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The Rho GTPase Rnd1 suppresses mammary tumorigenesis and EMT by restraining Ras-MAPK signalling

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Abstract

We identified the Rho GTPase Rnd1 as a candidate metastasis suppressor in basal-like and triple-negative breast cancer through bioinformatics analysis. Depletion of Rnd1 disrupted epithelial adhesion and polarity, induced epithelial-to-mesenchymal transition, and cooperated with deregulated expression of c-Myc or loss of p53 to cause neoplastic conversion. Mechanistic studies revealed that Rnd1 suppresses Ras signalling by activating the GAP domain of Plexin B1, which inhibits Rap1. Rap1 inhibition in turn led to derepression of p120 Ras-GAP, which was able to inhibit Ras. Inactivation of Rnd1 in mammary epithelial cells induced highly undifferentiated and invasive tumours in mice. Conversely, Rnd1 expression inhibited spontaneous and experimental lung colonization in mouse models of metastasis. Genomic studies indicated that gene deletion in combination with epigenetic silencing or, more rarely, point mutation inactivates *RND1* in human breast cancer. These results reveal a previously unappreciated mechanism through which Rnd1 restrains activation of Ras-MAPK signalling and breast tumour initiation and progression.

Introduction

Oncogenes such as Ras and BRAF deregulate mitogenesis but also induce senescence, which must be evaded through the acquisition of cooperating oncogenic mutations, such as loss of p53 or Rb (ref. 1). In the breast and other organs, progression to frank malignancy requires loss of epithelial adhesion and polarity and acquisition of an invasive phenotype[2]. In some cases, tumour cells hijack a developmental program of gene expression, the epithelial-to-mesenchymal transition (EMT), to gain an invasive capacity and disseminate[3]. The genetic or epigenetic alterations driving tumour initiation and progression in the most aggressive subtypes of breast cancer—basal-like and triple-negative—are incompletely understood.

Tumour initiation and EMT seem to be driven by distinct genomic alterations. Expression of mutant Ras from a knock-in allele or constitutive activation of ErbB2 coordinately disrupts epithelial adhesion, polarity and growth control, but does not induce full EMT in mammary epithelial cells[4, 5, 6]. In contrast, inactivation of the polarity proteins Scribble and Par 3 induces partial disruption of epithelial polarity, but not overproliferation[7, 8]. In addition, transcription factors, such as Snail and Twist, cause EMT but do not initiate transformation[4, 9, 10].

Rho GTPases regulate epithelial adhesion and polarity, cell migration, membrane traffic, and the cell division cycle[11]. Although infrequently mutated in most cancers, Rho, Rac and Cdc42 function downstream of mutant Ras to mediate transformation and to orchestrate the cytoskeletal changes required for tumour invasion[12]. As they govern several aspects of epithelial adhesion and polarity, Rho GTPases could also function as tumour suppressors. Here we show that inactivation of Rnd1 simultaneously induces mammary tumour initiation and EMT by activating oncogenic Ras-MAPK signalling.

RESULTS

Rnd1 is a potential suppressor of breast cancer progression

To identify Rho GTPases involved in breast tumour suppression, we used bioinformatic analysis and RNAi-mediated silencing. Kaplan–Meier analysis of the MSKCC DNA microarray data set, comprising predominantly advanced oestrogen-receptor-negative (ER^-) primary breast cancers[13], revealed that low levels of *RHOD* and *RND1*, but neither low nor high levels of other Rho GTPase transcripts, predict metastasis (Fig.1a and Supplementary Table1). Although Rnd1 and RhoD have not been implicated in tumorigenesis, 12q12-q13, which comprises *RND1*, is frequently deleted in pancreatic and adenoid cystic carcinomas[14, 15]. Silencing of Rnd1 induced scattering of cultured human mammary epithelial MCF-10A cells, disruption of adherens junctions, internalization and downregulation of E-cadherin, upregulation of N-cadherin and fibronectin, and a partial switch from the epithelial to the mesenchymal isoform of p120 catenin[16] (Fig. 1b–f). Quantitative PCR (qPCR) confirmed that E-cadherin was repressed by a transcriptional mechanism and potentially implicated the matrix metalloproteases MMP2 and MMP3 and the transcription factors Snail1, Zeb1, Zeb2 and Foxc2 in these phenotypic and functional changes (Fig. 1g). Control experiments confirmed the specificity and generality of these effects (Supplementary Fig. 1a–j). In contrast, silencing of RhoD induced apoptosis, but no scattering, as assessed by morphological criteria (Supplementary Fig. 1k, l). Thus, depletion of Rnd1, but not RhoD, causes an EMT.

Underexpression of Rnd1 correlated with expression of the 70-gene poor-prognosis signature[17] or the lung metastasis signature[13] in the MSKCC data set (Supplementary Fig. 1m) and was associated with increased risk of both lung and bone metastasis, suggesting that Rnd1 inhibits tissue invasion rather than organ-specific metastasis (Supplementary Fig. 1n, o). qPCR analysis of cancer cell lines and Oncomine analysis of breast cancer data sets revealed that *RND1* is expressed at significantly lower levels in the most aggressive subtypes of breast cancer (ER^-, basal-like and triple-negative; Fig. 1h and Supplementary Fig. 1p). Kaplan–Meier analysis of a data set comprising 2,324 patients[18] indicated that underexpression of Rnd1 correlates with a significantly reduced time to progression in ER^- but not ER^+ patients (Fig. 1i). Multivariate analysis indicated that underexpression of Rnd1 constitutes a strong, independent, negative prognostic factor (Supplementary Table 2). These observations identify Rnd1 as a potential suppressor of tumour progression and metastasis.

