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Caveolin elastin-like polypeptide fusions mediate temperature-dependent assembly of caveolar microdomains

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Abstract

Caveolae are membrane organelles formed by submicron invaginations in the plasma membrane, and are involved in mechanosensing, cell signaling, and endocytosis. Although implicated broadly in physiology and pathophysiology, better tools are required to elucidate the precise role of caveolar processes through selective activation and inactivation of their trafficking. Our group recently reported that thermally-responsive elastin-like polypeptides (ELPs) can trigger formation of ‘genetically engineered protein microdomains (GEPMs)’ functionalized with either Clathrin-light chain or the epidermal growth factor receptor. This manuscript is the first report of this strategy to modulate caveolin-1 (CAV1). By attaching different ELP sequences to CAV1, mild heating can be used to self-assemble CAV1-ELP microdomains inside of cells. The temperature of self-assembly can be controlled by tuning the ELP sequence. The formation of CAV1-ELP microdomains internalizes Cholera Toxin Subunit B, a commonly used marker of caveolae mediated endocytosis. CAV1-ELPs also colocalize with Cavin 1, an essential component of functional caveolae biogenesis. With the emerging significance of caveolae in health and disease and the lack of specific probes to rapidly and reversibly affect caveolar function, CAV1-ELP microdomains are a new tool to rapidly probe caveolae associated processes in endocytosis, cell signaling, and mechanosensing

Graphical Abstract

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Supporting Information

Cloning of CAV1-ELPs; cell culture and transfection; image acquisition and quantification; transition temperature determination

Conflict of Interest

JA MacKay is an inventor on patents describing applications of elastin-like polypeptides.

Keywords

caveolae-mediated endocytosis; CAV1; genetically engineered protein microdomains; GEPM; mechanobiology; quantitative fluorescence microscopy

The plasma membrane is a highly dynamic organelle comprised of domains that facilitate signaling, mechanosensing, and exchange of materials with the external environment. One of the most morphologically-distinct of these domains is constituted by caveolae, 50-100 nm bulb-shaped pits in the plasma membrane. The integral membrane protein Caveolin 1 (CAV1) is the essential structural protein constituent of caveolae, with individual caveolae containing approximately 140 oligomerized CAV1 proteins.¹⁻³ More recently, the peripheral membrane protein Cavin 1 has been shown to be crucial for functional caveolae regulation and biogenesis.⁴⁻⁶

Although CAV1 is an integral membrane protein, with its transmembrane domain completely buried in the lipid bilayer, both its N and C terminus face the cytosol. This unique molecular structure makes direct extracellular labeling or surface biotinylation of CAV1 impossible.⁷ In addition, caveolar abundance varies widely across different cell types and tissue. In adipocytes, muscle cells, and endothelial cells, caveolae can account for up to 50% of the plasma membrane surface area. In contrast, caveolae are present at low levels in the liver and apparently not at all in kidney proximal tubules.^{2, 3, 8} Furthermore, caveolae exhibit significant versatility in the structures they form, from individual caveolae to rosettes that extend deep into the cytoplasm.^{9, 10}

The considerable heterogeneity of caveolar abundance and structural versatility, combined with the lack of a direct extracellular domain for labeling, has made it difficult to discern the functions that caveolae play in health and disease. However, it is now clear that caveolae perform roles in signaling, plasma membrane dynamics, and trafficking. They regulate signal transduction by organizing signaling partners into discrete regions near the plasma membrane.¹¹⁻¹³ Not unlike the suspension system on an automobile, they can absorb mechanical or osmotic strain by flattening or assembling.^{2, 9, 14-16} Perhaps most famously, caveolae can pinch off from the plasma membrane to endocytose and traffic cargo in a process termed caveolae-mediated endocytosis (CAVME).¹⁷⁻¹⁹

ELPs are biocompatible, biodegradable polypeptides composed of a pentameric repeat of (VPGXG)_n. The X residue can be selected to control ELP hydrophobicity, and the number of repeats, *n*, determines the ELP molecular weight. ELPs can rapidly phase-separate into protein-rich domains in response to heating, whereby they form a secondary aqueous phase known as a coacervate. In cells, individual coacervate droplets are typically several hundred nanometers to several micrometers in diameter. This phase separation is thermodynamically-reversible, and ELP coacervates quickly resolubilize into bulk water upon cooling. The temperature of self-assembly, termed the transition temperature (*T_t*), is determined largely by the hydrophobicity of the guest residue and the ELP molecular weight.^{20, 21}

