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Distinct TP63 isoform-driven transcriptional signatures predict tumor progression and clinical outcomes

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Abstract

TP63 is required to maintain stem cell pluripotency and suppresses the metastatic potential of cancer cells through multiple mechanisms. These functions are differentially regulated by individual isoforms, necessitating a deeper understanding of how the distinct transcriptional programs controlled by these isoforms affect cancer progression and outcomes. In this study, we conducted a pan-cancer analysis of The Cancer Genome Atlas (TCGA) to identify transcriptional networks regulated by TAp63 and ΔNp63 using transcriptomes derived from epidermal cells of TAp63−/− and ΔNp63−/− mice. Analysis of 17 cancer developmental and 27 cancer progression signatures revealed a consistent tumor suppressive pattern for TAp63. In contrast, we identified pleiotropic roles for ΔNp63 in tumor development and found that its regulation of Lef1 was crucial for its oncogenic role. ΔNp63 performed a distinctive role as suppressor of tumor progression by cooperating with TAp63 to modulate key biological pathways, principally cell cycle regulation, extra cellular matrix remodeling, epithelial-to-mesenchymal transition, and the enrichment of pluripotent stem cells. Importantly, these TAp63 and ΔNp63 signatures prognosticated progression and survival, even within specific stages, in bladder and renal carcinomas as well as low-grade gliomas. These data describe a novel approach for understanding transcriptional activities of TP63

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isoforms across a large number of cancer types, potentially enabling identification of patient subsets most likely to benefit from therapies predicated on manipulating specific TP63 isoforms.

Keywords

The Cancer Genome Atlas; TAp63; ΔNp63; pluripotency; stem cells; extracellular matrix; epithelial mesenchymal transition; survival; staging

INTRODUCTION

TP53, TP63 and *TP73* family of genes transcriptionally regulate genomic programs to induce apoptosis, cell cycle arrest, senescence, metabolic reprogramming and stem cell maintenance(1–8). Manipulating specific isoforms of Tp63 and Tp73 is an effective approach to induce regression in Tp53-deficient and mutant tumors(9). Although Tp63 and Tp73 roles in cancer have been described(4,5,7,9,10), the transcriptional roles of TP63 and TP73 in tumor development and progression have been perplexing due to their variable expression in tissues, their complex interaction with each other and with TP53, and the lack of adequate antibodies to distinguish between their isoforms(3).

TP63 is of particular interest due to its high expression in epithelial tissues(11,12), its clinical use as a diagnostic marker (13–15), and its role in stem cell maintenance, tumor suppression, and metastasis (3,6,8,12,16). Further, its two isoforms, TAp63 and ΔNp63, exert various modulatory roles in stem cell maintenance, cancer metastasis, and miRNA biogenesis(6–9). Yet, the broad tissue specific roles of the genes regulated by TAp63 and ΔNp63 in cancer development and progression remain to be deciphered and are important for the proper interpretation of its use as a clinical diagnostic marker.

In this study, we identified the connection between the activity of TP63 isoforms in stem cell maintenance and in regulatory networks that control cancer development and progression. By applying integrative model for *TAp63* and *ΔNp63* signatures from mice into The Cancer Genome Atlas (TCGA) derived signatures, we found that TAp63 transcriptional activity is lost in 13 cancer development signatures, indicative of a tumor suppressor pattern, while ΔNp63 activity is pleiotropic in cancer development. TAp63 and ΔNp63 exerted tumor suppressor activity in 4 cancers via modulation of stem cell, extracellular matrix (ECM), epithelial to mesenchymal transition (EMT) and cell cycle pathways. Interestingly, we found that the ability of ΔNp63 to transcriptionally regulate Lef1 dictates its tumor suppressor or oncogenic activity. These signatures are not only predictive of survival across stages, but can stratify patients within the same stage into different survival groups. These findings can be exploited by further classifying patients into groups that can benefit from TP63-isoform targeted therapy (9).

MATERIALS AND METHODS

Bioinformatics analysis

The primary and processed data for 9578 tumor and 730 normal samples were downloaded from The Cancer Genome Atlas website between July 2014 and February 2015. We aligned

all TCGA sequencing data to Genome Reference Consortium Human Reference 38 (hg38) in order to identify all genes and TP63 isoforms (α, β, γ for TAp63 and Δ*Np63*). TAp63 signatures were derived from epidermal cells P1 *TAp63*$^{-/-}$ and wildtype mice at embryonic day 18.5 (6). For ΔNp63 murine gene signature, we used previously published transcriptome profiles (8). For putative tumor suppressor role of the TAp63 or ΔNp63 we identified genes that were significantly changing and in the same direction in both TAp63$^{-/-}$ (or Δ*Np63*$^{-/-}$) and the corresponding tumor signature. For oncogenic roles, an inverse approach was taken. PCSS was identified separately for each gene signature and for each role (eg. tumor suppressor or oncogene) using association of gene scores and survival that conforms with the predicted function. The highest-ranking genes were then combined to establish a robust PCSS. Z-scores were used for all activity scoring then ranked by the highest and lowest 25th percentile quartiles. We utilized BP, KEGG and Reactome compendiums for pathway analysis. All studies were approved by the Institutional Review Board. All patient studies were conducted in accordance with the U.S. Common Rule ethical guidelines.

In vitro assays

MCF10CA1D.cl1 breast cancer cell linem Human lung squamous cell carcinoma HCC95 cell line, and Human kidney clear cell carcinoma Caki-1 cell line were cultured in corresponding media. For western blot analysis, blots were probed with anti-ΔNp63 (619002, Biolegend), Lef1 (2230S, Cell Signaling), Myc-tag (9B11, Cell Signaling) and Flag-tag (A8592, Sigma) overnight at 4°C. Actin (A5441, Sigma) was used as loading control. For real-time PCR analysis, total RNA was isolated using miRNeasy Mini Kit and cDNA synthesized using SuperScript First-Strand Synthesis System (Invitrogen) and followed by qRT-PCR ran in triplicates using Taqman Universal PCR Master Mix (Applied Biosystems). Taqman probes included: human Lef1 (Hs01547250_m1) and human GAPDH (Hs03929097_g1) as internal controls. C_t values were normalized to GAPDH. For siRNA assays, 40 nM of siLef1 (SASI_Hs02_00349169 and SASI_Hs01_00151600, Sigma) was transfected into 0.5×10^6 cells on 6-cm dishes using Lipofectamine RNAi Max (Invitrogen). 1μg of plasmids was transfected into 0.5×10^6 cells using X-tremeGENE HP DNA transfection reagent. pcDNA3.1-DYK-Lef1 and pcDNA3.1-DYK-empty plasmids were obtained from Genscript. ΔNp63α ORF was cloned into pcDNA3.1-Myc-empty plasmid. Cells were collected 48 hours post-transfection. Cells were stained with Click-iT EdU microplate assay (Invitrogen) and proliferation quantification was done using Celigo and Incucyte. For CFU assays, colonies were stained with 0.005% crystal violet for 1 hour.

RESULTS

Gene Set Enrichment Analysis (GSEA) and signature activity reveal pleiotropic roles of TAp63 and ΔNp63 in tumor development and progression

We generated TAp63 and ΔNp63 RNA signatures from primary *TAp63*$^{-/-}$ (Supplementary File 1) and Δ*Np63*$^{-/-}$ (8) mouse epidermal keratinocytes. We then analyzed TP63 isoform transcriptomes across TCGA tumors. Because stem cell maintenance in epithelial tissues is intimately tied with tumorigenesis, our approach integrates pathways in normal stem cell biology and cancer.

