RESEARCH ARTICLE

CemOrange2 fusions facilitate multifluorophore subcellular imaging in *C. elegans*

Brian J. Thomas, Ira E. Wight, Wendy Y. Y. Chou, Marco Moreno, Zachary Dawson, Arielle Homayouni, Huiyan Huang, Hyori Kim, Hanna Jia, Justin R. Buland, Jennifer A. Wambach, F. Sessions Cole, Stephen C. Pak, Gary A. Silverman, Cliff J. Luke

1 Department of Pediatrics, Washington University School of Medicine and St. Louis Children's Hospital, St. Louis, MO, United States of America, 2 Division of Biology and Biomedical Sciences, Washington University in St. Louis, St. Louis, MO, United States of America, 3 Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO, United States of America, 4 Department of Genetics, Washington University School of Medicine, St. Louis, MO, United States of America

* gsilverman@wustl.edu (GAS); cjluke@wustl.edu (CJL)

Abstract

Due to its ease of genetic manipulation and transparency, *Caenorhabditis elegans* (*C. elegans*) has become a preferred model system to study gene function by microscopy. The use of *Aequorea victoria* green fluorescent protein (GFP) fused to proteins or targeting sequences of interest, further expanded upon the utility of *C. elegans* by labeling subcellular structures, which enables following their disposition during development or in the presence of genetic mutations. Fluorescent proteins with excitation and emission spectra different from that of GFP accelerated the use of multifluorophore imaging in real time. We have expanded the repertoire of fluorescent proteins for use in *C. elegans* by developing a codon-optimized version of Orange2 (CemOrange2). Proteins or targeting motifs fused to CemOrange2 were distinguishable from the more common fluorophores used in the nematode; such as GFP, YFP, and mKate2. We generated a panel of CemOrange2 fusion constructs, and confirmed they were targeted to their correct subcellular addresses by colocalization with independent markers. To demonstrate the potential usefulness of this new panel of fluorescent protein markers, we showed that CemOrange2 fusion proteins could be used to: 1) monitor biological pathways, 2) multiplex with other fluorescent proteins to determine colocalization and 3) gain phenotypic knowledge of a human ABCA3 orthologue, ABT-4, trafficking variant in the *C. elegans* model organism.

Introduction

Antibody staining techniques provide a sensitive means to visualize cellular dynamics such as protein disposition, vesicular and membrane trafficking and organellar morphogenesis and function. However, the images are static and may be distorted by fixation and permeabilization...
techniques. As an alternative to antibody staining, a transgene expressing a fluorescent protein (FP) fused to a protein of interest permits the assessment of cellular dynamics in real time [1]. Due to its transparency, small size and ease of genetic manipulation, *C. elegans* has become an ideal model system to visually study protein expression levels and subcellular dynamics within the context of a developing metazoan [2, 3]. Since Chalfie’s landmark description of using GFP reporters in *C. elegans* [4], a variety of spectrally variant FP tags have emerged [5]. However, their use in *C. elegans* has been limited due to the high levels of autofluorescence overlapping with the emission spectra of these different fluorophores [1, 6]. Moreover, the sensitivity of *C. elegans* to high-energy excitation wavelengths make dim FPs, such as blue fluorescent proteins, difficult to visualize in the nematode. Despite these limitations, CFP, GFP, YFP and mCherry FP reporters in *C. elegans* have proven utility in monitoring stress responses, cell death, redox states, cell division and serving as sensors for genetic screens [7–9].

As human pathological variants of unknown significance (VUS) are discovered, insight into their activity is gained by assessing their functions in model systems, like *C. elegans* [2, 3, 10, 11]. For example, by generating wild-type and VUS FP fusions, and expressing them in *C. elegans* strains harboring different FP tagged subcellular structures or organelles, multiplexing imaging studies can show a change in the disposition of the VUS (e.g., aggregation, change in subcellular localization) and confirm pathogenicity. *C. elegans* FP-tagged organelle makers, such as those for lysosomes and lysosome-related organelles (LRO’s), endoplasmic reticulum (ER) and autophagosomes already exist [12–16]. However, most of these markers contain the commonly used fluorophores (e.g. GFP and mCherry), making multiplexing with another FP that has overlapping excitation/emission (Ex/Em) spectra (e.g. YFP or mKate2) impractical. To circumvent this, newer FPs with a wider variety of Ex/Em spectra are now available (Table 1) [1, 17] which are more versatile, especially when coupled with non-conventional imaging technologies for enhanced spectral separation, such as supercontinuum white light lasers [18].

The goal of this work was to develop a toolbox of subcellular organelle markers with a fluorophore that could be spectrally separable from GFP, YFP and far-red fluorescent proteins, such as mKate2. Using expression vectors with multiple subcellular organelle targeting proteins or motifs fused with CemOrange2, we show that this FP was directed to the correct subcellular location in intestinal cells. Moreover, we show that transgenic animals expressing CemOrange2 can be multiplexed with other fluorophores to study different biological pathways.

### Materials and methods

#### Expression plasmids

All amplifications were performed using the Q5 high fidelity or Phuslon high fidelity PCR kits (NEB, Ipswich, MA). Restriction enzymes used for cloning procedures were purchased from New England Biolabs (NEB).

To generate *P*nhx-2 CemOrange2, *P*nhx-2 CemNeptune2.5, and *P*nhx-2 CemCardinal2 constructs (pJR2956, pJR2955 and pJR2953), minigene blocks with three synthetic introns (gblock) containing *C. elegans* codon optimized version of mOrange2 (CemOrange2), mNeptune2.5 (CemNeptune2.5) and mCardinal2 (CemCardinal2) with additional N- and C-terminal restriction sites were synthesized (IDT, Skokie, IL) and sub-cloned into the NheI/SacI restriction sites of the canonical expression vector, pPD49.26 [17, 19]. A 2kb *nhx*-2 promoter fragment was amplified (S1 Table, primer set 1) and ligated into the HindIII/XbaI restriction sites to yield the final constructs: pJR2956, pJR2955, and pJR2953, respectively.
Table 1. Reported fluorescence characteristics of the fluorescent proteins used in this study.

| fluorescent protein | Ex<sub>max</sub> | Em<sub>max</sub> | EC (M<sup>-1</sup> cm<sup>1</sup>) | QY | brightness | pKa | lifetime (ns) | photo-stability (s) | maturation (mins) |
|---------------------|----------------|----------------|-------------------------------|----|------------|-----|--------------|-------------------|-----------------|
| EGFP                | 488            | 507            | 55,900                        | 0.6| 33.5       | 6.0 | 2.6          | 174               | 25              |
| EYFP                | 513            | 527            | 83,400                        | 0.6| 50.9       | 6.9 | 3.1          | 60                | unknown         |
| mOrange2            | 549            | 565            | 58,000                        | 0.6| 34.9       | 6.5 | 6.5          | 270               | unknown         |
| mCherry             | 587            | 610            | 72,000                        | 0.2| 15.8       | 4.5 | 1.4          | 15                | 15              |
| mKate2             | 588            | 633            | 62,500                        | 0.4| 25.0       | 5.4 | 2.5          | 84                | 20              |
| mNeptune2.5        | 599            | 643            | 95,000                        | 0.2| 22.8       | 5.8 | unknown      | unknown           | 26              |
| mCardinal          | 604            | 659            | 87,000                        | 0.2| 16.5       | 5.3 | unknown      | 730               | 27              |

The excitation (Ex) and emission (Em) maxima, extinction coefficients (EC), quantum yields (QY), brightness, pKa, fluorescence lifetime, photosensitivity and maturation time of the indicated fluorescent proteins are reported on the fluorescent protein database (https://www.fpbase.org).

To generate expression constructs of cloned fragments the NEBuilder HiFi DNA Assembly Cloning Kit was used (NEB). For N-terminus CemOrange2 fusions, fragments were cloned into the KasI restriction site of pJR2956. P<sub>nhx-2</sub>sqst-1::CemOrange2 (pBT3037), P<sub>nhx-2</sub>aqp-1::CemOrange2 (pBT3102), P<sub>nhx-2</sub>lmm-1::CemOrange2 (pBT3035), P<sub>nhx-2</sub>Imp-1::CemOrange2 (pBT2999), P<sub>nhx-2</sub>aman-2::CemOrange2 (pLS2965), and P<sub>nhx-2</sub>glo-1::CemOrange2 (pBT3038) were generated by amplifying the sqst-1 (S1 Table, primer set 2), aqp-1 (S1 Table, primer set 3), lmm-1 (S1 Table, primer set 9), Imp-1 (S1 Table, primer set 11), aman-2 (S1 Table, primer set 15), and glo-1 (S1 Table, primer set 16) genomic DNA fragment and ligating the fragment into the Nhel site of P<sub>nhx-2</sub> CemOrange2 (pJR2956), respectively. P<sub>nhx-2</sub> CemOrange2::lgg-1 (pBT3043), P<sub>nhx-2</sub> CemOrange2::cup-5 (pBT3011), P<sub>nhx-2</sub> CemOrange2::rab-5 (pBT3007), P<sub>nhx-2</sub> CemOrange2::rab-7 (pBT3014), P<sub>nhx-2</sub> CemOrange2::tram-1 (pBT3000), and P<sub>nhx-2</sub> CemOrange2::psy-1 (pBT3003) were generated by amplifying the lgg-1 (S1 Table, primer set 4), cup-5 (S1 Table, primer set 8), rab-5 (S1 Table, primer set 12), rab-7 (S1 Table, primer set 13), tram-1 (S1 Table, primer set 14), and psy-1 (S1 Table, primer set 17) genomic DNA fragment and ligating the fragment into the KasI site of P<sub>nhx-2</sub> CemOrange2, respectively.

