HEALTH AND MEDICINE

The DNA cytosine deaminase APOBEC3B promotes tamoxifen resistance in ER-positive breast cancer

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Breast tumors often display extreme genetic heterogeneity characterized by hundreds of gross chromosomal aberrations and tens of thousands of somatic mutations. Tumor evolution is thought to be ongoing and driven by multiple mutagenic processes. A major outstanding question is whether primary tumors have preexisting mutations for therapy resistance or whether additional DNA damage and mutagenesis are necessary. Drug resistance is a key measure of tumor evolvability. If a resistance mutation preexists at the time of primary tumor presentation, then the intended therapy is likely to fail. However, if resistance does not preexist, then ongoing mutational processes still have the potential to undermine therapeutic efficacy. The antiviral enzyme APOBEC3B (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3B) preferentially deaminates DNA C-to-U, which results in signature C-to-T and C-to-G mutations commonly observed in breast tumors. We use clinical data and xenograft experiments to ask whether APOBEC3B contributes to ongoing breast tumor evolution and resistance to the selective estrogen receptor modulator, tamoxifen. First, APOBEC3B levels in primary estrogen receptor–positive (ER^+) breast tumors inversely correlate with the clinical benefit of tamoxifen in the treatment of metastatic ER^+ disease. Second, APOBEC3B depletion in an ER^+ breast cancer cell line results in prolonged tamoxifen responses in murine xenograft experiments. Third, APOBEC3B overexpression accelerates the development of tamoxifen resistance in murine xenograft experiments by a mechanism that requires the enzyme's catalytic activity. These studies combine to indicate that APOBEC3B promotes drug resistance in breast cancer and that inhibiting APOBEC3B-dependent tumor evolvability may be an effective strategy to improve efficacies of targeted cancer therapies.

INTRODUCTION

Improvements in the detection and therapy of operable breast tumors have contributed to a steady decline in mortality (*1*, *2*). Essentially all breast cancer deaths are caused by metastatic outgrowths that compromise vital organs, such as the brain, liver, or lungs. Adjuvant systemic therapies effectively reduce the risk of recurrence at these distant metastatic sites by treating preexisting, clinically undetectable, micrometastatic deposits. In estrogen receptor–positive (ER^+) breast cancer, a propensity for late recurrence more than 5 years after surgery is well documented and has resulted in recommendations to extend adjuvant endocrine therapy for a total of 10 years (*3*, *4*). Although endocrine therapy may be extended, it is evident that late recurrences occur even while the patient is taking appropriate therapy (*5*). The late recurrence of these apparently dormant metastatic breast cancer cells may be due to ongoing tumor evolution and acquisition of additional genetic aberrations.

Mutations are thought to be the major drivers of recurrence, metastasis, and therapeutic resistance. Recent studies on the molecular origins of mutations in breast cancer have implicated several molecular mechanisms, including both spontaneous and enzyme-catalyzed deamination of DNA cytosine bases (*6*–*10*) [reviewed by Swanton *et al.* (*11*), Roberts and Gordenin (*12*), and Helleday *et al.* (*13*)]. The former process correlates with aging and is mostly due to hydrolytic conversion of 5-methyl cytosine (mC) bases within 5′ NmCG motifs into thymines, which escape base excision repair and are converted into C-to-T transition mutations by DNA replication (N = A, C, G, or T). The latter process is attributable to single-stranded DNA cytosine-to-uracil (C-to-U) deamination catalyzed by one or more members of the APOBEC3 (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3) family of enzymes, characterized by C-to-T transitions and C-to-G transversions in 5′TCW motifs (W = A or T).

Human cells have the capacity to express up to seven distinct APOBEC3 enzymes, which function normally as overlapping innate immune defenses against a wide variety of DNA-based viruses and transposons [reviewed by Malim and Bieniasz (*14*), Stavrou and Ross (*15*), and Simon *et al.* (*16*)]. APOBEC3A (A3A) and APOBEC3B (A3B) are leading candidates for explaining APOBEC signature mutations in breast tumors because overexpression of these enzymes triggers DNA damage responses and inflicts chromosomal mutations in hallmark trinucleotide contexts (*7*, *17*–*21*). However, endogenous *A3A* is not expressed significantly, nor is its activity detectable in breast cancer cell lines (*7*, *22*) (see Results). The molecular relevance of A3A is therefore difficult to assess because the impact of the endogenous protein cannot be quantified. In comparison, endogenous A3B is predominantly nuclear and has been shown to be responsible for elevated levels of genomic uracil and mutation in multiple breast cancer cell lines (*7*, *22*). A3B is overexpressed in approximately 50%

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of primary breast tumors (*7*, *8*), and retrospective studies have associated elevated *A3B* mRNA levels with poor outcomes for adjuvant treatment–naïve ER$^+$ breast cancer cohorts (*23*, *24*). Our original studies relied on a retrospective prognostic analysis of a treatment-naïve ER$^+$ breast cancer cohort (*23*); therefore, the observed correlation between elevated *A3B* mRNA levels and poor clinical outcomes is consistent with a variety of therapy-independent intrinsic molecular mechanisms ranging from indirect models (such as A3B promoting tumor cell growth) to direct models (such as A3B causing the genomic DNA damage that results in mutations that fuel ongoing tumor evolution).

