

# Low Tristetraprolin Expression Is Associated with Lethal Prostate Cancer

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## Abstract

**Background:** Inflammation is linked to prostate cancer progression and is mediated by NF- $\kappa$ B. Tristetraprolin is a key node of NF- $\kappa$ B activation and we investigated its biological and prognostic role in lethal prostate cancer.

**Methods:** *In vitro* assays assessed the function of tristetraprolin and the association between low mRNA tristetraprolin levels and lethal prostate cancer (metastatic disease or death) was assessed across independent prostatectomy cohorts: (i) nested case-control studies from Health Professionals Follow-up Study and Physicians' Health Study, and (ii) prostatectomy samples from Cleveland Clinic, Mayo Clinic, Johns Hopkins and Memorial Sloan Kettering Cancer Center. Tristetraprolin expression levels in prostatectomy samples from patients with localized disease and biopsies of metastatic castration-resistant prostate cancer (mCRPC) were assessed in a Cornell University cohort.

**Results:** *In vitro* tristetraprolin expression was inversely associated with NF- $\kappa$ B-controlled genes, proliferation, and

enzalutamide sensitivity. Men with localized prostate cancer and lower quartile of tumor tristetraprolin expression had a significant, nearly two-fold higher risk of lethal prostate cancer after adjusting for known clinical and histologic prognostic features (age, RP Gleason score, T-stage). Tristetraprolin expression was also significantly lower in mCRPC compared with localized prostate cancer.

**Conclusions:** Lower levels of tristetraprolin in human prostate cancer prostatectomy tissue are associated with more aggressive prostate cancer and may serve as an actionable prognostic and predictive biomarker.

**Impact:** There is a clear need for improved biomarkers to identify patients with localized prostate cancer in need of treatment intensification, such as adjuvant testosterone suppression, or treatment de-intensification, such as active surveillance. Tristetraprolin levels may serve as informative biomarkers in localized prostate cancer.

## Introduction

Prostate cancer has a varied clinical course, with some patients having localized disease that does not require intervention whereas others present with metastatic disease that responds poorly to therapy (1, 2). Clinicopathologic staging such as higher Gleason score, larger primary, and findings of extension beyond the prostate at prostatectomy increase chances of developing metastatic and lethal disease (3, 4). In addition, commercially available assays of RNA transcript profiles representing biological processes such as cell cycle and apoptosis provide further prognostic infor-

mation on risk of relapse after prostatectomy or radiation for localized disease (5).

Biological drivers for the development of prostate cancer and its lethal subtypes are diverse. Epidemiologic and biological studies have implicated aberrant metabolism and inflammation (6). In the case of metabolism, obesity and markers of insulin resistance have been linked a greater chance of dying of prostate cancer (7). With respect to inflammation, elevation of serum cytokines such as IL6 have been associated with lethal prostate cancer and inflammatory changes have been associated with prostate cancer lesions in the prostate (6, 8). NF- $\kappa$ B activation is a transcription factor that controls inflammation and can either promote cancer progression or cancer cell death depending on its associated regulators (9). Despite numerous preclinical studies demonstrating a central role of NF- $\kappa$ B activation in promoting proliferation, development of metastases, and evasion of apoptosis in prostate cancer (10–12), biomarkers of NF- $\kappa$ B activation, such as presence of nuclear staining of the active subunit p65 in prostate cancer cells in localized disease have not been found to reliably identify patients with a higher risk of metastatic disease after a prostatectomy or radiation therapy given with curative intent (13–15). Identification of patients with loss of negative regulators of NF- $\kappa$ B activity which, in turn, promote aggressive or lethal prostate cancer, may therefore identify patients who need intensification of therapy such as adjuvant androgen deprivation therapy (ADT) with radiation or prostatectomy. Moreover, such patients may also benefit from adding drugs that block cancer-promoting NF- $\kappa$ B activity (9, 16).

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**Note:** Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (<http://cebp.aacrjournals.org/>).

