Nuclear interaction of the dynein light chain LC8a with the TRPS1 transcription factor suppresses the transcriptional repression activity of TRPS1

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The TRPS1 gene codes for a 1281 amino acids nuclear transcription factor with an unusual combination of different types of zinc finger motifs, including GATA-type DNA-binding and IKAROS-like zinc fingers. TRPS1 is a repressor of GATA-regulated genes and implicated in the human tricho-rhino-phalangeal syndromes. We found that two distinct regions of TRPS1 can physically interact with the dynein light chain 8 protein, LC8a, that are at least 458 amino acids apart from each other. Region A covers 89 amino acids (635–723), spanning three potential C₂H₂ zinc finger structures, and region B covers the 100 most C-terminal amino acids (1182–1281) containing the IKAROS-like motif. LC8a is known to interact with more than 10 different molecules, both proteins and nucleic acids. In most cases, LC8a was identified as a transport molecule in the cytoplasm. Interestingly, we found that LC8a co-localizes with TRPS1 in dot-like structures in the cell nucleus. In an electrophoretic mobility shift assay we could show that the interaction of LC8a and TRPS1 lowers the binding of TRPS1 to the GATA consensus sequence. In addition, GATA-regulated reporter gene assay indicated that LC8a is able to suppress the transcriptional repression activity of TRPS1.

INTRODUCTION

The TRPS1 gene on human chromosome 8q24.1 encodes the nuclear transcription factor TRPS1. It is the only known transcription factor that has nine potential zinc finger motifs of four different types (1). The first three motifs share a C₂X₁₄H₂ consensus sequence, and motifs 4, 5 and 6 share a C₂X₁₂H₅ consensus sequence. The function of these zinc finger domains is still unclear. The seventh is a C₂C₂-type zinc finger resembling the CXNCX₁₇CNXC consensus sequence. It has been shown that this domain is able to specifically bind to the GATA consensus sequence (2,3). The two C-terminal C₂H₂ zinc finger motifs share a high degree of similarity to the C-terminal zinc fingers of the IKAROS family of transcription factors. In IKAROS, these fingers are known to mediate protein–protein interaction between the family members to form homo- and heterodimers (4). Malik and colleagues performed the first functional studies on TRPS1 (2). They reported that TRPS1 exerts a transcriptional repression activity on GATA regulated genes. This activity is dependent on the presence of the C-terminal IKAROS-like zinc fingers. TRPS1 itself does not seem to have a transcription activation capability.

Mutations in or complete deletions of the TRPS1 gene cause the tricho-rhino-phalangeal syndromes. They are characterized by craniofacial and skeletal abnormalities. Three subtypes have been described. TRPS I is caused by deletions or nonsense mutations in TRPS1. TRPS II is a microdeletion syndrome affecting both the TRPS1 and EXT1 genes. The third form, TRPS III, shows severe brachydactyly due to short metacarpals and severe short stature, but without exostoses, and correlates with missense mutations in TRPS1, which exclusively affect the GATA DNA-binding zinc finger. These mutant TRPS1 proteins probably have a decreased affinity to DNA due to the altered GATA zinc finger structure and exert a dominant negative effect as a component of a multimeric...

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protein complex, in contrast to haploinsufficiency supposed for the remaining mutations (5).

Transcription factors, activators as well as repressors, enter into a variety of protein–protein interactions in transcription complexes in carrying out their biological functions. Here, we describe the identification of LC8a as the first interacting partner of the TRPS1 transcription factor. To date, LC8a has been mainly implicated in several cytoplasmic transport processes (6). We provide evidence for a nuclear function of LC8a and demonstrate that the interaction of both proteins reduces the binding of TRPS1 to the GATA consensus sequence and decreases the transcriptional repression activity of TRPS1.

RESULTS

Identification of potential TRPS1 binding proteins

We used the lexA yeast two-hybrid system (7,8) to identify proteins which interact with the TRPS1 transcription factor. Because a well-characterized mouse embryo cDNA expression prey library constructed by Hollenberg and colleagues (9) was available, we decided to use the mouse orthologue Trps1, which is 93% identical to the human TRPS1, as bait. The prey library contains size-selected (350–700 bp) cDNA fragments from 9.5 to 10.5-day-old whole mouse embryos fused to sequences encoding the lexA DNA-binding domain. Trps1 amino acids 635–1281 were fused to the lexA DNA-binding domain to yield the lexA–Trps1–F5 fusion protein (Fig. 1B). The bait plasmid encoding this fusion protein was transfected into the yeast strain L40 and the expression of lexA–Trps1–F5 was detected by western blot analysis using an anti-lexA antibody (data not shown). The yeast clone with the highest bait expression level was selected for transfection with the VP16 library. We obtained 23 yeast clones, which were able to grow in the absence of histidine, indicating an interaction between lexA–Trps1–F5 and a VP16 fusion protein. Twelve of these clones carried prey plasmids encoding the full length open reading frame of type a of the dynein light chain 8, Lc8a (6). Three out of 12 carried an insert of 369 bp and nine out of 12 were 571 bp in size. The other 11 clones represented five different proteins (data not shown) which are currently under investigation.

