SUPPLEMETARY INFORMATION

SUPPLEMENTARY MATERIALS AND METHODS

Generation and maintenance of the Ret/MTB mouse colony

We generated the pTetO-Ret expression vector by cloning a 3.3Kb *Cla*I/*Spe*I fragment encoding the human Ret51 (*hRet51*, NM_020975) from MCF7 cells into pTAMILA-PS 14.4 Kb [11], using the high fidelity enzyme (Finzyme). The vector also contains a plasmid pTet-Splice backbone (Gibco BRL) encoding the IRES-firefly luciferase expression cassette, to serve as a surrogate reporter, and a SV40 polyadenylation signal downstream of Ret. Founder lines were generated by injecting linearized plasmid DNA into fertilized oocytes collected from superovulated FVB/N mice. Transgenic mice carrying the TetO-Ret construct (Ret/-) were mated with mouse mammary tumor virus-reverse tetracycline transactivator (MMTV-rtTA) mice (MTB/-) [11] to obtain bitransgenic mice (Ret/MTB).

For genotyping, DNA was prepared from material collected at ear-punching biopsy and specific primers amplifying Ret (TetO-RetF: 5'ATCCACGCTGTTTTGACCTC3'; TetO-RetR: 5' CGAGAAGTAGAGGCCCAATG3') or MTB (MTBF: 5' ATCCGCACCCTTGATGACTCCG 3'; MTBR: 5'GGCTATCAACCAACACACTGCCAC3') sequences were used in PCR reactions to detect mice carrying either none (FVB/N background animals), one (single-transgenic animals, designated Ret/- or MTB/-) or both (Ret/MTB animals) transgenes.

During monitoring of Ret/MTB females by bioluminescence we observed an increase of luciferase activity in the mammary gland during the lactation period in comparison with other developmental stages. In addition, using bioluminescence

we observed luciferase activity in the area of the salivary glands of Ret/MTB animals under dox-food. In some animals under chronic induction, we observed swollen, irregular necks suggesting that these animals might have hyperplastic glands. We did not pursue this finding in these initial studies with the Ret/MTB model.

Generation of a collection of tumor pieces and tumor grafts

We have generated a collection of tumor pieces that can be successfully engrafted into Balb/c Nude, using both fresh and frozen tissue. We attempted to engraft tumor pieces in FVB/N mice, however, they were usually rejected, presumably because of the expression of luciferase and/or human Ret. This tumor collection was generated as follows: for the first *in vivo* passage, chronically dox-induced tumor-bearing Ret/MTB females were sacrificed, primary tumors were harvested and pieces were engrafted into the fad pad of estrogen (E2) pellet-carrying (0.025mg 17β-estradiol/90 days release; Innovative Research of America NE-121) or non pellet-carrying mice. Recipient animals were maintained under dox-food to ensure continued tumor graft growth. E2-pellets were implanted subcutaneously in the neck of the animals 3 days before engrafting tumor pieces. Dox-food was administrated 2 days before pellet implantation. After approximately 3 months, the arising tumors were engrafted in a second round using the same procedure. No significant differences were found between groups of E2 pellet-carrying and non pellet-carrying animal in the first, second and third passages. Over the course of passaging the tumor grafts began growing within 10-15 days of engraftment. Importantly, tumor grafts growing under dox-food keep the characteristics of the primary tumors until at least the sixth passage, the longest we have tested.

Analysis of the NGS data

RNA from independent primary tumor fragments, harvested before or after doxycycline withdrawal, was obtained using the RNeasy Mini Kit (Qiagen). After

quality control, sequencing libraries were prepared from 200ng total RNA using the TruSeq stranded mRNA library preparation kit (Illumina). Libraries were pooled and sequenced on an Illumina HiSeq2500. HCS 2.2.58 was used with default parameters for base calling and bcl2fastq 1.8.4 (Illumina) for demultiplexing and generating fastq files. Raw reads were preprocessed using the R(v3.3.0)/Bioconductor package QuasR(1.12.0). Reads with more than 2Ns were removed with the function preprocess reads. The remaining reads were aligned to mm10 using the qAlign with splicedAlignments=TRUE and default parameters. The QuasR function qCount was used to extract a table with raw read counts per gene for all Entrez genes for each sample. The data have been submitted to the Gene Expression Omnibus (GEO) and assigned the identifier GSE83897.

