EAF2 Associates with FOXA1 and EAF2 Alleviates FOXA1-Mediated Repression of Androgen Receptor Transactivation

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Anne Lipton Keener, PhD

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Prostate Cancer (PCa) is the most commonly diagnosed cancer and the second most common cause of cancer death in men in the United States. During the development of PCa, several androgen receptor (AR) regulated tumor-suppressors are down regulated, including ELL-associated factor 2 (EAF2), also known as androgen up-regulated 19 (U19), an RNA polymerase II transcription elongation factor. In order to determine how EAF2 is regulated and find other genes that interact with EAF2, an RNAi screen was performed in *C. elegans*. When *eaf-1*, the *C. elegans* ortholog to EAF2 was knocked out, the resulting worms had reduced fertility and the screen was designed to detect phenotypic enhancers, RNAis that increased the *eaf-1*KO reduced fertility phenotype. Knockdown of the gene *pha*-4, the *C. elegans* ortholog to Forkhead Box A1 (FOXA1), was found to cause egg degeneration rendering the *eaf-1*KO worms sterile. FOXA1, a pioneer factor for AR, is required for normal differentiation of prostatic epithelial cells and up-regulation of FOXA1 is associated with a worse prostate cancer prognosis in advanced prostate cancer. We hypothesized that EAF2 associates with FOXA1 to modulate AR transactivation. Transfected FOXA1 associated with EAF2 when co-immunoprecipitated and FOXA1 associated with both the N-terminus and C-terminus of EAF2. Co-expression of EAF2 and FOXA1 reduced EAF2 and FOXA1 protein levels relative to EAF2 or FOXA1 alone. FOXA1 protein level increased and PSA protein and mRNA levels decreased when EAF2 was knocked down in human PCa cell line LNCaP by siRNA. Similarly, EAF2 knockout also increased FOXA1 protein level in the mouse prostate. In a luciferase assay, FOXA1 reduced PSA-promoter expression in C4-2 prostate cancer cells, and EAF2 transfection alleviated FOXA1-mediated repression of PSA. In a colony formation assay performed in LNCaP cells, over-expression of EAF2 reduced the number of colonies, over-expression of FOXA1 increased the number of colonies, and over-expression of FOXA1 and EAF2 produced an intermediary number of

colonies. These findings suggest a potential functional and physical interaction of EAF2 and FOXA1 that may represent a new pathway and could potentially play a role in controlling growth in the normal prostate and prostate cancer.

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1.0 INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of cancer death in men in the US (*1*). Prostate cancer results from the accumulation of mutations that affect regulatory pathways controlling cell growth, differentiation, and death. ELL-associated factor 2, also known as androgen up-regulated 19, (EAF2/U19) is a putative transcription factor and was discovered to be a protein up-regulated by the androgen receptor in the normal prostate. Over-expression of EAF2 induces apoptosis in prostate cancer cell lines while EAF2 is down-regulated in the majority of human prostate cancer specimens (*2*). This shows that EAF2 plays a role in maintaining prostate homeostasis, which is dysregulated in prostate cancer. Furthermore, loss of EAF2 in mice results in prostatic intraepithelial neoplasia, the putative precursor of prostate cancer (*3*).

One important question is which proteins EAF2 interacts with and the effect of that interaction, on the prostate. An RNAi screen was performed in the *C. elegans* model to find genes that can enhance the reduced fertility phenotype caused by loss of *eaf-1*, the *C. elegans* ortholog of EAF2 *(4)*. These studies observed that knockdown of *pha-4* resulted in reduced fertility in wild-type worms, but knockdown of *pha-4* in *eaf-1* knockout worms resulted in sterility caused by egg degeneration as can be seen in Figures 7 and 8 in section 1.3.1. These results suggested that EAF2 and FOXA1 may functionally interact in mammals and the interaction may regulate cell growth, development, and differentiation. The mammalian ortholog

of *pha-4* is FOXA1 (*5*). FOXA1 is a pioneer factor for the androgen receptor (*6*); FOXA1 is required in the development of prostate epithelial cells due to the inability of Foxa1$^{-/-}$ prostate progenitor cells to give rise to luminal epithelial cells or luminal epithelial cell-lined ductal structures (*7*). Furthermore, up-regulation of FOXA1 in prostate cancer patients is associated with a poor prognosis (*8*). The combination of the experimental results and the known literature about EAF2 and FOXA1 suggested that EAF2 and FOXA1 interact and the interaction of FOXA1 and EAF2 affects cell growth and differentiation. We hypothesized that EAF2 associates with FOXA1 to modulate androgen receptor transactivation and that loss of EAF2 shifts androgen-receptor signaling towards increased growth via FOXA1, leading to prostatic intraepithelial neoplasia and prostate cancer. Figure 1 illustrates this hypothesis. This research will give a better understanding of the role of EAF2 in prostate development and prostate cancer.

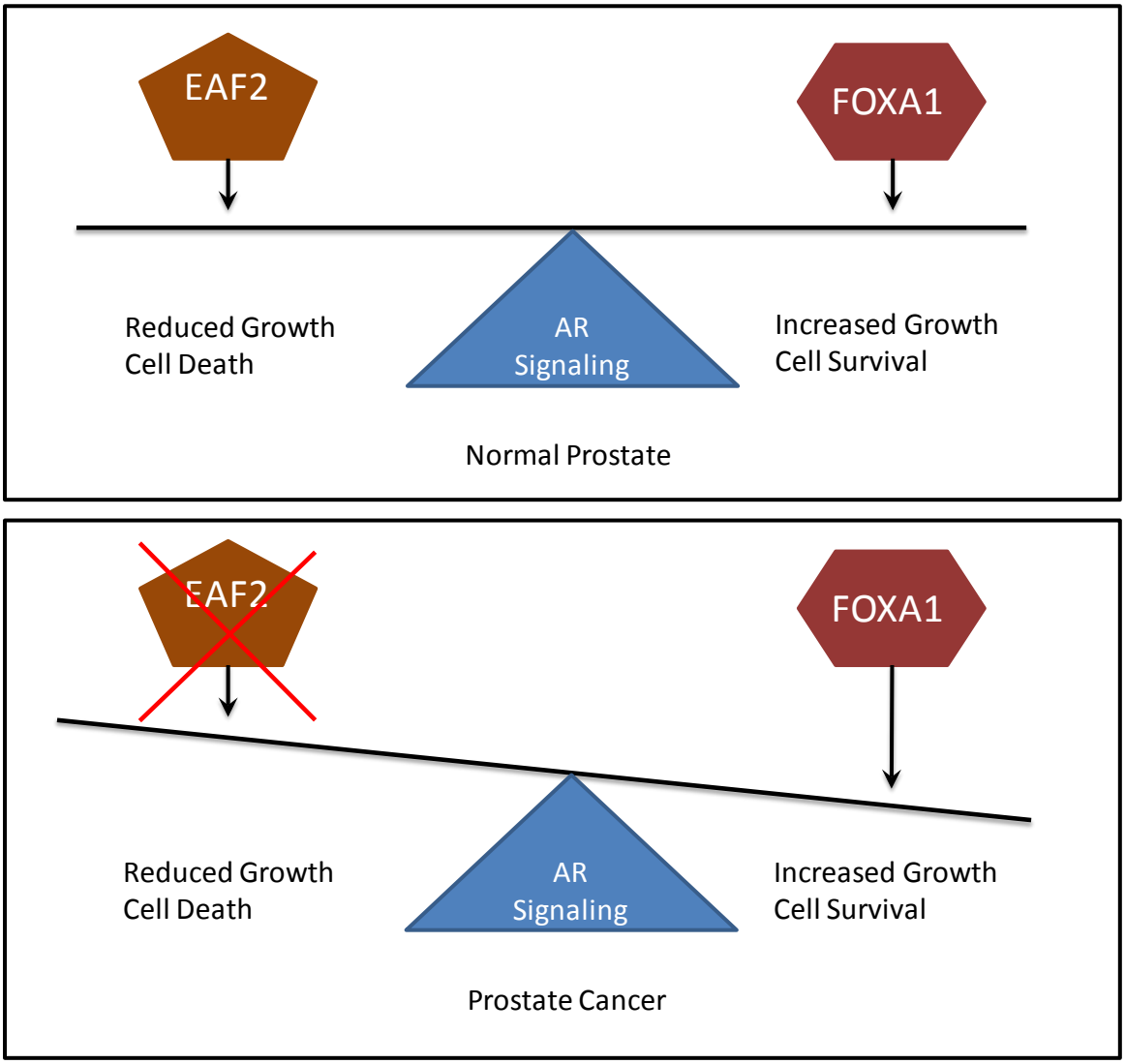

Figure 1. Hypothesized model of the interaction of FOXA1 and EAF2 modulating androgen receptor signaling

In the normal prostate epithelial cells, FOXA1 and EAF2 are both expressed. EAF2 suppresses growth and promotes cell death while FOXA1 promotes cell growth and suppresses cell death. The expression of both results in an intermediary phenotype and prostate homeostasis. In prostate cancer, EAF2 expression is reduced in early stage cancer and absent in advanced prostate cancer. Loss of EAF2 upsets the balance between increased proliferation signals and decreased proliferation signals, leading to increased cell growth and survival.

1.1 PROSTATE CANCER AND PROSTATE DEVELOPMENT

1.1.1 Prostate Cancer

The focus of this research was on furthering our understanding of the roles of EAF2 in prostate cancer and prostate development. Prostate cancer is one of the cancers that, like breast cancer, is reliant on a steroid receptor for growth and survival (*9, 10*). While PCa is characterized by origination in the prostate and is usually dependent on AR, PCa is heterogeneous in its sensitivity to androgen receptor and the outcomes of prostate cancer vary dramatically (*11*). Some tumors will be aggressive and proceed quickly to metastatic and castration-resistant prostate cancer, others are indolent. In addition, the standard first-line treatment for prostate cancer is prostatectomy or radiation supplemented with androgen deprivation therapy (ADT) which is achieved by physical or chemical castration (*9*). However, most prostate tumors that are treated with ADT eventually reach a state known as castration-resistant prostate cancer, where although the cancer is still dependent on AR for growth and survival, it is no longer dependent on circulating androgens (*12*). Common mechanisms by which prostate cancer escapes ADT include AR hyper-sensitization, AR gene amplification, AR over-expression, AR mutation which includes AR gene translocations, post-transcriptional modifications of AR, and intra-tumoral synthesis of androgen (*13*).

Prostate cancer is rarely diagnosed before age 40, when circulating androgen levels are highest (*10, 14, 15*). As has been reported previously, incidence of prostate cancer is sharply correlated with age and the rate of increase is the highest of all cancers after the age of 40 but decreases steadily with age (*10, 16*). Prostate cancer tends to be diagnosed at a time when the androgen/estrogen ratio is decreasing which means there is less circulating androgen available to

activate the androgen receptor (*10, 15*). As reported in Morley, J. E. *et al.*, 1997, the levels of circulating testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH), and sex hormone-binding globulin (SHGB) were measured in 39 subjects who were 61-87 years old at the time the New Mexico Aging Process Study began and took part in the study for 14 years (*15*). Testosterone levels were significantly decreased, while FSH, LH and SHGB levels were significantly elevated when compared to the original serum levels and FSH and LH levels correlated but testosterone levels did not (*15*). As reported in Isbarn, H. *et al.*, 2009, direct injections of testosterone did not increase a man's risk of developing prostate cancer, cause a recurrence of prostate cancer or significantly increase intraprostatic androgen levels (*13, 17*). Furthermore, prostate cancer can recur after androgen deprivation therapy, a condition known as castration resistant prostate cancer. (*18*). This led to the theory in the field that alterations in normal AR activity, such as duplication, mutation, increased bio-availability of androgen, or altered regulation of AR co-factors, were driving the development of prostate cancer, including castration resistant prostate cancer (*10, 12, 18*).

AR is a 113 kiloDalton (kDa) protein that is a member of the steroid receptor super-family and the AR gene is located on the long arm of the X chromosome (*6, 10*). AR has the traditional structure of a unique N-terminal domain, a DNA-binding domain, and a hormone binding domain (*18*). AR is localized in the cytoplasm bound to chaperones like heat-shock proteins in order to stabilize the protein and prevent degradation (*19, 20*). AR then binds to either testosterone, or to dihydrotestosterone (DHT), a more active form that is derived by the 5α-reductase from testosterone after testosterone enters the prostate cell (*10*). AR can then form a dimer and translocate into the nucleus (*18*). Upon entering the nucleus, pioneer factors like FOXA1 recruit AR to open chromatin, then other co-factors and the RNA pol II transcription

machinery are recruited, and the transcription of the early androgen-responsive genes, including PSA and EAF2, begins (*2, 6, 21*). However, in prostate cancer, particularly castration-resistant prostate cancer, AR binds to different stretches of DNA than it would in the normal prostate or in early-stage prostate cancer (*22*). As reported in Decker *et al.* 2012, AR mediates the expression of different set of genes in castration-resistant prostate cancer than in early-stage prostate cancer, and it was shown that there are distinct androgen-dependent AR occupied regions and androgen-independent AR occupied regions. These results showed that there are distinct alterations in androgen receptor signaling between in early-stage prostate cancer and castration-resistant prostate cancer (*23*).

AR signaling is required for normal prostate development. The prostate will not develop if males undergo androgen deprivation pre-puberty, and it will wither away if androgen deprivation occurs post-puberty (*24*). Figure 2 shows the activity of androgen receptor. FOXA1 is known to bind to AR and to recruit AR to the promoter (*6*). FOXA1 is one of the genes involved in the AR signaling pathway that is de-regulated in prostate cancer. FOXA1 has been found to be up-regulated in castration-resistant prostate cancer, and this up-regulation is an indicator of a poor prostate cancer prognosis (*25*).

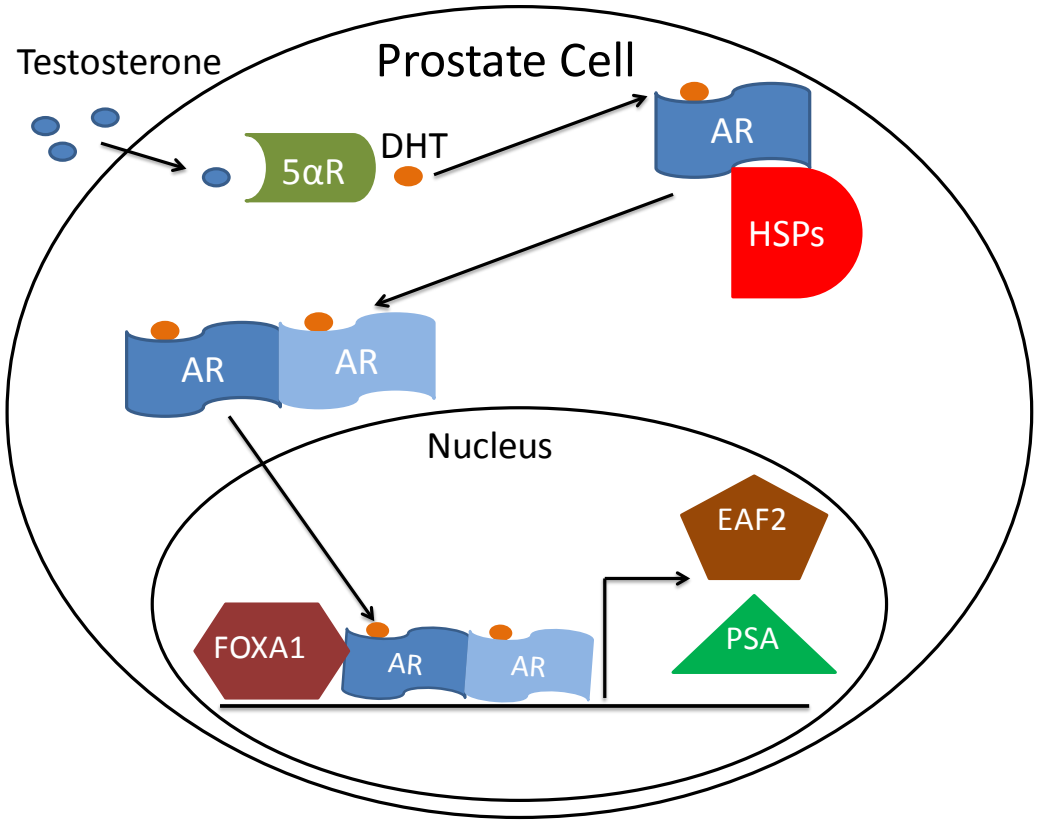

Figure 2. A simplified schematic of androgen receptor activation and activity in the prostate.

Testosterone enters the cell and is converted by 5-alpha reductase (5αR) to 5α-dihydrotestosterone (DHT). DHT binds to the androgen receptor (AR) in the cytoplasm, releasing AR from chaperoning heat-shock proteins (HSPs). AR dimerizes, translocates to the nucleus, binds to pioneer factors and co-factors (represented by FOXA1), and begins transcription of AR-upreglated genes (represented by PSA and EAF2).