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Using a bioinformatic analytical approach with Bayesian data integration of publicly available genomic datasets, we defined an NF- $\kappa$ B pathway that was enriched in patients with prostate cancer who relapsed with metastatic disease after a prostatectomy (17, 18). After reviewing the literature of potential candidates for known functions and biological data supporting a role in prostate cancer, tristetraprolin was considered the lead candidate and was taken forward for further evaluation. Other candidates included ATF3, CXCL2, DUSP5, JUNB, NEDD9, SELE, and TRIB1 (18) but tristetraprolin was considered to be the most promising and novel based on its known function and limited data in prostate cancer. Tristetraprolin homolog was found to be one of the key nodes in this pathway, and we postulated it is a regulator of NF- $\kappa$ B activity. We hypothesized that low tristetraprolin levels in prostatectomy specimens would be associated with a higher likelihood of metastasis or fatal disease.

Tristetraprolin (TTP), also known as ZFP36, is a member of the TIS11 (TPA-induced sequence) family, which binds specific mRNA sequences and leads to transcript deadenylation and degradation (19). The physiologic effects of tristetraprolin loss include inflammatory conditions such as arthritis and dermatitis (20) with increased levels of proinflammatory cytokines (21). Tristetraprolin control of NF- $\kappa$ B activity has been reported to occur via a number of mechanisms, including mediation of TNF $\alpha$  mRNA (an NF- $\kappa$ B activator) and IL1 $\beta$  (22) degradation, as well as blocking the nuclear translocation of the p65 subunit of NF- $\kappa$ B and physically interacting with histone deacetylases (HDAC), HDAC-1, HDAC-3, and HDAC-7 and these can also serve as co-repressors of NF- $\kappa$ B-dependent transcription (23). Tristetraprolin also controls other critical genes overexpressed in inflammation and cancer including c-myc, c-JUN, and p53 (19). Previous work adds further plausibility that loss of tristetraprolin function is a driver of cancer in general and prostate cancer specifically using *in vitro* modeling experiments and gene expression of human tissues showing association with biochemical relapse and decreased levels in metastatic deposits versus localized disease (24, 25).

We therefore sought to further define the biological activity of tristetraprolin and control of NF- $\kappa$ B-related genes within prostate cancer cells *in vitro*, and whether loss of this tumor suppressor in human tumors is prognostic for the more clinically relevant endpoints of metastatic or lethal prostate cancer.

## Materials and Methods

### In vitro

**Human cell lines.** LNCaP cell line was purchased from the ATCC. RWPE-1 cell line was obtained from Dr. Myles Brown lab in Dana-Farber Cancer Institute, Boston, Massachusetts. LNCaP cells were maintained in RPMI1640 and supplemented with 10% FBS. RWPE-1 cells were cultured in Keratinocyte-SFM with EGF and BPE from Life Technologies. All culture mediums include 100 IU of penicillin, streptomycin (100  $\mu$ g/mL). All cells were maintained at 37°C, 5% CO<sub>2</sub>, and 100% relative humidity and regularly screened for mycoplasma using a Venor GeM Mycoplasma Detection Kit (Sigma). RWPE-1 and LNCaP cell lines were originally obtained from ATCC. LNCaP was authenticated by whole genome sequencing. RWPE-1 was authenticated by determining the expression of a set of known genes and isoenzymes, specified by ATCC. These cell lines were routinely screened for mycoplasma contamination using the Mycoplasma Detection Kit from Sigma.

Freshly defrosted cell lines were used for experiments and experiments were completed within two or three passaging period. All our stocks are less than 10 passages.

**siRNA and overexpression.** Cell lines were cultured until approximately 80% confluence and then transfected with siRNAs or overexpression construct for tristetraprolin (Origene) using lipofectamine 2000. Knockdown or overexpression efficiency of tristetraprolin was detected by reverse transcription-polymerase chain reaction (RT-PCR; forward 5'- GCTATGTCGGACCTTCTCAGAG -3', reverse 5'- CCTGGAGGTAGAAGCTTGTGACAG -3') at 3 days after transfection. All RT-PCR experiments were performed in triplicate.

