A transposon-based analysis reveals RASA1 is involved in triple negative breast cancer

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ABSTRACT

RAS genes are mutated in 20% of human tumors, but these mutations are very rare in breast cancer. Here we used a mouse model to generate tumors upon activation of a mutagenic T2Onc2 transposon via expression of a transposase driven by the keratin K5 promoter in a p53+/- background. These animals mainly developed mammary tumors, most of which had transposon insertions in one of two RASGAP genes, neurofibromin1 (NF1) and RAS p21 protein activator (Rasa1). Immunohistochemical analysis of a collection of human breast tumors confirmed that low expression of RASA1 is frequent in basal (triple-negative) and ER-negative tumors. Bioinformatic analysis of human breast tumors in the TCGA database showed that although RASA1 mutations are rare, allelic loss is frequent, particularly in basal tumors (80%) and in association with TP53 mutation. Inactivation of RASA1 in MCF10A cells resulted in the appearance of a malignant phenotype in the context of mutated p53. Our results suggest that alterations in the Ras pathway due to the loss of negative regulators of RAS may be a common event in basal breast cancer.
INTRODUCTION

Ras genes are some of the most frequently mutated in human cancer. According to the catalogue of somatic mutations in cancer (COSMIC v77) (1), which represents the most comprehensive database on human cancer mutations currently available, around 20% of the analyzed tumors have activating mutations in any of the three Ras genes, with a maximum of 57% incidence for \textit{KRAS} in pancreatic tumors. Ras signaling may also be activated by other means, notably by inactivation of molecules that limit Ras activity, such as Ras GTPase-activating proteins (RasGAPs). Ras proteins are molecular switches that cycle between an inactive GDP-bound form and an active GTP-bound form. They signal through several effector pathways, and RasGAPs stimulate the weak intrinsic GTPase activity of normal (but not mutant) Ras proteins, effectively acting as suppressors of Ras function. Interestingly, less than 1% of the near 10,000 breast cancer samples sequenced in COSMIC have mutations in Ras genes, however the Ras pathway is significantly activated in a number of breast tumors, in particular of the triple-negative type (2).

Breast cancer is by far the most frequent tumor type in the female population worldwide (25% of all new cases in 2012), and although its mortality rate is not the highest, it is the most frequent cause of cancer death in women (14.7% of all deaths in 2012) (3). Triple negative breast cancers (TNBC, which are negative for HER2, ERα and the progesterone receptor) constitute a heterogeneous group of tumors which very often exhibit a basal-like signature (4). Although they represent approximately 15% of all breast cancers, they account for a much higher mortality: they are tumors with a poor prognostic, mainly due to the lack of specific targets for treatment. These triple-
negative tumors are enriched for mutations in TP53. Indeed, TP53 is mutated in 36% of all breast cancers, but this proportion rises to 86% in PAM50 basal-like tumors (5).

TNBC tumors also bear a highly variable number of genomic alterations, including the presence of a large number of somatic mutations and copy number aberrations (CNA) (6), suggesting that i) combinations of mutations interact to drive tumor formation, and ii) most of the mutations found are “passengers”, not related to development of the tumor.

Transposon-induced mutagenesis is an excellent method to identify cancer driver genes. For example, when mobilized by Sleeping Beauty (SB) transposase, the mutagenic T2Onc2 transposon integrates throughout the genome, and cells with insertions in a gene or combination of genes that favour tumorigenesis are positively selected. Using this method, genes involved in multiple tumor types have been identified (reviewed in (7)). This analysis has not yet been performed for breast cancer. In this manuscript, we report on generation of transposon-bearing mice that develop mammary tumors, identification of RasGAP genes as the major target of transposon-mediated mutagenesis, and identification of RASA1 hemizygous deletion in human triple negative breast cancer. Together with the identification of hemizygous deletions in NF1 and RASAL2 in breast cancer (8,9), our results highlight the importance of RasGAP gene hemizygosity as a driver of elevated Ras signalling and breast cancer in humans.
MATERIALS AND METHODS

Transgenic mice generation
Double K5-SB11/T2Onc2 (named as SB/T2) transgenic mice, containing both the SB11 and T2Onc2 transgenes were generated by interbreeding of heterozygous SB and T2 mice, as described (10). Heterozygous Trp53+/− transgenic mice were obtained by mating conditional mutants Trp53F2-10 mice (11), that carried floxed Trp53 alleles in a FVB genetic background, to K5-Cre transgenic female mice; in this breeding, the maternal K5-Cre transgene causes general cre-mediated deletion in early embryos (12), generating a Trp53-null allele. Triple transgenic K5-SB11/T2Onc2/Trp53+/− mice (SB/T2/p53+/−) were generated by mating heterozygous SB/T2 to heterozygous Trp53+/− mice. In these crosses, different genetic combinations lacking the transgenes T2Onc2 (SB/p53+/−), the transposase SB11 (T2/p53+/−) or both SB and T2Onc2 transgenes (p53 +/-) were also generated and used as control mice for the experiments. Animals were typed by PCR. All procedures involving mice were approved by the Institutional Organism for Animal Welfare (OEBA) and according to the European, Spanish and local regulations.

