The biophysical mechanism of mitochondrial pearling

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Abstract

Mitochondrial networks exhibit remarkable dynamics that are driven in part by fission and fusion events. However, there are other reorganizations of the network that do not involve fission and fusion. One such exception is the elusive, “beads-on-a-string” morphological transition of mitochondria. During such transitions, the cylindrical tubes of the mitochondrial membrane transiently undergo shape changes to a string of “pearls” connected along thin tubes. These dynamics have been anecdotally observed in many contexts and given disparate explanations. Here we unify these observations by proposing a common underlying mechanism based on the biophysical properties of tubular fluid membranes for which it is known that, under particular regimes of tension and pressure, membranes reach an instability and undergo a shape transition to a string of connected pearls. First, we use high-speed light-sheet microscopy to show that transient, short-lived pearling events occur spontaneously in the mitochondrial network in every cell type we have examined, including primary fibroblasts, T-cells, neurons, and budding yeast. We present evidence that transient mitochondrial pearling occurs during important biological events, particularly during T cell activation and neuronal firing. Using our high-temporal resolution data, we identify two distinct categories of spontaneous pearling, i) internal pressure-driven pearling regulated by ionic flux, and ii) external tension-driven pearling regulated by molecular motors. By applying live-cell STED microscopy we document the structural reorganization of inner cristae membranes during mitochondrial pearling. We then compare numerous methods for inducing pearling, including the ability to induce these dynamics with single mitochondrion precision. These methods include ionophores, channel activators, osmotic shock, detergents, laser stimulation, membrane intercalating molecules, chemical fixation, and micro-needle force. These disparate inducers establish three main physical causes of pearling, i) ionic flux amplifying internal osmotic pressure, ii) membrane packing lowering bending elasticity, and iii) external mechanical force increasing membrane tension. Pearling dynamics thereby reveal a fundamental biophysical facet of mitochondrial biology. We suggest that pearling should take its place beside fission and fusion as a key process of mitochondrial dynamics, with implications for physiology, disease, and aging.

Introduction

Inside the cell, mitochondrial units are arranged in complex networks that continually reorganize via dynamic processes including fission and fusion events [1,2]. A less appreciated dynamical behavior of mitochondria involves a transient shape transition from a cylindrical tubule to a “beads-on-a-string” morphology (**Figure 1A**), first observed in 1915 by Margeret and Warren Lewis [3]. This morphology has since been observed in numerous biological contexts and has been referred to as “beads-on-a-string” [4], “COMIC” [5], “MOAS” [6], “nodules”, “condensation”, “beading”, “fragmentation sites” [4,7], and “thread-grain transitions” [8]. These sightings include the muscle cells of *C. elegans* [9], the hypocotyl cells of Arabidopsis plants [10], and during the growth phase and meiosis of budding yeast [11,12]. Additionally, beads-on-a-string mitochondria have been observed *in vivo* including in postmortem brains of healthy, aged, Alzheimer's, and hypoxic-treated mice and humans [6,13,14].

One proposed explanation of this pearled morphology is that the mitochondrial fission machinery, largely through GTP hydrolysis by the dynamin-like protein, Drp1, could provide a force to constrict the mitochondrial membrane, forming the pinched regions between pearls. But such a model is not consistent with experiments in DRP1 knock-out cells, which have increased rather than decreased pearling rates that propagate across their highly fused mitochondrial networks [5,15]. Other hypothesized explanations for this phenomenon include reactive oxygen species (ROS) generation [16], ER constrictions around the mitochondrial tube, cytoskeleton remodeling, and non-conventional permeability transition pores (mPTP, [17]), but each fails to fully explain the experimental observations. More recently, the beads-on-a-string morphology was explained by contact-sites with the ER [18]. At these junctions, calcium release by the ER is followed by calcium influx into the mitochondria. However, axonal mitochondria undergo pearling spontaneously, as well as by laser axotomy [14], coinciding with matrix alkalization, depolarization, and increased oxidation, but without an influx in calcium. Similarly, during mitochondrial transplantation, pearl formation is independent of calcium influx [19]. Thus, the mechanism by which pearls form is unclear.

A radically different mechanism for these dynamics comes from the physics of the Rayleigh-Plateau instability governed by the Helfrich model of elastic membranes (eq. 1) [20–22]. The basis of this model is the fact that a cylinder is not actually the minimal surface area shape for an elongated membrane tube. If the surface is folded into sinusoidal curves, the same internal volume can be enclosed with a smaller surface area. The normally smooth cylindrical shape of membrane tubes results from the fact that the energy of bending a membrane is substantially larger than the energy of stretching them, thus the lowest energy state is one in which the surface is minimally curved (cylinder) at the expense of having to stretch to accommodate the internal volume. If membrane tension is increased, either by inflating the internal volume or else by

applying mechanical pulling forces, the energy balance shifts such that the stretching term becomes larger than the bending term, resulting in a lower energy state in which the membrane curves into pearls [22–25].

$$E = E_b + E_s + E_p \quad \text{(eq. 1)}$$

Equation (1) consists of a simplified version of the Helfrich model which determines the total energy of the membrane surface. This model consists of three energies; i) bending energy (Eb) derived from membrane's curvatures and bending modulus, ii) surface tension energy (E_s) capturing stretching or compressing of the membrane surface, and iii) pressure energy (E_p) representing the work done by the pressure difference across the membrane, proportional to the enclosed volume.

The Rayleigh-Plateau mechanism should further dictate the transitions of cellular structures. Any cylindrical tube of membranes, including double membranes such as cellular membranes, and reticulated organelle networks like mitochondria will undergo a spontaneous shape transition when the energy stored by membrane stretching exceeds the elastic energy stored by membrane bending. These energies are determined by the organelle's physical properties; in particular, membrane tension and elastic modulus. The membrane can reach this threshold from i) increased internal osmotic pressure, ii) increased external mechanical tension, or iii) reduced bending elasticity (i.e. stiffness) [22,26]. In accordance with this mechanism, we refer to this dynamical phenomenon as mitochondrial pearling.

Such pearling-like forms have also been described in other cellular structures such as plasma membranes [27,28], axons [29,30], microtubule-associated proteins [31], contractile vacuoles [32] and the endoplasmic reticulum (ER) [33–35]. However, because the mitochondrion has an inner membrane that is electrochemically coupled and highly structured into helicoid cristae [36], it has an elastic modulus an order of magnitude greater than typical double membranes of its size [20]. These properties make it particularly resistant to deformation and thereby physically unique among cellular organelles.

Here we propose that the Rayleigh-Plateau mechanism may provide a unified model of mitochondrial pearling that can account for the many circumstances in which it has been observed. Using a combination of live-cell imaging, high-resolution structural information, and perturbation experiments, we find that biological events that either apply osmotic pressure or mechanical tension, or alter the mitochondrial membrane, can induce a similar pearling instability. The ability of such disparate causes to drive mitochondrial pearling can most parsimoniously be explained if they all work by altering the balance of tension and bending energies in the membrane, thus avoiding the need to invoke a collection of distinct molecular processes.

Results

Spontaneous mitochondrial pearling encompasses two physically distinct events

Mitochondrial tubules generally take on cylindrical shapes interwoven into larger networks through fission and fusion processes. However, live-cell time lapse imaging of individual mitochondria tubules has revealed that mitochondria will occasionally transition into pearled units connected by thin membrane tubules, a shape that is held transiently before transitioning back to a uniform shape (**Figure 1B and Movie 1**). These events have previously been observed in standard imaging conditions in mammalian cells such as U2OS, HEK293, HepG2, MEFs, and cortical neurons [5,14,15,19]. These reports have documented events lasting 5 seconds to 2 minutes, with each mitochondrion having a 10%-50% chance of pearling every 10 minutes, depending on cell type. Here, we refine these reports with a single-objective light-sheet (SOLS) microscope (a.k.a. "Snouty", [37]) by imaging whole-cell mitochondrial networks in 3D at 2 Hz (i.e. 2 volumes/second) with minimal phototoxicity compared to standard 3D imaging. We found transient pearling events in every cell type we studied, which included U2OS, HEK293, COS7 (monkey), RPE1, Jurkat cells, primary human CD8 T cells, primary human fibroblasts, iPSC-derived neurons, and budding yeast (**Figure 1B, Methods Table 1 Supplementary Figure 1A-H**). Using the SOLS microscope, pearling events underwent a full transition (cylinder-pearl-cylinder) in as little as 2 seconds (0.5 Hz) **(Figure 1C & Movie 2**). Based on these dynamics, imaging these events with a typical confocal microscope (<0.2 Hz) would miss up to 27% of pearling events, depending on cell type. Additionally, imaging with SOLS microscopy at 1 Hz revealed the probability of a pearling event can vary widely between cell types: In a ten minute interval, it can be as low as 1.4% of mitochondria in iPSC-derived neurons or as high as 22.3% in U2OS cells (**Figure 1D & Supplementary Figure 1I**). Measuring the curvature of the mitochondrial profile throughout these videos revealed differential dynamics between cell types, with mitochondria that pearl tending to undergo 2nd and 3rd events within regular time intervals of 10-14 seconds (**Figure 1E**). Remarkably, the shape of these pearls across cell types was highly conserved with an average width (i.e cross-diameter) of 320-480 nm and peak-to-peak distance between pearls of 0.8-1.3 μm (**Figure 1F-G**).

In support of the physiological relevance of these dynamics, we have observed pearling events during reconstituted biological events. First, we culture iPSC-derived neurons and observed spontaneous pearling of individual mitochondrial tubules under baseline action potentials, and cell-wide pearling during chemically-induced (50 mM KCl) action potentials (**Supplementary Figure 1E, Supplementary Figure 2A-B, Movie 3**). Second, frequent transient pearls formed at the immune synapses of both primary human CD8 T cells and Jurkat cells when conjugated with Raji cells pulsed with Staphylococcus enterotoxin E (SEE) (**Supplementary**

Figure 2C-D, Movie 4-5). These vastly different biological contexts support the ubiquitous and physiological nature of mitochondrial pearling.

Further resolving this temporal transition revealed a wide range of intermediate pearl shapes shared across many cell types (**Supplementary Figure 1A-H, Movie 6**), in some cases with different temporal dynamics. For example, in a U2OS cell treated with the complex III inhibitor rotenone (5μM for 30min), a spontaneous pearling event was observed across a 25 μm long tubule (**Supplementary Figure 1J and Movie 7**). One minute later, the same tubule underwent a second transition to the pearled state. However, in this second event an additional fused 20 μm long tubule synchronously pearled with the original tubule. This second tubule had remained cylindrical during the first observed pearling event. This suggests that pearling can propagate across physically coupled units of mitochondria.

Co-labeling the mitochondrial inner membrane and mitochondrial calcium (Mito-R-GECO1, [38]) revealed that these spontaneous pearling events fall into two distinct classes of pearling events which occurred at similar frequencies. Class I included those preceded by a rise in calcium accompanied by shrinking of the tubule length (**Figure 1H**). Class II included those not preceded by a detectable increase in calcium uptake, but instead underwent elongation of the tubule (**Figure 1I**). Close examination of Class II elongation events revealed the tips of the mitochondria appeared to be pulled into thin protrusions, 180-300 nm in width, projected outwards 1-6 μm (**Movie 8**), and tended to be followed by fission events, consistent with the literature [5]. On the other hand, class I calcium-associated pearling events tended to shrink the mitochondria, such that all peripheral branches were rapidly compacted into a smaller area (**Movie 9**). These two classes can further be distinguished by tracking their structure and movement over time (**Figure 1J**), with class II elongation-associated pearling events recovering faster and maintaining a straight path throughout the event. It is worth noting there is a small subset of events that displayed both elongation and calcium uptake, but there were no observed events of shrinking without calcium uptake (**Figure 1K-L**). Additionally, the ratio of elongation-associated to calcium-associated event frequencies differed by cell type with the highest ratio in primary fibroblasts (8:2), followed by U2OS (7:4), and HEK (1:5) cells. Considering these observations in the context of the Rayleigh-Plateau instability, we hypothesized that these two classes of spontaneous events may reflect two different physical forces. We further hypothesize that class I involved changes to internal ionic pressure and class II involved external mechanical pulling. Each class is capable of increasing the energetic contribution of membrane tension until it exceeded the energetic contribution of bending elasticity, at which point the membrane transitions to the pearled shape to minimize its surface area.

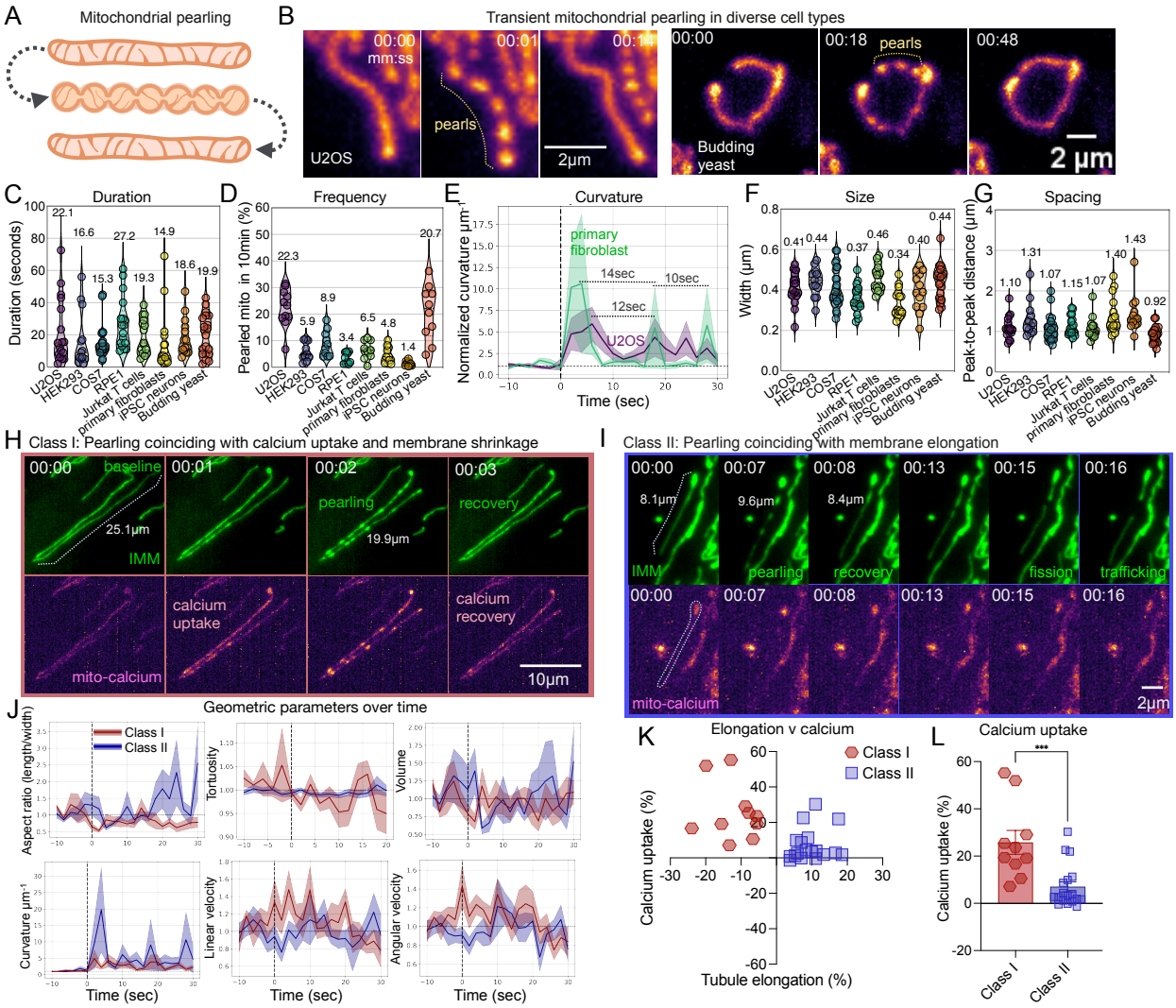

Figure 1. Spontaneous pearling is conserved and encompasses two distinct biophysical events. (**A**) Schematic of a transient mitochondrial pearling event. (**B**) Representative examples of transient pearling along the inner mitochondrial membrane of U2OS (COX8a-mEmerald), Jurkat T cells (COX8a-StayGold) and budding yeast (pre-COX4-mNeonGreen) (**C**) Duration of pearling transitions from uniform to pearl back to uniform transition amongst 8 different cell types (n= 13-25 events per cell type). (**D**) Rate of pearling events measured as the probability any mitochondria will pearl over a 10 minute period (n=10-11 videos per group). (**E**) Normalized curvature per µm across the length of mitochondrial tubule over time for primary fibroblasts (n=19 events) and U2OS cells (n=18 events). Each event is normalized to the average of the 3 initial timepoints. Dotted vertical line indicates the onset of pearling. (**F-G**) Mean cross-diameter width (**F**) and distance between the center of each neighboring pearl (**G**) for listed cell types (n=13-25 events per cell type). (**H-I**) Representative time series of tubule (G) shrinking and (H) elongation during a spontaneous pearling event in primary human fibroblasts co-labeled with the mitochondrial inner-membrane (COX8a-mStayGold) and mitochondrial calcium (genetically-encoded mito-R-GECO1). Dotted line indicates length of adjacent mitochondrial tubule. (**J**) Structural and dynamical metrics comparing pearls that undergo stretching (red) and shrinking (blue). All values normalized the average of the first three timepoints. Dotted line indicates the onset of pearling. Error bars = S.E.M. (**K**) Calcium uptake vs tubule elongation amongst spontaneous pearling events. Blue circles and red dots mark events under which elongation or shrinking occurred, respectively. (**L**) Comparison of calcium uptake between elongated and shrunk mitochondria during pearling. ** = $p < 0.01$ performed by non-parametric Mann-Whitney test. n = 10-20 events per group.