**Cell proliferation.** Cells were split into a 96-well plate with a confluence of approximately 40% after transfection for 24 hours. Cell proliferation assay was carried out at different days after splitting using the WST-1 assay (Roche) with the detection of the absorption at a wavelength of 450 nm, following the manufacture instruction. Each experiment was performed in triplicate.

**Human NF- $\kappa$ B signaling pathway PCR array.** Genes involved in the NF- $\kappa$ B signaling pathway were detected by the Human NF- $\kappa$ B Signaling Pathway PCR Array Kit from Sabioscience, following the manufacture instruction. Briefly, 1  $\mu$ g total RNA was extracted to synthesize cDNA for 96-well plate formats after cells were transfected by siRNA or overexpression construct of tristetraprolin for 3 days. Gene change was treated as significant when the fold change >1.5 and *P* value <0.05.

**Statistical analysis of in vitro studies.** Statistical analysis for all *in vitro* studies was performed with Prism 6.0 software (GraphPad Software Inc.). All values in figures are presented as means  $\pm$  SD. Statistical significance was calculated on the basis of Student *t* test (two-tailed) and the level of significance was set at *P* < 0.05.

**Pathoclinical outcome studies in newly diagnosed prostate cancer.** To assess the utility of tristetraprolin mRNA levels as a prognostic marker, we first leveraged data from a study nested in the Health Professionals Follow-up Study (HPFS) and Physicians' Health Study (PHS). Gene expression of tristetraprolin was quantified in archival surgical tumor tissue using Human Gene 1.0 ST microarrays (Affymetrix; GSE79021). Low and high tristetraprolin were defined as expression below the lower quartile and greater than or equal to the lower quartile, respectively, where the lower quartile cutoff was selected to discriminate tumors with very low tristetraprolin expression from those with normal levels. In this "extreme" case-control design (26), cases (*n* = 113) were men who died of prostate cancer or developed metastatic disease at any time over follow-up, and controls (*n* = 291) were men who lived at least 8 years after diagnosis and remained metastasis free. Further study details have been reported previously (27). ORs and 95% confidence intervals for lethal disease were estimated through logistic regression in R version 3.4.0.

Using the same definition of lethal cases and prostate cancer controls, the prognostic utility of tristetraprolin was further assessed in 788 NCCN intermediate and high-risk patients pooled from five cohorts in GenomeDx Bioscience's Genomics Resource Information Database (GRID). Tristetraprolin expression was quantified from radical prostatectomy specimens using the Affymetrix Human Exon 1.0 ST GeneChip (ref. 28; GSE79957), and

Gerke et al.

low and high tristetraprolin was again defined as below versus greater than or equal to the lower expression quartile. Because of differences among the five study cohorts, particularly in baseline tristetraprolin expression, analyses were stratified by cohort using conditional logistic regression in SAS v9.4 (29).

**Tristetraprolin levels in benign prostate and primary prostate cancer versus metastatic CRPC samples.** RNA-seq profiling data from the Weill Cornell Cohort have been previously published (dbGAPphs000909.v.p1; refs. 30, 31). They include 117 samples: 26 benign prostate, 39 localized prostate cancer, and 52 CRPC cases, processed according to the protocols described in those papers. For the purpose of this analysis, fragment per kilobase of exonic regions per million mapped reads (FPKM) values quantified tristetraprolin expression. Batch normalization with COMBAT (32) was used to account for differences in library preparation, specifically poly-A selection or ribosomal depletion. OncoPrint data were accessed to recapitulate the findings.

