Retina specific GCAPs in zebrafish acquire functional selectivity in \( \text{Ca}^{2+} \)-sensing by myristoylation and \( \text{Mg}^{2+} \)-binding

Stefan Sulmann*, Farina Vocke*, Alexander Scholten & Karl-Wilhelm Koch

Zebrafish photoreceptor cells express six guanylate cyclase-activating proteins (zGCAPs) that share a high degree of amino acid sequence homology, but differ in \( \text{Ca}^{2+} \)-binding properties, \( \text{Ca}^{2+} \)-sensitive target regulation and spatial-temporal expression profiles. We here study a general problem in cellular \( \text{Ca}^{2+} \)-sensing, namely how similar \( \text{Ca}^{2+} \)-binding proteins achieve functional selectivity to control finely adjusted cellular responses. We investigated two parameters of critical importance for the trigger and switch function of guanylate cyclase-activating proteins: the myristoylation status and the occupation of \( \text{Ca}^{2+} \)-binding sites with \( \text{Mg}^{2+} \). All zGCAPs can be myristoylated in living cells using click chemistry. Myristoylation does not facilitate membrane binding of zGCAPs, but it significantly modified the regulatory properties of zGCAP2 and zGCAP5. We further determined for all zGCAPs at least two binding sites exhibiting high affinities for \( \text{Ca}^{2+} \) with \( K_D \) values in the submicromolar range, whereas for other zGCAPs (except zGCAP3) the affinity of the third binding site was in the micromolar range. \( \text{Mg}^{2+} \) either occupied the low affinity \( \text{Ca}^{2+} \)-binding site or it shifted the affinities for \( \text{Ca}^{2+} \)-binding. Hydrodynamic properties of zGCAPs are more influenced by \( \text{Ca}^{2+} \) than by \( \text{Mg}^{2+} \), although to a different extent for each zGCAP. Posttranslational modification and competing ion-binding can tailor the properties of similar \( \text{Ca}^{2+} \)-sensors.

Calcium sensor proteins mediate signaling processes that respond to changing concentrations of \( \text{Ca}^{2+} \)-ions. The binding of \( \text{Ca}^{2+} \) to intracellular calcium sensor proteins can trigger conformational transitions, which constitute a crucial step to regulate further downstream signaling proteins. One family of \( \text{Ca}^{2+} \)-binding proteins named neuronal calcium sensor (NCS) proteins are predominantly expressed in neuronal tissue and are involved in diverse intracellular processes. All NCS proteins harbor four EF-hand \( \text{Ca}^{2+} \)-binding motifs, of which in most cases three (sometimes only two) motifs can bind micromolar to submicromolar \( \text{Ca}^{2+} \). One group of the NCS proteins is expressed in sensory cells and among them the guanylate cyclase-activating proteins (GCAPs) perform an important function in controlling the membrane bound guanylate cyclases (GCs) in retinal rod and cone cells.

In their \( \text{Ca}^{2+} \)-free, \( \text{Mg}^{2+} \)-bound form GCAPs activate GCs, but they switch to an inhibitory mode, when all \( \text{Ca}^{2+} \)-binding sites are filled with \( \text{Ca}^{2+} \). Changing levels of cytoplasmic \( \text{Ca}^{2+} \) in rod and cone outer segments are linked to changing levels of the intracellular messenger cGMP. After light activation of the photoreceptor cell the intracellular cGMP level is depleted, leading to a shutdown of cyclic nucleotide gated (CNG) channels in the outer segment of the cell. This stops the influx of \( \text{Ca}^{2+} \), which is however still extruded by the continuous operation of a \( \text{Na}^+ / \text{Ca}^{2+}, \text{K}^+ \) exchanger leading to a net decrease of cytoplasmic \( \text{Ca}^{2+} \). This decrease is sensed by GCAPs which in turn increase the GC activity,
leading to re-opening of the CNG-channels and is a necessary step for the recovery of the photoreceptor to the dark-adapted state 4–9.

Bovine and mice photoreceptor cells express two GCAP forms, GCAP1 and GCAP2, which bind to distant regions in the target GC and have different properties with respect to Ca\(^{2+}\)-sensitivity, impact on catalytic efficiency of the target GC and different structural implications of the N-terminally attached myristoyl group 10,11. Both GCAPs activate outer segment GCs in a Ca\(^{2+}\)-relay mode fashion, where GCAP1 is activated at higher free Ca\(^{2+}\), followed by GCAP2, which becomes active, when Ca\(^{2+}\)-levels have fallen to lower levels 9–11. This Ca\(^{2+}\)-relay system seems also to work in zebrafish rod and cone cells, where, however, the system is more complex due to the larger number of GCAP forms 12. Zebrafish photoreceptor cells express a total of six GCAPs (zGCAP1, 2, 3, 4, 5 and 7) 13,14 that differ in Ca\(^{2+}\)-binding properties, Ca\(^{2+}\)-sensitive GC regulation and spatial-temporal transcription/ expression profiles. Four zGCAPs, namely isoforms 3, 4, 5 and 7 are cone specific 12,14,15.

