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An *in vivo* gain-of-function screen identifies the Williams-Beuren Syndrome gene GTF2IRD1 as a mammary tumor promoter

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Summary

The broad implementation of precision medicine in cancer is impeded by the lack of a complete inventory of the genes involved in tumorigenesis. We screened in vivo ~1,000 genes, that are associated with signaling for positive roles in breast cancer, using lentiviral expression vectors in primary MMTV-ErbB2 mammary tissue. Gain-of-function of five genes, including RET, GTF2IRD1, ADORA1, LARS2, and DPP8 significantly promoted mammary tumor growth. We further studied one tumor promoting gene, the transcription factor GTF2IRD1. The misregulation of genes downstream of GTF2IRD1, including TβR2 and BMPR1b, also individually promoted mammary cancer development, and silencing of TβR2 suppressed GTF2IRD1-driven tumor promotion. In addition, GTF2IRD1 is highly expressed in human breast tumors, correlating with high tumor grades and poor prognosis. Our in vivo approach is readily expandable to whole genome annotation of tumor promoting genes.

Introduction

Breast cancer is a global health problem, which will likely grow as the at-risk population ages. Recent years have witnessed the development of precision medicine as a therapeutic strategy (Meric-Bernstam et al., 2013). Precision medicine formulates treatments based on the genetic changes in a specific cancer, and can dramatically enhance the response rate over conventional treatments. However, the broad application of precision medicine requires full knowledge of the primary driver genes and their functions, plus multiple other genes that can potentiate or accelerate tumorigenesis.

Cancer genes can be identified through unbiased forward genetics approaches, and shRNA-based gene libraries have been frequently used to screen for cancer suppressor genes in multiple systems (Wolf et al., 2014; Zender et al., 2008). Recently, in vivo screening using

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Author Contributions

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primary cells of origin (as opposed to cell lines) has shown great potential for the discovery of tumor suppressor genes under physiological conditions (Bric et al., 2009; Schramek et al., 2014; Beronja et al., 2013), although this approach has not yet been applied to breast cancer. Gene up-regulation, through epigenetic and other mechanisms, is an important additional driver of many aspects of cancer (Ding et al., 2013) but gain-of-function screening has not yet been widely implemented in vivo. In this study, we use mammary glands as an example, in which high throughput library screening has not previously been proven feasible, to functionally identify tumor promoting genes using a gain-of-function approach in vivo.

Results

Optimization of the mammary gland regeneration system to identify breast tumor promoting genes *in vivo*

Murine mammary stem cells can regenerate entire mammary glands when transplanted into syngeneic recipients (Shackleton et al., 2006; Stingl et al., 2006), which provides a facile approach to interrogate gene function in the mammary gland under physiological conditions, and to identify novel cancer genes (Figure 1A). MMTV-ErbB2 transgenic mice were used as the mammary stem cell donor in this study, because ErbB2 is a clinically relevant oncogene in breast cancer (van de Vijver et al., 1988). MMTV-ErbB2 transgenic mice consistently form mammary tumors with a latency of ~5 months (Siegel et al., 1999). However, after transplantation into the cleared fat pads of wild type syngeneic recipient mice, MMTV-ErbB2 cells did not form tumors for more than 9 months. Therefore, we could screen for genes that would significantly reduce tumor latency in this mammary cell transplantation model. We chose a cDNA lentiviral library over the more broadly used siRNA libraries because gain-of-function drivers are more accessible to drug targeting than are loss-of-function mutations (Wang et al., 2006). The library contains ~1,000 genes that encode proteins involved in signaling pathways. All open reading frames (ORFs) were constructed in a bicistronic lentiviral vector, in which each ORF is tagged with RFP, and a puromycin resistance gene is expressed downstream of the ORFs, to enable selection (Figure S1A, S1C). Two problems with using cDNA libraries are that, first, since lentivirus packaging efficiency decreases dramatically as vector size increases, genes will not be represented equally; and second, multiple genes might function collaboratively in one cell to promote tumorigenesis. Therefore, prior to packaging, we divided the library into 12 sub-groups based on the sizes of the open reading frames (Table S1). A multiplicity of infection of 2 was adopted as a compromise between the availability of primary cells and the necessity for low virus copy numbers in each cell (Figure S1B).

Identification of breast tumor promoting genes

Primary mammary gland stem/progenitor cells isolated from 6 – 8 week old MMTV-ErbB2 transgenic mice were transduced with the lentiviral cDNA library. Cells were selected by puromycin for 3 days to enrich for cells containing lentiviral vectors (Figure S1C). Protein distribution, as detected by the in-frame RFP fusion, showed distinct patterns in different cells (Figure S1D). These cells were transplanted into the cleared fat pads of 40 syngeneic recipients.

