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REGULAR ARTICLE

The vertebrate homologue of sulfide-quinone reductase in mammalian mitochondria

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Abstract Hydrogen sulfide (H_2S) is the first inorganic compound identified as both a substrate for mitochondrial oxidative phosphorylation and a transmitter in mammalian cells. H_2S seems to mediate effects that are correlated with those of nitric oxide (NO) by a reciprocal regulation. Moreover, H_2S is consumed by mitochondrial oxidation mediated by sulfide-quinone reductase-like protein (SQRDL)—the vertebrate homolog of sulfide-quinone oxidoreductase (SQR). There is evidence that SQR plays an essential role in regulating H_2S levels in fission yeast. To start understanding the role of SQRDL in the mammalian metabolism of H_2S, we examine rat tissues. Our results show that SQRDL protein is present in all tissues tested, albeit restricted to specific mitochondrial populations at the cellular level. We demonstrate a developmental regulation of *Sqrdl* transcription in the kidney, where SQRDL protein is detectable in glomerular podocytes and in tubular cells of the renal medulla. We also show that *Sqrdl* transcription in T cells is responsive to external H_2S. Taken together, our results suggest that *Sqrdl* transcription is adaptively regulated, probably to meet the need of H_2S oxidation. Thus far, SQRDL has only been studied in a limited set of tissues. The present report demonstrates the presence and specific localization of SQRDL in various mammalian tissues.

Keywords SQRDL · Hydrogen sulfide · Kidney · Mitochondria · Podocytes · Tubular cells

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Introduction

Sulfur is one of the six major elements in biological matter but hydrogen sulfide is an ambiguous molecule for life. On the one hand, it is highly toxic (Beauchamp et al. 1984), especially for aerobic organisms. Among other targets, it inhibits cytochrome *c*-oxidase, the key enzyme of aerobic respiration, almost as efficiently as cyanide, at micromolar concentrations (Nicholls 1975). On the other hand, it can be used as a hydrogen donor for energy conservation, either in respiratory or photosynthetic organisms. Since hydrogen sulfide is always present, not only in sulfidic environments but also in lower and higher animals (Abe and Kimura 1996; Julian et al. 2002; Kamoun 2004), adaptation to this ambiguous compound is required. Moreover, for mammals, it has recently been shown that hydrogen sulfide acts as a signaling substance. It is referred to as the third gaseous transmitter (gasotransmitter), besides nitric oxide (NO) and carbon monoxide (CO) (Abe and Kimura 1996; Boehning and Snyder 2003; Qu et al. 2008; Wang 2002). The numerous physiological effects of hydrogen sulfide often overlap with those of NO and CO. Like NO, sulfide affects smooth muscle relaxation and neuronal excitability. However, by opening ATP-dependent potassium channels, which leads to hyperpolarization, hydrogen sulfide triggers molecular events that are different from those triggered

by NO signaling (Wang 2002). As a consequence, the action of hydrogen sulfide as a transmitter requires subtle control mechanisms distinct from those controlling NO and CO. Noteworthy, several disorders, like Alzheimer's disease, Down's syndrome and ulcerative colitis, are associated with abnormal hydrogen sulfide levels (Kamoun et al. 2003; Qu et al. 2008; Roediger et al. 1997).

Detoxification of hydrogen sulfide seems to occur mainly by mitochondrial respiration, as has been documented for invertebrates (Ouml et al. 1997; Parrino et al. 2000), as well as for vertebrates (Furne et al. 2001; Goubern et al. 2007; Yong and Searcy 2001). Most significantly, oxygen consumption accompanied by ATP formation has been shown for mitochondria from colon epithelia, where hydrogen sulfide acts as a co-carcinogen (Furne et al. 2001; Goubern et al. 2007)). The colon mucosa is exposed to particularly high hydrogen sulfide concentrations arising from the anaerobic metabolism of enterobacteria (Florin et al. 1991; Gibson et al. 1988). Sulfide is oxidized by the mitochondrial sulfide-quinone reductase-like protein (SQRDL), a homologue of bacterial sulfide-ubiquinone oxidoreductase (SQR) (Vande Weghe and Ow 1999).

SQR is an ancient enzyme. It may be a relic from the ancient anoxic, "sulfidic" world (Anbar and Knoll 2002; Shen et al. 2001) and occurs throughout the prokaryotic domains (Theissen et al. 2003). The enzyme belongs to the disulfide oxidoreductases, a large flavoprotein family and exists in two main forms, type I and type II (Theissen et al. 2003). It was discovered and characterized in cyanobacteria (Arieli et al. 1994) and was studied in detail by molecular techniques in the purple bacterium *Rhodobacter capsulatus* (Schutz et al. 1997, 1999). A mechanism of action has been proposed based on site-directed mutants (Griesbeck et al. 2002),

An *Sqrdl* gene is present in the genomes of animals and fungi, which encodes for a homologue of type II of the bacterial enzymes (Shahak and Hauska 2008; Theissen et al. 2003). It seems to be absent from plants but was first isolated and characterized from fission yeast, where it has been called HMT2, because of its involvement in heavy metal tolerance (Vande Weghe and Ow 1999). Recently, it was described for the lug worm *Arenicola marina* (Theissen and Martin 2008).

The role of the mitochondrial HMT2 in heavy metal tolerance of fission yeast is to attenuate hydrogen sulfide to levels appropriate for the formation of defined metal–phytochelatin complexes in the cytoplasm, which can be taken up into the vacuole (Vande Weghe and Ow 2001). It is intriguing to consider a respective role of SQRDL in adjusting the hydrogen sulfide concentration in signaling of higher animals.

In view of these signaling functions of hydrogen sulfide and the hydrogen sulfide-oxidizing activity of SQRDL (Lagoutte et al. 2010), the question arises where and when it is synthesized on a histological and cellular level in mammals, including man. In this study, we demonstrate that SQRDL is a component of several mammalian tissues. Moreover, we show that *Sqrdl* transcription is developmentally regulated and adaptively responds to hydrogen sulfide levels.

Material and methods

SQRDL was investigated using a broad spectrum of biochemical, molecular and immunohisto- and cytochemical methods.

Primers

The sequences of primers used for detection of *Sqrdl* fragments are shown below:

SQR-I		SQR-II	
Forward	5'-GGTTTCGCTCATCC CAAAATAGG-3'	Forward	5'-CAGAGCCCTTGG AACAATTT-3'
Reverse	5'-GCTCCAGCACAC TTCACTG-3'	Reverse	5'-AGGCTTGTCCAG GTTCTCAA-3'

As a positive control, a fragment of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) cDNA was amplified, using the following primers:

GAPDH			
Forward	5'-GGTCGGTGTGAA CGGATTTG-3'	Reverse	5'-GTGAGCCCCAGC CTTCTCCAT-3'

For PCR reactions, 10 μM solution of the oligonucleotides was used.

Biomolecular procedures

Standard procedures were used for handling of nucleic acids and polymerase chain reactions (PCRs). Briefly, RNA was isolated using the RNeasy® Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All samples were digested with a DNase to avoid contamination by genomic DNA. Complementary DNA (cDNA) was produced from samples containing 1 μg RNA using the SuperScript™ II RNase H−Reverse Transcriptase (Invitrogen, Darmstadt, Germany) according to the manufacturer's instructions. Reverse transcription (RT) PCRs were performed using the SQR-I primer and the SAWADY Taq DNA Polymerase (peQLab, Erlangen, Germany), according to the manufacturer's instructions. In order to confirm band identity, PCR products were recovered using the MiniEluteTM Gel Extraction Kit (Qiagen) according to the manufacturer's instructions and sequenced by GeneArt, Regensburg, Germany, according to the Sanger method (Sanger et al. 1977). For semiquantitative real-time RT PCRs, the

QuantiTect SYBR Green PCR Kit (Qiagen) was used according to the manufacturer's instructions. Briefly, 1 μl of the 10 pmol/μl SQR-II or *Gapdh* primer solution and 1 μl cDNA (equivalent to 50 ng RNA) or 1 μl of a 1:20 dilution (equivalent to 2.5 ng RNA) were used, respectively. For each cDNA, three independent reactions were carried out. The reaction was carried out in a thermocycler (Mx3005p Real-Time PCR Systems; Stratagene, Santa Clara, USA) with 15 min activation time at 95 °C, 15 s denaturation at 94 °C, 30 s annealing at 56 °C, and 30 s elongation at 72 °C. Standard curves were used to determine the amount of target cDNA in arbitrary units (AU) using the measured cycle threshold values (Cts). For these standards, cDNA of all conditions studied was mixed and different concentrations of the resulting mixtures were used. The determined amounts of *Sqrdl* amplicons were averaged for each experiment. These values were divided by the average amount of *Gapdh* target cDNA of the same experiment. Finally, these ratios were averaged for each condition (Larionov et al. 2005; Rutledge and Cote 2003; Bustin et al. 2005).

