Impaired cell death and mammary gland involution in the absence of Dock1 and Rac1 signaling

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Throughout life, the tight equilibrium between cell death and the prompt clearance of dead corpses is required to maintain a proper tissue homeostasis and prevent inflammation. Following lactation, mammary gland involution is triggered and results in the death of excessive epithelial cells that are rapidly cleared by phagocytes to ensure that the gland returns to its prepregnant state. Orthologs of Dock1 (dedicator of cytokinesis 1), Elmo and Rac1 (ras-related C3 botulinum toxin substrate 1) in Caenorhabditis elegans are part of a signaling module in phagocytes that is linking apoptotic cell recognition to cytoskeletal reorganization required for engulfment. In mammals, Elmo1 was shown to interact with the phosphatidylserine receptor BAI1 and relay signals to promote phagocytosis of apoptotic cells. Still, the role of the RacGEF Dock1 in the clearance of dying cells in mammals was never directly addressed. We generated two mouse models with conditional inactivation of Dock1 and Rac1 and revealed that the expression of these genes is not essential in the mammary gland during puberty, pregnancy and lactation. We induced mammary gland involution in these mice to investigate the role of Dock1/Rac1 signaling in the engulfment of cell corpses. Unpredictably, activation of Stat3 (signal transducer and activator of transcription 3), a key regulator of mammary gland involution, was impaired in the absence of Rac1 and Dock1 expression. Likewise, failure to activate properly Stat3 was coinciding with a significant delay in the initiation and progression of mammary gland involution in mutant animals. By using an in vitro phagocytosis assay, we observed that Dock1 and Rac1 are essential to mediate engulfment in epithelial phagocytes. In vivo, cell corpses accumulated at late time points of invasion in Dock1 and Rac1 mutant mammary glands. Overall, our study demonstrated an unsuspected role for Dock1/Rac1 signaling in the initiation of mammary gland involution, and also suggested a role for this pathway in the clearance of dead cells by epithelial phagocytes.

Cell Death and Disease (2014) 5, e1375; doi:10.1038/cddis.2014.338; published online 14 August 2014

Programmed cell death is essential for embryonic development by contributing to tissue remodeling and elimination of transient cells such as during digit formation. Prompt clearance of apoptotic cells is necessary to prevent cellular leakage that could induce autoimmune reactions. A key ‘eat-clearance of apoptotic cells is necessary to prevent cellular remains unclear if a direct binding of CrkII to Dock1 is needed. Glycoprotein Mfge8 facilitates binding of the target cells to phosphatidylserine (PtdSer) on apoptotic cells by the phagocyte-secreted orthologs of Mfge8 in mammals was never directly addressed. We generated two mouse models with conditional inactivation of Dock1 and Rac1 and revealed that the expression of these genes is not essential in the mammary gland during puberty, pregnancy and lactation. We induced mammary gland involution in these mice to investigate the role of Dock1/Rac1 signaling in the engulfment of cell corpses. Unpredictably, activation of Stat3 (signal transducer and activator of transcription 3), a key regulator of mammary gland involution, was impaired in the absence of Rac1 and Dock1 expression. Likewise, failure to activate properly Stat3 was coinciding with a significant delay in the initiation and progression of mammary gland involution in mutant animals. By using an in vitro phagocytosis assay, we observed that Dock1 and Rac1 are essential to mediate engulfment in epithelial phagocytes. In vivo, cell corpses accumulated at late time points of invasion in Dock1 and Rac1 mutant mammary glands. Overall, our study demonstrated an unsuspected role for Dock1/Rac1 signaling in the initiation of mammary gland involution, and also suggested a role for this pathway in the clearance of dead cells by epithelial phagocytes.

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Received 11.12.13; revised 08.7.14; accepted 09.7.14; Edited by H-U Simon

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Abbreviations: Dock1, dedicator of cytokinesis 1; Rac1, ras-related C3 botulinum toxin substrate 1; Stat3, signal transducer and activator of transcription 3; NMuMG, normal murine mammary gland; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling

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The first phase, approximately 2 days and reversible, is characterized by the shedding of epithelial cells in the lumen of ducts. The second and irreversible phase is characterized by the secretion of active metalloproteases and the recruitment of macrophages to assist the MECs in removing dying cells.14

Signals promoting the initiation of involution converge toward the activation of the transcription factor Stat3 (signal transducer and activator of transcription 3) that is critical such that involution is delayed in the absence of its expression.15 The secreted factor Lif contributes to Stat3 activation in the gland during the first phase of involution to promote cell death through a lysosomal-mediated pathway.16 Activation of Stat3 by Lif leads to an upregulation of the Oncostatin receptor that promotes Stat3 activation in the second phase of involution by engaging its ligand Oncostatin.17 During this latter period, Stat3 uniquely promotes the death of MEC through apoptosis. While Stat3 activation in the mammary gland induces a specific gene response, the mechanisms governing Stat3 activation remain poorly defined.17–19

