The Breast Cancer Tumor Suppressor TRIM29 is Expressed via ATM-Dependent Signaling in Response to Hypoxia

Muzaffer Dükel1*, W. Scott Streitfeld1¶, Tsz Ching Chloe Tang1¶, Lindsey R.F. Backman1, Lingbao Ai1, W. Stratford May2, and Kevin D. Brown1

From the 1Department of Biochemistry and Molecular Biology and 2Department of Medicine, University of Florida College of Medicine, Gainesville, FL

Running title: TRIM29 expression is ATM-dependent

*Current Address: Department of Biology, Mehmet Akif Ersoy University, Burdur, Turkey.
¶These authors contributed equally

To whom correspondence should be addressed: Dr. Kevin D. Brown, Dept. of Biochemistry and Molecular Biology, University of Florida College of Medicine 1200 Newell Drive, Box 1000245, Gainesville, FL 32610. Telephone: (352) 273-5458; FAX: (352) 392-1445; Email: kdbrown1@ufl.edu

Keywords: Hypoxia, hypoxia-inducible factor (HIF), tumor suppressor gene, breast cancer, cell signaling

ABSTRACT

Reduced ATM function has been linked to breast cancer risk and the TRIM29 protein is an emerging breast cancer tumor suppressor. Here we show that, in cultured breast tumor and non-tumorigenic mammary epithelial cells, TRIM29 is upregulated in response to hypoxic stress but not DNA damage. Hypoxia-induced upregulation of TRIM29 is dependent upon ATM and HIF1α, and occurs through increased transcription of the TRIM29 gene. Basal expression of TRIM29 is also downregulated in cells expressing diminished levels of ATM and findings suggest this occurs through basal NF-κB activity as knockdown of the NF-κB subunit RelA suppresses TRIM29 abundance. We have previously shown that the activity of the TWIST1 oncogene is antagonized by TRIM29 and now show that TRIM29 is necessary to block the upregulation of TWIST1 that occurs in response to hypoxic stress. This study establishes TRIM29 as a hypoxia-induced tumor suppressor gene and provides a novel molecular mechanism for ATM-dependent breast cancer suppression.

INTRODUCTION

The ATM gene product is a high-molecular weight protein kinase that is catalytically activated in response to DNA double-strand breaks (DSBs). Upon activation, ATM phosphorylates numerous downstream substrates that activate a range of cellular responses to genotoxic stress such as cell cycle checkpoints, apoptosis, and DNA repair (1). Owing to its critical role in DNA damage response signaling and maintenance of genomic integrity, as well as the highly tumor-prone nature of both humans and mice with germline disruption of the ATM gene (2,3), ATM is roundly considered a tumor suppressor gene (TSG). While germline loss of ATM principally results in tumors of hematological origin (lymphoma and leukemia) (4), multiple studies have confirmed that female ATM heterozygotes possess an elevated breast cancer risk (5-7). Presently, it remains unresolved which facet(s) of ATM function are critical in breast cancer suppression.

ATM has been shown to respond to other forms of cellular stress such as redox imbalance (8) and hypoxic stress. Several studies documented that, in response to culture under low oxygen conditions, cells activate ATM-dependent signal transduction resulting in activation of the kinase Chk2 (9,10). More recently, ATM has been mechanistically linked to hypoxic response by directly phosphorylating the HIF1α protein (11), a subunit of the HIF1 transcriptional complex required to transactivate a spectrum of genes important in response to low oxygen conditions (12). While evidence indicates that ATM is activated during hypoxic response through a mechanism independent from the mechanism that
controls ATM activity in response to DNA DSBs (13), the impact of ATM-dependent signal transduction during response to this particular form of cellular stress remains unclear. This last point is also potentially paradoxical as hypoxic response, and activation of HIF1 in particular, is widely viewed as principally oncogenic in nature (12,14,15).

TRIM29, a member of the TRIM family of proteins (16), has proven an enigmatic protein in regards to its function in differing cancer types. Examination of TRIM29 expression in various tumor types has uncovered that increased expression of TRIM29 is associated with more aggressive forms of disease including bladder (17), colorectal (18), gastric (19), lung (20,21), and pancreatic cancer (22). Further, several studies have shown a significant association between elevated TRIM29 expression and reduced patient survival (17,19). In pancreatic cancer cells, investigators found that overexpression of TRIM29 promoted cell growth in vitro and metastatic activity in vivo (23). This property of TRIM29 stems from its ability to stimulate Wnt/β-Catenin/TCF signaling by binding to Dvl-2, a Wnt activator downstream of the Frizzled receptor. More recently, TRIM29 was found to bind to many molecular components of DNA damage response and to act as an important factor in response to genotoxic stress (24).

In breast cancer, a clearly contrasting view as to how TRIM29 impacts oncogenesis has emerged. The first report on this subject showed that TRIM29 was not commonly expressed in a small panel of breast cancer lines and that expression of recombinant TRIM29 in the breast cancer line BT-549 resulted in reduced growth and colony forming ability in soft agar (25). Later, Liu et al (26) observed that knockdown of TRIM29 in the non-tumorigenic MCF-10A line resulted in an increased growth rate and anchorage-independent growth, increased cell motility and invasiveness, and disrupted 3D acinar formation in vitro. In the MCF7 tumor line, which expresses low levels of TRIM29, expression of recombinant human TRIM29 had the opposite effect, namely, a slowing of growth and reduction in anchorage-independent growth. These findings led this group to conclude that TRIM29 was functioning in a growth suppressive role in MCF7 and MCF-10A cells, although the underlying mechanism(s) for this effect were not reported.

Our group (27) showed that TRIM29 knockdown in breast cancer and non-tumorigenic mammary epithelial lines resulted in increased cell motility, invasiveness, and altered gene expression patterns consistent with activation of the epithelial-to-mesenchymal transition (EMT). The molecular basis for this effect appears to be due, at least in part, to a mutually antagonistic relationship that TRIM29 has with the oncogenic transcription factor TWIST1 (27). We now show that TRIM29 expression is induced by hypoxia in an ATM and HIF1α-dependent manner, and that TRIM29 is necessary to block hypoxia-inducible TWIST1 expression.

RESULTS

TRIM29 expression is ATM-dependent and hypoxia-inducible

We initially uncovered the TRIM29 gene in an expression microarray screen for genes that were downregulated in breast cancer lines with knocked down (RNAi) ATM expression. To confirm these findings we knocked down ATM in two breast cancer cell lines that express TRIM29 (SKBr3 and MDA-MB-468) and an immortalized human mammary epithelial cell line (HMEC). As judged by RT-PCR on total RNA harvested from these lines transduced with control lentivirus or virus encoding two independent ATM-specific shRNA constructs (clones shATM-1 and shATM-2), diminished ATM expression resulted in a sharp drop in TRIM29 transcript abundance (Fig 1A). When quantitative RT-PCR (Q-PCR) was used to measure relative ATM and TRIM29 transcript abundance in these cells, we scored a >2-fold decrease in both ATM and TRIM29 mRNA in SKBr3, MDA-MB-468, and HMEC cells with ATM knockdown compared to control virus transduced cells. When immunoblotting was used to examine TRIM29 protein expression in these lines we again observed a clear decrease in TRIM29 in cells with ATM knockdown (Fig 1C). In contrast, immunoblotting with anti-TWIST1 showed a notable increase in TWIST1 protein in ATM knockdown cells.
ATM is critical in the activation of cellular response to DNA DSBs. As TRIM29 expression is controlled in an ATM-dependent manner, we sought to determine if genotoxic stress impacts TRIM29 abundance. To test this, we subjected SKBr3, MDA-MB-468, and HMEC cells to 10 Gy of γ-radiation, prepared extracts and immunoblotted with anti-TRIM29 and anti-γH2AX. We observed no change in TRIM29 expression 18 hr after γ-radiation exposure (Fig 1D). In contrast, abundant phosphorylation of the histone variant H2AX (γH2AX) was observable in these extracts consistent with a robust DNA DSB response (32). These results indicate that TRIM29 expression is not responsive to DNA damage, consistent with other recent studies (24).

