Functional expression and localization of HOPS/TMUB1 in mouse lens

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ABSTRACT

Transparency represents the functional phenotype of eye lens. A number of defined steps including quiescence, proliferation, migration and cell differentiation culminates in cell elongation and organelle degradation, allowing the light to reach the retina. HOPS/TMUB1 is a nucleo-cytoplasmic shuttling protein, highly expressed both in vivo and in vitro proliferating systems, bearing an ubiquitin-like domain. The present study shows HOPS expression during the phases of lens cell proliferation and fiber differentiation, and its localization in lens compartments. In lens, HOPS localizes mainly in the nucleus of central epithelial cells. During mitosis HOPS/TMUB1 shuttles to the cytoplasm and returns in the nucleus at the end of mitosis. The differentiating cells share distinct HOPS/TMUB1 localization in transitional zone depending on the differentiation phases. HOPS/TMUB1 is observed in lens cortex and nucleus. Here, it is attached to fibers, having a structural function with crystallin proteins, probably acting in the ubiquitin-proteasome system.

Keywords
HOPS/TMUB1, eye, lens, differentiation, UBL modifier, proliferation.
INTRODUCTION

HOPS (Hepatocyte Odd Protein Shuttling) or TMUB1 (Trans Membrane Ubiquitin-like containing protein 1), —hereafter HOPS— is a shuttling protein with an ubiquitin-like domain (UBL), three transmembrane domains and a proline-rich domain. In the nucleus of quiescent cells HOPS appears mainly as speckles and dots. Hops cDNA translates three different proteins with distinct molecular weights. The analysis of Hops coding sequence showed a first methionine encoding for a 245-amino acid (Aa) isoform of 27 KDa (long-HOPS, l-HOPS) and an alternative methionine, at 55 amino acids, acting as a second starting point, translating a 21 KDa isoform (short-HOPS, s-HOPS). A signal-peptide site at the N-terminus —a putative cleavage site— defines the 24 KDa intermediate form (intermediate-HOPS, i-HOPS) (1).

Originally, HOPS gene was identified as expressed in liver regeneration induced by partial hepatectomy (PH). During the first steps of proliferation HOPS migrates from nucleus to cytoplasm to be back into the nucleus at the end of mitosis (2). Many growth factors and molecules controlling proliferation of residual hepatocytes affect HOPS export. EGF and cAMP play an important role in HOPS nucleus/cytoplasm shuttling. HOPS export to the cytoplasm is CRM1-mediated (2). In tumour cells, HOPS overexpression acts as a suppressor of proliferation (2). HOPS localizes in centosome and its depletion leads to supernumerary centrosomes, abnormal spindles, multinucleated cells, and genomic instability in NIH-3T3 cells. HOPS depletion drastically reduces mitotic figures and arrests the cells in G0/G1 phase (3). Moreover, it has been demonstrated that HOPS affects p19Arf stability acting as a bridging protein in NPM-p19Arf interaction (4). Recently, HOPS has been identified as a regulator of cytoplasmic p53 function and fate. HOPS is involved in p53 stabilization, p53-mediated mitochondrial apoptosis and p53 nuclear import. This evidence suggests that HOPS acts as potential tumour suppressor by its Ubiquitin-like Domain, (5-7).

The lens is a transparent, avascular and innervation devoid organ, enclosed in a collagenous capsule, sited between cornea and retina (Figure 1A). In the eye, it guarantees transparency and refractive power. The lens is exposed to environmental insults such as U.V. light, smoke and other agents that can damage its structure, leading to cataract formation (8, 9).

The lens is considered a very good and simple experimental model to study the protein connections, simultaneously in cellular quiescence, proliferation, differentiation and apoptosis systems (10-12).

The lens epithelium is subdivided into three distinguish zones (Figure 1B): the anterior central zone (CZ), constituted of quiescent and undifferentiated cells with few organelles; the germinative zone (GZ) —a narrow zone underlying the ciliary body— defined by proliferating cells; the transitional zone (TZ) —near the equator plane— in which, lens postmitotic epithelial cells begin to differentiate in lens fibers, reserving transcription and translational activity (13).
Here, using the lens as physiological model, we observed that HOPS localization changes in different lens compartments relating to cell cycle and differentiation, and we evaluated its role in terminal phase of fibers differentiation. We assume that HOPS might play an important role in controlling proliferation and differentiation of lens epithelial cell.

