Suppression of CHK1 by ETS Family Members Promotes DNA Damage Response Bypass and Tumorigenesis

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ABSTRACT

The ETS family of transcription factors has been repeatedly implicated in tumorigenesis. In prostate cancer, ETS family members, such as ERG, ETV1, ETV4, and ETV5, are frequently overexpressed due to chromosomal translocations, but the molecular mechanisms by which they promote prostate tumorigenesis remain largely undefined. Here, we show that ETS family members, such as ERG and ETV1, directly repress the expression of the checkpoint kinase 1 (CHK1), a key DNA damage response cell-cycle regulator essential for the maintenance of genome integrity. Critically, we find that ERG expression correlates with CHK1 downregulation in human patients and demonstrate that Chk1 heterozygosity promotes the progression of high-grade prostatic intraepithelial neoplasia into prostatic invasive carcinoma in Pten+/− mice. Importantly, CHK1 downregulation sensitizes prostate tumor cells to etoposide but not to docetaxel treatment. Thus, we identify CHK1 as a key functional target of the ETS proto-oncogenic family with important therapeutic implications.

SIGNIFICANCE: Genetic translocation and aberrant expression of ETS family members is a common event in different types of human tumors. Here, we show that through the transcriptional repression of CHK1, ETS factors may favor DNA damage accumulation and consequent genetic instability in proliferating cells. Importantly, our findings provide a rationale for testing DNA replication inhibitor agents in ETS-positive TP53-proficient tumors. Cancer Discov; 5(5), 550–63. ©2015 AACR.
INTRODUCTION

The complex network of pathways known as DDR (DNA damage response) orchestrates the cellular response to damaged DNA (1, 2). One consequence of activation of these pathways is the induction of cell-cycle checkpoints that halt proliferation to repair the damaged DNA, or, if the damage is beyond repair, trigger apoptosis (3). A significant number of studies have demonstrated that derangement of the normal function of members of the DDR leads to unrepaired or misrepaired DNA, and consequent genomic instability, which is often a prelude to cancer (1, 3).

Checkpoint kinase 1 (CHK1) is a key regulator of the DDR best known for its involvement in various cell-cycle checkpoints and its monitoring of DNA integrity during replication (4–11). Loss of heterozygosity of 11q24, where CHK1 is located, has been reported along with frame-shift mutations in the CHK1 gene in genetically unstable stomach, colorectal, breast, colon, and endometrial cancers (12–16), whereas overexpression of PPM1D in breast cancer and other tumor types has been proposed to contribute to tumorigenesis by inappropriate inactivation of CHK1 (17). Variations of CHK1 mRNA and protein levels have been detected in lymphoid neoplasms in the absence of gene mutations, deletions, or promoter hypermethylation, suggesting the possibility of transcriptional and/or posttranscriptional downregulation mechanisms (18).

ERG is overexpressed in 50% of human prostate cancers as a result of chromosomal rearrangements involving mainly the TMPRSS2 and ERG genes (3, 19–23). Several studies have to some extent defined possible mechanisms by which ERG may promote prostate tumorigenesis (19–21, 24–27). We have previously shown that overexpression of ERG in combination with Pten heterozygosity in the mouse prostate converts Pten haploinsufficiency-dependent high-grade prostatic intraepithelial neoplasia (HGPIN) to invasive adenocarcinoma through the upregulation of genes involved in cell migration such as Adamts1 and Ccrr4 (24). Microarray analysis has likewise demonstrated that a significant number of genes involved in various pathways are deregulated by excess amounts of ERG (19–21, 24–27); however, the relationship of these genes to ERG-dependent oncogenesis is still unclear, and their in vivo genetic validation remains to be addressed.

RESULTS

CHK1 Is Downregulated upon ERG or ETV1 Induction

Like most of the transcription factors, ERG functions as an activator or a repressor on different target genes. Accordingly, microarray analysis from HEK-293 cells overexpressing
ERG (24) revealed downregulated expression of a number of interesting genes, including CHK1 (Supplementary Fig. S1A), which may represent a potential new link between ERG, DNA replication stress, DDR, and tumorigenesis. CHK1 downregulation was further confirmed using qRT-PCR and Western blot analysis in additional cell lines (Fig. 1A and Supplementary Fig. S1B) and in additional cell lines overexpressing exogenous ERG (Fig. 1B and Supplementary Fig. S1C). To test the ability of ERG to downregulate CHK1 endogenously, we treated VCaP cells (a prostate cell line characterized by the endogenous TMPRSS2–ERG translocation) with 10 nmol/L dihydrotestosterone (DHT) for 24 and 48 hours. qRT-PCR (Fig. 1C) and Western blot (Fig. 1D) data clearly showed a progressive downregulation of CHK1 with increasing amounts of ERG, whereas CHK2 levels remained unchanged (Supplementary Fig. S1D). Analysis of a publicly available expression profile dataset of VCaP cells (either untreated or treated with 10 nmol/L DHT for 16 hours) confirmed CHK1 downregulation upon ERG induction (Supplementary Fig. S1E; ref. 22). Importantly, expression profile data analysis of ERG-negative (ERG−, n = 57) and ERG-positive (ERG+, n = 47) human low Gleason untreated primary prostate cancer presented in the Taylor dataset (23) clearly shows a statistically significant reduction of CHK1 transcript in ERG+ compared with ERG− prostate cancer (Fig. 1E), which inversely correlates with the amount of ERG transcript (Fig. 1F).