Mitochondrial targeting (m), peroxisome targeting peptide (SKL) and nuclear localization sequences (NLS) were inserted into the P<sub>nhx-2</sub> CemOrange2 expression construct using the Q5 site-directed mutagenesis kit (NEB). P<sub>nhx-2</sub> mCemOrange2 (pBT3024) was generated using primer set 5 (S1 Table). P<sub>nhx-2</sub> NLS<sup>SV-40</sup> CemOrange2::NLS<sup>glo-1</sup> (pBT3047) was generated using primer sets 6 (SV-40) and 7 (egl-13) (S1 Table). P<sub>nhx-2</sub> CemOrange2::SKL (pBT3019) was generated using primer set 10 (S1 Table).

To generate P<sub>nhx-2</sub> sGFP::KDEL (pOL2184), the nhx-2 promoter was amplified (S1 Table, primer set 1) and sub-cloned into the HindIII/XbaI sites of pPD95.85 to create P<sub>nhx-2</sub> sGFP (pAV1771). The stop codon of GFP was mutated by Quikchange site-directed mutagenesis (S1 Table). P<sub>nhx-2</sub> CemOrange2::lgg-1 (pBT3047), P<sub>nhx-2</sub> CemOrange2::cup-5 (pBT3011), P<sub>nhx-2</sub> CemOrange2::rab-5 (pBT3007), P<sub>nhx-2</sub> CemOrange2::rab-7 (pBT3014), P<sub>nhx-2</sub> CemOrange2::tram-1 (pBT3000), and P<sub>nhx-2</sub> CemOrange2::psy-1 (pBT3003) were generated by amplifying the lgg-1 (S1 Table, primer set 4), cup-5 (S1 Table, primer set 8), rab-5 (S1 Table, primer set 12), rab-7 (S1 Table, primer set 13), tram-1 (S1 Table, primer set 14), and psy-1 (S1 Table, primer set 17) genomic DNA fragment and ligating the fragment into the KasI site of P<sub>nhx-2</sub> CemOrange2, respectively.

Mitochondrial targeting (m), peroxisome targeting peptide (SKL) and nuclear localization sequences (NLS) were inserted into the P<sub>nhx-2</sub> CemOrange2 expression construct using the Q5 site-directed mutagenesis kit (NEB). P<sub>nhx-2</sub> mCemOrange2 (pBT3024) was generated using primer set 5 (S1 Table). P<sub>nhx-2</sub> NLS<sup>SV-40</sup> CemOrange2::NLS<sup>glo-1</sup> (pBT3047) was generated using primer sets 6 (SV-40) and 7 (egl-13) (S1 Table). P<sub>nhx-2</sub> CemOrange2::SKL (pBT3019) was generated using primer set 10 (S1 Table).
Animals were allowed to recover for 2 hrs at room temperature or 16˚C overnight. Three irradiation, 100 mm NGM plate. Animals were exposed to 35 Gy radiation using an X-ray irradiator. allowed to lay eggs for 3 days. One-hundred L4s from the source plates were transferred to strain VK2266 (~7–10 days). Animals were collected in 1 ml of PBS and 10 µl was placed on 30 separate, 100

C. elegans strains and culture conditions

A list of worm strains and genotypes, along with the figures they correspond to is given in S2 Table for reference. All injection mixes were made to a final total DNA concentration of 100-150 ng/µl using pBluescript SK- (Agilent Technologies, Santa Clara, CA) as carrier DNA. The strain VK2266 (vkEx2266[Pnhx::mKate2::lgg-1::Pmyo-GFP]) was generated by co-injecting 80 ng/µl Pnhx::mKate2::lgg-1 with 5 ng/µl of Pmyo-GFP. The strains VK2664, VK2666, VK2671, VK2674, VK2688, VK2700, VK2702, VK2728, VK2732, VK2738, VK2755, VK2756, VK2757 and VK2883 were generated by co-injecting 50 ng/µl of the expression plasmids Pnhx::CemOrange2::tram-1, Pnhx::CemOrange2::rab-7, Pnhx::CemOrange2::rab-5, Pnhx::CemOrange2::psiy-1, Pnhx::CemOrange2::cup-5, Pnhx::CemOrange2::SKL, Pnhx::pisy-1, Pnhx::NLS-eGFP, Pnhx::pam2::CemOrange2, Pnhx::CemOrange2::lmp-1, Pnhx::NLS-eGFP, Pnhx::pam2::CemOrange2, Pnhx::CemOrange2::lmp-1, Pnhx::NLS-eGFP, Pnhx::pam2::CemOrange2, Pnhx::CemOrange2::lmp-1, Pnhx::NLS-eGFP, respectively. The strains VK2620 was generated by co-injecting 20 ng/µl of the expression plasmid Pnhx::aman-2::CemOrange2 with 5 ng/µl Pmyo-GFP. The strains VK2697, VK2734, VK2735 were generated by co-injecting 10 ng/µl of the expression plasmids Pnhx::Imp-1::CemOrange2, Pnhx::lmp-1::CemOrange2, and Pnhx::glo-1::CemOrange2, respectively, with 5 ng/µl Pmyo-GFP. The strains VK2748 and VK3007 were generated by co-injecting 15 ng/µl of Pnhx::GFP::KDEL with 35 ng/µl of Pnhx::CemOrange2::tram-1 and Pnhx::CemOrange2::psiy-1, respectively. The strain VK2838 was generated by co-injecting 50 ng/µl Pnhx::CemOrange2::SKL with 5 ng/µl Pmyo-GFP into VS11 [20]. The strains VK3160 and VK3161 were generated by co-injecting with 15 ng/µl Pmyo::CemOrange2::tram-1 with 15 ng/µl Pmyo::abt-4::mKate2 and Pnhx::abt-4::mKate2, respectively. The strains VK2697 and VK2734 were integrated spontaneously. The strains VK2288 (vkIs2288 [Pnhx::mKate2::lgg-1::Pmyo-GFP]), VK2797 (vkIs2797[vkIs2797[Pnhx::CemOrange2::rab-5::Pmyo-GFP]], VK2799 (vkIs2799[Pnhx::glo-1::CemOrange2::Pmyo-GFP]), VK2807 (vkIs2807[Pnhx::aman-2::CemOrange2::Pmyo-GFP]), VK2815 (vkIs2815[Pnhx::CemOrange2::rab-7::Pmyo-GFP]), and VK2878 were integrated via X-ray irradiation (vide infra) and derived from the strains VK2266, VK2671, VK2674, VK2680, VK2666, VK2728, and VK2738, respectively. The strain VK2617 (vkIs2617[Pnhx::GFP::ATZ;Pnhx::mKate2::lgg-1::Pmyo-GFP;Pmyo::mCherry]) was generated by crossing males from strain VK1882 [21] with hermaphrodites from strain VK2288. The strain VK2749 was generated by crossing males from strain VK2697 with hermaphrodites from strains VK2617. The worm strains VK2881 and VK2882 were generated by crossing males from strain VK2799, with hermaphrodites from strains VS17 and RT258, respectively [20, 22]. All strains were obtained from the Caenorhabditis Genetic Center (CGC) unless otherwise stated. Males were generated by heat shocking at 27˚C for 18 hrs or 30˚C for 6 hrs. Animals were routinely cultured at 20˚C on nematode growth media (NGM) plates seeded with E. coli strain OP50 unless otherwise stated.

Transgene integration

Ten transgenic adult animals were placed on 2 separate, 100 mm NGM source plates and allowed to lay eggs for 3 days. One-hundred L4s from the source plates were transferred to one, 100 mm NGM plate. Animals were exposed to 35 Gy radiation using an X-ray irradiator. Animals were allowed to recover for 2 hrs at room temperature or 16˚C overnight. Three irradiated L4 animals were placed on 30 separate, 100 mm NGM plates and grown to starvation (~7–10 days). Animals were collected in 1 ml of PBS and 10 µl was placed on 30 separate, 100
mm NGM plates. These plates were grown to starvation (~5–7 days), washed with 1 ml of PBS and 10 μl of the wash was placed onto 30 separate, 60 mm NGM plates seeded with OP50 and allowed to grow for 2 days. Six transgenic animals from each plate were transferred to 6 separate, 35 mm NGM plates and grown for 3 days. Animals that had 100% transmission of the transgenic marker were considered integrated and stocked.

**E. coli OP50 preparation**

A single colony of *E. coli* (OP50) was placed in 5 ml LB broth and incubated at 37˚C with shaking (250 rpm) overnight. This culture was then added to 500 ml of LB broth and incubated at 37˚C with shaking until reaching an OD<sub>600</sub> = 0.75–1.0. The bacteria were collected by centrifugation and washed once with 1:10 original volume of PBS and resuspended in 1:25 original volume in PBS. An equal volume of 50% glycerol was added for long-term storage at -80˚C. After thawing, the bacteria were concentrated by centrifugation, washed and re-suspended in 1:25 original volume in PBS.

**Autophagic flux assay**

Twenty late L4 animals were placed into 100 μl PBS supplemented with OP50 and the following conditions: no treatment (diluent control), 25 μM fluphenazine (Millipore-Sigma, St. Louis, MO), or 10 mM 3-methyladenine (3-MA; Millipore-Sigma). The animals were incubated for 16 hrs at RT and recovered on an NGM plate for 30 min. Animals were transferred to a 35 mm coverglass bottom petri dish (MatTek, Ashland, MA) for confocal imaging.

**Lysosomal membrane permeability (LMP) assay**

Twenty adult animals were placed in a 10 μl solution of either PBS alone, 5% tert-butyl hydroperoxide (t-BOOH; Millipore-Sigma) or 500 mM Leu-Leu-OMe (LLOMe; Bachem Torrance, CA) in PBS. Animals were incubated for 15 min and transferred to a 35 mm coverglass bottom petri dish (MatTek) for confocal imaging.

