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Loss of RALT/MIG-6 expression in *ERBB2*-amplified breast carcinomas enhances ErbB-2 oncogenic potency and favors resistance to Herceptin

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An emerging paradigm holds that loss of negative signalling to receptor tyrosine kinases (RTKs) is permissive for their oncogenic activity. Herein, we have addressed tumor suppression by RALT/MIG-6, a transcriptionally controlled feedback inhibitor of ErbB RTKs, in breast cancer cells. Knockdown of RALT expression by RNAi enhanced the EGF-dependent proliferation of normal breast epithelial cells, indicating that loss of RALT signalling in breast epithelium may represent an advantageous condition during ErbB-driven tumorigenesis. Although mutational inactivation of the *RALT* gene was not detected in human breast carcinomas, *RALT* mRNA and protein expression was strongly and selectively reduced in *ERBB2*-amplified breast cancer cell lines. Reconstitution of RALT expression in *ERBB2*-amplified SKBr-3 and BT474 cells inhibited ErbB-2-dependent mitogenic signalling and counteracted the ability of ErbB ligands to promote resistance to the ErbB-2-targeting drug Herceptin. Thus, loss of RALT expression cooperates with *ERBB2* gene amplification to drive full oncogenic signalling by the ErbB-2 receptor. Moreover, loss of RALT signalling may adversely affect tumor responses to ErbB-2-targeting agents.
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Introduction

Net signal output by receptor tyrosine kinases (RTKs) depends on the dynamic equilibrium between signal generation (positive signalling) and signal attenuation (negative signalling). Perturbation of this balance has deleterious consequences on cell and tissue homeostasis, as demonstrated by developmental studies in invertebrate organisms (Perrimon and McMahon, 1999; Moghal and Sternberg, 2003). Recently, attention has also been drawn on loss of negative signalling as a potential mechanism of oncogenic activation of RTKs. For instance, transforming mutations of MET and CSF-1R prevent c-Cbl from binding to these RTKs (Peschard and Park, 2003). Along this line, the increased c-Src activity associated to oncogenic EGFR signalling drives degradation of c-Cbl (Bao *et al*., 2003). In aggregate, these events uncouple c-Cbl from RTK signalling, thus allowing oncogenic receptors to escape downregulation via the internalization/degradation pathway (Bache *et al*., 2004; Polo *et al*., 2004).

Dramatic overexpression of the ErbB-2 RTK, most often caused by gene amplification, is detected in 20–30 % of human breast carcinomas and is causally linked to the aggressive clinical behaviour of this tumor subset (Slamon *et al*., 1989). Although unable to bind ligands directly, ErbB-2 is the hierarchically dominant receptor in the combinatorial assembly of ligand-driven hetero-dimeric complexes between ErbB family members, namely ErbB-1 (EGFR), ErbB-3 and ErbB-4 (Olayioye *et al*., 2000; Yarden and Sliwkowski, 2001). Among these signalling complexes, the ErbB-2.ErbB-3 combination is most remarkable in terms of oncogenic potency, due to the fact that ErbB-2 and ErbB-3 are strong activators of the Ras-ERK and PI-3K-AKT pathways, respectively (Olayioye *et al*., 2000; Yarden and Sliwkowski, 2001). Consistently, overexpression of ErbB-3 is linked to oncogenic activation of ErbB-2 in both human and mouse mammary tumors (Siegel *et al*., 1999; Holbro *et al*., 2003). Not surprisingly, although ErbB-2 homo-dimers may form and signal in *ERBB2*-amplified cancer cells, genetic evidence indicates that ErbB-3 acts as an essential partner of oncogenic ErbB-2 in *ERBB2*-amplified breast tumors (Holbro *et al*., 2003), possibly in conjunction with autocrine/paracrine ligand stimulation (Mincione *et al*., 1996).

An unresolved issue concerns negative signalling to ErbB-2-containing dimers in normal cells and its possible subversion in cancer cells. Negative signalling

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to RTKs is primarily provided by the internalization/degradation pathway (IDP), which is immediately triggered upon receptor occupancy (Bache *et al*., 2004; Marmor and Yarden, 2004; Polo *et al*., 2004) and therefore serves as a housekeeping-type mechanism of negative signalling to RTKs (Fiorini *et al*., 2001). IDP is integrated by feedback inhibition (FI). In the FI scenario, RTK signalling drives transcription of genes whose products feedback onto activated receptors and inhibit their signalling activity via diverse mechanisms (Fiorini *et al*., 2001). Owing to ErbB-2 refractoriness to downregulation via the IDP (Marmor and Yarden, 2004), FI could represent a primary element of regulation of ErbB-2 signalling. We (Fiorentino *et al*., 2000) and others (Hackel *et al*., 2001) have identified RALT/MIG-6 as a feedback inhibitor whose activity is restricted to receptors of the ErbB family via still undefined mechanism/s. RALT expression is triggered by ErbB signalling via activation of the Ras-ERK pathway (Fiorini *et al*., 2002). The RALT protein is in turn able to complex with activated ErbB RTKs and inhibit their signalling function in cultured cells (Hackel *et al*., 2001; Fiorini *et al*., 2002; Anastasi *et al*., 2003). In addition, a skin phenotype similar to that generated by a dominant-negative *EGFR* allele (Murillas *et al*., 1995) is observed in mice carrying a *RALT* transgene expressed by the K14 promoter (Costanza Ballarò, Oreste Segatto and Stefano Alemà, manuscript in preparation). The available data support a 'rheostat' model whereby the transcription rate of the *RALT* gene is proportional to incoming ErbB signals and the ensuing levels of RALT protein generate suppressive signals commensurate to ErbB activity (Fiorini *et al*., 2002; Anastasi *et al*., 2003). An extension of the above model postulates that maximal RALT activity could be an effective means of buffering oncogenic perturbations generated by aberrant ErbB signalling. As RALT is the only feedback inhibitor of ErbB-2 identified to date, the question arises whether RALT exerts oncosuppressor activity in tumors dependent on oncogenic ErbB-2 signalling, namely whether loss of RALT signalling (a) occurs during ErbB-2-driven tumorigenesis; and (b) effectively relieves a constraint on ErbB-2 oncogenic signalling.

