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Toolbox

A Functional GFP Fusion for Imaging Clathrin-Mediated Endocytosis

Joshua Z. Rappoport[1,2,*] and Sanford M. Simon[1,*]

[1]Laboratory of Cellular Biophysics, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA
[2]Current address: School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK
**Corresponding authors: Joshua Z. Rappoport, j.rappoport@bham.ac.uk and Sanford M. Simon, simon@rockefeller.edu*

The ability to localize proteins of interest in live cells through imaging inherently fluorescent protein tags has provided an unprecedented level of information on cellular organization. However, there are numerous cases where fluorescent tags alter the localization and/or function of the proteins to which they are appended. Clathrin-mediated endocytosis from the plasma membrane is a physiologically important process evolutionarily conserved from yeast to humans. Some proteins that are associated with the machinery of clathrin-mediated endocytosis have been tagged with fluorescent proteins. However, it has not yet been possible to study this process through a protein marker that is specific to this step and still fully functional when linked to a fluorescent protein. In this study, we present the first demonstration that one of these proteins, in this case a green fluorescent protein (GFP) fusion to α-adaptin, a marker of the adaptor protein-2 complex, functionally complements knockdown of endogenous protein through small interfering RNA silencing. GFP–α-adaptin, as well as the techniques used to test the fusion protein, represents an important contribution to the cell biologist's toolbox, which will permit a greater understanding of vesicle trafficking in live cells.

Key words: AP-2, clathrin, endocytosis, GFP, siRNA

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The ability to localize a protein with antibodies in fixed samples has provided tremendous insight into the organization and the diversity of organization of cells. The ability to fluorescently tag a protein adds the additional capability of following the movement of a protein in live cells and in response to stimuli. The earliest technologies used fluorescent antibodies to tag a protein or proteins that were purified, chemically labeled and then reinjected back into cells. Such studies helped to reveal details of the dynamics of the cytoskeleton (1). The observation that these chemically labeled proteins were reincorporated back into actin or microtubules suggested that these approaches were, at a minimum, not lethal to the cells.

A significant advance has been the ability to genetically modify a protein so that it is fluorescent. This approach has a number of distinct advantages: the protein no longer needs to be purified, chemically labeled and reintroduced back into cells; the location of the fluorophore on the protein is determined by the experimenter, unlike chemical labeling that often modifies multiple different sites; there is one fluorophore for each expressed protein, whereas in chemical labeling, the number of fluorophores on to protein is often variable, even within one labeling reaction; The protein can be expressed by its endogenous promoter.

Genetic encoding of fluorescence could be accomplished by expressing the protein as a fusion to a fluorescent protein (2), by expressing the protein with a tag that can bind a fluorophore (3), by expressing the protein with a peptide tag that can be enzymatically tagged with a fluorophore (4,5) or by expressing the protein in a fusion to one part of a split intein where the fluorophore to be conjugated is on the other half of the intein (6). Such studies have revolutionized the study of protein and organelle function.

A critical question that needs to be analyzed is whether the fluorescently tagged protein is a useful reporter of the activity of the endogenous protein. There are two major types of concerns. First, the presence of the fluorescent tag may affect the activity of the protein. Second, even if the activity of the fluorescently tagged protein is normal, it may not reflect the localization of the endogenous protein. The localization of many proteins is the consequence of binding to other proteins. If the protein of interest is not expressed at wild-type (wt) levels, the localization of the protein may be altered.

The use of endogenously fluorescent protein tags, such as the green fluorescent protein (GFP), has become commonplace (7). In most cases, appending a GFP to a protein of interest does not seem to produce deleterious effects. Importantly, however, direct demonstration that a GFP-tagged protein retains wt functionality has not been widely applied in the field of mammalian cell biology.

A few examples of the kinds of problems that have been identified with fluorescently tagged proteins are given below.

