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A Golgi-localized peptide encoded by the CENP-R transcript provides a valuable tool for live cell imaging

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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 (Raykhel et al., 2007; Bear et. al., 2000; Kim & Hwang, 2013). 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 act as a valuable tool for cell biology experimentation to visualize the Golgi for both fixed and live cell analyses.

Results:

A CENPR altORF peptide localizes to the Golgi compartment

Alternative open reading frame (altORF) are sequences present in transcribed mRNAs that 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 (Orr, Mao, Storz, & Qian, 2020). 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 (Samandi et al. 2017). 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 (data not shown).

Based on this observed localization, we hypothesized that the altORF likely localizes to the Endoplasmic Reticulum (ER) or Golgi apparatus given its localization to the nuclear periphery. To test this, we sought to compare this 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 (Nakamura et. al., 1995; Kain et. al., 1998). 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

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peptide (Fig. 1C). Together, we conclude that this altORF peptide localizes to Golgi compartment in HeLa cells.

The CENPR altORF localizes to the Golgi in diverse human and mouse cell lines

We next sought to test whether the observed localization behavior for the CENPR altORF also occurred in other 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, where we observed a similar localization behavior (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 sought to observe the association of the altORF with the Golgi under contexts where Golgi morphology is dynamic. First, we visualized altORF localization as a readout of Golgi behavior throughout the cell cycle. 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 (Fujiwara et. al., 1988). As expected, 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 (Fig. 2B). Thus, the altORF localizes robustly to the Golgi and provides a live cell reporter for Golgi dynamics under diverse conditions.

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-localized constructs (Fulton et al. 2004; Kjer-Nielson et. al., 1999; Komatsu et al., 2010).

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 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. First, we are working to understand how this peptide is targeted to the Golgi by assessing the sequence requirements for Golgi localization. Unlike targeting/retention sequences for other organelles such as the ER, mitochondrion, or nucleus, there is no established consensus sequence requirement for Golgi localization. The current understanding of the Golgi-localizing domains indicates that hydrophobic and aromatic residues play an important role in Golgi protein localization (Banfield, 2011). We are probing the sequence requirements for the Golgi targeting of this altORF by performing alanine swap mutations as well as truncations of the sequence to identify residues and/or regions of the peptide responsible for its targeting to the Golgi. We also plan to identify which other proteins interact with this altORF through immunoprecipitation-mass spectrometry (IP-MS) analysis. Knowing which proteins this peptide interacts with will 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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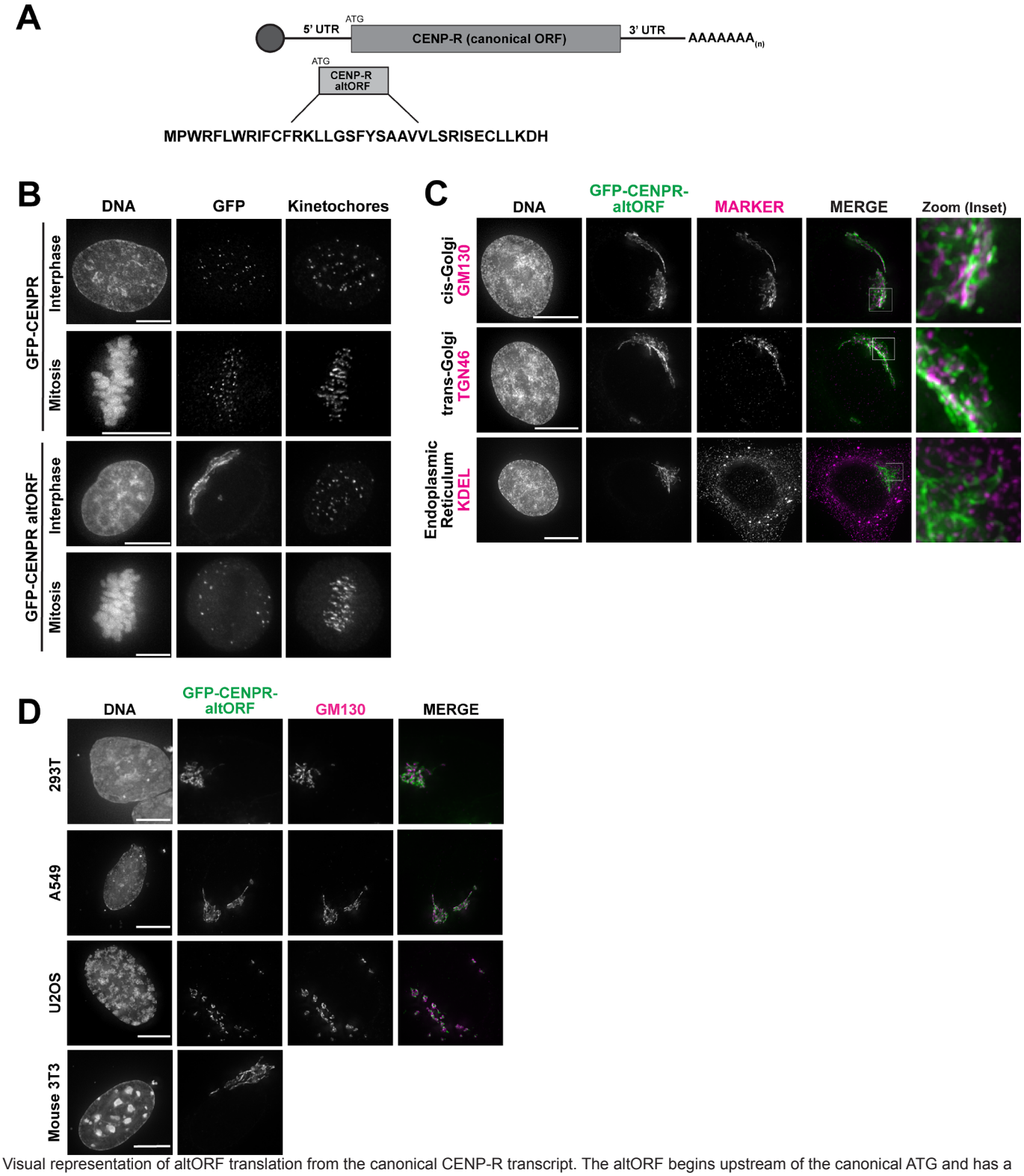
Methods:

