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In Vivo Imaging and Differential Localization of Lipid-Modified GFP-Variant Fusions in Embryonic Stem Cells and Mice

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Summary

The visualization of live cell behaviors operating in situ combined with the power of mouse genetics represents a major step toward understanding the mechanisms regulating embryonic development, homeostasis, and disease progression in mammals. The availability of genetically encoded fluorescent protein reporters, combined with improved optical imaging modalities, have led to advances in our ability to examine cells in vivo. We developed a series of lipid-modified fluorescent protein fusions that are targeted to and label the secretory pathway and the plasma membrane, and that are amenable for use in mice. Here we report the generation of two strains of mice, each expressing a spectrally distinct lipid-modified GFP-variant fluorescent protein fusion. The *CAG::GFP-GPI* strain exhibited widespread expression of a glycosylphosphatidylinositol-tagged green fluorescent protein (GFP) fusion, while the *CAG::myr-Venus* strain exhibited widespread expression of a myristoyl-Venus yellow fluorescent protein fusion. Imaging of live transgenic embryonic stem (ES) cells, either live or fixed embryos and postnatal tissues demonstrated that glycosylphosphatidyl inositol- and myristoyl-tagged GFP-variant fusion proteins are targeted to and serve as markers of the plasma membrane. Moreover, our data suggest that these two lipid-modified protein fusions are dynamically targeted both to overlapping as well as distinct lipid-enriched compartments within cells. These transgenic strains not only represent high-contrast reporters of cell morphology and plasma membrane dynamics, but also may be used as in vivo sensors of lipid localization. Furthermore, combining these reporters with the study of mouse mutants will be a step forward in understanding the inter- and intracellular behaviors underlying morphogenesis in both normal and mutant contexts.

Keywords

mouse; transgenic; ES cell; GFP; YFP; Venus; GPI; myristoyl; confocal; live imaging

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INTRODUCTION

A suite of genetic methodologies and the availability of the near complete genome sequence have established the mouse as the premier mammalian system for investigating mechanisms of development and disease progression. Genetic approaches have contributed greatly to our understanding of the roles of specific gene products; however, a dynamic multidimensional understanding of development and disease progression is often lacking due to the static and two-dimensional (2D) nature of established protocols for dissecting spatially and temporally regulated cellular phenotypes underlying overt morphological defects (Palade, 1964).

In Vivo Imaging of Fluorescent Proteins

Genetically encoded fluorescent proteins, such as the green fluorescent protein (GFP) (Chalfie *et al.*, 1994; for review, see Tsien, 1998) and its variants (Cormack *et al.*, 1997; Zhang *et al.*, 2002), exhibit high signal-to-noise ratios and currently offer the most attractive option for the real-time examination of dynamic cellular events operating in situ (reviewed in Hadjantonakis *et al.*, 2003; Megason and Fraser, 2003). A significant step toward transferring this genetically encoded reporter-based optical imaging technology to a mammalian organismal context was achieved first through the generation of transgenic mouse strains expressing GFP under widespread regulatory elements, thereby confirming developmental neutrality of the reporter (Okabe *et al.*, 1997; Hadjantonakis *et al.*, 1998), and subsequently under the control of regionally restricted regulatory elements (Anderson *et al.*, 1999; Rodriguez *et al.*, 1999). Incorporation of GFP into transgenic and targeted regimes in mice was followed by the combinatorial use of spectrally distinct GFP variants to simultaneously label and visualize distinct cell populations (Feng *et al.*, 2000; Hadjantonakis *et al.*, 2002).

However, the inability of transgenic lines expressing native GFP (or any of its variants) to clearly demarcate individual cells within a cohort has often limited our understanding of the cellular behaviors driving morphogenesis. Subcellularly localized fluorescent protein fusions (Rizzuto *et al.*, 1996) when expressed in mice provide the highest resolution visualization of dynamic cellular events and permit the identification of individual cells, or subcellular structures, within a three-dimensional (3D) field of view (reviewed in Hadjantonakis *et al.*, 2003; Megason and Fraser, 2003; Passamaneck *et al.*, 2006). We previously reported the development and use of human histone H2B GFP-variant fusions that label active chromatin. These are developmentally neutral, and therefore ideal for tracking cell position, division, and death in vivo in mice (Hadjantonakis and Papaioannou, 2004; Fraser *et al.*, 2005; Plusa *et al.*, 2005). However, to date no system that permits the routine visualization and quantitation of dynamic cell morphology and cell behavior has been established for use in embryonic stem (ES) cells and mice.

Plasma Membrane

The plasma membrane is a dynamic lipid bilayer (Palade, 1975; Edidin, 2003) that constitutes part of the secretory pathway (Schekman, 2004). It is comprised of several distinct domains (Brown and London, 1998) and serves as the outer boundary of the cell. Plasma membrane components are sorted and targeted to the membrane through the trans-Golgi network (Mogelsvang *et al.*, 2004; Polishchuk and Mironov, 2004), and are continually recycled through distinct endocytic pathways (Lippincott-Schwartz *et al.*, 2000). Biochemical fractionation has shown that the plasmalemma can be separated into microdomains that are either susceptible or resistant to nonionic detergent solubilization. The detergent-resistant fraction (referred to as lipid rafts) is enriched in cholesterol, sphingolipids, and lipid-modified proteins (Simons and Toomre, 2000). Due to their affinity for lipid-modified proteins, lipid rafts are believed to act as platforms for receptor-mediated signaling interactions (Helms and Zurzolo, 2004; Anderson and Jacobson, 2002; Simons and Ikonen, 1997). Although the small

size of lipid rafts has prevented direct visualization using conventional light microscopy, studies carried out in cell culture systems, including biophysical measurements (Varma and Mayor, 1998) and fluorescence recovery after photobleaching (FRAP) assays (Kenworthy *et al.*, 2004) have suggested that rafts organize into clusters that diffuse throughout the plasma membrane (reviewed in Mayor and Reizman, 2004).

Even though direct visualization of plasma membrane microdomains using optical techniques has been evasive, GFP fusion proteins, when used in combination with molecular genetic and pharmacological perturbations, have proved valuable for examining a variety of intracellular membrane behaviors and interactions (Lippincott-Schwartz *et al.*, 2000) and have led to the identification of distinct intracellular trafficking pathways (Nichols *et al.*, 2001; Sabharanjak *et al.*, 2002). However, due to the context-dependent plasticities observed in the behaviors of these targeted fluorescent reporters (Kenworthy *et al.*, 2004; Levine *et al.*, 2005), it will be necessary to extend any observations made in cell culture systems by performing analyses *in vivo* in whole animals. The examination of multiple cell types in their native milieu will allow a better understanding of the influence of cell and environmental context in organizing intracellular membrane interactions.