Bencokova et al. (13) observed that ATM is activated in response to hypoxia but that ATM activation under these conditions occurred independent of the MRN complex, a required component for ATM activation in response to DNA DSBs (33). This led these investigators to conclude that hypoxic stress activates ATM through a mechanism independent from that governing ATM activation in response to genotoxic stress. To test the effects of low oxygen conditions on TRIM29 abundance we cultured SKBr3, MDA-MB-468, and HMEC cells in a 1.0% O2 atmosphere for 18 hr. Subsequently, these cells, as well as those cultured under normoxic conditions were harvested and total RNA isolated. RT-PCR indicated a sharp increase in TRIM29 transcript abundance in cells cultured under hypoxic conditions (Fig 2A). Consistent with activation of a robust hypoxic response we observed increased levels of the hypoxia-inducible CAIX transcript. When Q-PCR was used to measure relative transcript abundance in ATM knockdown SKBr3 and MDA-MB-468 lines (Fig 3B), we observed either abrogated or notably blunted upregulation of TRIM29 in response to hypoxia. Consistent with previous studies suggesting that phosphorylation of HIF1α by ATM may stabilize HIF1α in response to hypoxia (11), we also observed either absent or severely limited increases in HIF1α protein in these ATM knockdown lines in response to hypoxia.

To assess a role for HIF1α in the hypoxia-induced TRIM29 upregulation we knocked down HIF1α in SKBr3 and MDA-MB-468 cells and measured a >3-fold decrease in HIF1α transcript in each line transduced with either of two independent shRNA-encoding lentivirus (Fig 4A). RT-PCR indicated that knockdown of HIF1α in either SKBr3 or MDA-MB-468 resulted in a coordinate reduction in basal TRIM29 transcript levels (Fig 4B). When these lines were examined for TRIM29 mRNA abundance in response to hypoxia, Q-PCR indicated a blunted upregulation of the TRIM29 transcript in HIF1α knockdown lines. Specifically, in this set of experiments, controls showed a 3.6-fold increase in TRIM29 mRNA in SKBr3 and 2.8-fold in MDA-MB-468 in response to hypoxia (Fig 4C). HIF1α knockdown blunted the hypoxia-induced upregulation of TRIM29 in SKBr3 (2.1 and 1.7-fold in shHIF1α-2 and shHIF1α-3, respectively) and MDA-MB-468

Hypoxia-inducible expression of TRIM29 is ATM and HIF1α-dependent

Following the discovery that TRIM29 expression is upregulated following hypoxia, we sought to understand the signaling responsible for this response. Hypoxia activates ATM (9,10), and more recently it was discovered that ATM directly phosphorylates HIF1α during hypoxic stress response (11). Given these facts, we examined hypoxic response in ATM knockdown SKBr3 and MDA-MB-468 cells. As judged by Q-PCR, we measured ~4.5-fold increase in TRIM29 mRNA in control SKBr3 cells but no significant increase in TRIM29 transcript in ATM knockdown SKBr3 cells following hypoxia (Fig 3A). Similarly, in the MDA-MB-468 line we measured that ATM knockdown abrogated the ~3.6-fold increase in TRIM29 mRNA observed in control cells (Fig 3A).
shHIF1α-3 cells (1.8-fold) where MDA-MB-468 shHIF1α-2 cells showed no significant increase in TRIM29 mRNA in response to hypoxia. Rises in TRIM29 mRNA measured in lines with knocked down HIF1α may be attributable to residual HIF1α expression in these cells or may suggest an additional HIF1α-independent mechanism(s) that drive increased TRIM29 mRNA levels in response to hypoxia.

Results obtained by immunoblotting paralleled Q-PCR results showing that HIF1α knockdown reduced the steady-state levels of TRIM29 and blocked the increase in TRIM29 protein in response to hypoxia in both SKBr3 and MDA-MB-468 cells (Fig 4D). Of note, we have been unable to demonstrate that the TRIM29 gene is directly regulated by HIF1α and, therefore, we cannot rule out that this gene is indirectly controlled through another HIF1α-dependent mechanism.

Under normoxic conditions, prolyl hydroxylases (primarily PHD2) use ambient oxygen to hydroxylate proline residues within HIF1α that subsequently promote association with the VHL protein (pVHL) (34,35). pVHL itself is a subunit of an E3 ubiquitin ligase complex that drives proteosomal degradation resulting in low steady-state levels of HIF1α (36,37). Given this, we knocked down VHL in SKBr3 cells using RNAi to determine if stabilization HIF1α under normoxic conditions would result in increased TRIM29 abundance. SKBr3 cells transduced with VHL-specific shRNA-encoding lentivirus displayed marked reduction in VHL mRNA abundance as determined by RT-PCR (Fig 4E), and by Q-PCR we measured ~3-fold reduced levels of VHL transcript in these cells compared to controls (data not shown). When these cells were immunoblotted we also observed the shRNA expression produced a marked reduction in VHL protein abundance in SKBr3 (Fig 4F). Of note, immunoblotting revealed a detectable increase in HIF1α and TRIM29 in VHL knockdown cells, leading us to conclude that stabilization of the HIF1α under normoxic conditions results in increased TRIM29 abundance.

HIF1 activity is primarily controlled posttranslationally through the stabilization of the labile HIF1α protein. To test if nascent transcription or translation is required for the hypoxia inducibility of TRIM29 we treated SKBr3 or MDA-MB-468 cells with either the RNA PolII inhibitor actinomycin D or the protein synthesis inhibitor cycloheximide prior to culturing these cells for 18 hr under 1% oxygen conditions. We observed that either cycloheximide or actinomycin D ablated the hypoxia-induced upregulation of TRIM29 in both lines (Fig 4G). In contrast, actinomycin D had no marked effect on the upregulation of HIF1α during hypoxic response, but inhibition of nascent protein synthesis by cycloheximide fully ablated HIF1α upregulation in both SKBr3 and MDA-MB-468 cells. From these experiments we conclude that, unlike HIF1α, the upregulation of TRIM29 during hypoxic response is principally controlled at the translational level.

**RelA is required to maintain basal TRIM29 expression**

In addition to observing the effects of ATM or HIF1α knockdown on hypoxia-responsiveness of TRIM29, we also observed that knockdown of either of these genes resulted in markedly reduced basal expression of TRIM29 in both SKBr3 and MDA-MB-468 cells. Further, we measured reduced steady-state levels of HIF1α transcript in ATM knockdown SKBr3 and MDA-MB-468 cells (Fig 5A). Transcription of the HIF1α gene has been demonstrated to be under the control of the NF-κB transcriptional complex (38,39), and our group has documented that ATM supports basal NF-κB activity in cultured breast cancer lines (30,40). To test a role for NF-κB in supporting basal expression of TRIM29 we knocked down the NF-κB subunit RelA (i.e., p65). Using two independent shRNA constructs in both SKBr3 and MDA-MB-468 cells, RT-PCR indicated not only the predicted decrease in RelA and HIF1α mRNA, but clear diminishment in TRIM29 mRNA as well (Fig 5B). Similarly, we observed a clear decrease in basal TRIM29 protein abundance in both SKBr3 and MDA-MB-468 cells in response to RelA knockdown (Fig 5D). These findings clearly indicate that RelA is necessary for the basal expression of TRIM29 in cultured breast cancer lines.
To determine if increased RelA expression is sufficient to drive TRIM29 expression, SKBr3 cells were transduced with adenovirus encoding full-length human RelA (Ad-p65) or a control (Ad-GFP) adenovirus as previously outlined (28). As judged by RT-PCR, infection of SKBr3 cells with Ad-p65 resulted in a dramatic increase in RelA mRNA abundance, as well as increased abundance of SOD2 mRNA, a known NF-κB target gene (41) (Fig 5E). In contrast, no increase in either HIF1α or TRIM29 mRNA was observed in SKBr3 infected with Ad-p65. Q-PCR indicated that infection of cells with Ad-GFP did not significantly alter the transcript abundance of either HIF1α, TRIM29, or SOD2 when compared to uninfected cells. We observed a slight rise in both TRIM29 and HIF1α mRNA levels after Ad-p65 infection, but these increases failed to reach statistical significance when compared cells infected with Ad-GFP. These results sharply contrast with the ~15-fold increase in SOD2 transcript measured following infection of SKBr3 cells with Ad-p65 (Fig 5F).

