Research paper

TP63 isoform expression is linked with distinct clinical outcomes in cancer

Armand Bankhead III[a,b], Thomas McMaster[c], Yin Wang[c], Philip S. Boonstra[a], Phillip L. Palmbos[c,*]

[a] Department of Biostatistics, School of Public Health, University of Michigan, Ann Arbor, MI 48109, USA
[b] Department of Computational Medicine and Bioinformatics, University of Michigan Medical School, Ann Arbor, MI 48109, USA
[c] Department of Internal Medicine, Hematology/Oncology Division, University of Michigan Medical School, Ann Arbor, MI 48109, USA

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A B S T R A C T

Background: Half of muscle-invasive bladder cancer patients will relapse with metastatic disease and molecular tests to predict relapse are needed. *TP63* has been proposed as a prognostic biomarker in bladder cancer, but reports associating it with clinical outcomes are conflicting. Since *TP63* is expressed as multiple isoforms, we hypothesized that these conflicting associations with clinical outcome may be explained by distinct opposing effects of differential *TP63* isoform expression.

Methods: Using RNA-Seq data from The Cancer Genome Atlas (TCGA), *TP63* isoform-level expression was quantified and associated with clinical covariates (e.g. survival, stage) across 8,519 patients from 29 diseases. A comprehensive catalog of *TP63* isoforms was assembled using gene annotation databases and *de novo* discovery in bladder cancer patients. Quantifications and un-annotated *TP63* isoforms were validated using quantitative RT-PCR and a separate bladder cancer cohort.

Findings: DNp63 isoform expression was associated with improved bladder cancer patient survival in patients with a luminal subtype (HR = 0.89, CI 0.80$-$0.99, Cox p = 0.034). Conversely, *TAp63* isoform expression was associated with reduced bladder cancer patient survival in patients with a basal subtype (HR = 2.35, CI 1.64$-$3.37, Cox p < 0.0001). These associations were observed in multiple TCGA disease cohorts and correlated with epidermal differentiation (*DNp63*) and immune-related (*TAp63*) gene signatures.

Interpretation: These results comprehensively define *TP63* isoform expression in human cancer and suggest that *TP63* isoforms are involved in distinct transcriptional programs with opposing effects on clinical outcome.

1. Introduction

More than 17,000 patients will die from bladder cancer in the United States this year [1]. 50% of patients with muscle-invasive bladder cancer (MIBC) will develop lethal metastatic relapse despite aggressive multi-modal therapy. Therefore, identification of prognostic biomarkers which identify patients at risk of relapse and death is critical.

Next generation sequencing has now allowed identification of MIBC molecular subtypes which correlate with clinical outcomes and behavior [2$-$4]. This subtyping analysis based on gene level expression (RNA-sequencing), gene mutations, DNA methylation and non-coding RNA has identified basal, luminal (luminal, luminal papillary, luminal infiltrated) and neuroendocrine subtypes each with distinct survival and treatment response dynamics [5,6]. Basal subtype tumors have similar molecular features to tumors which arise in the lung, breast, head and neck and ovaries and share a *TP63*-driven gene transcriptional program [7]. These results suggest that *TP63* may itself be an important prognostic marker in bladder and other cancer types.

TP63, a paralog of *TP53* and *TP73*, has been proposed to act both as a tumor suppressor and an oncogene, depending on cellular context [8$-$10] and SNPs associated with *TP63* have been suggested to increase risk of development of multiple cancer types [11$-$13]. We have recently shown that *TP63* regulates a transcriptional program which contributes to bladder tumor invasive progression [14], but it was unclear how *TP63* contributed to bladder cancer patient outcome, whether this association holds for basal and non-basal bladder cancer subtypes and if this association is observed in other similar cancer types. Such pan-subtype and pan-disease insights will provide a robust framework for identifying common transcriptional programs involving *TP63* and patient outcome and may also lead to identification of a prognostic biomarker.

Importantly, TP63 exists in multiple functionally distinct isoforms [10]. These isoforms have two distinct amino terminal regions,

* Corresponding author.
E-mail address: ppalmbos@med.umich.edu (P.L. Palmbos).

Research in context

Evidence before this study

TP63 is commonly expressed in bladder and other types of cancer but its role in tumor biology and as a prognostic marker remains unclear. *TP63* is expressed as multiple unique isoforms which can be grouped into *DNp63* and *TAp63* categories based on which amino terminal domain they express. Prior studies have suggested that these different *TP63* isoforms have distinct roles in tumor biology and patient outcomes, but *TP63* isoform expression has not been systematically profiled and correlated with clinical outcomes.

Added value of this study

Here, we utilize next generation transcriptome data derived from The Cancer Genome Atlas (TCGA) cohorts and other sources to systematically describe the spectrum of *TP63* isoform expression in bladder cancer and other tumors. By correlating *TP63* isoform expression with clinical outcomes, we find that while the *DNp63* isoforms correlated with improved patient prognosis, the *TAp63* isoforms correlated with worse patient prognosis in bladder, breast and lung cancers.

Implications of all the available evidence

These results suggest that differential *TP63* isoform expression is associated with opposing effects on patient clinical outcome and suggest the importance of isoform level profiling to inform prognostic test development.

harboring either TA (TransActivation) or DN (deltaN) domains, which utilize distinct promoters and likely reflect distinct biological functions. Likewise, the carboxyl domains of *TP63* isoforms are diverse, with α, β, γ, δ isoforms each reflecting unique splicing events and domain inclusion. Multiple isoforms including various permutations of these amino and carboxyl domains have previously been described in the literature and are represented in gene annotation databases such as RefGene. While *TP63* is commonly expressed in human bladder cancer, the exact spectrum of *TP63* isoforms expressed in human disease has not been fully characterized.

Expression of *TP63* isoforms has been suggested to correlate with clinical outcomes in patients with cancer, but the role of specific isoforms has been controversial [10,15]. Both TAp63 and DNp63 isoforms have been shown to regulate transcriptional programs related to cell differentiation, cell cycle and apoptosis through selective transcriptional activation and suppression of gene targets. Abbas et al. defined TAp63 and DNp63-related gene programs and identified both tumor promoting and suppressive functions of these programs as well as prognostic implications in a variety of TCGA cohorts [16]. These diverse or conflicting effects of TP63 isoform expression are also observed in bladder cancer studies. In patients with high-grade T1 urothelial carcinomas, protein expression of DNp63 (also known as p40) was associated with lower risk of progression [8]. In MIBC, TP63 expression has been shown to be correlated with induction of EMT and worse patient outcomes [9] and expression of DNp63 was shown to identify basal-subtype bladder cancers with aggressive clinical courses and poor prognosis [17]. Thus the prognostic significance of *TP63* isoform expression in bladder and other cancer types remains incompletely understood.

Here we comprehensively characterize *TP63* isoform expression and its association with patient survival in bladder cancer and across other human tumors, using data from The Cancer Genome Atlas (TCGA). We describe a prime *TP63* isoform variant that is commonly expressed in human disease and which is not included in existing isoform definitions. Using previously annotated, as well as, un-annotated *TP63* isoforms, we show that *DNp63* is the most commonly expressed isoform type in bladder cancer and most other cancer types in the TCGA and find that high expression is generally associated with improved patient outcomes. Conversely, although less commonly expressed, *TAp63* is associated with worse patient outcomes in bladder and other tumor types. In bladder cancers, the favorable association of *DNp63* was selectively observed in luminal tumors, whereas, the negative association of *TAp63* was observed specifically in basal squamous tumor subtypes. Further, we found that *DNp63* expression was associated with epithelial differentiation genes and that *TAp63* was associated with immune regulatory gene expression in bladder, lung and breast tumors. Taken together, these results define the spectrum of *TP63* isoform expression within human bladder and other cancers while highlighting distinct isoform prognostic insight not provided by gene level expression.

2. Materials and methods

2.1. RNA-Seq data processing and analysis

TCGA RNA-Seq paired-end read data was downloaded for 33 diseases from the GDC data portal after obtaining dbGaP NIH controlled-access. The data/analyses presented in the current publication are based on the use of study data downloaded from the dbGaP web site, under phs000178.v10.p8 (phs000001.v1.p1/https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000001.v3.p1). Patient clinical metadata was sourced from the GDAC Firehose [18,19]. For clarity, TCGA disease abbreviation definitions are provided as they are introduced in the text; for a complete list of definitions please refer to Table S1. Four TCGA diseases profiled with single-end reads were excluded for quantification comparability reasons: colon adenocarcinoma (COAD), rectum adenocarcinoma (READ), stomach adenocarcinoma (STAD), uterine corpus endometrial carcinoma (UCEC). Unique tumor samples were selected per patient by ordering patient barcodes alphanumerically and selecting the first. Data was analyzed using two different pipelines. The first pipeline mapped reads to GRCh38 using STAR v2.5.2 and used Cufflinks v2.2.1 run in GTF guide mode to discover potentially novel TP63 isoforms not represented in current annotation [20,21]. The second pipeline mapped and quantified reads to GRCh38 using Salmon v0.11.3 with sequence, GC, and position bias correction parameters turned on [22]. The first pipeline was only run on the bladder urothelial carcinoma (BLCA) cohort; the second pipeline was run on all 29 disease cohorts. Refgene gene and transcript annotations were sourced from UCSC genome browser 6/5/2017 [23]. Ensembl gene and transcript annotations were sourced from Gencode GRCh38 release 26 [24]. Transcripts per kilobase million (TPM) values were upper quartile normalized per disease cohort and z-scores were calculated per patient using log2 TPM values for gene and transcript level quantifications separately. Normalized TPM quantifications highlighted throughout this manuscript were derived using Salmon with bias correction (second pipeline). Multiple isoform quantification algorithms (Salmon, Kallisto, and Cufflinks) were considered, but quantification predictions derived using Salmon with bias correction were most consistent with splice junction spanning read support.

