Premammalian origin of the sperm-specific \textit{Slo3} channel

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Regulation of membrane potential (\(E_m\)) is vital for sperm function and, consequently, for fertility. Membrane hyperpolarization is an essential physiological change in sperm that enable it to acquire fertilization capacity [1,2]. This signalling event is conserved in phylogenetically distant species such as mammals [3] and marine invertebrates [4]. Such hyperpolarization is principally caused by \(K^+\)-selective channels. Nonetheless, the identity and functional properties of \(K^+\) channels as well as the molecular targets of sperm membrane hyperpolarization vary greatly among lineages. For instance, in the sea urchin, sperm membrane hyperpolarization is caused by a cyclic nucleotide-gated \(K^+\) channel (TetraCNGK) expressed in sperm flagellum [4,5], triggering a calcium (\(Ca^{2+}\)) influx via activation of Catsper, a sperm-specific \(Ca^{2+}\) channel that controls sperm chemotaxis [6]. TetraCNGK-mediated hyperpolarization also occurs in the sperm of the freshwater fish \textit{Danio rerio}, but in this species the TetraCNGK channel is located in the sperm head, and its activity is controlled by intracellular pH (\(pH_i\)) rather than by cyclic nucleotides [7]. In mammals, the principal \(K^+\) current that triggers the hyperpolarization of sperm membrane during capacitation, an essential process that prepares sperm to fertilize the ova, is generated by Slo3, a \(pH_i\)-sensitive \(K^+\) channel located in the principal piece of sperm flagellum [8–13]. In mouse, it has been demonstrated that the activity of the Slo3 channel is essential for male fertility [10,11] and that membrane hyperpolarization is necessary and sufficient to prepare sperm for acrosome reaction [14]. Slo3 belongs to the family of \textit{Slo3} is a sperm-specific potassium (\(K^+\)) channel essential for male fertility. \textit{Slo3} channels have so far been considered to be specific to mammals. Through exploratory genomics, we identified the \textit{Slo3} gene in the genome of terrestrial (birds and reptiles) and aquatic (fish) vertebrates. In the case of fish, \textit{Slo3} has undergone several episodes of gene loss. Transcriptomic analysis showed that vertebrate \textit{Slo3} transcript orthologues are predominantly expressed in testis, in concordance with the mammalian \textit{Slo3}. We conclude that the \textit{Slo3} gene arose during the radiation of early vertebrates, much earlier than previously thought. Our findings add to the growing evidence indicating that the phylogenetic profiles of sperm-specific channels are intermittent throughout metazoan evolution, which probably reflects the adaptation of sperm to different ionic milieus and fertilization environments.
high-conductance potassium channels, also known as big potassium (BK) or Slo channels [15]. The most studied member of this family is Slo1, a Ca2+ and voltage-activated K+ channel essential for excitability of both neuronal and non-neuronal cells [16]. Slo1 is widely distributed across metazoan and is the closest parologue to Slo3 [15]. Given that mammals are the sole species that undergo sperm capacitation, it has been widely assumed that Slo3 channels are only found in mammalian sperm membrane. Nonetheless, the phylogenetic distribution of the Slo3 channel has not been rigorously explored to date. Therefore, to better understand the functional significance of the Slo3 channel, in this study, we assess the evolutionary origin and species distribution of the Slo3 gene using a phylogenomic approach that involves comparative genomics and transcriptomic analyses.

Materials and methods

Genomic databases mining

Search of the Slo3 gene (annotated in genomic databases as Kcnvl) in amniotes was performed in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). Slo3 gene sequences from Homo sapiens and Gallus gallus were used as initial queries in BLAST searches against genome databases from Ensembl, NCBI and the Joint Genome Institute (http://www.jgi.doe.gov/). Protein sequences were employed for BLASTp and tBLASTn searches against amino acid and nucleotide (i.e. EST, mRNA, genome assembly) databases respectively. Nucleotide-coding sequences were used for BLASTn and tBLASTx searches as only RNA sequences read archives were available. To identify potential distant homologues, we performed BLAST analysis employing the Ensembl Compara toolkit and the Genomicus database [20].

Identification of Slo3 orthologues in aquatic species

In order to determine the evolutionary origin of the Slo3 gene, we further searched in genomic databases of amphibians and fishes (Table S2). We only found a
Slo1 homologue gene in the genome of the frog *Xenopus tropicalis* (Fig. S1), the amphibian species whose complete genome has been sequenced. We then screened the genomic sequence presumably comprising the Slo3 locus and interestingly we identified three small intronic sequences highly conserved with human Slo3 (Fig. S1). This suggests that the Slo3 gene could have developed in Xenopus, but rapidly degenerated.

