p62/SQSTM1 regulates cellular oxygen sensing by attenuating PHD3 activity through aggregate sequestration and enhanced degradation

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Summary

The hypoxia-inducible factor (HIF) prolyl hydroxylase PHD3 regulates cellular responses to hypoxia. In normoxia the expression of PHD3 is low and it occurs in cytosolic aggregates. SQSTM1/p62 (p62) recruits proteins into cytosolic aggregates, regulates metabolism and protein degradation and is downregulated by hypoxia. Here we show that p62 determines the localization, expression and activity of PHD3. In normoxia PHD3 interacted with p62 in cytosolic aggregates, and p62 was required for PHD3 aggregation that was lost upon transfer to hypoxia, allowing PHD3 to be expressed evenly throughout the cell. In line with this, p62 enhanced the normoxic degradation of PHD3. Depletion of p62 in normoxia led to elevated PHD3 levels, whereas forced p62 expression in hypoxia downregulated PHD3. The loss of p62 resulted in enhanced interaction of PHD3 with HIF-α and reduced HIF-α levels. The data demonstrate p62 is a critical regulator of the hypoxia response and PHD3 activity, by inducing PHD3 aggregation and degradation under normoxia.

Key words: Autophagy, Aggresome, HIF, Hypoxia, LC-3, LIR domain, NBR1, Prolyl hydroxylase, pVHL, Sequestosome1

Introduction

Hypoxia is a key component in the progression of many diseases such as stroke, inflammatory diseases and cancer (De Bock et al., 2011; Pugh and Ratcliffe, 2003). Hypoxia and the following reoxygenation pose cells to severe stress and reduce cell viability. However, carcinoma cells can exploit hypoxia to gain more aggressive features. Hypoxia causes genetic instability, activates autophagy, regulates protein translation and degradation and together with ROS generated by reoxygenation causes protein damage and misfolding (Jaakkola and Pursiheimo, 2009; Koshiji et al., 2005; Koumenis and Wouters, 2006; Liu et al., 2006; Rifkind et al., 1991; Zhang et al., 2008).

Mammalian cells are equipped with a multitude of mechanisms to overcome the viability reducing stress elicited by hypoxia. The main hypoxia response is mediated through hypoxia-inducible factor (HIF) and the family of oxygen- and iron-dependent HIF hydroxylating dioxygenases named prolyl hydroxylases 1–3 (PHD1−3) that sense the concentration of intracellular oxygen. Under sufficient oxygen availability PHDs hydroxylate two prolyl residues in the HIF-alpha subunit (HIF-α; HIF-1α and -2α) earmarking it to be recognized by the von Hippel-Lindau (pVHL) protein that targets HIF-α to proteasomal degradation (Bruick and McKnight, 2001; Cockman et al., 2000; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001; Ohh et al., 2000). Since PHDs have an absolute requirement for molecular oxygen, under hypoxia PHD activity is reduced and the degradation of HIF-α is blocked. This in turn capacitates HIF-α to transcriptionally activate hypoxia-responsive target genes (De Bock et al., 2011; Kaelin, 2005).

PHD3 that is expressed at low levels in normoxia but strongly upregulated by hypoxia has been suggested to be the primary regulator of HIF-α under prolonged hypoxia (Appelhoff et al., 2004). PHD3 has multiple functions and also other hydroxylation targets in addition to HIF-α (Jokilehto and Jaakkola, 2010; Schlisio, 2009; Wenger et al., 2009). PHD3 has been shown to mediate the stability of activating transcription factor 4 (ATF4) (Köditz et al., 2007), β2-adrenergic receptor (Xie et al., 2009) and to prevent degradation of myogenin (Fu et al., 2007). It has been reported to bind IKKβ and repress IKK/NF-κB signaling (Xue et al., 2010) and to be crucial for neutrophil-mediated inflammation (Walmsley et al., 2011). PHD3 has recently been implicated in the regulation of glycolytic metabolism by pyruvate kinase M2 (PKM2) (Chen et al., 2011; Luo et al., 2011). We have demonstrated that under hypoxia PHD3 enhances G1 to S transition of carcinoma cells (Högel et al., 2011). Moreover, PHD3 has been shown to enhance apoptosis of neuronal cells. Knockout studies show that PHD3 increases apoptosis of sympathoadrenal ganglia, an event required for maintaining their normal development and function (Bishop et al., 2008; Lee et al., 2005; Schlisio et al., 2008). We have previously shown that under normoxia PHD3 aggregates in cytoplasmic/perinuclear speckles that contain chaperones and proteasomal components (Rantanen et al., 2008) and PHD3 can homomultimerize in complexes that might enhance its degradation by Siah2 E3

ubiquitin ligase (Nakayama et al., 2004; Nakayama et al., 2007). Noticeably, in some carcinoma cells the aggregation associates with enhanced apoptosis (Rantanen et al., 2008).

Sequestosome 1 (SQSTM1/p62, herein designated as p62) is a versatile protein implicated in the regulation of several signal transduction pathways regulating osteoclastogenesis, inflammatory reactions, and adipocyte differentiation (Moscat and Diaz-Meco, 2009a; Moscat and Diaz-Meco, 2009b). p62 may be required for Ras-induced cell transformation by regulating the NF-κB pathway (Duran et al., 2008; Geetha et al., 2005) and modulate cellular oxygen consumption by downregulating phosphorylation of ERK1/2 (Rodriguez et al., 2006).

p62 is expressed in cytosolic speckles or aggregates, which may represent several subtypes of diverse functions (Moscat and Diaz-Meco, 2009a). p62 aggregates contain a number of proteins including components of the proteasomal system. p62 transports polyubiquitylated proteins to the autophagosome to be degraded by autophagy (Johansen and Lamark, 2011; Klionsky et al., 2012). It may also function as a cargo receptor shuttling some ubiquitin-conjugated proteins for proteasomal degradation (Seibenhener et al., 2004). In line with these, p62 interacts with several polyubiquitylated proteins such as aPKC and caspase-8 and besides regulating their degradation it also controls their activity. p62 localizes to autophagosome formation site and is itself a substrate for autophagy (Bjørkøy et al., 2005; Ichimura et al., 2008; Itakura and Mizushima, 2011; Mizushima et al., 2008; Pankiv et al., 2007). Various types of stress and deficient autophagy can upregulate p62 (Takamura et al., 2011). An upregulation in p62 protein level is detected in e.g. breast cancer, glioblastoma cells, hepatocellular carcinomas and TSC-deficient renal cancers (Galavotti et al., 2012; Inami et al., 2011; Parkhitko et al., 2011; Takamura et al., 2011; Thompson et al., 2003). In line, p62 can support autophagy in tumors and enhance tumor survival. Sustained 62 expression may promote tumorigenesis by activating NF-kB pathway (Duran et al., 2008; Ling et al., 2012; Mathew et al., 2009), anchorage-independent growth (Inami et al., 2011), cell invasion (Galavotti et al., 2012) and tumor metabolism (Guo et al., 2011; Rodriguez et al., 2006) and its expression correlates with aggressive progression of breast cancers (Thompson et al., 2003). However, we have previously shown that hypoxia, although commonly present in tumors, is an efficient downregulator of p62 expression. This suggests that selectively under hypoxia p62 may not be advantageous for cancer cell survival (Jaakkola and Pursiheimo, 2009; Pursiheimo et al., 2009).

Here we show that p62 controls PHD3 activity. p62 colocalizes and physically interacts with PHD3. We further show that p62 regulates the function of PHD3 oxygen sensor by trapping it into aggregates and enhances the degradation of PHD3. This control is released under hypoxia as p62 levels are rapidly and efficiently downregulated upon reduced oxygen tension.

