

Structural profiling of conformational transitions in recoverin orthologs

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ABSTRACT

The neuronal calcium sensor recoverin is essentially involved in the termination and recovery of the photo-response by calcium-dependent inhibition of rhodopsin kinase (GRK1). The interaction with GRK1 is facilitated by a pronounced conformational change upon calcium binding, the calcium-myristoyl switch. In contrast to mammalian rod and cone photoreceptors which share the same recoverin isoform, the retina of the zebrafish, an emerging model organism for cone phototransduction, expresses four recoverin isoforms of which only one is expressed in rods. The abundance of cone zRec isoforms with high sequence identity raised questions regarding their physiological significance and individual functions. Recent studies suggested functional differences indicated by their individual subcellular localization and calcium-sensitivities. However, structural data explaining functional differences between zRec isoforms is lacking. We evaluated calcium-dependent changes in cysteine accessibility and intrinsic fluorescence of myristoylated and non-myristoylated recoverins and supplemented the experimental data with structural modelling approaches. These approaches were employed to provide a structural interpretation of the experimentally determined accessibilities and identify the individual cysteine residues most likely responsible for the experimental observations. The accessibility of the conserved residue C39 that is closely associated with the myristoyl-binding pocket showed only slight variability among myristoylated isoforms whereas non-myristoylated isoforms displayed considerable individual differences. Bicelles and GRK1 individually affected cysteine accessibility. Pronounced differences between zRec isoforms were observed in their intrinsic fluorescence spectra, pointing to different calcium-dependent conformational changes between recoverin isoforms. The differences observed in intrinsic fluorescence and cysteine accessibilities indicate slight structural variations that could impact target regulation.

1. Introduction

Intracellular signalling involving changes in cytoplasmic Ca^{2+} -concentration $[\text{Ca}^{2+}]_c$ is often mediated by Ca^{2+} -sensor proteins. Prominent examples are calmodulin (CaM), STIM1 or the group of neuronal calcium sensor (NCS) proteins that sense changes in $[\text{Ca}^{2+}]_c$ via EF-hand binding motifs [1–3]. The helix-loop-helix structure of an EF-hand binding site is present in more than 500 proteins and fulfils critical tasks by decoding single signalling steps in an intricate network of proteins [4]. Such a network can be controlled by ubiquitously expressed Ca^{2+} -sensors like CaM or more specifically expressed sensors like the NCS proteins group. CaM shows a form of conformational

plasticity allowing different modes of protein-protein interaction by regulating diverse protein functions [5,6]. It is not well understood how widespread this phenomenon of conformational plasticity is and how it corresponds to differences in conformational transitions in a group of proteins that share a high degree of amino acid sequence identity. This aspect is particularly interesting when a genome duplication occurred during evolution as this was the case for teleost fish such as zebrafish (*Danio rerio*) [7].

The zebrafish retina expresses four recoverin genes, *rcv1a*, *rcv1b*, *rcv2a* and *rcv2b* coding for proteins zRec1a, zRec1b, zRec2a and zRec2b, respectively [8]. Immunohistochemical labelling showed presence of zRec1a in all rod cell compartments, but another study reported also

Abbreviations: DTNB, (5,5'-dithiobis-(2-nitrobenzoic acid)); EGTA, Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; bRec, bovine recoverin; zRec, zebrafish recoverin; GRK, G protein-coupled receptor kinase; HIC, hydrophobic interaction chromatography; PMSF, phenylmethylsulfonyl fluoride; pTM, predicted template modelling; RMSD, root mean square deviation; rSAS, relative Solvent-Accessible Surface.

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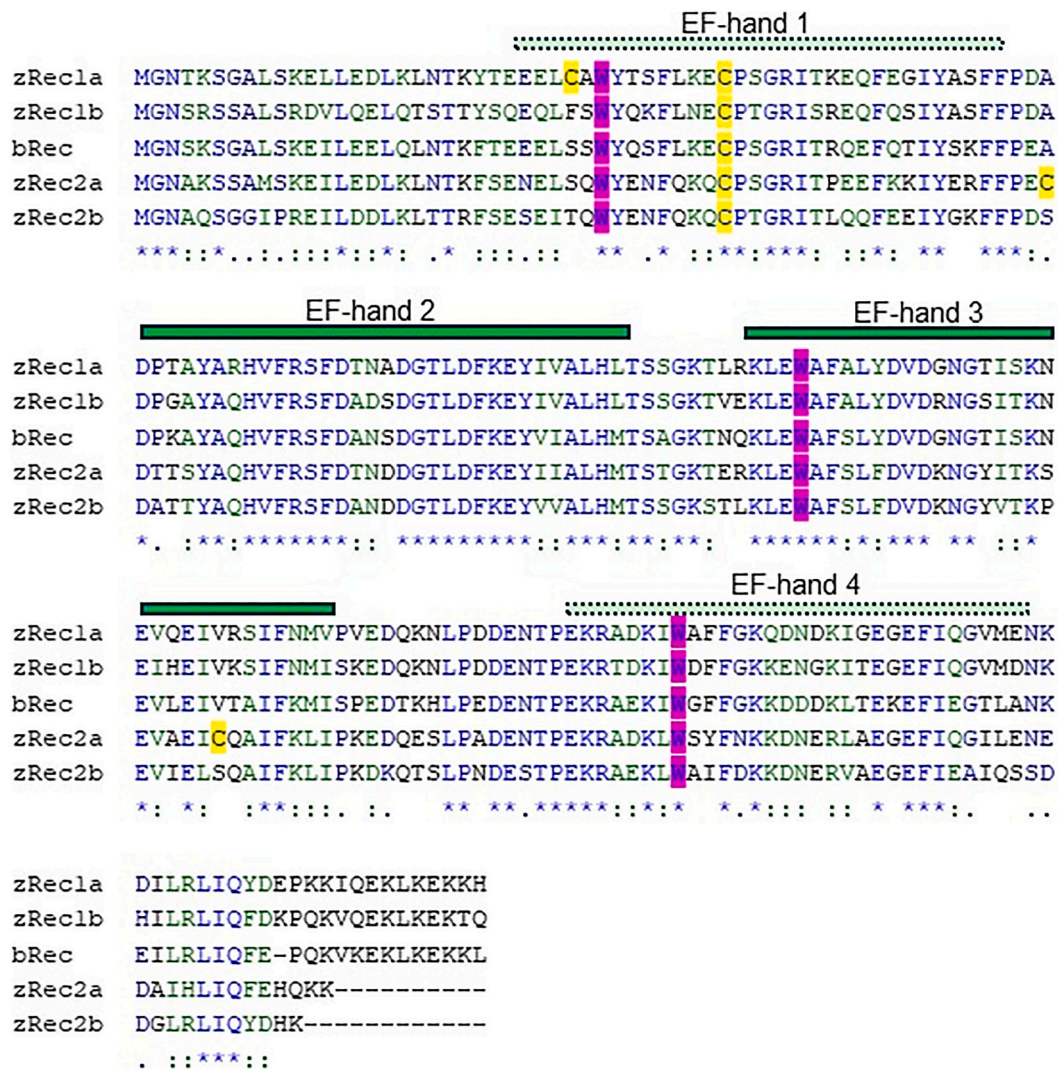


Fig. 1. Sequence alignment of bovine recoverin and zebrafish recoverin isoforms.

The dark green bar above the sequence alignment indicates functional EF-hands 2 and 3, while the light green bar with dotted frame marks non-functional EF-hands 1 and 4. Tryptophans are highlighted with a magenta background, cysteines with a yellow background. Consensus is indicated as follows: * - single, fully conserved residue; - conservation of strong groups; . - conservation of weak groups; - no consensus. Accession numbers are: NM_001030248.1 (zRec1a), NM_001317755.1 (zRec1b), NM_199964.1 (zRec2a), BC060902.1 (zRec2b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression in UV-cones [8]. The same authors also reported that other zRec forms are present in all cone photoreceptors. Immunohistochemical staining detected zRec2a further in bipolar cells. In contrast, Ahrens et al. [9] detected zRec2b only in double cones and cone pedicles and the immunohistochemical staining occasionally overlapped with localization of axonemes. Semi-quantitative immunoblotting showed a 30- to 60-fold lower amount of zRec1b in homogenized retinae in comparison to other zRec forms in agreement with the lack of immunoreactivity in retinal cryosections [9].

The functional role of recoverin was mainly investigated with the bovine ortholog revealing rhodopsin kinase or GRK1 as the main target. GRK1 is inhibited by Ca^{2+} -bound recoverin thereby preventing rhodopsin phosphorylation and the inhibition is relieved when $[\text{Ca}^{2+}]_c$ drops after illumination [10–13]. In the zebrafish retina there are four different paralogs of GRK (zGRK1a, zGRK1b, zGRK7a and zGRK7b) present, encoded by the genes *grk1a*, *grk1b*, *grk7a* and *grk7b* [14,15]. Recombinant zGRK forms can phosphorylate bovine rhodopsin in a light-dependent manner [15] indicating a critical role of zGRKs in rod and cone photoresponse termination. These studies are in broad agreement with electroretinogram (ERG) analyses of zebrafish larvae

harboring knockdowns of zGRK1b and zGRK7a [14,16]. Furthermore, data indicate regulation of GRK1b and GRK7b by zRec2b [8,14] and flash response data shows operation of zRec2a and zRec2b under different light regimes. Circadian expression profiles of *rcv1a* and *rcv2a* differ in larval and adult stages and an oscillating pattern was observed for *rcv1a* and *rcv2b* [17]. In addition to this functional diversity, all zRec paralogs change their conformation in a Ca^{2+} -dependent manner, but only zRec1a and zRec2a undergo the prototypical Ca^{2+} -myristoyl-switch mechanism. The exposure of hydrophobic patches during conformational changes is also not uniform among zRec paralogs [18].

