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ORIGINAL ARTICLE

EAF2 regulates DNA repair through Ku70/Ku80 in the prostate

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Androgens are known to protect prostate cancer cells from DNA damage. Recent studies showed regulation of DNA repair genes by androgen receptor signaling in prostate cancers. ELL-associated factor 2 (EAF2) is an androgen-regulated tumor suppressor and its intracellular localization can be modulated by ultraviolet light, suggesting a potential role for EAF2 in androgen regulation of DNA repair in prostate cancer cells. Here we show that knockdown of EAF2 or its homolog EAF1 sensitized prostate cancer cells to DNA damage and the sensitization did not require p53. EAF2 knockout mouse prostate was also sensitized to γ-irradiation. Furthermore, EAF2 knockdown blocked androgen repression of LNCaP or C4-2 cells from doxorubicin induction of γH2ax, a DNA damage marker. In human prostate cancer specimens, EAF2 expression was inversely correlated with the level of γH2ax. Further analysis showed that EAF2 and EAF1 are required for the recruitment and retention of Ku70/Ku80 to DNA damage sites and play a functional role in nonhomologous end-joining DNA repair. These findings provide evidence for EAF2 as a key factor mediating androgen protection of DNA damage via Ku70/Ku80 in prostate cancer cells.

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INTRODUCTION

Prostate cancer is the most common malignancy and the second leading cause of cancer deaths in US males.[1] The standard treatment for patients with advanced prostate cancer is androgen deprivation therapy that is known to enhance radiation therapy of prostate cancer and prolong patient survival.[2,3] Although the mechanisms of enhancement are poorly understood and studies on interaction between androgen receptor (AR) signaling and DNA damage repair are limited, several recent studies have provided new insight into the mechanisms of androgen regulation of DNA repair. Androgen deprivation therapy can sensitize AR-positive prostate cancer cells to DNA damage in both culture and xenografts.[4–6] Using RNA-sequencing and chromatin immunoprecipitation-sequencing, Polkinghorn *et al.*[5] showed that AR directly regulates the expression of DNA repair genes. In addition, some AR target genes such as *NKX3.1* can modulate DNA damage response.[7–10] Interestingly, DNA repair proteins such as PARP1 can also act as AR cofactors regulating AR signaling, suggesting a potential positive feedback loop between AR signaling and DNA repair genes. The regulation of DNA repair by AR signaling appears to be complex and identification of factors involved would help further understanding of the enhancement of radiotherapy by androgen deprivation therapy for prostate cancer patients.

Our previous studies[11–13] identified an androgen upregulated gene 19 (U19), also known as ELL-associated factor 2 (EAF2), as a tumor suppressor in the prostate that is frequently downregulated in prostate cancer. Multiple strains of EAF2 knockout mice developed prostatic defects including epithelial hyperplasia and high-grade prostatic intraepithelial neoplasia,[14–16] the putative precursor of prostate cancer. In mice, EAF2 inactivation on a Pten heterozygous background induced prostate cancer development and progression, and EAF2 and Pten co-downregulation occurred in over 50% clinical prostate specimens with higher Gleason scores.[17] The ultraviolet light-induced EAF2 intracellular localization and EAF2 knockout mouse lens cells displayed increased sensitivity to ultraviolet radiation-induced apoptosis,[18,19] implicating a potential role for EAF2 in DNA damage response. However, the relationship between EAF2 and DNA damage response and repair remains to be determined.

Here, we report a novel function of EAF2, along with its homolog EAF1, in DNA damage repair via the recruitment and retention of nonhomologous end-joining (NHEJ) pathway proteins Ku70/Ku80 to damaged DNA. Loss of EAF2 sensitized human prostate cancer cells and mouse prostate to DNA damage. In human prostate cancer specimens, EAF2 expression was inversely correlated with the level of DNA damage marker γH2ax. Our studies also suggested that EAF2 plays an important role in androgen regulation of DNA damage repair. These findings provide new insights into the role of EAF2 in prostate carcinogenesis as well as in androgen regulation of DNA damage repair in the prostate.

RESULTS

Knockdown of EAF2 and/or its homolog EAF1 sensitizes prostate cancer cells to DNA damage

To understand whether EAF1 and EAF2 play protective roles for prostate cancer cells upon damage, we depleted EAF1 and EAF2 individually or in combination by small interfering RNA (siRNA) in

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LNCaP prostate cancer cells before γ-irradiation which induces DNA damage mainly in the form of double-stranded breaks (DSBs).[20] Depletion of EAF1 and/or EAF2 induced elevated phospho-Histone H2ax (γH2ax) levels, a marker of DNA damage,[21,22] with higher γH2ax levels in the double knockdown than in individual knockdown of EAF1 or EAF2 (Figure 1a and Supplementary Figures S1A and B). Neutral comet assay also showed that knockdown of EAF1 and/or EAF2 in LNCaP cells resulted in longer comet tails with double knockdown exhibiting slightly longer comet tails (Figure 1b). Similar results were