Verification and regional assignment of the Trps1–Lc8a interaction

To verify that Trps1 and LC8a indeed interact and to narrow down the interaction region in the Trps1 protein, we performed another yeast in vivo assay. For this purpose, we used the original lexA–Trps1–F5 bait construct (see above) and designed three additional constructs coding for truncated Trps1 proteins. Fragment F6 (Trps1 amino acids 635–1184) lacks the IKAROS-like double zinc finger motif. Trps1 amino acids 635–1281 were fused to the carboxy terminal end of the lexA DNA binding domain to yield the lexA–Trps1–F5 fusion protein (Fig. 1B). The bait plasmid encoding this fusion protein was transfected into the yeast strain L40 and the expression of lexA–Trps1–F5 was detected by western blot analysis using an anti-lexA antibody (data not shown). The yeast clone with the highest bait expression level was selected for transfection with the VP16 library. We obtained 23 yeast clones, which were able to grow in the absence of histidine, indicating an interaction between lexA–Trps1–F5 and a VP16 fusion protein. Twelve of these clones carried prey plasmids encoding the full length open reading frame of type a of the dynein light chain 8, Lc8a (6). Three out of 12 carried an insert of 369 bp and nine out of 12 were 571 bp in size. The other 11 clones represented five different proteins (data not shown) which are currently under investigation.

Figure 1. Predicted structures of the human and mouse TRPS1 proteins (A) and of the constructs used for yeast two-hybrid, β-galactosidase and GST pull-down assays (B). (A) The human and mouse TRPS1 proteins are over 91% identical in their amino acid sequence. TRPS1 has 1281 amino acids with nine putative zinc finger motifs. Zinc fingers are indicated by black boxes. The seventh zinc finger has a high homology to GATA-type DNA-binding zinc fingers. The nuclear localization signal (NLS, shadowed) is located C-terminally to the GATA zinc finger. At the C-terminus, there are two C2H2 zinc fingers with a high degree of similarity to the IKAROS-like double zinc finger domain. (B) The Trps1 fragments fused to the lexA DNA-binding domain are illustrated. Fragment F5, containing zinc fingers 4–9, was used for the yeast two-hybrid assay. Fragments F6, F9, F10, F1 and F13 were used in β-galactosidase assays to verify the yeast two-hybrid results and to narrow down the Trps1 interaction domain. Fragments F9 and F10 were also used for in vitro GST pull-down assays (see Fig. 3).
inserted into the lexA bait plasmid to yield constructs lexA–Trps1–F6, lexA–Trps1–F9 and lexA–Trps1–F10 (Fig. 1B). As a reporter construct, we used the full-length murine Lc8a cDNA fused in frame to the VP16 coding part in a galactose-inducible vector (VP16–Lc8a). Each specific bait construct was co-transfected with VP16–Lc8a or with the lexA–F5 construct, alone, did not activate lac–Z gene expression. Because we had suspected an interaction between the Ikaros-like zinc fingers and Lc8a, we used two additional Trps1 constructs for further analysis (F1 and F13, experiment B). The fragment F1 shows an interaction whereas F13 does not. (B) These results indicate that Lc8a interacts with two distinct regions of Trps1. Region A spans Trps1 amino acids 635–723, containing three potential zinc finger (zf 4–6) motifs, and region B (amino acids 1182–1281), which contains the Ikaros-like zinc fingers.

We had expected that the Ikaros-like zinc finger domain would present a moiety to interact with other proteins. To test this hypothesis and to narrow down the already identified region of interaction, we engineered two additional constructs, lexA–Trps1–F1 and lexA–Trps1–F13. F1 codes only for the 100 most C-terminal amino acids (1182–1281) of Trps1 including the Ikaros-like motif, and F13 covers 458 amino acids (724–1181) between zinc fingers 6 and 8 containing the Gata DNA-binding zinc finger and the nuclear localization signal (NLS; Fig. 1). Interaction potentials of constructs lexA–Trps1–F1 and lexA–Trps1–F13 were investigated in β-galactosidase assays as described above. As expected, fragment F1 interacts with Lc8a, whereas fragment F13 does not (Fig. 2A, experiment B). These results indicate that Lc8a interacts with two distinct regions of Trps1. Region A spans amino acids 635–723, containing three potential zinc finger (zf 4–6) motifs, and region B (amino acids 1182–1281), which contains the Ikaros-like motif (Fig. 2B).