A current debate in the cancer field is whether the mutations that cause therapy resistance preexist in primary tumors (that is, exist even before diagnosis) or continually accumulate (even after treatment initiation). In support of the former view, primary tumors are often composed of billions of cells that are highly heterogeneous, and deep-sequencing studies have found known drug resistance mutations before therapy initiation [for example, (*25*–*27*)]. However, many studies also support the latter view of ongoing tumor evolution. For instance, primary tumor deep-sequencing studies often fail to find evidence for preexisting resistance mutations [for example, (*26*, *28*)]. Recurrent breast tumors also often have many more somatic mutations compared to corresponding primary tumors, suggesting ongoing and cumulative mutational processes (*29*, *30*). In addition, the subclonal nature of most mutations in breast cancer, as well as many other cancer types, provides strong evidence for ongoing tumor evolution, including significant proportions of APOBEC signature mutations (*28*, *31*, *32*). Moreover, at the clinical level, the fact that remission periods in breast cancer can last for many years strongly suggests that additional genetic changes are required for at least one remaining tumor cell to manifest as recurrent disease (*3*, *4*). Here, we test the hypothesis that A3B contributes to ongoing tumor evolution and to the development of drug resistance mutations in ER$^+$ breast cancer.

RESULTS

Primary breast tumor *A3B* mRNA levels predict therapeutic failure upon tumor recurrence

To determine whether A3B contributes to endocrine therapy resistance, we evaluated the predictive potential of *A3B* expression in primary breast tumors from a total of 285 hormone therapy–naïve breast cancer patients who received tamoxifen as a first-line therapy for recurrent disease (*33*). A schematic of the study timeline is shown in Fig. 1A, and detailed patient characteristics are shown in table S1. Archived fresh-frozen primary tumor specimens were used to prepare total RNA, and reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used to quantify *A3B* mRNA levels. These gene expression results were divided into four quartiles for subsequent clinical data analysis, with primary tumors of the upper quartile expressing an average of fourfold to sixfold more *A3B* mRNA than those in the lower quartile (dark blue versus red histogram bars, respectively, in Fig. 1B).

The progression-free survival (PFS) durations following recurrence and subsequent first-line tamoxifen therapy were compared for each of the four *A3B* expression groups. This analysis revealed a dose-response relationship, with the highest *A3B*-expressing group associating with the shortest PFS and with the lowest *A3B*-expressing group associating with the longest PFS (Fig. 1C; log-rank, $P < 0.0001$). The median PFS was 6.2 months for the highest *A3B*-expressing group and 14.5 months for the lowest *A3B*-expressing group [hazard ratio (HR) 2.40 (1.69 to 3.41); log-rank, $P < 0.0001$]. This result remained significant for high versus low A3B levels in

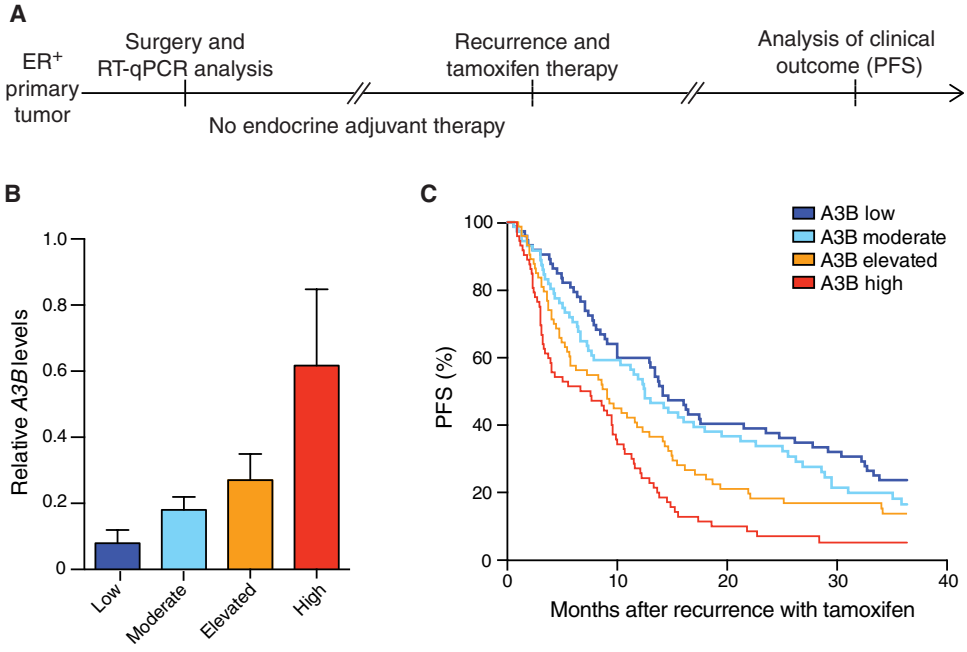

Fig. 1. High *A3B* levels in primary ER$^+$ breast tumors predict poor response to tamoxifen therapy after tumor recurrence. (**A**) Schematic of the clinical time course. Timeline breaks depict variable intervals between clinical milestones. (**B**) Relative *A3B* expression levels in each observation group [mean ± SD of $n = 72$ (quartiles 1 and 3), $n = 70$ (quartile 2), and $n = 71$ (quartile 4)]. (**C**) Kaplan-Meier curves showing the periods of PFS after initiating tamoxifen therapy for patients whose primary tumors expressed *A3B* at low (dark blue line), intermediate (light blue and orange lines), or high levels [red line; patient groups and color scheme match those in (B)].

a multivariate analysis after including the known clinical pathological predictors of age, disease-free interval, dominant site of relapse, adjuvant chemotherapy, and ER and progesterone receptor mRNA levels measured in the primary tumor [HR 2.19 (1.51 to 3.20); log-rank, $P < 0.0001$; table S2]. These data indicate that primary tumor *A3B* mRNA levels are strong and independent predictors of PFS for recurrent ER$^+$ breast cancer treated with tamoxifen. These observations do not support models in which resistance-conferring mutations preexist in primary tumors—or disease outcomes would have had no correlation with *A3B* expression levels and the data for each quartile group would have superimposed. Rather, the data support a model in which A3B promotes the ongoing diversification of residual primary tumor cells (micrometastatic deposits) that ultimately manifest in the recurrent setting as acquired resistance, failed tamoxifen therapy, and disease progression.

