April 3rd, 2006

Dr. Elaine Fuchs
Editor
The Journal of Cell Biology
The Rockefeller University Press
1114 First Avenue, 3rd Floor
New York, NY 10021-8325
USA

Re: JCB #200511027

Dear Dr. Fuchs,

We have considered the comments of the reviewers of our manuscript entitled "**Rhamm -/- mice are defective in skin wound repair due to aberrant ERK1,2 signaling in fibroblast migration"** and are resubmitting a revised manuscript that takes into consideration the helpful comments of reviewers. We have enclosed our responses to each of the reviewer's critique.

Thank you for your consideration.

Best Regards,

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Response to Reviewer's Critique:

Reviewer #1

Comment: The paper is hard to read. It is the terminology/style used and also the content. For example, on page 11 top paragraph they refer to the stress fibers being "remodeled" but one who is not an expert in stress fibers must look at the figure to understand what they mean. Likewise in that same paragraph, they state that actin stress fiber remodeling was "much more restricted". This terminology is not easy for a general audience to understand. Many of the paragraphs, such as the first one in the Introduction contain many different facts and long sentences with multiple points being made.

Response: We have re-written most of the manuscript to present concepts and data in a more straightforward and simplified manner. In the text, we have replaced ambiguous descriptions or jargon (e.g."actin remodeling", "much more restricted") with what we hope is clearer language (e.g. "disassembly of actin stress fibers" and "reduced"). We have also quantified the disassembly of actin stress fibers using image analysis to reduce ambiguity about the changes that Rhamm loss has on the actin cytoskeleton organization.

Comment: The authors comment on reduced clot formation (in the Abstract and Results on page 5) but do not indicate how it relates to their findings on cell migration. Also they claim reduced clot formation and show it nicely in the supplemental figure but in figure 1A clot formation looks increased in the KO cells. A more representative figure should be shown if this was their general finding. Unless they have more quantitative information and a proposed mechanism relative to their findings in this paper, they may want to omit this information.

Response: We agree with Reviewer 1's comments that the data on clot formation shown in the original manuscript were not well documented and were peripheral to the main focus of the manuscript on cell migration. We have omitted these data and descriptions in the revised manuscript.

Comment: Figure 1B. The number on the panel should be 2.50 not 2.25.

Response: This graph has been removed and Figure 1 completely modified in deference to comments by several of the reviewers.

Comment: Figure 3B. The Rh-/- in the upper and lower panel should be similar but they appear very differently. Some comment is needed.

Response: This point was a concern of all of the reviewers and was partly due to the inclusion of two types of ERK1,2 activators (PDGF and serum) in one figure. To address this, we have made the following modifications. Figure 3 now contains only the histochemical staining for active ERK1,2. Figure 4 shows a more comprehensive *in vitro* analysis of ERK1,2 activation in fibroblasts responding to one type of stimulus (serum). *In vitro* data showing ERK1,2 activation in response to PDGF have been placed in a new Supplemental Figure (III).

The reviewers concerns also brought up an important point about the semi-quantitative nature of the western blot assays previously shown in Fig. 3B. We have therefore now included results of ELISA assays that quantify ERK1,2 activation in

cultured cells. These data are presented as Figure 4A. We have now used our western blot analyses to re-quantify ERK1 activation in Rh-/- and Rh-rescued fibroblasts (new Fig. 4B). We performed new confocal analyses of ERK1,2 activation Rh-/- and Rh-rescued fibroblasts in response to serum (instead of PDGF). We quantified ERK1,2 activation in response to PDGF and this is now presented as a graph in Supplemental Fig. IIIb. We believe that these changes now clearly and consistently show a similar pattern for aberrant ERK1,2 activation in Rh-/- vs. Rh-rescued (or Wt) fibroblasts.

Comment: *Figure 5. They should omit the diagrams. The paper has so many bar graphs so they could put these data in a table.*
Response: We agree with the reviewer that this figure should be simplified. However, we feel strongly that the timelapsed images of the wounds provide data that is difficult to convey as a table. We have therefore removed Figure 5A (depicting wildtype and Rhamm-/- fibroblasts) and now include migration of Rh-/- and Rh-rescued fibroblasts in wound gap migration assays as well as collagen gel assays. Data showing the migration and invasion of primary Wt and Rh-/- fibroblasts have been moved to a new Supplemental Figure IV (a,b).

Comment: *Figure 6 would greatly benefit from photos of the cells for the elongation and roundness claims. Invasion is usually associated with protease activity. Has protease activity been examined?*
Response: Reviewer 3 also asked for clarification of this Figure. We have deleted Figure 6B since Reviewer 3 did not find the assay compelling, and as noted above included the collagen gel assay in Figure 5. We agree that assessing protease activity is an interesting avenue to pursue. However, given the complexity of this field, we feel that inclusion of such data is beyond the scope of the present manuscript.

Comment: *Figure 7. Is HA in serum? Some comment is needed.*
Response: The reviewer is of course correct. Hyaluronan is present in serum at a concentration of <10 pg/L. In our experience, this concentration is not sufficient to stimulate cell motility nor, to our knowledge, has this concentration of HA been reported to promote motility. We therefore consider it unlikely that serum HA contributes to the motility response to fetal calf serum. For quantifying the effect of HA, Fibroblast cultures were serum-starved prior to exposure to exogenous HA.

Comment: *Figure 8A. The symbols need to be larger as this figure is hard to read. Part B would make a better table.*
Response: Figure 8 has been revised to accommodate the other reviewers' comments. We have also made the graphs of ERK1,2 activity larger for clarity.

Comment: *In the Methods section, they should indicate how many times each experiment was done and the number of replicates. This is especially important for the wound healing in vivo.*
Response: We have now included this information both in the Methods section.

Reviewer #2

Comment 1a: *While the authors have used a lot of statistical analysis to show the defects in wound healing in the KO animals, the pictures are not of high quality. It is difficult to interpret what is being stated, and it seems rather subjective.*
Response: This is also a concern of other reviewers. We have therefore replaced the images shown in Figure 1B as a new Figure 1A. This shows a collage of sections of the wound site that has been stained for tenascin so that granulation tissue can be easily observed. We have quantified the area of tenascin positive granulation tissue in Wt and Rh-/- wounds and present these data as Figure 1B. We repeated this experimental series using sections 2 and 6 of serial sections starting at the wound center to ensure that the same wound position was used for both Wt and Rhamm-/- samples. We have omitted the data the reviewer is concerned about (previous Figure 1B and 2A).
For example the higher magnification image of the Rh-/- mice in fig 2B (should be Figure *1B?) seems to be of the lower part of the skin, where the fat pad is (as we can see a lot of vacuoles).*
Response: Rhamm-/- wounds aberrantly accumulate adipocytes within granulation tissue accounting for the large number of vacuolated cells depicted in the previous Figure 1B. We have retained this micrograph, as it is representative of the appearance of Rhamm-/- granulation tissue (now Figure 2A) but have provided additional data showing that Rhamm-/- granulation tissue contains adipocytes (new Figure 2C).

The authors state that the fibroblasts are less dense in the KO compared to the WT, but this is not reflected in the pictures shown, in fact, there are areas in the WT shaded area of the wound that look less dense (on the left hand side of the wound).
Response: We agree with the reviewer that Rhamm-/- wounds exhibit areas of high fibroblast density as well as areas of lower fibroblast density, and this micrograph (now Figure 2A) is representative of this heterogeneity. The quantification of fibroblast density (now Figure 2A) is based on number of fibroblasts per granulation tissue area in each field of view and therefore represents an average value (N= 4 sections from 8 animals for each genotype now described in the new Figure 2 legend). However, we did not note the heterogeneity in the previous manuscript and thank the reviewer for making this point. We have now included this in the new manuscript in the Figure 2 legend.

Comment 1b: *The authors should show the resolution of the actual wound on the back of the animals, at different times to give a better picture of how these wounds heal.*
Response: We have repeated the wounding experiments and have taken photographs of wound sites on the animal's backs from day 0 to day 7. These photographs shown as Supplemental Figure Ib and the wound area quantified from these experiments are presented in a lower panel of this Figure.
The pictures that are presented in figure 2 and supplementary fig1 do not convincingly represent the defects that have been described in the text.
Response: As noted above, we have repeated the wounding experiments and stained granulation tissue with tenascin. Using this method, we are confident that the data now presented as Figure 1A,B and Supplemental Figure Ia are representative of, and accurately quantify the defective wound repair of Rhamm-/- mice that we describe in this manuscript.

Comment 1c: *The authors should use overlapping pictures to show the extent of the wound. The use of a molecular marker, like αv or ß6 integrin to delineate wound edge would also be helpful.*
Response: As requested, we have presented wound sites as overlapping pictures (Figure 1A). We have replaced data quantifying granulation tissue formation/resolution from previous images with data using tenascin-positive areas to quantify granulation tissue formation/resolution (Figure 1A). We originally used Masson's trichrome staining to detect wound bed edges for quantifying wound contraction (now Supplemental Figure Ic). These wound contraction data supplement those shown in the wound photographs (Supplemental Figure Ib) and are now presented as Supplemental Figure Ic.

Comment 1d: *What is the brown staining that is seen in the sections in figure 1?*
Response: Sections were stained for vimentin. Only the higher magnification micrographs have been retained in the present manuscript.

Comment 2a: *In figure 3 the section of wounded 3d Rh-/- animal again have a lot of vacuoles in them.*
Response: As noted above, these are adipocytes, which accumulate to a greater extent in Rh-/- vs. Wt wound sites.
The 7d KO wound looks very washed out, and there seem to be fewer nuclei, which is at odds with the data that Rh-/- wounds have more cells per field compared to WT wounds both at 3d and 7d wounds.
Response: Panel day 7 Rh-/- in Figure 3 has been replaced with an image where cell nuclei were more clearly counter stained. The fibroblast density in day 7 Rh-/- wounds (Figure 3) is lower than WT and this is consistent with both the previous and new micrograph of day 7 Rh-/- granulation tissue.

Comment 2b: *What is the correlation between the low levels of ERK expression and the miscuing of granulation tissue formation?*
Response: Both Wt and Rh-/- granulation tissue fibroblasts *in vivo* ("unpublished data") and Rh-/- and Rh^{FL}-rescued fibroblasts *in vitro* express similar levels of ERK1,2 protein (Figure 4A,B). Activation of ERK1,2 is less sustained in Rh-/- fibroblasts. This low level of activation is associated *in vivo* with sparse fibroblast density and abnormal differentiation, and with reduced migration *in vitro*. This correlation is predicted by published reports noting an effect of ERK1,2 on migration, adipogenesis and myofibroblast differentiation. This relationship is noted on page 12 of the Discussion section in the revised manuscript.

Comment 3: *In figure 4 the confocal data looking at the activation of phospho-ERK after PDGF stimulation does not correlate with the western blot analysis in figure 3B. According to the blots, the Rh-/- cells show sustained activation for 30' after stimulation, and only then does it drop off. How do the authors reconcile this discrepancy?*
Response: We agree with the reviewer that the confocal images did not reflect the western blot data shown in the previous Figure 3B. As per our comments to Reviewer 1, we have repeated all of the ERK1,2 activation data in Rh-/- and Wt or Rh^{FL}-rescued fibroblasts and these are now presented as Figure 4. We have concentrated on presenting

activation of these MAP kinases in response to serum to improve data clarity and deleted or moved data showing responses to PDGF-BB to the supplemental figures. We have deleted confocal images in the previous Figure 4 (which showed ERK1,2 activation in response to PDGF-BB) and replaced these with a panel of confocal micrographs showing ERK1,2 activation in response to serum (Figure 4C).

Comment 4: With the scratch wound assays (figure 5), it is not clear to this reviewer, what the red and black squiggly lines represent. It would be simpler to show images from the time-lapse movies that the authors have done to show the defective migration.
Response: The lines represent the migration trajectories of individual cells. We feel that this provides important information discussed in the Results section (pages 8 and 9) that is difficult for readers to distill from either static or timelapsed images.
Also it does not seem surprising that the Rh-/- cells have a migration defect, given that it has been shown by these authors that "HA:Rhamm interactions promote cell locomotion via a protein tyrosine kinase signal transduction pathway that targets focal adhesions" and that "tyrosine kinase pp60c-src is associated with Rhamm in cells and is required for Rhamm mediated cell motility" (Hall and Turley 1995).
Response: We agree that our results showing an effect of Rhamm loss on cell migration is predicted by previous data from cultured cells. However, *in vitro* analyses often do not predict *in vivo* functions and our previous studies also did not address whether other genes (e.g. CD44) can compensate for Rhamm in regulating migration and whether Rhamm-regulated migration is involved in a physiological process. We had expected CD44 to compensate for Rhamm loss since we have previously published that these receptors have overlapping functions and that Rhamm can compensate for CD44 to mediate migration and invasion during disease processes such as arthritis (Nedvetski et al., 2004, Proc. Natl. Acad. Sci. U.S.A.101:18081). Our results suggesting that Rhamm is involved in regulating fibroblast migration during the physiological process of wound repair is therefore noteworthy. These points of view are now emphasized in the Discussion section (page 13).
There is no attempt to address the mechanism for changes in cell migration. What is the correlation between Rhamm and MAP Kinase signaling? We apologize for this omission.
Response: Our results in the present manuscript showing an effect of Rhamm on MAP kinase signaling are consistent with our and other's previous data providing evidence that Rhamm can act at the level of receptor protein tyrosine kinases to activate upstream effectors of ERK1,2 including src, and Ras. We have now included these important points in the Discussion section of the revised manuscript (page 12).

Comment 5: The authors' state in the text on page 9 that migration defect was displayed by primary dermal and "other" cells. What are the other "type of fibroblasts" that show the migration defect?
Response: In this study, we used primary embryonic fibroblasts (MEF), immortalized embryonic fibroblasts (which were also transfected with full length Rhamm and/or mutant active Mek1), and primary dermal fibroblasts from littermatched Wt and Rh-/- mice. We apologize for the poor documentation of this in the previous manuscript. We have attempted to make the origin of the fibroblasts used for each experiment more clear

by presenting the data using immortalized Rh-/- and Rh^{FL}-rescued fibroblasts in text Figures while retaining the data using primary Wt and Rh-/- MEF in the Supplemental Figures.

Comment: *The fact the rescue of Rh-/- phenotype was done using immortalized cells was only indicated in the figure legends and not in the text. It is not clear if these rescued cells are being compared to primary cells from the KO mice or with transformed KO cells in the migration assays.*
Response: We compared only primary Wt with primary KO fibroblasts and immortalized KO with Rh^{FL}-rescued KO fibroblasts. We have clarified this and apologize for the lack of labeling.

Comment: *Why was the rescue not done using the primary cells?*
Response: The transfection efficiency of primary fibroblasts is very low (<10%) and precluded their use in many analyses presented in this manuscript. We deliberately isolated immortalized Rh-/- and Rh^{FL}-rescued fibroblast lines to circumvent this difficulty since these are much more easily transfect (transfection efficiency with lipofectamine Plus is 60-70%).

Comment: *Do the transformed KO and rescued cells have the same response in the migration and invasion assays as the primary WT and KO cells?*
Response: The cell lines we used are not transformed but rather immortalized (they do not grow in soft agar or form foci in focus forming assays that would indicate transformation). Both cell lines exhibit similar phenotypes although the rates of migration, for example, differ slightly. We have separated these data as noted above to prevent reader confusion.

Comment: *If not these two cell types should not be compared.*
Response: We agree it would never be appropriate to compare primary to immortalized lines.
Minor Comment: *It would be good to see a picture of non-wounded WT and KO skin for comparison.*
Response: We agree that this is informative and have now included micrographs of uninjured skin as a new Supplemental Figure IIa.

