Title: A novel genetic fluorescent reporter to visualize mitochondrial nucleoids

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Abstract:

Mitochondria have their own genome (mtDNA), which is present in hundreds of copies per cell and organized into nucleoid structures that are distributed throughout the dynamic mitochondrial network. Beyond encoding essential protein subunits for oxidative phosphorylation, mtDNA can also serve as a signalling molecule when it is present into the cytosol. Despite the importance of this genome, there are still many unknowns with respect to its regulation. To study mtDNA dynamics in living cells, we have developed a genetic fluorescent reporter, mt-HI-NESS, which is based on the HI-NESS reporter that uses the bacterial H-NS DNA binding domain. Here, we describe how this reporter can be used to image mtDNA nucleoids for live cell imaging without affecting the replication or expression of the mtDNA. In addition to demonstrating the adaptability of the mt-HI-NESS reporter for multiple fluorescent proteins, we also emphasize important factors to consider during the optimization and application of this reporter.

Introduction

Mitochondria are endosymbiotic organelles that harbour their own genome (mtDNA), evidence of their bacterial origins. In humans, mtDNA is ~16.5 kbp and is located in the mitochondrial matrix; it encodes 2 ribosomal RNAs, 22 tRNAs, and 13 proteins that are essential components of oxidative phosphorylation complexes [1]. Unlike the nuclear genome, mtDNA is circular and is present in hundreds of copies per cell. Although the mtDNA copy number varies in different cell types, the mechanisms regulating this variability are not fully understood. The packaging, maintenance, and expression of mtDNA is carried out by a dedicated set of proteins that are encoded in the nucleus, translated in the cytosol, and imported into mitochondria. The mtDNA is packaged into nucleoid structures, in large part by the mtDNA binding protein TFAM [2], which also acts as a transcription factor [3].

Nucleoids typically contain ~1.4 mtDNA molecules [4] and are distributed evenly throughout the dynamic mitochondrial network. Impairments in mitochondrial fission and fusion can impact both mtDNA copy number, and the distribution of nucleoids [5–8]. Fission events are implicated in licensing mtDNA replication [9,10], while fusion is thought to be important for an even distribution of the machinery required for mtDNA replication and maintenance [11]. Meanwhile, impairments to either fission [12–15] or fusion [11,16,17] can also lead to clustering of nucleoids into larger aggregates. Further, reductions in mitochondrial fusion can also lead to individual mitochondrial that lack nucleoids entirely [18]. More recently, it is recognized that mtDNA can be released into the cytosol, where it can act as a signaling molecule that triggers innate immune inflammatory pathways [19].

The direct visualization of mtDNA distribution and dynamics in living cells is essential for decoding their function. Several tools are available to image mtDNA nucleoids, including immunofluorescence, DNA dyes, and fluorescent protein tags [20]. However, these tools are limited in their applicability for live-cell imaging. For example, immunofluorescence, against either DNA or nucleoid proteins requires fixation, and can thus only provide only a snapshot of mtDNA dynamics. Meanwhile, DNA specific dyes, such as PicoGreen [21,22] or SYBR Gold [23,24] can label mtDNA for live cell imaging. However, these dyes are not specific for mtDNA as they will bind any DNA in the cell, and can sometimes bind RNA. Moreover, due to their DNA intercalating activity, these dyes can impair mtDNA replication and transcription [20]. Finally, the binding affinity of these dyes can be impacted by the degree of mtDNA packaging, which may prevent intercalation [25]. On the other hand, when considering genetic constructs to label nucleoids, selectivity is a barrier with respect to using fluorescently tagged proteins. Although several nucleoid proteins have been identified, they are not all exclusive to nucleoids (*e.g.*, ATAD3A [25]) or do not label all nucleoids (*e.g.*, TFAM [26], TWINKLE [27], mtSSB [27]). Moreover, overexpression

of nucleoid proteins can impact mtDNA regulation (*e.g.*, TFAM [28,29], TWINKLE [30,31], M19 [32]). Thus, using dyes or overexpressing nucleoid proteins tagged with a fluorescent protein can have adverse effects on mtDNA that preclude observing nucleoids in their natural state.

To avoid nucleoid protein overexpression artifacts, it may be possible to introduce a fluorescent protein tag into a particular gene via CRISPR technology, enabling endogenous expression of the tagged nucleoid protein. However, this complex approach is limited in its applicability. It is also possible that adding a tag could impact the function of the tagged nucleoid protein. For example, many fluorescent proteins tend to oligomerize [33], which is known to introduce artefacts [34]. Meanwhile, tagging TFAM, the most well-characterized nucleoid protein may be problematic, as it is targeted to mitochondria via an N-terminal mitochondrial targeting sequence (MTS), as are many proteins that localize to the mitochondrial matrix. Finally, TFAM also has a critical C-terminal region required for its transcription stimulating activity [35]. Thus, better tools are required to study mtDNA dynamics.

With the goal of developing a non-invasive genetic fluorescent marker to visualize mtDNA nucleoids for live-cell imaging, we have adapted the recently described HI-NESS genetic reporter [36], which fuses a fluorescent protein to the bacterial H-NS DNA binding domain, and labels nuclear DNA without any reported adverse effects [37]. Notably, H-NS-like proteins have a preference for AT-rich sequences [38,39], which should make them ideal for the AT-rich human mtDNA. Despite the bacterial history of mitochondria, the core machinery for their transcription and replication does not resemble typical bacterial machinery, but instead is derived from T-odd phages [40], and has acquired additional unique regulatory factors [41–43]. Therefore, a HI-NESS reporter is not expected to impact mitochondrial DNA replication or transcription by interacting with this mitochondrial machinery. Thus, as a novel approach to monitor mtDNA nucleoid dynamics, we designed and tested several modified versions of the HI-NESS reporter targeted to mitochondria.

Results

Construct Design – mt-TagRFP-HI-NESS

As with the original HI-NESS construct [36], we started with the DNA binding domain of the *E. coli* H-NS protein (amino acids 80-137; GenBank: EIS4692233.1), which we fused to the C-terminus of a fluorescent protein. We chose to start with the TagRFP fluorescent protein derived *from Entacmaea quadricolor* [44] (GenBank: ABR08320.1), which is established to function in the environment of the mitochondrial matrix [45]. To facilitate immunodetection of the reporter, we added an HA epitope tag

(YPYDVPDYA) to the C-terminus of the H-NS DNA binding domain, which has previously been shown not to interfere with DNA binding of the H-NS protein [46]. Next, a double Cox8 MTS [47], shown to improve the mitochondrial import of fluorescent proteins [48], was added to N-terminus of the construct to target the reporter to mitochondria. The MTS is followed by an unstructured linker sequence (GDPPVAT) used previously for fluorescent proteins localized to the mitochondrial matrix [49].

The organization of this initial open reading frame, which we refer to as mt-TagRFP-HI-NESS, is shown in Figure 1, along with a predicted 3D model generated using AlphaFold 2 [50]. The nucleotide and protein sequences for this reporter can be found in the supplemental material (Fig S1). While the full-length protein is predicted at 40.6 kDA, the MTS is often cleaved and processed by a series of mitochondrial proteases [51]. Using existing programs available to predict the MTS cleavage site, the mt-TagRFP-HI-NESS protein is predicted to be cleaved either after residue L43 (MitoFates [52]) or K56 (TargetP2.0 [53]), resulting a mature protein predicted of 34.7 kDa or 36 kDa, respectively.

VectorBuilder (Chicago, IL, USA) was commissioned to synthesize the mt-HI-NESS-TagRFP open reading frame and insert it into a lentiviral mammalian gene expression vector. The lentivirus vector expresses the mt-TagRFP-HI-NESS open reading frame under an hPGK promoter and contains puromycin and ampicillin selection markers. The vector ID for mt-TagRFP-HI-NESS is VB221222-1228fqm, which can be used to retrieve detailed information about the vector of vectorbuilder.com. A vector map (Fig S2) and the DNA sequence (Fig S3) is provided in the supplemental material.