We derived tumor development signatures by comparing the expression profile of tumors to normal tissues. Tumor progression signatures were derived by comparing expression profile of high stage/grade tumors to low stage/grade tumors (Supplementary File 2). We characterized TP63 isoforms transcriptomes in cancer development and progression by employing the GSEA algorithm (17) in 17 tumor development and 27 tumor progression signatures. Identification of putative tumor suppressor and oncogene activities of TP63 isoforms in cancer development and progression was based on significance, directionality, and enrichment concordance. Specifically, when both upregulated and downregulated cancer signature genes are significantly enriched in concordant direction (positive normalized enrichment scores (NES) for upregulated genes, and negative NES for downregulated genes) in $TAp63^{-/-}$ or $\Delta Np63^{-/-}$ signatures, this suggests a concordance with the TAp63 or ΔNp63-deficient genotypes, thus a potential tumor suppressor activity. Alternatively, discordant enrichment in $TAp63^{-/-}$ or $\Delta Np63^{-/-}$ signatures suggests a predominant wildtype phenotype, and hence a potentially corresponding oncogenic activity. We then identified TP63 isoforms-derived signatures to measure corresponding transcriptional activity among patients and across stages, and to assess for survival (Fig. 1a).

TAp63 consistently exhibited a tumor suppressor pattern in 13 cancer development signatures, while ΔNp63 exhibited a pleiotropic role in tumorigenesis: oncogenic pattern in 7 signatures and a tumor suppressive pattern in 3 cancer development signatures ($q<0.0001$ for all) (Fig. 1b, Supplementary Table 1).

TAp63 and ΔNp63 signatures predict patient survival

To enable pan-cancer clinical assessment applications informed by TAp63 and ΔNp63 transcriptional activities, we refined TAp63 tumor suppressive and ΔNp63 oncogenic signatures (Supplementary File 3.1–3.2) based on a survival-predictive model dubbed Pan Cancer Survival Signature ($PCSS^{TAp63}$ and $PCSS^{\Delta Np63}$, respectively). $PCSS^{TAp63}$ activity significantly decreased across various Stages I to IV cancers including breast (BRCA), kidney clear cell (KIRC), and kidney papillary (KIRP), lung adenocarcinoma (LUAD), and lung squamous cell (LUSC) carcinoma (ANOVA<0.0001–0.032) exhibiting a tumor suppressor pattern (Fig. 1c). However, $PCSS^{\Delta Np63}$ activity increased significantly from Stage I to IV in BRCA and LUAD (ANOVA<0.0001) patterning an oncogenic role (Fig. 1d). Patients with increased activity of $PCSS^{TAp63}$ and $PCSS^{\Delta Np63}$ had significantly better and worse overall survival, respectively, within stages and across all patients ($p<0.0001–0.038$) (Fig. 1e–f). Corresponding enriched pathways are summarized in Supplementary Figure 1a–b and File 3.3–3.4.

ΔNp63 activates an oncogenic program through transcriptional regulation of its downstream target, *Lef1*

To explore the functional pleiotropic role of ΔNp63, we first manipulated ΔNp63 expression in breast and LUAD cell lines, where we found ΔNp63 to be oncogenic. Knockdown of ΔNp63 in MCF10-CA1D breast cancer cells, where ΔNp63 is overexpressed (Supplementary Fig. 2a), reduced proliferation and soft agar colony formation (Fig. 2a–d, and Supplementary Fig. 2b). In HCC95 LUAD cells, where ΔNp63 is overexpressed (Supplementary Fig. 2a), knockdown of ΔNp63 decreased soft agar colony formation but not

proliferation (Fig. 2e–h, and Supplementary Fig. 2b). To assess tumor suppressor activities of ΔNp63, we overexpressed ΔNp63α in Caki-1 human renal cancer cells (Supplementary Fig. 2b), where we found ΔNp63 as tumor suppressive (Fig. 1b) and while we did not observe significant differences in proliferation (Fig. 2i–j), the number of colonies decreased by 2-fold compared to control (Fig. 2k–l). These data further support that ΔNp63 plays a pleiotropic role depending on tissue of origin.

To identify the pleiotropic mechanism exerted by ΔNp63 in tumorigenesis, we overlapped previously published human ΔNp63 Chip-Seq data (18) with our TCGA derived signatures, which revealed enrichment for EMT genes. Next, we conducted hypergeometric analysis to identify transcription factors that regulate these EMT genes and overlap ΔNp63 keratinocyte signatures. We identified Lef1 targets (*INHBA, USP2, COL5A1, BMP1, PMEPA1*) to be highly significant (p=0.0009). To assess whether the oncogenic role of ΔNp63 functions through *Lef1*, we assessed *Lef1* mRNA expression in MCF10-CA1D and HCC95 cell lines after ΔNp63 knockdown. *Lef1* expression decreased (Fig. 3a–b) indicating that *Lef1* is downstream of ΔNp63. We then knocked down Lef1 in both MCF10-CA1D and HCC95 cell lines (Fig. 3c), and measured soft agar colony growth. Similarly to the results where ΔNp63 was knocked down, the Lef1 knockdown reduced MCF10-CA1D and HCC95 colony formation (Fig. 3d–g). In addition, overexpressing Lef1 in ΔNp63-knocked down cells (Supplementary Fig. 3a) significantly increased MCF10-CA1D and HCC95 colony formation (Fig. 3h–k), and MCF10-CA1D cell proliferation (Supplementary Fig. 3b–c), but not HCC95 proliferation (Supplementary Fig. 3d–e). Together, these data demonstrated that ΔNp63 functions as an oncogene via regulating *Lef1.* Importantly, the ΔNp63-overexpressing Caki-1 cells had increased Lef1 mRNA and protein expression (Supplementary Fig. 4a–b), which further confirmed that ΔNp63 regulates Lef1 expression. To assess whether the tumor suppressive role of ΔNp63 functions through *Lef1*, we overexpressed Lef1 in Caki-1 cells, and noted a non-statistically significant decrease in soft agar colony formation (Supplementary Fig. 4c–e). When Lef1 was knocked down in Caki-1 cells where we overexpressed ΔNp63 (Supplementary Fig. 4f), no significant difference was noted with respect to cell proliferation and colony formation (Supplementary Fig. 4g–j), suggesting the involvement of other factors in addition to Lef1 in the tumor suppressor roles of ΔNp63. Hence, ΔNp63 regulation of *Lef1* dictates its oncogenic roles in different tissues while the tumor suppressor pathways of ΔNp63 are still not identified.

TAp63- and ΔNp63-regulated transcriptional activities are lost in higher stages and can predict survival of BLCA, KIRC, KIRP and LGG

GSEA analysis revealed a suppressive role for both TAp63 and ΔNp63 in the progression of 4 tumors: bladder cancer (BLCA), KIRC, KIRP and low grade glioma (LGG) (Fig. 4a). To explore the role of TP63 isoforms in tumor progression, we first identified TAp63- and ΔNp63-regulated genes that were enriched in cancer progression signatures and the corresponding isoform signature, hereafter dubbed $TAp63^{BLCA}$, $\Delta Np63^{BLCA}$, $TAp63^{KIRC}$, $\Delta Np63^{KIRC}$, $TAp63^{KIRP}$, $\Delta Np63^{KIRP}$, $TAp63^{LGG}$ and $\Delta Np63^{LGG}$ (Supplementary File 4.1–4.2 and Supplementary Figure 5a–b).