Endogenous *A3B* depletion does not alter the phenotype of MCF-7L ER$^+$ breast cancer cells in culture

MCF-7 has been used for decades as a unique cell-based model for ER$^+$ breast cancer research [reviewed by Lee *et al.* (*34*)]. Engrafted MCF-7 tumors are dependent on ER function and therefore are sensitive to selective ER modulators, including tamoxifen. Furthermore, tamoxifen-induced tumor dormancy (indolence) in this model system, which can last for several months, frequently leads to drug-resistant and highly proliferative cell masses. For further studies, including animal experiments below, we elected to use the derivative line MCF-7L because it is tumorigenic in immunodeficient mice [Ibrahim *et al.* (*35*), Sachdev *et al.* (*36*), and references therein] and expresses endogenous *A3B* mRNA at levels approximating those found in many primary breast tumors (*7*). Like most other breast cancer cell lines, MCF-7L cells have very low levels of *A3A* and variable levels of other *APOBEC3* mRNAs, which have not been implicated in breast cancer mutagenesis (fig. S1).

We initially asked whether endogenous *A3B* depletion alters molecular or cellular characteristics of MCF-7L. Cells were transduced with an *A3B*-specific short hairpin RNA (shRNA) construct (shA3B) or a nonspecific shRNA construct as a control (shCON) (*7*), and uniform shRNA-expressing pools were selected using the linked puromycin resistance gene. In all shA3B-transduced pools, a robust >25-fold depletion of endogenous *A3B* mRNA was achieved (Fig. 2A). Moreover, the depletion of *A3B* mRNA was mirrored by a corresponding ablation of all measurable DNA cytosine deaminase activities from whole-cell and nuclear extracts (Fig. 2B). Although several other *APOBEC* family member genes are expressed in MCF-7L, their protein levels are likely too low to detect using this assay (A3A, A3D, A3G, and A1), the enzyme is not active on DNA (A2), and/or their single-stranded DNA cytosine deaminase activity is not evident in cellular extracts (A3C and A3F) (*7*, *22*). At the microscopic level, shA3B- and shCON-expressing cells were visibly indistinguishable (Fig. 2C). The two cell populations showed nearly identical growth rates and doubling times in cell culture (Fig. 2, D and E). These results are consistent with *A3B* knockdown data using the same shRNA construct in other breast cancer cell lines (*7*, *22*) and with the observation that *A3B* is a nonessential human gene (*37*).

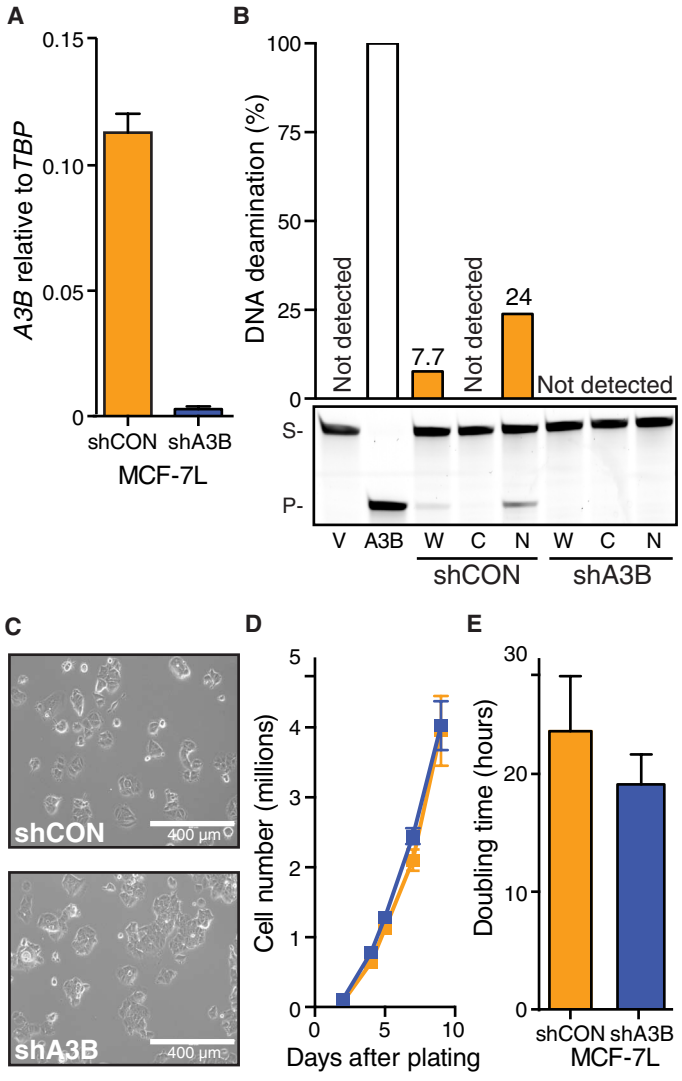

Fig. 2. Endogenous *A3B* depletion does not alter MCF-7L ER$^+$ breast cancer cells in culture. (**A**) *A3B* mRNA levels in MCF-7L cells expressing shA3B or shCON constructs (*TBP*, TATA-binding protein mRNA; each bar represents the mean ± SD of three RT-qPCR assays). (**B**) A3B DNA cytosine deaminase activity in soluble whole-cell (W), cytoplasmic (C), and nuclear (N) extracts of MCF-7L cells expressing shA3B or shCON constructs. Vector (V) and A3B-transfected 293T cell lysates were used as controls (S, substrate; P, product). (**C**) Light microscopy images of shA3B and shCON expressing MCF-7L pools. (**D** and **E**) Growth kinetics and doubling times of cultured MCF-7L cells expressing shA3B versus shCON constructs (mean ± SD of $n = 6$ cultures per condition).