Although zRec paralogs are functionally and conformationally diverse [9,18], they share a high degree of sequence identity (Fig. 1) thereby indicating forms of structural plasticity. Therefore, they raise questions about the structural features that cause these differences. For example, the three tryptophan (Trp) residues present in zRec paralogs and in bovine Rec are in the same position and would allow to monitor and compare changes in Trp fluorescence. The number of cysteines (Cys) differs among Rec orthologs (zebrafish and bovine, Fig. 1), but one position in the first non-functional EF-hand (Cys39) is conserved, whereas zRec1a and zRec2a harbor additional Cys in non-conserved

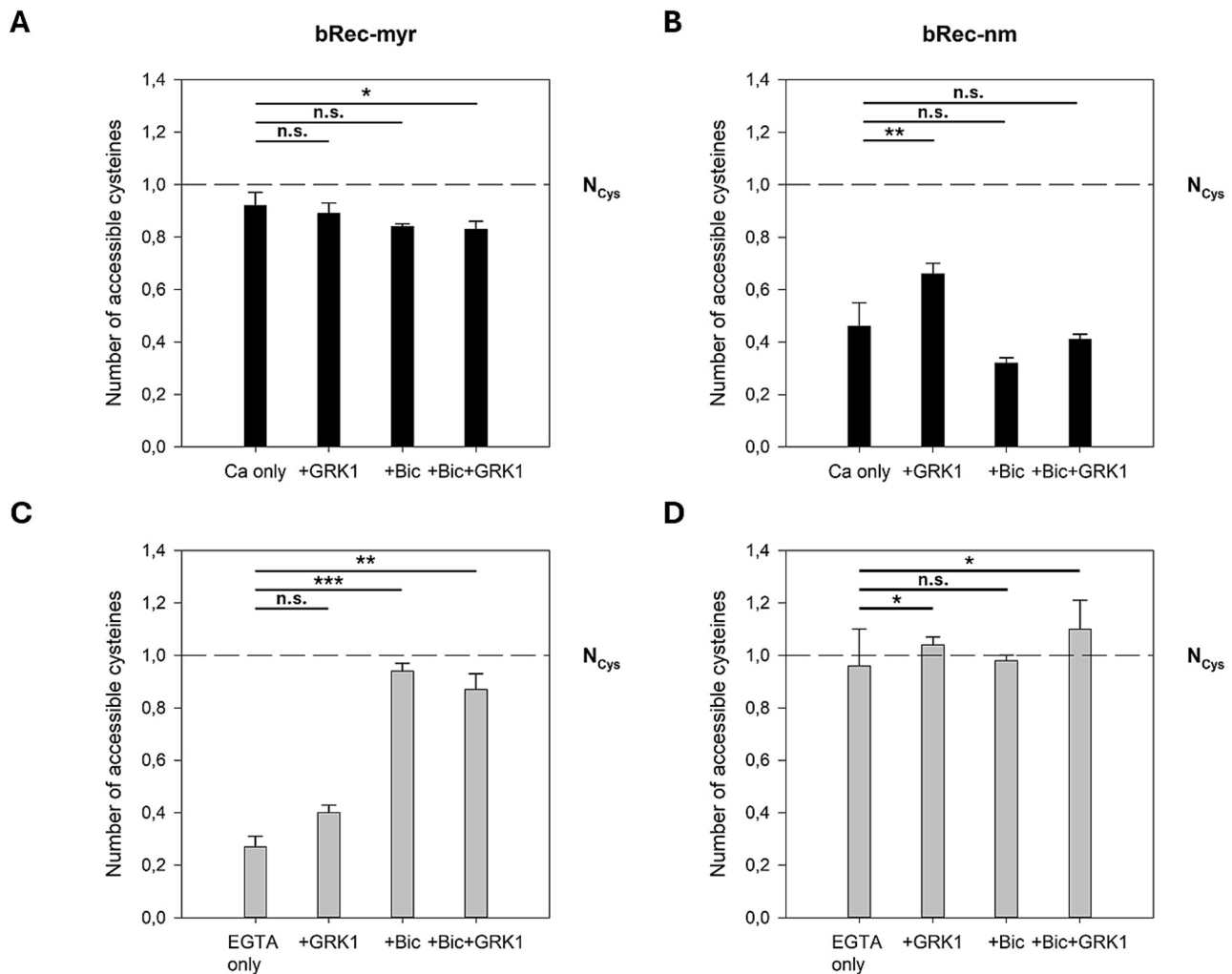


Fig. 2. Cysteine accessibility of bovine recoverin. A and C: cysteine accessibility in myristoylated bRec; B and D: non-myristoylated bRec. Black bars (upper panels) represent the number of accessible cysteines of bRec in calcium-saturated state (1 mM Ca^{2+}), grey bars (lower panels) represent the accessibilities in the calcium-free state (2 mM EGTA). The dashed line indicated with N_{Cys} represents the absolute number of cysteines present in the protein, here $N_{\text{Cys}} = 1$ (Cys39). “Ca/EGTA only”: 5 μM bRec at the respective $[\text{Ca}^{2+}]$ without any further additives. – “+GRK1”: with 7.5 μM GRK1 peptide – “+Bic”: with 10 mM bicelles – “+Bic + GRK1”: with 10 mM bicelles and 7.5 μM GRK1 peptide. Statistical analysis was performed in SigmaPlot 13.0 using Student's *t*-test and Mann-Whitney Rank Sum test. A comprehensive summary is provided in Table S1. $p \geq 0.05 = \text{n.s.}$; $p < 0.05 = *$; $p < 0.01 = **$; $p < 0.001 = ***$.

positions. Cysteine residues might have different accessibilities as this has been reported for guanylate cyclase-activating proteins (GCAPs), a group of NCS proteins related to zRecs [19,20]. Thus, bovine Rec and zRecs provide a unique opportunity to compare conformational changes in a set of homologous and highly conserved proteins. In the present paper we investigated differences in cysteine accessibility and Trp fluorescence emission when changing the free $[\text{Ca}^{2+}]$. We correlate the experimental results with structural features obtained from homology modelling.

2. Experimental procedures

2.1. Expression and purification of recoverin isoforms

Recoverin isoforms were heterologously expressed in BL21(DE3) cells carrying the plasmid pET11d(+) coding for the respective isoform. Forms of myristoylated recoverin were produced using standard protocols [18,21] in BL21(DE3) cells that were additionally transformed with pBB131-yNMT encoding yeast N-myristoyl transferase. Bacterial cells were grown under shaking (160 rpm) at 37 °C and fed with 50 $\mu\text{g}/\text{ml}$ myristic acid at an OD_{600} of 0.4. Protein expression was induced by

adding 1 mM IPTG at an OD_{600} of 0.6. After induction, the cultures were incubated for 4–5 h. The cells were pelleted by centrifugation at 5000 $\times g$ for 10 min. The cell pellets were resuspended in 50 mM Tris pH 7.5 and lysed with 100 $\mu\text{g}/\text{ml}$ lysozyme and 5 U/ml DNaseI for 30 min at 30 °C in a water bath. The lysates were supplemented with 1 mM DTT and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), then centrifuged for 30 min at 100,000 $\times g$ and 4 °C. The resulting supernatants were used for chromatographic protein purification. The pellets were homogenized in 6 M guanidinium hydrochloride and transferred into dialysis tubes (12,000 Da MWCO). The denatured proteins (~20 ml) were dialyzed twice against 3 l 20 mM Tris pH 8.0, 0.15 M NaCl, 0.1 mM DTT. Dialysates were centrifuged at 100,000 $\times g$, 4 °C for 30 min and the supernatants were used for further purification by hydrophobic interaction chromatography (HIC) [18].

2.2. Hydrophobic interaction chromatography (HIC)

Recoverin isoforms were purified with an ÄKTA pure™ chromatography system (Cytiva) using a 50 ml 26/10 Phenyl Sepharose HP column (Cytiva). Cell lysates containing zebrafish recoverins were adjusted to a calcium concentration of 6 mM and those containing bovine recoverin to

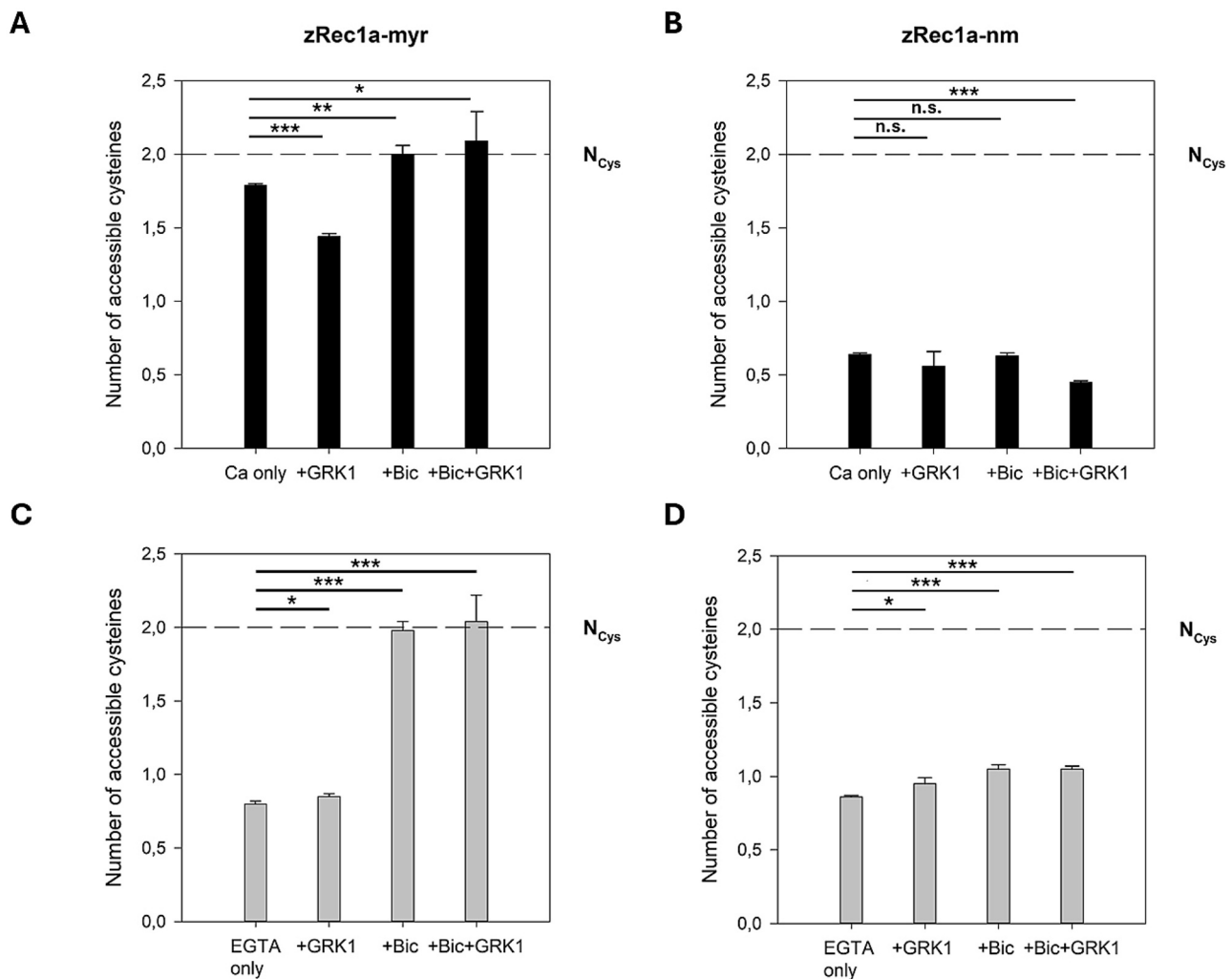


Fig. 3. Cysteine accessibility of zebrafish recoverin 1a. A and C: myristoylated zRec1a. B and D: non-myristoylated zRec1a. Black bars: 1 mM Ca²⁺ (Ca²⁺-saturated state). Grey bars: 2 mM EGTA (Ca²⁺-free state). $N_{Cys} = 2$ (Cys29, Cys39). “Ca/EGTA only”: 5 μ M zRec1a at the respective [Ca²⁺] without any further additives. – “+GRK1”: with 7.5 μ M GRK1 peptide – “+Bic”: with 10 mM bicelles – “+Bic + GRK1”: with 10 mM bicelles and 7.5 μ M GRK1 peptide.

0.5 mM. Both the soluble and insoluble fractions of recoverins were used for purification, with the exception of bovine recoverin of which only the soluble fraction was used. The column was equilibrated with 2.5 column volumes (CV) of HIC buffer A (20 mM Tris, 20 mM CaCl₂, 1 mM DTT, pH 8.0) at a flow rate of 2 ml/min. Samples were applied via the sample pump at a flow rate of 1 ml/min. After completing sample application, the column was washed with 4 CV HIC buffer A. Recoverin was eluted with 100% HIC buffer B (20 mM Tris, 20 mM EGTA, 1 mM DTT, pH 8.0). The fractions containing recoverin were combined and concentrated at 7000 x g, 4 °C in a Pall concentrator tube (10,000 Da MWCO). Chromatography profiles are provided in Figs. S1 and S2. The elution buffer was exchanged at least three times against 50 mM (NH₄) HCO₃. Recoverin was frozen in liquid nitrogen in aliquots of 500 μ g to 1 mg, lyophilized for 6–8 h and stored at –70 °C until further use. Purification of expressed bovine and zebrafish recoverin is a routine operation in our laboratory for several years with very similar outcome. Previous validation of myristoylation by high performance liquid chromatography yielded high percentages such as 96% for bRec, 89% for zRec1a, 96% for zRec2a and 73% for zRec2b [18]. Validation was performed by intrinsic fluorescence emission spectroscopy (see paragraph 2.4), far-UV CD spectroscopy and thermal denaturation profiles (see paragraph 2.5).

