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Tracking exogenous intracellular casp-3 using split GFP

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Abstract

Cytosolic protein delivery promises diverse applications from therapeutics, to genetic modification and precision research tools. To achieve effective cellular and subcellular delivery, approaches that allow protein visualization and accurate localization with greater sensitivity are essential. Fluorescently tagging proteins allows detection, tracking and visualization in cellulo. However, undesired consequences from fluorophores or fluorescent protein tags, such as nonspecific interactions and high background or perturbation to native protein's size and structure, are frequently observed, or more troublingly, overlooked. Distinguishing cytosolically released molecules from those that are endosomally entrapped upon cellular uptake is particularly challenging and is often complicated by the inherent pH-sensitive and hydrophobic properties of the fluorophore. Monitoring localization is more complex in delivery of proteins with inherent protein-modifying activities like proteases, transacetylases, kinases, etc. Proteases are among the toughest cargos due to their inherent propensity for self-proteolysis. To implement a reliable, but functionally silent, tagging technology in a protease, we have developed a caspase-3 variant tagged with the 11th strand of GFP that retains both enzymatic activity and structural characteristics of wild-type caspase-3. Only in the presence of cytosolic GFP strands 1-10 will the tagged caspase-3 generate fluorescence to signal a non-endosomal location. This methodology facilitates easy screening of cytosolic vs. endosomallyentrapped proteins due to low probabilities for false positive results, and further, allows tracking of the resultant cargo's translocation. The development of this tagged casp-3 cytosolic reporter lays the foundation to probe caspase therapeutic properties, charge-property relationships governing successful escape, and the precise number of caspases required for apoptotic cell death.

KEYWORDS

apoptosis, caspase, caspase-3, intracellular protein delivery, nanogel, split GFP

Abbreviations: C3-11, casp-3 tagged with GFP11; C3^{KO}-GFP11, catalytically inactive casp-3 tagged with GFP11; Casp, caspase cysteine aspartate protease; CQ, chloroquine diphosphate; DEVD-amc, N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; DTT, dithiothreitol; GFP11, superfolder GFP strand 11; GFP1-10, superfolder GFP strands 1-10 optimized; GSH, glutathione; HEKS1-10, HEK293T stably expressing GFP1-10; M3, M3 mutations to GFP11th strand L221H, F223Y, T225N; NG, nanogel; PBS, phosphate-buffered saline; PDS, pyridyl disulfide; PEG, polyethylene glycol; PEG-PDS, polymer of PEG and PDS; SEE, split-complementation endosomal escape; TNG, 20 mM Tris, 100 mM NaCl, 10% Glycerol, pH 7.5 buffer; WT, wild-type; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

1 | INTRODUCTION

Caspases, or cysteine aspartate proteases, are a tightly regulated enzyme family involved in cell death and inflammation pathways.¹ Specifically, apoptotic initiator (casp-8 and -9) and executioner (casp-3 and -7) caspases play distinct roles in programmed cell death.² Perturbation of their vital apoptotic function implicates caspases in many diseases such as cancer³ and neurodegeneration,^{4,5} characterized by a decrease or increase in cell death, respectively.⁶

In an effort to explore caspases as cytotoxic therapeutics to combat inappropriate cell survival,⁶ executioner casp-3 has been used to induce cell death upon intracellular delivery.^{7–13} The executioner, casp-3, has immense therapeutic potential as it irreversibly propagates the apoptotic cascade at a high catalytic rate through cleavage of substrates critical for maintaining cytoskeletal structure and regulating gene expression.¹⁴⁻¹⁶ Successful delivery of casp-3 has been achieved using a number of delivery vehicles including polymeric nanoparticles,⁷ inverse gold materials,9,10 cellemulsion nanoparticles,⁸ penetrating transporter peptides,^{11,12} or lipids,¹³ yet none of these delivery technologies have made it into therapeutic use. This is likely because the details of intracellular delivery: location, concentration, method of uptake, kinetics of intracellular redistribution and release have been difficult to pinpoint.

To fully exploit casp-3 and other cytotoxic enzymes as delivered therapeutics, it is essential that the details of their delivery and activation can be accurately localized and quantified. Typically, successful casp-3 delivery has been characterized using random chemical or sitespecific proteinaceous fluorescent tags or by assessing the downstream readout of cell death. Unfortunately, both of these approaches' present drawbacks. The use of conjugated fluorophores often introduces confounding factors, such as influencing uptake due to dve-membrane association or pH sensitivity, leading to over- or under-enhanced interpretations of cytosolic localization.¹⁷⁻¹⁹ Further, as casp-3 is known to have activity in the cell cytosol, mitochondria or nucleus,²⁰ successful cell death only indirectly implies casp-3 cytosolic localization and resultant casp-3 activity. To unambiguously monitor cytosolic localization, as opposed to its presence in other organelles or adventitious association with cell membranes, we employed the split GFP technology to develop a casp-3 variant that generates a fluorescent signal only in the cytosol.

The principal bottleneck for cytosolic protein delivery is the dependence on endosomal escape.^{21–23} GFP-based reporters have been developed to elucidate caspase activity,^{24–27} but to our knowledge, no such development has allowed detection of exogeneous, active or inactive, cytosolic casp-3. Split GFP enables the spontaneous assembly of the GFP beta strands 1-10 (GFP1-10) and the GFP beta strand 11 (GFP11). Split GFP has been widely used for protein detection, folding, and localization²⁸⁻³¹ and has recently been used to assess the degree of endosomal escape.32-36 Split GFP offers several advantages including a small tag (16 amino-acid residues), minimizing undesired effects to the protein of interest. Second, each fragment alone contributes no background fluorescence. Third, signal is gained only upon fragment reassembly characterized by low probabilities for false positive results and lastly, fluorophore maturation rates and signal intensity of the split fragments are comparable to full-length GFP.^{37,38} Ideally, all protein cargos could be facilely tagged with GFP11 with low impact on function of the tagged cargo. While this is assumed to be true even in the absence of functional characterization,³²⁻³⁶ in other proteins, appendage of GFP11 leads to adverse functional impacts, which can complicate or even nullify experimental interpretation. Therefore, herein we outline a characterization approach that enables development of properly folded, functional, GFP11 tagged protein. We undertook this to develop a panel of casp-3 variants tagged with GFP11, termed C3-11, that closely resemble wild-type (WT) casp-3 in fold and function.