We found that high grade/stage KIRC, KIRP and LGG tumors had significantly lower activities of their corresponding TAp63-driven signatures compared to low grade/stage tumors ($p<0.0001$) (Fig. 4b). KIRC ($p<0.0001$), KIRP ($p=0.023$) and LGG ($p<0.0001$) patients with higher corresponding TAp63 activity had significantly better survival than patients with lower corresponding TAp63 activity (Fig. 4c). Similarly, high grade/stage BLCA, KIRC, KIRP and LGG tumors had significantly lower activities of their corresponding ΔNp63-driven signatures compared to low grade/stage tumors ($p<0.0001$) (Fig. 4d), and KIRC ($p<0.0001$) and LGG ($p<0.0001$) patients with higher corresponding ΔNp63 activities had better survival (Fig. 4e). These data suggest that higher grade/stage tumors lose TAp63 and ΔNp63 activities when progressing. Association between activity and survival was also evident within the same grade/stage of tumors (Fig. 4f and 4g). This is of particular importance as it suggests that there is significant heterogeneity in the activity of ΔNp63 within high stages of KIRC, KIRP and LGG that may warrant different therapy. $TAp63^{BLCA}$ consisted of 1 gene only (*COL8A1*) and was not assessed.

We then used independent cohorts to validate our findings. With respect to TAp63, high grade (19) and stage (20) KIRC, high stage KIRP (21) and high grade LGG (22) tumors had significantly lower corresponding TAp63-signature activities (Fig. 5a and Supplementary Figure 6a). Also, KIRP patients (21) with higher $TAp63^{KIRP}$ activity had significantly better survival than KIRP patients with lower $TAp63^{KIRP}$ activity (Fig. 5b). Similarly, high stage BLCA (23), high grade (19) and stage (20) KIRC, high stage KIRP (21), and high grade LGG (22) patients had significantly lower corresponding ΔNp63-signature activities (Fig. 5c and Supplementary Figure 6b). These findings also translated into significantly better survival for BLCA and KIRP patients with higher corresponding ΔNp63-signature activities (Fig. 5d). These findings indicate that TAp63 and ΔNp63 derived signatures can predict survival across cancers and within specific stages in different cohorts and can identify patients with better survival among advanced tumors. Further, the large number of genes and pathways identified can be exploited in the clinic to identify therapies that could target these downstream effectors and can explain the heterogeneity in responding to certain treatments.

Patient prognosis correlates with expression profiles of TAp63 and Δ*N*p63 regulated signatures

We conducted unsupervised hierarchal clustering on BLCA, KIRC, KIRP and LGG patients with respect to corresponding TP63 isoform signatures. We identified multiple clusters (C) corresponding to TAp63 and ΔNp63 derived signatures (Fig. 6a–b). The corresponding activity and survival pattern was significantly different across the clusters identified in all cancers (Fig. 6c–d). Specifically, patients with the highest TAp63 and Δ*N*p63 activity had significantly better survival than the other clusters (Fig. 6e–f). These findings provide strong evidence of distinct TAp63 and Δ*N*p63 transcriptome profiles that identify distinct groups of patients within cancers and can predict their survival. This is of major significance given tumor heterogeneity and the difficulty of identifying patients with different prognosis within the same stage or group.

Cell cycle activity, pluripotency potential, EMT and ECM remodeling in high stage tumors modulated by TAp63 and ΔNp63 signatures

Cell cycle, sister chromatid/chromosome and organelle regulatory pathways were the most highly enriched pathways in TAp63 signatures (Supplementary Figure 7a and Supplementary File 4.3). For ΔNp63-derived signatures, there were 2 genes (*CTHRC1 and COL5A1* both are major ECM constituents) and 14 pathways that were common among $\Delta Np63^{BLCA}$, $\Delta Np63^{KIRC}$, $\Delta Np63^{KIRP}$ and $\Delta Np63^{LGG}$ (Supplementary File 4.4). The highest enrichments in ΔNp63 signatures were developmental and ECM remodeling pathways (Supplementary Figure 7b) which were of significant interest as these were indicative of stem cell pluripotency, EMT, and metastatic mechanisms. Because TAp63 and ΔNp63 were both acting as suppressors of tumor progression in the same cancers, we investigated whether these roles are exerted via common or distinct transcriptome networks. Interestingly, there were few overlapping genes regulated by corresponding TAp63 and ΔNp63 signatures in KIRC, BLCA, LGG and KIRP progression (Supplementary Figure 7c–f).

CTHRC1 and *COL5A1* are ΔNp63-regulated genes common to BLCA, KIRC, KIRP and LGG progression signatures. In addition to the ECM remodeling role of CTHRC1 and COL5A1, COL5A1 is also highly expressed in known stem cell signatures. Hence, we investigated whether the Δ*N*p63 activity of these two genes, hereafter dubbed $\Delta Np63^{CTHRC1/COL5A1}$, could predict staging and survival. In addition to the ECM remodeling role of CTHRC1 and COL5A1, COL5A1 is also highly expressed in known stem cell signatures (24). We found significantly lower $\Delta Np63^{CTHRC1/COL5A1}$ activity in the higher grade/stage of BLCA, KIRC, KIRP and LGG ($p<0.0001$ for all) (Supplementary Figure 8a). Further, KIRC ($p=0.0009$), KIRP (0.0086) and LGG ($p=0.0241$) patients with higher activity of $\Delta Np63^{CTHRC1/COL5A1}$ had significantly better survival than patients with lower activity (Supplementary Figure 8b). We then assessed whether single gene expression profile of COL5A1 and CTHRC1 correlates with survival in BLCA, KIRC, KIRP and LGG tumors. Interestingly, patients with higher expression of COL5A1 or CTHRC1 almost consistently had worst prognosis ($p< 0.05$) (Supplementary Figure 9a–b). These findings show that a Δ*N*p63-regulated signature driven by 2 genes that are known to modulate ECM and stem cell pluripotency can predict progression and survival of patients.

We then investigated whether previously published stem cell signatures were enriched in high grade/stage tumors and $\Delta Np63^{-/-}$ keratinocytes. GSEA analyses identified a significant enrichment of cancer invasive and primary stem cells(25,26), mouse and human embryonic stem cell signatures(24,27), and Nanog, Oct4 and Sox2 (NOS) targets(27) in high grade/stage tumors and $\Delta Np63^{-/-}$ epidermal cells signatures (Fig. 7a). Similarly, there was significant enrichment of primary tumor and metastatic ECM signatures(28) in high stage/grade and $\Delta Np63^{-/-}$ epidermal cell signatures (Fig. 7b). We found a significant enrichment of EMT in $\Delta Np63^{-/-}$ keratinocytes and progression signatures of BLCA, KIRC and KIRP (Fig. 7c), which is interesting given the role of TP63 isoforms in stem cell maintenance.

