

Loss of CHCHD2 and CHCHD10 activates OMA1 peptidase to disrupt mitochondrial cristae
phenocopying patient mutations

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Abstract

Dominant mutations in the mitochondrial paralogs CHCHD2 (C2) and CHCHD10 (C10) were recently identified as causing Parkinson's disease and ALS/FTD/myopathy, respectively. The mechanism by which they disrupt mitochondrial cristae, however, has been uncertain. Using the first C2/10 double knockout (DKO) mice, we report that C10 pathogenesis and the normal function of C2/10 are intimately linked. Similar to patients with C10 mutations, we found that C2/10 DKO mice have disrupted mitochondrial cristae, due to cleavage of the mitochondrial shaping protein L-OPA1 by the stress-induced peptidase OMA1. OMA1 was found to be activated similarly in affected tissues of mutant C10 knock-in (KI) mice, demonstrating that L-OPA1 cleavage is a novel mechanism for cristae abnormalities due to both C10 mutation and C2/C10 loss. Using OMA1 activation as a functional assay, we found that C2 and C10 are partially functionally redundant, and some but not all disease-causing mutations have retained activity. Finally, C2/10 DKO mice partially phenocopied mutant C10 KI mice with the development of cardiomyopathy and activation of the mt-ISR in affected tissues, tying mutant C10 pathogenesis to C2/C10 function.

Introduction

Autosomal dominant mutations in the mitochondrial paralogs C2 and C10 were recently shown to cause, respectively, Parkinson's disease (PD) and a spectrum of neuromuscular disorders (that includes myopathy and Amyotrophic Lateral Sclerosis [ALS]) (Bannwarth et al., 2014; Funayama et al., 2015; Ajroud-Driss et al., 2015; Penttilä et al., 2015). Most mutations with proven pathogenicity in C2/C10 cluster in a common hydrophobic α -helix N-terminal to the coiled-helix-coiled-helix (CHCH) domain, consisting of dual CX₉C motifs. These include the C10 S59L substitution, which was originally identified in a family with ALS, frontotemporal dementia, and myopathy, and substitution of the neighboring residue, G58R, identified in a family with myopathy (Bannwarth et al., 2014; Ajroud-Driss et al., 2015).

C2 and C10 are co-expressed in relevant cellular subtypes, such as *substantia nigral* neurons, pyramidal neurons, and myocytes, where they directly interact within a ~200 kDa complex in the mitochondrial intermembrane space (Straub et al., 2018; Burstein et al., 2018; Huang et al., 2018). Although their precise function remains unknown, cristae abnormalities have been observed in muscle tissue and cell lines from patients with C10 mutations, as well as a *Drosophila* lacking the C2/C10 ortholog (Bannwarth et al., 2014; Ajroud-Driss et al., 2015; Meng et al., 2017). To explain cristae abnormalities in C2/C10 patients, C10 and later C2 were proposed to be structural components of the mitochondrial contact site and cristae organizing system (MICOS) complex (Bannwarth et al., 2014; Genin et al., 2016; Zhou et al., 2019). However, recent reports have challenged this model, suggesting that C2/C10 mutation or loss may lead to cristae abnormalities by another as of yet unidentified mechanism (Straub et al., 2018; Burstein et al., 2018; Huang et al., 2018).

Cristae are infoldings of the inner mitochondrial membrane and can be considered the fundamental bioenergetic unit within mitochondria. Studded with respiratory complexes and rimmed with dimers of the F₁F₀ ATP synthase, they increase surface area for oxidative phosphorylation (Davies et al., 2011). Cristae are shaped by at least three protein complexes: the MICOS complex, bending the cristae membrane at its junction with the boundary membrane; the GTPase OPA1, which mediates inner

membrane fusion and supports cristae structure by bridging apposing membranes in the cristae fold; and ATP synthase dimers, which bend the membrane at the cristae edge (Paumard et al., 2002; von der Malsburg et al., 2011; Harner et al., 2011; Hoppins et al., 2011; Frezza et al., 2006; Dudkina et al., 2006; Davies et al., 2012; Ban et al., 2017).