Reviewer #3

Comment 1: *P. 7 and Discussion p. 13: Neither proliferation nor apoptosis in granulation tissue of Rh-/- mice was significantly different then Wt granulation tissue despite difference in ERK 1, 2 expression (*Figure *3A). The author point out (p 13) that they previously reported that "Rhamm regulates cell proliferation in both fibroblasts and fibromatosis cell under conditions of low density, but not high density." As presented, this sounds like a correction. If it is not a correction, then additional comments should be made to explain or justify the differences in results.*
Response: This comment was not meant as a correction of previous results and we thank the reviewer for pointing this out. We have modified the discussion to include possible

reasons for the discrepancy in our present results obtained *in vivo* vs. these previous results that were obtained *in vitro* (Discussion, second paragraph, page 11).
For example, does Rhamm have effects on expression of p27 that regulates both migration and proliferation.
Response: This is a very interesting suggestion that we will certainly consider for future experiments. However, we feel that this is beyond the scope of the present manuscript.

Comment 2: Figure *1 B and Supplement* Figure *1*, Figure *2A. These figures are inadequate to explain how the areas of the granulation tissue were established. This is particularly true since the area of the granulation tissue differs at the center and edge of the wound. The data as presented does not make it clear how the authors arrived at their numbers. Do markers of dermal remodeling (thrombospondin, Tenascin) help in identifying the granulation tissue for the reader?*

Response: We agree with the reviewer. We have redone the wounding experiments and have restricted our analyses to the wound center, which is most easily identified. We also matched age (18 months), gender (male) and wound placement (left flank) to facilitate data interpretation. We also thank the reviewer for the excellent suggestion to use tenascin as a marker for granulation tissue. We have now used tenascin-staining to measure granulation tissue boundaries in Wt and Rh-/- wounds. These new data are presented in a new Figure 1A,B.

Comment: Do differences in migration of the fibroblasts that are described in the rest of the manuscript account for the differences in granulation tissue?
Response: This is an important point that we have addressed indirectly in this manuscript in the sense that we did not observe differences in either proliferation or apoptosis *in vivo* leaving migration as a reasonable alternative. Defective migration is consistent with our *in vitro* data and with the differences in ERK1,2 activity observed both *in vivo* and *in vitro*. We are currently using knock-in approaches and *ex vivo* image analysis to directly address this point.

Comment: Where does Rhamm localize in the granulation tissue of the WT?
Response: We (Savani et al., 1995 Proc West Pharmacol Soc 38:131), and others (Lovoorn et al., 1998, J Pediatr Surg 33:1062), have previously published that Rhamm is expressed in granulation tissue fibroblasts as well as in white cells present in granulation tissue.

Comment: Figure *3. It is not clear from* Figure *3A that ERK1,2 expression is different in the Wt and Rh-/- tissue.*
Response: The figure shows ERK1,2 activity and not total ERK1,2 expression. Quantification of phospho-ERK1,2 was done using image analysis of 15 separate areas in 3 serial tissue sections/mouse used for the experiments. The values indicate significant differences in phospho-ERK1,2 activity although no detectable differences in ERK1,2 protein expression were detected in parallel sections stained for total ERK1,2 protein (data not shown). We have attempted to clarify this in the Figure legend (Figure 3 in revised manuscript).

Comment: What happened to panel Day 7 Rh-/-, it looks bleached out?
Response: We thank the reviewer for noting this. This panel has been replaced with one where the counter staining for the nucleus was similar to that of the other images in the panel (now Figure 3).

Comment: Figure *3B, published studies have shown that adhesion via integrins activates ERK. Does the contribution of Rhamm to ERK activation require adhesion via integrins?*
Response: We agree with the reviewer that this is an interesting and appropriate question given our data showing a role for Rhamm in focal adhesion turnover and FAK de-phosphorylation. Therefore part of our future research focus is directed towards identifying the pools of ERK1 that are affected by Rhamm including those in focal adhesions. However, this is a complex field and we feel is beyond the scope of the present manuscript.

Comment: Do differences in concentration of PDGF-BB compensate for the reported differences in ratios of P-ERK/total ERK (Figure 3B). This would suggest that Rhamm may increase the relative concentration of PDGF.
Response: This is again an interesting question but complicated to address using PDGF-BB as a stimulus. The highest PDGF-BB concentration we used was 50ng/ml and the lowest concentration was 2.5ng/ml. 25-50ng/ml of PDGF-BB are typically motogenic for fibroblasts while smaller concentrations are mitogenic. We have attempted to address the reviewers question using concentrations of PDGF-BB that vary between 20-50ng/ml. We did not observe significant differences in stimulation but feel that other stimuli might be more appropriately used for these experiments. Since this will require further in depth analyses, we have not included these data in the present manuscript but thank the reviewer for this excellent suggestion.

Comment: Figure *6B. Reporting the invasion of the fibroblasts as "Elongation" or "Roundness" factors is not adequate. Did the cells invade or not and how far?*
Response: We agree with the reviewer's opinion of using "roundness" as a measure of invasion and have deleted this from the manuscript. We have retained the collagen gel analysis where we measure the number of fibroblasts that migrate into the central "plug" containing collagen/PDGF-BB/HA (new Figure 5B).

Comment: Figure *7C. The reported differences in FAs are not apparent from the panels present.*
Response: We agree and have removed these data from the manuscript. We have retained the micrographs showing differences in Rh-/- vs. Rh^{FL}-rescued actin stress fibers, and have quantified this difference (new Figure 7) to provide evidence that Rhamm is required for disassembly of the actin cytoskeleton.

Rhamm-/- mice are defective in skin wound repair due to aberrant ERK1,2 signaling in fibroblast migration.

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Condensed title: Repair of skin wounds is aberrant in Rhamm-/- mice

Key words: hyaluronan, Rhamm, ERK1, fibroplasia, wound repair

Number of characters (not including Materials and Methods): 48568

Number of characters in Materials and Methods: 8549

Number of words in Abstract: 150

ABSTRACT

Rhamm is a hyaluronan (HA) binding protein with limited expression in normal tissues and high expression in advanced cancers. Here we have identified a physiological function for Rhamm during excisional skin wound repair. Genetic deletion of Rhamm impairs wound contraction and granulation tissue formation/resolution. This is associated with enhanced inflammation, sparse fibroplasia and aberrant differentiation as indicated by reduced myofibroblast conversion and increased adipocyte accumulation. Sparse fibroplasia is associated with an inability of Rhamm-/- (Rh-/-) fibroblasts to resurface large (>3mm) scratch wounds or invade HA-supplemented ECM gels to the extent of wild-type or Rhamm-rescued fibroblasts. These effects result from altered activation kinetics and subcellular targeting of ERK1,2 since stable introduction of constitutively active Mek1 rescues these defects in Rh-/- fibroblasts. Our results identify Rhamm as a fibrogenic factor required for appropriate cueing of migration/differentiation necessary for repair, and as an essential regulator of ERK1,2 motogenic-signaling pathways required for wound repair.

INTRODUCTION

Rhamm is a HA-binding protein that is either not expressed or expressed at low levels in normal adult tissues but is highly expressed in aggressive human tumors (Adamia et al., 2005; Tammi et al., 2002; Toole, 2004). Analyses of animal models have confirmed instructive roles for Rhamm in tumorigenesis and in other disease processes such as arthritis. Roles for Rhamm in these diseases are consistent with its well-documented *in vitro* functions in migration and proliferation/apoptosis (Turley et al., 2002). Since migration and proliferation/apoptosis are essential functions for morphogenesis and tissue homeostasis, it is surprising that genetic deletion of Rhamm does not affect embryogenesis or adult homeostasis. Indeed, to date, a physiological function for Rhamm has remained elusive.

Rhamm was originally isolated from subconfluent migrating fibroblasts (Turley, 1982) and subsequently cloned from mesenchymal progenitor cells (Hardwick et al., 1992). Antibodies prepared against a shed form of Rhamm block HA-stimulated-fibroblast motility, suggesting that Rhamm is a cell surface protein that transduces motogenic signaling pathways *in culture* (Turley et al., 2002). Rhamm-bound HA is detected in cancer cell lines (Adamia et al., 2005) and shown to exist also in intracellular compartments/structures including the actin and microtubule cytoskeletons, nucleus and cytoplasm (Adamia et al., 2005). These results suggest that Rhamm has extracellular and intracellular functions. However, whether or not Rhamm acts as a cell surface receptor for HA became controversial partly because cloning of the human (Crainie et al., 1999; Hofmann et al., 1998; Wang et al., 1996) and mouse genes (Hofmann et al., 1998) revealed an absence of both a signal peptide required for export through the golgi/ER and membrane spanning domain(s) common to most cell surface receptors. In this and other characteristics, Rhamm resembles a group of intracellular proteins (e.g. epimorphin/syntaxin-2, autocrine motility factor/phosphoglucose isomerase) that also lack these signature characteristics of cell membrane receptors but which are nevertheless found at the cell surface and transmit signals across the cell membrane to regulate a number of cellular functions (Radisky et al., 2003).

We have shown that Rhamm expression is high in aggressive fibromatoses (desmoid) tumors (Tolg et al., 2003). We further demonstrated that genetic deletion of Rhamm strongly reduced desmoid tumor initiation and invasion in a mutant APC and β-catenin-driven mouse model of this mesenchymal tumor. Fibroproliferative processes such as aggressive fibromatosis

resemble proliferative/migratory stages of wound healing (Cheon et al., 2002). The expression of Rhamm is modulated during wounding (Lovoorn et al., 1998) and by fibrogenic cytokines such as TGF-β (Samuel et al., 1993). Since factors that regulate fibroblast function play dual roles in wound repair and tumorigenesis (Bissell, 2001; Park et al., 2000), we have assessed in the current study whether Rhamm is involved in repair of excisional skin wounds using Rh-/- mice. The results show that Rhamm loss results in defects in early phases of skin repair, in particular in granulation tissue formation and resolution. This defect is associated with impaired migration/motility of fibroblasts and is due to aberrant kinetics of ERK1,2 activation/subcellular targeting.

RESULTS

Rhamm expression is required for granulation tissue formation and resolution in skin wounds.

Rhamm expression increases during repair of excisional wounds on human skin xenografts in immune compromised mice (Lovvorn et al., 1998) and following scratch wound of smooth muscle cell monolayers *in culture* (Savani et al., 1995). In the present study, Rhamm expression was followed during the first 7 days after excisional wounding of mouse skin. RT-PCR analysis of wounds showed that Rhamm expression was low in uninjured skin (Suppl. Fig. Ia). A marked increase in Rhamm mRNA was obvious one day after injury and expression was increased until day 3 when mRNA levels began to drop. By day 7 Rhamm mRNA levels were only slightly higher than those observed in uninjured skin. These results indicate that Rhamm is expressed during the early stages of excisional skin wound repair, which include wound contraction, re-epithelialization and granulation tissue formation. We therefore next assessed the consequences of Rhamm loss to the integrity of these early processes by photographing wound sites and by analyzing serial cross sections cut through wound centers.

Wt and Rh-/- wounds both contracted by day 1-3 after injury, but contraction of day 3 Rh-/- wounds was significantly reduced compared to Wt wounds (Suppl. Fig. Ib). Differences in wound contraction were not detected at later times when wound areas were measured from photographs (Suppl. Fig. Ib). By day 14, Wt and Rh-/- wound sites both appeared resolved at the macroscopic level ("unpublished data"). However, when the distance between wound edges was measured using tissue sections cut through wound centers, significant reductions in the contraction of Rh-/- wounds could be detected at days 1 and 3 but also at day 13 (Suppl. Fig. Ic). Since granulation tissue myofibroblasts contribute to wound contraction, since loss of Rhamm results in a significant decrease in the thickness of the dermis before injury and since resolution of Rh-/- dermis was delayed, as indicated by continued fibroplasia and reduced differentiation of dermal structures (day 21, Suppl. Fig. IIa,b), we next focused upon the consequences of Rhamm loss on granulation tissue formation/resolution.

A temporal spatial defect in the formation and resolution of granulation tissue was observed in Rh-/- vs. Wt wounds (Fig. 1A, B). Tenascin-positive granulation tissue was abundant in day 3 Wt wounds and began to decrease by day 7 (Fig. 1A). At day 14, wound granulation tissue was largely resolved in Wt mice (Fig. 1A, B). In contrast, the area of tenascin-positive granulation tissue in day 3 and 7 Rh-/- wounds was significantly smaller than in Wt wounds. Day 14 wounds of Rh-/- mice were

still strongly tenascin-positive, although the areas of these regions were highly variable between Rh-/- mice (Fig. 1A, B). Interestingly, the pattern of tenascin staining in day 14 Rh-/- wounds was abnormal as the staining was "patchy", in contrast to day 14 Wt wounds (Fig. 1A). An additional difference in Rh-/- wounds was the transient appearance of a thick layer of subcutaneous adipocytes in day 1-3 Rh-/- wounds (Fig. 2A and "unpublished data"). These results indicate that a prominent effect of Rhamm deficiency during wound repair is a miscuing of signals required for the temporal regulation of granulation tissue formation and resolution.

Fibroplasia is a particularly prominent feature of granulation tissue in excisional skin wounds. The biological activities of fibroblasts and other mesenchymal cells, such as myofibroblasts, are key factors in the formation of early granulation tissue architecture (Reid et al., 2004). Robust fibroplasia, as quantified by the density/unit area of granulation tissue fibroblasts, was apparent in day 3 Wt wounds and was increased by day 7 (Fig. 2A). Myofibroblasts, detected by smooth muscle actin staining, were also abundant in Wt wounds by day 7 (Fig. 2B). Fibroplasia was observed in day3/7 Rh-/- granulation tissue but was blunted appreciably in comparison to Wt wounds (Fig. 2A) and there was a significant decrease in the number of myofibroblasts in day 7 Rh-/- wounds compared to Wt (Fig. 2B). Furthermore, Rh-/- granulation tissue was confirmed to contain abundant adipocytes, particularly at the wound edge, as indicated by the presence of vacuolated cells (Fig. 2A, arrows) which stained strongly with the lipophylic dye, BODIPY493/503 (Gocze and Freeman, 1994) ("unpublished data "). Rh-/- cells explanted from normal skin (day 0) and from day 7 wounds expressed less smooth muscle actin and accumulated more lipid than explanted Wt cells (Fig. 2C). Thus, deletion of Rhamm results in lower fibroblast density and aberrant differentiation in Rh-/- granulation tissue.

A number of factors can affect fibroplasia as granulation tissue forms. For example, a chronic inflammatory response at the wound site is required to initiate fibroplasia and functions to provide growth factors and cytokines that attract fibroblasts into the wound site. These factors regulate fibroblast migration, survival and proliferation (O'Leary et al., 2002). Rhamm regulates white cell trafficking *in vivo* and proliferation/ apoptosis *in culture* (Adamia et al., 2005; Turley et al., 2002). Surprisingly, *in vivo* analysis revealed a significantly *greater* percentage of polymorphonuclear cells (cell/field) in Rh-/- day 3 and day 7 granulation tissue (65 ± 6; 40 ± 12) compared to Wt (42 ± 8 and 8 ± 1, respectively) suggesting that Rhamm loss results in prolonged acute inflammation within excisional wounds. However, proliferation, as measured by the number of murine Ki-67-positive nuclei in granulation tissue, was not significantly different

from Wt, nor was the rate of apoptosis significantly different in Rh-/- vs. Wt wounds when measured by ApopTag staining ("unpublished data"). While these data do not rule out a role for fibroblast proliferation/apoptosis in the blunted fibroplasia observed in Rh-/- wounds, they suggest that these are not dominant factors.

Rhamm expression is required to sustain ERK1,2 activation during granulation tissue formation in vivo and in fibroblasts responding to growth factors in culture.