Some GFP fusions are dominant negative

Some GFP fusion proteins override and block the function of the native protein. For example, GFP fused to the amino terminus of caveolin1 is a dominant-negative inhibitor of viral entry (e.g. SV40) (8,9). Similarly, yeast cells grow more slowly when a component of the spindle pole body, nuclear filament-related (NUF1), is expressed as a fusion to GFP [observation by T. N. David published in Cubitt et al. (10)].

Some GFP fusions are not functional

Expression of syncollin inhibits secretion, but a GFP fused to the carboxyl terminus of syncollin has no effect on secretion (11). For example, actin fused to GFP cannot function as the sole actin source in yeast (12). GFP fused to the carboxyl terminus of synaptotagmin cannot rescue a synaptotagmin deletion, whereas expression of wt synaptotagmin can rescue (13). GFP fusions to either the amino or the carboxyl terminus of breast cancer resistance protein (BCRP), a drug resistance protein, are not functional (unpublished data).

Some GFP fusions alter function

Any tag (from a small myc tag to a GFP) put on the amino terminus of the cystic fibrosis transmembrane regulator disrupts the function of the protein (14). Any tag on the carboxyl terminus disrupts sorting (D. Gadsby, personal communication, The Rockefeller University, New York City).

Some GFP fusions induce an immune response from the animal

There have been reports that when GFP is expressed in an animal outside the cell (not inside the cytosol), an immune response is triggered (15).

Some GFP fusions induce aggregation

Some fluorescent proteins (wt GFP and tdTomato) are dimers and others (e.g. dsRed) are tetramers. These can result in inappropriate localization or aggregation of appended proteins of interest.

Given these problems, the onus is upon the researcher to ensure that due diligence is applied when expression of fluorescent proteins is employed. There are a number of independent criteria that can and should be used.

Does the expression of the fluorescent-tagged protein rescue a functional loss of the wt?

One potential concern is that the presence of the XFP (any color fluorescent protein) attached to our protein of interest may reduce or eliminate the activity of the protein. This can be tested by deliberately reducing the activity of the endogenous protein and testing if the XFP can restore function. There are many ways to produce a functional loss in a protein. Genetics can be used to generate a deletion or mutation that eliminates either the protein or its function. Molecular biology can be used to reduce the levels of messenger RNA expression in the cell. Toxins can be used to compromise the activity of the native protein (e.g. botulinum toxin to cleave endogenous SNAREs). In each case, the critical test is to determine if expression of the XFP fusion protein restores a functional activity after loss of the activity of the endogenous protein. This test determines if the XFP fusion protein can function sufficiently to compensate for loss of the wt protein. However, this does not resolve what fraction of the fusion protein is functional or properly localized.

Does the fluorescently tagged protein demonstrate a localization that is indistinguishable from the wt protein?

A XFP fusion protein may fully complement for loss of the wt protein, but its fluorescence may not reflect the localization of the endogenous protein. This could happen if the XFP fusion is synthesized at levels greater than the endogenous protein. A small percentage of the XFP fusion protein may properly localize and complement function, while the bulk of the XFP fusion protein may not be properly localized. Thus, it is essential to compare the fluorescence localization of the XFP fusion protein with static fixed images using antibodies to localize the endogenous protein. The advantage of studying the XFP fusion proteins is that they add the capability of following the dynamics of the protein.

Does expression of the fluorescent protein result in any negative effects on other cellular processes?

The XFP fusion protein may functionally complement loss of the endogenous protein, it may localize similarly to the endogenous protein, but if it is expressed at 'other than physiological levels', it may have adverse effects on physiology through binding of other cellular components. Such effects can potentially be detected by a quantitative assay of the physiological responses at various levels of expression of the XFP fusion protein. Flow cytometry analysis is a very powerful way to gather large amounts of information about the effects of varying levels of synthesis of a XFP fusion protein on a cellular process.

No single one of these approaches is sufficient to test if the expression of an XFP fusion protein is innocuous.

