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Metabolic Reprogramming of Stromal Fibroblasts through p62-mTORC1 Signaling Promotes Inflammation and Tumorigenesis

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SUMMARY

The tumor microenvironment plays a critical role in cancer progression, but the precise mechanisms by which stromal cells influence the epithelium are poorly understood. Here we show that p62 levels were reduced in the stroma of several tumors and that its loss in the tumor microenvironment or stromal fibroblasts resulted in increased tumorigenesis of epithelial prostate cancer cells. The mechanism involves the regulation of cellular redox through an mTORC1/c-Myc pathway of stromal glucose and amino acid metabolism, resulting in increased stromal IL-6 production, which is required for tumor promotion in the epithelial compartment. Thus, p62 is an anti-inflammatory tumor suppressor that acts through the modulation of metabolism in the tumor stroma.

INTRODUCTION

Primary tumors are initiated as a result of the stepwise acquisition of genetic alterations within the epithelial compartment (Shen and Abate-Shen, 2010). However, increasing evidence supports the notion that the tumor microenvironment also plays a critical role in cancer progression in many types of neoplasias, including prostate cancer (PCa), although relatively little is known about the signaling pathways that mediate communication between the stromal and epithelial compartments (Ammirante et al., 2010; Erez et al., 2010; Santos et al., 2009; Trimboli et al., 2009). Inflammation and metabolism are two critical factors contributing to the protumorigenic properties of the stroma (DeBerardinis and Thompson, 2012; Grivennikov et al., 2010; Hanahan and Coussens, 2012; Metallo and Vander Heiden, 2013; Vander Heiden, 2013). Although not totally understood, some evidence suggests that the metabolic state of the tumor stroma can decisively influence the tumorigenic potential of the tumor epithelial compartment (Lisanti et al., 2013). Here we have addressed this fundamental biological question in the context of p62 deficiency in the nonepithelial tumor compartment. Our laboratory initially identified p62, also known as sequestosome-1, as a scaffold protein for the atypical protein kinase C isozymes and later implicated p62 in other cell stress responses (Diaz-Meco and Moscat, 2012; Moscat and Diaz-Meco, 2012; Moscat et al., 2007; Sanchez et al., 1998). p62 binds Raptor, a key component of the mTOR-orchestrated nutrient-sensing complex and an important activator of anabolic pathways that are instrumental in metabolic reprogramming during cell transformation (Duran et al., 2011; Moscat and Diaz-Meco, 2011). Nonetheless, nothing is known about the signaling cascades that p62 regulates in stromal cells or to

Significance

Inappropriate activation of the stroma as a consequence of the tumorigenic process can potentiate the growth and transformation of epithelial tumor cells, thus facilitating the progression of cancers toward more malignant stages. Using prostate cancer as a model system, we show that the loss of the signaling adaptor p62 in stromal cells triggers an inflammatory response that leads to activation of cancer-associated fibroblasts that enhances tumorigenesis in vitro and in vivo. Deficiency in p62 results in reduced mTORC1 activity and deregulation of metabolic pathways controlling inflammation. Because the stroma is increasingly recognized as a potential source of therapeutic targets, this study suggests that targeting stromal metabolic reprogramming can decisively influence the tumorigenic potential of the tumor epithelial compartment.

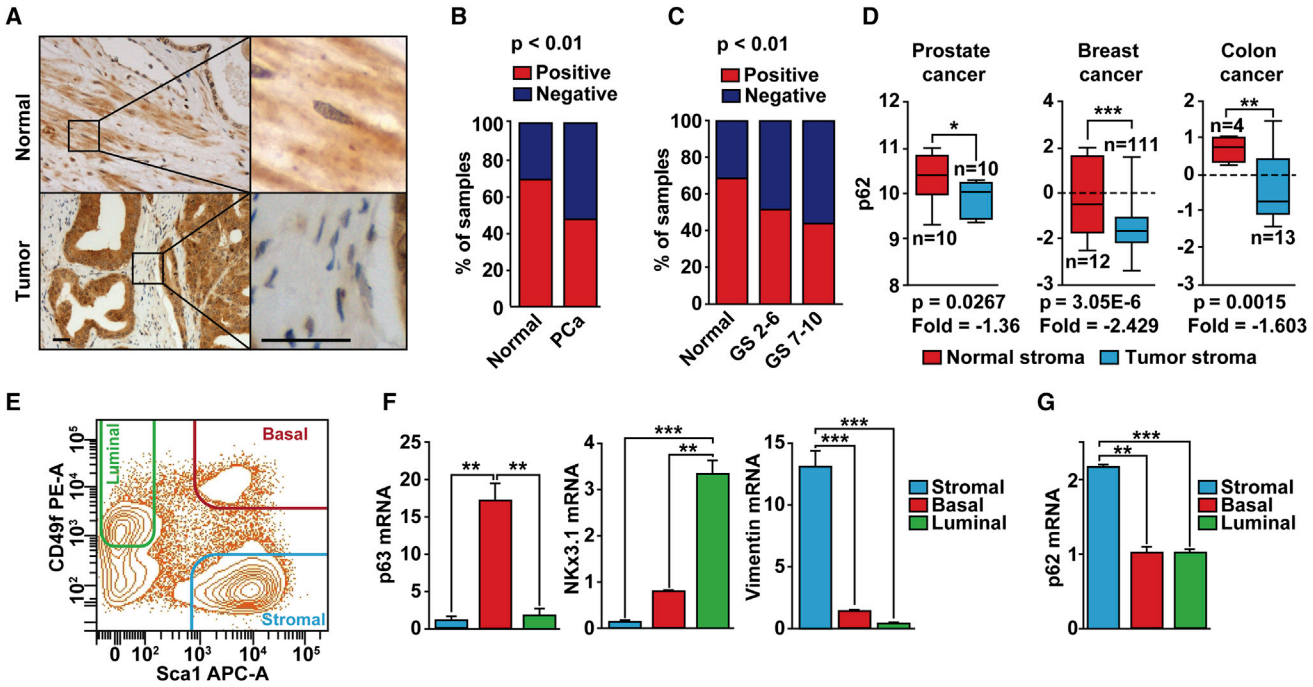

Figure 1. p62 Levels Are Reduced in the Stroma of Human Prostate Tumors

(A) Representative examples of p62 staining of normal and primary prostate cancer (tumor) samples. The scale bars represent 25 μm.

(B) Quantification of p62 staining in the stroma of primary PCa tumors compared with normal; n = 22 (normal), n = 202 (PCa). Fisher's exact test, $p < 0.01$.

(C) p62 levels are reduced upon PCa progression; n = 22 (normal), n = 70 (GS 2–6), n = 132 (GS 7–10). Chi-square test, $p < 0.01$.

(D) p62 mRNA levels in stroma of human cancer samples. Data were collected from public data sets of gene expression in the tumor stroma of several human cancers: GSE34312 (prostate cancer), GSE9014 (breast cancer), and GSE35602 (colon cancer).

(E) FACS-sorted adult murine prostate cell lineages. Prostate basal, luminal, and stromal cells are $Lin^{-}Sca\text{-}1^{+}CD49f^{Hi}$, $Lin^{-}Sca\text{-}1^{-}CD49f^{Low}$, and $Lin^{-}Sca\text{-}1^{+}CD49f^{-}$, respectively.

(F) RT-PCR of specific markers for each prostate cell population (n = 3): p63 (basal), Nkx3.1 (luminal), and vimentin (stromal).

(G) RT-PCR for p62 in prostate cell populations (n = 3).

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Results are presented as mean ± SEM. See also Figure S1.

what extent these pathways influence the epithelial-stromal interaction in the tumor microenvironment. Cancer-associated fibroblasts (CAFs) have been proposed to be key mediators of the crosstalk between malignant tumor cells and their microenvironment (Barron and Rowley, 2012; Franco and Hayward, 2012). CAFs and the complex set of signaling molecules they secrete generate an environment conducive to inflammation, and this in turn maintains the protumorigenic status of the stromal cells. Among these proteins, interleukin-1β (IL-1β), interleukin-8, and interleukin-6 (IL-6) have been implicated as part of the proinflammatory signature of the PCa stroma (Erez et al., 2010; Franco and Hayward, 2012; Schauer et al., 2008). Furthermore, IL-6 has received increasing attention as a key proinflammatory and protumorigenic molecule in many types of cancer, including PCa (Azevedo et al., 2011; De Marzo et al., 2007; Guo et al., 2012; Schafer and Brugge, 2007). Here we address the role of p62 in the stroma in the control of the inflammatory environment in PCa.

