VHL promotes immune response against renal cell carcinoma via NF-κB–dependent regulation of VCAM-1

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Vascular cell adhesion molecule 1 (VCAM-1) is an adhesion molecule assigned to the activated endothelium mediating immune cells adhesion and extravasation. However, its expression in renal carcinomas inversely correlates with tumor malignancy. Our experiments in clear cell renal cell carcinoma (ccRCC) cell lines demonstrated that von Hippel Lindau (VHL) loss, hypoxia, or PHD (for prolyl hydroxylase domain–containing proteins) inactivation decreased VCAM-1 levels through a transcriptional mechanism that was independent of the hypoxia-inducible factor and dependent on the nuclear factor κB signaling pathway. Conversely, VHL expression leads to high VCAM-1 levels in ccRCC, which in turn leads to better outcomes, possibly by favoring antitumor immunity through VCAM-1 interaction with the α4β1 integrin expressed in immune cells. Remarkably, in ccRCC human samples with VHL nonmissense mutations, we observed a negative correlation between VCAM-1 levels and ccRCC stage, microvascular invasion, and symptom presentation, pointing out the clinical value of VCAM-1 levels as a marker of ccRCC progression.

Introduction

Kidney cancer is among the top 10 most common cancers in the world. The most frequent type of renal neoplasm is renal cell carcinoma (RCC), which accounts for 85% of all renal malignancies. It is estimated that 320,000 new cases will be diagnosed in 2016, with the number of deaths ∼140,000 worldwide (Kabaria et al., 2016). RCC is the most common malignant tumor of the adult kidney, and tumor resection is the only effective treatment (Garcia and Rini, 2007) because of its resistance to chemotherapy (Hartmann and Bokemeyer, 1999) and radiotherapy (Blanco et al., 2011). Clear cell RCC (ccRCC) is a subtype of RCC that comprises 75–88% of cases (Linehan et al., 1995). It is typically characterized by malignant epithelial cells with clear cytoplasm and a compact-alveolar (nested) or acinar growth pattern interspersed with intricate, arborizing vasculature. Somatic gene mutations in the von Hippel-Lindau (VHL) tumor suppressor protein are the main cause in most primary sporadic ccRCC (Kaelin, 2002). Moreover, VHL loss in animal models has clearly shown a role of VHL as a gatekeeper gene in the pathogenesis of ccRCC (Gossage et al., 2015; Pritchett et al., 2015).

VHL's best-known function is to act as the recognition subunit of a complex with ubiquitin ligase activity, which targets hypoxia-inducible factor α (HIF-α) subunits for proteasomal degradation (Maxwell et al., 1999). The molecular basis for VHL recognition and degradation of HIF is the hydroxylation of key proline residues in the conserved motif LXXLAP (Jaakkola et al., 2001). This hydroxylation is mediated by a family of oxygen and 2-oxoglutarate–dependent dioxygenases termed PHD (for prolyl hydroxylase domain–containing proteins; Bruick and McKnight, 2001). Therefore, under hypoxia, PHD activity is significantly decreased, and HIF is stabilized, promoting transcription of multiple target

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Abbreviations used: ccRCC, clear cell RCC; DMOG, dimethyloxalylglycine; HIF, hypoxia-inducible factor; hnRNA, heterogeneous nuclear RNA; HUVEC, human umbilical vein endothelial cell; IKKα, inhibitor of NF-κB kinase; NF-κB, nuclear factor κB; NIK, NF-κB–inducing kinase; RCC, renal cell carcinoma; VCAM-1, vascular cell adhesion molecule 1; VHL, von Hippel Lindau.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 216 No. 3 835–847
https://doi.org/10.1083/jcb.201608024

genes (Semenza, 2003). Hypoxia is a characteristic of almost all types of solid tumors, and it has been associated with poor outcome in several human malignancies (Höpfl et al., 2004; Brahimi-Horn et al., 2007). However, although the role of VHL and hypoxia in the regulation of HIF has proven to be important for tumor growth, other VHL functions independent of HIF help to explain why loss of VHL leads to renal cancer (Maranchie et al., 2002; Hu et al., 2003; Stickle et al., 2004; Calzada et al., 2006).

Our present results have identified a new target gene that is regulated by VHL and hypoxia in ccRCC cell lines and may help to explain why these tumors are highly invasive. Vascular cell adhesion molecule 1 (VCAM-1), as this new target, is a member of the immunoglobulin gene superfamily first described as a cytokine-inducible endothelial adhesion molecule (Osborn et al., 1989; Carlos et al., 1990). This molecule is highly expressed in activated endothelium and participates in the recruitment of inflammatory cells to sites of tissue injury when it binds monocytes and lymphocytes expressing the integrins α4β1 (VLA-4) and α4β7 (Elices et al., 1990; González-Amaro and Sánchez-Madrid, 1999). The interaction between VCAM-1 and VLA-4 is crucial to many immunological processes, including lymphocyte-mediated cell lysis (van Seventer et al., 1991). VCAM-1 is also expressed in renal epithelium (Seron et al., 1991), and it has been considered as a predictor of cancer-free survival in renal carcinomas (Vasselli et al., 2003; Shioi et al., 2006; Yao et al., 2008). However, the mechanisms underlying VCAM-1 regulation in these tumors are yet unknown.

It is well established that nuclear factor κB (NF-κB) is one of the main regulators of VCAM-1 in many different cell types (Lin et al., 2015). NF-κB describes various dimeric complexes with members of its protein family, which comprises Rel (c-Rel), Rel A (p65), Rel B, NF-κB1 (p50), and NF-κB2 (p52; Ghosh et al., 1998). NF-κB is activated by TNF, a cytokine produced by activated leukocytes and many other cell types that is involved in systemic inflammation (Dempsey et al., 2003). Previous studies demonstrate that VHL loss induces heightened activity of the NF-κB classical pathway (Qi and Ohh, 2003; An et al., 2005); however, the molecular mechanisms underlying VHL-mediated suppression of NF-κB have not been completely elucidated. In addition, NF-κB activation is a critical component in the transcriptional response to hypoxia (Culver et al., 2010). Taking this into account, we hypothesized that decreased levels of VCAM-1 in ccRCC, which has been clearly related with a worse prognosis, might be caused by the effects of VHL loss or hypoxia on the NF-κB signaling pathway.

Our present results proved that VHL-deficient ccRCC cells and cells subjected to hypoxia had decreased VCAM-1 levels. We also demonstrated that, under such conditions, VCAM-1 levels decreased by a transcriptional mechanism in which the NF-κB signaling pathway was involved. Although we demonstrated that both VHL loss and hypoxia similarly decreased VCAM-1 levels, this effect was independent of HIF. Interestingly, we observed that inhibition or suppression of PHD activity also affected VCAM-1 levels, indicating that a cross talk between all these pathways might be responsible for the regulation of VCAM-1 in these tumors. Furthermore, our functional studies indicated that VCAM-1 decrease in these tumor cells contributed to decrease the antitumoral immune response.

Results

VHL loss and hypoxia regulate VCAM-1 levels in ccRCC cells

Although previous studies have suggested the significance of VCAM-1 levels in renal carcinomas, little is known about the mechanisms that regulate this adhesion molecule in ccRCC. We aimed to study whether VHL loss, the most characteristic event occurring in these tumors, affected VCAM-1 expression. To this aim, first, we analyzed VCAM-1 mRNA and protein expression levels in ccRCC cell lines that expressed a nonfunctional or aberrant VHL protein (786-O and RCC4) and that were stably transfected with empty vector (786-O-pRv and RCC4-pRv) or with wild-type VHL (786-O-VHL and RCC4-VHL), respectively; we also analyzed cells that expressed normal VHL (Caki-1) and cells with a nonfunctional mutated VHL (Caki-2). Our results proved that VCAM-1 mRNA and protein levels were significantly decreased in cells lacking functional VHL (Fig. 1, A and B). To confirm that this regulation was not a side effect caused by the overexpression of VHL, we analyzed the effect of VHL loss in the ACHN cell line, which expresses functional VHL, and knocked down its expression using siRNA. Our results confirmed that VHL loss significantly affected VCAM-1 mRNA levels (Fig. S1). Because hypoxia is considered a hallmark for tumor progression and is associated with disease progression and poor prognosis, we also analyzed the effects of hypoxia in the levels of VCAM-1 in these tumor cells. Similar to VHL loss, hypoxic conditions decreased VCAM-1 mRNA and protein levels (Fig. 1, A and B). Furthermore, the decrease in mRNA levels was observed at the short time of 6 h, whereas protein changes were observed at 12 h (Fig. S2).