In PCa, like other cancers, pathways used in development and wound healing are dysregulated. Genes that would normally inhibit the growth of the prostate like PTEN, p53, and EAF2 are down regulated (*26*). PTEN is a phosphatase that inhibits the PI3K-AKT signaling pathway and is considered a classical tumor-suppressor (*26*). The tumor-suppressor p53 is one of the most commonly mutated genes in cancer, and mutation of p53 is associated with metastatic and castration resistant prostate cancer (*26*). EAF2 is an androgen up-regulated tumor-suppressor

that is down-regulated in 100 percent of known prostate cancer cell lines and 83 percent of human prostate cancer tumor samples (*2*). Genes that promote the growth of the prostate like AR and FOXA1 are up-regulated in PCa (*26*). AR is a transcription factor that is required for prostate development and growth and is often duplicated or mutated (*13*). There have been reports of AR mutation in 8-25% of metastatic prostate cancer. One of the most common point mutations is T877A, which occurs in the ligand-binding domain of AR and has been reported to broaden ligand specificity, including allowing the anti-androgen flutamide to serve as a partial agonist and allow other steroids to activate AR (*13, 27*). One important marker of increased AR activity is the level of PSA, an androgen up-regulated protein, in the serum, due to PSA containing several androgen response elements (ARE) (*28*). FOXA1 is an androgen receptor pioneer factor that is up-regulated in metastatic and castration resistant prostate cancer (*13, 25*). Pioneer factors bind to condensed chromatin, and displace repressive histones (*29*).

1.1.2 Prostate Development

Prostate cancer is believed to be a result of a de-regulation of the normal prostate growth pathways. In order to understand how those pathways normally function, it is important to understand prostate development. The prostate, unlike most of the male accessory sex glands, derives from the endodermal urogenital sinus, not the mesodermal Wolffian ducts (*30*). As mentioned above, androgens are required for the prostate to develop. Initially, androgen receptors are present only in the urogenital sinus mesenchyme and appear in the epithelium a few days before the epithelium begins producing prostate-specific secretory proteins (*31, 32*). Prostate epithelial cells will develop if there is wild-type AR in the urogenital sinus mesenchyme and mutant AR in the urogenital sinus epithelium but not if there is mutant AR in the urogenital

sinus mesenchyme and wild-type AR in the urogenital sinus epithelium, showing that the development of the prostate requires crosstalk between the mesenchymal cells and the epithelial cells (*33*). Likewise, urogenital sinus mesenchyme can induce seminal vesicle epithelium and bladder epithelium to differentiate into prostate epithelium (*34*). One of the important proteins involved in this crosstalk is FOXA1. FOXA1 is highly expressed in the urogenital sinus epithelium and wild-type urogenital mesenchyme cannot rescue prostate epithelial development in FOXA1$^{-/-}$ epithelial cells (*7, 32*). The FOXA1$^{-/-}$ epithelial cells fail to develop the normal ductal structure of the prostate (*7*). EAF2 is involved in prostate growth. EAF2 is expressed in the prostate epithelial cells, and in EAF2$^{-/-}$ mice prostate abnormalities are observed beginning at 3 months old (*3*). The prostates of the EAF2$^{-/-}$ mice exhibit hypertrophy and the prostate epithelium exhibits hyperplasia and dysplasia (*3*). There are no abnormalities in the mesenchyme in EAF2$^{-/-}$ mixed genetic background mice, but Black 6 and FVB strain EAF2$^{-/-}$ mice show stromal abnormalities that include increased vascularization and stromal inflammation and it is unknown whether the stromal abnormalities are due to epithelial cell signaling or to the expression of EAF2 in other cell types (*3, 35, 36*). This leads to the conclusion that EAF2 is expressed in human prostate epithelial cells and in the mouse prostate. These results suggest that EAF2, an androgen up-regulated gene, plays a role in inhibiting AR-mediated growth in the normal prostate (*2, 3*).

1.2 EAF2 AND FOXA1

EAF2 and FOXA1 are two transcription factors that are known to play a role in regulating prostate growth (*3, 7*). They have also been shown to play a role in the development of prostate

cancer, although these are opposite roles. Loss of EAF2 is associated with late stage prostate cancer, development of prostatic intraepithelial neoplasia, and 33% $EAF2^{-/-}$ $PTEN^{+/-}$ Black 6 mice developed prostate cancer while no $EAF2^{-/-}$ $PTEN^{+/+}$ or $EAF2^{+/+}$ $PTEN^{+/-}$ Black 6 mice developed prostate cancer (*2, 3, 37*). FOXA1 promotes the development of prostate cancer. High levels of FOXA1 in primary prostate cancer indicates a poor outcome, FOXA1 is highly expressed in prostatic intraepithelial neoplasia, and FOXA1 is over-expressed in castration-resistant and metastatic prostate cancer compared to primary prostate cancer or the normal prostate (*8, 25, 38, 39*). A *C. elegans* screen that will be discussed further in chapter 3 section 1 suggested that EAF2 and FOXA1 interact and led to the work in this thesis.

1.2.1 EAF2

ELL-associated factor 2 (EAF2), also known as androgen-upregulated 19 (U19) is a member of the EAF family of transcription elongation factors (*40*). EAF2 was simultaneously discovered as a transcription elongation factor that was bound to eleven-nineteen lysine-rich leukemia factor (ELL), a transcription elongation factor that binds to RNA polymerase II (*40*) and as an androgen up-regulated tumor suppressor that is down-regulated in prostate cancer (PCa) (*2*). EAF2 is known to have an N-terminus ELL interaction domain located in amino acids 17-104 and a C-terminus transactivation domain that interacts with TCEA1 and TCEA2 located in amino acids 117-260 (*40-42*). EAF2 also possesses a poly-glutamine region located in amino acids 126-130 and a serine-rich region located in amino-acids 174-207 (*40*). EAF2 also has two serine residues that have been found to be phosporylated; these are serine residues 151 and 154 (*43*). EAF2 also possesses a homolog in mammals, ELL-associated factor 1 (EAF1) which has 74 percent amino

acid conservation with EAF2 and is involved in several of the same transcription complexes (*40, 44*). EAF2 has recently been implicated in several important roles.

EAF2 is a tumor suppressor (*2, 3*). EAF2 is down-regulated in PCa cell lines and human prostate cancer tissue samples (*2*). Furthermore, over-expression of EAF2 in prostate cancer cell lines causes apoptosis (*2*). Also, EAF2 is associated with and its transcriptional activity inhibited by FB1, which is a fusion partner with E2A in leukemia (*45*). Eaf2-knockout mice develop hepatocellular carcinoma, lung adenocarcinoma and B-cell lymphoma (*3*). EAF2 knockout mice also develop prostatic intraepithelial neoplasia (PIN), a hypothetical precursor to prostate cancer (*3*). In addition, although mice characterized by heterozygous loss of PTEN or homozygous loss of EAF2 alone do not display prostate tumors, thirty percent of $PTEN^{+/-}$ $EAF2^{-/-}$ mice develop prostate tumors when 12 months old (*37*). Four out of nine $PTEN^{+/-}$ and nine out of nine $PTEN^{+/-}$ $EAF2^{-/-}$ mice had prostate epithelial cell hyperplasia at 19 weeks old, compared to zero wild-type or $EAF2^{-/-}$ mice (*37*). Nine out of nine $PTEN^{+/-}$ $EAF2^{-/-}$ have developed prostatic intraepithelial neoplasia at 12 months old, compared to 8 out of 9 $PTEN^{+/-}$ mice, two out of nine $EAF2^{-/-}$ mice, and zero out of nine wild-type mice (*37*). When EAF2 and PTEN mRNA levels were measured in human prostate cancer samples, EAF2 was down-regulated in one out of nine and PTEN down-regulated in zero out of seven Gleason grade 6-7 prostate tumor samples while down-regulation of both EAF2 and PTEN was observed in six out of eleven Gleason grade 8-9 prostate tumor samples (*37*). These findings all provide evidence that EAF2 is a tumor suppressor that is down-regulated in prostate cancer.

EAF2 is a protein involved in growth and development of the prostate and other organs. EAF2 is expressed in the epithelium during development in the central nervous system and in tissues that require reciprocal tissue-tissue inductions between epithelial and mesenchymal cells,

like the kidneys, lungs and pancreas (*46*). EAF2 has been shown to be important for non-canonical wnt signaling and the development of several organs, including the eyes and neural tissue (*47-49*). The *Xenopus laevis* homolog of EAF2, was discovered to be a downstream target of wnt4, a non-canonical Wnt protein, in *Xenopus* eye development and over-expression of EAF2 can rescue eye development in wnt4 knockdown *Xenopus laevis* embryos (*47*). The zebrafish homolog of EAF2 (eaf2) was shown to form a negative feedback loop with wnt4a that is formed by eaf2 suppressing wnt4a through actions on the wnt4a promoter while eaf2 is up-regulated by wnt4a signaling (*48*, *49*). Figure 3 shows the feedback loop of EAF2 and Wnt-4.

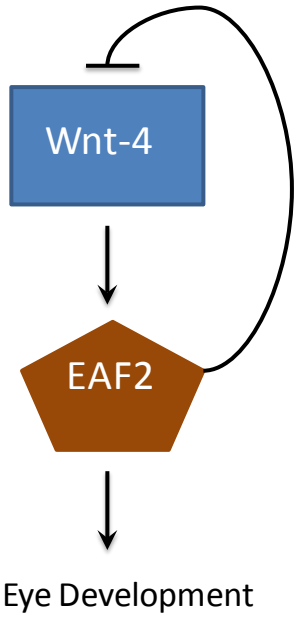

Figure 3. EAF2 is in a negative feedback loop with Wnt-4

EAF2 is up-regulated by non-canonical Wnt-4 signaling during eye development, and EAF2 inhibits expression of Wnt-4, forming a negative feedback loop during eye development.

Eaf2 has been found to regulate extension movements required for development of the heart and pancreas through upstream regulation of wnt5 and wnt11 signaling (*48*). EAF2 is also involved in immunoglobulin secretion by plasma cells through its interaction with eleven-nineteen lysine-rich leukemia factor 2 (ELL2) and is up-regulated in plasma cells compared to B-

cells (*50, 51*). EAF2 has also been implicated in regulating collagen formation and maintenance of the extracellular matrix, due to knockdown of the *C. elegans* homolog of EAF2, *eaf-1*, in *C. elegans* with mutations in the genes responsible for collagen formation rescuing the mutants (*4*). Loss of EAF2 has been shown to result in prostate hypertrophy, epithelial cell hyperplasia and the development of murine PIN lesions (*3*).

EAF2 is a transcription elongation factor that binds to eleven-nineteen lysine-rich leukemia factors 1 and 2 (ELL1 and ELL2) and is stabilized by ELL1 (*40, 42*). ELL1 and ELL2 are transcription elongation factors and ELL1 is frequently fused to MLL in acute myelogenous leukemia which disrupts the binding of EAF2 and ELL1 (*40, 52*). EAF2 also interacts with transcription elongation factor S-II to mediate RNA PolII transcription of DNA (*41*). EAF2 is a positive regulator of RNA polII and part of the super elongation complex (SEC) and the little elongation complex (LEC) (*53-55*). The SEC is a transcription complex that is involved in acute myelogenous leukemia due to several members of the complex, including EAF2 binding partner ELL, undergoing fusion with MLL and is involved in HIV due to HIV Tat recruiting the SEC to transcribe HIV genes (*44*). The major proteins in the SEC are ELL1 or ELL2, EAF1 or EAF2, AFF 1 or AFF4, AFF9 or ENL, and P-TEFb (*44*). The LEC is composed of ICE1, ICE2, ELL1, and EAF1 or EAF2 and is involved in the transcription of snRNA (*55*). EAF2 binds to MED26, linking the SEC and the Mediator complexes involved in gene transcription (*56*). The Mediator complex recruits the SEC to gene promoters during gene transcription (*56*).

EAF2 has been shown to play a role in angiogenesis and hypoxia response. EAF2 was shown to bind to and stabilize pVHL, an important inhibitor of hypoxia response and loss of EAF2 in a pVHL heterozygous background resulted in increased angiogenesis (*35, 57*). The protein pVHL modulates hypoxia response through inhibiting hypoxia-inducible factor 1 alpha

(HIF1α) by hydroxylation of HIF1α prolines P402 and P564 and HIF1α is observed to be up-regulated in the EAF2 KO mouse (*57*). EAF2 has also been shown to promote the transcription elongation of the anti-angiogenic protein TSP-1 through interactions with ELL and p53 (*58, 59*).

There is also evidence that EAF2 plays a role in DNA-damage repair. EAF2 has been shown to localize to the nucleus after DNA damage is induced by ultraviolet (UV) light (*60*). Loss of EAF2 has been shown to reduce apoptosis after cells have been exposed to UV through increased expression of p53 (*61*), providing further evidence of a role in DNA-damage repair. Additionally, ELL, a known binding partner of EAF2, binds to TFIIH and mediates the re-start of transcription after nucleotide excision repair (NER) (*62*).

Although several EAF2-interacting proteins had already been noted in the literature, it was important to find more EAF2-interacting proteins and to determine if that interaction affects growth and development in the normal prostate and in PCa. For these reasons, a screen was performed using the *C. elegans* homolog of EAF2, *eaf-1*, which revealed FOXA1 as a potential interacting protein (*4*).

1.2.2 FOXA1

Forkhead box protein A1 (FOXA1) was originally discovered in 1989 as a transcription factor named hepatocyte nuclear factor 3α (HNF3α) that bound to the promoters of transthyretin and α1-antitrypsin in hepatocytes, and then was distinguished from its homologs FOXA2/HNF3β and FOXA3/HNF3γ in 1990 (*63, 64*). HNF3α was renamed FOXA1 in recognition of the prominent winged-helix domain known as a forkhead domain that marked it as a homolog of the drosophila gene *fkh* and for being the first discovered member of the FOXA subfamily (*6, 65*).

FOXA1 is an important protein in the growth and development of many organs, including the liver, pancreas, kidney, lung, brain, gastrointestinal tract, breast and prostate (*39, 66*). Each of these tissues displays developmental abnormalities if FOXA1 is knocked out in a tissue-specific fashion or in a rescue experiment (*39, 66*). FOXA1 knockout mice survive to birth, but suffer neonatal mortality 2-14 days after birth (*67*). The cause of death was hypoglycemia due to a defect in insulin secretion, despite the fact that the pancreatic islets appeared normal until challenged with glucose and severe dehydration due to kidney defects (*39, 67*). In the pancreas, lung, brain, and gastrointestinal tract, FOXA2 can compensate for the absence of FOXA1 in a conditional knockout, but not in the breast or prostate (*39*). FOXA1 localizes with the estrogen receptor (ER) and GATA3 at a specific promoter sequence next to the ER binding site, and is necessary in the breast for normal development of the mammary gland because there is impaired ductal invasion in rescued $FOXA1^{-/-}$ breast tissue (*39*). FOXA1 also plays an important role in prostate development. Epithelial expression of FOXA1 is required for the normal glandular structure of the prostate and for luminal epithelial cells to develop (*7*). FOXA1 localizes with AR and binds to the promoter of AR-responsive genes right next to the androgen response element that AR binds to. FOXA1 also acts as a pioneer factor for AR and ER, by binding to stretches of chromatin, recruiting other proteins that modify the chromatin to enable transcription, and then by binding to AR and ER after they are activated by androgen and estrogen respectively (*68*).

FOXA1 also plays an important role in cancer, particularly in breast and prostate cancer. FOXA1 is required for breast cancer cells to respond to tamoxifen in ER^+ breast cancer, so the presence of FOXA1 is associated with a positive outcome in ER^+ breast cancer. (*69*). In ER^- apocrine breast carcinoma, FOXA1 and AR are over-expressed and promote tumor growth and survival. Furthermore, several studies have shown that FOXA1 recruits AR to sites that are

associated with ERα-mediated transcription and are dependent on AR in apocrine carcinoma (*69*). The results observed in breast cancer show that FOXA1 can serve as an oncogene or tumor suppressor, depending on which pathways FOXA1 interacts with. In prostate cancer (PCa), FOXA1 modulates AR activity, which is required for PCa tumor growth and survival. FOXA1 is observed to be necessary for some AR-mediated gene transcription (*6*). FOXA1 also regulates AR-mediated transcription in castration-resistant prostate cancer and is a driver of cancer cell growth (*70*). FOXA1 expression levels are indicative of PCa patient outcome, with a higher level of FOXA1 expression indicating a poorer outcome, particularly in metastatic and castration resistant prostate cancer (*8, 71*).

1.3 PROJECT DESIGN

The goal of this project was to confirm that a functional interaction between the *C. elegans* orthologs of EAF2 and FOXA1 occurred in mammals. After confirming the presence of the interaction, the next goal was to determine the effects of the interaction on PCa and prostate development.

1.3.1 *C. elegans* screen to find EAF2-interacting proteins

EAF2 is a conserved protein and does possess a *C. elegans* homolog, *eaf-1* (*4, 72*). When *eaf-1* is knocked out, the resulting *C. elegans* worms have a short and stubby body, also known as a dumpy phenotype, a shortened life expectancy, and reduced fertility (*4*). Compared to the wild-

type worms, the *eaf-1*KO worms produce two-thirds fewer offspring (*4*). EAF2 has ~63% similarity to *eaf-1* (*4*). Figure 4 shows the similarity of the EAF2 and *eaf-1* protein sequences.