Our group previously reported that ELPs can be fused to effector proteins to reversibly assemble cytosolic fluorescent microdomains both in live cells and in the single cells of living zebrafish embryos.^{22, 23} In addition, we have cloned ELPs with Clathrin Light Chain (CLC), and used temperature mediated self-assembly of the resulting CLC-ELPs to reversibly inhibit Clathrin-mediated endocytosis.²⁴ Most recently, we reported that fusion of an ELP to the transmembrane receptor tyrosine kinase known as epidermal growth factor receptor 1 promoted temperature-dependent receptor internalization and phosphorylation of ERK1/2.²⁵

In addition to work performed by our group, Ge et al. reported that recombinant ELP-GFP proteins can form microcompartments inside *E. Coli* or tobacco cells.²⁶ More recently, Jang et al. have reported that recombinant ELPs can be used for the tunable creation of protein vesicles containing functional, globular domains.²⁷ Relatedly, Voegle et al. have reported that elastin-like peptides can create self-assembled peptide vesicles. Furthermore, the growth of these vesicles can be genetically encoded by encapsulating a cell-free transcription-translation system together with the peptide DNA template inside the vesicles.²⁸ Such studies demonstrate how ELPs and related materials can be used to probe and to model important biological questions in a surprising variety of systems.

This manuscript reports that ELPs can be cloned to the C-terminus of CAV1 to create thermally-responsive CAV1-ELP fusion proteins. By changing ELP length and sequence, a library of CAV1-ELPs were created that display different transition temperatures. To determine the precise transition temperature of these CAV1-ELPs in live cells, we refined a novel technique developed in our lab to visualize and quantify their assembly through coassembly with coexpressed fluorescent ELPs.²⁵ We found that when CAV1-ELPs are incubated above their *T_t*, they self-assemble intracellular microdomains which partially

overlap with internalized Cholera Toxin Subunit B (CTXB), a commonly used marker of caveolae-mediated endocytosis. In addition, CAV1-ELPs display broad colocalization with Cavin1 at all temperatures, which is consistent with their cellular biology.

To determine whether temperature mediated ELP self-assembly could produce caveolar microdomains, a library of different CAV1-ELPs was first cloned (Figure 1A, Table 1), and their identity was confirmed using Western-blotting (Figure 1B). In this western blot, smaller bands are seen which are consistent with blots for detergent resistant membrane proteins and with the degradation reported for other CAV1 fusion proteins.²⁹ By changing ELP length and sequence, CAV1-ELPs were designed with transition temperatures above and below a physiological temperature of 37 °C. Based on previous studies, three ELP sequences were chosen: V96, V72, and A96. CAV1-V96 was intended to display near maximal self-assembly at 37 °C. Purified A96 displays a T_i above 80°C, thus CAV1-A96 is intended as a negative control (i.e. a CAV1-ELP with no assembly at physiological temperatures). Based on our previous work with membrane bound EGFR, it was estimated that CAV1-V72 would self-assemble within 5°C above the transition temperature of CAV1-V96.²⁵ As a control for overexpressed CAV1, wild-type CAV1 with a C-terminus myc epitope was also developed.

Because CAV1-ELPs are not fluorescent, their intracellular T_i cannot be directly visualized without fixation and indirect immunofluorescence. To circumvent this limitation, a live cell imaging technique was refined, which was previously applied to visualize the self-assembly of EGFR-ELP microdomains.²⁵ This technique exploits the co-assembly of non-fluorescent ELP fusion proteins with fluorescent GFP-V60 to visualize ELP microdomain formation in live cells (Figure 2). This fluorescent fusion reporter protein consists of the ELP V60 fused to the C-terminus of Green fluorescent Protein (GFP). GFP-V60 typically undergoes phase separation above physiological temperature. Below the intracellular T_i of GFP-V60, this protein is therefore soluble and GFP fluorescence is detected homogeneously throughout the cytoplasm. However, as temperature is increased above the T_i of GFP-V60, ELP mediated self-assembly produces fluorescent GFP microdomains. With the aid of an epifluorescent microscope, these fluorescent microdomains are readily visible. Accordingly, by tracking live cells undergoing a temperature ramp, a blinded observer can visually determine the temperature at which microdomain assembly occurs for individual cells. This temperature is defined as the intracellular T_i .

