A toolkit for testing membrane localisation tags across species

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ABSTRACT

Transgenic markers and tools have revolutionised how we study cells and developing organisms. Some of the elements needed to construct those tools are universally applicable (e.g. fluorescent proteins), while others are species-specific (e.g. *cis*-regulatory elements driving transcription). Membrane localisation signals targeting proteins to the plasma membrane have been identified in several model organisms. Unfortunately, these do not work equally well in different species, developmental stages and cell types. To generate a toolkit of membrane localisation tags that can be screened rapidly in diverse species, we collected 11 membrane localisation tags –known to localise proteins to the plasma membrane through different mechanisms– and fused them to the red fluorescent protein mScarlet3. Each tagged construct was placed downstream of a T7 promoter, allowing the *in vitro* production of tagged reporter mRNAs, which can be readily delivered to embryos or cell types of interest. The tags in this kit include signals for trafficking to the plasma membrane through the exocytic pathway with a transmembrane domain (SP-CD8tm) or a GPI anchor (SP-GPI), lipid-binding domains (PH, LactC2), signals for prenylation, palmitoylation and myristoylation (H-Ras, K-Ras, K-Ras-6R, GAP43, Lyn, Src64B), and a domain that targets proteins to the plasma membrane independently of exocytosis or lipid modification (RitC). We demonstrate the use of this toolkit in early embryos of the crustacean *Parhyale hawaiensis* and primary polyps of the hydrozoan *Clytia hemisphaerica*.

Keywords: plasma membrane targeting, non-conventional model organisms, evo devo, comparative cell biology

INTRODUCTION

A range of localisation signals have been found that target proteins to specific parts of the cell. These are often peptide sequences that interact with the cells' transport machinery, or direct chemical modifications that enable that targeting (von Heijne 1990; Casey 1995). The targeting peptides can often be fused with heterologous proteins, targeting them to specific cellular locations such as the nucleus, the plasma membrane or mitochondria. This approach has been used to localise a wide variety of marker and effector proteins in different parts of the cell, for live imaging, biochemical labelling, functional studies, gene editing, etc. Many targeting peptides function across eukaryotic species, which has helped to spread cellular markers and tools from established model organisms to new species of interest.

Various signals that target proteins to the plasma membrane have been discovered and applied successfully across species (e.g. Hadjieconomou et al. 2011; Kanca et al. 2013; Guignard et al. 2020; Özpolat et al. 2017; Onken et al. 2006; Greco, Hannus, and Eaton 2001), but there is little consensus on which membrane localisation signals work best. As researchers studying non-conventional model organisms, we have found that several commonly used membrane localization tags do not work sufficiently well in our species of interest.

A common problem is that membrane-tagged proteins do not localise exclusively to the plasma membrane, but also appear in endomembrane compartments, such as the endoplasmic reticulum, the Golgi, or endosomes. This is not surprising, as there is extensive trafficking between the plasma membrane and these compartments. The mechanisms that transport and anchor proteins selectively to the plasma membrane depend on interactions with other proteins and lipids in the plasma membrane. These may be influenced by signalling events, membrane composition and cell-cell contacts (e.g. Halet 2005; Morinaga et al. 2017; Gulyás et al. 2017), which can vary across species, developmental stages and cell types. Thus, there may not be a universal protein tag for targeting the plasma membrane in every species and cell type.

In this context, achieving plasma membrane localisation in new experimental systems requires testing of different tags to identify ones that work. Here, we establish a toolkit of 11 membrane localisation tags, which can be readily tested in new species. These tags have been shown to drive heterologous proteins to the plasma membrane in at least some organisms. The sequences encoding these tags were fused with sequences encoding a red fluorescent protein, mScarlet3 (https://www.fpbase.org/protein/mscarlet3/; Gadella et al. 2023), and placed downstream of a T7 promoter to facilitate the *in vitro* production of mRNA. Once transcribed, these reporter mRNAs can be readily injected in embryos or cell types of interest.

The tags we include in this toolkit employ several different mechanisms to reach the plasma membrane. They include a signal peptides for trafficking to the plasma membrane through the exocytic pathway, tags that direct the covalent attachments of lipids, a domain that targets proteins to the plasma membrane independently of exocytosis or lipid modification, and lipid-binding domains that bind lipids which are predominantly located in the plasma membrane. The tags are summarised in Table 1 and described in more detail below.

RESULTS AND DISCUSSION

Selection of membrane localisation tags and reporter design

To build this toolkit we selected membrane localisation tags by three criteria: first, we selected tags that have been shown to localise heterologous proteins to the plasma membrane in different model

organisms; second, we preferred tags that have been shown to work across different species; and third, we selected tags that employ different of mechanisms localisation and different modes of attachment to the plasma membrane. They are summarised in Table 1 and Figure 1.

One group of tags (constructs **#1 HRas**, **#2 KRas** and **#3 KRas6R**) includes C-terminal peptides that direct prenylation of the targeted proteins, i.e. the covalent attachment of isoprenoid lipids, typically geranyl or farnesyl lipid chains (Wang and Casey 2016). Prenylation tags typically carry a C-terminal CAAX motif and neighbouring sequences that may help to anchor the targeted protein to the plasma membrane. From this category, we include C-terminal peptides from the mammalian H-Ras and K-Ras proteins. The H-Ras tag carries a CAAX prenylation motif and a palmitoylation signal that together direct proteins to the plasma membrane through the exocytic pathway (Apolloni et al. 2000). The H-Ras tag has been used as a membrane tag beyond mammals (e.g. Kanca et al. 2013; Özpolat et al. 2017).

The tag from K-Ras isoform B (including exon 4B) carries a CAAX motif and a 'polybasic' domain, which includes positively-charged lysine residues that are thought to interact electrostatically with the negatively-charged lipids on the cytoplasmic side of the plasma membrane (Hancock et al. 1991; Apolloni et al. 2000; Gulyás et al. 2017). Previous studies have shown that a stretch of 6 lysine residues can be replaced by similarly charged arginine residues; the resulting variant K-Ras-6R also drives robust localisation to the plasma membrane (Hancock et al. 1991). Interestingly, K-Ras and K-Ras-6R appear to interact with different sets of lipids in the plasma membrane (Zhou et al. 2017), so we thought both should be included in this kit.

Besides canonical Ras proteins, a C-terminal fragment of the mammalian Ras-like protein Rit (construct **#4 RitC**) has been shown to target proteins to the plasma membrane in the absence of a CAAX prenylation motif (C.-H. J. Lee et al. 1996). Membrane targeting by RitC has been shown to work in mammalian cells and in fission yeast; it is thought to occur independently of the exocytic pathway and without a post-translational attachment of lipids (Onken et al. 2006).