A3B is required for the development of tamoxifen-resistant tumors in mice

The clinical data reported in Fig. 1 support a model in which A3B is responsible for precipitating the mutations that promote tamoxifen resistance. To directly test this model, we performed a series of xenograft experiments using MCF-7L pools in which endogenous *A3B* was left intact (shCON) or was depleted with the specific shRNA described above (shA3B). For each condition, 5 million cells were injected subcutaneously into the flank regions of a cohort of 5-week-old immunodeficient mice, and tumors were allowed to reach a volume of approximately 150 mm^3. At this point, typically 40 to 50 days after engraftment, the mice in each experimental group were randomly assigned into two subcohorts, one to receive daily tamoxifen injections and the other to be observed in parallel as a control (schematic of experimental design in Fig. 3A).

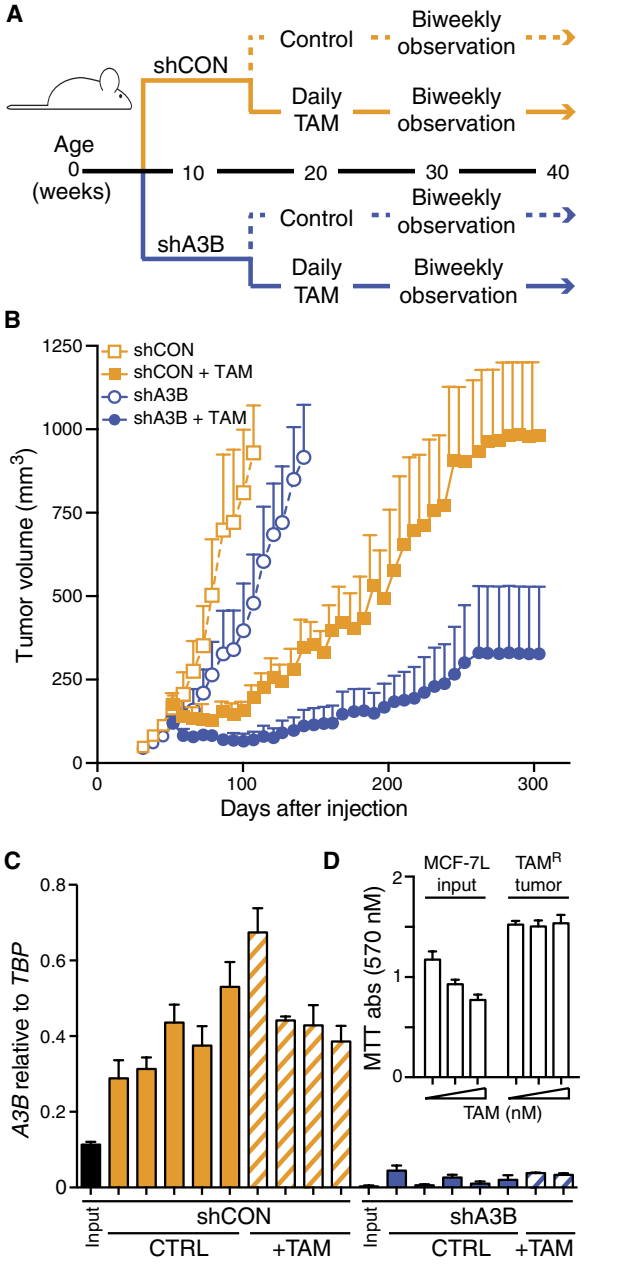

Fig. 3. A3B is required for the development of tamoxifen-resistant tumors in mice. (**A**) Schematic of the *A3B* knockdown xenograft study design and time course (see text for details). (**B**) Growth kinetics of engrafted MCF-7L cells expressing shA3B or shCON in the absence or presence of tamoxifen (TAM) treatment. Tumor volumes were measured weekly (mean + SEM shown for clarity of data presentation). (**C**) *A3B* mRNA levels in xenografted tumors recovered from the experiment shown in (B) (*TBP* mRNA; each bar represents the mean ± SD of three RT-qPCR assays). (**D**) MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] data comparing tamoxifen susceptibility of input MCF-7L cells versus tamoxifen resistance of a representative MCF-7L shCON tumor [tamoxifen (10, 100, and 1000 ng/ml)].

Control-transduced MCF-7L cells formed large 1000-mm^3 tumors within 100 days after engraftment and, interestingly, *A3B* knockdown caused a modest delay in tumor growth (open blue versus open orange symbols in Fig. 3B; linear mixed model, *F* test, $P = 0.002$). This result differed from the near-identical growth rates in cell culture (Fig. 2, D and E) and may be due to the likelihood that additional adaptations/mutations are required for monolayer/plastic-conditioned cells to be able to grow optimally as tumors in mice. As expected, tamoxifen treatment attenuated the growth of both engineered pools (filled orange and blue symbols in Fig. 3B). However, control-transduced cells rapidly developed resistance to tamoxifen and grew into large tumors, whereas the growth of the *A3B*-depleted cell masses was mostly suppressed by tamoxifen over the year-long duration of this representative experiment (filled orange versus blue symbols in Fig. 3B; linear mixed model, *F* test, $P < 0.0001$). Similar outcomes were observed in additional experiments (for example, fig. S2).

