of KIT. This question could be pursued further by probing with antibodies for specific phosphorylation sites of the protein or a mass spectrometry analysis for phospho-tyrosine. It is well established that different phosphorylation sites interact with different adapters and effectors causing diversity in biological output (reviewed in (7)).

We had hypothesized that the changes in migration when RIN3 levels are altered were due to modifications in the cell's ability to recycle the KIT receptor back to the membrane post stimulation. Using flow cytometry we cannot detect any different in KIT recycling. However, it is important to point out that although flow cytometry is the best way available to measure recycling the conditions of the migration assay and the recycling assay are quite different. In the migration assay, the cells have to sense the SCF in the lower well, bind to fibronectin, and squeeze through the pores in the chamber. The recycling assay involves treating the cells with SCF in the media, which is much less rigorous of a test of sensitivity compared to sensing a gradient of SCF. Also, the migration phenotype may be due to differences in the cell's ability to adhere to fibronectin which is also mediated by SCF signaling (9). In the future, it would be important to test the cell's ability to bind to fibronectin when RIN3 is silenced or overexpressed.

Although other members of the RIN family cause increased degradation of RTKs post stimulation, RIN3 only minimally increased degradation of KIT when robustly overexpressed. This subtle change in degradation may be partly responsible for the decreased migration toward SCF in RIN3 overexpressing cells. However, the reduced levels of KIT on the cell surface are likely to be the main culprit. The 30% reduction of surface KIT compromises the cell's ability to sense and respond to growth factor (Chapter 3).

References

1. Platta, H. W., and Stenmark, H. *Current opinion in cell biology* **23**(4), 393-403

2. Chen, Y. G., Wang, Z., Ma, J., Zhang, L., and Lu, Z. (2007) *The Journal of biological chemistry* **282**(13), 9688-9695

3. Liao, W., Xiao, Q., Tchikov, V., Fujita, K., Yang, W., Wincovitch, S., Garfield, S., Conze, D., El-Deiry, W. S., Schutze, S., and Srinivasula, S. M. (2008) *Curr Biol* **18**(9), 641-649

4. Ali, K., Bilancio, A., Thomas, M., Pearce, W., Gilfillan, A. M., Tkaczyk, C., Kuehn, N., Gray, A., Giddings, J., Peskett, E., Fox, R., Bruce, I., Walker, C., Sawyer, C., Okkenhaug, K., Finan, P., and Vanhaesebroeck, B. (2004) *Nature* **431**(7011), 1007-1011

5. O'Laughlin-Bunner, B., Radosevic, N., Taylor, M. L., Shivakrupa, DeBerry, C., Metcalfe, D. D., Zhou, M., Lowell, C., and Linnekin, D. (2001) *Blood* **98**(2), 343-350

6. Kajiho, H., Fukushima, S., Kontani, K., and Katada, T. *PloS one* **7**(1), e30575

7. Ronnstrand, L. (2004) *Cell Mol Life Sci* **61**(19-20), 2535-2548

8. Vecchione, A., Cooper, H. J., Trim, K. J., Akbarzadeh, S., Heath, J. K., and Wheldon, L. M. (2007) *Proteomics* **7**(24), 4565-4578

9. Dastych, J., and Metcalfe, D. D. (1994) *J Immunol* **152**(1), 213-219

Chapter 5

Concluding Remarks

5.1 Working model for role of RIN3 in mast cells

The number of people with allergies and asthma is on the rise in the US. This trend has been attributed to a number of factors such as increased pollution, increased personal and household hygiene, as well as increased awareness and diagnosis (1,2). Whatever the reasons may be, a deeper understanding of how mast cell responses are regulated is needed to better treat these diseases.

In this work, we identified RIN3 as a negative regulator of SCF-mediated responses in mast cells. RIN3 regulates the internalization and expression of KIT in mast cells as well as the cell's ability to migrate toward its growth factor SCF. As a working model (Fig 5-1), I believe that after SCF stimulation, RIN3 is recruited to the plasma membrane by binding to activated RAS. It can then deposit BIN2 at the membrane to begin bending to internalize the KIT receptor. RIN3 activation of RAB5 aids in early endosome fusion which influences the duration of KIT signaling causing increased migration. The amount of RIN3 present in the cell may also influence whether internalized KIT is degraded or recycled to the plasma membrane. RIN3 may be binding directly to KIT through its SH2 domain.

5.2 Future Directions

More experiments are necessary to better flesh out the relationship between RIN3 and KIT signaling. The initial steps of internalization are dependent on the location of RIN3 and BIN2. Experiments using the RIN3 antibody for confocal microscopy have