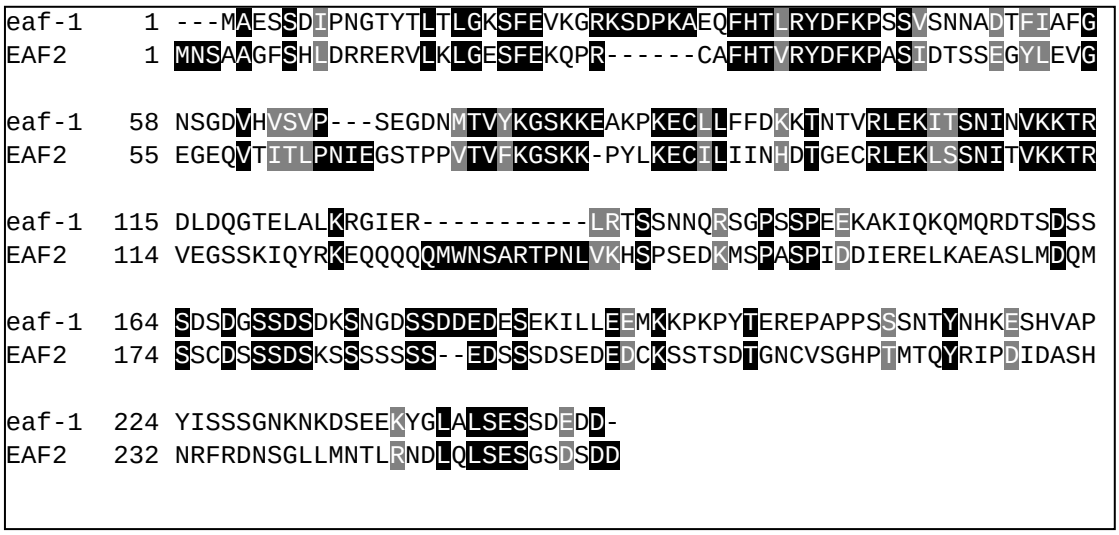

Figure 4. Sequence similarity of human EAF2 and *C. elegans eaf-1*.

Sequence analysis of human EAF2 (EAF2) and *C. elegans eaf-1* (eaf-1) was performed using ClustalW and BOXSHADE from Swiss EMBnet and protein sequences from the NCBI protein database. Identical amino acids are shaded black, similar amino acids are shaded grey, and a - represents amino acid stretches not present in one of the proteins.

Because *eaf-1* and EAF2 are so similar, a screen was conducted for phenotypic enhancers using *eaf-1* in *C. elegans*. *C. elegans* is an excellent organism to perform screens due to the ease of performing RNAi (*73*). A phenotypic enhancer is a gene that increases the severity of a phenotype from another gene. This enhancement of the phenotype is then considered to be functional synergism and is interpreted as a sign that the two genes functionally interact (*74, 75*). The *eaf-1*KO *C. elegans* worms were noted to have reduced fertility and this phenotype was used as the readout for the screen. Reduced fertility was the chosen readout because of the ease to

determine *C. elegans* fertility. The design of the screen is illustrated in Figure 5. Briefly, wild-type and *eaf-1*KO worms were treated with control RNAi and RNAi from a library of nuclear factors. It was then determined if the number of *eaf-1KO* + RNAi knockdown offspring were fewer than either *eaf-1*KO or RNAi knockdown alone.

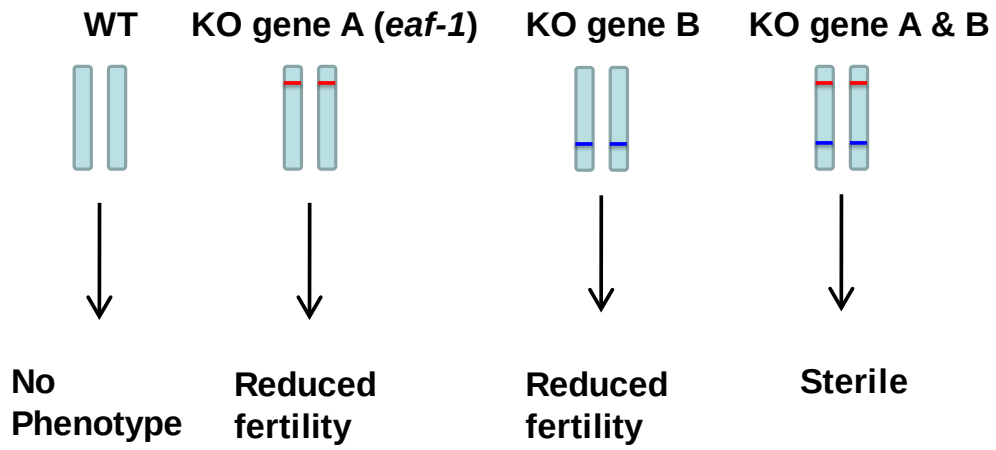

Figure 5. Schematic of the design of phenotypic enhancer screen.

Wild-type *C. elegans* (WT) will have normal fertility (no phenotype). *eaf-1*KO *C. elegans* (KO gene A) will have reduced fertility. Treatment of wild-type *C. elegans* with double-stranded RNA corresponding to a *C. elegans* gene (KO gene B) will result in the reduced fertility phenotype or no phenotype. Treatment of *eaf-1*KO *C. elegans* with the gene B RNAi will result in a severe phenotype like sterility (KO gene A&B).

The screen produced several hits and among these were *pha-4*, the *C. elegans* homolog of FOXA1 (*5*, *76*). Rat FOXA1 has 75-78% similarity to *pha-4* showing that *pha-4* is closely related to FOXA1 (*76*). Figure 6 shows the similarity between the *C. elegans pha-4* protein and human FOXA1. In *C. elegans*, *pha-4* is expressed in the pharynx, and is required for the pharynx

to develop (*77*). An abnormal pharynx was not noted in the *pha-4* knockdown *C. elegans* but was not the primary focus of the screen.

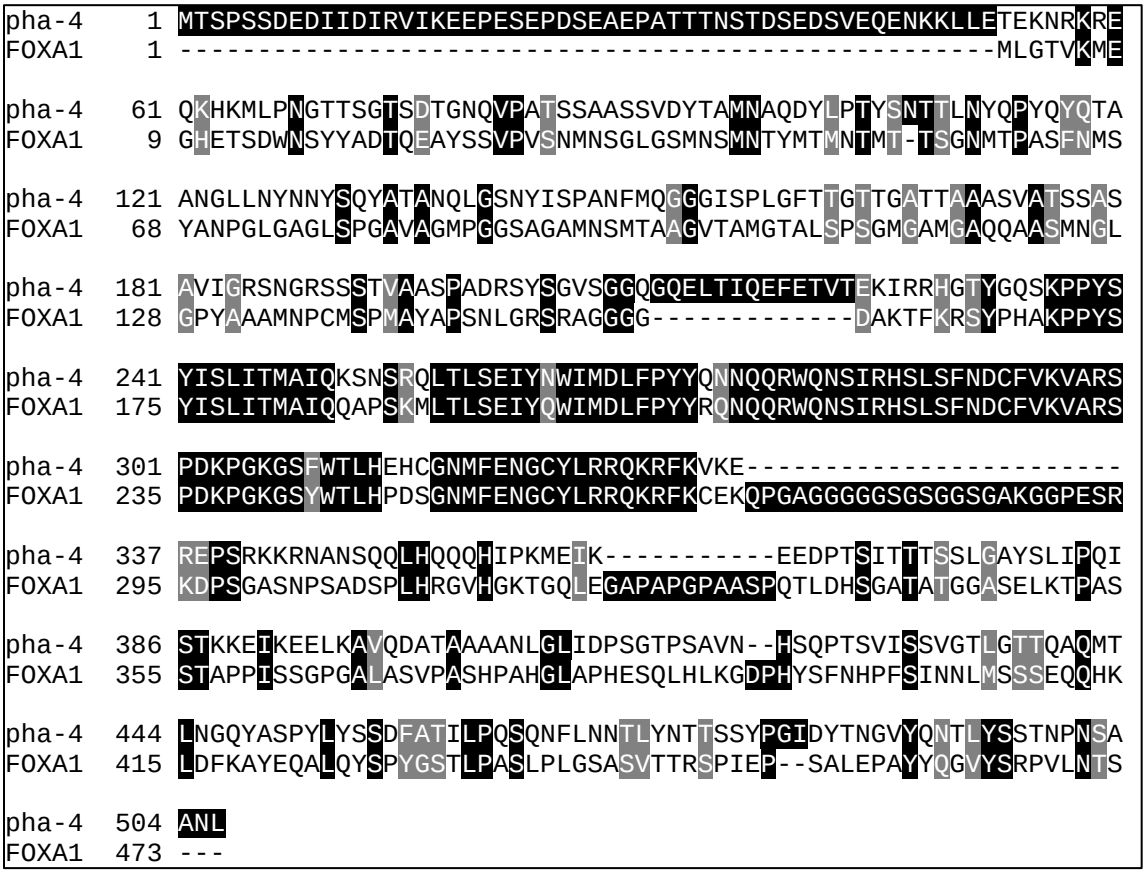

Figure 6. Sequence similarity of *pha-4* and FOXA1 proteins

Sequence analysis of human FOXA1 (FOXA1) and *C. elegans pha-4* (pha-4) was performed using ClustalW and BOXSHADE from Swiss EMBnet and protein sequences from the NCBI protein database. Identical amino acids are shaded black, similar amino acids are shaded grey, and a - represents amino acid stretches not present in one of the proteins.

RNAi knockdown of *pha-4* reduced the number of offspring to 10% of what was produced by wild-type *C. elegans*. When *pha-4* was knocked down in *eaf-1*KO worms, the resulting worms were sterile, with zero viable eggs produced. Figure 7 shows these results. When the worms were examined, the wild-type worms and the single mutants showed egg

development, but in the double mutants, the eggs appeared to degenerate during development (Figure 8). These results suggested functional synergism between *pha-4* and *eaf-1*, indicating that *pha-4* was a phenotypic enhancer of *eaf-1*.

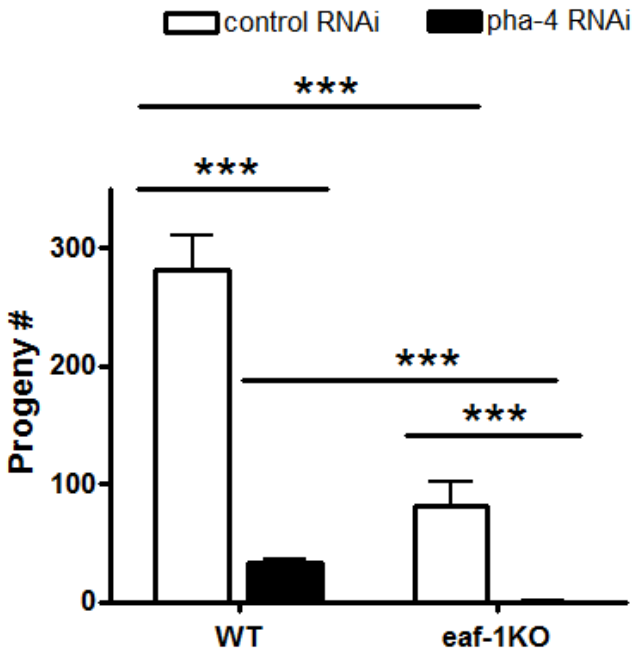

Figure 7. Knockdown of *pha-4* in *eaf-1* knockout *C. elegans* results in sterility

A single L2/L3 wild-type (WT) or *eaf-1* KO (eaf-1KO) *C. elegans* worm was placed on a plate treated with *E. coli* transformed with control or *pha-4* RNAi and the number of offspring was counted. The experiment was performed 4 times with each sample in triplicate. ***=$p \leq 0.001$.

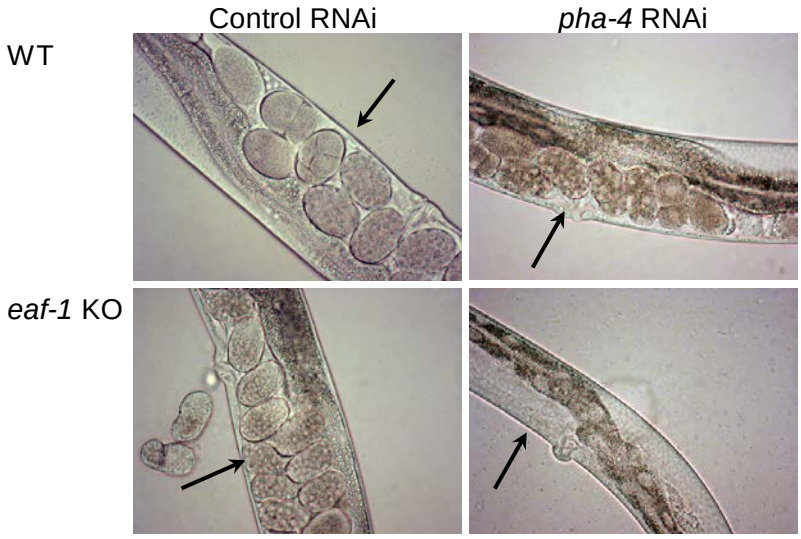

Figure 8. Loss of *pha-4* in *eaf-1* knockout *C. elegans* results in egg degeneration

Representative images showing egg development in adult wild-type (WT) and *eaf-1* KO (*eaf-1* KO) *C. elegans* that have been treated with control or *pha-4* RNAi. The arrows indicate the gonads in which egg development occurs. Egg development is absent in the *eaf-1* KO *C. elegans* treated with *pha-4* RNAi.

1.3.2 From *C. elegans* to mammals

The *C. elegans* screen showed that homologs of EAF2 and FOXA1 had a functional synergism, which could be due to 1) *eaf-1* and *pha-4* being two steps in the parallel pathways that both perform the same function, 2) *eaf-1* and *pha-4* being in two different pathways that converge for a specific function (*74*). The first scenario is most commonly found in homologous genes where the functions of the two genes are partially or completely redundant (*74*). For instance, FOXA1 is known to be partially redundant with FOXA2 because Foxa1$^{-/-}$ mice with lung-specific knockouts of Foxa2 show abnormal lung development, but loss of FOXA2 results in an embryonic lethal phenotype while loss of FOXA1 results in a postnatal lethal phenotype (*78*). Likewise EAF2 is known to be partially redundant with EAF1 due to both proteins binding to the

N-terminus of ELL, while EAF1 also binds to the C-terminus of ELL (*40*). In addition to the functional synergism of their *C. elegans* homologs revealed by the screen, EAF2 and FOXA1 both have roles in prostate cancer (PCa) and regulating prostate growth (*2, 3, 7, 38, 79*). The results of the *C. elegans* screen and the literature on FOXA1 and EAF2 led to the hypothesis that FOXA1 and EAF2 should interact at the molecular level or at the functional level. Therefore, experiments were performed to test for molecular interactions and functional interactions between FOXA1 and EAF2 in human prostate cancer cell lines and murine prostate tissue samples.

2.0 MATERIALS AND METHODS

The reagents and the experimental methods used for this project are described below to determine whether EAF2 and FOXA1 interact.

2.1 C. ELEGANS

N2 *C. elegans* or tm3976 (*eaf-1*KO) were used for the *C. elegans* experiments (*4*). The *C. elegans* were maintained on NGM plates on an *E. coli* OP50 lawn until they were used for an experiment (*80*). Eggs were harvested from N2 or *eaf-1*KO worms lysed by hypochlorite solution (3.5mL 10M KOH, 4mL NaClO solution (Chlorox), 35mL dH_2O) then washed 3x with S-BASAL (5.85g NaCL, 6g KH_2PO_4, 1g K_2HPO_4, ddH_2O to 1L) (*4*). The eggs were then plated on RNAi plates with *E. coli* transformed by control RNAi or *pha-4* RNAi obtained from the Ahringer RNAi library through the Fisher lab (diluted 1:3 with control RNAi) (*4, 81, 82*). The number of offspring was determined by moving a single L2/L3 *C. elegans* to individual RNAi plates containing the appropriate RNAi transformed *E. coli* and then counting the resulting number of L2/L3 offspring. Comparison analysis was then carried out with a *Student's t* test. Bright-field images were taken on a Leica microscope (Leica Microsystems, Buffalo Grove, IL, USA) using the offspring once adulthood was reached.

2.2 SEQUENCE ANALYSIS

A comparison of FOXA1 and *pha-4* protein sequences and EAF2 and *eaf-1* protein sequences were performed using ClustalW, BOXSHADE, and LALIGN from Swiss EMBnet.

