

Fluorescent Proteins in Cellular Organelles: Serious Pitfalls and Some Solutions

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Fluorescent proteins (FPs) have been powerful tools for cell biologists for over 15 years. The large variety of FPs available rarely comes with an instruction manual or a warning label. The potential pitfalls of the use of FPs in cellular organelles represent a significant concern for investigators. FPs generally did not evolve in the often distinctive physicochemical environments of subcellular organelles. In organelles, FPs can misfold, go dark, and even distort organelle morphology. In this minireview, we describe the issues associated with FPs in organelles and provide solutions to enable investigators to better exploit FP technology in cells.

Introduction

EUKARYOTIC CELLS contain membrane-bound compartments, which often have chemically distinct environments to perform specialized tasks. Different organelles within the secretory pathway have lumina with increasingly acidic pH values (Fig. 1A) (Paroutis *et al.*, 2004). In addition, proteins can form disulfide bonds in the endoplasmic reticulum (ER), the intermembrane space of mitochondria, and the periplasm of bacteria (Fig. 1B, C). Secretory proteins containing consensus glycosylation sequences (Hebert *et al.*, 2005; Schwarz and Aebi, 2011; Hanover *et al.*, 2012) can be glycosylated on asparagines and serines/threonines (Fig. 1B). Regardless of whether a protein is native to an organelle, the protein will be susceptible to these modifications.

Fluorescent proteins (FPs) were first identified in the cytoplasm of jellyfish (Shimomura *et al.*, 1962). The ability of the investigators to express the green fluorescent protein (GFP) in non-native species (bacteria, *Caenorhabditis elegans*, human cells, etc.) ushered in a new era of cell biology in which investigators could genetically label cellular proteins and compartments with fluorophores (Chalfie *et al.*, 1994). However, FPs appear to work more or less well in different cell compartments. In some cases, FPs work (or are obviously fluorescent), but as shown below, the fraction of successfully folded and fluorescent molecules may be quite low. Worse, FPs can even distort cellular organelle structures. How can FPs be made truly inert and fully exploited in all cellular compartments?

FP Essentials

All FPs share the common properties of forming a β -barrel and formation of the barrel is necessary before an autocatalytic reaction converts a tripeptide into a fluorophore (Tsien,

1998). Different FPs form fluorophores at rates ranging from minutes to several hours (Shaner *et al.*, 2007). Relevant to this minireview, perturbation of the β -barrel or failure to form the barrel will result in a nonfluorescent protein. Once the protein achieves a barrel formation and produces a fluorophore, the fluorophore properties can be modified by the environment. Most commonly in cells, the major environmental change that impacts fluorescence is pH. This is true for fluorescent dyes, such as FITC, as well. Fortunately, most FPs have been extensively characterized and the pH range that impacts a particular FP is described as the FP's pKa (Kneen *et al.*, 1998). Specifically, the pKa defines the pH at which the brightness of a fluorophore decreases by one half. The fluorophore will continue to become darker at decreasing pH values. Thus, in the progressively more acidic endocytic pathway of a mammalian cell (Fig. 1A), an FP may be fluorescent at the cell surface, dimmer in an early endosome (pH 5.5), and dark in a late endosome or lysosome (pH 4.5) (Paroutis *et al.*, 2004). Therefore, it is important to choose an FP that will be suitable for studies in a particular cellular pH.

FPs can be used by themselves as inert probes or reporters (Daye *et al.*, 1999; Nehls *et al.*, 2000; Snapp *et al.*, 2006) in biosensor fluorescence resonance energy transfer reporters (Zadran *et al.*, 2012), or as fusions with a protein of interest. In the latter case, placement of the FP relative to the protein of interest is essential. For example, proteins in the secretory pathway contain sequences critical for targeting to the ER (i.e., the signal sequence) and sequences involved in retrieval or retention of a protein in a compartment (i.e., a KDEL sequence). Both types of sequence are positional and must be at the absolute NH₂ or COOH terminus of a protein. Placement of the FP before or after the start or end of the protein will result in significant mistargeting. Issues of FP placement in fusion proteins are discussed more extensively elsewhere (Snapp, 2005, 2009).