RESULTS

p62 Expression Levels in the Tumor Microenvironment

The initial evidence suggesting that p62 plays a role in the regulation of the tumor microenvironment in PCa came from the histological analysis of a tissue panel comprising 202 primary human PCa tumors, 8 metastases, and 22 adjacent normal prostate tissue samples. This study revealed that p62 was expressed in the prostate epithelium and also in the stroma (Figure 1A). p62 protein levels were downregulated in the stroma of human primary PCa tumors compared with the stroma of normal samples (Figures 1A and 1B). Furthermore, when the tumor samples were grouped on the basis of low Gleason score (GS) (2–6) or high GS (7–10), p62 levels in the stroma were significantly reduced upon progression to the most aggressive stage (Figure 1C). p62 was also overexpressed in the epithelial compartment of the PCa human samples (Figure 1A; Figures S1A and S1B available online). This is consistent with previous observations suggesting that p62 is upregulated in many cancers, including lung cancer (Duran et al., 2008; Inoue et al., 2012), liver cancer (Inami et al., 2011), glioblastoma (Galavotti et al., 2013), breast cancer (Rolland et al., 2007; Thompson et al., 2003), and kidney cancer (Li et al., 2013). However, because those studies did not report on expression in the stromal component, it is not clear whether p62 was downregulated in the stroma in those samples, as we have shown in the samples analyzed here. Moreover, bioinformatics analysis of public data sets of stromal gene expression also demonstrated that p62 was significantly downregulated in the tumor stroma, compared

with normal stroma, in several types of cancers, including prostate, breast, and colon cancers (Figure 1D). In addition, fluorescence-activated cell sorting (FACS) analysis of adult mouse prostates showed that p62 is more highly expressed in cells of the stroma than in those of basal or luminal lineages (Figures 1E–1G). Quantitative RT-PCR analyses of the sorted prostate cell populations showed that transcripts for the basal marker p63, the luminal cell marker Nkx3.1, and the stroma marker vimentin were enriched in their corresponding cell populations, demonstrating successful cell fractionation (Figures 1E and 1F). Of note, p62 expression was highly enriched in the stromal compartment compared with the other two cell populations (Figure 1G). These results suggest that p62 could exert its effect as a tumor suppressor in the tumor microenvironment, likely in the stroma.

p62 Is a Suppressor of Inflammation and the CAF Phenotype in the Tumor Microenvironment

To test whether p62 deficiency in the tumor microenvironment is relevant to the transforming properties of epithelial cells, we performed orthotopic injections of syngeneic murine PCa cells (TRAMP-C2Re3) (Olson et al., 2006) into the prostates of wild-type (WT) and p62 knockout (KO) mice and then assessed tumor growth. The resulting tumors were bigger in the prostates of p62 KO mice than in those of WT mice (Figures 2A–2C), supporting the notion that a loss of p62 in the tumor microenvironment promotes PCa growth. We next carried out transcriptomic profiling of the orthotopic tumors in the WT and p62 KO mice. NextBio analysis revealed important correlations between genes upregulated in the p62 KO orthotopic tumors with a gene signature in the category of "response to wounding" (Figure S2A). In addition, gene set enrichment analysis (GSEA) also identified "response to wounding" as significantly enriched of the gene ontology (GO) biological-process categories (Figure 2D; Figures S2B and S2C) and "stromal stimulation" in the C2 curated gene set library (Figures S2D and S2E). Because CAFs acquire an "activated phenotype" during tumor progression that resembles that of fibroblasts during the wound-healing repair process, these results suggested that the p62 KO stroma is likewise activated (Barron and Rowley, 2012; Bissell and Radisky, 2001; Franco and Hayward, 2012; Schäfer and Werner, 2008) and has a more CAF-like phenotype than the WT stroma. In support of this notion, we observed an increase in the expression of α smooth muscle actin (α-SMA) in sections from orthotopic tumors in p62 KO mice compared to WT controls (Figure 2E), as well as an increase in transforming growth factor β (TGF-β) transcripts as determined by RT-PCR in the same samples (Figure 2F). TGF-β and α-SMA are two bona fide markers of the CAF phenotype (Barron and Rowley, 2012; Franco and Hayward, 2012). Consistent with this, Ingenuity Pathway Analysis identified TGF-β1 as a predicted upstream regulator in the p62 KO orthotopic tumors ($p = 1.47 \times 10^{-7}$, activation Z score = 3.890). To determine the potential cell-autonomous effect of p62 in this important function, we used FACS to isolate prostate stromal cells from mice of both genotypes, as described in Figures 1E and 1F. Interestingly, we found that p62-deficient stromal cells also showed characteristics of CAFs, as determined by increased expression levels of α-SMA, TGF-β, and vimentin (Figure 2G). To facilitate subsequent studies, we generated prostate fibroblasts from WT and p62 KO mice and determined their "CAF activation" state. In these cells, the loss of p62 resulted in increased CAF transcript markers (Figure 2H), as well as in the secretion of TGF-β, as determined by ELISA (Figure 2I). This is important because TGF-β is essential for the acquisition and maintenance of the CAF/myofibroblast phenotype (Kojima et al., 2010; Ostman and Augsten, 2009). Therefore, p62 loss modifies the stroma by inducing a CAF phenotype, which in turn drives tumor progression.

Further bioinformatics GSEA revealed a hyperinflammatory phenotype in the p62 KO orthotopic tumors. That is, we found "humoral immune response" and "inflammatory response" as second GO categories enriched in the p62 KO transcriptome profile (Figure 2J; Figures S2F and S2G). RT-PCR analysis of the tumors from p62 KO mice showed increases in the transcripts of inflammatory cytokines such as IL-6, IL-1β, and keratinocyte chemoattractant (Figure 2K), as well as in the secretion of IL-6 as determined by ELISA (Figure 2L). We hypothesized that IL-6 could be an important mediator of the stromal p62-dependent signals that influence PCa progression in the epithelium. To test this possibility, we carried out an orthotopic injection experiment using p62/IL-6 double-KO (DKO) mice as hosts. Notably, the increased tumor growth observed in p62 KO mice was completely reversed in the DKO mice (Figures 2M and 2N), demonstrating that p62 plays a tumor-suppressive role in the tumor microenvironment during PCa progression by inhibiting CAF activation and blocking inflammation.

p62 in Stromal Fibroblasts Regulates an IL-6/TGF-β Cascade Essential for Tumor Invasion

We next set up a 3D organotypic culture model that recapitulates, in a genetically accessible system, the tumor microenvironment and its interactions with the tumor epithelial cell, closely mimicking the physiological situation and the cellular architecture (Gaggioli et al., 2007; Kim et al., 2013; Nyström et al., 2005; Ridky et al., 2010). Because our genome-wide transcriptomic analysis suggested that the loss of p62 in the tumor microenvironment is associated with a CAF-like signature, and because fibroblasts are a critical component of the stroma, we next tested whether p62 KO prostate fibroblasts were able to recapitulate the in vivo phenotype in 3D organotypic cultures. To do this, we cocultured in this organotypic system prostate fibroblasts from p62 KO and WT mice with TRAMP-C2Re3 PCa cells (Figure 3A). Importantly, p62 KO prostate fibroblasts (versus WT counterparts) enhanced the invasiveness and proliferation index of PCa epithelial tumor cells (Figures 3B–3D). Similar results were obtained with other PCa cell lines, such as mouse Myc-CaP (Figure S3A), or human PC3 cells (Figure S3B). Mouse fibroblasts from p62 KO mice also enhanced the invasiveness and proliferation index of human normal prostate epithelial cells compared with similar organotypic cultures with WT fibroblasts (Figure S3C). Altogether, this indicates that p62 deficiency in the stromal fibroblasts has a pivotal role in mediating cancer cell proliferation and invasion.

To follow up on our findings that IL-6 levels were increased in orthotopically injected tissues and that increased IL-6 expression was associated with enhanced tumorigenicity in vivo (Figures 2K–2N), we further investigated the role of this cytokine in the protumorigenic microenvironment created by p62 deficiency