C3-11 can be used to verify cytosolic localization of delivered casp-3 upon endosomal escape through a splitcomplementation endosomal escape (SEE) assay (Scheme 1). This method promises advantages over traditional methods, such as fluorescent labeling (e.g., small molecule or protein tags) and colocalization studies, pH-sensitive studies (e.g., pH-sensitive cargo release or pH-sensitive probes) or signaling studies (e.g., FRET), as signal generation is solely generated for material that has escaped the endosome.^{21,22} Here, we report the development of a split GFP-tagged casp-3 that retains properties to allow tracking, quantification of this protein family and also provides a method by which other therapeutic cytotoxic proteins can be delivered and tracked.

2 | RESULTS

2.1 | Rational design of a split-GFP casp-3

Typically, generating fluorescently tagged proteins is considered to be a foolproof undertaking as many proteins can be successfully tagged at either termini, or even internally, without any impact on function. Tagging caspases, while retaining native levels of function, is much more challenging as the termini play critical functional roles. Caspases are produced as inactive, dimeric procaspase





SCHEME 1 Split-

complementation endosomal escape (SEE) pathways for tagged casp-3 (C3-11). (a) Following endocytosis, only once nanogels (NGs) escape the endosome into the cytosol will C3-11 be released by cytosolic glutathione, leading to reassembly with cytosolically expressed GFP1-10 and fluorescence generation. Chloroquine diphosphate (CQ) can be added to the cell-culture medium to liberate endosomally entrapped NG. (b) Upon endocytosis of unencapsulated but cationically tagged C3-11, only when the protein is able to reach the cytosol will fluorescence be generated by reassembly with GFP1-10

TABLE 1 Nomenclature, graphical representation, and detailed descriptions of constructs used

	Name	Representation	Description
Catalytically active	WT C3		Wild-type casp-3, C-terminal His-tag
	C3		Casp-3 ^a
	C3-11 no linker	M3	Casp-3 ^a with no linker to GFP11 ^{M3}
	C3-11		Casp-3 ^a with linker to GFP11
	C3-11 ^{M3}	— M3	Casp-3 ^a with linker to GFP11 ^{M3}
	[+]C3-11 ^{M3}		Casp- 3^{a} with <i>N</i> -term Arg ⁶ with linker to GFP11 ^{M3}
Catalytically inactive (KO)	C3 ^{KO}	КО	Casp-3 C163S, C-terminal His-tag
	C3 ^{KO} -11	KO –	Casp-3 ^a C163S with linker to GFP11
	C3 ^{KO} -11 ^{M3}	КО — МЗ	Casp-3 ^a C163S with linker to GFP11 ^{M3}
	[+]C3 ^{KO} -11 ^{M3}	KO – M3	Casp-3 ^a C163S with <i>N</i> -term Arg ⁶ with linker to GFP11 ^{M3}
	GALA-C3 ^{KO} -11 ^{M3}	КО — МЗ	Casp-3 ^a C163S with <i>N</i> -term GALA with linker to GFP11 ^{M3}

^aWith N-terminal His-tag, prodomain mutations D9A D28A.

zymogens. Active caspase heterotetramers only exist after proteolytic cleavage of the two procaspase dimer chains, which produces two large and two small subunits. The *Nt*ermini of caspases contain the prodomain, which are the most diverse regions within the family and required for substrate recognition. The large subunit *C*-termini and the small subunit *N*-termini form the substrate binding groove.³⁹ Casp-3 can be expressed and purified with a *C*terminal His₆-tag⁴⁰ however, GFP11 typically functions optimally when attached at a protein's *C*-terminus.^{31,36} As such, to make C3-11, we engineered a C3 expression construct that moved the His₆-tag to the *N*-terminus with two cleavage site substitutions, D9A and D28A, to prevent proteolytic removal of the prodomain and His-tag (all variants used herein described in Table 1).^{41,42} We then encoded the GFP11 peptide at the *C*-terminus connected via a glycine-rich linker (Figure 1a). To prevent induction of apoptosis during fluorescence inspection, we also generated a catalytically dead C3-11 variant via substitution of the active site cysteine residue with serine, C163S termed C3^{KO}-11 (Figure 1b). Casp-3 D9A D28A (retaining the prodomain), has a melting temperature ($T_{\rm m}$) of 57°C and demonstrated an increased stability of 12.9°C upon binding irreversible active site inhibitor z-VAD-fmk (Figure 1c, S1).⁴³ The melting profile of casp-3 D9A D28A with the GFP11 tag showed similar properties (increased stability of 10°C upon z-VAD-fmk binding), suggesting that casp-3 structure and stability has not been



FIGURE 1 C3-11 constructs retain WT C3 function. (a) The active C3-11 construct contains an *N*-terminal His tag followed by an uncleavable prodomain (D9A, D28A-white dots), large subunit, small subunit and the GFP11 tag attached through a GGGGSGG linker (..GG..). (b) Catalytically inactive C3^{KO}-11 with active-site substitution C163S (KO). (c) Melting temperatures (T_m) for casp-3 variants (all variants used herein described in Table 1) with and without irreversible active site inhibitor z-VAD-fmk, and ΔT_m . GFP11 labeled with M3 indicates mutations L221H, F223Y, T225N. n/a: not applicable due to C163A substitution at the active site. (d) Activity of C3-11 variants against fluorogenic peptide substrate DEVD-amc improved upon addition of linker and M3 mutations; Ex. 365 nm, Em. 495 nm. (e) Activity of C3-11^{M3} against native protein substrate FL casp-3 C163S is comparable to WT casp-3. (f) C3-11^{M3} and WT casp-3 cleavage of FL C3^{KO}-11^{M3}, demonstrating C3^{KO}-11^{M3} can be recognized and function as a substrate of casp-3

compromised by the addition of GFP11. $C3^{KO}$ -11, which lacks the catalytic cysteine (C163S) and cannot bind z-VAD-fmk, also has a similar T_m to its untagged counterpart, 65 and 67°C, respectively (Figures 1c and S1).

