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The PKC-NFκB Signaling Pathway Induces APOBEC3B Expression in Multiple Human Cancers

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Abstract

Overexpression of the antiviral DNA cytosine deaminase APOBEC3B has been linked to somatic mutagenesis in many cancers. HPV infection accounts for APOBEC3B upregulation in cervical and head/neck cancers, but the mechanisms underlying non-viral malignancies are unclear. In this study, we investigated the signal transduction pathways responsible for APOBEC3B upregulation. Activation of protein kinase C (PKC) by the diacylglycerol (DAG) mimic phorbol-myristic acid (PMA) resulted in specific and dose-responsive increases in APOBEC3B expression and activity, which could then be strongly suppressed by PKC or NFκB inhibition. PKC activation caused the recruitment of RELB, but not RELA, to the *APOBEC3B* promoter implicating non-canonical NFκB signaling. Notably, PKC was required for APOBEC3B upregulation in cancer cell lines derived from multiple tumor types. By revealing how APOBEC3B is upregulated in many cancers, our findings suggest that PKC and NFκB inhibitors may be repositioned to suppress cancer mutagenesis, dampen tumor evolution, and decrease the probability of adverse outcomes such as drug resistance and metastases.

Keywords

APOBEC3B; cancer mutagenesis; DNA cytosine deamination; NFκB; PKC

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Introduction

Somatic mutations are essential for nearly every hallmark of cancer (1). Mutations occur when DNA damage escapes repair. Cancer genome deep sequencing studies are confirming previously known sources of mutation as well as helping to discover new ones (2–4). Established sources of mutation include ultraviolet light in skin cancer, tobacco carcinogens in lung cancer, and hydrolytic deamination of methyl-cytosine as a function of age in nearly all cancers. One newly discovered source is the plant derived dietary supplement aristolochic acid, which causes A-to-T transversion mutations in liver and bladder cancers (5). A second and larger source of mutation is the APOBEC family of DNA cytosine deaminases, which cause signature C-to-T transition and C-to-G transversion mutations in breast, head/neck, bladder, cervical, lung, and ovarian cancers (2–4,6–11). The majority of these mutational events are dispersed throughout the genome, but an interesting minority is found in strand-coordinated clusters termed kataegis (2,12).

Expression profiling and functional studies independently discovered APOBEC as a major source of mutation in cancer (6,8). Human cells have the potential to express up to seven distinct antiviral APOBEC3 enzymes (13). APOBEC3B is the only family member clearly upregulated in breast and ovarian cancer cell lines and primary tumors (6,8). APOBEC3B is predominantly nuclear, and knockdown experiments demonstrated that it accounts for all measurable DNA cytosine deaminase activity in cancer cell line extracts and, likewise, is also responsible for elevated levels of genomic uracil and higher mutation rates (6,8). In addition, APOBEC3B levels correlated with higher C-to-T and overall base substitution mutation loads. Importantly, the biochemical preference of recombinant APOBEC3B deduced *in vitro* closely resembles the actual cytosine mutation bias in breast cancer as well as in several of the other tumor types listed above (*i.e.*, strong bias toward 5′-TC dinucleotides) (6–10).

HPV infection was recently shown to induce APOBEC3B expression in cell culture experiments, which helps explain APOBEC3B upregulation and mutation biases in virus-positive cervical and head/neck tumors (14,15). However, the mechanism responsible for APOBEC3B upregulation in other tumor types (*i.e.*, non-HPV cancers) is presently unknown, but not due to obvious processes such as chromosomal translocation, gene amplification, or promoter demethylation (6). Here, we show that the PKC/NFκB pathway specifically induces APOBEC3B expression, providing the first mechanistic link between a major signal transduction pathway and cancer mutagenesis. Multiple experimental approaches combined to demonstrate direct transcriptional upregulation of APOBEC3B by a signal transduction pathway involving the classical PKC isoform PKCα and the non-canonical NFκB transcription factor RELB. PKC inhibition also leads to APOBEC3B downregulation in several cancer cell lines suggesting that existing compounds may be repurposed to control mutagenesis in cancer.

Materials and Methods

Cell lines

Cell line information is in Table S1.

Reverse transcription quantitative PCR

RNA was extracted from healthy cells using the High Pure RNA Isolation Kit (Roche), and triplicate cDNA reactions were made using Transcriptor RT (Roche). qPCR was performed using primer-probe combinations for each *APOBEC* (16). The primers for PKCα were 5′-TGGTTTTGGTTCCCATTTCT and 5′-CATCCGGGTTTCCTGATTC and the probe was Roche UPL 1. The primers for TNFα were 5′-CAGCCTCTTCTCCTTCCTGAT and 5′-GCCAGAGGGCTGATTAGAGA, respectively and the probe was Roche UPL 29.

Immunoblotting

The development of the rabbit mAb against APOBEC3B will be described elsewhere (Brown *et al*., *in process*). The mAb used here is 10-87-13, and it recognizes endogenous APOBEC3B in a variety of assays including immunoblotting as demonstrated in several experiments. This mAb crossreacts with APOBEC3A and APOBEC3G but these proteins can be easily distinguished by faster and slower SDS-PAGE migration differences, respectively (*e.g*., Fig. 4). The anti-tubulin (Covance, MMS-407R) and anti-PKCα (Cell Signaling, 2056P) antibodies were used according to company specifications.

Deaminase activity assays

Single-stranded DNA cytosine deaminase activity assays were performed as reported (14). 4 pmol 5′-ATTATTATTATTCAAATGGATTTATTTATTTATTTATTTATTT-fluorescein was treated with cell extract containing 0.025 U/rxn UDG (New England BioLabs), UDG buffer, and 1.75 U/rxn RNase A (Qiagen) for 2 hours. Abasic sites were cleaved by treatment with 100 mM NaOH at 95°C for 10 min. Substrate was separated from product using 15% TBE-urea gel electrophoresis. Gels were analyzed using a FujiFilm Image Reader FLA-7000.

PMA induction and PKC-NFκB Inhibitors

2.5×10^5 cells were plated in a 6-well plate 1 day prior to drug treatment. PMA was then added to the media and incubated at 37°C with 5% CO_2 for 6 hours unless otherwise indicated. For experiments utilizing inhibitors, cells were pretreated with inhibitors 30 minutes prior to PMA induction (25ng/mL). PMA (Fisher Scientific), cyclohexamide (Acros Organics), Gö6983 (Cayman Chemical), LY294002 (EMD Chemicals), UO126 (EMD Chemicals), BIM-1 (Cayman Chemical), Gö6976 (Enzo Life Sciences), AEB071 (Medchem Express), BAY 11-7082 (R&D Systems), MG132 (Fisher Scientific), and TPCA-1 (Cayman Chemical) were stored as recommended.

PKC knockdowns

pLKO.1-based lentiviruses were produced in 293T cells as reported (6). MCF10A cells were transduced with PKCα #1 (Open Biosystems, TRCN0000001691), PKCα #2 (Open Biosystems, TRCN0000001692), PKCα #3 (Open Biosystems, TRCN0000001690), or a control lentivirus. 96 hours later the transduced pools were treated with 25ng/mL PMA for 3 (RNA) or 6 (protein) hours and then harvested and analyzed as described above.

RNA sequencing

Parallel sets of MCF10A cells were treated every 8 hours with media supplemented with PMA or DMSO for 48 hours. RNA was extracted using an RNeasy Mini Kit (Qiagen). Total RNA was submitted to the University of Minnesota Genomics Center for sequencing on the Illumina HiSeq 2000 platform. Raw reads were analyzed using both DESeq2 (17) and the Tuxedo suite (18) to identify changes in mRNA expression in PMA treated versus untreated cells.

