LSm4 Associates with the Plasma Membrane and Acts as a Co-factor in Cell Volume Regulation

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Key Words

LSm4 • ICln • IClswell • Cell volume regulation • FRET • RVD

Abstract

ICln is a ubiquitous, multifunctional protein with functions in cell volume regulation and RNA processing, and is thus part of an intricate protein network critically involved in the homeostasis of cells. To better understand this vital protein network in health and disease it is fundamental to characterize the interactions between the physiological pathways in which ICln is involved, as well as the spatio-temporal regulation of these interactions. In this study, we focused on the interaction between the two best studied pathways in which ICln is involved - regulatory volume decrease and RNA processing - and asked, whether or not the RNA processing factor and ICln interaction partner LSm4 may also have a function in cell volume regulation in NIH3T3 fibroblasts or HEK293 Phoenix cells. To address this question, we studied in isotonic and hypotonic conditions by FRET, biochemistry and electrophysiology, the intracellular distribution of the RNA processing factor LSm4, its interaction with ICln, as well as the involvement of

LSm4 in the activation of the swelling dependent anion and osmolyte channel IClswell. In isotonic conditions, LSm4 associates with ICln, and the plasma membrane. Hypotonic cell swelling leads to the dissociation of LSm4 from the plasma membrane, and from ICln. Over-expression of LSm4 affects the translocation of ICln to the cell membrane and markedly inhibits the activation kinetics and current density of IClswell. These findings indicate that LSm4 not only acts in RNA processing, but also as a co-factor in cell volume regulation.

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Introduction

Cell volume regulation is essential for the maintenance of cellular homeostasis, and is involved in important physiological processes like cell migration [1], cell proliferation [2], or apoptosis [3], amongst others. There is mounting evidence that cell volume regulatory mechanisms, and specifically the swelling dependent anion and osmolyte channel IClswell may be exploited for the therapy of pathophysiological events, for example in the heart [4, 5], in exercise induced asthma [6], viral

infections [7], pulmonary hypertension [8], ulcers [9], or cancer [10].

The ubiquitous and evolutionary highly conserved protein ICln has been identified as an important player in regulatory volume decrease (RVD), a process that enables cells to counteract swelling [11-14]. ICln is water soluble, and in isotonic conditions the majority of the protein is located in the cytosol while only a small fraction is associated with the cell membrane. In hypotonic conditions an appreciable fraction (up to ~45% in neonatal rat heart cells) of the total cellular ICln content travels from the cytosol to the cell membrane in some, but not all, of cultured cells [15-20]. Osmolarity dependent differences in the cytosol *versus* membrane distribution pattern of ICln were also observed in the kidney, *in vivo* [15, 21, 22]. The involvement of ICln in RVD is further supported by the findings that over-expression of ICln leads to an accelerated activation and an increased current amplitude of the swelling dependent anion and osmolyte channel IClnswell in hypotonic conditions, and that limiting ICln activity with ICln specific antibodies or antisense oligodeoxynucleotides shows the opposite effect [23-27]. In cultured neonatal rat cardiac myocytes, ICln translocation to the cell membrane is paralleled by a hypotonicity-stimulated taurine efflux [28]. Moreover, ICln can directly insert into artificial lipid bilayers from its water soluble form to build ion channels [29-32]. The specific role of ICln in cell volume regulation is controversially discussed, but based on above mentioned data, ICln can be viewed as a molecular candidate of IClnswell, a regulator of IClnswell, or as part of a channel complex constituting IClnswell [11, 13, 14, 33-36].

Fascinatingly, also other functions of ICln beside its role in RVD have been suggested, for example in the regulation of cell morphology [37], the activation of platelets [38], in angiogenesis [39] and, most notably, in RNA processing by binding to Sm and LSm proteins [40-45]. The current hypothesis on the function of ICln in RNA processing is 1) that ICln sequesters Sm and LSm proteins in the cytosol to prevent premature assembly of the heptameric Sm or LSm core of uridin-rich small nuclear ribonucleoprotein particles (U snRNPs); and 2) that ICln directs Sm and LSm proteins to the methylosome, where some of the Sm and LSm proteins are methylated by the protein arginine methyltransferase PRMT5 [40-45].

Taken together, these findings imply that ICln is part of an intricate protein network that is vital for cellular homeostasis, a notion that is underlined by the finding that knock-out of the ICln gene is lethal in early

development [46], and that decreased ICln protein levels cause motor axon degeneration in an animal model for spinal muscular dystrophy [47].

Given the essential role of ICln in the physiology of cells, a characterization of both the interactions between the different physiological pathways in which ICln is involved *per se*, and the spatio-temporal regulation of these interactions is fundamental for a better understanding of this vital protein network.

In this study, we focused on the interaction between the two best studied pathways in which ICln is involved - regulatory volume decrease and RNA processing - and asked, whether or not the RNA processing factor and ICln interaction partner LSm4 may also have a function in cell volume regulation.

Materials and Methods

Cloning

Starting from human (*Homo sapiens* (hs)) cDNA, the open reading frames of full length hsLSm4 and hsICln were amplified by PCR using standard protocols. The PCR products were cloned in frame into the mammalian expression vectors pECFP-C1, pEYFP-C1, pECFP-N1 or pEYFP-N1 (Clontech, USA) as indicated, to produce fusion proteins suitable for FRET [45]. HsLSm4 was also cloned into the HindIII and KpnI restriction sites of the mammalian expression plasmid pFLAG-CMV4 (Sigma, Austria) for the over-expression of n-terminally FLAG (DYKDDDDK)-tagged human LSm4 in HEK293 Phoenix cells. For electrophysiology experiments, hsLSm4 was cloned into the XhoI and SmaI site of the bicistronic mammalian expression vector pIRES2-EGFP (Clontech, Europe). All plasmids were sequenced for verification.

Cell culture and transient transfection

HEK293 Phoenix cells were cultured in Minimum Essential Eagle Medium (Sigma, Italy) supplemented with 10% fetal bovine serum (Cambrex Bio Science), 2 mM L-glutamine, 100 µg/ml penicillin, 100 U/ml streptomycin, 1 mM pyruvic acid (sodium salt). NIH3T3 fibroblasts were cultured in Minimal Essential Medium (Earles salts; Biochrom, Germany) supplemented with 10% fetal calf serum (Biochrom, Germany), 4 mM L-Alanyl-L-Glutamine (Sigma, Austria), and 100 µg/ml penicillin, 100 U/ml streptomycin (Sigma, Austria). The cells were maintained at 37 °C, 5% CO₂, 95% air, 100% humidity. Subcultures were routinely established every second to third day by seeding the cells into Petri dishes (Ø 100 mm) after trypsin/EDTA treatment. For patch-clamp experiments, HEK293 Phoenix cells were seeded into Ø 30 mm Petri dishes and transfected with plasmid DNA by the calcium phosphate method. Eight hours post transfection, cells were seeded on glass coverslips (Ø 10 mm) for electrophysiology.