Antibodies

For western blots and for the immunohisto- or cytochemical detection, the following primary antibodies were used (α stands for anti): mouse monoclonal α rat CD3 (0.006 mg/ml; RDI Research Diagnostics, USA; Nicolls et al. 1993), mouse monoclonal α rat CD11b (0.01 mg/ml; Serotec, UK; Robinson et al. 1986), mouse monoclonal α rat CD43 (0.008 mg/ml; Serotec; Brown et al. 1981), mouse monoclonal α COX IV (Cytochrome *c*-oxidase subunit IV) (0.000143 mg/ml; Abcam, UK; Kumarasamy et al. 2013), goat polyclonal α PECAM-1 (Platelet/endothelial cell adhesion molecule-1; 0.01 mg/ml; Santa Cruz Biotechnology, USA; Ilan et al. 2001), rabbit polyclonal α SQRDL (0.0017 mg/ml for immunohistochemistry, 0.00034 mg/ml for western blotting; GeneArt, Germany; Ackermann et al. 2011), mouse monoclonal α rat synaptopodin (0.002 mg/ml; kind gift from R. Witzgall, University of Regensburg, Germany; Mundel et al. 1991), sheep polyclonal α vWF (von Willebrand factor) (0.005 mg/ml; Serotec; Blades et al. 2002). The following secondary antibodies were used (IgG stands for immunoglobulin G): donkey α mouse IgG-Alexa 488 (0.002 mg/ml; Invitrogen;), donkey α goat IgG-Fluorescein isothiocyanate (FITC) (0.00375 mg/ml; Dianova, Hamburg, Germany), donkey α sheep IgG-FITC (0.003333 mg/ml; Dianova;), donkey α rabbit IgG-rhodamine red (RhoX) (0.002 mg/ml; Dianova;), goat α rabbit IgG-Horseradish peroxidase (HRP) (0.0001 mg/ml; Dako, Hamburg, Germany), goat α mouse IgG-HRP (0.0001 mg/ml; Chemicon, Temecula, USA).

Immunohistochemistry

For all immunohistochemical stainings, IgG and preadsorption controls were included. A 100-fold molar excess of antigen (SQRDL peptide) was incubated with the antibody overnight at 4 °C. After centrifugation at 20,000*g*, the resulting supernatant was used as the preadsorption control. As a vehicle control, the antibody was incubated with a 100-fold excess of bovine serum albumin (BSA).

Tissue cryo sections, ca. 7 μm, were fixed for 10 min at 4 °C with 4 % formaldehyde freshly prepared from paraformaldehyde. After washing with PBS (pH 7.4, 0.1 M) containing 0.1 % Triton X-100, slices were blocked for 1 h at room temperature in 5 % milk in the same PBS-Triton buffer. Incubation with primary antibodies was performed over night at 4 °C in a humidified chamber. Slices were then washed with PBS containing 0.1 % Triton X-100 prior to incubation for 1 h at room temperature with the secondary antibody. Slices were washed and mounted using PVA-DABCO [2.5 % (w/v) 1,4-Diazabicyclo-[2,2,2]-octane, in 24 ml Tris pH 8 containing 12 g glycerol and 4.8 g polyvinylalcohol].

Mitochondria were visualized using MitoTracker® Red FM (Invitrogen) according to the manufacturer's instructions. Briefly, cell pellets were resuspended in growth medium containing MitoTracker® Red FM and incubated for 30 min. After staining, cells were fixed in 4 % paraformaldehyde and permeabilized in PBS containing 0.1 % Triton X-100. Immunodetection of SQRDL was performed in analogy to the immunohistochemical protocol.

Microscopy

Immunohisto- and immunocytochemical stainings were analyzed on a fluorescence (microscope BX51) or confocal microscope (scanning laser microscope DMRX) and documented using analySIS software.

Isolation of mitochondria

Mitochondria were isolated using the Mitochondria Isolation Kit from Pierce (Thermo Fisher Scientific, Rockford, USA). For this purpose, option A (Mitochondria Isolation from Soft Tissues) from the manufacturer's protocol 1 (Reagent-based Method for Soft Tissues) was used. Sperm cells were frozen in liquid nitrogen prior to being subjected to the protocol. Mitochondria were analyzed by western blotting using standard procedures. Antibodies were applied in 5 % milk dissolved in PBS containing 0.05 % Tween. Bands were visualized using the ECL solution from Pierce (Thermo Fisher Scientific) according to the manufacturer's instructions.

Isolation of leukocytes

Leukocytes were purified from peripheral blood mononuclear cells (PBMCs) using Magnetic Cell Sorting (MACS). For this purpose, the following kits and additives (MiltenyiBiotec, Bergisch Gladbach, Germany) were used according to the manufacturer's instructions: Pan T Cell Isolation Kit II, CD4 MicroBeads, CD8 MicroBeads and B Cell Isolation Kit II. For isolation of PBMCs, buffy coats (Bayerisches Rotes Kreuz, Regensburg, Germany) were centrifuged in 50-ml tubes for 8 min at 450*g* (22 °C) and resulting pellets were resuspended in PBS/Heparin (5000 IE/ml Heparin in PBS, final volume approx. 35 ml). In each tube, 10 ml Ficoll(1,077 g/ml; Lymphoprep™; Progen Biotechnik, Heidelberg, Germany) were layered at the bottom of the cell suspension. After centrifuging for 20 min at 200*g* (22 °C), the supernatant was aspirated except for 25 ml and another centrifugation step was performed. Then, the PBMC-containing interphase was removed carefully and washed twice with PBS/EDTA (1 mM EDTA in PBS). In order to remove erythrocytes, the cell mixture was incubated with 5 ml 0.9 % (w/v) NaCl solution and 20 ml erythrocyte lysis buffer (155 mM ammonium chloride, 10 mM sodium hydrogencarbonate, 0.1 mM EDTA) for 10 min at 4 °C. Unlysed cells were harvested (10 min at 300*g*, 22 °C), washed in full medium (RPMI-1640, 10 % dialyzed fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin) and resuspended in medium without FCS. Then, macrophages and monocytes were allowed to sediment for 90 min at 37 °C in a cell culture flask. Unsedimented cells were collected at 300*g* for 10 min (22 °C), washed in MACS buffer (2 mM EDTA, 0.5 % BSA in PBS) and resuspended in 40 μl MACS buffer per 10^7 cells. Thereafter, 10 μl biotinylated antibody cocktail was added per 10^7 cells. After an incubation of 10 min at 4 °C, 30 μl MACS buffer and 20 μl anti-biotin MicroBeads were added per 10^7 cells and the mixture was incubated for another 15 min at 4 °C. Then, cells were washed by addition of 20 times the starting volume, harvested (10 min, 300*g*, 22 °C) and resuspended in 500 μl MACS buffer per 10^8 cells. Thereafter, cell sorting was carried out as outlined by the manufacturer.

