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Cyclin C Mediates Stress-Induced Mitochondrial Fission and Apoptosis

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Cyclin C mediates stress-induced mitochondrial fission and apoptosis

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ABSTRACT **Mitochondria are dynamic organelles that undergo constant fission and fusion cycles. In response to cellular damage, this balance is shifted dramatically toward fission. Cyclin C–Cdk8 kinase regulates transcription of diverse gene sets. Using knockout mouse embryonic fibroblasts (MEFs), we demonstrate that cyclin C directs the extensive mitochondrial scission induced by the anticancer drug cisplatin or oxidative stress. This activity is independent of transcriptional regulation, as Cdk8 is not required for this activity. Furthermore, adding purified cyclin C to unstressed permeabilized MEF cultures induced complete mitochondrial fragmentation that was dependent on the fission factors Drp1 and Mff. To regulate fission, a portion of cyclin C translocates from the nucleus to the cytoplasm, where it associates with Drp1 and is required for its enhanced mitochondrial activity in oxidatively stressed cells. In addition, although HeLa cells regulate cyclin C in a manner similar to MEF cells, U2OS osteosarcoma cultures display constitutively cytoplasmic cyclin C and semifragmented mitochondria. Finally, cyclin C, but not Cdk8, is required for loss of mitochondrial outer membrane permeability and apoptosis in cells treated with cisplatin. In conclusion, this study suggests that cyclin C connects stress-induced mitochondrial hyperfission and programmed cell death in mammalian cells.**

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INTRODUCTION

Mitochondria are dynamic organelles that undergo fusion and fission cycles that are controlled by conserved molecular machines consisting of dynamin-like GTPases (for review, see Westermann, 2010b). Under normal growing conditions, mitochondria are usually observed in a connected, reticular morphology. Mitochondrial fusion requires two GTPases, mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2), located in the mitochondrial outer membrane (MOM; for review, see Chan, 2012). The mitochondrial inner membrane fusion is mediated by a third GTPase, OPA1 (Olichon *et al.*, 2003). Another GTPase, termed Drp1, mediates mitochondrial fission. For fission, GTP-bound Drp1 is recruited to the mitochondrial outer membrane, where it forms atypical spiral filaments around the mitochondria. Then, GTP hydrolysis induces ring constriction and subsequent scission of the mitochondria (Smirnova *et al.*, 2001; Mears *et al.*, 2011). Drp1 is predominantly cytosolic under normal growth conditions but is recruited to mitochondria via membrane-bound receptors hFis1 (Yoon *et al.*, 2003) and Mff (Otera *et al.*, 2010), although Mff appears to play the dominant role (Loson *et al.*, 2013; Richter *et al.*, 2014). In addition, MiD49 and MiD51 (Palmer *et al.*, 2011a) also can recruit Drp1 to the mitochondria (Loson *et al.*, 2013; Richter *et al.*, 2014). Of interest, MiD49 or MiD51 overexpression exhibited a dominant-negative effect on the fission machinery, resulting in elongated mitochondria (Palmer *et al.*, 2011a; Liu *et al.*, 2013), which is dependent on either mitofusin (Palmer *et al.*, 2013). These findings indicate that mitochondrial dynamics is finely tuned, and this balance can be dramatically shifted in either direction through manipulation of the fission or fusion machinery.

Mitochondrial dynamics play an important role in many physiological processes, including the overall health of the organelle. For example, fission is required to eliminate defective mitochondria via mitophagy, whereas fusion facilitates mitochondrial DNA and membrane repair (Chan, 2012). In addition, loss of mitochondrial fission

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Abbreviations used: FRAP, fluorescence recovery after photobleaching; GST, glutathione *S*-transferase; HA, hemagglutinin; MEF, mouse embryonic fibroblast; PCD, programmed cell death; PES, 2-phenylethynesulfonamide; ROS, reactive oxygen species; TMRM, tetramethylrhodamine methyl ester; YFP, yellow fluorescent protein.

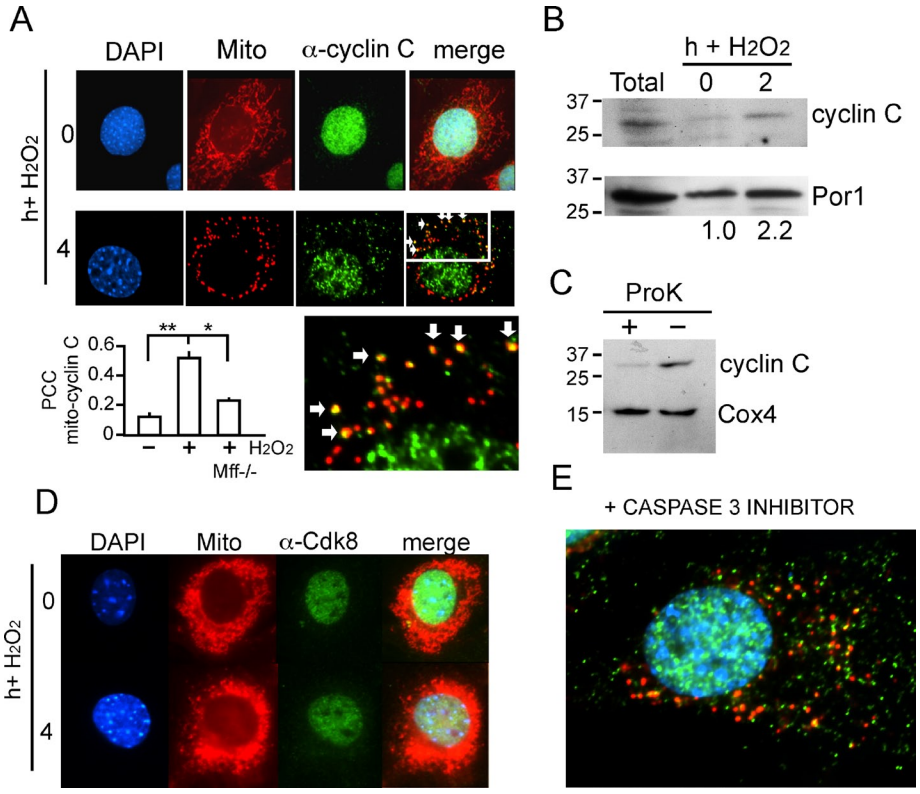

FIGURE 1: Cyclin C relocalizes to the mitochondria after stress. (A) Representative images of cyclin C localization as monitored by indirect IF in MEF cultures before and after H_2O_2 treatment (0.4 mM for 4 h). Mitochondria and nuclei were visualized using MitoTracker Red and DAPI staining, respectively. Mitochondria–cyclin C colocalization was calculated using the PCC under the conditions indicated. Bars indicate mean (±SEM) from three experiments. *$p = 0.05$, **$p = 0.001$. Inset, 3× magnified image; arrows indicate mitochondria–cyclin C colocalization. (B) Western blot analysis of similar cell equivalents of mitochondrial fractions prepared from immortalized MEF cells before and after 0.4 mM H_2O_2 treatment. Por1 levels were monitored to determine mitochondrial loading. Cyclin C signal intensity relative to Por1 averaged from three experiments (SEM ≤10%) is indicated below, with untreated sample set to 1. Molecular weight standards (kilodaltons) are indicated on the left. (C) The mitochondrial fractions described in B were treated with proteinase K (+) or buffer alone (–) as indicated. Cox4 levels served as loading control for mitochondria. (D) Cdk8 localization in MEF cells treated as described in A. (E) A wild-type MEF culture was incubated with the caspase inhibitor AC-DEVD-CHO (20 μM) for 1 h and then treated with cisplatin (20 μM) for 24 h. MitoTracker Red was added 30 min before fixation. Cyclin C and nuclei were identified as in A.

in mouse knockout models is attributed to reduced neural activity and increased sensitivity to cell death stimuli (Ishihara *et al.*, 2009; Wakabayashi *et al.*, 2009). Moreover, fusion defects are associated with the human diseases optic atrophy (Alexander *et al.*, 2000; Delettre *et al.*, 2000) and the neurodegenerative disorder Charcot–Marie–Tooth type 2 (for review, see Kageyama *et al.*, 2011). In addition to normal cellular development, extensive mitochondrial fission is also an early event in the intrinsic mitochondria-dependent programmed cell death (PCD) pathway (Cheung *et al.*, 2007). For example, inactivation of Drp1, or expression of a dominant-negative allele of this GTPase, protected cells from PCD (Frank *et al.*, 2001; Arnoult, 2007) even though the proapoptotic protein Bax was still efficiently recruited to the mitochondria (Karbowski *et al.*, 2002). Consistent with a proapoptotic role for fission, cell lines lacking fusion machinery components exhibit both constitutive mitochondrial fragmentation and hypersensitivity to cell death signals (Lee *et al.*, 2004).

The cyclin protein family was initially identified as promoters of cell cyclin progression that bound and activated cyclin-dependent kinases (Cdks; Glotzer, 1995). As indicated by their name, cyclins display a transient expression pattern, with their levels peaking at specific stages during mitotic cell division (reviewed in Murray, 2004). However, additional cyclin–Cdk kinases (e.g., cyclin C–Cdk8, cyclin H–Cdk7, and cyclin T–Cdk9) were subsequently discovered that regulate transcription rather than cell cycle progression (Dynlacht, 1997; Bregman *et al.*, 2000). This group shares several characteristics, including association with the RNA polymerase II machinery (Bjorklund and Kim, 1996; Conaway and Conaway, 2011) and the fact that the cyclin levels do not fluctuate during the cell cycle (Lew *et al.*, 1991; Cooper *et al.*, 1997). Of these cyclin–Cdk pairs, cyclin C–Cdk8 shares the most sequence conservation from yeast to human (Lolli, 2010). The present study reports that in response to oxidative stress or anticancer drug treatment, a portion of cyclin C translocates from the nucleus to the mitochondria. Further studies revealed that cyclin C is both necessary and sufficient for stress-induced hyperfission in a manner independent of Cdk8 activity. These results identify a new role for cyclin C as a key mediator of stress-activated mitochondrial fragmentation.