Addition of GFP11 has been demonstrated to decrease solubility in some proteins.^{8,33} Upon generation of C3-11, we likewise noticed a significant decrease in protein yield and activity. However, a series of established GFP11 mutations, L221H, F223Y, and T225N, which improve stability and solubility in some fusions has been termed GFP11M3.²⁹ C3-11 with these M3 mutations (C3-11^{M3}) yielded increased protein expression and displayed significantly more self-processing upon purification (Figure S2), compared to C3-11. This increase in self-processing was likewise manifested in an increase in activity against casp-3 peptide substrate DEVD-amc (Figure 1d), with a dependence on the presence of the glycine-rich linker

(GGGGSGG) between the casp-3 protein and GFP11 peptide. Previous split GFP studies suggest that linkers favorably allow freedom-of-movement, but varying linker length has little influence.³¹

Caspases utilize sites distal from the substrate binding groove, or exosites, to recruit protein substrates.^{44,45} Thus, to properly characterize C3-11^{M3} in vitro, it is necessary to validate cleavage of protein substrates as well as peptide substrates. C3-11^{M3} cleaved the inactive fulllength (FL) casp-3 zymogen, casp-3 C163S, at a rate comparable to WT casp-3 (Figure 1e). Further, $C3^{KO}$ -11^{M3} itself can be recognized and cleaved into two distinct bands, prodomain + large and small subunit, by both C3-11^{M3} and WT casp-3 (Figure 1f). These data demonstrate that C3-11^{M3} variants maintain casp-3 structure and activity against peptide and protein substrates and retains the ability to be recognized and processed by native casp-3.

2.2 | C3-11 efficiently reassembles with GFP1-10 in vitro

To be useful, C3-11 variants should allow assembly of the GFP holo-protein in the presence of the larger component of GFP, GFP1-10 (Figure 2a). Individually titrating either the active or inactive C3-11 variants into a solution of GFP1-10 in vitro demonstrated a dose-dependent increase in reassembled GFP fluorescence (Figure 2b,c) as a function of time (Figure S3). The lower fluorescence levels observed for active C3-11^{M3} compared to inactive C3^{KO}-11^{M3} (Figure 2b,c) is due to proteolytic cleavage of full-length GFP1-10 by C3-11^{M3} (Figure S4), presumably at the VELD sequence within the *N*-terminus of GFP1-10.⁴⁶ Therefore, approximately 25% less fluorescence was observed for C3-11^{M3} compared to C3-11



FIGURE 2 Characterization of C3-11 reassembly with GFP1-10 in vitro. (a) C3-11 reassembly with GFP1-10 results in a GFP fluorescence signal with maxima at 510 nm. (b) Fluorescence of recombinant C3-11^{M3} and recombinant GFP1-10 after 24 hr incubation at RT shows a dose-dependent increase in fluorescence; Ex. 430 nm, Em. 460–560 nm. (c) Fluorescence of recombinant C3^{K0}-11^{M3} and recombinant GFP1-10, is higher overall compared to the active variant, after 24 -hr incubation at RT; Ex. 430 nm, Em. 460–560 nm. (d) Effect of M3 mutations on reassembly for active and inactive constructs titrated into lysate from HEK293 cells stably expressing GFP1-10 (HEKs1-10). RFU at 509 nm reflecting C3-11 reassembly with GFP1-10 set to 100% for comparison with M3 variants monitored identically. (e) Fluorescence of recombinant C3^{K0}-11^{M3} in HEKs1-10 lysate after 24 -hr incubation at RT; Ex. 430 nm, Em. 430 nm, Em. 460–560 nm

(Figure 2d), due to increased casp-3 activity (Figure 1d). Comparatively, $C3^{KO}$ -11 benefited from the M3 mutations (Figure 2d) and thus, constructs were able to support GFP reassembly and attain a fluorescent state. GFP reassembly was also observed when $C3^{KO}$ -11^{M3} was titrated into lysate from HEKs1-10, HEK293 cells stably expressing GFP1-10 (Figure 2e).³¹ In all cases, the observed dose-dependent response indicated that when appended to the *C*-terminus of casp-3, GFP11 binds in a functional manner to GFP1-10.

2.3 | C3^{KO}-11 efficiently reassembled with GFP1-10 *in cells* upon transfection

We transfected plasmid DNA (pDNA) for each C3^{KO}-11 variant into HEK293 cells to evaluate the increase in fluorescence. The inactive C3^{KO} were transfected to prevent inducing cell death due to casp-3 activity during this stage of characterization. Initially, C3^{KO}-11^{M3} was cotransfected with GFP1-10 into HEK293T cells. This led to a notable population of GFP-positive cells by microscopy (Figure 3a). This fluorescence was observed throughout the cell, surrounding the nucleus, demonstrating a cytosolic distribution. Unfortunately, transient transfection of GFP1-10 lead to batch-to-batch variation in GFP levels. Therefore, all subsequent studies were executed in HEK cells stably transfected with GFP1-10 (HEKs1-10).³¹ Transfection of the inactive C3^{KO}-11 variants resulted in a significant shift in the population's fluorescence (Figure 3b,c) with similar cytosolic distribution. Interestingly, both the GFP11 and GFP11M3 variants displayed comparable fluorescence in these experiments, likely due to abundant cytosolic levels of each protein from high transfection efficiencies. After 24-h protein expression of either construct, a ~90% GFP positive population (Figure 3d) and approximately 100-fold increase in overall GFP mean fluorescence intensity (MFI) was observed (Figure 3e). These data suggest that the GFP11-tagged casp-3 is able to robustly bind to GFP1-10 cytosolically and that these reagents should allow accurate assessment of casp-3 cytosolic delivery using SEE.