Two parameters are of critical importance for the trigger and switch function of NCS proteins in general and GCAPs in particular: the myristoylation status and the occupation of EF-hand Ca\(^{2+}\)-binding sites with Mg\(^{2+}\) 7,8,10,16,17. We have previously shown that zGCAP3 and 4 are myristoylated, when co-expressed with yeast N-myristoyltransferase (NMT) in E.coli 15,18. Furthermore, zebrafish NMT is expressed in the developing larval retina at 5 days post fertilization (dpf) and is active as shown by modification of zGCAP3 after 7 dpf. In the present study we investigated in a comparative manner, whether all zGCAPs can be myristoylated in living cells using an approach of in vivo and in vitro click chemistry in combination with fluorescence microscopy. Revealing that all zGCAPs can exist in a myristoylated and non-myristoylated form we investigated its impact for target regulation and Ca\(^{2+}\)-dependent membrane interaction. We further asked whether the presence of physiological Mg\(^{2+}\) can influence the binding of Ca\(^{2+}\) to zGCAPs and how Ca\(^{2+}\)-induced conformational transitions in zGCAPs are influenced.

Our results indicate that myristoylation has a strong impact on the regulatory properties of two zGCAPs (2 and 5), but it does not facilitate Ca\(^{2+}\)-dependent membrane binding for all zGCAPs. Further Mg\(^{2+}\) ions control the Ca\(^{2+}\)-affinity as well as the Ca\(^{2+}\)-induced conformational changes in zGCAPs.

Results

Acylation of zGCAPs in living cells. Green-fluorescent protein (GFP) constructs of NCS proteins in general and GCAPs in particular: the myristoylation status and the occupation of EF-hand Ca\(^{2+}\)-binding sites with Mg\(^{2+}\) 7,8,10,16,17. We have previously shown that zGCAP3 and 4 are myristoylated, when co-expressed with yeast N-myristoyltransferase (NMT) in E.coli 15,18. Furthermore, zebrafish NMT is expressed in the developing larval retina at 5 days post fertilization (dpf) and is active as shown by modification of zGCAP3 after 7 dpf. In the present study we investigated in a comparative manner, whether all zGCAPs can be myristoylated in living cells using an approach of in vivo and in vitro click chemistry in combination with fluorescence microscopy. Revealing that all zGCAPs can exist in a myristoylated and non-myristoylated form we investigated its impact for target regulation and Ca\(^{2+}\)-dependent membrane interaction. We further asked whether the presence of physiological Mg\(^{2+}\) can influence the binding of Ca\(^{2+}\) to zGCAPs and how Ca\(^{2+}\)-induced conformational transitions in zGCAPs are influenced.

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for zGCAP5 and zGCAP7. Despite differences in protein loading, these results indicated also different expression levels after transfection. The band above 75 kDa visible in all lanes except in that of biotinylated recoverin resulted from a labeled protein that was already modified in Mock transfected control cells (Fig. 1, note that the corresponding band in bGCAP2-GFP is only partially visible). Purified recombinant wildtype recoverin without a fusion part was biotinylated via covalent linkage to a primary amine and served as a positive control. It was intensely labeled (Fig. 1). These results showed that all zGCAPs (as well as bovine GCAP2 and recoverin) were posttranslationally acylated in living cells.

In a second alternative approach we employed a copperless cycloaddition suitable for introducing a fluorescent dye (DIBO-TAMRA-dye) to the fatty acyl group of the NCS proteins in living cells. Thus we were able to colocalize the putative N-terminal attached fatty acyl chain and the C-terminal attached GFP to the protein in transfected cells. Figure 2 gives an overview of the results obtained with fluorescence microscopy for recoverin, zGCAP3 and 5. GFP and TAMRA fluorescence mainly overlapped for cytoplasmic regions indicating the presence of a covalently attached acylgroup on fluorescently labeled zGCAP3 and 5 (Fig. 2a–h). A similar localization pattern was observed with the other zGCAPs (data not shown). Cells expressing GFP-labeled zGCAP3 and recoverin (Fig. 2q–x) that were not modified by the azido-modified myristic acid substitute appear normal in shape comparable to previous results obtained with mammalian GFP-labelled GCAPs18,20. However, we observed no uniform spreading to the nucleus. Further, when we added the myristic acid substitute allowing the subsequent cycloaddition with the DIBO-TAMRA dye cell shape was affected leading to the round form visible in Fig. 2a–l (see also Figure S1 in supplement). Myristoylated recoverin was found in the vicinity to membranes, but it was also detected in restricted cytosolic regions (Fig. 2j–l and r–t). This might indicate partial association of recoverin with membrane structures at low Ca2+-concentration, which had been observed in previous studies and is mainly due to hydrophobic/electrostatic interactions21,23.