Fibroblast migration also contributes to fibroplasia and requires appropriate temporal regulation of signaling pathways such as ERK1,2, which provide cues for promoting and sustaining migration/invasion (Krueger et al., 2001). Furthermore, these MAP kinases have been implicated in fibroblast differentiation into adipocytes, the most prominent being ERK1 (Bost et al., 2005). Since we have shown that Rhamm associates with ERK1 in fibroblasts and that this association is required for PDGF-BB stimulated ERK1,2 activation (Zhang et al., 1998), we hypothesized that the activity of these MAP kinases may be deficient in Rh-/- wound granulation tissue and contribute to aberrant fibroblast migration/differentiation (Hornberg et al., 2005). Wt granulation tissue fibroblasts exhibited strong staining for the active forms of these kinases, as assessed with anti-phospho-ERK1,2 antibodies, at day 3 after wounding (Fig. 3). At this time, levels of phospho-ERK1,2 were also similar for Rh-/- vs. Wt granulation tissue, when standardized against total ERK1,2 levels (Fig. 3, graph). Staining intensity for phospho-ERK1,2 in Wt granulation tissue fibroblasts increased 6-fold by day 7 and did not drop significantly until day 13, whereas staining intensity of phospho-ERK1,2 had prematurely decreased in Rh-/- granulation tissue by day 7 (Fig. 3). These changes in active ERK1,2 of Rh-/- vs. Wt granulation tissue fibroblasts were not due to decreases in total ERK1,2 protein levels since immunoblot analyses revealed that Rh-/- fibroblasts expressed similar amounts of ERK1,2 protein compared to Wt fibroblasts *in vivo* ("unpublished data"). These results indicate that ERK1,2 activity in Rh-/- granulation tissue fibroblasts is aberrant and may contribute to the miscuing of granulation tissue formation/resolution of Rh-/- excisional wounds.

To determine whether the aberrant ERK1,2 activity observed in Rh-/- granulation tissue fibroblasts *in vivo* is a cell autonomous or micro-environmental defect, we quantified the response of isolated Rh-/- vs. Rh^{FL} (full length Rhamm)-rescued Rh-/- fibroblasts to serum (FCS) (Fig. 4). ELISA analysis of active (phospho)-ERK1,2 revealed that both Rh^{FL}-rescued and Rh-/- fibroblasts activated ERK1,2 in response to serum but activity declined more rapidly in Rh-/- fibroblasts (Fig. 4A). ERK1 activity in response to serum also prematurely decreased as

assessed using Western blot analyses (Fig. 4B). In contrast, Rh^{FL}-rescued fibroblasts sustained ERK1 activity for greater than 60 min, analogous to the Wt fibroblasts stimulated with PDGF-BB (Fig. 4B, Suppl. Fig. IIIa).

Confocal analysis showed that both Rh^{FL}-rescued and Rh-/- fibroblasts activate and target ERK1,2 to the cell nucleus (Fig. 4C). However, activated ERK1,2 accumulated to a lesser extent and disappeared more rapidly from the nucleus of Rh-/- fibroblasts than the nucleus of the Rh^{FL}-rescued counterparts (Fig. 4C, Suppl. Fig. IIIb). Importantly, very little activated ERK1,2 accumulated at the membrane of Rh-/- cell processes while these MAP kinases were clearly activated and targeted to cellular processes in Rh^{FL}-rescued fibroblasts or in Wt fibroblasts (Fig. 4C and "unpublished data"). These results suggest that Rhamm is required both for sustaining ERK1,2 activity in different subcellular compartments and for the appropriate temporal regulation of trafficking active ERK1,2, both of which could result in motility and differentiation defects (Hornberg et al., 2005).

Rhamm expression is required for fibroblast migration and invasion in culture assays.

To assess whether or not Rh-/- fibroblasts have an inherent migration defect, their motogenic behaviour was compared with Rh^{FL}-rescued fibroblasts using scratch wound and 3D collagen gel assays designed to mimic aspects of migration within the wound microenvironment (Reid et al., 2004). Significantly fewer Rh-/- than Rh^{FL}-rescued fibroblasts migrated across 3mm scratch wounds *in culture* (Fig. 5A), as quantified both by the number of fibroblasts present in the wound gap and by time-lapse cinemicrography of fibroblasts migrating from the wound edge into the wound. A similar difference in migration was also exhibited when comparing Rh-/- fibroblasts to litter-matched Wt fibroblasts (Suppl. Fig. IVa). Vector analysis of time-lapse wound images revealed that Rh-/- fibroblasts were not restrained to a vertical orientation towards the wound gap, as was characteristic of the Rh^{FL}-rescued fibroblasts or Wt fibroblasts (Fig. 5A, Suppl. Fig. IVa). These results indicate that loss of Rhamm expression results in an inherent migration defect related to a reduced ability of fibroblasts to orient towards haptotactic cues. The invasive properties of Rh-/- vs. Wt fibroblasts were also compared using 3D collagen type I gels. Gels were constructed with central plugs composed of collagen type I, PDGF-BB and HA, surrounded by fibroblasts enmeshed in the surrounding collagen gel (Fig. 5B). Migration of primary Rh-/- dermal fibroblasts into the central collagen gel plug containing PDGF-BB and HA was reduced by almost 90% compared to that of litter-matched Wt fibroblasts (Fig. 5B). A similar result was obtained with primary fibroblasts (Suppl. Fig. IVb) confirming that Rh-/-

fibroblasts exhibited intrinsic and severe defects in haptotaxis and invasion *in vitro*. Cell surface Rhamm [also designated CD168 (Mason et al., 2002)] is important in this migration since anti-Rhamm antibodies blocked the haptotaxis/invasion of Wt fibroblasts but not Rh-/- fibroblasts (Suppl. Fig. IV b).

Efficient repair of excisional wounds also depends upon the speed of motility in response to motogens present in the microenvironment (Li et al., 2004; Ridley et al., 2003). The velocity of migration stimulated by HA (Fig. 6A) or FCS (Suppl. Fig. Va,b) was assessed directly using time-lapse cinemicrography of cells cultured on 2D tissue culture surfaces. All experiments measuring the effects of HA on motility rates were conducted using cells plated onto fibronectin-coated culture surfaces, as described previously (Hall et al., 1996). Since both higher molecular weight HA (avg. 300kDa) and HA oligosaccharides (avg. 10kDa) promote cell motility (Turley et al., 2002), a mixture of these HA sizes was used for the motility assays. To render non-transformed cells sensitive to HA, fibroblasts can be pre-treated with PMA, which is required to activate protein kinase C-dependent processes, permitting motogenic responses to HA (Hall et al., 2001). The HA mixture significantly promoted random motility of Wt fibroblasts when PMA was present compared to PMA-treated cells alone (Fig. 6A), which is blocked by anti-Rhamm antibodies (Fig. 6A). In contrast, the HA formulation did not enhance Rh-/- fibroblast random motility; motility rates of these cells remained significantly lower than HA-stimulated Wt fibroblasts. As expected, anti-Rhamm antibodies had no effect on the motility rates of the Rh-/- fibroblasts (Fig. 6A). The inability of Rh-/- fibroblasts to increase random motility in response to HA was not due to lack of expression of CD44, which was expressed at equal levels in both Rh-/- and Wt fibroblasts (Fig. 6B). A similar result was observed when Rh-/- and Wt fibroblasts were stimulated with serum and motility of Wt fibroblasts was significantly inhibited by a Mek1 inhibitor, PD98059, or anti-Rhamm antibodies (Suppl. Fig. Va). The motility of Rh-/- fibroblasts was not affected by these inhibitors (Suppl. Fig. Va). These results indicate that Rhamm expression is required for a motogenic response to HA and serum, and that serum-induced motility is also dependent upon ERK1,2.

Cell migration and invasion requires the rapid assembly/disassembly of the actin cytoskeleton. Since intracellular Rhamm forms associate with the actin cytoskeleton (Turley et al., 2002), we compared the ability of Rh-/- fibroblasts with Rh^{FL}-rescued fibroblasts to disassemble phalloidin-positive actin stress fibers in response to PDGF-BB. Unstimulated Rh-/- fibroblasts exhibited similar actin stress fibers to Rh^{FL}-rescued fibroblasts (Fig. 7).

Immunofluorescent brightness of these structures, which provides a measure of disassembly, was also similar (Fig. 7). However, following exposure to PDGF-BB, Rh-/- fibroblasts exhibited a significantly reduced ability to disassemble actin fibers compared to Wt fibroblasts (Fig. 7). The Mek1 inhibitor, PD98059, also significantly blocked actin disassembly in response to PDGF-BB Wt fibroblasts (Fig. 7). These results indicate that reduced migration is linked to defective mechanisms of disassembly of the actin cytoskeleton and that Rhamm-regulated disassembly requires ERK1,2 activity.

Expression of mutant active Mek1 rescues the migration defect of Rhamm-/- fibroblasts.

Migratory behavior, including random motility, migration in scratch wound assays, invasion and cytoskeleton disassembly, have been shown previously to require ERK1,2 activity (Juliano et al., 2004; Reddy et al., 2003). Therefore, we asked whether these aberrant migratory properties of Rh-/- fibroblasts resulted directly from deficient ERK1,2 activity. Expression of mutant-active Mek1 restored the ability of Rh-/- fibroblasts to sustain activation of ERK1,2 in response to FCS (Fig. 8A, B) and this effect was not enhanced further by co-expression of Rh^{FL} (Fig. 8A). As well, expression of active Mek1 in Rh-/- fibroblasts restored both their ability to migrate across wound gaps and to invade collagen at rates similar to that of Rh^{FL}-rescued fibroblasts (Fig. 9A, B). Expression of mutant active Mek1 also re-established the ability of Rh-/- fibroblasts to disassemble actin stress fibers and to increase random motility in response to PDGF-BB (Fig. 7, Suppl. Fig. Vb), to levels similar to that of Rh^{FL}-rescued fibroblasts. Co-expression of Rh^{FL} and Mek1 did not further stimulate migration compared to Rh^{FL} or Mek1 alone (Fig. 9A, Suppl. Fig. Vb). These results suggest that Rhamm and Mek1 act on the same motogenic signaling pathway since Mek1 can compensate for Rhamm in restoring motile and invasive behavior. Collectively, the results demonstrate that a major motogenic effect of Rhamm is to regulate activation of the Mek1/ERK1,2 complexes, since co-expression of Rh^{FL} and Mek1 does not enhance ERK1,2 activity further.

DISCUSSION

This study identifies Rhamm as a fibrogenic factor that is required for appropriate timing and spatial regulation of granulation tissue formation and resolution. A major consequence of Rhamm loss on granulation tissue formation/resolution is reduced/delayed fibroplasia associated with sparse fibroblast density, enhanced neutrophil accumulation and aberrant mesenchymal differentiation as indicated by reduced myofibroblast conversion and increased adipocyte accumulation within wound granulation tissue. Our studies suggest further that an underlying defect associated with these repair deficiencies in Rh-/- wounds is de-regulated ERK1,2 activation that, in particular, impacts upon signaling pathways promoting fibroblast migration. This conclusion is supported by the demonstration that Rh-/- fibroblasts retain their inability to appropriately activate ERK1,2 *in culture* and result in migration defects, as measured by several locomotion assays. These defects are rescued by expression of mutant active Mek1, an ERK1,2 kinase activator.

We have previously reported that Rh-/- fibroblasts exhibit reduced proliferation *in culture* (Tolg et al., 2003). However, our current data do not provide support for an essential role of Rhamm in mitotic spindle formation or cell cycle regulation during wound repair in dermal fibroblasts *in vivo,* as judged by the lack of detectable differences in proliferation or apoptotic indices within Rh-/- vs. litter-matched Wt wound sites. Nevertheless, the slightly disorganized migration of Rhamm-/- fibroblasts from scratch wound assays on tissue culture plastic is consistent with a possible centrosomal defect that could contribute to aberrant migration (Watanabe et al., 2005) and merits further experimentation. A role for Rhamm in collagen contraction has also been controversial (Bagli et al., 1999; Travis et al., 2001). Unexpectedly, however, our studies have revealed a role for Rhamm in recruitment/differentiation of myofibroblasts and contraction of the wound bed. As is increasingly reported and recognized (Bissell et al., 2003), both of these results emphasize the importance of context and the microenvironment in regulating cell signaling. Thus, data obtained *in culture*, especially on two-dimensional (2D) substrata, need to be confirmed *in vivo* or at least in relevant microenvironments.

ERK1 and 2 are closely related MAP kinase isoforms that perform different physiological functions. For example, ERK2 is required for normal embryogenesis (Yao et al., 2003), whereas ERK1 plays more subtle and specific roles in adult physiology including adipogenesis (Bost et al., 2005). Both MAP kinases are activated by Mek1 or 2 and regulate signaling pathways that

control cell motility, invasion and cytoskeleton remodeling during migration *in culture*. Our results show that these defects of Rh-/- fibroblasts result from an inability to sustain and maximally activate ERK1,2 following growth factor stimulation. These results are consistent with our previous evidence that Rhamm is required for PDGF-BB stimulated ERK1,2 activity in mesenchymal stem cells and for promoting migration by regulating signaling through upstream activators of ERK1,2 including HA, Src, Ras and FAK (Hall et al., 1996; Hall et al., 1995; Turley et al., 2002).

Loss of Rhamm results in an abnormal temporal regulation of the subcellular localization of ERK1,2 in response to certain fibrogenic factors. We show that appropriate activation and compartmentalization of active ERK1,2 is required for generating highly motile cells and for repairing large wound gaps. ERK1,2 regulates motility by both transcriptional and post-transcriptional mechanisms (Huang et al., 2004). Initiation and early phases of migration during wound repair do not appear to require transcription (Providence and Higgins, 2004). Rhamm may therefore affect the ability of ERK1,2 to regulate transcription of motility- and invasion-related genes. ERK1,2 kinases are activated in response to a wide variety of stimuli and phosphorylate multiple substrates that mediate such disparate cellular functions as proliferation, apoptosis and differentiation (Lewis et al., 2000). The consequences of ERK1,2 signaling to these functions depends upon activation kinetics and subcellular compartmentalization (Colucci-D'Amato et al., 2003; Hendriks et al., 2005; Hornberg et al., 2005). These factors are determined by receptor dimerization, receptor internalization, "cross-talk" with other receptors, association of ERK1,2 with adaptor proteins, and activation of other kinases or phosphatases that modify ERK1,2 activity (Colucci-D'Amato et al., 2003; Hornberg et al., 2005). The mechanisms by which Rhamm controls the sustained ERK1,2 activation and compartmentalization are currently unknown, but intracellular Rhamm may act as an adaptor protein analogous to MP1 or IQGAP1 (Kolch, 2005) while cell surface Rhamm forms may act as co-receptors for growth factors and their cognate receptors (Zhang et al., 1998) to separately regulate ERK activity.

Motogenic responses to PDGF-BB or FCS are reduced but are not ablated completely in the absence of Rhamm, suggesting that other factors can partially compensate for loss of Rhamm. However, our data show that Rhamm expression is essential for a motogenic response of fibroblasts to HA even when CD44, a major HA receptor, is present (Toole, 2004), implying that a principle function of Rhamm *in vivo* is as an HA receptor. The molecular mechanisms by which polysaccharides in general, and HA in particular, regulate physiological processes such as

tissue repair are not yet well understood (Adamia et al., 2005; Toole, 2004). However, much like the closely related heparan sulfate, HA can impact signaling via receptors such as CD44 and Rhamm through their associations with growth factor receptors involved in repair such as TGF-βR and PDGFR (Turley et al., 2002). One way in which HA has been shown to affect signaling is by localizing growth factor receptors to specific cell surface membrane compartments. For example, HA and CD44 promote localization of fibrogenic receptors such as TGF-βR into lipid-rich rafts (Ito et al., 2004). This type of compartmentalization impacts the rate of internalization and intracellular trafficking of receptor/signaling complexes to endosomes and lysosomes for inactivation vs. recycling back to the cell surface. These parameters, in turn, have an effect on the kinetics of receptor activation including receptor-mediated activation of downstream effectors such as ERK1,2 (Hendriks et al., 2005). CD44, furthermore, links growth factor receptors such as TGF-βR to the cortical cytoskeleton (Turley et al., 2002). Like CD44, Rhamm has been shown to mediate signaling through TGF-β and PDGF-BB (Turley et al., 2002). Furthermore, the loss of Rhamm has similar effects on fibroblast migration and ERK1,2 activation *in culture* as does loss of PDGFR (Gao et al., 2005). How Rhamm directs signaling by HA and fibrogenic cytokines such as PDGF-BB remains to be determined.

