Macrophages Require Skap2 and Sirpα for Integrin-Stimulated Cytoskeletal Rearrangement

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SUMMARY

Macrophages migrate to sites of insult during normal inflammatory responses. Integrins guide such migration, but the transmission of signals from integrins into the requisite cytoskeletal changes is poorly understood. We have discovered that the hematopoietic adaptor protein Skap2 is necessary for macrophage migration, chemotaxis, global actin reorganization and local actin reorganization upon integrin engagement. PIP3 binding to the Skap2 PH domain, which relieves its conformational auto-inhibition, is critical for this integrin-driven cytoskeletal response. Skap2 enables integrin-induced tyrosyl phosphorylation of Src-family kinases (SFKs), Adap, and Sirpα, establishing their roles as signaling partners in this process. Furthermore, macrophages lacking functional Sirpα unexpectedly have impaired local integrin-induced responses identical to those of Skap2−/− macrophages, and Skap2 requires Sirpα for its recruitment to engaged integrins and for coordinating downstream actin rearrangement. By revealing the positive-regulatory role of Sirpα in a Skap2-mediated mechanism connecting integrin engagement with cytoskeletal rearrangement, these data demonstrate that Sirpα is not exclusively immunoinhibitory, and illuminate previously unexplained observations implicating Skap2 and Sirpα in mouse models of inflammatory disease.
INTRODUCTION

To participate in inflammatory responses, macrophages and their precursors must migrate to perceived sites of infection or insult (Geissmann et al., 2010; Hume, 2006). These cells rely on cues from the local environment delivered through integrins, receptors critical for cell-matrix and cell-cell interactions (Berton and Lowell, 1999; Hynes, 2002). Inflammatory stimuli can induce “inside-out” signals that control integrin cell surface expression and affinity for extracellular ligands. In turn, upon ligand binding, integrins initiate “outside-in” signals that control multiple downstream pathways required for cell adhesion, migration, and chemotaxis, all key components of innate and adaptive immune responses (Berton and Lowell, 1999; Gahmberg et al., 1998). These integrin-mediated responses also depend on the macrophage’s ability to remodel its cytoskeleton, thereby allowing changes in cell shape, motility, and directionality (Vicente-Manzanares and Sanchez-Madrid, 2004; Worthylake and Burridge, 2001). Despite their central role in macrophage function, the mechanisms by which integrin signals lead to cytoskeletal reorganization remain unclear.

In leukocytes, adaptor proteins participate in integrin-driven signaling. One such protein, Src kinase-associated phosphoprotein 2 (Skap2, a.k.a. Skap-hom), interacts with Adhesion and Degranulation-Promoting Adapter Protein (Adap, a.k.a. Fyb), an established integrin-responsive protein (Kasirer-Friede et al., 2007). Moreover, integrin engagement induces tyrosyl phosphorylation of Skap2 and Adap (Timms et al., 1999). The structurally similar Skap1 is expressed specifically in T cells and also binds Adap. However, Skap2 is expressed broadly in lymphoid and myeloid cells (Kouroku et al., 1998; Liu et al., 1998; Marie-Cardine et al., 1998) and cannot substitute for Skap1 in regulating T cell inside-out signals (Griffiths et al., 2001; Jo et al., 2005; Moog-Lutz et al., 2001), suggesting that Skap2 may perform a distinct role during integrin signaling. Studies have suggested an adhesive/migratory role for Skap2 in B cells and dendritic cells, but have provided no mechanism or relationship to integrin engagement or cytoskeleton modulation (Reinhold et al., 2009; Togni et al., 2005). Indeed, how Skap2 functions at the molecular level is not well understood in any biological system.

Skap2 also binds the transmembrane protein Signal Regulatory Protein-α (Sirpα, a.k.a. SHPS-1), which is generally regarded as an immune inhibitory receptor (Barclay, 2009; Kharitonenko et al., 1997; Veillette et al., 1998). Like Skap2, Sirpα is tyrosyl phosphorylated in response to integrin
engagement in macrophages (Inagaki et al., 2000; Johansen and Brown, 2007; Motegi et al., 2003; Timms et al., 1999). Importantly, Skap2- and Sirpα-deficient mice are resistant to autoimmune processes, such as experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis, which serve as models for human disease (multiple sclerosis and rheumatoid arthritis) that have integrin-dependent components (Okuzawa et al., 2008; Ransohoff, 2007; Togni et al., 2005; Tomizawa et al., 2007). Indeed integrins have emerged as important therapeutic targets in multiple sclerosis, rheumatoid arthritis, and other autoimmune diseases, such as inflammatory bowel disease (Parikh et al., 2011; Peters et al., 2011; Ransohoff, 2007; Rutgeerts et al., 2009). Thus, determining whether Skap2 and Sirpα regulate macrophage responses to integrin signals could significantly enhance our mechanistic understanding of inflammatory responses and diseases, thereby facilitating the identification of new therapeutic targets. Here, we find that Skap2 and Sirpα drive the transduction of integrin-evoked signals that lead to the cytoskeletal rearrangement required for macrophage migration.
RESULTS

Skap2 is required for macrophage migration, chemotaxis, and spreading

To determine whether Skap2 is required for migration, we tested the ability of bone marrow-derived macrophages (BMMs) from Skap2⁻/⁻ mice to migrate into scratches introduced into densely populated cultures. Skap2⁻/⁻ BMMs exhibited a pronounced migration defect in this assay (Fig. 1A). Likewise, these cells showed decreased migratory responses driven by M-CSF or the chemokines CCL2 and CXCL4 in transwell assays (Fig. 1B-D). Skap2⁻/⁻ BMMs also spread poorly on glass, which binds BMMs predominantly through their β2 integrins (Brown, 1991; Yakubenko et al., 2002), and impaired BMM spreading was accompanied by reduced F-actin content as measured by phalloidin staining (Fig. 1E,F). Together, these results suggest a mechanism in which Skap2 is necessary for eliciting the cytoskeletal changes that drive macrophage motility.

The spreading and migratory defects were observed in the absence of chemokine/cytokine gradients, suggesting that processes downstream of integrin engagement, not cytokine or chemokine detection per se, were affected by the loss of Skap2. Indeed, Skap2⁻/⁻ BMMs proliferate normally in response to M-CSF and GM-CSF (Togni et al., 2005), and M-CSF-induced tyrosyl phosphorylation was not globally perturbed by Skap2 deficiency (Fig. 2A). Although lysates from M-CSF-stimulated Skap2⁻/⁻ BMMs showed the absence of a ~50kD phosphotyrosyl band that was observed in M-CSF-treated Skap2⁺/⁺ BMMs, this band primarily corresponded to Skap2 itself (Bourette et al., 2005), as demonstrated by immunodepletion experiments (Fig. S1). Skap2⁻/⁻ BMMs also had normal integrin expression, so their defective migration and spreading were not due to decreased integrin availability (Fig. 2B). Furthermore, flow cytometric analysis with the activation-specific antibody 9EG7 showed similar levels of basal and phorbol 12-myristate 13-acetate (PMA)-evoked β1 integrin activation in WT and Skap2⁻/⁻ BMMs, suggesting that inside-out integrin activation was not impaired in these cells (Fig. S2).