Do zGCAPs perform a Ca2+-myristoyl switch? Our results showed that zGCAPs are modified by acylation (in the experiments above by a pseudo-myristoylation), which was tested in living and in disrupted cells. This prompted us to ask, whether zGCAPs can perform a Ca2+-myristoyl switch, which is typically observed in other NCS proteins like recoverin, neurocalcin δ, VILIP or hippocalcin24–27. In order to obtain a quantitative assessment on this topic we performed an equilibrium centrifugation assay. For this purpose we co-expressed zGCAPs and recoverin with yeast NMT in E.coli and purified them afterwards. Principal attachment of the myristoyl group was verified by a click chemistry reaction involving azido-dodecanoic acid and the alkyne derivative of biotin as described above. We then incubated myristoylated zGCAPs and recoverin with isolated photoreceptor outer segment membranes in the absence and presence of Ca2+ (Fig. 3). No zGCAP form showed a significant difference in binding to membranes under these conditions. Thus, no zGCAP performed a Ca2+-myristoyl switch thereby confirming our previous results that we reported for zGCAP3 and 4 resembling those obtained earlier with mammalian GCAP1 and GCAP228,29. Recoverin as the prototype of a Ca2+-myristoyl switching NCS protein served as positive control (Fig. 3).

Target regulation by zGCAPs. Nonmyristoylated zGCAPs exhibit different activity profiles when targeting membrane bound GCs12. We compared previously published data on nonmyristoylated zGCAPs with those obtained with myristoylated zGCAPs in the present study. A characteristic parameter for activity regulation is the [Ca2+] at which the GC activity in the presence of a GCAP molecule is halfmaximal denoted as IC50 value and listed in Table 1. Interestingly, large differences (> 2-fold) were only visible for zGCAP2 and zGCAP5. With a myristoyl group attached the Ca2+-sensitivity decreased about 4.7-fold for zGCAP2 and increased 5.7-fold for zGCAP5.

Ca2+-binding to myristoylated zGCAPs. All zGCAPs contain four EF-hand motifs in their primary structure, where the first one is suggested to bind no Ca2+ under physiological conditions as it was observed for mammalian GCAP1 and 2. So far no precise values of Ca2+-binding to myristoylated zGCAPs are available and furthermore it is not known, whether Ca2+-binding is affected by physiological concentrations of Mg2+. We used a calorimetric approach (ITC) to analyze the energetics of Ca2+-binding to zGCAPs in the presence and absence of 1 mM Mg2+, which allows us to determine precise values of apparent dissociation constants (Kd) for multiple binding sites and the associated changes in binding enthalpy (ΔH). For each titration Ca2+-free zGCAP was kept in a temperature controlled compartment, in which a series of small volumes of CaCl2 was injected. Representative examples for all zGCAPs are shown in Figs 4,5. Heat pulses were in almost all cases exothermic with a maximum heat release between −14 kcal per mol of CaCl2 (zGCAP3) and −17 kcal per mol of CaCl2 (zGCAP7). Small endothermic responses were only observed with zGCAP1 (Figs 4a,5a). However, despite numerous repetitions we could not get reproducible results for zGCAP1. Only once we detected a response pattern as seen in Fig. 4a. In the presence of Mg2+ only two titrations out of six were successful (Fig. 5a). We interpret these findings with the tendency of some NCS proteins to form higher order oligomers, a phenomenon we have previously observed30,31. Data could be fit by a sequential three site binding model yielding three distinct Kd values except for zGCAP7, where only a two site model was applicable (Table 2). In all zGCAPs at least two binding sites exhibited high affinities for Ca2+ with Kd values in the submicromolar range.
Figure 2. Acylation of zGCAPs in living cells by double fluorescence detection. HEK 293 cells were transfected with GFP constructs of zGCAPs and recoverin, which were acylated in vivo and labeled with the DIBO-TAMRA dye. Nuclear DAPI staining is displayed in left most column (a,e,i,m,q,u), GFP fluorescence in second left column (b,f,j,n,r,v), DIBO-TAMRA fluorescence in the third column (c,g,k,o) and an overlay of all signals in fourth column (d,h,l,p,t,x). GFP and DIBO-TAMRA fluorescence mainly overlaps for cytoplasmic regions. Pictures in panels s and w (column three) are bright field images of HEK cells that were not treated with the azido myristic acid substitute. Mock transfected cells are shown as controls. Figures were taken by using the LUCP PlanFi 40 x/0.60 olympus objective and the DAPI/Fitc/TexasRed Filter Set (Olympus). Scale bars: 20 μm.
(K_{D1} and K_{D2} in Table 2), for zGCAP3 also the third site (K_{D3}) showed high affinity for Ca^{2+}, whereas for other zGCAPs the affinity of K_{D3} was in the micromolar range.