METHODS

Animals

One month old SVJ 129 mice (Charles River Laboratories, Milan, Italy) were housed in animal house facility of University of Perugia. The mice were maintained in a pathogen-free barrier area on standard 12h/12h light/dark cycle with ad libitum food and water in ~25°C and 40–60% humidity. Untreated and treated animals were sacrificed by cervical dislocation. For EGF treatment, mice were sacrificed at 0, 30 and 60 minutes after intraperitoneal injection of 10 μg/gr body weight of EGF (Sigma Aldrich, St. Louis, MO, USA). For each experimental point, the lenses were collected and O.C.T.–embedded for immunofluorescence analysis (14, 15).

Isolation of lens epithelium

Eyes were explanted and quickly included in Tissue-Tek® O.C.T. Compound (Sakura Finetek, Torrance, CA, USA). Eye was sectioned by coronal incision in the sclera. Retina and vitreous were discarded, the posterior side of the lens was exposed and the suspensory ligaments were severed to release the lens. For lens epithelium whole mounts, the lens was placed with capsular side down on a Superfrost-Plus™ slide (Thermo Fisher Scientific, Waltham, MA, USA). Fiber mass with posterior capsule was discarded. The slides were dried at 30°C and stored at -80°C (10, 16).

Primary lens epithelial cells (LEC)

For primary lens epithelial cell (LEC) cultures, the epithelia explants with the capsular side downwards, were placed in a 35 mm Petri dishes containing DMEM medium with Foetal Bovine Serum (FBS, EuroClone, Milan, I). LEC were placed in a 37°C incubator, 5% CO₂ for 14 days, to allow epithelial cells to spread to Petri dishes. The preparations were cultured until epithelial cells recolonized the cell-denuded areas of the lens capsule and migrated from the lens capsule onto the dish. At confluence, cells were collected and 1 X 10⁶ cells were plated in 60 mm dish in differentiating medium, containing 100 ng/ml of b-FGF (Sigma-Aldrich). The culture medium was changed every 3 days until the monolayer confluence (17, 18).
Histological analyses and immunofluorescence

Lens epithelium whole mount and lens cryosection (7 μm) were performed as previously described (2). Anti-vimentin and anti-γ-tubulin (Sigma Aldrich) primary antibodies and Cy3 goat anti-rabbit (H+L) and Alexa 488 goat anti-mouse (H+L) secondary antibodies (Thermo Fisher Scientific) were used. DNA was DAPI stained (Sigma Aldrich). Images were captured with a Zeiss Axioplan fluorescence microscope controlled by a Spot 2-cooled camera (Diagnostic Instruments, MI, USA).

Cell proliferation analysis.

BrdU (Sigma Aldrich) was intraperitoneal injected in mice at a concentration of 1.5 mg/30 gr body weight. After 1 hour, mice were sacrificed and lens epithelium whole mount was isolated and treated as previously described (2). Proliferating cells were revealed with anti-BrdU antibody (Abcam, Cambridge, UK) and Alexa 488 goat anti-mouse (H+L) (Thermo Fisher Scientific) as secondary fluorescent antibody (19).

Western blot analyses.

Whole eye and dissected iris, cornea, retina, optic nerve, lens, lens epithelium, cortex and nucleus were immediately homogenized in Laemmli 1X buffer and boiled at 95°C for 5 minutes for protein analysis. The samples were sonicated and the supernatants stored at -80°C. Cortex and nucleus were separated from lens mass and homogenized in Tris-HCl 10 mM pH 7.4 + PMSF + PIC (Sigma Aldrich) and sonicated. Then the samples were centrifuged at 100000 g for 10 minutes at 4°C. The resulting pellets were resuspended in the same buffer and centrifuged at 10000 g for 10 minutes at 4°C. The pellets were then dissolved in Urea 7M (Sigma Aldrich) in 10 mM Tris-HCl pH 7.4 and centrifuged twice at 100000 g for 10 minutes at 4°C. The final pellets were dissolved in Tris-HCl pH 7.4 10 mM, sonicated and stored at -80°C (20).

Extracted proteins were subjected to Western blot analysis with anti-HOPS polyclonal antibody (2) and the appropriate HRP conjugated secondary antibody (Bio-Rad, Hercules, CA, USA) and visualized with ECL (GE Healthcare Life Sciences, Little Chalfont, UK).

