Biochemical and Biophysical Research Communications 447 (2014) 292–298

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Liquan Cai [a], Dan Wang [a], Alfred L. Fisher [b,c,d,*], Zhou Wang [a,d,*]

[a] Department of Urology, The University of Pittsburgh, 5200 Centre Avenue, Pittsburgh, PA 15216, United States
[b] Division of Geriatrics, Gerontology, and Palliative Medicine, Department of Medicine, UTHSCSA, San Antonio, TX 78229, United States
[c] Center for Healthy Aging, UTHSCSA, San Antonio, TX 78229, United States
[d] GRECC, STVAHCS, San Antonio, TX 78229, United States

ARTICLE INFO

*Article history:*
Received 25 March 2014
Available online 12 April 2014

*Keywords:*
The tumor suppressor EAF2
Retinoblastoma protein
*lin-53*
*RNAi* library screen
*C. elegans*
Apoptosis

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

* Corresponding authors. Address: Division of Geriatrics, Gerontology, and Palliative Medicine, Department of Medicine, UTHSCSA, San Antonio, TX 78229, United States. Fax: +1 210 617 5312 (A.L. Fisher). Address: Department of Urology, The University of Pittsburgh, 5200 Centre Avenue, Pittsburgh, PA 15216, United States. Fax: +1 412 623 3907 (Z. Wang).
*E-mail addresses:* fishera2@uthscsa.edu (A.L. Fisher), wangz2@upmc.edu (Z. Wang).

http://dx.doi.org/10.1016/j.bbrc.2014.03.138
0006-291X/

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-