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Identification of a genetic interaction between the tumor suppressor EAF2 and the retinoblastoma protein (Rb) signaling pathway in *C. elegans* and prostate cancer cells

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ABSTRACT

The tumor suppressor EAF2 is regulated by androgen signaling and associated with prostate cancer. While EAF2 and its partner ELL have been shown to be members of protein complexes involved in RNA polymerase II transcriptional elongation, the biologic roles for EAF2 especially with regards to the development of cancer remains poorly understood. We have previously identified the *eaf-1* gene in *Caenorhabditis elegans* as the ortholog of EAF2, and shown that *eaf-1* interacts with the ELL ortholog *ell-1* to control development and fertility in worms. To identify genetic pathways that interact with *eaf-1*, we screened RNAi libraries consisting of transcription factors, phosphatases, and chromatin-modifying factors to identify genes which enhance the effects of *eaf-1(tm3976)* on fertility. From this screen, we identified *lin-53*, *hmg-1.2*, *pha-4*, *ruvb-2* and *set-6* as hits. LIN-53 is the *C. elegans* ortholog of human retinoblastoma binding protein 4/7 (RBBP 4/7), which binds to the retinoblastoma protein and inhibits the Ras signaling pathway. We find that *lin-53* showed a synthetic interaction with *eaf-1(tm3976)* where knockdown of *lin-53* in an *eaf-1(tm3976)* mutant resulted in sterile worms. This phenotype may be due to cell death as the treated worms contain degenerated embryos with increased expression of the *ced-1:GFP* cell death marker. Further we find that the interaction between *eaf-1* and *lin-53/RBBP4/7* also exists in vertebrates, which is reflected by the formation of a protein complex between EAF2 and RBBP4/7. Finally, overexpression of either human EAF2 or RBBP4 in LNCaP cells induced the cell death while knockdown of EAF2 in LNCaP enhanced cell proliferation, indicating an important role of EAF2 in controlling the growth and survival of prostate cancer cells. Together these findings identify a novel physical and functional interaction between EAF2 and the Rb pathway.

1. Introduction

We previously reported the identification of the EAF2 ortholog *eaf-1* in *Caenorhabditis elegans*. The EAF-1 protein forms a protein complex with the ELL ortholog ELL-1, and together these proteins control fertility, body size, and the expression of cuticle collagens in worms [1]. Despite these important functions, genetic deletion of the *eaf-1* gene in the worm is not lethal but leads to a reduction in fertility, where *eaf-1* mutants produce ~150 offspring instead of the normal 300. The preserved viability and fertility of the *eaf-1* mutants could indicate the existence of the parallel pathways which are partially redundant with *eaf-1*.

In this study, we used a targeted RNAi screen to search for genetic pathways that could compensate for the loss of *eaf-1* to the preserved fertility. Via this approach we identified a novel genetic interaction between *eaf-1* and *lin-53*, the worm homolog of retinoblastoma (Rb) binding proteins 4 and 7 (RBBP4/7). The Rb signaling pathway is involved in the development of the ovary and testis [2,3], as well as inhibiting the formation of prostate and breast cancer [4–6]. Like *Eaf2*, the loss of *Rb1* alone gives rise to an early stage of prostate cancer in the mouse, whereas a loss of both *Rb1* and *p53* yields a more significant effect [7]. The tumor suppressor genes, such as *p53*, *Rb1* and *BRCA2* [7,8], can interact with other

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genetic factors to control the tumor progression. Hence, it is possible that EAF2 functionally and/or physically interacts with the Rb pathway, and that this interaction is conserved from *C. elegans* to humans and plays important roles in *C. elegans* development and human cancer. We have validated this idea by testing the protein–protein interactions between the human orthologs RBBP4 and EAF2. Also, we have discovered their overlapping roles in controlling cell death in prostate cancer cells, implicating their paralleled functions in mammals. This is the first time *C. elegans* was used as a model system to identify pathways that intersect with the tumor suppressor gene *EAF2*. The discovery of EAF2-Rb1 genetic interaction might open a new avenue for understanding the genetic control of cancer progression.

2. Materials and methods

2.1. C. elegans strain, worms strains and breeding

Wild type N2; MR142 (*cdc-25.1(rr31) I; rrIs1(elt-2p:NLS-GFP)*; and DZ325 (*ezIs2 III; him-8(e1489) IV, ezIs2[fkh-6::GFP + unc-119(+)]*) were obtained from the *C. elegans* Genetics Center, which is supported in part by NIH funding. ALF50 (*eaf-1(tm3976)*) was described previously [1]. ZH231 *unc-76*(*e911*) *V*; *enIs7* [*Pced-1 ced-1*::*GFP* + *unc-76*(+)] *X* [9] was generously provided by Zheng Zhou (Baylor College of Medicine, Houston, TX). The *rrIs1(elt-2p:NLS-GFP)* transgene in a wild-type background was generated by outcrossing MR142 with N2.

