Mitochondrial morphology and activity regulate furrow ingression and contractile ring dynamics in *Drosophila* cellularization

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Abstract

Mitochondria are maternally inherited in many organisms. Mitochondrial morphology and activity regulation is essential for cell survival, differentiation, and migration. An analysis of mitochondrial dynamics and function in morphogenetic events in early metazoan embryogenesis has not been carried out. In our study we find a crucial role of mitochondrial morphology regulation in cell formation in *Drosophila* embryogenesis. We find that mitochondria are small and fragmented and translocate apically on microtubules and distribute progressively along the cell length during cellularization. Embryos mutant for the mitochondrial fission protein, Drp1 (dynamin-related protein 1), die in embryogenesis and show an accumulation of clustered mitochondria on the basal side in cellularization. Additionally, Drp1 mutant embryos contain lower levels of reactive oxygen species (ROS). ROS depletion was previously shown to decrease myosin II activity. Drp1 loss also leads to myosin II depletion at the membrane furrow, thereby resulting in decreased cell height and larger contractile ring area in cellularization similar to that in myosin II mutants. The mitochondrial morphology and cellularization defects in Drp1 mutants are suppressed by reducing mitochondrial fusion and increasing cytoplasmic ROS in superoxide dismutase mutants. Our data show a key role for mitochondrial morphology and activity in supporting the morphogenetic events that drive cellularization in *Drosophila* embryos.

Introduction

Organelles such as mitochondria are predominantly maternally inherited in metazoan embryos (Ukeshima and Fujimoto, 1984; Pepling and Spradling, 2001; Wilding et al., 2001; Dumollard et al., 2006; Zhang et al., 2008). Mitochondria are semiautonomous organelles that are involved in energy production and calcium buffering in the cell. They produce reactive oxygen species (ROS) as a byproduct of the electron transport chain and important metabolites that interact with various processes in a eukaryotic cell.

Mitochondria are small and fragmented in the early blastoderm vertebrate and invertebrate embryos (Bavister and Squirrell, 2000; Sathananthan and Trounson, 2000; Van Blerkom et al., 2002; Dumollard et al., 2007; Chowdhary et al., 2017). Mitochondrial size and shape are regulated by dedicated fission and fusion machinery. Fission protein Drp1 (dynamin-related protein 1) oligomerizes around the outer mitochondrial membrane to form smaller daughter mitochondria. Opa1 (optic atrophy 1) and Mfn/Mfn2 (mitofusins) (mitochondrial assembly–regulating factor; Marf in *Drosophila*) are involved in the fusion of inner and outer membranes, respectively (van der Blik et al., 2013). Mitochondrial shape and cristae architecture have been correlated with their metabolic output, ROS, and calcium buffering (Yu et al., 2006; Hom et al., 2010; Chen et al., 2012; Cogliati et al., 2013; Mishra and Chan, 2016; Toyama et al., 2016). Loss of proteins regulating mitochondrial morphology during embryogenesis leads to abrogation of development. Drp1, Mfn, and Opa1 knockout mice are embryonic lethal (Chen et al., 2003; Ishihara et al., 2009; Wakabayashi et al., 2009; Moore et al., 2010).

Mitochondrial morphology regulation is important for efficient mitochondrial transport within cells. Mitochondria travel on microtubules that interact with various processes in a eukaryotic cell.
FIGURE 1: Mitochondria accumulate in apical regions above nuclei during cellularization. (A) Schematic showing the Drosophila embryo at the end of cellularization with a single layer of epithelial cells at the cortex. The plasma membrane extends basally during cellularization, and microtubules (red) emanate from apical centrosomes. Early, mid, and late stages represent successive increase in plasma membrane (black) length and change in furrow architecture at the base from polygonal (at early) to circular (at mid) followed by constriction (at late). Myosin II (yellow) is present at the furrow and gets enriched during mid cellularization. (B–K) Mitochondria enrich apically during cellularization. Optical sections at depths represented for early, mid, and late cellularization obtained from live imaging of embryos expressing Mito-GFP (gray) and Sqh-mCherry (red) show increase in MitoGFP (gray) signal in apical sections (5 μm) during mid and late cellularization. Membrane extension during these stages is visualized using Sqh-mCherry (red) and is marked by yellow.
mitochondria with the help of specific motors to distinct locations in the cell (Yaffe et al., 1996; Hollenbeck and Saxton, 2005; Saxton and Hollenbeck, 2012; Schwarz, 2013). Their transport to distinct locations in neurons is required for local energy supply and calcium buffering, which is essential for neuronal synapse function (Morris and Hollenbeck, 1993; Mironov and Symonchuk, 2006; Rice and Gelfand, 2006; Saotome et al., 2008; Wang and Schwarz, 2009). Fused mitochondria in Drp1 mutant neurons accumulate in the cell body and axon and lead to lowered synaptic activity (Verstreken et al., 2005; Rikhy et al., 2007).

The literature altogether points to the involvement of mitochondrial shape and function in morphogenetic processes in metazoan embryogenesis. However, the mechanism and function of mitochondrial morphology regulation during embryogenesis remain to be studied. In this study, we aim to discern the function of mitochondrial morphology and distribution during cell division in Drosophila embryogenesis. The Drosophila blastoderm embryo is a syncytium where nuclear cycles (NC) 1–13 occur in a common cytoplasm. Cell formation occurs in the prolonged interphase of NC14 in a process called cellularization. During cellularization, the short cell membranes of approximately 3–5 μm length extend basally in a synchronous manner in the entire embryo. This forms a cortically arranged layer of approximately 6000 tall epithelial cells of 40–45 μm height in about 45–50 min (Lee and Wieschaus, 2000; Mazumdar and Mazumdar, 2002). The membrane extension is accompanied by the assembly of actomyosin contractile structures at ingressing membrane fronts (Warn and Robert-Nicoud, 1990; Young et al., 1991; Schejter and Wieschaus, 1993; He et al., 2016), along with cytoskeletal remodeling proteins (Afshar et al., 2000; Field et al., 2005; Grosshans, 2005; Mavrikis et al., 2014) to constrict the membrane at the base and enclose the cytoplasmic contents within the cells.

We examined whether mitochondrial morphology and distribution are regulated during Drosophila cellularization. Mitochondria are small, fragmented, and abundant in the syncytial Drosophila embryos (Chowdhary et al., 2017). We found that mitochondria were fragmented and enriched basally at the start of cellularization. Their distribution became pronounced apically during cellularization by microtubule-based transport. The apical transport of mitochondria failed upon their clustering in mitochondrial fission protein Drp1 mutant embryos. Drp1 depletion resulted in a decrease in cytoplasmic ROS and defects in furrow ingression and contractile ring constriction, similar to that in myosin II mutants. These defects were suppressed upon forced mitochondrial fission and elevation of cytoplasmic ROS in Drp1 mutant embryos. Our study proposes a role for mitochondrial morphology and activity in maintaining a threshold of active myosin II function to drive cell formation and contractile ring constriction during Drosophila embryogenesis.

RESULTS
Mitochondria translocate apically and distribute throughout the cell during cellularization

The Drosophila blastoderm embryo consists of a single layer of nuclei present at the embryo cortex at the start of cellularization in the interphase of NC14 (Figure 1A). Myosin II is assembled at the base of the membrane furrow during this stage and reaches a peak during midcellularization (He et al., 2016; Xue and Sokac, 2016). Myosin II aids in circularization and constriction of the membrane at the furrow tip as the furrow progresses to the basal side (Figure 1A). Mitochondria are fragmented and are distributed toward the basal regions during NC10–13 in the syncytial Drosophila embryos (Chowdhary et al., 2017). To analyze the mitochondrial distribution along the apicobasal axis during cellularization, we imaged live embryos expressing UASP-Mito-GFP with nanos-Ga4 (Mito-GFP) and Sqh-mCherry tagged myosin regulatory light chain (Sqh) under the sqh promoter (Sqh-mCherry) up to 40 μm depth from the cortex at 25°C (Figure 1B). We quantified the mean Mito-GFP fluorescence intensity from apical to basal sections with 1 μm increments at three different time points during cellularization: early (0 min), mid (~22 min), and late (~45 min) (Figure 1, B and C, and Supplemental Movie S1).