RNA extraction and qPCR

RNA extraction and qPCR were performed as previously described (21, 22). Total RNA was extracted from lens epithelium, cortex and nucleus using TRIzol® reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. cDNA was reverse-transcribed from 1 μg of RNA using the iScript™ kit (Bio-Rad). qPCR was performed by SYBR® Green qPCR Master Mix (Thermo Fisher Scientific). Primer sequences used for Hops detection are: Hops Forward 5’-
GCCTCAGGACACCATTGG-3’; Hops Reverse 5’-CTAGCAGTTGACCTTGGTAGATG-3’. The relative amount of mRNA was normalized to Gusb gene.

Statistical analyses
Analyses were performed using the Excel software and presented as means ± SEM. Images are representative of three experiments at least performed in triplicate.

RESULTS
HOPS is expressed in lens epithelium
HOPS is expressed in all tissues examined (1). So far, HOPS in the eye has been detected only in retina (Protein Atlas https://www.proteinatlas.org). To evaluate and verify its presence in all lens components, we dissected and extracted proteins from the eye compartments and analysed HOPS protein expression through Western blotting analysis. HOPS expression was investigated in: iris, lens epithelium and whole lens, cornea, optical nerve, retina, lens nucleus, cortex and whole eye. Except for lens nucleus and cortex, we found the three HOPS isoforms in all the compartments examined (Figure 1C left). To assess whether HOPS absence was determined by technical aspects – due to difficult extraction of fiber proteins, attributable to the intricate intertwine between the proteins in the fibrous structure– or to lack of HOPS in the fibers, we performed protein extraction from lens nucleus and cortex using high salt concentration and strong chaotropic agent. The data obtained indicated that HOPS is present in lens fibers and is tightly linked to its structure (20) (Figure 1C right).
In addition, qPCR analysis confirmed the presence of Hops transcripts in the epithelium, cortex and nucleus lens (Figure 1D).

HOPS localization in different compartments of the lens cells
Once validated HOPS expression in the eye compartments, we focussed our attention on studying HOPS localization in different zones of the lens cells. As described above the lens represents a very interesting experimental model to analyse, in a single structure, cell quiescence, proliferation and differentiation. Because HOPS shuttles from nucleus to cytoplasm and vice versa, depending on cell cycle progression and stress stimuli, we analysed HOPS localization in different regions of lens in relation to proliferating and/or differentiating areas.
HOPS distribution was analysed in whole mount epithelium. HOPS appears mainly in nucleus in quiescent cells of the central area. In GZ (Figure 2A), characterized by the presence of proliferating
cells, HOPS assumes a perinuclear and cytoplasmic localization while, in TZ, HOPS is mostly cytoplasmic (Figure 2B).

The three different regions of the lens epithelium were analysed by transverse cryosections observation. In CZ, cells are quiescent and HOPS is highly expressed in the nucleus. Moving towards GZ we observed that HOPS is mainly localized in the perinuclear zone. Proceeding to TZ, where the differentiation of cells into fibers differentiation takes place, the localization of HOPS is mainly cytoplasmic (Figure 2C).

Identification of GZ by BrdU

To assess HOPS localization in the lens epithelium and to better identify the mitotically active cell population associated to GZ, we injected BrdU in the mice as marker for replicating cells, before proceeding to dissection. Indeed, the mitotic index in adult lens is very low and GZ is restricted in a small district near the equatorial zone (13).

In germinative zone of whole mount specimens, HOPS is mainly localized in the cytoplasm, according to the proliferative state of the cells. The analysis of GZ with BrdU positive cells identifications showed a diffuse localization of HOPS, mainly perinuclear and cytoplasmic, concerning to the specific cell cycle phase (Figure 3A).

The data obtained confirmed that, at the beginning of the proliferative program, HOPS progressively leaves the nucleus to migrate to the cytoplasm while in proliferating cells HOPS resides in the cytoplasm.

Identification of TZ by Vimentin

Vimentin is an abundant intermediate filament protein of lens epithelium and superficial cortex whose distribution and structure have been shown to be zone specific (23).

Vimentin was used to identify germinative and transition zone in the lens epithelium. In GZ Vimentin takes the form of defined basket-like structures that persist in the TZ median cells. These structures are no longer visible in the sidelong cells of TZ (14).

The obtained data, analysing epithelium whole mount, show that in the GZ, HOPS assumes a perinuclear localization, while in the TZ it becomes predominantly cytoplasmic (Figure 3B).

