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COMMENTARY

Salt-secreting ionocytes in marine fishes: new dimensions and evolutionary implications of a fundamental model

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ABSTRACT

To reside in marine habitats, marine fishes must actively eliminate the excess Na^+ and Cl^- they acquire from their environment. These ions are passively absorbed across body surfaces and through the ingestion of seawater (SW), which is necessary to maintain water balance. Salt secretion occurs through the actions of mitochondrion-rich 'SW-type ionocytes' in the gills or other specialized salt-secreting organs. For nearly 50 years, the SW-type ionocyte model proposed by Patricio Silva and colleagues has proven remarkably enduring. The Silva model guided researchers toward identifying key molecular components of SW-type ionocytes, such as cystic fibrosis transmembrane conductance regulator 1 (Cftr1), $Na^+/K^+/2Cl^-$ cotransporter 1 (Nkcc1) and the inwardly rectifying K^+ channel (Kir). However, emerging findings indicate that alternative molecular mechanisms may complement, or in some cases, operate in lieu of those included in the Silva model. In this Commentary, we argue that it is time to critically evaluate whether ionocyte-based strategies for salt secretion are more diverse than currently recognized. We highlight recent developments regarding the operation of Cftr-independent ionocytes in lamprey, the emerging role of anoctamin 1 (Ano1) in lamprey and bony fishes, and various 'new' pathways for Cl^- and K^+ to enter and exit ionocytes. Additionally, we propose future research directions for identifying novel salt-secretory mechanisms in various lineages of marine fishes. We conclude this Commentary by presenting three hypotheses for the divergence of ionocyte-mediated salt secretion in marine fishes and outlining a conceptual framework for considering the evolutionary homology of salt-secreting ionocytes across vertebrates.

KEY WORDS: Ionocyte, Osmoregulation, Fishes, Cftr, Ano1

Introduction

Regardless of environmental salinity, nearly all fishes maintain extracellular Na^+ and Cl^- concentrations around 100–200 mmol l^{-1}, with variation among taxa (Marshall and Grosell, 2006). This is notable considering that freshwater (FW) and seawater (SW) environments present starkly different challenges to the salt and water balance of the organism; NaCl concentrations can range from <0.001 mol l^{-1} in FW to >0.5 mol l^{-1} in SW. In FW-adapted fishes, the gill epithelium facilitates the uptake of Na^+ and Cl^- from the relatively dilute environment. In marine actinopterygians, the gill functions as the primary site for the active secretion of Na^+ and Cl^- into the salt-concentrated environment (Kaneko et al., 2008). In marine chondrichthyans, the rectal gland, not the gill, is responsible for the active secretion of Na^+ and Cl^- (Marshall and Grosell, 2006).

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For nearly five decades, the model describing how fishes actively secrete Na^+ and Cl^- into marine environments has remained remarkably unchanged (see Fig. 1). The salt-secretory function of the gills and rectal gland is mediated by specialized cells coined 'SW-type ionocytes' (also referred to as 'chloride cells' or 'mitochondrion-rich cells'). This fundamental model of SW-type ionocytes was established through a series of pioneering studies by Patricio Silva and colleagues in the 1970s (Silva et al., 1977a,b; Epstein and Silva, 1985). Despite nearly 50 years having passed, this model has endured. However, its longevity raises an important question: has the staying power of the Silva model (see Glossary) discouraged critical reevaluation? Or, perhaps from a more conservative perspective: what aspects of SW-type ionocyte function warrant renewed attention?

Given recent findings and technological advancements, the time appears ripe for a renewed critical examination and exploration of the Silva model of salt secretion. New findings in agnathans, elasmobranchs and teleost fishes indicate that complementary molecular mechanisms may operate in addition to, or in some cases, in lieu of those included in the Silva model. Additionally, recent investigations in taxa outside fishes have revealed the presence of ionocyte-mediated salt-secretory pathways that are similar to those described by the Silva model. Here, we aim to provide an up-to-date assessment of the Silva model. We begin by presenting a brief history of the model and the molecular processes it involves. Subsequently, we synthesize new findings and advocate for a broader, comparative and technologically driven approach to understanding the unity, diversity and evolution of mechanisms for epithelial Na^+ and Cl^- secretion in marine fishes and other vertebrates.

Some historical perspective on the 'SW-type ionocyte' model

Our mechanistic understanding of salt secretion by marine fishes traces back to the seminal work of the late 1970s conducted by multiple groups at the Mount Desert Island Biological Laboratory (MDIBL) in Maine, USA. During this period, a series of studies on euryhaline teleosts (see Glossary) – including the Atlantic killifish (*Fundulus heteroclitus*) and American eel (*Anguilla rostrata*) – and a marine elasmobranch, the spiny dogfish (*Squalus acanthias*), established the fundamental mechanisms of salt secretion by SW-type ionocytes.

