Conditional deletion of ELL2 induces murine prostate intraepithelial neoplasia

Laura E Pascal1,*, Khalid Z Masoodi1,2,*, June Liu1,4,*, Xiaonan Qiu5, Qiong Song1,4, Yujuan Wang1, Yachen Zang1,5, Tiejun Yang1,4, Yao Wang1,2, Lora H Rigatti6, Uma Chandran9, Leandro M Colli10, Ricardo Z N Vencio11, Yi Lu12,13, Jian Zhang12,13 and Zhou Wang1,14,15

1Department of Urology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA
2Transcriptomics Lab, Division of Plant Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, Shalimar, Srinagar, Jammu and Kashmir, India
3School of Medicine, Tsinghua University, Beijing, China
4Department of Urology, The Second Affiliated Hospital of Soochow University, Suzhou, China
5Department of Urology, Henan Cancer Hospital, Affiliated Cancer Hospital of Zhengzhou University, Zhengzhou, China
6Department of Urology, China-Japan Hospital of Jilin University, Changdun, Jilin, China
7Division of Laboratory Animal Resources, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA
8Department of Biomedical Informatics, University of Pittsburgh, Pittsburgh, Pennsylvania, USA
9Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto-SP, Brazil
10Division of Computing and Mathematics FFCLRP-USP, University of São Paulo, Ribeirão Preto, Brazil
11Key Laboratory of Longevity and Aging-related Diseases, Ministry of Education, China and Center for Translational Medicine Guangxi Medical University, Nanning, Guangxi, China
12Department of Biology, Southern University of Science and Technology School of Medicine, Shenzhen, Guangdong, China
13University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA
14Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

*(L E Pascal, K Z Masoodi and J Liu contributed equally to this work)
†(J Liu is now at Lovelace Respiratory Research Institute, Albuquerque, New Mexico, USA)

Abstract

Elongation factor, RNA polymerase II, 2 (ELL2) is an RNA Pol II elongation factor with functional properties similar to ELL that can interact with the prostate tumor suppressor EAF2. In the prostate, ELL2 is an androgen response gene that is upregulated in benign prostatic hyperplasia (BPH). We recently showed that ELL2 loss could enhance prostate cancer cell proliferation and migration, and that ELL2 gene expression was downregulated in high Gleason score prostate cancer specimens. Here, prostate-specific deletion of ELL2 in a mouse model revealed a potential role for ELL2 as a prostate tumor suppressor in vivo. Ell2-knockout mice exhibited prostatic defects including increased epithelial proliferation, vascularity and PIN lesions similar to the previously determined prostate phenotype in Eaf2-knockout mice. Microarray analysis of prostate tissues from Ell2-knockout and wild-type mice on a C57Bl/6J background at age 3 months and qPCR validation at 17 months of age revealed a number of differentially expressed genes associated with proliferation, cellular motility and epithelial and neural differentiation. OncoPrint analysis identified combined downregulation or deletion in prostate adenocarcinoma cases from the Cancer Genome Atlas (TCGA) data portal. These results suggest that ELL2 and its pathway genes likely play an important role in the development and progression of prostate cancer.

Key Words

- ELL2
- prostatic intraepithelial neoplasia
- prostate cancer
- EAF2
- HIF1α
Introduction

ELL2 (elongation factor, RNA polymerase II, 2; previously eleven-nineteen lysine-rich leukemia 2) is an RNA Pol II elongation factor with functional properties similar to ELL and ELL3 (Shilatifard et al. 1997, Miller et al. 2000). ELL2 suppresses transient pausing of RNA polymerase II activity along the DNA strand and facilitates the transcription process (Shilatifard et al. 1997). The ELL family proteins are components of the super elongation complex (SEC), which regulate HOX gene expression in MLL-based hematological malignancies by controlling genes involved in early development and in immediate early gene transcription (Lin et al. 2010, 2011, Smith et al. 2011, Takahashi et al. 2011). ELL was recently identified as a component in the little elongation complex (LEC), which is involved in RNA polymerase II transcription of small nuclear RNA (snRNA) genes (Smith et al. 2011). ELL and EAF proteins also bind to MED26, a component of the human mediator that plays a key role in transcriptional activation (Takahashi et al. 2011). ELL2 was also reported to direct immunoglobulin secretion in plasma cells by stimulating alternative RNA processing associated with histone methylation (Martincic et al. 2009, Milcarek et al. 2011).