Intrinsic subtype classification of tumors into LuminalA-like, LuminalB-like Basal-like, Her2-enriched and Normal-like groups was performed using the 50-gene (PAM50) predictor bioclassifier R script (used by Parker et al [17]).

In silico analysis of Ret expression in human breast carcinomas was classified by expression of ER, PR and HER2. Comparative analysis of Ret mRNA expression profiles in human breast samples was done using the TCGA breast cancer project at the cBioPortal resource (http://www.cbioportal.org/). Normalized Ret microarray expression profiles were compared according the differential expression of ER, PR and HER2 in 1985 breast carcinomas samples from METABRIC repository. Kruskal-Wallis chi-squared was used as a statistic test.

In vivo treatments

Ret/MTB tumor fragments (1-2 mm in diameter) were engrafted in female Balb/c nude mice, with 4-10 mice per group. Recipient animals were maintained under doxycycline-food starting 2 days before performing implants, to ensure continued tumor graft growth. When Ret/MTB tumors reached ~ 100mm^3 (15-20 days after implanting) mice were randomized into treatment groups that received vehicle, NVP-AST487, fulvestrant or AEE788, as indicated. Other groups received the corresponding vehicles as controls. We used the Stratified Sample randomization

method to allocate the experimental groups. The selection of this method is based in the fact that in the population of tumor-bearing mice exist very often subpopulations differing in tumor size. The individuals were organized by this aspect into two different strata: smaller (≤100 mm^3) and middle (>100 mm^3 and <200 mm^3) tumors. From each stratum, an animal is then selected at random for the different groups with the intent of representing the overall population as closely as possible. Treatments were carried out for 2 weeks. NVP-AST487 (Novartis) was formulated and used as previously described. [7] Fulvestrant (Sigma) was prepared by dissolving the powder in 10% ethanol corn oil (Sigma) to obtain 1mg/100µl and given three times per week by s.c. injection (3mg/week). AEE788 (Novartis) was prepared by dissolving the powder in water and was administered orally three times per week (40mg/Kg/injection). No changes in the body weight of the inhibitor-treated mice were measured. Calipers were used to measure tumor area in two dimensions. Tumor volumes were determined according to the formula: length x diameter 2 x π/6. The average of the tumor volumes was plotted for each group ± s.e.m. Tumors were collected at the end of the experiment, 1 hour and 3 hours after the last drug administration, and they were weighed.

For experiment of drug administration, we used the statistics of a preliminary experiment to give the appropriate sized sample for our desired power, 80%. Groups of n=7 tumor graft-bearing mice each were vehicle- or NVP-AST487-treated for 14 days. We determined that the mean (μ) of tumor size of the vehicletreated group (μVeh) is 1226.85 mm3 with a standard deviation (σ) of 847.67 mm3. We determined that the treatment with NVP-AST487 give us 952.21 mm3 of reduction in tumor size. Then, with this effect, the μ of the NVP-AST487-treated group (μAst) is 274.64 mm3. Assuming normal distribution of the data and taking a significance of α=0.05 and a desired power of 1-β=0.80 we perform the sample size calculation (http://powerandsamplesize.com/Calculators/Compare-2-Means/2-Sample-Equality) obtaining a n=13 mice. Thus, for the pre-clinical studies we used a minimum of 13 mice/group considering at least 2 independent experiments.

For ovariectomy, tumor fragments were transplanted into the inguinal fat pads of BALB/c Nude females. When the established tumors reached a measurable size, the animals were either sham-operated or ovariectomized (OVX). Success of the OVX was checked by microscopic observation of the vaginal epithelium. Tumor growth was monitored as described above.