Critically, when cells are dually-transfected with either CAV1-V96 or CAV1-V72 in addition to GFP-V60, microdomain assembly occurs at a lower temperature in comparison to cells transfected solely with GFP-V60. In addition, co-transfected cells display GFP microdomains that are fewer in number and larger relative to those produced by GFP-V60 alone, which allows the distinction of singly- transfected cells from dually- transfected cells. Individual cells transfected with only GFP-V60 were evaluated for their microdomain T_i , microdomain number per cell, and average area per microdomain (Figure 2B-E). Using this technique, cells transfected with GFP-V60 alone displayed a median intracellular T_i of 42°C. In contrast, cells transfected with both GFP-V60 and either CAV1-V96 or CAV1-V72 exhibit a significant reduction of intracellular T_i to 36.3°C or 38.5°C, respectively. (Figure 2E, Table 1). Although the CAV1-V72 + GFP-V60 group did exhibit a trend towards a

higher intracellular T_t compared to CAV1-V96 + GFP-V60, the difference between the groups was not statistically significant (p-value = 0.85). In contrast, our previous work did find differences in T_t between V72 and V96 fusion proteins of GFP, Clathrin light Chain, and EGFR.^{22, 24, 25} This result highlights how the cellular location (e.g. membrane vs. cytosolic) and idiosyncrasies of the wild-type protein affect the transition temperature of its ELP fusion. In future work, experimenters may further fine tune the CAV1-ELP T_t by selecting pentameric repeats and guest residues beyond what we have examined. As there was no significant difference between CAV1-V96 and CAV1-V72, and because CAV1-V96 had a median T_t below 37°C, all subsequent characterization in this manuscript was performed using CAV1-V96.

Using a confocal laser scanning microscope equipped with an Airy scan super-resolution detector and an indirect immunofluorescence approach, we next determined whether CAV1-V96 self-assembly would disrupt internalization of cholera toxin subunit B (CTXB), a commonly used marker of caveolar lipid rafts and caveolar-mediated endocytosis.^{30, 31} HEK293T cells were transfected with either CAV1-V96, CAV1-A96, or CAV1-WT. After an initial incubation at 4°C, which was necessary to resolubilize any already assembled CAV1-V96 and also to broadly inhibit ongoing caveolar endocytosis, cells were surface-labeled with fluorescent CTXB at 4°C, washed, and then incubated at 37°C or kept at 4°C for 50 minutes.

Surprisingly, at 37°C CAV1-V96 self-assembled into microdomains which directly overlapped with CTXB (Figures 1C, 3, 4). The formation of these microdomains produced a statistically significant increase in colocalization with CTXB at 37°C vs. 4°C (p-value = 0.0014). In contrast, CAV1-WT and temperature insensitive CAV1-A96 did not assemble microdomains and did not exhibit a statistically significant increase in colocalization with CTXB at 37°C compared to 4°C. These results suggest that CAV1-V96 self-assembly internalizes CTXB in a mechanism perhaps like caveolae-mediated endocytosis.

To further explore if CAV1-V96 self-assembly was responsible for internalization of CTXB, 60 slice z-stacks were collected in fixed cells using indirect immunofluorescence. Representative images are shown in Figure 4, where the axial views reveal that CAV1-V96 microdomains overlap with CTXB detected inside the cell, in agreement with CAV1-V96 self-assembly internalizing CTXB. These results suggest that CAV1-V96 self-assembly does internalize CTXB, but the specificity of microdomain assembly to internalize caveolar lipid rafts remained unknown. Since CTXB binds to the ganglioside GM1, which is preferentially found in caveolar and non-caveolar lipid rafts, we hypothesized that if CAV1-V96 self-assembly was responsible for internalization of caveolar lipid rafts, then cells transfected with CAV1-V96 and induced to self-assemble before CTXB labeling would display a fractional decrease in CTXB binding. This decrease would be due to CAV1-V96 mediated internalization of caveolar lipid rafts and subsequent reduction of extracellular GM1 available for CTXB binding.

