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Functionally Recurrent Rearrangements of the MAST Kinase and Notch Gene Families in Breast Cancer

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Abstract

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Breast cancer is a heterogeneous disease, exhibiting a wide range of molecular aberrations and clinical outcomes. Here we employed paired-end transcriptome sequencing to explore the landscape of gene fusions in a panel of breast cancer cell lines and tissues. We observed that individual breast cancers harbor an array of expressed gene fusions. We identified two classes of recurrent gene rearrangements involving microtubule associated serine-threonine kinase (MAST) and Notch family genes. Both MAST and Notch family gene fusions exerted significant phenotypic effects in breast epithelial cells. Breast cancer lines harboring Notch gene rearrangements are uniquely sensitive to inhibition of Notch signaling, and over-expression of *MAST1* or *MAST2* gene fusions had a proliferative effect both *in vitro* and *in vivo*. These findings illustrate that recurrent gene rearrangements play significant roles in subsets of carcinomas and suggest that transcriptome sequencing may serve to identify patients with rare, actionable gene fusions.

Recurrent gene fusions and translocations have long been associated with hematologic malignancies and rare soft tissue tumors as driving genetic lesions [1–3]. Over the last few years, it is becoming apparent that these genetic rearrangements are also found in common solid tumors including a large subset of prostate cancers [4,5] and smaller subsets of lung cancer, among others [6]. Secretory breast cancer, a rare subtype of breast cancer, is characterized by recurrent gene fusions of *ETV6-NTRK3* [7]. While a number of breast cancer genomes have been sequenced [8,9], and complex somatic rearrangements observed [10], "driving" recurrent gene fusions have thus far not been identified.

We employed paired-end transcriptome sequencing on a panel of 89 breast cancer cell lines and tumors (Fig. S1), and applied our previously developed chimera discovery pipeline [11,12]. This represented a spectrum of breast carcinoma, including 42 estrogen receptor (ER) positive, 21 ERBB2 positive, and 27 triple negative ($ER^-/PR^-/ERBB2^-$) samples (Table S1). Fusion transcript discovery led to the identification of 384 expressed gene fusions, an average of nearly five per breast cancer sample, with a slightly higher number of gene fusions in the cell lines compared to primary tumors (Fig. S1b and Table S2). Remarkably, only *SEC16A-NOTCH1* was found to be recurrent in our compendium, even as several fusion genes did appear in combination with different fusion partners. Overall, 24 genes were found to be recurrent fusion partners, highlighted in Table S2. In order to focus on potentially tumorigenic 'driver' fusions, we prioritized the gene fusions based on the known cancer-associated functions of component genes. While there were many singleton fusions in our compendium that met these criteria, we identified 5 cases of fusions of MAST family kinases and 8 cases of fusions of Notch family genes (Fig. 1 and Fig. S2).

MAST kinase family genes are characterized by the presence of a serine/threonine kinase domain, a 3' MAST domain with some similarity to kinase domains, and a PDZ domain [13]. Little is known about the biological role of MAST kinases and somatic alterations have not been described in cancer. Initially, three independent cases of MAST gene fusions were identified by transcriptome analyses-*ARID1A-MAST2, ZNF700-MAST1*, and *NFIX-MAST1* (Fig. 1a). We devised a targeted sequencing approach to screen additional samples for MAST gene fusions. A transcriptome library of 74 pooled breast carcinoma RNAs was

generated and captured with baits encompassing *MAST1* and *MAST2*. After sequencing, two new MAST gene fusions were discovered, *TADA2A-MAST1* and *GPBP1L1-MAST2* (Fig. 1a). The samples harboring MAST gene fusions are distinct from those with Notch family gene fusions (Fig. 1b), discussed later.

The fusions were confirmed by fusion-specific PCR (Fig. 2a). All five MAST fusions encoded contiguous open reading frames, some retaining the canonical serine/threonine kinase domain and all retaining the PDZ domain and the 3' kinase-like domain (Fig. 2b). Thus overall, we have discovered five novel gene fusions encoding *MAST1* and *MAST2* in a cohort of a little over 100 breast cancer samples and more than 40 cell lines, suggesting that the novel serine/threonine kinase family gene fusions represent a subset of 3–5% of breast cancers.