We did not use blinding procedure for the individual conducting the treatments. However, those analyzing the results were blinded to the experimental groups.

Immunohistochemistry (IHC), staining and antibodies

IHC analysis of Ret/MTB-mammary glands and Ret/MTB-evoked primary tumors or their grafts, for different markers was carried out in paraffin sections using the Ventana DiscoveryUltra instrument (Roche Diagnostics, Manheim). The procedure RUO Discovery Universal was used with 40 minutes CC1 pre-treatment and incubation for 1 hour at 37°C with the following primary antibodies: phosphoS10-Histone H3 (pS10H3, 9701 Cell Signaling, 1:100), D175-cleaved caspase 3 (CC3, 9661 Cell Signaling, 1:100), Ret E1N8X (14556 Cell Signaling, 1:50, anti-human and mouse) ErbB2 21N (Cell Signaling, 1:1000), CK8/18 GP11 (Progene, 1:400), CK14 RB-9020 (Thermo Scientific, 1:200), SMA (RB-9010, Thermo Scientific, 1:200), ER MC-20 (sc-542 Santa Cruz, 1:1000), PR SP2 (RM-9102 Thermo Scientific, 1:200), pY701Stat1 58D6 (9167Cell Signaling, 1:100), pY705Stat3 D3A7 (9145 Cell Signaling, 1:100), pY694Stat5 D47E7 (4322 Cell Signaling, 1:50), pT202/Y204Erk D13.14.4E (4370 Cell Signaling, 1:200), pS240/244S6 (2215 Cell Signaling, 1:150) The corresponding secondary antibodies (ImmPRESS reagent kit peroxidase, Vector) were applied manually (200 microliters) and incubated for 32 min at 37°C. ChromoMap DAB kit (Roche Diagnostics, Mannhein) was used for detection. Finally slides were counterstained with Hematoxylin II and Bluing Reagent (Roche Diagnostics, Mannheim) for 8 min.

For hyperplastic and control mammary gland tissue, pS10H3-positive and -negative cells were counted under the microscope (600X) in 10 independent ducts from the glands of 7 different mice per group, corresponding to 2 independent experiments (n=7). Results were expressed as a % of pH3-positve cells/counted

cells in 10 ducts ± s.d. For tumors, pS10H3-positive and -negative cells were counted under the microscope (600X) in 7 fields corresponding to different areas of the tumor. Results were expressed as a % of pH3-positve cells/7 fields ± s.d (n=6-8). For pY701Stat1 and pY705Stat3, the quantification was done by microscopic counting (400X) of pY701Stat1- or p705Stat3-positive and –negative epithelial cells in 5 fields corresponding to different areas of the tumor and the results were expressed as a % of pStat-positive cells/field ± s.d (n=5-8). Counting was performed in healthy area of the tumors, avoiding tumor edge and necrosis. For CC3, pictures (200X) covering the whole area of the tumor were taken (n=5-7) and quantification was done using ImageJ software and expressed as positive area fraction/field ± s.d. for each staining. H&E-staining was done by standard protocols. For hyperplastic and control mammary gland tissue, quantification of the area covered by ducts was done on pictures of carmine-stained whole mounts by AxioVison software.

Protein extraction and Western blot (WB)

Tissue was lysed in a buffer containing 50mM Hepes (pH7.4), 150mM NaCl, 25mM b-Glycerophospate, 25mM NaF, 5mM EGTA, 15mM PPI, 1% NP40 and 1mM DTT, containing the inhibitors, 1mM PMSF, 10µg/ml aprotinin, 10µg/ml leupeptin, 2mM sodium orthovanadate and 10mM sodium molybdate. The tumor tissues were homogenized for 15 seconds in 10 volumes of lysis buffer using a polytron PT 1600 (Kinematica).