Osmotic pressure drives chemically-induced mitochondrial pearling

We next investigated the mechanism of class I pearling events characterized by ionic flux. The most likely source of internal pressure resulting from uptake of ions into the mitochondrial interior is osmotic pressure, whereby water molecules surge into the mitochondrial matrix. Osmotic shock is known to result in pressure-driven pearling instability in membrane tubes [32]. To confirm that internal pressure is capable of pearling mitochondria, we replicated the 1915 osmotic shock experiment of Margaret Lewis using repetitive media changes between hyper and hypo-osmotic media. By replacing the cell medium (280 mOsm) with a hypotonic solution of 0.1x PBS (28 mOsm i.e. low-salt water) pearling was induced across the entire mitochondrial network (**Movie 10**). This was subsequently reversed by swapping media back to the original medium or a balanced salt solution of 1x PBS (280 mOsm) (**Figure 2A-B, Supplementary Figure 3A**). Additionally, swapping media with hypertonic solution of 10x PBS (2800 mOsm) resulted in the mitochondrial network becoming flaccid spaghetti-like cylinders with minimal movement (presumably from Brownian motion) (**Movie 11**). When the hyper-osmotic media was replaced with hypo-osmotic media, pearls appeared. Remarkably, repetition between these two conditions could convert the mitochondrial network between flaccid (hypertonic) to pearled (hypotonic) states up to five times until the cells detached from the cover glass (**Supplementary Figure 3B, Movie 12**). Surprisingly, a 20 second burst of hyperosmotic shock, followed by a return to normal media did not return mitochondria to baseline morphology, but instead resulted in cell wide pearling that took 10 minutes to recover back to a uniform state (**Movie 13**). Additionally, hypotonic induced pearling occurred even if the mitochondria were depolarized with the proton ionophore FCCP, though depolarized mitochondria did not recover baseline curvature after isotonic rescue (**Supplementary Figure 3C, Movie 14**). Under hypotonic shock, pearled mitochondria were accompanied by shrinking of the tubule length and an influx of calcium, which were reversed by a return to an isotonic environment (**Supplementary Figure 3D-F**). Class I spontaneous pearling events may therefore share a common mechanism with hypotonic shock induced by osmotic pressure.

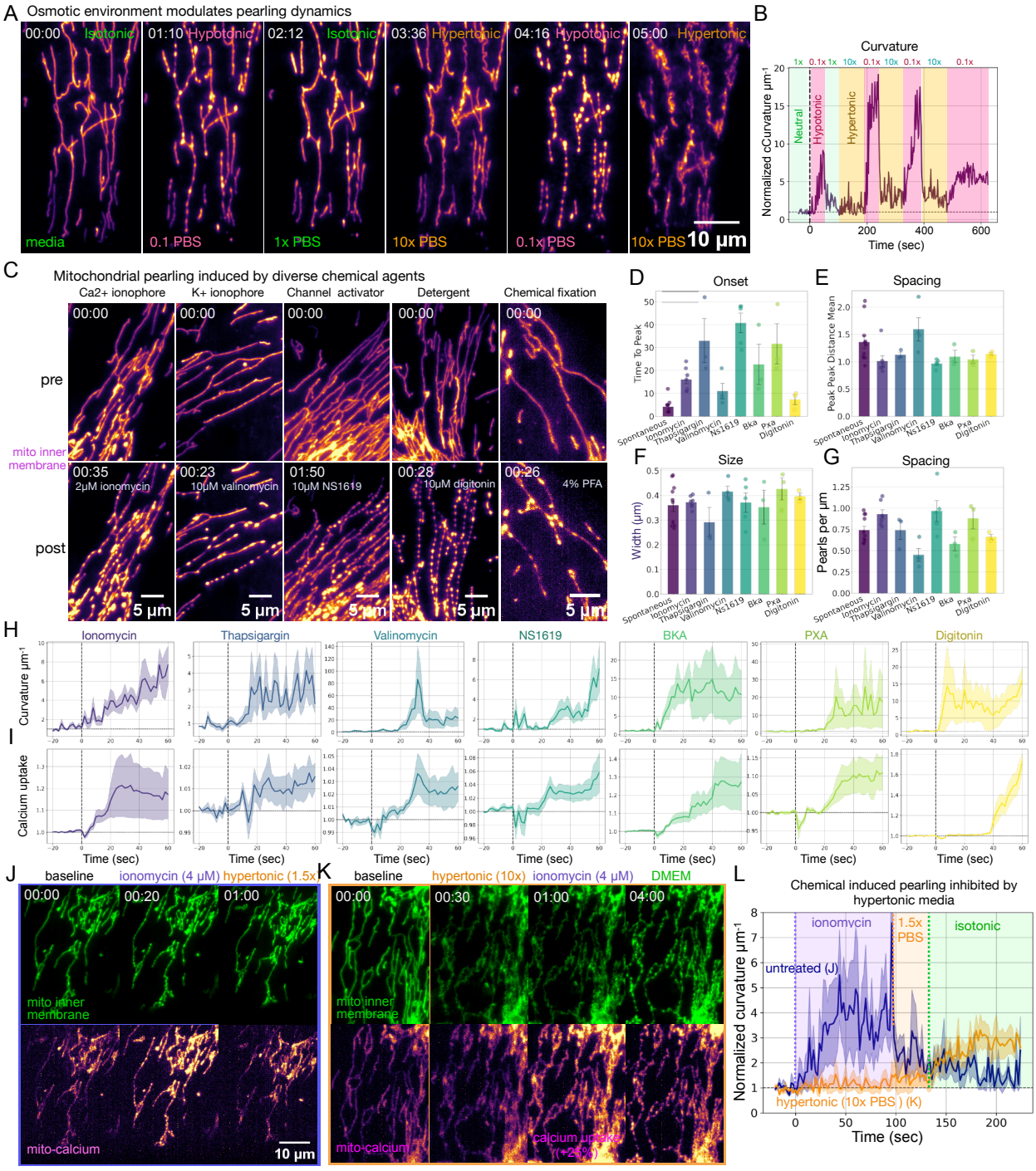

Figure 2. Osmotic pressure underpins chemically induced mitochondrial pearling (**A**) Osmotic shock of primary human fibroblasts. Media is repeatedly swapped between isotonic (DMEM), hypotonic (0.1x PBS) and hypertonic (10x PBS) conditions. (**B**) Quantifying pearling based on normalized curvature per µm over time during different osmotic shock regiments corresponding to (A). (**C**) Classes of chemical inducers of mitochondria pearling encompassing calcium and potassium ionophores, potassium channel activator (BKCa), membrane permeabilization, and chemical fixation. (**D-G**) Comparison of chemical agents along with spontaneous pearling of primary human fibroblasts for the (D) time to the peak of pearling curvature (E) the distance between the centers of neighboring pearls at the peak of pearling, (F) the cross-diameter width of pearls, and (G) the number of pearls per micron of tubule length before the onset of pearling. (**H**) Panel of mitochondrial curvature per µm normalized to first time points over time. Dotted line indicates the point of injection of the chemical agent. (**J**) Rescue of tubular morphology induced by ionomycin (2 µm) by addition of 1.5x hyper-

osmotic PBS to the media. (**K**) Inhibition of ionomycin-induced pearling (2 μM) by preconditioning the cell with hypertonic media (10x PBS). Three minutes after ionomycin addition, media is swapped back to untreated isotonic DMEM. (**L**) Intensity-based curvature per μm normalized to first time points over time for primary human fibroblasts without pre-treatment (blue) or placed in hypertonic 10x PBS (orange). Cells then underwent chemical-induction by 4μM ionomycin and recovery by either 1.5x hypertonic solution or isotonic media. Vertical dotted lines indicate injection of 4 μM ionomycin (purple), injection of hypertonic 1.5x PBS (orange), or media swap to isotonic media (green). n=7 cells per group.

To determine whether there is a specific ion or channel regulating mitochondrial osmotic pressure, we performed perturbation experiments using several chemical agents capable of inducing mitochondrial pearling (**Table 1, Supplementary Figure 4**). Both calcium and potassium ionophores and channel activators induced cell-wide mitochondrial pearling within a minute of treatment (**Figure 2C, Movie 15**). To confirm that these agents were acting through their respective ionic species, we co-labeled cells with mitochondrial calcium marker Mito-R-Geco1. As expected, the calcium ionophore ionomycin, as well as thapsigargin, induced an uptake in mitochondrial calcium that preceded the onset of pearling. The potassium ionophore, valinomycin, and the BKCa channel activator, NS1619, caused cell-wide pearling that did not always accompany calcium uptake (**Supplementary Figure 3H**), suggesting multiple ion species can induce mitochondrial pearling.

Compounds with less direct ionic flux targets such as bonkeric acid (BKA), and phomoxanthone A (PXA) also caused cell-wide pearling. Interestingly, close examination of the intermediate time points of NS1619, BKA, PXA and valinomycin immediately before the formation of pearls, revealed that mitochondria tubules entered a flaccid shape identical to hyperosmotic shock (**Supplementary Figure 3G, Movie 16**). These compounds may have caused hyperosmotic pressure on mitochondria which later pearled when recovering back to isotonic balance.

In mammalian cells, the plasma membrane maintains a negative ionic differential of -50 to -70mV between the inside and outside of the cell by pumping out and sequestering positively charged ions like calcium and sodium. The membrane permeabilization agent, digitonin, resulted in the fastest onset of global pearling (**Figure 2D, right-most graph, Movie 17**) which can be explained as an increase in osmotic pressure as water followed the ionic gradient into the cell. Further, the widely used fixative paraformaldehyde (PFA, 4%), caused cell-wide pearling, consistent with earlier reports of it being osmotically active [39] (**Supplementary Figure 3I, Movie 18**). Pearl formation was inhibited by fixation with a combination of 3% PFA and >0.5% glutaraldehyde (GA), but this was cell type dependent. (**Supplementary Figure 3J, Movie 19**). This inhibition is likely due to the faster fixation rate of GA (see discussion). Other reported inducers of mitochondrial pearling not tested here include histamine, tunicamycin, A23187, and glutamate [18,40–42]. Direct comparison of pearl properties of these diverse chemical inducers with

spontaneous pearling events revealed similar width, peak-to-peak distances, and pearls per μm within the initial onset of pearl formation (**Figure 2D-G**). Further, all of these chemical agents led to a shrinking of tubule length (**Supplementary Figure 4C**), again reminiscent of Class I spontaneous events.

Confirming that all of these compounds were acting via osmotic pressure, the addition of 1.5x PBS (420 mOsm/L) into the media containing the chemical agent rescued pearled morphology (**Figure 2J, Movie 20**). Finally, and most importantly, when cells were placed in a hyperosmotic solution of 10x PBS (2800 mOsm/L) the calcium ionophore, ionomycin, still caused calcium uptake in mitochondria but tubules remained flaccid, without any visible pearling or rise in membrane curvature (**Figure 2K-L, Movie 21**). Only after removal of the hypertonic media did the mitochondria display pearling. This is direct evidence that osmotic pressure mediated the effect of ionic flux. This shared property amongst diverse chemical inducers of pearling indicated that it is unlikely that a single molecular ligand-binding mechanism can explain the shared dynamics of such experiments. Instead, osmotic pressure itself, being the common feature in all cases, is likely to be the driving force of these pearl events.

Pearling inducer	Concentration (μM)	Time of onset (sec)	Known target	FCCP dependent	Hyperosmotic recovery
ionomycin	2-20	15-45	calcium ionophore	no	yes
thapsigargin	0.2-10	20-50	inhibitor of calcium ATPase (SERCA) pump	no	yes
valinomycin	10	20-50	potassium ionophore	no	yes
NS1619	10-30	60-90	BKCa channel activator	yes	yes
PXA	10	30-60	fungal toxin	no	yes
BKA	10	15-45	ATP/ADP translocase and mPTP inhibitor	no	yes
digitonin	10	5-35	membrane permeabilization	no	yes
PFA	4%	30-60	fixative	NA	yes

Table 1: List of chemical agents that induce cell-wide mitochondrial pearling transitions. NA indicates the combination was not tested or is not experimentally plausible.

Mitochondrial pearling can be induced with single-mitochondrion precision.

To experimentally manipulate pearling dynamics of individual mitochondria, we exposed cells to focused laser light (0.3 mW for 200 milliseconds) pinpointed to individual mitochondrial tubules (**Figure 3A**. This stimulation photobleached fluorophores within a <1 μm diameter (**Supplementary Figure 5A**), while inducing pearled morphology in all mitochondrial tubules connected to the tubule targeted by the laser, but not in unconnected tubules. In primary fibroblasts, with long and fused mitochondrial tubules, laser stimulation induced pearls as far as 140 μm away from the point of stimulation, again highlighting that connected components of mitochondrial network are biophysically coupled (**Figure 3B, Movie 22**). Stimulating a random selection of mitochondria tubules revealed that a subset of mitochondria would not respond to the stimulation at all, including in some cases all mitochondria of some cells. In the reverse case, cells with fully fused mitochondrial networks would pearl simultaneously across the entire cell, despite stimulation of a small fraction of an individual mitochondrial branch in the cell (**Figure 3C, Movie 23**). Reminiscent of spontaneous pearling events, laser-induced pearls recovered a cylindrical morphology on average 15 seconds post laser stimulation (**Figure 3D**). However, stimulated tubules that recovered back to baseline did on occasion revert back to a pearl morphology within 60 seconds post-stimulation (**Supplementary Figure 5B, Movie 24**). Additionally, pearls induced by laser stimulation tended to have farther peak-to-peak distances than spontaneous events, but otherwise maintained similar pearl structure and dynamics to spontaneous events (**Figure 3E**). Colabeling with Mito-R-GECO1 revealed a rise in mitochondrial calcium uptake that sometimes preceded the onset of pearls (**Supplementary Figure 5D, Movie 25**). Further, these pearling events were all marked by retraction of branching tubules that were stimulated, suggesting a shared mechanism to class I spontaneous pearling events that underwent calcium uptake and shrinkage (**Figure 3F**). Like chemical inducers of pearling, laser stimulation did not pearl mitochondria when cells were placed in hypertonic media (**Figure 3D**), suggesting osmotic pressure is also the downstream driver of pearling resulting from laser-stimulation.

Next, we sought to confirm that pearls maintained matrix connection during pearl formation by using a FRAP laser to deplete the fluorophores of individual pearls and track fluorophore recovery (**Figure 3G**). Recovery time of fluorescence within depleted pearls of spontaneous and laser-induced pearls took 9.6 seconds and 7.1 seconds, respectively ($p = 0.98$), (**Figure 3H-I**). The majority (>70%) of pearls recovered from depletion, indicating that most pearled mitochondria maintain matrix flow between connected inner membranes. The few events that did not recover were all located at the tips of mitochondrial tubules, had independent movements from the adjacent tubule, and therefore likely underwent complete fragmentation during the pearling event.

To uncover what properties regulate mitochondrial pearling, we applied a series of functional markers and chemical inhibitors and then induced pearling with laser stimulation (**Methods Tables 4-5**). Like other inducers of pearling, depolarizing mitochondria with FCCP reduced pearl formation after laser stimulation by 94% (**Figure 3J, Movie 26**). Similarly, inhibiting electron transport chain (ETC) complex I, III, and V with rotenone, antimycin A, or oligomycin, mitigated pearl curvature by 76%, 79%, and 59% respectively. Note, that these ETC inhibitors independently raised baseline curvature by 2-fold before laser stimulation (i.e. caused partial pearl formation) (**Supplementary Figure 5C**). Overall, depolarization with FCCP had the most dramatic reduction in formation of pearls by laser stimulation. Interestingly, while untreated cells took on average 10 seconds to recover back to a uniform morphology, oligomycin-treated cells delayed recovery to 35 seconds ($p<0.01$). This may be because inhibiting the ATP synthase causes mild hyperpolarization on the inner membrane [43], or due to off-target effects on actin polymerization [44].

Similar to oligomycin, laser stimulation of mitochondrial tubules following depolymerization of the actin cytoskeleton with latrunculin B, resulted in a permanent transition to pearl morphology that could not recover back to the cylindrical shape (**Figure 3K, Movie 27**). These pearls often would progressively accumulate into larger pearls as was seen in the downstream chemically-induced pearls. In contrast, with microtubule polymerization inhibited, pearls had reduced curvature by ~10% and recovered back to the baseline state 10 seconds faster than controls. Further, the microtubule stabilizing agent, taxol, mimicked the behavior of latrunculin B, whereby pearled mitochondria did not recover back to a uniform morphology. Taken together, these inhibitors reveal that both electron transport chain activity as well as cytoskeleton structure influence pearling dynamics.

Colabeling with the membrane potential marker, TMRE, revealed that the IMM depolarized after the onset of pearling and recovered back to baseline after 70% of laser-induced pearling events (**Figure 3L-M, Movie 28**). This recovery often occurred on only specific portions of the original tubule that had separated during pearl formation. As previously reported, pearl shapes recovered back to a cylindrical shape faster than the recovery of membrane potential (**Figure 3N**) [15]. Interestingly, membrane potential recovery was necessary for a second induction of pearling on the same tubule by laser stimulation. Overall, laser-induced pearling matched the behavior of DRP1-KO pearls [15], in that both were supported by electron transport chain activity and resulted in transient depolarization of the inner membrane.

