Oxomers - a novel oxygen-dependent signalling in cell physiology?

A. Baum, A. D. Dorsch, C. C. Scholz

Institute of Physiology, University Medicine Greifswald

Deutsche Forschungsgemeinschaft

Grant number: SCHO 1856/1-1

Universitätsmedizin Greifswald

Introduction:

Oxygen is essential for cellular metabolism. In reduced oxygen availability (hypoxia), cellular oxygen sensors are required to adapt the cell metabolism to the altered conditions. In normoxia, Prolyl-4-hydroxylase domain 1-3 (PHD1-3) proteins and Factor Inhibiting HIF (FIH), act as oxygen sensors and belong to the Fe^{2+}-/ and 2-oxoglutarate (2-OG)-dependent dioxygenase superfamily. PHD1-3 use molecular O_2 as co-substrate to hydroxylate two proline residues of the Hypoxia Inducible Factor (HIF) 1α-subunit. The Hippel-Lindau (VHL) protein subsequently recognizes HIF-1α and recruits an E3 ubiquitine ligase. Consequently, HIF-1α is polyubiquitinated, which leads to proteasomal degradation. FIH hydroxylates an asparagine residue in HIF-1α, preventing transcriptional co-activators/histone acetyl transferases p300/CBP from being recruited. After hydroxylation of HIF-1α by PHD1-3 or FIH, HIF is degraded and inactive and HIF target genes are not transcribed (Fig.1). In hypoxia, PHD1-3 and FIH are inactive, thus HIF-1α stabilized and active. Therefore, HIF target genes are transcribed. FG-4592, a PHD inhibitor (PHI), pharmacologically inhibits hydroxylation of HIF-1α and thereby promotes HIF-1α stabilization. FG-4592 is clinically approved for treatment of renal anemia.

Outside the HIF signaling pathway, FIH was recently shown to form a likely covalent bond with the deubiquitinase ovarian tumor domain-containing ubiquitin aldehyde binding 1 (OTUB1), identifying an alternate substrate of FIH. The formation of the protein complex is oxygen-dependent and is referred to as oxomer (**ox**ygen-dependent stable protein olig**omer**s). The oxygen dependency is due to the necessity of FIH activity for oxomer formation, which depends on oxygen availability. These insights indicate the possibility of other cellular oxygen sensors forming oxomers as well. In previous studies potential target proteins for the interaction with PHD1-3 were identified by mass spectrometry. This project aims to identify PHD2 dependent oxomer formation.

Methods: HEK 293 cells were transiently co-transfected with V5-PHD2 and C- or N-terminally FLAG-tagged target proteins. Starting at 5 h post transfection, transfected cells were treated for 43 h with FG-4592 and harvested 48 h post transfection. The cells were lysed and protein expression levels were analyzed in immunoblotting. V5-FIH and FLAG-OTUB1 were used as positive control for oxomer formation.