The *ZNF700-MAST1* fusion transcript encodes a truncated MAST1 protein that retains the 3' kinase-like and PDZ domains. We cloned the open reading frame of the *ZNF700-MAST1* fusion to test phenotypic effects and used a full-length *MAST2* expression construct to mimic the function of *ARID1A-MAST2* over-expression. To assess the potential oncogenic functions of MAST genes, we ectopically over-expressed epitope tagged- truncated MAST1 and full length MAST2 in the benign breast cell line, TERT-HME1 (Fig. S3a–h). We next cloned and expressed all five MAST1/MAST2 fusions. Consistent with the earlier observations, TERT-HME1 cells overexpressing the five MAST fusions (Fig. 2c) also displayed higher rates of cell proliferation (Fig. 2e). Overall, these results suggest that ectopic expression of the MAST fusions impart growth and proliferative advantage in benign breast epithelial cells.

As the endogenous *ARID1A-MAST2* fusion is present in the breast cancer cell line MDA-MB-468, we used multiple independent *MAST2* or *ARID1A-MAST2* fusion-specific siRNAs to achieve a marked knockdown of the *ARID1A-MAST2* fusion protein (Fig. S3i–s). Knockdown of MAST2 showed significant growth inhibitory effects in MDA-MB-468 cells but not in the fusion negative line BT-483, or benign breast cells, TERT-HME1 (Fig. 2d). To further characterize the effects of the *ARID1A-MAST2* fusion in MDA-MB-468 cells, we used shRNA targeting *MAST2*, which displayed efficient knockdown of *ARID1A-MAST2* fusion transcript and protein (Fig. S3k–l). MDA-MB-468 cells treated with *MAST2* shRNA exhibited a dramatic reduction in growth as demonstrated in a colony formation assay (Fig. 2f), as well as showed increased apoptosis with S-phase arrest (Fig. S3m–n). In the mouse xenograft model, MDA-MB-468 cells transiently transfected with *MAST2*-shRNA failed to establish palpable tumors over a time course of 4 weeks (Fig. 2g). The knockdown studies show that the *ARID1A-MAST2* fusion is a critical driver fusion in MDA-MB-468 cells.

In addition to MAST fusions, a total of 8 rearrangements involving either *NOTCH1* or *NOTCH2* were discovered (Fig. 1b and Fig. S2). All were found in ER negative breast carcinomas ($p = 0.008$), and all but one in triple negative breast carcinomas. We focused on one ER negative tumor and three ER negative breast cancer cell lines with 3' *NOTCH1* or *NOTCH2* fusion transcripts for functional studies. The Notch fusion transcripts are abundantly expressed and are specific to samples harboring DNA rearrangements (Fig. 3a). All fusion transcripts retain the exons encoding the Notch intracellular domain (NICD),

responsible for inducing the transcriptional program following Notch activation (Fig. 3b). The DNA breakpoints associated with Notch fusions were characterized by mate-pair genomic library sequencing, or long-range genomic PCR (Fig. S4a,b).

The predicted open reading frames for the *NOTCH1* and *NOTCH2* fusion transcripts fall into two classes (Fig. 3b). For both the *SEC16A-NOTCH1* fusions and the intragenic *NOTCH1* fusion in HCC1599, the predicted ORFs initiate after the S2 cleavage site, but before the S3 γ-secretase cleavage site, similar to the *TCRB-NOTCH1* fusion in the T cell adult lymphocytic leukemia line CUTLL1[14]. In contrast, the *SEC22B-NOTCH2* fusion ORF is predicted to initiate just after the γ-secretase S3 cleavage site. The resultant protein would be nearly identical to NICD itself, and would be predicted to be highly active and independent of cleavage by γ-secretase (Fig.3b).

We observed substantially higher Notch responsive transcriptional activity in the three cell lines containing Notch fusions, compared with other breast cell lines using a Notch luciferase reporter (Fig.3c). Thus, each of the three Notch fusions is capable of activating the expression of Notch responsive genes. Using an antibody specific to the γ-secretase cleaved active form of the NOTCH1-NICD, both HCC1599 and HCC2218 exhibit high levels of NICD, consistent with the fusion protein acting as a substrate for activation by γ-secretase (Fig.3d). HCC1187, which contains a *NOTCH2* fusion gene, exhibits little NOTCH1-NICD. Most breast cancer lines express wild-type *NOTCH1* (Fig. 3d, middle panel), however, only the two cell lines with *NOTCH1* fusion alleles show high levels of activated NICD. Each of the three fusion alleles, co-transfected with a Notch reporter plasmid, induced Notch-responsive transcription equivalent to NICD itself (Fig.3e).