Chromatin immunoprecipitations (ChIP)

MCF10A cells were treated with either DMSO or 25 ng/mL PMA for 2 hours. Cross-linking was performed with 1% formaldehyde for 10 min at room temperature and quenched with 150 mM glycine. Cells were then lysed in Farnham Lysis Buffer at 4°C for 30 minutes. Nuclei were pelleted, resuspended in RIPA Buffer, and sonicated (Diagenode Pico Sonicator) to generate approximately 600 bp DNA fragments. Immunoprecipitations were done using Protein G Dynabeads (Invitrogen) and 2 μg antibody per sample. Samples were washed in 1 mL low salt wash buffer, 1 mL high salt wash buffer, 1 mL LiCl wash buffer, and eluted at 65°C for 30 minutes. Samples were reverse cross-linked using 200 mM NaCl and treated with Proteinase K for 12 hours at 65°C. DNA was purified using a ChIP DNA Clean and Concentrator Kit (Zymo Research) and qPCR was performed with SYBR Green master mix (Roche) on a Roche LightCycler 480. Values represent the percentage of input DNA immunoprecipitated (IP DNA) and are the average of three independent biological replicates. All ChIP reagents are listed in Table S2.

Results

Specific upregulation of APOBEC3B by PMA

The first cDNAs representing *APOBEC3A* and/or *APOBEC3B* were cloned from PMA-treated primary human keratinocytes (19). PMA is a DAG analog known to trigger PKC signaling as well as activate a number of other cellular processes (20–23). Due to high levels of homology between *APOBEC3A* and *APOBEC3B* (92%) including stretches of perfect identity, it is not clear which gene may have been represented by these original cDNAs. Moreover, the primary tissues used in this study consisted of multiple epithelial cell types and most likely also infiltrating immune cells making it unclear where the cDNAs may have originated. These distinctions are important given the fact that APOBEC3A (not APOBEC3B) is upregulated >100-fold by interferon-α treatment of myeloid cell types (24,25), and that APOBEC3B (not APOBEC3A) is upregulated by HPV infection of keratinocytes (14,15).

To resolve these issues and get a molecular handle on *APOBEC3B* transcriptional regulation, a panel of cell lines was treated with PMA or equal amounts of DMSO as a negative control, and the mRNA levels of all eleven human *APOBEC* family members were quantified by RT-qPCR (16). *APOBEC3B* mRNA was induced at least 2-fold in all lines by PMA treatment (except 293T), with the greatest magnitude occurring in the immortalized normal breast epithelial cell line MCF10A (Fig. S1). Under standard cell culture conditions MCF10A expresses low levels of *APOBEC3B* and *APOBEC3F*, even lower levels of

APOBEC3G and *APOBEC3H*, high levels of *APOBEC3C*, and undetectable levels of all other *APOBEC* family members. PMA treatment caused a specific 100-fold upregulation of *APOBEC3B* mRNA, with no detectable changes in the expression levels of any other *APOBEC* family members (Fig. 1A and S2).

APOBEC3B was induced with as little as 1 ng/mL PMA, and its induction was dose responsive and near maximal at 25 ng/mL PMA (Fig. 1B, histogram). *APOBEC3B* mRNA levels correlated with a rise in steady-state protein levels as measured by immunoblotting (Fig. 1B, immunoblot) and enzymatic activity as measured by a gel-based single-stranded DNA cytosine deamination assay (Fig. 1B, polyacrylamide gel). Moreover, significant *APOBEC3B* mRNA induction was detected 30 minutes after PMA treatment and maximal levels were observed by 3 hours post-treatment (Fig. 1C, histogram). APOBEC3B protein and activity levels lagged shortly behind mRNA levels and persisted through the duration of the 6-hour time course (Fig. 1C, immunoblot and polyacrylamide gel). An extended time course revealed that *APOBEC3B* mRNA levels begin to decrease by 12 hours and return to near basal levels by 24 hours post-PMA treatment (Fig. S3). Importantly, *APOBEC3B* upregulation is likely to be a direct result of signal transduction as the kinetics of mRNA upregulation were not affected by simultaneously treating cells with the protein translation inhibitor cyclohexamide (Fig. 1D). Cycloheximide treatment was effective as evidenced by disrupted APOBEC3B protein accumulation. Altogether, these data demonstrate that *APOBEC3B* is strongly and specifically upregulated by a PMA-induced signal transduction mechanism in multiple cell lines and most strongly in the immortalized normal breast epithelial cell line MCF10A. Notably, *APOBEC3B* upregulation can be as high as 100-fold and this mRNA level is on par with those observed in many different cancer cell lines and tumor types including a large fraction of breast and ovarian cancers [*i.e.*, mRNA levels 2- to 5-fold higher than those of the housekeeping gene *TBP* (6–8,14)].

PKC is required for APOBEC3B induction by PMA

PMA is a known agonist of PKC signaling, but is also capable of affecting other cellular processes (20–23). To determine whether *APOBEC3B* induction by PMA occurs through PKC signal transduction or an alternative mechanism, MCF10A cells were pre-treated for 30 minutes with varying concentrations of the pan-PKC inhibitor Gö6983 (26) and then incubated for 6 hours with an optimal amount of PMA (25 ng/mL). In comparison to strong *APOBEC3B* upregulation observed with PMA treatment alone, pretreatment with Gö6983 caused a dose responsive suppression of *APOBEC3B* induction (Fig. 2A). *APOBEC3B* was suppressed to background levels by as little as 5 μM Gö6983 (Fig. 2A). Moreover, no morphological defects or viability issues were observed at these concentrations of Gö6983 (Fig. S4). As additional controls, MCF10A cells were pretreated in parallel with the phosphoinositol 3 kinase (PI3K) inhibitor, LY294002, and the mitogen-activated protein kinase (MEK) inhibitor, UO126, prior to PMA induction (Fig. 2B–C). In both instances, *APOBEC3B* was still induced fully by PMA.

Human cells can express up to 9 different *PKC* genes (20,21,23). The resulting PKC proteins (conventionally called isoforms) are divisible into 3 classes based primarily on activation mechanism: classical PKC (cPKC) isoforms require both DAG and increased

levels of intracellular calcium, novel PKC (nPKC) isoforms require only DAG, and atypical PKC (aPKC) isoforms are activated by other signals. To test which class of PKC isoforms is responsible for *APOBEC3B* upregulation, we utilized two additional inhibitors known to have potency similar to Gö6983, but greater selectivity for certain PKC classes. First, we pretreated MCF10A cells with bisindolylmaleimide-1 (BIM-1), which is known to inhibit both the cPKC and nPKC classes (27), and then induced with optimal PMA concentrations. A nearly identical dose dependent suppression of *APOBEC3B* induction was observed (Fig. 2D). This result was expected as DAG mimics do not generally activate aPKCs. Second, we pretreated MCF10A cells with Gö6976, which is an inhibitor of cPKC proteins (28). The dose responsiveness of *APOBEC3B* repression was again similar to Gö6983 (Fig. 2E). Taken together, these chemical inhibition data strongly implicated a cPKC isoform in *APOBEC3B* induction by PMA.

One of the most potent and clinically advanced PKC inhibitors is AEB071, which selectively inhibits cPKC and nPKC isoforms (29,30). AEB071 has shown results in preclinical studies and phase I clinical trials for treatment of uveal melanoma (31–34). To fortify the pharmacologic approaches elaborated above, we asked whether pretreatment of MCF10A cells with AEB071 would produce a similar reductive effect on PMA induced APOBEC3B expression as the above PKC inhibitors. Indeed, a clear dose dependent response was observed and, importantly, AEB071 caused a complete suppression of *APOBEC3B* expression at 500 nM, which is approximately 10-fold more potent than Gö6983, BIM-1, or Gö6983, consistent with reported lower IC50 values for this molecule (26–29,35) (Fig. 2F).