For cell fractionation experiments, HEK293 Phoenix cells were seeded into 100 mm Petri dishes and transfected with the

pFLAG-CMV4-LSm4 plasmid (6 µg/plate) using linear polyethylenimine (PEI, 25 kD, Polysciences Inc, Germany). For FRET experiments, NIH3T3 cells were seeded on Ø 30 mm glass cover slips, cultured over night and then transfected with plasmid DNA (3 µg/plate) using linear polyethylenimine.

All experiments were performed within 24 h–56 h following transfection.

Electrophysiology

Whole cell patch clamp recordings. HEK293 Phoenix cells were transiently transfected by the calcium phosphate method with a bicistronic mammalian expression plasmid (pIRES2-EGFP, Clontech, Europe) coding for the enhanced green fluorescent protein (EGFP) alone (controls), or EGFP and LSm4 as separate proteins. Twenty-four to fifty-six hours after transfection, single cells expressing EGFP were selected by fluorescence microscopy and voltage-clamped using the whole cell patch-clamp technique. The resistance of the glass pipettes was 3–8 MΩ when filled with the pipette solution (in mM: CsCl 125, MgCl₂ 5, EGTA 11, raffinose 50, ATP 2, HEPES 10, pH 7.2 (adjusted with CsOH)). The hypertonic bath solution was composed of (in mM): NaCl 125, CaCl₂ 2.5, MgCl₂ 2.5, HEPES 10, mannitol 100, pH 7.4 (adjusted with NaOH). Fast exchange of the hypertonic bath solution with a hypotonic bath solution (in mM: NaCl 125, CaCl₂ 2.5, MgCl₂ 2.5, HEPES 10, pH 7.4) was obtained using a perfusion system with a flow rate of 5 ml/min and a bath volume of ~300 µl. All experiments were carried out at room temperature. For data acquisition, an EPC-9 amplifier (HEKA Elektronik, Germany) controlled by a Macintosh computer running the PULSE (HEKA Elektronik, Germany) software was used. Access resistance as well as fast and slow capacitance were compensated and monitored throughout the recordings. All current measurements were filtered at 2.9 kHz and digitized at 2 kHz. The cells were held at 0 mV and step pulses of 400 ms duration were applied from 0 mV to 40 mV every 20 s to monitor the activation and the steady state current density of the swelling activated chloride current. These data were fitted by linear regression in the data range 320 s – 920 s using PRISM (GraphPad, USA) to determine the activation kinetics of IClswell. To establish the current to voltage (IV) relationship of IClswell, step pulses of 500 ms from -120 mV to 100 mV in 20 mV increments from a holding potential of 0 mV duration were applied every 10 min. For data analysis, PULSE-FIT (HEKA Elektronik, Germany) and EXCEL (Microsoft, USA) software were used.

Fluorescence resonance energy transfer (FRET)

The acceptor photobleaching technique employed in this study is described in detail in [15, 45]. In brief, transfected cells were superfused with either an isotonic control solution (in mM: NaCl 90, KCl 5, CaCl₂ 2, MgCl₂ 2, glucose 5, mannitol 80, HEPES 10, pH 7.4; ~300 mOsm) or a hypotonic solution (omission of mannitol) of ~33% lower tonicity. CFP- and/or YFP-expressing cells were imaged on an Olympus IX70 inverted microscope equipped with a monochromator (Polychrome 4, TILL Photonics, Germany) and a cooled CCD camera (TILL Imago SVGA, TILL Photonics, Germany) controlled by the TILLvisION software (version 4.0.1, TILL Photonics, Germany).

Experiments were performed by switching between two separate Olympus BX cubes equipped with the appropriate filter combinations for CFP (excitation bandpass filter 436/20; beamsplitter 455DCLP; emission bandpass filter 480/40) and YFP (emission bandpass filter 510/20; beamsplitter 530DCLP; emission bandpass filter 560/40). Optical filters were purchased from AHF Analysentechnik, Germany. All images were corrected for background, which was then clamped to zero.

Extrapolated FRET efficiencies (exFRETeff) were calculated for each individual experiment according to [48] from the increase in CFP intensity after an acceptor photobleaching interval corresponding to a YFP bleaching of ~50%.

To label the plasma membrane for FRET measurements, the plasmids pECFP-Mem and pEYFP-Mem (Clontech, Europe) were used. In these constructs, EYFP or ECFP are fused to the 20 N-terminal amino acids of GAP-43, which contain a signal for posttranslational palmitoylation that provides a sorting signal to the inner leaflet of the plasma membrane [49, 50].

Number of experiments (n) corresponds to the number of individual experiments (cells) in separate dishes on at least three different experimental days.

Western blotting

HEK293 Phoenix cells were transfected with a mammalian expression plasmid coding for FLAG-hsLSm4 (pFLAG-CMV4-LSm4). Transfected cells were incubated on the cell culture plate for 15 min in a hypotonic (in mM: NaCl 90, KCl 5, CaCl₂ 2, MgCl₂ 2, glucose 5, HEPES 10, pH 7.4) or isotonic buffer (in mM: NaCl 90, KCl 5, CaCl₂ 2, MgCl₂ 2, glucose 5, HEPES 10, mannitol 80, pH 7.4), followed by the extraction of cytosolic, total membrane or plasma membrane fractions (Plasma Membrane Protein Extraction Kit, MBL International Corp, USA).

In brief, cells were scraped off the plates and lysed in homogenization buffer by one freeze/thaw cycle followed by repeated passage through a 29G needle. Cell lysates were centrifuged at 700–800 g to remove cell debris. The cleared supernatant was then centrifuged at 10000 g to pellet the total membrane fraction (t.m.) and to recover the cytosolic (cyt.) fraction. Pellets containing total cellular membranes were carefully washed with ice cold PBS to remove cytosolic contaminants. After resuspension of total membrane fractions in a low density buffer (upper phase solution), an equal volume of a high density buffer (lower phase solution) was added and the mix centrifuged at 1000 g to separate intracellular membranes (which accumulate at interface between upper and lower phase) from plasma membranes (upper phase). Upper phases were collected, and plasma membranes were pelleted by centrifugation (20000 g).

Protein concentrations were determined by using either the Bradford (Bio-Rad, Germany) or an amido black assay [51]. The following amounts of protein samples were separated by SDS-PAGE and electroblotted onto PVDF membranes: 2–5 µg each for cytosolic and membrane fractions (Fig. 1D upper 4 panels; Fig. 2), or 2 µl cytosol and 10 µl for plasma membrane fractions (Fig. 1D bottom panel; Fig. 4A). In quantitative Western blot experiments, samples were run on the same gel/blot.