2.3. Cysteine accessibility

To assess the number of accessible cysteines in recoverin isoforms depending on myristoylation and presence or absence of calcium ions, the Ellman method was used. Ellman's reagent, or DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)), binds free thiol groups to form a mixed disulfide and TNB²⁻. The formation of TNB²⁻ can be monitored at 412 nm and can thus be used for spectrophotometric quantification of thiol reactivity [22]. Prior to the analyses, a calibration line was generated with 0, 5, 10, 15, 20, 25 and 30 μ M L-cysteine, of which the slope was used to quantify the number of accessible cysteines. Before each measurement, a fresh stock solution of 12 mM DTNB in 0.1 M Tris pH 8.0 was prepared. The solution was sonicated for 3–5 min. Lyophilized recoverin was dissolved in 50 mM HEPES pH 7.4 by ultrasonication for 5 min on ice. 5 μ M of recoverin were incubated with either 2 mM EGTA or 1 mM CaCl₂ and 60 μ M DTNB for 10 min at room temperature. Then, the absorbance at 412 nm was measured in a Specord200 plus spectrophotometer in a 500 μ l OS glass cuvette (Hellma). The number of accessible cysteines was calculated using the formula $N_{Cys} = E_{412} / (a * c)$ with E_{412} being the absorbance of the sample at 412 nm, a the slope of the calibration curve, and c the concentration of recoverin in solution. Subsequently, the reaction setup was expanded to include a peptide containing the first 25 amino acids of bovine GRK1 [23], hereafter referred to as “GRK1 peptide” or simply “GRK1”, which was added to the reaction in a molar ratio

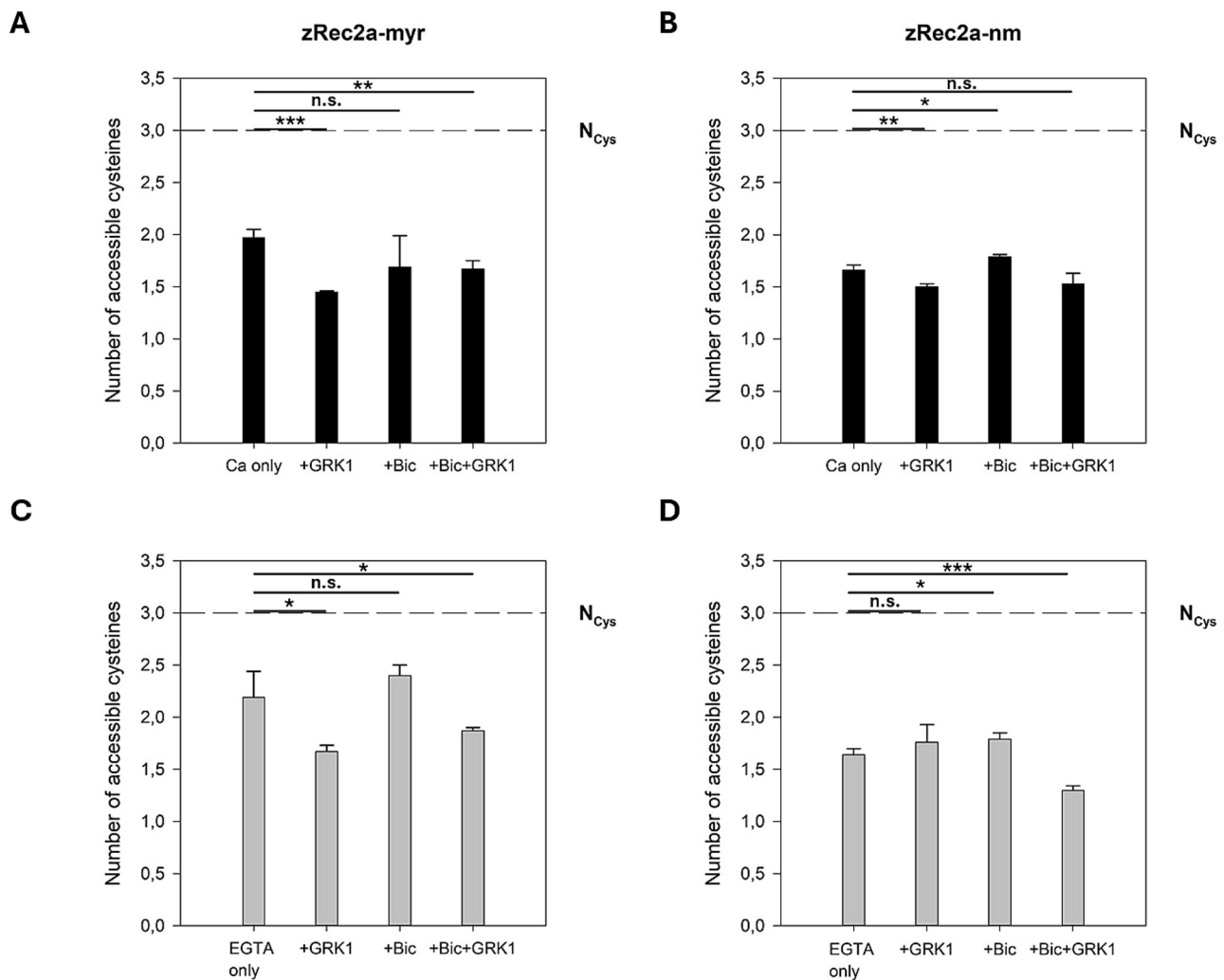


Fig. 4. Cysteine accessibility of zebrafish recoverin 2a. A and C: myristoylated zRec2a. B and D: non-myristoylated zRec2a. Black bars: 1 mM Ca^{2+} (Ca-saturated state). Grey bars: 2 mM EGTA (Ca-free state). $N_{\text{Cys}} = 3$ (Cys39, Cys60, Cys126). “Ca/EGTA only”: 5 μM zRec2a at the respective $[\text{Ca}^{2+}]$ without any further additives. – “+GRK1”: with 7.5 μM GRK1 peptide – “+Bic”: with 10 mM bicelles – “+Bic + GRK1”: with 10 mM bicelles and 7.5 μM GRK1 peptide.

of 1.5:1 (GRK1:Rec). In addition, the effect of a lipid bilayer was tested using 10 mM DMPC:DHPC bicelles, which were produced according to recently established protocols [24,25]. In a third setting, both were combined. These additional conditions were measured sequentially with recoverin alone as a control.

2.3.1. Statistical analysis

The absorbance at 412 nm was measured for each sample and condition in multiple technical replicates. The measurements were repeated using samples from additional cell batches, which were considered biological replicates. Statistical analysis was performed in SigmaPlot 13.0. For each isoform, all three conditions (+bicelles, +GRK1, +bicelles+GRK1) were compared in a paired *t*-test with “Ca only” as the control for the calcium-bound forms and “EGTA only” as the control for the apo-forms of recoverins. The pairwise comparisons against the control samples are indicated by the bars in the respective graphs (Figs. 2–5). The test types used, the calculated *p*-values, and the number of technical and biological replicates for each isoform are summarized in Supplementary Table S1.

2.4. Intrinsic fluorescence spectroscopy

Intrinsic fluorescence assays were performed on a fluorescence spectrophotometer (Photon Technology International) using a protocol

similar to previous measurements on recoverin mutants and GCAP variants [26,27]. Intrinsic fluorescence was excited at 280 nm and the emission interval was set to 300–420 nm. The assays were performed in 500 μl quartz cuvettes (Hellma) with 10 mm path length. In a preliminary experiment, fluorescence spectra of the calcium-free (2 mM EGTA) and saturated (1 mM Ca^{2+}) states of myristoylated and non-myristoylated recoverin isoforms were compared. Bovine recoverin exhibited different intrinsic fluorescence emission spectra depending on the myristoylation status as reported previously [28,29] serving as a check for the presence of the attached myristoyl group (Fig. S3). The spectral differences between myristoylated and non-myristoylated forms were consistent with the expected effects of N-terminal myristoylation and with previous chromatographic validation of proteins produced using the same expression protocol (Fig. S3). These recordings were followed by calcium titrations to determine the calcium concentration for each isoform that leads to a half-maximal change in intrinsic fluorescence intensity. The titrations were performed in a range from <1 nM to 500 μM Ca^{2+} . For all experiments, 2 μM recoverin in fluorescence buffer (80 mM HEPES, 40 mM KCl, pH 7.4) was used. For this purpose, lyophilized recoverin was dissolved in fluorescence buffer by ultrasonication for 5 min on ice. Following concentration determination by the absorbance of the sample at 280 nm, the recoverin solution was distributed to reagent vessels with pre-added calcium/EGTA to a final recoverin concentration of 2 μM . After ten minutes of incubation at room