Next, we asked whether VCAM-1's decreased mRNA levels were caused by transcriptional regulation or mediated by posttranscriptional events (e.g., processing or degradation). To this aim, first, we analyzed mRNA stability by treating 786-0-VHL cells with the transcriptional inhibitor actinomycin D and then subjected them to normoxia or hypoxia for different times. Our results proved that hypoxia did not affect the stability of VCAM-1 mRNA (Fig. 1 C). To further confirm that a transcriptional mechanism was involved, we analyzed the heterogeneous nuclear RNA (hnRNA) mRNA before splicing modification, which proves to be useful to determine gene transcriptional state. We found that hnRNA was similarly decreased under hypoxia or in VHL-negative cells (Fig. 1 D). Therefore, these data provide support for a transcriptional repression of VCAM-1 mediated by VHL loss or hypoxia.

Because VCAM-1 is a cell membrane protein, we asked whether a decrease in total protein levels resulted in decreased expression in the cell membrane. Analysis of VCAM-1 membrane protein by flow cytometry revealed a significant decrease in VHL-deficient cells compared with their counterparts stably transfected with VHL and in VHL-positive cells subjected to hypoxic conditions compared with normoxia (Fig. 1 E). These results indicated that VHL loss or hypoxia affects VCAM-1 exposition in the cell surface, which in turnmight also have functional effects.

Transcriptional regulation of VCAM-1 under hypoxia or in the absence of VHL is not mediated by HIF

VCAM-1 transcriptional regulation in the absence of VHL or in hypoxia suggested that the HIFs might be involved in downregulating VCAM-1 in ccRCC cell lines. To address this aim, we knock down their expression by using specific siRNA to HIF-1α

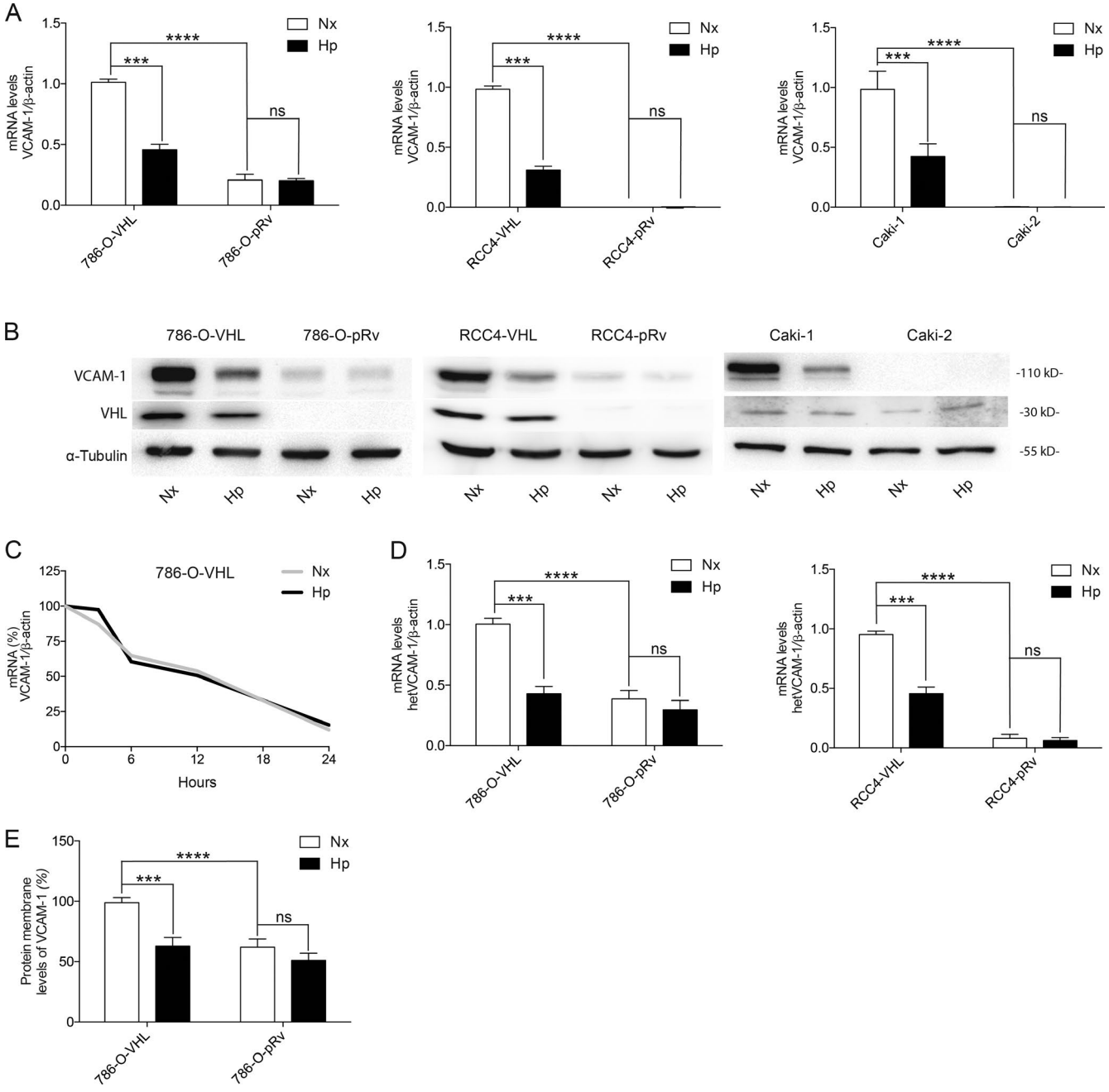

Figure 1. **Effect of VHL loss and hypoxia on VCAM-1 levels in ccRCC cell lines.** (A) VCAM-1 mRNA levels were determined by quantitative RT-PCR in 786-O and RCC4 transfected with empty vector pRv (786-O-pRv and RCC4-pRv) or with pRv-VHL (786-O-VHL and RCC4-VHL), Caki-1, and Caki-2 and exposed to normoxia (Nx) or hypoxia 0.5% O_2 (Hp) for 24 h. mRNA levels are expressed as fold-change and normalized over the levels of VHL-positive cells in normoxic conditions and controlled with *β-Actin* as the housekeeping gene. Statistical analysis was done using two-way ANOVA followed by Bonferroni's posthoc test. *n* = 6 experiments. (B) VCAM-1 and VHL protein levels were analyzed in total cell lysates from 786-O-pRv, 786-O-VHL, RCC4-pRv, RCC4-VHL, Caki-1, and Caki-2 cell lines exposed to normoxia or hypoxia 0.5% O_2 for 24 h. A representative Western blot (*n* = 6) is shown. As a loading control, α-tubulin was used. (C) 786-O-VHL cells were treated with 2 µg/ml actinomycin D and then cultured under normoxia or hypoxia for the indicated times. Gene expression is represented as fold-change and normalized over the levels of VHL-positive cells in normoxic conditions (considered 100%) and controlled with *β-Actin* gene expression levels. *n* = 4 experiments. (D) VCAM-1 mRNA levels were determined by quantitative RT-PCR before splicing (measuring nuclear heterogeneous RNA) in 786-O-pRv, 786-O-VHL, RCC4-pRv, and RCC4-VHL exposed to normoxia or hypoxia 0.5% O_2 for 24 h. Gene expression is represented as fold-change over the levels of VHL-positive cells in normoxic conditions and controlled with *β-Actin* as the housekeeping gene. Statistical analysis between different conditions was done using two-way ANOVA followed by Bonferroni's posthoc test. *n* = 4 experiments. (E) VCAM-1 levels at the cell membrane were determined by flow cytometry in 786-O-VHL and 786-O-pRv cells exposed to normoxia or hypoxia 0.5% O_2 for 24 h. Quantification of the fluorescence mean in each condition is shown. Statistical analysis was done using two-way ANOVA followed by Bonferroni's posthoc test. *n* = 4 experiments. The values represent the mean ± SEM. ***, P < 0.001 and ****, P < 0.0001 were considered significant.

or HIF-2α in these cells. HIF-2α interference in 786-O cells, which have lost HIF-1α expression (Raval et al., 2005), did not prevent the decrease in VCAM-1 mRNA levels in VHL-negative cells or in hypoxia (Fig. 2 A, top). However, the expression levels of the HIF-2α target gene *phd3* were significantly decreased (Fig. 2 A, bottom). Similar results were observed when