The three breast cell lines containing the Notch fusions exhibit decreased cell-matrix adhesion and grow in suspension, or as weakly adherent clusters, unlike the majority of breast carcinoma cell lines. When *NOTCH1* and *NOTCH2* fusion alleles were transduced to create stable pools of TERT-HME1 cells, striking morphological changes were observed (Fig. 3f). TERT-HME1 cells exhibit adherent epithelial properties, while the Notch fusion expressing cells lose adherence and propagate as weakly attached clusters, similar to the index lines harboring the Notch fusions and consistent with previously reported effects of NICD expression in MCF10A cells[15]. Furthermore, the fusion alleles dramatically induced expression of the Notch target genes, *MYC*, *HES1*, and *HEY1* (Fig. 3g).

The Notch fusions represent two functional classes with respect to dependence on γ-secretase activity. BrCa10040, HCC2218, and HCC1599 fusions are dependent on S3 cleavage for activity and sensitive to γ-secretase inhibitors (GSIs). The HCC1187 fusion class is independent of S3 cleavage. Stable Notch reporter lines were established from each of the three Notch fusion index lines and treated with the γ-secretase inhibitor DAPT [16]. A dramatic reduction of Notch reporter activity upon DAPT treatment was seen with HCC1599 and HCC2218 fusion alleles (Fig. 4a). On the other hand, Notch reporter activity is only slightly diminished by DAPT in HCC1187, expressing a γ-secretase independent Notch fusion allele. DAPT treatment also dramatically reduced NICD protein levels in both sensitive cell lines (Fig. 4b). Furthermore, index cell lines exhibit dependence on Notch signaling for proliferation and survival (Fig. 4c). The HCC1599 and HCC2218 cell lines

exhibit dramatic reductions in proliferation following DAPT treatment. HCC1187, which expresses a GSI-independent *NOTCH2* fusion, shows no reduction in proliferation upon DAPT treatment, as do breast cell lines not expressing Notch fusion alleles.

Treatment with DAPT repressed expression of the Notch targets *MYC* and *CCND1* (Fig. 4d), two genes demonstrated to play a key role in mouse mammary tumorigenesis induced by Notch [17,18], further supporting the possibility GSIs may be useful in treating cancers harboring activated Notch alleles. Consistent with this, treatment with DAPT significantly reduced tumor volume in a xenograft tumor model of HCC1599 (Fig. 4e).

Since the discovery of the *TMPRSS2-ERG* gene fusion in approximately 50% of prostate cancers, emerging evidence suggests that recurrent gene fusions play a more significant role in common solid tumors than previously appreciated. The MAST and Notch aberrations in breast cancer represent novel classes of rare but functionally recurrent gene fusions with therapeutic implications (similar to the ALK fusions in lung cancer). MAST kinase and Notch gene rearrangements are mutually exclusive aberrations in the samples tested, and together, may represent up to 5–7% of breast cancers. The discovery of functionally recurrent MAST and Notch fusions in a subset of breast carcinomas illuminates a promising path for future research and treatment in breast cancer and illustrates the power of next-generation sequencing as a tool in the development of personalized medicine.

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

Methods

Cell lines and specimen collection

Breast cancer cell lines were purchased from the American Type Culture Collection. This study was approved by the respective Internal Review Boards and breast cancer samples were obtained from the University of Michigan and the Breakthrough Breast Cancer Research Centre, Institute of Cancer Research (London, UK).

Paired end transcriptome sequencing

Total RNA was extracted from normal and cancer breast cell lines and breast tumor tissues and the quality of RNA was assessed with the Agilent Bioanalyzer. Transcriptome libraries from the mRNA fractions were generated following the RNA-SEQ protocol (Illumina. Each sample was sequenced in a single lane with the Illumina Genome Analyzer II (40–80 nucleotide read length) or with the Illumina HiSeq 2000 (100 nucleotide read length). Paired-end transcriptome reads passing filter were mapped to the human reference genome (hg18) and UCSC genes with Illumina ELAND software (Efficient Alignment of Nucleotide Databases). Sequence alignments were subsequently processed to nominate gene fusions using the method described earlier [11,12].

Quantitative RT-PCR and long-range PCR

Quantitative RT-PCR assays using SYBR Green Master Mix (Applied Biosystems) were carried out with the StepOne Real-Time PCR System (Applied Biosystems). Relative mRNA levels of each chimera shown were normalized to the expression of *GAPDH*. To detect the genomic fusion junction in HCC1187 cells, primers were designed flanking the predicted fusion position and PCR reactions were carried out to amplify the fusion fragments.

Immunoblot detection of MAST2 fusion protein and NOTCH1 protein

Immunoblot analysis for MAST2 was carried out using MAST2 antibody from Novus Biologicals. Human β-actin antibody (Sigma) was used as a loading control. For NOTCH1 protein detection, cells were lysed in RIPA buffer containing protease inhibitor cocktail (Pierce). Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with antibodies recognizing total NOTCH1 (Cell Signaling), γ-secretase-cleaved NOTCH1 (NICD, Cell Signaling), or beta-actin (Santa Cruz).