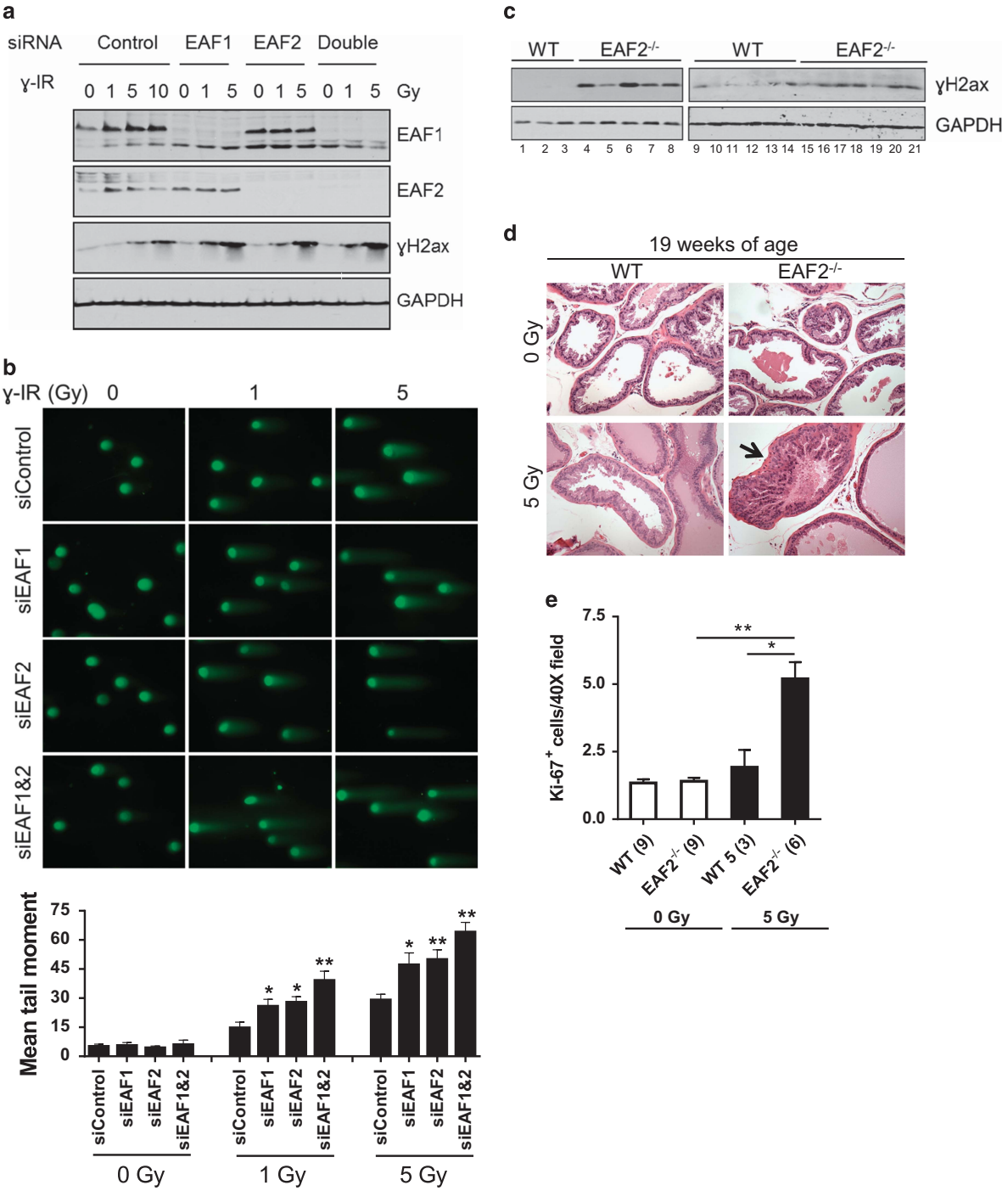


Figure 1. Knockdown of EAF1 and EAF2 sensitizes prostatic cells to DNA damage. (**a**) Effect of siEAF1 and/or siEAF2 on γH2ax induction by γ-irradiation in LNCaP cells. Cells were irradiated at indicated dosages 48 h after siRNA knockdown and collected 5 h after irradiation. (**b**) Influence of siRNA knockdown of EAF1 and/or EAF2 on single-cell neutral gel electrophoresis (COMET) assay of LNCaP cells treated with γ-irradiation as in (**a**). Mean value of tail moments of cells with or without irradiation shown below. (**c**) Knockout of EAF2 sensitized prostate to γ-radiation-induced DNA damage. γH2ax expression in anterior prostate lobes of wild-type (WT) and EAF2$^{-/-}$ male mice at the age of 5–7 months at 24 h after γ-irradiation (8 Gy). Blots were reprobed with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody to provide loading control. Numbers indicate lanes. (**d**) Decreased latency of prostatic intraepithelial neoplasia (PIN) lesions (black arrow) induced by γ-radiation in EAF2$^{-/-}$ mice. (**e**) Ki-67 immunostaining of EAF2$^{-/-}$ mouse prostate tissues at 19 weeks of age treated with 5 Gy of γ-radiation at 8 weeks of age. *$P < 0.05$, **$P < 0.01$. See also Supplementary Figure S1.

obtained using doxorubicin hydrochloride (Dx) to induce DSBs[23] (Supplementary Figures S1C and D). These data indicate that both EAF1 and EAF2 play a role in the repair of DNA DSBs in prostate cancer cells.

Knockout of EAF2 sensitizes mouse prostate to DNA damage

The role of EAF2 in protecting cells upon damage was next analyzed in the mouse prostate. EAF2 knockout[24] and wild-type C57BL/6J mice were irradiated at a whole-body dose of 8 Gy γ-irradiation and γH2ax levels were evaluated. As shown in Figure 1c, EAF2 knockout anterior prostates exhibited higher γH2ax levels than wild-type controls 24 h after irradiation, indicating that EAF2 knockout enhanced susceptibility of the prostate to DNA damage. Furthermore, EAF2 knockout mice subjected to 5 Gy whole-body irradiation at 8 weeks of age exhibited dramatically increased epithelial proliferation, atypical hyperplasia and decreased the latency of prostatic intraepithelial neoplasia lesions from 12 to 24 months of age[15,17] to as early as 19 weeks of age when compared with nonirradiated controls (Figures 1d and e). Thus, EAF2 is important for radiation protection *in vivo*.

EAF2 downregulation correlates with γH2ax upregulation in human prostate cancer specimens

To determine the potential association of EAF2 downregulation with increased DNA damage response in prostate carcinogenesis, we tested whether EAF2 downregulation correlated with γH2ax expression by immunostaining human prostate cancer specimens with Gleason scores 6–9 and matched nonmalignant adjacent tissues and in a tissue microarray (Figure 2a). We found downregulation of EAF2 and enhanced γH2ax in prostate cancer specimens compared with nonmalignant adjacent tissues (Figures 2b and c). Semiquantitative analysis of immunostaining intensity revealed an inverse relationship between EAF2 and γH2ax in human prostate specimens (Figure 2d). Increased γH2ax (H-Score $\geqslant 5$) immunostaining was detected in 29 of 122 prostate cancer specimens with decreased EAF2 (H-Score < 150), whereas γH2ax immunostaining was detected in only 1 of the 95 specimens with EAF2 expression (H-Score $\geqslant 150$) (Figure 2d); the difference was statistically significant ($P < 0.0001$). These findings suggest that EAF2 downregulation was required for the increased γH2ax expression.

Overexpression of EAF1 protects prostate cancer cells from DNA damage

As downregulation of EAF family proteins sensitized cells to DNA damage, we examined the effects of overexpression of EAF family proteins on LNCaP cells exposed to DNA damage. We established LNCaP cells with stable overexpression of GFP-EAF1 (LNCaP/GFP-EAF1) or GFP (LNCaP/GFP). When compared with LNCaP/GFP in a Dx dose–response study using γH2ax as a marker of DNA damage, LNCaP/GFP-EAF1 cells were less sensitive to Dx-induced DNA damage (Figure 3a). EAF2 is a multifunction protein that induces apoptosis,[13] and hence we were unable to stably overexpress GFP-EAF2 in the LNCaP cell line.[13] As the critical DNA damage sensor p53[25,26] binds to EAF2,[24] we examined whether EAF1 overexpression could modulate p53 induction in response to DNA damage. However, no difference was detected in the dose response of Dx induction of p53 protein between LNCaP/GFP and LNCaP/GFP-EAF1 cells (Figure 3a), suggesting that EAF1 overexpression does not influence p53 protein stabilization upon DNA damage induction. EAF1 knockdown in p53-negative PC3 cells enhanced γ2ax levels upon Dx treatment (Figure 3b), further suggesting that EAF1 can enhance DNA repair and that EAF family protein modulation of DNA repair does not require p53. Knockdown of GFP-EAF1 in LNCaP/GFP-EAF1 cells using two different siRNAs separately enhanced γH2ax levels when cells were treated with 0.4 μg/ml of Dx (Figure 3c), indicating that GFP-EAF1 overexpression was indeed responsible for protecting LNCaP/GFP-EAF1 cells from DNA damage.

Decreased γH2ax levels in response to Dx in GFP-EAF1-expressing cells might be associated with efficient repair of DNA damage. To test whether this was the case, DSBs were induced by Dx in LNCaP/GFP and LNCaP/GFP-EAF1 cells that were then allowed to recover for 25 h. As shown in Figure 3d, at the same dosage, Dx induced less γH2ax expression in LNCaP/GFP-EAF1 cells compared with controls. γH2ax virtually disappeared 25 h after cells were switched to fresh medium, consistent with GFP-EAF1 enhancement of DNA damage repair. When LNCaP/GFP and LNCaP/GFP-EAF1 cells were treated with Dx at 0.5 and 0.8 μg/ml, respectively, the γH2ax level induced in LNCaP/GFP-EAF1 cells was higher than that in LNCaP/GFP cells (Figure 3e). However, γH2ax still disappeared faster (within 48 h) in LNCaP/GFP-EAF1 cells after Dx removal when compared with LNCaP/GFP cells (over 60 h) (Figure 3e).