**Intracellular organelle labeling**

LysoTracker Deep Red (LTDR; Life Technologies Corp, Carlsbad, CA) were diluted to 1 μM in PBS. About 20–30 adult stage animals were placed in the solution and incubated at room temperature for 30 min. To chase excess dye prior to imaging, animals were placed on a fresh NGM plate seeded with OP50 for 15 min.

MitoTracker Deep Red (Life Technologies Corp) was resuspended in DMSO to a stock concentration of 1.25 mM. Prior to use, the stock solution was diluted to 500 nM in PBS. About 20–30 adult stage animals were placed in 10 μl staining solution and incubated at room temperature for 30 min. To chase excess dye prior to imaging, animals were placed on a fresh NGM plate seeded with OP50 for 15 min.

Bovine serum albumin (BSA)-Alexa Fluor conjugate (Life Technologies Corp) was resuspended in PBS to a concentration of 5 mg/ml. About 20–30 adult stage animals were placed in 10 μl BSA and 5 μl OP50 and incubated for 16hrs. To chase excess dye prior to imaging, animals were placed on a fresh NGM plate seeded with OP50 for 60 min.

BODIPY FL C₅-Ceramide (C5 FL) complexed to BSA (Life Technologies Corp) was resuspended in water to a stock concentration of 500 μM. Prior to use, the stock solution was diluted to 50 μM with PBS supplemented with OP50. About 20–30 adult stage animals were placed in 10 μl staining solution and incubated at room temperature for 1hr. To chase excess dye prior to imaging, animals were placed on a fresh NGM plate seeded with OP50 for 30 min.
CellMask Deep Red (Life Technologies Corp) plasma membrane stain was diluted 1:100 in PBS. About 20–30 adult stage animals were placed in 10 μl staining solution and incubated at room temperature for 1 hr. To chase excess dye prior to imaging, animals were placed on a fresh NGM plate seeded with OP50 for 30–60 min.

Microscopic imaging and analysis
To prepare animals for imaging, 6–10 μl of 100 mM NaN₃ (Millipore-Sigma) in PBS was placed on the middle of a 35 mm coverglass bottom dish (MatTek). Approximately, 10–15 adult stage animals were transferred to the NaN₃ solution and covered with a 12 mm circular coverslip and then a 25 mm square coverslip.

Confocal images were taken with a Leica SP8X tandem scanning confocal microscope with a white light laser using either a 40x 1.3 NA or 63x 1.4 NA oil PlanApo objective over ≥20 z-planes and a pinhole size of 1.00 (Leica Microsystems, Buffalo Grove, IL). Images were displayed as single XY planes, except nuclear markers which were displayed as maximum intensity projections. Images were rendered and analyzed using LASX (Leica Microsystems) and Volocity (v6.3; Quorum Technologies, CAN) software.

Spectral data were obtained using a supercontinuum white light laser and prism-based spectral detector. Excitation spectra were determined by measuring the intensity of the sample while exciting every 3–5 nm. Sample excitation was started ~100 nm lower of reported excitation maximum (Ex_max), up to a detection range (~40–50 nm higher than the emission maxima (Em_max)). The Em spectra were determined by exciting the sample 50–100 nm lower than the Ex_max and measuring in a range of 20 nm in 5 nm steps.

Colocalization and quantification of data were obtained using the Volocity cellular imaging and analysis software (v6.3; Quorum Technologies). Either Pearson correlation or Manders correlation coefficient (MCC) was used to assess the positional relationship between two objects [23]. Colocalization and quantification of vesicular or punctate structures were obtained using the Volocity Colocalization or Volocity Quantification modules, respectively, with thresholding of fluorescence intensity and size to remove background noise. A minimum of 5 individual animals was used in the analysis.

Statistical analyses
Two-tailed (heteroscedastic) t-tests were performed on quantified data using Microsoft Excel. Experiments were repeated at least two times to ensure reproducibility.

Results
CemOrange2 excitation and emission spectra in vivo
Detectable differences in the Ex/Em spectra of individual FPs permit the visualization of multiple fluorophores within the same physical space (Table 1). Three FPs with different spectra, GFP (Ex488 nm/Em505 nm), YFP(Ex515 nm/Em526 nm) and mKate2(Em585 nm/Ex605 nm) [24–26], have been utilized in C. elegans within our laboratory [27, 28]. To broaden our repertoire for multiplexing, we synthesized minigene gblocks for mOrange2, mNeptune2.5 and mCardinal2 [17, 29]. To enhance expression of these FPs in C. elegans, we codon optimized the cDNA sequence and introduced 3 synthetic introns using the C. elegans Codon Adapter software, with a Codon Adaption Index of 1.0 (https://worm.mpi-cbg.de/codons/cgi-bin/optimize.py) [19, 30]. The C. elegans (Ce) optimized CemOrange2, CemNeptune2.5 and CemCardinal2 gblocks were ligated into a backbone expression vector containing the intestinal promoter nhx-2 in pPD49.26 [31, 32]. Correct plasmid construction was confirmed by DNA
sequencing. These expression plasmids, along with a co-injection marker (P
myo-2:GFP), were introduced to the germline of N2 (wild-type) animals via microinjection. Individual transgenic animals were selected using the co-injection marker, discernable via a fluorescent stereoscope and placed onto single plates. Lines that passed the marker onto the second generation were selected for imaging and spectral analysis. The $E_{\text{max}}/E_{\text{max}}$ spectra in the intestinal cytosol were determined using a confocal laser-scanning microscope equipped with a supercontinuum white light laser and prism-based spectral detector. The $E_{\text{max}}/E_{\text{max}}$ were: 1) GFP, $E_{\text{max}} 488 \text{ nm}/E_{\text{max}} 505 \text{ nm}$; 2) YFP, $E_{\text{max}} 515 \text{ nm}/E_{\text{max}} 526 \text{ nm}$; 3) CemOrange2, $E_{\text{max}} 555 \text{ nm}/E_{\text{max}} 562 \text{ nm}$; 4) mKate2, $E_{\text{max}} 585 \text{ nm}/E_{\text{max}} 605 \text{ nm}$; 5) CemNeptune2.5, $E_{\text{max}} 595 \text{ nm}/E_{\text{max}} 620 \text{ nm}$; and 6) CemCardinal2, $E_{\text{max}} 595 \text{ nm}/E_{\text{max}} 642 \text{ nm}$ (Fig 1A). Spectral characteristics show that the $E_{\text{max}}/E_{\text{max}}$ spectra of CemOrange2 were between those of GFP, YFP and mKate2. CemNeptune2 and CemCardinal2 had $E_{\text{max}}$ spectra shifted ~10 nm higher than that of mKate2 and $E_{\text{max}}$ spectra shifted ~15 nm and ~37 nm higher than that of mKate2, respectively (Fig 1A). Moreover, CemOrange2 was distinct from autofluorescence found in wild-type N2 animals (S2 Fig) These data suggest that CemOrange2 could be utilized for multicolor imaging with other commonly used FPs.

Excitation and emission spectra of fluorescent proteins (FPs) expressed within the cytoplasm of C. elegans intestinal cells: GFP (green), YFP (yellow), CemOrange2 (orange), mKate2 (red), CemNeptune2 (dark red), CemCardinal2 (magenta) FPs were expressed from transgenes driven by the intestinal specific promoter, P
nhx-2 (A). Dotted and solid lines represent excitation and emission curves, respectively. Spectral scans in both the excitation (470 nm–670 nm) and emission (480 nm–770 nm) wavelengths were obtained using a supercontinuum white light laser and spectral detectors on a confocal microscope. Intensity was normalized to the maximum fluorescence intensity value ($I/I_{\text{max}}$). The inset table indicates the maximal excitation ($E_{\text{max}}$) and emission wavelengths ($E_{\text{max}}$) determined for each fluorophore. (B-M) Representative confocal maximum intensity projections of transgenic animals expressing a single FP using the $E_{\text{max}}/E_{\text{max}}$ (± 20 nm) determined from Fig 1A. Fluorescent and DIC (a single XY-plane) images are displayed in the left and right columns, respectively. Scale bar = 25 μm. Different FPs exhibit a range of characteristics (Table 1), such as brightness, aggregation and cellular toxicity, depending on the cell or organism in which they are expressed [33]. Interestingly, the three related far-red FPs, mKate2, CemNeptune2.5 and CemCardinal2, had lower $E_{\text{max}}$ in vivo (Fig 1A) than previously reported. Transgenic animals expressing different cytosolic FPs within the intestine were visualized qualitatively using confocal laser-scanning microscopy at the previously determined $E_{\text{max}}/E_{\text{max}}$. GFP, YFP, CemOrange2 and mKate2 were bright, diffuse and uniform (Fig 1B, 1D, 1F and 1H). CemNeptune2.5 expression was diffuse but non-uniform within the cytosol (Fig 1J). CemCardinal2 was neither bright nor uniform in its distribution and exhibited punctate structures suggestive of FP aggregation or concentration within a subcellular structure (Fig 1L). Corresponding DIC images confirmed expression was restricted to intestinal cells. (Fig 1C, 1E, 1G, 1I, 1K and 1M). Taken together, these data suggest that CemOrange2 would be the FP of choice for subcellular imaging.

