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Tumorigenesis and Neoplastic Progression

The NADPH Oxidase Subunit p22^{phox} Inhibits the Function of the Tumor Suppressor Protein Tuberin

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Mutations in the von Hippel-Lindau (*VHL*) gene give rise to renal cell carcinoma. Reactive oxygen species, generated by Nox oxidases, are involved in tumorigenesis. We have previously demonstrated that in VHL-deficient cells, p22^{phox}-dependent Nox1 and Nox4 oxidases maintain hypoxia inducible factor-2α (HIF-2α) protein expression through an Akt-dependent translational pathway. Phosphorylation of tuberin, by Akt, results in its inactivation. Here we show that diphenyleneiodonium chloride, an inhibitor of Nox oxidases, and small-interfering RNA-mediated down-regulation of p22^{phox} inhibit Akt-dependent phosphorylation of tuberin and stabilizes tuberin protein levels in VHL-deficient renal carcinoma cells. p22^{phox}-mediated inactivation of tuberin is associated with an increase in ribosomal protein S6 kinase 1 and eukaryotic initiation factor 4E-binding protein-1 (4E-BP1) phosphorylation as well as HIF-2α stabilization. Importantly, we find that marked up-regulation of p22^{phox} in human renal cell carcinoma correlates with increased tuberin phosphorylation, decreased tuberin protein levels, and increased phosphorylation of 4E-BP1. Our data provide the first evidence that p22^{phox}-based Nox oxidases maintain HIF-2α protein expression through inactivation of tuberin and downstream activation of ribosomal protein S6 kinase 1/4E-BP1 pathway. *(Am J Pathol 2010, 176:2447–2455; DOI: 10.2353/ajpath.2010.090606)*

Epithelial tumors comprise the majority of renal cell carcinomas (RCCs), in which ~75% are histologically of the clear cell type. Biallelic inactivation of the von Hippel-Lindau (*VHL*) tumor suppressor gene is linked to the development of hereditary and sporadic RCC. Mutations within the *VHL* gene maintain the expression of hypoxia inducible factor-2α (HIF-2α) and up-regulates of genes involved in autonomous cell growth,[1] survival,[2] and metastasis,[3,4] major features of RCC. NADPH oxidases of the Nox family are major sources of reactive oxygen species (ROS) in cancer, including renal cancer.[5–12] Selected Nox isoforms form a heterodimer with p22^{phox}, and the dimers facilitate ROS-generating activity of the Nox subunits.[13–16] We and others have shown that p22^{phox}, Nox1, and Nox4 contribute to the maintenance of HIF-2α protein expression and are up-regulated in VHL-deficient cells.[10,11] Moreover, Nox-derived ROS maintain HIF-2α protein levels through a phosphoinositide 3-kinase/Akt-dependent translational mechanism.[11] Activation of the phosphoinositide 3-kinase/Akt pathway promotes translation through inactivation of tuberin.[17] Inhibition of tuberin function occurs through Akt-dependent phosphorylation at residues S^{939}, Thr^{1462} leading to the dissociation of the harmatin/tuberin complex and ubiquitination and degradation of the free tuberin.[17,18] Tuberin negatively regulates mRNA translation by inhibiting mammalian target of rapamycin (mTOR) and subsequent phosphorylation of the translational repressor, eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), and the ribosomal protein S6 kinase 1 (S6K). In this study we show for the first time that p22^{phox}, a key component in the activation of Noxes, results in HIF-2α protein accumulation via inactivation of tuberin and subsequent activation of S6K/4E-BP1 pathway. Furthermore, we report that in human RCC, expression of p22^{phox}, Nox1, and Nox4 is elevated and correlates with increased tuberin phosphorylation on Thr^{1462} and decreased tuberin

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protein levels together with increased phosphorylation of 4E-BP1.

Materials and Methods

Reagents

N-Acetyl-L-cysteine (NAC), diphenyleneoidonium (DPI), Tiron, and PEG-Catalase were purchased from Sigma (St. Louis, MO). PD 98059 and Akt X were purchased from Calbiochem (Gibbstown, NJ).

Cell Lines, Transfection/Infection, Treatment, and Western blot Analysis

Normal immortalized proximal tubular epithelial cells (HK2) and the human VHL-deficient renal carcinoma cell line RCC 786-O (American Type Culture Collection, Manassas, VA) were maintained as described in Block et al.[11] Small-interfering RNA (siRNA) knockdown of p22^{phox} or scrambled control was performed as previously described.[11] All chemical treatments and transfections, unless otherwise noted, were performed in complete media. RCC 786-O cells were treated with indicated inhibitors for short (T0 to T24 hours) or long (T48 to T72 hours). Preparation of lysates for Western blot analysis is described in Block et al.[11] Human RCC or adjacent normal tissue was homogenized in radioimmunoprecipitation assay buffer supplemented with “complete” protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), and the homogenate was rotated at 4°C for 2 hours and centrifuged to remove debris. For Western analysis, protein concentrations were determined by the Bradford protein assay by using bovine serum albumin (Pierce, Rockford, IL) as a standard. Aliquots (30 to 70 μg) of total protein were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The Western blots were blocked with 5% dry milk in 0.1% Tris-buffered saline Tween-20 then incubated with the indicated antibodies: p22^{phox}, Nox1 (Santa Cruz Biotechnology, Santa Cruz, CA), Nox4 (Spring Biosciences, Pleasanton, CA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), HIF2α (Novus Biologicals, Littleton, CO), Actin, β-tubulin (Sigma), p4E-BP1, 4E-BP1, Tuberin, pTuberin Thr^{1462}, pS6K, S6K, pAkt S^{473}, Akt (Cell Signaling, Danvers, MA), and HA (Roche Applied Science, Indianapolis, IN). Antigen-antibody complexes were detected by using the appropriate secondary antibody linked to horseradish peroxidase (BioRad, Hercules, CA) followed by chemiluminescence using Enhanced Chemiluminescence (ECL) reagent (Amersham Biosciences, Pittsburgh, PA).