To answer this hypothesis, HEK293T cells were transfected with either CAV1-V96 or temperature insensitive CAV1-A96. After an initial incubation at 4°C to resolubilize CAV1-ELPs, cells were incubated at either 4°C or 37°C for 50 minutes and then labeled with

fluorescent CTXB-AF555 at 4°C before fixation and analysis of indirect immunofluorescence to detect CAV1-ELPs. We then collected 60 slice Airy scan confocal n z-stacks of over 100 cells and measured the CTXB-AF555 fluorescence from these z-stacks (Figure 5).

Figure 5B displays 95% confidence intervals for the ratio of fluorescent CTXB signal at 4°C vs 37°C within each transfection. Indeed, CAV1-V96 displayed a statistically-significant temperature dependent reduction of CTXB signal ($p = 0.00675$). Figure 5B reveals that for CAV1-V96, a pre-incubation at 4°C vs 37°C leads to an approximate 1.8-fold change in CTXB signal/binding (i.e. equivalently, for CAV1-V96 transfected cells pre-incubation at 37°C leads to a ~44.4% decrease in CTXB binding compared to a 4°C pre-incubation). In contrast, temperature insensitive CAV1-A96 does not display a statistically significant temperature dependent difference in CTXB binding, as its 95% confidence interval contains a ratio of 1, the null value. This result occurs because CAV1-A96 does not self-assemble microdomains and therefore does not internalize caveolar GM1.

Representative cells from each transfection and temperature are shown in Figure 5A. The cells chosen are from images at the approximate 50% percentile of CTXB signal from within each treatment. The CAV1-V96 37°C cell (second row) displays a staining pattern consistent with CAV1-V96 microdomain formation, however there is limited overlap with CTXB. This result is consistent with CAV1-V96 self-assembly internalizing caveolar GM1, thus making these regions unavailable for subsequent CTXB binding. However, while pre-incubating CAV1-V96 transfected cells at 37°C fractionally decreases CTXB binding, it does not abolish it. This result is consistent with reports in the literature of CTXB entry into cells via non-caveolar routes, the fact that caveolar GM1 represents a fraction of the GM1 pool found on the plasma membrane, and our observation that non-transfected HEK293T cells, which are CAV1 negative, display robust CTXB binding.³⁰⁻³²

Since Cavin 1 is required for functional caveolar biogenesis and because it colocalizes with wild-type CAV1 near the plasma membrane, it was next determined whether CAV1-ELPs would colocalize with Cavin 1 and whether this colocalization was temperature-dependent.³³ Accordingly, HEK293T cells were transfected with Cavin 1, in addition to either CAV1-V96, CAV1-A96, or CAV1-WT (wild type CAV1 with a C-terminal myc tag). The representative images shown in Figure 6 reveal broad overlap between transfected Cavin 1 and CAV1, regardless of ELP sequence or temperature. These results are consistent with CAV1-ELPs retaining partial wild-type CAV1 functionality and plasma membrane localization.

In the near future, we envision a wide variety of recombinant polypeptide fusions as synthetic biological triggers. Among these, the ELPs are particularly well-suited due their lack of association with other proteins and their abrupt, reversible phase separation. It is likely that other polypeptides could provide similar control; furthermore, these may have diverse applications to perturb and study many cellular-pathways.

We foresee CAV1-ELPs primarily as a tool for cell culture studies of endocytosis, cell signaling, and mechanosensing. As our manuscript reports, the formation of CAV1-ELP

microdomains induces a process reminiscent of endocytosis. Accordingly, CAV1-ELP microdomain formation could be used to study the internalization of receptors, pathogens, and drugs. We also note that by using viral transfection methods, the range of CAV1-ELP transfectable cells could be extended to primary cells or patient derived iPSCs to explore the role of CAV1 in a variety of pathological states.

In addition to endocytosis, caveolae also regulate signal transduction by organizing signaling partners into discrete regions near the plasma membrane.¹¹⁻¹³ Accordingly, CAV1-ELP microdomains may cluster caveolar cell surface receptors. The ability to indirectly control receptor clustering on the plasma membrane could be exploited by the cell biology community to control and dissect cell signaling.