Another group of tags (constructs **#5 GAP43**, **#6 Lyn** and **#7 Src64B**) includes N-terminal peptides that direct the myristoylation and/or palmitoylation of the targeted proteins, i.e. the covalent attachment myristic or palmitic fatty acids (Resh 1999), which direct targeting to the exocytic pathway and the plasma membrane. C-terminal myristoylation and palmitoylation tags have been used to label plasma membranes in vertebrates, tunicates, annelids and arthropods (Haas and Gilmour 2006; Roure et al. 2007; Lauri et al. 2014; Vopalensky et al. 2019; Pfeiffer et al. 2010; Hadjieconomou et al. 2011; Alwes, Enjolras, and Averof 2016; Gilles et al. 2019).

Two tags (constructs **#8 PH** and **#9 LactC2**) consist of protein lipid-binding domains, which bind lipids that are enriched in the plasma membrane. The N-terminal PH domain of human PLCdelta1 has a strong affinity and specificity for PtdIns(4,5)P2, a phosphoinositide that resides primarily in the plasma membrane (Halet 2005). It has been used to label cell membranes in tunicate embryos (Guignard et al. 2020). The C-terminal domain of bovine Lactahedrin (LactC2) binds specifically phosphatidylserine (PS), a phospholipid that is enriched in the inner leaflet of plasma membranes (Yeung et al. 2008). LactC2 has been used as a sensor of PS in the plasma membranes of mammalian cells, budding and fission yeast (Yeung et al. 2008; Fairn et al. 2011; Kay et al. 2012; Haupt and Minc 2017).

Our toolkit also includes two constructs with canonical signal peptides, expected to reach the plasma membrane through canonical exocytosis, via the endoplasmic reticulum and the Golgi. One of these constructs carries the transmembrane domain of the mammalian CD8 protein, placing the tagged fluorescent on the cytoplasmic side of the plasma membrane (construct **#10 SP-CD8tm**); it

has previously been used as a membrane localisation tag in *Drosophila* (T. Lee and Luo 1999; Hadjieconomou et al. 2011; Stapornwongkul et al. 2020; Rujano et al. 2022). The second construct carries a glycosylphosphatidylinositol (GPI) attachment signal (construct **#11 SP-GPI**); during transit through the endoplasmic reticulum, GPI is covalently attached at this site (Orlean and Menon 2007; Kinoshita 2020). Unlike the other membrane anchoring tags, the GPI anchor tethers the fluorescent protein on the extracellular side of the plasma membrane. The GPI tag we selected has been used previously in *Drosophila* and mammalian cells (Greco, Hannus, and Eaton 2001; Keller et al. 2001).

In addition to these tagged constructs, we generated a negative control in which the mScarlet3 is expressed without a localisation tag (construct **#0 Control**).

Each of these tags was placed at the N- or C-terminus (see Table 1) of the mScarlet3 fluorescent protein, flanked with 5' and 3' UTR sequences and placed downstream of the T7 promoter (see Materials and Methods). The plasmids carrying those constructs were used as templates for *in vitro* transcription, to produce the corresponding mRNAs.

Screening of membrane tags in the crustacean *Parhyale*

We tested the ability of these tags to localise the mScarlet3 fluorescent protein in early embryos of *Parhyale hawaiensis* (Paris et al. 2022) by microinjecting each of the mRNAs into ~100 embryos at the 1- or 2-cell stage. We screened the embryos for red fluorescence 1 day post injection on an epifluorescence stereoscope and a confocal microscope: 5 out of the 11 tagged constructs gave strong fluorescence localised to the plasma membrane (H-Ras, K-Ras, K-Ras-6R, Lyn and Src64B) and 4 gave weaker signals on the plasma membrane (GAP43, PH, LactC2 and GPI). Some fluorescence could also be seen in speckles inside the cells, possibly in endosomes. The SP-CD8tm fusion appeared to localise to the endoplasmic reticulum or the Golgi, and the RitC fusion gave no fluorescence above background. The untagged control gave fluorescence in the cytoplasm and the nucleus. This initial screen was conducted by one person in 4 days. The results were subsequently confirmed by imaging on a light-sheet microscope (Figure 2).

Among the membrane-localised constructs, H-Ras, K-Ras, K-Ras-6R and Src64B gave the most robust and specific signals on the plasma membrane.

Screening of membrane tags in the hydrozoan jellyfish *Clytia*

We also tested these tags in early primary polyps of *Clytia hemisphaerica* (Houliston et al. 2022) by microinjecting each of the mRNAs into ~100 eggs prior to fertilisation. 3 days after injection, planula larvae were induced to metamorphose and were observed using a confocal microscope within a few hours of settlement (Figure 3). 6 out of the 10 constructs tested yielded embryos with clear fluorescence signal localised at the plasma membrane of the epidermis (ectoderm). Weaker fluorescence was also observed in the gastrodermis (endoderm). The H-Ras, K-Ras, K-Ras-6R, Lyn and Src64B tags gave clear membrane localisation with moderate signal intensity. A weaker mislocated signal was also detectable in vesicular structures in the cytoplasm, likely in endosomes. GAP43 gave a stronger signal at the plasma membrane, but also stronger fluorescence in the cytoplasm. The tree C-terminal Ras tags showed the best localisation to the plasma membrane. LactC2 gave a weak and more diffuse signal at the plasma membrane. The SP-CD8tm tag did not give specific signals at the plasma membrane, but fluorescence was localised in the cytoplasm. The RitC and PH tags gave no visible fluorescence. The SP-GPI tag has not yet been tested in *Clytia*.

Comparing membrane localisation between tags and across species

Our results show that several membrane-localisation tags work robustly in a wide range of animals (summarised in Figure 4, in the context of previous studies). The C-terminal H-Ras, K-Ras and K-Ras-6R tags are efficient in localising mScarlet3 to the plasma membrane in early *Parhyale* embryos and in *Clytia* primary polyps, as previously shown in mammalian cells, insects and annelids (Hancock et al. 1991; Kanca et al. 2013; Özpolat et al. 2017). The same appears to be true for the N-terminal GAP43, Lyn and Src64B tags, although the performance of some of these tags – particularly GAP43 – varies considerably across species.

The phosphoinositide-binding PH domain, which has been exceptionally good in localising fluorescent proteins to the plasma membrane in tunicate embryos (Guignard et al. 2020), is less effective in other species: it gives weak/modest mSCarlet3 fluorescence that is localised to the plasma membrane in *Parhyale* embryos, but no fluorescence in the primary polyps of *Clytia*. Previous work has shown that this tag can localise fluorescent proteins to the plasma membrane in the early blastula stage of *Clytia* (Uveira et al. 2024), but the same construct had shown no localised signal in the planula or in primary polyps. The phosphatidylserine-binding LactC2 tag, which has been successful in fungi (Fairn et al. 2011; Haupt and Minc 2017), gives weak and partly localised fluorescence in *Parhyale* and in *Clytia*. In *Parhyale*, the signal delineates cell boundaries clearly.