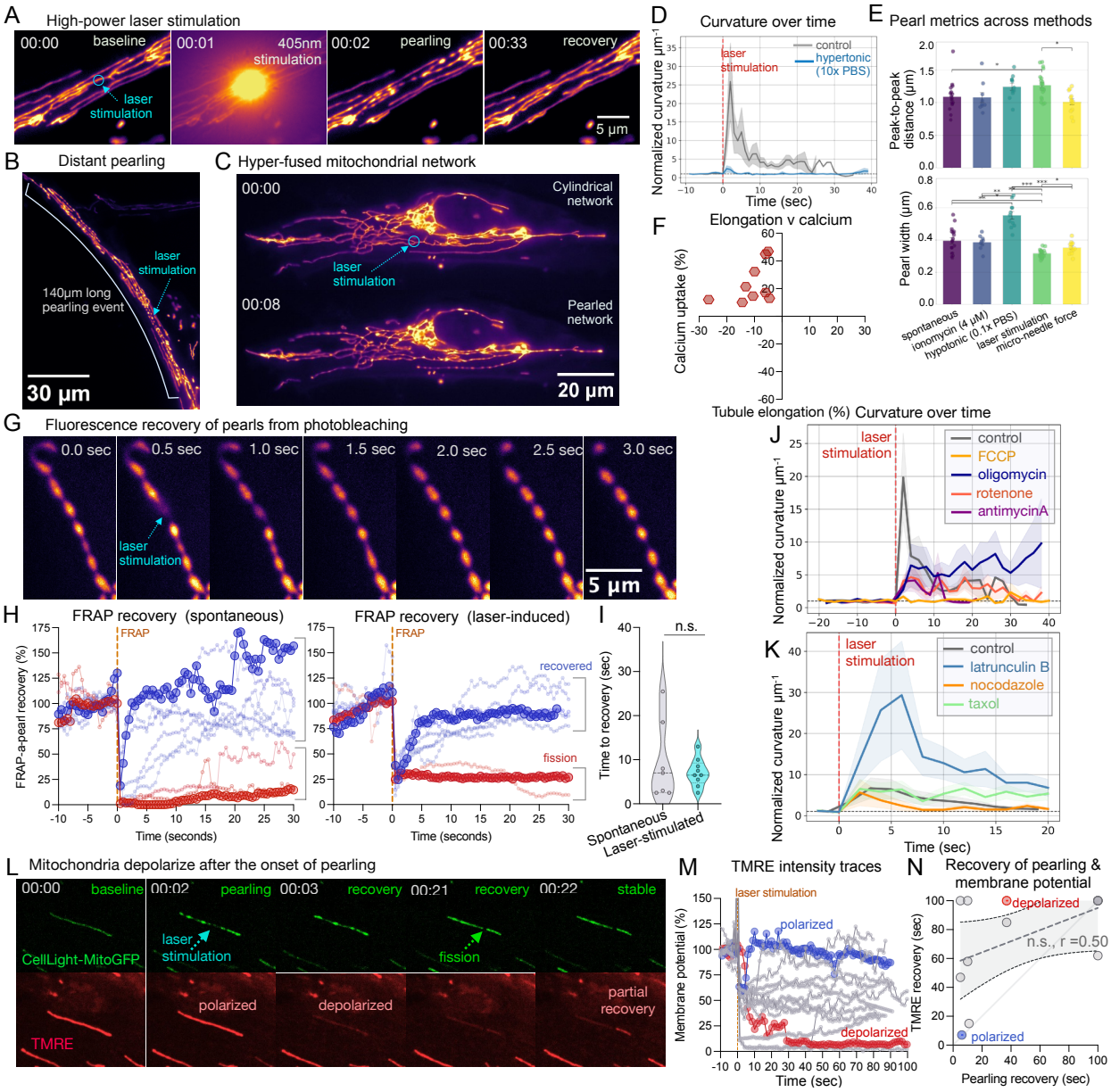

Figure 3. Laser stimulation induces pearling of individual mitochondrial tubules. (**A**) Pearling induced on primary fibroblast by a pinpointed 405nm stimulation laser. The 405 nm laser was fired at 20mW (0.3mW reaching sample) for 200 milliseconds pointed directly on the mitochondrial tubule (blue arrow). (**B**) Primary fibroblast with elongated mitochondria tubule pearled by laser stimulation. (**C**) Hyper-fused mitochondrial network of a primary fibroblasts before and after laser stimulation to a region marked by a blue circle. (**D**) Curvature per µm over time for laser stimulated untreated cells (n=22, gray) and cells placed in 10X PBS (n=15, blue). (**E**) Comparison of peak-to-peak distance between pearls and pearl width (minor-axis) amongst different methods to induce mitochondrial pearling. One-way anova model used. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. (**F**) Scatter plot of calcium uptake vs tubule elongation amongst laser-stimulated pearling events. Red dots mark events under which shrinking occurred. n = 10 events. (**G**) Spontaneous mitochondrial pearls depleted with laser and fluorescence recovery tracked over time. (**H**) Fluorescence recovery after photobleaching of individual pearls of spontaneous and laser stimulated pearling events (n = 10 events/group). Each line indicates an individual event. Blue indicates recovery, while red indicates minimal recovery. (**I**) Comparison of FRAP recovery times between different methods of induction, Mann-Whitney non-parametric t-test. (**J**) Curvature per unit µm comparing electron transport chain inhibitors, n=21 oligomycin (1 uM) events, n=18 FCCP (4 µM) events, n=18 rotenone (5 uM) events, n=18 antimycin

A (5 uM) events. Red dotted line indicates the point of laser stimulation. All data normalized to the average of the first five timepoints for each treatment group. (**K)** Curvature per µm over time for laser stimulated untreated cells, latrunculin B (1 µM), nocodazole (200 nM), and taxol (100 nM) (n=8-10 events per treatment). Data normalized to the first three timepoints of each treatment group. (**L**) Representative time series of membrane potential collapse and recovery after laser-stimulated pearling. CellLight MitoGFP labels the IMM and mitochondrial matrix. TMRE labels mitochondrial membrane potential. (**M**) Normalized TMRE intensity over time. Each line indicates an individual event. Blue indicates an event that recovered back to baseline intensity. Red indicates an event which does not recovery within the duration of imaging. (**N**) Spearman correlation between time for pearls to recovery back to cylindrical shape and time of TMRE signal to return to stable signal. Red and blue dots match individual events highlighted in (M). Faded gray line indicates an ideal correlation of 1.0. Dotted line indicates linear regression with 95% confidence lines.

Endogenous fluorescence predicts pearling response to laser stimulation

Dim-emission detection of the 488-525 nm channel revealed an endogenous fluorescent signal coming from mitochondrial inner matrix that accumulated after the onset of both spontaneous and laser-stimulated pearling events (**Supplementary Figure 6A-B, Movies 29-30**). This endogenous molecule that fluoresces in the green channel accumulates in the mitochondrial matrix after pearling occurs and sometimes recovers. This autofluorescent signal was amplified by depolarization with FCCP (4 µM) or cholesterol depletion by atorvastatin (50 µM) (**Supplementary Figure 6C**). The identity of the endogenous fluorophore could be the endogenous metabolite FAD and NAD/H, whose autofluorescent properties have been widely reported [45–47]. Whatever its identity, this fluorescence predicted whether an individual mitochondrial tubule would pearl from laser stimulation (**Supplementary Figure 6D**).

STED reveals swollen cristae ultrastructure in mitochondrial pearls

Using super-resolution STED imaging, we then characterized mitochondrial cristae ultrastructure of pearls. The cristae of laser-induced pearls (class I) under STED had a similar structure to the helicoidal ramp structures to the Mic60 complex maintaining crista junctions [48,49] (**Figure 4A, Movie 31**). Comparison of mitochondrial size before and after laser stimulation under STED confirmed an 20.4% increase in cross-sectional area and 70.7% increase in inter-cristae distance at the onset of laser-induced pearling (**Figure 4B-C**). Optical sectioning at STED resolution revealed 3D pearled mitochondria with swollen regions containing nucleoids at the center of most pearls and continuous inner membrane between pearls (**Figure 4D-E**).

To investigate a potential functional role of cristae organization in pearling we targeted the cristae junction complex, MICOS, and generated Mic60 knockout cells in budding yeast with the inner mitochondrial membrane protein TIM50 labeled with the fluorophore mNeonGreen. Pearling was induced with laser stimulation (**Figure 4F, Movie 32**). The resulting pearling events showed more homogeneously spaced pearls with 21% reduction of inter-pearl distance ($p<0.001$) and a 50% reduction in pearl volume ($p<0.01$) (**Figure 4G-H**). This suggested that the MICOS complex

influenced the shapes of pearls through its architectural support to the mitochondrial tubule, but is not itself required for pearl formation [5].

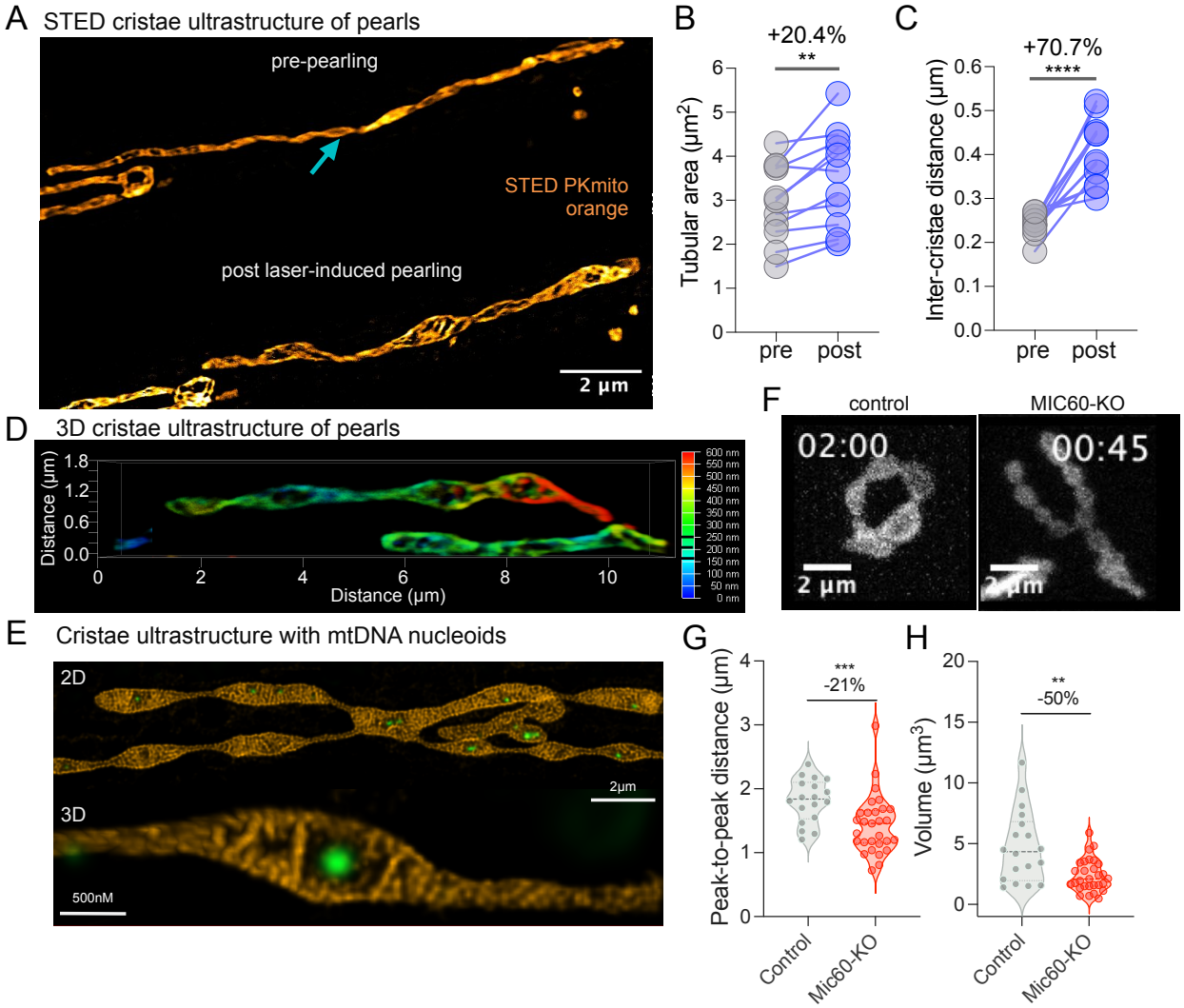

Figure 4. Mitochondrial cristae ultrastructure during pearling. (**A**) Stimulated emission depletion (STED) microscopy of mitochondrial cristae labelled with PKmito Orange dye, before and after laser stimulation of mitochondrial tubule. Two-way anova, ** = $p < 0.01$. (**B**) Comparison of tubular area before and after laser-induced pearling. n=11 mitochondria. (**C**) Inter-cristae distance before and after laser-induced pearling. n=11 mitochondria. Two-way anova, **** = $p < 0.0001$. (**D**) Cristae architecture in 3-dimensions using tauSTED. Depth-encoded color mapping. (**E**) Deconvolved STED imaging of mitochondrial inner membrane (orange, MitoOrange) along with mitochondrial DNA (green, Sybr Gold) during laser-induced pearling event. An example 2D image is shown in the top panel and a separate 3D image in the bottom panel. (**F**) Laser-induced pearling of budding yeast labelled with TIM50 mNeon-green (inner membrane) in wild-type and MIC60-knockout cells. Pearling induced by a 405nm stimulation laser at 0.3mW for 200 ms. (**G**) Distance between centers of neighboring pearls, and (**G**) the pearl volume amongst laser-induced pearling of mitochondria in wildtype (n=20 events) and MIC60-KO yeast (n=25 events).

Mitochondrial membrane elasticity regulates pearling dynamics

The molecules that compose the mitochondrial membrane influence the elastic properties of the tubule (**Figure 5A**), and thus we sought to investigate the consequences of their modulation

to laser-induced pearling. These molecules include cholesterol, cardiolipin, and the MICOS complex. Depleting cholesterol with the statin atorvastatin (24hr of 50μM) resulted in mitochondria that did not pearl in response to laser-stimulation (**Figure 5B-C**). Without sufficient cholesterol content the mitochondrial membrane may have been too elastic to hold tension and undergo a pearling transition. On the other hand, knockout of the MICOS complex genes, MIC10, MIC12, MIC60 all increased the frequency of spontaneous pearling events by 6.9 to 8.4% ($p<0.01$) (**Figure 5D**). We suspect that disruption of cristae lamella folding decreases the membrane's exceptionally high bending modulus, and thereby basal levels of tension more easily exceed its elasticity and cross the pearling instability. We further tested the removal of the cardiolipin synthesis gene, TAZ1, or CRD1. Cardiolipin is a unique lipid of the IMM and supports cristae invaginations in the inner membrane [50]. However, knockout of TAZ1 and CRD1 did not show a statistically significant increase in the frequency of spontaneous pearling events with a +2.813% and -1.5% change compared to wildtype, respectively. We attempted to measure mitochondrial membrane tension with the membrane intercalating dye, Mito Flipper-TR (1 μM), a reporter of mitochondrial membrane tension (Goujon et al. 2019). Although we were unable to capture a consistent fluorescence lifetime shift in membrane tension during pearling, we were surprised to see that even mild exposure to confocal laser light to cells labelled with Mito-Flipper-TR caused cell-wide mitochondrial pearling (**Figure 5E-F, Movie 33**). We speculate that similar to the presence of cholesterol, membrane intercalating dyes stiffened the IMM, resulting in an increased rate of pearling. We next ruled out other potential mechanistic explanations of spontaneous pearling including fission proteins, ER constrictions, actin or microtubule polymerization, ROS generation, and mtDNA presence.

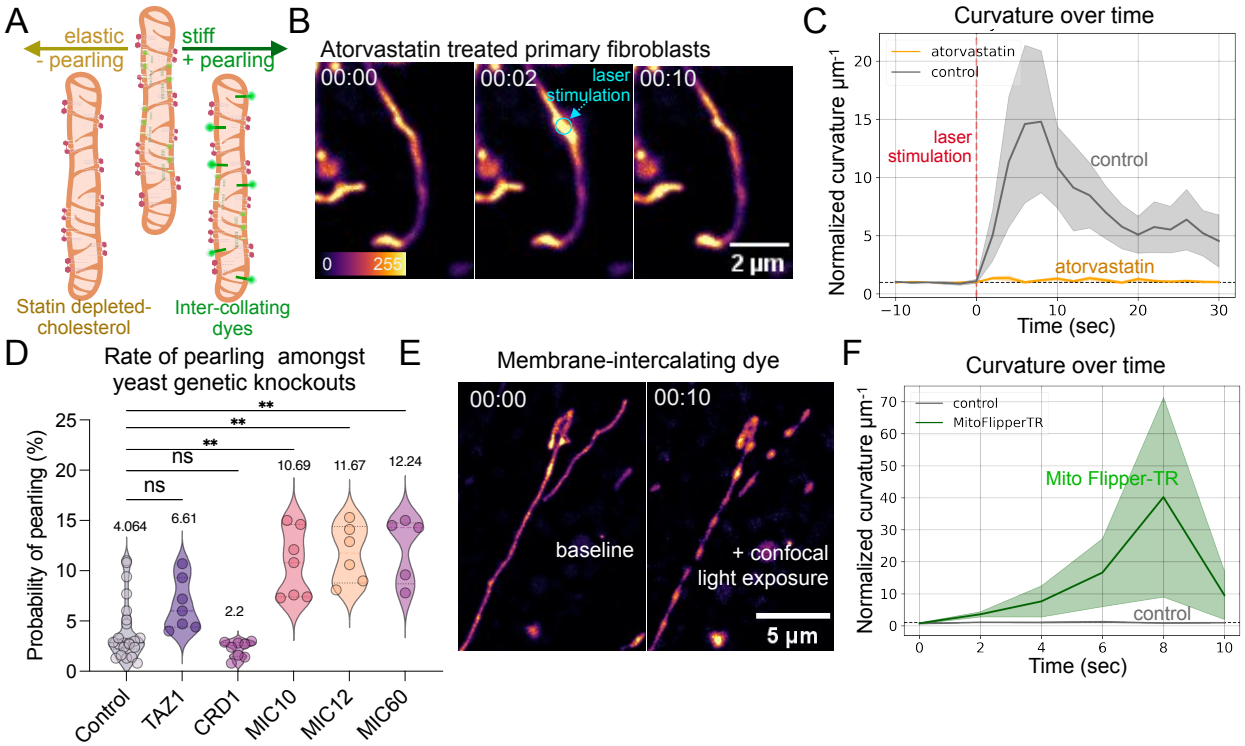

Figure 5. Altering mitochondrial membrane elasticity regulates pearling dynamics. (**A**) Schematic of how membrane content affects the biophysical properties of the mitochondria and influence its likelihood to pearl. (**B**) Mitochondria of a primary fibroblast treated overnight with 50 µM atorvastatin before and after 405nm laser stimulation (blue circle). Cells are labelled with PKmito Orange. (**C**) Time series of the membrane curvature along the mitochondrial tubule of control (n=11) and overnight atorvastatin-treated (24hr of 50µM, n=13) primary human fibroblasts. (**D**) Probability of a pearling occurring in a yeast cell over 100 volumes of imaging for wild-type, and genetic knockouts of TAZ1 & CRD1 (cardiolipin synthesis), and MIC10, MIC12, MIC19, MIC26, MIC27, and MIC60 (MICOS complex). Non-parametric Kruskal-Wallis test performed. ** = $p < 0.01$. (**E**) Images of mitochondria labelled with the intercalating membrane dye, Mito Flipper-TR (1 µM), before and after exposure to confocal laser light. Note: no stimulation laser was fired, rather just the field of view was illuminated to image the cell. (**F**) Time series of the membrane curvature along the mitochondrial tubule of control (n=9) and cells dyed with Mito-Flipper-TR (15-60 minutes 1 µM, n=10) primary human fibroblasts.