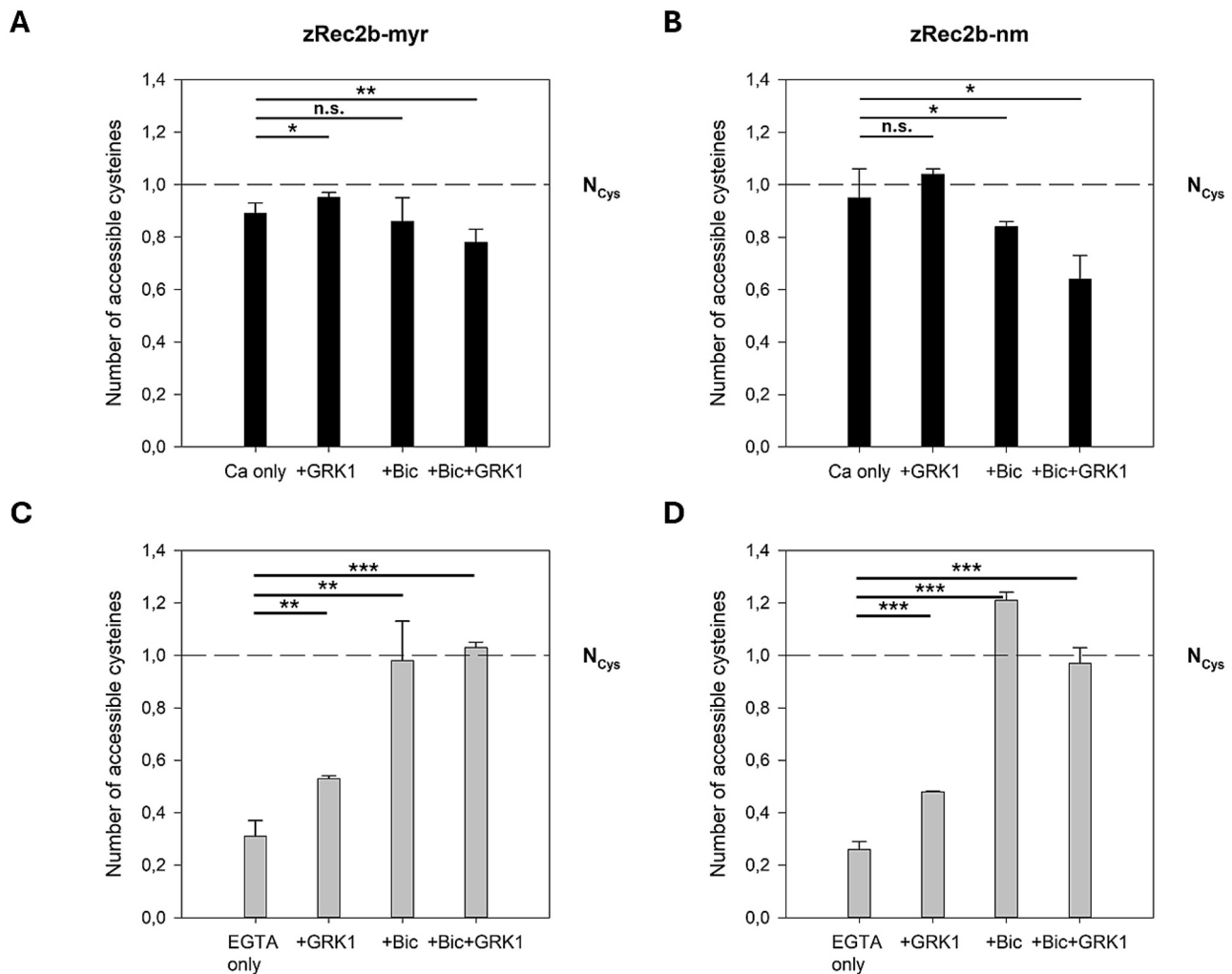


Fig. 5. Cysteine accessibility of zebrafish recoverin 2b. A and C: myristoylated zRec2b. B and D: non-myristoylated zRec2b. Black bars: 1 mM Ca^{2+} (Ca-saturated state). Grey bars: 2 mM EGTA (Ca-free state). $N_{\text{Cys}} = 1$ (Cys39). “Ca/EGTA only”: 5 μM zRec2b at the respective $[\text{Ca}^{2+}]$ without any further additives. – “+GRK1”: with 7.5 μM GRK1 peptide – “+Bic”: with 10 mM bicelles – “+Bic + GRK1”: with 10 mM bicelles and 7.5 μM GRK1 peptide.

temperature to allow equilibrium to be established, the samples were measured with the above-mentioned parameters. Between measurements, the cuvette was washed thoroughly with 70% ethanol and double distilled water. Measurements were performed in triplicates. Since the recoverin isoforms did not show fluorescence changes below 1 μM Ca^{2+} , concentrations below this were omitted, with the exception of 2 mM EGTA to represent the calcium-free state. For each calcium concentration, the fluorescence intensity and the wavelength of the emission maximum were obtained. To ensure that changes in fluorescence between the isoforms were comparable, the fluorescence intensities were normalized to their respective maximum values. The half-maximal change in intrinsic fluorescence intensity and changes of the wavelength where the fluorescence spectrum reached its maximal emission were determined by means of a Hill 4 parameter fitting in SigmaPlot 13.0.

2.5. Far-UV circular dichroism spectroscopy and thermal denaturation profiles

Far-UV CD spectroscopy was employed to monitor variations in secondary structure and thermal stability of Rec isoforms upon Ca^{2+} -binding, thus allowing the assessment of their functionality. CD data was collected on a Jasco J-710 spectropolarimeter equipped with a Peltier-type thermostat. Lyophilized Rec isoforms were dissolved in 80 mM

Tris-HCl pH 7.4, 40 mM KCl buffer and diluted to 7–9 μM (with the exception of the Ca^{2+} -bound myr zRec1a sample for thermal denaturation profiles, in which protein concentration was 1 μM) after measuring concentration with Bradford assay and a calibration curve based on bRec. Far-UV CD spectra (200–250 nm) of Rec isoforms were recorded in the presence of 200 μM EGTA and after addition of 400 μM Ca^{2+} , leading to a free Ca^{2+} concentration of 200 μM . The parameters for spectra recording were the following: 1 nm data pitch, 4 s integration time, 1 nm bandwidth, 20 $^{\circ}\text{C}$ temperature, 50 nm/min scan rate, 5 accumulations. Thermal denaturation profiles were obtained with the same EGTA and Ca^{2+} concentrations as for far-UV CD spectra; the ellipticity at 222 nm (one of the typical minima of all α -helical proteins) was monitored between 20 and 96 $^{\circ}\text{C}$, the parameters for profile recording were the following: 1 $^{\circ}\text{C}$ data pitch, 1 nm bandwidth, 1.5 $^{\circ}\text{C}/\text{min}$ scan rate, 4 s integration time. Data was fitted to a polynomial function of the sixth order, the lowest degree which ensured the convergence of the estimation of the melting temperature of all Rec isoforms according to the criteria elucidated in [30].

2.6. Molecular modelling of rec isoforms and surface analysis

The three-dimensional structures of apo non-myristoylated bRec, zRec1a, zRec2a and zRec2b were predicted using the AlphaFold3 [31] server (<https://alphafoldserver.com/>) after providing the sequences of

Table 1

Relative solvent-accessible surface (rSAS) of Cys residues in Rec isoforms.

Isoform	Residue	Myristoylated			Non-myristoylated	
		Apo	Ca ²⁺	Ca ²⁺ + GRK1	Apo	Ca ²⁺
bRec	C39	23.2 ± 11.5	35.6 ± 9.8	39.8 ± 8.2	29.7 ± 3.4	14.5 ± 14.6
zRec1a	C29	40.0 ± 3.3	33.4 ± 8.4	48.4 ± 5.3	39.9 ± 0.6	44.4 ± 1.2
	C39	23.4 ± 11.2	38.0 ± 13.0	38.3 ± 15.4	0	5.1 ± 11.3
zRec2a	C39	25.1 ± 12.4	30.3 ± 13.6	46.1 ± 7.4	7.8 ± 7.7	24.3 ± 14.8
	C60	15.3 ± 10.7	26.7 ± 6.0	16.5 ± 5.8	6.8 ± 2.6	16.9 ± 7.7
	C126	7.4 ± 6.8	6.1 ± 7.4	12.7 ± 7.4	0	0
zRec2b	C39	32.4 ± 12.8	45.8 ± 12.3	38.2 ± 21.0	6.9 ± 9.4	7.2 ± 16.1

bovine Rec (UniProt: P21457), zebrafish Rec1a (UniProt: Q4V8S2), Rec2a (UniProt: Q6PC38), and Rec2b (UniProt: Q1LUD3) as input. The Ca²⁺-bound forms of non-myristoylated Rec isoforms were also modelled using AlphaFold3 server by adding 2 Ca²⁺-ions to the input sequences. The server generated five structural models ranked according to a score accounting for the mean predicted local distance difference test (pLDDT), calculated as the arithmetic mean of the pLDDT values of all atoms belonging to the model, and the predicted template modelling (pTM) scores. The difference in the ranking scores was not large enough to identify the most reliable model for each variant, thus all 5 models were subjected to surface analysis. Furthermore, residue-level pLDDT values were calculated for all modelled structures as the arithmetic mean of the atoms belonging to each residue. Ramachandran analysis was performed to assess the stereochemical plausibility of the models predicted by AlphaFold3.

The three-dimensional structure of myristoylated zRec variants in their apo forms were predicted by homology modelling using all 22 conformers of the NMR-solved structure of apo myristoylated bovine recoverin (PDB: 1IKU) [32] as template. Analogously, the myristoylated Ca²⁺-bound forms of zRec variants were modelled using all 24 conformers of the NMR solved structure of Ca²⁺-bound myristoylated bovine recoverin (PDB: 1JSA) [33] as template. As myristoylation could not be directly considered as a post-translational modification by the homology modelling pipeline, the covalent bond between the N-terminal Gly and the carbonyl of the myristoyl moiety was manually generated for all myristoylated variants.

The Ca²⁺- and GRK1-bound forms of myristoylated zRec variants were modelled over the 10 conformers of the NMR solved structure of Ca²⁺- and GRK1-bound myristoylated bovine recoverin (PDB: 2194) [34] as template in which, however, the first 7 residues at the N-terminus were not resolved. The atomic coordinates of GRK1 peptide (encompassing residues 1–25) were imported by the original structure (PDB: 2194) after C α -superimposition of zRec variants onto myristoylated Rec template. Homology modelling was performed using Maestro/BioLuminate (Schroedinger, New York, USA) suite, which generated an energy-based model for each variant by preserving the rotamers of conserved residues, optimizing the side chain of non-conserved residues and employing distance constraints between the ions and/or the myristoyl group and the protein.

All modelled myristoylated and non-myristoylated zRec and bRec structures, as well as the experimentally solved structure of Ca²⁺-bound bRec in complex with the peptide encompassing the first 25 residues of GRK1, were then subjected to BioLuminate's *Protein preparation* pipeline, which consisted of: i) assignment of bond orders based on the Chemical Components Dictionary database (www.pdb.org), wwPDB foundation, Piscataway NJ, USA); ii) addition of H-atoms; iii) creation of disulfide bonds and zero-order bonds to metals according to *Prime*; iv) prediction of the charge of ionizable side chains and heteroatoms at pH 7.5 using *PROPKA* and *Epik*, respectively; v) assignment and optimization of H-bonds; vi) energy minimization using OPLS4 forcefield (Schroedinger, New York, USA), using 0.3 Å Root Mean Square Deviation of heavy atoms as threshold. The solvent accessibility of the side chains of residues was calculated by the *Protein Surface Analysis* tool provided by

BioLuminate, data reported in Table 1 is presented as average $\pm$ standard deviation of the results obtained on all conformers/models available for the respective variant, namely 22 for apo myristoylated Rec, 24 for Ca²⁺-bound myristoylated Rec, 10 for the complex between Ca²⁺-bound Rec and GRK1 peptide, and 5 for non-myristoylated Rec isoforms. To determine whether the residues were solvent-exposed, the relative Solvent-Accessible Surface (rSAS), representing the percentage of exposure with respect to the total SAS in a x-residue-x tripeptide, was calculated and a threshold of 25% was considered for exposed [35], <5% for buried [36], and 5% < rSAS < 25% for partially exposed residues, which was adopted as an operative threshold to capture residues that could not unambiguously be assigned to either category.

3. Results and discussion

3.1. Cysteine accessibility of rec orthologs

Myristoylated and non-myristoylated forms of bovine Rec, zRec1a, zRec2a and zRec2b were purified as recombinant proteins from *E. coli* lysates yielding sufficient amounts for conformational investigations. First, we monitored cysteine accessibility of all Rec variants in the presence and absence of Ca²⁺, with and without the target peptide derived from bovine GRK1 [23]. A further variation in the test was the presence of bicelles. Results for bovine Rec, zRec1a, zRec2a and zRec2b are described in detail below.

3.1.1. Bovine recoverin

Bovine Rec contains one cysteine residue, Cys39, which is located in EF-hand 1 and is highly conserved among NCS proteins. In the myristoylated form it was fully accessible at saturating [Ca²⁺] (1 mM) (Fig. 2A). The presence of the GRK1 peptide did not affect the accessibility of Cys39 under these conditions. Bicelles alone had no effect on the accessibility but in combination with the GRK1 peptide, it was slightly reduced. At low calcium concentrations (Fig. 2C), which were established with 2 mM EGTA, Cys39 became inaccessible to the reagent. Here too, the presence of GRK1 had no effect on the accessibility, but the cysteine became exposed in presence of bicelles despite the low calcium level.