To detect cytosolic or membrane bound proteins, the following primary antibodies were used for Western blotting as indicated: monoclonal anti-FLAG M2 (Sigma, Austria); polyclonal rabbit anti-ICln [24]; polyclonal chicken anti-LSM4 (Abcam, UK); monoclonal anti-Na⁺/K⁺-ATPase α 1 subunit (Millipore, Italy), polyclonal rabbit anti-GFP (Invitrogen, Europe), polyclonal rabbit anti-Actin (Sigma, Austria) as well as monoclonal anti-GAPDH (Abcam, UK). After extensive washing with TBST, incubation with primary antibody was

Fig. 2. Hypotonic cell swelling leads to the release of LSm4 from cell membranes. A) Acceptor photobleaching experiments in NIH3T3 fibroblasts expressing YFP-mem (FRET acceptor) and CFP-LSm4 (FRET donor). In unpaired experiments, NIH3T3 fibroblasts were either incubated in isotonic buffer (closed symbols), or 15 min in hypotonic buffer of ~33% lower tonicity (open symbols), followed by acceptor photobleaching for 1, 2 and 3 minutes. B) Summary of the exFRETeff values derived from photobleaching experiments as described in (A). Cells were transiently transfected with either the test FRET pair “YFP-mem/CFP-LSm4” or the negative control FRET pairs “YFP-mem/LSm4-CFP” and “YFP-mem/CFP”. The FRET pair “YFP-mem/CFP-mem” served as positive control for FRET within the plane of the cytoplasmic leaflet of the plasma membrane. C) Western blots and densitometric analysis of cytosolic and total membrane fractions prepared from control and FLAG-LSm4 expressing HEK293 Phoenix cells following treatment with either an isotonic or hypotonic buffer (~33% lower tonicity) for 15 min. *Upper panel, left:* Anti-LSm4 Western blot of cytosolic (cyt.) and total membrane (t.m.) fractions of two representative experiments in control cells; *upper panel, right:* Anti-Flag Western blot of cytosolic (cyt.) and total membrane (t.m.) fractions from two representative experiments in FLAG-LSm4 expressing cells; *bottom panel:* Summary of the densitometric analysis of LSm4 bands in Western blots of cytosolic and total membrane fractions prepared from control or FLAG-LSm4 over-expressing cells. The ratio of the LSm4 contents in total membrane fractions ($LSm4_{t.m.}$ or $FLAGLSm4_{t.m.}$) over those in cytosolic fractions ($LSm4_{cyt.}$ or $FLAGLSm4_{cyt.}$) was calculated as an indicator of LSm4 redistribution in isotonic or hypotonic conditions. A decrease in the ratio of membrane associated over cytosolic LSm4 corresponds to a lower content of these proteins in membrane fractions, i.e. redistribution towards the cytosol of HEK293 Phoenix cells.

Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2007) was used, while VisionWorksLS software (version 6.3.3, UVP, UK) was used for the analysis of chemiluminescence signals recorded with the BioSpectrum 500 imaging system.

Statistical Analysis

All data are expressed as arithmetic means $\pm$ SEM. For statistical analysis, SigmaStat (SPSS, USA) or PRISM (GraphPad, USA) software was used. All data were tested for normal distribution before testing differences in the means by unpaired Student's t-test or ANOVA (two-way). If the normality test failed, the Mann-Whitney rank sum test was used for statistical comparison. To test differences in the current to voltage relation of IC_{lswell} in control and hsLSm4 over-expressing cells, two-way ANOVA was applied separately to

the data in the positive or negative voltage ranges, respectively.

Statistically significant differences were assumed at $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Results

The RNA processing factor LSm4 interacts with ICln [45], a small (~26 kDa) ubiquitous protein with functions in at first sight unrelated physiological pathways such as RNA processing and regulatory volume decrease (see introduction). Given the proposed functions as well as the number of identified interaction partners of ICln, reciprocal interactions between pathways in which ICln

is involved, may likely occur. To get insight into the spatio-temporal dynamics of ICln interactions during regulatory volume decrease after hypotonic cell swelling, where an appreciable amount of ICln travels from the cytosol to the cell membrane [15-19], we asked, whether or not cell swelling leads to a redistribution of the RNA processing factor LSm4, whether or not the interaction between ICln and the RNA processing factor LSm4 is affected by cell swelling, and whether or not LSm4 is a regulator of IClnswell.

A fraction of LSm4 is associated with the plasma membrane in isotonic conditions.

As a first step towards the understanding of potential functions of the RNA processing factor LSm4 in cell volume regulation, we investigated the intracellular distribution of LSm4 in NIH3T3 fibroblasts and HEK293 Phoenix cells by fluorescence resonance energy transfer (FRET) and subcellular fractionation experiments, respectively. To label the plasma membrane for FRET experiments, NIH3T3 fibroblasts were transfected with a membrane targeted enhanced yellow fluorescent protein (YFP-mem) that serves as the FRET acceptor in these experiments. In the YFP-mem construct, enhanced YFP is fused to the 20 N-terminal amino acids of Neuromodulin/GAP-43, which contain a signal for posttranslational palmitoylation that provides a sorting signal to the inner leaflet of the plasma membrane [49, 50].

Figure 1A shows an image of NIH3T3 fibroblasts transfected with YFP-mem demonstrating predominant plasma membrane localization of this membrane targeted YFP fusion protein (Figure 1A, upper left and upper right panels).

Plasma membrane localization is also evident in Western blots (Fig. 1A, bottom) of cytosolic, total membrane and plasma membrane fractions prepared from NIH3T3 fibroblasts 24 h following transfection with YFP-mem. YFP-mem can be detected in the total as well as plasma membrane fractions, and is only weakly visible in the cytosolic (cyt.) fraction.