Because of the many caveats to be considered in analyzing most of the publicly available expression profile datasets, to further investigate the status and a possible correlation of ERG and CHK1 in human prostate cancer, we created a highly controlled tissue microarray (TMA) dataset with matched clinical information, including 130 biopsied specimens from prostatectomized human primary prostate cancer, and stained serial sections for ERG or CHK1. ERG and CHK1 staining was scored independently on 89 specimens as a percentage of tumor cells expressing the marker. The histopathologic analysis defined a significant inverse correlation between ERG and CHK1 expression in human primary prostate cancer (Supplementary Fig. S1F), fully confirming the expression profile analysis (Fig. 1E and F).

Because ERG expression in human prostate cancer depends on androgen receptor (AR) activity, we overexpressed AR in HEK-293 cells, either untreated or treated with 10 nmol/L DHT for 24 hours, to exclude a possible direct role of AR in CHK1 transcriptional repression. No sizable differences in CHK1 levels were detected between control and AR-overexpressing cells (Supplementary Fig. S2A). Next, we silenced ERG in VCaP cells untreated or treated with 10 nmol/L DHT for 24 hours. As shown in Fig. 1G, ERG knockdown consistently increased CHK1 levels in untreated conditions (Fig. 1G, line 1 compared with lines 3 and 5), whereas, unexpectedly, DHT treatment increased the CHK1 amount in ERG-silenced VCaP cells instead of decreasing it (Fig. 1G, lines 1–2 compared with 3–4 and 5–6). Finally, we treated a panel of AR-proficient or AR-deficient prostate cell lines (VCAp, LNCaP, RWPE1, 22Rv1, and PC3) with 10 nmol/L DHT for 24 hours (Fig. 1H and Supplementary Fig. S2B). As expected, VCaP cells showed a robust induction of ERG and a consequent downregulation of CHK1 (Fig. 1H). Importantly, CHK1 levels remained unchanged in ERG-negative, AR-proficient (RWPE1 and 22Rv1 cells) (Fig. 1H and Supplementary Fig. S2B), as well as in ERG-negative, AR-null PC3 prostate cell lines (Fig. 1H).

Intriguingly, however, CHK1 downregulation was observed at the protein and RNA level in ERG-negative AR-proficient LNCaP cells (Fig. 1H and Supplementary Fig. S2B), as well as in ERG-negative, AR-null PC3 prostate cell lines (Fig. 1H).

To identify the ERG binding sites on the CHK1 promoter, we took advantage of a recently published chromatin immunoprecipitation sequencing (ChIP-seq) dataset mapping, genome-wide, the ERG binding sites and the putative ERG-regulated genes in VCaP cells (26). Supporting our thesis, this dataset lists two different sequences belonging to the CHK1 promoter, both immediately upstream of the ATG (ERG 25631, ERG 25632; Supplementary Fig. S3A; ref. 33), which prompted us to clone and study the first 1,290 nucleotides of the regulatory region of the human CHK1 gene (Fig. 2A; Supplementary Fig. S3B). A luciferase assay in HEK-293T cells clearly showed the ability of ERG to repress the CHK1 promoter in a dose-dependent manner (Fig. 2A). Through an in silico analysis, we identified a total of 20 core motif consensus DNA-binding sites GGA(A/T) for ETS factors spread along the 1290-bp sequence of the CHK1 promoter, suggesting 20 putative ERG binding sites (red bars in Fig. 2A; highlighted in red in Supplementary Fig. S3B). Among them, according to Nihili and colleagues’ study (34), only two (positions −1050 bp and −314 bp) were characterized by strong flanking
Figure 1. CHK1 downregulation upon ERG overexpression. A, qRT-PCR and Western blot analyses showing reduction in CHK1 levels in response to overexpression of ERG in HEK-293 cells. B, CHK1 mRNA and protein downregulation 24 hours following overexpression of ERG in the indicated cell lines. C, increasing amount of ERG transcript in the VCaP cells inversely correlates with CHK1 mRNA levels after 24 and 48 hours of DHT (10 nmol/L) treatment. D, ERG and CHK1 protein levels in samples described in C. E, box plot showing mean, SD, and range of z-scores of CHK1 expression in normal prostate tissue (N), ERG-positive (ERG+), or ERG-negative (ERG–) prostate tumors. P < 0.05 calculated using two-tailed t test. F, dot plot of CHK1 mRNA expression versus ERG mRNA expression. Each dot represents an individual patient. A blue line shows the linear regression of the data. The slope of the line is significantly nonzero (r = –0.22, P = 0.006). Error bars in bar graphs, mean ± SD. G, Western blot analysis of ERG, CHK1, and PSA in VCaP cells transduced with two independent shRNAs targeting ERG and treated for 24 hours with 10 nmol/L DHT. VCaP cells transduced with a scramble shRNA sequence were used as control. H, Western blot analysis of ERG, CHK1, AR, and PSA in VCaP, LNCaP, PC3, and RWPE1 prostate cell lines after 24 hours of DHT (10 nmol/L) treatment. GAPDH was used as loading control. Error bars, mean ± SD. Experiments were performed in triplicate; data were analyzed using unpaired t test. Values of P < 0.05 were considered statistically significant (*, P < 0.05; **, P < 0.01).
**Figure 2.** ERG binds the CHK1 promoter and represses its transcription. **A,** left, luciferase reporter assay construct showing the 20 core consensus sequences GGA(A/T) for ERG binding (red bars). Asterisks, the most probable ERG binding sites according to Nhili et al. (34). Purple arrows, two regions of CHK1 promoter identified by Yu and colleagues in a ChIP-seq analysis for ERG (26). Right, ERG represses CHK1 regulatory region activity in a dose-dependent manner. ERG expression levels are shown in the bottom panel. WT, wild-type. **B,** left, mutational scheme of the luciferase constructs F10, F11, and F10/11 with mutagenized ERG binding sites indicated by red asterisks. Right, dose-dependent ERG repression of both the wild-type and mutant CHK1 regulatory region. **C,** ChIP assay from VCaP prostate cell lines both untreated and treated with DHT (10 nmol/L) for 24 hours, showing levels of direct binding of endogenous ERG in specific sites of the promoter region of human CHK1. PLA1A promoter was used as positive control (19). Error bars, mean ± SD. **D,** ChIP assay from VCaP prostate cell lines both untreated and treated with DHT (10 nmol/L) for 24 hours, showing levels of H3K27 Ac in the promoter region of human CHK1 corresponding to −1050 bp described in **C**. **E,** left, scheme of WT, −1290–490, Mut−1156, Mut−1050, and Mut−975 luciferase constructs. Core consensus sequences GGA(A/T) for ERG binding are indicated by red bars; mutant sites are in orange. Right, ERG-dependent downregulation of basal reporter activity is abolished only when the core consensus motif GGAT at position −1050 bp is mutated to GT. Transfected ERG levels are shown below the bar graph. RLU, relative luciferase units. Experiments were performed in triplicate; data were analyzed using an unpaired t test. Values of P < 0.05 were considered statistically significant (*, P < 0.05; **, P < 0.01).
sequences (black asterisks in Fig. 2A). A mutational strategy transforming ACC*GGAT*GCC at position –1050 bp in ACC*GT*GCC (F10 mutant) and GCC*GGAA*AGC at position –314 bp in GCC*AA*AGC (F11 mutant) was used to eliminate the two putative ERG binding sites both individually (F10 and F11 mutants) or in combination (F10/11 mutant; Fig. 2B, red asterisk indicates the mutated region). In a luciferase assay in HEK-293T cells, however, ERG overexpression still repressed in a dose-dependent manner the two single mutants (F10 and F11) as well as the double mutant (F10/11) similar to the wild-type promoter of CHK1 (Fig. 2B).