CemOrange2 fused to targeting motifs or proteins localized to subcellular structures and organelles

CemOrange2 exhibited $E_{\text{max}}/E_{\text{max}}$ spectra that were distinguishable from those of GFP, YFP and mKate2. Also, CemOrange2 expressed in intestinal cells yielded a diffuse cytoplasmic pattern (i.e., no evidence of aggregation). Taken together, these characteristics suggested that this FP would facilitate multiplexing with unknown FP-tagged proteins by targeting known
Fig 1. Excitation and emission spectra and cytosolic expression patterns of fluorescent proteins in *C. elegans*.

https://doi.org/10.1371/journal.pone.0214257.g001
subcellular structures. Thus, the P_nhx-2::CemOrange2 construct (pJR2945) was used to generate a transgene expressing the FP fused to different targeting motifs or a C. elegans protein known to localize to specific organelles/structures (Table 2). These structures included the nuclear lamina [34]; the rough ER [15]; the Golgi [35]; early and late endosomes [36]; the lysosome [12, 14]; autophagosomes [16]; ubiquitinylated cargos [37, 38]; lysosomal related organelles (LRO) [13, 39]; the apical, lateral and basal plasma membrane [40]; the nucleus [41]; the mitochondrial matrix [42]; and peroxisomes [43]. All genes were amplified by PCR from wild-type genomic DNA using gene-specific primers and cloned in frame with CemOrange2 at either the N- or C-termini (Table 2). Subcellular targeting subsequences were introduced at either the N- or C-termini by site-directed mutagenesis. The identities of all constructs were confirmed by DNA sequencing.

The subcellular localizations of the CemOrange2 fusion proteins in transgenic wild-type animals were visualized using confocal laser-scanning microscopy with an Ex555 nm/Em565-590 nm. The nucleus marker, NLS^{SV-40::CemOrange2::NLS}_{egl-13}, was restricted to the nucleus (Fig 2A and 2B). The nuclear envelope marker, LMN-1::CemOrange2, showed characteristic highlighting of the nuclear lamina (Fig 2C and 2D). However, there was evidence of areas of CemOrange2 accumulation on the lamina, possibly due to overexpression artifact (white arrowhead). The ER markers, CemOrange2::TRAM-1 and CemOrange2::PISY-1, respectively, showed bright reticular fluorescence characteristic of the ER (Fig 2E and 2F and Fig 2G and 2H, respectively). The golgi localizing protein, AMAN-2::CemOrange2, showed punctate structures (~10-20/cell) characteristic of Golgi stacks (Fig 2I and 2J, white arrowheads). Similar to previous studies, CemOrange2::RAB-5 (Fig 2K and 2L) and CemOrange2::RAB-7 (Fig 2M and 2N) showed multiple sized clusters of puncta, consistent with endosomes (white arrowheads; RAB-5* early and RAB-7* late endosomes, respectively, cannot be distinguished under these conditions) [13, 36, 45]. Unlike previous studies, the lysosome marker, LMP-1::CemOrange2 showed discrete and variably sized punctate structures (Fig 2O and 2P, white arrowheads). The lysosome-related organelle (LRO) marker, GLO-1::CemOrange2, demonstrated punctate structures that appeared qualitatively larger than those structures demarcated by the lysosome makers (Fig 2S and 2T, white arrowheads). The peroxisome marker, CemOrange2::SKL, showed multiple clusters of small puncta (Fig 2U and 2V, white arrowheads). The clusters of puncta localized close to basolateral membrane. The mitochondrial probe, CemOrange2::mt showed densely packed small, oblong structures, that are characteristic of mitochondria (Fig 2W and 2X, white arrowheads).

The selective autophagy receptor, SQST-1::CemOrange2, is a diffuse cytosolic protein with few puncta detectable at lower resolution under normal conditions (Fig 2Y and 2Z, white arrowheads). Similarly, the autophagy marker, CemOrange2::LGG-1, also showed mainly a cytosolic expression pattern with a few puncta in well-fed animals (Fig 2AA and 2BB, white arrowheads). The characteristics of these makers change depending on the state of autophagic flux (vide infra).

The water channel, AQP-1::CemOrange2, localized to the intestinal plasma membrane (Fig 2CC and 2DD) with bright fluorescence evident at both the apical (Fig 2DD, white asterisk) and basolateral membranes (Fig 2DD, white arrowhead).

**Trafficking of CemOrange2 fusion proteins**

The transgenic lines described above demonstrated subcellular distribution profiles consistent with those described in previously published reports. However, we sought an independent
means to confirm that CemOrange2 markers trafficked to the correct positions by performing colocalization studies with a different set of previously reported genes, target peptides or fluorescent stains known to localize to specific organelles. Colocalization was assessed using the Manders Correlation Coefficient (MCC) with a value $\leq 0.5$ showing no colocalization, $0.5–0.8$ demonstrating partial colocalization and $\geq 0.8$ corresponding to high colocalization [23].

To confirm that LMP-1::CemOrange2 (Fig 3A) and CUP-5::CemOrange2 (Fig 3D) localized to lysosomes, transgenic animals expressing either fusion protein were labeled with LTDR, a lysosomotropic probe that fluoresces in acidic conditions (Figs 3B and 2E). Transgenic C. elegans expressing LMP-1::CemOrange2 partially colocalized with LTDR staining (Fig 3C, merge, white arrowheads) with an average MCC = 0.52 (Fig 3BB). However, the majority of the CUP-5::CemOrange2 positive vesicles colocalized under the same staining conditions (Fig 3F, merge, white arrowheads) with an average MCC = 0.87 (Fig 3BB). The GLO-1::CemOrange2 positive vesicles in C. elegans did not colocalize with LTDR (S1A–S1C Fig) consistent with the protein localization to LRO’s rather than lysosomes. To further confirm these findings, plasmids containing $\text{P}_{\text{nhx-2}}\text{glo-1}::\text{CemOrange2}$ were injected into transgenic animals expressing either LMP-1::GFP under the $\text{vha-6}$ intestinal specific promoter (a kind gift

Table 2. C. elegans proteins and target peptides.

| protein/ target peptide | human homolog (best BLASTP match) | organelle | reference |
|-------------------------|-----------------------------------|-----------|-----------|
| TRAM-1                  | TRAM1 (translocating chain-associated membrane protein 1) | rough endoplasmic reticulum | [15] |
| PISY-1                  | CDIPT (isoform 1 of CDP-diacylglycerol—inositol 3-phosphatidytransferase) | endoplasmic reticulum (some golgi structures) | [15] |
| RAB-7                   | RAB7A (ras-related protein Rab-7a) | endosome (late) | [36] |
| RAB-5                   | RAB5B (ras-related protein Rab-5B) | endosome (early) | [36] |
| LMP-1                   | LAMP1 (lysosomal associated membrane protein 1) | lysosome | [14] |
| CUP-5                   | MCOLN3 (isoform 1 of Mucolipin-3) | lysosome | [12] |
| AMAN-2                  | MAN2A1 (alpha-mannosidase 2) | Golgi | [35] |
| GLO-1                   | RAB32 (ras-related protein Rab-32) | lysosome-related organelle | [13] |
| SKL$^*$                 | | peroxisome | [43] |
| mt$^4$                  | | mitochondria | [42] |
| LGG-1                   | GABARAP (gamma-aminobutyric acid receptor-associated protein) | cytoplasmic/autophagosome | [16] |
| SQST-1                  | SQSTM1 (sequestosome-1) | cytoplasmic/autophagosome | [44] |
| LMN-1                   | LMNB1 (lamin B1) | nuclear lamina | [34] |
| NLS$^\dagger$           | | nucleus | [41] |
| AQP-1                   | AQP10 (aquaporin 10) | plasma membrane | [40] |

$^8$ www.wormbase.org
$^\dagger$ Peroxisome target sequence = SKL
$^4$ mt (mitochondria target sequence) = MLSLRQSIRFFKPATRTCLSRTTL
$^\dagger$ NLS$^{SV-40}$ = MAPKKRKVK; NLS$^{\text{egl-13}}$ = M5RKKRANPTKISENAKKLAKEVEN

https://doi.org/10.1371/journal.pone.0214257.t002
Fig 2. CemOrange2 subcellular expression patterns within C. elegans. Representative confocal images of transgenic C. elegans strains expressing CemOrange2 fused with: the N-terminal SV-40 and C-terminal egl-13 nuclear localization signals (A, B); LMN-1, a nuclear lamin (C, D); TRAM-1, a rough ER protein (E, F); PISY-1, ER and Golgi protein (G, H); AMAN-2, a Golgi protein (I, J); RAB-5, an early endosomal protein (K, L); RAB-7, a late endosomal protein (M, N); LMP-1 and CUP-5, lysosomal membrane proteins (O-R); GLO-1, lysosomal related organelle protein (LRO) (S, T);
from Dr. B. Grant) or GLO-1::GFP under the ges-1 intestinal specific promoter (VS17). Transgenic animals expressing LMP-1::GFP (S1D Fig) and GLO-1::CemOrange2 (S1E Fig) did not colocalize (S1F Fig) consistent with the LTDR data (S1A–S1C Fig). Transgenic animals expressing GLO-1::GFP (S1G Fig) and GLO-1::CemOrange2 (S1H Fig) colocalized (S1I Fig) suggesting that CemOrange2 did not affect the trafficking of the GLO-1 protein. Taken together, these data suggested that GLO-1::CemOrange2 did not localize to the lysosome and that CemOrange2 did not affect the trafficking of the GLO-1 protein.

Alexa Fluor 647 conjugate (BSA647) is a high molecular weight fluorescent tracer taken up by endocytosis and traffics through early (RAB-5+) and then late (RAB-7+) endosomes to lysosomes (Fig 3H and 3K) [48]. Both CemOrange2::RAB-5 (Fig 3G) and CemOrange2::RAB-7 (Fig 3J) colocalized with BSA647 (Fig 3I and 3L, white arrowheads) with an average MCC = 0.86 and 0.82, respectively (Fig 3BB).

daf-22, a homologue to the human sterol carrier protein, along with GFP::SKL, localizes to the peroxisome in wild-type animals [49]. To determine if the CemOrange2 FP altered trafficking of the SKL motif, DAF-22::GFP transgenic C. elegans were injected with the P
\[\text{nhx-2}\] CemOrange2::SKL plasmid. CemOrange2::SKL (Fig 3M) and GFP::DAF-22 (Fig 3N) colocalized (Fig 3O, white arrowheads) with an average MCC = 0.96 (Fig 3BB).