Constructs

Adenovirus vector expressing Hemagglutinin, HA-tagged dominant-negative Akt (Akt T^{308}A; S^{473}A) and adenovirus vector expressing 4E-BP1 with mutations in all four mTOR phosphorylation sites (T^{35}A, T^{45}A, T^{69}A, S^{64}A) were used as described.[11,19] Flag-tuberin mutant (Flag-tuberin SATA) harboring mutations at the predominant Akt phosphorylation sites (Ser^{939}A and Thr^{1462}A) was generated as described and was a kind gift from Lewis C. Cantley.[17] Ad GFP, containing green fluorescence protein, was used as a control.[11] For short hairpin loop (shp22^{phox}) knockdown, the siRNA was cloned into a p*Silencer* 4.1-CMV neo (Ambion, Austin, TX) as a 54-nucleotide hairpin loop, between restriction sites BamHI and HindIII using the oligos: sense, 5′-GATCCCCATGTGGGCCAACGAACATTCAAGAGATGTTCGTTGGCCCACATGGTTA-3′; antisense, 5′-AGCTTAACCATGTGGGCCAACGAACATCTCTTGAATGTTCGTTGGCCCACATGGG-3′. Insert sequence was confirmed by DNA sequencing. Vector containing nontargeting short hairpin RNA was used as a control (Ambion). For p22^{phox} overexpression, human cDNA for p22^{phox} was subcloned into pCS2^{+} as described in Block et al.[20]

Immunohistochemistry

Specimens containing both tumor and adjacent normal tissue were fixed in formalin and embedded in paraffin block. Four-micrometer-thick sections were baked, deparaffinized in xylene, and rehydrated through a series of ethanol treatment. Antigen retrieval was performed by incubating the sections at 100°C in 10 mmol/L sodium citrate buffer (pH 6.0) for 20 minutes. Endogenous peroxidase activity was quenched by treating with 0.6% hydrogen peroxide solution for 5 minutes. Slides were incubated with 2% bovine serum albumin to block nonspecific antibody binding and then reacted with the primary rabbit polyclonal anti-p22^{phox} (Santa Cruz Biotechnology) at 4°C overnight. After washing in PBS, slides were incubated with the streptavidin-biotin-peroxidase complex (Dako Co., Carpinteria, CA). Sections were visualized by 3,3′-diaminobenzidine chromagen (Dako Co.). Normal rabbit IgG at the same concentration as the primary antibody served as a negative control. Sections were counterstained with H&E.

Human Tumor Specimens

Tumor samples and normal corresponding tissue from patients with RCC were obtained from the Department of Urology at the University of Texas Health Science Center at San Antonio. The tumors for this study were histologically classified as clear cell renal carcinoma and staged according to the TNM classification. The collection and handling of human samples was performed according to a protocol approved by the University of Texas Health Science Center at San Antonio, Institutional Review Board.

Results

p22^{phox} Inactivates Tuberin through an Akt-Dependent Mechanism

Inactivation of tuberin occurs through Akt-dependent phosphorylation of tuberin at residue Thr^{1462} with subsequent dissociation from hamartin.[18] p22^{phox} is up-regulated in VHL-deficient RCC 786-O cells where Akt activity is constitutively active.[2,11] To examine if NADPH oxidases