Characterization of mt-TagRFP-HI-NESS

We generated lentivirus that was used to transduce U2OS cells for stable expression of mt-Tag-RFP-HI-NESS. Flow cytometry was used to select cells expressing TagRFP, and these cells were imaged using live-cell confocal microscopy. Notably, the expression level of TagRFP was variable among different cells (Fig 2A). Nonetheless, we consistently observed TagRFP signal localizing to punctate spots that were confirmed to be nucleoids through co-staining with SYBR gold to label mtDNA (Fig 2B), or MitoTracker Green to label the mitochondrial network (Fig 2C). While this initial imaging suggested proof of principal that mt-TagRFP-HI-NESS could be used for live cell imaging of nucleoids, we also noted that in a significant number of cells there was non-nucleoid specific TagRFP signal that was distributed throughout the mitochondrial network. Notably, through single cell cloning, we were able to isolate cell lines mitochondrial nucleoid-specific TagRFP signal that lacked any non-specific mitochondrial network signal (Fig 3A,B). To test if the non-nucleoid mitochondrial TagRFP signal was due to excess mt-Tag-RFP-HI-NESS protein expression, essentially swamping out the available DNA binding sites, we used flow

cytometry to sort cells based on levels of TagRFP expression into four bins: low, medium-low, medium-high, and high (Fig 4A & S4A). Confocal imaging across all expression levels again showed some cells with underlying TagRFP signal that is not specific for nucleoids though it was specific for the mitochondrial network (Fig S4B), suggesting this non-nucleoid localization was not an overexpression artefact.

Next, we tested if mt-TagRFP-HI-NESS expression impacted mtDNA maintenance and expression. To this end, we took advantage of our cell populations sorted by TagRFP expression. Western blotting with anti-HA confirmed the variable protein expression in our cell populations, and revealed no changes in expression of VDAC1 or HSP60 mitochondrial proteins often used to measure mitochondrial abundance (Fig 4A). In addition, the expression of protein subunits for all five oxidative phosphorylation complexes (CI-CV), including mtDNA-encoded MTCOI, was unaltered. When we proceeded to look at mtDNA copy number by quantitative PCR we saw no significant differences in most lines (Fig 4B). However, there was a significant reduction (~30%) in mtDNA copy number in cells expressing the highest levels of mt-TagRFP-HI-NESS, which suggests caution is warranted when the reporter is highly expressed. Finally, we examined steady-state levels of mtDNA-encoded transcripts via q-RT-PCR (Fig 4C). While we did not see any significant differences in transcript levels, it is notable that the results were variable, which may reflect a technical issue with this approach.

Double HI-NESS

Given that sorting by TagRFP expression level did not mitigate the non-specific mitochondrial labelling in some cells, we reasoned that the mt-TagRFP-HI-NESS protein may not be binding to mtDNA efficiently. Thus, we set out to modify our original construct by adding a second H-NS DNA binding domain to increase binding efficiency. We considered two possibilities (Fig 5A,B). The first option was adding a second H-NS domain immediately after the K56 cleavage site in the MTS, such that one H-NS domain was at the N-terminus and the second was at the C-terminus (N/C). The second option was to add a second H-NS domain at the C-terminus, immediately after the HA tag, such that both H-NS domains were at the C-terminus (C/C). Ultimately, we decided to test the N/C double HI-NESS construct. To avoid repetitive sequences in the open reading frame that might have adverse effects on plasmid and/or viral propagation, we took advantage of the degeneracy in codon usage to change the nucleotide sequence without changing the encoded protein domain. To this end, the codon sequence for the N-terminal H-NS domain was 'humanized' by choosing codons more commonly used in the human genome. This modified reporter, mt-TagRFP-N/C-HI-NESS, has a predicted full-length size of 47 kDa, and a predicted processed size of 43.3 kDa (MitoFates) or 41 kDa (TargetP 2.0). The vector ID for mt-TagRFP-

N/C-HI-NESS is VB230529-1292kdj, which can be used to retrieve detailed information about the vector of vectorbuilder.com. A vector map and the DNA sequence is provided in the supplemental material (Fig S2&3). When we tested the mt-TagRFP-N/C-HI-NESS reporter expressed in U2OS following stable viral transduction, we observed a significant improvement in nucleoid-specific staining, with much less non-specific mitochondrial localization (Fig 5C).

Mt-Kaede-HI-NESS

Given the initial success of the mt-TagRFP-HI-NESS reporter in labelling mtDNA nucleoids, we wanted to take advantage of the palette of available fluorescent proteins available for live-cell imaging, including photoconvertible properties that can be taken advantage of to better understand biological processes. To this end, we started with Kaede, a photoconvertible fluorescent protein derived from *Trachyphyllia geoffroyi*, which when activated by UV or violet light, permanently switches its fluorescence emission from red to green [54]. By replacing the TagRFP domain with Kaede, in our original mt-TagRFP-HI-NESS construct, we generated a mt-Kaede-HI-NESS reporter with a predicted full-length size of 40.2 kDa, and a predicted processed size of 35.6 kDa (MitoFates) or 34.2 kDa (TargetP 2.0) (Fig 6A,B). The vector ID for mt-Kaede-HI-NESS is VB230322-1526htn, which can be used to retrieve detailed information about the vector of vectorbuilder.com. A vector map and the DNA sequence is provided in the supplemental material (Fig S2&3).

Initial imaging with U2OS cells stably transduced to express mt-Kaede-HI-NESS revealed specific punctate signal within the mitochondrial network, as imaged with MitoTracker Deep Red (Fig 6C). Remarkably, we observed only puncta with the mt-Kaede-HI-NESS reporter, without any of the mitochondrial network labelling sometimes seen with the original mt-mTagRFP-HI-NESS reporter. This observation suggests that the TagRFP protein properties hinder the DNA binding capacity of the H-NS domain. Highlighting the utility of the mt-Kaede-HI-NESS reporter, we were able to successfully photoconvert a subset of nucleoids and follow these nucleoids over time with live cell imaging (Fig 6D).

Discussion

Building on the original HI-NESS genetic reporter for labeling DNA [36], we generated a modified mt-HI-NESS reporter that can be used for live cell imaging of mtDNA. Importantly, we show that when this construct is expressed at lower levels, it does not impair mtDNA replication or transcription, and thus will be a valuable tool to study mtDNA via live cell imaging. In addition, using the photoconvertible Kaede fluorescent protein, we show that the

fluorescent protein domain can be swapped in the mt-HI-NESS reporter to make use of fluorescent proteins with different properties.

While the first iteration of the mt-mTagRFP-HI-NESS reporter was able to label mtDNA nucleoids, some unexpected non-nucleoid labelling of the mitochondrial network was observed. We demonstrated that the non-nucleoid signal was not due to overexpression, as it was also observed in cells expressing low levels of mt-TagRFP-HI-NESS. We suggest that the non-nucleoid signal is a result of poor DNA binding, which is reminiscent of the observations with the original HI-NESS reporter when expressed in certain bacteria [36]. In this work, when the HI-NESS reporter was expressed in bacterial cells expressing endogenous H-NS, the HI-NESS reporter stained the bacterial cytosol, likely because it was outcompeted by the full-length H-NS protein.

Despite the non-nucleoid signal in some cells, it is still possible to visualize mtDNA in live cells. Moreover, it is possible to identify cells that have specific nucleoid labelling. Although why more specific labelling was observed in only certain cells within the same samples remains unclear. One way to circumvent this limitation is to only study cells without non-nucleoid staining. To this end, we showed that it is possible to select for cells that have specific mt-TagRFP-HI-NESS signal that colocalizes more strongly with mtDNA. Nonetheless, to improve the efficacy of nucleoid labelling, we generated a modified reporter with a second H-NS DNA binding domain, mt-TagRFP-N/C-HI-NESS, which has superior mtDNA specificity, and we recommend using this construct for imaging mtDNA.

