

Identification of a Mammalian Silicon Transporter

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29 **Abstract:**

30 Silicon (Si) has long been known to play a major physiological and structural role in certain
31 organisms, including diatoms, sponges, and many higher plants, leading to the recent
32 identification of multiple proteins responsible for Si transport in a range of algal and plant
33 species. In mammals, despite several convincing studies suggesting that silicon is an
34 important factor in bone development and connective tissue health, there is a critical lack of
35 understanding about the biochemical pathways that enable Si homeostasis. Here we report the
36 identification of a mammalian efflux Si transporter, namely Slc34a2 (also termed NaPiIIb) a
37 known sodium-phosphate co-transporter, which was upregulated in rat kidney following
38 chronic dietary Si deprivation. Normal rat renal epithelium demonstrated punctate expression
39 of Slc34a2 and when the protein was heterologously expressed in *Xenopus laevis* oocytes, Si
40 efflux activity (i.e. movement of Si out of cells) was induced and was quantitatively similar
41 to that induced by the known plant Si transporter *OsLsi2* in the same expression system.
42 Interestingly, Si efflux appeared saturable over time, but it did not vary as a function of
43 extracellular HPO_4^{2-} or Na^+ concentration, suggesting that Slc34a2 harbors a functionally
44 independent transport site for Si operating in the reverse direction to the site for phosphate.
45 Indeed, in rats with dietary Si depletion-induced upregulation of transporter expression, there
46 was increased urinary phosphate excretion. This is the first evidence of an active Si transport
47 protein in mammals and points towards an important role for Si in vertebrates and explains
48 interactions between dietary phosphate and silicon.

49

50 **Key words:**

51 Silicon, transport, Slc34a2, *Xenopus laevis* oocytes, rat kidneys

52 **Introduction:**

53 Silicon (Si) is the second most abundant element in the Earth's crust, and is ubiquitous in the
54 diet, but the role it plays in mammalian physiology remains unclear. There is substantial
55 evidence for its importance in the normal health and development of bone and connective
56 tissues of vertebrates (6, 25, 43, 45) but a specific physiological and/or metabolic function
57 has not been identified. In particular, proteins responsible for Si transport in mammals remain
58 elusive. Silicon is essential for many algae (*e.g.* diatoms) to produce their exoskeleton and to
59 complete their cell cycle (5, 21). It is also important in many species of plants, with both
60 structural and physiological roles identified (12, 13, 27).

61 The first Si-transporter to be identified (*CjSIT1*) was in the diatom species *Cylindrotheca*
62 *fusiformis* (22), and SITs are now known from a wide range of diatoms (51),
63 choanoflagellates (32) and haptophytes (11). In plants, Si transport occurs through a
64 collaboration of two individual transporter types, one of which is responsible for influx
65 (movement of Si into cell) and the other for efflux (movement of Si out of cell). Influx occurs
66 through an aquaporin (AQP) channel (*e.g.* *Lsi1*, *Lsi6*) whereas efflux occurs through an
67 energy-dependent active transport process driven by a proton gradient (*e.g.* *Lsi2*) (29, 30).
68 Despite the characterization of multiple Si transporters in algae and plants as described, no
69 Si-transporting homologs have been reported in mammals yet (29, 30, 32).

70 Previously, we reported that Sprague Dawley rats on a Si depleted diet massively reduced
71 their urinary Si output to maintain serum and tissue Si levels (24). This was at the expense of
72 phosphorus, which was decreased in serum and bone (24). These findings suggested that the
73 kidney may be actively involved in Si conservation under chronic Si-deprivation and that,
74 somehow, phosphate is lost in the process. Here we report on the mammalian phosphate
75 transport protein, *Slc34a2*, which was upregulated in the kidney of the rats deprived of
76 dietary Si. This protein was found to induce Si efflux activity when expressed in *Xenopus*

oocytes and to exhibit structural similarity with Lsi2 in many plants. Identification that Slc34a2 can transport Si provides new evidence for a biological role for this element in mammals and establishes another distinct gene family of Si transporters.

80

81 **Materials and Methods:**

82 ***Silicon depletion study.*** Kidneys were obtained from the study of Jugdaohsingh *et al.* (24)
83 following 6 months of dietary Si intervention. Three-week old female Sprague-Dawley rats
84 were maintained for 26 weeks on a formulated low-Si feed ($\sim 3 \mu\text{g Si/g feed}$), with either low
85 Si drinking water ($\sim 15 \text{ ng Si/g water}$; Si deplete group, $n=20$) or with orthosilicic acid
86 (H_4SiO_4) supplemented in the drinking water ($53 \mu\text{g Si/g water}$; Si replete group, $n=10$). A
87 reference group of rats received a normal laboratory maintenance chow diet (B&K Rat and
88 Mouse Standard Diet; B&K Universal Ltd) which is naturally high in Si ($322 \mu\text{g Si/g feed}$)
89 and tap water ($5 \mu\text{g Si/g water}$); see reference (24) for diet compositions. This third group of
90 rats is referred to as Si-high reference group. Total Si intakes were $0.17 \text{ mg Si/kg body}$
91 weight/day in the Si deplete group, 4.1 mg Si/kg/day in the Si replete group and 18.5 mg
92 Si/kg/day in the Si-high reference group. After 26 weeks, rats were sacrificed by asphyxiation
93 with carbon dioxide gas as previously described (24). Rats were killed and processed one at a
94 time, with one rat from each group on the same day. Tissues were then harvested, as
95 previously described (24) and stored at -20°C immediately following harvesting and then at $-$
96 80°C for long term storage. All groups of rats and their tissues were treated in precisely the
97 same fashion. Spot urine samples were collected from fasted rats (24). Urinary Si and P
98 analysis was by inductively coupled plasma optical emission spectrometry (ICP-OES), as
99 described below and data were corrected for urinary creatinine (24). As previously described
100 (24) all animal procedures were carried out in accordance with the UK Home Office Animal

Scientific Procedures Act 1986 (Scientific Procedures on Living Animals). Use of laboratory animals was approved by King's College London (UK) Animal Ethics Committee and the UK Home Office. For this study, the left kidney from n=10 Si deplete, n=8 Si replete and n=5 Si-high reference rats were ground in liquid nitrogen and total RNA was extracted with the Qiagen RNeasy Maxi kit for microarray and quantitative PCR analysis. This part of the study was carried out in 2008.

Gene array analysis. Five μ g total RNA per sample were hybridized to Affymetrix GeneChip Rat Genome 230 2.0 arrays (n=4 Si deplete and n=4 Si replete kidneys). Gene chip robust multi-array analysis (gcRMA) was used to normalize the data including a summarization step based on m-estimator values for the probe sets (58). Modified T-statistics were used to calculate significance of differential gene expression (10, 44) between the Si replete versus Si deplete groups. Genes were selected as 'differentially expressed' when false discovery rate $q < 0.1$ (49). [The microarray dataset has been submitted to the NCBI Gene Expression Omnibus: Accession number: GSE58404.]

Expression studies. Quantitative Real-Time PCR was used to investigate the expression of relevant transcripts, including that of an internal control (18S), in the full cohort of rat kidney RNAs (n=10 Si deplete, n=8 Si replete, and n=5 Si-high reference group). Transcripts were amplified with the TaqMan Universal protocol for real-time RT-PCR. The primers were TaqMan Gene Expression Assays consisting of a FAM reporter and TaqMan MGB probes. Differences in gene expression between groups were statistically analyzed by unpaired t-test.

Immunohistochemistry. Kidneys from a normal laboratory maintenance chow fed rat were excised immediately after necropsy and then fixed in 4% PBS buffered paraformaldehyde. The samples were then cryo-protected via sucrose gradient and snap-frozen in iso-pentane cooled on dry ice. The frozen samples were then embedded in Optimal Cutting Temperature

compound (VWR, UK). Tissue sections were subsequently cryo-sectioned at 12 μ m thickness, collected on SuperFrost® slides (Thermo Scientific, USA) and allowed to air dry overnight at room temperature. Sections were blocked with normal serum in PBS. Samples were then incubated with primary antibody against Slc34a2 (Genetex) or an appropriately matched isotype control. Primary antibody was then detected by incubation with goat anti-rabbit IgG (H+L) Alexa Fluor® 488 conjugate (Invitrogen, UK) prior to counter-staining the nuclei with Hoechst 33342 (Invitrogen, UK) and the cytoskeleton (f-actin) with phalloidin CF633 (Biotium, USA). Imaging was carried out on Leica SP2 confocal microscope using a 1.2NA 63x water immersion lens. Images were collected using Leica Application Suite software. Alongside Slc34a2-antibody stained sections, images of the isotype controls were also collected under identical settings and in ‘matched’ parallel tissue sections. A threshold removing any minor non-specific signal in the isotype controls was then defined, with this threshold subsequently applied identically across all collected images to robustly identify Slc34a2. Staining for Slc34a2 was distinctly punctate so, as well as presentation in as-collected intensity format, images are also presented in binary format (i.e. all Slc34a2 signal that is brighter than isotype threshold shown at maximum intensity). This ‘view’ was included to facilitate visualisation of Slc34a2 locality within the limits of printed image size.

Urinary P and Si analyses. Fasting spot urine samples collected from 6-h fasted rats (n=8 Si deplete, n=5 Si replete and n=6 Si-high reference rats) were digested (in 1:1 mixture of 69% nitric acid and 40% hydrogen peroxide), diluted (1:100) and analysed for total phosphorus by inductively coupled plasma optical emission spectrometry (ICP-OES; Jobin Yvon 2000-2) at 214.914 nm with sample-based standards. Urinary Si was analysed by ICP-OES as previously described (24).

Inter-organism homology of Si-transporters. Homology search was performed with BLASTp (3) against plant and diatom sequences in the EMBL/Genbank non-redundant

150 protein database using the default settings (www.ncbi.nlm.nih.gov). BLASTp and tBLASTn
151 were also used to identify homologs in a range of fully sequenced vertebrate genomes from
152 the EMBL/Genbank and Ensembl databases, and also to identify homologs in selected
153 phylogenetically relevant groups where complete genomes were not available (see
154 Supplemental Table 1). An alignment of homologs was generated using MUSCLE
155 (<http://www.ebi.ac.uk/Tools/msa/muscle/>) under the default settings, producing a final
156 alignment of 38 sequences from 17 species. ProtTest (1) found that the JTT+G+I model
157 provided the best fit to the data under the Akaike Information Criterion. Maximum likelihood
158 analysis was carried out using PhyML (19). Starting trees were generated by BioNJ, with tree
159 searching using the NNI heuristic methods, and topology and branch lengths optimized in
160 ML calculations. One hundred bootstrap datasets were analyzed using the same model and
161 method as for the PhyML analysis, with bootstrap proportions added as numbers to the nodes
162 of the PhyML tree. The alignment was also used for Bayesian MCMC analysis using
163 Phylobayes 3.3 (26), under the CAT +G+I model until convergence (maximum discrepancy
164 <0.3, effective size >100), for 15 parallel chains with sampling every 100 cycles and a burn-
165 in equal to one-fifth the total size of the chain. Posterior probabilities were used to express
166 the support for the nodes in the Bayesian phylogeny. The trees generated were viewed using
167 FigTree v1.3.1 (Andrew Rambaut, Institute of Evolutional Biology, University of Edinburgh
168 2006-2009).

169 ***Calculated Oxoacid volumes.*** The structure for each oxoacid/oxoanion was optimized using
170 the PBE0 functional (2, 38, 39) and 6-31++g(d,p) atomic orbital basis set (4, 9, 15, 16, 20, 41,
171 42). The electron density corresponding to these optimized structures was used to estimate
172 the molecular volume that describes the solvent accessible surface, defined as the volume
173 bounded by a density contour of 0.001 electrons/Bohr³. An increased density of points was
174 used to ensure a more accurate integration so that the computed molecular volumes are

quantitative (37, 56). Since these species are in an aqueous environment, structures were optimized within a solvent field using the integral equation formalism variant of the polarizable continuum model (7, 52, 57) to account implicitly for the effects of an aqueous environment on the solvent accessible surface. The Gaussian09 suite of programs (17) was used in these determinations.

Transport activity in *Xenopus laevis* oocytes

Cloning the gene of interest and oocyte preparation. A cDNA sequence verified *Rattus norvegicus* IMAGE clone pExpress-1/Slc34a2 (Unigene ID: Rn.16933, Entrez Gene: 84395 in DH10BTonA) was purchased from Source BioScience LifeSciences (Cambridge, UK).

For synthesis of capped RNA, the open reading frame (ORF) was amplified by PCR with the following primers: 5'- GAGGATCCATGGCTCCTTGGCCCGAGTTG-3' and 5'- GAGGATCCTAGAACACTGTAGTGTGGACA-3'. The fragment containing the ORF was inserted into the *Bgl*III site of a *Xenopus* oocytes expression vector pX β G-ev1 (a pSTP64 T-derived pBluescript type vector into which *Xenopus* β -globin 5' and 3' UTR had been inserted) (40). Capped RNA was then synthesized from linearized pX β G-ev1 plasmids by *in vitro* transcription with mMESSAGE mMACHINE High Yield Capped RNA Transcription Kit (Ambion) according to the manufacturer's instructions.