EAF2 knockdown inhibits androgen protection against DNA damage

Androgen signaling has been shown to accelerate the repair of DNA damage.[5] EAF2 knockdown in LNCaP cells resulted in an increased sensitivity to Dx-induced cytotoxicity (Figure 3f). As EAF2 is encoded by an androgen-responsive gene,[12,13] the effects of androgens on DNA damage repair following the knockdown of EAF2 in prostate cancer cells was determined. Knockdown of EAF2 in both LNCaP and C4-2 cells resulted in higher γH2ax levels in cells treated with Dx in both the presence and absence of androgens (Figures 3g and h).

EAF family proteins protect LNCaP cells from doxorubicin-induced cell death

Cells overexpressing EAF1 exhibited lower γH2ax levels compared with controls in response to DNA damage, indicating they might be resistant to Dx-induced cytotoxicity. As expected, Dx treatment induced a lower level of cleaved poly ADP-ribose polymerase (c-PARP), an apoptosis marker,[27] in LNCaP/GFP-EAF1 cells as compared with LNCaP/GFP controls (Supplementary Figure S2A). This was consistent with lower γH2ax levels detected in LNCaP/GFP-EAF1 cells (Supplementary Figure S2A). Knockdown of EAF1 in PC3 cells reversed the above phenotype (Supplementary Figure S2B). Flow cytometry analysis further confirmed that knockdown of EAF1 and/or EAF2 sensitized LNCaP cells to Dx-induced cell death, with double knockdown having the most dramatic effect (Supplementary Figure S2C).

Knockdown of EAF1 impairs NHEJ repair

Mammalian cells have evolved the NHEJ- and the homologous recombination (HR)-directed pathways to repair DSBs in DNA.[28–31] Thus, we utilized the previously established H1299dA3-1#1 and Hela pDR-GFP[32] cell models to test the role of EAF proteins in these two DSB repair pathways. Briefly, H1299 dA3-1#1 cells carrying a stably integrated DNA fragment with two recognition sites for the I-SceI endonuclease were subjected to DSB induction by transient expression of I-SceI. Restriction digestion at the two I-SceI sites followed by NHEJ excision of the TK gene enables expression of the enhanced green fluorescent protein (EGFP) gene driven by an upstream cytomegalovirus (CMV) promoter[32] that provides a quantitative measurement of DNA repair events. In the Hela pDR-GFP HR assay,[32] two differentially mutated GFP genes, a GFP gene harboring a single I-SceI site with in-frame stop codons, SceGFP, and a downstream internal GFP fragment (iGFP), can undergo a gene conversion event upon I-SceI transfection, resulting in the expression of intact GFP protein that can be measured by C6 flow cytometry. Endogenous EAF2 expression was very low in H1299dA3-1#1 and was not detectable in Hela pDR-GFP cells. Fortunately, both cell lines expressed EAF1 at levels

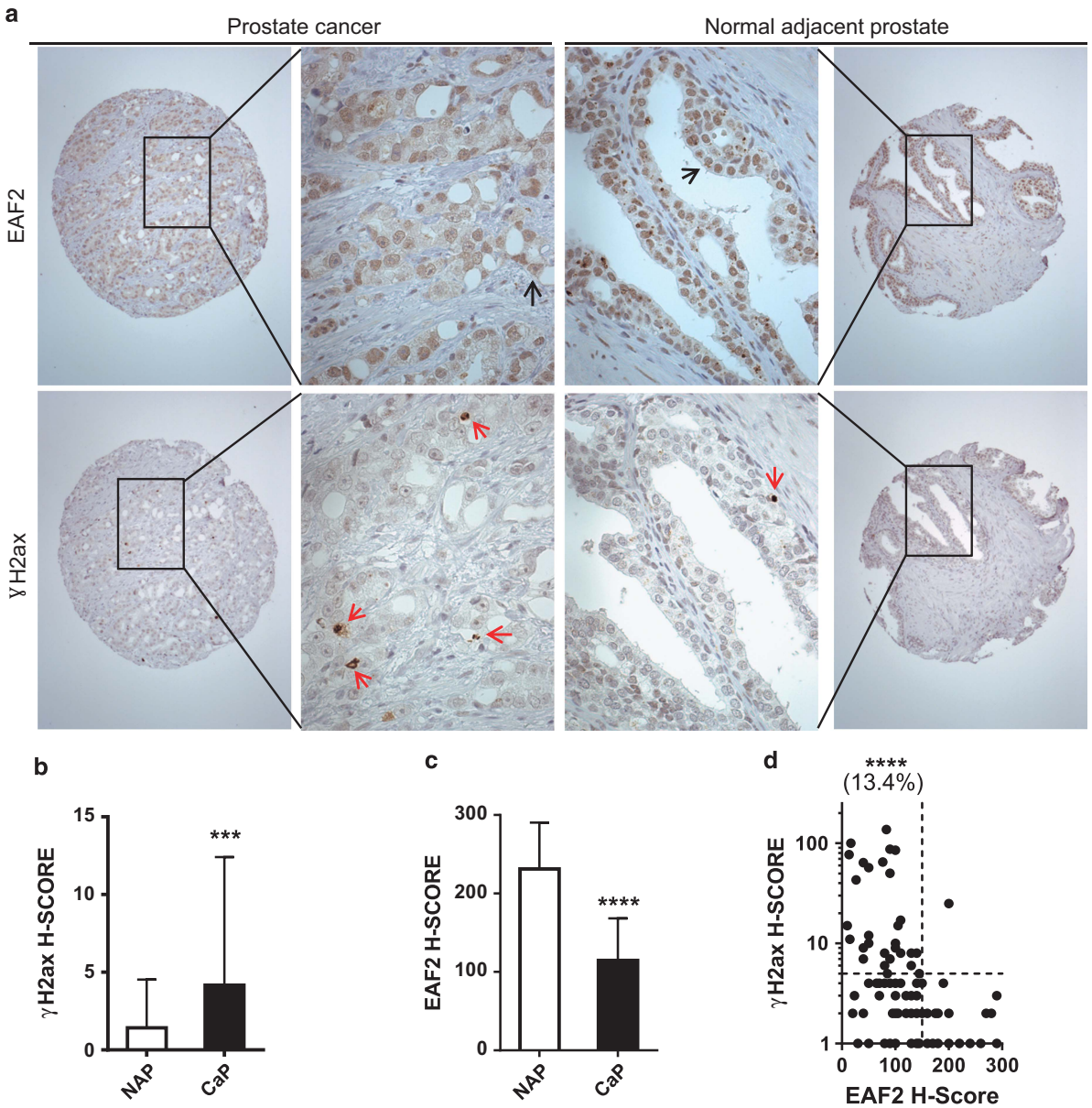


Figure 2. Inverse correlation of EAF2 and γH2ax expression in human prostate cancer specimens. (**a**) Immunostaining of EAF2 and γH2ax in normal adjacent prostate and prostate cancer. Black and red arrows indicate staining of EAF2 and γH2ax, respectively. (**b**) Quantification of EAF2 immunostaining intensity H-Score in normal adjacent prostate (NAP) and prostate cancer (CaP) tissue specimens. (**c**) Quantification of γH2ax staining intensity H-Score in matched NAP and CaP tissue specimens ($n = 233$). Scale bars: 100 μm, ×40. (**d**) Scatter plot of EAF2 and γH2ax immunostaining intensity in human prostate tissue specimens. Positive γH2ax staining (H-Score $\geqslant 5$) in specimens with EAF2 downregulation (H-Score < 150) was significantly higher than in those with EAF2 expression (H-Score $\geqslant 150$) according to Fisher's exact test ($P < 0.0001$). ***$P < 0.001$, ****$P < 0.0001$.