Identity of cell preparations was verified using FACS (fluorescence activated cell sorting) analysis. Briefly, cells were washed twice with PBS, resuspended in FACS buffer (1 % BSA, 0.05 % sodium azide in PBS) and incubated in the dark for 30 min at 4 °C with the respective conjugated antibody (1:100 dilution) in polysterene round-bottomed tubes (Becton Dickinson Biosciences, Franklin Lakes, USA). The following antibodies were used: rat α human CD3 (Serotec; Jones et al. 1993), FASTIMMUNE CD4 FITC/CD69 PE/CD3 PerCP, FASTIMMUNE CD8 FITC/CD69 PE/CD3 PerCP, FASTIMMUNE CD19 FITC/CD69 PE/CD45 PerCP, and FASTIMMUNE Control γ_1 FITC/γ_1 PE/CD3 PerCP (Becton Dickinson Biosciences). Then, cells were washed in FACS buffer and analyzed by flow cytometry (FACS Calibur; Becton Dickinson Biosciences). For evaluation, the WinMDI 2.8 software (http://winmdi.software.informer.com/2.8/) was used.

RNA from LPS-treated and untreated human leukocytes (Gastpar et al. 2005; Hähnel et al. 2002; Kranzer et al. 2004, 2005; Pham et al. 2007) was a kind gift from Michael Rehli (University Clinic of Regensburg, Germany). Monocytes had a purity of >85 % as determined by expression of CD14 (Pham et al. 2007). Macrophages were generated as previously described (Pham et al. 2007).

Isolation of renal cells

RNA from human kidney cells was a kind gift from B. Banas (Department of Nephrology, Clinic of Regensburg, Germany). Human mesangial cells (hMC) were obtained from glomeruli prepared from the cortex of the intact pole of kidneys removed for circumscribed tumor and confirmed as mesangial using established markers (Rodriguez-Barbero et al. 2006). Conditionally immortalized human podocytes (hP) were developed by M.A. Saleem (Saleem et al. 2002) and were reported to behave as in vivo podocytes at the permissive temperature of 33 °C. HK-2 (Ryan et al. 1994) is a proximal tubular cell line derived from human kidney and is commercially available (ATCC® Catlog No. CRL-2190).

Cell culture

The leukemia T cell line CEM-C7H2 (Strasser-Wozak et al. 1995) is a subclone of the T-ALL cell line CCRF-CEM C7 and was a kind gift from K. Renner (University of Regensburg, Germany). The cell line is glucocorticoid-sensitive and deficient in functional p53 and p16. The cells were cultured as described previously (Renner et al. 2002) at 5 % CO_2 and 37 °C in RPMI (Roswell Park Memorial Institute)-1640 cell culture medium (PAA, Cölbe, Germany) supplemented with 10 % dialyzed fetal calf serum (dFCS, PAA, Cölbe, Germany), 2mM L-glutamine (Sigma-Aldrich, Taufkirchen, Germany), 100 units/ml penicillin (PAA, Cölbe, Germany), 100 μg/ml streptomycin (PAA) in round-bottom 96-well plates. T cell-enriched populations were cultured in the presence of 5 ng/ml IL-15 (R&D Systems, Wiesbaden-Nordenstadt, Germany) or without any interleukin addition. Prior to RNA isolation, the medium was adjusted to 100 μM Na_2S (Sigma-Aldrich) in 96 wells, while 96 wells were treated with medium without Na_2S. After an incubation time of 12 h, 24 h and 4 days, cells were harvested, washed twice in PBS and frozen in liquid nitrogen.

The colonic adenocarcinoma cell line HT-29 (commercially available at ATCC®) was a kind gift from K. Renner (University of Regensburg, Germany). The cell line was isolated from a primary tumor. Under standard growth

conditions, HT-29 cells "form a multilayer of nonpolarized cells that display an undifferentiated phenotype" (Cohen et al. 1999). The cells were cultured as described previously (Goubern et al. 2007) at 10 % CO_2 and 37 °C in DMEM (Dulbecco's modified eagle medium) cell culture medium (PAA) supplemented with 10 % dialyzed fetal calf serum (dFCS; PAA), 2mM L-glutamine (Sigma-Aldrich). Cells were harvested at 60–70 % confluence.

Results

SQRDL protein is present in multiple tissues

Recent studies (Lagoutte et al. 2010; Linden et al. 2012) have demonstrated SQRDL activity in mitochondrial preparations from kidney, liver, heart and colon of the mouse. These observations added credence to the notion that "there is within the mammalian body a significant amount of mitochondria in different cell phenotypes which are able to oxidize sulfide" (Lagoutte et al. 2010; p.1506). We wanted to extend this notion and examined SQRDL in tissues that have not so far been addressed.

SQRDL protein was detected with a polyclonal rabbit antibody in tissue slices. To demonstrate the specificity of the SQRDL labeling, preadsorption of the antibody with an excess of antigen was performed. No labeling was observed (Fig. 1b, d). As a positive control (Fig. 1a), we used colonic tissue from mouse where SQRDL is detectable in epithelial cells of the mucosa (Linden et al. 2012). Immunoreactivity with SQRDL was also observed in epithelial cells of rat colon (Fig. 1c). We observed SQRDL-immunoreactive signals in myocard cells and lung cells (Fig. 2a–d). Since these organs are able to oxidize sulfide (Lagoutte et al. 2010; Olson et al. 2010), the presence of SQRDL was expected. Nonetheless, our observations allow for detailed analyses the first time. In the heart, SQRDL is prominent in all myocard cells. In the lung, by contrast, only a discrete population of cells harbors SQRDL. Furthermore, we extend previous reports by including the thyroid and the genital tract to our analyses. We detected SQRDL protein in the thyroid (Fig. 2e, f), penis (Fig. 2g, h) and testicles (Fig. 2i, j).

SQRDL protein is present in sperm

In the testicle, SQRDL seemed to be localized to sperm cells in the seminiferous tubules. In order to verify this observation, we examined ejaculated, human sperm by western blotting (Fig. 3a) and confocal microscopy (Fig. 3b, c) and detected SQRDL protein. As positive controls, we used liver and kidney samples of the rat because SQRDL activity is evident in both organs (Lagoutte et al. 2010; Linden et al. 2012; Yong and Searcy 2001). All samples were subjected to a fractionation experiment separating mitochondrial from cytosolic

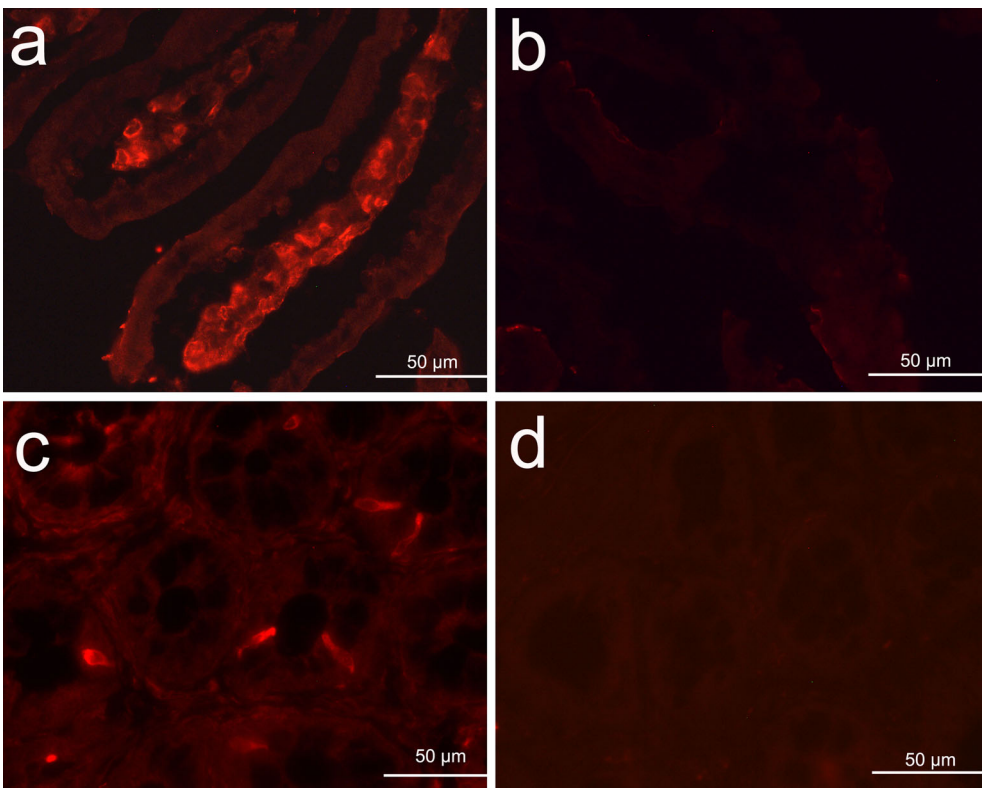

Fig. 1 Immunohistological detection of SQRDL and controls in sections of adult mouse and rat. **a** SQRDL-immunopositive signals in the mouse colon as a positive control, **c** SQRDL-immunopositive signals in the rat colon, **b**, **d** similar areas as (**a**) and (**c**), respectively, showing the preadsorption control for the SQRDL antibody preincubated with a 100-fold molar excess of SQRDL peptide