Although results obtained by luciferase assay seemed to disprove the prediction, further evidence indicating the distal part of the –1290-bp CHK1 promoter sequence as a potential important region for ERG repression was obtained by a ChIP assay analysis in VaCaP cells either untreated or treated for 24 hours with 10 nmol/L DHT. Different sets of primers were designed to amplify, respectively, the promoter regions around the putative ERG binding sites at –1050 bp (two-headed red arrows) and at –314 bp (two-headed black arrows), and in the 3′ untranslated region (3′-UTR; two-headed blue arrows) of the CHK1 gene (Fig. 2C). PLA1A was used as a positive control for the ChIP assay (19). Notably, ChIP results fully confirmed the ability of ERG to bind the putative CHK1 promoter around the putative ERG binding site at –1050 bp in normal growth conditions (DMEM plus 10% FBS; Fig. 2C, black bars), and, consistently, showed an increase in the amount of ERG binding after its inducing treatment with 24-hour DHT treatment (Fig. 2C, red bars). On the other hand, the region of the CHK1 promoter around the putative ERG binding site at –314 bp and the one in the 3′-UTR were not enriched in the ERG ChIP compared with control (Fig. 2C). Finally, in line with the repressive activity of ERG on the CHK1 promoter, the ChIP assay for H3K27Ac showed a significant decrease in H3K27Ac after 24 hours of DHT treatment (Fig. 2D). Conclusive evidence for the functional role of the –1050-bp ERG binding site in the transcriptional regulation of the CHK1 promoter was obtained from a luciferase assay with the region of the CHK1 promoter included from –1290 bp to –890 bp (WT; Fig. 2E). Indeed, although ERG overexpression significantly repressed the basal reporter activity of both wild-type WT, –1290 to –890, and mutant constructs Mut –1156 and Mut –975 (where core motifs of ERG consensus DNA binding sites located at –1156 bp “GGAT” and at –975 bp “GGAA” were mutated to “GT” and “GA,” respectively; Fig. 2E), a robust suppression of the downregulation of the reporter activity was evident when the ETS binding DNA core motif “GGAT” located at position –1050 bp was mutated to “GT” (Fig. 2E).

Overall, these results strongly suggest that the ERG binding site at position –1050 bp of the CHK1 promoter is functional for CHK1 transcriptional repression. However, the generation of two additional luciferase constructs progressively losing the distal part of the CHK1 promoter (SacI and KpnI in Supplementary Fig. S3C) demonstrated the ability of ERG to still repress CHK1 transcription in the absence of the –1050-bp binding site (Supplementary Fig. S3C), thereby suggesting the existence of other ERG consensus sequences among the 20 predicted that can probably be used by ERG to repress the CHK1 promoter.

Lastly, to further exclude a potential contribution of AR in CHK1 transcriptional regulation, we analyzed the luciferase activity of the wild-type CHK1 promoter construct (WT) in HEK-293T cells overexpressing AR, ERG, or an empty vector as control. An AR reporter construct (4xARE) was used as positive control for AR activity. As shown in Supplementary Fig. S3D, AR robustly induced the transcription of 4xARE in a dose-dependent manner, whereas it did not affect the basal transcription of the CHK1 promoter. An interesting question that remains to be addressed, however, regards the possible role of ERG and AR in the transcriptional regulation of CHK1 through their binding in the 3′-UTR region of the gene. Indeed, the ERG binding site identified by Yu and colleagues (26) on the 3′-UTR of CHK1 (ERG 25633; Supplementary Fig. S3A) falls in the same region where two independent datasets mapped an AR binding site (Supplementary Fig. S3A), even though the ERG site was not enriched in our ChIP assay (26, 35).