The molecular pathways implicated in the clearance of dying epithelial cells are being unraveled. The PtdSer-glycoprotein Mteg8 decorates dying epithelial cells for rapid clearance by phagocytes during involution.20,21 The molecular intermediates necessary for engulfment during involution remain largely uncharacterized. Here, we investigated the role of Dock1/Rac1 (ras-related C3 botulinum toxin substrate 1) in the clearance of epithelial cells during mammary gland involution. By using mouse models with inactivation of Dock1 and Rac1 in mammary gland epithelial cells, we reveal an unsuspected role for these genes in the activation of Stat3 during involution, which coincide with a delay in the initiation of mammary gland involution. Moreover, we observed that Dock1 and Rac1 mediate engulfment of apoptotic corpses by epithelial phagocytes.

Results

Ablation of Dock1 and Rac1 in the mammary gland. Orthologs of Dock1, Elmo and Rac are part of one of two signaling cascades that control engulfment in C. elegans.3 We generated mice with inactivation of Dock1 and Rac1 in the mammary gland epithelial compartment by crossing animals carrying floxed (flx) alleles of these genes with MMTVCre+ transgenic mice to examine their roles during cell clearance using mammary gland involution as an experimental model. We confirmed that expression of Cre led to the recombination of the Dock1 and Dock1 flx alleles in the mammary gland (Supplementary Figures S1a and S1b) and that Rac1 and Dock1 are expressed in wild-type mammary glands at lactation day 10 (Figures 1a and 2a). Importantly, we observed that Cre-mediated genetic ablation of Rac1 and Dock1 reduced their levels of expression in the mammary glands of MMTVCre+ Rac1flx/flx and MMTVCre+ Dock1flx/flx animals, respectively, as verified by western blot (Figures 1a and 2a).

Rac1 and Dock1 mutant mice have normal mammary gland development. Before addressing the importance of Dock1 and Rac1 in cell clearance during involution, we investigated whether these genes are required during mammary gland puberty, pregnancy and lactation. The role of Dock1 during mammary gland development was previously assessed and it was shown that it is not required for proper mammary gland development.22 Whole-mount mammary gland outgrowth analyses of MMTVCre+ Rac1wt/wt and MMTVCre+ Rac1flx/flx animals at 9, 12 and 15 weeks confirmed that Rac1 is not required for mammary gland development during puberty (Supplementary Figure S2). Furthermore, whole-mount analyses confirmed that Dock1 and Rac1 are not required for mammary gland remodeling during pregnancy (Supplementary Figure S3). To exclude the possibility that Dock1 and Rac1 are required for the progression to the lactating state, we stained Lac10 (10 days of lactation) glands of both genotypes for the lactation marker pStat5.23 These analyses demonstrated a robust activation of pStat5 in epithelial cell in both Dock1 and Rac1 mutants that was comparable to control glands (Supplementary Figure S4). The staining of pStat5 was rapidly lost upon entering the involution state (Supplementary Figure S4). Whole-mount staining of Lac10 glands demonstrated an identical gross morphology for both genotypes in comparison with controls (data not shown). Both MMTVCre+ Rac1flx/flx and MMTVCre+ Dock1flx/flx models were fertile. In addition, pups derived from MMTVCre+ Rac1flx/flx and MMTVCre+ Dock1flx/flx females were viable and reached adulthood without any signs of deterioration in health or weight. These results confirm the normal lactating state of Dock1 and Rac1 mutant animals. We investigated by semiquantitative RT-PCR if the expression of Rac1 or Dock1 is regulated during involution. We found that these genes are expressed during both lactation and involution (Supplementary Figure S5).