In this study, we show that the *RALT* gene does not undergo mutational inactivation in breast cancer. Loss of RALT expression was nevertheless found to occur selectively in *ERBB2*-amplified breast cancer cell lines. Reconstitution of RALT expression in *ERBB2*-amplified tumor cells was sufficient to inhibit ErbB-2 oncogenic signalling and negated the ability of ErbB ligands to rescue tumor cells from Herceptin-mediated growth inhibition. These data, coupled to the finding that RNAi-mediated knockdown of RALT signalling in normal breast epithelial cells enhanced their mitogenic response to ErbB ligands, provide evidence that RALT may (i) exert tumor suppressor activity in *ERBB2*-amplified breast carcinomas; and (ii) be a determinant of responses to ErbB-2-targeting agents, such as Herceptin.

Results

Knockdown of RALT expression in breast epithelial cells enhances mitogenic signalling by ErbB RTKs

MCF-10A cells are immortalized nontransformed breast epithelial cells that are strictly dependent upon EGF supplementation for their growth and proliferation. This cell line is widely used to investigate the biology of normal breast epithelial cells and to model mechanisms of cell transformation (reviewed in Debnath *et al*., 2003). Thus, we used RALT RNAi in MCF-10A cells to investigate whether reduced RALT signalling impacts on the proliferation of normal breast epithelium.

Expression of the shRNA *RALT 4* by the pSuper retro vector was found to inhibit accumulation of ectopic human RALT protein in HEK 293 cells (Supplemental Figure 1A). This effect was specific, as a control sh*GFP* sequence did not affect human RALT expression. Owing to nucleotide sequence divergence between the human and rat mRNA species, sh*RALT 4* is not predicted to target the rat *RALT* mRNA. Consistently, sh*RALT 4* did not reduce the accumulation of coexpressed rat RALT protein in HEK 293 cells (Supplemental Figure 1B). Next, we generated MCF-10A derivatives via infection with either pSuper retro or pSuper retro-shRALT 4 retrovirus stocks (henceforth referred to as pSuper and RALT 4 cells, respectively). While readily induced in pSuper cells, RALT protein accumulated at much lower levels in RALT 4 cells stimulated with either optimal (Figure 1a) or suboptimal (Figure 1b) doses of EGF. In contrast, c-Fos immunoreactivity was comparable in pSuper and RALT 4 cell lysates (Figure 1a), an indication that expression of sh*RALT 4* did not cause a generalized impairment of ERK-driven gene transcription.

The knockdown of RALT expression imposed by sh*RALT 4* expression led to a significant enhancement of the duration of EGF signals (Figure 1b). Thus, AKT activation was similar in pSuper and RALT 4 cells at early time points of EGF stimulation, while from the 2 h time point onwards it was 1.6–2-fold higher in RALT 4 cells. In comparison to pSuper controls, ERK activity was marginally higher in RALT 4 cells at early time points (possibly due to complete loss of RALT expression in quiescent cells), while showing a robust reinforcement (2–3-fold increase over pSuper cells) past the 2 h time point (Figure 1b). Importantly, ectopically expressed rat RALT, but not RALT ΔEBR, was still able to suppress EGF-driven ERK activity in RALT 4 cells (Supplemental Figure 2), thus confirming the specificity of our observations. In aggregate, the above data indicate that the notable effects of RALT knockdown on EGF signalling were confined to the timeframe which, in control cells, coincided with maximal accumulation of the RALT protein.

Knockdown of RALT expression increased the proliferation rate of MCF-10A cells over a wide range of EGF concentrations (Figure 2a), without affecting cell viability (data not shown). This phenotype was specific, since it could be reversed by ectopic expression

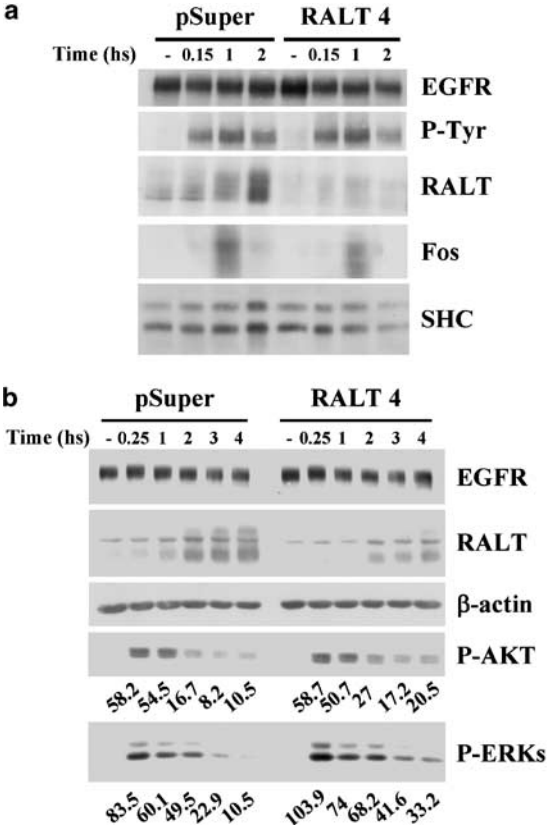

Figure 1 Knockdown of RALT increases the duration of EGF signals in MCF-10A cells. (**a**) Lysates were made from quiescent pSuper and RALT 4 cells either before or after stimulation with 1 ng/ml EGF for the indicated time (h) and immunoblotted as indicated. (**b**) pSuper and RALT 4 cells were made quiescent and stimulated for the indicated time (h) with 0.3 ng/ml EGF. Cell lysates were immunoblotted with the indicated antibodies. Anti-P-AKT and anti-P-ERK immunoreactivity was quantified using the Quantity One software (Biorad) after normalization for protein loading (as assessed by anti-β-actin stain). Optical density values, expressed as arbitrary units, are reported for each lane of the P-AKT and P-ERKs autoradiographs