Oocytes were isolated from *Xenopus laevis* frogs purchased from NASCO (Nasco-Fort Atkinson, WI, USA) and from Watanabe Zosyoku (Hyogo Pref, Japan). Procedures for defolliculation, culture condition and selection were the same as described previously (35). A volume of 50 nl of the *in vitro* cRNA transcripts (1 ng/nl) was injected into stage V oocytes using a Nanoject II automatic injector (Drummond Scientific Co.). Water-injected oocytes were used as a negative control, *OsLsi1*-injected oocytes as positive controls while testing for influx activity and *OsLsi2*-injected oocytes while testing for efflux activity. Ethical approval

was obtained (permit number 21031043) from the Animal Care Committee of Laval University (Canada).

Influx transport activity. After incubation in a Modified Barth's Saline (MBS) solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 15 mM Tris-HCl at pH 7.6, 0.3 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 10 µg/ml sodium penicillin, and 10 µg/ml streptomycin sulfate) at 18 °C overnight, the cRNA-injected oocytes were exposed to the MBS solution supplemented with 1 mM H₄GeO₄, 0.1 mM HAsO₄²⁻ or 1 mM HPO₄²⁻ at pH 7.6. Following 30 min incubation at 18 °C, the oocytes were washed five times with MBS alone and digested with concentrated (61%) HNO₃. The Ge, As and P concentrations in the digested solutions were determined by ICP-MS (7700X; Agilent Technologies) with appropriate standards, QCs and sample blanks.

To investigate the Si influx and its dependence on extracellular [Na⁺] or [HPO₄²⁻], oocytes were incubated for three days at 18 °C in MBS5 (84 mM Na⁺ and 2 mM HPO₄²⁻) supplemented with 100 µM each of penicillin and streptomycin. Then a set of 10 oocytes for each condition was exposed to MBS2 (1.7 mM H₄SiO₄, 10 mM Na⁺ and 0.5 mM HPO₄²⁻) or MBS3 (1.7 mM H₄SiO₄, 84 mM Na⁺ and 2 mM HPO₄²⁻) solution for 2 h. After exposure, oocytes were rinsed in a solution containing 0.32 M sucrose and 5 mM HEPES (pH 7.4) and then digested in 25 µl concentrated nitric acid, dried at 82 °C for 2 h, reconstituted in plasma grade water (100 µl) and 10 µl analyzed by atomic absorption spectroscopy (see below).

Efflux transport activity. To investigate the efflux transport activity for H₄GeO₄ by *RnSlc34a2*, 50 nl 1 mM H₄GeO₄ in MBS was directly injected into *RnSlc34a2* transfected oocytes. The oocytes were then washed five times with MBS and transferred to 200 µl of fresh MBS at 18 °C. H₄GeO₄ was allowed to efflux into the incubation medium. After 30 min and 2 h, the incubation medium was carefully sampled, and at the end of the experiment, the

223 oocytes were digested with concentrated HNO_3 and the samples were analyzed for Ge by
224 ICP-MS (7700X; Agilent Technologies) with appropriate standards, QCs and sample blanks.

225 To investigate the efflux transport activity for Si by *RnSlc34a2*, oocytes were injected with
226 25 nl of 500 ng/nl cRNA of *RnSlc34a2* or *OsLsi2* or an equal volume of H_2O as a negative
227 control. Pools of 10 oocytes were then loaded with Si by incubation for three days at 4 °C in
228 MBS1 or MBS2, both containing 2 mM Si but different concentrations of Na^+ and HPO_4^{2-}
229 (Supplemental Table 2). These were then exposed to fresh MBS without added Si but with
230 different concentrations of Na^+ and HPO_4^{2-} (MBS, MBS3, MBS4 or MBS5 solution;
231 Supplemental Table 2) for zero, one or two hours. After exposure, oocytes were rinsed in a
232 solution containing 0.32 M sucrose and 5 mM HEPES (pH 7.4), digested with concentrated
233 HNO_3 (25 μl for each pool of 10 oocytes) and dried at 82 °C for 2 h. Plasma-grade water
234 (100 μl) was then added, and samples were incubated for 1 h at room temperature. Samples
235 were vortexed and then centrifuged for 5 min at 13,000 g. 10 μl of samples were then
236 analysed by Zeeman atomic absorption spectrometer (Varian AA240Z;
237 <http://www.varian.com>) equipped with a GTA120 Zeeman graphite tube atomizer, to
238 determine the intracellular Si concentration. Silicon levels in the samples and sample blanks
239 were quantified using appropriate standards prepared using 1,000 ppm ammonium
240 hexafluorosilicate solution (Fisher Scientific, <http://www.fishersci.com>). Data were analyzed
241 with SpectrA software (Varian).

242 **Statistical analysis.** Results are reported as means \pm SD unless otherwise stated. Linear
243 relationships between dietary Si exposure and the relative renal expression of *Slc34* genes
244 were assessed at a significance of $p \leq 0.05$. Thereafter, individual differences were assessed
245 by Independent (unpaired) Samples 2-Tailed T-test. Where there were multiple group
246 comparisons a Bonferroni correction was applied to the p value (i.e. p/n), and significance

was taken as $p \leq 0.05/n$. All statistical analysis was conducted in GraphPad Prism (Version 6.0b) or IBM SPSS version 21 (IBM Corporation).

Results and Discussion:

Identifying *RnSlc34a2* as a candidate for Si transport. Data from our study of Si deficiency in rats strongly suggested active urinary conservation of Si during dietary Si depletion (24). The present investigation utilized kidneys harvested during this study to investigate Si regulatory genes. Gene arrays (Affymetrix GeneChip Rat Genome 230 2.0 arrays containing over 16,000 Entrez IDs and 11 probes per gene) were performed on RNA extracted from the kidney tissues of Si deplete and Si replete rats ($n=4$ for each group) and the data were interrogated for differential regulation of potential transporters (Fig. 1a). The gene array findings^{Footnote1} were confirmed by Real-Time RT-PCR analysis and this technique was also subsequently used to investigate a larger cohort of samples from the Si deplete ($n=10$), Si replete ($n=8$) and a reference group ($n=5$) which were rats kept on a normal laboratory chow diet that is naturally high in Si (referred to as Si-high reference group). *Slc34a2* (type II sodium-phosphate co-transporter), commonly referred to as NaPi-IIb, was expressed especially highly in the kidneys of rats on the Si deplete diet (2.8 and 4.8 fold higher than for kidneys from rats on the Si replete and Si-high reference diets, respectively; Fig. 1b). mRNA expression of other *Slc34* family members, namely *Slc34a1* and *Slc34a3*, were unchanged with dietary Si intervention (inset, Fig. 1b).

Correlation between *Slc34a2* expression and urinary Si concentration, showed an inverse exponential relationship between fasting urinary Si level and the relative expression of *Slc34a2* (Fig. 2), implying that *Slc34a2* is involved in the reabsorption of H_4SiO_4 from the

270 pre-urine under dietary Si deprivation. No such relationship was observed for Slc34a1 and
271 Slc34a3, or other candidate transporters identified in the gene arrays (Fig. 1a).

272 Only a few reports have demonstrated the renal expression of Slc34a2. The original paper
273 characterising the transporter demonstrated its presence in murine kidney at the mRNA level
274 (23). Suyama *et al.* confirmed this more recently by *in situ* hybridization as well as protein
275 expression and localisation by antibody staining (50). The kidney samples from our study
276 were not adequately collected for immunohistochemical analysis, but were for RNA analysis.
277 Thus we confirmed with appropriately collected kidneys from a control rat that, as previously
278 published (50), Slc34a2 protein is expressed by the tubular epithelial cells of the kidney
279 cortex (Fig. 3). Here, as previously reported (50), Slc34a2 showed distinct punctate staining:
280 some of which was basolateral within the cell and some of which was apical/cytosolic (Fig.
281 3). Whether silicate deficiency dictates only the level of expression of Slc34a2 (Fig. 1) or,
282 also its precise location in the cell, as excess dietary phosphate does (50), should be
283 investigated in future work.

284 Finally, to translate these observations (i.e. that Slc34a2 has some basolateral expression in
285 kidney cells and is upregulated in Si deplete diets) we measured urinary P excretion in the
286 three groups. The Si-high reference group diet was higher in P than the Si replete group diet,
287 being 7.0 versus 2.3 mg/g respectively, and so, as expected, urinary P excretion was
288 significantly reduced in the latter (by 89 mg/mmol creatinine for the medians; $p = 0.008$; $n = 6$
289 and 5 respectively (Fig 4)). However, the Si deplete group (with the same dietary P level as
290 the Si replete group) showed no difference in urinary P levels compared to the reference
291 group (Fig. 4), showing that in this group, phosphate was being (relatively) wasted as a
292 consequence of Si being conserved.

293 **RnSlc34a2 transport activity.** The ubiquitous nature of Si makes transport studies of soluble
294 silicic acid (H_4SiO_4) challenging. It is well known that related oxoacids may ride the same

transport systems (22, 29, 36, 54) owing to similarities in their structure and solvated molecular volume (Table 1). Germanic acid (H_4GeO_4), the closest structural analogue of silicic acid, is therefore often employed as a proxy for Si transport, thereby avoiding all background and contamination issues with Si and facilitating analysis (22, 29). Recently, however, graphite furnace atomic absorption spectrometry (GFAAS) was shown to be effective for directly measuring Si influx/efflux in *Xenopus laevis* oocytes transfected with Si-transporting aquaporins from plants (8, 18). Hence, both methods of characterizing Si transport – indirect and direct – were used in the present investigation.

The Slc34a2 coding sequence was inserted into a *Xenopus laevis* expression vector, and cRNA synthesized from this construct was injected into oocytes. Initial expression and plasma membrane localization were verified using an eGFP tagged Slc34a2 construct. Slc34a2 is recognised as a sodium phosphate importer, especially in the brush border of small intestine membrane cells (14, 31, 36, 55), and arsenate also rides this transport system (36, 54). Therefore, both of these oxoacids were utilized as easily-measured probes to confirm Slc34a2 influx activity (Fig. 5a & 5b). The rice Si importer, *OsLsi1*, was used as a positive control and was found to promote both H_4SiO_4 (Fig. 5c) and H_4GeO_4 (Fig. 6a) influx. By contrast, no influx of either H_4SiO_4 or H_4GeO_4 by Slc34a2-expressing oocytes was observed (Fig. 5c & Fig. 6a). On the other hand, efflux of both H_4SiO_4 and H_4GeO_4 was detected for oocytes expressing Slc34a2 (Fig. 5e & Fig. 6b, respectively) as well as those expressing rice Si exporter *OsLsi2*, which was employed as a positive efflux control. Of note, the magnitude of fractional H_4SiO_4 efflux after two hours was quantitatively similar between Slc34a2 and *OsLsi2*.

Given that inward phosphate (HPO_4^{2-}) transport by Slc34a2 is coupled to the inward transport of three sodium ions (i.e. it is electrogenic (14, 55)), we investigated how varying the concentrations of Na^+ and HPO_4^{2-} in the external medium might influence Si influx and

efflux in Slc34a2-expressing oocytes. No significant effects were observed at the broad concentrations investigated (Fig. 5d & Fig. 5f). These findings suggest that Si is not translocated across the membrane through the Na^+ or HPO_4^{2-} transport site, but through an independent transport site that is potentially involved in Si efflux primarily. In keeping with this possibility is the presence of multiple, often independent binding sites in a number of ABCD family members (47). Alternatively, Slc34a2 could cooperate with accessory proteins to promote Si efflux. In this regard, the Na^+/K^+ -ATPase gamma subunit FXYP2 appears to play a role in Mg^{2+} transport, while the alpha and beta subunits alone do not exhibit such transport capabilities (46).

Homology between Slc34a2 and Si transporters. Comparative sequence analysis of Slc34a2 indicated no significant homology with known plant or algal Si transporters. However, marked similarities were revealed upon pairwise alignment of the transmembrane domains of Slc34a2 and the plant Si efflux transporter Lsi2 (Fig. 7), thereby suggesting a conserved structure among Si efflux proteins.

Phylogenetic analysis of the Slc34 family. Sequence alignment within the rat Slc34 gene family led to the identification of a ~30-residue stretch that is only present in Slc34a2. Given that Slc34a1 and Slc34a3 were not upregulated under Si deprivation, this finding points towards the possibility that the ~30-residue stretch conveys Si-transport activity to Slc34a2 (Fig. 1c and Table 2).

Phylogenetic analysis of the Slc34a genes from a range of vertebrates (Supplementary Table 1 & Fig. 8) showed that the family underwent an expansion relatively early in vertebrate evolution, resulting in three distinct main groups (a1, a2, and a3) among the modern jawed vertebrates. At least one member of the a2-group was found in all of the jawed vertebrate genomes searched. In contrast, losses of the a1- and a3-group genes were observed in several

fully-sequenced genomes (e.g. zebrafish, chicken). This would suggest that the Slc34a2-group genes have a unique or important role whose loss cannot be complemented for by other transporters, and that this function is conserved across the jawed vertebrates. Common to all members of the Slc34a2 group, and to the homologous Slc34a2 gene in the lamprey, is a motif containing three positive amino acid residues (R, H or K) separated by smaller uncharged residues (commonly C or S) (Fig. 9). This motif aligns with the unique predicted transmembrane domain noted above in the rat Slc34a2 gene (Fig. 1c and Table 2), and points towards an important functional role. Conserved positively charged amino acids have been noted in other Si-related proteins, such as the GRQ motifs of the SIT active Si transporters (32, 51). It may be postulated that these residues interact with local negative charges on the silicic acid molecule as part of a general biochemical basis for transmembrane Si transport.