RNA sequencing data from primary T1 or T2 bladder human bladder tumors and bladder cancer cell lines was provided by Dr. Scott Tomlins. Detailed characteristics of these patient samples and bladder cancer cell lines have been previously published in [25,26], respectively. Data was processed using pipelines described above.

TP63 isoform groups (e.g. DNp63, TAp63) were constructed by adding transcriptional signal from all isoforms making up a particular sub-set of related isoforms for each patient. For example, TAp63 isoform expression was calculated by adding together all TPM values for all TP63 isoforms with a 5p trans-activation domain (TAp63alpha, TAp63alphaP, TAp63beta, TAp63gamma, TAp63delta).

2.1.1. Statistical methods

Statistical data analysis and visualizations were generated using the R statistical programming language. We used Cox proportional hazards regression to measure the association between TP63 gene and isoform expression (as a continuous variable) and patient survival in the pan-cancer TCGA dataset [27−29]. Initial investigation using Cox regression showed evidence of an association with survival in BLCA, BRCA, and LUSC patient cohorts with expression of TP63, DNp63, and TAp63 isoforms. To illustrate these associations with Kaplan Meier (KM) plots, patient cohorts were stratified into high and low TP63 gene and isoform expression groups by evaluating five thresholds: 5th, 10th, 50th, 90th, and 95th percentile of patient cohort expression. The same percentile threshold was used across multiple diseases when significant; otherwise the most significant threshold was used.

GSEA v2.2.3 PreRanked was used with v6 gene sets sourced from MSigDB using 10,000 permutations in weighted mode [30]. Genes were rank-ordered by negative $\log_{10}$ p-value from a Wilcoxon test to quantify differences in patient gene expression for patients with high or low TP63 isoform expression with negative fold changes represented as a negative score for ranking. Gene sets with an FDR adjusted p-value $\leq$ 0.05 were considered significant. Literature-curated protein interactions from the Human Protein Reference Database (HPRD) were sourced from Pathway Commons v10 [31−33].

2.1.2. Cell culture and ectopic overexpression

253J, UM-UC5, UM-UC14, UM-UC13 and UM-UC10 human bladder cancer cell lines were obtained and fingerprinted as previously described [34]. Cells were passaged in DMEM according to standard cell culture conditions as previously described [35]. Empty vector or dNp63α lentiviral overexpression vectors were transduced into UM-UC10 as previously validated and described [14].

2.1.3. Generation of cDNA

RNA from human bladder cancer cell lines was purified from 1×10^6 cells using PureLink RNA Minikit (Invitrogen, Thermo Fisher Scientific, Grand Island, NY). RNA was stored at −80 C and then converted to cDNA using High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific, Grand Island, NY).

2.1.4. PCR TP63 isoform confirmation

To confirm the presence of unannotated prime TP63 isoforms (dNp63αP and dNp63P we designed forward PCR primers which specifically annealed to the unique prime exon 8b-9 (TCGGACAGTACAAA-GAACGGTGATGCG) or nonprime (canonical) exon 8a-9 junctions (GAACGGTGATGGTACGAAGCGCC) and reverse PCR primers corresponding the unique regions of TP63 alpha (exon 12−13) (GCCCAACCTCGCTAAGAAACTGACAATGC), beta (exon 12−14)(AGG-GATTTTCAGACTTGCCAGATCCTG) and delta (exon 11a-14)(CAGG-GATTTTCAGACTTGCCAGATCTGTTG). Pairing of the nonprime or prime forward primer with the alpha, beta or gamma reverse primer allowed amplification of nonprime or prime alpha, beta or gamma PCR product of (567, 572 and 427 base pairs) respectively if these transcripts were present in the cDNA pools used as template. PCR reactions utilized Applied Biosystems Taqman Universal PCR Master Mix (Thermo Fisher, Grand Island, NY). PCR amplification cycle conditions were: 95C 30s, 58C 30s and 72C for 1 min for 30 cycles. Products were run on a 1% agarose gel and visualized.

TP63 Isoform Quantification by qPCR. To perform quantitative real-time PCR, the prime exon 8b-9 or nonprime exon 8a-9 forward primers were paired with a reverse primer (TCATAAGTCT-CACGGCCCCTCACTGG) which generated a 95 or 110 base pair product for prime or nonprime transcripts, respectively. To perform quantitative PCR for TP63 alpha, beta or delta reverse primers used above were paired with alpha and beta forward primer (CAATGGCTGGAGACATGAATGGACTCA) or a delta forward

primer (CTGCCTTCTGTGAGCCAGCTTATCAACC). GAPDH forward and reverse primers were used as a control (AAGGTCGGAGT-CAACGGATTTGGTCG and GCCATGGGTGGAATCATATTGGAACA). Quantitative PCR reactions utilized Applied Biosystems Sybr Green PCR Master Mix (Thermo Fisher, Grand Island, NY) and were carried out as previously described [14].

2.1.5. Immunoblot

Immunoblotting was carried out as previously described [36]. Total TP63 SC-8431 (Santa Cruz Biotechnology, Dallas, TX) or p63-alpha 13109S (Cell Signaling Technology, Danvers, MA) primary antibodies were used at 1:500 dilutions. Beta actin A1978 (Sigma-Aldrich, St. Louis, MO) was used at a 1:5000 dilution. IRDye 800CW Donkey anti-Rabbit IgG and IRdye 680LT Goat anti-Mouse IgG were obtained from LI-COR Biosciences (Lincoln, NE) and used at 1:10,000 dilution. Blots were imaged on a LICOR system and the protein bands were quantified by ImageJ software (NIH).

3. Results

3.1. Pan-disease gene-level TP63 expression and associations with survival

To comprehensively assess TP63 gene expression and its association with patient outcome among bladder and other cancer patients, we downloaded, quantified per gene and z-score normalized RNA-Seq and clinical metadata from the 8,519 patients of 29 disease cohorts in the TCGA. TP63 has been shown to be commonly expressed in tumors with squamous histology and, as expected, the cohorts with predominantly squamous histology, head and neck squamous (HNSC), lung squamous cell (LUSC), cervical and endocervical cancers (CESC), esophageal carcinoma (ESCA), showed the highest expression followed by bladder cancer (BLCA) (Fig. 1(a)) [37−40].

To determine if TP63 expression correlated with patient outcomes in the pan-cancer data set, we performed Cox regression analysis. Higher expression of TP63 was significantly associated with reduced survival in the lower grade glioma (LGG, HR 1.95, CI 1.53−2.49, Cox p = 0.0001), skin cutaneous melanoma (SKCM, HR 1.2, CI 1.05−1.31, Cox p = 0.011), and pancreatic adenocarcinoma (PAAD, HR 1.24, CI 1.04−1.48, Cox p = 0.023) cancer cohorts (Fig. 1(b)). In contrast, breast carcinoma BRCA (HR 0.86, CI 0.76−0.96, Cox p = 0.008), LUSC (HR 0.95, CI 0.89−1.00, Cox p = 0.071) and BLCA (HR 0.94, CI 0.88−1.01, Cox p = 0.088) cohorts displayed trends towards improved survival with increasing TP63 expression. No significant association was observed in other tumors demonstrating uniformly high expression of TP63 (e.g., HNSC) or diseases with relatively low TP63 expression (e.g., lung adenocarcinoma, LUAD). Interestingly, these results suggest that BLCA, BRCA, and LUSC patients with higher TP63-expressing tumors may have a survival advantage while LGG, SKCM or PAAD patients with higher TP63-expressing tumors are likely to have reduced survival.

To further explore how TP63 expression related to patient outcomes in the TCGA BLCA cohort, we examined the distribution of TP63 expression as a function of log2 TPM. TP63 expression was bimodal in the BLCA cohort, with a large sub-set of patients expressing high TP63 and a smaller sub-population with little or no evidence of expression roughly corresponding to the lowest 10% quantile (Fig. 1(c)). BLCA patients with low/absent TP63 expression had significantly worse median OS as compared to those with higher TP63 expression (median OS 19.8 mos. vs. 44.9 mos., respectively, log-rank p = 0.001) (Fig. 1(d)). These results suggest that there are at least two populations (TP63 high and low) in the TCGA BLCA cohort with differential survival outcomes.