The SP-CD8 tag, which is used extensively in *Drosophila* (T. Lee and Luo 1999; Hadjieconomou et al. 2011; Stapornwongkul et al. 2020; Rujano et al. 2022), does not traffic to the plasma membrane in *Parhyale* or *Clytia*. Confocal images from *Parhyale* embryos suggest that it may be retained in the endoplasmic reticulum or the Golgi (not shown). In contrast, the signal peptide in the SP-GPI tag is capable of driving mScarlet3 to the plasma membrane in early *Parhyale* embryos.

The C-terminal RitC domain, which has worked well in mammalian cells and in fission yeast (C.-H. J. Lee et al. 1996; Onken et al. 2006), showed no detectable fluorescence in *Parhyale* and *Clytia*. This could be due to inefficient translation, misfolding or instability of our mScarlet3 fusion protein.

Outlook

The toolkit we present here was made to facilitate the discovery of effective plasma membrane localisation tags in organisms where such tags have not yet been identified. Testing multiple tags is needed because there is, so far, no universally established tag for localising proteins to the plasma membrane in every experimental species. Tags that work well in some species are not always useful in others (see Figure 4).

The reasons for these inter-species differences are unclear, as many of the mechanisms that insert proteins to the plasma membrane (e.g. signals for trafficking or attachment to lipids) appear to be widely conserved. The differences could be explained, in part, by variation in how localisation tags anchor proteins to the plasma membrane, by interacting with other proteins or lipids in the membrane; protein-protein interactions and the membrane's lipid composition can vary between species.

Protein localisation to the plasma membrane does not only vary between species, but also between cell types and cell states within a species, as observed in *Clytia* for membrane localisation of the PH domain protein in embryos vs primary polyps. These intra-species differences could be due to variations in the way protein trafficking is organised in different cell types (e.g. in polarised epithelial versus unpolarised mesenchymal cells or blastomeres), the effects of signalling and cell-cell contacts on protein anchoring to membranes, or variations in the lipid composition of membranes between

cell types or developmental stages (e.g. Halet 2005; Carvalho et al. 2012; Morinaga et al. 2017). Differences in protein concentration and turnover may also contribute to variation between experimental conditions (e.g. cell types, levels of the tagged proteins). If proteins accumulate to levels well above saturation for available binding sites or protein modification machinery, the normal localisation, anchoring or recycling mechanisms may fail, leading to accumulation in the wrong cellular compartments.

It is interesting to note that attachment of a single myristyl, palmityl or prenyl lipid chain to a protein is insufficient to confer robust anchoring to the plasma membrane. Additional elements are required to increase affinity for the plasma membrane, such as the attachment of a second lipid or the presence of positively charged amino acids that interact with negative charges on the plasma membrane (Shahinian and Silvius 1995; Resh 1999). Protein palmitoylation, in particular, is reversible and could be modulated across cell types, cell states and developmental stages (Resh 1999; Baekkeskov and Kanaani 2009; Conibear and Davis 2010). [In contrast, a single GPI chain is sufficient for anchoring proteins to the membrane; and the addition of myristyl, prenyl and GPI groups are constitutive and irreversible.]

Differences in membrane localisation between cell types suggest that screening these reporters in one cell type might not necessarily predict membrane localisation in another. Screening our toolkit by microinjection in early embryos can therefore serve as a rapid initial screen, from which promising candidates may be selected for further testing at later stages, in other cell types of interest.

The main requirement for testing this toolkit is the ability to deliver mRNA into cells. In many organisms this can be achieved by microinjection of early embryos, but it might also be possible to deliver these constructs by other methods, e.g. by transfecting cells, electroporation or biolistics.

We expect that this toolkit will primarily serve the evo-devo community, allowing researchers to label and track cells during early embryonic development, after mRNA injection, in a wide range of non-conventional model organisms. In more genetically tractable species, like *Parhyale* and *Clytia*, this kit will help to generate transgenic reporters for labelling the outlines of cells, to study the morphologies and behaviours of diverse cell types. The ease-of-use of this toolkit could also make it an attractive toolkit to test during practical courses, e.g. embryology courses with access to embryos from diverse species. Some of the tags (e.g. the lipid sensors, tags localising to the endoplasmic reticulum and Golgi) could also be used in comparative cell biology.

We hope that researchers working in a wide variety of organisms and cell types will use this toolkit to identify tags that are useful in their system. Comparing the results obtained in different species will help to identify tags that work well in a wide range of eukaryotes. We invite researchers who would like to participate in a comparative screen to contact us. We would be interested to extend this manuscript, integrating data from a wider range of species.

MATERIALS AND METHODS

Plasmids

The membrane localisation tags for constructs #1 to #11 were selected as described earlier. These were fused with the N- or C-terminus of the mScarlet3 coding sequence (https://www.fpbase.org/protein/mscarlet3/; Gadella et al. 2023). The tagged (#1 to #11) and untagged/control (#0) constructs were flanked by a ~110 bp 5'UTR fragment taken from the

Drosophila hsp70Bb gene and a ~703 bp 3'UTR fragment taken from the *Drosophila inflated* gene (Kapetanaki et al. 2002), and placed under the T7 promoter. In each of these constructs, a unique NotI restriction site was placed after the 3' UTR to allow linearisation of the plasmid prior to in vitro transcription, and unique KasI and AsiSI restriction sites were placed on either side of the tagged mScarlet3 coding sequences, to facilitate subsequent subcloning. An AxxATG Kozak sequence was placed at the start codon, to ensure efficient translation (Kozak 1984).

The membrane-localisation tags used in each construct are: (#1) 29 amino acids from the C-terminus of human H-Ras; (#2) 22 amino acids from the C-terminus of human K-Ras isoform B; (#3) same as in #2, replacing the stretch of 6 lysines in the polybasic domain by 6 arginines (Hancock et al. 1991); (#4) 62 amino acids from the C-terminus of human Rit; (#5) 20 amino acids from the N-terminus of mouse GAP43; (#6) 18 amino acids from the N-terminus of mouse Lyn; (#7) 85 amino acids from the N-terminus of *Drosophila* Src64B; (#8) 174 amino acids from the N-terminus of human PLC delta 1; (#9) 158 amino acids from the C-terminus of bovine Lactahedrin; (#10) 38 amino acids from the N-terminus of *Drosophila* Wingless (signal peptide), 39 amino acids corresponding to the transmembrane domain of human CD8, and 39 amino acids including the transmembrane domain of mouse CD8, and a 6 amino acid linker (Stapornwongkul et al. 2020); (#11) 24 amino acids from the N-terminus of rabbit lactase-phorizin hydrolase/LPH (signal peptide), a 15 amino acid linker, the mScarlet3 protein, a 14 amino acid linker, and 31 amino acids from the C-terminus of human LFA-3/CD58 (Keller et al. 2001; Shelby et al. 2023). The constructs were produced by gene synthesis in the pTwist vector (Twist Bioscience), a high copy plasmid carrying an ampicillin resistance gene.