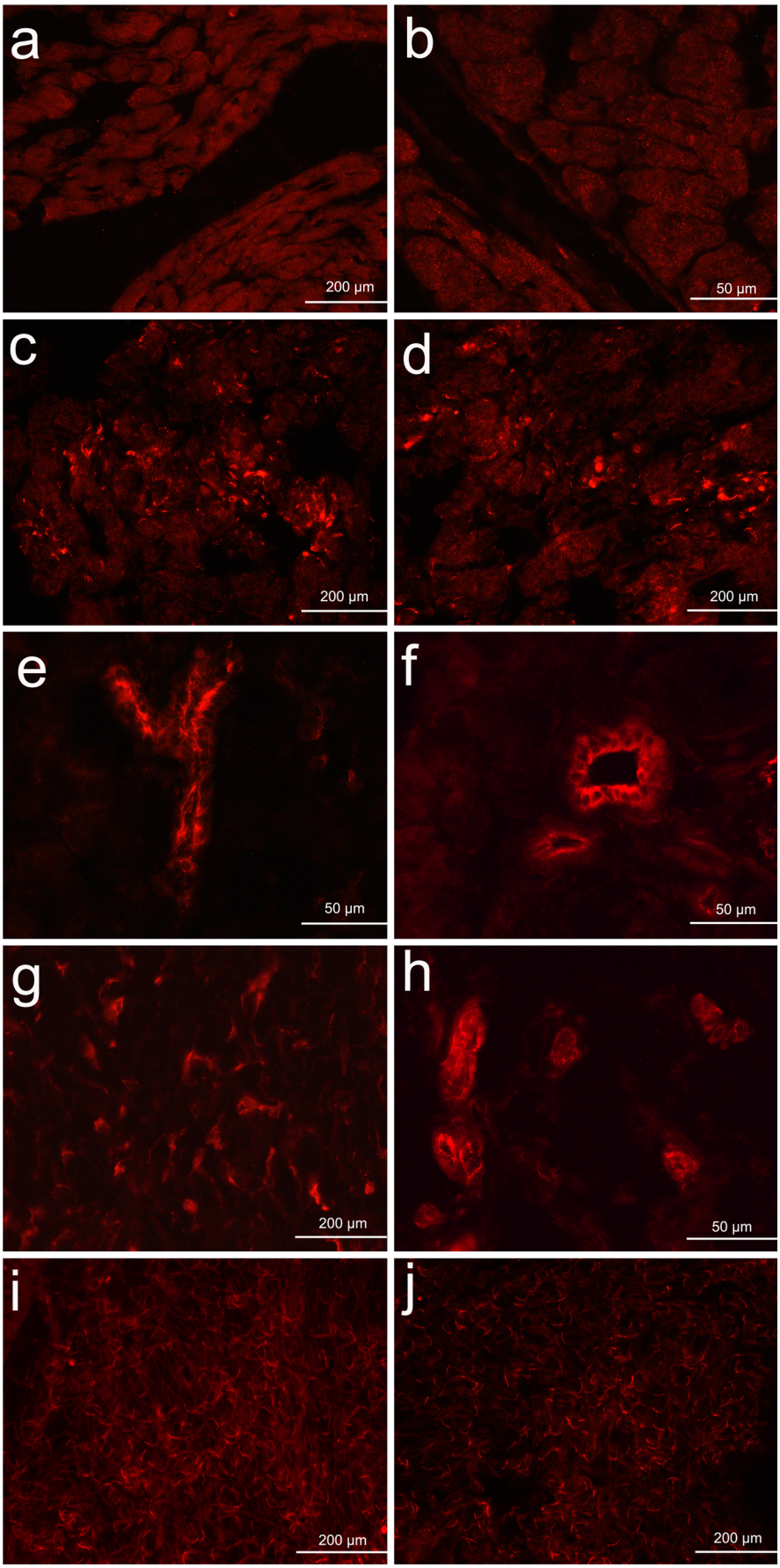

Fig. 2 Immunohistological detection of SQRDL in adult rat sections. **a**, **b** SQRDL-immunopositive signals in heart tissue, **c**, **d** lung tissue, **e**, **f** thyroid tissue, **g**, **h** penis tissue, **i**, **j** testicle tissue

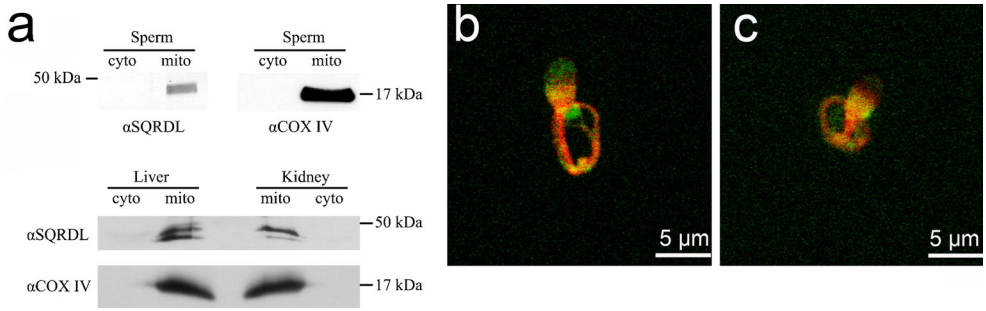

Fig. 3 Immunodetection of SQRDL in sperm cells, liver and kidney. **a** Immunodetection of SQRDL in mitochondrial (*mito*) and cytosolic (*cyto*) fractions of rat liver and kidney (*lower panel*) and of human sperm (*upper panel*). Subunit IV of cytochrome *c*-oxidase (*COX IV*) was used as a mitochondrial marker. **b**, **c** Confocal micrographs showing human sperm cells. SQRDL protein was labeled fluorescently (*green signal*) and mitochondria were labeled with MitoTracker (*red signal*)

fractions. In agreement with previous findings (Ackermann et al. 2011), our results demonstrate that the SQRDL protein is localized to mitochondria as evidenced by the mitochondrial marker cytochrome *c*-oxidase subunit IV (COX IV). In line with this localization, SQRDL-immunoreactive bands run at approximately 46 kDa, which is in agreement with the proposed cleavage of a mitochondrial targeting sequence from the 50kDa full-length precursor (Shahak and Hauska 2008).

The results presented so far demonstrate that SQRDL is present in several mammalian tissues. However, only a discrete population of cells harbors SQRDL-containing mitochondria. Next, we asked whether SQRDL is a permanent requirement in mitochondria within this cell population or whether its expression and synthesis is regulated and responsive to external stimuli. Sperm cells stored in testicles differ from those in the ejaculate with respect to their differentiation status. SQRDL protein was found in sperm cells of both status (Figs. 2i, j, 3). In order to address our question, we had to choose alternative experimental paradigms. Since sulfide oxidation in colonic cells has been studied to some extent (Goubern et al. 2007; Linden et al. 2012), we chose two different paradigms where prominent sulfide oxidation was observed (Lagoutte et al. 2010). On the tissue level, we examined kidney and, on the cellular level, we examined leukocytes. Both paradigms are of interest because, being exposed to plasma sulfide, both kidney and leukocytes might be important for maintaining blood sulfide levels below a critical threshold. Recent studies (Furne et al. 2008; Lagoutte et al. 2010) argue in favor of such a critical threshold.