One limitation intrinsic to this type of in vivo screen is the lack of knowledge of the mammary cell-of-origin from which tumors might arise, and the heterogenous population of cells being transduced, which makes any calculation of coverage problematic. The potential range of coverage is ~1.5 (if only stem cells can generate tumors) to about 2,500 (if all cells can generate tumors). However, since ErbB2+ tumors have luminal characteristics, we speculate that the cell-of-origin might be a luminal progenitor, which constitute about 14% of total mammary epithelium at 6 – 10 weeks of age, suggesting a maximum coverage of ~350 (Giraddi et al, 2015). Nonetheless, given the uncertainty regarding the actual coverage achieved in vivo in this screen, we cannot exclude the possibility that some genes in the library were not queried for their involvement in tumor promotion.

Importantly, the transplanted cells, which over-express ErbB2, formed normal mammary ductal trees within 6 weeks. After another 8 months latency, small tumors along the mammary ducts were detected in 4/40 mice transplanted with cells that had been infected with the lentiviral sublibraries (Figure 1B). No tumors were found at this time in 10 control mice transplanted with control MMTV-ErbB2 cells. We extracted genomic DNA from the tumors and identified individual genes by sequencing across the integrated lentivirus. Five genes were identified: *RET*, *GTF2IRD1*, *ADORA1*, *LARS2*, and *DPP8*. No tumor expressed multiple lentiviral genes; but one mouse had 2 tumors each of which expressed a different gene (Figure 1B). Among the hits, *RET* is known to promote mammary tumorigenesis (Mulligan, 2014), and served to validate our approach. The other 4 genes had never been shown to function as breast cancer genes.

One potential problem is that the integration of lentivirus might activate an endogenous oncogene, or inactivate a tumor suppressor to promote ErbB2-driven tumor formation. Therefore, we next expressed the 4 candidates individually in mammary cells from MMTV-ErbB2 transgenic mice (Figure 1C). Each of the 4 genes promoted breast tumor formation. LARS2 over-expressing tumors were of the invasive papillary carcinoma phenotype, while tumors with the other 3 genes, *GFT2IRD1, ADORA1,* and *DPP8*, showed invasive ductal carcinoma phenotypes (Figure 1D). These results demonstrated that all four genes individually can promote tumor growth.

One interesting gene is general transcription factor 2I repeat domain-containing protein 1 (*GTF2IRD1*, also called Ben). Haploinsufficiency of GTF2IRD1 causes Williams-Beuren syndrome (Franke et al., 1999; Tassabehji et al., 2005). Patients with this syndrome suffer from a variety of symptoms, including hypertension, diabetes, osteoporosis, and anxiety (Pober, 2010). A detailed analysis of the tumors driven by *GTF2IRD1* showed that about 70% of the tumors were invasive ductal carcinomas and the rest ranged from ductal hyperplasia, lobular carcinoma, to invasive papillary carcinoma (Figure S1E), which is similar to the prevalence of histological subtypes in human breast cancer (Viale, 2012). We next asked if *GTF2IRD1* is expressed in human cancers. We analyzed existing database and found that *GTF2IRD1* expression is significantly increased not only in invasive breast ductal carcinoma, but also in lung and ovarian cancers (Figure S2A, C, E) (Gaglio et al., 1995; Selamat et al., 2012). High *GTF2IRD1* correlates to poor overall survival of patients with breast cancer, lung cancer, or ovarian cancer (Figure S2 B, D, F) (Gyorffy et al., 2010; Gyorffy et al., 2012; Gyorffy et al., 2013). These data suggest that *GTF2IRD1* is a bona fide

tumor-promoting gene relevant to human cancer. Together, these experiments demonstrate the efficacy of gain-of-function screens in the mammary gland system under physiological conditions.

GTF2IRD1 functions through downstream genes *TβR2* and *BMPR1b*

We next sought to understand how *GTF2IRD1* contributes to breast cancer development. First, we asked if the lentivirus-driven expression of GTF2IRD1 in the tumors was within a physiologically relevant range, and found a 2.5 fold increase compared to that in normal mammary gland tissues (Figure 2A). This is comparable to the observed increases in human invasive breast cancers, which have a ~1.8 fold increase in *GTF2IRD1* mRNA compared to that in normal human mammary tissues (Figure S2A).

We then examined downstream effectors of GTF2IRD1. Several genome-wide studies have uncovered genes that are regulated by GTF2IRD1 in mice, including *Tβ R1, TβR2, SMAD5, OPN*, and *BMPR1b* (Chimge et al., 2008; Enkhmandakh et al., 2009; Lazebnik et al., 2008). We initially tested these genes using the untransformed human mammary gland cell line, MCF10A. Over-expression of GTF2IRD1 in MCF10A cells caused small but significant increases in *OPN* and *TβR2* transcript levels and decreased *BMPR1b* mRNA level, compared to the vector control (Figure 2B). However, no significant changes were detected for *TβR1* and *SMAD5*. We also examined the protein levels of OPN, TβR2, and BMPR1b by immunoblot, and found qualitatively similar responses to the changes in mRNA (Figure 2C, D).