## Results

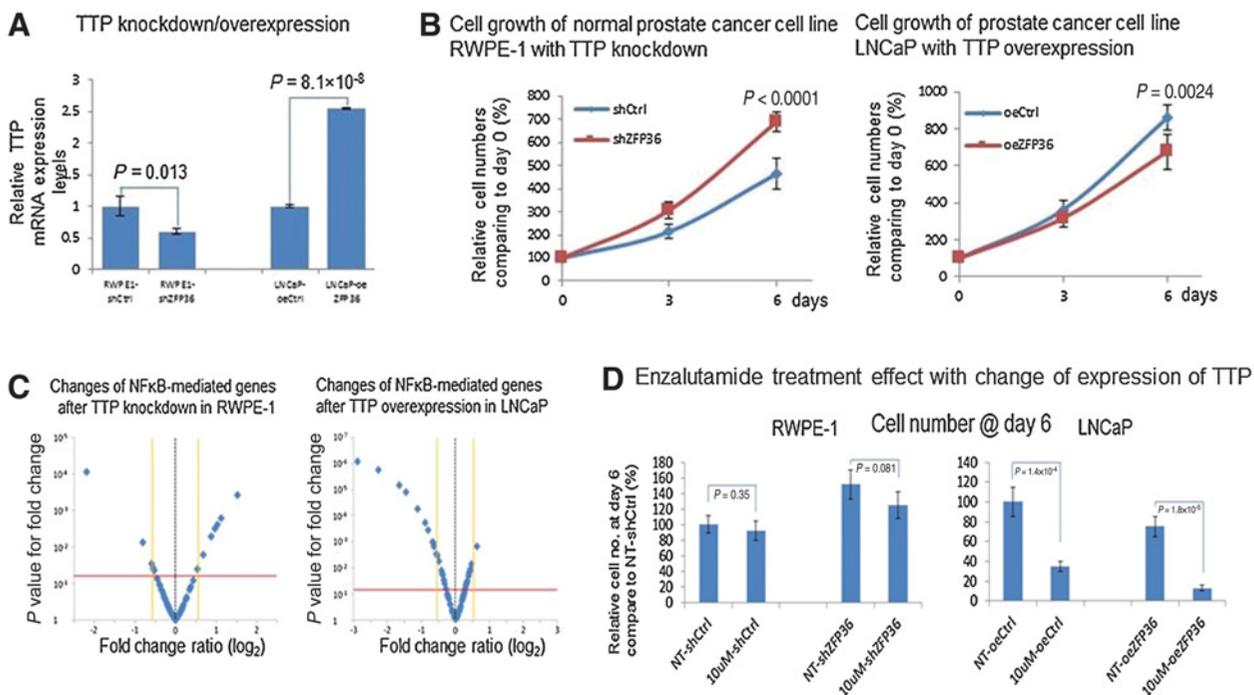
*In vitro* silencing and overexpressing of tristetraprolin was undertaken in the RWPE1 and LNCaP cell lines, respectively (Fig. 1A). Silencing tristetraprolin in the RWPE-1 cell line increased the proliferative potential whereas restoring the tumor suppressor in LNCaP resulted in decreased proliferation (Fig. 1B). Silencing tristetraprolin resulted in increased expression of many NF- $\kappa$ B-related genes and overexpression of tristetraprolin decreased levels of NF- $\kappa$ B-related genes (Fig. 1C). Notably, increasing tristetraprolin led to decreased BCL2A1, CSF2, and

CSF3, and decreasing tristetraprolin led to increases in these same NF- $\kappa$ B-regulated genes (Supplementary Table S1). Decrease of the NF- $\kappa$ B inhibitor, NFKBIA, with overexpression of tristetraprolin in LNCaP presumably relates to decreased NF- $\kappa$ B activity, and silencing tristetraprolin results in increased NF- $\kappa$ B activity and compensatory increase in the inhibitor, NFKBIA as part of tightly controlled feedback loops (9). It was also noted that overexpression of tristetraprolin augmented the sensitivity of LNCaP cells to enzalutamide, whereas silencing tristetraprolin again increased proliferation of the RWPE1 cells and notably the enzalutamide had only a mild effect on decreasing the proliferation (Fig. 1D).