2.2. RNAi libraries and individual RNAi clones

Three RNAi libraries used in this study were ordered from Source Bioscience (Table 1). The RNAi clones for *hmg1.2*, *lin-53* and *ruvb-1* were retrieved from the Ahringer RNAi library [10]. The *ell-1* RNAi clone was previously described [1]. All RNAi clones were validated by DNA sequencing.

2.3. RNAi screening and treatment

The RNAi screen using each library was performed in 12-well plates containing NGA agar plus 1 mM IPTG and 200 mg/ml ampicillin. Pairs of wells were then spotted with individual RNAi clones from a fresh overnight culture grown in LB media containing 200 mg/ml ampicillin. This design resulted in each RNAi clone being screened in duplicate to demonstrate reproducibility of the screen results. Hits were defined by the reduction in F2 progeny numbers produced by RNAi treated *eaf-1* mutants compared to N2. RNAi treatment using individual RNAi clones was performed as previously described [1]. Nomarski and fluorescent imaging was performed using a BX51 Olympus microscope and DP70 digital camera.

2.4. Generation of DNA constructs, protein co-immunoprecipitation and Western blot

The *RBBP4-GFP* construct was generated by traditional DNA cloning, and the resulting plasmid DNA was validated by DNA sequencing. The *EAF2-Myc* and *GFP-EAF2* DNA constructs were previously described [1]. Transfection of HEK293 cells, cell lysate co-immunoprecipitation, PAGE electrophoresis and Western blotting procedures were previously described [1]. The antibodies used in this study are listed as follows: Anti-Myc tag antibody agarose (Abcam, ab1253); Anti-Myc antibody (Thermo Scientific, clone 9E10); Anti-GFP antibody (Torrey Pines Biolabs, TP401).

Table 1
RNAi libraries used in this study.

Phosphotase library (166 clones)
Transcription factors library (387 clones)
Chromatin factors library (257 clones)

2.5. Immunostaining of transfected cells and BrdU staining

The LNCaP cells were grown in glass coverslips inserted into 12-well plates. The antibody immunostaining was carried out as previously described [11]. Briefly, the transfected LNCaP cells were fixed with 4% paraformaldehyde at 4 °C overnight. On next day, the fixed cells were rinsed with 1× PBS with 0.1% Triton-100 for three times, followed by blocking with 10% normal goat serum for 1 h at room temperature. Subsequently, the first antibody, the secondary antibody incubation, and DAPI staining were applied. Photoimages were taken via a Zeiss compound fluorescent microscope. The cleaved caspase-3 (Asp175) antibody was purchased from Cell Signaling (#9664) and the Alexa Fluor® 488 Goat anti-Rabbit antibody from Life Technologies (#A-11008).

We used a BrdU incorporation assay to study the effect of the human *EAF2* gene on cell proliferation. Briefly, LNCaP cells were transfected with control (Santa Cruz, sc-37007) or EAF2 siRNA (Santa Cruz, sc-62251) in the presence of 1 nM methyltrienolone (R1881) (Perkin Elmer, CAS# 965-93-5) for 48 h. These cells were then allowed to incorporate 10 μM 5-bromo-2′-deoxyuridine, BrdU (Sigma, B5002) for 6 h. BrdU staining was performed using a standard protocol with a primary anti-BrdU antibody from Sigma (#B-2531) and a Cy3 conjugated-goat anti-mouse secondary antibody from Life technologies (A10521). SYTOX® Green (Life technologies, S7020) was used to label the nuclei.

2.6. Colony formation in LNCaP cells

The colony formation assays were performed as previously described [12]. 1 μg of DNA plasmids (*GFP-EAF2*, *GFP-RBBP4* and *GFP*) were transfected into LNCaP cells in a 6-well plate. After 24 h, transfected cells were distributed into four 10-cm dishes with 1 mg/ml G418 (Gemini Bio-Products, catalog# 400-111P). The culture media was changed twice per week. After three weeks, the visible colonies formed (around 1 mm in diameter) were counted and the GFP signals were visualized using a fluorescent microscope.

3. Results

3.1. RNAi library screen and identification of eaf-1 synthetic enhancers

We treated both *eaf-1(tm3976)* mutants as well as the wild type N2 worms with RNAi in 12-well plates spotted with clones from these three RNAi libraries (Table 1). Fertility was assessed by noting the number of F2 progeny and comparing the difference between the N2 and *eaf-1(tm3976)* mutant worms (Fig. 1A). Based on these criteria, we identified five candidate genes which markedly reduced the fertility of *eaf-1(tm3976)* compared to N2, which are *lin-53*, *pha-4*, *hmg-1.2*, *ruvb-1* and *set-6*. All of the five genes encode the nuclear proteins involved in chromatin remodeling and the activation of gene transcription (Table 2), which is consistent with the known role of EAF2 in transcriptional regulation.