MitoTracker Deep Red is a fluorescent probe that accumulates within mitochondria. Transgenic animals expressing CemOrange2 with the N-terminal mitochondrial localization tag (\(\text{mt-CemOrange2}, \text{Fig 3P}\)) were stained with MitoTracker Deep Red and examined by confocal microscopy. The \(\text{mt-CemOrange2}\) protein colocalized with MitoTracker Deep Red (Fig 3R; white arrowheads) with an average MCC = 0.82 (Fig 3BB).

The ER retention signal, KDEL, fused to the C-terminus of secreted FPs is often used as an ER and Golgi localization marker. We generated transgenic C. elegans strains by injecting the plasmids P
\[\text{nhx-2}\] GFP::KDEL (Fig 3T) and P
\[\text{nhx-2}\] CemOrange2::tram-1 (Fig 3S). CemOrange2::TRAM-1 colocalized with the GFP::KDEL (Fig 3U, yellow) with an average MCC = 0.88 (Fig 3BB).

Golgi stacks were labeled using C5 FL (Fig 3W). Almost all AMAN-2::CemOrange2 (Fig 3V) colocalized with C5 FL (Fig 3X, white arrowheads) with an average MCC = 0.92 (Fig 3BB). The apical plasma membrane is labeled using CellMask, an amphipathic molecule that exhibits both lipophilic and hydrophilic moieties. AQP-1::CemOrange2 (Fig 3Y) and CellMask (Fig 3Z) colocalized (Fig 3AA, magenta) with an average MCC = 0.91 (Fig 3BB). Taken together, these data suggested that CemOrange2 did not affect the trafficking of fusion proteins or peptides described in this report.

The biological application of CemOrange2 fusion proteins

To assess the bioapplicability of the subcellular organelle markers, transgenic strains expressing a CemOrange2 fusion protein were exposed to different stressors. Lysosomal membrane permeabilization (LMP) is the hallmark of necrotic cell death in the C. elegans enterocyte [50, 51]. LMP was induced in wild-type animals expressing LMP-1::CemOrange2 by the ROS
misfold in the ER and accumulate as polymers and aggregates [55, 56]. Using a modified mutant form of the human serpin, three different FPs; mKate2::LGG-1, LMP-1::CemOrange2, and sGFP::ATZ [27]. ATZ is a

Three-color imaging

To determine the utility of the CemOrange2 FP in multifluorophore imaging, we generated a transgenic line harboring three different nhx-2-driven transgenes expressing proteins fused to three different FPs; mKate2::LGG-1, LMP-1::CemOrange2, and sGFP::ATZ [27]. ATZ is a mutant form of the human serpin, μ1-antitrypsin/SERPINA1. The Z mutation causes ATZ to misfold in the ER and accumulate as polymers and aggregates [55, 56]. Using a modified
CemOrange2 spectra in both the excitation and emission spectrum \((\text{Ex} \, 545 \text{ nm/ Em} \, 560-590 \text{ nm})\) and sequential scanning, the CemOrange2 FP was distinguished from both GFP and mKate2 (Fig 6A–6H). This combination of probes also confirmed several features related to the degradation of ATZ. Macroautophagy is a major degradation pathway for ER-retained sGFP::ATZ. At higher resolution, sGFP::ATZ was detected in LGG\(^+\) puncta (i.e., autophagosomes) as evident by the merge of GFP and mKate2 signals (Fig 6G and 6H, yellow arrowheads). The colocalization of autophagosomes with lysosomes is evident in the merge of mKate2 and CemOrange2 signals (Fig 6G and 6H; white arrowheads). The absence of GFP from these latter structures likely reflects the instability of GFP in acidic vesicles.

In another example, a transgenic \(C. \, e\, l\, e\, g\, a\, n\, s\) strain expressing both \(P_{\text{vha-6}} \, \text{LMP-1::GFP}\) and \(P_{\text{nhx-2}} \, \text{GLO-1::CemOrange2}\) was stained with LTDR and imaged as described above (Fig 6I–6P). Representative images show there is minimal overlap between the fluorophores. As expected, there was extensive overlap between LTDR\(^+\) vesicles merged with GFP\(^+\) lysosomes (Fig 6O and 6P; arrowheads). Taken together, these data demonstrated that CemOrange2 was useful for live-animal multifluorophore imaging.

**Pathological variant validation by using co-localization markers**

Model organisms, such as \(C. \, e\, l\, e\, g\, a\, n\, s\), \(D. \, r\, e\, r\, i\, o\) and \(D. \, m\, e\, l\, a\, n\, o\, g\, a\, s\, t\, e\), have proven to be essential systems in determining whether human variants of unknown significance (VUS) are pathologic [10, 11]. In \(C. \, e\, l\, e\, g\, a\, n\, s\), genotype-pathologic phenotype correlations can be determined by comparing wild-type versus mutant human transgenes or by generating homologous mutations in orthologous genes. One means to assess these phenotypes is by using microscopic analysis to determine whether the variant of interest perturbs cellular dynamics or by altering...
the proteins subcellular distribution [2, 3]. The generation of the CemOrange2 organelle marker toolbox described here will be useful in identifying the subcellular phenotypic changes associated with a pathological VUS. As an example, human ABCA3 is a phospholipid transporter required for assembly of pulmonary surfactant in lamellar bodies and lamellar body biogenesis in type 2 pulmonary alveolar epithelial cells [57]. Different types of ABCA3 mutations disrupt surfactant synthesis and cause neonatal respiratory failure or childhood interstitial lung disease (chILD) in older infants and children [57–61]. One mutation, L101P, results in protein misfolding and accumulation within the ER. To determine if this pathologic variant can be detected in 

C. elegans, a mutated transgene (the conserved leucine is at position 162 in 

C. elegans) containing the worm orthologue of ABCA3, abt-4, fused to the N-terminus of mKate2 was introduced into wild-type animals (Fig 7A, asterix). Wild-type ABT-4::mKate2 was trafficked to the intestinal cell membrane (Fig 7B, 7D and 7F) however, similar to that

Fig 5. SQST-1:: and ::LGG-1 CemOrange2 fusion proteins monitor autophagic flux. P

nhx-2::sqst-1::CemOrange2 (A, C, E; red) and P

nhx-2::CemOrange2::lgg-1 (B, D, F; red) transgenic animals were treated with a diluent control (0.1% DMSO; A and B), 3-methyladenine (10 mM 3-MA, C and D), or 25 μM fluphenazine (E and F) for 16 hrs in liquid culture and analyzed using confocal microscopy over ≥ 20 z-planes Ex555 nm/Em565-590 nm. Representative confocal images of animals shown as a single XY plane. Scale bar = 25 μm. Multiple confocal images of transgenic animals (n ≥ 5 animals) expressing SQST-1::CemOrange2 (G) or CemOrange2::LGG-1 (H) after treatment with the above compounds, were quantified for number of puncta per μm² imaged using the Quantification module in the Volocity image analysis software (v6.3). Statistical analysis of the drug-treated animals relative to diluent control was performed using an unpaired, 2-tailed t-test (’p<0.05).

https://doi.org/10.1371/journal.pone.0214257.g005
Fig 6. CemOrange2 and three-color imaging. $P_{\text{nhx-2} \text{mp-1}}: \text{CemOrange2}; P_{\text{nhx-2} \text{GFP}}: \text{ATZ}; P_{\text{nhx-2} \text{mKate2}}: \text{lgg-1}$ transgenic C. elegans expressing sGFP::ATZ (A and B; green; Ex488 nm/Em500-540 nm), LMP-1::CemOrange2 (C and D; pseudocolored blue; Ex545 nm/Em560-590 nm) and mKate2::LGG-1 (E and F; red; Ex594 nm/Em605-645 nm) were examined by confocal microscopy over >20 z-planes using a 40x PlanApo oil immersion objective (N.A. 1.3). Maximum intensity projections are shown (A, C, E, G). Scale bars = 25 μm. Magnified single XY regions (dashed box) are included to highlight colocalization events.
observed in mammalian cell lines, ABT-4\textsuperscript{L162P} was retained in the ER of intestinal cells of \textit{C. elegans} (Fig 7C, 7E, 7G and 7H). These results confirm that \textit{C. elegans} can be used to assess human VUSs.