OPA1, in particular, is highly regulated by mitochondrial bioenergetics and proteostatic stress to dynamically shape the inner membrane in response to changing conditions within the mitochondrial network. The peptidase OMA1 is activated by mitochondrial stressors to cleave the active long form of OPA1 (L-OPA1) from its membrane anchor, leading to mitochondrial fragmentation and alterations in cristae structure (Head et al., 2009; Ehses et al., 2009). L-OPA1 processing by OMA1 also occurs upon loss of quality control proteases, such as YME1, AFG3L2, and SPG7, as well as inner membrane scaffolding proteins of the SPFH family, namely, prohibitins and SLP-2, that organize quality control proteases within inner membrane microdomains (Anand et al., 2014; Wai et al., 2016; Merkwirth et al., 2008; Ehses et al., 2009; Magri et al., 2018). Thus, OMA1 provides an escape mechanism for cristae with failed of protein quality control, separating dysfunctional mitochondrial units from the network for degradation by autophagy (MacVicar and Lane, 2014).

Very recently, mice null for C10 and knock-in for the pathogenic S59L mutation have been reported (Genin et al., 2019; Anderson et al., 2019; Burstein et al., 2018). Whereas C10^{-/-} mice have a normal lifespan, C10^{S59L/+} knock-in (KI) mice develop a cardiomyopathy by 23 weeks, which is uniformly fatal at 13 months, supporting a gain of function mechanism of pathogenesis. Anderson et al. (2019), in particular, observed C10 levels increase and co-aggregate with C2 in affected tissues, leading them to propose a toxic aggregate model of pathogenesis, in which C2/C10 toxic aggregates trigger the integrated mitochondrial stress response (mt-ISR) and tissue degeneration. However, it remains unclear in this aggregate model whether the pathogenesis of mutant C10 relates to C2/C10 physiologic function.

Here, we evaluate the first C2/C10 DKO mouse model with two novel findings. First, we identify OPA1 cleavage by the stress-induced peptidase OMA1 to be at least partially responsible for cristae abnormalities in absence of C2/C10. We further show that OMA1 activation is a key event in mutant C10

pathogenesis *in vivo*. Thus, we establish for the first time OPA1 cleavage as potential mechanism for cristate abnormalities in patients with C10 mutations and C2/C10 models. Second, we observe that C2/10 DKO mice unlike C10 KO mice partially phenocopies the recently reported mutant C10 model, including activation of the mt-ISR and development of cardiomyopathy. This suggest that C10 pathogenesis and C2/10 physiological function are intimately linked. We propose that C2/C10 levels are required in a narrow range of expression for physiologic processing of L-OPA1. Mutations in C10 may lead to OMA1 activation by a dominant negative action (e.g., through co-aggregation with WT C2/C10) or by lowering the concentration at which mutant C10 expression triggers OMA1 activation.

Results

C2/C10 double knockout leads to cristae abnormalities due to increased L-OPA1 processing by the stress-induced protease OMA1 in cell culture

To investigate the physiological role of C2 and C10 in mammalian primary cells, we generated C2 and C10 single knockout (KO) and double knockout (DKO) mice (Figure 1A). Whereas cristae structure was normal for WT and C10 single knockout cell lines by Transmission Electron Microscopy (TEM) (Supplemental Figure 1A), primary fibroblasts from DKO mice exhibited abnormalities in mitochondrial ultrastructure, including convoluted cristae, which were sometimes circular and detached from boundary membrane, as well as a decrease in cristae number (8.232 vs. 16.43 cristae/ μm^2 , $p < 0.0001$) (Supplemental Figure 1A and Figure 1B – D). Cristae number was also mildly decreased for C10 single KO cells compared to WT (14.87 vs. 16.43 cristae/ μm^2 , $p = 0.030$) (Figure 1B – D). By confocal microscopy, mitochondrial area was also found to be slightly reduced in C2/C10 DKO compared to two WT primary fibroblasts (Supplemental Figure 1B).

As the cristae abnormalities in C2/C10 DKO fibroblasts resembled those with OPA1 KO (Figure 1E), we assessed OPA1 processing by immunoblotting (Figure 1F – H). There are five OPA1 isoforms visible by immunoblotting corresponding to the membrane-bound long OPA1 (L-OPA1) forms (*a* and *b*), and their proteolytic cleavage products, the short OPA1 (S-OPA1) forms (*c* – *e*), generated by YME1 (*d*) or OMA1 cleavage (*c* and *e*), respectively (Figure 1H) (Ehse et al., 2009; Head et al., 2009). In C2/C10 DKO fibroblasts, L-OPA1 forms decreased and OMA1-dependent S-OPA1 forms (*c* and *e*) increased, consistent with OMA1 cleavage (Figure 1F and G). Notably, L-OPA1 processing in C2 single KO cells was between WT and C2/C10 DKO cells, and normal in C10 KO cells, consistent with partial functional redundancy of the paralogs.

