Molecular Biology of the Cell
Vol. 19, 2231–2240, May 2008

Prolyl Hydroxylase PHD3 Activates Oxygen-dependent Protein Aggregation

Krista Rantanen,* Juha Pursiheimo,* Heidi Högel,* Virpi Himanen,* Eric Metzen,† and Panu M. Jaakkola*‡

*Turku Centre for Biotechnology, Turku University and Åbo Akademi University, FIN-29521 Turku, Finland; †Institute of Physiology, University of Luebeck, D-23538 Luebeck, Germany; and ‡Department of Oncology and Radiation Therapy, Turku University Hospital, FIN-20520 Turku, Finland

Submitted November 12, 2007; Revised February 12, 2008; Accepted March 3, 2008
Monitoring Editor: Jonathan Weissman

The HIF prolyl hydroxylases (PHDs/EGLNs) are central regulators of the molecular responses to oxygen availability. One isoform, PHD3, is expressed in response to hypoxia and causes apoptosis in oxygenated conditions in neural cells. Here we show that PHD3 forms subcellular aggregates in an oxygen-dependent manner. The aggregation of PHD3 was seen under normoxia and was strongly reduced under hypoxia or by the inactivation of the PHD3 hydroxylase activity. The PHD3 aggregates were dependent on microtubular integrity and contained components of the 26S proteasome, chaperones, and ubiquitin, thus demonstrating features that are characteristic for aggresome-like structures. Forced expression of the active PHD3 induced the aggregation of proteasomal components and activated apoptosis under normoxia in HeLa cells. The apoptosis was seen in cells prone to PHD3 aggregation and the PHD3 aggregation preceded apoptosis. The data demonstrates the cellular oxygen sensor PHD3 as a regulator of protein aggregation in response to varying oxygen availability.

INTRODUCTION

Hypoxia forms a key component of multiple diseases, including stroke, inflammatory diseases and the progression of solid tumors. Hypoxia and in some cases the following reoxygenation pose considerable stress to cells. These include increased production of reactive oxygen species (ROS) and changes in protein translation, as well as exposure of cells to protein damage and misfolding (Rifkind *et al.*, 1991; Koumenis and Wouters, 2006; Liu *et al.*, 2006; Thuerauf *et al.*, 2006). Mammalian cells have evolved a molecular machinery to determine whether cells attempt to survive or go into apoptosis in these conditions. The best characterized molecular responses to hypoxia are mediated through a family of dioxygenases that use O_2 as a cosubstrate (Bruick and McKnight, 2001; Epstein *et al.*, 2001) and are termed prolyl hydroxylase domain proteins (PHD), also known as HIF prolyl hydroxylases or Egl-9 homologues (EGLN). Three known PHD (PHD1-3) isoforms hydroxylate the α-subunits of hypoxia-inducible factors 1 and 2 (HIF-1 and -2) under normoxic conditions at two proline residues (Pro402 and 564; Ivan *et al.*, 2001; Jaakkola *et al.*, 2001; Yu *et al.*, 2001; Masson *et al.*, 2004). The hydroxylated HIF-1α is recognized by the von Hippel-Lindau tumor suppressor protein (pVHL) that subsequently leads to ubiquitination and proteosomal destruction of HIF-1α (Kallio *et al.*, 1999; Maxwell *et al.*, 1999; Cockman *et al.*, 2000; Ohh *et al.*, 2000; Tanimoto *et al.*, 2000). Under restricted O_2 availability the PHD activity decreases and the degradation of HIF-1α is blocked, activating the transcription of a wide range of genes (Semenza, 2001; Harris, 2002; Pugh and Ratcliffe, 2003; Schofield and Ratcliffe, 2004).

All PHD isoforms have been reported to hydroxylate HIF and to have similar requirements for O_2 and cosubstrates FeII and 2-oxoglutarate at least in vitro (Hirsila *et al.*, 2003). However, their function and characteristics also differ in several aspects. Two isoforms, PHD2 and PHD3, are upregulated transiently by hypoxia, PHD3 showing the most robust induction (Berra *et al.*, 2003; D'Angelo *et al.*, 2003; Appelhoff *et al.*, 2004; Aprelikova *et al.*, 2004; Marxsen *et al.*, 2004). PHD2 demonstrates most abundant mRNA expression across tissues, whereas PHD3 is expressed at mRNA level in nonstressed conditions only in the cardiac and neural tissue (Lieb *et al.*, 2002; Cioffi *et al.*, 2003; Hirsila *et al.*, 2003). Their subcellular localization also differs (Metzen *et al.*, 2003). PHD2 is mainly cytoplasmic, although during tumor progression PHD2 can translocate to the nucleus (Jokilehto *et al.*, 2006). PHD3 in contrast, is expressed both in the nucleus and the cytoplasm. Moreover, a recent report suggests a non-HIF hydroxylation target, ATF-4, for PHD3 (Koditz *et al.*, 2007). Besides hypoxia, PHD3 or the murine homolog SM-20 is up-regulated, e.g., by vascular tissue injury (Wax *et al.*, 1996), ageing of cells (Rohrbach *et al.*, 2005) and nerve growth factor (NGF) removal in neural cells (Lipscomb *et al.*, 1999, 2000; Straub *et al.*, 2003; Lee *et al.*, 2005). Unlike the two other PHD isoforms, PHD3 has been reported to bear apoptotic function. In neural cells PHD3 is required and is sufficient to induce apoptosis after growth factor removal (Lipscomb *et al.*, 1999, 2000; Straub *et al.*, 2003; Lee *et al.*, 2005).

Protein aggregation is induced in response to various cellular stress and when a cell's capacity to degrade misfolded proteins is exceeded as well as during degenerative processes (Wojcik *et al.*, 1996; Johnston *et al.*, 1998; Kopito, 2000; Garcia-

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07–11–1124) on March 12, 2008.

Address correspondence to: Panu M. Jaakkola (panjaa@utu.fi).

Mata *et al.*, 2002). The aggregates generally contain components of the 26S proteasome, diverse ubiquitylated proteins and chaperones such as HSP70 and TRiC. Both cytotoxic and cytoprotective functions have been reported for these structures. In some situations the accumulation of protein aggregates impairs the function of the ubiquitin-proteasomal system (Bence *et al.*, 2001) and may lead to caspase-dependent apoptosis (Kristiansen *et al.*, 2005). Protein aggregation has been implicated in the pathogenesis of several neurodegenerative diseases, such as Parkinson's, Huntington's, and Alzheimer's diseases and amyotrophic lateral sclerosis (ALS) as well as alcoholic liver disease (Kopito and Sitia, 2000; Garcia-Mata *et al.*, 2002; Ardley and Robinson, 2004; Olanow *et al.*, 2004).

The protein aggregation is a tightly regulated and dynamic process. The number, size, subcellular localization, and protein content of the aggregates vary depending on the aggregating protein and cell type. Multiple aggregates varying in size and scattered around the cytoplasm are in many instances referred to as aggresome-like structures. The small cytoplasmic protein aggregates can be transported along the microtubules (MT) toward the perinuclear region where they converge and form large structures at the microtubule organizing center (MTOC) and are termed aggresomes. Aggresomes may disrupt the organization of cytoskeleton, leading to formation of vimentin cage around the aggresome. Aggresomes are induced by various proteins such as α-synuclein (Lee and Lee, 2002) and HDAC6 deacetylase in Parkinson's disease (Kawaguchi *et al.*, 2003) and prions in prion-associated disease (Kristiansen *et al.*, 2005). Smaller scattered protein aggregates or aggresome-like structures are detected, for example, with PLIC-1 (Heir *et al.*, 2006) and p62/SQSTM1 (Zatloukal *et al.*, 2002; Paine *et al.*, 2005; Pankiv *et al.*, 2007).

PHD3 is undetectable or expressed at low level in normoxic cancer cells. The expression is strongly induced during hypoxia (Appelhoff *et al.*, 2004; Aprelikova *et al.*, 2004; Marxsen *et al.*, 2004). However, in hypoxia PHD3 remains mainly inactive and the full activity is restored upon reoxygenation. Here we have studied the expression and function of PHD3 in oxygenated conditions. We show that PHD3 forms subcellular aggregates. The aggregation is dependent on both oxygen and PHD3 catalytic activity. Forced PHD3 expression induced the formation of protein aggregates in an oxygen- and hydroxylase activity–dependent manner. Furthermore, we show that the PHD3-induced aggregates bear an apoptosis-inducing potential in HeLa cells.

MATERIALS AND METHODS

Cell Culture, Plasmids, Transfection, and Chemicals

HeLa cells were obtained from the American Type Culture Collection (Rockville, MD). UT-SCC2 cell line have been described previously (Jokilehto *et al.*, 2006). Cells were cultured in DMEM, supplemented with 10% FCS, penicillin-streptomycin, and L-glutamine. Cells were cultured in humidified air containing 5% CO_2 at 37°C. For hypoxia treatments air was replaced by nitrogen to reach 1% oxygen in a hypoxia workstation (In vivo$_2$, Ruskinn Technology, Bridgend, United Kingdom).

Cells were transfected at optimal confluence using either FuGene HD (Roche Applied Science, Lewes, East Sussex, United Kingdom) or Effectene transfection reagent (Qiagen, Chatsworth, CA) according to manufacturer's protocols.