RESULTS

Cyclin C relocalizes to the mitochondria after stress

We recently described a new role for the yeast cyclin C in the induction of mitochondrial fission after exposure to hydrogen peroxide (Cooper *et al.*, 2012, 2014). This role was direct, as cyclin C translocated from the nucleus to sites of fission at the mitochondria. However, given the significant differences between the yeast and mammalian fission processes (Adachi and Sesaki, 2014), it was unclear whether cyclin C performed a similar function in cells of higher organisms. To determine whether this role is conserved, we monitored the subcellular localization of cyclin C by indirect immunofluorescence (IF) in immortalized mouse embryonic fibroblast (MEF) cultures. As expected, cyclin C localized predominantly in the nuclei in the absence of stress (Figure 1A, top). However, by 4 h after 0.4 mM H_2O_2 treatment, 77% (±9; $n = 6$) of the culture exhibited a portion of cyclin C in the cytoplasm (Figure 1A, bottom). To determine whether cyclin C was directed to a particular cytoplasmic address, we also treated the cells with a mitochondrion-specific stain (MitoTracker Red). As expected, the mitochondrial morphology changed from reticular to fragmented after H_2O_2 treatment in 93% (±5, $n = 4$) of the cells. Of importance, this analysis revealed that 100% of the cells exhibiting cytoplasmic cyclin C demonstrated its partial colocalization with the mitochondria (arrows, Figure 1A, bottom). Quantifying cyclin C-mitochondrial colocalization revealed a statistically significant increase in stressed cells. In addition, cyclin C signals were observed independent of the mitochondria, suggesting that cyclin C has additional cytoplasmic destinations and/or transiently associates with this organelle.

To further investigate cyclin C–mitochondria interaction, we conducted subcellular fractionation in extracts prepared from MEF cells before and after H_2O_2 treatment. These studies revealed a modest (2.2-fold) enrichment of cyclin C in the mitochondrial fraction only in the H_2O_2-treated cells (Figure 1B). A similar enrichment was observed for the yeast cyclin C (Cooper *et al.*, 2012, 2014). However, unlike the yeast cyclin C (Cooper *et al.*, 1997), the mouse protein is not subjected to proteolysis after stress (Supplemental Figure S1). Cyclin C was sensitive to proteinase K digestion in these mitochondrial fractions (Figure 1C), suggesting that it localized to the outer mitochondrial membrane. However, some residual cyclin C was still detected after proteinase K treatment, leaving open the possibility that a portion of mitochondrial cyclin C is internalized. In addition, indirect immunofluorescence revealed a very low cytoplasmic Cdk8 signal after stress application (Figure 1D). These studies indicate that cyclin C, but not Cdk8, exhibits stress-induced relocalization to the mitochondria and perhaps other cytoplasmic addresses.

One possible explanation of the results just described is that, due to its relatively small size (33 kDa), cyclin C may simply leak out of the nucleus due to nuclear breakdown associated with the apoptotic response. To test this possibility, we treated MEF cells with a caspase 3 inhibitor (Ac-DEVD-CHO) to prevent H_2O_2-induced PCD progression and loss of nuclear integrity. Similar to the results in Figure 1A, mitochondrial fragmentation (97% ± 0.5; $n = 3$) and cyclin C relocalization (83% ± 4; $n = 3$) were observed in these cells (see Figure 1E for a representative image). These results indicate that cyclin C relocalization and mitochondrial fragmentation do not require caspase activity.

Cyclin C is required for stress-induced mitochondrial fission

The mitochondrial localization of cyclin C prompted the question of whether this factor was involved in the extensive mitochondrial remodeling that occurs in stressed cells. To address this question, we constructed a floxed allele of cyclin C ($CCNC^{fl}$) with Cre recombination sites flanking exons 2–4 that encode most of the cyclin box domain responsible for Cdk8 interaction (Supplemental Figure S2; see *Materials and Methods*). An immortalized homozygous CCNC deletion ($CCNC^{-/-}$) MEF cell line was generated (Figure 2A). Gross analysis of this $CCNC^{-/-}$ cell line did not reveal any significant differences in growth rate, cell morphology, or return to growth kinetics after dilution and replating (unpublished data). Therefore cyclin C is dispensable for mitotic cell division.

To trigger mitochondrial hyperfission, the cells were treated with the anticancer drug cisplatin. Cisplatin was chosen because it provides a reproducible cellular response but still operates by generating reactive oxygen in the nucleus to induce DNA damage (for review, see Deavall *et al.*, 2012). In the wild-type cell line, an eightfold increase in cells containing fragmented mitochondrial was observed after drug treatment (Figure 2B). Conversely, no significant increase in the percentage of cells exhibiting the hyperfission phenotype was observed in $CCNC^{-/-}$ MEF cells compared with the untreated control. However, mitochondrial morphology in the stressed $CCNC^{-/-}$ cells appeared less regular than in the untreated control, with potential constrictions (arrows, Figure 2C).

To quantify the extent of mitochondrial fission in a different way, we conducted a fluorescence recovery after photobleaching (FRAP) assay. This approach measures mitochondrial connectivity by determining the rate at which a photobleached mitochondrial section gets repopulated by unbleached fluorescent molecules from connected mitochondria (Cleland *et al.*, 2011). The faster the photobleached region is repopulated, the more connected are the mitochondria. Wild-type or $CCNC^{-/-}$ MEF cultures were maintained in a temperature/atmosphere-controlled chamber and subjected to FRAP analysis after H_2O_2 treatment. In two separate trials, the H_2O_2-treated $CNCC^{-/-}$ MEF cells exhibited a faster recovery after photobleaching compared with the control cells (Figure 2D). Taken together, these experiments indicate that cyclin C is required for extensive mitochondrial fission after stress. To determine whether Cdk8 also mediates stress-induced mitochondrial fragmentation, we repeated these experiments with wild-type MEF cells knocked down for Cdk8 (Figure 2E). The analysis of mitochondrial morphology revealed no reduction in stress-induced hyperfission when Cdk8 levels were reduced compared with wild type (Figure 2F). These results indicate that cyclin C, but not Cdk8, is required for stress-activated mitochondrial fragmentation.

Cyclin C is sufficient to induce mitochondrial fragmentation

The foregoing results indicate that cyclin C is necessary for stress-induced hyperfission. The next question we addressed was whether cyclin C was sufficient to induce fission or whether additional stress-specific signals were required. To examine this issue, we used permeabilized $CCNC^{-/-}$ MEF cells and *Escherichia coli*–purified human glutathione *S*-transferase (GST)–cyclin C (Hs GST-cyclin C). The $CCNC^{-/-}$-null cell line was used to avoid any contribution from endogenous cyclin C. GST-cyclin C or GST (~4 nM) was added to digitonin-treated cells, and mitochondrial morphology was monitored in living cells by confocal microscopy. Images were collected for 18 min at 2-min increments. Before addition of the fusion proteins, the percentage of cells exhibiting predominantly fragmented mitochondria was calculated for each dish. Cells were considered to have fragmented mitochondria if they did not possess ≥10 mitochondria with a length >10 μM. As indicated in Figure 1, 8–10% of the cells exhibited a fragmented morphology before treatment. Deconvolved images reveled little detectable changes in mitochondrial morphology in the GST-treated cultures up to 18 min (9.3%; Figure 3A, left). However, significant fragmentation of the mitochondria was observed when the human GST-cyclin C was added to cells beginning by 10 min, with 94% of the culture exhibiting complete fragmentation by 18 min (arrows, Supplemental Figure S3, middle, for a full-field view). These results indicate that cyclin C is sufficient to induce mitochondrial fission without an added stress signal. To determine whether this function is conserved, we also examined the activity of the yeast cyclin C fused to GST (GST-cyclin C Sc). The yeast fusion protein was as efficient in inducing mitochondrial fission as the human cyclin C (Figure 3A, right), indicating that this activity is conserved. To determine whether the quantity of GST-cyclin C added was rate limiting, we repeated this experiment with twice the GST-cyclin C Hs concentration as before. These studies revealed a more rapid response, with total mitochondrial fragmentation occurring by 8 min (Figure 3B). These results suggest that the relocalization rate of cyclin C may help the cell regulate the kinetics of mitochondrial fission.

Previous studies found that Drp1 function is controlled by several posttranslational modifications, such as phosphorylation (Palmer *et al.*, 2011b), SUMOylation (Braschi *et al.*, 2009; Guo *et al.*, 2013), and ubiquitylation (Horn *et al.*, 2011). For phosphorylation, both inhibitory (Chang and Blackstone, 2007; Cribbs and Strack, 2007) and stimulatory (Taguchi *et al.*, 2007) modifications were identified. For example, cyclin B–Cdk1 phosphorylation at the G2/M boundary stimulates Drp1 activity to induce fission to promote mitochondrial partitioning during mitosis (Taguchi *et al.*, 2007). This modification is clearly observed in cells arrested at the G2/M boundary after addition of the microtubule-destabilizing agent nocodazole (Figure 3C). Therefore one possibility is that cytoplasmic cyclin C is now free to