To test a potential membrane association of LSm4 in NIH3T3 fibroblasts by FRET, the FRET acceptor YFP-mem was over-expressed together with the FRET donor CFP-LSm4. Using the acceptor photobleaching technique, FRET was mostly observed in the periphery of cells (Fig. 1B), a strong indication that LSm4 localizes to within <10 nm to the plasma membrane in isotonic conditions. Figure 1C shows a summary of FRET experiments between YFP-mem and CFP-LSm4 in NIH3T3 fibroblasts in isotonic conditions, as demonstrated by the

Fig. 3. Hypotonic cell swelling leads to the release of ICln from LSm4. **A)** Acceptor photobleaching experiments in NIH3T3 fibroblasts expressing YFP-ICln (FRET acceptor) and CFP-LSm4 (FRET donor). In unpaired experiments, NIH3T3 fibroblasts were either incubated in isotonic buffer (closed symbols), or 15 min in hypotonic buffer of ~33% lower tonicity (open symbols), followed by acceptor photobleaching for 1, 2 and 3 minutes. **B)** Summary of the exFRETeff values derived from photobleaching experiments as described in (A), of cells transiently transfected with either the FRET pair “YFP-ICln/CFP-LSm4” or the negative control FRET pairs “YFP-ICln/LSm4-CFP” or “YFP/CFP”.

acceptor photobleaching technique and calculation of the extrapolated FRET efficiency (exFRETeff) [48] as an indicator of interaction. An exFRETeff of 0.152 ± 0.030 ($n=6$) between YFP-mem and CFP-LSm4, which is statistically significantly different from the exFRETeff measured in NIH3T3 fibroblasts transfected with either the control FRET pairs “YFP-mem/CFP” (exFRETeff:

Fig. 4. Over-expression of LSm4 leads to an inhibition of ICls well in HEK293 Phoenix cells and to a decrease in membrane associated ICln. A) Western blots (probed with an ICln specific antibody (α ICln)) of cytosolic (cyt.) and plasma membrane (p.m.) fractions of two representative experiments in control (upper panel) or FLAG-LSm4 over-expressing (FLAG-LSm4, lower panel) HEK293 Phoenix cells following 15 min of treatment in a hypotonic buffer of ~33% lower tonicity. B) Summary of densitometric analysis of ICln bands in Western blots such as described in (A). The ratio of plasma membrane bound ICln ($ICln_{p.m.}$) over cytosolic ICln ($ICln_{cyt.}$) was calculated as an indicator of ICln transposition to the cell membrane upon hypotonic cell swelling in control and FLAG-LSm4 expressing HEK293 Phoenix cells. C,D,E) For electrophysiological measurements, cells were kept in a hypertonic (375 mOsm) extracellular solution and voltage-clamped using the whole cell patch-clamp technique. Subsequent to recording of the current (I) to voltage (V) relation (IV) in hypertonic conditions, the extracellular solution was rapidly replaced with a hypotonic (275 mOsm) extracellular solution to activate ICls well. C) Current to voltage relation of ICls well in HEK293 Phoenix cells over-expressing EGFP alone (closed symbols; IRES-EGFP; n=49), or EGFP and LSm4 (open symbols; LSm4-IRES-EGFP; n=47), in hypertonic or hypotonic conditions. To establish the current to voltage (IV) relationship of ICls well, step pulses of 500 ms duration were applied every 10 min from -120 mV to 100 mV in 20 mV increments from a holding potential of 0 mV. D) Activation kinetics of ICls well in LSm4 over-expressing ($\circ$ LSm4-IRES-EGFP) and control ($\bullet$ IRES-EGFP) cells. To monitor the activation and the steady state current density of ICls well, cells were held at 0 mV and step pulses of 400 ms duration were applied from 0 mV to 40 mV every 20 s. E) Original tracings demonstrating the decrease in the ICls well current amplitude in LSm4 expressing cells.

0.042 ± 0.023 ; $n=11$; $p < 0.05$; Fig. 1C, inset) or “YFP-mem/LSm4-CFP” (exFRETeff: 0.030 ± 0.001 ; $n=8$; $p < 0.001$; Fig. 2B) demonstrates that a fraction of LSm4 is located within <10 nm to the plasma membrane.

To verify this finding, membrane and cytosolic fractions of HEK293 Phoenix cells were prepared and probed by Western blotting using antibodies against LSm4, ICln, GAPDH, actin and the trans-membrane protein Na^+/K^+ -ATPase (Fig. 1D). In isotonic conditions, both LSm4 and its interaction partner ICln were detected in cytosolic fractions (Na^+/K^+ -ATPase negative, GAPDH positive (Fig. 1D, cyt)) as well as in the total (t.m.) and plasma membrane (p.m.) fractions (Na^+/K^+ -ATPase

positive (Fig. 1D)) of HEK293 Phoenix cells, verifying the plasma membrane localization of LSm4 observed in NIH3T3 fibroblasts by FRET.

Hypotonic cell swelling leads to a redistribution of LSm4.

Given the established findings that 1) LSm4 interacts with ICln in isotonic conditions [45], 2) ICln is located in the cytosol while only a minor fraction is associated with the cell membrane in isotonic conditions [17, 21, 25, 28], and 3) that hypotonic cell swelling leads to a translocation of ICln from the cytosol to the plasma membrane [15, 21, 28], we next asked whether or not

hypotonic cell swelling also affects the distribution of LSm4, i.e. influences association of LSm4 with the plasma membrane.

As demonstrated by FRET experiments (acceptor photobleaching technique) between the cell membrane label YFP-mem and CFP-LSm4 (Fig. 2A, B), hypotonicity led to a release of LSm4 from the cell membrane of NIH3T3 fibroblasts, which is evident from a statistically significant ($p < 0.05$) decrease of the exFRETeff between YFP-mem and CFP-LSm4 from 0.152 ± 0.030 ($n=6$) in isotonic conditions to 0.074 ± 0.011 ($n=10$) 15 min after establishing hypotonic conditions.

No statistically significant effect of cell swelling was observed on the exFRETeff in NIH3T3 fibroblasts transfected with the control FRET pairs “YFP-mem/CFP” (iso: 0.042 ± 0.023 ($n=11$); hypo: 0.041 ± 0.006 ($n=9$); $p=0.447$), “YFP-mem/LSm4-CFP” (exFRETeff: iso 0.030 ± 0.001 ($n=8$); hypo: 0.023 ± 0.006 ($n=8$); $p=0.283$) and “YFP-mem/CFP-mem” (iso: 0.102 ± 0.014 ($n=11$); hypo: 0.082 ± 0.015 ($n=11$); $p=0.344$) (Fig. 2B), demonstrating that the decrease of FRET between YFP-mem and CFP-LSm4 is not a consequence of either a dilution of the cytosol or membrane stretch due to cell swelling.

To verify release of LSm4 from the plasma membrane during hypotonic conditions, membrane and cytosolic fractions were prepared from HEK293 Phoenix cells in isotonic and hypotonic conditions and analyzed by quantitative densitometric analysis of Western-blots against either native or FLAG-tagged LSm4. FLAG-tagged LSm4 was chosen for over-expression due to the availability of well characterized and specific antibodies against the FLAG tag, that allow to distinguish over-expressed from native LSm4 in Western blots.