In recent years it has become clear that caveolae act as suspension system for the plasma membrane by flattening and assembling in response to mechanical and osmotic pressure.^{2, 9, 14-16} Therefore, another potential application would be the use of CAV1-ELP microdomains to control plasma membrane curvature and surface area. This would be of great practical use in the emerging field of mechanobiology. In combination with near infra-red laser-irradiation of nanoparticles or dyes, such as the FDA-approved indocyanine green, it may be possible to locally trigger transfected CAV1-ELP phase separation *in vivo*.^{34, 35}

The ability to precisely control temperature at the tissue or organism level does represent a challenge for this technique. However, given our previous results with zebrafish embryos, in which ELP length and sequence, incubation temperature, and mRNA injection concentration were controlled to achieve tunable ELP assembly, it is likely that this model system could be used for *in vivo* studies of CAV1-ELPs.²² Given the emerging significance of CAV1 in health and disease and the remaining major questions regarding caveolar function, CAV1-ELPs will provide the life science community with an innovative method to control CAV1.

In summary, this manuscript demonstrates for the first time a tunable temperature-dependent strategy to self-assemble intracellular CAV1 using ELPs. Self-assembly of CAV1-ELPs induces internalization of CTXB in a mechanism reminiscent of caveolae-mediated endocytosis. The temperature of CAV1-ELP self-assembly can be fine-tuned by adjusting the ELP sequence. CAV1-ELPs also retain the colocalization with Cavin 1 that is characteristic of endogenous CAV1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Construction and confirmation of a library of CAV1-ELP fusions.

A) Genes encoding three different ELP sequences (V72, V96, A96) were cloned to Caveolin 1. A myc-tag was placed in-between CAV1 and ELP sequences for convenient immunofluorescent detection. Each ELP sequence confers a unique transition temperature (T_t). CAV1 sequence with myc-tag and no ELP sequence (bottom row) is referred to as CAV1-WT. B) CAV1-ELPs were expressed in HEK 293T cells and their size was confirmed using a caveolin 1 antibody. GAPDH is displayed at the bottom. NT = No transfection. The entire gel picture for the GAPDH protein loading control can be found in the supporting information (Supplementary Figure S1).

Figure 2. CAV1-ELP Transition Temperature (T_t) can be visually determined in live cells.

A) Schematic of the live cell imaging technique which was used to determine CAV1-ELP T_t . B) Live cells transfected with either GFP-V60 or GFP-V60 + CAV1-V96 and subjected to a temperature ramp to assemble distinct microdomains (also termed coacervates) at different temperatures. Images were acquired with epifluorescence microscopy. Scale bar is 10 μm C) Indirect immunofluorescence using Airy scan confocal microscopy in fixed cells confirms that CAV1-V96 (myc antibody) co-assembles with GFP-V60 (GFP antibody) at 37°C. These CAV1-V96 + GFP-V60 microdomains also colocalize with internalized fluorescent cholera toxin subunit B (CTXB-AF555). Scale bar is 5 μm . D) Cells dually-transfected with GFP-V60 + CAV1-V96 or CAV1-V72 + GFP-V60 display microdomains which are larger and fewer in number compared to cells transfected with GFP-V60 alone. Each circle is data for one cell above its T_t . Horizontal dashed line is at 23 μm^2 , the max size recorded among all GFP-V60 only transfected cells. This line served as the filter for distinguishing true dual transfected cells. E) Boxplots of T_t between different transfections after filtering shown in panel D. Each dot is the T_t for a single cell. P-values calculated from a two-sided Tukey's HSD post hoc-test following a statistically significant one-way ANOVA. ***p-value < 0.001. Magnified panels A-E are in the supporting information.

Figure 3. CAV1-V96 microdomains colocalize with CTXB.

A) HEK293T cells transfected with indicated constructs were labeled with CTXB-AF555 at 4°C and then placed at the indicated temperatures for 50 min before fixation and immunostaining via myc epitope. Airy scan confocal microscopy reveals that temperature sensitive CAV1-V96 self-assembles into microdomains which colocalize with fluorescent CTXB (2nd from bottom row and corresponding magnified panel). This colocalization was not present in cells transfected with CAV1-WT or temperature insensitive CAV1-A96. Scale bar is 5 μ m. Images shown are representative from dozens of images acquired within each treatment group. **B)** CAV1-V96 colocalization with fluorescent CTXB is temperature dependent. Dozens of transfected cells in each group were imaged to quantify colocalization of CAV1-ELP/CAV1-WT with CTXB in response to temperature increase from 4°C to 37°C. Each diamond represents one magnified image containing 1-3 transfected cells. Error bars are standard deviation. Pairwise p-values were calculated from a two-sided Tukey's HSD post hoc-test following a statistically significant one-way ANOVA. * p-value < 0.05, ** p-value < 0.01, ***p-value < 0.001

Figure 4. Temperature mediated self-assembly of CAV1-V96 induces internalization of fluorescent Cholera Toxin Subunit B (CTXB).