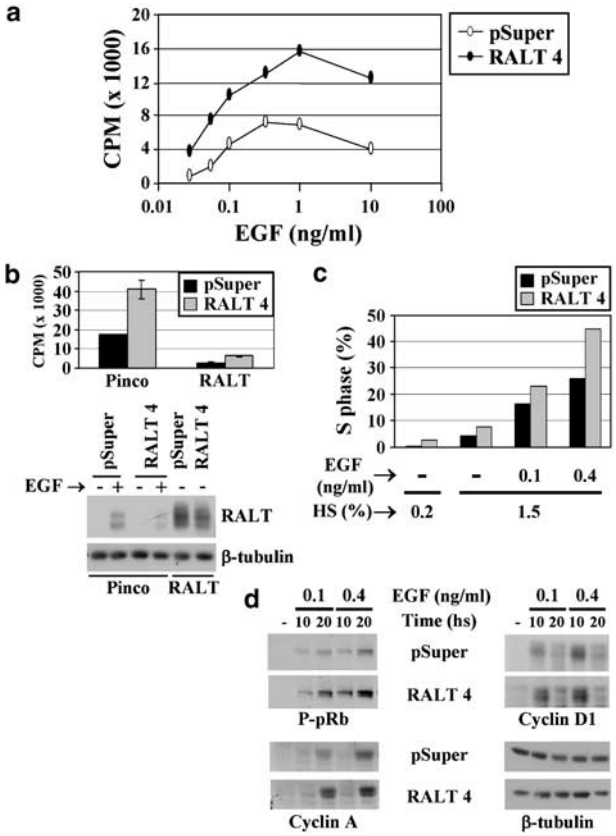

Figure 2 Knockdown of RALT amplifies EGF mitogenic signals in MCF-10A cells. (**a**) pSuper and RALT 4 cells were seeded in 48-well plates (5×10^3 cells/well) in medium containing escalating EGF concentrations. Cell proliferation was assessed after 48 h by measuring the radioactivity incorporated during a 4-h pulse with [^{3}H]methyl-thymidine. Data are expressed as cpm after subtraction of the radioactivity incorporated by cells grown without EGF. Assays were performed in quadruplicate wells, with variability not exceeding 15%. (**b**) upper panel: pSuper and RALT 4 cells were seeded in 48-well plates and infected with either Pinco or Pinco-RALT retrovirus. At 24 h postinfection, cells were switched for 48 additional hours to medium containing 1 ng/ml EGF. Proliferation was assessed as described in (**a**). Expression of endogenous (as detected in quiescent Pinco cells stimulated with 1 ng/ml EGF for 2 h) and ectopic RALT proteins was assessed by parallel immunoblot analysis (lower panel). (**c**) pSuper and RALT 4 cells were rendered quiescent by a 24 h incubation in mitogen-free medium containing 0.2% horse serum (HS). Cells were subsequently stimulated with medium containing 1.5% HS and the indicated doses of EGF, harvested after 24 h (at which time cells had completed exit from G1) and processed for cell cycle analysis. Data are expressed as percentage of cells in S phase. (**d**), Quiescent pSuper and RALT 4 cells were stimulated with the indicated doses of EGF for either 10 or 20 h. Lysates were immunoblotted with the indicated antibodies

of rat RALT (Figure 2b). Enhanced proliferation of RALT 4 cells was due to increased recruitment of cells into the cell cycle, since quiescent RALT 4 cells that were able to transit into S phase upon EGF stimulation exceeded by 25–50% the number of their pSuper counterpart (Figure 2c). Under these experimental conditions, EGF-stimulated RALT 4 cells had a higher content of cyclin D1, cyclin A and hyper-phosphorylated pRb when compared to pSuper cells (Figure 2d).

Collectively, these data indicate that knockdown of RALT expression in breast epithelial cells releases ErbB receptors from an important element of negative regulation, thus increasing cell recruitment into the mitotic cycle at suboptimal doses of EGF.

Mutational analysis of the RALT gene in breast cancer

The *RALT/MIG-6* gene maps to chromosome 1p36.12–36.33 (NCBI Accession NT_021937). It consists of four exons spread over 14 562 bp (Figure 3a). Loss of heterozygosity (LOH) at loci in the 1p chromosome has been recorded in 40–60% of breast tumors. In particular, commonly deleted regions were mapped to 1p36.3, 1p36.1, 1p35, 1p32, 1p31 (Farabegoli *et al*., 1996; Tsukamoto *et al*., 1998; Bieche *et al*., 1999; Ragnarsson *et al*., 1999) and 1p36 under-representation in human breast cancer was found to be associated with high ErbB-2 expression (Farabegoli *et al*., 1996). Owing to its chromosomal location and the biological function