Conclusions

The identification that Slc34a2 can transport Si in mammals establishes another distinct gene family of Si-transporters that could be involved in regulation of Si homeostasis and that bears no sequence similarity with known Si-related genes in plants, sponges, choanoflagellates or diatoms, although it shows strong structural similarities to silicon exporters in plants. Crucially, our work is also one of the first pieces of evidence for a functionally relevant Si-responsive gene in mammals. In parallel with this work, Garneau *et al.* (18) and Deshmukh *et al.* (8) have recently identified Si-permeable aquaporins that appear to play an important role in Si influx. Coupled with the active efflux transporter that is reported herein, we propose a Si transport model in mammals that mirrors that known in plants (28), i.e., a model in which an influx and efflux transporter must be present to allow Si movement through cells. Here, Slc34a2 is effluxing H_4SiO_4 from the renal tubular epithelial cell into the circulation, i.e. it is

involved in the reabsorption of H_4SiO_4 in the kidneys. As an inevitable consequence of this expression at this cellular location, phosphate will be moved in the opposing direction. Dietary Si-P interactions have been noted, with animals on a Si deficient diet showing conserved bone Si levels but depleted bone P levels (24). Assuming Slc34a2 is similarly involved in bone-conservation of Si as it is in the kidney then our results explain these observation (24). Finally, it is also interesting to note that Lsi2's are equally upregulated in plants in conditions of Si deprivation (34), a phenomenon that was instrumental in identifying Slc34a2 in this work. Collectively our data provide indication that, rather than being a biochemically-inert element, Si in fact plays a role in vertebrate physiology deserving of its preservation under exposure conditions of deprivation.

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404 **Author Contributions:**

405 J.J.P, R.J, and S.R devised the competitive renal gene expression approach in Si deficiency
406 and sufficiency and strategically led the overall hypothesis with input from S.D.K. M.V.B
407 and M.M performed transcriptomic analysis and interpretation of transcriptomics data.
408 N.M.U and J.F.M designed and performed the Ge, As, and P influx experiments. R.R.B, R.D,
409 and J.V identified that Slc34a2 could have Si export activity and undertook the Si transport
410 experiments in *Xenopus* oocytes with input from P. I. S.R and A.M produced the Slc34a2-
411 GFP-pT7TS plasmid construct, verified *Xenopus* expression by fluorescent microscopy, and
412 performed the phylogenetic and bioinformatics analysis. J.R and J.W performed the IHC
413 analysis and helped with interpretation of the data. R.C.M. undertook the oxoacid and
414 oxoanion calculations. All authors contributed towards writing the manuscript.

415 **Footnote:**

416 Gene array data has been submitted to the GEO repository and assigned the reference
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Figure Captions and Tables:

Figure 1. Identifying *RnSlc34a2* as a candidate for Si transport. (a) Relative expression of solute-like carriers in the kidney of *Rattus norvegicus* from Si deplete (n=4) compared with Si replete (n=4) animals. Data was analysed by gene array. Red indicates up-regulation and blue down-regulation of expression; Si replete versus Si deplete group. Multiple probe sets per gene can be present as was the case for *Slc13a1*. (b) Quantitative PCR analysis of *Slc34a2* and of family members (inset) in the kidneys of Si-high reference, Si replete and Si deplete rats. Overall, the relative expression of *RnSlc34a2* was inversely related to dietary Si exposure ($p < 0.05$), but there was no relationship with *Slc34a1* or *Slc34a3* ($p = 0.5$ and 0.4 respectively). Gene expressions are relative to the Si-high reference group. (c) Sequence alignment of the *Rattus norvegicus* *Slc34* gene family. *Slc34a2* is characterized by a ~30-residue stretch (highlighted in yellow) that is not present in *Slc34a1* and *Slc34a3*. Asterisks (*) below sequence indicate identical amino acids, colons (:) indicate functionally similar amino acids and dashes (-) indicate gaps in the alignment.

Figure 2. Correlation between renal *RnSlc34a2* expression (by quantitative RT PCR analysis) and fasting urinary Si excretion. Urinary Si excretion in the rats (Si deplete (*squares*), Si replete (*circles*) and lab chow reference group (Si-high reference; *triangles*) showed an inverse relationship with *Slc34a2* expression in the kidneys; $r = 0.47$.

Figure 3. Immunohistochemistry analysis of *Slc34a2* in freshly harvested rat kidney cortex. Sections of freshly harvested kidneys from a healthy wild-type rat were analysed by immunohistochemistry with anti-*Slc34a2* (*green*) antibody (**this figure**) or the appropriate isotype control (**see Supporting Figure 1**). Cell nuclei were counterstained (*blue*) with Hoescht 33342 and cell cytoskeleton (f-actin, *red*) with phalloidin CF633. Antibody stained

sections and isotype controls were collected under identical settings, as stated in Methods. A threshold removing all Slc34a2 attributable signal was defined on the isotype controls and uniformly applied to all images (i.e. antibody stained images). Staining for Slc34a2 within the tubular epithelial cells was distinctly punctate so, as well as the signal above the isotype control being presented in an as-collected 'intensity' format (i.e. the more secondary antibody that is bound, the brighter the signal) (**A & B**), it is also displayed as a binary format (i.e. all signal that is brighter than isotype threshold is given the maximum intensity value) as this aids visualisation (**C & D**). All images are of the kidney cortex and scale-bars are 50 μ m. (**B**) As-collected 'intensity' format without actin staining. (**D**) A high power image (x63 magnification) of the area within the quadrant in image (**C**).

Figure 4. Fasting urinary phosphorus excretion. (**a**) Urinary P excretion was measured in the lab chow reference group (Si-high reference; n=6), Si replete (n=5) and Si deplete (n=8) rats by ICP-OES and corrected for creatinine concentration. The higher P excretion in the lab chow reference group is due to the higher P content of the diet (see (**b**)). However, the difference in urinary P excretion between the Si replete and Si deplete rats cannot be explained by a difference in dietary P content, but rather due to the upregulation of Slc34a2 in the latter group mediated by Si deficiency in the diet and drinking water (**b**).

Figure 5. Transport activity in *RnSlc34a2*-expressing oocytes. Influx transport activity of *Rattus norvegicus* Slc34a2 for (**a**) arsenate, HAsO_4^{2-} (p= 0.0001), (**b**) phosphate, HPO_4^{2-} (p=0.0008), and (**c**) silicic acid, H_4SiO_4 (p= 0.66). Rice transporter Lsi1 was used as a positive control for H_4SiO_4 influx (p <0.0001). The concentrations of sodium and phosphate in the medium did not influence H_4SiO_4 influx by *RnSlc34a2*-expressing oocytes (**d**), nor that by *OsLsi1*-expressing oocytes (p<0.0001 in both instances). Water-injected oocytes were

used as a negative control. In H_4SiO_4 efflux studies **(e)** rice transporter Lsi2 was used as a positive control. Data were corrected against water-injected control oocytes. **(f)** Changes in sodium and phosphate concentration did not affect H_4SiO_4 efflux by Slc34a2 expressing oocytes. Data are shown as means \pm SE (n=15).

Figure 6. Germanium transport activity in *RnSlc34a2*-expressing oocytes. Transport activity for H_4GeO_4 showing **(a)** a lack of influx ($p=0.14$) but significant efflux **(b)** following a 2 h incubation ($p=0.004$). Efflux was not significant at 30 minutes ($p=0.14$) for Slc34a2 expressing oocytes. The rice Si transporters Lsi1 and Lsi2 were used as positive controls for influx and efflux activity, respectively ($p<0.0001$ in both cases compared to negative control, water-injected oocytes).

Figure 7. Pairwise alignment of transmembrane domains of Si efflux transporters. Pairwise alignment of transmembrane domains predicted in *RnSlc34a2* rat protein (*red*) with the four Si efflux transporters in plants (*green*). Transmembrane domains were predicted by OCTOPUS (56) and subsequent alignment was performed by AlignMe tool (51). **(a)** *OsLsi2* (rice) **(b)** *ZmLsi2* (maize), **(c)** *HvLsi2* (barley), **(d)** *CmLsi2-1* (pumpkin).

Figure 8. Phylogeny of Slc34a gene family member in vertebrates. **(a)** The tree was produced using PhyML maximum likelihood analysis with the JTT +G+I model from an alignment of 880 positions. Numbers at nodes are a percentage of 100 bootstrap replicates, with nodes having less than 70% bootstrap support being collapsed. **(b)** The tree was produced using Phylobayes Bayesian MCMC analysis under the CAT +G+I model (15 parallel chains with sampling every 100 cycles, burn-in one-fifth the total size of the chain) from an alignment of 880 positions. Numbers at nodes indicate posterior probabilities, with

nodes having less than 0.95 support being collapsed. The scale bar indicates the average number of amino acid substitutions per site. The Slc34a1 clade is in green, the Slc34a2 clade in *blue* and the Slc34a3 clade in *red*. The trees are rooted using the single Slc34a homolog identified from the Lamprey genome. The Slc34a gene phylogeny largely agrees with the species phylogeny for vertebrates (33), with incongruent branches (e.g. the basal branches of the a2 clade) only having low statistical support. The maximum likelihood phylogenetic analyses resolve that the Slc34a clade evolved from a single ancestor in jawless vertebrates, and likely involved two main duplication events, initially producing the a3 and a1+2 clades, with a further divergence of the a1 and a2 clades. A teleost-specific duplication event resulted in the evolution of Slc34a2a and Slc34a2b, as found in stickleback and zebrafish. The Bayesian analysis had poor phylogenetic resolution at the base of the a2 clade, but still resolves the a1 and a3 groups as distinct monophyletic clades, and is not incongruous with the maximum likelihood analysis results. For full details of the species and sequences used see Supplemental Table 1.

Figure 9. Alignment of vertebrate Slc34a protein sequences showing characteristic motif conserved across members of the Slc34a2 group. The alignment shows the region around the portion identified as unique to rat Slc34a2 in comparison to rat Slc34a1 or a3 (red lettering, see Fig. 1c). Highlighted in yellow are the homologous regions in other vertebrate Slc34a2 proteins, and in the Slc34a-type lamprey sequence. The characteristic Slc34a2 motif identified within this region contains at least three positive residues uninterrupted by any negatively charged residues, with the positive residues regularly spaced apart by at least four small residues (primarily cysteines). A Slc34a sequence containing this motif was found in all vertebrate species investigated. The only members of the Slc34a2 clade (see Fig. 8) where this motif was incomplete was are in the zebrafish and stickleback SLC34a2b (highlighted in

673 *blue*). Positively charged residues are shown in bold and underlined, small amino acids are in
674 italics. Sequence names correspond to the species and gene identifiers given in Supplemental
675 Table 1 and to the phylogeny shown in Fig. 8. The incomplete spiny shark and skate NaPi-IIb
676 sequences are omitted due to this region being missing from the EMBL/Genbank data. The
677 alignment was generated using MUSCLE (<http://www.ebi.ac.uk/Tools/msa/muscle/>).

678 **Table 1. Oxoacid and oxoanion volumes**

Oxoacid/Oxoanion	Gas phase	Implicit aqueous solvent
	V_M (cm ³ /mol)	V_M (cm ³ /mol)
HCO ₃ ⁻	41.9	39.1
H ₃ BO ₃	43.4	43.6
H ₄ BO ₄ ⁻	52.5	55.8
H ₄ SiO ₄	63.2	56.5
H ₄ GeO ₄	61.3	57.8
HPO ₄ ²⁻	62.0	59.2
HAsO ₄ ²⁻	65.0	63.1

679 PBE0/6-31++g(d,p) calculated solvent accessible surface molar volumes (V_M) for various
680 main group oxoacids and oxoanions at physiological pH, showing the similarities between
681 H₄SiO₄, H₄GeO₄ (two oxoacids that are effluxed by Slc34a2), HPO₄²⁻ and HAsO₄²⁻
682 (oxoanions that are influxed by Slc34a2).

683 **Table 2. Predicted transmembrane domains of Slc34a2**

Helices No.	N-terminal	Transmembrane region	C-terminal	Helices type	Length
1	95	FQGIGKFILLLGFLYLFVCSLDV	117	1°	23
2	137	NSIMSNPVAGLVIGVLVTVMVQS	159	2°	23
3	166	IIVSMVASSLLSVRAAPIIMGA	188	2°	23
4	374	LILCGCLIMIVKLLGSVLRGQVA	396	1°	23
5	422	VGAGMTFIVQSSSVFTSAMTPLI	444	1°	23
6	459	LGSNIGTTTTAILAALASPGNTL	481	2°	23
7	527	WFAVFYLIFFLLTPLTVFGLSL	549	1°	23
8	555	LVGVGVPIILLILLVLCLRMLQA	577	1°	23
9	618	CCCCRVCCRVCCMVCGCKCCRC	640	2°	23

684 The transmembrane domains were predicted using SOSUI software. The sequence
685 highlighted (*yellow*) through multiple sequence alignment of the three *Rattus norvegicus*
686 Slc34 family members (Fig. 1c) is present in the ninth transmembrane helix of Slc34a2. The
687 C-terminal and N-terminal amino acid for each transmembrane domain are indicated, as is
688 the type of alpha-helical structure (i.e. primary or secondary helices, denoted as 1° and 2°
689 respectively).