The plasmids are available from Addgene, at https://www.addgene.org/browse/article/28251646/; annotated sequences are available as 'Supplemental Documents' at Addgene.

In vitro transcription

Plasmids were digested with NotI. After heat-inactivating NotI (15 min at 65˚C) and plasmid purification, the plasmid was *in vitro* transcribed by the T7 RNA polymerase using the mMESSAGE mMACHINE T7 Transcription Kit (Thermo Fisher Scientific #AM1344). The mRNA was precipitated in (2.8M LiCl, overnight at -20˚C), washed in 70% ethanol, and resuspended in DEPC-treated water at a concentration of 600 ng/µl.

Alternatively, the constructs were amplified by PCR using the Phusion DNA polymerase (Thermo Scientific #F530L) and primers GCAATTAACCCTCACTAAAGGTACAAGAAGAGAACTCTGGGCG (including a T3 promoter) and AGAGTACACTGCAAGAAGTCGG. PCR products purified with Wizard SV PCR purification kit (Promega) was in vitro transcribed by the T3 RNA polymerase using the mMESSAGE mMACHINE T3 Transcription Kit (Thermo Fisher Scientific #AM1348). The mRNA was purified using the MegaClear kit (Thermo Scientific #AM1908).

Microinjection and screening in *Parhyale*

One- and two-cell stage embryos of *Parhyale hawaiensis* were microinjected with 480 ng/µl of mRNA, as described previously (Kontarakis and Pavlopoulos 2014). The embryos were allowed to recover for ~24 h in filtered artificial seawater at 26˚C. Approximately 100 embryos were injected per construct. Of these, 70 to 80% survived and a large number showed fluorescence a day after injection. Surviving embryos were initially screened on a Leica MZ16F epifluorescence stereoscope. They were observed in greater detail on a Zeiss LSM 800 confocal microscope using a Plan-Apochromat 20 x/0.8 M27 objective, and a Zeiss LightSheet 7 microscope using a W

Plan-Apochromat 20x/1.0 DIC M27 objective. For confocal microscopy, live embryos were observed in 35mm Ibidi Glass Bottom Dishes (Ibidi µ-Dish 35 mm, #81158) in filtered artificial seawater. For light sheet microscopy, samples were mounted in 1% low-melt agarose in filtered artificial seawater.

Microinjection and screening in *Clytia*

mRNA was diluted in distilled water to 250 ng/µl and microinjected into naturally spawned eggs before fertilisation, as described previously (Momose and Houliston 2007). The injected mRNA solution was about 2-3% of the egg volume. Embryos were cultured in plastic dishes with artificial seawater (Red Sea Salt, 37 ppt) containing 1:2000 diluted penicillin-streptomycin solution (Sigma #P4333) at 21°C. Metamorphosis was induced by transferring planula larvae on a fluorinated ethylene propylene film (FEP, 150 µm thickness) with a drop of seawater containing GLWamide-6 (Lechable et al. 2020). The settled planulae on the film were mounted on a glass slide and observed on a Leica Stellaris 5 confocal microscope, focusing on the epidermal layer.

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Author contributions

IK and MA designed the experiments; MA selected the tags and designed the constructs; IK and TM performed the microinjections, screened the embryos and analysed the results in *Parhyale* and *Clytia*, respectively; MZ performed the light-sheet microscopy in *Parhyale*.

Competing interests

The authors declare no competing interests.

References

Alwes, Frederike, Camille Enjolras, and Michalis Averof (2016) Live imaging reveals the progenitors and cell dynamics of limb regeneration. *eLife* 5: 73.

Apolloni, Prior, Lindsay, Parton, and Hancock (2000) H-Ras but Not K-Ras Traffics to the Plasma Membrane through the Exocytic Pathway. *Molecular and Cellular Biology* 20 (7): 2475–87.

Baekkeskov and Kanaani (2009) Palmitoylation Cycles and Regulation of Protein Function (Review). *Molecular Membrane Biology* 26 (1): 42–54.

Carvalho, Sampaio, Palm, Brankatschk, Eaton, and Shevchenko (2012) Effects of Diet and Development on the Drosophila Lipidome. *Molecular Systems Biology* 8:600.

Casey (1995) Protein Lipidation in Cell Signaling. *Science* 268: 221–25.

Conibear and Davis (2010) Palmitoylation and Depalmitoylation Dynamics at a Glance. *Journal of Cell Science* 123: 4007–10.

Fairn, Hermansson, Somerharju, and Grinstein (2011) Phosphatidylserine Is Polarized and Required for Proper Cdc42 Localization and for Development of Cell Polarity. *Nature Cell Biology* 13: 1424–30.

Gadella, Weeren, Stouthamer, Hink, Wolters, Giepmans, Aumonier, Dupuy, and Royant (2023) mScarlet3: A Brilliant and Fast-Maturing Red Fluorescent Protein." *Nature Methods* 20 (4): 541–45.

Gilles, Schinko, Schacht, Enjolras, and Averof (2019) Clonal Analysis by Tunable CRISPR-Mediated Excision. *Development* 146, dev170969.

Greco, Hannus, and Eaton (2001) Argosomes: : a potential vehicle for the spread of morphogens through epithelia. *Cell* 106: 633–45.

Guignard, Fiuza, Leggio, Laussu, Faure, Michelin, Biasuz, et al. (2020) Contact area-dependent cell communication and the morphological invariance of ascidian embryogenesis. *Science* 369, eaar5663.

Gulyás, Radvánszki, Matuska, Balla, Hunyady, Balla, and Várnai (2017)cPlasma Membrane Phosphatidylinositol 4-Phosphate and 4,5-Bisphosphate Determine the Distribution and Function of K-Ras4B but Not H-Ras Proteins. *Journal of Biological Chemistry* 292: 18862–77.

Haas and Gilmour (2006) Chemokine Signaling Mediates Self-Organizing Tissue Migration in the Zebrafish Lateral Line. *Developmental Cell* 10: 673–80.

Hadjieconomou, Rotkopf, Alexandre, Bell, Dickson, and Salecker (2011) Flybow: genetic multicolor cell labeling for neural circuit analysis in Drosophila melanogaster. *Nature Methods* 8: 260–66.

Halet (2005) Imaging Phosphoinositide Dynamics Using GFP-Tagged Protein Domains. *Biology of the Cell* 97: 501–18.