PHD3-EGFP construct, where enhanced green fluorescent protein (EGFP) is fused to the C-terminus of PHD3, has been described previously (Metzen *et al.*, 2003). PHD3R206K-EGFP was made by QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) using 5′-CCCTCTTACGCAACCA*A*ATATGCTATGACTGTCTGG-3′ oligonucleotide (the mutated residue is underlined). For red light-emitting EGFP variant PHD3-dsRed, complete PHD3 ORF was acquired by PCR and cloned into pDsRed-Monomer-N1 vector (Clontech, Heidelberg, Germany) using HindIII and SmaI sites. PCR primer oligonucleotides used were 5′-GAGCAAGCTTTTATGCCCCTGGGACAC-3′ and 5′-CCTCCCGGGTCAGTCTTCAGTGAGG-3′.

Hydroxylase inhibitor dimethyloxaloylglycine (DMOG) was obtained from Cayman Chemical (Ann Arbor, MI) and used at 40 μM or as indicated. Cobalt chloride (Sigma-Aldrich, Poole, Dorset, United Kingdom) was used as 100 μM final concentration. Nocodazole (Sigma-Aldrich) was used at 5 μM. The proteosomal inhibitor MG132 (Z-leu-leu-Al; Sigma-Aldrich) was used at 10 μM.

Protein Detection, Cell Imaging, and Fluorescence-activated Cell Sorting Analysis

For Western blotting, cells were harvested in 1× SDS sample buffer. Proteins were detected by enhanced chemiluminescence (Pierce, Bonn, Germany). For immunocytochemistry, cells were fixed on cover slips with PTEMF (100 mmol/L PIPES, pH 6.8, 10 mmol/L EGTA, 1 mmol/L MgCl, 0.2% Triton X-100, and 4% formaldehyde). Fixed cells were then treated with primary and secondary antibodies and mounted with Mowiol-DABCO (Carpinteria, CA) on cover glass. Histograms of colocalization were generated with the LSM META software.

Endogenous PHD3 was detected by immunocytochemistry using two independent specific primary antibodies at 1:2500 dilution: Rabbit polyclonal PHD3 (cat. no. BET-A300-327A, Bethyl Laboratories, Montgomery, TX) was used in Supplementary Figure 1 and Figure 6B and rabbit polyclonal PHD3 (cat. no. NB100-139, Novus Biologicals, Littleton, CO) in all other experiments. Other primary antibodies used with indicated dilutions were as follows: rabbit polyclonal PHD2 at 1:2500 dilution (cat. no. NB100-137, Novus Biologicals), rabbit polyclonal activated caspase-3 antibody at 1:400 dilution (cat. no. G748, Promega, Madison, WI), mouse monoclonal HSP70 at 1:1000 (clone SPA-810, Stressgen, San Diego, CA), rabbit polyclonal anti-20S proteasome core subunits at 1:2000 (cat. no. PW8155, Biomol, Hamburg, Germany) and beta-Actin (AC-40, Sigma). Hemagglutinin (HA)-ubiquitin was detected using antibody against HA at 1:1000 dilution (clone 3F10, Roche Diagnostics, Mannheim, Germany). Cy3- or Cy5-conjugated secondary antibodies were used as at 1:1000 dilution (Jackson Immunochemicals, West Grove, PA). Cell nuclei were stained with Hoechst 33342 (Sigma).

Quantification of the EGFP amount (Figure 1D) was done using ImageJ image analysis software (NIH, http://rsb.info.nih.gov/ij/) after imaging the cells with LSM510 Meta confocal microscope. Cell perimeter was defined manually and the mean intensity of the region of interest (the entire cell) was measured using ImageJ analysis option. Flow cytometric analysis (FACS) was done using FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). To study the cell cycle and in particular the amount of sub-G1 population (apoptotic cells), the cells were fixed with methanol and stained with propidium iodide solution. Fluorochromes (GFP) were excited with 488 nm laser and GFP was measured using 530/30 bandpass filter. Propidium iodide was measured using 670 longpass filter. For FACS analysis CellQuest software (Becton Dickinson) was used. The sub-G1 population was studied from the GFP-positive and -negative populations separately.

Live-Cell Microscopy and Fluorescence Recovery after Photobleaching

For live-cell microscopy, cells were grown on glass-bottomed dishes (MatTek, Ashland, MA) and visualized with Zeiss LSM 510 META confocal laser scanning microscope with Physiology software (Carl Zeiss, Jena, Germany) in a humidified cell culture chamber with 5% CO_2 at 37°C. For detection of mitochondrial membrane potential tetramethyl rhodamine (TMRM, Invitrogen, Karlsruhe, Germany) was used at 50 nM.

The experimental conditions for FRAP have been described previously elsewhere (Phair and Misteli, 2000). Briefly, from cells transiently expressing PHD3-EGFP a region of interest (ROI) was selected and imaged (prebleach) followed by photobleaching of the ROI with argon laser 488 nm at maximum power for 100 iterations. The recovery of the bleached region was imaged with 2% laser power with 2-s intervals. The mobile fraction and the half-life of the recovery was calculated using FRApCalc program (Dr. Rolf Sara, Turku Centre for Biotechnology).

RESULTS

PHD3 Forms Subcellular Aggregates in an Oxygen and Hydroxylase-dependent Manner

In most cancer cells PHD3 is expressed at low or nondetectable level in normoxia, but is strongly induced by hypoxia (del Peso *et al.*, 2003; Appelhoff *et al.*, 2004; Aprelikova *et al.*, 2004; Marxsen *et al.*, 2004). In hypoxia, however, PHD3 remains mainly inactive (Hirsila *et al.*, 2003) and is reactivated after reoxygenation. Using squamous cancer cells (SCC2) as a model, we studied the expression pattern of PHD3 in normoxia and hypoxia and after short reoxygen-

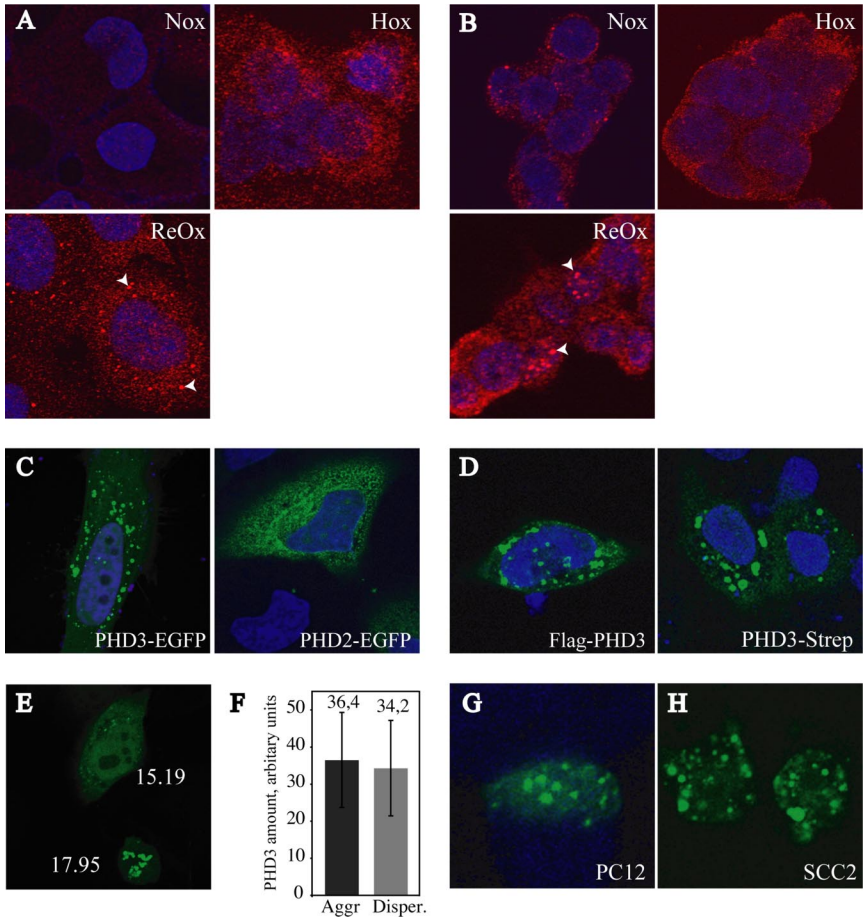

Figure 1. PHD3 forms subcellular aggregates. (A) Immunostaining for endogenous PHD3 after 48-h normoxia (nox), hypoxia (hox) and 1-h reoxygenation (reox) in low passage head and neck squamous carcinoma cells (SCC2). Arrowheads point to cytoplasmic aggregates in reoxygenated conditions. (B) Immunostaining for endogenous PHD3 after 48-h normoxia (nox), hypoxia and 1-h reoxygenation in PC12 cells. Arrowheads point to PHD3 aggregates in reoxygenated conditions. (C) HeLa cells transfected with PHD3-EGFP or PHD2-EGFP and visualized 24 h after transfection by confocal microscopy. (D) HeLa cells transfected with two different PHD fusion proteins: N-terminal Flag (Flag-PHD3) and C-terminal Streptacting (PHD3-Strep). (E) Quantification of PHD3-EGFP expression by confocal microscopy. An example demonstrates expression values obtained in a cell displaying dispersed PHD3 expression (top cell) and in a cell showing aggregation of PHD3 (bottom cell). (F) Quantification of PHD3 expression (means ± SD) level in 15 cells with either aggregated or dispersed PHD3, indicating similar expression levels regardless of the subcellular localization of PHD3. Subcellular localization of PHD3-EGFP in PC12 (G) and SCC2 (H) cells. All magnifications, ×630.