In the presence of 1 mM Mg^{2+} the multiphasic binding isotherm for Ca^{2+}-binding gave a best fit with a two site model for zGCAP1, 2, and 3. This result can be best interpreted as having two high affinity sites filled with Ca^{2+}, but the lower affinity site being occupied by Mg^{2+} (Fig. 5 and Table 2). However, zGCAP5 and 7 showed a somewhat different behavior: the binding isotherm of zGCAP5 still obeyed to a sequential three site model, but all three K_{D} values were shifted to lower affinity (Table 2). In contrast, zGCAP7 displayed almost equal values for K_{D1} and K_{D2} indicating no binding of Mg^{2+} to either of these binding sites.

Surprisingly, we were unable to record any response with zGCAP4 in the presence of Mg^{2+} (Fig. 5d), although reliable binding isothemers were recorded without Mg^{2+} (Fig. 4d and Table 2).

Size exclusion chromatography of myristoylated zGCAPs revealed that the monomeric form was the dominant species for all zGCAPs except for zGCAP5 and 7. A Ca^{2+}-dependent shift in the monomer-dimer equilibrium was only observed for zGCAP2, but the monomeric zGCAP was with approx. 80% the dominant form. High molecular mass oligomers were not detected except occasionally for zGCAP7 and to a lower extent for zGCAP5 (data not shown).

Ca^{2+}-induced conformational changes of myristoylated zGCAPs. The lack of a Ca^{2+}-myristoyl switch operation in zGCAPs (Fig. 3) led us suggest that Ca^{2+}-induced conformational changes in zGCAPs might be less pronounced than in recoverin that undergoes a large rearrangement of its three-dimensional fold during the exposure of its myristoyl chain. We recently developed a technique to detect subtle changes in protein conformation during conformational transitions of Ca^{2+}-sensors by applying SPR spectroscopy. The technique allowed us to correlate even small changes in conformation with both a rearrangement of the protein hydration shell and protein hydrodynamic properties. Thus, we applied
this technique for the detection of conformational transitions in zGCAPs in the absence and presence of Mg\(^{2+}\). For this purpose zGCAPs were immobilized at high density on a dextran-coated sensor chip surface and pulses of CaCl\(_2\) were injected into the flow cell system of the SPR device resulting in a pattern of increasing amplitudes, when the [Ca\(^{2+}\)] is increased (Fig. 6). Evaluation of the titration revealed a K\(_{1/2}\)SPR in the micromolar range (Table 3) as previously determined and discussed for mammalian GCAPs, recoverin and other Ca\(^{2+}\)-sensors\(^{30-32}\). Performing the same titration series in the presence of 1 mM Mg\(^{2+}\) (Fig. 6, open circles) shifted the K\(_{1/2}\)SPR to higher values, in particular for zGCAP2 (Table 3). However, we lack data for zGCAP3, since flushing a zGCAP3-coated surface with Mg\(^{2+}\)-containing buffer diminished any response during titration. Apparently, Mg\(^{2+}\) increased the instability of zGCAP3 on the chip surface.

Finally, the maximal amplitudes that were reached at the end of the Ca\(^{2+}\)-titration (in the absence of Mg\(^{2+}\)) differed significantly among the proteins exhibiting the following order: zGCAP5 > zGCAP2 > z

Figure 4. Ca\(^{2+}\)-binding to myristoylated zGCAP-isoforms. Representative ITC measurements with 10.2 μM zGCAP1 (a), 12.8 μM zGCAP2 (b), 18.7 μM zGCAP3 (c), 10.2 μM zGCAP4 (d), 19.5 μM zGCAP5 (e), 18.5 μM zGCAP7 (f). The upper part shows the heat pulse for every injection, the lower part shows the corresponding molar enthalpy change. Data analysis by curve fitting to three or two Ca\(^{2+}\) binding sites yielded dissociations constants (K\(_{D}\)) and and enthalpy changes (ΔH) given in Table 2.
GCAP7 > zGCAP3 = zGCAP4. No signals were observed for zGCAP1, although the purified protein was functional shown by the GC activation assay. We conclude from these results that all zGCAPs undergo Ca\(^{2+}\)-induced conformational changes, which however have different consequences for the protein hydrodynamic properties indicating differences in the extent of conformational transitions.