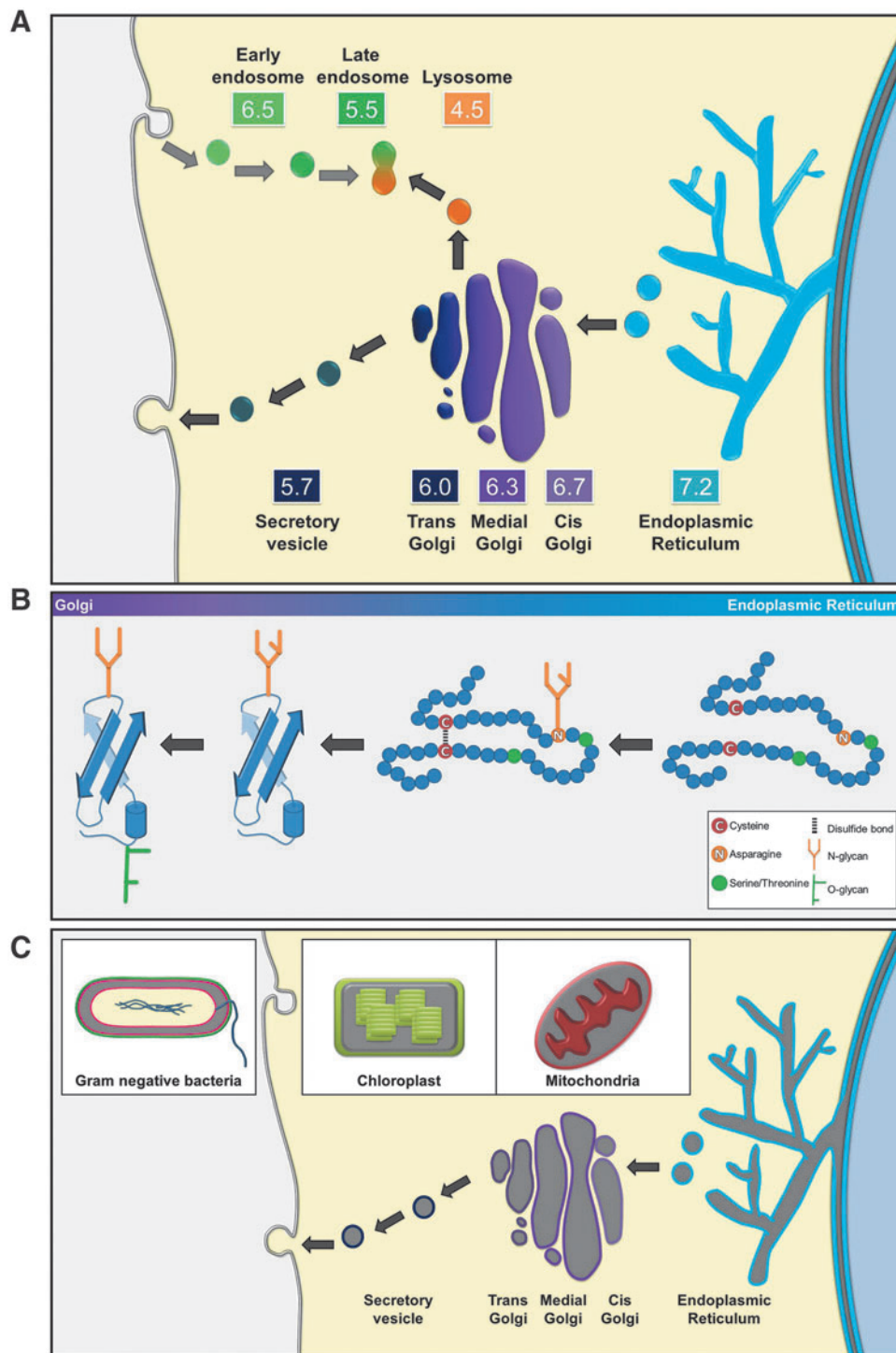


FIG. 1. The diverse chemical environments within cellular organelles pose undesirable consequences for fluorescent proteins (FPs). **(A)** The organelles of the eukaryotic secretory pathway maintain distinct luminal pH values. FPs targeted to the secretory or endosomal pathways will encounter pH values that range from 7.2 to 5.7 or 6.5 to 4.5, respectively. Therefore, it is important to consider the pKa values of FPs in these environments. **(B)** Secretory proteins with cysteine(s) and/or glycosylation consensus sequences are modified as they traverse through the secretory pathway. In the endoplasmic reticulum (ER), N-linked sugars are added covalently, and as proteins within the secretory pathway continue to the Golgi complex, N-linked sugars are modified and potential O-linked glycosylation can be added as well. Cysteines have the potential to form disulfide bonds, primarily within the ER, although the entire secretory pathway is effectively oxidizing. **(C)** Eukaryotic (secretory pathway and the intermembrane space of chloroplasts and mitochondria) and prokaryotic (gram-negative bacteria periplasm) cellular environments with oxidizing environments, depicted with gray coloring.

Assuming one has proper placement within a fusion protein of an FP appropriate to an organelle's pH, what other issues might an investigator face? Surprisingly, there are at least three major issues that can dramatically impact the outcome and interpretation of FP-fusion experiments in cells: (1) disulfide bond formation, (2) glycosylation, and (3) oligomerization.

Oxidizing Environments and Disulfide Bond Formation

Multiple cellular compartments contain proteins with covalently disulfide-bonded cysteines, even the cytoplasm

(Cumming *et al.*, 2004). Disulfide bonds form inter- and intrachain between cysteines in proteins exposed to oxidizers, especially in environments with strong oxidizing potentials, including the ER, the intermembrane space of chloroplasts and mitochondria, and the periplasm of gram-negative bacteria (Fig. 1C). Proteins native to these environments have evolved sequences and folds that promote the formation of specific disulfide bonds and this process is further guided by chaperones such as protein disulfide isomerase family members. However, any protein with cysteines potentially can be a substrate for disulfide bond formation