**Chk1 and Pten Compound Haploinsufficiency Triggers the Transition from HGPIN to Invasive Prostate Carcinoma**

TPRRESS2–ERG fusion and the consequent oncopogenic activity of ERG are early events in human prostate cancer and are frequently accompanied by heterozygous loss of PTEN (23, 24). To assess the in vivo relevance of our observations, we took advantage of the Pten+/–;PB-ERG mouse model (21, 24). Our previous study demonstrated that Pten+/–;PB-ERG mice [in which ERG is specifically expressed in the epithelial prostate cells through the AR-responsive mouse Probasin (PB) promoter] develop invasive adenocarcinoma with 100% penetrance by the age of 6 months, whereas Pten+/– mouse prostate is characterized by HGPIN at this age with no signs of stroma invasion (24, 36). The function of CHK1 as a guardian of DNA integrity and replication fork number and stability becomes particularly important in a proliferative state in which cells are rapidly replicating their DNA. Bearing in mind our *in vitro* data, we therefore hypothesized that one of the critical mechanisms by which ERG promotes progression to a more aggressive stage of prostate cancer in Pten+/–;PB-ERG mice is through downregulation of CHK1 levels. To test this hypothesis, we examined Chk1 mRNA amounts in prostates from 7-week-old wild-type, Pten+/–;PB-ERG, and Pten+/–;PB-ERG mice. qRT-PCR analysis revealed that the relative levels of Chk1 mRNA were decreased in the prostate of PB-ERG and Pten+/–;PB-ERG mice compared with those of age-matched Pten+/– or wild-type mice (Fig. 3A). Chk1 downregulation upon ERG induction was also observed in the prostate of R26ERG;PB-Cre4 and Ptenfloflo;R26ERG;PB-Cre4 mouse models recently published by Chen and colleagues (Fig. 3B; ref. 21). Interestingly, in both cases, downregulation of Chk1 was more pronounced when overexpression of ERG was combined with loss of PTEN (Fig. 3A and B). Although these data might simply reflect the relative amount of Chk1 mRNA belonging to the contaminating ERG-negative stroma component of the prostate (sizable in PB-ERG and R26ERG;PB-Cre4 normal prostates, but minimal in Pten+/–;PB-ERG and Ptenfloflo;R26ERG;PB-Cre4 prostate epithelial tumors), a recent publication describes ERG transcriptional function positively modulated by AKT activation (32). Transient
knockdown of PTEN, however, did not affect CHK1 levels in VCaP cells (Supplementary Fig. S4A).

Given the high frequency of concomitant PTEN loss and TMPRSS2-ERG translocation in human primary prostate cancer, to determine whether overexpression of ERG also leads to DDR in vivo in a PTEN-deficient context as previously demonstrated in vitro in prostate cell lines (19, 37), the status of γH2AX was investigated in immunoblot and IHC analyses using prostate samples of wild-type, Pten+/−; and Pten+/−;PB-ERG mice. Increased levels of γH2AX were detected in all genetically modified mice, although it was most prominently found in Pten+/−;PB-ERG mice (Fig. 3C and D and Supplementary Fig. S4B). Impairment of CHK1 function has been described in PTEN-null contexts as a consequence of AKT phosphorylation of CHK1 on S280, promoting a partial delocalization of CHK1 from the nucleus to the cytoplasm (10).

To understand if this was also the case in prostate lesions developed by Pten+/− mice, we fractionated the cytosolic cellular component of wild-type and Pten+/− mouse prostates and analyzed CHK1 by Western blot. As shown in Supplementary Fig. S4C, the amount of the cytosolic fraction of CHK1, compared with the total, was comparable in wild-type and Pten+/− mouse prostates, likely suggesting the requirement of a marked AKT activation, exclusive of a PTEN-null condition, for the cytosolic delocalization of CHK1 or otherwise the species/context specificity of the AKT-dependent phosphorylation of CHK1.

These results indicate that downregulation of CHK1 is a possible mechanism by which overexpression of ERG contributes to prostate cancer progression. However, it is interesting...
to note that CHK1 was always downregulated but never completely repressed in the Pten+/−;PB-ERG and in Pten−/−;R26lox/lox;PB-Cre+ mouse prostates, as well as in human prostate cancer and in human prostate cell lines endogenously (VCAP and LNCaP) or exogenously (HEK-293, LNCaP, 22Rv1, PC3, U2OS, or RWPE1) overexpressing TMPRSS2–ERG or ETV1, respectively (Figs. 1 and 2; Supplementary Figs. S1 and S2).