2.3 CELL CULTURE

Prostate cancer cell lines PC3 and LNCaP were obtained from ATCC (Mannasas, VA, USA) and the prostate cancer cell line C4-2 was a generous gift from Dr. Leland Chung. Please see Table 1 for a characterization of the cell lines and the experiments each cell line was used in. The cell lines were cultured in RPMI-1640 with 10% fetal bovine serum (FBS), penicillin/streptomycin, and L-glutamine (RPMI+ media). LNCaP cells were treated with siRNA for 18 hours in OptiMEM medium. Then cells were cultured in RPMI 1640 medium containing 1nM of R1881 for additional 24 hours. Cells were collected in RIPA buffer for Western blotting using the indicated antibodies. Control siRNA and siRNA EAF2-1 were purchased from Santa Cruz. siRNA EAF2-2 was purchased from Qiagen. C4-2 shScr and shEAF2 cells were generated using lentivirus supplied by the UPCI Vector Core Facility and were cultured in RPMI-1640 with 10% FBS, penicillin/streptomycin, L-glutamine and 1 μg/mL puromycin. PC3, LNCaP, and C4-2 cells were transfected using PolyJet (SignaGen, Gaithersburg, MD, USA).

Table 1. Cell Lines and Experiments

Cell Line	Experiments	AR status/androgen dependence	FOXA1 status	EAF2 status
PC3	Co-transfection and co-immunoprecipitation, cycloheximide assay	AR - / androgen independent	FOXA1 positive	EAF2 negative
LNCaP	Endogenous co-immunopreciptation, EAF2 siRNA cell lines, QPCR, colony formation assay	AR + / androgen dependent	FOXA1 positive	EAF2 positive
C4-2	Luciferase assay, shRNA	AR+ / castration resistant	FOXA1 positive	EAF2 positive

Characterization of cell lines used for this project and the experiments they were used in.

2.4 PLASMIDS

CMV-Myc, CMV-HA and pEGFP-N3 vectors were purchased from Clontech (Mountain View, California, USA) (*42*). Human EAF2 cDNA was amplified by PCR and cloned into the pEGFP-N3, CMV-HA and CMV-Myc vectors, producing pEGFP-EAF2-N3, CMV-HA-EAF2, and CMV-Myc-EAF2 (*2*, *42*). EAF2 deletion mutants were generated by PCR and cloned into the CMV-Myc vector (Figure 9) (*83*). pCMV-Renilla was obtained from Promega (Madison, Wisconsin, USA) and pGL-PSA was a generous gift from Dr. Marianne Sadar, Ph.D. (BC Cancer Agency, British Columbia, Canada). pCMV6-FOXA1 was purchased from Origene (Rockville, Maryland, USA) (RC20604); pCMV6, pEGFP-N3-FOXA1, and FOXA1-untagged were generated from pCMV6-FOXA1 by PCR subcloning. Full-length cDNAs were verified by sequencing.

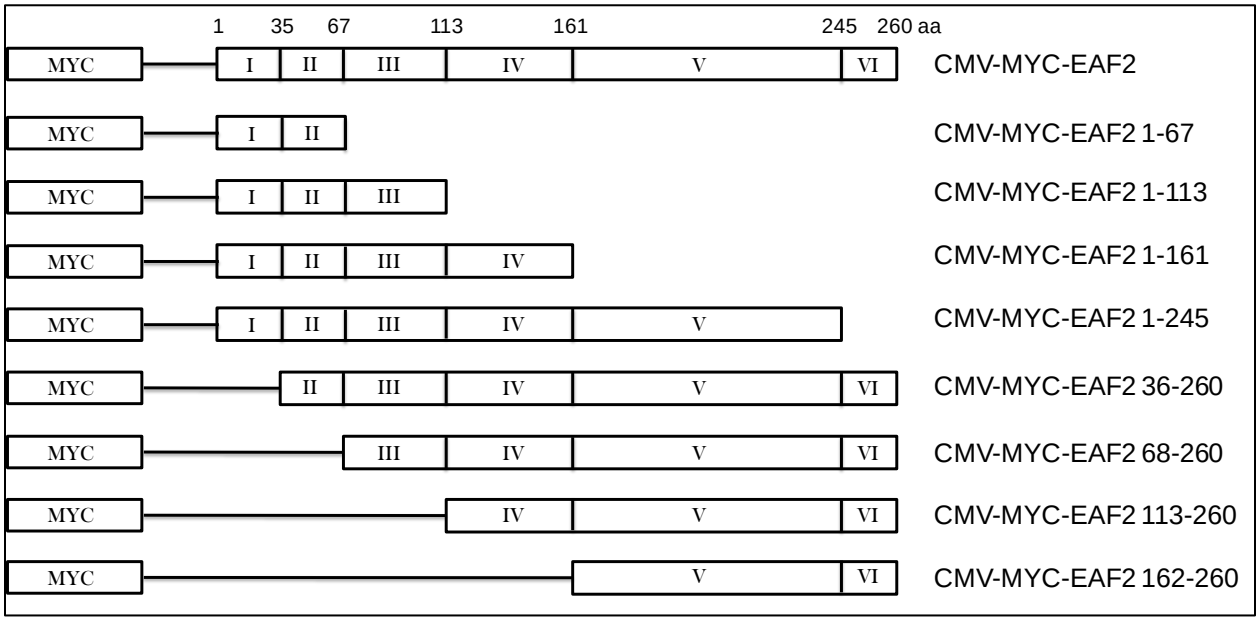

Figure 9. Structure of EAF2 and EAF2 deletion mutants

Map of full-length EAF2 and EAF2 deletion mutants. EAF2 is 260 amino acid residues long and encoded by 6 exons, I, II, III, IV, V, and VI. The numbers at the top indicate amino acids corresponding to each exon below.

2.5 ANTIBODIES

The following antibodies were used for the co-immunoprecipitations and Western Blots: polyclonal rabbit anti-FOXA1 (ab23738, 1:1500 dilution for western blot) and agarose-conjugated polyclonal goat anti-Myc (ab1253, 10-20uL for co-immunoprecipitation) were obtained from Abcam (Cambridge, United Kingdom), polyclonal rabbit anti-GFP (TP401 (1:2000–1:3000 dilution for western blot) from Torrey Pines Biolabs (Houston, Texas, USA), monoclonal mouse anti-Myc (MMS-150R, 1:1000 dilution for western blot) and monoclonal mouse anti-HA (MMS-101R, 1:1000 dilution for western blot) from Covance (Princeton, New

Jersey, USA), monoclonal mouse anti-EAF2 generated by the Wang lab (1:1000 dilution for western blot), and polyclonal rabbit anti-GAPDH (sc-25778, 1:3000 dilution for western blot) from Santa Cruz Biotechnology (Dallas, Texas, USA), protein A/G-conjugated agarose beads (sc-2003, 20 µL for pre-clearing lysates), goat anti-rabbit-HRP (sc-2004, 1:2500-1:5000 dilution for western blot) or goat anti-mouse-HRP (sc-2005, 1:2500 dilution for western blot) secondary antibodies from Santa Cruz Biotechnology, light-chain specific mouse anti-rabbit (211-032-171, 1:4000-1:5000 dilution for western blot) and light-chain specific goat anti-mouse (115-035-174, 1:2500-1:4000 dilution for western blot) antibodies from Jackson ImmunoResearch (West Grove, Pennsylvania, USA).

2.6 CO-IMMUNOPRECIPITATION

PC3 cells were plated into 10 cm dishes. When the cells reached 60-80% confluency, they were transfected with pEGFP-N3 and CMV-Myc, pEGFP-N3 and CMV-Myc-EAF2, pEGFP-FOXA1 and CMV-Myc, pEGFP-FOXA1-N3 and CMV-Myc-EAF2, or pEGFP-FOXA1-N3 and the CMV-Myc-EAF2 deletion mutants. Cells were lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mMEDTA, 1% (v/v) NP-40, 0.1% SDS, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM PMSF, 1:100 dilution of protease inhibitor cocktail (P8340, Sigma-Aldrich, St Louis, MO, USA). Protein concentration was determined by BCA Protein Assay (Thermo Scientific, Rockford, IL, USA). Pre-clearing was performed using 20 µL of protein A/G-agarose and the lysates from the experiments to determine which regions of EAF2 are required for FOXA1 and EAF2 to associate. The beads and lysates were incubated at 4^{o}C for 1 hour. Pre-clearing was not performed in the experiments to determine if FOXA1 and EAF2

associate. Immunoprecipitation was performed using agarose-conjugated mouse anti-Myc. 20 µL were used for the co-immunoprecipitations to determine whether FOXA1 and EAF2 associate the beads were incubated with the lysates overnight at 4^{o}C. 10 µL were used for the co-immunoprecipitations to determine which regions of EAF2 are responsible for the association of EAF2 and FOXA1 and the incubation was 4-6 hours at 4^{o}C.

Immunoprecipitates and whole cell lysate (WCL) were boiled with SDS sample buffer for five minutes, separated on a 10% SDS-PAGE gel (4.8 mL ddH_2O, 2.5 mL 1.5 M Tris pH 8.8, 100 µL 10% SDS, 100 µL 10% APS, 2.5 mL 40% Bis-Tris acrylamide, 10 µL TEMED) with a 4% SDS-PAGE stacking gel (2920 µL ddH_2O, 500 µL 1M Tris pH 6.8, 40 µL 10% SDS, 40 µL 10% APS, 500 µL 40% tris-bis acrylamide, 4 µL TEMED) under reducing conditions and transferred onto a nitrocellulose membrane. The membranes were then blotted with the above primary antibodies. Signals were visualized using chemiluminescence (ECL Western Blotting Detection Reagents, GE Healthcare, Little Chalfont, United Kingdom) and were exposed to X-ray film (Fuji film, Valhalla, NY, USA).

2.7 WESTERN BLOTS OF CELL LINES

After co-transfection using the appropriate plasmids as mentioned in the figure, cells were lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mMEDTA, 1% (v/v) NP-40, 0.1% SDS, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM PMSF, 1:100 dilution of protease inhibitor cocktail (P8340, Sigma-Aldrich, St Louis, MO, USA). Protein concentration was determined by BCA Protein Assay (Thermo Scientific, Rockford, IL, USA). Cell lysates were boiled with SDS sample buffer for five minutes, separated on a 10% SDS-

PAGE gel (4.8 mL ddH_2O, 2.5 mL 1.5 M Tris pH 8.8, 100 µL 10% SDS, 100 µL 10% APS, 2.5 mL 40% Bis-Tris acrylamide, 10 µL TEMED) with a 4% SDS-PAGE stacking gel (2920 µL ddH_2O, 500 µL 1M Tris pH 6.8, 40 µL 10% SDS, 40 µL 10% APS, 500 µL 40% tris-bis acrylamide, 4 µL TEMED) under reducing conditions and transferred onto a nitrocellulose membrane. The membranes were then blotted with the above primary antibodies and secondary antibodies. Signals were visualized using chemiluminescence (ECL Western Blotting Detection Reagents, GE Healthcare, Little Chalfont, United Kingdom) and were exposed to X-ray film (Fuji film, Valhalla, NY, USA). The signals were quantified using ImageJ (NIH, Bethesda, Maryland, USA) and statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, California, USA).

2.8 CYCLOHEXIMIDE ASSAY

PC3 cells in 10cm dishes at 60-80% confluency were transfected with pCMV6-FOXA1 + HA, pCMV6-FOXA1 + HA-EAF2, or pCMV-Myc + HA-EAF2 using Polyjet (Signagen, Gaithersburg, MD, USA). At 24 hours post transfection, each 10 cm dish was split into 5 wells on a 6-well plate. 40 hours post transfection, 4 of the 5 wells were treated with 1uL of 50ug/uL cycloheximide. The fifth well of each 6-well plate was lysed to provide the 0 hour time point. Every two hours one well from each 6-well plate was lysed. The last time point was 8 hours post transfection. The lysates were then analyzed by western blot as described above.

2.9 QRT-PCR

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed as described (*84*). Briefly, after siControl and siEAF2 cells were lysed and RNA isolation, reverse transcription, and real-time PCR were performed using CellsDirect^{TM} One-Step qRT-PCR Kit (Invitrogen, Carlsbad, California, USA). The PSA, and GAPDH primers described in (*84*) were used and are listed in Table 2. The FOXA1 qRT-PCR primers were proprietary primers from Applied Biosystems (Foster City, California, USA, catalogue number 4331182). Efficiencies were incorporated into calculations for qRT-PCR using the ΔCp (crossing point) method ($R = 2^{[Cp\ sample - Cp\ control]}$) in which the relative expression ratio (R) for each sample was to GAPDH. All assays were run on an ABI Step-One Plus thermocycler (Applied Biosystems Inc., Carlsbad, California, United States) (*36*).

Table 2. qRT-PCR primer sequences

Gene	Forward Sequence	Reverse Sequence	Probe	Species
PSA	5'-CATCAGGAACAAAAGCGTGA-3'	5'-AGCTGTGGCTGACCTGAAAT-3'	5'-6FAMCACAGCCTGTTTCATCCTGAAGACACATAMRA-3'	Mouse
GAPDH	5'-CATGTTCGTCATGGGTGTGA-3'	5'-GTAGACCATGTAGTTGAGGTCA-3'	5'-6FAMACAGCCTCAAGATCATCAGCAATGCCTCTAMRA-3'	Mouse

Sequences of the primers and Taqman probes used for qRT-PCR.

2.10 ANIMALS

U19/EAF2 heterozygous mice (U19/EAF2$^{+/-}$) were previously generated as described in (*3*). The heterozygous mice were then backcrossed to the C57BL/6J strain (The Jackson Laboratory, Bar Harbor, ME, USA) for >12 generations to generate U19/EAF2 knockout (KO) mice with a pure C57BL/6J background under approval by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Genotyping was determined by PCR analysis of mouse tail genomic DNA as described earlier (*3*). A group of 3 wild-type and 5 EAF2$^{-/-}$ C57BL/6J mice at 5-7 months of age were euthanized and the anterior prostates were isolated for western blot analysis 24 hours after the mice were treated with 8 gray of whole body γ-radiation. Anterior prostate lobes were micro-dissected from the urogenital tract and stored at -80^{o}C. The tissue was lysed and the protein lysates were analyzed by Western blot using rabbit anti-FOXA1 (ab23738, Abcam, Cambridge, USA) at a 1:1000 dilution and rabbit anti-GAPDH (sc-25778, Santa Cruz Biotechnology, Santa Cruz, USA) at a 1:1000 dilution. Protein quantification was performed using ImageJ and comparison analysis was carried out with a *Student's t* test.

2.11 LUCIFERASE ASSAY

C4-2 prostate cancer cell line cells were plated into 24-well plates and then transfected at 60-80% confluency with 250 ng pGL-PSA, 25 ng pCMV-Renilla, 250ng total of FOXA1-untagged and pCMV6 at varying ratios, and 250 ng total of HA-EAF2 and HA at 1:1, 1:5, 1:15 and 1:25 ratios of FOXA1 to EAF2, with pCMV6 balancing FOXA1. Luciferase activity was measured using Dual-Luciferase Reporter Assay (Promega, Madison, Wisconsin, USA) and luminescence

was assessed with LMax II/LMax II 384 Luminometer (Molecular Devices, Sunnyvale, CA, USA). Background was subtracted and fold induction calculated. Each sample was performed in triplicate. Luciferase activities were normalized to the expression of Renilla. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., La Jolla, California, USA).

2.12 COLONY FORMATION ASSAY

LNCaP cells were plated into five 10cm dishes. When the cells reached 60-80% confluency, four dishes were transfected with 0.83 µg pEGFP-N3 and 4.17 µg CMV-HA plasmids, 0.83 µg pEGFP-N3 and 4.17 µg CMV-HA-EAF2 plasmids, 0.83 µg pEGFP-FOXA1-N3 and 4.17 µg CMV-HA plasmids, or 0.83 µg pEGFP-FOXA1-N3 and 4.17 µg CMV-HA-EAF2 plasmids, respectively using Polyjet (SignaGen, Gaithersburg, MD, USA). The fifth plate was mock transfected and remained an un-transfected control. Eighteen hours after transfection the media was changed with fresh RPMI+ media in order to remove the transfection reagents. Twenty-four hours after transfection each 10 cm dish was split into three 10 cm dishes. The samples were not normalized relative to each other. Forty-eight hours after transfection, the media was changed over to RPMI+ media supplemented with 1mg/mL G418. The cells were grown for three weeks, changing media about once a week. After 3 weeks, the colonies were stained with a 0.5% crystal violet solution. The colonies were counted using the Adobe Photoshop CS5 counting tool (Adobe, San Jose, California, USA). Comparison analysis was performed by *Student's t* test using GraphPad Prism (GraphPad Software Inc., La Jolla, California, USA).

3.0 RESULTS

The *C. elegans* screen identified a phenotypic interaction between the *C. elegans* homologs of EAF2 and FOXA1. Loss of *eaf-1* or *pha-4* alone resulted in reduced fertility, and loss of *pha-4* in *eaf-1*KO *C. elegans* resulted in sterility. FOXA1 had been reported to be a pioneer factor for the androgen receptor and EAF2 had been reported to be up-regulated by androgen in the normal prostate (*2, 6*). Loss of FOXA1 had been reported to result in the absence of prostate luminal epithelial cells and loss of EAF2 resulted in epithelial cell hypertrophy and hyperplasia in three month old mice (*3, 7*). It was hypothesized that FOXA1 and EAF2 interact and that interaction affects androgen receptor signaling, cell growth, and cell development. The following experiments were performed to test that hypothesis.