Notably, Skap2⁺/⁻ BMM responses were identical to those of wild-type (WT, Skap2⁺/⁺) BMMs in our assays; this was likely due to the role Adap plays in binding and stabilizing Skap1 and Skap2 against proteolysis (Huang et al., 2005; Kasirer-Friede et al., 2007), thereby limiting Skap2’s intracellular expression (Togni et al., 2005), which was equivalent in WT and Skap2⁺/⁻ BMMs (Fig. 2C). Indeed, immunodepletion of Skap2 from lysates of Skap2⁺/⁻ cells also resulted in depletion of
Adap (Timms et al., 1999; Fig. S1), suggesting that the level of protein produced by a single Skap2 allele was able to saturate the available Adap,

*Skap2 is required for integrin-dependent actin cytoskeletal rearrangement*

Because Skap2 is crucial for macrophage migration, chemotaxis, and spreading, we hypothesized that it is required for integrin-dependent actin cytoskeletal rearrangement. After spreading on glass, *Skap2*+/− BMMs developed pronounced actin ruffles, or curvilinear accumulations of polymerized F-actin (Fig. 3A). By contrast, *Skap2*−/− BMMs developed fewer and shorter ruffles (Fig. 3B). In *Skap2*+/− BMMs, Skap2 colocalized with ruffles preferentially at the leading edges and apical portions of the cells (Fig. 3C), often within punctate structures associated with the edges of ruffles. Trails of subjacent actin were observed to emanate from the ruffles’ leading edges (Supplementary Material, Video 1).

Cell adhesion, migration, and chemotaxis are complex processes that integrate changes in integrin modulation and engagement, cytoskeletal reorganization, cell polarization, and cell shape through interconnected signaling pathways (Berzat and Hall, 2010; Chen et al., 2003; Jones, 2000). In the face of such complexity, it is advantageous to study integrin-mediated events through spatially and temporally focused approaches in model systems that isolate and control receptor engagement in order to measure local, early responses. Therefore, we employed a bead assay that incorporated integrin ligands and monoclonal antibodies to examine further the role of Skap2 in integrin-mediated cytoskeletal rearrangement. Polystyrene beads were coated with polyRGD (pRGD) (Alenghat et al., 2009; Miyamoto et al., 1995), which binds to a broad range of integrins, including β1, β2, and β3 (Plow et al., 2000). *Skap2*+/− and *Skap2*−/− BMMs bound these beads similarly, consistent with their equivalent levels of surface integrin expression (Fig. 2B). However, whereas pRGD beads evoked pronounced actin ruffling in *Skap2*+/− BMMs (Fig. 3D), *Skap2*−/− BMMs generated markedly less ruffling in proximity to the beads (Fig. 3E, F). This cytoskeletal response occurred within 20 minutes of integrin engagement, and beads coated with a control protein (albumin) failed to elicit ruffling in BMMs of either genotype (Fig. 3F). Finally, a monoclonal antibody directed against the extracellular portion of αv integrin, which, by clustering integrins, evokes signaling, yielded similar results (Fig. 3F). As with the ruffles seen in plated cells, Skap2 was concentrated at the leading edges of these integrin-induced actin structures (Supplementary Material, Video 2).
Manganese (Mn2+), which activates integrins non-selectively (Dransfield et al., 1992), enhanced the cytoskeletal response to control beads in Skap2-replete cells, but had no detectable additional effect on local integrin-mediated ruffling in response to pRGD-coated beads in either Skap2+/+ or Skap2−/− cells (Fig. 3G). Moreover, the requirement for Skap2 for integrin-induced actin ruffling was generalizable across several other α and β integrins found on macrophages (Fig. 3H). From these studies, we concluded that Skap2 plays a general and essential role in integrin-mediated actin rearrangements in BMMs.

**Skap2 structural domains determine integrin-induced signaling**

Structural and functional studies have shown that unstimulated Skap2 exists in an auto-inhibited state, wherein the pleckstrin-homology (PH) domain binds to a four-helix bundle created by the interaction of N-terminal dimerization domains (DM) of Skap2 monomers, and that this auto-inhibitory interaction is relieved upon phosphatidylinositol [3,4,5]-triphosphate (PIP3) binding (Swanson et al., 2008). To probe the role of specific Skap2 domains in regulating integrin-mediated cytoskeletal responses, we reconstituted Skap2−/− BMMs with Skap2 mutants that either disrupt the auto-inhibitory DM/PH domain interaction (D129K) or impair PIP3 binding (R140M) (Swanson et al., 2008). WT Skap2 rescued the deficient actin ruffling of Skap2−/− BMMs (Fig. 4A). However, expression of the R140M mutant in Skap2−/− BMMs failed to rescue integrin-induced ruffling (Fig. 4A). Furthermore, expression of this mutant, which dimerizes with normal Skap2 in Skap2-replete BMMs (Swanson et al., 2008), completely prevented the normal response in a dominant negative fashion (Fig. 4B). Preventing PIP3 production with the PI3K inhibitor GSK2126458 (Leung et al., 2011) also ablated the ruffling response (Fig. S3A). By contrast, superimposing the D129K mutation on R140M (D129K/R140M) led to hyperactive actin polymerization beyond the level generated by WT Skap2 (Fig. 4A), and even generated a significant response to control beads (likely through non-specific integrin interactions with polystyrene (Brown, 1991; Yakubenko et al., 2002)). This hyperactive response occurred despite the inability of this mutant to bind PIP3 (as a consequence of the R140M mutation), indicating that the auto-inhibitory DM/PH domain interaction is a critical regulator of Skap2 action. Consistent with this conclusion, mutation of D129K alone also evoked hyperactive actin polymerization (Fig. S3B). These data indicated that relief of auto-inhibition via PIP3 binding to the Skap2 PH domain is critical for Skap2 function in integrin-mediated cytoskeletal reorganization in BMMs; they further showed that the normal auto-
inhibitory interaction—between the Skap2 PH and DM domains—is required for regulating the integrin-driven actin response in these cells.

