

Conditional inactivation of HIF-1 using intrabodies

Arjan J. Groot^a, Eelke H. Gort^a, Elsken van der Wall^b, Paul J. van Diest^a and Marc Vooijs^{a,*}

^a *Department of Pathology, University Medical Center Utrecht, 3508 GA, Utrecht, The Netherlands*

^b *Department of Internal Medicine, University Medical Center Utrecht, 3508 GA, Utrecht, The Netherlands*

Abstract. Hypoxia is a hallmark of solid cancers and triggers the transcription of genes responsible for cell survival. The transcription factor Hypoxia-Inducible Factor 1 (HIF-1) is a key regulator in this response and frequently activated in human cancer. HIF-1 activation is associated with tumor aggressiveness and poor clinical outcome and, therefore, may provide an attractive therapeutic target. Here we provide a novel approach for HIF-1 targeted therapy using single-domain llama antibodies directed against the HIF-1 α oxygen dependent degradation domain which encompass the N-terminal transactivation domain. Conditional expression of HIF intrabodies in mammalian cells interfered with binding to pVHL and inhibited hypoxia induced activation of endogenous target genes. Inducible intrabody targeting is a highly specific strategy for temporal protein inactivation and may have applications for disease treatment.

Keywords: Cancer, HIF, hypoxia, intrabody, single-chain antibody fragment, VHH

1. Introduction

Normal tissues maintain a balance between growth and proliferation and oxygen supply. This balance is altered during solid tumor growth where focal regions of low oxygen (hypoxia) arise and tumor growth disrupts normal vasculature. Cancer cells undergo genetic and adaptive changes that allow them to survive and proliferate under hypoxic conditions. These genetic changes contribute to aggressive tumor behaviour and clinical response. For example, hypoxic tumors have a higher resistance to radiotherapy [1,46,48], chemotherapy [39] acquire genetic instability [22,25] and intratumoral hypoxia selects for a more metastatic phenotype [16,47]. Cancer cells adapt to hypoxic conditions by converting to glycolytic energy metabolism, induction of angiogenesis and other cellular survival programs [33]. Thus intratumoral hypoxia can be considered as a major hallmark of solid tumor growth [27, 34] important in carcinogenesis [3,17] and prognosis of cancers [2]. The cellular response to hypoxia is mediated by the Hypoxia Inducible Factor (HIF) family of transcription factors [11]. HIF complexes are heterodimers of an α - and β -subunit, both of which belong to the basic helix–loop–helix (bHLH)-PAS (Per, ARNT, SIM) protein family. Mammalian cells have

three α -subunits HIF-1 α , HIF-2 α and HIF-3 α . All heterodimerize with the ubiquitously expressed bHLH protein HIF-1 β or ARNT (aryl hydrocarbon receptor nuclear translocator) [31]. The stability of the alpha subunit is tightly regulated by oxygen concentration via the ubiquitin–proteasome system. Under normal oxygen tension ($pO_2 \sim 21\%$), HIF α modification by (prolyl- and asparaginyl)hydroxylation [9] and acetylation [20] increases the affinity of HIF α for the VHL (von Hippel–Lindau) tumor suppressor gene product that acts as an E3 ubiquitin ligase [37] and targets HIF α for proteasomal degradation. Under hypoxic conditions ($pO_2 < 5\%$) the activity of oxygen-dependent prolyl-hydroxylases (PHD) is attenuated, dramatically increasing the half-life of HIF α proteins [36]. Stabilization of HIF α proteins results in nuclear translocation and the formation of HIF α /ARNT heterodimers that bind to an enhancer element called the Hypoxia Responsive Element (HRE) in the promoters of target genes [28]. HIF α can also be stabilized under normal oxygen tension by oncogene or tumor suppressor gene mutation. For example, in hereditary and sporadic renal cell carcinoma loss of VHL leads to HIF α stabilisation, which at least partly accounts for tumor development [23,24]. Moreover, hypoxic HIF activation is highly relevant for solid tumor progression [32]. Therefore, silencing of HIF activity in tumor cells may provide an attractive therapeutic target in cancer treatment. Anti-cancer therapies using antibodies have been

*Corresponding author. E-mail: m.vooijs@umcutrecht.nl.

extremely successful in targeting cell surface molecules [30,35]. Standard antibody mediated therapy is, however, not amendable to targeting cytoplasmic or nuclear proteins.

VHH are small (15 kDa), naturally occurring single chain polypeptides from camellids with full antigen-binding capacity and specificity [15]. Because of their small size, specificity, low toxicity and immunogenicity and ease of molecular engineering, VHH antibodies have the potential to act like small molecule drugs. VHH can be expressed in mammalian cells and retain full antigen binding capacity and are also referred to as intrabodies [29]. Intrabodies have been shown to be able to block viral replication [7], prevent polyA-binding protein aggregation [43], and to protect cells from apoptosis after oxidative stress [14]. We recently reported the identification of VHH antibodies against HIF-1 α [13]. Epitope mapping revealed that antibody VHH-AG2 mapped within the HIF oxygen-dependent degradation domain (ODDD) in close proximity to the hydroxyl acceptor residue: Proline 564 critical for HIF-1 α degradation [18,19]. We hypothesized that such HIF intrabodies could be used to conditionally express and bind HIF *in vivo* and provide a selective and inducible approach to interfere with HIF transcriptional activity. Here, we provide evidence for this rationale by showing how nuclear localized HIF intrabodies bind HIF on active promoters and inhibit transcriptional activation. These intrabodies provide a highly specific strategy and universally applicable approach for temporal regulation of endogenous protein activity in mammalian cells. When applied to oncogenes, such targeted protein inactivation may provide complementary strategies for anti cancer treatment.

2. Material and methods

2.1. Construction of expression plasmids

To generate our IB-AG2 expressing construct, the cDNA of VHH-AG2 (kindly provided by Unilever Research Vlaardingen, The Netherlands) was retrieved by *Pst*I and *Bst*EII digestion from the previously described plasmid X22 [13] and cloned in an eukaryotic expression vector for intrabody expression, a kind gift of Verheesen [43]. The cDNA of VHH-AG2 was in frame with the SV40 T-antigen nuclear localization signal (NLS), green fluorescent protein (GFP) and HIS6 tag, for detection and pull down purposes. An unrelated VHH (G5) specific for the nuclear pro-

tein PABPN1 was used as a control: IB-UR [42]. A construct with *Bam*HI restriction sites flanking full-length HIF-1 α was kindly provided by Simos [5] and cloned into the p3XFLAG-CMVTM-10 expression vector (Sigma-Aldrich Co., St. Louis, MO, USA), resulting in FLAG-HIF-1 α [40]. Mutants for HIF-1 α P564A were generated using the QuickChange[®] XL Site directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol with forward primer 5'-CTTGGAGATGTTAGCTGCCTATATCCCAATGGATG-3' and reverse primer 5-CATCCATTGGGATATAGGCAGCTAACATCTCC AAG-3' on templates of FLAG-HIF-1 α and pET λ HIS coding HIF-1 α fragment B (aa375-605), as described in [13]. This resulted in plasmids FLAG-HIF-P564A and recombinant fragment B P564A. Recombinant HIF-2 α (aa508-591) resulted from a PCR with primers forward 5'-CGGGATCCGACACAGAGGCCAAGGCAATGC-3' and reverse 5'-GGGAAGCTTCTGCTGCTGAAACTTGTCCAGG-3' on FLAG-HIF-2 α [10] as template. Recombinant HIF-3 α (aa468-531) was PCR cloned using primers forward 5'-CGGGATCCGCACTGAGGCAGTGGAGACAG-3' and reverse 5'-GGGAAGCTTTGACAGGCCATGGAAGCTCCGAG-3' from pcDNA3.1 hHIF3a1 kindly provided by Dr. Michael Ohh. Both fragments were cloned after *Bam*HI-*Hind*III digestion in pET λ HIS. Recombinant fragments were purified with immobilized metal ion affinity chromatography (IMAC) as described in [42]. To make an inducible intrabody construct, under control of the Tet-ON system, IB-AG2 was digested with *Not*I and *Xho*I and cloned into pcDNA4/TO (Invitrogen, Carlsbad, CA, USA) Restriction enzymes and buffers were purchased from ROCHE (Basel, Switzerland). Cloning was controlled by restriction analysis and verified by sequencing.