Initially, although Na^+/K^+-ATPase (Nka) – which moves Na^+ out of and K^+ into cells, providing the driving force for secondary ion transport processes – was thought to be involved in salt secretion, there was a debate about whether it was expressed in the basolateral or apical membrane (see Glossary) of branchial ionocytes. If Nka were expressed on the apical surface, it would provide an obvious route for Na^+ to travel from the ionocyte interior to the ambient environment (Evans and Cooper, 1976; Maetz, 1969). However, in the late 1970s, two groups at MDIBL definitively localized Nka to the basolateral membrane. In opercular membranes from SW-acclimated killifish mounted in Ussing chambers (see Glossary), Cl^- secretion was inhibited by the basolateral addition of ouabain

Journal of Experimental Biology (2025) 228, jeb251075. doi:10.1242/jeb.251075

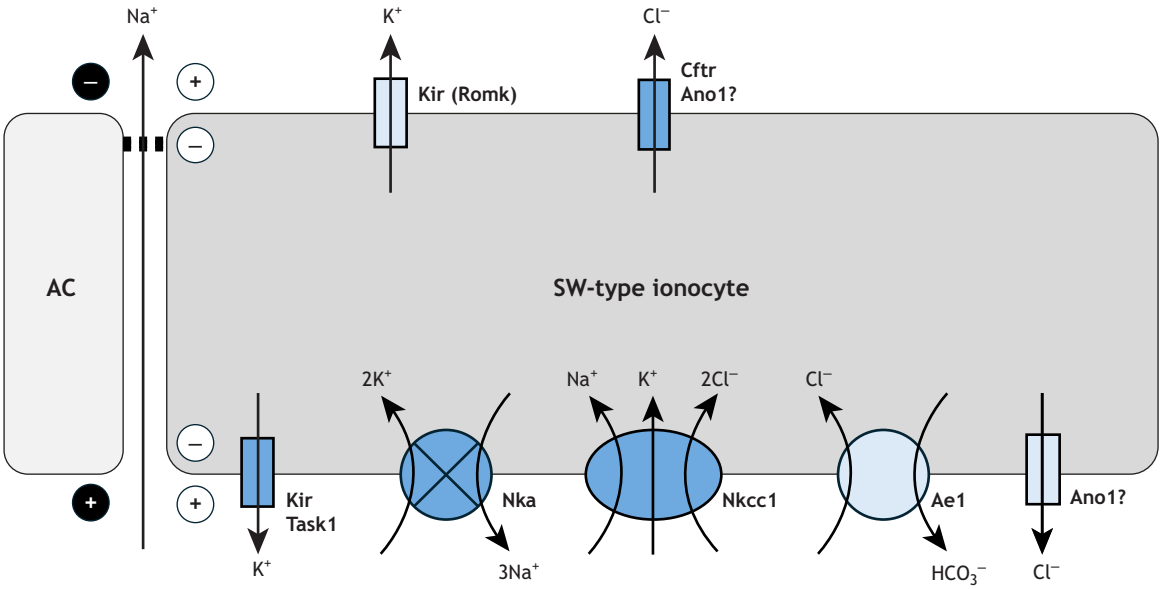

Fig. 1. The model for Na^+ and Cl^- secretion by SW-type ionocytes in the marine fish gill and rectal gland. Patricio Silva and colleagues originally proposed the model for salt secretion in marine fishes represented by the dark blue shading; the identities of the key transporters involved in this model were determined after its original description. Transporters with light blue shading have recently been proposed to operate in the SW-type ionocytes of some marine fishes. Apical and basolateral sides are presented at the top and bottom of cells, respectively. Positive and negative indicators describe transmembrane (white) and transepithelial (black) potential differences. AC, accessory cell; Ae1, anion exchanger 1; Ano1, anoctamin 1; Cftr, cystic fibrosis transmembrane conductance regulator; Kir, inwardly rectifying K^+ channel; Nka, Na^+/K^+-ATPase; Nkcc1, $Na^+/K^+/2Cl^-$-cotransporter 1; Romk, renal outer medullary K^+ channel; SW, seawater; Task1, two-pore acid-sensitive K^+ channel 1.

(an Nka inhibitor) or furosemide (known at the time as a Na^+ transport inhibitor) (Karnaky et al., 1977). Short-circuiting the killifish operculum by neutralizing transepithelial voltage in Ussing chambers inhibited Na^+ secretion; this suggested that the mechanism for Na^+ transport is a passive process dependent on transepithelial electrical potential difference (Degnan et al., 1977).

Glossary

Apical
The surface of a cell facing the external environment or a body cavity.
Basolateral
The side of a cell facing the internal environment and blood supply.
Euryhaline
Capable of tolerating a wide range of environmental salinities, from freshwater to seawater.
Ionocyte (chloride cell, mitochondrion-rich cell)
A specialized epithelial cell that actively transports ions (e.g. Na^+, Cl^-, Ca^{2+}, and K^+).
Osmoconformation
Maintaining the total solute and water composition of the body similarly to the external environment.
Osmoregulation
Active regulation of the total solute and water composition of the body to be different from that of the external environment.
Silva model
The classic framework for salt secretion in marine fishes that describes basolateral Na^+ and Cl^- loading, transcellular Cl^- secretion, and paracellular Na^+ secretion.
Ureotelism
Excreting nitrogen waste mainly as urea, a strategy used by sharks, rays, and some other vertebrates. Teleosts excrete nitrogen as ammonia.
Ussing chamber
An experimental apparatus used to measure ion transport across epithelial tissues by short-circuiting (i.e. neutralizing the transmembrane voltage) and recording transepithelial current.

