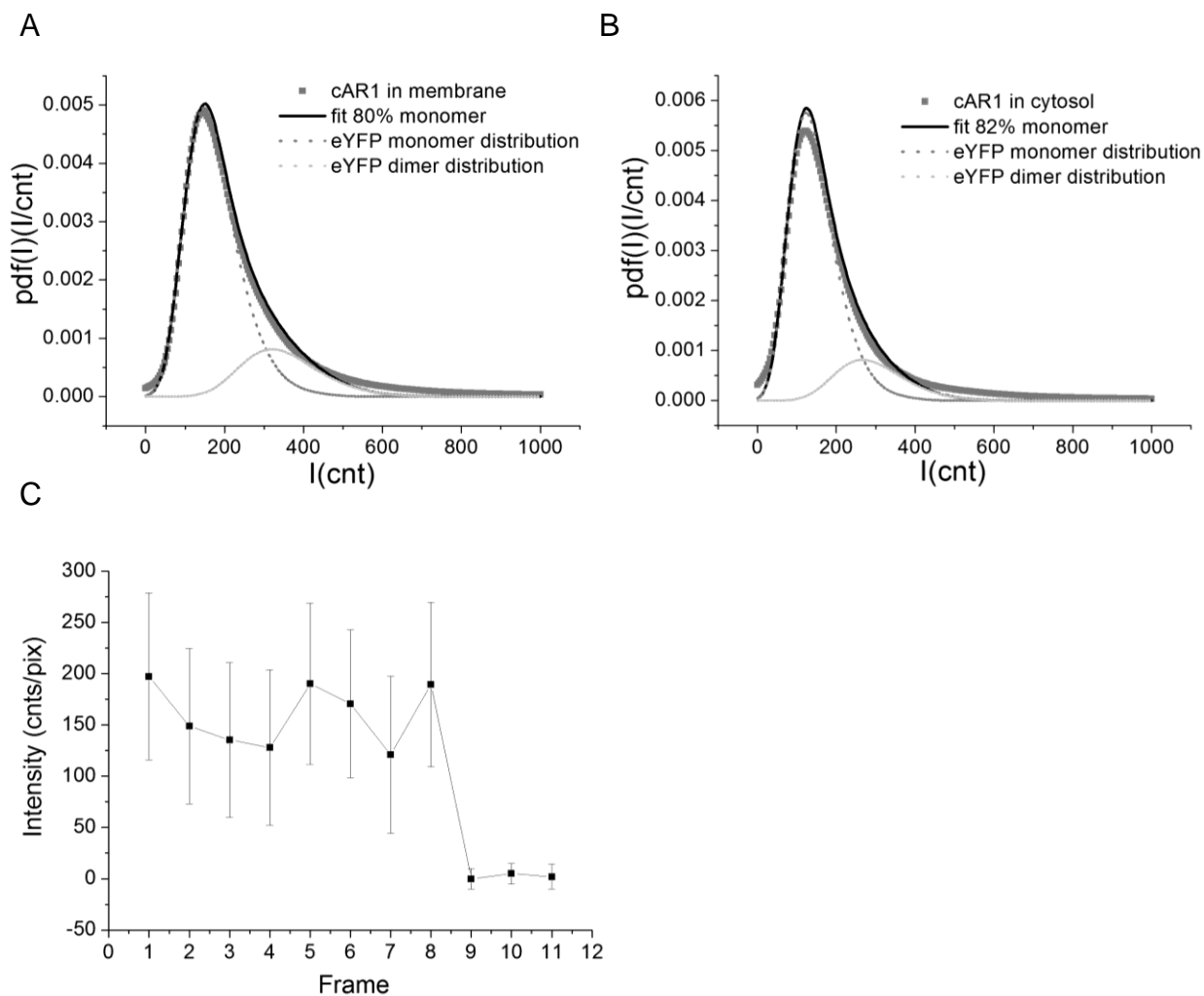
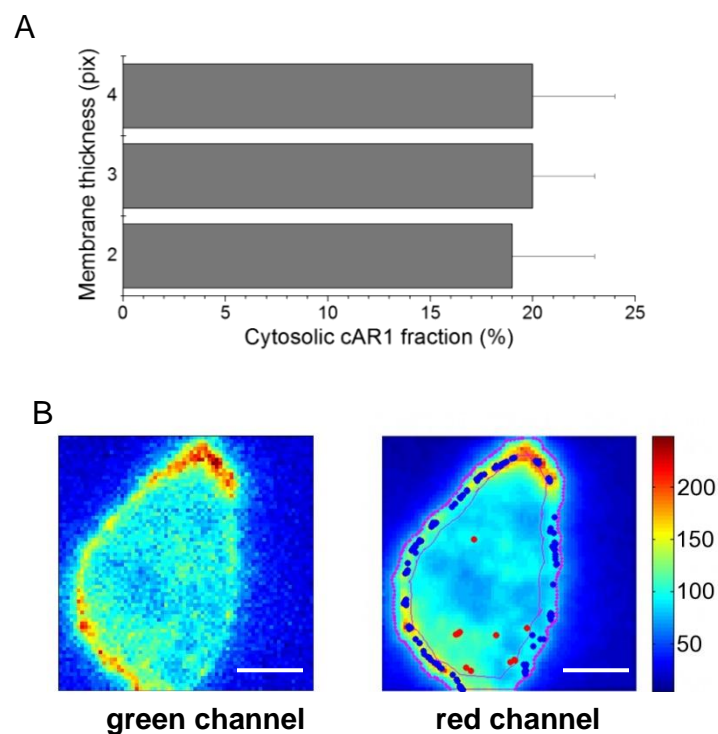