RNAseq data revealed that *PKCα* (*PRKCA*) is the only cPKC isoform expressed in MCF10A cells (Fig. 2G). *PKCα* mRNA levels were unchanged by PMA treatment consistent with a mechanism by which PMA signals through pre-existing PKCα to ultimately stimulate *APOBEC3B* transcription (Fig. 2G). To further test the involvement of PKCα in this regulatory pathway, we depleted PKCα expression using 3 independent shRNA-encoding lentiviral constructs. In each case, PKCα knockdown resulted in a corresponding reduction in the level of *APOBEC3B* mRNA induced by PMA (Fig 2H). Immunoblots confirmed PKCα knockdown and proportional reductions in APOBEC3B (Fig 2I). Altogether, the pharmacologic and genetic approaches provide a strong case for PKCα as the predominant PKC isoform driving PMA-mediated upregulation of APOBEC3B.

NFκB is required for APOBEC3B induction by PMA

We next asked which downstream transcription factor is responsible for driving *APOBEC3B* upregulation in response to PMA. PKC is known to signal through several different transcription factors, including ERK, JNK, NFκB, and others (20,21,23). We therefore started at the DNA level and examined the *APOBEC3B* promoter region for binding sites of known PKC-regulated transcription factors. Interestingly, these *in silico* analyses revealed several NFκB binding sites within 2.5 kb of the *APOBEC3B* transcriptional start site (5′-GGRRNNYYCC). NFκB is known to have multiple roles in immunity and inflammation (36), and a direct NFκB-mediated relay to *APOBEC3B* expression could be physiologically beneficial, given APOBEC3B's known roles in innate immunity.

To test for a mechanistic link between NFκB and *APOBEC3B* transcription, we used two compounds known to block NFκB signaling through independent mechanisms. First, we treated MCF10A cells with varying amounts of BAY 11-7082, which is an NFκB inhibitor that acts by inhibiting upstream ubiquitin assembly (37). This small molecule caused strong dose-responsive drops in *APOBEC3B* induction by PMA treatment (Fig. 3A). Second, we pretreated MCF10A cells with a titration of the proteasome inhibitor, MG132, prior to PMA stimulation. It is well known that both the canonical and non-canonical NFκB signaling pathways require proteasome-mediated degradation of IκB and processing of p100, respectively, for efficient signal transduction and that MG132 blocks these events (36). Consistent with this mechanism of action, MG132 treatment caused a dose-dependent decrease in *APOBEC3B* expression (Fig. 3B). As above, neither BAY 11-7082 nor MG132 caused cell cycle or morphological changes through the durations of these experiments (Fig. S4).

RNAseq data revealed that MCF10A expresses both the canonical NFκB components, *RELA* and *NFKB1*, and the non-canonical NFκB components, *RELB* and *NFKB2*, and levels of these mRNAs are unaffected by PMA treatment (Fig. 3C). Canonical signaling is known to require IKKβ, whereas non-canonical NFκB signaling is strictly dependent on IKKα-catalyzed phosphorylation of p100 (36). To distinguish between these pathways, we used TPCA-1, which is known to have a 22-fold selectivity for IKKβ (canonical) over IKKα (non-canonical) (38). MCF10A cells were pretreated with a titration of TPCA-1 concentrations spanning the IC50 values of both proteins, and then PMA was used to induce *APOBEC3B* upregulation. *APOBEC3B* expression was inhibited closer to the reported IC50 of IKKα, consistent with involvement of the non-canonical NFκB pathway (Fig 3D). As an additional control, we also analyzed *TNFα*, which is regulated by the canonical pathway (39,40). As expected, *TNFα* expression was inhibited by much lower concentrations of TCPA-1 confirming the differential selectively of this compound and further implicating the non-canonical NFκB pathway (Fig 3D).

RELB and p100/p52 are recruited to the *APOBEC3B* promoter region in response to PMA

We next performed ChIP experiments to further test whether the non-canonical NFκB pathway is responsible for upregulating *APOBEC3B*. Primer sets were designed for each of the predicted NFκB binding sites near the *APOBEC3B* transcriptional start site (Fig. 3F). As a control, an additional primer set was made for the promoter region of *NFKBIA*, which contains NFκB binding sites and is also upregulated by PMA with similar kinetics as *APOBEC3B* (*NFKBIA* encodes IκB; Figs. 3E and F). ChIP was performed for RELA, RELB, p100/p52, RNA POL II (positive control), and isotype matched IgG (negative control). As expected, RELA, RELB, p100/p52, and RNA POL II were all bound to the *NFKBIA* promoter following PMA treatment (Fig. 3G). We also found RNA POL II bound to the *APOBEC3B* gene near the transcriptional start site and throughout the gene body in response to PMA (Fig. 3G). Interestingly, both RELB and p100/p52 were recruited to the same sites as RNA POL II following PMA treatment, indicating that these factors are also involved in driving *APOBEC3B* expression in response to PMA (Fig. 3G). An expanded ChIP experiment replicated these data and showed that RNA POL II, RELB, and p100/p52 binding are dependent on PKC signaling as treatment with AEB071 completely ablated all

binding to the *APOBEC3B* promoter (Fig. S5). These ChIP data strongly implicate the non-canonical NFκB pathway, specifically the RELB and p100/p52 heterodimer (and not RELA and p105/p50), in directly inducing *APOBEC3B* transcription in response to PMA activation of PKC.

Endogenous *APOBEC3B* expression requires PKC in multiple cancer cell lines

We next asked whether the constitutively high levels of endogenous *APOBEC3B* observed in many human cancer cell lines occurs through the PKC pathway (6,8,14). For this series of experiments, we selected 4 breast, 4 ovarian, 4 bladder, and 4 head/neck cancer cell lines expressing a 10-fold range of endogenous *APOBEC3B* mRNA levels (Fig. 4A). Each line was treated for 48 hrs with 10 μM AEB071, the most potent PKC inhibitor identified above, and then *APOBEC3B* mRNA and protein levels were quantified by RT-qPCR and immunoblotting. As above, no effects on the cell cycle or cell viability were observed (Fig. S6). This is important since higher concentrations of AEB071 are known to cause cell cycle perturbations and apoptosis in certain cell types (31–33). *APOBEC3B* mRNA levels were reduced by more than half in 7/16 cell lines, including the breast cancer cell lines MDA-MB-468, MDA-MB-453, and HCC1806, the ovarian cancer cell line OVCAR5, and the head/neck lines SQ-20B, JSQ3, and TR146 (Fig. 4B, histogram). Changes of protein levels largely mirrored the mRNA results (Fig. 4B, immunoblot). Interestingly, several cell lines including all of the bladder cancer cell lines showed little decrease in APOBEC3B expression upon treatment with AEB071, suggesting that at least one additional induction mechanism exists. Altogether, these data demonstrate that the PKC axis is responsible for the constitutive upregulation of endogenous *APOBEC3B* in a variety of cancer cell lines representing multiple distinct cancer types.

Discussion

These studies are the first to establish mechanistic linkages between the PKC-NFκB signal transduction pathway and upregulation of the DNA mutating enzyme APOBEC3B in cancer. Our studies suggest a model in which PKCα activation signals through the non-canonical NFκB pathway and results in the recruitment of RELB to the *APOBEC3B* gene and its transcriptional activation (Fig. 5). This mechanism is remarkably specific to *APOBEC3B*, as expression of the related *APOBEC* family members is not affected. This specificity is concordant with our prior studies indicating that APOBEC3B is the only DNA deaminase family member upregulated in these and other cancer types in comparison to normal tissues (6–8,14). Moreover, PKC inhibitor studies with breast, head/neck, and ovarian cancer cell lines demonstrate that the PKC-NFκB pathway contributes to the constitutively high levels of endogenous APOBEC3B associated with cancer mutagenesis. Additional studies will be needed to determine the precise proportions of each tumor type affected by this APOBEC3B upregulation mechanism that, based on prior studies from our laboratory and others, is expected to endow cancer cells with mutational fuel for accelerated tumor evolution.