We next asked if related expression changes occur in mouse breast tumors. *TβR2* and *bmpr1b* expression were regulated the same way as in MCF10A cells. However, *opn* expression was significantly decreased in tumors expressing GTF2IRD1 compared to the control tumors (Figure 2E). This opposite behavior might reflect species differences or a difference in the tumor environment or gene regulatory network, as compared to a cell line in culture. The surprise was TβR2, because it is widely known as a tumor suppressor gene (Guasch et al., 2007; Novitskiy et al., 2011). Nonetheless, loss of TβR2 can increase breast tumor latency in a MMTV-Neu transgenic mouse model (Novitskiy et al., 2014), suggesting that it may have paradoxical effects in tumors as compared to normal tissues.

TGF-β signals are transmitted via phosphorylation and nuclear translocation of the Smad family members. To further test if TβR2 is a downstream target of GTF2IRD1, we measured phospho-Smad2 levels in MCF10A cells over-expressing GTF2IRD1 or TβR2. Immunoblot analysis showed that phospho-Smad2 levels were significantly higher in the cells expressing GTF2IRD1, or TβR2, than in cells with the empty vector control (Figure 2F, G). These data demonstrate GTF2IRD1 up-regulates membrane TβR2 levels, which mediate an elevated TGF-β activity.

Do *OPN, TβR2,* and *BMPR1b* act downstream of GTF2IRD1 gene to promote breast cancer development? We asked whether these genes promote tumorigenic behavior in MCF10A cells *in vitro* and in mouse mammary glands *in vivo*. MCF10A stable cell lines were established to over-express control vector, GTF2IRD1, OPN or TβR2, or shRNA against *BMPR1b*. These cell lines were then used to assess proliferation rates, soft agar colony

formation, and tumorsphere growth. Notably, GTF2IRD1 and its downstream target genes all suppressed cell proliferation compared to the control vector, for cells grown in 2D culture (Figure S3A). However, they all promoted colony formation in soft agar, and tumorsphere formation under anchorage independent growth conditions (Figure S3B, S3C).

We also manipulated gene expression in mouse mammary stem/progenitor cells to study their impact on mammary cancer development *in vivo*. Lentiviruses to over-express *GTF2IRD1*, *OPN* or *TβR2*, or shRNA against *BMPR1b,* were transduced into mammary gland stem/progenitor cells isolated from MMTV-ErbB2 transgenic mice. These cells were then transplanted into cleared fat pads of syngeneic recipients to regenerate mammary glands. Mice were palpated once a week to detect breast tumors until tumors had formed in mice with the control vector. *GTF2IRD1*, *TβR2*, and shRNA against *BMPR1b* accelerated tumor incidence significantly compared to the control vector (Figure 3A). OPN had no significant effect. We conclude that TβR2 and BMPR1b act in opposite ways downstream of GTF2IRD1 to promote breast tumor formation.

We next analyzed the histology of mouse breast tumors driven by GTF2IRD1, TβR2 and shRNA against *BMPR1b*. The majority of tumors were invasive ductal carcinomas, with low levels of ductal or lobular hyperplasia (Figure 3B, S3D). Quantitative analysis showed that *GTF2IRD1, TβR2*, and the shRNA against *BMPR1b* did not change the sizes of the primary tumors and lung metastases significantly from those of the control tumors, indicating that the over-expression of *GTF2IRD1*, *TβR2*, or loss of *BMPR1b* gene did not increase tumor cell proliferation in primary tumors or at metastatic sites (Figure 3C). Moreover, dysregulation of TβR2 or BMPR1b drove high-grade tumor formation, which is consistent with the GTF2IRD1 tumors (Figure 3D).

To determine whether the tumor promoting activity of GTF2IRD1 is dependent on the dysregulation of these downstream genes, we asked whether reversal of the expression of one of the key downstream genes, in combination with GTF2IRD1 over-expression, would impact tumor incidence. Using shRNA, TβR2 expression was silenced in ErbB2 mammary cells that over-express GTF2IRD1, and transplanted into recipient mice. Tumor latency was significantly increased when TβR2 expression was suppressed, compared to GTF2IRD1 over-expression alone (Figure 3E).

