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Identification of a Golgi-localized peptide encoded by the CENP-R transcript

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Understanding cell biological behaviors requires the ability to visualize different cellular structures and compartments. Excellent markers and tagged proteins exist to detect key cellular structures such as the ER and mitochondrion, where minimal targeting motifs can be used to image and target proteins to these specific organelles [1-3]. These markers make visualizing these organelles robust and easy, and provide insights into the requirements for targeting proteins to these subcellular compartments. In the course of our ongoing work, we identified a 37 amino acid peptide that is encoded by a hypothetical alternative open reading frame (altORF) within the mRNA produced for the CENP-R gene. Unlike the centromere-localized canonical CENP-R protein, we find that this small peptide localizes specifically to the Golgi compartment. Our studies demonstrate that this altORF peptide can serve as a valuable tool for cell biology experimentation to visualize the Golgi for both fixed and live cell analyses.

A CENPR altORF peptide localizes to the Golgi compartment

Alternative open reading frame (altORF) are sequences present in transcribed mRNAs that are distinct from the canonically-defined open reading frames. Such sequences have alternative translation start sites that result in the translation of a novel protein sequence. AltORFs exist in a variety of proteins, and many have unknown functional roles and behaviors [4]. In the course of our ongoing analysis of the CENP-R gene, we identified a potential altORF that initiates upstream of the defined CENP-R open reading frame (Fig. 1A). Although hypothetical, this altORF has been identified based on ribosome profiling data and was included in a proteomic database of small peptides produced by altORFs with a cutoff of less than 100 amino acids [5]. This altORF corresponds to a peptide sequence of 37 amino acids with a predicted molecular weight of 4.5 kDa and a pI of 9.84. The predicted translation of this altORF in the CENP-R mRNA begins upstream of the canonical start for full length CENP-R and has a unique reading frame (Fig. 1A).

To assess whether this peptide could have cellular functions, we tested its localization by ectopically expressing this peptide with an N-terminal GFP tag in human HeLa cells. Strikingly, we found that this peptide localized outside of the nucleus in what appeared to be membrane-bound structures during Interphase (Fig. 1B). These membranous structures localized peripherally to the nucleus and were typically compact, clustering adjacent to the nucleus. In mitosis, the altORF localized to distinct puncta throughout the cytoplasm of the dividing cell (Fig. 1B). This localization behavior contrasts with that of the canonical CENP-R protein, which localizes to centromere regions in interphase and mitosis (Fig. 1B). Similar localization was also observed when the altORF was tagged at its C-terminus (altORF-GFP; Fig. 1E).

Based on this observed localization, we hypothesized that the altORF likely localizes to the Endoplasmic Reticulum (ER) or Golgi apparatus given its organization relative to the nuclear periphery. To test this, we sought to compare altORF localization to established markers for the (ER) – using an anti-KDEL antibody - and Golgi compartment – using an anti-GM130 antibody. We found that the altORF peptide did not co-localize with ER markers, but overlapped closely with GM130 in fixed cells (Fig. 1C). The Golgi network is further divided into cis-, medial-, and trans- compartments. To define the localization behavior of this altORF peptide, we additionally compared its localization to that of GM130, a marker of the cis-Golgi, and TGN46, a marker of the trans-Golgi [6, 7]. In each case, we observed approximate co-localization of the altORF peptide with these antibodies, but the resolution limits of these images did not allow us to precisely define which sub-compartment is labeled by this peptide (Fig. 1C). Together, we conclude that this altORF peptide localizes to Golgi compartment in HeLa cells.

We next sought to test whether the observed localization behavior for the CENPR altORF also occurred in other

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cell lines. To test this, we transfected the N-terminal GFP construct into a panel of human cell lines including A549, U2OS, and 293T cells. We observed similar localization to the nuclear periphery as in HeLa cells, as well as co-localization with GM130 (Fig. 1D). In addition to these human cell lines, we tested at the localization of the human altORF in mouse fibroblast NIH3T3 cells. We observed a similar localization of the altORF to the Golgi in NIH3T3 cells (Fig. 1D). We therefore conclude that the altORF robustly localizes to the Golgi across a variety of mammalian cell lines.