**Discussion**

Calcium sensor proteins like the group of NCS proteins are involved in unique patterns of cellular regulatory pathways and therefore mediate various physiological responses including ion channel function, enzyme activity control and cellular trafficking\(^{5,3}\). NCS proteins are able to specifically recognize their targets despite the fact that they share a high degree of amino acid sequence homology and that the overall three-dimensional folding is nearly identical in the core regions (e.g. sequential order of EF-hand
work has shown 1) that all zGCAPs are expressed in photoreceptor cells in the larval and adult stages. In order to work on these issues we chose to compare zGCAPs representing one subfamily of NCS proteins. These NCS proteins are well suited for a comparative analysis, since previous

indicating competition with Ca\textsuperscript{2+} in this NCS protein group was zGCAP7: fitting ITC data to a two site-binding model was sufficient for

\[ \text{Ca}^{2+} \]

sensing properties of myristoylated zGCAPs were broadly consistent with the primary sequences that contain three predicted functional EF-hand Ca\textsuperscript{2+}-binding sites as described above and presented in Table 2. Further, ITC data showed that Mg\textsuperscript{2+} can bind to zGCAP1, 2 and 3 by occupying one EF-hand and leaving the other two for binding Ca\textsuperscript{2+}. Indirect evidence of Mg\textsuperscript{2+}-binding was obtained for zGCAP5, in which the low affinity of one Ca\textsuperscript{2+}-binding site decreased by the presence of Mg\textsuperscript{2+} (Table 2) indicating competition with Ca\textsuperscript{2+}. ITC measurements with zGCAP4 were only possible in the absence of Mg\textsuperscript{2+} showing also small endothermic responses, in the absence of Mg\textsuperscript{2+} only one set of data was reliable (see main text).

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Figure 6. SPR responses of myristoylated zGCAP isoforms at increasing free \([\text{Ca}^{2+}]\). Examples of original SPR recordings are shown in the left panels, evaluation of data without Mg\(^{2+}\) (●) and with 1 mM Mg\(^{2+}\) (O) are shown on the right panels. Data fitting was obtained by a sigmoidal Hill curve resulting in \(K_{1/2}\) values listed in Table 3.
known to provide an invariant oxygen ligand for coordinating Ca$^{2+}$ or Mg$^{2+}$. Val is present in zGCAP7 at −Y (EF-hand 2) and it does also not belong to the amino acids that are frequently observed in EF-hands in this position. The same position −Y is also peculiar in EF-hand 3 that contains a Lys (common) in zGCAP3, 4 and 7, but again an uncommon Cys in zGCAP1, 2 and 5. These amino acid substitutions in EF-hands 2 and 3 could therefore be the molecular determinants for tuning the different Ca$^{2+}$ affinities. Ca$^{2+}$-induced conformational changes in zGCAPs are thought to trigger target activation and are therefore key control steps in Ca$^{2+}$-mediated feedback loops. Probing these changes by a recently established surface plasmon resonance approach (Fig. 6 and Table 3) we observed distinct changes in the hydrodynamic properties of zGCAPs triggered by Ca$^{2+}$. The $K_{1/2}^{SPR}$ value that is estimated from these titrations is an empirical parameter, which describes a concerted action of a binding step involving a conformational switch. Therefore, the apparent dissociation constants listed in Table 2 are a different set of parameters, which are not identical to the $K_{1/2}^{SPR}$ values. Instead, the $K_{1/2}^{SPR}$ values might reflect a major structural reorganization in zGCAPs as we observed and discussed for mammalian GCAPs and recoverin before. For isoforms zGCAP2, 4, 5 and 7 we determined $K_{1/2}^{SPR}$ values in the lower micromolar range matching the low affinity Ca$^{2+}$-binding site in zGCAP2 and zGCAP5 and to some extent also in zGCAP4.

In contrast, we measured for zGCAP3 and zGCAP7 only three or two high affinity Ca$^{2+}$-binding sites and the meaning of the $K_{1/2}^{SPR}$ values is less obvious for these proteins. At the moment we can only speculate that the rearrangement of the protein hydration shell, which is reflected and measured by our SPR approach, is differentially affected in zGCAP3 and 7 indicating different surface properties. For example, a Ca$^{2+}$-dependent or Ca$^{2+}$-independent monomer-dimer equilibrium like it is observed for mammalian GCAP2 or GCAP1, respectively, could have an impact on the formation of the hydration shell around zGCAP3 or 7. Interestingly, zGCAP7 undergoes a shift in monomer-dimer rearrangement, when [Ca$^{2+}$] is changed, an observation that might also account for other peculiarities of zGCAP7 (e.g. two Ca$^{2+}$-binding sites instead of three, no apparent effect of Mg$^{2+}$).