Inactivation of Rnd1 causes hyperproliferation followed by senescence

Rnd proteins are constitutively activated Rho GTPases, which exert cell-type-specific effects on signalling and cytoskeletal organization by interacting with multiple target-effectors[19]. As we could not identify or generate high-affinity antibodies reacting specifically with endogenous Rnd1 (Fig. 1b; Methods), we used qPCR to examine whether its expression varies during the cell cycle. Mitogens caused a progressive decline of Rnd1 messenger RNA, whereas exposure to TGF-β, contact inhibition, and ultraviolet light (UV) treatment exerted the opposite effect, suggesting that Rnd1 inhibits cell cycle progression (Supplementary Fig. 2a). Consistently, depletion of Rnd1 elevated the levels of phospho-Rb, Cyclin A and phospho-Histone H3 and the expression of cell cycle genes and increased cell cycle progression at day 3 post-infection, whereas ectopic expression counteracted these effects (Fig. 2a, b and Supplementary Fig. 2b–e and Table 3). At later time points, the percentage of Rnd1-silenced cells in S phase declined below control values, whereas the percentage of those in G2/M remained elevated (Fig. 2b). Following replating, the Rnd1-silenced cells underwent complete growth arrest and senescence (Fig.2c–f). Thus, inactivation of Rnd1 provokes transient hyperproliferation followed by premature senescence, as seen in strong oncogenic lesions.

Oncogene-induced senescence arises from aberrant DNA replication as well as production of reactive oxygen species (ROS), which cause DNA damage[20]. Rnd1-silenced MCF-10A cells exhibited activated CHK2, phosphorylated H2A.X, and elevated expression of genes involved in the DNA damage response, Ras-induced mitotic stress, and EMT (ref. 21; Fig. 2g and Supplementary Fig. 2d, f and Table 3). These cells accumulated elevated levels of ROS and treatment with glutathione (GSH) rescued them from senescence, suggesting that ROS-mediated DNA damage contributes to their senescence (Fig.2h, i). MCF-10A cells do not express p16 and p15 because of homozygous deletions at the *CDKN2A* and *CDKN2B* loci[22]. Rnd1 silencing led to accumulation of p27, but not p53 or p21, in these cells, and silencing of p27 rescued them from senescence, causally linking p27 to the induction of senescence in this setting (Supplementary Fig.2g and Fig.2j).

Myc rescues the Rnd1-silenced cells from senescence and enables their neoplastic conversion and invasive outgrowth

Prolonged culture enabled us to isolate clones of Rnd1-silenced MCF-10A cells, which had escaped senescence and were able to grow in soft agar (Supplementary Fig. 3a, b). These cells exhibited elevated c-Myc but not p27, suggesting that Myc may rescue the Rnd1-silenced cells from senescence by suppressing p27 (ref. 23; Supplementary Fig. 3c). Indeed, ectopic expression of Myc downregulated p27 and enabled the MCF-10A cells to escape from senescence and undergo transformation following inactivation of Rnd1 (Fig. 3a–d and Supplementary Fig. 3d–f). HMLE cells did not undergo senescence following Rnd1 depletion, presumably because they overexpress telomerase and lack Rb and p53 function[24], but instead exhibited robust soft agar growth (Supplementary Fig. 3g, h), confirming that loss of Rnd1 induces neoplastic transformation.

Expression of Myc enabled MCF-10A cells to form filled pseudo-acini with a significant degree of epithelial organization in three-dimensional (3D) Matrigel, as anticipated[8] (Fig. 3e, f). Additional inactivation of Rnd1 produced disorganized, expansive multi-acinar structures surrounded by a halo of dispersed cells (Fig. 3e, f). These structures had partially disorganized adherens junctions (E-cad) and a discontinuous or absent basement membrane (Lam-5). Isolated cells and groups of cells had detached from the multi-acinar structures consistent with basement membrane defects and invaded through Matrigel (Lam-5, arrow). These cells and those still within the structures had re-oriented their Golgi apparatus towards the Matrigel (GM130, small arrows). Whereas the pseudo-acinar structures induced by Myc underwent limited expansion because Myc induces modest overproliferation balanced by apoptosis[5,8], the multi-acinar structures formed by Rnd1-silenced cells underwent significant expansion as a result of significant overproliferation (Supplementary Fig. 3i–l). Thus, inactivation of Rnd1 promotes hyperproliferation, disruption of epithelial adhesion and polarity, and invasion.

Depletion of Rnd1 activates Ras signalling

Rnd proteins indirectly inhibit the activation of Rho and Ras GTPases, in a cell-context-dependent manner[19]. Rnd1 silencing, however, did not activate Rho proteins in MCF-10A cells (Supplementary Fig. 4a). Considering the similarity of the effects induced by inactivation of Rnd1 and activation of Ras[25], we examined the possibility that Rnd1 suppresses Ras signalling. GST–RBD pulldown indicated that Ras is robustly activated in MCF-10A cells at 2 and 5 days after short hairpin RNA (shRNA)-mediated depletion of Rnd1 (Fig. 4a). Immunoblotting with isoform-specific antibodies demonstrated activation of both H-Ras and K-Ras (Fig. 4b). Ras activation was particularly evident in the absence of mitogens and following peak stimulation with mitogens. This was also observed in HMLE cells, human umbilical vein endothelial cells (HUVECs) and HEK 293-T cells, attesting to its generality (Supplementary Fig. 4b, c).

In spite of its ability to activate Ras, depletion of Rnd1 did not rapidly activate the Raf–ERK cascade or AKT. Instead, short-term depletion of Rnd1 suppressed MEK and ERK activation in MCF-10A cells (Fig. 4a; compare days 2 and 5). Acute depletion of the Ras-GAP NF1 induces a similar effect because excessive Ras signalling is antagonized by strong negative feedback loops, which contribute to the induction of senescence26. In agreement with this interpretation, Rnd1 silencing upregulated several Ras target genes, which encode negative regulators of the Ras–ERK pathway, such as Sprouty, Spred and MAPK phosphatases (Fig. 4c). Furthermore, MEK and ERK inhibition correlated with CHK2 activation in Rnd1-silenced cells (Fig.

4a). Cells escaping from senescence after NF1 depletion are no longer restrained by negative feedback loops acting on Ras signalling and exhibit hypersensitivity to mitogenic stimulation26, 27. Similarly, Rnd1 depletion sustained the activation of the Raf–ERK pathway and AKT in response toEGF in Rnd1-silenced MCF-10A cells that had bypassed senescence (Fig. 4d). Thus, inactivation of Rnd1 induces robust activation of Ras and, following disengagement of negative feedback loops, enhances sensitivity of the Raf–ERK cascade to mitogenic stimulation.