**Discussion**

The purpose of this study was to generate a panel of FP markers for subcellular structures that could be used for multiplex imaging in \textit{C. elegans}, one of the premier model organisms for studying cell biological and developmental processes in real-time \cite{2, 3, 11}. The challenge was to select a FP that did not aggregate spontaneously \textit{in vivo}, and displayed an Ex/Em spectra that was distinct from the popular FPs already adapted for use in \textit{C. elegans} (e.g., GFP, YFP, CFP and mCherry) \cite{1, 5, 7–9}. The ideal fluorescent protein should also have a fast maturation time, high brightness and high photostability. Of the fluorescent proteins that we chose to study, mCardinal has the highest photostability and a fast maturation time (Table 1). However, it possesses approximately half the brightness of EGFP (Table 1). While the maturation time of mNeptune2.5 is similar to EGFP, it has approximately 70% the brightness and the photostability is unknown. Additionally, the reported \(Ex_{\text{max}}/Em_{\text{max}}\) of mNeptune2.5 is closest to both mCherry and mKate2, which would make it more difficult to spectrally separate from these 2 commonly used fluorophores. While mOrange2 has a reported slow maturation time of 270 minutes, it has a brightness similar to EGFP and has a high photostability of 228 seconds (Table 1). After codon adaptation, we found that CemOrange2, had an \(Ex_{\text{max}}/Em_{\text{max}}\) spectra \textit{in vivo} that placed it between those of YFP and mKate2, and that the signal from this FP was easily distinguished from \textit{C. elegans} autofluorescence (S2 Fig). While CemOrange2 could be spectrally distinguished from the far red mKate2 \textit{in vivo}, this FP spectra would overlap with other commonly used red FPs, such as DsRed (\(Ex_{\text{max}}/Em_{\text{max}}\) 558/553) and dTomato (\(Ex_{\text{max}}/Em_{\text{max}}\) 554/581). In contrast, both the \textit{in vivo} CemNeptune2.5 and CemCardinal emission and excitation spectra overlapped significantly with mKate2, which would confound colocalization studies. Moreover, the CemOrange2 FP demonstrated a diffuse and homogenous cytoplasmic distribution when expressed in \textit{C. elegans} intestinal cells. In contrast, CemCardinal2, a monomeric FP, showed both a diffuse and granular distribution pattern. We did not determine whether CemCardinal2 formed aggregates \textit{in vivo}, or associated with a vesicular structure due to a unique aspect of its structure. Regardless, we were reluctant to utilize CemCardinal2 as this characteristic could confound its use in colocalization studies. Another advantage of CemOrange2 is that it also avoids the phototoxicity associated with the distinct \(Ex/Em\) spectra of BFPs \cite{62}. While the confocal setup described here uses a supercontinuum white light laser source, more traditional confocal and widefield systems can be used to to visualize CemOrange2. Indeed, CemOrange2 was detectable using a 561nm wavelength excitation laser (S3D Fig). Additionally, there was no observable cross talk with spectral channels visualizing the GFP or mKate2 fluorophore excitation and emission settings (\(Ex488\) nm/\(Em500-540\) nm and \(Ex594\) nm/\(Em605-640\) nm, respectively; S3A–S3F Fig). Moreover, tuning the white light laser
Fig 7. A CemOrange2 marker co-localizes a mutant ABC transporter protein to the ER. Alignment of the primary amino sequence from human (Hsa) ABCA3 (Accession: AAH20724.1) and C. elegans (Cel) ABT-4 (Accession: NP_503175.1) using the ClustalW algorithm (A). Only residues 100–136 of HsaABCA3 aligned with residues 161–200 from CelABT-4 are shown. The asterisk marks L101 (human numbering) or L162 (C. elegans numbering) in the alignment that is conserved between species. P_{nhx-2}::abt-4::mKate2; P_{nhx-2}::TRAM-1::CemOrange2 (B, D, F) or P_{nhx-2}::abt-4^{L162P}::mKate2; P_{nhx-2}::TRAM-1::CemOrange2 (C, E, G) transgenic C. elegans strains were imaged by confocal microscopy using a 40x PlanApo oil immersion objective (N.A. 1.3) over >20 z-planes. Representative single XY regions are shown. ABT-4::mKate2 (D, pseudocolored blue) or ABT-4^{L162P}::mKate2 (E, pseudocolored blue; Ex 594 nm/Em 605-645 nm) and TRAM-1::CemOrange2 (C, orange; Ex 549 nm/Em 560-580 nm), showed that ABT-4::mKate2 trafficked normally to the apical membrane in intestinal cells (F), whereas the single point mutation in ABT-4^{L162P} triggered retention within the ER (G). Scale bar = 25 μm. (H) Colocalization between ABT-4 or ABT-4^{L162P} and TRAM-1 (F and G, respectively) was determined using the Pearson correlation in Volocity image analysis software (v6.3) (** p<0.001; n>10).

https://doi.org/10.1371/journal.pone.0214257.g007
source to a more conventional single line laser imaging system using excitation wavelengths of 488nm, 561nm and 594nm, we were still able to separate GFP, CemOrange2 and mKate2 FPs in the P
\textit{nhx-2}::\textit{CemOrange2};P
\textit{nhx-2}::\textit{GFP};P
\textit{nhx-2}::\textit{mKate2};lgg-1 transgenic \textit{C. elegans} (S3G–S3L Fig).

We generated a series of plasmids containing a CemOrange2 minigene fused to a targeting sequence or another gene expressing a protein known to target to a unique subcellular organelle or location. Using confocal microscopy, the subcellular distribution patterns of these markers suggested that they were targeted to the correct location. However, FPs can affect the folding and conformation of their fusion partner so we sought an independent means to assure that the CemOrange2 markers were targeting the correct cellular address [63]. In all nine cases tested, the CemOrange2 fusions showed a significant colocalization with a non-overlapping fluorophore targeting the same structure (MCC>0.5). Taken together, these studies showed that CemOrange2 FPs were directed to the correct subcellular addresses and should prove useful for multicolor live-cell imaging. Indeed, this functionality was demonstrated by a transgenic strain expressing human alpha-1 antitrypsin with the Z mutation fused to the C-terminus of GFP. This aggregation-prone protein, is retained in the ER and is partially degraded by macroautophagy [27, 28, 32, 56]. Examination of these animals showed colocalization of ATZ in LGG-1+ (a \textit{C. elegans} orthologue of LC3) structures (i.e., autophagosomes). The identification in of autophagosomes with a specific cargo is difficult in real-time and underscores the sensitivity of multifluorophore imaging technology.

Knowledge about human variants of unknown significance can be obtained by determining whether the phenotype of the variant differs from the wild-type gene when expressed in model organisms, such as \textit{C. elegans}, \textit{D. rerio}, \textit{D. melanogaster} or \textit{S. cerevisiae} [2, 3, 10, 11]. Some abnormal phenotypes are straightforward in their presentation at the whole organism level, if they result in, for example, abnormal development of decreased longevity. However, many abnormal phenotypes are subtler in their presentation and are manifest only after the application of a cellular stress or by examining subcellular functions; for example, protein misfolding disorders. Many of these proteostasis disorders only manifest after the aggregation-prone or misfolded proteins accumulate over time. Classical examples are neurodegenerative disorders associated with the aberrant accumulation of Huntingtin, A\textbeta, alpha-synuclein and/or tau [64–67]. In many cases, alterations in the subcellular distribution of these proteins are detected long before a pathologic phenotype emerges. For this reason, multifluorophore imaging in \textit{C. elegans} can be a useful adjunct to the analysis of VUS, especially those associated with the subtle effects associated with protein misfolding. We showed that a mutation in the \textit{C. elegans} ABC transporter, \textit{abt-4}, resulted in ER retention, which is exactly what occurs in the human orthologous gene, ABCA3, with an identical mutation at a single conserved amino acid [57–61]. Similar results were observed in \textit{C. elegans} expressing the human pathologic variant Z of alpha-1 antitrypsin [27, 28, 32]. Taken together, these studies show that the addition of CemOrange2 to the \textit{C. elegans} FP toolbox expands their ability to assess human VUS behavior by multifluorophore, real-time subcellular imaging.

**Supporting information**

S1 Table. PCR primer pairs used for transgene construction. (DOCX)

S2 Table. \textit{C. elegans} strains used in this study. (DOCX)
S1 Fig. The lysosomal related organelle marker, GLO-1, does not colocalize with lysosomes. A-C) Transgenic C. elegans expressing GLO-1::CemOrange2 (A; red, Ex549 nm/Em560-585 nm) were stained with LTDR (B, blue; Ex647 nm/Em660-700 nm) and examined by confocal microscopy over >20 z-planes using a 40x PlanApo oil immersion objective (N.A. 1.3). Note the lack of colocalization as shown by discrete blue and red puncta (C, merge). The MCC of this representative image was 0.18, indicating the absence of colocalization. P_{nhx-glo-1}::CemOrange2;P_{vha-6}::GFP (D and E) transgenic C. elegans were imaged by confocal microscopy over >20 z-planes using a 63x PlanApo oil immersion objective (N.A. 1.4). LMP-1::GFP puncta (D and F; green; Ex488 nm/Em500-540 nm) do not colocalize with the GLO-1::CemOrange2 (E and F; red; Ex555 nm/Em565-590 nm). (G-I) CemOrange2 did not affect the trafficking of GLO-1. P_{nhx-glo-1}::CemOrange2;P_{ges-glo-1}::GFP (G and H) transgenic animals showed GLO-1::GFP positive (G, green; Ex488 nm/Em500-540 nm) and GLO-1::CemOrange2 puncta (H, red; Ex555 nm/Em565-590 nm) colocalized (I, merge yellow). Scale bars = 5 μm.

S2 Fig. CemOrange2 fluorescence is distinct from background autofluorescence in wild-type C. elegans. Either transgenic (A) P_{nhx-2}::CemOrange2 or (B) wild-type (N2) C. elegans posterior intestines were imaged using a confocal microscope fitted with a white light laser and spectral detectors at varying excitation (range 470 nm-670 nm in 5 nm steps) and emission wavelengths (range 485–785 in 5 nm steps and a detection window of 20 nm). The fluorescence intensity (0–255) of each 8 bit image at each excitation and emission wavelength was plotted using the 2D bilinear excitation and emission lambda scan algorithm in the LASX software (Leica Microsystems, Buffalo Grove, IL) using the color gradient of fluorescence intensity indicated. The inset in B shows the autofluorescence of the wild-type C. elegans with a the look up table (LUT) rescaled to between 0 and 20 to show that autofluorescence is exhibited in the blue light range with minimal overlap with the CemOrange2 fluorescence spectrum.