2.2. Cell culture, transfections

U2OS and HeLa cells were cultured with DMEM. LS174TR1 cells expressing the tetracycline repressor (kind gift of van de Wetering [41]), were cultured with RPMI (Gibco BRL, Paisley, UK). All media were supplemented with 10% Fetal Calf Serum (Gibco), 100 U/ml penicillin-streptomycin (Gibco) and 100 U/ml L-Glutamine (Gibco) and cells were cultured at 5% CO₂, 21% O₂ for normoxia and 1% O₂ for hypoxia in an Invivo₂ Hypoxia Workstation 1000 (Biotrace International, UK) at 37°C. For the induction of HIF-1 α with desferrioxamine (DFO), cells were stimulated with 0.1 mM DFO. Cells were transfected with linear polyethylenimine (P-PEI, Polysciences Inc., Warrington, PA, USA). Se-

lection of mono/polyclonal U2OS cells expressing IB-AG2 were co-transfected with puromycin resistance (pSuper-PURO) and IB-AG2 plasmids. Selection was for 1 week in medium supplemented with 1 µg/ml puromycin (Sigma-Aldrich). For the IB-AG2 selection of the inducible cell line LS174TR1/IB-AG2, the medium was supplemented with 500 µg/ml zeocin and 10 µg/ml blasticidin, and for induction 1 µg/ml of doxycyclin was added to the medium. For luciferase assays, U2OS cells were seeded in 12-well plates in triplicate for each treatment group. Cells were cotransfected with a HIF-1 α -dependent 3 \times HRE-EPO-luciferase reporter plasmid (kind gift of Dr. R. Bernards), and expression a vector encoding TK-renilla [40], NLS-GFP, IB-AG2 and IB-UR. 24 h after transfection, cells were exposed to normoxic or hypoxic conditions (1% O₂) for 24 h. A microplate luminometer (Veritas, Turner BioSystems Inc. Sunnyvale, CA, USA) has been used to determine the Firefly and Renilla luciferase reporter activity as described by the manufacturer (Promega, Madison, WI, USA). Luciferase activity was normalized for Renilla luciferase activity to correct for transfection efficiency. As a control for transfection we used H2B-GFP (kind gift of Dr. A. Shvarts). For our reoxygenation experiment with LS174TR1/IB-AG2, with or without doxycyclin, we exposed those cells to hypoxic conditions (1% O₂) for 24 h and then reoxygenated at normoxic conditions for variable times.

2.3. Pull down and immunoprecipitations

Pull down and immunoprecipitation (IP) were done in immunoprecipitation buffer (IPB): 40 mM Tris pH 8.0, 1% Triton X-100, 10% glycerol, 280 mM NaCl, 10 mM PMSF supplemented with a protease inhibitor cocktail (Roche). Methods for IP were performed as previously described with bivalent VHH-AG2 antibodies (AG-1N2C-MYC) from transfected U2OS cells [13]. For the pull down, from HeLa cells with intracellular expressed IB-AG2 or IB-UR, Ni-NTA Magnetic Agarose Beads were used (QIAGEN, Hilden, Germany). For the GST pull down we used Glutathione Sepharose™ 4B (GE Healthcare, Uppsala, Sweden) where we coexpressed FLAG-HIF-1 α , H2B-GFP and VHL-GST a kind gift of Dr. B. van de Sluis. 48 h after transfection cells were lysed in IPB and incubated O/N, at 4°C, with the sepharose. The next day sepharose was washed for 8 times and sepharose was incubated with no VHH, bivalent VHH AG-1N2C-VSV [13] or an unrelated (VHH EME7E [44]) bivalent VSV tagged VHH O/N, at 4°C. Next, immunoprecipitated complexes were washed three times with IPB and sup-

plemented with Laemmli loading dye and analysed by Western blotting.

2.4. ELISA with VHH-AG2 on wild type and mutant recombinant HIF-1 α /2 α /3 α

For a comparison between binding of bivalent AG-1N2C-MYC to HIF-1 α and HIF-2 α and HIF-3 α , we immobilised HIF-1 α fragment C (aa543-605), recombinant HIF-2 α (aa508-591) and recombinant HIF-3 α (aa468-531) at a concentration of 500 ng/well, 100 µl per well, O/N, at 4°C in a NUNC MAXISORP (NUNC, Roskilde, Denmark). As a negative control we used recombinant HIF-1 α fragment E (aa375-455). Wells were blocked with 1% BSA in PBS/0.1% Tween-20 (BSAT) for 1 h at 27°C at 500 rpm in a TERMOstar incubator (BMG LABTECH, Offenburg, Germany). All following incubation steps were performed under these conditions in this incubator. Wells were incubated with VHH AG-1N2C-MYC in BSAT (0–7.5 µM). After three rapid washes with PBST, bound VHH was detected by incubation with HRP-conjugated mouse anti-*Myc* (Invitrogen) 1:5000 in BSAT for 20 min, followed by staining using the ImmunoPure TMB Substrate kit (Pierce Co) according to fabricant instructions. The signal intensities were measured using an ELISA reader (BioRad) at a wavelength of 450 nm. Determination whether the mutation of P564A influenced binding of VHH-AG2 we performed an ELISA experiment as previously described above. We used wild type and mutant recombinant HIF-1 α fragment B (aa375-605) and VHH-AG4, specific for amino acids 375-455, as an internal control [13].

2.5. Western blotting

Laemmli loading dye was used in preparation of samples for all Western blot procedures, 6 and 12% SDS-PAGE gels were used for separation of the proteins. Proteins were transferred onto PVDF membranes. Protein detection was performed with subsequently primary antibodies: anti-Lamin A (Abcam, Cambridge, MA, USA), anti-Flag (SIGMA), anti HIF-1 α and ARNT (BD Transduction Laboratories San Diego, CA, USA), anti-VSV-G, anti-GFP antibodies (both ROCHE), anti GST (Santa Cruz Biotech, Santa Cruz, CA, USA) and anti- β -actin (USBiological), Bivalent VHH AG-1N2C-MYC [13], HRP-conjugated mouse anti-T7 (Novagen, Madison, WI, USA) and HRP-conjugated mouse anti-*Myc* antibody (Invitrogen). Anti hydroxylated HIF-1 α polyclonal rabbit antiserum (anti-HYP-564) was a kind gift of Y. Tian [21]. Secondary antibodies used were: goat anti rabbit IgG

(H + L) (BioRad, Hercules, CA) and goat anti mouse IgG + IgM (Biosource, Camarillo, CA, USA) both HRP conjugated. ECL (Amersham Biosciences, Buckinghamshire, UK) was used for visualization as described by the manufacturer. Band intensities were determined with *Phoretix* TotalLab software version 2003.03 (Nonlinear Dynamics Ltd., Durham, NC, USA) and timepoint zero was set to a 100% for different conditions.

2.6. Immunofluorescence microscopy

Transfected U2OS, with IB-AG2 and IB-UR or stable U2OS cell lines expressing IB-AG2 were grown on glass slides. Slides were rinsed with PBS, and fixed with 3.7% formaldehyde-PBS for 10 min, blocked with 50 mM glycine-PBS for 10 min and rinsed with PBS. Next, nuclear DNA was stained with TO-PRO Iodide (Molecular Probes Inc., Eugene, USA) in PBS, rinsed and VectaShield (Vector Laboratories Inc., Burlingame, CA, USA) mounted microscope slides were assessed by confocal microscopy (LEICA DMRXA2, Leica Geosystems, Mannheim, Germany).