Adipocyte repopulation is delayed in Rac1 and Dock1 mutant mice. To investigate the role of Rac1 during involution, pups were removed from the nursing mother after Lac10 to induce involution in a synchronous manner. Histologic analysis was carried on in MMTVCre+ Rac1wt/wt and MMTVCre+ Rac1flx/flx animals to observe the progression of morphologic changes after 0 to 4 days of forced involution (Lac10, Inv1, Inv2, Inv3 and Inv4). At Lac10, majority of the gland in MMTVCre+ Rac1wt/wt and MMTVCre+ Rac1flx/flx animals was composed of alveolar structures filled with milk produced by the epithelial cells and no difference was observed between genotypes, which correlates with the ability of mutants mice to nurse their progeny (Figure 1b). After 1 day of involution, and more noticeable after 2 days, fat cell repopulation could be observed in the mammary gland parenchyma of MMTVCre+ Rac1wt/wt animals (Figure 1b). Unexpectedly, the mammary glands of MMTVCre+ Rac1flx/flx animals retained the appearance of a lactating gland after 1 day of involution (Figure 1b). The presence of adipocytes could be detected after 2 days of involution but was significantly delayed in the absence of Rac1 expression (Figure 1b). These results were confirmed by detecting adipocytes with anti-Perilipin, a marker protein found in fat droplets24 (Figure 1d). Notably, adipocytes accounted for only a small fraction of cells, present in same quantities, in both genotypes at Lac10. Quantification of the area occupied by adipocytes in the mammary glands of
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Figure 1  Mammary gland involution is delayed in MMTVCre<sup>+</sup> Rac<sup>flx/flx</sup> mice. (a) Western blot analysis demonstrating the absence of Rac1 expression in Lac10 mammary glands of MMTVCre<sup>+</sup> Rac<sup>flx/flx</sup> mice. (b) H&E staining of mammary glands at 10 days of lactation and after 1, 2, 3 or 4 days of involution showing delayed repopulation of adipocytes in MMTVCre<sup>+</sup> Rac<sup>flx/flx</sup> mice (scale bar: 100 μm, × 20). (c) Average percentage of the area occupied by adipocytes at the indicated lactation and involution days in MMTVCre<sup>+</sup> Rac<sup>wt/wt</sup> and MMTVCre<sup>+</sup> Rac<sup>flx/flx</sup> mammary gland. Twenty random sections were analyzed and quantified from each mouse (n = 6). Data were shown as mean ± S.E.M. The indicated P-values were calculated by using a two-tailed Student’s t test. NS, not significant, *P ≤ 0.05 and **P ≤ 0.01. (d) IHC analysis showing Perilipin staining in mammary glands at 10 days of lactation and after 1, 2, 3 and 4 days of involution showing delayed repopulation of adipocytes in MMTVCre<sup>+</sup> Rac<sup>flx/flx</sup> mice (scale bar: 100 μm, × 20x). (e) Average percentage of the area occupied by Perilipin-positive cells at the indicated lactation and involution days in MMTVCre<sup>+</sup> Rac<sup>wt/wt</sup> and MMTVCre<sup>+</sup> Rac<sup>flx/flx</sup> mice. Ten random sections were analyzed and quantified from each mouse (n = 3). Data were shown as mean ± S.E.M. The indicated P-values were calculated by using a two-tailed Student’s t test. *P ≤ 0.05

21 kDa — Rac1
50 kDa — Tubulin
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Figure 2  Mammary gland involution is delayed in MMTVCre⁺ Dock/+ mice. (a) Western blot analysis demonstrating the absence of Dock1 expression in Lac10 mammary glands of MMTVCre⁺ Dock/+ mice. (b) H&E staining of mammary glands at 10 days of lactation and after 1, 2, 3 or 4 days of involution showing delayed repopulation of adipocytes in MMTVCre⁺ Dock/+ mice (scale bar: 100 μm, × 20). (c) Average percentage of the area occupied by adipocytes at the indicated lactation and involution days in MMTVCre⁺ Dock/+ and MMTVCre⁺ Dock/+ mammary gland. Twenty random sections were analyzed and quantified from each mouse (n = 6). Data were shown as mean ± S.E.M. The indicated P-values were calculated by using a two-tailed Student’s t-test. NS, not significant, *P ≤ 0.05 and ***P ≤ 0.001. (d) IHC analysis showing Perilipin staining in mammary glands at 10 days of lactation and after 1, 2, 3 and 4 days of involution showing delayed repopulation of adipocytes in MMTVCre⁺ Dock/+ mice (scale bar: 100 μm, × 20). (e) Average percentage of the area occupied by Perilipin-positive at the indicated lactation and involution days in MMTVCre⁺ Dock/+ and MMTVCre⁺ Dock/+ mice. Ten random sections were analyzed and quantified from each mouse (n = 3). Data were shown as mean ± S.E.M. The indicated P-values were calculated by using a two-tailed Student’s t-test. NS, not significant, *P ≤ 0.05 and **P ≤ 0.01.
MMTVCre⁺ Rac1wt/wt and MMTVCre⁺ Rac1flx/flx animals at the indicated time points further confirmed the presence of a delay in the initiation of involution in mutant animals (Figures 1c and e).

Similarly, the progression of involution was studied in MMTVCre⁺ Dock1wt/wt and MMTVCre⁺ Dock1flx/flx animals. At Lac10, the majority of the gland in MMTVCre⁺ Dock1wt/wt and MMTVCre⁺ Dock1flx/flx animals was occupied by adipocytes in the mammary glands of animals after synchronous removal of the pups (Figure 2b). The mammary gland of MMTVCre⁺ Dock1flx/flx animals remains the entrance of a lactating gland after 1 day of involution (Figure 2b). Moreover, a delay in the re-emergence of adipocytes in the gland was observed at all time points analyzed in Dock1 mutants (Figure 2b). No difference in adipocytes was detected between genotypes at Lac10. These results were confirmed when adipocytes were stained with anti-Perilipin (Figure 2d). Quantification of the area occupied by adipocytes in the mammary glands of MMTVCre⁺ Dock1wt/wt and MMTVCre⁺ Dock1flx/flx animals confirmed the significant delay observed in the initiation and progression of involution in the absence of Dock1 expression (Figures 2c and e). Collectively, these results revealed an unsuspected requirement of Dock1 and Rac1 for the initiation of mammary gland involution.