machinery. The original FP molecules and their derivatives all evolved in the relatively nonoxidizing environment of a cell and the bacterial cytoplasm. In such an environment, there was little selection against the accumulation of cysteines. GFP and its derivatives [BFP (Heim and Griesbeck, 2004), CFP (Ormo *et al.*, 1996), cerulean (Rizzo *et al.*, 2004), EGFP (Yang *et al.*, 1996), emerald (Cubitt *et al.*, 1999), YFP (Tsien, 1998), Venus (Nagai *et al.*, 2002), Citrine (Griesbeck *et al.*, 2001)] all contain two cysteines. When expressed in the secretory pathway of eukaryotic cells, a significant population of the FPs forms interchain disulfide bonds, as evidenced by the presence of a ladder pattern on nonreducing SDS-PAGE immunoblots (Fig. 2A) (Jain *et al.*, 2001; Aronson *et al.*, 2011). The cysteines of EGFP normally face the inside of the β -barrel, which means that interchain disulfide bond

formation must occur before a β -barrel forms. Equally curious is the implication that despite all of the different proteins in oxidizing environments that EGFP could form a random disulfide with, there appears to be a strong tendency for the non- β -barrel form of GFP to oligomerize (Fig. 2A).

Despite the absence of an obvious structural role for the two cysteines, mutation of either causes the GFP to dim considerably and mutation of both to relatively conservative serines makes the mutant FP dark (Jain *et al.*, 2001). Obviously, such an FP is useless for imaging. Even the visible FP is suboptimal as a significant fraction (in some cases greater than 50% of the total FP population) is misfolded, dark, and of unclear functionality (Fig. 2A). To maximize the fluorescent signal, minimize the amount of an FP fusion that needs to be expressed to visually detect it, and to minimize