Figure 2C shows that both endogenous LSm4 and over-expressed FLAG-tagged LSm4 could be detected in membrane fractions, and relocated from the cell membrane to the cytosol of HEK293 Phoenix cells in hypotonic conditions. The ratio of membrane associated over cytosolic LSm4 decreased statistically significantly ($p < 0.05$) from 0.591 ± 0.123 ($n=6$) in isotonic conditions, to 0.273 ± 0.054 ($n=7$) in hypotonic conditions (Fig. 2C, left panel). In HEK293 Phoenix cells over-expressing FLAG-LSm4, the ratio of membrane associated over cytosolic FLAG-LSm4 decreased statistically significantly ($p < 0.05$) from 1.364 ± 0.280 ($n=5$) in isotonic conditions to 0.487 ± 0.100 ($n=5$), in hypotonic conditions (Fig. 2C, right panel). This effect of hypotonicity on the intracellular distribution of either native or FLAG-tagged LSm4 is specific for LSm4, since hypotonic cell swelling did not influence the distribution of the cytosolic protein GAPDH

(data not shown), a contaminant in the membrane fractions (Fig. 1D).

Taken together, these findings clearly indicate that both over-expressed FLAG-LSm4 and endogenous LSm4 behave similarly in HEK293 Phoenix cells, i.e. a fraction associates with the cell membrane in isotonic conditions and travels from the cell membrane to the cytosol in hypotonic conditions.

The interaction of LSm4 with ICln is affected by hypotonic cell swelling.

The findings that ICln and LSm4 interact in isotonic conditions [45] and move in opposite directions in hypotonic conditions - LSm4 from the plasma membrane to the cytosol (Fig. 2) and ICln from the cytosol to the plasma membrane [15-19] - imply that the interaction of ICln with LSm4 is affected by hypotonic cell swelling.

To test this hypothesis, FRET experiments in NIH3T3 fibroblasts over-expressing the FRET pair “YFP-ICln/CFP-LSm4” were performed in isotonic and hypotonic conditions. As previously shown [45] and also demonstrated here (Fig. 3), LSm4 interacted robustly with ICln in isotonic conditions (exFRETeff: 0.196 ± 0.011 ; $n=13$). Upon hypotonic cell swelling, the exFRETeff between CFP-LSm4 and YFP-ICln (Fig. 3) dropped statistically significantly ($p < 0.01$) to 0.140 ± 0.015 ($n=10$). Hypotonic cell swelling did not show a statistically significant effect on the exFRETeff of the control FRET pairs “YFP/CFP” (iso: 0.031 ± 0.009 ($n=13$); hypo: 0.023 ± 0.009 ($n=13$); $p=0.526$) and “YFP-ICln/LSm4-CFP” (iso: 0.077 ± 0.014 ($n=11$); hypo: 0.071 ± 0.013 ($n=7$); $p=0.753$), verifying that the decrease of FRET between YFP-ICln and CFP-LSm4 is not a consequence of a dilution of the cytosol due to cell swelling. The finding that the exFRETeff between YFP-ICln and CFP-LSm4 decreased during hypotonic cell swelling is therefore a clear indication of diminished interaction and hence release of ICln from LSm4 in hypotonic conditions.

Over-expression of LSm4 inhibits ICln translocation to the cell membrane.

As demonstrated in figures 1-2, LSm4 is located both at the cell membrane as well as in the cytosol in isotonic conditions. The finding that LSm4 is released from the plasma membrane and from cytosolic ICln during hypotonic conditions suggests that LSm4 may act as a negative regulator of ICln in isotonic conditions, for example by inhibiting the translocation of ICln to the plasma membrane in isotonic conditions. If this is indeed the case, one would expect that intracellular excess of

LSm4 due to over-expression would limit ICln translocation to the plasma membrane in hypotonic conditions. Since FLAG-tagged LSm4 shows a similar distribution pattern and behavior to native LSm4 in both isotonic and hypotonic conditions, FLAG-tagged LSm4 was over-expressed in HEK293 Phoenix cells to study the effect of increased intracellular LSm4 levels on ICln distribution in response to hypotonic cell swelling. FLAG-specific antibodies were used in each Western blot to test for successful over-expression of LSm4.

As demonstrated in figure 4A and figure 4B, quantitative densitometric analysis of Western blots of cytosolic and plasma membrane fractions prepared from HEK293 Phoenix cells (Fig. 4B) after 15 min of hypotonic treatment, showed a ~5 fold decrease in the ratio of membrane bound to cytosolic ICln in FLAG-LSm4 expressing cells. While in isotonic conditions, no effect of FLAG-LSm4 on the ratio of plasma membrane bound over cytosolic ICln was observed (data not shown), hypotonic treatment led to a statistically highly significant decrease ($p < 0.001$) of the ratio of plasma membrane bound over cytosolic ICln from 1.160 ± 0.189 ($n=9$) in native HEK293 Phoenix cells to 0.218 ± 0.065 ($n=8$) in cells over-expressing FLAG-LSm4 (Fig. 4B).

Over-expression of LSm4 down-regulates ICls well in HEK293 Phoenix cells.

Given the well documented finding that ICln is involved in the activation of the swelling dependent anion channel ICls well [23-26], we next asked if over-expression of LSm4 which limits ICln translocation to the cell membrane in hypotonic conditions (Fig. 4A, B) affects the activation and current density of the swelling dependent anion channel ICls well.

As demonstrated in Fig. 4, over-expression of LSm4 led to a statistically significant ($p < 0.0001$; two-way ANOVA) decrease of the ICls well current amplitude after 10 min of hypotonicity (Fig. 4C; control (+100 mV): 109.23 ± 15.24 pA/pF ($n=49$); LSm4 over-expressing cells (+100 mV) 73.73 ± 11.48 pA/pF ($n=47$)), an attenuation of the ICls well steady state current density (Fig. 4D; control: 87.82 ± 11.41 pA/pF, $n=32$; LSm4 over-expressing cells: 59.39 ± 8.13 pA/pF, $n=29$; $p < 0.0001$ by two-way ANOVA), as well as to a significantly slower activation of ICls well (Fig. 4D; derived by linear regression in the data range 320 s - 920 s; control: slope 0.103 ± 0.004 (pA/pF) s^{-1} ; quality of fit: $r^2=0.967$; LSm4 over-expressing cells: slope 0.069 ± 0.003 (pA/pF) s^{-1} ; quality of fit: $r^2=0.937$; $p < 0.0001$), consistent with an inhibitory role of LSm4 on ICls well.

Discussion

Here we report for the first time on 1) the plasma membrane association of the RNA processing factor LSm4, 2) the effect of hypotonic cell swelling on the intracellular distribution pattern of LSm4 and its interaction with the RNA processing and cell volume regulatory factor ICln, and 3) on the influence of LSm4 on the activation and current density of the swelling dependent anion and osmolyte channel ICls well.