Presence of Mg$^{2+}$ caused less than two-fold shifts in $K_{1/2}^{SPR}$ values (Table 3), but also a distinct decrease in response amplitudes (Fig. 6, right panels). Only the $K_{1/2}^{SPR}$ of zGCAP2 was more affected and shifted by a factor of 2.4. We made similar observations recently on mammalian GCAP1 and on retinal disease related mutants of GCAP1. Collectively, these results indicated that the hydrodynamic properties of zGCAPs are more influenced by Ca$^{2+}$ than by Mg$^{2+}$-binding. Recent molecular dynamics simulations further showed that mammalian GCAP1 in its Mg$^{2+}$-bound forms has a less solvent exposed surface than the Ca$^{2+}$-saturated GCAP1 state. This finding would be consistent with the smaller response amplitudes observed for zGCAPs in the presence of Mg$^{2+}$, which mirror changes of hydrodynamic diameters and of the rearrangement of the water-protein interface.

### Methods

**Cloning of zGCAPs and GFP-zGCAP constructs.** For heterologous expression in *E.coli* the coding sequences of all zGCAP forms were amplified by PCR and ligated into a pET21-vector earlier. In order to obtain N-terminal acylated zGCAPs in *E.coli*, point mutation of zGCAP1, 4 and 5 was necessary to create a consensus sequence for yeast NMT. The cloning of the point mutants zGCAP4-A5S and zGCAP5-D7N were described before. Accordingly, we prepared a zGCAP1-G5S-mutant employing primers 5′-AACATATGGGCAATTCAGGACGCAGC-3′ and 5′-AAGGATTCTTTAAACGCTGCTCTGGTATTG-3′.

ZGCAP-GFP-fusions were obtained by PCR on wildtype sequences adding restriction sites (NheI and XhoI) and a Kozak sequence in front of the start codon and removing the stop codon. Primer sequences are given in the supplement. The amplified PCR products were ligated into the pTurboGFP-N vector (Evrogen) according to standard protocols. Sequences of all zGCAPs were verified by DNA sequencing.

**Culture and transfection of HEK 293 cells.** HEK-293 cells were cultivated in Dulbecco’s modified Eagle’s medium, pH 7.4 (DMEM and GlutaMaxTM-I, Gibco®) including 10% (v/v) fetal bovine serum (Gibco®) and antibiotics/antimycotic (Gibco®) at 37°C and 5% CO₂. Cells were transfected with the

| GCAP-Isoform (myristoylated) | $K_{1/2}^{SPR}$(μM) | $K_{1/2}^{SPR}$ (μM) (± 1 mM Mg$^{2+}$) |
|-----------------------------|---------------------|--------------------------------------|
| zGCAP1                      | N.D.                | N.D.                                 |
| zGCAP2                      | 7.7 ± 0.5           | 18.7 ± 0.3                           |
| zGCAP3                      | 40.3 ± 1.3          | N.D.                                 |
| zGCAP4                      | 16.3 ± 0.6          | 24.1 ± 0.4                           |
| zGCAP5                      | 8.3 ± 0.4           | 13.0 ± 0.4                           |
| zGCAP7                      | 14.7 ± 0.6          | 20.9 ± 0.4                           |

Table 3. Halfmaximal SPR response value $K_{1/2}^{SPR}$ in the presence and in the absence of Mg$^{2+}$.
corresponding plasmid DNA of NCS proteins (GCAPs and recoverin) or their GFP fusion constructs. Transfection was performed by electroporation essentially as described before41. Briefly, the transfection was tested with different DNA concentrations of 5–50μg at a cell density of 9 × 10^6 for obtaining high transfection rates. After electroporation the mixture was spread on a 6 well (2.5 ml DMEM media) or 24 well plate (325μl DMEM media) and incubated for 1 to 2 days at 37°C. Alternative transfection was performed by lipofection using the PolyFect reagent exactly according to the protocol of the manufacturer (Quiagen, Venlo, Netherlands).

For in vitro analysis cells were centrifuged for 5 min at 1000 × g, the pellet was stored at –80°C or immediately used for further experiments.