Following 30 minutes on ice, lysates were cleared by centrifugation, then the supernatants were collected and stored at -80°C. Before freezing, a sample aliquot was diluted at 1:1000 in water for determination of the protein concentration with a commercially available protein Bradford assay (Bio-Rad) using bovine serum albumin as standard. Sixty ug of each sample was separated by SDS-PAGE and blotted onto a PVDF immobilon membrane (Millipore). After 1 hour blocking with 20% horse serum (HS) in PBS, and 0.1% Tween 20, filters were probed overnight (4°C) with specific primary antibodies (dissolved 1:1000 in 10% HS in PBS 0.1%

Tween 20). The antigen-antibody complexes were visualized using horseradish peroxidase-conjugated anti-mouse or rabbit IgG secondary antibody (Amersham, GE Healthcare) and the enhanced chemiluminescence was detected using ECL Western blotting System (Amersham, GE Healthcare). The membrane was stripped and re-probed.

The following antibodies were used for WB: anti-phosphoT202/Y204-Erk 4370, anti-Erk 9102, anti-phosphoY705-Stat3 9131, anti-human and mouse Ret 3223, anti-mTor 2972 (Cell Signaling); anti-mouse and human phosphoY1062-Ret sc-0252-R (Santa Cruz); anti-tubulin MS-581-P1 (Neomarkers); anti-Stat3 610190 (Transduction Labs); anti-phosphoS2481-mTor 09-343SP (Milipore).

Statistical analyses

Data were analyzed using the indicated test in the corresponding figure legend by GraphPad Software and considered significant when p-value < 0.05.

Normality was assessed by Shapiro-Wilk test to ensure validity of using the parametric tests described. If the data generated was found to deviate significantly from a normal distribution, we instead used Non-parametric test (e.g. Mann-Whitney Rank Sum Test) for statistical analysis. Otherwise, we used two-sided two sample T tests to compare measurements between experimental and control groups

Specially for *in vivo* experiments, omitting data points is not a good practice as this may encourage individuals to removing data points to reach a p value < 0.05. An outlier robust rank based nonparametric test was performed when outliers were suspected. When death of a single animal occurred due to a known technical mistake, this animal was removed from the experiment.

SUPPLEMENTARY TABLES

Supplementary Table S1. Sequence (5’-3’) corresponding to the primers par used in qRT-PCR to detect specific genes.

Gene	Primer par 5'-3' sequence
Human Ret	TCATATGTGGCCGAGGAGGCG
	CAGTCCTGAGGGCAAATGTTGATG
Mouse GFRα1	CTGTGTGCTCCTATGAAGAACGA
	TTGCTGCAATCGCACCACGGC
Mouse GDNF	AGGAGGAACTGATCT TTCGATAT
	TGGCCTACTTTGTCACTTGTT
Mouse Nrtn	CTGGAAGGCAGCGGCCC
	CCCTGGAGCAGAGCGCG
Mouse Artn	TGGAACTGGGACTTGCAGAG
	CTGCGGGACATTGGGTCC
Mouse Prsn	TGGCTGCAGGAAGACTTCG
	TGAGAGCTTATCTGCCACAG
Mouse Gapdh	AGAAGGTGGTGAAGCAGGCATC
	CGAAGGTGGAAGAGTGGGAGTTG

Supplementary Table S2. Total counts for Ret network partners in the NGS data.