When uninfected SKBr3, cells infected with Ad-p65 or Ad-GFP virus were assayed for TRIM29 levels by immunoblotting we detected no change in TRIM29 but, as expected, we noted a substantial increase on RelA expression in response to Ad-p65 infection (Fig 5G). Of note, we found no detectable HIF1α in SKBr3 cells infected with Ad-p65 virus while extracts from hypoxic SKBr3 cells run in parallel showed clear upregulation of HIF1α (data not shown).

To further explore the relationship between NF-κB, HIF1α, and TRIM29 we cultured SKBr3 and MDA-MB-468 cells on the antioxidant N-acetyl cysteine (NAC). Previous work from our group has demonstrated that culturing breast cancer cells on NAC will diminish basal ATM-dependent activation of NF-κB (40). To determine if culturing these lines on NAC would have a similar effect on TRIM29 transcript levels we treated SKBr3 and MDA-MB-468 cells with 10 mM NAC and measured both TRIM29 and HIF1α transcripts by Q-PCR. This analysis revealed that co-culturing either SKBr3 or MDA-MB-468 cells on NAC significantly reduced both TRIM29 and HIF1α transcript abundance in these cell lines (Fig 5H). These findings suggest that basal activity of the ATM / NF-κB pathway supports baseline expression of the TRIM29 gene.

In sum, these studies establish that RelA is necessary, but not sufficient, for basal TRIM29 abundance in SKBr3 cells. As ectopic expression of RelA results in markedly increased NF-κB activity, as scored by transcriptional reporter assays and increased SOD2 expression, these results may suggest that TRIM29 is not a direct transcriptional target for NF-κB. Alternatively, TRIM29, like HIF1α, may be NF-κB responsive but cannot respond solely to increased RelA expression/activity. Moreover, decreased TRIM29 expression observed when RelA or ATM is knocked down is possibly an indirect effect due to decreased basal HIF1α expression.

**TRIM29 blocks TWIST1 upregulation during hypoxic response**

Studies indicate that the oncogenic TWIST1 transcription factor is upregulated in response to hypoxia (42) and our previous work documented that TRIM29 blocks TWIST1 activity and expression (27). To better understand the relationship between TWIST1 and TRIM29 during hypoxic response we examined TWIST1 expression in response to hypoxia in two breast cancer cell lines that express TRIM29 (SKBr3 and MDA-MB-468) and two lines previously determined to have epigenetically silenced the TRIM29 gene (MDA-MB-231 and BT-549) (27). We observed a robust response to hypoxia as judged by the multi-fold upregulation of the CAIX transcript in each line (Fig 6A). Similarly, the TWIST1 mRNA also showed significant increases in MDA-MB-231 and BT-549 cells but no significant change in TWIST1 mRNA was observed in either SKBr3 or MDA-MB-468 cells following hypoxia. When immunoblotting was used to judge TWIST1 protein abundance in each line following hypoxia, clear increases in TWIST1 were observed in MDA-MB-231 and BT-549 cells but, paralleling the Q-PCR results, no change in TWIST1 was protein was observed in SKBr3 and MDA-MB-468 cells cultured under 1% O2 (Fig 6B). Of note, no expression of TRIM29 was observed in either MDA-MB-231 or BT-549 cells cultured under low oxygen conditions, indicating that hypoxic response could not reverse transcriptional silencing of the TRIM29 gene in these lines.
Finally, we examined the role that TRIM29 plays in modulating TWIST1 abundance in response to hypoxia. To conduct this experiment we engineered SKBr3 cells to express reduced TRIM29 by using either gene editing (ie, CRISPR/Cas9) or RNAi (lentiviral shRNA) approaches. Both approaches resulted in markedly diminished basal expression of TRIM29 (Fig 6C). When TWIST1 transcript was examined in these lines we measured a ~2-fold increase in TWIST1 mRNA in the TRIM29-depleted lines (Fig 6D), consistent with previous work (27). Moreover, when TRIM29-depleted lines were cultured under low-oxygen conditions we observed a further increase in TWIST1 mRNA but this response was absent in the control line. Parallel results were observed when TWIST1 protein abundance was examined in these lines by immunoblotting (Fig 6E). Taken together, these findings clearly indicate that TRIM29 is necessary to block the upregulation of the TWIST1 oncogene in response to hypoxic stress.

DISCUSSION

We originally identified TRIM29 as a gene whose expression was downregulated following ATM knockdown. Work outlined in this report has confirmed and extended this finding by documenting that not only is basal TRIM29 expression ATM-dependent, but that TRIM29 is also responsive to hypoxic stress through an ATM/HIF1 signaling pathway. ATM and RelA knockdown significantly reduced the basal level of HIF1α and, along with HIF1α knockdown, reduced TRIM29 mRNA levels. Taken together, these findings suggest that reduced ATM and RelA activity lead to diminished basal HIF1 activity that, in turn, lowers basal TRIM29 expression. While low oxygen conditions dramatically increase HIF1 activity by forcing stabilization of the labile HIF1α protein, HIF1 transcriptional activity can be activated via mechanisms independent of oxygen tension (43). For example, Zundel et al demonstrated that Akt signaling can induce stabilization of HIF1α (44), and Lu and colleagues showed that the glucose metabolites pyruvate and oxaloacetate block HIF1α degradation (45). Others showed that reactive oxygen species (ROS) generated in response to hypoxia (46,47) activate HIF1 by promoting the oxidation of Fe(II), a necessary cofactor for oxygen-dependent proline hydroxylases (PHDs), to Fe(III) and thus promoting reduced turnover of the labile HIF1α protein (48). Of note, ROS activates ATM through oxidation of an internal cysteine residue that, in turn, promotes disulfide bridge formation and ATM dimerization that activates ATM kinase activity (8). We have previously documented that culturing cells on the antioxidant N-acetyl cysteine (NAC) decreases ATM-dependent activation of NF-κB (40) and, moreover, we show here that HIF1α and TRIM29 transcript levels are sensitive to antioxidant treatment in both SKBr3 and MDA-MB-468 cells. These findings suggest that ROS can promote basal HIF1 activity and this, in turn, drives basal TRIM29 expression.

Cam et al (11) observed that ATM-deficiency severely attenuated HIF1α stabilization and upregulation of the hypoxia-inducible REDD1 gene. Moreover, these investigators discovered that hypoxia results in phosphorylation of HIF1α at the Ser696 residue, a consensus phosphorylation site for ATM. Similarly, we observed that ATM knockdown blunted the accumulation of HIF1α and TRIM29 in response to hypoxia. As HIF1α levels in response to hypoxia are principally controlled at the post-transcriptional level (34,49,50 and see Fig 4G), our findings support the proposal of Cam and colleagues that ATM-dependent phosphorylation of HIF1α functions in stabilizing HIF1α and promoting HIF1 activity.