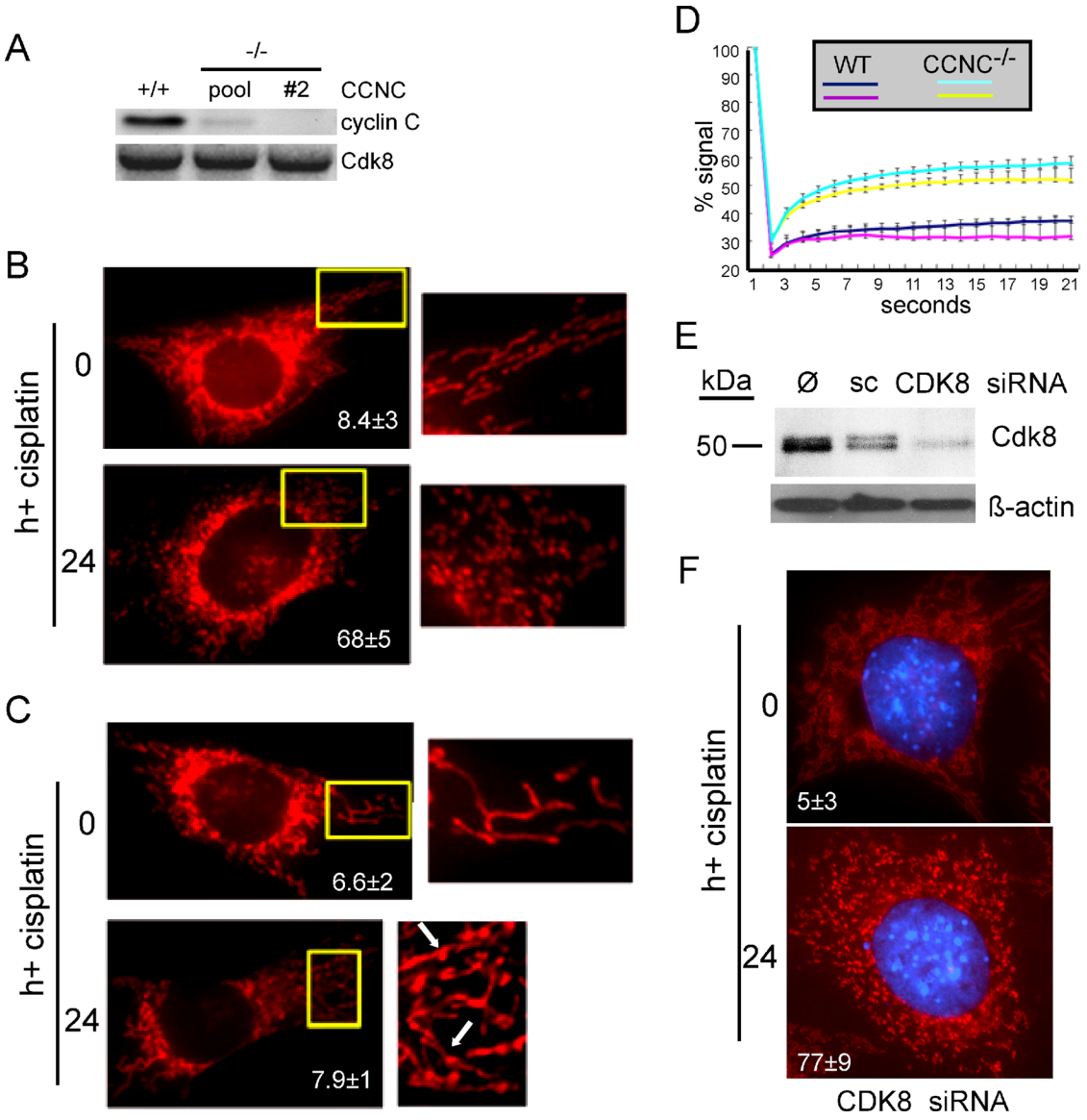

FIGURE 2: Cyclin C is required for stress-induced mitochondrial fission. (A) Western blot analysis of cyclin C levels in wild-type and CCNC$^{-/-}$ MEF cells (see Supplemental Figure S2). Cdk8 levels were monitored as a loading control. Mitochondrial morphology was monitored in CCNC$^{+/+}$ (B) or CCNC$^{-/-}$ (C) MEF cells before and 24 h after 20 μM cisplatin treatment. Percentages of cells with highly fragmented mitochondria (mean ± SEM, $n = 3$). Enlarged images are indicated by the boxes. Arrows indicate regions of intermediate phenotype. (D) FRAP studies were conducted on wild-type and CCNC$^{-/-}$ MEF cells after H_2O_2 treatment (0.4 mM). Curves represent separate experiments in which at least 40 individual mitochondria were sampled (mean ± SEM). (E) Western blot analysis of wild-type MEF cells treated with either scrambled (sc) or CDK8-specific siRNAs. β-Actin levels were used as a loading control. Ø, mock transfection. (F) Mitochondrial morphology of CDK8 siRNA–transfected cells analyzed as described in B.

interact with another Cdk to modify Drp1 and induce fission. However, we find no increase in Drp1 phosphorylation at the activating serine (Ser-616) in HeLa cells subjected to H_2O_2 or in permeabilized CCNC$^{-/-}$ MEF cells treated with GST-cyclin C (Figure 3D). These results indicate that enhanced Ser616 phosphorylation is not required for elevated Drp1 activity in stressed cells. Quantitation of the Ser-616 phosphorylation signal indicated that this modification was reduced in permeabilized cells treated with GST-cyclin C (Figure 3D). Although we have no clear role for cyclin C in controlling Drp1 phosphorylation, one possible explanation is that the accelerated fission in these experiments results in dephosphorylation of Ser-616, helping to attenuate mitochondrial fragmentation.

Cyclin C–induced fission operates through the Drp1-Mff pathway

Next we assessed whether GST-cyclin C was functioning though the known mitochondrial fission pathway. As outlined earlier, fission requires recruitment of the dynamin-like GTPase Drp1 to the mitochondria. To determine whether Drp1 is necessary for cyclin C-induced fission, we added GST-cyclin C to permeabilized cells treated with the reported IC_{50} concentration of Mdivi-1, a specific inhibitor of the GTPase Drp1 (Cassidy-Stone *et al.*, 2008). This compound effectively inhibits Drp1 function in human cell lines (Cui *et al.*, 2010), as well as in MEF cultures (Vazquez-Martin *et al.*, 2012). Compared to untreated controls, fragmentation was reduced in these cells,

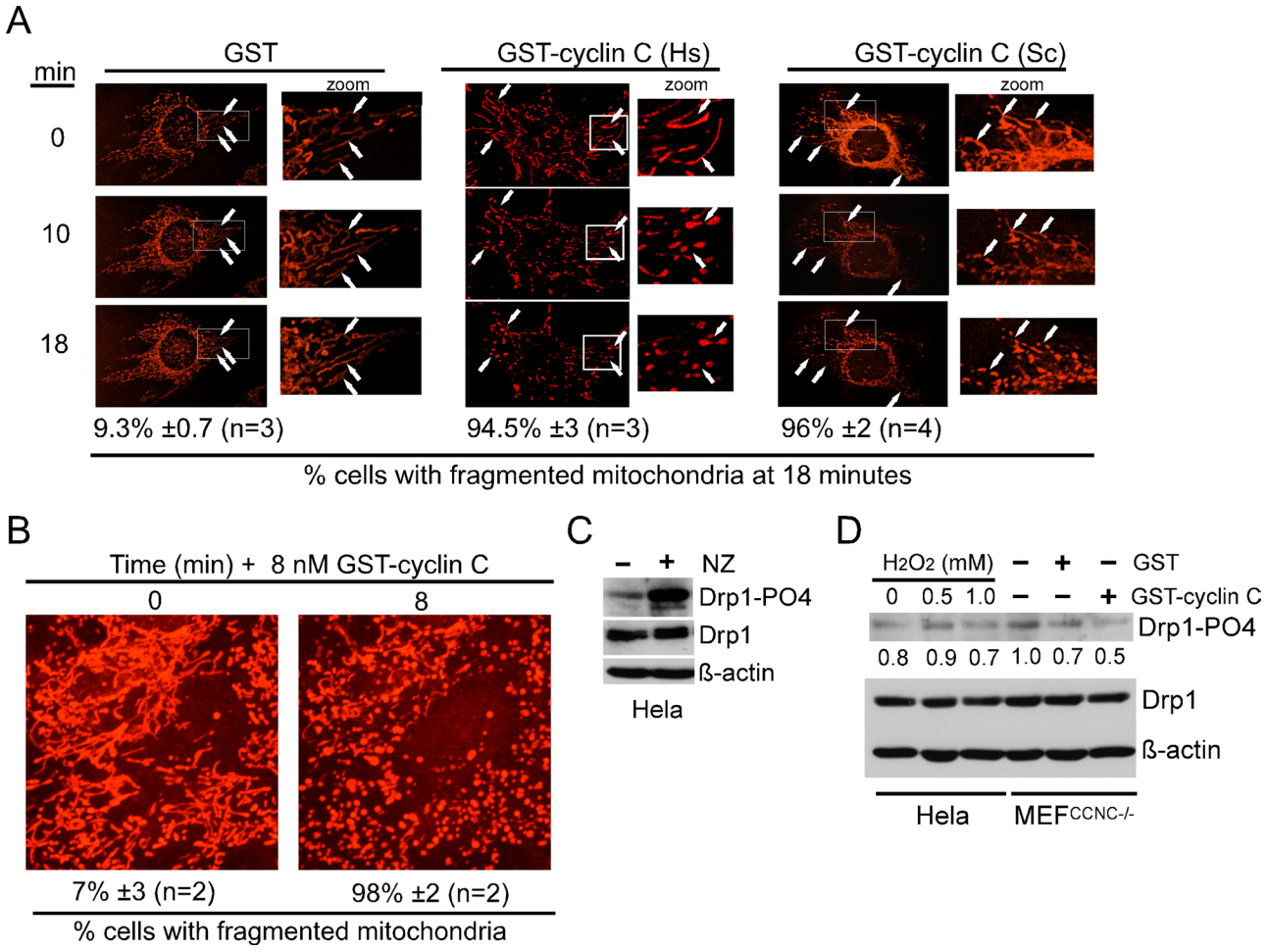

FIGURE 3: Cyclin C is sufficient to induce mitochondrial fission in the absence of stress. (A) Live-cell imaging of CCNC$^{-/-}$ MEF cells permeabilized by digitonin and then incubated with ~4 nM of GST or human (Hs) or yeast (Sc) GST-cyclin C for the times indicated. Magnified regions (zoom) are indicated by the boxes. Arrows indicate regions of interest described in the text. GST alone controlled for the effects of permeabilization on mitochondrial morphology. Percentage of each population exhibiting a fragmented phenotype by 18 min (mean ± SEM). (B) Experiment in A repeated with 8 nM GST-cyclin C. (C) HeLa cells after a thymidine block/release protocol were treated with nocodazole (NZ; 0.1 μg/ml) for 12 h to arrest cells in G2. Western blot analysis of whole-cell extracts probed with the antibodies indicated to determine Ser-616 phosphorylation status. β-Actin levels were used as a loading control. (D) Western blot analysis of Ser-616 phosphorylated Drp1 in HeLa cells exposed (4 h) to differing concentrations of H_2O_2 or permeabilized CCNC$^{-/-}$ MEF cells incubated 20 min with GST or GST-cyclin C as indicated. The blot was stripped and reprobed for Drp1 and β-actin for loading controls. The intensities of the phospho-Drp1 signals relative to Drp1 were determined by phosphorimaging for each lane.

with long mitochondrial filaments still observed in nearly half of the cells after 18 min (white arrows, Figure 4A). These results indicate that Drp1 is required for GST-cyclin C–induced fission.