In the past few years, a large number of papers have been written using fluorescent fusion proteins and live cell imaging to study the process of clathrin-mediated endocytosis (16–24). One potentially confounding aspect of these studies is that clathrin itself is not solely localized to endocytic vesicles originating from the plasma membrane. Indeed, clathrin plays important functional roles at several cellular locations including the *trans* Golgi network, certain populations of endosomes and even the mitotic

spindle (25–27). Thus, a protein that only marks sites of clathrin-mediated endocytosis could be an extremely valuable addition to the cell biologist's toolbox, provided it fulfilled each of the criteria listed above. Interestingly, most proteins involved in clathrin-mediated endocytosis play roles in other cellular processes (e.g. dynamin) (28), act as a dominant negative when overexpressed (e.g. clathrin assembly lymphoid myeloid leukemia) (29) or do not cause a discernable phenotype when silenced (e.g. epsin) (30).

The adaptor protein-2 (AP-2) complex both links the clathrin coat to cargo sorting signals and serves as a hub for protein–protein interactions (31). Furthermore, there is currently no evidence that the AP-2 complex functions beyond roles in clathrin-mediated endocytosis. Several recent reports have demonstrated that knockdown of components of the AP-2 complex results in potent inhibition of clathrin-mediated endocytosis (32,33). As the AP-2 complex is a heterotetramer, there are numerous ways in which florescent proteins could be appended for use in imaging studies. However, the search for an appropriate fluorescent marker for the AP-2 complex has been challenging. GFP-tagged subunits have localized to plasma membrane clathrin-coated pits but have also been observed in localizations where the endogenous protein is not found [e.g. the seeming incorporation of overexpressed GFP-tagged β2 adaptin into AP-1 complexes (20) and the localization of σ subunit in the nucleus (21)]. More recently, an inability to rescue the endocytic phenotype resulting from silencing an endogenous component has also been observed (e.g. α-adaptin with a GFP on the carboxyl terminus, α-GFP) (34).

Each of our previous analyses of the AP-2 complex has employed a fusion protein of α-adaptin with a GFP on the amino terminus (GFP–α) (35). Our initial analyses demonstrated that GFP–α colocalized at the plasma membrane with markers for clathrin-mediated endocytosis. Furthermore, we also showed that this construct did not mislocalize into AP-1 complexes. Finally, we did not detect any dominant-negative effect in endocytosis assays performed following expression of GFP–α.

Presently, we have set out to determine whether GFP–α is capable of functionally substituting for endogenous α-adaptin. To ensure that our results were comparable with other studies, we have employed the same small interfering RNA (siRNA) as used in previous studies (30,32). The study that revealed that α-GFP was incapable of rescuing a knockdown phenotype had to employ site-directed mutagenesis to make their complementary DNA construct 'siRNA resistant' (34). In the case of GFP–α, our construct contained a single non-coding polymorphism in the key 'seed region' required for effective siRNA binding and subsequent silencing (36,37).

Our results demonstrate that GFP–α can indeed functionally complement following silencing of endogenous α-adaptin. When taken in the context of our previous study, this observation serves to directly demonstrate that GFP–α represents the first functional marker specific for clathrin-mediated endocytosis. Thus, GFP–α should remain a very useful addition to the cell biologist's toolbox.

Results and Discussion

To test the efficacy of the siRNA suppression of α-adaptin, we tested its effects on endocytosis of transferrin. After 10 min, fluorescently tagged transferrin is observed in the endocytic pathway (Figure 1). In contrast, in cells first pretreated with two rounds of siRNA to α-adaptin, no fluorescent transferrin is internalized (Figure 1). Thus, siRNA against α-adaptin can suppress the endocytic phenotype.