SQRDL is present in glomeruli and the medulla of rat kidney

Kidney is an organ that might be crucial in regulating blood H_2S levels. H_2S produced in the gut or in body cells can cross cell membranes and enter the blood. Although erythrocytes are able to detoxify H_2S by methylation (Roediger et al. 1997), renal oxidation might be needed to increase the hydrophilic character of the hydrogen sulfide molecule. Moreover, filtration, reabsorption and catabolism of amino acids could release significant amounts of H_2S in the kidney. In line with this reasoning, highest rates of sulfide oxidation were observed in kidney as compared with other organs (Lagoutte et al. 2010). For this reason, it was interesting to inspect SQRDL in the kidney more closely.

In kidney slices, SQRDL-immunopositive signals were observed in the renal cortex (Fig. 4a) and in the medulla (Fig. 4c). Signal intensity was higher in the medullary regions. In the cortex, immunohistological signals were mostly confined to glomeruli. In the medulla, thin limbs of Henle's loop were found to be positive.

SQRDL is present in podocytes

In order to identify the cells labeled for SQRDL in more detail, double labeling was performed with cell type-specific antibodies. For the detection of endothelial cells, anti-von Willebrand Factor (vWF) and anti-platelet/endothelial cell adhesion molecule-1 (PECAM-1) were used. Co-localization of SQRDL and PECAM-1 was not observed while co-localization of SQRDL and vWF was observed to a minor degree. Thus, synthesis of SQRDL in endothelial cells cannot be excluded.

Anti-synaptopodin antibodies were used to localize podocytes. Double staining against SQRDL and synaptopodin provided evidence for some co-localization of both proteins (Fig. 5a, c, d). Signals were not overlapping in the entire podocyte as synaptopodin is restricted to the foot process while SQRDL protein is located in the cell body (Fig. 5d). Expression of *Sqrdl* in human podocytes was confirmed by PCR (Fig. 5e). As a positive control, *Sqrdl* transcription was tested for several rat tissues including kidney and colon. Moreover, *Sqrdl* expression was evident in human mesangial cells and proximal tubule cells (Fig. 5e). This suggests that immunoreactivity with SQRDL can be ascribed to tubular cells in the renal cortex and to both podocytes and mesangial cells within glomeruli.

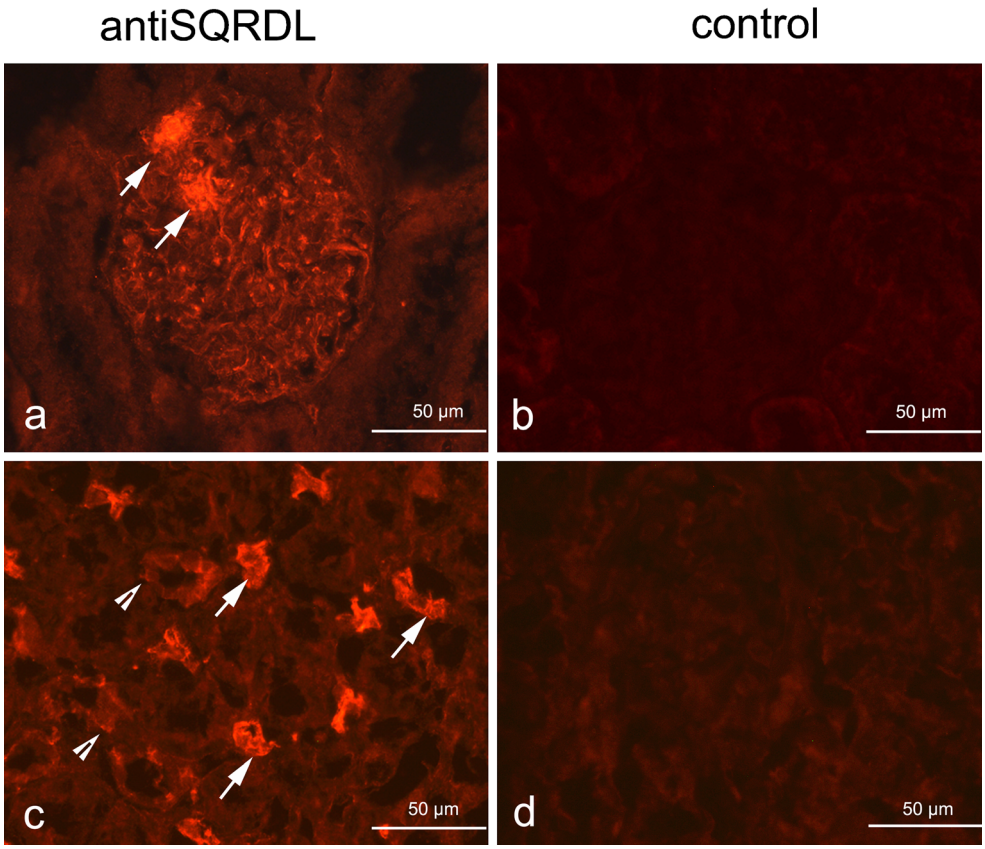

Fig. 4 Immunohistological detection of SQRDL and controls in adult kidney sections. **a** Kidney cortex with glomerulus showing a regular staining against SQRDL. *Arrows* indicate intensely labeled endothelial cells (vascular pole) and glomerular cells. **b** Same area as in (**a**) showing the IgG control. **c** Renal medulla showing a regular staining against SQRDL. Thin loops of Henle were found to be labeled with highest intensity (*arrows*). Other tubular structures were also positive for the enzyme (*arrowheads*) but the label was weaker as for the thin loops. **d** Same area as in (**c**) showing the preadsorption control for the SQRDL antibody preincubated with a 100-fold molar excess of SQRDL peptide

Sqrdl transcription increases during kidney maturation

To date, several studies have addressed sulfide oxidation. However, so far, no study has addressed the question whether the ability of tissues and cells to oxidize sulfide changes over time. We examined the amount of *Sqrdl* transcript during development. Relative *Sqrdl* mRNA levels in the kidney at various developmental stages were determined by semi-quantitative real-time PCR. The mRNA levels of an invariant reference gene, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), were used as a control. The ratio of *Sqrdl*/*Gapdh* mRNA levels was used as a normalized outcome (Fig. 6). As the rat kidney is not completely developed at the time of birth, our results provide the first evidence for an increase of *Sqrdl* expression during maturation of the organ. Furthermore, an increase in *Sqrdl* mRNA levels was observed in the aging animal.

Sqrdl is expressed in leukocytes

As a second paradigm, we chose leukocytes because they show prominent sulfide oxidation (Lagoutte et al. 2010). We tested several tissues for infiltration of leukocytes. Surprisingly, within leukocytes, immunoreactivity with SQRDL could only be observed to a minor degree (Fig. 7a–c) or not at all (Fig. 7d–i). On the transcriptional level, however, we obtained evidence for *Sqrdl* mRNA in human monocytes, macrophages, granulocytes, dendritic cells, natural killer cells, B cells and T cells (Fig. 7j). *Sqrdl* transcription in T cells was confirmed in the leukemia cell line C7H2 (Fig. 7j). As a positive control, *Sqrdl* transcription was tested in the colonic adenocarcinoma cell line HT-29 (Fig. 7j), where SQRDL protein is synthesized (Ackermann et al. 2011).

