Characterization of Eukaryotic Aquaporin Regulation

Jessica Glas

PhD thesis

Department of Chemistry and Molecular Biology
University of Gothenburg

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Cover illustration: Predicted complex of human AQP4 with nicastrin (AlphaFold2), shown as a BiFC complex (YFP PDB ID: 1MYW)

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Jessica.glas@gu.se

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Department of Chemistry and Molecular Biology
Division of Biochemistry and Structural Biology
University of Gothenburg
SE-405 30 Göteborg, Sweden

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ABSTRACT

Aquaporins, as transmembrane proteins essential for cellular water balance maintenance, are increasingly recognized for their modulation by protein-protein interactions, often implicated in various human pathologies. This thesis explores the function and regulation of aquaporins in human, plant and fish, focusing on human aquaporin 4 (hAQP4), spinach aquaporin (SoPIP2;1) and climbing perch aquaporin (cpAQP1aa), which function in different environments with different osmolarity challenges. Employing bimolecular fluorescence complementation and flow cytometry *in vivo*, we established standardized sample preparation methods, distinguishing constructive interactions from randomly formed complexes. This approach, utilized across various studies, included screening for the interaction of the key regulator CaM with human aquaporins as well as screening a human brain expression library for interaction with hAQP4. By employing FACS and an established sorting gate to capture strong fluorescence signals, we efficiently isolated novel interaction candidates to water channel in the brain.

While extensive insight into the structural gating mechanism of SoPIP2;1 was gained through X-ray crystallography, reproducing such crystal quality for other membrane proteins remains challenging. Hence, alternative techniques, such as continuous diffraction, were explored to obtain structural data. Furthermore, this thesis delves into the molecular mechanism of AQP1 from the fish *Anabas testudineus*. Through structural, mutational and functional studies, key residues responsible for the osmoregulatory mechanisms have been discovered. Integration of stop-flow assays and molecular dynamics simulations revealed a previously unknown extracellular gating mechanism for this aquaporin isoform involving phosphorylation.

Altogether, the work summarized here presents a new robust method for evaluation and screening of membrane protein complexes, with potential importance for regulation. Furthermore, high-resolution structures are unbeatable tools for analysing protein function, and in this thesis, alternative approaches as well as the first structure of an aquaporin from fish are presented, shedding new light on eukaryotic aquaporin regulation.

SAMMANFATTNING PÅ SVENSKA

Aquaporiner är transmembranproteiner som är viktiga för den cellulära vattenbalansen i alla levande organismer. Vi får alltmer förståelse för hur dessa vattenkanaler moduleras genom protein-protein interaktioner, mekanismer som ofta kopplas samman med olika sjukdomar hos människor. Den här avhandlingen undersöker funktionen och regleringen hos aquaporiner från människa, växtriket och fisk, med fokus på humant aquaporin 4 (hAQP4) samt aquaporin från spenat (SoPIP2;1) och klätterfisk (cpAQP1aa), vilka ansvarar för vattenflödet i celler som omges av olika osmotiska utmaningar. Med hjälp av bimolekylär fluorescence komplementering (BiFC) och flödescytometri (FC) *in vivo*, har vi etablerad en standardiserad metod för provberedning där vi kan särskilja konstruktiva interaktioner från slumpmässigt formade membranprotein komplex. Denna metodik har tillämpats i flera av de ingående studierna, vilket inkluderar utvärdering av komplex mellan nyckelregulatorn calmodulin och aquaporiner från människa screening av ett proteinbibliotek från human hjärna där vi tittar efter interaktionspartners specifikt till hAQP4. Genom att kombinera fluorescensaktiverad cellsortering (FACS) med en definierad sorteringsram för effektiv identifiering av starka fluorescerande signaler har vi isolerat nya möjliga interaktionsproteiner till vattenkanalen i hjärnan. När det gäller SoPIP2;1 har omfattande insikter rörande den strukturella regleringsmekanismen erhållits med hjälp av röntgenkristallografi, men det är fortfarande en utmaning att erhålla sådan kvalitet på kristallint material för andra membranproteiner. Vi har därför utvärderat alternativa tekniker, såsom kontinuerlig diffraktion, för att erhålla strukturdata. Därtill har denna avhandling detaljstuderat den molekylära mekanismen hos cpAQP1aa. Genom strukturella och funktionella studier, vilka även inkludera mutationsanalys, har nyckelpositioner i den osmoregulatoriska mekanismen hos detta aquaporin identifierats. Därtill har studier av vattenflödet hos isolerat aquaporin kombinerats med molekylär simulering av strukturdata vilket har genererat en tidigare okänd extracellular regleringsmekanism för detta aquaporin vilken styrs av fosforylering. Sammantaget presenterar arbetet i den här avhandlingen en ny robust metod för att utvärdera och screena membranproteinkomplex, vilka har en möjlig betydelse för proteinreglering. Därtill är högupplösta proteinstrukturer oslagbara verktyg för att analysera proteinfunktion, och här presenteras alternativ metodik samt den första strukturen av ett aquaporin från fisk, vilket bidrar med nya insikter rörande regleringen av eukaryota aquaporiner.

LIST OF PUBLICATIONS

I. Schmitz F*****, **Glas J***, Neutze R, Hedfalk K (2021) A bimolecular fluorescence complementation flow cytometry screen for membrane protein interactions. *Sci Report*, 10.1038/s41598-021-98810-

II. **Glas J**, Landén J, Hedfalk K (2024) Evaluating human aquaporin-calmodulin protein complexes using bimolecular fluorescence complementation. *Manuscript*

III. **Glas J,** Panagiotidis J, Hedfalk K (2024) A high-stringency method to screen membrane protein interaction partners – focusing on human AQP4 in the brain. *Manuscript*

IV. Hagströmer C J, Yefanov, O, **Glas J**, Kreida, S, Hedfalk K, Chapman H, Törnroth-Horsefield S, (2023) Continous diffraction from membrane protein crystals. *Manuscript*

V. Zeng J, Schmitz F, Isaksson S, **Glas J,** Arbab O, Andersson M, Sundell K, Eriksson L, Swaminathan K, Törnroth-Horsefield S, Hedfalk K (2022) High-resolution structure of a fish aquaporin reveals a novel extracellular fold. *Life Sci Alliance*, 10.26508/lsa.202201491.

***** These authors contributed equally

CONTRIBUTION REPORT

Referred to publications by Roman numerals

I. I performed the majority of the experiments and was involved in the development of the flow cytometry assay for membrane complexes. I took large part in curating the data, formal analysis, methodology and visualization. I took part in the editing of the manuscript.

II. I performed the majority of the experiments and supervised the master student in the laboratory. I performed the data analysis and methodology, visualization, and I wrote the actual draft of the manuscript.

III. I performed the majority of the experiments and developed the method for screening interaction of the human brain library, also supervising bachelor and master students in the laboratory. I performed the data analysis, methodology and visualization, and I wrote the actual draft of the manuscript.

IV. I produced, purified and crystallized SoPIP2;1 with the secondary detergents. I collected some of the structural data in MaxIV synchrotron.

V. I cloned, produced and purified some mutated variants of cpAQP1aa and performed functional experiments on water transport. I took part in curating the data.

TABLE OF CONTENTS

ABBREVIATIONS

AQP	Aquaporins
Ar/R	Aromatic/Arginine restriction site in aquaporins
BiFC	Bimolecular Fluorescence Complementation
Ca^{2+}	Calcium Ions
CFU	Colony forming units
CMC	Critical micelle concentration
CNS	Central nervous system
DA	Dalton
DDM	n-dodecyl-beta-maltoside
DM	n-decyl-beta-maltoside
FACS	Fluorescence Activated Cell Sorting
FSC	Forward scattering
GFP	Green fluorescent protein
HOLE	A program for the analysis of pore dimensions
IEC	Ion-exchange Chromatography
IMAC	Immobilized Metal Affinity Chromatography
LDAO	Dimethyldodecylamine N-oxide
MAS	Mag angle spinning
MD	Molecular dynamics
mRNA	Messenger RNA

MST	Microscale thermophoresis
NaCl	Sodium chloride
NG	n-Nonyl-Beta-D-Glucopyranoside
NMR	Nuclear magnetic resonance
NPA	Asparagine, Proline, Alanine signature motif
OD	Optical density
OG	Octylglucopyranoside
OGNG	octyl glucose neopentyl glycol
PDB	Protein data bank
PPI	Protein:protein interaction
SC	Synthetic complete
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
SSC	Side scattering
YFP	Yellow fluorescent protein
Å	Ångström

1. INTRODUCTION

Water, the elixir of life, serves as a cornerstone in the intricate processes of all living organisms. From the vast ecosystems to the tiniest cells, water's presence is essential for the functioning and survival of every living being. Its significance cannot be overstated, for without water, it would be impossible to sustain life. While we can survive for weeks without food, our very survival depends on access to water, as our bodies are composed of 50 to 80 percent of it. Yet, cells cannot store water indefinitely. Constant loss occurs through excretions such as urine, faeces, sweat, and even through respiration. As a result, we must rehydrate through consumption, ensuring a delicate balance of water within our cells. This balance is vital for upholding the structural integrity of cells, supporting enzymatic reactions, and facilitating the transport of essential nutrients and waste products. To maintain water equilibrium within cells and regulate solute concentrations, water homeostasis operates as a tightly controlled and rapid process. Osmotic pressure acts as the driving force, influenced by the concentrations of solutes both inside and outside the cells. However, the hydrophobic core of the cell membrane hinders efficient movement of water. To overcome this barrier, cells employ specialized channels that enable the swift and selective diffusion of water across the membrane, the aquaporins, a topic we will explore in the upcoming chapters.

1.1 THE CELL MEMBRANE AND MEMBRANE PROTEINS

In the cellular domain, the lipid membrane takes centre stage, acting as a vital boundary that separates the cell's inner environment from the surrounding extracellular fluid. The cell membrane regulates the selective movement of molecules in and out of the cell[1]. It serves as a barrier, communicator, and regulator that ensures the maintenance of cellular homeostasis and facilitates essential cellular processes.

The prevailing model used to illustrate membrane structure and function is the "fluid mosaic model," first proposed by Singer and Nicolson in 1972[2]. In contrast to earlier assumptions portraying the cell membrane as static, uniform, and with proteins randomly embedded throughout, the fluid mosaic model presents membranes as dynamic and flexible entities, much like a fluid mosaic comprised of various components, including lipids, proteins, and carbohydrates.
While the fluid mosaic model remains a foundational concept, our understanding of cell membranes has evolved over time with the advent of advanced imaging technologies, providing deeper insights into the dynamic and complex nature of cell membranes[3]. Today, we recognize that the cell membrane is far more intricate than previously thought, exhibiting heterogeneity and variability in composition and organization.
The basic framework of the cell membrane is a lipid bilayer, consisting of two layers of phospholipid molecules. Each phospholipid molecule consists of a hydrophilic head and two hydrophobic tails. The hydrophilic heads face outward, interacting with the watery extracellular and intracellular environments, while the hydrophobic tails point inward, creating a hydrophobic core that restricts the movement of water-soluble molecules. While small non-polar molecules, like oxygen and carbon dioxide, can effortlessly diffuse through this region due to their hydrophobic nature, polar molecules and ions rely on the assistance of transport proteins to traverse this hydrophobic barrier.
Membrane proteins are structurally diverse and can span the lipid bilayer of the cell membrane (transmembrane proteins), be anchored to the membrane surface (peripheral proteins) or be covalently attached to lipid molecules within the cell membrane (lipid-anchored proteins) (**Fig. 1**). Transmembrane proteins often have hydrophobic regions that anchor them within the lipid bilayer, while their hydrophilic regions interact with water and cellular components on both sides of the membrane. Transmembrane proteins include ion channels, transporters and receptors[4].

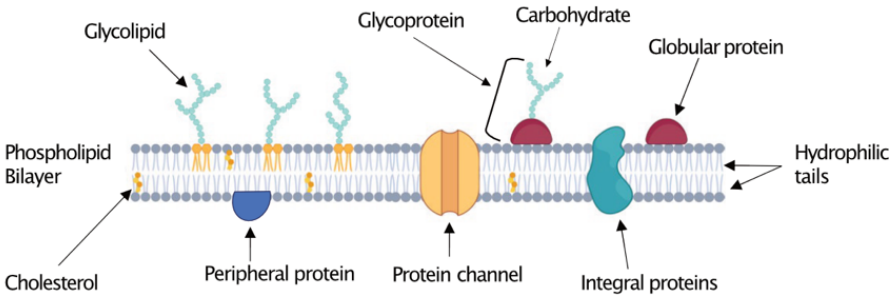


Figure 1. Schematic presentation of the cell membrane. Figure was created with Biorender.com.

When the membrane protein research began, transmembrane proteins had not been isolated in a pure state, nor had their amino acid sequences been determined[5]. This lack of knowledge persisted until 1978 when the first integral membrane protein, human erythrocyte glycophorin, was successfully characterized[6]. As time progressed, advancements in electron microscopy enabled researchers to directly observe the structure of cell membranes, providing visual confirmation of proteins spanning the lipid bilayer [7]. Additionally, techniques such as X-ray crystallography provided scientists with high-resolution insights into the molecular structure of integral membrane proteins.
Today, thousands of transmembrane proteins have been identified and studied and they continue to be a focal point of research due to their critical roles in cellular physiology. While comprising only 20-30% of the human proteome[8], membrane proteins constitute a significant 60% of drug target[9].

In the large field of transmembrane proteins, we focus in this thesis on one particular class with a crucial function: aquaporins (AQPs). While membrane proteins are central to various cellular processes such as molecule/ion transport, signal transduction and enzymatic reactions, AQPs are characterized by their role in facilitating water transport across the hydrophobic membrane, thus ensuring the cell's water balance[10].

1.2 AQUAPORINS

1.2.1. DISCOVERY OF AQUAPORINS

The exploration into AQPs began with a fundamental question: Was passive diffusion sufficient, or were specialized water transporters necessary for water traversing the cell membrane?[11] In the 50s, researchers, using a device capable of measuring the swelling and shrinking of red blood cells revealed that water had two pathways across membranes. Monitoring osmotic swelling and shrinking at various temperatures, a slow, temperature-sensitive process alongside a rapid, temperature-independent process has been discovered. The slow process indicated diffusional permeability of water through the lipid bilayer, whereas the fast process suggested the presence of aqueous pores in the membrane, facilitating rapid, osmotically driven water passage[12]. Further investigations by Macey and Farmer[13] demonstrated that mercuric chloride could inhibit the fast component of this process, implying the presence of a protein pore, or water channel, within the membrane. Despite numerous efforts to identify the protein responsible for forming water channels, progress in the field remained stagnant for many years[14]. In 1991, during the study of Rhesus factor proteins in red cell membranes, Peter Agre and his colleagues at John Hopkins University made a surprising discovery. During their SDS-Page experiments, a persistent contaminant with a molecular weight of 28 kDa appeared. Instead of disregarding this discovery, they speculated that this could indeed be the elusive water channel in red cells. To explore further their discovery, the Agre group subjected oocytes injected with the mRNA encoding the putative water channel to a hypotonic buffer. *Xenopus* oocytes typically possess impermeable membranes to water. Unexpectedly, this experiment resulted in a significant increase in water influx, leading to oocyte swelling and eventual rupture[15]. This outcome provided strong evidence that the injected mRNA prompted the production of a novel protein that rendered the membrane highly permeable to water. This unexpected finding marked the identification of a new protein channel CHIP28 (channel-forming integral membrane protein of 28 kDA), later named Aquaporin-1 (AQP1)[16, 17]. The discovery of AQP1 validated the existence of water-transporting proteins, finally putting an end to the long-standing debate on this topic. The molecular mechanisms of water transport across cell membranes were finally revealed [18, 19], which led to the award of the Nobel Prize in 2003 for their discovery[20].

As research progressed, more AQPs isoforms were discovered, expanding the AQP family. To date, hundreds of AQP isoforms have been identified in various organisms, each with specific tissue distributions and functional properties[21, 22]. In the human body there are 13 AQP homologues (AQP0-AQP12) expressed in a large variety of tissues such as the kidneys, lungs, where water regulation is of utmost importance. According to their substrate specificity, they can be sub grouped into classical AQPs or aquaglyceroproteins. The classical AQPs (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6 and AQP8) transport only water, while the aquaglyceroproteins AQP3 and AQP7, AQP9, AQP10, transport small solutes such as glycerol, urea and ammonia in addition to water. AQP11 and AQP12 belong to the group of superaquaporins[23, 24]. However, the research on superaquaporins is still relatively limited and their functional characteristics as wells as their biological roles are not yet fully understood (**Table 1**).

Table 1. Functional properties of mammal AQPs with its major tissue distribution and substrate specify[25, 26]

	Major tissue distribution	**Substrate**
AQP0	Eye lense	Water (low transport), Ions
AQP1	Brain, kidney, eye, lung, muscle, erythrocytes	Water, hydrogen peroxide, carbon dioxide, ammonia, Ions, nitric oxide
AQP2	Kidney collecting duct	Water
AQP3	Kidney, digestive tract, erythrocytes, lymphocytes, macrophages, dendritic cells, skin	Water, glycerol, urea, arsenic, antimony, silicon, polyols
AQP4	Astrocytes in the CNS, the kidney, skeletal muscle, digestive tract	Water, carbon dioxide
AQP5	Sweat, lacrimal and salivary glands, lungs	Water, carbon dioxide
AQP6	Renal epithelia	Water, carbon dioxide, urea, ammonia, glycerol, anions
AQP7	Liver, kidney, male reproductive system, cardiac muscle	Water, glycerol, urea, ammonia, arsenic, antimony, silicon
AQP8	Kidney, liver, pancreas, colon, trachea,testis	Water, hydrogen peroxide, urea, glycerol, ammonia
AQP9	Hepatocytes, epididymal cells and several types of immune cell such as neutrophils, macrophages and lymphocytes	Water, glycerol, carbon dioxide, urea, ammonia, arsenic, antimony, silicon, lactic acid, polyols, purines, pyrimidines, carbamides
AQP10	Adipose tissue, gastrointenstinal tract	Water, urea, glycerol, arsenic, antimony, silicon
AQP11	Kidney, liver, testes, brain, and adipose tissue	Water, glycerol
AQP12	Intestine, pancreas, stomach, and tongue	Unknown

1.2.2 STRUCTURAL FEATURES

The unique structure of AQPs is the key to their specialized function, setting them apart from other proteins[20, 25]. The initial high-resolution structure of human AQP1 in 2000 unveiled the characteristic "hourglass" shape of the central pore[27,28]. Generally, AQPs share similar structural features, with a molecular weight about 30 kDa in the non-glycoslyated form, and varying glycosylation states leading to higher molecular weights[14]. AQPs typically arrange themselves as tetramers, comprised of individual functional monomeric subunits. They can arrange into homotetramers **(Fig. 2A),** where all subunits are identical, or heterotetramers, composed of different splicing variants or mutants, as observed in AQP4[29, 30].