To identify specific ΔNp63-regulated genes that promote stem cell pluripotency in cancer progression, we compared $\Delta Np63^{BLCA}$, $\Delta Np63^{KIRP}$, $\Delta Np63^{KIRC}$ and $\Delta Np63^{LGG}$ signatures to the known stem cell signatures that were enriched in our progression cancer model shown

in Figure 7a(24–27). We found 11, 17, 43, and 23 ΔNp63-regulated stem cell pluripotency genes to be upregulated in Δ*N*p63^{BLCA}, Δ*N*p63^{KIRP}, ΔNp63^{KIRC} and ΔNp63^{LGG} and at least one previously published stem cell signature, respectively (Fig. 7d). We also identified overlapping genes between the different signatures (Fig. 7d). Interestingly, one stem cell pluripotency gene *COL5A1* is regulated by Δ*N*p63, common to all of ΔNp63^{BLCA}, Δ*N*p63^{KIRP}, ΔNp63^{KIRC} and ΔNp63^{LGG} signatures, and is also a major ECM remodeling factor; we showed it to be a major predictor of staging and survival when combined with CTHRC1. To note, the role of ΔNp63 in pluripotency in the different tumors is exerted via a large network of genes as evident from the little overlap among the stem cell pluripotency genes of ΔNp63^{BLCA}, ΔNp63^{KIRP}, ΔNp63^{KIRC} and ΔNp63^{LGG}. The suppressive activity of ΔNp63 in tumor progression is consistent with previous reports indicating that ΔNp63 serves to promote differentiation of epithelial tissues and that loss of ΔNp63 leads to pluripotency(8,29). The progression of these tumors entails loss of TAp63 and ΔNp63 activities, which leads to increased stem cell pluripotency, ECM remodeling, and EMT via ΔNp63, and disruption of normal cell cycle regulation by TAp63, which ultimately leads to progression and metastasis (Fig. 7e). These findings identify patients within different stages that could benefit from treatments that specifically target these pathways.

DISCUSSION

In this study, we conducted a pan-cancer analysis using TCGA and informed by unique transcriptome profiles of TP63 isoforms, TAp63 and ΔNp63. These unique transcriptome profiles were generated from *TAp63*$^{-/-}$ and *ΔNp63*$^{-/-}$ epidermal cells exhibiting profound phenotypes in epithelial stem cell maintenance and renewal, and thus, allowed for an assessment of p63 isoform-driven stem cell biology in cancer development and progression (Fig 1a)(6,8,12,29). TP63 alterations have been previously implicated in many cancer(30–33) and is frequently used as a diagnostic marker(34,35). Our mechanistic understanding and the clinical significance of the underlying mechanisms of TP63 isoforms in tumor suppression and oncogenesis are still poorly defined. Here, we found that while TAp63 activity follows a suppressive pattern in tumor development and progression, the activity of ΔNp63 was pleiotropic in cancer development. Importantly, we identified Lef1 as a major downstream target of ΔNp63 that mediates the switch between the tumor suppressive and oncogenic activities of ΔNp63 in a tissue specific manner. The ΔNp63-Lef1 axis seems to be crucial for its role as an oncogene while the underlying factors that dictate its role as a tumor suppressor gene remain to be elucidated. Importantly, we found no evidence that TAp63 regulates Lef1 neither in our genomic analysis nor via chromatin immunoprecipitation assay (ChiP) (Napoli and Flores, unpublished data).

This study constitutes the largest analysis from TCGA platform in terms of number of cancers analyzed and in light of important epidermal stem cell and cancer regulators, TAp63 and ΔNp63, and their transcriptional networks. We unveiled TAp63 and ΔNp63 driven signatures that can predict tumor development, progression, and patient survival. Although ΔNp63 was previously thought to act primarily an oncogene, Δ*Np63* has more recently been found to be lost during tumor progression and proposed to be able to suppress metastasis(36,37). Our results demonstrate that ΔNp63 plays a dual role as a tumor suppressor or oncogene in cancer development. Its regulation of Lef1 is crucial for its

oncogenic roles. In addition, ΔNp63 acts as a suppressor of tumor progression. Using integrative bioinformatics analyses, we identified TAp63-driven and ΔNp63-driven transcriptional networks that can predict survival and tumor progression within and across cancer stages. Our findings identify patient populations that may benefit from targeting TAp63 and ΔNp63 pathways in order to improve their survival. Further, the TAp63 and ΔNp63 signatures establish an expression profile that can identify different clusters of patients with differential potential to express stem cell pluripotency, ECM and EMT markers that can confer resistance to treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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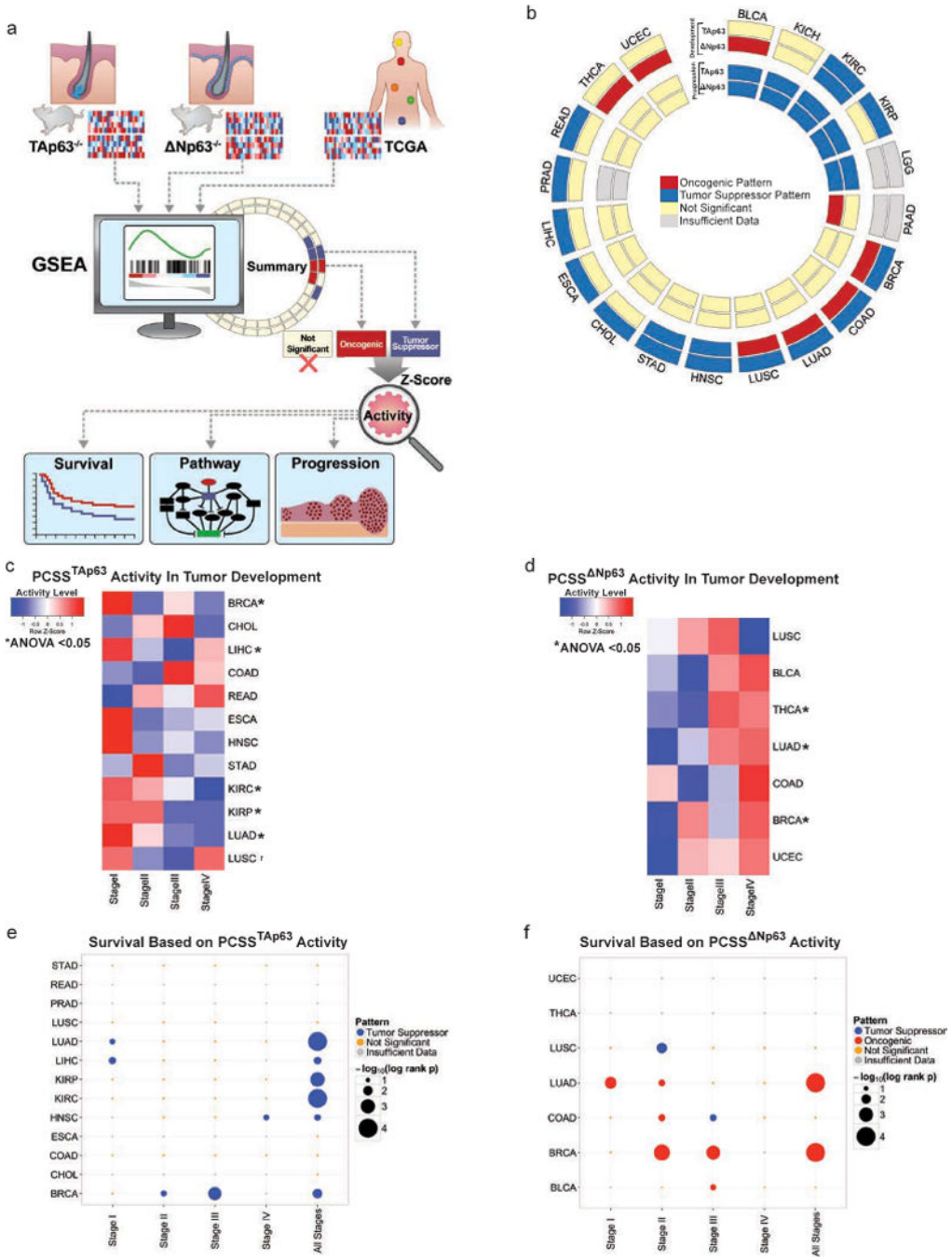