Rhamm does appear to be required as a mediator of HA/cytokine signaling. However, Rhamm does not perform all the functions attributed to HA. For example, HA is essential for embryogenesis, whereas Rhamm is not (Tolg et al., 2003). Therefore Rhamm, like CD44 (which also is not required for embryogenesis), must mediate only a subset of HA-regulated functions *in vivo*. Most probably these include some HA-mediated functions that are distinct from CD44 but also some that are shared with CD44. For example, Rhamm can compensate for HA-driven collagen-induced arthritis when CD44 is absent (Nedvetzki et al., 2004). Furthermore, while loss of either CD44 or Rhamm have consequences on wound repair (Toole, 2004), we have shown in the present study that aberrant granulation tissue formation and resolution resulting from Rhamm loss cannot be compensated for by CD44 expression.

To date, most of the reported effects of Rhamm *in vivo* are limited to mesenchymal and immune cells. Loss of Rhamm appears to have no appreciable effect on the appearance of pre-neoplastic polyps in the upper intestinal tract of APC-mutant transgenic mice, although it profoundly affects the initiation and invasion of mesenchymal tumors (Tolg et al., 2003). Furthermore, in CD44-/- mice, splenocytes utilize Rhamm for homing to damaged joints and Rhamm hyperexpression contributes to aggressive behavior of multiple myeloma subsets

assessed *ex vivo* (Adamia et al., 2005). Here, we have noted that a dominant effect of Rhamm loss is on granulation tissue fibroblast and polymorphonuclear cell accumulation. Only minor and transient differences were observed in the keratinocyte layer of Rh-/- vs. Wt wounds ("unpublished data"). Intriguingly, the loss of Rhamm expression affects wound repair in a manner that most closely resembles the loss of another mesenchymal protein, vimentin (Eckes et al., 2000), which is also predominantly expressed in fibroblasts and cells of hematopoietic lineage (Adamia et al., 2005; Eckes et al., 2000; Turley et al., 2002). Like Rhamm, vimentin has been shown also to be essential for cell migration. Importantly, a prominent phenotype of vimentin knockouts is the aberrant formation/resolution of granulation tissue that results from impaired fibroblast migration. Collectively, these results are consistent with the conclusion that Rhamm is a fibrogenic factor expressed predominantly in cells that do not necessarily form parenchymal units. These physiological functions may provide a basis for understanding and further dissecting the importance of Rhamm hyperexpression in the invasion and metastasis of malignant tumors, processes that are also regulated by vimentin.

MATERIALS AND METHODS

Reagents

Medical grade HA prepared from bacterial fermentation was the kind gift of Skye Pharma (London UK) and was free of detectable proteins, DNA or endotoxins (Filion and Phillips, 2001). The average molecular weight range and polydispersity of HA was 276.7kDa and 1.221kDa, respectively. HA oligosaccharides (MW_{avg} 10kDa) were a kind gift of Dr. F. Winnik (University of Montreal, QC) and were prepared by partial digestion with testicular hyaluronidase and purification by gel filtration. Human plasma fibronectin (BRL) , Ki67 (pAb, DAKO), α-smooth muscle actin (pAb, Santa Cruz), tenascin (pAb, Chemicon) and vimentin (pAb, Santa Cruz) antibodies and Oregon Green phalloidin (Molecular Probes) were used according to the manufacturer's instructions. Function blocking, affinity purified anti-Rhamm antibodies (Zymed) were confirmed to be specific by western blot and immunofluorescence assays of Rh-/- fibroblasts. Anti-CD44 antibodies (mAb, KM114 and IM7, Pharmingen) were confirmed to be specific using western and immunofluorescence analyses of CD44-/- dermal fibroblasts. Phospho-ERK1,2 antibodies (pAb, Cell Signaling Technology) were used for immunohistochemistry and immunofluorescence and phospho-ERK1,2 (mAb, Sigma) and pan-ERK1 antibodies (pAb, Santa Cruz,) were used for western blot analyses. Secondary antibodies were anti-rabbit Alexa 555 (Molecular Probes), Texas-Red or FITC labeled goat anti-mouse/goat anti-rabbit (Jackson laboratories), HRP-goat anti-mouse (Biorad), HRP-goat anti-rabbit (Pharmingen), HRP-rabbit anti-goat (Santa Cruz). All antibodies were used according to manufacturer's instructions. ABC staining system (Santa Cruz) was used for immunohistochemistry and ApoTag peroxidase *in situ* apoptosis detection kit (Chemicon) was used for quantification of apoptosis. FACE ERK1/2 ELISA kit (Active Motif) was used according to manufacturer's instructions to quantify ERK1/2 activation in response to FCS in Rh-/-, Rh^{FL}-, Mek1- and Mek1/Rh^{FL}-rescued cell lines. Mounting medium for immunofluorescence contained DAPI (Vectashield) while Cytoseal 60 (Richard-Allan Scientific) was used for mounting of tissue sections. The Mek1 inhibitors, PD98059 and U0126 (50μM and 10μM respectively, Biosciences), were used according to manufacturer's instructions. BODIPY 493/503 was purchased from Invitrogen and was used according to manufacturer's instructions.

Rh-/- mice; mouse embryonic fibroblasts and dermal fibroblasts

All animal experiments were performed in accordance with regulations of the animal use subcommittee at the University of Western Ontario, London, Ontario, Canada. The preparation

of Rh-/- mice and mouse embryonic fibroblasts (MEF), as well as genotyping of mice and fibroblasts, have been described (Tolg et al., 2003). CD44-/- mice have been described (Schmits et al , 1997). Dermal fibroblasts were isolated from explanted skin from newborn mice. For the isolation of cells from granulation tissue, wound punches were cut into small pieces and cultured with the dermal side facing down in complete cell culture medium (10% FCS, DMEM, antibiotic-antimycotic).

RT-PCR analysis of Rhamm and CD44 mRNA

Rhamm mRNA was amplified as previously described (Tolg et al., 2003) and PCR products were detected by Southern analysis using Rhamm exons 14-16 as a radioactive probe. CD44 mRNA was amplified as previously reported (Schmits et al., 1997). Amplification of *β-actin* mRNA was used as a loading control (Tolg et al., 2003).

Western blots

Western analyses of CD44, phospho-ERK1,2 and total ERK1,2 proteins were performed as described (Schmits et al., 1997; Tolg et al., 2003; Zhang et al., 1998). Densitometry was performed using Image Quant 5.1 software (Molecular Dynamics).

Cell culture and transfection

Cell culture medium and culture conditions were described previously (Tolg et al., 2003; Zhang et al., 1998). PDGF-BB (25ng/ml), HA (500ng/ml-1mg/ml) or FCS (10%) were added to 24hrs serum-starved, 50% sub-confluent fibroblasts on fibronectin (25µg/ml)-coated dishes (Hall et al., 1996; Zhang et al., 1998). To obtain a response to HA, cells were pre-treated with 5nM PMA (Sigma) (Hall et al., 2001). Immortalized Rh-/- cells were transfected with Rh^{FL} murine Rhamm and/or mutant active Mek1 (kind gift of N. Ahn, U. Colorado, Boulder) in the presence of Lipofectamine Plus (Invitrogen) as described previously (Zhang et al., 1998). All transfectants were selected in G418 (1-5mg/ml for 2-3 weeks).

Excisional wounds and histology

Wt and Rh-/- mice (3-18 month old) were anaesthetized by Halothane inhalation. Two full thickness wounds were placed on denuded back skin using a 4mm metal punch. Mice were housed in individual cages for the experimental period. Wounds were harvested at varying times using a 8mm metal punch from similar locations on the backs of mice of the same gender and age. Harvested wounds were fixed overnight in 4% paraformaldehyde and paraffin embedded as described (Tolg et al., 2003). Numbered serial sections were cut perpendicular to the wound edge starting at the wound center. The first and last sections were stained with Masson's

trichrome and non-stained sections were used for immunohistochemistry. To ensure that serial sections were cut starting at the wound center, wound samples were cut in half through the wound center prior to embedding

In total, five experimental series were performed. In each experiment, wounds were harvested at four different time points (1, 3, 7, and 14 days after wounding). For each time point, four age and gender matched mice were used (two Rh-/- and two Wt mice). In total, for each time point, ten Rh-/- and ten Wt mice were analyzed.

Immunohistochemistry of tissue sections and immunofluorescence of cultured cells

Tissue sections were stained for collagen (Masson's trichrome), α-smooth muscle actin, vimentin and tenascin following manufacturer's recommendations. Staining was quantified after counter-staining with Harris Hematoxylin (EM SCIENCE) and mounting in Cytoseal 60 (Tullberg-Reinert and Jundt, 1999). Immunofluorescence of phospho-ERK1,2 was done as previously published (Avizienyte et al., 2004). For the detection of droplets of neutral lipids, paraformaldehyde-fixed cells were stained with BODIPY 493/503 (25*u*g/ml) (Gocze and Freeman, 1994). Acton stress fibers were detected with Oregon-green phalloidin.

In vitro wound and invasion assays

Confluent cell monolayers on fibronectin coated dishes were serum starved overnight Scratch wounds (1 or 3 mm) were made using sized cell scrapers, then covered with medium containing 10% FCS or 25ng/ml PDGF-BB for 24-48hrs. Monolayers were fixed (3% paraformaldehyde), washed, stained with methylene blue (0.1% in methanol) then photographed using a Nikon inverted Eclipse TE 300 microscope. Images were analyzed for cell number per unit area of wound gap using Simple PCI (Compix). For 3D assays, collagen (Vitrogen100, Cohesion) or Matrigel (BD) gels were prepared according to manufacturers instructions. Plastic inserts were placed in the gel center. Fibroblasts were ($5x10^5$cells/ml) added to the outer gel ECM solution. After 24-48hrs, plastic inserts were removed and the cell free space was filled with collagen containing 25ng/ml PDGF-BB, 100μg/ml HA, and 25ng/ml fibronectin. Gels were fixed and analyzed 72hrs later for cell numbers/unit area..

Time-lapse cinemicrography

For experiments assessing the motogenic effects of HA and PDGF-BB, cells were plated onto fibronectin-coated tissue culture flasks at 50% sub-confluence overnight then serum-starved for 24hrs. PDGF-BB or HA were added prior to filming as described (Hall et al., 2001; Zhang et al., 1998). For quantifying the effect of FCS, fibroblasts were plated at 50% sub-confluence

overnight onto tissue culture dishes that had been pre-coated with serum proteins. FCS was added after a 24hr period of serum-starvation and cells were filmed as above.

Image acquisition, image enhancement and image analysis

Masson's trichrome and eosin/hematoxylin stained tissue sections as well as vimentin, tenascin and phospho-ERK1,2 stained tissue section images were taken with air objectives (4X, NA=0.16; 20X NA=0.7) using an Olympus AX70 Provis microscope equipped with a Cooke SensiCam color camera (CCD Imaging) and Image Pro Plus Version 4.5.1.2.9 software (Media Cybernetics, Inc.). For quantification of pERK1,2 staining, images were saved as tiff files and quantification of histology staining was done using Photoshop 6.0 (Adobe). The area of blue Hematoxylin staining, representing number of cells, was quantified by selecting and counting blue pixels (select, color range, blue, Image, Histogram). After deletion of the selected blue pixels, the area stained by the peroxidase substrate DAB was identified by selecting shadows (select, color range, shadows) and quantified by measuring the number of pixels (Image, Histogram). The area stained with tenascin was quantified using Simple PCI imaging software (Compix).

Images in Fig. 1A are composites of images taken with a 4x objective. The colors were enhanced using Photoshop 6.0 (Adobe, adjust, auto levels).

Scratch wound images were taken with air objectives (4x Nikon objective, air, NA=0.1, equipped with Hoffman modulation Contrast optics) using a Nikon Eclipse TE300 microscope equipped with a Hamamatsu digital camera (Hamamatsu) and Simple PCI imaging software (Compix). Images of the wounds were acquired using a Conica/Minolta Dimage Z3 digital camera equipped with 12x optical zoom. The wound area was quantified using Simple PCI imaging software (Compix). Immunofluorescent images of actin fluorescence (10x Nikon objective, air, NA=0.25) were also acquired using the Nikon Eclipse TE300 microscope and quantified using Photoshop 6.0 as above. Confocal images were taken using a 63X oil objective (Zeiss, NA=1.4) with a Zeiss 510 LSM Meta Confocal microscope using LSM 5 imaging software (Zeiss). Fluorescence intensity of images was measured using LSM 5 imaging software (Zeiss).

ACKNOWLEDGEMENTS

The technical assistance of Ms. Jenny Ma is gratefully acknowledged. This study was funded by a CIHR grant to E.T. (MOP-57694). Additional salary support was provided by The Breast Cancer Society of Canada (EAT), a CIHR fellowship and Breast Cancer Society of Canada fellowship (SRH, UST-63811), and a Postdoctoral Fellowship from the Translational Breast Cancer Research Traineeship Program (CT). JBM is supported in part by a grant from DA/DAMD 17-02-1-0102. MJB is supported by the USDOE (Office of the Biological and Environmental Research), by the USNCI and by an Innovator award from the USDOD Breast Cancer Program. ET and MJB are also the recipients of a BCRP-CDMRP grant (BC044087).

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ABBREVIATIONS

bFGF-2	Basic fibroblast growth factor-2
2D	2-dimensional culture
3D	3-dimensional culture
ECM	Extracellular matrix
ERK1,2	Extracellular regulated kinases 1,2
FAK	Focal adhesion kinase
HA	Hyaluronic Acid / Hyaluronan
Matrigel	Basement membrane matrix
MEFs	Mouse embryonic fibroblasts
Mek1	Mitogen activated kinase kinase 1
MMPs	Matrix metalloproteinases
MW	Molecular weight
Mw_{avg}	Average molecular weight
PDGF-BB	Platelet derived growth factor-BB
PDGFR	Platelet derived growth factor receptor
PMNs	Polymorphonuclear cells
Rhamm	Receptor for Hyaluronic Acid Mediated Motility
Rh^{FL}	Full-length Rhamm
Rh-/-	Rhamm -/-
TE	Tris-EDTA
TGF-β	Transforming growth factor-β
TGF-βR	Transforming growth factor-β receptor
Wt	Wild-type

FIGURE LEGENDS

Figure 1: Loss of Rhamm delays and alters the pattern of granulation tissue formation in skin wounds. *A. Tenascin protein expression in wound sections:* Wound granulation tissue is abundant in day 7 Wt wounds as indicated by positive and homogenous staining for tenascin. Granulation tissue has largely resolved in Wt wounds by day 14 as indicated by restricted tenascin staining. The area of tenascin-positive Rh-/- granulation tissue is less in days 3 and 7 than Wt and has become aberrantly "patchy" by day 14, indicating delayed and abnormal patterning of granulation tissue resolution. Paraffin processed tissue sections were prepared perpendicular to the wound surface and cut at the wound center then stained for tenascin as a marker for granulation tissue. *B. Areas of tenascin-positive granulation tissue:* The area of Wt granulation tissue is significantly greater than Rh-/- at both day 3 and 7 after wounding ($p<0.01$ for both time points). High standard errors of tenascin-positive areas of Rh-/- granulation tissue reflect aberrant resolution patterns. Values represent the Mean and S.E.M. N= 4 tissue sections from 8 male mice for each genotype.