ation (Figure 1). To validate the specificity of the PHD3 staining, we evaluated two commercial antibodies raised against PHD3 and performed small interfering RNA (siRNA) experiments. The two anti-PHD3 antibodies showed an indistinguishable staining pattern (Supplementary Figure 1A). Moreover, transfection of a previously validated PHD3 siRNA (Berra *et al.*, 2003) before PHD3 immunocytochemistry caused complete loss of the PHD3 signal compared with control siRNA (Supplementary Figure 1B). Normoxic SCC2 cells showed negligible PHD3 expression. After 48-h hypoxia PHD3 demonstrated expression within the nucleus and cytoplasm as reported previously (Metzen *et al.*, 2003). Interestingly, the SCC2 cells also showed a punctuate or aggregating PHD3 expression pattern in hypoxia. After the subsequent 1-h reoxygenation, a subset of the cells demonstrated increased aggregation of PHD3 seen as an increase in the amount and size of the aggregates (Figure 1A). Besides SCC2 cells, subcellular PHD3 aggregates were also detected in PC12 cells and these were increased in size and amount in a subset of cells in normoxic conditions as compared with hypoxia (Figure 1B).

To study the nature of the aggregates we used HeLa cells, which have a low or undetectable normoxic PHD3 level, with ectopically expressed PHD3-EGFP. In addition to cells that showed a dispersed PHD3 expression pattern, a large subset of the cells demonstrated subcellular aggregates (Figure 1C). Costaining with laminA/C (not shown) demonstrated that the aggregates were mainly located in the cytoplasmic or perinuclear compartments. The aggregation of PHD3 did not depend on the tag used because other PHD3 fusion proteins with different, both N- and C-terminal, tags displayed similar aggregates (Figure 1D). Quantification of the PHD3-EGFP expression level from aggregate-forming and nonforming cells showed that the aggregation did not directly correlate to the amount of the expressed PHD3 within a given cell (Figure 1, E and F). Similarly to HeLa cells, the aggregation of PHD3-EGFP was detected in PC12 (Figure 1G) and SCC2 cells (Figure 1H). These aggregates were termed PHD3 bodies.

Next we asked whether the formation of the PHD3 bodies depends on oxygen availability (Figure 2). HeLa cells expressing ectopical PHD3-EGFP were exposed to hypoxia (1% O_2) or normoxia (21% O_2) for 8 h. The normoxic aggregation was strongly reduced in hypoxic conditions. Hypoxia reduced the size of the aggregates and the amount of aggregates within the cells as well as the amount of cells displaying aggregates (Figure 2, B, E, and F).

Prompted by the requirement of oxygen for PHD3 body formation, we further investigated if this required the hydroxylase activity of PHD3. First, HeLa cells expressing PHD3 were subjected to a well-characterized hydroxylase inhibitor DMOG for 8 h. The inhibition of hydroxylase activity by DMOG clearly reduced the cells displaying PHD3 bodies (Figure 2, C and E). Similarly to hypoxia and DMOG, two hypoxia-mimicking compounds, desferrioxamine (DFO) and cobalt chloride ($CoCl_2$), attenuated the aggregate formation (not shown). To study whether the hydroxylase activity of PHD3 is required for the aggregation, we introduced a catalytic activity abrogating point mutation to PHD3. Here, the 2-oxoglutarate coordinating arginine (R206) is mutated into

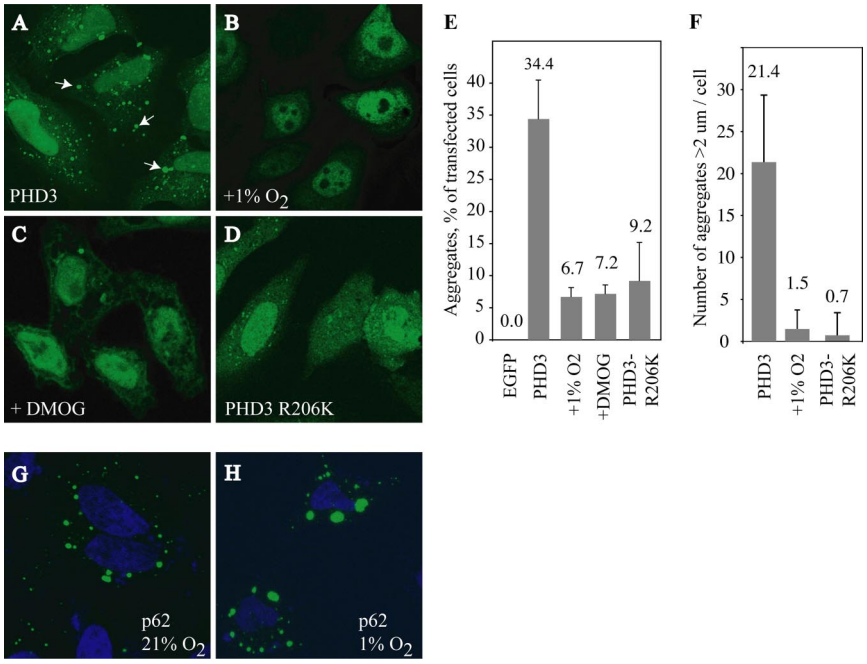

Figure 2. PHD3 aggregation is oxygen- and hydroxylase-dependent. (A–D) Subcellular localization of PHD3 under hypoxia and hydroxylase inhibition. HeLa cells with wild-type PHD3-EGFP (A) or mutant PHD3R206K and PHD3-EGFP (D) exposed to 8-h hypoxia (B) or 8-h DMOG (D). Aggregation of PHD3 (arrows) was attenuated by the inhibition of hydroxylase activity. (E) Quantification of cells demonstrating visible PHD3 aggregates (>2 μM). Cells, n = 200–300, were studied and judged either to have or not to have PHD3 aggregates. Means ± SE for three independent experiments are shown. (F) Quantification of the number of PHD3 aggregates (>2 μM) within cells at indicated conditions. Cells, n = 200–300, were studied and judged either to have or not to have PHD3 aggregates. Means ± SE of 15 cells for each condition are shown. (G) SCC2 cells transfected with EGFP-p62, exposed to normoxia, and visualized 24 h after transfection. (H) EGFP-p62 exposed to 8-h hypoxia before visualization. Magnifications, ×630.

lysine (PHD3R206K). Similarly to DMOG, PHD3R206K showed strongly diminished aggregation. This was seen as reduced aggregate size as well as amount of aggregation, measured either by the amount of cells showing aggregation or the amount of cytoplasmic aggregates within the cells (Figure 2, D–F). The results indicated that besides sufficient oxygen level, PHD3 aggregation depends on its prolyl hydroxylase activity.

The PHD3 bodies strongly resembled some proteins that form aggresome-like structures, such as p62/SQSTM1. We asked whether the oxygen dependency is a more general feature of protein aggregation by using EGFP-p62. Cells transfected with EGFP-p62 were exposed to normoxia or 8-h hypoxia (Figure 2, G and H). p62 formed cytoplasmic aggregates in normoxia as described previously (Zatloukal *et al.*, 2002; Pankiv *et al.*, 2007). The formation of p62 aggregates were not altered by hypoxic exposure, suggesting that oxygen is not in general required for protein aggregation.

PHD3 Bodies Colocalize with Proteosomal Components

The aggresome-like structures contain components of the 26S proteasome. To study whether PHD3 localizes with the proteosomal components, cells were cotransfected with PHD3 and a structural component of the 20S proteasome, LMP2 (Reits *et al.*, 1997). A red variant of EGFP fused to PHD3 (PHD3-dsRed) was used to detect the colocalization of PHD3 with LMP2-EGFP. Cells expressing both constructs showed nearly complete colocalization 1 d after transfection (Figure 3, A and B). We further studied the localization of the endogenous proteasomal components using an antibody against the 20S proteasome together with ectopic PHD3-EGFP. Similarly to LMP2, the endogenous 20S demonstrated colocalization with PHD3 (Figure 3, C and D, Supplementary Video 1). Besides proteasomal components, the protein aggregates may contain various chaperones and ubiquitin. In line with this, we detected colocalization of PHD3 with endogenous HSP70 chaperone (Figure 3E) as well as with ectopically expressed HA-ubiquitin (Figure 3F). However, we did not detect vimentin reorganization upon PHD3 expression and the PHD3 bodies were not surrounded by a vimentin cage (Supplementary Figure 2).

We next asked whether the endogenous PHD3 colocalizes with the proteosomal structures. SCC cells (Figure 3G) were transfected with LMP2-EGFP. This was followed by 48-h hypoxic exposure to induce PHD3 expression and a short reoxygenation to retain ambient oxygen level. In accordance with the ectopical PHD3 expression, partial colocalization of LMP2 and endogenous PHD3 was detected (Figure 3G), albeit the endogenous PHD3 expression was clearly lower than that of PHD3-EGFP. Finally, to study the localization of PHD3 outside the aggregates, we used low concentration DMOG (40 μm) to partially disperse the PHD3 protein. The data demonstrated that PHD3 did not colocalize with 20S proteasome outside the aggregates (Figure 3H).