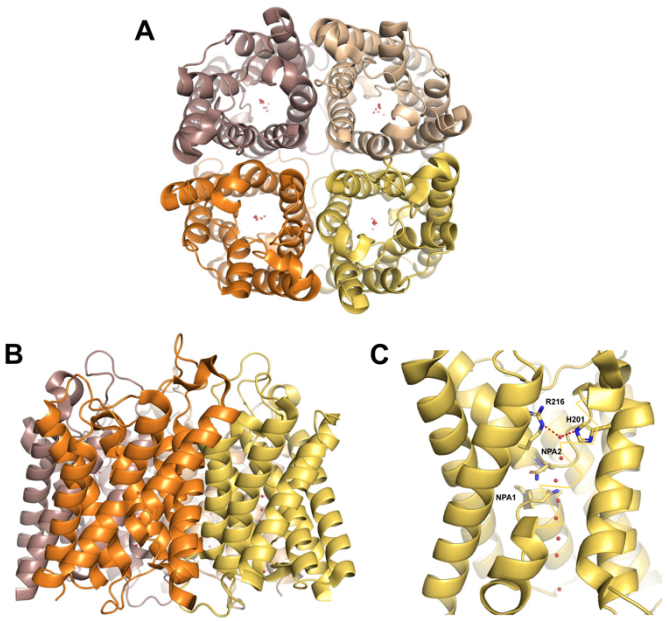


Figure 2. Extracellular view (A) and side view (B) of a typical AQP monotetramer, here hAQP4 (PDB: 3GD8). C) Close-up of the constriction region, containing the R/Arg selectivity filter and the NPA motifs.

Each AQP monomer comprises of six membrane-spanning regions connected by five loops and two short helical segments, which enter the membrane midway, thereby forming a seventh pseudotransmembrane and contributing to the formation of a central pore. The pore is lined predominantly with hydrophilic amino acids,

forming an aqueous channel where water molecules pass through in single file (**Fig. 2B**). Within the water-conducting pore, two copies of the highly conserved asparagine-proline-alanine (NPA) motifs are situated, constraining the orientation of water molecules and creating an electrostatic barrier that prevents proton movement through the Grotthuss-type mechanism[31-33]. On the extracellular side of the pore, an aromatic-arginine region acts as the narrowest part of the pore, preventing the entry of large molecules, and also contributing to the proton exclusion (**Fig. 2C**). The amino acid composition of the ar/R selectivity filter differs between the AQP subgroups, thereby conferring different solute specificity. In aquaglyceroproteins, this region is slightly wider to allow the passage of larger solutes[34]. The amino (N)- and carboxy (C)-termini are located on the cytosolic side of the membrane. The composition and length of these termini vary significantly between the isoforms, adding to the diversity of AQP functionality and regulation (**Fig. 3**).

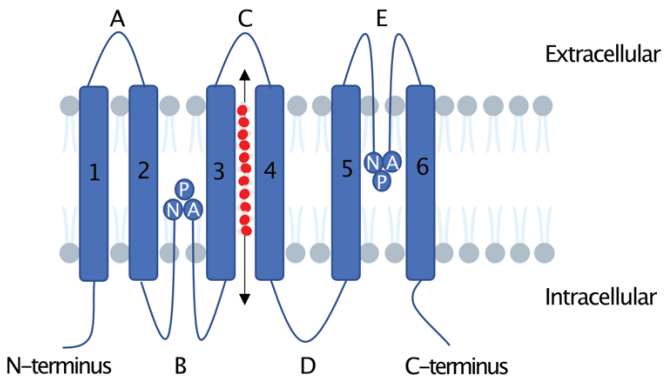


Figure 3. Aquaporin Topology**.** All AQPs exhibit a shared topology characterized by six transmembrane α-helices and five connecting loops.

1.2.3 REGULATION BY GATING AND TRANSLOCATION

Different environmental conditions or cellular needs necessitate intrinsic control of water flow. While the flow itself cannot be actively controlled in response to environmental signals, eukaryotic AQPs undergo tight regulation in response to various cellular and environmental factors. Fluctuations in osmotic conditions, hormone levels (e.g., vasopressin, adrenaline, histamine), and cellular stress

influence AQP activity[35]. Additionally, post-translational modifications such as phosphorylation, binding of divalent cations, and interactions with other proteins impact AQP targeting to different cellular membranes (trafficking or translocation)[36] or directly regulate their function within the membrane (gating)[32] (**Fig. 4**).
AQP translocation refers to the dynamic movement of AQP proteins to or from the cell membrane, controlling their availability for water transport. When required, AQPs are transported via small vesicles to the plasma membrane, where they are inserted to serve as functional water channels. This happens usually as a response to stimuli such as hormonal signals. In the kidneys, for instance, the hormone vasopressin stimulates a shift in equilibrium by promoting the translocation of aquaporin-2 (AQP2) channels to the apical membrane of renal collecting duct cells, thereby reducing their presence inside the cells. This process enhances water reabsorption and facilitates urine concentration[37, 38]. However, this presence at the plasma membrane is not permanent; when AQPs are no longer needed, they are internalized into storage vesicles and returned to the cell interior. Regulation by gating have also been observed in human AQPs, involving conformational changes in their structure that can either open or close the channel. This provides a rapid mechanism to modulate membrane permeability in response to sudden changes in water balance. One mechanism involves the narrowing of the central pore in the transmembrane region, known as the "pinching effect," as seen in AQP0, particularly in ocular lenses[39]. Alternatively, another mechanism involves the insertion of an external loop of the protein into the central pore, effectively blocking it. This latter mechanism is commonly observed in the gating process of plant AQPs[40].

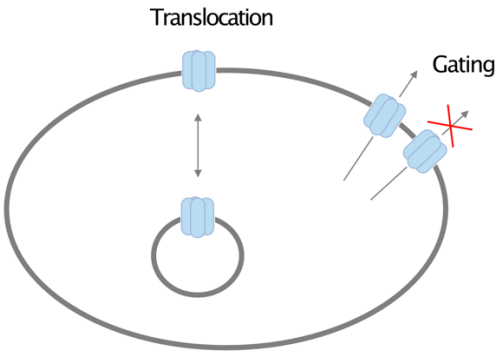


Figure 4. Schematic illustration of the two post- translational regulation mechanism of AQPs: Trafficking/Translocation and Gating.

1.3. SCOPE OF THE THESIS

The main objective of this work is a thorough investigation of the functionality and regulation of AQPs, which includes one member each from the three kingdoms of eukaryotic life: human, plant and fish species. Specifically, this thesis focuses on human aquaporin 4 (hAQP4), spinach aquaporin (SoPIP2;1) and climbing perch aquaporin (cpAQP1aa), which operate in different environments with different osmolarity requirements. The knowledge base regarding these three representative AQP homologues varies significantly (**Fig. 5**) forming a suitable basis for further studies of the eukaryotic AQP family on the whole. Insight into their respective functionalities and regulatory mechanisms will shed light on how AQPs adapt and respond to different environmental factors and ultimately contribute to a comprehensive understanding of their role in biological systems.

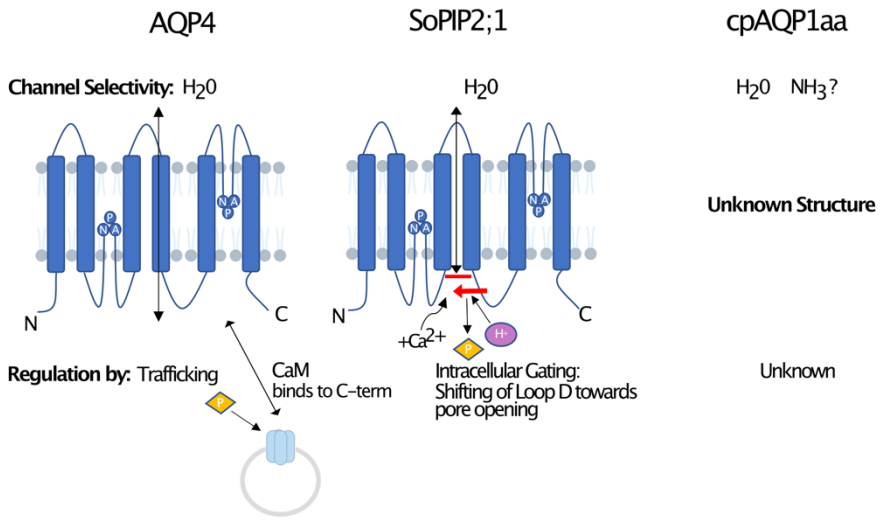


Figure 5. Comparison of the targets of interest for this thesis: human aquaporin 4, fish aquaporin cpAQP1aa and plant aquaporin SoPIP2;1 regarding channel selectivity and regulation mechanism.

1.4. HUMAN AQUAPORINS

Gating and translocation mechanisms in human AQPs are often initiated through interactions with regulatory proteins. Protein-protein interactions (PPIs) are important for organizing and regulating human AQPs, forming complexes with various partners and contributing to the functional regulation of both AQPs and their interacting counterpart[41]. These interactions play crucial roles in diverse physiological and pathological processes. Regulatory interactions typically occur at the less-conserved N-terminal and C-terminal domains of AQPs (**Fig. 3**), which contain multiple sites for post-translational modifications such as phosphorylation by protein kinases[42, 43]. The C-terminal domain, in particular, is frequently associated with protein-protein interactions, with its proximal part forming a short amphipathic alpha helix in water-specific AQPs, serving as a common interaction site[44].

One protein which has been demonstrated to bind and regulate several human AQPs is calmodulin (CaM)[45, 46]. CaM is a versatile calcium-binding protein and its structure resembles a dumbbell, composed of two globular domains connected by a flexible linker. These domains are categorized into N-terminal and C-terminal lobes, each containing two distinct calcium binding-sites referred to as EF-hand (helix-loop-helix) motifs. Its regulatory role extends across diverse cellular processes, including muscle contraction, neurotransmitter release, enzyme activity, and ion channel function[47, 48]. The binding and regulation of AQP0 by CaM have been extensively studied [45, 49, 50]. AQP0 is the most abundant membrane protein in lens fiber cells and plays a crucial role in cell adhesion[51]. Additionally, AQP0 is essential for maintaining lens transparency in the eye[52-54]. The function of AQP0 is regulated by pH and CaM binding at residues 256-275 of its C-terminal region. This interaction reduces water permeability, as demonstrated by early liposome swelling assays[55]. Molecular dynamic simulations suggest that this interaction alters a pore constriction site, decreasing water permeability[50]. Electron microscopy reveals a 1:2 stoichiometry in the AQP0-CaM complex, a non-canonical interaction[56, 57]. Building upon our detailed understanding of the AQP0-CaM complex, we developed a high-throughput method for identifying membrane protein interactions *in vivo.* This complex serves as an exemplary model of a thoroughly investigated AQP interaction, providing a reliable control for our methodology in testing protein interactions **(Paper I).** Furthermore, given CaM's significant role in regulating certain AQPs, our study aims to explore its potential influence on the complete set of human AQPs,

addressing interactions that may not have been extensively investigated *in vivo* before (**Paper II**).

1.4.1 AQUAPORIN 4

Astrocytes play vital roles in the central nervous system, fulfilling essential metabolic and signalling functions within brain cell networks. They possess intricate morphological structures characterized by extensive and branching projections that closely interact with synapses. The movement of water across their plasma membranes is believed to be a primary mechanism through which astrocytes regulate their volume and control the mobility of their processes[58]. Notably, astrocytes demonstrate rapid swelling or shrinking in response to changes in osmolytes and water levels between the extracellular and intracellular compartments[59].
Within astrocytes, the transportation of water is primarily facilitated by AQP4 water channels[59, 60] , which is a central target for this thesis. These channels are predominantly situated at perivascular endfeet[61] and, to a lesser extent, at projections opposite excitatory synapses[62]. Its crucial role lies in facilitating water movement across the blood-brain and blood-spinal cord barriers, thereby contributing to brain water content regulation, ion balance and neurotransmitter and waste clearance[63].
AQP4 exist in two major isoforms of AQP4, M1 and M23, differing in their translation start sites from methionine M1 (323 amino acids) or methionine M23 (301 amino acids). Tetramers comprised of M23 form higher-order structure known as orthogonal arrays of particles (OAP) in the plasma membrane. Although both isoforms exhibit similar water transport capacities, they differ in aggregation properties and cellular distribution onto astrocytes[64, 65]. AQP4-M1 forms small aggregates contrasting with the large AQP4-M23 clusters, particularly near glutamatergic synapses[30, 66].The homotetramers of AQP4-M1 can move freely in the membrane whereas the AQP4-M23 show low mobility. The M23 isoform of AQP4 is believed to help keep the polarized expression of AQP4 in astrocyte end-feet.

Alterations in AQP4 expression or polarization are associated with various diseases focusing on three well-known and extensively studied conditions: neuromyelitis optica spectrum disorders (NMOSD), central nervous system (CNS) edema, and Alzheimer's disease (**Fig. 6**).
NMOSD is a rare autoimmune disease primarily affecting the optic nerves and spinal cord. In NMOSD, the body's immune system

mistakenly targets AQP4 on astrocytes, producing autoantibodies against AQP4, notably AQP4-IgG antibodies. These autoantibodies disrupt the dynamic distribution of AQP4-M23, leading to a decrease in M23 clustering and consequently affecting astrocyte processes and synaptic activity. OAPs are the primary target of anti-AQP4 antibodies in NMOSD, rather than monomeric AQP4-M1. This condition can result in severe visual impairment, paralysis, and other neurological symptoms[67, 68].

Cytotoxic brain edema poses a significant challenge in neurological conditions such as traumatic brain injury or stroke, where uncontrolled water influx leads to swelling of the brain and spinal cord, primarily due to an enrichment of AQP4 at the astrocyte endfeet[46].
Additionally, a growing body of evidence suggest that AQP4 may be involved in the pathogenesis of Alzheimer's disease.
Altered AQP4 expression and distribution in the brains of individuals with Alzheimer's disease have been observed, potentially contributing to a breakdown in the blood-brain barrier. AQP's role in facilitating waste clearance from the CNS in the glymphatic systems could be related. Reduced AQP4 polarization might hinder efficient waste clearance, leading to the accumulation of beta-amyloid, a hallmark of Alzheimer's disease[63]. Currently, the critical regulatory partners for this redistribution of AQP4 proteins remain unknown. Understanding the molecular mechanisms underlying this translocation could provide valuable insights into potential pharmaceutical targets for Alzheimer's disease.

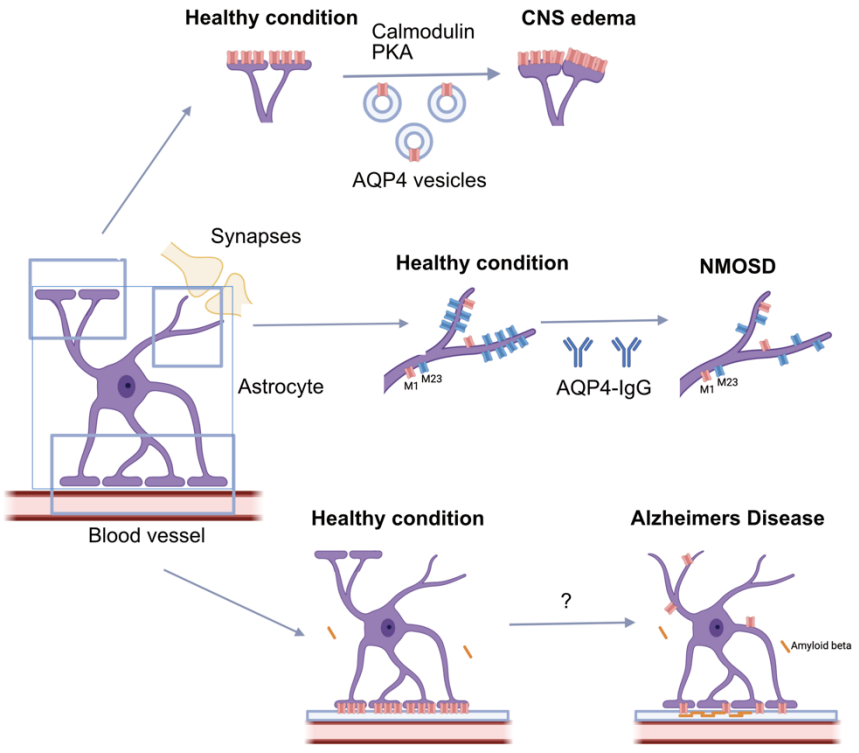


Figure 6. Changes of AQP4 distribution pattern in astrocytes in different diseases. Figure was created with Biorender.com.

Taken together, altered polarized localization of AQP4, particularly at the perivascular endfeet, is of special interest as an increasing number of neurological conditions are associated with changes in AQP4 expression or localization. However, the regulation of polarized AQP4 expression at the perivascular endfeet remains poorly understood. To comprehend the link between AQP4 and diseases, there is a pressing need for a deeper understanding of its actual regulatory mechanisms. Several studies have suggested a potential role of regulatory proteins in modulating AQP4 functionality and numerous proteins have been identified as interaction partners of AQP4. Among these, CaM emerges as a key regulator, playing a crucial role in regulating the trafficking of AQP4, facilitating its positioning to astrocyte endfeet in response to calcium levels along with phosphorylation by PKA. Alongside CaM, several other proteins have been identified as potential interactors. Predominantly, these include ion channels and pumps localized in the

plasma membrane of astrocytes, along with kinases that exert functional impacts on AQP4 through phosphorylation events. Together, these interaction partners might play an important part in controlling the functional network of AQP4 in astrocytes. However, the functionality of many interactions with AQP4 remains unknown[43].

Table 2. Overview of the current known interaction partners of AQP4. Information was taken mainly from[43] and was supplemented by information from [69-71]

Interaction Partner	Biological Function	AQP4 Functionality
Transient Receptor Potential Vanilloid 4 (**TRPV4**)	Role in hypotonicity-induced calcium increase and volume decrease inhibition	Modulates AQP4-mediated **water movement**
Transient Receptor Potential Cation Channel Subfamily M Member 4 (**TRPM4**)	Non-selective cation channel activated by intracellular calcium	Forms ternary complex with AQP4 and SUR1 involved in **cell volume regulation**
Sulfonylurea receptor 1 (**SUR1**)	ATP-binding cassette transporter regulating pore-forming units	Forms ternary complex with AQP4 and TRPM4 involved in **cell volume regulation**
Dystrophin-glycoprotein complex (**DGC**)	Support of muscle fibers	Important **for polarized expression** of AQP4 in astrocytes (mediated by alpha-syntrophin)
Glutamate transporter 1 (**GLT1**)	Sodium and glutamate co-transporter	Interaction with AQP4; functionality unknown
Mu-opioid receptor (**MOR**)	Binding of opioids, neuromodulation of physiological functions	Interaction with AQP4; functionality unknown
Na,K-ATPase	Active pump for sodium and potassium	Regulates AQP4 **water permeability**
Metabotropic glutamate receptor 5 (**mGluR5**)	Neuronal signalling and synaptic transmission	Regulates AQP4 **water permeability**
Clathrin assembly protein complexes 2 and 3 (**µAP2** and **µAP3**)	Involved in endocytosis and lysosomal targeting	Modulates AQP4 **lysosomal targeting**, Phosphorylation by casein-kinase-II
Calmodulin (**CaM**)	Neuronal response to changes in intracellular calcium concentration	Modulates AQP4 **targeting to plasma membrane** upon phosphorylation by PKA
Cystic fibrosis transmembrane conductance regulator (**CFTR**)	ATP gated chlorid channel	Binds AQP4 in Sertoli cells, functionality unknown

Understanding the complex interactions between AQP4 and its partners is crucial for clarifying their involvement in diseases and to get valuable insights into therapeutic strategies. However, establishing methods to screen and verify novel interaction partners in its natural environment presents challenges. This work aims to lay the groundwork for methods to screen and verify novel interaction partners of hAQPs, advancing our understanding of hAQP4 regulation and function in neurological disorders (**Paper III**).