Figure 1. TAp63 and ΔNp63 have tissue-dependent activity in tumor development

a, Working model for identification of transcriptional networks enriched in TCGA tumors **b,** Summary of GSEA concordant findings across in cancer development and cancer progression signatures. **c,** Heatmap of the activity of $PCSS^{TAp63}$ across different stages of tumorigenesis. **d,** Heatmap of the activity of $PCSS^{\Delta Np63}$ across different stages of tumorigenesis. **e,** Dot plot of the survival analysis of different tumors with respect to $PCSS^{TAp63}$ activity. **f,** Dot plot of the survival analysis of different tumors with respect to $PCSS^{\Delta Np63}$ activity.

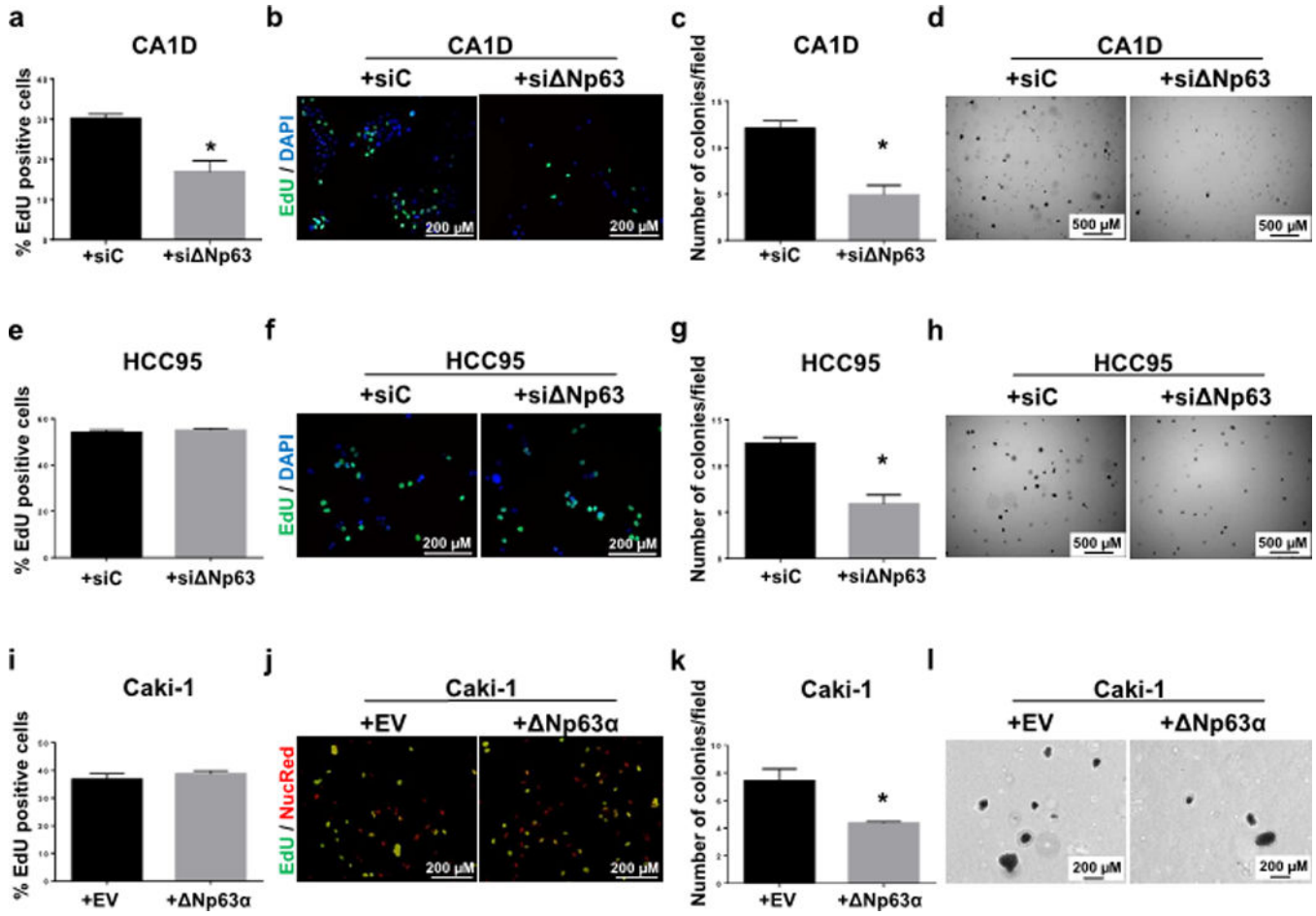

Figure 2. ΔNp63 plays a pleiotropic role as a tumor suppressor or oncogene

a–b, e–f,i–j, Quantification and fluorescence representative images of EdU (green) incorporation MCF10-CA1D (a–b), HCC95 (e–f) and Caki-1 (i–j) cell lines. **c–d, g–h, k–l,** Quantification and bright field representative images of anchorage-independent colony formation of MCF10-CA1D (c–d), HCC95 (g–h) and Caki-1 (k–l) cell lines in soft agar assay (per 10× field). Data are mean ± SD, n = 3. Asterisks indicate statistical significance, $p < 0.005$ versus siRNA scramble control (siC) or empty vector (EV), two-tailed t test.