Xenograft tumor *A3B* mRNA levels were analyzed by RT-qPCR, and, in all instances, the intended knockdown or control mRNA level was found to be durable and maintained through the entire duration of the experiment (Fig. 3C). This series of control experiments also revealed that endogenous *A3B* mRNA levels increase in control shRNA–transduced tumor masses in comparison to the same cells before engraftment (Fig. 3C). The mechanism for *A3B* induction in immunodeficient mice is not known but is unlikely to be due to estrogen (figs. S3 and S4), as suggested by a recent report (*38*). Representative xenografts were recovered in culture, and the tamoxifen-resistant phenotype was reconfirmed (for example, Fig. 3D). These results are fully supportive of a mechanism in which endogenous A3B causes an inheritable drug resistance phenotype (addressed further below). It is notable that endogenous *A3B* mRNA levels in this system are comparable to those observed in a large proportion of primary tumors [approximately 0.1 to 0.2 relative to *TBP* mRNA levels in cultured MCF-7L cells (Fig. 2B), 0.4 relative to *TBP* in animal tumors described here (Fig. 3C and fig. S3), and a range of 0 to 1.25 and a median of 0.25 relative to *TBP* in primary breast tumors previously documented using the same RT-qPCR assay (*7*)].

A novel lentivirus-based system enables A3B overexpression in any cell type

We next developed a conditional A3B overexpression system to further test the A3B mutagenesis model. A conditional approach is required because A3B expression in virus-producing cells causes lethal mutagenesis of retroviral complementary DNA intermediates during reverse transcription (*39*–*42*), and excessive levels of cellular A3B have the potential to inflict genomic DNA damage that ultimately leads to cytotoxicity (*7*, *18*, *19*). We therefore developed a novel lentiviral construct that will only express A3B upon transduction into susceptible target cells (Fig. 4A). This construct mitigates viral toxicity issues because it is inactive in virus-producing cells as a result of disruption of the antisense *A3B* open reading frame with a sense strand intron, and it is only expressed after intron removal by splicing in the virus-producing cells and reverse transcription and integration of the full proviral DNA in susceptible target cells. It also mitigates toxicity issues for target cell populations because expression levels are not excessive (see below). In parallel, an A3B catalytic mutant derivative (E255Q) was created by site-directed mutagenesis to serve as a negative control.

Transducing viruses were made by plasmid transfection into 293T cells with appropriate retroviral helper plasmids encoding Gag, Pol, and Env (vesicular stomatitis virus glycoprotein). As anticipated, no producer cell toxicity was observed, and A3B and A3B-E255Q viral titers were equivalent by RT-qPCR. MCF-7L cells were transduced

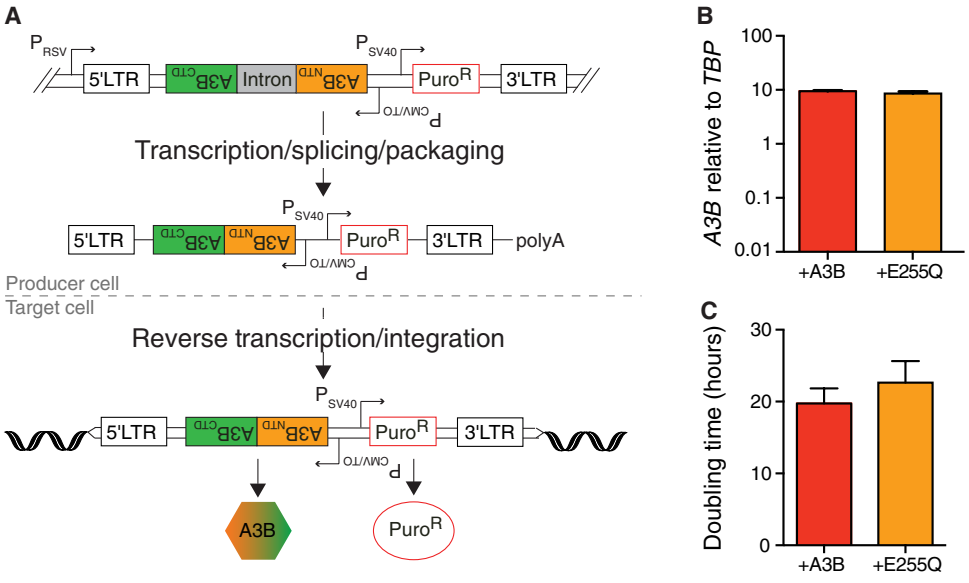

Fig. 4. Novel lentivirus-based system for conditional A3B overexpression. (**A**) Schematic of the lentiviral construct for conditional *A3B* overexpression (see text for details). LTR, long terminal repeat; RSV, Rous sarcoma virus; CTD, C-terminal domain; NTD, N-terminal domain; CMV, cytomegalovirus; SV40, simian virus 40. (**B**) *A3B* mRNA levels relative to *TBP* in MCF-7L cells expressing lentivirus-delivered *A3B* or a catalytic mutant derivative (E255Q) as well as endogenous *A3B* (mean ± SD of three RT-qPCR assays). (**C**) Doubling times of cultured MCF-7L cells overexpressing A3B or A3B-E255Q (mean ± SD of four replicates).

with each virus stock, and puromycin selection was used to eliminate nontransduced cells and to ensure 100% transduction efficiencies. *A3B* quantification by RT-qPCR showed that each construct elevates mRNA expression to levels approximately 10-fold higher than those of the reference gene *TBP* (Fig. 4B), which equate to levels approximately 50-fold higher than those of the endogenous *A3B* expressed in this system. These *A3B* mRNA levels are similar to those found in the top fraction of breast tumors and cancer cell lines [Burns *et al.* (*7*), Leonard *et al.* (*22*), Sieuwerts *et al.* (*23*), and this study]. As for the *A3B* knockdown experiments above, A3B- and A3B-E255Q–overexpressing MCF-7L populations showed no overt signs of toxicity and indistinguishable growth rates (Fig. 4C).