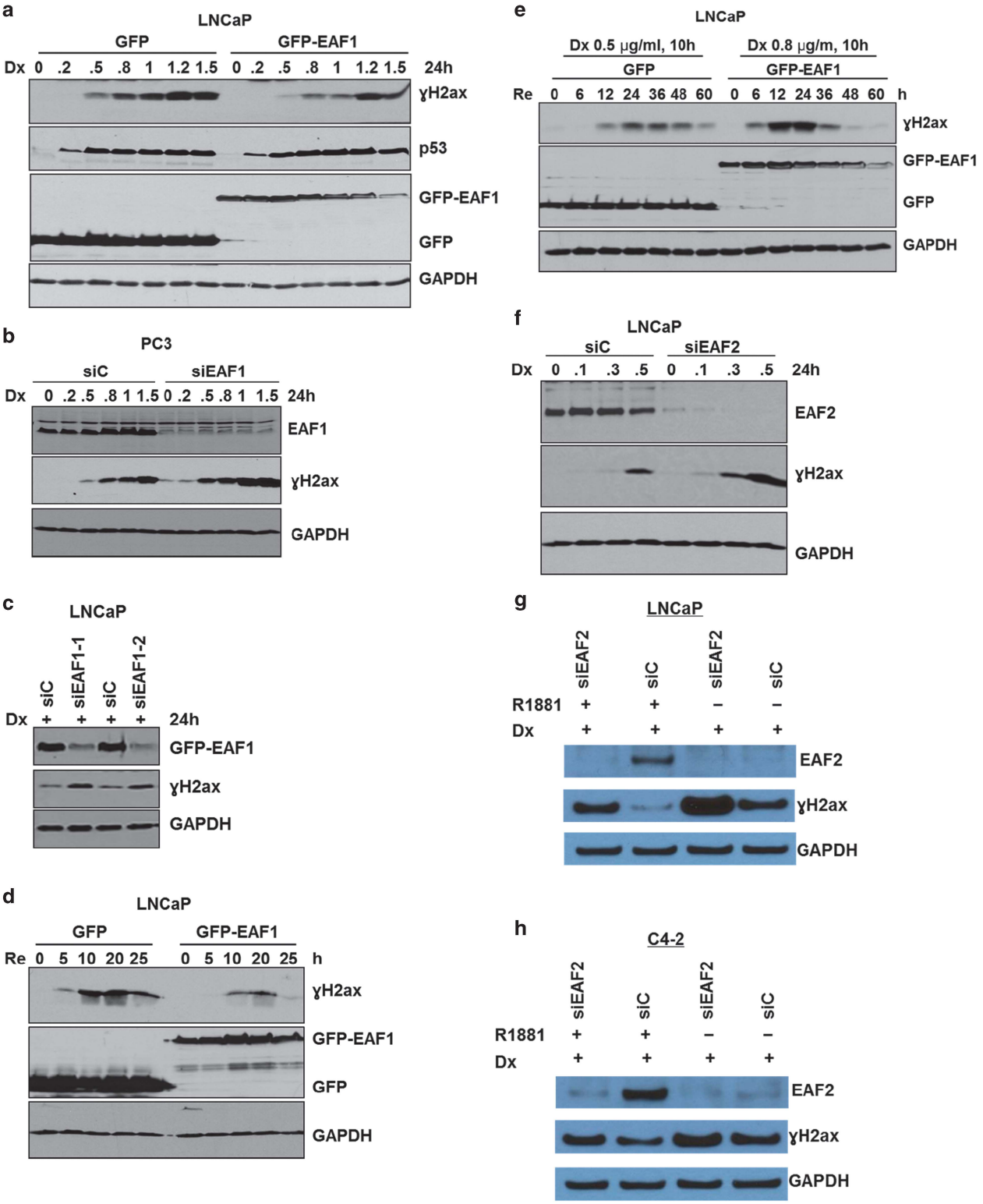
comparable to LNCaP cells (data not shown), providing an opportunity to test the effect of EAF1 depletion on DSB repair in chromosomal DNA. Knockdown of EAF1 reduced the percentage of GFP-positive cells compared with cells treated with siRNA control (Figure 4a), indicating that EAF1 was required for efficient NHEJ. Using the Hela pDR-GFP-based assay for HR repair,[33] no significant difference was detected in the percentage of GFP-positive cells between siEAF1-treated and siRNA control groups (Figure 4b), indicating that EAF1 may not affect the HR repair pathway.

EAF family proteins bind to and co-accumulate with Ku70/Ku80 proteins at sites of DNA damage

As EAF family proteins could modulate NHEJ of DSBs and prostate epithelial cells have been shown to undergo repair predominantly by the NHEJ pathway in response to irradiation,[31] we tested whether EAF1 and EAF2 accumulated at DSBs induced by laser microirradiation in LNCaP cells. GFP-EAF1 and GFP-EAF2 protein accumulated at sites of DNA damage induced by 405 nm laser microirradiation (Figure 5) and the accumulation occurred very rapidly in ~10 s after the microirradiation (Supplementary Figure S3). The core NHEJ machinery consists of DNA-dependent protein kinase and the ligase IV/XRCC4/XLF complex.[34,35] The Ku70/80 heterodimer is the DNA binding component of DNA-dependent protein kinase, and forms a ring that can specifically bind to exposed broken DNA ends.[36,37] Formation of the Ku heterodimer is an early and upstream event of NHEJ.[38] Inactivation of Ku70 or Ku80 in mice leads to multiple defects including hypersensitivity to radiation and malignant transformation.[39,40] Considering the importance of Ku70 and

Ku80 heterodimers in NHEJ repair, we also tested whether EAF family proteins and Ku proteins co-accumulated at DSB sites. As shown in Figure 5, both GFP-EAF1 and GFP-EAF2 co-accumulated at DSB sites with RFP-Ku70 and RFP-Ku80. All four proteins responded to microirradiation within seconds, preventing us from distinguishing the order of their accumulation at DSBs.

Colocalization of proteins is often indicative of their physical interaction. Using HEK293 cells transfected with various combinations of GFP-Ku70, GFP-K80, Myc-EAF1 and HA-EAF2 plasmids, we found that both Ku70 and Ku80 co-immunoprecipitated with transfected EAF1 and EAF2 proteins, both in the absence and presence of Dx (Figures 6a and b). Nuclear extracts prepared from LNCaP cells with and without Dx



treatment were also immunoprecipitated with EAF1 or EAF2 that showed endogenous Ku70 and Ku80 co-precipitation with endogenous EAF proteins (Figures 6c and d). These results further suggest that EAF1 and EAF2 physically interact with Ku70 and Ku80 in the nucleus.