2.4 | C3-11 variants can be encapsulated, released and delivered by PEG-PDS redoxresponsive polymeric nanogels

Given that C3-11 variants retain activity and WT-like structural characteristics, we anticipated that they should likewise retain their ability to be reactively encapsulated within redox-responsive polymeric nanogels (NGs) similarly to WT casp-3. C3-11 NG were formulated with





FIGURE 3 C3^{KO}-11 allows reassembly with GFP1-10 and emergence of fluorescence in cells. (a) Co-transfection of C3^{KO}-11^{M3} and GFP1-10 in HEK293 cells demonstrated cytosolic GFP fluorescence after 24 h; ×20 objective, BF (bright-field), Nucleus (NucBlueTM stain, 405 nm), GFP (reassembly of C3^{KO}-11 and GFP1-10, 488 nm), scale bars indicate approximately 60 µm. (b) Flow cytometry analysis of C3^{KO}-11 transfection mediated reassembly in HEKs-10 shows fluorescence appearing in a dose-dependent manner; 488 nm laser used to analyze the viable single-cell population. (c) Flow cytometry analysis of C3^{KO}-11^{M3} mediated reassembly. (d) Quantification of GFP positive cells generated by C3^{KO}-11 and C3^{KO}-11^{M3} reassembly with GFP1-10 upon transfection, gating for untreated HEKs1-10. (e) The change in GFP mean fluorescence intensity (MFI) after normalizing to the untreated HEKs1-10 population, was similar for C3^{KO}-11 and C3^{KO}-11^{M3} indicating that the M3 mutations did not significantly impact fluorescence

similar procedures to those used for WT casp-3.⁷ Key functionalities in the polymer include a hydrophilic polyethylene glycol (PEG) side chain to impart dispersibility in aqueous conditions and a hydrophobic pyridyldisulfide (PDS) moiety to generate covalent crosslinks that stabilize the NG (Figure 4a).⁷ The PDS unit also directly facilitates protein encapsulation via reactive self-assembly of the disulfide moiety with native, solvent exposed cysteine residues on the casp-3 surface (Figure 4a). Following protein encapsulation, crosslinking to lock-in the NG formation and purification, in vitro protein release was visualized using millimolar concentrations of the reductant dithiothreitol (DTT, Figure 4b).

It is critical that delivery vehicles release proteins that remain active. We analyzed whether the NG-released C3^{KO}-11 variants were able to reassemble with GFP1-10 in HEKs1-10 lysate. Independent of whether the protein was pre-released from the NG, then added to GFP1-10, or released in the presence of GFP1-10 (simultaneous), efficient reassembly was observed (Figure S5). As designed, no signal was generated in the absence of reductant, which confirmed that all free protein had been removed after NG purification and demonstrated the requirement of NG disassembly and protein liberation for GFP reassembly.

Immunoblot analysis of total cell lysate allowed visualization of inactive C3^{KO}-11^{M3} delivery to MCF7 breast cancer cells, following subsequent washes to remove all non-endocytosed NGs (Figure 4c). As a critical control, NGs housing inactive C3^{KO}-11^{M3} cargo showed minimal



FIGURE 4 Inactive and active C3-11^{M3} NG formation, delivery, and cell death. (a) Reaction between pyridyl disulfide (PDS) amphiphilic random copolymer (PEG-PDS) and solvent exposed cysteine residues of casp-3 yield a nanogel (NG). (b) C3^{KO}-11^{M3} encapsulation in NG and release visualized by SDS-PAGE in the absence or presence of the reductant dithiothreitol (DTT). (c) Visualization of NG mediated delivery of C3^{KO}-11^{M3} to MCF7 cells. (d) C3^{KO}-11^{M3} NG complexes are nontoxic up to 1 mg/mL across multiple cell lines. (e) C3-11^{M3} encapsulation in NG and release visualized by SDS-PAGE in the absence or presence of DTT. (f) Visualization of NG mediated delivery of C3-11^{M3} to A549 cells after 24-hr incubation. (g) C3-11^{M3} NG induce cell death after 24-hr incubation. (h) Poly (ADP-ribose) polymerase 1 (PARP) cleavage in HEKs1-10 cells, indicating C3-11^{M3} mediated apoptosis, compared to the empty NG lacking PARP cleavage after 24-hr incubation. (i) WT C3 NG induced cleavage of PARP is decreased by treatment with 20 μM z-VAD-fmk

cell death across multiple cell lines, demonstrating that apoptosis was only induced by catalytically active cargo, but not by catalytically dead cargo or the NGs themselves (Figure 4d). In addition, active C3-11^{M3} was efficiently encapsulated and released in PEG-PDS NGs (Figure 4e) and delivery was likewise visualized by immunoblot (Figure 4f). Favorably, C3-11^{M3} NG maintained an effective cell killing ability (Figure 4g). During apoptosis, casp-3 cleaves hallmark substrates such as poly (ADP-ribose) polymerase 1 (PARP).^{14,15} C3-11^{M3} NGs effectively induced significant disappearance of full-length PARP as well as appearance of cleaved PARP (Figure 4h), suggesting an efficient apoptosis cascade had been activated. To demonstrate that this cleavage is in

fact due to casp-3 activity by monitoring cleavage of PARP in the presence of irreversible pan-caspase inhibitor z-VAD-fmk (Figure 4i).

2.5 | Free and delivered $[+]C3^{KO}-11^{M3}$ demonstrated fluorescence in cells with chloroquine

We assessed fluorescence generation by C3^{KO}-11^{M3} NG in HEKs1-10. However, no significant GFP positive population was detected after gating against cells with properties that mirror that of the untreated population. We hypothesizd that low levels of GFP fluorescence were observed due to low levels of NG endosomal escape. When HEK cells were treated with dual fluorescently labeled NG, in which the NG polymers are labeled with Cy3 (Cy3-PEG-PDS) and the casp-3 is labeled with Cy-5 (Cy5 C3KO), significant NG and casp-3 fluorescence colocalized with lysotracker signal, suggesting the NG were endosomally entrapped (Figure 5a). Therefore, we investigated whether modification of the protein cargo could increase endosomal escape. Previously, we utilized arginine-NG modification to improve the cytosolic distribution of exogeneous casp-3⁷ as arginine is extensively used to facilitate impermeable macromolecule penetration into cells.^{22,48} Notably, the cellentry mechanism of arginine-based materials is not fully understood. Many studies hypothesize direct translocation across the membrane but, other efforts appear to indicate an endocytotic mechanism.^{47–50}

We appended an arginine tag onto the *N*-terminus of C3-11^{M3} but found that it had reduced activity (denoted as [+]C3-11^{M3}, Figure S6), indicating that additional engineering would be required before it could be used to induce cell death. For that reason, we aimed to determine if the arginine modification could enhance endosomal escape by monitoring the inactive version's distribution upon delivery. We expressed C3^{KO}-11^{M3} with an Arg⁶ peptide present just after the penta-histidine tag on the *N*-terminus (HHHHHPRRRRR; denoted as [+]C3^{KO}-11^{M3}, Figure 5b). This variant can likewise be encapsulated and released within PEG-PDS NG (Figure S7).