Overexpression of catalytically active A3B accelerates the development of tamoxifen-resistant tumors

To further test the model in which A3B provides mutagenic fuel for tumor evolution and drug resistance, we performed a series of xenograft experiments using MCF-7L cells transduced with the aforementioned constructs and thereby overexpressing wild-type A3B or the catalytic mutant derivative A3B-E255Q (Fig. 5A). Immunodeficient animals were injected subcutaneously with 5 million cells and, upon palpable tumor growth (150 mm^3), randomly divided into groups for tamoxifen injections or control observation. Remarkably, most of the cell masses overexpressing *A3B* developed rapid resistance to tamoxifen (filled red symbols in Fig. 5B). In comparison, MCF-7L cells expressing equivalent levels of *A3B-E255Q* mutant mRNA showed resistance kinetics similar to those of the shCON engraftments described above (filled orange symbols in Fig. 5B; linear mixed model, F test, $P = 0.015$). An independent experiment yielded similar results (fig. S5). These data demonstrate that A3B overexpression accelerates the kinetics of the development of tamoxifen resistance and, notably, that this phenotype requires catalytic activity.

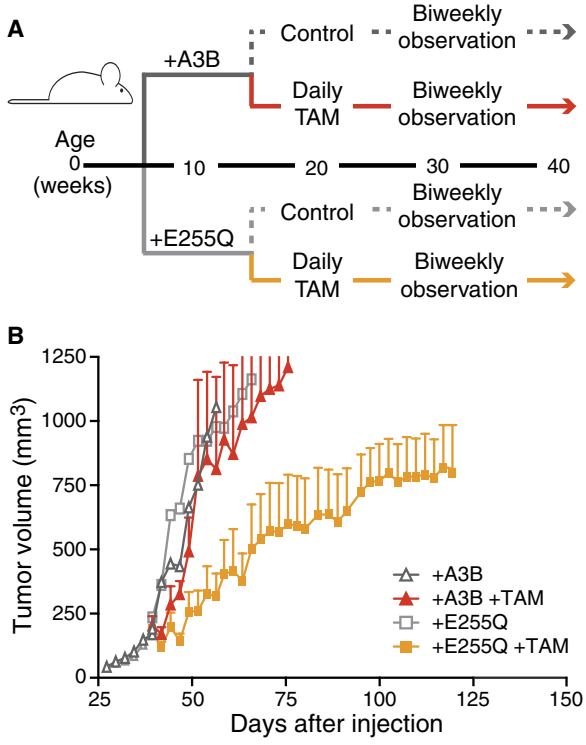

Fig. 5. Overexpression of catalytically active A3B accelerates the development of tamoxifen-resistant tumors in mice. (**A**) Schematic of the *A3B* overexpression xenograft study design and time course (see text for details). (**B**) Growth kinetics of engrafted MCF-7L cells overexpressing A3B or A3B-E255Q in the absence or presence of tamoxifen treatment. The graph reports tumor volumes measured weekly (mean + SEM shown for clarity of data presentation). Average tumor volumes from the untreated control arms are shown by gray symbols, and overlapping error bars are omitted for clarity of presentation.

ESR1 mutations are not responsible for tamoxifen resistance in the MCF-7L model for ER$^+$ breast cancer

Although the development of tamoxifen-resistant breast tumors is a major clinical problem, in most cases the molecular basis for resistance is unknown. A small fraction of treated patients develop tumors with *ESR1* exonic mutations that cause amino acid changes in the hormone-binding domain of the ER. These mutations have been seen mostly in tumors resistant to aromatase inhibitors and not as frequently in tumors resistant to tamoxifen [reviewed by Clarke *et al.* (*43*) and Jeselsohn *et al.* (*44*)]. To determine whether *ESR1* mutations are also part of the tamoxifen resistance mechanism in MCF-7L cells, we performed DNA exome sequencing on 9 independent tamoxifen-resistant xenografts and 10 independent control tumor masses. The *ESR1* gene contained no mutations under either condition (see table S3 for a full list of base substitution mutations). Resistant tumor *ESR1* mRNA levels were somewhat variable but still similar to those present in the original MCF-7L cell populations (fig. S6). Together with the data presented above indicating heritable resistance to tamoxifen (Fig. 3D), these results suggest that at least one other resistance mechanism occurs in the MCF-7 model system for ER$^+$ breast cancer.