If SQRDL is not present in all leukocytes, what triggers its synthesis? It is well known that gene expression and RNA stability in leukocytes are strongly influenced by external stimuli. One of the most potent stimuli is lipopolysaccharide (LPS) (Liang et al. 2007; Mandal and Hamilton 2007; Mandal et al. 2005; Rhee et al. 2007; Solis et al. 2007; Tassiulas et al. 2007). For this reason, we examined *Sqrdl* transcription after LPS treatment. However, the amounts of *Sqrdl* mRNA seemed unchanged in LPS-treated compared with untreated cells (Fig. 7j). This observation was confirmed by real-time PCR (data not shown).

Next, we asked whether *Sqrdl* transcription was responsive to external hydrogen sulfide concentrations. Human blood cells were depleted of erythrocytes that do not harbor mitochondria and, hence, are devoid of SQRDL. The cell population to be analyzed was enriched for T cells because comparison of PCR efficiencies revealed the best match with the reference gene *Gapdh* that was used for relative quantification.

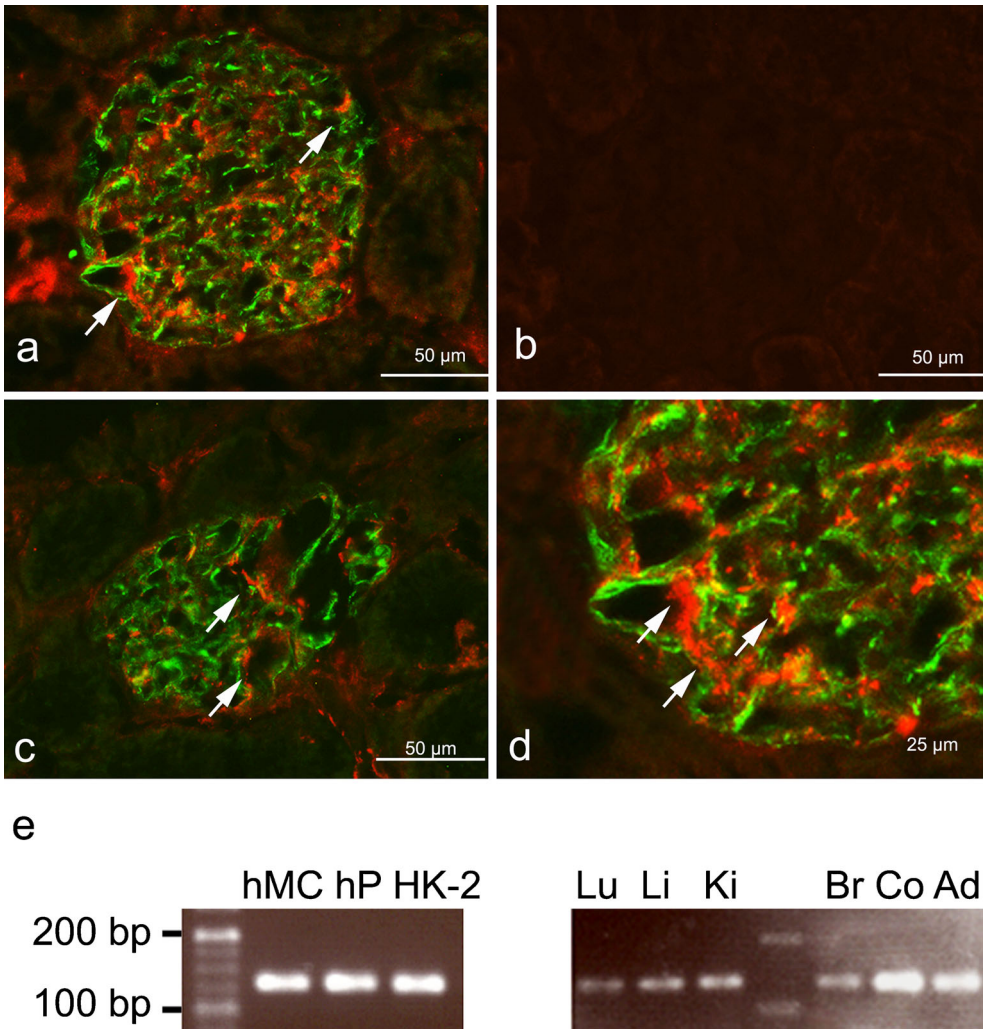

Fig. 5 Immunohistochemical co-localization of SQRDL and synaptopodin in the adult rat kidney.**a**, **c**, **d** Double stainings against SQRDL (*red signal*) and synaptopodin (*green signal*). *Arrows* indicate podocyte co-localization. **b** Preadsorption control. **e** *Sqrdl* fragments amplified from cDNA obtained from human mesangial cells (*hMC*), human podocytes (*hP*), the human kidney cell line HK-2, rat lung (*Lu*), liver (*Li*), kidney (*Ki*), brain (*Br*), colon (*Co*) and adipose tissue (*Ad*). The expected product size is 134 bp

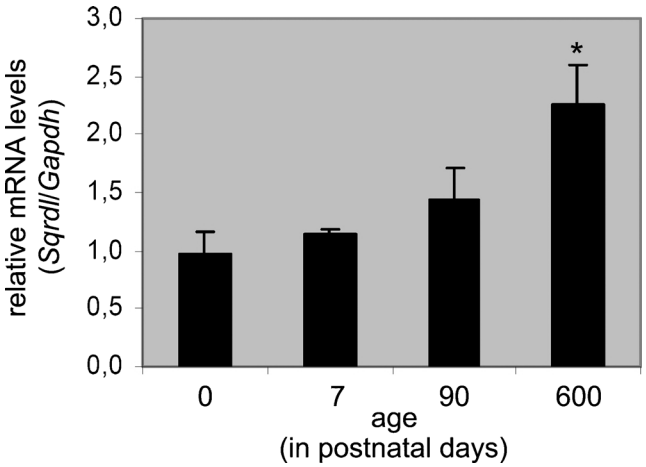

Fig. 6 Graphical representation of age-specific variations in *Sqrdl* transcription in the kidney. On the *x-axis*, ages are given in postnatal days. On the *y-axis*, the ratios of Sqrdl/Gapdh mRNA levels are plotted. *Error bars* represent the standard deviation of the ratios of at least three independent experiments. T*$p<0.01$

Exposure to sulfide enhances Sqrdl transcription

A T cell-enriched population was grown in 96-well plates in the presence of interleukin 15 (IL-15) or without interleukin addition. For each culture condition (IL-15, no interleukin addition), 96 wells were adjusted to 100 μM Na_2S and 96 wells were filled with regular medium. After 12 and 24 h and 4 days of Na_2S treatment, the relative amount of *Sqrdl* mRNA was significantly increased as compared with untreated cells (Fig. 8e).

Discussion

In this study, we demonstrate that SQRDL is a component of several mammalian tissues. Interestingly, at the histological level, we observed that SQRDL is restricted to specific areas