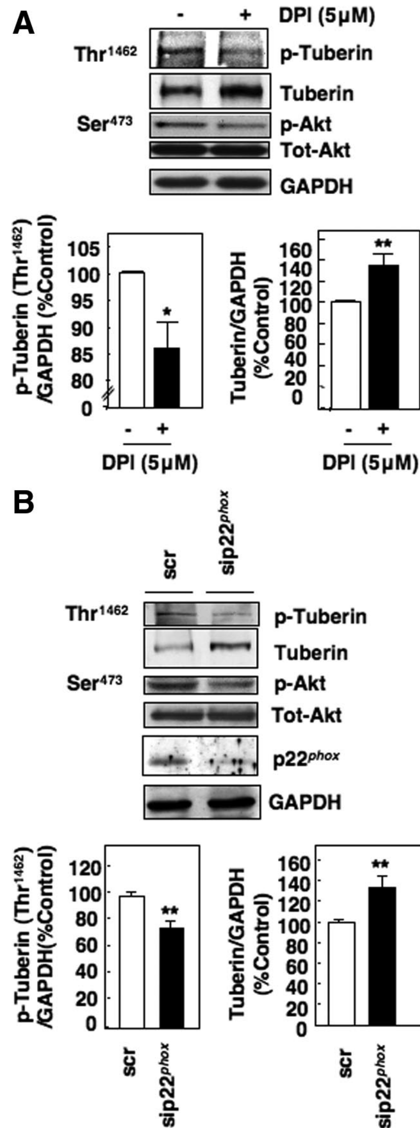

Figure 1. p22^{phox} inactivates tuberin through an Akt-dependent mechanism. **A:** RCC 786-O cells were treated with (+) or without (−) DPI. Equivalent amounts of cell lysates were analyzed for Akt-dependent phosphorylation of tuberin at Thr^{1462}, total tuberin protein expression, Akt phosphorylation, and total Akt protein expression. GAPDH was used as a loading control. Phosphorylation of tuberin at Thr^{1462} or total tuberin protein expression was measured as the ratio of pTuberin (Thr^{1462}) or total tuberin to GAPDH and normalized as percentage of control (% control). The results are expressed as the means ± SE. $*P < 0.05$ and ± SE. $**P < 0.01$, respectively, compared with control (−). **B:** Lysates of RCC 786-O cells transfected with scrambled control (scr) or small-interfering RNA to p22^{phox} (sip22^{phox}) for 48 hours were analyzed by Western blot for tuberin phosphorylation on the Akt-dependent Thr^{1462} site, total tuberin protein expression, Akt phosphorylation, and total Akt protein expression. Knockdown of p22^{phox} was analyzed by using p22^{phox} antibodies, and GAPDH was used as a loading control. Quantification of Akt-dependent phosphorylation of tuberin at Thr^{1462} or total tuberin was measured as in **A**. The results are expressed as the means ± SE. $**P < 0.01$, respectively, compared with control (−). The data (**A** and **B**) are representative of three independent experiments; $n = 3$.

play a role in Akt-dependent inactivation of tuberin, VHL-deficient cells were treated with or without the Nox inhibitor DPI. Treatment with DPI decreased phosphorylation of Akt with concomitant reduction in tuberin phosphorylation at Thr^{1462}. DPI did not alter total Akt protein levels (Figure 1A). To directly test the involvement of p22^{phox},

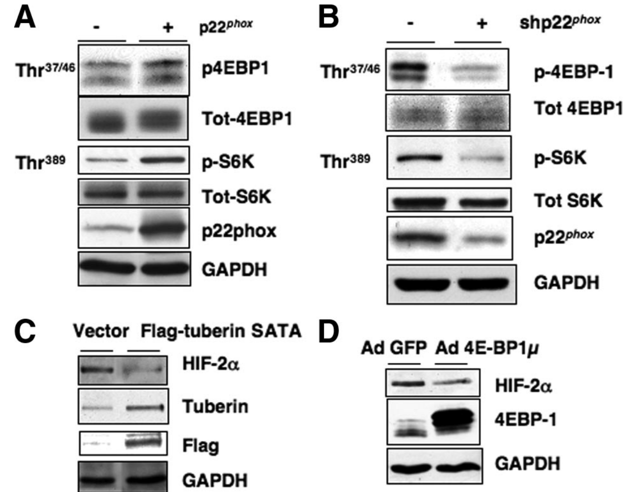

Figure 2. p22^{phox} activates mTOR activity. **A:** Renal proximal tubular epithelial cells expressing VHL (HK2) were mock-transfected or transfected with a mammalian expression plasmid encoding p22^{phox}. Cells were serum starved for 24 hours and cell lysates were prepared. Equivalent amounts of cell lysates were analyzed by Western blot for p4E-BP1, total 4E-BP1, pS6K, Total S6K, p22^{phox}, or GAPDH loading control where $n = 3$. **B:** Lysates of RCC 786-O cells transfected for 48 hours with empty vector or short hairpin RNA-p22^{phox} (shp22^{phox}) were analyzed by Western blot for p4E-BP1, total 4E-BP1, pS6K, Total S6K, p22^{phox}, or GAPDH loading control. Knockdown of p22^{phox} was analyzed as in **B** where $n = 3$. **C:** RCC 786-O cells were transfected with a mammalian expression plasmid expressing Flag-tuberin$^{S939/T1462A\ (SATA)}$, a mutant lacking the major Akt phosphorylation sites. The cell lysates were immunoblotted by using HIF-2α antibody. Expression of Flag-tuberin mutant (tuberin SATA) was evaluated by Western analysis by using Flag and tuberin antibodies. **D:** RCC 786-O cells were infected with an adenovirus vector expressing the mutant form of 4E-BP1 (Ad 4E-BP1μ). The cell lysates were immunoblotted by using HIF-2α antibody. GAPDH was used as a loading control. Expression of 4E-BP1μ was evaluated by Western blot analysis by using 4E-BP1 antibody. The data are representative of three independent experiments.

we used siRNA targeting. siRNA-mediated down-regulation of p22^{phox} decreased Akt phosphorylation as well as tuberin phosphorylation on residue Thr^{1462} (Figure 1B). Importantly, tuberin protein levels are higher in the cells treated with DPI or transfected with sip22^{phox} (Figure 1, A and B, respectively). Effective knockdown of p22^{phox} protein expression was confirmed by p22^{phox} immunoblot analysis (Figure 1B). These results indicate that p22phox-dependent Nox oxidases regulate Akt-mediated phosphorylation of tuberin.

p22^{phox}-Dependent Nox Oxidases Regulate Phosphorylation of S6K and 4E-BP1

Akt-dependent phosphorylation/inactivation of tuberin leads to mTOR activation and subsequent phosphorylation of its downstream targets, S6K and 4E-BP1.[21,22] Therefore, the phosphorylation status of 4E-BP1 (Thr37/46) and S6K (Thr389) was used as a surrogate for mTOR-dependent activation. Overexpression of p22^{phox} in renal proximal tubular epithelial cells that express VHL (HK2) resulted in increased phosphorylation of mTOR targets, 4E-BP1 and S6K (Figure 2A). Conversely, short hairpin RNA-mediated knock-down of p22^{phox} in VHL-deficient cells results in decreased phosphorylation of 4E-BP1 and S6K (Figure 2B). Overexpression or knockdown of p22^{phox} was monitored by p22^{phox} immunoblot analysis

(Figure 2, A and B, respectively). Taken together, these results indicate that p22*phox*-dependent Nox oxidases are upstream regulators of mTOR activity and initiation of translation-dependent pathways.