Robertson et al. used unsupervised learning of RNA-Seq data to stratify TCGA bladder cancer patients into five molecular subtypes shown to be associated with survival outcome while also confirming
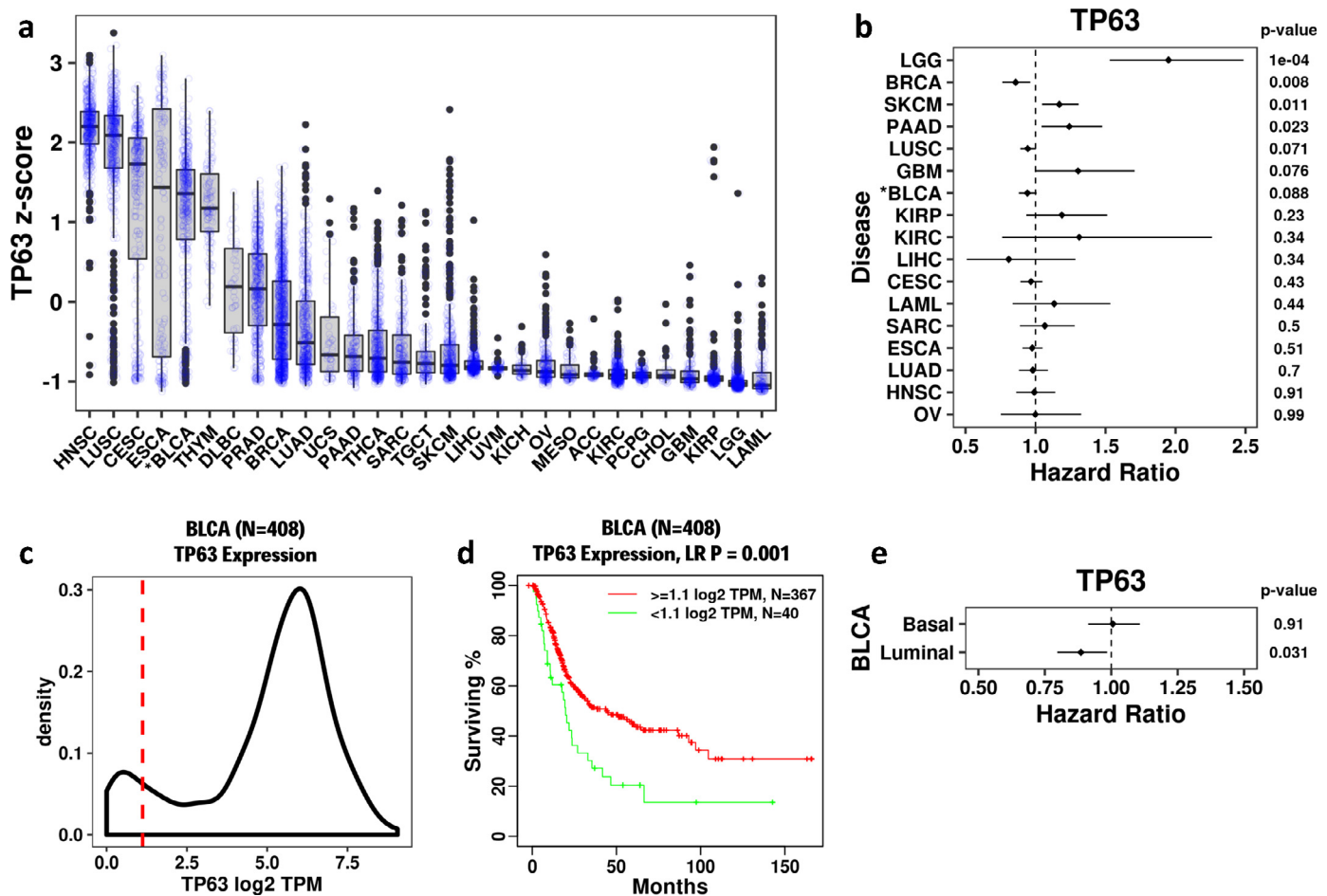
Fig. 1. Pan-cancer survey of *TP63* expression and associations with survival: (a) *TP63* expression shown for 8,519 TCGA patients from 29 disease cohorts where expression has been z-score normalized per patient for comparability. (b) Pan-cancer hazard ratios calculated using a Cox regression. Diseases with less than 100 patients or fewer than 10% patients events were excluded. (c) Bladder cancer patient TP63 expression distribution was bi-modal with a larger proportion (90%) having expression greater than 1 log2 TPM. Dashed line indicates 1 log2 TPM cut off. (d) Kaplan−Meier visualization comparing survival of bladder cancer patients with low TP63 expression versus patients with increased TP63 expression using a 10th percentile threshold. (e) Bladder cancer patients were stratified using bladder cancer molecular subtypes from Robertson et al. with luminal subtypes combined as a single class. Hazard ratios and maximum likelihood statistics calculated using Cox regression. Upper/lower quartiles and medians represented as boxplots with whiskers indicating +/− 1.5 IQR. Forest plot HR error bars represent 95% CI. (Robertson et al. [4]).

previously identified major luminal and basal subtypes [4]. When evaluated separately we did not observe significant associations between *TP63* expression and survival outcome for each of the five molecular subtypes (Supplemental Fig. S1). However, after combining luminal molecular subtypes (Luminal Papillary, Luminal Infiltrated, and Luminal) we observed a significant association with *TP63* expression and decreased hazard (HR = 0.89, CI 0.80−0.98, Cox $p = 0.031$) (Fig. 1(e)). Associations with survival outcome were not significant in the basal squamous sub-population (HR = 1.005, CI 0.91−1.1, Cox $p = 0.91$). These results support the idea that *TP63* gene expression may result in differential effects depending on molecular subtype and that, in bladder cancer, *TP63* expression is associated with a survival benefit in the luminal subtypes.

3.2. Identification of TP63 isoforms expressed in bladder cancer

Given the complex relationship between *TP63* and patient outcomes in our analysis and in the literature, we postulated that differential effects of *TP63* might be related to diversity in *TP63* isoform expression [10,16]. To assemble a complete view of *TP63* isoform expression in bladder cancer we first constructed a catalog of all *TP63* isoforms. Two strategies were used to collect a comprehensive set of *TP63* isoform annotations: (1) annotations were sourced from the gene definition databases Refgene and Gencode (2) *de novo* isoform

discovery was used to identify un-annotated expressed *TP63* isoforms. A total of 14 *TP63* isoforms were identified between Refgene and Gencode databases; 13 in both references and one unique to Gencode (ENST00000460036.1). One isoform (NM_001329148/ ENST00000440651.6) with an alternative first exon was excluded because it was not expressed in TCGA BLCA cohort and confounded isoform quantitation due to aberrant read assignments.

The Cufflinks program was used to perform *de novo* discovery of expressed and potentially novel transcripts not represented in current *TP63* isoform annotations [21]. Two additional *TP63* transcripts were identified in at least 7% (28 patients) of the BLCA cohort and were supported by exon junction spanning reads. These un-annotated isoform variants are closely related to DNp63alpha and DNp63beta but harbor a 4 amino acid alternative splice junction acceptor site at the 3′ end of exon 8 resulting in an alternative exon 8−9 junction (Fig. 2(a)). This alternative exon 8−9 junction is present in *TAp63alpha* and *DNp63delta* (NM_001329148 and NM_001329149) forms, but is not present in Refgene or Encode gene definitions as a variant of *DNp63alpha* or *DNp63beta* isoforms. Here we will refer to these isoforms as *DNp63alphaP* (*DNp63alpha prime*) and *DNp63betaP* (*DNp63beta prime*). Although unannotated, these two isoforms have previously been described [41]. To confirm that the *DNp63alphaP* and *DNp63betaP* isoforms were truly expressed in human bladder cancer, we designed PCR primers specific to their unique 8a-9 or 8b-9 exon junctions and
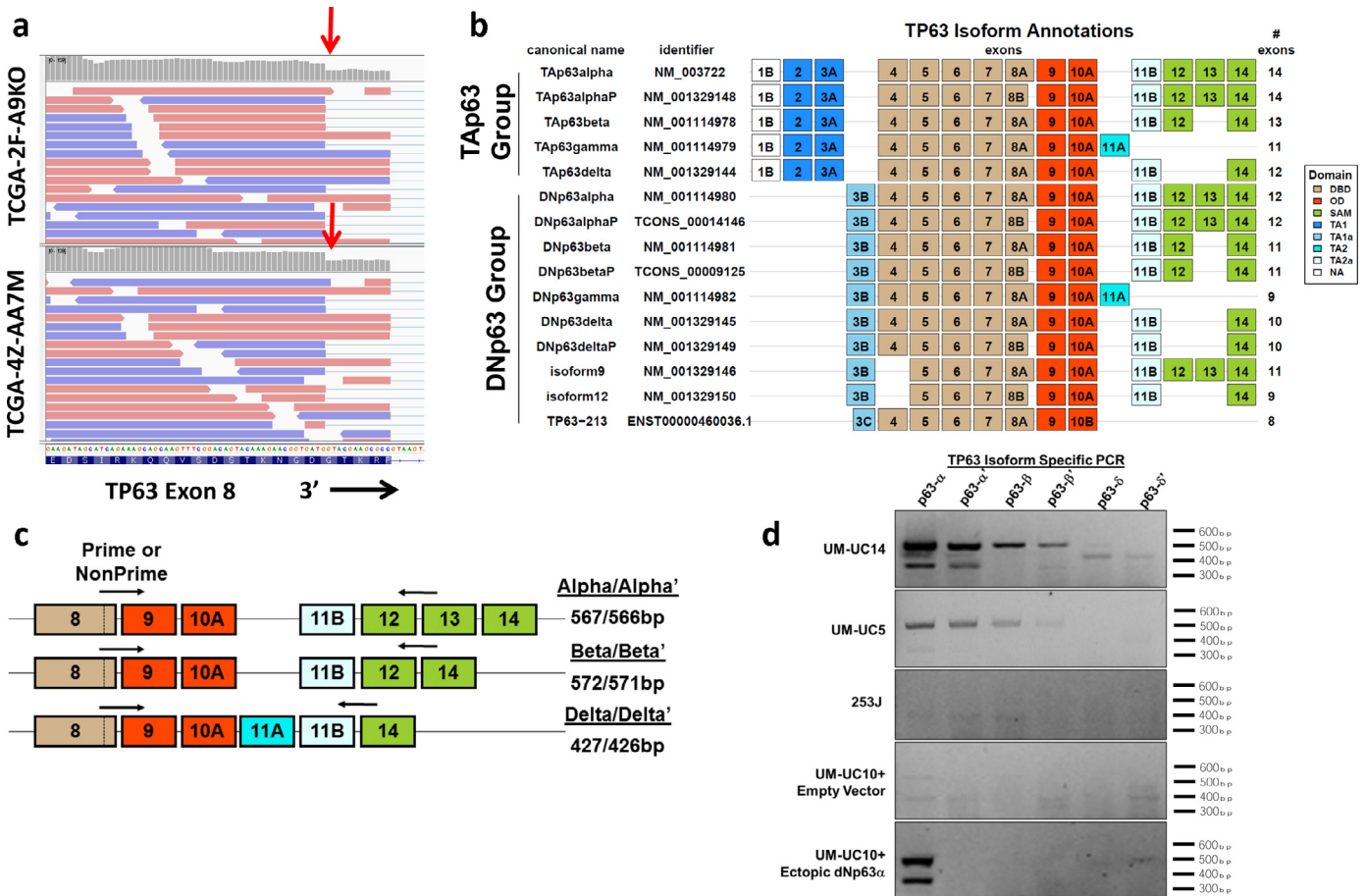
Fig. 2. Identification of *TP63* Isoforms Expressed in Bladder Cancer: (a) RNA-Seq evidence of un-annotated *DNp63* isoforms with alternative splice junction expressed in TCGA bladder cancer patients. Drop-off in expression (red arrows) indicates that both forms of exon 8 are expressed. TCGA-4Z-AA7M and TCGA-2F-A9KO are two bladder cancer patients represented in TCGA BLCA cohort. (b) Catalog of *TP63* isoforms derived from Refgene, Gencode, and *de novo* isoform discovery using Cufflinks. DBD = DNA Binding Domain, OD = C-terminal oligomerization domain, SAM = Sterile Alpha Motif, TA = Trans-activation, NA =none. (c) Schematic of primers used to detect prime and nonprime TP63 isoform expression. 5′ was specific for either prime (8A-9) or nonprime (8B-9) exon junction. There was a 1 bp difference between prime and nonprime products. (d) PCR confirms expression of prime isoforms in human bladder cancer cell lines. 253J and UM-UC10 have low endogenous *TP63* expression and serve as negative controls. dNp63alpha (non-prime) ectopic expression serves as a positive control for p63-alpha PCR.