1.5. PLANT AQUAPORINS

Plants, being stationary organisms, heavily rely on environmental factors for sustenance compared to mobile organisms like humans. Water is essential not only for hydration but also for nutrient transportation, respiration, and growth processes within plants. Osmosis, facilitated by AQPs, enables water transport between plant cells, maintaining cell turgor pressure essential for their strength and shape. This pressure allows plants to withstand external forces like wind and gravity[72]. Additionally, plants utilize AQPs in their roots to uptake various solutes, including urea, glycerol, formamide, ammonia and others. The diversity of plant AQPs homologues and their substrate specificities reflect the diverse life of plants, resulting in a wider array of AQPs compared to humans. For instance, the model plant *Arabidopsis thaliana* alone possesses 35 AQPs homologues[73]. These AQP types are expressed in different parts of the plant, and phylogenetic analysis divide them into four main subfamilies: tonoplast intrinsic protein (TIPs), plasma membrane intrinsic proteins (PIPs), NOD26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs) and uncategorized X intrinsic protein (XIPs)[73-78]. Our focus will rely mainly on PIP AQPs, which are separate in two distinct phylogenetic groups (PIP1 and PIP2).

The crucial ability plants must possess for survival is the rapid adjustment to changes in water availability, particularly vital when faced with sudden environmental shifts like flooding or drought to prevent dehydration or overhydration of the plant[79, 80]. In such situations, AQPs can undergo gating to restrict water flow through the central pore of the channel. The closure of water channels in response to these challenges can occur through two distinct mechanisms: dephosphorylation of specific serine residues activated during drought stress or protonation of a specific histidine residue in response to cytoplasmic pH drop and addition of Ca^{2+} caused by flooding-induced anoxia[81-83].

1.5.1. SOPIP2;1

A comprehensive understanding of the pH dependence, serine phosphorylation, and calcium dependence of PIP gating was established in 2005 with the elucidation of the X-ray structure for the open and closed conformations of the AQP isoform of spinach, known as SoPIP2;1 (Spinacia oleracea Plasma membrane Intrinsic Protein 2;1)[84, 85]. In the closed conformation, loop D acts as a cap, obstructing

the channel from the cytoplasm and creating a hydrophobic barrier that occludes the pore. This closed state is stabilized by hydrogen and ionic bonds with cadmium, though likely involving Ca^{2+} ions *in vivo*, anchoring loop D to the N-terminus. Consequently, the pore constricts to less than 1 Å near the cytoplasmic vestibule, significantly limiting water passage[84, 85]. Conversely, under neutral or alkaline pH conditions, the channel can transition to an open state by displacing loop D up to 16 Å away from the cytoplasmic entrance, enabling increased water permeability. Additionally, phosphorylation at two sites (Ser115 in loop B and Ser274 at the C-terminus) disrupts the network of hydrogen bonds anchoring loop D to the N-terminus, leading to the movement of loop D from its closed to its open form. This results in channel opening and a subsequent 50-fold increase in water transport rate compared to the closed conformation[84, 85].

In this study, SoPIP2;1 was chosen as a well-characterized model of plant AQP gating, with a specific focus on its closed conformation. Crystals of this closed conformation were utilized for an investigation employing a novel approach to protein structure determination using continuous scattering data (**Paper IV**).

1.6. FISH AQUAPORINS

Moving from our understanding of the regulation of AQPs in humans and plants, we shift our focus to fish, going deeper into the challenges of osmoregulation that they face. Fish inhibit an underwater world, which comes with its own set of demands. Fish need to carefully control water gain or loss due to their constant contact with water[86, 87]. Additionally, they must regulate the levels of ions in their bodies to maintain a stable internal environment. Unlike terrestrial organisms, fish are presented two primary osmoregulatory challenges: hypoosmotic (lower salt concentration) and hyperosmotic (higher salt concentration) environments. Fish living in freshwater environments face the risk of water diffusing into their bodies, leading to dilution of body fluids. On the other hand, marine fish must prevent water loss to the more saline surroundings. In response to these challenges, fish have developed various osmoregulatory strategies. Freshwater fish actively absorb water through their gills and skin to compensate for the water lost due to osmosis. They excrete diluted urine and actively move ions like sodium and chloride out through their gills to regulate their internal ion

balance. Specialized cells in their gill epithelium, called chloride cells, play a crucial role in ion transport. Marine fish lose water through their gills and skin due to the higher osmolarity of the water. To maintain their water balance, they drink seawater and excrete excess salt through their specialized kidneys and gills. Their kidneys efficiently concentrate urine, conserving water. The chloride cells in their gill epithelium actively transport ions inward to maintain internal ion levels[86] (**Fig. 7).**

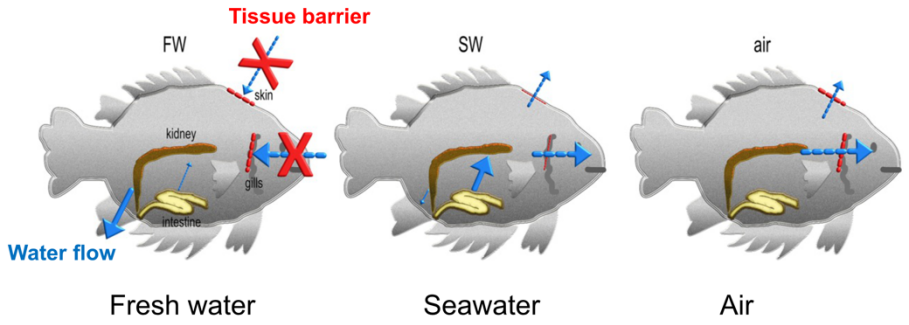


Figure 7: Schematic figure of the water circulation for climbing perch in fresh water, seawater (SW), and air. Figure adapted from **Paper V**.

Overall, fish have evolved remarkable adaptations to cope with varying water conditions and maintain water and ionic balance. AQPs are essential components of the osmoregulatory machinery of fish, enabling them to adapt to different aquatic environments and varying salinity levels. They play a critical role in maintaining fluid volume in the body and preventing excessive water loss by osmosis. The presence of specific AQP isoforms such as AQP1, AQP3, and AQP8, found in the gill epithelium, kidney, and skin, contributes to the maintenance of proper osmotic balance[88, 89].

1.6.1. CPAQP1AA

This thesis focuses on one representative of the euryhaline fish species, the highly adaptable *Anabas testudineus,* commonly known as the climbing perch, which is native to Far East Asia.
This species possesses a range of remarkable abilities that allow it to live in diverse environmental conditions.
It is known to survive in low-oxygen environments and even to leave the water temporarily to migrate overland using specialized gill

chambers, enabling it to endure almost complete desiccation. Furthermore, the climbing perch has the ability to actively excrete ammonia, thus maintaining low ammonia concentrations during periods of emersion[90 91]. And last, this species can acclimate from fresh water to seawater in a progressive manner[92], showing its extreme adaptability. Due to these abilities, the climbing perch serves as an exceptional model organism for the investigation of osmoregulation in fish.

However, at the molecular level, our knowledge of climbing perch AQPs is currently quite limited. Previous research has so far identified a single AQP isoform, cpAQP1aa, which is mainly found in the skin and gills of climbing perch[90]. Considering the significance of these barriers in regulating water flow rates under varying circumstances, and the important role of osmoregulation in aquatic organisms, it's reasonable to speculate that cpAQP1aa plays a vital role in osmoregulation.

Previous studies have also indicated a major involvement of cpAQP1aa in active ammonia excretion in climbing perch[90].

While previous research has mainly concentrated on its identification, localization, and expression, less attention has been directed towards its structural or functional aspects. Unraveling the structural-function relationship of AQPs in fish, especially through obtaining atomic structures of cpAQP1aa, could shed light on its function and specificity. Unfortunately, such structural information is currently lacking, not only for cpAQP1aa but also for other AQPs in fish. Such insights could significantly contribute to understanding the climbing perch's ability to thrive in diverse aquatic habitats and its distinctive semi-terrestrial lifestyle. Addressing this gap in knowledge, this study aims to provide structural insights into cpAQP1aa, ultimately enhancing our comprehension of osmoregulation of AQPs in fish (**Paper V**)

2. THEORY OF METHODS

2.1. PROTEIN-PROTEIN INTERACTION SCREENING

Proteins display high connectivity in the cell. In other words, proteins never act alone; on the contrary, they associate with other proteins to form stable or transient multiprotein complexes that execute a defined function. Importantly, aberrant protein interactions are related to many diseases and therefore, they have become important targets for the development of new chemical drugs. Overall, there is a requirement of techniques that allow studying protein binding as well as their specific inhibition *in vivo* because the cellular environment highly affects the establishment of these interactions[93]. Protein interactions within living cells are intricately influenced by the dynamic cellular environment, including extracellular signals and protein expression levels. Research conducted in living cells offers a more representative snapshot of conditions akin to those found in living organisms. Additionally, conducting experiments in intact cells avoids potential disruptions to protein interactions caused by cell lysis or fixation.
Protein-fragment complementation assays (PCA) provide a valuable approach to studying PPis because in their natural cellular environment, where factors such as cellular localization, post-translational modifications, and other cellular processes can influence interactions. PCAs involve fusing potential binding partners to two carefully designed fragments of a reporter protein. When the bait and prey proteins interact, they bring split reporter fragments close enough for non-covalent and specific reassembly, resulting in the recovery of the reporters native structure and activity, thereby generating a detectable signal[41]. Variants of this method include yeast two-hybrid (YTH) systems YTH uses a reporter gene whose transcription and expression are triggered by interactions between the proteins of interest, resulting in a detectable protein[94].

PCAs find broad applications in studying *in vivo* PPis at various levels, from investigating specific bindings within living organisms to screening novel PPis across different species, including bacteria, animals, and plants. Various proteins have been used as reporter proteins, however PCAs have been significantly expanded through the availability of fluorescent and luminescent proteins. When fluorescent proteins are employed as reporters, it's referred to as bimolecular fluorescent complementation (BiFC).

2.1.2. BIMOLECULAR FLUORESCENCE COMPLEMENTATION

BiFC is a potent technique utilized to visualize PPis within cells. Its principle is straightforward: two proteins, suspected to interact, are each fused to a non-fluorescent fragment of a fluorescent protein, typically GFP or YFP. When these fusion proteins come together and form a complex, the non-fluorescent fragments unite, resulting in the reconstitution of a functional fluorescent protein[95, 96]. This re-association is greatly facilitated if the proteins have a strong interaction[97].

The evaluation of BiFC complexes traditionally involves fluorescence microscopy[98]. Previous assessment of AQP0 and CaM interaction has been evaluated manually using a fluorescence microscope[79]. However, this thesis proposes a novel approach using flow cytometry (**Paper I**). To ensure specificity, the design of the fluorescent protein fragments is critical to prevent spontaneous reconstitution.
The careful selection of fragments aims to prevent unintended reconstitution of the fluorescent protein without interaction between the fusion proteins. Fragmentation of the fluorescent protein typically occurs within beta-strands or loops to minimize background fluorescence[97, 99].
In YFP, these two sites are found either within the loop connecting β-sheets 8 and 9, resulting in YFP_N-1-173 and YFP_C-174-239, or within the loop between β-sheets 7 and 8, yielding YFP_N-1-154 and YFP_C-155-239. For this study, the latter variant was chosen, as it demonstrates low background fluorescence in the absence of interaction[96] **(Fig. 8**).

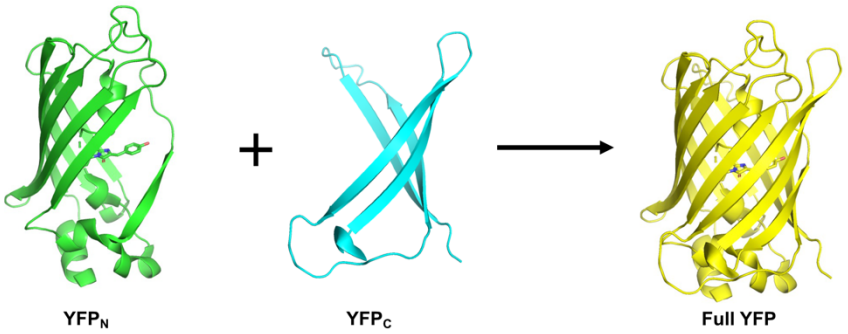


Figure 8. Fluorescence protein complementation of YFP (PDB ID: 1MYW). YFP is divided into two fragments: YFP_N (1-154) and YFP_C (155-239). Notably, the chromophore region, responsible for YFP's fluorescence properties, is highlighted at the center of the protein.

In this study, a variant of YFP called Super Yellow Fluorescent Protein 2 (SYFP2) was employed. SYFP2 boasts enhanced brightness and photostability, making it particularly suitable for applications requiring prolonged imaging or detection of weak signals in living cells[100]. The careful selection of fragments aims to prevent unintended reconstitution of the fluorescent protein without interaction between the fusion proteins. While YFP is commonly used in BiFC assays, there are various fluorescent proteins with different properties available, offering flexibility in experimental design with up to 15 options for fluorescence complementation assays[98].

While BiFC presents numerous advantages, it's crucial to acknowledge potential limitations. The fusion or tagging of proteins can disrupt natural processes and interactions, potentially leading to inaccurate results. False positives or negatives may arise due to factors such as spatial proximity rather than direct physical contact[101].To address this, negative controls, such as mutated interaction sites, are essential to differentiate false positives. Despite these drawbacks, BiFC offers several advantages. It enables *in vivo* experimentation, allowing researchers to observe proteins in their native state with natural modifications. Moreover, BiFC has the potential to detect short-term interactions, in addition to more stable complexes, providing insights into transient PPIs[95] that may be involved in processes like AQP regulation. BiFC is particularly useful for investigating weaker interactions and transient PPIs, which can be captured and studied. This capability extends to detecting PPIs involved in phosphorylation events, which are significant in AQP regulation.

However, BiFC's irreversibility can be limiting for studying dynamic interactions, and its reliance on fluorescent protein maturation

precludes real-time detection[41, 102]. These considerations should be taken into account when designing experiments and interpreting results in BiFC studies.

2.1.3. FLOW CYTOMETRY AND FACS

Population analysis in BiFC relies on the statistical evaluation of numerous samples and demands rapid and precise detection of fluorescent cells. While conventional light microscopes with fluorescence detection systems are widely used, they lack the efficiency needed for high-throughput analysis due to manual handling limitations. In this study, we utilized flow cytometry as an automated method for analyzing and separating cell populations based on their physical characteristics. Flow cytometry, developed in the 1950s and 1960s, has become the keystone technique for analyzing cell populations and offers the ability to quantify multiple characteristics of individual cells at a high throughput. It is widely used in research, clinical diagnostics and industry, simplifying tasks such as counting and sorting cells and detecting biomarkers[103]. A typical flow cytometer consists of three main components: the fluidic system, an optical system and the signal detection and processing unit[104,105]. In the fluidic system, a liquid cell solution is introduced, and cells are directed through a narrow nozzle to form a single-line stream of small droplets, each containing one cell [106]. It includes components such as the sample injection system, sheath fluid and flow cell. These droplets then pass through the optical system, where they encounter a laser beam. The process during which a cell passes the beam is called an event.

Depending on the physical attributes of the event, such as size and granularity, the laser beam scatters in different directions. The scattered light is collected, converted into electrical pulses, and interpreted by the signal detection and processing system. Two types of scattered light are analyzed: forward scatter (FSC) and side scatter (SSC), providing information about cell size and complexity, respectively. The optical system includes components such as lenses, filters, and detectors, which collect and redirect emitted light from individual cells to photodetectors for recording and analysis. The resulting digital signals are typically displayed in histograms or scatter plots, enabling visualization and analysis of fluorescence intensities and cellular properties. Gating may be necessary to exclude anomalies such as debris or unwanted particles, involving selecting an area on the

acquired dot plot corresponding to the cell population of interest and adjusting parameters accordingly. In one measurement, we recorded 100,000 events and further analyzed healthy yeast cells by applying a gate based on size and granularity. A sample size of at least 10,000 cells is recommended in the final gating process to ensure valid results[107] (**Fig. 9**).

For high-throughput screening of protein interactions, FACS is a specialized form of flow cytometry used for live cell sorting. This method separates a cell population into sub-populations based on fluorescent labeling, scatter characteristic or other parameters. To sort the cells, specific features of interest are gated, and if a cell meets the selected parameters, a charge is applied to the droplet containing the cell. With the assistance of electrostatic deflection plates, negatively charged droplets are sorted into designated collection tubes, while uncharged droplets, such as debris, are discarded. Depending on the sorting method, cells can be collected individually or in bulk. The sorted cell populations are then analyzed to confirm successful cell sorting, and the sorted cells can be cultured for further experimentation. This method offers several significant advantages. Firstly, it provides high precision in cell sorting, allowing for sorting based on multiple characteristics simultaneously and yielding a highly pure cell solution. Secondly, it enables the analysis and sorting of living cells, thus allowing the sorted cells to be utilized further in experiments after recovery.

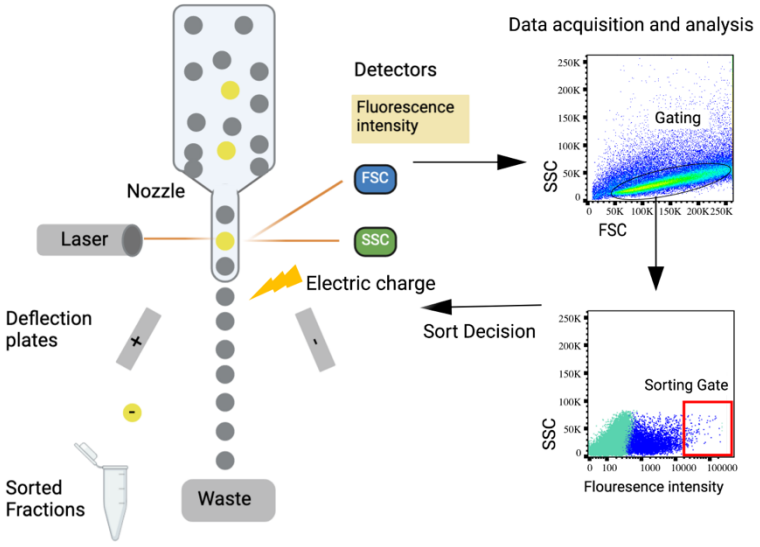


Figure 9. Schematic illustration of the FACS process for evaluating and sorting BiFC complexes. SSC and FSC data from a stream of single cells are collected and displayed as histograms or scatter plots for evaluation. Our approach focuses on analyzing healthy, living cells based on their fluorescence properties, such as intensity and frequency. Cells exhibiting a high fluorescence intensity signal are sorted by applying an electric charge and separated into individual tubes.