In our previous studies, we have employed a mouse α-adaptin construct tagged at the amino terminus with GFP (35,38,39). Starting at nucleotide 150, mouse α-adaptin

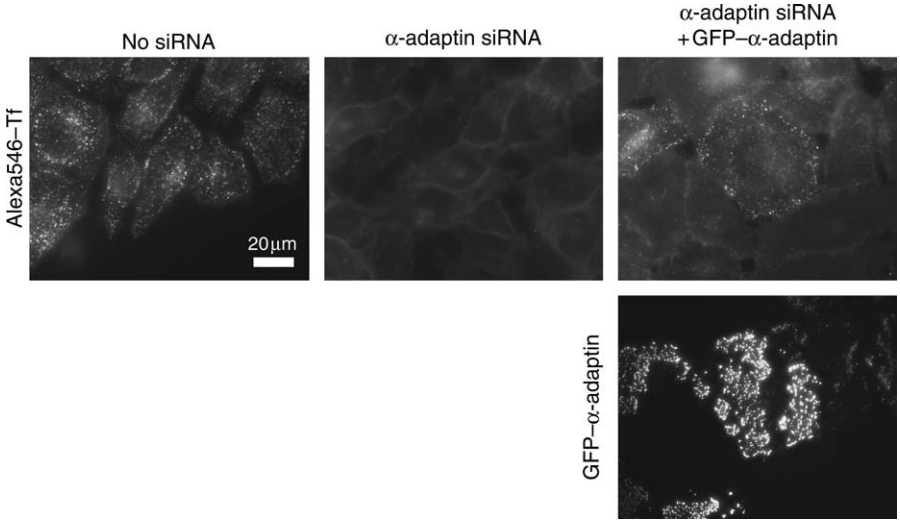

Figure 1: Imaging of transferrin uptake in HeLa cells: non-transfected (left), transfected with siRNA against α-adaptin (middle) or siRNA plus GFP–α (right). Top row shows fluorescence of transferrin (Tf) and bottom row of GFP–α-adaptin. Endocytosis of transferrin was decreased by siRNA, and this effect was reversed by coexpression of GFP–α-adaptin.

(NM_007459.2) reads GAGCATGTGCACGCTGGCCA. This is 100% identical to human α-adaptin (NM_130787) and the siRNA employed (GAGCAUGUGCACGCUGGCCAdTdT). However, the mouse α-adaptin employed in the construction of GFP–α has the following non-coding polymorphism at position 8 in the antisense strand (GAGCATGTGCAC**A**CTGGCCA) and T354 is left intact. Thus, the siRNA should be incapable of silencing GFP–α.

To test if the GFP fusion protein was functional, cells that had been treated with siRNA against α-adaptin were transiently transfected with GFP–α-adaptin yielding a mixed population: some cells expressing GFP–adaptin and some not (Figure 1). Fluorescent transferrin was endocytosed in cells that expressed GFP–α-adaptin. Thus, as shown in this field of cells, expression of GFP–α-adaptin can rescue the loss of α-adaptin by siRNA.

To quantify the effects of expressing GFP–α-adaptin on rescuing the suppression by siRNA, we used flow cytometry. The transferrin uptake (*y*-axis) was quantified as a function of the GFP–α expression (*x*-axis) in cells that were transfected with α-adaptin siRNA as well as GFP–α-adaptin. In wt cells, expression of GFP–α-adaptin did not affect the uptake of fluorescent transferrin (Figure 2, left panel). This is consistent with our previous observations that GFP–α-adaptin does not alter clathrin-mediated endocytosis (35). Consistent with the observations from microscopy (Figure 1), cells transfected with siRNA against α-adaptin exhibited a striking reduction in transferrin endocytosis (Figure 2, center panel), similar to previously published reports from other groups (30,32). Expression of GFP–α-adaptin in siRNA-treated cells markedly increases the level of transferrin uptake in a dose-dependent manner (Figure 2, right panel). These results suggest that GFP–α-adaptin can functionally substitute for endogenous α-adaptin.

There are two explanations for the effects of expression of GFP–α-adaptin on the rescue of the endocytic phenotype in siRNA-treated cells. One possibility is that the GFP-tagged marker for the AP-2 complex can functionally complement silencing of the endogenous protein. Alternatively, it is possible that the siRNA was limiting and the GFP-α-adaptin mRNA bound the siRNA causing expression of more endogenous α-adaptin in cotransfected cells. In addition to a reduction in the endocytosis phenotype, this would result in two other observable effects. The green fluorescence associated with cotransfected cells would then be less than in cells only transfected with GFP–α-adaptin alone. However, this was clearly not apparent in our flow cytometry data (Figure 2).