The fact that the non-specific mitochondrial labelling was only observed with the TagRFP fluorescent protein, and not the Kaede fluorescent protein was surprising. This observation suggests that there is something inherently different between the structures of these fluorescent proteins that impacts the ability of the H-NS domain to bind DNA. With respect to differences between the TagRFP and Kaede fluorescent proteins, they are 48% identical and 63.6% similar at the amino acid level. Notably, compared to Kaede, TagRFP has some amino acid insertions at the C-terminus (Fig S5), which is adjacent to the H-NS DNA binding domain. In this regard, it is also worth noting that the original HI-NESS DNA reporter used the mEOS3.2 fluorescent protein, which is more similar to the Kaede fluorescent protein

at the C-terminus. It is also notable that TagRFP and Kaede have different tendencies to form oligomers, with TagRFP modified to reduce oligomerization [44], while Kaede is known to form tetramers [54]. Meanwhile, though mEos3.2 is listed as monomeric, it is notable that even monomeric fluorescent proteins can form oligomers under certain conditions [55]. Regardless of the exact underlying mechanism for the non-specific nucleoid signal, this observation highlights the importance of carefully testing modified mt-HI-NESS reporters when different fluorescent proteins are substituted. If non-specific signal is observed for a desired fluorescent protein, we would recommend testing it with the double N/C HI-NESS reporter backbone.

When using the mt-HI-NESS reporters, it will be important to consider the specific fluorescent protein used, as well as to consider the possible effects of overexpression. To this end, experimental conditions may need to be optimized and determined for each reporter in each cell line. Nonetheless, we propose that the novel mt-HI-NESS fluorescent proteins developed and described here will be valuable tools to study the dynamics of mtDNA nucleoids in live cell imaging experiments. These reporters will allow researchers to address questions about how nucleoids are distributed throughout the mitochondrial network, how mtDNA replication is coordinated with mitochondrial fission and fusion events, and to study release of mtDNA into the cytosol or its endosomal/vesicle-mediated transport to autophagosomes for degradation [56–58].

Methods

Cell culture

HEK-293 and U2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, 11965092) supplemented with 10% fetal bovine serum (FBS) (Fisher, 12483020). All cells were maintained at 37 °C and 5% CO2 incubator.

Protein Structure Modeling

To model the predicted structures of our novel chimeric mt-HI-NESS proteins, we used Alphafold 2 [59,60] (https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/beta/AlphaFold2_advanced.ipynb), and the 3D structures were visualized using Chimera software [61]. To model predicted interactions between our mt-HI-NESS proteins and mitochondrial DNA (mtDNA) we used the online application HADDOCK (YYY) [62]. The 3D co-ordinates of the mtDNA mitochondrial light strand promotor sequence (5'TTAACAGTCACCCCCCAACTAA3') were obtained from a previously reported crystal structure [63]. A range of active site residues YSYVDENGETKTWTGQGRTP was provided to the server based on previous reports [64] and all other parameters were set to default settings within the HADDOCK web application (https://wenmr.science.uu.nl/haddock2.4/). The complex with the highest binding energy score was used to generate the figures, which were visualized using the Chimera software.

HI-NESS plasmids

The mt-HI-NESS reporters were synthesized by VectorBuilder (Chicago, IL, USA) and cloned into a mammalian gene expression lentiviral vector following the hPGK Promoter. The vector contains ampicillin and puromycin resistance markers. Vector maps and DNA sequences are provided in the supplemental material, and the vector IDs, which can be used to retrieve detailed information about the vector of vectorbuilder.com are as follows:

mt-TagRFP-HI-NESS	VB221222-1228fqm
mt-TagRFP-N/C-HI-NESS	VB230529-1292kdj
mt-Kaede-HI-NESS	VB230322-1526htn

Lentivirus Production

HEK 293T were cells seeded into 100 mm dishes and incubated until they reached approximately 80% confluency. The plasmids containing mt-HI-NESS constructs (2µg) were transfected into HEK cells along with the packaging (psPAX2; 1.5µg) and envelope (pMD2.G; 0.5 µg) plasmids using either FuGENE 6 or Lipofectamine 3000 transfection reagent (both from Invitrogen, Thermo Fisher Scientific, Burlington, Ontario, Canada) according to the

manufacturer protocol. The medium was changed after cells were incubated at 37 °C and 5% CO2 for about 16 hrs. The culture medium containing lentivirus particles were collected at 48-96 hrs post-transfection.

Generation of stable cell lines

U2OS cells were seeded in a 6-well plate (50,000 cells/well) and cultured to reach 70% confluency (1-2 days). Fresh medium containing 8 µg/mL polybrene was added to cells, along with 100-500 µL of media containing lentivirus particles, for a total of 2 mL. Infected cells were incubated at 37 °C and 5% CO2 overnight at which point fresh culture medium without polybrene was added. After 24 hrs, the was media replaced again, this time containing puromycin (2 µg/mL) to select for stably transduced cells. Cells were grown for two weeks of selection, with puromycin-containing media changed every 2-3 days. To establish a genetically homogeneous population of cells, individual cells were obtained by serial dilution and single colonies picked.

Flow cytometry

Cells were sorted into four groups based on expression level of mTagRFP using a Sony SH800 cell sorter. Briefly, trypsinized, washed cells were suspended at a concentration of 1×10^6 cells/mL in the growth medium and filtered using a 40 µm cell strainer (VWR: 10199-655). Cells were then sorted into 15 mL collection tubes using a sterile 100 µm chip (Sony: LE-C3210).

Sample preparation for live-cell imaging

Unless otherwise indicated, all reagents and cell culture media components were obtained from ThermoFisher Scientific (Markham, Ontario, Canada). Transduced cells expressing different variants of the HI-NESS constructs were cultured in 35 mm dishes with a 1.5 glass coverslip bottom (Cellvis, D35-20-1.5-N). Imaging was performed either 1 or 2 days after seeding the cells in the dishes. For some experiments, an additional extrinsic fluorescent label was also applied to the cells (*e.g.*, SYBR Gold (S11494), MitoTracker Green FM (M7514) or

MitoTracker Deep Red FM (M22426)). Stock solutions of each probe were prepared in sterile anhydrous DMSO (D12345) (stock concentrations: SYBR Gold 1:100; MitoTracker Green 1 mM; MitoTracker Deep Red 100 uM). To label the cells, media was aspirated, and fresh and pre-warmed media containing 1:1000 dilutions of SYBR Gold, MitoTracker Green, or MitoTracker Deep Red stock solutions were added. Cells were incubated for 1 hr, washed once with sterile PBS, and fresh media was then added to the wells. Cells were then imaged on an LCI system stage (Quorum Technologies, Guelph, Ontario, Canada), where temperature, humidity, and pH (5% CO2) were maintained.

Confocal live-cell imaging

Confocal images were acquired using a Nikon A1R system equipped with a Ti2 motorized microscope and Elements acquisition software version 5.42.01 and 5.42.03 (Nikon Canada, Mississauga, Ontario, Canada). The confocal system was equipped with lasers emitting at 405 nm (Ch1), 488 nm (Ch2), 562 nm (Ch3), and 639 nm (Ch4), and with standard PMTs (Ch1, Ch4) and GaAsP (Ch2, Ch3) detectors. Imaging was performed using a Plan Apo 60x 1.4 NA oil immersion lens. Spectral separation was effected using a primary dichroic (405/488/561/640) and additional filter cubes with center transmission wavelength and FWHM bandpass as follows (in nm): Ch1 452/45; 525/50; 595/50. One or more channels were selected, depending on the fluorophores used in the imaging experiment. Galvo-galvo scanning (unidirectional, 1.1 µs dwell time, sequential, laser switching per line) was used to record z-stacks at single time points. Lateral (x,y) sampling ranged from 0.1 to 0.3 mm, while axial sampling (z) ranged from 0.125 to 0.3 µm. The confocal pinhole diameters ranged from approximately 28 to 40 µm.
For imaging the photoconvertible Kaede variant, cells were labelled with MitoTracker Deep Red FM. Sequential images were acquired from Ch2 (green emission) and Ch3 (red emission) to monitor the shift in fluorescence emission green to red after activation with the 405 nm laser. The MitoTracker Deep Red FM fluorescence was monitored using Ch4 (far-red emission). Sequential Images were acquired 10 s for 2 min as a baseline. At 2 min, 405 nm laser was swept over a defined sub-cellular region-of-interest for 2 s. After the photostimulation pulse, images were acquired as for the baseline for up to 30 min. The 405 nm laser power was measured

using the similar illumination conditions using a S170C sensor and PM100D power meter console (Thorlabs, NJ USA). A relative power of 5%, corresponding to 6.5 µW, was used to photoconvert the Kaede fusion protein. The laser exposure was about 6.5 µW x 2000 µs, for a total power of 13 µJ delivered to the region-of-interest (ROI). The photostimulated ROI had an area of 3.8 μm^2, corresponding to a power density of 340 $\mu J/cm^2$.