2.2 PROTEIN PRODUCTION IN YEAST

Protein production using yeast offers a versatile and well-established platform. It finds extensive use across diverse industries, including biopharmaceuticals and biotechnology, where large quantities of proteins are often needed. In scientific research, particularly for tasks like understanding protein structure and function, substantial protein quantities are essential[108]. In this thesis, both yeast organisms *S.cerevisiae* and *P. pastoris* has been used for different approaches by using its specific advantages being eukaryotic cells. It thereby shares many cellular processes and pathways with higher eukaryotes, making it a valuable model organism.

2.2.1 PRODUCTION IN *S. CEREVISIAE* FOR FUNCTIONAL EVALUATION

S. cerevisiae, or baker's yeast was used in this thesis for investigating PPis [109]. It serves as an exemplary model for analyzing PPIs due to its well-characterized genetics and was as one of the first organisms to have its complete genome sequenced and annotated [110, 111]. With its compact and relatively simple genome, genetic manipulation becomes straightforward. This yeast variety offers the flexibility to introduce reporter genes, aiding in interaction study. There's a wide array of yeast strains and vectors at disposal, each designed for specific applications. For instance, the S288C strain, utilized in this thesis, is prototrophic, meaning it can grow on basic media without extra supplements. Yeast expression vectors feature robust promoters for controlling gene expression, with options for either constitutive (such as ADH1 or GPD) or inducible expression (such as GAL). Culturing and managing yeast cultures is easy and conducive to high-throughput screening, crucial when investigating numerous potential interactions, like in our studies **(Paper I- III**).

2.2.2 HIGH-YIELD PRODUCTION IN *P. PASTORIS*

In choosing an overproduction system for proteins, it's crucial to consider whether the host organism can perform specific protein modifications that match the requirements of the protein of interest. *P.pastoris* shares some advantages with higher eukaryotic expression systems, such as the ability to perform post-translational modifications. This is particularly important for membrane proteins, as many of them require glycosylation[112], disulfide bond formation, and other modifications to fold properly and function correctly[108].
P. pastoris is a very popular production host for membrane proteins, often required in substantial quantities for further analysis. It is a methylotrophic yeast, meaning it possesses the unique capability to utilize methanol as its sole carbon source. This metabolic switch is intricately linked to the strong and highly regulated alcohol oxidase 1 promoter (AOX1). In the presence of methanol, the yeasts's alcohol oxidase initiates a metabolic process. This process involves the conversion of methanol into formaldehyde and hydrogen peroxide as initial byproducts by consuming significant amounts of oxygen. By this we can drive high-level expression of the protein of interest[113]. *P.pastoris* can be grown to high cell densities in bioreactors,

making it a scalable system for the production of large quantities of membrane proteins[114].
For membrane proteins that need to be accurately localized to the cell membrane or other membrane compartments, *P. pastoris* possesses the necessary cellular machinery. Additionally, *P. pastoris* has lower proteolytic activity compared to other expression hosts like *E. coli*, which helps maintain the stability of membrane proteins during production and purification[115, 116].
Over time, numerous protocols, tools, commercial kits and expression vectors have been developed for working with *P.pastoris*, making it a preferred choice for membrane protein research[117].

Initially, AQP structures were primarily obtained from their native sources. However, over time, recombinant protein production has emerged as the predominant source of AQP protein for structural biology studies[118, 119]. For most structural biology projects, protein production in *P. pastoris* is the preferred method due to its high expression levels of recombinant proteins. In this thesis, we utilized. *P.pastoris* for producing membrane proteins, namely SoPiP2;1 and cpAQP1aa (including mutants), essential for structural and functional studies (**Paper IV – V**).
The overall strategy for protein production and purification in *P. pastoris* is outlined in **Fig 10**, although not all steps were carried out for each protein target. The process involves numerous experimental steps to optimize conditions and protocols for each protein. However, the workflow for protein production in *P. pastoris* has been optimized and is readily available from Invitrogen.

The initial step involves cloning the gene of interest into a pPICZ vector and amplifying it in *Escherichia coli (E. coli).* For generating mutants of cpAQP1aa, single point mutations were introduced in the PCR step using overlapping primers. By using this method, the use of ligase is avoided and the whole process of generating mutants get simplified. After linearization, the plasmid is transformed into a *P. pastoris* X-33 strain by electroporation, where it is incorporated into the genome downstream of the AOX promoter by homologous recombination. The Zeocin™ selection marker aids in clone selection, with higher resistance correlating with increased plasmid incorporation and higher protein expression[120]. Clones with high Zeocin™ resistance are selected for further evaluation. In a small-scale expression test, Zeocin™-resistant clones are compared for high target expression[114]. Immunoblot analysis of cell lysates helps to identify clones suitable for large-scale growth in bioreactors[121] and after selecting clones with the

highest protein production level, large-scale production is carried out in 3L fermenters. The use of bioreactors is advisable due to their ability to tightly control environmental factors, such as oxygen supply, temperature and pH, resulting in improved yields compared to shaking flasks[122]. Each fermentation batch typically yields around 500g of cells.

2.2.3 PROTEIN PURIFICATION

The first step in purifying membrane proteins is to isolate the membranes from the cells. This is usually done by lysing the cells using methods suitable for yeast, such as French press homogenization or a bead beater with glass beads. After lysis, a series of centrifugation steps, including centrifugation at 10,000 xg, is performed to separate cell debris and unbroken cells from the membranes. The crude membranes containing the target protein are then separated from the supernatant by ultracentrifugation at 100,000 xg[119].

Many purification protocols include washing steps with salt, urea, or sodium hydroxide to remove peripheral and integral proteins from the membranes as much as possible. Once the membranes are isolated, they are solubilized in detergent. This step is crucial as it covers the hydrophobic parts of the protein, which are otherwise buried in the phospholipid membrane. The detergent concentration must exceed the critical micelle concentration (CMC) to initiate micelle formation and solubilize the protein out of the membrane. Typically, an excess of detergent is added to maximize solubilization efficiency[123].
Membrane solubilization is a critical step as it transfers the protein from the lipid environment to the water environment. The objective is to extract as much protein as possible from the membranes without compromising its integrity. The choice of detergent depends on the specific membrane protein target and often requires an initial screening to determine the most suitable option[114]. In this thesis, we primarily used OG detergent for SoPIP2;1 and NG detergent for cpAQP1aa.
After solubilization, the next step involves extracting the protein target from the mixed protein solution. This is typically achieved using chromatography techniques such as Immobilized Metal Affinity Chromatography (IMAC) or Ion Exchange Chromatography (IEC), depending on the design of the expression construct for the target protein. These chromatographic methods exploit different properties of the protein, such as its charge or the presence of specific tags like histidine to facilitate purification. It is important to note that the presence of these tags may interfere with crystal formation in downstream

applications, particularly in structural biology studies. For most applications the purity of the protein post IEC/IMAC step is sufficient for further analysis. In the preparation of proteins for crystallization, where achieving a high-purity sample is critical, an additional purification step is often required. Many purification protocols conclude with a size exclusion chromatography (SEC) step, which is particularly essential in this context[124].

In SEC, proteins are separated on the basis of their size, with smaller proteins being retained longer in the column. In addition, SEC serves as a quality control for sample homogeneity and can detect significant protein aggregation during the run.

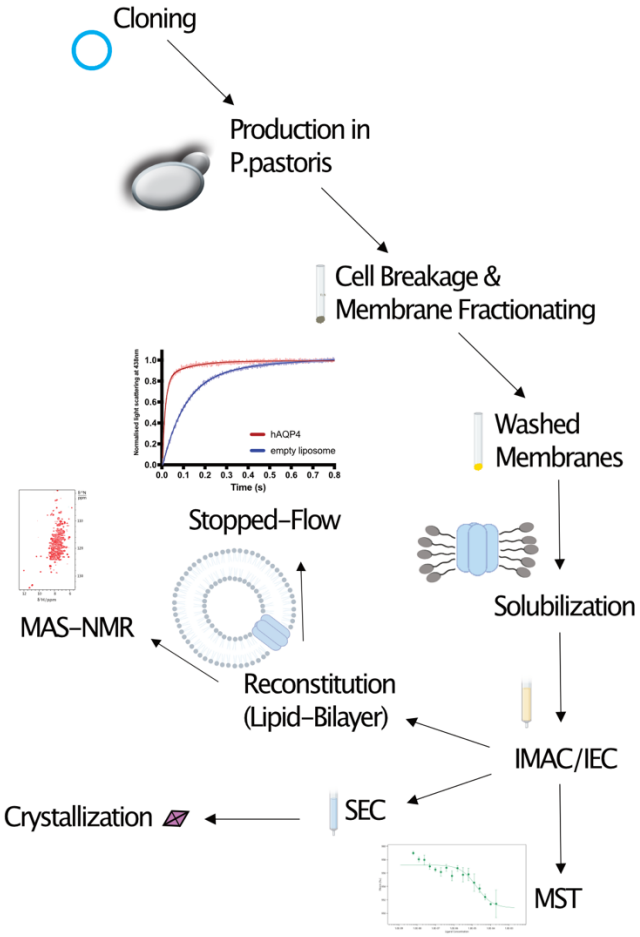


Figure 10: Overview over the different steps towards structural and functional studies of an AQP. Although MST and MAS-NMR are not discussed in detail in this thesis, they represent possible approaches for future investigations.

2.3 FUNCTIONAL ANALYSIS

2.3.1 STOPPED FLOW

Understanding the functionality of AQPs is crucial for biophysical studies, as they are integral to rapid biological processes. However, observing the transport of water molecules through AQPs presents challenges due to the rapid and shielded nature of the process. While experiments can be conducted *in vivo* using systems like oocytes or spheroplasts, or in artificial membrane mimics such as liposomes, the former may struggle to reliably measure low water transport and contend with inherent variations in protein production levels that are challenging to standardize. Hence, in this thesis, functional investigations of cpAQP1aa were conducted by incorporating purified protein into liposomes. Stopped-flow measurements emerged as a solution, enabling the analysis of water transport kinetics indirectly.
The passive transport of water across cell membranes relies on osmotic gradients. Utilizing this dependency, a natural environment for membrane proteins can be created to detect indirect signals for kinetic measurements. Incorporating AQPs into liposomes and exposing them to hyperosmotic gradients enables the detection of vesicle shrinkage, which manifests as variations in light scattering intensity.
To measure AQP activity, purified proteins are solubilized in detergent micelles and subsequently transferred into liposomes. This transfer facilitates the observation and analysis of AQP-mediated water transport, providing insights into their functional dynamics. The reconstitution of AQPs into liposomes involves transferring the protein from its micellar environment into artificial lipid vesicles. To achieve this, a mixture comprising lipids, detergent, buffer, and protein is prepared. It's crucial that the detergent concentration is sufficiently high to completely solubilize the lipids into micelles[125].
The detergent can then be removed through methods such as dialysis, dilution, or the use of polystyrene beads[126], known as BioBeads, which selectively absorb detergent molecules via hydrophobic interactions[127]. As the detergent concentration decreases, the lipids spontaneously assemble into protein-containing liposomes, known as proteoliposomes [128]. This process allows for the creation of lipid vesicles encapsulating the AQP protein, facilitating further study of its function and behavior in a more native-like membrane environment. Since water transport through AQPs is bidirectional and passive, the random orientation of

the protein within the liposomes typically does not pose a significant issue.

The quantification of water transport rate in proteoliposomes is conducted using stopped-flow spectroscopy (**Fig. 11**). In this method, an osmotic gradient serves as the driving force for the kinetic measurements of water transport across lipid bilayers. The experimental setup involves filling one syringe with liposome solution and another syringe with hyperosmotic buffer. When these solutions are mixed rapidly, the osmotic gradient generates an outward force that leads to rapid shrinkage of the vesicles as water flows out of the proteoliposomes. Interrupting the flow after a predetermined mixing time defines the time frame of the measurements. The shrinkage of vesicles is recorded as light scattering, typically measured at a 90° angle. Multiple proteoliposome preps with each several mixing experiments are replicated, averaged, normalized, and time-truncated. Subsequently, the data is fitted with a one or two exponential function. This fitting process enables the derivation of constants representing the water transport rate, allowing for quantitative comparison between different AQP species[129].

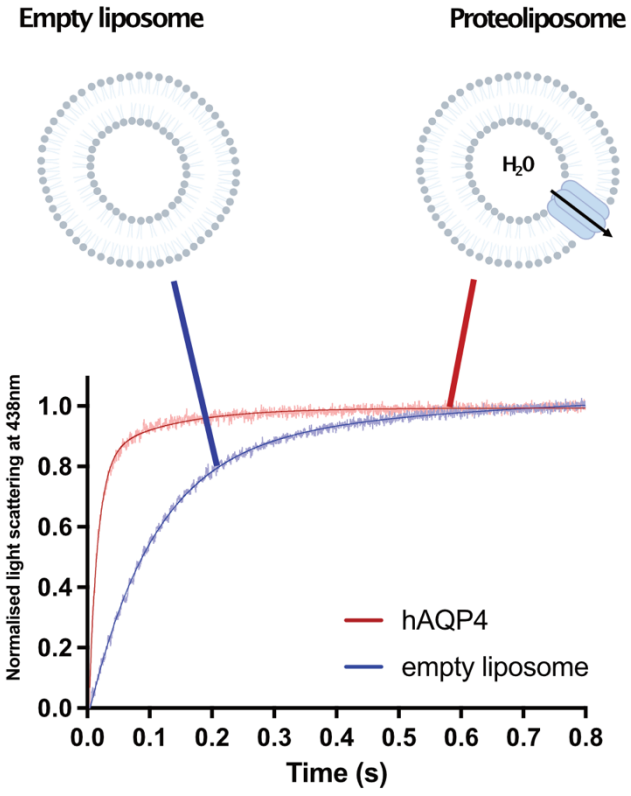


Figure 11: Representive curve for water transport of hAQP4 and empty liposomes. Water transport of empty liposomes is slower than proteoliposomes containing hAQP4.

Moreover, in addition to water, the transportation of glycerol can also be evaluated with the liposome system. This is achieved by suspending the liposomes in a buffer containing glycerol that is isotonic to the sucrose buffer utilized to facilitate transport. When the liposomes are mixed with the sucrose buffer, glycerol is transported out of the cells along the concentration gradient. The accumulation of solutes outside the liposomes induces the co-transport of water, resulting in the shrinkage of the vesicles.

2.4 STRUCTURAL ANALYSIS

2.4.1. X-RAY CRYSTALLOGRAPHY

X-ray crystallography is a pillar of structural biology, providing unparalleled insight into the atomic details of proteins. Its efficiency in solving static protein structures at high resolution, typically at the atomic level, has made it essential for structural studies. Remarkably, it has contributed to the solution of about 80 % of membrane protein structures, although the handling of such proteins is challenging[130]. Issues such as low expression levels, difficulties in protein extraction from cell membranes, and the use of detergents can impact protein stability and function[131]. The historic milestone achieved in 1985 with the determination of the first membrane protein structure, bacterial rhodopsin, marks the start of X-ray crystallography in the field of membrane proteins[132]. Despite subsequent advances, the number of membrane protein structures deposited in the Protein Data Bank (PDB) as of March 2024 is still relatively low (3345 membrane protein structures have been deposited in the PDB, with only 1690 representing unique structures http://blanco.biomol.uci.edu/mpstruc/), underlining the urgent need for comprehensive efforts in determining membrane protein structures.

The first condition for investigating the three-dimensional structure of a protein in an X-ray diffraction experiment is obtaining protein crystals. These crystals, consisting of atoms, ions, or molecules arranged in a repeating, organized pattern, retain the native structure and function of proteins in a stable crystalline form. Unlike small molecules, like NaCl, protein crystallization is a highly challenging process. The unit cell, forming the fundamental building block of the crystal lattice (**Fig. 12**), creates a lattice defined by its dimensions (a, b, c) and angles (α, β, γ), determining the spatial arrangement of atoms or molecules within the crystal. Symmetry within the unit cell, characterized by rotation, translation, and reflections, is defined by the space group, influencing the packing density of molecules and solvent content. Protein crystals rely on weak interactions for stabilization, with solvent molecules occupying a significant portion of the crystal volume.
Interactions between neighbouring protein molecules occur at the crystal interface. Membrane protein crystals typically have a high solvent content due to detergent micelles, resulting in weaker crystal contacts, fragility and increased disorder[131, 133].

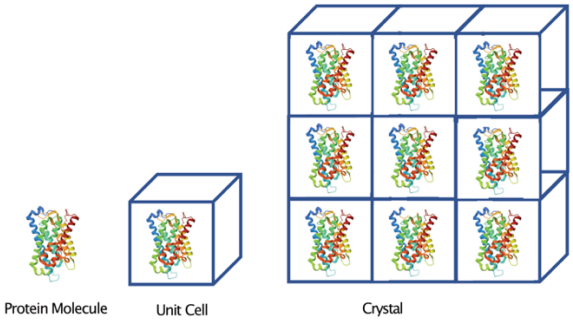


Figure 12: Simplified illustration of a unit cell using a SoPIP2;1 protein molecule.

Typically, protein crystals are grown using vapor-diffusion experiments, which are commonly performed as *hanging drops* (**Figure 13)** or *sitting drops***.** As the drop evaporates, the concentration of reagents decreases, causing the protein to enter *supersaturation and nucleation phase.* Under ideal conditions, the protein concentration enters the *metastable zone*, existing nuclei continue to grow, resulting in the formation of crystals suitable for X-ray diffraction studies (**Figure 14**).

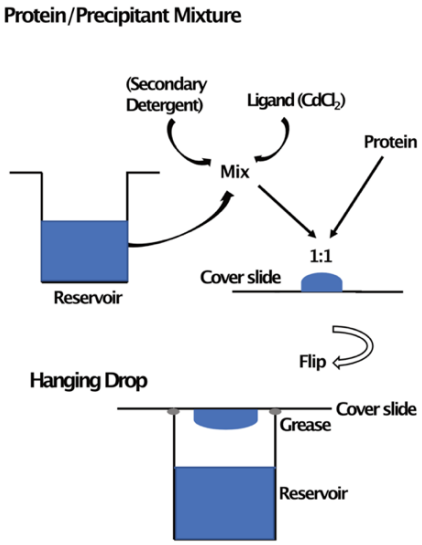


Figure 14. Vapor diffusion method (Hanging Drop) illustrated using SoPIP2;1 crystals as an example. The protein is pre-mixed in a 1:1 ratio with a reservoir/ligand/additive mixture. This mixture is applied onto a coverslip. The coverslip is then inverted so that the protein/precipitant mixture hangs over the reservoir solution.

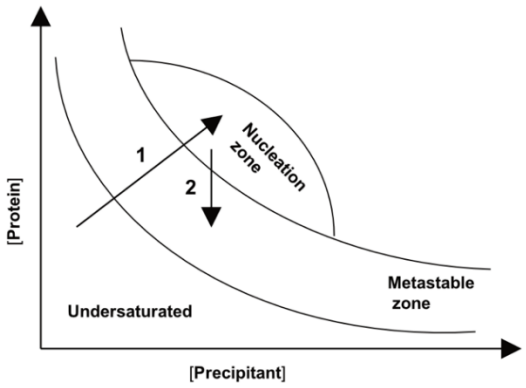


Figure 13. Crystallization phase diagram illustrating the path for the formation of large crystals in a vapor diffusion experiment, indicated by arrows.

In conventional X-ray experiments, a single crystal mounted on a cryo-loop is exposed to an X-ray beam, and diffraction patterns are captured using an X-ray detector (**Fig. 15)**.
Synchrotron facilities are commonly utilized for such experiments.