S3 Fig. CemOrange2 can be visualized using standard excitation laser lines. Either P_{nhx-2}::Imp-1::CemOrange2 (A-F) or P_{nhx-2}::Imp-1::CemOrange2;P_{nhx-2}::GFP::ATZ;P_{nhx-2}::mKate2::lgg-1 (G-L) transgenic C. elegans were imaged using a confocal microscope fitted with a white light laser set at either optimized 488, 545 and 594 nm (A, C, E, G, I, K) or standard 488, 561 and 594 nm (B, D, F, H, J, L) excitation wavelengths in sequential imaging mode over >30 z-planes. At either optimized or standard imaging excitation wavelengths, P_{nhx-2}::Imp-1::CemOrange2 was detected only with the 545 nm (C; blue) or 561 nm (D; blue) excitation laser settings with similar punctate distribution with no cross talk with the 488 nm (A, B; green) and 594 nm (E, F; red) excitation laser lines. P_{nhx-2}::Imp-1::CemOrange2;P_{nhx-2}::GFP::ATZ;P_{nhx-2}::mKate2::lgg-1 transgenic C. elegans imaged under the same conditions showed that all three fluorophores were readily detected using optimized or standard imaging settings; GFP::ATZ (G, H; green) using the Ex488 nm laser line, LMP-1::CemOrange2 (I, J; blue) with either Ex545 nm (I) and Ex561 nm (J) laser line and mKate2::LGG-1 (K, L; red) with the Ex594 nm laser line indicating that CemOrange2 can be utilized with more standard confocal imaging systems. Scale bar = 25 nm.

Acknowledgments

This work was supported by National Institute of Health grants R01DK104946 (https://www.niddk.nih.gov/, GAS), R01DK114047 (https://www.niddk.nih.gov/, CJL, GAS), UDN U01HG010215 (https://www.genome.gov/, FSC, JAW), the Children’s Discovery Institute of...
St. Louis Children’s Hospital Foundation (http://www.childrensdiscovery.org, CJL, SCP). Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (https://orip.nih.gov/, P40 OD010440). The plasmid constructs described in this manuscript (including CemOrange2, CemCardinal2 and CemNeptune2) were deposited with Addgene (https://www.addgene.org/). C. elegans strains with subcellular structures labeled by CemOrange2 fusion proteins were deposited in the Caenorhabditis Genetics Center (https://cgc.umn.edu/).

Author Contributions

Conceptualization: Brian J. Thomas, Huiyan Huang, Justin R. Buland, Jennifer A. Wambach, F. Sessions Cole, Stephen C. Pak, Gary A. Silverman, Cliff J. Luke.

Formal analysis: Brian J. Thomas, Cliff J. Luke.

Funding acquisition: Gary A. Silverman.

Investigation: Brian J. Thomas, Ira E. Wight, Wendy Y. Y. Chou, Marco Moreno, Zachary Dawson, Arielle Homayouni, Hyori Kim, Cliff J. Luke.

Methodology: Ira E. Wight, Wendy Y. Y. Chou, Marco Moreno, Zachary Dawson, Arielle Homayouni, Huiyan Huang, Hyori Kim, Hanna Jia.

Supervision: Stephen C. Pak, Gary A. Silverman, Cliff J. Luke.

Writing – original draft: Brian J. Thomas, Gary A. Silverman, Cliff J. Luke.

Writing – review & editing: Brian J. Thomas, Huiyan Huang, Justin R. Buland, Jennifer A. Wambach, F. Sessions Cole, Stephen C. Pak, Gary A. Silverman, Cliff J. Luke.

References

1. Heppert JK, Dickinson DJ, Pani AM, Higgins CD, Steward A, Ahringer J, et al. Comparative assessment of fluorescent proteins for in vivo imaging in an animal model system. Mol Biol Cell. 2016; 27(22):3385-94. https://doi.org/10.1091/mbc.E16-01-0063 PMID: 27385332

2. O’Kane CJ. Modelling human diseases in Drosophila and Caenorhabditis. Semin Cell Dev Biol. 2003; 14(1):3–10. PMID: 12524001

3. Silverman GA, Luke CJ, Bhatia SR, Long OS, Vetica AC, Perlmutter DH, et al. Modeling molecular and cellular aspects of human disease using the nematode Caenorhabditis elegans. Pediatr Res. 2009; 65(1):10–8. https://doi.org/10.1203/PDR.0b013e31819009b0 PMID: 18852689

4. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. Science. 1994; 263(5148):802–5. PMID: 8303295

5. Day RN, Davidson MW. The fluorescent protein palette: tools for cellular imaging. Chem Soc Rev. 2009; 38(10):2887–921. https://doi.org/10.1039/b901966a PMID: 19771335

6. Pincus Z, Mazer TC, Slack FJ. Autofluorescence as a measure of senescence in C. elegans: look to red, not blue or green. Aging (Albany NY). 2016; 8(5):889–98.

7. Green RA, Audhya A, Pozniakovsky A, Dammermann A, Pemble H, Monen J, et al. Expression and imaging of fluorescent proteins in the C. elegans gonad and early embryo. Methods Cell Biol. 2008; 85:179–218. https://doi.org/10.1016/S0091-679X(08)85009-1 PMID: 18155464

8. Hutter H. Fluorescent protein methods: strategies and applications. Methods Cell Biol. 2012; 107:67–92. https://doi.org/10.1016/B978-0-12-394620-1.00003-5 PMID: 22226521

9. Miller DM 3rd, Desai NS, Hardin DC, Piston DW, Patterson GH, Fleenor J, et al. Two-color GFP expression system for C. elegans. Biotechniques. 1999; 26(5):914–21. https://doi.org/10.2144/99265rr01 PMID: 10337485

10. Splinter K, Adams DR, Bacino CA, Bellen HJ, Bernstein JA, Chee-Jarvela AM, et al. Effect of genetic diagnosis on patients with previously undiagnosed disease. N Engl J Med. 2018; 379(22):2131–9. https://doi.org/10.1056/NEJMoa1714458 PMID: 30304647
11. Wang J, Al-Ouran R, Hu Y, Kim SY, Wan YW, Wangler MF, et al. MARRVEL: Integration of human and model organism genetic resources to facilitate functional annotation of the human genome. Am J Hum Genet. 2017; 100(6):843–53. https://doi.org/10.1016/ajhg.2017.04.010 PMID: 28502612

12. Campbell EM, Fares H. Roles of CUP-5, the Caenorhabditis elegans orthologue of human TRPML1, in lysosome and gut granule biogenesis. BMC cell biology. 2010; 11:40. https://doi.org/10.1186/1471-2121-11-40 PMID: 20504742

13. Hermann GJ, Schroeder LK, Hieb CA, Kershner AM, Rabbits BM, Fonarev P, et al. Genetic analysis of lysosomal trafficking in Caenorhabditis elegans. Mol Cell. 2005; 16(7):3273–88. https://doi.org/10.1016/j.molcel.2005.05.022 PMID: 15843430

14. Kostich M, Fire A, Fambrough DM. Identification and molecular-genetic characterization of a LAMP/CDD6-like protein from Caenorhabditis elegans. J Cell Sci. 2000; 113 (Pt 14):2595–606.

15. Rolls MM, Hall DH, Victor M, Stentz AJ. Visible continuum generation in air-silica microstructured optical fibers with anomalous dispersion at 800 nm. Opt Lett. 2000; 25(1):25–7. PMID: 18059770

16. Redemann S, Schloissinger S, Ernst S, Pozniakowsky A, Ayloo S, Hyman AA, et al. Codon adaptation-based control of protein expression in Caenorhabditis elegans. Nat Methods. 2011; 8(3):250–2. https://doi.org/10.1038/nmeth.1565 PMID: 21278743

17. Shaner NC, Lin MZ, McKeown MR, Steinbach PA, Hazelwood KL, Davidson MW, et al. Improving the photostability of bright monomeric orange and red fluorescent proteins. Proc Natl Acad Sci U S A. 2010; 107(45):19043–8. https://doi.org/10.1073/pnas.1010758107 PMID: 21070744

18. Patterson GH, Knobel SM, Sharif WD, Kain SR, Piston DW. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. Biophys J. 1997; 73(5):2782–90. https://doi.org/10.1016/S0006-3495(97)78307-3 PMID: 9370472

19. Shcherbo D, Murphy CS, Ermakova GV, Solovieva EA, Chepurnykh TV, Shcheglov AS, et al. Far-red fluorescent tags for protein imaging in living tissues. Biochem J. 2009; 418(3):567–74. https://doi.org/10.1042/BJ20081949 PMID: 19143658

20. Tsien RY. The green fluorescent protein. Annu Rev Biochem. 1998; 67:509–44. https://doi.org/10.1146/annurev.biochem.67.1.509 PMID: 9759496

21. Wang J, Al-Ouran R, Hu Y, Kim SY, Wan YW, Wangler MF, et al. MARRVEL: Integration of human and model organism genetic resources to facilitate functional annotation of the human genome. Am J Hum Genet. 2017; 100(6):843–53. https://doi.org/10.1016/ajhg.2017.04.010 PMID: 28502612

22. Campbell EM, Fares H. Roles of CUP-5, the Caenorhabditis elegans orthologue of human TRPML1, in lysosome and gut granule biogenesis. BMC cell biology. 2010; 11:40. https://doi.org/10.1186/1471-2121-11-40 PMID: 20504742

23. Hermann GJ, Schroeder LK, Hieb CA, Kershner AM, Rabbits BM, Fonarev P, et al. Genetic analysis of lysosomal trafficking in Caenorhabditis elegans. Mol Cell. 2005; 16(7):3273–88. https://doi.org/10.1016/j.molcel.2005.05.022 PMID: 15843430

24. Kostich M, Fire A, Fambrough DM. Identification and molecular-genetic characterization of a LAMP/CDD6-like protein from Caenorhabditis elegans. J Cell Sci. 2000; 113 (Pt 14):2595–606.
32. Gosai SJ, Kwak JH, Luke CJ, Long OS, King DE, Kovatch KJ, et al. Automated high-content live animal drug screening using C. elegans expressing the aggregation prone serpin α1-antitrypsin Z. PLoS One 2010; 5(11):e15460. https://doi.org/10.1371/journal.pone.0015460 PMID: 21103396

33. Ettinger A, Wittmann T. Fluorescence live cell imaging. Methods Cell Biol. 2014; 123:77–94. https://doi.org/10.1016/B978-0-12-420138-5.00005-7 PMID: 24974023