Tumor collection
Tumors were excised from the mice and were fractionated. One part was included in formaldehyde for subsequent paraffin embedding for immunohistochemistry analysis, and the rest was snap frozen in liquid N2 for protein/nucleic acid extraction.

Human Tumors
Samples and data from patients included in this study were provided by the Biobanco i+12 in the Hospital 12 de Octubre integrated in the Spanish Hospital Biobanks Network (RetBioH; www.redbiobancos.es) following standard operation procedures.
with appropriate approval of the Ethical and Scientific Committees. Patient’s clinical
data are shown in Supplementary Table SII

**Cell lines**

MCF10A cells were purchased from ATCC. T47D, HCC1954 and BT474 breast carcinoma
cell lines were obtained from Dr. Cristina Sánchez (University Complutense, Madrid, Spain), who purchased them from ATCC and provided vials after first division. All cells
lines were authenticated by ATCC by isoenzyme analysis and STR profiles, and were
kept in culture for less than six months. Cells were periodically analyzed for
contamination with a mycoplasma detection kit. All cells were passaged and cultured
using the media and recommendations provided by ATCC. MCF10A-p53
R175H/shRasa1 cells were grown in plates coated with 0.1% gelatin due to their
limited adhesion. For experimentation, all other types of MCF10A cells were also
grown in gelatin-coated plates to avoid substrate effect.

**Immunohistochemistry**

Mouse tissues were fixed in 10% buffered formalin and embedded in paraffin. Five μm-
厚 sections were used for H&E staining or immunohistochemical preparations. Most
of the tumors originated in the mice were fixed and classified by morphology after
sectioning and staining with H&E. For immunohistochemistry, slides were
deparaffinized and antigen retrieval was performed in microwave with citric acid
buffer (pH 6) for mouse tissues, and in pressure cooker with Dako Target Retrieval
Solution (pH 9) (Dako, Agilent Technologies) for human tissues. Endogenous
peroxidase was inhibited with hydrogen peroxide (0.3 %) in methanol. Unspecific
epitopes were blocked with PBS containing 10% of horse serum. The antibodies used
in the immunohistochemical analysis were against keratin K5 (PRB-160P; 1:1000)
(Covance, San Diego, CA); RASA1 (sc-63; 1:100), NF1 (sc-67; 1:400), ERα (sc-542; 1:200) (Santa Cruz Biotechnology), P53 (M7001; 1:50, Dako) and SB transposase (MAB2798; 1:50) (R&D Systems, Minneapolis, MN). Signal was amplified with avidin/biotin technology (VECTASTAIN Elite ABC system) and was visualized with DAB peroxidase substrate kit (Vector Laboratories).

**Statistic analysis**
For western blotting, bands were quantified by Quantity One software and normalized with respect to beta-actin, total ERK or total AKT expression. P values were determined by using the unpaired, two-tailed Student t test. For immunohistochemistry, samples were assessed in a blind manner by two experts. RASA1 and NF1 staining was quantified from 0 to 3 comparing with normal tissue. When staining was heterogeneous within tumors, five different areas were assessed and an average value was calculated. P values were determined by using the Chi-square test. In both cases, p values < 0.05 were considered significant and data are expressed as mean ± SEM.
RESULTS

SB/T2/p53+/- mice develop spontaneous mammary tumors.

To generate tumors, double transgenic mice bearing both a concatemer of T2Onc2 mutagenic transposons and the SB11 transposase under control of the keratin K5 promoter (10) were mated with heterozygous Trp53+/- mice (Fig. 1A). As expected, these Trp53+/- animals are prone to the development of lymphoma. Also, as K5 is expressed in the skin, they also develop skin tumors (10). Interestingly, SB/T2/p53+- triple transgenic mice preferentially developed mammary tumors (Figure 1B). This occurs at a higher frequency (41% vs. 19%) and shorter latency (49 vs. 60 weeks) than in control Trp53+- mice lacking transposition (Fig. 1C). Keratin K5 is expressed in the myoepithelial layer of the mammary gland and SB11 transposase was detected in this layer of transgenic animals (Fig. 1D). Likewise, mammary tumors showed concurrent expression of K5 and SB11 transposase (Fig. 1D). Thus, transposition of T2Onc2 in K5-expressing cells facilitates development of mammary gland tumors in a Trp53+- background.

We collected 33 mammary gland tumors from 26 SB/T2/p53+/- females. Classification of these tumors based on histology revealed that most lesions were either acinar carcinoma or adenocarcinoma (~60%). Mixed pattern acinar-solid carcinoma (~19%) and papillary carcinoma or cystadenocarcinoma (6%) were also frequently seen. Other subtypes, such as solid, tubular and spindle cell carcinoma occurred in a small number of animals (Fig. 1E, F). Most (84%) tumors generated from SB/T2/p53+/- mice were ER-
positive (Fig. 1G). It is interesting to note that mammary tumors generated by KRas activation are mainly ER-positive (13).