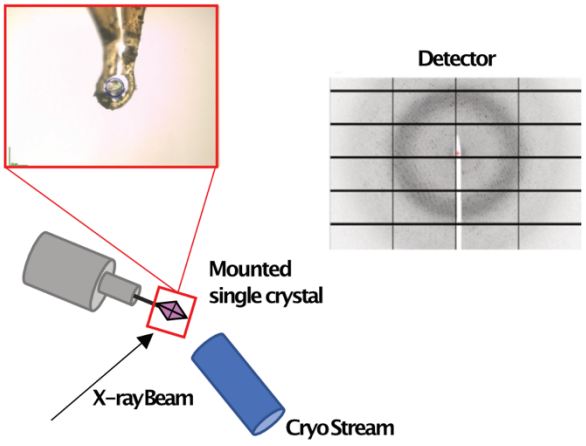


Figure 15. Schematic illustration of a protein crystallography setup**,** showing the example of a mounted SoPIP2;1 crystal and its corresponding diffractions.

X-ray crystallography relies on the interaction between X-rays and atoms within a crystal lattice, leading to the formation of a unique diffraction pattern known as reflections. These reflections occur as X-rays strike various unit cells within the crystal and scatter coherently. Bragg's law describes the relationship between the wavelength of incident X-rays, the spacing between atomic planes, and the angle of incidence, leading to constructive interference and the generation of diffraction peaks.

$$n\lambda=2d\sin(\theta)$$

The equation states that for a given order of reflection n, the product of the wavelength λ and the path difference between two adjacent reflecting planes 2d is equal to n times the wavelength pattern[134].

After reaching the detector, we analyze the intensities of diffracted waves, but crucial phase information remains unseen, posing the phase problem. Overcoming this challenge involves determining phase values, typically through methods like phase determination or calculation. Approaches to address the phase problem include isomorphous replacement, anomalous scattering, and molecular replacement. Molecular replacement, using a known protein structure as a template, is commonly employed to estimate the phase of the target structure[135]. Achieving high-resolution Bragg peaks typically necessitates a well-organized, highly symmetric crystal. However, imperfections within the crystal lattice, common when working with membrane proteins, can lead to irregularities and affect diffraction patterns. Continuous diffraction, where diffraction peaks are spread continuously rather than appearing as discrete spots, is often observed due to these imperfections[136]. Continuous diffraction patterns, historically associated with disorder, now reveal valuable structural insights. When combined with Bragg peaks, continuous diffraction yields higher resolution data. This approach enabled obtaining a static image of the membrane protein complex photosystem II at 3.5Å resolution using an XFEL source. Continuous diffraction also allows direct phasing of the pattern (ab initio phasing) without requiring a prior model[136]. Given challenges in obtaining high-resolution crystals of membrane proteins, this alternative approach offers a crucial opportunity for studying their three-dimensional structure. This work aims to explore alternative approaches for studying membrane protein structures, aiming to overcome challenges of traditional X-ray crystallography.

3. RESULTS

3.1. A HIGH THROUGHPUT APPROACH FOR ANALYSING AQUAPORIN INTERACTIONS

It is increasingly evident that protein-protein interactions play important roles in controlling and modulating the function of AQPs. To gain a deeper understanding of these regulatory processes, particularly at the molecular level, it is crucial to reveal the tissue-dependent regulation of AQPs and their role in health and disease. For comprehensively understanding the molecular mechanisms underlying membrane protein interactions, a robust methodology is essential.

In **Paper I** we addressed this challenge by establishing a higher - throughput screening approach utilizing flow cytometry to characterize membrane protein interaction partners in the host *S. cerevisiae*. Our objective was to create a dependable platform for screening and evaluating membrane protein interactions *in vivo* with statistical confidence. Using the well-established BiFC assay with construct from a prior investigation[79], we analysed known interaction partners such as YFP_N-AQP0 and YFP_C-CaM as well as YFP_N-AQP0ΔC and YFP_C-CaM representing an unconstructive complex where the CaM binding site is deleted. Additionally, we utilized tetramerization (YFP_N-AQP0 and YFP_C-AQPO) as a positive control. To complete the set, a single plasmid negative control (YFP_C-CaM) devoid of any fluorescence signal, and the full-length chromophore fused to human AQP0, YFP-AQP0, served as a positive control (**Fig. 16**).

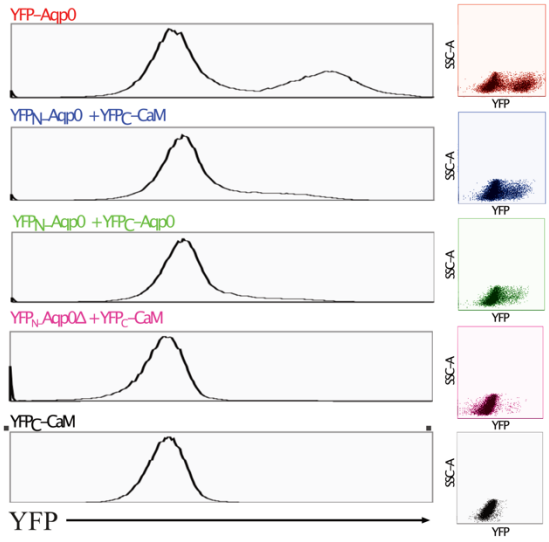


Figure 16. Histograms (left) and Dotplots (right) compare fluorescence intensity of a single representative colony across different BiFC complexes with the plasmid negative control (YFP_C-CaM). Dotplots display individual events as dots. Histograms show fluorescence intensity distribution of gated events, with the first peak indicating autofluorescence and the second peak representing BiFC signal.

Our protocol for interaction studies includes cultivating *S. cerevisiae* cells, ensuring equal expression levels of constructs via Immunoblot analysis, and confirming the correct subcellular localization of BiFC complexes through fluorescence microscopy. Fluorescence intensity signal, indicating the strength of the YFP signal upon complex reconstitution, and the percentage of events exhibiting a YFP signal above autofluorescence level, known as the fluorescence frequency signal, were measured. Additionally, a robust protocol was developed for optimized growth conditions of yeast cells prior to flow cytometry measurements. This involved regrowing single transformants to log phase (approximately an OD_{600} = 0.5) in liquid media instead of measuring fluorescence intensity and frequency directly from the growth plate. This approach effectively reduced BiFC background, leading to statistically robust data with low standard deviation (**Fig. 17**).

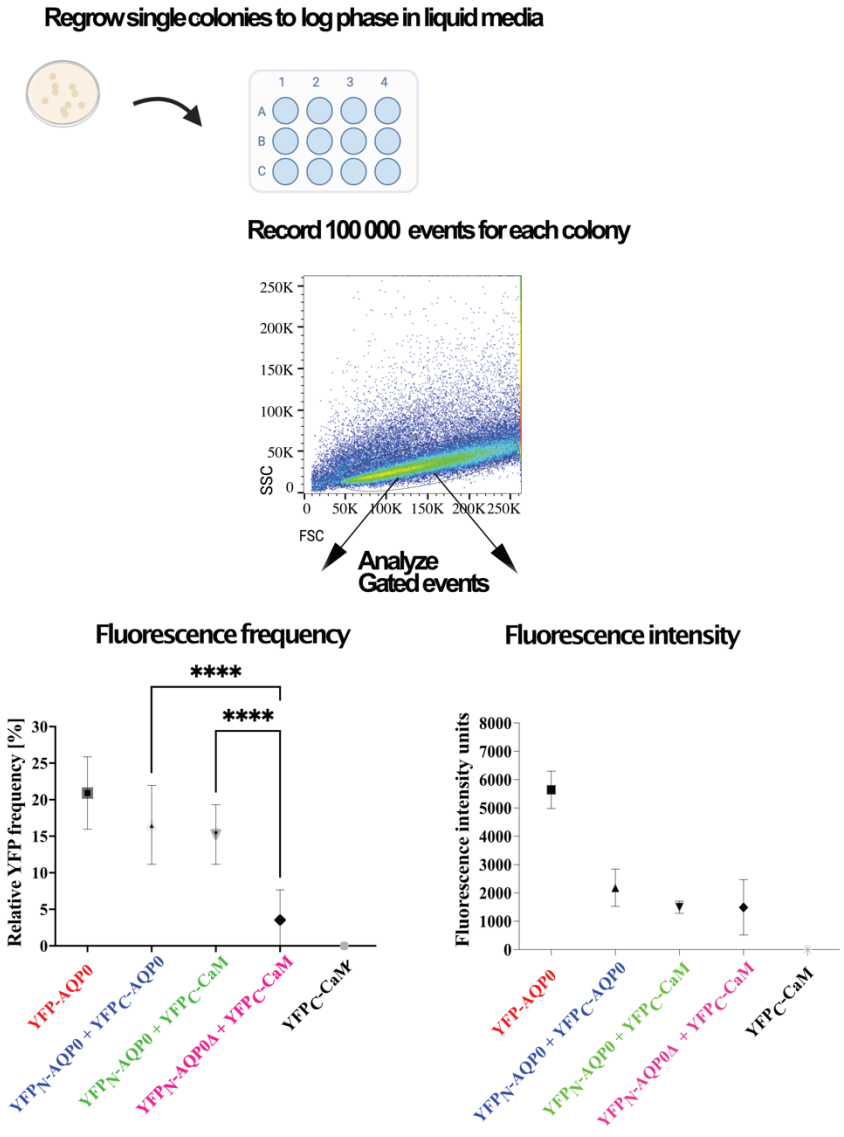


Figure 17. Fluorescence frequency signals and fluorescence intensity signals shown for the different BiFC pairs after regrowing single colonies in liquid media to log phase. For each construct, at least 30 measurements were performed to evaluate fluorescence data.

Data was collected for each BiFC complex from three biological replicates, with each replicate consisting of 10 technical repeats. This approach accounted for biological variability by working with living cells.

By utilizing the fluorescence frequency value as the primary criterion for authentic complex formation, we were able to differentiate between constructive PPIs and false positives with high statistical confidence. Establishing this methodology is crucial for our investigation into AQP interaction complexes, as it offers a foundation for library screenings and interaction studies. This method will aid in validating unknown interactions and furthering our understanding of regulatory mechanisms.

3.2. PROTEIN COMPLEXES OF HUMAN AQPS TO CAM

Building on the methodology we established, we conducted a comprehensive screening of all human AQPs to investigate their interaction with the regulatory protein CaM (**Paper II**). Literature research indicated CaM's significant influence on the translocation of hAQP1[137] and hAQP4[46], and in the case of hAQP0, its involvement in channel gating[50]. Additionally, a potential binding between CaM and hAQP6 was suggested[138]. Despite the pivotal role of CaM in various regulatory mechanisms, no known interactions have been reported for other hAQPs, indicating a significant gap in research in this area. In addition, only interaction between hAQP1 and CaM, hAQP0 and CaM and hAQP4 and CaM has been reported in living cells[43]. Our approach aimed to address this gap by investigating the binding of the complete set of human hAQPs *in vivo*, using our established BiFC and flow cytometry screening method from **Paper I.**

To initiate the identification of potential hAQP-CaM binding sites, we employed a computational approach using the Calmodulin Target Database. This method aimed to find potential binding sites located on either the N- or C-terminus (**Table 3**) and compared the outcomes with previously confirmed experimental results. The results from our computational analysis predominantly corroborated the confirmed interactions. Specifically, we found that hAQP1 and hAQP6 harboured their binding sites on the N-terminus, while hAQP0 and hAQP4 exhibited C-terminal binding sites. For the unexplored interaction partners, we obtained interesting results from the theoretical predictions: hAQP2 showed a C-terminal binding site hit, hAQP7 displayed a N-terminal binding site hit, and both hAQP8 and hAQP9 indicated N-terminal binding sites.

Table 3. Summary of theoretical and experimentally confirmed binding sites as well as their investigated role in regulation

AQPs	Theoretical Evaluation Binding site for CaM	Experimentally confirmed Binding site for CaM	Regulation by CaM
AQP0	C–terminal	C–terminal	Gating
AQP1	N–terminal	not specified	Translocation
AQP2	C–terminal	–	–
AQP3	–	–	–
AQP4	C–terminal	N– and C–terminal	Translocation
AQP5	–	–	–
AQP6	N–terminal	N–terminal	Unknown
AQP7	N–terminal	–	–
AQP8	N–terminal	–	–
AQP9	N–terminal	–	–
AQP10	–	–	–
AQP11	–	–	–
AQP12	–	–	–

For our cloning strategy, we tagged human AQPs with the larger YFP_N fragment at the N-terminus, while CaM was labeled with the YFP_C fragment also at the N-terminus, based on previous studies showing a constructive complex formation with this labeling strategy[79]. However, we remained mindful that a different labeling approach might introduce steric hindrance for other complexes. Following the procedure outlined in **Paper I,** we quantified BiFC intensity and the frequency of events showing a YFP signal above the pre-set threshold, comparing them to the previously used non-interaction control hAQP0ΔC and CaM. Additionally, we captured fluorescence and bright-field images of the constructs to verify correct YFP signal localization and cell health. We also assessed the expression levels of the different hAQPs from a small-scale membrane preparation using an antibody specific to the YFP_N part. Our primary observation revealed a distinct correlation between hAQP expression levels and YFP fluorescence frequency signal. Specifically, highly expressed hAQP0 and hAQP4 exhibited the strongest interaction signal with CaM. In contrast, the previously confirmed hAQP6 demonstrated the lowest frequency signal, likely due to very low production levels, rendering this assay unsuitable for confirming this specific interaction.

To visually represent and correlate the measured fluorescence frequency signal with hAQP production levels, we sorted them from

highest to lowest (left to right) and applied different colors to indicate high protein producers (red) and low protein producers (green). The production level of the mutant hAQP0ΔC was not included in this investigation and is represented in grey (**Fig. 18).** However, it was previously examined in **Paper I** and demonstrated production levels comparable to those of hAQP0.

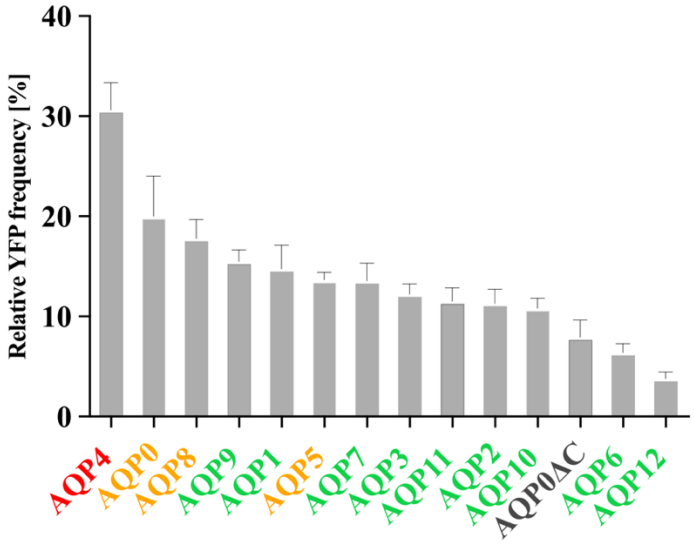


Figure 18. Arrangement of fluorescence frequency signals of hAQP-CaM interaction from high to low. Red represents very high expression levels, yellow indicates high expression levels, green denotes low expression levels, and black signifies data not measured.

Based on our conclusions and considering the *in silico* prediction of CaM binding sites, hAQP0, hAQP4, hAQP8, hAQP9, and hAQP1 emerged as potential interaction candidates, exhibiting significantly higher YFP frequency signals than the negative control, particularly noteworthy given the low protein expression levels of hAQP9 and hAQP1.
However, while hAQP5 showed a similar high YFP frequency level to hAQP1, we considered it a false positive due to its similar high production level to hAQP0 and the absence of predicted binding. Biologically, the mechanisms of trafficking for hAQP8 remain largely unknown, including whether phosphorylation, as observed in other human AQPs like hAQP1 and hAQP4, plays a significant role. hAQP8 is distributed in various tissues such as the liver, pancreas, colon, and salivary glands, while hAQP9, despite its widespread presence in

different tissues, is most abundant in the testis. Regarding interaction partners, hAQP8 interacts with human T-lymphotropic virus type 1 (HPLV1), whereas hAQP9 has three confirmed interaction partners[43]. To date, no interaction study has been reported for hAQP8 and hAQP9 with CaM and therefore we were able to give first insights into these largely uninvestigated hAQPs.

3.3. SCREENING FOR NOVEL INTERACTION PARTNERS TO AQP4

Several interaction partners of AQP4 have been identified, suggesting potential roles in regulating its function[43]. Despite the significance of these interactions, many aspects regarding AQP4's involvement in pathological processes, especially neurodegenerative diseases, remain elusive.
In **Paper III**, we aimed to address the notable gap in understanding the regulators and interaction partners of hAQP4 in the brain. Given the vast genetic complexity of the human brain, comprising over 15,000 coding genes, our study entailed the construction of a human brain expression library using total RNA from human brain tissue. Our objective was to generate a diverse library encompassing a wide array of human brain proteins, which we subsequently screened for interactions with hAQP4 in *S. cerevisiae*.

Expanding upon the established platform outlined in **Paper I**, which predominantly uses flow cytometry for evaluating membrane protein interactions, we refined our screening methodologies by integrating FACS as an additional screening tool. We implemented a sorting gate to isolate events exhibiting exceptionally strong fluorescence signals from the library mixture. Subsequently, these selected events were recultivated for further analysis. This approach enabled us to rapidly isolate individual colonies from our brain protein library based on their fluorescence intensity, enhancing the efficiency and throughput of our screening process. Consequently, it allowed more effective enrichment of candidates displaying a high proportion of cells exhibiting a fluorescence signal, which presents our primary criteria for identifying real complex formation. By integrating controls from our previous study (**Paper II**) and implementing stringent thresholds, initially through the sorting gate during FACS, and subsequently by comparing fluorescence frequency signals with robust controls, we obtained direct indications of strong interactions and visualized direct protein-protein interactions. A significant finding from our library screening was the

consistent identification of CaM, obtained rapidly. This discovery validated the efficiency of our newly developed library approach in identifying interaction candidates.

3.3.1. FUTURE DIRECTIONS – PRELIMINARY RESULTS

3.3.1.1 THE CAM AND HAQP4 INTERACTION

The interaction between CaM and hAQP4, as investigated in **Paper II**, revealed a notable fluorescence frequency signal. The recurrence of CaM detection in our library screen further suggests this interaction, providing additional support to prior findings[46]. However, the exact binding mechanism and stoichiometry of this interaction remain uncertain. Experimental validation of the C-terminal binding site, particularly between amino acids 256 – 275, aligns with hits obtained from the Calmodulin Target Database. Nevertheless, recent research using AQP4 N- and C-terminal peptides identified CaM-binding domains within both termini. Specifically, the N-terminus was found to interact with the N-lobe of CaM, while the C-terminus interacted with both the C- and N-lobes of CaM. Additionally, both peptides were capable of simultaneous interaction with CaM, forming a ternary complex[139]. Whether this phenomenon occurs in the full-length context remains to be established.