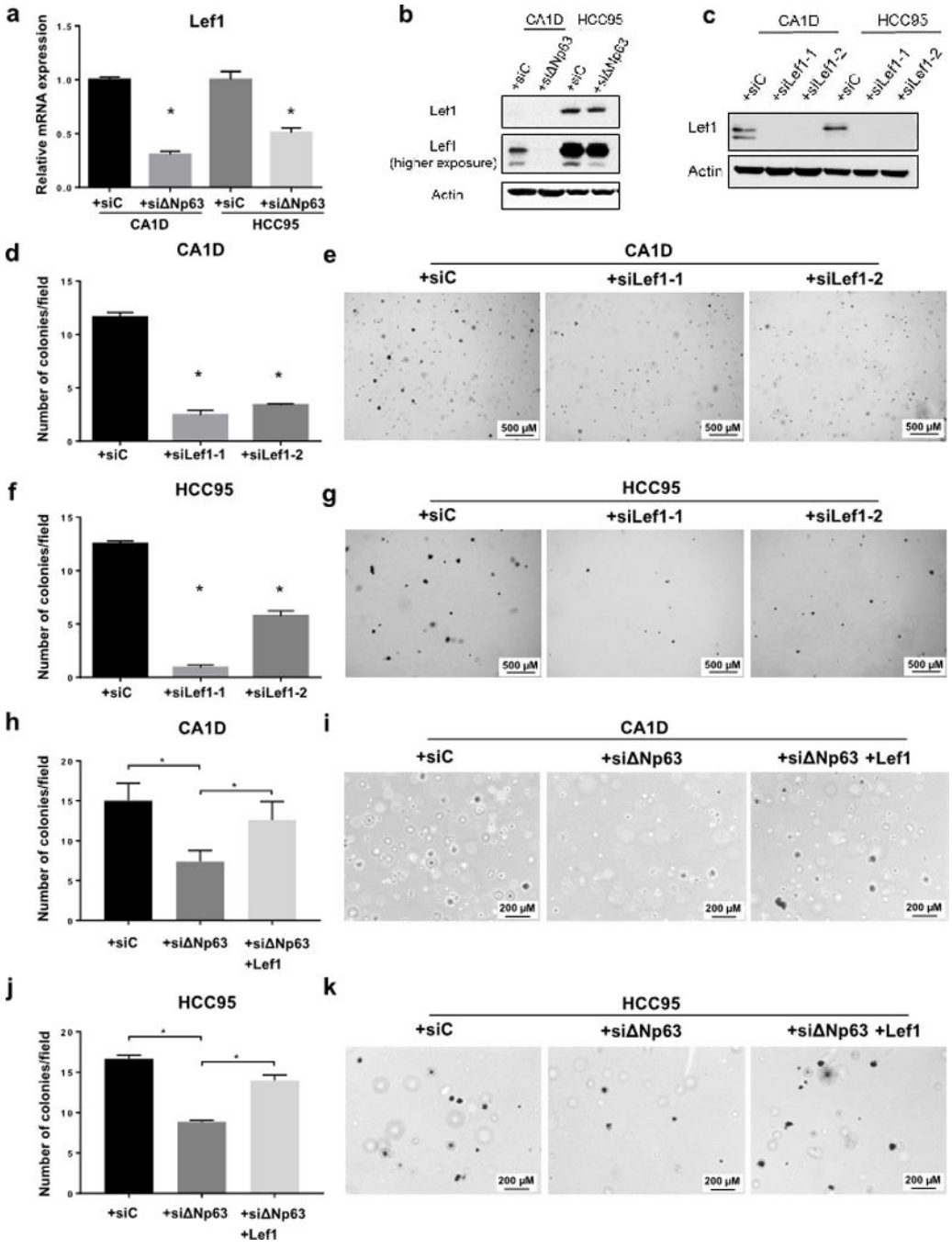

Figure 3. Lef1 is downstream of ΔNp63 and dictates the function of ΔNp63 as a tumor suppressor or oncogene

a, qRT-PCR for *Lef1* in MCF10-CA1D and HCC95 cells expressing either siC or siΔNp63. Asterisks indicate statistical significance, $p < 0.005$ versus siC, two-tailed t test. **b–c,** Western blot analysis for: Lef1 expression in MCF10-CA1D and HCC95 cells expressing either siC or siΔNp63 (**b**) or in MCF10-CA1D and HCC95 cells treated with siC, siLef1-1 or siLef1-2 (**c**). Actin was used as a loading control. **d–k,** Quantification and bright field representative images of anchorage-independent colony formation of indicated cell lines in

soft agar assay (per 10× field). Data are mean ± SD, n = 3. Asterisks indicate statistical significance, $p < 0.005$ versus siC or siΔNp63, two-tailed t test.

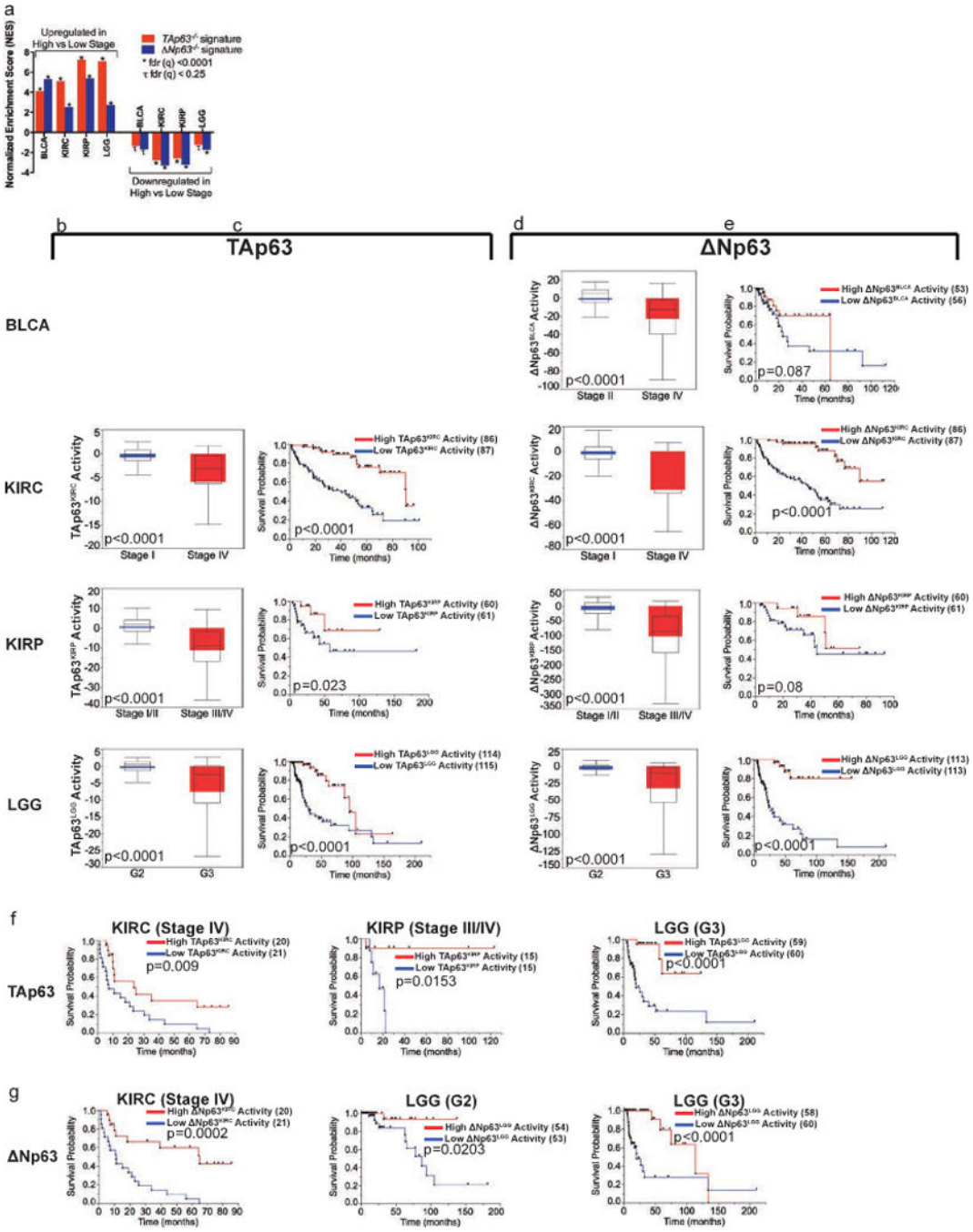

Figure 4. Tumor suppressor activity of TAp63 and ΔNp63 in cancer progression

a, Normalized enrichment score (NES) of upregulated and downregulated cancer progression signatures in *TAp63*$^{-/-}$ and ΔNp63$^{-/-}$ signatures. **b,** Activity score of TAp63^{KIRC}, TAp63^{KIRP} and TAp63^{LGG} in corresponding cancer patients at high and low grade/stages. **c,** Kaplan-Meier survival of kidney clear cell (KIRC), kidney papillary (KIRP) and low grade glioma (LGG) patients with respect to the corresponding TAp63 signature activity. **d,** Activity score of ΔNp63^{BLCA}, ΔNp63^{KIRC}, ΔNp63^{KIRP} and ΔNp63^{LGG} in corresponding cancer patients at high and low grade/stages. **e,** Kaplan-Meier survival of

bladder cancer (BLCA), KIRC, KIRP and LGG patients with respect to the corresponding ΔNp63 signature activity. **f,** Kaplan-Meier survival within Stage IV KIRC, Stage III/IV KIRP and Grade (G) 3 LGG with respect to $TAp63^{KIRC}$, $TAp63^{KIRP}$ and $TAp63^{LGG}$ activities. **g,** Kaplan-Meier survival within Stage IV KIRC, and G2 and G3 LGG patients with respect to $\Delta Np63^{KIRC}$ and $\Delta Np63^{LGG}$ activities, respectively. Number of cases in each survival group is listed between parenthesis where indicated.