ELL is frequently translocated with the MLL gene on chromosome 11q23 in acute myeloid leukemia (Thirman et al. 1994, Mitani et al. 1995); and homozygous deletion of ELL is embryonic lethal in the mouse as well as in Drosophila (Mitani et al. 2000, Eisenberg et al. 2002). ELL and ELL2 interact with ELL-associated factors 1 and 2 (EAF1 and EAF2) (Simone et al. 2003) resulting in enhanced ELL elongation activity (Kong et al. 2005). ELL and ELL2 expression ratios vary in different human tissues, suggesting tissue-specific roles for these genes (Shilatifard et al. 1997). Since high expression of ELL2 has been reported in the prostate (Uhlen et al. 2005), ELL2 may be important for prostate homeostasis. ELL2 was also found to be an androgen response gene (Nelson et al. 2002, Bolton et al. 2007) that is upregulated in response to chronic prostate inflammation in rats (Funahashi et al. 2015) and was upregulated in human benign prostatic hyperplasia (BPH) (O’Malley et al. 2009). The specific role of ELL2 in the prostate has not been fully elucidated; however, transfected ELL2 protein has been shown to interact with the potential prostate tumor suppressor gene ELL-associated factor 2 (EAF2) (Simone et al. 2003). In this study, Simone and coworkers showed that endogenous EAF2 coimmunoprecipitated with transfected ELL2 in 293 cells and that like the MLL–ELL fusion protein (Lavau et al. 2000), MLL–EAF2 could immortalize hematopoietic progenitor cells in vitro. Recently, eaf-1 and eaf-1, worm orthologs of EAF1, EAF2 and ELL1 and ELL2, were shown to have overlapping function in the regulation of fertility, survival and cuticle formation in C. elegans (Cai et al. 2011). In advanced prostate cancer, EAF2 protein was downregulated (Xiao et al. 2003, Ai et al. 2013, Pascal et al. 2013); and, overexpression of EAF2 in prostate cancer cell lines induced apoptosis and inhibited the growth of xenograft tumors (Xiao et al. 2003). Eaf2-knockout mice developed high-grade murine prostatic intraepithelial neoplasia (mPIN) and increased vascularity in several murine strains (Xiao et al. 2008, Pascal et al. 2013). Recently, we reported that siRNA knockdown of ELL2 in combination with RB knockdown enhanced proliferation, migration and invasion in prostate cancer cell lines LNCaP, C4-2 and 22RV1, while ELL2 knockdown alone enhanced migration and invasion but did not induce a statistically significant increased proliferation (Qiu et al. 2017). ELL2 expression was downregulated in high Gleason score prostate cancer specimens. Cumulatively, these studies suggest that ELL2 may play a significant role in maintaining prostate homeostasis similar to EAF2.

In the current study, the potential role of ELL2 in the prostate was explored in a murine knockout model. Conditional prostate epithelial cell-specific ELL2-knockout mice were generated and examined for histologic defects. Microarray analysis of mouse prostates was performed to identify potential target genes of ELL2. Differentially expressed genes identified by microarray analysis in animals at 3 months of age were validated by qPCR in animals at 17–20 months of age.

Materials and methods

In situ hybridization

Before hybridization, murine prostate tissue cryosections (ProbeOn, Fisher Biotech, Pittsburgh, PA) were washed with PBS, fixed in 4% paraformaldehyde, digested with proteinase K at 20 µg/mL in PBS, refixed in 4% paraformaldehyde, rewash in PBS and then acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0. Full-length mouse ELL2 cDNA was inserted into the EcoRI and XhoI site between T3 and T7 promoters in pBluescript II SK plasmid vector. The plasmid was purified by CsCl double banding, linearized with EcoRI or XhoI, and protease K treated. Purified linear DNA...
templates were used in the synthesis of both sense and antisense digoxigenin-labeled riboprobes using either T3 or T7 RNA polymerase (Promega) as previously described (Cytic et al. 2002). Riboprone size was reduced to approximately 250 bp using limited alkaline hydrolysis.

For hybridization, the probe was diluted in hybridization solution (5× SSC, 1× Denhardt’s, 100 µg/mL salmon testis DNA, 50% formamide and 250 µg/mL yeast transfer RNA), and slides were hybridized overnight at 67°C in a sealed chamber humidified with 5× SSC/50% formamide. Coverslips were removed, and slides were washed in 0.2× SSC at 72°C for 1 h. After washing in buffer (0.1 M Tris (pH 7.6), 0.15 M NaCl), slides were blocked in 10% horse serum at room temperature for 1 h. Slides were then incubated overnight at 4°C with antidigoxigenin-AP Fab fragments (1:2000, Boehringer Mannheim, Mannheim, Germany) in 1% horse serum. Slides were washed and then developed with nitro blue tetrazolium or T7 RNA polymerase (Promega) as previously described.