3.1 FOXA1 AND EAF2 ASSOCIATE

Since FOXA1 and EAF2 are known transcription factors, and knowing that FOXA1 and EAF2 both show nuclear localization, it is possible that EAF2 and FOXA1 associate within the same complex (*42, 85*). In order to test this, PC3 cells were transfected with FOXA1 and EAF2 plasmids, then an immunoprecipitation was performed for EAF2 and the immunoprecipitation was then blotted for FOXA1. The PC3 cell line was chosen because it is a cell line derived from prostate cancer, fast-growing when compared to the LNCaP and C4-2 cell lines, and has higher

transfection efficiency when compared to the LNCaP and C4-2 cell lines when using the PolyJet transfection reagent (Signagen). However, the PC3 cell line is AR^-, so it was not used for the experiments to test signaling or survival. As shown in Figure 10, EAF2 does specifically immunoprecipitate FOXA1 when over-expressed, which suggested EAF2 may associate in the same complex endogenously.

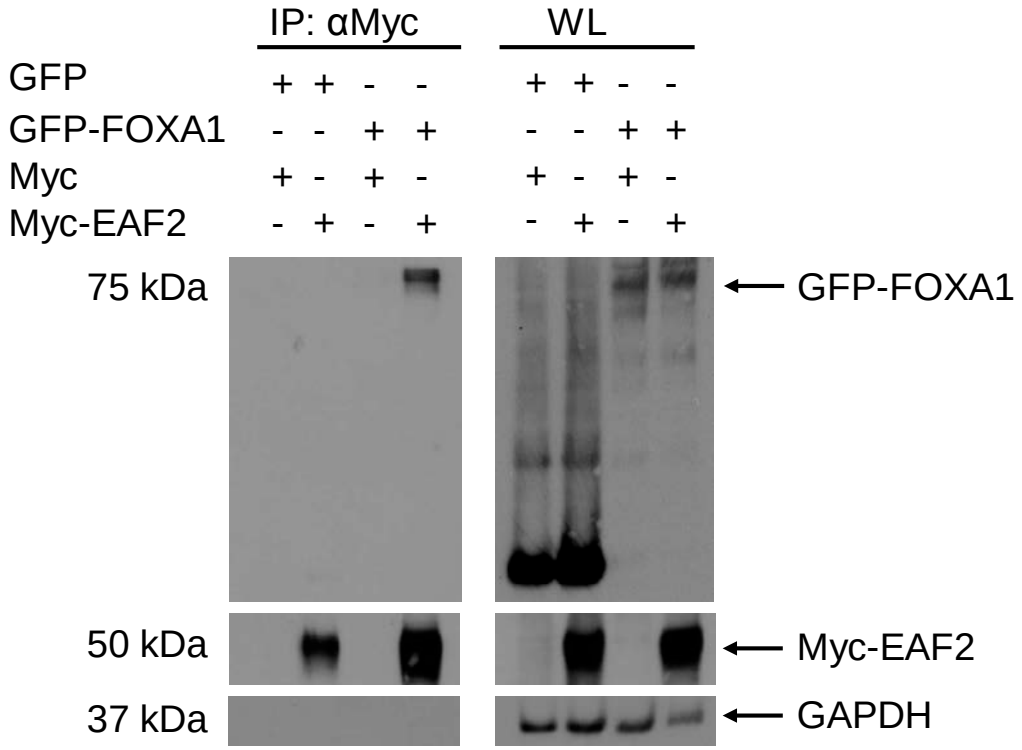

Figure 10. FOXA1 and EAF2 associate when overexpressed in a prostate cancer cell line

GFP-tagged FOXA1 (GFP-FOXA1) and the GFP-tagged vector (GFP) were co-transfected with either Myc-tagged EAF2 (Myc-EAF2) or the Myc-tagged empty vector (Myc) into PC3 cells. Cell lysates were immunoprecipitated (IP) using anti-Myc antibody-conjugated agarose beads, and blots of the IP lysates and whole cell lysates (WL) were probed using anti-GFP antibody (top images), anti-Myc antibody (middle images), and anti-GAPDH antibody (bottom images). Four replications were performed.

After showing that FOXA1 and EAF2 associate when over-expressed, the next question was which regions of EAF2 are required for EAF2 to bind to FOXA1. For this purpose, N-terminus deletion mutants and C-terminus deletion mutants of EAF2 were transfected with full length FOXA1 and then an immunoprecipitation was performed to pull down the EAF2 deletion mutants and the immunopreciptation was then blotted for FOXA1 to determine which regions of EAF2 are required for EAF2 to associate with FOXA1. As can be seen in Figures 11 & 12, FOXA1 bound to the N-terminus deletion mutants and the C-terminus deletion mutants. The N-terminus of EAF2 is known to bind to ELL, and the C-terminus of EAF2 is involved in DNA transactivation and the results of the co-immunoprecipitations shown in Figures 11 and 12 suggest that FOXA1 associates with both the N-terminus and C-terminus of EAF2 and may be associating with EAF2 in a transcription complex (*41*, *42*).

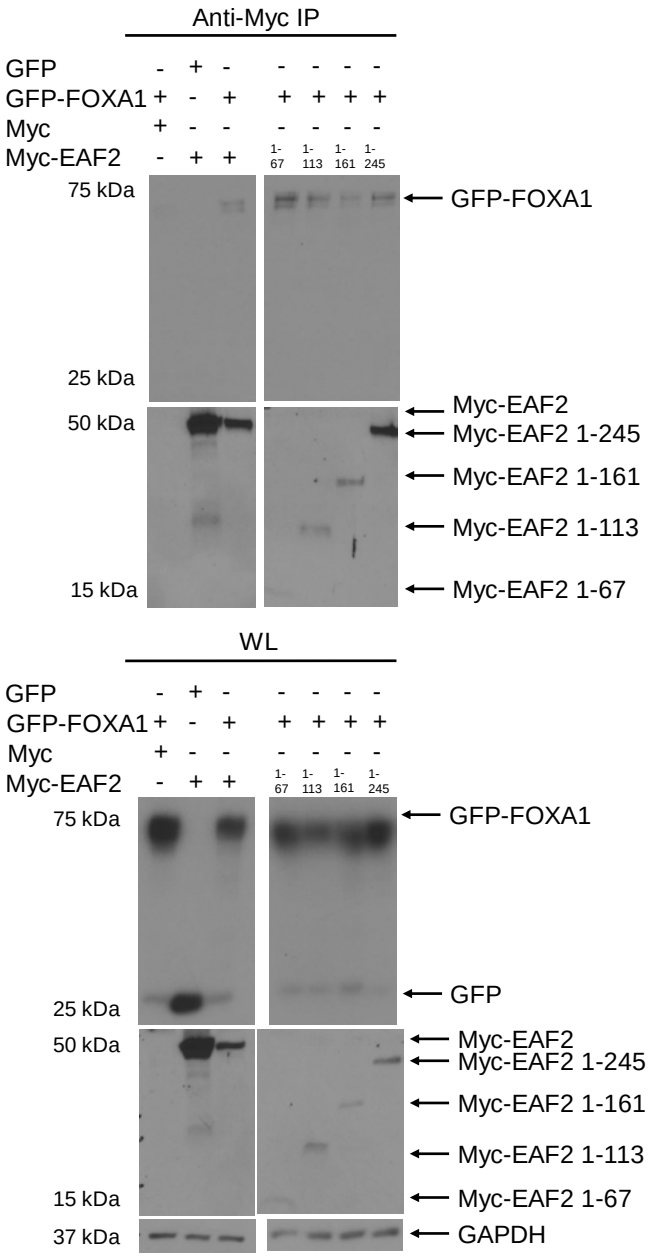

Figure 11. FOXA1 associates with the NH_2-terminus of EAF2

GFP-tagged FOXA1 (GFP-FOXA1) was co-transfected with full length Myc-tagged EAF2 (Myc-EAF2), Myc-tagged EAF2 COOH-terminal deletion mutants (1-67, 1-113, 1-161, and 1-245) or Myc-tagged empty vector (Myc) into PC3 cells. Myc-EAF2 was co-transfected with the GFP-tagged empty vector (GFP) as a negative control. Cell lysates were immunoprecipitated

with anti-Myc antibody-conjugated agarose beads and blots were probed with anti-GFP antibody (1st and 3rd blot), anti-Myc antibody (2nd and 4th blot) or anti-GAPDH antibody (5th blot).

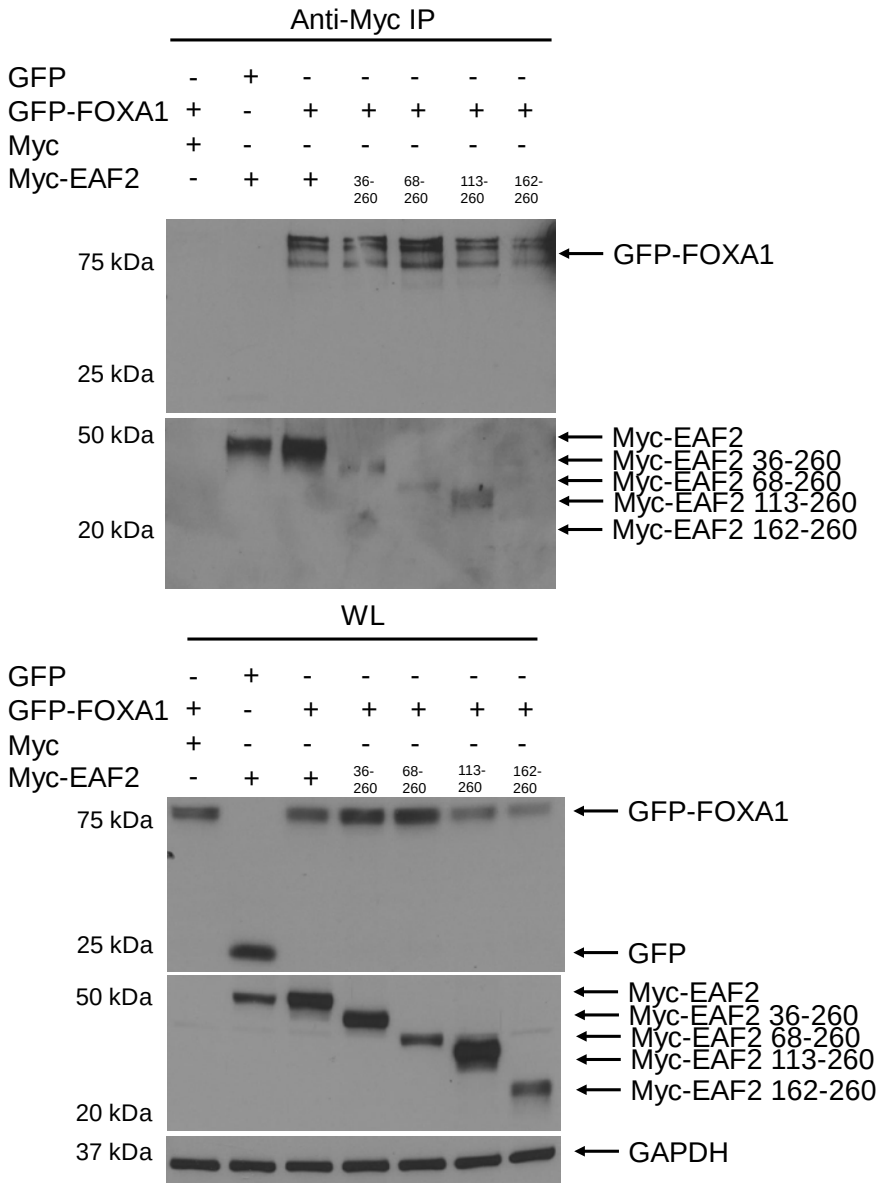

Figure 12. FOXA1 associates with the COOH-terminus of EAF2

GFP-tagged FOXA1 (GFP-FOXA1) was co-transfected with full length Myc-tagged EAF2 (Myc-EAF2), Myc-tagged EAF2 NH_2-terminal deletion mutants (36-260, 68-260, 113-260, and 162-260) or Myc-tagged empty vector (Myc) into PC3 cells. Myc-EAF2 was co-transfected with

the GFP-tagged empty vector (GFP) as a negative control. Cell lysates were immunoprecipitated with anti-Myc antibody-conjugated agarose beads and blots were probed with anti-GFP antibody (1st and 3rd blot), anti-Myc antibody (2nd and 4th blot) or anti-GAPDH antibody (5th blot).

3.2 EAF2 PROTEIN LEVEL IS REDUCED BY FOXA1 AND FOXA1 PROTEIN LEVEL IS REDUCED BY EAF2

While performing the co-immunoprecipitations of FOXA1 and EAF2, it was noted that FOXA1 altered EAF2 protein levels and EAF2 altered FOXA1 protein levels. Therefore a series of experiments was performed to confirm whether the presence of FOXA1 reduced EAF2 protein levels and that the presence of EAF2 reduced FOXA1 protein levels.

3.2.1 FOXA1 and EAF2 protein levels are reduced when co-expressed

In order to test whether FOXA1 reduces EAF2 protein level and EAF2 reduces FOXA1 protein level, untagged FOXA1 and GFP-tagged EAF2 were co-expressed in LNCaP cells, which do express both FOXA1 and EAF2 endogenously; a western blot was performed, and the protein level of EAF2 and FOXA1 was quantified (Figure 13). FOXA1 protein levels were slightly reduced in the presence of EAF2 and EAF2 protein levels were reduced in the presence of FOXA1. The results were not statistically significant, due to differences between the degree of EAF2 and FOXA1 expression between the three different experiments. Table 3 shows the relative levels of Myc-DDK-FOXA1 and Table 4 shows the relative levels of GFP-EAF2 (normalized to GAPDH).

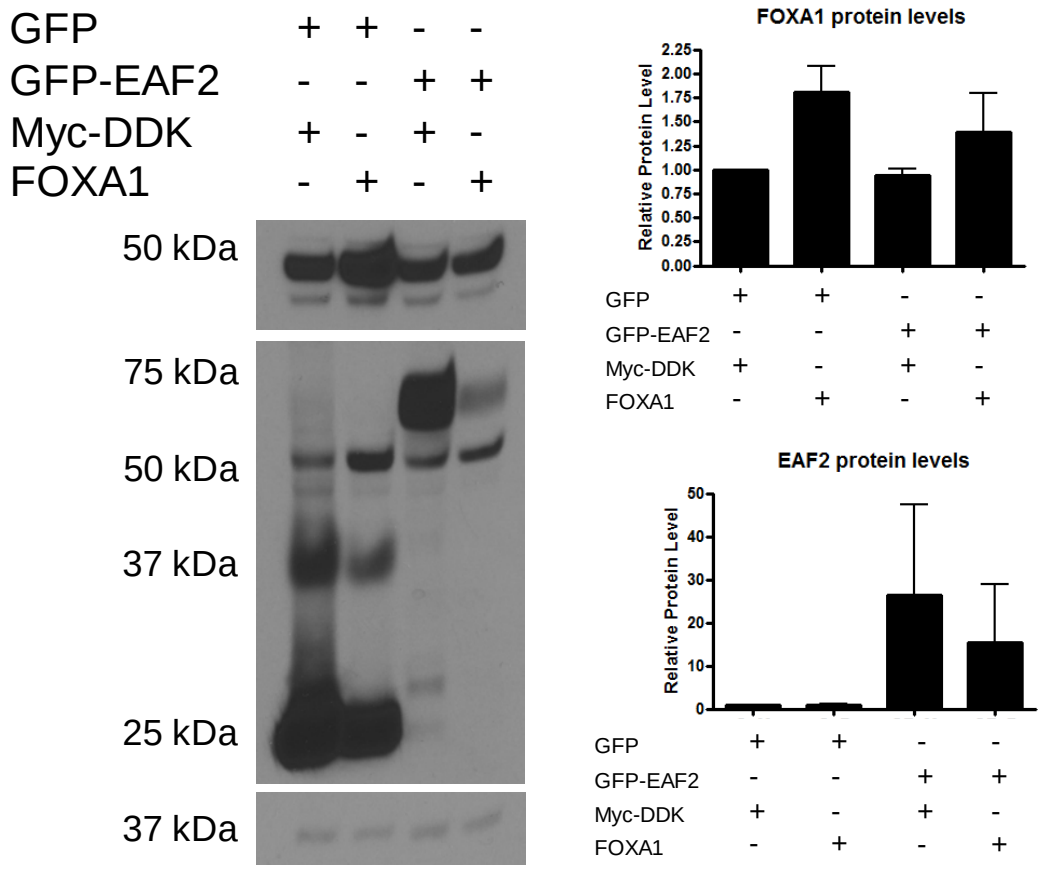

Figure 13. FOXA1 and EAF2 protein levels are reduced when co-expressed

Western blot of whole cell lysate from LNCaP cells co-transfected with GFP-tagged EAF2 (GFP-EAF2), GFP-tagged empty vector (GFP), untagged FOXA1 (FOXA1), or Myc-DDK-tagged empty vector (Myc-DDK) suggests EAF2 and FOXA1 protein levels are reduced when co-expressed. The left panel is a blot that is representative of the 3 replications and was blotted with anti-FOXA1 antibody (top blot), anti-GFP antibody (middle blot), or anti-GAPDH antibody (bottom blot). The upper right panel is a graphical summary of the densitometric analysis of FOXA1 expression normalized to GAPDH used as a loading control. The lower right panel is a graphical summary of the densitometric analysis of EAF2 expression normalized to GAPDH used as a loading control. Bars represent the means of 3 independent experiments ± SEM.