**Skap2 directs a subset of integrin-induced Sirpα-associated signaling events**

The observed defects in integrin mobility, cytoskeletal rearrangement, and migration in Skap2−/− BMMs led us to investigate the effect of Skap2 deficiency on integrin-stimulated signaling. BMMs were plated onto fibrinogen-coated surfaces in order to trigger integrin (primarily β2 and β3) activation. Plating-induced protein tyrosyl phosphorylation was globally decreased in the absence of Skap2, consistent with a defect in an early event in integrin signal transduction (Fig. 5A). Indeed, activation of SFK was abrogated in the absence of Skap2 (Fig. 5A). Although there was a mild decrement in the residual low level of SFK activation seen in suspended Skap2−/− cells compared with control cells, this did not appear to affect the basal integrin activation state (Fig. S2). Notably, Mn2+ restored global integrin-evoked tyrosyl phosphorylation as well as SFK activation in Skap2−/− BMMs (Fig. 5A); this is significant because Mn2+ was unable to restore integrin-induced actin ruffling in these cells (Fig. 3G). Therefore, the Mn2+-induced tyrosyl-phosphorylated proteins in Skap2-deficient cells likely represent participants in pathways distinct from those leading to the Skap2-dependent actin ruffling response. Likewise, whereas basal Syk phosphorylation was mildly increased in Skap2−/− cells, plating-induced tyrosyl phosphorylation of Syk, an established integrin effector required for cytoskeletal rearrangement (Vines et al., 2001), was not affected significantly by the absence of Skap2, and, like SFK activity, was amplified by Mn2+ treatment in both Skap2+/− and Skap2−/− cells (Fig. 5B). These results demonstrate that, while activated integrins can stimulate SFKs and Syk, even forced activation of these proteins with Mn2+ was insufficient to drive actin rearrangement in the absence of Skap2.

Adap, a known binding partner of Skap2, failed to become tyrosyl phosphorylated in response to integrin engagement in Skap2−/− BMMs; moreover, in contrast to the SFK and Syk results, Mn2+-induced Adap phosphorylation was minimal and failed to reach the levels of Adap phosphorylation found in control cells (Fig. 5C). Therefore, although Skap2 participates in inside-out integrin activation of SFKs, activation of a specific Skap2 signaling partner (Adap), along with downstream actin remodelling, is driven primarily through Skap2-mediated outside-in signaling. Similar results were obtained when BMMs were plated on fibronectin (not shown), which primarily engages β1 and β3 integrins, again indicating that Skap2 propagates signals downstream of multiple integrins.
We have shown previously that the transmembrane receptor Sirpα forms complexes with Skap2 and Pyk2 independently of Sirpα tyrosyl phosphorylation and Shp1/2 binding (Timms et al., 1999). Furthermore, inhibition of Sirpα function can lead to defects in cell migration (Inagaki et al., 2000; Motegi et al., 2003). Whereas both Sirpα and Sirpα-associated Pyk2 became tyrosyl phosphorylated in Skap2+/− BMMs plated on integrin ligands, plating-induced phosphorylation of Sirpα was minimal in Skap2−/− BMMs (Fig. 5D). By contrast, activation of Pyk2, another binding partner of Sirpα (Timms et al., 1999), was not affected by Skap2 deficiency. As with Adap, Sirpα phosphorylation, which occurs within the immunoreceptor tyrosine-based inhibitory motifs (ITIMs), was not salvaged by Mn²⁺ treatment of Skap2−/− cells (Fig. 5D), suggesting that this event, but not Pyk2 activation, is part of a Skap2-dependent outside-in pathway. This model is consistent with the finding that Skap2 and Pyk2 associate with separate pools of Sirpα (Timms et al., 1999), and with our observation that plating-induced activation of Akt, Erk, and Paxillin (all Pyk2 effectors) was not altered in Skap2−/− BMMs (Fig. 5E) (Blaukat et al., 1999; Williams and Ridley, 2000). Interestingly, Lyn−/− BMMs showed a similar defect in Sirpα phosphorylation with normal Pyk2 phosphorylation upon plating-induced integrin engagement (Fig. 5F, also see Fig. S4), implicating Lyn as the primary SFK involved downstream of Skap2 in pathways leading to Sirpα phosphorylation (Hibbs et al., 2002).

Sirpα also is necessary for the Skap2-dependent cytoskeletal response

Sirpα binds to Sirpα and directs its integrin-dependent phosphorylation, but does not control other aspects of integrin-dependent Sirpα signaling (see above). These results led us to further probe Sirpα’s role in integrin-induced cytoskeletal rearrangement. As in Skap2−/− cells, BMMs from mice homozygous for a Sirpα mutant lacking its cytoplasmic tail (SirpαΔ/Δ, (Inagaki et al., 2000)) exhibited deficient actin ruffling in response to integrin ligation by beads coated with either pRGD or α−αV (Fig. 6A). Like Skap2−/− BMMs, SirpαΔ/Δ BMM responses were similar to that seen for WT cells. Furthermore, just as in Skap2-deficient cells, the response to integrin ligation was not augmented significantly by Mn²⁺ in SirpαΔ/Δ BMMs (Fig. 6B). The normal response of SirpαΔ/Δ cells suggested that the mutant Sirpα fragment did not interfere with the function of endogenous wild-type Sirpα. To test this further, we identified two shRNA sequences that depleted Sirpα expression to approximately 25% of wild-type levels, as demonstrated by
immunofluorescence microscopy and by immunoblotting (Fig. 6C). Importantly, cytoskeletal rearrangements in response to bead-based integrin engagement exhibited similar impairment to that seen in the SirpαΔΔ BMMs (Fig. 6D). These results show that the Sirpα mutation behaves as a null allele for these integrin-dependent functions, and, along with the Skap2-dependent phosphorylation of Sirpα (Fig. 5D), support a cooperative mechanism between Skap2 and Sirpα in driving the actin response.