If downregulation of CHK1 is an important downstream mediator of ERG-dependent prostate oncogenesis, we would expect Pten+/−;Chk1+/− mice to develop a more aggressive prostate phenotype than their age-matched Pten+/− littermates. To investigate this possibility, we generated a Pten+/−;Chk1+/− cohort of mice by crossing Pten+/− mice (38) with Chk1+/− mice (39–41). Pten+/−;Chk1+/− male compound mutants behave and age in a similar manner to their Pten+/− littermates (Supplementary Fig. S4D–S4G), although they are generally characterized by an exacerbation of classical Pten+/− phenotypes, such as benign lymphoproliferation, spleen enlargement, and pheochromocytoma (Supplementary Fig. S4F and S4G; ref. 38). As for the Pten+/− mice (38), the same phenotypes were far more pronounced in the Pten+/−;Chk1+/− female compound mutant cohort, which dictated euthanization in some cases (Supplementary Fig. S4E). To study the possible oncogenic role of Chk1 downregulation in Pten heterozygous mouse prostates, a cohort of age-matched Pten+/−;Chk1+/− (n = 24), Pten+/− (n = 15), Chk1+/− (n = 12), and wild-type (n = 16) mice were sacrificed and characterized histopathologically at 5 months of age, and another large cohort was analyzed at 12 months (see below). At 5 months, 8 of 24 Pten+/−;Chk1+/− mouse prostates showed a robust increase of HGPIN (>40%) in the tissue of at least one of the three lobes (Fig. 3E). The remaining 16 Pten+/−;Chk1+/− mice were characterized by HGPIN in approximately 20% of their prostate tissue, a condition fully comparable with the prostate phenotype found in the 14 of 15 age-matched Pten+/− mice (Fig. 3E). All wild-type and Chk1+/− prostates were normal. Importantly, Western blot analysis of wild-type, Pten+/−, Chk1+/−, and Pten+/−;Chk1+/− mouse prostates showed the presence of γH2AX only in the Pten+/−;Chk1+/− prostates (Fig. 3F). No substantial differences were detected in Akt activation (Fig. 3F). We further proved the increased amount of DDR in Pten/Chk1 double heterozygous mouse prostatic epithelial cells by performing IHC analysis for γH2AX and phospho-53BP1 on prostate sections from Pten−/− (n = 3) and Pten+/−;Chk1+/− (n = 3) mice. Prostate epithelial cells presenting regular nuclear morphology accompanied by γH2AX or phospho-53BP1 nuclear dots staining were counted. As shown in Fig. S4H and Supplementary Fig. S4I, the number of prostate epithelial cells showing evidence of DDR was two times higher in Pten+/−;Chk1+/− than in Pten+/− prostates, thereby validating our hypothesis. These results suggest that by reducing the levels of CHK1, ERG can favor genomic instability by increasing the presence of unrepaired DNA damage, and, stochastically, accelerating tumor progression in PTEN-deficient human prostate cancer.

As previously reported (36), 12-month-old Pten+/− mice rarely develop invasive prostatic adenocarcinomas. Accordingly, to determine whether CHK1 downregulation in a Pten heterozygous context was sufficient to transform the Pten+/−-driven HGPIN into invasive prostate carcinoma, as is the case for ERG (24), a further cohort of age-matched Pten+/−;Chk1+/− mice (n = 16), Pten+/− (n = 22), Chk1+/− (n = 16), and wild-type (n = 15) mice were sacrificed at 12 months of age. Indeed, histopathology analysis carried out on serial sections of the three prostate lobes of all the mice revealed the presence of invasive prostate carcinoma in 8 of the 16 (50%) Pten+/−;Chk1+/− mice, whereas only 3 of the 20 (15%) Pten+/− showed tumor epithelial cells invading the surrounding stroma (Fig. 3H; Fisher exact test, P = 0.033). All the wild-type and Chk1+/− mice were characterized by normal prostate epithelium with sporadic signs of hyperplasia.

Overall, these results strongly support the hypothesis that ETS family members might favor tumorigenesis by lowering CHK1 levels, thus promoting DNA damage accumulation, aberrant G1–S cell-cycle checkpoint, and, in turn, genomic instability (6, 10, 42, 43).

Lowering of CHK1 Levels in Prostate Cancer Cells Bearing Functional TP53 Enhances Sensitivity to Etoposide, but Not to Docetaxel

Found in more than half of patients, TMPRSS2–ERG translocation is one of the most studied oncogenic lesions in prostate cancer. Although a growing body of evidence describes ERG overexpression involved in different aspects of prostate tumor progression, its correlation with specific clinical and pathologic parameters is still a matter of debate (44), whereas very little effort has been devoted, until now, to translate the knowledge acquired on the oncogenic activity of ERG in rationalized therapeutic strategies.

Intriguingly, we frequently found fragmented nuclei in cells overexpressing high levels of exogenous ERG (Supplementary Fig. S5A), whereas immunoblot analysis showed high levels of TP53, its phosphorylation on serine 15, and the upregulation of p21, suggesting the presence of extensive DNA damage in these cells and the expected TP53 response (Supplementary Fig. SSB, refs. 45, 46). Consistent with these results, excess amounts of ERG prevented colony formation in LNCaP and 22Rv1 cells bearing a functional TP53 pathway (see below), but not in PC3 cells completely lacking TP53 expression (Supplementary Fig. SSC). Therefore, to study (i) the possible effect of lowering CHK1 levels in different human prostate cancer cell lines and (ii) if this downregulation might influence sensitivity to specific drug treatments, we stabilized a panel of human immortalized and prostate cancer cell lines (RWPE-1, VCaP, LNCaP, 22Rv1, and PC3), with two different shRNAs (shCHK1100 and shCHK1200) targeting the CHK1 transcript with different efficacy (Fig. 4A). In all four prostate cancer cell lines (LNCaP, 22Rv1, PC3, and VCaP), with the exclusion of the immortalized RWPE1, shCHK1200 robustly reduced CHK1 levels (Fig. 4A). Consistent with the literature (47–50), however, the extremely low levels of CHK1 in shCHK1100 stable LNCaP, 22Rv1, PC3, and VCaP cell lines caused an excessive accumulation of unrepaired DNA damage (γH2AX in Fig. 4A) and, in turn, a persistent apoptotic response (cleaved PARP in Fig. 4A) that made it impossible to maintain the stable clones for more than few passages. On the other hand, the lower efficacy of shCHK1100 in targetting CHK1 (Fig. 4A) permitted the generation and propagation of stable clones for all of them. Because the ATR–CHK1 pathway is primarily activated by replicative stress during the S-G2 phases of the cell cycle, and CHK1 activity is essential
to genomic stability by maintaining replication fork integrity (42), suppressing inappropriate firing of late or cryptic DNA replication origins (43), and promoting homologous recombination DNA damage repair (11), we tested whether reduction of CHK1 levels might be detrimental for prostate tumor cells when treated with agents directly targeting the replication machinery (9, 51). RWPE-1, VCaP, LNCaP, 22RV1, and PC3 prostate cell lines stably expressing shControl and shCHK1 were treated with 20 μmol/L etoposide for 48 hours. Untreated cells served as the control. Annexin V/7-AAD flow cytometric analysis defined the PC3 cell line as completely resistant to etoposide treatment, whereas VCaP, LNCaP, 22RV1, and RWPE1 cells showed a robust apoptotic response (Fig. 4B, upper and lower quadrants on the right in the plots; orange and green bars in the graphs). RWPE1 was the most sensitive to etoposide among the prostate cell lines tested, probably as a consequence of the fact that RWPE1 cells are immortalized normal prostate epithelial cells and not tumor cells. CHK1 downregulation, however, did not change the amount of apoptotic RWPE1 cells after etoposide treatment.