If expression of GFP–α-adaptin prevented silencing of the endogenous protein, the level of endogenous α-adaptin in cells cotransfected with siRNA and GFP–α-adaptin would be higher than in cells transfected with siRNA alone. This possibility was tested with western blots using an antibody specific for α-adaptin as well as anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control. These studies analyzed samples from each group studied in the above flow cytometry studies (Figure 2) as well as non-transfected cells. The levels of endogenous α-adaptin were nearly equivalent in naïve cells and cells transfected with GFP–α-adaptin (Figure 3, lanes 1 and 4). In cells treated with siRNA, the endogenous α-adaptin level was reduced ∼40% in this experiment (lane 2) and still further reduced by transfection with GFP–α-adaptin (∼90% less than non-transfected control). While performing these studies, we noted that while endogenous human α-adaptin in HeLa cells was readily detected, in these blots, with these levels of loading, antibody concentrations and expression levels, GFP–α evidently was not.

These results confirm that expression of GFP–α-adaptin does not in fact prevent silencing of endogenous α-adaptin. Thus, GFP–α-adaptin is responsible for functionally complementing loss of the endogenous protein. Indeed, these results also demonstrate that the levels of endogenous α-adaptin can be decreased by expression of GFP–α-adaptin. This might reflect something similar to the previously published observation that endogenous clathrin expression is reduced in cells transfected with GFP-tagged clathrin (40).

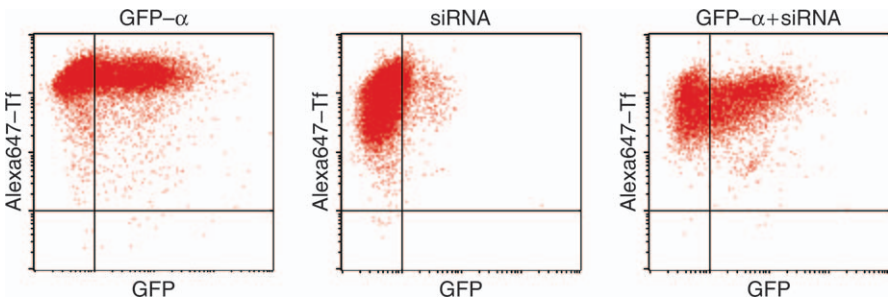

Figure 2: Flow cytometry analysis of transferrin uptake. Cells transfected with GFP–α, siRNA against α-adaptin or both together were analyzed by flow cytometry following incubation in Alexa647—transferrin (Tf). In each panel, the *y*-axis reflects transferrin endocytosis (red channel) and the *x*-axis shows GFP–α expression (green channel). This analysis demonstrates that GFP–α expression has no effect of Tf uptake. siRNA treatment decreased transferrin endocytosis, and this effect was reversed by GFP–α coexpression. Gates were set with unlabeled and single-color controls.