Alternative mechanisms of mitochondrial pearling

Other potential mechanisms of mitochondrial pearling include dynamin-like fission machinery, ER constriction events, ROS oxidative stress, actin waves, and mtDNA nucleoid colocalization. In regard to the idea that DRP1 constrictions create the pearls, several studies reported increased pearling when DRP1 and related fission machinery was removed [5,15,16], clearly showing that DRP1 mediated fission is not required. Regarding the idea that ER rings squeeze the mitochondrion into pearls, we colabelled ER and mitochondrial networks and saw ER network structures often did not form ring shapes around pearl constriction points (**Figure 6A-C, Movies 34-37**), with 25% of mitochondrial pearl constriction points forming without any nearby ER signal labeled with KDEL and calreticulin probes (**Figure 6D**).

To rule out the possibility of phototoxic initiation of pearling events we examined the distribution of pearling events across all cell types (**Figure 6E**). We did not see any increase of pearling events with time, and additionally observed 9 events at the first volume of imaging showing that pearls were already formed in the absence of illumination (**Figure 6G**). Regarding the idea that ROS-related oxidative stress drives pearling, treatments of cells with antioxidants mitoTEMPO (20 nM) and N-acetyl cysteine (NAC, 5 μM) failed to inhibit the induction of pearling by laser stimulation (**Figure 6F**). Additionally, co-labeling with the mitochondrial-specific ROS markers, MitoSOX green and MitoSOX, red did not increase at the onset of laser-stimulated pearling, with a change in +0.4% and -1.2%, respectively (**Supplementary Figure 5E**). Mitochondrial intermembrane ROS sensor, Hyper7 did increase by 18.3% directly after laser-stimulation but this increase occurred across the all mitochondria of the cell, and was not specific to tubules that underwent pearling (**Supplementary Figure 5F-H, Movie 38**).

Colabeling with a microtubule marker, Sir-Tubulin, revealed that microtubule structure remains intact during laser induced pearling (**Figure 6H**). Further, treatment with microtubule depolymerization with 10 μM nocodazole did not stop the induction of pearling by laser-stimulation (Figure 3K) or by chemical-induction with 4 μM ionomycin (**Supplementary Figure 5I)**. Regarding the idea that actin mediated forces drive pearling, actin depolymerization did not stop spontaneous pearling events (**Figure 6I**) or pearling induced by 4 μM ionomycin (**Supplementary Figure 5J**). Instead, the lack of actin inhibits pearls from recovering back to the tubule form (Figure 3K).

Another explanation of pearls could be a reorganization of mtDNA nucleoids to discrete spheres marking membrane pearls. In U2OS cells, 77% of pearls marked by the mitochondrial inner membrane colocalized with mtDNA nucleoids, while in primary fibroblasts only 20% of pearls colocalized with nucleoids (**Figure 6J-L**). Further depletion of mtDNA with ethidium bromide did not stop the induction of pearling by laser-stimulation (**Figure 6K-L**). This ruled out the possibility that pearl shapes on the membrane were downstream of nucleoid structures.

Given the inability of these molecular mechanisms to account for the pearling that we observed, we instead turned to a biophysical mechanism, whereby ionic flux and tension forces within the cell can explain the onset spontaneous pearling that coincides with either class I calcium waves passing across cells and amplified by many different experimental contexts [40,51–53] or class II molecular motors pulling on the mitochondrial membrane [54,55]. These forces could be the source of osmotic pressure and mechanical tension on the mitochondrial tubule that cause spontaneous pearling events.

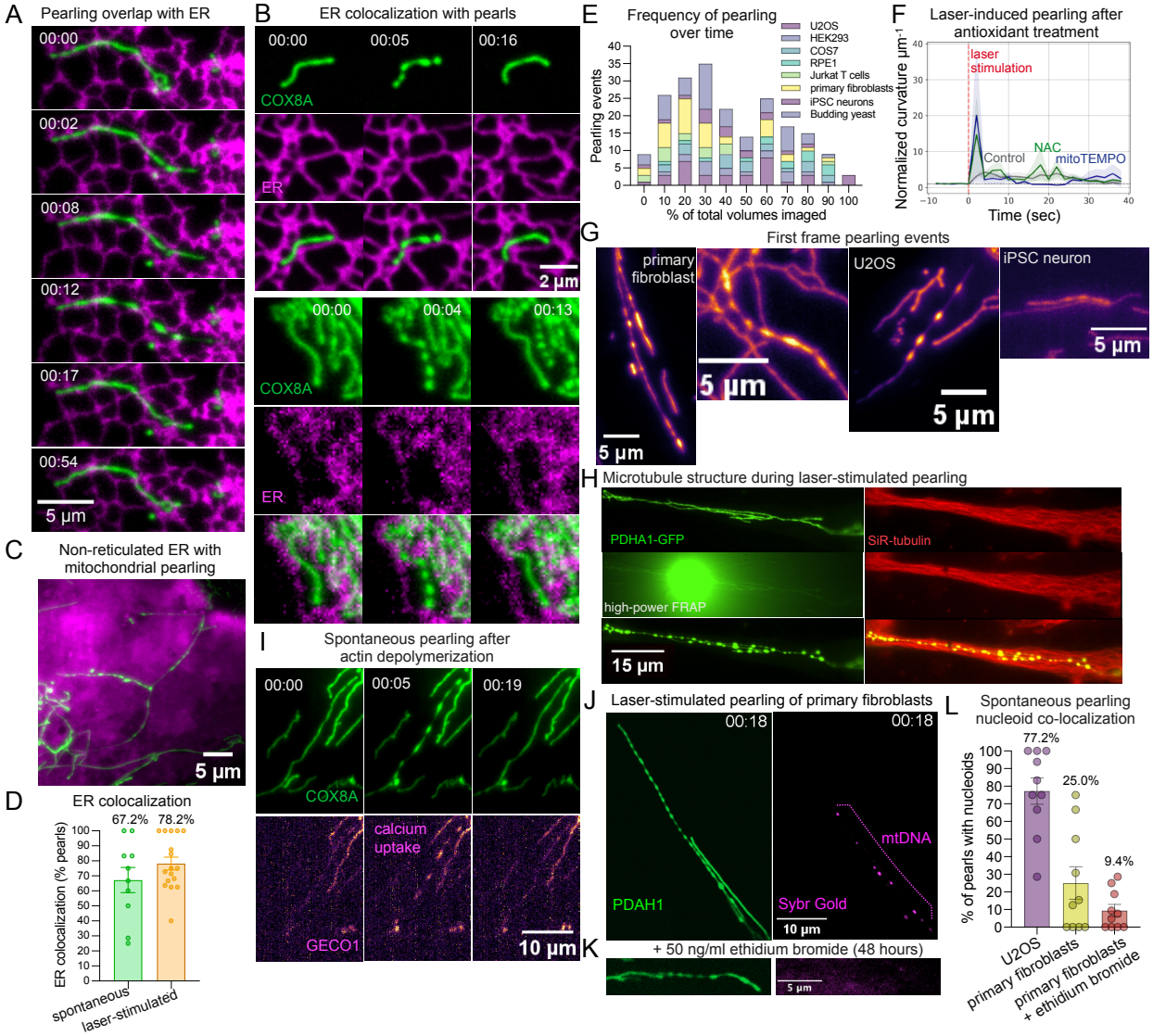

Figure 6. ER constrictions, oxidative stress, microtubule organization, actin polymerization, and nucleoid localization fail to regulate mitochondrial pearling dynamics (**A-B**) Spontaneous pearling events with both mitochondria (COX8A-mEmerald) and ER (KDEL, CellLight-ER-RFP) labelled in U2OS cells. (**C**) A cloudy ER network surrounding mitochondrial pearls in a U2OS cell. Mitochondria (COX8A-mEmerald) and ER (KDEL, CellLight-ER-RFP) labelled. (**D**) Percentage of pearl constriction points with or without overlapping ER fluorescence signal from both U2OS and primary fibroblasts either from spontaneous events (green, n=10 events) or laser-induction (orange, n=17 events). (**E**) Frequency distribution of the frame at which a pearling event was first observed normalized to the total number of frames (i.e. 3D volumes) taken in a video. (**F**) Mean curvature per µm over time for primary fibroblasts treated with antioxidants 5 µM NAC and 20 nM mitoTEMPO (n=10-14 events per treatment group). Red line indicates point of laser stimulation. (**G**) Mitochondrial pearls imaged in the first frame of a timelapse video. (**H**) Laser-induced pearling with both the mitochondrial inner membrane (green, CellLight MitoGFP) and microtubules (red, Sir-tubulin) labelled. (**I**) A spontaneous pearling event observed in U2OS cells treated with 1 µM latrunculin B for 1 hr. Top panel contains the mitochondrial inner membrane (COX8A-mStayGold) and the bottom panel contains mitochondrial calcium (Mito-R-GECO1). (**J**) Mitochondrial pearls overlapping with mtDNA (Sybr Gold) induced by laser-stimulation of a primary fibroblast mitochondria in (I) untreated, and (**K**) DNA intercalating ethidium bromide (50 ng/mL) for 48 hours. IMM = CellLight MitoGFP, mtDNA = Sybr Gold. (**L**) Quantification of the number of pearls with overlapping mtDNA signal for U2OS cells (purple),

primary fibroblasts (yellow) and primary fibroblasts treated with 50 ng/ml ethidium bromide for 48 hours. n=10 events per group.

Micro-needle manipulation induces mechanical force-based pearling on individual mitochondria tubules.

The chemical and laser induction of pearling described above can all be explained by the influx of ions resulting in mitochondrial shrinkage, swelling, and osmotic pressure. An alternative mechanism for pearling via the Rayleigh-Plateau instability could involve mechanical forces pulling on the membrane such as class II stretching-associated spontaneous pearling events observed above. To test this idea, mechanical forces were applied to individual mitochondrial tubules within the cell using a fluorescently-labeled glass microneedle with a triple axis (X,Y,Z) 56 nm step-motor (**Figure 7A**)[56]. Bringing the micro-needle within a micron of the mitochondrial tubule induced a rapid and transient pearling transition on the specific mitochondria of choice (**Figure 7B, Movie 39**). Like laser-induced pearling, depolarizing the mitochondria with the proton ionophore FCCP inhibited the ability of needle force to convert tubules to pearls (**Figure 7E, Movie 40**). Attempting to further manipulate the tubule by pulling the depolarized tubule largely resulted in cutting (i.e. mechanical fission) at the point of needle manipulation. Regardless of whether the mitochondria were polarized or not, the insertion of the needle into the cell often induced a rise in cell-wide mitochondrial calcium uptake, which was quickly followed by mitochondrial pearling across the entire cell (**Figure 7C-D, Movie 41**). These global pearling events may be a result of actin waves caused by the introduction of the needle in the cell. Previous work has shown the calcium ionophore, ionomycin, induces actin waves followed by calcium uptake into the mitochondria and an increase the rate of pearling events [18].

To overcome the needle's tendency to cause global pearling, the actin polymerization inhibitor, Latrunculin B, was applied (1 μM for 30-60 minutes). In actin inhibited cells, manipulated mitochondria tubules were more likely to undergo localized pearling at the targeted mitochondria, while non-manipulated remained stably cylindrical (**Figure 7C-D**) and calcium uptake remained low in non-manipulated mitochondria (**Figure 7G**). This further dispels the idea that actin is itself the constrictive actor that forms mitochondrial pearls. Remarkably, with actin inhibited, the needle could pull on the mitochondrial strand resulting in a transition to a pearled morphology specifically on the portion of the tubule undergoing deformation (**Figure 7H, Movie 42**). Further, in some cases this same force-induced pearling preceded a rise in calcium uptake by the mitochondria (**Supplementary Figure 7, Movie 43**). These experiments highlight a scenario in which calcium on its own was not sufficient to induce pearling. Rather, only with an added mechanical force on the membrane was a pearling instability reached. Thus, both the internal osmotic pressure of ionic flux as well as external mechanical tension of the membrane can result in a pearling instability across the mitochondrial tubule.

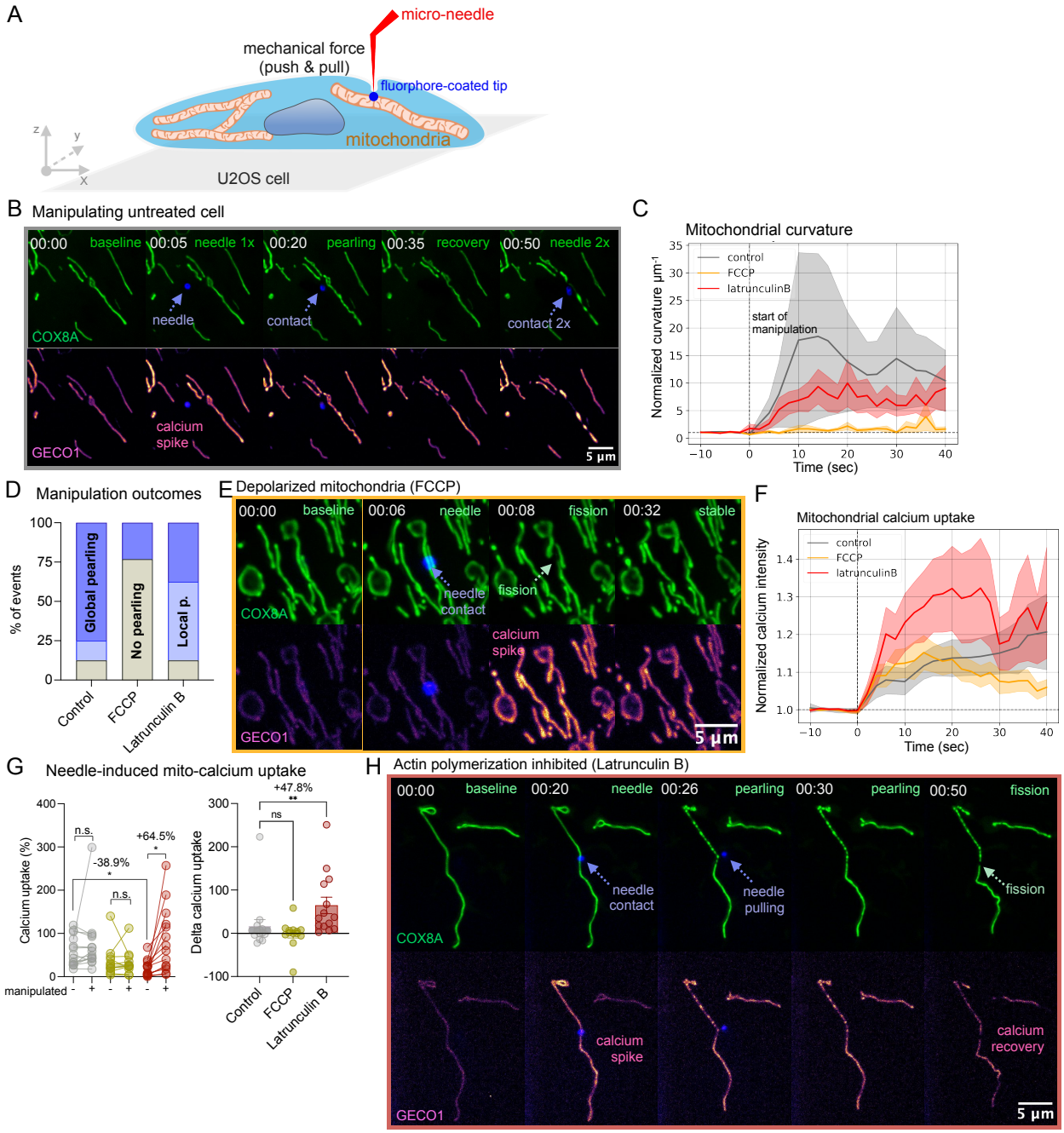

Figure 7. Micro-needle mechanical force triggers localized mitochondrial pearling.
(**A**) Schematic of micro-needle experimental design. Red needle is manipulated in x, y, z dimensions with a 56 nm step-size control. The tip is coated with fluorescent pigment (far-red channel, labeled dark-blue in figure). Pico-nano newton forces are then applied to individual mitochondrial tubules with push and pull forces. (**B**) Representative time series of untreated U2OS cell mitochondria manipulated with micro-needle. Mitochondrial inner membrane labeled with COX8A-mStayGold (green). Mitochondrial calcium flux labeled with Mito-R-GECO1 (red). Needle was pushed against tubule before being removed from the cell and then reapplied for a repeated manipulation of the same tubule. (**C**) Curvature over time for single mitochondria tubules manipulated with micro-needle in untreated controls (n=14 cells), FCCP (10µM, 10-60 minutes, n=13 cells) and Latrunculin B (10µM, 30-60 minutes, n=16 cells). Thick lines represent mean weighted curvature per µm normalized to the first 3 timepoints before manipulation. Error bars indicate standard error of the mean. Dotted vertical line indicates the beginning of needle manipulation on the tubule. Curvature values are weighed per µm of distance and normalized to the mean of the first three pre-pearl timepoints. (**D**) Categorical outcome

from attempted micro-needle manipulation of mitochondria. No pearling (gray) indicates that curvature of all mitochondria tubules remained stable throughout the experiment. Local pearling (light-blue) indicates pearling was uniquely induced on the tubule targeted by the needle. Global pearling (dark-blue) indicates that all other tubules in the cell underwent pearling whether or not it was targeted by the micro-needle. 13-16 cells per treatment group. (**E**) Representative time series of U2OS cell mitochondria depolarized with 10μM FCCP for 30 minutes and manipulated with micro-needle. (**G**) Calcium uptake derived from Mito-R-GECO1 maximum intensity values after the needle is present in the cell for non-manipulated (-) and manipulated (+) mitochondria. Right-panel shows delta calcium uptake between the mitochondria manipulated by the needle and those untouched. (**H**) Representative time series of manipulated mitochondria from a U2OS cell with actin polymerization inhibited by 10μM Latrunculin B for 45 minutes.