Moving from GZ towards the equatorial edge, epithelial dividing cells begin the differentiating program into fiber cells. HOPS detection at equatorial rim showed a group of proliferating cells, where HOPS is retained in cytoplasm. Furthermore, we used vimentin as marker of lens cell differentiation. In the same fields, Vimentin showed the classical fiber-like aspect, indicating a cell switch towards differentiation (24).
In GZ, the analysis carried out in transverse sections confirms perinuclear and cytoplasmic HOPS localization and highlights its colocalization with vimentin at fibers in cortex and nucleus regions (CR and NR). (Figure 3C). The data obtained from immunofluorescence analysis, qPCR and Western blotting, tightly confirm the presence of HOPS also at the level of the cortex fibers and the nucleus of the lens (Figures 1C and D).

**EGF treatment**

Since HOPS localization in lens is determined by cell cycle phases, we assessed whether HOPS shuttling was sensitive to growth factors. As many researchers assessed the role of growth factors in driving lens differentiation (14, 15), we injected EGF in mouse in order to evaluate HOPS localization.

Of note, just 30 minutes after injection, EGF treatment induced HOPS migration to cytoplasm even in CZ, showing a dynamic and sensitive HOPS shuttling in proliferating cells arrested among exposure to EGF. 60 minutes after EGF inoculation, HOPS has moved back to nucleus (Figure 4A).

**HOPS in the lens primary cell culture**

To evaluate HOPS behaviour during differentiation, we examined HOPS localization in the lens primary cell cultures. The cell culture is able of mimicking lens development as it occurred *in vivo*, forming lens-like structures known as lentoids. Lens primary cell cultures show many biological characteristics similar to the *in vivo* differentiated cells of the lens. Use of primary cell cultures allowed to observe lens cell differentiation *in vitro* (25).

To this end, we prepared lens primary cells from mice eye explants. We induced cells to differentiate by adding FGF to the culture medium. Keeping cells in culture with the same culture medium for several days, differentiation areas were identified by the presence of the lentoid bodies (Figure 4B) (26).

HOPS localization was observed in both proliferating and differentiating cells of lens epithelial cells using γ-tubulin as marker (27). In proliferating cells, far from lentoids, HOPS was revealed both in the cytoplasm and in the nucleus depending on the cell cycle phase (Figure 4C). Instead, in differentiating cells, HOPS was associated to the lens fibers. These results agree with the *in vivo* data (Figure 4D).

**DISCUSSION**
The lens is a simple experimental model to study cellular processes in a single structure, presenting simultaneously cells in quiescence, proliferation and differentiation state. Indeed, all these events are well separated in distinct lens compartments.

As previously published, we describe HOPS as a shuttling protein that moves from nucleus to cytoplasm depending on cell cycle phases both in regenerating liver and tumour cells (2). Recently, we highlighted that HOPS ability to act as tumor suppressor not only relies on its expression, but also on its localization. In nucleus HOPS overexpression stabilizes p19\(^{A\text{rf}}\), which in turn activates p53 that arrests the proliferation (4). Interestingly, similar results have been observed in lens. Here, we show for the first time HOPS in the nucleus of quiescent epithelial cells of CZ, while in GZ and TZ –proliferative and differentiative regions respectively– HOPS is prevalent in cytoplasm. Since now no results have been published about HOPS localization in differentiating cells. The lens offers an excellent model to study HOPS localization in differentiation. Our results indicate that during cell differentiation HOPS shuttles from cytoplasm to nucleus, inducing proliferative arrest and starting differentiation. These data agree with the results obtained in vitro using cultured lens epithelial cells where the progressive accumulation of HOPS in nucleus is accomplished with cell differentiation.

An important role in controlling proliferation and differentiation is played by p53. Indeed, p53 controls proliferation by activating p21 (28), and differentiation by triggering apoptotic signalling in the programmed removal of organelles from differentiating lens fiber cells (29).

This apoptotic program is fundamental to sustain lens transparency through formation of an organelle-free zone. We can speculate that HOPS localization in nucleus and the differentiation of lens cells are accompanied by a progressive arrest of proliferation, which triggers the differentiation. Recently, we demonstrated that HOPS UBL plays a notable role, as protein modifier, regulating p53 stability and triggering the p53-mediated apoptosis (5). In such a complex structure as lens is the role of protein modifiers and ubiquitin is fundamental and the machinery involved in proteasomal pathway execution is strategic in the shift from proliferation to differentiation (30, 31).