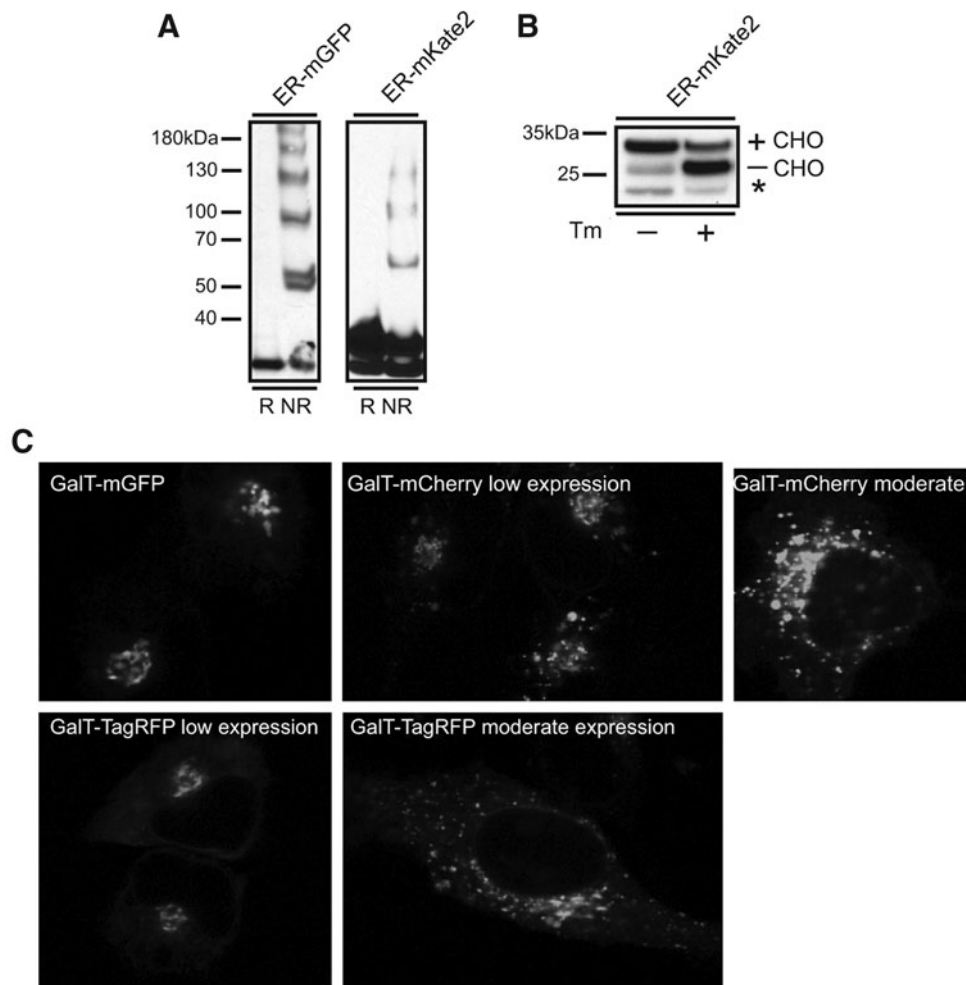


FIG. 2. FPs localized to the secretory pathway can form disulfide bonds, become *N*-glycosylated, and oligomerize. ER localized mGFP and mKate2 (ER-mGFP and ER-mKate2, respectively) form inappropriate disulfide bonds. Representative immunoblots (A) reveal the presence of higher molecular weight oligomers in nonreducing (NR) conditions. Oligomers form due to non-native interchain disulfide bond formation. mKate2 also contains a potential *N*-glycosylation consensus sequence. (B) When localized to the ER, ER-mKate2 is *N*-glycosylated (+CHO). Treatment with the *N*-glycosylation inhibitor, tunicamycin (Tm), shifts the majority of ER-mKate2 to a lower molecular weight band, indicating the nonglycosylated population of FP (-CHO). Asterisk (*) denotes band corresponding to a cleavage product due to sample preparation. (C) Different FPs fused with the integral membrane Golgi complex marker Galactosyltransferase (GalT) can distort Golgi complex morphology. Representative images of cells expressing GalT-mGFP, -mCherry, or -TagRFP reveal fusion proteins, GalT-mCherry and -TagRFP expressed at moderate levels significantly disrupt the normal organelle structure. Compared to the low expression levels, GalT fusions retain typical compact Golgi complex morphology.

the formation of misfolded proteins, investigators need to use FPs resistant to disulfide bond formation.