		8 weeks dox-induced transgenic glands								Ret/MTB-tumors						Control transgenic glands					
		Control Ret/- glands				Hyperplastic Ret/MTB glands				Growing under dox-food				Regressed after dox-withdrawal		dox-induced Ret/- glands				Ret/MTB glands in normal-food	
SYMBOL	Entrezid	TG5_R	TG5_R	TG5_RM	TG5_RM	TG5_R	TG5_R	TG5_RM	TG5_RM	T	T	T	T	T_OUT	T_OUT	MG_R	MG_R	MG_R	MG_R	MG_RM	MG_RM
Gfra1	14585	100	3	13	72	23	46	50	28	36	56	52	74	61	90	82	114	75	91	78	88	3	1	0
Gfra2	14586	72	43	30	27	36	41	160	216	203	232	158	215	343	336	433	304	286	207	530	488	20	43	35
Gfra3	14587	2	0	0	3	1	1	13	8	10	17	11	8	26	21	80	53	41	35	21	22	0	0	0
Gfra4	14588	44	451	208	15	9	6	55	40	33	32	53	55	145	108	169	144	139	92	81	104	551	576	1237
Gdnf	14573	2	0	0	0	0	1	1	3	5	8	4	3	4	0	11	2	1	6	1	3	0	0	0
Nrtn	18188	2	2	6	19	15	10	5	0	1	6	3	1	15	16	66	50	44	86	3	43	117	53	137
Artn	11876	5	0	1	1	1	0	1	3	4	5	5	7	4	2	5	9	8	4	0	3	2	2	3
Pspn	19197	1	2	4	1	1	1	0	0	3	3	0	1	1	4	1	0	0	1	1	2	0	0	6

Supplementary Table S3. PAM50 correlation score. ROR-S: Risk of relapse subtype alone; ROR-P: Risk of relapse predicted by program.

Tumor	Basal	Her2	LumA	LumB	Normal	Call	Confidence	ROR-S (Subtype Only)	ROR-S Group (Suptype Only)	Proliferation Score	ROR-P (Suptype + Proliferation)	ROR-P Group (Subtype + Proliferation)
T1	0,34	0,05	-0,44	-0,14	0,04	Basal	0,98	59,34	high	0,50	65,70	high
T11	-0,10	-0,16	0,28	-0,31	0,35	Normal	0,98	21,52	low	-0,71	6,76	low
T12	-0,29	-0,07	0,43	-0,07	0,22	LumA	1,00	19,60	low	-0,68	8,15	low
T15	-0,19	-0,30	0,55	-0,33	0,45	LumA	1,00	7,46	low	-1,11	-13,69	low
T16	-0,44	-0,26	0,59	-0,03	0,19	LumA	1,00	10,31	low	-0,60	8,41	low
T18	-0,37	-0,21	0,52	-0,02	0,20	LumA	1,00	14,51	low	-0,70	5,65	low

Supplementary Table S4. GSEA index report

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Name GS follow link to MSigDB	Size	ES	NES	NOM p-val	FDR q-val
CGGAARNGGCNG_UNKNOWN	50	0,4728	1,7266	0,0072	0,0741
KRCTCNNNNMANAGC_UNKNOWN	59	0,4382	1,6559	0,0000	0,0721
TMTCGCGANR_UNKNOWN	154	0,3509	1,5866	0,0000	0,0820
V$NRF2_01	258	0,3235	1,5096	0,0000	0,1201
GATGKMRGCG_UNKNOWN	62	0,3744	1,4471	0,0353	0,1733
ACTAYRNNNCCCR_UNKNOWN	430	0,2862	1,4198	0,0000	0,1775
V$GABP_B	248	0,2978	1,4144	0,0000	0,1601
V$STAT1_01	66	0,3565	1,3884	0,0245	0,1731
TCCCRNNRTGC_UNKNOWN	206	0,2921	1,3497	0,0096	0,2125
GCCATNTTG_V$YY1_Q6	394	0,2735	1,3492	0,0000	0,1924

gsea_report_for_TumorON_ vs_Tumor OUT_1424447312132 transcription factor					
Name GS follow link to MSigDB	Size	ES	NES	NOM p-val	FDR q-val
GGCNNMSMYNTTG_UNKNOWN	70	0,4015	1,5699	0,0059	0,3520
V$STAT1_01	66	0,3969	1,5532	0,0066	0,2150
TMTCGCGANR_UNKNOWN	154	0,3453	1,5472	0,0021	0,1509
V$E2F_Q2	163	0,3398	1,5408	0,0000	0,1213
V$E2F_Q6_01	232	0,3283	1,5361	0,0000	0,1019
V$XBP1_01	127	0,3430	1,4878	0,0081	0,1438
V$E2F1_Q4	234	0,3058	1,4499	0,0000	0,1879
V$E2F_Q4_01	227	0,3058	1,4353	0,0039	0,1910
MCAATNNNNNGCG_UNKNOWN	80	0,3503	1,4304	0,0202	0,1790
AACYNNNNTTCCS_UNKNOWN	92	0,3450	1,4304	0,0161	0,1612