690 Supplemental Table 1. Sequences used for the vertebrate Slc34a phylogeny

Species Name	Common Name	Gene Name	Accession No.	Notes
<i>Rattus norvegicus</i>	Rat	Slc34a1	gi 6981544	
<i>Rattus norvegicus</i>	Rat	Slc34a2	gi 16758110	
<i>Rattus norvegicus</i>	Rat	Slc34a3	gi 21326473	
<i>Mus musculus</i>	Mouse	Slc34a1	gi 66793411	
<i>Mus musculus</i>	Mouse	Slc34a2	gi 66793411	
<i>Mus musculus</i>	Mouse	Slc34a3	gi 224994177	
<i>Homo sapiens</i>	Human	Slc34a1	gi 156627569	
<i>Homo sapiens</i>	Human	Slc34a2	gi 295789158	
<i>Homo sapiens</i>	Human	Slc34a3	gi 25014088	
<i>Bos taurus</i>	Cow	Slc34a1	gi 157073970	
<i>Bos taurus</i>	Cow	Slc34a2	gi 27807195	
<i>Bos taurus</i>	Cow	Slc34a3	gi 528970641	
<i>Loxodonta africana</i>	Elephant	Slc34a1	gi 344265327	
<i>Loxodonta africana</i>	Elephant	Slc34a2	gi 344298758	
<i>Loxodonta africana</i>	Elephant	Slc34a3	gi 344309900	
<i>Monodelphis domestica</i>	Opossum	Slc34a1	gi 126291054	
<i>Monodelphis domestica</i>	Opossum	Slc34a2	gi 126331900	
<i>Monodelphis domestica</i>	Opossum	Slc34a3	gi 126302869	
<i>Ornithorhynchus anatinus</i>	Platypus	Slc34a1	gi 149423843	
<i>Ornithorhynchus anatinus</i>	Platypus	Slc34a2	gi 345307737	
<i>Anolis carolinensis</i>	Lizard	Slc34a1	gi 327265671	
<i>Anolis carolinensis</i>	Lizard	Slc34a2	gi 327282884	
<i>Gallus gallus</i>	Chicken	Slc34a1	gi 513206663	
<i>Gallus gallus</i>	Chicken	Slc34a2	gi 46048932	
<i>Xenopus tropicalis</i>	Frog	Slc34a2	gi 46195785	Multiple hits, several duplicates or redundant sequences
<i>Xenopus tropicalis</i>	Frog	Slc34a3	gi 301622941	Multiple hits, several duplicates or redundant sequences
<i>Latimeria chalumnae</i>	Coelacanth	Slc34a2	ENSLACP00000002539	Genome not yet searchable within EMBL/Genbank database
<i>Latimeria chalumnae</i>	Coelacanth	Slc34a3	ENSLACP000000014496	Genome not yet searchable within EMBL/Genbank database
<i>Takifugu rubripes</i>	Pufferfish	Slc34a1	gi 410915152	
<i>Takifugu rubripes</i>	Pufferfish	Slc34a2	gi 410920832	
<i>Danio rerio</i>	Zebrafish	Slc34a2	gi 33504533	
<i>Danio rerio</i>	Zebrafish	SLC34 2a	gi 18859377	
<i>Squalus acanthias</i>	Spiny Shark	NaPi-IIb1	gi 11494391	No genome available, Single sequence deposit, not full length transcript
<i>Squalus acanthias</i>	Spiny Shark	NaPi-IIb2	gi 11494393	No genome available, Single sequence deposit, not full length transcript
<i>Callorhynchus milii</i>	Elephant Shark	SLC34a	AAVX01126234.1	Draft genome, not yet searchable within EMBL/Genbank database, not full length transcript
<i>Leucoraja erinacea</i>	Skate	NaPi-IIb1	gi 11494395	No genome available, Single sequence deposit, not full length transcript
<i>Leucoraja erinacea</i>	Skate	NaPi-IIb2	gi 11494399	No genome available, Single sequence deposit, not full length transcript
<i>Petromyzon marinus</i>	Lamprey	SLC34A	ENSPMAP00000003321	Genome not yet searchable within EMBL/Genbank database

The Slc34a sequences were selected on the basis of taxonomic coverage of relevant vertebrate groups, availability of fully sequenced genomes and sequence completeness and homology to rat Slc34a genes. Where multiple homologs were recovered from the same genome project, the sequences were inspected to remove any duplicated or erroneous sequences. Taxonomic classifications are based on the EMBL/Genbank or genome sequencing project annotations as relevant. The common names shown are used in the Slc34a phylogeny in Fig. 8. Gene names are given according to the relevant BLASTp or tBLASTn hit to rat Slc34a1, a2 or a3. In cases where the sequences were recovered from incompletely sequenced genomes or where the homology was unclear a more general gene name classification was used. In the case of the Spiny Shark and Skate sequences the gene names were taken directly from the EMBL/Genbank annotations and refer to "Type II Sodium-Phosphate co-transporter", a synonym of Slc34a.

04 **Supplemental Table 2. Modified Barth's solutions**

05 (i) Modified Barth's Saline (MBS) solution

	Na ⁺	Cl ⁻	NO ₃ ⁻	K ⁺	Ca ²⁺	Mg ²⁺	HPO ₄ ²⁻	SO ₄ ²⁻	HCO ₃ ⁻	HEPES	Osm
	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mmol/L)
NaCl	82	82	82								164
KCl	1		1	1							2
Ca(NO ₃) ₂	0.33				0.33						0.99
CaCl ₂	0.41		0.82		0.41						1.23
MgSO ₄	0.82					0.82		0.82			1.64
NaHCO ₃	2.4	2.4							2.4		4.8
Na HEPES	10	10								10	20
Osm (mmol/L)		94.4	83.82	0.66	1	0.74	0.82	0	0.82	2.4	195

06 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); Osm (osmolarity)

07 (ii) MBS1 (MBS supplemented with 1.7 mM H₄SiO₄)

	Na ⁺	Cl ⁻	Rb ⁺	Ca ²⁺	Mg ²⁺	HPO ₄ ²⁻	SO ₄ ²⁻	HEPES	HCO ₃ ⁻	NO ₃ ⁻	Si(OH) ₄	Osm
	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mmol/L)
NaCl	82	82	82									164
RbCl	1		1	1								2
Ca(NO ₃) ₂	0.33			0.3						0.7		1
CaCl ₂	0.41		0.8	0.4								1.2
Na HEPES	10							5				5
MgSO ₄	0.82				5		5					10
NaHCO ₃	2.4	5							5			10
Na ₂ O ₃ Si	1.7	4									2	6
Osm (mmol/L)		91	83.82	1	0.74	5	0	5	5	0.66	2	199

08 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); Osm (osmolarity)

09 (iii) MBS2 (1.7 mM H₄SiO₄, 10 mM Na⁺ & 0.5 mM HPO₄²⁻)

	Na ⁺	Cl ⁻	NO ₃ ⁻	NMG	K ⁺	Ca ²⁺	Mg ²⁺	HPO ₄ ²⁻	SO ₄ ²⁻	HCO ₃ ⁻	HEPES	Si(OH) ₄	Glu	Osm
	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mmol/L)
NMG-Cl pH 7.4	74		74	74										148
NaCl	6	6	6											12
KCl	1		1		1									2
Ca(NO ₃) ₂	0.33			0.66		0.33								0.99
CaCl ₂	0.41		0.82			0.41								1.23
NMG HPO ₄	0.5			0.5				0.5						1
NMG HEPES	15			15							15			30
MgSO ₄	0.82						0.82		0.82					1.64
Na ₂ O ₃ Si	1.7	4										2		6
Ouabain	0.1													
NMG gluconate	1.5			1.5									1.5	3
Osm (mmol/L)		10	81.82	0.66	91	1	0.74	0.82	0.5	0.82	15	2	1.5	206

10 NMG (N-methyl-D-glucamine); HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); Osm (osmolarity); Glu (gluconate)

11 (iv) MBS3 (0 mM Na⁺ & 0 mM HPO₄²⁻)

		Na ⁺	Cl ⁻	NO ₃ ⁻	K ⁺	Ca ²⁺	Mg ²⁺	HPO ₄ ²⁻	SO ₄ ²⁻	HCO ₃ ⁻	HEPES	Osm
	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mmol/L)
NMG-Cl pH 7,4	80		80									160
KCl	1		1		1							2
Ca(NO ₃) ₂	0.33			0.66		0.33						0.99
CaCl ₂	0.41		0.82			0.41						1.23
NMG HEPES	15										15	30
MgSO ₄	0.82						0.82		0.82			1.64
Osm (mmol/L)		0	81.82	0.66	1	0.74	0.82	0	0.82	0	15	196

12 NMG (N-methyl-D-glucamine); HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); Osm (osmolarity)

13

14

15 (v) MBS4 (10 mM Na⁺, 0.5 mM HPO₄²⁻)

		Na ⁺	Cl ⁻	NO ₃ ⁻	NMG	K ⁺	Ca ²⁺	Mg ²⁺	HPO ₄ ²⁻	SO ₄ ²⁻	HEPES	Glu	Osm
	mM	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mmol/L)
NMG-Cl pH 7,4	74		74		74								148
NaCl	6	6	6										12
KCl	1		1			1							2
Ca(NO ₃) ₂	0.33			0.66			0.33						0.99
CaCl ₂	0.41		0.82				0.41						1.23
NMG HPO ₄	0.5				0.5				0.5				1
NMG HEPES	15				15						15		30
MgSO ₄	0.82							0.82		0.82			1.64
Ouabain	0.1												
NAG gluconate	4	4										4	8
Osm (mmol/L)		10	81.82	0.66	89.5	1	0.74	0.82	0.5	0.82	15	4	205

16 NMG (N-methyl-D-glucamine); HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]; Osm (osmolarity); Glu (gluconate)

17

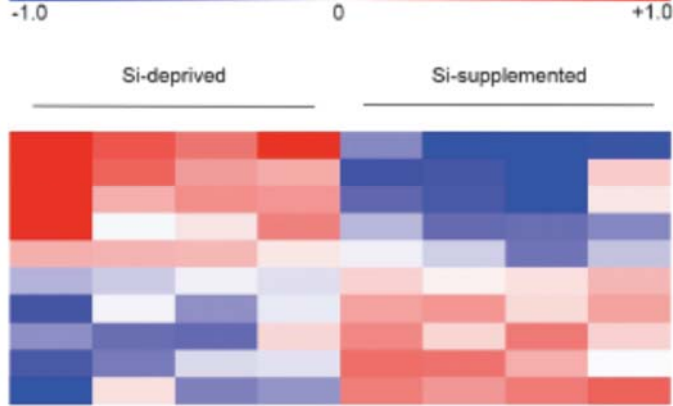
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19 (vi) MBS5: 84 mM Na⁺ & 2 mM HPO₄²⁻

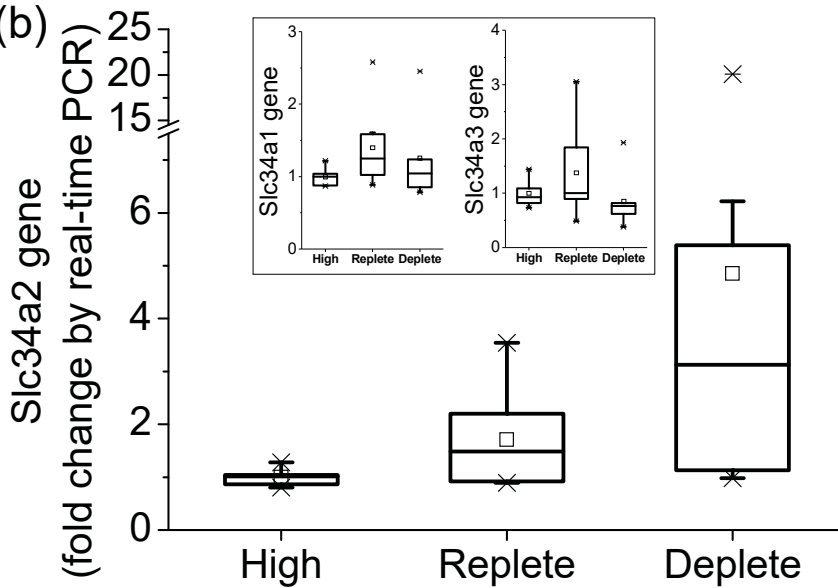
		Na ⁺	Cl ⁻	NO ₃ ⁻	NMG	K ⁺	Ca ²⁺	Mg ²⁺	HPO ₄ ²⁻	SO ₄ ²⁻	HCO ₃ ⁻	HEPES	Glu	Osm
	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mM)	(mmol/L)
NaCl	80	80	80											160
KCl	1		1			1								2
Ca(NO ₃) ₂	0.33			0.66			0.33							0.99
CaCl ₂	0.41		0.82				0.41							1.23
NMG HPO ₄	2				2				2					4
NMG HEPES	15				15							15		30
MgSO ₄	0.82							0.82		0.82				1.64
Ouabain	0.1													
NAG gluconate	4	4											4	8
Osm (mmol/L)		84	81.82	0.66	17	1	0.74	0.82	2	0.82	0	15	4	208

20 NMG (N-methyl-D-glucamine); HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]; Osm (osmolarity); Glu (gluconate)

(a)