The non-myristoylated form of bRec displayed the opposite behavior (Fig. 2B and D): at high [Ca²⁺], Cys39 was inaccessible to the Ellman reagent, whereas it became fully accessible at low [Ca²⁺], indicating a difference in thiol reactivity between myristoylated and non-myristoylated recoverin. When GRK1 was added, access slightly increased at both high and low calcium levels. Bicelles did not have a significant effect on the accessibility under these conditions and appeared to eliminate the small effect of the GRK1 peptide. At low [Ca²⁺], Cys39 was largely accessible under all conditions.

3.1.2. Zebrafish recoverin 1a

Zebrafish recoverin isoform 1a contains two cysteine residues at positions 29 and 39 located in EF1. In the myristoylated form, the two cysteines were accessible at saturating [Ca²⁺] (Fig. 3A). Addition of

GRK1 led to a small decrease by about 0.5. When bicelles were present, full accessibility was observed. At low calcium concentration (Fig. 3C), one of the two cysteines became inaccessible. Addition of GRK1 slightly increased the access, but the two cysteines became fully reactive to DTNB in the presence of bicelles.

In the non-myristoylated form (Fig. 3B), the accessibilities at saturating $[Ca^{2+}]$ pointed to a restricted exposure of one cysteine while the other one was not reactive towards DTNB. When bicelles and GRK1 were added, both cysteines became nearly inaccessible. At low $[Ca^{2+}]$ (Fig. 3D), one of the two cysteines was accessible. The presence of bicelles led only to a small increase of accessibility.

3.1.3. Zebrafish recoverin 2a

This isoform contains three and therefore the highest number of cysteines of all zRec isoforms. In addition to Cys39, these are position 60, which is located between EF1 and EF2, and 126, which is part of EF3 (Fig. 1). Of the three, at least one cysteine was always accessible, but no more than two were fully accessible (Fig. 4). Myristoylated zRec2a exposed two cysteines at high $[Ca^{2+}]$ (Fig. 4A), but addition of the GRK1 peptide reduced the thiol reactivity. Bicelles alone did not have a significant effect but the combination with GRK1 decreased the access. At low $[Ca^{2+}]$, the presence of GRK1 peptide led to a significant decrease of accessibility and only one cysteine remained fully accessible, either in the absence or presence of bicelles (Fig. 4C).

The cysteine accessibility did not change much between high and low $[Ca^{2+}]$ in non-myristoylated zRec2a (Fig. 4B and D). At high $[Ca^{2+}]$, the numbers ranged between 1.5 and 2. At low $[Ca^{2+}]$, the most significant change was observed in the combination of GRK1 and bicelles showing an access of about one cysteine.

3.1.4. Zebrafish recoverin 2b

The access of Cys39 in myristoylated zRec2b resembled those of myristoylated bovine Rec under all conditions (Fig. 5A and C), which was not surprising as both proteins have one cysteine at the same position. However, a major difference was evident between the non-myristoylated isoforms (Figs. 2 and 5, B and D). Cys39 in zRec2b was reactive at high $[Ca^{2+}]$ in both the myristoylated (Fig. 5A) and non-myristoylated form (Fig. 5B) and the combination of bicelles and GRK1 reduced the thiol reactivity of the cysteine. In case of the myristoylated form, the GRK1 peptide had a minor positive effect on the accessibility. But at low $[Ca^{2+}]$ (Fig. 5C), the cysteine exhibited a very low accessibility. The GRK1 peptide slightly increased the accessibility to 0.5 and addition of bicelles made it fully accessible. In contrast to the other isoforms, this was also the case for non-myristoylated zRec2b where the presence of the lipids led to a strong increase in accessibility (Fig. 5D).

3.2. Structural analysis of zRec isoforms

The functionality of purified recombinant Rec isoforms (Supplementary Fig. S1 and S2) as Ca^{2+} -sensor proteins was assessed by recording intrinsic fluorescence spectra and analyzing the resulting emission curves in terms of both the maximal fluorescence intensity and the wavelength where the emission intensity was maximal. In line with previous results on bovine Rec [28,29], zRec isoforms exhibited peculiar spectral differences upon Ca^{2+} -addition (Supplementary Fig. S3) which allowed to verify not only the conformational change expected by Ca^{2+} -sensors, but also to distinguish between myristoylated and non-myristoylated isoforms. On the same line, far-UV CD spectra (Supplementary Fig. S4) confirmed that all isoforms acquired an all α -helix secondary structure susceptible to Ca^{2+} , as shown by the variations in spectral shape ($\theta_{222}/\theta_{208}$, Supplementary Table S2) upon Ca^{2+} -binding. Indeed, $\theta_{222}/\theta_{208}$ of myristoylated bRec increased from 0.79 to 0.93, while that of the non-myristoylated isoform increased from 0.84 to 0.90, substantially in line with previous results (0.77 to 0.87 for myristoylated bRec and 0.83 to 0.88 for non-myristoylated bRec) [29] collected at

25 °C and with higher ionic strength (150 mM vs 40 mM in this study).

The analysis of the thermal stability (Fig. S5) also confirmed that Ca^{2+} -binding induced an increase of the melting temperature (T_m , estimated as explained in [30]) of all Rec isoforms exception made for zRec1a. Indeed, Ca^{2+} -bound myristoylated zRec1a exhibited almost the same melting temperature as the apo form (72.3 vs 71.7 °C), while the non-myristoylated isoform exhibited a unique pattern among all tested variants, as in the apo form a double transition at 44.6 and 86.2 °C could be identified, while upon Ca^{2+} -addition a single transition could be detected at a T_m of 74.5 °C. On the other hand, the melting temperatures of bRec aligned with previous results [29], with myristoylated bRec showing a T_m of 73.3 and 82.7 °C in the apo and Ca^{2+} -bound form, respectively (73.1 and 78.8 °C in [29]), and the non-myristoylated isoform displaying T_m of 74.4 and 84.9 °C in the apo and Ca^{2+} -bound form, respectively (73.8 and 79.4 °C in [29]). Interestingly, non-myristoylated bRec was found again to be more stable than the myristoylated isoform, moreover, both Ca^{2+} -bound forms were found >4 °C higher compared to the values published in [29]. Overall, fluorescence and far-UV CD spectra, together with thermal denaturation profiles, demonstrate that all isoforms functioned as Ca^{2+} -sensor proteins with isoform-specific differences between the myristoylated and non-myristoylated forms.

3.2.1. Cysteine accessibility

To investigate the accessibility of cysteine residues of zRec isoforms at atomic resolution we predicted the structural models for apo and Ca^{2+} -bound zRec1a, zRec2a and zRec2b by homology modelling for myristoylated forms, and by AlphaFold3 server prediction for non-myristoylated variants. Additionally, to provide comparison of cysteine solvent exposure with the bovine ortholog, we predicted the structural models of non-myristoylated bRec in their apo and Ca^{2+} -bound forms with AlphaFold3 and compared them with the experimentally solved myristoylated forms (Supplementary Fig. S6). Quality metrics describing the models generated with AlphaFold3 showed overall good quality of all non-myristoylated Rec variants, with ranking scores spanning between 0.77 and 0.88 (Supplementary Table S3 and S4), pTM varying between 0.7 and 0.82 (Supplementary Table S3 and S4), and mean pLDDT values ranging between 77.1 and 83.8, with the exception of apo zRec2a, which exhibited the lowest values of pTM (0.56–0.58), ranking score (0.57–0.6), and mean pLDDT (72.3–73.4, Supplementary Table S3 and S4). Nevertheless, the differences in ranking score did not allow to univocally identify the most reliable model, thus all AlphaFold3 generated models of non-myristoylated Rec isoforms were subjected to the analysis of Cys accessibility. Ramachandran analysis indicated good stereochemical quality for all AlphaFold3 models, as among the 40 structures modelled 97–100% of residues with defined ϕ and ψ angles occupied favored regions, with only five isolated outliers were detected in five different models. No systematic differences were observed between apo and Ca^{2+} -bound states. Notably, although zRec2a models did not show any Ramachandran outliers, favorable dihedral combinations do not resolve the lower pLDDT and higher PAE observed, which evaluate different aspects of model quality.

Residue-level pLDDT analysis (Supplementary Data) indicated generally high local confidence for the cysteine residues of bRec, zRec1a and zRec2b, although the confidence of Cys39 decreased moderately in the Ca^{2+} -bound models (Supplementary Table S5). The most pronounced state-dependent differences were observed for zRec2a. Indeed, while Cys126 consistently displayed high confidence, regardless of the state, in the apo models Cys39 and Cys60 displayed moderate (pLDDT range: 75.1–76.7) and low confidence (pLDDT range: 64.3–65.8). Ca^{2+} addition increased the local confidence of both residues, albeit Cys60 remained less confidently modelled (Supplementary Table S5). Consequently, rSAS estimates involving Cys60, and to a lesser extent Cys39, in apo zRec2a should be regarded as model-based hypotheses with substantial local structural uncertainty.

The structural analysis of myristoylated bovine Rec highlighted that

the only cysteine present, namely Cys39, went from partially to totally exposed upon Ca^{2+} -binding, as shown by the ~ 1.5 -fold increase in solvent accessibility ($23.2 \pm 11.5\%$ vs $35.6 \pm 9.9\%$, Table 1), while further binding to GRK1 did not substantially affect solvent exposure (35.9 ± 9.9 vs $39.8 \pm 8.2\%$, Table 1), in line with experimental results (Fig. 2). On the other hand, non-myristoylated bRec exhibited the opposite trend, with Cys39 being solvent-exposed in the apo form ($29.7 \pm 3.4\%$, Table 1) but only partially exposed in the Ca^{2+} -bound form ($14.5 \pm 14.6\%$, Table 1), although the difference was less pronounced compared to the experimental results (Fig. 2).

It must be noted that, due to the intrinsic limitations of AlphaFold3 models in predicting the structure of proteins undergoing major conformational changes, the superimposition of the backbone of non-myristoylated bRec bound to GRK1 in the absence and in the presence of Ca^{2+} yielded an RMSD of $1.3 \text{ \AA}$, meaning that the two structures were very similar to one another. Moreover, although the solved structure of the complex between Ca^{2+} -bound bRec and GRK1 (PDB: 2I94) [34] was obtained with myristoylated Rec, the N-terminal part of the structure, which contained the post-translational modification (amide bond between myristic acid-COOH and $\text{NH}_3\text{-Gly}$) was not present in the experimental structure. Thus, to avoid overinterpretation due to AlphaFold3 modelling artifacts, the only data reported on Rec isoforms complexed with GRK1 refer to the myristoylated and Ca^{2+} -bound variants [34,37,38].