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. (**a**) Ret/MTB or control mammary glands (MTB/-) after 1 week of doxycycline-induction were analyzed by H&E-staining. (**b**) IHC staining for the proliferation marker pS10H3 was performed in mammary tissue from the indicated mice after 1 or 4 weeks of induction. Scale bars, 50μm. (**c**) mRNA levels of the co-receptor GFRa1 and the indicated Ret ligands were analyzed by qRT-PCR using specific primers. The values were normalized to GAPDH expression and represented as absolute values. Columns, means of the values ± s.e.m. from 4 independent females. (**d**) IHC staining for Ret and pT202/Y204Erk were performed in Ret/MTB-tumors and surrounding non-neoplastic tissue under the indicated conditions. Scale bars, 50μm. T: Tumor. Hyp: hyperplastic surrounding tissue.

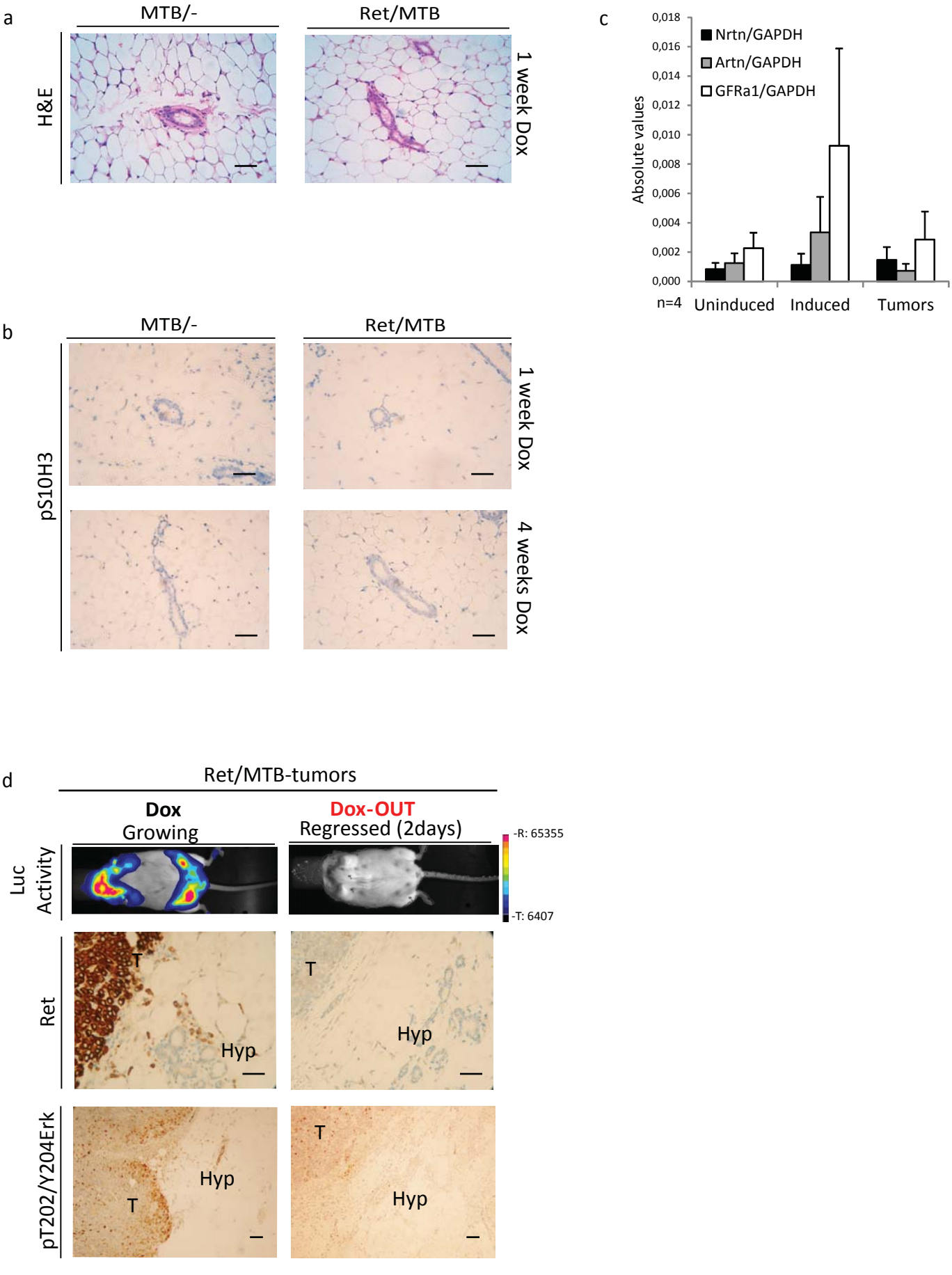
Supplementary Figure S2. Immunostaining for ER was performed in the Ret/MTB independent primary tumors (T1, T11, T12, T15, T16, T18) used for the RNA-seq. Φ: area of hemosiderin laden inflammatory cells.