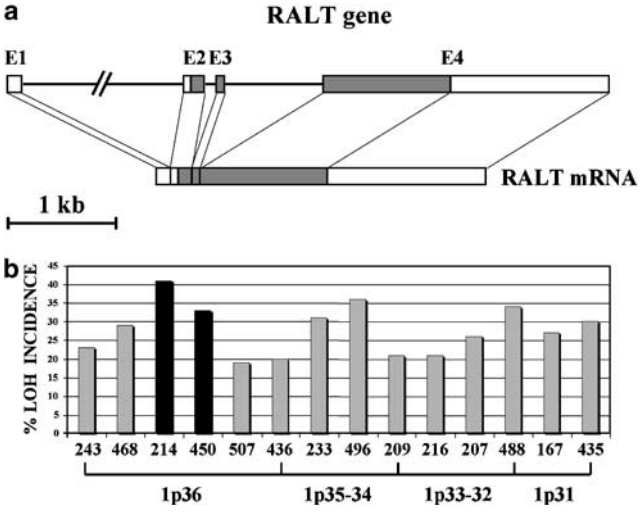

Figure 3 Analysis of the *RALT* gene in breast cancer. (**a**) Schematic representation of the human *RALT* gene and mature *RALT* mRNA. Exons (E1–E4) are represented by boxes, introns by lines. White and gray areas correspond to noncoding and coding exon sequences, respectively. (**b**), Incidence of LOH at D1S microsatellite markers in the 1p31–1p36 region. D1S214 and D1S450 flanking the *RALT* gene are indicated by dark columns

assigned to its product, *RALT*/*MIG-6* is a candidate tumor suppressor gene in breast cancer.

We searched for allelic loss of *RALT* in a cohort of breast carcinomas with documented LOH in the 1p31-pter region (as defined by the D1S435–D1S243 interval) (Ragnarsson *et al*., 1999). As indicated in Figure 3b, the *RALT* locus maps in the interval defined by D1S214 (telomeric) and D1S450 (centromeric). Out of a total of 75 tumor samples, those informative for either D1S214 or D1S450 were 57 (76%) and 52 (69.3%), respectively. We found that the incidence of LOH at D1S214 was 41.3% (31/75), whereas LOH at D1S450 was found in 33.3% of the cases (25/75) (Figure 3b). A total of 37 tumor samples were informative for both D1S214 and D1S450; 13 of these had LOH at both markers. Thus, 17.3% of the tumors in our cohort had combined LOH at D1S214 and D1S450. In the large majority of the cases, this was caused by very large deletions of 1p sequences (data not shown) and the *RALT* gene did not fall within a minimal commonly deleted region. Consistently, we did not find mutations of the *RALT* coding sequence in genomic DNAs obtained from 92 breast carcinomas (including the 75 tumors with documented LOH at 1p), as determined by single-strand conformation polymorphism (SSCP) analysis. In three patients, we detected a non synonymous single nucleotide polymorphism (SNP) at codon 109 (Supplemental Figure 3). This SNP appeared with similar frequency in a panel of randomly selected healthy donors (7/190, data not shown). Direct sequencing of RT–PCR-amplified *RALT* cDNAs from a panel of breast carcinoma cell lines (MCF-7, MDA MB453, MDA MB231, MDA MB436, MDA MB361, SKBr-3, BT20, MDA MB415, BT549, T47D, BT474, MDA MB175) also did not identify mutations in the *RALT* coding sequence (data not shown). Collectively, these data indicate that the *RALT* gene does not undergo mutational inactivation in sporadic breast carcinomas.

Analysis of RALT expression in breast cancer cell lines

Since expression of RALT is subjected to a tight transcriptional and post-translational control (Fiorini *et al*., 2002), we postulated that mechanisms other than genetic inactivation could abrogate RALT function in breast tumors. Western blot analysis of lysates prepared from a panel of several breast carcinoma cell lines stimulated with Neuregulin1 (NRG1) indicated that, in general, expression of RALT was not lower than that detected in MCF-10A cells and roughly proportional to ErbB-2 expression. The only notable exception was represented by tumor cells displaying the most extreme degree of ErbB-2 overexpression, namely the *ERBB2*-amplified SKBr-3 and BT474 cell lines. A representative analysis of five cell lines is shown in Figure 4a. SKBr-3 (Figure 4b) and BT474 cells (data not shown) displayed poor RALT expression also upon exposure to cortisol, PMA and sorbitol-mediated osmotic shock. Conversely, all of these stimuli induced high RALT expression in both MCF-10A (Figure 4b) and MCF-7 cells (data not shown), as previously reported for murine cells (Fiorini *et al*., 2002). Low levels of RALT protein in SKBr-3 and BT474 cells correlated with reduced accumulation of *RALT* mRNA, as detected by both Northern blotting (Figure 4c) and quantitative real-time RT–PCR (data not shown).

As previously documented in murine fibroblasts (Fiorini *et al*., 2002), ERK activity was required for RALT expression in MCF-7 and MDA MB361 cells, since blockade of ERK activation by the MEK-1 inhibitor UO126 prevented accumulation of RALT protein following stimulation with either NRG1 or PMA (Figure 4d, upper panel). Notably, ERK activity in SKBr-3 and BT474 cells was higher than that detected in MCF-7 and MDA MB361 cells, both under basal conditions and following agonist stimulation (Figure 4d, lower panel). Thus, poor expression of the *RALT* gene in *ERBB2*-amplified cells is not caused by inadequate ERK signalling.

Restoration of RALT expression in ERBB2*-amplified breast cancer cells: impact on mitogenic signalling*

We next investigated whether restoration of RALT expression in *ERBB2*-amplified breast cancer cells affects ErbB-2 signalling. To this aim, we introduced in SKBr-3 and BT474 cells either wt RALT or its ΔEBR derivative. This mutant lacks the ErbB binding region (EBR) and is therefore unable to complex with ErbB RTKs (Anastasi *et al*., 2003). Recombinant Pinco, Pinco-RALT and Pinco-RALT ΔEBR retrovirus stocks were used to infect SKBr-3 cells and FACS-select populations expressing similar levels of green fluorescent protein (GFP) (referred to as Pinco, $RALT_{HIGH}$ and RALT ΔEBR cells). We also FACS-selected RALT cells