The same analysis was performed on zRec1a, which has two cysteine residues in position 29 and 39. Structural modelling led us to predict that in the apo form Cys29 was solvent-exposed ($40.0 \pm 3.3\%$), while Cys39 was only partially accessible ($23.4 \pm 11.2\%$). Ca^{2+} -binding significantly improved the accessibility of Cys39 (38.0 ± 13.0 vs $23.4 \pm 11.2\%$, Table 1) which became completely accessible, while that of C29 was slightly reduced (40.0 ± 3.3 vs $33.4 \pm 8.4\%$, Table 1), yet without affecting its status of being solvent-exposed, in line with experimental results (Fig. 3). Surprisingly, GRK1 binding to Ca^{2+} -bound zRec1a further increased the accessibility of Cys29 ($33.4 \pm 8.4\%$ vs $48.4 \pm 5.3\%$, Table 1), while Cys39 did not exhibit appreciable changes (38.0 ± 13.0 vs $38.3 \pm 15.4\%$, Table 1, Fig. 7), at odds with the results presented in Fig. 3A, where GRK1 binding decreased the apparent number of accessible cysteine residues to ~ 1.5 . Such discrepancy may arise from the higher structural flexibility exhibited in the presence of GRK1, as shown by the ~ 1.5 -fold increase in the standard deviation of rSAS. Non-myristoylated zRec1a, on the other hand, did not show significant variations in accessibility upon Ca^{2+} -binding either for Cys29, which was always solvent-exposed (39.9 ± 0.6 vs $44.4 \pm 1.2\%$, Table 1), or for Cys39, which was completely buried (0 vs $5.1 \pm 11.3\%$, Table 1). Even though experimental results (Fig. 3) showed a slightly different trend, that is a minor decrease in cysteine accessibility in the presence of Ca^{2+} , it must be noted that the number of exposed cysteine residues ranged between 0.6 and 0.8, suggesting that one Cys was always exposed (Fig. 7). Based on our computational predictions we suggest that this is Cys29.

Out of all isoforms investigated in this study, zRec2a is the only one presenting three cysteines in position 39, 60, and 126. In line with experimental results (Fig. 4), no more than two cysteines were solvent-accessible under all conditions tested (Fig. 8), as in myristoylated zRec2a residue Cys126 was only partially accessible both in the apo form ($7.4 \pm 6.8\%$) and in the Ca^{2+} -bound form ($6.1 \pm 7.4\%$), as well as upon binding to GRK1 ($12.7 \pm 7.4\%$), while in the non-myristoylated variant Cys126 was completely buried regardless of the presence of Ca^{2+} . The other Cys specific of zRec2a, namely Cys60, exhibited small Ca^{2+} -dependent variations in accessibility in the myristoylated form, indeed its exposure increased from 16.5 ± 10.4 to $26.7 \pm 6.0\%$ (Table 1), thus shifting from a partially to a totally exposed side chain upon Ca^{2+} -binding. Interestingly, GRK1 binding to Ca^{2+} -bound zRec2a resulted in a decrease in accessibility of Cys60 from 26.7 ± 6.0 to $16.5 \pm 5.8\%$ (Table 1), rendering the residue only partially solvent-exposed. A similar behavior could be observed when considering the non-myristoylated

variant, that is an increase of solvent-accessibility upon Ca^{2+} -binding, although to a lesser extent, as rSAS went from 7.8 ± 7.7 to $16.9 \pm 7.7\%$ (Table 1), thus going from almost buried to partially exposed. Finally, Cys39 was found in the myristoylated form to be always solvent-exposed (Fig. 8), and to be increasingly more accessible upon binding to Ca^{2+} (25.1 ± 12.4 to $30.3 \pm 13.6\%$, Table 1) and GRK1 ($46.1 \pm 7.4\%$, Table 1). In non-myristoylated zRec2a, on the other hand, Cys39 was only partially exposed in the absence of Ca^{2+} ($7.8 \pm 7.7\%$, Table 1), but the conformational change associated with Ca^{2+} -binding induced the complete exposure of the residue ($24.3 \pm 14.8\%$, Table 1). It must be noted, though, that Cys39 and especially Cys60 of non-myristoylated zRec2a exhibited lower residue pLDDT values compared to all Cys residues of the other isoforms, indicating structural uncertainty. Moreover, a quantitative correlation of the rSAS of cysteine residues (calculated as a percentage of the total SAS in a x-C-x tripeptide) with the number of exposed Cys (based on the relative stoichiometry between DTNB and Rec concentration) could not be identified due to high variability of rSAS and the different nature of the data.

Therefore, rSAS was employed to provide a structural interpretation of the experimental results on Cys accessibility (with the limitations due to structural uncertainty within the models), rather than a predictive model.

Concerning zRec2b, the only cysteine present, namely Cys39, was estimated to be significantly solvent-exposed in the myristoylated form both in the absence ($32.4 \pm 12.8\%$, Table 1) and in the presence of Ca^{2+} ($45.8 \pm 12.3\%$, Table 1), as well as upon binding to GRK1 ($38.2 \pm 21.0\%$, Table 1). On the contrary, in the non-myristoylated variant Cys39 exhibited limited accessibility both in the absence ($6.9 \pm 9.4\%$, Table 1) and in the presence of Ca^{2+} ($7.2 \pm 16.1\%$, Table 1 and Fig. 9), thus significantly lower than its counterpart in non-myristoylated bRec ($29.7 \pm 3.4\%$ and $14.5 \pm 14.6\%$, respectively). It is worth noting, though, that the structural heterogeneity of the models predicted by both AlphaFold3 and homology modelling gave rise to high rSAS variability, as shown by the standard deviation of the predicted values, which was on average the highest among all tested cases.

3.2.2. Influence of bicelles on cysteine accessibility

In all myristoylated recoverin isoforms except zRec2a the cysteine accessibility at low $[\text{Ca}^{2+}]$ increased in the presence of bicelles (Figs. 2–5, bottom). This can be explained by a shift of the equilibrium from tense to relaxed state since the extrusion of the myristoyl group might be more favorable in presence of bicelles. In zRec2a (Fig. 4), however, the maximum number of accessible cysteines was already present at low $[\text{Ca}^{2+}]$ as residue Cys126 was, according to the structural models, not accessible under any of the tested conditions.

At high calcium concentration, bicelles slightly increased cysteine accessibility of myristoylated zRec1a (Fig. 3A) and non-myristoylated zRec2a (Fig. 4B) and zRec2b (Fig. 5B). At low $[\text{Ca}^{2+}]$, there were significant differences observed between presence and absence of bicelles, especially in the case of zRec1a-myr (Fig. 3C) and zRec2b (Fig. 5C and D). The accessibility of myristoylated bovine recoverin at low $[\text{Ca}^{2+}]$ became considerably larger when bicelles were added ($+0.6$) (Fig. 2C and D) whereas in non-myristoylated bRec, there was no further increase in accessibility observed (Fig. 2D). Due to the lack of the myristoyl group, non-myristoylated recoverins would be expected to not bind to bicelles and thus not show a change in cysteine accessibility. Indeed, there was almost no change observed in the non-myristoylated isoforms, except in apo zRec2b (Fig. 5D). It was exceptional among non-myristoylated recoverins as its single cysteine became fully exposed upon addition of bicelles. Non-myristoylated recoverin can also bind to ROS membranes but to a lesser extent than myristoylated recoverin [39]. This could explain the small but significant increase in cysteine accessibility when non-myristoylated apo zRecs are exposed to excess bicelles (Figs. 3–5, D). Non-myristoylated zRec2b might expose a more hydrophobic surface than the other isoforms and therefore display a higher affinity towards the artificial membrane.

3.2.3. Influence of GRK1 on cysteine accessibility

In SPR experiments, all (myristoylated) recoverin isoforms showed binding to bGRK even at low calcium concentrations, albeit to a lesser extent. In zRec2b-myr, however, the binding signal was almost identical under both conditions [9]. Although the change in cysteine accessibility in the presence of GRK is not equivalent to a GRK binding signal, it can at least be used to compare the isoforms and may provide clues about steric or electrostatic effects.

In myristoylated bRec, there was no fundamental difference in cysteine accessibility when GRK was added to the mixture (Fig. 2A and C), although it would be expected to be bound by recoverin at high $[Ca^{2+}]$ [10–13]. Presumably, the binding of DTNB to cysteine is not influenced by the peptide in this case. In contrast, the non-myristoylated variant showed an increase in cysteine accessibility of about 0.2 in the presence of GRK1, regardless of the calcium concentration (Fig. 2B and D).

In myristoylated zRec1a, presence of calcium reduced cysteine accessibility by about 0.5 when the GRK1 peptide was present (Fig. 3A) which could be explained by a slight steric blocking of Cys39 or Cys29 by the peptide's α -helix or a torsion of the structure as result of the interaction with the peptide. However, the structural modelling yielded the contrary result in that the accessibility of Cys29 increased, while that of Cys39 remained almost unchanged.

In myristoylated zRec2a, cysteine accessibility was also reduced by about 0.5 in the presence of calcium and GRK (Fig. 4A), but this was also the case at low $[Ca^{2+}]$ (Fig. 4C). In non-myristoylated zRec2a, it decreased by about 0.2 at high $[Ca^{2+}]$ and in the presence of GRK1 (Fig. 4B). No difference was observed at low $[Ca^{2+}]$. When myristoylated zRec2b was incubated with GRK1 peptide at high $[Ca^{2+}]$, there was a slight increase in cysteine accessibility (Fig. 5A). At low $[Ca^{2+}]$, accessibility increased by about 0.2 (Fig. 5C). Non-myristoylated zRec2b showed no difference in the accessibility of the individual cysteine at high $[Ca^{2+}]$ (Fig. 5B), but at low $[Ca^{2+}]$ it increased again by about 0.2 (Fig. 5D).

Thus, bicelles and GRK1 influenced cysteine accessibility of zRec paralogs to different extents indicating conformational plasticity in these related proteins of high similarity in primary, secondary and tertiary structure.

3.3. Intrinsic fluorescence emission

Ca^{2+} -binding to myristoylated bovine Rec leads to a pronounced red-shift of fluorescence emission and to an increase in fluorescence intensity [26,40]. These previous studies served as a benchmark control for our experiments showing that all zRec paralogs displayed similar basic features of changes in fluorescence emission, however to different extent (Table 3). All zRec isoforms showed an increase in intensity of fluorescence emission with increasing free $[Ca^{2+}]$. Notable differences were detected in the net fluorescence intensity change between apo and calcium-bound forms. For example, the change was larger in the non-myristoylated variants of zRec2a and zRec2b than in their myristoylated counterpart, whereas in zRec1a, the myristoylated variant showed a considerably larger net change than its non-myristoylated variant. A substantial red-shift in the emission spectrum was only observed in the myristoylated variants of zRec1a and zRec2a, with zRec1a exhibiting the largest shift of 16 nm. Only slight shifts were observed in case of both myristoylated and non-myristoylated zRec2b and non-myristoylated zRec2a. The least shift was observed for non-myristoylated zRec1a (Table 3).