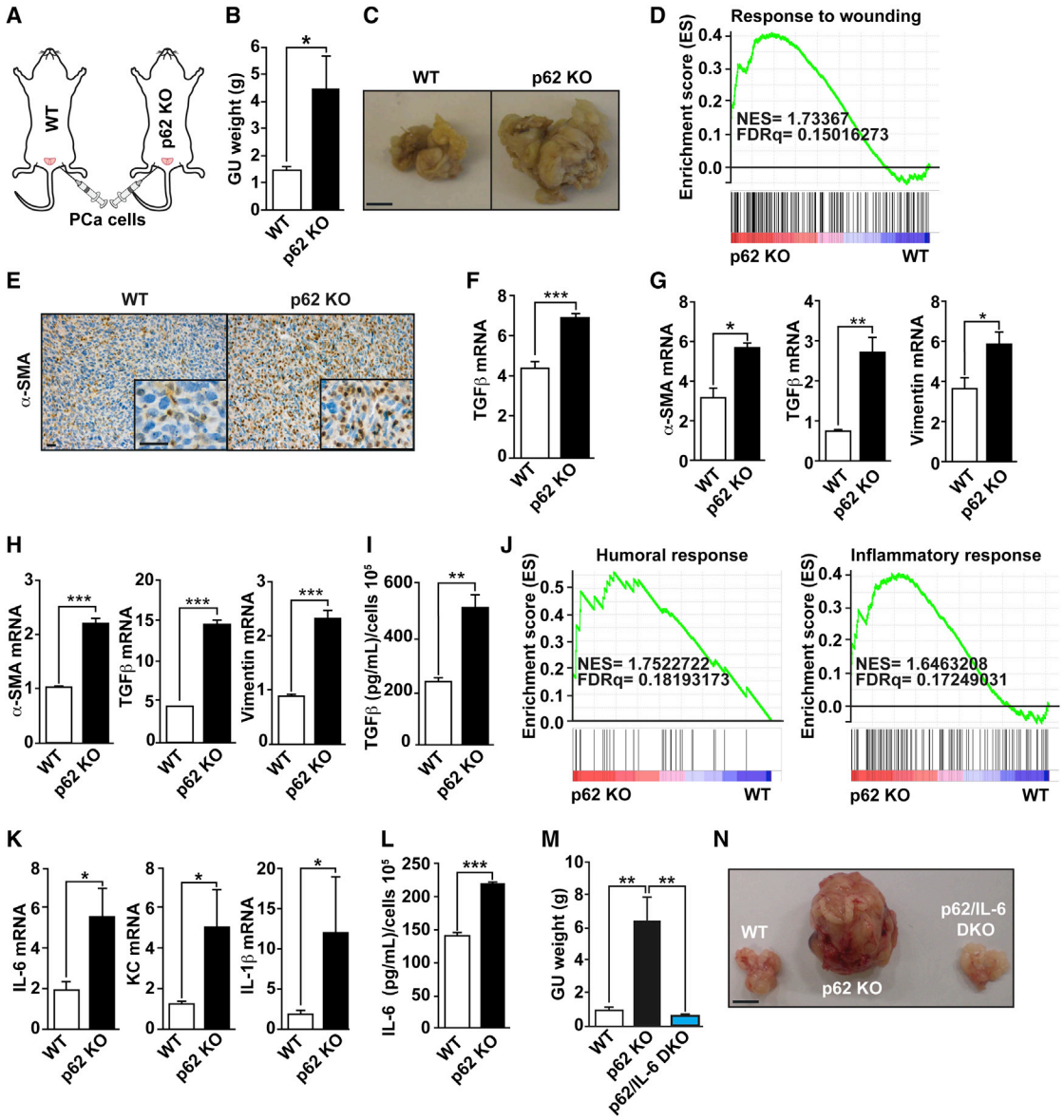

Figure 2. IL-6 Is Required for p62's Role in the Tumor Microenvironment

(A) Orthotopic injection of TRAMP-C2Re3 cells into the prostates of syngeneic WT and p62 KO mice. Orthotopic tumors were allowed to grow for two months.

(B and C) GU tract weight (B) and pictures (C) from (A); n = 5 or 6 mice per genotype. The scale bar represents 1 cm.

(D) "Response to wounding" GSEA plot of enrichment of gene expression in p62 KO orthotopic tumors.

(E) α-SMA staining of orthotopic tumors from WT and p62 KO mice. The scale bars represent 25 μm.

(F) RT-PCR of TGF-β in orthotopic tumors from WT and p62 KO mice (n = 3).

(G and H) RT-PCR of CAF markers (α-SMA, TGF-β, and vimentin) in FACS-sorted prostate stromal fraction from WT and p62 KO mice (G) and in WT and p62 KO prostate fibroblasts (H); n = 3.

(I) TGF-β production in prostate fibroblasts was determined by ELISA.

(J) GSEA plots of enrichment of gene expression in p62 KO orthotopic tumors.

(K) RT-PCR of inflammatory cytokines in orthotopic tumors of WT and p62 KO mice; n = 5 or 6 animals per group versus WT.

(L) IL-6 ELISA in fibroblasts.

(M and N) Orthotopic injection of TRAMP-C2Re3 cells into the prostates of mice of different genotypes (n = 5 or 6 mice). GU weights (M) and pictures (N). The scale bar represents 1 cm.

*p < 0.05, **p < 0.01, ***p < 0.001. Results are presented as mean ± SEM. See also Figure S2.

in the stroma. The two major sources of IL-6 in the tumor microenvironment are macrophages and stromal fibroblasts (Hanahan and Coussens, 2012). Notably, 3D organotypic culture experiments established that fibroblasts (Figures 3B and 3C), but not macrophages (Figure S3D), from p62 KO mice recapitulated the p62 KO phenotype in the orthotopic tissue grafting

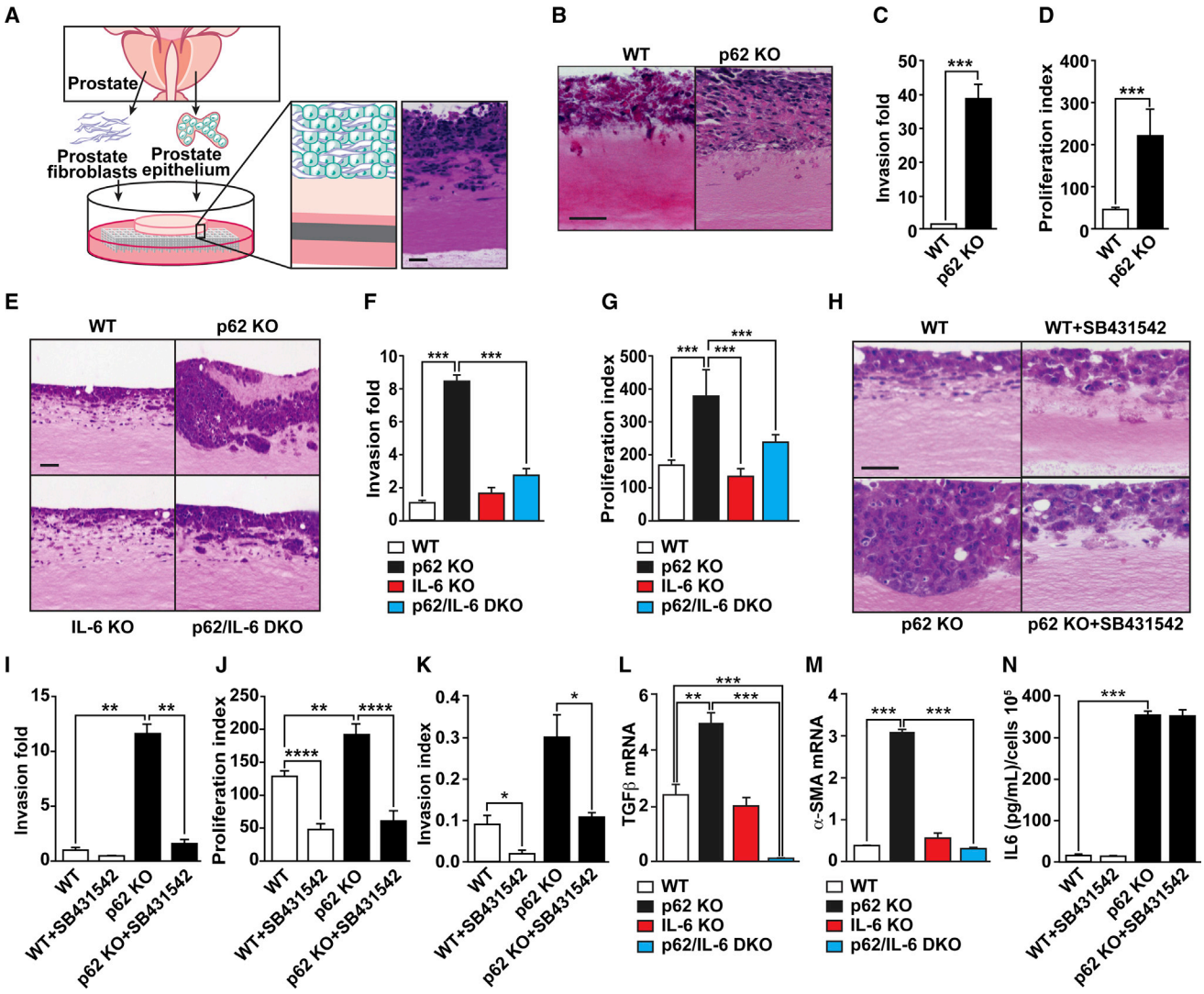

Figure 3. p62-Deficient Stroma-Mediated Invasion Is IL-6 and TGF-β Dependent

(A) Schematic representation of 3D organotypic cultures.

(B) H&E-stained sections of TRAMP-C2Re3 cells cultured in an organotypic system in the presence of primary prostate fibroblasts from WT and p62 KO mice.

(C and D) Quantification of PCa cell invasion (C) and proliferation index (D) of experiment shown in (B); n = 4.

(E) H&E staining of organotypic gels combining Myc-CaP cells with prostate fibroblasts from mice of different genotypes (n = 4).

(F and G) Quantification of PCa cell invasion (F) and proliferation index (G) of experiment shown in (E); n = 4.

(H) H&E staining of organotypic gels combining Myc-CaP cells with prostate fibroblasts from mice WT and p62 KO mice in the presence or absence of the TGF-β inhibitor SB431542 (10 μM).

(I and J) PCa cell invasion quantification (I) and proliferation index (J) of (H); n = 4.

(K) Invasion index determined by modified Boyden chamber assay with conditioned media from WT and p62 KO fibroblasts in the presence or absence of SB431542 (10 μM); n = 3.

(L and M) RT-PCR of TGF-β (L) and α-SMA (M) mRNA levels in fibroblasts of mice of different genotypes (n = 4).

(N) IL-6 production by WT and p62 KO fibroblasts in the presence or absence of the TGF-β inhibitor SB431542 (n = 4).