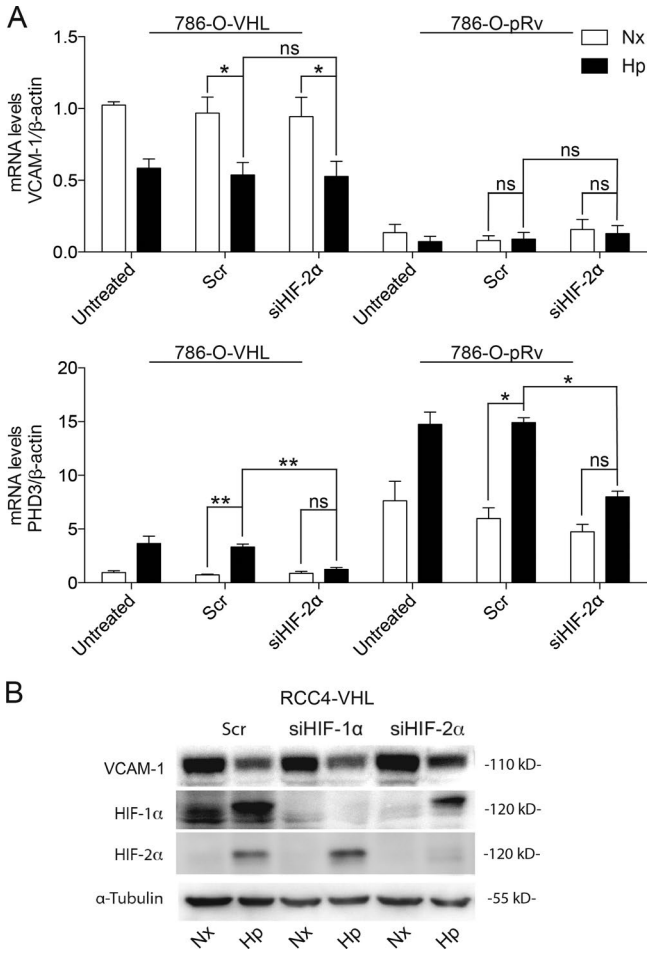

Figure 2. **Role of HIFα in VCAM-1 regulation in ccRCC cell lines.** (A) VCAM-1 and PHD3 mRNA levels in 786-O-VHL or 786-O-pRv cells untreated or transfected with a scrambled siRNA (Scr) or siRNAs specific for HIF-2α (siHIF-2α) were analyzed after 24 h under normoxia (Nx) or hypoxia 0.5% O_2 (Hp). Gene expression was represented as fold-change over the levels of VHL-positive cells in normoxic conditions and controlled with *β-Actin* as the housekeeping gene. Statistical analysis was done using two-way ANOVA followed by Bonferroni's posthoc test. The values represent the mean ± SEM. $n = 3$ experiments. *, $P < 0.05$ and **, $P < 0.01$ were considered significant. (B) VCAM-1, HIF-1α, and HIF-2α protein levels were analyzed in total cell lysates from RCC4-VHL transfected with a scrambled siRNA or siRNAs specific for HIF-1α (siHIF-1α) or HIF-2α (siHIF-2α) and then subjected to 24 h under normoxia or hypoxia 0.5% O_2. A representative Western blot is shown. $n = 3$.

VCAM-1 protein levels were analyzed in RCC4-VHL cells in which HIF-1α or HIF-2α had been interfered (Fig. 2 B).

Additionally, HIF-independent regulation of VCAM-1 in ccRCC cells was also confirmed in cells expressing a mutant form of VHL that has been previously reported to regulate HIF normally but is defective in promoting extracellular fibronectin matrix assembly, the naturally occurring type 2C VHL mutant L188V (Ohh et al., 1999; Hoffman et al., 2001). Our results demonstrated that VCAM-1 mRNA and protein levels in VHL mutant L188V under normoxic or hypoxic conditions were significantly decreased to levels resembling those in VHL-negative cells (Fig. 3, A and B). Conversely, mRNA levels of a well known HIF target gene, *glut-1*, were similarly regulated in VHL and VHL mutant L188V (Fig. 3 A). Collectively, these data demonstrate that VCAM-1 transcriptional regulation in ccRCC cell lines is independent of HIF.

VHL and PHDs control VCAM-1 levels in ccRCC cells via NF-κB pathway

It is well established that the NF-κB pathway regulates VCAM-1 expression (Iademarco et al., 1992; Zerfaoui et al., 2008). To ascertain how hypoxia or VHL loss affected the NF-κB signaling pathway and whether this was involved in VCAM-1 regulation in ccRCC cells, we cultured the cells under hypoxia or treated them with dimethyloxalylglycine (DMOG; a PHD inhibitor) in the absence or presence of the NF-κB agonist TNF. Our results proved that the hypoxia-mediated decrease of VCAM-1 mRNA levels in VHL-positive cells was completely recovered in TNF-treated cells, and those in normoxia were further increased (Fig. 4 A). Conversely, TNF-dependent induction of VCAM-1 was lost in the absence of VHL (Fig. 4 A). To further prove that the NF-κB signaling pathway was involved in keeping high levels of VCAM-1 in VHL-positive cells under normoxia, we treated the cells with an inhibitor of the NF-κB pathway (SM7368), alone or in the presence of TNF. Our results demonstrated that SM7368 treatment significantly decreased VCAM-1 mRNA and protein levels (Fig. 4 B). Similarly, VCAM-1 levels remained low in cells treated with DMOG alone or in combination with TNF (Fig. 4 B). These results prompted us to think that hypoxic decrease of PHD activity was probably involved in this signaling pathway. To ascertain this, we knocked down the three PHDs, PHD1, PHD2, and PHD3, in our VHL-positive cells and analyzed VCAM-1 mRNA and protein levels. We observed that VCAM-1 mRNA and protein levels were decreased when specific siRNA for PHD1, 2, or 3 were used, although this regulation only reached significance when PHD2 or PHD3 were interfered (Fig. 4 C). Altogether, these results indicated that VHL is an essential component in the NF-κB–mediated regulation of VCAM-1 in these cell lines and that PHDs might be a switch that enables VHL to increase VCAM-1 levels.

VHL and hypoxia affect noncanonical NF-κB pathways in ccRCC cells

Our results from Fig. 4 indicated that VCAM-1 repression in ccRCC cells might be caused by decreased NF-κB signaling in cells lacking VHL or in hypoxia. Conversely, previous results demonstrate that VHL loss or hypoxia activates canonic NF-κB signaling in RCC cell lines (Qi and Ohh, 2003; Cummins et al., 2006; Taylor and Cummins, 2009). To reconcile our results with previously published ones, we aimed to analyze whether changes on different members of the noncanonical NF-κB pathways were affected in ccRCC cells and whether these could explain the regulation of VCAM-1 in these cells. Interestingly, protein levels of central signaling components of the noncanonical NF-κB pathway, the NF-κB–inducing kinase (NIK) and its downstream kinase, inhibitor of NF-κB kinase α (IKKα), demonstrated a consistent decrease in cells lacking VHL (Fig. 5 A). In addition, protein levels of the transcriptional activator complex Rel B–p52 were significantly decreased in the absence of VHL or in hypoxic condition, whereas protein levels of the canonical NF-κB transcription factor p65 (Rel A) remained stable in all conditions (Fig. 5 A). To further confirm that defects in the noncanonical pathway were responsible for the decrease in VCAM-1 levels, we knocked down the expression of several components of this pathway in VHL-positive cells. Our results proved that interference of IKKα, NIK, or p52 in VHL-positive cells resulted in a significant decrease of VCAM-1 levels. Conversely, the interference of the canonic component p50 had only minimal effects on VCAM-1 protein levels (Fig. 5 B). These

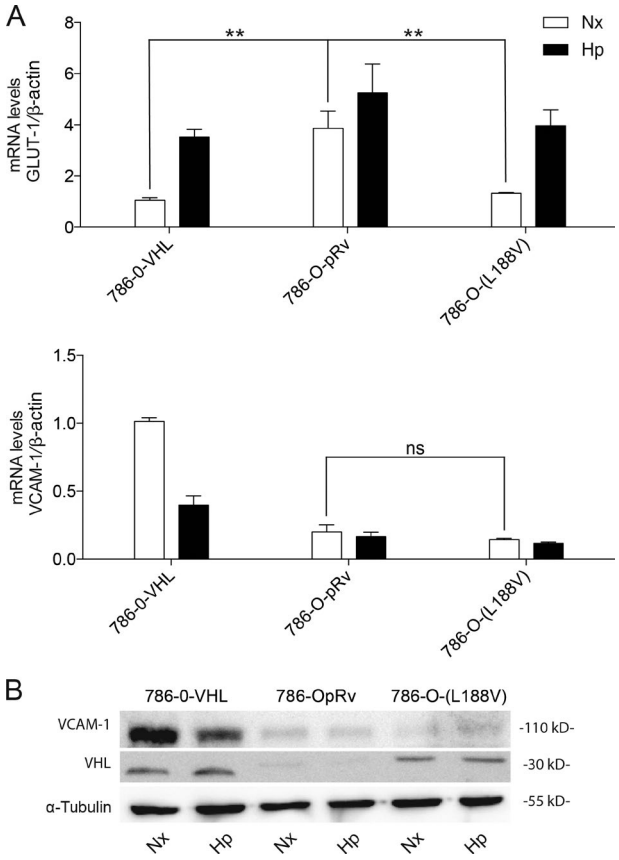

Figure 3. **Effect of VHL mutation L188V in VCAM-1 regulation.** (A) GLUT-1 and VCAM-1 mRNA levels were quantified by RT-PCR in 786-O-VHL, 786-O-pRv, or 786-O-(L188V) (L188V; cells stably expressing the VHL mutant L188V) cultured under normoxia (Nx) or hypoxia 0.5% O_2 (Hp) for 24 h. mRNA levels are represented as fold-change over the levels of VHL-positive cells in normoxic conditions and controlled with *β-Actin* as the housekeeping gene. Statistical analysis was done using two-way ANOVA followed by Bonferroni's posthoc test. The values represent the mean ± SEM. $n = 4$ experiments. **, $P < 0.01$ was considered significant. (B) VCAM-1 and VHL protein levels were analyzed in total cell lysates of 786-O-VHL, 786-O-pRv, or 786-O-(L188V) cells. As a loading control, α-tubulin was used. A representative Western blot is shown. $n = 3$.

results clearly prove that in the absence of VHL or in hypoxia, the noncanonical NF-κB pathway is affected, and this contributes to the regulation of VCAM-1 in these conditions.