The CENPR altORF provides a live cell marker to visualize Golgi dynamics

To assess the ability of the altORF to act as a live cell marker for Golgi localization and dynamics, we used the altORF construct to generate a cell line that stably expresses the N-terminally tagged GFP-altORF using retroviral integration. Stable expression of the altORF had no apparent effect on cell viability, behavior, or Golgi morphology. With this cell line, we analyzed the association of the altORF with the Golgi under contexts where Golgi morphology is dynamic. First, we visualized the localization of the altORF peptide throughout the cell cycle. The Golgi is known to undergo a cycle of assembly and disassembly as the cell progresses through the cell cycle [8]. Using time lapse imaging, we observed the rapid fragmentation of the Golgi upon entry into mitosis, with the altORF appearing as small puncta throughout the cytoplasm of the dividing cell, consistent with prior fixed cell analysis. As cells exited mitosis in telophase, we observed the reformation of larger stacks and ultimately the full reformation of the Golgi ribbon-like structures in G1 (Fig. 2A). Next, we sought to observe altORF localization and Golgi behavior by inducing morphological changes to the Golgi using treatment with Brefeldin A (BFA). BFA is an inhibitor of the secretory pathway that causes the Golgi to disassemble and redistribute into the Endoplasmic reticulum [9]. Upon treatment with Brefeldin A, within 15 min we observed the initiation of the breakdown of the ribbon-like structures of the Golgi into small puncta that move freely throughout the cytoplasm of the cell (Figure 2B). In conclusion, the altORF peptide remains robustly associated with the Golgi membrane under conditions where the organelle is disassembled, providing a valuable tool for the live cell imaging of Golgi dynamics.

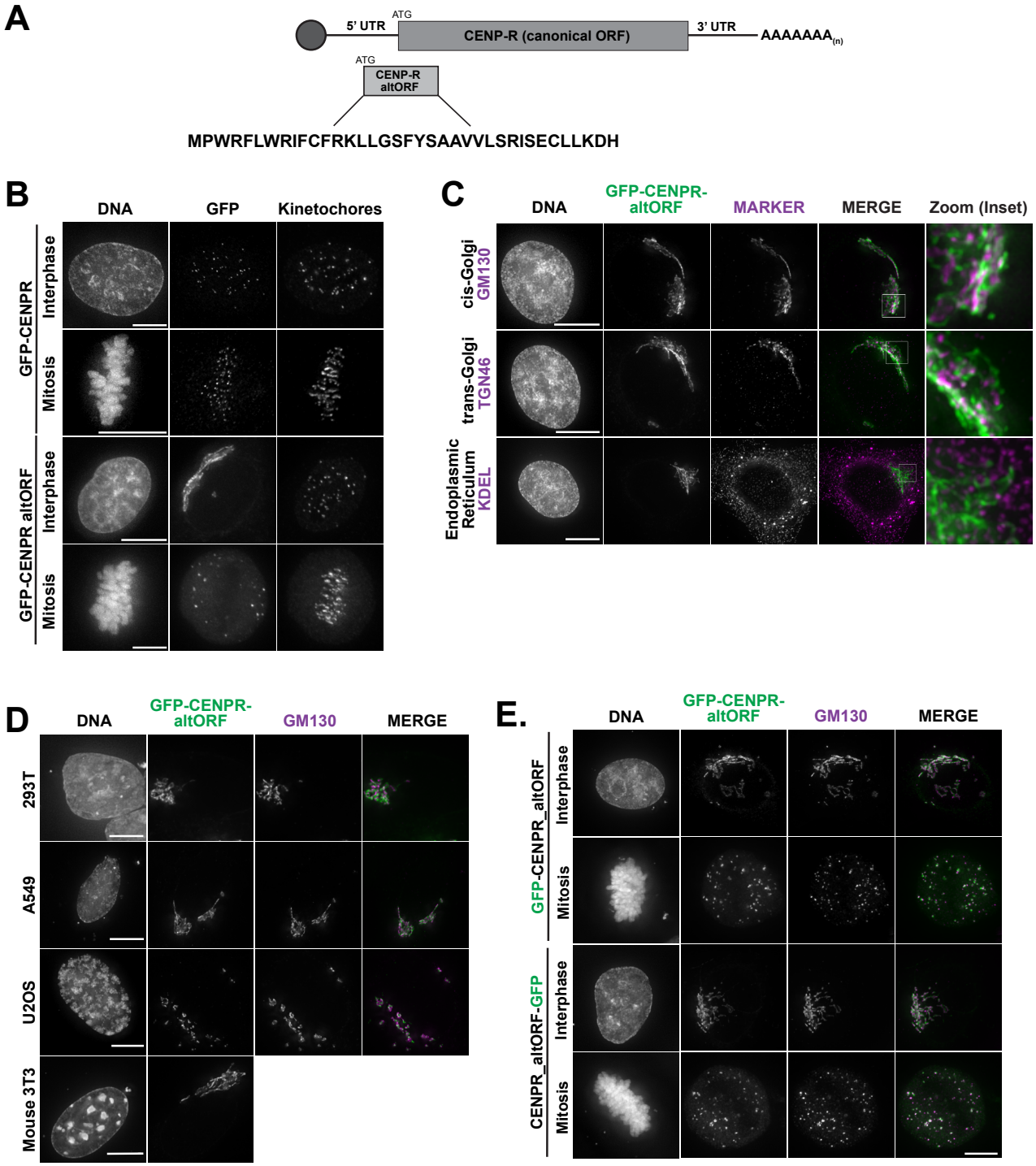
Cysteine residues are important for localization of altORF peptide to the Golgi