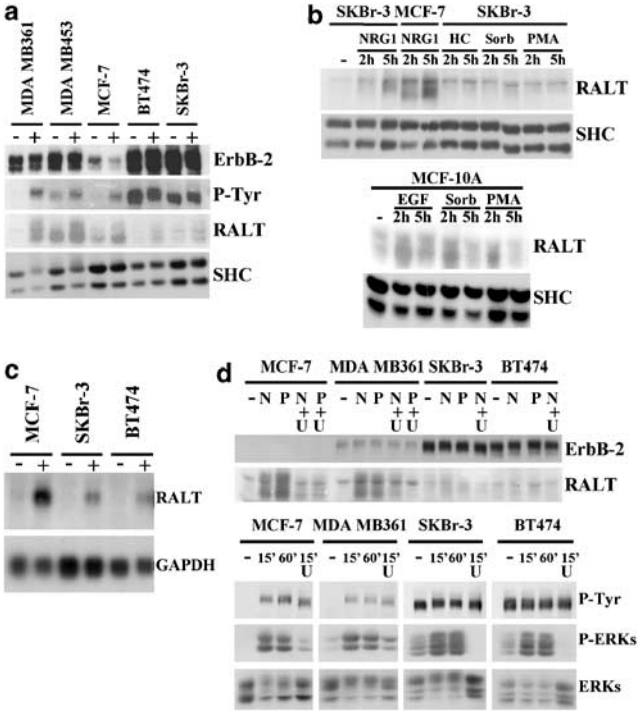

Figure 4 Analysis of RALT expression in *ERBB2*-amplified breast cancer cells. (**a**) The indicated cell lines were cultured for 24 h in mitogen-free medium and stimulated for 3 h with either carrier (−) or 10 ng/ml NRG1 (+). Lysates were immunoblotted with the indicated antibodies. (**b**) The indicated cell lines were made quiescent by 24 h mitogen deprivation and stimulated for the indicated time with NRG1 (10 ng/ml), EGF (10 ng/ml), PMA (6 ng/ml), hydrocortisone (HC, 1 μM). To induce osmotic shock, cells were exposed to 300 mM sorbitol (Sorb) for 15 min, washed and cultured for the remaining time in mitogen-free medium. Lysates were immunoblotted with the indicated antibodies. (**c**) The indicated cells were made quiescent and harvested for RNA extraction either before (−) or after (+) stimulation for 90 min with 10 ng/ml NRG1. Total RNAs were hybridized to the indicated cDNA probes. Detection was by autoradiography. (**d**) The indicated cell lines were serum-deprived and subsequently stimulated for 3 h (top panel) or the indicated time (min, bottom panel) with either 10 ng/ml NRG1 (N) or 60 ng/ml PMA (P). Where indicated, 10 μM UO126 (U) was added 1 h before stimulation. Lysates were immunoblotted with the indicated antibodies

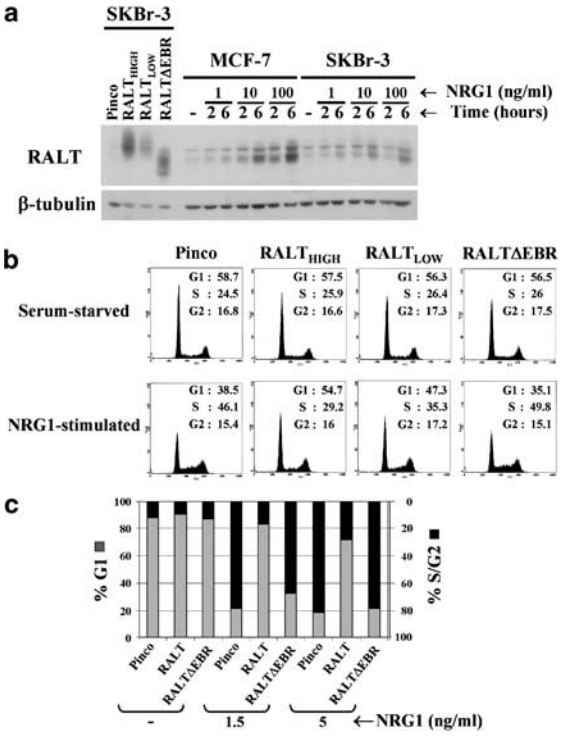

Figure 5 Reconstitution of RALT activity suppresses mitogenic signals in *ERBB2*-amplified breast cancer cells. (**a**) Lysates were made from the indicated SKBr-3 derivatives as well as from quiescent and NRG1-stimulated MCF-7 and parental SKBr-3 cells. Immunoblot analysis was performed with the indicated antibodies. (**b**) The indicated SKBr-3 derivatives were rendered quiescent by serum deprivation and harvested either before or after stimulation with 1.5 ng/ml NRG1 for 24 h. Percent cell distribution through the G1, S and G2/M phases of the cell cycle was determined by flow cytometry. (**c**) BT474 cells were infected with the indicated recombinant retrovirus stocks. Infection efficiency, as assessed by GFP imaging, was 70–80%. At 36 h post-infection, cells were rendered quiescent by serum deprivation and harvested either before or after stimulation with the indicated doses of NRG1. Cell cycle distribution was assessed by flow cytometry. Gray and dark columns refer to cells in G1 and S/G2, respectively

expressing 3–4-fold lower GFP levels (RALT$_{LOW}$). RALT$_{HIGH}$ and RALT ΔEBR cells expressed ectopic RALT proteins at levels 2–3-fold higher than those detected in MCF-7 cells following maximal NRG1 stimulation (Figure 5a) and roughly similar to those expressed by NRG1-stimulated MDA MB361 cells (not shown, see also Figure 4a). RALT expression in RALT$_{LOW}$ cells, instead, was comparable to that of the endogenous protein in MCF-7 cells stimulated with 10–100 ng/ml NRG1 (Figure 5a). Unlike RALT ΔEBR, ectopic RALT coupled efficiently to ErbB receptors, as assessed by its ability to coimmunoprecipitate with ErbB-2 and ErbB-3 (Supplemental Figure 4). Remarkably, while mitogenic responses to optimal doses of NRG1 were comparable in Pinco and RALT ΔEBR cells, both RALT$_{HIGH}$ and RALT$_{LOW}$ cells were severely impaired in their ability to respond to NRG1 (Figure 5b). Similar results were obtained upon reconstitution of RALT expression in BT474 cells (Figure 5c). Reconstitution of RALT expression inhibited also the proliferation of SKBr-3 and BT474 cells in serum-containing medium (Figure 7a,b).