VHL loss or hypoxia diminishes monocytic cell adhesion to ccRCC cells

It is well known that VCAM-1 through its binding to the integrins α4β1 and α4β7 mediates binding of circulating monocytes and lymphocytes to the endothelium (Elices et al., 1990). Similarly, VCAM-1 expression on the surface of tumor cells mediates interaction of several cell types of the hematopoietic lineage that express α4 integrins (Fogler et al., 1996). To study whether VCAM-1 decrease in ccRCC cells played any functional role in the biology of these tumor cells, we analyzed their capacity to mediate monocytic cell adhesion. To this aim, we used two monocytic cell lines labeled with fluorescent markers. We incubated them with our ccRCC cell lines and let them interact for 20 min, and afterward, we quantified cell–cell adhesion by counting fluorescent cells. Our results proved that monocytes' binding to ccRCC cells was VHL dependent because adhesion to VHL-negative cells was significantly lower compared with the adhesion to VHL-expressing cells (Fig. 6, A and B). Similarly, hypoxia significantly decreased monocytes binding to ccRCC cells (Fig. 6 C).

Monocytic ccRCC cell adhesion through VCAM-1–α4β1 interaction promotes a cytotoxic immune response

Given that VHL loss or hypoxia regulated the adhesion of monocytic cells to ccRCC, we asked whether this was mediated by VCAM-1 interaction with its cognate receptor the integrin α4β1 (VLA4). To this aim, we knocked down VCAM-1 expression in VHL-positive cells and then tested ccRCC–monocyte cell adhesion. We observed that cell adhesion was significantly decreased when VCAM-1 levels were decreased (Fig. 7, A and B). Interestingly, this adhesion was similar to that observed in VHL-negative cells (Fig. 7 B). More importantly, monocytic cell adhesion to VHL-positive cells was also inhibited in the presence of anti–VCAM-1 receptor–blocking antibodies, like those blocking the α4 or β1 subunits of the integrin α4β1, whereas no effects were observed with an αL-blocking antibody as a control (Fig. 7 C). However, cell adhesion to VHL-negative cells was not further decreased in the presence of these blocking antibodies (Fig. 7 C). These results demonstrate that RCC cells are able to elicit immune cell binding, and this is specifically mediated by VCAM-1 interaction with the integrin α4β1.

U937 cells have been widely used as a model to investigate a variety of biological processes related to monocyte and macrophage function. Our results indicating that VCAM-1 expression on the surface of ccRCC cells allowed interaction with monocytic cells made conceivable that this interaction might trigger an immune response against tumor cells. Therefore, we performed cytotoxicity assays in co-cultures of ccRCC with human myeloid cells activated toward an M1 phenotype. We observed that loss of VHL conferred a cell advantage against the cytotoxic effects of activated monocytic cells because it reduced ccRCC death (Fig. 7 D). These results indicate that VCAM-1 decrease in tumor cells lacking VHL or in hypoxia adversely affects the antitumor immune response.

Analysis of VCAM-1 levels in human ccRCC

To address a possible clinical value of our data, we analyzed VCAM-1 expression levels in nephrectomy samples from patients with ccRCC. We analyzed a cohort with a total of 127 tumor samples that were divided into two groups according to the type of VHL mutation. One group included missense mutations, and the other group included nonmissense mutations, including nonsense, insertions, deletions, and methylations. In these two groups, we analyzed the association between VCAM-1 expression levels and several clinicopathologic variables. Our results indicated that VCAM-1 levels were higher in samples included in the group of VHL missense mutations compared with nonmissense mutations, although this difference did not reach statistical significance. However, we observed a negative association between VCAM-1 levels and ccRCC stage, microvascular invasion, and symptom presentation that was statistically significant in the group with nonmissense mutations ($P \leq 0.05$; Mann-Whitney *U* test; Table 1). Overall, our results point out the clinical value of VCAM-1 levels as a marker of ccRCC progression. In addition, they also point to effects on the regulation of VCAM-1 that appear to be dependent on the type of VHL mutation.

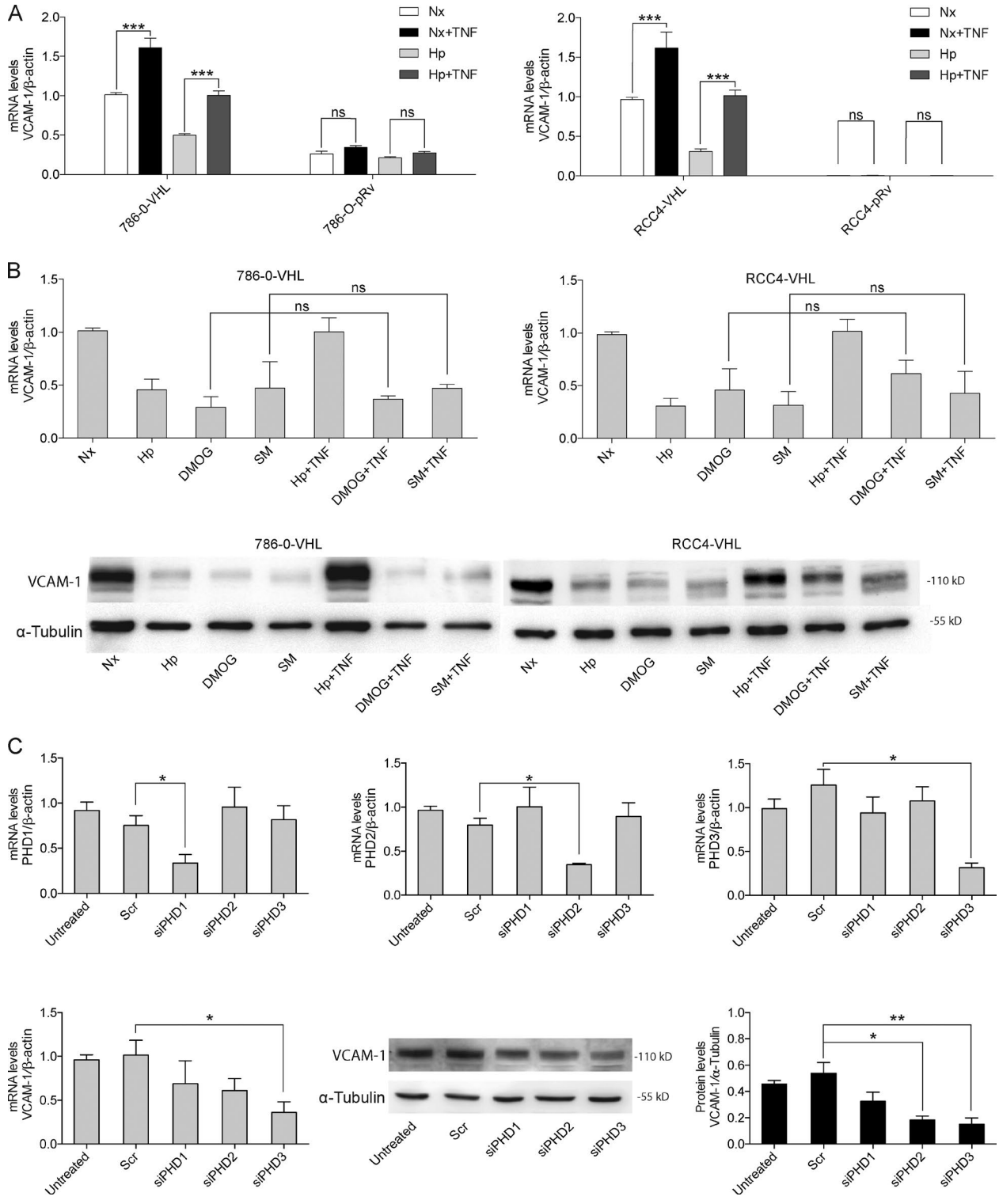

Figure 4. **VHL and PHDs limit VCAM-1 regulation via NF-κB in ccRCC cells.** (A and B) VCAM-1 mRNA (A) and protein (B) were analyzed in 786-O– or RCC4-positive or -negative cells under different stimuli for 24 h: normoxia (Nx), hypoxia 0.5% O_2 (Hp), 1 mM DMOG, or 20 µM SM7368 (SM) alone or in combination with 20 ng/ml TNF. *n* = 6 experiments. A representative Western blot is shown (*n* = 4). As a loading control, α-tubulin was used. (C) PHD1, PHD2, PHD3, and VCAM-1 mRNA levels in 786-O-VHL cells untreated or transfected with a scrambled siRNA (Scr) or siRNAs specific for each PHD (siPHD1, siPHD2, or siPHD3) were analyzed. Gene expression is represented as fold-change over the levels of untreated cells and controlled with *β-Actin* as the housekeeping gene. *n* = 4. VCAM-1 protein levels were detected in the same samples, and densitometry quantification of protein bands controlled with α-tubulin is represented. Protein levels are expressed as fold-change over untreated cells. *n* = 4. A representative Western blot is shown. Statistical analysis was done using one-way ANOVA followed by Bonferroni's posthoc test. The values represent the mean ± SEM. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 were considered significant.