We chose to focus on *lin-53* for further study since the *C. elegans* LIN-53 protein acts as part of the worm RB complex and is the homolog of human RB binding proteins RBBP4/7 [13]. In *C. elegans*, LIN-53 is a member of the Class B SynMuv protein family, and is also part of several distinct transcriptional repressor protein complexes, such as NuRD (the nucleosome remodeling and deacetyl-

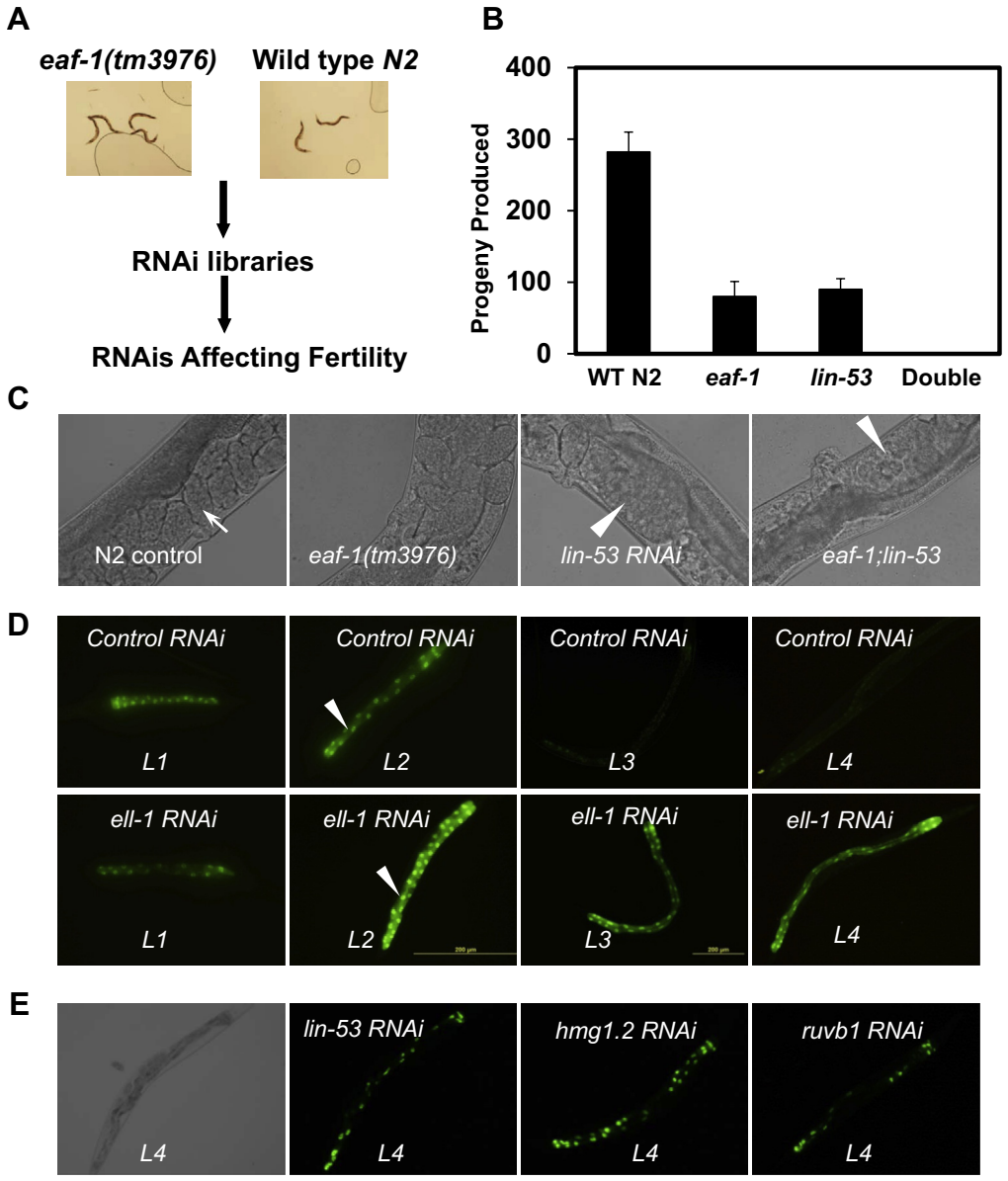

Fig. 1. Synthetic interaction between *eaf-1* and *lin-53* affects *C. elegans* fertility and gene expression in the intestine. (A) A genetic screen using RNAi libraries (RNAis specific for the transcription factors, chromatin factors and phosphatase/kinases) was designed to identify the genetic enhancers working with *eaf-1* in the regulation of *C. elegans* fertility. (B) Treatment of the *eaf-1* mutant with RNAi against *lin-53*, one of the candidates identified, resulted in a greater reduction in fertility. (C) The *eaf-1(tm3976); lin-53 RNAi* treated worms showed extensive embryo degeneration which was seen to a lesser degree in the *lin-53 RNAi* treated worms. The white triangle points the degenerated embryos while the arrow points the intact embryo. (D) The genetic enhancers identified from the screen share the same feature of regulating the expression of an *elt2:GFP* transgene in the intestine. The expression of the *elt-2:GFP* transgene in *C. elegans* decreases during larval development and is strongly down-regulated in L4 larval animals. Treatment of *elt-2:GFP* transgenic worms with *ell-1 RNAi* increases *elt-2:GFP* expression with GFP expression now being observed in all larval stages. The *eaf-1* enhancers (*lin-53*, *hmg1.2*, *ruvb1*, *set-6* and *pha-4*) observed in our RNAi screen have similar effects as *ell-1(RNAi)* on the expression of *elt-2:GFP* expression in the intestine. The white triangles in the panel D point the intestinal cells expressing nuclear-localized GFP.

Table 2
Genetic enhancers identified by the RNAi library screen.

lin-53/rba-2: Nucleosome remodeling factor, subunit CAF1/NURF55/MSI1
hmg-1.2: HMG box-containing protein
pha-4: Forkhead/HNF-3-related transcription factor
ruvb-2: DNA helicase TIP49, TBP-interacting protein
set-6: Histone H3 (Lys9) methyltransferase SUV39H1/Clr4, required for transcriptional silencing

ase) and DRM (DP, Rb and MuvB complex). In parallel with the Class A SynMuv protein family, LIN-53 regulates vulva formation through the EGFR/RAS/MAPK signaling pathway [14,15]. Of note, both of these tumor suppressor complexes are conserved in humans and control cancer invasiveness [16,17].