In SW-acclimated American eel, the inhibition of Nka with ouabain nearly abolished Na^+ and Cl^- efflux, with a more pronounced effect observed when ouabain was applied to the basolateral side (Silva et al., 1977a). Similarly, perfusing the rectal gland of spiny dogfish with ouabain or furosemide sharply reduced Cl^- secretion (Silva et al., 1977b). It was later hypothesized that the large influx of K^+ through Nka required the activity of K^+ efflux pathways to maintain the electrochemical gradients necessary for continued Na^+ and Cl^- secretion. The killifish opercular membrane was leveraged once again, this time to demonstrate that blocking K^+ transport across the basolateral side inhibited Cl^- secretion (Degnan, 1985).

Based on these observations, Silva and colleagues proposed a model in which basolateral Nka activity drives the secondary active, transcellular secretion of Cl^-, which enters the ionocyte via a basolateral Na^+-linked cotransport pathway (Fig. 1) (Epstein and Silva, 1985). Na^+ secretion was proposed to occur passively through paracellular junctions between ionocytes and adjacent cells, driven by the electrical potential across the epithelium and local Na^+ concentrations created by the high activity of the basolateral Nka. Lastly, the model proposed that passive K^+ efflux occurs across the basolateral membrane.

In subsequent decades, several key molecular components of SW-type ionocytes were identified. The Na^+-linked cotransport pathway for Cl^- was ascribed to the $Na^+/K^+/2Cl^-$ cotransporter 1 (Nkcc1), which simultaneously moves Na^+, K^+ and Cl^- into the cell across the basolateral membrane of SW-type ionocytes (Lytle and Forbush, 1992; Pelis et al., 2001; Shaughnessy and McCormick, 2020; Xu et al., 1994). The apical conduit for Cl^- was identified as cystic fibrosis transmembrane conductance regulator 1 (Cftr1) (Marshall et al., 1991; Riordan et al., 1994; Singer et al., 1998), a transmembrane, phosphorylation-activated Cl^- channel located in the apical membrane of ionocytes. A pathway for basolateral K^+ efflux was then associated with the inwardly rectifying K^+ channel (Kir5.1) (Suzuki et al., 1999).

The long-standing and widely accepted framework for how SW-type ionocytes function, particularly when compared with the numerous and evolving models of ion-absorptive FW-type ionocytes (Kovac and Goss, 2024), may reflect that a basic strategy for epithelial salt secretion is remarkably conserved across vertebrate evolution. This is undoubtedly true to some extent. Still, this does not preclude the exploration of novel molecular strategies for Na^+ and Cl^- secretion across a broader range of marine fish lineages than has been studied previously. Indeed, recent studies have made progress by critically examining the SW-type ionocyte across diverse taxa, yielding findings that could help to update our understanding of ion-transporting epithelia in fishes and other vertebrates. Here, we do not intend to challenge the fundamental framework of the Silva model, which includes basolateral Cl^- loading, apical Cl^- efflux and paracellular Na^+ movement. Instead, we aim to explore the molecular mediators of these transport pathways and the evolutionary homology of salt-secreting ionocytes across vertebrates.

A Cftr-independent, Ano1-rich SW-type ionocyte in lamprey

Agnathan and gnathostome fishes diverged over 500 million years ago; agnathans are represented by the extant lineages of lampreys and hagfishes, and gnathostomes include all osteichthyans and chondrichthyans. It has been known for a decade that the sea lamprey (*Petromyzon marinus*) genome includes a *cftr* homolog that is differentially expressed across tissues (Ren et al., 2015; Cui et al., 2019). However, our recent studies on sea lamprey have revealed a surprising departure from the canonical Cftr-dependent SW-type ionocyte model proposed by Silva and colleagues. Using functional genomics, immunofluorescence microscopy and pharmacology, we demonstrated that SW-type branchial ionocytes in sea lamprey, which express Nka and Nkcc1 (Reis-Santos et al., 2008; Shaughnessy and McCormick, 2020), do not richly co-express Cftr (Shaughnessy et al., 2025). Our conclusion was primarily based on transcriptomic and targeted PCR analyses showing low or absent *cftr* mRNA in the gill. The lack of immunological detection and the outcomes of our pharmacological studies, although supportive of this interpretation, were less compelling owing to limitations inherent in these approaches. Therefore, we cannot rule out the possibility that Cftr protein is present at low levels or is undetectable with available antibodies.

However, there are other reasons to suspect that lamprey Cftr may not be involved in SW-type ionocyte function in this species. Although the lamprey Cftr has Cftr-like structural domains and functions as an ion channel, it is <50% similar to gnathostome Cftrs. It also exhibits reduced activation by protein kinase A, reduced ion permeability and altered pharmacological sensitivity compared with gnathostome Cftrs (Cui et al., 2019). Together, these results suggest that even if Cftr is present in lamprey gills, its low expression and reduced functional capacity may preclude it from being the principal apical Cl^- channel in ionocytes. If this is indeed the case, it would be the first known instance of a SW-type ionocyte in a marine fish that does not use Cftr; however, if it is simply the case that some other Cl^- channel functions instead of Cftr, then the Silva model essentially describes SW-type ionocytes in lamprey.