Mito-GFP fluorescence was enriched basally in the early cellularization time point, similar to their distribution in syncytial embryos (Chowdhary et al., 2017) (Figure 1, B, early, and C, red). At the midcellularization time point, mitochondrial fluorescence accumulated apically, showing two peaks of mitochondrial fluorescence, apically and basally (Figure 1, B, mid, and C, green). By the end of cellularization, mitochondrial fluorescence showed a single peak toward the apical side (Figure 1, B, late, and C, blue). The mitochondrial fluorescence increased in apical regions with time as also seen in sagittal sections of Mito-GFP::Sqh-mCherry embryos (Figure 1D and Supplemental Movie S2). We quantified the total Mito-GFP fluorescence across 40 μm during cellularization and found that the fluorescence did not change significantly (Supplemental Figure S1, A and B). This suggested that there was no significant biogenesis of mitochondria during this time period and mitochondria were likely to be

dotted lines (B). Mean fluorescence intensity of Mito-GFP is quantified across optical sections in 1 μm depth increments and plotted with depth at early (red), mid (green), and late (blue) cellularization; n = 3 embryos, 200 cells (C). Sagittal images of Mito-GFP::Sqh-mCherry embryos at represented membrane ingression lengths (arrowheads) show increase in apical mitochondrial fluorescence and presence along the ingressing furrow (D). Total Mito-GFP fluorescence intensity above the furrow tips (D, bracketed region; E, black, left Y-axis) plotted along with membrane ingestion (gray, right Y-axis) across time display slow and fast phases. The transition point between slow and fast phases is marked with a yellow dotted line. n = 4 embryos (approximately 40 cells each), three embryos (five furrows each) for mitochondrial intensity and membrane ingestion measurements, respectively (E). (F–H) Apical translocation of mitochondria is observed using photoactivation. Schematic of photoactivation shows photoactivated basal ROI (red) and nonphotoactivated apical ROI (green) (F). A rainbow colored intensity scale is used to depict the sagittal images showing photoactivation of Mito-PAGFP at the basal ROI (red) during early cellularization. The mean fluorescence intensity decreases at the basal region (red) and increases at the apical region (green) with time during cellularization (G, H). Normalized mean fluorescence intensity is plotted for basal (red) and apical (green) regions for n = 4 embryos (approximately three cells each) across time (H). (I–K) TEM images of early, mid, and late cellularization stages show punctate mitochondria (I). Embryo stages are identified by furrow length (I, furrow tips–red arrowheads). Basal regions are marked by yellow dashed lines. Number of mitochondria in the apical region per section (I, black rectangle, and K) increases gradually from early (red, K) to mid (green, K) and late (blue, K) cellularization. n = 3, 4, 4 embryos and total 49, 26, 66 cells for early, mid, and late stages, respectively are quantified. ***, P ≤ 0.001, Mann–Whitney test. Scale bar = 5 μm (B, D, and G); 2 μm (I); and 1 μm (J).
enriched in apical regions due to translocation from the basal regions. The membrane ingress is known to occur in two phases: a slow phase of membrane ingress followed by a fast phase (Warn and Magrath, 1983; Merrill et al., 1988; Lecuit and Wieschaus, 2000; Royou et al., 2004; Figard et al., 2013; He et al., 2016). We measured membrane length across time during cellularization and obtained a similar trend (Figure 1E, gray). We also quantified the Mito-GFP fluorescence in the area above the furrow tips (Figure 1D, bracketed area) with respect to time. We observed an increase in Mito-GFP in the forming cells as the furrow ingressed during cellularization (Figure 1E, black).

Mitochondria and other organelles in the Drosophila blastoderm embryo are compartmentalized to one nucleocyttoplasmic domain with very little exchange between the neighboring syncytial cells (Frescas et al., 2006; Chowdhary et al., 2017). Therefore, the apical redistribution of mitochondria seen in cellularization is likely due to their transport from the basal side. We tested this possibility by photoactivating mitochondrionally localized photoactivatable GFP (Mito-PAGFP) expressed using nanos-Gal4. We photoactivated Mito-PAGFP, highlighting mitochondria below the nuclei in a region of interest (ROI) (red) at 12–15 μm from the apical surface in early cellularization stage embryos imaged sagittally (Figure 1F) and followed the fluorescent signal across successive time points during cellularization (Figure 1G). We measured the mean fluorescence intensity in apical (green) and basal (red) regions as the cellularization progressed (Figure 1H). Fluorescence rapidly moved apically (green) from the basal region (red) within 1 min after photoactivation. The fluorescence intensity kept increasing in the apical regions for 40 min during the entire cellularization process (Figure 1, G and H, and Supplemental Movie S3). By the end of cellularization, fluorescence was almost entirely lost from the basal regions and was enriched apically (Figure 1, G and H).

We visualized mitochondrial distribution in cellularization by imaging embryo sections of early, mid, and late stages using transmission electron microscopy (TEM) (Figure 1, I and J). Mitochondria were seen as electron dense structures in the EM sections in early, mid, and late cellularization (Figure 1, I and J). We quantified the visible mitochondria in apical regions above the nuclei in TEM sections and found that their numbers significantly increased in mid and late cellularization compared with the early stage (Figure 1K). Therefore we conclude that mitochondria are transported to the apical region from the basal region during cellularization and this translocation occurs coincidently with membrane ingress.

**Mitochondrial redistribution during cellularization is regulated by microtubule-based transport**

During cellularization, microtubules are oriented in the apicobasal axis with centrioles present above the nuclei and plus ends pointing toward the basal regions (Karr and Albers, 1986). Microtubule-based apical transport of Golgi complexes (Sisson et al., 2000; Papoulas et al., 2005) and basal transport of lipid droplets have been reported during late cellularization (Welte et al., 1998; Arora et al., 2016). Bidirectional transport of mitochondria on microtubules has been well characterized in axons (Morris and Hollenbeck, 1993). The kymograph from the photoactivation movie (Supplemental Movie S3) of Mito-PAGFP during cellularization identified linear tracks of mitochondrial transport toward the apical direction (Supplemental Figure S1C, red arrowheads), suggesting that mitochondria are likely to be transported in a microtubule-based manner. To explore the link between microtubules and mitochondrial distribution, we expressed Mito-GFP and Tubulin-mCherry (Tub-mCherry) with nanos-Gal4 to image mitochondria and microtubules together. Microtubules were organized like an inverted basket around the nuclei, and mitochondria were juxtaposed to microtubule filaments (Figure 2A). We could visualize mitochondria moving adjacent to microtubules to apical regions with time (Figure 2A, sagittal, white arrowheads, and Supplemental Movie S4). Mitochondria (green) and tubulin (red) showed overlapping peaks of fluorescence intensity (Figure 2, A and B, white dashed line, 6 μm).

To study the role of microtubule motors in regulating the mitochondrial distribution, we expressed RNA interference (RNAi) to knock down kinesin heavy chain (khc) and dynein heavy chain (dhc) with nanos-Gal4 and stained the embryos with fluorescently coupled streptavidin to mark mitochondria (Hollinshead et al., 1997; Chowdhary et al., 2017). Mitochondria were predominantly present in the basal regions in the syncytial division cycles (Chowdhary et al., 2017). Thus during early cellularization, few distinct and sparse mitochondria were observed at the subapical region in control embryos (Figure 2, C and D). Similar to our observations in the syncytial blastoderm embryo (Chowdhary et al., 2017), mitochondria were clustered subapically in the early cellularization stages in 100% of the khc embryos (Figure 2D, sagittal, white arrowheads), consistent with a role of kinesin in regulating plus-ended movement of mitochondria in syncytial cycles and early cellularization. Mitochondria translocated apically toward the minus ends of microtubules during cellularization, and their distribution was predominant in apical regions in late cellularization. The mitochondrial intensity was seen at the apical regions in sagittal sections, and comparatively fewer, distinct mitochondria were observed at the basal regions near the contractile rings in surface views in control embryos (Figure 2, E and F). We observed basal accumulation of mitochondria in contractile rings in late cellularization stages in 93% of the dhc embryos (Figure 2F, sagittal, white arrowheads), with few mitochondria present at the apical regions (Figure 2F, sagittal, yellow arrowheads), suggesting that minus end–directed microtubule transport is mediating the translocation of mitochondria to the apical region.

Mitochondrial Rho GTPases (mira) interact with microtubule motors kinesin (Fransson et al., 2006) and dynein (Morlino et al., 2014), as well as adapter protein Milton (Morlino et al., 2014; Melkov et al., 2016), to facilitate bidirectional transport of mitochondria (Guo et al., 2005; Russo et al., 2009). We assessed the role of microtubule motors in the apical mitochondrial transport during cellularization using an RNAi-based approach to knock down Miro (mira) using nanos-Gal4. In 86.4% of the mira embryos, mitochondria accumulated in basal regions (Figure 2F, sagittal, white arrowheads) near contractile rings during early as well as late cellularization and were absent apically (Figure 2F, sagittal, yellow arrowheads). These results together showed that apical transport of mitochondria during cellularization depends on minus end–directed microtubule transport mediated by dynein motors and Miro.