the exon 12−13 junction (unique to alpha isoforms), exon 12−14 junction (beta isoforms) and for the exon 11b-14 junction (delta isoforms) (Fig. 2(c)). Using these primer combinations, we performed PCR using cDNA derived from human bladder cancer cell lines with high (UM-UC5 and UC14) or low (253 J, UM-UC10) expression of *TP63* and from UM-UC10 cells with ectopic expression of *DNp63alpha*. PCR products for both *p63alpha, p63beta* and *p63delta* non-prime and prime isoforms were detectable in UM-UC14 and UM-UC5 but not 253J or UM-UC10 control bladder cancer cells (Fig. 2(d)), confirming the presence of both prime and non-prime isoforms in these cells. As expected, a PCR product corresponding to *p63alpha* non-prime was only robustly detectable in UM-UC10 when this isoform was ectopically expressed (Fig. 2(d)), supporting the specificity of our prime PCR products. Taken together, these results support the presence of *DNp63alphaP* and *DNp63betaP* as isoform variants in human bladder cancer. The resulting catalog of 15 *TP63* isoforms was used in our isoform expression survey and is represented in Fig. 2(b) to illustrate differences between isoforms and annotated protein domains [42].

3.3. Quantification of TP63 isoform expression in bladder and other cancers

After establishing the spectrum of *TP63* isoforms expressed in human bladder cancer, Salmon was used to quantify individual isoform expression in the TCGA and other human bladder cancer

cohorts [22]. In bladder cancer, *TP63* expression was dominated by *DNp63* group isoforms (*DNp63alpha, DNp63alphaP, DNp63beta*, and *DNp63betaP, DNp63gamma, DNp63delta, DNp63 deltaP*) whereas *TAp63* group isoforms (*TAp63alpha, TAp63alphaP, TAp63beta, TAp63-gamma* and *TAp63delta*) were expressed in only a minority of patients (Fig. 3(a)). We observed a similar pattern of isoform expression in an independent set of bladder cancer primary tumor samples and bladder cancer cell lines (Fig. 3(b) and (c)) [25,26,34]. In all three data sets, prime versions of *DNp63alpha* and *DNp63beta* demonstrated slightly less expression than non-prime analogs, although they were among the most highly expressed transcripts. The similarity in the landscape of expressed *TP63* isoforms observed among in vitro model systems and in non-TCGA bladder cancer patient cohorts provided confidence that *TP63* isoform quantifications were not dataset or sample type specific.

To confirm that our *TP63* isoform quantifications derived using Salmon accurately reflected the relative expression of *TP63* isoforms in bladder cancer, we performed quantitative PCR using primers specific to non-prime or prime *TP63* or specific for alpha, beta or delta isoforms and compared with Salmon *TP63* isoform quantifications derived from RNA-Seq from the same cell lines. Quantitative PCR confirmed that prime isoforms were highly expressed in UM-UC14 and UM-UC5, and in similar proportions as seen by RNA-Seq isoform quantification (Fig. 3(d) and (e)). Similarly, to confirm that Salmon was accurately quantitating the proportion of alpha, beta and delta
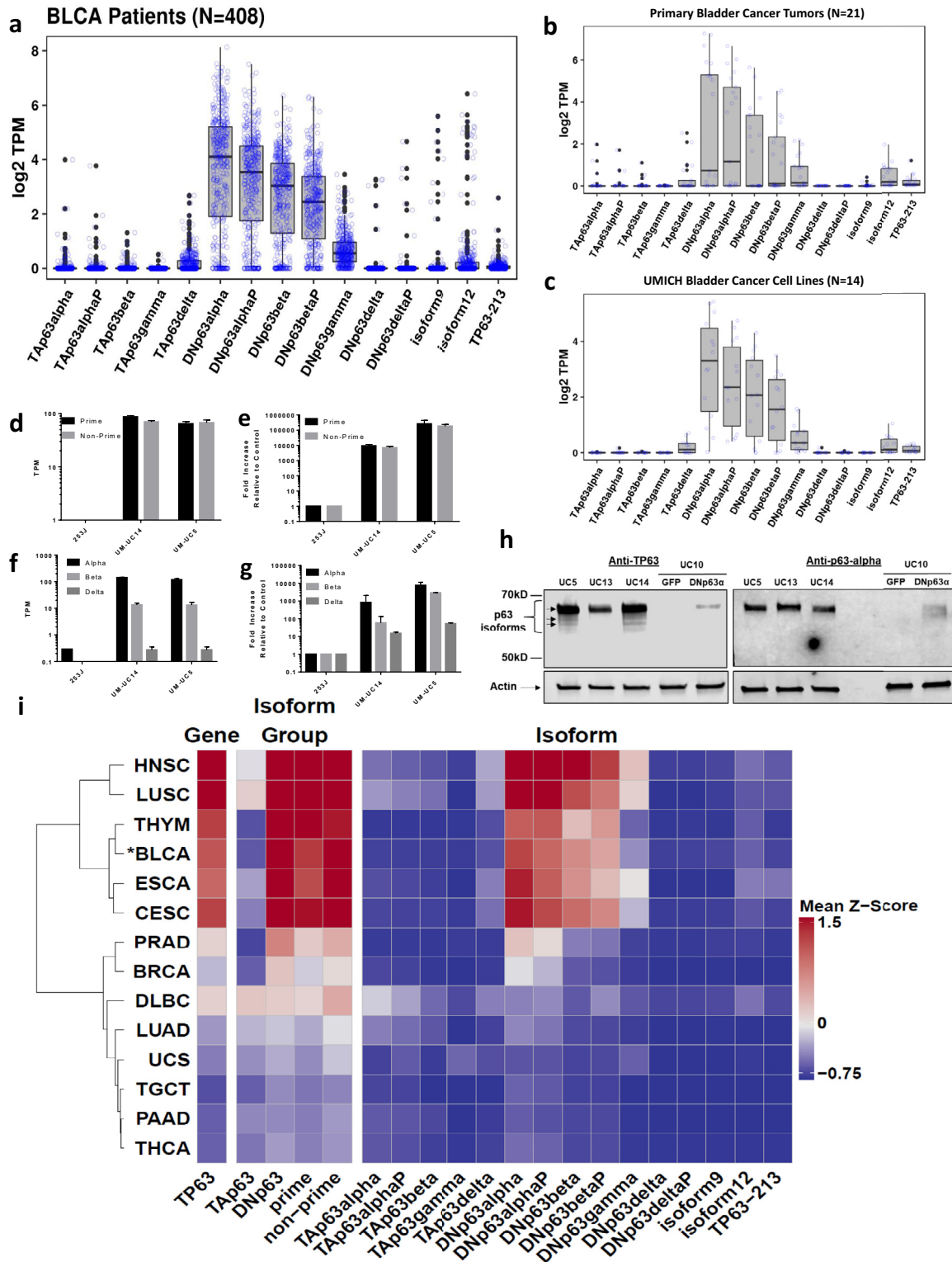
Fig. 3. Quantification of TP63 Isoform Expression. (a) TCGA bladder cancer expression of 15 *TP63* isoforms was dominated by *DNp63* expression with a subset of patients exhibiting low levels of *TAp63* expression. Independent cohort of primary bladder cancer tumors (b) and bladder cancer cell lines exhibited similar isoform expression patterns (c). (d) Quantification of Prime vs. Non-prime *TP63* isoform expression in human bladder cancer cell lines based on RNA-sequencing and Salmon quantification. (e) Quantification of Prime and Non-Prime *TP63* isoform expression using RT-PCR. (f) Quantification of Alpha, Beta and Delta isoform expression based on Salmon quantification. (g) Quantification of Alpha, Beta and Delta *TP63* isoform expression using RT-PCR. (h) Immunoblot of TP63 protein expression in bladder cancer cell lines. UM-UC5, UC13, UC14 and UC10 are shown. UM-UC10 has no endogenous TP63 expression and was transfected with GFP vector control or DNp63alpha expression vector. Left panel = immunoblot with anti-TP63 antibody. Right panel = immunoblot with antibody specific for alpha isoform. Arrows indicate 3 distinct TP63 isoforms. (i) Pan-cancer *TP63* isoform expression summarized as mean z-score per disease. On average *TP63* isoform expression is dominated by DNp63alpha, DNp63alphaP, DNp63beta, DNp63betaP with moderate levels of TAp63 isoform expression. TA, DN, prime, and non-prime columns represent the sum of isoform expression signal for each isoform group as quantified by Salmon and then normalized as a z-score using the mean and standard deviation of all quantified transcripts. Only diseases with at least 1 log2 TPM average TP63 gene expression are shown. Upper/lower quartiles and medians represented as boxplots with whiskers indicating +/− 1.5 IQR. Barplot error bars represent mean +/− standard deviation.