These findings, which suggest that Cftr is not operating in lamprey gills, were unexpected, as it has generally been assumed that lampreys leverage the same Cftr-dependent mechanisms for Cl^- secretion as other marine fishes (Bartels and Potter, 2004). If Cftr does not provide the apical conduit for Cl^- secretion in lamprey SW-type ionocytes, then what does? Currently, this question remains unanswered. However, our recent investigations have determined that anoctamin 1 (Ano1), a Ca^{2+}-activated Cl^- channel, is abundantly expressed in sea lamprey SW-type ionocytes (Shaughnessy et al., 2025). Ano1 belongs to a family of transmembrane proteins that function as Ca^{2+}-activated Cl^- channels (Pedemonte and Galietta, 2014). As discussed below, the available information regarding the structural and functional features of Ano1 is skewed toward studies involving mammalian models. However, in sea lamprey, the robust expression of Ano1 in the gills is further enhanced following SW acclimation. Although immunofluorescence microscopy shows that Ano1 localizes to Nka- and Nkcc1-rich branchial ionocytes, it appears to be expressed in the basolateral membrane (Shaughnessy et al., 2025).

The apparent basolateral localization of Ano1 raises an important and somewhat familiar question: if lamprey SW-type ionocytes lack apical Cftr, and Ano1 is not localized to the apical membrane, how does Cl^- exit these cells? Several possibilities exist: first, limitations in antibody resolution might have obscured apical Ano1 signal, and higher-resolution or stacked imaging could reveal more about its expression pattern. Second, Ano1 may function in concert with another yet-unidentified apical Cl^- channel in sea lamprey ionocytes. Third, Cl^- secretion might happen through less conventional pathways, such as paracellular routes or unknown transporter families. The mode of apical Cl^- exit from lamprey ionocytes certainly remains unresolved and will be a focus of future studies. Nonetheless, these results suggest that branchial Cl^- secretion in sea lamprey is independent of Cftr and potentially involves Ano1. If Ano1 is indeed expressed basolaterally in lamprey ionocytes, it cannot serve as the canonical apical Cl^- secretory pathway envisioned by the Silva model. Instead, it may serve as a shunt pathway for Cl^- recycling to stabilize intracellular Cl^- or help regulate cell volume, or it might be linked to Ca^{2+}-dependent signaling pathways that affect intracellular Cl^- homeostasis. Determining whether Ano1 plays a direct role in net Cl^- secretion or a complementary role by supporting other functions of SW-type ionocytes remains an important unresolved question.

We must also consider recent findings in hagfishes, the sister taxon to lampreys, when describing ‘new’ aspects of agnathan ionoregulation. As osmoconforming species (see Glossary), hagfishes are unusual among vertebrates, maintaining extracellular ion concentrations close to those in the marine environment (Bellamy and Jones, 1961; Currie and Edwards, 2010). Nevertheless, because they ingest a high salt load while feeding, they are likely to have a strategy to excrete excess salts. Nka is highly expressed in branchial mitochondrion-rich cells in Pacific hagfish (*Eptatretus stoutii*) (Tresguerres et al., 2006) and is transcriptionally responsive to salinity changes in the gills of the inshore hagfish (*Eptatretus burgeri*) (Yamaguchi et al., 2024). However, in the inshore hagfish, branchial *nkcc1* expression does not respond to salinity changes and *cftr* is seemingly absent from the genome entirely (Yamaguchi et al., 2024). A preliminary analysis of the inshore hagfish genome indicates that hagfishes do appear to contain members of the anoctamin family, including an *ano1*-like gene (C.A.S., unpublished results). Given their notable physiological and molecular differences from lampreys, hagfish represent an important, as yet underexplored, lineage for understanding the evolution of salt-secreting ionocytes in vertebrates.

Ano1 in SW-type ionocytes of teleosts

While our investigations into lamprey ionoregulation were ongoing, parallel work conducted by our labs and others revealed that *ano1* is expressed in the gills of Atlantic killifish and Japanese medaka (*Oryzias latipes*). In killifish, *ano1* is expressed alongside *cftr1* in

the gills and is upregulated after exposure to both SW and hypersaline (>100 ppt) conditions (Breves et al., 2024; Tao and Breves, 2024). Similarly, *ano1* mRNA is detected in the ionocytes of medaka and is also upregulated during SW acclimation (Konno et al., 2024). The exposure of SW-acclimated medaka to Ca^{2+}-activated Cl^- channel inhibitors ($T16A_{inh}$-A01, $CaCC_{inh}$-A01 and niclosamide) disrupts ionoregulatory homeostasis (Konno et al., 2024). Furthermore, as in both killifish and medaka, *ano1* expression is higher in SW- versus FW-acclimated threespine stickleback (*Gasterosteus aculeatus*) (Taugbøl et al., 2022). These observations across multiple teleost lineages suggest that Ano1 may play a significant role in the operation of SW-type ionocytes in euryhaline and marine teleosts, including those known to express Cftr.