While the *eaf-1* mutation or *lin-53* RNAi treatment reduced the fertility of worm, the treatment of the *eaf-1* mutant with *lin-53* RNAi resulted in complete sterility of the treated worms (Fig. 1C). The developing embryos in the *lin*-53 RNAi treated *eaf-1* mutants were degenerated showing largely acellular clumps instead of intact embryos nicely demarked and aligned in the ventral region of the wild-type N2 worms (Fig. 1C). To a lesser degree, the single *eaf-1* mutants or the worms treated with *lin-53* RNAi also developed these degenerated embryos which might account for the reduced progeny numbers when each individual gene was inhibited (Fig. 1C).

Since both *C. elegans* LIN-53 and human RBBP4/7 can change chromatin structure and subsequently alter gene expression [18,19], we explored whether inhibiting *eaf-1* function via treating worms with RNAi against the *eaf-1* partner *ell-1* or *lin-53* RNAi could change gene expression. To test this hypothesis, we used the *elt-2::GFP* transgenic worms as the marker for gene expression. This *elt-2::GFP* transgene is exclusively expressed in the nucleus of the intestinal cells, and normally *elt-2::GFP* expression gradually vanished during larval development (Fig. 1D). Interestingly, *ell-1 RNAi* treatment enhanced *elt-2::GFP* expression in the intestine indicating an essential role of *ell-1* in silencing of *elt-2* expression (Fig. 1D). We have tested all five candidates identified from this screen for their effects on *elt-2::GFP* expression. Surprisingly, all of the *eaf-1* genetic enhancers showed the same effects as *ell-1 RNAi*. In particular, L4 larval RNAi treated worms retained highly expressed *elt-2::GFP* (Fig. 1D and E). This results support the hypothesis that *C. elegans eaf-1* and its genetic enhancers work in the same regulatory pathway to control chromatin structure and gene expression at least in the *C. elegans* intestine.

3.2. Human EAF2 and RBBP4/7 physically interact in human cells and form nuclear speckles

To explore whether interactions between the tumor suppressors genes *EAF2* and *RBBP4/7* might also occur in vertebrate cells, we performed a co-immunoprecipitation experiment. As shown in Fig. 2, the transfected EAF2 and RBBP4 proteins formed a complex and were co-immunoprecipitated. We have found a similar complex with the human EAF2 and the RBBP7 protein, which is closely related to RBBP4, in transfected PC3 cells (Fig. 2B). These findings suggest that there are physical interactions between the tumor suppressors EAF2 and RBBP4/7 in human cells.