Figure 2: Loss of Rhamm reduces fibroblast density and increases granulation tissue cell heterogeneity. *A. Fibroblast Density in granulation tissue:* The density of fibroblasts is significantly reduced in Rh-/- granulation tissue at both day 3 ($p<0.0001$) and 7 ($p<0.001$) after wounding. Arrows indicate the presence of vacuolated cells, which are adipocytes. Fibroblast density is heterogeneous in Rh-/- granulation tissue (e.g. dotted circle is sparse; filled line circle is dense) but fibroblast density shown in graph was averaged per microscope field. Paraffin processed tissue sections were stained for vimentin. Values in graphs represent the Mean and S.E.M., N=4 sections from 8 animals for each genotype. *B. Smooth muscle actin-positive fibroblasts in wounds:* The number of wound myofibroblasts is significantly reduced in day 7 Rh-/- wounds compared to Wt granulation tissue ($p<0.0001$). Paraffin processed tissue sections were stained for α–smooth muscle actin as described in Methods. Values in graphs represent the Mean and S.E.M., N=4 sections from 8 animals for each genotype. *C. Smooth muscle actin- and lipid-positive fibroblasts (adipocytes) in granulation tissue explants in culture*: The numbers of smooth muscle actin-positive fibroblasts are significantly reduced and the numbers of lipid-containing cells are significantly increased in Rh-/- granulation wound tissue when compared to

Wt ($p<0.0001$). Cell outgrowths were stained with anti α–smooth muscle actin and BODIPY493/503 as described in Methods. In confocal images, red staining is smooth muscle actin and green staining is BODIPY493/503 taken up into lipid droplets within cells. Laser settings were kept constant for Wt day 0 and Rh-/- day 0 images and for Wt day 7, Rh-/- day 7, IgG day 0 and acetone extracted day 0 images.

Figure 3: Loss of Rhamm alters ERK1,2 activation in granulation tissue fibroblasts. Both Wt and Rh-/- granulation tissue fibroblasts are positive for activated (phospho)-ERK1,2 at day 3 after wounding. Staining for activated ERK1,2 significantly increases in Wt granulation tissue by day 7 ($p<0.001$), then drops by day 14. In contrast, staining drops to near background in day 7 Rh-/- wound granulation tissue ($p<0.00001$), and remains significantly lower than Wt at day 14 ($p<0.01$). Paraffin sections were stained with anti-phospho-ERK1,2 antibodies. Staining was quantified using image analysis and averaged per unit area of granulation tissue as described in Methods. Values represent the Mean and S.E.M., N=15 images of 3 tissue sections for each genotype (5 mice each).

Figure 4: Loss of Rhamm reduces serum activation of ERK1,2 in fibroblasts *in culture.* *A. ELISA analysis of phospho-ERK1,2:* Rh^{FL}-rescued fibroblasts sustain significantly higher levels of ERK1,2 activity at 30-60 min post serum stimulation than Rh-/- fibroblasts ($p<0.05$ for both time points). Phospho-ERK1,2 in serum starved Rh^{FL}-rescued and Rh-/- fibroblasts exposed to serum were quantified as described in Methods. Values at 0 min were subtracted from values at 30 and 60 min. Values represent the Mean and S.E.M., N=3 samples. *B. Western blot analysis of phospsho-ERK1:* Quantification of ERK1 activity shows that Rh^{FL}-rescued fibroblasts sustain activity for 10-50 min post serum stimulation while activity drops in Rh-/- fibroblast 10 min after stimulation. Anti-phospho-ERK1,2 antibodies were used to detect active ERK1,2 in western blots. Phospho-ERK1 and total ERK1 were quantified by densitometry. Images and values for the graph are taken from one of 4 typical experiments. *C. Confocal micrographs of phospho-ERK1,2*: Micrographs of Rh^{FL}-rescued and Rh-/- fibroblasts stained with anti-phospho-ERK1,2 antibodies confirm the more rapid drop in activated ERK1,2 observed in Rh-/- compared to Rh^{FL}-rescued fibroblasts, and show that targeting of phospho-ERK1,2 (red fluorescence) to cell processes and the nucleus (blue fluorescence) is also reduced in Rh-/- fibroblasts. Micrographs are taken from one of 4 similar experiments.

Figure 5: Loss of Rhamm reduces fibroblast migration and invasion. *A. Migration into scratch wounds:* Transfection of immortalized Rh-/- fibroblasts with Rh^{FL} cDNA restores their ability to efficiently migrate into 3mm scratch wounds in response to PDGF-BB; significantly more Rh^{FL}-rescued fibroblasts migrate into the wound gap than Rh-/- fibroblasts ($p<0.0001$). Values represent the Mean and S.E.M., N=6 randomly chosen fields. Time-lapse analysis of wound edges shows that re-expression of Rh^{FL} permits Rh-/- fibroblasts to migrate longer distances over a 24 hr period. Values represent the Mean and S.E.M. N=3 experiments. *B. Invasion into collagen gels*: Diagram shows the construction of a collagen gel invasion assay where HA and PDGF-BB is present only in the central plug. A significantly greater number of Wt dermal fibroblasts migrate into gel centers than do Rh-/- dermal fibroblasts ($p<0.00001$). Values represent the Mean and S.E.M., N=4 experiments.

Figure 6: Loss of Rhamm ablates migration of fibroblasts in response to HA: *A. Random motility in response to HA:* In contrast to Wt, Rh-/- fibroblasts do not increase random motility in response to HA. Further, Rhamm antibodies reduce motility of Wt but not Rh-/- fibroblasts in the presence of HA. Fibroblasts were first exposed to PMA to generate responsiveness to HA. The values represent the Mean and S.E.M., N=30 cells and are the results of one of 4 similar experiments. *B. CD44 protein expression:* Rh-/- fibroblasts express similar levels of CD44 proteins as Wt, assessed by western blot analysis. β-actin is used as a protein loading control.

Figure 7: Loss of Rhamm and inhibition of Mek1 activity reduce actin cytoskeleton disassembly. Rh-/- fibroblasts form phalloidin-positive actin stress fibers similar in structure and immunofluorescent brightness (graph) to Rh^{FL}-rescued fibroblasts but do not disassemble these structures to the same extent as rescued fibroblasts in response to PDGF-BB. The ability of Rh^{FL}-rescued fibroblasts to disassemble actin stress fibers in response to PDGF-BB requires ERK1,2 activation since the Mek1 inhibitor, PD98059, inhibits this process. Conversely, expression of mutant active Mek1 in Rh-/- fibroblasts restores an ability to disassemble actin stress fibers in response to PDGF-BB. Significant differences in fluorescence of actin are marked by asterisks ($p<0.0001$). Values represent the Mean and S.E.M., N=30 images/treatment.

Figure 8: Mutant active Mek1 rescues aberrant ERK1,2 activity in Rhamm-/- fibroblasts. *A. ELISA analysis of ERK1,2 activity:* Expression of mutant active Mek1 in Rh-/- fibroblasts restores ERK1,2 activation ($p< 0.05$ for 30 and 60 min) in response to serum stimulation and activity is not further increased by co-expression of Rh^{FL} and mutant active Mek1 Phospho-ERK1,2 in serum starved Rh-/-, Mek1-rescued and Mek1/Rh^{FL}-rescued fibroblasts exposed to serum was quantified as described in Methods. Values at 0 min were subtracted from values at 30 and 60 min. Values represent the Mean and S.E.M., N=3 samples. *B. Western blot analysis of ERK1 activity:* Western blot analyses show that expression of mutant active Mek1 rescues the ability of Rh-/- fibroblasts to activate ERK1. The graph is a typical result of 3 separate experiments.

Figure 9: Mutant active Mek1 rescues migration and invasion defects in Rhamm-/- fibroblasts. *A. Scratch wound migration assay:* Expression of mutant active Mek1 in Rh-/- fibroblasts restores their ability to migrate into a 3 mm wound gap in response to PDGF-BB to a similar extent as expression of full-length Rhamm. Co-expression of Mek1 and Rh^{FL} has no additive effect on migration. Asterisks indicate $p<0.01$. Values represent the Mean and S.E.M., N=3 experiments. *B. Collagen gel invasion assay:* Expression of mutant active Mek1 in Rh-/- fibroblasts rescues the ability of Rh-/- fibroblasts to invade into collagen gel centers (see Figure 5B) to a similar extent as expression of Rh^{FL}. Asterisks indicate $p<0.00001$. Values represent the Mean and S.E.M., N=3 experiments.

Supplemental Figure I. Rhamm expression is regulated during early phases of wound repair and loss of Rhamm reduces wound contraction. *a. Rhamm mRNA expression in excisional wounds:* Rhamm mRNA expression is transiently up-regulated following excisional skin injury. Rhamm mRNA was amplified by RT-PCR and PCR products were visualized by Southern analysis using a Rhamm-specific probe. The band (arrow) represents the full-length Rhamm PCR product. RT-PCR of β-actin mRNA was used as a loading control. *b. Macroscopic quantification of wound contraction:* Analyses of excisional areas from photographs of wounds show that Wt wounds contract more rapidly than Rh-/- wounds with a significant difference observed at day 3 after wounding ($p<0.05$). The circled area denotes forming granulation tissue, which is reduced in Rh-/- vs. Wt wounds. *c. Microscopic quantification of wound contraction.* Analyses of tissue sections of wounds reveal a significant reduction in contraction at day 1

($p<0.0001$), day 3 ($p<0.05$) and day 13 ($p<0.001$) in Rh-/- vs. Wt wounds. Wound contraction was measured as the distance between the edges of each wound site using tissue sections cut at the center of the excisional wound. Values represent the Mean and S.E.M. of 3 sections from 3 wounds of each genotype.

Supplemental Figure II. Loss of Rhamm expression results in aberrant dermal structure and thickness in both uninjured and repaired skin. *a. Dermis of uninjured skin:* The dermis of uninjured Wt skin (day 0) is significantly thicker than uninjured Rh-/- skin ($p<0.0001$). Sections were stained with Masson's Trichrome. *b. Dermis of resolved wounds:* The dermis at both the center and edges of Wt wounds at day 21 are resolved in that histology is similar to uninjured skin. Wt dermis is significantly thinner than Rh-/- wounds ($p< 0.0001$ and $p<0.01$). Rh-/- wounds have not fully resolved, as indicated by increased dermal thickness suggesting continuing fibroplasias as well as reduced dermal differentiation compared to Wt wounds (e.g. hair follicles have not formed shafts, subcutaneous lipid layer is not formed and muscle layer is not continuous at the underside of wounds). Paraffin-processed tissue sections were stained with Mason's trichrome to visualize dermal collagen (a, green stain) or hematoxylin/eosin to visualize cells (b). The thickness of the dermal layer was measured as the distance between the keratinocyte layer and the subcutaneous fat layer. Solid arrowheads mark the still discernable wound site in Rh-/- skin. Solid arrows mark undifferentiated hair follicles and open arrow heads indicate ongoing fibroplasia and incomplete muscle formation observed at the underside of Rh-/- wounds. Values represent the Mean and S.E.M. of 5 areas from 3 separate tissue sections for each experimental condition.

Supplemental Figure III. Rhamm loss reduces ERK1,2 activation in primary fibroblasts. *a. Western blot analysis of ERK1 activation in response to PDGF-BB:* Loss of Rhamm results in a more rapid drop in ERK1 activation following exposure to PDGF-BB than in litter-matched Wt fibroblasts. ERK1 activity was quantified using western blot assays and anti-phospho-ERK1,2 antibodies. The western blot and graph values are one of three typical experiments. *b. Quantification of confocal analysis of ERK1,2 activation in response to FCS:* Loss of Rhamm reduces accumulation of activated ERK1,2 in the nuclear/perinuclear area ($p<0.05$) compared to Wt. Immunofluorescence of phospho-ERK1,2 in the nucleus and peri-nucleus was quantified at

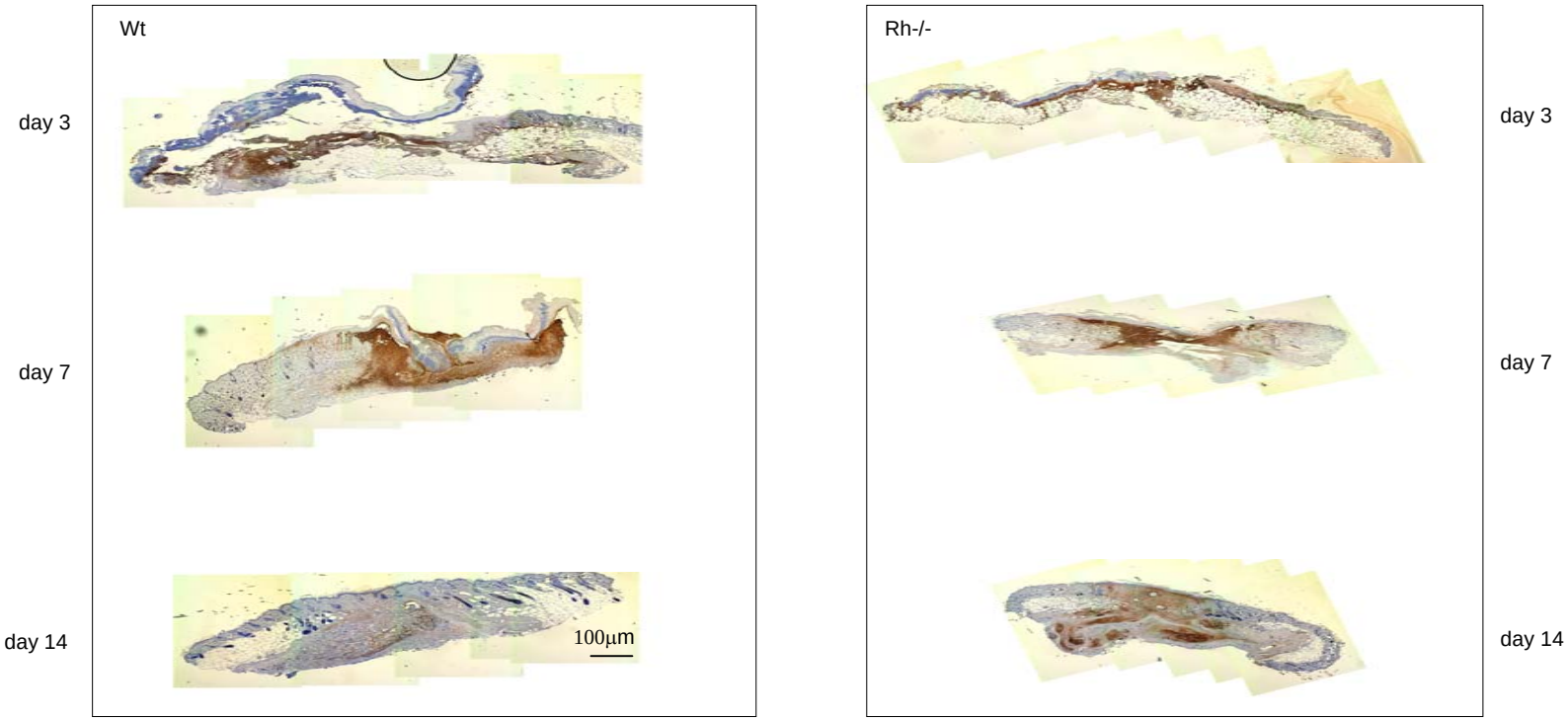
30 min post-stimulation with FCS using image analysis as described in Methods. Values represent the Mean and S.E.M., N=10 cells.

Supplemental Figure IV. Loss of Rhamm reduces migration and invasion of primary fibroblasts. *a. Migration into Scratch wounds:* Significantly more Wt fibroblasts migrate into wound gaps than Rh-/- fibroblasts ($p<0.0001$). Values represent the Mean and S.E.M., N=6 randomly chosen areas. Time-lapse analysis of wound edges shows that Wt fibroblasts migrate longer distances than Rh-/- fibroblasts over a 24 hr period ($p<0.0001$). Values represent the Mean and S.E.M., N=3 experiments. *b. Invasion into collagen gels*: A significantly greater number of Wt fibroblasts invade into the center of collagen gels than Rh-/- fibroblasts ($p<0.00001$). Furthermore, anti-Rhamm antibodies significantly block invasion of Wt fibroblasts ($p<0.00001$) but not Rh-/- fibroblasts. Non-immune IgG was used as a control for the anti-Rhamm antibody. Values represent the Mean and S.E.M., N=4 experiments.