Nkcc1-independent pathways for Cl^- entry into SW-type ionocytes

For decades, Nkcc1 has been widely regarded as the sole pathway for the basolateral entry of Cl^- into ionocytes. However, alternative pathways for Cl^- transport, including through basolateral Cl^-/HCO_3^- exchangers, warrant inclusion in the SW-type ionocyte model (Kovac and Goss, 2024) (Fig. 1). In killifish opercular epithelia mounted in Ussing chambers, the inhibition of Cl^-/HCO_3^- exchangers by broad pharmacological inhibitors of anion transporters, such as stilbene disulfonates, reduces transepithelial Cl^- secretion (Zadunaisky et al., 1995). However, these pharmacological agents are well described to have limited specificity among certain classes of anion transporters; experiments using them should be interpreted cautiously and future studies should confirm the roles of particular transporters using more selective tools or heterologous expression systems. In medaka, paralogs of anion exchanger 1 (Ae1) mediate basolateral Cl^-/HCO_3^- exchange in SW-type ionocytes (Liu et al., 2016). The pharmacological inhibition of Nkcc1 with bumetanide reduces Cl^- secretion by 63%, whereas inhibiting Ae1 with DIDS lowers Cl^- secretion by 47%. Combined inhibition leads to an 81% reduction in Cl^- secretion. Ae1 is also transcriptionally expressed in the SW-type ionocytes of Atlantic salmon (West et al., 2021). Therefore, Nkcc1 does not seem to be the only basolateral pathway for Cl^- to enter SW-type ionocytes. Furthermore, this Ae1-mediated pathway for Cl^- entry functionally links acid–base regulation to a process supporting ion secretion: apical Cl^- secretion supported by Ae1 on the basolateral membrane becomes more pronounced during acidosis, whereas Cl^- secretion mediated by Nkcc1 becomes more prominent during alkalosis (Liu et al., 2016). Further investigation across species is necessary to determine whether this pH-dependent dual transport mechanism is a conserved feature of SW-type ionocytes.

K^+ efflux channels in SW-type ionocytes

More-recent studies have identified additional inwardly rectifying channel proteins responsible for K^+ efflux from SW-type ionocytes, in addition to the originally identified Kir5.1 (Suzuki et al., 1999). Our ongoing analyses of the sea lamprey branchial transcriptome suggest that Kir4.1 may be the main K^+ channel in the SW-type ionocytes of this species (C.A.S., unpublished results). Single-cell transcriptomic analysis of Atlantic salmon (*Salmo salar*) indicates that Kir4.2 is the primary transmembrane K^+ channel in the SW-type ionocytes of salmon (West et al., 2021). In Mozambique tilapia (*Oreochromis mossambicus*), the renal outer medullary K^+ channel a (Kir1.1/Romka) is expressed in the apical membrane of SW-type ionocytes (Furukawa et al., 2012). Romka is also present in the apical membrane of two subtypes of tilapia FW-type ionocytes (Furukawa et al., 2014). Romka expression in tilapia ionocytes increases substantially following exposure to high environmental K^+ levels, suggesting that it helps maintain K^+ homeostasis of the whole organism (Furukawa et al., 2012, 2014). In the Silva model, K^+ efflux is necessary to sustain the electrochemical gradients needed for Na^+ and Cl^- secretion. This efflux is typically presumed to occur basolaterally so that recycled K^+ can support Nka and Nkcc1 function (Degnan, 1985). Whether the operation of an apical K^+ channel such as Romka in SW-type ionocytes affects apical membrane or transepithelial potential differences in a way that has an impact on Na^+ or Cl^- secretion warrants further investigation.

Other K^+ channels, besides those in the Kir family, are involved in branchial Na^+ and Cl^- secretion (or are at least expressed by SW-type ionocytes). For example, in Atlantic salmon, a Ca^{2+}-activated K^+ channel is transcriptionally upregulated in the gills after SW exposure (Loncoman et al., 2018). A study on the shark rectal gland identified yet another type of K^+ channel operating in SW-type ionocytes; specifically, two-pore acid-sensitive K^+ channel 1 (Task1) (Telles et al., 2016). Similarly to the Kir K^+ channels described in teleost gills, Task1 is located in the basolateral membrane of shark rectal gland ionocytes, and acts as a K^+ efflux pathway essential for maintaining a favorable electrochemical gradient for apical Cl^- secretion. Together, the recent characterizations of Task1 and Romka indicate that previous descriptions of the SW-type ionocyte did not fully capture the diversity of K^+ channels that may support Na^+ and Cl^- secretion by these cells.

Toward a functional framework for Ano1 in SW-type ionocytes

As summarized above, recent findings suggest that the model for Na^+ and Cl^- secretion is more complex than initially presented, especially given the discovery of 'new' molecular pathways for Na^+, Cl^- and K^+ to enter and exit SW-type ionocytes. Here, we focus on how the field might explore the pathways for the apical exit of Cl^- from SW-type ionocytes, given recent discoveries of Ano1 in the SW-type ionocytes of agnathans and teleosts, which suggest a fundamental, yet previously unknown, role for Ano1.

Ano1 has been extensively studied in mammals, where it functions as an apical Cl^- channel in various epithelial tissues (Pedemonte and Galietta, 2014). However, little is known about the properties of fish Ano1 homologs, such as their ion selectivity, activation mechanisms and interactions with other proteins. Comparative genomic analyses promise to unveil the evolutionary history of Ano1 in vertebrate salt-secretory epithelia and highlight potentially significant structural differences between fish and mammalian Ano1 homologs. One area of particular interest is the functional domains involved in Ca^{2+}/calmodulin binding and ion selectivity, which may have evolved to meet the specific physiological demands of marine osmoregulation (see Glossary).