In order to further verify that Lc8a interacts with Trps1, we performed an in vitro assay. For this purpose, we cloned the Trps1 fragments F9 and F10, which contain the interaction region A into the expression vector pCDA3. Unfortunately, we could not obtain expression plasmids representing fragments F1 or F5, which encode interaction region B. The entire open reading frame of Lc8a was cloned into the bacterial expression plasmid pGEX-4T2, which codes for glutathione-S-transferase (GST–Lc8a) and the Trps1 fragments F9 and F10 were synthesized in vitro and radiolabelled by Promega’s TNT reticulocyte lysate. The GST–Lc8a fusion protein was coupled to a sepharose matrix and incubated with the labelled Trps1 fragments F9 or F10. After washing, proteins were released from the sepharose beads, separated by SDS–PAGE, and exposed to an X-ray film. Lanes 1 and 4 identify fragments F9 and F10 as Lc8a binding partners in these eluates. Lanes 3 and 6 aliquots of the in vitro syntheses are separated for comparison. To exclude unspecific protein pull down by GST or the sepharose matrix, we incubated the Trps1 fragments F9 and F10 with GST coupled sepharose. Lanes 2 and 5 show that neither F9 nor F10 interact with this matrix.

**TRPS1–Lc8a interaction in eukaryotic cells**

The yeast two-hybrid and all other assays described above were performed with the mouse orthologues Trps1 and Lc8a. For further analyses, we used the human TRPS1 and Lc8a. The entire coding sequences of TRPS1 and Lc8a were cloned into green fluorescent protein (GFP)- or FLAG-expression plasmids, respectively, to obtain the fusion proteins...
TRPS1 and LC8a interact in the cell nucleus

Since TRPS1 is a nuclear protein but LC8a was first described to reside in the cytoplasm (10), we wanted to determine precisely the intracellular localization of both proteins and the localization of their interaction. For this purpose, we analysed primary human fibroblasts by confocal laser scanning microscopy (CLSM). TRPS1 was detected by using a rabbit anti-TRPS1 antiserum and a purified secondary, Alexa-tagged anti-rabbit antibody. Each analysed cell showed strong TRPS1 signals in the nucleus (Fig. 5A). Interestingly, TRPS1 localizes in large dot-like structures, which varied numerically between one and four per cell. TRPS1 could also be detected in the cytoplasm, but to much lesser extent than in the nucleus. This is in agreement with the results of the western blot experiment (Fig. 4D). We used the rat anti-LC8a antibody and a purified, Cy3-labelled secondary anti-rabbit antibody to visualize the LC8a protein. Each cell showed a homogeneous distribution of LC8a in the cytoplasm (Fig. 5B). Interestingly, LC8a could also be found in the nucleus of ~60% of all analysed fibroblasts. Where it was present in the nucleus, it was localized in dot-like structures like the TRPS1 protein. An overlay of the TRPS1- and the LC8a-signals illustrated that TRPS1 and LC8a are co-localized in the nucleus, but not in the cytoplasm (Fig. 5C), suggesting a function of this protein complex in the nucleus.

LC8a reduces the binding of TRPS1 to the GATA consensus sequence

Recently, it was demonstrated that the seventh zinc finger of TRPS1 binds to the GATA consensus sequence (2,3). We analysed whether the TRPS1-LC8a interaction has an influence on the DNA binding affinity of TRPS1 using electrophoretic mobility shift assays (EMSA). Sequences that contain the entire open reading frames of TRPS1 and LC8a were inserted

Figure 4. Expression and co-precipitation of TRPS1 and LC8a. Proper expression of TRPS1–GFP and LC8a–FLAG in COS-1 cells was monitored by SDS–PAGE and western blot analysis using a mouse αFLAG antibody or a rabbit αTRPS1 antiserum. (A) LC8a–FLAG expression was detectable in the presence of a GFP expressing plasmid (lane 1) or the TRPS1–GFP construct (lane 2). (B) TRPS1–GFP expression was visible in the presence of the pFLAG vector (lane 1) or LC8a–FLAG (lane 3). (C) TRPS1–GFP and LC8a–FLAG can be co-precipitated from COS-1 cells. Immunoprecipitations were performed with αFLAG antibodies. To detect TRPS1–GFP, the precipitates were analysed by SDS–PAGE and western blotting using a αTRPS1 antiserum. Lanes 1 and 2 are negative controls using empty pFLAG vector (lane 1) or LC8a–FLAG (lane 2). (D) LC8a–FLAG–pGFP double transfected cells were analysed in lane 3. The TRPS1–GFP fusion construct could be detected as co-precipitated protein from TRPS1–GFP/pLC8a–FLAG co-transfected cells (lane 4). (E) The expression of TRPS1 and LC8a in primary human fibroblasts was analysed by western blot analysis using a rabbit anti-TRPS1 antibody and a rat αLC8a antibody. (F) TRPS1 was detectable in the nucleus (lane 2) and to much lesser extent in the cytoplasm (lane 1). Each lane was loaded with 60 μg of total protein of each respective fraction. (E) The expression of LC8a (arrow) was monitored by using an αLC8a antibody. In lane 1, 50 μg of total cell extract were analysed and LC8a was detectable at the expected size. Lane 2 contains an aliquot of the LC8a IP-sediment to check if LC8a was precipitated, and lane 3 represents the supernatant to determine the efficiency of the precipitation. (F) Presence of TRPS1 (arrow) in the αLC8a IP-sediment could be proven by western blot analysis using a rabbit αTRPS1 antiserum (lane 2), whereas the additional bands could be shown as non-specific using an irrelevant rabbit antiserum (αX) in lane 1. Detected immunoglobulin chains of the αLC8a antibody (lgM) are indicated.