Hancock, Cadwallader, Paterson, and Marshall (1991) A CAAX or a CAAL Motif and a Second Signal Are Sufficient for Plasma Membrane Targeting of Ras Proteins. *The EMBO Journal* 10: 4033–39.

Haupt αvδ Minc (2017) Gradients of Phosphatidylserine Contribute to Plasma Membrane Charge Localization and Cell Polarity in Fission Yeast. *Molecular Biology of the Cell* 28: 210–20.

Heijne (1990) Protein Targeting Signals. *Current Opinion in Cell Biology* 2: 604–8.

Houliston, Leclère, Munro, Copley, and Momose (2022) Past, Present and Future of Clytia Hemisphaerica as a Laboratory Jellyfish. *Current Topics in Developmental Biology* 147: 121–51.

Kanca, Caussinus, Denes, Percival-Smith, and Affolter (2013) Raeppli: a whole-tissue labeling tool for live imaging of Drosophila development. *Development* 141: 472–80.

Kapetanaki, Loukeris, Livadaras, and Savakis (2002) High Frequencies of Minos Transposon Mobilization Are Obtained in Insects by Using in Vitro Synthesized mRNA as a Source of Transposase. *Nucleic Acids Research* 30: 3333–40.

Kay, Koivusalo, Ma, Wohland, and Grinstein (2012) Phosphatidylserine Dynamics in Cellular Membranes. *Molecular Biology of the Cell* 23: 2198–2212.

Keller, Toomre, Díaz, White, and Simons (2001) Multicolour Imaging of Post-Golgi Sorting and Trafficking in Live Cells. *Nature Cell Biology* 3: 140–49.

Kinoshita (2020) Biosynthesis and Biology of Mammalian GPI-Anchored Proteins. *Open Biology* 10: 190290.

Kontarakis and Pavlopoulos (2014) Transgenesis in Non-model Organisms: The Case of Parhyale. *Methods in Molecular Biology* 1196: 145–81.

Kozak (1984) Compilation and Analysis of Sequences Upstream from the Translational Start Site in Eukaryotic mRNAs. *Nucleic Acids Research* 12: 857–72.

Lauri, Brunet, Handberg-Thorsager, Fischer, Simakov, Steinmetz, Tomer, Keller, and Arendt (2014) Development of the annelid axochord: Insights into notochord evolution. *Science* 345: 1365–68.

Lechable, Jan, Duchene, Uveira, Weissbourd, Gissat, Collet, et al. (2020) An Improved Whole Life Cycle Culture Protocol for the Hydrozoan Genetic Model Clytia Hemisphaerica. *Biology Open* 9: bio051268.

Lee, Della, Chew, and Zack (1996) Rin, a Neuron-Specific and Calmodulin-Binding Small G-Protein, and Rit Define a Novel Subfamily of Ras Proteins. *Journal of Neuroscience* 16: 6784–94.

Lee and Luo (1999) Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* 22: 451–61.

Momose and Houliston (2007) Two Oppositely Localised Frizzled RNAs as Axis Determinants in a Cnidarian Embryo. *PLoS Biology* 5: e70.

Morinaga, Yanase, Okamoto, Yamaguchi, and Yamaguchi (2017) Recruitment of Lyn from Endomembranes to the Plasma Membrane through Calcium-Dependent Cell-Cell Interactions upon Polarization of Inducible Lyn-Expressing MDCK Cells. *Scientific Reports* 7: 493.

Onken, Wiener, Philips, and Chang (2006) Compartmentalized Signaling of Ras in Fission Yeast." *Proc National Acad Sci USA* 103 (24): 9045–50.

Orlean and Menon (2007) GPI Anchoring of Protein in Yeast and Mammalian Cells, or How We Learned to Stop Worrying and Love Glycophospholipids. *Journal of Lipid Research* 48: 993–1011.

Özpolat, Handberg-Thorsager, Vervoort, and Balavoine (2017) Cell Lineage and Cell Cycling Analyses of the 4d Micromere Using Live Imaging in the Marine Annelid Platynereis Dumerilii. *eLife* 6, e30463.

Paris, Wolff, Patel, and Averof (2022) The Crustacean Model Parhyale Hawaiensis. *Current Topics in Developmental Biology* 147: 199–230.

Pfeiffer, Ngo, Hibbard, Murphy, Jenett, Truman, and Rubin (2010) Refinement of Tools for Targeted Gene Expression in Drosophila. *Genetics* 186: 735–55.

Ramos, Gustafsson, Labert, Salecker, Nilsson, and Averof (2019) Analysis of the genetically tractable

crustacean Parhyale hawaiensis reveals the organisation of a sensory system for low-resolution vision. *BMC Biology* 17: 67.

Resh (1999) Fatty Acylation of Proteins: New Insights into Membrane Targeting of Myristoylated and Palmitoylated Proteins. *Biochimica et Biophysica Acta* 1451: 1–16.

Roure, Rothbächer, Robin, Kalmar, Ferone, Lamy, Missero, Mueller, and Lemaire (2007) A Multicassette Gateway Vector Set for High Throughput and Comparative Analyses in Ciona and Vertebrate Embryos. *PloS One* 2: e916.

Rujano, Briand, Đelić, Marc, and Spéder (2022) An Interplay between Cellular Growth and Atypical Fusion Defines Morphogenesis of a Modular Glial Niche in Drosophila. *Nature Communications* 13: 4999.

Shahinian and Silvius (1995) Doubly-Lipid-Modified Protein Sequence Motifs Exhibit Long-Lived Anchorage to Lipid Bilayer Membranes. *Biochemistry* 34: 3813–22.

Shelby, Castello-Serrano, Wisser, Levental, and Veatch (2023) Membrane Phase Separation Drives Responsive Assembly of Receptor Signaling Domains. *Nature Chemical Biology* 19: 750–58.

Stapornwongkul, de Gennes, Cocconi, Salbreux, and Vincent (2020) Patterning and Growth Control in Vivo by an Engineered GFP Gradient. *Science* 370: 321–27.

Uveira, Donati, Léria, Lechable, Lahaye, Vesque, Houliston, and Momose (2024) Planar Cell Polarity Coordination in a Cnidarian Embryo Provides Clues to Animal Body Axis Evolution. *bioRxiv*, https://doi.org/10.1101/2024.09.05.609312.

Vopalensky, Tosches, Achim, Handberg-Thorsager, and Arendt (2019) From Spiral Cleavage to Bilateral Symmetry: The Developmental Cell Lineage of the Annelid Brain. *BMC Biology* 17: 81.

Wang and Casey (2016) Protein Prenylation: Unique Fats Make Their Mark on Biology. *Nature Reviews Molecular Cell Biology* 17: 110–22.