Protein aggregates may be transported along the microtubules, and in the case of aggresome formation they are transported toward the perinuclear region. Inhibition of microtubule polymerization by nocodazole abrogates the transportation. Supporting the hypothesis of PHD3 transport along the microtubuli, 5 μM nocodazole nearly completely inhibited the aggregation of PHD3 (Figure 4A). Moreover, suggesting the requirement of proteasomal function for the localization of the PHD3 bodies, the inhibition of proteosomal function by MG132 caused PHD3 to cluster along the microtubuli (Figure 4A).

Next we studied the dynamics of the PHD3 bodies using the FRAP technique. FRAP was performed within both cytoplasmic and perinuclear aggregates. In general, the cytoplasmic aggregates showed rapid recovery of PHD3 within the aggregates indicating fast movement of PHD3 into these structures (Figure 4, B and C, and Supplementary Video 2). The live-cell imaging studies further demonstrated cytoplasmic movement and convergence of the PHD3 aggregates (Supplementary Video 2). The dynamics within the perinuclear aggregates demonstrated overall slower recovery of PHD3 (Figure 4C) and some very stable structures. The range of the mobile fraction within the cytoplasmic aggregates ranged from 23 to 96% and within the perinuclear

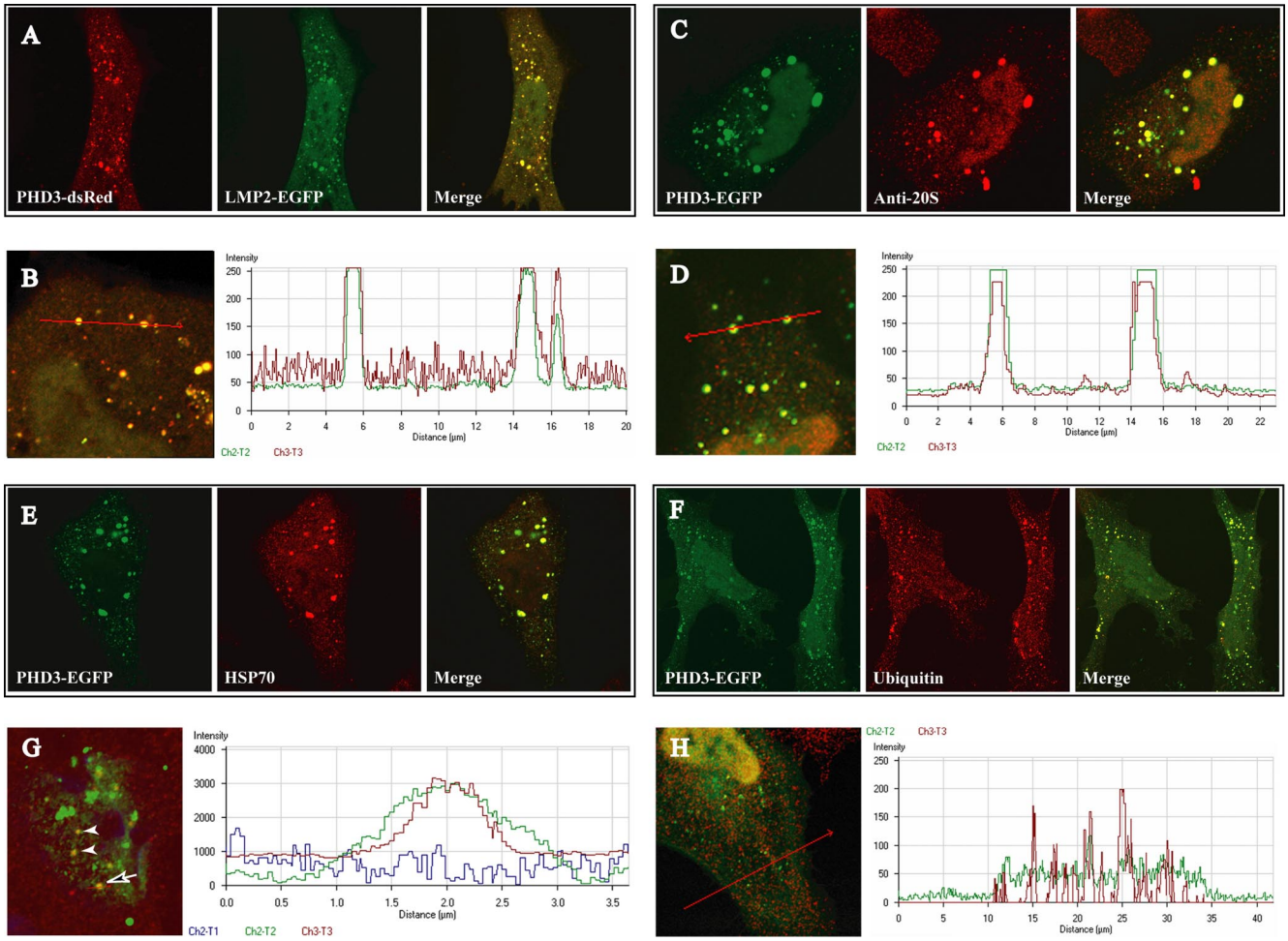

Figure 3. PHD3 colocalizes with aggresome-like structures. (A) PHD3-dsRed (red) and the proteasomal component EGFP-LMP-2 (green) transfected into HeLa cells and detected by fluorescence confocal microscopy 24 h after transfection. (B) Profiling of the localization demonstrated near complete colocalization of LMP-2 with PHD3 within the aggregates. The red line indicates the profile location. (C) PHD3-EGFP detected by fluorescence together with the endogenous large subunit (20S) of the proteasome by immunocytochemistry. (D) Profiling demonstrating colocalization of 20S and PHD3. (E) Colocalization of PHD3-EGFP and endogenous HSP70 (red) by immunocytochemistry. PHD3 and HSP70 show near complete colocalization. (F) Colocalization of PHD3-EGFP and HA-ubiquitin (red). (G) Colocalization of endogenous PHD3 (red) with LMP2-EGFP (green) after 2-d hypoxia and 1-h reoxygenation. The endogenous PHD3 partially colocalizes with the proteosomal marker LMP2 (arrowheads). The large arrow indicates the aggregate used in the adjacent profiling. (H) The endogenous PHD3 (red) with LMP2-EGFP (green) after 8-h DMOG indicates no colocalization outside the aggregates. Magnifications, ×630.

structure from 8 to 30% (Figure 4C). Moreover, exposure of cells to a low concentration of nocodazole (5 μM) after the aggregates had formed showed reduced PHD3 movement (not shown). Taken together this demonstrated that the PHD3 bodies are highly dynamic structures and show features of aggresome-like structures.

PHD3 Expression Activates Aggregation of Proteasomal Components in a Hydroxylase Activity–dependent Manner

The experiments with forced PHD3 expression suggested that PHD3 could activate the aggregation of proteasomes. To study this more closely, we compared the aggregation induced by proteosomal inhibition to that induced by PHD3 expression. Forced PHD3 expression activated aggregation of the 20S proteasomal subunit, and visible 20S aggregates were detected only within cells with aggregating PHD3 (Figure 5, C and D). The 20S aggregation was comparable to that induced by MG132 (Figure 5B). Importantly, the inhibition of the PHD3 hydroxylase activity either by DMOG (Figure 5, E and F) or by the hydroxylase activity inhibiting mutation (Figure 5, G and H) inhibited the aggregation of 20S by PHD3. We further compared PHD1, -2, and -3 in their ability to induce proteasomal aggregation. In contrast to EGFP-PHD3, neither EGFP-PHD1 nor EGFP-PHD2 expression elicited the formation of visible 20S aggregates in normoxic conditions (Supplementary Figure 3).

A small amount of PHD3 aggregation was retained after inhibition of the PHD3 hydroxylase activity either by application of the chemical hydroxylase inhibitor or by expression of the inactivated PHD3 (Figure 2E). However, neither of these was able to induce the aggregation of the 20S proteasome. We therefore asked whether the small amount of aggregates formed by the mutant PHD3 colocalized with 20S proteasome (Figure 5I). Interestingly, the mutant PHD3 did not show colocalization with the proteosomal components. Similar results were obtained with wild-type PHD3 using DMOG inhibition (not shown). This indicated that the small amount of aggregates seen when PHD3 hydroxylase

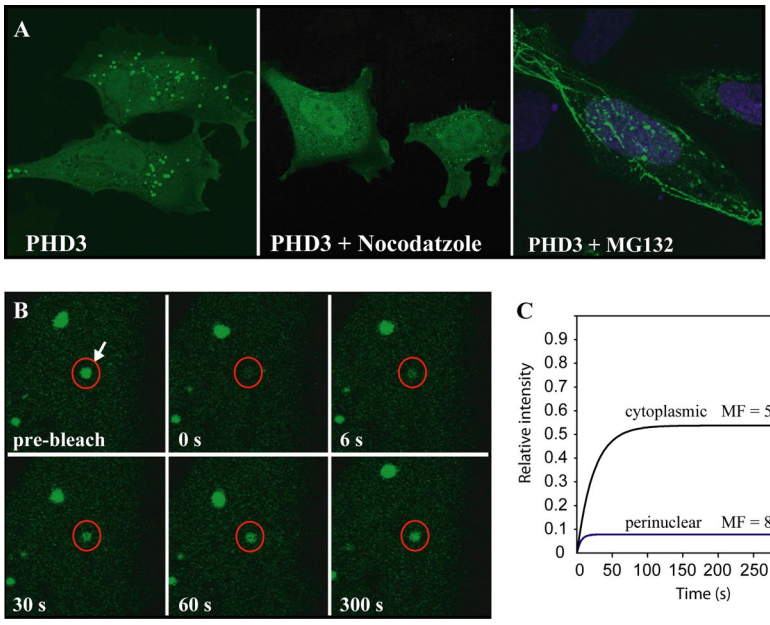

Figure 4. Dynamics of PHD3 aggregation. (A) PHD3-EGFP expression in cells exposed to nocodazole (5 μM) or MG132 (20 μM) for 6 h. (B) PHD3-EGFP (green) was used for FRAP. A representative study of a cytoplasmic aggregate is shown. The red circles mark the photobleached aggregate. Reappearance of PHD3-EGFP expression within the aggregate demonstrated fast movement of PHD3 into the aggregate. (C) Histogram of the FRAP analysis from representative cytoplasmic and perinuclear aggregates. MF, mobile fraction.