Patient characteristics for the HPFS and PHS case-control design are summarized in Table 1. Mean tristetraprolin expression was significantly lower in the lethal cases compared with the nonlethal controls ( $P < 0.001$ ). The crude OR for lethal disease comparing those with low versus high tristetraprolin expression was 3.52 (95% CI, 2.18–5.69;  $P < 0.001$ ). This association persisted with adjustment for Gleason category (<7, 3+4, 4+3, 8, 9–10), age at diagnosis, and clinical stage (T1/T2 N0/Nx M0/Mx, T3 N0/Nx M0/Mx, T4/N1/M1), with an adjusted OR of 1.83 (95% CI, 0.99–3.33;  $P = 0.05$ ).

Similar associations were observed in the pooled GenomeDX cohorts (Table 2). The crude OR for metastatic disease comparing those with low to those with high tristetraprolin expression was 1.93 (95% CI: 1.26–2.44;  $P < 0.001$ ). The OR adjusted for Gleason score, diagnostic age, and clinical stage was 1.84 (1.24–2.75;  $P = 0.003$ ).

In the further independent cohort from Weill Cornell Medicine, tristetraprolin expression levels from RNA-Seq were



**Figure 1.**

Knockdown of tristetraprolin in noncancer prostate cell line (RWPE-1) and overexpression in prostate cancer cell line (LNCaP) resulted in significantly increased and decreased proliferation respectively (B) and made LNCaP more sensitive to enzalutamide (E) and also impacted NF- $\kappa$ B-related genes (C and D).

**Table 1.** Clinical characteristics of the HPFS and PHS stratified by lowest tristetraprolin quartile

	Tristetraprolin <sup>Low</sup> (n = 101)	Tristetraprolin <sup>High</sup> (n = 303)	P value
Cohort, n (%)			0.55
HPFS	66(26)	188(74)	
PHS	35(23)	115(77)	
Age at diagnosis, median (SD; range)	67.9 (7.0; 47-81)	65.0 (6.2; 49-79)	0.003
Pathologic RP			<0.001
Gleason score, n (%)			
2-6	10 (18)	47 (82)	
7	49 (20)	192 (80)	
8	13 (30)	30 (70)	
9-10	29 (46)	34 (54)	
Clinical stage, n (%)			0.001
T1/T2	78 (22)	272 (78)	
T3	7 (26)	20 (74)	
T4	12 (60)	8 (40)	
Total PSA at diagnosis, ng/mL, n (%)			0.11
0-4	4 (10)	37 (90)	
4-10	37 (19)	159 (81)	
10-20	16 (25)	47 (75)	
>20	12 (29)	29 (71)	
Not available	32 (51)	31 (49)	
Case status (%)			<0.001
Lethal	49 (43)	64 (57)	
Indolent	52 (18)	239 (82)	

Abbreviation: RP, radical prostatectomy.

approximately the same in benign and localized disease specimens, but significantly decreased in the metastatic castration-resistant prostate cancer tissues compared with each ( $P = 7.1 \times 10^{-10}$  and  $P = 9.0 \times 10^{-14}$ , respectively; Fig. 2). These findings were confirmed by analysis of seven publicly available datasets in OncoPrint (Fig. 3).