Yeung, Gilbert, Shi, Silvius, Kapus, and Grinstein (2008) Membrane Phosphatidylserine Regulates Surface Charge and Protein Localization. *Science* 319: 210–13.

Zhou, Prakash, Liang, Cho, Gorfe, and Hancock (2017) Lipid-Sorting Specificity Encoded in K-Ras Membrane Anchor Regulates Signal Output. *Cell* 168: 239–51.

Table 1. Plasma membrane localisation tags included in the toolkit

Name	Type of tag, mechanism	Previous use
H-Ras	C-terminal prenylation (CAAX) and palmitoylation sites; attached on the cytoplasmic side of the plasma membrane	Mammalian cells (Hancock et al. 1991), Drosophila (Kanca et al. 2013), Parhyale neurons (Ramos et al. 2019), Platynereis (Özpolat et al. 2017), fission yeast (Onken et al. 2006)
K-Ras	C-terminal prenylation site (CAAX) and polybasic domain; attached on the cytoplasmic side of the plasma membrane	Mammalian cells (Hancock et al. 1991; Zhou et al. 2017), fission yeast (Haupt and Minc 2017)
K-Ras-6R	Same as KRas, with 6K polybasic domain replaced by 6R; potentially interacting with different lipids	Mammalian cells (Hancock et al. 1991; Zhou et al. 2017)
RitC	C-terminal non-CAAX, non-exocytic membrane targeting domain; attached on the cytoplasmic side of the plasma membrane	Mammalian cells (C.-H. J. Lee et al. 1996), fission yeast (Onken et al. 2006)
GAP43	N-terminal peptide carrying two palmitoylation sites; attached on the cytoplasmic side of the plasma membrane	Ciona (Roure et al. 2007), Tribolium (Gilles et al. 2019), Platynereis (Lauri et al. 2014)
Lyn	N-terminal myristoylation and palmitoylation sites; attached on the cytoplasmic side of the plasma membrane	Mammalian cells (Gulyás et al. 2017), zebrafish (Haas and Gilmour 2006), Drosophila (Hadjieconomou et al. 2011), Parhyale (Alwes et al. 2016), Platynereis (Vopalensky et al. 2019)
Src64B	N-terminal myristoylation site from *Drosophila* Src64B; attached on the cytoplasmic side of the plasma membrane	Drosophila neurons (Pfeiffer et al. 2010)
PH	N-terminal mammalian PH domain, binding PtdIns(4,5)P2 lipids, enriched in the plasma membrane	Mammalian cells (Halet 2005), tunicates (Guignard et al. 2020), hydrozoan embryos (Uveira et al. 2024)
LactC2	C-terminal phosphatidylserine (PS) binding domain, a lipid found in the inner leaflet of the plasma membrane	Mammalian cells (Yeung et al. 2008; Kay et al. 2012), budding yeast (Fairn et al. 2011), fission yeast (Haupt and Minc 2017)
SP-CD8tm	N-terminal signal peptide and transmembrane domain; trafficked in the exocytic pathway, attached on cytoplasmic side	Drosophila (T. Lee and Luo 1999; Hadjieconomou et al. 2011; Stapornwongkul et al. 2020; Rujano et al. 2022)
SP-GPI	N-terminal signal peptide and C-terminal GPI attachment site; trafficked in the exocytic pathway, attached on extracellular side	Mammalian cells (Keller et al. 2001; Shelby et al. 2023), Drosophila (Greco, Hannus, and Eaton 2001)

Figure 1. Toolkit of membrane-tagged constructs

Illustration of the eleven membrane-tagged constructs (#1-11) and the untagged control (#0), highlighting the mScarlet fluorescent protein shown (in red), C-terminal lipid addition tags (in blue), N-terminal lipid addition tags (in purple), lipid binding domains (in green), signal peptides (in yellow), transmembrane domain (in orange) and a C-terminal GPI attachment tag (in cyan).