**Maternal depletion of mitochondrial fission protein Drp1 leads to embryo lethality and accumulation of clustered mitochondria basally during cellularization**

Since we observed that mitochondria were small and fragmented in early Drosophila embryos, we assessed the distribution of mitochondrial fission protein Drp1 in cellularizing embryos. Immunostaining against Drp1 showed that it was localized to mitochondria in cellularization (Supplemental Figure S2A). We depleted Drp1 maternally in two ways: we overexpressed a GTPase domain mutant of Drp1, Drp1SG, and an RNAi against Drp1 (drp1) with nanos-Gal4::UASp-Mito-GFP. When the nanos-Gal4 was crossed to both the Mito-GFP and each Drp1 mutant transgenes, 84% (±7.8) Drp1SG and 73% (±14.7) drp1 embryos were lethal and did not hatch to.
produce larvae at 24 h after laying (n = 300 each). The embryonic lethality of Drp1<sup>SG</sup> and drp1<sup>i</sup> increased to 99% (±1.7) and 100%, respectively (n = 300), where the nanos-Gal4 was crossed to the Drp1 mutants alone. Thus, maternal depletion of Drp1 led to embryonic lethality.

Both Drp1<sup>SG</sup> and drp1<sup>i</sup> embryos had clustered mitochondria in cellularization (Figure 3A). We quantified the mean area of optically resolvable fluorescent punctae of mitochondria per embryo and found that control embryos had a mean area of 0.25 (±0.075) μm². Mitochondrial fluorescence was spread over a larger area in Drp1<sup>SG</sup> and drp1<sup>i</sup>, with mean areas of 0.7 (±0.4) μm² and 2.2 (±1.4) μm², respectively, indicating clustering or elongation or fusion of mitochondria in the Drp1 mutant embryos (Figure 3B). Mitochondrial fusion and electrical continuity has been noted on loss of Drp1 in Drosophila follicle cells in previous studies (Mitra et al., 2012).

Mitochondrial fission is important for the translocation of mitochondria on microtubules in neurons (Chen and Chan, 2009), and Drp1 mutant neurons show an accumulation of mitochondria in the cell body and axon (Verstreken et al., 2005; Rikhy et al., 2007; Choi et al., 2013). We assessed the basal to apical mitochondrial transport in Mito-GFP–expressing Drp1<sup>SG</sup> embryos during cellularization (Figure 3C). Drp1<sup>SG</sup> embryos containing Mito-GFP had reduced fluorescence (Figure 3C) as compared with controls (Figure 1B), and Mito-GFP fluorescence had to be acquired at a higher laser power as compared with controls. Mitochondria in Drp1<sup>SG</sup> embryos were visible more distinctly and clearly in streptavidin-stained embryos compared with Mito-GFP–containing embryos, thus allowing us to quantitate their architecture (Figure 3B). However, like controls, we obtained relative Mito-GFP fluorescence intensity estimates within each Drp1<sup>SG</sup>-expressing living embryo to analyze mitochondrial localization during cellularization and furrow extension. The relative mean mitochondrial fluorescence was obtained at three time points: early, mid, and late cellularization, along the apicobasal axis with 1 μm depth increments for a total 30 μm depth. Similar to the control embryos (Figure 1B), the Mito-GFP intensity was basally pronounced in the early cellularization (Figure 3, C, top panel, and D, red). However, unlike controls, the apical regions in Drp1<sup>SG</sup> did not gain fluorescence at the following time points at mid (Figure 3, C, middle panel, and D, green) cellularization. At late cellularization there was a mild increase in the apical intensity, but the peak of mitochondrial intensity remained in basal regions (Figure 3, C, bottom panel, and D, blue). Similar to Drp1<sup>SG</sup>, drp1<sup>i</sup> embryos also showed a lack of change in the Mito-GFP signal distribution from basal to apical from early to mid and late stages during cellularization (Supplemental Figure S2B). The mean mitochondrial fluorescence intensity remained at a peak in basal regions in these embryos during cellularization (Supplemental Figure S2C).

We further measured the total mitochondrial fluorescence incorporated within the forming cells above the furrow tip as the furrow extended basally. The membrane extension took place in the slow and fast phase in Drp1<sup>SG</sup> mutant embryos similar to controls (Figure 1E). However, unlike the control embryos, mitochondrial fluorescence did not show an increase with time, remaining lower than in controls (Figure 3E). The mitochondrial fluorescence intensity remained higher below the furrow (marked with Sqh-mCherry in drp1 mutant embryos and did not get effectively incorporated in the forming cell likely due to lack of their transport (Figure 3C).

We confirmed the decreased apical transport in Drp1<sup>SG</sup> embryos by photoactivation using Mito-PAGFP. We photoactivated
FIGURE 3: Apical translocation of mitochondria is decreased in Drp1<sup>SG</sup>. (A, B) Drp1 mutant embryos have clustered mitochondria. Mitochondria (green) labeled using fluorescent streptavidin are clustered in Drp1<sup>SG</sup> and drp1<sup>i</sup> embryos compared with control Mito-GFP embryos (A). Optically resolvable mitochondrial area per embryo is quantified (B). Mitochondrial sizes are significantly larger in Drp1<sup>SG</sup> (deep red) and drp1<sup>i</sup> (red) embryos compared with Mito-GFP (light red) embryos (B). Each data point represents mean mitochondrial size from one embryo. n = 37, 28, and 20 embryos for control, Drp1<sup>SG</sup>, and drp1<sup>i</sup>, respectively; approximately 40 cells and 15,000 optically resolvable fluorescent mitochondrial spots were counted per embryo. ***, P ≤ 0.001, Mann–Whitney test (B). Scale bar: 5 μm (A). (C–H) Apical mitochondrial translocation is reduced in Drp1<sup>SG</sup> embryos. Live imaging of Mito-GFP–containing Drp1<sup>SG</sup> embryos shows mitochondrial clusters in basal regions of cells in early (C, top panel), mid (C, middle panel), and late (C, bottom panel) cellularization. Mitochondria are missing in the apical regions (C). Sqh-mCherry signal is a readout of the membrane length at each time point. Mean Mito-GFP fluorescence across depth is plotted with SEM for n = 3 embryos and a total of 200 cells for early, mid, and late cellularization stages (D). Mito-GFP fluorescence peaks in basal regions at early (red), mid (green), and late (blue) cellularization stages (D). Total intensity of Mito-GFP signal above the furrow is plotted with membrane length across time during cellularization. Yellow dotted line represents the slow–fast phase transition time point (E). Scale bar: 5 μm (C). (F–H) Absence of apical translocation of mitochondria in Drp1<sup>SG</sup> embryos is observed using photoactivation. Schematic of photoactivation experiment showing basal photoactivated ROI (red) and nonphotoactivated apical ROI (green) (F). Sagittal images of Mito-PAGFP–containing Drp1<sup>SG</sup> embryos, represented with rainbow intensity color map, show no change in the localization of activated Mito-PAGFP signal at the basal regions (red), and the apical region (green) does not gain significant fluorescence signal with time during cellularization (G). Normalized mean fluorescence intensity plotted with SEM for the basal (red) and apical (green) regions for n = 3 embryos (approximately three cells each) shows no change across time (H). Scale bar: 10 μm (G).
fluorescence in early cellularization embryos in a region below the nuclei (Figure 3F, red) and followed the fluorescence in the apical region (Figure 3F, green) as depicted. The photoactivated mitochondrial fluorescence in basal regions of Drp1SG embryos remained basal and was not transported to the apical region (Figure 3G and Supplemental Movie S5). We quantified the mean fluorescence intensity in the basal (red) and apical (green) regions. There was no significant depletion in the fluorescence intensity signal of the basal region, and the apical region did not gain any fluorescence either, further demonstrating the lack of apical transport of mitochondria (Figure 3H). Our data together show that the loss of mitochondrial fission by Drp1 depletion leads to larger mitochondria that do not translocate apically and accumulate at the basal regions during cellularization.