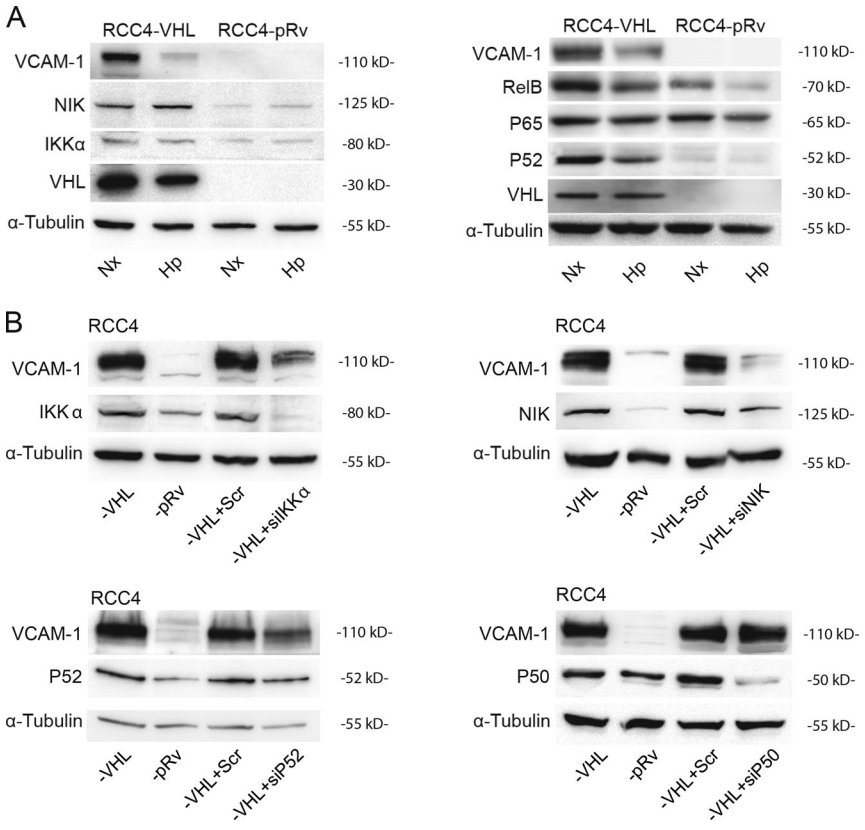

Figure 5. **VHL and hypoxia affect noncanonical NF-κB pathways in ccRCC cells.** (A and B) Western blots of RCC4-VHL–positive or –negative cells under normoxia (Nx) or hypoxia 0.5% O_2 (Hp); A) or cells transfected with a scrambled siRNA (Scr) or siRNAs specific for IKKα, NIK, p52, or p50 (B). Images show protein levels of VCAM-1, NIK, IKKα, Rel B, p65, p52, p50, VHL, and α-Tubulin as a loading control. A representative Western blots are shown. $n = 3$.

Discussion

ccRCC is one of the most abundant cancers in the world (Kabaria et al., 2016). Remarkably, 50–80% of sporadic RCCs and the most frequent form with clear cell histological features (ccRCC) present inactivating mutations or epigenetic silencing of VHL (Kaelin, 2002). Although the role of VHL in the regulation of HIF proves to be important for tumor growth, other VHL functions independent of HIF have been reported and help to explain why loss of VHL leads to renal cancer (Calzada, 2010). Previous studies report the relevance of VHL expression on VCAM-1 levels in renal carcinoma (Staller et al., 2003; Vanharanta et al., 2013), and other studies correlate higher levels of VCAM-1 expression in ccRCC with a better prognosis (Vasselli et al., 2003; Shioi et al., 2006; Yao et al., 2008). However, the mechanism underneath VCAM-1 regulation in these tumors remains unknown. Our experiments demonstrate that VHL loss or hypoxia down-regulates VCAM-1 mRNA and protein levels in ccRCC cells. This regulation was at the transcriptional level but was independent of HIF. Interestingly, ccRCC cells' treatment with TNF reverted VCAM-1 decrease in VHL-positive cells under hypoxia, although this was not reproduced in VHL-negative cells. In agreement with these results, previous findings demonstrate a VHL-dependent sensitization of RCC cells to TNF-mediated effects (Caldwell et al., 2002). This regulation does not appear to be exclusive to tumor cells, as TNF-induced expression of VCAM-1 in human umbilical vein endothelial cells (HUVECs) was significantly decreased under hypoxia or when treated with PHD or NF-κB inhibitors, as well as in cells treated with VHL siRNA (Fig. S3). This is of especial relevance in the setting of tumor migration and immune cell trafficking.

Although VHL's most accepted function is as an ubiquitin ligase that targets various proteins for degradation by the proteasome, it has also been observed that VHL has an opposite role increasing the half-life of several proteins such as BIMEL (Bcl2-interacting mediator of cell death extra long; Guo et al., 2009), p53 (Roe et al., 2006), and Jade-1 (Zhou et al., 2004). Previous results demonstrate a constitutive activation of the NF-κB pathway in RCC cell lines. However, VHL expression abolishes the canonical NF-κB pathway and the induction of antiapoptotic genes in those cells (Oya et al., 2001; Qi and Ohh, 2003; An et al., 2005). Furthermore, another study has shown that PHD2 controls TNF effects by positively regulating NF-κB signaling (Li et al., 2015), and PHD3 serves as a coactivator of NF-κB signaling activity (Fujita et al., 2012). Our results demonstrate that both VHL and PHD activity are required to maintain VCAM-1 levels in ccRCC cells and that they are essential for TNF-mediated induction of VCAM-1 through an NF-κB noncanonical pathway. These results are relevant considering the study published by Cummins et al. (2006), in which they show that kinases of the NF-κB signaling pathway, IKKα and IKKβ, contain the LXXLAP motif for proline hydroxylation. These authors demonstrate that IKKβ coimmunoprecipitates with PHD1 and VHL. Interestingly, this interaction does not result in ubiquitination or proteasomal degradation of IKKs. Although we were unable to demonstrate a direct interaction of VHL with IKKα or IKKβ in ccRCC cell lines, probably because of the lability of this interaction, our results clearly prove that, in the absence of VHL or in hypoxia, the noncanonical NF-κB pathway is affected, and this contributes to decrease VCAM-1 levels.

The NF-κB noncanonical pathway requires IKKα-dependent NF-κB2 (p100) processing in the proteasome to generate the binding subunit p52. Then, this subunit is associated to the transcriptional activator Rel B, enters the nucleus, and binds to