FIGURE 5 Arg⁶ functionalization to C3KO-11M3 significantly influences SEE. (a) Endosomal entrapment of $C3^{KO}$ NG in HEK cells, visualized by fluorescent confocal microscopy (×60 objective, Bright-Field, Nucleus, (NucBlue[™] stain) Ex. 405 nm, Cy3-PEG-PDS polymer Ex. 562 nm, Lysotracker-Green Ex. 488 nm, Cy5-C3^{KO} Ex. 632 nm, scale bars indicate 21 µm. (b) Inactive [+]C3^{KO}-11^{M3}. (c) SEE after 24-h incubation with C3^{KO}-11^{M3} NG complexes at 1 mg/mL polymer concentration (10 µM protein) in HEKs1-10 cells with CQ titrated from 25 to 100 μ M. (d) [+] C3^{KO}-11^{M3} NG at 1 mg/mL polymer concentration shows increase in GFP positive population with CQ. (e) Free C3^{KO}-11^{M3} does not result in significant GFP positive population (20 μ M protein) while (f) [+]C3^{KO}-11^{M3} demonstrates fluorescence with 100 µM CQ. [+] indicates the presence of the Arg⁶ peptide on C3^{KO}-11^{M3}

Upon NG formulation and SEE analysis (Scheme 1), we observed a 2% GFP positive population with $[+]C3^{KO}$ -11^{M3} NG, compared to the C3^{KO}-11^{M3} NG (Figure 5c.d). To test the hypothesis that NG are significantly trapped in endosomes, we added chloroquine diphosphate (CO), an endosome-permeable small molecule that disrupts endosomal membranes using a proton-sponge mechanism.^{51,52} Interestingly, upon the addition of CO, we observed a notable difference in the behavior of these identical NG with distinctive cargo. With CQ, [+]C3^{KO}-11^{M3} NGs demonstrated a clear population tail shift (Figure 5d) in contrast with C3^{KO}-11^{M3}, which produced no noticeable change. Quantitatively, approximately 20% (50 µM CQ) and 40% (100 µM CQ) GFP positive cells were observed for $[+]C3^{KO}-11^{M3}$ NG, with a threefold increase in GFP mean fluorescence intensity (MFI; Figure S8). This suggests that Arg⁶ functionalization to the protein cargo directly may have significantly increased the concentration of NG per endosome.

We then tested the ability of the *N*-terminal Arg⁶ sequence to facilitate cell-permeation of the free (unencapsulated) protein. Without CQ, free $C3^{KO}-11^{M3}$ and $[+]C3^{KO}-11^{M3}$ again demonstrated negligible GFP positive signal, ~1 and ~3%, respectively (Figure 5e,f). Conversely, upon the addition of CQ (Scheme 1), free $C3^{KO}-11^{M3}$ and $[+]C3^{KO}-11^{M3}$ yielded approximately 14 and 90% GFP positive cells, respectively (Figure 5e,f) and $[+]C3^{KO}-11^{M3}$ generated a 12-fold change in GFP MFI (Figure S8).

These data suggest that arginine-mediated cell entry for free-caspases and potentially other proteins may also occur via endocytosis, trapping these exogeneous proteins, leading to their subsequent lysosomal degradation.⁵³

As the endosome is an acidic compartment, pHsensitive groups could beneficially exploit the inherent pH decrease exhibited upon endocytosis. We were interested in introducing pH-sensitive moieties to C3^{KO}-11^{M3} in an effort to enhance SEE. For example, GALA, a soluble amphipathic peptide composed of glutamic acid-alanineleucine-alanine repeats has a random coil structure at neutral pH. But at acidic pH, GALA can bind bilayer membranes upon conversion to an amphipathic α -helix.⁵⁴ This peptide has been exploited to facilitate endosomal escape of nanomaterials.^{55,56} Introducing 15-amino acid truncated GALA57 on the N-terminus of C3KO-11M3 rendered casp-3 insoluble (GALA- C3^{KO}-11^{M3}, Figure S9) even with alterations to the induction conditions. Although GALA was incompatible with caspase folding and function, exploring other modifications to casp-3 that would promote endosomal escape while maintaining proper casp-3 fold and function hold therapeutic potential.

To visualize the strong fluorescence signal generated from free $[+]C3^{KO}-11^{M3}$, we utilized fluorescence microscopy. After 24-h incubation with free $C3^{KO}-11^{M3}$ negligible GFP fluorescence was observed with or without CQ (Figure 6a,b). In contrast, incubation of $[+]C3^{KO}-11^{M3}$ led to strong GFP fluorescence in the presence of CQ



FIGURE 6 $C3^{KO}-11^{M3}$ and $[+]C3^{KO}-11^{M3}$ reassembly with GFP1-10 visualized by fluorescence microscopy. Recombinant proteins (7 μ M) were added to HEKs1-10 cells and allowed to enter cells and subsequently reassemble with stably transfected GFP1-10 for 24 h. (a) $C3^{KO}-11^{M3}$ (b) $C3^{KO}-11^{M3}$ in the presence of 100 μ M CQ resulted in low-intensity diffuse GFP fluorescence (c) $[+]C3^{KO}-11^{M3}$ and (d) $[+]C3^{KO}-11^{M3}$ in the presence of 100 μ M CQ resulted in significant cytosolic fluorescence and co-localization with the nuclear stain. ×20 objective, BF (bright-field), Nucleus (NucBlueTM stain, 405 nm), GFP (reassembly of C3^{KO}-11 and GFP1-10, 488 nm), scale bars indicate approximately 50 μ m

(Figure 6c,d). Notably, significant overlap between the reassembled GFP and the nuclear stain was observed, indicating subsequent translocation of the reassembled GFP to the nucleus. This result is consistent with previous observations that GFP itself can translocate to the nucleus due to its low molecular weight (27 kDa).⁵⁸ Further, GFP nuclear accumulation has been shown to be significantly enhanced by incorporation of nuclear localization signals⁵⁹ or even six arginine residues alone.⁶⁰ Thus, these data demonstrate that the split GFP system developed herein can be utilized to elucidate endosomal escape following endocytosis, but also permits specific protein tracking following organelle translocation after reaching the cytosol.