In sum, our findings argue that that ATM impacts HIF1α and HIF1 activity via two distinct mechanisms (Fig 7). First, under normoxic conditions ATM promotes basal HIF1 activity by supporting NF-κB activity and downstream transactivation of the HIF1α gene. Second, ATM is required for hypoxia-induced accumulation of the HIF1α protein and HIF1-induced transcription of the TRIM29 gene. While our study further supports an important role for ATM in response to low oxygen conditions, understanding the full spectrum of ATM-dependent effects on hypoxia response awaits further clarification. Further, although an attractive notion, it also remains unknown if response to increased oxidative radicals drives ATM activation during hypoxic response.
Response to low oxygen tension within a growing tumor promotes a range of responses that are pro-tumorigenic and pro-metastatic in nature (12). For example, HIF1 stimulates upregulation of factors that promote neo-vascularization, such as VEGF and SDF-1 (51,52). Similarly, increased TRIM29 expression is associated with more aggressive forms of disease including bladder (17), colorectal (18), gastric (19), lung (20,21), and pancreatic cancer (22,53). In contrast, Liu et al (26) and our group (27) observed that TRIM29 reduces cell growth, motility, and invasiveness in cultured breast cancer and non-tumorigenic mammary epithelial cell lines, and low expression was associated with poor patient outcomes. These findings strongly suggest that TRIM29 can act either as an oncogene or a tumor suppressor depending on tumor origin. Here we show that TRIM29 blocks the hypoxia-induced upregulation of TWIST1 in breast tumor lines, thus providing a clear tumor suppressive mechanism for the TRIM29 protein in this cell type during response to hypoxic stress. It awaits further clarification if tumor hypoxia is the mechanism that drives increased TRIM29 expression observed in other tumor types.

ATM plays a critical role in the maintenance of genome integrity and is a well-established tumor suppressor. Epidemiologic studies on female obligate ATM heterozygotes documented significantly higher breast cancer incidence rates over controls (7,54,55). Recent synopses of these studies calculated a relative risk (RR) of 2.5 for breast cancer in these individuals (5,56), and several groups have uncovered mutations in ATM associated with increased familial breast cancer risk (57-61). Despite the general lack of truncating ATM mutations in sporadic breast cancer (62-66), several studies (67,68) including one from our group (69) documented that ATM expression is commonly (30-50% of tumors examined) diminished in sporadic breast tumors. In this present study, we have demonstrated that expression of the TRIM29 tumor suppressor gene is controlled through an ATM-dependent mechanism. This provides strong evidence for a previously unknown ATM-dependent signaling pathway in breast cancer suppression.

Finally, this work highlights an intriguing and somewhat paradoxical aspect for ATM function in breast tumorigenesis; specifically, why is the ATM tumor suppressor important in the activation of two prominent oncogenic pathways, namely NF-κB and HIF1, in breast cancer cells? The answer to this question appears to be that while ATM is necessary for maintaining genomic integrity by activating cell cycle checkpoints and DNA repair (1), ATM also functions in parallel to activate survival signaling in response to cellular insults. For example, ATM-dependent activation of NF-κB and AKT in response to ionizing radiation are essential pro-survival mechanisms triggered following DNA damage (70-72). Similarly, as we and others (11) have shown, ATM is required to activate HIF1, a critical adaptive response key to survival in low oxygen environments (73). Thus, while ATM function is certainly crucial in limiting cancer development arising from genotoxic insult, ATM also activates some prominent oncogenic mechanisms during its role in promoting cellular survival to this and other stressors. Moreover, activation of ATM stemming from the chronic presence of stressors, such as elevated ROS, can negatively impact breast cancer behavior (40). In sum, evidence supports the notion that ATM can exert both positive and negative effects on breast tumorigenesis, making this a compelling molecule for further study.

EXPERIMENTAL PROCEDURES

Cell Culture and Drug Treatment

SKBr3 and MDA-MB-468 cell lines were obtained from ATCC (Manassas, VA) and hTERT-immortalized human mammary epithelial cells (HMEC) were the generous gift of Dr. Jianrong Lu (Univ. of Florida). All lines were cultured using established protocols (27) at 37°C in a humidified 5% CO2 environment. Where indicated, cells were exposed to 10 Gy of γ-radiation using a 137Cs source irradiator (Gammacell 1000).

Cells were exposed to hypoxic conditions by placing in a sealed chamber and purging the atmosphere with gas containing 1% O2, 5% CO2, and 94% N2. After purging, the chamber was placed in a 37°C incubator for 18 hr before cell harvest and subsequent analysis. Where indicated, cycloheximide (final concentration = 10 μg/ml) or actinomycin D (final concentration = 1.0 μg/ml) were added to the tissue culture media immediately prior to placement in the hypoxia
TRIM29 expression is ATM-dependent

chamber. Both cycloheximide and actinomycin D were purchased from Sigma-Aldrich (St. Louis, MO) and stocks stored at -20°C prior to use.

RT-PCR and Q-PCR Analysis

Total RNA was isolated from cultured cells using TRI Reagent (Ambion, Austin, TX) per manufacturer’s instructions. 2 µg of RNA was then used in 20 µl first strand reactions with the High Capacity RNA-to-cDNA Kit (Life Technologies, Grand Island, NY). For Q-PCR a 15 µl reaction was assembled containing 1.0 µl of cDNA reaction, 1.0 µl of forward and reverse primers (5 µM each), and 7.5 µl of SYBR Green master mix (Applied Biosystems, Norwalk, CT). PCR was carried out in an Applied Biosystems StepOnePlus thermocycler, fold changes in transcript abundance were calculated by the 2-ΔΔCt method (28) using GAPDH as the internal standard. For RT-PCR, reactions were subjected to agarose gel electrophoresis, stained with ethidium bromide, and photographed. Primers used for RT-PCR and Q-PCR are listed in Table I.

Immunoblot Analysis

SDS-PAGE and immunoblotting was performed using established protocols (29). Nitrocellulose membranes were probed with anti-TRIM29 (sc-33151; Santa Cruz Biotechnology, Santa Cruz, CA), TWIST1 and VHL (GTX12310 and GTX101087, respectively; GeneTex, Irvine, CA), γH2AX (#9718, Cell Signaling Technologies, Danvers, MA), HIF1α (NB100-499; Novus Biologicals, Littleton, CO), CAIX (a gift from Dr. Susan Frost, Dept of Biochem and Mol. Biol., Univ of Florida). To assure even protein loading, parallel blots were probed with anti-Actin (sc-47778; Santa Cruz Biotechnology,) or Tubulin (E7) obtained from the Developmental Studies Hybridoma Bank (Univ. of Iowa). Immunoblot signals were developed using chemiluminescence and recorded on X-Ray film. Where indicated, immunoblot signals were quantified using ImageJ.

shRNA gene knockdown

For RNAi-mediated gene knockdown, shRNA lentiviral vectors were obtained from a human shRNA library (Open Biosystems) maintained by the UF Health Cancer Center. ATM, p65 (RelA), and TRIM29 shRNA constructs were previously described (27,30). For HIF1α, two independent pLKO.1 plasmids encoding HIF1α-specific shRNA inserts were used (TRCN0000010819 (clone #2) and TRCN0000003810 (clone #3)). For VHL knockdown constructs TRCN000039624 (clone #2) and TRCN0000039625 (clone #3) were used. Lentivirus encoding shRNA or pLKO.1 vector containing non-specific shRNA insert sequence were packaged in HEK-293FT cells (ATCC, Manassas, VA) by co-transfection with the packaging plasmids psPAX2 and pMD2.G (Addgene, Cambridge, MA). Two days after transfection spent medium was collected, polybrene (4 µg/ml final conc.) added, and applied to cultured cells. Selection with 2 µg/ml puromycin was conducted for approximately 2-3 weeks prior to analysis of the resultant polyclonal cell populations.

Gene editing (CRISPR/Cas9)

The endogenous TRIM29 gene was mutated in SKBr3 cells using the lentCRISPRv2 plasmid (Addgene #52961) previously described by the Zhang lab (31). Briefly, using an online design tool (http://crispr.mit.edu), a guide RNA (designated guide-13) within the open reading frame of TRIM29 exon 1 (nt# +664-683; transcriptional start site = nt#1) was chosen based on the location of a PAM site (5’-TGG-3’) within the TRIM29 gene and the low predicted probability of off-site targeting within the human genome. Partially complementary oligonucleotides (Forw: 5’-CACCGGCCCGCAAGTGTCCCGTGCA-3’; Rev: 5’-AAA CTGCAAGGGACACTTGCGGC-3’) were annealed and resultant double-stranded DNA was ligated into BsmBI-digested lentCRISPRv2 as instructed (Addgene). Recombinant clones were identified by restriction mapping and confirmed by automated Sanger sequencing. Lentivirus were created using the TRIM29 guide-13 plasmid or empty lentCRISPRv2 as outlined above, and SKBr3 were transduced with these viruses and selected using puromycin for three weeks at which point polyclonal populations of stably transduced cells were harvested and tested for TRIM29 expression by immunoblotting.