Reconstitution of RALT expression in SKBr-3 cells reduced the basal activity of ERKs and limited both strength and duration of NRG1-dependent ERK and AKT activity (Figure 6a). When compared to their Pinco counterpart, RALT-reconstituted SKBr-3 cells showed the most prominent reduction of NRG1-induced ERK and AKT activity from the 2 h time point onwards (Figure 6a). This alteration was biologically relevant, as delayed pharmacological interruption of either ERK or AKT signalling was sufficient to arrest NRG1-stimulated SKBr-3 cells in G1 (Figure 6c). Consistent with its inability to suppress the proliferation of SKBr-3 cells, ectopically expressed RALT ΔEBR suppressed neither basal nor NRG1-induced ERK and AKT activity (Figure 6b).

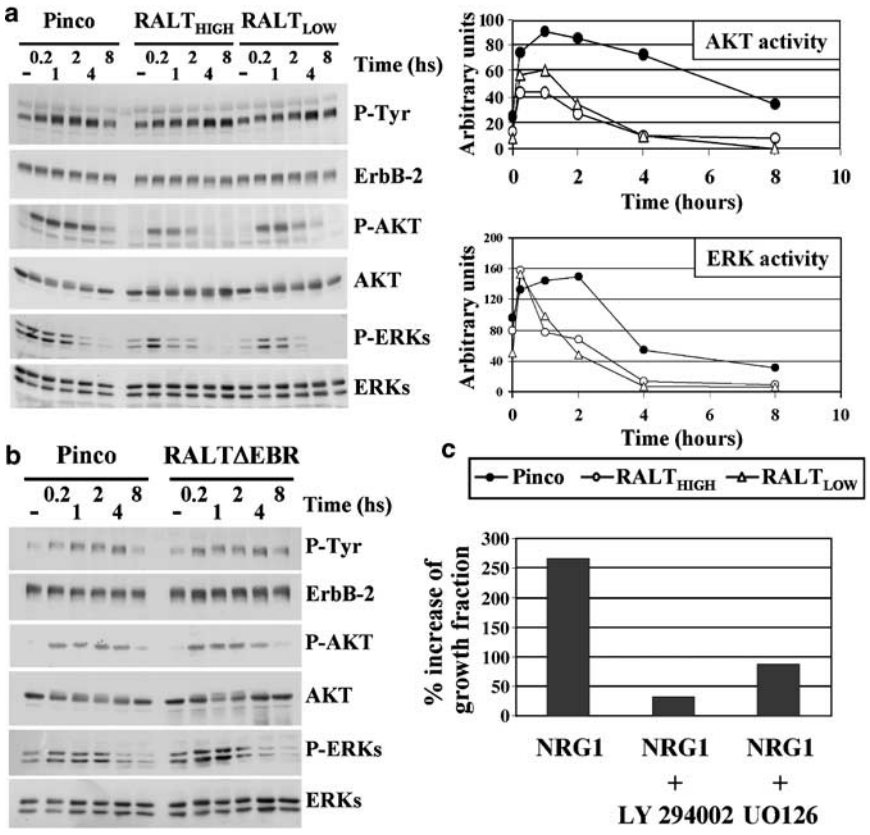

Figure 6 Impact of RALT reconstitution on signalling pathways triggered by NRG1 in SKBr-3 cells. (**a**) Pinco and RALT derivatives of SKBr-3 cells were mitogen-deprived for 24 h and subsequently challenged with 1.5 ng/ml NRG1 for the indicated time (h). Lysates were immunoblotted with the indicated antibodies (left panel). Anti-P-AKT and anti-P-ERK immunoreactivity was quantified using the Quantity One software (Biorad) and plotted after normalization for total AKT and ERK immunoreactivity (right panels). (**b**), Pinco and RALT ΔEBR derivatives of SKBr-3 cells were analysed as described in (**a**). (**c**) Quiescent SKBr-3 cells were stimulated for 24 h with 1.5 ng/ml NRG1. Where indicated, pharmacological inhibitors of PI-3 K (LY 294002, 25 μM) or MEK1 (UO126, 10 μM) were added 2.5 h past NRG1 stimulation. Data are expressed as % increase of cells in S/G2 in NRG1-stimulated samples versus untreated control

The above results indicate that restoration of RALT activity in *ERBB2*-amplified breast cancer cells inhibits mitogenic signals generated by receptor dimers containing ErbB-2. These effects are elicited by doses of RALT not dissimilar from those detected in either normal breast epithelial cells or non-*ERBB2*-amplified breast cancer cells. Finally, RALT exerts its suppressive activity in these cells at a receptor-proximal cellular location, as demonstrated by the ΔEBR phenotype.

Loss of RALT function as a determinant of resistance to Herceptin

The humanized anti-ErbB-2 monoclonal antibody Herceptin is an effective treatment for *ERBB2*-amplified breast carcinomas. However, clinical responses to Herceptin are limited to a minority of treated patients, indicating that ErbB-2 overexpression is not sufficient to confer sensitivity to treatment. It has been proposed that growth factors available in the tumor microenvironment could rescue Herceptin-targeted tumor cells via the vicarious activation of signalling pathways, that is, the PI-3K-AKT and Ras-ERK pathways that would normally be activated by ErbB-2 but become silent upon Herceptin administration (Motoyama *et al*., 2002). Accordingly, stimulation of Herceptin-targeted tumor cells with ErbB ligands induces resistance to Herceptin (Motoyama *et al*., 2002). The recently described neutralization of PTEN activity by Herceptin (Nagata *et al*., 2004) might also be counteracted by vicarious activation of ErbB→Src signalling.