As demonstrated in figures 1 and 2, LSm4 is located both at the plasma membrane and in the cytosol of cells in isotonic conditions. Membrane localization of LSm4 has to our knowledge not been described before and suggests that LSm4 is not only involved in RNA processing (for a review see [52]), but may also have a function in membrane physiology, for example in cell volume regulation. This hypothesis is based on the finding that LSm4 interacts with ICln [45], a protein with dual functions in RNA processing [40-45] and in cell volume regulation [17, 23, 24, 28].

ICln shows a similar distribution pattern (Fig. 1D) to that of LSm4 (Fig. 1D, 2), i.e. predominantly cytosolic with a small fraction located at the plasma membrane in isotonic conditions. The finding that ICln travels in a substantial portion from the cytosol to the plasma membrane in hypotonic conditions - a shift from $<10\%$ in isotonic conditions to $\sim 45\%$ of the cellular ICln content during hypotonic cell swelling was observed in neonatal rat cardiac myocytes [28] - raises the questions, what kind of effects hypotonic cell swelling exerts on either the intracellular distribution pattern of LSm4 or the interaction of ICln with LSm4, since alterations in these two factors may in consequence affect either of the two pathways in which LSm4 or ICln are involved. In this study we have focused on the potential role of LSm4 in cell volume regulation as a first step towards the understanding of the mutual interactions within the vital ICln protein interaction network.

To test the hypothesis that LSm4 is involved in cell volume regulation we first studied the intracellular distribution pattern and dynamics of LSm4 in isotonic and hypotonic conditions by cell fractionation of HEK293 Phoenix cells and by FRET in NIH3T3 fibroblasts (Fig. 1, 2).

The occurrence of FRET between YFP-mem and CFP-LSm4 (Fig. 1B, C) clearly suggests that a fraction of LSm4 is located within <10 nm to the plasma membrane. This finding was confirmed by the identification of endogenous as well as FLAG-tagged

L_{Sm}4 in plasma membrane enriched fractions of HEK293 Phoenix cells (Fig. 1, 2) by Western blotting. Since there is no indication that L_{Sm}4 itself is a transmembrane protein, the most likely explanation for the finding that L_{Sm}4 is located at the plasma membrane appears to be that L_{Sm}4 is a part of a membrane associated protein complex. Whether this association occurs via the cytoskeleton - actin was co-purified with the membrane fractions (Fig. 1D) - or any other interaction, remains to be established. The identification and biochemical characterization of this putative membrane associated complex containing L_{Sm}4 is subject of current investigations.

Upon hypotonic cell swelling, L_{Sm}4 travels from the plasma membrane to the cytosol (Fig. 2). This hypotonicity induced redistribution of L_{Sm}4 occurs in the opposite direction to ICln, which travels from the cytosol to the cell membrane in hypotonic conditions [15-19, 21, 28]. This oppositely directed movement of ICln and L_{Sm}4 upon cell swelling appears at first sight contradictory, but can be explained by the finding that ICln is released from L_{Sm}4 during hypotonic cell swelling, as demonstrated by a decrease of FRET between YFP-ICln and CFP-L_{Sm}4 in hypotonic conditions (Fig. 3).

Taken together, these findings suggest that L_{Sm}4 may act as a cytosolic scavenger of ICln in isotonic conditions, and that hypotonic cell swelling leads to a release of ICln from L_{Sm}4 which would allow relocation of ICln to the plasma membrane.

If this is indeed the case, one would expect that over-expression and hence intracellular excess of L_{Sm}4 limits the translocation of ICln to the cell membrane also in hypotonic conditions due to a surplus of cytosolic binding sites for ICln. Figure 4 shows that over-expression of FLAG-L_{Sm}4 indeed inhibits the redistribution of ICln to the plasma membrane in hypotonic conditions.

In addition to interfering with ICln translocation to the cell membrane, over-expression of L_{Sm}4 partially impedes the activation of IC_{lswell}, which is evident from the delayed activation and the lower steady state current density of IC_{lswell} in hypotonically swollen L_{Sm}4 expressing HEK293 Phoenix cells (Fig. 4C, D).

While the involvement of ICln in the activation of IC_{lswell} is well established [23-27], the molecular identity of IC_{lswell} is subject of a controversial debate which

led to the current perception that the molecular correlate of IC_{lswell} has not yet been unequivocally identified (reviewed in [13, 36, 53]). Due to the involvement of L_{Sm}4 in RNA degradation and splicing (for a review see [52]) one explanation for the finding that over-expression of L_{Sm}4 affects IC_{lswell} activation kinetics and current densities may be that L_{Sm}4 over-expression alters the expression level of an as yet unidentified molecular entity/complex constituting IC_{lswell} per se.

The mechanisms by which ICln is involved in the activation of IC_{lswell} *in vivo* have not been studied in great detail yet, several hypotheses however, have been put forward (for reviews see [11, 13, 14, 36, 53]): 1) ICln is a molecular candidate of IC_{lswell}; 2) ICln is part of an IC_{lswell} channel complex; and 3) ICln regulates IC_{lswell} either directly or through participation in cytoskeletal remodeling upon hypotonic cell swelling. In the light of the finding that targeting ICln to the plasma membrane augments the anion permeability of NIH3T3 fibroblasts in hypotonic conditions [15], another explanation for the finding that over-expression of L_{Sm}4 partially inhibits IC_{lswell} appears to be that L_{Sm}4 limits the amount of ICln at the plasma membrane (Fig. 4A, B) and may thus affect IC_{lswell} through any of the proposed roles of ICln in the activation of IC_{lswell} described above.

In summary, based on the findings that 1) L_{Sm}4 associates with the cell membrane in isotonic conditions; 2) hypotonic cell swelling leads to a redistribution of L_{Sm}4 from the cell membrane to the cytosol as well as to a release of ICln from L_{Sm}4; 3) over-expression of L_{Sm}4 partially prevents translocation of ICln to the cell membrane in hypotonic conditions; and 4) that over-expression of L_{Sm}4 delays the activation and decreases the current density of the swelling dependent anion and osmolyte channel IC_{lswell}, we suggest that in addition to its function in RNA processing, L_{Sm}4 is a co-factor in cell volume regulation.