Supplemental Figure V. ERK1,2 activity is required for a motogenic response to PDGF-BB and mutant active Mek1 rescues the motogenic defect of Rhamm-/- fibroblasts. *a. Effects of Mek1 inhibition on random motility:* Primary Wt fibroblasts increase random motility in response to FCS to a significantly greater extent than litter-matched Rh-/- fibroblasts. Both a Mek1 inhibitor, PD98059, and anti-Rhamm antibodies significantly reduce motility of Wt (but not Rh-/- fibroblasts) in the presence of FCS. Asterisks denote significance levels of $p<0.0001$. The values represent the Mean and S.E.M., N=30 cells and are the results of one of 4 similar experiments. *b. Motility of Rh-/- fibroblasts expressing mutant active Mek1*: Random motility of Rh-/- fibroblasts is significantly increased by either expression of Rh^{FL} or mutant active Mek1 ($p<0.0001$). Co-expression of Rh^{FL} and mutant active Mek1 does not further increase cell motility. Values represent the Mean and S.E.M., N=30 cells/treatment.

Figure 1. (Tolg et al.) Loss of Rhamm delays and alters the pattern of granulation tissue formation in skin wounds

A. Tenascin protein expression in wound sections



B. Areas of tenascin-positive granulation tissue

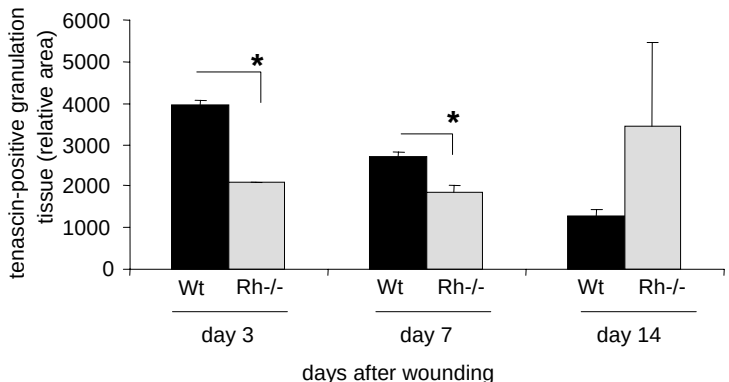
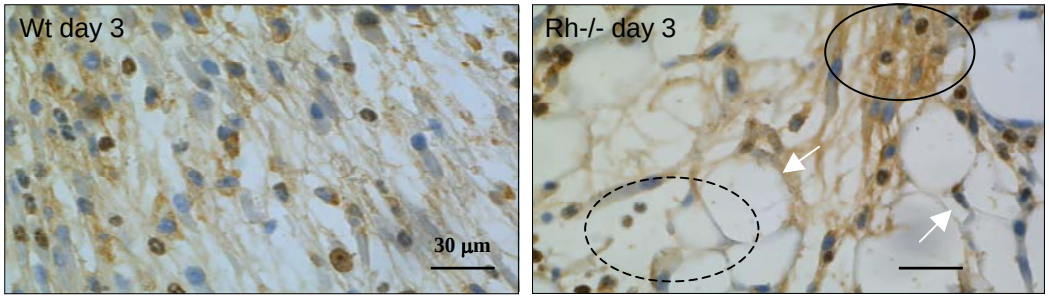
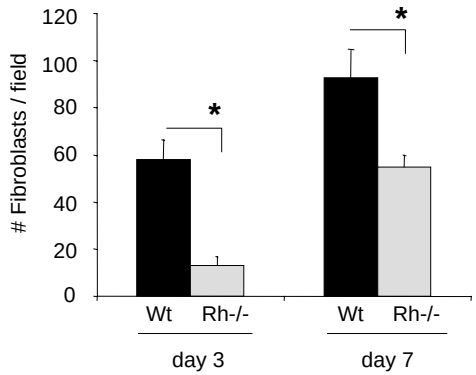


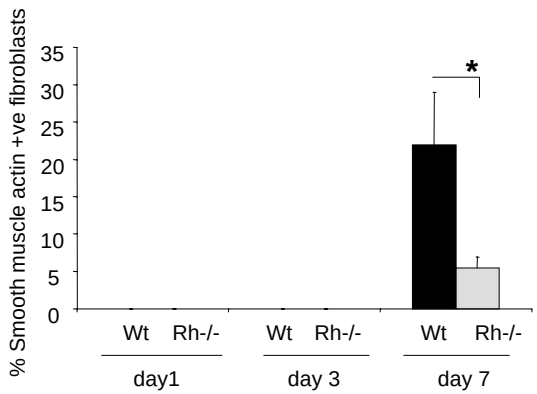
Figure 2. (Tolg et al.) Loss of Rhamm reduces fibroblast density and increases granulation tissue cell heterogeneity

A. Fibroblast density in granulation tissue





B. Smooth muscle actin-positive fibroblasts in wounds



C. Smooth muscle actin and lipid-positive fibroblasts (adipocytes) in granulation tissue explants *in culture*.

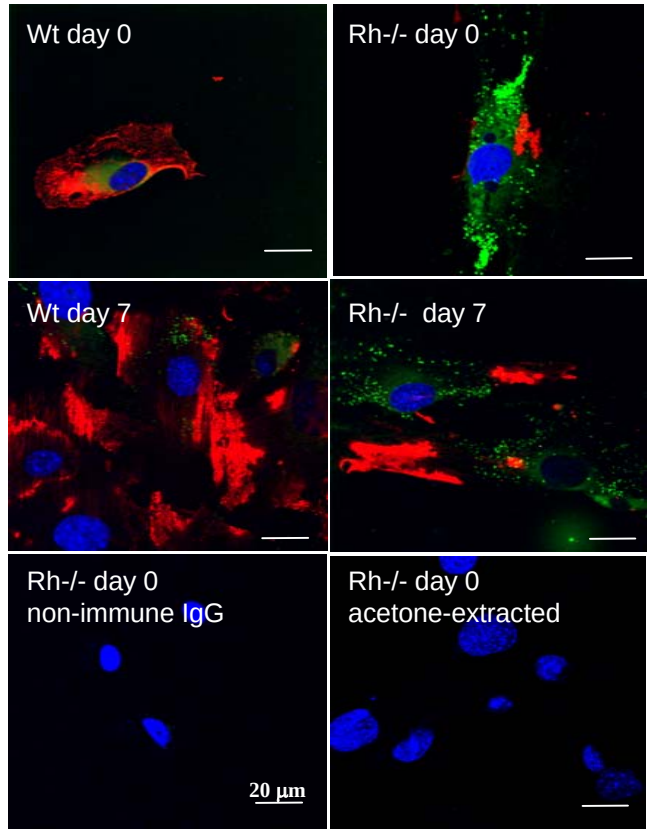
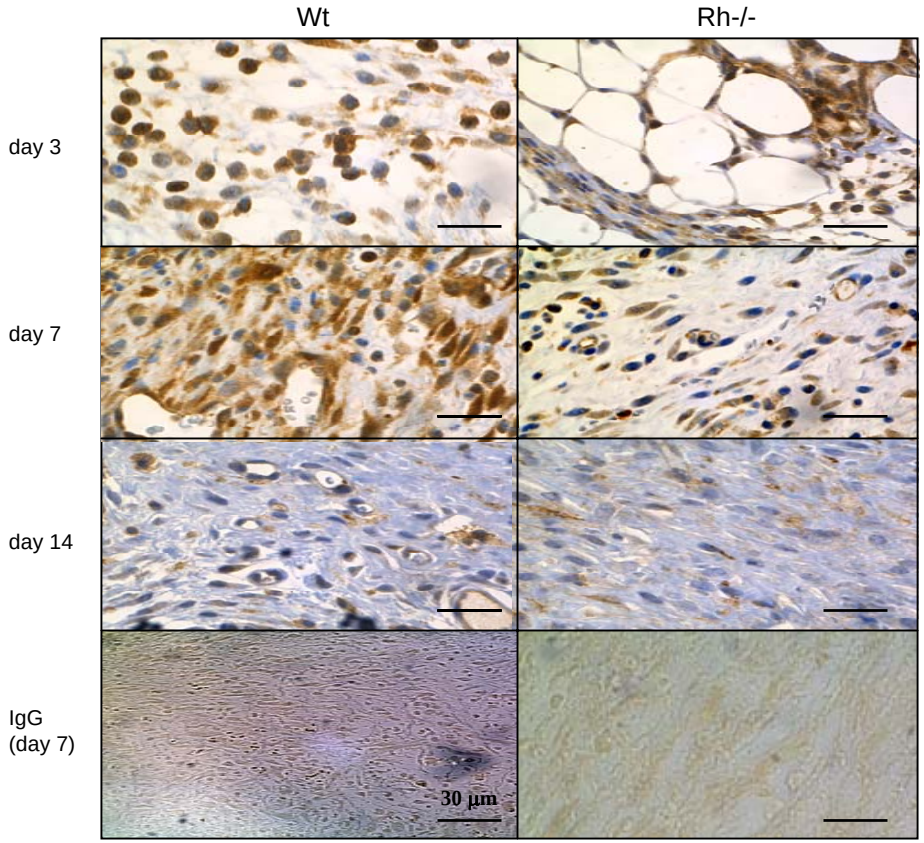


Figure 3. (Tolg et al.) **Loss of Rhamm alters ERK1,2 activation in granulation tissue fibroblasts**



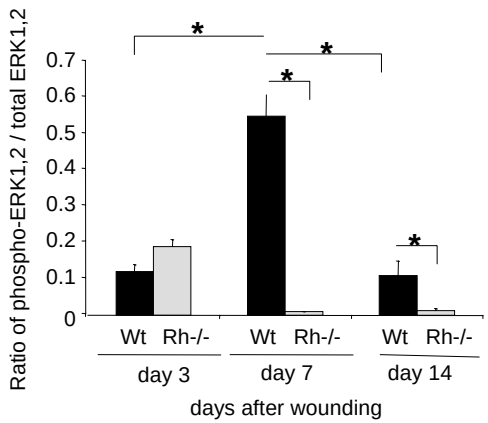
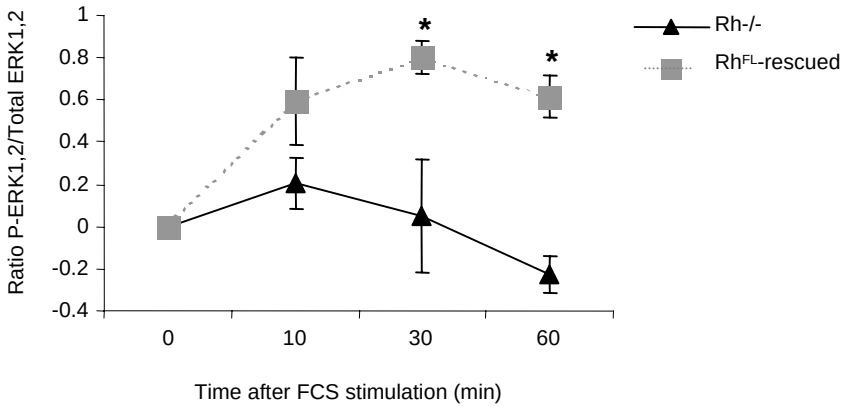
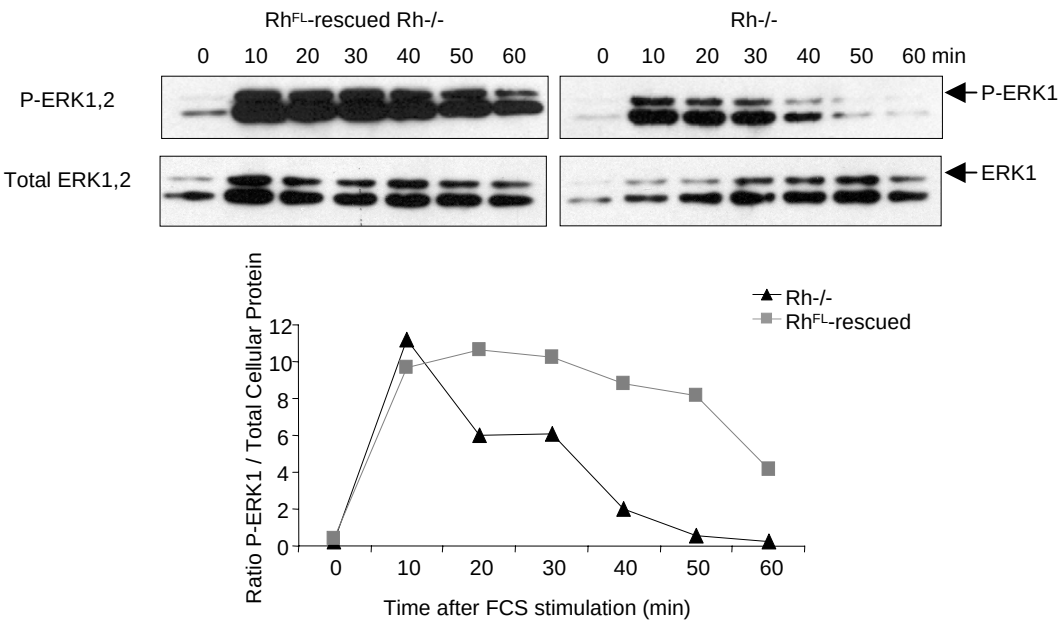


Figure 4. (Tolg et al.) Loss of Rhamm reduces serum activation of ERK1,2 in fibroblasts *in culture*

A. ELISA analysis of phospho-ERK1,2



B. Western blot analysis of phospho-ERK1



C. Confocal micrographs of phospho-ERK1,2

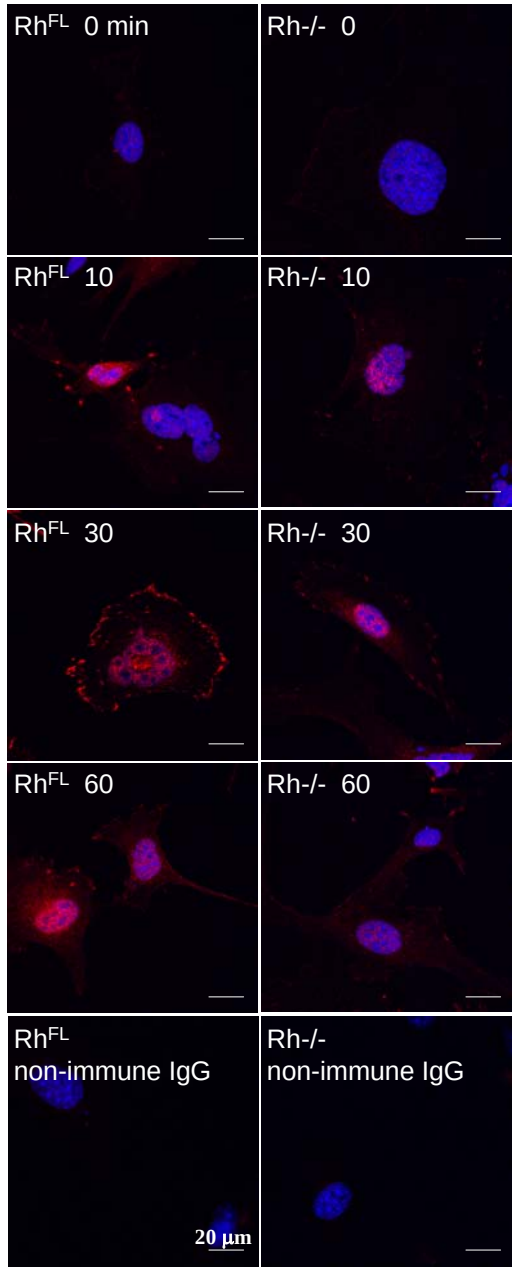
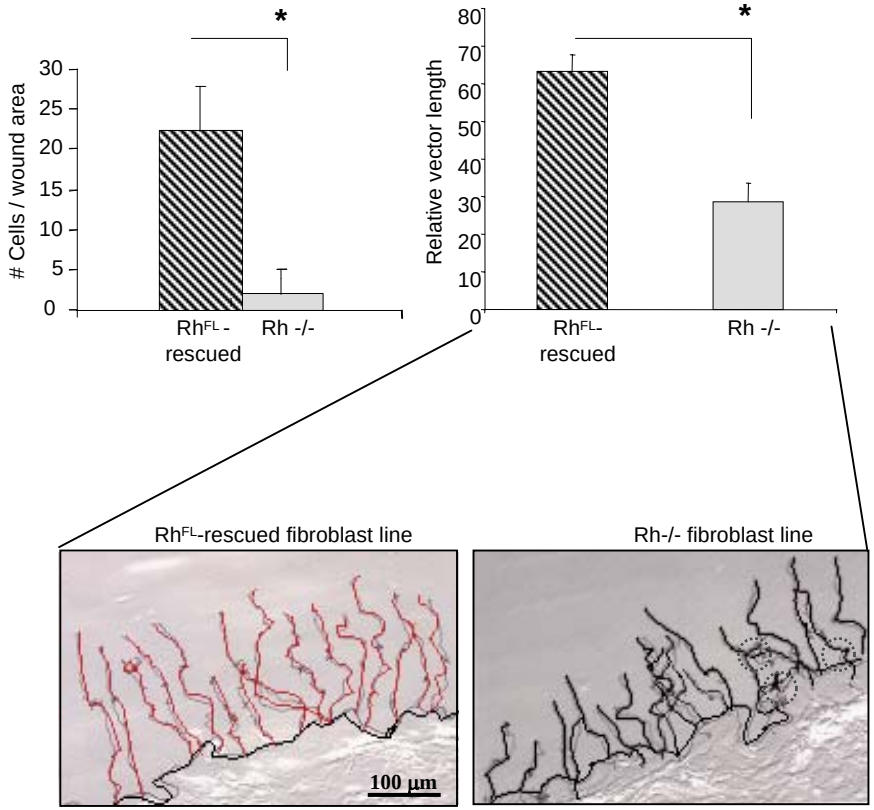