To test this possibility further, we quantified the recruitment of these signaling proteins to local sites of integrin ligation. Skap2 recruitment to integrin-directed beads was impaired in SirpαΔΔ cells; by contrast, Sirpα recruitment to engaged integrins was not significantly reduced in Skap2+/− BMMs (Fig. 7A). Indeed, in these cells, the Sirpα staining pattern showed small accumulations that were deficient in actin (Fig. 7A). As expected, Adap, which binds stochiometrically to Skap2, was not recruited to sites of integrin engagement in Skap2+/− BMMs (Fig. 7A). To further test the relationship between Sirpα and Skap2, we determined whether the constitutively active D129K/R140M variant of Skap2 could bypass the requirement for Sirpα to rescue the phenotype rendered by Sirpα deficiency. Unlike Skap2-deficient or Skap2-replete BMMs, SirpαΔΔ BMMs expressing D129K/R140M Skap2 could not mount a hyperactive cytoskeletal response to integrin stimulation (Fig. 7B). Together, these data demonstrate that Sirpα is required to recruit the Skap2/Adap complex to sites of integrin engagement and, in turn, that recruited Skap2/Adap directs both Sirpα ITIM phosphorylation and downstream actin polymerization.
This study uncovers a previously unrecognized signaling mechanism required for integrin-mediated signal transduction leading to cytoskeletal reorganization (Fig. 8). Specifically, we show that Skap2 and Sirpα, the latter previously regarded mainly as an inhibitory receptor, are both required for driving integrin-induced cytoskeletal reorganization in macrophages. Our results further indicate that these proteins act in concert—and in proximity to integrins—to transmit signals necessary for the early steps of a novel pathway required for cell migration. Notably, this pathway utilizes a distinct mechanism from IgG- and complement-mediated phagocytosis, which are not affected by the absence of Skap2 and are hyperactive in the absence of Sirpα (Okazawa et al., 2005; Togni et al., 2005). Whereas SFK activation is defective in plated Skap2−/− cells, forcing integrins into an active conformation (with Mn2+) stimulates both Syk and SFK activation. However, Mn2+ treatment does not restore Adap or Sirpα phosphorylation or integrin-stimulated actin ruffling. Therefore, Syk and SFKs are necessary (Vines et al., 2001), but not sufficient, for integrin-stimulated cytoskeletal rearrangement; their activation via integrin engagement does not obviate the need for Skap2 as a major early mediator downstream of PI3K but upstream of Adap and Sirpα phosphorylation. Skap2 signaling may also be involved in feedback regulation triggered by downstream events including branched actin formation (Coppolino et al., 2001); such a mechanism could be responsible for the Skap2-dependent, plating-induced SFK activation we observed.

Interestingly, Adap is regarded as a driver of inside-out signaling, such as that stimulated by antigen receptor engagement (Wang et al., 2009). However, in our experiments, β1 integrin activation is not dependent on Skap2, the major Adap binding protein. We also find that Mn2+, which promotes the general activation of integrins, thus bypassing inside-out pathways, is unable to drive normal actin cytoskeletal responses in Skap2−/− BMMs. In these experiments, Mn2+ treatment does restore the tyrosyl phosphorylation of selected proteins, consistent with rescue of early integrin functions. Therefore, while we do not exclude the possibility of subtle differences in inside-out signaling in Skap2−/− BMMs not detectable by our assays, defects in inside-out integrin signaling pathways are not likely the cause of defective actin ruffling in these cells. Rather, it is clear that additional, Skap2-dependent processes are required to couple early integrin-evoked phosphorylation events to downstream cytoskeletal rearrangements. We propose a model in which a key outside-in
integrin-stimulated signal requires the Sirpα/Skap2/Adap complex for actin remodeling in macrophages.

Skap2 is recruited to the plasma membrane by binding Sirpα through a ‘back-side’ interaction with its C-terminal SH3 domain (Timms et al., 1999). Accordingly, the PH domain is not required to localize Skap2 to the membrane. Instead, interaction between the PH and dimerization domains of Skap2 imposes auto-inhibition that is relieved upon PIP3 binding (Swanson et al., 2008). Consistent with these findings, we report that a Skap2 variant unable to bind PIP3 (R140M) fails to promote integrin-stimulated actin polymerization. Because functional Skap2 homodimerizes, the dominant-negative effect of this variant is likely due to the mutant Skap2 forming hemi-complexes with endogenous Skap2. However, when coupled with a mutation that perturbs the auto-inhibitory binding interface between the PH and dimerization domains, the resulting variant drives hyperactive actin polymerization in response to integrin ligation and in the absence of PIP3 binding. Taken together, these data place Skap2 as an early mediator — just downstream of PI3K but upstream of SFK, Adap and Sirpα — in pathways leading to integrin-driven actin cytoskeletal rearrangement, and also identify the key role of the PH domain in coupling Skap2 activation to phosphoinositide generation.

Prior studies of Skap2, which was discovered as a SFK-associated protein, have provided little mechanistic insight into Skap2 function, aside from its implication in adhesion/migration events (Black et al., 2000; Reinhold et al., 2009; Timms et al., 1999; Togni et al., 2005). The present work provides a mechanistic context in which Skap2 drives integrin-stimulated cytoskeletal rearrangement. As further support for Skap2’s role in normal macrophage biology, it is important to note that the Yersinia pestis virulence factor, YopH, targets the Skap2/ADAP complex for dephosphorylation in macrophages as part of its immunosuppressive strategy to interfere with normal adhesion-mediated signaling (Black et al., 2000).

Sirpα is a member of the so-called immune inhibitory receptor family. In addition to binding Skap2, it also binds Shp1/2, and many studies have focused on Shp1/2 interactions with Sirpα’s ITIMs to drive negative-regulatory signals (Fujioka et al., 1996). However, we find that both Sirpα and Skap2 are required to transmit signals from ligand-stimulated integrins and to promote local cytoskeletal rearrangement in macrophages. Although there is prior evidence that Sirpα ligation can stimulate nitric oxide pathways (Adams et al., 1998), our finding that Sirpα contributes to
integrin outside-in signaling comes as an unexpected and surprising finding, as it demonstrates that Sirpα acts not only as an inhibitory receptor but also as a facilitator of the Skap2-containing pathways downstream of integrin engagement, with significant impact on the cytoskeleton. The findings that Sirpα is recruited to sites of integrin ligation and that a constitutively hyperactive Skap2 mutant cannot salvage actin ruffling in SirpαΔ/Δ BMMs support a mechanism in which Skap2, Adap, and Sirpα cooperate in a single signaling module.

Our results have important implications for understanding the role of Skap2 in inflammatory disorders. For example, Skap2- and Sirpα-deficient mice are resistant to EAE, a mouse model for multiple sclerosis (MS) (Reinhold et al., 2009; Togni et al., 2005; Tomizawa et al., 2007). Both EAE and MS progression depend on antigen-presenting cells such as macrophages, and integrins are an established therapeutic target in MS (Ransohoff, 2007). Additionally, Sirpα-deficient mice are resistant to rheumatoid arthritis, which is partially macrophage-dependent (Okuzawa et al., 2008; Tanaka et al., 2008). We therefore suspect that both Skap2 and Sirpα play important roles in other settings of acute and chronic inflammation, particularly in highly integrin-dependent and macrophage-centric processes such as atherosclerosis and inflammatory bowel disease (Parikh et al., 2011; Rutgeerts et al., 2009; Travis et al., 2007). These roles, along with finer details of downstream signaling promoted by Sirpα and Skap2, are a focus of current and future investigation.