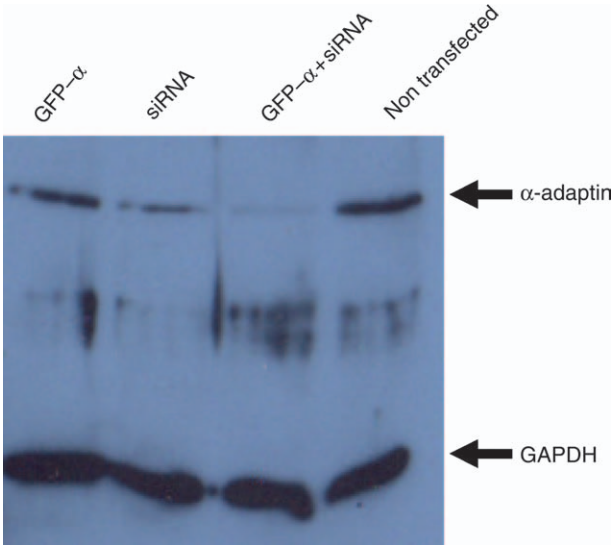

Figure 3: Western blots of protein from cells employed in flow cytometry studies. Non-transfected cells and cells transfected with GFP–α-adaptin, siRNA against α-adaptin or both together were analyzed by western blotting. Primary antibodies against α-adaptin and GAPDH (a housekeeping gene product) were employed. The band intensities demonstrate that transfection with GFP–α-adaptin did not prevent effective silencing of endogenous α-adaptin expression.

These results, taken together with our previous analyses of GFP–α-adaptin, fulfill the criteria listed above for full utility of an XFP-tagged protein (35). Thus, the GFP–α-adaptin represents a fluorescent-tagged protein marker, which can be used effectively as a marker for aspects of clathrin-mediated endocytosis and as a reporter for the function of the AP-2 complex (35). Indeed, GFP–α (i) localizes properly, (ii) does not mislocalize, (iii) does not function as a dominant negative and (iv) can functionally substitute for the endogenous protein. Finally, this study should set an example of the criteria required to claim that observations made with fluorescent-tagged fusion proteins reflect functions of the endogenous proteins. However, in our opinion in the absence of successful siRNA rescue studies, fusion proteins can still be viewed as innocuous markers, which can still provide useful functional insights.

Methods

Cells and transfections

HeLa cells were obtained from American Type Culture Collection and were cultured in DMEM with 10% FBS and grown at 37°C with 5% CO_2.

The production of mouse αC-adaptin with a GFP on the amino terminus was previously described (35). siRNA against α-adaptin was obtained from Dharmacon and matched that previously employed (30,32) (GAGCAUGUGCACGCUGGCCAdTdT).

Transfections were performed with Lipofectamine 2000 (Invitrogen) according to the supplier's protocols. The first round of transfection was performed the day after cell plating. About 48 h later, the cells were transfected again. About 48 h later, assays (e.g. flow cytometry and western blotting) were performed.

Transferrin uptake flow cytometry studies

Cells were incubated at 37°C in serum-free DMEM for 15 min and then for another 15 min at 37°C in Alexa647–transferrin (Molecular Probes) diluted 1:500. Following one rinse in acid PBS (pH 5.0) and two more in PBS, cells were processed and analyzed as previously described (35,39).

Cells were detached following incubation for 5 min at 37°C in 0.5 mL cell stripper (Mediatech Cellgro) by vigorous pipetting after the addition of 1.5 mL PBS. About 2 mL of detached cells were added to 2 mL of 8% paraformaldehyde (Electron Microscopy Sciences) and incubated for 5 min at room temperature. Following fixation, cells were rinsed by centrifugation twice in phosphate buffered saline (PBS) and then brought to The Rockefeller University Flow Cytometry Resource Center. GFP and Alexa647 emissions were collected simultaneously in a flow cytometer (FACSort; BD Biosciences) after gating with unlabeled and single-color controls. Identical flow cytometry studies were replicated three times.

Western blots

Cell lysates were prepared in radio immuno-precipitation assay (RIPA) buffer, clarified by centrifugation and then run on 10% SDS–PAGE. After transfer and blocking in 5% milk, blots were probed with polyclonal rabbit anti-α-adaptin (M300) and polyclonal rabbit anti-GAPDH (Santa Cruz Biotechnology). Bands were visualized with enhanced chemiluminescence+ (Amersham) following detection with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma). α-adaptin band intensities were quantified and normalized relative to GAPDH band intensities. Local background intensities were subtracted from all bands. Western blots were performed from three independent protein preparations.