In an attempt to decrease mitochondrial clustering in Drp1SG embryos, we expressed RNAi against mitochondrial fusion protein Opa1 (opa1) in Drp1SG embryos (Drp1SG,opa1). Flies expressing opa1 alone did not lay embryos; however, flies expressing the Drp1SG,opa1 combination gave embryos that were lethal (87.5% unhatched, n = 180 embryos). We measured the mean area of optically separable fluorescent mitochondrial structures per embryo in apical (Supplemental Figure S3A, apical, 3 μm depth) and basal (Supplemental Figure S3A, basal, depth ≥15 μm) sections near the contractile rings (Supplemental Figure S3A) in streptavidin-stained control, Drp1SG and Drp1SG,opa1 embryos. The area of apical mitochondrial fluorescent punctae was similar in all the combinations (Figure S3, A and B, apical). There was no significant difference between apical and basal mitochondrial sizes in controls (Supplemental Figure S3B). The mitochondrial punctae at the basal region in Drp1SG were much larger at an average of 0.9 (±0.5) μm² in size, compared with the control mitochondria that were 0.3 (±0.2) μm² in size (Supplemental Figure S3B, red, black, respectively). The basal mitochondrial size defect seen in Drp1SG embryos was completely suppressed in Drp1SG,opa1 (0.34, ±0.05 μm²) embryos (Supplemental Figure S3B).

We further quantified the apical density of mitochondria in late cellularization stage embryos as a measure of mitochondrial apical translocation during cellularization. For this we quantified the relative mitochondrial area occupied in apical regions above the nucleus in mid–late cellularization stage embryos with furrow length above 12 μm. On average, mitochondria occupied 7.2 (±2.8)% of the apical area in control embryos (Supplemental Figure S3C, black). The mean relative area decreased to 0.3 (±0.4)% in Drp1SG (Supplemental Figure S3C, red) embryos and was consistent with the analysis of mitochondrial distribution done using Mito-GFP (Figure 3, C and D) and Mito-PA-GFP (Figure 3, G and H) embryos that showed the presence of mitochondrial clusters at the basal region and lack of apical translocation. Compared to Drp1SG, the relative mean mitochondrial area increased to about 6.1 (±2.2)% in Drp1SG,opa1 embryos (Supplemental Figure S3C, blue). Thus, an increase in mitochondrial size led to basal accumulation of mitochondria in Drp1SG embryos, and this phenotype was suppressed in Drp1SG,opa1 embryos. Together these data show that maintenance of mitochondrial fission is essential for mitochondrial translocation to apical locations during cellularization.

Drp1 depletion leads to decrease in cytoplasmic ROS
Mitochondrial shape changes lead to alterations in mitochondrial metabolic activity and ATP production (Mishra and Chan, 2016). Decrease in ATP production leads to accumulation of AMP, which triggers phosphorylation of AMPK (pAMPK) (Sakamoto et al., 2005; Hardie and Sakamoto, 2006). Mitochondria are the major source of ATP in Drosophila blastoderm embryos (An et al., 2014; Chowdhary et al., 2017). Mutations in electron transport chain components lead to a decrease in ATP and an elevation of pAMPK. Electron transport chain mutant embryos showed a reduction in the metaphase furrow lengths in the syncytial Drosophila embryos (Chowdhary et al., 2017). We tested for ATP stress response in Drp1SG embryos using pAMPK staining. Both control and Drp1SG embryos were imaged using identical laser settings. During cellularization, pAMPK showed a cytoplasmic distribution with enrichment in punctae in the apical region (Figure 4A). The quantification of mean pAMPK intensity in apical sections in Drp1SG did not show a significant difference as compared with the controls, indicating that the embryos did not have increased AMP and ATP stress (Figure 4B).

ROS is produced as a byproduct of the electron transport chain activity (Li et al., 2002; Bell et al., 2007). ROS has been implicated in the regulation of differentiation and morphogenetic movements in embryos (Li et al., 2010; Muliyil and Narasimha, 2014; Hunter et al., 2018). We assessed ROS levels in the cytoplasm using fluorescein dihydroethidium (DHE) staining in control and Drp1SG embryos (Figure 4C). DHE oxidation due to the presence of ROS leads to its fluorescence at 561 nm, thus providing a quantitative readout for the amount of ROS in cells and allowing the comparison of different genotypes when imaged with the same laser settings (Figure 4, C and D). Drp1SG embryos showed significantly lowered mean fluorescence compared with controls (Figure 4, C and D). These data are in agreement with other studies that show reduction of ROS in fission mutants (Yu et al., 2006; Röth et al., 2014; Son et al., 2015; Kim et al., 2018). We overexpressed mutant human superoxide dismutase 1 (hSOD1A4V) as a positive control to test for increased ROS in embryos. hSOD1A4V expression does not have any SOD activity (Watson et al., 2008; Islam et al., 2012). We observed an approximately threefold increase in the mean cytoplasmic DHE fluorescence in hSOD1A4V-expressing embryos as compared with controls (Figure 4, C and D). Thus, Drp1SG embryos containing clustered mitochondria have decreased ROS without any ATP stress.

Drp1 depletion leads to defects in contractile ring constriction and membrane extension in cellularization
ROS levels are important for regulating myosin II–based apical constriction of epithelial cells in dorsal closure and wound healing (Muliyil and Narasimha, 2014; Hunter et al., 2018). Since mitochondria were clustered and ROS was decreased in Drp1SG embryos, we analyzed the distribution and function of myosin II during cellularization. During cellularization, the furrow ingresses basally, along with an assembly of an actomyosin ring at the furrow tip followed by ring constriction (Figure 1A). We imaged sqh-sqh-mCherry (Sqh-mCherry) embryos in the sagittal orientation for visualizing the furrow ingression with time from the start of cellularization until gastrulation at the same laser power and gain settings (Figure 5A and Supplemental Movie S6). We measured furrow membrane length by Sqh-mCherry enrichment at furrow tips every 2 min in control (black) and Drp1SG (red) embryos (Figure 5B) as it ingressed basally during cellularization. Both genotypes exhibited similar ingestion kinetics with an initial slow phase up to 20 min, followed by a fast phase until the completion of cellularization (Figure 5, A and B). This ingestion trend has been reported before in cellularization (Memili et al., 1988; Lecuit and Wieschaus, 2000; Figard et al., 2013; He et al., 2016; Xue and Sokac, 2016), leading to the formation of columnar epithelial cells of approximately 40 μm height. The membrane ingestion process stopped, and Sqh-mCherry signal reduced from furrow tips at approximately 40 min in Drp1SG embryos (Figure 5, A, arrowhead,
and B. red, and Supplemental Movie S7) and resulted in significantly shorter cells at an average height of 29.3 (±1.3) μm (Figure 5, B, red, and C) compared with 40.8 (±1.9) μm in controls (Figure 5, B, black, and C).

We imaged Sqh-mCherry control and Drp1SG mutant embryos in three dimensions over time to analyze the Sqh levels and the area of rings to estimate constriction at the furrow tip in cellularization with the same laser power and gain settings. Cross-sectional images of the growing furrow tips allowed us to monitor both Sqh-mCherry localization and membrane architecture at the tips as the furrow grows deeper with time (Figure 5D). The base of the furrow was polygonal at the start of cellularization (Figure 5D, 5 μm). During the slow-to-fast-phase transition, the membrane organization changed from polygonal to circular. During the fast phase, rings constricted rapidly (Figure 5D, 12 μm onward). We measured the meanSqh-mCherry intensity at the furrow tips with respect to the length of membrane furrow in control and Drp1SG embryos. Control embryos showed an initial increase in the Sqh-mCherry intensity until approximately 15 μm of furrow length, after which the signal gradually dropped (Figure 5E, black). Drp1SG embryos showed an overall decrease in Sqh-mCherry intensity at the membrane front (Figure 5E, red). Constriction was calculated by measuring the contractile ring area with respect to the length in cellularizing embryos (Figure 5F). The area of contractile rings in Drp1SG (Figure 5F, red) was similar to that in control embryos (Figure 5F, black) at the beginning of cellularization, but it remained larger compared with controls (Figure 5F). The area of contractile rings at the end of cellularization was significantly larger in Drp1SG embryos (19, ±4.3 μm²) compared with controls (10.2, ±1.1 μm²) (Figure 5G).

Since mitochondrial morphology and apical transport defects seen in Drp1SG were suppressed on inhibiting mitochondrial fusion in Drp1SG; opa1i, we asked whether the decrease in cell height and contractile ring defect observed in Drp1SG embryos could also be suppressed on additional depletion of Opa1. To analyze membrane ingestion dynamics, we imaged sagittal sections of Drp1SG; opa1i embryos using sqh-Sqh-GFP (Sqh-GFP) (Supplemental Figure S4A and Supplemental Movie S8). The relative fluorescence levels in Sqh-GFP and Sqh-mCherry embryos were comparable across cellularization (Supplemental Figure S5). Membrane length was estimated with Sqh-GFP localized at the furrow tips (Supplemental Figure S4, A and B). Furrow ingress rates in Drp1SG; opa1i (blue) embryos were similar to those in controls (black) (Supplemental Figure S4B). The membranes reached 36 (±2.3) μm in Drp1SG; opa1i embryos (Supplemental Figure S4C, blue), and this was significantly more compared with Drp1SG embryos (Supplemental Figure S4C, red, and Supplemental Movie S8).