## Discussion

We previously applied an integrated Bayesian and systems biology approach to interrogate (18) the network signaling of inflammatory processes implicated in prostate cancer development. This identified tristetraprolin as a potential regulator of NF- $\kappa$ B signaling. Tristetraprolin was chosen as the lead candidate based on prior literature, indicating it may have a role in prostate cancer biology. In this work, we first confirmed using *in vitro* studies that tristetraprolin was biologically relevant in prostate cancer by at least partially controlling NF- $\kappa$ B-related genes and proliferation in prostate noncancerous and cancerous cell lines and partially modulating resistance to androgen receptor inhibition. The latter observation is consistent with previous work reporting that overexpression of tristetraprolin represses multiple steroid nuclear receptors including androgen receptor (AR) transactivation in breast cancer cell lines (33). As such, LNCaP with overexpressed tristetraprolin may be made more sensitive to enzalutamide treatment by dual mechanisms of AR signaling inhibition both pharmacologically with enzalutamide and by the effects of tristetraprolin suppression on NF- $\kappa$ B-regulated AR transcription and blocking NF- $\kappa$ B-mediated resistance mechanisms.

Having confirmed tristetraprolin has a biological role in prostate cancer, we then assessed the hypothesis that mRNA levels of

tristetraprolin would be low in human prostate tissue, which is associated with lethal or metastatic disease after a prostatectomy. The results of the present gene expression profiling across several prostatectomy cohorts reproducibly and convincingly showed that tristetraprolin gene expression is lower in patients who develop metastatic disease. Analyses of an additional cohort and seven publicly available cohorts confirmed that tristetraprolin levels are lower in metastatic deposits. The relationship has important prognostic features, in particular that the association of low tristetraprolin gene levels with a significant increased risk of lethal prostate cancer after treatment with curative intent is independent of Gleason score. It is also noted that beyond its prognostic role for lethal disease, tristetraprolin may additionally serve as a predictive biomarker for patients who might benefit from NF- $\kappa$ B inhibition.

Strengths of this study include use of the clinically relevant endpoint of metastatic and lethal prostate cancer from prostate cancer cohorts with long-term prospective follow-up and consistent effect estimates across a variety of patient populations. These findings further the findings from prior work which used less clinically relevant endpoints such as biochemical recurrence and

**Table 2.** Patient characteristics of the GenomeDX cohorts stratified by lowest tristetraprolin quartile

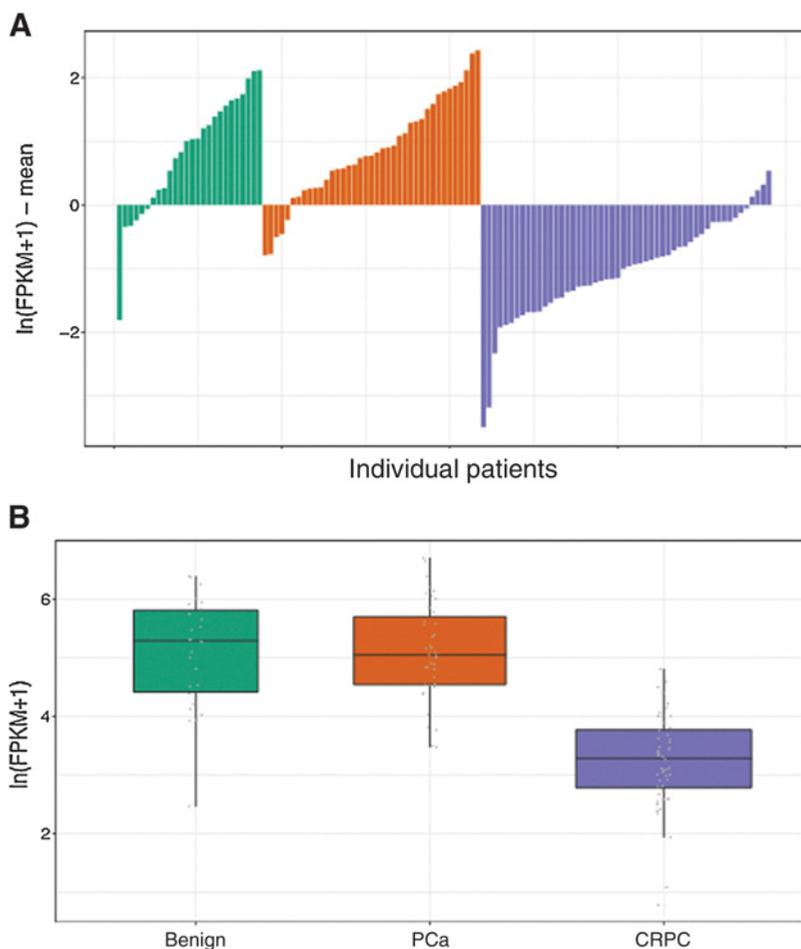
	Tristetraprolin <sup>Low</sup> (n = 197)	Tristetraprolin <sup>High</sup> (n = 591)	P value
Study cohort, n (%)			<0.001
CCF	6 (6)	101 (94)	
JHU	96 (36)	170 (64)	
MSK	2 (13)	14 (88)	
Mayo I <sup>a</sup>	78 (30)	186 (70)	
Mayo II	15 (11)	120 (89)	
Age at diagnosis, median (SD; range)	63.0 (6.8, 38-77)	62.5 (6.9, 42-79)	0.22
Pathologic RP			0.043
Gleason score, n (%)			
2-6	12 (14)	72 (86)	
7	68 (24)	217 (76)	
8	33 (25)	99 (75)	
9-10	84 (29)	203 (71)	
Clinical stage, n (%)			0.31
T1/T2	173 (24)	534 (76)	
T3	24 (30)	57 (70)	
Adjuvant ADT, n (%) <sup>b</sup>			0.91
No	62 (23)	202 (77)	
Yes	31 (23)	104 (77)	
Adjuvant RT, n (%) <sup>b</sup>			0.63
No	80 (23)	269 (77)	
Yes	13 (26)	37 (74)	
Total PSA at diagnosis, ng/mL, n (%)			0.036
0-4	20 (26)	57 (74)	
4-10	84 (24)	271 (76)	
10-20	47 (23)	155 (77)	
>20	44 (34)	85 (66)	
Not available	2 (8)	23 (92)	
Case status, n (%)			<0.001
Lethal	120 (30)	278 (70)	
Indolent	77 (20)	313 (80)	