Lipid Modifications of Proteins

Lipid modifications often serve to direct proteins to the plasma membrane. Several classes of lipid modification have been identified, including: N-myristoylation, *s*-acylation (palmitoylation), prenylation (farnesylation and geranylgeranylation), glycosylphosphatidylinositol (GPI) anchoring, and cholesterol modification. To generate and evaluate fusion proteins that would label either the inner or outer leaflet of the plasma membrane, we tagged GFP-variant fluorescent proteins with sequences that direct the addition of different lipid moieties. These included addition of a myristoylation sequence to the amino (N)-terminus of a fluorescent protein (to label the inner leaflet; Resh, 1999), and a GPI anchor to the carboxy (C)-terminus (to label the outer leaflet; Kondoh *et al.*, 1999; Mayor and Riezman, 2004).

Myristoylation occurs cotranslationally (Wilcox *et al.*, 1987); the initiator methionine is removed by methionyl peptidase, then myrostatic acid is added through an amide linkage to the exposed N-terminus of a glycine residue (Resh, 1999). Approximately 10 amino acid residues are often sufficient to direct this modification (van't Hof and Resh, 2000). Sequence requirements for myristoylation include a glycine at position 2 (after the initiator methionine), amino terminal basic residues, and a Ser/Thr at position 6 (Sigal *et al.*, 1994; Resh, 1999). In contrast, the addition of a GPI anchor occurs in the endoplasmic reticulum, where a GPI transamidase generates an amide linkage between the phosphoethanolamine unit of GPI and the C-terminus of a protein (Mayor and Riezman, 2004).

Lipid-Modified Fluorescent Protein Fusions in Transgenic Mice

We used ES cell-mediated transgenesis to evaluate GFP-variant myristoyl and GPI fusions first in ES cells and then in mice. We generated two mouse strains: one exhibited widespread expression of GFP-GPI (referred to as *CAG::GFP-GPI*), and second, exhibited widespread expression of myristoyl (myr)-Venus (referred to as *CAG::myr-Venus*). Our results demonstrated that these transgenic animals are viable and fertile and exhibit no altered phenotype when compared to nontransgenic littermates. Furthermore, we showed that both *CAG::GFP-GPI* and *CAG::myr-Venus* strains maintained widespread transgene expression throughout embryonic development and adulthood. Interestingly, fixed samples displayed the same localization as live samples. Our data demonstrated the sufficiency of the sequences directing N-terminal myristoylation and/or C-terminal GPI anchor addition to target GFP-variant fluorescent proteins to domains within the plasma membrane, trans-Golgi network,

endosomes, and perinuclear regions. Moreover, by examining a live embryo that expresses both reporters, we demonstrated that the fluorescent reporter fusion proteins label distinct organelles. Therefore, the generation of mouse transgenic lines that express widespread subcellularly targeted fluorescent reporters will allow for the examination of dynamic intracellular behavior in multiple cell and tissue types in vivo.

RESULTS AND DISCUSSION

Lipid-modified fluorescent protein fusions that label the secretory pathway including the plasma membrane were developed to provide dynamic information on 1) cell morphology and cell behavior, and 2) secretory pathway itineraries. To construct the GFP-GPI fusion, the N-terminal signal sequence from the sperm secretory protein acrosin and the C-terminal GPI-anchor sequence of Thy-1 were added to the N- and C-terminus of enhanced green fluorescent protein (EGFP; Cormack *et al.*, 1996), respectively, as described previously (Kondoh *et al.*, 1999). The myr-fluorescent proteins fusion were generated by adding the sequence encoding the N-terminal nine amino acids from avian c-Src (Thomas and Brugge, 1997) to the 5' end of EGFP and Venus (Nagai *et al.*, 2002). Venus was chosen over enhanced yellow fluorescent protein (EYFP) as it is currently the brightest and fastest-maturing GFP-variant exhibiting a red-shifted yellow fluorescence (Nagai *et al.*, 2002). The resulting GFP-GPI, myr-GFP, and myr-Venus fusions were then placed under the regulation of the CAG promoter (Niwa *et al.*, 1991) to generate the plasmids *pCX::GFP-GPI*, *pCX::myr-GFP*, and *pCX::myr-Venus*. The CAG promoter comprises the CMV immediate early enhancer, chicken beta actin promoter, 5' UTR, and first intron and has previously been shown to drive robust widespread transgene expression in ES cells, embryos, and adult mice (Niwa *et al.*, 1991; Hadjantonakis *et al.*, 1998, 2002).

Plasmid constructs were tested for fluorescent fusion protein expression and subcellular localization by transient transfection of Cos-7 cells. Since myr-GFP and myr-Venus were shown to exhibit an identical subcellular localization (data not shown), and since Venus is spectrally distinct from GFP and can therefore be combinatorially imaged with GFP, we chose *pCX::myr-Venus* and *pCX::GFP-GPI* for further analysis in ES cell-mediated transgenesis experiments. Plasmids *pCX::GFP-GPI* and *pCX::myr-Venus* were used to generate lines of *CAG::GFP-GPI* and *CAG::myr-Venus* transgenic ES cells as described previously (Hadjantonakis and Papaioannou, 2004). Fluorescent colonies were identified and picked under an epifluorescence stereo dissecting microscope. Clones were passaged in 96-well plates in the absence of drug selection and monitored for maintenance of transgene-encoded fluorescence. ES cell lines exhibiting constitutive expression of either GFP-GPI or myr-Venus while retaining normal morphology when grown either in the presence of LIF or when induced to differentiate into embryoid bodies were further analyzed for level and heterogeneity of fluorescence by flow cytometry as described previously (Hadjantonakis and Nagy, 2000). Only clones satisfying these criteria were used for further investigation.

To determine the subcellular distribution of each fluorescent fusion at high resolution, two different ES cell clones for each transgenic construct were examined using confocal laser scanning microscopy. The lipid-modified fluorescent protein fusions retained both fluorescence and subcellular localization after fixation of specimens in paraformaldehyde. No differences in level of expression or subcellular compartmentalization were observed between ES cell clones expressing any given fusion. Furthermore, treatment of transgenic ES cells with cycloheximide did not alter the localization of fluorescence, suggesting that the observed fluorescent protein distribution was likely due to targeted localization of the fusion rather than buildup of excess newly synthesized protein (data not shown). The cellular distributions for each construct in live ES cells are shown in Figure 1. GFP-GPI was localized on plasma membranes (yellow arrowhead, Fig. 1a), Golgi membranes (red arrowhead, Fig. 1a), and some

secretory vesicles (blue arrowhead, Fig. 1a). myr-Venus is concentrated in perinuclear regions (green arrowhead, Fig. 1b) and vesicular structures located inside the nucleus (white arrowhead, Fig. 1b), in addition to the plasma membrane (yellow arrowhead, Fig. 1b), endosomes of the secretory pathway (blue arrowhead, Fig. 1b), and the Golgi (red arrowhead, Fig. 1b). With the exception of the nuclear localization of myr-Venus, which we attribute to either the increased resolution or live context of our analysis, these distributions are consistent with previous reports of GPI localization in F9 cells and Src localization in fibroblasts (Kaplan *et al.*, 1992).