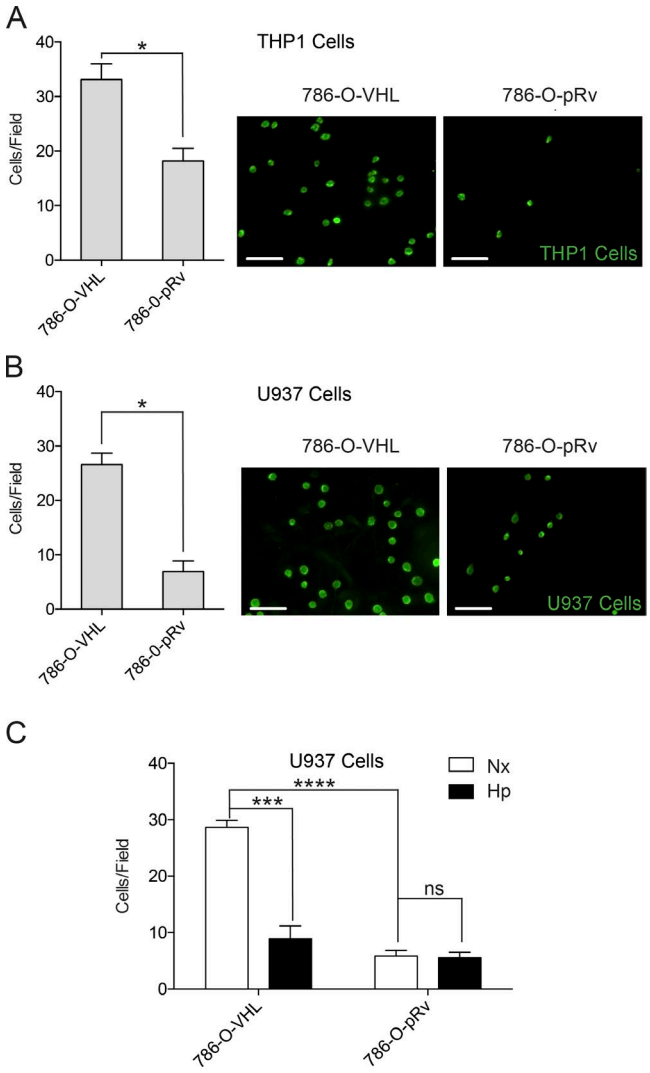

Figure 6. **Effects of VHL loss and hypoxia on monocytic cell adhesion to ccRCC cells.** (A and B) 786-O-VHL or 786-pRv cells were grown at confluence in a 24-multiwell plate and cultured under normoxic conditions. Then, THP1 (A) or U937 (B) monocytic cell lines (60 × 10^3 cells/well) previously labeled with 10 mM calcein-AM (green) were added. Cell adhesion was performed for 20 min at 37°C, and afterward, attached fluorescent cells were counted under the microscope. Two-tailed Student's *t* test was performed. Representative images of attached THP1 or U937 monocytic cell (green) are shown. Bars, 50 µm. (C) U937–calcein-AM–labeled cell adhesion on 786-O-VHL or 786-O-pRv cells previously cultured under normoxia (Nx) or hypoxia 0.5% O_2 (Hp) for 24 h. Cell adhesion was performed for 20 min at 37°C, and afterward, fluorescent cells were counted under the microscope. Statistical analysis was done using two-way ANOVA followed by Bonferroni's posthoc test. Data are represented as number of cells ± SEM. $n = 5$. 10 random fields were analyzed per condition. *, $P < 0.05$; ***, $P < 0.001$; and ****, $P < 0.0001$ were considered significant.

DNA–NF-κB binding sites in certain NF-κB–responsive genes (Senftleben et al., 2001; Xiao et al., 2001; Dejardin et al., 2002). In our ccRCC cell lines, we observed that IKKα and the upstream activator NIK (Ling et al., 1998) were decreased in the absence of VHL. Furthermore, Rel B and p52 were notably decreased under hypoxia or in the absence of VHL, whereas no changes in the canonic binding subunit p65 were observed. The possibility exists that PHD-mediated hydroxylation of the noncanonical activator IKKα promotes VHL binding or an intermediate protein that is also regulated by VHL. This interaction activates the noncanonical pathway, whereas its interaction with IKKβ represses the canonical pathway. Alternatively, an upstream regulation of the noncanonical pathway might occur after direct hydroxylation of NIK by PHDs. In this respect, NIK contains an LXXXLAP sequence that is similar to the LXX LAP consensus hydroxylation sequence present in IKKs (Cummins et al., 2006). Although these results demonstrated that the noncanonical pathway was involved in VCAM-1 regulation in these cells, we cannot discard that the canonical NF-κB pathway might also contribute. The NF-κB signaling pathways have a diverse spectrum of effects, and the NF-κB canonical pathway represents only a fraction of the whole range of genes that are regulated by this family of transcription factors. Although it is well established that NF-κB regulates VCAM-1 in many different cell types (Lin et al., 2015), the binding of noncanonical dimers to the described VCAM-1 NF-κB binding sites is highly probable because of the high similarity among these sequences. Our model proposes that VHL-PHD signaling promotes the NF-κB noncanonical pathway that increases VCAM-1 levels, also down-regulating the NF-κB canonical pathway (Fig. 8). Future studies will be required to clarify whether canonical and noncanonical NF-κB pathways are interconnected or differentially activated in ccRCC, contributing to tumor growth and progression. Although the two pathways usually cooperate in their biological functions, negative interplays have also been identified. The positive and negative interplays between the two NF-κB pathways may serve to modulate the kinetics and magnitude of expression of NF-κB target genes.

The biological roles of the noncanonical NF-κB pathway have been extensively studied (Dejardin, 2006; Zhu and Fu, 2010; Novack, 2011). However, how this pathway functions in specific cell types is still unclear. VCAM-1 has been generally correlated with activated endothelium, although its expression has also been detected in tumor models in which the function attributed to VCAM-1 depends on the type of tumor. Thus, recent studies in breast cancer and gastric carcinoma have shown that increased expression of VCAM-1 in these tumors favors metastasis to organs such as lung and bone (Chen et al., 2011; Lu et al., 2011). Conversely, other studies have highlighted the important role of VCAM-1 in the development of kidney tumors, in which an inverse correlation between the expression of VCAM-1 and tumor malignancy has been observed (Vasselli et al., 2003; Shioi et al., 2006; Yao et al., 2008). In agreement with these results, we have shown that VCAM-1 expression levels were decreased in a cohort of human ccRCC carrying VHL nonmissense mutations compared with those with missense mutations. In addition, a negative correlation between VCAM-1 levels and ccRCC stage, microvascular invasion, and symptom presentation was only statistically significant in the group with nonmissense mutations. These results suggest the existence of differential effects on VCAM-1 regulation and functions that might depend on the type of mutation affecting VHL. This is also supported by our in vitro results in which we observed that TNF-mediated regulation of VCAM-1 levels was differentially affected in various VHL mutants (Fig. S4). In addition, our results demonstrated that VCAM-1 expression in VHL-positive ccRCC cells promotes adhesion to monocytic cells through its interaction with the integrin α4β1 and elicits an M1 cytotoxic response. Conversely, VHL loss or hypoxia disrupts this interaction, probably because of the decrease in VCAM-1 levels.

Collectively, these findings reveal pathways that may be critical in ccRCC tumorigenicity and identify novel candidates

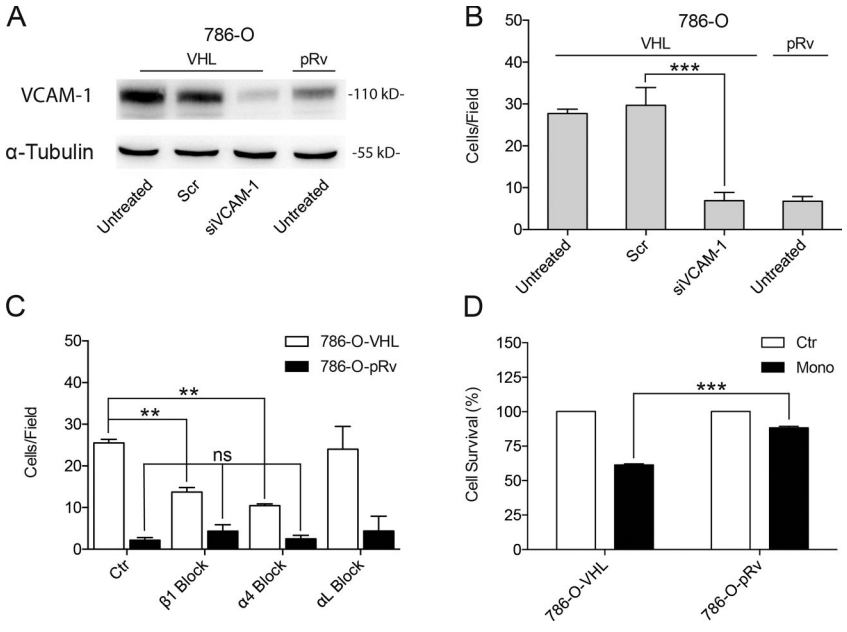

Figure 7. **Role of VCAM-1/α4β1 binding in monocyte adhesion to ccRCC.** (A) Analysis of VCAM-1 protein levels in 786-O-pRv or 786-O-VHL cells untreated or transfected with a scrambled siRNA (Scr) or siRNAs specific for VCAM-1. α-Tubulin was used as a loading control. A Western blot representative of three experiments is shown. (B) U937–calcein-AM–labeled cell adhesion on 786-O-pRv or 786-O-VHL cells untreated or transfected with a scrambled siRNA or siRNAs specific for VCAM-1. Cell adhesion was performed for 20 min at 37°C, and afterward, fluorescent cells were counted under the microscope. $n = 5$. (C) U937–calcein-AM–labeled adhesion experiment on 786-O-pRv or 786-O-VHL untreated (control [Ctr]) or treated with 10 µg/ml of blocking antibodies against β1, α4, or αL integrin subunits. Cell adhesion was performed for 20 min at 37°C, and afterward, fluorescent cells were counted under the microscope. $n = 4$. (B and C) Data are represented as number of cells ± SEM. 10 random fields were analyzed per condition. Statistical analysis was done using two-way ANOVA followed by Bonferroni's posthoc test. (D) Effects of VHL loss in monocytic cell-mediated cytotoxicity against ccRCC cells. 786-O-pRv or 786-O-VHL target cells were seeded into 16-well sensor plates, and activated human monocytes treated with IFN-γ and LPS were directly added into wells at a 60:1 ratio, monocytes (Mono)/ccRCCs. Cell survival measurements were automatically collected every 5 min by the real-time cell electronic-sensing analyzer system (xCELLigence System) for up to 96 h. Cellular index results were expressed as cell survival percentage mean ± SEM at 96 h. Data are representative of three different experiments. Statistical analysis between different conditions was done using two-way ANOVA followed by Bonferroni's posthoc test. **, $P < 0.01$ and ***, $P < 0.001$ were considered significant.

that could serve as targets for future therapeutic intervention or as diagnostic/prognostic biomarkers for patients with advanced ccRCC. In addition, the findings described herein may help to understand how VHL acts as a gatekeeper gene in the kidney and provide an insight into the existence of VHL-regulated functions through HIF-independent mechanisms. A better understanding of the molecular mechanisms that allow tumors to escape immune response will be a great benefit to the development of strategies for cancer treatment.