EAF2 and its binding partner ELL were reported to regulate chromatin structure and gene expression [20,21]. Previously we observed the nuclear speckle formation in the cells overexpressing EAF2 and ELL [22]. To further this finding, we overexpressed the proteins in 293 cells. Interestingly, double expression of RBBP4 and ELL gave rise to the nuclear speckle formation (Fig. 2C), while single expression of RBBP4, ELL or EAF2 alone did not form any speckles. This suggests that the protein–protein interaction/complex is essential for this nuclear structure formation. Moreover, the nuclear speckles by the RBBP4-ELL overexpression consisted of one or two bigger GFP fluorescent dots, compared to the multiple smaller dots by the EAF2-ELL overexpression in 293 cells (Fig. 2C). The difference of the nuclear speckles might implicate diverse roles of these proteins in the cell.

3.3. EAF2 and RBBP4 induce the cell death in prostate cancer cells

Our previous studies demonstrated that EAF2 acts as a tumor suppressor by promoting tumor cell apoptosis [23]. We also found that the transfection of RBBP4 can induce cell death in LNCaP cells

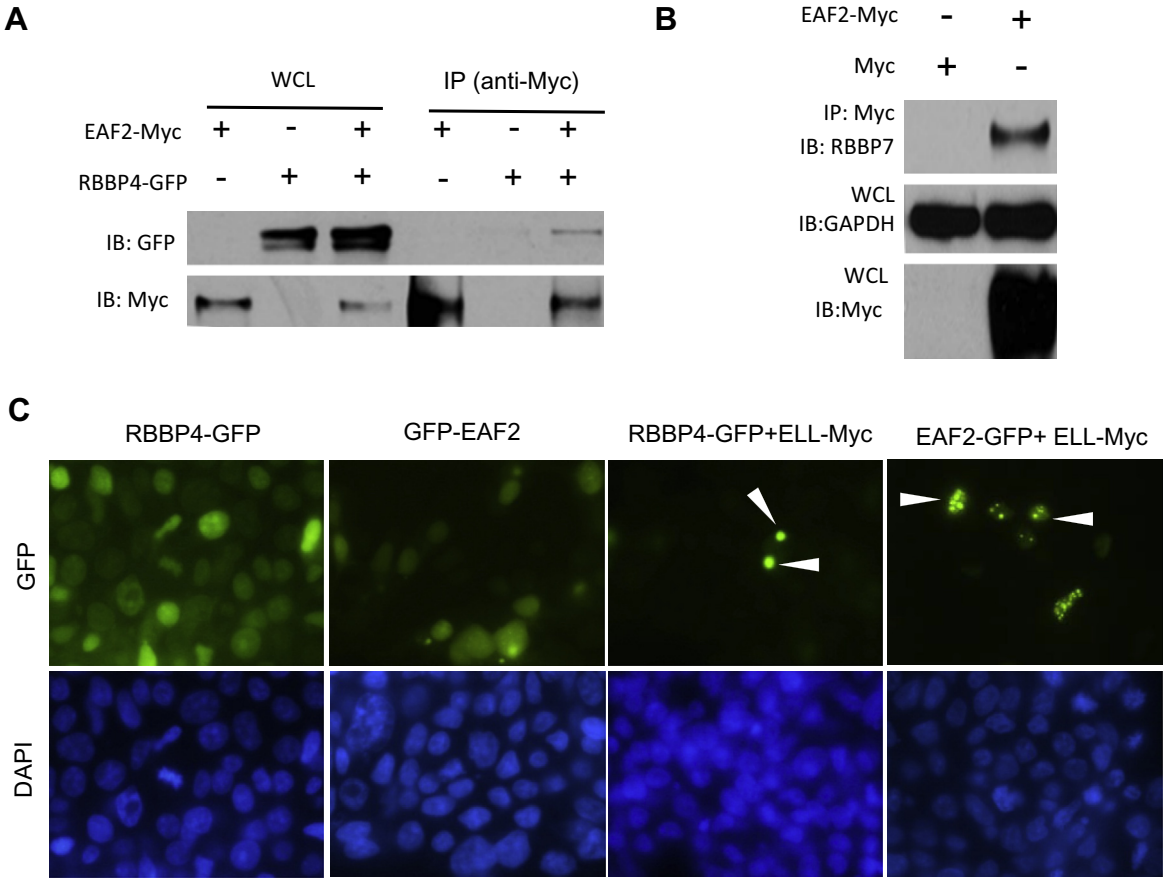

Fig. 2. Human EAF-2 and RBBP4/7 form a protein complex in the nucleus. (A) Western blot showing the co-immunoprecipitation of RBBP4 with EAF2 from transfected 293 cells. The cells were transfected with the human *EAF2-Myc* and *RBBP4-GFP*. The cell lysates were incubated with anti-Myc antibody coupled to agarose and after washing the precipitates were run on an SDS–PAGE gel and used for Western blotting with either anti-GFP or Myc antibodies. WCL: whole cell lysate. (B) Western blot showing the co-immunoprecipitation of EAF2 and RBBP7 from PC3 cells. The whole cell lysate from the PC3 cells transfected with the *EAF2-Myc* DNA was incubated with the anti-Myc antibody linked to beads, washed, and then used for a Western blot with anti-RBBP7 antibody. (C) Nuclear speckles are observed in human RBBP4-GFP and ELL-Myc co-transfected 293 cells indicating the RBBP4-ELL protein interaction might alter chromatin structure. In contrast, single expression of RBBP4-GFP or GFP-EAF2 did not give rise to these nuclear speckles. The white triangles point the nuclear speckles. Note the speckles with two bigger dots in RBBP4-ELL overexpressed cells while the speckles with multiple smaller dots in EAF2-ELL overexpressed cells.