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References

1. Wadsworth P, Sloboda RD. Microinjection of fluorescent tubulin into dividing sea urchin cells. J Cell Biol 1983;97:1249–1254.
2. Gerdes HH, Kaether C. Green fluorescent protein: applications in cell biology. FEBS Lett 1996;389:44–47.
3. Griffin BA, Adams SR, Jones J, Tsien RY. Fluorescent labeling of recombinant proteins in living cells with FlAsH. Methods Enzymol 2000;327:565–578.
4. Johnsson N, George N, Johnsson K. Protein chemistry on the surface of living cells. Chembiochem 2005;6:47–52.
5. Chen I, Howarth M, Lin W, Ting AY. Site-specific labeling of cell surface proteins with biophysical probes using biotin ligase. Nat Methods 2005; 2:99–104.
6. Muralidharan V, Muir TW. Protein ligation: an enabling technology for the biophysical analysis of proteins. Nat Methods 2006;3:429–438.
7. Lippincott-Schwartz J, Patterson GH. Development and use of fluorescent protein markers in living cells. Science 2003;300:87–91.
8. Daecke J, Fackler OT, Dittmar MT, Krausslich HG. Involvement of clathrin-mediated endocytosis in human immunodeficiency virus type 1 entry. J Virol 2005;79:1581–1594.
9. Pelkmans L, Kartenbeck J, Helenius A. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. Nat Cell Biol 2001;3:473–483.