Dock1 and Rac1 are required to activate Stat3 during involution. Activation of the transcription factor Stat3 is essential to initiate involution.25 Notably, mammary gland involution is delayed in Stat3 mutants.15 We previously reported that Stat3 activation is reduced in mammary neoplastic lesions induced by the HER2/Neu oncogene in Dock1 mutant mice.22 We hypothesized that a failure to maximally activate Stat3 upon milk accumulation in the mammary gland could contribute to the involution delays observed in the absence of Rac1 and Dock1 expression. We investigated the phosphorylation status of Stat3 during involution in control and mutant animals by performing immunohistochemistry (IHC) and immunofluorescence (IF) analyses. Stat3 activation, measured by detecting phospho-(p)-Stat3Y705, was induced and sustained in MECs after lactation and involution days in MMTVCre⁺ Stat3wt/wt mice (scale bar: 100 µm, × 20). (b) Average number of the p-Stat3-positive MECs normalized by the total number of nuclei (%) at the indicated lactation and involution days in MMTVCre⁺ Stat3wt/wt and MMTVCre⁺ Stat3flx/flx mice. Ten random sections were analyzed and quantified from each mouse (n = 3). Data were shown as mean ± S.E.M. The indicated P-values are calculated by using a two-tailed Student’s t-test. NS, not significant, *P < 0.05, **P < 0.01 and ***P < 0.001.

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Figure 3 Stat3 activation is delayed in the absence of Rac1 expression during involution. (a) IHC analysis showing p-Stat3 staining in mammary glands at 10 days of lactation and after 1, 2, 3 and 4 days of involution in MMTVCre⁺ Stat3wt/wt and MMTVCre⁺ Stat3flx/flx mice (scale bar: 100 µm, × 20). (b) Average number of the p-Stat3-positive MECs normalized by the total number of nuclei (%) at the indicated lactation and involution days in MMTVCre⁺ Stat3wt/wt and MMTVCre⁺ Stat3flx/flx mice. Ten random sections were analyzed and quantified from each mouse (n = 3). Data were shown as mean ± S.E.M. The indicated P-values are calculated by using a two-tailed Student's t-test. NS, not significant, *P < 0.05, **P < 0.01 and ***P < 0.001.

animals (Figure 4a). We found a decrease in the levels of p-Stat3 at 1 and 2 days of involution in the absence of Dock1 expression (Figure 4a). Similar observations were made when p-Stat3 positive cells were analyzed by IF (Supplementary Figures S6 and S7). Quantification of the amount of p-Stat3-positive cells normalized on the total number of nuclei further confirmed the initial delay observed in the activation of Stat3 when Dock1 is absent (Figure 4b and Supplementary Figure S8). At Inv4, levels of p-Stat3 reached a maximum in mutant animals. These results indicate that Dock1 and Rac1 are required to activate Stat3 during involution.
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**Dock1 and Rac1 contribute to the engulfment of dead corpses in epithelial cells.** We hypothesize that Dock1 and Rac1 could contribute to dying cell clearance during involution. Because of the complexity of the in vivo phenotypes with respect to cell death initiation, we first addressed directly this issue by testing the requirement of Dock1 and Rac1 during engulfment by MECs. Normal murine mammary gland (NMuMG) epithelial cells were transfected with a CTRL siRNA or siRNAs targeting Dock1 or Dock1. Western blot analyses confirmed the downregulation of these proteins (Figures 7a and b). Following transfection, cells were presented with apoptotic thymocyte targets labeled with CFSE (Supplementary Figure S12). The engulfment of apoptotic targets was observed using confocal microscopy (Figure 7c) and the percentage of engulfed apoptotic thymocytes was quantified (Figure 7d). After 2 h of incubation, most apoptotic thymocytes were engulfed in CTRL cells. In contrast, only a small proportion of the thymocytes was engulfed by siRac1 or siDock1 NMuMG cells (Figure 7d). The same tendency was observed after 5 h of engulfment (data not shown). These results demonstrate that Dock1 and Rac1 are required to promote dying cell clearance by MECs in vitro.

To investigate if Dock1 and Rac1 contribute to removing superfluous cells in vivo, we stained late time points of involution for TUNEL for the presence of persistent dying cells. We found a significant accumulation of TUNEL-positive cells at Inv6 and Inv8 in Rac1 mutant mammary glands (Supplementary Figure S10). Likewise, a persistent detection of TUNEL-positive cells was observable at Inv8 in Dock1 mutant glands (Supplementary Figure S11). These data suggest that Dock1 and Rac1 contribute to promote dying cell clearance by MECs in vivo.