We measured Sqh intensity in the contractile rings in Drp1SG; opa1i embryos and compared it to that in Sqh-GFP controls (Supplemental Figure S4, D and E). The Sqh-GFP intensity in Drp1SG; opa1i embryos (Supplemental Figure S4E, blue) increased as compared with Drp1SG embryos (Supplemental Figure S4E, red) but was lower than in controls (Supplemental Figure S4E, black). The area of contractile rings in Drp1SG; opa1i (Supplemental Figure S4F, blue) embryos decreased faster compared with that in Drp1SG alone (Figure 5F and Supplemental Figure S4F, red), with the area being comparable to that in control embryos at 35 μm membrane length (Supplemental Figure S4F, black). The contractile ring area, measured at the end of cellularization in Drp1SG; opa1i embryos (12.6, ±5 μm²), was smaller than in Drp1SG (Supplemental Figure S4G).

We overexpressed a nonphosphorylatable version of sqh (pUAsg-SqhAA) (Vasquez et al., 2014) with nanos-Gal4 to analyze the effects of myosin II inactivation on the furrow extension and ring shape dynamics (Figure 5A). Previous studies show that SqhAA, when expressed in the background of the hypomorphic sqh1 allele, leads to loss of contractility in cellularization (Xue and Sokac, 2016). SqhAA has also been shown to localize to sites of actomyosin function and cause loss of actomyosin contractility in a dominant
FIGURE 5: Drp1SG embryos have shorter cells, decreased myosin II, and decreased area of contractile rings. (A–C) Drp1SG embryos have shorter cells at the end of cellularization. Sqh-mCherry–expressing control, Drp1SG, and SqhAA embryos are imaged in the sagittal plane. Sqh-mCherry, localized to the membrane tips, shows the extent of membrane ingestion during cellularization (A). Membrane length quantified across time during cellularization shows a similar trend of the slow and fast phase of ingestion in control (black), Drp1SG (red), and SqhAA (light red) (B). The plots for control and Drp1SG are repeated from Figures 1E and 3E, respectively, for comparison. Final cell length achieved postcellularization (A, red arrowheads) in Drp1SG (red) and SqhAA (light red) is significantly smaller compared with control (black) (A, C). n = 4, 3, 6 embryos (five furrows each) (B); 6, 5, 7 embryos (C) for control, Drp1SG, and SqhAA, respectively. Numbers on the plot represent total furrows analyzed for each data set (C). ***, P ≤ 0.001, Mann–Whitney test (C). Scale bar: 5 μm (A). (D–G): Drp1SG embryos have lowered Sqh-mCherry at the furrow tips during cellularization (Figure 5, A, D, and E, light red) and decreased ring constriction (Figure 5F, light red) with a significantly larger contractile ring area 25.1 ± (9.1) μm² at the end of cellularization (Figure 5G, light red).

These data together show that shorter cell height and impaired actomyosin constriction in Drp1SG embryos occurs due to reduced myosin II function.

Increasing ROS suppresses mitochondrial clustering defects in Drp1 mutant embryos

ROS was previously shown to regulate mitochondrial fragmentation in cells undergoing dorsal closure in Drosophila embryogenesis (Muliyil and Narasimha, 2014). We therefore assessed the mitochondrial morphology and distribution in hSOD1A4V, and Drp1SG;hSOD1A4V expressing embryos. Mitochondria were depleted in apical regions (Figure 6A, apical) and accumulated in basal regions in Drp1SG embryos (Figure 6A, basal and sagittal white arrowheads) as compared with controls. The mitochondrial organization in apical and basal regions was negative manner when expressed in the wild-type background (Jordan and Karess, 1997; Sen et al., 2012; Zhang et al., 2018; Das Gupta and Narasimha, 2019; Mishra et al., 2019). Furrow extension in SqhAA embryos occurred in a slow and fast phase similar to that in control and Drp1SG embryos (Figure 5, A and B). Uneven furrow extension in the fast phase was shown previously for sqh1 (Royou et al., 2004) and the sqh1;sqhAA combination (Vasquez et al., 2014). We observed shorter cells at an average length of 28.2 (±4.6) μm in SqhAA-expressing embryos (Figure 5, A–C, light red, and Supplemental Movie S9). Reduced constriction of the contractile ring has been observed in embryos with lowered myosin II activity previously (Wenzl et al., 2010; Mavrikis et al., 2014; He et al., 2016; Xue and Sokac, 2016). Expectedly, SqhAA embryos had lowered Sqh-mCherry at the furrow tips during cellularization (Figure 5, A, D, and E, light red) and decreased ring constriction (Figure 5F, light red) with a significantly larger contractile ring area 25.1 ± (9.1) μm² at the end of cellularization (Figure 5G, light red).

These data together show that shorter cell height and impaired actomyosin constriction in Drp1SG embryos occurs due to reduced myosin II function.
that in controls (Figure 6B, black) and significantly smaller than in Drp1SG (Figure 6B, red).

We also quantified the relative apical mitochondrial area to estimate apical translocation of mitochondria. The area occupied by mitochondria apically in hSOD1AV (Figure 6C, green) and Drp1SG,hSOD1AV (Figure 6C, blue) was 7.4 (±3.4)% and 5.9 (±2.1)% respectively, which was comparable to that in controls (Figure 6C, black) and significantly greater than that in Drp1SG (Figure 6C, red). These data suggest that an increase in ROS leads to a change in mitochondrial morphology in Drp1-depleted embryos, thereby reversing the mitochondrial distribution defect in the apical and basal regions.

**Contractile ring constriction and membrane ingestion defects of Drp1SG embryos are suppressed in Drp1SG;hSOD1AV embryos**

An increase in phosphorylation of myosin II subunits by Src kinases enhances their recruitment and activity at the contractile ring (Thomas and Wieschaus, 2004; Strong and Thomas, 2011). Also, Src kinase activity increases due to oxidation by elevated mitochondrial ROS during wound healing (Hunter et al., 2018). Since ROS levels were reduced in Drp1SG with a decrease in the myosin II function, we analyzed the furrow ingestion and contractile ring dynamics in hSOD1AV and Drp1SG; hSOD1AV embryos in order to test the possible role of ROS in regulating myosin II during cellularization.

First, we analyzed the membrane ingestion in hSOD1AV and Drp1SG; hSOD1AV embryos during cellularization (Figure 7A). Interestingly, the slow phase became shorter and the fast phase started earlier at around 16 min in hSOD1AV embryos (Figure 7B, light green dotted line) in contrast to 20 min in controls (Figure 7B, yellow dotted line) (Supplemental Movie S11). The membrane ingestion also occurred faster in hSOD1AV embryos (Figure 7B, green, and Supplemental Movie S10) compared with controls (Figures 5B and 7B, black, and Supplemental Movie S6). Drp1SG;hSOD1AV embryos also showed faster ingestion (Figure 7B, blue, and Supplemental Movie S11) as compared with controls (Figures 5B and 7B, black, and Supplemental Movie S6) during the fast phase. The average length of cells at the end of cellularization in hSOD1AV embryos (44.7, ±3.4)% and 5.9 (±2.1)% significantly greater than that in controls (Figure 7C, green) whereas the length in Drp1SG; hSOD1AV embryos (Figure 7C, blue) (38.6, ±3.7)% was similar to that in controls, thus suppressing the membrane ingestion defects of Drp1SG embryos (Figure 7C, red, and Supplemental Movie S11).
and their activity during port of mitochondria during cellularization. Our findings and previ
5F and 7F, black), and the final ring area (9.4 ± 1.9 μm²) was also comparable to that of controls (Figure 7G, black). Thus, contractile ring constriction and membrane ingression defects of Drp1
1.97 μm) was also significantly lower compared with the control embryos (Figure 7G, green and black, respectively). The decrease in contractile ring area with respect to membrane length in Drp1
emtions of cells has been implicated in neurodegeneration (Costa et al., 2010; Wang et al., 2011; Rawson et al., 2014). Drp1
A4V, 2005) suggest a significant regulation of microtubule motors facilitated by increased myosin-II activity by phosphorylating Sqh and inhibiting ROCK. ROCK increases myosin II activity by phosphorylating Sqh and inhibiting m1l phosphatase (MBS) (Kimura et al., 1996). Inhibition of Rok affects myosin II recruitment (Royou et al., 2002; Xue and Sokac, 2016) and results in shortening of furrow length during cellularization (Royou et al., 2002, 2004). On the other hand, optogenetic activation of Rho-GEF2 at the basal membrane increases myosin II levels, leading to increased ring constriction (Krueger et al., 2018). Notably, hSOD1
A4V embryos alone (Figure 7, D and E, green) was elevated compared with that of Sqh-mCherry controls (Figures 5, D and E, and 7, D and E, black). Sqh-GFP intensity in Drp1
mg levels are developmentally regulated so as to maintain balanced myosin-II activity in morphogenetic processes.