10. Cubitt AB, Heim R, Adams SR, Boyd AE, Gross LA, Tsien RY. Understanding, improving and using green fluorescent proteins. Trends Biochem Sci 1995;20:448–455.
11. Wasle B, Hays LB, Rhodes CJ, Edwardson JM. Syncollin inhibits regulated corticotropin secretion from AtT-20 cells through a reduction in the secretory vesicle population. Biochem J 2004;380: 897–905.
12. Doyle T, Botstein D. Movement of yeast cortical actin cytoskeleton visualized in vivo. Proc Natl Acad Sci U S A 1996;93:3886–3891.
13. Han W, Rhee JS, Maximov A, Lin W, Hammer RE, Rosenmund C, Sudhof TC. C-terminal ECFP fusion impairs synaptotagmin 1 function: crowding out synaptotagmin 1. J Biol Chem 2005;280:5089–5100.
14. Chan KW, Csanady L, Seto-Young D, Nairn AC, Gadsby DC. Severed molecules functionally define the boundaries of the cystic fibrosis transmembrane conductance regulator's NH(2)-terminal nucleotide binding domain. J Gen Physiol 2000;116:163–180.
15. German-Retana S, Candresse T, Alias E, Delbos RP, Le Gall O. Effects of green fluorescent protein or beta-glucuronidase tagging on the accumulation and pathogenicity of a resistance-breaking Lettuce mosaic virus isolate in susceptible and resistant lettuce cultivars. Mol Plant Microbe Interact 2000;13:316–324.
16. Gaidarov I, Santini F, Warren RA, Keen JH. Spatial control of coated-pit dynamics in living cells. Nat Cell Biol 1999;1:1–7.
17. Rappoport J, Simon S, Benmerah A. Understanding living clathrin-coated pits. Traffic 2004;5:327–337.
18. Rappoport JZ, Simon SM. Real-time analysis of clathrin-mediated endocytosis during cell migration. J Cell Sci 2003;116:847–855.
19. Merrifield CJ, Feldman ME, Wan L, Almers W. Imaging actin and dynamin recruitment during invagination of single clathrin-coated pits. Nat Cell Biol 2002;4:691–698.
20. Keyel PA, Watkins SC, Traub LM. Endocytic adaptor molecules reveal an endosomal population of clathrin by total internal reflection fluorescence microscopy. J Biol Chem 2004;279:13190–13204.
21. Ehrlich M, Boll W, Van Oijen A, Hariharan R, Chandran K, Nibert ML, Kirchhausen T. Endocytosis by random initiation and stabilization of clathrin-coated pits. Cell 2004;118:591–605.
22. Merrifield CJ, Perrais D, Zenisek D. Coupling between clathrin-coated-pit invagination, cortactin recruitment, and membrane scission observed in live cells. Cell 2005;121:593–606.
23. Yarar D, Waterman-Storer CM, Schmid SL. A dynamic actin cytoskeleton functions at multiple stages of clathrin-mediated endocytosis. Mol Biol Cell 2005;16:964–975.
24. Lee DW, Wu X, Eisenberg E, Greene LE. Recruitment dynamics of GAK and auxilin to clathrin-coated pits during endocytosis. J Cell Sci 2006; 119:3502–3512.
25. Abazeed ME, Blanchette JM, Fuller RS. Cell-free transport from the trans-golgi network to late endosome requires factors involved in formation and consumption of clathrin-coated vesicles. J Biol Chem 2005;280:4442–4450.
26. Goda Y, Pfeffer SR. Cell-free systems to study vesicular transport along the secretory and endocytic pathways. FASEB J 1989;3:2488–2495.
27. Royle SJ, Bright NA, Lagnado L. Clathrin is required for the function of the mitotic spindle. Nature 2005;434:1152–1157.
28. McNiven MA, Cao H, Pitts KR, Yoon Y. The dynamin family of mechanoenzymes: pinching in new places. Trends Biochem Sci 2000;25:115–120.
29. Tebar F, Bohlander SK, Sorkin A. Clathrin assembly lymphoid myeloid leukemia (CALM) protein: localization in endocytic-coated pits, interactions with clathrin, and the impact of overexpression on clathrin-mediated traffic. Mol Biol Cell 1999;10:2687–2702.
30. Huang F, Khvorova A, Marshall W, Sorkin A. Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. J Biol Chem 2004;279:16657–16661.
31. Schmid EM, Ford MG, Burtey A, Praefcke GJ, Peak-Chew SY, Mills IG, Benmerah A, McMahon HT. Role of the AP2 beta-appendage hub in recruiting partners for clathrin-coated vesicle assembly. PLoS Biol 2006;4:e262.
32. Motley A, Bright NA, Seaman MN, Robinson MS. Clathrin-mediated endocytosis in AP-2-depleted cells. J Cell Biol 2003;162:909–918.
33. Hinrichsen L, Harborth J, Andrees L, Weber K, Ungewickell EJ. Effect of clathrin heavy chain- and alpha-adaptin-specific small inhibitory RNAs on endocytic accessory proteins and receptor trafficking in HeLa cells. J Biol Chem 2003;278:45160–45170.
34. Motley AM, Berg N, Taylor MJ, Sahlender DA, Hirst J, Owen DJ, Robinson MS. Functional analysis of AP-2 alpha and mu2 subunits. Mol Biol Cell 2006;17:5298–5308.
35. Rappoport JZ, Taha BW, Lemeer S, Benmerah A, Simon SM. The AP-2 complex is excluded from the dynamic population of plasma membrane-associated clathrin. J Biol Chem 2003;278:47357–47360.
36. Schwarz DS, Ding H, Kennington L, Moore JT, Schelter J, Burchard J, Linsley PS, Aronin N, Xu Z, Zamore PD. Designing siRNA that distinguish between genes that differ by a single nucleotide. PLoS Genet 2006;2:e140.
37. Haley B, Zamore PD. Kinetic analysis of the RNAi enzyme complex. Nat Struct Mol Biol 2004;11:599–606.
38. Rappoport JZ, Benmerah A, Simon SM. Analysis of the AP-2 adaptor complex and cargo during clathrin-mediated endocytosis. Traffic 2005; 6:539–547.
39. Rappoport JZ, Kemal S, Benmerah A, Simon SM. Dynamics of clathrin and adaptor proteins during endocytosis. Am J Physiol Cell Physiol 2006;291:C1072–C1081.
40. Moskowitz HS, Heuser J, McGraw TE, Ryan TA. Targeted chemical disruption of clathrin function in living cells. Mol Biol Cell 2003;14: 4437–4447.