Table 3. Relative protein levels of over-expressed FOXA1 in the FOXA1 and GFP-EAF2 over-expression experiments.

Sample	GFP + Myc-DDK	GFP + FOXA1	GFP-EAF2 + Myc-DDK	GFP-EAF2 + FOXA1
15 March 2013	1.00	1.94	0.94	1.07
21 March 2013	1.00	2.19	1.06	0.93
3 April 2013	1.00	1.30	0.84	2.19
Average	1.00	1.81	0.95	1.40

Numerical summary of the densitometric analysis of FOXA1 expression normalized to GAPDH used as a loading control. Numbers are in densitometric units relative to the empty vector control.

Table 4. Relative protein levels of over-expressed GFP-EAF2 in the GFP-EAF2 and Myc-DDK-FOXA1 co-expression experiments

Sample	GFP + Myc-DDK	GFP + FOXA1	GFP-EAF2 + Myc-DDK	GFP-EAF2 + FOXA1
15 March 2013	1.00	0.51	2.06	1.39
21 March 2013	1.00	0.40	9.27	2.40
3 April 2013	1.00	1.64	68.1	42.4
Average	1.00	0.85	26.5	15.4

Numerical summary of the densitometric analysis of EAF2 expression normalized to GAPDH used as a loading control. Numbers are in densitometric units relative to the empty vector control.

3.2.2 FOXA1 protein levels rise when EAF2 is lost

The co-transfection results suggested that EAF2 could affect FOXA1 protein stability so the next set of experiments tested FOXA1 protein and mRNA levels in the presence and absence of EAF2. The first experiment, shown in Figure 14, tested the level of FOXA1 in the prostates of wild-type and $EAF2^{-/-}$ mice. As can be seen in Figure 14, FOXA1 protein levels were elevated 3-fold in the $EAF2^{-/-}$ mice when compared to the wild-type mice, and this was a significant elevation ($p \leq 0.05$). However, the mice used in the experiment had been irradiated as part of a separate project examining the role of EAF2 in DNA-damage response. It may be that while absence of EAF2 increases FOXA1 protein levels in an irradiated mouse prostate, absence of EAF2 will have no effect on FOXA1 protein levels under normal biological conditions. This mechanism could be due to the published finding that EAF2 promotes apoptosis after UV exposure (*61*). It is possible that the reduction in FOXA1 protein levels is due to EAF2-induced apoptosis. Therefore, additional experiments were performed to confirm the finding that FOXA1 protein levels rise in the absence of EAF2.

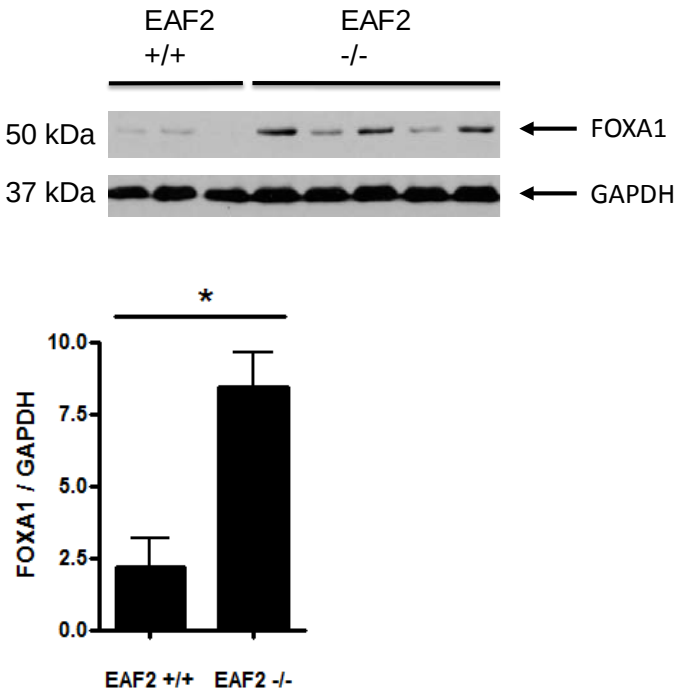

Figure 14. FOXA1 protein levels are elevated in the prostates of EAF2 knockout mice

Western blot of murine prostate tissue reveals FOXA1 is elevated when EAF2 is knocked out. Protein lysates harvested from the anterior prostates of 3 wild type (EAF2 +/+) and 5 Eaf2 knockout (EAF2 -/-) mice 24 hours after the mice were exposed to 8 gray of whole-body γ-radiation were blotted with anti-FOXA1 antibody (upper blot) or GAPDH antibody (lower blot). The lower panel is a graphical summary of the densitometric analysis of XA1 expression normalized to GAPDH used as a loading control. Bars represent the mean of the different samples ± SEM. A t-test was performed to determine significance. *=$p \leq 0.05$

Having observed that FOXA1 protein levels were elevated when EAF2 was knocked out in irradiated mice, the next experiment tested if FOXA1 protein levels were elevated when EAF2 is knocked down in a human prostate cancer cell line. This experiment avoided the problem of irradiation adding an extra variable to the results. As shown in Figure 15, a reduction in EAF2 protein level caused by treatment with siEAF2 in LNCaP cells increased FOXA1 protein level

(p=0.08). The experiment was reproduced three times, and the same results were observed in all three. Figure 15 shows a representative experiment. Interestingly, the level of PSA protein, a protein used as a marker of androgen receptor activity, was also reduced in the EAF2 siRNA knock-down cells ($p \leq 0.01$). However, androgen receptor protein level was not altered in the EAF2 knock-down cells, showing that the decrease in PSA protein levels was not due to a reduction in androgen receptor protein levels.

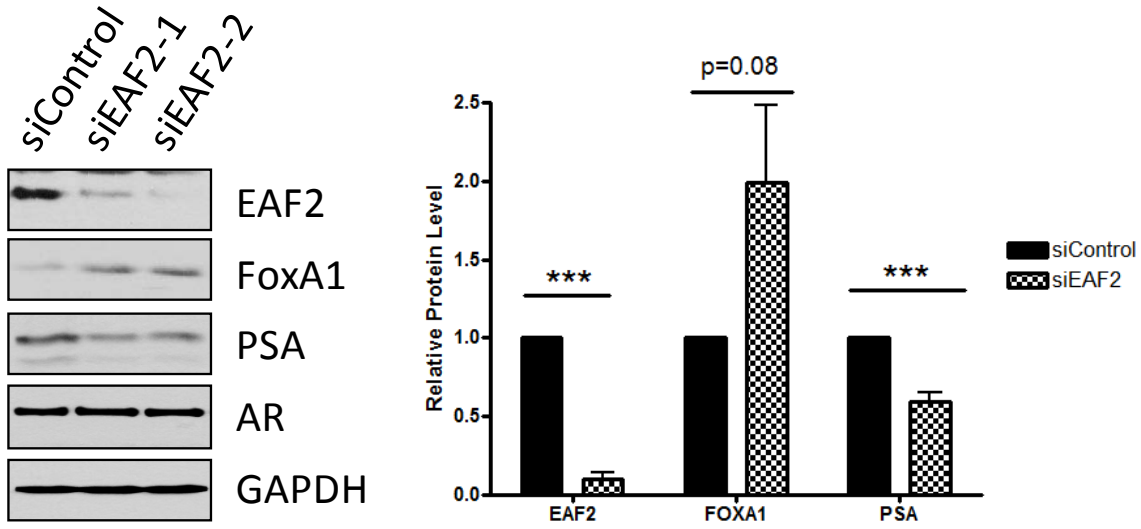

Figure 15. FOXA1 protein levels are elevated and PSA protein levels are reduced in EAF2 knockdown LNCaP cells

Western blot of EAF2 knockdown LNCaP cells reveals loss of EAF2 increases FOXA1 and decreases PSA protein levels after treatment with control siRNA (siControl) and EAF2 siRNA (siEAF2-1 and si-EAF2-2). The left panel is a blot that is representative of the 3 replications and was blotted with anti-EAF2 antibody (1st blot), anti-FOXA1 antibody (2^{nd} blot), anti-PSA antibody (3^{rd} blot), anti-AR antibody (4^{th} blot) or anti-GAPDH antibody (5th blot). The right panel is a graphical summary of the densitometric analysis of EAF2, FOXA1, and PSA

expression normalized to GAPDH used as a loading control. Bars represent the mean of the different samples ± SEM. A t-test was performed to determine significance. ***=$p \leq 0.001$.

3.3 FOXA1 PROTEIN LEVELS ARE ELEVATED AND PSA RNA LEVELS ARE REDUCED UPON EAF2 KNOCKDOWN

Having observed that loss of EAF2 altered FOXA1 and PSA protein levels, the next question was whether the effect occurred at the protein level or at the mRNA level and if the increase of FOXA1 and the decrease of PSA occurs at the mRNA level, does overexpression of FOXA1 and EAF2 affect PSA-promoter activity. In order to test this, qRT-PCR was performed on RNA isolated from the control and EAF2 knockdown LNCaP cells. As shown in Figure 16, there was a statistically significant 1.3 fold reduction of PSA mRNA levels ($p \leq 0.01$) in the EAF2 knockdown cell lines compared to the control. FOXA1 mRNA levels showed a trend of elevation, but the result was not statistically significant ($p \leq 0.1295$).

After observing that FOXA1 protein was elevated and PSA mRNA was reduced when EAF2 was knocked down, the next experiment tested, by luciferase assay, the effect of FOXA1 and EAF2 on PSA promoter activity. When FOXA1 was over-expressed in C4-2 cells, PSA promoter activity was reduced in a dose-dependent manner, as can be seen in Figure 17 and which matches previous reports (*86*). This led to the hypothesis that loss of EAF2 reduced PSA expression due to increased FOXA1 protein inhibiting PSA promoter activity.

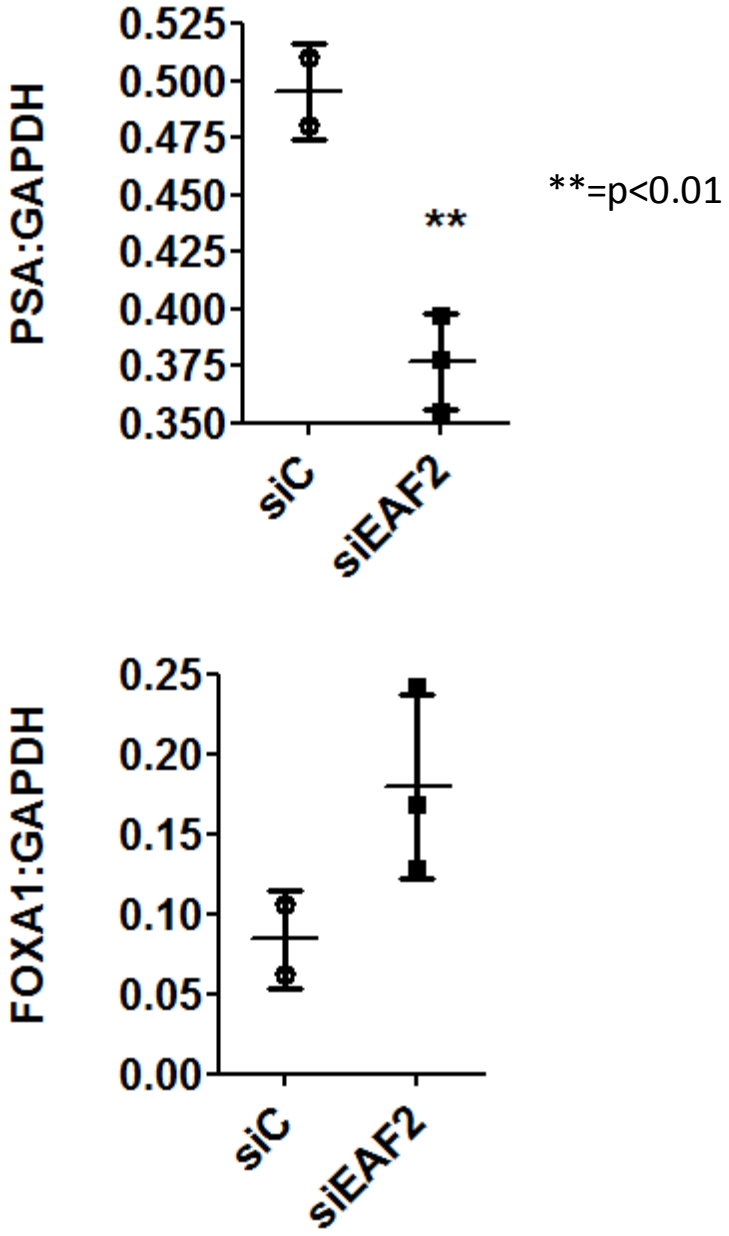

Figure 16. PSA mRNA levels are reduced in EAF2 knockdown LNCaP cells

LNCaP cells were treated with EAF2 siRNA (siEAF2) or control siRNA (siC). The cells were then subjected to reverse transcription and quantitative real-time PCR for PSA mRNA levels (top) and FOXA1 mRNA levels (bottom). Data represents the mean ± SEM of 2 siC samples and 3 siEAF2 samples. A t-test was performed to determine significance. **= $p \leq 0.01$.

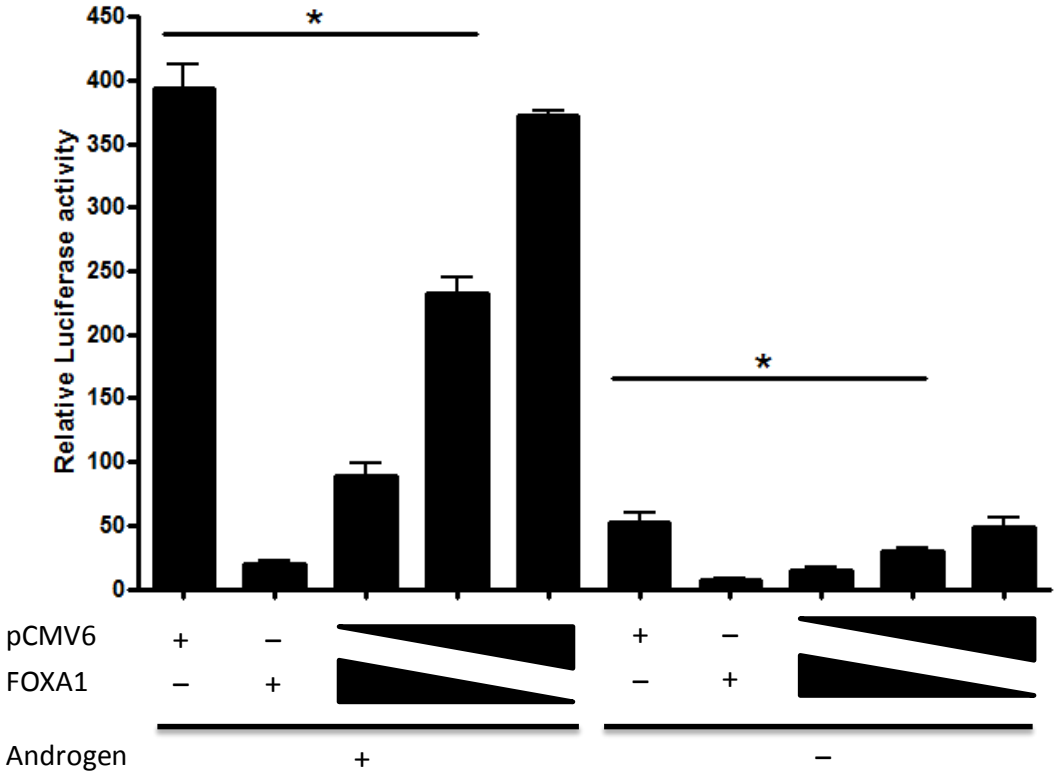

Figure 17. FOXA1 represses PSA promoter activity

C4-2 cells were transfected with 250 ng of PSA-promoter driven luciferase, 25 ng of CMV-promoter driven renilla and 250 ng of FOXA1-untagged plasmid or pCMV6 empty vector alone or with 1:1, 1:5, 1:15, and 1:25 parts FOXA1 plasmid to pCMV6 plasmid, totaling 250 ng of DNA. Cells were lysed 24 hours after treatment with charcoal-striped RPMI+ media with (+ androgen) or without (-androgen) 1 nM supplemental R1881 administered 16 hours before lysis. Luciferase expression was calculated relative to renilla expression. Significance determined by t-test. *=$p \leq 0.05$

3.4 CO-EXPRESSION OF EAF2 AND FOXA1 COUNTERACTS EACH OTHER'S EFFECTS ON CELL GROWTH

Over-expression of EAF2 has been previously shown to reduce growth when over-expressed in PC3 cells and knockdown of FOXA1 in LNCaP and PC3 cell lines reduces growth (*2*, *25*) When EAF2 is overexpressed in LNCaP cells, a colony formation assay shows that cell growth is reduced. Likewise, when FOXA1 is over-expressed in LNCaP cells, cell growth is increased. When EAF2 and FOXA1 are over-expressed together, an intermediary growth phenotype similar to the control cells is observed (Figure 18). Compared to the empty-vector control average colony number of 52, the EAF2-alone average colony number was 24 which is a 2-fold reduction colony number ($p \leq 0.01$ by t-test), the FOXA1-alone average colony number is 83 colonies which is a 1.6 fold increase in colony number ($p \leq 0.01$), and the average number of FOXA1+EAF2 colonies is 36 which is a 1.3 fold reduction in colony number ($p \leq 0.05$). The FOXA1+EAF2 average colony number is reduced 2.3 fold compared to the FOXA1-alone colony number ($p \leq 0.001$). The FOXA1+EAF2 average colony number is elevated 1.5 fold compared to the EAF2-alone average colony number which was significant, but with a $p=0.06$. This data shows that while FOXA1 increases cell survival and EAF2 decreases cell survival, expression of both together results in an intermediate phenotype.