We have previously demonstrated that p22*phox* maintains HIF-2α protein expression, in part, through the phosphoinositide 3-kinase/Akt-dependent translational pathway.[11] To determine whether Akt-dependent phosphorylation of tuberin play a role in maintaining HIF-2α, a Flag-tuberin mutant (Flag-tuberin SATA) lacking the major Akt phosphorylation sites (Ser^{939}A and Thr^{1462}A) was overexpressed in RCC 786-O cells.[17] Expression of the tuberin mutant resulted in a decrease in HIF-2α protein levels (Figure 2C). Successful transfection of the tuberin mutant was monitored by Western blot analysis using tuberin and Flag antibodies. To confirm the role of mTOR-mediated translation in HIF-2α protein accumulation, the cells were infected with an adenovirus vector expressing 4E-BP1 mutant (4E-BP1μ) that harbors mutations in all four mTOR phosphorylation sites (T35A, T45A, T69A, and S64A).[19] HIF-2α protein expression is decreased in cells expressing 4E-BP1μ (Figure 2D). 4E-BP1μ protein expression was monitored by using an antibody that recognizes 4E-BP1 (Figure 2D). Together, these results indicate that p22*phox* maintains HIF-2α through Akt-dependent inactivation of tuberin and subsequent mTOR activation.

Akt Is Required for Activation of mTOR, Downstream of Tuberin

We have shown that p22*phox*-mediated Akt-dependent tuberin phosphorylation and subsequent mTOR activation are required for maintaining HIF-2α protein expression in the absence of VHL[11] (Figures 1 and 2). We next examined whether Akt modulates mTOR signaling. Inhibition of Akt with pharmacological reagent or through infection of the cells with an adenovirus vector expressing a HA-tagged dominant-negative mutant of Akt (Ad DN Akt) resulted in a decrease in phosphorylation of mTOR downstream targets, 4EBP-1 and S6K (Figure 3, A and B). Ad GFP was used as control. Expression of the dominant-negative HA-Akt was confirmed by Western blot analysis using antibodies against HA and total Akt. This suggests that Akt mediates mTOR-dependent phosphorylation of 4EBP-1 and S6K in VHL-deficient cells. Similarly, we assessed whether tuberin stabilization is mediated through Akt. To this end, expression of dominant-negative Akt significantly increased the expression of tuberin (Figure 3C). These observations suggest Akt-mediated regulation of tuberin abundance.

Superoxide is the Predominant ROS Involved in the Regulation of Signaling Pathways Necessary for Maintaining HIF-2α Protein Expression

Nox oxidases generate superoxide anion (O_2^-), which can be dismutated to hydrogen peroxide (H_2O_2). To determine whether superoxide or hydrogen peroxide is in-

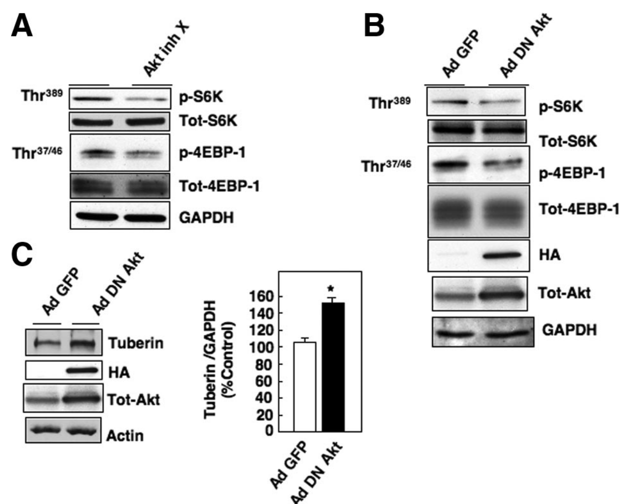

Figure 3. Akt is required for activation of mTOR targets, p4EBP-1 and S6K. **A:** RCC 786-O cells were treated without (−) or with (+) the Akt inhibitor X (Akt inh X-2 μmol/L) for 24 hours. Equivalent amounts of cell lysates were analyzed by Western blot analysis for mTOR downstream targets, pS6K, total S6K, p4E-BP1, total 4E-BP1, or GAPDH loading control. **B** and **C:** RCC 786-O cells were infected 48 hours with Ad DN Akt or Ad GFP control. Equivalent amounts of cell lysates were analyzed by Western blot analysis for mTOR downstream targets, pS6K, Total S6K, p4E-BP1, total 4E-BP1, or GAPDH loading control (**B**) or tuberin and Actin loading control (**C**). Expression of DN-Akt was evaluated by Western analysis for HA or Akt antibody. GAPDH was used as a loading control. Tuberin protein expression was measured as the ratio of total tuberin to GAPDH and normalized as percentage of control (% control). The results are expressed as the means ± SE. $^*P < 0.05$ compared with control (Ad GFP). The data are representative of three independent experiments.

volved in maintaining HIF-2α expression, RCC 786-O cells were treated with vehicle or antioxidants that scavenge or inhibit H_2O_2, (PEG-Catalase, NAC) or antioxidants that scavenge O_2^- (Tiron). There is only a modest decrease in HIF-2α expression using PEG-Catalase or NAC, whereas Tiron and DPI resulted in marked decrease in HIF-2α (Figure 4, A and B, respectively). Tiron also inhibited phosphorylation of tuberin, Akt, and 4E-BP1 with concomitant increase in tuberin protein expression (Figure 4C). Collectively, these data suggest that superoxide anion, or a downstream product of superoxide, is the predominant species implicated in HIF-2α regulation.