DISCUSSION

The clinical and xenograft results presented here strongly support a model in which A3B drives tamoxifen resistance in ER$^+$ breast cancer. Clinically, resistance to endocrine therapies has been defined as primary or secondary, depending on the length of time a patient benefits from ER-targeted therapy. Our data suggest that A3B may have a role in both kinds of resistance and particularly in the development of secondary, acquired resistance. Suppression of endogenous levels of A3B enhances tamoxifen benefit (Fig. 3), whereas overexpression of A3B eliminates almost all benefits from tamoxifen therapy (Fig. 5). Because the only known biochemical activity of A3B is single-stranded DNA cytosine deamination [for example, (*7*, *42*, *45*)] and the tamoxifen resistance phenotype is heritable (Fig. 3D), the most likely mechanism is A3B-catalyzed DNA C-to-U editing coupled to the processing of these uracil lesions into somatic mutations by normal DNA repair processes [reviewed by Swanton *et al.* (*11*), Roberts and Gordenin (*12*), and Helleday *et al.* (*13*)]. In further support of this mechanism, the catalytic glutamate of A3B (E255) is required for accelerated tamoxifen resistance kinetics upon enzyme overexpression.

Because *ESR1* mutations were not observed in MCF-7L tamoxifen-resistant tumors, the identity of the resistance-conferring mutations in this system will require significant future studies and possibly even whole-genome sequencing if the predominant causal lesions lie outside the exomic fraction of the genome. The intrinsic signature of A3B may help to identify candidate (frequently mutated) sites for mechanistic follow-up. Then, for instance, genetic knock-in experiments could be used to unambiguously establish a cause-effect relationship. However, the resistance-conferring mutations (such as gene translocations, amplifications, or deletions) could also be complex and difficult to recapitulate precisely because DNA repair enzymes can readily process genomic uracil lesions into single- and double-stranded breaks (*46*, *47*).

A3B has been implicated as a dominant source of mutation in breast, head/neck, lung, bladder, and cervical cancers and—to a lesser but still significant extent—in many other tumor types (*7*–*10*, *28*, *32*, *48*, *49*). The fundamental nature of the DNA deamination mechanism, together with the data presented here, strongly suggests that A3B may be a general mechanism of therapeutic resistance to cancer therapy. At this point, potential mutagenic contributions from other APOBEC3 family members, such as A3A, cannot be excluded fully, but they do not appear to manifest in the MCF-7L system, nor are these potential contributions large enough to prevent the significant association between *A3B* expression levels and clinical outcomes for ER$^+$ breast cancer patients [treatment-naïve data in the studies by Sieuwerts *et al.* (*23*) and Cescon *et al.* (*24*) and post-recurrence tamoxifen resistance data in Fig. 1]. Thus, strategies to down-regulate A3B activity or expression, as reported here using a specific shRNA knockdown construct in a model system for ER$^+$ breast cancer, may be beneficial as chemotherapeutic adjuvants to "turn down" the mutation rate, decrease the likelihood of evolving drug resistance, and prolong the clinical benefit of therapy for the many cancers that are likely to be driven by this ongoing mutational process.

MATERIALS AND METHODS

Clinical studies

The clinical characteristics of the 285 patients [225 from Rotterdam (Erasmus University Medical Center) and 60 from Nijmegen (Radboud University Medical Center)] whose primary tumor specimens and data were used here have been described previously by Sieuwerts *et al.* (*33*). The protocol to study biological markers associated with disease outcome was approved by the medical ethics committee of the Erasmus University Medical Center (Rotterdam, Netherlands) (MEC 02.953); for Nijmegen, coded primary tumor tissues were used in accordance with the Codes of Conduct of the Federation of Medical Scientific Societies in the Netherlands (www.federa.org/codes-conduct). Thirty-two patients presented with distant metastasis at diagnosis or developed distant metastasis (including supraclavicular lymph node metastasis) within 1 month following primary surgery (M1 patients). These 32 patients and the 253 patients who developed a first recurrence during follow-up (25 patients with local-regional relapse and 228 patients with distant metastasis) were treated with first-line tamoxifen. All patients were ER$^+$ and anti–hormonal therapy–naïve, but 38 patients received adjuvant chemotherapy. The median time between the primary surgery and the start of therapy was 24 months (range, 0 to 120 months). The median follow-up of patients alive at the end of follow-up was 98 months (range, 9 to 240 months) after the primary surgery and 45 months (range, 3 to 178 months) after the start of first-line tamoxifen therapy. For 182 patients (64%), disease progression occurred within 6 months of the start of the first-line therapy being controlled by tamoxifen. At the end of the follow-up period, 268 (94%) patients had developed tumor progression, and 222 (78%) patients had died.

Total RNA was extracted with RNA Bee (Tel Test, Thermo Fisher Scientific Inc.) from 30-μm fresh-frozen primary tumor tissue sections containing at least 30% invasive tumor cell nuclei, and mRNA transcripts were quantified by RT-qPCR as described previously by Sieuwerts *et al.* (*23*). The median *A3B* expression level in the group of 285 breast cancers was 0.22 relative to the normalized average of three reference genes [*HPRT1*, *HMBS*, and *TBP* (*23*)].

DNA constructs

A3B knockdown and control shRNA constructs were described and validated previously by Burns *et al.* (*7*) and Leonard *et al.* (*50*). The A3B and A3B-E255Q lentiviral expression constructs were based

on the pLenti4TO backbone (Life Technologies). Overlapping PCR was used to place a sense-encoded intron between an antisense-encoded A3B open reading frame (primers available on request). A cytomegalovirus promoter drove A3B expression, and a simian virus 40 early promoter drove puromycin resistance. Constructs were verified by DNA sequencing.