**Generation of prostate-specific ELL2 deletion mice**

Mice with prostate-specific deletion of the ELL2 gene were generated by cross-breeding Ell2^{loxP/loxP} mice with probasin-Cre4 (Wu et al. 2001) mice. Ell2^{loxP/loxP} mice were cloned by homologous recombination between Ell2^{loxP/loxP} targeting vector and the Ell2 genomic locus (Park et al. 2014). Briefly, a conditional targeting vector in which Ell2 exon 3 was flanked by a single upstream loxP site and a downstream <FRT/neomycin resistance/ FRT/loxP> cassette was constructed. Correctly targeted embryonic stem cells contained the cko allele with an 8.6 Sacl band, in addition to a 14 kb wild-type band, following hybridization with the 5′ probe. These cko clones also contained a 13.9 kb EcoRI-targeted band as well as a 12 kb wild-type band, following hybridization with the 3′ probe. Mice carrying the Ell2-cko allele were maintained on a C57BL/6J background and cross-bred with the 3′-ATGCATCGTCGAACAGGAGTTCAAGGT-3′ and Ell2ko-SaclI-5′-CTGATACCGCGGGTGAAATCACTCC-3′ (reverse) and Cre-upstream, 5′-TTGCCTGCATTACCGGTGATG-3′ and Cre-downstream, 5′-TCCAGCCACCGCTTGGATG-3′ (forward) and 5′-CGGGTCCCCGTGAAATGAC-3′ (reverse). Prostate tissue necropsy was performed and organs were cleaned of excess fat and membrane with phosphate-buffered saline; mass of each prostate lobe was determined after blotting with filtration paper to remove excess water.

**Histopathologic analysis**

Samples were fixed in 10% formalin for at least 24 h, and then embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin. All tissues were examined by a board-certified animal pathologist in a blinded fashion (LHR, V M D). Lesions were identified as epithelial hyperplasia, stromal hyperplasia and mPIN per the criteria published by Shappell and coworkers commonly used to score prostate lesions in transgenic mouse models (Shappell et al. 2004). Epithelial hyperplasia was recognized as an increase in epithelial cells within normal-appearing gland profiles, reflected by stratification of epithelial cells (Shappell et al. 2004). Stromal hyperplasia was identified as a non-neoplastic increase in the cellularity of the stromal component of the prostate compared with age-matched controls (Shappell et al. 2004). mPIN ranged from low grade, characterized by glands lined by 1–3 layers of epithelial cells displaying minimal pleomorphism or hyperchromasia, slight nuclear enlargement with little atypia, infrequent mitosis, and essentially, normal glandular profiles with only occasional hints of papillary epithelial proliferation to high grade, characterized by extensive intraglandular epithelial proliferation, formation of papillary or cribriform structures consisting of epithelial cells displaying significant nuclear atypia and hyperchromasia, cellular pleomorphism and increased frequency of mitoses (Shappell et al. 2004).
Tissue preparation and microarray hybridization

All lobes of the prostates from 6 WT and 6 Ell2-cko virgin male mice littermates at 3 months of age were microdissected from the urogenital tract in phosphate-buffered saline with the aid of a dissecting Carl Zeiss Stemi 2000 Stereomicroscope (Zeiss) and stored in 1 mL of RNA later at −80°C. Expression profiling experiments were performed by the High-Throughput Genome Center at the Department of Pathology, University of Pittsburgh. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA quantity and quality were measured by obtaining A260 and an A260/A280 wavelength ratio of 1.8–2.1 via Agilent 2100 bioanalyzer (Agilent), respectively. Microarray hybridization was performed with a pre-equilibrated Mouse Genome 430 2.0 Genechip array (Affymetrix Inc., Santa Clara, CA). For each sample, 8 µg total RNA was used for retro-transcription and 15 µg cRNA to the chip for hybridization. Hybridization data were normalized to an average target intensity of 500 per chip using Affymetrix GeneChip Operating Software (GCOS 1.4) and were converted to a Microsoft Excel spreadsheet text file.

Bioinformatics data analysis

The differential expression result was achieved fitting a lognormal distribution to all 6 Affymetrix intensity signals of each group, WT and Ell2-cko and asking what is the probability that \( \text{Ell2-cko} > \text{WT} \) (and conversely \( \text{WT} > \text{Ell2-cko} \)). Along with probabilistic support, the magnitude of the effect was also considered taking into account the average fold-changes. The final gene list considered was obtained from filtering the gene list at two simultaneous thresholds: (i) log2-ratios greater than -2 and (ii) probability \( \text{Pr(\text{Ell2-cko} > \text{WT}) > 95\%} \), for upregulated, or \( \text{Pr(\text{WT} > \text{Ell2-cko}) > 95\%} \), for downregulated. Functional and ontology enrichment analysis was performed using the DAVID web-based tool (Dennis et al. 2003) and Ingenuity Pathways Analysis (IPA) 5.0 (Ingenuity Systems, Redwood City, CA) as described by Haram and coworkers (Haram et al. 2008).