Abbreviation: RP, radical prostatectomy.

<sup>a</sup>Mayo I and II refer to distinct patient cohorts that were used in the original discovery and validation, respectively, of GenomeDX Decipher genomic classifier (Erho, 2013).

<sup>b</sup>Based on total  $n = 399$  (93 Tristetraprolin<sup>Low</sup>, 309 Tristetraprolin<sup>High</sup>).

Gerke et al.

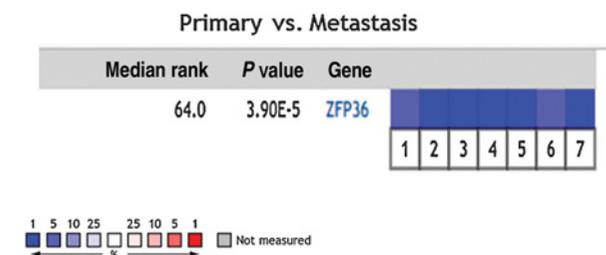


**Figure 2.** Tristetraprolin expression in WCM prostate cancer cohorts. The top (A) depicts the expression of tristetraprolin for all samples in the cohort where each bar corresponds to a sample. The bottom (B) summarizes the data in whisker plots. Tristetraprolin expression is significantly downregulated in CRPC compared with benign prostate (Wilcoxon test  $P = 7.1e-10$ ) and localized prostate cancer ( $P = 9.0e-14$ ).

are bolstered by *in vitro* work both detailed in this manuscript and prior publications (24, 34–36). Importantly, addition of tristetraprolin expression improves the prognostic accuracy of known clinical factors, as evidenced by the magnitude of the

tristetraprolin effect on metastatic progression in the multivariable models across the HPFS/PHS and GenomeDX cohorts. Additional AUC analyses were not possible in the GenomeDX data, due to the need to stratify by cohort. When modeled as a continuous variable in a logistic regression for prognosis in HPFS/PHS, tristetraprolin improved the AUC of the clinical factor model from 0.84 (95% CI, 0.80–0.88) to 0.85 (95% CI, 0.81–0.89). Although this improvement was not statistically significant ( $P = 0.23$ ), tests for AUC improvement are known to have low power, and it has been shown that the ability of a biomarker such as low tristetraprolin to improve the clinical model can more powerfully be inferred from its effect in the multivariable logistic regression presented in Results (37).