Figure 4. CHK1 levels dictate etoposide sensitivity in prostate cancer cell lines bearing a functional p53 pathway. A, Western blot analysis comparing the levels of PARP (total and cleaved), CHK1, and γH2AX in shControl (shC), shCHK1, and shCHK1+ETO stable LNCaP, 22RV1, PC3, VCaP, and RWPE1 prostate cell lines. GAPDH was used as loading control. B, FACS analysis of Annexin V/7-AAD staining showing the amount of apoptotic cells in shControl (shCTR) and shCHK1+ETO VCaP, RWPE1, PC3, 22RV1, and LNCaP stable cell lines treated with 20 μmol/L of etoposide for 48 hours. Experiments were performed in triplicate; data were analyzed using an unpaired t test. Values of P < 0.05 were considered statistically significant (*, P < 0.05; **, P < 0.01). 7-AAD, 7-Aminoactinomycin D.
ETS-Dependent DNA Damage Response Bypass

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(4B). VCaP, 22RV1, and LNCaP showed comparable sensitivity to etoposide (orange bars); CHK1 downregulation, however, significantly increased the percentage of apoptotic cells in LNCaP and 22RV1, whereas it did not affect the response to etoposide in VCaP (Fig. 4B, quadrants on the right in the plot; red and green bars in the graphs). The differential sensitivity to etoposide after CHK1 knockdown of LNCaP and 22RV1 compared with VCaP cells might be reconciled by the status of TP53 among these three different prostate cell lines. Indeed, whereas LNCaP cells have both TP53 alleles wild-type and 22RV1, one allele wild-type and one expressing a mutant form of TP53 (Q331R) mildly affecting TP53 activity, VCaP cells are characterized by a dysfunctional TP53 pathway expressing a classic hotspot mutation of TP53 (R248W; ref. 52).

Finally, to understand if CHK1 levels might influence sensitivity to other types of drug, we treated LNCaP and VCaP cells stably expressing shControl and shCHK1 with docetaxel, a widely used standard-of-care therapy for prostate cancer (Fig. 5).

Figure 5. CHK1 downregulation does not improve docetaxel efficacy. A, FACS analysis of Annexin V/7-AAD staining showing the amount of apoptotic cells in shControl (shCTR) and shCHK1 LNCaP and VCaP stable cell lines treated with 0.2 nmol/L of docetaxel for 48 hours. Experiments were performed in triplicate, and data were analyzed using an unpaired t test. Values of P < 0.05 were considered statistically significant. B, model summarizing the cooperative contribution of PTEN heterozygosity and ERG expression to prostate cancer progression and the potential sensitivity of these tumors to specific drug regimens.
cancer. As shown in Fig. 5A, downregulation of CHK1 did not sensitize either LNCaP or VCaP cells to docetaxel treatment. Overall, these results strongly support the concept that reduction of CHK1 levels renders prostate tumor cells bearing functional TP53 specifically sensitive to genotoxic agents targeting the DNA replication machinery and causing stalling of replication forks, such as etoposide.