3D and 4D information was generated by computer-assisted rendering of the z-stacks of xy images. Rendered images of *CAG::GFP-GPI* and *CAG::myr-Venus* ES cells are shown in Figure 1c–f (blue, red, and yellow arrowheads highlight similar structures). Post-Golgi carriers fusing with the membrane were identified by comparison of the face-on (red arrowhead, Fig. 1c) and rotated (Fig. 1d) views of the rendered stacks. Higher density of myr-Venus-labeled structures in the cytoplasm (Fig. 1e,f) as compared to GFP-GPI was also evident. These images also provided data with respect to the direction of membrane secretion occurring within a single cell in a non-polarized cluster, also discerned with respect to the position of the nucleus (white arrowhead, Fig. 1e).

Having established the neutrality of widespread lipid-modified fusion protein expression in ES cells, we next investigated the extent of individual transgene expression in embryos. We generated 4n (tetraploid) wildtype $\leftrightarrow$ *CAG::GFP-GPI* and equivalent *CAG::myr-Venus* chimeras, as described previously (Hadjantonakis *et al.*, 1998, 2002). We then documented transgene expression (as determined by fluorescence) at embryonic day (E)9.5. Three independent ES cell lines were tested for each transgenic construct. All *CAG::GFP-GPI* ES cell-derived embryos exhibited widespread GFP-GPI expression, indicating that: 1) the level of expression was sufficiently strong to be visualized *in vivo*; 2) that the transgene was not silenced; and 3) development was able to proceed normally to midgestation (data not shown).

For *CAG::myr-Venus* transgenic ES cell clones, all embryos exhibited normal morphology, with two lines exhibiting widespread myr-Venus expression. However, one line consistently produced mosaic myr-Venus expression around E9.5, which became more widespread at later stages. Our past experience in imaging cells *in vivo* in mouse embryos has shown that mosaic reporter expression, for example, in diploid chimeras, yields more information about cell morphology, since cells can be imaged in relative “isolation,” providing information on plasma membrane projections and dynamics. Since we have also generated strains of mice, exhibiting widespread expression of myristoyl-monomeric red fluorescent protein variants throughout embryonic development (G.S. Kwon, C.S.L., and A.-K.H., unpubl. data), we chose to use the latter midgestational mosaic *CAG::myr-Venus* ES cell clone for generating germline transmitting chimeras.

ES cell-mediated transgenesis was used for the generation of equivalent transgenic strains of mice. Chimeras transmitted their transgenes to F1 progeny in a Mendelian fashion, suggesting that widespread transgene expression is compatible with normal development and fertility. Hemizygous (*CAG::GFP-GPI*^{Tg/+} or *CAG::myr-Venus*^{Tg/+}) and homozygous (*CAG::GFP-GPI*^{Tg/Tg} or *CAG::myr-Venus*^{Tg/Tg}) animals were indistinguishable from each other and from wildtype littermates; they exhibited sustained widespread expression of the lipid-modified fusions and were viable and fertile, demonstrating the developmental neutrality of the lipid-modified fusion proteins. Homozygous animals exhibited identical sub-cellular distribution but increased fluorescence as compared to hemizygotes.

We examined the distribution of the two fusion reporters *in vivo* in hemizygous animals in different cells and at different developmental stages using laser scanning confocal microscopy.

The onset of the respective zygotic transgene expression was observed in early (uncompacted) morulae and was easily visualized as morulae compacted (Fig. 2a–d,j–m). Both transgenes were widely expressed and clearly labeled the plasma membrane, with a high signal-to-noise ratio, such that inner cells of the morula fated to form the inner cell mass and primitive endoderm could be distinguished from outer cells fated to form the trophectoderm. Widespread expression of both transgenes was maintained as development proceeded (Fig. 2). By E3.5 the blastocoelic cavity could be detected in both single optical sections (Fig. 2f,g,o,p) and in rendered stacks (Fig. 2h,i,q,r). In addition, close inspection of GFP-GPI revealed localization to other subcellular organelles, including the Golgi of the mural trophectoderm (yellow arrowhead, Fig. 2r).

Sagittal sections through embryos expressing the respective reporters demonstrated widespread expression at postimplantation stages (Fig. 3a,b). Even though the *CAG* regulatory elements drive widespread expression in ES cells, embryos, and adult mice (Niwa *et al.*, 1991; Okabe *et al.*, 1997; Hadjantonakis *et al.*, 1998), specific populations consistently expressed higher levels of the reporter (Hadjantonakis and Nagy, 2000; Lickert *et al.*, 2004), including the heart (Fig. 3a,b, arrowhead) and notochord (Fig. 3c–f). The GFP-GPI fusion was predominantly located at the plasma membrane of cells in the ventral region of the neural tube (Fig. 3e), whereas myr-Venus also exhibited additional diffuse cytosolic expression (Fig. 3f), which most likely reflects inherent differences in localization to different subcellular organelles.

The C-terminal region of Thy-1 incorporated into the GFP-GPI fusion has previously been shown to be sufficient to target basolateral proteins to the apical surface in MDCK cells (Brown *et al.*, 1989). Moreover, previous characterization of GFP-GPI localization in a transgenic mouse strain generated by pronuclear injection of DNA into zygotes revealed a tissue-inherent apical distribution of the protein (Kondoh *et al.*, 1999). Consistent with these reports, we observed increased GFP-GPI signal in the apical membranes of specific cells at different developmental stages in different tissues. For example, GFP-GPI expression was evident throughout the plasma membrane, but was more intense at the apical membranes of the endoderm (white arrowhead, Fig. 3g) at E9.5. In contrast, myr-Venus distribution was not polarized (white arrowhead, Fig. 3h), as it was present throughout the plasma membrane and cytosol, yet consistently excluded from the nucleus.

While our work supported and confirmed previous observations, previous work imaging GFP-GPI in a strain of transgenic mice generated by pronuclear DNA injection supports the apical localization of the GFP-GPI fusion that we observed in many tissues (Kondoh *et al.*, 1999). In contrast, we also detected basolateral localization. The apparent disparity in the localization of the GFP-GPI fusion between past and current investigations might be attributed to: 1) A difference in the level of transgene expression, such that if the current strain exhibits higher levels of protein expression a wider repertoire of localization may be observed. This is unlikely, since all three GFP-GPI ES cell lines evaluated in 4n chimeras displayed identical localization. 2) The difference in the method used for transgenesis (transfection of DNA into ES cells versus microinjection of DNA into zygotes). We believe that this is unlikely, as the product of both methodologies (the transgenic strain) should yield comparable results. Furthermore, our past experience has demonstrated that reporter localization does not correlate with mode of transgenesis. 3) A difference in the coding sequence of the transgene. Previously, the C-terminal sequence KLEN (where N is the omega-site) was used to encode the GPI attachment sequence, whereas in the fusion used here the lysine (K) was changed to a glutamine (Q), because it was found that the lysine residue was sensitive to some proteases (such as kallikrein), which may cause artificial release of GFP-GPI from the cell membrane. We therefore favor the latter hypothesis.