Gene expression validation

The anterior prostates of an independent set of WT \((n = 5)\) or Ell2-cko male mice \((n = 3)\) at 17 months of age were used for total RNA isolation using the RNeasy minikit (Qiagen). Animal tissues were homogenized with a Kontes pellet pestle for 30 s twice (Thermo Fisher Scientific). RNA quality was analyzed by bleach gel electrophoresis (Aranda et al. 2012). qPCR verified expression scored by cDNA arrays of ventral prostate tissue and expression levels in anterior prostate tissue (SYBR Green/ROX, Thermo Scientific). PCR amplification was carried out using Applied Biosystems StepOnePlus Real-Time PCR Systems (Applied Biosystems). PCR amplification of various genes was normalized to the housekeeping gene Gapdh using the comparative \( C_{\text{t}} \) method (Schmittgen & Livak 2008). Primer sequences are listed in Table 1. Gapdh was chosen as an internal control because there was no difference in Gapdh expression between WT and Ell2-cko prostate in the microarray data. Also Gapdh has been used as a normalization control in murine prostate research (Ai et al. 2009). Each experimental sample was assayed in triplicate from a minimum of 3 animals per group.

Cell culture experiments

Human C4-2 prostate cancer cells (kind gift from Leland K Chung) were maintained in RPMI-1640 (10-040-CV, Corning cellgro) supplemented with 10% fetal bovine serum (FBS) (S11150, Atlanta Biologicals) and 5% antibiotics. LNCaP prostate cancer cells were purchased from ATCC and maintained in RPMI-1640. ELL2 response to androgen was analyzed by culturing cells over a concentration range of R1881 (0–2 nM). After treatment, cells were lysed in modified radioimmune precipitation assay buffer (50 mm Tris (pH 7.4), 1% Igepal CA-630, 0.25% Na-deoxycholate, 150 mm NaCl, 1 mm EDTA (pH 8.0), 1 mm NaF, 2 mm phenylmethylsulfonylfluoride, 1 mm Na3VO4, and protease inhibitor cocktail (Sigma)). Western blot analysis of ELL2, HIF1α and GAPDH was as described previously (Saporita et al. 2007). Antibodies used were rabbit polyclonal anti-ELL2 (1:1000, A302-505A, Bethyl Laboratories, Inc., Montgomery, TX, USA), HIF1α (1:1000, 54/HIF-1α, 610959, BD Transduction Laboratories, San Jose, CA, USA) and rabbit polyclonal anti-GAPDH (1:6000, FL-335, sc25778, Santa Cruz Biotechnology). All experiments were performed in triplicate.

The effects of ELL2 knockdown were determined in C4-2 cells. Small-interfering RNAs (siRNAs) targeting ELL2 (siELL2, 5′-AUUUACAAUCUGAGGAGGAUUGAGAU, 3′-TAAUGUAGACUCCUCUAGACUC) and EAF2 (siEAF2, 5′-GGAGAUGUCGGCUAGAAAAT, 3′-GACCUCUUACAGCGAUCUUU) were purchased from Integrated DNA Technologies (Coralville, IA, USA),
and negative control (siSCR) siRNAs were purchased from Santa Cruz (Control siRNA-A:sc-37007). Cells were transfected with siRNA for 48 h using DharmaFECT Transfection Reagents (GE Healthcare) following the manufacturer’s instructions. Experiments were performed in triplicate.

**Immunohistochemistry**

A group of mice including 10 WT and 19 Ell2-cko mice was generated and examined for histological defects in the prostate at age 17–20 months. Immunohistochemical stains were performed on five-micron sections of paraffin-embedded murine tissue specimens as described previously (Pascal et al. 2011). Briefly, sections were deparaffinized and rehydrated through a graded series of ethanol. Heat-induced epitope retrieval was performed using a decloaker, followed by rinsing in TBS buffer for 5 min. Primary antibodies for immunostaining of murine tissue sections were rabbit polyclonal anti-ELL2 (bs-6993R, Biox Antibodies, Woburn, MA, USA), rat monoclonal anti-Ki-67 (TEC-3, M7249, Dako, Carpinteria, CA, USA) and rat monoclonal anti-CD31 (MEC 13.3, 550274, BD Biosciences, San Jose, CA, USA) (working dilution 1:100 for ELL2 and 1:400 for Ki-67 and CD31). Slides were then counterstained in hematoxylin and coverslipped.