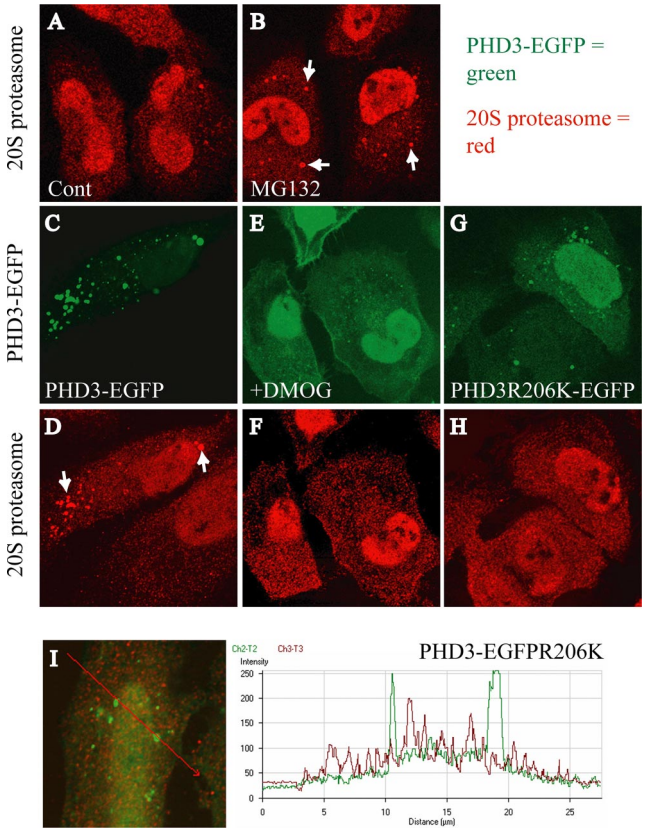

Figure 5. PHD3 induces aggregation of the 20S proteasome in a hydroxylase-dependent manner. HeLa cells were left untransfected (A and B) or transfected with PHD3-EGFP construct (green) (C–H) and stained for endogenous 20S proteasome (red). (B) Proteosomal inhibition by MG132 caused aggregation of 20S proteasome (arrows). (C and D) The forced expression of PHD3 led to the development of comparable aggregates (arrows). (E and F) PHD3-expressing cells exposed to 8-h DMOG (750 μm) showed reduction of 20S aggregation. (G and H) Expression of the mutant PHD3 did not demonstrate visible 20S aggregation. (I) Localization of PHD3R206K-EGFP with endogenous 20S. Aggregates formed by PHD3R206K did not colocalize with the proteasomes. Magnifications, ×630.

activity is inhibited do not represent aggresome-like structures. Taken together this demonstrated that PHD3 induces the formation of proteasome containing structures that bear close resemblance to aggresome-like structures. Moreover, the data shows that sufficient oxygen together with the intact hydroxylase activity of PHD3 is required for this.

PHD3-induced Aggregates Activate Apoptosis in an Oxygen- and Hydroxylase Activity–dependent Manner

PHD3 induces caspase-dependent apoptosis under oxygenated conditions in neural cells when accumulated after NGF withdrawal (Lipscomb *et al.*, 2000; Straub *et al.*, 2003). Moreover, proteins that induce proteasomal aggregation have been reported to activate caspase-3–dependent apoptosis, and aggresomes can impair the proteosomal function (Bennett *et al.*, 2005; Kristiansen *et al.*, 2005). Using HeLa cells as a model, we asked whether PHD3 has apoptosis-inducing capacity when forced to be expressed upon reoxygenation. Apoptotic cell death, as measured by nuclear fragmentation, was detected with forced PHD3 expression during normoxia and after long reoxygenation (24 h), but not after short reoxygenation (4 h; Figure 6A) after overnight hypoxia. In contrast, PHD2 expression did not lead to cell death under any of these conditions. In line with the fact that HeLa cells are resistant to reoxygenation-induced cell death, the endogenous hypoxia-induced PHD3 was rapidly degraded during reoxygenation, reaching normoxic background levels in less than 2 h (Figure 6B).

We next asked whether the hydroxylase activity of PHD3 is required for its apoptotic activity as has previously been shown for neural cells (Lipscomb *et al.*, 2000; Lee *et al.*, 2005). HeLa cells were transfected with PHD3-EGFP and exposed for 8 h to DMOG followed by the detection of apoptosis by nuclear fragmentation and caspase-3 activation. Both the nuclear fragmentation and the caspase-3 activation induced by PHD3 were strongly inhibited by DMOG (Figure 6, C–E), indicating that intact cellular hydroxylase activity is required for PHD3 to activate apoptosis. Similarly to DMOG, the genetic inactivation of PHD3 (PHD3R206K) led to the loss of the apoptotic activity as measured by nuclear fragmentation (Figure 6C) or caspase-3 activation (Figure 6, D

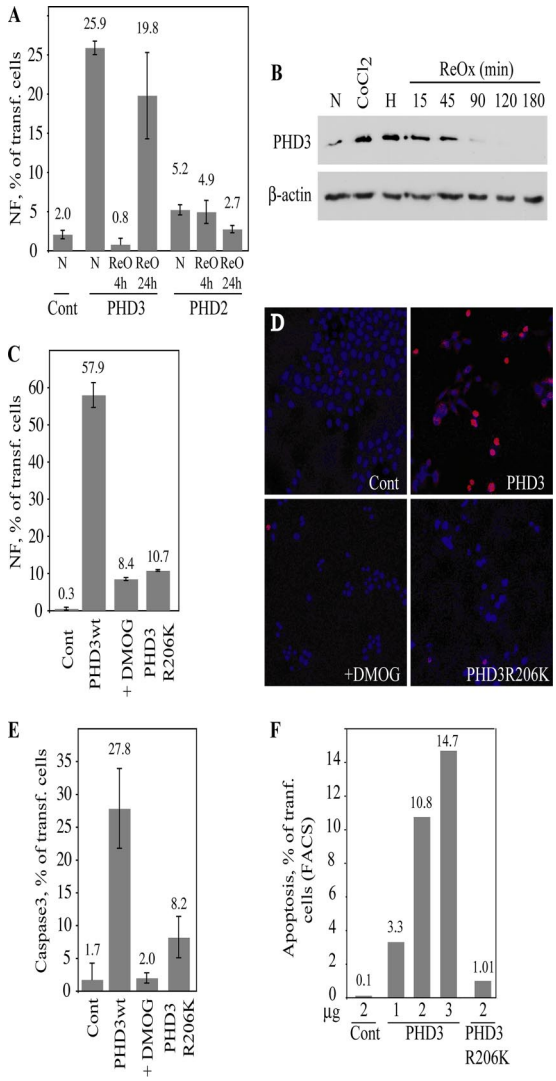

Figure 6. PHD3 induces apoptosis in a hydroxylase-dependent manner in HeLa cells. (A) HeLa cells expressing EGFP (cont), PHD3-EGFP, or PHD2-EGFP exposed to normoxia (N) or reoxygenation (ReO) at two time points after overnight hypoxia. Apoptosis was analyzed by nuclear fragmentation in ~300 cells. Means ± SD of three experiments are shown. (B) Western blotting analysis of endogenous PHD3 in normoxia (N), 48-h hypoxia (H), or cobalt chloride treatment and reoxygenation after 48-h hypoxia at the indicated time points. (C) HeLa cells expressing control plasmid, PHD3-EGFP, mutant PHD3 (PHD3R206K-EGFP) or PHD3-EGFP exposed to 8-h hydroxylase inhibition (DMOG) were analyzed for apoptosis by nuclear fragmentation (NF). (D) The same conditions were used to study the activated caspase-3 by immunocytochemistry (red). Caspase-3 activation induced by PHD3 was suppressed by the inhibition of PHD3 hydroxylase activity (DMOG and PHD3R206K). Hoechst was used to counterstain the nuclei (blue). Magnification, ×400. (E) Quantification of caspase-3 activation from three independent experiments. For each experiment ~300 cells were counted. Means ± SD of three experiments are shown. (F) Apoptosis measured by FACS analysis in HeLa cells transfected with PHD3 at indicated concentrations. A representative study is shown. The complete FACS analysis data are shown as Supplementary Figure 4.

and E), indicating that the apoptosis-activating capacity requires PHD3's own enzymatic activity. FACS studies further showed that by increasing the amount of the transfected PHD3, the number of cells undergoing apoptosis was augmented (Figure 6F and Supplementary Figure 4). The data demonstrated that elevated normoxic PHD3 levels activate apoptosis in HeLa cells in an oxygen- and PHD3 hydroxylase activity–dependent manner.