Constructs used for over-expression studies

The *ZNF700-MAST1* fusion ORF from BrCa00001 was cloned into Gateway pcDNA-DEST40 mammalian expression vector (Invitrogen) using LR Clonase II. A plasmid with C-terminus V5 tag was generated and tested for protein expression following transfection in HEK293 cells. A full-length expression construct of *MAST2* with DDK tag was obtained from Origene.

Establishment of stable pools of TERT-HME1 cells

The five MAST fusion alleles were cloned with an amino terminal FLAG epitope tag into the lentiviral vector pCDH510-B (SABiosciences). Lentivirus was produced by cotransfecting each of the MAST plasmids with the ViraPower packaging mix (Invitrogen) into 293T cells using FuGene HD transfection reagent (Roche). Thirty-six hours post-transfection the viral supernatants were harvested, centrifuged, and then filtered through a 0.45 micron Steriflip filter unit (Millipore) TERT-HME1 were infected at an MOI of 20 with polybrene at 8 μg / ml. Forty-eight hours post-infection, the cells were split and placed into puromycin-selective media. Stable pools of TERT-HME1 cells expressing the NOTCH fusion alleles, as well as a control NOTCH1 intracellular domain were generated using the same procedures.

Knockdown assay

For siRNA knockdown experiments, multiple independent MAST2 siRNAs from Thermo were used (J-004633-06, J-004633-07, and J-004633-08). All siRNA transfections were carried out using Oligofectamine reagent (Life Sciences. Similar experiments were performed with multiple custom siRNA sequences targeting the ARID1A-MAST2 fusion (Thermo). Lentiviral particles expressing the MAST2 shRNA (Sigma, TRCN0000001733) were transduced using polybrene, according to the manufacturer's instructions.

Colony formation assay

MDA-MB-468 cells transduced with scrambled or *MAST2* shRNA lentivirus particles were plated and selected using puromycin. After 7–8 days the plates were stained with crystal violet to visualize the number of colonies formed. For quantitation of differential staining, the plates were treated with 10% acetic acid and absorbance was read at 750nm.