Heterologous expression systems offer powerful experimental approaches for characterizing the functional properties of fish Ano1 proteins. Patch-clamp electrophysiological analyses of fish Ano1 expressed in *Xenopus* oocytes would enable detailed channel-specific functional studies, including the determination of ion selectivity, conductance and voltage dependence. Ussing chamber electrophysiological analyses of mammalian epithelial cell models expressing fish Ano1 proteins could elucidate their functional interactions with cell signaling pathways and other membrane proteins. The isolated expression of fish ion transporters in heterologous systems would also help validate the use of pharmacological activators or inhibitors in *ex vivo* or *in vivo* experiments.

Although heterologous expression systems may provide insight into the intrinsic properties of fish Ano1, studies in native tissues

are crucial for understanding how Ano1 operates within the physiological context of SW-type ionocytes. Several experimental approaches, reminiscent of pioneering studies on ionocytes from decades ago, are particularly well-suited for these investigations. For example, when mounted in Ussing chambers, the opercular epithelium of Atlantic killifish can be exposed to selective Ano1 inhibitors to investigate the necessity of Ano1 for Cl^- secretion. Primary gill epithelial cell culture models, which contain ionocytes, also provide a tractable *in vivo*-like system for testing transport mechanisms and pharmacological responses under controlled conditions. It is important to note that most pharmacological modulators of Ano1 were developed for mammalian homologs and may have different potencies or specificities for fish Ano1 proteins; however, this can be assessed using heterologous expression systems as described above.

Given that mammalian Ano1 and Cftr are activated by distinct signaling pathways (Ca^{2+}- versus phosphorylation-dependent activation, respectively), it is tempting to propose that the co-expression of both Ano1 and Cftr in the apical membrane of fish ionocytes allows for the nuanced regulation of salt secretion through various cell signaling pathways. Intracellular ion imaging using fluorescent indicators may offer insight into the dynamics of ion movements within SW-type ionocytes. For example, monitoring changes in intracellular Cl^- or Ca^{2+} levels after manipulating Ano1 activity could uncover its role in Cl^- homeostasis and its interaction with Ca^{2+} signaling pathways.

Advances in genome editing technologies have made it increasingly feasible to manipulate gene expression in non-model fish species. CRISPR/Cas9-mediated knockout of Ano1 could yield evidence of its functional importance in SW adaptation. Unfortunately, the most commonly used genetic fish model, the zebrafish (*Danio rerio*), is not physiologically suitable for this work because it lacks SW tolerance. However, Japanese medaka provide a euryhaline model for gene-manipulative approaches. For taxa where genetic manipulation is challenging, such as elasmobranchs and lampreys, antisense morpholino oligonucleotides or RNA interference (RNAi) methods may offer alternative strategies to disrupt Ano1 expression. Although morpholinos or interfering RNA are typically administered during embryonic stages, a vivo-morpholino approach, in which membrane-penetrating morpholinos are administered systemically or directly to targeted tissues at post-embryonic stages, has been applied in fish (Notch et al., 2011). Morpholino and RNAi methods generally offer only transient knockdown rather than complete knockout; however, they can still provide valuable insights into the functions of proteins in comparative physiological studies.

Working hypotheses for the divergence of salt-secretory mechanisms in marine fishes

The presence of a Cftr-independent SW-type branchial ionocyte in sea lamprey highlights the potential for discovering novel salt-secretory mechanisms across various lineages of marine fishes. With the addition of the sea lamprey, representatives from the three major lineages of fishes (i.e. osteichthyans, chondrichthyans and agnathans) have now been examined for how their SW-type ionocytes facilitate Na^+ and Cl^- secretion. Critically, the marine representatives from these three lineages appear to accomplish Na^+ and Cl^- secretion differently – the agnathans through a Cftr-independent SW-type ionocyte in the gills, the chondrichthyans through a Cftr-dependent SW-type ionocyte in the rectal gland, and the actinopterygians through a Cftr-dependent SW-type ionocyte in the gills. Here, we present three hypothetical scenarios to explain these evolutionary differences in salt-secretory organs and ionocytes in marine fishes (Fig. 2). It is important to acknowledge that the ionoregulatory mechanisms at work in marine sarcopterygians are currently unknown. However, the West Indian Ocean coelacanth (*Latimeria chalumnae*) exhibits elevated levels of serum osmolality, urea and trimethylamine oxide, along with high levels of Nka activity in the rectal gland. Thus, it seems likely that the ionoregulatory physiology of marine sarcopterygians is homologous to that of chondrichthyans; resolving this uncertainty in marine sarcopterygians would provide critical insight into the evolutionary diversification of ionoregulatory strategies in fishes.

A first hypothesis, the ‘ancestral ionocyte’ hypothesis (H1) proposes that an ionoregulating ancestral vertebrate (the common ancestor to agnathans and gnathostomes) utilized a Cftr-independent, salt-secretory branchial ionocyte, which remains present in extant lampreys. Following this hypothesis, during the early radiation of gnathostomes, an osmoconforming, ureotelic strategy (ureotelism; see Glossary) emerged and salt secretion shifted to a newly acquired salt-secretory rectal gland. As a result, the salt-secretory pathways in the gills became dormant. Then, in the early radiation of actinopterygians, an osmoregulatory strategy re-emerged in this lineage; the salt-secretory rectal gland was lost, and the gills regained their primary role in salt secretion. At this point, Cftr assumed its role as the primary pathway for apical Cl^- secretion by branchial ionocytes.