Results

PHD3 and p62 colocalize in aggresome-like structures in normoxic carcinoma cells

We have previously described that exogenous PHD3 forms subcellular bodies in normoxic cells (Rantanen et al., 2008). To investigate the possible significance of PHD3 bodies in cancer the subcellular expression pattern of PHD3 was studied in clinical cancer samples using breast cancer and squamous cell carcinomas of head and neck region (HNSCC; Fig. 1A). Both of these cancer types are known to overexpress PHD3 (Högel et al., 2011). In line with the cell culture data, large cytoplasmic PHD3 aggregates were detected in the clinical samples of both cell types suggesting a physiological role for PHD3 aggregation in cancer cells. In normal tissue with high basal PHD3 expression, such as placenta, the aggregates were clearly less abundant and smaller (Fig. 1A).

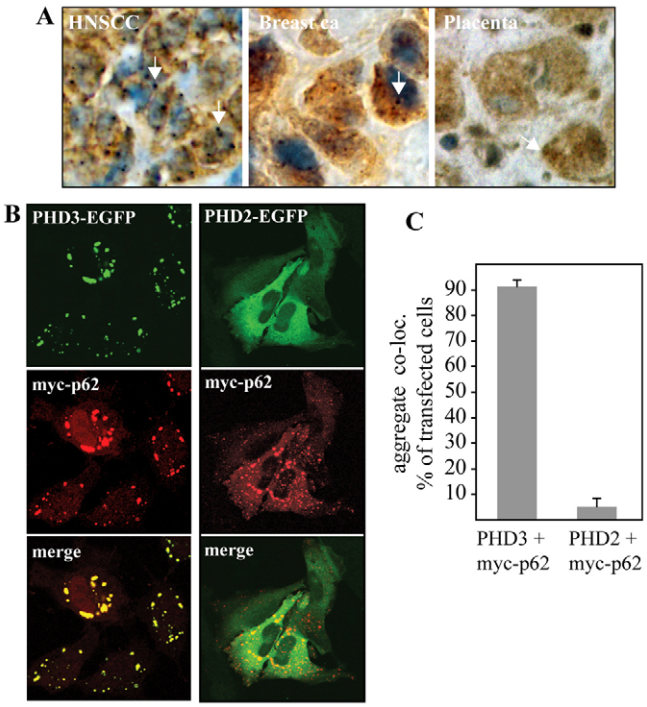

Fig. 1. Colocalization of p62 with PHD3. (**A**) PHD3 forms juxtanuclear aggresome-like structures (arrows) in carcinomas. Immunohistochemical analysis of PHD3 expression in paraffin-embedded tissue sections of head and neck squamous cell carcinoma, breast cancer and normal placental tissue sections. (**B**) HeLa cells were co-transfected either with PHD3-EGFP or PHD2-EGFP (green) and myc-p62 (red) and grown under normoxia (21% O_2) for 24 hours prior to visualization by confocal microscopy for EGFP (green) or myc (red). (**C**) Colocalization of p62 and PHD3–EGFP or PHD2–EGFP in aggregates was quantified. Values are means and s.d. of five optical fields.

The PHD3 bodies strongly resemble some aggresome-like structures that contain components of the 26S proteasome, chaperones and ubiquitin (Rantanen et al., 2008). p62 has also been reported to reside in aggresome-like structures (Moscat and Diaz-Meco, 2009a; Pankiv et al., 2007). To study whether p62 and PHD3 proteins colocalize in the same structures we ectopically expressed EGFP-tagged PHD3 (PHD3–EGFP) and myc-tagged p62 (myc–p62) in HeLa cells. 24 hours after transfection cells were fixed and visualized for PHD3 and p62 expression. Colocalization of PHD3 and p62 in the aggregates was seen in practically all cells while PHD2 failed to colocalize with p62 (Fig. 1B). Quantification demonstrated colocalization of PHD3 and p62 in over 90% of cells expressing both proteins whereas PHD2–EGFP colocalization was seen in less than 10% of cells (Fig. 1C).

Similarly to the exogenous p62, endogenous p62 colocalized with PHD3–EGFP in aggregates (Fig. 2A). As the expression level of PHD3 in normoxic conditions is normally low,

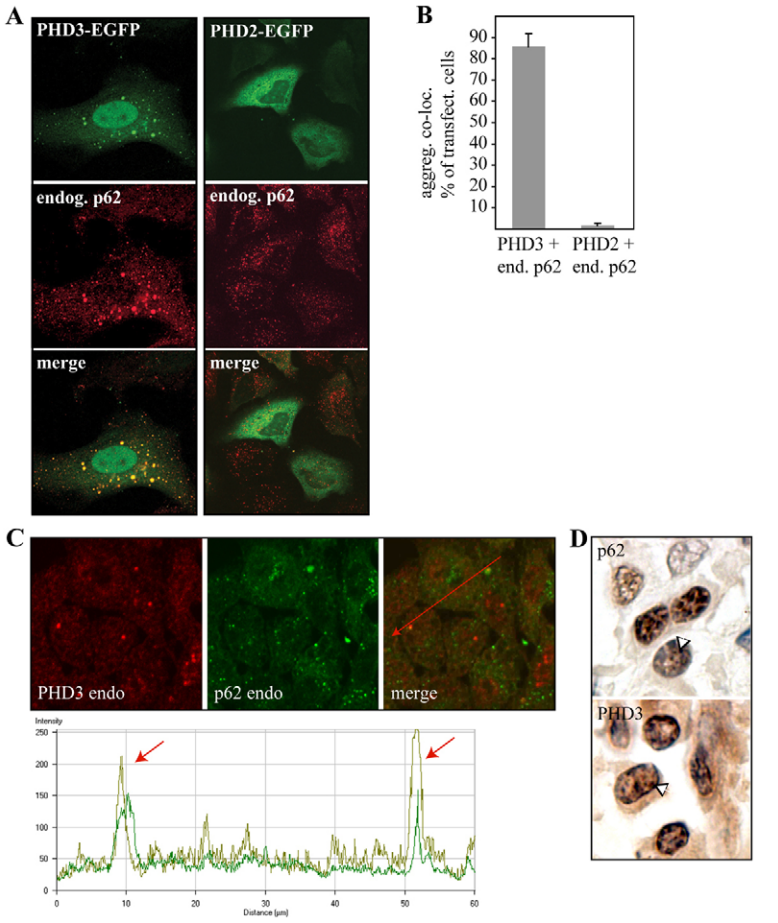

Fig. 2. Colocalization of endogenous p62 with PHD3. (**A**) Immunofluorescence analysis of endogenous p62 (red) and PHD3–EGFP or PHD2–EGFP (green) under normoxia. (**B**) Quantification of p62 colocalization with PHD3 and PHD2. Values are means and s.d. of three visual fields. (**C**) HeLa cells grown on slides were stained for both endogenous PHD3 and p62 and their expression pattern studied by confocal microscopy. Images from both PHD3 and p62 were studied separately as well as from overlay images (merge). A histogram showing colocalization of the two proteins along the line of the red arrow was generated from the image using LSM510 software (lower panel). Arrows indicate colocalization in aggregates. (**D**) Consecutive HNSCC tissue sections stained for p62 and PHD3 show aggregates for both proteins in same regions. Examples of aggregates are indicated by arrows.

colocalization studies were performed by overexpressing PHD3. Most of the aggregates were either cytoplasmic or perinuclear as shown for PHD3 previously (Rantanen et al., 2008). Again, scoring of the colocalization revealed that in almost 90% of cells p62 and PHD3 were found in same structures (Fig. 2B). To further validate the *in vivo* colocalization of endogenous PHD3 and p62, normoxic HeLa cells were studied with co-immunostaining using high resolution confocal microscopy. While the endogenous expression of p62 clearly exceeded that of PHD3, the endogenous PHD3 partially colocalized with p62 in cytoplasmic speckles in similar pattern to that seen in overexpression studies (Fig. 2C). Besides HeLa cells, colocalization of PHD3–EGFP and endogenous p62 was seen in several other cell types including primary squamous carcinoma cells derived from HNSCC, MCF breast cancer cell line and partially also in immortalized non-cancerous HaCaT keratinocytes (supplementary material Fig. S1). Supporting the colocalization p62 and PHD3 were found to aggregate in same regions when studied from consecutive human HNSCC samples (Fig. 2D). Moreover, PHD3 demonstrated colocalization with another autophagic adapter protein, NBR1 (supplementary material Fig. S2).