Acknowledgements: We thank Dr. Mary Law (Dept of Pharmacology and Therapeutics, Univ. of Florida) for the kind gift of Ad-p65 and Ad-GFP viral stocks. M.D. was supported by a research fellowship from the Scientific and Technological...
TRIM29 expression is ATM-dependent

Research Council of Turkey (TUBITAK). This work was supported by funding from the NIH (R03 CA125824), the Florida Department of Health, and the Violet Brownhill Trust (to KDB).

Conflict of Interests: The authors declare that they have no conflicts of interest with the contents of this article.

Footnote: The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author contributions: MD, WSS, TCCT, LA, LRFB, and KDB designed the study, KDB wrote and WSM edited the manuscript. MD conducted all experiments except Figs 4E, 5E and 5F which were conducted by WSS and TCCT, Fig 4F was conducted by KDB, Fig 5G that was conducted by LA, and Fig 5H which was conducted by LRFB. All authors analyzed the results and approved the final version of the manuscript.

REFERENCES

1. Shiloh, Y. (2003) ATM and related protein kinases: safeguarding genome integrity. Nat Rev Cancer 3, 155-168

2. Xu, Y., Ashley, T., Brainerd, E. E., Bronson, R. T., Meyn, M. S., and Baltimore, D. (1996) Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, immune defects, and thymic lymphoma. Genes Dev 10, 2411-2422.

3. Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., Shiloh, Y., Crawley, J. N., Reid, T., Tagle, D., and Wynshaw-Boris, A. (1996) Atm-deficient mice: a paradigm of ataxia telangiectasia. Cell 86, 159-171

4. Taylor, A. M., Metcalfe, J. A., Thick, J., and Mak, Y. F. (1996) Leukemia and lymphoma in ataxia telangiectasia. Blood 87, 423-438

5. Renwick, A., Thompson, D., Seal, S., Kelly, P., Chaghtai, T., Ahmed, M., North, B., Jayatilake, H., Barfoot, R., Spanova, K., McGuffog, L., Evans, D. G., Eccles, D., Easton, D. F., Stratton, M. R., and Rahman, N. (2006) ATM mutations that cause ataxia-telangiectasia are breast cancer susceptibility alleles. Nat Genet 38, 873-875

6. Stankovic, T., Kidd, A. M., Sutcliffe, A., McGuire, G. M., Robinson, P., Weber, P., Bedenham, T., Bradwell, A. R., Easton, D. F., Lennox, G. G., Haites, N., Byrd, P. J., and Taylor, A. M. (1998) ATM mutations and phenotypes in ataxia-telangiectasia families in the British Isles: expression of mutant ATM and the risk of leukemia, lymphoma, and breast cancer. Am J Hum Genet 62, 334-345.

7. Swift, M., Morrell, D., Massey, R. B., and Chase, C. L. (1991) Incidence of cancer in 161 families affected by ataxia-telangiectasia. N Engl J Med 325, 1831-1836.

8. Guo, Z., Kozlov, S., Lavin, M. F., Person, M. D., and Paul, T. T. (2010) ATM activation by oxidative stress. Science 330, 517-521

9. Gibson, S. L., Bindra, R. S., and Glazer, P. M. (2005) Hypoxia-induced phosphorylation of Chk2 in an ataxia telangiectasia mutated-dependent manner. Cancer Res 65, 10734-10741

10. Freiberg, R. A., Hammond, E. M., Dorie, M. J., Welford, S. M., and Giaccia, A. J. (2006) DNA damage during reoxygenation elicits a Chk2-dependent checkpoint response. Mol Cell Biol 26, 1598-1609

11. Cam, H., Easton, J. B., High, A., and Houghton, P. J. (2010) mTORC1 signaling under hypoxic conditions is controlled by ATM-dependent phosphorylation of HIF-1alpha. Mol Cell 40, 509-520

12. Semenza, G. L. (2010) Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene 29, 625-634

13. Bencokova, Z., Kaufmann, M. R., Pires, I. M., Lecane, P. S., Giaccia, A. J., and Hammond, E. M. (2009) ATM activation and signaling under hypoxic conditions. Mol Cell Biol 29, 526-537

14. Chan, D. A., and Giaccia, A. J. (2007) Hypoxia, gene expression, and metastasis. Cancer Metastasis Rev 26, 333-339

15. Gilkes, D. M., and Semenza, G. L. (2013) Role of hypoxia-inducible factors in breast cancer metastasis. Future Oncol 9, 1623-1636

16. Hatakeyama, S. (2011) TRIM proteins and cancer. Nat Rev Cancer 11, 792-804

17. Fristrup, N., Birkenkamp-Demtroder, K., Reinert, T., Sanchez-Carbajo, M., Segersten, U., Malmstrom, P. U., Palou, J., Alvarez-Mugica, M., Pan, C. C., Ulhøi, B. P., Borre,
TRIM29 expression is ATM-dependent

M., Orntoft, T. F., and Dyrskjot, L. (2013) Multicenter validation of cyclin D1, MCM7, TRIM29, and UBE2C as prognostic protein markers in non-muscle-invasive bladder cancer. *Am J Pathol* **182**, 339-349

18. Glebov, O. K., Rodriguez, L. M., Soballe, P., DeNobile, J., Cliatt, J., Nakahara, K., and Kirsch, I. R. (2006) Gene expression patterns distinguish colonoscopically isolated human aberrant crypt foci from normal colonic mucosa. *Cancer Epidemiol Biomarkers Prev* **15**, 2253-2262

19. Kosaka, Y., Inoue, H., Ohmachi, T., Yokoe, T., Matsumoto, T., Mimori, K., Tanaka, F., Watanabe, M., and Mori, M. (2007) Tripartite motif-containing 29 (TRIM29) is a novel marker for lymph node metastasis in gastric cancer. *Ann Surg Oncol* **14**, 2543-2549

20. Tang, Z. P., Dong, Q. Z., Cui, Q. Z., Papavassiliou, P., Wang, E. D., and Wang, E. H. (2013) Ataxia-telangiectasia group D complementing gene (ATDC) promotes lung cancer cell proliferation by activating NF-kappaB pathway. *PLoS One* **8**, e63676

21. Xiao, Z., Jiang, Q., Willette-Brown, J., Xi, S., Zhu, F., Burkett, S., Back, T., Song, N. Y., Datla, M., Sun, Z., Goldszmid, R., Lin, F., Cohoon, T., Pike, K., Wu, X., Schrump, D. S., Wong, K. K., Young, H. A., Trinchieri, G., Wiltrout, R. H., and Hu, Y. (2013) The pivotal role of IKKalpha in the development of spontaneous lung squamous cell carcinomas. *Cancer Cell* **23**, 527-540

22. Iacobuzio-Donahue, C. A., Ashfaq, R., Maitra, A., Adsay, N. V., Shen-Ong, G. L., Berg, K., Hollingsworth, M. A., Cameron, J. L., Yeo, C. J., Kern, S. E., Goggins, M., and Hruban, R. H. (2003) Highly expressed genes in pancreatic ductal adenocarcinomas: a comprehensive characterization and comparison of the transcription profiles obtained from three major technologies. *Cancer Res* **63**, 8614-8622

23. Wang, L., Heidt, D. G., Lee, C. J., Yang, H., Logsdon, C. D., Zhang, L., Fearon, E. R., Ljungman, M., and Simeone, D. M. (2009) Oncogenic function of ATDC in pancreatic cancer through Wnt pathway activation and beta-catenin stabilization. *Cancer Cell* **15**, 207-219

24. Masuda, Y., Takahashi, H., Sato, S., Tomomori-Sato, C., Saraf, A., Washburn, M. P., Florens, L., Conaway, R. C., Conaway, J. W., and Hatakeyama, S. (2015) TRIM29 regulates the assembly of DNA repair proteins into damaged chromatin. *Nature communications* **6**, 7299