To determine the distribution of the respective lipid-modified fusion reporters at a later stage during embryogenesis and organogenesis, we examined their distribution at E13.5. Transverse sections through a *CAG::GFP-GPI* or *CAG::myr-Venus* hemizygous transgenic embryos revealed widespread distribution of the respective lipid-modified fluorescent fusions (Fig. 3i,j). Higher-magnification views of the ventral region of the developing spinal cord (Fig. 3k,l) revealed high levels of expression in the floorplate extending into the ependymal layer in both *CAG::GFP-GPI* and *CAG::myr-Venus* animals. Furthermore, differences in cell morphology were easily visualized in the differentiating ventral motor and interneurons within the mantle layer at these later stages (Fig. 3i,k) compared to the E9.5 neural tube (Fig. 3e,f). In addition, distinct staining throughout the marginal layer marking the future white matter was observed (Fig. 3i,k).

In the developing lung, the bronchioles revealed a polarized localization of GFP-GPI that was enriched within apical regions of the plasma membrane (white arrowhead, Fig. 3m). This is in contrast to the nonpolarized distribution of myr-Venus. Moreover, stromal cells surrounding some, but not all, bronchioles consistently expressed high levels of the respective fusions (red arrowhead, Fig. 3m,n). Optical sections of transverse cuts through the developing heart (Fig. 3o,p) revealed an enrichment of fluorescent membranes within the myocardium. GFP-GPI was observed throughout the plasma membrane, including clusters of fluorescence present in some cells (yellow arrowhead, Fig. 3o), in the Golgi (red arrowhead, Fig. 3o), and intracellular secretory vesicles (blue arrowhead, Fig. 3o). In addition to these populations, myr-Venus was also observed in perinuclear regions (green arrowhead, Fig. 3p) and microtubule organizing centers (MTOCs, red arrowhead, Fig. 3p). These data demonstrate the dynamic and cell type dependent subcellular distribution of the GFP-GPI and myr-Venus fusions during embryogenesis.

Consistent with our previous findings using the *CAG* regulatory elements (Hadjantonakis *et al.*, 1998; Hadjantonakis and Papaioannou, 2004; Long *et al.*, 2005), low-magnification wide-field microscopic examination of organs obtained from hemizygous (Tg/+) 4-day-old (P4), 6-week-old, and 3-month-old animals of both sexes revealed widespread expression of both GFP-GPI and myr-Venus (data not shown). Confocal images were acquired from sections of organs isolated from postnatal day (P)4 animals. Low-magnification images of transgenic kidneys revealed widespread expression of both fusion proteins in respective transgenic embryos (Fig. 4a,b). As previously reported (Hadjantonakis *et al.*, 1998), the *CAG* promoter drives increased expression in the glomeruli (red arrowheads, Fig. 4a–d). Apical localization in arterioles (white arrowhead, Fig. 4c), nonpolarized membrane distribution in Bowman's capsule (yellow arrowhead, Fig. 4c), and diffuse cytoplasmic staining in the collecting ducts (blue arrowhead, Fig. 4c) demonstrated the diverse, cell context dependent distribution of GFP-GPI. The distribution of myr-Venus was similar to that of GFP-GPI in the cells of Bowman's capsule (yellow arrowhead, compare Fig. 4c,d) but was not apically localized in arterioles (white arrowhead, compare Fig. 4c,d).

Strong fluorescence was detected within the P4 heart of both *CAG::GFP-GPI* and *CAG::myr-Venus* transgenic lines (Fig. 4e,f). Optical sections revealed that GFP-GPI was localized at the plasma membrane (yellow arrowhead, Fig. 4g), perinuclear regions (blue arrowhead, Fig. 4g), and intracellular vesicles (red arrowhead, Fig. 4g). Higher-magnification views revealed the absence of cell borders in fused cardiomyocytes (Fig. 4h). Fluorescence was observed in vesicles distributed throughout the syncytium, the sarcoplasmic reticulum (blue arrowhead, Fig. 4h), on the Golgi (red arrowhead, Fig. 4h), and on the membranes of presumably unfused cells (yellow arrowhead, Fig. 4h).

In polyploid cells of the P4 liver, neither of the two distinct reporter fusions were concentrated at cell boundaries (Fig. 4i,j). Rather, they showed similar diffuse distributions throughout the

cytoplasm. Furthermore, 3D rendering of a field of liver tissue revealed that GFP-GPI was present on the plasma membrane of mononuclear hepatocytes (yellow arrowhead, Fig. 4k), microsomes (blue arrowhead, Fig. 4k), and Golgi (red arrowhead, Fig. 4k). The position of the Golgi with respect to the nucleus (white arrowhead, center panel, Fig. 4k,) was illustrated by obscuring of the structure in the merged panel. myr-Venus was localized to the plasma membrane (yellow arrowhead, Fig. 4l), nucleus (red arrowhead, Fig. 4l), and throughout the cytoplasm of hepatocytes. However, myr-Venus was absent from vacuolar structures within these cells (white arrowhead, Fig. 4l). The nucleus of a cell expressing myr-Venus is marked with a red arrowhead to highlight its position in the merged panel (center panel, Fig. 4l).

Sections through the P4 small intestine demonstrated that GFP-GPI was strongly expressed on membranes of cells within the lamina propria (yellow arrowhead, Fig. 4m) and muscularis (white arrowhead, Fig. 4m). However, the signal was diffuse throughout the mucosal epithelium (blue arrowhead, Fig. 4m). In contrast, myr-Venus expression was clearly present in the lateral membranes of the mucosal epithelium (green arrowhead, Fig. 4n) and apical brush borders (red arrowhead, Fig. 4n), in addition to punctate nuclear staining (blue arrowhead, Fig. 4n). Similar to the pattern observed in hepatocytes, myr-Venus was excluded from vacuolar structures inside the cytosol of cells of the mucosal epithelium (white arrowhead, Fig. 4n). We also examined fixed coronal sections of P4 brains to demonstrate widespread distribution of both fluorescent proteins (Fig. 4o,p). These fluorescent reporters were subcellularly localized, suggesting that these transgenic lines can be used for the study of neuronal cell fate and behavior, similar to previous reports (Rodriguez *et al.*, 1999; Feng *et al.*, 1999).