Many other FPs have three or more cysteines. The recently developed FusionRed (Shemiakina *et al.*, 2012) contains four cysteines. Kusabira orange derivatives even have a cysteine that forms part of the fluorophore (Karasawa *et al.*, 2004). At \$400 or more for some FP expression plasmids, selection of an FP unsuitable for a cellular environment of interest can be a costly mistake. Fortunately, alternatives exist and we describe these at the end of this minireview.

Glycosylation

Within the secretory pathway of eukaryotic cells, two types of sugars, asparagine linked (*N*-linked) and serine/threonine linked (*O*-linked) can be attached to proteins. *N*-linked are added in the ER as nascent proteins emerge from the translocon channel if the proteins contain an N-X-S/T consensus sequence (where X is any amino acid except proline) (Fig. 1B). *O*-linked sugars are added to serines and threonines in the Golgi complex by *N*-acetylgalactosaminyltransferases (Fig. 1B). In contrast to *N*-linked glycosylation, *O*-linked consensus sites remain poorly defined, although flanking residues are often enriched in proline, serine, threonine, and alanine (Potter *et al.*, 2006). There are additional forms of *O*-linked glycosylation, but these tend to be highly specialized for a small subset of proteins and are unlikely to be a significant concern for FPs.

The consequences of sugar additions can be substantial. First, *N*-linked sugars are added to the primary sequence of the protein still in the translocation channel, often before significant folding has occurred. If the sugar is placed on a residue that faces the inside of the β -barrel, then the ~ 1.3 nm sugar could potentially interfere with β -barrel folding and/or fluorophore formation. On the outside of the β -barrel, the sugar will dramatically increase the size of the ~ 4.6 nm diameter molecule. The size increase could cause steric interference blocking interactions of the fusion protein with a binding partner. More problematic on a global scale, the sugar marks the FP as a client for the glycosylation quality control machinery, including the lectin chaperones calnexin and calreticulin, UDP-glucose glycoprotein:glucosyltransferase, and the ER-associated degradation pathway (Hebert and Molinari, 2012). The overexpressed glycosylated FP will potentially titrate the glycosylation quality control machinery and could upset ER homeostasis or the half-life of the fusion protein could be shortened. If one is performing biophysical measurements (i.e., fluorescence recovery after photobleaching to determine a protein's diffusion coefficient in a cell) of a reporter FP or a fusion protein, then the sugar can substantially alter the FP's physical properties (Costantini *et al.*, 2013).

Many of the newer evolved FPs contain *N*-linked glycosylation consensus sites. TagRFP contains one, TagBFP contains three, and mKate2 contains one. Figure 2B illustrates that, when localized to the ER, mKate2 is *N*-glycosylated. The GFP family members and the DsRed family members typically lack *N*-linked glycosylation consensus sites.

FP Oligomerization

Some FPs, such as DsRed, are obligate oligomers. FP-mediated protein oligomerization is generally highly unde-

sirable for fusion proteins, as oligomerization can lead to activation of the fusion protein, inappropriate localization of the fusion protein, or can even alter organelle architecture. For example, standard EGFP is a weak dimer ($K_d = 0.11$ mM) (Zacharias *et al.*, 2002). In the context of a fusion protein in a confined or constrained space, this modest affinity becomes more than sufficient to promote interactions with the same protein on an apposing ER membrane, which in turn leads to membrane stacking and whorl formation and global reorganization of the ER (Snapp *et al.*, 2003). Constraint of EGFP to a membrane protein restricts the tumbling movement of EGFP in a solution to a two-dimensional plane and increases the effective concentration, leading to an increasing probability of membrane stacking. Thus, it is critical to avoid oligomerizing FPs. Unfortunately, this has proven difficult because there is no agreed upon standard in the field for distinguishing oligomeric from monomeric FPs. The definitions of monomeric range from quantitative to experimental, although the criteria for monomeric FPs vary between laboratories. With the goal of establishing a quantitative standard for monomeric FPs, we developed a visual assay that reports on the tendency of an FP to oligomerize under physiologic conditions, when attached to an integral membrane protein (Costantini *et al.*, 2012). Using this tool, we found that TagRFP is not, as previously reported (Shaner *et al.*, 2008) monomeric. In fact, TagRFP is the FP most prone to oligomerizing that we have identified to date. The Cy-TERM construct is freely available from our laboratory and can be used to test any FP for oligomerizing tendencies.