Discussion

Mechanism of pearling induction

Our biophysical model of mitochondrial pearling describes the organelle as a network with electrically coupled membranes under tension that exists near a critical threshold of instability. Under sufficient tension these tubules collapse and reorganize into thinly connected spherical units. This instability is regularly crossed due to changes in three physical forces. First, the internal osmotic pressure resulting from the ionic flux of cations entering the mitochondrial matrix. Second, from changes to elasticity of the membrane such as addition of cholesterol or removal of cristae junctions. Third, from external forces pulling on the membrane. Ultimately, all three forces can result in the membrane tension surpassing its bending energy (**Figure 8**). This biophysical mechanism unifies mitochondrial pearling events observed in diverse chemical, physical, and genetic, and physiological scenarios (**Supplementary Figure 8**). In physiological scenarios such as neuronal action potentials, and T cell synapse formation, pearling is likely a result of ionic flux, which results in an influx of water molecules, matrix swelling, and osmotic pressure on the inner membrane thereby collapsing the mitochondrial tubule into pearls.

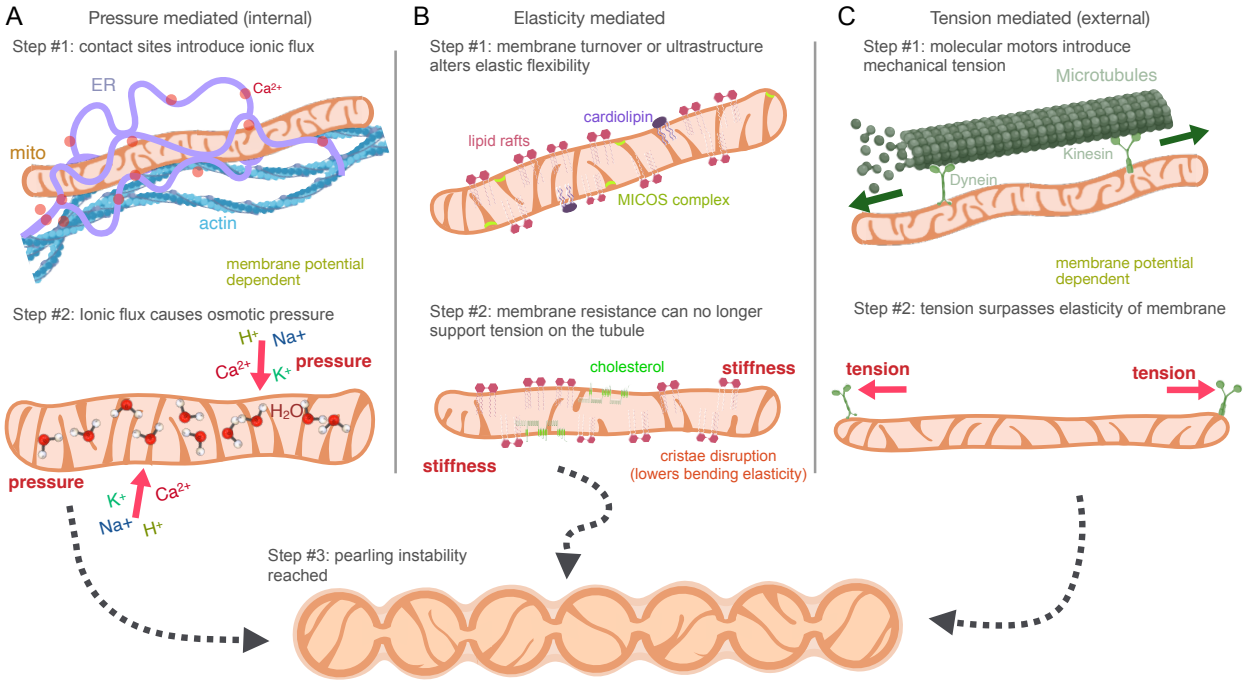

Figure 8. Biophysical mechanism of mitochondrial pearling dynamics. (**A-C**) Pressure, tension, and elasticity are the three main physical properties that influence the pearling instability of the mitochondria membrane. (**A**) Contact sites with other organelle systems introduce ionic flux on the mitochondrial membrane. Ionic flow into the mitochondria causes an osmolarity difference between the inside of the mitochondria causing an influx in water. Osmotic pressure increases tension on the membrane which exceeds its bending elasticity and collapses the mitochondrial tubule into pearls. (**B**) Alteration to components of the mitochondrial membrane such as cholesterol, cardiolipin, and cristae organization change the elasticity of the membrane and can collapse the tubular mitochondria into pearls. (**C**) Molecular motors traffic mitochondria and introduce external mechanical tension through

pulling forces on the membrane. Mechanical tension exceeds the bending elasticity of the membrane and collapses the mitochondria tubule into pearls, following a Rayleigh-plateau 'pearling' instability.

Spontaneous pearling triggered by multiple physical forces

With this biophysical framework, the two different types of spontaneous pearling we have uncovered, stretching and calcium associated pearling, respectively, can be driven by two distinct biophysical causes. On one hand, stretching can be sourced from motor proteins on the cytoskeleton network tethered to mitochondria and pulling the membrane [57]. This is directly validated by activation of these motors with optogenetically-activated motors, which results in force-dependent elongation of mitochondrial tubules followed by network wide mitochondrial pearling events within the stimulated cell [58]. On the other hand, spontaneous events that coincide with calcium uptake and membrane shrinkage may be explained by calcium waves propagating through the cell that are soaked up by mitochondria's buffering capacity [59] leading to internal osmotic pressure. We further identified genetic and metabolic perturbations that validate the importance of the mitochondrial inner membrane's high elastic modulus supported by cristae ultrastructure. Removal of lipid packing components, cholesterol or cardiolipin, as well as essential junction proteins of the MICOS complex alters the mitochondria's capacity for crossing a pearling instability. Therefore, only by characterizing the state of individual mitochondrial tubules in terms of the osmotic pressure, mechanical tension, and its membrane's elastic and electrochemical state can one properly predict a pearling transition.

Electrochemical coupling supports mitochondrial pearling

Our findings indicate that the membrane polarization maintained by the IMM is a primary regulator of pearling transitions. This suggests that the flux of ions and water that build tension on the IMM are supported by the electrical coupling of the IMM. A future direction of this work would refine our biophysical model of mitochondrial pearling to explain how the inner membrane's electrochemical coupling is capable of regulating pearling events. There remains open questions, such as why depolarized mitochondria are unable to undergo a pearling transition spontaneously or by laser stimulation and micro-needle force? Yet, depolarized mitochondria still pearl from osmotic pressure, such as 0.1x PBS or ionomycin. We postulate that membrane polarization reinforces lipid organization of the mitochondrial inner membrane. Polarized inner membranes may induce a conformation of lipids that supports increased tension on the membrane. Without the electrochemical gradient, mitochondrial membranes become "soft" with disorganized lipids and thereby unable to maintain higher tension states. Further, like a neuronal action potential, the electrical pulse resulting from membrane depolarization should be accompanied by a mechanical wave that propagates across the membrane [60]. This electrically-driven mechanical wave may drive the structural reorganization necessary for pearl formation.

Ionic flux, not oxidative stress, explains the onset and frequency of pearling transitions

We report that the frequency of spontaneous pearling events is dependent on the amount of light exposure on a given sample. A common explanation of this phototoxic effect [16] is an increase in ROS which causes oxidative damage on macromolecules. Downstream it is speculated that the mitochondria induce mitophagy to recycle these damaged materials. With this logic, pearling may therefore represent a damage-response to isolate dysfunctional materials for autophagy. We attempted to inhibit pearling by addition of antioxidants NAC and MitoTEMPO. However, none of them were capable of altering the pearling induced by laser stimulation (Figure 6K). Additionally, using the mitochondrial ROS sensor, mitoSOX green and red, no detectable increase of the fluorophore was observed. The intermembrane ROS sensor, Hyper7-IMS, revealed increased H2O2 directly following laser-stimulation but was not specific to tubules that underwent a pearling transition. Regardless of the role of ROS, we contend that this hypothesis does not adequately explain the structure and dynamics of the observed pearling event. Why do the constriction sites appear at regular intervals? Why is it a modular event affecting connected tubules? Why does the tubule transiently recover back to a cylindrical morphology? Without an understanding of the biophysical properties of the mitochondrial membranes themselves these questions remain unanswered. That being said, our work does corroborate the link between increased light exposure and increased pearling transitions. We report that the frequency of pearling events is increased on higher-power imaging systems. We are confident that these pearling events, although amplified by exposure to laser power, occur spontaneously for several reasons. i) we observe these spontaneous events at the beginning of imaging an individual cell, without previous exposure to light (Figure 6F). ii) the distribution of spontaneous pearling events does not increase the longer the sample is imaged (Figure 6E). iii) The events are transient and rapidly revert back to tubular shapes unlike direct exposure to high power-laser or LED light which results in a permanent pearled state that aggregate into larger spherical shapes. iv) phase-contrast imaging of cells still reveals pearling events (Landoni et al., 2024).

Chemical fixation induces pearling artifacts

Paraformaldehyde has previously been reported to be osmotically active [39]. Others have found that the addition of glutaraldehyde can mitigate this artifact through its faster fixation rate [61–63]. It is worth noting that in addition to a pearling artifact, the reverse 'hyper-osmotic' artifact of flaccid tubules of mitochondria is also visible at higher levels of glutaraldehyde (**Supplementary Figure 3D-E**). It is for this reason that a titration of PFA:GA should be performed under the microscope to maintain ideal ultrastructure. Alternatively, switching to high-pressure freezing or cryo-fixation should achieve the same aim [30]. With this in mind, it is possible that earlier reports

of mitochondrial pearling in tissue samples [6,13] may be an artifact of the osmotic pressure exerted by 4% PFA during perfusion of the animal.

Functional utility of mitochondrial pearling

Beyond generating ATP, one of the core functions of mitochondria is to buffer ions undergoing flux within cells. Mitochondrial membranes contain many species of ionic pumps regulating the flux of H+ (ATP synthase), Ca2+ (calcium uniporter, MCU), K+ (mitoKATP, , mitoBKCa, mitoKv, mitoTASK, mitoIKCa), Na+ (mitoSLO2), Mg2+ (MRS2), and Cl- (CLIC4&5, Ano-1). The fact that the presence of each channel varies from tissue to tissue and we have observed pearling in diverse cell types across evolutionary distinct species, hints that it is unlikely for a specific ion channel to regulate the entirety of pearling events. However, it is still possible that many of these pores evolved in order to mitigate or potentiate pearling events for cellular homeostasis. We have yet to establish how pearling affects mitochondrial function, particularly energy production. This is largely because current readouts of bioenergetic output, namely Seahorse extracellular flux analyzer, measures oxygen consumption on the order of minutes and pearling occurs on the order of seconds. Future bioenergetic instrumentation may enable access to energy production on the same timescale as a pearling event. We hypothesize that energy production will follow a biphasic effect with increased OXPHOS as the IMM increases in tension and approaches instability followed by OXPHOS precipitously declines upon crossing the instability. Supporting evidence is the fact that membrane potential collapses after the onset of pearling (Figure 3H-I). This leads us to believe that in energetically demanding cells, such as muscle cells, mitochondria are driven to the threshold of membrane tension to maximize energy production. Pearling, in this context, represents a physical barrier or breakpoint that these cells strive to avoid. However, in other cell types such as neurons or cardiomyocytes, mitochondria's primary function is to buffer ions to help potentiate action potentials periodically moving across the cell [59,64]. In this context, pearling dynamics may be a necessary event to support ionic buffering and ionic channels are tuned to ensure rapid pearl recovery for periodic neuronal activity.

Fission machinery reduces network tension

We hypothesize that the reason why mitochondria of Drp1 knock-out cells have an increased rate of pearling event [5,15] is because with a hyperfused network pearling events will spread along all physically coupled segments of material, as we have shown with laser-stimulated induced pearling. With fission blocked, the spontaneous pearling events, whether triggered by calcium microwaves or motor protein pulling, will spread through the interconnected tubules, instead of the typical event occurring along a relatively isolated segment of mitochondria. We further argue that without fission, hyperfused mitochondria will enter a higher tension regime,

similar to senescent fibroblasts, and thereby more readily cross a pearling instability from exposure to normal imaging light. Molecular fission machinery may thereby serve to mitigate the physical coupling of pearling, isolating these transitions to smaller regions of the cell.

In conclusion, our experimental results indicate that mitochondrial pearling is governed by the biophysical forces of elasticity, pressure, and tension. The ubiquitous presence of mitochondrial pearling in diverse biological contexts across evolutionary scales, places pearling as a core dynamic event of mitochondria alongside fission and fusion processes. Our unified biophysical mechanism bridges mitochondria dynamics with ionic flux, molecular motors, and cellular energetics. Pearling is thereby a prime example justifying mitochondria's role as the central processor of the cell [65] simultaneously integrating both biochemical and biophysical signals. Other mitochondrial dynamics such as fission and fusion were previously implicated in aging and age-related disease [66]. Mitochondrial pearling may similarly advance our understanding of key health transitions. With that in mind, this paper establishes a unified biophysical model to understand the driving forces of mitochondrial pearling.

Methods

Mammalian cell culture

Cell lines include U2O2, HEK293, COS7, RPE1, human primary fibroblasts (hFB12, hFB13), Jurkat cells, Raji cell, primary human CD8 T cells, and iPSC-derived neurons (**Methods Table 1**). All cells were maintained in normal 37°C incubator conditions. Immortalized cells were grown in DMEM (GlutaMax, 25mM glucose) supplemented with 10% FBS, passaged weekly, and maintained for less than 20 passages. Primary human fibroblasts were grown in DMEM (GlutaMax, 5.5mM glucose).

Non-adherent Jurkat cells were grown in RPMI media supplemented with 10% FBS. Cells were transduced with lentivirus labeled with COX8A-mitoStayGold using a spin-transduction protocol for 1.5 hours at 2400 rpm. Plating was performed in an 8-well chamber slide coated with 10 µg/ml fibronectin and 5 µg/ml ICAM-1 for 3 hours at 37°C. Jurkat cells were then co-cultured with SEE-pulsed Raji cells labeled with Sybr Gold (1:10,000x) in the chamber for 30 min at 37°C before imaging.

Primary human CD8 T cells were grown in X-VIVO 15 media (Lonza #04-418Q) with gentamicin and phenol red, supplemented with 5% FBS, 4 mM n-cysteine, 55 mM 2-mercaptoethanol (Thermo #21985023). Primary cells were activated by three day incubation with DynaBeads Human T-Activator CD3/CD28 (Thermo #11131D). Beads were magnetically removed and cells imaged with 8 hours of activation. For synapse formation, Raji cells labeled with Sybr Gold (1:10,000x for 30 min) were co-cultured with primary T cells labeled with PKmito Orange (500 µM for 1hr). Synapse formation between T cells and Raji cells were found by scanning imaging plates for adjacent cells with distinct fluorescent labels.

iPSCs were cultured in mTESR Plus media (Stem Cell Technologies #100-0276) on hESC qualified matrigel (Corning #354277) coated tissue culture dishes. Differentiation to ventral midbrain identity was adapted from a previously described method [67] with modifications for 3D embryoid body based differentiation instead of 2D differentiation. On Day 0, iPSCs were dissociated into single cells with accutase and seeded into ultra low attachment 96 well plates at 5K cells per well to form embryoid bodies in Neural Differentiation Media (1:1 Neurobasal (Thermo #A3582901)/Advanced DMEM F12 (Thermo #12634010), N2 (Thermo #17502048), B27 -vitA (Thermo #12587-010), Glutamax (Thermo #35050-061), Sodium Pyruvate (Thermo #11360070), NEAA (Thermo #11140050), Pen/Strep (Thermo #15140-122) and 55 µM b-ME (Thermo #21985-023) supplemented with 10uM SB431542 (Selleck Chemicals #S1067), 100 nM LDN193189 (Selleck Chemicals #S2618), 0.75 µM CHIR99021 (Thermo #S1263), 500nM SAG (Thermo #S6384), 100 µM Ascorbic Acid (Sigma #A4034-100G) and 10 µM Y-27632 (BioTechne #1254). After 2 days media was exchanged to remove Y-27632. On day four of differentiation media was exchanged and CHIR99021 concentration was increased to 7.5 µM. On Day 7 of differentiation

embryoid bodies were collected and transferred to 10 cm low attachment plates using wide bore pipet tips and incubated for the remainder of differentiation on an orbital shaker at 40 rpm. Media was changed to Neural Differentiation Media supplemented with 7.5 μM CHIR99021, 500 nM SAG, and 100 μM AA until Day 10. On Day10 CHIR99021 and SAG were removed and 0.5 ng/mL TGF-b (Bio-Techne #243-B3-002/CF) and 200 μM dbcAMP (Sigma #D0627- 1G) was added. On Day 12 media was exchanged and supplemented with 10 μM DAPT (BioTechne #2634/50). On Day 15 media was exchanged to the same conditions with an additional supplement of 10 ng/ml BDNF (Bio-Techne 11166-BD-01M) and GDNF (Bio-Techne #212-GD-01M/CF). Media was maintained the same changing every 3-4 days until dissociation. On Day 20-22 of differentiation EBs were collected and dissociated at 37°C in Accumax(~15 EBs/ml of Accumax). EBs were incubated at 37°C with agitation. Following 15 mins EBs were pipetted with p1000 narrow bore tips to check state of dissociation. If still intact then EBs were returned to the shaker. Following 30 mins of enzymatic digestion 25 μg/ml DNase1 was added and EBs were manually triturated 5-10 times to begin manual dissociation. If EBs begin to dissociate then they are manually triturated ~10-20 times, if not they are returned to the shaker for an additional 10 mins. EBs were dissociated by manual trituration into single cells with one volume of media. After dissociation neurons were pelleted by centrifugation and then filtered through a 30 μm cell strainer before plating to remove clumps. Neurons were plated in Neuronal Maintenance Media (Neurobasal Plus, B27 plus, Glutamax, Pen/Strep and 55 μM b-ME) supplemented with 10 ng/ml BDNF, 10 ng/ml GDNF, 100 μM Ascorbic Acid, 200 μM dbcAMP, 10 μM DAPT and 20 mM each of Uridine (Sigma #U3750) and Fluorodeoxyuridine (Sigma #F0503) for 1.5 weeks. Neurons were plated on 8-well chamber slides pre-coated with poly-ornithine, laminin and fibronectin. For imaging experiments, neuronal cultures were labelled with PKmito Orange (500 μM for 1 hr) and CalBryte (1 μM for 1hr) followed by a 3x media wash and a final addition of probenecid (1 mM).