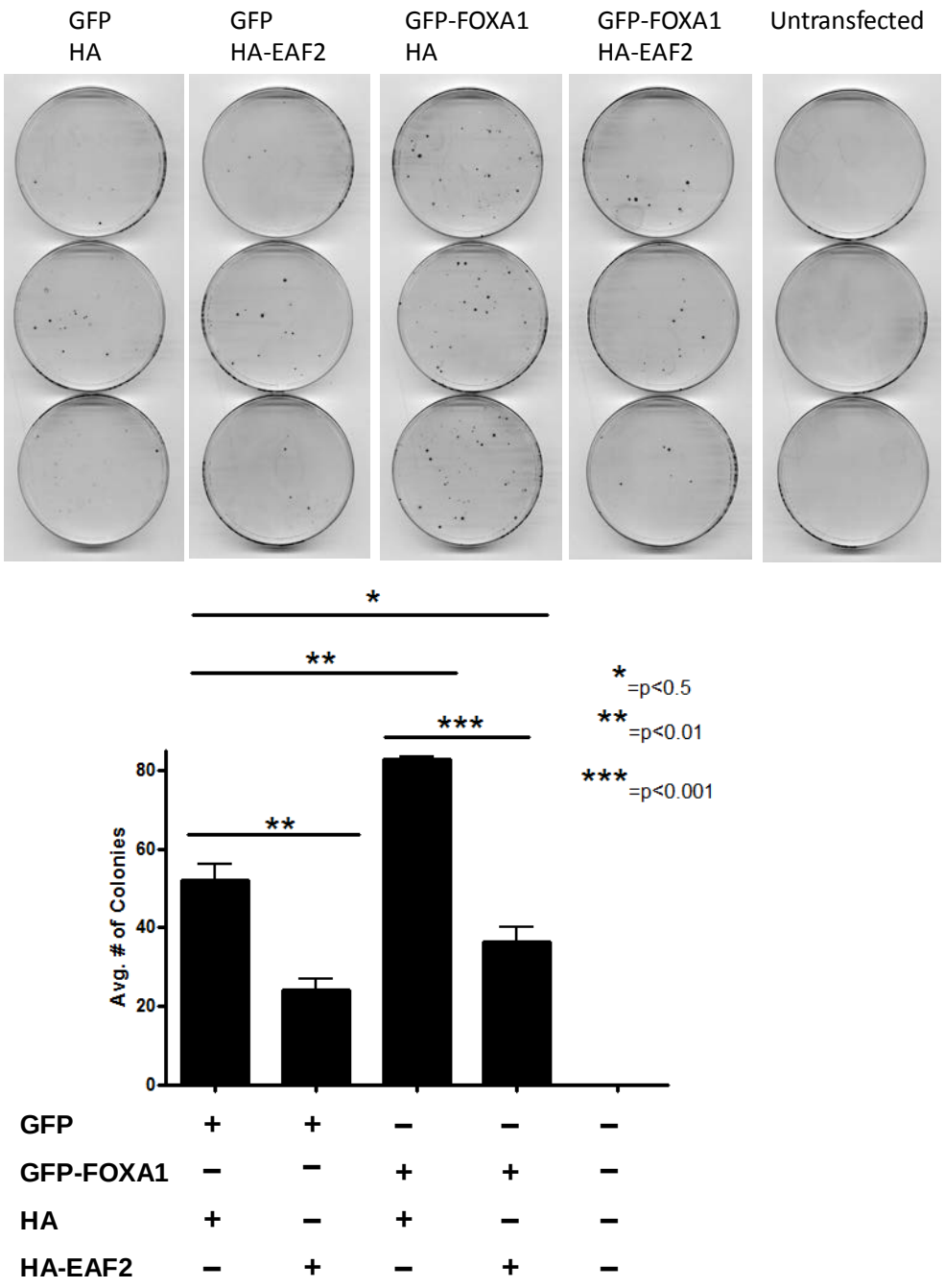

Figure 18. Co-expression of FOXA1 and EAF2 results in an intermediary growth phenotype

Effects of FOXA1 and EAF2 on colony formation. Surviving LNCaP cells co-transfected with HA-EAF2 and/or GFP-FOXA1 at 3 weeks stained with crystal violet (top image). Empty vector controls GFP and HA. Data depicted as mean ± SEM. Significance determined by t-test. *=p≤0.05, **=p≤0.01, ***=p≤0.001.

4.0 DISCUSSION

4.1 OVERVIEW OF RESULTS

The research presented here demonstrates an interaction between FOXA1 and EAF2 that appears to alter FOXA1-mediated androgen receptor signaling. Specifically, FOXA1 and EAF2 appear to be part of a feedback loop that modulates androgen receptor (AR) signaling in the normal prostate, which is de-regulated in prostate cancer (Figure 1). The first evidence of the interaction was a genetic screen performed in *C. elegans*, which showed that loss of the *C. elegans* FOXA1 homolog in *C. elegans* where the EAF2 homolog had been knocked out resulted in sterility due to egg degeneration (Figures 7&8).

Then human FOXA1 and EAF2 were shown to associate when over-expressed in prostate cancer (PCa) cell lines. Specifically, when FOXA1 and EAF2 are over-expressed, EAF2 is able to specifically immunoprecipitate FOXA1, suggesting that FOXA1 and EAF2 are associating in the same complex (Figure 10). The association appears to be mediated by both the N-terminus and C-terminus of EAF2 (Figures 11&12).

Next, it was shown that FOXA1 and EAF2 mutually reduce each other's protein level. Specifically, FOXA1 protein levels are reduced when co-expressed with EAF2 and EAF2 protein levels are reduced when co-expressed with FOXA1 (Figure 13, Tables 3&4).

Furthermore, FOXA1 protein levels are elevated in prostate tissue samples from EAF2 knockout mice (Figure 14) and in prostate cancer cell lines when EAF2 is knocked down (Figure 15).

In addition, loss of EAF2 increases FOXA1-mediated repression of the PSA promoter. First, PSA mRNA levels are reduced when EAF2 is knocked-down in PCa cell lines (Figure 16), and FOXA1 has been shown to inhibit PSA promoter activity (Figure 17), while loss of EAF2 increases FOXA1 (Figures 14&15).

Finally, over-expression of FOXA1 and EAF2 in a prostate cancer cell line results in an intermediary growth phenotype, when compared with expression of FOXA1 or EAF2 alone (Figure 18). This result indicated that FOXA1 and EAF2 mediate cell growth, with over-expression of FOXA1 promoting cell growth and over-expression of EAF2 promoting cell death.

4.2 ROLE OF FOXA1 AND EAF2 INTERACTION IN AR SIGNALING

As prostate cancer (PCa) is the most common non-cutaneous cancer diagnosed and the second most common cause of cancer death in men in the United States, it is important to explore the mechanisms of PCa development and progression (*1*). PCa, like other cancers, is believed to be caused by a dysregulation of various signaling pathways, in particular the androgen receptor (AR) signaling pathway that the prostate is dependent on for growth and survival (*34*). However, the genes upregulated and down-regulated by AR in androgen dependent prostate cancer and castration resistant prostate cancer are different (*87*). Therefore it is important to study how prostate homeostasis is maintained in response to androgen receptor signaling and to determine the signaling pathways that are dysregulated in prostate cancer. The interaction of FOXA1 and

EAF2 appears to play a role in maintaining prostate homeostasis and is dysregulated in PCa due to the down-regulation of EAF2 (Figure 1).

One hypothesized way in which growth is controlled in the normal prostate is the regulation by androgen receptor of growth-suppressive genes (*88*). EAF2, initially discovered in 1997 as U19 and in 2003 as EAF2, is an androgen up-regulated tumor suppressor and appears to play a role in maintaining prostate homeostasis (*2, 3, 40, 89*). One of the key pieces of evidence that EAF2 plays a role in maintaining prostate homeostasis is that when EAF2 was knocked out in mice, prostate epithelial hypertrophy, hyperplasia, and dysplasia was observed (*3*). Therefore, it is possible that EAF2, which is known to be a transcription elongation factor that promotes the up-regulation of genes which inhibit the vascularization required for tumor growth and a pro-apoptotic protein, could be promoting the transcription of a set of genes that inhibit prostate growth and promote cell differentiation (*2, 35, 59*).

FOXA1 also plays a role in androgen receptor signaling, as an androgen receptor pioneer factor (*6, 29*). FOXA1 binds to chromatin right next to an androgen response element (ARE), and then AR binds to FOXA1 and to the ARE to begin transcription (*6*). FOXA1 has also been shown to modulate AR signaling, with over-expression of FOXA1 inhibiting PSA promoter activity (Figure 17) (*86*). FOXA1 is required for prostate epithelial cells to develop, and FOXA1 has been observed to be over-expressed in castration-resistant prostate cancer (*7, 25*). Furthermore, over-expression of FOXA1 results in a castration-resistant prostate cancer phenotype in the androgen-dependent LNCaP prostate cancer cell line, likely due to changes in chromatin accessibility (*90*).

FOXA1 and EAF2 appear to associate in the same complex (Figure 10). In addition, over-expression of FOXA1 reduces EAF2 protein levels and over-expression of EAF2 reduces

FOXA1 protein levels, while FOXA1 protein levels rise when EAF2 is absent or reduced (Figures 13-15, Tables 3&4). This data supports the interaction of FOXA1 and EAF2 and hints towards a possible mechanism by which FOXA1 and EAF2 form a feedback loop that modulates AR activity and prostate cell growth and survival. In addition, over-expression of FOXA1 was shown to promote cell growth and survival in a PCa cell line, while over-expression of EAF2 decreased cell growth and survival, which matches previous reports of the roles of FOXA1 and EAF2 in cell growth and survival (Figure 18) (*2, 90*). However, when FOXA1 and EAF2 are over-expressed together, the cell growth resembles that of the cells transfected with the empty vector control (Figure 18). This data again shows that FOXA1 and EAF2 modulate prostate cell growth. Both are present in the normal prostate, but EAF2 expression is reduced in prostate cancer, while FOXA1 expression increased, suggesting a dysregulation of the AR-mediated pathways that control PCa growth (*2, 25*). Furthermore, down-regulation of EAF2 may up-regulate FOXA1 expression, or enhance FOXA1 function, which promotes PCa development and progression.

FOXA1 and EAF2 may regulate AR signaling through co-factor recruitment to androgen response elements on the promoters of androgen-responsive genes. For instance, FOXA1 has been shown to recruit the Groucho/TLE co-repressor complex to chromatin (*91*). Groucho/TLE represses gene transcription through multiple mechanisms including interactions with histone deacetylases and promoter binding (*92*). FOXA1-mediated recruitment of co-repressors has been shown to alter AR signaling and may be responsible for the different gene expression patterns observed in androgen-dependent prostate cancer and castration-resistant prostate cancer (*8*). EAF2 may inhibit FOXA1-mediated repression of AR signaling by blocking the recruitment of co-repressors to the promoters of AR-dependent genes. EAF2 has also been shown to inhibit

gene transcription by blocking the recruitment of CBP/p300 (*93*). CBP/p300 is a transcription co-activator that acetylates chromatin, which then shifts DNA into a conformation that is permissive to gene transcription (*94*). EAF2 may inhibit FOXA1-mediated transcription of genes that promote growth by inhibiting the recruitment of CBP/p300 to the promoter. FOXA1 and EAF2 have both been reported to alter gene transcription by recruiting or inhibiting the recruitment of transcription co-activators and co-repressors and may regulate AR-mediated gene transcription through this mechanism.

4.3 MECHANISM OF FOXA1 AND EAF2 INTERACTIONS

There are multiple possibilities how FOXA1 and EAF2 could be interacting to affect each other and prostate cell growth. Because EAF2 and FOXA1 are both transcription factors with nuclear localization (*40, 42, 63*), it was hypothesized that the two proteins could associate in the same complex. Another sign that FOXA1 and EAF2 could interact either directly or indirectly within the same complex was that FOXA1 and EAF2 both associate with subunits of the Mediator Complex with FOXA1 associating with MED1 and EAF2 associating with MED26 (*56, 95, 96*). This suggested that EAF2 and FOXA1 could be components of the same transcription complex. Furthermore, FOXA1 is known to recruit MED1 to the promoter of UBE2C, an oncogene that is over-expressed in castration-resistant prostate cancer (*95*). Therefore, co-immunoprecipitations were performed to test if FOXA1 and EAF2 associate. As Figure 10 shows, FOXA1 and EAF2 co-immunoprecipitated when over-expressed together in the PC3 cell line. Likewise, as shown in Figures 11 and 12, FOXA1 associates with both the N-terminus and C-terminus of EAF2. The N-terminus of EAF2 binds to ELL and joins EAF2 to the Super Elongation Complex, while the

C-terminus binds to TCEA1 and TCEA2, two transcription elongation factors that associate with RNA polymerase II (*40, 41, 54*). These results indicate that FOXA1 and EAF2 are associating in the same complex, potentially a transcription complex. It is possible that FOXA1 and EAF2 affect cell growth and survival by altering gene transcription, either in conjunction with AR or independently of AR. For instance, it has been found that glucocorticoids up-regulate EAF2 mRNA expression, and FOXA1 binds to the promoters of glucocorticoid receptor regulated genes (*87*). In addition, it has been found that down-regulation of EAF2 can be mediated by the epidermal growth factor receptor in PCa (*97*). These results indicate that FOXA1 and EAF2 can function independently of AR.

Another potential mechanism by which FOXA1 and EAF2 may affect each other is through modulating the protein and mRNA levels of each other, which then affects AR-mediated transcription of PSA and prostate cell growth and survival. As seen in Figure 13 and Tables 3&4, over-expression of FOXA1 reduced EAF2 protein levels and over-expression of EAF2 reduces FOXA1. In this experiment, the cells were transfected with CMV promoter-driven FOXA1 and EAF2 plasmid constructs. Therefore, the effect occurred at the post-translational level, not the transcriptional level. EAF2 could affect FOXA1 protein stability through direct interactions where binding of FOXA1 to EAF2 promotes degradation of both. EAF2 could promote FOXA1 degradation by binding to and stabilizing a second protein which then promotes FOXA1 degradation. This would be similar to how EAF2 reduces protein levels of HIF1α by binding to and stabilizing pVHL which then binds to HIF1α by hydroxylation of HIF1α prolines and recruits ubiquitin ligases that ubiquitinate HIF1α, leading to HIF1α degradation by the proteoseome (*57*). It could also be a chain of protein-protein interactions leading ultimately to FOXA1 degradation. This would be similar to how NFκB is activated, where a chain of kinase-

mediated phosphorylation steps are required which ultimately leads to the ubiquitination and degradation of IκB and NFκB translocation into the nucleus (*98*).

Another way EAF2 could reduce FOXA1 protein levels is by mediating the transcription of another protein that then reduces FOXA1 protein levels and FOXA1 could use a similar mechanism to reduce EAF2 protein levels. For instance, EAF2 binds to and is stabilized by ELL2, which is another androgen-responsive gene (*40, 42, 84*). Over-expression of CMV-promoter-driven FOXA1 may reduce CMV-promoter-driven EAF2 protein levels by reducing transcription of ELL2 mRNA, which then leads to reduced ELL2 protein levels. With less available ELL2 to bind to and stabilize EAF2, EAF2 protein degradation is promoted.

Use of CMV-promoter driven FOXA1 and EAF2 indicated that FOXA1 and EAF2 regulate each other at the post-transcriptional level, regulation at the post-transcriptional level does not rule out the possibility that FOXA1 can reduce transcription of EAF2 mRNA and EAF2 can reduce transcription of FOXA1 mRNA. For instance, while knockdown of EAF2 significantly increased FOXA1 protein levels, FOXA1 mRNA levels were also increased, although the result was not statistically significant (Figure 16). Over-expression of FOXA1 reduced the expression of PSA mRNA (Figures 16&17) and may reduce the expression of EAF2 mRNA as well.