In this study we demonstrate that HOPS is engaged in regulating proliferation and differentiation of the lens texture having a possible role, as UBL modifier, in the final constitution of the differentiated structure of the lens. Together with the crystallin superfamily and the other lens proteins, HOPS may contribute to warrant the transparency of the structure by regulating various proliferative or apoptotic events to avoid the cataract formation (10, 32).
Data Availability
The authors agree to make any materials, data, code and associated protocols (relating to their published research) available to bona fide researcher or reader requests without undue delay or qualifications.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

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Author contributions
D.B., D.P., M.C., S.P. and M.A.D-F performed the in vivo experiments, D.S., S.F. and N.D-I. performed the experiments and analysed the data. D.B., M.A.D-F. and G.S. designed the study, analysed the data and wrote the manuscript.

Ethics Approval
The study was carried on in accordance with ethical principles of the latest version of the Declaration of Helsinki. All animal experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines (EU Directive 2010/63/EU for animal experiments). All experiments involving animals were done according to the guidelines of the University of Perugia Ethical Committee and the European Communities Council Directive 2010/63/EU.

Abbreviations
HOPS: Hepatocyte Odd Protein Shuttling; TMUB1: Trans Membrane Ubiquitin-like containing protein 1; UBL: ubiquitin-like; PH: partial hepatectomy; b-FGF: basic Fibroblast growth factor; EGF: epidermal growth factor; cAMP: cyclic AMP; BrdU: Bromo Deoxyuridine; CRM-1: Chromosome Region Mantainance-1; EF-1A: elongation factor 1A; HSP70: Heat Shock protein 70; NPM: nucleophosmin; CZ: central zone; GZ: germinative zone; TZ: transitional zone; LEC: lens epithelial cell; CR: central region; NR: nuclear region.
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Figure 1. Mouse eye and lens structure

(A, B) Mouse eye (A) and lens (B) schematic overview with main components indication. (C) WB analysis of HOPS protein expression in eye and lens compartments (left). The fibers proteins of lens cortex and nucleus obtained upon Tris-HCl (lane 1, 2) and urea treatment (lane 3, 4) evaluated by WB (right). Ponceau S staining was used to evaluate protein amount. Images are representative of one mouse eye proteins. (D) qPCR showing Hops-mRNA in lens epithelium, cortex and nucleus. mRNA in lens epithelium was assumed as 1 and samples were normalized to Gusb gene. Representative images are shown.

Figure 2. HOPS localization in mouse lens epithelium

(A) Graphical overview of mouse lens transverse sections (dashed lines) and whole-mount view (blue bold arrow) displayed in (B) and (C). (B) Lens epithelium whole-mount showing HOPS (red) cellular localization. Nuclei were DAPI stained (blue). Bars, 10 μm. (C) Lens transverse sections at the CZ, GZ and TZ evidenced HOPS localization (red) in the lens epithelium. Nuclei were DAPI stained (blue). For each zone, images at two magnifications are shown. Bars, 10 μm (left) and 5 μm (right). Merged images are shown. Representative images are shown.

Figure 3. HOPS localization in nuclear and cortical regions of the lens epithelium

(A) HOPS (red) cellular localization in whole-mount lens epithelium specimens of BrdU treated mice. BrdU (green) was used as proliferation marker to discriminate GZ from CZ. Nuclei were DAPI stained (blue). Bars, 10 μm. For each panel an image insight is reported on the right. (B) HOPS cellular localization (red) observed in whole-mount lens epithelium. Vimentin (green) was used as marker to identify TZ. Nuclei were DAPI stained (blue). Bars, 5 μm. (C) Lens transversal sections at GZ were analysed for HOPS localization (red) in nuclear (NR) and cortical (CR) regions. Vimentin (green) was used to identify cells layer and fibers of the lens epithelium. Nuclei were DAPI stained (blue). Bars, 5 μm. Merged images are reported. Representative images are shown.

Figure 4. HOPS localization in LEC

(A) HOPS (red) nucleo-cytoplasmic shuttling was observed after EGF treatment. Nuclei were DAPI stained (blue). Bars, 5 μm. (B, C, D) HOPS localization (red) in differentiating lens primary cell cultures mimicking in vivo development. HOPS immunolocalization was analysed in lentoid (B), proliferating (C) and differentiating cultured cells (D). Nuclei were DAPI stained (blue). γ-tubulin
(green) was used as marker. Bars, 200 \( \mu m \) in (B) and 5 \( \mu m \) in (C) and (D). Merged images are reported. Representative images are shown.