**Discussion**

Upon accumulation of milk in lactating mammary glands, the death of excessive epithelial cells is induced and these cells are promptly removed by neighboring epithelial cells and by infiltrating phagocytes. Mammary gland involution is essential to ensure that the tissue returns to a prepregnancy state and that it conserves its ability to initiate another cycle. Orthologs of Dock1, Elmo and Rac in *C. elegans* were
described as part of a signaling module promoting engulfment. Here we investigated the role of Dock1 and Rac1 in the engulfment of dead corpses in mammals by using mammary gland involution as a model. While we revealed that Dock1 and Rac1 expression contribute in epithelial phagocytes to mediate dead cell clearance in vitro and in vivo, our studies unexpectedly revealed a role for Dock1 and Rac1 in the initiation of involution. Dock1- and Rac1-null mammary glands failed to activate properly Stat3 such that a delay in the morphologic changes associated with involution was observed in mutant animals.

Most studies on Dock1 and Rac1 with respect to their roles in apoptosis focused on signaling in engulfing cells. However, inactivation of Dock1 or Rac1 promotes cell death in epiblasts. Likewise, downregulation of Dock1 in cardiomyocytes predisposes them to apoptosis. In contrast, our study highlights a role for Dock1 and Rac1 as proapoptotic proteins. A complex formed of CrkII-Dock1 relocates to the nucleus to...
promote cell death in fibroblasts. Whether such a relocalization of Dock1 also occurs in predying MECs should be explored. The ability of a phagocyte to engage a preapoptotic cell is a required step for full execution of apoptosis; a possibility in our model is that impaired engulfment lengthens the survival of cells destined to die.

We observed that the delay in adipocyte repopulation during involution is more robust in Dock1 than in Rac1 mutant mammary glands. We speculate that Rac2 or Rac3 could be acting redundantly or could be compensating for the loss of Rac1. Further expression profiling and generation of double or triple Rac mutants could confirm this hypothesis. Likewise, it is possible that Dock5 in part compensates for the loss of Dock1; we reported a genetic interaction between these RacGEFs during myoblast fusion.

Stat3 is both an orchestrator of mammary gland involution and an inducer of cell proliferation and invasion in breast cancer cells. This transcription factor is classically activated downstream of a variety of receptors including cytokines receptors, growth factor receptors and G-protein-coupled receptors. Although the mechanisms still remain unclear, signaling by Rac contributes to Stat3 activation. Binding of the MgcRacGAP/Rac1 complex to Stat3 promotes Stat3 phosphorylation on Y705. MgcRacGAP is also proposed to work as a nuclear chaperone and facilitates nuclear translocation of the MgcRacGAP/Rac1/Stat3 complex. Moreover, cadherin engagement was reported to increase the levels of activated Rac1 that promotes IL-6 production. In this context, signaling by Rac acts upstream of IL-6 production and indirectly contributes to Stat3 activation. We previously reported that Stat3 activation in small mammary gland neoplasic lesions induced by HER2 is reduced in the absence of Dock1. Here, we observed that Stat3 activation at the onset of involution is diminished in the absence of Rac1 and Dock1. We propose that Dock1 is an important GEF-activating Rac1 and promoting Stat3 activation in both physiologic and pathologic conditions. It will be important to identify the molecular intermediates that act upstream/downstream of Dock1/Rac1 to connect to Stat3 signaling pathway during mammary gland involution and breast cancer. One candidate that could relay the signals induced by milk accumulation to Dock1/Rac1/Stat3 is p130Cas. It was previously shown that mechanical stretching of cells at focal adhesion induced a conformational change in p130Cas that allow sensing of the mechanical force to be converted into a biochemical signaling. Through its binding with the adaptor protein Crk, p130Cas forms a complex with Dock1 and could relay mechanical stimuli to Rac1. As milk is proposed to induce mechanical stretching in the duct, it will be interesting to investigate the contribution of p130Cas to mammary gland involution. A previous study revealed that overexpressing p130Cas in the mammary gland induced a delay in mammary gland involution.

Our study also revealed that Dock1/Rac1 signaling is required in epithelial phagocytes to promote engulfment of dead cells. This is the first study to address and demonstrate a possible conservation of the function of Dock1 in the clearance of dead cells in mammals. Although we observed an accumulation of apoptotic cells in late involution time points in Dock1/Rac1 glands, it is possible that professional