*p22*phox *Protein Levels are Increased in Human Renal Carcinoma*

We have previously demonstrated that p22*phox* is overexpressed in VHL-deficient cells. We therefore examined the protein expression of p22*phox* in human clear cell RCC. Levels of p22*phox* protein were significantly upregulated in ~90% of the tumors compared with the normal adjacent epithelium as examined by immunoblot analysis (Figure 5A). Immunohistochemical analysis of renal tumors and normal adjacent epithelium in paraffin embedded tissue using p22*phox* antibody showed significantly increased levels of p22*phox* (Figure 5B). In the bottom panels (Figure 5B), the tumor and normal adjacent tissue were probed with nonimmune IgG to demonstrate specificity. Figure 5C shows representative histo-

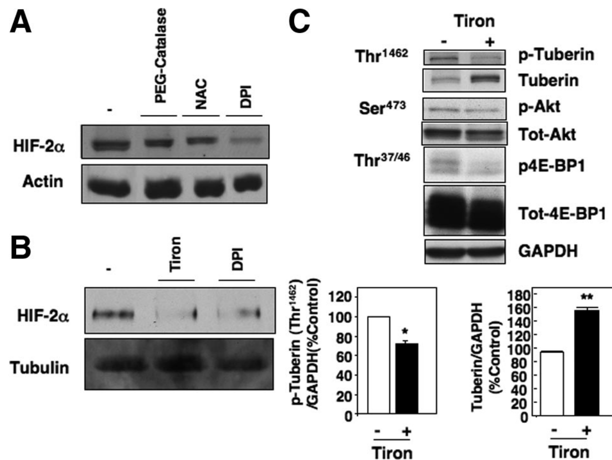

Figure 4. Superoxide is the major ROS involved in maintaining HIF-2α in the absence of VHL. **A:** Where indicated, antioxidants targeting hydrogen peroxide, PEG-Catalase (500 U/ml), NAC (100 mmol/L), or vehicle alone (−) were added to RCC 786-O cells for 24 hours. DPI (5 μmol/L) was used as a control. Western blot analysis for HIF-2α was performed on equivalent amount of cell lysates. Actin was used as a loading control. **B:** Where indicated, antioxidants targeting superoxide anion or with vehicle alone (−), Tiron (10 mmol/L) was added as described in **A**. Western blot analysis was performed on equivalent amount of cell lysates for HIF-2α protein expression. Tubulin was used as a loading control. **C:** RCC 786-O cells were treated with (+) or without (−) Tiron. Equivalent amounts of cell lysates were analyzed for p-tuberin at Thr^{1462}, total tuberin, p-Akt, total Akt p4E-BP1, and total 4E-BP1. GAPDH was used as a loading control where $n = 3$. Phosphorylation of tuberin at Thr^{1462} or total tuberin protein expression was measured as the ratio of pTuberin (Thr^{1462}) or total tuberin to GAPDH and normalized as percentage of control (% control). The results are expressed as the means ± SE. $^{*}P < 0.05$ and ± SE. $^{**}P < 0.01$, respectively, compared with control (−).

logical sections of RCC and normal adjacent tissue samples used in these studies.

Proximal tubular epithelial cells predominantly express the Nox catalytic subunits Nox4 and Nox1.[11] Supplemental Figures S1, A and B, show that Nox4 and Nox1 proteins are significantly elevated in human clear cell RCC as compared with adjacent control tissue (Supplemental Figure S1, A and B, see *http://ajp.amjpathol.org*).

Tuberin is Inactivated in Human Renal Carcinomas

Because p22*phox* protein expression modulates tuberin phosphorylation and protein expression *in vitro*, we examined tuberin phosphorylation in the human clear cell renal tumors compared with normal adjacent tissue. Phosphorylation of tuberin at Thr^{1462} was significantly increased concomitant with significantly decreased tuberin protein expression in human RCC compared with normal adjacent tissue (Figure 6, A and B). This observation was supported by the finding that phosphorylation of Akt was significantly increased in the tumors compared with the normal adjacent tissue (Figure 7, A and B). Taken together these results indicate that high levels of p22*phox* protein expression in human clear cell RCC play an important role in inhibiting tuberin function, at least in part, through Akt-dependent mechanisms.