Drp1 is recruited to the mitochondria by a collection of outer membrane receptors (Mff, hFis1, MiD49, MiD51; Otera *et al.*, 2010; Loson *et al.*, 2013; Palmer *et al.*, 2013), with Mff playving the major role in this process. To determine whether cyclin C-induced fission operates through one or more of these receptors, the we repeated permeabilized cell assay with MEF cell lines deleted for hFis1, Mff, or both genes. Without added GST-cyclin C, these cell lines exhibited extended mitochondrial morphology, with this phenotype being most pronounced in the double mutant (Loson *et al.*, 2013; Figure 4B, top). The addition of GST-cyclin C induced robust fission in the hFis1$^{-/-}$ cell line within 20 min (white arrows, Figure 4B, left). Similarly, fission was observed in the Mff$^{-/-}$-knockout cell line (Figure 4B, middle), although the total amount of fragmentation was significantly reduced. However, the mitochondria were protected from GST-cyclin C–induced fission in the double-mutant cell line (Figure 4B, right). These results indicate that cyclin C uses both hFis1 and Mff to mediate fission in this assay, with Mff playing the predominant role. In support of this model, cyclin C–mitochondria colocalization is significantly reduced in stressed (0.4 H_2O_2) cells lacking Mff and hFis1 function (Figure 1A). Taken together, our results indicate that cyclin C operates predominantly through Mff-Drp1 to direct mitochondrial fragmentation in these assays.

Cyclin C is required for stress-induced mitochondrial accumulation of Drp1

The results just described indicate that cyclin C induces mitochondrial fission through a Drp1-dependent pathway. We next addressed whether cyclin C controls mitochondrial fission by regulating Drp1. In response to stress, Drp1 localization changes from

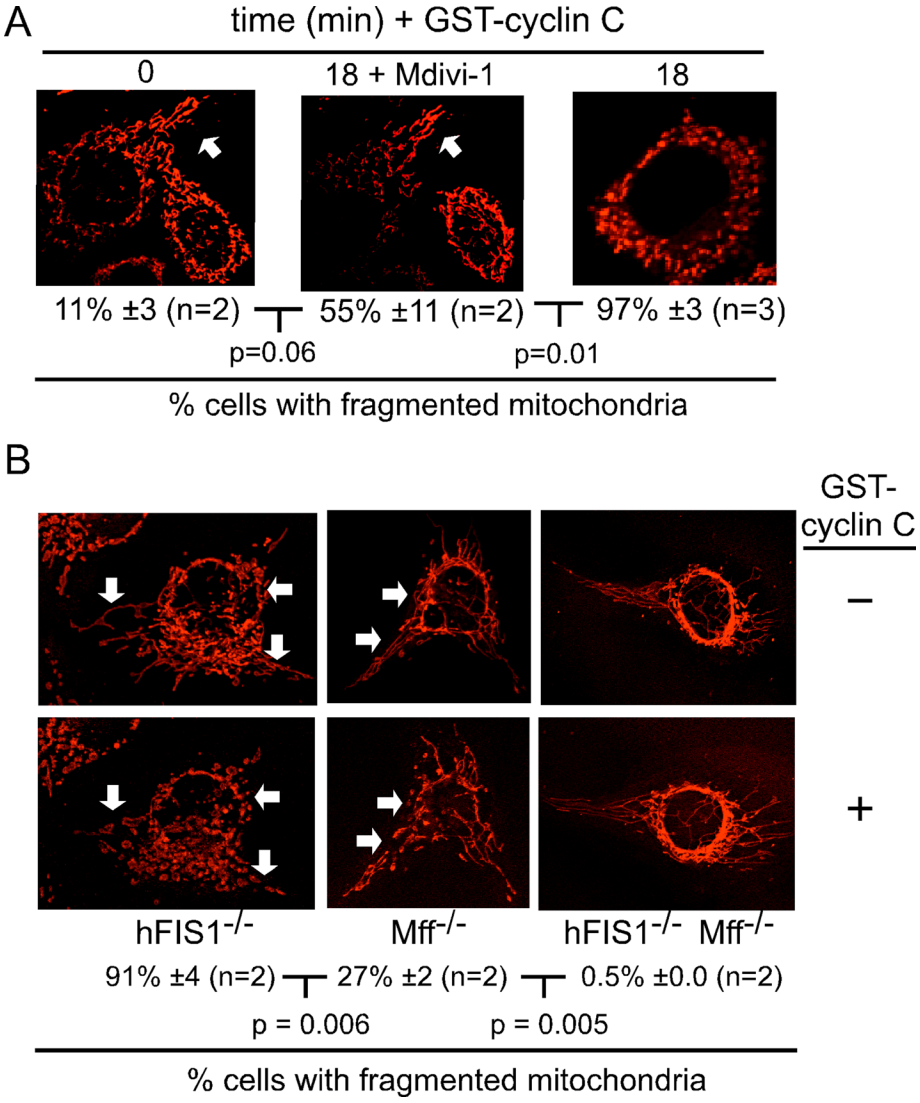

FIGURE 4: Cyclin C induces mitochondrial fragmentation through the normal fission machinery. (A) CCNC$^{-/-}$ MEF cells with our without Mdivi-1 treatment (50 μM) for 30 min before digitonin permeabilization and incubation with human GST-cyclin C for the times indicated. Arrows indicate regions of the mitochondria refractory to fission. (B) Representative images of MEF cells with the indicated genotypes before (top) and after (bottom) addition of GST-cyclin C. Arrows indicate regions of the mitochondria undergoing fission. Percentage of cells exhibiting a fission phenotype is indicated with corresponding *p* values.

mostly cytosolic to distinct foci at sites of fission (Frank *et al.*, 2001). As shown in Figure 5A, wild-type CCNC$^{+/+}$ MEF cells treated with cisplatin displayed Drp1 accumulation at the mitochondria, especially those ringing the nucleus. This change in mitochondrial colocalization can be visualized by profiling the relative Drp1 and mitochondrial signals (Figure 5A, right). Not only do the mitochondrial and Drp1 signals demonstrate a more positive correlation after cisplatin treatment, the widths of the foci increased as well, suggesting Drp1 aggregation. This increase in Drp1–mitochondria colocalization was confirmed calculating the Pearson colocalization coefficient (PCC) for these experiments (Figure 5C).

Next we followed Drp1–mitochondria association in CCNC$^{-/-}$ MEF cells. Before cisplatin treatment, Drp1 displayed mitochondrial colocalization similar to wild type (Figure 5, B, top, and C). In cisplatin-treated CCNC$^{-/-}$ cultures, a significant reduction in Drp1–mitochondria colocalization was observed compared with wild type (Figure 5C). This reduced association was also observed using fluorescence microscopy (Figure 5B, bottom). Similarly, Drp1 enrichment in mitochondrial fractions derived from stressed cells was present, but reduced, in CCNC$^{-/-}$ cells compared with wild type (Figure 5D). Taken together, these results indicate that cyclin C is required for efficient relocalization of Drp1 from the cytoplasm to the mitochondria. However, it is important to note that a statistically significant increase in Drp1 association with the mitochondria was still observed in stressed CCNC$^{-/-}$ cells. This observation suggests that cyclin C may be promoting mitochondrial fission through a mechanism other than simple targeting of Drp1 to the mitochondria (see *Discussion*).

Stress-activated subcellular relocalization of cyclin C in HeLa cells

The physiology of transformed versus nontransformed cells differs markedly. For example, elevated internal reactive oxygen species (ROS; Trachootham *et al.*, 2006) and alternative carbon utilization pathways (Warburg, 1956) often distinguish transformed from normal cells. To determine whether stress-induced cyclin C localization occurred in human tumor cell lines, we visualized endogenous cyclin C localization by IF in H_2O_2-stressed HeLa cells. In the absence of stress, cyclin C displayed diffuse nuclear staining, as expected for a transcription factor (Figure 6A, top). However, unlike MEF cultures, a portion of cyclin C was observed as small cytoplasmic punctate foci even under normal culture conditions (Figure 6A, boxed region). Increased magnification revealed that cyclin C was associated with the mitochondria (arrow), but much of the signal observed was independent of the mitochondria. H_2O_2 addition enhanced mitochondrial fragmentation, although not as dramatically as observed in MEF cells (Figure 6A, bottom). Moreover, cyclin C foci were more aggregated in the cytoplasm and displayed clear association with the ends of the mitochondria (arrows). Similar to MEF cultures, Cdk8 remained nuclear in H_2O_2-treated HeLa cells (Figure 6B). These findings indicate that cyclin C relocalization to the mitochondria after stress is also observed in HeLa cells.