Cell culture studies

MCF-7L cells were cultured at 37°C under 5% CO_2 and maintained in improved minimum essential medium (Richter's modification medium) containing 5% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 11.25 nM recombinant human insulin. These cells were originally obtained from C. Kent Osborne (Baylor College of Medicine, Houston, TX) and are subject to short tandem repeat analysis yearly to confirm their identity with the original MCF-7 cell line. Cells were transduced with the lentivirus-based shRNA or conditional expression constructs described above and selected with puromycin (1 μg/ml; United States Biological) for 72 hours to generate uniformly transduced pools. Cell growth experiments were performed by plating 100,000 cells per six-well plate and incubating them at 37°C for the indicated days. Cells were trypsinized, diluted 1:2 in trypan blue (Invitrogen), and counted via a hemocytometer (six biological replicates per day per condition). Cell proliferation rates were determined using the xCELLigence real-time cell analyzer dual-plate instrument according to the manufacturer's instructions (ACEA Biosciences).

The mRNA level of each *APOBEC* family member gene was quantified using previously described RT-qPCR protocols and primer/probe combinations and presented relative to the housekeeping gene *TBP* (*7*, *51*, *52*). *ESR1* and *C-MYC* RNA were quantified by RT-qPCR using intron-spanning primers 5′-ATGACCATGACCCTCCACACC and 5′-TCAGACCGTGGCAGGGAAACC (UPL24) and 5′-GCTGCTTAGACGCTGGATTT and 5′-TAACGTTGAGGGGCATCG (UPL66), respectively, and manufacturer-recommended protocols (LightCycler 480, Roche). *C-MYC* is an established estrogen-responsive gene (*53*).

DNA deaminase activity was measured in soluble whole-cell, nuclear, and cytoplasmic fractions of MCF-7L cultures using established protocols (*7*, *54*). The single-stranded DNA substrate contained a single target cytosine (5′-ATTATTATTATTCGAATGGATTTATTTATTTATTTATTTATTT-fluorescein); deamination, uracil excision, and backbone cleavage resulted in a single faster-migrating product on SDS–polyacrylamide gel electrophoresis and image analysis (Typhoon FLA 7000 and ImageQuant software, GE Healthcare Life Sciences).

Xenograft studies

The University of Minnesota Institutional Animal Care and Use Committee approved the animal protocols used here (1305-30638A). MCF-7L cells were harvested at 70% confluence, counted, and resuspended in serum-free medium (without phenol red) at a concentration of 5 million cells per 50 μl of final volume. Ovariectomized, athymic mice (Harlan) were injected subcutaneously in the left flank with 50 μl of cell suspension at approximately 5 weeks of age. Each experiment was initiated with 5 or 10 mice per experimental condition. One week before injection and at all times following, the mice were provided with drinking water supplemented with 1 μM β-estradiol (Sigma-Aldrich) (except for the subset of mice used in the experiment shown in fig. S3). Tumors were measured bidirectionally twice weekly, and tamoxifen treatment began when the average tumor volume reached 150 mm^3. Tamoxifen citrate (500 μg; Sigma-Aldrich) emulsified in 50 μl of peanut oil was administered subcutaneously 5 of 7 days each week. Tumor volumes were calculated using the following formula: length × $breadth^2/2$.

MCF-7L exome sequencing

Genomic DNA was prepared from tumor cell masses (~20 mg per sample) via the Gentra Puregene Tissue DNA isolation protocols (Qiagen). Samples were diluted to 100 ng/μl and assessed further for quality and purity by SYBR Green PCR on a 197-bp fragment of *A3H* using primers 5′-CATGGGACTGGACGAAGCGCA and 5′-TGGGATCCACACAGAAGCCGCA. Samples with no amplification were excluded from the analysis. One microgram of total genomic DNA per sample was subjected to whole-exome sequencing on the Complete Genomics platform to an average target depth of 100× (BGI). Reads were aligned by BGI using its in-house pipeline, and the alignments in bam format were used for variant calling. Somatic variants were called for each tumor alignment by VarScan 2 (*55*) using an estrogen-treated shA3B sample as the normal control. The variants were filtered with a minimum overall coverage depth of 20 reads and a minimum coverage depth of 4 reads for the alternate allele. Any variant occurring at any frequency above 0 at the same position in more than one sample was considered a common mutation in the input pool and was removed. A full list of base substitution mutations is provided in table S3.

Statistics

Comparisons of the PFS of hormone-naïve breast cancer patients following treatment for first recurrence with tamoxifen, by *A3B* expression level (divided into quartiles), were conducted using log-rank tests; HRs and 95% confidence intervals are presented for pairwise comparisons. Clinical data were analyzed using SPSS Statistics version 23.0 (IBM). In the xenograft studies, repeated measures of tumor volume over time were compared by treatment group using linear mixed models with fixed effects for treatment, days, and interaction between treatment and days and with random intercept and slope effects for each mouse. P values <0.05 were considered statistically significant. Xenograft data were analyzed using Prism 6 and SAS 9.3.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/10/e1601737/DC1

fig. S1. APOBEC family member expression in MCF-7L cells.
fig. S2. Replica *A3B* knockdown xenograft experiment.
fig. S3. Estrogen does not affect *A3B* mRNA levels in engrafted MCF-7L cells.
fig. S4. *A3B* is not estrogen-inducible.
fig. S5. Replica *A3B* overexpression xenograft experiment.
fig. S6. *ESR1* mRNA levels in tamoxifen-resistant MCF-7L cells.
table S1. Patient characteristics and median and interquartile range of *APOBEC3B* mRNA levels.
table S2. Cox univariate and multivariate analyses for PFS after initiating first-line tamoxifen.
table S3. Single-base substitution mutations in tamoxifen-resistant tumors (separate Microsoft Excel file).

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