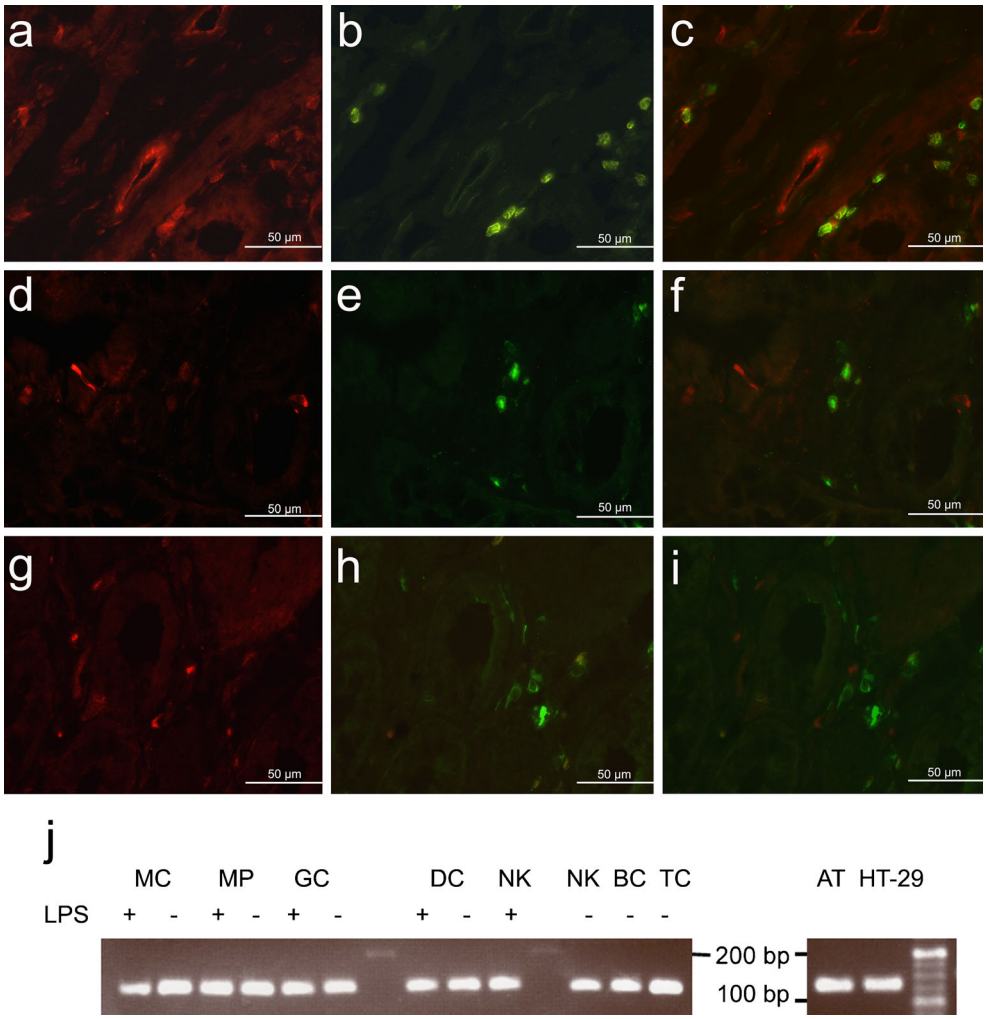

Fig. 7 Inspection of leukocytes. **a**–**i** Sections of the rat colon. **a**, **d**, **g** Stainings against SQRDL, **b**, **e**, **h** stainings against CD11b, CD43 and CD3, respectively, **c**, **f**, **I** the respective overlays. CD11b is present on macrophages, including peritoneal macrophages, as well as dendritic cells and granulocytes. CD43 and CD3 are antigens found on thymocytes. **j** *Sqrdl* fragments amplified from cDNA obtained from human leukocytes. The expected product size is 134 bp. The lanes were loaded as indicated. *LPS* lipopolysaccharide, *MC* monocytes, *MP* macrophages, *GC* granulocytes, *DC* dendritic cells, *NK* natural killer cells. *BC* B cells, *TC* T cells, *AT* T-ALL cell line CCRF-CEM C7 (C7H2), *HT-29* the adenocarcinoma cell line HT-29

and cells, e.g., podocytes of the glomeruli and in tubular cells of the renal medulla. Moreover, analyses of *Sqrdl* transcription by semi-quantitative real-time PCR revealed a developmental and adaptive regulation. In kidney, *Sqrdl* mRNA levels increase with age. In T cells, *Sqrdl* transcription is responsive to exogenous hydrogen sulfide levels. Taken together, these results point to a scenario where SQRDL function is required at specific sites of the organism and at specific times. This notion adds further credence to the assumption that SQRDL plays an essential role in regulating H_2S levels (Lagoutte et al. 2010).

In the light of accumulating data on sulfide oxidation in a variety of paradigms (Furne et al. 2001; Goubern et al. 2007; Lagoutte et al. 2010; Linden et al. 2012; Olson et al. 2010; Yong and Searcy 2001), we asked whether SQRDL might be much more common in mammalian tissues than previously thought. In addition to previous analyses of SQRDL production at the histological level (Linden et al. 2012), we detected SQRDL protein in heart (Fig. 2a, b) and lung (Fig. 2c, d) tissue. Both organs are known to perform mitochondrial sulfide oxidation (Lagoutte et al. 2010; Olson et al. 2010). In order to extend previous studies, we chose to examine the thyroid as an organ that had not been previously associated with SQRDL activity nor with H_2S signaling. Indeed, we detected SQRDL protein in tissue slices of the thyroid (Fig. 2e, f). In a reciprocal approach, we chose the penis as a system where signaling of gasotransmitters, including signaling of H_2S (d'Emmanuele di Villa Bianca et al. 2009; Srilatha et al. 2006, 2007), has received extensive attention. Intriguingly, we did not only observe SQRDL in the penis (Fig. 2g, h) but also in the testicles (Fig. 2i, j). Together with our data for the brain (Ackermann et al. 2011), the presented data establish the notion that SQRDL is not a peculiar component of selected tissues like the colon (Goubern et al. 2007) but instead exists in several tissues.

This raises the question as to what the specific function of SQRDL in these tissues and cells might be. Oxidation of hydrogen sulfide by colonic cells is well established (Goubern et al. 2007). Still, it is not understood in a physiological context. Strong immunoreactivity with SQRDL has been demonstrated in epithelial cells and the myenteric plexus of the mouse (Linden et al. 2012; Fig. 1a) and the rat (Fig. 1c)

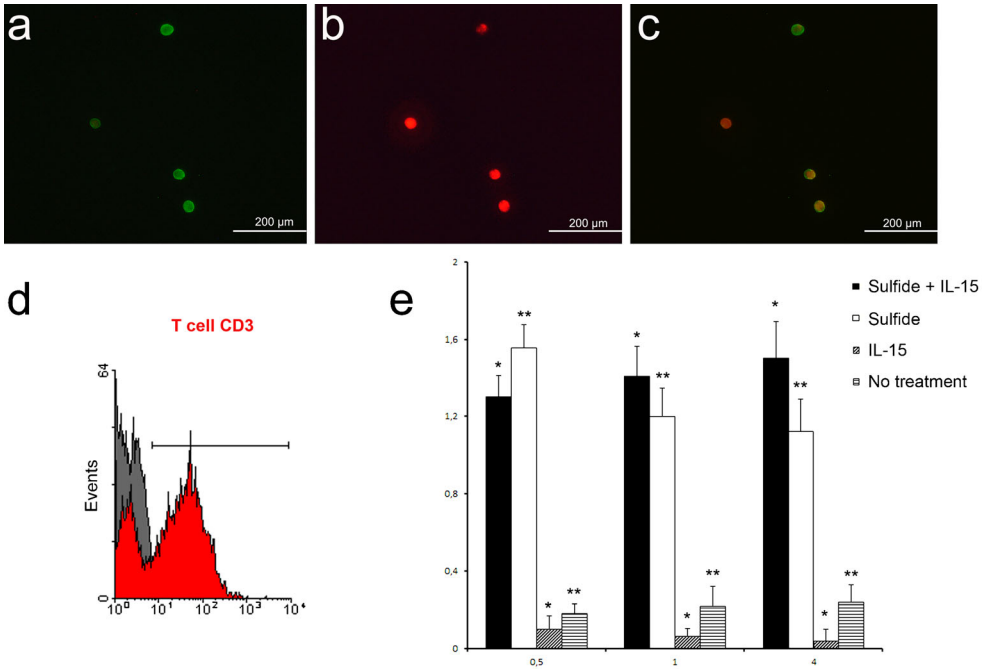

Fig. 8 Expression of *Sqrdl* in leukocytes **a**–**c** Confocal micrographs showing untreated leukocytes of a T cell-enriched population (see **d**). SQRDL protein was labeled fluorescently (*green signal*) and mitochondria were labeled with MitoTracker (*red signal*). **d** The purity of the T cells is 67 % as determined by FACS analysis of immuno-labeled CD3 antigen. **e** Variations in *Sqrdl* transcription induced by Na_2S treatment in a T cell-enriched population. Culture conditions are indicated on the *x-axis*. Cells were treated with 100 µM Na_2S in 96 wells, while 96 wells were treated with medium without Na_2S. After an incubation time of 12 h, 24 h or 4 days, cells were harvested and processed for RNA isolation. The ratios of Sqrdl/Gapdh mRNA levels are plotted on the *y-axis*. *Error bars* standard deviation of the ratios of at least three independent experiments. *$p<0.05$, **$p<0.05$

colon. In the mouse, however, immunoreactivity with SQRDL is present in more epithelial cells as compared with the rat. What might be the reason why the number of cells synthesizing the SQRDL protein is different? Differences might not only arise from the specie's tissue itself but also from the microbes present in that tissue, i.e., the colon. Since certain microbes produce hydrogen sulfide while others do not or do so to a lesser extent, the exposure to hydrogen sulfide in the colon can vary drastically depending on the microbes present. Therefore, it is a challenge for future studies to investigate colonic SQRDL in the context of different microbiota. This is all the more intriguing because cross-fostering and cohousing experiments suggest that certain microbiota can "displace" others during life, thereby ameliorating or exacerbating disease phenotypes (Elinav et al. 2011).