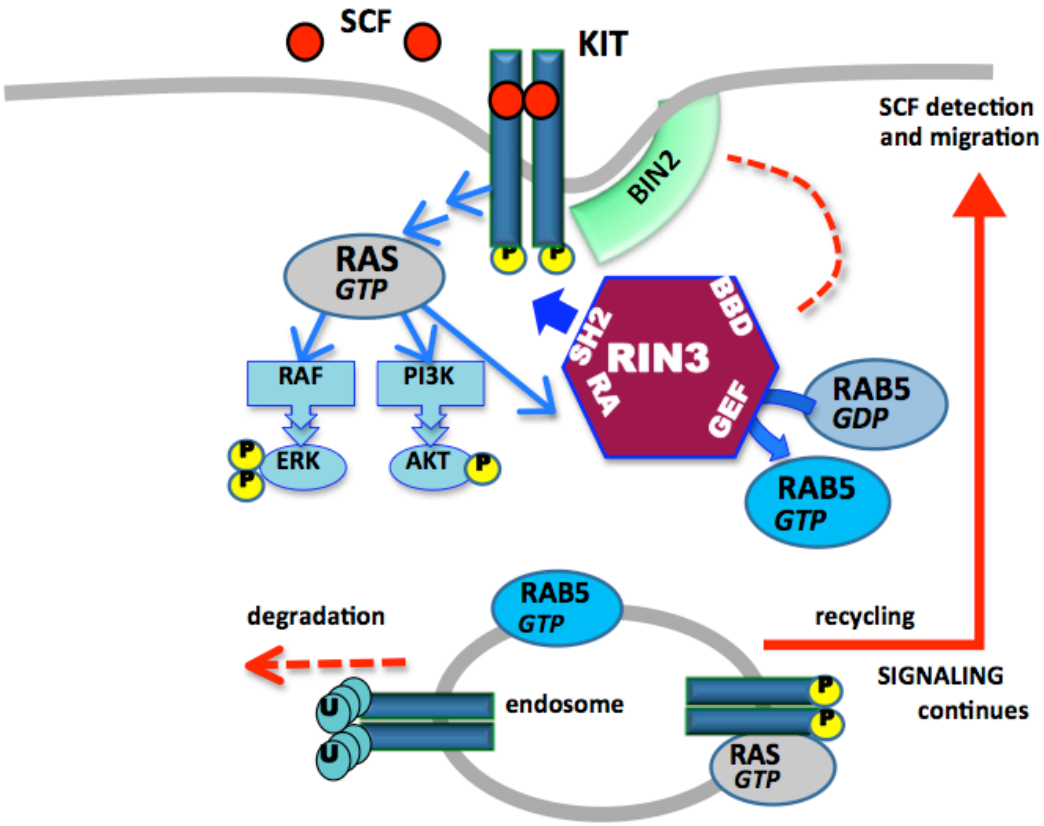


Figure 5-1. ***Working model.***

yielded images with very high background and little positive staining. We have also tried unsuccessfully to locate RIN3 with sub-cellular fractionation. A better way to determine the location of RIN3 post-stimulation would be to infect LAD2 cells with a fluorescently tagged RIN3 and BIN2 constructs. We could then use live cell imaging to determine if RIN3 is depositing BIN2 at the plasma membrane post SCF treatment. Due to the downregulation of KIT with RIN3 overexpression, these experiments may benefit from an inducible system for RIN3 expression.

Also, we know that RIN3 negatively regulates migration toward SCF, but a molecular mechanism still remains to be elucidated. Migration is a complex process that involves the cell sensing a growth factor, binding to fibronection, and squeezing through

a pore on the bottom chamber. We hypothesized that RIN3 may be affecting KIT recycling and therefore the cell's ability to sense the gradient. Yet, we did not detect a difference in KIT recycling back to the membrane post stimulation. However, this experiment was performed with cells floating in media with SCF added, which is different from a gradient of SCF. Also, the surface expression was measured using flow cytometry, which measures surface expression over the entire surface of the cell. To better understand if RIN3 is involved in sensing an SCF gradient, a similar experiment could be done using confocal microscopy. Cells could be loaded on one side of a chamber with SCF on the other and a porous membrane in between. After staining for KIT, images could be scored on how KIT is distributed on the membrane. In addition to probing for KIT recycling, we could also test if RIN3 affects the cell's ability to bind to fibronectin.

Overexpression of RIN3 causes a striking reduction of KIT on the surface and in the whole cell lysate. We show that this reduction can be partially rescued by treating with the proteasome inhibitor MG132 (Fig 3-6). This result suggests that RIN3 is causing increased degradation of the receptor. However, we have yet to uncover how RIN3 accomplishes this. We also observed increased RAB5 activity. Another group (3) has shown that increased RAB5 activity leads to decreased RTK levels. I hypothesize that the increased RAB5 activity caused by RIN3 overexpression causes the receptor to be targeted for degradation. This could be tested by measuring the levels of ubiquitination of KIT post stimulation. It is also known that CBL phosphorylation and recruitment to KIT leads to KIT's ubiquitination and degradation (4). We could also test if RIN3 overexpression causes increased CBL activity and recruitment to KIT.

Lastly, we would next like to test the affects of mutations in RIN3 on mast cell function. We have made knock out mutations to the Proline rich, SH2, and GEF domains of RIN3. It is possible that overexpression of one or more of these mutants may serve as a domain negative, which would enhance our ability to test our hypotheses in cell lines. In particular, we saw increased surface expression and delayed internalization of KIT when RIN3-ΔPR was expressed in HMC1.1 cells. I hypothesize that this mutant would show the same phenotypes in LAD2 cells, and additionally, I'd expect these cells would be better able to migrate toward SCF and be less sensitive to imatinib treatment. Also, the RIN3-ΔPR construct could be used in an unbiased mass spectrometry experiment to determine which proteins interact with RIN3's Proline rich region.

References

1. Platts-Mills, T.A.E., Erwin, E., Heymann, P., and Woodfolk, J. (2005) *Allergy* 60: 25-31
2. Martinez, F.D. (1994) *Thorax* **49**(12), 1189–1191
3. Dinneen, J.L., Ceresa, B.P., (2004) *Traffic* **5**(8), 606-615
4. Masson, K., Heiss, E., Band, H., and Ronnstrand, L. (2006) *Biochem. J.* **399**(1), 59-67