A recent publication implicated both the interferon response and the canonical and non-canonical NFκB pathways in *APOBEC3A* and *APOBEC3B* upregulation and clearance of

HBV episomes from infected cells (41). Activation of the lymphotoxin-β receptor through treatment of infected hepatocytes with bivalent or tetravalent antibodies led to the nuclear translocation of both RELA and RELB and the activation of known NFκB pathway genes. These antibody treatments also led to the upregulation of *APOBEC3A* and/or *APOBEC3B* and to the gratuitous deamination of HBV cccDNA cytosines, viral DNA degradation, and long-term virus suppression. Taken together with our results presented here, it is tempting to speculate that both the PKC and the lymphotoxin-β receptor signaling mechanisms converge upon the non-canonical RELB-dependent NFκB pathway in order to activate *APOBEC3B* expression. Thus, our work suggests additional strategies such as PMA treatment to induce APOBEC3B upregulation and clearance of HBV from infected hepatocytes. However, these strategies may induce collateral damage through genomic DNA mutagenesis and should be approached carefully.

Clear evidence for APOBEC3B overexpression and mutation signatures in cervical and head/neck cancers suggested that HPV infection might trigger an innate immune response that includes DNA deaminase upregulation (7,9). Subsequent work demonstrated that infection by high-risk (not low-risk) HPV types causes the specific upregulation of APOBEC3B, suggesting that this is not simply a gratuitous response to viral infection (14,15). Moreover, the E6 oncoprotein alone from high-risk types was sufficient to trigger APOBEC3B upregulation (14). It is notable that the overall fold induction by HPV is lower than that described here, due partly to higher background and partly to a smaller magnitude of induction. The mutator phenotype induced by HPV infection is likely fueling tumor evolution as the pattern of PI3K-activating mutations in HPV-positive tumors is biased toward cytosine mutations in APOBEC-like motifs in the helical domain of the kinase, whereas the pattern in HPV-negative tumors is split between the helical and kinase domains of the enzyme (10,11). Obviously, HPV-mediated upregulation of APOBEC3B only impacts cervical cancers and a proportion of head/neck and bladder carcinomas. In contrast, a larger number of tumor types is likely to be using the mechanism described here.

It will also be interesting to determine the relationship between APOBEC3B upregulation and immunotherapy responsiveness, as recent reports have suggested that increased tumor mutation loads correlate with stronger anticancer immune responses (42,43). It may therefore be useful to induce APOBEC3B, as described here, to increase tumor neoantigens in order to boost efficacies of current immunotherapies.

Although *PKC* mutations are rare in cancer, altered expression of several PKC isoforms is observed and associated with poor clinical outcomes (20,21). In addition, mutations in *GNAQ* and *GNA11* occur in approximately half of all uveal melanoma samples [(44,45); illustrated as Gq in Fig. 5]. Inhibition of PKC in these uveal tumors leads to clinical benefits attributed to cell cycle arrest and apoptosis (31–34). It is possible that downregulation of APOBEC3B and a subsequent decrease in tumor evolution through lowered mutation rates may also contribute to these encouraging clinical responses. Based on substantive prior work from our lab and others demonstrating a major role for APOBEC3B in cancer mutagenesis and correlating high levels of APOBEC3B with poor prognoses for ER-positive breast cancers (46,47), together with the studies presented here, we propose that existing inhibitors of the PKC-NFκB axis such as AEB071 may be repurposed to treat primary tumors in

combination with existing therapies and help prevent detrimental outcomes such as drug resistance and metastases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011; 144:646–74. [PubMed: 21376230]
2. Nik-Zainal S, Alexandrov LB, Wedge DC, Van Loo P, Greenman CD, Raine K, et al. Mutational processes molding the genomes of 21 breast cancers. Cell. 2012; 149:979–93. [PubMed: 22608084]
3. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SAJR, Behjati S, Biankin AV, et al. Signatures of mutational processes in human cancer. Nature. 2013; 500:415–21. [PubMed: 23945592]
4. Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature. 2013; 499:214–8. [PubMed: 23770567]
5. Poon SL, Pang S-T, McPherson JR, Yu W, Huang KK, Guan P, et al. Genome-wide mutational signatures of aristolochic acid and its application as a screening tool. Sci Transl Med. 2013; 5:197ra101.
6. Burns MB, Lackey L, Carpenter MA, Rathore A, Land AM, Leonard B, et al. APOBEC3B is an enzymatic source of mutation in breast cancer. Nature. 2013; 494:366–70. [PubMed: 23389445]
7. Burns MB, Temiz NA, Harris RS. Evidence for APOBEC3B mutagenesis in multiple human cancers. Nat Genet. 2013; 45:977–83. [PubMed: 23852168]
8. Leonard B, Hart SN, Burns MB, Carpenter MA, Temiz NA, Rathore A, et al. APOBEC3B upregulation and genomic mutation patterns in serous ovarian carcinoma. Cancer Research. 2013; 73:7222–31. [PubMed: 24154874]
9. Roberts SA, Lawrence MS, Klimczak LJ, Grimm SA, Fargo D, Stojanov P, et al. An APOBEC cytidine deaminase mutagenesis pattern is widespread in human cancers. Nat Genet. 2013; 45:970–6. [PubMed: 23852170]
10. Henderson S, Chakravarthy A, Su X, Boshoff C, Fenton TR. APOBEC-mediated cytosine deamination links PIK3CA helical domain mutations to human papillomavirus-driven tumor development. Cell Rep. 2014; 7:1833–41. [PubMed: 24910434]
11. Cancer Genome Atlas Network. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature. 2015; 517:576–82. [PubMed: 25631445]
12. Roberts SA, Sterling J, Thompson C, Harris S, Mav D, Shah R, et al. Clustered mutations in yeast and in human cancers can arise from damaged long single-strand DNA regions. Mol Cell. 2012; 46:424–35. [PubMed: 22607975]
13. Refsland EW, Harris RS. The APOBEC3 family of retroelement restriction factors. Curr Top Microbiol Immunol. 2013; 371:1–27. [PubMed: 23686230]