While the presence of SQRDL in the colon had been established, it was surprising that the protein is also present in sperm cells (Figs. 2i,j, 3a–c). Interestingly, biochemical analyses of the human SQRDL protein even suggest that concentrations "below the threshold value (≤1 µM) can be maintained" (Lagoutte et al. 2010). Attenuation of cellular hydrogen sulfide reduces the amount of S-sulfhydration (Mustafa et al. 2009), possibly including the reduction of disulfide bridges. Intriguingly, reducing agents compromise chromatin stabilization in sperm (Menezo et al. 2007) because disulfide bridges contribute to the protection of sperm DNA from oxidative stress (Bjorndahl and Kvist 2011; Enciso et al. 2011). On the other hand, H_2S oxidation might simply share in mitochondrial ATP production, thus fueling the flagellar motor. It is well established that electrons originating from H_2S enter the respiratory chain, thus driving ATP synthase (Goubern et al. 2007; Lagoutte et al. 2010; Linden et al. 2012; Yong and Searcy 2001). Last but not least, sperm might be exposed to elevated hydrogen sulfide levels in the vagina and uterus because of its role as a smooth muscle relaxant (Srilatha et al. 2009).

Lagoutte et al. (2010) showed that SQRDL activity cannot be boosted to meet an immediate sulfide overload. Instead, they reported that the amount of SQRDL protein was the "limiting factor" (p. 1503) for sulfide oxidation. Based on this finding, we asked whether, in physiological contexts, H_2S oxidation might be regulated at the level of *Sqrdl* gene expression. To address this question, we used kidney and leukocytes as experimental paradigms. SQRDL activity has been studied in both paradigms (Lagoutte et al. 2010).

Using semi-quantitative real-time PCR, we demonstrated that *Sqrdl* mRNA levels in T cells increase after exposure to external sulfide. Recent evidence (Furne et al. 2008; Lagoutte et al. 2010) suggests that plasma hydrogen sulfide concentrations are much lower than previously thought. Instead of 40 µM, latest estimates favor levels of approximately 1 µM. According to the current opinion (Lagoutte et al. 2010), this

threshold is maintained by SQRDL activity in leukocytes. While gene expression has been tested at NaHS concentrations of 300 μM (Wen et al. 2013) to 1.0 mM (Kloesch et al. 2011), we used 100 μM sodium sulfide. In aqueous solution, sodium sulfide yields hydrosulfide (HS^-) and hydrogen sulfide (H_2S). While the former (HS^-) is the predominant species (Millero 1986; Morse et al. 1987), the latter (H_2S) can develop into a gas that is volatile in cell culture (Hu et al. 2011). Despite such volatilization, the concentration used in our assay (100 μM) was by far above physiological concentrations. However, a comparable overload must have accumulated in the study of Lagoutte et al. (2010). They challenged cells with increasing injection rates (e.g., 132 pmol/s ml, which yields a micromolar concentration within a few minutes) and examined immediate SQRDL activity. In this study, we challenged T cells with sulfide and examined *Sqrdl* transcription . While immediate SQRDL activity "hardly" (Lagoutte et al. 2010, p. 1502) increased upon sulfide challenge, we observed a significant increase in *Sqrdl* transcription. Our observations complement previous studies demonstrating increased gene expression of pro-inflammatory genes (Kloesch et al. 2011) and protein synthesis of antioxidant enzymes (Wen et al. 2013) upon administration of NaHS.

In another approach using semi-quantitative real-time PCR, we demonstrated that *Sqrdl* mRNA levels in the kidney increase with increasing age. Embryonic kidney cells are unable to oxidize hydrogen sulfide (Lagoutte et al. 2010) and even at the time of birth, kidneys are not mature (Chen et al. 2009). In agreement with this, we observed the lowest level of *Sqrdl* transcription at postnatal day 0 (Fig. 6). As nephrogenesis proceeds in postnatal development, specific genes are differentially expressed (Chen et al. 2009). These genes are downregulated once nephrogenesis is completed around postnatal day 21. In contrast to this, we observed an ongoing increase in *Sqrdl* mRNA. Together with the observation that *Sqrdl* transcription is responsive to hydrogen sulfide, this result suggests an increasing need for H_2S oxidation independent of kidney maturation.

Which physiological needs might be met by this increased transcription? In rat kidney, cystathionine gamma-lyase (CSE) mainly accounts for hydrogen sulfide synthesis (Kamoun 2004; Stipanuk and Beck 1982). In addition, H_2S filtrated from the blood or released from reabsorbed amino acids can also be expected in the kidney. Interestingly, high concentrations of cysteine and prominent activity of CSE were reported for rat kidney (Awata et al. 1989). H_2S acts as a transmitter regulating smooth muscle cell (SMC) relaxation in the vascular system (Wang 2002). Importantly, kidney dysfunction is believed to result from "increased capillary pressure", ultimately leading to interstitial fibrosis (Palm and Nordquist 2011). Moreover, H_2S has been studied in the context of inflammation (Li et al. 2005). We propose that maintenance of H_2S concentrations might become more and more important in the course of life. Biochemical data (Lagoutte et al. 2010) are available to argue that SQRDL has the capacity to fulfil such a requirement.

Interestingly, NO is a transmitter that targets SMCs, albeit using fundamentally different mechanisms than H_2S. However, H_2S and NO mediated effects seem to be correlated by a reciprocal regulation (Wang 2002), balancing their frequently antagonistic effects. H_2S has been reported to downregulate the vascular NO pathway (Geng et al. 2007). NO is known to be vasoprotective, thus preventing vascular fibrosis. In the kidney, low levels of NO inhibit SMC growth and extracellular matrix (ECM) production (Eberhardt and Pfeilschifter 2007). However, high NO levels are associated with pathological events like vascular collapse and tissue remodeling during chronic inflammation. Thus, vascular integrity largely depends on balancing NO activity. It is tempting to speculate that this balance might be maintained by attenuating H_2S at sites where NO production and action are desired. SQRDL protein was detected in podocytes and tubular cells. Podocytes are important for glomerular filtration and the filtration rate is affected by NO produced in vascular endothelial cells (Palm et al. 2009). NO synthesized in tubular cells, on the other hand, regulates mitochondrial respiration and tubular transport capacity (Palm et al. 2009). The present study demonstrates the presence and specific localization of SQRDL in a variety of rat tissues, with a special focus on kidney and leukocytes. Our results suggest that renal transcription of *Sqrdl* is regulated in a spatial and temporal manner. First, SQRDL is specifically localized to tubular cells and podocytes. Second, *Sqrdl* transcription is developmentally regulated. In order to substantiate the notion that *Sqrdl* transcription adapts to the physiological need of H_2S disposal, we used a human T cell enriched population as an independent experimental paradigm. Here, we provide strong evidence that *Sqrdl* transcription is responsive to sulfide levels.

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