Knockdown of EAF family proteins impairs recruitment of Ku70/Ku80 at sites of DSBs

Recruitment of Ku70 and Ku80 heterodimers to DSBs is a key step to initiate NHEJ.[36,37] As EAF1 knockdown impaired NHEJ (see Figure 4), we examined the effect of EAF1 and EAF2 knockdown on Ku70 and Ku80 recruitment in response to laser microirradiation. Knockdown of EAF1 and/or EAF2 inhibited Ku70 accumulation and retention at DSB sites dramatically in LNCaP cells (Figure 7a). Similar results were obtained for Ku80 recruitment and retention at DSBs, although the inhibition was less dramatic than that for Ku70 (Figure 7b). As a control, we showed that knockdown of EAF1 and/or EAF2 did not affect the levels of Ku70 and Ku80 proteins (Supplementary Figure S4A). The impaired recruitment of Ku proteins to DNA damage sites upon EAF1 knockdown was rescued by re-expression of an EAF1 transgene insensitive to siEAF1 in LNCaP cells (Figures 7c–e), suggesting that the effect of siEAF1 on recruitment of Ku proteins was indeed mediated through EAF1 knockdown. Similar results were obtained when another independent siEAF2 sequence was used to knock down EAF2, further arguing that the siEAF2 effect on Ku proteins was mediated through EAF2 knockdown (Supplementary Figures S4B–D). Taken as a whole, these data strongly indicate that EAF family proteins work together with Ku proteins in regulation of DNA damage response.

DISCUSSION

Our studies here provide evidence for EAF2 and its homolog EAF1 being key regulators of DNA damage repair in the prostate. First, knockdown of EAF family proteins sensitized prostate cancer cells and murine prostate to DNA damage (Figure 1) and the sensitization did not require p53 (Figure 3). Second, down-regulation of EAF2 protein in prostate cancer specimens was correlated with elevated expression of DNA damage response marker γH2ax (Figure 2). Third, EAF2 knockdown inhibited androgen protection against DNA damage (Figure 3). Finally, we found that EAF family proteins could modulate NHEJ of DSBs, and were required for the recruitment and retention of DNA repair proteins Ku70/Ku80 at DNA damage sites (Figures 4–7).

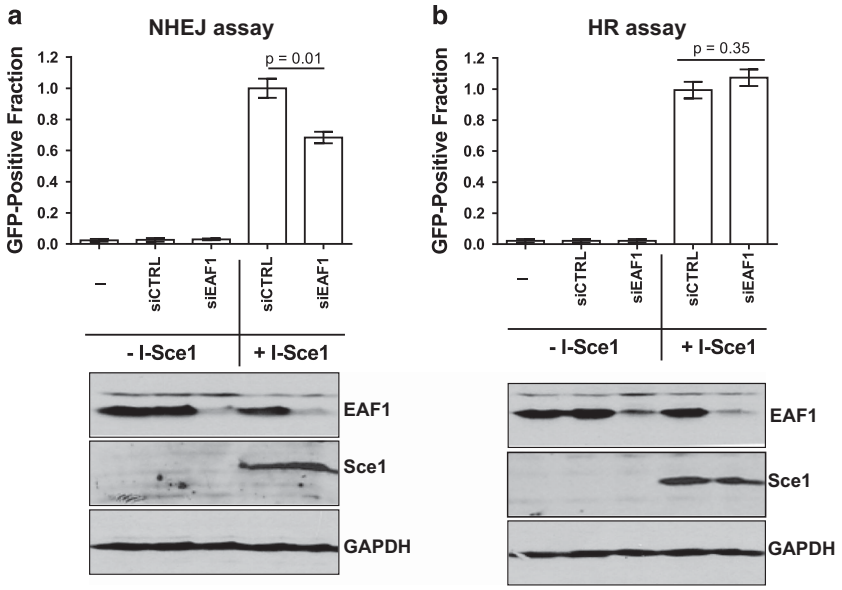


Figure 4. Knockdown of EAF1 impairs NHEJ but not HR of double-strand breaks (DSBs) in chromosomal DNA. (**a**) Assay for NHEJ of chromosomal DSBs in H1299 (dA3-1) cells. Transiently expressed I-SceI protein cleaves the I-SceI sites and produces DSBs with incompatible ends in the substrate. NHEJ of two broken DNA ends of chromosomal DNA results in EGFP expression. GFP-positive fraction of cells treated with siEAF1 or siCTRL indicates frequency of NHEJ repair of chromosomal DNA. Western blot analysis was performed to confirm knockdown of EAF1 and Sce1 expression. (**b**) Assay for HR frequency of chromosomal DNA containing a recombination substrate DR-GFP in HeLa cells. GFP-positive fraction of cells treated with siEAF1 or siCTRL indicate frequency of HR repair of chromosomal DNA. Western blot confirmed knockdown of EAF1 and Sce1 expression. Blots were reprobed with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody to provide protein loading control.

Figure 3. EAF1 enhances DNA repair and EAF2 knockdown inhibits androgen protection against doxorubicin-induced DNA damage. (**a**) LNCaP/GFP and LNCaP/GFP-EAF1 cells treated with indicated doses of doxorubicin (Dx, μg/ml) for 24 h. (**b**) Knockdown of EAF1 sensitized p53-negative PC3 cells to Dx-induced DNA damage. (**c**) After siRNA knockdown of EAF1 in LNCaP/GFP-EAF1 cells for 48 h, cells were treated with Dx (0.4 μg/ml) for additional 24 h. (**d**) LNCaP/GFP and LNCaP/GFP-EAF1 cells were treated with 0.4 μg/ml Dx for 12 h. Cells were then washed with PBS twice and cultured in fresh medium to recover (Re) for indicated time in hours. (**e**) LNCaP/GFP and LNCaP/GFP-EAF1 cells were treated with Dx (0.5 or 0.8 μg/ml) for 10 h and then allowed to recover as in (**d**). (**f**) LNCaP cells treated with siRNA targeting EAF2 for 48 h and then with Dx at indicated concentrations for an additional 24 h in the presence of 1 nM dihydrotestosterone (DHT) to enhance EAF2 expression. (**g**) LNCaP cells treated with siRNA targeting EAF2 in both the presence and absence of androgens (1 nM R1881). (**h**) C4-2 cells treated with siRNA targeting EAF2 in both the presence and absence of androgens (1 nM R1881). Cells were cultured in charcoal-stripped RPMI-1640 with and without 1 nM R1881 (**g** and **h**). LNCaP or C4-2 cells treated with siRNA targeting EAF2 for 48 h and then treated with 0.5 μg/ml of Dx for an additional 24 h. Blots were reprobed with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody to confirm equal protein loading.

These findings suggest that EAF family proteins work with Ku70/Ku80 to protect cells from DNA damage via NHEJ, a major repair pathway for DSBs in mammalian cells, providing mechanistic insights into androgen regulation of DNA repair in prostate cancer cells.

Our study testifies further that phosphorylated H2ax (γH2ax) is a reliable marker for evaluating DNA damage in prostate cancer. The effect of siRNA knockdown of EAF1 or EAF2 on γH2ax levels was consistent with observations using the neutral comet assay that directly measures chromosomal DNA degradation. The