Aggregation of PHD3 Coincides with Apoptosis

Both the emergence of the PHD3 bodies (Figure 2) and the PHD3-induced apoptosis (Figure 6) were strictly dependent on oxygen availability and PHD3 hydroxylase activity. We asked whether the aggregation of PHD3 coincides with PHD3-induced apoptosis. First, FACS analysis showed that the PHD3-induced apoptosis is time-dependent. Twenty-four hours after transfection a clearly increased apoptosis was detected, but maximal apoptosis was seen 48 h after transfection (Figure 7A). Next, apoptosis was determined by nuclear fragmentation in PHD3-EGFP expressing HeLa cells. Twenty-four hours after transfection 60% of the transfected cells that showed nuclear fragmentation displayed aggregated PHD3. Forty-eight hours after transfection nearly all apoptotic cells displayed aggregated PHD3. In contrast, within the cells with no visible signs of nuclear fragmentation, the PHD3 aggregation was negligible (Figure 7B). Similarly to the nuclear fragmentation, 48 h after transfection a clear correlation between caspase activation and PHD3 aggregation was seen. Cells with visible PHD3 aggregates showed activated caspase-3 in contrast to the cells with dispersed PHD3 (Figure 7C). The two cell populations (cells with aggregated and dispersed PHD3) showed a mirror image 48 h after transfection; more than 90% of the cells with PHD3 aggregates displayed activated caspase-3. Vice versa, <5% of cells with nonaggregating PHD3 demonstrated activated caspase-3 (Figure 7D).

We further performed live-cell imaging of HeLa cells with forced PHD3-EGFP expression to detect the temporal correlation between PHD3 aggregation and cell death (Figure 7E). The loss of mitochondrial membrane potential and blebbing of the cells were used as a marker for apoptosis. A population of nine cells was followed from 12 to 24 h after transfection. Of the nine cells, seven cells showed PHD3 expression and five of these cells aggregating PHD3. All aggregate forming cells (5/5) showed signs of cell death during the follow-up time. In each cell the aggregation of PHD3 preceded cell death. In contrast, none of the untransfected or nonaggregating cells (4/4) demonstrated signs of cell death (Figure 4E and Supplementary Video 3).

DISCUSSION

Here we have shown that PHD3 forms proteasome component–containing bodies that closely resemble aggresome-like structures. The activation of the PHD3-induced protein aggregation is strictly dependent on sufficient oxygen availability and moreover, requires the hydroxylase activity of PHD3. We have further shown that the activation of protein aggregation by PHD3 correlates with its ability to induce apoptosis in HeLa cells.

Several lines of evidence imply that the ability of PHD3 to activate apoptosis and to form aggresome-like structures are crucial biological mechanisms of the oxygen-sensing pathway to regulate cell fate. First, the activation of protein aggregation has been recognized in the past years as an important and tightly controlled response to regulate cell survival upon stressed conditions. In keeping with this, only few proteins have been reported to bear a proteasomal aggregation–inducing capacity. Second, the wild type, but not the mutated PHD3 induces aggregation. If this were a cyto-

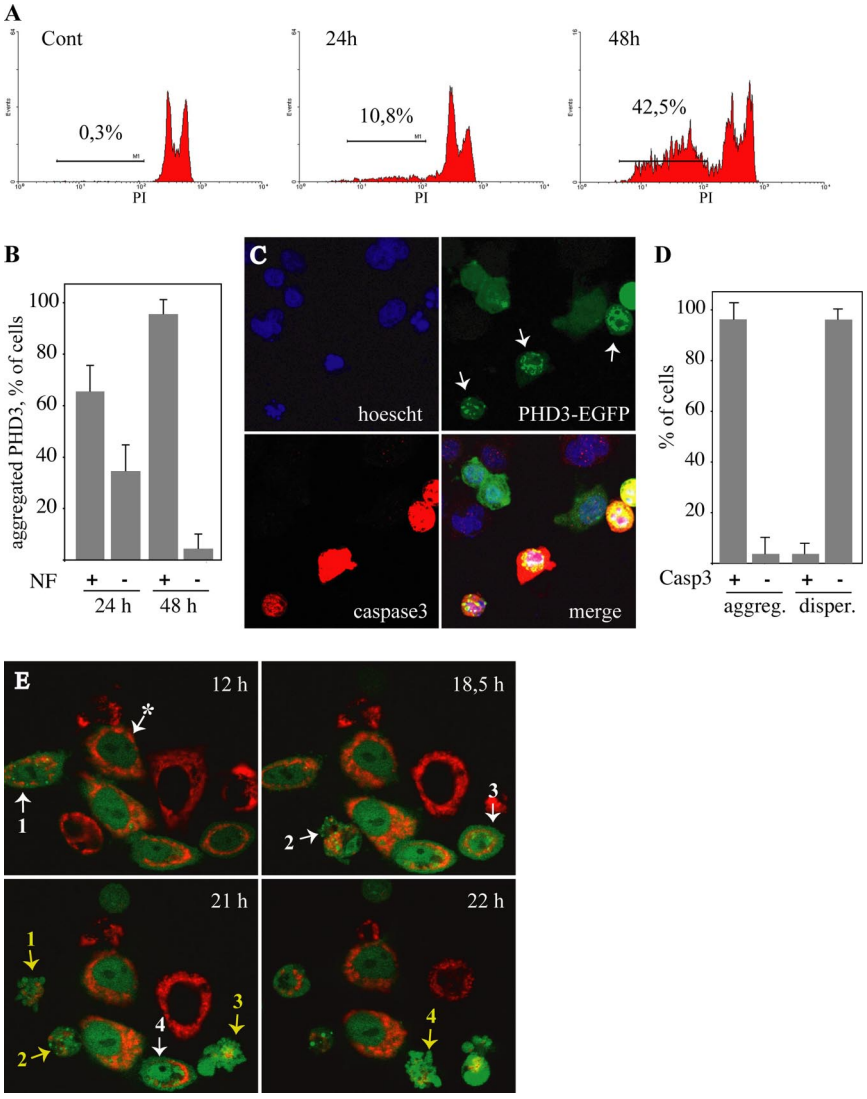

Figure 7. PHD3 aggregation coincides with apoptosis. (A) Apoptosis measured by FACS analysis in HeLa cells expressing control plasmid (EGFP, 24 h) or PHD3-EGFP for 24 and 48 h. Line and percentage indicates cells in sub-G1 (apoptotic) phase. (B) Quantification of cells demonstrating visible aggregates in cells with (+) or without (−) nuclear fragmentation (NF) at 24 and 48 h. Cells with nuclear fragmentation demonstrated simultaneous PHD3 aggregation in almost all cells 48 h after transfection. (C) HeLa cells transfected with PHD3-EGFP (green) and stained for activated caspase-3 (red) 24 h after transfection. Cells with PHD3 aggregation show activated caspase3 (arrows), whereas no caspase-3 activation was seen in the cells with dispersed PHD3 expression. (D) Quantification of caspase-3 activation from cells showing either aggregation of PHD3 (aggr) or dispersed expression (disper.) 48 h after transfection. (E) Selected still images from an 12-h live-cell imaging of PHD3-EGFP (green) and TRMR as an indicator of intact mitochondrial membrane potential (red). White arrows point to cells when PHD3 aggregation was first detected. Yellow arrows point to same numbered cells when the first signs of apoptosis were visible, as judged either by the loss of cell respiration of by the blebbing of the cells. Asterisks point to cells with no visible aggregate formation. These are low-resolution images derived from live-cell imaging. A full video best demonstrating the temporal correlation between PHD3 aggregation and apoptosis is provided as Supplementary Video 3.

toxic response to misfolded overexpressed protein, either both proteins or the mutant form only, would be expected to activate aggregation and cell death. Third, both the PHD3 body formation and its apoptotic function are prevented by hypoxia or by inhibiting the hydroxylase activity. In the case of a plain nonspecific cytotoxicity one would expect hypoxia or the hydroxylase inhibition to have an additive effect, rather than reversal, on the PHD3-induced cell death. Finally, the endogenous PHD3 when aggregated upon reoxygenation partially colocalizes with proteasomal aggregates. However, unlike in stressed neural cells, the endogenous PHD3 does not activate apoptosis after reoxygenation in cancer cells. This is in line with the fact that cancer cells are resistant to reoxygenation-induced cell death and with the rapid oxygen-induced degradation of PHD3 in these cells. It needs to be emphasized that even with forced expression PHD3 requires 24–48 h to activate cell death. One may argue that PHD3 could block the proteosomal system when artificially overexpressed as it is degraded by the proteasomal system (Nakayama *et al.*, 2004). Several lines of evidence argue against this. The vast majority of proteins degraded by the proteasome, such as HIF-1α, HIF-2α, and PHD2 in the oxygen-sensing pathway, are not aggregated even when forced to express (Kallio *et al.*, 1998; Metzen *et al.*, 2003; Acker *et al.*, 2005; Figure 2). Moreover, if the overexpressed PHD3 sterically blocked the proteasome, the PHD3R206K should have a similar effect, which was not the case.