To investigate the utility of combinatorially imaging GFP-GPI and myr-Venus for investigating mechanisms of subcellular organelle trafficking, we examined their combined distributions in cells of live double transgenic (*CAG::GFP-GPI/+*; *CAG::myr-Venus/+*) E9.5 embryos. Due to the similarity in GFP and Venus spectra, linear unmixing (Dickinson *et al.*, 2001; Hadjantonakis *et al.*, 2003) was used to separate GFP and Venus fluorescence in vivo (Fig. 5). As expected from examinations of single transgenic embryos, we found both distinct and overlapping domains of fluorescent protein localization. An example of a distinct myr-Venus domain is shown in an epithelial cell of a live transverse view of somites (yellow arrowhead, Fig. 5a). As observed in fixed sections of E9.5 embryos, GFP-GPI is concentrated in the apical plasma membrane domain of live double transgenic gut, whereas the myr-Venus reporter does not exhibit a polarized distribution (white arrowhead, Fig. 5b). Finally, an image of live intermediate mesoderm is presented to highlight overlapping distributions on the plasma membranes (blue arrowhead, Fig. 5c). These data confirmed our ability to segregate distinct lipid moieties (Munro, 2004) in a single double transgenic animal in vivo, where cells exist in the context of their normal microenvironment (Bissell and Labarge, 2005).

To extend our observations of live ES cells and early embryos to verify labeling of the secretory pathway, and in particular the Golgi apparatus, the subcellular localization of the GFP-GPI and myr-Venus fusions was determined with respect to Golgi markers. Markers used included antibodies directed against a 58K microtubule binding protein associated with the cytoplasmic surface of the Golgi apparatus (Fig. 6) and β -COP (data not shown). Cells were processed for indirect immunofluorescence, with fluorescent protein expression visualized directly. The protocol used required both fixation of cells and subsequent detergent permeabilization and resulted in a more diffuse localization of the lipid-modified fluorescent proteins compared to live cells.

Since ES cells grow in clumps and have an unusual morphology, with a large nucleus and minimal cytoplasm, we were unable to unequivocally confirm colocalization of the lipid-modified fluorescent proteins with secretory pathway markers (Fig. 6a,c). However, GFP-variant fluorescence was covisualized with indirect immunofluorescence using the 58K Golgi-

specific antibodies in ES cells that had been induced to differentiate (regions appearing yellow in merged panels, Fig. 6b,d). The colocalization of GFP-GPI and myr-Venus fluorescence with Golgi markers confirmed the localization of these fusions to the Golgi apparatus, suggesting that these fusion reporters could be used to monitor Golgi dynamics. Furthermore, the ability to visualize the position of the Golgi within individual cells in vivo should permit the determination of polarity in certain cell types.

A current challenge in the fields of cell and developmental biology is to directly visualize the spatial and temporal organization of intracellular events, including the dynamic and polarized nature of vesicle transport (Rothman, 2002). Vesicles represent the compartmentalization of modular components that are critical for the regulation of different processes, including cell polarity (Nelson, 2003) and cell fate (LeBorgne, *et al.*, 2005; Wang and Struhl, 2005). To illustrate the utility of the *CAG::GFP-GPI* and *CAG::myr-Venus* transgenic lines for this purpose, we examined individual vesicular populations in live ES cells and embryonic tissues (Fig. 7).

To label recycling endosomal populations, we cultured ES cells and E9.5 embryos in media supplemented with Alexa-Fluor 568-conjugated 10K Dextran and imaged them. We observed both distinct (green or red arrowheads, Fig. 7a,b) and overlapping (yellow arrowheads, Fig. 7a,b) distributions of the lipid-modified fluorescent protein-tagged proteins and moieties in ES cells having taken up and therefore been tagged with 10K Dextran (red arrowheads, Fig. 7a,b). Furthermore, to demonstrate these properties in cells operating in the context of their normal microenvironment, we next examined these two distributions in somites (Fig. 7c–h). Lateral views at low (Fig. 7c,d) or high (Fig. 7e–h) magnification are presented to show the gross and fine distributions of both the fluorescent protein fusions and 10K Dextran in live tissues. We found that 10K Dextran became trapped in the extracellular matrix of live tissue, resulting in high background fluorescence (red, Fig. 7c,d). High-magnification views of somites show that 10K Dextran is localized in discrete vesicles, which appear similar to those observed in ES cells. These Dextran-positive vesicles both overlap (yellow arrowheads, Fig. 7e–h) and are distinct (red arrowheads, Fig. 7g,h) with the respective fluorescent protein fusions (green arrowheads, Fig. 7e–h). Combined, these data suggest that GPI-GFP and myr-Venus localize to distinct vesicles and overlap with endosomes of the secretory pathway.

CONCLUSIONS

It is widely recognized that dynamic cell behaviors, including the orientation of cell division, and regulation of cell morphology, and cell polarity, underlie tissue morphogenesis. Fluorescent proteins represent important optical imaging tools that can be used for the elucidation of dynamic cell behaviors in vivo in diverse systems. Moreover, fluorescent protein fusions that are subcellularly localized permit investigations at subcellular resolution. Here we report the generation and preliminary characterization of two transgenic mouse strains that exhibit widespread expression of lipid-modified GFP-variant fusions (GFP-GPI and myr-Venus) that target the respective fluorescent proteins to the outer and inner leaflet of the plasma membrane. We demonstrated the developmental neutrality of these GFP fusions in ES cells and mice, paving the way for their use in targeted and transgenic regimes.

While fluorescent reporter fusion proteins have served as invaluable tools for investigating dynamic inter- and intracellular events in cell culture-based assays, these interactions must ultimately be examined in vivo. Lipid-modified fluorescent protein fusion expressing strains of mice will be useful for: 1) labeling the plasma membrane and visualization of dynamic cell morphologies, cell behaviors, and intercellular interactions, and 2) investigating dynamic intracellular behaviors such as vesicular dynamics, in vivo in transgenic animals, chimeras or transplantation models, and in explant cultures.

High-resolution imaging of live and fixed samples revealed a distinct and dynamic subcellular localization of these two fluorescent protein fusions to different lipid-enriched compartments that are both cell type- and time-dependent. Both fusion reporters were found on plasma membranes. However, GFP-GPI was localized more prominently on membranes than myr-Venus. Additionally, GFP-GPI was observed at increased levels in apical plasma membrane domains of epithelial tissues. Both fluorescent protein fusions localized at the Golgi complex, revealing the highly dynamic nature of this organelle. Finally, each fusion protein was targeted to distinct organelles of different sizes.