PHD3 and p62 interact in normoxic carcinoma cells

As PHD3 and p62 colocalized in cytoplasmic speckles, their possible physical interaction was studied. FLAG-tagged PHD3 and PHD2 (FLAG-PHD3, FLAG-PHD2) were co-expressed with EGFP–p62 in normoxic HeLa cells for 24 hours (Fig. 3A). FLAG antibody used as a bait co-immunoprecipitated p62 in cells transfected with PHD3 (Fig. 3A, beads) and no p62 immunoprecipitation was detected without antibody. The immunoprecipitation demonstrated specificity for PHD3 as only a small fraction of the amount of p62 was pulled down by FLAG–PHD2 as compared to FLAG–PHD3. The interaction was tag independent since, similarly to the EGFP tag, myc-tagged p62 interacted with PHD3 (Fig. 3B). To further evaluate the PHD3–p62 interaction, HeLa cells were transfected either with FLAG-PHD2 or FLAG-PHD3 and the pull down of endogenous p62 was studied (Fig. 3C). Similarly to exogenous p62, immunoprecipitation experiments showed that the endogenous p62 interacted with PHD3 but not with PHD2 implying a physical *in vivo* interaction between p62 and PHD3. To further validate the PHD3–p62 interaction, we performed immunoprecipitation from cells transfected with FLAG-PHD3 and wild-type (wtp62) or LC-3 interaction-domain-deleted p62 (delLIRp62) (Fig. 3D). The data indicated that the LC-3 domain of p62 is required for PHD3 interaction validating the interaction.

Finally, the *in vivo* interaction of endogenous p62 and PHD3 was validated using an *in situ* proximity ligation assay (PLA, Duolink®) that can detect protein interactions in single cells (Fig. 3E) (Söderberg et al., 2006). For technical controls omission of either primary or secondary antibody were used (supplementary material Fig. S3). The PLA assay revealed close proximity of PHD3 and p62 and was seen mainly in the cytoplasm. As expected, attenuation of p62 expression by

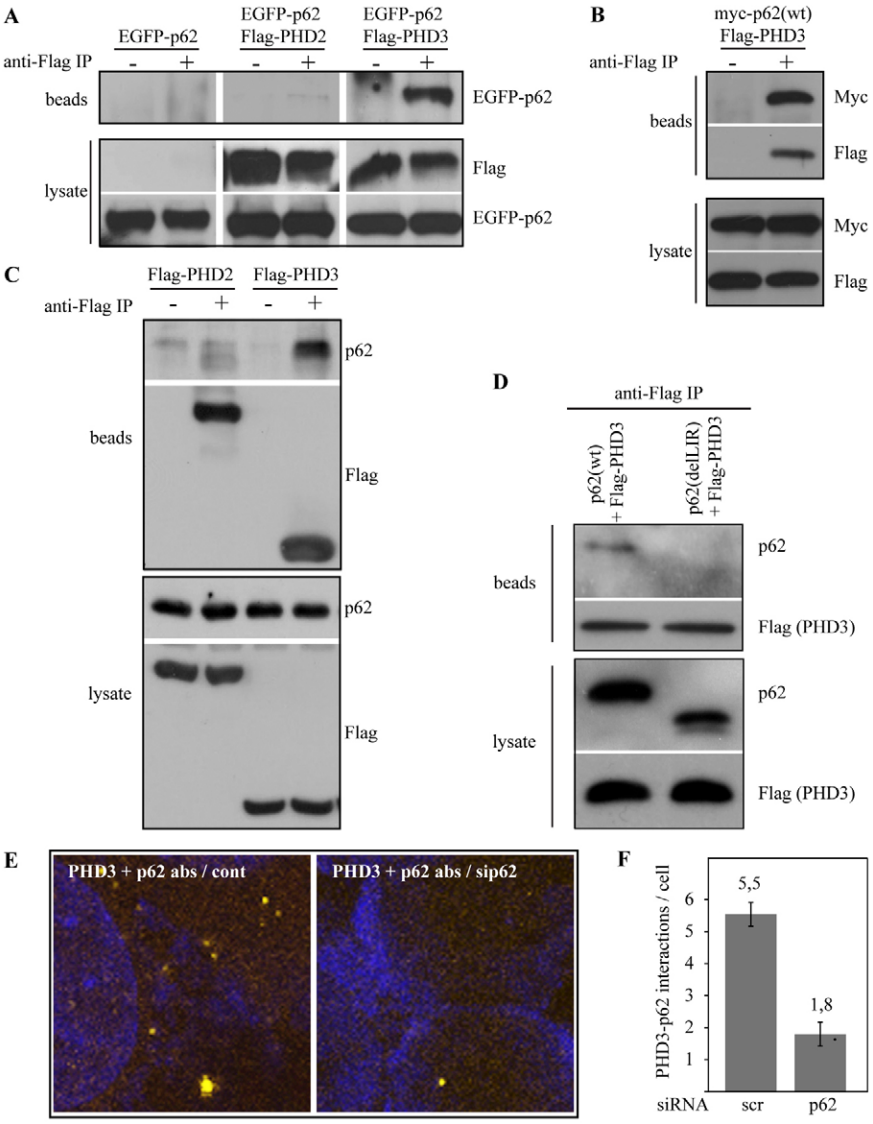

Fig. 3. p62 interacts with PHD3.
(**A**) Immunoprecipitation analysis of PHD3 and p62 interaction under normoxia. HeLa cells grown under normoxia were transfected with either EGFP-p62 alone or together with FLAG-PHD2 or FLAG-PHD3. Anti-FLAG antibody was used for co-immunoprecipitation (+) or was omitted (−) and captured p62 was analyzed by western blotting. (**B**) HeLa cells were transfected with FLAG-PHD3 and myc-tagged wild-type p62 (myc-p62). Co-immunoprecipitation analysis using FLAG antibody was performed. (**C**) HeLa cells were transfected with either FLAG-PHD2 or FLAG-PHD3. Endogenous p62 co-immunoprecipitated by FLAG antibody was detected by western blot analysis. PHD3, but not PHD2, captured endogenous p62. (**D**) HeLa cells were transfected with either wild-type p62 or p62 with LIR domain deletion [p62(delLIR)] together with FLAG-PHD3. p62 co-immunoprecipitated by FLAG antibody was detected by western blot analysis. PHD3 captured wild-type p62 but not p62(delLIR). (**E**) The proximity of endogenous PHD3 and p62 proteins were examined on HeLa cells using a commercial *in situ* PLA kit. As a control p62 depletion with siRNA was used. (**F**) Quantification of p62–PHD3 interactions detected as in E in control and p62 siRNA-treated cells. Means and s.d. of five optical fields are shown.

exposure of cells to p62 siRNA (sip62) resulted in a reduced interaction between PHD3 and p62 (Fig. 3E). The interaction reduced to approximately one third of that seen with intact p62 expression (Fig. 3F). sip62 was validated by transfection and p62 western blot analysis (not shown). Unlike PHD3, PHD2 and p62 demonstrated little proximity (not shown). Taken together, the data demonstrated that p62 and PHD3 physically interact in cytoplasmic/perinuclear aggregates under normoxic conditions.