Cell line	Cell type	Species	Media	Source
U2OS	epithelial	human	DMEM (25mM glucose)	ATCC HTB-96
HEK293	kidney	human	DMEM (25mM glucose)	ATCC CRL-1573
RPE1	fibroblast	epithelial	DMEM (25mM glucose)	ATCC CRL-4000
COS7	fibroblasts	monkey	DMEM (25mM glucose)	ATCC CRL-1651
hFB12	fibroblasts	human	DMEM (5.5mM glucose)	Lifeline CellTech FC-0024 Lot # 03099
hFB13	fibroblasts	human	DMEM (5.5mM glucose)	Lifeline CellTech FC-0024 Lot # 00967
Jurkat	T cells	human	RPMI	ATCC TIB-152
Raji	B cells	human	RPMI	ATCC CCL-86
CD8 T cells	primary T cell	human	X-VIVO 15	in-house
iPSC	dopaminergic neurons	human	Neurobasal/Advanced DMEM F12 mTESR Plus	in-house

Methods Table 1. List of mammalian cell types images for mitochondrial pearling dynamics

Yeast culture

Budding yeast were grown in synthetic complete medium supplemented with 2% glucose and 2% methyl-alpha-D-mannopyranoside (MMP, Sigma #M6882) to the stationary phase. Cells were plated in pre-coated overnight with 1:100 concanavalin A 8-well glass-bottom chamber slides (CellVis #C8-1.5H-N). Yeast knockout strains are listed in **Methods Table 2**.

Knockout	Yeast strain	Target	Label
HO (Control)	CGY14.31xYKO <ho>	HO	preCox4 mNeon-green
CRD1	CGY14.31xYKO <crd1>	cardiolipin synthesis	preCox4 mNeon-green
TAZ1	CGY14.31xYKO <taz1>	cardiolipin synthesis	preCox4 mNeon-green
MIC10	CGY14.31xYKO <mic10>	MICOS complex subunit	preCox4 mNeon-green
MIC19	CGY14.31xYKO <mic19>	MICOS complex subunit	preCox4 mNeon-green
MIC60	CGY14.31xYKO <mic60>	MICOS complex subunit 60	preCox4 mNeon-green
Control	CGY49.77	wildtype	TIM50-mNeon-green
MIC60	CGY57.57	MICOS complex subunit 60	TIM50-mNeon-green

Methods Table 2. Yeast knockout strains screened for pearling

Fluorescent Labeling

All fluorescent labels used in the current study are listed in **Methods Table 3**. Transiently transfection of cells for Mito-R-GECO1 plasmid (Addgene #46021) was performed overnight using 0.2 µl lipofectamineTM 3000 (Thermo #L3000008) with 250 ng of plasmid DNA and 0.2 µl P3000™ reagent following the manufacturer's protocol. Stable cell line of COX8a-mEmerald was made by transfection with 1 µg of DNA using the SE Cell Line Nucleofector Kit (Lonza #V4XC-1032) following the manufacturer's protocols. Transfected cells were cultured on collagen-coated plates to facilitate the recovery process. After two days, cells were selected with 1mg/ml Geneticin Selective Antibiotic (G418 Sulfate, ThermoFisher #10131035) for a week. Fluorescence signals were continuously monitored during the selection process. Upon recovery, G418 at a concentration of 0.5 mg/ml was used for stable cell line maintenance. FACS-sorting for moderate-expression cells was performed to enhance the homogeneity of cells containing labeled mitochondria.

Lentivirus construction of COX8A-mStayGold and Mito-R-GECO1 was constructed with an RSV promoter, EF-1a promoter, and puromycin resistant gene using VectorBuilder custom pipeline. Virus was added at 20 particles per cell (ppc) with 10 µg/ml polybrene (PL200) and incubated overnight. Cells then recovered in normal media for 4-7 days. Selection was performed with 1 µg/ml Puromycin Dihydrochloride (Thermo #A1113803). FACs-sorting was used to filter for high-expression cells (top 10%) to enhance homogeneity and maximize fluorescent intensity signals. Cells infected with both constructs were engineered in sequential transductions.

Dye staining was performed to cells as follows; PKmito Orange was applied at 500 nM for 1 hour at 37°C standard incubation, followed by 2x media wash, two hour incubation, and another 2x media wash. Sybr Gold staining was performed 10,000x dilution for 30 minutes at 37°C standard incubation, followed by a 3x wash. Fluo4 was added 2 µM for 30 minutes at 37°C and then addition of 0.02% Pluronic™ F-127 (Thermo #P3000MP). Calbryte™ 520 AM was added at 1 µM for 1 hour at 37°C followed by a 3x media wash and addition of the anion transporter inhibitor, probenecid (1mM). Fluo4 was added for 30 min at 1 µM followed by a 1x media swap and addition of 0.02% Pluronic™ F-127 (Thermo #P3000MP).

Label	Category	Target	Properties	Source
PKmito Orange	dye	IMM	membrane potential dependent	Cytoskeleton Inc #CY-SC053
Sybr Gold	dye	mtDNA intercalation	membrane potential dependent (partial)	Thermo #S11494
TMRE	dye	mitochondrial inner matrix	Mitochondrial membrane potential dependent	Thermo # T669
CellLight Mito-GFP	transient plasmid	PDHA1 IMM and matrix	Marker of mitochondrial structure	Thermo #C10600
CellLight Mito-RFP	transient plasmid	PDHA1 IMM and matrix	Marker of mitochondrial structure	Thermo #C10601
CellLight ER-RFP	transient plasmid transfection	ER	Marker of ER structure	Thermo #C10591
COX8A-mEmerald	genetically encoded sequence	COX8A protein on the IMM and mitochondrial matrix	geneticin selection, FACS-sorted	Addgene #54160
COX8A-mStayGold	lenti-transduction	COX8A protein on the IMM and mitochondrial matrix	lentivirus package, puromycin selection, FACS-sorted	https://doi.org/10.1038/s41592-023-02085-6
Mito-R-GECO1	lenti-transduction	mitochondrial calcium	ratiometric intensity mitochondrial calcium flux	Addgene #46021
MitoSOX red	dye	mitochondrial ROS	mitochondrial superoxide indicator	Thermo #M36008
MitoSOX green	dye	mitochondrial ROS	Superoxide indicator	Thermo #M36005
Hyper7-IMS	dye	mitochondrial H_2O_2 ROS	mitochondrial intermembrane space hydrogen peroxide indicator	Addgene #136469
Calbryte™ 520 AM	dye	intracellular calcium	cytoplasmic calcium flux	AAT Bioquest #20651
Mito Flipper-TR	dye	mitochondrial membrane tension	mitochondrial membrane tension	Cytoskeleton #CY-SC023

Methods Table 3: IMM = inner mitochondrial membrane

Inhibitor	[ ] (μM)	Duration (min)	Known target	Pearling effect	Source
FCCP	10	10-60	mitochondrial membrane potential	full inhibition	Sigma #C2920
Oligomycin	1	10-60	ATP synthase inhibitor	inhibits recovery	Sigma #75351
Rotenone	4	10-60	complex II inhibitor	partial inhibition	Sigma #R8875
Antimycin A	2	10-60	complex II inhibitor	partial inhibition	Sigma #A8674
Latrunculin B	10	30-60	actin polymerization	inhibits recovery	Sigma #L5288
Nocodazole	10	30-60	microtubule polymerization	partial inhibition	Sigma #M1404-2MG
Taxol	10	30-60	microtubule stabilization	inhibits recovery	Sigma #PHL89806
Cyclosporin A	10	10-60	mPTP inhibitor	increased	Sigma #30024
Atorvastatin	50	240	cholesterol depletion	full inhibition	Selleck Chemical # S207750MG
NAC	5	24 hour	cytoplasmic antioxidant	n.s.	Thermo #A15409.36
MitoTEMPO	0.02	24 hour	mitochondrial-specific antioxidant	n.s.	Sigma #SML0737

Methods Table 4. Chemical inhibitors of mitochondria

Live cell imaging

Live cell imaging was performed on several systems including an in-house single-objective light-sheet (SOLS) microscope, a Nikon CSU-W1 spinning disk microscope with laser stimulation, and a Leica Stellaris TauSTED microscope. For all microscopes, cells were plated on fibronectin coated (Sigma #F1141-1mg) 8-well glass-bottom chamber slides (CellVis #C8-1.5H-N) and incubated overnight prior to imaging.

SNOUTY Light Sheet

The in-house single-objective light-sheet (SOLS) microscope [37] was equipped with a 100x NA 1.35 Silicone primary objective, a fast optical scanner (galvo) for quickly taking 3D data (up to ~100x100x30μm XYZ field of view), a Gaussian light-sheet (~12μm Rayleigh range) and a stage-

top incubator for maintaining a temperature of 37°C with 5% CO_2 throughout. The majority of the imaging was acquired at a frequency of 1 volume/second (1 ms exposures per image) using 5% 488 nm and 12% 561 nm laser powers (20.67 uJ and 70.47 uJ per volume on the sample, respectively) and a quad emission filter (zET405/488/561/635m). Max projected 2D image previews were generated from the 3D data. Deskewing of the 3D light-sheet data was computed using Snouty Viewer (v0.2.5).

CSU-W1 Spinning Disk and FRAP Laser-stimulation

Laser stimulation was performed on Nikon CSU-W1 spinning-disk confocal system paired with Andor Zyla sCMOS camera (5.5 megapixels), Nikon Perfect Focus system, Oko stage top incubator, and Vortran 405 nm photoactivation and photobleaching laser. Imaging was performed with 488 nm and 561 excitation lasers using a Plan Apo VC 100x/1.4 oil objective and a zET405/488/561/635m quad filter. FRAP laser was calibrated before each experiment using a 0.1mg/ml fluorescein-coated coverslip (Thermo #L13251.36). Laser power was recorded with a Microscope Power Slide Meter Sensor (ThorLabs #S175C). Full 3D z-stacks were acquired with 250 nm slices for a total of 5.25 µm depth across the cell. Imaging 2D time series videos were collected at 0.33 frames sec-1. For autofluorescence detection the camera was swapped to Andor DU-888 EMCCD camera ideal for amplifying low-emission signals.

Stimulated Emission Depletion Microscopy (STED)

For cristae labeling, cells were incubated with 500nM PKmito orange (PKMO, #CY-SC053, Cytoskeleton Inc. [68] for 1 hour at 37°C, followed by 4 successive washes over a two hour period. Additionally, mtDNA nucleoid staining was performed with 1:10,000x SybrGold (#S11494, ThermoFisher) for 30 min incubation at 37°C followed by a 3x wash. Images were acquired using an 86x water objective, taken at 7x zoom, 16x line averaging, and 1024x524 resolution with PKMO acquired with a 5% 591 nm white light laser excitation line, a 70% 775 nm STED laser depletion line, and SybrGold at 4% 488 nm excitation. Tau-Sted lifetime enhancement was applied at 100%.

Micro-needle manipulation

Microneedle manipulation was adapted for interphase U2OS cells based on previous techniques detailed in Suresh et al. Microneedles were made from glass capillaries with an inner and outer diameter of 0.58 mm and 1 mm respectively (1B100-4 or 1B100F-4, World Precision Instruments). Glass capillaries were pulled via micropipette puller (P-87, Sutter Instruments, Novato, CA), bent and polished using a microforge (Narishige International, Amityville, NY) according to the same specifications, parameters, and geometries described in detail in Suresh et al. These parameters allowed for the needle to approach cells orthogonal to the imaging plane and conduct manipulations without rupturing the cell (Suresh et al.) Prior to imaging microneedles

were coated with BSA Alexa Fluor 647 (A34785, Invitrogen) by soaking them in solution for 60s. The solution was created via dissolving BSA-Alexa dye and Sodium Azide (Nacalai Tesque, Kyoto, Japan) in 0.1 M phosphate-buffered saline (PBS) at a final concentration of 0.02% and 3 mM, respectively (Sasaki et al., 2012). The solution allows needles to be visualized via fluorescence imaging, aiding in positioning of the needle along a single mitochondrion. It should be noted, that due to technical constraints involved with manipulation experiments, the imaging chamber lid had to be removed. As such, the sample dish was only able to achieve a stable temperature of 30 C.

Cells for microneedle manipulation were chosen based on the following criteria: chosen cell was in interphase and cell possessed one or more mitochondria that are well separated from the surrounding mitochondria network. These criteria were important for perturbing single mitochondrion without affecting the entire network.

The micromanipulator was mounted to the scope body and positioned above samples as described in Suresh et al. Manipulations were performed in 3D using an x-y-z stepper-motor micromanipulator (MP-225, Sutter Instruments, Novato, CA). A 3-axis-knob (ROE-200) connected to the manipulator via a controller box (MPC-200, Sutter Instruments). Prior to manipulation, the needle was positioned via phase imaging at the approximate x-y position of the intended manipulation site ~ 10 μm above the cell in z. While imaging every 2 seconds at 3 z-planes (0.0 +/- 0.5 μm) the needle was manually lowered into place near a single mitochondrion. If necessary, the needle's position in x-y was changed by first raising the needle above the cell and repositioning in x-y before approaching the intended mitochondrion. Once properly positioned, the needle was manually moved to tap the mitochondrion to apply a brief mechanical load. After, the needle was slowly removed from the cell.

To be analyzed, the manipulated cells had to demonstrate all listed attributes: cell health was not significantly impacted via manipulation (ie cell rupture), mechanically challenged mitochondrion was clearly visible throughout the entirety of manipulation, the mitochondrion demonstrated slight deformation due to the applied mechanical load, a single mitochondrion was contacted by the needle.

Image Analysis

Image analysis was performed in fiji software as well as an updated version of MitoMeter [69] called Nellie [70] and available at https://github.com/aelefebv/nellie. This pipeline enables automated segmentation, tracking, and feature extraction of individual organelles over time. Metrics derived from Nellie include mitochondrial counts, tubule length, tubule width, aspect ratio, linear velocity, linear acceleration, angular velocity, angular acceleration. A separate pearl

detection script was added on the end of Nellie specifically to identify pearls and measure pearl shape. First a polygon mask was drawn over the pearled region. Pearls were then detected by applying Frangi filtering to enhance tubular structure within each image. The filtered images were thresholded to binary masks, followed by KDtree-based distance transformation and maximum filtering to identify local maxima for potential pearls. Candidate peaks were labeled and refined using a proximity-based region growing algorithm, followed by removal of small objects, and those touching image edges to eliminate false positives.

Frequency of pearling events

The frequency of pearling events was calculated with two approaches. The first method counted the number of pearling events in each video and normalized to the duration of imaging. The total number of mitochondria was measured using the Nellie software object detection, yielding the number of events per mitochondria per second. The event probability over 10 minutes was then calculated. The second method measured the total length of pearled mitochondria across a minute of time, and divided by the sum of lengths across all mitochondria in the cell outputted by Nellie software. To distinguish pearling from other dynamics such as fission events, inclusion criteria for pearling included, i) the mitochondria underwent a full transition from cylinder to pearls to cylinder. ii) the pearl formation contained at least three pearls, and iii) the pearls remained tethered to one another during the transition.

Intensity-based curvature

Curvature metrics were calculated on tracked skeleton line profiles of individual mitochondrial tubules over time outputted by Nellie software. Curvature was calculated using a 2D parametric curve formula to a spline fitted line across the skeleton. Smoothing factors were optimized for each time series to maximize the difference in curvature between a pearled morphology and the frame before the onset of pearling. Curvature was weighed by the length of the skeleton and normalized to the first 3 timepoints before the onset of pearling. Other extracted metrics include peak-to-peak distance, volume, length, width, height, duration of event, pre/post tortuosity, the duration of the full transition, time to peak of event, time of recovery, number of pearls, length of pearled segment, pearls per micron, width/length of tubule before and after pearling, number of pearling events per tubule.