TP63, we performed qPCR using primers specific for the alpha, beta or delta isoforms. This experimental investigation confirmed that alpha was most abundant followed by beta and delta and was similar to the RNA-Seq quantification predicted by Salmon (Fig. 3(f) and (g)).

To examine whether TP63 isoform protein levels were similar to the mRNA expression profile, we immunoblotted for total TP63 and the alpha isoform of TP63 (Fig. 3(h)). As expected, multiple bands corresponding to various TP63 isoforms were observed. All bands corresponded to DNp63 isoforms consistent with the complete lack of TAp63 isoform mRNA expression in these cell lines (Fig. 3(b)). Salmon quantifications of DNp63alpha isoform expression indicated that DNp63alpha corresponded to 90 and 91% of total TP63 mRNA in UM-UC5 and UC14, respectively. Consistent with this finding, immunoblot for the alpha isoform of TP63 and quantification of the corresponding band by densitometry indicated that DNp63alpha corresponded to 84 and 80% of total TP63 protein expression in UM-UC5 and UM-UC14, respectively. These results confirm that isoform quantifications using Salmon accurately predicted expression of TP63 isoforms and that DNp63alpha is the dominant TP63 isoform expressed in bladder cancer.

This landscape of relative *TP63* isoform expression was also observed across most TCGA tumor cohorts with average $\log_2$ TPM expression ≥ 1 of *TP63*. Consistent with the spectrum of *TP63* isoform expression observed in bladder cancer cohorts, *DNp63* expression was higher than *TAp63* in all but diffuse large B-cell lymphoma (DLBC). Diseases with the highest *TP63* gene level expression had, on average, the highest amounts of *DNp63alpha, DNp63alphaP, DNp63beta* and *DNp63betaP* with lower to moderate expression of *TAp63* isoforms (Fig. 3(i)). Consistent with previous in vitro findings by Sethi et al.,

DLBC expressed the highest average levels of *TAp63* and was the only disease to express more *TAp63* than *DNp63* isoforms [41].

3.4. TP63 isoform expression in bladder cancer sub-types

Transcriptomic profiling of human bladder cancer has identified multiple molecular subtypes [2−4]. *TP63* has been specifically linked to the basal squamous subtype [7,14]. To determine whether *TP63* isoform expression varied according to molecular subtype, we examined *TP63* isoform expression in basal squamous, luminal papillary, luminal infiltrated, luminal and neuroendocrine subtypes (Fig. 4(a)). We observed significantly higher expression of the *DNp63* isoform group in luminal papillary and basal squamous subtypes as compared to the luminal, luminal infiltrated or neuronal subtypes (Wilcoxon $p < 0.0001$) (Fig. 4(b)). In contrast, basal squamous patients were found to have significantly higher *TAp63* group expression compared with luminal (Wilcoxon $p < 0.0001$) (Fig. 4(c)) and luminal papillary patients expressed significantly less *TAp63* (Wilcoxon $p = 0.001$) than basal squamous tumors. These results suggest that *DNp63* and *TAp63* may have distinct expression profiles based on TCGA molecular subtype and that these differences may contribute to distinct patient outcomes.

TP63 Isoform Expression is Associated with Patient Survival

After observing an association with overall *TP63* gene expression and improved survival in bladder cancer patients, we next tested whether individual *TP63* isoforms or the *DNp63* and *TAp63* isoform groups were similarly associated with patient survival. To examine this, we stratified BLCA patients using five quantile thresholds per isoform and determined significance using a log-rank statistic FDR-
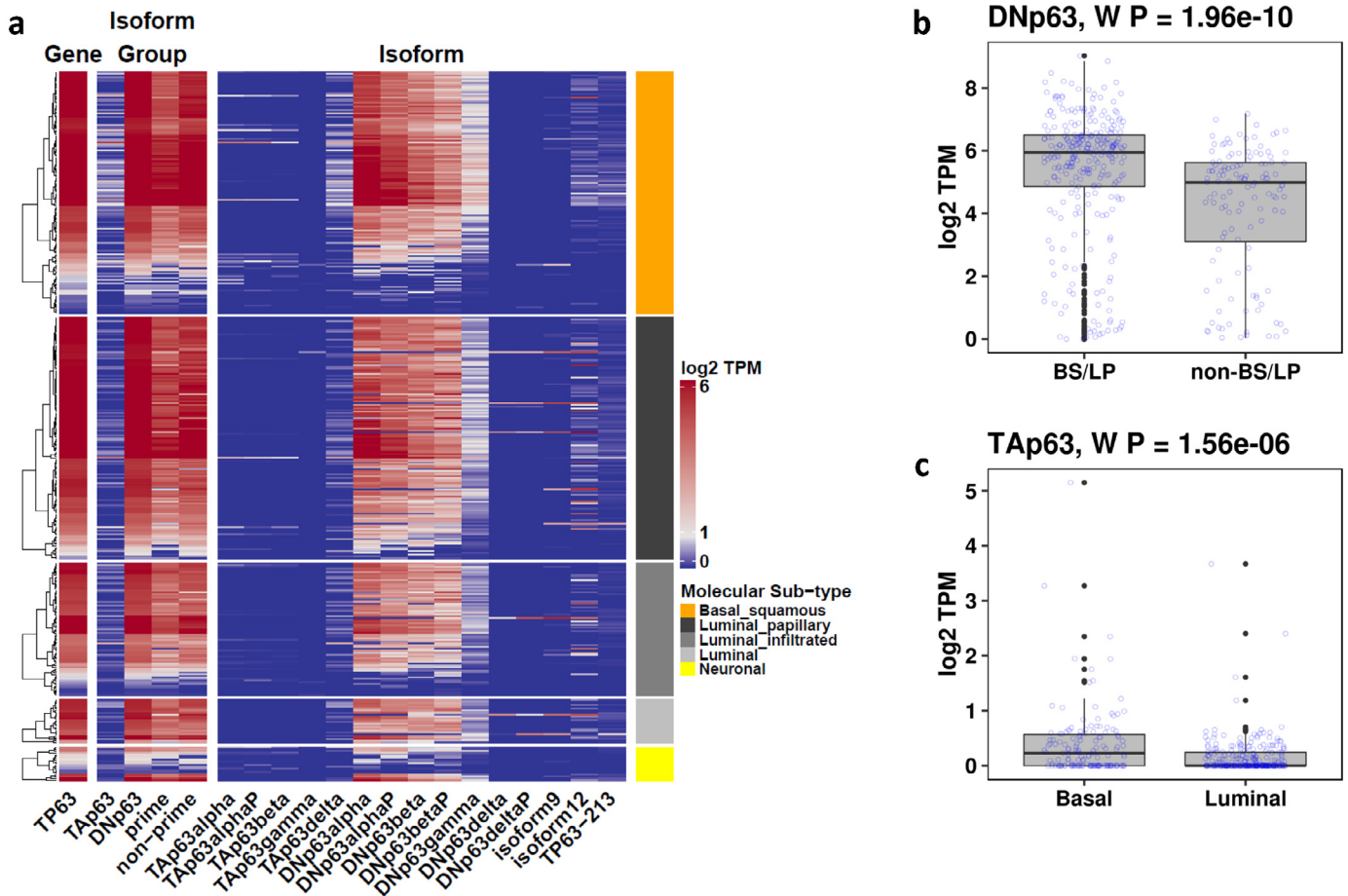