p62 induces PHD3 aggregation

p62 is known to transport diverse proteins into aggregates that contain proteasomal protein components. PHD3 forms aggregates in over 80% of transfected carcinoma cells under normoxia (Rantanen et al., 2008). To study if p62 is needed for the aggregation of PHD3, normoxic HeLa cells were transiently transfected with PHD3-EGFP simultaneously with either control siRNA (scr siRNA) or siRNA directed against p62 (sip62) (Fig. 4A). Simultaneous transfection with PHD3-EGFP and p62 siRNA resulted in marked attenuation in the aggregate-bound expression of PHD3 thus allowing PHD3 to be expressed diffusely in the cytoplasm and nucleus. Quantification demonstrated almost complete abolishment of PHD3 aggregation under p62 depletion (Fig. 4B) indicating that p62 is required for PHD3 aggregation in normoxia in carcinoma cells. In contrast, knockdown of PHD3 did not affect the ability of p62 to form aggregates (supplementary material Fig. S4).

p62 protein requires UBA and PB1 domain to form cytoplasmic bodies or to transport proteins into them. We co-transfected PHD3-EGFP with wild-type myc-p62 or two mutated p62 constructs; myc-p62(D69A) and myc-p62(1–358) (Fig. 4C). D69A is a mutant that abrogates PB1 domain-mediated polymerization of p62. Myc-p62(1–358) has a deletion of the UBA domain which is needed for non-covalent binding of ubiquitin domains. Both mutants are known to disrupt the aggregation of p62 (Bjørkøy et al., 2005; Lamark et al., 2003). Wild-type p62 co-transfected with PHD3-EGFP showed expected punctuate expression pattern for both p62 and PHD3. As expected, both mutated p62 plasmids failed to form aggregates. Importantly, neither of them was able to induce PHD3 aggregation. Quantification showed practically complete abolishment of PHD3 speckles when expressed with either of the non-aggregating p62 forms (Fig. 4D). The data demonstrated

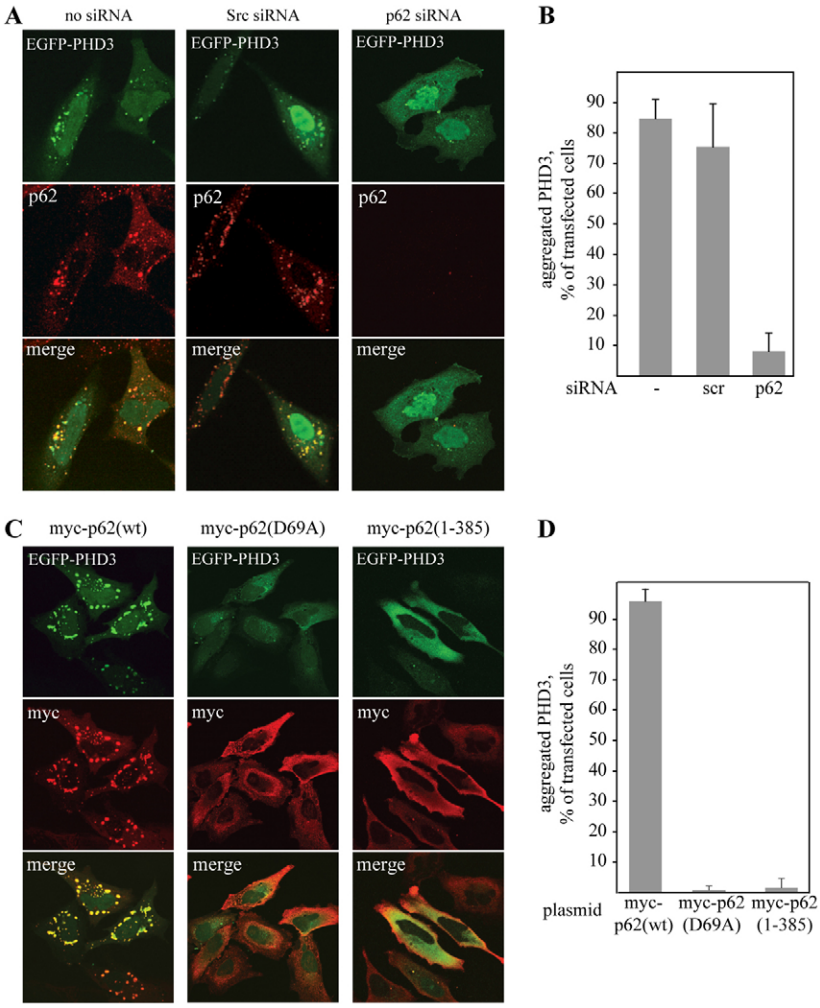

Fig. 4. p62 is required for PHD3 aggregation in normoxia. (**A**) HeLa cells grown under normoxia were transfected with PHD3-EGFP (green) followed by transfection with either control siRNA (Scr siRNA) or with p62 siRNA and imaged for colocalization with endogenous p62 (red). (**B**) Quantification of p62 colocalization with PHD3 after transfection with the indicated siRNAs. Values are means and s.d. of five visual fields. (**C**) HeLa cells transfected with PHD3-EGFP (green) and with either wild-type p62 (myc-p62), a point mutated PB1 domain [myc-p62(D69A)] or UBA-domain deletion [myc-p62(1–358)] plasmids, were immunostained for myc tag (red). Both the UBA domain and PB1 domain of p62 were required for aggregation of PHD3. (**D**) Quantification of p62 colocalization with PHD3 after treatment as in C. Values are means and s.d. of five optical fields.

that p62 is sufficient and necessary to drive PHD3 into subcellular aggregates in normoxic conditions.

We have previously shown that PHD3 aggregation is lost in hypoxia resulting in evenly distributed expression throughout the cytoplasm (Rantanen et al., 2008). p62 expression is abundantly expressed in carcinoma cells but efficiently downregulated in response to hypoxia (Fig. 5A) (Pursiheimo et al., 2009). To see whether p62 retains its ability to form aggregates and to induce PHD3 aggregation also in hypoxia we used forced p62 expression. PHD3-EGFP and myc-p62 were co-transfected into HeLa cells followed by exposure to hypoxia. After 24 hours hypoxia forced p62 expression demonstrated aggregation comparable to that seen in normoxia (Fig. 5B). Importantly, forced expression of p62 completely restored the aggregation of PHD3. Similarly to normoxia, PHD3 and p62 colocalized in the aggregates. The data indicated that p62 is able to drive PHD3 into aggregates independently from the oxygenation status.

Under reoxygenation p62 level is rapidly restored. To study the association of p62 and PHD3 expression we analyzed PHD3 with p62 upon reoxygenation at two time points (Fig. 5C). p62 levels that were strongly reduced under hypoxia were restored after 1–6 hours of reoxygenation. Simultaneously the high hypoxic PHD3 level reduced to basal normoxic level after 6 hours. This suggested that there may be a causal relationship between PHD3 and p62 expression.

p62 controls normoxic protein levels of PHD3

Many cancer cell types express p62 abundantly whereas normoxic PHD3 expression level is generally low. PHD3 is strongly induced by hypoxia simultaneously with downregulation of p62, a setup that is reversed upon reoxygenation (Fig. 5C). Since PHD3 was forced to aggregate by p62, we asked whether p62 contributes to the scarce expression of PHD3 under oxygenated conditions. First we used siRNA to deplete p62 and subsequently measured the PHD3 expression at 24 hours and 48 hours. Depleting p62 resulted in markedly elevated levels of PHD3 (Fig. 6A). Quantification of the western blot analyzes demonstrated consistent and strong upregulation of PHD3 levels by p62 inhibition at 24 hours post-transfection (Fig. 6B). In line with this, visualization of normoxic PHD3 under p62 depletion showed enhanced PHD3 expression in most cells together with reduced PHD aggregation (Fig. 6C).