Loss of Rnd1 promotes neoplastic transformation and EMT through Raf–ERK signalling

To examine whether loss of Rnd1 promotes neoplastic transformation and EMT by activating Ras signalling, we used pharmacological inhibition. Inhibition of MEK with PD98059 or UO126 blocked the disassembly of adherens junctions in Rnd1-silenced cells, but inhibition of PI(3)K with wortmannin or Rac with NSC23766 did not (Fig. 4e and Supplementary Fig. 4d). Inhibition of the Rho effectors Rho-kinase and Myosin II with Y-27632 and blebbistatin, respectively, caused partial disassembly of adherens junctions in control cells, as anticipated from the requirement for Rho signalling in their assembly[28], and did not interfere with their disruption following Rnd1 depletion (Supplementary Fig. 4e). Furthermore, inhibition of MEK suppressed the ability of Rnd1-silenced cells expressing Myc to grow in soft agar, and to invade through Matrigel, whereas inhibition of PI(3)K inhibited these processes to a lesser extent (Fig. 4f, g and Supplementary Fig. 4f). These results indicate that Rnd1 inactivation promotes neoplastic transformation and EMT by activating Raf–ERK signalling, consistent with the involvement of this pathway in both processes[29].

To examine the clinical relevance of our observations, we analysed the EMC192, EMC286 and MSKCC99 breast cancer DNA microarray data sets[13]. Gene set enrichment analysis (GSEA) indicated that underexpression of *RND1* correlated with expression of a Ras signature in all three data sets and with expression of a Myc signature in two of them, but not with expression of a Src or a β-catenin signature in any data set (Fig. 4h and Supplementary Fig. 4g). Underexpression of *RND1* correlated with overexpression of *MYC* in all of the data sets, corroborating the hypothesis that loss of Rnd1 cooperates with deregulation of c-Myc in human breast cancer (Supplementary Fig.4h). These findings suggest that inactivation of Rnd1 underlies aberrant activation of Ras signalling and cooperates with deregulated Myc in human breast cancer.

Rnd1 inhibits Ras signalling by activating the GAP domain of plexin B1

Rnd proteins bind to p190-RhoGAP and activate its GAP activity towards RhoA, causing disassembly of stress fibres and focal adhesions in fibroblasts[30]. In addition, Rnd1, but not Rnd2 or 3, combines with and activates the cytoplasmic GAP domain of Plexin B1 to mediate growth cone collapse in neurons treated with Sema4D (refs 31, 32). To examine the mechanism by which Rnd1 suppresses proliferation, we mutated four evolutionarily conserved residues in its effector loop and identified two loss-of-function mutations, T45A and E48A (Fig. 5a, b). These mutations disrupted the association of Rnd1 with Plexin B1 but not p190-RhoGAP. In contrast, the other two mutations did not affect binding to Plexin B1 or p190-RhoGAP (Fig. 5c). These results suggest that Rnd1 suppresses cell proliferation by activating the GAP domain of Plexin B1.

Plexin B1 is expressed at similar levels in both normal and neoplastic breast epithelial cells, whereas the levels of its ligand Sema4D are upregulated in breast cancer cells (Supplementary Fig. 5a), consistent with the hypothesis that Sema4D promotes tumour angiogenesis by engaging Plexin B2 on endothelial cells[33]. Plexin B1 silencing caused robust activation of Ras and phenotypic and functional changes associated with the EMT in MCF-10A cells (Fig. 5d–f and Supplementary Fig. 5b), consistent with the hypothesis that Rnd1 suppresses Ras by activating Plexin B1. Although Plexin B1 has been reported to combine with ErbB2 and c-Met to influence their signalling capacity[34, 35], treatment with Sema4D did not modify c-Met signalling in

HMLE cells or HER2 signalling in ZR751 cells (Supplementary Fig. 5c, d). These results suggest that Rnd1 suppresses Ras signalling and the EMT by activating the GAP domain of Plexin B1.

Plexin B1 inhibits Rap1 and activates p120 Ras-GAP

As Plexin B1 exerts a potent GAP activity towards Rap1, but not R-Ras, M-Ras or H-Ras (refs36, 37), we reasoned that the Rnd1–Plexin B1 complex suppresses Ras by inhibiting Rap1. In agreement with a requirement for Rnd1 in Plexin B1 function, siRNA-mediated depletion of Rnd1 caused activation of Rap1 in MCF-10A cells (Fig. 6a, b). Moreover, wild-type Rap1 and, even more so, constitutively active Rap1-V12 induced GTP loading on Ras and ensuing activation of CRAF, MEK and ERK (Fig. 6c). Finally, simultaneous inactivation of all Rap1 and Rap2 isoforms through expression of Rap1-GAP attenuated the basal activation of Ras in control MCF-10A cells and reversed the overactivation of Ras induced by depletion of Rnd1 (Fig. 6d). These results suggest that depletion of Rnd1 activates Ras by increasing GTP-loading on Rap1.

Intriguingly, GTP-Rap1 binds tightly to p120 Ras-GAP (encoded by *RASA1*) without undergoing GTP hydrolysis, making Rap1 an effective inhibitor of p120 Ras-GAP (refs 38, 39). Thus, the Rnd1–Plexin B1 complex may inhibit multiple Ras isoforms by inhibiting Rap1 and thereby activating p120 Ras-GAP. In agreement with this hypothesis, TCGA data sets indicated that *RND1*, *PLXNB1* and *RASA1* are underexpressed in largely non-overlapping and sizeable subsets of basal-like, but not luminal, breast cancers (Supplementary Fig. 6a). Furthermore, Rap1-V12 associated with p120 Ras-GAP, whereas wild-type Rap1 and Rap1-N17 did not (Fig. 6e). Finally, p120 Ras-GAP depletion caused robust activation of Ras, but not CRAF, MEK or ERK, in MCF-10A cells, consistent with an engagement of negative feedback loops (Fig. 6f and Supplementary Fig. 6b). Collectively, these results suggest that the Rnd1–Plexin B1 complex inhibits Rap1 and, hence, de-represses p120 Ras-GAP, leading to inhibition of Ras (Fig.6g).