3 | DISCUSSION

Split GFP is a useful tool to visualize protein location. More recently, it has been implemented to measure endosomal escape of non-polymeric delivery systems,^{32–36} due to the aforementioned benefits over traditional methods using small molecule and whole protein tags.¹⁷⁻¹⁹ Herein we have developed a split GFP casp-3 variant (C3-11) that can generate GFP fluorescence in the cytosol upon treatment of HEKs1-10, as there is a pressing need for robust assays to assess nanoparticle endosomal escape.²¹ C3-11 can effortlessly replace the cargo in preexisting delivery systems and upon subsequent treatment to HEKs1-10, would allow for easy screening of endosomal escape through SEE, and concurrently, assess delivery vehicle efficacy through cell death evaluation.

Despite the development of C3-11 variants and their ability to robustly generate fluorescence with GFP1-10 in vitro and upon transfection in cellulo, little fluorescence was observed upon NG delivery. This result was somewhat unexpected because robust induction of apoptosis by casp-3 containing NG has been observed⁷ and because analysis using fluorescence microscopy as well as immunoblotting suggests substantial intracellular protein delivery and apoptosis efficacy. As mentioned, standard fluorophore labeling using either hydrophobic small molecules or fluorescent protein tags can improperly influence protein function, thereby unwittingly influencing interpretation. In addition, sample preparation for immunoblotting disrupts both the cellular and endosomal membranes, only allowing for combined quantification of the cytosolic and endosomal fractions together.¹⁷⁻¹⁹ Hence, we aimed to overcome these pitfalls and provide an independent, more sensitive measure of cytosolic habitat using split GFP. These data together do allow us to conclude that the small amount of casp-3

protein that successfully escapes the endosome is enough to effectively induce apoptosis. Most importantly, the tools presented here should allow quantification, for the first time, of precisely how many molecules of an active caspase (in this case casp-3) are required to induce cell death.

Herein we have demonstrated SEE's turn-on capability, with extremely low background prior to GFP reassembly, leading to more sensitive signals compared to traditional microscopy⁷ or immunoblot analysis of casp-NG cytosolic habitat. However, even with this increased sensitivity, we failed to observe significant cytosolic translocation of C3-11 by NG, suggesting that NG escape form the endosome is limiting. Here, we observed that endosomal escape could be enhanced with pHsensitive molecules such as CQ. As endosomal escape efficiencies of delivery systems in general are routinely extraordinarily low (approximately 1%),⁶¹ SEE assays help to elucidate trends and help suggest when modifications to delivery systems would be advantageous.³³⁻³⁶ Lipid bilayer leakage assays^{61,62} can be used to similarly reveal endosomal escape capabilities and when engineering may be needed but notably, they do not completely mimic biological contexts.²¹ This is imperative to note as endosomal escape is truly the bottleneck of deliverv.²² and the lack of easy assays to evaluate structure-activity relationships hinder delivery vehicle optimization and progress. Notably, supplementary assays to evaluate endosomal escape capabilities through other analytical methods are being developed for more comprehensive assessment but are not necessarily easily translatable to These technologies other systems. include dexamethasone-labeled conjugates and GFP-labeled glucocorticoid receptor colocalizations,63 concentrationdependent FRET⁶⁴ and combining correlative light and electron microscopy with fluorescent nanobody colocalization.⁶⁵ Thus, the SEE approach we describe here, which can be applied even to challenging protein cargos such as proteases, fills an important void in the drug delivery assessment tool box.

Adding an arginine patch to the casp-3 variant enabled $[+]C3^{KO}-11^{M3}$ to generate a significant GFP positive population. This result suggests that cationically tagged $[+]C3^{KO}-11^{M3}$ proteins are endocytosed, as previously reported for charged GFPs^{53,66} and other proteins which have been functionalized with arginine or charged peptide motifs.^{46,48,67} As charged proteins can alter the ionic composition of the endosomal lumen facilitating endosomal escape, they have application as therapeutics alone or, even as delivery agents themselves.^{66,68} Furthermore, these data spark future directions to investigate engineered casp-3 as a therapeutic. Specifically, chargemediated uptake and charge-mediated endosomal escape relationships would reveal charge requirements and tolerances for potent $[+]C3-11^{M3}$ induced programmed cell death. These investigations would result in optimized [+]C3-11^{M3} variants capable of entering cells on their own, inducing cell death and self-reporting fluorescence translocation.

Intracellular delivery of protein therapeutic systems has tremendous potential; however, assays to evaluate their cytosolic distribution are still lacking. This work has demonstrated that while it is possible to harness the utility of split GFP for SEE even with challenging protein cargos such as casp-3, it is naïve to do so in the absence of thorough biochemical characterization of the tagged cargo proteins. We have demonstrated an approach for evaluating split-GFP tagged protein cargos that can be applied to a broad range of functional proteins. These approaches will allow continued SEE evaluation of casp-3 modifications and resultant cytosolic protein distribution. For delivery, using CQ significantly increased the amount of casp-3 that could escape the endosome. Due to the toxicity of CQ,³⁴ alternative non-toxic approaches for enhancing endosomal escape should be investigated in future work, including modifications to delivery vehicle structure and overall system composition. Finally, our C3-11 SEE system can now be used to rapidly evaluate endosomal escape as well as therapeutic effect, moving us closer to the goal of optimally delivering caspases cytosolically, as therapies.

4 | MATERIALS AND METHODS

Generation of Casp-3 expression constructs

Typically, WT casp-3 is expressed with a C-terminal His₆-tag in pET 23b.⁴⁰ For the casp-3 split GFP constructs, the His₆-tag was required to be on the N-terminus so that the GFP11 could be appended onto the *C*-terminus. Using site-directed mutagenesis, we encoded the His₆-tag attached to the N-terminus and installed two prodomain mutations, D9A and D28A, to prevent cleavage of the prodomain and His₆ tag. Next, the 11th strand of superfolder GFP (GFP11), with or without the M3 mutations, was added to the Cterminus of the NH₆ casp-3 D9A D28A construct with a GGGGSGG linker to generate C3-11 and C3-11^{M3}. Sequence of GFP11M3: RDHMVLHEYVNAAGIT and GFP11: RDHMVLLEFVTAAGIT. To generate inactive versions of these variants, we utilized site-directed mutagenesis to replace the active site cysteine with serine, C163S (C3^{KO}-11, C3^{KO}-11^{M3}). The expression and purification procedure for these casp-3 variants are identical to that of WT casp-3.