**RasGAPs are the most frequently mutated genes in transposon-induced mammary tumors.**

DNA from these 33 mammary tumors was extracted, and sequences flanking the transposon insertion sites were amplified by PCR and then subjected to next-generation Illumina sequencing to identify all transposon integrations. Scrutiny of these integrations using gene-centric Common Insertion Site (gCIS) analysis (14) resulted in identification of 16 CIS or Common Insertion Sites, which represent specific sites in the genome that accumulate transposon insertions in independent tumors at a rate significantly higher than expected by chance, and are therefore likely the result of positive selection during tumor development (Table I). 26 of the 33 tumors had transposon insertions in at least one of these CIS. Most of the genes found in these CIS are related to cancer, and the human counterparts of five of them (PTEN, NF1, NFIB, SMAD3 and GATA3) are already defined as cancer genes in the Cancer Genes Census (cancer.sanger.ac.uk/census). Moreover, several of the identified genes have already been implicated in breast cancer. For instance, PTEN and GATA3 are two of the most frequently mutated genes in human breast cancer (15-18), and NFIB, which is disrupted by SB in 40% (13/33) of tumors undergoes translocations in breast cancers (19). Gene ontology analysis showed selection for insertion into genes related to cell matrix adhesion, oncogenesis, apoptosis, cell migration and angiogenesis (Table II).
In our murine tumors, the RasGAPs Nf1 and Rasa1 were among the most frequently mutated genes. Of note, 18 of the 33 analyzed tumors (54%) had transposon insertions in either one or both of the RasGAP genes Nf1 and Rasa1, strongly suggesting activation of the Ras pathway in generation of these tumors. The position and orientation of T2Onc2 insertions in each target gene hints at the type of alteration produced (20); when orientation of the transposon was analyzed, we found that 100% of the transposon insertions in Nf1 and 70% of insertions in Rasa1 were in the opposite orientation, suggesting that gene inactivation has occurred. Both in Nf1 and Rasa1, all insertions were located before or into the functional RASGAP domain (Fig. 2A), effectively disrupting the function of these proteins and resulting in the activation of Ras signalling.

**Mouse mammary tumors show reduced RasGAP expression**

To validate these results, we analyzed a number of tumors by western blot (Fig. 2B). In spite of the histological heterogeneity of these lesions, there was an excellent correlation between presence of transposon insertions in Rasa1 and the amount of detected RASA1 protein (Fig. 2C, t-test: p<0.0001). In the case of Nf1, all tumors with insertions showed reduced expression of the protein by western blot. Interestingly, some tumors showed reduced NF1 expression, even in the absence of SB-mediated disruption of Nf1 (Fig. 2B and 2D), suggesting inactivation of Nf1 expression by other mechanisms independent of transposon insertion.
Since disruption of *Rasa1* or *Nf1* should result in enhanced Ras signaling, we checked for activation of ERK (a known Ras effector) and AKT (an effector of Ras and PI3K) in these tumors. ERK activity (measured as pERK accumulation) was present in 85% (6/7) of tumors with *Rasa1* and/or *Nf1* insertions, as compared to 38% (3/8) of tumors without insertions in either gene (Fig. 2B and 2E). A correlation was also detected between activation of AKT and diminution of RASA1 (Fig. 2B and 2F), suggesting activation of the Ras pathway in tumors with *Rasa1* or *Nf1* insertions. These partial correlations may well be associated with ERK activation through oncogenic pathways distinct from elevated Ras signaling associated with disruption of *Rasa1* or *Nf1*.

Interestingly, in some tumors with high pERK but lacking insertions in *Rasa1* or *Nf1* we found insertions (not included in our gCIS list, since they were found in less than three tumors) in other Ras-related genes, such as *Rasgrf1* (see Supplementary Table SI). We did not find any correlation between insertions in *Rasa1* or *Nf1* and expression of EGFR, ERBB2 or ESR1 (data not shown).

**RASA1 and NF1 genes are frequently lost in triple negative breast cancer**

While *NF1* is a well-known tumor suppressor gene, and *NF1* deletions and mutations have been reported in breast cancer (8,18), *RASA1* has not been previously considered as a breast cancer gene. We used immunohistochemistry to assess expression of RASA1 in 32 human breast tumors. Intensity of RASA1 staining in the tumor area was compared to intensity in the normal mammary tissue and quantified in a blind manner using an arbitrary scale from 0 to 3 (fig. 3A and supplementary Table SIII). Expression of RASA1 was reduced in basal or triple negative tumors versus other types (p<0.05, fig
3C and supplementary Tables S III and S IV). This trend was even more evident when ER-negative tumors were compared to ER-positive tumors (p<0.01, Fig 3D). For NF1 staining, a trend towards lower expression in ER-negative tumors was also seen (supplementary Tables S III and S IV). TP53 is frequently mutated in triple negative breast cancer, and TP53 staining of these tumors allowed us to identify a significant correlation between low or absent RASA1 staining and mutation in TP53 (p<0.05, Fig 3A and 3B and supplementary Tables S III and S IV). Low or absent RASA1 staining also correlated with high Ki67 staining (p<0.05), and was associated to high grade (G3) tumors (p<0.01) and stage III tumors (p<0.05) (supplementary table S IV).