We further investigated this interaction using Microscale Thermophoresis (MST). While this study had been previously conducted [46] we introduced a modification to the experimental setup by labeling the lysines of the hAQP4 protein instead of the soluble protein CaM. The MST experiment confirmed a binding event of hAQP4 to CaM. However, our preliminary results suggested a dual-binding event with two distinct KD values: The first slope was observed at KD 10-100 nM whereas the second slope was observed at KD 29 µM, consistent with the published KD value[46]. Whether this result might indicate a binding of CaM to both termini of hAQP4 needs further exploration.

Furthermore, we used AlphaFold2 to predict a potential complex of the hAQP4 and CaM proteins. In our first model (**Fig. 19A**) utilizing one hAQP4 and one CaM protein, we observed the well-established binding of the CaM molecule to the C-terminus of hAQP4, particularly involving amino acids 256-274 (**Fig. 20A**). Surprisingly, when we run the program with one hAQP4 molecule and two CaM molecules, we predicted binding of one CaM molecule to the N-terminal region of hAQP4

instead, specifically involving the amino acids 16 – 30. The binding pattern of the CaM molecule to hAQP4 closely resembles other sequence motifs involved in canonical CaM binding. In these motifs, an amphiphilic alpha-helical structure is situated between the N- and C-lobes of CaM[140]. However, the second CaM molecule in the prediction performed contacts to hAQP4 but without displaying the canonical binding pattern, and also showing a high error in the relative position of the amino acids. We concluded that the second CaM does not demonstrate an acceptable Predicted Aligned Error (PAE) and is not depicted in the figure (**Fig. 19B**).

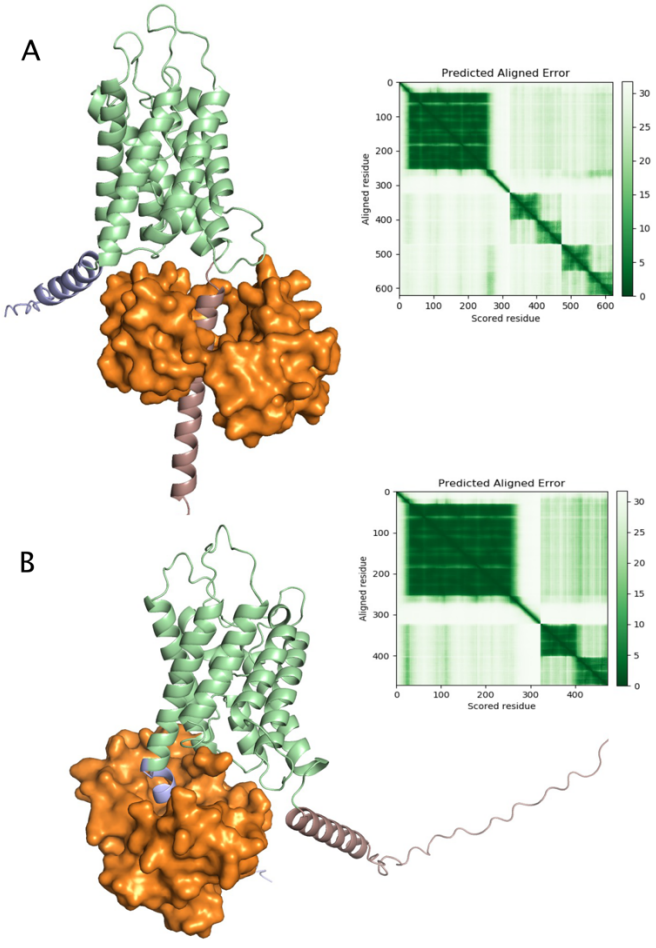
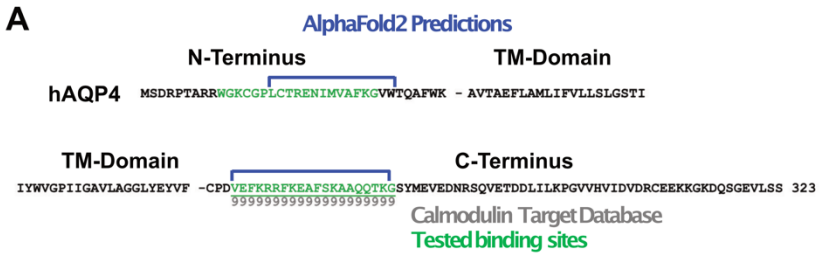


Figure 19. Structure hAQP4 (green) -CaM (orange) complex predicted by using AlphaFold2 together with Error Map. A) Model of CaM binding to the C-terminus of AQP4 (red). B) Model of CaM binding to the N-terminus of hAQP4 (purple).

To experimentally determine whether CaM binds to both termini of hAQP4 also *in vivo,* as indicated by the AlphaFold2 predictions, we generated variants of hAQP4 lacking either the N-terminal binding site, the C-terminal binding site, or both (**Fig. 20B**). These mutations were based on the findings of the study by Ishida et al[139] and we employed our established BiFC assay to evaluate any interaction with CaM.



B

Cloned Mutants	N-Terminus	C-Terminus
hAQP4ΔC(Δ256)	No changes	-CPD*
hAQP4ΔN (Δ1-27)	MVWTQAFWK -	No changes
hAQP4ΔNΔC	MVWTQAFWK -	-CPD*

Figure 20. A) Amino acids of hAQP4 involved in a binding to CaM according to AlphaFold2 predictions (blue), Calmodulin Target Database (grey) and experimentally validated binging sites (green). B) Created deletions in the N- and C-terminus of hAQP4 for our study.

Furthermore, we conducted an analysis of expression levels and confirmed the correct localization of the signal on the cell membrane using fluorescence microscopy (**Fig. 21)**. The control of hAQP4 lacking either the N- or the C-terminus still exhibited a high frequency signal upon co-transformation with CaM in *S. cerevisiae* cells. However, we observed a significant difference in the fluorescence frequency signal between the three control and the hAQP4-CaM complex. This suggests that the interaction between CaM and hAQP4, even after deleting either the N- or C-terminus, is still possible, albeit less frequent, supporting our hypothesis that both termini are involved in the interaction. Nonetheless, the ΔNΔC control displayed a similarly high fluorescence frequency signal but with a high standard error of mean, indicating a

potential complex formation. Expression level analysis of the controls revealed that the hAQP4ΔC control exhibited similar expression levels to hAQP4, while the other two controls surprisingly showed very low expression levels.

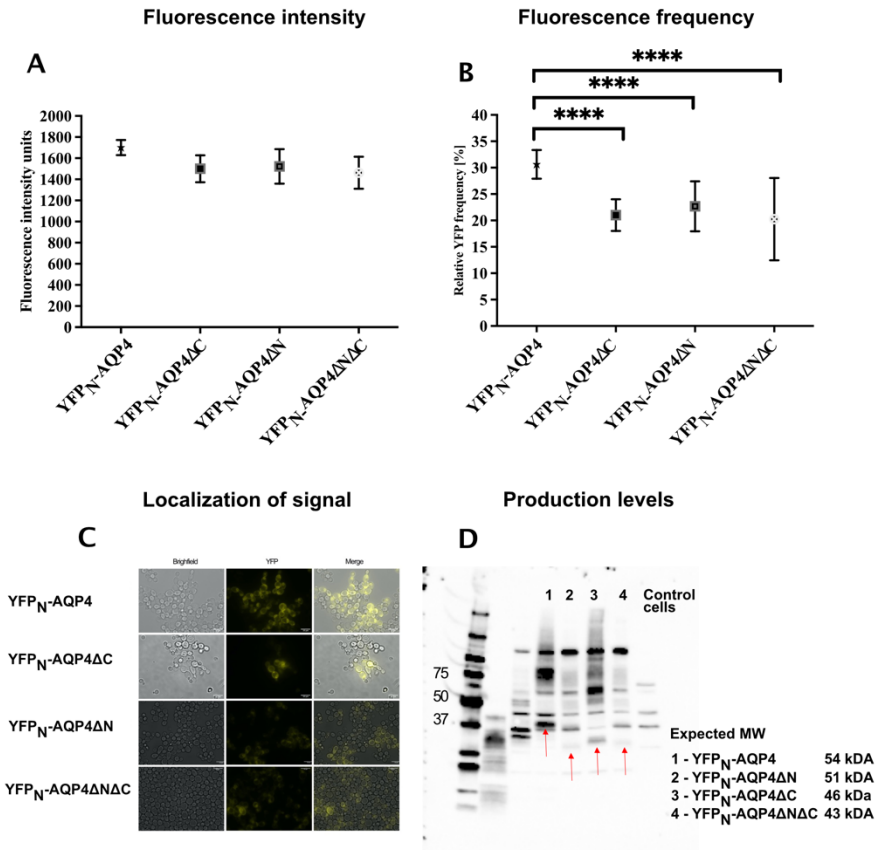


Figure 21. Datasets of *S.cerevisiae* cells co-producing CaM and the hAQP4 controls. Quantification of BiFC yields through flow cytometry (Fluorescence intensity (A) and YFP frequency (B), C) Fluorescence and Brightfield Images and D) Immunoblot analysis of production levels. Bands corresponding to hAQP4 are indicated with arrows. The first two lanes display different samples that are unrelated to this study.

3.3.1.2. NOVEL INTERACTION PARTNERS TO HAQP4

Throughout the screening process of the library, we identified additional hits to CaM (**Fig. 22**). However, there was significant variation in the length of the fragments identified. While the CaM fragment encompassed the entire protein (148 amino acids), the stretches for other candidates were notably shorter and covered only the 3' end of the protein. Some of these stretches, such Leydig cell tumor 10kDa protein homologue (14 amino acids), were exceedingly short and consequently excluded from further investigations. It appears that the fluorescence frequency signal tends to be higher for shorter fragments, potentially leading to false positives. Therefore, careful interpretation is required. For nicastrin, although the peptide length of the protein was longer (68 amino acids out of 709 total), and for signal peptidase complex subunit 1 (27 amino acids out of 169 total), they still did not cover the entirety of the protein. Thus, further investigation is required to confirm the validity of the identified hits, including assessment of their binding affinity and biological relevance.

Identity (Blast)	Number of Hits	Fluorescence Frequency	Fragment length	Protein length
CaM	3	18%, 17%, 25%	148 aa	148 aa
Leydig cell tumor (LCT)	2	17% , 21%	14 aa	99 aa
Nicastrin	1	15 %	68 aa	709 aa
Signal Peptidase complex subunit 1	1	22 %	27 aa	169 aa

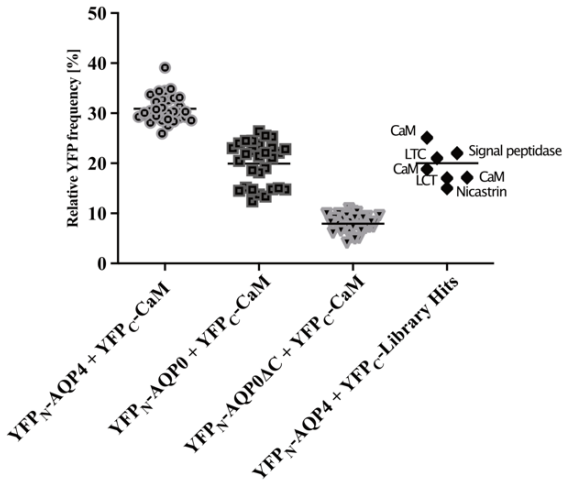


Figure 22. Evaluation of potential library hits by comparing fluorescence frequency signal and amino acid length.

As a next step, we employed AlphaFold2 to predict the structure of a complex consisting of an hAQP4 tetramer with nicastrin. Nicastrin, a component of the gamma-secretase complex, serves as a receptor for gamma-secretase substrates, such as the amyloid precursor protein, implicated in Alzheimer's disease pathology[141]. The prediction indicated a confident interaction between hAQP4 and nicastrin (**Fig. 23**). In this predicted complex, the C-terminus of hAQP4 is implicated in the interaction. Specifically, the peptide sequence of hAQP4 spanning amino acid positions 290 to 304 (DLILKPGVVHVIDV) was predicted to bind to the same pocket where the amyloid precursor protein typically binds (**Fig. 24**).

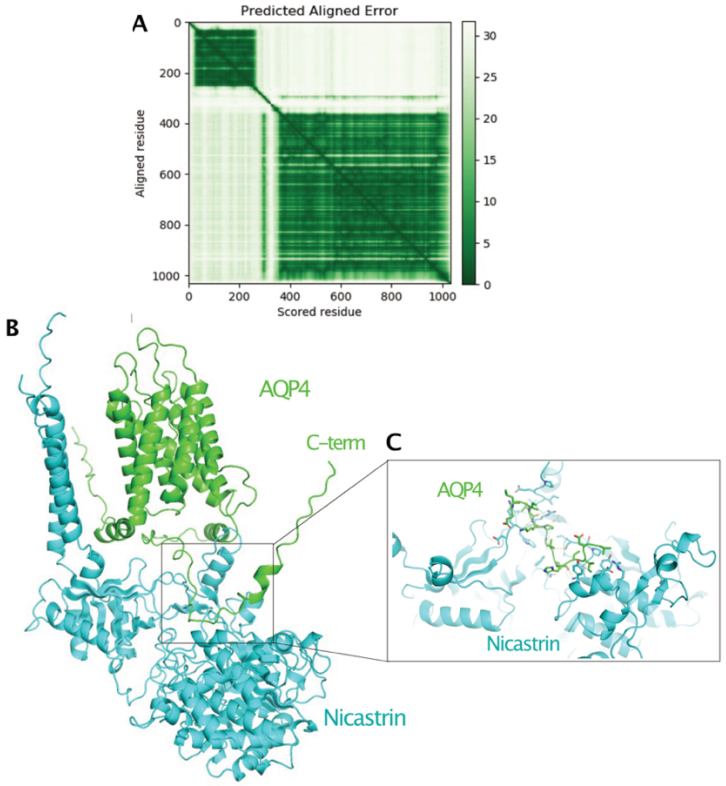


Figure 23. Structure of hAQP4-nicastrin complex achieved by using AlphaFold2. A) Error Map, showing with high confidence a predicted interaction of AQP4 to nicastrin involving the binding peptide of 290-DLILKPGVVHVIDV-304 in the C-terminus of hAQP4 B) Predicted complex of hAQP4-nicastrin with C) Zoom-in into the direction of nicastrin and the C-terminus of hAQP4. (hAQP4 = green; nicastrin = cyan).

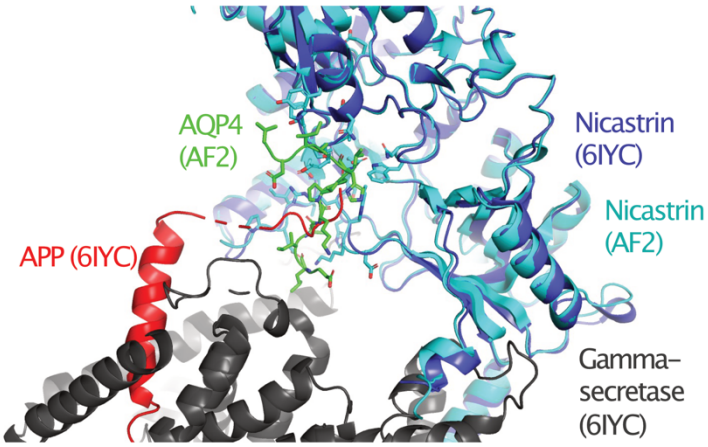


Figure 24. Overlay of the AlphaFold2 complex hAQP4-nicastrin (hAQP4: green and nicastrin: cyan) to the PDB structure of the recognition of the amyloid-precursor protein APP by gamma-secretase complex (APP: red; gamma-secretase: black; nicastrin: dark blue) (PDB: 6IYC).

3.3.1.3. MAGIC ANGLE SOLID STATE NMR OF HAQP4-COMPLEXES

One prospective direction of this work is to develop methodologies for resolving the structures of hAQP4 interaction complexes, especially those identified from the library screening. One such method we are establishing is MAS solid-state NMR, known for its efficacy in studying membrane protein structures and dynamics. MAS NMR operates by swiftly rotating the sample during measurement, typically at speeds reaching up to 130 kHz, around an axis tilted at the "magic angle" (approximately 54.74 degrees) relative to the external magnetic field[142]. This rotational motion serves to average out dipolar interactions, resulting in sharper and more defined spectral lines[143]. MAS NMR offers valuable insights into protein behaviour under physiological conditions, serving as a complementary technique to X-ray crystallography. Moreover, it proves particularly advantageous for investigating protein complexes that pose challenges for crystallization[144, 145].
Here we are presenting preliminary results studying the interaction of hAQP4 tetramerization. Our initial data includes 2D (^{1}H-^{15}N) and 3D (^{1}H-^{13}C-^{15}N) correlation spectra of isotopically labelled ^{13}C-^{15}N-hAQP4. This protein, produced in *P. pastoris*, has been purified in high yields and reconstituted into lipid bilayers for MAS solid-state NMR

measurements. This process involved mixing the protein with preformed 1-palmitoyl-2-oleoyl-sn-phosphatidylcholine (POPC) liposomes at a protein-to-lipid ratio of 2:1 (w/w). The detergent was removed by dialysis against a detergent-free Tris-HCl buffer. Subsequently, the sample was harvested and transferred into MAS NMR rotors after ultracentrifugation. For ^{15}N- and ^{13}C-labeled hAQP4, 0.5 mg was utilized in a 0.7 mm MAS NMR rotor.

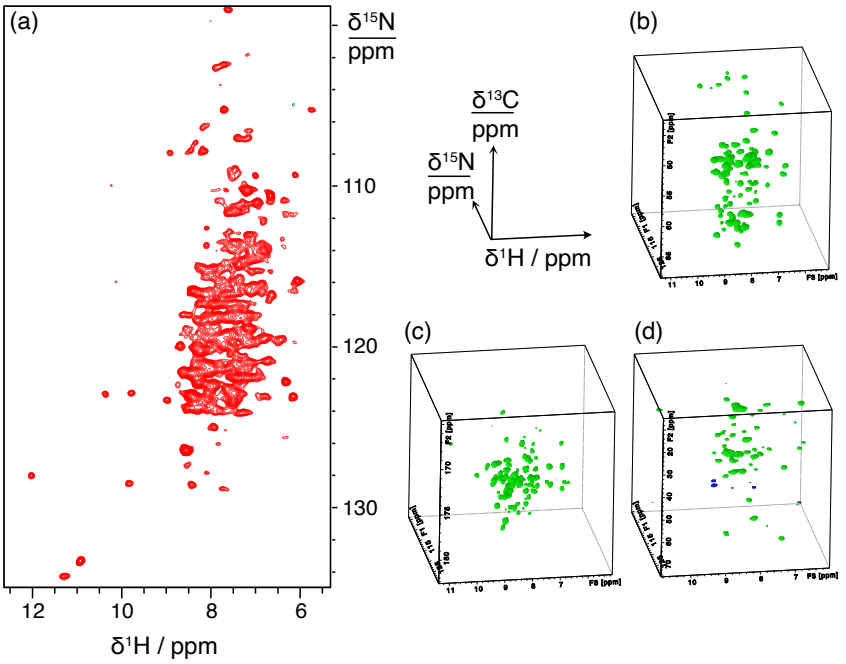


Figure 25. illustrates the 2D ^{1}H-^{15}N correlation spectrum of [^{15}N, ^{13}C]-labeled hAQP4, where each correlation corresponds to a backbone amide or a side chain amine/amide site. The dispersion of 1H resonances indicate a well-folded helical protein. Notably, under similar conditions for other membrane proteins (*[146]*), we obtained a high-quality NMR spectrum, suggesting a homogeneous environment for the protein within lipids. The spectra were obtained and analyzed by Tanguy Le Marchand at the Centre de RMN à Très Hauts Champs.