(b)



(c)

Slc34a2 | MAPWPELENA---HPNPNK---FIEGASG---PQSSIPDKDKG-TSKTNDSGTPVAKIEL
 Slc34a1 | MMSYSERLGGPAVSPPLVVRGRHMVHGAFAFYVSPQVLHRIPGTTTYAISSLSPVALTEH
 Slc34a3 | M---PNSLAGDQ-VPNPRLD---AIGLVDWSLRNAGTSGSTPG-----

Slc34a2 | LPSYSALVLEEPPPEGNDPDLPELQDNGIKWSEKSGKILCFQIGKFIILLGFLYL
 Slc34a1 | SCPYGEVL-----ECHDPLPAKLAQEEQKPEPRLSQ-KLAQVGTLLKVLPLMLGFLYL
 Slc34a3 | -----LEEG--GTDPTWTSQKNTDQKKEVGTAS-KLHQVVSGLKACGLLGSLYF

Slc34a2 | FVCSLDVLSSAFQLVGGKMGAGQFFSNNSIMSNPVAGLVIGVLTVMVQSSSTSSSIIVSM
 Slc34a1 | FVCSLDVLSSAFQLVGGKMGAGQFFSNNSIMSNPVAGLVIGVLTVMVQSSSTSSSIIVSM
 Slc34a3 | FICSLDILSSAFQLVGGKMGAGQFFSNNSIMSNPVAGLVIGVLTVMVQSSSTSSSIIVSM

Slc34a2 | VASSLLSVRAAIPIMGANGTISITNTIVALMQAGDRNEFRRAFAGATVHDFFNWLSVLV
 Slc34a1 | VSSGLLEVSSAIPIMGANGTISITNTIVALMQAGDRNEFRRAFAGATVHDFCNWLSVLV
 Slc34a3 | VASKSLTVQASVPIIMGVNVTGITSITLVMSAQSGDRDEFQRAFSGSAVHGIFNWLTVLV

Slc34a2 | LLPLEAATHYLEKLTNLVLETFQNGEDAPDILKVITDPFTKLIQLDKKVIQIIMGD
 Slc34a1 | LLPLEAATGYLHHVTGLVVASFNIRGGRDAPDLKVITEPFTKLIQLDKSVITSIIVGD
 Slc34a3 | LLPLENATAALERLSELALGAASLPQGQAPDILKALTRPFTHLIIQLDSSVVTSSITSN

Slc34a2 | SEAQNKSLIKIWCKTISNVIEENVTVSPDNCTSPSYCWTGDIQTWTIQNVTEKENIAKC
 Slc34a1 | ESLRNHSLIRIWQCP-----ETKEASTSMSRVEAIG---SLANTT---MEKC
 Slc34a3 | TT--NSSLIKHWCGRG-----ETPQG--SSECDLSGSCTERNSSASPGEDRLLC

Slc34a2 | QHIFVNFSLPDLAVGIILLTVSLLILCGCLIMIVKLLGSVLRGQVATVIKKTINTDFPFP
 Slc34a1 | NHIFVDTGLPDLAVGLILLAGSLVLCCLILVLMNLSLLKGQVANVIQKVINTDFPAP
 Slc34a3 | HHLFAGSELTDLAVGFIILLAGSLVLCVCLVLIVKLLNSVLRGRIQAVKTVINADFPFP

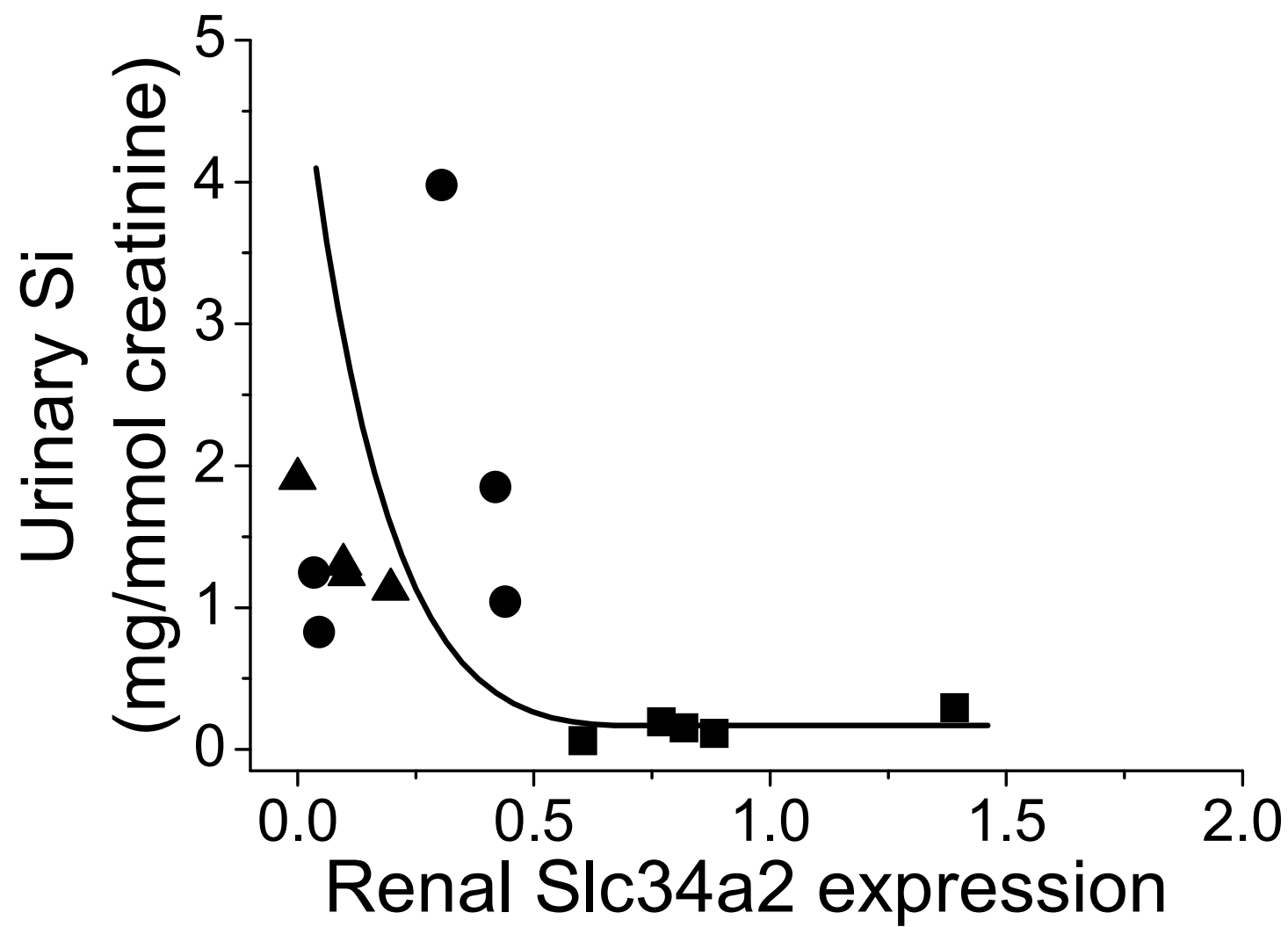
Slc34a2 | FAWLTGYLAILVGAGMTFIVQSSSVFTSAMTPLIGIVISIERAYPLTLGSGNIGTTTTAI
 Slc34a1 | FTWVTGYFAMVVGASMTFVQSSSVFTSAITPLIGLGVISIERAYPLTLGSGNIGTTTTAI
 Slc34a3 | FGWLSGYLAILVGAGLTFLQSSSVFTAAIVPLMGVGVINLERAYPLFLGSGNIGTTTTAL

Slc34a2 | LAALASPGNTLRSSQLIALCHFFFNISGILLWYPIPFTRLPRLAKGLGNISAKYRWFAV
 Slc34a1 | LAALASPREKLSSSQIALCHFFFNISGILLWYPLPCTRLPIRMALGKRTAKYRWFAV
 Slc34a3 | LAALASPADTLLFAVQVALIHFFFNLAGILLWYLPVLRPLPIPLAKRFGDLTAQYRWFAI

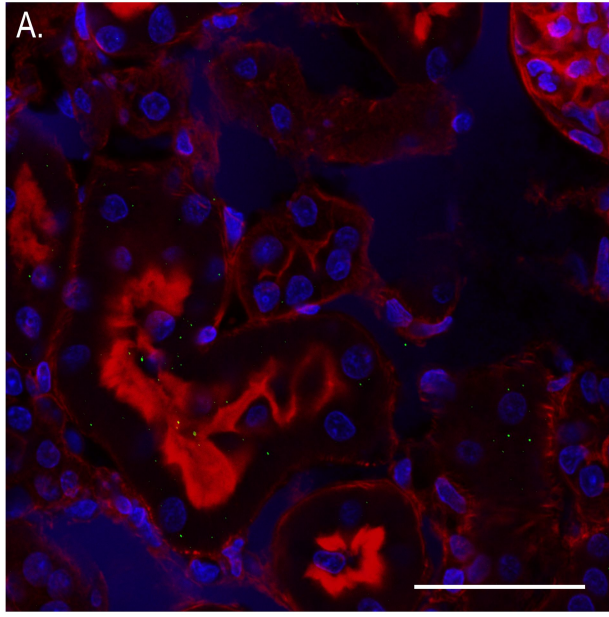
Slc34a2 | FYLIFFFLTLPLTVFGLSLAGWPVLVGVGVPIILLIILLVLCRLMLQARCPRIPLPLKLRDW
 Slc34a1 | LYLLVCFLLLPSLVFGISMAGWQAMVGVGTPFGALLAFVVLNVNLQSRSPGHLPKWLQTF
 Slc34a3 | VYLLLTFLLLPLAAGFLSLAGGSVLAAGVGPLVGLVLLIILVNVLQRRHPSWLPRLQSW

Slc34a2 | NFLPLWMHSLKPDWNIISLATSCFQRRCCCCRVCCRVCCMVCGCKCCRCCKCNLEEE
 Slc34a1 | PRSPQLPVRV-----FLEELPAPTSPR--LALPAHHNATRL
 Slc34a3 | -----PFKA---YNSHMTSKVACHYENPQ-VIASQQL-----

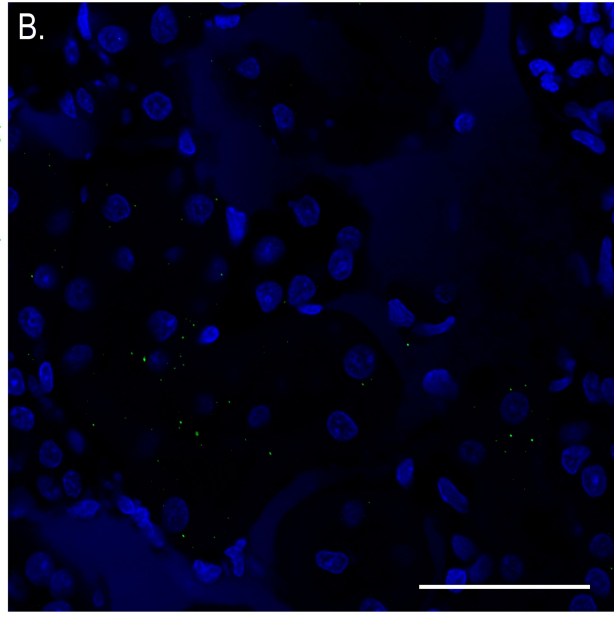
Slc34a2 | EKEQDVPEKASGGFDNTAMSEKQDEGKGQVEVLGMKALSNTTVF
 Slc34a1 | PRSPQLPVRV-----FLEELPAPTSPR--LALPAHHNATRL
 Slc34a3 | -----PFKA---YNSHMTSKVACHYENPQ-VIASQQL-----



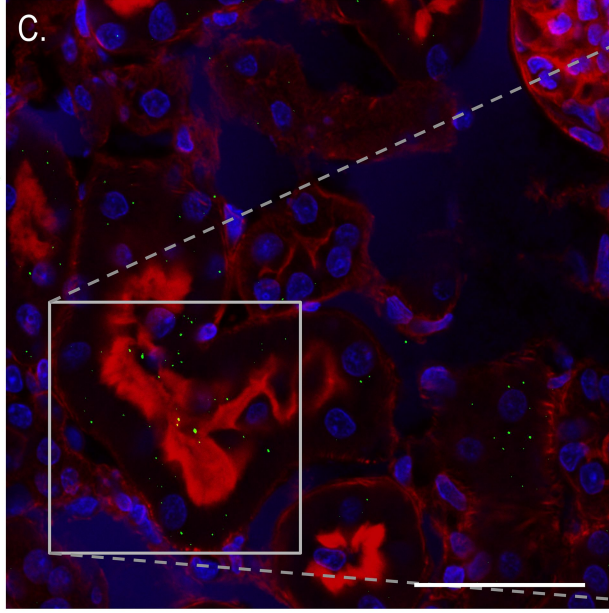
Nuclei / Actin / SLC34A2 (intensity)