4E-BP1 Phosphorylation is Increased in Human Clear Cell RCC

We have shown *in vitro* that increased phosphorylation of tuberin coincides with increased phosphorylation of 4E-BP1 in VHL-deficient cells. Therefore, we examined the phosphorylation status of 4E-BP1 as a surrogate for mTOR-dependent translational activation in human clear cell RCC. Figure 8, A and B, shows enhanced phosphorylation of 4E-BP1 in clear cell RCCs compared with normal adjacent tissue. This highly correlated with clear cell RCCs that overexpress p22*phox*. These results suggest that up-regulation of the p22*phox*-based Nox oxidases

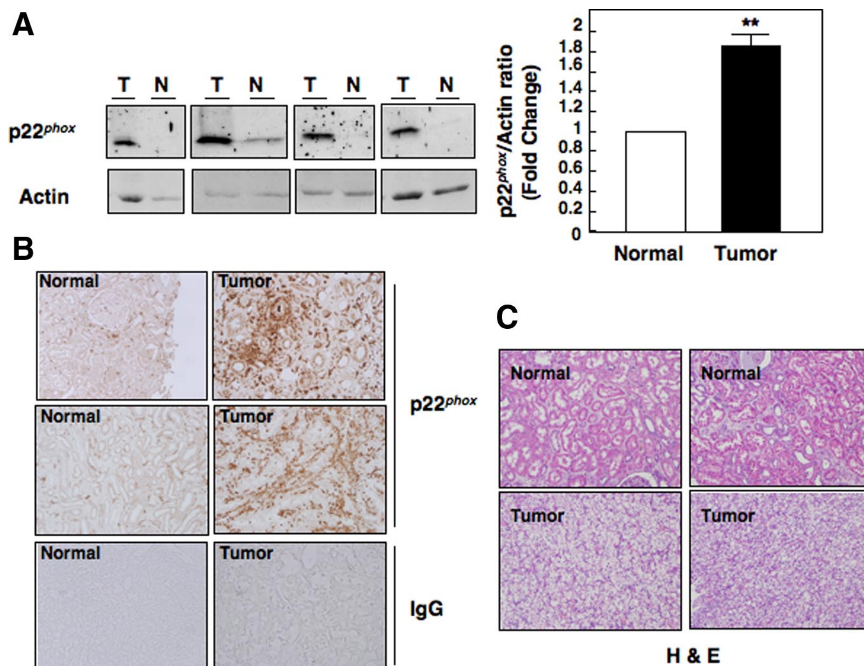

Figure 5. p22*phox* protein expression is elevated in RCC tumors compared with normal adjacent renal tissue. **A:** Expression of p22*phox* was determined by direct immunoblotting in cell lysates prepared from human clear cell RCC and normal adjacent control tissue. Actin was used as a loading control. The histogram in the **right panel** represents the ratio of the intensity of p22*phox* bands quantified by densitometry factored by the densitometric measurements of the actin band. The data are expressed as fold change where the ratio in the control was defined as 1. Values are the means ± SE ($n = 13$); $^{**}P < 0.01$. **B:** p22*phox* expression was detected by immunoperoxidase staining in clear cell RCC (tumor) and normal adjacent tissue samples. In the **bottom panel**, tissue sections were probed with IgG as a negative control (IgG). Representative images are shown ($n = 17$). **C:** H&E staining of representative human clear cell RCC and normal adjacent renal tissue used for these studies.

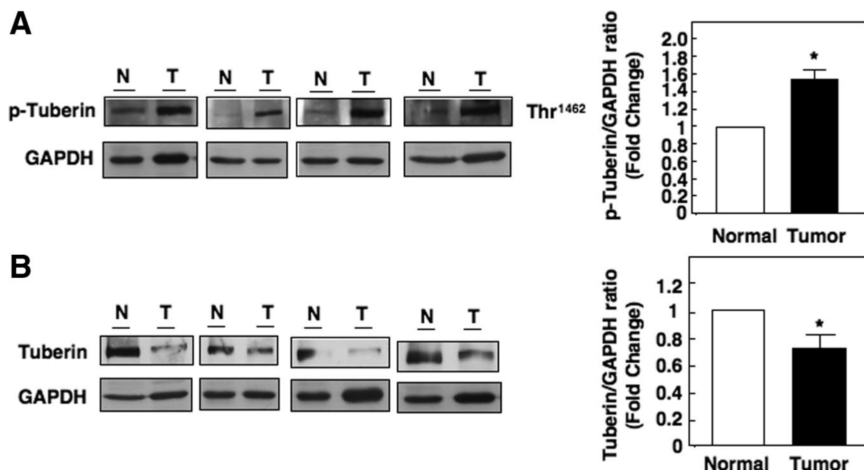

Figure 6. Increased tuberin phosphorylation and decreased tuberin protein expression in RCC. **A:** Expression of pTuberin (Thr^{1462}) was determined by direct immunoblotting in cell lysates prepared from human clear cell RCC and normal adjacent control tissue. GAPDH was used as a loading control. The histogram in the **right panel** represents the ratio of the intensity of ptuberin bands quantified by densitometry factored by the densitometric measurements of the GAPDH band. The data are expressed as fold change where the ratio in the control was defined as 1. Values are the means ± SE (n = 7); *$P < 0.05$. **B:** Expression of tuberin was determined by direct immunoblotting in cell lysates prepared from human clear cell RCC and normal adjacent control tissue. GAPDH was used as a loading control. The histogram in the **right panel** represents the ratio of the intensity of tuberin bands quantified by densitometry factored by the densitometric measurements of the GAPDH band. The data are expressed as fold change where the ratio in the control was defined as 1. Values are the means ± SE (n = 7); *$P < 0.05$.

correlates with the activation of the translational pathway in human clear cell RCC.

Discussion

In this study we identified a major role for p22^{phox}-dependent Nox oxidases as predominant positive regulators of translational signaling pathways necessary for maintaining HIF-2α protein expression in VHL-deficient cells. We find that p22^{phox}-dependent Nox oxidases promote tuberin inactivation through Akt phosphorylation, leading to the phosphorylation of mTOR targets, S6K and 4E-BP1, and subsequent stabilization of HIF-2α protein expression (Figure 9). Moreover, we provide evidence that this pathway is similarly dysregulated in human RCC. Our data indicate that in tumor samples of RCC there is a positive correlation between the expression of p22^{phox} and inactivation of tuberin that results in activation of mTOR-dependent signaling pathway.