Casp-3 expression and purification

pET 23b plasmid encoding human WT casp-3 was transformed into BL21(DE3) Escherichia coli cells via electroporation and plated on agar plates containing ampicillin. Single colony cultures were grown in 50 mL LB media with the corresponding antibiotic at 37°C overnight. The following day 8 L of LB was inoculated with ~5 mL per L of the small seed culture and grown at 37°C until an OD₆₀₀ of ~0.6 was achieved. The incubation temperature was then reduced to 25°C and cells were induced with a final concentration of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and left to express for ~3 h. Cells were then harvested by centrifugation at $4,700 \times g$ for 10 min and stored at -80° C. Cell pellets were thawed and lysed using a microfluidizer (Microfluidics, Inc.) in a buffer containing 50 mM Na₃PO₄, 300 mM NaCl, 2 mM imidazole, pH 8. Lysed cells were centrifuged at $30,600 \times g$ for 55 minutes to remove cellular debris. The lysate supernatant was then loaded onto a pre-charged 5 mL HiTrap Ni-affinity column (GE Healthcare) and the column was subsequently washed with lysis buffer. Following the lysis wash, the column was further washed with an increased imidazole buffer, 50 mM, and the protein was finally eluted using a linear gradient to 300 mM imidazole. The eluted protein was diluted seven-fold in a buffer containing 20 mM Tris and 2 mM DTT, pH 8. This ~175 mL solution was then loaded onto a 5 mL HiTrap Q column (GE Healthcare) and finally eluted using a linear NaCl steep gradient in 20 mM Tris, 2 mM DTT, pH 8. The Q-fractions were analyzed for purity via SDS-PAGE and concentration concluded via A_{280} absorbance, using molar extinction coefficients ~25,900 M⁻¹ cm⁻¹ and subsequently stored at −80°C.

C3-11 variant characterization

To ensure that the addition of the GFP11 tag did not significantly perturb the activity or structure of casp-3, casp-3 variants were characterized using several approaches. *Thermal stability using Sypro Orange*: Recombinant casp-3 variants were individually diluted in a buffer containing 5 mM DTT, 20 mM Tris and 100 mM NaCl to a final concentration of 10 μ M. Fifty-five microliters of each protein solution with or without benzyloxycarbonyl-*Val-Ala-Asp-fluoromethylketone* (z-VAD-fmk, Enzo Life Sciences, 50 μ M) was aliquoted in a 96-well RT-PCR plate and 10 μ L of a ×10 Sypro Orange stock in the same buffer were added. The samples were assayed using a CFX Connect RT-PCR detection system (BioRad Laboratories, Inc.). The resulting fluorescence

intensity was recorded as a function of temperature at 0.5°C intervals from 25 to 95°C. Thermal melting points $(T_{\rm m})$ were calculated using Prism 5 (GraphPad) software with Boltzman Sigmoidal curve fittings where V50 is equal to the $T_{\rm m}$. For the samples without inhibitor, DMSO was supplemented. Activity against small fluorogenic peptide substrates: The cleavable peptide N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-amc, Enzo Life Sciences) was used as a fluorogenic substrate. Recombinant casp-3 variants were individually diluted in casp-3 activity assay buffer (20 mM HEPES pH 7.5, 150 mM NaCl, CaCl₂ and 10% PEG 400) in the presence of 10 mM DTT and simultaneously added to 10 µL of DMSO containing varying concentrations of DEVD-amc. Plates were immediately assayed over a 10-min time course at 37°C in a 96-well plate. Fluorescence of cleaved coumarin was measured in a Spectramax M5 spectrometer (Molecular Devices, excitation 365 nm; emission 495 nm). The final DMSO concentration was 10% of the overall solution of 100 µL. The resulting kinetic curves were plotted using Prism 5 (GraphPad Prism). Activity against protein substrates: Full-length casp-3 active site knockout, C163S, was used as a protein substrate for C3-11^{M3} and WT casp-3. Casp-3 C163S was diluted to 10 µM in casp-3 activity buffer (20 mM HEPES pH 7.5, 150 mM NaCl, CaCl₂ and 10% PEG 400) in the presence of 10 mM DTT. Five hundred nanomolar of C3-11^{M3} or WT was added and the reaction was incubated at 37°C. At different time points (0, 0.5, 1, 3, 6 h), 30 µL of the reaction was removed and added to 10 μ L of SDS-PAGE \times 3 dye. The samples were immediately boiled at 95°C for 5 min to halt enzyme activity. Samples were then loaded onto a 16% SDS-polyacrylamide gel and electrophoresis was executed at 175 V for 60 min to separate cleaved bands and allow analysis of cleavage of the zymogen substrate. The same procedure for zymogen cleavage was adapted using C3^{KO}-11^{M3} as the substrate for WT casp-3 and C3-11^{M3}.

Recombinant protein reassembly assay

Active C3-11 and inactive C3^{KO}-11 variants were serially diluted in TNG buffer (20 mM Tris, 100 mM NaCl, 10% Glycerol, pH 7.5) and 20 μ L was aliquoted to wells in a black 96-well microliter plate (12–0.1 μ M final range). Using a multichannel pipette, 80 μ L of recombinant, purified GFP1-10 (18 μ M stock, diluted in TNG, 14 μ M final) was added and fluorescence emission was immediately measured in a Spectramax M5 spectrometer (Molecular Devices) from 450 to 650 nm after excitation at 435 nm. The plate was sealed with an adhesive aluminum sticker and left at RT to monitor fluorescence as a function of time. At subsequent time points the sealant sticker was removed and the plate was analyzed using the same conditions. Reassembly of active and inactive WT GFP11 and GFP11M3 variants were directly compared.

Lysate reassembly assay

A similar protocol as recombinant protein reassembly assay was adapted but instead of recombinant GFP1-10, lysates prepared from HEK cells stably expressing GFP1-10 (HEKs1-10) were used. Final lysate concentration was 1,200 μ g/mL total protein, 100 μ L added. Final concentration of C3-11 variants range from 25–3 μ M, 150 μ L added.