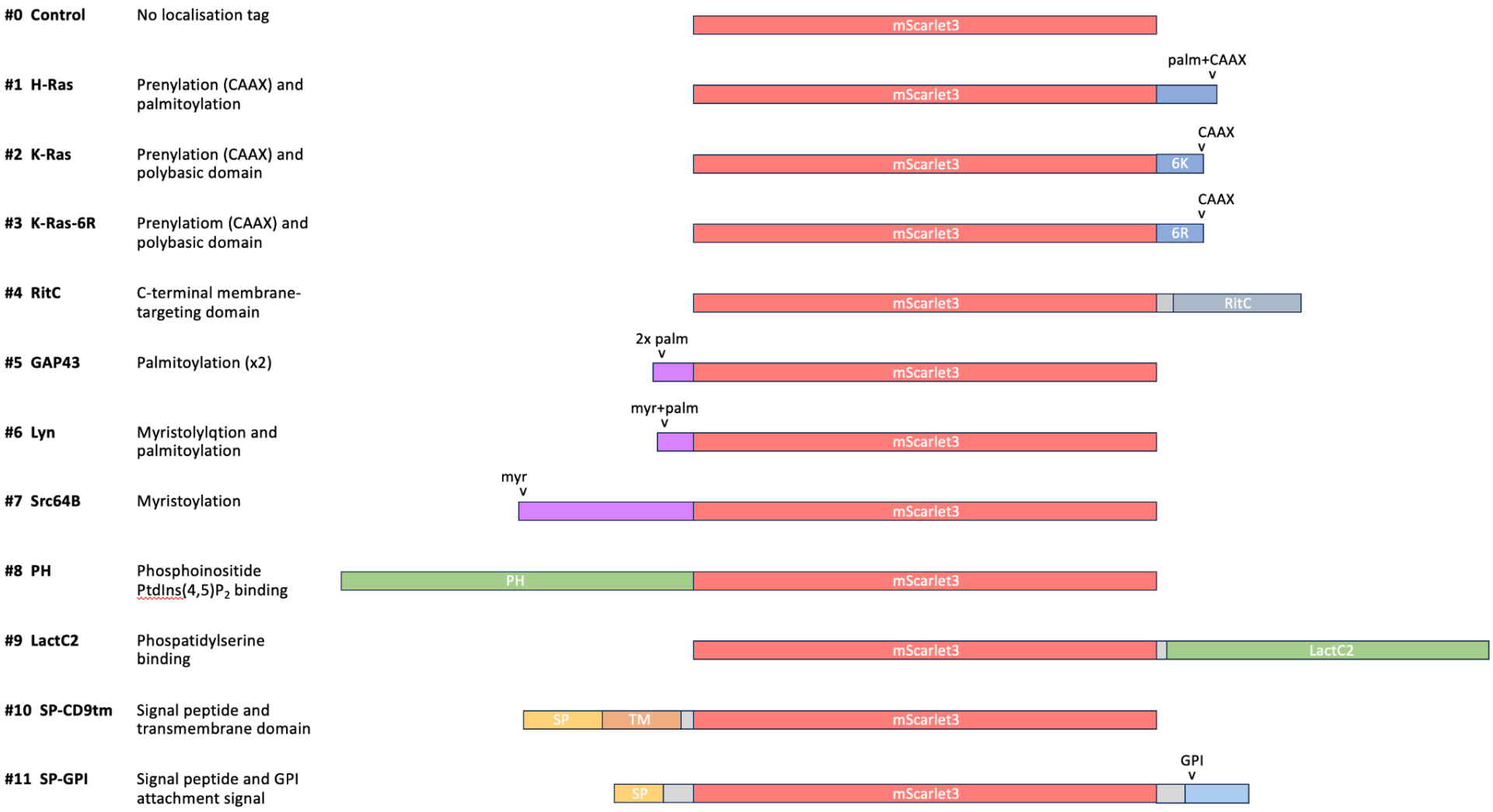

Figure 2. Localisation of membrane-tagged reporters in early embryos of *Parhyale hawaiensis*

mScarlet3 fluorescence in 1-day old *Parhyale* embryos, injected with mRNA of the membrane-tagged and control constructs. Only parts of each embryo express the reporter, due to uneven distribution of the injected mRNA. Images show maximum projections from light-sheet microscopy captured with different exposure times (except #4, which gave no signal above background and was captured with conventional fluorescence microscopy). Scale bar, 50 µm.

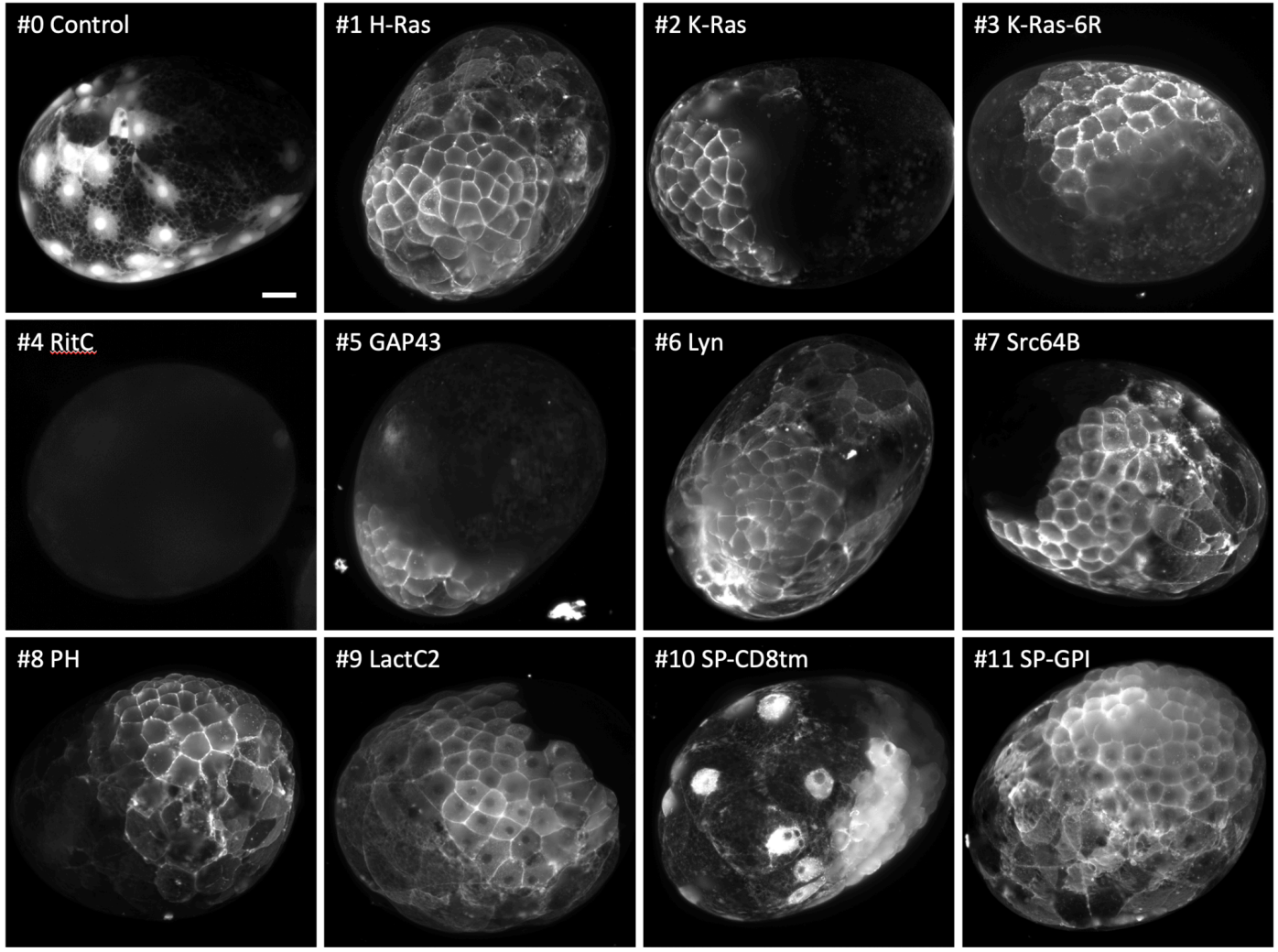

Figure 3. Localisation of membrane-tagged reporters in early primary polyps of *Clytia hemisphaerica*

mScarlet3 fluorescence in *Clytia* primary polyps a few hours after settlement (3 days after mRNA injection into oocytes). The images are from a single confocal plane capturing the outer epidermal layer of the polyp, on the oral side. They were captured with the same settings, so fluorescent intensities are comparable across panels. Scale bar, 10 µm.