Western blotting

Cell pellets (~2,000,000 cells) were collected after trypsinization, washed with phosphate buffered saline (PBS) and lysed with RIPA buffer (ThermoFisherScientific, 89900) containing protease inhibitors. The cell lysates (20-50 µg of proteins) were resolved on SDS-PAGE gels (12%) and transferred onto PVDF Membranes in 25mM Tris, 192mM glycine and 20% (vol/vol) methanol at 30V overnight at 4°C. After blocking with 5% nonfat milk in TBST (25mM Tris, pH7.4, 150mM NaCl , 0.05% Tween-20), the blots were washed and incubated at RT for 2-3hrs with the following primary antibodies at the indicated dilutions; OxPhos antibody cocktail (Abcam, ab110411; 1:1000 dilution), anti-Actin (Sigma, A5316; 1:1000), anti-HSP60 (Cell Signaling Technology; 1:1000), anti-VDAC1 (Abcam, ab14734; 1:1000), anti-HA tag (Santa Cruz Biotechnology, sc-7392; 1:1000). Appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies were used as follows: goat anti-rabbit IgG HRP linked Antibody (Cell Signaling Technology, 7074S, 1:2000), or goat anti-mouse IgG HRP (Santa Cruz Biotechnology, sc-2055;1:2000). After washing with TBST (4x) the blots were developed with SuperSignal West Femto enhanced chemiluminescence substrate (Pierce Chemical, Rockford, IL). The signal was detected and quantified with a chemiluminescence imaging analyzer (LAS3000 mini, Fujifilm).

Quantification of mtDNA copy number

Total DNA was isolated from cell pellets (~1,500,000 cells) using the E.Z.N.A. Tissue DNA Kit, Omega Bio-tek (VWR Scientific, D6905-03), according to the manufacturer's protocol. Isolated DNA (50-100 ng) was used for quantitative PCR (QPCR) to determine mtDNA copy number, as reported previously [16]. Primers used are as bellow: MT forward: CACCCAAGAACAGGGTTTGT; Reverse: TGGCCATGGGTATGTTGTTAA; 18S forward:

TAGAGGGACAAGTGGCGTTC reverse:CGCTGAGCCAGTCAGTGT. Briefly, mtDNA and 18S DNA were amplified using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, A25742) in the QuantStudio 6 Flex Real-Time PCR system (Thermo Fisher Scientific) machine, and the delta delta Ct method was used to determine mtDNA copy number relative to the 18S. Reactions were performed in triplicate and mtDNA copy number analysis was performed on six independent biological replicates.

Quantification of mitochondrial RNA transcripts

RNA was isolated from cell pellets (~150,000 cells) using the E.Z.N.A. HP Total RNA Kit, Omega Bio-tek (VWR Scientific, CA101414-850), according to the manufacturer's protocol. Following isolation, cDNA was generated from 7.5 μg of RNA using the iScript™ Advanced cDNA Synthesis Kit (Biorad, 1725038), and 20-30 ng of cDNA was then used for quantitative PCR with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, A25742) in the QuantStudio 6 Flex Real-Time PCR system (Thermo Fisher Scientific) machine. The delta delta Ct method was used to determine the relative expression of various transcripts relative to actin using the primers indicated below:

COX I	Forward:	GACGTAGACACACGAGCATATTTCA
COX1	Reverse:	AGGACATAGTGGAAGTGAGCTACAAC
ND1	Forward:	AAGTCACCCTAGCCATCATTCTAC
ND1	Reverse	GCAGGAGTAATCAGAGGTGTTCTT
ATP6	Forward:	TAGCCATACACAACACTAAAGGACGA
ATP6	Reverse:	GGGCATTTTTAATCTTAGAGCGAAA
12S	Forward:	TAGAGGAGCCTGTTCTGTAATCGAT
12S	Reverse:	CGACCCTTAAGTTTCATAAGGGCTA
Actin	Forward:	AAGACCTGTACGCCAACACA
Actin	Reverse:	AGTACTTGCGCTCAGGAGGA

Reactions were performed in triplicate and three independent sample groups were tested. Data is presented as mean ± SD and unpaired, 2-tailed Student's t-tests were used to determine statistical significance.

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Conflict of interest statement:

The authors declare that they have no conflict of interest.

Author contributions:

JD designed, performed, and analyzed experiments, and prepared figures. LS designed, performed, and analyzed experiments, prepared figures, and edited the manuscript. FS performed and analyzed experiments, prepared figures, and edited the manuscript. AJ performed experiments and prepared figures. CB performed and analyzed experiments, and edited the manuscript. PC conceived and designed the study, designed and analyzed experiments, supervised the study, prepared figures and wrote and revised the manuscript. TES conceived and designed the study, analyzed experiments, prepared figures, supervised the study, and wrote and revised the manuscript.

Figure Legends

Figure 1. Overview of mt-TagRFP-HI-NESS reporter. A) Depiction of the domain organization of the mt-TagRFP-HI-NESS protein. **B)** Predicted 3D structure of the mature protein in A generated with AlphaFold 2 bound to mtDNA as predicted via HADDOCK. Colours indicate mitochondrial targeting sequence (green), linker (black), TagRFP (red), H-NS DNA binding domain (blue), HA tag (yellow) and DNA (dark green).

Figure 2. mt-TagRFP-HI-NESS labels mtDNA nucleoids. A) U2OS cells transduced with mt-TagRFP-HI-NESS were imaged in the red channel and show variable RFP expression levels. U2OS cells transduced with mTagRFP and co-stained with SYBR Gold to label DNA (**B)** or MitoTracker Green to label mitochondrial networks (**C**), reveal that the mt-TagRFP protein localizes with mitochondrial nucleoids. Note some non-nucleoid mt-TagRFP-HI-NESS signal can be seen throughout the mitochondrial network in some cells (see arrowhead in B)

Figure 3. Clonal selection for improved mt-TagRFP-HI-NESS nucleoid imaging. Representative images from a selected clone U2OS cells transduced with mTag-RFP with more specific nucleoid staining as revealed by co-staining with SYBRGold to label DNA (**A**) or MitoTracker Green to label mitochondrial networks (**B**).

Figure 4. Characterization of mtDNA function. A) Immunoblotting of U2OS control cells, and cells sorted by flow cytometry for TagRFP expression into low, medium-low, medium-high and high groups. Blots were probed with the indicated antibodies. CI-CV denotes the specific oxidative phosphorylation complexes to which the indicated proteins belong. **B)** Quantitative PCR of mtDNA relative to 18S from the lines described in A, normalized to U2OS control (n=6 independent biological replicates). **C)** Quantitative RT-PCR of mtDNA-encoded transcripts (COX1, ND1, ATP6, 12S) relative to actin from the lines described in A and normalized to U2OS control. (N=3 independent biological replicates). Error bars represent SD. * denotes p-value<0.05 relative to WT cells, as determined via Student's T-test.

Figure 5. mt-TagRFP-N/C-HI-NESS labels mtDNA nucleoids. A) Depiction of the domain organization of the N/C and N/N double HI-NESS proteins. **B)** Predicted 3D structure of the mature proteins in A generated with AlphaFold 2 bound to mtDNA as predicted via HADDOCK.

Colours in A&B indicate mitochondrial targeting sequence (green), linker (black), TagRFP (red), H-NS DNA binding domain (blue), and HA tag (yellow). Representative images show improved mtDNA localization of mt-TagRFP-N/C-HI-NESS in U2OS cells co-stained with SYBR Gold to label DNA **(C)** or MitoTracker Green to label mitochondrial networks **(D)**.