The few tumors that arose from cells expressing GTF2IRD1 and depleted of TβR2 are similar histologically to those expressing GTF2IRD1, with invasive ductal carcinoma phenotypes. However, all tumors showed apparent hemorrhaging, in contrast to those tumors that expressed GTF2IRD1 alone (Figure S3E). This observation is consistent with a previous study showing that suppression of TβR2 increases vasculogenesis and blood vessel leakage (Novitskiy et al., 2014). These data support a molecular mechanism in which GTF2IRD1 induces *TβR2*, which, together with reduced BMPR1b expression, promotes mammary tumorigenesis.

GTF2IRD1 is a clinical relevant breast tumor promoting gene

Finally we asked what clinico-pathological traits *GTF2IRD1* contributes to breast cancer in patients. We evaluated the association between GTF2IRD1 protein levels and cancer

characteristics in patients in the Nashville Breast Health Study, a population-based case-control study (Zheng et al., 2009). The specificity of the GTF2IRD1 antibody was tested by both western blots and immunohistochemistry staining in multiple tissues, including breast cancer tissues (Figure S4). We found that higher GTF2IRD1 expression was significantly associated with higher tumor grade (P=0.04). Additionally, ER-negative tumor ($P = 0.01$) and PR-negative ($P = 0.05$) tumors have higher GTF2IRD1 expression compared to ER-positive and PR-positive tumors (Table 1). However, GTF2IRD1 expression was not associated with TNM stage or tumor size (Table 1, S2). Age at diagnosis, menopausal status, or family history of breast cancer has no association with GTF2IRD1 protein levels in the tumor (Table S2). These data demonstrate high levels of GTF2IRD1 correlate with poor prognosis in patients with breast cancer.

Discussion

In this study, we establish a system to functionally identify gain-of-function effects on tumorigenesis in the mammary gland, study the mechanisms through which these genes promote tumor formation, and investigate their clinical relevance. We identified four tumor-promoting genes through this approach that had not previously been associated with cancer. One of these genes, *GTF2IRD1*, was further studied for its mechanism and clinical relevance. It significantly accelerates mammary tumor formation in an oncogenic background, and correlates with increased tumor grade and poor prognosis in human patients.

We demonstrate that induction of TβR2 is essential for GTF2IRD1-driven tumorigenesis and that over-expression of TβR2 is sufficient to accelerate mammary tumor formation. GTF2IRD1 or TβR2 each activate TGFβ signaling, and promote soft agar colony formation in vitro, but reduce cell proliferation. Therefore, these genes would not have been revealed as tumor promoters using a classical in vitro screen, highlighting the importance of using clinically relevant in vivo approaches. Consistent with these paradoxical effects, TβR2 was earlier demonstrated to be a tumor suppressor gene in early tumor stages, but can also behave as a tumor promoter (Massague, 2012). Loss of TβR2, for example, promotes metastatic squamous cell carcinoma and aggressive tumor behavior (Lu et al., 2006; Malkoski et al., 2012), whereas in HER2 mammary carcinogenesis, a dominant-negative TβR2 mutant delayed tumor onset (Novitskiy et al., 2014). The other downstream target of GTF2IRD1 is *BMPR1b*, which has not been directly linked to tumorigenesis. In our study, this receptor functions as a mammary tumor suppressor. It will be instructive in the future to determine if these genes can promote tumor formation independent of any specific driver, as seems likely from the human breast cancer data, or whether they are ErbB2/HER2-specific. Finally, our in vivo approach can be easily expanded to a genome–wide gain of function screen using a pooled CRISPR gene activation library, which we expect will reveal additional gain-of-function genes that can promote breast cancer and that will be of clinical relevance.

Experimental Procedures

Mice

ErbB2 transgenic mice MMTV-NEU-NDL were kindly provided by Dr. William Muller. Male ErbB2 transgenic mice were bred to female FVB mice purchased from the Jackson Laboratory. No randomization method was used for inbred mice. All experiments were carried out in accordance with AAALAC guidelines and with Vanderbilt University Institutional Animal Care and Use Committee approval.