TRPS1–GFP and LC8a–FLAG. To investigate whether TRPS1 and LC8a interact also in eukaryotic cells, we first performed co-immunoprecipitation studies. Both plasmids were co-transfected into COS-1 cells and proper expression of the fusion constructs was monitored by western blot analyses using a commercial anti-FLAG antibody (Fig. 4A) and a rabbit anti-TRPS1 serum (Fig. 4B). To identify interaction of LC8a and TRPS1, we incubated cell extracts after transfection with a Sepharose-coupled anti-FLAG antibody. After washing, the eluates were analyzed by western blot analysis. The anti-TRPS1 antiserum identified TRPS1–GFP only in those cells, in which TRPS1–GFP was co-expressed with LC8a–FLAG (Fig. 4C, lane 4), but not in cells, in which it was co-expressed with the empty pFLAG vector (Fig. 4C, lane 2). These results indicate that TRPS1 and LC8a form stable complexes in eukaryotic cells, at least if both genes are expressed ectopically.

By using a rabbit anti-TRPS1 antiserum (SN652) (3) we were able to detect the endogenous TRPS1 in the nucleus and in a much lower amount in the cytoplasm of primary human fibroblasts (Fig. 4D). In the same cells, we detected the endogenous LC8a with a rat anti-LC8a antibody (Alexis, Fig. 4E). Therefore, we used human fibroblasts to verify the interaction of the endogenous TRPS1 and LC8a proteins. Extracts of fibroblasts were incubated with an anti-LC8a antibody, immuno-precipitated and analysed by western blot. The successful precipitation of LC8a could be verified by showing LC8a in the sediment (Fig. 4E, lane 2), whereas no signal was detectable in the supernatant (Fig. 4E, lane 3). In the same immuno-sediments, TRPS1 was detectable by using the anti-TRPS1 antiserum (Fig. 4F, lane 2), indicating that both endogenous TRPS1 and LC8a proteins form stable complexes in human fibroblasts.
LC8a suppresses the transcriptional repression activity of TRPS1

TRPS1 is able to repress GATA-dependent, XGATA4-induced luciferase reporter gene activity. The degree of repression was proportional to the amounts of the transfected TRPS1 encoding plasmid (2). We used this assay to determine whether LC8a has any influence on the repressory function of TRPS1. We transfected HepG2 cells with plasmids encoding XGATA4, TRPS1 and LC8a. As a reporter, we used the zD3 (11) construct which contains multiple repeats of the sequence AGATAA upstream of the firefly luciferase cDNA (see Materials and Methods). The luciferase activity in cells transfected with the zD3 reporter construct alone, was set as 1 (Fig. 7, column 1). Cells transfected with XGATA4 and the reporter show a 5.7-fold higher luciferase activity (Fig. 7, column 2). When TRPS1 was added, the XGATA4 induced transactivation of the reporter gene was almost completely suppressed (Fig. 7, column 3). This transcriptional repression by TRPS1 was considerably relieved when an LC8a expression construct was co-transfected (Fig. 7, columns 4 and 5). We used different amounts of LC8a to analyse whether the strength of this effect depends on the ratio of TRPS1 and LC8a. When TRPS1 and LC8a expression constructs were co-transfected in equal amounts, the repressory function of TRPS1 was strongly reduced to a luciferase activity of 4.4 (Fig. 7, column 4). A relative reduction of LC8a (60 w/w compared to TRPS1) led to a decrease of this effect (Fig. 7, column 5), indicating a dose-dependent regulation of the repressional function of TRPS1. This is in agreement with the results of the electrophoretic mobility shift assays (Fig. 6). Control transfections could exclude a trans-activational or repressional function of LC8a, alone (Fig. 7, column 7) or in combination with XGATA4 (column 6).

DISCUSSION

Transcription factors enter into a variety of protein–protein interactions in carrying out their biological functions. Since interacting partners for the recently identified transcription factor TRPS1 (1) are not yet known, we have set out to identify such molecules. Northern blot and mRNA in situ hybridization experiments had revealed that the TRPS1 gene is expressed in many different tissues (1,2,12,13). Furthermore, TRPS1 contains beside the DNA-binding GATA zinc finger—another eight zinc finger motifs which might interact with other proteins. We had therefore expected to find more than one interacting protein. In a yeast two-hybrid screen with the C-terminal half of the Trps1 protein as a bait, we have identified six different potential binding partners. We had expected homodimerization of TRPS1 because of the high degree of similarity (72%) between the C-terminal double zinc finger motifs of TRPS1 and the IKAROS protein family, which are known to mediate formation of homo- and heterodimers (4). However, we did not find a Trps1-expressing prey plasmid when using lexA–Trps1–F1 and lexA–Trps1–F5 as bait...