14. Vieira VC, Leonard B, White EA, Starrett GJ, Temiz NA, Lorenz LD, et al. Human papillomavirus E6 triggers upregulation of the antiviral and cancer genomic DNA deaminase APOBEC3B. MBio. 2014; 5:e02234–14. [PubMed: 25538195]
15. Warren CJ, Xu T, Guo K, Griffin LM, Westrich JA, Lee D, et al. APOBEC3A functions as a restriction factor of human papillomavirus. Journal of Virology. 2015; 89:688–702. [PubMed: 25355878]
16. Refsland EW, Stenglein MD, Shindo K, Albin JS, Brown WL, Harris RS. Quantitative profiling of the full APOBEC3 mRNA repertoire in lymphocytes and tissues: implications for HIV-1 restriction. Nucleic Acids Res. 2010; 38:4274–84. [PubMed: 20308164]
17. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014; 15:550. [PubMed: 25516281]
18. Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc. 2012; 7:562–78. [PubMed: 22383036]
19. Madsen P, Anant S, Rasmussen HH, Gromov P, Vorum H, Dumanski JP, et al. Psoriasis upregulated phorbolin-1 shares structural but not functional similarity to the mRNA-editing protein apobec-1. Journal of Investigative Dermatology. 1999; 113:162–9. [PubMed: 10469298]
20. Rosse C, Linch M, Kermorgant S, Cameron AJM, Boeckeler K, Parker PJ. PKC and the control of localized signal dynamics. Nat Rev Mol Cell Biol. 2010; 11:103–12. [PubMed: 20094051]
21. Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. Nat Rev Cancer. 2007; 7:281–94. [PubMed: 17384583]
22. Spitaler M, Cantrell DA. Protein kinase C and beyond. Nat Immunol. 2004; 5:785–90. [PubMed: 15282562]
23. Mackay HJ, Twelves CJ. Targeting the protein kinase C family: are we there yet? Nat Rev Cancer. 2007; 7:554–62. [PubMed: 17585335]
24. Stenglein MD, Burns MB, Li M, Lengyel J, Harris RS. APOBEC3 proteins mediate the clearance of foreign DNA from human cells. Nat Struct Mol Biol. 2010; 17:222–9. [PubMed: 20062055]
25. Thielen BK, McNevin JP, McElrath MJ, Hunt BVS, Klein KC, Lingappa JR. Innate immune signaling induces high levels of TC-specific deaminase activity in primary monocyte-derived cells through expression of APOBEC3A isoforms. Journal of Biological Chemistry. 2010; 285:27753–66. [PubMed: 20615867]
26. Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller HJ, Johannes FJ. Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase c isoenzymes. FEBS Lett. 1996; 392:77–80. [PubMed: 8772178]
27. Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, et al. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. Journal of Biological Chemistry. 1991; 266:15771–81. [PubMed: 1874734]
28. Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, et al. Selective inhibition of protein kinase C isozymes by the indolocarbazole Gö 6976. Journal of Biological Chemistry. 1993; 268:9194–7. [PubMed: 8486620]
29. Evenou J-P, Wagner J, Zenke G, Brinkmann V, Wagner K, Kovarik J, et al. The potent protein kinase C-selective inhibitor AEB071 (sotrastaurin) represents a new class of immunosuppressive agents affecting early T-cell activation. J Pharmacol Exp Ther. 2009; 330:792–801. [PubMed: 19491325]
30. Wagner J, Matt von P, Sedrani R, Albert R, Cooke N, Ehrhardt C, et al. Discovery of 3-(1H-indol-3-yl)-4-[2-(4-methylpiperazin-1-yl)quinazolin-4-yl]pyrrole-2,5-dione (AEB071), a potent and selective inhibitor of protein kinase C isotypes. J Med Chem. 2009; 52:6193–6. [PubMed: 19827831]
31. Wu X, Li J, Zhu M, Fletcher JA, Hodi FS. Protein kinase C inhibitor AEB071 targets ocular melanoma harboring GNAQ mutations via effects on the PKC/Erk1/2 and PKC/NF-κB pathways. Mol Cancer Ther. 2012; 11:1905–14. [PubMed: 22653968]
32. Chen X, Wu Q, Tan L, Porter D, Jager MJ, Emery C, et al. Combined PKC and MEK inhibition in uveal melanoma with GNAQ and GNA11 mutations. Oncogene. 2014; 33:4724–34. [PubMed: 24141786]

33. Musi E, Ambrosini G, de Stanchina E, Schwartz GK. The phosphoinositide 3-kinase α selective inhibitor BYL719 enhances the effect of the protein kinase C inhibitor AEB071 in GNAQ/GNA11-mutant uveal melanoma cells. Mol Cancer Ther. 2014; 13:1044–53. [PubMed: 24563540]
34. Piperno-Neumann S, Kapiteijn E, Larkin JMG, Carvajal RD, Luke JJ, Seifert H, et al. Phase I dose-escalation study of the protein kinase C (PKC) inhibitor AEB071 in patients with metastatic uveal melanoma. J Clin Oncol. 2014; 32:9030.
35. Wagner J, Matt von P, Faller B, Cooke NG, Albert R, Sedrani R, et al. Structure-activity relationship and pharmacokinetic studies of sotrastaurin (AEB071), a promising novel medicine for prevention of graft rejection and treatment of psoriasis. J Med Chem. 2011; 54:6028–39. [PubMed: 21797275]
36. Vallabhapurapu S, Karin M. Regulation and function of NF-kappaB transcription factors in the immune system. Annu Rev Immunol. 2009; 27:693–733. [PubMed: 19302050]
37. Strickson S, Campbell DG, Emmerich CH, Knebel A, Plater L, Ritorto MS, et al. The anti-inflammatory drug BAY 11-7082 suppresses the MyD88-dependent signalling network by targeting the ubiquitin system. Biochem J. 2013; 451:427–37. [PubMed: 23441730]
38. Podolin PL, Callahan JF, Bolognese BJ, Li YH, Carlson K, Davis TG, et al. Attenuation of murine collagen-induced arthritis by a novel, potent, selective small molecule inhibitor of IkappaB Kinase 2, TPCA-1 (2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide), occurs via reduction of proinflammatory cytokines and antigen-induced T cell Proliferation. J Pharmacol Exp Ther. 2005; 312:373–81. [PubMed: 15316093]
39. Foxwell B, Browne K, Bondeson J, Clarke C, de Martin R, Brennan F, et al. Efficient adenoviral infection with IkappaB alpha reveals that macrophage tumor necrosis factor alpha production in rheumatoid arthritis is NF-kappaB dependent. Proceedings of the National Academy of Sciences. 1998; 95:8211–5.
40. Liu H, Sidiropoulos P, Song G, Pagliari LJ, Birrer MJ, Stein B, et al. TNF-alpha gene expression in macrophages: regulation by NF-kappa B is independent of c-Jun or C/EBP beta. J Immunol. 2000; 164:4277–85. [PubMed: 10754326]
41. Lucifora J, Xia Y, Reisinger F, Zhang K, Stadler D, Cheng X, et al. Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. Science. 2014; 343:1221–8. [PubMed: 24557838]
42. Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. N Engl J Med. 2014; 371:2189–99. [PubMed: 25409260]
43. Lo JA, Fisher DE. The melanoma revolution: from UV carcinogenesis to a new era in therapeutics. Science. 2014; 346:945–9. [PubMed: 25414302]
44. Van Raamsdonk CD, Bezrookove V, Green G, Bauer J, Gaugler L, O'Brien JM, et al. Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. Nature. 2009; 457:599–602. [PubMed: 19078957]
45. Van Raamsdonk CD, Griewank KG, Crosby MB, Garrido MC, Vemula S, Wiesner T, et al. Mutations in GNA11 in uveal melanoma. N Engl J Med. 2010; 363:2191–9. [PubMed: 21083380]
46. Sieuwerts AM, Willis S, Burns MB, Look MP, Meijer-Van Gelder ME, Schlicker A, et al. Elevated APOBEC3B correlates with poor outcomes for estrogen-receptor-positive breast cancers. Horm Cancer. 2014; 5:405–13. [PubMed: 25123150]
47. Cescon DW, Haibe-Kains B, Mak TW. APOBEC3B expression in breast cancer reflects cellular proliferation, while a deletion polymorphism is associated with immune activation. Proceedings of the National Academy of Sciences. 2015; 112:2841–6.