Mammary stem/Progenitor cell isolation and transplantation

Mammary gland stem/progenitor cells were isolated from 6–8 week old nulliparous female mice. The third to fifth pairs of mammary glands were removed and minced. After 45 min digestion at 37 °C (DMEM/F12, 2mg/ml collagenase I (Roche), 5mg/ml insulin (Sigma), 200U/ml Nystatin (Sigma), and 100U/ml penicillin/streptomycin) epithelial organoids were washed in 5ml of DMEM/F12 containing DNase I and centrifuged at 1,000 rpm for 5 min. The pellet was resuspended in 5ml DMEM/F12 with 10% fetal bovine serum followed by 15 sec of centrifugation at 1,500 rpm for 5×. Organoids were dissociated in 1ml of fresh 0.05% trypsin/EDTA (Invitrogen) for 12 min to obtain a single-cell suspension of mammary gland cells. Freshly isolated mammary gland cells were transduced by lentivirus in a final volume of 200 μl for 1 h. Cells were cultured for 3 d in mammary epithelial cells media (DMEM/F12, 5% FBS, 1% ITS (Gibco), 5ng/ml EGF (Invitrogen), 100U/ml penicillin/streptomycin) and selected by puromycin (invitrogen, 2.5μg/ml) for another 3 d. 20,000 mammary cells were transplanted into cleared fats of 3 week-old FVB mice for all experiments except the initial screening. Incisions were made around the fourth pair of nipples and endogenous mammary glands were removed. Cells were injected into each cleared fat pad for mammary gland regeneration. All mice were examined each week for palpable tumors. Experiments were terminated once palpable tumors formed in control mice or the tumor volume reaches $2mm^3$. Tissues were fixed in formalin and processed at Vanderbilt Translational Pathology Shared Resource.