The absence of a predicted mitochondrial signal sequence indicated that translocation of cyclin C to the mitochondria occurred through a nonsecretory pathway. The tumor suppressor p53 also displays mitochondrial translocation in response to DNA damage (Murphy *et al.*, 2004) through a mechanism sensitive to the Hsp70 chaperone inhibitor pifithrin-μ (PES; Strom *et al.*, 2006; Leu *et al.*, 2009). To determine whether a similar strategy is used to relocalize cyclin C from the nucleus to the cytoplasm, we added PES to HeLa cells before H_2O_2 addition. Without PES, cyclin C demonstrated enhanced colocalized with the mitochondria after H_2O_2 treatment (Figure 6C). However, pretreatment with PES reduced mitochondrial

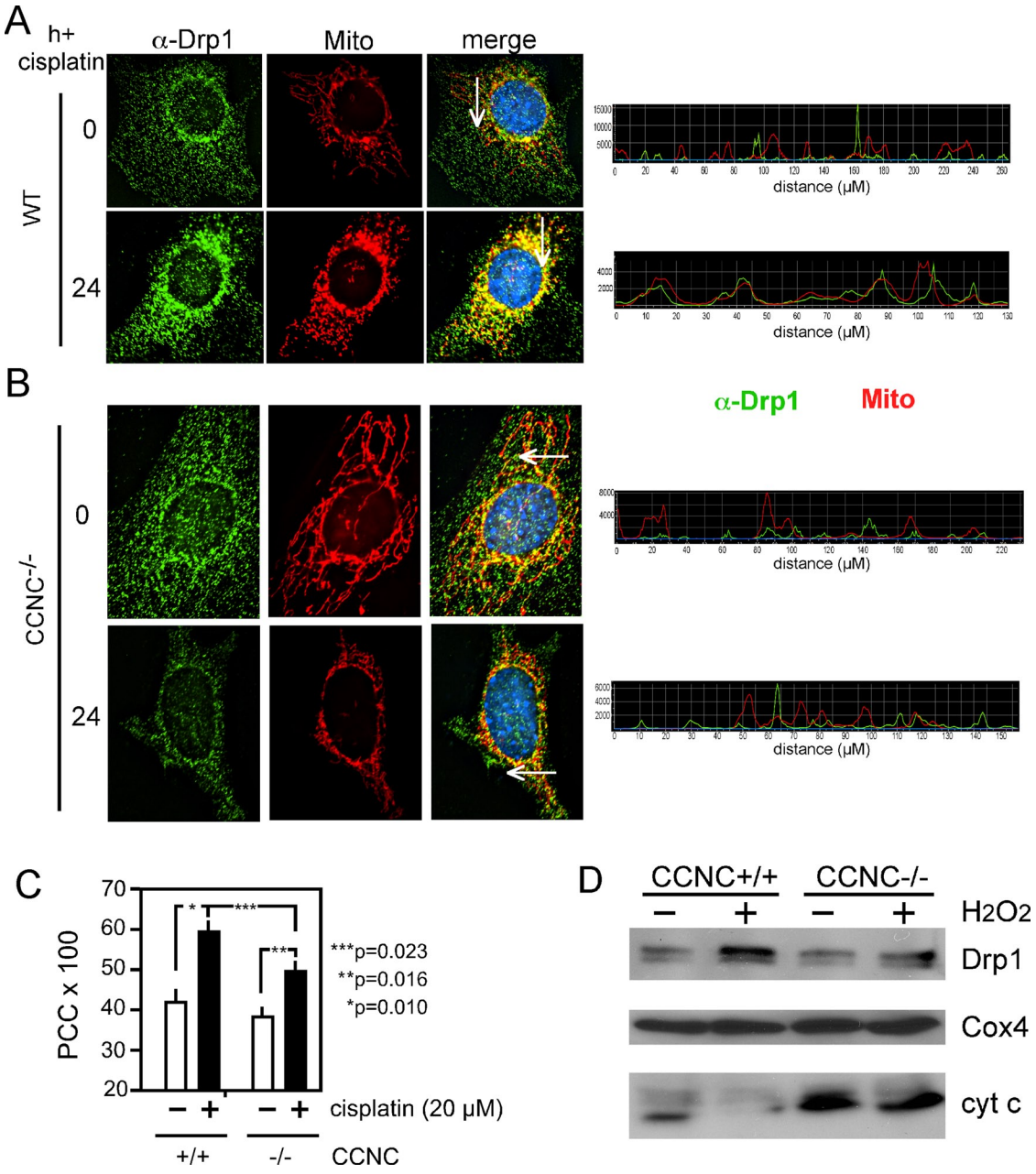

FIGURE 5: Cyclin C is required for normal stress-induced mitochondrial accumulation of Drp1. (A) Representative images of Drp1 in wild-type MEF cells before and after cisplatin (20 μM) treatment by IF. Arrows indicate the cytoplasmic paths used for pixel profiling of Drp1 and mitochondrial signals shown on the right. (B) Experiments described in A repeated with CCNC$^{-/-}$ cells. (C) PCC for Drp1–mitochondria colocalization determined from three independent experiments. Mean (±SEM) and *p* values. (D) Enriched mitochondrial fractions prepared from wild-type and CCNC$^{-/-}$ cells before and after H_2O_2 exposure (0.4 mM, 2 h) were probed for the presence of Drp1 and cytochrome *c*. Cox4 levels were monitored to ensure equal mitochondrial loading.

association of cyclin C after H_2O_2 stress (Figure 6C, bottom). These findings suggest that a similar pathway promotes cyclin C and p53 mitochondrial localization after cellular damage.

Cyclin C displays constitutive cytoplasmic localization in U2OS cells

To determine whether cyclin C translocation was a more general aspect of the oxidative stress response, we examined its subcellular localization in the U2OS osteosarcoma cell line. We chose this cell line because we found that individual mitochondria are more easily visualized than in HeLa cells. Somewhat similar to HeLa cells, a portion of cyclin C was observed in the cytoplasm before H_2O_2 addition (Figure 7A). However, when the U2OS cells were counterstained with MitoTracker Red, cyclin C exhibited extensive association with the mitochondria (arrows, Figure 7A). This observation was consistent throughout the culture (see Supplemental Figure S4 for full-field image). In addition, cyclin C associated with regions within this linear structure representing either potential sites of fission (white arrows) as determined by constriction of the MitoTracker signal or actual scission points (yellow arrows). These findings suggest that

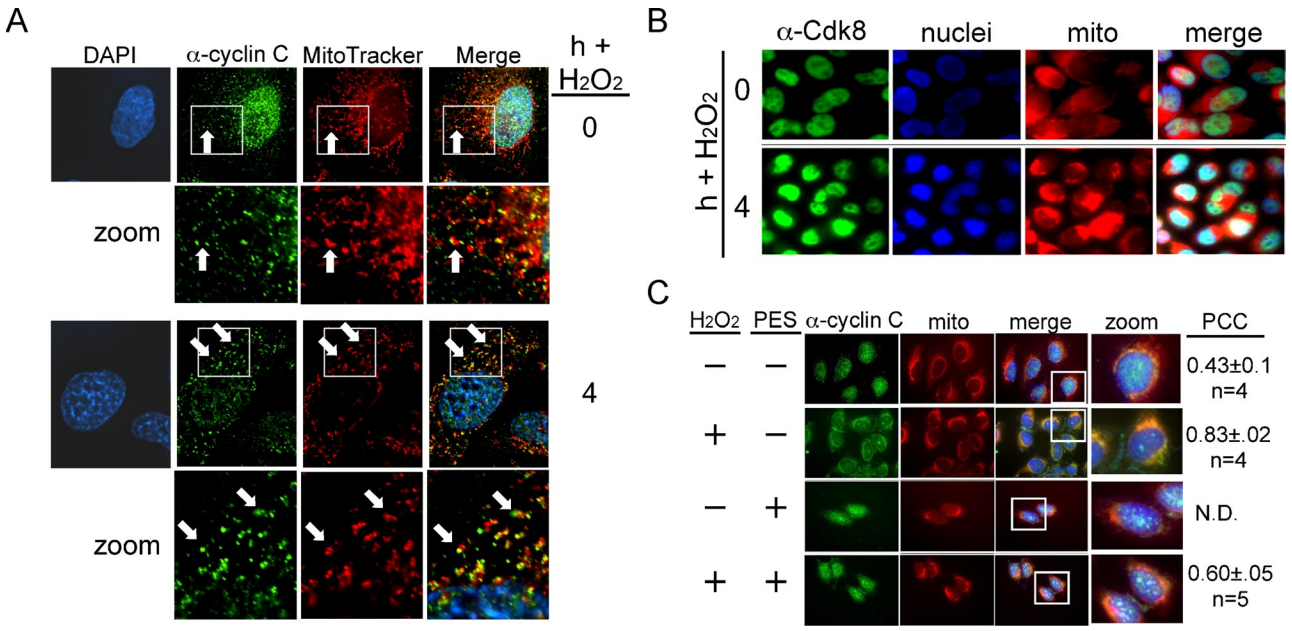

FIGURE 6: Characterization of cyclin C relocalization in stressed HeLa cells. (A) Subcellular localization of cyclin C, the nucleus, and mitochondria was determined in HeLa cell cultures before and after H_2O_2 addition (0.4 mM, 4 h). Arrows in the deconvolved images indicate points of interest discussed in the text. The zoom images are indicated by the boxes. (B) Cdk8, nuclear, and mitochondria subcellular localization in HeLa cells before and after H_2O_2 (0.4 mM) stress as determined by fluorescence microscopy. (C) The Hsp70 chaperone inhibitor PES (10 μM) and/or H_2O_2 (0.4 mM) was added to HeLa cultures as indicated. Representative images of cyclin C, mitochondrial, and nuclear localization were examined by fluorescence microscopy. Merged and enlarged (indicated by boxes) images. PCC was determined for cyclin C–mitochondria colocalization in the cytoplasm (mean ±SEM). $p = 0.01$ for stressed cells with and without PES; *n* indicates number of fields examined.

cyclin C mediates mitochondrial fission in U2OS cells without adding an exogenous stressing agent. However, the mitochondria appeared to be retained in an overall structure, as suggested by the linear distribution of the MitoTracker Red signal. However, after oxidative stress, the mitochondria become more fragmented and exhibit more disorganized puncta typical for stress-induced hyperfission (yellow arrow, Figure 7B). In addition, more independent mitochondrial (red arrow) and cyclin C (green arrow) signals are seen in these samples compared with untreated cells. These results suggest that cyclin C association with the mitochondria is not sufficient for complete fragmentation in this cell line. However, the addition of a stress signal promotes fission and perhaps cyclin C release from the mitochondria.