Both protective and survival-impairing functions have been described for aggresome-like structures. In the HeLa cell strain used in this study, the aggregation induced by PHD3 clearly functioned as an apoptosis-inducing factor. However, we also detected HeLa cell strains that did not undergo apoptosis with elevated PHD3 (not shown). Interestingly, although in these strains PHD3 did aggregate, it neither activated the aggregation of the proteasomal system nor was PHD3 localized to aggresome-like structures. Furthermore, these aggregates did not respond to hypoxia nor to hydroxylase inhibition (not shown), which, along the fact that PHD3 aggregation was seen only in a subpopulation of HeLa cells (Figure 2E), indicated that PHD3 aggregation is a cell type–specific response and that other regulatory components are required for the aggregation. Moreover, it is also clear that PHD3 requires the hydroxylase activity to activate aggregation. This suggests the existence of PHD3 hydroxylation targets that are expressed in normoxic conditions. Previous findings have demonstrated an interaction between PHD3 and the TRiC chaperone (Masson *et al.*, 2004), which is a known component of protein aggregates. How-

ever, rather than being a hydroxylation target of PHD3, TRiC may regulate the function of PHD3. Whether TRiC takes part in targeting PHD3 into the aggregates is an open question. One non-HIF target for PHD3, ATF-4, has been recently characterized (Koditz *et al.*, 2007). Interestingly, ATF-4 is a transcription factor that mediates the unfolded protein response. However, although ATF-4 is an intriguing target for PHD3 in the aggregates, it is a nuclear protein and PHD3 bodies are mainly found in the cytoplasm.

The PHD3 bodies differ from classical aggresomes such as the cystic fibrosis transmembrane regulator, presenilin-1 (Johnston *et al.*, 1998) or HDAC6 (Kawaguchi *et al.*, 2003). First, we did not detect complete localization of PHD3 to the MTOC, which is seen with aggresomal proteins. Even at the late stages of PHD3-induced apoptosis, several aggregates, which were localized mainly at the perinuclear region, were detected. Second, aggresomes are surrounded by a vimentin cage and this was not detected with the PHD3 bodies. The PHD3 bodies more resembled some aggresome-like structures, such as p62/SQSTM1, that show multiple scattered cytoplasmic proteasome- and chaperone-containing structures (Paine *et al.*, 2005). It is unclear whether the PHD3 bodies represent functionally similar class to, for example, the p62/SQSTM1 bodies but respond differentially to the oxygen levels.

Nearly complete colocalization of PHD3 bodies with proteasomal components was detected with ectopical PHD3 and only partial with the endogenous PHD3. This implies that strongly elevated normoxic PHD3 is required for global PHD3-induced proteasomal aggregation. Moreover, it suggests that only a subset of the endogenous PHD3 aggregates are associated with proteasomes and may represent the larger endogenous PHD3 aggregates that are more pronounced in oxygenated conditions. LMP-2, which partially colocalized with the endogenous PHD3, is a component of immunoproteasomes (Puttaparthi *et al.*, 2007). Therefore it is an intriguing possibility that a subset of the PHD3 bodies could in fact represent immunoproteasomes.

To our knowledge the only physiological event in which the endogenous PHD3 has been reported to induce apoptosis, is in stressed neural cells. Noticeably, in these conditions PHD3 is expressed under normoxia for long time periods (72 h), and the PHD3-activated apoptosis requires both oxygen and intact hydroxylase activity (Lee *et al.*, 2005). Whether the activation of protein aggregation by PHD3 plays a role either in stressed neural cells or during hypoxia, reoxygenation injury in some other cell types remains to be investigated. Perhaps an even more tempting function for PHD3 exists in the neurodegenerative processes. Aggresomes or aggresome-like structures are components of a number of neurodegenerative disorders including Parkinson's, Alzheimer's, and Huntington's diseases as well as motoneuronal diseases (Garcia-Mata *et al.*, 2002; Ardley and Robinson, 2004; Olanow *et al.*, 2004). It is not completely clear whether the aggregates represent a protective response or whether they are the actual cause of the diseases potentially by impairing the proteasomal system. However, it is tempting to speculate that the aggregation activating function of PHD3 is a feature of neurodegenerative disorders.

ACKNOWLEDGMENTS

Raisa Vuorinen and Taina Kalevo-Mattila are acknowledged for expert technical assistance. We thank Jouko Sandholm for assistance in image analysis and Perttu Terho for assistance in FACS analysis. Dr. Carina Holmberg (Helsinki University, Finland) kindly provided the EGFP-LMP2 construct, Dr. Lea Sistonen (Turku Centre for Biotechnology) the HA-ubiquitin construct, and Dr. Terje Johansen (Tromso University, Norway) the EGFP-p62 construct. The work was supported by The Academy of Finland (Grants 200779, 210282, and 8212695), Sigrid Juselius Foundation, and Emil Aaltonen Foundation. P.M.J. is a research fellow of the Finnish Cancer Institute.

REFERENCES

Acker, T. *et al.* (2005). Genetic evidence for a tumor suppressor role of HIF-2alpha. Cancer Cell *8*, 131–141.

Appelhoff, R. J., Tian, Y. M., Raval, R. R., Turley, H., Harris, A. L., Pugh, C. W., Ratcliffe, P. J., and Gleadle, J. M. (2004). Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. J. Biol. Chem. *279*, 38458–38465.

Aprelikova, O., Chandramouli, G. V., Wood, M., Vasselli, J. R., Riss, J., Maranchie, J. K., Linehan, W. M., and Barrett, J. C. (2004). Regulation of HIF prolyl hydroxylases by hypoxia-inducible factors. J. Cell Biochem. *92*, 491–501.

Ardley, H. C., and Robinson, P. A. (2004). The role of ubiquitin-protein ligases in neurodegenerative disease. Neurodegener. Dis. *1*, 71–87.

Bence, N. F., Sampat, R. M., and Kopito, R. R. (2001). Impairment of the ubiquitin-proteasome system by protein aggregation. Science *292*, 1552–1555.

Bennett, E. J., Bence, N. F., Jayakumar, R., and Kopito, R. R. (2005). Global impairment of the ubiquitin-proteasome system by nuclear or cytoplasmic protein aggregates precedes inclusion body formation. Mol. Cell *17*, 351–365.

Berra, E., Benizri, E., Ginouves, A., Volmat, V., Roux, D., and Pouyssegur, J. (2003). HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. EMBO J. *22*, 4082–4090.

Bruick, R. K., and McKnight, S. L. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. Science *294*, 1337–1340.

Cioffi, C. L., Liu, X. Q., Kosinski, P. A., Garay, M., and Bowen, B. R. (2003). Differential regulation of HIF-1 alpha prolyl-4-hydroxylase genes by hypoxia in human cardiovascular cells. Biochem. Biophys. Res. Commun. *303*, 947–953.

Cockman, M. E., Masson, N., Mole, D. R., Jaakkola, P., Chang, G. W., Clifford, S. C., Maher, E. R., Pugh, C. W., Ratcliffe, P. J., and Maxwell, P. H. (2000). Hypoxia inducible factor-alpha binding and ubiquitylation by the von hippel-lindau tumor suppressor protein. J. Biol. Chem. *275*, 25733–25741.

D'Angelo, G., Duplan, E., Boyer, N., Vigne, P., and Frelin, C. (2003). Hypoxia up-regulates prolyl hydroxylase activity: a feedback mechanism that limits HIF-1 responses during reoxygenation. J. Biol. Chem. *278*, 38183–38187.

del Peso, L., Castellanos, M. C., Temes, E., Martin-Puig, S., Cuevas, Y., Olmos, G., and Landazuri, M. O. (2003). The von Hippel Lindau/hypoxia-inducible factor (HIF) pathway regulates the transcription of the HIF-proline hydroxylase genes in response to low oxygen. J. Biol. Chem. *278*, 48690–48695.

Epstein, A. C. *et al.* (2001). *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell *107*, 43–54.

Garcia-Mata, R., Gao, Y. S., and Sztul, E. (2002). Hassles with taking out the garbage: aggravating aggresomes. Traffic *3*, 388–396.

Harris, A. L. (2002). Hypoxia—a key regulatory factor in tumour growth. Nat. Rev. Cancer *2*, 38–47.

Heir, R., Ablasou, C., Dumontier, E., Elliott, M., Fagotto-Kaufmann, C., and Bedford, F. K. (2006). The UBL domain of PLIC-1 regulates aggresome formation. EMBO Rep. *7*, 1252–1258.

Hirsila, M., Koivunen, P., Gunzler, V., Kivirikko, K. I., and Myllyharju, J. (2003). Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor. J. Biol. Chem. *278*, 30772–30780.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., and Kaelin, W. G., Jr. (2001). HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science *292*, 464–468.

Jaakkola, P. *et al.* (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science *292*, 468–472.

Johnston, J. A., Ward, C. L., and Kopito, R. R. (1998). Aggresomes: a cellular response to misfolded proteins. J. Cell Biol. *143*, 1883–1898.