The availability *CAG::GFP-GPI* and *CAG::myr-Venus* animals represent reagents that can be used for in vivo imaging of cell morphology. Moreover, when combined with additional spectrally distinct fluorescent reporters they will permit 4D analysis of cellular and molecular dynamics in vivo. For example, covisualization of fluorescent proteins labeling the plasma membrane with histone H2B fusions labeling chromatin should allow simultaneous tracking of cell position, division, and death in conjunction with dynamic changes in cell morphology. Furthermore, these lipid-modified fluorescent protein-expressing strains will now allow dynamic investigations of the secretory pathway in an organismal context by permitting analyses of cellular dynamics in vivo. These animals represent a unique reagent for characterizing discrete and dynamic subcellular features integral to cell behavior and cell fate specification (Le Borgne *et al.*, 2005; Lippincott-Schwartz *et al.*, 2000). For example, the endocytic itineraries of these fluorescent reporters can be investigated. Therefore, these novel strains of mice should permit the in vivo characterization of plasma membrane to Golgi cycling pathways. As a result, we can now begin to elucidate the genetic basis for basic cell biological questions and dynamic cell behaviors by observing them in vivo during development, homeostasis, and disease progression, in both wildtype and mutant contexts.

MATERIALS AND METHODS

Vector Construction

The *pCX::GFP-GPI* plasmid has been described previously (Kondo *et al.*, 1999) and contains the N-terminal membrane translocation signal sequence from acrosin (MVEMLPTVAVLVLAHSVVA), and a C-terminal sequence derived from the Thy-1 N-terminal GPI-linked signal sequence (KDNTTLQEFAT-LAN).

To generate myristoylated fluorescent fusion proteins, an N-terminal myristoylation tag derived from Src was generated by adding the sequences MGSSKSKPK to the N-terminus of any given fluorescent protein. This was achieved by using the following oligonucleotide: 5' GFP-MYR-Eco (5'-CTT GAA TTC GCC ACC ATG GGA AGC AGC AAG AGC AAG CCA AAG GTG AGC AAG GGC GAG GAG CTG). The GFP and Venus coding sequences were amplified from pEGFP-N1 (BD Biosciences, San Jose, CA) and pCS2-Venus (Nagai *et al.*, 2002) to generate myr-GFP and myr-Venus, respectively, by high-fidelity polymerase chain reaction (PCR) using *Pfx* Polymerase (Invitrogen, La Jolla, CA) with the 5' myristoylation primer combined with a 3'-GFP primer (5'-GTC ATG AAT TCT TAC TTG TAC AGC TCG TCC) primer, respectively. The resulting product was cloned into the *EcoRI* site of *pCAGGS* to generate *pCX::myr-EGFP* and *pCX::myr-Venus*.

Cos-7 cells were transiently transfected using the Eugene 6 Transfection Reagent according to the manufacturer's recommendations (Roche, Nutley, NJ). Cells were imaged 24–36 h after transfection.

Generating Transgenic ES Cells

Transgenic ES cell lines constitutively expressing myr-GFP, myr-Venus, or GFP-GPI were generated by coelectroporation of a *Sall* or *ScaI* linearized *pCX-myr-EGFP*, *pCX::myr-Venus* or *pCX::GFP-GPI* construct along with a circular *PGK-Puro-pA* plasmid conferring transient puromycin resistance (Tucker *et al.*, 1996). Puromycin selection was carried out as described previously (Hadjantonakis *et al.*, 1998, 2002). Fluorescent colonies were identified and picked under an epifluorescence stereo dissecting microscope. Clones were passaged in 96-well plates according to standard protocols (Nagy *et al.*, 2002), and scored for the maintenance, subcellular localization, and level of fluorescence upon passage in culture in the absence of selection.

Chimera Production and Mouse Breeding

Tetraploid (4n) chimeras were used to evaluate trans-gene expression and developmental potential and were generated as described previously (Hadjantonakis *et al.*, 1998, 2002). ES cell clones exhibiting robust fluorescent protein expression in vivo in tetraploid embryo chimeras were selected for germline transmission. Subsequently, one *CAG::myr-Venus* and two *CAG::GFP-GPI* ES cell lines were used for diploid chimera generation. Chimeras were mated to outbred ICR and inbred 129/Tac mice (Taconic, Germantown, NY) for germline transmission. Since each of the two clones *CAG::GFP-GPI* lines exhibited comparable expression of each transgene, thereafter only one line was maintained for further analysis. Animals from both lines exhibited widespread fluorescence in all subsequent generations tested ($n = 5$), and therefore data were pooled. All transgenes were homozygous viable (*CAG::GFP-GPI*^{Tg/Tg} or *CAG::myr-Venus*^{Tg/Tg}) on an ICR background.

Embryo Collection

Preimplantation embryos were recovered in M2 media and subsequently cultured in KSOM media under mineral oil in an incubator at 5% CO₂. Postimplantation embryos and organs were dissected either in HEPES buffered DMEM containing 10% fetal calf serum (FCS) or phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) or 10% FCS, and cultured in media comprising 50% rat serum, 50% DMEM/F12 supplemented with L-glutamine. Embryos were fixed in 4% paraformaldehyde in PBS. All data presented are from hemizygous (Tg/+) animals. For determining colocalization of the two fluorophores in vivo, hemizygous double transgenic embryos were generated by intercrossing hemi- or homozygous adults.

Vibrating Microtome Sectioning

Specimens were either embedded and sectioned immediately after dissection or fixed in 4% paraformaldehyde for 4–72 h, washed 3× in PBS, and embedded in a mixture of 5% sucrose, 4% low melting point agarose in PBS. Blocks were trimmed using a scalpel blade, then mounted onto a vibrating microtome (Leica VT1000) chuck using superglue. Sections were cut at a thickness of 50–200 μm.

Vital Dye Counterstaining

Live ES cells were placed in a 1:400 dilution of Draq5 (DAKO, Carpinteria, CA) in DMEM + 10% FCS for 10 min prior to imaging, washed, and imaged in DMEM + 10% FCS. Vibrating microtome sections were placed in a 1:200 dilution of Hoechst 33342 (Molecular Probes, Eugene, OR) overnight at room temperature in PBS prior to imaging, then washed and imaged in PBS.

Immunofluorescence

Samples were fixed in 2% paraformaldehyde in PBS for 20 min at room temperature, washed three times in PBS, then once in PBS + 1% BSA + 2% goat serum + 0.2% Triton X-100, then incubated in a solution of PBS + 1% BSA + 2% goat serum + 0.2% Triton X-100 containing primary antibody for ~3 h. The primary antibody solution was rinsed off and then replaced with Alexa-Fluor 543 secondary antibody (Molecular Probes) solution for 60 min. Samples were then washed three times in PBS + 1% BSA + 2% goat serum + 0.2% Triton X-100, stained with DAPI (Molecular Probes), and mounted in ProLong anti-fade (Molecular Probes) for imaging. Primary antibodies used were anti-Golgi 58K (Sigma, St. Louis, MO) and anti- β -cop (Sigma).

Dextran Uptake Assay

Live ES cells or embryos were washed in DMEM containing no FCS. Samples were then cultured in DMEM containing of 0.5 mg/ml Alexa-Fluor 568 conjugated 10K Dextran (Molecular Probes) for 15 min. Samples were then washed 2 $\times$ in DMEM and placed in culture media containing 10% FCS (cells) or 50% rat serum (embryos). Samples were placed back in a heated and humidified incubator for an additional 15–30 min and then placed on the microscope stage for live imaging.