Immunostained sections were imaged with a Leica DM LB microscope (Leica Microsystems Inc., Bannockburn, IL, USA) equipped with a QImaging MicroPublisher 3.3 RTV digital camera (QImaging, Surrey, BC, Canada).

For murine tissues, Ki-67-positive cell density was determined by analysis of sections from at least 6 independent mice from each genotype. Slides stained with Ki-67 were scanned and digitized using the Aperio ScanScope CS scanner (Aperio, Vista, CA, USA) to capture digital whole slide images (WSI) using the ×20 objective lens at a spatial sampling period of 0.47 μm per pixel. The digital WSI was analyzed using Aperio ImageScope software (http://www.aperio.com/pathology-services/image-scope-slide-viewing-software.asp). The manufacturer’s (Aperio Technologies, Inc.) algorithms were used to quantify nuclear staining. Composite images were constructed with Photoshop CS (Adobe Systems). Assessment of microvessel density was determined based on CD31-positive blood vessel count as previously described (Pascal et al. 2011). Briefly, microvessel density was determined from at least 20 fields imaged at 10× magnification for prostate with no overlap and identified by evaluating histological sections, and CD31-positive vessels were counted to determine the average vessel numbers per field for each section.
Data analysis using cBioPortal

The cBioPortal was utilized to determine the percentage of alterations and co-occurrence between ELL2 and its potential target genes in two data sets of prostate cancers. In cBioPortal, OncoPrints are generated for visualizing gene and pathway alterations across a set of cases. Individual genes are represented as rows, and individual cases or patients are represented as columns. We studied the alterations in a set of genes potentially regulated by ELL2 identified by microarray analysis of murine Ell2-cko prostate at age 3 months and validated by qPCR analysis of murine prostate at age 17 months. The cBioPortal for Cancer Genomics site (http://cbioportal.org) provides a web resource for exploring, visualizing and analyzing multidimensional cancer genomics data and is subjected to scheduled updates (Cerami et al. 2012, Gao et al. 2013).

Statistical analysis

For non-microarray data, straightforward established biostatistics analyses were used. Comparisons between groups were calculated using the Student’s t-test or two-tailed Fisher exact test as appropriate. A P value of <0.05 was considered significant. GraphPad Prism, version 4 was used for graphics (GraphPad Software). Values are expressed as means ± s.e.m.

Results

Conditional deletion of ELL2 in the murine prostate epithelial cell results in murine prostatic intraepithelial neoplasia (mPIN)

The cell type-specific expression of Ell2 mRNA in the WT murine prostate was determined by in situ hybridization. Ell2 expression was localized to prostate epithelial cells of all lobes of the murine prostate (Fig. 1). Expression of Ell2 was also evident in smooth muscle cells and fibroblasts in the stroma. In order to determine the function of Ell2 in the murine prostate epithelial cell, mice with conditional deletion of Ell2 were generated by crossing PbsnCre4 mice to mice harboring a floxed Ell2 allele on a pure C57BL/6J background. Genotyping to confirm Ell2-cko was determined by performing PCR analysis of mouse tail genomic DNA at 21 days of age and confirmed on muscle DNA when animals were killed (Fig. 2A). A group of 19 male Ell2-cko mice were generated and examined at 17–20 months of age for prostatic defects. Since Eaf2-knockout mice on a pure C57BL/6J background did not develop epithelial hyperplasia and neoplasia until ~20 months (Pascal et al. 2013), we examined Ell2-cko mice at a similar timepoint. Loss of ELL2 protein in prostate epithelial cells was verified by immunostaining (Fig. 2B). Nuclear and cytoplasmic immunoexpression of ELL2 was evident in the epithelial cells of the WT murine prostate. The prostate epithelial cells of Ell2-cko mice only had background staining. Ell2-cko mice at 17–20 months displayed a statistically significant increased incidence in epithelial hyperplasia, stromal hyperplasia and murine prostatic intraepithelial neoplasia (mPIN) compared to WT controls (Fig. 2C and Table 2). No WT animals displayed mPIN lesions.

In addition to histologic defects, the prostates of Ell2-cko mice had increased mass in all lobes of the prostate compared to age-matched WT controls, further suggesting that Ell2 loss could induce increased epithelial proliferation (Fig. 3A). The proliferative marker Ki-67 was used to detect dividing cells in the prostates of Ell2-cko and WT mice. In agreement with the increased mass, the number of Ki-67-positive epithelial cells was significantly increased in all lobes of the Ell2-cko mice compared to WT controls (Fig. 3B and C). These results suggest that Ell2 loss in the murine model could induce
epithelial proliferation, contributing to the development of mPIN lesions. ELL2 was previously identified as androgen-responsive gene in the prostate cancer cell line LNCaP (Nelson et al. 2002, Bolton et al. 2007). We also recently showed that knockdown of ELL2 in C4-2 and LNCaP cells significantly enhanced the invasion and migration and induced a slight increase in BrdU incorporation (Qiu et al. 2017). Here, knockdown of ELL2 in LNCaP prostate cancer cells induced an increase in cellular proliferation (Fig. 3D). In the prostate cancer cell line C4-2, ELL2 expression following exposure to increasing concentrations of R1881 was determined by Western blot (Fig. 3E). As expected, ELL2 protein levels were increased by androgens in a dose-dependent manner. Cumulatively, these results suggest that ELL2 loss could contribute to an increase in prostate cancer proliferation, invasion and migration.