RASA1 mutations are unusual. According to the catalogue of somatic mutations in cancer (COSMIC v77, cancer.sanger.ac.uk), as of 17/05/2016 RASA1 mutations have been found in only 256 out of 26,161 tumor samples (1 %). RASA1 mutations in breast are even less frequent, with only 8 cases found out of 1571 samples sequenced. To expand our results, and given that in our mouse tumors T2Onc2 does not cause point mutations but rather disrupts one copy of Rasa1, yielding tumors with low but detectable levels of RASA1 protein, we searched for RASA1 hemizygous loss using the cBio Cancer Genomics Portal (www.cbioportal.org) (18,21). Analysis of breast cancer samples from TCGA (5) confirmed that 27% of breast tumors (289/1080) showed RASA1 gene loss, most of which represented low-level or hemizygous loss (Fig. 4A and supplementary table S V). This percentage rose to 84% (90/107) when only PAM50 basal tumors were considered. PAM50 HER2-enriched tumors also had allelic losses in 49% (25/51) of cases, while losses in PAM50-luminal A and B tumors were much less frequent. In general, RASA1 allelic loss was more common in ER-negative (70%) than in
ER-positive tumors (14%) (p<0.0001, Fisher’s exact test, Fig. 4A and supplementary table S V). As expected, there was a clear correlation between copy number and expression of RASA1 mRNA (Fig 4B). Interestingly, we also detected a correlation between copy number of RASA1 and ESR1 expression, both at mRNA and protein levels (Figs. 4C and D), and also between high methylation of ESR1 and hemizygous deletion of RASA1 (Fig. 4E). A similar correlation was found between RASA1 copy loss and PGR1 (but not ERBB2) expression (data not shown). In addition, 75% of PAM50 basal-like tumors showed coincident allelic loss at RASA1 and mutation of TP53 (vs 21% when all tumors are considered). Enrichment analysis on 974 tumors also revealed a significant correlation between RASA1 copy number loss and mutation of TP53 (p= 3.50E-60, Fisher’s exact test, Supplementary Table S VI). Similarly, heterozygous deletions in NF1 were more prominent in PAM50 basal (62%, 66/107) and HER2-enriched (59%, 30/51) tumors as compared to breast cancer when considered as a whole (31%, 334/1080) (supplementary table S VII). While allelic loss of NF1 in PAM50-basal and ER-negative tumors was statistically significant, no correlation could be found with ESR1 expression (not shown).

We also performed expression meta-analysis using the GOBO online database (http://co.bmc.lu.se/gobo) (22), which includes more than 1800 patients from 10 breast cancer studies. Again, in agreement with our results, expression of NF1 and RASA1 was significantly reduced in ER-negative tumors, and in particular in basal tumors (p<0.00001), (Fig. 4F-I). Moreover, low expression of NF1 and, in particular of RASA1, correlated with high grade (G3) tumors (p<0.0001, data not shown).
Concomitant TP53 mutation and inactivation of RASA1 malignize human breast cells