25. Hosoi, Y., Kapp, L. N., Murnane, J. P., Matsumoto, Y., Enomoto, A., Ono, T., and Miyagawa, K. (2006) Suppression of anchorage-independent growth by expression of the ataxia-telangiectasia group D complementing gene, ATDC. *Biochem Biophys Res Commun* **348**, 728-734

26. Liu, J., Welm, B., Boucher, K. M., Ebbert, M. T., and Bernard, P. S. (2012) TRIM29 functions as a tumor suppressor in nontumorigenic breast cells and invasive ER+ breast cancer. *Am J Pathol* **180**, 839-847

27. Ai, L., Kim, W. J., Alpay, M., Tang, M., Pardo, C. E., Hatakeyama, S., May, W. S., Kladde, M. P., Helderson, C. D., Siegel, E. M., and Brown, K. D. (2014) TRIM29 suppresses TWIST1 and invasive breast cancer behavior. *Cancer Res* **74**, 4875-4887

28. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408

29. Ai, L., Kim, W. J., Demircan, B., Dyer, L. M., Bray, K. J., Skehan, R. R., Massoll, N. A., and Brown, K. D. (2008) The transglutaminase 2 gene (TGM2), a potential molecular marker for chemotherapeutic drug sensitivity, is epigenetically silenced in breast cancer. *Carcinogenesis* **29**, 510-518

30. Ai, L., Skehan, R. R., Saydi, J., Lin, T., and Brown, K. D. (2012) Ataxia-Telangiectasia, Mutated (ATM)/Nuclear Factor kappa light chain enhancer of activated B cells (NFkappaB) signaling controls basal and DNA damage-induced transglutaminase 2 expression. *J Biol Chem* **287**, 18330-18341

31. Sanjana, N. E., Shalem, O., and Zhang, F. (2014) Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods* **11**, 783-784
32. Pilch, D. R., Sedelnikova, O. A., Redon, C., Celeste, A., Nussenzweig, A., and Bonner, W. M. (2003) Characteristics of gamma-H2AX foci at DNA double-strand breaks sites. Biochem Cell Biol 81, 123-129

33. Lee, J. H., and Paull, T. T. (2005) ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. Science 308, 551-554

34. Huang, L. E., Gu, J., Schau, M., and Bunn, H. F. (1998) Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A 95, 7987-7992

35. Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., von Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 292, 468-472

36. Iwai, K., Yamanaka, K., Kamura, T., Minato, N., Conaway, R. C., Conaway, J. W., Klausner, R. D., and Pause, A. (1999) Identification of the von Hippel-lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. Proc Natl Acad Sci U S A 96, 12436-12441

37. Kim, W., and Kaelin, W. G., Jr. (2003) The von Hippel-Lindau tumor suppressor protein: new insights into oxygen sensing and cancer. Curr Opin Genet Dev 13, 55-60

38. Belaiba, R. S., Bonello, S., Zahringer, C., Schmidt, S., Hess, J., Kietzmann, T., and Gorlach, A. (2007) Hypoxia up-regulates hypoxia-inducible factor-1alpha transcription by involving phosphatidylinositol 3-kinase and nuclear factor kappaB in pulmonary artery smooth muscle cells. Mol Biol Cell 18, 4691-4697

39. van Uden, P., Kenneth, N. S., and Rocha, S. (2008) Regulation of hypoxia-inducible factor-1alpha by NF-kappaB. Biochem J 412, 477-484

40. Alpay, M., Backman, L. R., Cheng, X., Dukel, M., Kim, W. J., Ai, L., and Brown, K. D. (2015) Oxidative stress shapes breast cancer phenotype through chronic activation of ATM-dependent signaling. Breast Cancer Res Treat 151, 75-87

41. Pahl, H. L. (1999) Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 18, 6853-6866

42. Yang, M. H., Wu, M. Z., Chiou, S. H., Chen, P. M., Chang, S. Y., Liu, C. J., Teng, S. C., and Wu, K. J. (2008) Direct regulation of TWIST by HIF-1alpha promotes metastasis. Nat Cell Biol 10, 295-305

43. Denko, N. C. (2008) Hypoxia, HIF1 and glucose metabolism in the solid tumour. Nat Rev Cancer 8, 705-713

44. Zundel, W., Schindler, C., Haas-Kogan, D., Koong, A., Kaper, F., Chen, E., Gottschalk, A. R., Ryan, H. E., Johnson, R. S., Jefferson, A. B., Stokoe, D., and Giaccia, A. J. (2000) Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev 14, 391-396

45. Lu, H., Dalgard, C. L., Mohyeldin, A., MeFate, T., Tait, A. S., and Verma, A. (2005) Reversible inactivation of HIF-1 prolyl hydroxylases allows cell metabolism to control basal HIF-1. J Biol Chem 280, 41928-41939

46. Chandel, N. S., Maltepe, E., Goldwasser, E., Mathieu, C. E., Simon, M. C., and Schumacker, P. T. (1998) Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc Natl Acad Sci U S A 95, 11715-11720

47. Chandel, N. S., McClintock, D. S., Feliciano, C. E., Wood, T. M., Melendez, J. A., Rodriguez, A. M., and Schumacker, P. T. (2000) Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing. J Biol Chem 275, 25130-25138

48. Gerald, D., Berra, E., Frapart, Y. M., Chan, D. A., Giaccia, A. J., Mansuy, D., Pouyssegur, J., Yaniv, M., and Mecha-Grigoriou, F. (2004) JunD reduces tumor angiogenesis by protecting cells from oxidative stress. Cell 118, 781-794

49. Salceda, S., and Caro, J. (1997) Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome...
system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem 272, 22642-22647

50. Kallio, P. J., Wilson, W. J., O'Brien, S., Makino, Y., and Poellinger, L. (1999) Regulation of the hypoxia-inducible transcription factor 1alpha by the ubiquitin-proteasome pathway. J Biol Chem 274, 6519-6525

51. Buchler, P., Reber, H. A., Buchler, M., Shrinkante, S., Buchler, M. W., Friess, H., Semenza, G. L., and Hines, O. J. (2003) Hypoxia-inducible factor 1 regulates vascular endothelial growth factor expression in human pancreatic cancer. Pancreas 26, 56-64

52. Ceradini, D. J., Kulkarni, A. R., Callaghan, M. J., Tepper, O. M., Bastidas, N., Kleinman, M. E., Capla, J. M., Galiano, R. D., Levine, J. P., and Gurtner, G. C. (2004) Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat Med 10, 858-864

53. Wang, L., Yang, H., Abel, E. V., Ney, G. M., Palmbos, P. L., Bednar, F., Zhang, Y., Leflein, J., Waghray, M., Owens, S., Wilkinson, J. E., Prasad, J., Ljungman, M., Rhim, A. D., Pasca di Magliano, M., and Simeone, D. M. (2015) ATDC induces an invasive switch in KRAS-induced pancreatic tumorigenesis. Genes Dev 29, 171-183

54. Swift, M., Reitnauer, P. J., Morrell, D., and Chase, C. L. (1987) Breast and other cancers in families with ataxia-telangiectasia. N Engl J Med 316, 1289-1294.

55. Pippard, E. C., Hall, A. J., Barker, D. J., and Bridges, B. A. (1988) Cancer in homozygotes and heterozygotes of ataxia-telangiectasia and xeroderma pigmentosum in Britain. Cancer Res 48, 2929-2932.