The robust localization of this 37 amino acid altORF peptide to the Golgi raises questions as to how this peptide associates with and is retained at the Golgi. We first wanted to understand whether this altORF peptide associated with the cytoplasmic or luminal side of the Golgi membrane. To test this, we utilized a linker that contains a TEV protease cleavage site between the altORF and GFP [10]. We then expressed mCherry tagged TEV protease in cells stably expressing the GFP-TEV-altORF peptide. If the altORF peptide faces the cytoplasm, then the TEV cleavage site should be exposed and susceptible to cleavage by the cytoplasmic TEV protease resulting in a loss of GFP signal at the Golgi. Indeed, upon TEV expression, we observed a substantial increase in cytoplasmic GFP fluorescence and a loss of Golgi localization (Figure 3F).

Next, we sought to elucidate the sequence requirements for targeting the alt-ORF peptide to the Golgi. Unlike targeting/retention sequences for other organelles such as the ER, mitochondria, or nucleus, there is no established consensus sequence that is sufficient for Golgi localization [11]. Resident Golgi proteins can associate with the Golgi membrane as transmembrane or peripheral membrane proteins. Golgi-associated transmembrane domains typically span 20 aa in length and are enriched in aromatic and hydrophobic amino acids [12]. To determine whether this altORF peptide could act as a transmembrane protein at the Golgi, we utilized the Phobius online transmembrane topology tool to test for the presence of predicted transmembrane domains [13, 14]. Analysis of the altORF sequence alone found a low probability for the presence of a transmembrane domain (Figure 3B).