To validate in vitro these observations, we permanently inactivated RASA1 by lentiviral-mediated shRNA interference in several breast cell lines. In T-47D, BT-474 and HCC-1954 cells, inactivation of RASA1 with two different shRNAs (sh1 and sh2) resulted in activation of ERK and/or AKT, confirming that partial deletion of RASA1 is enough to activate the Ras pathway (Fig. 5A). Interestingly, in SUM159 or MDA-MB-231 cells, which already have constitutive activation of the Ras pathway, this effect was not seen (data not shown). Since all the breast cancer cell lines tested had mutations in TP53, and most triple negative tumors present concomitant deletion of RASA1 and mutations in the TP53 gene, we next investigated the relation of TP53 and RASA1 using MCF10A cells, a TP53-wild type immortalized non-malignant mammary epithelial cell line which is often used as a model of normal human mammary gland. In these cells, interference using sh1 almost completely suppressed the expression of RASA1, while sh2 reduced its expression to around 50% (Fig. 5B). For both shRNAs, phosphorilation of ERK was weak, as also was upon introduction of a R175H TP53 mutation (the most frequent mutation in breast tumors). However, combination of p53R175H expression and RASA1 knockdown caused a strong increase in the activation of the Ras pathway, as seen by strong phosphorilations of ERK and AKT (Fig. 5B). Cells in which only RASA1 had been inactivated did not show signs of increased malignancy, but co-occurrence of TP53 mutation and RASA1 inactivation resulted in increased malignancy, as indicated by several endpoints reminiscent of epithelial-mesenchymal transition (EMT): cells abandoned their cobblestone-like appearance, lost adherence and acquired an elongated, fibroblastoid aspect (Fig. 5C). Cytometry analysis confirmed that both types of p53R175H/shRASA1 cells strongly reduced EpCAM and
CD49f expression (Fig. 5D), which is indicative of a transition from an epithelial to a mesenchymal phenotype (23). Moreover, p53R175H/shRASA1 cells lost E-cadherin and upregulated N-cadherin expression (Fig. 5B and E). Finally, double p53R175H/shRASA1 cells also acquired invasive properties, as seen by invasion chamber assays using matrigel (Fig. 5F). Interestingly, the two interfering shRNAs exerted similar but not identical effects on MCF10A cells in the context of the p53 mutation: almost total inactivation of RASA1 by sh1 resulted in almost total disappearance of EpCAM and E-cadherin, while partial inactivation of RASA1 by sh2 resulted in partial EpCAM loss and partial inactivation of E-cadherin, with the remaining E-cadherin expressing cells exhibiting a spiky, discontinuous pattern of E-cadherin, instead of the continuous staining seen in the control cells (Fig. 5E). On the contrary, both total and partial inactivation of RASA1 coupled with p53R175H resulted in activation of the Ras pathway, induction of N-cadherin and invasivity to similar extents, indicating that loss of RASA1, even if it is not complete, results in increased malignancy of human mammary cells when in coexistence with mutated TP53.

Collectively, our results indicate that decreased RASA1 expression associated with transposon insertion leads to mammary tumor formation in Trp53+/- mice, and strongly suggest that hemizygous loss of RASA1 (frequently associated to TP53 mutation) is a common oncogenic driver in basal and other triple negative breast cancers.
DISCUSSION

Transposon-mediated generation of tumors in a Trp53-heterozygous background has allowed us to identify inactivation of RasGAP genes as a frequent event in murine mammary tumors. Since its initial development as a tool for identification of cancer-promoting genes in transgenic mice (24,25), transposon technology has been successfully used by many laboratories to identify genes causing cancer in a variety of tissues, and many of these have proved to be of clinical significance (reviewed in (7)).

Our screen has identified a number of gCIS that are known to function as tumor suppressor genes, and are subject to loss-of-function mutations in human breast cancer. 30% of the genes identified in our screen are included in the cancer gene census, among them PTEN, which is mutated in a high percentage of human breast tumors, in particular in TNBC (26). Combined deletion of Pten and Trp53 in mouse mammary epithelium results in the development of claudin-low type tumors (27).

Ras genes are some of the most frequently mutated genes in human cancer, but mutations in breast cancer are rare. In spite of this, several lines of evidence suggest involvement of elevated Ras signaling in a subset of breast tumors: i) The Ras transcriptional signature is highly prevalent in triple negative or basal-like breast cancer. Indeed, integrative analysis suggest widespread activation of the Ras pathway in triple negative breast cancer (2,26); ii) Breast cancer cell lines of the basal type have an activated Ras-like transcriptional program and are particularly sensitive to MAPK/ERK inhibitors (28,29); iii) Active, mutant Ras can transform mammary cells: human mammary cells transduced with a mutant KRAS gene generate invasive ductal carcinomas (30) and MMTV-RAS or WAP-RAS transgenic mice develop mammary
tumors, which appear earlier and are more malign in a Trp53+/- background (reviewed in (31)). Furthermore, there are several reports that hint in particular to RASA1 as a breast cancer gene: for instance, downregulation of RASA1 is associated with poor survival of breast invasive ductal carcinoma patients (32), and loss of Chr 5q14 (where the RASA1 gene is located) has been noted before in breast tumors (33-35). Moreover, chromosome 5q loss is a characteristic marker of the integrative cluster IntClust 10. This IntClust 10 is one of 10 groups that result from the characterization of human breast tumors according to genomic and transcriptomic landscapes (36,37), and includes mostly triple negative tumors from the core basal-like intrinsic subtype. Interestingly, these tumors have the highest rate of TP53 mutations (37).

More than 50% of the murine tumors that we have analyzed showed transposon insertions in either one or both of the RasGAP genes Nf1 and Rasa1, suggesting a strong selective pressure towards RasGAPs inactivation for tumorigenesis. There are some classical studies that have already established a relationship between NF1 and human breast cancer (38-41), and recently, high throughput studies have confirmed involvement of NF1 mutations in this disease (8,18), so it is conceivable that RASA1 (which is a protein that shares its main function with NF1) is also involved in the development of breast tumors. Our results also synergize with recent reports on RasGAPs alterations in other tumor types, as for instance RASA2 in melanoma (42), RASAL1 in colorectal cancer (43) and RASAL2 also in breast cancer (9,44). RASA1 itself has been linked to colorectal and prostate tumorigenesis (43,45,46) (See also Maertens and Cichowski (47) for a recent review). In addition, other molecules downstream of Ras could also facilitate breast oncogenesis: for example, DUSP4, a
negative regulator of ERK activity, acts as a tumor suppressor in basal-like breast cancer (48). Interestingly, a recently reported breast cancer transposon screen has also identified Rasa1 as a potential breast tumor suppressor gene in a Pten-mutant background (49).