*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Results are presented as mean ± SEM. The scale bars represent 100 μm. See also Figure S3.

experiment, and this effect was abolished when p62/IL-6 DKO fibroblasts were used in the 3D system (Figures 3E–3G). Furthermore, when TGF-β signaling was inhibited by incubating the organotypic cultures with the TGF-β inhibitor SB431542 (Inman et al., 2002), the protumorigenic phenotype of p62-deficient fibroblasts was reverted, consistent with the notion that TGF-β is important for the CAF phenotype and PCa proliferation (Figures 3H–3J). Likewise, the TGF-β inhibitor reverted the increased invasion index of PCa cells incubated with p62-deficient fibroblast conditioned medium in a Boyden chamber invasion assay (Figure 3K). Results shown in Figure S3E demonstrate the effectiveness of this inhibitor to block the TGF-β pathway. Moreover, the enhanced TGF-β production observed in the p62 KO fibroblasts, as well as that of α-SMA, was completely abrogated in

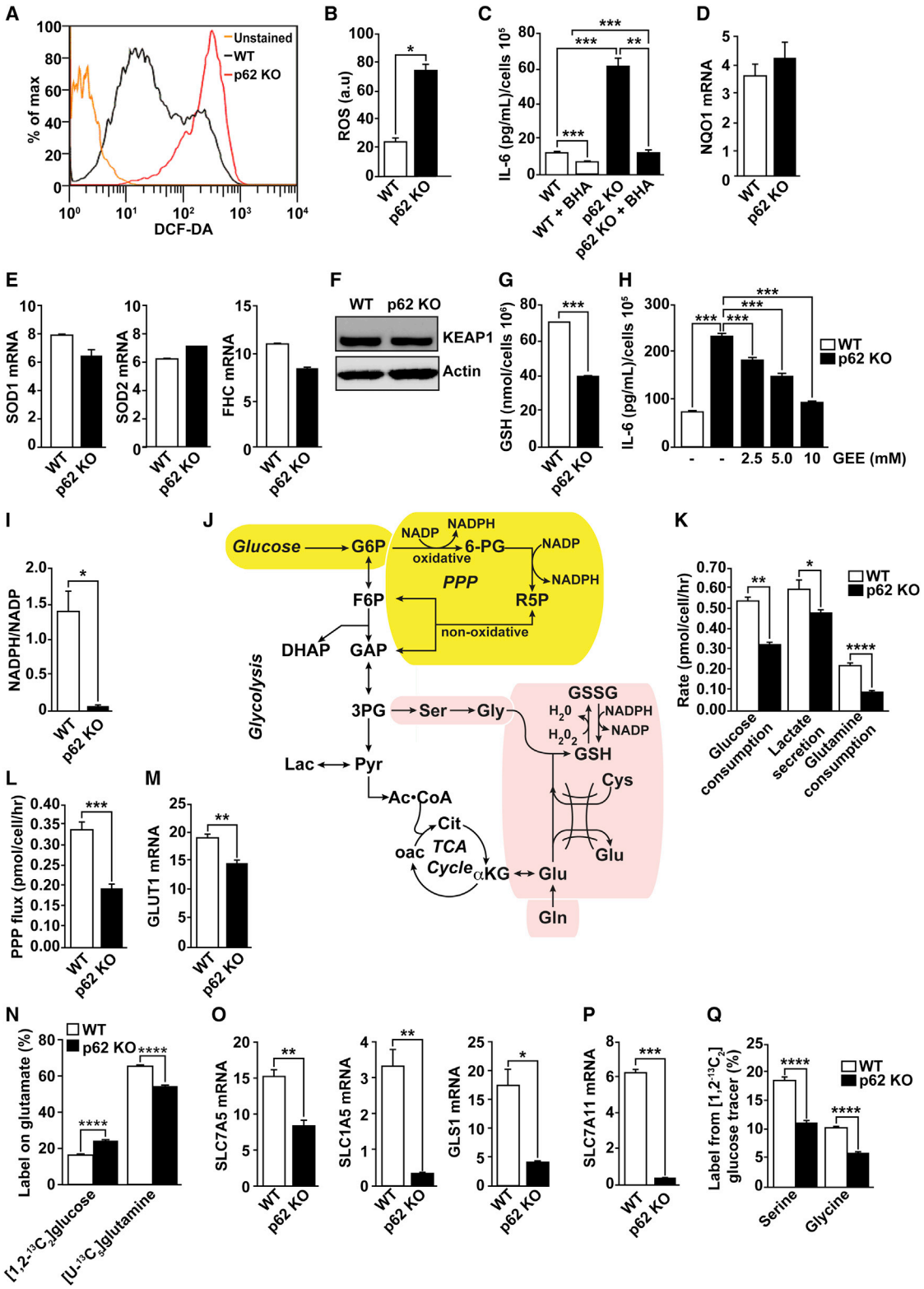

Figure 4. Metabolic Reprogramming in p62-Deficient Stroma

(A and B) Total intracellular levels of ROS in WT and p62 KO fibroblasts (A) and quantification (B); n = 4.

(C) IL-6 ELISA of WT and p62 KO fibroblasts treated with vehicle or the ROS scavenger BHA (100 μM) for 12 hr (n = 4).

(D and E) RT-PCR of NQO1 (D) and SOD1, SOD2, and FHC (E) mRNA levels in fibroblasts (n = 4).

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the DKO fibroblasts (Figures 3L and 3M). Consistently, the knockdown of IL-6 in p62 KO fibroblasts impaired IL-6 secretion and, more important, also reverted TGF-β production and PCa invasion (Figures S3F–S3H). Furthermore, incubation of p62/IL-6 DKO fibroblasts with exogenously added IL-6 restored TGF-β levels to those of p62 KO cells as well as PCa invasion (Figure S3I and S3J). All this is consistent with a cell-autonomous role of the p62-IL-6 axis in the control of the CAF phenotype. However, incubation of p62 KO fibroblasts with the TGF-β signaling inhibitor SB431542 did not affect the overproduction of IL-6 in p62 KO fibroblasts (Figure 3N). These are important observations that establish a sequential p62/IL-6/TGF-β axis in the tumor fibroblastic compartment contributing to the control of epithelial tumorigenesis during PCa progression.

p62 Controls IL-6 Levels by Repressing Reactive Oxygen Species Production through Metabolic Reprogramming

We next sought to determine how p62 controls IL-6 production in fibroblastic stromal cells and whether the mechanisms mediating IL-6 production are relevant to stroma-driven tumorigenesis. It should be noted that p62 KO fibroblasts have increased levels of reactive oxygen species (ROS) (Figures 4A and 4B) and that the inhibition of ROS production (by the ROS scavenger butylated hydroxyanisole [BHA]) completely reverts the IL-6 hyperproduction phenotype (Figure 4C). This indicates that the mechanism whereby p62 represses IL-6 production in fibroblasts involves the control of ROS levels. It has previously been reported that p62 can activate NF-κB and NRF2 (Duran et al., 2008; Komatsu et al., 2010; Moscat and Diaz-Meco, 2009), which suggests that these molecules could play a role in the ability of p62 to repress ROS production and the subsequent activation of IL-6. The expression of critical detoxifying NF-κB- or NRF2-dependent genes (Figures 4D and 4E), as well as the levels of the NRF2 inhibitor Keap1 (Figure 4F), was not affected by the loss of p62 in fibroblasts. However, we found that p62 KO fibroblasts displayed lower levels of reduced glutathione (GSH) than the WT controls (Figure 4G). These are important observations because GSH is central to the control of ROS levels. In fact, treatment of p62 KO fibroblasts with the GSH analog GSH-reduced ethyl ester (GEE) reduced IL-6 to levels comparable with those of WT fibroblasts (Figure 4H). These results demonstrate that the loss of p62 results in lower GSH levels, thus promoting ROS accumulation, which is required for IL-6 overproduction in p62-deficient fibroblasts.

We observed a striking decrease in the reduced nicotinamide adenine dinucleotide phosphate (NADPH)/nicotinamide adenine dinucleotide phosphate (NADP) ratio in p62-deficient fibroblasts (Figure 4I). This ratio provides additional information on the cellular redox status, as the relative concentration of GSH versus oxidized GSH depends on the cellular content of NADPH. Glycolytic metabolism plays a critical role in maintaining NADPH production through the oxidative pentose phosphate pathway (PPP) (Figure 4J, yellow shading). Indeed, p62 KO cells exhibited decreased glucose uptake and lactate secretion (Figure 4K). This reduction in glycolytic rate resulted in decreased flux through the oxidative PPP, as determined by stable isotope tracing with [1,2-$^{13}C_2$]glucose (Figure 4L). These metabolic changes correlated with a reduction in GLUT1 levels in p62-deficient fibroblasts (Figure 4M), providing evidence that transcriptional changes associated with p62 loss influence metabolic flux.