ELL2 gene deletion is associated with increased vascular density in the prostate

To investigate the effects of ELL2 loss on microvessel density in the prostate, we examined the number of CD31-positive blood vessels by immunostaining in a subset of animals (Fig. 4). The normal prostate is characterized by prominent stromal vasculature and rarely intraductal vessels, whereas there is a noticeable migration of vessels into the prostatic duct within PIN lesions (Huss et al. 2001). In agreement with the increased incidence of mPIN lesions, the number of CD31-positive intraductal vessels as well as total microvessel density increased significantly in all lobes of the Ell2-cko prostate as compared to WT control animals (Fig. 4A, B and C). As EAF and ELL proteins have overlapping functions, and both EAF2 (Xiao et al. 2009,
Chen et al. 2014) and ELL (Liu et al. 2010) interact with HIF1α, we examined its expression in response to ELL2 loss. Hif1α gene expression was significantly increased in Ell2-cko mice compared to WT controls (Fig. 4D), while VEGF levels were not significantly different (Fig. 5). siRNA knockdown of ELL2 in C4-2 cells also induced an increase in HIF1α protein levels compared to siSCR (Fig. 4E). ELL2 could regulate prostate vascularity through the HIF1α pathway directly or through its interaction with EAF2 protein.

Microarray analysis and qPCR validation of genes differentially expressed in the prostate of ELL2-knockout mouse

To identify target genes of ELL2 prior to the development of histological defects, we performed cDNA microarray analysis of the prostate from 6 WT and 6 Ell2-cko mice at 3 months of age. The most differentially regulated genes are listed in Supplementary Table 1 (see section on supplementary data given at the end of this article).
The most upregulated genes identified in the ELL2 knockout mice included Sfi1, Mac4, Cd209b, Zfp786, Armc9, Plcd3 and Ctse. The most downregulated genes identified included L3mbtl3, Rbm41, S1pr1, Hps1, Scarb1, Mepce, Atl2 and Lrpap1.

qPCR analysis of anterior prostates isolated from WT (n=5) and Ell2-cko (n=3) mice at 17 months of age was used to validate several genes identified by cDNA microarray as up- or downregulated by ELL2 knockout in the mouse prostate at 3 months of age. Insufficient samples for qPCR analyses were available for mice at 3 months of age; therefore, genes that were altered at both 3 months of age as well as at 17 months of age were considered. Fifteen genes were chosen randomly or because they encode products that are important in tumorigenesis for qPCR validation. In ELL2-knockout prostates at age 17 months, Sfi1 and Cxcl10 were significantly upregulated compared to WT controls, while Pds5a, Rtn4, Olig2, Rho bt3 and Gbp2 were significantly decreased in agreement with the cDNA microarray results from mice at age 3 months (Fig. 5). Additionally, Pbsn and Vegf were not significantly altered, also in agreement with the microarray data. Several of the genes analyzed by qPCR in mice at 17 months of age did not agree with the microarray results at 3 months of age. These included Muc4, Ctte and S1pr1, which were not differentially expressed; as well as, Arsk, S1pr3 and Snai3, which were downregulated in mice at age 3 months, but upregulated in the mice at 17 months of age (Fig. 6A). ELL2 knockdown in C4-2 cells induced a statistically significant decrease in PDS5A, while the effects of ELL2 knockdown on RHOBTB3 and ARSK were not significant (Fig. 6B). Fold-changes from the microarray platforms were listed in Supplementary Table 1.
Functional classification of differentially expressed genes was analyzed for significant enrichment using the DAVID annotation tool (Dennis et al. 2003) (http://david.abcc.ncifcrf.gov/). Gene ontology-based analysis performed using DAVID identified 65 Annotation Clusters with gene count >2 (Supplementary Table 2). Annotation clusters 1, 2 and 3 had enrichment scores >1.4 and included terms involving golgi apparatus, intracellular transport, protein transport, protein localization, protein targeting, organelle membrane and mitochondrion. IPA (http://www.ingenuity.com) was also used to identify networks of interacting genes. The most significant network was associated with differentiation of neurons and the generation of cells (Fig. 7A and B).