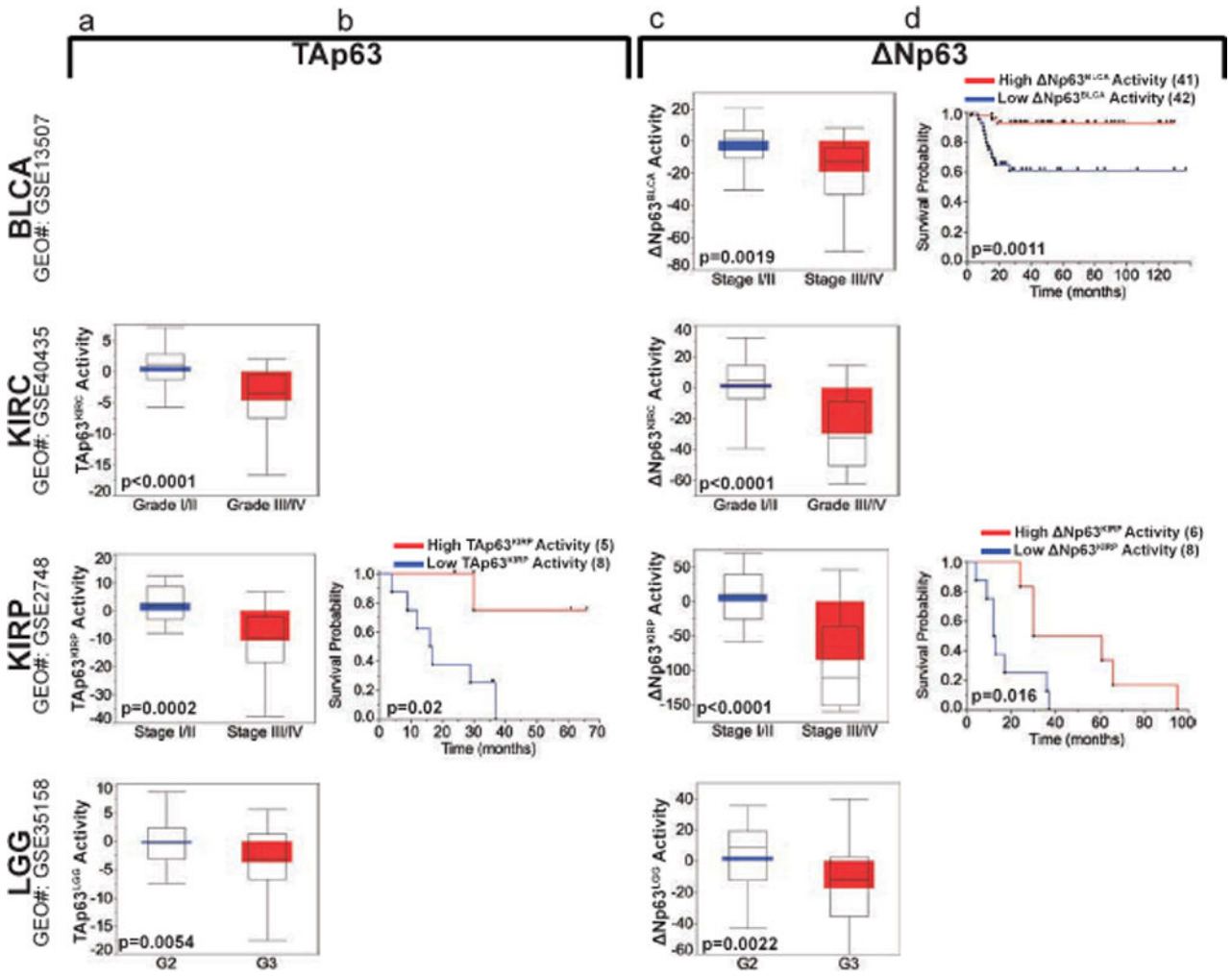

Figure 5. Validation of TAp63 and ΔNp63 in independent cohorts

a, Validation of corresponding TAp63 activities in independent cohorts of kidney clear cell (KIRC), kidney papillary (KIRP) and low grade glioma (LGG). **b,** Survival of KIRP in independent cohorts with respect to $TAp63^{KIRP}$ activity. **c,** Validation of corresponding ΔNp63 activities in independent cohorts of bladder cancer (BLCA), KIRC, KIRP and LGG. **d,** Survival of BLCA and KIRP in independent cohorts with respect to $\Delta Np63^{BLCA}$ and $\Delta Np63^{KIRP}$ activities, respectively. Number of cases in each group is listed between parenthesis where indicated.