Amino acids are critical for the production of GSH, a peptide composed of glutamate, cysteine, and glycine (Figure 4J, pink shading). Glutamine serves as an important precursor for glutamate, and loss of p62 in fibroblasts leads to lower glutamine consumption compared with WT cells (Figure 4K). We also observed a decrease in the direct conversion of [U-$^{13}C_5$] glutamine to glutamate in p62 KO fibroblasts, with a relative increase in the fraction of glutamate derived from [1,2-$^{13}C_2$]glucose (Figure 4N). In good agreement with these changes in glutamine metabolism, we observed reduced levels of the glutamine transporters SLC7A5 and SLC1A5, as well as glutaminase-1 (GLS1) (Figure 4O), a critical enzyme in the pathway that catalyzes the conversion of glutamine into glutamate (Figure 4J). Consistent with reduced levels of GSH, p62 KO fibroblasts also exhibit a dramatic reduction in the levels of SLC7A11, the xCT cystine/glutamate antiporter, which is the major driver of cystine uptake, a critical and rate-limiting step in the synthesis of GSH in several cell types, including fibroblasts (Figure 4P) (Bannai and Tateishi, 1986; Gout et al., 1997). Finally, we observed significant decreases in labeling of both serine and glycine from [1,2-$^{13}C_2$] glucose (Figure 4Q). Serine serves as a precursor to glycine and cysteine (when synthesized from methionine), so this decrease in label transfer provides evidence that there is less demand for GSH synthesis in p62-deficient cells. These results collectively demonstrate that loss of p62 in fibroblasts influences metabolic pathways controlling cellular redox, including NADPH production in the PPP and GSH synthesis.

p62 Is a Critical Regulator of c-Myc Levels

Previous data from other laboratories have established the critical role of c-Myc in the regulation of glutamine and glucose metabolism (Dang, 2012). We found significantly reduced levels of c-Myc in p62 KO fibroblasts as well as in WT fibroblasts in which p62 has been knocked down by small hairpin RNA (shRNA)

(F) Immunoblot analysis of KEAP1 in cell lysates from WT and p62 KO fibroblasts. Results are representative of three experiments.
(G) Cellular GSH levels in WT and p62 KO fibroblasts (n = 4).
(H) IL-6 ELISA of fibroblasts treated with increasing concentrations of the GSH analog GEE (n = 4).
(I) Cellular NADPH/NADP levels in WT and p62 KO fibroblasts (n = 4).
(J) Metabolic scheme depicting biosynthetic routes to NAPDH (yellow shading) and GSH (pink shading) from glucose and glutamine.
(K) Glucose consumption, lactate secretion, and glutamine consumption rates determined by spent medium analysis from WT and p62 KO fibroblasts (n = 3).
(L) PPP flux estimates from metabolic flux analysis in WT and p62 KO fibroblast cultures labeled with [1,2-$^{13}C_2$]glucose (n = 3).
(M) RT-PCR of GLUT1 mRNA (n = 4).
(N) Glutamate labeling in WT and p62 KO fibroblasts grown in either [1,2-$^{13}C_2$]glucose and unlabeled glutamine or [U-$^{13}C_5$]glutamine and unlabeled glucose (n = 3).
(O and P) RT-PCR of SLC7A5, SLC1A5, and GLS1 (O) and SLC7A11 (P) mRNA levels in WT and p62 KO fibroblasts (n = 3).
(Q) Labeling of serine and glycine from [1,2$^{13}C_2$]glucose (n = 3).
*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Results are presented as mean ± SEM.

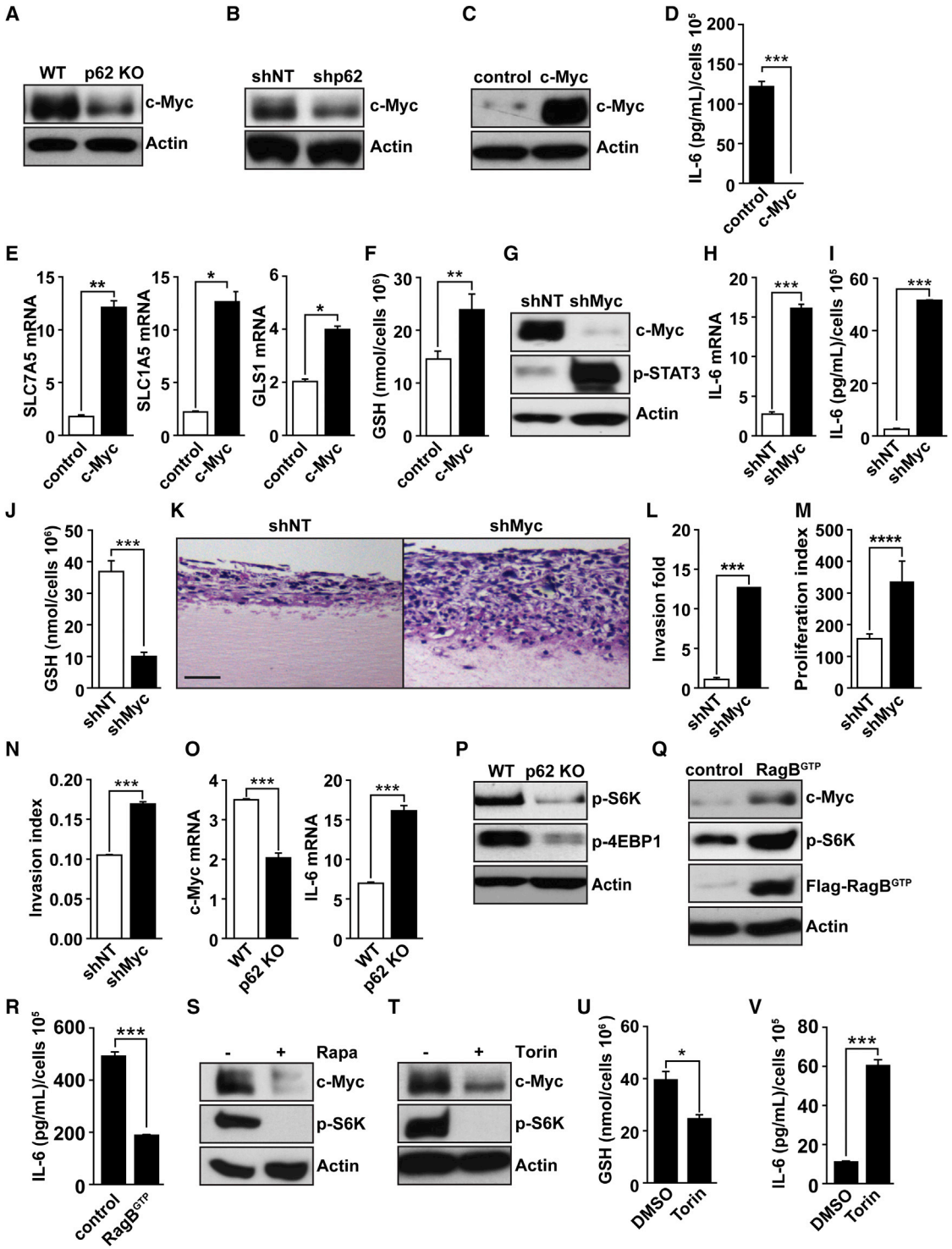

Figure 5. c-Myc-Mediated Metabolism in p62-Deficient Stroma

(A–C) Immunoblot analysis of c-Myc levels in WT and p62 KO fibroblasts (A), in WT fibroblasts lentivirally infected with shRNA nontargeted control (shNT) or shRNA specific for p62 (shp62) (B), and in p62 KO fibroblasts retrovirally infected with control vector (control) or with c-Myc expression vector (c-Myc) (C). Results are representative of three experiments.

(D) IL-6 ELISA in control and c-Myc cells (n = 4) as in (C).

(E) RT-PCR of SLC7A5, SLC1A5, and GLS1 mRNA in control and c-Myc cells (n = 3).

(F) Intracellular GSH levels in control and c-Myc cells (n = 3).

(G) Immunoblot analysis of c-Myc and p-STAT3 in WT fibroblasts infected with shNT or shRNA for c-Myc (shMyc).

(legend continued on next page)

(Figures 5A and 5B) and reductions in the levels of the key glutamine transporters SLC7A5 and SLC1A5, and GLS1 (Figure 4O), which are targets of c-Myc (Dang, 2012). Interestingly, ectopic expression of c-Myc in p62 KO fibroblasts (Figure 5C) reverted the p62-deficient phenotype in terms of IL-6 production (Figure 5D) and the levels of glutamine transporters and GLS1 (Figure 5E) and GSH (Figure 5F). On the contrary, c-Myc knockdown in WT fibroblasts (Figure 5G) resulted in increased IL-6 production at the mRNA and protein levels (Figures 5H and 5I). c-Myc knockdown in fibroblasts led to decreased levels of GSH (Figure 5J), as well as enhanced PCa cell invasion and proliferation in organotypic cell cultures (Figures 5K–5M). Also, the knockdown of c-Myc in fibroblasts resulted in increased PCa cell invasion index in a Boyden chamber assay (Figure 5N). Of note, this cause-and-effect correlation between p62 deficiency, c-Myc expression, and IL-6 production was also found in FACS-isolated prostate stromal cells from WT and p62 KO mice (Figure 5O). Collectively these results demonstrate that p62 repression of c-Myc expression in the stroma fibroblasts accounts for its tumor suppressive role in PCa.