Materials and methods

Cell culture

The 786-O, RCC4, Caki-1, and Caki-2 cell lines from ATCC were cultured in DMEM containing 10% FBS (vol/vol) and 100 U/ml penicillin/100 µg/ml streptomycin. 786-O and RCC4 were stably transfected with vectors pRc/CMV or HA-VHL-pRc/CMV (in this paper, named as pRv or VHL, respectively), provided by W. Kaelin (Dana-Farber Cancer Institute, Boston, MA) through Addgene (plasmids nos. 20814

Table 1. **Effects of VHL mutations on VCAM-1 expression levels and its association with clinicopathologic characteristics in human ccRCCs**

VHL alteration	*n*	VCAM-1 levels	P	Clinicopathologic characteristics		*n*	VCAM-1 levels	P[a]
Missense	32	2.622	0.739	Tumor stage	I + II	12	2.943	0.613
					III + IV	20	1.933	
				Tumor grade	1 + 2	22	2.943	0.223
					3 + 4	10	1.484	
				Tumor size	≤7	19	3.042	0.173
					7.1≥	13	1.775	
				Microvascular invasion	neg	19	2.846	0.863
					pos	13	1.775	
				Symptom presentation	neg	12	3.577	0.018
					pos	20	1.225	
Nonmissense	95	2.099		Tumor stage	I + II	55	2.481	0.010
					III + IV	40	1.562	
				Tumor grade	1 + 2	70	2.309	0.001
					3 + 4	25	1.336	
				Tumor size	≤7	77	2.250	0.118
					7.1≥	18	1.492	
				Microvascular invasion	neg	57	2.524	0.009
					pos	38	1.650	
				Symptom presentation	neg	57	2.481	0.004
					pos	38	1.588	

Abbreviations used: neg, negative; pos, positive.
[a]Mann-Whitney *U* test was used.

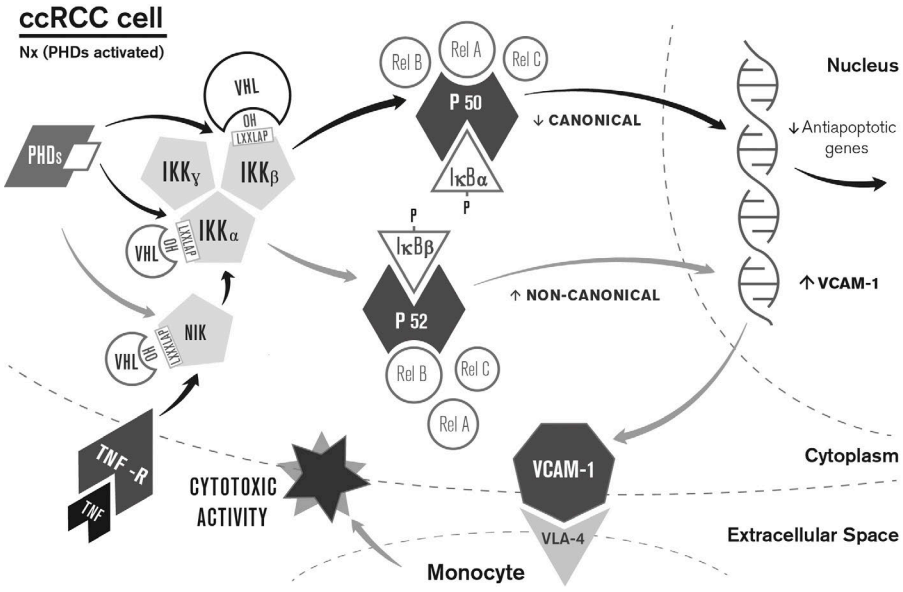

Figure 8. **Proposed molecular model of VCAM-1 regulation in ccRCC-VHL–expressing cells.** IKKα and IKKβ hydroxylation by active PHDs in normoxic (Nx) conditions allows VHL binding to IKKs. VHL-dependent regulation of IKKα promotes the noncanonical pathway, whereas regulation of IKKβ represses the canonical pathway. Alternatively, an upstream regulation of the noncanonical pathway might occur after PHD direct hydroxylation of NIK. These events regulate transcriptional effectors of the noncanonical pathway (p52–Rel B) that ultimately lead to the up-regulation of VCAM-1 in normoxia. VCAM-1 increase in the ccRCC cell membrane promotes binding to monocytes VLA-4 (α4β1 integrin) and activates their antitumoral effects. In the absence of VHL or in hypoxia, this pathway is nonfunctional, and VCAM-1 transcriptional induction is repressed. Black arrows indicate previously published data. Gray arrows indicate the proposed model based on results from this paper.

and 19999, respectively). Cells were selected with 1 mg/ml G418 sulfate. Additionally, we used 786-O expressing the naturally occurring type 2C VHL mutant L188V or a nonneddylateable version of VHL termed RRR (in which lysines 159 [K159], K171, and K196, have been substituted by an arginine [R], with K159 being an essential residue for neddylation). As a control for this mutant, we used 786-O cells transfected with another version of VHL (KRR) that carries mutations only at Lys171 and Lys196 (which do not interfere with the normal VHL function; Stickle et al., 2004). These mutants were provided by M. Ohh (University of Toronto, Toronto, Ontario, Canada). The human kidney adenocarcinoma cell line ACHN from ATCC (CRL-1611) was grown in DMEM containing 10% (vol/vol) FBS and 100 U/ml penicillin/100 µg/ml streptomycin. Cells were maintained in culture at 37°C in the presence of 5% CO_2 and 21% O_2 (normoxia). HUVECs from ATCC (CRL-1730) were grown following the manufacturer's specifications. For hypoxic experiments, cells were incubated at 37°C in a hypoxia workstation (InvIvO_2 400; The Baker Company) in the presence of 5% CO_2 and 0.5% oxygen, for the times indicated.

Human tumor tissue and ethics statements

Tumor samples were collected from nonselected patients who underwent nephrectomy at Yokohama City University Hospital and its affiliated hospitals. All specimens were snap frozen with liquid nitrogen and stored at −80°C until nucleic acid extraction. All tumors were confirmed to have occurred sporadically according to the patients' medical records. The histopathology of the tumors was classified according to the classifications recommended by the Union Internationale Contre le Cancer and the American Joint Committee on Cancer (Störkel et al., 1997). Tumor stage and grade were determined after surgical treatment according to the tumor node metastasis classification (Guinan et al., 1997). Written informed patient consent was obtained for studying gene expression profiles, and the study protocol was approved by the institutional ethics committee.

Antibodies and reagents

Monoclonal anti–VCAM-1 (H-276; sc-8304), monoclonal anti-VHL (VHL40; sc-135657), polyclonal anti–HIF-2α (H-310; sc-28706), polyclonal anti-NIK (H-248; sc-7211), polyclonal anti–NF-κB p65 (C-20; sc-372), polyclonal anti–NF-κB p52 (k-27; sc-298), monoclonal anti–NF-κB p50 (E-10; sc-8414), and monoclonal anti–NF-κB Rel B (D-4; sc-48366) antibodies were from Santa Cruz Biotechnology, Inc. Monoclonal anti–HIF-1α antibody (241809; mab1536) was from R&D Systems. Monoclonal anti-IKKα (3G12; no. 2682) was from Cell Signaling Technology, and monoclonal anti–α-tubulin antibody (T6199) was from Sigma-Aldrich. The antibodies were used at the concentrations suggested by the manufacturers. HRP-conjugated secondary polyclonal (NA9340V; GE Healthcare) or monoclonal (P0260; Dako) antibodies were used. DMOG (89464-63-1) and 3-chloro-4-nitro-*N*-(5-nitro-2-thiazolyl)-benzamide (SM7368; 380623-76-7) were obtained from Sigma-Aldrich. TNF (210-TA-005 RD) was obtained from Transduction Laboratories.