(Fig. 3). We performed a time course study to follow the death of LNCaP cells transfected with *GFP-EAF2*, *RBBP4-GFP* or *GFP* control plasmid DNA. On Day 3 post transfection, there were a great number of fluorescent cells lost in among both *GFP-EAF2* and *RBBP4-GFP* transfected LNCaP cells, while GFP expressing LNCaP cells remained as relatively stable percentage of the total cell number (Fig. 3A). Moreover, on Day 6 of transfection, the fluorescent cells in both *GFP-EAF2* and *RBBP4-GFP* transfection groups were essentially lost, compared to a larger number of fluorescent cells seen in the *GFP* transfected LNCaP cells. To further determine if the loss of *EAF2* and *RBBP4* expressing cells was due to apoptosis, we performed immunostaining with the active caspase-3 antibody. We found that the anti-caspase-3 positive cells both showed *GFP-EAF2* or *RBBP4-GFP* expression and also contained abnormal nuclei as seen by DAPI staining (Fig. 3B). We also confirmed the findings by using Western blotting of caspase-3 on lysates from LNCaP cells expressing *GFP-EAF2* or *RBBP4-GFP* 48 h after transfection (Fig. 3C).

To examine whether *eaf-1* and *lin-53* affect cell death in *C. elegans*, we examined the expression of CED-1 in *ced-1p:ced-1:GFP* transgenic worms. CED-1 is a transmemberane protein involved in the apoptosis pathway and specifically labels the dying cells [24]. *lin-53 RNAi* treatment produced an increase in cell death in the developing embryos (Fig. 4A), indicating an essential role for *lin-53* during embryogenesis in *C. elegans*. The *eaf-1(tm3976)* mutation produced little change in CED-1:GFP expression (Fig. 4A). However the treatment of *eaf-1* mutants with *lin-53 RNAi* lead to the prominent expression of CED-1:GFP in the degenerated embryos (Fig. 4A).

We also performed a colony formation assay aiming to investigate the effects of this functional interaction on tumor cell growth.