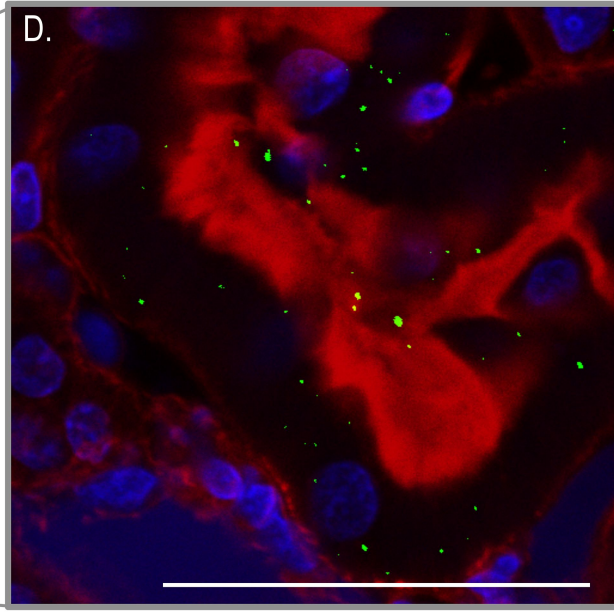
Nuclei / SLC34A2 (intensity)

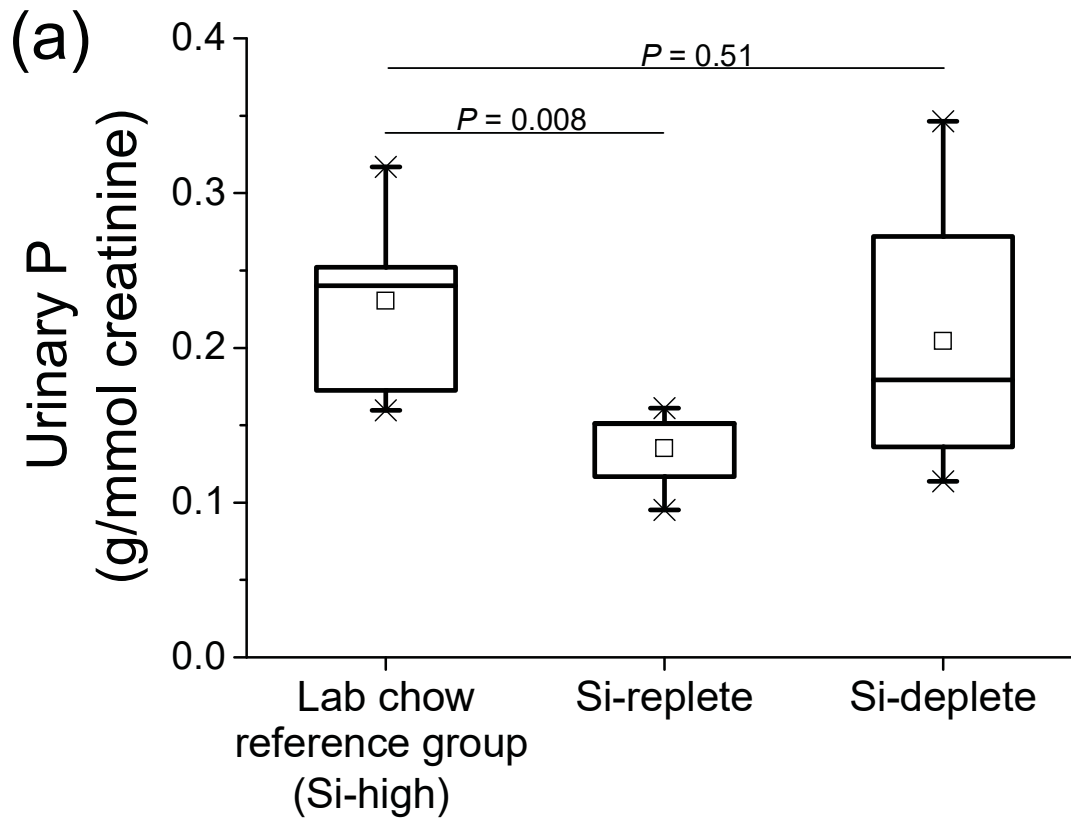


Nuclei / Actin / SLC34A2 (binary)



Inset: Nuclei / Actin / SLC34A2 (binary)

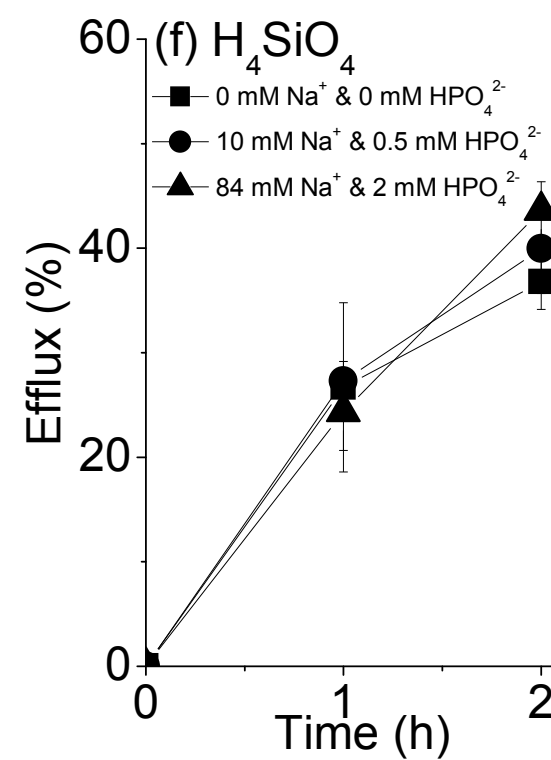
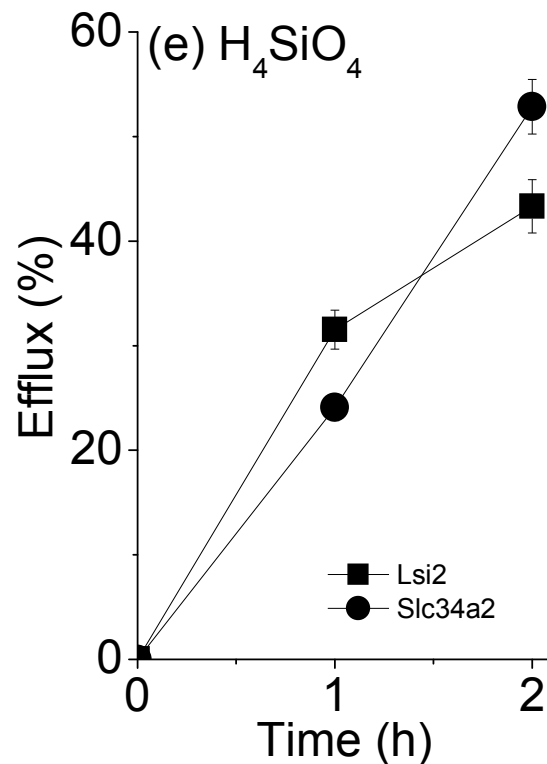
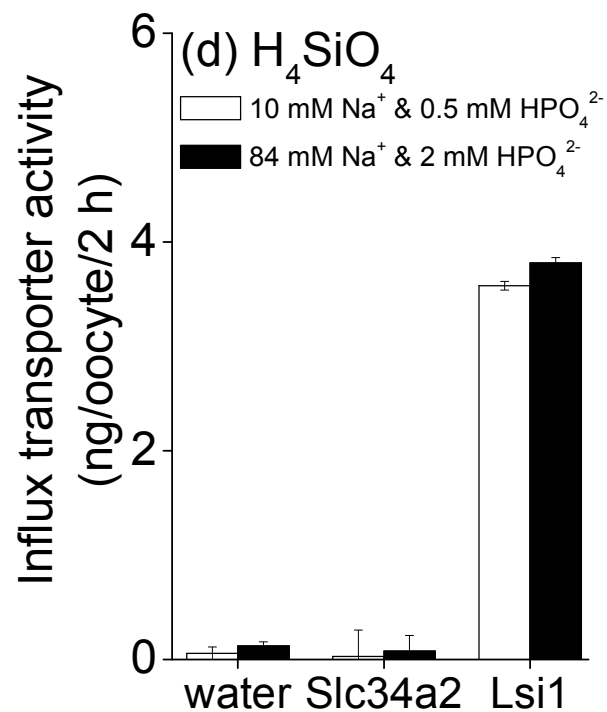
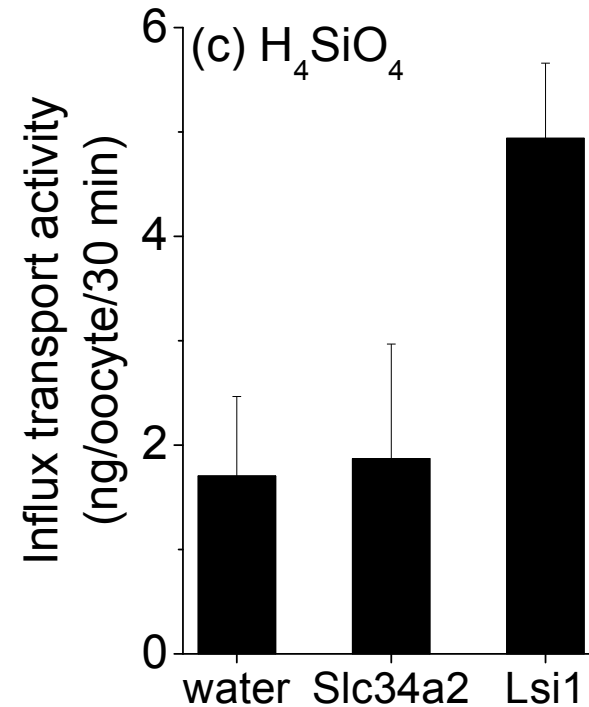
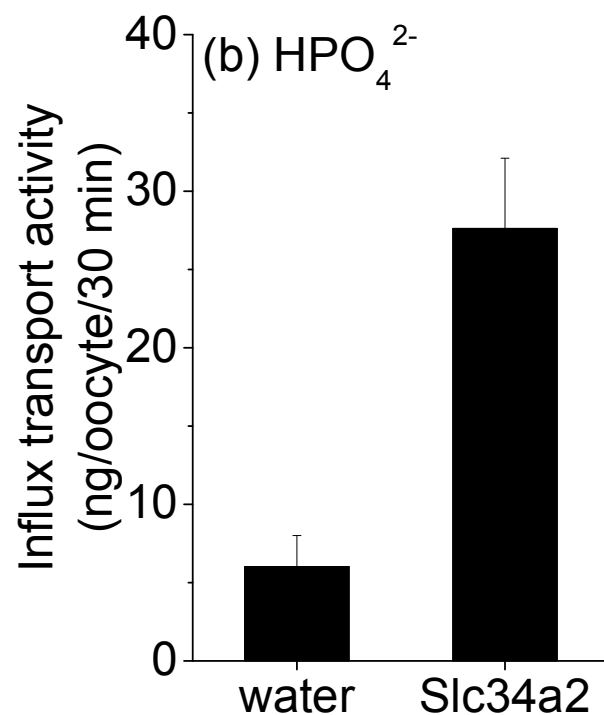
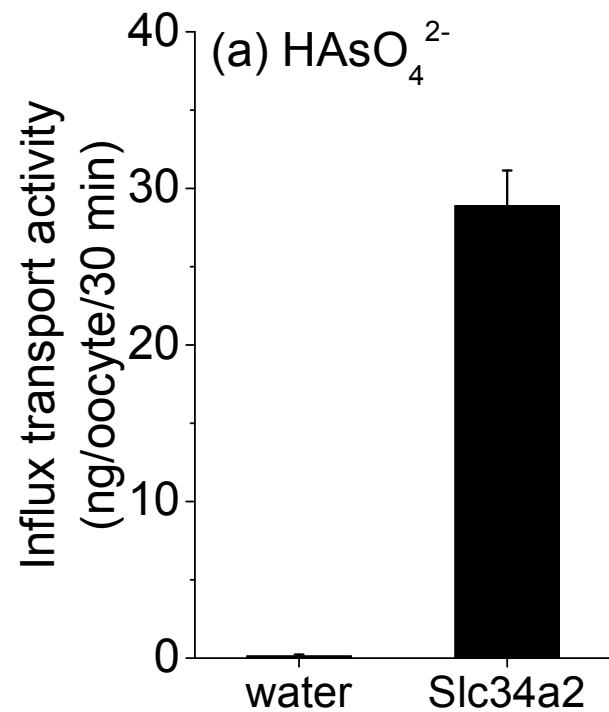