Fig. 4. Bladder Cancer *TP63* Isoform Expression in Molecular Subtypes: (a) isoform expression shown for all 408 bladder cancer tumor samples grouped by Robertson molecular sub-types. (b) Significantly higher *DNp63* expression observed in Basal Squamous (BS) and Luminal Papillary (LP) sub-types while (c) *TAp63* expression was significantly higher in Basal Squamous as compared to luminal patients. Upper/lower quartiles and medians represented as boxplots with whiskers indicating +/− 1.5 IQR.

adjusted p-value. Similar to total *TP63* expression, *TP63* isoform expression also exhibited a bimodal distribution of expression, which could be separated by using a 10th percentile cut-off for *DNp63* isoforms or a 95th percentile cut-off for *TAp63* isoform group (Supplemental Fig. S2). KM survival plots of the BLCA *DNp63* positive and negative groups demonstrated that the *DNp63* positive group had significantly improved overall survival (median OS 44.3 mos. vs. 20.5 mos., log-rank p = 0.006) (Fig. 5(a)). Likewise, grouping patients by presence or absence of *DNp63alphaP* demonstrated improved overall survival in *DNp63alphaP* positive patients (median OS 38.2 mos. vs. 20.2 mos., log-rank p = 0.0002) (Fig. 5(a)). Similar results were observed in the BRCA and LUSC cohorts using identical percentile-based cut-offs suggesting this finding is true across tumor types and validating this observation.

Stratification of BLCA patients into those with *TAp63* isoform expression (5%) and those without (95%) demonstrated that patients with high *TAp63* or *TAp63beta* expressing tumors had significantly worse OS than those with low/no expression (median OS 13.4 mos. vs. 38.2 mos., log-rank p = 0.0007) (Fig. 5(b)). Similar trends were observed in the BRCA and LUSC patient cohorts suggesting this finding to have broad applicability.

The relationship between *TP63* gene expression and survival was dependent on bladder cancer molecular subtypes (Fig. 1(e)). Similarly, *DNp63* expression was significantly associated with reduced hazard in luminal patients (HR = 0.89, CI 0.80−0.99, Cox p = 0.034), but did not show a significant association in patients with a basal squamous subtype (HR = 1.00, CI 0.91−1.10, Cox p = 0.95) (Fig. 5(c)). *TAp63* was significantly associated with increased hazard in basal squamous subtype patients (HR = 2.35, CI 1.64−3.37, Cox p < 0.0001), but not in patients with luminal tumors. Taken together, these results suggest that *DNp63* plays a protective role in luminal bladder cancer patients whereas *TAp63* is associated with poor risk in basal bladder cancers (Fig. 5(c)).
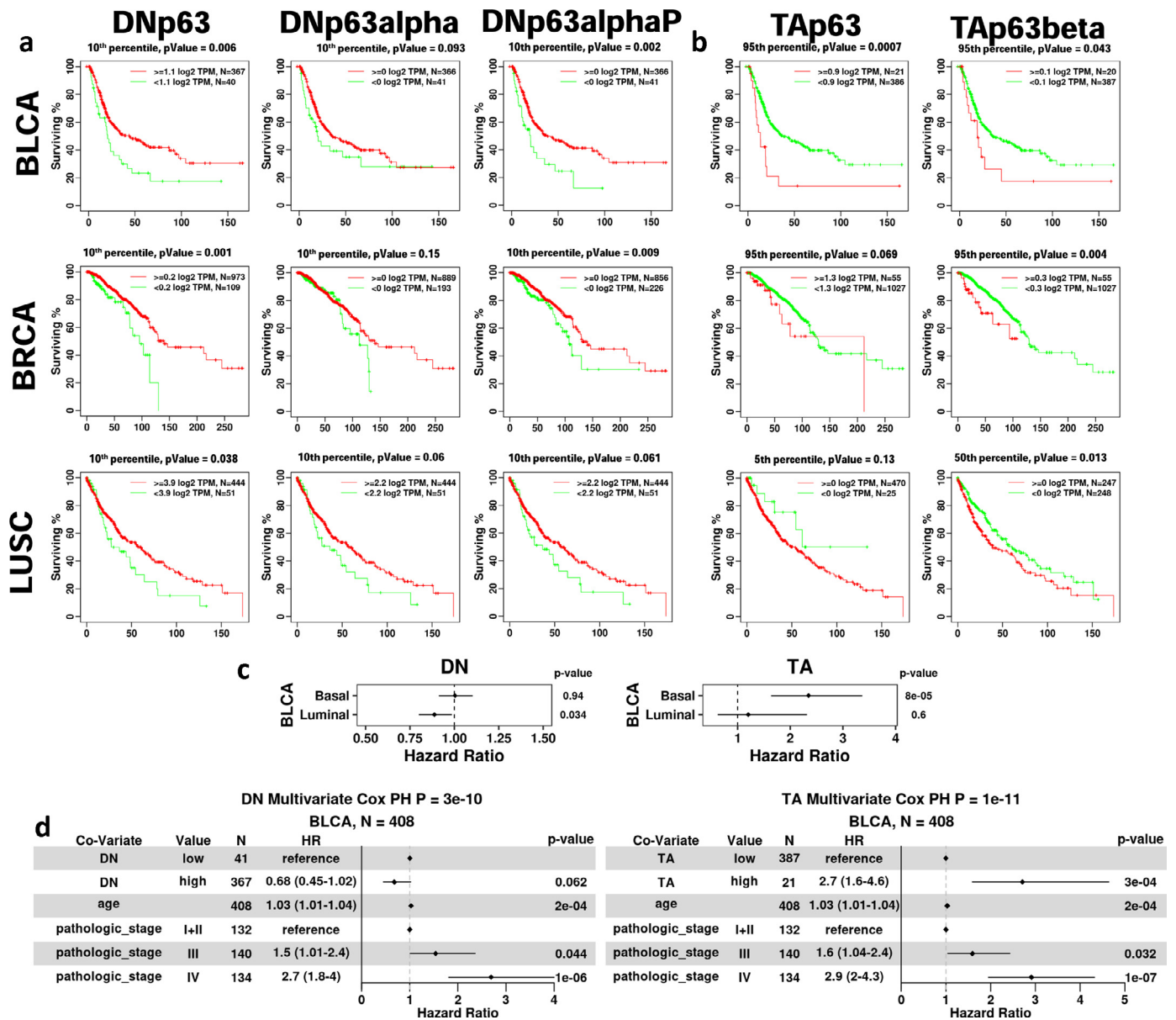
Fig. 5. *TP63* isoform expression is associated with survival: Higher expression of *DNp63* was associated with improved survival (a) and higher expression of *TAp63* was associated with reduced patient survival (b). Similar *TP63* isoform-specific survival associations were observed in bladder cancer, breast cancer, and lung squamous carcinoma patient cohorts. (c) Bladder cancer patients were grouped by molecular subtype and evaluated for association of *DNp63* and *TAp63* isoform expression with survival using univariate Cox regression. (d) Multivariate Cox regression analysis. High *DNp63/TAp63* expression associated with reduced/increased hazard ratio, respectively. Forest plot HR error bars represent 95% CI.

To determine if the prognostic importance of *TAp63* or *DNp63* was independent of other known factors associated with bladder cancer patient prognosis, we performed multivariable Cox regression to adjust for potentially relevant clinical attributes (age, gender, grade, pathologic stage). Only age and pathologic stage were significantly associated with survival and were considered in multivariable survival modeling with *TP63* isoform expression. Multivariable Cox regression analysis confirmed that high *DNp63* expression continued to trend with increased survival when controlling for age and pathologic stage (HR 0.68, CI 0.45−1.0, Cox *p* = 0.062) (Fig. 5(d)). Likewise, high *TAp63* expression was significantly associated with an increased risk of death when controlling for age and pathologic stage (HR 2.7, CI 1.6−4.6, Cox *p* = 0.0002). These results implicate *DNp63* and *TAp63* expression as markers of good or poor prognosis in bladder cancer independent of patient stage or age.

Overall, survival data implicated *DNp63* and *TAp63* isoform group expression as positive or negative prognostic indicators in BLCA, BRCA and LUSC. To more broadly examine the implications of expression of these isoforms across tumor type and to account for confounding effects associated with our 10 or 95% cut offs, we next performed univariate Cox proportional-hazards regression analyses on the entire TCGA pan-cancer cohort (Fig. 6(a) and (b)). This analysis confirmed *DNp63* association with improved HR for BRCA (HR 0.86,

CI 0.77−0.96, Cox *p* = 0.007) and demonstrated trends toward improved HR for BLCA and LUSC (Fig. 6(a)). Likewise, these analyses confirmed that *TAp63* was associated with reduced survival for BLCA (HR 1.8, CI 1.4−2.4, Cox *p* = 0.0002). Interestingly, in SKCM patients, although total *TP63* was associated with worsened outcome (Fig. 1(b)), the isoform level *DNp63* expression was associated with worse survival (HR 1.2, CI 1.1−1.4, Cox *p* = 0.0003) and *TAp63* associated with improved survival (HR 0.70, CI 0.51−0.96, Cox *p* = 0.020) which was also in the opposite direction of BLCA, BRCA and LUSC.