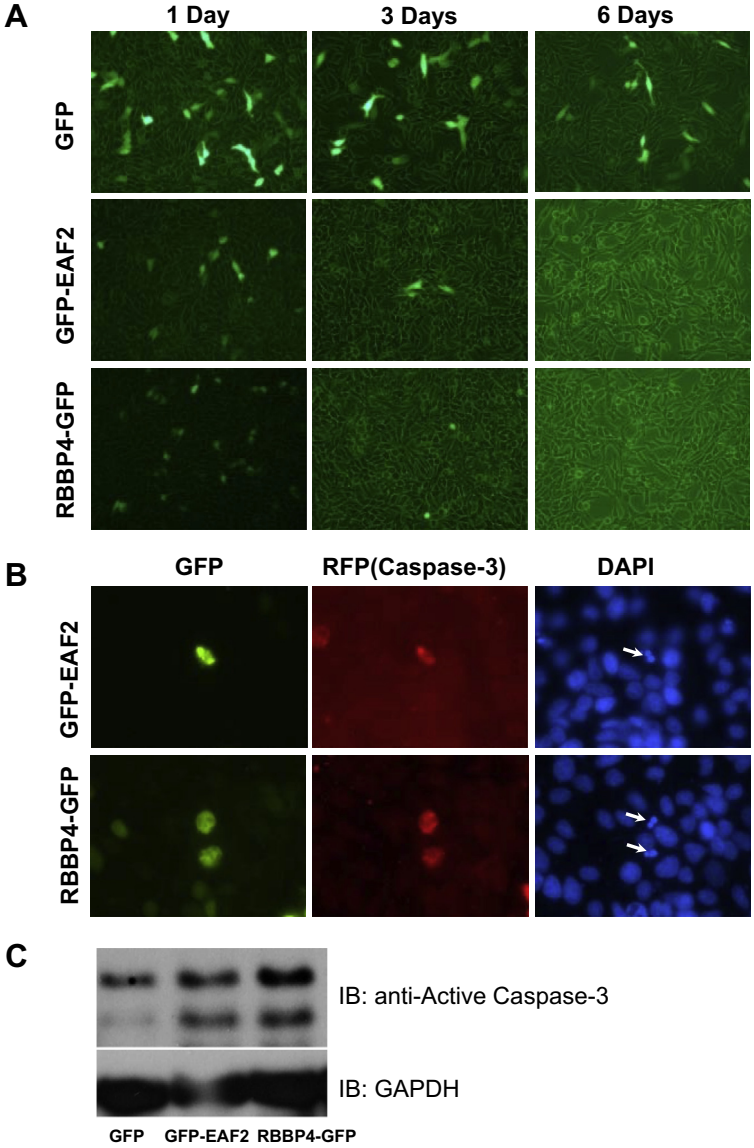

Fig. 3. EAF2 and RBBP4 induce cell death in LNCaP cells. (A) LNCaP cells were transfected with *GFP-EAF2*, *RBBP4-GFP* and *GFP* empty vector plasmids and the GFP expressing cells were visualized 1 day, 3 days and 6 days after transfection via fluorescence microscopy. GFP positive cells were readily seen even 6 days after transfection among the *GFP* transfected cells, whereas GFP positive cells were progressively lost starting 3 days after transfection among the *GFP-EAF2* and *RBBP4-GFP* transfected cells. (B) Immunostaining with an anti-active caspase-3 antibody demonstrated the co-localization of active caspase-3 with *GFP-EAF2* or *RBBP4-GFP* expression, indicating the induction of apoptosis in these transfected LNCaP cells 48 h after transfection. Moreover, these caspase-3 positive cells showed DNA condensation as visualized by DAPI staining (Arrows point the condensed nuclei). (C) Western blotting with the anti-caspase-3 antibody further demonstrated that active caspase-3 protein expression was increased in LNCaP cells transfected with *GFP-EAF2* or *RBBP4-GFP* after 48 h, compared to the *GFP* empty vector control transfected cells.