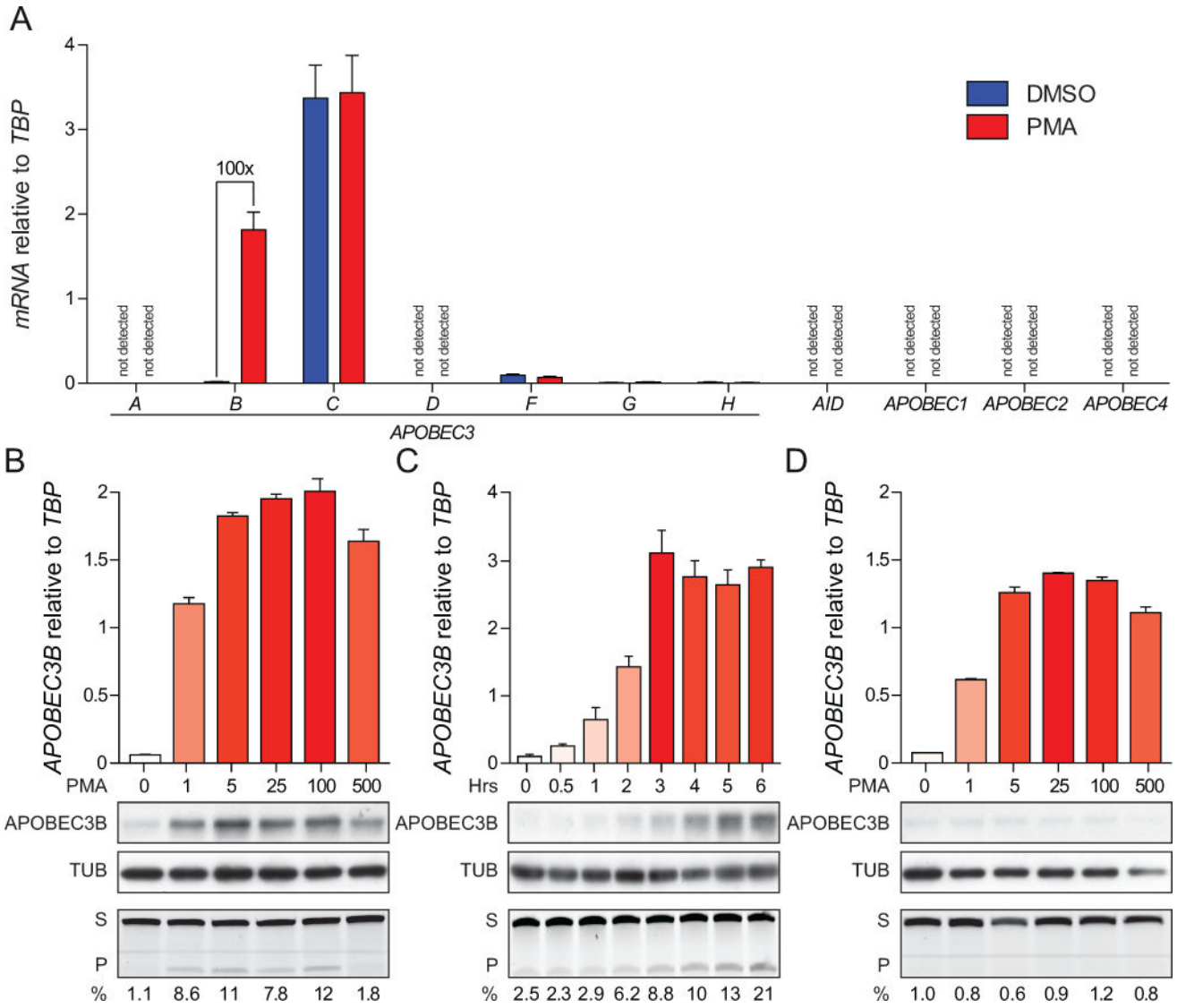

Figure 1.

APOBEC3B upregulation by PMA.

A. A histogram showing the specific upregulation of *APOBEC3B* mRNA by PMA. MCF10A cells were treated with PMA (25 ng/ml) or vehicle control for 6 hrs, and mRNA levels were measured by RT-qPCR (mean and SD are shown for triplicate RT-qPCR reactions normalized to *TBP*). The same data points are shown in the context of a larger PMA dose response experiment in Fig. S1.

B. A histogram demonstrating the dose responsiveness of *APOBEC3B* upregulation by PMA. Normalization and quantification were calculated as in Fig. 1A. The middle images show immunoblots for corresponding APOBEC3B and TUBULIN proteins levels, and the lower image shows DNA cytosine deaminase activity for the corresponding whole cell extracts (S, substrate; P, product; percent deamination quantified below each lane).

C. A histogram depicting the rapid kinetics of APOBEC3B upregulation following PMA treatment. MCF10A cells were treated with a single concentration of PMA (25 ng/ml), and mRNA, protein, and activity levels are reported as in Fig. 1B.

D. New protein synthesis is dispensable for *APOBEC3B* mRNA upregulation by PMA. Representative dose response experiment for MCF10A cells treated with the indicated concentrations of PMA following a 30 min pretreatment with 10 μg/mL cyclohexamide. mRNA, protein, and activity levels are reported as in Fig. 1B

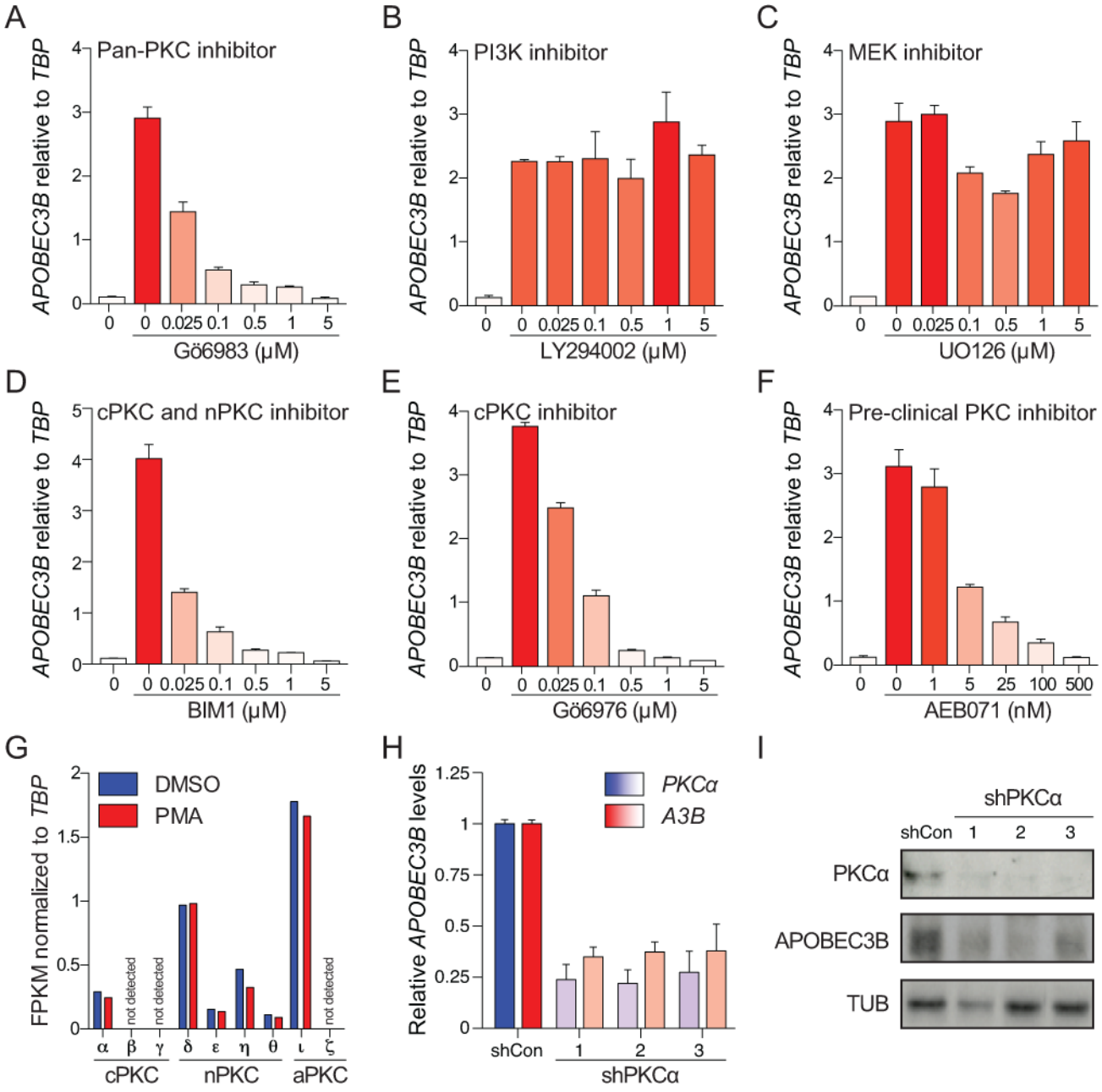

Figure 2.

APOBEC3B upregulation by PMA is dependent on PKC.