4.4 FOXA1 AND EAF2 REPRESENT A NOVEL FEEDBACK LOOP IN ANDROGEN RECEPTOR SIGNALING

The interaction of EAF2 and FOXA1 appears to be part of a newly identified feedback loop in androgen receptor signaling. The over-expression of FOXA1 reduces the protein levels of the

androgen up-regulated genes like PSA and reduces the protein level of over-expressed EAF2. Likewise, over-expression of EAF2 reduced FOXA1 protein levels. In addition, FOXA1 and EAF2 associate in the same complex. Together the data suggests an interaction loop between FOXA1 and EAF2 as illustrated in Figure 19. EAF2 reduces FOXA1 protein levels and inhibits FOXA1-mediated repression of PSA and cell growth, and FOXA1 reduces EAF2 protein levels and inhibits EAF2-mediated cell death. These results suggest that the interaction of FOXA1 and EAF2 represents an interesting target for future therapies of prostate cancer. Pharmaceuticals that promote EAF2 expression or which mimic the effects of the interaction of EAF2 and FOXA1 could be used to treat prostate cancer by restoring normal androgen receptor signaling. Restoration of normal androgen receptor signaling would return the prostate back to normal homeostasis and inhibit tumor growth.

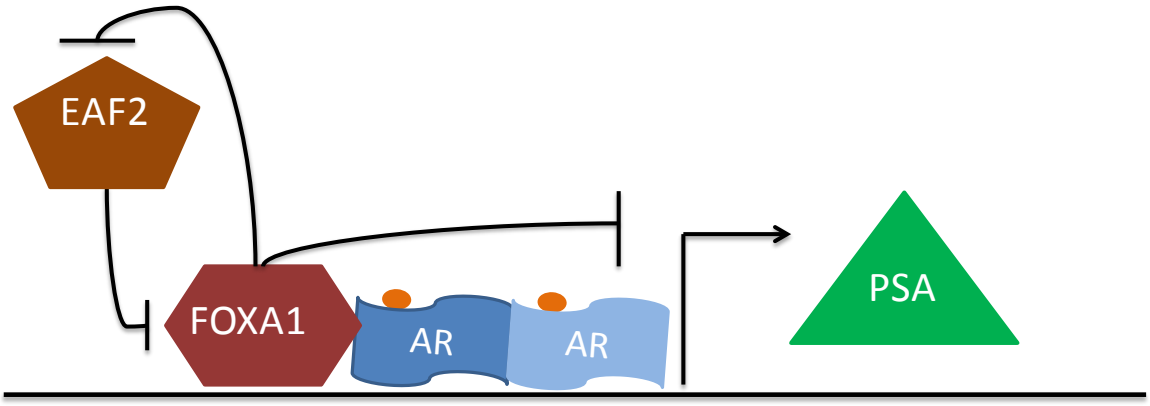

Figure 19. FOXA1 and EAF2 form a feedback loop in androgen receptor signaling

Schematic of the proposed mechanism based on the current observations of the interaction of FOXA1 and EAF2. FOXA1 binds to the androgen receptor (AR) and to a gene promoter and inhibits transcription of androgen-responsive genes like PSA. EAF2 inhibits FOXA1-mediated

repression of androgen-receptor transcription and reduces FOXA1 protein levels and FOXA1 reduces EAF2 protein levels and inhibits EAF2.

4.5 FUTURE DIRECTIONS

The interaction of FOXA1 and EAF2 may be a promising target for future pharmaceuticals that will either stabilize EAF2 by inhibiting the interaction of FOXA1 and EAF2 or will reduce FOXA1 protein levels by mimicking the interaction of FOXA1 and EAF2. However, for such pharmaceuticals to be practical, the mechanism of the interaction of FOXA1 and EAF2 needs to be further elucidated, as well as the ramifications of inhibiting the interaction on prostate cell growth and proliferation.

One key step is to further elucidate the association of FOXA1 and EAF2. Co-immunoprecipitations will be performed to determine if the association of FOXA1 and EAF2 occurs when FOXA1 and EAF2 are expressed endogenously in addition to when they are over-expressed. Also, further co-immunoprecipitations using FOXA1 deletion mutants and EAF2 deletion mutants will be used to determine which regions of FOXA1 and EAF2 are required for FOXA1 and EAF2 to associate. Once it is possible to generate mutants of FOXA1 and EAF2 that do not associate, it is possible to determine if FOXA1 and EAF2 reduce the protein levels of each other via association in the same complex or if another mechanism like transcription of an intermediary protein is required. If FOXA1 and EAF2 reduce the protein levels of each other through the association shown in Figure 10, FOXA1 and EAF2 deletion mutants lacking the

regions required for association will not be affected by co-transfection with wild-type EAF2 and FOXA1 respectively.

Another question that should be answered is if FOXA1 and EAF2 promote the degradation of each other. One method would be to perform pulse-chase experiments to determine if FOXA1 protein levels are different when EAF2 is knocked-down and if EAF2 protein levels are different when FOXA1 is knocked-down. Then it can be determined if the proteins reduce the stability of each other or if somehow more FOXA1 and EAF2 protein is expressed when the other protein is absent.

It will also be important to determine if EAF2 alters the availability of FOXA1 and AR at the PSA promoter. Chromatin immunoprecipitations would be performed to determine if the presence or absence of EAF2 affects the localization of AR and FOXA1 to the PSA-promoter. This would provide evidence that EAF2 is directly altering PSA-promoter activity by altering the presence of AR at the PSA-promoter.

It is also important to determine if FOXA1 changes EAF2 mRNA levels and if EAF2 reduces FOXA1 mRNA levels. First, it should be determined if loss of EAF2 increases FOXA1 mRNA levels significantly and if loss of FOXA1 alters EAF2 mRNA levels. This can be performed by knocking down FOXA1 and EAF2 in LNCaP or C4-2 cells, which endogenously express FOXA1 and EAF2. If loss of EAF2 increases FOXA1 mRNA levels or if loss of FOXA1 alters EAF2 mRNA levels, chromatin immunoprecipitations will be performed to determine if EAF2 is associated with the FOXA1 promoter or if FOXA1 is associated with the EAF2 promoter.

Another interesting question to answer would be if EAF2 binding partners like ELL1, ELL2, or pVHL also affect FOXA1 protein levels and FOXA1-mediated transcription of PSA in

a manner similar to EAF2. It would be hypothesized that if ELL1 or ELL2 interact with FOXA1 in a manner similar to EAF2, the observed interaction involved either the super elongation complex or little elongation complex of which EAF2, ELL, ELL2 are components (*55*). If pVHL affects FOXA1 in a manner similar to EAF2, it would be hypothesized that the observed interaction would involve the hypoxia-response signaling pathways (*57, 99*).

The research here suggests that in the normal prostate there is a balance between AR signaling that promotes cell proliferation and that which promotes cell death (*100, 101*). During the development and progression of prostate cancer, the balance between cell proliferation and cell death becomes skewed towards cell proliferation due to the loss of tumor suppressors like EAF2 (*2, 26*). More work is needed to elucidate the interaction of FOXA1 and EAF2, but it represents a new potential way to restore normal AR signaling and to treat prostate cancer.

5.0 SUPPLEMENTARY FIGURES

5.1 FOXA1 AND EAF2 PROTEIN STABILITY ASSAY

It is possible that FOXA1 and EAF2 could affect the stability of the other protein. Therefore assays were performed in LNCaP cells to determine if EAF2 protein stability was reduced in the presence of FOXA1 or if FOXA1 stability was reduced in the presence of EAF2. As shown in Figure 12, EAF2 degraded too quickly to be able to determine if FOXA1 affected EAF2 protein stability or if EAF2 affected FOXA1 protein stability. EAF2 had been previously reported to be 50% degraded within 6 hours when over-expressed Cos-7 cells and 50% degraded within 2 hours when over-expressed in 293 cells (*42*). A pulse-chase experiment using radioactively-labeled EAF2 followed by another using radioactively-labeled FOXA1 will avoid the complications caused by EAF2 degradation due to continuous expression of replacement EAF2 and will be performed in the future.

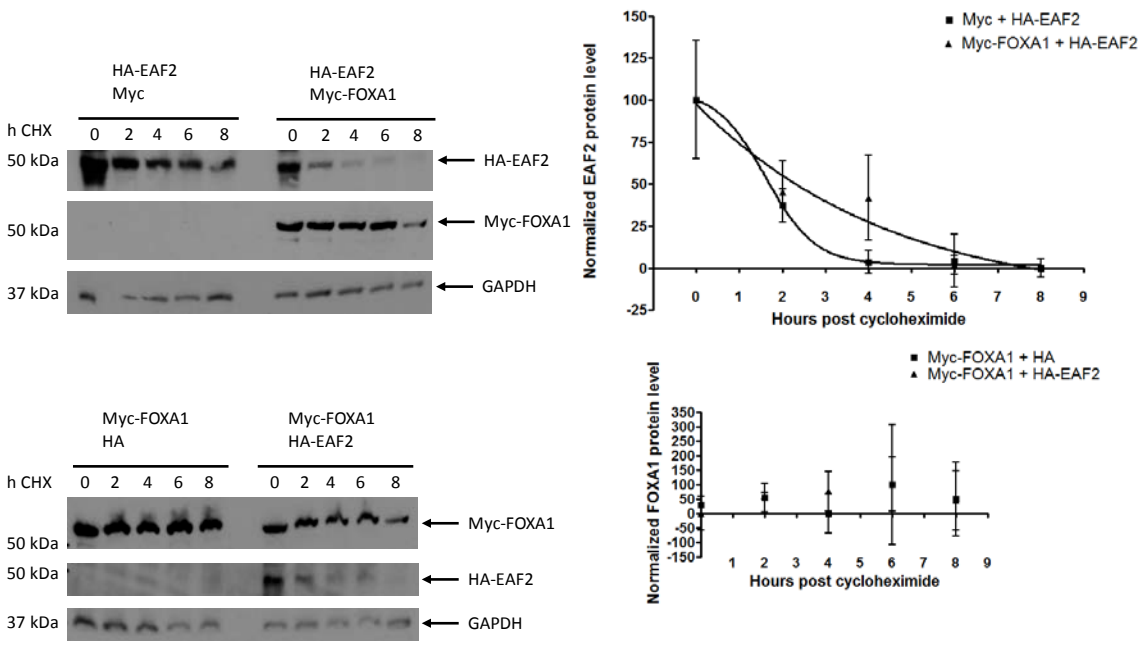

Figure 20. The effect of co-expression of EAF2 and FOXA1 on FOXA1 and EAF2 protein stability is inconclusive

The effect of FOXA1 on EAF2 protein stability (top images). Two micrograms of HA-EAF2 were co-transfected with 2.0 µg of Myc-DDK-FOXA1 (Myc-FOXA1) or Myc empty vector (Myc) into PC3 cells. Forty hours after transfection, cells were treated with cycloheximide (CHX) at 50 µg/µL for the indicated number of hours. A graphical display of the densitometric analysis of EAF2 protein levels normalized to GAPDH protein levels was made. Points represent the mean of the same time point from three different experiments ± SEM. The effect of EAF2 on FOXA1 protein stability (bottom images). Two micrograms of Myc-DDK-FOXA1 were co-transfected with 2.0 µg of HA-EAF2 or HA empty vector (HA) into PC3 cells. Forty hours after transfection, cells were treated with cycloheximide (CHX) at 50 µg/µL for the indicated number of hours. A graphical display of the densitometric analysis of EAF2 protein levels normalized to GAPDH protein levels was made. Points represent the mean of the same time point from three different experiments ± SEM.

5.2 EFFECT OF OVER-EXPRESSION OF FOXA1 AND EAF2 ON PSA-PROMOTER ACTIVITY

The results of over-expressing FOXA1 with EAF2 were mixed. The initial experiments, as seen in Figure 17, showed that EAF2 alleviated FOXA1-mediated repression PSA promoter in C4-2 cells. There were three replications with these results. However, as seen in Figure 18, the later set of experiments had 4 replications that showed EAF2 enhancing FOXA1-mediated repression of the PSA promoter. These results could be due to impurities in the plasmids used to perform the transfections, although the plasmids were checked by nanodrop and agarose gel, cell confluency at time of transfection (60% vs. 80%), or variations in the cell cycle at the time of transfection.

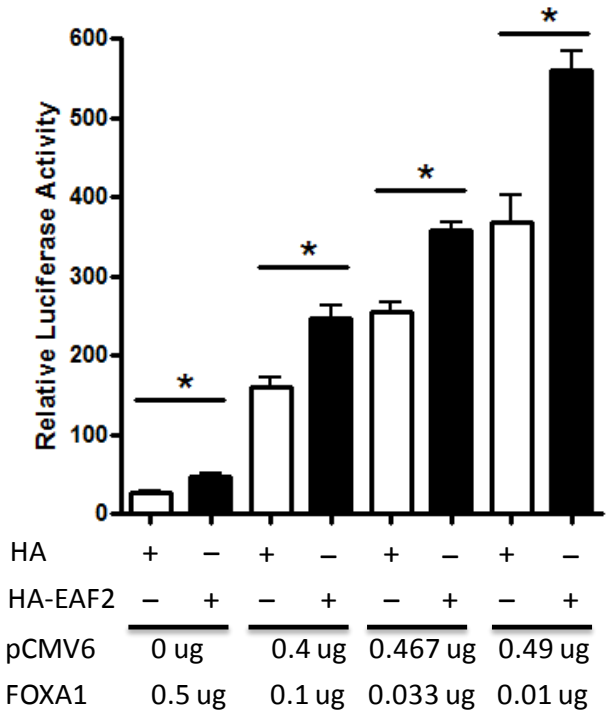

Figure 21. EAF2 alleviated FOXA1-mediated repression of the PSA promoter

C4-2 cells were transfected with 250 ng of PSA-promoter driven luciferase, 25 ng of CMV-promoter driven renilla, 250 ng of HA-EAF2 or HA empty vector (HA) and 250 ng of FOXA1-untagged plasmid alone or with 1:1, 1:5, 1:15, and 1:25 parts FOXA1 plasmid to pCMV6 plasmid, totaling 250 ng of DNA. Cells were lysed 24 hours after treatment with charcoal-striped RPMI+ media with 1 nM supplemental R1881 administered 16 hours before lysis. Luciferase expression was calculated relative to renilla expression. Significance determined by t-test. *=p≤0.05

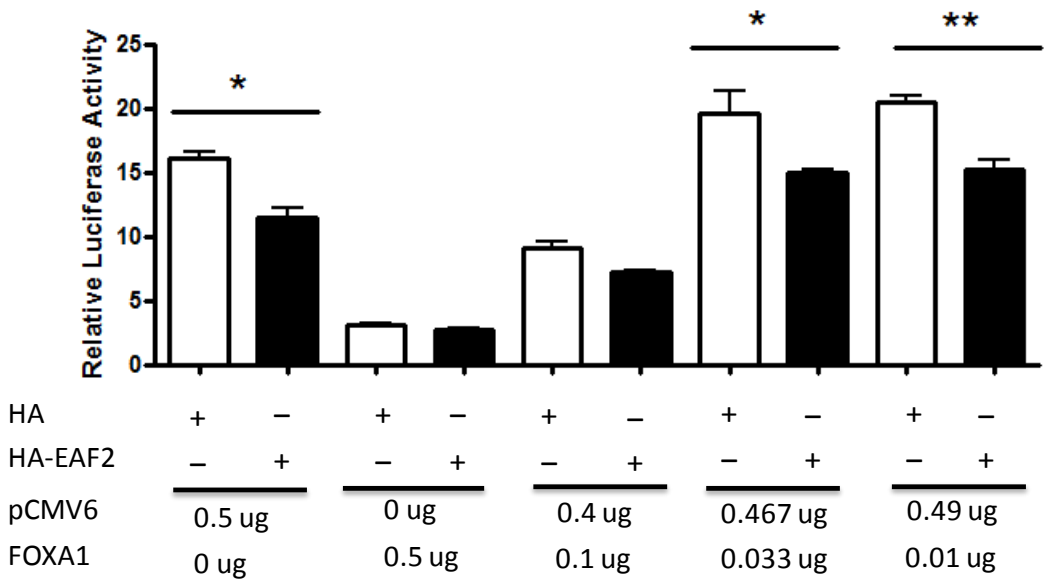

Figure 22. EAF2 enhanced FOXA1-mediated repression of the PSA promoter

C4-2 cells were transfected with 250 ng of PSA-promoter driven luciferase, 25 ng of CMV-promoter driven renilla, 250 ng of HA-EAF2 or HA empty vector (HA) and 250 ng of FOXA1-untagged plasmid alone or with 1:1, 1:5, 1:15, and 1:25 parts FOXA1 plasmid to pCMV6 plasmid, totaling 250 ng of DNA. Cells were lysed 24 hours after treatment with charcoal-striped RPMI+ media with 1 nM supplemental R1881 administered 16 hours before lysis. Luciferase

expression was calculated relative to renilla expression. Significance determined by t-test. *=$p \leq 0.05$

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