Figure 5. Intracellular localization of TRPS1 and LC8a. To determine the intracellular distribution of TRPS1 and LC8a, we used confocal laser scanning microscopy. TRPS1 was detected by using the rabbit zTRPS1 antiserum and a secondary, Alexa-tagged (green) antibody. LC8a was shown using the rat zLC8a antibody and an anti-rat, Cy3-labelled (red), secondary antibody. The nuclei were stained by DAPI and appear in blue. (A) TRPS1 was detectable in the nuclei of every analysed cell. It is localized in one to four dot-like structures which vary in size. A weak TRPS1 signal was also found in the cytoplasm. (B) LC8a is homogeneously distributed throughout the cytoplasm of all investigated cells. In addition, ~60% of all cells show an additional LC8a signal in the nucleus where it was found to be associated with dot-like structures similar to TRPS1. (C) An overlay of (A) and (B) revealed that the complexes between TRPS1 and LC8a are restricted to the nucleus and are not found in the cytoplasm.
constructs. Furthermore, we could not prove an interaction between these constructs and a VP16–Trps1–F1 prey construct in a β-galactosidase assay (not shown).

We have chosen LC8a to start with the analysis of interacting partners. The yeast two-hybrid results were confirmed and the TRPS1/Trps1 binding regions were narrowed down using full-length recombinant LC8a and different truncated Trps1 constructs in yeast β-galactosidase and in vitro GST pull-down assays. TRPS1 and LC8a were co-immunoprecipitated from COS-1 cell extracts overexpressing both proteins. And the interaction could be confirmed in primary human fibroblasts by co-immunoprecipitation, co-localization and DNA binding and functional assays. Thereby, our conclusion that LC8a and TRPS1 form stable complexes in the nucleus is supported by several independent lines of experimental evidence.

The 89 amino acid protein LC8a is the mammalian orthologue of the M, 8000 light chain of the Chlamydomonas reinhardtii flagellar outer arm dynein, initially found in bovine and rat brain as an essential component of the cytoplasmic dynein complex and associated with other cytoplasmic molecules (10). It is identical at least in mouse, rat, pig, cow and human (6). Synonyms for Lc8a in the literature are DLC8 (14), Dlc-1 (15), hdlc1 (16) and protein inhibitor of neuronal nitric oxide synthase, PIN (17). To date, more than 10 different molecules of extremely diverging sizes and different biological functions, with no apparent connection between them, have been found to interact with LC8a. Some of these interactions seem to be associated with minus-end directed microtubule-based intracellular transport. For instance, LC8a seems to be involved in the transport of neuronal nitric oxide synthase (nNOS) (18) and IκBα, an inhibitor of the NFκB transcription factor (15). In parathyroid cells LC8a interacts with the 3’-untranslated region of the parathyroid hormone (PTH) mRNA to translocate it along microtubules for its further processing or translation (19). Even Lyssa RNA-viruses and the African swine fever DNA-virus (ASFV) have been reported to use the cytoplasmic dynein shuttle via LC8a (20–23). Further evidence for the involvement of LC8a in transport events is its association with the chicken brain myosin-Va, an actin-based molecular motor (24).

However, LC8a does not seem to be a simple link molecule in intracellular transport. It interacts with Bim, a member of the

Figure 6. LC8a decreases the binding of TRPS1 to the GATA consensus sequence in vitro. To demonstrate the influence of the interaction between TRPS1 and LC8a on the DNA binding, we performed an electrophoretic mobility shift assay. Full-length TRPS1 and LC8a proteins were synthesized in vitro and incubated with a radioactively labelled oligonucleotide which contains the GATA consensus sequence (see Materials and Methods). Protein–DNA complexes were separated on a native polyacrylamide gel and visualized by autoradiography. Incubation of TRPS1 with increasing amounts (0.1, 0.5, 1.0 and 5.0 μl) of in vitro synthesized LC8a decreases the binding of TRPS1 to the GATA oligonucleotide (lanes 4–7) as compared to non-competitive incubation without LC8a (lanes 2 and 8). Lane 3 demonstrates the mobility shift of the TRPS1–DNA complex after addition of the TRPS1 antiserum SN652. Lane 1 shows the incubation of a control lysate with the GATA oligonucleotide. Positions of the protein–DNA complexes and free GATA oligonucleotide are indicated by arrows.