Finally, Sirpα is the receptor for CD47 (Jiang et al., 1999), which constitutes a “don’t eat me” signal on both healthy cells and malignant cells evading immune surveillance (Jaiswal et al., 2009; Oldenborg et al., 2001; Takenaka et al., 2007). Although this CD47-driven inhibition has been attributed to the ability of Sirpα to recruit Shp1 to down-regulate signals leading to phagocytosis (Oldenborg et al., 2001), our data lead us to speculate that, in addition, CD47 on opposing membranes may interfere with the Sirpα/Skap2 mechanism described here to alter the integrin-mediated cytoskeletal rearrangement necessary for the mechanics of phagocytosis. Therefore, along with the insights the present findings provide on mechanisms of inflammation, our results also could have implications for cancer biology and macrophage-mediated maintenance of homeostasis.

In summary, we have shown that integrin signaling in macrophages involves Sirpα acting via the Skap2/Adap adaptor molecules. Our study significantly changes the current view of integrin outside-in signal transduction in these cells and also provides new insights that could lead to the
identification of novel therapeutic targets to regulate both immune surveillance and inflammatory disease.
MATERIALS AND METHODS

**Antibodies and reagents.** Anti-pY416 Src, anti-pY118 Paxillin, and anti-pSirpα were obtained from Cell Signalling Technologies. Anti-Pyk2 was purchased from Santa Cruz. Anti-pY352 Syk was purchased from BioSource. 4G10 was a gift from Dr. Tom Roberts. Anti-Sirpα and Anti-Adap were purchased from Millipore. Anti-Skap2 was purchased from Proteintech Group. The following integrin antibodies were used in bead assays: RMV7 anti-αv and 9EG7 anti-β1 from BD Biosciences, R1-2 anti-α4, 5H10-27 anti-α5, M1-70 anti-αM, M18/2 anti-β2, and 2C9.G2 anti-β3 from Biolegend. pRGD and rat polyclonal IgG were purchased from Sigma-Aldrich.

**Mice, cell culture, and retroviral gene transduction.** Skap2−/− mice (Balb/c) were previously described (Togni et al., 2005), and were maintained under pathogen-free conditions and used at 8–12 weeks. BMMs were differentiated ex vivo as described (Tushinski et al., 1982), and analyzed after 7 days in culture. HEK293T/17 cells were maintained in 10% FCS and 10% CO2 at 37°C. Retroviruses were produced by co-transfecting pEcoPAK (Clonetech) and pMXs-Puro constructs bearing the inserts indicated in the figure legends into HEK293T/17 cells, using polyethylenimine (Polysciences) (Godbey et al., 2000; Swanson et al., 2008). For shRNA knockdown experiments, virus was produced in 296FT cells by co-transfection of the shRNA hairpin in Mission pLKO.1, pCMVD8.9 and p-CMV-VSV-G. shRNA vectors (Sigma-Aldrich) targeted either CTGTCTAACTTCATCCCGAGTT (shRNA 1) or TGGTTCAAAGATGGGCAAGAA (shRNA 2). Viruses were harvested 36 hours post-infection and used to infect bone marrow cultures, and cells were then differentiated into macrophages in the presence of 2 μM puromycin. All studies were approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center and Harvard Medical School.

**Cell migration assays.** For modified Boyden-chamber assays, BMMs were suspended in growth media and 5 X 10⁴ cells were placed in transwell chambers (Neuro Probe) with 8-μm pore size and the indicated concentrations of chemokine in the bottom chamber. For chemotaxis to M-CSF, cells were suspended in media containing 10% fetal calf serum. After four hours of incubation, cells were fixed in ethanol, the cells on the upper surface were removed, and the remaining cells on the underside of the membrane were stained with crystal violet and counted subsequent to photomicroscopy at 200X. For wound healing assays, BMMs were plated onto Petri dishes at confluence in growth media and then scratch wounds were introduced into the cultures. The
resulting wounds were photographed at 200X, then incubated at 37 C for eight hours and re-
photographed.

**Bead preparation and binding.** 10-µm diameter carboxylated polystyrene beads (Polysciences) were washed twice in PBS and mixed with 2 µg of antibody against the specific integrin, rat polyclonal IgG, human serum albumin, or polyRGD overnight at 4 C. This mixture was then washed twice with PBS and blocked with 1% albumin in PBS for 30 minutes. Non-confluent cells plated on No. 1.5 glass coverslips were placed on ice, and beads were added (~10 beads/cell) and allowed to settle onto the cell surface for 10 minutes before returning to 37 C for 20 minutes. For PI3K inhibition, 5 µg/ml GSK2126458 was incubated with cells for 30 minutes prior to bead binding. Cells were washed three times with PBS and then fixed and stained as described below.

**Biochemical and FACS analysis.** Cells were detached from polystyrene plates by incubation for 30 minutes in Versene (1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, 2.7 mM KCl, 120 mM NaCl, 1 mM glucose, 0.8 mM EDTA) at 4 C. Cells were harvested into DMEM (GibcoBRL), split into equivalent pools and kept in suspension for 2 hours with gentle agitation at room temperature. Equivalent numbers of cells were plated onto polystyrene plates coated with 10 µg/ml bovine fibrinogen for the indicated times and then lysed in Nonidet P-40 (NP-40) lysis buffer (2% NP-40, 100 mM Tris-HCl [pH 7.4], 300 mM NaCl, 200 µM perva
date, protease inhibitor cocktail [final concentrations, 10 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 µg/ml antipain]). A sample was also kept in suspension and collected by centrifugation and lysed in lysis buffer. Whole cell extracts were prepared by washing cells in phosphate buffered saline (PBS) and lysing them in NP-40 lysis buffer. Lysates were clarified in a microcentrifuge at 4 C for 10 minutes and protein concentrations in the resulting supernatant were determined using a bicinchoninic acid protein assay reagent kit (Pierce) or Bradford reagent (Biorad). Immunoprecipitations were performed by adding the indicated antibodies and protein A-Sepharose beads to lysates and incubating at 4 C for 2 hr. Immune complexes were washed with lysis buffer, resolved by SDS-PAGE, and transferred onto Immobilon-FL membranes (Millipore). Immunoblots were blocked with 5% BSA or 5% milk in TBS with 0.05% Triton X-100 (TBST) for 1 hr, incubated for 1 hr with primary antibodies in TBST, washed three times for 10 min each in TBST, then incubated for 1 hr with IR 680-labeled anti-mouse IgG (Invitrogen) or IR 800-labeled anti-rabbit IgG (Rockland, Gilbertsville, PA) and detected using the LiCor Odyssey fluorescence reader (Lincoln, NB). FACS analysis for basal
integrin expression was performed with the indicated antibodies, and at least 5000 cells per
condition were analyzed using FACSCalibur (BD Biosciences). Integrin activation was measured
by incubating suspended cells with antibody for 15 minutes at 37°C in the presence or absence of
1.5 nM PMA (Lenter et al., 1993), washing three times, labeling with anti-rat secondary antibody,
and performing FACS analysis.