The expression of genes associated with ELL2 loss was also examined in several large-scale genomics data sets available through the cBioPortal for Cancer Genomics (Cerami et al. 2012, Grasso et al. 2012). Interestingly, several of the genes identified by microarray analysis of the Ell2-knockout mouse as potentially regulated by ELL2 were frequently altered in patients with ELL2 changes in two prostate cancer data sets. In the data set neuroendocrine prostate cancer (Trento/Cornell/Broad 2016), ELL2 was amplified or upregulated in 17% (13 of 77 sequenced) of patients (Fig. 8A). Genes PDSSA, RHOBTB3, CTSE, ARSK, S1PR3, HIF1A and EAF2 were most frequently amplified or upregulated in conjunction with ELL2. This dataset included whole exome and RNA Seq data of castration-resistant adenocarcinoma and castration-resistant neuroendocrine prostate cancer (somatic mutations and copy number aberrations) (Beltran et al. 2016). Conversely, the TCGA, Provisional dataset generated by the TCGA Research Network: http://cancergenome.nih.gov/ identified several patients with gene downregulation coinciding with deep deletion or mRNA upregulation of ELL2. In this dataset, 9% (43 of 492 sequenced cases) of patients had ELL2 alterations. RHOBTB3, GBP2 and ARSK were most frequently altered in combination with ELL2 (Fig. 8B). One missense mutation was identified in the occludin homology domain of one patient (1 out of 112 sequenced cases) in the dataset prostate adenocarcinoma (Broad/Cornell 2012) (Barbieri et al. 2012). These results suggest that ELL2 alterations in prostate cancer are most commonly copy number alteration or mRNA dysregulation rather than somatic mutations.

**Discussion**

ELL and EAF family proteins play important roles in both development and tumorigenesis. ELL2 is a binding partner of EAF2, which is androgen responsive and acts as a prostate
tumor suppressor (Xiao et al. 2003, 2008). EAF2-knockout mice have increased incidence in neoplastic PIN lesions, increased epithelial proliferation and increased vascularity (Xiao et al. 2008, Pascal et al. 2013). Here, prostate-specific deletion of ELL2 in the murine prostate also induced an increased incidence in epithelial proliferation and mPIN lesions in older mice. EAF2 can induce increased HIF1α through the stabilization of VHL protein (Xiao et al. 2009).

Figure 7
(A) Highest scoring interactive network generated by mapping differentially expressed genes to Ingenuity Pathway Knowledge Base information. Node color intensity indicates degree of upregulation (red) or downregulation (green) in Ell2-cko mice compared to wild-type mice at 3 months of age. (B) Schematic pathways activated by downregulation of several genes. Downregulation of genes (green) leading to the activation of generation of cells (orange) and inhibited the differentiation of neurons (blue).

Figure 8
Oncoprint data represent samples from two published sets from the cBioPortal for cancer genomics (Cerami et al. 2012, Gao et al. 2013). (A) The queried samples represent ELL2-enriched tumors from neuroendocrine prostate cancer (107 samples, whole exome and RNA Seq data of castration-resistant adenocarcinoma and castration-resistant neuroendocrine prostate cancer (somatic mutations and copy number aberrations)) (Beltran et al. 2016). (B) ELL2 enriched tumors from prostate adenocarcinoma tumor samples with sequencing and CNA data (492 samples, TCGA Research Network: http://cancergenome.nih.gov/). Individual genes are represented as rows, and individual cases or patients are represented as columns. The types of genetic alteration included in both sets are: amplification, deep deletion, mRNA upregulation, mRNA downregulation and missense mutation. cBioPortal data are subjected to scheduled updates.
and can decrease the expression of anti-angiogenic TSP1 (Su et al. 2010). ELL has also been shown to influence the HIF1α pathway by modulating expression of VEGF and Glut-1, and knockdown of ELL increased HIF1α protein expression (Liu et al. 2010). Here, we show that Ell2 loss in the murine prostate was also associated with an increase in microvessel density and recruitment of intraductal vessels to mPIN lesions compared to WT controls (Figs 2 and 3). The prostates of Ell2-knockout mice had increased mRNA expression of Hif1α (Fig. 4). Knockdown of ELL2 in C4-2 prostate cancer cells also resulted in an increase in HIF1α protein. Furthermore, Rhobtb3 mRNA was identified by cDNA microarray as one of the most differentially downregulated genes in ELL2-knockout mouse prostate in mice at 3 months of age (Supplementary Table 1) as well as in the anterior prostates of mice at 17 months of age (Fig. 5). RHOBTB3 was recently shown to promote the hydroxylation, ubiquitination and degradation of HIF1α (Zhang et al. 2015). Increased expression of HIF1α has been reported as a potential early ‘angiogenic event’ in the development of prostate cancer in the TRAMP model (Huss et al. 2001) and is negatively regulated by EAF2 (Chen et al. 2014). ELL2 and EAF2 appear to similarly regulate prostate angiogenesis in part through modulation of HIF1α. Future studies will be required to determine if ELL2 and EAF2 interaction is critical for the regulation of prostate vascularity.