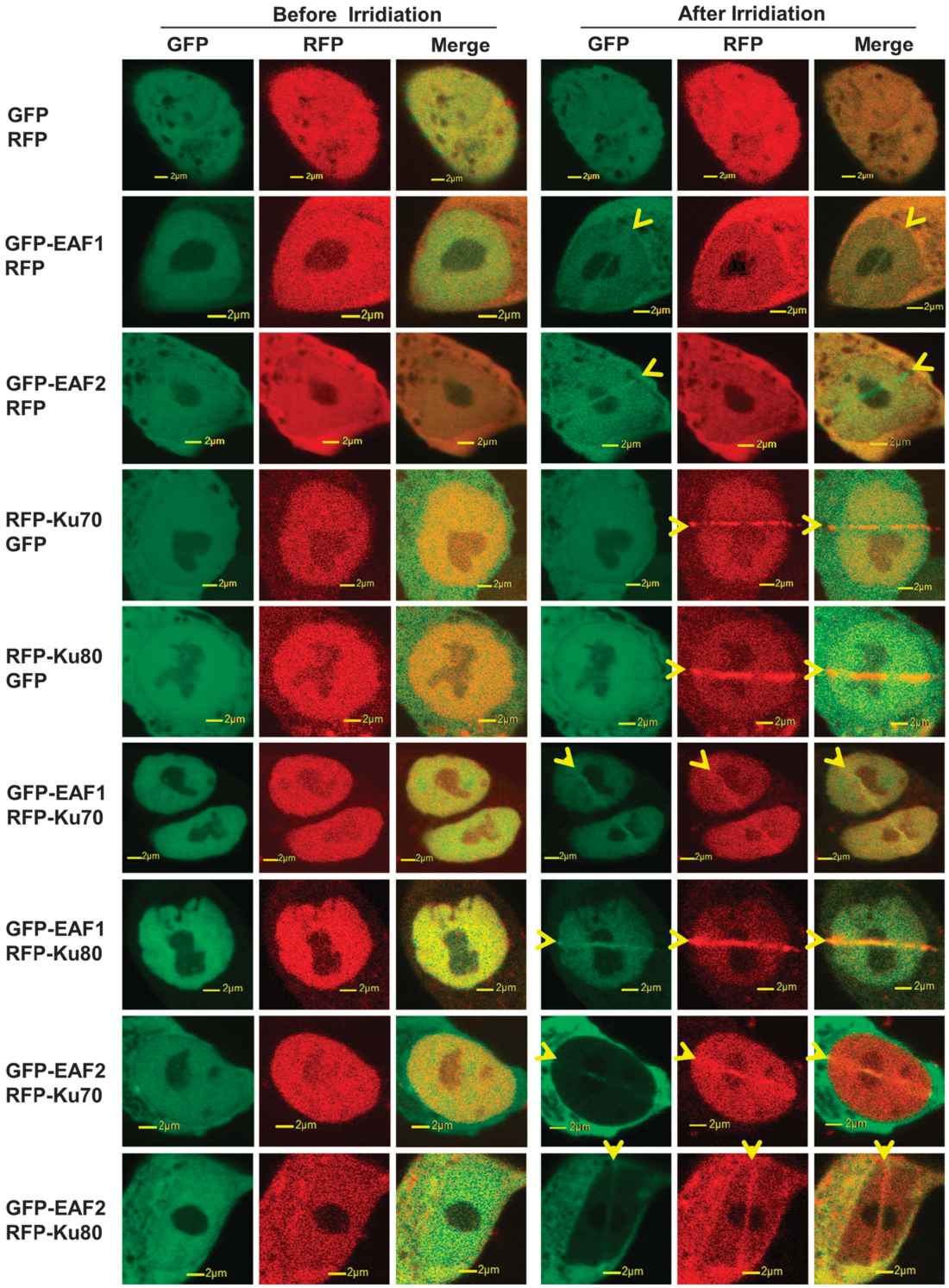


Figure 5. Response and co-accumulation of EAF and Ku family proteins at laser microirradiation-induced DSBs sites. LNCaP cells were transiently transfected with GFP or GFP-tagged EAF1, EAF2 and red fluorescent protein (RFP) or RFP-tagged Ku70 and Ku80 expression vectors and treated with laser microirradiation to induce DSBs 24 h after transfection before and after irradiation. Accumulation of the transfected proteins was indicated by GFP (green) or RFP (red) fluorescence at laser-irradiated sites. Yellow arrowheads indicate direction of laser irradiation. Co-accumulation was visualized in merged images.

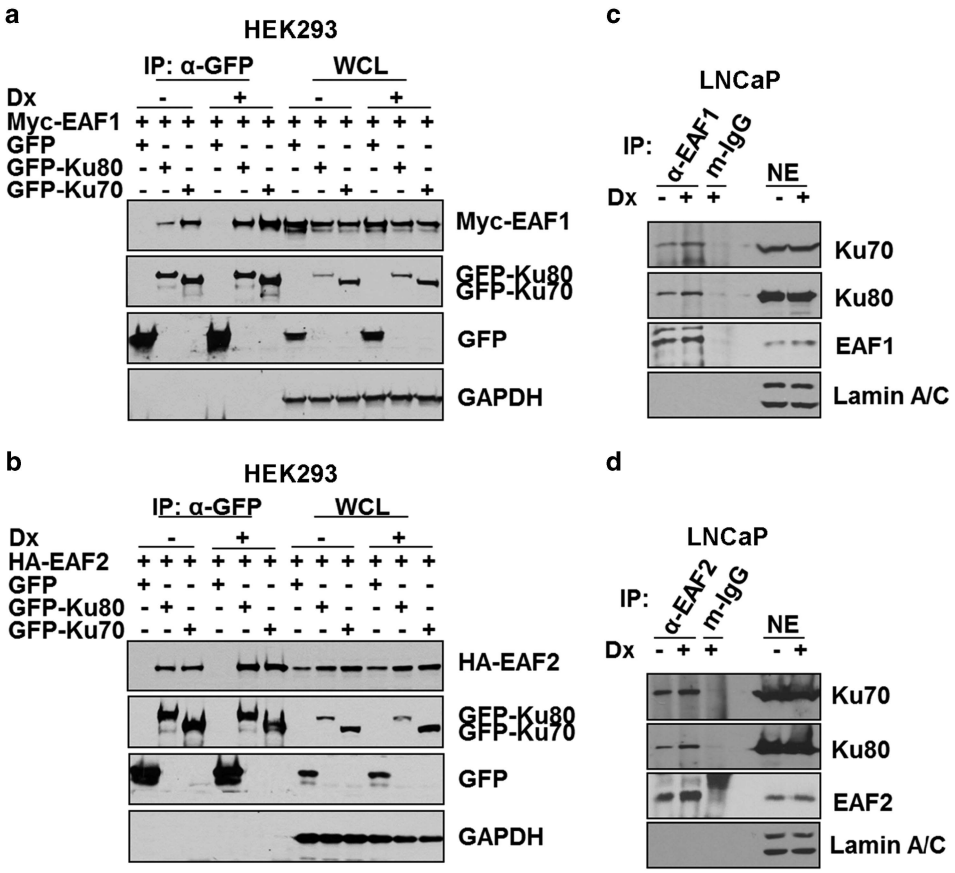


Figure 6. EAF1 and EAF2 bind to DNA repair proteins Ku70 and Ku80. (**a**) Co-immunoprecipitation of transfected EAF1 with Ku70 and Ku80. HEK293 cells were transiently transfected with the mammalian expression vectors for Myc-EAF1 and/or GFP-Ku70 and GFP-Ku80. Whole-cell lysates (WCLs) were immunoprecipitated with immobilized anti-GFP protein G beads and immunoblotted with anti-Myc or anti-GFP. (**b**) Co-immunoprecipitation of transfected EAF2 with Ku70 and Ku80 in HEK293 cells transiently transfected with mammalian expression vectors HA-EAF2 and/or GFP-Ku70 and GFP-Ku80. Cell lysates were immunoprecipitated with immobilized anti-GFP protein G beads and immunoblotted with anti-HA or anti-GFP. (**c**) Co-immunoprecipitation of endogenous EAF1 with Ku70 and Ku80. LNCaP cells were cultured in the presence of 1 nM dihydrotestosterone (DHT) for 24 h and then treated with and without doxorubicin (Dx, 1.0 μg/ml, 5 h) before harvest and nuclear extract preparation. Nuclear extracts (NEs) were precipitated using immobilized anti-EAF1 antibody and immunoblotted with anti-Ku70, anti-Ku80 and anti-EAF1 antibodies. (**d**) Co-immunoprecipitation of endogenous EAF2 with Ku70 and Ku80. The same LNCaP cell nuclear extracts as in (**c**) were precipitated using immobilized anti-EAF2 antibody and immunoblotted with anti-Ku70, anti-Ku80 and anti-EAF2 antibodies.

increased DNA damage-induced cell death upon knockdown of EAF proteins further supports this conclusion. Thus, the elevated γH2ax level induced by γ-irradiation in EAF2 knockout prostate (Figure 1c) would reflect that EAF2 knockout prostate was more sensitive to γ-irradiation-induced DNA damage. Similarly, the inverse correlation of EAF2 expression with γH2ax protein level in human prostate cancer specimens (Figures 2a and b) suggests that downregulated expression of EAF2 would permit elevated DNA damage in prostate cancer cells when these cells were subjected to genotoxicity.