A–F. Histograms reporting the impact of the indicated small molecules on PMA-induced *APOBEC3B* upregulation. *APOBEC3B* induction was inhibited by Gö6983 (pan-PKC inhibitor), BIM-1 (classical and novel PKC inhibitor), Gö6976 (classical PKC selective inhibitor), and AEB071 (preclinical PKC inhibitor) but not by LY294002 (PI3K inhibitor) or UO126 (MEK inhibitor). MCF10A cells were treated with PMA following a 30 min pretreatment with the indicated concentrations of each inhibitor. mRNA expression is reported as the mean of 3 independent RT-qPCR reactions normalized to *TBP* (error bars report SD from triplicate assays).

G. Histogram depicting *PKC* isoforms expressed in MCF10A cells treated with PMA or vehicle control. mRNA expression was determined by RNA-seq and is reported as fragments per kilobase of exon per million fragments mapped (FKPM) and normalized to *TBP*.

H. Histogram showing that PKCα knockdown inhibits *APOBEC3B* induction by PMA. MCF10A cells were treated with PMA following PKCα knockdown using 3 independent PKCα specific shRNA encoding lentiviruses and a control. mRNA levels for both *PKCα* (blue) and *APOBEC3B* (red) are reported.

I. Immunoblots confirming PKCα knockdowns and proportional reductions in APOBEC3B protein levels.

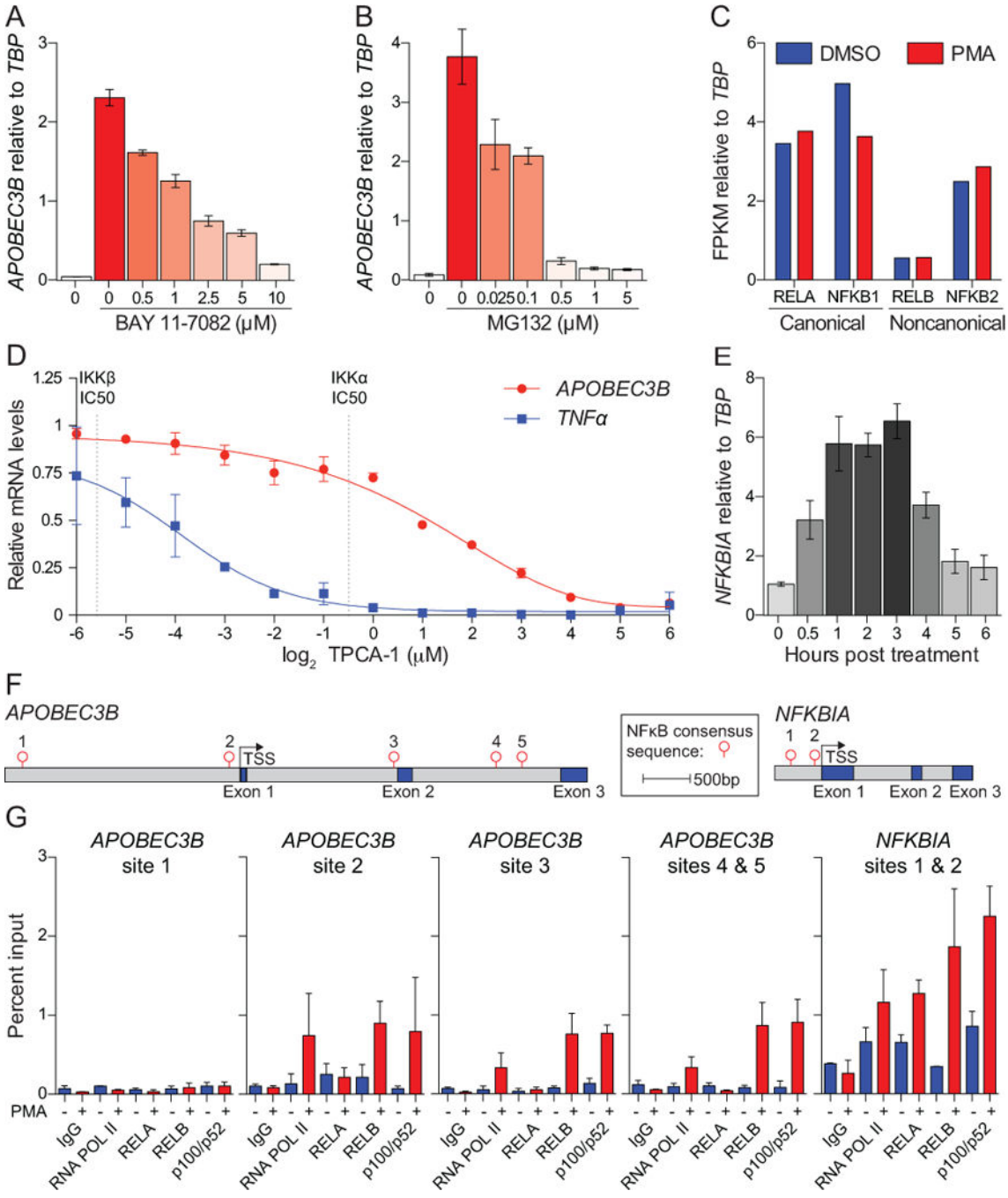

Figure 3.

Non-canonical NFκB signaling is responsible for *APOBEC3B* upregulation by PMA.

A–B. Histograms depicting the dose responsive inhibition of PMA-induced *APOBEC3B* upregulation by BAY 11-7082 (ubiquitination inhibitor) and MG132 (proteasome inhibitor). MCF10A cells were treated with PMA following a 30 min pretreatment with the indicated concentrations of each inhibitor. *APOBEC3B* mRNA expression is reported as the mean of 3 independent RT-qPCR reactions normalized to *TBP* (error bars report SD from triplicate assays).

C. Histogram depicting *NFκB* subunit mRNA levels in MCF10A cells treated with PMA or vehicle control. Expression was determined by RNA-seq and is reported as FKPM and normalized to *TBP*.

D. Plot depicting inhibition of PMA-induced *APOBEC3B* expression by the IκB kinase (IKK) inhibitor, TPCA-1, near the IC50 for IKKα, not IKKβ. MCF10A cells were treated with PMA following treatment with varying concentrations of TPCA-1. *TNFα* (blue) and *APOBEC3B* (red) mRNA levels are reported as the mean of 3 independent RT-qPCR reactions normalized to *TBP* (error bars report SD from triplicate assays). The dotted lines denote previously reported *in vitro* IC50 values for IKKα and IKKβ inhibition by TPCA-1 (38).

E. Histogram showing the kinetics of *NFKBIA* upregulation PMA. MCF10A cells were treated with PMA for the indicated times and mRNA values were quantified as in Fig. 3A.

F. The *APOBEC3B* and *NFKBIA* promoter regions contain several putative NFκB binding sites (TSS, transcriptional start site).

G. RELB and p105/p52 are specifically and robustly recruited to the *APOBEC3B* promoter region by PMA. ChIP was performed after a treatment with PMA or vehicle control for 2 hrs. *APOBEC3B* sites 4 & 5 and the two *NFKBIA* sites are reported together because they are too close to each other to be distinguished by this procedure. qPCR results are reported as percent of the total chromatin input.