Since RALT is a pan-ErbB inhibitor (Anastasi *et al*., 2003), we reasoned that loss of RALT expression could render tumor cells more responsive to ErbB ligands and thus favor resistance to Herceptin. This hypothesis was tested by evaluating the activity of Herceptin in control and RALT-reconstituted cells. Cultivation with either NRG1 or TGF-α enhanced the basal proliferation of Pinco derivatives of SKBr-3 and BT474 cells, respectively, and negated the cytostatic activity of Herceptin (Figure 7a,b). RALT signalling cooperated with Herceptin in limiting basal cell proliferation (Figure 7a,b). Most importantly, RALT signalling neutralized the ability of TGF-α and NRG1 to rescue *ERBB2*-amplified cells from antimitogenic activity of Herceptin (Figure 7 a,b).

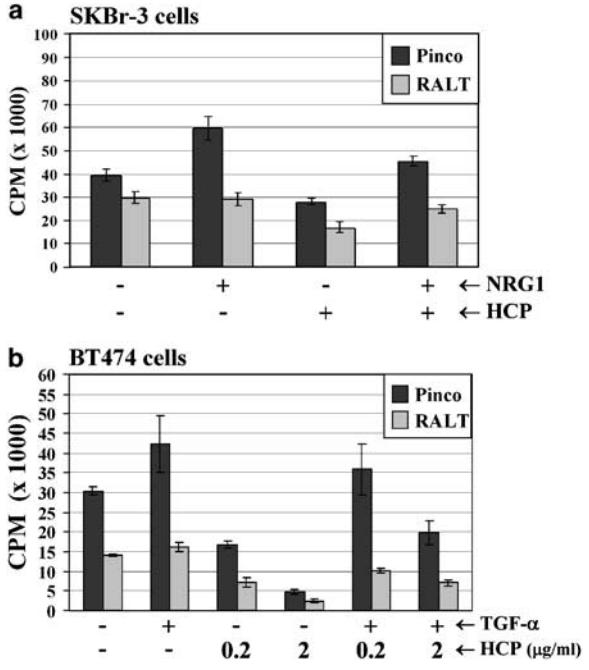

Figure 7 Loss of RALT signalling favors resistance to Herceptin. (**a**) SKBr-3 cells were seeded in 48-well plates (1×10^4 cells/well) and infected with the indicated recombinant retrovirus. At 24 h post-infection, cells were switched to medium containing the indicated additions. NRG1 and Herceptin (HCP) were used at 0.3 ng/ml and 2 μg/ml, respectively. Cell proliferation was assessed after 48 hours by measuring the radioactivity (expressed as cpm) incorporated during a 4-h pulse with [^{3}H]methyl-thymidine. Each experimental point was determined in quadruplicate wells. (**b**) BT474 cells were plated in 48-well plates (2×10^4 cells/well) and infected with the indicated retrovirus. Following infection cells were treated and processed as in (**a**) except that TGF-α (10 ng/ml) was used instead of NRG1

Discussion

Tumor suppressor genes/proteins antagonize oncogenic perturbations that drive tumor initiation and/or maintenance. Consequently, their function needs to be ablated during tumor progression (Hanahan and Weinberg, 2000). In this study, we have used a combination of genetic and cell biological approaches to address whether RALT, a feedback inhibitor of ErbB oncoproteins, exerts tumor suppressor activity in breast cancer.

RNAi-mediated knockdown of RALT expression increased the duration of EGF signals and conferred to normal mammary epithelial cells the ability to proliferate robustly at EGF concentrations that instead stimulated marginal proliferation of control cells. Such hypersensitivity to low doses of ErbB agonists is particularly relevant to oncogenesis, since it may grant growth autonomy under conditions of restricted availability of mitogens (Hanahan and Weinberg, 2000). As strength is an element of signal identity, loss of RALT function could alter also the quality of cellular responses to ErbB ligands: for instance, mitogenic signals could be generated inappropriately at low receptor occupancy, that is, under conditions that normally would solely support cell survival.

Our RNAi experiments provided initial biological validation of the candidacy of RALT as a tumor suppressor gene/protein in mammary epithelium. Consistently, RALT was poorly expressed at the mRNA and protein level in *ERBB2*-amplified breast cancer cell lines. Moderate overexpression of the ErbB-2 protein in non-*ERBB2*-amplified breast cancer cells was not associated with loss of RALT expression. Thus, loss of RALT signalling is confined to the subset of *ERBB2*-amplified breast cancer cell lines, that is, those in which the extreme ErbB-2 overexpression is causally linked to the transformed status. Our analysis of the *RALT* locus in 92 breast tumor samples ruled out genetic inactivation of RALT function in breast carcinomas. A CpG island extends from the 5' upstream region of the *RALT* gene through exon 1 and the adjacent intron. However, we did not observe re-expression of RALT upon treatment of BT474 and SKBr-3 cells with 5-Aza-dC alone or in combination with Trichostatin A (data not shown). Thus, mechanism/s other than epigenetic silencing is/are likely to be responsible for transcriptional repression of the *RALT* gene in *ERBB2*-amplified tumor cells. Remarkably, loss of expression of Sprouty proteins, involved in transcriptionally controlled feedback inhibition of FGFs signals (Kim and Bar-Sagi, 2004), has been recently described in breast carcinomas, in the absence of genetic or epigenetic alterations of *SPRY* genes (Lo *et al*., 2004).