DISCUSSION
ETS family members have been implicated in tumors of various histologies, including leukemia, sarcoma, and prostate cancer (53–55). In particular, ERG and ETV1 have been found translocated and ectopically expressed in a large proportion of human prostate cancers (60%), a tumor type that still represents the second leading cause of cancer-related deaths in males in the United States (23, 27, 55, 56). Thus, defining the molecular mechanisms underlying the contribution of ETS family members to tumorigenesis is of great relevance, as it may offer new opportunities for therapeutic intervention. Ectopic and aberrant overexpression of ETS proteins may conceivably mediate their proto-oncogenic role in tumorigenesis through the mis-expression of their transcriptional target genes. The data presented here suggest that CHK1 may represent such a critical gene. ERG most likely does not initiate tumorigenesis in the prostate, because overexpression of ERG alone does not affect proliferation in a postmitotic tissue such as the prostate gland (21, 24, 57). Nonetheless, expression of ERG in a PtEN heterozygous or null background causes a more aggressive stage of cancer, suggesting that the genes deregulated by ERG become functionally relevant when cells are proliferating (21, 24, 57). In accordance with this notion, ERG overexpression in human patients is more frequently observed in conjunction with mutations that are known to trigger proliferation, such as partial PTEN loss (24, 57, 58). Recently, Brenner and colleagues (19) have associated ERG or ETV1 overexpression in prostate cell lines with increased numbers of H2AX nuclear foci, which offers evidence of DNA damage in these cells. They have also described ERG in a multiprotein complex with PARP1 and DNAPK and have shown that this complex regulates ERG activity (19). However, the mechanism responsible for the increased DNA damage remained elusive. Our findings provide a compelling transcriptional mechanism explaining how ERG and other ETS family members could cause genomic instability. We propose that ERG overexpression may facilitate the persistence of mild levels of DNA damage through the downregulation of CHK1. Reductions in CHK1 levels have been shown to cause DNA damage, most likely due to impairment of the monitoring of DNA integrity during replication (5–7, 11, 43, 59–62). Thus, it is conceivable that ERG-dependent DNA damage and consequent genomic instability might be due at least in part to reduced CHK1 levels and, consequently, an impaired cell-cycle checkpoint process during cellular proliferation. This particular condition, when associated with a hyper-proliferative context such as the one triggered by PTEN loss, may dictate increased genomic instability and the acquisition of further genetic lesions, which can drive prostate tumor progression and resistance to treatment (Fig. 5B).

Importantly, our study also offers straightforward therapeutic implications. Inactivating CHK1 function has been given serious consideration as a way to abrogate the cell-cycle checkpoints believed to account for the survival of cancer cells in response to chemotherapy and radiotherapy (63–68), and a number of CHK1 inhibitors are currently in clinical trials. Our data suggest that tumors harboring elevated levels of ERG, or other ETS family members, may be more sensitive to specific classes of drugs targeting the replication machinery than, for instance, to agents such as taxanes hitting microtubule polymerization, in a TP53 functional context. Finally, CHK1 inhibitors, due to already reduced levels of CHK1, may be administered at very low doses in ETS-positive tumors and therefore would be extremely effective in a synthetically lethal approach in combination with radiotherapy/chemotherapy and PARP inhibitors (69).

METHODS
In Vitro Experiments
HEK-293, VCaP, LNCaP, 22Rv1, PC3, RWPE1, and U2OS human cell lines were purchased from the ATCC. Cells were tested and authenticated by the ATCC (DNA fingerprinting, karyotyping, and morphology) and additionally by Western blot and qRT-PCR for specific markers in our laboratory. Cells were cultured in DMEM or RPMI medium (as indicated by the ATCC) supplemented with 10% FBS and tested for Mycoplasma contamination every month. For DHT treatments, the regular medium was replaced with regular medium supplemented with 10 nmol/L DHT. Lipofectamine 2000 (Invitrogen) was used as transfection reagent.

Microarray Analysis
The human prostate cancer dataset has been previously described (23). Briefly, copy-number data were generated on Agilent 244KaCGH arrays, and mRNA expression data were obtained on Affymetrix Human Exon 1.0 ST arrays. The complete genome dataset and analytic methods are reported separately (23). Specimens were classified as harboring ERG aberrant expression with a z-score greater than 4.0. This correlated with ERG genetic rearrangements as identified by comparative genomic hybridization analysis. CHK1 levels (z-score) were analyzed according to ERG aberrant expression status, and a t test was used to compare the mean z-score between tumors with ERG aberrant expression and those without. Publicly available expression data GSE39388 (22), GSE21032 (23), GSE14595 (24), GSE14097 (26), and GSE46799 (21) were obtained from the Gene Expression Omnibus database. Differential expression and statistical significance have been evaluated by the Bioconductor limma package. Values of P < 0.05 were considered statistically significant.

Generation of Plasmid Vectors
The pCMV-SPORT6-ERG (human) was purchased from Life Technologies (MHS6278-202759378). ERG cDNA was recovered by digestion with XhoI/SalI from pCMV-SPORT6-ERG and cloned in pBabe-puro by using SalI site. Human ETV1 cDNA was cloned into EcoRI site in pBabe-3xFlag-puro. ERG target sequences in pLKO were as follows: 5′-CGACATCTTCTTCTCACAT-3′ (ERG-shRNA#1) and 5′-GATGTTGTTAAGCCCTTA-3′ (ERG-shRNA#2). ShRNAs targeting human CHK1 were purchased from Thermo Fisher Scientific (RHS4533-EG1111). pLKO-shScramble (#1864) was purchased from Addgene and used as a control. Smart pool siRNA for PTEN was purchased from Dharmacon (siGENOME siPTEN M-003023-02-0005). Addgene and used as a control. Smart pool siRNA for PTEN was purchased from Dharmacon (siGENOME siPTEN M-003023-02-0005). chaser from Dharmacon (siGENOME shPTEN M-003023-02-0005). pCDNA3.1-AR was kindly provided by Dr. Steven Balk (Beth Israel Deaconess Medical Center, Boston, MA). The wild-type (WT) construct used in luciferase reporter activity was generated by amplifying 1290 bp upstream of the translation initiation site of CHK1 using the following primers: CCGGACGCGTGCACTCCAGGATCCGAGTCTACGAGG and CCGGAGATCTGACTCCACGAGGACCTCGG. Sequences in
bold indicate recognition sites for MluI and BglII, respectively. This PCR fragment was subcloned into the MluI and BglII sites of the pGL2-enhancer luciferase reporter vector (Promega). The mutant forms of this construct were generated using the following primers and their reverse complementary oligos in site-directed mutagenesis (Strategene) PCR reactions: for mutant F10: AAATCTCTTCGACGCTTCCTCCTCCCCGACTGAAAG; for mutant F11: CTCCTCCCGGCTTGGCCAAGGAGATTTGTCTCCC; for mutant MutF10/Gut: GCCGCGACGGCCTGGATGGGCGCGGTTCG; for mutant MutF10/ETV1: AAATCTCTTCGACGCTTCCTCCTCCGACTGAAAG; and for mutant MutEtv1/Gut: GTCGACGGCTTCACACCGGCTCCACTCATATTTG. The mutated sites are shown in bold.