## Supplemental figures

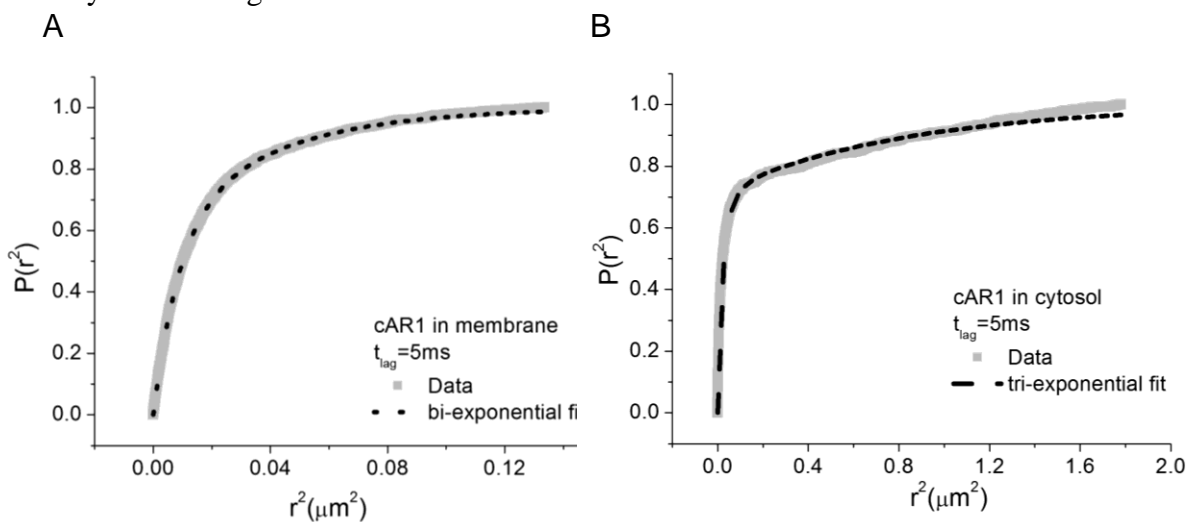
**Figure S1.** Single molecule observation of cAR1 *in vivo*. During data-analysis, typically fluorescent signals which exceed the noise by a factor of 1.5 were further investigated with a non-linear fitting procedure of two-dimensional Gaussian profiles to the original images. Cut-off criteria regarding the intensity, the width, and the relative errors in all fitting parameters were used to validate the signal as an individual emitting eYF molecule. The intensity distribution of the fluorescent signals (>20.000 analyzed) showed that the majority of the signals, localized in either the plasma membrane or the cytosolic compartment resemble the intensity distribution of a single emitting eYFP molecule. Fitting the signal distributions with a weighted sum of the predicted intensity distributions for eYFP-monomers and eYFP-dimers (de Keijzer et al. 2008) showed that 80% and 82% of the signals localized in the plasma membrane (A) and the cytosolic compartment (B) resp. originated from single cAR1-eYFP molecules. Furthermore, the intensity in time of the fluorescent signals fitted in this study, as illustrated with a typical example (C), showed single-step photobleaching, an event that provides evidence for single molecule observation.