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References

- Jakab M, Ritter M: Cell volume regulatory ion transport in the regulation of cell migration. *Contrib Nephrol* 2006;152:161-180.
- Klausen TK, Bergdahl A, Hougaard C, Christophersen P, Pedersen SF, Hoffmann EK: Cell cycle-dependent activity of the volume- and Ca^{2+} -activated anion currents in Ehrlich lettré ascites cells. *J Cell Physiol* 2007;210:831-842.
- Lang F, Ritter M, Gamper N, Huber S, Fillon S, Tanneur V, Lepple-Wienhues A, Szabo I, Gulbins E: Cell volume in the regulation of cell proliferation and apoptotic cell death. *Cell Physiol Biochem* 2000;10:417-428.
- Clemo HF, Stambler BS, Baumgarten CM: Swelling-activated chloride current is persistently activated in ventricular myocytes from dogs with tachycardia-induced congestive heart failure. *Circ Res* 1999;84:157-165.
- Baumgarten CM, Clemo HF: Swelling-activated chloride channels in cardiac physiology and pathophysiology. *Prog Biophys Mol Biol* 2003;82:25-42.
- Paulmichl M, Norris AA, Rainey DK: Role of chloride channel modulation in the mechanism of action of nedocromil sodium. *Int Arch Allergy Immunol* 1995;107:416.
- Gschwentner M, Susanna A, Woll E, Ritter M, Nagl UO, Schmarda A, Laich A, Pinggera GM, Ellemunter H, Huemer H, et al.: Antiviral drugs from the nucleoside analog family block volume-activated chloride channels. *Mol Med* 1995;1:407-417.
- Dai YP, Bongalon S, Hatton WJ, Hume JR, Yamboliev IA: CIC-3 chloride channel is upregulated by hypertrophy and inflammation in rat and canine pulmonary artery. *Br J Pharmacol* 2005;145:5-14.
- Schmarda A, Dinkhauser P, Gschwentner M, Ritter M, Furst J, Scandella E, Woll E, Laich A, Rossmann H, Seidler U, Lang F, Paulmichl M: The gastric $\text{H}_2\text{K-ATPase}$ blocker lansoprazole is an inhibitor of chloride channels. *Br J Pharmacol* 2000;129:598-604.
- Davies AR, Belsey MJ, Kozlowski RZ: Volume-sensitive organic osmolyte/anion channels in cancer: Novel approaches to studying channel modulation employing proteomics technologies. *Ann N Y Acad Sci* 2004;1028:38-55.
- Paulmichl M, Li Y, Wickman K, Ackerman M, Peralta E, Clapham D: New mammalian chloride channel identified by expression cloning. *Nature* 1992;356:238-241.
- Gschwentner M, Susanna A, Schmarda A, Laich A, Nagl UO, Ellemunter H, Deetjen P, Frick J, Paulmichl M: ICln: A chloride channel paramount for cell volume regulation. *J Allergy Clin Immunol* 1996;98:S98-101; discussion S105-106.
- Furst J, Botta G, Saino S, Dopinto S, Gandini R, Dossena S, Vezzoli V, Rodighiero S, Bazzini C, Garavaglia ML, Meyer G, Jakab M, Ritter M, Wappler-Kornherr E, Paulmichl M: The ICln interactome. *Acta Physiol (Oxf)* 2006;187:43-49.
- Okada Y: Volume expansion-sensing outward-rectifying Cl^- channel: Fresh start to the molecular identity and volume sensor. *American Journal of Physiology* 1997;273:C755-C789.
- Ritter M, Ravasio A, Jakab M, Chwatal S, Furst J, Laich A, Gschwentner M, Signorelli S, Bartscher C, Eichmüller S, Paulmichl M: Cell swelling stimulates cytosol to membrane transposition of ICln. *J Biol Chem* 2003;278:50163-50174.
- Meyer G, Rodighiero S, Guizzardi F, Bazzini C, Botta G, Bertocchi C, Garavaglia L, Dossena S, Manfredi R, Sironi C, Catania A, Paulmichl M: Volume-regulated Cl^- channels in human pleural mesothelioma cells. *FEBS Lett* 2004;559:45-50.
- Musch MW, Luer CA, Davis-Amaral EM, Goldstein L: Hypotonic stress induces translocation of the osmolyte channel protein pICln in embryonic skate (*Raja eglanteria*) heart. *J Exp Zool* 1997;277:460-463.
- Jakab M, Grundbichler M, Benicky J, Ravasio A, Chwatal S, Schmidt S, Strbak V, Furst J, Paulmichl M, Ritter M: Glucose induces anion conductance and cytosol-to-membrane transposition of ICln in INS-1E rat insulinoma cells. *Cell Physiol Biochem* 2006;18:21-34.
- Tamma G, Procino G, Strafino A, Bononi E, Meyer G, Paulmichl M, Formoso V, Svelto M, Valenti G: Hypotonicity induces aquaporin-2 internalization and cytosol-to-membrane translocation of ICln in renal cells. *Endocrinology* 2007;148:1118-1130.
- Emma F, Breton S, Morrison R, Wright S, Strange K: Effect of cell swelling on membrane and cytoplasmic distribution of pICln. *Am J Physiol* 1998;274:C1545-1551.
- Laich A, Gschwentner M, Krick W, Nagl UO, Furst J, Hofer S, Susanna A, Schmarda A, Deetjen P, Burckhardt G, Paulmichl M: ICln, a chloride channel cloned from kidney cells, is activated during regulatory volume decrease. *Kidney Int* 1997;51:477-478.
- Tao GZ, Komatsuda A, Miura AB, Kobayashi A, Itoh H, Tashima Y: pICln predominantly localizes at luminal surface membranes of distal tubules and Henle's ascending limbs. *Biochem Biophys Res Commun* 1998;247:668-673.
- Chen L, Wang L, Jacob TJ: Association of intrinsic pICln with volume-activated Cl^- current and volume regulation in a native epithelial cell. *Am J Physiol* 1999;276:C182-192.
- Gschwentner M, Nagl UO, Woll E, Schmarda A, Ritter M, Paulmichl M: Antisense oligonucleotides suppress cell-volume-induced activation of chloride channels. *Pflügers Arch* 1995;430:464-470.
- Krapivinsky GB, Ackerman MJ, Gordon EA, Krapivinsky LD, Clapham DE: Molecular characterization of a swelling-induced chloride conductance regulatory protein, pICln . *Cell* 1994;76:439-448.
- Ackerman MJ, Krapivinsky GB, Gordon E, Krapivinsky L, Clapham DC: Characterization of a native swelling-induced chloride current, $\text{ICl}_{\text{swell}}$, and its regulatory protein, pICln, in *Xenopus* oocytes. *Jpn J Physiol* 1994;44 Suppl 2:S17-24.
- Hubert MD, Levitan I, Hoffman MM, Zraggen M, Hofreiter ME, Garber SS: Modulation of volume regulated anion current by I(Cln). *Biochim Biophys Acta* 2000;1466:105-114.
- Musch MW, Davis-Amaral EM, Vandenberg HH, Goldstein L: Hypotonicity stimulates translocation of ICln in neonatal rat cardiac myocytes. *Pflügers Arch* 1998;436:415-422.
- Garavaglia ML, Rodighiero S, Bertocchi C, Manfredi R, Furst J, Gschwentner M, Ritter M, Bazzini C, Botta G, Jakab M, Meyer G, Paulmichl M: ICln channels reconstituted in heart-lipid bilayer are selective to chloride. *Pflügers Arch* 2002;443:748-753.
- Garavaglia M, Dopinto S, Ritter M, Furst J, Saino S, Guizzardi F, Jakab M, Bazzini C, Vezzoli V, Dossena S, Rodighiero S, Sironi C, Botta G, Meyer G, Henderson RM, Paulmichl M: Membrane thickness changes ion-selectivity of channel-proteins. *Cell Physiol Biochem* 2004;14:231-240.
- Furst J, Bazzini C, Jakab M, Meyer G, König M, Gschwentner M, Ritter M, Schmarda A, Botta G, Benz R, Deetjen P, Paulmichl M: Functional reconstitution of ICln in lipid bilayers. *Pflügers Arch* 2000;440:100-115.