Inactivation of Rnd1 induces mammary tumour initiation and progression *in vivo*

To study the tumour suppressor function of Rnd1 *in vivo*, we silenced its expression in mouse Comma-D cells, which possess a subpopulation of ductal–alveolar progenitor cells and can regenerate seemingly normal mammary glands on injection in the mammary fat pad[40]. On silencing of Rnd1, these cells underwent phenotypic and functional changes consistent with EMT and acquired the ability to grow in soft agar but did not become senescent, presumably because they are *Tp53* mutant[41] or overexpress c-Myc (Supplementary Fig. 7a–g). Intriguingly, whereas control cells formed seemingly normal glandular structures following orthotopic injection *in vivo*, the Rnd1-silenced cells produced highly undifferentiated and locally invasive tumours characterized by defective deposition of laminin, loss of expression of E-cadherin, and increased expression of vimentin (Fig.7a, b). In spite of their invasive ability, the Rnd1-silenced Comma-D cells did not colonize the lung within 6 weeks after tail-vein injection, consistent with the hypothesis that the EMT opposes metastatic reactivation[42]. We thus examined whether expression of Rnd1 could inhibit tumour cell dissemination and metastatic colonization. Bioluminescent imaging indicated that ectopic expression of Rnd1 suppresses the ability of ErbB2-transformed and 4T1 mammary tumour cells to colonize the lung following tail-vein injection (Fig. 7c and Supplementary Fig. 7h–j; >99% inhibition). To examine the ability of Rnd1 to inhibit spontaneous metastasis, we injected control and Rnd1-expressing 4T1 cells orthotopically. Having found that Rnd1 delays primary tumour growth (Fig.7d), we surgically removed bilateral tumours that had grown to a similar cumulative size in both cohorts and evaluated lung colonization after 1 week (Fig.7e). The results indicated that Rnd1 suppresses spontaneous metastasis to the lung (Fig.7f and Supplementary Fig. 7k). These experiments indicate that Rnd1 suppresses tumour initiation and progression *in vivo*.

Deletion and epigenetic inactivation of *RND1* in human breast cancer

Fluorescence *in situ* hybridization (FISH) analysis revealed allelic losses at the *RND1* locus in 17% of primary breast carcinomas from the University of Torino (Fig. 8a and Supplementary Fig. 8a). In addition to hemizygous losses, we detected reduced copy numbers in the context of Chr. 12 polysomy, suggesting that selective pressures drive deletion of *RND1* even within an amplicon[43]. The percentage of tumour cells with hemizygous deletions at the *RND1* locus was in many instances large (up to 73%) and in 55% of the cases >20% of the remaining tumour cells exhibited Chr. 12 monosomy (Supplementary Fig. 8a and Table 4). However, we did not identify homozygous deletions, suggesting that additional mechanisms contribute to inactivate *RND1* in breast cancer.

To examine the contribution of epigenetic mechanisms, we treated a subset of luminal and basal-like cell lines expressing very low levels of Rnd1 with the DNA methylation inhibitor 5-aza-2-deoxycytidine (5-Aza), the histone de-acetylase (HDAC) inhibitor SAHA, or both compounds. Simultaneous exposure to both inhibitors led to a large increase in expression of *RND1* in the basal-like lines, indicating that both promoter methylation and histone deacetylation suppress expression of *RND1* in these lines (Supplementary Fig. 8b). In contrast, the inhibitors were largely ineffective, even when combined, in most luminal lines (Supplementary Fig. 8b). SAHA alone rescued the expression of *RND1* in the HCC1428 cells, which are ER$^+$ but carry mutant *BRCA2*, a lesion typically occurring in triple-negative cancers[44]. Epityper analysis confirmed that the promoter of *RND1* is methylated extensively in HCC1569 cells and to a more limited degree in MBA-MB468 and BT474 cells (Supplementary Fig. 8e). 3-deazaneplanocin A (DZNep; ref45), which induces specific degradation of Polycomb Repressor Complex 2 components, cooperated with SAHA to rescue expression of *RND1* in the MDA-MB468, HCC1428 and BT474 cells (Fig. 8b). Other cell lines underwent rapid apoptosis with DZNep (ref.45). Silencing of the PRC2 component EZH2 produced similar effects (Supplementary Fig. 8f, g). Finally, EZH2 bound to the *RND1* promoter and deposited its repressive mark, H3K27me3, in HCC1569 and HCC1428 cells, but not ZR751 and MCF-10A cells (Fig.8c). These results suggest that promoter methylation and PRC2-mediated silencing contribute to suppressed expression of *RND1* in breast cancer cells.

Mutations of *RND1* in human breast cancer

To examine whether missense or nonsense mutations contribute to clonal inactivation of *RND1* in breast cancer, we subjected 96 breast cancers from the University of Hiroshima to targeted deep sequencing by SOLiD and identified 4 missense mutations, which replaced evolutionarily conserved amino acids of Rnd1 (Fig. 8d and Supplementary Table5). Structural considerations suggest that the *G70R* mutation, which replaces a switch II residue that is highly conserved among Ras-like GTPases, disrupts GTP binding and possibly interaction with the GAP domain of Plexin B1 (Fig.8d, e). Furthermore, the *F180C* mutation, which also maps to a highly conserved residue, may impair the folding of Rnd1, reducing guanine nucleotide binding (Fig. 8d, e). BrdU incorporation experiments indicated that G70R, E98D and F180C do not possess growth inhibitory activity, whereas M185V retains partial growth inhibitory activity (Fig. 8f).