2.7. Chromatin immunoprecipitation with intrabodies

U2OS cells were transiently transfected with IB-AG2 or IB-UR. 24 h after transfection, cells were exposed to normoxic or hypoxic conditions for 24 h. Cells were washed at room temperature (RT) with PBS and cross-linked at RT for 20 min in 11% HCHO, 0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA and 20 mM HEPES. Reaction was stopped with 1.27 M glycine. Cells were scraped, collected and spun down at 500g for a minute at 4°C. Cells were resuspended in the above described buffer supplemented with a protease inhibitor cocktail (ROCHE). DNA was fragmented by sonication using a Bioraptor sonicator (Diagenode, Liège, Belgium): power – high, temperature of water 0°C, intervals – 30 s sonication/2 min pause, 30 min in 15 ml polypropylene tubes. Samples were centrifuged at 14,000 rpm for 5 min. Chromatin IP (ChIP) was then performed with protein G Agarose (Upstate Cell Signaling Solution, Lake Placid, NY) anti GFP antibodies (ROCHE) or a anti-acetyl-histone H3 rabbit polyclonal IgG (Upstate Cell Signaling Solution) as a positive control, overnight at 4°C in 0.15% SDS, 1% triton X-100, 150 mM NaCl, 1 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 20 mM HEPES pH 8.0, supplemented with protease inhibitors cocktail and BSA (final concentration 0.1%). The next day, immunoprecip-

itated complexes were washed twice with 0.1% SDS, 0.1% DOC, 1% triton X-100, 150 mM NaCl, 1 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 20 mM HEPES pH 8.0. Twice with 0.1% SDS, 0.1% DOC, 1% triton, 500 mM NaCl, 1 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 20 mM HEPES pH 8.0, twice with 0.27 M LiCl, 0.5% DOC, 0.5% NP-40, 1 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 20 mM HEPES pH 8.0, and twice with 1 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 20 mM HEPES pH 8.0. Protein complexes were eluted with 1% SDS, 0.1 M NaHCO₃ for 20 min, RT, being rotated. Beads were centrifuged and supernatants were transferred to a new tube. 5 M NaCl (final 200 mM) was added, mixed; incubated for 4 h at 65°C, while shaking. DNA was extracted with phenol–chloroform–isoamyl ethanol and chloroform–isoamyl ethanol. Glycogen was added together with 0.1 vol. 3 M NaAc, pH 5.2 in 2.5 vol. 100% ethanol and precipitated O/N at –20°C. The next day samples were centrifuged for 27 min and washed with 70% ethanol. DNA pellets were dissolved in water and stored at –80°C. PCR on DNA samples was performed with primers flanking the HRE in the *Erythropoietin* promoter as previous described [45]. PCR products were analysed by gel electrophoresis on a 2% ethidium bromide stained gel.

2.8. Quantitative PCR for HIF-1 α induced target genes

LS174TR1/IB-AG2 cells were cultured with or without doxycyclin and exposed to normoxic or hypoxic conditions (1% O₂) for 8 h. RNA from total cell lysates was prepared by homogenation in Trizol reagent (Invitrogen) followed by chloroform/phenol extraction. cDNA was prepared from 1 μ g of RNA using Reverse Transcriptase (ROCHE) and oligo-(dT) primers (Invitrogen). Quantitative Taqman PCR for *PGK1* was performed using commercially available assays for *PGK1* (Hs99999906_m1) and hydroxymethylbilane synthase (*HMBS*) (Hs00609297_m1) (Applied Biosystems, Foster City, CA, USA) using an ABI7900 analyzer (Applied Biosystems). *HMBS* was used for normalization. Quantitative PCR for *BNIP3* was performed using SYBR green (Applied Biosystems) according to manufacturers' protocol using primers BNIP3F 5'-AATATTCCTCCCAAGGAGTTCC-3' and BNIP3R 5'-CTGCAGAGAATATGCCCCCTTT-3' or GUSBF 5'-GAAAATATGTGGTTGGAGAGCTCATT-3' and GUSBR 5'-CCGAGTGAAGATCCCTTTTTTA-3'. *GUSB* was used for normalization. Data were analyzed using the SDS2.2.1 program (Applied Biosystems).

3. Results

We previously mapped the intrabody IB-AG2 to amino acid residues 543–605 of the human HIF-1 α [13] that encompasses the N-TAD domain and the regulatory Proline 564 (P564) (boxed in Fig. 1) that acts as a recognition and binding site for pVHL mediated proteasomal degradation (Fig. 1). Since this domain is highly conserved between HIF-1 α , HIF-2 α and HIF-3 α (Fig. 1), we investigated whether the AG2

could also directly bind HIF-2 α and HIF-3 α using recombinant purified proteins. Half maximum binding ($1/2 B_{max}$) of VHH-AG-1N2C-MYC (a VHH-AG2 bivalent) to recombinant HIF-1 α was approximately 600 fold more efficient than to recombinant HIF-2 α or HIF-3 α fragments. We calculated the apparent affinities at $1/2 B_{max}$ for HIF-1 α (5.0 nM), HIF-2 α (4.26 μ M) and HIF-3 α (3.10 μ M) and compared this with an N-terminal HIF-1 α fragment (aa375-455) not containing the epitope (285.44 μ M) (Fig. 2(a)).

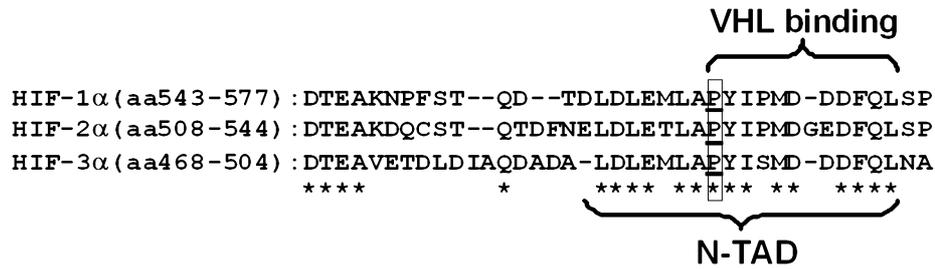


Fig. 1. Schematic representation of the minimal binding site of VHH-AG2 to HIF. HIF-1 α (aa543-577), HIF-2 α (aa508-544) and HIF-3 α (aa468-504). Identical residues are shown with asterisk. Indicated are residues defining the N-TAD and residues important for pVHL binding. The regulatory hydroxy-proline is boxed.

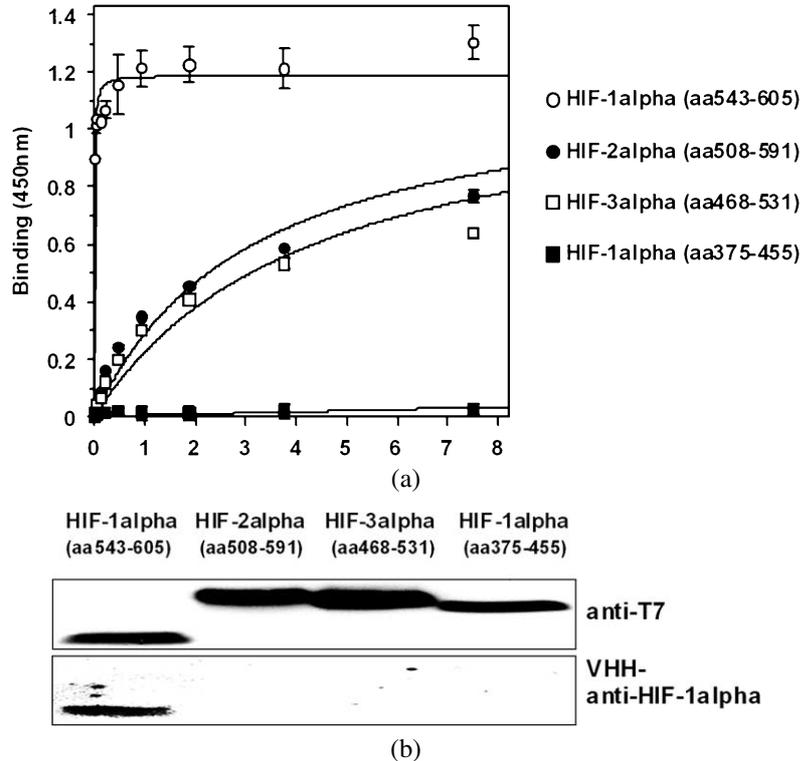


Fig. 2. VHH-AG2 binds specifically to HIF-1 α . (a) Binding of bivalent AG-1N2C-MYC to immobilized recombinant HIF-1 α fragment C (aa543-605), HIF-2 α (aa508-591), HIF-3 α (aa468-531) and HIF-1 α fragment E (aa375-455). The apparent affinity is approximated from the determined concentration yielding half-maximum binding to HIF-1 α (aa543-605) at B_{max} (OD450 nm) \approx 1.188. Data represent the mean + SD of three experiments. (b) Western blot analysis on recombinant HIFs with anti-T7 and AG-1N2C-MYC (VHH) antibodies.