In addition to oligomerization, FPs can fold correctly, but be sticky and aggregate into bright puncta (Yanushevich *et al.*, 2002; Snapp, 2005). This behavior can be so severe that it can disrupt organelles in the secretory pathway. For example, the integral membrane Golgi complex marker Galactosyltransferase (GalT) can be fused to mGFP and localizes to a typical compact Golgi complex structure in a live cell (Fig. 2C). In contrast, fusion to TagRFP disrupts the Golgi complex into a fragmented dispersed structure (Fig. 2C). Whereas red FPs have been successfully used for a number of studies, it is important to recognize the potential of these (and any FP) to distort organelles. Therefore, it remains critical to compare an FP fusion protein's localization pattern to the native endogenous protein (preferably) or at least to the distribution of an epitope tagged fusion (i.e., myc or HA tagged). Note that the latter option is suboptimal for uncharacterized proteins as the tag may mask position-dependent targeting sequences.

Solutions

Taken together, this sampling of post-translational modifications and environmental differences illustrates the potential of cellular organelle environments to complicate and compromise the use of FPs. Therefore, it is critical for investigators to recognize the potential caveats of using FPs evolved for the cytoplasm. Table 1 summarizes properties of commonly used FPs and, thus, illuminates their potential utilities in different cellular environments. Inertness of FPs should always be a primary goal when selecting an FP for experiments.

Fortunately, solutions are available. First, the monomerized derivatives of DsRed (i.e., mRFP and mCherry)

TABLE 1. PROPERTIES OF COMMONLY USED FLUORESCENT PROTEINS

FP	Monomeric	pKa	N-linked consensus seq.	Disulfide forming
EGFP ^a	No	6.0	No	Yes
mGFP ^{a-c}	Yes	6.0	No	Yes
mVenus ^d	Yes	6.0	No	Yes ^e
mCitrine ^f	Yes	5.7	No	Predicted
Cerulean ^{c,g}	Yes	3.2	No	Yes ^e
TagBFP ^{h,i}	Yes	2.7	Yes	Yes
TagRFP ^a	No	<4.0	Yes	Predicted
mCherry ^a	Yes, but sticky	<4.5	No	No
sfGFP ^{c,j}	Yes	5.5	No	No

^aShaner *et al.* (2007).

^bSnapp *et al.* (2003).

^cAronson *et al.* (2011).

^dNagai *et al.* (2002).

^eOur unpublished results.

^fGriesbeck *et al.* (2001).

^gMarkwardt *et al.* (2011).

^hCostantini *et al.* (2013).

ⁱSubach *et al.* (2008).

^jPedelacq *et al.* (2006).

FP, fluorescent protein; sfGFP, superfolder green fluorescent protein.

naturally lack cysteines and readily fluoresce in the oxidizing bacterial periplasm and ER (Campbell *et al.*, 2002; Shaner *et al.*, 2004; Snapp *et al.*, 2006). In addition, DsRed and its derivatives do not contain consensus sequences for N-linked glycosylation. Therefore, these proteins are reasonable choices, especially for fusions with soluble monomeric proteins. Other FPs should be used with integral membrane protein fusions or with naturally oligomerizing proteins due to the stickiness of monomeric DsRed family members.

What about truly inert FPs? Through educated guesses and directed mutagenesis, it has been possible to mutate the cysteines of TagBFP and a GFP variant and maintain much of the original fluorescence brightness of the original proteins (Suzuki *et al.*, 2012; Costantini *et al.*, 2013). Curiously, at least one FP with cysteines, superfolder GFP (sfGFP) (Pedelacq *et al.*, 2006), is resistant to disulfide bond formation in multiple environments (Aronson *et al.*, 2011). It is truly monomeric (Pedelacq *et al.*, 2006; Costantini *et al.*, 2012) and contains no N-linked glycosylation consensus sites. In our hands, sfGFP appears to be the most inert FP and we strongly recommend sfGFP for use in oxidizing environments. We anticipate that, as more investigators become aware of the problems of environmental impacts on FP functionality, improved inert FPs of a wider palette will be developed.

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No competing financial interests exist.

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