Microscopy. Cells were fixed in 4% PFA, 25 mM PIPES pH 6.8, 129 mM KCl, 20% sucrose, 5
mM EDTA, and permeabilized in 0.05% Triton X-100 in PBS, pH 7.4. For immunofluorescence,
cells were stained with phalloidin (Invitrogen), conjugated to either Alexa488 or Alexa568, and
rabbit anti-Skap2 (Upstate-Millipore) in 1X PBS containing 3% BSA. Bound antibodies were
visualized using Alexa568-conjugated anti-rabbit IgG (Invitrogen) and observed under oil
immersion using a Zeiss Axiovert 200M microscope with a 63X Plan-Apochromat objective with a
numerical aperture of 1.4. Confocal images were obtained with a Yokogawa spinning disk on a
Nikon Ti inverted microscope equipped with a 60X Plan Apo NA 1.4 objective lens. Fluorescence
was excited by 488 nm and 568 nm lines from a 100 mW Melles Griot argon krypton laser. Z-
stacks of images 0.5 µm apart, centered at the bead equator, were acquired with a Hamamatsu
ORCA ER cooled CCD camera controlled with MetaMorph 7 software. Images were analyzed
using the iterative deconvolution program in the Axiovision 4.5 and Metamorph software packages.
Using the raw deconvolved images, actin ruffles were quantified in an 8 µm annulus around the
bead (excluding the bead itself) at the z-level with largest bead diameter (equator). Brightness and
contrast were adjusted on displayed images (identically for compared image sets) using MetaMorph
7 and Adobe Photoshop software.

Statistical testing of differences was performed using the 2-tailed Student’s t-test.
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AUTHOR CONTRIBUTIONS

F.J.A. and K.D.S. designed research, performed research, analyzed data, and wrote the paper. N.T.R. performed research and analyzed data. Q.J.B. and L.I.P. performed research, analyzed data, and assisted in writing the paper. C.A.L., D.E.G., and B.G.N. helped design research and assisted in writing the paper.

CONFLICTS OF INTEREST

There are no conflicts of interest to report.
REFERENCES


**FIGURE LEGENDS**

**Figure 1:** Skap2-deficient BMMs exhibit defective migration, spreading, and actin polymerization. (A) Scratches were introduced into confluent cultures of Skap2+/− or Skap2−/− BMMs (upper panels). After 8 hours (lower panels), migration across the scratch wounds was impaired in cells lacking Skap2. (B-D) Chemotaxis of Skap2+/− and Skap2−/− BMMs in response to cytokine M-CSF (B) and chemokines CCL2 (C) and CXCL4 (D) in transwell migration assays. In (D), “haplo” denotes haplotaxis conditions where equal concentrations of CXCL4 are in both chambers. For (B-D), data are presented as mean ± S.E.M., n = 3, * p < 0.01 compared to Skap2−/− under the same condition. (E) Skap2+/− or Skap2−/− BMMs were suspended in DMEM for 3 hours, plated for 30 minutes on glass coverslips, then fixed and stained for F-actin with rhodamine-labeled phalloidin. Scale bar = 10 µm. (F) Quantification of plating-induced spreading area from (E) in µm² (left) and relative fluorescence intensity per pixel for rhodamine-labeled phalloidin (right) for Skap2+/− and Skap2−/− BMMs. Data presented as mean ± S.E.M., n = 30, ** p < 0.001 compared to Skap2+/− BMMs.

**Figure 2:** Skap2 is not required for M-CSF-induced signaling and does not affect integrin expression in macrophages. (A) Adherent Skap2+/− and Skap2−/− BMMs treated with M-CSF for the indicated times were lysed, electrophoresed, and immunoblotted for phosphotyrosine (p-Tyr) and phosphorylated Akt, Erk2, and Src, with total Adap and Vinculin as loading controls. (B) Flow cytometric analysis was performed on Skap2-replete (WT, except Skap2+/− for αV and β1) or Skap2−/− BMMs using antibodies against the indicated cell surface integrins. (C) Skap2+/+ and Skap2+/− BMMs express equal amounts of Skap2 protein; Syk is a loading control. For all panels, representative results from 2 independent experiments are shown.

**Figure 3:** Skap2 is required for integrin-dependent actin cytoskeletal rearrangement. (A) Confocal micrograph of a typical phalloidin-stained Skap2+/− BMM plated on glass, demonstrating curvilinear actin ruffles. (B) Skap2−/− BMMs typically exhibit less actin ruffle formation. (C) At the plane of the nucleus (top panels, with blue nucleus in focus), a Skap2+/− BMM has minimal cortical actin (green) and cytosolic Skap2 (red), without significant colocalization (yellow, in merge); in contrast, at the apical surface (bottom panels), Skap2 colocalizes strongly with cortical actin ruffles. See also Supplementary Video 1. (D) Upon binding to a Skap2+/− BMM for 20 minutes, a polystyrene bead (denoted by dotted outline) coated with anti-αV integrin antibodies recruits actin ruffles (green).
colocalized with Skap2 (red). See also Supplementary Video 2. (E) This response is markedly reduced in Skap2\(^{-/-}\) BMMs. For (A-E), scale bar = 10 \(\mu\)m. (F) Quantified actin ruffling responses, measured as average pixel intensity in an 8-\(\mu\)m annulus surrounding beads coated with integrin-engaging or control proteins, are shown for BMMs of both genotypes. Data are normalized against the albumin/Skap2\(^{+/-}\) condition as Relative Actin Ruffling and presented as mean \(\pm\) S.E.M., n=10 per condition, * p < 0.05 compared to albumin-coated beads on the same cells, ** p < 0.01 compared to same beads on Skap2\(^{+/-}\) BMMs. (G) Quantified actin ruffling responses, induced by binding to polystyrene beads coated with either albumin or polyRGD, is shown for Skap2\(^{+/-}\) and Skap2\(^{-/-}\) BMMs with or without treatment with 1 mM Mn\(^{2+}\). Data are normalized against the Mn\(^{2+}/\)albumin/Skap2\(^{+/-}\) condition and presented as mean \(\pm\) S.E.M., n=10 per condition, * p < 0.05 compared to albumin-coated beads, no Mn\(^{2+}\), on the same cells. (H) Quantified actin ruffling responses to subtype-specific integrin-engaging beads are shown for BMMs of both genotypes. Data are normalized against the rat IgG/Skap2\(^{+/-}\) condition and presented as mean \(\pm\) S.E.M., n=10 per condition, * p < 0.01 compared to the same beads on Skap2\(^{-/-}\) BMMs.