Our results also confirm that downregulation of RASA1, when accompanied by the presence of a mutated TP53, is sufficient to induce an EMT response in MCF10A cells. This response probably requires a mutation in TP53, since cells bearing wild-type TP53 did not show any sign of malignancy upon RASA1 inactivation, nor did cells with p53R175H and wild-type RASA1 (Figure 5 and data not shown). These results agree with the strong correlation between RASA1 loss and mutation of TP53 that we have detected (Supplementary Table S VI). Interestingly, partial suppression of RASA1 expression also induces significant traits of malignancy in MCF10A p53R175H cells, suggesting that haploinsufficiency of RASA1 can malignize breast cells in the context of a TP53 mutation.

The murine tumors generated in this study have arisen from cells that express or previously expressed keratin K5. While keratin K5 is associated with basal myoepithelial cells of the differentiated gland, it is also expressed by primitive cells within the epithelial hierarchy, including bipotential cells that can generate luminal and basal cell types (50-52). Indeed, our transposon-induced tumors are not myoepithelial tumors, and therefore they are likely to arise from some form of bipotent or multipotent progenitor cell. K5-positive mammary stem cells, which give rise both to luminal and basal populations are long-lived, and capable of considerable
expansion (50). The longevity of these cells makes them likely targets for acquisition of mutations associated with continuous mobilization of mutagenic transposon by K5 promoter-driven transposase. Moreover, the exacerbated expansion of clonogenic stem/progenitor cells in both luminal and basal mammary epithelial cell layers that results as a consequence of p53 inactivation (53) seems to facilitate transposon-induced tumorigenesis, since mammary tumors were not common in SB/T2 Trp53WT transgenic mice (10).

In conclusion, deletion of RASA1 (probably in association with TP53 mutation) is likely to promote development and/or progression of basal subtype breast cancer in humans. Together with the recently reported losses of other RasGAPs such as NF1 and RASAL2 (8,9), our results support the possible activation of the Ras pathway, independently of mutations in Ras, as a driver of tumorigenesis in human breast tumors.
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TABLES

Table I. Candidate cancer genes present in common insertion sites from murine mammary tumors. Only genes with transposon insertions in at least three different tumors are shown.

| Gene Symbol | Gene Name                          | P-value   | # of tumors | % Disrupt |
|-------------|------------------------------------|-----------|-------------|-----------|
| Nfib        | nuclear factor I/B                 | 0         | 13          | 80        |
| Nf1         | neurofibromatosis 1                | 0         | 13          | 100       |
| Rasa1       | RAS p21 protein activator 1        | 0         | 9           | 70        |
| Gata3       | GATA binding protein 3             | 0         | 4           | 0         |
| Ripk4       | receptor-interacting serine-threonine kinase 4 | 0     | 3           | 100       |
| Ube2f       | ubiquitin-conjugating enzyme E2F   | 8.83E-65  | 3           | 100       |
| Pten        | phosphatase and tensin homolog     | 6.71E-57  | 4           | 80        |
| Cul3        | cullin 3                           | 1.49E-46  | 4           | 75        |
| Smad3       | MAD homolog 3 (Drosophila)         | 5.37E-37  | 3           | 67        |
| Zfr         | zinc finger RNA binding protein    | 1.85E-31  | 3           | 67        |
| Tead1       | TEA domain family member 1         | 6.53E-27  | 4           | 0         |
| Flnb        | filamin, beta                      | 1.05E-24  | 3           | 0         |
| Pum1        | pumilio 1 (Drosophila)             | 6.60E-24  | 3           | 67        |
| Ankrd11     | ankyrin repeat domain 11           | 3.15E-20  | 3           | 25        |
| Ankrd28     | ankyrin repeat domain 28           | 4.26E-17  | 3           | 100       |
| Fam135b     | family with sequence similarity 135, member B | 9.87E-10 | 3           | 0         |
Table II. Gene Ontology analysis of the 16 candidate cancer genes using DAVID-NIH revealed enrichment in several canonical signaling pathways and processes.