To explore further the finding that cyclin C does not induce complete fission in U2OS cells, we next determined whether cyclin C associated with Drp1 in this cell type. Initially, the U2OS cell line was transiently transfected with a plasmid expressing yellow fluorescent protein (YFP)–Drp1. These cells were subjected to H_2O_2 stress or left untreated and cyclin C localization determined by IF. Fluorescence microscopy revealed a close association between cyclin C and YFP-Drp1 in unstressed cultures (yellow arrows, Figure 7C). In response to H_2O_2, the colocalization between these two proteins continued, with the foci becoming larger, suggesting a concentration of these factors. These results indicate that cyclin C associates with Drp1 in stressed and unstressed U2OS cells. We next tested Drp1–mitochondria colocalization in U2OS cells before and after H_2O_2 exposure. In the absence of stress, Drp1 was observed extensively localized to linearly organized mitochondrial filaments (Figure 7D). In many instances, Drp1 appeared to be at sites of fission, based on the constriction of the MitoTracker Red signal (yellow arrows). In addition, although mitochondrial fission is occurring in these unstressed cells, more extensive fragmentation is observed after H_2O_2 addition, suggesting that an additional stress signal is required for full fission to occur.

Drp1 and cyclin C associate in vivo

The finding that cyclin C is required for normal recruitment of Drp1 to the mitochondria raised the question of whether cyclin C regulated this process through a direct or an indirect mechanism. We next used coimmunoprecipitation studies to address this question. Our initial attempts using endogenously expressed proteins in MEF cells were unsuccessful at detecting cyclin C and Drp1 coimmunoprecipitation (unpublished data). Studies in both yeast and mammalian cells found that either overexpression or cross-linking was required to detect interactions between components of the fission machinery (Bhar *et al.*, 2006). However, cyclin C overexpression in MEF cells was toxic, requiring use of a different cell model. We were able to stably introduce a hemagglutinin (HA) epitope–tagged cyclin C expression plasmid into the U2OS osteosarcoma cell line. This cell line was transiently transfected with a plasmid expressing YFP-Drp1. Soluble extracts prepared from cultures before and after H_2O_2 treatment were immunoprecipitated with the HA monoclonal antibody (mAb) and the immunoprecipitates probed for the presence of YFP-Drp1. YFP-Drp1 was found in the HA immunoprecipitates dependent on the presence of HA-cyclin C (Figure 7E, compare lanes 6 and 7). These findings indicate that cyclin C associates with Drp1 in U2OS cells. Of importance, as indicated by the immunofluorescence studies, cyclin C–Drp1 interaction was detected in extracts prepared from both stressed and unstressed cultures (lanes 7 and 8). Consistent with our results in permeabilized MEF cultures, these

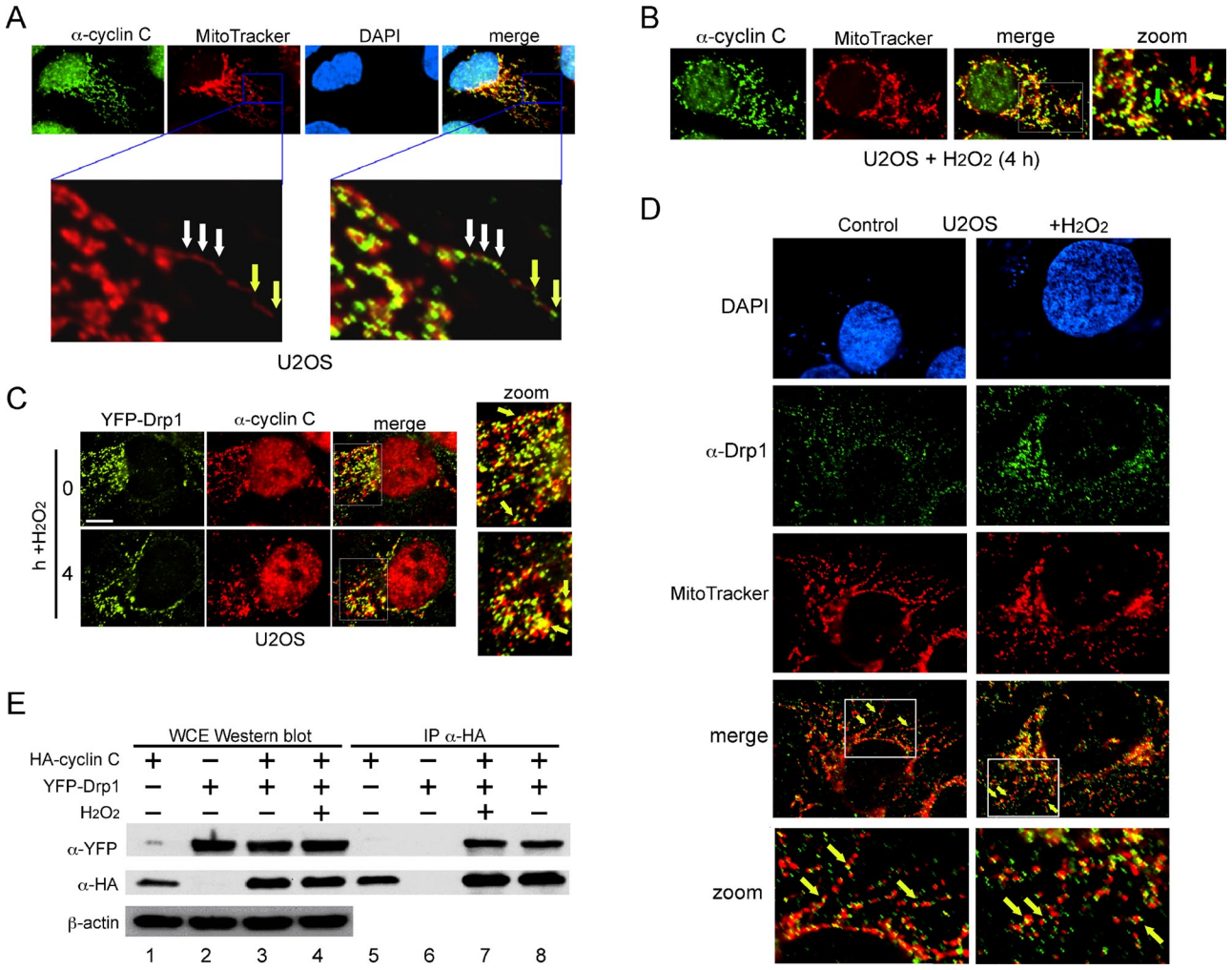

FIGURE 7: Constitutive cytoplasmic localization of cyclin C in U2OS cells. (A) Subcellular localization of cyclin C, mitochondria, and nuclei in unstressed U2OS cells. White and yellow arrows in enlarged images indicate cyclin C localization at potential sites of fission (constricted MitoTracker signal) and the ends of mitochondria, respectively. (B) Experiments in A after H_2O_2 treatment (0.4 mM). Green and red arrows indicate cyclin C and mitochondrial signals independent of each other. Yellow arrows indicate merger of the two signals. (C) Colocalization of YFP-Drp1 and cyclin C in U2OS cells before and after H_2O_2 stress as indicated. Yellow arrows indicate overlap between the two signals in both treated and untreated cells. (D) Endogenous Drp1 localization was monitored by IF before and after H_2O_2 (0.4 mM, 3 h) treatment. Boxed regions in the merged panels are enlarged in the bottom images. Yellow arrows indicate Drp1–mitochondria association at fission sites based on constriction of the MitoTracker Red signal. (E) Extracts prepared from stressed or untreated U2OS cells expressing HA-cyclin C and/or YFP-Drp1 as indicated were either subjected to straight Western blot analysis (50 μg) and probed with the antibodies indicated (lanes 1–4) or immunoprecipitated (IP, 250 μg) with HA mAb and the immunoprecipitates probed for the indicated proteins (lanes 5–8).

findings indicate that cytoplasmic localization is sufficient to promote cyclin C–Drp1 interaction and subsequent fission. Therefore the decision to release cyclin C from the nucleus represents an important control point regulating stress-induced mitochondrial fission.

Cyclin C is required for normal stress-induced apoptosis

Previous studies in yeast and mammalian cell culture systems found that failure to induce mitochondrial hyperfission is associated with resistance to exogenous cytotoxic compounds (Fannjiang *et al.*, 2004; Lee *et al.*, 2004; Cooper *et al.*, 2014). To determine whether the murine cyclin C regulates programmed cell death, we treated wild-type and CCNC$^{-/-}$ MEF cells with 20 μM cisplatin for 24 h. Before drug treatment, no differences in the percentage of the annexin V–positive cells were observed between CCNC$^{+/+}$ and CNCC$^{-/-}$ cultures, indicating that loss of cyclin C alone does not alter PCD rates (Figure 8A). However, after cisplatin treatment, CCNC$^{-/-}$ cells exhibited a significant reduction in annexin V signal compared with the CCNC$^{+/+}$ control. To verify that this phenotype was due to the loss of cyclin C activity, we repeated this experiment with the null CCNC$^{-/-}$ cell line transfected with an enhanced green fluorescent protein (EGFP)–cyclin C expression plasmid. The percentage of the population that was double positive for EGFP and annexin V staining was determined. These studies found that EGFP-cyclin C complimented the cisplatin resistance phenotype, indicating that cyclin C is required for normal PCD efficiency in MEF cells. Similarly, CCNC$^{-/-}$ MEF cells were more resistant to H_2O_2 exposure than wild-type controls (Figure 8B). These results indicate that cyclin C is required for normal PCD efficiency in response to two different stimuli.