Figure 6. mt-Kaede-HI-NESS labels mtDNA nucleoids. A) Depiction of the domain organization of the mt-Kaede-HI-NESS protein. **B)** Predicted 3D structure of the mature protein in A generated with AlphaFold 2 bound to mtDNA as predicted via HADDOCK. Colours in A&B indicate mitochondrial targeting sequence (green), linker (black), Kaede (red/green), H-NS DNA binding domain (blue), and HA tag (yellow). **C**) Representative image of U2OS cells transduced with mt-Kaede-N/C-HI-NESS and co-stained MitoTracker Deep Red to label mitochondrial networks reveals specific nucleoid signal. Moreover, 405nm laser activation of a subregion, indicated by the dashed box, photoswitches a subset of the Kaede label, which can be used to follow specific nucleoids over time, as seen in the zoomed images (**D**).

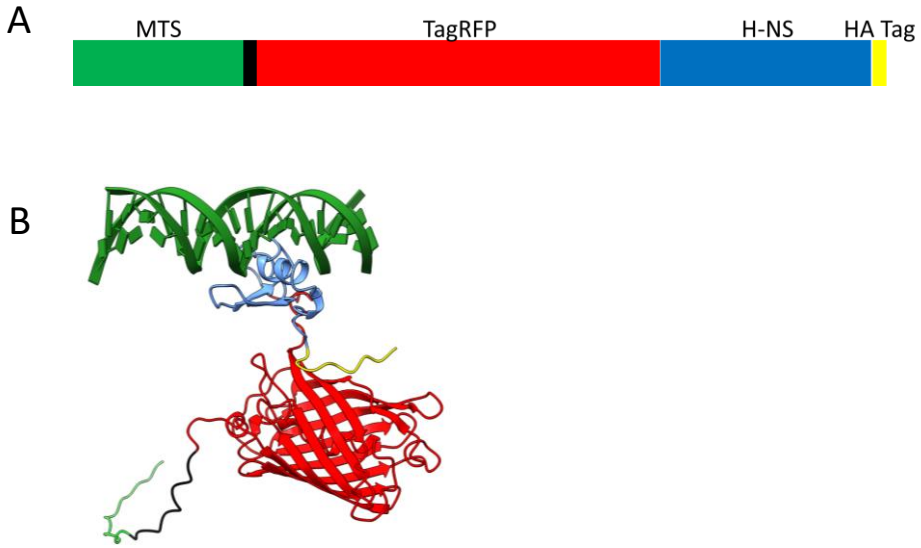


Figure 1. Overview of mt-TagRFP-HI-NESS reporter. A) Depiction of the domain organization of the mt-TagRFP-HI-NESS protein. **B)** Predicted 3D structure of the mature protein in A generated with AlphaFold 2 bound to mtDNA as predicted via HADDOCK. Colours indicate mitochondrial targeting sequence (green), linker (black), TagRFP (red), H-NS DNA binding domain (blue), HA tag (yellow) and DNA (dark green).

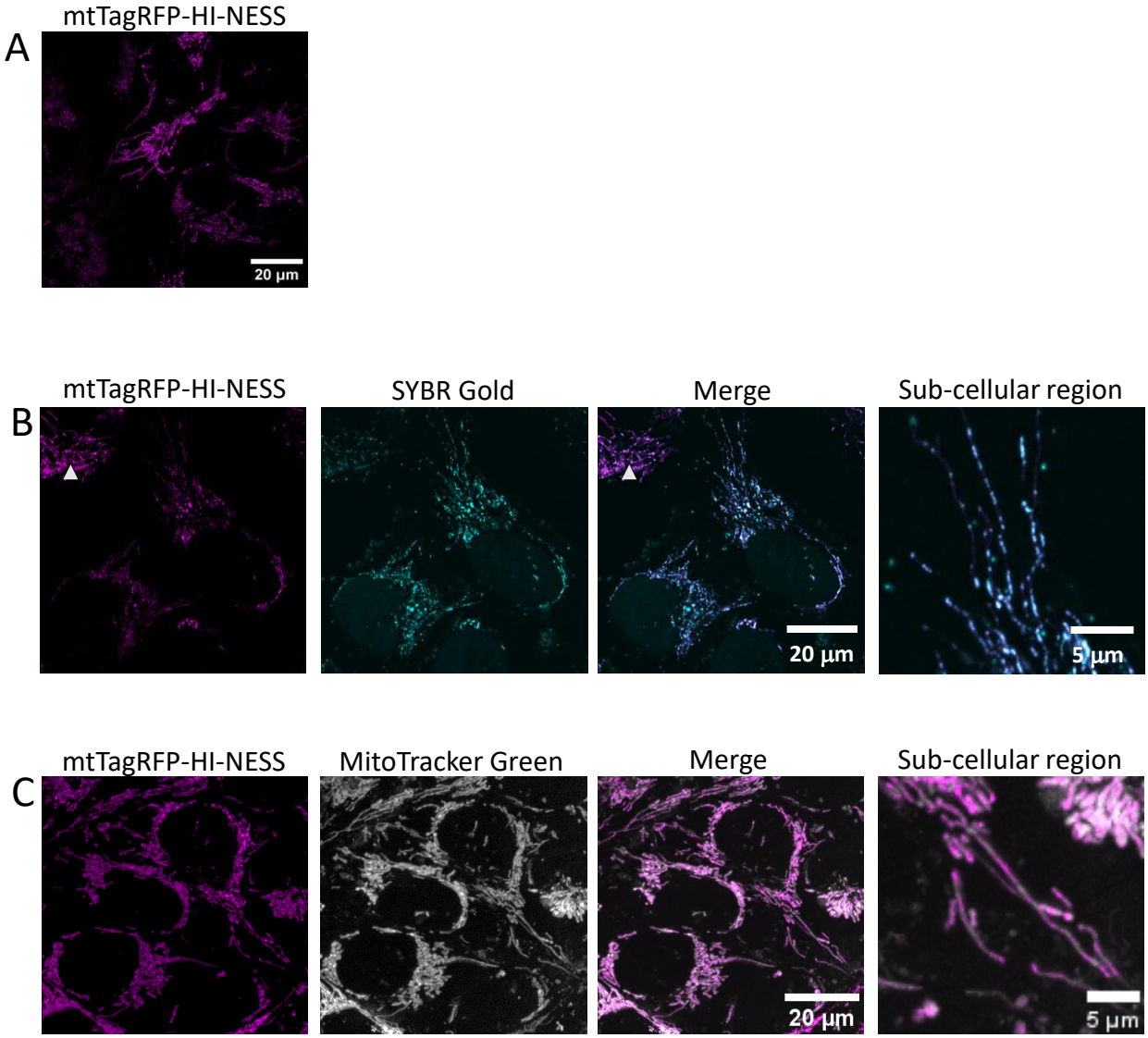


Figure 2. mt-TagRFP-HI-NESS labels mtDNA nucleoids. A) U2OS cells transduced with mt-TagRFP-HI-NESS were imaged in the red channel and show variable RFP expression levels. U2OS cells transduced with mTagRFP and co-stained with SYBR Gold to label DNA (**B)** or MitoTracker Green to label mitochondrial networks (**C**), reveal that the mt-TagRFP protein localizes with mitochondrial nucleoids. Note some non-nucleoid mt-TagRFP-HI-NESS signal can be seen throughout the mitochondrial network in some cells (see arrowhead in B)