| Category          | Term                                      | Count | PValue     | Genes                      |
|-------------------|-------------------------------------------|-------|------------|----------------------------|
| GOTERM_BP_FAT     | Regulation of cell-matrix adhesion        | 4     | 1.54E-06   | NF1, SMAD3, PTEN, RASA1    |
| GOTERM_BP_FAT     | Regulation of apoptosis                   | 5     | 0.004      | CUL3, NF1, SMAD3, PTEN,    |
|                   |                                           |       |            | RASA1                      |
| PANTHER_BP_ALL    | Oncogenesis                               | 4     | 0.006      | CUL3, NF1, SMAD3, PTEN     |
| GOTERM_BP_FAT     | Regulation of cell migration              | 3     | 0.009      | NF1, SMAD3, PTEN           |
| GOTERM_BP_FAT     | Blood vessel morphogenesis                | 3     | 0.014      | NF1, PTEN, RASA1           |
| GOTERM_BP_FAT     | Posttranscriptional regulation of gene    | 3     | 0.014      | PUM1, SMAD3, PTEN          |
|                   | expression                                |       |            |                            |
| GOTERM_CC_FAT     | Nuclear lumen                             | 5     | 0.028      | ANKRD28, TEAD1, SMAD3, ZFR,|
|                   |                                           |       |            | NFIB                       |
| SP_PIR_KEYWORDS   | Nucleus                                   | 8     | 0.032      | CUL3, ANKRD28, GATA3,      |
|                   |                                           |       |            | ANKRD11, TEAD1, SMAD3, ZFR,|
|                   |                                           |       |            | NFIB                       |
| KEGG_PATHWAY      | MAPK signaling pathway                    | 3     | 0.036      | NF1, FLNB, RASA1           |
| GOTERM_BP_FAT     | Positive regulation of transcription from  | 3     | 0.041      | TEAD1, SMAD3, NFIB         |
|                   | RNA polymerase II promoter                |       |            |                            |
FIGURES

Figure 1: Generation of mammary tumors in mice by transposon mobilization. A), Schematics of the animal crossings performed to generate the mice. B), Anatomical localization of tumors arisen in female mice for non-transposition (p53+/−, n=71) and transposition (SB/T2/p53+/−; n=66) mice. “Others” include Thymus, Uterus, Liver and Ovary. C) Kaplan-Meier analysis of mammary gland tumor-free survival in SB/T2/p53+/- (n = 66) and in control p53+/- mice (n = 71). D) Expression of K5 (upper row) and SB11 transposase (lower row) in normal mammary gland (left column) and SB/T2/p53+/- mammary tumor tissue (middle column), and lack of expression of SB11 in p53-only tumors (right column). All images are at the same magnification. E) Table indicating the histological adscription of the 32 SB/T2/p53+/- murine mammary tumors. F), examples illustrating the most common mammary tumor types obtained: Left column, acinar carcinoma. Middle column, solid carcinoma. Right column, papillary carcinoma. Magnifications are indicated. G), Examples of ER-negative (left) and ER-positive (center and right) tumors. Inset shows an ER-positive, normal mammary epithelia in the same histological preparation than the ER-negative tumor (bar: 50 μm).

Figure 2: Inactivation of Rasa1 and Nf1 genes by transposon insertions. A) Gene structure of the murine Nf1 and Rasa1 genes showing the localization of the transposon insertions. Arrows designate transcriptional starts. The arrowheads represent the orientation of the transposon for each detected insertion. The functional domains of each protein are represented below and the exons encompassing the
RasGAP domain are indicated. B) Protein expression in mammary tumors induced by transposon insertion. Samples are grouped according to insertions in Rasa1, Nf1, both Nf1 and Rasa1 or none. Insertions in other genes are not considered. C) Relative quantification of the expression of RASA1 protein in tumors without (left) and with (right) transposon insertion in the Rasa1 gene. D) Relative quantification of the expression of NF1 protein in tumors without (left) and with (right) transposon insertion in the Nf1 gene. E, F) Relative quantification of the phosphorylation of ERK in Thr202/Tyr204 (E) and AKT in Ser473 (F) in tumors without (left) and with (right) transposon insertion in the Rasa1 gene. Panels C and D were normalized using β-actin signal, panels E and F were normalized using total ERK and AKT signals, respectively.

Figure 3: Immunohistochemical staining of human breast tumors using RASA1 and TP53 antibodies. A) Examples of negative, low, moderate and intense staining (indicated by 0, 1, 2, and 3, respectively) for RASA1 in several human breast tumors. Insets represent different fields in the same slide. N: Normal, non-tumorigenic area. T: Tumor. Scale bar: 100 um. Tumor numbers are indicated in the lower right corner. B) TP53 staining in the same human breast tumors as in A. C) Association of RASA1 relative staining with triple negative subtype. Tumors classified as triple negative have lower staining of RASA1 than tumors from the other types. D) Association of RASA1 relative staining with ER-status. Tumors with ER-negative status present lower staining of RASA1 than tumors with ER-positive status.
Figure 4: Analysis of RASA1 and NF1 alterations in breast cancer databases. (A) Analysis of the GISTIC putative copy number alterations for RASA1 in the TCGA breast invasive carcinoma cohort (5). For each tumor subtype, the percentage of each alteration is shown in accumulative bars. IDC: Invasive ductal carcinoma. B-E: Analysis of the relation between putative copy number alterations (GISTIC) of the RASA1 gene in cBioportal and (B) the levels of RNA expression of RASA1 as measured by RNAseq; (C) RNA expression of ESR1; (D) protein expression of ESR1; and (E) methylation status of the ESR1 gene. For each column, the number of samples is indicated above. F-I: analysis of the mRNA expression level of (F) RASA1 and (G) NF1 in PAM50 classified breast tumors from the GOBO database. For each column, the number of samples is indicated above. Analysis of the mRNA expression level of RASA1 (H) and NF1 (I) in breast tumors from the GOBO database, classified according to the ER status.