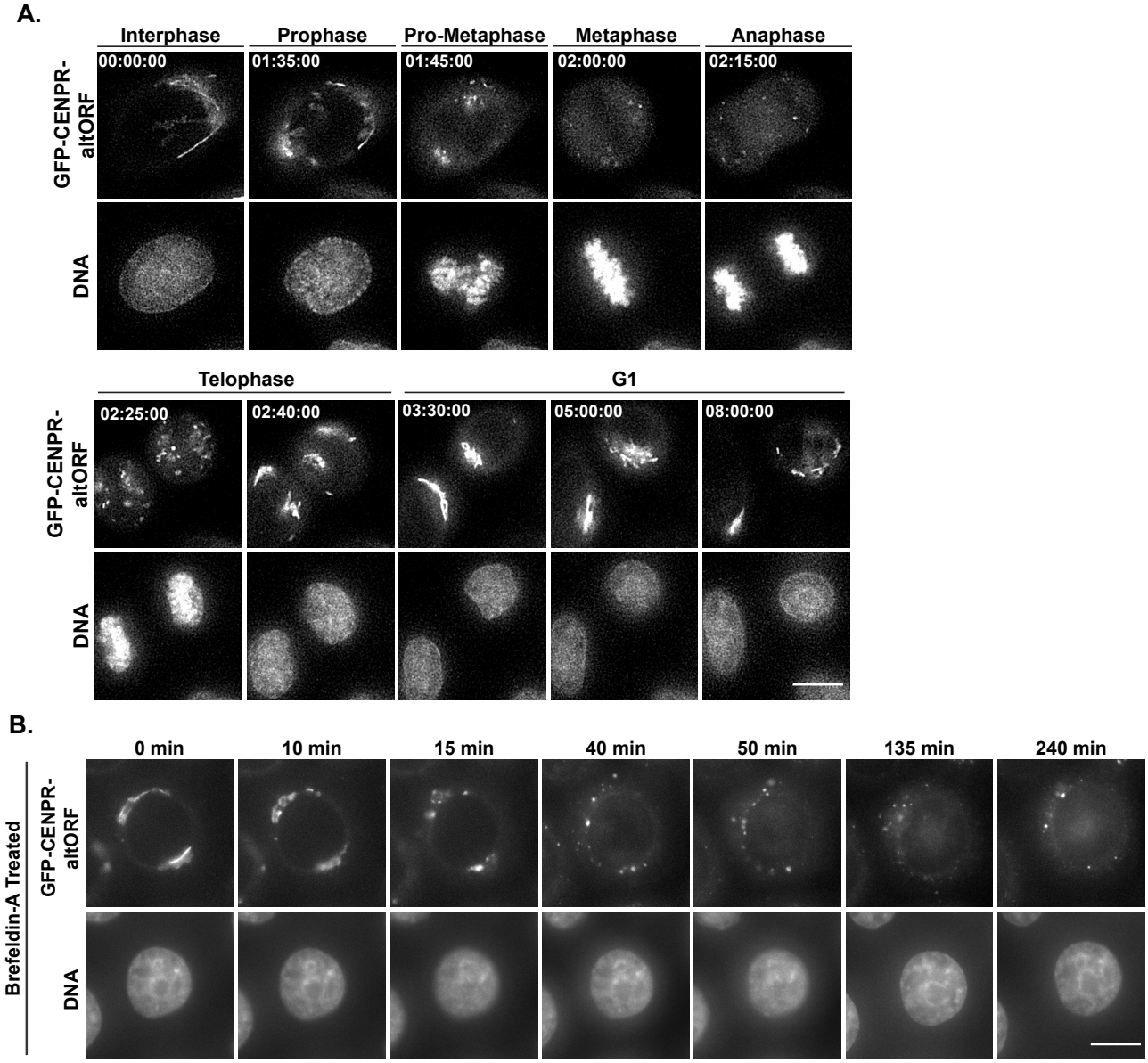
Proteins that are peripherally associated to the Golgi do so through protein/protein interactions and/or post-translational modifications such as lipidation, which frequently modify cysteine residues. Therefore, we sought to determine the sequence requirements that facilitate the robust localization of the altORF to the Golgi. To do this, we generated truncation mutants of the altORF peptide to test the potential contributions of key amino acids groups that contribute to its localization including hydrophobic amino acids, aromatic amino acids, and Cysteine residues (Figure 3A,C). Based on these truncations, we were able to identify a minimal 14 amino acids sequence that was sufficient to direct Golgi localization (Figure 3D). However, further truncating the altORF peptide to the first 9 amino acids resulted in a loss of Golgi localization (Figure 3D). Based on this result, we hypothesized that the Cys11 residue that is present in the altORF14aa mutant, but lost in the altORF9aa may play an important functional role in Golgi localization. To test this, we generated an altORF mutant where both Cysteine residues are mutated to Alanine, referred to as altORFC11A, C32A. The altORFC11A, C32A mutant failed to localize the Golgi (Figure 3E), suggesting that these amino acids play a critical role in the association of this peptide to he Golgi. Additionally, this supports the notion that post-translational modifications may contribute the localization behavior of this peptide. Together, our work identifies a short peptide sequence that is sufficient to localize to the cytoplasmic surface of the Golgi, likely through lipid modifications, providing a valuable tool for live-cell imaging.