IL-6 Is Regulated by a p62/mTORC1/c-Myc Cascade

Consistent with previously published observation (Duran et al., 2011), we found that p62 KO cells displayed reduced mTORC1 activity (Figure 5P). We hypothesized that the reduction in c-Myc levels found in p62 KO fibroblasts could be the consequence of mTORC1 inhibition. Importantly, we rescued c-Myc inhibition in p62 KO fibroblasts by expressing a permanently active mutant of the small-guanosine triphosphatase RagB, which is a critical activator of mTORC1 (Figure 5Q). IL-6 levels were likewise reduced under these conditions (Figure 5R). These results demonstrate that reduced mTORC1 activity in p62 KO fibroblasts accounts for the low levels of c-Myc and the subsequent increase in IL-6 production in these mutant cells. Treatment of WT fibroblasts with rapamycin or Torin, two different inhibitors of mTORC1, effectively reduced c-Myc levels (Figures 5S and 5T), promoting a significant reduction in GSH levels (Figure 5U) and a concomitant increase in IL-6 production (Figure 5V). Therefore, p62's ability to regulate mTORC1 in the stroma is essential for its control of the c-Myc/GSH/IL-6 axis.

p62 KO Mice Develop Prostate Hyperplasia and Prostatic Intraepithelial Neoplasia upon Aging

On the basis of these results, we hypothesized that the loss of p62 at an organismal level, which would include both the prostate stroma and epithelium, might be sufficient to drive prostate epithelium toward neoplasia. We characterized the prostates of p62 KO mice by histological analysis, which revealed no abnormalities in development or morphology at early stages. However, at 9 months of age, prostates from p62 KO mice developed hyperplasia, with a concomitant increase in Ki67 staining (Figures 6A and 6B). These lesions progressed to prostatic intraepithelial neoplasia (PIN) at 1 year of age (Figure 6C). This indicated that, whereas in xenograft experiments PCa epithelial cells with reduced p62 displayed inhibited tumorigenesis (Duran et al., 2011), the total loss of p62 in vivo promoted prostate epithelial cell growth. These observations are in good agreement with our model whereby p62 in the stromal fibroblasts normally acts as a tumor suppressor, and the total KO of p62 results in p62-deficient stromal fibroblasts that drive the prostate epithelium to a malignancy-prone state. To further test this hypothesis, we crossed total p62 KO mice with two well-established mouse models of PCa ($PTEN^{+/-}$ and $TRAMP^{+}$) (Di Cristofano et al., 1998; Greenberg et al., 1995) and asked whether total ablation of p62 inhibited or promoted prostate tumor development. Figures 6D and 6E show hematoxylin and eosin (H&E) analyses of $PTEN^{+/-}$/p62 KO prostrates demonstrating an increase in the percentage of glands with high-grade PIN at the age of 6 months. Furthermore, $TRAMP^{+}$/p62 KO mice had reduced survival (Figure 6F), increased percentages of poorly differentiated adenocarcinoma (Figure 6G) and neuroendocrine tumors (Figure 6H), as well as a larger number of metastases (Figure 6I), of which a higher percentage were in the liver (Figure 6J). Consistent with our model, prostate fibroblasts from $PTEN^{+/-}$/p62 KO mice showed increased IL-6 and reduced c-Myc expression compared with those from p62-proficent $PTEN^{+/-}$ mice (Figures S4A–S4C). Interestingly, immunohistochemical analysis of prostates from $PTEN^{+/-}$ mice confirmed reduced expression of p62 in the stromal compartment compared with those from WT mice (Figure S4D). To further support the role of p62 deficiency in the stroma in driving tumorigenesis in vivo, we coinjected syngeneic PCa cells (TRAMP-C2Re3) with WT or p62 KO fibroblasts and assessed the effect that fibroblasts exert on tumor growth. Tumors coinjected with p62 KO fibroblasts grew significantly faster and were larger than tumors in mice injected with WT fibroblasts, consistent with the cell-autonomous tumor-promoting activity of p62-deficient fibroblasts on epithelial PCa cells (Figures 6K and 6L). In agreement with this, bromodeoxyuridine (BrdU) incorporation was increased in the p62 KO fibroblast-driven tumors (Figures 6M and 6N).

(H and I) RT-PCR of IL-6 mRNA (H) and IL-6 ELISA (I) in the same cells (n = 3) as in (G).
(J) Quantification of intracellular GSH levels in WT shNT and shMyc cells (n = 3).
(K) H&E-stained organotypic gels of TRAMP-C2Re3 cells with shNT or shMyc fibroblasts. The scale bar represents 100 μm.
(L and M) Quantification of PCa cell invasion (L) and proliferation index (M) of experiment shown in (K).
(N) Invasion index determined by modified Boyden chamber assay of Myc-CaP cells cocultured with shNT and shMyc fibroblasts.
(O) RT-PCR of c-Myc and IL-6 mRNA levels in FACS-isolated prostate stromal cells from WT and p62 KO mice (n = 3).
(P) Immunoblot analysis with the indicated antibodies of cell lysates of WT and p62 KO fibroblasts.
(Q) Immunoblot analysis for the specified proteins of cell lysates from p62 KO fibroblasts retrovirally infected with control vector (control) or FLAG-$RagB^{GTP}$ expression vector ($RagB^{GTP}$). Results are representative of three experiments.
(R) IL-6 ELISA (n = 3) in cells shown in (Q).
(S and T) Immunoblot analysis of c-Myc and p-S6K in fibroblasts treated with rapamycin (S) or Torin1 (T) for 12 hr.
(U) Intracellular GSH levels in fibroblasts treated with Torin1.
(V) IL-6 ELISA in fibroblasts treated with Torin1 (n = 3).
*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Results are presented as mean ± SEM.

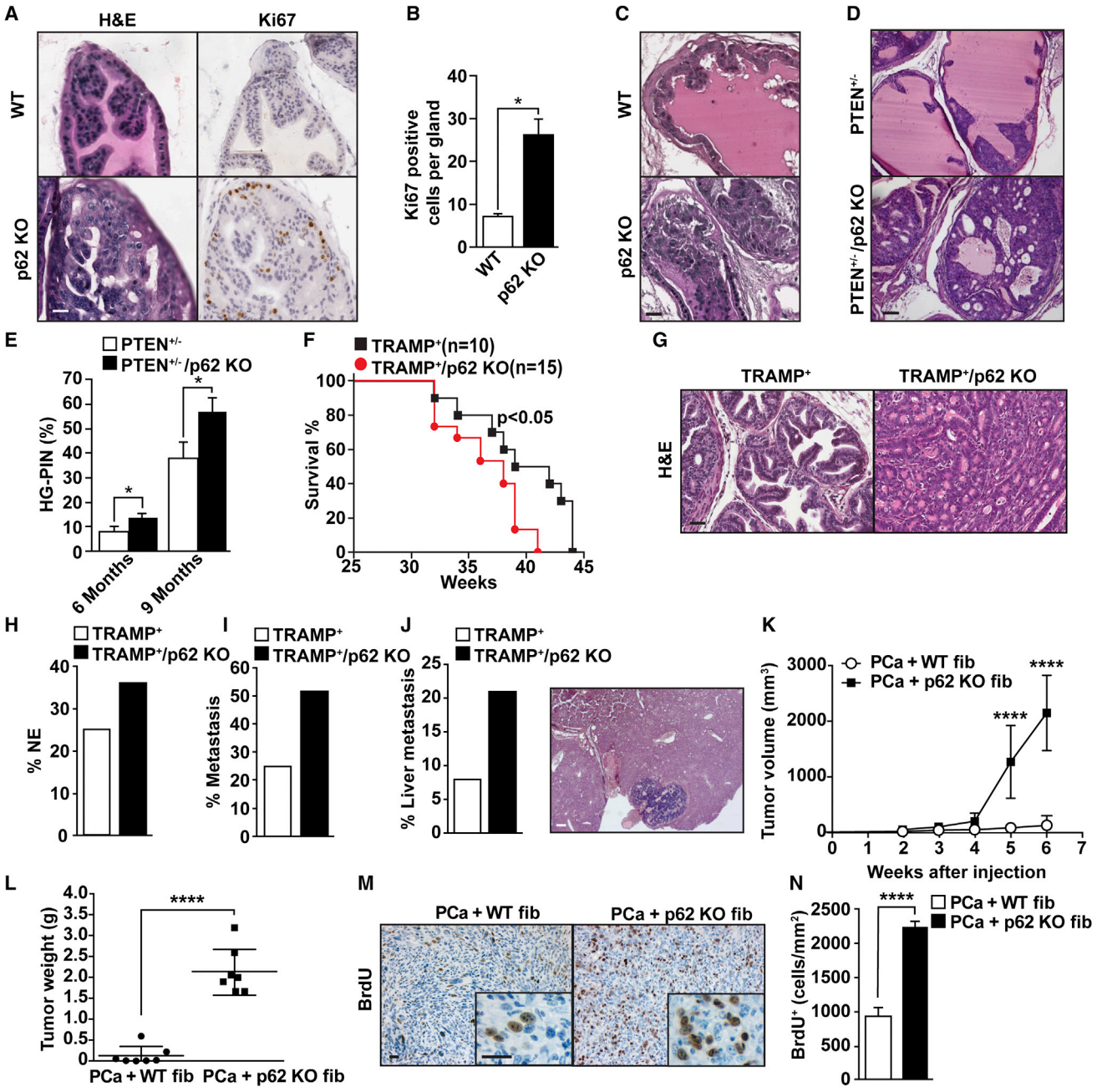

Figure 6. p62 Deficiency Accelerates Prostate Tumor Progression in Different Mouse Models of Prostate Cancer

(A) Hyperplasia in the prostatic anterior lobe of p62 KO mice. H&E and Ki67 staining of prostates from 9-month-old WT and p62 KO mice (n = 5).