Figure 5. (Tolg et al.) Loss of Rhamm reduces fibroblast migration and invasion

A. Migration into scratch wounds



B. Invasion into collagen gels

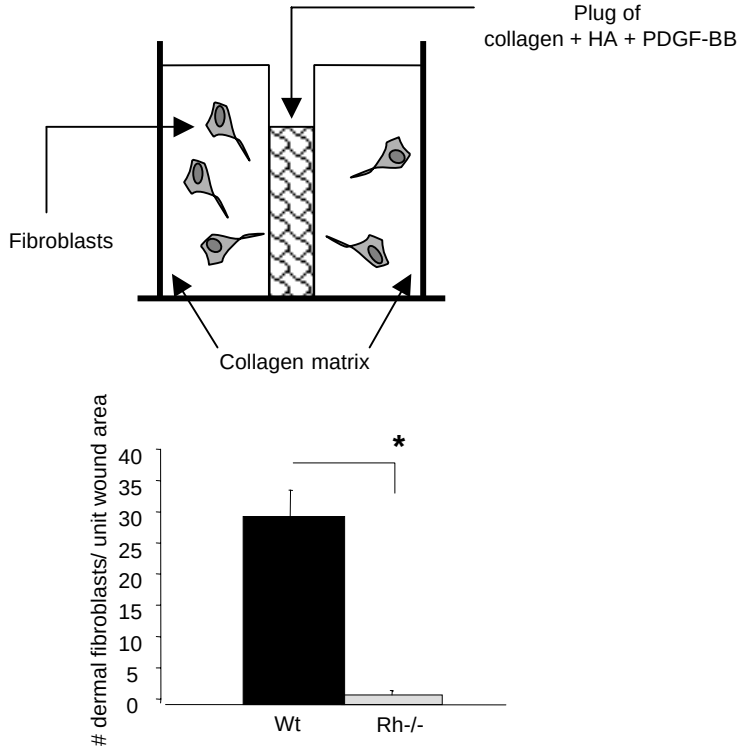
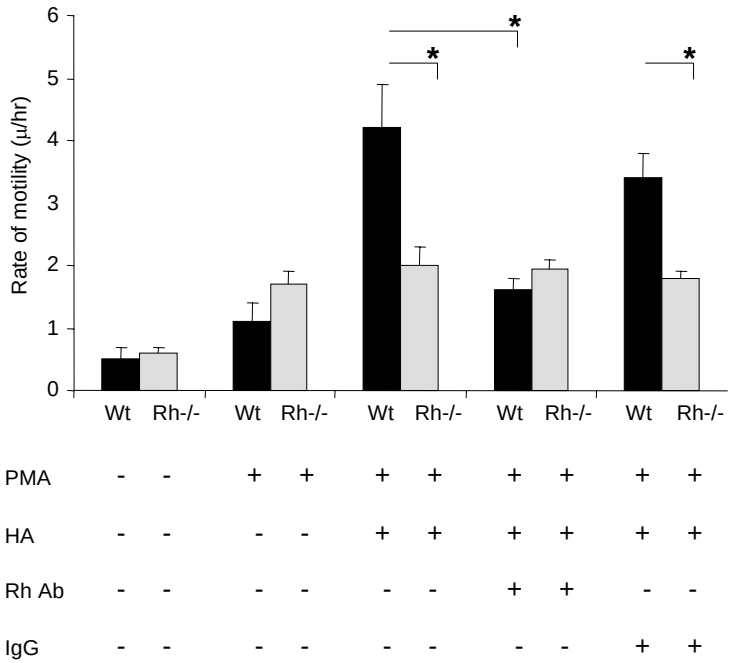


Figure 6. (Tolg et al.) Loss of Rhamm ablates migration of fibroblasts in response to HA

A. Random motility in response to HA



B. CD44 protein expression

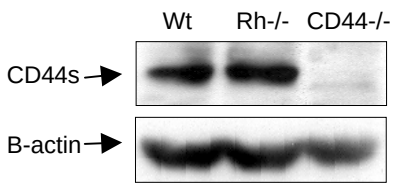


Figure 7. (Tolg et al.) Loss of Rhamm and inhibition of Mek1 activity reduce actin cytoskeleton disassembly

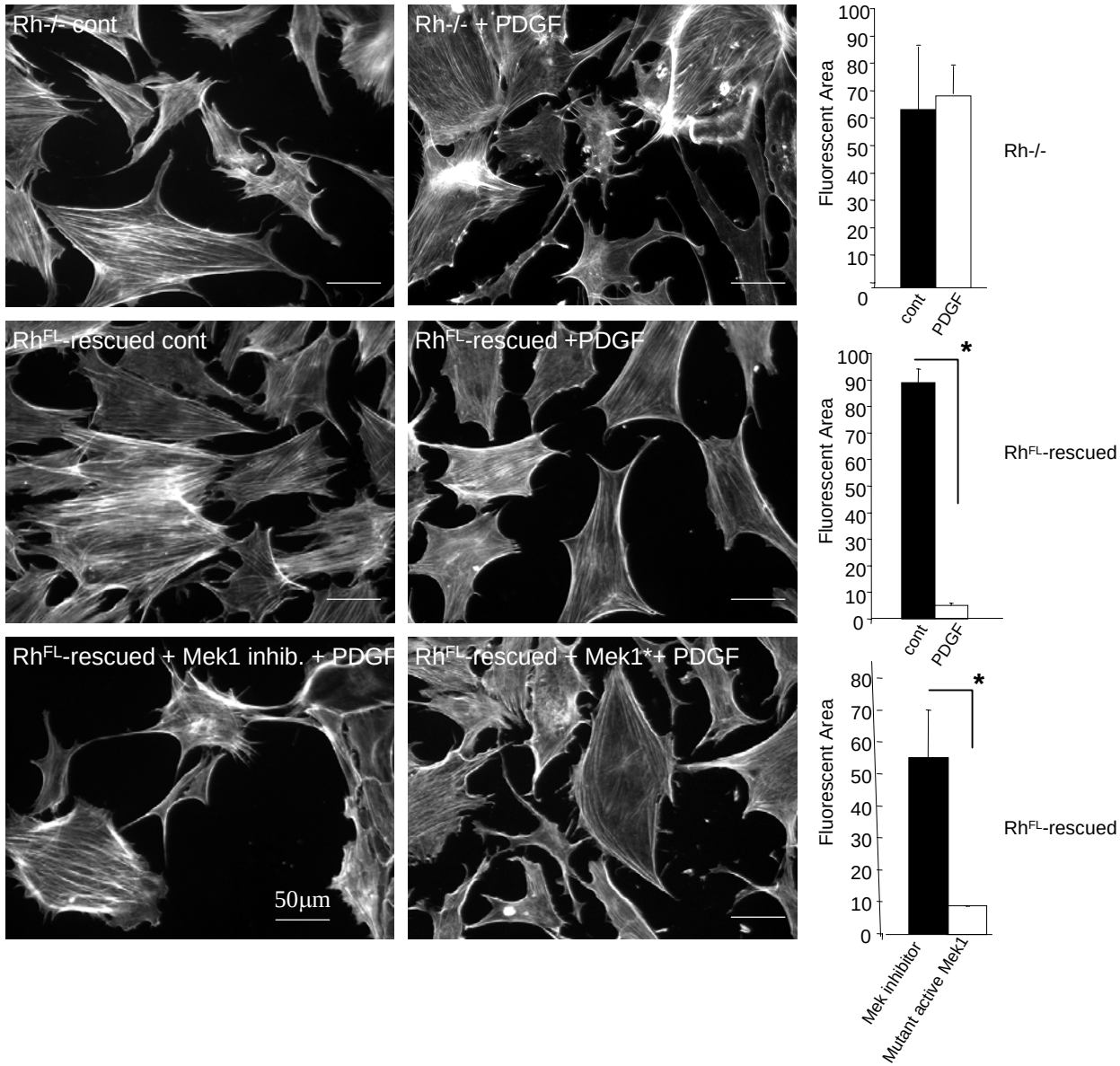
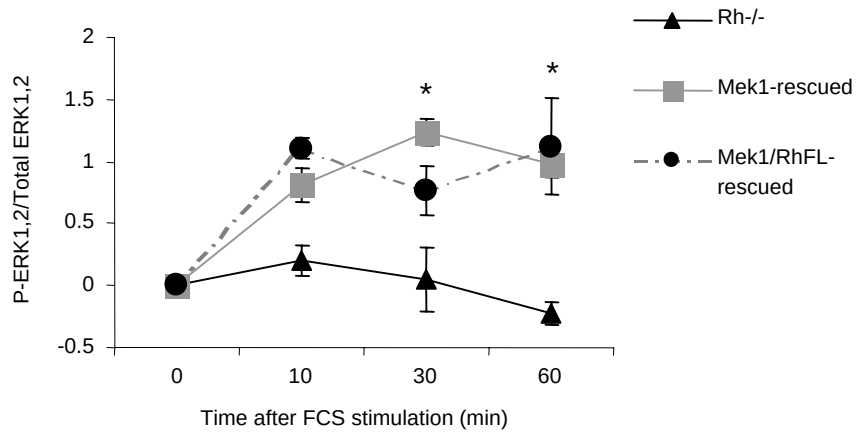


Figure 8. (Tolg et al.) Mutant active Mek1 rescues aberrant ERK1,2 activity in Rhamm-/- fibroblasts

A. ELISA analysis of ERK1,2 activity



B. Western blot analysis of ERK1,2 activity

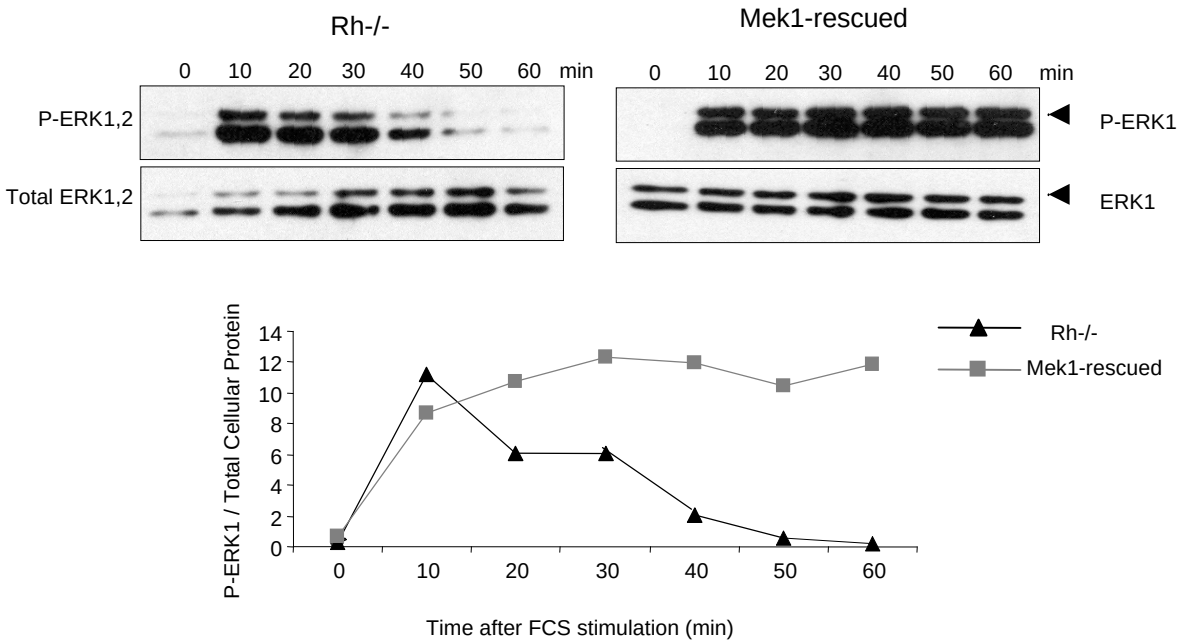
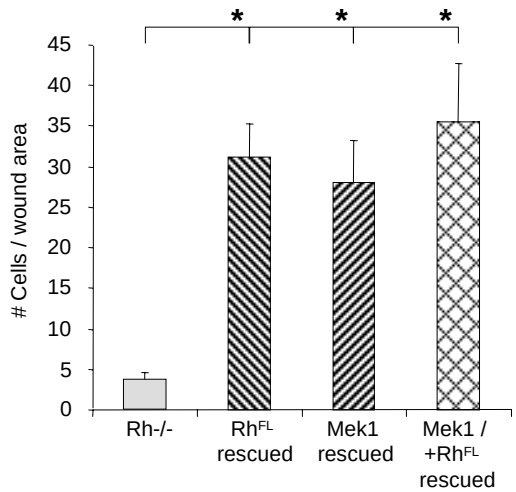
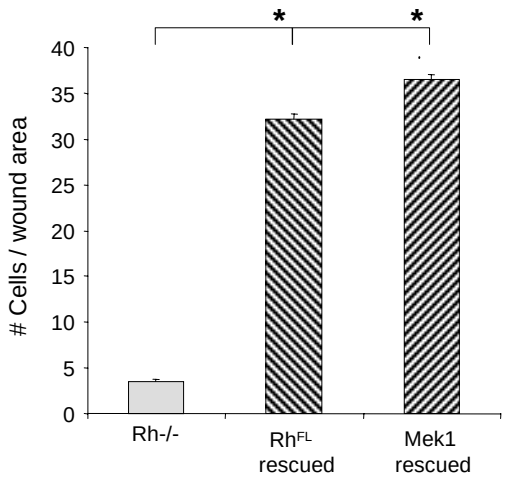


Figure 9. (Tolg et al.) Mutant active Mek1 rescues migration and invasion defects of Rh-/- fibroblasts

A. Scratch wound migration assay



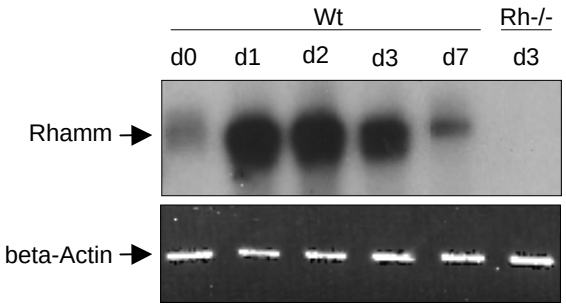
B. Collagen gel invasion assay



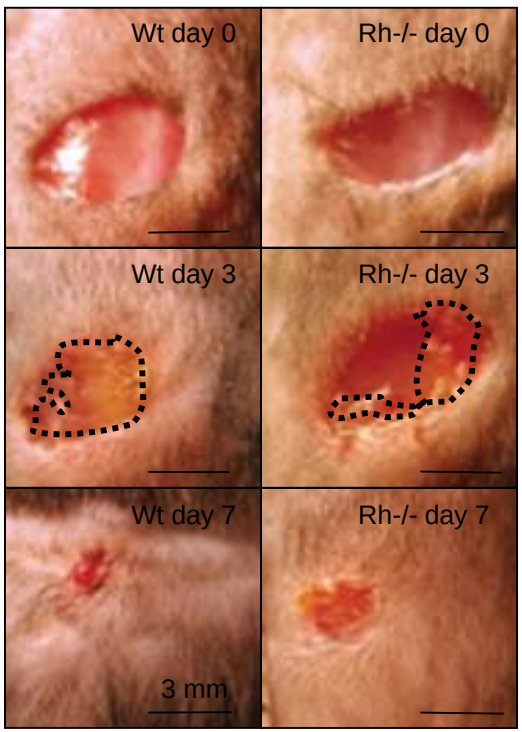
Supplemental Figure I. (Tolg et al.)