Figure 1: A CENP-R AltORF localizes robustly to the Golgi.



A. Visual representation of altORF translation from the canonical CENP-R transcript. The altORF begins upstream of the canonical ATG and has a different reading from the canonical protein.
B. Localization of CENP-R altORF during interphase and mitosis as compared to the canonical CENP-R after transfection of GFP tagged constructs in HeLa cells. The CENP-R altORF shows distinct localization compared to that of canonical CENP-R, which localizes to centromeres. GFP boost was used to amplify GFP signal and kinetochore are stained with ACA. Images in this figure are deconvolved. Scale bars, 10 µm.
C. The CENP-R altORF co-localizes with the Golgi markers GM130 and TGN46, but not with the Endoplasmic reticulum marker anti-KDEL. Images were deconvolved. Scale bar, 10 µm. Inset is 5 µm.
D. The CENP-R altORF localizes to the Golgi in a variety of cell lines, including mouse fibroblast 3T3 cells. The CENP-R altORF was transiently transfected into each cell lines and assessed for colocalization with Golgi marker GM130, except for mouse fibroblast as the human GM130 antibody did localize in mouse cells. Images are deconvolved. Scale bar, 10 µm.
E. The altORF peptide localizes to the Golgi whether it is tagged at the N- or C- terminus. Constructs were transiently transfected into HeLa cells and assessed for colocalization with Golgi marker GM130. Images are deconvolved. Scale bar, 10 µm.

Figure 2: The CENP-R altORF marks the Golgi throughout dynamic processes



A. Images from a time-lapse sequence in a cell line stably expressing the CENP-R altORF. DNA was stained using sIR-DNA. Cells were imaged for 8 hours in 5-minute intervals at 37°C in CO2 independent media. The time-lapse was deconvolved. Scale bar, 10 µm.
B. Images from time-lapse sequence of a cell line stably expressing the CENP-R altORF following treatment with 0.2 µM Brefeldin A. DNA was stained with sIR-DNA. Cells were imaged immediately after the addition of Brefeldin A for 4 hours, imaged every 5 minutes. Selected frames shows the breakdown of the Golgi and the association of the altORF to the Golgi throughout this process. Scale bar, 10 µm.

Discussion

This work identifies a small, 37 aa peptide from the CENP-R transcript that robustly localizes to the Golgi body. This small peptide, when tagged with a fluorescent protein, can be used to image the Golgi by either transient transfection or stable expression. The localization of this peptide is Golgi specific, as it co-localizes with established markers of the Golgi, but not of the ER. The altORF can also be used in a variety of cell lines, including mouse fibroblasts, for both fixed and live imaging of the Golgi. This easy-to-use Golgi labeling construct will be made available on Addgene, which we hope will provide a valuable tool for researchers conducting studies of Golgi localization and dynamics in addition to the existing Golgi labeling peptides [15-17].

In addition to providing a valuable fixed and live cell Golgi marker, the behavior of the altORF peptide raises a number of interesting open questions. Our goal is to address these questions through ongoing work, with the anticipation of updating this pre-printed work when additional data is available. The altORF peptide originates from an mRNA whose primary defined open reading frame is not canonically-associated with the Golgi. Despite this, the alternative ORF peptide localizes robustly to this organelle raising questions regarding the basis for

the localization of this peptide to the Golgi, the biology that can be revealed by this peptide, and the extent to which this can be used as a tool to study the Golgi body.