The fluorescence of Trp residues is essentially determined by the degree of solvent exposure and the polarity and charge of neighboring groups. Generally speaking, the exposure of Trp towards an aqueous solvent leads to a red-shift of the emission spectrum and a decrease in fluorescence intensity, whereas buried Trp residues in hydrophobic environment usually display a blue-shifted spectrum with higher fluorescence intensity [41,42]. However, the red-shift of the emission

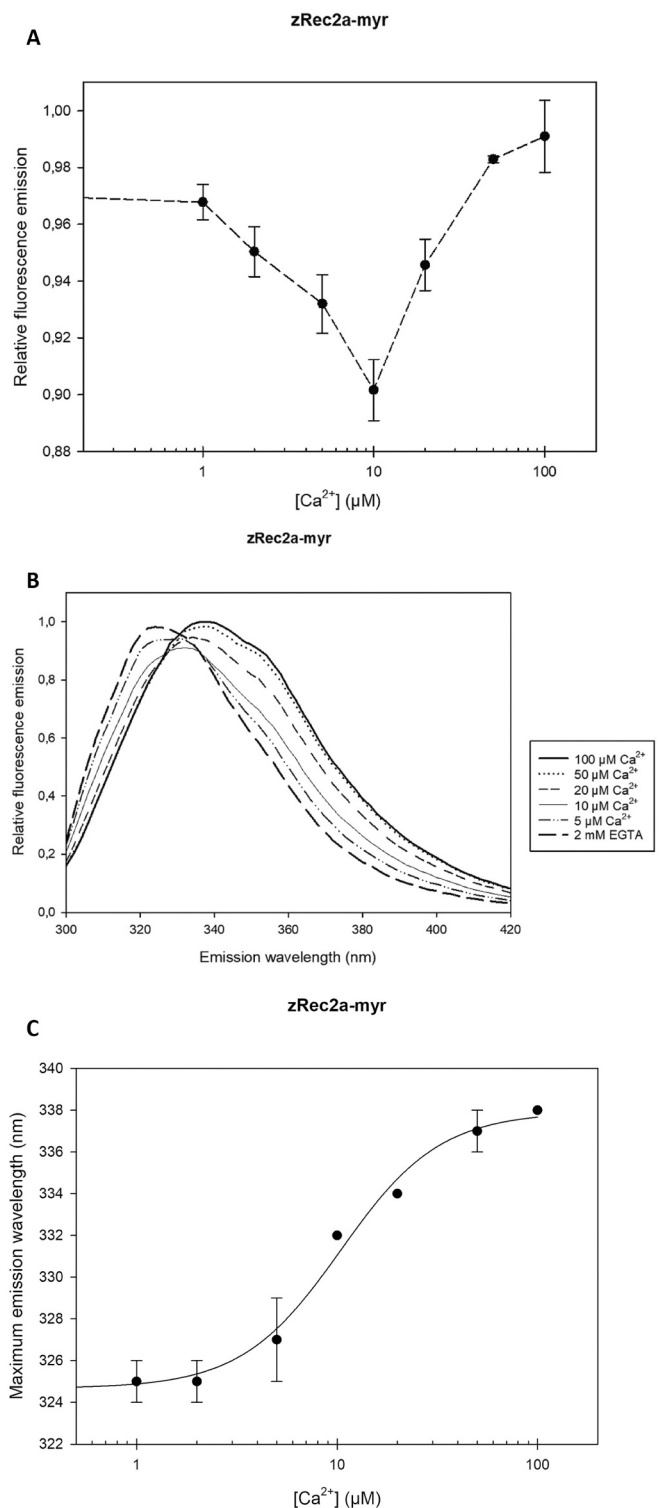


Fig. 6. Intrinsic fluorescence of zebrafish recoverin 2a.

A: Normalized Trp fluorescence of myristoylated zRec2a as function of $[Ca^{2+}]_{free}$ in μM . The two half-maximal values estimated for the atypical biphasic shift of Trp fluorescence emission are $K_{1/2}$ (I) = 3.8 μM and $K_{1/2}$ (II) = 20 μM . B: Shift of the Trp fluorescence spectra of myristoylated zRec2a visualized for different $[Ca^{2+}]_{free}$. C: Wavelength of the emission maximum (λ_{max}) of myristoylated zRec2a as function of $[Ca^{2+}]_{free}$. $K_{1/2}$: 10.5 $\pm$ 1 μM .

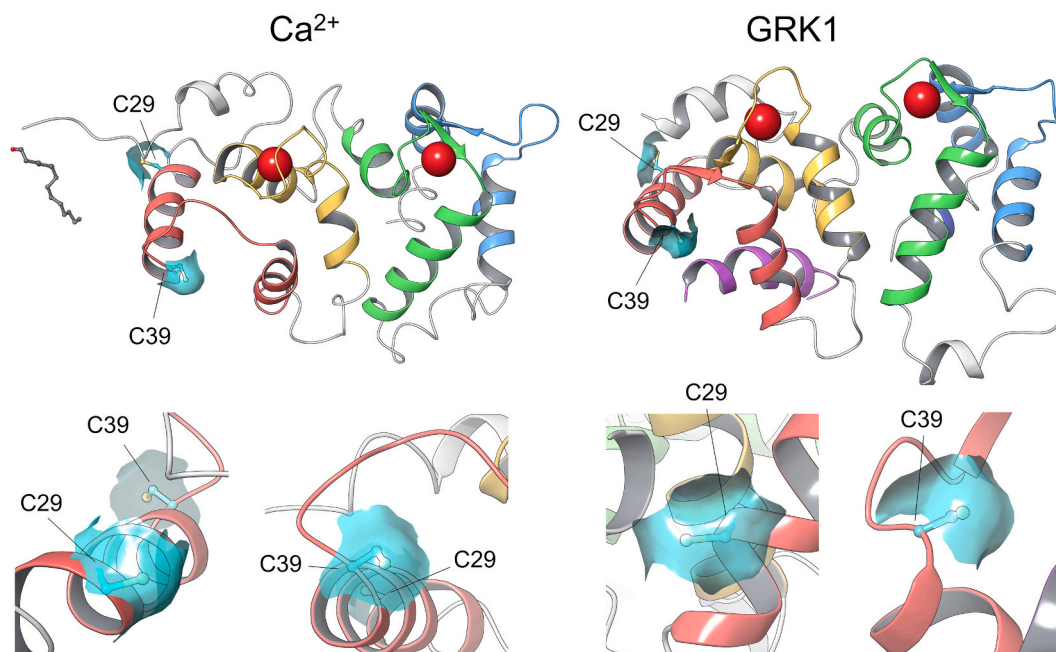


Fig. 7. Solvent accessibility of Cys residues of myristoylated zRec1a in the absence and in the presence of GRK1. Structural details of Cys residues of myristoylated zRec1a in its Ca²⁺-bound (left) and GRK1-bound form (right). The three-dimensional structure of zRec1a is shown in cartoon with EF1 colored in red, EF2 in yellow, EF3 in green, EF4 in azure and the C-terminal helix in purple, GRK1 peptide is represented in magenta. The myristoyl group is shown as grey sticks with O atoms in red, Cys residues are represented by cyan sticks with C atoms in yellow, Ca²⁺-ions are depicted as red spheres, the solvent-accessible surface of Cys residues is colored cyan and shown in transparency. Insets below show an alternative magnified view of Cys residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

spectrum and the increase of fluorescence intensity for all recoverin forms (bRec and zRec) indicate a complex overlay of Trp environments during the transition from the apo to the Ca²⁺-bound form. If extrusion of the myristoyl group and exposure of the hydrophobic binding pocket would allow water molecules or polar side chains to access previously buried Trp residues, one could predict that all myristoylated zRecs

display a red-shift with decreasing fluorescence intensity, which is in part opposite to what we observed. But the largest red-shift exhibited by bRec and zRec1a could indicate solvent exposure and change of Trp environment during extrusion of the myristoyl group, in agreement with Elbers et al. [18], who reported a Ca²⁺-myristoyl switch for zRec1a, whereas zRec2b did not show such a switch.

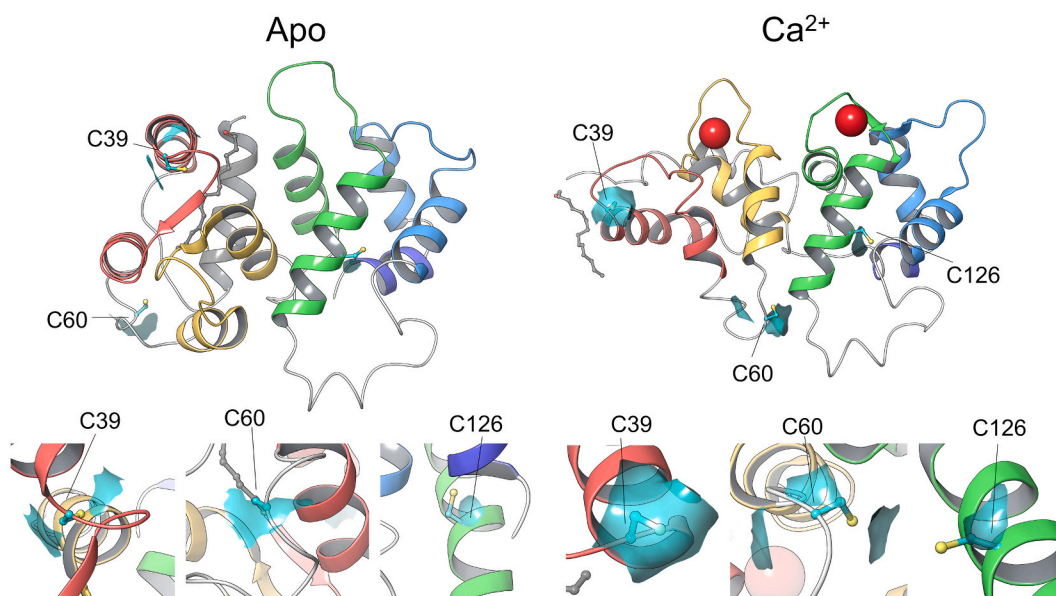


Fig. 8. Solvent accessibility of Cys residues of myristoylated zRec2a in the Ca²⁺-free and Ca²⁺-bound state. Structural details of Cys residues of myristoylated zRec2a in its apo (left) and Ca²⁺-bound (right) form. The three-dimensional structure of zRec2a is shown in cartoon with EF1 colored in red, EF2 in yellow, EF3 in green, EF4 in azure and the C-terminal helix in purple. The myristoyl group is shown as grey sticks with O atoms in red, Cys residues are represented by cyan sticks with C atoms in yellow, Ca²⁺-ions are depicted as red spheres, the solvent-accessible surface of Cys residues is colored cyan and shown in transparency. Insets below show an alternative magnified view of Cys residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

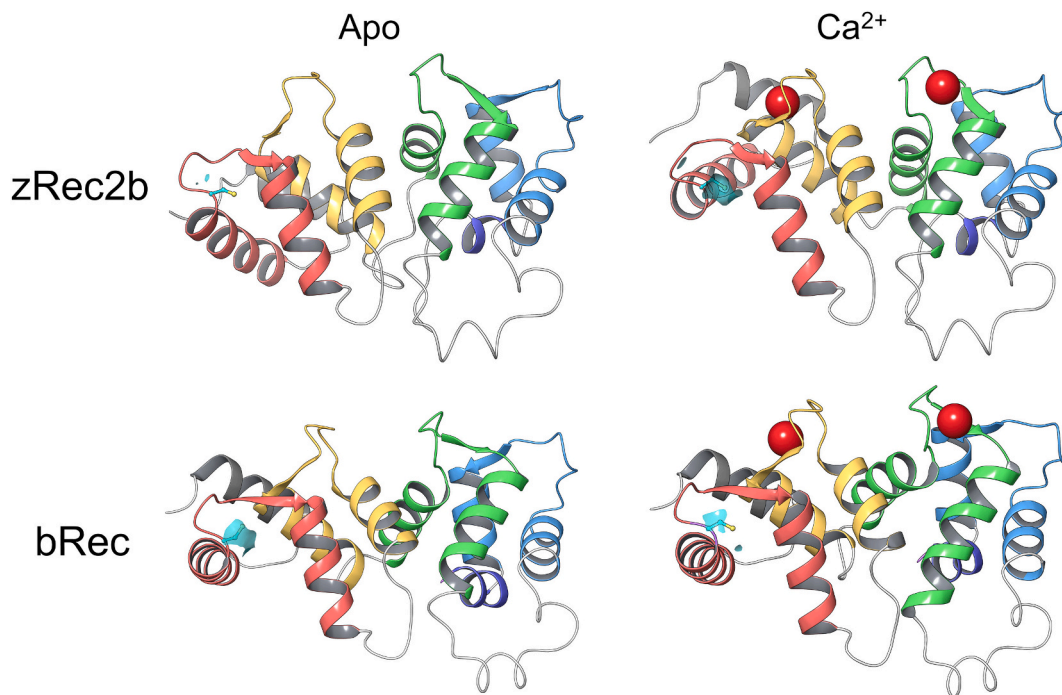


Fig. 9. Solvent accessibility of Cys residues of non-myristoylated zRec2b and bRec in the Ca^{2+} -free and Ca^{2+} -bound states.