Next, HeLa cells were cultured in hypoxia or normoxia for 24 hours either with or without forced expression of myc-p62. Whereas the expression levels of PHD2 remained unchanged, forced p62 expression in hypoxia reduced PHD3 expression in these cells (Fig. 6D). This indicated that besides regulating the subcellular localization of PHD3, p62 is also a major regulator of PHD3 protein expression.

To analyze whether the normoxic upregulation of PHD3 by p62 depletion was due to enhanced transcription we performed

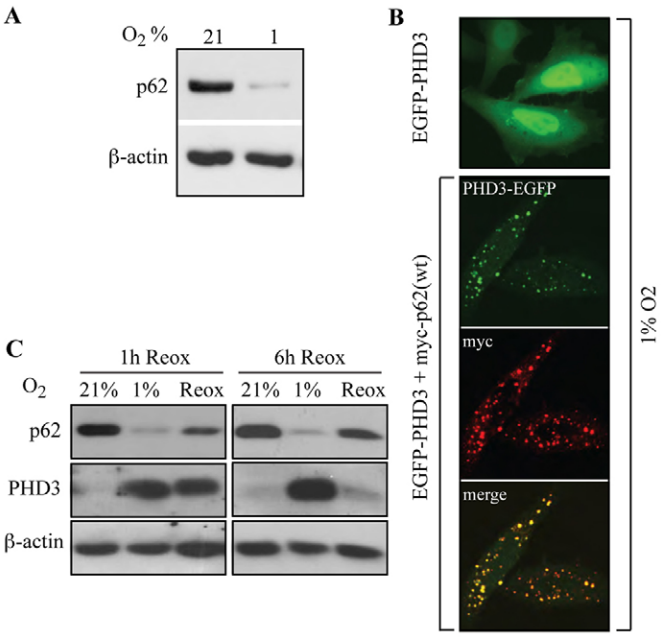

Fig. 5. p62 expression in hypoxia forces aggregation of PHD3. (**A**) p62 expression is lost in hypoxia. HeLa cells were grown either under normoxia (21% O_2) or hypoxia (1% O_2) for 24 hours and analyzed for p62 expression by western blotting. β-actin was used as loading control. (**B**) Forcing p62 expression under hypoxia restores the aggregation of PHD3. HeLa cells were transfected with PHD3-EGFP alone (top image) or together with wild-type myc-p62 (red; lower panels) and grown in hypoxic conditions (1% O_2) for 24 hours. (**C**) p62 expression was restored 6 h after reoxygenation, simultaneously with the disappearance of PHD3. HeLa cells were grown either under normoxia (21% O_2) or hypoxia (1% O_2) for 24 hours followed by 1 h or 6 h reoxygenation of hypoxia-treated cells. Western blot analysis was performed to determine the levels of p62 and PHD3. β-actin was used as a loading control.

RT-PCR analysis (Fig. 6E). In contrast to PHD2 siRNA, which expectedly increased PHD3 mRNA level, sip62 did not upregulate PHD3 mRNA indicating that the regulation of PHD3 expression by p62 is post-transcriptional. In line with this, under hypoxia with or without forced p62 expression proteasomal inhibition by MG-132 increased PHD3 expression suggesting enhanced degradation of PHD3 also in cells overexpressing p62 (Fig. 6F). Noticeably, none of the autophagosome inhibitors including 3-methyladenine (3-MA, 4 or 10 hours) that blocks the sequestration step of autophagy nor bafilomycin A1 or chloroquine (both at 10 hours) that block the fusion of autophagosomes with lysosomes were able to elevate PHD3 levels suggesting that the degradation occurs through proteasomes.

Finally, we used p62 depletion by siRNA to analyze the disappearance of PHD3 upon reoxygenation (Fig. 7A). The high PHD3 expression level in hypoxia began to decline after 1 hour re-oxygenation in cells exposed to control siRNA (left-hand panel). However, in cells exposed to sip62 the disappearance of PHD3 was markedly slower. After 1 hour no visible reduction in PHD3 was seen and after 3 hours the level was higher as compared to control. The data indicated that p62 is required for maximal rapid degradation of PHD3 after reoxygenation and further supported the effect of p62 in controlling PHD3 protein levels. In keeping with this, the reduced PHD3 aggregation in hypoxia was restored after reoxygenation and colocalization of PHD3 with p62 was detected mainly after short (1 hour) reoxygenation at the time when both proteins are expressed (Fig. 7B,C).

p62 regulates the PHD3 –HIF-α interaction

HIF-α is the best-characterized hydroxylation target of PHD3 although an *in vivo* interaction between the proteins has been notoriously difficult to demonstrate. In order to study the functional significance of PHD3 sequestration by p62 we used the proximity ligation assay (PLA) that is able to *in vivo* detect transient protein interactions (Söderberg et al., 2006). Under normoxia when PHD3 is sequestrated in aggregates and the free cytoplasmic levels of PHD3 are low, PLA demonstrated very low interaction between PHD3 and HIF-1α. However, the interaction was strongly enhanced under p62 depletion by siRNA (Fig. 8A). PHD3–HIF interaction sites within normoxic cells increased approximately threefold under p62 depletion as compared to control (Fig. 8B). Vice versa, under hypoxia with low p62 and high PHD3 expression the amount of PHD3–HIF interaction was reduced by forced p62 expression. Here, the PHD3–HIF interaction was clearly diminished in cells that expressed EGFP–p62 as compared to non-transfected adjacent cells (Fig. 8C,D). Similar p62-induced reduction in PHD3–HIF interaction was seen when comparing cells with forced EGFP–p62 and EGFP control expression (not shown). Moreover, under forced p62 expression the location of the remaining PHD3–HIF interaction was shifted from nucleus to cytoplasm (Fig. 8E). Under hypoxia without p62 expression approximately half of the PHD3–HIF interaction was seen in the nucleus and half in the cytoplasm. However, within p62 expressing cells 80% of the interactions occurred in the cytoplasm thus reducing the nuclear interactions. Since the PHD3 protein interactors, such as HIF or PKM2, operate mainly in the nucleus the finding further supported the view that p62 keeps PHD3 away from its nuclear targets. The significance of the reduction in PHD3–HIF-1α interaction by forced hypoxic p62 expression was further corroborated by illustration of increased HIF-1α level in these conditions (Fig. 8F; supplementary material Fig. S5).

Discussion

In the present study we have shown that p62, a multifunctional regulator of cell differentiation, metabolism and survival also controls the expression and localization of cellular oxygen sensor PHD3. Under normoxic conditions PHD3 is transported into aggresome-like cytoplasmic and perinuclear structures and the transportation is lost when cells are exposed to hypoxia (Rantanen et al., 2008). We now show that p62 is responsible for the trapping of PHD3 in the aggregates. The data also at least partially explains why p62 expression needs to be rapidly downregulated under hypoxia. We have demonstrated that PHD3 and p62 colocalize and interact in cytosolic aggregates in normoxic carcinoma cells and that the depletion of p62 results in loss of PHD3 aggregation. In contrast, under hypoxia where p62 is downregulated (Pursiheimo et al., 2009) the aggregation of PHD3 is greatly reduced and PHD3 is distributed evenly throughout the cytoplasm and nucleus. Forced expression of p62 in hypoxia relocalized PHD3 back into aggregates. This was further corroborated by the fact that mutations known to disrupt p62 aggregation also resulted in an inability of PHD3 to