Real-time PCR analysis

Analysis of mRNA to determine the gene expression or analysis of hnRNA to determine the transcriptional state of genes was performed by quantitative RT-PCR. Cells were grown to 95% confluence in 60-mm culture dishes, and the total RNA was isolated from cells using the TRIzol RNA Isolation system (Invitrogen). 1 µg/sample RNA was reverse transcribed to cDNA with Improm II reverse transcriptase (Promega) in a final volume of 20 µl. For quantitative RT-PCR, 1 µl cDNA was amplified with the specific primers pairs, and PCR amplifications were performed using Power SYBR green PCR Master Mix (Applied Biosystems). The following primer pairs used were: *β-Actin* (forward, 5′-CGATGCCTGAGGCTCTTT-3′ and reverse, 5′-TGGATG CCACAGGATTCCA-3′), *vhl* (forward, 5′-CGCCGCATCCACAGC TA-3′ and reverse, 5′-TGTGTCCCTGCATCTCTGAAGA-3′) *vcam-1* (forward, 5′-GTTCCTAGCGTGTACCC-3′ and reverse, 5′-GCTGAC CAAGACGGTTG-3′), *glut-1* (forward, 5′-TCAACCGCAACGAGG AGAA-3′ and reverse, 5′-CTGTCCCGCGCAGCTT-3′), *phd1* (forward, 5′-GCGCTGCATCACCTGTATCTAT-3′ and reverse, 5′-CCG CCATGCACCTTAACG-3′), *phd2* (forward, 5′-CCCTCATGAAGT ACAACCAGCAT-3′ and reverse, 5′-CATCTGCATCAAAATACC AAACAGT-3′), and *phd3* (forward, 5′-TGCATCACCTGCATCTAC TATCTG-3′ and reverse, 5′-TACATGGTGGGATCCTGCG-3′). Primers used for analysis of VCAM-1 hnRNA were: forward, 5′-CCACAG TAAGGCAGGCTGTAAA-3′ and reverse, 5′-GCAGCTTTGTGGATG GATTCA-3′. The data were analyzed using StepOne Plus Software (Applied Biosystems). All values were controlled with *β-Actin* gene expression levels. For the analysis of VCAM-1 in human samples, the following primers and a probe were used: 5′-CAAAGGCAGAGTACG CAAACAC-3′ (forward primer), 5′-CTGGCTCAAGCATGTCATATT CAC-3′ (reverse primer), and 5′-6FAM-CAGAGATACAACCGTCTT

GGTCAGCCCTTTAMRA-3′ (probe). PCR amplification was done using the iCycler iQ Real-Time PCR Detection system (Bio-Rad Laboratories). The amount of product was measured by interpolation from a standard curve. In each experiment, at least two independent RT-PCRs were done to obtain the mean expression signal values. Values were controlled with *β-Actin* gene expression levels.

Protein analysis by Western blotting

Lysates of snap-frozen ccRCC cells were prepared in radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.5, 1% NP-40, 1 mM EDTA, 125 mM NaCl, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and 1× phosphatase/protease inhibitors cocktail; Roche). Cell lysates were centrifuged at 17,000 *g* for 20 min. A bicinchoninic acid assay (Bio-Rad Laboratories) was used to quantify total protein. 30 mg/lane of lysates mixed with 1× reducing Laemmli buffer (Bio-Rad Laboratories) was boiled at 95°C for 5 min, electrophoretically separated on SDS-PAGE gels, and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). Blots were probed with primary antibody to the respective proteins and afterward with HRP-conjugated secondary antibodies. Proteins were visualized with HRP substrate (Luminata Forte; EMD Millipore) on a camera system (ImageQuant LAS 4000; GE Healthcare).

Flow cytometry analysis

VCAM-1 levels on the cell surface were measured by flow cytometry. 786-O cells and their counterparts stably expressing VHL were cultured under normoxia or hypoxia (0.5% O_2) for 24 h. Afterward, cells were washed and resuspended in PBS at 10^6 cells/ml and then incubated with anti–VCAM-1 antibody (sc-8304) for 1 h at 4°C. After this time, nonbound antibody was washed out, and the cells were incubated with Alexa Fluor 488 goat anti–mouse secondary antibody (R37120; Thermo Fisher Scientific) for 30 min at 4°C. VCAM-1 expression was quantified using a flow cytometer (FACSCalibur; BD).

Co-culture adhesion experiments

786-O cells stably transfected with VHL or empty vector (pRv) and with siScr or siVCAM-1 were grown at confluence in a 24-multiwell plate (30×10^3 cells/well). When indicated, cells were grown under hypoxia (0.5% O_2) for 24 h previous to adhesion experiments. THP-1 or U937 monocytic cell lines from ATCC were labeled with the fluorescent dye calcein AM (2.5 mM; 148504-34-1; Sigma-Aldrich) in serum-free medium for 20 min at 37°C. Then, cells were washed, resuspended in adhesion buffer (Hepes-buffered Hanks' balanced salt solution containing 1% bovine serum albumin, 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 0.2 mM $MnCl_2$), and added at 60×10^3 cells/well on top of the ccRCC monolayer, and both cell lines were allowed to interact for 20 min at 37°C. When indicated, cells were incubated with blocking antibodies anti-α4 (HP2/1; Sánchez-Madrid et al., 1986), anti-β1 (VJ1/14; Yáñez-Mó et al., 1998), or anti-αL (TS1/11; Sanchez-Madrid et al., 1982), provided by F. Sanchez-Madrid (Universidad Autonoma of Madrid, Madrid, Spain). After 15 min at 37°C, membranes were washed with adhesion buffer. Afterward, unbound THP-1 or U937 monocytic cells were gently removed, and bound monocytes were fixed with 3% paraformaldehyde and counted under a fluorescence microscope (DMR 020-525.024 fluorescence microscope; Leica Biosystems). Images were taken with a camera (D-35578 Wetzlar; DFC360 Fx [115470000]; Leica Biosystems) and objective (HCX PL APO 100×/1.400.7 oil CS; Leica Biosystems), and the imaging software used was Leica Application Suit (LAS v4.1).

siRNA-mediated gene silencing

siRNA experiments were performed with specific pools of siRNAs directed against human VCAM-1 (sc-29519), HIF-1α (sc-35561), HIF-2α (sc-35316), *vhl* (sc-36816), *phd1* (sc-45616), *phd2* (sc-45537), *phd3* (sc-45799), *nik* (sc-36065), *ikkα* (sc-29365), *p52* (sc-29409), and *p50* (sc-29407) or with a nontargeted pool of control scrambled siRNAs (sc-37007) from Santa Cruz Biotechnology, Inc. Cells were transfected with Lipofectamine 2000 (11668019; Invitrogen), according to the manufacturer's instructions. 2 d after transfection, silencing efficiency was analyzed, and cells were used for experiments.

Myeloid cell isolation and culture

Peripheral blood mononuclear cells were purified by Ficoll gradient centrifugation from pooled human buffy coats obtained from the Hospital Universitario de la Princesa. Myeloid cells were isolated from mononuclear cell populations by positive selection for CD14 by two rounds of magnetic bead (130-050-201) immune selection according to the manufacturer's directions (Miltenyi Biotec). All studies with human cells were conducted with the approval of the Human Subjects Institutional Review Board of the Hospital Universitario de la Princesa.

Cytotoxicity assays

These assays were performed with the xCELLigence System (Roche), which measures electrical impedance across microelectrodes integrated in the bottom of tissue culture plates. This provides quantitative information about the status of the adherent cells, including viability. Co-cultures of ccRCC (2,500 cells) with M1-differentiated myeloid cells (activated with 100 U/ml IFN-γ [CAA31639] from R&D Systems and 2 µg/ml LPS [297-473-0] from Sigma-Aldrich, in a proportion of 1:60, respectively) were monitored for 96 h with measures every 5 min. Cellular index, normalized with real-time cell analysis software (Roche), was expressed as percentage of living cells.

Statistical analysis

The data are presented as the mean + SEM for all the studies done with cells. ANOVA followed by Bonferroni's posthoc test was used when comparing three or more groups, and two-tailed Student's *t* test was used to compare two groups, according with the conditions of normality and homoscedasticity. Shapiro–Wilk normality test and Brown–Forsythe test were used to analyze these conditions. A maximum p-value of 0.05 was considered significant. Mann-Whitney *U* test was used to determine differences between groups from tumor samples.

Online supplemental material

Fig. S1 shows the effect of VHL loss and hypoxia on VCAM-1 mRNA levels in the renal adenocarcinoma cell line ACHN. Fig. S2 shows the effect of hypoxia on VCAM-1 protein levels in the ccRCC cell line 786-O-VHL. Fig. S3 shows the effect of VHL loss, hypoxia, and NF-κB inhibitors on VCAM-1 levels in HUVECs. Fig. S4 shows analysis of VCAM-1 levels in VHL mutants.

Acknowledgments

This work was supported by grants from the Instituto de Salud Carlos III (co-funded by the European Union and Fondo Europeo de Desarrollo Regional; grants PI16/02166 and PIE13/00041), a grant from Red Cardiovascular (RD12/0042/0065 to M.J. Calzada), and a grant from Ministerio de Economía y competitividad (SAF2015-64215R to R. Sánchez-Prieto).

The authors declare no competing financial interests.

Submitted: 5 August 2016
Revised: 21 December 2016
Accepted: 30 January 2017

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