Mitochondrial fusion and fission proteins are maternally dumped in significant quantities in the early Drosophila embryo. The mitochondrial shape changed to a more clustered and fused form upon maternally depleting Drp1. The mitochondrial morphology regulation is essential for mitochondrial transport in cells (Li et al., 2004; Verstreken et al., 2005; Chen and Chan, 2009; Saxton and Hollebeek, 2012). On the other hand, microtubule motor activity was recently found to be important for maintaining mitochondrial shape by allowing increased mitochondrial contact to facilitate fusion (Mehta et al., 2019). Loss of mitochondrial transport to distinct locations of cells has been implicated in neurodegeneration (Costa et al., 2010; Wang et al., 2011; Rawson et al., 2014). Drp1
s under conditions of increased ROS. Mitochondrial shape changed to a more clustered and fused form upon maternally depleting Drp1. The mitochondrial morphology regulation is essential for mitochondrial transport in cells (Li et al., 2004; Verstreken et al., 2005; Chen and Chan, 2009; Saxton and Hollebeek, 2012). On the other hand, microtubule motor activity was recently found to be important for maintaining mitochondrial shape by allowing increased mitochondrial contact to facilitate fusion (Mehta et al., 2019). Loss of mitochondrial transport to distinct locations of cells has been implicated in neurodegeneration (Costa et al., 2010; Wang et al., 2011; Rawson et al., 2014). Drp1
Drosophila embryo dorsal closure and zebrafish wound healing (Muliyil and Narasimha, 2014; Hunter et al., 2018). Consistent with these studies, Drp1
Drosophila embryos containing reduced ROS levels displayed a decrease in Sqh intensity leading to impaired constriction of contractile rings and shorter membrane length during cellularization. Interestingly, the Drp1
Drosophila embryos showed inhibition of mitochondrial transport to microtubule plus ends, leading to their accumulation in apical planes (Chowdhary et al., 2017). Kinesin knockdown showed premature apical accumulation of mitochondria during early cellularization. This is similar to previous analyses with lipid droplets that accumulate apically in the absence of kinesin motors (Shubeita et al., 2008). Mitochondria can bind to both dynein and kinesin adapters with the help of Miro (Saotome et al., 2008; Russo et al., 2009). The increased mitochondrial accumulation in basal sections in dhc and miro suggest their role in microtubule motor–based apical transport of mitochondria during cellularization. Our findings and previous work on apical transport of Golgi complexes and basal transport of lipid droplets (Welte et al., 1998; Sisson et al., 2000; Papoulas et al., 2005) suggest a significant regulation of microtubule motors and their activity during Drosophila cellularization.

DISCUSSION
Our study shows that mitochondria need to be fragmented in order to distribute uniformly along the apicolobasal axis, thereby enabling appropriate furrow ingestion and ring constriction in Drosophila cellularization (Figure 8). We find that the basal-to-apical translocation of larger, clustered mitochondria is inhibited in Drp1 mutant embryos, giving rise to defects in membrane ingestion and ring constriction. Given the similarity in the ring constriction and membrane ingestion defects between Drp1
AA and significantly lower levels of Sqh in Drp1
AA mutants, the defects are likely to be due to inhibition of myosin II during cellularization (Figure 8). The suppression of these defects by supplementing ROS indicates that ROS depletion in Drp1 mutants is a likely mechanism for regulation of myosin II activity. We discuss the implications of our findings on the following: 1) the regulation of microtubule motor activity and distribution of organelles during cellularization, 2) the role of mitochondrial size in distribution of mitochondria in distinct parts of the developing embryo, and 3) the role of ROS levels in maintaining a threshold of active myosin II for morphogenetic processes in cellularization.

Previous studies have shown that Golgi complexes associate with dynein with the help of adapter protein Lava Lamp and migrate apically during the late stages of cellularization (Sisson et al., 2000; Papoulas et al., 2005). Inhibition of Golgi activity leads to disruption of membrane extension during cellularization (Sisson et al., 2000). On the other hand, lipid droplets are transported to the basal regions by kinesin (Arora et al., 2016). Our previous analysis of depleting the plus-end motor, kinesin, during syncytial division cycles of Drosophila embryos showed inhibition of mitochondrial transport to microtubule plus ends, leading to their accumulation in apical planes (Chowdhary et al., 2017). Kinesin knockdown showed premature apical accumulation of mitochondria during early cellularization. This is similar to previous analyses with lipid droplets that accumulate apically in the absence of kinesin motors (Shubeita et al., 2008). Mitochondria can bind to both dynein and kinesin adapters with the help of Miro (Saotome et al., 2008; Russo et al., 2009). The increased mitochondrial accumulation in basal sections in dhc and miro suggest their role in microtubule motor–based apical transport of mitochondria during cellularization. Our findings and previous work on apical transport of Golgi complexes and basal transport of lipid droplets (Welte et al., 1998; Sisson et al., 2000; Papoulas et al., 2005) suggest a significant regulation of microtubule motors and their activity during Drosophila cellularization.
FIGURE 7: Contractile ring area and cell length defects in Drp1^{SG} are suppressed in Drp1^{SG}; hSOD1^{AV} embryos. 
(A–C) Cell length defects are suppressed in Drp1^{SG}; hSOD1^{AV} embryos. hSOD1^{AV} and Drp1^{SG}; hSOD1^{AV} embryos are imaged sagittally using Sqh-mCherry and Sqh-GFP, respectively (A). Membrane ingression, plotted with time during cellularization, is faster in hSOD1^{AV} (green, B) and Drp1^{SG}; hSOD1^{AV} (blue, B) compared with control (black, B, repeated from Figure 5B for comparison) embryos. Drp1^{SG}; hSOD1^{AV} (blue, B) shows a suppression compared with Drp1^{SG} (red, B, repeated from Figure 5B). n = 4, nine embryos for hSOD1^{AV} and Drp1^{SG}; hSOD1^{AV}, respectively (B). 
Average final cell height achieved postcellularization (A, red arrowheads) in hSOD1^{AV} (green, B and C) is significantly longer compared with control embryos (black, repeated from Figure 5C). Average final length in Drp1^{SG}; hSOD1^{AV}
It is possible that ROS, in order to fragment mitochondria, can reduce the activity of mitochondrial fusion proteins such as Opal and Marf by oxidizing them directly or indirectly (Yashima et al., 2018) when Drp1 itself is inactive in mutants like Drp1SG. We have previously shown that depletion of Marf in addition to Drp1 mutation leads to reversal of Drp1 defects in Drosophila follicle cell differentiation by reducing mitochondrial fusion (Mitra et al., 2012). Our results show that depletion of Opal in combination with Drp1SG suppresses the clustered mitochondrial morphology phenotype in Drosophila embryos. Thus, ROS-mediated inhibition of mitochondrial fusion in Drp1SG embryos may be the cause of mitochondrial fragmentation in the Drp1SG; hSOD1A4V embryos. Future experiments that focus on understanding how ROS can lead to mitochondrial fragmentation will provide insights into the regulation of mitochondrial shape by ROS.

In summary, we show that mitochondrial architecture, distribution, dynamics, and ROS generation activity play a crucial role in morphogenetic processes involved in cell formation during Drosophila embryogenesis. Our study motivates an analysis of how distinct mitochondrial functions of ATP and ROS generation, regulation of metabolites, and calcium buffering can regulate key morphogenetic processes in metazoan embryogenesis.