Figure 7. The transcriptional repression function of TRPS1 is suppressed by LC8a. HepG2 cells were transiently transfected with plasmids encoding a GATA-dependent luciferase reporter, the XGATA4 transcription factor, and the TRPS1 transcription factor and with different amounts of an LC8a encoding plasmid. The luciferase activity of cells transfected with the reporter construct alone was set as fold activation (FA) 1 (column 1). Co-transfection with XGATA4 results in an increase to FA 5.7 (column 2). Adding of TRPS1 reduces the luciferase activity to FA 1.4 (column 3). This TRPS1-induced repression could be partially released by the presence of LC8a. Equal amounts of LC8a and TRPS1 expression plasmids lead to an FA 4.4 (column 4), whereas lower amounts of LC8a cut down this effect to FA 3.4 (column 5). LC8a alone does not repress XGATA4-mediated luciferase activity (column 6), nor does it have any activating effect on the reporter construct (column 7).
Bcl-2 family, and is able to regulate its pro-apoptotic activity (25). LC8a also regulates the activity of neuronal nitric oxide synthase (nNOS) by modulating the phosphorylation status of nNOS (26). And finally, Herzig et al. (27) showed a nuclear interaction of LC8a with the structurally and functionally related transcription factors, the nuclear respiratory factor-1 (NRF-1) and the ecret wing gene product (EWG) of *Drosophila*.

### Analysis of the TRPS1–LC8a interaction

At a first glance, the literature provides contradictory data about the subcellular localization of the various LC8a containing protein complexes. Whereas this protein was originally described as an exclusively cytoplasmic molecule (10,16,17), Crépieux et al. (15) and Herzig et al. (27) found LC8a in both the cytoplasm and the nucleus. Since different cell types and conditions have been used in those experiments, the subcellular localization of LC8a may be dependent on cell type and the cell cycle phase. Our data show that, in primary human fibroblasts, LC8a is localized in the cytoplasm and additionally in the nucleus of ~60% of all analysed cells. When found in the nucleus, LC8a was organized in the same dot-like structures as TRPS1. Our co-localization data illustrate that the TRPS1–LC8a interaction is restricted to these structures, which indicates a nuclear function.

The LC8a binding domains of the various targets are confined to short stretches of amino acid residues. Rodriguez-Crespo et al. (28) identified two different consensus sequences in some of the LC8a binding proteins using a pepscan technique. They identified a GIQVD sequence which was recognized in the LC8a interacting region of nNOS and GKAP and a KSTQT stretch for example in Bim and swallow. Fan and colleagues (14) could also narrow down the LC8a interacting region of Bim to amino acids DKSTQT. We report here that LC8a interacts with two distinct domains of TRPS1. These two regions flank the seventh zinc finger motif of TRPS1, which has been demonstrated to specifically bind to the GATA consensus sequence (2,3). Region A (Fig. 2B) harbours the TRPS1 zinc finger motifs 4–6 but does not contain any of the identified signature motifs of LC8a interaction. However, region B (amino acids 1182–1281, Fig. 2B) includes the IKAROS-like double zinc finger and has a DRSTQ (amino acids 1206–1210) stretch which is highly similar to the minimal interacting region of Bim and swallow. Malik and colleagues (2) have shown that N-terminally truncated TRPS1 proteins, which lack the LC8a binding region A (amino acids 635–723), are still able to repress a GATA-regulated reporter gene, *in vivo*, and that this transcriptional repression activity is dependent on the presence of the 119 most C-terminal amino acids (2).

### LC8a affects the transcriptional repression activity of TRPS1

Interestingly, we found that LC8a significantly decreases the binding of TRPS1 to the GATA consensus sequence (Fig. 6), and that the presence of LC8a correlates with a reduction of TRPS1 activity as a repressor in a concentration dependent manner (Fig. 7). LC8a itself had neither a repressing effect on the XGATA4 mediated transcription (Fig. 7, column 6), nor a transactivating effect on the reporter construct (Fig. 7 column 7). Thus, the suppression of the TRPS1 function correlates with the LC8a/TRPS1 interaction and may be achieved by a conformational change of TRPS1 upon binding to LC8a. Such a chaperone-like function of LC8a has already been shown for its interaction with the intermediate chain 74 (IC74) of the dynein motor complex (29).

### Conclusion

As TRPS1 is a transcriptional repressor, the inhibition of its activity by LC8a is expected to result in the upregulation of TRPS1 target genes. These genes are still unknown, but they are likely to be involved in chondrocyte and hair follicle function. It is unclear which of the various intracellular processes involving LC8a is linked to these genes, but the Bcl-2 regulated apoptosis is an attractive candidate, because it plays a central role in bone development and hair follicle cycling (30,31). The investigation of this pathway and the identification of the target genes will be the next step in unravelling the pathogenesis of the tricho-rhino-phalangeal syndromes.