In this updated pre-print, we have determined that the altORF peptide likely localizes to the Golgi as a peripheral membrane protein. We propose that this is mediated by a post-translational modification that targets the cysteine residues in this peptide (Figure 3 D, E). We are currently working to determine what modifications may be responsible for this. We hypothesize that this modification is likely palmitoylation, given the absence of consensus motifs for myristoylation or prenylation, modifications that are also common for Golgi localization [11, 18]. Additionally, we are currently working to identify altORF peptide interacting proteins through immunoprecipitation-mass spectrometry (IP-MS) analysis. Knowing what proteins this peptide interacts with may provide information on how it is localized to the Golgi and also what region of the Golgi this peptide resides. We also welcome your feedback and suggestions for additional experiments to try.

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Methods:

Cell Culture, cell transfection, and cell line generation

HeLa, U2Os, A549, and 3T3 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (GE Healthcare), 100 U/ml penicillin and streptomycin, and 2 mM L-glutamine at 37C with 5% CO2. Stable cell line expressing altORF was generated by retroviral infection followed selection by Blasticidin at 2 ug/mL and single cell sorting to generate a clonal cell line. Transfection was performed with Xtremegene-9.

Immunofluorescence and Microscopy

Cells were plated on glass coverslips coated with poly-L-lysine (Sigma-Aldrich). Cells were fixed with 4% formaldehyde in PBS for 10 min at room temperature or with Methanol for 5 minutes at -20C. Blocking and all antibody dilutions were performed using Abdil (20 mM Tris, 150 mM NaCl, 0.1% Triton X-100, 3% BSA and 0.1% NaN3, pH 7.5). PBS plus 0.1% Triton X-100 (PBS-TX) was used for washes. GFP-Booster from Chromotek was used to amplify the fluorescence of the GFP-tagged transgenes at a dilution of 1:200. Microtubules were stained with DM1A antibody from Sigma-Aldrich at a dilution of 1:3000. For staining of Golgi for co-localization experiments GM130 antibody from Cell Signaling Technologies (#12480S) at a dilution of 1:3200 and TGN146 antibody from Abcam (ab50595) at a dilution of 1:500. For staining of the Endoplasmic Reticulum KDEL antibody from abcam (ab176333) was used at a dilution of 1:100. Cy3- and Cy5-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used at a 1:200 dilution in PBS plus 0.1% Triton X-100. DNA was visualized by incubating cells in 1 ug/ml Hoechst33342 (Sigma-Aldrich) in PBS plus 0.1% Triton X-100 for 10 min. Coverslips were mounted using PPDM (0.5% p-phenylenediamine and 20 mM Tris-Cl, pH 8.8, in 90% glycerol) and sealed with nail polish. For live-cell imaging, cells were seeded into 8-well glass-bottomed chambers (Ibidi) and moved into CO2-independent media (LifeTech) before imaging at 37°C. For certain movies, DNA was stained with sirDNA (Cytoskeleton Inc) at 0.2 µM. Cells were treated with 0.2uM Brefeldin A (Sigma B5936) diluted in DMSO; control cells were treated with DMSO. Images were acquired on a DeltaVision Core deconvolution microscope (Applied Precision) equipped with a CoolSnap HQ2 charge-coupled device camera (Photometrics). Images were maximally projected and deconvolved where noted.

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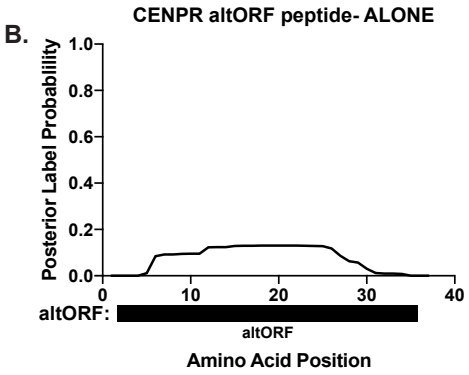
Figure 3: Sequence requirements for CENPR altORF Golgi localization

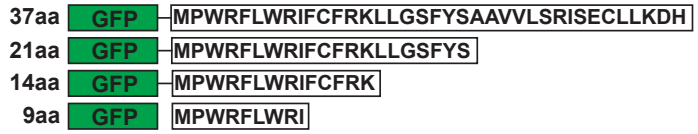
A.