Z-stacks from Airy scan confocal microscopy reveal that CAV1-V96 self-assembly internalizes surface labeled CTXB (top row). CAV1-ELP/CAV1-WT signal is from indirect immunofluorescence via myc tag. Lateral scale bar is 5 μm . Axial scale bars are 2 μm . Images shown are representative from dozens of images.

Figure 5. CAV1-V96 self-assembly removes a fraction of CTXB binding sites from the plasma membrane.

A) HEK293T cells transfected with CAV1-V96 or CAV1-A96 were incubated at 4°C or 37°C for 50 minutes and were then labeled with CTXB-AF555, fixed, and immunostained for CAV1-ELPs via myc epitope. Z-stacks were then collected and fluorescent CTXB signal was quantified. Cells shown are from images at the approximate 50% percentile of fluorescent CTXB signal from within each treatment. The CAV1-V96 37°C cell (second row) displays likely microdomain formation but without overlapping CTXB signal, consistent with CAV1-V96 self-assembly internalizing caveolar GM1, thus making these regions unavailable for subsequent CTXB binding. Scale bar is 5 μm . **B)** 95% confidence intervals for the ratio of fluorescent CTXB signal after a 4°C vs. 37°C pre-incubation. Dashed vertical line is at 1, the null value (i.e. no difference in signal). Corresponding p-values are also shown. For CAV1-V96, pre-incubation at 4°C vs. 37°C leads to a ~1.8-fold increase in CTXB binding (i.e. pre-incubation at 37°C leads to a ~44.4% decrease in CTXB binding compared to a 4°C pre-incubation). Twenty-seven ($n = 27$) z-stack images were acquired within each temperature/transfection combination, totaling 108 quantified images.

Figure 6. Cavin 1 exhibits broad colocalization with CAV1, regardless of ELP sequence or temperature.

HEK293T cells co-transfected with Cavin 1 and either CAV1-V96, CAV1-A96, or CAV1-WT were labeled with CTXB-AF555 and then incubated at either 4°C or 37°C for 50 minutes before fixation and indirect immunofluorescence. Broad colocalization between Cavin 1 and CAV1-ELPs/WT is evident in all treatments. Note the CAV1-V96 cell at 37°C (top row) displays stereotypical microdomain formation with CTXB, which also colocalizes with Cavin 1. Transfected Cavin 1 was detected with an HA antibody for C-terminus HA epitope. CAV1 constructs were detected with a myc antibody.

Table 1.

Nomenclature, amino acid sequence, and phase behavior of expressed proteins.

Protein Label	Amino acid Sequence ^a	MW ^c [kD]	Median T_t in cells ^d [°C]	Mean T_t in cells ^e [°C]	Percent of cells showing assembly $f_{[%]}$	Intended behavior
CAV1-V96	<u>CAV1</u> -myc ^b -(VPGVG) ₉₆ Y	62.5	36.3	35.9 (34.2, 37.7)	67	temperature-responsive
CAV1-V72	<u>CAV1</u> -myc-(VPGVG) ₇₂ Y	52.7	38.5	36.5 (34.8, 38.2)	43	temperature-responsive
CAV1-A96	<u>CAV1</u> -myc-(VPGAG) ₉₆ Y	59.8	NA	NA	NA	temperature-insensitive
CAV1-WT	<u>CAV1</u> -myc	24.9	NA	NA	NA	temperature-insensitive
GFP-V60	<u>GFP</u> -(VPGVG) ₆₀ Y	67.2	42	42.5 (41.6, 43.5)	6.4	live-cell imaging tool

Not applicable (NA). CAV1-A96 does not self-assemble at physiological temperatures.

^{a)} ORF amino acid sequences are found in the Methods section.

^{b)} myc-epitope amino acid sequence: EQKLISEEDL

^{c)} Estimated molecular weight from open reading frame and confirmed by western blot (Figure 1B).

^{d)} Median T_t obtained from live cell imaging technique described in Figure 2.

^{e)} Mean T_t (95% Confidence Interval) obtained from live cell imaging technique described in Figure 2.

^{f)} The percentage of cells with a T_t < 37°C. Percentages calculated from data shown in Figure 2E.