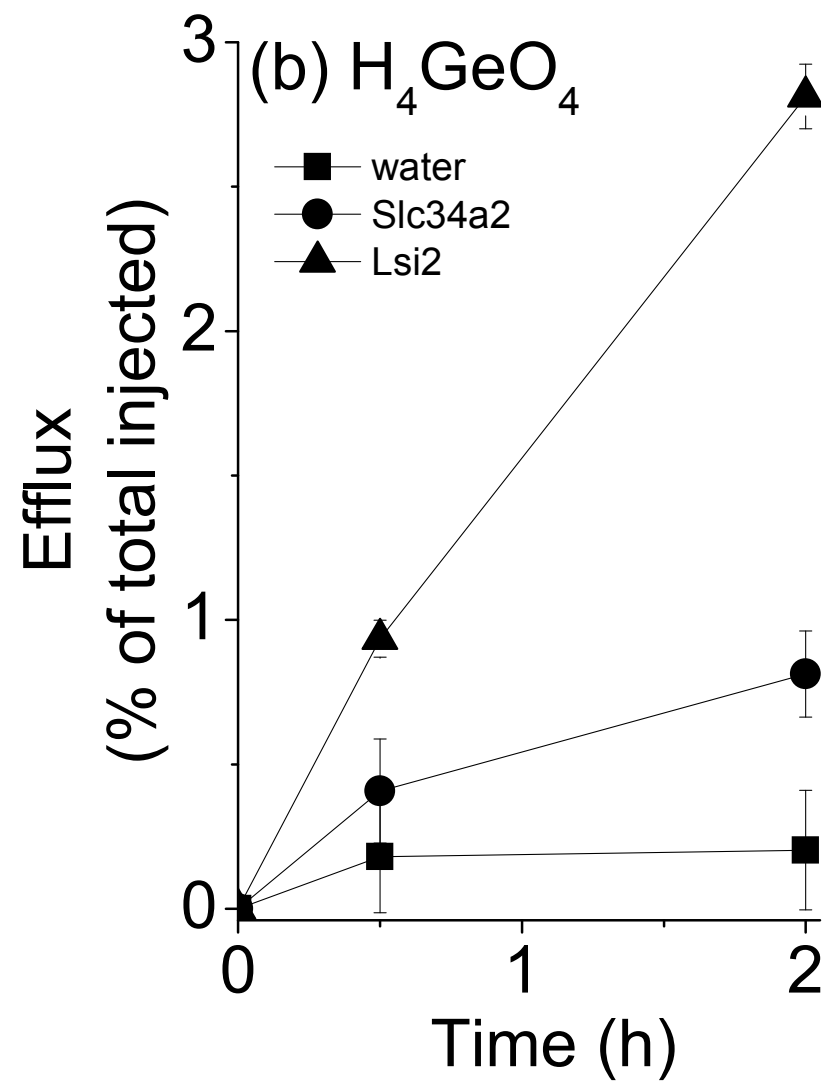
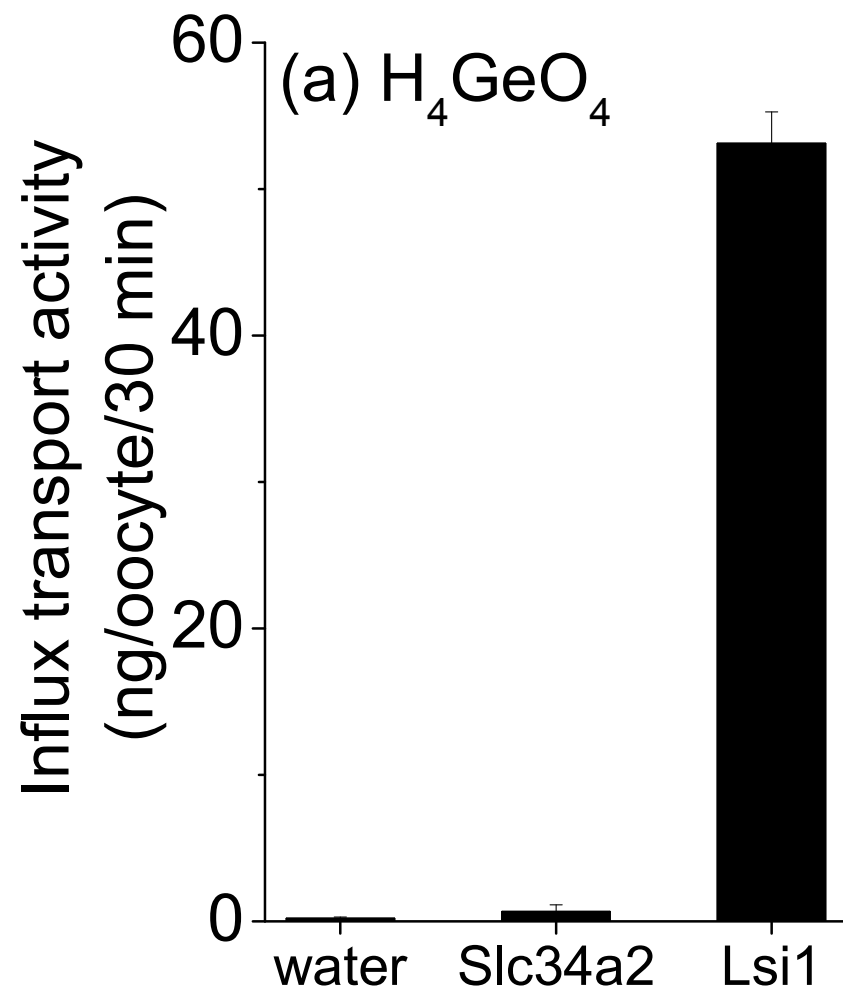


(b)

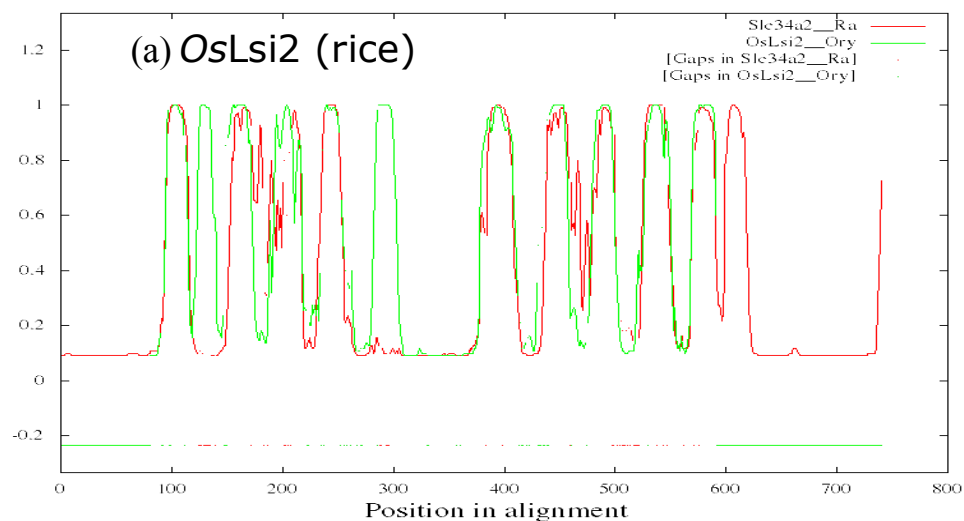
	Lab chow reference group (Si-high)	Si -replete	Si-deplete
Renal Slc34a2 (relative expression)*	0.336 ± 0.062	0.574 ± 0.308	1.63 ± 1.90
Si ($\mu\text{g/g}$ feed)	322 ± 47	3.2 ± 0.6	3.2 ± 0.6
Si ($\mu\text{g/g}$ drinking water)	5.04 ± 1.12	53.2 ± 0.6	0.015 ± 0.010
P (g/kg feed)	7.00	2.28	2.28

*Summarised from Figure 1b

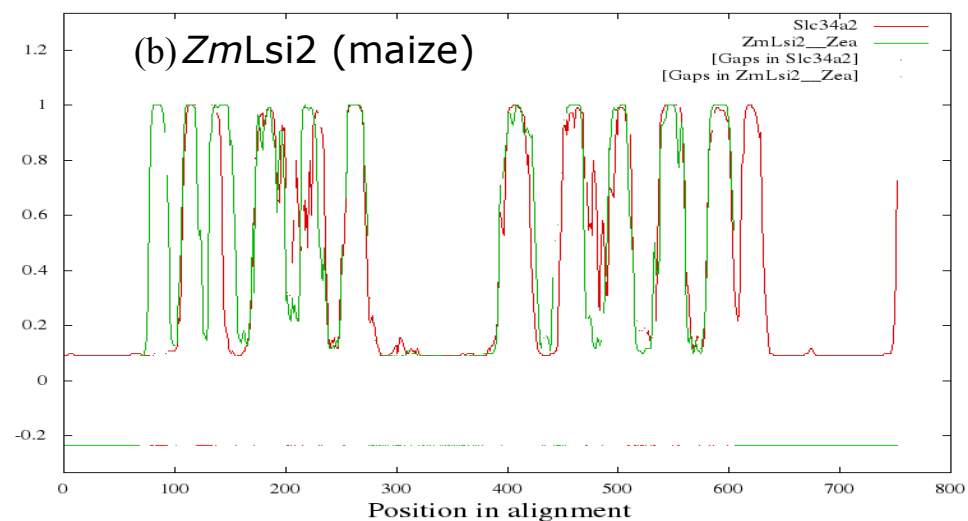




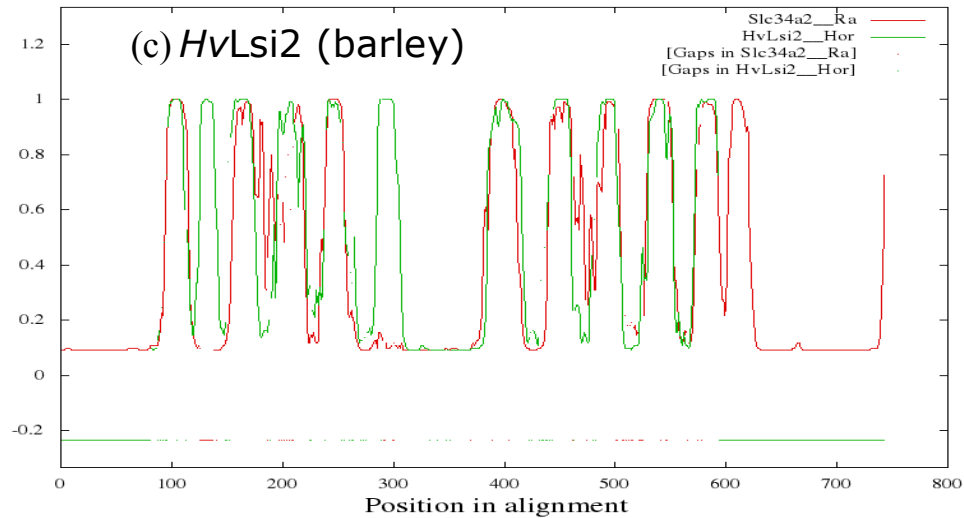
Membrane probabilities obtained with OCTOPUS



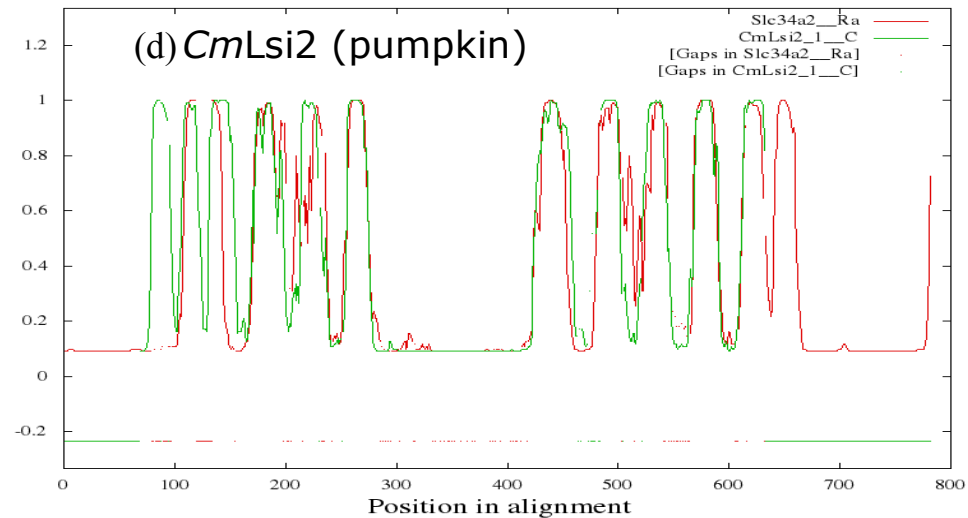
Membrane probabilities obtained with OCTOPUS