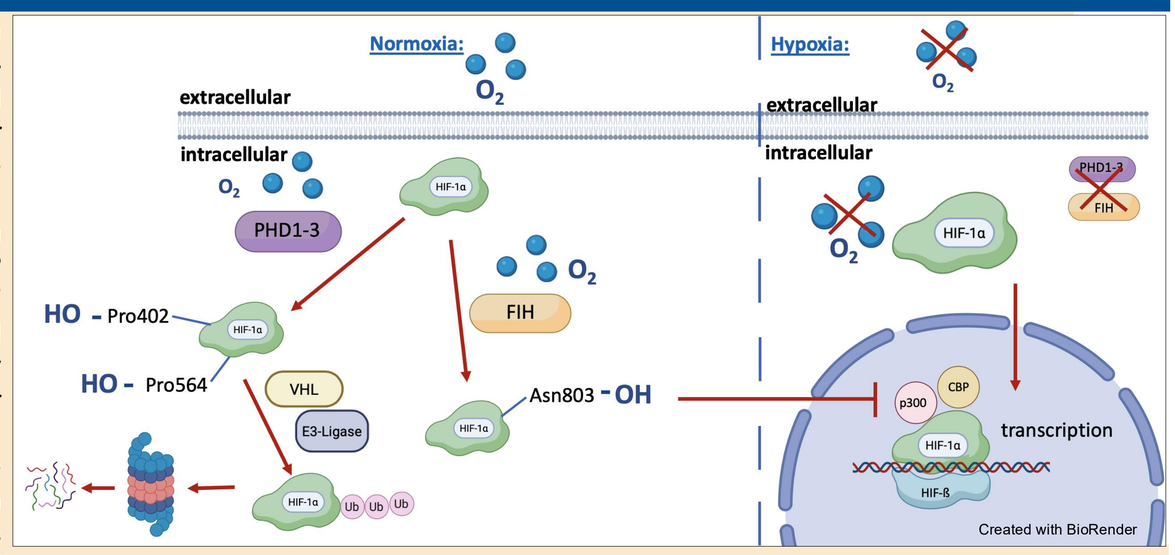

Fig. 1: HIF signaling pathway: HIF is a heterodimeric transcription factor, consisting of the subunits HIF-α and HIF-ß. In normoxia, HIF-1α is regulated by the cellular oxygen sensors PHD1-2 and FIH. PHD1-3 hydroxylate two prolin (Pro) residues, leading to enhanced proteosomal degradation, mediated by the von-Hippel Lindau (VHL) protein. FIH hydroxylates an asparagine (Asp) residue of HIF-1α, thereby preventing binding of co-transcription factors p300 and CBP, resulting in inactivity of HIF-1α. In hypoxia, the enzymatic activity of PHD1-3 and FIH is markedly reduced, leading to HIF-1α stabilization and transcription of target genes.

Results:

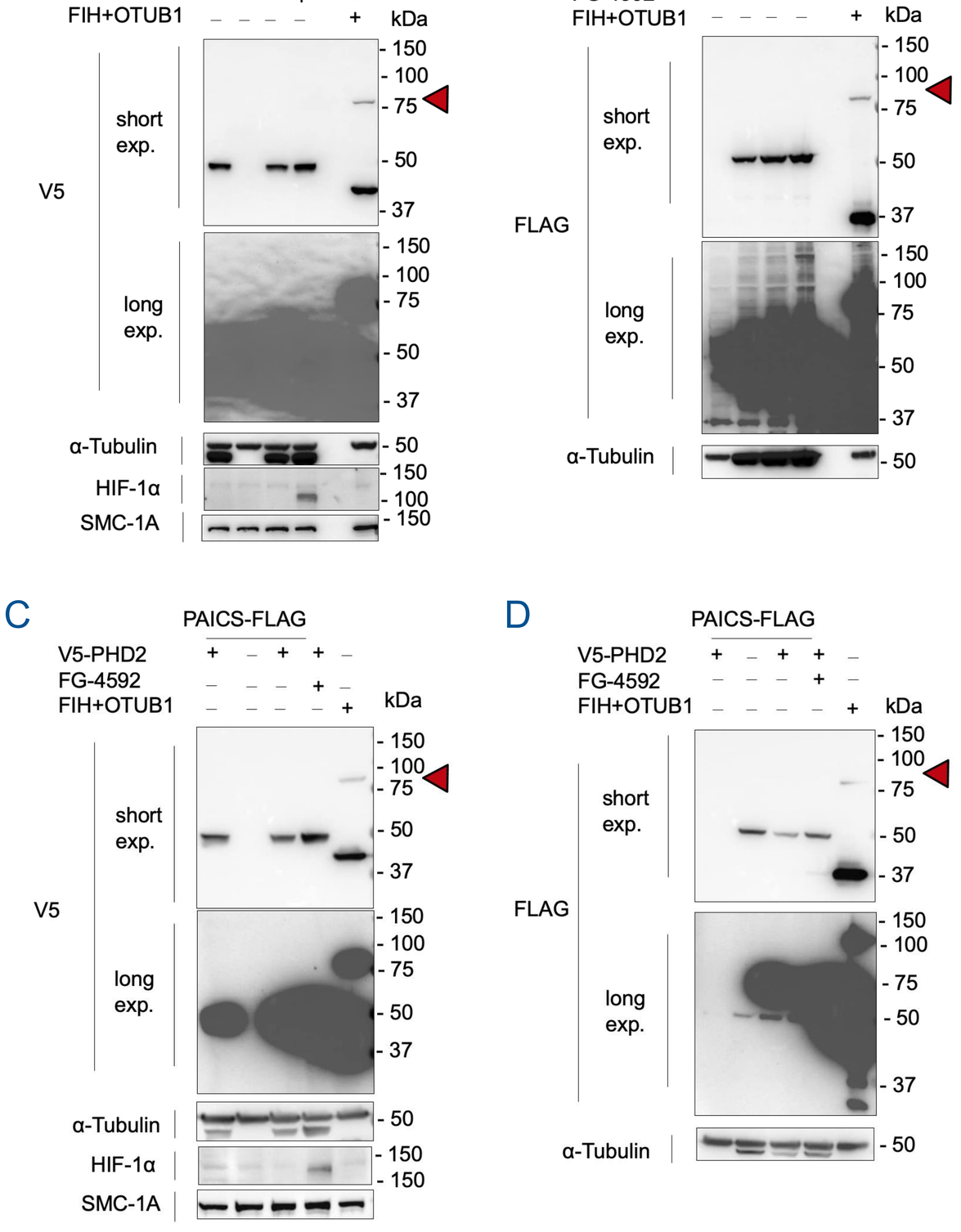

Fig. 2: Analysis of a potential oxomer formation between PHD2 and candidate protein PAICS. Representative immunoblot analysis of HEK 293 cells transiently co-transfected with V5-PHD2 (46 kDa) and FLAG-PAICS (A, B; 47 kDa) or PAICS-FLAG (C, D; 47 kDa). Transfected cells were treated with FG-4592 (100 µM) for 43 h before harvesting to stabilize HIF-1α (120 kDa). Protein expression was assessed either with anti-V5 (A, C) or anti-FLAG (B, D) antibodies. α-Tubulin (50 kDa) or SMC-1A (145 kDa) were used as loading control. Theoretical oxomer size is depicted with a red triangle at 93 kDa. exp., exposure. n=1