**ChIP Assays**

ChIP analysis was performed according to the Simple ChIP Enzymatic Chromatin IP Kit (9003; Cell Signaling Technology). Briefly, 5 × 10^6 VCaP cells per condition were fixed with 1% formaldehyde for 10 minutes at room temperature. Relief of crosslinking for mutant Mut –975 : GTCGAGCCTCACACC (Abcam; ab4729), and 3′ GCCCGCAGCCCCGCCT (Meridian Life Science), rabbit monoclonal anti-ERG (k92110R; 1:1,000; Santa Cruz Biotechnology), rabbit polyclonal anti-AR (PG21; 1:1,000; Cell Signaling Technology), rabbit polyclonal anti-PSA (k92110R; 1:1,000; Sigma Aldrich), rabbit polyclonal anti-PTEN (J886G; 1:1,000; Cell Signaling Technology), rabbit polyclonal anti-phospho-AKT(S473) (9271S; 1:1,000; Cell Signaling Technology), rabbit polyclonal anti-akt (9272S; 1:1,000; Cell Signaling Technology), rabbit polyclonal anti-GAPDH (14C10; 1:6,000; Cell Signaling Technology), and mouse monoclonal anti-FLAG (M2; 1:3,000; Sigma Aldrich). For IHC, tissues were fixed in 10% formalin and embedded in paraffin in accordance with standard procedures. Mouse prostate sections were stained for 4′,6-Diamidino-2-phenylindole (DAPI) (97188S; 1:250, Cell Signaling Technology) and phospho-53BP1 (ab22550; 1:500, Abcam). Human prostate TMA sections were stained for CHK1 (ab40866; 1:250, EP691Y; Abcam) and ERG (ab92513, 1:250, EPR3864; Abcam). For immunofluorescence, cells were fixed in paraformaldehyde 4% in PBS 1× for half an hour, washed in PBS 1× three times, treated with 0.1% Triton X-100 in PBS 1× for 1 hour, and permeabilized with 0.1% Triton X-100 in PBS 1× for 15 minutes. Rabbit polyclonal anti-4′,6-Diamidino-2-phenylindole (DAPI) (97188S; 1:500, Cell Signaling Technology) and mouse monoclonal anti-ERG (C-1; 1:500; Santa Cruz Biotechnology) were used in PBS 1×.

**Stable Cell Lines**

Stable cell lines were generated by transduction of the indicated shRNAs. Twelve hours after infection, cells were washed and puro- mycin (2 μg/mL) was added to fresh media. Selection was maintained for 2 weeks after transduction before cells were used for experiments. Stable clones were maintained under puromycin selection (0.5 μg/mL). Stable cell lines were tested and authenticated by Western blot and qRT-PCR for specific markers in our laboratory.

**Annexin V/7-AAD FACS Analysis**

PC3, LNCaP, 22RV1, VCaP, and RWPE1 shControl and shCHK1 cells stable cell lines were plated in 6-well/multwells (0.2 × 10^6) PC3 and 0.5 × 10^6 LNCaP, 22RV1, VCaP and RWPE1. Twenty-four hours later, 20 μmol/L of etoposide or 0.2 nmol/L of docetaxel was added to the medium. Cells were collected 48 hours later and processed following the manufacturer's instructions (Thermo Fisher Scientific; BDBS59763).

**Pten+/+, Pten−/−;PB-ERG, and Pten−/−;Chk1 Mutant Mice**

Pten+/+ (C57BL/6) and Pten−/−;PB-ERG (C57BL/6) mice were generated as previously described (24). Chk1+/+ (C57BL/6) mice were obtained from The Jackson Laboratory following permission given by Dr. Stephen Elledge. Pten−/−;Chk1M mice were generated by crossing Pten+/− and Chk1+/− mice. All mouse work was done in accordance with the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee protocol.

**Cytosolic Fractionation**

Twelve-month-old wild-type (n = 2) and Pten−/− (n = 2) mice were sacrificed, and prostate were immediately extracted and processed for cytosolic fractionation following the manufacturer's instructions (Pierce Biotechnology; 78833).

**B- and T-Cell Staining**

Mice were sacrificed at the age of 5 months, and the axillary, cervical, and inguinal lymph nodes were surgically excised. Organs were forced through a nylon screen to make single-cell suspensions, and cells were washed and resuspended in PBS with 2% FBS. Thereafter, cells were stained with fluorescent-labeled anti-CD3, Thy1.1, Ly6G, and anti-6220HTC (BioLegend). Analysis was performed with LSR II (BD bioscience).

**Histopathology**

Murine prostate tissues were harvested and fixed in formalin overnight, washed in PBS, and dehydrated in Et-OH. Paraffin embedding
and hematoxylin and eosin staining were performed by the Histology Core Facility at the Beth Israel Deaconess Cancer Center. Parts of the tissues were used for proteins and RNA extraction. Histopathologic analysis of human prostate TMA was performed by the Pathology Histology Core Facility at the Icahn School of Medicine of Mount Sinai.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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