Mouse Xenograft Models

Four week-old female SCID C.B17 mice were procured from a breeding colony at University of Michigan, maintained by Dr. Kenneth Pienta. Mice were anesthetized using a cocktail of xylazine (80 mg kg^{-1} IP) and ketamine (10 mg kg^{-1} IP) for chemical restraint. *MAST2* shRNA or scrambled shRNA knockdown MDA-MB-468 breast cancer cells (4 million) or *NOTCH1* fusion allele positive HCC1599 breast cancer cell line (5 million) were resuspended in 100ul of 1X PBS with 20% Matrigel (BD Biosciences) and implanted into right and left abdominal-inguinal mammary fat pads. Ten mice were included in each group. Two weeks after tumor implantation, HCC1599 xenograted mice were treated daily with γ-secretase inhibitor (DAPT) dissolved in 5% ethanol in corn oil (IP). All procedures involving mice were approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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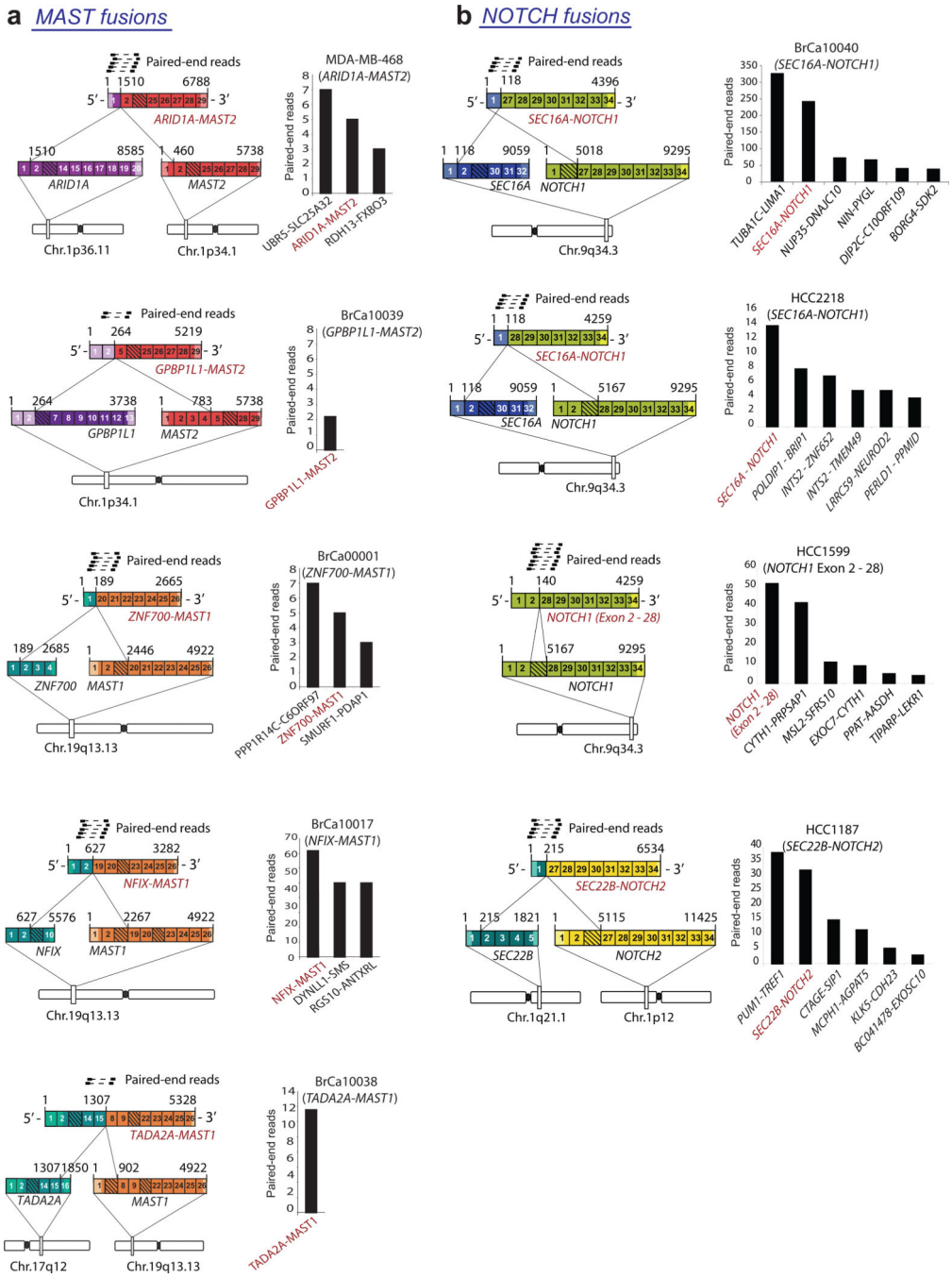

Figure 1. Discovery of the MAST kinase and Notch gene fusions in breast cancer identified by paired-end transcriptome sequencing

(**a**) MAST family gene fusions. (**b**) Notch family gene fusions. Fusion junctions with respective exon numbers (and nucleotide positions) comprising the chimeric transcripts are presented. Bar plots of top ranked gene fusions by number of paired-end reads supporting each nominated fusion in the index samples are shown on the right, with MAST or Notch fusion genes in red.