Image Acquisition

Live ES cells and embryos were imaged in coverslip-bottomed dishes (MatTek, Ashland, MA). Fixed samples prepared for immunofluorescence were imaged on glass coverslips placed on microscope slides. For live imaging experiments, embryos were maintained under physiological conditions in a temperature-controlled, humidified chamber in a 5% CO₂ atmosphere (Solent Sci, UK). Laser scanning confocal data was acquired in using a Zeiss LSM510 META on a Zeiss Axiovert 200M. Fluorophores were excited with a 405-nm diode laser (DAPI/Hoechst), 488-nm argon laser (EGFP/Venus), a 543-nm HeNe laser (Alexa543/Alexa568). Objectives used were a Plan-Neofluar 40x/NA1.3, C-apochromat 40x/NA1.2, plan-apochromat 20x/NA0.75 and a fluar 5x/NA0.25. Confocal images were acquired as z-stacks of x,y images taken at 0.2–2 μ m z-intervals.

Image Processing

Raw data were processed using Zeiss AIM software (Carl Zeiss Microsystems at <http://www.zeiss.com/>), and Volocity (Improvision at <http://www.improvision.com/>). GFP fluorescence was pseudocolored green, Venus fluorescence was pseudocolored yellow, green, or red depending on figure, Alexa548 and 633 were pseudocolored red, and Draq5 and Hoechst were pseudocolored blue in all figures. Reanimation and annotation of data to generate movies of time-lapses or rotations were performed using QuickTime Pro (Apple Computer at <http://www.apple.com/quicktime/>).

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FIG. 1.

Localization of GPI- and myristoyl-tagged fluorescent proteins in live mouse ES cells. Live imaging of *CAG::GFP-GPI* and *CAG::myr-Venus* hemizygous transgenic ES cells. The left column represents a single channel fluorescence image of either GFP-GPI or myr-Venus expression. The middle column represents nuclear fluorescence visualized using Draq5. The right column represents merged images of the GFP/Venus and Draq5 fluorescent channels.

a: Single *xy* section taken from a *z*-stack of *CAG::GFP-GPI*/+ ES cells. **b:** Single *xy* section taken from a *z*-stack of *CAG::myr-Venus*/+ ES cells. **c:** 3D rendered image of a *z*-stack of 30 *xy* sections taken at 0.3 μm intervals of *CAG::GFP-GPI* ES cells. **d:** Image in **c**, tilted towards the viewer to show lateral information. **e:** 3D rendered image of a *z*-stack of 30, 0.3 μm optical sections of *CAG::myr-Venus*/+ ES cells. **f:** Image in **e**, tilted towards the viewer to show lateral information. All images were taken using a Plan-Neofluar 40x/1.3 objective. The electronic zoom factors (ZF) were: a = 5.1, b = 3.6, c and d = 5.0, e and f = 5.2. See text for description of arrows. The RGB-colored vector on the bottom left of the 3D rendered images depicts the orientation of *x*-axis in green, *y*-axis in red, and *z*-axis in blue. Note that the 3D reconstructions (**c–f**) do not include the full height of cells, otherwise the plasma membrane would obscure the visualization of information from within each cell. In all panels GFP fluorescence is displayed in green, Venus fluorescence in yellow, and Draq5 fluorescence in blue.

FIG. 2.

GPI-linked and myristoylated fluorescent protein localization in live preimplantation mouse embryos. Living embryos hemizygous for the *CAG::myr-Venus* or *CAG::GFP-GPI* transgenes were imaged by confocal laser scanning microscopy. A single *xy* section taken from a *z*-stack of a morula stage embryo, brightfield image (**a**: *CAG::GFP-GPI*/+; **j**: *CAG::myr-Venus*/+), overlay of single confocal section or yellow fluorescence and brightfield (**b**: *CAG::GFP-GPI*/+; **k**: *CAG::myr-Venus*/+), yellow fluorescence channel only (left panel in **c**: *CAG::GFP-GPI*/+; **l**: *CAG::myr-Venus*/+). Single fluorescence (*xy*) images taken at intervals through the *z*-stack (**c**: *CAG::GFP-GPI*/+; **i**: *CAG::myr-Venus*/+). Rendered *z*-stack (3D reconstruction) of computationally bisected embryo rotated 180° around the *y*-axis depicted in five incremental steps (**h**: *CAG::GFP-GPI*/+; **q**: *CAG::myr-Venus*/+). A single *xy* section taken from a *z*-stack of a blastocyst stage *CAG::myr-Venus*/+ embryo, brightfield image (**e**: *CAG::GFP-GPI*/+; **n**: *CAG::myr-Venus*/+), overlay of single confocal section or yellow fluorescence and brightfield (**f**: *CAG::GFP-GPI*/+; **o**: *CAG::myr-Venus*/+), yellow fluorescence channel only (left panel in **g**: *CAG::GFP-GPI*/+; **p**: *CAG::myr-Venus*/+). Single fluorescence (*xy*) images taken at intervals through the *z*-stack (**g**: *CAG::GFP-GPI*/+; **p**: *CAG::myr-Venus*/+). Rendered *z*-stack (3D reconstruction) of computationally bisected embryo rotated 180° around the *y*-axis depicted in five incremental steps (**h**: *CAG::GFP-GPI*/+; **q**: *CAG::myr-Venus*/+). Rendered *z*-stack (3D reconstruction) of a thin (10 μ m) slice taken from the *z*-stack rotated 180° around the *y*-axis depicted in five incremental steps (**i**: *CAG::GFP-GPI*/+; **r**: *CAG::myr-Venus*/+). In all images Venus fluorescence is depicted in yellow and GFP fluorescence in green. All images

were taken using a Plan-Neofluar 40x/1.3 objective. The RGB-colored vector on the bottom left of the 3D reconstruction rotations (**d,h,i,m,q,r**) depicts the x -axis in green, y -axis in red, and z -axis in blue. Red arrowheads highlight the position of the blastocoelic cavity; orange arrowhead highlights the remnants of the second polar body, blue arrowhead highlights a cell in mitosis; yellow arrowhead highlights the Golgi apparatus of a mural trophoctoderm cell. In all panels GFP fluorescence is displayed in green and Venus fluorescence in yellow.

gut as marked in **d**. **i–p**: E13.5 embryos transgenic for and expressing either GFP-GPI or myr-Venus. **i**: Transverse section through the thoracic region of *CAG::GFP-GPI/+* embryo (5× objective, ZF 0.7×). **j**: Transverse section through the thoracic region of *CAG::myr-Venus/+* embryo. **k**: Higher magnification of ventral neural tube as marked in **i** (40× objective, ZF 0.7×). **l**: Higher magnification of ventral neural tube as marked in **j**. **m**: Higher magnification of lung as marked in **l** (40× objective, ZF 0.7×). **n**: Higher magnification of lung as marked in **j**. **o**: High magnification of a sagittal section through the heart of *CAG::GFP-GPI/+* embryo (40× objective, ZF 4×). A field of developing myocardium is shown. **p**: High magnification of a sagittal section through the heart of *CAG::myr-Venus/+* embryo. ht (heart), NT (neural tube), not (notochord), FP (floorplate), MA (mantle layer), ML (marginal layer), br (bronchiole), myo (myocardium). In all panels GFP fluorescence is displayed in green, Venus fluorescence in yellow, and Hoechst fluorescence in blue.