We next examined the transcriptome of the lungfish *Protopterus annectens*, representing the closest lineage relative to amniotes [23]. We identified four short reads that map to highly conserved regions between Slo1 and Slo3 with which we cannot determine the presence of Slo3 gene in lungfish unambiguously. In the genome of the coelacanth *Latimeria chalumnae*, we uniquely identified a Slo1 orthologue (Fig. 2, Table S1). Similar to that observed in Xenopus, we found three short sequences in the syntenic region of the putative *L. chalumnae* Slo3 locus highly conserved with human Slo3 (Fig. S2). Surprisingly, these sequences do not overlap with those found in Xenopus. This observation adds support to the hypothesis that the Slo3 gene could have been present in aquatic vertebrates. We next explored the genome of several Teleostei (bony fishes). Interestingly, we identified potential Slo3 orthologues in two teleost species: *Clupea harengus* and *Salmo salar* (Table S1). These sequences were robustly resolved within the Slo3 clade, lack the poly-aspartic repeat that defines the Slo1 calcium bowl (Fig. 2), and conserve the synteny with tetrapod genomes (Fig. 3). We did not find hints of the Slo3 gene in the remaining teleost genomes searched. Nonetheless, conserved synteny of the closest flanking genes suggest that Slo3 was lost in these lineages (Fig. 3). Teleost fishes have undergone at least a round of whole-genome duplication and several Slo1 gene copies have been identified (Table S1) [24]. Despite several copies of neighbour genes being detected in the
examined teleost genomes (Table S3), we found a single Slo3 copy in C. harengus and S. salar. This suggests that additional Slo3 copies originated by genome duplication have degenerated in these species. In the genome of the spotted gar (Lepisosteus oculatus), whose lineage diverged from teleost fishes before their genome duplication event, we identified two Slo-like homologues. Although one of them is a conserved Slo1 orthologue, the other sequence leads a robustly resolved branch within the Slo3 clade and lacks the poly-aspartic repeat that defines the Slo1 calcium bowl.

Fig. 2. Comparative analysis of Slo1 and Slo3 sequences. Cladogram representing the molecular phylogeny built with Slo1 and Slo3 protein sequences. Phylogenetic tree was built by Maximum Likelihood, and supporting values of posterior bootstrap analysis with 100 replicates are shown on each node. The model of protein evolution used was JTT+G (G fixed to 0.937). Sequence alignment of the calcium bowl region corresponding to Slo1 is shown on right. Amino acids are coloured indicating similarity with < 60% (white), 60–80% (light grey), 80–99% (dark grey) and 100% (black). The residues involved in Ca2+ sensing in Slo1 are indicated with a black line under alignment.

have no evidence to unambiguously identify a Slo3 orthologue. We further scanned the genome of the elephant shark (Callorhinchus milli), whose lineage – cartilaginous fishes – is phylogenetically the oldest of the living jawed vertebrates, and we found two genomic fragments that form a conserved Slo3 orthologue as they were assembled (Fig. 2, Table S1). We further searched in the genome of a jawless vertebrate, the sea lamprey (Petromyzon marinus), and we identified two partial Slo-like sequences after assembling several genomic scaffolds (Table S1). Nevertheless, both sequences align to a conserved region of Slo channels (Fig. S3) and were too short to be robustly resolved in the phylogenetic tree. In addition, scaffolds of sea lamprey genome are still poorly annotated, for which we could not perform a comparative genomics approach to locate the syntenic regions. Nonetheless, the observation that Metazoan generally have a single Slo1 copy, excepting teleost with duplicated genomes [22], suggests that jawless vertebrates might preserve an ancestral form of Slo3. Genomic searches of remaining chordates and deuterostomes – the cephalochordate...
Branchiostoma floridae, the urochordate Ciona intestinalis, the echinoderm Strongylocentrotus purpuratus and the hemichordate Saccoglossus kowalevskii – yielded solely Slo1 orthologues (Fig. 2, Table S1). Likewise, we found only a Slo1 copy in the invertebrate genomes of Caenorhabditis elegans and Drosophila melanogaster (Fig. 2, Table S1). Altogether, these data suggest that the Slo3 channel emerged at the time of the radiation of early vertebrates, which is much earlier than previously assumed.

Slo3 is predominantly expressed in testis of vertebrates

To assess whether the expression pattern of Slo3 channel, characterized by a predominant expression in testis [25], is conserved in nonmammalian lineages, we analysed the transcriptome of a range of representative vertebrate species (see Materials and methods). We determined that Slo3 gene is predominantly transcribed in the testis of model species of birds (chicken) and reptiles (anole lizard) (Fig. 4A). We also identified an enriched expression in the testis of the spotted gar, which was used as the representative fish species (Fig. 4B).

Discussion

In this study, we found that the emergence of the Slo3 gene, which encodes a sperm-specific K⁺ channel, dates to the radiation of ancestral vertebrates (Fig. 5). This finding radically changes the widely established assumption in the field of reproductive biology and sperm physiology that Slo3 is a channel exclusively found in mammals [10,11,25]. The fact that Slo3 gene is not only present in the genome of nonmammalian species but also has an active and prominent expression in testis, suggests that the function of Slo3 as a sperm potassium (K⁺) channel is conserved in vertebrates.