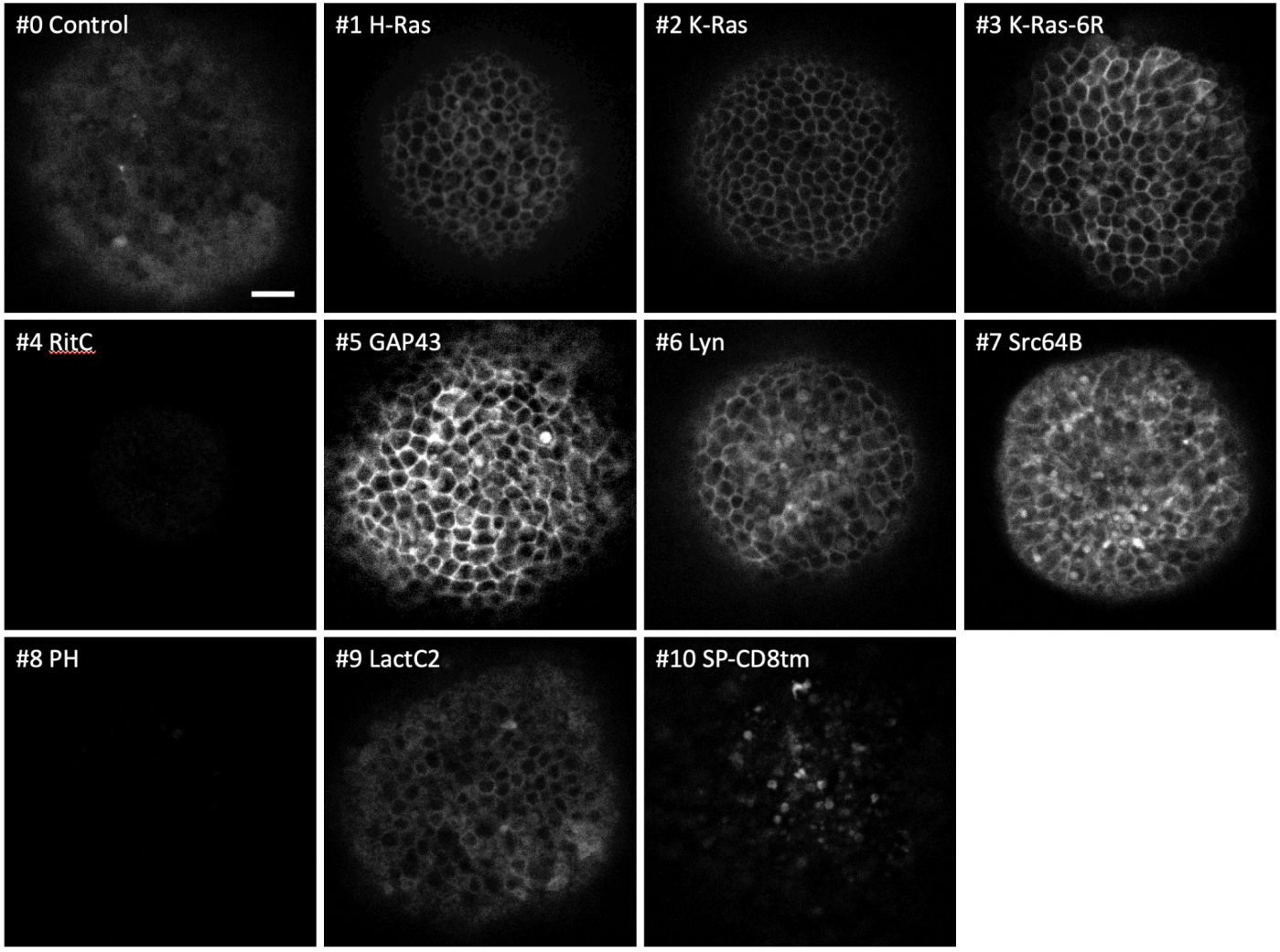

Figure 4. Overview of plasma membrane localisation in different species
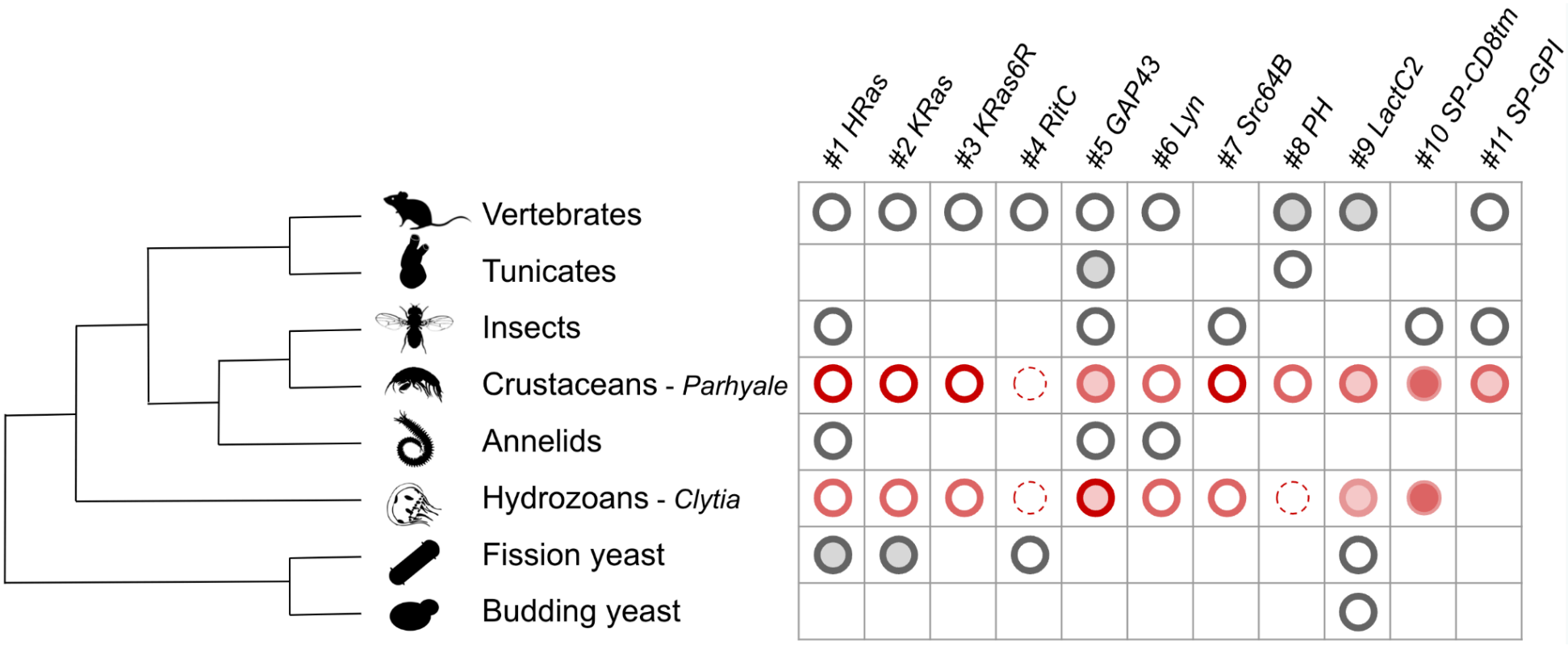
Illustration summarising results from the 11 membrane localisation tags across different organisms. The outline of each circle represents the intensity of plasma membrane localisation, while the filling of the circle represents signal inside the cell. Grey circles correspond to information taken from previous publications (see Table 1). Red circles represent data from this paper; the intensities of mScarlet3 fluorescence are indicated by different shades of red.