Figure 7 Phagocytosis of apoptotic cells by MECs requires Rac1 and Dock1 expression. (a) Western blot analysis demonstrating siRNA-mediated knockdown of Rac1 expression in NMuMG cells. (b) Western blot analysis demonstrating siRNA-mediated knockdown of Dock1 expression in NMuMG cells. (c) Representative confocal microscopy images showing defective engulfment of apoptotic thymocytes by NMuMG cells transfected with siRac1 and siDock1 (scale bar: 5 μm; red: phalloidin staining; blue: Hoechst; green: CFSE). (d) Average percentage of apoptotic thymocytes engulfed by NMuMG cells transfected with siCTRL, siRac1 and siDock1 after 2 h (n = 3). Data were shown as mean ± S.D. The indicated P-values are calculated by using one-way ANOVA, followed by a Bonferroni’s multiple comparisons test. ****P ≤ 0.0001
phagocytes have infiltrated the remodeling glands to assist the epithelial cells in the removal of dying cells. The Dock1-binding partner Elmo1 was previously shown to act downstream of the PtdSer receptor Ba1 to promote clearance of apoptotic cells and Elmo1 mutant animals have a defect in the clearance of apoptotic germ cells in the testis. \(^8,^9\) It will remain to be determined whether Ba1 is expressed and required by epithelial cells to promote activation of the Dock1/Elmo1/Rac1 module to induce clearance of dead epithelial cells during mammary gland involution. Alternatively, other phagocytic receptors such as integrins or Tyro3, Axl or MerTK could be critical to clear dying cells through Dock1/Rac1 signaling. Interestingly, MerTK inactivation, but not AXL-Tyro3 double deletion, prevents prompt clearance of dying MECs. \(^3^9\) Genetic inactivation of the integrin ligand Mgf8, which decorates dying MECs, leads to involution defects. \(^2^0,^2^1\) Dock1 operates downstream of this receptor–ligand module to promote engulfment by immature dendritic cells. \(^6\) Inactivation of genes coding for autophagy proteins, Becn and Apg7, impairs clearance of dying MECs during involution; it was reported that Mgf86-binding integrin Itgb5 and MerTK are poorly expressed in these mouse models, explaining impaired removal of dead corpses. \(^5^0\) The exact receptors using the Dock1/Rac pathway for clearance of dying MECs remain to be established.

We previously observed that higher levels of Dock1 expression are associated with a worst prognosis in basal and HER2-positive breast cancer patients and showed that the HER2 oncogene uses Dock1 to promote breast cancer progression. \(^2^2\) Several studies highlighted that the mammary gland stroma is prometastatic during involution and epimorphology studies suggest that the period surrounding pregnancy is transiently associated with an increase risk of breast cancer. \(^4^1,^4^2\) Breast cancer is still the most common cancer in women worldwide; therefore, a better comprehension of the molecular mechanisms regulating all aspects of the mammary gland life cycle could provide important insights into the mechanisms that mediate tumorigenesis.

**Materials and Methods**

**Mice strains and animal care.** Rac1flx/flx mice were acquired from Jackson Laboratory (Bar Harbor, ME, USA) (STOCK Rac1flx/flx, no. 005550). Dock1flx/flx mice were generated in our laboratory and described previously. \(^2^2\) Transgenic MMTVCre \(^1\) mice were described previously. \(^4^3\) All mouse experiments were conducted according to the Canadian Council of Animal Care rules and were previously approved by the Institut de Recherches Cliniques de Montreal Animal Care Committee.

**Involvement studies.** Female MMTVCre \(^1\) Racflx/flx, MMTVCre \(^1\) Racflx/flx MMTVCre \(^1\) Dockflx/flx and MMTVCre \(^1\) Dockflx/flx mice, immediately following the first pregnancy and ongoing lactation, were used for involuton studies. Pups were allowed to suckle for 10 days postpartum and were then removed from the cage to force mammary gland involution. The mammary glands were collected at either Lac10 or after 1, 2, 3 or 4 days following pups removal (Inv1, Inv2, Inv3 and Inv4). At the time of analysis, the right-side fourth mammary glands (Mg4) was collected, fixed in 4% paraformaldehyde (PFA), paraffin embedded, sectioned at 5 \(\mu\)m for IHC analysis and stained with H&E for histologic analysis, while the right-side second mammary gland (Mg2) was collected for the protein study.

**Adipocyte repopulation.** Sections of mammary glands at Lac10 or Inv1–4 of involution were stained with H&E. Adipocytes were also stained by immunohistochemistry with an anti-Perilipin2 antibody (see below). Average percentage of the area occupied by adipocytes was quantified using the ImageJ software.

**Immunohistochemistry.** Paraffin-embedded sections were deparaffinized in xylene for 20 min, and rehydrated in decreasing concentrations of ethanol, 100%, 95%, 90%, 70% and 50% respectively. They were then washed three times in phosphate-buffered saline (1 \(\times\) PBS) and subjected to antigen retrieval in 10 mM sodium citrate (pH 6.0) or 1 mM EDTA (pH 8.0) at 95°C. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide and sections were washed with PBS, permeabilized with PBSST (1 \(\times\) PBS, 0.5% Triton X-100 and 0.02% Tween), blocked in PBSST, 1% bovine serum albumin (BSA) at RT and then were incubated with primary antibody diluted in PBSST 1% BSA overnight. Sections were washed three times in PBSST and incubated with secondary antibody diluted in PBSST 1% BSA. They were then washed three times in PBSST, incubated with secondary antibody diluted in PBSST 1% BSA and washed three times in 1 \(\times\) PBS. Antigen localization of washed sections was performed with DAB Peroxidase Substrate Kit (no. SK 4100; Vector Laboratories, Burlington, ON, Canada).