Epitope tagging experiments indicated that Rnd1 co-localizes with H-Ras in the Golgi and at the plasma membrane and with K-Ras within a vesicular compartment surrounding the Golgi and at the plasma membrane, suggesting that Rnd1 follows a biosynthetic and transport route similar to that of Ras proteins[46] (Supplementary Fig. 8c, d). Although M185V localized in the Golgi and at the plasma membrane like wild-type Rnd1, G70R and F180C accumulated in large cytosolic aggregates and E98D exhibited an intermediate phenotype (Fig. 8g). Cycloheximide chase experiments indicated that the half-life of the mutants was decreased in a manner proportional to their functional inability to accumulate at the membrane (Fig. 8h). Transfection of fivefold excess expression vector resulted in clearly detectable steady-state levels of G70R, E98D and F180C but did not induce their association with Plexin B1 (Fig. 8i). In contrast, M185V combined with Plexin B1. These results suggest that G70R, E98D and F180C have lost growth suppressive activity as a result of defective folding, transport to the plasma membrane, and association with Plexin B1. Together, these findings indicate that hemizygous deletion in combination with silencing or, more rarely, mutation contributes to the inactivation of the tumour suppressor function of *RND1* in breast cancer.

DISCUSSION

We found that Rnd1 loss robustly activates Ras and provokes unscheduled proliferation and EMT in the mammary epithelium, and linked these events to activation of the Raf–ERK cascade. Similar to mammary epithelial cells overexpressing mutant Ras (refs 47, 48), those depleted of Rnd1 underwent senescence unless they were induced to overexpress c-Myc or had lost p53 function. In the presence of these cooperating oncogenic alterations, inactivation of Rnd1 simultaneously induced neoplastic conversion and EMT. These findings indicate that the inhibition of Ras by Rnd1 prevents unscheduled proliferation and disruption of epithelial adhesion and polarity in mammary epithelium.

Our mechanistic studies provided evidence that Rnd1 suppresses oncogenic Ras signalling by activating the GAP domain of Plexin B1, which inhibits Rap1 (ref.37). As GTP-loaded Rap1 binds to p120 Ras-GAP (ref. 36), blocking its ability to inhibit multiple Ras proteins[38, 39], we examined whether the Rnd1–Plexin B1 complex inhibits Ras by inactivating Rap1 and hence activating p120 Ras-GAP. Inactivation of Rap1 suppressed the activation of Ras induced by silencing of Rnd1. In addition, activated Rap1 combined with p120 Ras-GAP and activated Ras similar to silencing of p120 Ras-GAP. Thus, inactivation of Rnd1 and, hence, of Plexin B1 GAP domain activates Rap1, which in turn blocks p120 Ras-GAP, leading to activation of Ras. However, because Rap1 has an effector domain identical to that of Ras and activates shared target-effectors, including BRAF but not CRAF, it can buttress activation of ERK in mammalian cells expressing BRAF (refs 49, 50, 51) or in *Drosophila*, which express a single isoform of RAF similar to mammalian BRAF (ref. 52). It is therefore possible that inactivation of Rnd1 sustains signalling to ERK also through Rap1-mediated activation of BRAF. Of note, in general agreement with our results that Rap1 signals to ERK in mammary epithelial cells, overexpression of Rap1 activated ERK and induced disruption of epithelial adhesion and polarity and invasion in mammary epithelial HMT-3522 cells placed in 3D Matrigel[53]. Although inhibition of Ras provides a compelling explanation for the tumour suppressor function of Rnd1, we cannot formally exclude the possibility that Rnd1 opposes neoplastic conversion and EMT also by additional mechanisms.

Although *RAS* mutations are relatively infrequent in breast cancer[54], approximately 50% of breast tumours exhibit a constitutively activated Ras–ERK pathway[55]. Our results indicate that genetic and epigenetic inactivation of *RND1* underlies activation of the Ras–ERK pathway in a subset of these cancers. Notably, previous studies have indicated that Plexin B1 functions as a tumour suppressor in melanoma cells[56, 57] and genomic sequencing has identified missense mutations in *PLXNB1* and other Plexin genes in breast and prostate cancer[58, 59]. Two prevalent mutations disrupt the interaction of Plexin B1 with Rnd1 (ref. 60). Finally, TCGA analysis indicates that *RND1* suffers from potentially inactivating point mutations in melanoma (3.9%), and *PLXNB1* in melanoma (8.3%), colorectal cancer (11.1%), and lung adenocarcinoma (4.4%) (ref. 61), suggesting that the tumour suppressor pathway we have identified may be inactivated in several tumour types driven by aberrant Ras signalling. We propose that MEK inhibitors or other agents targeting the Ras–ERK signalling axis could potentially exhibit efficacy in cancers carrying inactivating alterations in *RND1* or *PLXB1*.

Methods

Cells and transfection.

HMLE cells were obtained from R. Weinberg (Whitehead Institute, MIT, Cambridge, Massachusetts) and MCF-10A cells from J. Brugge (Harvard University, Boston, Massachusetts). The COMMA-Dβ-geo cells from D. Medina (Baylor College of Medicine, Houston, Texas) were cultured in DMEM supplemented with 2% fetal calf serum, insulin (10 μg ml^{-1}), EGF (5 ng ml^{-1}) and PSFG. HMLE and MCF-10A cells were cultured in MEGM (Lonza). 4T1 cells from F. R. Miller (Wayne State University, Detroit, Michigan) were cultured in DMEHG supplemented with 10% FBS. Human breast cancer cell lines were cultured as described previously[69]. The mouse ErbB2-transformed mammary tumour cells were derived from an MMTV-*Neu(YD)* mouse and reconstituted with a wild-type form of the rat Neu oncogene under the control of the CMV promoter[6]. Lipofectamine 2000 (Invitrogen) and electroporation (Amaxa) were used to transfect HEK 293T cells and HUVECs, respectively.

Antibodies and reagents.