Reconstitution of RALT expression in SKBr-3 and BT474 cells restored a relevant pathway of negative signalling to ErbB-2 and inhibited cell proliferation. It also inhibited prolonged activation of ERK and AKT (Figure 6), in remarkable consonance with the loss of function studies reported in Figure 1. Protracted signalling via ERK and AKT couples RTKs to the cell cycle machinery (Vivanco and Sawyers, 2002; Coleman *et al*., 2004), thus promoting G1 traverse and entrance into S phase. We conclude that loss of RALT signalling complements *ERBB2* gene amplification: dramatic overexpression of ErbB-2 promotes its constitutive activation, while removal of RALT is permissive for unabated propagation of ErbB-2 oncogenic signals. Moreover, as *RALT* transcription is promiscuously induced by many extracellular stimuli, including hormones, growth factors and stress (Makkinje *et al*., 2000; Fiorini *et al*., 2002; Saarikoski *et al*., 2002), loss of RALT expression renders ErbB-2 oncogenic signalling resilient to cross-regulation by extracellular cues.

An example of such resilience is provided by our results on RALT signalling in Herceptin-targeted cells. Although not required for cells to respond to Herceptin, RALT modulated Herceptin responses. Thus, vicarious signalling by ligand-activated ErbB RTKs and the ensuing neutralization of Herceptin function (Motoyama *et al*., 2002) were facilitated by loss of RALT expression. Our data suggest that resistance to Herceptin is a likely outcome whenever loss of RALT expression in *ERBB2*-amplified tumors is concomitant to autocrine/paracrine production of ErbB ligands.

Conclusions

Our results reinforce the emerging paradigm that full expressivity of dominantly acting oncogenic lesions of RTKs may require the concomitant loss of negative signalling to RTKs (Peschard and Park, 2003; Bache *et al*., 2004). This paradigm may in fact be extended to non-tyrosine kinase receptor systems. Thus, full oncogenic signalling by the WNT-β catenin axis in colon carcinomas requires epigenetic loss of SFRPs (a family of WNT antagonists) even in tumors that carry activating downstream mutations (Suzuki *et al*., 2004).

Our data also postulate a role for loss of RALT signalling in the pathogenesis of *ERBB2*-amplified breast carcinomas. This will have to be formally validated by a thorough analysis of RALT expression in tumor samples. While this type of studies is presently hampered by the lack of antibodies suitable for immuno-histochemical detection of RALT protein, we note that downregulation of *RALT*/*MIG-6* mRNA in breast carcinomas has been recently found to correlate with poor survival (Amatschek *et al*., 2004). Collectively, these data make a cogent case for further investigations of RALT expression in tumors caused by oncogenic ErbB signalling.

Materials and methods

Cell culture and gene transfer procedures

Human breast cancer and HEK 293 cells were cultured in DMEM containing 10% foetal calf serum. MCF-10A cells were grown as described (Debnath *et al*., 2003). Retroviral stocks were generated by transfection of pVSVG (Clontech), pGag-pol (Clontech) and either Pinco-based (Fiorentino *et al*., 2000) (expressing GFP and foreign cDNA) or pSuper-based (Brummelkamp *et al*., 2002) vectors into HEK 293 cells. The pSuper-shRALT 4 vector targets nt 1323–1341 of human RALT mRNA (NCBI Accession NM_018948). Infection procedures were as described (Fiorentino *et al*., 2000). For cell cycle studies, cells were processed as described (Fiorentino *et al*., 2000), imaged with a Coulter Epics flow cytometer and analysed with the Multicycle software (Phoenix Flow System). For cell proliferation studies, cells were labelled for 4 h with 1 μCi/ml of [^{3}H]methyl-thymidine. Incorporation of label by proliferating cells was measured as described (Fiorentino *et al*., 2000).

Immunochemistry

Proteins were solubilized in HNTG buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 5 mM EGTA). Immunoblot was performed as described (Fiorentino *et al*., 2000). Rabbit polyclonal antibodies to cyclin A (H-432, Santa Cruz Biotechnology), c-Fos (UBI), p-Ser 807/811 pRb (Cell Signalling), SHC (Transduction Laboratories), p-Thr 202/p-Tyr 204 ERK, p-Ser 473 AKT (Cell Signalling) and MoAbs to P-Tyr (4G10, UBI), tubulin (D-10, Santa Cruz Biotechnology), β-actin (Sigma) and cyclin D1 (Zymed) were used as suggested by manufacturers. The anti-RALT S1 antiserum was described previously (Fiorentino *et al*., 2000).

LOH and SSCP analysis

Genomic DNA was extracted from tumor samples and from PBMC as described (Ragnarsson *et al*., 1999). DNAs were subjected to PCR amplification with DynaZyme polymerase (Finnzymes Oy, Espoo, Finland) for 35 cycles. PCR products were run on 6.5% polyacrilamide gels containing 8 M urea, transferred to Hybond-N$^+$ nylon membranes and hybridized as described (Ragnarsson *et al*., 1999). Hybridization probes for LOH analysis were generated by elongation of PCR primers with terminal transferase, followed by conjugation with HRP (Ragnarsson *et al*., 1999). Hybridization products were detected by ECL (Amersham). Absence or significant reduction ($>50\%$) of hybridization signal was scored as LOH. For SSCP analysis, PCR-amplified genomic DNAs were resolved in acrylamide gels, transferred onto Hybond N$^+$ nylon membranes and hybridized to probes generated by terminal transferase-dependent elongation of PCR primers (Ragnarsson *et al*., 1999).

Northern blot

Total RNA was extracted by Trizol (Life Technologies) and processed for Northern hybridization (Fiorentino *et al*., 2000). cDNA probes were labelled with [^{32}P]dCTP using a random priming procedure (Amersham Pharmacia Biotech).

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