**Post-translational modification of zGCAPs by fatty acylation detected by copper-free cycloaddition (Click chemistry) in living cells.** Acylation of heterologous expressed zGCAPs was detected in living cells and in lysed cell preparations. In both cases a 12-azido-dodecanoic acid was used as a myristic acid substitute. The labeling in living cells was done in adherent HEK-293 cells. Cover slips were inserted into one well of a 24 well chamber petri dish and coated with 0.1 mg/ml poly-L-lysine solution overnight to improve the adhesion of the HEK cells. After two washing steps with PBS (137 mM NaCl, 2.7 mM KCl, 12 mM phosphate, pH 7.4), the cells were seeded on the cover slips and incubated for 2–3 days. After one day of incubation 12-azido dodecanoic acid (40μM) was added and cells were further incubated. Afterwards cells were washed twice with PBS before adding 1.25μM of the alkynyl-dye reagent DIBO-TAMRA (Invitrogen, Eugene, USA). Cells were washed four times to remove all non-bound dye residues and fixed with 4% formaldehyde (15 min, RT). To remove the fixation solution, two washing steps with PBS, one with TBST (155 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.05% (v/v) Tween-20), one with TBS and a final one with PBS followed before the cover slips were used on a microscopic slide for fluorescence microscopy. Bovine recoverin® served as positive control.

**Detection of acylated zGCAPs in lysed cells using a Cu(I)-catalyzed Huisgen cycloaddition.** About 2.25 × 10^6 HEK cells were used in one transfection charge resulting in 10–30% successful transfection rate expressing zGCAPs with a N-terminal covalently attached 12-azido-dodecanoic acid. Cells were disrupted in 200μl lysis buffer (1% (w/v) SDS, 5U DNAse, mammalian protease inhibitor cocktail from Sigma containing AEBSF (104 mM), Aprotinin (80μM), Bestatin (4 mM), E-64 (1.4 mM), Leupeptin (2 mM), Pepstatin A (1.5 mM), 50 mM Tris-HCl, pH 8.0) and incubated for 30 minutes on ice. The cell suspension was centrifuged (13,000–18,000 × g; 4°C; 5 min) and the resulting supernatant was transferred to a new reaction tube and shock frozen with liquid nitrogen. Samples were stored at −20°C or used immediately.

To label the azido-dodecanoyl group in the NCS proteins we added to the respective lysed cell extract an alkynyl derivative of biotin, which reacts in a Cu (I) catalyzed cycloaddition with the azido group, following the protocol of the Click it® Protein Reaction Buffer Kit (Invitrogen, Eugene, USA). Briefly, we used 60μL of the lysed cell extract and added ten μl 40μM CuSO₄ (final concentration of 2μM). The reaction was allowed to proceed for 20 min in a test tube that rotated end-over-end. Subsequently, the suspension was filled into a concentrating device (3K; Amicon® Ultra; Millipore, Ireland) and centrifuged (15000 × g, 30 min, 18°C). Samples were run on a SDS polyacrylamide gel, blotted and the presence of the biotin-acyl group was tested by incubating the blot with peroxidase-coupled streptavidin for one hour. Acylated protein bands were visualized by the ECL system.

**Fluorescence microscopy.** The microscopic analysis was performed on an Olympus IX81 microscope with a LUCPPlanFl 40*/0.60 Olympus objective. For the detection of the TAMRA labeled azido-dodecanoyl moiety we used the Texas Red filter (excitation 568 nm). The GCAP-GFP constructs were detected with a LUCPPlanFi 40*/0.60 Olympus objective. For the detection of the TAMRA labeled azido-dodecanoyl moiety we used the Texas Red filter (excitation 568 nm). The GCAP-GFP constructs were detected with a LUCPPlanFi 40*/0.60 Olympus objective.

**Protein expression, purification and characterization.** For biophysical characterization of zGCAPs proteins were overexpressed in BL21 E.coli cells as described previously12,15,18. To obtain myristoylated zGCAPs, E.coli cells were cotransformed with the plasmid pBB131 containing a gene for the yeast (S. cerevisiae) NMT. A consensus sequence for yeast NMT is present in zGCAP 2, 3 and 7. For zGCAP1, 4 and 5 we used the point mutations described above.