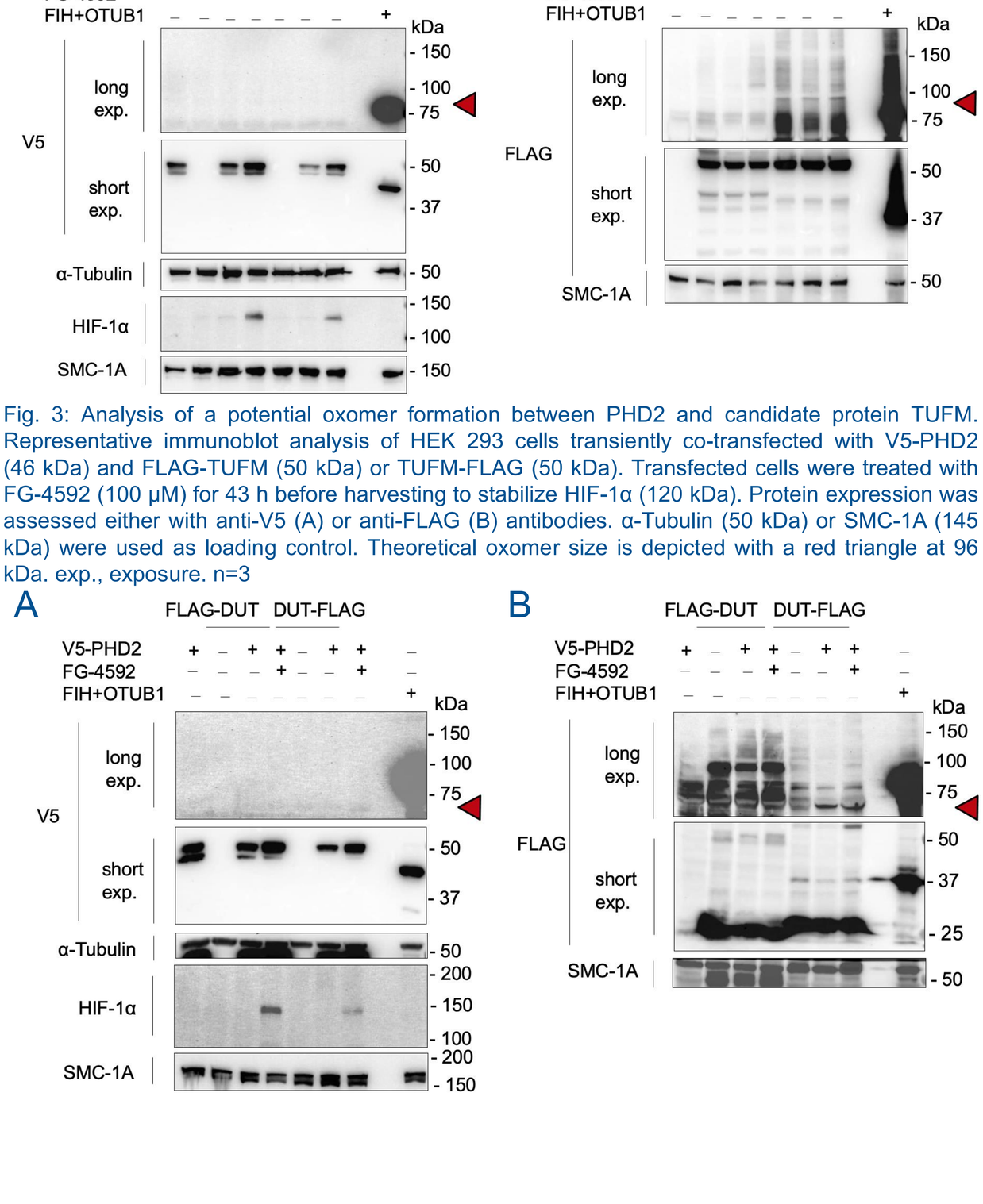

Fig. 3: Analysis of a potential oxomer formation between PHD2 and candidate protein TUFM. Representative immunoblot analysis of HEK 293 cells transiently co-transfected with V5-PHD2 (46 kDa) and FLAG-TUFM (50 kDa) or TUFM-FLAG (50 kDa). Transfected cells were treated with FG-4592 (100 µM) for 43 h before harvesting to stabilize HIF-1α (120 kDa). Protein expression was assessed either with anti-V5 (A) or anti-FLAG (B) antibodies. α-Tubulin (50 kDa) or SMC-1A (145 kDa) were used as loading control. Theoretical oxomer size is depicted with a red triangle at 96 kDa. exp., exposure. n=3

Fig. 4: Analysis of a potential oxomer formation between PHD2 and candidate protein DUT. Representative immunoblot analysis of HEK 293 cells transiently co-transfected with V5-PHD2 (46 kDa) and FLAG-DUT (27 kDa) or DUT-FLAG (27 kDa). Transfected cells were treated with FG-4592 (100 µM) for 43 h before harvesting to stabilize HIF-1α (120 kDa). Protein expression was assessed either with anti-V5 (A) or anti-FLAG (B) antibodies. α-Tubulin (50 kDa) or SMC-1A (145 kDa) were used as loading control. Theoretical oxomer size is depicted with a red triangle at 73 kDa. exp., exposure. n=1

Summary and Conclusions:

- The N-/or C-terminally tagged candidate target proteins TUFM, DUT, and PAICS were tested for oxomer formation with PHD2
- Neither of these candidate proteins indicated oxomer formation with PHD2
- The method is suitable for oxomer detection, as shown for the already published FIH-OTUB1 oxomer
- Other potential target proteins, like NAP1L4, EEF1D and RBBP4 will be investigated