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The GTPase RAB20 is a HIF target with mitochondrial localization mediating apoptosis in hypoxia

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ABSTRACT

Hypoxia is a common pathogenic stress, which requires adaptive activation of the Hypoxia-inducible transcription factor (HIF). In concert transcriptional HIF targets enhance oxygen availability and simultaneously reduce oxygen demand, enabling survival in a hypoxic microenvironment. Here, we describe the characterization of a new HIF-1 target gene, Rab20, which is a member of the Rab family of small GTP-binding proteins, regulating intracellular trafficking and vesicle formation. Rab20 is directly regulated by HIF-1, resulting in rapid upregulation of Rab20 mRNA as well as protein under hypoxia. Furthermore, exogenous as well as endogenous Rab20 protein colocalizes with mitochondria. Knockdown studies reveal that Rab20 is involved in hypoxia induced apoptosis. Since mitochondria play a key role in the control of cell death, we suggest that regulating mitochondrial homeostasis in hypoxia is a key function of Rab20. Furthermore, our study implicates that cellular transport pathways play a role in oxygen homeostasis. Hypoxia-induced Rab20 may influence tissue homeostasis and repair during and after hypoxic stress.

1. Introduction

Molecular oxygen is required for cellular respiration, where it is used as a terminal electron acceptor in generating adenosine triphosphate during oxidative phosphorylation. Thus, constant availability of molecular oxygen (O_2) is essential for energy homeostasis of all organisms of higher order. Mammalian systems have developed multiple strategies in response to reduced oxygen supply (hypoxia), such as an enhancement of respiratory rate and regional vasodilation. On the cellular level, one of the major mechanisms of transcriptional adaptation to hypoxia is the induction of the Hypoxia-inducible transcription Factor (HIF). HIF is a heterodimeric protein, consisting of a constitutively expressed β-subunit and an oxygen regulated α-subunit [1]. At least two oxygen dependent HIFα subunits have been identified, HIF-1α and HIF-2α, with incomplete understanding of their individual functions. Under low oxygen tension, HIFα is stabilized and translocates to the nucleus. This results in upregulation of numerous HIF target genes by binding to hypoxia response elements (HREs) within their promoter region. The activation of several targets in concert mediates the adaptation to hypoxia. Among those are glucose transporter-1 (Glut-1) [2], erythropoietin (EPO) [3] and vascular endothelial growth factor (VEGF) [4], which compensate reduced oxygen supply by increased glucose uptake, oxygen delivery or by promoting angiogenesis, respectively. On the other hand, processes like translation and proliferation which cause high energy consumption are reduced under hypoxic conditions [5]. Interestingly, these reactions are also at least in part regulated by HIF [6,7]. Thus, cells are capable of activating a wide range of adaptive molecular mechanisms to overcome hypoxic stress.

However, under conditions of severe or prolonged hypoxia protective cellular effects by HIF activation may not be beneficial for tissue integrity and apoptosis may be more advantageous, e.g. to induce repair. Indeed, it has been shown that hypoxia may induce apoptosis and that this may be HIF-dependent [8]. Early reports showed, that hypoxia induced apoptosis was consistently lower in tumors which derive from HIF-1α$^{-/-}$ ES cells compared with tumors from HIF-1α$^{+/+}$ cells [9]. Of note, several pro-apoptotic genes like BNIP3, NIX or Noxa are induced by hypoxia and appear to be regulated mainly by HIF-1 [10–12]. More recently, it has been shown that not only HIF-1α but also HIF-2α is able

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to induce apoptosis. Upon UV-irradiation, HIF-2α protein seems to be stabilized and regulates several pro-apoptotic genes, thereby contributing significantly to apoptosis induction [13]. Thus, under certain conditions of severe hypoxia the response to HIF accumulation may preferentially lead to cell death rather than cellular protection. However, the molecular mechanisms underlying this important hypoxia response remain poorly understood.

In this report we describe the characterization of a new HIF-1α target gene Rab20 (Ras related in brain 20) which we recently identified as being induced by hypoxia [14]. Interestingly, our results indicate that Rab20 is involved in hypoxia induced apoptosis. Rab20 belongs to the large family of Rab proteins, which represents one group of the superfamily of small GTPases. To date, more than 60 Rab proteins have been identified in humans and they mainly function in intracellular traffic processes. They localize to the cytosolic face of intracellular membranes and regulate vesicle formation, actin- and tubulin-dependent vesicle movement and membrane fusion [15]. Rab proteins act as molecular switches, cycling between an active GTP and an inactive GDP-bound conformation. In the active state, Rab proteins recruit their effectors (transported proteins) to the target membrane [16]. Interestingly, it was reported that some Rab proteins are overexpressed in cancer leading to the hypothesis that Rab proteins may participate in tumor progression [17]. Of note, the tumor microenvironment often displays areas of severe hypoxia, which leads to the activation of HIF. The latter is often discussed as being important in tumor progression, as well as therapy resistance [18].

Rab20 was first described as being expressed in the mouse kidney with predominant tubular expression [19]. It was speculated that Rab20 could be involved in proximal tubule bicarbonate reabsorption because it precipitates with vacuolar H^+-ATPases [20]. The human Rab20 protein was first cloned 2006 and it was suggested to localize in the vicinity of the Golgi apparatus in HeLa cells. The same study showed that Rab20 is upregulated in pancreatic tumor cell lines and primary pancreatic carcinomas [21].

Here we demonstrate that HIF-1α directly regulates the expression of Rab20 and that it colocalizes with mitochondria. Furthermore, knockdown of Rab20 by siRNA inhibits the induction of hypoxia induced apoptosis. It is well established, that mitochondria play a key role in the initiation and regulation of cell death. Of note, most HIF regulated pro-apoptotic target genes mediate their function in a mitochondria dependent manner. Mitochondria collect and process apoptotic signals and decide whether a cell has to die or not. Many different apoptotic stimuli bind to the mitochondrial membrane and cause mitochondrial membrane permeabilization, thereby leading to apoptosis mainly by activation of caspases and nucleases [22]. Several recent publications demonstrated an interesting interplay between HIF and mitochondria. For instance it was shown, that HIF-1 induces the enzyme pyruvate dehydrogenase kinase 1 (PDK1) resulting in the inactivation of pyruvate dehydrogenase (PDH) finally suppressing the Krebs cycle and mitochondrial respiration [23]. Furthermore, HIF directly regulates the expression of the cytochrome c oxidase subunit COX4 to optimize the efficiency of respiration at different O_2 concentrations [24].

Thus, many lines of evidence connect the Hypoxia-inducible transcription Factor with mitochondrial homeostasis. Our report is the first demonstration that Rab20, a member of the large group of Rab GTPases, could be a new link in this mechanism. We hypothesize that Rab20 could play an important role in mitochondrial homeostasis under hypoxic stress.

2. Materials and methods

2.1. Cell culture and reagents

HeLa, MCF-7 and NIH3T3 cells were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). RCC4 and RCC10 wildtype and VHL reconstituted cells were kind gifts from P. Ratcliffe, Oxford, U.K and T. Acker, Frankfurt, Germany, respectively. All cells were cultivated in DMEM, 1.0 g glucose/L, 10% fetal calf serum, 2 mmol/L L-glutamine, 100 U penicillin and 100 μg streptomycin/ml. HKC-8 cells (human renal tubular cells, described by Racusen et al. [25]) were cultivated in DMEM and HAM's-F12 in a 1:1 ratio, 10% FCS, 1× ITS liquid media supplement (Sigma) and 100 U penicillin and 100 μg streptomycin/ml. Human primary tubular cells (hPTs) were isolated and cultured as described in detail previously [26]. Medium and penicillin/streptomycin were supplied by PAN-Biotech (Aidenbach, Germany), fetal calf serum (standard "Gold") by PAA Laboratories (Coelbe, Germany). If not stated otherwise, all reagents were purchased from Sigma-Aldrich (Taufkirchen, Germany).

2.2. Animal procedures

For total RNA extracts, adult C57BL/6 mice at the age of 3–6 months were sacrificed by cervical dislocation and organs were snap frozen immediately in liquid nitrogen. RNA was extracted using RNAzolB (Biogenesis, Poole, UK). Hypoxic exposure of mice was performed in an INVIVO$_2$ 400 hypoxic chamber (Ruskinn, Bridgend, UK) at 8% O_2 for 6 h. All animal procedures were approved by the Institutional Review Board (file number 31-25/04).

2.3. Cell stimulation

Cells were stimulated for the indicated time periods in a hypoxic chamber (INVIVO$_2$ 400) at 1% O_2 or 0.1% O_2, respectively. Pharmacological HIF induction was performed by the use of DP (Dipyridyl 100 μM), DMOG (Dimethyloxaloxglycine 1 mM) or $CoCl_2$ (Cobaltchloride 100 μM).

2.4. Promoter analysis

Genomic sequences of human (NM_017817), mouse (NM_011277), rat (NM001109535), monkey (ENSMMUT00000031347) and dog (ENSCAFG00000006072) Rab20 gene were obtained from the NCBI and Ensembl databank, respectively. Putative HIF binding sites were identified using the online program MatInspector (www.genomatix.de), putative protein domains were identified by the use of the online database ensembl (www.ensembl.org).

2.5. Generation of Rab20 cDNA and fusion plasmids

The human Rab20 gene was amplified by standard PCR using cDNA from hypoxically stimulated HeLa mRNA extracts with a gene-specific primer pair (5′-GTGGTACCACCCAGCTCCTTC-3′ and 5′-ATCTCGAGTCCAACATTCTGCTG-3′; NCBI reference sequence NM_017817) and cloned into the vector pcDNA3 (Invitrogen). A Haemaglutinin-Tag (HA-Tag, YPYDVPDYA) separated by the two amino acids proline and glycine was fused to the C-terminal end of the human Rab20 cDNA by PCR-mutagenesis using the following primers: 5′-AGATGCTGGATGAGCAGGAT-3′, 5′-GGGCACGTCGTAGGGGTAGCCGGGGGCACAACACCCAGA-3′, 5′-TACGACGTGCCCGACTACGCCTGACTTTCGAGGGCCTCC-3′ and 5′-GATGGCTGGCAACTAGAAGG-3′. For generation of an N-terminal HA-Tag Rab20 cDNA following primers were used: 5′-CACCAAAATCAACGGGACTT-3′, 5′-TACGACGTGCCCGACTACGCCGGACCAAGGAAGCCCGACAGCAAG-3′, 5′-GGGCACGTCGTAGGGGTATCCTCCCATCTTCCCGTAAGAAC-3′ and 5′-TCTTTGCTGGCTGTGTCTGT-3′. Again, the HA-Tag was separated from the Rab20 protein by the addition of the amino acids glycine and proline. For the generation of GFP-Rab20 fusion plasmids, the human Rab20 wildtype cDNA was amplified with the following primers: 5′-TACTCGAGATGAGGAAGCCCGACA-3′ and 5′-TACTCGAGTGATGAGGAAGCCCGACA-3′ (containing the 5′ restriction sites *Xho*I and *Kpn*I) and cloned into the vector pEGFP-C_1 (Clontech, Mountain View, CA, USA).

2.6. Cloning and generation of Rab20 expressing HeLa cells

Cells were stably transfected with the plasmid pWHE146 coding a doxycycline transactivator (rtTA2s-M2, [27]) and selected with 0.5 mg/ml G418 (PAA Laboratories). rtTA expression was monitored using a luciferase gene under control of a Tet-responsive element (TRE; plasmid: pUHC13-3; [28]; data not shown). Subsequently, one positive clone was transfected with C-terminal HA-tagged Rab20 cDNA cloned into the TET on system plasmid pTRE2hyg (without hygromycin resistance cassette originally from BD Biosciences, Heidelberg, Germany) and the plasmid pSV2-hyg [29] containing a hygromycin resistance gene. Clones were selected by adding 250 μg/ml Hygromycin B (InvivoGen, San Diego, USA). Doxycycline (Fargon, Hamburg, Germany) was used to activate Rab20 expression.

2.7. siRNA and transfection procedures

Cells were transfected with siRNAs (final concentration 100 nmol/L) at 50% confluency by the use of Oligofectamine (Invitrogen) in Opti-MEM 1 medium (Invitrogen) according to the manufacturer's protocol. siRNAs against HIF-1α and HIF-2α were described before [14]. siRNAs targeting the human Rab20 mRNA (acc. no. NM_017817) (Rab20/1 sense 5'-GGCAAGCUGUGAUCUGAAAdTdT (catalog number SI00119700), Rab20/2 sense 5′-ACGUGUUCAUUUAUACAAAdTdT (catalog number SI00119707) were synthesized by Qiagen (Hilden, Germany). siRNA against GFP (sense 5′-GCUACCUGUUCCAUGGCCAdTdT, Eurogentec) was used as control. Generally, siRNAs were transfected 8 h prior to the start of an experiment, i.e. hypoxic exposure.

2.8. RNase Protection Assays (RPA)

Total RNA was extracted from cell culture using RNAzolB (Biogenesis, Poole, UK) according to the manufacturer´s instructions and for total RNA extracts from organs as described above. 32P-labeled riboprobes were synthesized using SP6 or T7 RNA polymerase (Roche). RPAs were performed essentially as described previously [30]. Ribroprobes for HIF-1α, HIF-2α, EPO and Glut-1 were described earlier [14,31]. For human and mouse Rab20 riboprobes were generated by PCR from cDNA of hypoxically stimulated HeLa cells or NIH3T3 mouse fibroblasts, respectively. Exon-spanning primer pairs were used for targeting human Rab20 mRNA (acc. no. NM_017817, 5′-GGGGTACCTACAACATCTCCATCTGG-3′ and 5′-CGCTCGAGGCAGTCTTTGCTGGCT-3′, protected fragment 190 bp) and mouse Rab20 mRNA (acc. no. NM_011227, 5′-GCGGTACCGTTCCTTCAACATC-3′ and 5′-CACTCGAGGCTGTTTCTGTCAGG-3′, protected fragment 185 bp). Restriction enzyme recognition sites *Xho*I and *Kpn*I were introduced into the specific primer sequences to facilitate cloning of cDNA fragments into the pcDNA3 vector. U6sn RNA served as an internal control.

2.9. Immunoblotting

HeLa and human primary tubular cells (hPTs) were stimulated as described above. As control, HeLa cells were transfected either with equal amounts of empty vector (pcDNA3), human Rab20 wildtype or HA-tagged cDNA (Rab20-C-HA) using jetPEI transfection reagent (Polyplus-transfection, Illkirch, France) according to the manufacturer's instruction. After stimulation or 24 h after transfection, cells were homogenized into extraction buffer (8 M urea, 10% glycerol, 1% SDS, 10 mM TrisHCl pH 6.8, protease inhibitor complete™ (Roche, Mannheim, Germany), 1 mM Sodium-Vanadate and 1 mM AEBSF). Enrichment of mitochondria was performed using the ProteoExtract Cytosol/Mitochondria Fractionation Kit (Calbiochem, Darmstadt, Germany). Equal amounts of protein were separated by SDS PAGE, transferred to PVDF membranes (Millipore, Bedford, MA, USA) and stained with following antibodies: Mouse monoclonal HIF-1α (Transduction Laboratories, Lexington, KY, USA), mouse polyclonal Rab20 (B01, Abnova, Taiwan, catalog-ID: H00055647-B01), rabbit polyclonal Rab20 (Ab-2, Amillet et al., 2006), mouse monoclonal OxPhos Complex V (subunit b, A21351, Molecular Probes, Eugene, OR, USA), rabbit polyclonal Cleaved PARP (Asp214, Cell Signaling Danvers, MA, USA), rat monoclonal HA antibody (High Affinity, clone 3 F10, Roche, Mannheim, Germany) and mouse monoclonal Caspase-3 (3 G2, Cell Signaling). As loading control, β-actin was stained using the monoclonal antibody AC-74 (Sigma).

2.10. Luciferase reporter assays

A 390 bp fragment upstream of the translational start site from the human Rab20 gene including the putative HRE (5′-CGACGTGG-3′, −209 bp from ATG) was amplified by PCR on genomic DNA using primer pairs where the restriction enzyme recognition sites *Kpn*I and *Xho*I were introduced (5′-GCGGTACCTGGAGAACTCGGATGCTTG-3′, 5′-CTCTCGAGCTTCCCGTAAGAACCCCCAG-3′) and cloned into the pGL2-Basic vector. By site directed mutagenesis, the putative HRE was mutated into 5′-CGACTTTG-3′. The generated plasmids were designated pGL2-hRab20-luc WT and pGL2-hRAb20-luc mut, respectively.

HeLa cells were seeded into 24-well plates and transfected with 200 ng of the indicated reporter constructs or equimolar amounts of empty vector pGl2-Basic and stimulated as described 8 h after transfection. For over-expression experiments, cells were co-transfected with equimolar amounts of either empty vector pcDNA3, a cDNA coding for HIF-1α (*tm*HIF-1α, [32]), or HIF-1α together with a cDNA coding for HIFβ. 24 h later, cells were lysed with reporter lysis buffer and luciferase activity of 30 μl cell extract was measured 10 s according to the manufacturer's instructions in a 96-well plate after injection of 40 μl luciferase assay reagent (Luciferase Assay System, Promega, Madison, USA) using a standard luminometer (Berthold, Bad Wildbad, Germany). Reporter assays were normalized by cotransfection of 50 ng β-Gal each well and the β-Gal enzyme assay system with reporter lysis buffer (Promega, Mannheim, Germany) according to the manufacturer's instructions. Results are mean values of 4 independent transfected wells of one representative experiment and show fold induction related to control or empty vector transfection.

2.11. Electrophoretic mobility shift assay (EMSA)

Recombinant protein for HIF-1α and HIF-1β was generated by *in vitro* transcription and translation using the TNT® Quick coupled transcription/translation system (Promega) and incubated with a 32P-labelled 27-bp Rab20 wild type HRE (sense strand; 5′-CCGGGCCCCCGACGTGGGCTCCGTGCT-3′) and a mutated Rab20 (sense strand; 5′-CCGGGCCCCCGACTTTGGCTCCGTGCT-3′) oligonucleotide probe. Oligonucleotides corresponding to the EPO enhancer were used as control. For supershift assays, anti-HIF-1α antibody (clone 54, Transduction Laboratories, 750 ng) was added and reactions were incubated for 20 min on ice before electrophoresis. EMSA was performed as described [33].

2.12. Immunocytochemistry

Cells were seeded onto cover slips into a 6-well plate. For over-expression experiments, cells were transfected with equal amounts of either empty vector (pEGFP-C_1, pcDNA3), pEGFP-C_1-Rab20 or pcDNA3/Rab20-N-HA. Visualisation of mitochondria was performed by staining of the cells with MitoTracker CMXRos (Invitrogen) for 30 min according to the manufacturer's protocol. After incubation, cells were washed with PBS and fixed with 3% PFA for subsequent staining. For antibody reactions, cells were permeabilized 10 min in PBS containing 0.1% Triton-X, blocked with FCS containing DMEM for 1 h and incubated with a rabbit polyclonal Rab20 antibody (Ab-2, [21]) diluted 1:100 in PBS or anti-HA antibody (High Affinity, clone 3 F10, Roche, Mannheim, Germany) over night at 4 °C. Secondary antibody was Alexa Flour 488 anti-rabbit IgG, Alexa Flour 488 anti-rat IgG or Rhodamine Red-X anti-rabbit IgG (all from Invitrogen), respectively. All secondary antibodies

were diluted 1:500 in PBS and incubated for 1 h at room temperature. Nuclei were stained with 4′,6-diamidine-2-phenylindole dihydrochloride (DAPI, 6.5 µg/ml, Roche) for 1 h. The stained cover slips were embedded in mowiol 4-88 reagent (EMD Biosciences, San Diego, CA, USA). Images were generated using a Nikon Eclipse 80i microscope, a CCD-camera (SPOT RT KE/SE, Visitron Systems, Puchheim, Germany) and the Spot Advanced 4.6 software (Visitron Systems).

2.13. Caspase-3/7 assay

Caspase-3/7 activity of 10.000 cells each well was measured using the Caspase-Glo 3/7 assay (Promega, Mannheim, Germany) according to the manufacturer's instructions. If not stated otherwise, results are mean values of at least 3 independent experiments.

2.14. AnnexinA5 binding assay

HKC-8 cells were seeded into 10 cm dishes and the following day transfected with siRNA against GPF, HIF-1α or Rab20, respectively. Two days after transfection, cells were stimulated with 0.1% hypoxia for 24 h and the exposure of phosphatidylserine (PS) was measured by binding of fluorescein isothiocyanate-labeled annexinA5 (Genethor, Berlin, Germany), while the discrimination of necrotic cells was performed by counterstaining with propidium podide (PI). In brief, at day of measurement, 100.000 cells were stained for 30 minutes at 4 °C with 500 ng/ml of annexinA5-fluorescein isothiocyanate and 20 ng/ml of propidium iodide in 500 µl of Ringer's solution (DeltaSelect, Dreieich, Germany) [34]. The samples were immediately measured using an EPICS XL flow cytometer (Coulter Inc., Hialeah, FL). Results are mean values of at least 3 independent experiments of total annexinA5 positive cells.

3. Results

3.1. Hypoxia, HIF stabilization and VHL deficiency induce Rab20

We recently observed that the small GTPase Rab20 (Ras related in brain 20) is induced by hypoxia and pharmacological HIF induction in Hep3B and HeLa cells [14]. Therefore, we here started to characterize the mode of Rab20 regulation by hypoxia in detail. Upon stimulation of HeLa and HKC-8 cells with 1% O_2, the amount of Rab20 mRNA increases rapidly indicating that Rab20 is induced directly by a factor activated or induced by hypoxia (Fig. 1A+B, left). We next stabilized the Hypoxia-inducible Factor (HIF) pharmacologically by treatment with DP, $CoCl_2$ or DMOG (Fig. 1A + B, right). All three substances diminish the degradation of HIF under normal oxygen tensions and resulted in increased Rab20 mRNA expression, suggesting that HIF is responsible for the hypoxic induction of Rab20.

HIFα subunits are targeted for oxygen dependent degradation by the tumor suppressor von Hippel Lindau (VHL) [33]. Therefore, biallelic inactivation of VHL leads to accumulation of HIFα. The renal clear cell carcinoma and VHL negative cell lines RCC4 and RCC10 showed no regulation of Rab20 mRNA after hypoxic stimulation (Fig. 1C). The reintroduction of VHL suppresses the expression in normoxia and restores the ability to induce Rab20 in hypoxia. The restored induction of HIF by hypoxia after reintroduction of VHL was confirmed by HIF-1α immunoblotting in these cells (Fig. 1C, two lower panels).

Altogether these data indicate that Rab20 mRNA expression could indeed be regulated directly by HIF.

3.2. Induction of HIF induces Rab20 protein

We analyzed the expression of Rab20 at the protein level using two different antibodies (B01 and Ab-2, Fig. 2A). The second antibody (Ab-2) was generated by Amillet and colleagues and described earlier [21]. With both antibodies, we were able to detect a clear induction of Rab20 protein level after DP treatment in HeLa cells. The induction of

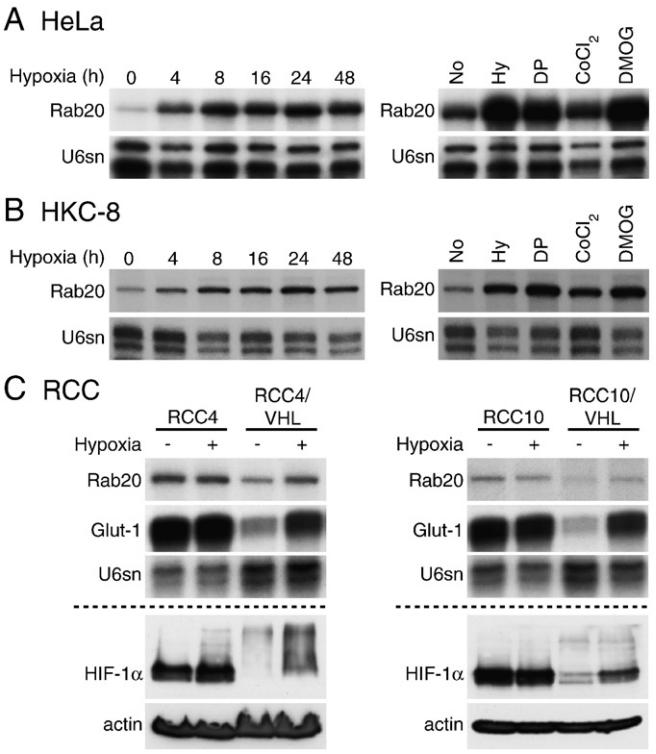

Fig. 1. Hypoxia and pharmacological HIF stabilization induces Rab20 mRNA. HeLa (A) and HKC-8 (B) cells were incubated for the indicated time periods in a hypoxic chamber at 1% O_2. Rab20 expression was analyzed by RNase protection assay (RPA). In addition, the expression of Rab20 was measured by RPA after pharmacological induction of HIF by DP (Dipyridyl), $CoCl_2$ and DMOG (Dimethyloxaloxylycine) after treatment for 16 h. (C, upper panels) The renal clear cell carcinoma cell lines RCC4 and RCC10 (both VHL deficient) and their VHL reconstituted clones were analyzed for Rab20 expression by RPA after hypoxic stimulation (16 h, 1% O_2). The mRNA regulation of the HIF target gene glucose transporter-1 (Glut-1) was measured as control. The induction of HIF-1α protein in RCC4 and RCC10 was analyzed by immunoblotting (C, below dotted lines).

Rab20 protein could be diminished by the use of two different siRNAs against Rab20, confirming that both antibodies specifically detect the human Rab20 protein. The specificity and efficacy of the Rab20 siRNAs were confirmed by RPA (Fig. 2B). To further evaluate the specificity of these two Rab20 antibodies, we transfected HeLa cells with equimolar amounts of Rab20 cDNA or C-terminal HA-tagged cDNA, each under the control of a CMV-promoter (Rab20 HA). After transfection, we were able to detect overexpressed Rab20 protein with both antibodies. The HA-tagged product migrates marginally slower in the gel, caused by the higher molecular mass as compared to the endogenous or untagged exogenous Rab20 protein.

Since previous studies indicated that Rab20 is predominantly expressed in the kidney, we studied the expression in non-transformed renal human primary tubular cells (hPTs, Fig. 2C). Again, both antibodies display protein accumulation of Rab20 under hypoxia and pharmacological stabilization of HIF with DP, where the induced species migrates comparably to the transfected product.

3.3. In vivo expression of Rab20

The mouse Rab20 gene was originally identified in the kidney where it was reported to be expressed at high levels [19], and variable expression in other organs. We were able to confirm these results by RPA in murine tissues. Rab20 mRNA is expressed in an organ specific manner with most prominent expression in the kidney and the heart. Interestingly, despite the fact that Rab proteins are named after their first characterization in brain, the Rab20 RNA seems to be absent in this organ (Fig. 3A). We next tested if Rab20 mRNA could be induced by hypoxia *in vivo*. C57BL/6 mice were exposed to 8% O_2 for 6 h or kept

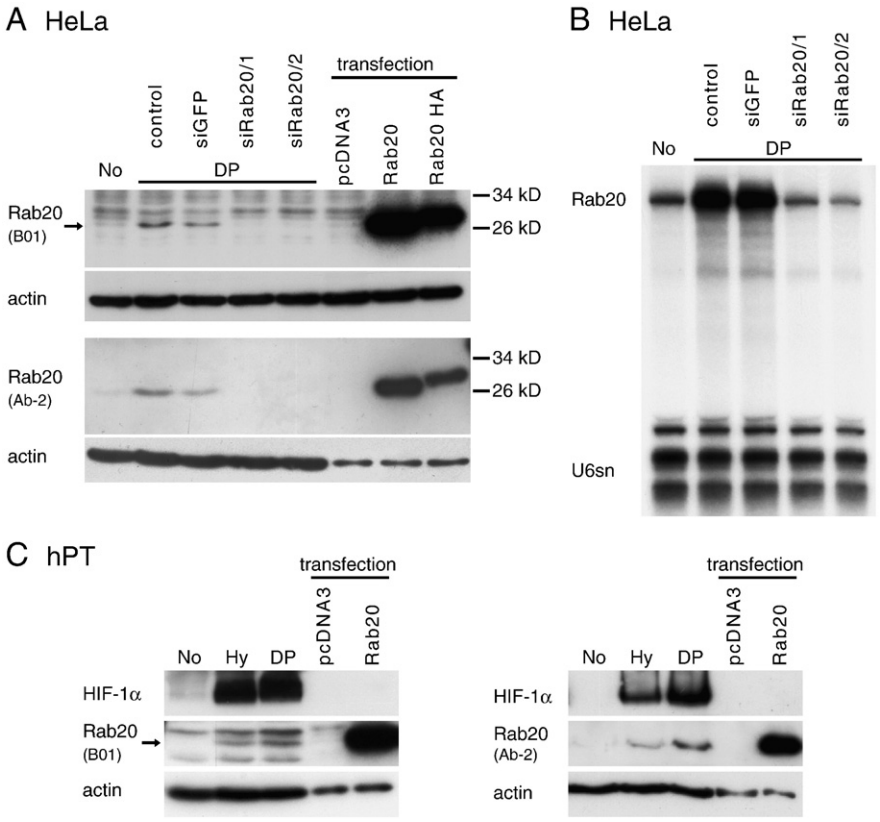

Fig. 2. Induction of Rab20 protein. (A) We analyzed the induction of Rab20 protein after HIF stabilization by the use of the iron chelator DP (16 h) in HeLa cells with two independent antibodies (B01 and Ab-2) by immunoblotting. The specificity of these antibodies was confirmed by the use of two different siRNAs against Rab20. Additionally, lysates from HeLa cells transfected with either empty vector (pcDNA3), the human Rab20 wildtype cDNA or HA-tagged cDNA of Rab20 under control of a CMV-Promoter were analyzed (A). The efficiency of the Rab20 siRNAs was confirmed on mRNA level after DP stimulation (16 h) by RPA (B) where siRNA against GFP was used as control. (C) Regulation of Rab20 protein by hypoxia and DP was also investigated in human renal primary tubular cells (hPTs) by immunoblotting. As above, lysates from Rab20 wildtype cDNA transfected HeLa cells were used as control.

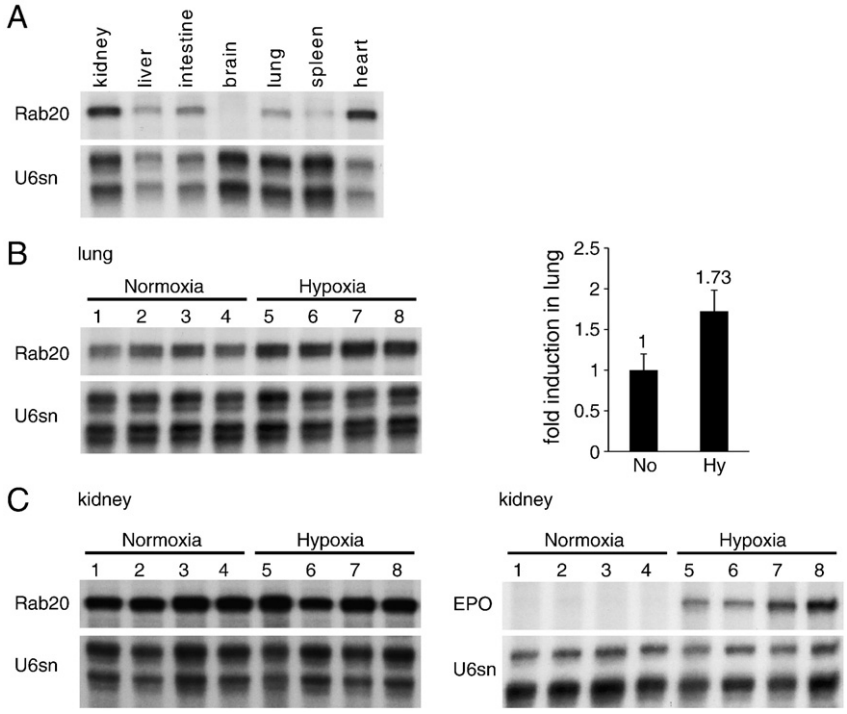

Fig. 3. *In vivo* expression of Rab20 mRNA by RPA. (A) We analyzed the expression and distribution of mouse Rab20 in different organs under normal oxygen tensions by RPA. (B + C) To analyze the hypoxic induction of Rab20 mRNA *in vivo*, mice (4 each group) were incubated in a hypoxic chamber at 6% O_2 for 8 h or under ambient air and extracts from selected organs were analyzed for expression. Rab20 mRNA was upregulated by hypoxia in the lung 1.7-fold after hypoxic stimulation (mean ± SEM). The kidney shows no consistent regulation on mRNA level. In order to confirm successful hypoxic stimulation of the mice, the kidney RNA extracts were also analyzed for EPO expression.

under normal oxygen tensions and organ extracts were analyzed for Rab20 expression by RPA. Rab20 mRNA increases approximately 1.7-fold in the lung after hypoxic stimulation (Fig. 3B). However, we were not able to detect a consistent hypoxic regulation of Rab20 mRNA in the kidney (Fig. 3C), or other organs investigated (liver and heart; data not shown), although EPO mRNA increased in the kidney under these conditions (Fig. 3C).

Although the two different antibodies used for Rab20 immunoblotting marked specific bands at the correct molecular length, immunohistochemistry with these two antibodies produced opposing results, in both kidney tissue and renal cancer (data not shown). Regrettably therefore, in the absence of a further independent antibody, we cannot reliably report the specific protein expression pattern (and regulation) at this stage.

3.4. Hypoxic induction of Rab20 is directly mediated by HIF-1α

The hypoxic induction of HIF target genes is mediated by the binding of HIF to so called Hypoxia-response element (HRE) [3]. We therefore compared the promoter region and the gene structure from the human, mouse, rat, monkey and dog Rab20 gene searching for potential HREs. As shown in Fig. 4A, all Rab20 genes analyzed exhibit a nearly identical gene structure, consisting of two exons separated by one large intron. Furthermore, the coding site has nearly the same size and all coded proteins consist of 232 to 235 amino acids. One putative HRE was identified almost at the identical position in every species, approximately −200 bp upstream of the translational start site (AUG). These findings strongly support the expectation that this highly conserved HRE could be of biological relevance.

To further verify the contribution of HIF in hypoxic Rab20 induction, we performed siRNA experiments against HIF-1α and HIF-2α in HeLa and HKC-8 cells (Fig. 4B). The following day, cells were exposed to 1% O_2 for 16 h in a hypoxic chamber. Hypoxic stimulation of both cell lines induced the expression of Rab20 mRNA, as observed before. Interestingly, the knockdown of HIF-1α reduces the mRNA level of Rab20 to an amount comparable to unstimulated cells. Knockdown of HIF-2α or the use of siRNA against green fluorescent protein (GFP) as control had no effect. In brief, this demonstrates that hypoxic Rab20 induction is regulated by HIF-1.

3.5. HIF-1 binds to the human Rab20 gene promoter

In order to analyse the functionality of the putative Rab20 HRE, we constructed luciferase-reporter plasmids containing a wildtype (WT) or mutated variant of the partial human Rab20 promoter. A 390 bp fragment starting directly upstream of the translational start codon from the human Rab20 gene including the putative −209 bp HRE was cloned into the multiple cloning site of a commercial pGL2-Basic luciferase reporter. In addition, the putative HRE was mutated by PCR mutagenesis. We next transfected equimolar amounts of both plasmids, designated as pGL2-hRab20-WT and pGL2-hRAb20-mutated, respectively (Fig. 5A), (and pGL2-basic vector as control) into HeLa cells and stimulated the cells for 16 h with 1% hypoxia or DP. Hypoxia as well as pharmacological HIF induction activated the reporter significantly (Fig. 5B). Furthermore, mutation of the putative HRE diminished the reporter activity indicating that the HRE is indeed responsible for hypoxic induction of the Rab20 gene.

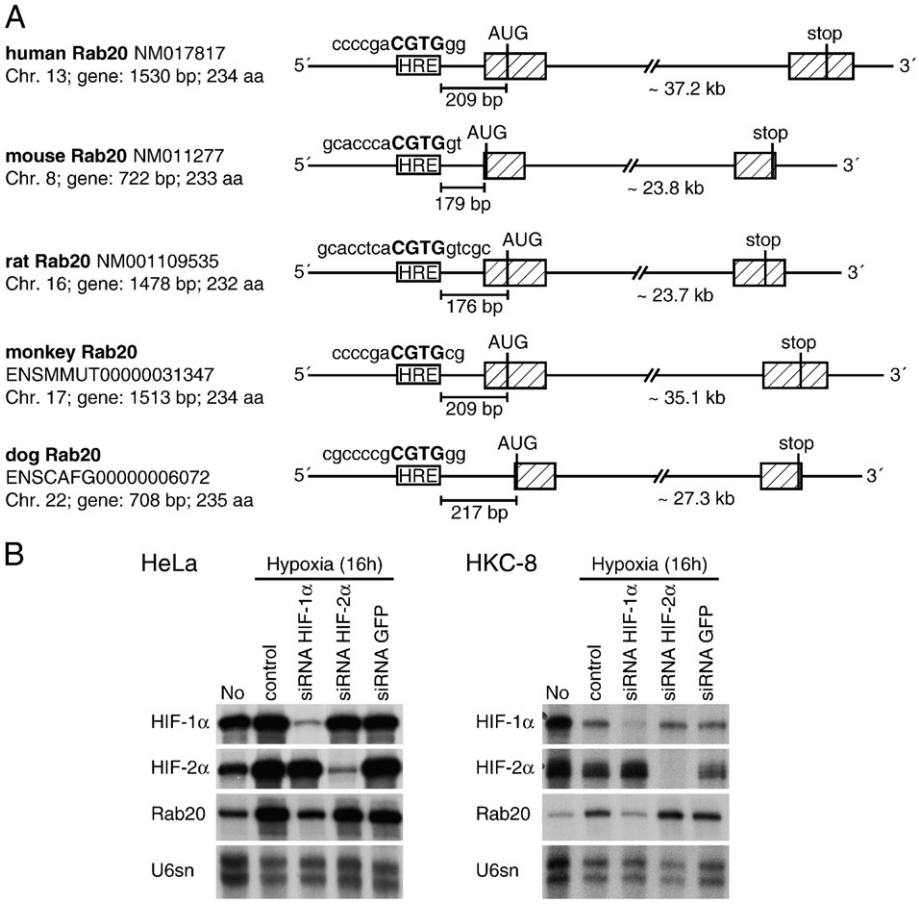

Fig. 4. HIF-1α dependent regulation of Rab20. (A) We analyzed the promoter region of the Rab20 gene from different species. Besides a highly conserved gene structure we could identify one potential Hypoxia-response element (HRE) at comparable positions in every species, approximately 200 bp upstream of the translational start site. (B) siRNA knockdown of HIF-1α and HIF-2α in HeLa and HKC-8 cells were performed, as well as GFP as control. After knockdown, cells were stimulated with 1% hypoxia for 16 h and Rab20 mRNA expression was analyzed by RPA. RPA for HIF-1α and HIF-2α display the efficacy and specificity of the respective HIF knockdown.

Of note, not only induction of endogenous HIF, but also the co-transfection of a cDNA coding for HIF-1α is able to induce the Rab20 luciferase reporter (Fig. 5C). Additional co-transfection with a cDNA coding for HIFβ (ARNT), probably resulting in more transcriptionally active HIF heterodimer complexes, further enhances the reporter activity. Again, the mutation of the identified HRE represses the reporter activity, at least partially.

We next analyzed the direct binding of HIF-1 to the human HRE by electrophoretic mobility shift assay (EMSA). Incubation of a radiolabeled oligonucleotide containing the wildtype Rab20 HRE with the HIF-1 heterodimer formed by *in vitro* translated HIF-1α and HIFβ protein, clearly retards migration in the acrylamide gel (Fig. 5E). Furthermore, incubation of HIF-1 together with an antibody against HIF-1α results in a supershift of the detected oligonucleotides due to the higher molecular mass of the formed complexes. The mutation of the putative HRE in the same way as for the luciferase reporter constructs avoids the binding of HIF-1. Radiolabeled oligonucleotides of an established EPO HRE were used as a positive control. Altogether these findings demonstrate that Rab20 is a direct target gene of HIF-1 and establish the HRE at −209 bp to be the functional binding site for HIF-1.

3.6. Rab20 protein colocalizes with mitochondria

To gain more insight into the intracellular localization and function of Rab20 protein, we cloned Rab20 fusion-tag plasmids with the wildtype human Rab20 cDNA (pEGFP-C_1-hRab20, Rab20-N-HA). In the literature comparable transfection experiments with Rab proteins were repeatedly performed in the human breast adenocarcinoma MCF-7 cells, which are readily transfectable [35,36]. We therefore initially used MCF-7 cells to overexpress GFP-Rab20, which demonstrated a cytoplasmic and dotted staining pattern in the majority of transfected cells by autofluorescence (Fig. 6A). In addition, this speckled pattern of Rab20 could also be observed after overexpression of an N-terminal HA-tagged cDNA of Rab20 (Rab20-N-HA) and subsequent staining with an anti-HA antibody (Fig. 6B). The specificity of the observed signals was verified by staining of the transfected cells with an antibody directed against Rab20 (Ab-2, introduced in Fig. 2). As shown, the antibody is able to detect identical staining patterns, as demonstrated by an almost complete overlay (merge) of the signals in both, the pEGFP-C_1-Rab20 as well as the Rab20-N-HA cDNA expressing cells. These results demonstrate that the Rab20 antibody Ab-2 specifically detects Rab20 protein in cytochemistry and probably no other unspecific proteins at relevant levels.

Importantly, focusing on the endogenous Rab20 protein in untransfected cells by increasing the exposure times ("high"), we also see the characteristic dotted staining pattern. As database analysis of the human Rab20 protein revealed sequence homology with Miro-1 (mitochondrial Rho), which was reported to be present at mitochondria [37], we again transfected the pEGFP-C_1-hRab20 plasmid into MCF-7 and subsequently stained the cells with the mitochondrial marker MitoTracker CMXRos (Fig. 7A). GFP-tagged Rab20

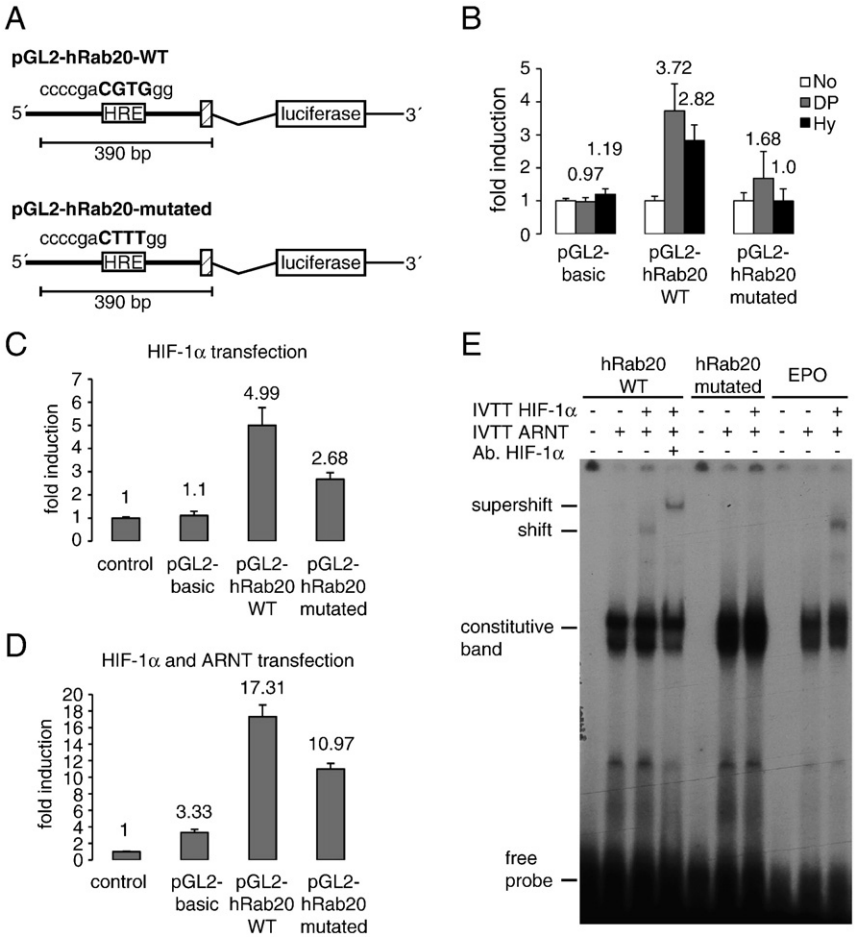

Fig. 5. Direct regulation of the Rab20 promoter by HIF-1. (A) WT and mutated luciferase-reporter constructs containing a 390 bp fragment upstream of the transcriptional start site from the human Rab20 gene including the putative −209 bp HRE were constructed. (B) HeLa cells were transfected with a pGL2-hRab20-WT or the mutated counterpart and stimulated with hypoxia or DP as indicated. Panel (C) represents reporter gene activation after cotransfection with a plasmid coding HIF-1α or HIF-1α together with ARNT (D). (E) Binding of recombinant HIF-1α and ARNT on the HRE located −209 bp in the human Rab20 promoter by EMSA. Radiolabeled oligonucleotides corresponding to the wildtype (WT) or mutated HRE located −209 bp in the Rab20 promoter were incubated with *in vitro* transcribed and translated (IVTT) HIFß alone (as control), HIFß and HIF-1α, or together with an anti-HIF-1α (Ab. HIF-1α) antibody, as indicated. Established radiolabeled EPO oligonucleotides were used as a positive control.

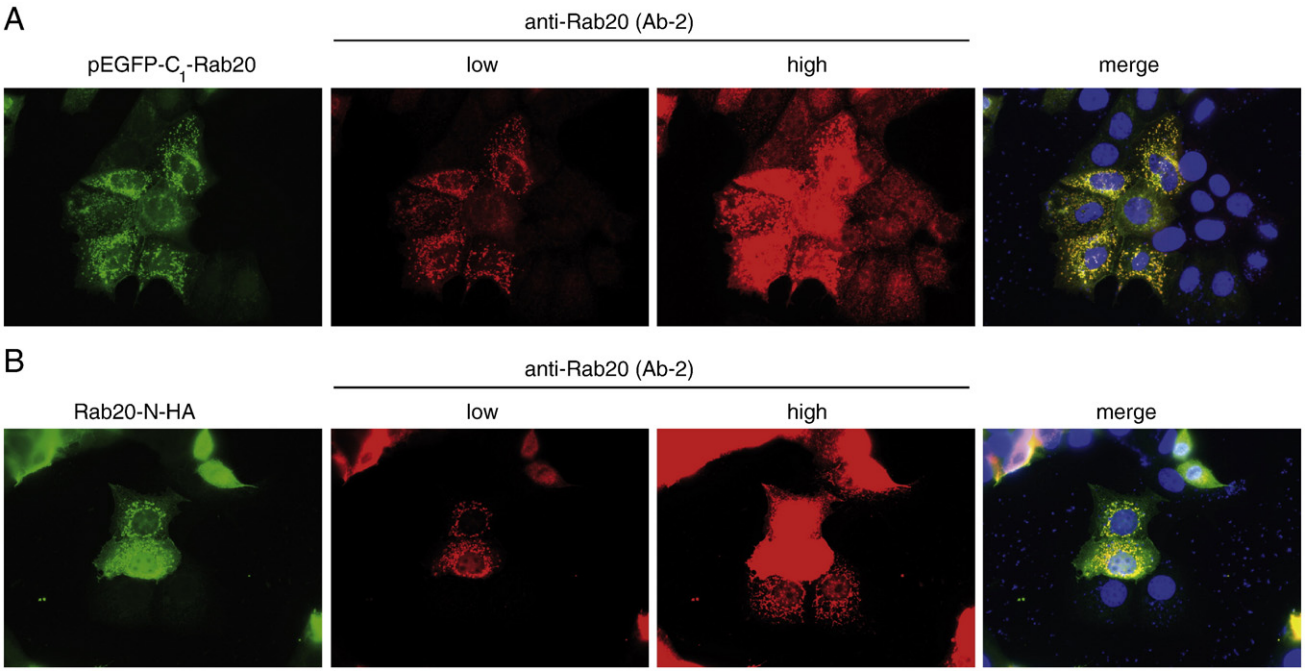

Fig. 6. Intracellular localization of overexpressed Rab20 protein. Immunofluorescence of MCF-7 cells that were transfected with GFP- (pEGFP-C_1-Rab20) (A) or HA-tagged (Rab20-N-HA) (B) human Rab20 cDNA. Both samples were subsequently stained with the Rab20 antibody Ab-2. Autofluorescence was detected for pEGFP-C_1-Rab20, whereas for Rab20-N-HA expressing cells (B), a double staining with an anti-HA antibody was performed.

protein and MitoTracker showed a clear colocalization. Of note, not all cells exhibit a signal for the transfected GFP-Rab20 construct but, of course, all cells were stained for mitochondria. This verifies our observation, because both fluorescence signals do not overlap. The demonstrated mitochondrial localization could also be detected after overexpression of the N-terminal HA-tagged Rab20 cDNA (Rab20-N-HA) as documented in Fig. 7B by visualization of mitochondria by MitoTracker and subsequent staining of Rab20 with an anti-HA antibody.

Finally, we investigated whether endogenous Rab20 protein also colocalizes with mitochondria, using MCF-7 and the previously studied cell lines HeLa and HKC-8 as well as human primary tubular cells (hPT). Again, mitochondria were visualized by the use of the mitochondrial marker MitoTracker and afterwards all cells were stained with the Rab20 antibody Ab-2. All cells show a colocalization with labeled mitochondria even under normal oxygen tensions (Fig. 8). Although immunoblotting showed strong induction of Rab20 protein, there was no difference in staining after hypoxic stimulation or DP treatment for 16 h (data not shown). This may be due to existing baseline expression of Rab20 even under normal oxygen tensions (compare to Fig. 1A and B, Figs. 2B and 4B). Noteworthy, immunocytochemistry is not a valid method to detect quantitative differences in protein expression, due to high amplification on cellular level.

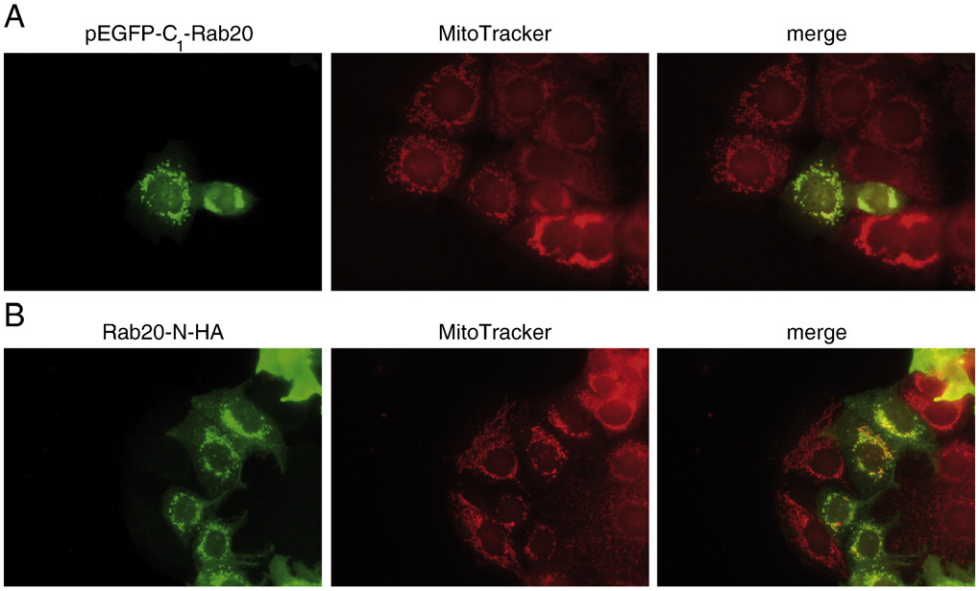

Fig. 7. Transfected Rab20 protein colocalizes with mitochondria. (A) MCF-7 cells were transfected with human Rab20 cDNA fused to a Green Fluorescent Protein (pEGFP-C_1-Rab20) and subsequently stained with the mitochondrial marker MitoTracker CMXRos. (B) MCF-7 cells were transfected with an HA-tagged variant of the human Rab20 cDNA and stained with an anti-HA antibody to detect the transfected cells. Again, mitochondria were visualized by the use of the MitoTracker.

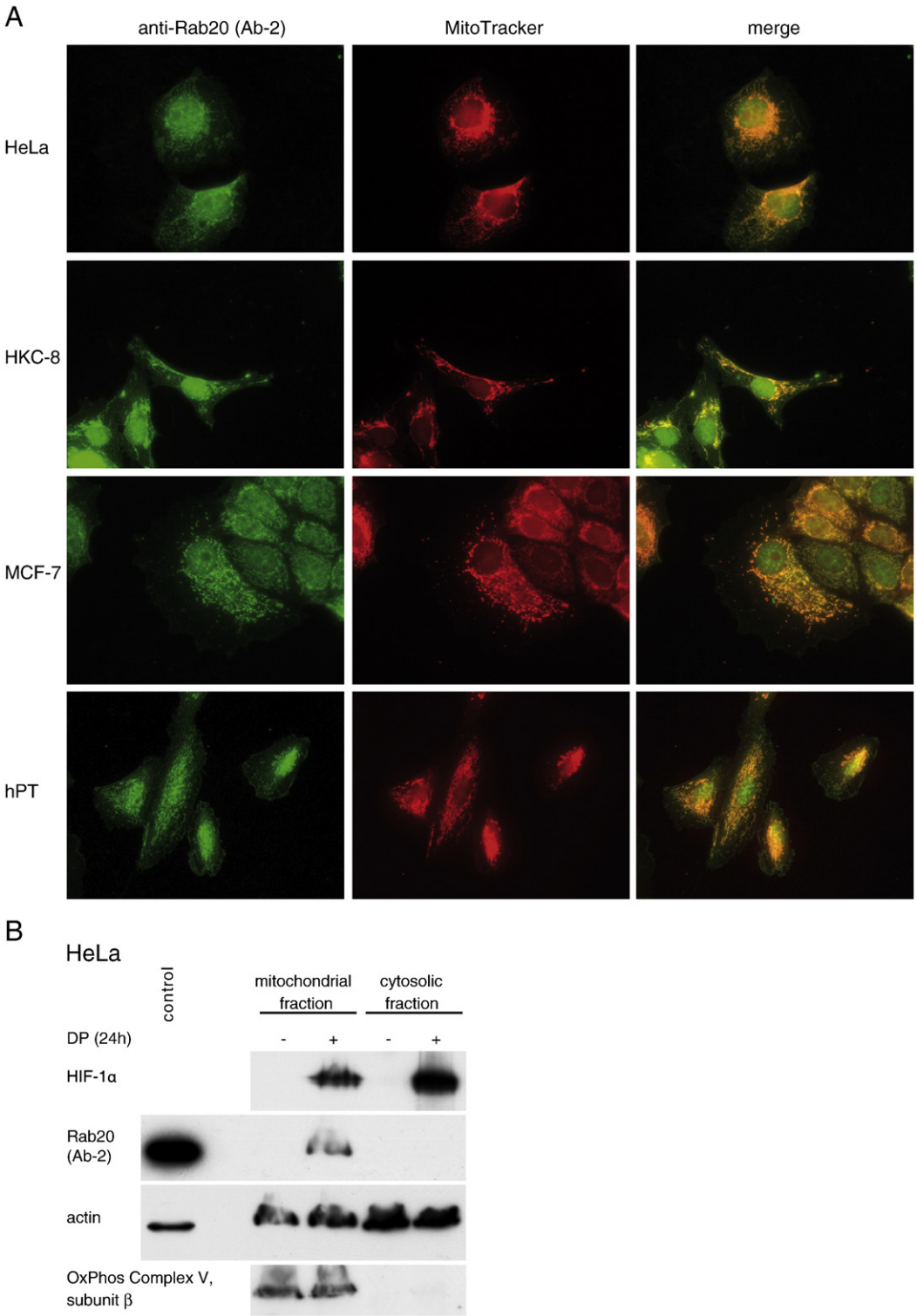

Fig. 8. Endogenous Rab20 colocalizes with mitochondria in different human cell lines and primary tubular cells. Immunofluorescence for endogenous Rab20 protein, stained in HeLa, HKC-8 and MCF-7 cells and in hPTs (human primary tubular cells) with the Rab20 antibody Ab-2. Mitochondria were visualized by MitoTracker Red CMXRos. (B) HeLa cells were stimulated with DP for 24 h and mitochondrial and cytosolic enrichment was performed. Extracts were used for immunoblotting against HIF-1α, Rab20 and OxPhos Complex V, subunit β. Rab20 cDNA transfected cells were used as control.

To further evaluate the mitochondrial localization, we performed cellular fractionation of HeLa cells after pharmacological activation of HIF (Fig. 8B). The induction of HIF results in the accumulation of Rab20 in the mitochondrial fraction of these cells, where it was not detectable in the cytoplasm. An antibody against ATP synthase (Oxidative phosphorylation (OxPhos) complex V, subunit β) was used as control for the mitochondrial enrichment.

Taken together, we were able to show that exogenous as well as endogenous Rab20 protein localizes at mitochondria in different cell lines.

3.7. Rab20 influences hypoxia induced apoptosis

Since a large body of evidence has shown that hypoxia is able to activate a pro-apoptotic pathway, predominantly via mitochondrial

apoptosis, we tested whether the hypoxia mediated apoptosis could be facilitated at least in part by Rab20 induction.

Hypoxic stimulation of HKC-8 cells increases the activity of caspase 3 and 7 approximately 2-fold. Interestingly, the induced caspase activity could be diminished by knockdown of either HIF-1α or Rab20 mRNA expression (Fig. 9A). We were also able do detect the cleaved caspase 3 fragment by immunoblotting after hypoxic stimulation in these cells (Fig. 9B). The cleaved caspase 3 fragment was reduced by knockdown of HIF-1α or Rab20. To confirm the effects of Rab20 on hypoxia-induced cell death we aimed to utilize annexinA5 binding as an independent assay technique. However, relatively mild hypoxia with 1% O_2 did not induce annexinA5 binding, with no apparent change of cellular morphology or increase of dead cells within the cell culture media (data not shown). In contrast, more severe hypoxia of 0.1% O_2 significantly changes the cell shape and the morphology of the HKC-8 cells to a more spindle like and apoptotic phenotype (data not shown), including increased exposure of phosphatidylserine. Fig. 9D shows representative dot plots of the annexinA5/propidium iodide binding of HKC-8 cells after exposure to 0.1% hypoxia for 24 h and Fig. 9 C the mean results of 3 independent experiments. Severe hypoxia increases the

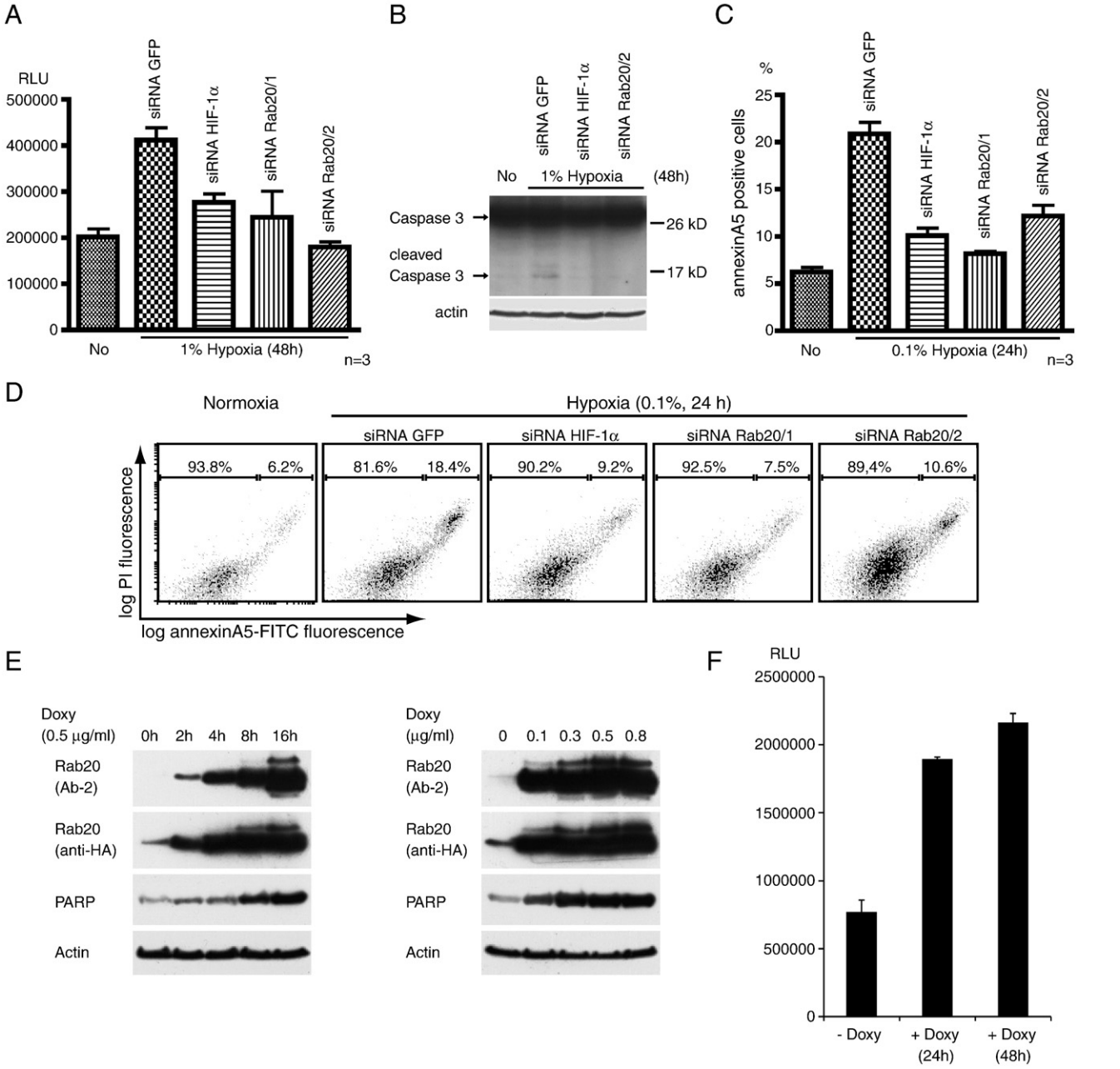

Fig. 9. Rab20 influences hypoxia induced apoptosis. (A) HKC-8 cells were transfected with siRNA against HIF-1α or two independent siRNA against Rab20 and subsequently incubated at 1% O_2 for 48 h. GFP siRNA was used as control. Bar chart shows mean result of three independent experiments measured in triplicates. (B) Caspase 3 cleavage was detected by immunoblotting, which is able to detect endogenous levels of full length (35 kDa) and the cleaved large fragment (17/19 kDa) of caspase 3 after knockdown of HIF-1α and Rab20 and subsequent hypoxic stimulation. (C) HKC-8 cells were treated with siRNA as described above and incubated at 0.1% O_2 for 48 h. At day of measurement, cells were counted and analyzed for annexinA5 binding. Bar chart shows mean results of 3 independent experiments measured in duplicates. Percentages represent the total number of annexinA5 positive cells without differentiating between apoptotic or necrotic cells. (D) Illustrates one representative dot plot of a flow cytometric analysis of annexinA5/propidium iodide binding of the HKC-8 cells. (E) Tetracycline inducible Rab20 HeLa cells were stimulated with 0.5 μg/ml doxycycline for different time periods or with different concentrations of doxycycline for 16 h. Activation of Rab20 expression was determined by immunoblotting against Rab20 and the HA-tag. Induction of apoptosis was monitored by stimulation of cleaved PARP. (F) Activation of caspase 3 and 7 after stimulation of inducible HeLa-Rab20-HA cells with 0.5 μg/ml doxycycline for 24 or 48 h, respectively. Bar chart shows mean results of one representative experiment measured in triplicates.

percentage of annexinA5 positive cells from 6% to approximately 20%. Again, the knockdown of either HIF-1α or Rab20 mRNA profoundly reduces this effect. The influence of Rab20 on apoptosis was further supported by the generation of tetracycline inducible Rab20 expressing HeLa cells (Fig. 9E). Activation of Rab20 in normoxic cells results in profound accumulation of the protein poly (ADP-ribose) polymerase which is one of the main targets of caspase 3 and serves as a marker of cells undergoing apoptosis [38,39]. In accordance to these findings, stimulation of Rab20 inducible HeLa cells results in activation of caspase 3 and 7 (Fig. 9 F).

Altogether our data clearly indicate that the induction of Rab20 by HIF is involved in the regulation of cellular apoptosis in hypoxically stressed cells. Since Rab20 is a predominant mitochondrial protein, we presume that it is the mitochondrial pathway that is primarily targeted.

4. Discussion

Rab proteins are small GTPases, which constitute the largest family within the Ras superfamily. So far, there are over 60 Rab proteins identified and most of them are highly conserved throughout different species. It is widely believed, that these monomeric small proteins function as molecular switches to regulate vesicle trafficking and transport pathways. Namely, they are involved in exocytotic and endocytotic trafficking [15] or membrane fusion [40]. Under pathological conditions, it has been reported that some Rab proteins may participate in tumor progression [17] or other diseases [41]. To date, little is known about the role of vesicle trafficking and Rab proteins in oxygen homeostasis. However, considering the great importance of vesicular transport for cellular integrity and function, as well as its high rate of energy consumption, it is plausible that oxygen deprivation would influence these processes.

In this report we show that Rab20 is a new and direct target of HIF-1. So far, there is very little known about this protein. The data reported to date are limited to the description of Rab20 as expressed in an organ specific manner with focus on the kidney [19], a possible colocalization with H^+-ATPases therein [20] and an upregulation of Rab20 in pancreatic tumor cell lines and primary pancreatic carcinomas [21].

Considering the high degree of interspecies homology of most Rab proteins the question arises, whether other Rab proteins are also regulated by hypoxia and what functional aspects this hypoxic regulation implicates. In a recently published gene array, where we searched for novel HIF targets induced by the iron chelator dipyridyl in Hep3B cells, we could not identify any Rab proteins other than Rab20 (data not shown and [14]). However, another study using microarray technology identified Rab20 as being upregulated by hypoxia in two different nasopharyngeal carcinoma cell lines [42], as well as another Rab protein, Rab40C. This might be a hint that the transcriptional control of oxygen deprivation on cellular transport processes goes beyond Rab20.

As already mentioned, database analyses revealed a sequence homology to a miro-like domain within the human Rab20 protein sequence. This domain was first characterized by Fransson et al. and is present in the Miro-1 and Miro-2 proteins which located to mitochondria [37]. Interestingly, we were able to show a colocalization of transfected and endogenous Rab20 with mitochondria in different cell lines (Fig. 7 and 8). Furthermore, HIF induced Rab20 protein accumulates in the mitochontrial fraction of HeLa cells (Fig. 8). As our database analysis did not reveal a transmembrane domain, the question arises how Rab20 could be anchored to mitochondria. Small G proteins usually have sequences at their C-terminal end that undergo posttranslational modifications. In the case of Rab proteins two cysteine residues are mostly geranylgeranylated which allows anchorage into a lipid membrane [16]. Of note, for Rab32 it was shown that two C-terminal cysteine residues are necessary to anchor the protein to mitochondria [43]. Indeed, there are also two cysteine residues conserved at the C-terminal end of the mouse, rat and human Rab20 protein sequence, indicating that these two amino acids might be necessary for mitochondrial targeting.

Amillet and colleagues [21] described the localization of Rab20 in the vicinity of the Golgi apparatus in HeLa cells. However, they did not further evaluate the localization with other organel markers. They showed a mostly perinuclear staining of Rab20 protein in HeLa cells, which is comparable to our results (Fig. 8). In our experiments immunolocalization of Rab20 perfectly colocalizes with mitochondria. We also investigated Rab20 localization in MCF-7, HKC-8 and hPT cells, where mitochondria appear to be more widely distributed through the cytosolic space.

Mitochondria play a key role in the regulation of cell death by many ways as thoroughly reviewed recently [22]. The induction of apoptosis is a critical event in all organisms of higher order, since propagation of cells has to be stopped if they are irretrievably damaged. There are many stress signals like nuclear DNA damage leading to the induction of p53 that induce apoptosis in order to prevent the accumulation or proliferation of abnormal cells [44]. Hypoxia also induces the stabilization of p53 protein [45] and a direct interaction between HIF-1α and p53 was reported [46]. Interestingly, HIF regulates many proteins which directly influence mitochondria either by upregulation of glycolytic enzymes to enforce anaerobic respiration [47], downregulation of mitochondrial oxygen consumption [23] or by modulating important components of the respiratory chain (COX4, [24]). Finally, Hypoxia is able to induce apoptosis [8] and HIF directly increases the expression of proapoptotic genes like NIX, BNIP3, BAX and Noxa [10–12]. Altogether this demonstrates the necessity of a well structured order of apoptosis regulation under hypoxic conditions.

Fransson et al. [37] demonstrated that overexpression of Miro-1 is able to induce an increased apoptotic rate. Our results indicate that induction of Rab20 protein (with a putative miro-like domain) by HIF may contribute to hypoxia-induced, mitochondrial apoptosis. Our experiments show that mild (1% O_2) or severe hypoxia (0.1%) increases the activity of caspases 3 and 7 or the number of annexinA5 positive cells, respectively (Fig. 9). In both cases, the knockdown of either HIF-1α or Rab20 reduces hypoxia induced apoptosis. In addition to the knockdown of Rab20 before hypoxia induced apoptosis, transient overexpression of Rab20 cDNA showed no effect (data not shown). It can be speculated that additional protein modification may be necessary to activate (transfected) Rab20 in hypoxia, particularly since Rab proteins are regulated by many different factors [16]. Alternatively, Rab20 may simply be required to shuttle other pro-apoptotic proteins towards the mitochondria, such as BNIP3 [48]. In that case, mere overexpression of Rab20 would not suffice to induce apoptosis. In contrast, strong and stable induction of Rab20 in every cell is indeed able to induce apoptosis as shown by us using tetracycline-inducible Rab20 expressing HeLa cells. Altogether, this implicates that the involvement of Rab20 in hypoxia-induced apoptosis is complex and may underlie different levels of regulation and yet unknown cofactors, which needs to be elucidated by further experiments.

Another aspect how mitochondria contribute to apoptosis is the process of fission and fusion of these organelles. During apoptosis, more and smaller mitochondria are formed and fission and fusion can actively participate in apoptosis induction [49]. It is possible that Rab proteins as organizers of vesicle trafficking and membrane fusion may participate in this process. However, we were not able to detect a difference of mitochondrial morphology or localization after pharmacological HIF stabilization or hypoxic treatment (data not shown). As described already by Papandreou and colleagues [23] the effects of HIF on mitochondria seems to be more functional and not structural. They could not observe a difference in mitochondrial localization in renal clear cell carcinoma cells with constitutive active HIF as compared to cells without activation of HIF protein. Nevertheless, it was reported recently that the newly identified HIF-1 target gene

HUMMR is directly involved in mitochondria motility [50]. This, once again, exhibits the strong connection between HIF, hypoxia and mitochondrial activity, where Rab20 may play a role.

Another known sequela of hypoxia is accumulation of protons and consecutive tissue acidosis. Maintenance of the cellular pH within narrow boundaries is vital to any of the cells functions and integrity. Already, an important compensatory mechanism by HIF-1 has been identified, which comprises strong upregulation of the carbonic anhydrase 9 (CA9) and thereby adaptation of cellular pH [51]. Interestingly, since Rab20 has been suggested to be involved in regulation of renal bicarbonate reabsorption by association with vacuolar H^+-ATPases [20], this may be an additional compensatory effect. Bicarbonate reabsorption is an important physiological task of the kidney, which might explain why there is relatively high basal expression in this organ and little regulation in hypoxia (Fig. 3). However, this effect might be functionally important in other hypoxic tissues, and/or tumors. To date, these considerations remain mere speculation and this context deserves further investigation.

In summary, we demonstrate that Rab20 is induced by HIF-1 and localizes to mitochondria where it may contribute to hypoxia induced apoptosis. In physiological cells and tissues hypoxia-induced apoptosis probably enables repair and restores tissue homeostasis. Whether classical transport activities are influenced by Rab20 under hypoxia is not known to us. However, it is conceivable that pro-apoptotic proteins are escorted by Rab20 towards the mitochondria in hypoxia. The effect of Rab20 activation in tumors would be more difficult to predict, but could also help to select damaged cells from viable cells under certain stress situations, such as hypoxia. Therefore, manipulation of HIF in malignant disease may result in selection of a more aggressive tumor phenotype by reducing growth arrest or apoptosis. Thus, more profound knowledge on the multiple avenues of HIF biology is required to estimate the effects of therapeutic manipulation.

Acknowledgments

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