Limitations of this work are the preliminary nature of the preclinical findings, which are correlative at this time. Nonetheless, the data completed to date is consistent with and adds to the prior but limited preclinical work and provides guidance for further studies. These include *in vivo* tumorigenesis and more detailed mechanistic and functional studies dissecting the role of tristetraprolin in controlling NF- $\kappa$ B versus other pathways such as myc and JUN and whether it also impacts inflammatory factors in the stroma. This could be achieved with silencing RNA of tristetraprolin RNA with and without inhibition of NF- $\kappa$ B via I $\kappa$ B super-repressor (38) or pharmacologically with dimethylaminoparthenolide (16). Other limitations include use of retrospective cohorts and limited prognostic variables for the clinical



**Figure 3.** Oncomine analysis: <https://www.oncomine.com/>. The expression of tristetraprolin in several Oncomine prostate cancer datasets show significant downregulation in metastatic samples compared with primary tissues. 1. Prostate Cancer - Metastasis; Grasso Prostate, Nature, 2012 (39); 2. Prostate Cancer - Metastasis; Holzbeierlein Prostate, Am J Pathol, 2004 (40) 3. Prostate Cancer - Metastasis LaTulippe Prostate, Cancer Res, 2002 (41); 4. Prostate Cancer - Metastasis; Ramaswamy Multi-cancer, Proc Natl Acad Sci U S A, 2001 (42); 5. Prostate Cancer - Metastasis Ramaswamy Multi-cancer 2, Nat Genet, 2003 (42); 6. Prostate Cancer - Metastasis Taylor Prostate 3, Cancer Cell, 2010 (43); 7. Prostate Cancer - Metastasis Yu Prostate, J Clin Oncol, 2004 (44).

association studies. These data support use of samples from prospective trials to address these limitations.

In conclusion, this work provides robust evidence that tristetraprolin is a potentially actionable prognostic and predictive biomarker that was chosen following an integrated Bayesian-systems biology approach to interrogating gene expression data. The next tranche of work is to further explore the biology of tristetraprolin in prostate cancer and assess whether patients with low tristetraprolin benefit from adjuvant androgen deprivation, and/or specific NF- $\kappa$ B inhibition to define its clinical utility.

### Disclosure of Potential Conflicts of Interest

R.J. Karnes reports receiving a commercial research grant from GenomeDx; has ownership interest (including stock, patents, etc.) in GenomeDx. E. Davicioni is a Chief Scientific Officer at GenomeDx, Inc. K. Yousefi and A.E. Ross have ownership interest (including stock, patents, etc.) in GenomeDX Biosciences. B.J. Trock reports receiving a commercial research grant from Myriad Genetics, Inc. and MDxHealth, Inc.; is a consultant/advisory board member of Myriad Genetic, Inc. and GenomeDx Biosciences, Inc.; and has provided expert testimony for Rochon Genova LLP. C.J. Sweeney, L.A. Mucci, T. Gerke, G.-S.M. Lee, D. Börmigen, X. Wang and C. Huttenhower have ownership interest (including stock, patents, etc.) in TTP as a biomarker in prostate cancer. C.J. Sweeney has ownership interest (including stock, patents, etc.) in a company developing dimethylaminoparthenolide. No other potential conflicts were disclosed by the other authors.

### Authors' Contributions

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**Study supervision:** G.-S.M. Lee, C.J. Sweeney

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Gerke et al.

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# Cancer Epidemiology, Biomarkers & Prevention

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