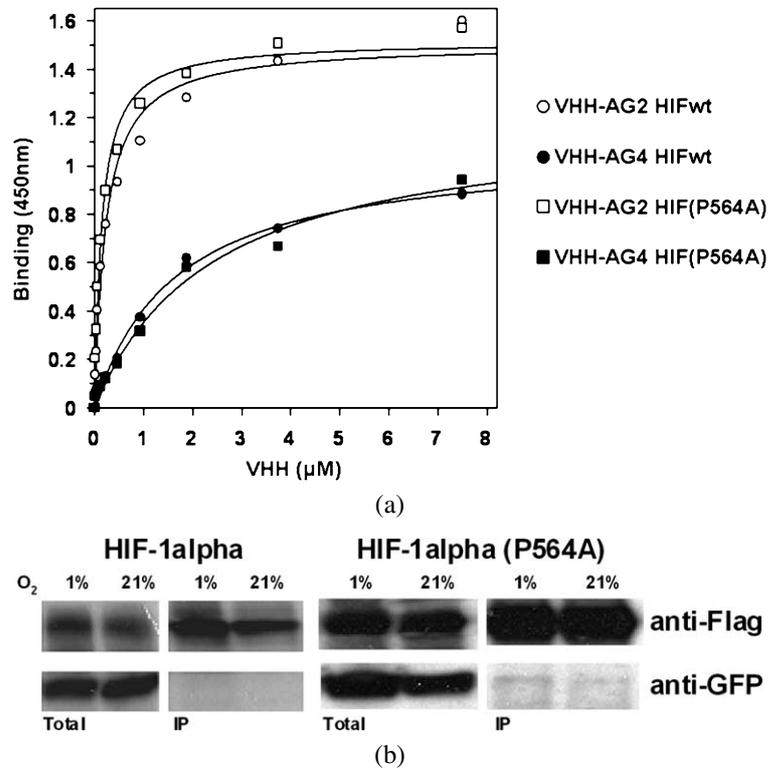


Fig. 3. P564 is not necessary for binding of VHH-AG2 to HIF-1 α . (a) ELISA VHH binding assay to wild type and mutant recombinant HIF-1 α . VHH-AG2 (binds to aa543-605), and as an internal control VHH-AG4 binds to (aa375-455), were incubated with immobilized recombinant wild type and mutant P564A HIF-1 α fragment B (aa375-605). Binding is expressed as OD at 450 nm and expressed as mean of duplicate experiments. (b) Immunoprecipitation of FLAG-HIF-1 α and mutant FLAG-HIF-1 α -P564A with bivalent VHH-AG2 (AG-1N2C-MYC) from U2OS transfected cells with H2B-GFP as a control. Cells were exposed to normoxic (21%) or hypoxic conditions (1% O₂) for 24 h after transfection. Total lysates and IPs were submitted to immunoblot analyses with anti FLAG and GFP antibodies.

This difference in affinity was confirmed by Western blot analysis with VHH-AG-1N2C-MYC using recombinant proteins where only the recombinant HIF-1 α fragment was detectable and not purified control and HIF-2/3 α fragments (Fig. 2(b)). Although we could immunoprecipitate overexpressed full length FLAG-tagged HIF-1, -2 and -3 α with VHH-AG-1N2C-MYC, we could only detect endogenous protein in the case of HIF-1 α [13] (data not shown). Since the regulatory P564 residue in HIF-1 α lies within the epitope recognized by AG2 we next investigated whether this proline was necessary for binding by testing whether AG2 still bound HIF-1 α -P564A a hydroxyl-deficient constitutive active HIF mutant [18]. We found that *in vitro* purified wild type and HIF-1 α -P564A proteins bound equally efficiently to AG2 as well as by immunoprecipitation of FLAG tagged proteins from transfected cells (Fig. 3).

To direct HIF intrabodies to the nucleus and to follow their fate we engineered VHH-AG2 with an

N-terminal Nuclear Localization Signal (NLS) and a C-terminal GFP and HIS6 tags constructing IB-AG2. After transfection of IB-AG2, strong nuclear GFP expression could be detected (Fig. 4(a)). To address whether IB-AG2 was still able to bind to endogenous stabilized HIF-1 α , we immunopurified HIS-tagged IB-AG2 from IB-AG2 transfected cells and confirmed that it was still functional in HIF-1 α binding. Empty vector and a control intrabody IB-UR did not pull down HIF-1 α (Fig. 4(b)). These results indicate that VHH are functional when expressed in mammalian cells and that the NLS and GFP tags do not impair HIF binding and permit visualization in living cells. To address whether the observed IB-AG2 binding of HIF-1 α did not occur post-lysis and whether it was able to detect HIF-bound DNA complexes, we performed ChIP making use of the GFP tag on the IB-AG2, using an anti-GFP antibody. IB-AG2 transfected cells showed enrichment for binding to the canonical HIF-1 α target gene erythropoietin (EPO), whereas an un-