Rhamm expression is regulated during early phases of wound repair and loss of Rhamm reduces wound contraction.

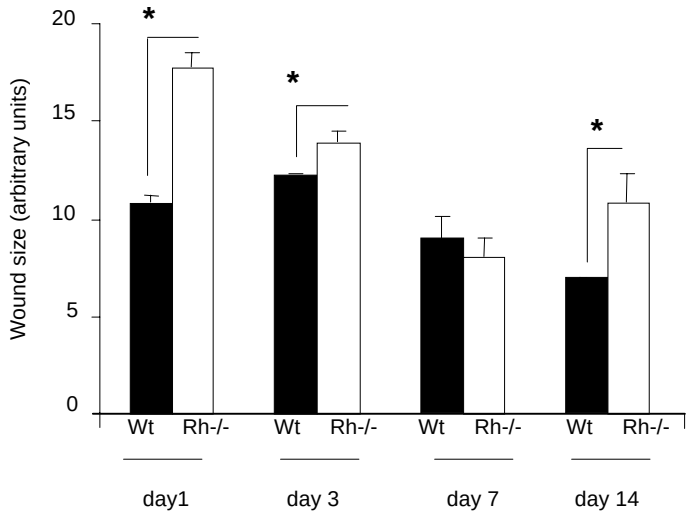
a. Rhamm expression in excisional wounds

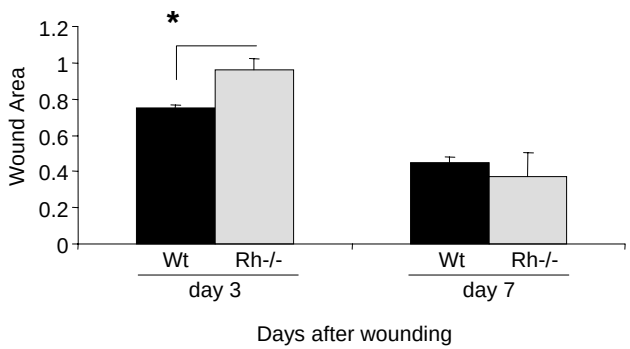


b. Macroscopic quantification of wound contraction



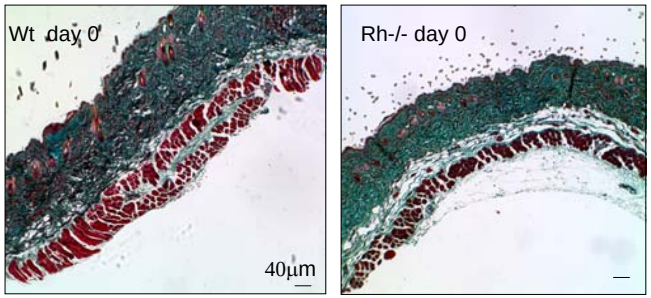
c. Microscopic quantification of wound contraction

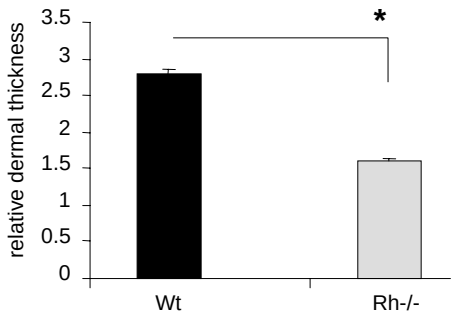




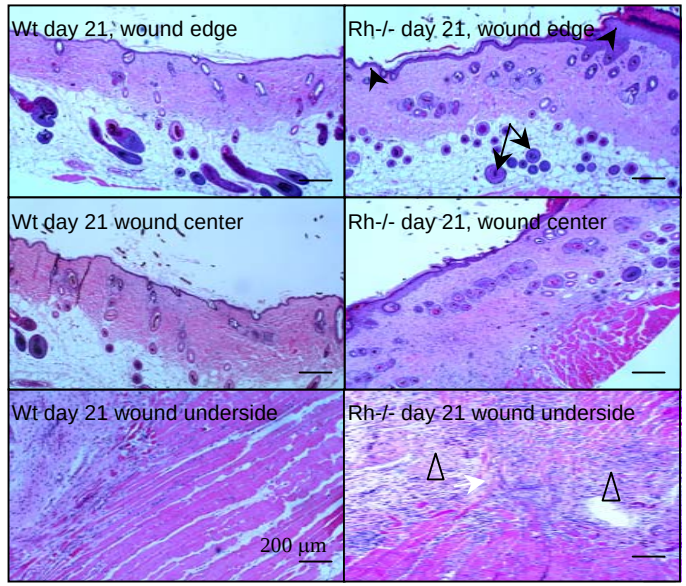
Supplemental Figure II. (Tolg et al.) Loss of Rhamm expression results in aberrant dermal structure and thickness in both uninjured and repaired skin.

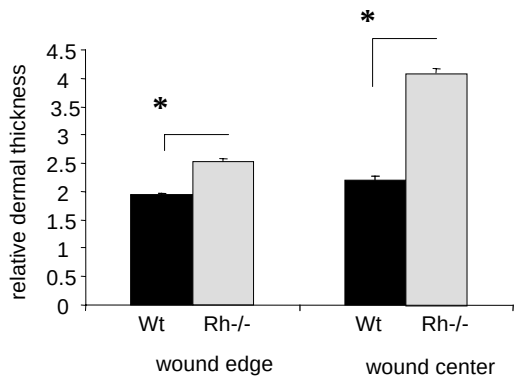
a. Dermis of uninjured skin





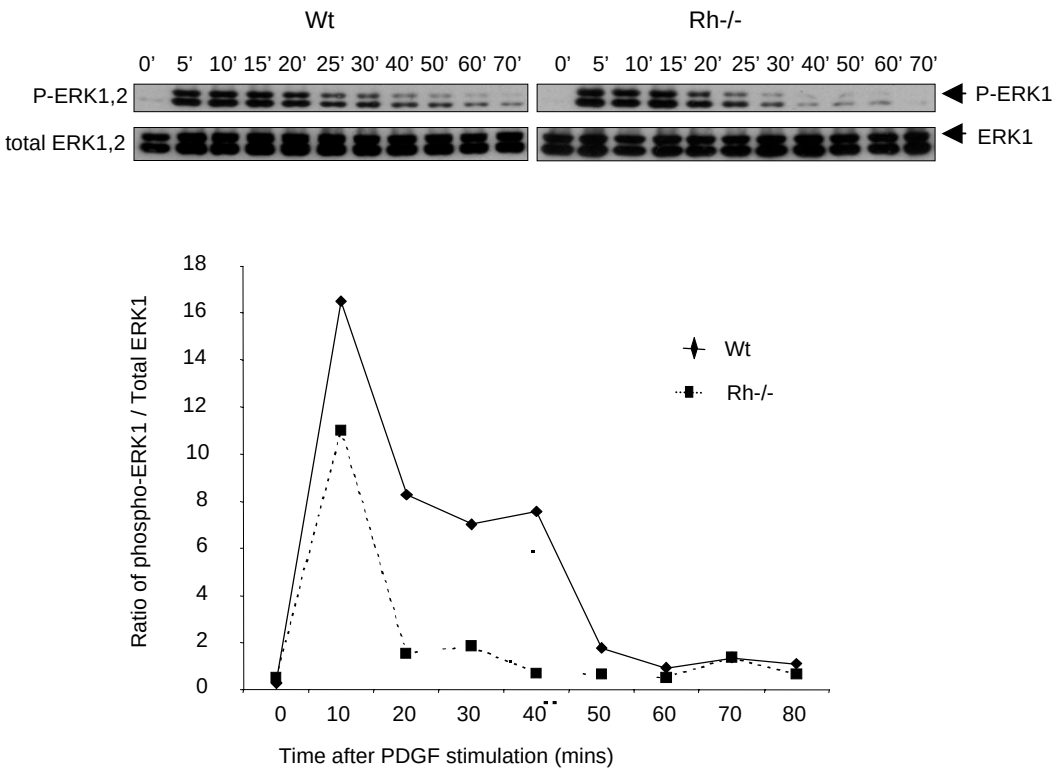
b. Dermis of resolved wounds



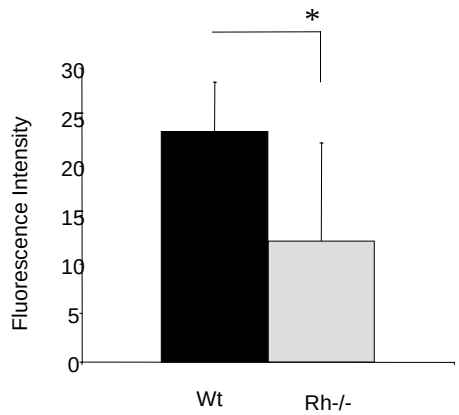


Supplemental Figure III. (Tolg et al.) Rhamm loss reduces ERK1,2 activation in primary fibroblasts

a. Western blot analysis of ERK1 activation in response to PDGF-BB

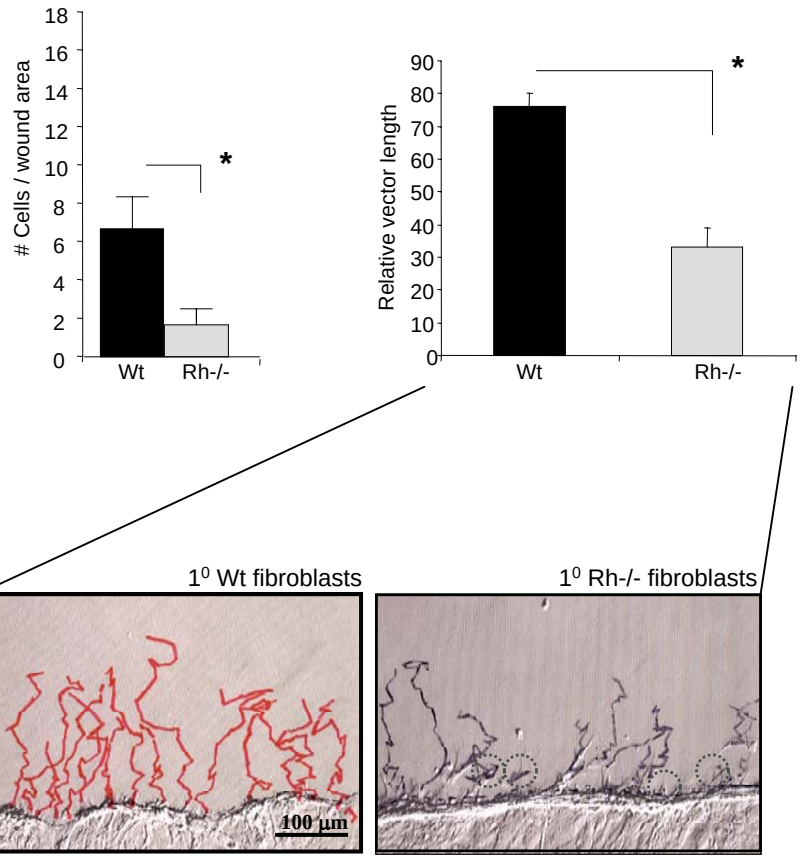


b. Quantification of confocal analysis of ERK1,2 activation in response to FCS

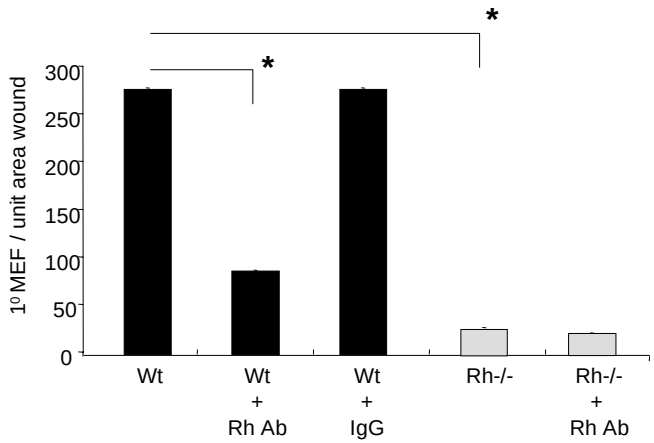


Supplemental Figure IV. (Tolg et al.) Loss of Rhamm reduces migration and invasion of Wt and Rh-/- primary fibroblasts

a. Migration into scratch wounds

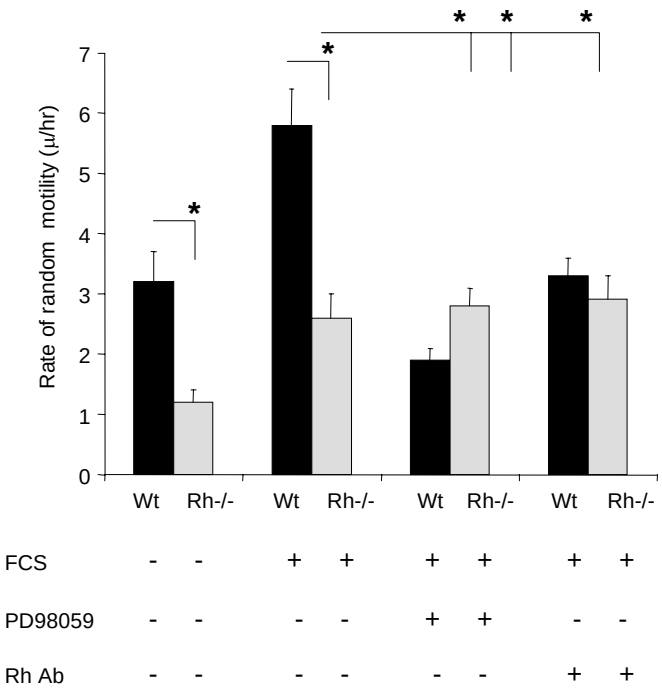


b. Invasion into collagen gels



Supplemental Figure V. (Tolg et al.) ERK1,2 activity is required for a motogenic response to PDGF-BB and mutant active Mek1 rescues the motogenic defect of Rh-/- fibroblasts.

a. Effects of Mek1 inhibition on random motility



b. Motility of Rh-/- fibroblasts expressing mutant active Mek1