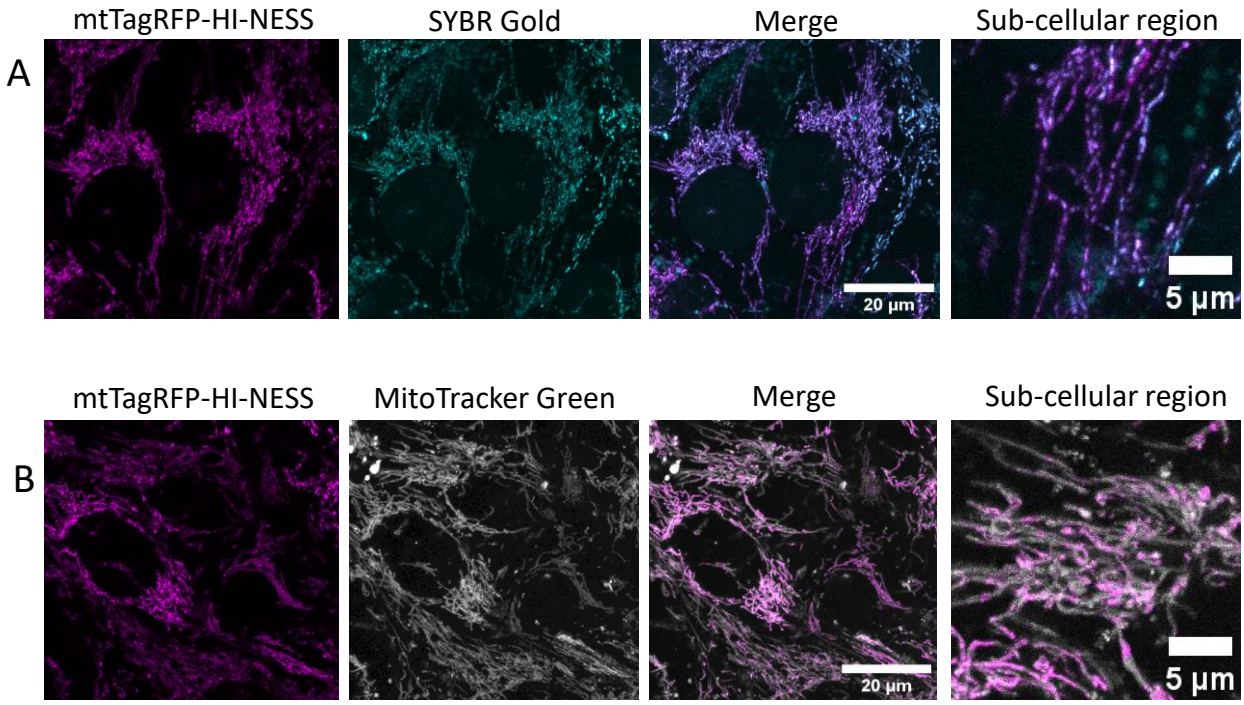
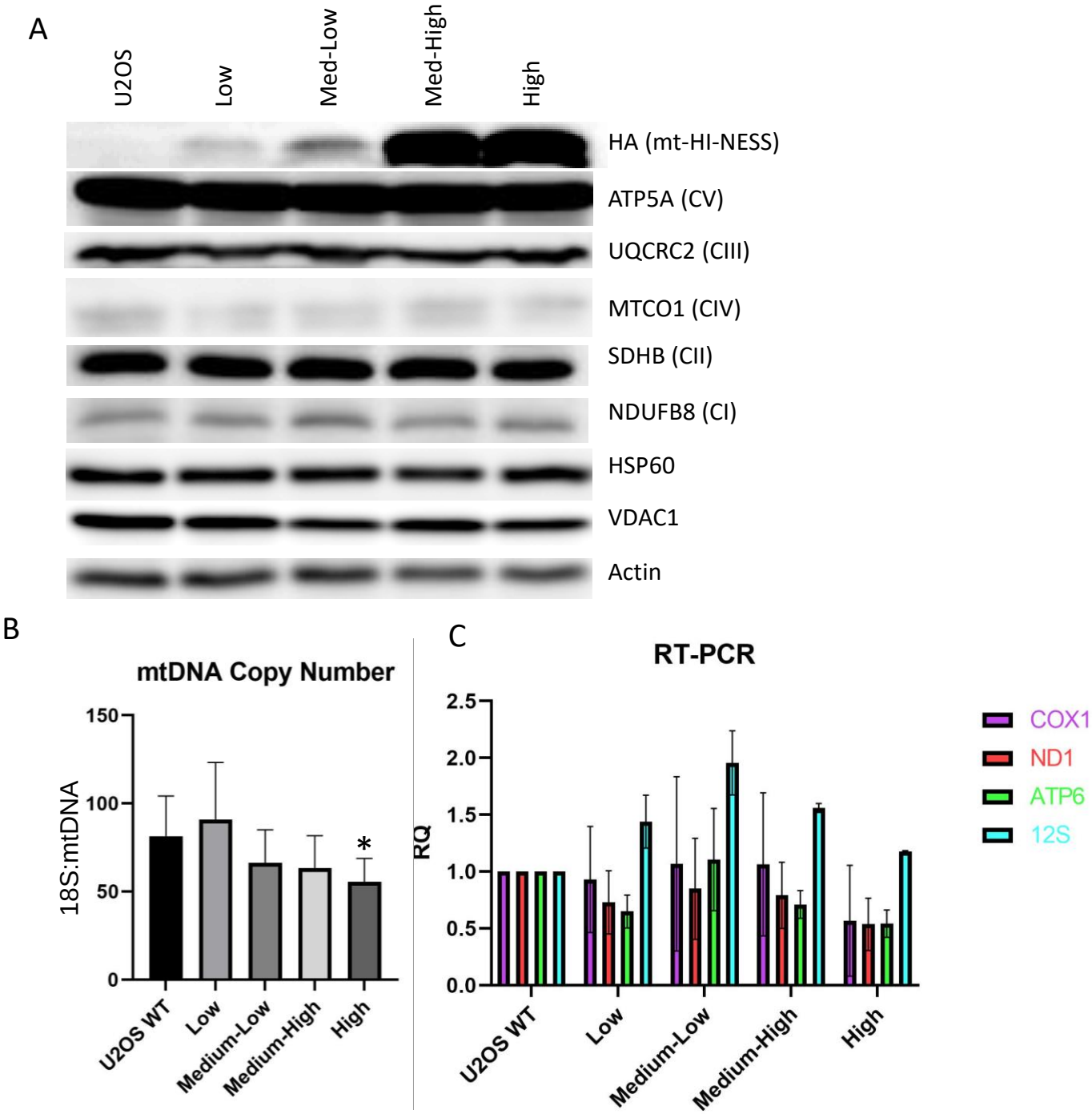


Figure 3. Clonal selection for improved mt-TagRFP-HI-NESS nucleoid imaging. Representative images from a selected clone U2OS cells transduced with mTag-RFP with more specific nucleoid staining as revealed by co-staining with SYBRGold to label DNA (**A**) or MitoTracker Green to label mitochondrial networks (**B**).



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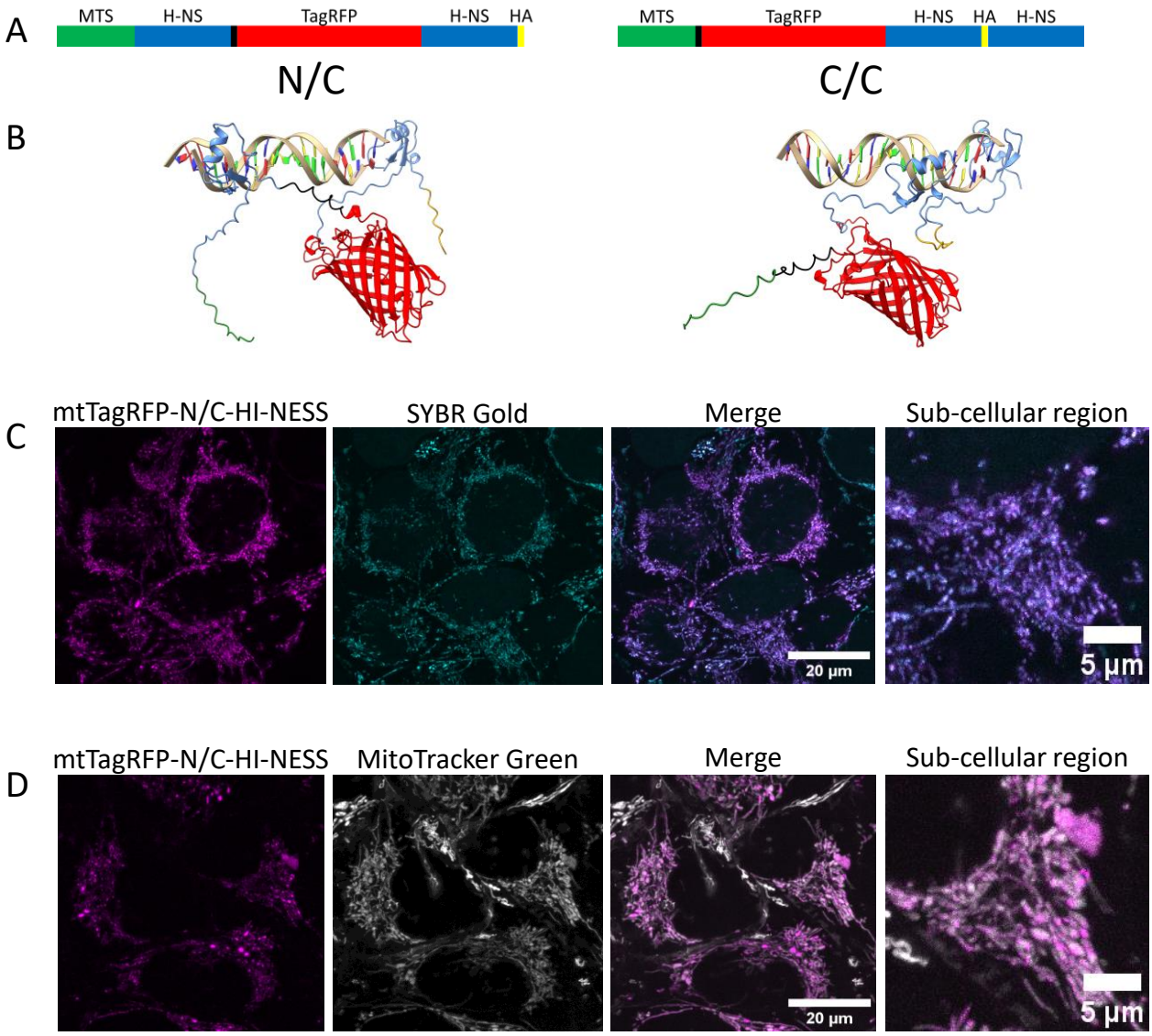