Protein levels of the MICOS subunits, Mic19 and Mic27 were also mildly reduced in C2/C10 DKO fibroblasts compared to WT fibroblasts, similar to what we observed previously in HEK293 C2/C10 DKO cells (Huang et al., 2018) (Supplemental Figure 1C). Mic60 levels were also decreased although the trend was not significant. A recent report found that OMA1 can cleave Mic19 on activation, and so we considered whether this could be the mechanism for the observed decrease in MICOS subunits in C2/C10 DKO cells (Tang et al., 2020). However, we did not detect cleaved Mic19 in HEK293 C2/C10 DKO cells, suggesting that this mechanism of crosstalk between OMA1 and the MICOS complex likely does not account for the decrease in MICOS subunits (Supplemental Figure 2A). In contrast to L-OPA1 levels and MICOS subunits, DRP1 and Mitofusin-2 levels were similar in all cell lines (Supplemental Figure 1C and D).

As OMA1 is activated in response to bioenergetic collapse, we investigated cellular respiration in DKO cells by oximetry. Consistent with our prior observations in HEK293 cells, basal and maximal oxygen consumption was comparable for DKO and WT primary fibroblasts under growth conditions in which L-OPA1 processing was disrupted (Figure 1H) (Huang et al., 2018). Mitochondrial membrane potential measured by TMRM fluorescence was also similar for C10 KO and C2/10 DKO fibroblasts (Supplemental Figure 1C). Membrane potential in C2/C10 single and double knockouts was slightly

reduced when compared to WT lines (Supplemental Figure 1C). Together these findings argue against OPA1 instability in C2/10 DKO cells resulting from disrupted mitochondrial bioenergetics.

To assess whether OPA1 processing was altered also in human cells, we evaluated the OMA1/OPA1 axis in HEK293 DKO cells that we described previously (Huang et al., 2018). Similar to DKO mouse fibroblasts, cristae number was significantly reduced in HEK293 DKO cells (Figure 2A and B). Consistently, L-OPA1 was found to be processed in a pattern reflective of OMA1 activation (Figure 2C). OMA1 levels were likewise decreased, as expected given OMA1 is degraded shortly after its activation (Head et al., 2009). Similar to primary fibroblasts we previously found only a mild oxygen consumption deficit in HEK293 DKO cells compared to wildtype cells (Huang et al., 2018), and basal TMRE fluorescence was slightly higher for DKO cells compared to WT cells, indicating increased membrane potential (Supplemental Figure 2C). TMRE fluorescence decreased in response to CCCP and increased in response to oligomycin in both WT and DKO cells, consistent with TMRE reflecting membrane potential in the unquenched mode. To test whether a moderate reduction of OXPHOS is sufficient to activate OMA1 in HEK293 cells, we inhibited mitochondrial translation in HEK293 cells with 2 μ g chloramphenicol for 5 – 7 days. This caused 35% decrease in membrane potential and a 78% decrease in expression of the Complex IV subunit, COX2 (Supplemental Figure 2D and E). By comparison, COX2 levels were 37% lower in untreated HEK293 C2/C10 DKO cells compared to WT cells (Supplemental Figure 2E). However, even the large reduction in COX2 expression with chloramphenicol treatment was insufficient to trigger L-OPA1 processing by OMA1. Together these data demonstrate that L-OPA1 processing in C2/C10 DKO cells is not driven by a bioenergetic deficit or the observed mild reduction in OXPHOS subunits.

Next, to test formally whether OMA1 is responsible for L-OPA1 cleavage in the absence of C2/C10, we generated OMA1 KO and C2/C10/OMA1 triple KO (TKO) HEK293 cell lines (Figure 2 A - C). Loss of OMA1 in HEK293 cells led to a mild decrease in total OPA1 (68.1% of WT levels, $p = 0.0006$) and CHCHD2 levels were slightly increased (128% of WT levels, $p = 0.0099$) (Supplemental Figure 3A). Crucially, in TKO cells L-OPA1 isoforms (*a* and *b*) were stabilized against cleavage to

isoforms *c* and *e*, whereas basal processing of L-OPA1 isoform *a* to *d* by the protease YME1 was unaffected (Figure 2C). Additionally, cristae density was partially restored to C2/C10 deficient cells following KO of OMA1 (Figure 2B). Together these findings demonstrate cristae abnormalities in the absence of C2 and C10 are caused at least in part by OMA1 cleavage of L-OPA1.