A second hypothesis, the ‘resurrected ionocyte’ hypothesis (H2), proposes that a branchial ionocyte in an ionoregulating ancestral vertebrate was rich in Cftr, akin to ionoregulatory strategy of extant marine teleosts. In this scenario, at some point in the agnathan lineage, the role of Cftr as the Cl^- channel in branchial salt-secreting ionocytes was lost. Additionally, assuming the ancestral ionoregulating vertebrate had a Cftr-rich branchial ionocyte, which was apparently lost during the acquisition of a salt-secretory rectal gland and the radiation of the gnathostomes, this Cftr-rich ionocyte was resurrected in the marine actinopterygians when the rectal gland was lost, and branchial salt secretion was reacquired.

A third hypothesis, the ‘independent ionocytes’ hypothesis (H3), proposes that the branchial SW-type ionocytes of lampreys and teleosts evolved independently. In this scenario, the last common vertebrate ancestor did not ionoregulate through branchial Na^+ and Cl^- secretion (H3a), but rather through salt secretory processes based in the rectal gland or another organ. A variant of this hypothesis proposes that the last common ancestor of agnathans and gnathostomes did not ionoregulate by Na^+ and Cl^- secretion at all (H3b). In either case, branchial Na^+ and Cl^- secretion was independently derived in lampreys and actinopterygians, which now utilize distinct molecular strategies for Cl^- secretion.

Unfortunately, the information available about branchial Cl^- secretory mechanisms in marine actinopterygians is limited, primarily because of the relatively small number of species studied, all of which are teleosts. Thus, Cftr-independent SW-type ionocytes may exist in the gills of marine actinopterygians that have simply not yet been examined. Such a case, particularly the presence of Cftr-independent Cl^--secreting ionocytes in non-teleost marine actinopterygians such as sturgeons, would highlight the derived nature of a Cftr-rich ionocyte and support H1 and H3, which state that a Cftr-dependent mechanism for branchial Na^+ and Cl^- secretion is a relatively recent feature among marine fishes.

Toward a unifying hypothesis for ionocyte-mediated salt-secretion beyond marine fishes

Among vertebrates, the Silva model seems to be broadly applicable. In reptiles and birds, salt-secretory ionocytes in lingual, orbital and