We have previously reported that Akt-dependent translation is critical for p22^{phox}-mediated HIF-2α protein accumulation in VHL-deficient cells. However, the role of downstream targets of Akt in p22^{phox}-induced translational regulation of HIF-2α has not yet been investigated. The hamartin/tuberin complex functions normally as a tumor suppressor through negative regulation of translation.[23] Tuberin is inhibited by Akt-dependent phosphorylation on Thr^{1462} and subsequent dissociation from hamartin. Moreover, it has been shown that phosphorylation of tuberin by Akt in hematopoietic cells triggers its ubiquitination and proteasome-dependent degradation.[18] We find that p22^{phox} inactivates tuberin through both mechanisms, as treatment of VHL-deficient cells with the Nox inhibitor, DPI, or down-regulation of p22^{phox} resulted in decreased tuberin phosphorylation on Akt-dependent residue Thr^{1462} and stabilized tuberin protein levels. The critical role for Akt phosphorylation of tuberin is confirmed by the observation that transfection of RCC 786-O cells with a tuberin mutant lacking the major Akt phosphorylation sites (S^{939}AT^{1462}A) results in a decrease in HIF-2α protein expression.

The negative effect of tuberin on translation is due to its inhibition of mTOR signaling. Hence, inactivation of tuberin by Akt leads to the phosphorylation of S6K and 4E-BP1 by mTOR.[21,22] Therefore, we examined the phosphorylation status of S6K and 4E-BP1 as surrogates for mTOR-dependent translational activation. 4E-BP1 acts as a translational repressor by binding and inhibiting the eukaryotic translation initiation factor 4E, which recognizes the 5′ end cap of eukaryotic mRNAs. Phosphorylation of 4E-BP1 by mTOR results in the dissociation of 4E-BP1 from eukaryotic translation initiation factor 4E, thereby relieving the inhibition of 4E-BP1 on eukaryotic translation initiation factor 4E-dependent translation initiation.[24,25] We also find that p22^{phox} is required for phosphorylation of the mTOR targets, S6K and 4E-BP1. This is confirmed by the observation that overexpression of p22^{phox} in cultured renal proximal tubular epithelial cells, which express VHL, is sufficient for phosphorylation of mTOR downstream targets 4E-BP1 and S6K. The impor-

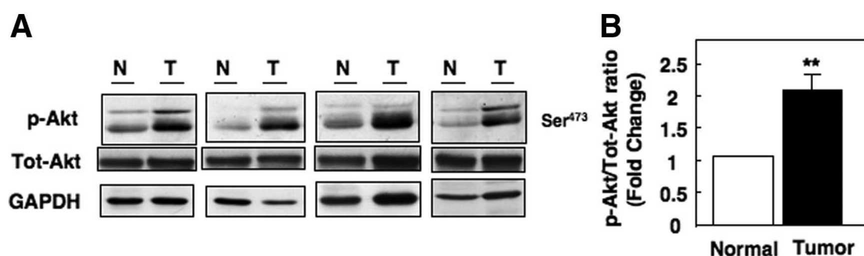

Figure 7. Phosphorylation of Akt is higher in RCC compared with normal adjacent renal tissue. **A:** Expression of pAkt and total Akt was determined by direct immunoblotting in cell lysates prepared from human clear cell RCC and normal adjacent control tissue. GAPDH was used as a loading control. The histogram in **B** represents the ratio of the intensity of pAkt bands quantified by densitometry factored by the densitometric measurements of the total Akt bands. The data are expressed as fold change where the ratio in the control was defined as 1. Values are the means ± SE (n = 7); **$P < 0.01$.

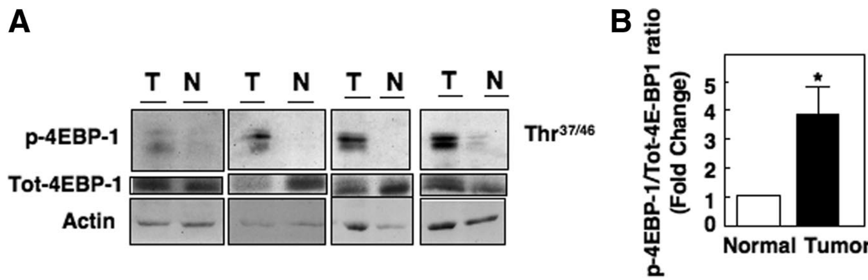

Figure 8. Phosphorylation of 4E-BP1 protein is higher in RCC compared with normal adjacent renal tissue. **A:** The phosphorylation status of 4E-BP1 on S37/46 and total 4E-BP1 were examined in lysates prepared from human clear cell RCC and normal adjacent control tissue by Western blot analysis. Actin was used as a loading control. The histogram in **B** represents the ratio of the intensity of the phosphorylated 4E-BP1 bands quantified by densitometry factored by the densitometric measurements of the total 4E-BP1 bands. The data are expressed as fold change where the ratio in the control was defined as 1. Values are the means ± SE ($n = 13$); $^*P < 0.05$.

tance of mTOR in the regulation of HIF-2α accumulation is further demonstrated by the finding that overexpression of nonphosphorylatable form of 4E-BP1 (DN 4E-BP1μ) resulted in decreased HIF-2α protein expression in VHL-deficient cells. Furthermore, inhibition of Akt with pharmacological inhibitor or dominant-negative Akt results in decreased phosphorylation of 4E-BP1 and S6K and decreased HIF-2α protein levels.[11]