**MATERIALS AND METHODS**

**Fly stocks** All Drosophila crosses were maintained at 28°C in standard cornmeal agar medium. nanoGal4 was used for inducing expression of all transgenes and RNAi. Tub-mCherry (P[UAsh-ChRFP::Tub2], Bloomington No. 25774), khc (y; sc v sev1); P[TRIP.MOS01519];attP2, Bloomington No.: 35770), dhc (y; sc v sev2); P[TRIP.GLO00543];attP40, Bloomington No.: 38538), mir6 (y; sc v sev1); P[TRIP.LO1583];attP2, Bloomington No.: 43973), drp1i (y; v); P[TRIP.HMC0323];attP40, Bloomington No.: 51483), opa1i (y; sc v sev2); P[TRIP.MOS00349];attP2, Bloomington No.: 32358), UAS-hSOD1A4V (w1118; P[UAsh-hSOD1.A4V9.1/Tm6B, Tb]; Bloomington No.: 33607), UAS-SqhA2A (w; P[UAsh-Sqh.A20A21];, Bloomington No.: 64114), sqh-Sqh-mCherry (w; P[sc-f-mCherry.M5, 90024], and sqh-Sqh-GFP (w1118; P[sgf-PRLC3], 57145) were obtained from the Bloomington Drosophila Stock Center, Bloomington, IN. UASp-Drp1SG and UASp-Mito-PAGFP (Chowdary et al., 2017) were generated in the R. R. lab. UASp-Mito-GFP was obtained from Rachel Cox (USUHS, Bethesda, MD). Recombinant fly stocks, nanoGal4::UASp-MitoGFP (Mito-GFP) and nano-Gal4::UASp-MitoGFP::sqh-Sqh-mCherry (Mito-GFP::Sqh-mCherry). were made using standard genetic crosses and were crossed with drp1i, Drp1SG, SqhA2A, and hSOD1A4V. Drp1SG; hSOD1A4V and Drp1SG;opa1i were crossed with either nanos-Gal4 or sqh-Sqh-GFP; nanosGal4. khc, dhc, and mir6 were crossed with nanosGal4. Embryos were obtained from F1 generation flies.

**Maintaining comparable Gal4 levels across genotypes** Care was taken to maintain comparable copies of transgenes across genotypes to enable comparative expression. The nano-Gal4; UASp-Mito-GFP and nano-Gal4::UASp-MitoGFP::sqh-Sqh-mCherry recombinant line was used for expressing single mutant transgenes of Drp1i, Drp1SG, SqhA2A, hSOD1A4V. Double mutants Drp1SG; hSOD1A4V and Drp1SG;opa1i were crossed to nanos-Gal4 or nano-Gal4; Sqh-GFP to compensate for the Gal4 amounts compared with the single mutant transgenes.

**Cloning of Drp1SG mutants** The conserved residue in the GTPase domain of Drp1, Ser-193, was mutated to glycine. Primers: 5’ GAC ATG GCC ACC GAG GCA CTA AAG melting temperature (TM) = 68, length = 25 and 5’ CTT GAG TTC CTC GCC GTG GGC CAT GTG Tm = 68, length = 27 containing flanking restriction enzyme site sequences against Drp1 with a mutation at the 193rd serine to mutate it to glycine. Mutated sites are underlined in the primer. Flanking primers at the N- and C-terminus of the coding region were used to extract the sequence from the vector pOTB7 and treated with Dpn1 to get rid of the methylated strand. We further transformed the mutated vector in DH5α cells, and the colonies obtained were sent for sequencing to allow correct identification of point mutation. Correct sequences with the point mutation were further cloned into the pUASP vector and sent for injections to the fly facility in National Center for Biological Sciences, Bangalore, India.

**TEM of embryos** Yw embryos were collected for 30 min at 25°C and aged for different times at 25°C to enrich in early, mid, and late cellularization stages. Embryos were dechorionated with bleach, rinsed with water, and fixed for 10 min in a mix of 1 ml glutaraldehyde 25% and 4 ml from the vector pOTB7 and treated with Dpn1 to get rid of the methylated strand. We further transformed the mutated vector in DH5α cells, and the colonies obtained were sent for sequencing to allow correct identification of point mutation. Correct sequences with the point mutation were further cloned into the pUASP vector and sent for injections to the fly facility in National Center for Biological Sciences, Bangalore, India.

**Number of unfurrows quantified are represented in the plot (C), ns, P > 0.05, ***, P ≤ 0.001, Mann–Whitney test (C), Scale bar: 5 μm (A). (D–G) Sqh intensity at the contractile rings is suppressed in Drp1SG; hSOD1A4V. Sections through the unfurrow tips in Sqh-mCherry-containing hSOD1A4V and Sqh-GFP-containing control and Drp1SG; hSOD1A4V embryos are shown (D). Mean Sqh-mCherry intensity in hSOD1A4V (green, E) and Sqh-GFP intensity in Drp1SG; hSOD1A4V (blue, E) are quantified with respect to Sqh-mCherry (Figure 5E, black) and Sqh-GFP (E, black) control embryos, respectively. Mean Sqh-mCherry fluorescence intensity in hSOD1A4V (green, E) is greater than that in control Sqh-mCherry (Figure 5E, black). Mean Sqh-GFP intensity in Drp1SG; hSOD1A4V (blue, E) is comparable to that in control Sqh-GFP (black, E) and suppressed compared with Drp1SG (red, repeated from Figure 5E); n = 6, 9 embryos for hSOD1A4V and Drp1SG; hSOD1A4V, respectively. 40 cells per embryo. Area of contractile rings quantified with respect to length shows increased constriction in hSOD1A4V (green, F) compared with control (black, repeated from Figure 5F). Contractile ring area across membrane depth in Drp1SG; hSOD1A4V (blue, F) is comparable to that in control (black, repeated from Figure 5F) and is suppressed compared with Drp1SG (red, repeated from Figure 5F). n = 6 embryos each for hSOD1A4V and Drp1SG; hSOD1A4V five rings each (F). Mean final ring area is significantly smaller in hSOD1A4V (green, F) compared with that in control and Drp1SG embryos (black, red, repeated from Figure 5G). Mean final area in Drp1SG; hSOD1A4V (blue, G) is comparable to that in control (black, repeated from Figure 5G) and is significantly smaller than that in Drp1SG (red, repeated from Figure 5G) embryos. n = 6 embryos for hSOD1A4V and Drp1SG; hSOD1A4V; number of contractile rings quantified is indicated in the plot (G), ns, P > 0.05, *** P ≤ 0.001, Mann–Whitney test (G). Scale bar: 5 μm (D).
Two-hour-old embryo collection cages at 28°C containing yeast-supplemented sucrose–agar medium. Embryos were dechorionated using 100% bleach for 1 min and washed. They were mounted in two-chambered cover glass dishes (LabTek, Germany) in PBS and imaged live at 25°C in a temperature-controlled microscope chamber using a 63×/1.4 NA objective on a confocal laser scanning microscope (Zeiss LSM 710/780 or Leica SP8). Z stacks with 1 μm intervals were acquired across 50 μm from apical to basal regions. Sagittal imaging was done at embryonic sections where nuclei were visibly aligned. Care was taken to maintain the imaging within the 0–255 range on an 8-bit scale to avoid saturation and enable image analysis.

**Phototoxication**

Embryos expressing Mito-PAGFP were phototoxicated at rectangular regions of interest (ROIs) of 10–15 μm² area in basal regions (below the nuclei) in sagittal sections in the early cellularization stage using a 405 nm laser at 100% power and 30 iterations using a 63×/1.4 NA objective on a confocal laser scanning microscope (Zeiss LSM 780). Images were obtained using 488 nm excitation wavelength. Mean fluorescence intensities in phototoxicated ROIs and reference ROIs were measured using ImageJ. They were normalized with the mean intensity of the phototoxicated ROI at the first time point. The averaged normalized intensity values for multiple embryos were plotted with SEM using GraphPad Prism 5.0 (Figures 1, G and H, and 3, G and H).

**Immunostaining**

Embryos 3.5–4 h old were obtained from collection cages, washed, dechorionated with bleach for 1 min, fixed using 1:1 heptane and 4% paraformaldehyde (PFA) in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄) for 20 min and devitellinized by shaking in 1:1 heptane:methanol or hand devitellinized using insulin needles. Hand-devitellinization was used for staining with the F-actin label, phalloidin. The embryos were then washed thrice with PBST (Trition X-100, 0.3%), blocked for 1 h using 2% BSA, and incubated with anti-pAMPK (Cell Signalling; rabbit, 1:200) or anti-Drp1 (Leo Pallanck, University of Washington Seattle, WA; rabbit, 1:500) diluted in BSA overnight at 4°C. The samples were imaged using a Plan Apochromat 63×/1.4 NA oil immersion objective on a confocal laser scanning microscope (Zeiss LSM 710 or 780). The samples were imaged using a Plan Apochromat 63×/1.4 NA oil immersion objective on a confocal laser scanning microscope (Zeiss LSM 710 or 780 or Leica SP8). Streptavidin is used as a mitochondrial marker since it probes biotinylated proteins enriched in them (Holinshead et al., 1997). It also colocalizes with Mito-GFP and Mito-PAGFP (Chowdhary et al., 2017).