To achieve sequential resonance assignment, a series of 3D ^{1}H-^{13}C-^{15}N correlation spectra was recorded*[147]*(**Fig. 25**). These measurements resulted in the detection of 150 peaks, from which 88 resonances were automatically assigned with high confidence (**Fig. 26**) using CYANA software*[148]*. The assigned residues primarily belonged to

transmembrane helices, while residues in tails or loops could not be assigned, indicating potential for future improvement. Additionally, residues in helices located at the outer part of the interface between monomers (residues 31 to 60 and 155 to 179) were not assigned, suggesting the presence of important local dynamics or transient interactions with lipids.

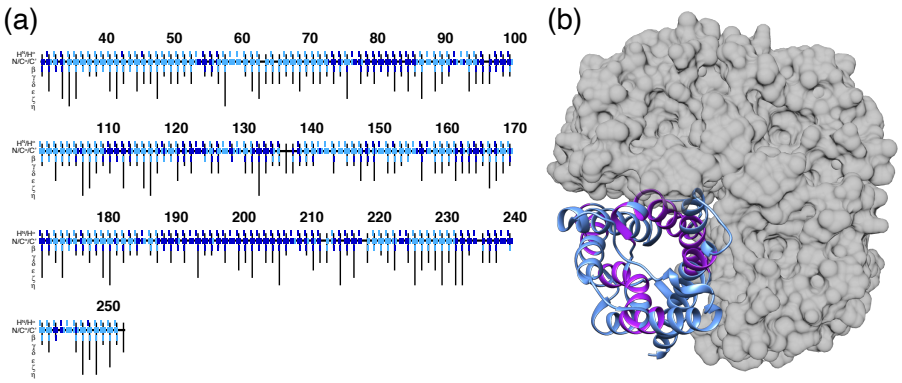


Figure 26. The automated resonance assignment of hAQP4. (a) The results of CYANA calculations depict the automatic assignment of 88 nuclei (dark blue), contrasting with non-assigned nuclei (light blue). (b) The assigned residues are depicted on a monomeric unit of hAQP4's 3D structure (PDB: 3GD8), while the surfaces of the three remaining monomers are displayed in grey. The spectra were obtained and analyzed by Tanguy Le Marchand at the Centre de RMN à Très Hauts Champs.

The obtained high-quality multidimensional NMR spectra validate this setup as a robust platform for analyzing the physiological implications of hAQP tetramerization. Our optimization of lipid reconstitution represents an initial step toward addressing challenges associated with unassigned tails and loops, with further refinement expected to yield enhanced insights. This advancement promises to provide valuable structural insights into hAQP4-interaction complexes, facilitating a deeper exploration of hAQP4's functional roles and regulatory mechanisms.

3.4. STRUCTURAL CHARACTERIZATION OF AQUAPORIN REGULATION

3.4.1 A NOVEL APPROACH USING CONTINUOUS DIFFRACTION

The availability of high-resolution structural data for membrane proteins is primarily attributed to the well-behaved crystal of this protein, resulting in sharp and high-diffracting Bragg peaks. However, this scenario is quite rare for membrane proteins, mainly due to the disruptive effects of detergent micelles on crystal contacts or their propensity to induce translational disorder. As previously noted, continuous diffraction observed in photosystem II has demonstrated its potential to enhance the information content and resolution of diffraction patterns. Our investigation aimed to explore whether this phenomenon could be applied to other systems, particularly AQPs (**Paper IV**).

We conducted experiments comparing SoPIP2;1 crystals, crystallized in its closed form, with AQP2 crystals. Our observations revealed that SoPIP2;1 crystals exhibited weaker continuous diffraction, suggesting a lesser degree of translational disorder compared to AQP2 crystals. The crystal packing within the normal space group I4 demonstrated a denser arrangement in comparison to AQP2 crystals (space group $P4_2$), resulting in lower solvent content and stronger crystal contacts. This correlation effectively explains the association between the presence of continuous diffraction and protein displacement caused by weak crystal contacts.
Our next goal was to induce continuous diffraction in the SoPIP2;1 crystals by adding a different micelle as a secondary additive to investigate the potential induction of translational disruption. We tested various detergents (OGNG, NG, LDAO, DM and DDM) at different concentrations (1xCMC, 2xCMC and 5xCMC), in addition to the ligand cadmium chloride, which is required for crystal formation. **Fig. 27** shows which conditions resulted in crystal formation and how the morphology of the crystal changed. Indeed, we noted significant changes in crystal morphology during our experiments. Typically, the crystals have sizes ranging from 100 to 400 µm without the addition of any secondary detergent. However, with increasing detergent concentrations, we observed the formation of smaller crystals and even noticed deformations in their shapes. Notably, crystals treated with 5x OGNG displayed a distinct needle-like morphology.

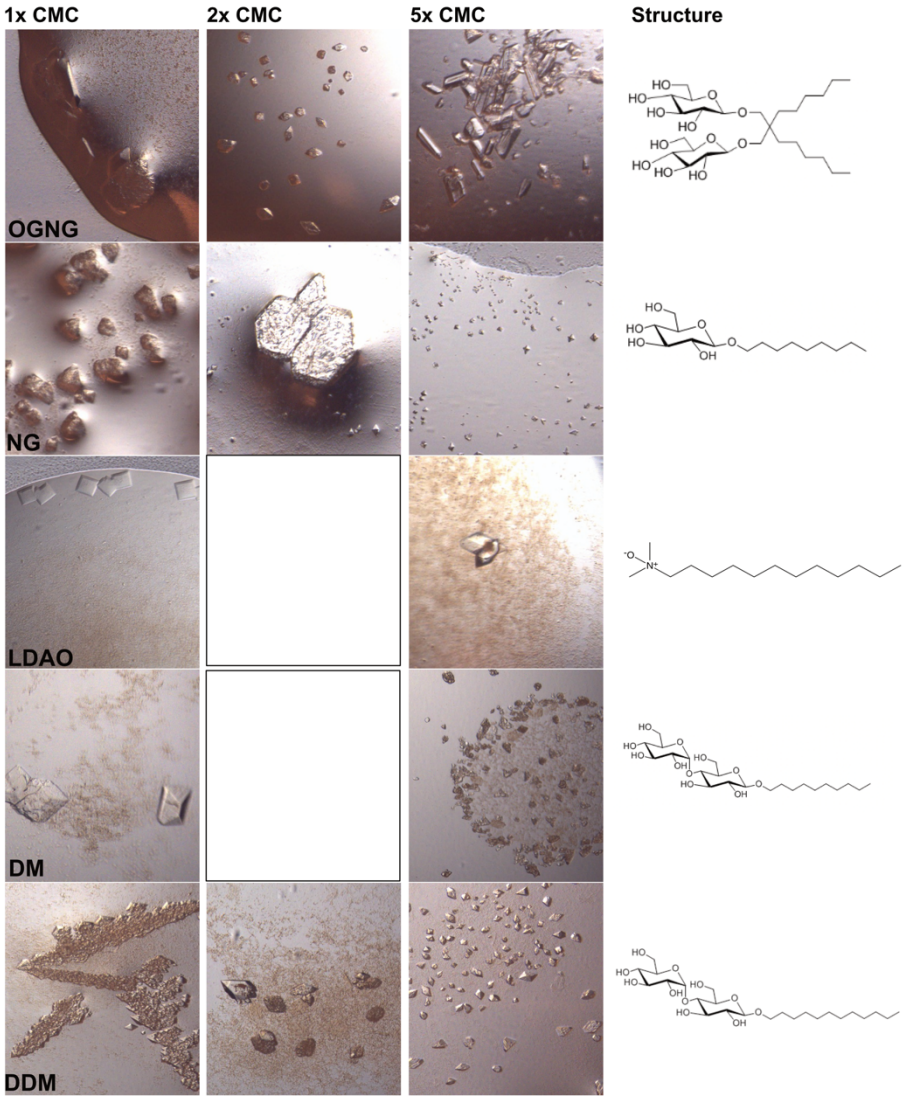


Figure 27. Pictures of the different crystals formed upon adding a secondary detergent at three different concentrations (1x CMC, 2x CMC, 5x CMC). Images of crystals formed with DM at 2x CMC were not captured. LDAO at 2x CMC did not lead to crystal formation.

Additionally, the incorporation of secondary detergents generally led to weaker Bragg diffractions compared to crystals without any secondary detergent (2.1 Å) (**Fig. 28**).

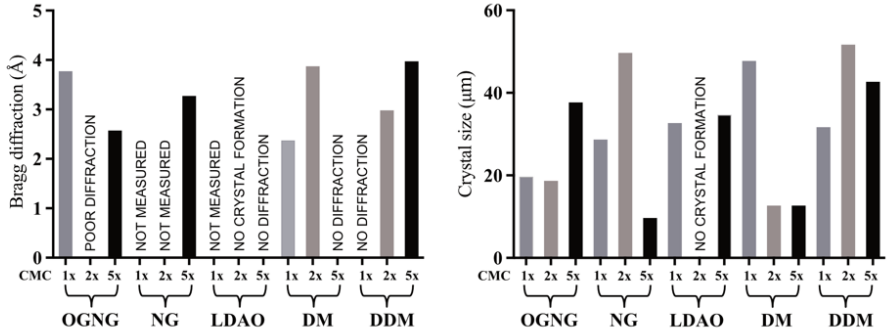
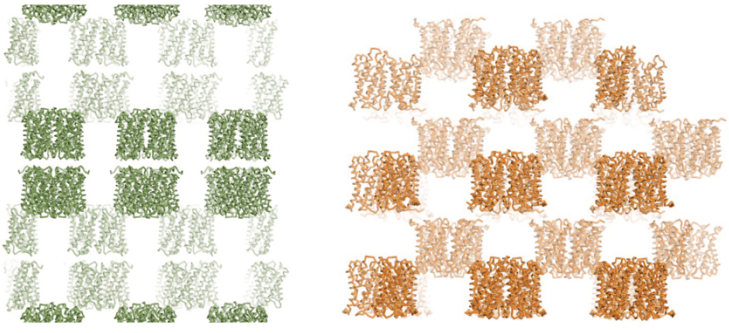


Figure 28. Overview of the various conditions tested, showing the Bragg diffractions (left side) and the corresponding crystal size (right side). The crystal size averaging the measurements of at least three individual crystals the Bragg diffractions are shown only for the best diffracting crystals.

The addition of a secondary detergent did not result in a notable enhancement of continuous diffraction in both SoPIP2;1 and AQP2 crystals, despite a decrease in Bragg diffractions. However, for SoPIP2;1 crystals, we noted an alteration in crystal packing upon the introduction of a secondary micelle, leading to a change in the space group. Particularly notable was the impact observed when using a detergent containing maltoside which changed the space group from I4 in the original structure to P4212 (**Fig. 29**).



Octyl glucoside (OG)
Bragg diffraction:2.1 Å
Space group: I4

Decyl maltoside (DM)
Bragg diffraction: 2.4 Å
Space group: P42 12

Figure 29. Comparison of crystal packing between SoPIP2;1 crystals with the original OG micelle and SoPIP2;1 crystals with the added secondary detergent DM. Figures were made by Susanna Törnroth-Horsefield, Lund University.

Continuous diffraction represents an intrinsic property of crystal systems that is not easily manipulated through crystallization condition optimization, as demonstrated in both SoPIP2 and AQP2 cases. This observation is significant, as it contrasts with the typical goal of structural biologists to enhance translational order by optimizing crystallization conditions.

3.4.2. FUTURE DIRECTIONS – PRELIMINARY RESULTS

3.4.2.1. A NEW REGULATION MECHANISM IN PLANT AQUAPORINS?

We have gained extensive insights into the structural gating mechanism of SoPIP2;1 in the past. The closed conformation of SoPIP2;1 involves structural adjustments in Loop D, which block the intracellular channel entrance and are stabilized by hydrogen and ionic bonds with cadmium ions[84]. Upon examining the effects of detergents containing maltoside, particularly DM detergent, we observed significant outcomes. Besides a shift in space group from I4 to P4212, we observed a structural alteration originating from the N-terminus of SoPIP2;1 in the presence of DM detergent. The DM molecule displaced the cadmium ion and occupied its position within the crystal lattice, potentially contributing to crystal contacts (**Fig. 30**). While previous studies have suggested that Ca^{2+} binds the regulatory site *in vivo*, we speculate that a molecule akin to maltoside could bind in its place. This discovery holds implications for the gating mechanism of SoPIP2;1, especially regarding the N-terminus, and suggests potential implications for the regulation of pore function by molecules with similar chemical structures, such as other disaccharides.

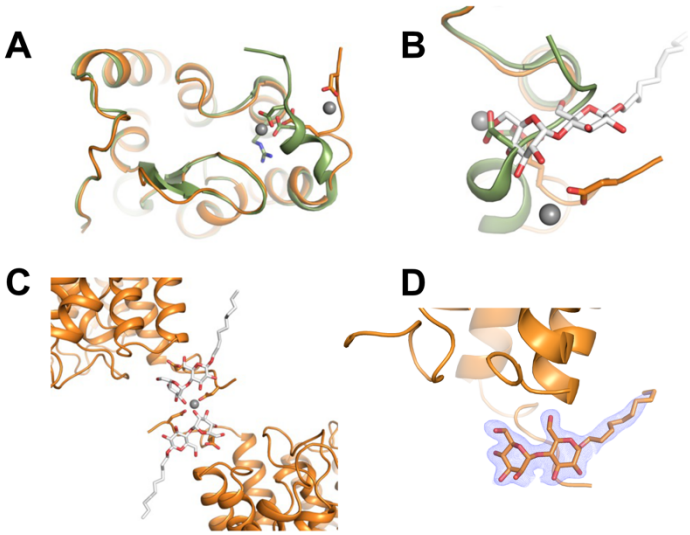


Figure 30. Structural comparison of the N-terminus of SoPIP2;1. Structural overlay of the structures of original SoPIP2;1 (green) and the structure with added DM (orange) showing structural differences at the N-terminus. Cadmium is shown as grey spheres. DM molecule is shown in stick representation. A) View on the N-terminus showing the main structural differences and B) Zoom-in on N-terminus showing significant structural differences between the two overlaid structures. Binding of the DM molecule to the N-terminus leads to a relocalization of the cadmium molecule. C) shows the involvement of cadmium and the DM molecule in the crystal packing in the new structure. Figures were made by Susanna Törnroth-Horsefield, Lund University. D) Electron density map of the DM molecule.

As a preliminary result, we explored the impact of different disaccharides (sucrose, trehalose, lactose, and maltose) at varying concentrations (0.1 mM, 1 mM, and 10 mM) added to the crystals directly. The resulting crystals exhibited similar deformed characteristics seen after the addition of detergents DDM and DM, along with weaker Bragg peaks ranging from 2.2 Å to 5.7 Å and twinning issues. Further investigations are warranted to obtain high-quality diffraction data for a closer examination of this phenomenon.

3.5 AQUAPORIN REGULATION IN CPAQP1AA

3.5.1. A NOVEL EXTRACELLULAR GATE REGULATED BY PHOSPHORYLATION

In contrast to the vast amount of knowledge gained from structural studies of AQPs in various organisms, including plants and mammals, which have provided invaluable insights into the intricate relationships between structure and function, **Paper V** aims to fill the current gap in understanding the regulatory mechanisms of AQPs in fish, the last kingdom of eukaryotic AQPs I addressed in this thesis.

In this study, we present the first high-resolution structure of a fish AQP, cpAQP1aa, achieved by truncating the protein to remove hindering C-terminal flexible regions. Produced in *P. pastoris*, crystallized, and solved to 1.9Å resolution, the structure revealed a distinct extracellular constriction region (**Fig. 31**). This structural feature is a complete novelty and has never been observed in any other AQP structure before.

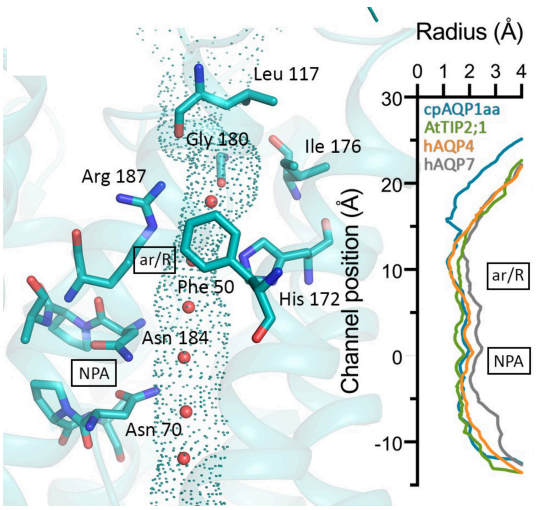


Figure 31. HOLE analysis was conducted on the water-conducting channel, with highlighted residues in the ar/R and NPA regions. The graph shows a comparison between the channel profiles of cpAQP1aa (teal), human AQP4 (orange, PDB code 3GD8), human AQP7 (gray, PDB code 6QZI), and Arabidopsis TIP2;1 (green, PDB code 5I32). Figure taken from **Paper V**.

It is attributed to structural differences in Loop C, notably the flipped orientation of Tyr107, inducing constriction. This unique feature creates a semi-open channel, specific for water transport. To investigate the potential regulation mechanism of cpAQP1aa, we aimed to determine if it functions as a gated channel and, if so, to identify the underlying gating mechanism. Rapid modulation of water transport rates in aquatic organisms through gating could offer a means of adapting to environmental conditions. Our initial investigation did not reveal any structural determinants related to pH in cpAQP1aa in contrast to gating in SoPIP2;1[84] or AQP0[149]. This suggests that the conformational gating regulation of extracellular loop C is not pH-dependent. Consequently, we redirected our focus to explore whether phosphorylation could potentially induce conformational changes in loop C, thereby altering pore dimensions. It's worth noting that directly mimicking phosphorylation by adding a phosphate group to an amino acid is not feasible for studying protein activation or deactivation. Instead, point mutations with similar characteristics are utilized to simulate the effects of phosphorylation and investigate their functional consequences[150]. Based on the high-resolution structure we identified putative phosphorylation sites near Loop C in advance and tested whether point mutations at these sites significantly affected water flow rate, indicating a potential impact of phosphorylation on channel gating (**Fig. 32**).

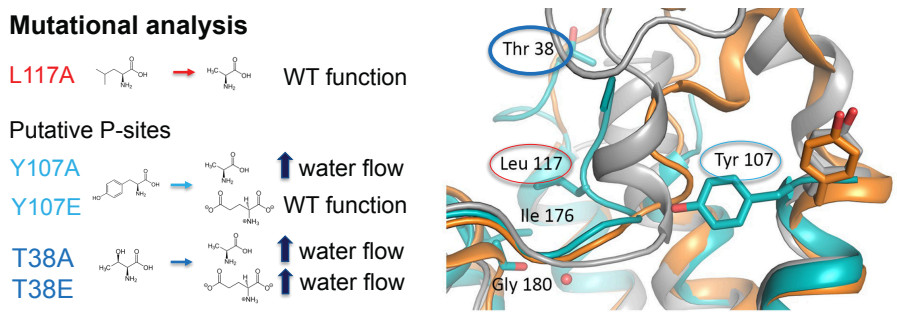


Figure 32. Overview of tested mutants showing their water flow rates compared to cpAQP1aa WT, with target amino acids near the constricted region highlighted in different colors.