We found that the Slo3 gene is conserved in birds and reptiles. The molecular changes that sperm undergo in the female genital tract are scarcely known in these lineages [26,27]. Our findings place the Slo3 channel as the first described molecular component putatively involved in sperm function of birds and reptiles. Although it is frequently assumed that sperm capacitation is a process confined to mammals, some studies have reported that nonmammalian species undergo sperm capacitation-like changes after ejaculation [28–31]. These observations, in conjunction with our results, suggest that Slo3 channels could mediate a primary K⁺ current essential to acquire fertilizing ability also in the sperm of nonmammalian vertebrates. Another possibility is that in nonmammalian species, Slo3 channels could have functions in sperm other than mediating membrane hyperpolarization during capacitation-like processes. For example, Slo3 channels may have a role in thermo- or chemosensation.
responding to sensory cues such as temperature or chemical substances. Functional studies on sperm of avian and reptilian species will be necessary to characterize the role of Slo3 channels in nonmammalian sperm.

An interesting finding of the present work is that the Slo3 channel shows an intermittent pattern of presence and absence across aquatic vertebrates as a result of different events of gene loss. We identified Slo3 orthologues in some species of bony and cartilaginous
fishes, while in a large number of teleost fish species the Slo3 channel appears to have degenerated. In some fishes, orthologues of TetraCNGK, an ancestral and sperm-specific cyclic nucleotide-gated K⁺ channel which is also found in marine invertebrates and unicellular eukaryotes [5,32], have been identified [7]. The alternative distribution of Slo3 and TetraCNGK observed across fishes could be the result of lineage-specific evolutionary conflicts between these two channels for being the principal sperm K⁺ channel. This would explain the presence of TetraCNGK and the absence of Slo3 in coelacanth, or the opposite in the case of elephant shark. Under this hypothetical framework, the conservation in the spotted gar genome of both Slo3 and TetraCNGK [7] is surprising. The coexistence of two sperm-specific K⁺ channels could still be explained by a subfunctionalization process whereby sperm membrane potential is regulated by the joint activity of both channels. Regarding fishes in which neither Slo3 nor TetraCNGK channels have been identified, it will be worth performing studies encompassing molecular and physiological approaches in order to characterize the K⁺ transporters in these species. The same is true for amphibian species, where the physiological changes that sperm undergo prior to fertilization are poorly understood, and consequently we do not have a clear interpretation about the functional and evolutionary significance of lacking both Slo3 and TetraCNGK channels.

The Slo3 gene likely originated from a duplication of its closest parologue Slo1 at the time of one of the two whole-genome duplications occurring before divergence of lamprey from the jawed vertebrates (Fig. 5) [33,34]. This seems reasonable as the complex genomic rearrangements that occurred during ancestral vertebrate evolution implied a further expansion of a large number of ion channel families [35,36]. On the other hand, lineage-specific gene loss seems to be an evolutionary characteristic of sperm-specific ion channels. Cai and Clapham [37] were the first to observe an heterogeneous phylogenetic profile of the Catsper channel complex in metazoan genomes. In addition, the sperm-specific sodium–hydrogen exchanger required for sperm motility [38] also shows such a mosaic distribution in metazoans (F. Romero & T. Nishigaki, personal communication). This evolutionary pattern is at odds with that of most ion channels expressed in somatic cells, which are highly conserved across metazoans [36,39–41]. As a recent review highlights, sperm from different species use distinct types of signalling molecules and highly conserved proteins may perform different tasks according to the fertilization strategy of a particular species [42]. Therefore, further evolutionary genomics studies on sperm ion channels will be of great importance to unravel the functional basis of lineage-specific sperm adaptations throughout metazoan evolution.

Conclusions

In this study, we established that Slo3, a sperm-specific channel thought to be mammalian specific, arose with the radiation of early vertebrates. In addition, we observed that the Slo3 gene has undergone different events of gene loss through evolution of aquatic vertebrates, a feature shared with other sperm-specific ion channels. The fact that Slo3 channel is widely preserved in terrestrial vertebrates, along with its importance for mammalian fertility, prompt us to suggest that Slo3 channels have a pivotal role in species with internal fertilization. Meanwhile, in aquatic vertebrates that use external fertilization, Slo3 could be evolving under more relaxed functional constraints, resulting in many cases in gene loss. Our findings, together with evidences from recent studies, establish a new picture where channel repertoires and functions greatly vary among species, which likely reflects sperm adaptations to species-specific fertilization environments.

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Author contributions

AV performed genomic database mining, phylogenetic and transcriptomic analysis. KL and RMG initiated the database mining analyses and participated in the drafting of study. DC performed transcriptomic analysis. CT participated in the design, drafting and
coordination of the study. All authors read and approved the final manuscript.

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Supporting information
Additional Supporting Information may be found online in the supporting information tab for this article:
Fig. S1. Exploration of Slo3 locus in Xenopus.
Fig. S2. Exploration of Slo3 locus coelacanth.
Fig. S3. Identification of Slo-like sequences in sea lamprey genome.
Table S1. Table of protein sequences collected in this study.
Table S2. Table with public genomic databases searched during this study.
Table S3. Copies of genes flanking Slo3 locus in teleost fishes.