- 32 Li C, Breton S, Morrison R, Cannon CL, Emma F, Sanchez-Olea R, Bear C, Strange K: Recombinant pICln forms highly cation-selective channels when reconstituted into artificial and biological membranes. *J Gen Physiol* 1998;112:727-736.
- 33 Duan D, Winter C, Cowley S, Hume J, Horowitz B: Molecular identification of a volume-regulated chloride channel. *Nature* 1997;390:417-421.
- 34 Rossow CF, Duan D, Hatton WJ, Britton F, Hume JR, Horowitz B: Functional role of amino terminus in CIC-3 chloride channel regulation by phosphorylation and cell volume. *Acta Physiol (Oxf)* 2006;187:5-19.
- 35 Furst J, Gschwentner M, Ritter M, Botta G, Jakab M, Mayer M, Garavaglia L, Bazzini C, Rodighiero S, Meyer G, Eichmuller S, Woll E, Paulmichl M: Molecular and functional aspects of anionic channels activated during regulatory volume decrease in mammalian cells. *Pflugers Arch* 2002;444:1-25.
- 36 Sardini A, Amey JS, Weylandt KH, Nobles M, Valverde MA, Higgins CF: Cell volume regulation and swelling-activated chloride channels. *Biochim Biophys Acta* 2003;1618:153-162.
- 37 Krapivinsky G, Pu W, Wickman K, Krapivinsky L, Clapham DE: pICln binds to a mammalian homolog of a yeast protein involved in regulation of cell morphology. *J Biol Chem* 1998;273:10811-10814.
- 38 Larkin D, Murphy D, Reilly DF, Cahill M, Sattler E, Harriott P, Cahill DJ, Moran N: ICln, a novel integrin α IIb β 3-associated protein, functionally regulates platelet activation. *J Biol Chem* 2004;279:27286-27293.
- 39 Li H, Yonekura H, Kim CH, Sakurai S, Yamamoto Y, Takiya T, Futo S, Watanabe T, Yamamoto H: Possible participation of pICln in the regulation of angiogenesis through alternative splicing of vascular endothelial growth factor receptor mRNAs. *Endothelium* 2004;11:293-300.
- 40 Pu WT, Krapivinsky GB, Krapivinsky L, Clapham DE: pICln inhibits snRNP biogenesis by binding core spliceosomal proteins. *Mol Cell Biol* 1999;19:4113-4120.
- 41 Meister G, Eggert C, Buhler D, Brahm H, Kambach C, Fischer U: Methylation of Sm proteins by a complex containing PRMT5 and the putative U snRNP assembly factor pICln. *Curr Biol* 2001;11:1990-1994.
- 42 Friesen WJ, Paushkin S, Wyce A, Massenet S, Pesiridis GS, Van Duyne G, Rappsilber J, Mann M, Dreyfuss G: The methylosome, a 20S complex containing JBP1 and pICln, produces dimethylarginine-modified Sm proteins. *Mol Cell Biol* 2001;21:8289-8300.
- 43 Friesen WJ, Wyce A, Paushkin S, Abel L, Rappsilber J, Mann M, Dreyfuss G: A novel WD repeat protein component of the methylosome binds Sm proteins. *J Biol Chem* 2002;277:8243-8247.
- 44 Azzouz TN, Pillai RS, Dapp C, Chari A, Meister G, Kambach C, Fischer U, Schumperli D: Toward an assembly line for U7 snRNPs: Interactions of U7-specific LSm proteins with PRMT5 and SMN complexes. *J Biol Chem* 2005;280:34435-34440.
- 45 Furst J, Schedlbauer A, Gandini R, Garavaglia ML, Saino S, Gschwentner M, Sarg B, Lindner H, Jakab M, Ritter M, Bazzini C, Botta G, Meyer G, Kontaxis G, Tilly BC, Konrat R, Paulmichl M: ICln159 folds into a pleckstrin homology domain-like structure: Interaction with kinases and the splicing factor LSm4. *J Biol Chem* 2005;280:31276-31282.
- 46 Pu WT, Wickman K, Clapham DE: ICln is essential for cellular and early embryonic viability. *J Biol Chem* 2000;275:12363-12366.
- 47 Winkler C, Eggert C, Gradl D, Meister G, Giegerich M, Wedlich D, Lagerbauer B, Fischer U: Reduced U snRNP assembly causes motor axon degeneration in an animal model for spinal muscular atrophy. *Genes Dev* 2005;19:2320-2330.
- 48 Jobin CM, Chen H, Lin AJ, Yacono PW, Igarashi J, Michel T, Golan DE: Receptor-regulated dynamic interaction between endothelial nitric oxide synthase and calmodulin revealed by fluorescence resonance energy transfer in living cells. *Biochemistry* 2003;42:11716-11725.
- 49 Liu Y, Fisher DA, Storm DR: Intracellular sorting of neuromodulin (GAP-43) mutants modified in the membrane targeting domain. *J Neurosci* 1994;14:5807-5817.
- 50 Moriyoshi K, Richards LJ, Akazawa C, O'Leary DD, Nakanishi S: Labeling neural cells using adenoviral gene transfer of membrane-targeted GFP. *Neuron* 1996;16:255-260.
- 51 Kaplan RS, Pedersen PL: Determination of microgram quantities of protein in the presence of milligram levels of lipid with amido black 10B. *Anal Biochem* 1985;150:97-104.
- 52 Wilusz CJ, Wilusz J: Eukaryotic LSm proteins: Lessons from bacteria. *Nat Struct Mol Biol* 2005;12:1031-1036.
- 53 Jentsch TJ, Stein V, Weinreich F, Zdebik AA: Molecular structure and physiological function of chloride channels. *Physiol Rev* 2002;82:503-568.