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Author Contributions

G.S. conceptualized the study, designed and ran the experiments, analyzed the data, and wrote the initial draft of the manuscript

K.H. assisted in project conceptualization and experimental design

A.Y.T.L developed image analysis software and assisted in analysis

C.R. and S.D. developed and assisted in microneedle experiments

D.I. assisted in running laser stimulation experiments

M.C. generated iPSC neurons

K.M.T. assisted in experimental design

W.L. designed and assisted in T cell activation experiments

B.R. and A.W. generated yeast knockout strains

J.C.L assisted in project conceptualization and protocol development

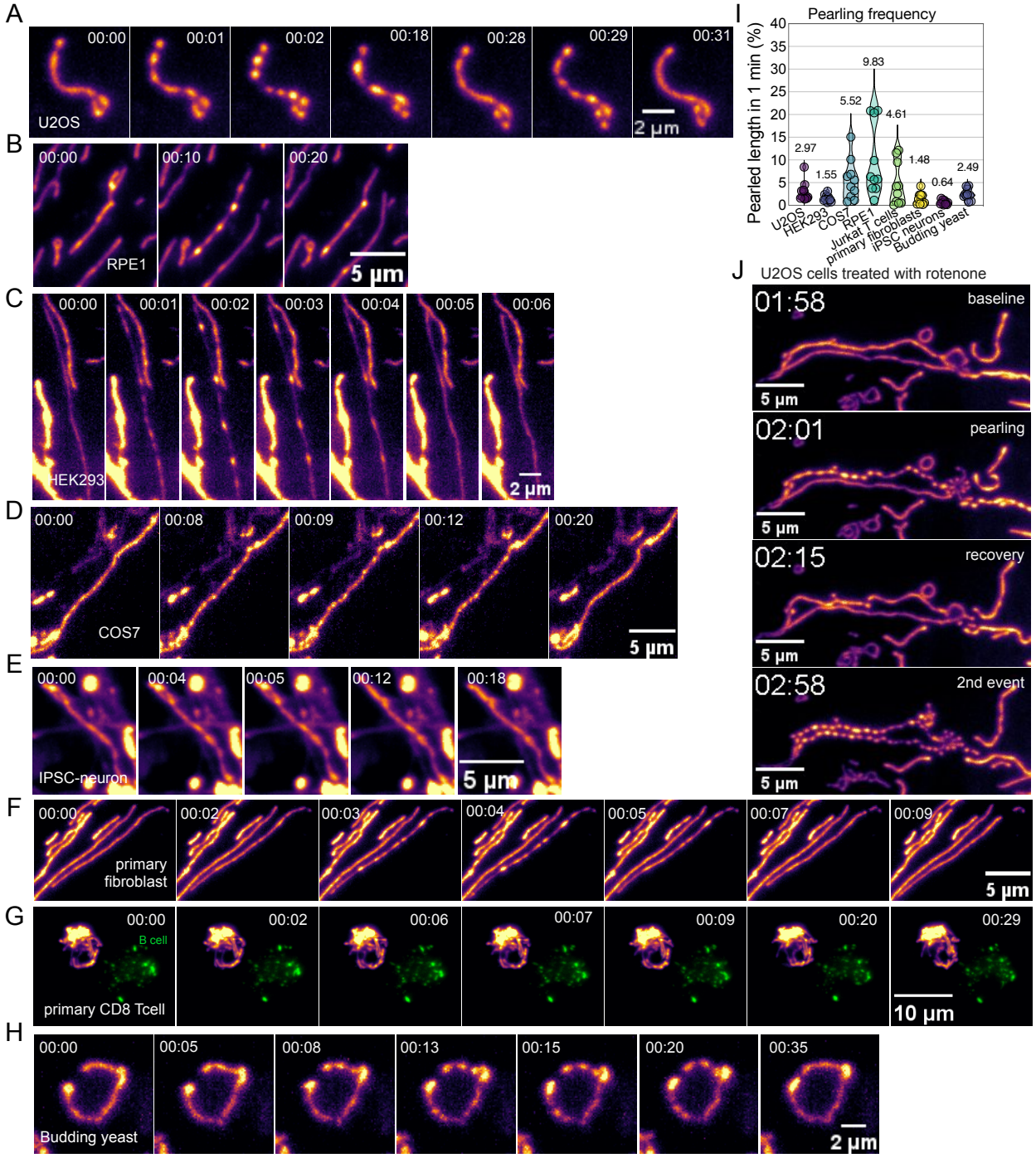
W.M. and S.M. secured funding and assisted in project conceptualization

All authors reviewed and edited the manuscript

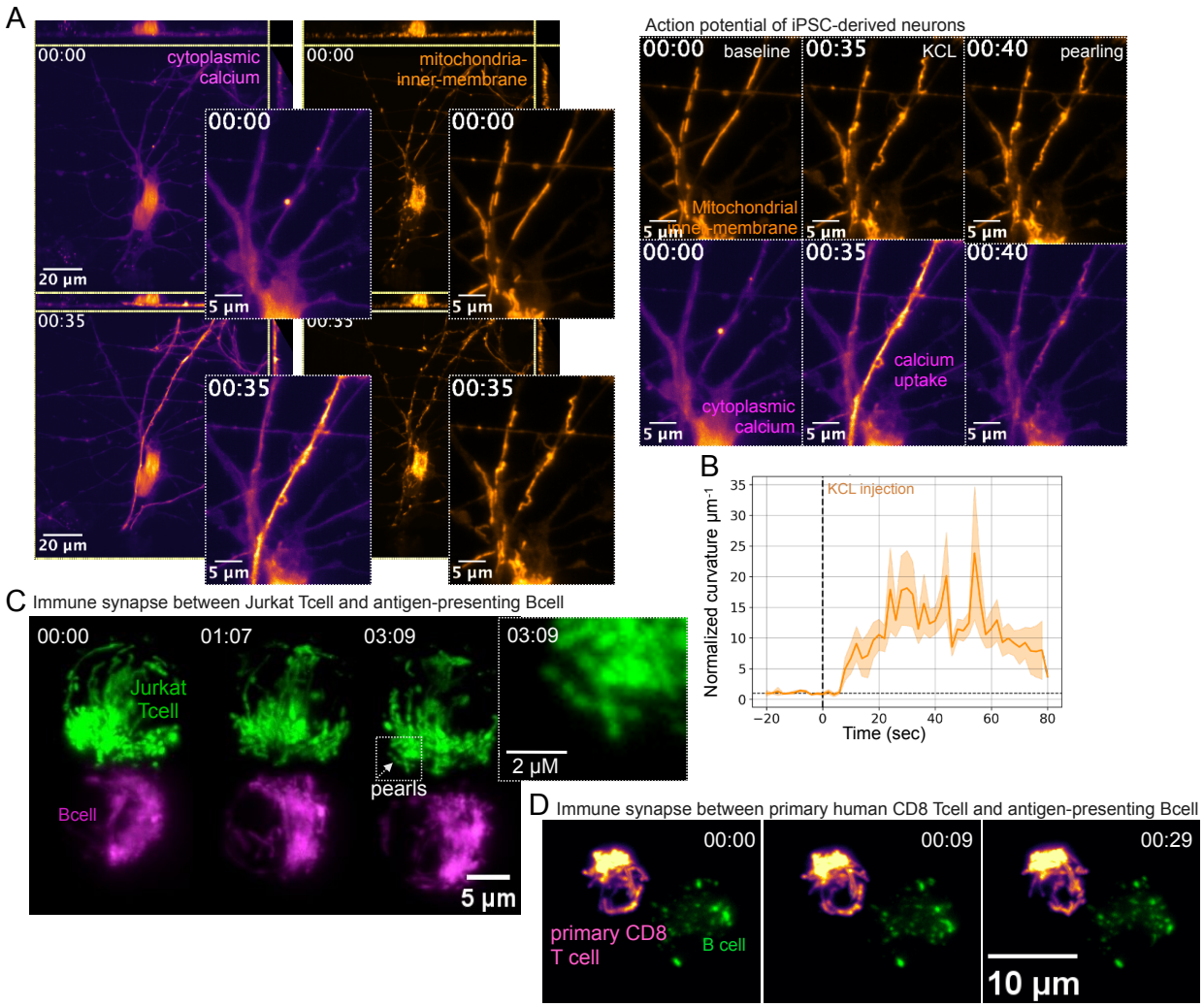
Supplemental Movies

Movies 1-43 along with supporting metadata are available on our public repository at https://github.com/gav-sturm/Mitochondrial_Pearling.

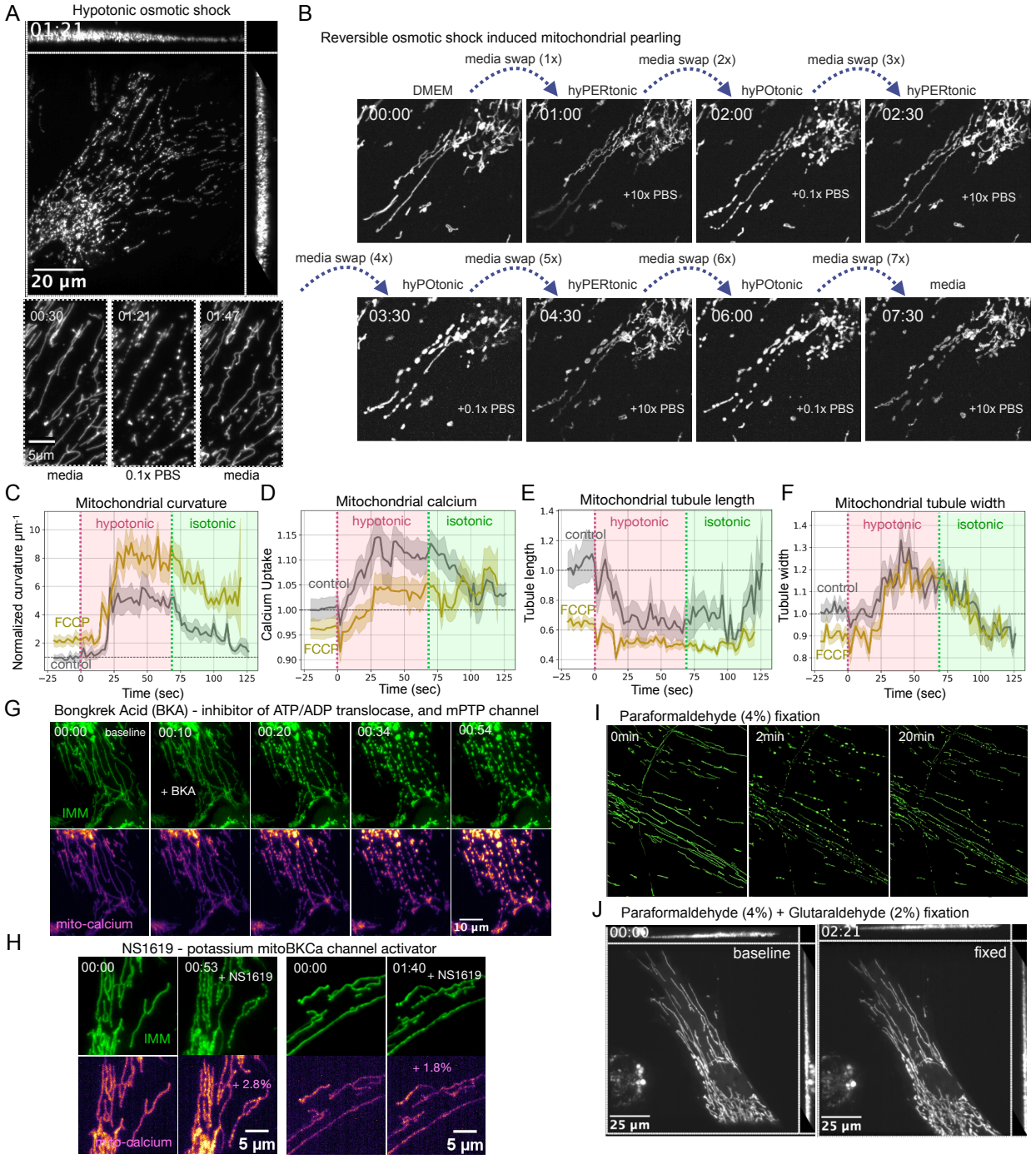
Supplementary Figures



Supplementary Figure 1. Panel of pearling by cell type (**A-H**) Transient mitochondrial pearling occurring spontaneously under light sheet microscopy (SNOUTY), imaging at 1 3D volume per second. (**I**) Rate of pearling per µm of length measured as the percent of mitochondrial length that has pearl within one minute period (n=10-11 videos per group). (**J**) Spontaneous pearling of mitochondria tubule of a U2OS cell. U2OS cells treated with rotenone (5 µM for 30min) undergo a pearling transition twice. Mitochondrial inner membrane (inferno LUT) labelled with genetically-encoded COX8A-mEmerald.

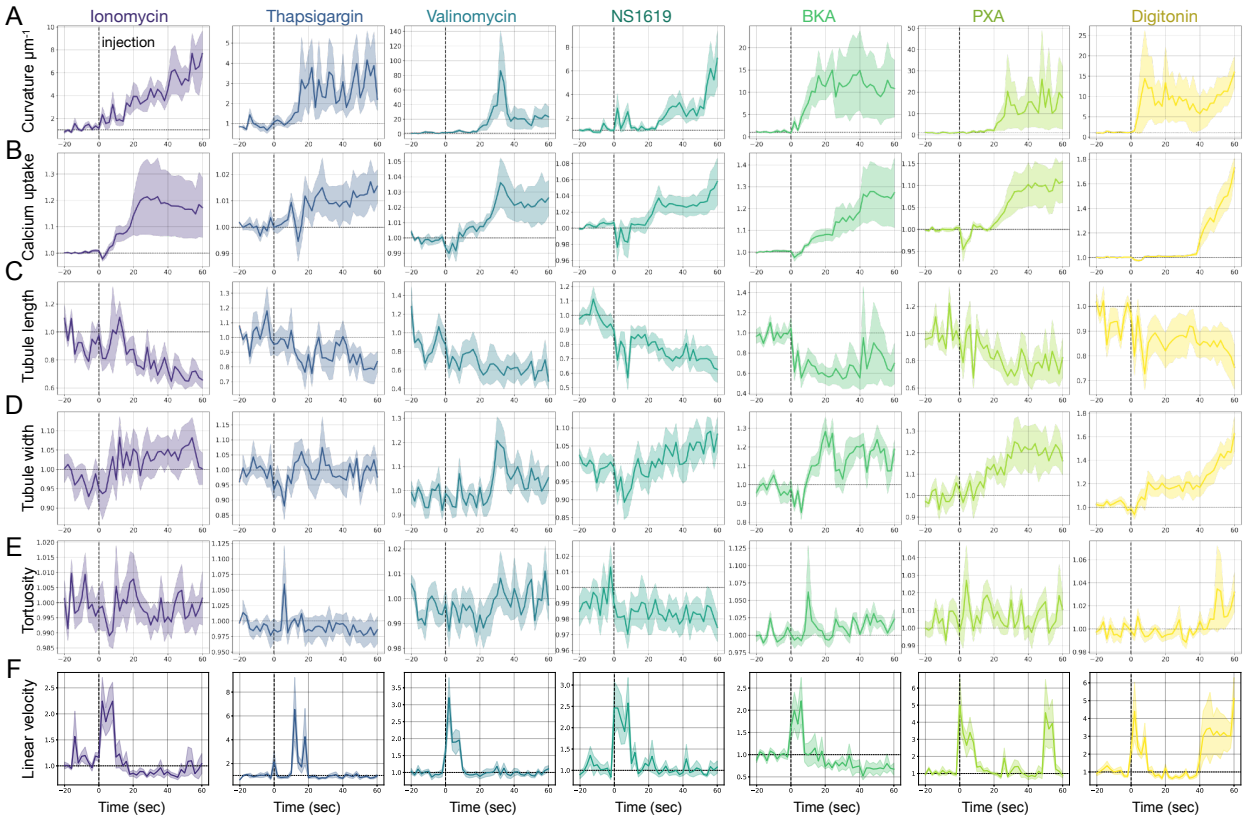


Supplementary Figure 2. Mitochondrial pearling observed across physiological events marked by ionic flux (**A**) Pearling observed during a KCL (50 mM) triggered action potential of iPSC-derived dopaminergic neurons. Top panel tracks the mitochondrial inner membrane using the dye PKmito Orange. Bottom-panel tracks global calcium levels using Calbryte™ 520 AM dye. (**B**) Curvature per µm over time normalized to the first three time points. Dotted line indicates the point of 50mM KCl injection (n=5 cells). (**C**) Mitochondrial pearling observed at the synapse between a Jurkat cell labelled with Mito-mStayGold targeting an SEE-pulsed Raji cell labelled with PKmito Orange. Both labels mark the mitochondrial inner membrane. (**D**) Mitochondrial pearling observed at the synapse between a primary human CD8+ T cell labelled with PKmito orange (IMM) targeting an SEE-pulsed Raji cell labelled with Sybr Gold (mtDNA).