FIG. 4.

Widespread expression and subcellular localization of GPI-linked and myristoylated fluorescent protein expression in adult mouse tissues. Tissues from P4 animals hemizygous for either transgenic were used for all images. Venus fluorescence is depicted in yellow, GFP fluorescence in green, and Hoechst in blue. **a:** Composite of longitudinal section through kidney of *CAG::GPI-GFP/+* animal (5× objective, ZF 0.7×). **b:** Composite of longitudinal section through kidney of *CAG::myr-Venus/+* animal. **c:** Higher magnification of region marked in **a** (40× objective, ZF 1.6×). **d:** Higher magnification of region marked in **b**. **e:** Cross section through a *CAG::GPI-GFP/+* heart (5× objective, ZF 0.7×). **f:** Cross section through a *CAG::myr-Venus/+* heart (5× objective, ZF 0.7×). **g:** High magnification of single optical

section from **e** (40× objective, ZF 4×). **h**: High magnification of single optical section from **f** (40× objective, ZF 4×). **i**: High magnification of single optical section through *CAG::GPI-GFP/+* liver (40× objective, ZF 4×). **j**: High magnification of single optical section through *CAG::myr-Venus/+* liver (40× objective, ZF 4×). **k**: 3D-rendered image of 34, 0.5 μm optical sections of *CAG::GPI-GFP/+* liver tissue (40× objective, ZF 4×). The RGB-colored vector on the bottom left of the 3D rendered images depicts the *x*-axis in green, *y*-axis in red, and *z*-axis in blue. **l**: High magnification of single optical section through *CAG::myr-Venus/+* liver (40× objective, ZF 4×). **m**: Cross section through a *CAG::GPI-GFP/+* small intestine (40× objective, ZF 0.7×). **n**: Cross section through a *CAG::myr-Venus/+* small intestine villi (40× objective, ZF 1.6×). **o**: Single optical section through the telencephalon of *CAG::GPI-GFP/+* animal (5× objective, ZF 0.7×). Low magnification inset represents a composite from 12 single images. **p**: Single optical section through the telencephalon of *CAG::myr-Venus/+*. Low magnification inset represents a composite of 12 single images. kid (kidney), si (small intestine), vi (villus), liv (liver).

FIG. 5.

Differential localization of GFP-GPI and myr-Venus fusion proteins in E9.5 *CAG::GFP-GPI/+; CAG::myr-Venus/+* double transgenic embryo. Linear unmixing of the independent GFP-variant fluorophores was used to identify unique and overlapping regions of reporter localization in different epithelial tissues. GPI-GFP fluorescence is depicted in green, myr-Venus fluorescence is false-colored red, and background residual pixels are in blue. **a:** Reporter expressions in E9.5 somites. Yellow arrowheads mark region of exclusive myr-Venus expression. **b:** Reporter expressions in E9.5 developing gut tube. White arrowheads mark exclusive GPI-GFP expression. **c:** Reporter expressions in E9.5 intermediate mesoderm. Blue arrowheads mark overlapping reporter expression.

FIG. 6.

Colocalization GFP-GPI or myr-Venus fusion proteins with Golgi markers in ES cells.

Undifferentiated GFP-GPI ES cells (**a**) were stained and visualized for DAPI to label nuclei (blue), GFP was visualized directly (green), 58K Golgi was visualized with indirect immunofluorescence (red). The three fluorescent channels were merged (panel on the right). GFP-GPI ES cells mixed with nontransgenic ES cells and induced to differentiate (**b**) were stained and visualized for DAPI (blue), GFP (green), 58K Golgi (red). The three fluorescent channels were merged (panel on the right). Yellow spots that are perinuclearly located in four cells reveal regions of fluorescent protein and Golgi colocalization. Undifferentiated myr-Venus ES cells (**c**) were stained and visualized for DAPI (blue), Venus was visualized directly (green), 58K Golgi was visualized with indirect immunofluorescence (red). The three fluorescent channels were merged (panel on the right). Diffuse regions of yellow coloring that are perinuclearly located reveal regions of colocalization. Myr-venus ES cells mixed with nontransgenic ES cells and induced to differentiate (**d**) were stained and visualized for DAPI (blue), Venus (green), 58K Golgi (red). The three fluorescent channels were merged (panel on the right). Yellow spots that are perinuclearly located in two cells reveal regions of colocalization. Scale bars on the bottom right of all merge panels = 20 μ m.

FIG. 7.

Differential vesicular distributions of GFP-GPI and myr-Venus fusion proteins in ES cells and in the somites of E9.5 transgenic embryos. Hemizygous GFP-GPI ES cells (**a**), myr-Venus ES cells (**b**), E9.5 GFP-GPI (**c,e,g**) or E9.5 myr-Venus (**d,f,h**) embryos were cultured in the presence of 0.5 mg/ml 10K Dextran to identify distributions to recycling endosomes of the secretory pathway. **a:** GPI-GFP ES cells treated with 10K Dextran show both distinct (green arrows, GPI-GFP-specific; red arrows, 10K Dextran specific) and common (yellow arrows, overlapping) distributions after treatment. **b:** myr-Venus ES cells treated with 10K Dextran show both distinct (green arrowheads, myr-Venus-specific; red arrowheads, 10K Dextran-specific) and common (yellow arrowheads, overlapping) distributions after treatment. **c:** Low magnification of lateral view of somite from E9.5 GFP-GPI embryo treated with 10K Dextran. 10K Dextran was observed in subcellular vesicles and trapped in the extracellular matrix after treatment. **d:** Low magnification lateral view of somite from E9.5 myr-Venus embryo treated with 10K Dextran. **e,g.** Higher magnification views of GPI-GFP somite show distinct (green arrowheads, GFP-GPI-specific; red arrowheads, 10K Dextran-specific) and common (yellow arrowheads, overlapping) distributions of the reporters. **f,h:** Higher magnification views of myr-Venus somite show distinct (green arrowheads, myr-Venus-specific; red arrowheads, 10K Dextran-specific) and common (yellow arrowheads, overlapping) distributions of the reporters. GFP-GPI and myr-Venus are both false-colored green, 10K Dextran is false-colored red.