Cell Culture, cell transfection, and cell line generation

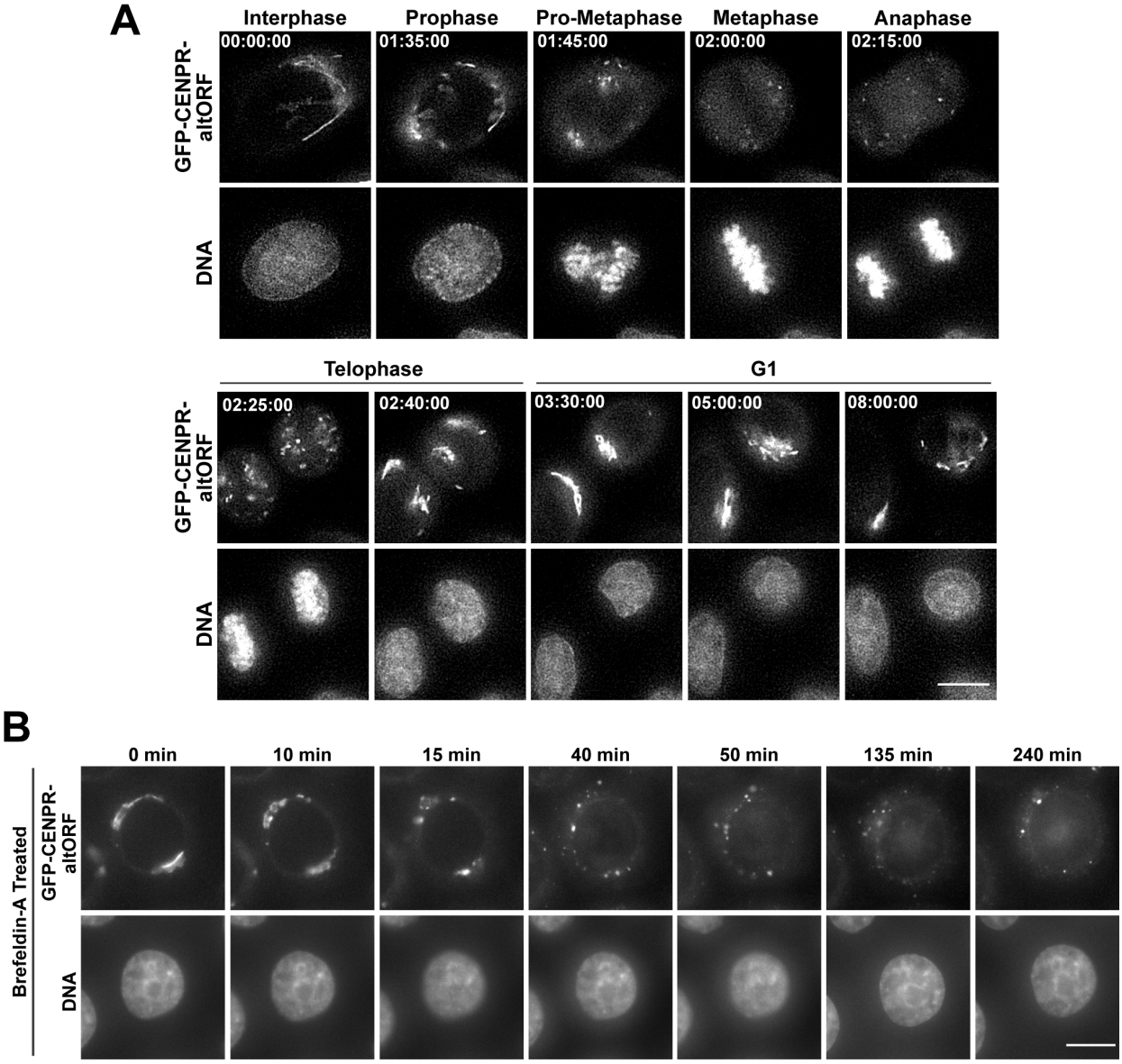
HeLa, U2Os, A549, and 3T3 cells were cultured in

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.

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.

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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