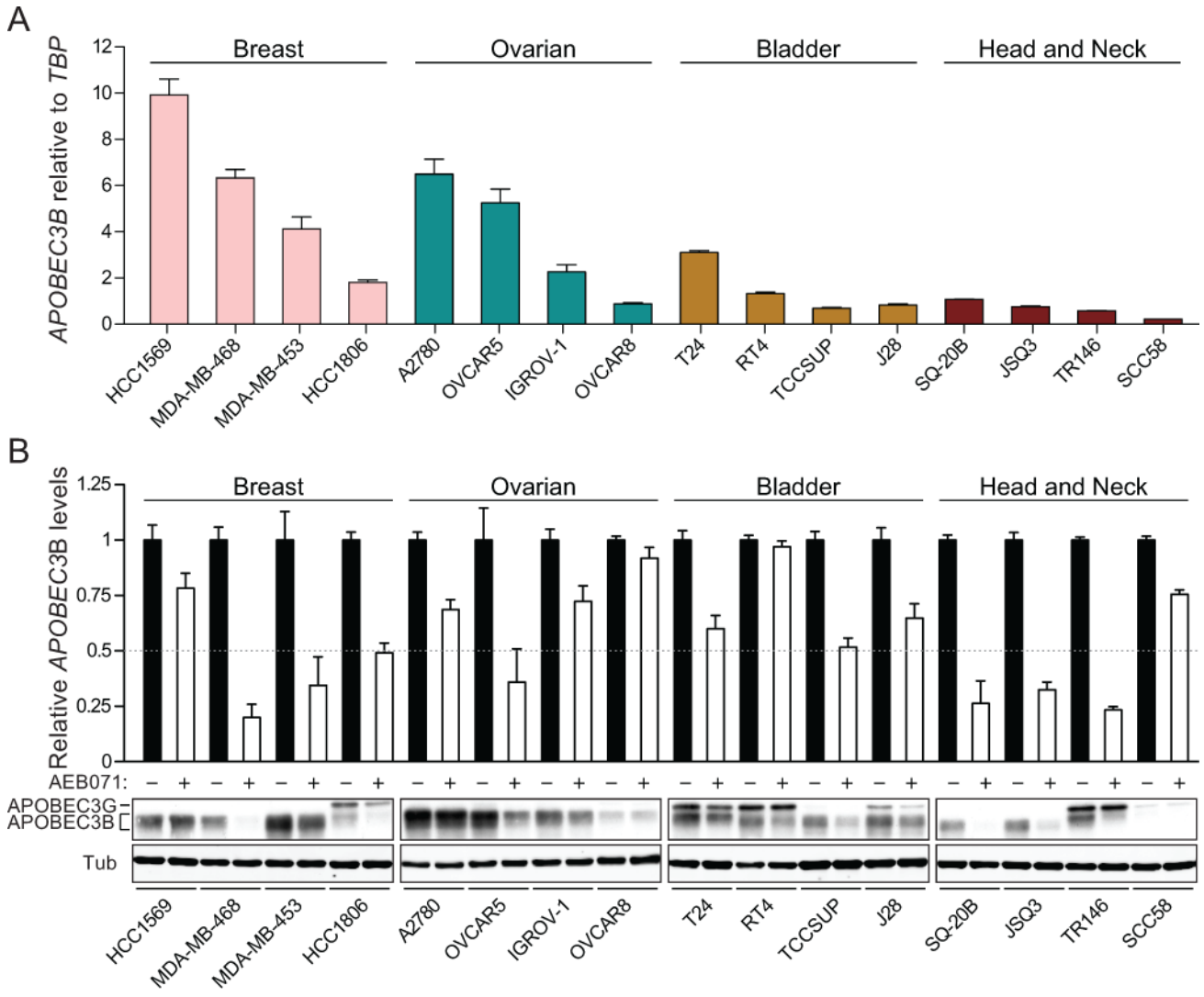

Figure 4.

The PKC pathway drives endogenous APOBEC3B expression in cancer cells.

A. *APOBEC3B* mRNA levels in representative breast, ovarian, bladder, and head/neck cancer cell lines. mRNA expression is reported as the mean of 3 independent RT-qPCR reactions normalized to *TBP* (error bars report SD from triplicate assays).

B. AEB071 downregulates APOBEC3B in multiple cancer cell lines. The histogram reports *APOBEC3B* mRNA levels normalized to the vehicle treated control for each line. The dotted line represents a 50% decrease of *APOBEC3B* expression due to AEB071. The corresponding immunoblots show APOBEC3B and TUBULIN levels. Each line was treated with AEB071 (10μM) or vehicle control for 48 hours prior to mRNA and protein analysis.

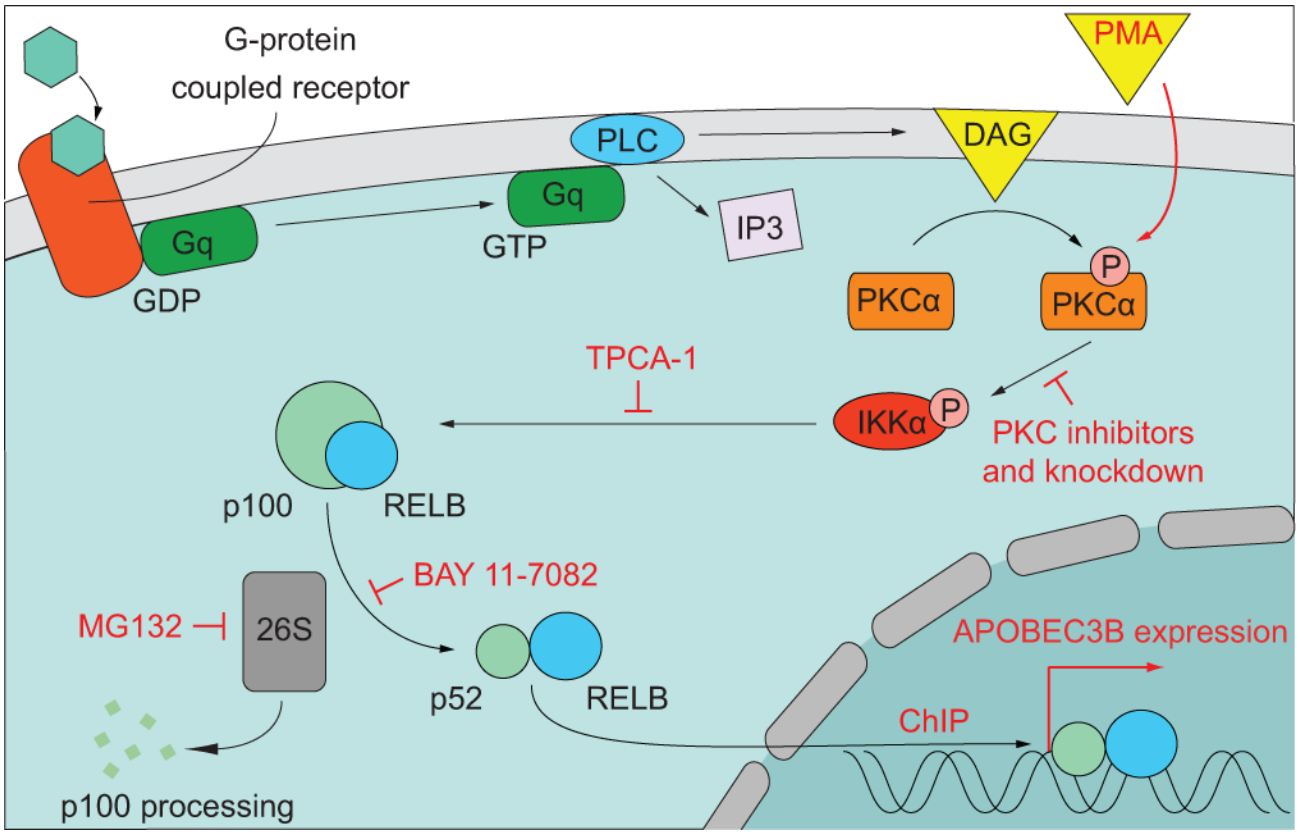

Figure 5.

Model for APOBEC3B upregulation by the PKC-NFκB pathway.

PKCα activation by DAG or PMA leads to IKKα phosphorylation and proteasome-dependent cleavage of NFκB subunit p100 into the transcriptionally active p52 form. The non-canonical NFκB heterodimer containing p52 and RELB is then recruited to the *APOBEC3B* promoter to drive transcription. Red labels represent the small molecules and approaches used to interrogate this signal transduction pathway.