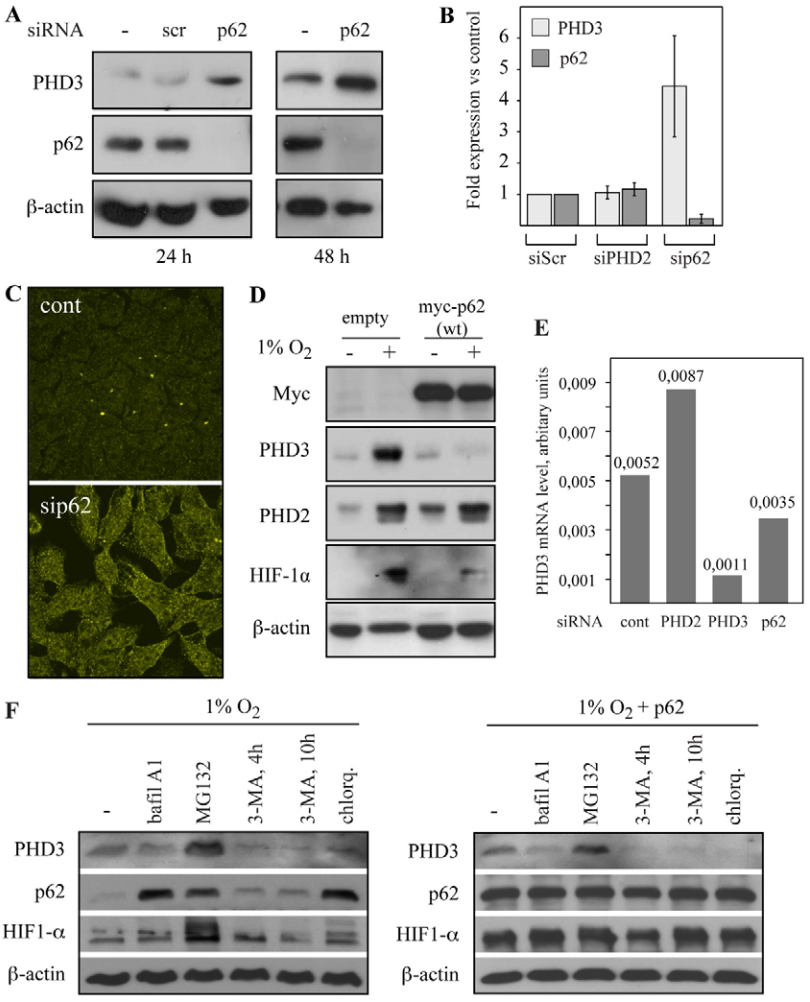

Fig. 6. p62 enhances the degradation of PHD3. (**A**) p62 depletion upregulates the PHD3 protein level. HeLa cells were left untreated or treated with either control siRNA (scr) or p62 siRNA. Cells were grown in normoxic conditions (21% O_2) for 24 hours or 48 hours and subsequently analyzed for PHD3 and p62 expression with western blotting. (**B**) Western blot intensities were analyzed with the MCID^{TM} imaging software system and the fold expression relative to control was calculated. Values are means and s.d. of three experiments. (**C**) HeLa cells were grown on coverslips in normoxia and either left untreated or transfected with sip62. Endogenous PHD3 was detected by immunostaining and imaged using confocal microscopy. sip62 exposure increased PHD3 expression. (**D**) Cells were transfected with control or wild-type p62 (myc-p62) plasmid and grown either under normoxia (−) or hypoxia (+) for 24 hours, followed by western blot analysis of myc, PHD3, PHD2 and HIF-1α. (**E**) HeLa cells were transfected with control or PHD2, PHD3 and p62 siRNAs. RNA was collected and the mRNA levels determined by quantitative RT-PCR. p62 siRNA did not induce PHD3 mRNA expression. (**F**) Western blot analysis of PHD3, p62 and HIF-1α under hypoxia and hypoxia with forced p62 expression. Cells were exposed to autophagosome inhibitors 3-MA (4 and 10 hours), bafilomycin A1, chloroquine or the proteasome inhibitor MG-132 (all 10 hours). MG-132 was the only inhibitor that increased PHD3 levels.

aggregate. Moreover, p62 regulated the expression level of PHD3 post-transcriptionally by enhancing the degradation of PHD3. Depletion of p62 in normoxia resulted in attenuation of PHD3 degradation and forced expression of p62 in hypoxia enhanced the degradation. Thus, the regulation of PHD3 activity by p62 under normoxia is attenuated through two mechanisms that both are released under hypoxic p62 loss. In keeping with this, p62 regulated the interaction of PHD3 with HIF-1α. Under normoxia, with very low PHD3 and high p62 level, the loss of p62 resulted in increased PHD3–HIF-1α interaction. Vice versa, under hypoxia, with elevated PHD3 and low p62, restoration of p62 expression led to strongly reduced PHD3–HIF-1α interaction and accordingly, to increased HIF-1α level. While HIF-α is the best-characterized hydroxylation target of PHD3, the alteration of subcellular localization, i.e. p62-induced trapping of PHD3 in the cytoplasm, imply that the interaction of PHD3 with other nuclear target proteins would be similarly affected by p62.

There are several ways as to how p62 can affect cell fate through PHD3 regulation. First, under normoxia p62 is required to restrict PHD3 activity by activating aggregation and keeping PHD3 away from its targets. These include HIF-α but also other reported PHD3 targets such as PKM2 (Chen et al., 2011; Luo et al., 2011), ATF4 (Köditz et al., 2007), β2-adrenergic receptor (Xie et al., 2009) and IKKβ (Xue et al., 2010). Under p62 depletion the increased normoxic PHD3 activity would result in suppression or malfunction of these pathways. This is highlighted by our data demonstrating vastly increased interaction between normoxic basal HIF-1α and PHD3 under p62 depletion. The normoxic HIF-1α expression is low but essential for normal cell function and is mainly kept low by PHD2. The HIF-1α and PHD3 interaction under p62 depletion was remarkable as it exceeded that of HIF-1α and PHD2. This indicated that p62 is a major regulator that keeps PHD3 from interacting with its targets. Second, during hypoxia the downregulation of p62 is required to adjust cellular energy metabolism to meet the diminished oxygen availability. This has been shown by the loss of p62 which led to constitutive upregulation of ERK phosphorylation and subsequently to altered expression of various genes involved in energy metabolism. Interestingly, two recent papers suggest that PHD3 is a crucial regulator of cell metabolism by regulating the function of a central glycolytic enzyme PKM2 either by regulating hypoxic PKM2 activity (Chen et al., 2011) or recruiting PKM2 into HIF complex and enhancing the expression of glycolytic genes among others (Luo et al., 2011). Therefore, it is feasible that the altered metabolism seen with p62 depletion could partially be due to enhanced PHD3 activity.