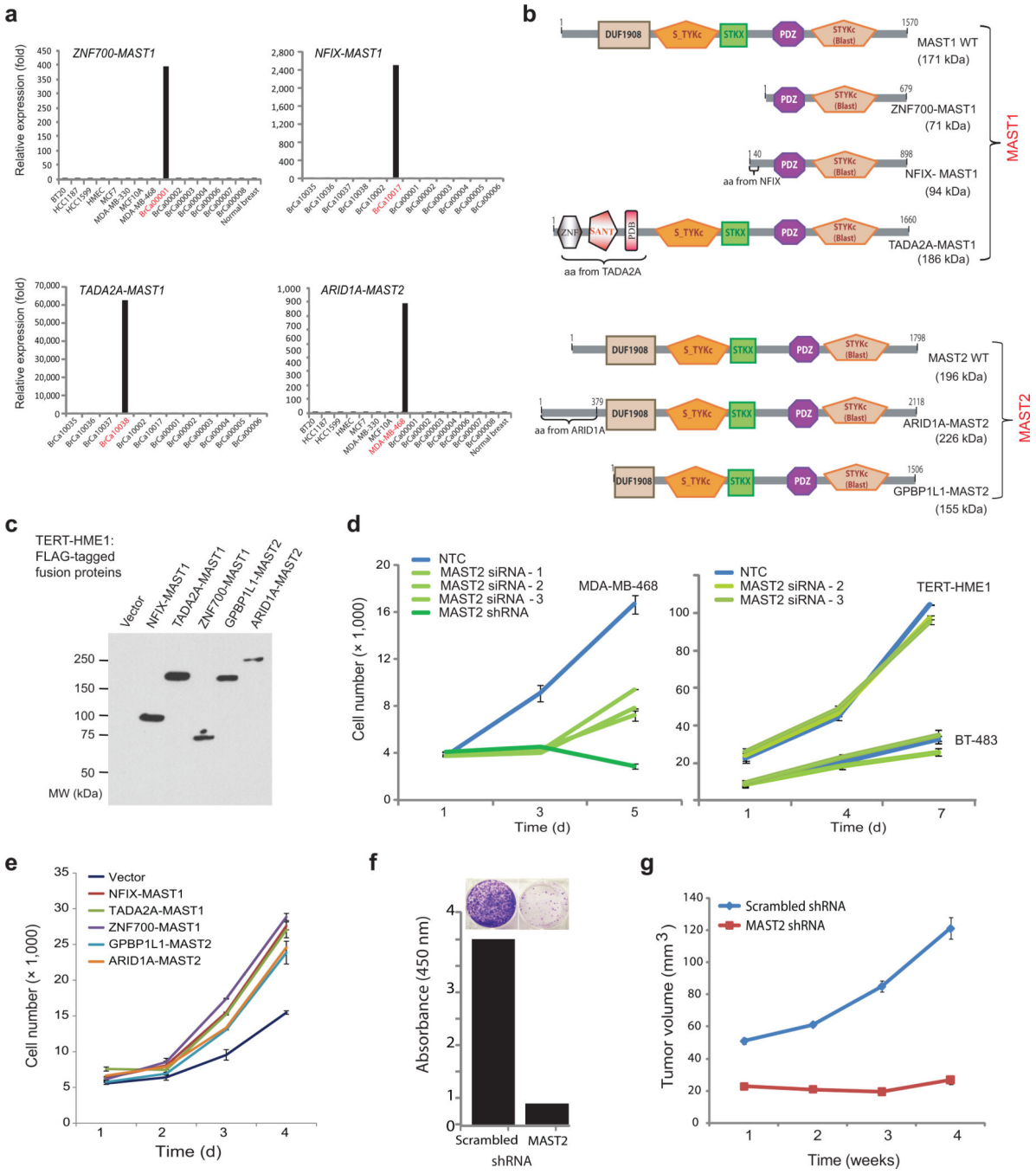

Figure 2. Characterization of MAST fusion genes

(**a**) Expression of *ZNF700-MAST1* gene fusion in breast cancer tissue BrCa00001, *NFIX-MAST1* in BrCa10017, *TADA2AMAST1* fusion in BrCa10038, and *ARID1A-MAST2* fusion in MDA-MB-468 was validated by real-time RT-PCR. (**b**) Schematic representation of functional domains retained in the putative chimeric proteins involving MAST1 and MAST2. (**c**) Stable expression of MAST fusion proteins in TERT-HME1 cells. The expression of the chimeric proteins was detected by anti-FLAG antibody. (**d**) *MAST2* knockdown reduces proliferation of MDA-MB-468 cells (left) but not fusion-negative

TERT-HME1 and BT-483 cells (right). (**e**) Overexpression of MAST chimera induces cell proliferation in TERT-HME1 cells. (**f**) Colony formation assay with MDA-MB-468 cells treated with *MAST2*-specific shRNA or control scrambled-shRNA. The inset shows crystal violet staining of cells treated with either scrambled or *MAST2* shRNA. (**g**) Reduced tumor growth by *MAST2* knockdown in a mouse xenograft model.