TP63 gene expression associations with TCGA survival varies across disease cohorts with some diseases having a high hazard ratio (LGG) while others have a low hazard ratio (LIHC) (Fig. 1(b)). Because of the inverse relationship of *DNp63* and *TAp63* expression to survival, we hypothesized that *TP63* association with high or low HR, might be explained by shifts in the relative abundance of these two isoforms. To investigate this hypothesis, we grouped entire TCGA tumor cohorts together and plotted the average proportion of *TAp63* or *DNp63* over total *TP63* for each population vs. *TP63* HR for the entire cohort (Fig. 6(c) and (d)). A significant negative correlation was observed between average proportion of *DNp63* per patient cohort and gene level *TP63* hazards ratio. This pan-cancer relationship indicates that diseases with higher *TP63* associated hazard are more likely to have patients with lower relative amounts of *DNp63*. As expected, since most TP63 is
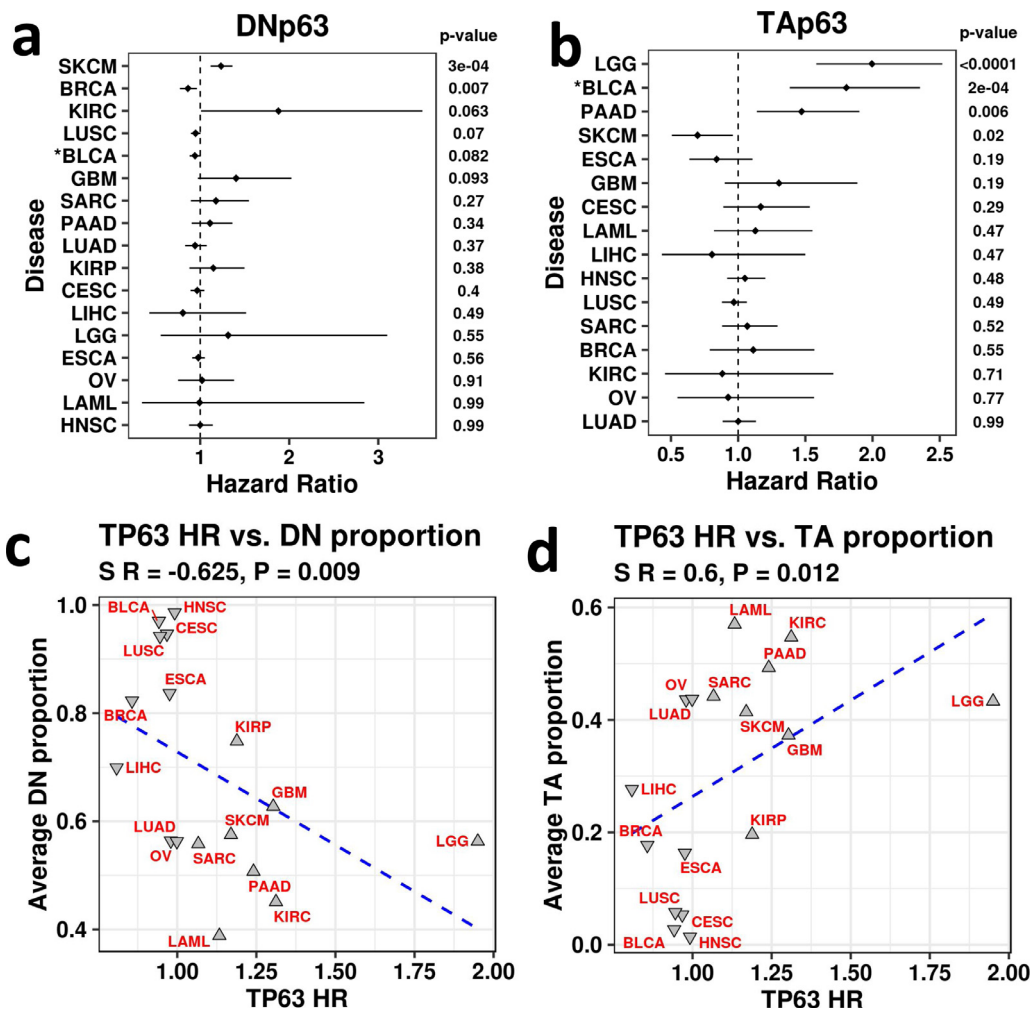
Fig. 6. Univariate Cox proportional-hazards regression used to quantify association of DNp63 (a) or TAp63 (b) with survival. Diseases with less than 100 patients, fewer than 10% dying patients, or no patients with isoform expression greater than log2 TPM > 1 were excluded from pan-cancer survival analysis. (c) Increased average DN proportion in each TCGA disease cohort correlated with reduced *TP63* specific HR. (d) Diseases with higher average TA proportion tended to have increased *TP63* hazard ratios. Proportions of expression per disease were calculated by dividing TA/DN group expression by TP63 gene expression per patient and then averaging proportions across all patients per disease. Forest plot HR error bars represent 95% CI.

either TA or DN, a positive correlation was observed between *TAp63* and gene level *TP63* hazard indicating that diseases with higher *TP63* hazard have relatively higher levels of *TAp63*. These results suggest that the relative proportion of *TAp63* or *DNp63* vs. total *TP63* associates with clinical outcome regardless of tumor type.

Taken together, these results demonstrate that *TP63* isoform expression provides additional prognostic information that is not available from gene level expression and further establishes *DNp63* and *TAp63* associations with positive or negative survival in BLCA and other patient populations.

3.5. Multi-disease DNp63 and TAp63 isoform signaling programs

Abbas et al. had previously shown that DNp63 and TAp63 promoted transcriptional programs of prognostic significance in various tumor types including bladder cancer [16]. Contrasting

isoform-specific associations with patient survival observed across multiple diseases lead us to investigate transcriptional signaling programs that distinguish patient populations with high levels of *DNp63* or *TAp63*. We hypothesized that DNp63 and TAp63 might induce distinct transcriptional programs similar to that seen in Abbas et al. Using the same expression thresholds described in Fig. 5(a) and (b), patients in BLCA, BRCA, and LUSC cohorts were stratified using *DNp63* and *TAp63* expression levels and a Wilcoxon test was used to identify genes changing in expression between patient populations. Signed Wilcoxon p-values were used to rank genes for GSEA and identify gene sets statistically enriched in groups with high levels of each isoform.

Patients with tumors with high levels of *DNp63* enriched for gene sets related to epidermal cell differentiation, keratinization, and skin development (Fig. 7(a)). These results were consistent with known *DNp63* functions and their enrichment in three patient cohorts
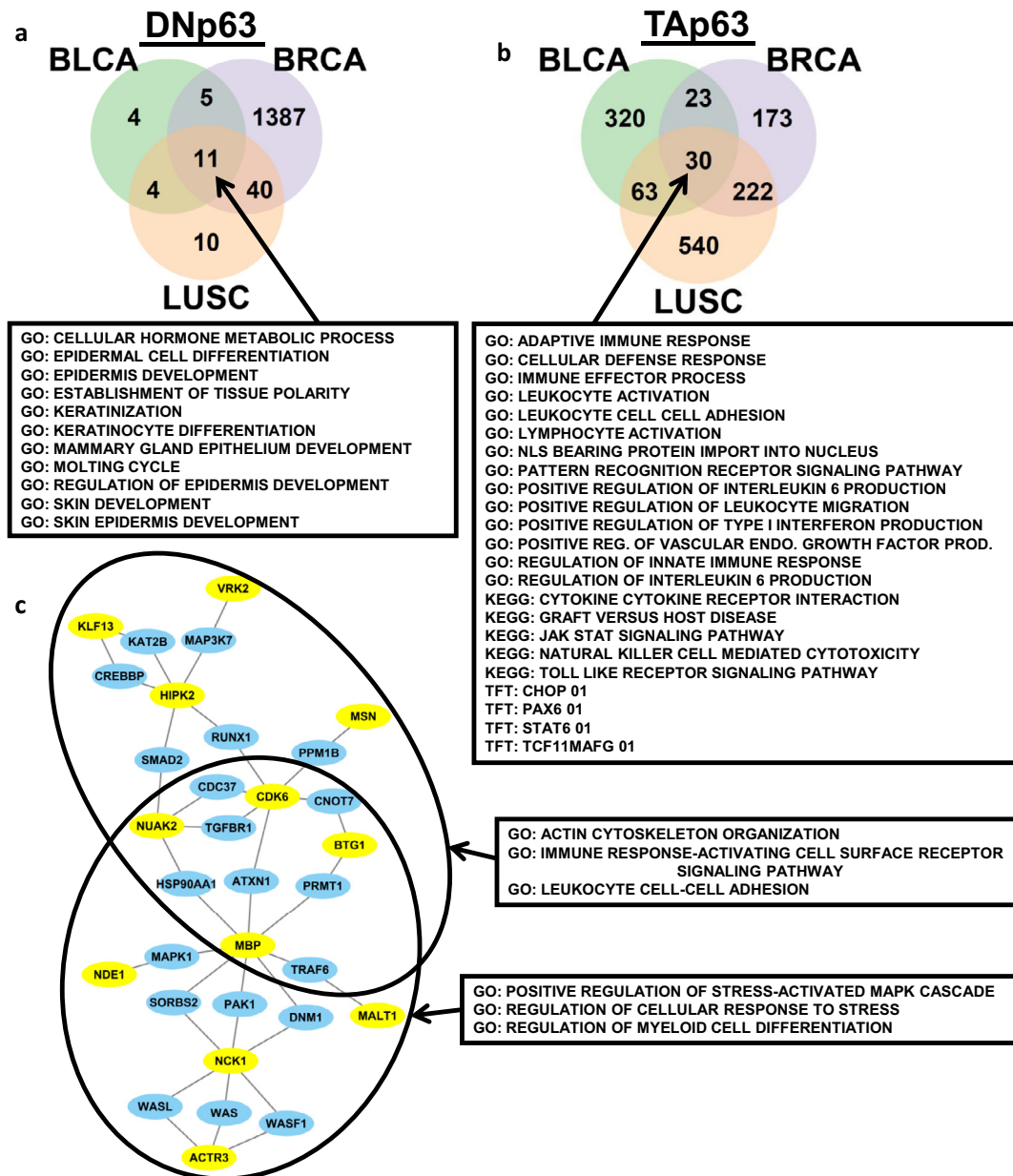
Fig. 7. TP63 Isoform Expression Signaling Programs: GSEA was used to identify gene sets enriched for genes with increased expression in patients with high levels of *DNp63* (a) and *TAp63* (b). 11 and 30 gene sets were commonly enriched in *DNp63* and *TAp63* high expressing patient cohorts, respectively. (c) A bladder cancer specific *TAp63* signaling network was constructed by connecting genes with the most significant differential expression (yellow) in *TAp63* patients with intermediate interacting partners (blue) identified using HPRD protein interactions. Fisher's exact gene set enrichment was used to identify gene ontology (GO) categories enriched in sub-networks.