Besides autophagosomes p62 may transport some proteins into the proteasomal machinery and is itself degraded by autophagosomes. In both cases p62 is expressed in cytoplasmic and perinuclear speckles. We have previously shown that under

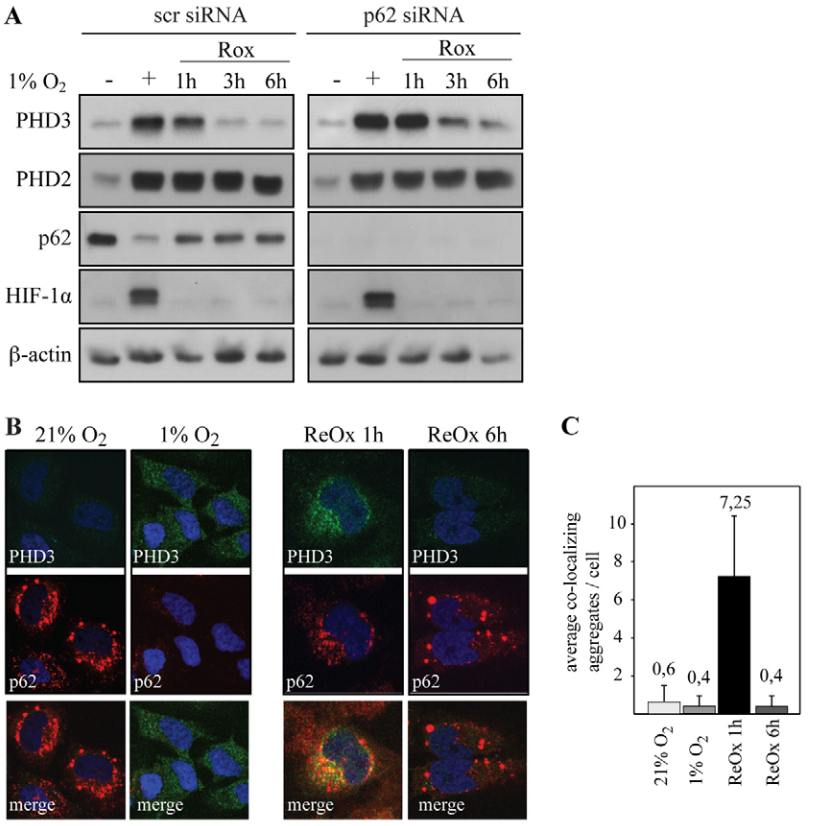

Fig. 7. Re-expression of p62 under reoxygenation restores the normoxic expression pattern of PHD3. (**A**) Western blot analysis of the effects of p62 siRNA treatment on the expression levels of PHD3, PHD2, p62 and HIF-1α in normoxia, hypoxia and reoxygenation (Rox) at three different time points (1–6 hours). p62 siRNA markedly slowed the disappearance of PHD3 expression. (**B**) Cells were imaged with confocal microscopy for the pattern and level of expression of PHD3 and p62 after normoxia, hypoxia and at two different time points after reoxygenation. (**C**) Quantification of p62–PHD3 interaction under the indicated conditions shown as colocalizing aggregates/cell. Values are means and s.d. of five cells.

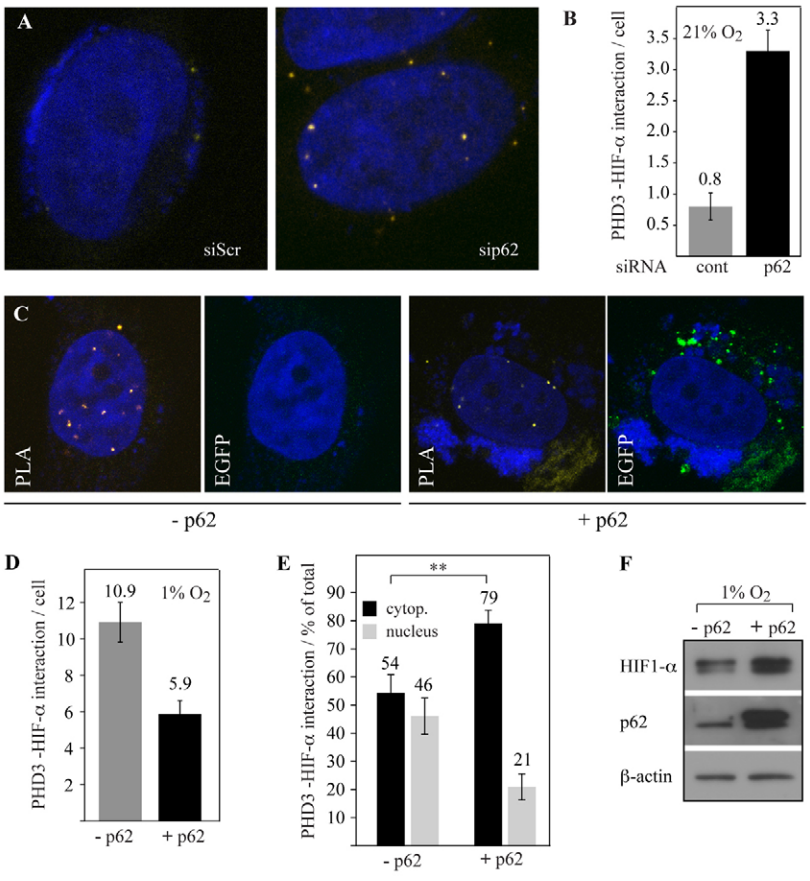

Fig. 8. p62 controls the *in vivo* interaction of PHD3 with HIF-α. (**A**) Proximity ligation assay for HIF-1α and PHD3 in control (siScr) and p62 depleted (sip62) cells. Following siRNA treatments, PLA analysis was performed and the interactions between PHD3 and HIF-1α (yellow) were visualized by confocal microscopy. Nuclei were stained with Hoechst (blue). A marked increase in the amount of interaction was seen with p62 depletion. (**B**) Quantification of PLA signals depicting PHD3–HIF-1α interactions in normoxia. Values are means and s.d. of 31 interactions. (**C**) PLA for HIF-1α and PHD3 under hypoxia in control (non-transfected) cells and cells with forced p62 expression (+p62, green). HeLa cells were transfected with EGFP-p62 and subsequently exposed to hypoxia for an additional 24 hours followed by examination of EGFP expression and PLA signals. (**D**) Quantification of PHD3–HIF-1α interactions in hypoxia with or without p62 expression. Values are means and s.d. of 23 interactions. (**E**) p62 overexpression influences the subcellular localization of PHD3–HIF-1α interactions. Quantification of PHD3–HIF-1α interactions with or without p62 expression in the cytoplasmic and nuclear compartments under hypoxia. (**F**) Forced expression of p62 in hypoxia elevates HIF-1α expression. HeLa cells were transfected with p62 or mock control followed by exposure to hypoxia for 24 hours and subsequently analyzed for HIF-1α and p62 expression by western blotting. β-actin was used as loading control.

normoxia both endogenously and exogenously expressed PHD3 accumulates in aggregates together with chaperones and components of the 20S proteasome (Rantanen et al., 2008). Here we have demonstrated that the LC-3 interacting domain of p62 is required for PHD3 interaction and that PHD3 also colocalizes with another autophagosome adapter NBR1. These suggest that in addition to proteasomes, PHD3 may partially localize to autophagosomes. Interestingly however, unlike MG-132 the autophagy inhibitors were unable to elevate PHD3 level implying that the degradation of PHD3 mainly occurs through proteasomes. This, together with the data showing that sip62 increases PHD3 protein but not mRNA level, strongly suggests that besides keeping PHD3 stored in the aggregates, p62 also enhances the proteasomal degradation of PHD3. Siah-2 E3 ubiquitin ligase has been shown to downregulate the hypoxic expression of PHD3 by proteasome-dependent degradation and to facilitate the assembly of PHD3 into complexes under hypoxia (Nakayama et al., 2004; Nakayama et al., 2007). Therefore Siah2 and p62 show similar function in restricting PHD3 activity. However, as Siah2 seems to operate mainly under hypoxia, p62 in contrast restricts the normoxic activity of PHD3.

p62 is overexpressed in some cancers due to reduced autophagosomal clearance (Galavotti et al., 2012; Parkhitko et al., 2011; Takamura et al., 2011; Thompson et al., 2003). Carcinoma cells may also benefit from increased p62 due to its ability to keep PHD3 activity low and restricting PHD3 interaction with HIF-1α and other targets under normoxia. This is likely to be cancer type specific since reduced PHD3 expression has been detected in some cancers such as colon carcinomas while some carcinomas such as HNSCC display elevated PHD3 (Högel et al., 2011). Moreover, the benefit of p62 for tumors may be restricted to normoxic conditions, while p62 may be suppressed in hypoxic regions. Therefore, it would be of interest to study whether the increased p62 levels are found only in well-oxygenated tumor regions or whether it is also upregulated in regions with acute hypoxia, e.g. by decreased autophagosomal clearance of p62. The latter would lead to enhanced hypoxic PHD3 expression and activate enhanced glycolysis resulting in metabolically altered phenotype.