Jokilehto, T., Rantanen, K., Luukkaa, M., Heikkinen, P., Grenman, R., Minn, H., Kronqvist, P., and Jaakkola, P. M. (2006). Overexpression and nuclear translocation of hypoxia-inducible factor prolyl hydroxylase PHD2 in head and neck squamous cell carcinoma is associated with tumor aggressiveness. Clin. Cancer Res. *12*, 1080–1087.

Kallio, P. J., Okamoto, K., O'Brien, S., Carrero, P., Makino, Y., Tanaka, H., and Poellinger, L. (1998). Signal transduction in hypoxic cells: inducible nuclear

translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha. EMBO J. *17*, 6573–6586.

Kallio, P. J., Wilson, W. J., O'Brien, S., Makino, Y., and Poellinger, L. (1999). Regulation of the hypoxia-inducible transcription factor 1alpha by the ubiquitin-proteasome pathway. J. Biol. Chem. *274*, 6519–6525.

Kawaguchi, Y., Kovacs, J. J., McLaurin, A., Vance, J. M., Ito, A., and Yao, T. P. (2003). The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. Cell *115*, 727–738.

Koditz, J., Nesper, J., Wottawa, M., Stiehl, D. P., Camenisch, G., Franke, C., Myllyharju, J., Wenger, R. H., and Katschinski, D. M. (2007). Oxygen-dependent ATF-4 stability is mediated by the PHD3 oxygen sensor. Blood *15*, 3610–3617.

Kopito, R. R. (2000). Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol. *10*, 524–530.

Kopito, R. R., and Sitia, R. (2000). Aggresomes and Russell bodies. Symptoms of cellular indigestion? EMBO Rep. *1*, 225–231.

Koumenis, C., and Wouters, B. G. (2006). "Translating" tumor hypoxia: unfolded protein response (UPR)-dependent and UPR-independent pathways. Mol. Cancer Res. *4*, 423–436.

Kristiansen, M., Messenger, M. J., Klohn, P. C., Brandner, S., Wadsworth, J. D., Collinge, J., and Tabrizi, S. J. (2005). Disease-related prion protein forms aggresomes in neuronal cells leading to caspase activation and apoptosis. J. Biol. Chem. *280*, 38851–38861.

Lee, H. J., and Lee, S. J. (2002). Characterization of cytoplasmic alpha-synuclein aggregates. Fibril formation is tightly linked to the inclusion-forming process in cells. J. Biol. Chem. *277*, 48976–48983.

Lee, S. *et al.* (2005). Neuronal apoptosis linked to EglN3 prolyl hydroxylase and familial pheochromocytoma genes: developmental culling and cancer. Cancer Cell *8*, 155–167.

Lieb, M. E., Menzies, K., Moschella, M. C., Ni, R., and Taubman, M. B. (2002). Mammalian EGLN genes have distinct patterns of mRNA expression and regulation. Biochem. Cell Biol. *80*, 421–426.

Lipscomb, E. A., Sarmiere, P. D., Crowder, R. J., and Freeman, R. S. (1999). Expression of the SM-20 gene promotes death in nerve growth factor-dependent sympathetic neurons. J. Neurochem. *73*, 429–432.

Lipscomb, E. A., Sarmiere, P. D., and Freeman, R. S. (2001). SM-20, a novel mitochondrial protein that causes caspase-dependent cell death in nerve growth factor-dependent neurons. J. Biol. Chem. *276*, 5085–5092.

Liu, L., Cash, T. P., Jones, R. G., Keith, B., Thompson, C. B., and Simon, M. C. (2006). Hypoxia-induced energy stress regulates mRNA translation and cell growth. Mol. Cell *21*, 521–531.

Marxsen, J. H., Stengel, P., Doege, K., Heikkinen, P., Jokilehto, T., Wagner, T., Jelkmann, W., Jaakkola, P., and Metzen, E. (2004). Hypoxia-inducible factor-1 (HIF-1) promotes its degradation by induction of HIF-alpha-prolyl-4-hydroxylases. Biochem. J. *381*, 761–767.

Masson, N., Appelhoff, R. J., Tuckerman, J. R., Tian, Y. M., Demol, H., Puype, M., Vandekerckhove, J., Ratcliffe, P. J., and Pugh, C. W. (2004). The HIF prolyl hydroxylase PHD3 is a potential substrate of the TRiC chaperonin. FEBS Lett. *570*, 166–170.

Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R., and Ratcliffe, P. J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis [see comments]. Nature *399*, 271–275.

Metzen, E. *et al.* (2003). Intracellular localisation of human HIF-1 alpha hydroxylases: implications for oxygen sensing. J. Cell Sci. *116*, 1319–1326.

Nakayama, K. *et al.* (2004). Siah2 regulates stability of prolyl-hydroxylases, controls HIF1alpha abundance, and modulates physiological responses to hypoxia. Cell *117*, 941–952.

Ohh, M., Park, C. W., Ivan, M., Hoffman, M. A., Kim, T. Y., Huang, L. E., Pavletich, N., Chau, V., and Kaelin, W. G. (2000). Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. Nat. Cell Biol. *2*, 423–427.

Olanow, C. W., Perl, D. P., DeMartino, G. N., and McNaught, K. S. (2004). Lewy-body formation is an aggresome-related process: a hypothesis. Lancet Neurol. *3*, 496–503.

Paine, M. G., Babu, J. R., Seibenhener, M. L., and Wooten, M. W. (2005). Evidence for p62 aggregate formation: role in cell survival. FEBS Lett. *579*, 5029–5034.

Pankiv, S., Clausen, T. H., Lamark, T., Brech, A., Bruun, J. A., Outzen, H., Overvatn, A., Bjorkoy, G., and Johansen, T. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J. Biol. Chem. *282*, 24131–24145.

Phair, R. D., and Misteli, T. (2000). High mobility of proteins in the mammalian cell nucleus. Nature *404*, 604–609.

Pugh, C. W., and Ratcliffe, P. J. (2003). Regulation of angiogenesis by hypoxia: role of the HIF system. Nat. Med. *9*, 677–684.

Puttaparthi, K., Van Kaer, L., and Elliott, J. L. (2007). Assessing the role of immuno-proteasomes in a mouse model of familial ALS. Exp. Neurol. *206*, 53–58.

Reits, E. A., Benham, A. M., Plougastel, B., Neefjes, J., and Trowsdale, J. (1997). Dynamics of proteasome distribution in living cells. EMBO J. *16*, 6087–6094.

Rifkind, J. M., Zhang, L., Levy, A., and Manoharan, P. T. (1991). The hypoxic stress on erythrocytes associated with superoxide formation. Free Radic. Res. Commun. *12–13*(Pt 2), 645–652.

Rohrbach, S., Simm, A., Pregla, R., Franke, C., and Katschinski, D. M. (2005). Age-dependent increase of prolyl-4-hydroxylase domain (PHD) 3 expression in human and mouse heart. Biogerontology *6*, 165–171.

Schofield, C. J., and Ratcliffe, P. J. (2004). Oxygen sensing by HIF hydroxylases. Nat. Rev. Mol. Cell Biol. *5*, 343–354.

Semenza, G. L. (2001). Hif-1, o(2), and the 3 phds. How animal cells signal hypoxia to the nucleus. Cell *107*, 1–3.

Straub, J. A., Lipscomb, E. A., Yoshida, E. S., and Freeman, R. S. (2003). Induction of SM-20 in PC12 cells leads to increased cytochrome c levels, accumulation of cytochrome c in the cytosol, and caspase-dependent cell death. J. Neurochem. *85*, 318–328.

Tanimoto, K., Makino, Y., Pereira, T., and Poellinger, L. (2000). Mechanism of regulation of the hypoxia-inducible factor-1alpha by the von Hippel-Lindau tumor suppressor protein. EMBO J. *19*, 4298–4309.

Thuerauf, D. J., Marcinko, M., Gude, N., Rubio, M., Sussman, M. A., and Glembotski, C. C. (2006). Activation of the unfolded protein response in infarcted mouse heart and hypoxic cultured cardiac myocytes. Circ. Res. *99*, 275–282.

Wax, S. D., Tsao, L., Lieb, M. E., Fallon, J. T., and Taubman, M. B. (1996). SM-20 is a novel 40-kd protein whose expression in the arterial wall is restricted to smooth muscle. Lab. Invest. *74*, 797–808.

Wojcik, C., Schroeter, D., Wilk, S., Lamprecht, J., and Paweletz, N. (1996). Ubiquitin-mediated proteolysis centers in HeLa cells: indication from studies of an inhibitor of the chymotrypsin-like activity of the proteasome. Eur. J. Cell Biol. *71*, 311–318.

Yu, F., White, S. B., Zhao, Q., and Lee, F. S. (2001). HIF-1alpha binding to VHL is regulated by stimulus-sensitive proline hydroxylation. Proc. Natl. Acad. Sci. USA *98*, 9630–9635.

Zatloukal, K., Stumptner, C., Fuchsbichler, A., Heid, H., Schnoelzer, M., Kenner, L., Kleinert, R., Prinz, M., Aguzzi, A., and Denk, H. (2002). p62 Is a common component of cytoplasmic inclusions in protein aggregation diseases. Am. J. Pathol. *160*, 255–263.