The dramatic decrease in latency of prostatic intraepithelial neoplasia lesions in EAF2 knockout prostate following γ-irradiation substantiated the importance of EAF2 in prostatic DNA damage repair. The hypersensitivity of EAF2 knockout prostate to γ-irradiation is unlikely mediated through neoplastic transformation secondary to EAF2 knockout because prostate carcinogenesis was shown to be insensitive to either low- or high-dose radiation in the TRAMP mouse model.[41] Furthermore, Ku protein deletion was reported to enhance sensitivity to irradiation in the mouse model.[42,43] Cumulatively, these findings suggest that hypersensitivity to irradiation in EAF2 knockout prostate is mediated through the Ku protein-dependent pathways.

Regulation of DNA repair by EAF family proteins through Ku70 and Ku80 proteins is supported by the association between EAF proteins and Ku70/Ku80 proteins in the same complex and their rapid co-accumulation at the DSB following microirradiation. This model is further indicated by the inhibition of Ku70/Ku80 recruitment at the DSB sites upon EAF1 and/or EAF2 knockdown (Figure 7). It is worth pointing out that knockdown of EAF1 and/or EAF2 did not change expression levels of Ku70 and Ku80 proteins (Supplementary Figure S4). This suggests that reduced Ku protein accumulation and retention at DSBs upon EAF knockdown was not due to reduced Ku protein expression. Given the demonstrated importance of Ku70/Ku80 complex in the NHEJ repair mechanism, when EAF2 is downregulated, inhibition of Ku protein recruitment at DSBs would impair DNA repair and subsequently lead to genomic DNA mutations and/or deletions that are key steps in carcinogenesis.

The present study suggests that EAF1 and EAF2 are not functionally redundant in the regulation of Ku proteins and DNA damage repair, although EAF1 and EAF2 are two closely related homologs. Knockdown of either factor impaired the recruitment and retention of Ku proteins and DNA damage response and repair. One potential mechanism for the non-redundant function of EAF1 or EAF2 is that both EAF1 and EAF2 are required

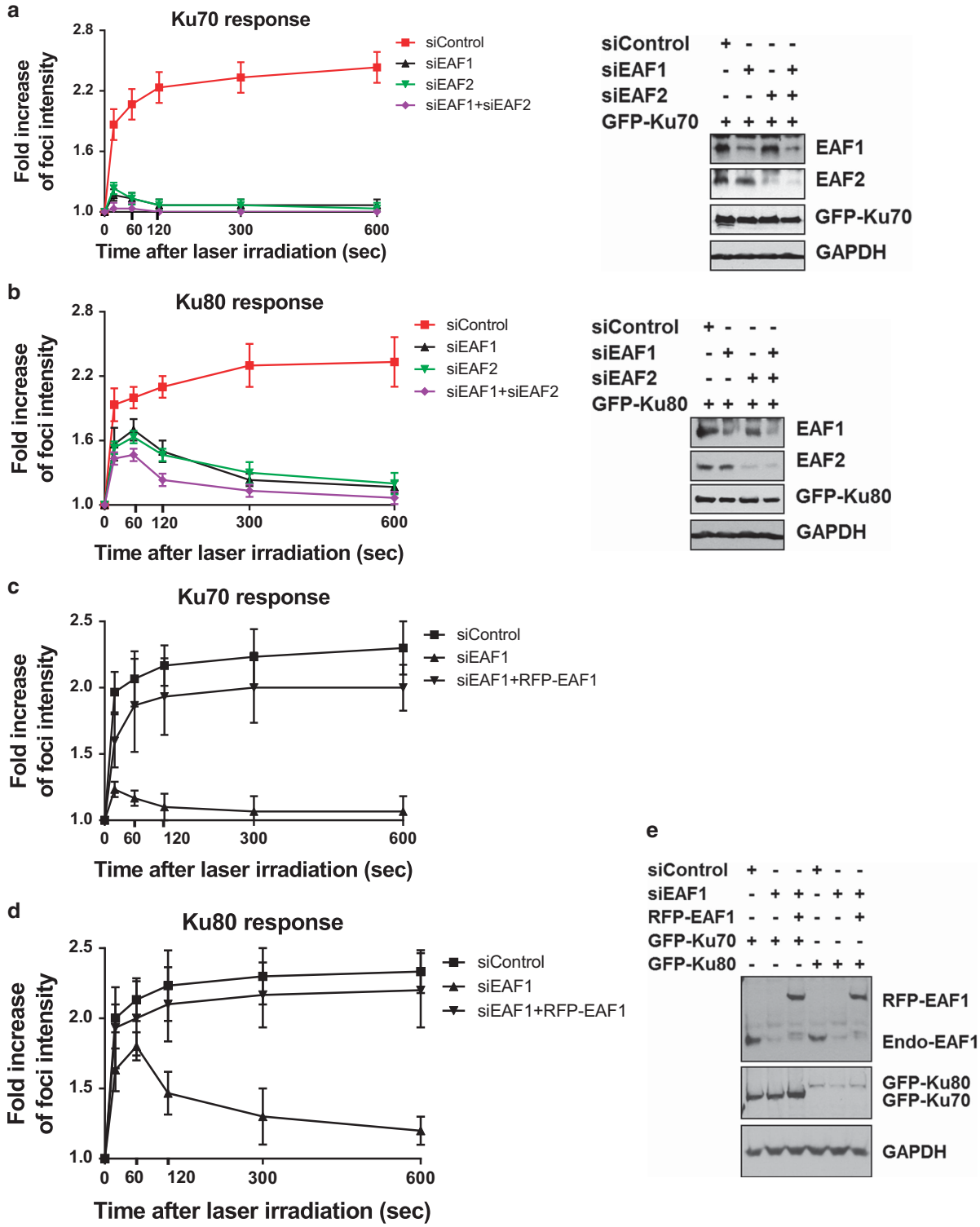


Figure 7. Accumulation of Ku70 and Ku80 proteins at laser microirradiation-induced damage sites after knockdown of EAF1 and/or EAF2. (**a**) Foci intensity of GFP-Ku70 accumulation at sites of laser microirradiation in LNCaP cells treated with indicated siRNA(s) for 48 h followed by transfection with GFP-Ku70 in the presence of 1 nM dihydrotestosterone (DHT) for 24 h. Mean foci intensity was measured every 20 s for 10 min and subtracted from background intensity. Right panel shows EAF1 and/or EAF2 knockdown and GFP-Ku70 expression. (**b**) Kinetics of GFP-Ku80 accumulation at laser-irradiated sites in LNCaP cells treated as in (**a**). Right panel shows EAF1 and/or EAF2 knockdown and GFP-Ku80 expression. (**c**) LNCaP cells were treated with siEAF1 for 48 h. Cells were then transfected with GFP-Ku70 alone or in combination of siEAF1-resistant GFP-EAF1 plasmids in the presence of 1 nM DHT. Kinetics of GFP-Ku70 accumulation at laser-irradiated sites was measured 24 h later. (**d**) Kinetics of GFP-Ku80 accumulation at laser-irradiated sites in LNCaP cells treated similarly to (**d**). (**e**) Protein expression of cells treated identically as in (**c**) and (**d**). Blots were reprobed with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody to confirm equal protein loading. See also Supplementary Figure S2.