As previously described antibodies did not possess sufficient affinity and specificity to allow detection of endogenous Rnd1, but not Rnd2 or 3, we generated antibodies against Rnd1 by immunizing 2 rabbits with a GST-fusion protein comprising full-length Rnd1. The rabbit antiserum that exhibited the highest affinity to recombinant Rnd1 and no crossreaction with Rnd2 or Rnd3 was adsorbed on GST–Sepharose and then affinity-purified on Sepharose-conjugated GST–Rnd1. These affinity-purified antibodies reacted selectively with recombinant Rnd1, but not Rnd2 or Rnd3, but did not detect endogenous Rnd1 in multiple cell lines. All other antibodies used are listed in Supplementary Table 6. ROS were detected using the fluorescent indicator carboxy-H_2DCFDA (dichlorodihydrofluorescein diacetate, BD Molecular Probe). The MEK inhibitor U0126 was from Promega and PD98059 from Calbiochem. The PI(3)K inhibitor LY294002 was from Cell Signaling and wortmannin from Sigma. The Rac inhibitor NSC23766 was from Calbiochem. The ROCK inhibitor Y-27632 dehydrochloride was from AXXORA. Glutathione monoethyl ester was from SIGMA. SA-β-galactosidase activity was detected using the Senescence β-Galactosidase Staining Kit from Cell Signaling. HGF was from GIBCO, Sema4D from Sino Biological, HRG from R&D. The demethylating agent 5-aza-2-deoxycytidine (5-Aza) was from Calbiochem, the HDAC inhibitor SAHA from Sigma, and DZNep from Cayman. Cells were plated onto tissue culture plates for 48 h and then treated with 5 or 10 µM 5-Aza for 72 h followed by SAHA for 24 h (5 µM), or with each agent alone, and collected for qPCR. HCC1428, MDA-MB468 and BT474 cells were treated with DZNep for 48 h (5 µM) followed by 5-Aza-2′ deoxycytidine for 72 h (10 µM) and HDAC inhibitor SAHA for 24 h (5 µM), or with various combinations thereof.

Viral transduction.

For silencing, viral supernatants were generated by transfecting 293-FT cells with the shRNA constructs in combination with the packaging vectors pVSVG and pDR2. pLKO.1 plasmids encoding short hairpin RNAs targeting human Rnd1 (clones TRCN0000018338 and TRCN0000039977) and targeting PlexinB1 (clones TRCN0000061533 and TRCN0000061537) were from Open Biosystems; shRNAs targeting mouse Rnd1 (clones TRCN0000089694 and TRCN0000089695) and p27 (clone TRCN0000039928 and TRCN0000039930) were from Sigma and those targeting RhoD (SKI-RSI-197446, SKI-RSI-246157) were from the HTSC of MSKCC. siRNAs targeting Rnd1 (J-008929-05), p120 Ras-GAP (M-005276-01) and non-targeting control (D-001810-10-05) were from Dharmacon. For ectopic expression, Rnd1 was subcloned from pRK5-Rnd1 from A. Ridley (King's College London, UK) into pBABE (neomycin selection), pBABE-HA (puromycin selection) and pQCIX (neomycin selection). pVSVG-PlexinB1 and pKH3-HA-P190-RhoGAP were from L. Tamagnone (University of Turin, Italy) and M. Resh (MSKCC, New York), respectively. The lentiviral vector pLV-DsRED-Myc was from R. Weinberg. pEGFP-H-Ras and pEF-BOS-Flag-K-Ras, pEF-BOS-HA-Rap1WT and Rap1-V12 were from T. Kataoka (Kobe University, Japan). pcDNA3-HA-Rap1-N17 was from X. Zhang (Southwestern University, Dallas, Texas). pLOC-Rap1-GAP was from Open Biosystems and pLV-Flag-P120 Ras-GAP from Genecopoeia. Mutagenesis was performed with Quick Change (Qiagen).

3D Matrigel and soft agar assays.

Cells were cultured in growth-factor-reduced reconstituted basement membrane (Matrigel; BD Biosciences) as described previously[70]. Acini were fixed in 4% PFA and subjected to immunostaining. Images were taken with a Leica inverted confocal microscope (Leica TCS AOBS SP2). Each assay consisted of four replicate wells. Sections from 4 to 5 organoids per well were examined to determine the percentage of cleaved-caspase 3- and Ki-67-positive cells. For invasion assays, 1×10^5 cells were placed in serum-free MEGM on Transwell inserts coated with 2 mg ml^{-1} Matrigel. After incubation for 24 h, the inserts were cleaned to remove non-invasive cells, fixed with 4% PFA, and stained with crystal violet. The absorbance of the eluted dye was measured at $A_{595\ nm}$. Each assay consisted of two or three replicate wells and was repeated at least twice. For soft agar assays, 2×10^4 or 1×10^5 MCF-10A, HMLE cells and Comma-D cells were suspended in 0.35% agar in 6-well Ultra Low Cluster Plates (Costar). Each assay consisted of two or three replicates and was repeated at least twice.

Tumorigenicity and metastasis assays.

Orthotopic injections in the mammary fat pad and experimental metastasis assays were performed as previously described[6]. Briefly, 1×10^6 Comma-D cells suspended in 50 µl of PBS and Matrigel (1:1) were injected into the mammary fat pad of 6–8-week-old female NOD/SCID mice. Tumour volumes were measured by calliper at indicated time points. To measure spontaneous metastasis, 4T1 cells transduced with TGL vector were re-suspended at 1×10^4 in 50 µl PBS and injected into the mammary gland no. 4 of Balb/C mice. Primary tumour growth was monitored weekly by taking measurements of tumour length (L) and width (W). Tumour volume was calculated using the formula Volume = $(\text{width})^2 \times \text{length}/2$.

For lung colonization experiments, cells transduced with TGL vector were trypsinized, washed with PBS twice, re-suspended at 5×10^5 (ErbB2 cells) or 1×10^5 (4T1 cells) in 50 µl of PBS, and injected in the tail vein of 5–6-week-old female nude mice. Bioluminescent imaging and HE staining were used to measure lung colonization. For bioluminescent imaging, mice were anaesthetized and injected retro-orbitally with 1.5 mg of D-luciferin at the indicated times after xenografting. Animals were imaged in an IVIS 100 chamber within 5 min after D-luciferin injection and data were recorded using Living Image software (Xenogen). To measure lung colonization, photon flux was calculated for each mouse by using a circular region of interest encompassing the thorax of the mouse after subtracting the background. ImageJ software was used to calculate the percentage area of whole lung with metastatic lesions.

Biochemical assays.