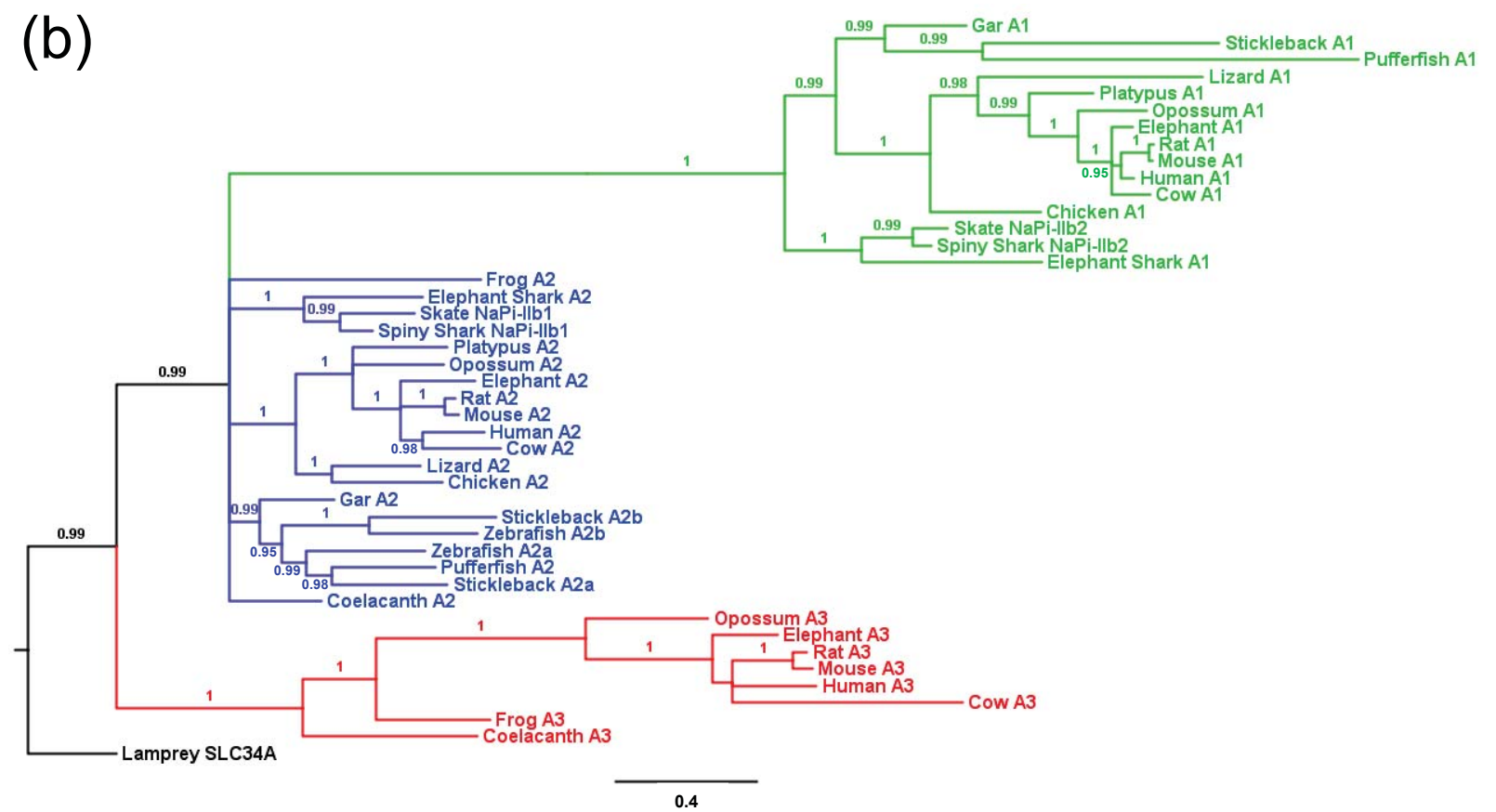
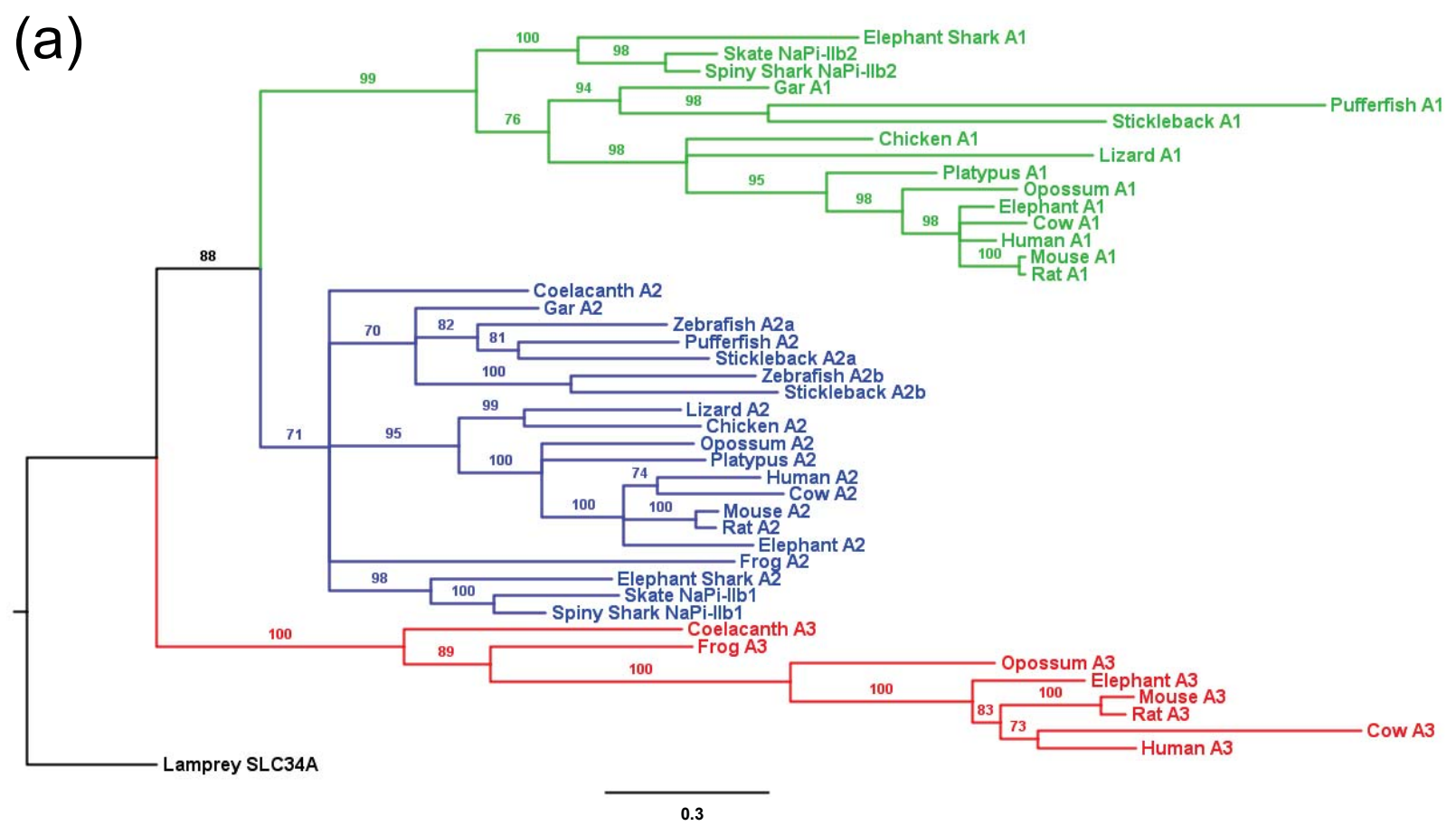


Membrane probabilities obtained with OCTOPUS



Membrane probabilities obtained with OCTOPUS





Pufferfish Slc34a1	NWDFFPiWMTSLQPIDDLITRMS-----RVCrQNrgWwRI--HrNRSTtPlERgTVHtVTN
Stickleback Slc34a1	SWDFLPKWMRS�KPLDRLiTKATA-----CGCS-----GHQDARGEDGGDGRMSTKEIVRESAQKME----QL
Lizard Slc34a1	SWDFLPFwLRS�QPMdGLITRtMR-----CCTV-----CCSYsREKHSATSPQMKaGLcNPslSfLGELSLPPKPSs
Gar Slc34a1	SWDFLPgWmHSLKPMdAFITNATL-----CCTV-----HCSTKNSdVQgQpVfSDSfAEKKAKMA-----
Elephant Shark Slc34a1	NWDFLPiWmHSLKPMdRvITNvTL-----YCT-----NHCRcSEKTDLKEVNCQqERSpQLKEK----AV
Chicken Slc34a1	SWDFLPaWmHSLQPLdSLITRATL-----CCT-----DRCrSPeGWD---EREAAARDKARLGldNPvL
Opossum Slc34a1	TWDFLPyWmHSLKPLdRLITRATL-----CCTH-----PEPRSPPLpTRVYLEELpTATpSPRLGvLPpDT
Platypus Slc34a1	TWEFLPRWmRS�KPLdGLITRATL-----CCAH-----ADPAKSPRLPARATyDNpGARvYLQEL----PR
Cow Slc34a1	TWDFLPŁWmHSLKPLdRLITRATL-----CCAR-----AEPRSPPLPARVfLEELPPATpSPHLA-----
Mouse Slc34a1	TWDFLPřWmHSLQPLdGLITRATL-----CYAR-----PEPRSPQLPPRVfLEELPPATpSPRLA-----
Rat Slc34a1	TWDFLPřWmHSLQPLdGLITRATL-----CYAR-----PEPRSPQLPPRVfLEELPPATpSPRLA-----
Elephant Slc34a1	TWDFLPQWmHSLKPLdHfITRATL-----CCAR-----PEPRSPMPRRVfLEELPPATpSPHLA-----
Human Slc34a1	TWDFLPřWmHSLKPLdHLITRATL-----CCAR-----PEPRSPPLPPRVfLEELPPATpSPRLA-----
Frog Slc34a2	NWDFLPKWMHSLKPDWScMGgASL-LCR HFCCCC -----CGKMCKPCKCCCKCHDK----EDEEYSIEPKQA----LE
Platypus Slc34a2	NWDFLPKWMHSLKPDWSVVSGLTG-SFR KCCCCC -----RiCCMLCGCPPCCRCs KCCR DSGTedeEPaKdIPVKG----GE
Opossum Slc34a2	NWHFLPLWmRS�QPDGIVSLTGnCCQLPCCWCC RACCRVCCLLCGCPRCRCsK CCDLL---EEENvKEIPIKV----PE
Cow Slc34a2	SWDFLPFwMRSLEPwDKLITSLTS-CFQM RCCCCCRVCCRLCCGLCGCSKCCRCtK CS----EDLEEGKDEPVKS----PE
Elephant Slc34a2	NWNFLPLWmHSLKPDWHLISLTS-CCQ RCCCC ---CCHLCCVLCGCP KCCRCM KYLEDL-EEGQEYSKDTPIKT----PN
Human Slc34a2	NWNFLPLWmRS�KPDWDAVVSKFTG-CFQM RCCCCCRVCCRACCLLCGCPKCCRCsK CCEDL--EEAQEGQDVpVKA----PE
Mouse Slc34a2	DWNFLPLWmHSLKPDWNVISLATT-CFQR RCCCCCRVCCRVCCMVCGC-KCCRCsKCCR DQGEeeeeEKQdIPVKA---SG
Rat Slc34a2	DWNFLPLWmHSLKPDWNIISLATS-CFQR RCCCCCRVCCRVCCMVCGC-KCCRCsKCC KNL---EEEEKEQDVpVKA---SG
Lamprey SLc34a	TWNFLPDWmHSMKPLdRvISAVCG---- RCKKc -----C-----KCVsKSGK-----NKQVEVVSIpDST-----
Zebrafish Slc34a2b	SWDFLPŁWAHSLDPWDRVVTVIAA---- RCCCC -----C-----KCCNSNEEDEKAKLENLANGIEINDNT-----
Stickleback Slc34a2b	SWDFLPŁWAHSLSPWdkVVGVFTA---- KCCCC -----C-----KCCQFADD-----DKETGETESLENNKSHTe
Pufferfish Slc34a2a	SWDFLPřPLHSLAPWDAVVTSMLG-FCGN RCCCC -----C-----KCSNCCQR-----NDEEAVR---KGS---LE
Stickleback Slc34a2a	SWDFLPřPMRS�APWDAVVTSAFG-FCGK HCCCC -----C-----KCKCKCRK-----KEDEKVMNQGRKS---LE
Zebrafish Slc34a2a	SWEFLPKPLHSLKPDWrvVTAGMS-FCRT RCCCC -----C-----KCCR-----NEEKNHMENNDRS---LE
Gar Slc34a2	TWEFLPKWMHSLKPDWrvITSMLS-FCRT RCCCC -----C-----KCCNKIS-----SEEDGTGKEKRGs---LE
Coelacanth Slc34a2	TWDFLPŁWmHSLHPWdkVITSMMG-YCGS HCKKc -----C-----KCCR-MVH-----AEDAGVKEKQVES---LH
Elephant Shark Slc34a2	TWKFLPIWLSLEPwDRVMQRFTD---IFCCCC-----K-----Kc-----SNKMNKKEKGIKS---FE
Lizard Slc34a2	NWGFLPKWMRSLEPwDNVvTSVSS-TCG RCCCCC -----C-----KCCR--RD---KGEDVAKEkPTKS---LE
Chicken Slc34a2	NWDFLPiWmHSLPwDNMIMSSLA-FCGK HCCGF -----C-----KCKVN-----AEQEGAKDNQLKT---ME
Cow Slc34a3	SWAWLPŁWLRSLEPwDLLVRR-----CCPC-----KACSPpQAVAKE-----TH
Opossum Slc34a3	SWAFLPLWLHSLQPDWRAITG-----CCPC-----CPRAHH-----EDSAGAAKE-----AQ
Elephant Slc34a3	SWAWLPŁWLHSLPwDGLVTH-----CCPC-----QACSAPhATTkK-----AH
Human Slc34a3	SWAWLPVWLHSLPwDRLVTR-----CCPC-----NVCSPPKATTKE-----AY
Mouse Slc34a3	SWAWLPŁWLHSLPwDRLVTA-----CCPC-----RACSNsPMtSKV-----AH
Rat Slc34a3	SWAWLPŁWLHSLPwDRLVTG-----CCPF-----KAYSNSHMTSKV-----AH
Coelacanth Slc34a3	TWNFLPMWmHSLKPDWRLfSA-----CCT-----NCFKCKKQEGKGnSTAAAATELETAFGYNEPH
Frog Slc34a3	DWGFLPTWmHSLAPLdRLfSSVCG-----CCC-----KCKNKS-----EEEKPEQLPADLSIGDMH