56. Thompson, D., Duedal, S., Kirner, J., McGuffog, L., Last, J., Reiman, A., Byrd, P., Taylor, M., and Easton, D. F. (2005) Cancer risks and mortality in heterozygous ATM mutation carriers. J Natl Cancer Inst 97, 813-822

57. Bernstein, J. L., Bernstein, L., Thompson, W. D., Lynch, C. F., Malone, K. E., Teitelbaum, S. L., Olsen, J. H., Anton-Culver, H., Boice, J. D., Rosenstein, B. S., Borresen-Dale, A. L., Gatti, R. A., Concannon, P., and Haile, R. W. (2003) ATM variants 7271T>G and IVS10-6T>G among women with unilateral and bilateral breast cancer. Br J Cancer 89, 1513-1516

58. Bernstein, J. L., Teraoka, S., Southey, M. C., Jenkins, M. A., Andrlulis, I. L., Knight, J. A., John, E. M., Lapinski, R., Wolitzer, A. L., Whittemore, A. S., West, D., Seminara, D., Olson, E. R., Spurde, A. B., Chenex-Trench, G., Giles, G. G., Hopper, J. L., and Concannon, P. (2006) Population-based estimates of breast cancer risks associated with ATM gene variants c.7271T>G and c.1066-6T>G (IVS10-6T>G) from the Breast Cancer Family Registry. Hum Mutat 27, 1122-1128

59. Brooks, A., Urbanus, J. H., Floore, A. N., Dahler, E. C., Klijn, J. G., Rutgers, E. J., Devilee, P., Russell, N. S., van Leeuwen, F. E., and van't Veer, L. J. (2000) ATM-heterozygous germline mutations contribute to breast cancer susceptibility. Am J Hum Genet 66, 494-500.

60. Chenex-Trench, G., Spurde, A. B., Gatei, M., Kelly, H., Marsh, A., Chen, X., Donn, K., Cummings, M., Nyholt, D., Jenkins, M. A., Scott, C., Pupo, G. M., Dork, T., Bendix, R., Kirk, J., Tucker, K., McCredie, M. R., Hopper, J. L., Sambrook, J., Mann, G. J., and Khanna, K. K. (2002) Dominant negative ATM mutations in breast cancer families. J Natl Cancer Inst 94, 205-215.

61. Langholz, B., Bernstein, J. L., Bernstein, L., Olsen, J. H., Borresen-Dale, A. L., Rosenstein, B. S., Gatti, R. A., and Concannon, P. (2006) On the proposed association of the ATM variants 5557G>A and IVS38-8T>C and bilateral breast cancer. Int J Cancer 119, 724-725

62. Wooster, R., Ford, D., Mangion, J., Ponder, B. A., Petio, J., Easton, D. F., and Stratton, M. R. (1993) Absence of linkage to the ataxia telangiectasia locus in familial breast cancer. Hum Genet 92, 91-94.

63. Cortessis, V., Ingles, S., Millikan, R., Diep, A., Gatti, R. A., Richardson, L., Thompson, W. D., Pagani-Hill, A., Sparkes, R. S., and Haile, R. W. (1993) Linkage analysis of
DRD2, a marker linked to the ataxia-telangiectasia gene, in 64 families with premenopausal bilateral breast cancer. Cancer Res 53, 5083-5086.

64. Bebb, D. G., Yu, Z., Chen, J., Telatar, M., Gelmon, K., Phillips, N., Gatti, R. A., and Glickman, B. W. (1999) Absence of mutations in the ATM gene in forty-seven cases of sporadic breast cancer. Br J Cancer 80, 1979-1981.

65. Shafman, T. D., Levitz, S., Nixon, A. J., Gibans, L. A., Nichols, K. E., Bell, D. W., Ishioka, C., Isselbacher, K. J., Gelman, R., Garber, J., Harris, J. R., and Haber, D. A. (2000) Prevalence of germline truncating mutations in ATM in women with a second breast cancer after radiation therapy for a contralateral tumor. Genes Chromosomes Cancer 27, 124-129.

66. FitzGerald, M. G., Bean, J. M., Hegde, S. R., Unsal, H., MacDonald, D. J., Harkin, D. P., Finkelstein, D. M., Isselbacher, K. J., Haber, D. A. (1997) Heterozygous ATM mutations do not contribute to early onset of breast cancer. Nature Genetics 15, 307-310.

67. Angele, S., Treilleux, I., Tanieer, P., Martel-Planche, G., Vuillaume, M., Bailly, C., Bremond, A., Montesano, R., and Hall, J. (2000) Abnormal expression of the ATM and TP53 genes in sporadic breast carcinomas. Clin Cancer Res 6, 3536-3544.

68. Kairouz, R., Clarke, R. A., Marr, P. J., Watters, D., Lavin, M. F., Kearsley, J. H., and Lee, C. S. (1999) ATM protein synthesis patterns in sporadic breast cancer. Mol Pathol 52, 252-256.

69. Vo, Q. N., Kim, W. J., Cvitanovic, L., Boudreau, D. A., Ginzinger, D. G., and Brown, K. D. (2004) The ATM gene is a target for epigenetic silencing in locally advanced breast cancer. Oncogene 23, 9432-9437.

70. Rashi-Elkeles, S., Elkon, R., Weizman, N., Linhart, C., Amariglio, N., Sternberg, G., Rechavi, G., Barzilai, A., Shamir, R., and Shiloh, Y. (2006) Parallel induction of ATM-dependent pro- and antiapoptotic signals in response to ionizing radiation in murine lymphoid tissue. Oncogene 25, 1584-1592.

71. Veuger, S. J., and Durkacz, B. W. (2011) Persistence of unrepaired DNA double strand breaks caused by inhibition of ATM does not lead to radio-sensitisation in the absence of NF-kappaB activation. DNA Repair (Amst) 10, 235-244.

72. Golding, S. E., and Valerie, K. (2011) MRE11 and ATM AKTivate pro-survival signaling. Cell Cycle 10, 3227.

73. Ke, Q., and Costa, M. (2006) Hypoxia-inducible factor-1 (HIF-1). Mol Pharmacol 70, 1469-1480.

FIGURE LEGENDS

Table I. Oligonucleotide primers used in this study.

Figure 1. TRIM29 expression is ATM-dependent. A. SKBr3, MDA-MB-468 and HMEC cells were transduced with control lentivirus or virus encoding two independent shRNAs specific for ATM. Following puromycin selection, total RNA was harvested, cDNA synthesized and analyzed by PCR for ATM, TRIM29, TWIST1, and GAPDH (loading control) transcript abundance. B. ATM knockdown and control cells were analyzed by Q-PCR for relative ATM and TRIM29 transcript abundance. C. ATM knockdown and control cells were analyzed for TRIM29, TWIST1 and Tubulin (loading control) protein abundance by immunoblotting. D. SKBr3, MDA-MB-468, and HMEC cells were either untreated or exposed to 10 Gy of ionizing radiation. Extracts were formed 18 hr post-IR and immunoblotted with anti-TRIM29, phosphorylated H2AX (γH2AX), or actin. Q-PCR assays were conducted at least in triplicate, graphed is the mean of all assays conducted. Error bars = SD; ***= P < 0.001; Students T-test.

Figure 2. TRIM29 expression is hypoxia-inducible. A. SKBr3, MDA-MB-468, and HMEC cells were cultured under hypoxic (1% O2) or normoxic conditions for 18 hr, subsequently harvested, total RNA
isolated, and cDNA synthesized. PCR using primers specific for TRIM29, CAIX or GAPDH was subsequently conducted. B. Relative TRIM29 transcript abundance in indicated cell lines was measured by Q-PCR. C. SKBr3, MDA-MB-468, and HMEC cells were cultured in 1% O₂ or normoxic conditions and 18 hr later analyzed by immunoblotting for TRIM29, HIF1α, CAIX, and Actin protein levels. Where indicated, relative immunoblot signal intensity within normoxic/hypoxic pairs is given. Q-PCR assays were conducted at least in triplicate, graphed is the mean of all assays conducted. Error bars = SD; ** = P < 0.001; Students T-test.

Figure 3. Hypoxia-induced upregulation of TRIM29 is ATM-dependent. A. Control and ATM knockdown SKBr3 and MDA-MB-468 cells were either cultured in normoxic (filled bars) or hypoxic (open bars) conditions for 18 hr. After this, total RNA was isolated and relative TRIM29 transcript abundance measured by Q-PCR. B. Control and ATM knockdown SKBr3 and MDA-MB-468 cells cultured in normoxic or hypoxic conditions for 18 hr were analyzed by immunoblotting for TRIM29, HIF1α, and Actin levels. Where indicated, relative immunoblot signal intensity within normoxic/hypoxic pairs is given. Q-PCR assays were conducted at least in triplicate, graphed is the mean of all assays conducted. Error bars = SD; ns = P > 0.05; ** = P < 0.01; Students T-test.