**DHE staining**

ROS estimation of embryos was performed using DHE (Molecular Probes, Lifetechnologies). DHE fluoresces when imaged at 561 nm upon oxidation due to the presence of ROS in the cells and is a quantitative readout for the amount of ROS. Embryos were fixed using heptane:4%PFA (1:1), washed with PBS, and incubated with 30 nM DHE for 7 min in PBS. The embryos were then washed with PBST, stained with Hoechst 33342 (Molecular Probes; 1:1000), and

**FIGURE 8:** Summary. Mitochondria (green) translocate on microtubules toward apical regions synchronously with furrow ingression and ring constriction (myosin, yellow) during cellularization. Clustered and presumably fused mitochondria (depicted as green filaments) in drp1 mutants lead to low ROS and are not transported apically, leading to defects in furrow ingression and ring constriction, due to loss of active myosin II (dotted yellow) from the contractile rings. These defects are suppressed either by forced fragmentation of mitochondria using opa1Δ or by supplementing ROS by expressing hSOD1ΔAV along with Drp1SG. The data suggest that myosin II activity during cellularization is regulated by optimal levels of ROS maintained by mitochondrial shape and/or localization.

containing 0.1% Tween 20 and 0.1% bovine serum albumin (BSA). Embryos were hand-devitellinized on double-stick tape with a hypodermic needle (BD Microlance 3–30Gx1/2”, BD 304000), and embryos of the desired stages were transferred to a freshly prepared fixative solution (2.5% glutaraldehyde in 50 mM cacodylate buffer, pH 7.2–7.4, 0.1% tannic acid) and then stored at 4°C in the same solution overnight. Embryos were postfixed for 2 h in 1% osmium, 2% glutaraldehyde in 50 mM cacodylate buffer at 4°C. Embryos were washed in cacodylate buffer, dehydrated in a graded ethanol series, and finally embedded in epon. Ultrathin isagittal sections (80 nm) were prepared with an ultramicrotome, mounted onto copper grids, contrasted with 1% uranyl acetate, and examined with a FEI Tecnai G2 200 kV electron microscope. Images were acquired with a FEI Eagle charge-coupled device (CCD) camera (4096 × 4096 pixels) for 1700× magnifications and an Olympus Veleta CCD camera (2048 × 2048 pixels) for 31000× and 85000× magnifications. For a given sagittal section and stage, the acquired images covered the whole region from the apical cortex down to yolk spheres.

**Live imaging**

Two-hour-old nano-Gal4::UASp-Mito-GFP, nano-Gal4::UASp-Mito-GFP::sqh-Sqh-mCherry, or sqh-Sqh-GFP; nano-Gal4--contain-
mounted on slides using Slowfades Gold (Life Technologies). The samples were imaged using a Plan Apochromat 63×/1.4 NA oil immersion objective on a confocal laser scanning microscope (Zeiss LSM 780) at the same laser and gain settings.

**Image analysis**

**Mean and total mitochondrial fluorescence intensity in Z stacks.** Mean Mito-GFP intensity was measured in Z stacks with 1 μm increments in live Mito-GFP cellularizing embryos using ImageJ. The mean intensity was normalized to the highest intensity value in each set. The average of normalized intensities from three embryos was plotted with SEM using GraphPad Prism 5.0 (Figures 1C and 3D and Supplemental Figure S2C).

To estimate mitochondrial incorporation in cells as the furrow ingressed, mean Mito-GFP intensity was measured from sum-projected Z stacks from apical to the section where furrow had reached during cellularization (Figures 1E and 3E). The intensity is plotted relative to the first cellularization time points of control and Drp1SG (Figures 1E and 3E). Total Mito-GFP during cellularization was quantified by measuring the mean intensity of sum-projected 40 Z stacks for each embryo across time in cellularization (Supplemental Figure S1B).

**Mitochondrial number quantification in EMs.** Mitochondria in the EMs were identified based on structure and electron density. The electron dense particles in the cortical nuclei above the nuclei in each cell were counted manually for early, mid, and late cellularization stage embryos. The total number of apical mitochondria per cell was plotted as mean with SEM using GraphPad prism 5.0 (Figure 1K).

**Mitochondrial area measurement in apical and basal sections and relative mitochondrial area measurement in apical sections.** Images obtained from fluorescently streptavidin-stained embryos were intensity thresholded by subtracting 1.5 times the mean intensity value of a single image plane and the mean intensity of projected images for 2 stacks. Fluorescent particles of size greater than 0.05 μm² were marked using the “particle analyser” tool in ImageJ (Figures 3B and 6B and Supplemental Figure S3B). Mean particle size was obtained for each embryo. To estimate relative apical mitochondrial area (Figure 6C and Supplemental Figure S3C), the total area of threshold-selected fluorescent particles was divided by the area of the imaging field and expressed as a percentage. The data were plotted and analyzed with a Mann–Whitney test using GraphPad Prism 5.0.

**pAMPK and DHE intensity quantification.** Control and mutant embryos were processed and imaged at the same time using the same laser power and gain settings. Differences in the levels of pAMPK antibody signals (Figure 4B) and DHE fluorescence (Figure 4D) between control and experimental embryos were estimated based on fluorescence intensity. The mean intensities were measured from single planes using ImageJ. All intensity values in control and Drp1 were divided by the average of all the mean intensity values obtained for control embryos to get normalized values to represent as a fold change with respect to the control embryos and to show variability across control embryos. The data were plotted and compared with a Mann–Whitney test using GraphPad Prism 5.0.

**Furrow membrane length measurements in cellularization.** Furrow membrane tips were identified by Sqh-mCherry or Sqh-GFP signal, and the length from the apical plasma membrane was measured by using the line tool in ImageJ (Figures 1E, 2E, 5, B and C, and 7, B and C, and Supplemental Figure S4, B and C). Readings were taken every 2 min for three to five furrows in each embryo. Mean lengths with SEM were plotted across time using GraphPad Prism 5.0. The final lengths were compared using a Mann–Whitney test on GraphPad Prism 5.0.

**Sqh-GFP and Sqh-mCherry fluorescence intensity and contractile ring area measurements.** Identical laser power and gain settings were used for imaging control and experimental embryos. Sum-intensity images for Sqh-mCherry or Sqh-GFP embryos (Figures 5E and 7E and Supplemental Figure S4E) were obtained for the basalmost optical sections showing the brightest signal and two stacks above and below it (total Z depth 4 μm). Mean fluorescence intensities of these projections were measured at different membrane ingress lengths using ImageJ and background corrected. A central region in a section below the nuclei-containing uniform signal in the first cellularization time point (membrane length 3 μm) was chosen to measure the background intensity (He et al., 2016). The values obtained were normalized using the average mean intensity value at 15 μm depth for control embryos in every imaging set; this is a point at which myosin II intensity peaks during cellularization. Note that the same trend of relative fluorescence changes was seen in Sqh-mCherry and Sqh-GFP embryos (Supplemental Figure S5). Normalized mean intensities across membrane lengths were plotted using GraphPad Prism 5.0.

**Contractile ring area (Figures 5, F and G, and 7, F and G, and Supplemental Figure S4, F and G) was measured at the basalmost membrane section from five contractile rings per embryo for fixed images or per time point for live samples. The ring area was marked manually in sum-projection images of Sqh-mCherry or Sqh-GFP–containing embryos using the polygon drawing tool in ImageJ. The average area was plotted with membrane length using GraphPad Prism 5.0. The final areas of contractile rings were compared using a Mann–Whitney test on GraphPad Prism 5.0.

**ACKNOWLEDGMENTS**

We thank Girish Ratnaparkhi for discussions on the data and manuscript. We thank R. R. lab members for their critical input on the data in the manuscript. We thank the Indian Institute of Science Education and Research (IISER), Pune, Drosophila and Microscopy facilities for help with experiments. We thank the Bloomington Drosophila Stock Center, Bloomington, IN, for stocks and Developmental Studies Hybridoma bank (DSHB) for antibodies. We thank Leo Pallanck for anti-Drp1 antibody. S. C. thanks UGC, S. M. thanks IISER, Pune, D. T. thanks University Grants Commission (UGC), and R. R. thanks the Department of Biotechnology (DBT), Department of Science and Technology (DST) and IISER, Pune, for funding. We thank J-P. Chauvin, A. Aouane, and F. Richard at the Institut de Biologie du Développement de Marseille (IBDM) electron microscopy facility (Marseille, France) for help with embryo processing and image acquisition.

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