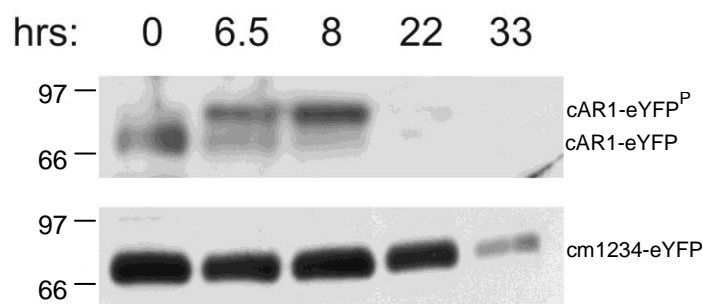
**Figure S2.** The chosen membrane thickness is not affecting the fraction of cytosolic (and membrane) localized receptors significantly. We used an estimated membrane thickness of 3 pixels (660nm) to analyze the localization of cAR1 receptors in the plasma membrane or cytosolic compartment and confirmed this estimation to be correct in two ways. First, we analyzed the localization of cAR1 molecules with different membrane thicknesses (440-880 nm) and showed that this not significantly influences the fraction (A). Secondly, the estimated plasma membrane compartment agreed with concanavalin A-Alexa 647 staining of the plasma membrane (B). The plasma membrane of cAR1-eYFP cells was stained with concanavalin A-Alexa 647 and images were taken in the median plane of the cell after photobleaching. The cAR1-eYFP data shown in the left panel (green channel, one image of the image sequence that was fitted) was analyzed and the peaks were localized in the plasma membrane and cytosolic compartment. The right panel shows the fitted cAR1-eYFP peaks in the plasma membrane and cytosolic compartments on top of the concanavalin A-Alexa 647 (red channel) staining in the same cell. The scale bar is 3  $\mu\text{m}$  long.



**Figure S3.** Dynamics of majority of cytosolic receptors resemble that of plasma membrane localized receptors. Displayed are the cumulative probability distribution of the 1-step displacements ( $t_{lag} = 5ms$ ) of cAR1-eYFP molecules in the plasma membrane compartment (A) and cytosolic compartment (B). Fitting the data of cAR1-eYFP molecules in the plasma membrane with a bi-exponential fit (de Keijzer et al, 2008) showed that 40 % of the molecules had a mean square displacement (MSD) of  $0.04 \mu m^2$  and 60 % of  $0.01 \mu m^2$  ( $t_{lag} = 5ms$ ). The data from the cytosolic compartment was not correctly fitted with a bi-exponential but rather with a model describing three populations, showing that 29% of the molecules had a MSD of  $0.84 \mu m^2$ , 36% had a MSD of  $0.04 \mu m^2$  and 35% and MSD of  $0.01 \mu m^2$ . This analysis showed that the mean square displacements ( $t_{lag} = 5ms$ ) of the majority of cAR1-eYFP molecules in the cytosolic compartment are in the same order of magnitude as the mean square displacements for molecules localized in the plasma membrane indicating that the molecules are not diffusing freely but probably localized on vesicles. For further analysis of the dynamics of receptors in the plasma membrane versus the cytosolic compartment requires the observation of longer trajectories of the molecules. In order to image longer trajectories, a labeling strategy is necessary that allows for longer observation of the cAR1 molecules and 3D detection and analysis of the signals.



**Figure S4.** Expression and phosphorylation of cAR1 and the phosphorylation deficient mutant, cm1234 during development. cAR1 and cm1234 expressed in *car1-/car3-* cells were plated on DB-agar plates ( $2 \times 10^7$  cells/plate) and harvested in SDS-PAGE sample buffer (1 ml/plate) at the indicated times. To assess total receptor amounts and phosphorylation, samples ( $10^6$  cell equivalents/well) were analyzed by SDS-PAGE and immunoblotting using cAR1 antiserum. cAMP stimulation induced an electrophoretic mobility shift characteristic for the wildtype cAR1 receptor protein (upper panel) due to phosphorylation. The cAMP induced shift did not occur for the cm1234 mutant, indicating that this receptor can't be phosphorylated as described before. Furthermore, cm1234 receptor mutant is for a longer period of time expressed during the developmental program than wildtype cAR1.



**Supplemental movie M1.** The localization and the behavior of the receptors was followed on a single cell by recording image stacks at different time points; before ( $t=0$ ) and upon continuous stimulation with  $10 \mu\text{M}$  cAMP ( $t=5$  and  $t=15$  min). *De novo* protein synthesis was inhibited by supplementing the medium with  $90 \mu\text{M}$  cycloheximide 1 h before and during the experiment.