We accessed the specificity of activated OMA1 to L-OPA1 in HEK293 DKO cells, by examining another substrate of activated OMA1, PGAM5. PGAM5 processing was similar WT and DKO cells both under basal conditions and following mitochondrial uncoupling with CCCP, suggesting that OMA1 activation with C2/C10 DKO may preferentially affect L-OPA1 processing (Supplemental Figure 3B) (Sekine et al., 2012). Consistent with our previously reported observations, C2 and C10 increased in response CCCP treatment in cell culture (Supplemental Figure 3b) (Huang et al., 2018). These findings suggest that OMA1 activation in response to C2/C10 loss may be relatively specific for L-OPA1.

As the MICOS complex is reported to interact with OPA1 and we observe a mild decrease in some MICOS subunits in the absence of C2/C10 (Ding et al., 2015; Darshi et al., 2011; Genin et al., 2016; Zhou et al., 2019), we tested whether the reduction in MICOS subunits could account for the observed OMA1 activation and L-OPA1 cleavage. As was found in primary fibroblasts and we reported previously in HEK293 cells (Huang et al., 2018), levels of MICOS subunits, MIC19, MIC27, and MIC60, were mildly reduced in HEK293 DKO cells compared to WT cells (77.4% for MIC19, 69.8% for MIC27, and 80.1% for MIC60; Figure 2D and Supplemental Figure 3C). As expected, depletion of a central subunit of the MICOS complex, MIC60, further destabilized MIC19 and MIC27, components of the respective MICOS subcomplexes in both DKO and WT cells (Figure 2D). OPA1 processing, by contrast, was unaffected under these conditions, demonstrating that MICOS complex instability alone cannot account for OPA1 cleavage by OMA1.

We additionally assessed the relative abundance of the MICOS and other complexes previously found to affect OMA1 activation and OPA1 cleavage (Wai et al., 2016; Merkwirth et al., 2008), using LC-MS/MS-based complexomics in WT and DKO HEK293 cells. Detected subunits of the SPY,

Prohibitin, and MICOS complexes were of similar abundance in WT and DKO cells (Figure 2E, Supplemental Figure 3D, and Table S1).

Loss of mitochondrial proteostasis is known to activate OMA1. To assess whether there is a general loss in mitochondrial proteostasis in the absence of C2/C10, we fractionated mitochondria from WT and DKO cells into soluble and insoluble fractions. Consistent with a general loss in mitochondrial proteostasis, HEK293 cells lacking C2/C10 had a 64% increase in total protein in the insoluble mitochondrial fraction (Figure 2F).

We next assessed for interactions among C2, OPA1, OMA1, and proteins forming the SPY complex involved in quality control proteostasis, SLP-2, PARL, and YME1 (Wai et al., 2016). As reported, previously, C10 robustly co-immunoprecipitates with C2-Flag (Supplemental Figure 4A and B). By contrast, OPA1 and OMA1 failed to co-immunoprecipitate with C2 under the same conditions. Interactions between C2 and PARL and C10 and YME1 of the SPY complex have been identified, previously, in LC-MS/MS co-immunoprecipitation experiments (Floyd et al., 2016; Wai et al., 2016). We were able to confirm an interaction between C2-Flag and YME1. However, we were not able to detect interactions between C2-Flag and PARL or C2-Flag and SLP-2 under the conditions used (Supplemental Figure 4A and B). Similarly, whereas PARL-Flag pulled down endogenous SLP-2, it failed to pull down endogenous C2 or C10. Together these findings could be consistent with weak transient interactions with the SPY complex (and in particular with YME1) but argue against strong physical interactions between C2 and OMA1, the SPY complex, or OPA1.

Given YME1 loss activates OMA1 and C2 interacts with YME1, we assessed whether decreased YME1 activity might be mediating the effect of C2/C10 DKO on OMA1. As expected, loss of either YME1 in HeLa cells or C2/C10 in HEK293 cells led to OMA1 activation, with a decrease in L-OPA1, an increase in OMA1 cleaved S-OPA1 (bands *c* and *e*), and a decrease in OMA1 (Supplemental Figure 4C). Activation of OMA1 appeared to be even stronger in C2/C10 DKO cells than YME1 KO cells, although this may also reflect a difference in cell type. Whereas YME1 KO led to the expected increase in its substrates TIM23 and PRELID, loss of C2/C10 resulted in slightly reduced levels of these YME1

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substrates (Supplemental Figure 4C and D) (Potting et al., 2013; Rainbolt et al., 2013). This suggests that YME1 activity may be slightly increased in C2/C10 DKO cells. Taken together these results suggest that decreased YME1 activity does not account for OMA1 activation in C2/C10 DKO cells.