	Sequence
Hyrdophobic aa	MPWRFLWRIFCFRKLLGSFYSAAVVLSRISECLLKDH
Aromatic aa	MPWRFLWRIFCFRKLLGSFYSAAVVLSRISECLLKDH
Cysteine PTM	MPWRFLWRIFCFRKLLGSFYSAAVVLSRISECLLKDH

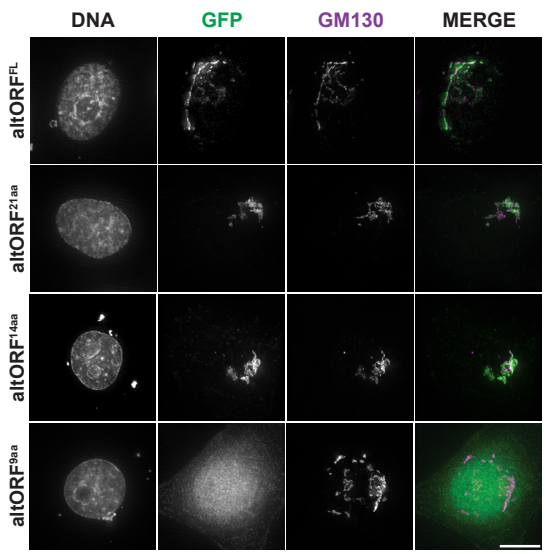
B.



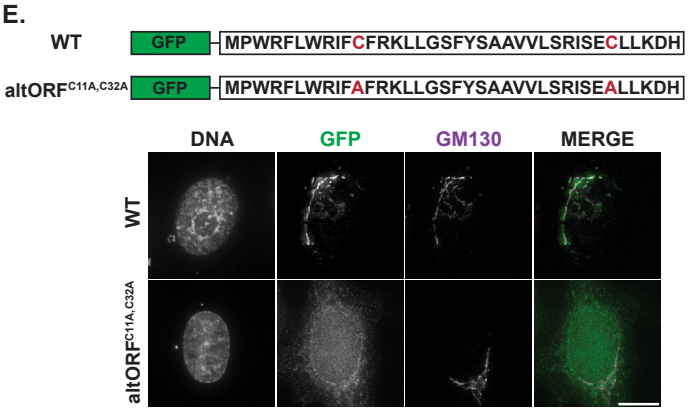
C.



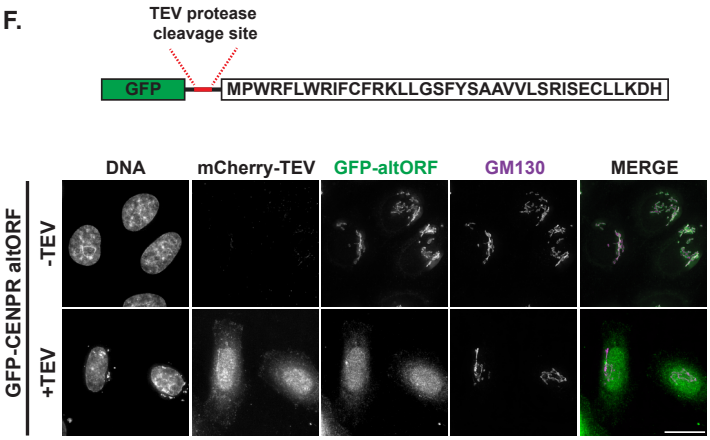
D.



E.



F.



A. Table representing the altORF peptide highlighting the amino acids belonging to each group of interest.
B. Hydrophobicity plot of the CENPR altORF peptide sequence. Sequence was run through the Phobius program (https://phobius.sbc.su.se/poly.html), probability values assigned to amino acids to determine transmembrane topology are plotted.
C. Figures representing the truncation mutants generated and tested for localization.
D. Localization of the truncated altORF constructs transfected in HeLa cells. Localization to the Golgi was determined based on colocalization with Golgi marker GM130. Scale bar, 10 μm.

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