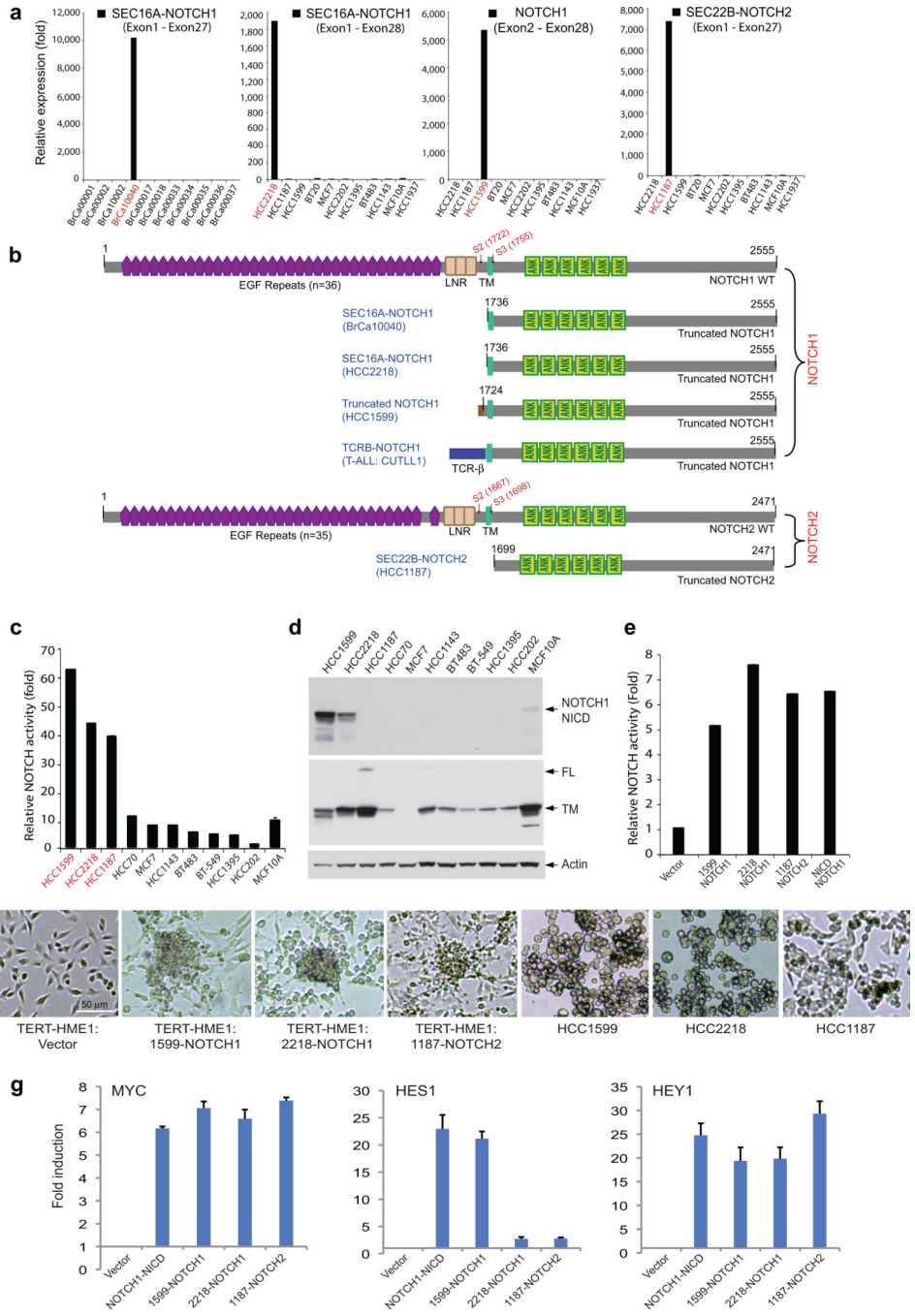

Figure 3. Identification and characterization of Notch gene aberrations in breast carcinomas

(**a**) Detection of novel Notch transcripts by quantitative RT-PCR. (**b**) Schematic presentation of the predicted protein structures of the aberrant Notch genes. (**c**) Notch reporter activities are elevated in Notch fusion index lines. Fold activation of Notch pathway was calculated using HCC202 as the reference. All the data was normalized to Renilla luciferase activity. (**d**) Western blot analysis of NOTCH1-NICD expression, detected with an antibody specifically recognizing the active NOTCH1-NICD protein after γ-secretase cleavage. FL, full length NOTCH1; TM, transmembrane NOTCH1. (**e**) Activation of Notch

signaling pathway in 293T cells by transient Notch expression. (**f**) Notch fusion alleles induce morphological change when expressed in benign TERT-HME1 cells. Bright-field images of control vector, Notch fusion allele expressing TERT-HME1 cells. Notch fusion positive breast cancer lines are shown for comparison. (**g**) Activation of Notch signaling pathway in TERT-HME1 cells stably expressing Notch fusions. The expression levels of three Notch target genes were measured by quantitative RT-PCR.