For the initial screen, 500,000 cells were injected into one fat pad each of 3 – 4 mice (~2×10^6 cells total per subpool). Each sub-group contains about 80 genes and the lentiviral infection efficiency was about 10%. However, given the heterogeneity of the primary mammary cell population, and the lack of information available on cell-of-origin for the tumors, we cannot reliably estimate actual coverage. If all mammary cells are equally capable of forming tumors, our library coverage is ~2,500; but if only stem cells act as the tumor cell of origin, then the coverage is only ~1.5, assuming 1/2000 cells is a stem cell (Huo and Macara, 2014). However, even stem cell frequency can vary over >2 orders of magnitude depending on the duration of growth in culture and the culture conditions (Prater et al., 2014). Since ErbB2+ tumors have luminal characteristics, we speculate that the cell-of-origin might be a luminal progenitor. Sca1−Cd49b+ progenitors constitute about 9% and Sca1+CD49b+ progenitors about 5% of the total mammary epithelium in 10-week old virgin mice, and the ratio is similar at 6 weeks (Giraddi et al., 2015), suggesting a maximum coverage of about 350×. Forty mice in total were transplanted. The parental vector pLEX was used as a control in 10 mice.

Statistical Analysis

ANOVA was used in the analysis for differences of characteristics and clinico-pathological parameters compared to GTF21RD1 expression. The missing indicator method was used for missing covariate information. All the tests were performed using SAS (version 9.3; SAS Institute, Inc., Cary, North Carolina). The significance levels were set at $P < 0.05$ and based on two-sided probability. For other experiments a two-tailed paired Student t-test was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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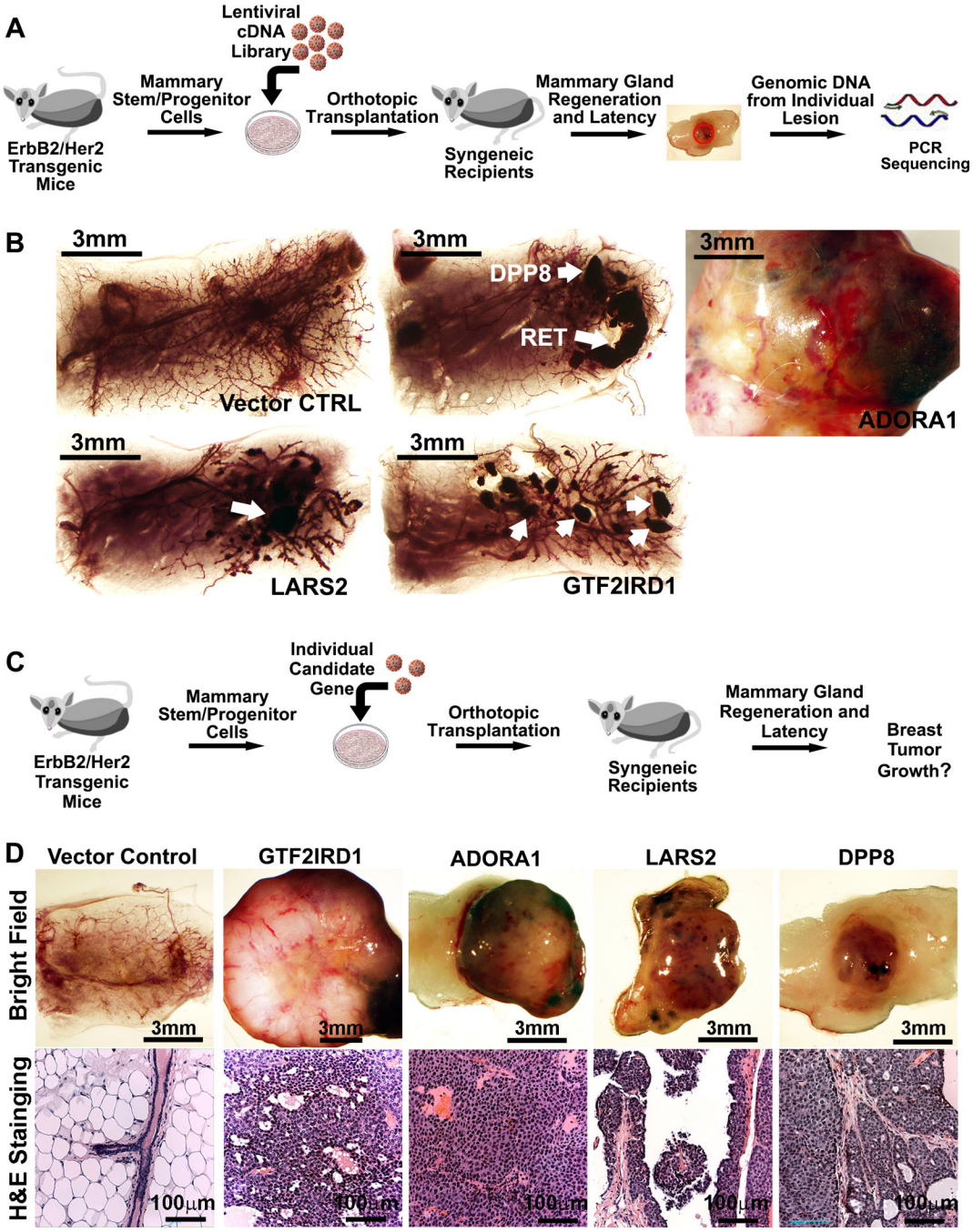