**Figure 4:** Integrin-induced cytoskeletal reorganization requires a functional Skap2 PH domain. (A) Actin ruffling induced by beads coated with rat IgG (control) or anti-\(\alpha_c\) bound to Skap2\(^{-/-}\) BMMs infected with empty vector (pMXs), WT Skap2 (WT), or Skap2 mutants with an R140M substitution or with combined D129K and R140M substitutions. Data are presented as mean \(\pm\) S.E.M., n=10 per condition, * p < 0.01 compared to pMXs, ** p < 0.05 compared to pMXs. (B) Skap2\(^{-/-}\) and Skap2\(^{+/-}\) BMMs infected with empty vector (pMXs), WT Skap2 (WT), or Skap2 mutants with an R140M substitution or with combined D129K and R140M substitutions were bound to polystyrene beads coated with antibodies directed against \(\alpha_c\) integrin, and stained for actin (phalloidin) and for Skap2. n = 10 per condition. Scale bar = 10 \(\mu\)m.

**Figure 5:** Skap2 is required for specific integrin-induced signaling events. (A) Skap2\(^{+/-}\) and Skap2\(^{-/-}\) BMMs in suspension (S) or plated on fibrinogen for 20 or 60 minutes, either in the absence (left six lanes) or presence (right six lanes) of 1 mM Mn\(^{2+}\), were lysed, electrophoresed and immunoblotted for phosphotyrosine (p-Tyr; upper panel), Src pY416 (middle panel), and Adap as a loading control (lower panel). (B) Skap2\(^{-/-}\) and Skap2\(^{+/-}\) BMMs were kept in suspension or plated on fibrinogen for 30 minutes in the absence or presence of Mn\(^{2+}\), lysed, and immunoblotted for pSyk and vinculin as a loading control. Baseline quantified pSyk/vinculin is \(~\)2-fold higher in Skap2\(^{-/-}\).
cells. Plating increases levels ~2-fold and Mn$^{2+}$ increases levels ~5-fold in both genotypes. (C) 

$Skap2^{+/+}$ and $Skap2^{-/-}$ BMMs were kept in suspension or plated on fibrinogen for 30 minutes in the absence or presence of Mn$^{2+}$, lysed, immunoprecipitated for Adap, and immunoblotted for phosphotyrosine (pTyr; upper panel) and Adap (lower panel). Baseline quantified pTyr/Adap is ~ 5-fold higher in $Skap2^{-/-}$ cells. Plating increases levels ~5-fold in $Skap2^{+/+}$ and ~1.2-fold in $Skap2^{-/-}$ cells. Mn$^{2+}$ increases levels ~6-fold in $Skap2^{+/+}$ cells and 3-fold in $Skap2^{-/-}$ cells. (D) Sirp$\alpha$ was immunoprecipitated from lysates of BMMs in suspension (S) or after plating for the indicated times on fibrinogen, and phosphotyrosine (p-Tyr) was analyzed by immunoblotting (left panels), with prominent bands corresponding to Sirp$\alpha$ and Pyk2. Similar lysates of suspended, plated, or 1 mM Mn$^{2+}$-treated BMMs (right panels) were probed for Sirp$\alpha$ phosphorylation. (E) Akt, Erk, and Paxillin phosphorylation were probed in $Skap2^{+/+}$ and $Skap2^{-/-}$ BMMs in suspension (S) or after plating for the indicated times on fibrinogen. (F) BMMs from $Hck^{-/-}$, $Fgr^{-/-}$, and $Lyn^{-/-}$, as well as $Hck/Fgr$ double knockout ($HF^{-/-}$) and $Hck/Fgr/Lyn$ triple knockout ($HFL^{-/-}$) mice, were lysed after plating on fibrinogen, subjected to immunoprecipitation for Sirp$\alpha$, and immunoblotted for p-Tyr and Pyk2. All panels are representative of at least 3 independent experiments.

**Figure 6:** Integrin-induced cytoskeletal reorganization is also Sirp$\alpha$-dependent. (A) Actin ruffling induced by beads coated with albumin, rat polyclonal IgG, pRGD, or anti-$\alpha_v$ bound to WT, $Skap2^{-/-}$, Sirp$\alpha^{+/+}$, and Sirp$\alpha^{-/-}$ BMMs. (B) Actin ruffling induced by beads coated with albumin or pRGD with or without 1 mM Mn$^{2+}$. For (A-B), data are presented as mean ± S.E.M., n=10 per condition, * p < 0.01 compared to either homozygous mutant cell under same conditions. (C) Immunofluorescence staining for Sirp$\alpha$ in representative WT BMMs treated with scrambled vs. Sirp$\alpha$ shRNA, along with quantified relative staining intensity in untreated cells and cells treated with scrambled shRNA vs. the two different Sirp$\alpha$ shRNA. Inset shows Western analysis of cell lysates of the same cell populations, blotted for Sirp$\alpha$. (D) Immunofluorescence staining for actin and Sirp$\alpha$ in response to $\alpha_v$-directed beads on WT BMMs treated with indicated shRNA, along with quantified actin ruffling induced by these beads. Data are presented as mean ± S.E.M., n=10 per condition, * p < 0.05 compared to scrambled shRNA and untreated conditions.

**Figure 7:** Sirp$\alpha$ and Skap2 cooperate in response to integrin engagement. (A) Quantified Skap2 recruitment to $\alpha-\alpha_v$ beads bound to Sirp$\alpha^{+/+}$ and Sirp$\alpha^{-/-}$ BMMs, and Adap and Sirp$\alpha$ recruitment
to α–α₉ beads bound to Skap₂⁺/⁻ and Skap₂⁻/⁻ BMMs. Data are normalized to the respective heterozygous condition and presented as mean ± S.E.M., n=10-20 per condition, * p < 0.05. Representative beads, co-stained for actin, are shown. (B) Actin ruffling induced by beads coated with rat polyclonal IgG or anti-α₉, bound to Skap₂⁺/⁻ and SirpαΔ/Δ BMMs infected with empty vector (pMXs) or D129K/R140M Skap2. Data are presented as mean ± S.E.M., n=10-15 per condition; † p = 0.1 compared to SirpαΔ/Δ and p < 0.01 compared to pMXs under the same conditions; * p < 0.01 compared to SirpαΔ/Δ under the same conditions.

**Figure 8:** Skap2 and Sirpα in integrin signaling. A Sirpα/Skap2/Adap signaling module drives actin cytoskeleton reorganization downstream of integrin engagement in macrophages.
SUPPLEMENTARY MATERIAL

Figure S1: There are no significant changes in M-CSF-driven tyrosyl phosphorylation conferred by Skap2 deficiency, except for the lack of phosphorylation of Skap2 itself. Adherent Skap2+/− and Skap2−/− BMMs, with or without 5 minutes of M-CSF treatment, were lysed, and half of each lysate was subjected to Skap2 immunoprecipitation. The original lysate, the Skap2-depleted lysate, and the immunoprecipitate were electrophoresed and immunoblotted for phosphotyrosine (p-Tyr), Skap2, Adap, and Vav as a loading control. Skap2 is effectively immunodepleted, and removing Skap2 also depletes Adap from Skap2+/− cells. Note that the IgG heavy chain used for immunoprecipitation migrates at the same molecular weight as Skap2.