Figure 5: Effects of the inactivation of RASA1 RNA in human mammary cell lines. Two different shRNAs for RASA1 (sh1 and sh2) and a non-specific shRNA (n-s) were used. (A) Western Blot showing downregulation of RASA1 protein and phosphorylation of ERK 1/2 (Thr202/Tyr204) and AKT (Ser473) by RASA1 shRNAs in HCC-1954, BT-474 and T-47D cell lines. (B) Western blot showing the effect of RASA1 inactivation in MCF10A cells. Both sh1 and sh2 shRNAs reduced RASA1 expression to different extents. Cells infected with n-s, sh1 or sh2 constructs had no significant changes, while co-introduction of these shRNAs with p53R175H caused activation of ERK and AKT, downregulation of E-cadherin and upregulation of N-cadherin. (C). Photographs of exponentially growing MCF10A cells. Cells expressing non-specific (not shown), sh1 or sh2 shRNAs did not show any difference with controls. Cells expressing p53R175H had a
more rounded appearance, cells coexpressing p53<sup>R175H</sup> and either sh1 or sh2 RASA1 shRNAs adopted a fibroblastoid appearance and lost adhesion to the substrate. Bar: 100 μm. (D). Flow cytometry analysis for EpCAM and CD49f (integrin α6) for each cell type. MCF10A control cells, as well as sh1, sh2 and non-specific sh (not shown) present almost exclusively a EpCAM<sup>+</sup>/CD49f<sup>hi</sup> phenotype. Introduction of p53<sup>R175H</sup> partially shifts cells towards an EpCAM<sup>-</sup>/CD49f<sup>med/low</sup> phenotype, whereas cointroduction of p53<sup>R175H</sup> and sh1 or sh2 resulted in a majority of EpCAM<sup>-</sup>/CD49f<sup>med/low</sup> cells. Data are showed as mean ± SEM. (E) Expression of E-cadherin or N-cadherin in the different MCF10A cells was detected by immunofluorescence. Cells infected with non-specific, sh1 or sh2 RASA1 shRNAs (not shown) behaved as control, expressing only E-cadherin. Cells expressing p53<sup>R175H</sup> slightly diminished E-cadherin expression and showed small patches of N-cadherin expression, cells with both p53<sup>R175H</sup> and sh1 or sh2 greatly increased N-cadherin expression and reduced E-cadherin expression, in particular p53<sup>R175H</sup>/sh2 cells, which totally reversed the control phenotype. Bar: 100 μm. (F) Invasion assays on matrigel. Graphic shows invasion indexes normalized to control cells.
**A**

K5-SB11 × T2Onc2

K5-SB11 × p53 +/-

K5-SB11 T2Onc2 p53 +/-

**B**

p53 +/-

SB/T2/p53 +/-

**C**

Tumor-free survival (%)

Age (in weeks)

p = 0.0002

**D**

Normal gland T2/SB11

Tumor T2/SB11 p53 +/-

Tumor p53 +/-

**E**

| Histopathology classification          | n = 32 |
|---------------------------------------|-------|
| Acinar carcinoma                      | 19    |
| Solid-acinar carcinoma                | 6     |
| Papillary carcinoma                   | 2     |
| Papillary cystadenocarcinoma          | 2     |
| Solid carcinoma                       | 1     |
| Tubular carcinoma                     | 1     |
| Spindle-cell carcinoma                | 1     |

**F**

SB/T2/p53 +/-

**G**

Figure 1
Figure 3

A

RASA1

| 0 | 0 | 0 |
|---|---|---|
| T23 | T29 | T2 |
| 1 | 2 | 3 |
| T | T | T |
| N | N | N |
| T1 | T19 | T8 |

B

TP53

| T23 | T29 | T2 |
|-----|-----|----|
| T1 | T19 | T8 |

C

RASA1 Relative staining

| TNBC | Non-TNBC |
|------|----------|
| ![Staining](image1) | ![Staining](image2) |

* p=0.0105

D

RASA1 Relative staining

| ER- | ER+ |
|-----|-----|
| ![Staining](image3) | ![Staining](image4) |

** p=0.0098
Figure 4
Figure 5
A transposon-based analysis reveals RASA1 is involved in triple negative breast cancer

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