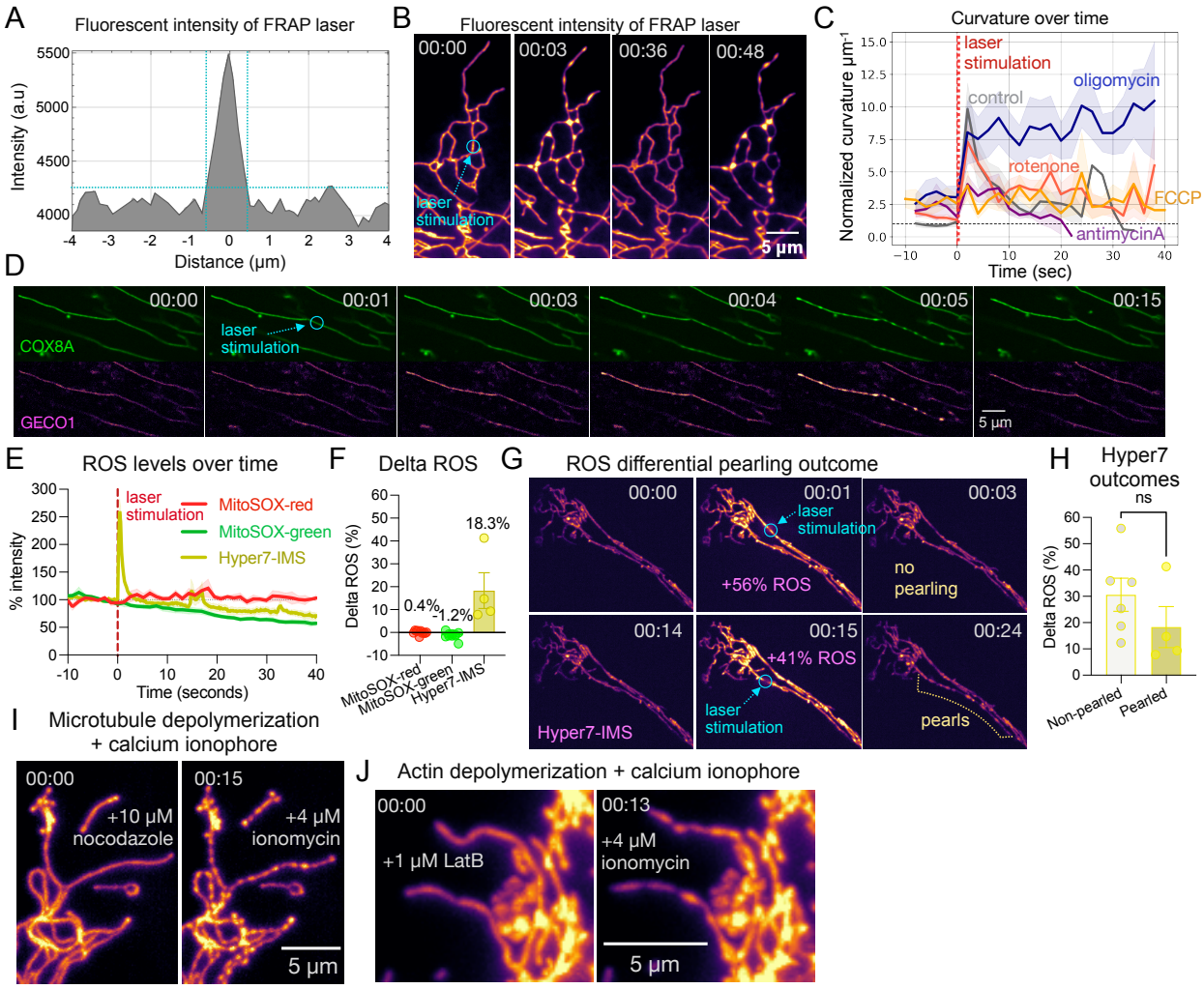


Supplementary Figure 3. Osmotic shock and chemical induction of mitochondrial pearling. (**A**) Mitochondrial pearling induced by replacing DMEM with hypotonic medium, 0.1x PBS and then recovered by replacing medium back to DMEM. (**B**) Reproduction of the 1915 experiment (Lewis & Lewis, AJA, 1915) showing reversible pearling morphology via altering from hypo-osmotic (0.1x PBS) to hyper-osmotic (10x PBS) media conditions. The mitochondria of human epithelial U2OS cells are marked with genetically encoded COX8A-mEmerald. (**C-F**) Time series of untreated (gray) and cells pre-treated with 10 µM FCCP for 15min (yellow) with (F) Normalized curvature per µm (COX8a-mStayGold), (G) mitochondrial calcium uptake (Mito-R-GECO1) and (H) mitochondrial tubule length (COX8a-mStayGold). n = 12 cells per group. Data normalized the mean of the first 5 control timepoints. Red vertical dotted line indicates media swap to hypotonic shock (0.1x PBS). Green line indicates media swap to isotonic DMEM. (**G**) Time series of 10 µM BKA induced pearling with a transient 'hypertonic' signature visible at 10 seconds, which precedes the rise in calcium. Top panel is labelled

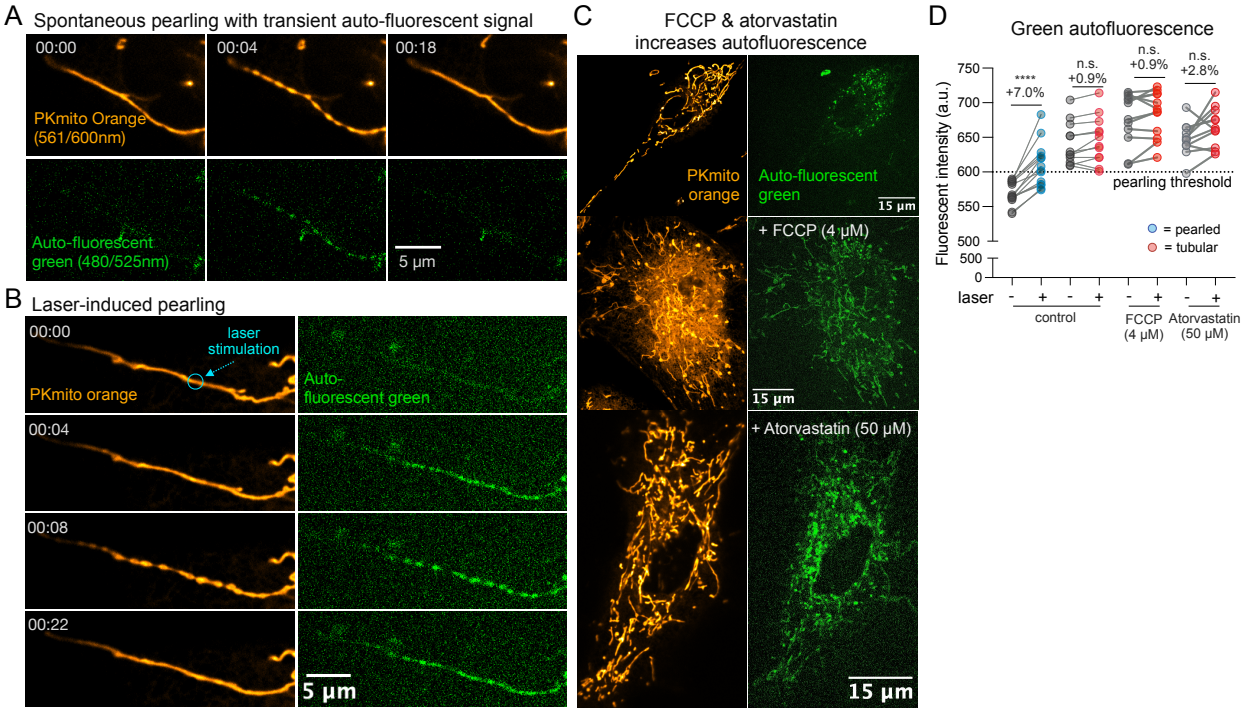
with IMM (COX8A-mStayGold), bottom-panel is labelled with mitochondrial calcium (Mito-R-GECO1). (**H**) Time series of the potassium BKCa channel activator NS1619 (10 μM) induced pearling with or without calcium uptake. Top panel is labelled with IMM (COX8A-mStayGold), bottom-panel is labelled with mitochondrial calcium (Mito-R-GECO1). (**I**) Pearling induced by fixation with 4% paraformaldehyde (PFA) and remains pearled indefinitely. (**J**) Stable mitochondrial network maintained after fixation by combining 4% PFA with 2% glutaraldehyde (GA).



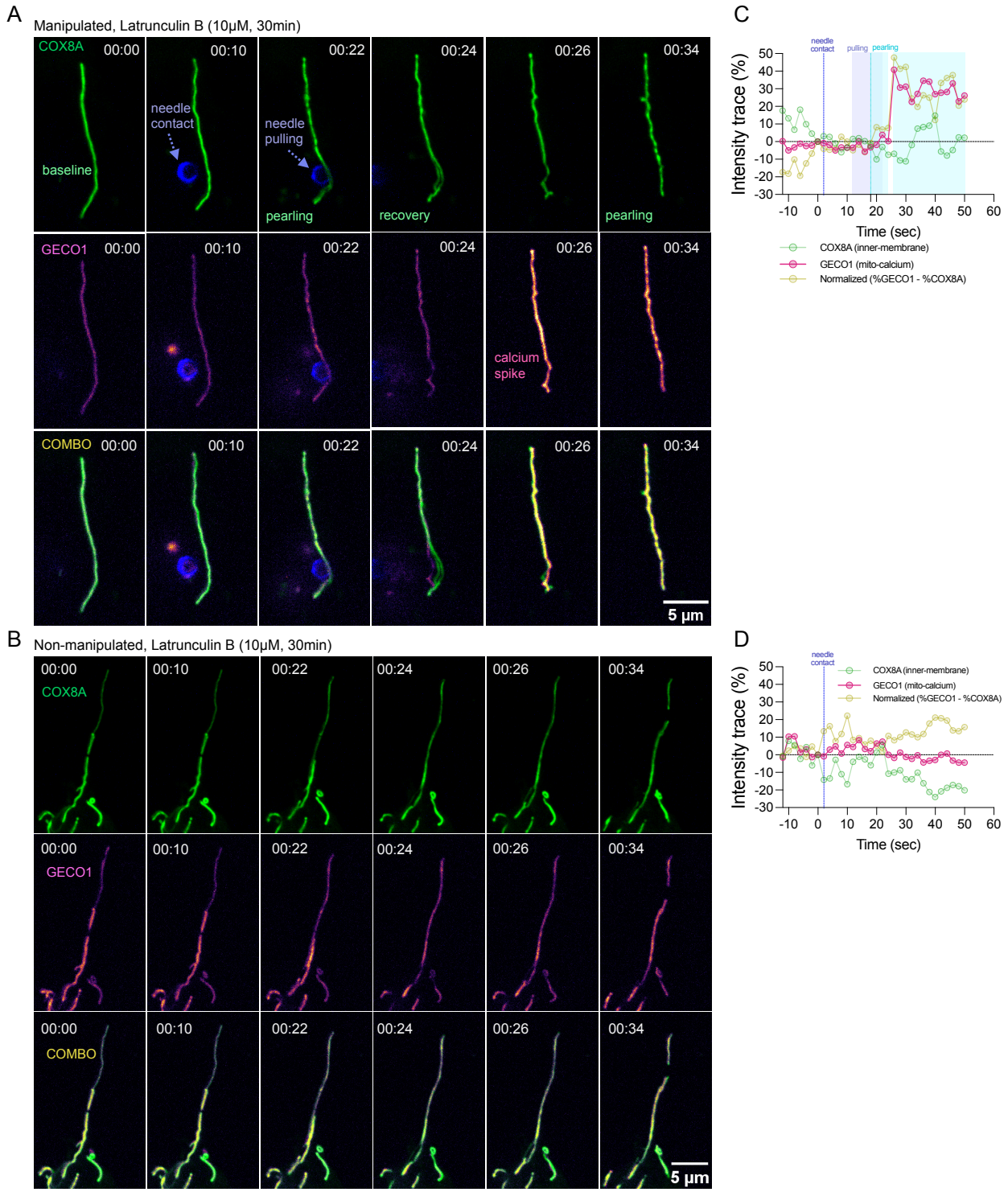
Supplementary Figure 4. Dynamical metrics tracked after chemically induced pearling. (**A-F**) Tracked feature of mitochondrial pearling induced, from left to right, by ionomycin (4 μM), thapsigargin (10 μM), valinomycin (10 μM), NS1619 (10 μM), BKA (10 μM), PXA (10 μM), and digitonin (10 μM). All data is normalized to the average of the first 5 timepoints of that treatment. Tracked metrics include; (**A**) normalized curvature per μm measured on IMM marker COX8A-MitoStayGold, (**B**) calcium uptake as measured by the change in mean fluorescence intensity of Mito-R-GECO1, (**C**) mean tubule length, (**D**) mean tubule width, (**E**) tortuosity (**F**) linear velocity (μm^2). Black dotted line indicates the time at which the chemical was added.



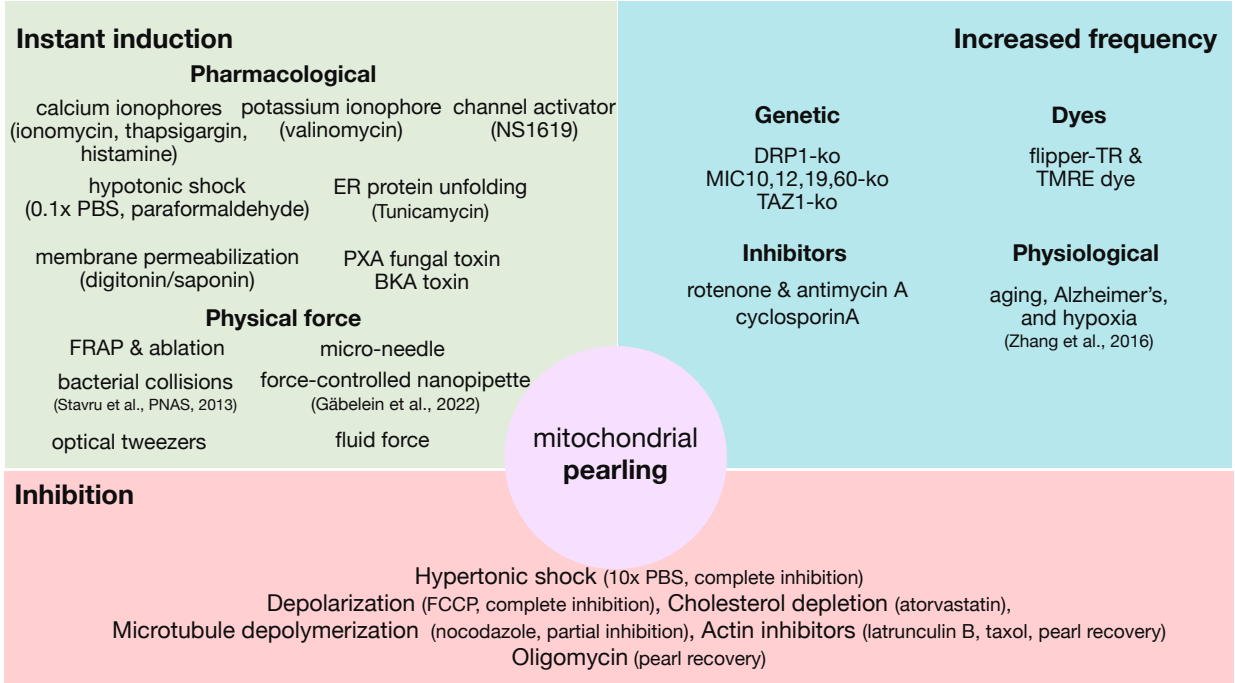
Supplementary Figure 5. Characterizing laser-induced pearling events. (A) Precision of 405nm stimulation laser, Crest-C2, with 100x oil immersion objective, 20mW (0.3mW reaching sample) for 200 milliseconds. (**B**) Laser-induced pearling recovers back to cylindrical shape but then spontaneously returns back to the pearled shaped. (**C**) Curvature per unit μm comparing electron transport chain inhibitors, n=21 oligomycin (1 uM) events, n=18 FCCP (4 μM) events, n=18 rotenone (5 uM) events, n=18 antimycin A (5 uM) events. Red dotted line indicates point of laser stimulation. All data normalized to the average of the first five timepoints of the control group. (**D**) High-temporal tracking of calcium rise that precedes the onset of laser-induced pearling. COX8A = IMM and GECO1 = mitochondrial calcium. (**E**) ROS fluorescent sensors tracked over time after laser-induced pearling event (n=10 events per sensor). Data normalized to the average of the 5 timepoints before stimulation. (**F**) ROS sensors percent change in fluorescent intensity after laser-stimulation that induced pearling events. (**G**) Representative example of Hyper7-IMS ROS sensor increasing across mitochondrial network after laser stimulation, regardless of pearling event occurring. (**H**) Hyper7-IMS percent change in fluorescent intensity after laser-stimulation for tubules that remained uniform (left bar) and those that underwent a pearling event (right bar). Mann-Whitney non-parametric t-test performed. (**I-J**) U2OS cell treated with 10 μM of nocodazole (I) or 1 μM latrunculin B (J) for 1 hour and mitochondria labeled with COX8A-mStayGold (IMM). Pearling is then induced by injection of 4 μM ionomycin.



Supplementary Figure 6. Green autofluorescence predicts mitochondrial pearling transition. (**A**) Spontaneous pearling event is followed by a rise in green-shifted autofluorescence that recovers back to background as the tubule reforms. (**B**) Laser-induced pearling similarly results in a rise in green-shifted autofluorescence within the mitochondrial tubule. (**C**) Cells treated with FCCP (4 µM) and atorvastatin (50 µM) increase green-shifted autofluorescence and do not respond to the laser but rather remain in the tubular state. (**D**) Level of green-shifted autofluorescence within the mitochondrial tubule before and after firing the laser. Mitochondria are split left to right by those that pearl (blue-dots) and those that remain in the tubular state (red-dots). Dotted-line indicates fluorescent threshold at which mitochondria will pearl after firing a 405nm stimulation laser. Mixed-effects anova performed with multiple comparison. **** = 0.00001, n.s. = 0.10 p value.



Supplementary Figure 7. Micro-needle induced pearling precedes calcium uptake. (**A**) Glass micro-needle manipulation of mitochondria in a U2OS cell treated with 1 μm latrunculin B for 30 minutes. (**B**) A tubule from the same cell that was not manipulated by the needle. Cells are labelled with COX8A-mStayGold (inner-membrane) and Mito-R-GECO1 (**C-D**) Intensity trace (R1/R0) over time normalized to the point prior to the presence of the needle in the cell for the (C) manipulated tubule of A and (D) a non-manipulated tubule of B.



Supplementary Figure 8. Experimental perturbations that influence mitochondrial pearling. Perturbations are characterized by those that induce pearling across the entire mitochondrial network in the cell (light-green box), those that increase the frequency of spontaneous pearling events (light-blue box), and those that inhibit either the induction or recovery of pearling events (light-red box).

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