Collectively, the data indicate that p22^{phox}-containing Nox oxidases are an upstream regulator of a translational pathway leading to the accumulation of HIF-2α. This effect of p22^{phox} involves Akt-mediated tuberin inactivation and subsequent phosphorylation of the mTOR targets S6K and 4E-BP1. Nox oxidase regulation of HIF-2α is additionally supported by the finding of Maranchie and Zhan[10] that down-regulation of Nox4 decreases HIF-2α mRNA levels. The mTOR pathway has recently been linked to transcriptional regulation of several genes.[22] Whether p22^{phox} regulates HIF-2α not only through translation but also through a putative mTOR-dependent transcriptional mechanism remains to be determined.

We also explored the predominant species of ROS involved in HIF-2α translational regulation. Our data suggest that superoxide anion, or a downstream product of superoxide, is the predominant species involved in regulation of signaling pathways necessary for maintaining HIF-2α protein expression as antioxidants targeting superoxide, but not hydrogen peroxide, mimic the effect of p22^{phox} down-regulation.

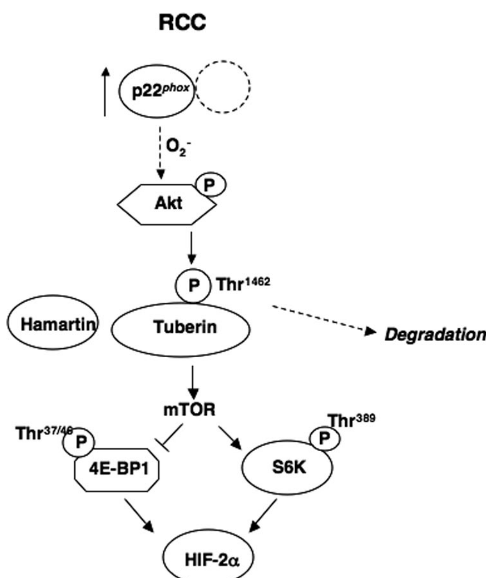

Figure 9. Proposed mechanism of p22^{phox}-dependent regulation of HIF-2α mRNA translation in RCC.

The mechanism by which ROS activate Akt is currently unknown. One possibility is that Akt activation is due to ROS-dependent inhibition of PTEN, a protein phosphatase that dephosphorylates PIP_3, the upstream activator of Akt.[26,27] However, PTEN expression is absent in VHL-deficient RCC 786-O cells, suggesting that ROS are unlikely to act at the level of the phosphatase.[28,29] Further studies are needed to determine the mechanism by which ROS activate Akt in RCC 786-O cells.

To determine the relevance of our observations in the cultured cells to human RCC, we examined the expression of these signaling molecules in RCC samples. We demonstrate for the first time that the levels of p22^{phox} are significantly elevated in ~90% of human clear cell RCC samples compared with normal adjacent tissue. We also observed that the increase in p22^{phox} is associated with increased expression of Nox4 and Nox1 proteins. The mechanism by which VHL-deficiency results in an increase in p22^{phox} and Nox4/Nox1 protein expression remains to be determined. VHL-deficiency may result in concomitant up-regulation of p22^{phox} and the Nox catalytic subunits. Alternatively, up-regulation of p22phox may result in stabilization of the catalytic subunits.[13–15] Note that the *in vivo* data are in agreement with our previous work showing that p22^{phox} and Nox4 protein expression are up-regulated in RCC 786-O cells.[11]

Nox subunits are regulated and activated by growth factors, cytokines,[16,30–32] and hypoxia,[33–35] parameters involved at various stages during the development of RCC. Expression of p22^{phox} protein in RCC was independent of tumor grade or stage examined in our study (data not shown). Taken together, our findings suggest that up-regulation p22^{phox}-based Nox oxidases are an early event possibly involved in the initiation and development of human clear cell RCC through the accumulation of HIF-2α.

Interestingly, we also report that tuberin is inactivated through post-translational modification in human clear cell RCC. We detect a significant increase of tuberin phosphorylation on the Akt-dependent residue, Thr^{1462}, and decreased tuberin protein levels in RCC tumors compared with normal adjacent tissue of the same patients. Our current findings that tuberin function is inhibited by a p22^{phox}-based Nox oxidase mechanism is of importance because germline mutation of the tuberin gene (*TSC2*) has been associated with RCC; however, sporadic mu-

tations in the *TSC2* gene have not been identified in VHL-deficient RCC.[36,37] Importantly, the Eker rat model, which harbors inactivating mutation of the *TSC2* gene, while retaining wild-type *VHL*, demonstrates increased HIF-2α protein expression and gives rise to vascularized tumors.[38] To our knowledge, this is the first study demonstrating tuberin inactivation via post-translational mechanisms in RCC.

In conclusion, specific catalytic Nox subunits, Nox1 and Nox4, and their regulatory component, p22*phox*, are up-regulated in renal cell carcinoma. Our findings reveal a pivotal role of p22*phox*-containing Nox oxidases as proximal regulators of a translational pathway leading to the accumulation of HIF-2α in renal cell carcinoma. Thus pharmacological inhibition of flavoprotein oxidases, and in particular blockade of p22*phox* binding to the Nox catalytic subunits, are potential alternative approaches to down-regulate HIF-2α expression and its target pathways involved in renal carcinogenesis.

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