### MATERIALS AND METHODS

#### Yeast two-hybrid assay

We used *Trps1* fragments (F5) representing the 647 C-terminal amino acids of *Trps1* as a bait for the yeast two-hybrid assay. The fragment was generated by using specific primers (‘5’-CATGGATCCGACCCTGATGATCTCCAAAG-3’; ‘5’-CAAG CTATTGAATTCCATCAAGAAAAAC-3’) for PCR based amplification from a marathon-ready mice embryo library (Clontech, catalogue no. 7460-1). The fragments were BamHI/EcoRI cloned into the LexA bait plasmid (p414M25LexNO) and sequenced using appropriate primers and the BigDye terminator sequencing kit (PE Applied Biosystems). The bait construct was transfected into the yeast strain *S. cerevisiae* L40 by the Li-acetate method and the expression was monitored by SDS–PAGE and western blotting analysis using an anti-LexA antibody (Santa Cruz). We screened against an expression library with size selected cDNA fragments. They were derived from randomly primed amplification products from 9.5 to 10.5-day-old mouse embryo RNA and inserted into a pVP16 vector (9). The library plasmids were introduced into the yeast L40 clones already carrying the bait-fragment by a modified Li-acetate method (32), and clones were selected for growth on histidine-deficient plates. Positive clones were selected, the plasmid DNAs were isolated from the yeast clones (QIAprep 8 kit, QIAGEN) and used to transform competent *E. coli* DH5a. The plasmid DNAs were extracted by standard procedures and sequenced using a VP16 primer (‘5’-GTTGATCCTGAGTACGCTCAATTCC-3’) and the BigDye terminator cycle sequencing reaction kit (PE Applied Biosystems). Homology searches were done by BlastN and BlastP analyses.
β-Galactosidase assay

The entire Lc8a ORF was sub-cloned into a galactose-inducible VP16 vector (p415GALNVP0) and co-transfected with truncated Trps1 fragments fused to the bait vector. Transfected L40 yeast cells were grown in selective medium to an OD600 of 0.4–0.5, pelleted and resuspended in 1.2 ml of buffer Z (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 50 mM 2-mercaptoethanol, pH 7.0). From this suspension, 100–150 μl (V E) were diluted to 1 ml with buffer Z and cells were lysed by the addition of 15 μl chloroform and 10 μl 0.1% SDS. The mixture was equilibrated at 30°C for a few minutes and the reaction was started by adding 200 μl of ONPG (4 mg/ml o-nitrophenol galactose in 0.1 mM K2PO4, pH 7.0). After a defined reaction period (tR, e.g. 1–3 h) the reaction was stopped by the addition of 500 μl of 1 M Na2CO3. The reaction was cleared of insoluble material by centrifugation and the OD was measured at 420 and 550 nm. The β-galactosidase activity (A) was calculated in units by the following equation: A = 1000 × OD420 − 1750 × OD550/OD600 × V E × tR (33).

In vitro GST pull-down assay

The truncated Trps1 fragments were subcloned into pcDNA3 (Invitrogen). In vitro translation using T7 polymerase was performed in a single step transcription–translation system using TNT-coupled reticulocyte lysate (Promega) according to the supplier’s information. The reaction contained 1 μg of plasmid DNA and 40 μCi of [35S]-labelled methionine in a total volume of 50 μl. To generate a GST–Lc8a fusion protein, the entire Lc8a coding region was subcloned into the expression vector pGEX-4T2 (Clontech), transfected into REP4 bacteria, and expressed under isopropyl-β-D-thiogalactopyranoside (IPTG) induction. The fusion protein was isolated via affinity chromatography using glutathione-coupled Sepharose beads according to the supplier’s instructions. The eluates from Sepharose beads showed a specific band on SDS–PAGE analysis and Coomassie staining, indicating successful purification. For the analysis of protein–protein interactions 0.5 μg of GST fusion protein or as a control 3 μg of GST protein was incubated with 2.5–10 μl of an in vitro translation reaction in 200 μl binding buffer [50 mM Tris–HCl pH 8.0, 120 mM NaCl, 1 mM dithiothreitol (DTT), 0.5% v/v NP-40] for 1 h at 4°C. Glutathione–Sepharose beads were washed several times in binding buffer and were added to the binding reaction, which was then further incubated for 30 min at 4°C. The beads were spun down and washed five times in binding buffer containing 0.5 M NaCl. Bound proteins were eluted from the beads by incubation with SDS sample buffer and were analysed by SDS–PAGE.

Co-immunoprecipitation

For immunochemical analysis the entire coding region of the human TRPS1 was cloned into the green fluorescent protein (GFP) expression vector pEGFP-N3 (Clontech). The full-length LC8a cDNA amplified from a human fetal brain marathon-ready cDNA library (Clontech, catalogue no. 7402-1) was cloned into a FLAG expression plasmid (pFLAG-N3). Both plasmids were co-transfected into COS-1 cells by electroporation.