Microarray analysis identified several other differentially expressed genes in the Ell2-cko-knockout mouse at 3 months of age in addition to Rhobtb3. Functional classification of these genes utilizing DAVID and IPA revealed alteration of genes involved in golgi apparatus, intracellular transport and cell-cell signaling and cancer. qPCR validation confirmed the persistent altered expression of several of these genes in animals at 17 months of age, including Sf1, Cxcl10, Pds5a, Rtn4, Olig2, Rhobtb3 and Gbp2 suggesting that these genes and their associated pathways might contribute to the development of the histological defects identified in the prostates of aged animals with ELL2 loss. SF1 is a key regulator of normal endocrine steroidogenesis, which is upregulated in castration-resistant prostate cancer (Lewis et al. 2014). PDSSA, also known as SCC-112, was recently reported as a translocation partner of MLL (Put et al. 2012) and was initially characterized as a cell cycle regulator and promoter of apoptosis (Kumar et al. 2004). Persistent alteration of these genes likely contributes to the proliferative and highly vascularized preneoplastic phenotype observed in the prostates of Ell2-knockout mice at age 17–20 months.

RTN4, OLIG2, S1PR1 and S1PR3 interact with several pathways previously associated with prostate carcinogenesis and are involved in neural differentiation (see Fig. 7). Eaf2 loss was previously shown to activate the ERK pathway (Su et al. 2013). RTN4 is a neurite outgrowth inhibitor (Spillmann et al. 1998) that was shown to inhibit proliferation and promote apoptosis when transfected into human hepatocellular carcinoma cells (Chen et al. 2005). OLIG2 is a neural stem cell transcription factor that regulates the differentiation of oligodendrocytes (Zhou & Anderson 2002). S1PR1 and S1PR3 are involved in dendritic remodeling (Willems et al. 2016). As these genes were also frequently co-upregulated with ELL2 in the neuroendocrine prostate cancer dataset (Fig. 8A), ELL2 may play a role in the differentiation and proliferation of neuroendocrine cells through the regulation of RTN4, OLIG2, S1PR1 and S1PR3. Overexpression of ELL2 and its target genes was associated with prostate cancers with a neuroendocrine phenotype, whereas downregulation of ELL2 and ELL2 target genes was associated with prostate adenocarcinoma. Future studies will be required to fully elucidate the role of ELL2 interaction with these genes in prostate carcinogenesis.

Cumulatively, these results show that similar to EAF2 loss, ELL2 loss in the prostate induced increased epithelial proliferation, increased microvessel density and preneoplastic lesions. Several pathways were identified that are potentially regulated by ELL2 in the prostate, and many of these genes were found to be altered in conjunction with ELL2 in patient tumors in the cBioPortal database. Interestingly, ELL2 and its regulated genes were upregulated in neuroendocrine prostate tumors and downregulated in prostate adenocarcinoma. These findings provide a strong foundation for further studies to elucidate the mechanisms by which ELL2 loss promotes prostate carcinogenesis.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-17-0112.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was funded in part by NIH grants R01CA186780, P50 CA180995, T32 DK007774 and 1R50 CA211242 and scholarships from the Tippins...
Foundation (L.E.P) and the Mellam Foundation (K.Z.M). The microarray work was performed in the Genomics Research Core at the University of Pittsburgh. This project used the UPCI Animal Facility and was supported in part by award P30CA047904.

Author contribution statement

Acknowledgements
The authors are grateful to Marie Acquafondata, Megan Lambert, Katie Leschak, and Aiyuan Zhang for technical support and Karen Seisek for reading the manuscript.

References
Eissenberg JC, Ma J, Gerber MA, Christensen A, Kennison JA & Shliatfard A 2002 dELL is an essential RNA polymerase II elongation factor with a general role in development. PNAS 99 9894–9899. (doi:10.1073/pnas.152193699)
Milcarek C, Albring M, Langer C & Park KS 2011 The eleven-nineteen lysine-rich leukemia gene (ELL2) influences the histone H3 protein...
modifications accompanying the shift to secretory immunoglobulin heavy chain mRNA production. Journal of Biological Chemistry 286 33795–33803. (doi:10.1074/jbc.M111.272096)


Received in final form 18 July 2017
Accepted 4 August 2017