Mammalian cell transfection

The day before transfection, cells were plated at a density of ~5 x 10^4 in a 24-well plate and left to adhere for ~24 h. Upon reaching confluency, cells were transfected using JetPrime (Polyplus transfection reagent) in serum media. The transfection complexes were prepared according to the manufacture's suggestions. GFP1-10 construct was purchased from addgene (#70219). Casp-3 transfection DNA constructs were prepared by cloning C3^{KO}-11 and C3^{KO}-11^{M3} sequences into pcDNA3.1 vectors. The cells were incubated for 24 h and the transfection monitored by microscopy or flow cytometry analysis using 488 nm GFP laser lines. Note: significant GFP fluorescence was also achieved in HEK293T cells via co-transfection of GFP1-10 and C3^{KO}-11^{M3} using JetPrime.

Mammalian cell studies for apoptosis validation

Briefly, all cells were grown in 100 mm \times 15 mm tissue culture dishes in the presence of DMEM (Gibco, Thermofisher) supplemented with 10% FBS (Gibco, Thermofisher) and 1%penicillin/streptomycin (Gibco, Thermofisher) at 37°C, 5% CO₂ until ready for use. For cell viability experiments: 2 days prior to the assay, cells were plated at a density of $\sim 1 \times 10^4$ in a 96-well plate and left to adhere for ~24 h. The day before the assay, purified NG samples were diluted in a media and ×1 PBS, pH 7.4 mixture where the ×1 PBS composes 10% of the final solution volume. The cell culture media was removed and 100 µL of the NG-containing media was added to the appropriate wells. Plates were left to incubate for 24 h unless otherwise noted. When time elapsed, 10 μ L of a sterile filtered ~5 mg/mL MTT/media solution was added to every well. The plates were left to incubate for 2.5-3 h and then centrifuged at $3,000 \times g$ at $4^{\circ}C$ for 5 min to adhere the insoluble product to the bottom of the plate. The media was removed and 100 µL of DMSO was added to each well to solubilize the MTT-assay product. Plates were then characterized by the difference of the absorbance at 550 and 630 nm using a Spectramax M5 spectrometer (Molecular Devices). For whole cell extract experiments: 2 days prior to the assay, cells were plated at a density of $\sim 5 \times 10^4$ in a 24-well plate and left to adhere for ~24 h. The day before the assay, purified NG samples were diluted in a media and 1× PBS, pH 7.4 mixture where the $1 \times PBS$ composes 10% of the final solution volume. For C3 NG treatment in the presence of z-VADfmk, cells were treated with 20 μ M inhibitor for 1 h (added from a 20 mM inhibitor stock) prior to C3 NG addition. Plates were then left to incubate for the appropriate amount of time. When time elapsed, cells were washed twice with ×1 PBS (Gibco, Thermofisher) and then lysed by incubation with 60 µL of freshly prepared and sterile filtered lysis buffer. Lysis buffer contained 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton-x 100 and 10 mM DTT with ×1 Halt Protease/Phosphatase inhibitor cocktail (Pierce, Thermofisher). Cell plates were incubated at 4°C for 25 min with constant rotation. Lysates were then extracted and clarified by centrifugation for 20 min at 16,000 \times g, 4°C. Lysates were immediately used, or frozen overnight then thawed.

SEE FACS experiments

Two days prior to the assay, HEKs1-10 cells were plated at a density of $\sim 5 \times 10^4$ in a 24-well plate and left to adhere for ~24 h. Upon reaching confluency, cells were treated with purified NP diluted in a mixture of complete DMEM media and 10% v/v ×1 PBS, pH 7.4. A 100 mM CQ stock was diluted into media to produce samples ranging from 12.5 to 100 µM and NG were diluted into these CQ-containing samples. 100 millimolar CQ stocks were freshly prepared and used within 1 week. After ~24 h (unless otherwise noted), the cell incubation media was removed and cells were washed twice with ×1 PBS and then incubated with 100 μ L of \times 1 trypsin solution (prepared from 0.5% Trypsin-EDTA) for ~5 min at 37°C. Two hundred microliter of FACS buffer (sterile filtered 0.5% BSA in \times 1 PBS, pH 7.4) was added to the wells and the entire solution was mixed then transferred to FACS compatible tubes. Samples were immediately assayed using flow cytometry with 488 nm GFP laser line. Samples were analyzed first using a live cell forward scatter area vs. side scatter area gate (FSC-A vs. SSC-A) followed by single cell gate (FSC-A vs. FSC-H). Finally, this livesingle-cell population was gated against the media only (un-treated, cells only) control to have ~0.5–1% GFP positive cells (histogram of FITC-A). Percent GFP positive values were reported as is. For GFP MFI, samples were normalized to the media only control.

Fluorescence microscopy

Two days prior to the assay, cells were plated at a density of $\sim 1 \times 10^4$ in glass-bottom confocal dishes and left to adhere for 24-36 h. NG were added at a dose of 1 mg/mL and incubated for 12 or 24 h. To generate Cy5-labeled C3^{KO} NG, C3^{KO} was labeled with Cy5 (Lumiprobe) prior to NG formation. C3^{KO} was diluted in 0.1 M NaHCO₃, pH 8.5 to protonate all lysine residues and mixed with 1.5 equivalents of Cy5-NHS ester (in 10% DMSO of the final reaction volume) for 3 h at room temperature. Cy5-labeled protein was separated from unreacted Cy5 using a Nap5 column equilibrated in 0.1 M NaHCO₃, pH 8.5. Following NG incubation, cells were then stained with NucBlueTM Live ReadProbesTM (Hoechst, Thermo-Fisher) and LysotrackerTM (ThermoFisher) according to the manufacturer's recommendations followed by two 0.5 mL ×1 PBS washes to remove any non-endocytosed NG and excess staining reagents. Cells were then replenished with Live Cell Imaging Solution (Molecular Probes by Life Technologies[™]) prepared with 10% FBS and imaged using fluorescence microscopy.

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AUTHOR CONTRIBUTIONS

Francesca Anson: Conceptualization; data curation; formal analysis; investigation; methodology. **Pintu Kanjilal:** Data curation. **Sankaran Thayumanavan:** Conceptualization; funding acquisition; project administration; resources; supervision; writing-review and editing. **Jeanne Hardy:** Conceptualization; funding acquisition; project administration; resources; supervision; writing-review and editing.

CONFLICT OF INTEREST

The authors declare no conflict interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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