(B) Quantification of Ki67-positive cells in the prostate sections shown in (A). Results are the means ± SD of counts from 10 different fields per mouse (n = 5).

(C) PIN in the dorsolateral lobes of prostates from 12-month-old p62 KO mice.

(D) Representative examples of H&E staining of dorsolateral lobes of prostates from $PTEN^{+/-}$ and $PTEN^{+/-}$/p62 KO mice at 6 months of age (n = 5).

(E) Percentage of glands with HG-PIN (n = 5 mice).

(F) Kaplan-Meier survival curve of $TRAMP^{+}$ mice (n = 10), compared with $TRAMP^{+}$/p62 KO mice (n = 15).

(G) Representative H&E staining of mouse prostate sections from $TRAMP^{+}$ and $TRAMP^{+}$/p62 KO mice (n = 10).

(H) Incidence of neuroendocrine tumors in $TRAMP^{+}$ (n = 12) and $TRAMP^{+}$/p62 KO mice (n = 19).

(I and J) Incidence of metastasis (I) and liver metastasis (J) in $TRAMP^{+}$ compared with $TRAMP^{+}$/p62 KO mice.

(K–M) Coinjection of syngeneic TRAMPC2-Re3 PCa cells with either WT or p62 KO fibroblasts in C57BL/6 mice. (K) Tumor volume assessed at different time points after injection (n = 7 mice). (L) Tumor weight at 6 weeks after injection (n = 7 mice). (M) BrdU staining in tumor sections.

(N) Quantification of BrdU positive cells of (M).

Results are presented as mean ± SEM (n = 10). *p < 0.05, ****p < 0.001. The scale bars represent 25 μm. See also Figure S4.

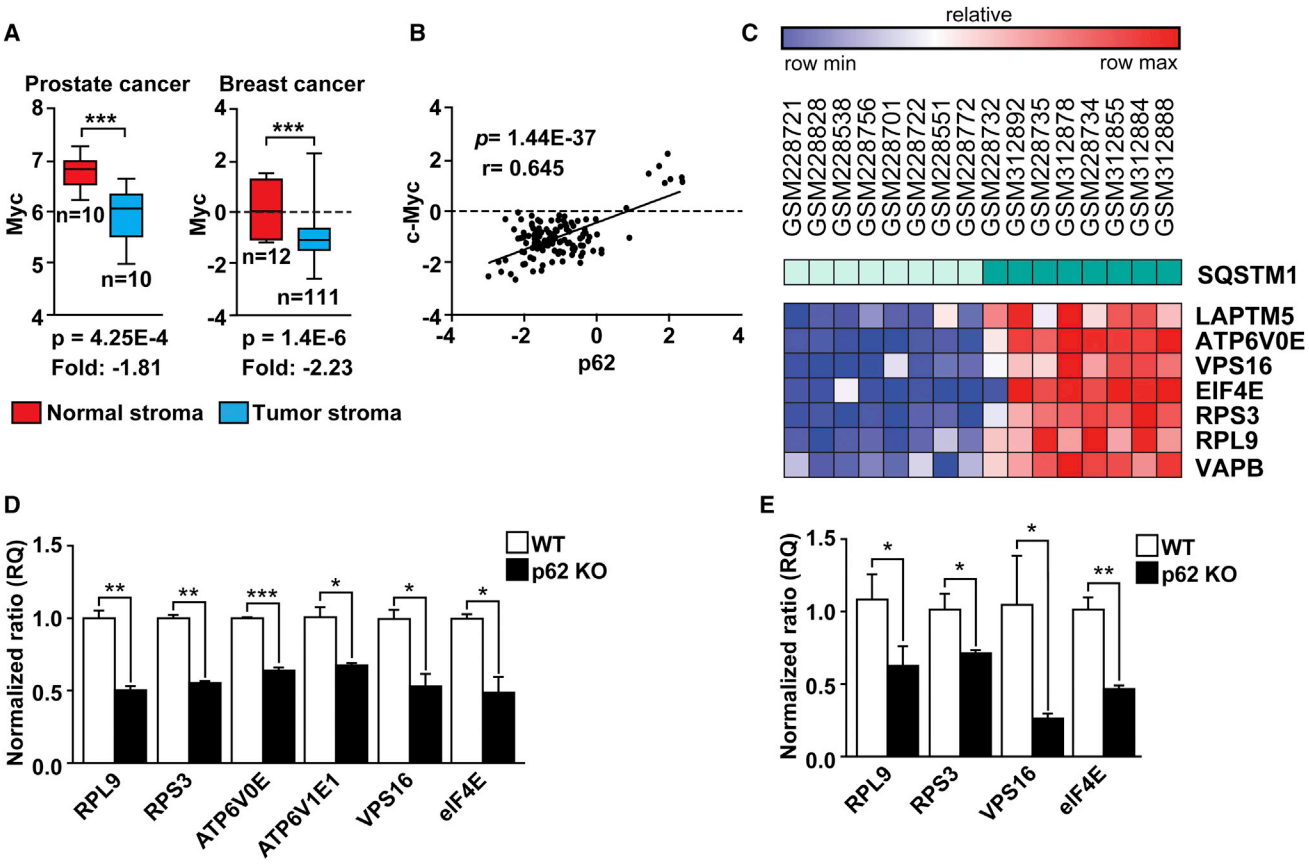

Figure 7. The p62-Myc-mTORC1 Cassette Is Downregulated in Prostate Tumor-Associated Stroma in Human Samples

(A) Myc levels are downregulated in the tumor stroma of human tissue samples. Data were collated from public data sets of gene expression in tumor stroma in several human cancers: GSE34312 (prostate cancer) and GSE9014 (breast cancer). The p value, fold change of expression, and size of the sample (n) for each study are indicated in the corresponding panels.

(B) Positive correlation between p62 and Myc levels in the stroma.

(C) Heatmap of mTORC1 signature selected from p62 neighboring genes in human stroma. p62 levels are indicated as SQSTM1.

(D and E) RT-PCR analysis of mTORC1 genes in WT and p62 KO fibroblasts (D) and in FACS sorted mouse prostate stromal fraction (E).

*p < 0.05, **p < 0.01, ***p < 0.001. Results are presented as mean ± SEM (n = 3). See also Figure S5.

p62/mTORC1/c-Myc Connection in Human Cancer Stroma

To determine whether the identified link between p62 and c-Myc through mTORC1 has relevance to the role of the stroma in human cancer, we used bioinformatics to analyze c-Myc transcript levels in two sets of human gene-expression arrays from prostate and breast cancer stroma. Stroma of human tumors displayed reduced levels of c-Myc (Figure 7A), and there was a statistically significant correlation between c-Myc and p62 expression in tumor stroma (Figure 7B), emphasizing the clinical relevance of the p62-Myc connection in the stroma. To further explore the link between p62 and mTORC1 in the tumor stroma of human cancers, we identified expression neighbors of p62. We developed this gene signature by using the human cancer stroma data set shown in Figure 7A, in which we classified tumors on the basis of p62 expression levels and selected for analysis only those samples in the top and bottom 25%. Interestingly, this analysis revealed a statistically significant correlation between p62 expression and that of genes previously reported to be controlled by mTORC1 activity (Figure 7C; Figure S5) (Peña-Llopis et al., 2011). We determined the expression levels of a selection of these genes by RT-PCR and found that their expression was reduced in p62 KO fibroblasts compared with WT (Figure 7D). The same results were obtained when these were analyzed in prostate stromal cell preparations from p62 KO and WT mice (Figure 7E). Furthermore, we found a clear statistically significant correlation between p62 expression and that of these genes in human cancer stroma (Figures S5B–S5H). Altogether, these results demonstrate that the p62/mTORC1/c-Myc connection is not only relevant in the mouse prostate stroma but it is also important in human cancer stroma.