simultaneously for the efficient recruitment and retention of Ku70/Ku80. Alternatively, EAF1 and EAF2 are functionally identical in the recruitment of Ku70/Ku80 and the efficient recruitment and retention of Ku70/Ku80 requires a certain threshold level of EAF proteins. siRNA knockdown of either EAF protein would reduce the level of total EAF proteins below the required threshold. We were unable to investigate whether EAF1 downregulation correlated with γH2ax upregulation in human prostate cancer specimens (Figure 2) because of the lack of an EAF1 antibody suitable for immunohistochemistry. Although the role of EAF1 in prostate carcinogenesis remains to be established, our study demonstrates the importance of EAF1 as well as EAF2 in regulating DNA repair in prostate cells. The contributions of EAF1 and EAF2 individually and in combination in DNA damage repair and in prostate carcinogenesis will be further analyzed in future studies.

EAF2 can affect multiple important pathways in prostate carcinogenesis via various binding partners. EAF proteins play important roles in transcription and transcription elongation.[44] Regulation of Ku protein recruitment and retention at the DSB sites by EAF family proteins suggests a potential link between DNA damage response and transcription. This also provides an opportunity to explore the crosstalk of DNA repair with the transcription and transcription elongation machinery. ELL/EAF proteins also bind to MED26, a component of the human mediator that plays a key role in transcriptional activation of multiple genes.[45] EAF proteins have additional binding partners, including pVHL and p53.[24,46] EAF2 deletion upregulated the protein level of Hif-1α, a pVHL target protein, and enhanced angiogenesis[46,47] that also could contribute to prostate carcinogenesis. As p53 is a key tumor suppressor involved in multiple cancers including prostate cancer,[48] inactivation of EAF2 could also modulate p53 signaling pathways and subsequently promote prostate carcinogenesis. Furthermore, EAF2 knockout enhanced phosphorylation of ERK, another key regulator of carcinogenesis.[24,46] EAF2 could also negatively regulate canonical Wnt/β-catenin signaling.[49] Therefore, impaired DNA damage repair and multiple altered signaling pathways upon EAF2 deletion/downregulation could act synergistically to promote prostate carcinogenesis.

Our studies reveal an important role of EAF2 in androgen regulation of DNA repair in the prostate. As EAF2 expression is androgen dependent, EAF2 provides a mechanistic link between androgen signaling and DNA repair via Ku70/Ku80. This is supported by the observation that androgen protection against DNA damage in LNCaP and C4-2 cells was inhibited by EAF2 knockdown. Given the importance of EAF2 in DNA damage repair, downregulation of EAF2 is likely an important mechanism leading to radiation sensitization by androgen deprivation therapy.

MATERIALS AND METHODS

siRNAs, transfection and immunoblotting

EAF1 and EAF2 knockdown in cells was performed using siRNA transfected in OptiMEM medium (Life Technologies, Paisley, UK) via Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the recommended siRNA dosages by the manufacturer's instructions. Western analyses were conducted as described in Supplementary Experimental Procedures with antibodies listed in Supplementary Table 1.

Comet assay

LNCaP cells were treated with siRNA for 48 h, then treated with γ-irradiation or doxorubicin and collected for DNA damage analysis. DNA damage was quantified using a neutral comet assay (Comet Assay Kit, Trevigen, Gaithersburgh, MD, USA).

Flow cytometry

LNCaP cells were treated with siRNA for 48 h followed by treatment with various concentrations of Dx for additional 20 h, in the presence of 1 nM of dihydrotestosterone to induce EAF2 expression. Cells were then collected and washed with cold phosphate-buffered saline (PBS) twice and fixed in 70% ethanol at 4 °C for overnight. Cells were washed again twice with cold PBS and resuspended in PBS containing RNase (100 μg/ml) and propidium iodide (50 μg/ml) 30 min before analysis. Experiments were repeated a minimum of two times.

NHEJ and HR assay

H1299 dA3-1#1, a subline of human lung cancer cells generated by transfecting a plasmid DNA containing two I-SceI sites into H1299 cells, was used as a model to assay for NHEJ of chromosomal DSBs. HeLa pDR-GFP cells containing a recombination substrate DR-GFP were used to assay for HR frequency of chromosomal DNA.[50] pCMV-NLS-I-SceI expression vector was introduced by transfection with Lipofectamine 2000 reagent into 1.5×10^5 H1299 dA3-1#1 cells (for NHEJ) or 3.0×10^5 HeLa pDR-GFP cells (for HR) pre-transfected with siRNA for 48 h using Lipofectamin 2000 (Invitrogen). For fluorescence-activated cell sorting (FACS) analysis, cells were harvested by trypsinization, washed with PBS and applied to the FACS caliber apparatus (Becton Dickinson, Franklin Lakes, NJ, USA). EGFP-positive cells were counted using Cellquest software (Cellquest Company, San Jose, CA, USA).

Laser microirradiation

LNCaP cells were treated with 100 μM 8-methoxypsoralen and subjected to laser irradiation as previously described.[51]

Immunoprecipitation

To immunoprecipitate GFP-tagged proteins, whole-cell lysates and anti-GFP tag agarose beads (MBL, Woburn, MA, USA) were combined and incubated. Nuclear extracts were incubated with anti-EAF1 or anti-EAF2 antibodies, or normal mouse IgG and protein A/G PLUS-Agarose beads. Eluted proteins were electrophoresed and analyzed by immunoblot as described previously[52] and in Supplementary Experimental Procedures with antibodies listed in Supplementary Table 1.

γ-Irradiation of mice

Wild-type and EAF2$^{-/-}$ male mice on a C57BL/6J background[24] were subjected to whole-body γ-irradiation. The use and care of mice were approved by the institutional animal care and use committee. See Supplementary Experimental Procedures for details.

Histological and pathological analysis

Human primary prostate specimens included 16 specimens with Gleason scores 6–9 and normal adjacent tissues as well as a tissue microarray composed of 120 prostate cancer specimens with Gleason scores 6–9, 20 benign prostatic hyperplasia specimens and 80 matched normal adjacent specimens. Slides were immunostained and blindly evaluated by a pathologist using a semiquantitative analysis of EAF2 and γH2ax staining as previously described.[15] See Supplementary Experimental Procedures with antibodies listed in Supplementary Table 1 for details.

Statistics

Comparisons between groups were calculated using Student's *t*-test, the two-tailed Fisher's exact test method of summing small *P*-values, the one-way and two-way analysis of variance and Bonferroni's multiple comparison test as appropriate. A value of $P < 0.05$ was considered significant. GraphPad Prism version 6 was used for graphics (GraphPad Software, San Diego, CA, USA). Values are expressed as means ± s.e.m. or mean ± s.d.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)