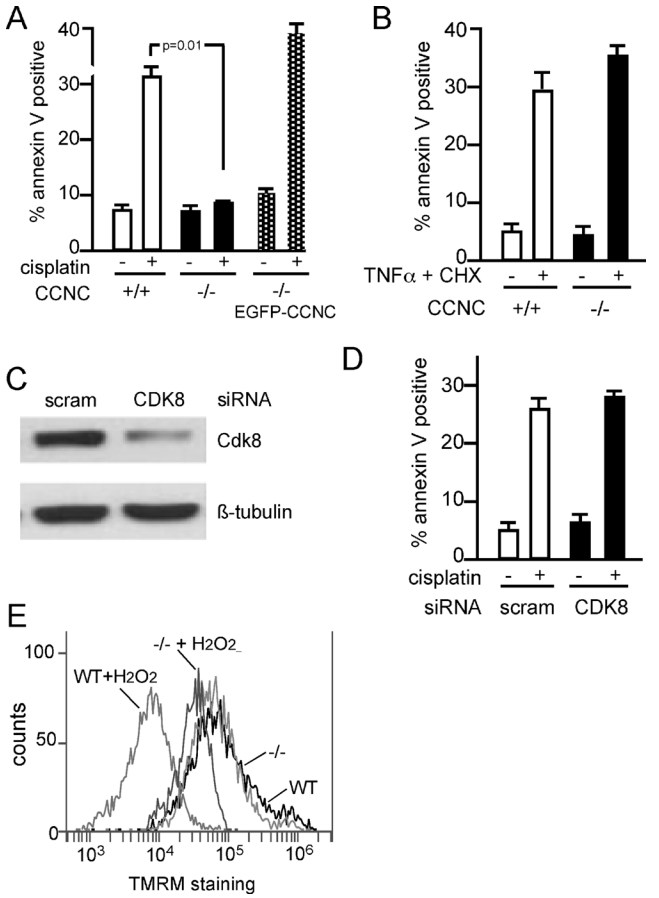

FIGURE 8: Cyclin C is required for intrinsic programmed cell death. (A) MEF cultures with the indicated genotypes were treated with cisplatin for 24 h and then assayed for annexin V positivity by FACS analysis. EGFP-cyclin C expression plasmid was transiently transfected into the CCNC$^{-/-}$ culture and the experiment was repeated, sorting for EGFP- and annexin V–positive cells. The mean (±SEM) from at least three independent experiments. (B) MEF cultures with the indicated genotypes were treated with TNF-α and CHX for 24 h and then assayed as in A. (C) Western blot of extracts prepared from cultures treated with CDK8-specific siRNA or a scrambled control. β-Tubulin levels were determined as a loading control. (D) Annexin V values were determined as described in A for wild-type MEF cultures treated with the siRNA constructs as indicated. (E) TMRM staining was evaluated in wild-type and CCNC$^{-/-}$ MEF cultures before and after H_2O_2 treatment (0.4 mM for 4 h) as indicated.

One model consistent with these results proposes that cyclin C relocalization to the mitochondria promotes hyperfission and subsequent PCD. An alternative hypothesis is that loss of cyclin C causes misregulation of a gene (or genes) that in turn controls sensitivity to cisplatin or H_2O_2. To test these two models, we used small interfering RNA (siRNA) to knock down Cdk8 in wild-type MEF cells (Figure 6C). As shown in Figure 6D, no difference in the sensitivity of these cells to cisplatin was observed compared with the scrambled siRNA–transfected controls. Taken together, these data argue that cyclin C regulates ROS-induced PCD independent of its role as a transcription factor. We next determined how general a role cyclin C played in controlling PCD pathways. Wild-type and CCNC$^{-/-}$ MEF cultures were subjected to extrinsic death receptor–mediated cell killing. No difference in annexin V staining was observed between the wild-type and mutant cultures (Figure 6E), indicating that cyclin C regulation was restricted to the mitochondrial intrinsic pathway.

Next we explored where in the PCD pathway cyclin C operated. Given its role in inducing mitochondrial fission, we first examined the ability of CCNC$^{-/-}$ cells to execute MOM permeabilization (MOMP). For these studies, MOMP was measured in two ways. First, wild- type and CCNC$^{-/-}$ MEF cells were loaded with a dye (tetramethylrhodamine methyl ester [TMRM]) that readily accumulates in active, intact mitochondria but is released after MOMP. In these studies, we found that both wild-type and CCNC$^{-/-}$ MEF cells incorporated TMRM into their mitochondria to similar extents, indicating that the mitochondrial membranes were functioning similarly in both cell lines (Figure 6F). In response to H_2O_2, TMRM was rapidly lost from the wild-type cells, but only a slight reduction was observed in the mutant cultures. These results indicate that cyclin C is required for MOMP. This conclusion is supported by the finding that the inner membrane protein cytochrome *c* is absent in wild-type MEF mitochondria after stress but still present in the stressed mutant preparations (Figure 5D). Taken together, these results indicate that cyclin C is required for mitochondrial fission, efficient loss of mitochondrial outer membrane integrity, and PCD execution in response to cytotoxic signals.

DISCUSSION

In all organisms examined, exposure to exogenous stress shifts the balance between mitochondrial fission and fusion dramatically toward fission (Igaki *et al.*, 2000; Frank *et al.*, 2001; Karbowski *et al.*, 2002; Vieira *et al.*, 2002; Breckenridge *et al.*, 2003; for review, see Hoppins and Nunnari, 2012). Although the basic fission machinery is required for the extensive mitochondrial fragmentation observed after stress, the signal that triggers this response has been unknown. This study finds that the nuclear transcription factor cyclin C is both necessary and sufficient for stress-induced mitochondrial fragmentation. First, MEF cells deleted for cyclin C fail to undergo mitochondrial hyperfission in response to an anticancer drug or H_2O_2. Second, the addition of purified cyclin C to nonstressed permeabilized cultures rapidly induces mitochondrial fragmentation. This role appears to be direct, as cyclin C interacts with the dynamin-like fission GTPase Drp1 and enhances its association with the mitochondria. In addition, knocking down CDK8 did not affect mitochondrial fission in response to cellular damage, suggesting that cyclin C induces mitochondrial fission independent of its transcriptional regulatory role. Although not common, Cdk-independent roles for other cyclins have been reported. For example, cyclin D1, which drives G1 progression by inducing Cdk activity, also binds transcription factors and chromatin-remodeling machines to control gene expression (Fu *et al.*, 2004). In addition, cyclin E can stimulate DNA replication independent of Cdk2 through direct interaction with the MCM initiation complex (Geng *et al.*, 2007). However, this is the first example of a cyclin involved in transcriptional regulation, rather than cell cycle progression, to have a function independent of its Cdk.

A previous study revealed that the budding yeast cyclin C also translocates to the mitochondria and is required for stress-induced fission (Cooper *et al.*, 2014). In addition, we demonstrate in the present study that the addition of the yeast cyclin C to permeabilized MEF cultures is sufficient to induce fission. These results indicate that the role cyclin C plays in controlling mitochondrial fission is an ancient process. Although the outcomes are similar, the underlying mechanisms, both in regulating cyclin C and how it controls mitochondrial morphology, appear different in yeast and mammalian cells. In yeast, several stressors, including oxidative stress, induce complete relocalization of cyclin C to the cytoplasm, where it is destroyed after mitochondrial fission (Cooper *et al.*, 1997, 2012). However, in stressed MEF and HeLa cultures, only a portion of cyclin C

relocates to the cytoplasm, and no significant loss in protein levels was detected. This observation may indicate that cyclin C maintains its function as a transcriptional regulator even in stressed mammalian cells. In addition, we observed elevated cytoplasmic cyclin C in unstressed HeLa cells that was more dramatic in U2OS cultures. This may be the result of the high endogenous ROS load associated with tumor cells, which in turn triggers partial cyclin C release (Trachootham *et al.*, 2006). Alternatively, Med13p, a conserved component of the Cdk8 module, was recently demonstrated to retain cyclin C in the nucleus in unstressed yeast cells (Khakhina *et al.*, 2014). Therefore U2OS cells may be defective in Med13-dependent cyclin C nuclear retention or contain an activated signal transduction pathway that constitutively signals cyclin C release. This question is being examined.

Another difference between yeast and mammalian cyclin C is in how they stimulate fission. In yeast, cyclin C associates with Mdv1p (Cooper *et al.*, 2014), the adaptor protein that bridges the dynamin protein Dnm1p to the outer membrane receptor Fis1p (Westermann, 2010a). The role of Mdv1p or cyclin C in stressed yeast cells is not to recruit Dnm1p to the mitochondria. Instead, Mdv1p and cyclin C stimulate Dnm1p to form productive complexes (Schauss *et al.*, 2006; Cooper *et al.*, 2014). However, in mammalian cells, no adaptor proteins similar to Mdv1p have been identified. Instead, cyclin C appears to interact directly with Drp1 and functions primarily through the Mff mitochondrial receptor. Previous studies revealed elevated Drp1 association with the mitochondria in stressed cells (Frank *et al.*, 2001). This study found that cyclin C is required for this enhanced association, suggesting a role in Drp1 mitochondrial recruitment and/or its retention on the organelle. However, we still detect an elevated, although reduced, association of Drp1 with the mitochondria in stressed CCNC$^{-/-}$ cells. Therefore the role of cyclin C may not be solely to help target Drp1 to the mitochondria but also to enhance its activity once associated at the organelle. Although many potential roles for cyclin C could be envisioned, one possible function is based on the finding that stable Drp1 filament formation occurs in its GTP-bound state (Ingerman *et al.*, 2005; Naylor *et al.*, 2006; Lackner *et al.*, 2009). However, the complex itself possesses intrinsic GTPase activity, with GTP hydrolysis causing ring constriction and filament dissolution. Therefore the ability of cyclin C to stabilize the GTP-bound form of Drp1 would enhance filament stability and therefore its retention on the mitochondria. Alternatively, cyclin C may enhance Drp1-Mff association, again leading to enhanced mitochondrial retention. Additional biochemical studies are required to test these possibilities. In conclusion, this article describes a conserved role for cyclin C in directing stress-induced mitochondrial fragmentation in mammalian cells.