Materials and Methods

Cell culture, plasmids and transfections

Cells were routinely cultured in DMEM (Sigma-Aldrich) supplemented with 10% FCS, penicillin, streptomycin and L-glutamine. Transfections were performed with FuGene HD (Roche Applied Science) according to manufacturer's protocols. Hypoxic treatments were performed in Invivo2 400 incubator (Ruskinn Technology Ltd, UK) in 1% O_2, 5% CO_2, 90% humidity. Oxygen was replaced with 99.5% pure N_2 (AGA, Finland). For siRNA experiments double-stranded siRNA oligonucleotides were used at 200 nM final concentration on 6-well cell culture plates. Transfections were performed with Oligofectamine (Invitrogen) following manufacturer's protocol. The siRNAs (MWG Biotechnology) used were: PHD2, 5′-GACGAAAGCCAUGGUUGCUUG(dTdT)-3′; PHD3, 5′-GUCUAAGGCAAUGGUGGCUUG(dTdT)-3′; p62, 5′-GUAAGCCUAGGUGUUGUCATT-3′; HIF-1α, 5′-AACUAACUGGACACAGUGUGU(dTdT)-3′; non-target (scr) siRNA, 5′-CCUACAUCCCGAUCGAUGAUG(dTdT)-3′.

Plasmids used (pDestmyc-p62 and PHD2/3-EGFP) have been described in detail elsewhere (Bjørkøy et al., 2005; Rantanen et al., 2008).

Autophagy inhibitors 3-methyladenine (3-MA, Sigma-Aldrich), Bafilomycin A1 (LC Laboratories), erythro-9-[3-(2-hydroxynonyl)] (Sigma-Aldrich) and proteasome inhibitor MG-132 (Calbiochem) were used at the indicated concentrations.

Detection of proteins

For protein analysis cells were harvested in SDS–Triton lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM NaF, 0.5% Triton X-100, 1% SDS, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride and Complete Protease Inhibitor cocktail) followed by addition of 2-mercaptoethanol, sonication and boiling prior to loading. Protein concentrations were measured with Bio-Rad DC-protein assay prior to addition of mercaptoethanol or boiling and equal amounts of protein were loaded and run on SDS-PAGE in a mini-gel chamber (Bio-Rad) and transferred to a PVDF membrane (Millipore). Western blot analyses with the following antibodies were performed with the indicated dilutions: guinea-pig polyclonal p62 (Progen Biotechnik) at 1:5000, mouse monoclonal Hif-1α (BD Transduction Laboratories, San Jose, CA) at 1:1500, and mouse monoclonal AC-40 actin antibody (Sigma-Aldrich) at 1:5000 dilution and mouse monoclonal anti-Myc (Sigma) 1:1500. All primary antibody incubations were done overnight in +4℃ followed by secondary antibody treatment for 1 hour at room temperature. Anti-mouse-HRP, anti-rabbit–HRP (DAKO) and anti-guinea-pig–HRP (Sigma) antibodies were used at 1:5000 dilutions. Proteins were detected with enhanced chemiluminescence reagent (Pierce).

For fluorescence microscopy cells were grown on coverslips and fixed with PTEMF buffer (4% formaldehyde, 10 mM EGTA, 1 mM $MgCl_2$, 20 mM PIPES pH 6.8, 0.2% TX-100) containing 4% formaldehyde. The guinea-pig anti-p62 antibody was used at a 1:2000 dilution. Alexa-Fluor-555-labeled goat anti-guinea-pig antibody (Invitrogen) was used as the secondary antibody. For detection of endogenous PHD3 1:1000 dilution was used followed by treatment with Cy3-conjugated secondary antibody. For detection of Myc-tagged p62 rabbit polyclonal anti-Myc tag was used in 1:1000 dilution (Abcam). Cy3-conjugated anti-mouse IgG was used as secondary antibody.

Following antibody treatments cell nuclei were stained with Hoechst-33342 (Invitrogen) at a 1:10,000 dilution in PBS. Cells were visualized with an LSM 510 confocal microscope (Zeiss). The objectives used for image acquisition were: 20×/0.5 Plan neofluar, 40×/1.3 oil Plan neofluar, 63×/1.4 oil Plan apochromat and 100×/1.4 oil Plan apochromat. Subsequent to visualization Zeiss LSM5 software was used to generate protein colocalization histograms.

Immunoprecipitation

For immunoprecipitation HeLa cells overexpressing FLAG–PHD3 or FLAG–PHD2 and the indicated p62 constructs were collected in ice cold lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 0.1% SDS, Complete Protease Inhibitor cocktail). After overnight incubation at +4℃ with anti-FLAG (Sigma) antibody, protein G magnetic beads (Amersham Biosciences) were added and the mixture was incubated at +4℃ for 2 hours. Following incubation beads were washed with lysis buffer and bound proteins were detached from beads with boiling in TXLB buffer for 10 min. Immunoprecipitated proteins were analyzed by western blotting.

Quantitative RT-PCR

Primers for RT-PCR were obtained from Oligomer and probes from Roche (PHD3: No. 62, cat nr 04688597001). The sequence for *PHD3* primers are: fwd 5′-ATCGACAGGCTGGTCCTCTA-3′, rev 5′-GATAGCAAGCCACCATTGC-3′. Samples were collected in RLT buffer and RNA was extracted using NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Reverse transcription was performed in the presence of RNase inhibitor rRNAsin (Promega, Madison, WI, USA) using M-MuLV RNase H-reverse transcriptase (Finnzymes, ThermoFisher, Waltham, MA, USA). RT-PCR reactions were run using Applied Biosystems 7900HT Fast Sequence Detection System and TaqMan Universal Master Mix II, no UNG (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The data were normalized using β-actin as a reference gene.

Immunohistochemistry

HNSCC and breast cancer tissue samples were obtained from anonymous retrospective diagnostic samples at Turku University Hospital, Department of Pathology. Paraffin embedded tissue sections were stained with PHD3 antibody (Novus Biologicals, Littleton, CO) at 1:500 dilution. Primary antibody was visualized with Bright Vision plus Poly-HRP-anti-mouse/rabbit/rat IgG. Cells were counterstained with Hematoxylin. Heat-mediated antigen retrieval was used. Tissue sections were viewed with an Olympus BX60 phase-contrast microscope with the indicated objectives.

Proximity ligation assay

Cells were grown on coverslips, fixed with PTEMF, blocked WITH 5% BSA-TBST and incubated with primary antibodies at the following dilutions (anti-PHD3 1:1000, anti-p62 1:1000, anti-HIF-1 1:1000, anti-PHD2 1:1000). Duolink *in situ* proximity ligation assay was performed according to manufacturer's protocol (Olink Bioscience). Briefly, following primary antibody treatment cells were incubated with PLA probes for 1 h at 37℃ followed by hybridization, ligation and amplification. Cell nuclei were stained and slides mounted on coverslips using the manufacturer's mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI). Slides were analyzed with confocal microscopy (Zeiss LSM510). As a negative control secondary-antibody-omitted as well as PLA-probe-omitted slides were used.

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Author contributions

K.R. and J.P. designed the experiments with P.J., performed most of the experiments and data analyses, and took part in writing the manuscript. H.H. performed statistical analyses. P.M. assisted in performing and analyzing immunocytochemistry. J.S. provided clinical samples and took part in their analysis. P.J. designed the experiments with K.R. and J.P., and was mainly responsible for writing the article and finalizing the figures.

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