After cell lysis zGCAP 3 and 4 were isolated from the soluble fraction, but zGCAP1, 2, 5 and 7 were extracted from the insoluble fraction by homogenization in 6 M guanidinium hydrochloride and dialytic refolding against Tris-buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mMDTT pH 7.5). After one buffer change insoluble material was removed by centrifugation (100,000 × g for 30 min). The purification was a combination of size exclusion chromatography (SEC) and anion-exchange chromatography (AEC). Prior to chromatography steps the volume of zGCAP solutions was reduced by ammonium sulfate precipitation. Afterwards, zGCAPs were resolved in Tris-buffer and were applied onto the SEC column (Superdex 75,GE Healthcare, Germany) in the presence of either 2 mM EGTA (zGCAP1, 3, 4) or 2 mM CaCl₂ (zGCAP2, 5, 7). Fractions containing zGCAPs were further purified using an AEC column (HiLoad 26/10 Q Sepharose; GE Healthcare, Germany) equilibrated in Tris-buffer with 2 mM EGTA. Chromatography was performed with a gradient of 200–550 mM NaCl in 70 mL. Analytical size
lysine residues. Typical immobilization densities ranged from 3.5 ng to 10.5 ng/mm².

Surface plasmon resonance experiments. SPR experiments, we used exactly the same decalciﬁed buffer conditions as for the ITC experiments, except that Tween20 was added to a ﬁnal concentration of 0.005% (v/v). The Ca²⁺-titration experiments and data analysis were performed as outlined elsewhere and repeated six to eight times, with immobilized zGCAP3 four times. In brief CaCl₂ of the highest grade available was dissolved in the decalciﬁed buffer to a ﬁnal concentration of 46 mM. This stock solution was used to obtain Ca²⁺ samples of 0.4 μM, 0.7 μM, 0.9 μM, 1.1 μM, 1.6 μM, 2.5 μM, 4.8 μM, 14 μM, 37 μM and 46.2 μM. All buffers were ﬁltered (0.22 μm) and degassed for at least 1 h before use. Immobilization of protein samples was achieved by attaching them to the carboxy-methyl dextran matrix of CM5 sensorchips (GE Healthcare) via the terminal amino group or via internal accessible

Isothermal titration calorimetry (ITC). ITC experiments with zGCAP-isofoms were performed on a VP-ITC from MicroCal (Northhampton, MA) exactly as described previously for mammalian GCAP1 variants. Brieﬂy, puriﬁed zGCAP-isofoms were present in the recording cell in titration buffer (5 mM Tris/HCl, pH 7.5; 150 mM KCl) at 10–21 μM and were titrated with 3–5 μL of a 1–2 mM CaCl₂ stock solution at T = 25°C. The titration buffer was decalciﬁed using a self-packed gravity ﬂow Chelex 100 column (Bio-Rad). The remaining Ca²⁺ concentration was determined by a BAPTA absorption assay and was found to range between 30 and 100 nM. All buffers were ﬁltered (0.22 μm) and degassed at least 1 h before use. At least three independent replications were made for each titration set, if not stated otherwise.

Reference injections of Ca²⁺ into decalciﬁed buffer without any zGCAP was performed, but did not show signiﬁcant heat changes in the recording cell. Each titration was analyzed by a model implemented in the software Origin (MicroCal) assuming either three or two Ca²⁺ binding sites. The best ﬁtting results out of these models were used to obtain dissociation constants Kᵦ and enthalpy changes (ΔH).

Surface plasmon resonance experiments. For SPR experiments, we used exactly the same decalciﬁed buffer conditions as for the ITC experiments, except that Tween20 was added to a ﬁnal concentration of 0.005% (v/v). The Ca²⁺-titration experiments and data analysis were performed as outlined elsewhere and repeated six to eight times, with immobilized zGCAP3 four times. In brief CaCl₂ of the highest grade available was dissolved in the decalciﬁed buffer to a ﬁnal concentration of 46 mM. This stock solution was used to obtain Ca²⁺ samples of 0.4 μM, 0.7 μM, 0.9 μM, 1.1 μM, 1.6 μM, 2.5 μM, 4.8 μM, 14 μM, 37 μM and 46.2 μM. All buffers were ﬁltered (0.22 μm) and degassed for at least 1 h before use. Immobilization of protein samples was achieved by attaching them to the carboxy-methyl dextran matrix of CM5 sensorchips (GE Healthcare) via the terminal amino group or via internal accessible lysine residues. Typical immobilization densities ranged from 3.5 ng to 10.5 ng/mm².

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**Author Contributions**

S.S., F.V. and A.S. performed experiments and collected data. All authors analysed data. K.W.K. wrote the manuscript. S.S., F.V. and A.S. corrected the manuscript. All authors reviewed the final version of the manuscript.

**Additional Information**

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Erratum: Retina specific GCAPs in zebrafish acquire functional selectivity in Ca\(^{2+}\)-sensing by myristoylation and Mg\(^{2+}\)-binding

Stefan Sulmann, Farina Vocke, Alexander Scholten & Karl-Wilhelm Koch

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In the Supplementary Information file originally published with this Article, the primer sequences for cloning the constructs were omitted. This error has now been corrected in the Supplementary Information that now accompanies the Article.

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