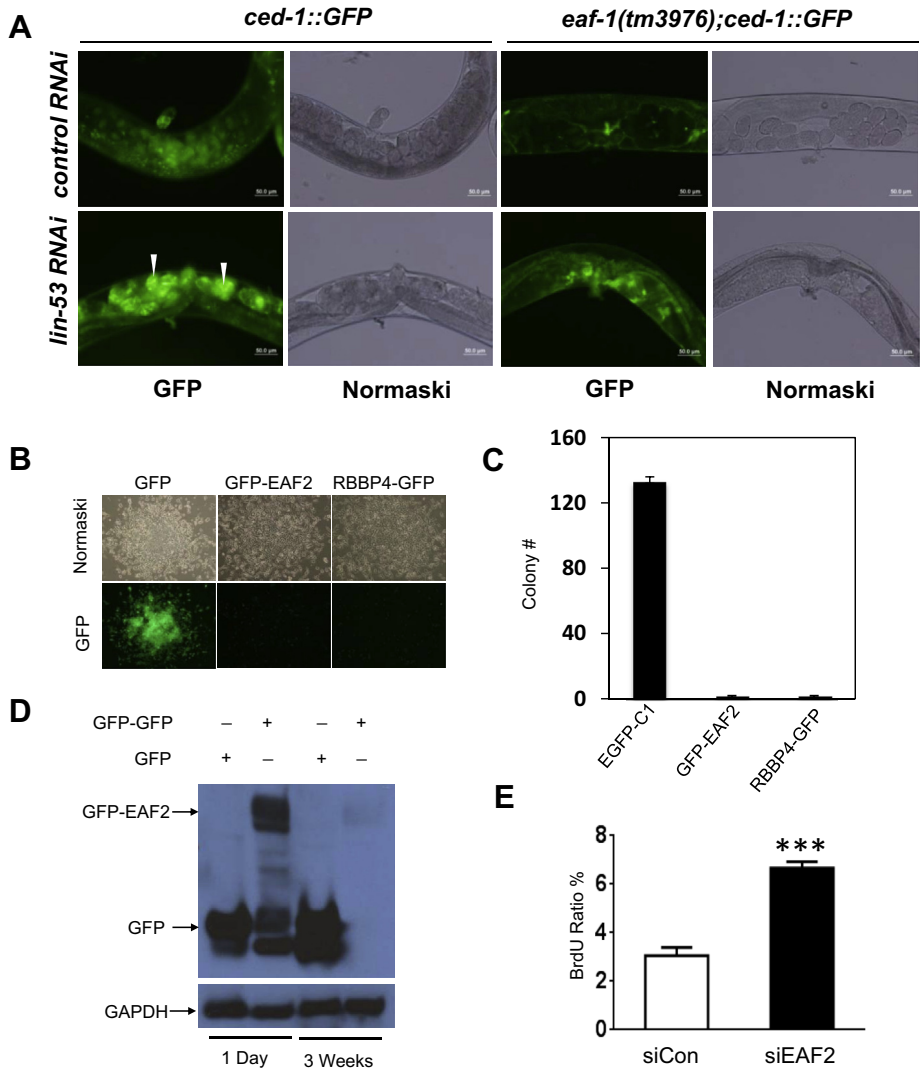

Fig. 4. The conserved effects on cell death for EAF2 and RBBP4 in prostate cancer cells and *C. elegans*. (A) The *ced-1p:ced-1:GFP* worm strain was used to visualize the cell death. Control RNAi treated and *eaf-1(tm3976)* mutant worms showed little expression of CED-1:GFP. In contrast, *lin-53* RNAi treated and particularly *lin-53* RNAi treated *eaf-1(tm3976)* mutant worms show a marked increase in CED-1:GFP expression consistent with an increase in cell death. The white triangles point to the dying embryos. (B) A colony formation assay performed using LNCaP cells showed no GFP positive colonies among the *GFP-EAF2* or *RBBP4-GFP* transfected LNCaP cells after 3 weeks of selection, while many GFP expressing colonies were observed among the *GFP* transfected LNCaP cells. (C) Quantification of the colony numbers from the colony formation assay. (D) The loss of GFP expression was also seen by Western blotting using lysates from the colonies observed after 3 weeks of selection. (E) Knockdown of *EAF2* with siRNA increases LNCaP cell proliferation. $^{***}P \leqslant 0.001$.

LNCaP cells were transfected with the *GFP-EAF2*, *RBBP-GFP* or *GFP* DNA plasmids and subsequently treated with G418 (neomycin) to selectively grow the transfected cells. After three weeks, The *GFP* vector alone transfected LNCaP cells formed colonies expressing GFP, while the *GFP-EAF2* and *RBBP4-GFP* transfected LNCaP cells formed fewer colonies, but none of them were fluorescent (Fig. 4B and C). This could reflect the selective loss of the *GFP-EAF2* or *RBBP4-GFP* genes compared to the neomycin resistance gene, however these DNA fragments are located on the same plasmid while being driven by different promoters. Further, the expression of the GFP-*EAF2* protein was lost in these colonies; compared to the highly expressed GFP protein in the colonies derived from the GFP transfected LNCaP cells as shown by Western blot (Fig. 4D). This absence of *GFP-EAF2* expressing cells is consistent with our finding that EAF2 functions as a cell death inducer [25]. To further characterize the role of EAF2, we tested the effect EAF2 knockdown on cell proliferation in LNCaP model. Knockdown of *EAF2* via siRNA increased the BrdU staining positive LNCaP cells which indicates that *EAF2* normally acts to inhibit cell proliferation perhaps through promoting apoptosis (Fig. 4E).

4. Discussion

This study identified a conserved interaction between EAF2 and RB1 which occurs in both *C. elegans* and prostate cancer cells. Human EAF2 and RB1 are tumor suppressor proteins, with the RB1 gene reported to be frequently mutated in various cancers including prostate cancer [26]. Meanwhile, mutant animals lacking only one of these two genes can only show the early stages of cancer development [5,23]. Therefore, the genetic interaction between human *EAF2* and *RB1* might block the progression to the later stages of prostate cancer development. Further data from clinical evidence and animal models will be very useful to solidify this hypothesis.

How these two tumor suppressors interact in human cancer cells remains elusive. We showed that each protein can promote cell death, which is consistent with previous studies involving the retinoblastoma protein [27], RBBP7 [28], and EAF2 [29]. It is highly possible that in the prostate, these two pathways interact to control the cell cycle progression and/or the cell death.

Alternatively, EAF2 and RB1 might alter chromatin structure and gene expression. The nuclear speckles were proposed in this study as a possible marker for the gene transcription or the RNA polymerase II activity. This concept is supported by several studies involving the ELL and EAF family proteins, including the Drosophila ELL [21] and human EAF2 [20]. It will be important to elucidate the biological roles of these nuclear speckles, especially in the setting of cancer progression.

Acknowledgments

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