For immunoprecipitation analysis using the lysate of transfected COS-1 cells, a volume equivalent to 100 μg of protein was dissolved in 1 ml of Dignam D buffer (20 mM HEPES pH 7.9, 20% glycerol, 0.1% NP-40, 75 mM NaCl, 100 mM KCl, 0.2 mM EDTA, 3% BSA). For the studies of the endogenous proteins, a volume equivalent to 1 mg of total protein extracts from primary human fibroblasts was dissolved in 1 ml of incubation buffer (20 mM HEPES pH 7.9, 75 mM KCl, 2.5 mM MgCl2, 1 mM DTT, 0.1% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 mM Na3VO4). The solutions were precleared with 30 μl of pre-equilibrated protein Sepharose slurry for 30–60 min. The supernatant of the COS-1 cell extracts was then incubated with 0.2 μg of an anti-FLAG antibody (Santa Cruz) for 30 min and 30 μl of pre-equilibrated 50% (v/v) protein A-Sepharose slurry for another 90 min. The fibroblast extracts were incubated for 12–16 h with 20 μl of the anti-LC8a antibody (Alexis). Afterwards, 70 μl of the pre-equilibrated Sepharose slurry was added and the mixture was incubated for another 90 min. The Sepharose beads were precipitated, washed three times with incubation buffer and resuspended in 4× SDS-gel loading buffer [62 mM Tris pH 6.8, 2% (v/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.005% (w/v) bromophenol blue]. All the incubations mentioned above were carried out at 4°C and with constant movement using a head-over-tail rotor. The precipitates were analysed by SDS–PAGE and western blotting using the rabbit anti-TRPS1 antisemur SN652 (3) and a rat anti-PIN antibody (catalogue no. 804-340-C100, Alexis).

Intracellular localization

For immunofluorescence studies and the detection of TRPS1 and LC8a, primary human fibroblasts were seeded in 6 cm plates (1 × 105 cells/plate) on glass coverslips. The cells were harvested 24–48 h later in cold phosphate-buffered saline (PBS), fixed with methanol, washed with PBS and with IF buffer [10 mM Tris pH 7.5, 300 mM NaCl, 0.05% (v/v) Tween 20]. The slides were blocked for 30 min in 0.5% bovine serum albumin (BSA) in IF buffer, incubated with primary antibody for 1 h, washed twice with IF buffer. Cells were incubated with a fluorescence-labelled secondary antibody, washed with IF buffer and finally mounted on slides with aquamount (BDH Laboratories). Where indicated, cell nuclei were stained with DAPI blue. For the detection of LC8a a rat anti-PIN antibody (Alexis) and an affinity-purified anti-rat Alexa-labelled secondary antibody (Molecular Probes) were used. TRPS1 was visualized by using a rabbit anti-TRPS1 antisemur (SN653) (3) and an affinity-purified Cy3 labelled anti-rabbit antibody (Molecular Probes). The cells were analysed by confocal laser scanning microscopy (CLSM).

Electrophoretic mobility shift assay

Recombinant cDNA of human TRPS1 and human LC8a were cloned into pcDNA4.0 (Invitrogen) and used as templates for
in vitro synthesis with Promega’s TNT lysate assay. Electrophoretic mobility shift assays were performed as described earlier (34). Briefly, in vitro synthesized TRPS1 (2 µl of the translation mixture) was pre-incubated with increasing amounts (0.1, 0.5, 2.0 or 5.0 µl) of in vitro synthesized LC8a for 20 min at room temperature. A total volume of 7 µl in each reaction was achieved by adding appropriate amounts of control lysate. Next, the proteins were incubated with 10 fmol of 32P-labelled oligonucleotides containing the GATA consensus sequence (5’-GGCGGAGTTGA TAAGGGGAGGCACGC-3’) for 20 min at room temperature. The formed protein–DNA complexes were separated on a 5% polyacrylamide gel (acrylamide:bisacrylamide = 80:1) in 0.5× TBE buffer, and were visualized by exposure to a Fuji RX X-ray film.

Transcriptional activation assays

Transient transfection assays were performed in the hepatocellular carcinoma HepG2 cells as described before (11). The zD3 promoter construct, provided by Shivdasani, contains multiple repeats of the sequence AGATAA upstream of the firefly luciferase cDNA. Transfections were performed using FuGENE6 (Roche), 0.25 µg of the zD3 reporter plasmid, 0.25 µg of the TRPS1 and the GATA4 expression plasmids and 0.15–0.25 µg of the LC8a expression plasmid. After 48 h, cells were lysed at 4°C in 25 mM Tris (pH 7.8), 2 mM 1,2-diaminocyclohexane-N,N,N’,N’-tetraacetic acid, 10% glycerol, 1% Triton X-100, 2 mM DTT, 0.3 mM PMSF and 2 µg/ml aprotinin. The luciferase activity was measured by luminometry after diluting the lysate 1:3 or 1:5 in 25 mM glycyl glycine, 15 mM MgSO4, 15 mM K2HPO4, 4 mM EGTA, 40 µM ATP, 40 µM DTT and 0.3 µg/ml of luciferin. Assays were repeated at least six times.

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