Journal of Experimental Biology (2025) 228, jeb251075. doi:10.1242/jeb.251075

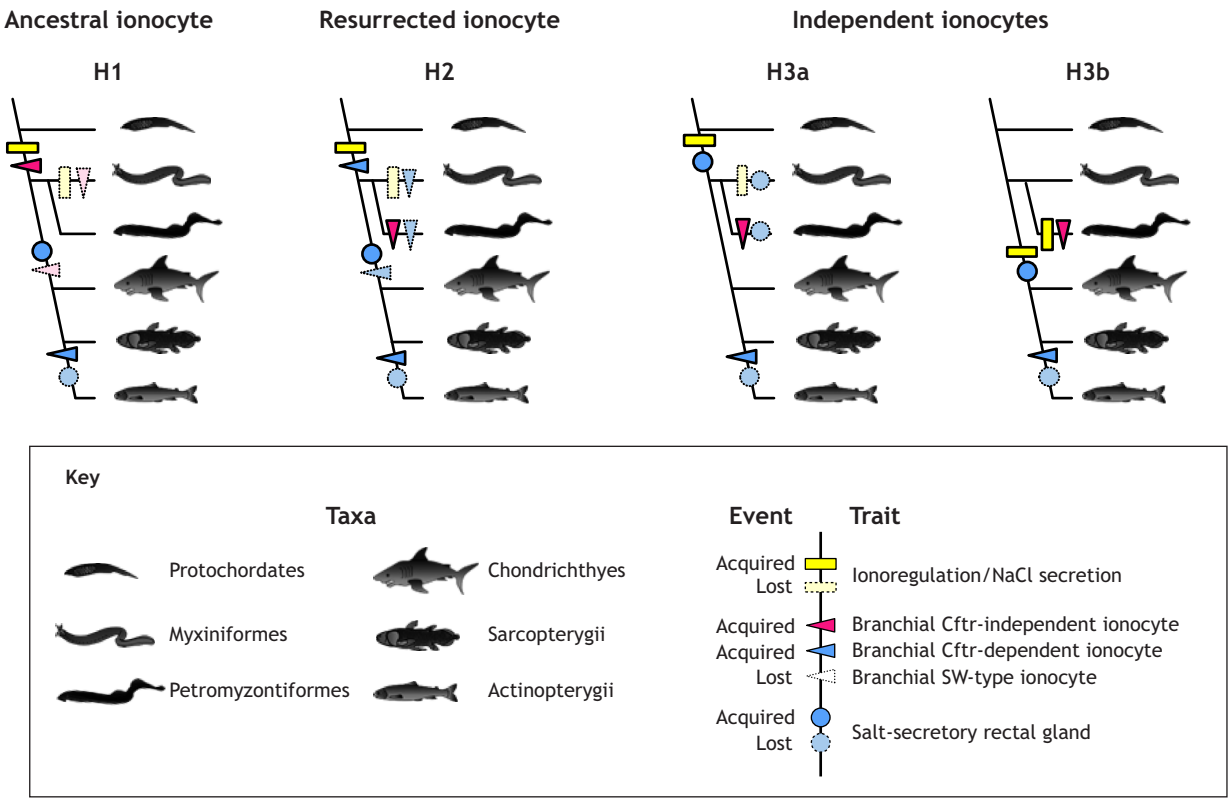

Fig. 2. Hypothetical evolutionary scenarios for the acquisition and loss of ionoregulatory strategies in fishes. Three hypotheses for the evolution of ionoregulation in fishes are presented: (H1) the 'ancestral ionocyte' hypothesis; (H2) the 'resurrected ionocyte' hypothesis; and (H3a,b) the 'independent ionocytes' hypothesis. See text for details.

nasal glands co-express Nka, Nkcc1 and Cftr, and are thought to work similarly to the ionocytes in the gills and rectal glands of marine fishes (Komnick, 1986). This demonstrates the broad taxonomic relevance of the Silva model in explaining how specialized salt-secreting epithelia function beyond fishes. However, the Silva model of ionocyte-mediated Na^+ and Cl^- secretion may not extend beyond vertebrates. The model has been tested in the salt-secretory gills of brine shrimp (*Artemia salina*) and fiddler crabs (*Uca pugilator*), but measurements of transepithelial potential in these species reveal an opposite transepithelial polarity compared with that of marine fishes, which would preclude the secretion of Na^+ as described by the Silva model (Evans et al., 1976; Holliday et al., 1990). Therefore, how crustaceans secrete Na^+ and Cl^- remains an area for future research.

The broad applicability of the Silva model among vertebrates raises an intriguing evolutionary question: are the salt-secreting cells contained within the elasmobranch rectal gland, marine fish gill, reptilian lingual gland and the avian nasal gland homologous, even though the organs that contain them are not? A conceptual framework for understanding how cells originate and evolve proposes that homology can be inferred when cell types share functional, mechanistic and, critically, developmental regulatory processes. In contrast, convergence should be inferred when cell types share functional and mechanistic processes but are produced by distinct developmental programs (Arendt et al., 2016). The recent identification of conserved transcription factors, such as forkhead box I (Foxi) proteins, as markers for ionocyte lineages may provide a way to investigate whether vertebrate salt-secreting ionocytes are homologous. Foxi proteins serve as transcription factors involved in promoting the differentiation of the mammalian pulmonary ionocyte, which is yet another example of a salt-secretory ionocyte that richly co-expresses Nka, Nkcc1 and Cftr (Montoro et al., 2018; Plasschaert et al., 2018; Yuan et al., 2023). Foxi proteins have also been found in the Nka- and Nkcc1-rich branchial ionocytes of protochordates (Sackville et al., 2022), and *foxi* gene expression is more prominent in ionocytes than in other branchial cell types of Atlantic salmon (West et al., 2021). Ultimately, comparative single-cell transcriptomics, guided by the expression of markers such as Foxi, may help determine whether salt-secreting ionocytes in vertebrates (and possibly invertebrates) share a deeply conserved differentiation program or if they instead represent independently evolved, convergent solutions for epithelial salt secretion. In this context, SW-type/salt-secreting ionocytes can serve as a valuable model for understanding not only how Na^+ and Cl^- are secreted, but also how conserved regulatory programs shape epithelial differentiation and cellular fate programming more broadly.

Conclusions

As we approach the 50th anniversary of Silva and colleagues' foundational work, comparative physiologists are well-positioned to lead new investigations into the diversity of ionoregulatory mechanisms in marine fishes. The Silva model has proven to be remarkably enduring and will continue to inform the field in this area. Indeed, the lasting strength of this model may contribute to assumptions about its universality. However, it is time for a renewed examination, including broader taxonomic surveys to determine whether Cftr functions universally as the apical Cl^- channel in ionoregulating marine gnathostomes, and whether other ion transporters, such as Ano1 or yet-unidentified candidates, play essential roles in ion secretion by SW-type ionocytes. Such efforts

are likely to reinforce the robustness of the Silva model of salt-secretory mechanisms in marine fishes, while also promising to yield new mechanistic insights. By integrating comparative molecular, cellular and physiological approaches, we can achieve a deeper understanding of adaptive ionoregulatory strategies. Although gaps in extant taxa may obscure the evolutionary origins of vertebrate salt secretion, recent discoveries underscore the need for more extensive comparative studies of Ano1 and Cftr in basal lineages, including protochordates, hagfishes, lampreys, elasmobranchs and marine osteichthyans. We propose that future research should integrate an evolutionary and developmental framework – including consideration of the role of Foxi proteins or other conserved regulators in driving ionocyte formation – into the Silva model. This will offer the greatest potential to advance our understanding of both the physiology of extant species and the evolutionary history of epithelial ionoregulatory processes in marine fishes.

Competing interests
The authors declare no competing or financial interests.

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