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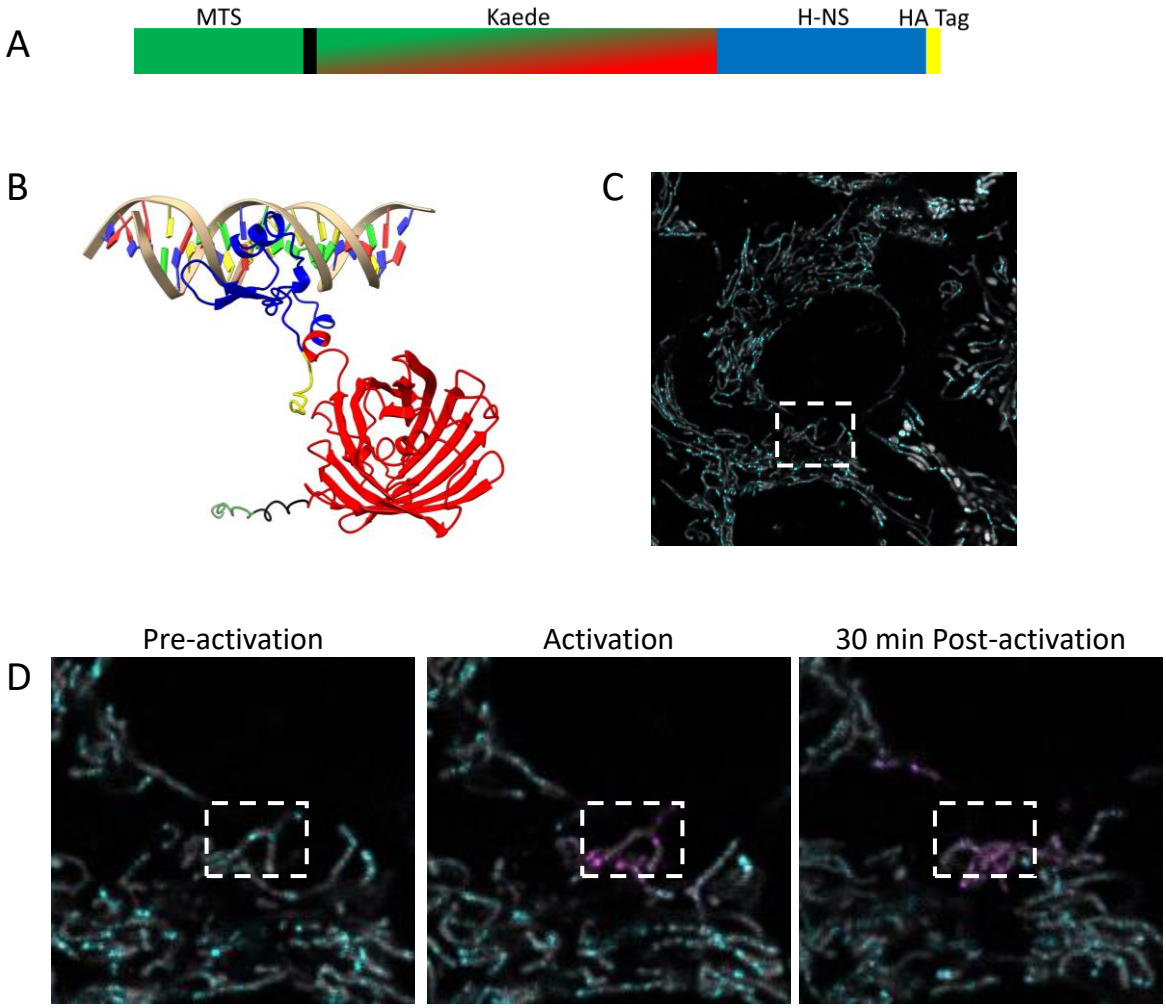

Figure 6. mt-Kaede-HI-NESS labels mtDNA nucleoids. A) Depiction of the domain organization of the mt-Kaede-HI-NESS protein. **B)** Predicted 3D structure of the mature protein in A generated with AlphaFold 2 bound to mtDNA as predicted via HADDOCK. Colours in A&B indicate mitochondrial targeting sequence (green), linker (black), Kaede (red/green), H-NS DNA binding domain (blue), and HA tag (yellow). **C**) Representative image of U2OS cells transduced with mt-Kaede-N/C-HI-NESS and co-stained MitoTracker Deep Red to label mitochondrial networks reveals specfic nucleoid signal. Moreover, 405nm laser activation of a subregion, indicated by the dashed box, photoswitches a subset of the Kaede label, which can be used to follow specific nucleoids over time, as seen in the zoomed images (**D**).

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Supplemental Figure 1: Nucleotide and protein sequences of mt-HI-NESS constructs. The text is colour coded according to the protein domains as follows: green indicates the double Cox8a mitochondrial targeting sequence; black indicates the linker sequence; red indicates the fluorescent protein open reading frame; blue indicates the H-NS DNA binding domain; yellow indicates the HA tag. Bolded protein residues in the protein sequences indicate predicted cleavage sites of the mitochondrial targeting sequence.

>mt-TagRFP-HI-NESS cDNA

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>mt-TagRFP-HI-NESS Protein

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VTTYEDGGVLTATQDTSLQDGCLIYNVKIRGVNFPSNGPVMQKKTLGWEANTEMLYPADGGLEGRSDMALKLVGGG
HLICNFKTTYRSKKPAKNLKMPGVYYVDHRLERIKEADKETYVEQHEVAVARYCDLPSKLGHKAAVKSGTKAKRAQRPA
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>mt-TagRFP-N/C-HI-NESS cDNA

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>mt-TagRFP-N/C-HI-NESS Protein
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> mt-Kaede-HI-NESS cDNA
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ACGCTtaa