Structural details of Cys39 of non-myristoylated zRec2b (top panels) and bRec (bottom panels) in their apo (left) and Ca^{2+} -bound (right) form. The three-dimensional structure of zRec2b is shown in cartoon with EF1 colored in red, EF2 in yellow, EF3 in green, EF4 in azure and the C-terminal helix in purple. The myristoyl group is shown as grey sticks with O atoms in red, Cys residues are represented by cyan sticks with C atoms in yellow, Ca^{2+} -ions are depicted as red spheres. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

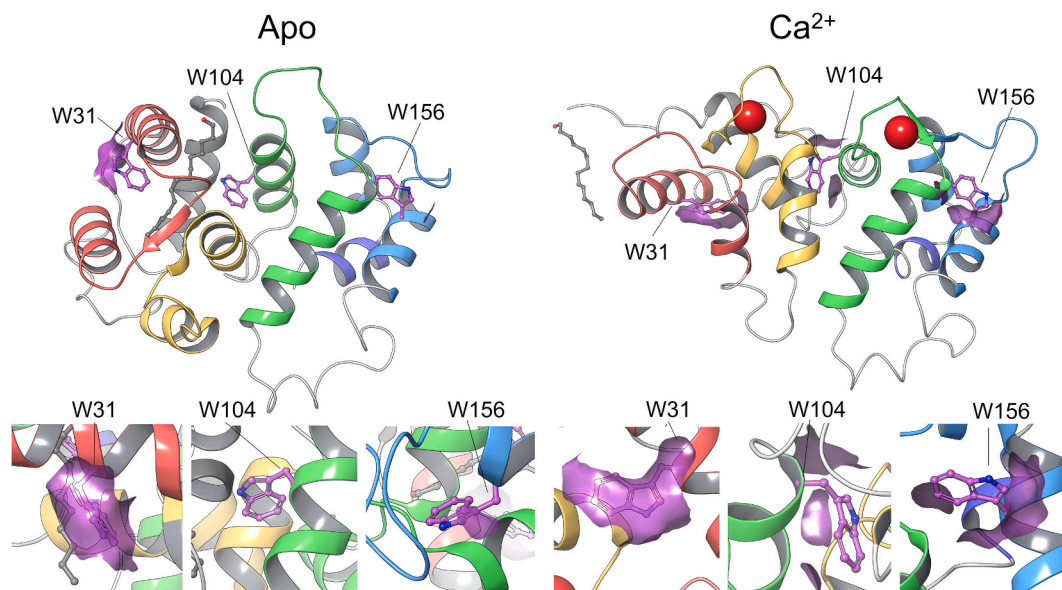


Fig. 10. Solvent accessibility of Trp residues of myristoylated zRec2a highlighting Trp residues.

Structural details of Trp residues of myristoylated zRec2a in its apo (left) and Ca^{2+} -bound (right) form. The three-dimensional structure of zRec2a is shown in cartoon with EF1 colored in red, EF2 in yellow, EF3 in green, EF4 in azure and the C-terminal helix in purple. The myristoyl group is shown as grey sticks with O atoms in red, Trp residues are represented by violet sticks with N atoms in blue, Ca^{2+} -ions are depicted as red spheres, the solvent-accessible surface of Trp residues is colored purple and shown in transparency. Insets below show an alternative magnified view of Trp residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

A further exception was found in the Ca^{2+} -titration for myristoylated zRec2a, which resulted in an atypical biphasic shift of the Trp fluorescence emission (Fig. 6A and B). Such a profile has been observed for GCAPs [43,44] indicating involvement of different Trp residues, but has not been seen for recoverin orthologs so far. The first phase starts in

myristoylated zRec2a with the highest overall intensity value at low $[\text{Ca}^{2+}]$ (2 mM EGTA). When free $[\text{Ca}^{2+}]$ was raised, the intensity value decreased until the minimum was reached at $10 \mu\text{M} \text{Ca}^{2+}$. The estimated $K_{1/2}$ for this phase is $3.8 \mu\text{M}$. The second phase ranged from $10 \mu\text{M}$ to $100 \mu\text{M}$ where the Trp emission intensity reached saturation. The $K_{1/2}$ of

Table 2Average relative solvent-accessible surface (rSAS) of aromatic residues of Rec isoforms. Δ rSAS is calculated as $(\text{rSAS}^{\text{Ca}^{2+}} - \text{rSAS}^{\text{apo}}) / \text{rSAS}^{\text{apo}}$.

Isoform	Residues	Myristoylated			Non-myristoylated		
		Apo	Ca^{2+}	Δ rSAS	Apo	Ca^{2+}	Δ rSAS
zRec1a	PHE	13.8 ± 12.4	14.9 ± 13.8	8.0	7.9 ± 8.7	10.8 ± 10.6	36.7
	TYR	15.3 ± 13.8	16.8 ± 13.4	9.8	6.7 ± 7.7	12.1 ± 7.1	80.6
	TRP	10.7 ± 12.1	16.2 ± 13.2	51.4	8.6 ± 6.7	11.2 ± 8.8	30.2
	ALL	13.8 ± 12.6	15.7 ± 13.3	13.8	7.6 ± 6.1	11.3 ± 9.3	48.7
zRec2a	PHE	27.0 ± 10.7	27.0 ± 14.5	0	10.3 ± 9.7	11.7 ± 10.9	13.6
	TYR	24.7 ± 9.9	26.6 ± 11.0	7.7	18.0 ± 12.1	14.3 ± 11.6	-20.5
	TRP	14.8 ± 10.1	26.3 ± 7.2	77.7	16.6 ± 13.8	14.5 ± 12.2	-12.6
	ALL	24.9 ± 11.1	26.8 ± 13.0	7.6	12.9 ± 10.6	12.7 ± 11.7	-1.5
zRec2b	PHE	26.2 ± 10.9	25.8 ± 14.5	-1.5	10.0 ± 11.5	11.5 ± 12.3	15.0
	TYR	29.9 ± 10.7	27.1 ± 12.6	-9.4	14.0 ± 10.5	16.4 ± 10.7	17.1
	TRP	17.6 ± 11.0	27.9 ± 7.1	58.5	16.2 ± 13.8	16.1 ± 13.0	-0.6
	ALL	26.1 ± 11.4	26.4 ± 13.2	1.1	11.9 ± 12.6	13.4 ± 13.6	12.6

Table 3Calcium-dependent shifts in intrinsic fluorescence intensity (ΔF) and of the wavelength at maximal fluorescence emission ($\Delta\lambda$) of recoverin isoforms. ΔF is calculated as $F_{\text{highCa}^{2+}} - F_{\text{lowCa}^{2+}}$ and $\Delta\lambda$ as $\lambda_{\text{highCa}^{2+}} - \lambda_{\text{lowCa}^{2+}}$.

Isoform		ΔF (relative)	$\Delta\lambda$ (absolute)
zRec1a	myr	0.270 ± 0.003	16.0 ± 0.0 nm
	non-myr	0.182 ± 0.015	0.66 ± 0.9 nm
zRec2a	myr	0.103 ± 0.012	13.3 ± 0.9 nm
	non-myr	0.110 ± 0.009	4.0 ± 0.0 nm
zRec2b	myr	0.218 ± 0.001	4.7 ± 0.9 nm
	non-myr	0.243 ± 0.002	2.7 ± 0.9 nm

phase II was estimated to be 20.5 μM . The maximum of the emission intensity exhibited a red-shift of 13 nm. Plotting the wavelength at maximal fluorescence emission against the free $[\text{Ca}^{2+}]$ led to a $K_1/2 = 10.5 \pm 1 \mu\text{M}$ (Fig. 6C), exactly at the minimum of the biphasic profile in Fig. 6A. Using Trp > Phe mutants Peshenko and Dizhoor [44] observed that phase II depends on Trp at position 94 in GCAP1 and concluded that the Ca^{2+} -specific increase in fluorescence intensity indicates Trp moving into a less polar environment. Trp94 in bovine GCAP1 aligns with Trp104 in recoverin orthologs, which might account for a similar Ca^{2+} -specific increase in fluorescence emission in zRec paralogs. The fluorescence representing phase I in GCAP1 is weakened by mutation in Trp21, which would align with Trp31 in zRec forms. The phase I response that we specifically observed for zRec2a could point to a movement of Trp31 to a more polar environment at $[\text{Ca}^{2+}]$ below 10 μM and distinguishes zRec2a from other zRec paralogs. Further, the significant red-shift of 13 nm also pointed to a more prototypical Ca^{2+} -myristoyl switch for this zebrafish paralog than in other zRec forms in agreement with Elbers et al. [18] and our structural prediction (Fig. 10).

The experimental results seemed in contrast to the average rSAS values determined in the structural modelling: among the myristoylated isoforms, zRec2a displayed the largest Δ rSAS between apo and calcium-loaded form and zRec1a the lowest (Table 2). However, the solvent accessibility is not the only determinant for Trp fluorescence and as it is only the average of all three Trp, it does not account for individual changes in the Trp microenvironment. A complicating factor is that excitation at 280 nm is not exclusive to Trp: Tyr residues are also excited and emit fluorescence over a comparable wavelength range. Since the average rSAS of Tyr in the modelled zRec structures was similarly affected by Ca^{2+} binding (Table 2), the interpretation of the experimental fluorescence data becomes less straightforward.

4. Conclusion

Despite their high sequence identity, zebrafish recoverins display subtle structural differences which are indicated by the differences in cysteine accessibilities depending on presence and absence of GRK1 and a lipid bilayer that was mimicked by bicelles. Even more remarkably,

they exhibited considerable differences in their intrinsic fluorescence that can be attributed to three highly conserved tryptophan residues and their individual local environment. The ability of isoforms zRec1a and zRec2a to undergo a calcium-myristoyl switch was reflected by the pronounced red-shifts of their spectra but the underlying mechanisms of the myristoyl switch are likely to differ and remain to be elucidated. Previous studies indicated partly overlapping functions and structural plasticity of recoverin isoforms [18], which is reflected by the similar basic features of calcium-dependent Trp fluorescence changes and accessibilities of the conserved cysteine 39. The observed differences, especially in presence of GRK1 and bicelles, indicate distinct calcium-driven conformational changes, which are possibly related to the individual target regulation and subcellular localization of the isoforms.

CRedit authorship contribution statement

Luisa Hintze: Methodology, Investigation, Formal analysis, Conceptualization. **Valerio Marino:** Methodology, Investigation, Formal analysis. **Daniele Dell'Orco:** Methodology, Formal analysis. **Karl-Wilhelm Koch:** Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbapap.2026.141190>.

Data availability

The data referred to in this manuscript are present in the manuscript and in the supporting information. Unprocessed data are deposited on a server of the University of Oldenburg in accordance with the data policy of the University of Oldenburg and can be made available on request from the corresponding author.

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