**Immunofluorescence.** Paraffin-embedded sections were deparaffinized in xylene for 20 min, and rehydrated in decreasing concentrations of ethanol, 100%, 95%, 90%, 70% and 50% respectively. They were then washed three times in PBS and subjected to antigen retrieval in 1 mM EDTA (pH 8.0) at 95°C. Sections were washed with PBS, permeabilized with PBSST, blocked in PBSST 1% BSA at RT and then were incubated with primary antibody diluted in PBSST 1% BSA overnight. Sections were washed three times in PBT (1 \(\times\) PBT, 0.2% Triton X-100) and incubated with secondary antibody diluted in BBT (1% BSA) for 30 min. They were then washed three times in PBT and incubated with Alexa Fluor 633 phalloidin (5:200 dilution) (A22824; Invitrogen, Burlington, ON, Canada) in PBT for 30 min, followed by three washes with PBT. Hoechst (33342, Invitrogen) was applied to fluorescently labeled sections and a coverslip was sealed using nail polish. Images were taken using a Zeiss LSM710 confocal microscope (Carl Zeiss, Toronto, ON, Canada) and analyzed using the Zen 2009 software (Carl Zeiss).

**Whole-mount analysis of mammary glands.** Whole-mount analyses of mammary glands were realized as described elsewhere. \(^2^2\) Briefly, Mg4s from MMTV-Cre \(^1\) Racflx/flx and MMTV-Cre \(^1\) Racflx/flx mice at 9, 12 and 15 weeks of puberty or Mg4s from MMTVCre \(^1\) Racflx/flx MMTVCre \(^1\) Racflx/flx MMTVCre \(^1\) Dockflx/flx and MMTVCre \(^1\) Dockflx/flx mice at 12 days of pregnancy were collected, fixed in 4% PFA, washed with 70% ethanol, defatted two times with acetone and stained in carmine red solution overnight. Tissues were rehydrated in increasing concentrations of ethanol, 70%, 50% and 100% respectively. They were then cleared in xylene and mounted with Permount (no. SP15-500; Fisher, Ottawa, ON, Canada). Main and side branches from the duct were quantified from each virgin mammary gland by counting each branch in the mammary gland section as described elsewhere. \(^4^4\) Alveolar lobules were quantified from each pregnant mammary gland at 12 days of pregnancy.

**Cell culture and siRNA transfection.** NMuMG cells were grown in DMEM supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine, 20 mM HEPES (pH 7.4), 10 \(\mu\)g/ml insulin and penicillin/streptomycin. NMuMG cells at 70% of confluence were transfected with siCTRL, siDock1 or siRacl (180 nM, siGENOME Smartpool Dharmacon, Lafayette, LA, USA) using Lipofectamine 2000.

**Western blot analyses.** Total tissue extracts from mammary glands were prepared as described elsewhere. \(^4^5\) Briefly, frozen mammary glands were homogenized using a tissue grinder (no. 358003; Wheaton, Millville, NJ, USA) in 2 ml extraction buffer (25 mM Tris (pH 7.4), 150 mM NaCl). They were then centrifuged and fat was removed from the supernatant. The homogenates were next lysed in Radio ImmunoPrecipitation Assay (RIPA) buffer (50 mM Tris (pH 7.5), 0.1% SDS, 0.5% deoxycholic acid, 1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA). NMuMG cells were directly lysed in RIPA. Equal amount of proteins were subjected to SDS-PAGE and proteins of interest were detected by immunoblotting as described previously. \(^4^6\)

**Phagocytosis assay and microscopy.** Thymus from 5-week-old mice were dissected, cut into small pieces, transferred into a culture dish containing RPMI, 10% FBS and washed two times with the same medium. Freshly isolated thymocytes were then incubated with 10 \(\mu\)M dexamethasone (no. D2915; Sigma-Aldrich, Oakville, ON, Canada) at 37°C for 6 h in RPMI containing 10% FBS to induce apoptosis. Annexin V-FITC/propidium iodide (PI) staining was performed to evaluate apoptosis levels. Apoptotic thymocytes (2 \(\times\) 10\(^3\) cells) were labeled with CFSE (no. C34554; Invitrogen) according to the manufacturer's instructions.
CFSE-labeled apoptotic thymocytes were then added to 5.0 × 10^4 NMuMG cells transfected with siCTRL, siDock1 or siRac1 that were replated in four-well culture slides at 24 h posttransfection (no. 354104; BD Biosciences) and reached 50% confluence. Apoptotic targets were incubated with epithelial cells for 2 h to allow phagocytosis. Cells were washed gently with 1 × PBS, fixed in 4% PFA for 10 min, washed three times with PBT and blocked with BLOT overnight. They were washed three times with PBT, incubated with Alexa Fluor 633 phalloidin (5:200 dilution) (A22824; Invitrogen) in PBT for 30 min, followed by three washes with PBT. Hoechst (33342) (Invitrogen) was applied to fluorescently labeled sections and the coverslip was sealed using nail polish. Images were taken using a Zeiss LSM710 confocal microscopy (Carl Zeiss) and analyzed using the Zen 2009 software (Carl Zeiss).