>mt-Kaede-HI-NESS Protein
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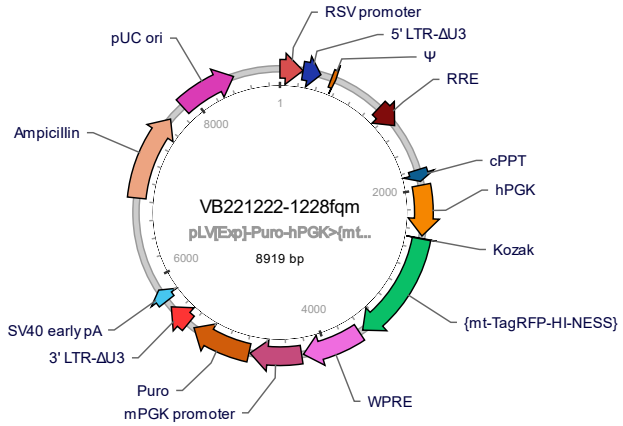
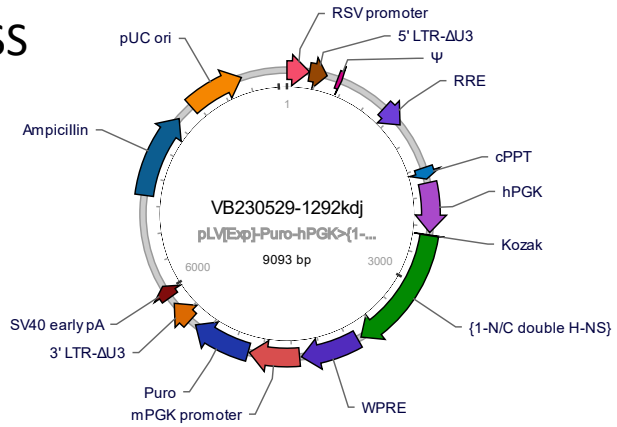
Supplemental Figure 2

mt-TagRFP-HI-NESS



mt-TagRFP-N/C-HI-NESS



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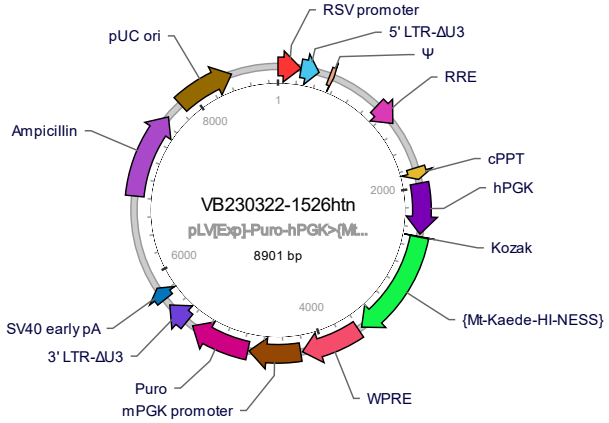
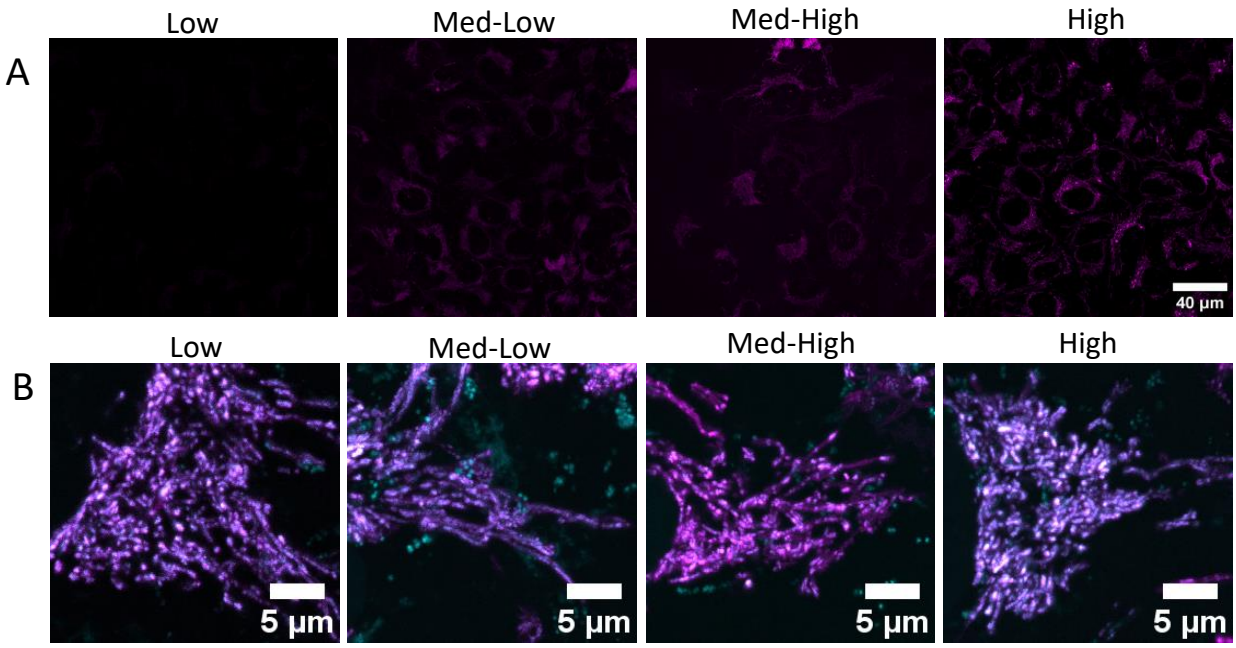


Sup Fig 3 – Sorted by Expression Levels



Supplemental Figure 4. Non-nucleoid mt-TagRFP-HI-NESS. A) Representative images of U2OS cells transduced with mTag-RFP selected for different expression levels of TagRFP and imaged with the same microscope settings. **B)** Sorted cells as in A can show non-nucleoid mitochondrial network signal, even at low expression levels. Imaging of the mt-TagRFP-HI-NESS signal was optimized for each condition.

Supplemental Figure 3: Nucleotide sequences of the plasmids used in this study. The indicated vector IDs can be used to retrieve detailed information about the vector of vectorbuilder.com.

mt-TagRFP-HI-NESS VB221222-1228fqm

```
>VB221222-1228fqm pLV[Exp]-Puro-hPGK>{mt-TagRFP-HI-NESS}
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CGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTT
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AAAAGCTGGAGCTGCAAGCTT

```
CLUSTAL O(1.2.4) multiple sequence alignment

TagRFP       MSELIKENMHMKLYMEGTVNNHHFKCTSEGEGKPYEGTQTMRIKVVEGGPLPFAFDILAT 60
Kaede        -MSLIKPEMKIKLLMEGNVNGHQFVIEGDGKGHPFEGKQSMDLVVKEGAPLPFAYDILTT 59
mEos3.2      -MSAIKPDMKIKLRMEGNVNGHHFVIDGDGTGKPFEGKQSMDLEVKEGGPLPFAFDILTT 59
               . ** :*::** ***.**.*:*   .:* *:*:**.*:* : * **.*****:***:*

TagRFP       SFMYGSRTFINHTQGIPDFFKQSFPEGFTWERVTTYEDGGVLTATQDTSLQDGCLIYNVK 120
Kaede        AFHYGNRVFAKYPDHIPDYFKQSFPKGFSWERSLMFEDGGVCIATNDITLKGDTFFNKVR 119
mEos3.2      AFHYGNRVFAKYPDNIQDYFKQSFPKGYSWERSLTFEDGGICNARNDITMEGDTFYNKVR 119
             :* **.*.* :: : * *:******:*::***   :****:  * :* :::.. :  :*:

TagRFP       IRGVNFPSNGPVMQKKTLGWEANTEMLYPADGGLEGRSDMALKLVGGGHLICNFKTTYRS 180
Kaede        FDGVNFPPNGPVMQKKTLKWEASTEKMYLRDGVLTGDITMALLLKGDVHYRCDFRTTYKS 179
mEos3.2      FYGTNFPANGPVMQKKTLKWEPSTEKMYVRDGVLTGDIEMALLLEGNAHYRCDFRTTYKA 179
             : *.*** ********** ** .** :*  ** * *   *** * *. *  *:*:***::

TagRFP       KKPAKNLKMPGVYYVDHRLERIKEADKETYVEQHEVAVARYCDLPSKLGHK        231
Kaede        RQ--EGVKLPGYHFVDHCISILRHDKDYNEVKLYEHAVAH-SGLPDNVK--        225
mEos3.2      KE--KGVKLPGAHFVDHCIEILSHDKDYNKVKLYEHAVAH-SGLPDNARR-        226
             ::  :.:*:** ::*** :. : . .. . *: :* ***: ..**.:
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Supplemental Figure 5. Fluorescent protein sequence alignment. Protein alignment of mTagRFP, Kaede, and mEos3.2 fluorescent proteins sequences downloaded from fpbase.org and generated using CLUSTAL 0 (1.2.4) through the EMBL-EBI online server (https://www.ebi.ac.uk/Tools/msa/clustalo/).