Supplementary Figure S3. (**a**) Box Plot shows a comparative analysis of the Ret mRNA expression according to breast cancer subtypes based on ER/PR and Her2 detection. Inside each box the number of cases is indicated. (**b**) GSEA shows the correlation between indicators of proliferation (REACTOME_DNA_REPLICATION) and gene sets regulated in Ret/MTB-tumor (TumorDox) after 48 hours of doxycycline withdrawal (TumorDox-OUT). n=3 each group. NES: normalized enrichment score; *q*: false discovery rate. *p*-value was computed on the basis of comparison against random stimulation as implemented in GSEA.

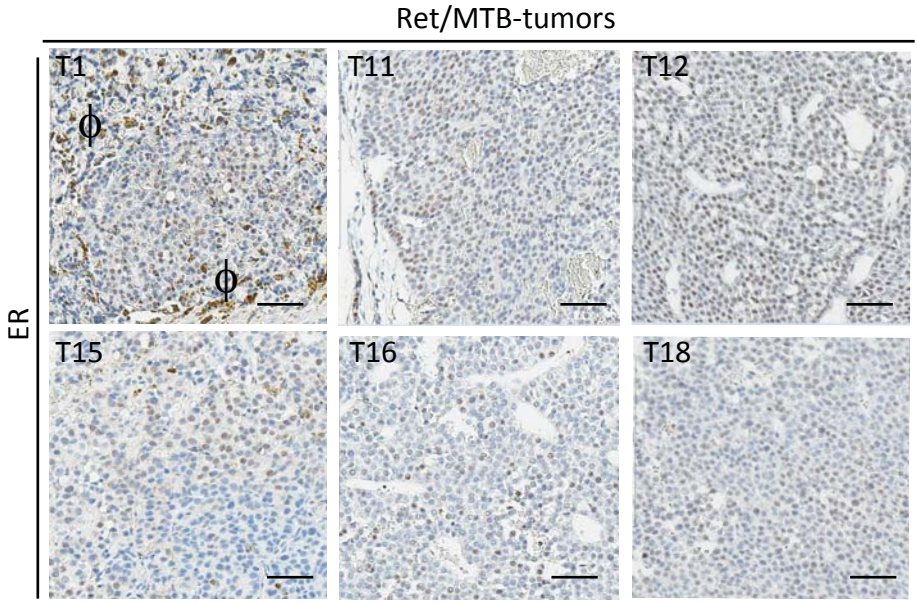
Supplementary Figure S4. Histological features of Ret/MTB-tumor grafts generated in Balb/c Nude mice. IHC for Ret, ErbB2, ER and PR receptors was performed in tumor grafts. Representative pictures are shown. Scale bars, 50μm.