Immunoprecipitation and immunoblotting experiments were conducted as described previously[6]. To analyse Ras pathway activity, MCF-10A cells were starved in MEBM with 0.2% FBS for 24 h, and then stimulated with 5 ng ml^{-1} of EGF for the indicated times. Cells were lysed in SDS-buffer, protein concentration was measured, and equal amounts of protein were loaded on SDS–PAGE and subjected to immunoblotting. To measure the active forms of Ras and RhoGTPases, the GTP-bound forms of each small

GTPase were pulled down from 200 μg of total proteins by using 30 μg of GST–Raf–RBD for Ras and GST–RalGDS–RID for Rap1. Eight hundred micrograms of total proteins were used to pull down active Rac and Cdc42 with GST–Pak67-150 and active Rho with GST–RBD of Rhotekin. Bound fractions were subjected to immunoblotting with antibodies against relevant Ras and Rho GTPases.

Immunostaining.

Paraffin-embedded sections were subjected to immunohistochemistry and immunofluorescent staining using the automated Leica staining system at the MSKCC Molecular Cytology Core Facility.

qPCR and DNA microarray analysis.

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN) and reverse transcribed using SuperScript III, First Strand Synthesis Super Mix (Invitrogen). qPCR was performed with the Taqman Universal PCR Master Mix and probes from the Taqman Gene Expression Assay system (Applied Biosystems) in triplicate. See Supplementary Table 7 for taqman probes. The MSKCC data set and the complete clinical data file were downloaded from the Gene Expression Omnibus (GEO) website (http://www-ncbi-nlm-nih-gov.offcampus.dam.unito.it/geo/) by using accession number GSE2603. Affymetrix CEL files were imported in Partek Genomic Suite 6.4 software (Partek) by using the Robust Multichip Average (RMA) method. Log_2-transformed data of the probe sets for RhoGTPases (Rnd1 included) and Myc (202431_s_at) were used for the analysis. The 70-gene prognostic profile (van't Veer signature)[17] was used to divide the population into 'Good prognosis' and 'Poor prognosis' groups, and the histological classification was used to divide the tumours into 'Triple negative' versus 'Non triple-negative'. The Beer lung metastasis risk index[13] was used to classify the tumours as lung metastasis signature negative or positive. The correlation between Rnd1 mRNA level and oestrogen receptor (ER) status, tumour transcriptomic subtype or triple-negative status was studied in 10 microarray data sets[13, 62, 63, 64]. Data were obtained from Oncomine[71]. Univariate survival analysis of metastasis-free survival (MFS), lung MFS and bone MFS has been estimated according to the Kaplan–Meier method and analysed by Log-rank (Mantel–Cox) and Gehan–Breslow–Wilcoxon test. Hazard ratios and 95% confidence intervals (CIs) were calculated by use of a stratified Cox regression analysis. In the initial RhoGTPase screening, the patients' tumours were divided in two groups (low and high) on the basis of the median of all the values for the probe sets for each RhoGTPase. For the survival analysis, 82 samples possessed complete clinical annotations and/or more than three years of clinical follow. Multivariate analysis was performed using the Cox proportional hazards model (R programming language). MCF-10A cells were plated in triplicate at subconfluent density and infected with lentiviruses encoding control shRNA or Rnd1 shRNA (no. 1 and no. 2). cDNAs were hybridized to the Human HT-12 Expression BeadChip. Microarrays data analysis was performed with Partek Genomic Suite 6.4 software (Partek). Data were $\log_2$-transformed and normalized using scale to median. Principal component analysis was used to verify the quality of data. Rnd1-regulated probe sets consensually and significantly regulated by Rnd1 knockdown with a fold change ≥1.5 both in the comparison of either shRNA no. 1 and shRNA no. 2 versus scramble (total of 233 probe sets) were used for hierarchical clustering and pathway analyses. A total of 233 genes (126 downregulated and 117 upregulated genes) were grouped in biological functions and canonical pathways by Ingenuity Pathway Analysis (IPA) Software (2009 Ingenuity Systems). Only significantly regulated ($P \leq 0.05$, obtained by right-tailed Fisher Exact test) pathways are shown. A subset of biologically interesting genes upregulated after Rnd1 silencing was selected for functional validation by qPCR (*CDC2*, *PLK1*, *CCNB2*, *CDCA1*).

Kaplan–Meier analysis of relapse-free survival.

Kaplan–Meier analysis of relapse-free survival was generated using the online resource http://kmplot.com/analysis and gene set for breast cancer patients[18].

Gene set enrichment analysis.

To examine whether underexpression of RND1 correlates with activation of Ras, Myc, β-catenin or Src, we used oncogene signature probe sets from primary human mammary epithelial cells transduced with oncogenic forms of the above signalling proteins (data set GSE3151, ref.72). Superman correlations between the *RND1* probe and the oncogene signature probes were calculated for the MSKCC-99 (GSE2603), EMC-286 (GSE2034) and EMC-192 (GSE12276) data set. The ranks of these correlations were divided by the total number of probes in the array (22,215 for Affymetrix U133a and 54,612 for Affymetrix U133plus2) to range them between 0 and 1. The ranks of the signature sets were used to calculate the CDF (cumulative distribution function) of the signature set ranks. If the ranks of the signature set are randomly distributed, the CDF will be along the diagonal. A departure from the diagonal indicates over- or under-representation. The correlation of low Rnd1 with activation of Ras signalling was confirmed by using an additional Ras signature[73].

FISH analysis.