The connection between mitochondrial hyperfission and PCD is not clear, although it has been observed in many systems (Martinou and Youle, 2011). The localization of proapoptotic factors that induce MOMP (e.g., Bax) to sites of fission (Karbowski *et al.*, 2002) has been suggested to be an intersection of these two processes. One possibility is that loss of stress-induced fission sites such as observed in CCNC$^{-/-}$ cells may reduce the frequency at which these proapoptotic factors can associate with the MOM, as observed previously (Tondera *et al.*, 2009). This possibility incorporates a model proposing that, during the fission process, transient hemifission intermediates are produced that are more easily breeched (Katsov *et al.*, 2004). The combination of Bax recruitment and the formation of these intermediate membrane structures may accelerate the release of proapoptotic factors (Martinou and Youle, 2011). It is important to note, however, that mitochondrial fission is not always a prerequisite for cytochrome *c* release or PCD. These observations may indicate that many factors, including the particular system studied and/or the stressors applied, may influence the complex interaction between mitochondrial dynamics and execution of the programmed cell death pathway. For example, conditions just able to attain the threshold required to evoke PCD may require fission to facilitate the process. However, harsher treatments may be able to generate enough relocalization of proapoptotic Bcl-2 proteins to induce MOMP and subsequent cell death. Therefore the final decision of whether to execute PCD may depend on the magnitude of the apoptotic signal and the sensitivity of the system. The presence of cyclin C may result in a lower threshold to trigger MOMP and subsequent cell death.

MATERIALS AND METHODS

Cell culture, transfection, and siRNA

U2OS and HeLa cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. For localization and mitochondrial morphology experiments in MEF cultures, the cells were maintained in 5% CO_2, 5% O_2. CCNC$^{+/+}$ and CCNC$^{fl/fl}$ MEF cell lines were established from 13.5-d postcoitum embryos using standard protocols. These cell lines were subsequently immortalized through introduction of the SV40 T-antigen (Tevethia, 1984). The immortalized CCNC$^{fl/fl}$ MEF cells were then infected with retrovirus (pMIY2-Cre-IRES-YFP) expressing Cre-YFP recombinase. Positive transfectants were obtained by fluorescence-activated cell sorting (FACS) to generate a CCNC$^{-/-}$ MEF pool. Single clones were further isolated by dilution plating, with the deletion allele verified by PCR and Western blots (see Supplemental Figure S2 for details). Thymidine/nocodazole arrest/release/arrest protocol was conducted with 2 mM thymidine for 24 h, followed by release into normal medium containing 0.1 μg/ml nocodazole (Sigma-Aldrich, St. Louis, MO). DNA plasmids were transfected into cells with Lipofectamine 2000 (Invitrogen). Cdk8 siRNA (Cell Signaling Technology, Danvers, MA) was introduced into cells using siPORT transfection reagent (Invitrogen) according to the manufacturer's instructions. The GST-cyclin C fusion genes were constructed by inserting the human cyclin C cDNA (a gift from S. Reed, Scripps Research Institute, La Jolla, CA) or the yeast coding sequence into pGEX-2T (GE Healthcare, Pittsburgh, PA). The fusion proteins, as well as the GST control, were expressed and purified from *E. coli* as suggested by the manufacturer. GST fusion protein concentrations were approximated by comparing Coomassie-stained bands to known BSA standards.

PCR genotyping of MEF cells

The primers used to genotype the MEF cells, CCNC2 (5′-TAA TCG ACC AGA CAG TAC GGG AGT C-3′), SDL2 (5′-GGT AGT TTA TCT GAA CTG ATG AAA ACA CAT C-3′), and Lox1 (5′-GGA AGC AGA AGC AAC AGG AAT CTG-3′), are indicated in Supplemental Figure S2A.

Indirect immunofluorescence

Cells were cultured on coverslips and then fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 15 min, blocked with 2% BSA, and incubated with antibodies as indicated. The cells were mounted with 4′,6-diamidino-2-phenylindole (DAPI)–containing medium (Vector Labs, Burlingame, CA), and the images were acquired with Nikon (Melville, NY) Eclipse 90i microscope equipped with a Retiga Exi charge-coupled device (CCD) camera and NIS software for data analysis. Data were collected using Autoquant and processed using Image

Pro software. Primary antibodies (cyclin C [Thermo Scientific], Drp1 [BD Transduction Laboratories, San Jose, CA], Cdk8 [Santa Cruz Biotechnology, Santa Cruz, CA], phosphorylated Drp1 [#3455; Cell Technologies, Shaker Heights, OH], HA epitope [Roche, Indianapolis, IN], GFP [Clontech, Mountain View, CA], and β-actin [Sigma-Aldrich]) were used according to suppliers' suggestions. Fluorescein isothiocyanate–conjugated secondary antibodies were from Jackson Laboratories. Mitochondrial staining was accomplished using MitoTracker Red CMXRos as described by the manufacturer (Molecular Probes, Grand Island, NY). Caspase 3 was inhibited in $CCNC^{+/+}$ MEF cells as described previously (Naarmann-de Vries *et al.*, 2013) with 10 μM (final concentration) of Ac-DEVD-CHO (BD PharMingen, San Jose, CA) for 1 h before addition of 20 μM cisplatin treatment for 24 h. MitoTracker Red was added 30 min before fixing the cells. The Hsp70 chaperone inhibitor PES was added (10 μM) to cultures 30 min before H_2O_2 treatment.

Mitochondrial fragmentation assays

FRAP assays were conducted essentially as described (Cleland *et al.*, 2011), with the following modifications. $CCNC^{+/+}$ and $CCNC^{-/-}$ MEF cells were treated with 0.4 mM H_2O_2 for 4 h. Mitochondrial morphology was monitored by MitoTracker Red staining. The cells were imaged with the 60× objective on the Nikon Eclipse C1T*i* confocal microscope equipped with a Ds-Qi1MC CCD camera. Data were collected before and after a 2-μm circle containing multiple mitochondria was photobleached with a 633-nm laser. Forty individual FRAP samples were analyzed from each experiment that displayed an approximate 30% signal bleaching to allow better comparisons of the resulting recovery curves. The GST-cyclin C addition experiments were conducted with MEF cells seeded 1 d before in 35-mm glass-bottom culture dishes. The cells were preloaded with MitoTracker Red for 30 min and then washed with phosphate-buffered saline (PBS) twice before transfer to membrane maintenance buffer (DMEM, 10% FBS, 1% penicillin/streptomycin, 110 mM sucrose, and 1 mg/ml BSA; Verma *et al.*, 2013). The cells were then placed in a CO_2- and temperature-controlled chamber on the confocal microscope. Digitonin, 1.0 μg/ml, was added for 2 min to permeabilize the cells, and then another 1 ml of membrane maintenance buffer was added containing human (Hs) or yeast (Sc) GST-cyclin C fusion proteins or GST alone (approximate 4 nM final concentration). Permeabilization conditions were determined by monitoring the ability of propidium iodide to enter digitonin- treated cells. Cells were monitored by confocal microscopy with a 561-nm laser (1% power). Mdivi-1 (50 μM) was added to the cells for 30 min and then washed away with PBS before the permeation step.

Subcellular fractionation, extract preparation, and colocalization analysis

Mitochondrion-enriched fractions were obtained using the Thermo Scientific mitochondrial isolation kit from ~1×10^7 log-phase cells. Whole-cell extracts were prepared from cells harvested in lysis buffer (250 mM NaCl, 50 mM Tris-HCl, pH 7.8, 0.5% NP-40, plus protease inhibitors) and then incubated for 30 min on ice before disruption. Whole-cell lysates were centrifuged at 13,000 × *g* for 10 min at 4°C to separate soluble proteins from aggregates and cell debris. Protein concentrations were determined by Bradford microassays (Bio-Rad). Western blot analysis was conducted with antibodies directed against cyclin C (Bethyl, Montgomery, TX), Por1/VDAC (Clontech), Cox4 (Molecular Probes), HA (Roche), and GFP (Clontech), used as recommended by the suppliers. Western blot signals were visualized by secondary antibody conjugated to alkaline phosphatase and chemiluminescence. These signals were quantified using a CCD imager (Kodak, Rochester, NY). To determine fold enrichments, the cyclin C signal was compared with Por1 levels. To calculate colocalization values, the PCC was obtained using NIS software, examining the cytoplasm of at least 10 cells from three separate experiments. Mitochondrial fragmentation was quantitated by calculating the percentage of the population with >10 mitochondria with a length of ≥10 μM. SDs of the means were calculated using the Student's *t* test.

Survival and stress assays

MEF cells were seeded in 12-well plates at a density of 0.5 × 10^5 cells/well at 1 d before stress treatment. H_2O_2 was added to cells immediately after a switch to serum-free medium. For cisplatin treatment, the drug was added to normal culture medium. Activation of the extrinsic pathway was accomplished with addition of tumor necrosis factor-α (TNF-α; 5 ng/ml) and cycloheximide (CHX; 10 μg/ml) for 24 h. Annexin V (BD Biosciences) assays were conducted as described by the manufacturer. MOMP studies were performed as described (Verma *et al.*, 2013), with the following modifications. Exponential wild-type or $CCNC^{-/-}$ MEF cultures were treated with H_2O_2 (0.4 mM) for 3 h before staining with TMRM (Molecular Probes).

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