The molecular dynamics (MD) simulation of Tyr107 phosphorylation induced a significant shift of loop C towards the channel opening, further narrowing the pore. Integrating MD simulations with mutational analysis, we established a model emphasizing the essential role of phosphorylation in modulating water flux through cpAQP1aa (**Fig. 33**).

The crystal structure obtained shows the non-phosphorylated state, featuring a constricted loop C region and a semi-open channel. Phosphorylation of Tyr107 might lead to closing the channel on the extracellular side while phosphorylation of Thr38 leads to an open channel state.

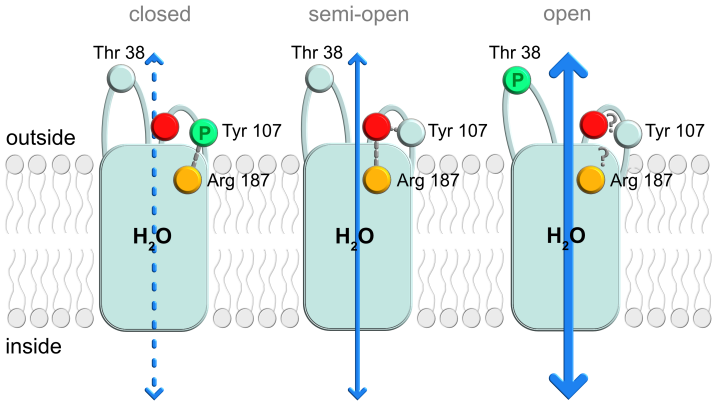


Figure 33. Possible regulation mechanism for cpAQP1aa showing a schematic extracellular gating mechanism depending on the phosphorylation of Tyr107 and Thr38. Figure taken from **Paper V**.

The lack of detectable phosphorylation of Tyr107 and Thr38 in the crystal structure poses a challenge for supporting our model. However, it's important to note that the absence of phosphorylation in the crystal structure does not rule out the possibility of *in vivo* phosphorylation. It's conceivable that these amino acids are phosphorylated by extracellular kinases[151, 152], which may not be present in the yeast system. Therefore, while the crystal structure may not capture the phosphorylation events, evidence from related systems supports the notion that cpAQP1aa could undergo phosphorylation in its native environment, potentially influencing its functional properties as proposed in our model.

3.5.2. FUTURE DIRECTIONS

Further investigations are necessary to validate the proposed model of extracellular phosphorylation as a novel gating mechanism for fish AQP function. Potential kinase candidates have been identified for phosphorylating the putative sites. Notably, PKDCC, an extracellular tyrosine kinase, have implicated in phosphorylating osteopontin at a site analogous to Tyr107[153]. Additionally, FAM69 (DIA1) is a Ser/Thr kinase[154] which could potentially phosphorylate Thr38. To assess

whether these kinases phosphorylate Tyr107 and Thr38, an *in vitro* phosphorylation assay could be conducted. Recombinant expression of the kinases, followed by incubation with cpAQP1aa and ATP, would enable analysis of the phosphorylated status using immunoblotting or mass spectrometry. Subsequent verification of *in vivo* phosphorylation could be achieved by expressing cpAQP1aa in a human cell line that naturally expresses extracellular kinase candidates. Transfecting *Xenopus* oocytes is another potential option. However, it's worth noting that the extracellular kinases mentioned are not inherently present in fish egg cells. This assertion has been confirmed through database validation obtained from xenbase.org. In this case, the kinase of interest could be introduced either by co-transfection or co-expression into *Xenopus* oocytes to investigate their phosphorylation of cpAQP1aa *in vivo*. *Xenopus* has been shown to work as an enzyme expression system previously[155]. As a final step, to confirm extracellular phosphorylation occurs biologically, using biological sources, preferably climbing perch itself, tissue samples of the gills and skin could be prepared. cpAQP1aa could then be isolated directly from these tissues, and its phosphorylation status tested by mass spectrometry[156].

3.6. SUMMARY OF RESULTS

To understand human AQP regulation within cellular contexts, interpreting interaction partners and their structural changes is crucial. To address this, we established a higher-throughput interaction screening method (**Paper I**), building upon previous research utilizing fluorescent BiFC complexes assessed via fluorescence microscopy. This advancement optimized screening efficiency by employing flow cytometry to evaluate fluorescent membrane protein complexes.
Utilizing this methodology, in **Paper II**, I conducted a comprehensive study of the CaM–hAQP interaction, a thorough examination not previously undertaken. We have reasons to suggest that CaM may be an important regulator for several hAQPs, as evidenced by its interactions with hAQP1, hAQP8, and hAQP9, in addition to the well-established interaction complexes hAQP0-CaM and hAQP4-CaM.
Our ultimate goal is to gain novel insights into hAQP4 function and regulation, which involves interaction with other proteins. In **Paper III**, I describe a method for producing and screening a human brain expression library for interaction with hAQP4. This method serves as a potent tool for identifying novel hAQP4 interaction partners in future

research. Furthermore, our preliminary results suggest that there is a potential CaM binding site in the hAQP4 N-terminus and we have also identified some potentially interaction with nicastrin, which has been evaluated further using AlphaFold2. In addition, we present a preliminary solid state NMR structure of hAQP4, providing an interesting tool in future studies of AQP4 complexes.

Structural investigations of membrane proteins at high resolution have proven to be difficult, primarily because well-ordered, large crystals do not form easily. In **Paper IV**, we take advantage of a novel approach for determining protein structures using the well- studied plant AQP SoPIP2;1. Preliminary results being outcome of this work, shows an intriguing result from the binding of DM to the SoPIP2;1 N-terminus, having implications on the regulatory mechanism.

To address the lack of structural information on fish AQPs, particularly relevant for understanding osmoregulation, we conducted the first high-resolution structural analysis of cpAQP1aa. Our study not only revealed the first high-resolution structure of a fish AQP but also identified the first example of an extracellular gate in AQPs, presumably regulated by phosphorylation (**Paper V**). Altogether, the research outcomes presented in this thesis represents a significant advancement in understanding AQP function and regulation in various eukaryotic organisms (**Fig. 34**).

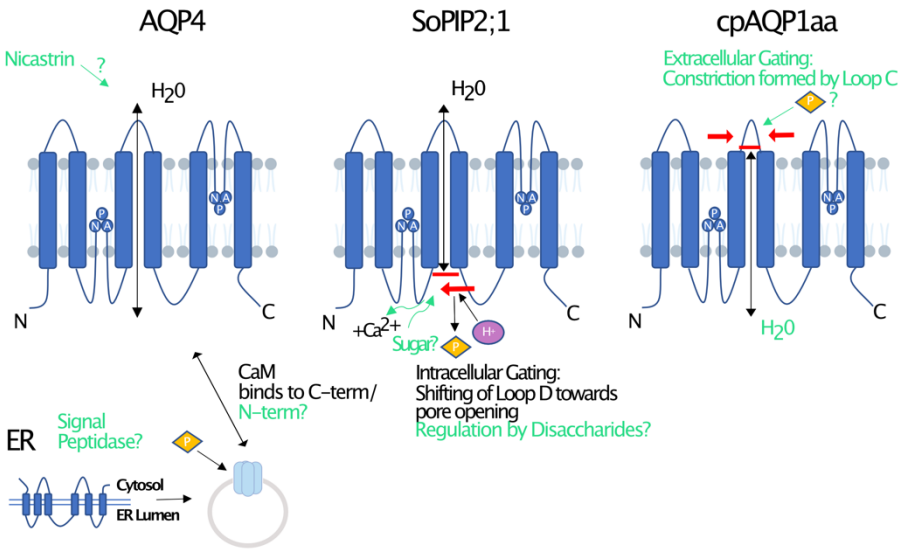


Figure 34: New insights into the regulation of different AQP channels**.** In green the new insights are highlighted.

4. DISCUSSION

4.1. CONFIRMATION OF AQP4-COMPLEXES

Our study introduces a robust platform that addresses the limitations of conventional PPI methods and paves the way for future investigations into the structural determinants of membrane protein interaction complexes. Utilizing BiFC in our library screening enables the detection of stable complexes as well as short-term interactions, including weaker or transient interactions such as phosphorylation events, crucial for regulating AQP function. This approach stands in contrast to traditional methods like Co-immunoprecipitation (Co-IP) and yeast two-hybrid screening, which may overlook such weak or transient interactions due to their reliance on strong interactions[157]. This contrast is evident in previous studies on AQP4 interactions, where Co-IP experiments predominantly identified stable multi-protein complexes with AQP4, comprising various channels and pumps fused together, with minimal detection of soluble protein interactions[43]. We are confident that the combination of BiFC and flow cytometry offers the capability to capture a broader spectrum of PPIs in a higher throughput manner. By applying fluorescence-activated cell sorting (FACS) with a specific set gate, we can selectively screen the few candidates reaching a predefined threshold of high fluorescence, enhancing the efficiency of our screening process. Potential enhancements could involve optimizing the quality of the library to improve fragment sizes and CFU. However, even without these optimizations, we have already identified several promising hits.

While CaM's abundance in the brain increases the likelihood of its detection as an interaction partner, resulting in multiple identifications

in our library screen, we have also identified new hits, such as nicastrin or signal peptidase. Those hits have to be confirmed using various approaches, particularly to understand if the hit was just a specific protein domain rather than the full-length protein. Various biophysical methods can be employed to characterize interaction complexes, including SEC, MAS NMR and MST. SEC, when equipped with a fluorescence detector, enables the monitoring of tagged proteins, such as those labelled with a fluorescent marker or BiFC complexes, throughout the purification process[158]. This method has been shown to effectively purify BiFC complexes due to the stability of the reconstituted split YFP molecule, thus facilitating the purification of stable membrane protein complexes suitable for further structural characterization[79]. Additionally, combining SEC with Multi-Angle Light Scattering (MALS) enhances protein characterization by providing estimates of protein weight and oligomerization state[159]. This can be particularly useful for estimating the size of the complex, which is of interest for interactions involving multiple molecules of both proteins, as observed in previous studies. Crosslinking of the proteins may be necessary beforehand to increase stability[160].

MST provides a method to assess binding affinities between the library hits and hAQP4. Specific peptides involved in the interaction of full-length proteins, could be tested for their binding. Additionally, investigating the binding sites of hAQP4 to CaM, particularly at the N- and C-termini, could include testing hAQP4 controls (AQP4ΔN, AQP4ΔC, and AQP4ΔNΔC) for their binding affinity to both complete CaM and CaM variants containing only the N-lobe or C-lobe. To characterize the structural complexes of these interactions, future work may include co-crystallization of both proteins, followed by structure determination using X-ray crystallography. Additionally, exploring alternative methods, such as utilizing continuous diffraction data in addition to Bragg peaks, holds promise for obtaining structural information on AQPs and their complexes more efficiently. Furthermore, our recently established MAS ssNMR technique enables the study of large complexes within a lipid bilayer environment, providing valuable insights into their organization and function, particularly given the involvement of AQP4 in multi-protein complexes[43]. Cryo-electron microscopy (Cryo-EM) serves as another complementary method for confirmation, allowing the direct visualization of multi-protein complexes as well as providing critical information about their overall architecture and organization.

Furthermore, understanding the involvement of hAQP4 in both healthy cells and disease states is crucial. Constructing libraries from different materials, such as brain samples from individuals with diseases like Alzheimer's disease, where hAQP4 plays an active role, could shed light on the regulatory mechanisms involved. Investigating the hAQP4 interactome may also open new therapeutic avenues for treating human diseases in which hAQPs play a significant role and the regulatory protein could be the target for treatment.

4.2. BIOLOGICAL SIGNIFICANCE OF AQUAPORIN REGULATION

The initial focus on exploring hAQP4's involvement in neurodegenerative diseases like Alzheimer's disease, especially with the identification of nicastrin as part of the gamma-secretase complex, is compelling. Similar to the interaction between AQP1 and the beta-secretase complex in the brain, where AQP1 inhibits beta-secretase and amyloidogenesis[161], there might be a comparable interaction with the gamma-secretase complex and AQP4. Despite nicastrin's extracellular location and the cytosolic positioning of the C-terminal peptide of AQP4, the AlphaFold2 model predicts a high likelihood of their interaction. One plausible scenario for AQP4 binding to nicastrin extracellularly involves the release of certain AQPs into the extracellular space via extracellular vesicles (EVs)[162], where AQP4-EV may be distributed into the interstitial fluid, cerebrospinal fluid and finally to the bloodstream of the brain[26, 163]. Within EVs, AQPs exhibit a reversed topology, exposing their N- and C-termini to the extracellular environment[164]. This hypothesis suggests a potential mechanism for how AQP4 and nicastrin might encounter each other in nature, despite their distinct endogenous subcellular locations. Although research on AQP4 EVs is currently limited, both AQP4 and EVs have been implicated in various pathologies related to CNS injury[165]. In the case of Alzheimer's disease, recent studies indicate a potential functional role of AQP4 within EVs as recent studies have demonstrated elevated levels of AQP4-positive EVs in the blood serum of mice afflicted with Alzheimer's disease[162].

Literature investigations of the other obtained library hit revealed that AQP4 harbours a cryptic signal peptidase recognition site that can be cleaved by signal peptidase[166]. This recognition site may not be readily apparent from the primary sequence alone but could become accessible under specific conditions, such as conformational changes

induced by interactions with other proteins or fusion partners like YFP. Therefore, the biological role of signal peptidase binding might lie in AQP4 maturation or alternative splicing. Additionally, recent insights into the human signal peptidase complex have revealed its function in quality control[167], cleaving faulty membrane proteins to maintain a healthy membrane proteome. This could involve interactions with cryptic recognition sites or other sequences within AQP4, regulating its abundance in the plasma membrane.

Translocation of AQP4 has been reported to be controlled by an interaction between CaM and AQP4[46]. Comprehending the factors contributing to the altered translocation of hAQP4 in cytotoxic edema is crucial and knowledge about the exact stoichiometry and binding sites of hAQP4 and CaM can provide valuable insights into the regulation of translocation from the storage vesicles to the plasma membrane. It could also shed light on the structural complexes associated with the translocation event identifying potential targets for intervention in the case of cytotoxic edema.

The discovery of sugar molecules stabilizing the closed conformation of plant AQPs, ¨represents a novel concept in AQP gating. This regulatory mechanism's biological significance may stem from scenarios where sugar production exceeds the plant's capacity for translocation and utilization during the photoperiod, akin to stress situations. Previous studies have hinted at sugars' influence on plant AQPs, affecting gene expression and reducing leaf water conductance, likely through a feedback mechanism[168]. It's conceivable that sugars directly impact AQP gating, thus coordinating sugar levels with water management.

The identification of a novel extracellular fold in cpAQP1aa has provided valuable insights into potential regulatory mechanisms involving extracellular phosphorylation in osmoregulation. Further research is required to determine whether the regulatory mechanism of extracellular phosphorylation is exclusively, if confirmed, restricted to gating of proteins such as cpAQP1aa, or whether extracellular phosphorylation additionally affects translocation as observed for intracellular phosphorylation. Once considered uncommon, extracellular phosphorylation has emerged as a significant regulatory mechanism in various physiological processes[169], including osmoregulation[170]. Recent studies have uncovered extracellular kinases capable of phosphorylating serine, threonine, and tyrosine residues of extracellular and transmembrane proteins[171], suggesting a role in driving protein interactions and contributing to nervous system

development and function. While the involvement of extracellular phosphorylation in osmoregulation has been reported in forebrain regions[172] its role in aquatic organisms remains yet to explore. Despite the evolution of diverse osmoregulatory strategies in aquatic animals, the specific contribution of extracellular phosphorylation to osmotic homeostasis remains unclear. Given the lack of structural data on AQPs in other freshwater and saltwater fish, it would be intriguing to explore whether the extracellular gate is a common feature in aquatic species, where osmoregulation plays a crucial role in survival.

In conclusion, through in-depth studies, combining molecular biology and biochemistry methods with theoretical predictions, the work included in this thesis has uncovered novel insights into AQP regulatory mechanisms in the eukaryotic AQP members in human, plant and fish (**Fig. 34**). In a larger context, this study aims to improve our understanding of regulatory processes in healthy cells and their impact on disease development as well as sustainable aquatic cultures.

5. ACKNOWLEDGMENTS

Before I come to an end, I would like to say that I have very much enjoyed writing this book. Despite the countless trials and errors along the way, it has been extremely satisfying to see the results of my efforts. Although my contributions may seem modest in the broad field of aquaporins, I hope that they will ultimately enrich our understanding of aquaporin regulation in both normal and pathological cellular states, particularly in the context of Alzheimer's disease - a topic that is of great personal importance to me, as it runs unfortunately in my family.

When I think about how quickly the five years since I started my PhD have passed, I am amazed. This time has shaped me in ways I could hardly have imagined. The rollercoaster of emotions during this journey has been intense, but the growth - both personally and professionally - has been deep. Living in a foreign country and adapting to a new environment has given me invaluable insights. When I look back at the person I was five years ago, I hardly recognize myself.
At the beginning of my PhD, I had many doubts about my ability to obtain a doctorate, especially after being out of academia for two years. However, your great confidence in me, **Kristina**, was there from the very beginning. I still remember our interview, when we were discussing how the FACS machine works over a fika in the sun. The way you motivated me during these years, especially after making mistakes (of which there were many in the beginning) with the phrase "good that you found the problem" is an example of your ability to encourage and motivate others. For me, you represent the ideal balance between work and life, and I am very grateful to you for guiding me on my path to my PhD.
I would also like to express my gratitude to **Gisela** for her role as my co-supervisor. If you were to ask me now which part of research I enjoy the most - planning, conducting experiments or analyzing data - it would undoubtedly be analyzing data!
Richard, I want to extend my thanks for being my examiner during the first part of my PhD. Your constructive feedback and valuable inputs

during our paper writing process were very helpful. I'm also grateful for your positivity and for all the pleasant singing in the corridor.
And to **Gergely**, thank you for stepping in as an examiner, especially on such short notice before my halftime examination. Your support has been invaluable. I would also like to thank **Susanna** for your help and expertise, through you I learned a lot about the structural part of aquaporins. I would also like to thank all our collaborators: **Carl Johan**, **Oleksandr**, **Stefan** and **Henry** from the RÅC project, I really enjoyed our meetings and learning from your expertise and **Jiao, Simon, Martin, Kristina, Leif** and **Swaminathan**, it was great to be able to participate in this interesting study on fish aquaporins. Thanks to **Göran** for all your support and ideas for upcoming projects and to **Tanguy** for our collaboration and for explaining the basics of NMR spectra to me in a very simple way.
Florian, not the first one to mention but definitely not the least ☺, the Lundberg lab hasn't been the same since you left. You are no doubt a remarkable person - an outstanding colleague and a valued friend. I have no doubt that you will find your place and achieve great things.
Ulrika, I had simply hoped that you would have joined our group much earlier. I value your professional advice immensely. I have the utmost respect for all that you contribute to the PPS platform.
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