Figure S2: β1 integrin activation is not affected by Skap2. Skap2+/− and Skap2−/− BMMs in suspension were incubated for 15 minutes at 37 C with either activation-specific anti-β1 (9EG7) or non-selective anti-β1 antibody in the presence or absence of PMA. The cells were then subjected to FACS analysis for this antibody staining.

Figure S3: PI3K inhibition blocks, whereas D129K mutation promotes, integrin-induced actin ruffling. (A) Actin ruffling induced by beads coated with anti-αv bound to Skap2+/− and Skap2−/− BMMs with or without 30 minutes of pre-treatment with the PI3K inhibitor GSK2126458. Data are presented as mean ± S.E.M., n=10 per condition, * p < 0.05 compared to all other conditions. (B) Actin ruffling induced by beads coated with rat IgG (control) or anti-αv bound to Skap2−/− BMMs infected with empty vector (pMXs), WT Skap2 (WT), or Skap2 mutants with a D129K substitution or with combined D129K and R140M substitutions. Data are presented as mean ± S.E.M., n=10-20 per condition, * p < 0.05 compared to pMXs, ** p < 0.05 compared to WT.

Figure S4: BMMs from Hck−/−, Fgr−/−, and Lyn−/−, and Hck/Fgr/Lyn triple knockout (HFL−/−) mice were lysed after plating on fibrinogen, subjected to immunoprecipitation for Sirpα, and immunoblotted for p-Tyr and Sirpα, with ratiometric band quantification as indicated.

Video 1: Skap2 localizes to the ends of actin ruffles. (A) Fluorescence micrograph of a typical phalloidin- and Skap2-immunostained Skap2+/− BMM, demonstrating curvilinear actin ruffles
(green) with Skap2 (red) colocalization (yellow). (B) Video 3-D reconstruction from the region in the inset of (A) shows that Skap2 is localized at the ends of ruffles, with trails of actin extending away from it. The apical surface of the cell is at the top. (C-E) Skap2 antibody does not stain Skap2\(^{-/-}\) cells, providing evidence of its specificity (C – Skap2, D – actin, E – merge).

**Video 2:** Confocal micrographs of representative 10 µm-diameter beads bound to \(\alpha_\nu\) integrins. Cells are stained for actin (green) and Skap2 (red). In a Skap2\(^{+/+}\) BMM, actin ruffles emanate from the integrin-directed bead, and Skap2 is concentrated at their leading edges, as shown at the bead equator (A) and as a video 3-D reconstruction of the bead (B). Skap2\(^{-/+}\) cells do not have integrin-mediated actin ruffling, as shown at the bead equator (C – Skap2, D – actin, E – merge).
Figure 1

Panel A: Images of cells labeled Skap2^+/^- and Skap2^-/-.

Panel B: Bar graph showing Fluorescence/Pixel with M-CSF concentration. The graph shows a significant increase in fluorescence with increasing M-CSF concentration for Skap2^+/-. Black bars represent Skap2^+/-, white bars represent Skap2^-/-.

Panel C: Bar graph showing Cell Number (x10^2) for CCL2. Black bars represent Skap2^+/-, white bars represent Skap2^-/-.

Panel D: Bar graph showing Cell Number (x10^2) for CXCL4. Black bars represent Skap2^+/-, white bars represent Skap2^-/-.

Panel E: Images of cells labeled Skap2^+/^- and Skap2^-/- under fluorescence microscopy.

Panel F: Bar graph showing Area and Fluorescence/Pixel. The graph shows a significant difference in both Area and Fluorescence/Pixel for Skap2^+/-. Black bars represent Skap2^+/-, white bars represent Skap2^-/-.
Figure 2

A. Western blot analysis showing the expression of various proteins over time.

B. Flow cytometry data for Skap2 stained or unstained samples.

C. Western blot analysis of Skap2 and Syk in different genotypes.
Figure 3

A B

C

D E

F G H
Figure 4

A

![Graph showing relative actin ruffling](image)

- **pMXs**
- **WT**
- **R140M**
- **D129K/R140M**

**Y-axis:** Relative Actin Ruffling

- **X-axis:** rat IgG, α-αν

B

![Images of actin bead-level and whole cell](image)

- **pMXs**
- **WT**
- **R140M**
- **D129K/R140M**

**Legend:**
- Skap2 +/−
- Skap2 −/−
- Bead-level
- Whole cell

**Actin:**
- **Skap2**
- **Actin**

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**Figure 5**

A. Western blots showing p-Tyr and pY416-Src levels in Skap2+/+ and Skap2−/− cells with and without 1 mM Mn2+.

B. Western blots showing pSyk and vinculin levels in Skap2+/+ and Skap2−/− cells with and without Mn2+.

C. Western blots showing p-Tyr and Adap levels in Skap2+/+ and Skap2−/− cells with and without Mn2+.

D. Western blots showing p-Tyr and Sirpα levels in Skap2+/+ and Skap2−/− cells with and without Mn2+.

E. Western blots showing pAkt, pErk, pY118-Paxillin, and vinculin levels in Skap2+/+ and Skap2−/− cells with and without Mn2+.

F. Western blots showing p-Tyr and Sirpα levels in Pyk2+/+ and Pyk2−/− cells with and without Mn2+.

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Figure 6

(A) Relative Actin Ruffling

- WT
- Skap2⁻/⁻
- Sirpα⁺/Δ
- Sirpα⁻/Δ

albumin  rat IgG  pRGD  αν

(B) Relative Actin Ruffling

- WT
- Skap2⁻/⁻
- Sirpα⁺/Δ
- Sirpα⁻/Δ

albumin  albumin + Mn  pRGD  pRGD + Mn

(C) Relative Sirpα Staining Intensity

no shRNA  scrambled  Sirpα shRNA 1  Sirpα shRNA 2

Sirpα

(D) Relative Actin Ruffling

- rat IgG
- αν

no shRNA  scrambled  Sirpα shRNA 1  Sirpα shRNA 2

Sirpα

Scrambled shRNA

Sirpα shRNA 1
Figure 7

A

![Bar charts showing relative actin ruffling for different genotypes of Sirpα, Skap2, and Adap.](image)

B

![Graph showing relative actin ruffling with different conditions.](image)
Figure 8

The diagram illustrates the interaction between ECM (extracellular matrix) and integrin, with key molecules such as Skap2, Adap, Shp1/2, and Syk involved. This interaction leads to actin rearrangement.