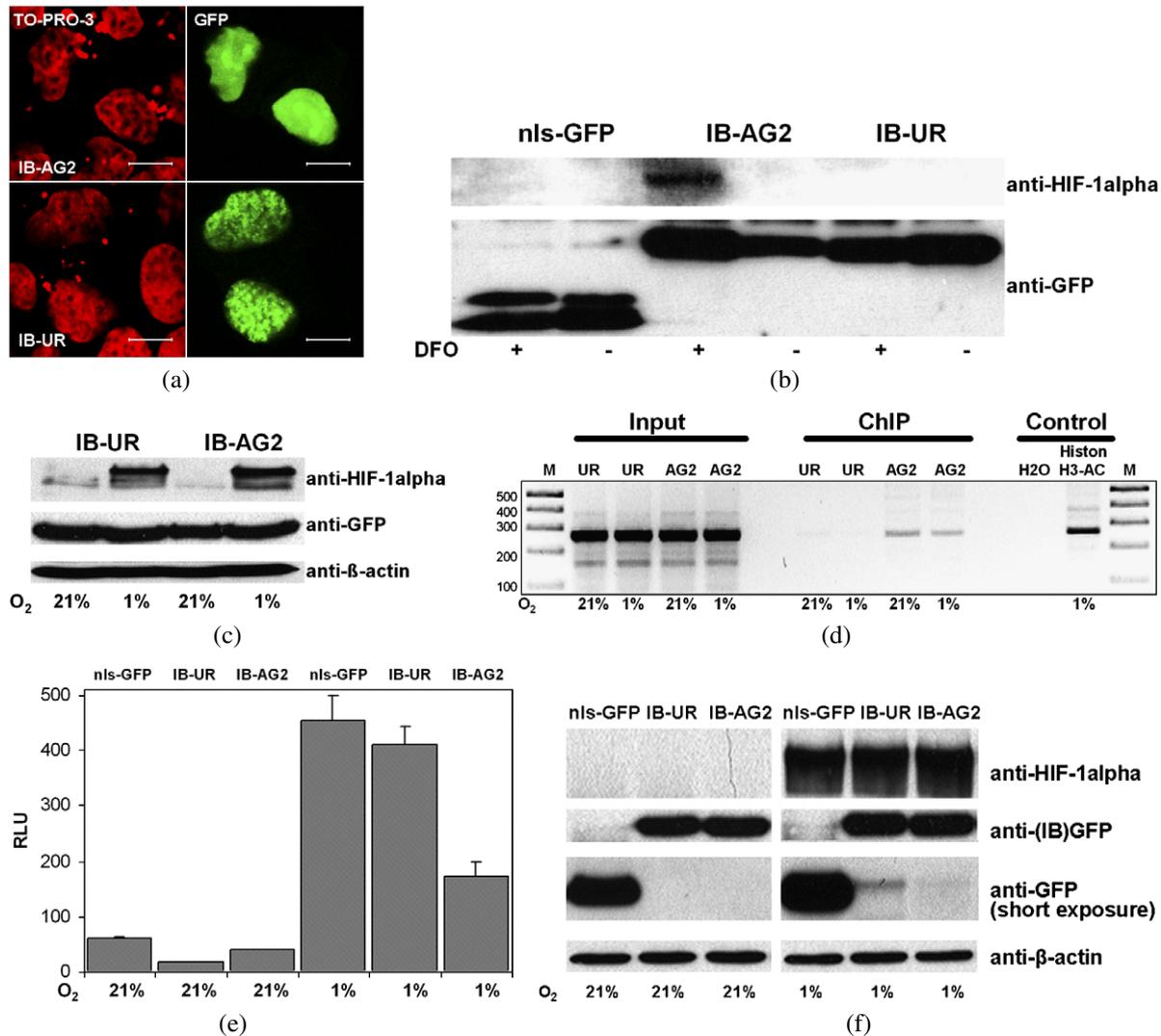


Fig. 4. Characterization of HIF intrabodies. (a) Immunofluorescence confocal microscopy with cultured U2OS cells transfected with either nuclear localized GFP fused IB-AG2 or control IB-UR intrabodies, DNA is stained with TO-PRO-3 (dark-grey/red*), scale bar = 10 μm. (b) Western blot analysis of Ni-NTA intrabody pull down of HeLa cell lysates transfected with IB-AG2, IB-UR or nls-GFP un- or stimulated with DFO. (c) U2OS cells were transfected with either IB-AG2 or IB-UR and 24 h after transfection exposed to normoxia (21% O₂) or hypoxia (1% O₂) for 24 h. Separate culture dishes were used for either protein expression analysis for input with HIF-1α, GFP and β-actin, or subjected to ChIP. (d) Ethidium bromide stained gel with PCR products from GFP ChIP isolates from IB-AG2 or control IB-UR transfected cells on endogenous EPO promoter. (H₂O as a negative control and as positive control ChIP with anti-acetyl-Histone H3 antibody.) (e) HIF reporter assay with HRE-EPO-luc in U2OS cells transfected with IB-AG2 or control intrabody, IB-UR. IB-AG2 inhibits hypoxia induced HRE activity. All luciferase experiments were performed in triplicate and values were corrected for TK-renilla expression. Error bars represent mean + SD. (f) Corresponding immunoblot analyses to (e) of HIF-1α and GFP fusions, with β-actin as a loading control.

related intrabody did not (Fig. 4(c and d)). We also confirmed binding to two other known HIF targets carbonic anhydrase IX (CAIX) and vascular endothelial growth factor (VEGF) (data not shown). To address the functional consequence of IB-AG2 binding to en-

dogenous HIF-1α on transcriptional activation we conducted luciferase based reporter gene assays. IB-AG2 markedly reduced hypoxia induced reporter activity on a synthetic reporter with optimized HRE sites, whereas NLS-GFP or an irrelevant intrabody did not (Fig. 4(e and f)). Thus, IB-AG2 directly binds to HIF/DNA transcriptional complexes and attenuates transcriptional

*The colors are visible in the online version of the article.

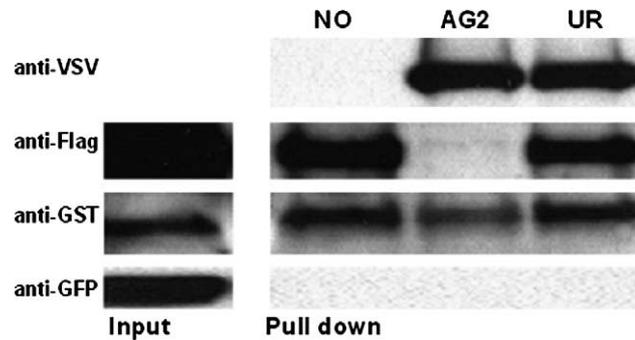


Fig. 5. VHH-AG2 competes for pVHL binding to HIF-1 α . GST pull down for VHL-GST and FLAG-HIF-1 α . Pull downs were incubated with antibodies, VHH AG-1N2C-VSV an AG2 bivalent or an unrelated (UR) VSV tagged bivalent. Supernatant of the pull down was immunoblotted for VSV as a control for antibodies added. Input and pull down was immunoblotted for H2B-GFP, FLAG-HIF-1 α and VHL-GST.

activation. To investigate the effects of prolonged IB-AG2 expression in mammalian cells we derived several monoclonal and polyclonal cell lines expressing IB-AG2, that bound to HIF-1 α and reduced hypoxia induced transcriptional activity in reporter gene assays (data not shown). Importantly, cell lines with constitutive IB-AG2 were maintained in culture for several months indicating little toxicity.

Unexpectedly, IB-AG2 cells showed high levels of endogenous HIF-1 α under normal oxygen tension compared to parental wild type U2OS cells (Suppl. Fig. S1: <http://www.qub.ac.uk/isco/JCO>). Additionally, transient transfected cells with IB-AG2 showed an inhibitory effect on endogenous HIF-1 α degradation upon oxygenation when compared to control IB-UR (Suppl. Fig. S2: <http://www.qub.ac.uk/isco/JCO>). We, therefore, hypothesized that normoxic HIF-1 α could be explained by interference of IB-AG2 with pVHL binding. To address this we performed a pull down for GST-tagged pVHL and FLAG-HIF-1 α from transfected cells, and added purified bivalent AG2 or an unrelated bivalent VHH to the precipitated GST complexes. Whereas adding a control VHH to GST purified complexes did not affect binding of pVHL to HIF-1 α in normoxic cell lysates, AG2 almost completely blocked recovery of FLAG tagged HIF-1 α from GST purified pVHL, indicating high affinity of AG2 for the pVHL binding site on HIF-1 α (Fig. 5).

To further refine this system of (HIF-1 α) protein inactivation, we generated doxycycline (dox) inducible cell lines expressing IB-AG2. Dox rapidly induced nuclear IB-AG2 expression, which was reversible upon removal of dox (Fig. 6(a)). In these conditional cells dox-regulated IB-AG2 inhibited both normoxic and hypoxia induced HRE- reporter activity (Fig. 6(b and c)) as well as hypoxic induction of

endogenous HIF-1 α target genes; PGK1 and BNIP3 (Fig. 6(d and e)). Interestingly, we never observed normoxic build up of HIF-1 α after dox administration, in contrast to the stable transfected U2OS cell lines. To further understand the mechanism of IB-AG2 interference with HIF-1 activity we addressed whether the kinetics of HIF-1 α degradation were affected by expression of IB-AG2. Whereas HIF-1 α accumulated in hypoxic cells is rapidly degraded upon oxygenation, this was markedly impaired in IB-AG2 expressing cells (Fig. 7). To directly address if IB-AG2 inhibited HIF- α degradation through inhibition of prolyl-hydroxylation we monitored prolyl-hydroxylation using antibodies specific for P-564-hydroxy-proline. Whereas IB-AG2 expression resulted in an inhibition of HIF- α degradation this was not accompanied by a decrease in prolyl-hydroxylation (Fig. 7(a)). Immunoblot quantitation showed stabilization of prolyl-hydroxylated HIF- α during this time course when compared to a reduction in controls [21] (Fig. 7).

4. Discussion

Hypoxia is a hallmark of solid cancer development and unfavourably affects therapeutic response. The transcription factor HIF-1 α is the principal mediator of this response and high expression and activity of HIF-1 α correlates with poor clinical outcome. Inactivation of HIF-1 α in tumor cells may therefore provide an attractive therapeutic target to halt tumor progression. Currently, small molecule inhibitors specific for HIF-1 α are scarce [26]. Antibody therapeutics provide highly specific drugs that can be used in targeting tumor antigens at the cell surface. To date, however, only a handful of HIF targets have been iden-

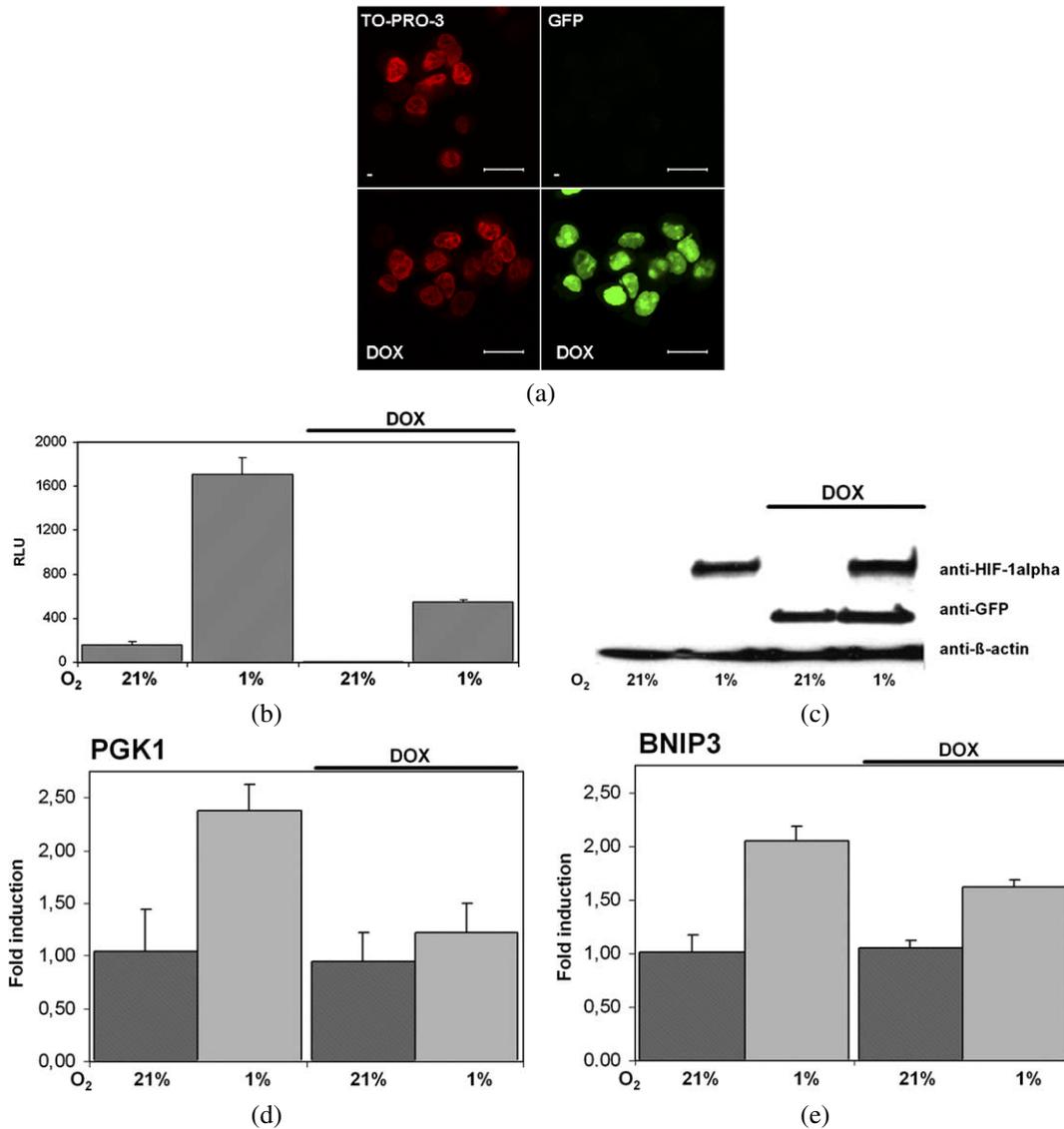


Fig. 6. Functional consequences of intrabody expression. (a) Immunofluorescence confocal microscopy with cultured LS174TR1/IB-AG2 cells with or without doxycyclin (dox), DNA is stained with TO-PRO-3 (dark-grey/red), GFP in green (bright-grey), scale bar = 20 μm. (b) HRE-luc reporter assay in LS174TR1/IB-AG2 cells exposed to normoxic (21%) or hypoxic conditions (1% O₂) for 24 h. Luciferase activities in the lysates were measured and expressed as relative light units (RLU). IB-AG2 attenuates hypoxia induced HRE-activity. All luciferase experiments were performed in triplicate. Error bars represent mean + SD. (c) Corresponding immunoblot analyses to (b) of HIF-1α and IB-AG2 GFP fusion, with β-actin as a loading control. (d, e) LS174TR1/IB-AG2 cells were cultured with or without dox and were exposed to normoxic (21%) or hypoxic conditions (1% O₂) for 8 h. Q-PCR for endogenous HIF-1α targets PGK1 and BNIP3 on prepared cDNA was performed. IB-AG2 inhibits the hypoxic induction of both endogenous targets PGK1 and BNIP3. Experiments were performed in triplicate. Error bars represent mean + SD.

tified at the cell surface that can be used for such approaches. With the development of single variable chain antibodies highly specific antibodies can be generated, using high throughput approaches that can be expressed in mammalian cells. Here we demonstrate that llama derived single variable heavy chain antibodies (VHH) against HIF can be inducibly expressed and

inactivate hypoxia inducible transcription of endogenous HIF-1 targets in mammalian cells. Our data show that the inhibitory mode of action of HIF intrabodies is likely by occupying the pVHL binding region or N-TAD in HIF-1α thereby causing increased stabilization of HIF-1α. Our data clearly show that despite robust prolyl-hydroxylation HIF intrabody binding atten-

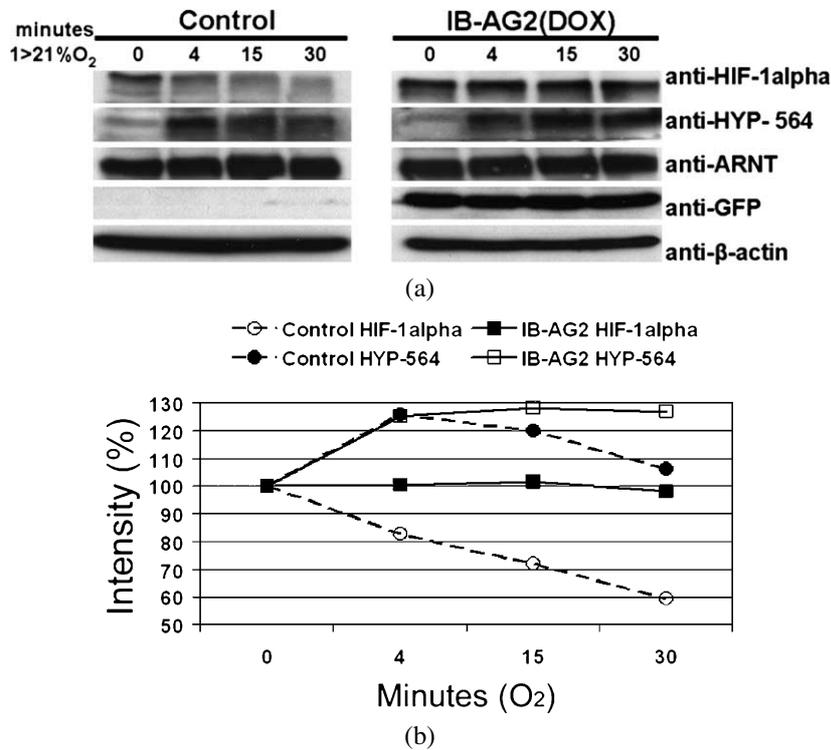


Fig. 7. Prolyl-hydroxylated HIF is stabilized by IB-AG2. (a) Reoxygenation experiment with inducible LS174TR1/IB-AG2 cells with or without doxycyclin. Cells were exposed to hypoxic conditions (1% O₂) for 24 h and then reoxygenated (21% O₂) for the indicated timepoints after which cell lysates were prepared. Total cell lysates were immunoblotted and analysed for HIF-1 α , prolyl-hydroxylated HIF-1 α (HYP-564), ARNT and GFP. β -actin is used as loading control. (b) Plotted curves of band intensities for HIF-1 α and hydroxylated HIF-1 α corresponding to immunoblots shown in (a).

uates proteosomal degradation and transcriptional activation, suggesting that binding of intrabody not only interferes with pVHL binding but also might impair in part the proper assembly of transcriptional complexes, although HIF-1 α and HIF-1 β dimers are formed in the presence of the intrabody, as our ChIP experiment demonstrates. Besides pVHL that interacts with the N-TAD of HIF-1 α , the redox regulatory protein Ref-1 is capable to functionally and physically interact with the N-TAD as well as the C-TAD of HIF-1 α . The hypoxia-inducible activity of both these TADs can independently be enhanced by a combination of SRC-1, CREB-binding protein (CBP)/p300 and Ref-1 coactivators but only in complex with HIF-1 β [4]. One possibility is that our intrabody interferes with the assembly of the SRC-1-CREB-Ref-1 coactivational complex with HIF-1 in a similar fashion as it does with pVHL by competing with Ref-1. This may explain why our intrabody is capable of attenuating but not inhibiting HIF-1 transcriptional activation. This may provide an approach to block N-TAD transcription while maintaining C-TAD transcription. Such experiments may

be useful to discriminate between N-TAD and C-TAD mediated transcription regulation of full-length HIF-1 proteins.

Several strategies can be envisioned to deliver therapeutic intrabodies to tumors *in vivo* [38]. For example adenoviral transduction may enable tumor cell infection and intracellular delivery of intrabodies. In such an approach non-hypoxic tumor cells and wild type cells are infected but lack stabilized HIF and therefore IB-AG2 is not expected to have any effect. The fact that we have maintained IB-AG2 constitutive expressing cells in culture for several months without any apparent detrimental consequence supports this. We have recently explored an alternative approach termed *Clostridium*-directed antibody therapy (CDAT) [12] using the obligate anaerobic oncolytic bacterium *C. Novyi*-NT that has been shown to home specifically into hypoxic tumors and induce non-specific oncolysis [6]. Expression of HIF intrabodies in such *C. Novyi* strains may further enhance such approaches for the treatment of hypoxic tumors if such antibodies can be made to enter hypoxic cells. We are currently investi-

gating whether intrabody expression in tumor cells affects their tumorigenic potential *in vivo*.

Small single chain antibodies like those reported here have several advantages over traditional antibodies. They are not very immunogenic and because of their small size (15 kDa) are more likely to target complex folded proteins and catalytic pockets [8]. Furthermore high throughput selection using phage-display and mutagenesis allows selection and enrichment for high affinity binders. Intrabodies can be easily cloned and modified with epitope tags and expressed and visualized in living cells as shown here.

More generally inducible protein inactivation using intrabodies provides a powerful highly specific alternative to other loss of function strategies like RNAi with temporal and spatial control. The ability to use GFP-fused intrabodies offers the possibility for combining highly specific protein inactivation with *in vivo* imaging. Such approaches may be helpful in pre-clinical models where the therapeutic efficacy of targeted knockdown of diseases-related gene products can be quantitatively monitored and visualized at the cellular or organism level.

Acknowledgements

The authors would like to thank Dr. N. Bovenschen for his help with preparing some of the figures. This work was supported by The Dutch Cancer Society (UU2003-2825) and in part by the 2nd AEGON international Scholarship in Oncology.

References

- [1] B. Bachtary, M. Schindl, R. Potter, B. Dreier, T.H. Knocke, J.A. Hainfellner, R. Horvat and P. Birner, Overexpression of hypoxia-inducible factor 1alpha indicates diminished response to radiotherapy and unfavorable prognosis in patients receiving radical radiotherapy for cervical cancer, *Clin. Cancer Res.* **9** (2003), 2234–2240.
- [2] R. Bos, P. van der Groep, A.E. Greijer, A. Shvarts, S. Meijer, H.M. Pinedo, G.L. Semenza, P.J. van Diest and E. van der Wall, Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma, *Cancer* **97** (2003), 1573–1581.
- [3] R. Bos, H. Zhong, C.F. Hanrahan, E.C. Mommers, G.L. Semenza, H.M. Pinedo, M.D. Abeloff, J.W. Simons, P.J. van Diest and E. van der Wall, Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis, *J. Natl. Cancer Inst.* **93** (2001), 309–314.
- [4] P. Carrero, K. Okamoto, P. Coumailleau, S. O'Brien, H. Tanaka and L. Poellinger, Redox-regulated recruitment of the transcriptional coactivators CREB-binding protein and SRC-1 to hypoxia-inducible factor 1alpha, *Mol. Cell. Biol.* **20** (2000), 402–415.
- [5] G. Chachami, E. Paraskeva, E. Georgatsou, S. Bonanou and G. Simos, Bacterially produced human HIF-1alpha is competent for heterodimerization and specific DNA-binding, *Biochem. Biophys. Res. Commun.* **331** (2005), 464–470.
- [6] L.H. Dang, C. Bettgowda, D.L. Huso, K.W. Kinzler and B. Vogelstein, Combination bacteriolytic therapy for the treatment of experimental tumors, *Proc. Natl. Acad. Sci. USA* **98** (2001), 15155–15160.
- [7] S. Dekker, W. Toussaint, G. Panayotou, T. de Wit, P. Visser, F. Grosveld and D. Drabek, Intracellularly expressed single-domain antibody against p15 matrix protein prevents the production of porcine retroviruses, *J. Virol.* **77** (2003), 12132–12139.
- [8] A. Desmyter, S. Spinelli, F. Payan, M. Lauwereys, L. Wyns, S. Muyldermans and C. Cambillau, Three camelid VHH domains in complex with porcine pancreatic alpha-amylase. Inhibition and versatility of binding topology, *J. Biol. Chem.* **277** (2002), 23645–23650.
- [9] A.C. Epstein, J.M. Gleadle, L.A. McNeill, K.S. Hewitson, J. O'Rourke, D.R. Mole, M. Mukherji, E. Metzen, M.I. Wilson, A. Dhanda, Y.M. Tian, N. Masson, D.L. Hamilton, P. Jaakkola, R. Barstead, J. Hodgkin, P.H. Maxwell, C.W. Pugh, C.J. Schofield and P.J. Ratcliffe, C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl-hydroxylation, *Cell* **107** (2001), 43–54.
- [10] E.H. Gort, G. van Haften, I. Verlaan, A.J. Groot, R.H. Plasterk, A. Shvarts, K.P. Suijkerbuijk, T. van Laar, E. van der Wall, V. Raman, P.J. van Diest, M. Tijsterman and M. Vooijs, The TWIST1 oncogene is a direct target of hypoxia-inducible factor-2alpha, *Oncogene* **27** (2008), 1501–1510.
- [11] A.E. Greijer, P. van der Groep, D. Kemming, A. Shvarts, G.L. Semenza, G.A. Meijer, M.A. van de Wiel, J.A. Belien, P.J. van Diest and E. van der Wall, Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1), *J. Pathol.* **206** (2005), 291–304.
- [12] A.J. Groot, A. Mengesha, E. van der Wall, P.J. van Diest, J. Theys and M. Vooijs, Functional antibodies produced by oncolytic clostridia, *Biochem. Biophys. Res. Commun.* **364** (2007), 985–989.
- [13] A.J. Groot, P. Verheesen, E.J. Westerlaken, E.H. Gort, P. van der Groep, N. Bovenschen, E. van der Wall, P.J. van Diest and A. Shvarts, Identification by phage display of single-domain antibody fragments specific for the ODD domain in hypoxia-inducible factor 1alpha, *Lab. Invest.* **86** (2006), 345–356.
- [14] D. Gueorguieva, S. Li, N. Walsh, A. Mukerji, J. Tanha and S. Pandey, Identification of single-domain, Bax-specific intrabodies that confer resistance to mammalian cells against oxidative-stress-induced apoptosis, *FASEB J.* **20** (2006), 2636–2638.
- [15] C. Hamers-Casterman, T. Atarhouch, S. Muyldermans, G. Robinson, C. Hamers, E.B. Songa, N. Bendahman and R. Hamers, Naturally occurring antibodies devoid of light chains, *Nature* **363** (1993), 446–448.

- [16] M. Hockel, K. Schlenger, B. Aral, M. Mitze, U. Schaffer and P. Vaupel, Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix, *Cancer Res.* **56** (1996), 4509–4515.
- [17] N. Horree, P.J. van Diest, P. van der Groep, D.M. Sie-Go and A.P. Heintz, Hypoxia and angiogenesis in endometrioid endometrial carcinogenesis, *Cell. Oncol.* **29** (2007), 219–227.
- [18] M. Ivan, K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J.M. Asara, W.S. Lane and W.G. Kaelin Jr., HIF- α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing, *Science* **292** (2001), 464–468.
- [19] P. Jaakkola, D.R. Mole, Y.M. Tian, M.I. Wilson, J. Gielbert, S.J. Gaskell, A. Kriegsheim, H.F. Hebestreit, M. Mukherji, C.J. Schofield, P.H. Maxwell, C.W. Pugh and P.J. Ratcliffe, Targeting of HIF- α to the von Hippel–Lindau ubiquitylation complex by O₂-regulated prolyl-hydroxylation, *Science* **292** (2001), 468–472.
- [20] J.W. Jeong, M.K. Bae, M.Y. Ahn, S.H. Kim, T.K. Sohn, M.H. Bae, M.A. Yoo, E.J. Song, K.J. Lee and K.W. Kim, Regulation and destabilization of HIF-1 α by ARD1-mediated acetylation, *Cell* **111** (2002), 709–720.
- [21] Y. Kageyama, M. Koshiji, K.K. To, Y.M. Tian, P.J. Ratcliffe and L.E. Huang, Leu-574 of human HIF-1 α is a molecular determinant of prolyl-hydroxylation, *FASEB J.* **18** (2004), 1028–1030.
- [22] M. Koshiji, K.K. To, S. Hammer, K. Kumamoto, A.L. Harris, P. Modrich and L.E. Huang, HIF-1 α induces genetic instability by transcriptionally downregulating MutS α expression, *Mol. Cell.* **17** (2005), 793–803.
- [23] S.J. Mandriota, K.J. Turner, D.R. Davies, P.G. Murray, N.V. Morgan, H.M. Sowter, C.C. Wykoff, E.R. Maher, A.L. Harris, P.J. Ratcliffe and P.H. Maxwell, HIF activation identifies early lesions in VHL kidneys: evidence for site-specific tumor suppressor function in the nephron, *Cancer Cell* **1** (2002), 459–468.
- [24] P.H. Maxwell, M.S. Wiesener, G.W. Chang, S.C. Clifford, E.C. Vaux, M.E. Cockman, C.C. Wykoff, C.W. Pugh, E.R. Maher and P.J. Ratcliffe, The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis, *Nature* **399** (1999), 271–275.
- [25] V.T. Mihaylova, R.S. Bindra, J. Yuan, D. Campisi, L. Narayanan, R. Jensen, F. Giordano, R.S. Johnson, S. Rockwell and P.M. Glazer, Decreased expression of the DNA mismatch repair gene Mlh1 under hypoxic stress in mammalian cells, *Mol. Cell. Biol.* **23** (2003), 3265–3273.
- [26] N.G. Nickols, C.S. Jacobs, M.E. Farkas and P.B. Dervan, Modulating hypoxia-inducible transcription by disrupting the HIF-1-DNA interface, *ACS Chem. Biol.* **2** (2007), 561–571.
- [27] J. Pouyssegur, F. Dayan and N.M. Mazure, Hypoxia signalling in cancer and approaches to enforce tumour regression, *Nature* **441** (2006), 437–443.
- [28] C.W. Pugh, C.C. Tan, R.W. Jones and P.J. Ratcliffe, Functional analysis of an oxygen-regulated transcriptional enhancer lying 3' to the mouse erythropoietin gene, *Proc. Natl. Acad. Sci. USA* **88** (1991), 10553–10557.
- [29] U. Rothbauer, K. Zolghadr, S. Tillib, D. Nowak, L. Schermelleh, A. Gahl, N. Backmann, K. Conrath, S. Muyltermans, M.C. Cardoso and H. Leonhardt, Targeting and tracing antigens in live cells with fluorescent nanobodies, *Nat. Methods* **3** (2006), 887–889.
- [30] N.G. Schaefer, B.C. Pestalozzi, A. Knuth and C. Renner, Potential use of humanized antibodies in the treatment of breast cancer, *Exp. Rev. Anticancer Ther.* **6** (2006), 1065–1074.
- [31] G.L. Semenza, Regulation of mammalian O₂ homeostasis by hypoxia-inducible factor 1, *Annu. Rev. Cell Dev. Biol.* **15** (1999), 551–578.
- [32] G.L. Semenza, Targeting HIF-1 for cancer therapy, *Nat. Rev. Cancer* **3** (2003), 721–732.
- [33] G.L. Semenza, Angiogenesis in ischemic and neoplastic disorders, *Annu. Rev. Med.* **54** (2003), 17–28.
- [34] G.L. Semenza, Hydroxylation of HIF-1: oxygen sensing at the molecular level, *Physiology (Bethesda)* **19** (2004), 176–182.
- [35] R.M. Sharkey and D.M. Goldenberg, Targeted therapy of cancer: new prospects for antibodies and immunoconjugates, *CA Cancer J. Clin.* **56** (2006), 226–243.
- [36] M.C. Simon, Siah proteins, HIF prolyl-hydroxylases, and the physiological response to hypoxia, *Cell* **117** (2004), 851–853.
- [37] C.E. Stebbins, W.G. Kaelin Jr. and N.P. Pavletich, Structure of the VHL-ElonginC-ElonginB complex: implications for VHL tumor suppressor function, *Science* **284** (1999), 455–461.
- [38] C.H. Swan, B. Buhler, P. Steinberger, M.P. Tschan, C.F. Barbas 3rd and B.E. Torbett, T-cell protection and enrichment through lentiviral CCR5 intrabody gene delivery, *Gene Ther.* **13** (2006), 1480–1492.
- [39] B.A. Teicher, Hypoxia and drug resistance, *Cancer Met. Rev.* **13** (1994), 139–168.
- [40] B. van de Sluis, P. Muller, K. Duran, A. Chen, A.J. Groot, L.W. Klomp, P.P. Liu and C. Wijmenga, Increased activity of hypoxia-inducible factor 1 is associated with early embryonic lethality in Commd1 null mice, *Mol. Cell. Biol.* **27** (2007), 4142–4156.
- [41] M. van de Wetering, E. Sancho, C. Verweij, W. de Lau, I. Oving, A. Hurlstone, K. van der Horn, E. Batlle, D. Coudreuse, A.P. Haramis, M. Tjon-Pon-Fong, P. Moerer, M. van den Born, G. Soete, S. Pals, M. Eilers, R. Medema and H. Clevers, The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells, *Cell* **111** (2002), 241–250.
- [42] S. van Koningsbruggen, H. de Haard, P. de Kievit, R.W. Dirks, A. van Remoortere, A.J. Groot, B.G. van Engelen, J.T. den Dunnen, C.T. Verrips, R.R. Frants and S.M. van der Maarel, Llama-derived phage display antibodies in the dissection of the human disease oculopharyngeal muscular dystrophy, *J. Immunol. Methods* **279** (2003), 149–161.
- [43] P. Verheesen, A. de Kluijver, S. van Koningsbruggen, M. de Brij, H.J. de Haard, G.J. van Ommen, S.M. van der Maarel and C.T. Verrips, Prevention of oculopharyngeal muscular dystrophy-associated aggregation of nuclear polyA-binding protein with a single-domain intracellular antibody, *Hum. Mol. Gen.* **15** (2006), 105–111.
- [44] P. Verheesen, A. Roussis, H.J. de Haard, A.J. Groot, J.C. Stam, J.T. den Dunnen, R.R. Frants, A.J. Verkleij, C. Theo Verrips and S.M. van der Maarel, Reliable and controllable antibody fragment selections from Camelid non-immune libraries for target validation, *Biochim. Biophys. Acta* **1764** (2006), 1307–1319.

- [45] F. Wang, R. Zhang, T.V. Beischlag, C. Muchardt, M. Yaniv and O. Hankinson, Roles of Brahma and Brahma/SWI2-related gene 1 in hypoxic induction of the erythropoietin gene, *J. Biol. Chem.* **279** (2004), 46733–46741.
- [46] K.J. Williams, B.A. Telfer, D. Xenaki, M.R. Sheridan, I. Desbaillets, H.J. Peters, D. Honess, A.L. Harris, G.U. Dachs, A. van der Kogel and I.J. Stratford, Enhanced response to radiotherapy in tumours deficient in the function of hypoxia-inducible factor-1, *Radiother. Oncol.* **75** (2005), 89–98.
- [47] U. Woelfle, J. Cloos, G. Sauter, L. Riethdorf, F. Janicke, P. van Diest, R. Brakenhoff and K. Pantel, Molecular signature associated with bone marrow micrometastasis in human breast cancer, *Cancer Res.* **63** (2003), 5679–5684.
- [48] A. Wouters, B. Pauwels, F. Lardon and J.B. Vermorken, Review: implications of *in vitro* research on the effect of radiotherapy and chemotherapy under hypoxic conditions, *Oncologist* **12** (2007), 690–712.



Hindawi
Submit your manuscripts at
<http://www.hindawi.com>