Figure 1. ***See also*** **Figure S1, S2, Table S1. An** ***in vivo*** **screening identifies** ***RET*****,** ***DPP8*****,** ***ADORA1*****,** ***LARS2*****, and** ***GTF2IRD1*** **as breast tumor promoting genes**

(A) Schematic view of the *in vivo* screening system. Mammary gland stem/progenitor cells were isolated from 6–8 week old ErbB2 transgenic mice and transduced with a lentiviral cDNA library. After transplantation into the cleared fat pads of syngeneic hosts, these cells regenerated mammary glands. Tumors formed in 4/40 mice after 8 months. DNA was isolated from the tumors and the lentiviral genes identified by sequencing. (B) *RET*, *DPP8*, *ADORA1*, *LARS2*, and *GTF2IRD1* were identified from tumors in the regenerated mammary glands. (C) Schematic of the validation process. Mammary gland stem/progenitor

cells from MMTV-ErbB2 mice were transduced with individual candidate genes expressed from lentiviruses. (D) *DPP8*, *ADORA1*, *LARS2*, and *GTF2IRD1* each drives tumor formation in mammary glands. The LARS2 driven tumors show an invasive papillary carcinoma phenotype and the rest are invasive ductal carcinomas.

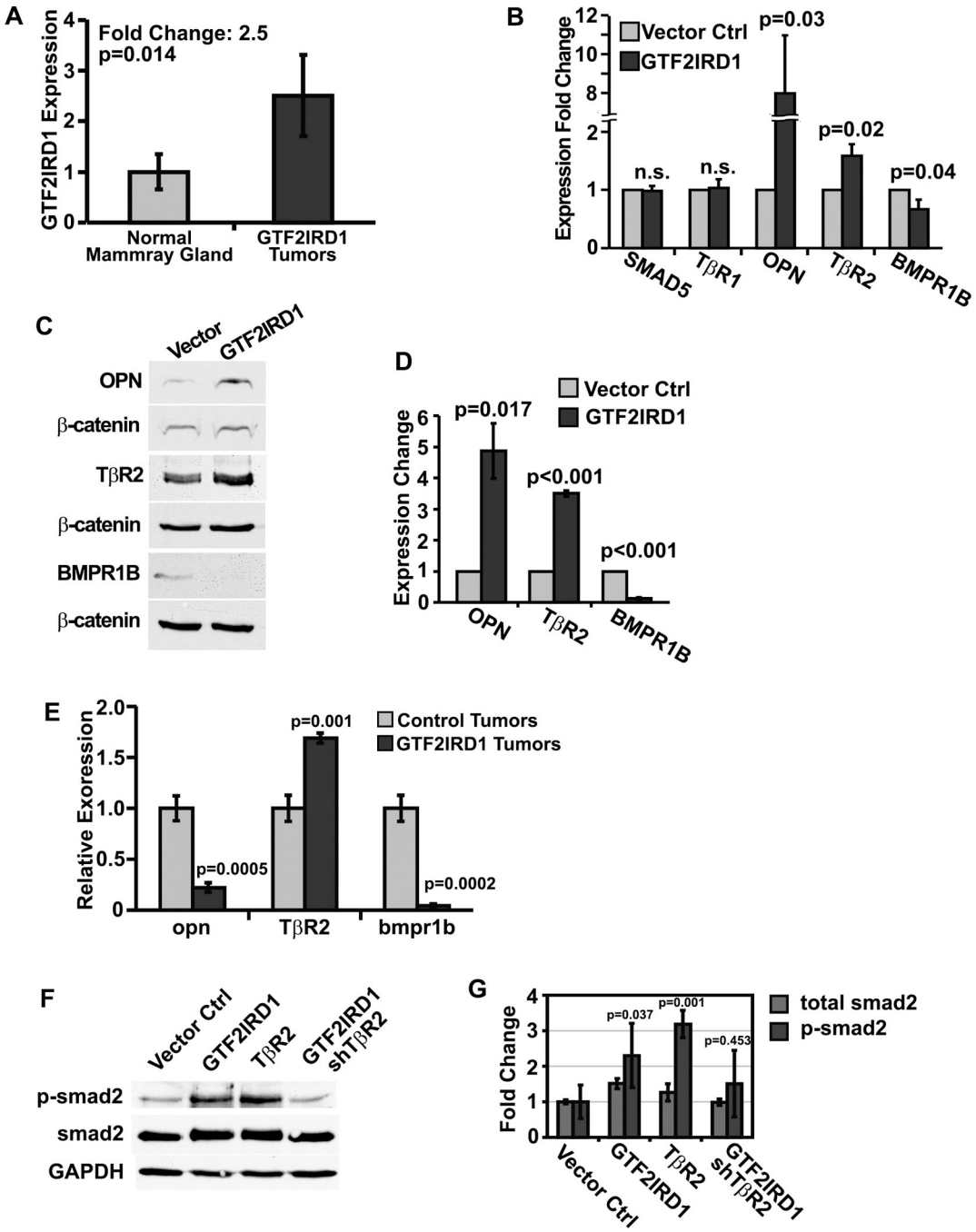

Figure 2. GTF2IRD1 regulates expression of *OPN*, *TβR2*, and *BMPR1b* in MCF10A cells and breast tumors

(A) GTF2IRD1 expression in tumors was increased 2.5-fold compared to normal mammary gland tissues. Error bars represent mean ± SD, n=4. (B) Over-expression of GTF2IRD1 in MCF10A cells increased *OPN* and *TβR2* transcript levels and decreased *BMPR1B* mRNA levels significantly compared to the vector control, but had no effects on *SMAD5* and *TβR1* mRNA levels, as assayed by quantitative PCR (n = 3). (C, D) Immunoblot analysis confirmed that over-expression of GTF2IRD1 in MCF10A cells up-regulated OPN and TβR2 and down-regulated BMPR1b protein levels. Immunoblots were quantified and results

are summarized in panel C (n= 3). (E) In tumors, GTF2IRD1 over-expression up-regulated TβR2 and down-regulated OPN and BMPR1b levels significantly. Error bars represent mean ± SEM. n=3. (F,G) Phospho-Smad2 levels were measured in MCF10A cells by immunoblot. Protein levels were quantified and results are summarized in panel G. Error bars represent mean ± SD, n=3. p values were calculated by a two-tailed paired Student *t* test.