demonstrates consistent association with DNp63 gene programs [43]. We applied the same method to *TAp63* and found commonly enriched gene sets related to adaptive and innate immune response, VEGF signaling, Jak-Stat signaling and several other transcription factors (CHOP, PAX6, TCF11, MAFG) (Fig. 7(b)). In addition to GSEA, we mapped genes that were most significantly increased in patients with high levels of *TAp63* (*N* = 32) and used experimentally derived protein interactions from HPRD to identify candidate signaling pathways which might be associated with the *TAp63* effect (Fig. 7(c)). The resulting network represents genes that are differentially expressed and candidate protein interaction partners in downstream signal transduction cascades. This 32 gene network contains a total of 40 protein interactions and is enriched for signaling related to immune response, cell stress response, and actin cytoskeleton organization. These findings imply that *DNp63* and *TAp63* expressing tumors are associated with unique transcriptional and signaling programs, which may drive patient outcomes.

4. Discussion

The prognostic implication of TP63 expression in bladder and other cancers was previously unclear. While some groups have found that higher *TP63* mRNA predicted worse survival in patients with MIBC [9] as well as contributing to invasive progression in MIBC [14], others demonstrated that TP63 protein levels did not correlate with prognosis following cystectomy [44]. Therefore, to clarify the role of TP63 in cancer patient prognosis, we comprehensively profiled *TP63* expression across TCGA tumor groups and then correlated *TP63* expression with patient outcomes with specific focus on the bladder cancer cohort. We found that BLCA had the fifth highest level of *TP63* expression and that *TP63* expression actually correlated with increased survival in the BLCA cohort, while predicting worse survival in the SKCM, PAAD and LGG cohorts (Fig. 1(b)). Interestingly, the increased survival association seemed to be limited to the luminal bladder cancer subtypes (Fig. 1(e)). This suggested that the relationship of TP63 with patient outcome might depend on factors beyond gene level expression.

We hypothesized that heterogeneity of *TP63* isoform expression might be associated with survival in bladder cancer patients and in patients with other tumor types. This hypothesis is supported by recent work from Abbas et al. which showed that DNp63- and TAp63-driven transcriptional programs are associated with different clinical outcomes [16]. To comprehensively quantify *TP63* isoform expression in the TCGA BLCA, we inspected previously defined isoforms and identified two isoforms−here called *DNp63alphaP* and *DNp63betaP*, which had not previously been annotated as TP63 isoforms. Quantification using this more comprehensive list of isoform definitions demonstrated that *DNp63* group isoforms represented the predominate isoform type expressed in bladder cancer and most other tumor types with high levels of *TP63* expression (Fig. 3(i)). As far as we are aware of, this is the first pan-cancer analysis of the landscape of *TP63* isoform expression. In this context, *DNp63* isoforms appear to be the predominate cancer-associated isoform.

Molecular subtyping based on gene level RNA expression has identified five distinct bladder cancer cohorts exhibiting different survival and propensity to respond to therapy [4,6]. Interestingly, quantification of *TP63* isoforms by bladder cancer molecular subtypes, suggested that *TP63* isoform expression may be distinct between subtypes. Although both basal and luminal subtype tumors have high gene level expression of *TP63* and *DNp63* isoforms, the basal subtype expressed increased levels of *TAp63* as well. We theorize that this may be why increased TP63 was associated with increased survival in luminal, but not basal subtype tumors.

Similar to the published data on *TP63* expression, the effect of individual *TP63* isoforms on bladder cancer patient prognosis is also controversial. Genome-wide association studies (GWAS) have found that

bladder cancer risk is associated with a sequence variant of an enhancer specifically controlling *DNp63* expression [12,13]. In contrast, *DNp63* in NMIBC did not correlate with tumor risk of relapse [45] and was associated with reduced risk of invasive progression in T1 bladder tumors [8]. Further, high expression of DNp63 (p40) protein was associated with reduced patient survival in MIBC patients [46]. Interestingly, analysis of TAp63 or DNp63 transcriptional signatures demonstrated that these signatures can also correlate with patient outcomes [16]. Our analysis of the TCGA cancer cohorts support a *DNp63* expression association with improved patient survival in bladder cancer as well as BRCA and LUSC and link increased *TAp63* isoform expression with reduced patient survival. There are many potential reasons for the discrepancies observed in these data sets. First, most prior studies relied on antibody-based IHC quantification by pathologists in a limited number of patients which introduced observer and staining technique variation. Second, given the limited number of patients, it is also possible that there were different proportions of molecular subtypes. Since the effect of DNp63 is related to subtype, differential proportions of luminal or basal subtypes (for example) could have significantly impacted the study outcomes. Finally, we hypothesize that patient prognosis may be determined by the proportion of *TP63* that is expressed as *DNp63* vs. *TAp63*. This idea is supported by examination of *DNp63* and *TAp63* isoform expression across the entire TCGA cohort, which demonstrated a significant association between proportionally higher expression of TA with increased HR and proportionally higher expression of DNp63 with lower HR. Since many prior studies focused on detection of one isoform type, they might miss changes in relative proportion of isoform expression. These results also imply that quantification of the entire spectrum of *TP63* isoforms may be necessary to understand how TP63 contributes to tumor biology and patient outcomes and that gene level or individual isoform quantification may be inadequate for prognostic determination. It also remains unclear whether DNp63 or TAp63 expression determines therapeutic response; this is an important area for future investigation.

Immune-related transcriptional programs associated with TAp63 (Fig. 6(b)/(c)) combined with absence of TAp63 mRNA in bladder cancer cell lines and relatively high expression of TAp63 in lymphoma patients (Fig. 3) suggest the possibility that the observed levels of TAp63 expression might be derived from immune cell infiltrate in BLCA, BRCA, and LUSC patient tumors. Whether or not this is the case, the enrichment of immune pathways in the TAp63 expressing tumors suggests a link between TAp63 and tumor immune infiltrates. Given the emerging importance of antitumor immunity in bladder cancer, future studies using single-cell sequencing to de-convolute TAp63 expression from tumor and immune cell types may increase insight into how TAp63 contributes to worse patient outcomes. Since the prognostic value of infiltrating immune cells has been shown to be either a positive and negative indicator of patient outcome, it remains unclear if the reduced patient survival we observe could be solely explained by immune infiltrate [47]. Regardless, our results strengthen the potential of TAp63 as a biomarker of immune cell interactions and highlight relevance to recent check-point inhibitor therapeutic studies [48].

Although prime variant isoforms have previously been described [41], here we characterize two commonly expressed variants, *DNp63alphaP* and *DNp63betaP*, which are not currently part of the TP63 isoform definitions present in Refgene and Gencode. Since these isoforms together represent 39.9% of average *TP63* expression in the BLCA cohort and 37.0% of average *TP63* expression in the entire TCGA cancer cohort, these definitions should be considered when analyzing *TP63* isoform expression. Further exploration regarding this exon 8 variant and it's impact on the DNA-binding domain may help elucidate the importance of this region to overall TP63 function.

Overall, these results illustrate the importance of differential isoform expression to biology and patient outcomes. It is likely that

TP63 is not unique in its isoform diversity and that other genes may display isoform switching events which drive biological and clinical outcomes. Further investigation of isoform switching events may allow us to refine the bladder cancer molecular signatures and subtypes and to develop better prognostic tests.

Declaration of Competing Interest

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2019.11.022.

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