DISCUSSION

Tumorigenesis is a slow process that is initiated by the successive accumulation of genetic and epigenetic changes that result in the activation of cell growth and survival genes and the inactivation of tumor suppressors (Hanahan and Weinberg, 2011). However, for tumor development to take place, initiation is not sufficient. Other signals are required to drive tumor promotion

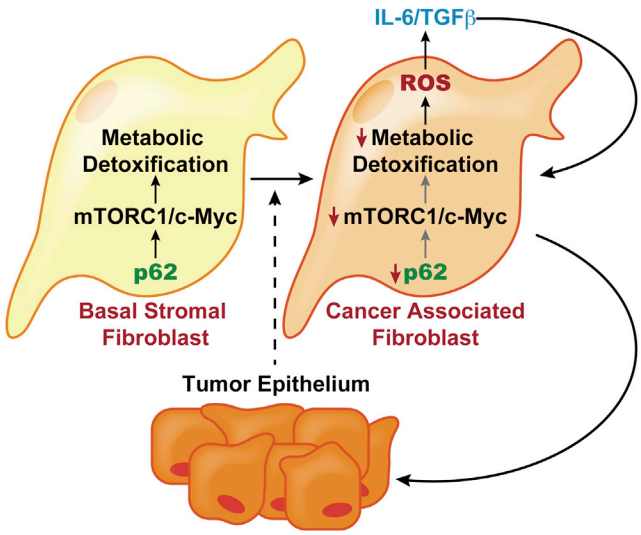

Figure 8. Stromal Activation by p62 Deficiency in Cancer
Tumor epithelium promotes the downregulation of p62 in stromal fibroblasts, leading to reduced mTORC1 activity and c-Myc expression, which results in impaired metabolic detoxification and the subsequent release of ROS and IL-6. An autocrine pathway promotes TGF-β and the induction of CAF phenotype, which further increases epithelial invasion and tumorigenesis.

and progression and the development of the fully malignant stage. The progression phase is most likely orchestrated via the tumor microenvironment by nonepithelial cells in which metabolic stress and inflammation create an environment in which epithelial tumor-derived cells propagate and acquire more aggressive phenotypes (Hanahan and Coussens, 2012; Hanahan and Weinberg, 2011). Immune cells, such as tumor-associated macrophages, are among the cell types in the tumor microenvironment that contribute to inflammation (Coussens and Werb, 2002; Johansson et al., 2008). On the other hand, a crosstalk between metabolic pathways in the stromal and epithelial compartments of the tumor may drive the survival and growth of epithelial cancer cells (Lisanti et al., 2013). However, it has not been thoroughly investigated whether metabolic reprogramming in the stromal cells of the tumor microenvironment exerts any control over inflammation and the malignant characteristics of the transformed epithelium.

Here we demonstrate that the inactivation of mTORC1 in p62-deficient stromal fibroblasts results in metabolic reprogramming through c-Myc inactivation (Figure 8). This reprogramming leads to increased levels of IL-6, which promotes epithelial cell invasion and proliferation. Therefore, because of its regulation of mTORC1, p62 emerges as a tumor suppressor that acts by regulating c-Myc and thus inducing an inflammatory response. These results are in marked contrast to the role played by p62 and mTORC1 in epithelial cancer cells. That is, we have recently demonstrated that p62 inactivation in PCa and lung adenocarcinoma epithelial cells inhibits the proliferation and tumorigenic properties of these cells and correlates with decreased mTORC1 activation. Moreover, the increased IL-6 phenotype can be reverted by expression of a permanently active mutant of the mTORC1 activator RagB. This has important implications from a therapeutic point of view because inhibition of p62 and/or mTORC1 may result in opposite effects in the stroma and the epithelium of the tumor, thus reducing the efficacy of broadly applied mTORC1-based chemotherapeutic approaches. In this regard, these results are reminiscent of the dual role that mTORC1 might play as a regulator of autophagy, which can have a tumor-suppressing or a tumor-promoting effect, depending of the stage of the tumor (Guo et al., 2013; Levine and Kroemer, 2008), and also on whether the manipulation takes place in the epithelium or in the stroma (Lisanti et al., 2013). Our data using KO mice clearly reveal that p62 deficiency creates a protumorigenic environment for p62-proficient PCa cells in orthotopic experiments and also show that, even under normal conditions, it drives PIN formation in the endogenous epithelium in the absence of any other induced mutations. Furthermore, in two PCa models, the lack of p62 at an organismal level results in increased tumorigenesis, despite the fact that p62 is absent not only in the stroma but also in the transformed epithelium. These results are very important because they demonstrate that even though p62 is required for epithelial cancer cells to proliferate in vitro and in xenografts (Duran et al., 2011), the p62-deficient tumor microenvironment overrides the requirement for p62 in the epithelium. Our in vitro and in vivo findings establish that increased IL-6 levels generated by stromal fibroblasts are a critical event in that process. Therefore, it can be predicted that total ablation of p62 at an organismal level, either genetically or pharmacologically, may increase tumorigenesis, rather than inhibiting it, depending on the contribution of the stroma and the ability of p62 deficiency to reprogram stromal metabolism to generate ROS and inflammation. Our data shown here demonstrate that this is the case in prostate tumorigenesis and suggest that it could be a relevant mechanism in other tumor types as well. The proinflammatory microenvironment in the p62-deficient stroma results in a CAF-activated phenotype that is maintained by stromal TGF-β production. This is consistent with previous results in colon cancer demonstrating that a TGF-β-activated tumor microenvironment is critical for fully aggressive cancer cells to metastasize (Calon et al., 2012).

Metabolic reprogramming in cancer is emerging as a central process in tumor cell survival and growth (DeBerardinis and Thompson, 2012; Metallo and Vander Heiden, 2013; Vander Heiden, 2013). The so-called Warburg effect supports the importance of an atypical glucose metabolism tailored to the cancer cell's need for efficient anabolic utilization of nutrients (Vander Heiden et al., 2009). More recently, different types of reprogramming events have been unveiled that constitute specific responses of the tumor cell to a nutrient-deficient environment. These include the metabolism of serine or the utilization of the PPP to alleviate oxidative stress conditions during tumorigenesis (Locasale, 2013; Ma et al., 2013; Possemato et al., 2011; Vander Heiden et al., 2010). In the current study, we show that metabolic reprogramming triggered by p62 deficiency in the tumor stroma is critical for the creation of a protumorigenic inflammatory environment driven by IL-6. Moreover, we have shown that this involves an mTORC1/c-Myc/ROS cascade that is controlled by p62. In this regard, previous results from our and other laboratories have shown that p62 represses ROS by inducing the activation of NF-κB- or NRF2-dependent detoxifying molecules (Duran et al., 2008; Komatsu et al., 2010; Ling et al., 2012). Surprisingly, neither of these two transcription factors nor Keap1

levels were affected in p62-deficient stromal fibroblasts, indicating that p62 may use diverse cascades in different cellular compartments of the tumor. Interestingly, our previous data demonstrate that under conditions of Ras-induced transformation, p62 deficiency leads to increased cell death and reduced tumorigenesis due to enhanced ROS production (Duran et al., 2008). In contrast, we show here that the enhanced ROS observed in the untransformed stromal fibroblasts does not result in increased cell death but rather in the creation of a proinflammatory phenotype. The main conclusion of these results is that increased ROS production induced by p62 deficiency has different outcomes depending on the cell type and the mechanisms whereby ROS is produced. The outcome also depends on whether or not the levels of ROS are high enough to engage a c-Jun N-terminal kinase-driven cell-death pathway, as found in the Ras-tumor cell, as opposed to increased IL-6 production and a protumorigenic effect on epithelial cells, as we demonstrated in the stromal nontransformed fibroblasts.

Importantly, we were able to show that the implication of the p62/mTORC1/c-Myc cascade is relevant not only in mouse model systems but also in human samples, in which this pathway is inactivated in the tumor stroma. Therefore, our findings support a more comprehensive approach when devising therapeutic strategies in cancer, which should take into account not only the altered pathways in the transformed epithelial compartment but also how the inhibition of these cascades might affect the surrounding stroma. Our observations suggest that pharmacological inhibition of IL-6 and/or TGF-β to target stromal activation could be beneficial in combination with epithelial-targeted therapies.

EXPERIMENTAL PROCEDURES

Mice

WT, p62 KO, PTEN$^{+/-}$, and TRAMP^{+} mice were previously described (Durán et al., 2004; Di Cristofano et al., 1998; Greenberg et al., 1995). All mouse strains were generated in a C57BL/6 background. All mice were born and maintained under pathogen-free conditions. All genotyping was done by PCR. Mice were sacrificed and genitourinary (GU) sections were dissected. Mice were injected with 5-bromo-2′-deoxyuridine intraperitoneally and sacrificed 2 hr after injection. Animal handling and experimental procedures conformed to institutional guidelines (Sanford-Burnham Medical Research Institute Institutional Animal Care and Use Committee).

Cell Lysis and Western Immunoblotting

Cells were rinsed once with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenyl methyl sulfonyl fluoride, and protease inhibitors). Cell extracts were denatured, subjected to 8% to 14% SDS-PAGE, transferred to nitrocellulose-enhanced chemiluminescence membranes (GE Healthcare), and immunoblotted with the specific antibodies. Chemiluminescence was used to detect the proteins (Thermo Scientific).

Statistical Analysis

Significant differences between groups were determined using Student's t test. Scoring of immunostaining of human prostate tissue microarrays was analyzed using Fisher's exact test. The significance level for statistical testing was set at $p < 0.05$.

ACCESSION NUMBERS

The Gene Expression Omnibus accession number for the microarray data reported in this paper is GSE55587.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2014.05.004.

AUTHOR CONTRIBUTIONS

T.V., J.Y.K., S.A.-B. and A.D. performed experiments. J.M.-P. and M.R.-C. performed bioinformatics analysis. E.A.C. provided pathologist expertise for histological analysis. C.S.A. and T.V. performed the metabolic experiments. C.M.M., M.D.M., and J.M. designed and analyzed metabolic data. M.D.M. and J.M conceived and supervised the project with equal contribution. M.D.M. and J.M. wrote the manuscript with assistance from all the authors.

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