To examine whether *RND1* is inactivated by deletion in human breast cancer, we conducted 2-colour FISH on 124 primary breast carcinomas from the Department of Pathology of the University of Torino using a probe spanning the RND1 locus located at Chr12q12-13 and a probe complementary to a centromeric sequence. Diagnoses were assigned according to the WHO classification by at least two experienced pathologists. Informed consents were obtained following the recommendations of local ethical committees (Protocol number 0081521 and 0072468). All locus-specific probes were developed using bacterial artificial chromosomes (BAC). The RP11-270J9 probe (CHORI), positioned centromeric (71 kbp) to the RND1 gene and RP11-302B13 (CHORI) positioned telomeric (94 kbp) to the RND1 gene were directly labelled with spectrum orange dUTP (Nick Translation Kit, Vysis). RP11-502N13 (CHORI), a control probe located on the p arm of chromosome 12, was labelled with spectrum green dUTP (Nick Translation Kit, Vysis). A probe specific for the centromeric region of chromosome 12 (Abbott Laboratories) was used to enumerate chromosome copy number. Probes and hybridization conditions were applied according to the Vysis manual instructions. Hybridization signals were analysed using a fluorescence microscope (Olympus BX 51 Olympus America or Zeiss AxioImage) with appropriate filters. Images were captured using the CytoVision imaging system (Applied Imaging) and/or analysed using MetaSystem software. Tumour sections were first scanned at low power with a DAPI filter to identify areas of optimal tissue digestion and non-overlapping nuclei. Only intact nuclei were scored. The assay was considered positive for RND1 deletion if the signal revealed two green and one orange foci. The hybridization efficiency of the FISH probes was >95%, based on the hybridization signals in normal elements of cytological smears. At least one hundred nuclei per sample were analysed and the highest number of tumour nuclei was collected for each individual slide. The neoplastic nuclei were defined on the basis of cytological and histopathological features. Cutoff values for deletion were determined using 7 fibroadenoma breast tissues and 5 normal lymphoid tissues (tonsil and lymph nodes). Deletion-positive samples were defined when loss of RND1 signals was greater than 20% for paraffin-embedded tissue section of primary breast adenocarcinoma.

DNA methylation analysis.

The methylation of the promoter region of the *RND1* gene was examined by using the Epityper system from Sequenom. Specific PCR primers for bisulphite-converted DNA were designed using the EpiDesigner software (www.epidesigner.com). Primer sequences, target chromosomal sequence, and Epityper specific tags are available on request.

ChIP assay.

Chromatin immunoprecipitations were performed by using the ChIP assay kit from Millipore following standard protocols. The Rnd1 primers were as follows: promoter Fwd-5′ 5′-CTCCAGCCTGACCTCACTTC-3′ and Rev-5′ 5′-CCCTTCCTCCCTTCTCTTTC-3′; intron Fwd-5′ 5′-TGCCCACTTCCAGTGAATTT-3′ and Rev-5′ 5′-TGTGATGGGAGGATCACTTG-3′. GAPDH primers are from the kit.

Structural analysis.

The GTP-loaded structure of RND1 was obtained from protein database PDB number 2CLS. The PyMOL program was used to predict and analyse the various mutational variants affecting effector loop and GTP loading.

SOLiD sequencing.

Analysis of TCGA data sets revealed that *RND1* suffers from point mutation in a small percentage of melanomas, colorectal carcinomas, endometrial carcinomas, and melanomas[54, 61]. Most point mutations identified are likely to be pathogenic because they truncate Rnd1 (*A6fs*28*, *G70_splice*, *E162**, *Y164**, *R201**) or replace amino acid residues that are predicted to be necessary for its function (*E48K*, *D75N*, *S85L*, *W107G*, *R130Q*, and *L165P*). In addition, some individual mutations were identified in different tumour types (*D75N* and *R130Q*) and one patient tumour carried two missense mutations (*R130Q* and *A154T*). As we found only one missense mutation in *RND1* in breast cancer (*I217M*), we reasoned that the filters that had to be applied to these large data sets might have removed mutations present in a subpopulation of tumour cells. To examine whether missense or nonsense mutations contribute to clonal inactivation of *RND1* in breast cancer, we divided 96 breast cancers from the University of Hiroshima into 10 pools and subjected them to targeted deep sequencing by SOLiD. Allelic variants present in the dbSNP137 database (>1,000 normal genomes) were excluded and putative pathogenic mutations were validated by SOLiD re-sequencing. Fresh frozen breast samples were obtained from the Department of Surgery of the University of Hiroshima following a protocol approved by the Institutional Review Board of the University. Informed consent was obtained for all samples. Genomic DNA samples extracted from 96 breast tumours and 8 normal breast tissues were amplified by PCR and the amplicons spanning the *RND1* exons from groups of 10 tumour samples or 8 normal breasts were pooled. One microgram of pooled amplicons was concatenated, sheared for 5 min (covaris), end-repaired, and ligated with barcoded SOLiD sequencing adaptors. Eight cycles of PCR were performed and 2 ng of the final 10 libraries were then pooled for one full-size emulsion PCR using the EZbead system. The enriched beads were sequenced in an octet of a SOLiD4 sequencing system for 50 bp run with the Exact Call Chemistry module (ECC). An average of 6.2 million reads were generated per pool; the mapping rates ranged from 86.9% to 89.1%. The colourspace CSFASTA and QUAL files are first converted to double-encoded FASTQ files, which are then mapped to the target genome (hg19) using BWA (ver. 0.5.9-r16) with default options plus the colour space mode option (-c) and the genome index is built with the colourspace option. Variants were then called using a VarScan (ver 2.2)-based pipeline. Reads were first filtered for those that had a MAPQ > 40 (using samtools view—q

40) and also reads with undefined bases ('N') were removed. A pileup was generated with samtools mpileup with default depth set to 100,000 (-d 100000) and the resulting pileup was piped to varscan. Only variants in the *RND1* gene region were retained. Allelic variants present in the dbSNP 137 database (>1,000 normal genomes) were excluded and putative pathogenic mutations were validated by SOLiD re-sequencing. Informed consents were obtained and the protocol was approved by Internal Review Board (IRB) No. 299 from Hiroshima University.

Repeatability of experiments.

All of the results documented by immunoblots or micrographs are representative of experiments that were repeated independently at least twice with similar results. The number of independent experiments is specified in each figure legend and one representative experiment is shown.

Statistical analysis.

Each experiment was repeated two or three times or more as mentioned in each figure legend. Data are presented as mean ± s.d., unless stated otherwise. Student's *t*-test (unpaired, two-tailed) was used to compare two groups for independent samples. Meade's resource equation was used to predetermine sample size for primary tumour growth experiments (E:10-20). For lung colonization experiments, we used $n \geq 5$ as this sample size easily detects a large difference driven by a biologically robust event. No statistical method was used to predetermine sample size for *in vitro* experiments. On arrival mice were randomly allocated to experimental groups. Investigators were not blinded to allocation during experiments and outcome assessment.

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