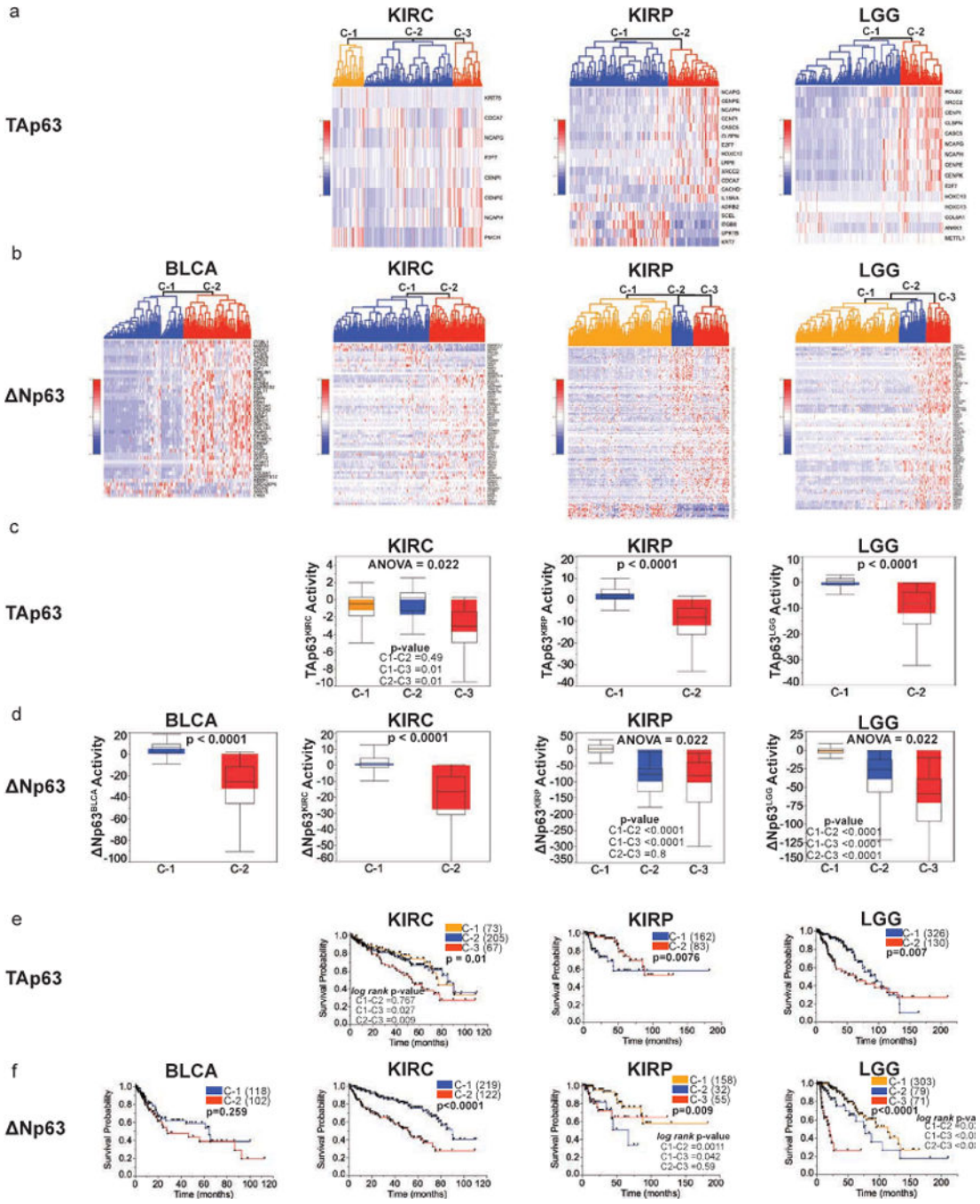

Figure 6. Expression profiles of TAp63 and ΔNp63 regulated signatures in identify different groups of BLCA, KIRC, KIRP and LGG patients with different prognosis

a–b, Unsupervised hierarchal clustering of bladder cancer (BLCA), kidney clear cell (KIRC), kidney papillary (KIRP) and low grade glioma (LGG) patients based on expression profile of corresponding (a) TAp63 and (b) ΔNp63 signatures. **c–d,** Activity score of (c) $TAp63^{KIRC}$, $TAp63^{KIRP}$ and $TAp63^{LGG}$ and (d) $\Delta Np63^{BLCA}$, $\Delta Np63^{KIRC}$, $\Delta Np63^{KIRP}$ and $\Delta Np63^{LGG}$ in corresponding cancer patients of different clusters. **e–f,** Kaplan-Meier survival of different clusters of BLCA, KIRC, KIRP and LGG patients with respect to corresponding

(e) TAp63 and (f) ΔNp63 expression profile. Number of cases in each survival group is listed between parenthesis where indicated.

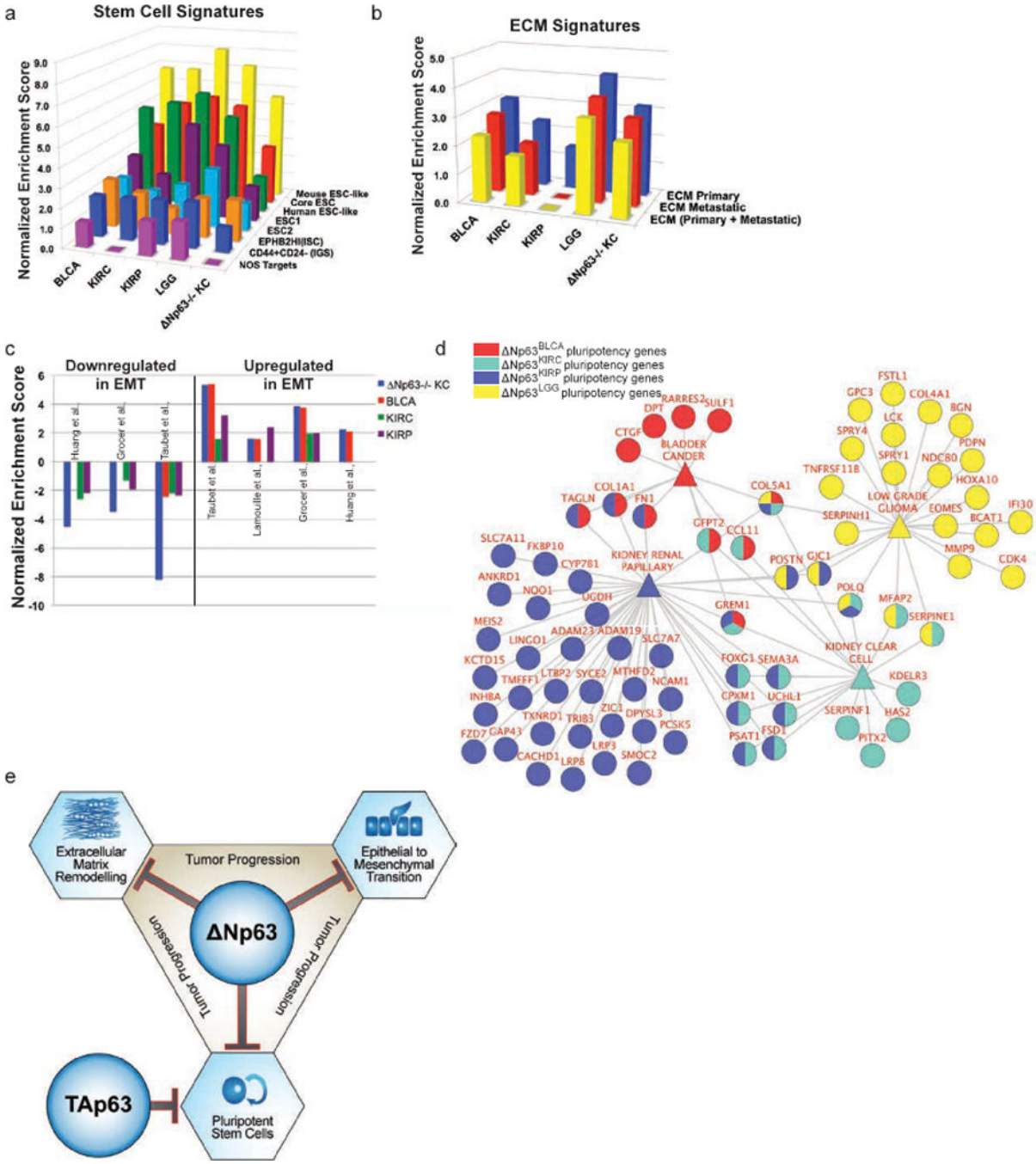

Figure 7. Enrichment of pluripotency, ECM and EMT in ΔNp63 driven progression signatures, and cooperation between TAp63 and ΔNp63 to suppress progression

a–c, Normalized enrichment scores (NES) of bladder cancer (BLCA), kidney clear cell (KIRC), kidney papillary (KIRP) and low grade glioma (LGG) progression and *ΔNp3*$^{-/-}$ signatures in (a) known stem cells and pluripotency, (b) ECM and (c) EMT signatures. **d,** ClueGO diagram representing enrichment for pluripotency genes in ΔNp63 driven signatures of BLCA, KIRC, KIRP and LGG. **e,** Suggested model of cooperativity between

TAp63 and ΔNp63 to suppress tumor progression via modulation of EMT, ECM, pluripotency and cell cycle activity.