Figure 4. HIF1α is required for TRIM29 upregulation in response to hypoxia. A. SKBr3 or MDA-MB-468 cells were transduced with control lentivirus or virus encoding two independent HIF1α-specific shRNAs (clone shHIF1α-2 and shHIF1α-3). Following puromycin selection total RNA was isolated and analyzed by Q-PCR for relative HIF1α transcript levels. B. Control and HIF1α knockdown SKBr3 and MDA-MB-468 cells were analyzed for HIF1α and TRIM29 mRNA abundance by RT-PCR followed by agarose gel electrophoresis. C. Control and HIF1α knockdown SKBr3 and MDA-MB-468 cells cultured under normoxic and hypoxic conditions and relative TRIM29 transcript abundance was analyzed by Q-PCR. D. TRIM29 and Actin protein levels were probed by immunoblot analysis in control and HIF1α knockdown SKBr3 and MDA-MB-468 cells cultured under normoxic and hypoxic conditions. E. SKBr3 cells were transduced with control lentivirus or virus encoding two independent VHL-specific shRNAs (clone shVHL-2 and shVHL-3). After puromycin selection, cells were harvested, total RNA isolated, and used in RT-PCR reactions using VHL or GAPDH-specific primers. F. Control and VHL knockdown SKBr3 cells were immunoblotted with anti-VHL, anti-HIF1α, anti-TRIM29 or anti-Actin as indicated. G. SKBr3 and MDA-MB-468 cells were either untreated or treated with cycloheximide or actinomycin D prior to placement in a hypoxia chamber or cultured under normoxic conditions. Following an 18 hr incubation cells were harvested, lysates prepared, and immunoblotted with anti-TRIM29, HIF1α, or Actin. Where indicated, relative immunoblot signal intensity within normoxic/hypoxic pairs is given. Q-PCR assays were conducted at least in triplicate, graphed is the mean of all assays conducted. Error bars = SD; ns = P > 0.05; *= P < 0.05; ** = P < 0.01; *** = P < 0.001; Students T-test.

Figure 5. Basal TRIM29 expression is NF-κB-dependent. A. Relative HIF1α transcript levels were measured in control and ATM knockdown SKBr3 and MDA-MB-468 cells by Q-PCR. B. SKBr3 or MDA-MB-468 cells were transduced with control lentivirus or virus encoding two independent RelA-specific shRNAs (clone shRelA-1 and shRelA-2). Following puromycin selection total RNA was isolated and analyzed by PCR with primers specific for RelA, TRIM29, HIF1α, and GAPDH. C. Control and RelA knockdown SKBr3 and MDA-MB-468 cells were analyzed by Q-PCR for relative RelA, HIF1α and TRIM29 transcript abundance. D. Control and RelA knockdown SKBr3 and MDA-MB-468 cells were analyzed by immunoblotting for RelA, TRIM29, and Actin. E. SKBr3 cells were either uninfected (control), infected with adenovirus (MOI=10) encoding full-length human RelA (Ad-p65) or adenovirus created from a control virus (Ad-GFP). 48 hr post-infection cells were harvested and analyzed by RT-PCR with primers specific for RelA, SOD2, TRIM29, HIF1α or GAPDH. F. Q-PCR analysis was conducted on uninfected SKBr3 cells and cells infected with either Ad-p65 or Ad-GFP. Relative TRIM29, HIF1α, and SOD2 transcript abundance was measured. G. Uninfected SKBr3 cells and cells infected with either Ad-p65 or Ad-GFP were immunoblotted with anti-TRIM29, RelA, HIF1α, or
Tubulin. H. SKBr3 or MDA-MB-468 cells were either untreated or treated with 10 mM N-acetyl cysteine (NAC) for 18 hr, total RNA isolated and assayed for either HIF1α or TRIM29 mRNA abundance by Q-PCR. Q-PCR assays were conducted at least in triplicate, graphed is the mean of all assays conducted. Error bars = SD; ns = P > 0.05; *** = P < 0.001, Students T-test.

Figure 6. TRIM29 blocks hypoxia-induced TWIST1 upregulation. A. SKBr3, MDA-MB-468, MDA-MB-231, and BT-549 cells were cultured under normoxic or hypoxic conditions for 18 hr. After this, cells were harvested, total RNA isolated, and subjected to Q-PCR to measure relative levels of CAIX or TWIST1 transcript. B. Cells were treated as outlined in (A) but protein extracts were formed and immunoblotted with anti-TWIST1, TRIM29, or Actin. C. SKBr3 cells were either subjected to CRISPR/Cas9 gene editing to mutate the TRIM29 gene or transduced with control lentivirus or virus encoding a TRIM29-specific shRNA. Following puromycin selection cells were assayed for TRIM29 or actin protein levels by immunoblotting. D. Control SKBr3 or cells with either mutated or knocked down TRIM29 were cultured in normoxic or hypoxic conditions for 18 hr. Following this, cells were analyzed by Q-PCR for relative TWIST1 abundance. E. Cells were treated as outlined in (D) but were analyzed by immunoblotting for TWIST1 and Actin. Q-PCR assays were conducted at least in triplicate, graphed is the mean of all assays conducted. Error bars = SD; ns = P > 0.05; ** = P > 0.01; *** = P < 0.001; Students T-test.

Figure 7. Proposed signaling pathways controlling both basal and hypoxia-induced TRIM29 expression.
| GENE   | FORWARD                      | REVERSE                           |
|--------|------------------------------|-----------------------------------|
| ATM    | 5’-CAGGGTAGTTTAGTGAGGATCTGAG-3’ | 5’-CTATACTGGTGTCATGGGCAAGTAAC-3’  |
| HIF-1α | 5’-CCCTCTGCGCTTGTCTCCAAT-3’   | 5’-TCCACCTCTTGGGCAAGCA-3’         |
| RelA   | 5’-GTGCCGGGTGCCTGACGAAAC-3’   | 5’-GTGGGTCGGCTGAAAGGACT-3’        |
| TRIM29 | 5’-GAGGCGGAGCAGCAGAAGAAT-3’   | 5’-CAAGGACACAAATTCCTGCAGAAAC-3’   |
| TWIST1 | 5’-CAGTCTTACGGAGGCAGCTCAGAC-3’| 5’-CTGGAAACAAAGCTCATCAGGGTCGTC-3’|
| CAIX   | 5’-GTCTGCTTTGGAAGAAATCG-3’    | 5’-AGAGGCTGAGCTGCTTTA            |
| GAPDH  | 5’-ACCCAGAAGACTGTGGATGG-3’    | 5’-TTTCAAGCAGGGATGCCTT-3’         |
| ACTIN  | 5’-GCACAGAGCCTCCTCCT-3’       | 5’-GGTGGTCAACGAGAGGG-3’           |
| SOD2   | 5’-GCTCTTACGGAGCAGCTCAGAC-3’  | 5’-CGTGTCCACACATCAATC-3’          |
| VHL    | 5’-GCTCTTACGGAGCAGCTCAGAC-3’  | 5’-ATCCGTTGATGTCACATGC-3’         |

**TABLE I**

Dukel et al
Figure 1
Dukel et al
Figure 2
Dukel et al
Figure 3
Dukel et al
Figure 4
Dukel et al
Figure 5
Dukel et al
Figure 6
Dukel et al
Figure 7
Dukel et al
The Breast Cancer Tumor Suppressor TRIM29 is Expressed via ATM-Dependent Signaling in Response to Hypoxia
Muzaffer Dükel, W. Scott Streitfeld, Tsz Ching Chloe Tang, Lindsey R. F. Backman, Lingbao Ai, W. Stratford May and Kevin D. Brown

J. Biol. Chem. published online August 17, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M116.730960

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts