TSGA10 prevents nuclear localization of the hypoxia-inducible factor (HIF)-1α

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Abstract The hypoxia-inducible factor (HIF)-1 is a transcriptional regulator of genes involved in oxygen homeostasis. We previously described testis-specific isoforms of HIF-1α (mHIF-1αI.1 and hHIF-1αTe). Using mHIF-1α exon I.1 knock-out mice we confirmed the specific expression of mHIF-1αI.1 in the sperm tail. A protein–protein interaction between HIF-1α and the testis specific gene antigen 10 (TSGA10) was identified by yeast two-hybrid screening. TSGA10 is expressed in testis but also in other organs and malignant tissues. Immunofluorescence analysis indicated that the C-terminal part of TSGA10 accumulates in the midpiece of spermatozoa, where it co-localizes with HIF-1α. HIF-1α nuclear localization and HIF-1 transcriptional activity were significantly affected by overexpressed TSGA10.
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1. Introduction

The testis represents a unique environment where profound differentiation processes occur within a small compartment at a high rate. Spermatogenesis is a process by which the undifferentiated spermatogonia begin to differentiate to mature spermatozoa. Spermatogonia divide to primary spermatocytes, which go through the first meiotic division and thereby become secondary spermatocytes. These cells complete the second meiotic division resulting in haploid round spermatids. At this stage, chromatin is compacted and transcription is stopped. Specific mRNAs are stored for later translation of sperm-specific proteins [1]. During mouse spermiogenesis, round spermatids mature in 16 distinct steps via elongated spermatids to mature spermatozoa. After about 13.5 days, mature spermatozoa are appearing in the lumen of the seminiferous tubuli [2]. Interestingly, the oxygen partial pressure in the seminiferous

tubuli are low compared to the conditions found in other tissues under resting conditions [3]. Regarding the high division and differentiation rate as well as the environmental challenges a unique set of specific genes or splice variants of common genes are expressed exclusively in the testis. For example, testis-specific isoforms of glycolytic enzymes are expressed during the haploid stages of spermatogenesis and are still active in mature spermatozoa, indicating the importance of testis-specific regulation of the glycolytic flux [4].

The hypoxia-inducible factor-1 (HIF-1) is the ubiquitously expressed transcriptional regulator for the induction of hypoxia-inducible gene expression (reviewed by Wenger et al. [5]). Since HIF-1 target genes include among others erythropoietin, vascular endothelial growth factor as well as glycolytic enzymes, HIF-1 is regulating core processes like oxygen transport, angiogenesis and glycolytic capacity [6]. HIF-1 comprises an oxygen-dependently expressed α subunit and the constitutively expressed β subunit. Under normoxic conditions, the von Hippel–Lindau tumor suppressor protein (pVHL) targets the HIF-1α subunit for very rapid ubiquitination and proteasomal degradation [7]. pVHL binding requires HIF-1α prolyl hydroxylation by a family of oxygen-dependent prolyl hydroxylases [8–10].

Cloning of the mouse HIF-1α gene demonstrated that its expression is driven by two different promoters located 5′ to two alternative first exons designated exon I.1 and exon I.2 [11,12]. The upstream exon I.1 promoter exhibits tissue-specific features driving testis-specific expression of HIF-1α, the downstream exon I.2 promoter is a typical housekeeping-type promoter driving the ubiquitous transcription [13]. The predicted mouse mHIF-1αI.1 differs in 12 lacking amino acids from the predicted mHIF-1αI.2 protein. However, the actual N-terminus of neither mHIF-1αI.2 nor mHIF-1αI.1 is known. At least *in vitro*, this N-terminal deletion does not affect DNA-binding efficiency, despite its vicinity to the basic-helix–loop–helix DNA binding domain [14]. Recently, we also described a testis-specific human isoform of HIF-1α termed hHIF-1αTe [15,16] in contrast to the predicted mHIF-1αI.1 protein, hHIF-1αTe acts as a dominant negative regulator of normal HIF function [16]. Mouse mHIF-1αI.1 and human hHIF-1αTe are expressed in the postacrosomal region and the midpiece of spermatozoa [16,17]. Here, we demonstrate that

HIF-1α interacts with the filament-building sperm protein TSGA10 [18], which modulates the intracellular localization of HIF-1α. Since TSGA10 expression was recently demonstrated also in embryonic tissue, different adult organs like brain, kidney and heart as well as malignant tissues, this finding may have also implications for tissue-dependent regulation of HIF-1 activity [19].

2. Materials and methods

2.1. Antibodies and chemicals

Antibodies were purchased from the suppliers indicated in parentheses: mouse anti-HIF-1α (Transduction Laboratories, Heidelberg, Germany), mouse anti-HIF-1α (Novus/Acris, Hiddenhausen, Germany), mouse anti-V5 (Invitrogen, Karlsruhe, Germany), rabbit anti-MBP (New England BioLabs, Frankfurt, Germany), mouse anti-β-actin (Sigma, Deisenhofen, Germany). For generating anti-TSGA10 antibodies, peptides derived from the TSGA10 N-and C-terminal regions (aa 206–219 and 676–689, respectively) were synthesized, conjugated to keyhole limpet hemocyanin and used to immunize two rabbits. Resulting antisera thus contained antibodies directed against both the TSGA10 N- and C-terminal peptides. In indicated experiments, antibodies were used which were affinity purified against either the C- or the N-terminal peptide. Appropriate horseradish peroxidase-labeled or fluorescence-labeled secondary antibodies were purchased from SantaCruz (Santa Cruz Biotechnology, Santa Cruz, CA). If not stated otherwise, all other chemicals were obtained from Roth (Karlsruhe, Germany).

2.2. Animals

HIF-1αI.1 null mice were developed by deleting a ~500 bp *Bam*HI–*Hin*dIII fragment surrounding exon I.1 using a ~1.5 kb *Xho*I–*Bam*HI fragment as 5′ arm and ~3 kb *Hin*dIII–*Eco*RI as 3′ arm by homologous recombination in ES cells of 129 strain origin. Chimeric mice were generated by injection of ES cells into C57BL/6 blastocysts. A detailed description will appear elsewhere (D. Lukashev and M. Sitkovsky, manuscript in preparation). Experimental protocols regarding those animals were performed following the Swiss Animal Protection Law and were supervised by the Veterinary Department of the Kanton Zürich (approval number 192/2003).

2.3. Plasmid constructions

All cloning work was carried out using Gateway technology (Invitrogen). Entry vectors were generated by cloning PCR fragments into *Nco*I–*Eco*RV-digested pENTR4 or pENTR/D-TOPO vectors. Full-length TSGA10 (Accession No. AF530050) was amplified by PCR from pEGFP2mTSGA10 [18]. Fragments of TSGA10 corresponding to the indicated amino acids were amplified by PCR from the full-length entry vector. Fragments of mHIF-1αI.1 or mHIF-1αI.2 were amplified by PCR from plasmids pcDNA3mHIF-1αI.1 and pcDNA3mHIF-1αI.2, respectively. Fragments of hHIF-1α and hHIF-1αTe were amplified from pBShHIF-1α (kindly provided by G.L. Semenza, Baltimore, MD) and pcDNA3.1hHIF-1αTe [16], respectively. The inserts of all entry vectors were verified by DNA sequencing. To generate plasmids expressing fusion proteins, DNA inserts were transferred from entry clones to destination vectors using LR Clonase recombination enzyme mix (Invitrogen). Destination vector pcDNA3.1/nV5-Dest was used to express N-terminal V5-tagged proteins in mammalian cells or in rabbit reticulocyte lysates (TNT T7 quick coupled transcription translation system, Promega, Mannheim, Germany). To generate expression plasmids for yeast two-hybrid analysis, destination vectors pDEST32 (Gal4-BD) and pDEST22 (Gal4-AD) were used. Vector pMal-c2x (NewEngland Biolabs, Frankfurt, Germany) was converted to a destination vector by ligation of the gateway vector conversion cassette B (Invitrogen) into the *Eco*RI site (blunt ended by Klenow fill-in) of pMal-c2x, allowing to express MBP-fusion proteins in *E. coli* after recombination. The green fluorescent protein EGFP was amplified by PCR and cloned into the gateway compatible *Hin*dIII–*Eco*RV digested pcDNA3.1nV5DEST (Invitrogen) vector. These entry vectors were used to perform LR Clonase recombinations to obtain fluorescence-coupled expression of TSGA10

or HIF-1α. For generating the red fluorescent TSGA10 fusion protein, mTSGA10 was amplified by PCR and cloned in the Ecl136II and *Bam*-HI digested pHcRed1c1 vector (Invitrogen).

2.4. Cell culture and transient transfection

All cell lines were cultured in Dulbecco's modified Eagle's medium (high glucose). Oxygen partial pressures in the hypoxic incubator (Binder, Tuttlingen, Germany) were either 140 mm Hg (20% O_2 vol/vol, normoxia) or 7 mm Hg (1% O_2 vol/vol, hypoxia). HeLa cells were purchased from ATCC. Transformed and immortalized MEF-HIF-1α+/+ and MEF-HIF-1α−/− cells (kind gift from R.S. Johnson, San Diego, CA) have been described previously [20].

For determining filament formation of TSGA10, HeLa and MEF cells were transiently transfected with pEGFPc2mTSGA10, pHcRedC1mTSGA10 or pcDNA3.1GFPHIF-1α [18]. In brief, cells were seeded on sterilized glass coverslips in 6-well plates at a concentration of 4×10^4 cells/well (MEF) or 2×10^5 cells/well (HeLa). One day after seeding, cells were transfected with 3.5 μg pEGFPc2TSGA10, pHcRed1c1mTSGA10 or pcDNA3.1EGFPHIF-1α by the calcium phosphate co-precipitation method and exposed to 20% O_2 or 1% O_2 for 4 or 24 h. Subsequently, the cells were fixed with 3% paraformldehyde in PBS, mounted in Mowiol (Calbiochem, Darmstadt, Germany) and analyzed by fluorescence microscopy (Axioplan 2000, equipped with an Axiocam digital camera and Axiovision software; Carl Zeiss Vision, Mannheim, Germany).

2.5. Bacterial protein expression and purification

MBP-fusion proteins were expressed in the *E. coli* strain TB1 (New-England Biolabs) transfected with pMal-c2x-Dest and purified using the pMal purification system according to the manufacturer's instructions (NewEngland Biolabs).

2.6. In vitro pulldowns

Purified MBP-TSGA10 aa556-688 (15 μg) was bound in 20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA to amylose-resin (New-England Biolabs) for 1 h at 4 °C. After washing, bound proteins were incubated with *in vitro* translated V5-HIF-1α aa 1–401 in 500 μl of 20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA for 1 h at room temperature. The resins were washed 5 times, bound proteins were eluted with SDS–PAGE loading buffer and subjected to immunoblot analysis.

2.7. Yeast two-hybrid analysis

Yeast two-hybrid analyses were performed using the ProQuest system according to the manufacturer's instructions (Invitrogen). As a bait, mHIF-1αI.1 aa 1–401 fused to the Gal4 DNA-binding domain (BD) was expressed from pDEST32-mHIF-1αI.1 aa 1–401 in *Saccharomyces cerevisiae* strain MaV203 (Invitrogen). To test for self-activity, co-transformants of pDEST32-mHIF-1αI.1 aa 1–401 and pExp-AD502, encoding the Gal4 activation domain (AD), were examined on selection plates. The library was prepared from total RNA extracted from testes of two adult male C57BL/6 mice by Gene Discovery Service (Invitrogen). RNA was reverse transcribed using a Biotin-attB2-αdT first strand primer with SuperScriptII (Invitrogen). Resulting cDNAs were inserted into the pEXpAD502 vector. The library contained 3.38×10^7 primary clones with an average insert size of 1.878 kb and >95% plamids with inserts. The library was transformed into MaV203 pDEST32-mHIF-1α aa 1–401 and transformants were screened on synthetic dropout medium lacking tryptophan, leucine and histidin and containing 12.5 mM 3-amino-1,2,4-triazole (3-AT). Positive clones were further assayed for growth on synthetic dropout medium lacking tryptophan, leucine and uracil and for β-galactosidase activity to characterize the strength of interaction. The pExpAD502 plasmids encoding putative mHIF-1αI.1-interacting proteins were isolated, retested for interaction and tested for self-activity. Inserts of confirmed interactors were sequenced to ensure in-frame coding sequence with the AD of Gal4.

2.8. Reporter gene analysis and protein expression

Hela cells were transiently transfected with the HIF-dependent firefly luciferase reporter gene construct pH3SVL, containing a total of 6 HIF-1 DNA-binding sites derived from the transferrin gene [21] by the calcium phosphate co-precipitation method. Cells were seeded in 6-

well plates at a concentration of 5×10^4 cells/well. One day after seeding, cells were co-transfected with 1.0 µg pH3SVL together with 3.5 µg pcDNA3.1 or pcDNA3.1/nV5-Dest containing full length TSGA10 or different fragments of TSGA10 and 0.15 µg renilla luciferase control plasmid pRL-SV40 (Promega, Mannheim, Germany). Cells were subsequently exposed to normoxic or hypoxic conditions for 24 h. Luciferase activities were determined using the dual-luciferase assay kit (Promega). Results were normalized to the pcDNA3.1-transfected
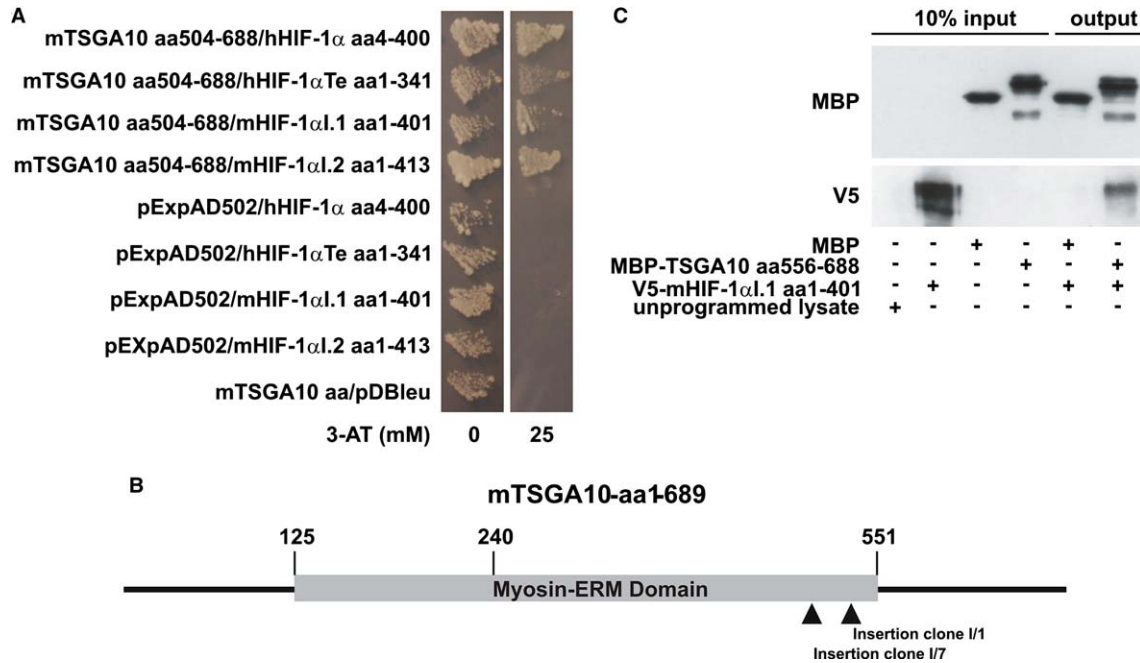

Fig. 1. Interaction of HIF-1α with TSGA10. (A) Yeast reporter strain MaV203 expressing Gal4-BD-TSGA10 and Gal4-AD-mHIF-1αI.1 aa 1–401, Gal4-AD-mHIF-1αI.2 aa 1–413, Gal4-AD-hHIF-1α aa 4–400 or Gal4-AD-hHIF-1αTe aa 1–341 was assayed for histidine auxotrophy. (B) Schematic drawing of TSGA10 showing the localization of the myosin-ERM domain. Arrows indicate the different insertions of the two independent TSGA10 clones found to interact with mHIF-1αI.1 in the yeast two-hybrid screen. (C) Binding of *in vitro* translated V5-mHIF-1αI.1 aa 1–401 to MBP-TSGA10 aa 556–688 attached to amylose resin. Antibodies against MBP or V5 were used for immunoblot detection as indicated.
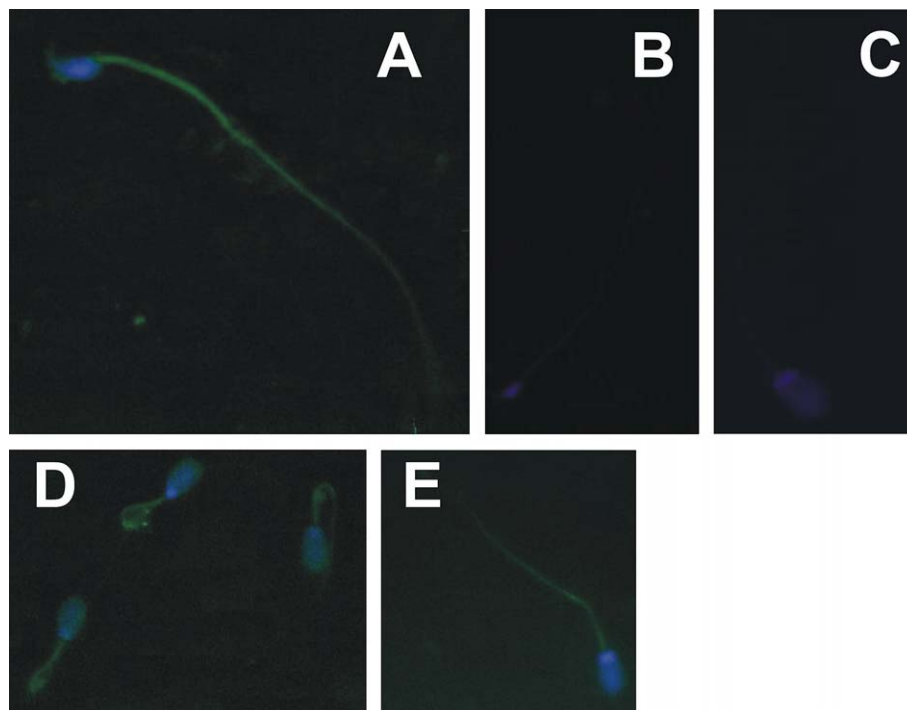

Fig. 2. Immunolocalization of the TSGA10 C-terminus to the midpiece of spermatozoa. Mouse (A and B) and boar (C–E) epididymal spermatozoa were fixed on glass slides and stained with anti-TSGA10 antiserum, which was affinity purified against the C-terminal peptide aa 676–689, followed by a FITC-coupled secondary antibody (A,D,E). (B and C) controls blocked with the TSGA10 aa 676–689 peptide. Nuclei were stained with Hoechst 33258.

normoxic control values which were arbitrarily defined as 1. The same protein samples were analyzed for expression of HIF-1α, V5 and β-actin. Protein concentrations were determined by the Bradford method using BSA as a standard [22]. For immunoblot analysis, cellular protein (50 μg) was electrophoresed through 7.5% SDS–polyacrylamide gels and electrotransferred onto nitrocellulose membranes (Amersham, Freiburg, Germany) by semi-dry blotting (BioRad, München, Germany). Membranes were stained with Ponceau S (Sigma) to confirm equal protein loading and transfer. HIF-1α, V5 and β-actin were detected using the primary antibodies indicated above followed by HRP-labeled anti-mouse antibodies raised in goats. Chemiluminescence detection of horseradish peroxidase was performed by incubation of the membranes with 100 mM Tris–HCl, pH 8.5, 2.65 mM H_2O_2, 0.45 mM luminol and 0.625 mM coumaric acid for 1 min followed by exposure to X-ray films (Amersham).

2.9. Immunofluorescence

Collection of human ejaculated spermatozoa was approved by the ethics committee of the University of Leipzig (approval number 067-2005). Mouse spermatozoa were collected from the epididymides of HIF-1αI.1+/+ or HIF-1αI.1−/− mice as described previously [17]. All spermatozoa and cells were fixed with methanol for 5 min, and the non-specific binding sites were blocked with 3% BSA in PBS for 30 min. The cells were incubated for 1 h with mouse monoclonal anti-HIF-1α IgG antibodies (anti-human HIF-1α, Transduction Laboratories; anti-mouse HIF-1α, Novus, Acris), diluted 1:10, with or without the anti-C-terminal TSGA10 rabbit antiserum diluted 1:10 in PBS containing 3% BSA, followed by TexasRed-coupled secondary anti-mouse (Dako, Hamburg, Germany) antibody diluted 1:100 and FITC-coupled secondary anti-rabbit (Dako) antibody diluted 1:100 in PBS containing 3% BSA. Subsequently, nuclei were stained with Hoechst 33258 dye for 5 min. After extensive washings with PBS, the slides were mounted and analyzed by fluorescence microscopy as above.

For detection of TSGA10 in mouse or boar spermatozoa, cells were collected from epididymides and washed in PBS. Spermatozoa were spread on slides and incubated in demembranization buffer (5 mM DTT, 50 mM Tris–HCl, pH 9.0, and 2% Triton-X100) followed by fixation in 4% formaldehyde. Cells were spread on slides treated with poly-L-lysine and blocked with 3% BSA. Subsequently, coverslips were incubated with rabbit anti-TSGA10 antisera diluted 1:25 for 1 h at 37 °C. After washing with PBS and incubation with secondary FITC-coupled anti-rabbit antibody, cells were stained with DAPI and analyzed by fluorescence microscopy.

3. Results

3.1. Identification of TSGA10 as interacting partner of HIF-1α

To identify novel interaction partners of the testis-specific mouse HIF-1α isoform mHIF-1αI.1, we screened a mouse testis cDNA library fused to Gal4-AD with mHIF-1αI.1 aa 1–401 fused to Gal4-BD as bait. A fragment of mHIF-1αI.1 was used since screening with full-length mHIF-1αI.1 was not possible due to self activity (data not shown). In contrast, the mHIF-1αI.1 aa 1–401 bait alone was unable to confer yeast growth after transformation together with pExpAD502 containing no insert (Fig. 1A). In total, 40 interactions were identified in this screen, encompassing 11 independent prey clones. Four prey clones contained the cDNA for TSGA10 (Accession No. AF530050). TSGA10 comprises a myosin ERM-domain (aa 125–551) and can be posttranslationally processed to a 27 kDa protein, which corresponds to aa 1–240 (Fig. 1B). The four preys included two independent clones located close to the C-terminal end of the myosin-ERM domain (aa 503 and aa 532) of TSGA10.

Testing for direct protein–protein interaction in yeast, we determined that TSGA10 was also able to interact with the ubiquitous mouse isoform mHIF-1αI.2 as well as with the ubiquitously expressed human hHIF-1α and the recently described human testis-specific isoform hHIF-1αTe (Fig. 1A).

To verify the interaction of mHIF-1αI.1 and TSGA10 by pull-down assays, we purified TSGA10 aa 556–688 from bacteria as a MBP-fusion protein. In the following MBP-pull-down, V5-tagged *in vitro* translated mHIF-1αI.1 aa 1–401 specifically interacted with MBP-TSGA10 aa 556–688 whereas no interaction was detectable with MBP alone (Fig. 1C).

3.2. TSGA10 and HIF-1αI.1 are located in the midpiece of spermatozoa

Previously it has been described that TSGA10 is a component of sperm tail structures [18]. TSGA10 antibodies raised against the N-terminus (including the processed 27 kDa protein) resulted in immunofluorescence signals in the principal piece of the sperm tail, which also harbors the fibrous sheath. In the present study, we used TSGA10 antibodies raised against the C-terminus (including the HIF-1α interaction site). Applying these antibodies in immunofluorescence studies resulted in fluorescence signals in the midpiece of the tail of mature mouse and boar spermatozoa in addition to the
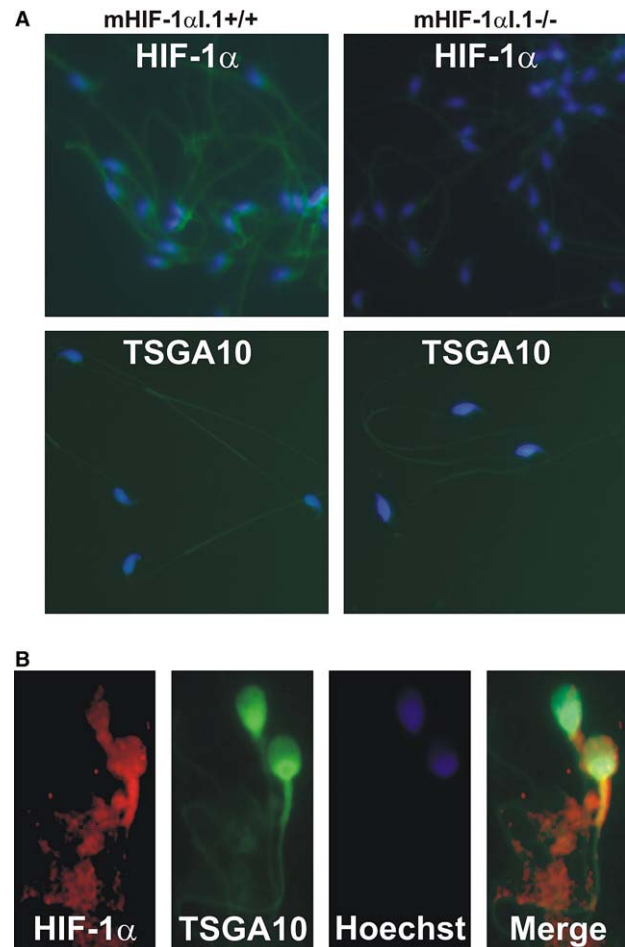

Fig. 3. HIF-1α co-localizes with TSGA10 in sperm cells. (A) HIF-1α immunofluorescence analysis of spermatozoa isolated from epididymides and vas deferens of mHIF-1αI.1+/+ or mHIF-1αI.1−/− mice. (B) HIF-1α and TSGA10 immunofluorescene analysis of ejaculated human sperm cells. TSGA10 immunofluorescence was performed with anti-TSGA10 antiserum, which was affinity-purified against the TSGA10 aa 676–689 C-terminal peptide.

principal piece (Fig. 2A–F) as described also recently [19]. In this context it should be noted, that applying the antibody against the C-terminus for Western blot analysis has also been reported to result in the detection of TSGA10 in cytoplasmic sperm protein extracts [18]. In contrast, the 27 kDa processed protein, which can be detected with the antibody against the N-terminus, was just detectable in fibrous sheath protein extracts [18]. The different signals, which were obtained with the N-terminal and C-terminal TSGA10 antibodies, indicate that after processing the C-terminal and N-terminal parts of TSGA10 may have different functions.

We described HIF-1α expression in mouse and human sperm cells previously [16,17]. As demonstrated for the C-terminal part of TSGA10, mHIF-1αI.1 and hHIF-1αTe were mainly localized to the midpiece of the sperm flagellum. Originally, HIF-1α in mouse sperm cells was identified by immunofluorescence studies employing antibodies which can not differentiate between mHIF-1αI.1 and mHIF-1αI.2. To confirm isoform-specific expression in mouse sperm cells, HIF-1α immunofluorescence was performed in spermatozoa isolated from the epididymides of wild-type mHIF-1αI.1+/+ and newly generated knock-out mHIF-1αI.1−/− mice (Fig. 3A). Most interestingly, HIF-1α immunofluorescence could be detected in mHIF-1αI.1+/+ but not in mHIF-1αI.1−/− spermatozoa,

supporting the specificity of the signal and demonstrating that mHIF-1αI.1 is the major HIF-1α isoform present in mature spermatozoa.

To further examine the protein–protein interaction between TSGA10 and HIF-1α *in vivo*, double-immunofluorescence studies were performed in human spermatozoa. As shown in Fig. 3B, HIF-1α and TSGA10 co-localize in the flagellum.

3.3. Hypoxia and HIF-1α do not alter TSGA10 filament formation

It has been reported that TSGA10 forms filaments in NIH3T3 mouse fibroblasts, mediated by its myosin/ERM domain [18]. To determine filament formation of TSGA10 in HeLa cells, cells were transfected with GFP-TSGA10 and incubated at normoxic conditions. As described in the literature for fibroblasts, ectopic TSGA10 formed cytoplasmic short thick filaments (Fig. 4B). To determine if hypoxia can influence filament formation, we overexpressed GFP-TSGA10 in HeLa cells and subsequently exposed the cells for 24 h to normoxic (20% O₂) or hypoxic (1% O₂) conditions (Fig. 4A). Formation of filaments was not altered by incubation under hypoxic conditions. To further explore the specific effect of HIF-1α on filament formation, we transfected GFP-TSGA10 in MEF-HIF-1α+/+ and MEF-HIF-1αI.1−/− cells (Fig. 4A). Oxygen-independent filament formation could be observed in
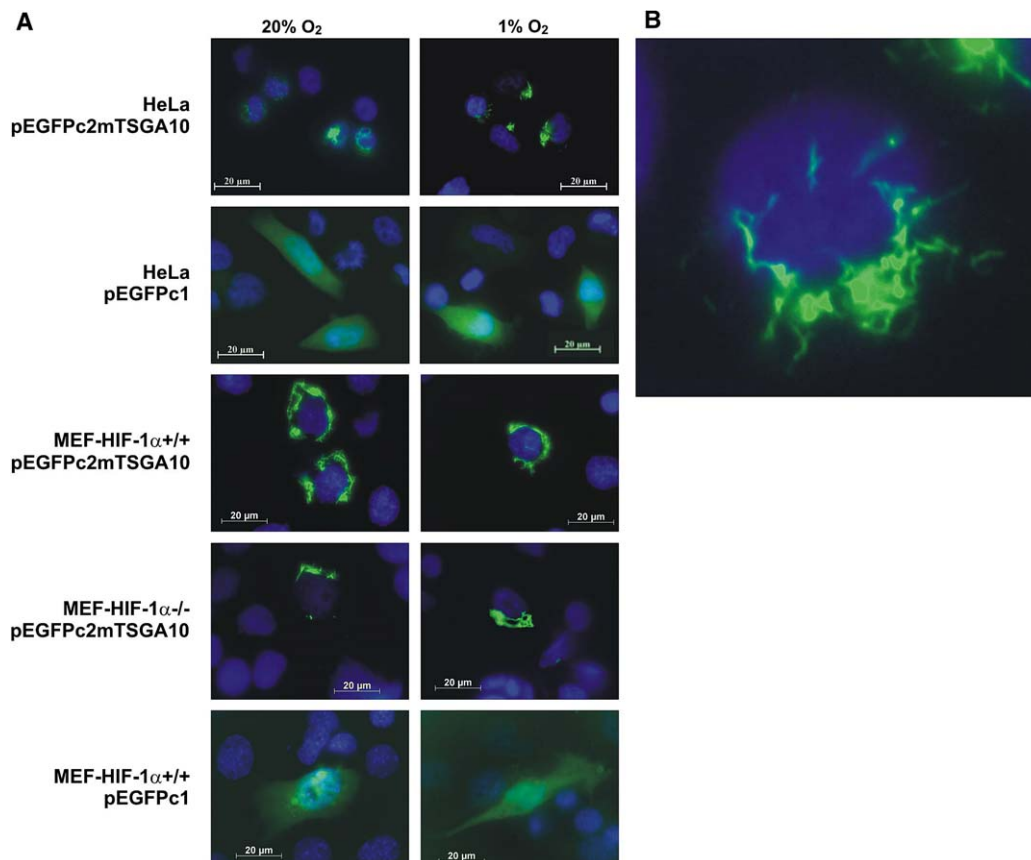

Fig. 4. Hypoxia or HIF-1α do not alter filament formation of TSGA10. (A) HeLa, MEF-HIF-1α+/+, or MEF-HIF-1α−/− cells were transiently transfected with EGFP-TSGA10 fusion protein or EGFP alone. After transfection, the cells were incubated for 24 h at 20% O₂ or 1% O₂. Cells were fixed, stained with Hoechst33258, mounted and analyzed by fluorescence microscopy. (B) HeLa cells were transiently transfected as in A with EGFP-TSGA10 and incubated for 24 h at 20% O₂. Cells were fixed, stained with Hoechst 33258, mounted and analyzed by fluorescence microscopy with high magnification to demonstrate cytosolic filament formation.

both cell lines, demonstrating that TSGA10 function is not altered by hypoxia or HIF-1α.

3.4. TSGA10 prevents nuclear accumulation of HIF-1α

To explore the effect of TSGA10 on HIF-1 function, we determined HIF-1 transcriptional activity in the presence or absence of TSGA10 in a reporter gene assay. Exposure of cells to hypoxia (1% O_2 for 24 h), co-transfected with a HIF-dependent reporter gene together with the empty expression vector pcDNA3.1, induced reporter gene activity by 49-fold (Fig. 5A). Co-expression of TSGA10 significantly reduced HIF-1 activity. This effect was not due to reduced HIF-1α protein levels, since total HIF-1α protein did not change as demonstrated by immunoblot analysis (Fig. 5B). To identify the mechanism responsible for the TSGA10 mediated reduction in HIF-1 activity, different fragments of TSGA10 were co-transfected together with the reporter gene. Besides the full-length protein no other fragment (TSGA10 aa 1–240, aa 237–688 or aa 556–688) had a similar strong effect on HIF-1 activity. Of note, filament formation was impaired when

TSGA10 fragments were transfected compared to the full-length TSGA10 (Fig. 6). Therefore, TSGA10 filament formation appears to be necessary for the modulation of HIF-1 activity.

To this end, we determined whether the cytoplasmic TSGA10 filaments affect the cellular localization of HIF-1α. As shown in Fig. 7A, transfection of GFP-hHIF-1α fusion protein and subsequent exposure of the cells to hypoxia resulted in green fluorescence mainly in the nucleus. However, co-transfection of HcRed1-TSGA10 fusion protein changed the cellular localization of mHIF-1α whose nuclear accumulation was hindered. Instead, mHIF-1α co-localized with the filaments in the cytoplasm (Fig. 7B).

4. Discussion

The fibrous sheath (FS) is a unique cytoskeletal structure surrounding the axoneme and outer dense fibre. It defines the extent of the principal region of the sperm flagellum. The FS is assembled during spermiogenesis. In recent years many proteins have been described to be located in the FS, including TSGA10 [18,23]. TSGA10 is predominantly expressed in the postmeiotic phase of spermatogenesis. It is synthesized as a 82 kDa protein and becomes post-translationally processed to a 27 kDa FS protein most likely during spermiogenesis [18]. Previously, it has been shown by Western blot and immunofluorescence analysis that the processed 27 kDa mature TSGA10 is located in the FS [18]. The localization of the rest of the protein after the separation of the mature 27 kDa TSGA10, however, has not been described so far. Using peptide antibodies we show that the C-terminal part of TSGA10 is located in the midpiece of mouse and boar spermatozoa. Most interestingly, a co-localization of HIF-1α with TSGA10 could be detected in the flagellum using an anti-C-terminal TSGA10 antibody. For a long time the FS has been regarded just as structure without regulatory function. The importance of the FS for several signal transduction pathways besides its mechanical function has been described recently, including the anchoring and modulation of protein kinase A, glycolytic enzymes as well as components of the Rho signaling pathway [24,25]. The importance of the FS for adequate sperm function is also exemplified by male infertility due to dysplasia of the fibrous sheath [26]. Regarding our data, HIF-1α can be added as a signal transduction molecule which might be regulated by a flagellum protein.

In the present study we demonstrated that TSGA10 is directly interacting with HIF-1α and influences HIF-1 transcriptional activity by anchoring HIF-1α partly into the cytoplasm. Both proteins are expressed during the postmeiotic stages of spermiogenesis [16,18]. By using a genetically altered mice, we also provided the first demonstration that mHIF-1αI.1 is the predominant isoform in spermatozoa, whereas mHIF-1αI.2 is ubiquitously expressed. In these mice the testis-specific first exon of the mHIF-1αI.1 allele was deleted, while the ubiquitously expressed mHIF-1αI.2 isoform remained intact. mHIF-1αI.1 knock-out mice do not show any obvious phenotype and were normally fertile and viable. A detailed description of these mice will be provided elsewhere (D. Lukashev and M. Sitkovsky, manuscript in preparation).
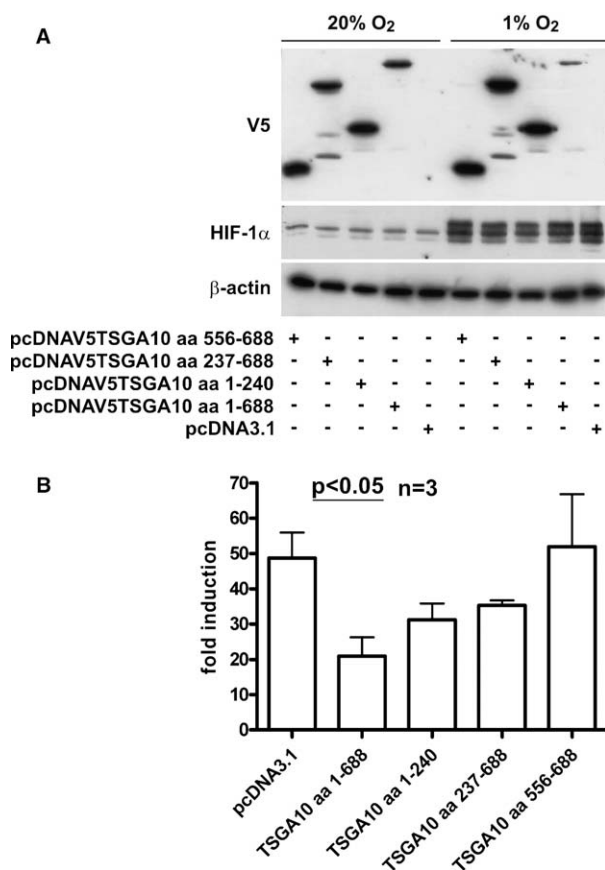

Fig. 5. Inhibition of HIF-1-dependent reporter gene induction by TSGA10. (A) The pcDNA3.1nV5 expression vector, containing or not containing TSGA10 aa 1–688, TSGA10 aa 1–240, TSGA10 aa 237–688 or TSGA10 aa 556–688, was cotransfected together with a HRE-driven firefly luciferase reporter gene and a constitutive renilla luciferase expression vector. 24 h after incubation at normoxic or hypoxic conditions cells were lysed. Protein extracts were analyzed for the expression of HIF-1α or V5-fusion proteins as described in Section 2. (B) Same extracts as described in A were used for luciferase activity assays. Shown are means ± S.D. of relative values normalized to the corresponding renilla activities.
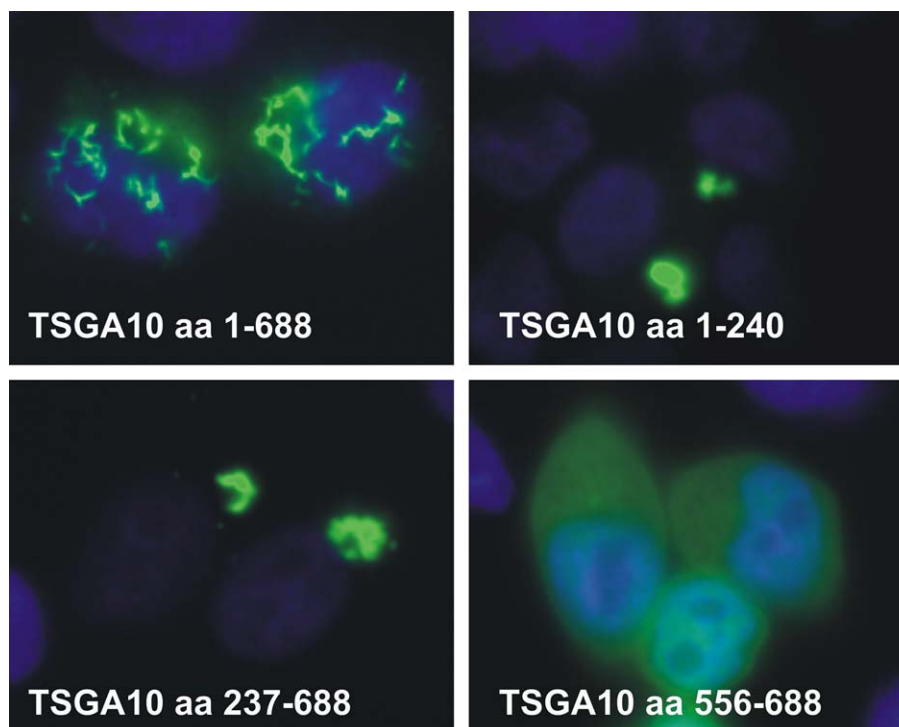
Fig. 6. Full-length TSGA10 is necessary for filament formation. HeLa cells were transfected with pcDNA3.1EGFPmTSGA10 aa 1–688, pcDNA3.1EGFPmTSGA10 aa 1–240, pcDNA3.1EGFPmTSGA10 aa 237–688, or pcDNA3.1EGFPmTSGA10 aa 556–688. After transfection, the cells were incubated for 24 h at 20% O$_2$. Cells were fixed, stained with Hoechst 33258, mounted and analyzed by fluorescence microscopy.
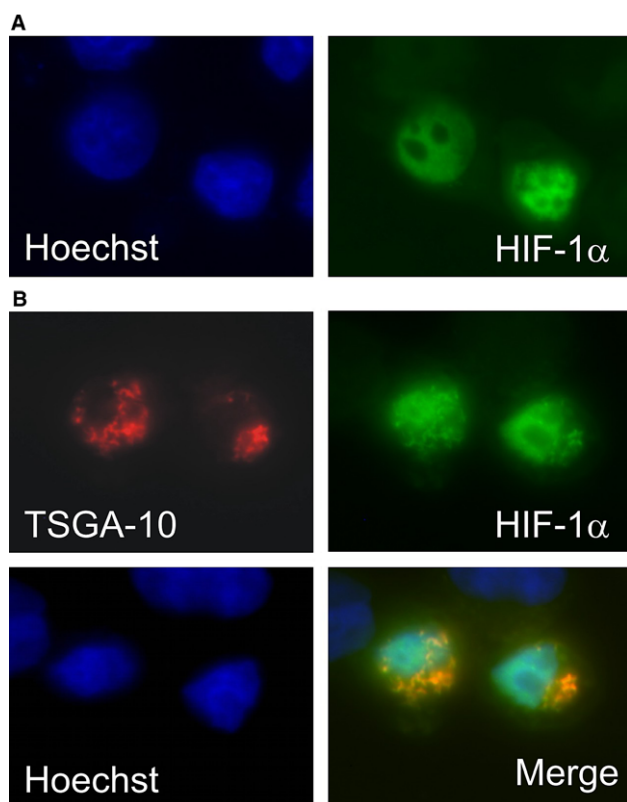

Fig. 7. TSGA10 prevents nuclear accumulation of HIF-1α. HeLa cells were transiently transfected with GFP-mHIF-1αI.1 together with the empty expression vector pcDNA3.1 (A) or together with pHcRed1mTSGA10 (B). After transfection, the cells were incubated for 20 h at 20% O$_2$ and subsequently for 4 h at 1% O$_2$. Cells were fixed, mounted and analyzed by fluorescence microscopy.

Accumulation of HIF-1α in the nucleus is based on a bipartite-type nuclear localization signal (NLS) [27,28]. Nuclear import, however, does not rely on specific regulatory mechanism [29,30]. However, preventing nuclear accumulation of HIF-1α by mutation of the NLS results in decreased transactivation activity [28]. Interestingly, in sperm cells HIF-1α is excluded from the nucleus despite the presence of a NLS [16,17]. Here we demonstrate that the fibrous sheath protein TSGA10 prevents nuclear accumulation of HIF-1α by binding it to its filaments, resulting in decreased HIF-1α transactivation activity. Since TSGA10 is expressed in postmeiotic spermatozoa, this mechanism may be involved in organ-specific regulation of hypoxic gene expression during sperm maturation. Conditions, in which oxygen supply for the testis is limited like ischemic injury or varicocele, have been demonstrated to increase HIF-1α expression in the rat testis [31,32]. Future studies have to address under which conditions the protein interaction between TSGA10 and HIF-1α plays a functional role especially under the consideration that nuclear and chromatin structure in spermatozoa are quite different from somatic cells.

Besides testis recently TSGA10 expression was also described for different tumor entities like hepatocellular carcinoma, ovarian cancer, bladder cancer, T-cell lymphoma and lymphoblastic leukemia [33–35]. Moreover, the C-terminal 55 kDa TSGA10 has been found in different organs like retina, brain, kidney and heart. Therefore, TSGA10 mediated regulation of HIF-1 activity may also play a role in those malignancies and in tissue specific regulation of HIF-1 activity.

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