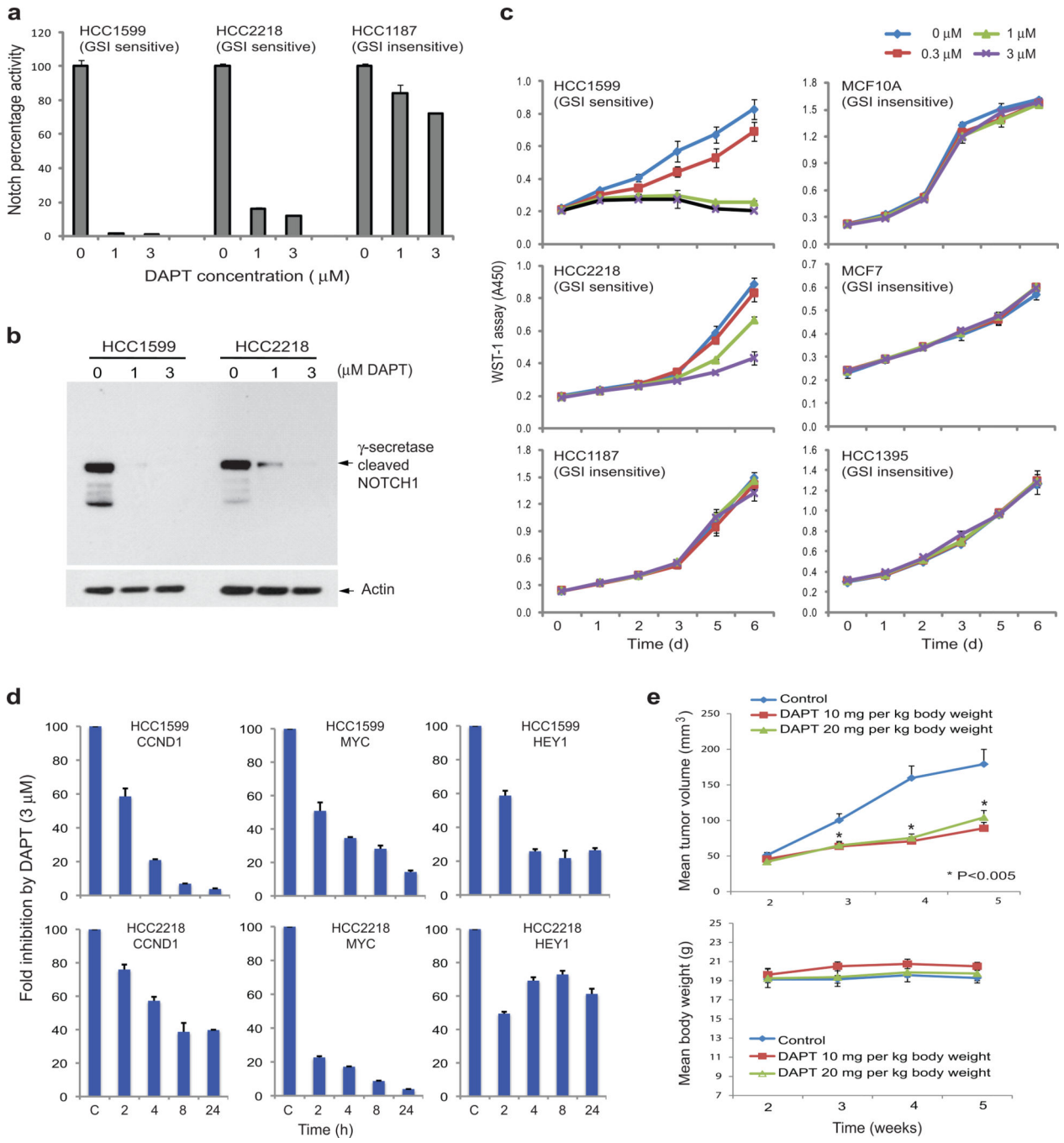

Figure 4. γ-secretase inhibitor DAPT blocks Notch-dependent cell proliferation

(**a**) Inhibition of the Notch signaling pathway by DAPT. Breast cancer cells were co-infected with a Notch-reporter construct Lenti-RBPJ-firefly luciferase and the internal control Lenti-Renilla luciferase and treated with DAPT. Twenty-four hours after DAPT treatment, luciferase activities were measured. (**b**) Reduction of NICD production after DAPT treatment, detected with an antibody specific to the active NOTCH1-NICD after γ-secretase cleavage. (**c**) Inhibition of cell proliferation by DAPT. HCC1599 and HCC2218 express a form of NOTCH1 which requires γ-secretase-dependent cleavage for activity.

HCC1187 cells express a NOTCH2-fusion mutant that is independent of γ–secretase processing. MCF10A, MCF7, and HCC1395 express wild type NOTCH1 and NOTCH2. GSI: γ-secretase inhibitor. (**d**) Diminished expression of Notch target genes following DAPT treatment, measured by quantitative RT-PCR. (**e**) Inhibition of tumor growth by DAPT in a mouse xenograft model. Mice xenografted with HCC1599 cells were treated with DAPT after tumors were formed, and the tumor size was monitored.