34. Liu J, Rolef-Shahar T, Riemer D, Treinin M, Spann P, Weber K, et al. Essential roles for Caenorhabditis elegans lamin gene in nuclear organization, cell cycle progression, and spatial organization of nuclear pore complexes. Mol Biol Cell. 2000; 11(11):3937–47. https://doi.org/10.1091/mbc.11.11.3937 PMID: 11071918

35. Paschinger K, Hackl M, Gutierrez M, Kretschmer-Lubich D, Stemmer U, Jantsch V, et al. A deletion in the golgi alpha-mannosidase II gene of Caenorhabditis elegans results in unexpected non-wild-type N-glycan structures. J Biol Chem. 2006; 281(38):28265–77. https://doi.org/10.1074/jbc.M602878200 PMID: 16864579

36. Chen B, Jiang Y, Zeng S, Yan J, Li X, Zhang Y, et al. Endocytic sorting and recycling require membrane phosphatidylinerine asymmetry maintained by TAT-1/CHAT-1. PLoS Genet. 2010; 6(12):e1001235. https://doi.org/10.1371/journal.pgen.1001235 PMID: 21170358

37. Guo B, Huang J, Wu W, Feng D, Wang X, Chen Y, et al. The nascent polypeptide-associated complex regulates the activity of autophagy. 2014; 10(10):1738–48. https://doi.org/10.4161/auto.29638 PMID: 25126725

38. Tian Y, Li Z, Hu W, Ren H, Tian E, Zhao Y, et al. C. elegans screen identifies autophagy genes specific to multicellular organisms. Cell. 2010; 141(6):1042–55. https://doi.org/10.1016/j.cell.2010.04.034 PMID: 20550938

39. Hermann GJ, Scavarda E, Weis AM, Saxton DS, Thomas LL, Salesky R, et al. C. elegans BLOC-1 functions in trafficking to lysosome-related gut granules. PLoS One. 2012; 7(8):e43043. https://doi.org/10.1371/journal.pone.0043043 PMID: 22916203

40. Huang CG, Lamitina T, Agre P, Strange K. Functional analysis of the aquaporin gene family in Caenorhabditis elegans. Am J Physiol Cell Physiol. 2007; 292(5):C1867–773. https://doi.org/10.1152/ajpcell.00514.2006 PMID: 18072588

41. Lyssenko NN, Hanna-Rose W, Schlegel RA. Cognate putative nuclear localization signal effects strong nuclear localization of a GFP reporter and facilitates gene expression studies in Caenorhabditis elegans. Biotechniques. 2007; 43(5):596–600. https://doi.org/10.2144/000112615 PMID: 17229810

42. Labrousse AM, Zappaterra MD, Rube DA, van der Bliek AM. C. elegans dynamin-related protein DRP-1 controls severing of the mitochondrial outer membrane. Mol Cell. 1999; 4(5):815–26. PMID: 1083/jcb.200511103 PMID: 100514.2006 PMID: 17229810

43. Motley AM, Hettema EH, Ketting R, Plasterk R, Tabak HF. Caenorhabditis elegans has a single pathway to target matrix proteins to peroxisomes. EMBO Rep. 2000; 1(1):40–6. https://doi.org/10.1093/embo-reports/kvd010 PMID: 11256623

44. Guo B, Huang J, Zhang P, Qi L, Liang Q, Zhang X, et al. Genome-wide screen identifies signaling pathways that regulate autophagy during Caenorhabditis elegans development.

45. Sato M, Konuma R, Sato K, Tomura K, Sato K. Fertilization-induced K63-linked ubiquitylation mediates clearance of maternal membrane proteins. Development. 2014; 141(6):1324–31. https://doi.org/10.1242/dev.103044 PMID: 24595290

46. Artal-Sanz M, Samara C, Symthichaki P, Tavernarakis N. Lysosomal biogenesis and function is critical for necrotic cell death in Caenorhabditis elegans. J Cell Biol. 2006; 173(2):231–9. https://doi.org/10.1083/jcb.200511103 PMID: 16636145

47. Schroeder LK, Kremer S, Kramer MJ, Currie E, Kwan E, Watts JL, et al. Function of the Caenorhabditis elegans ABC transporter PGP-2 in the biogenesis of a lysosome-related fat storage organelle. Mol Biol Cell. 2007; 18(3):995–1008. https://doi.org/10.1091/mbc.E06-08-0685 PMID: 17202409

48. Weaver DJ Jr., Voss EW Jr. Analysis of rates of receptor-mediated endocytosis and exocytosis of a fluorescent hapten-protein conjugate in murine macrophage: implications for antigen processing. Biol Cell. 1998; 90(2):169–81. PMID: 9691434

49. Joo HU, Yim YH, Jeong PY, Jin YX, Lee JE, Kim H, et al. Caenorhabditis elegans utilizes dauer pheromone biosynthesis to dispose of toxic peroxisomal fatty acids for cellular homeostasis. Biochem J. 2009; 422(1):61–71. https://doi.org/10.1042/Bj20090513 PMID: 19496754

50. Coburn C, Allman E, Mahanti P, Benedetto A, Cabreiro F, Pincus Z, et al. Anthranilate fluorescence marks a calcium-propagated necrotic wave that promotes organismal death in C. elegans. PLoS Biol. 2013; 11(7):e1001613. https://doi.org/10.1371/journal.pbio.1001613 PMID: 23935448
51. Luke CJ, Pak SC, Askew YS, Naviglia TL, Askew DJ, Nobar SM, et al. An intracellular serpin regulates necrosis by inhibiting the induction and sequelae of lysosomal injury. Cell. 2007;130(6):1108–19. https://doi.org/10.1016/j.cell.2007.07.013 PMID: 17889653

52. Aits S, Kricker J, Lui B, Ellegaard AM, Hamalisto S, Tvangsholm S, et al. Sensitive detection of lysosomal membrane permeabilization by lysosomal galectin puncta assay. Autophagy. 2015;11(8):1408–24. https://doi.org/10.1080/15548627.2015.1063871 PMID: 26114578

53. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, et al. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem. 2007;282(33):24131–45. https://doi.org/10.1074/jbc.M702824200 PMID: 17580304

54. Blommaart EF, Krause U, Schellen JP, Vreeling-Sindelarova H, Meijer AJ. The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. Eur J Biochem. 1997;243(1–2):240–6. PMID: 9030745

55. Perlmutter DH. The cellular response to aggregated proteins associated with human disease. J Clin Invest. 2002;110(12):1219–20. https://doi.org/10.1172/JCI16780 PMID: 12417557

56. Perlmutter DH. The role of autophagy in alpha-1-antitrypsin deficiency: a specific cellular response in genetic diseases associated with aggregation-prone proteins. Autophagy. 2006;2(4):258–63. PMID: 16874089

57. Shulenin S, Nogee LM, Annio T, Wert SE, Whitsett JA, Dean M. ABCA3 gene mutations in newborns with fatal surfactant deficiency. N Engl J Med. 2004;350(13):1296–303. https://doi.org/10.1056/NEJMo/a032178 PMID: 15044640

58. Cheong N, Madesh M, Gonzales LW, Zhao M, Yu K, Ballard PL, et al. Functional and trafficking defects in ATP binding cassette A3 mutants associated with respiratory distress syndrome. J Biol Chem. 2006;281(14):9791–800. https://doi.org/10.1074/jbc.M507515200 PMID: 16415354

59. Matsumura Y, Ban N, Ueda K, Inagaki N. Characterization and classification of ATP-binding cassette transporter ABCA3 mutants in fatal surfactant deficiency. J Biol Chem. 2006;281(45):34503–14. PMID: 16959783

60. Wambach JA, Yang P, Wegner DJ, Heins HB, Kaliberova LN, Kaliberov SA, et al. Functional characterization of ATP-binding cassette transporter A3 mutations from infants with respiratory distress syndrome. Am J Respir Cell Mol Biol. 2016;55(5):716–21. https://doi.org/10.1165/rcmb.2016-0008OC PMID: 27374344

61. Weichert N, Kaltenborn E, Hector A, Woischnik M, Schams A, Holzinger A, et al. Some ABCA3 mutations elevate ER stress and initiate apoptosis of lung epithelial cells. Respir Res. 2011;12:4. https://doi.org/10.1186/1465-9921-12-4 PMID: 21214890

62. Godley BF, Shamshi FA, Liang FQ, Jarrett SG, Davies S, Boulton M. Blue light induces mitochondrial DNA damage and free radical production in epithelial cells. J Biol Chem. 2005;280(22):21061–6. Epub 2005/03/31. https://doi.org/10.1074/jbc.M502194200 PMID: 15797866

63. Lee S, Lim WA, Thorn KS. Improved blue, green, and red fluorescent protein tagging vectors for S. cerevisiae. PLoS One. 2013;8(7):e67902. https://doi.org/10.1371/journal.pone.0067902 PMID: 23844123

64. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer’s disease: progress and problems on the road to therapeutics. Science. 2002;297(5580):353–6. https://doi.org/10.1126/science.1072994 PMID: 12130773

65. Ross CA, Poirier MA. Opinion: What is the role of protein aggregation in neurodegeneration? Nat Rev Mol Cell Biol. 2005;6(11):891–8. https://doi.org/10.1038/nrm1742 PMID: 16167052

66. Rubinsztein DC. The roles of intracellular protein-degradation pathways in neurodegeneration. Nature. 2006;443(7113):780–6. https://doi.org/10.1038/nature05291 PMID: 17051204

67. Tillement JP, Lecanu L, Papadopoulos V. Amyloidosis and neurodegenerative diseases: current treatments and new pharmacological options. Pharmacology. 2010;85(1):1–17. https://doi.org/10.1159/000259044 PMID: 19923873