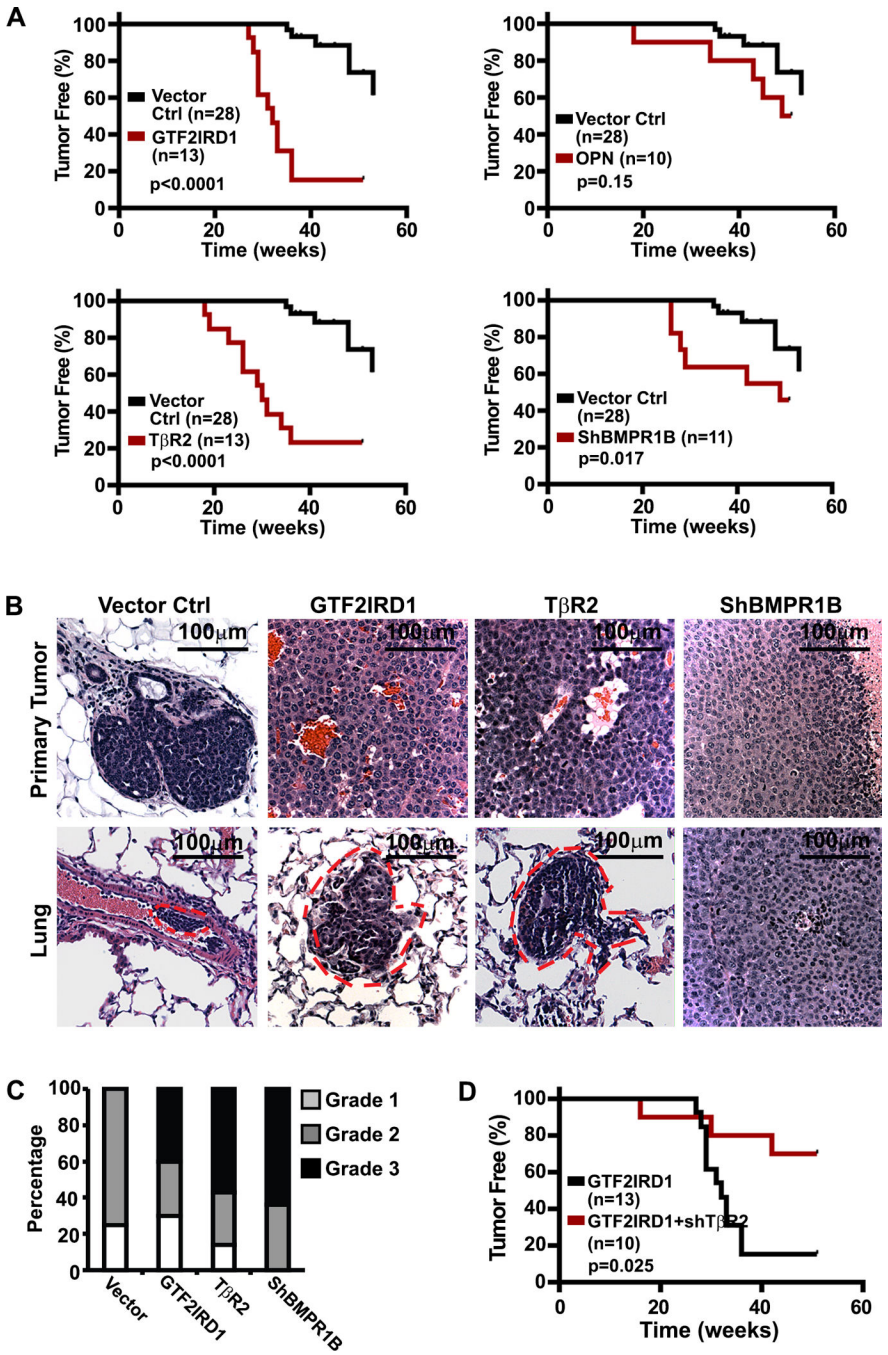

Figure 3. ***See also*** **Figure S3. GTF2IRD1 promotes breast tumor formation through and its downstream genes *TβR2* and *BMPR1b***

(A) GTF2IRD1 and TβR2 over-expression or BMPR1b silencing each decreased tumor formation latency significantly compared to the vector control. Tumor free survival curves were generated using Prism software (Version 6.0, GraphPad Software Inc.). *P* values were calculated for the experimental groups compared to the vector control group. (B) Histology shows that over-expression of GTF2IRD1 or TβR2, or depletion of BMPR1b promotes formation of invasive ductal carcinomas and lung metastases in mice. (C) GTF2IRD1 and its downstream effectors TβR2 and BMPR1B drive high grade tumor development. (D)

Depletion of TβR2 in GTF2IRD1 over-expressing mammary gland stem/progenitor cells significantly increased tumor latency compared to over-expression of GTF2IRD1 alone.

Table 1

Correlation of GTF2IRD1 protein expression with clinic-pathological parameters of breast cancer

		N	GTF2IRD1 expression		*P*-value
			Mean	SD	
Tumor size	≤ 2 cm	133	72.5	27.0	0.14
	> 2 cm	69	78.6	29.3	
Tumor grade	I	36	69.6	26.1	
	II	73	70.3	30.3	0.04
	III	93	80.0	25.8	
ER/PR/Her-2 status	ER-positive	111	70.8	27.7	0.01
	ER-negative	50	82.1	24.0	
	missing	41	75.8	31.3	
	PR-positive	85	70.4	26.4	0.05
	PR-negative	76	78.6	27.3	
	missing	41	75.8	31.3	
	Her-2-positive	36	79.3	21.2	0.11
	Her-2-negative	102	70.8	28.8	
	missing	64	78.1	29.3	
Molecular subtype[a]	Luminal A	75	67.5	29.5	
	Luminal B	19	78.4	19.8	0.09
	HER2	16	80.4	24.1	
	Triple negative	26	79.7	25.3	
	unknown	66	78.1	28.8	

[a]Luminal A: ER-positive and/or PR-positive, Her-2-negative; Luminal B: ER-positive and/or PR-negative, Her-2-postitive; HER2: ER-negative and PR-negative, Her-2-positive; Triple negative: ER-negative, PR-negative, Her-2-negative