Supplementary Figure S5. (**a**) Groups of Ret/MTB-tumor graft-bearing Balb/c Nude mice (n=10) were randomized and treated thrice weekly with vehicle (H_2O) or the ErbB2 inhibitor AEE788 (40mg/kg/injection). Tumor volume was determined every 2 days for 2 weeks. Points represent mean ± s.e.m. (**b**) Spider plots show that transplanted tumors grow at similar rates in controls or after ovarian hormone deprivation. Fragments of secondary (circles or triangles, upper graph) or primary (squares and rhombuses, lower graph) passages from Ret/MTB-evoked mammary tumors were transplanted into the mammary fat pads of Balb/c Nude mice. When the established tumors reached 1-600 mm^3 in volume, the animals were either sham-operated (Sham, black curves; n=4) or ovariectomized (OVX, blue curves; n=4). Each line represents an individual tumor. (**c**) Groups of Ret/MTB-tumor graft-bearing mice were randomized and treated with vehicle (EtOH 10% corn oil) or fulvestrant (3 mg/week). There were no changes in tumor volume as measure every 2 days. Tumor weight was measured at the end of the experiment. Bars represent tumor mass mean ± s.e.m.

Supplementary Figure S1.

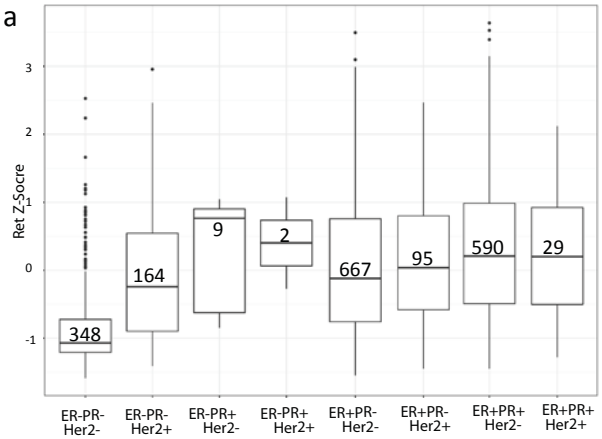


Supplementary Figure S2.



Supplementary Figure S3.

a



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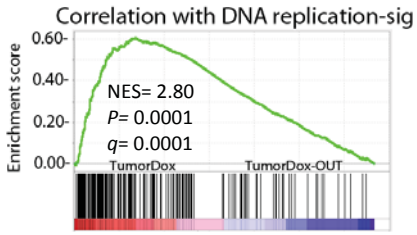
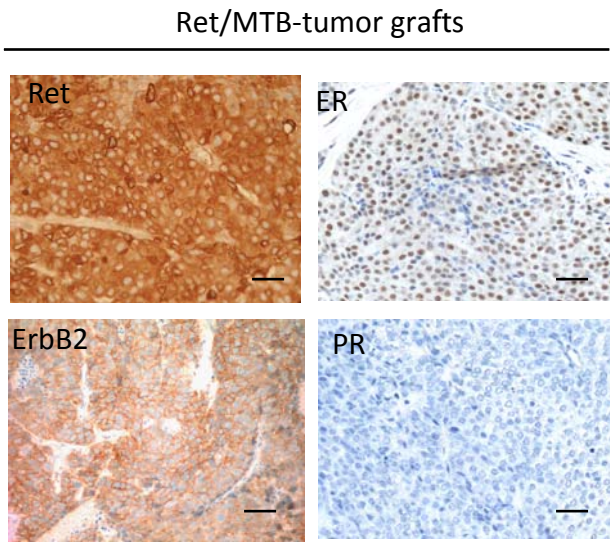


Supplementary Figure S4.



Supplementary Figure S5.