Antibodies for immunohistochemistry, IF and western blotting.

Antibodies used for IHC were as follows: Perilipin (1:100; no. 3470S; Cell Signaling Technology, Danvers, MA, USA) p-Stat3 (1:100; no. 9145S; Cell Signaling Technology), cleaved caspase-3 (1:200; no. 96618; Cell Signaling Technology), p-Stat3 (1:200; no. 9314S; Cell Signaling Technology). Antibodies used for IF were as follows: p-Stat3 (1:100; no. 9145S; Cell Signaling Technology). Antibodies used for western blotting were as follows: Rac1 (1:3000; no. 05-398; Upstate Biotechnology, Lake Placid, NY, USA); Dock1 (1:1000; no. sc-6043; Santa Cruz Biotechnology, Dallas, TX, USA); tubulin (1:10,000; no. T5168; Sigma-Aldrich); p-Stat3 (1:700; no. 9132S; Cell Signaling Technology).

RNA isolation and RT-PCR from mammary gland tissues. Total RNA from mammary gland tissues was extracted using RNeasy Mini Kit (no. 74104; Qiagen) according to the manufacturer’s instructions. The purity of the RNA samples was determined by visualizing intact 18S and 28S rRNA bands by agarose gel electrophoresis. One microgram of total RNA was used for each RNA samples was determined by visualizing intact 18S and 28S rRNA bands by agarose gel electrophoresis. One microgram of total RNA was used for each sample. cDNA synthesis was carried out using M-Mulv Reverse Transcriptase (M0233S; New England Biolabs) according to the manufacturer’s instructions. Primers for RT-PCR were as follows: Rac1 (forward: 5'-CTGAAGTGCGACAC-CTGCTG-3' and reverse: 5'-CTGCTCCGCAAGTT-CTGAAGTGCGACAC-3'); Dock1 (forward: 5'-GGCTTACAGTTGCTTCG-3' and reverse: 5'-TACTGCTCCGCAAGTT-CTGAAGTGCGACAC-3'); and β-Actin (forward: 5'-GGCTTATTCCTCCTGC-3' and reverse: 5'-CCAGTTGGACACACTGTCATCT-3').

TUNEL assay. Paraffin-embedded mammary gland tissues were subjected to TUNEL assay using DeadEnd Fluorometric TUNEL System Kit (no. G3250; Promega, Madison, WI, USA). Briefly, paraffin-embedded sections were deparaffinized in xylene for 20 min, and rehydrated in decreasing concentrations of ethanol, 100%, 95%, 70%, 50% and 10% respectively. Sections were washed in 0.85% NaCl for 5 min, rinsed in PBS for 5 min, fixed in 4% PFA for 10 min and washed again with PBS for 5 min. The liquid from the tissue section was removed and 100 µl of the 20 µg/ml Proteinase K solution was added onto each section and incubated for 10 min at RT. Sections were then washed with PBS, fixed in 4% PFA for 10 min and washed again with PBS. Sections were then incubated with 100 µl of equilibration buffer for 10 min. The liquid was removed and was incubated with 50 µl of rtTdT incubation buffer at 37 °C for 60 min inside a humidified chamber covered with aluminum foil. Sections were then washed with 2 × SSC (sodium-sodium citrate) buffer for 15 min at RT to stop the reaction, and then rinsed three times in PBS. Hoechst (33342, Invitrogen) was applied to sections and a coverslip was sealed using nail polish. Images were taken using a Zeiss LSM710 confocal microscopy (Carl Zeiss) and analyzed using Zen 2009 software (Carl Zeiss).

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. We thank Dr Morag Park and Dr Josie Ursini-Sigel for critical reading of the manuscript. We also thank Dr I Rhee and Dr A Veilette for their help with the phagocytosis assays. This work was funded by grants from the Canadian Cancer Society (no. 19104; and CIHR (MOP-77591) to JFC. WMJ is a recipient of a Canada Research Chair in Molecular Oncology. ML was supported by a scholarship of U Montreal and JH was a recipient of an Invitrogen-IRCM scholarship. JFC is a recipient of a Senior Investigator Award from Fonds de Recherche du Québec-Santé.

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Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)