Exogenous C2 and C10 and some (but not all) pathogenic mutants rescue OMA1 processing in C2/C10 DKO cells

We next addressed whether OMA1 activation in C2/C10 DKO cells can be rescued by exogenous expression of either C2 or C10, as would be expected if the paralogs are functionally redundant. Consistent with functional redundancy between C2 and C10, stable expression of either C2 or C10 reduced OMA1 specific S-OPA1 cleavage products in HEK293 cells lacking endogenous C2 and C10.

We next examined whether disease-causing mutations in C2 or C10 disrupted their activity, using OMA1 activation as a functional readout. We generated HEK293 DKO lines that were stably expressing mutations previously demonstrated to cause familial PD (C2 T61I) or neuromuscular disorders (C10 S59L or G66V) (Funayama et al., 2015; Bannwarth et al., 2014; Penttilä et al., 2015) (Figure 3A and B). We also attempted to generate a stable cell line expressing the C10 G58R mutation at a similar level but failed due to toxicity, which was investigated further below (data not shown). The C2 T61I and C10 G66V mutants rescued L-OPA1 processing, similar to their WT counterparts (Figure 3A and B). By contrast, the C10 S59L mutant did not suppress OMA1 activation, suggesting that the S59L mutation disrupts C10 activity (Figure 3A and B). This analysis demonstrated that some but not all pathogenic mutations retain activity and is consistent with a dominant negative or toxic gain-of-function mechanism of disease for the dominant mutations.

Mitochondrial fragmentation by C10 mutant depends on L-OPA1 cleavage by OMA1

Having identified a functional interaction between C2/C10 and OMA1/OPA1, we next asked whether OMA1 activation is responsible for mitochondrial fragmentation that we and others observed

previously following overexpression of the pathogenic C10 mutation G58R in human cells (Ajroud-Driss et al., 2015; Huang et al., 2018). Over-expression of C10 G58R caused severe mitochondrial fragmentation on a WT background, as was expected (intermediate or fragmented mitochondria in 96.7% of C10 G58R cells vs. 23.3% of untransfected cells, $p < 0.0001$) (Figure 3C and D). Notably, this fragmentation was blocked by OMA1 KO (intermediate or fragmented in 29.67% C10 G58R expressing HeLa^{OMA1 KO} cells vs. 28.67% of untransfected cells, $p > 0.9999$). Overexpression of WT C10 also resulted in a trend toward increased mitochondrial fragmentation, although this trend did not reach statistical significance (Figure 3A and B). These findings demonstrated that C10 G58R overexpression causes mitochondrial fragmentation by triggering cleavage of L-OPA1 by OMA1.

The G58R substitution lies within a conserved hydrophobic α -helix of C10 and contributes to a GXXXGXXXG motif that often mediates protein-protein interactions (Huang et al., 2018; Russ and Engelman, 2000) (Figure 3A, schematic at top). The G58R substitution, which introduces a positive charge into the predicted hydrophobic glycine cleft of the GXXXGXXXG helix, is likely to disrupt the α -helix and/or its interactions. To test this idea further we deleted the α -helix of C10 ($\Delta\alpha$ H). Similar to C10 G58R, C10 ($\Delta\alpha$ H) induced the fragmentation of mitochondria in an OMA1-dependent manner (Supplemental Figure 5A and B). These findings are consistent with the notion that the C10 G58R mutation disrupts the α -helix of C10 to induce OMA1/OPA1 dependent mitochondrial fragmentation.

Forced C2 and C10 expression activates OMA1, and C10 mutant activates OMA1 at a lower concentration than C10 WT

Consistent with the observed OMA1-dependent mitochondrial fragmentation with C10 G58R overexpression, C10 G58R strongly stimulated OMA1 dependent processing of L-OPA1 to S-OPA1 (Figure 3E, left lanes). Unexpectedly, overexpression of WT C10 also induced L-OPA1 processing by OMA1 (Figure 3E, right lanes). To more directly compare C10 WT and C10 G58R expression in HeLa

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cells, we established tetracycline-inducible cell lines. Treatment with 100 ug doxycycline induced expression of C10 WT and C10 G58R to 9.5 and 7.2 times endogenous levels, respectively ($p = 0.90$ for WT vs. G58R comparison). 1000 ug doxycycline further increased expression of C10 WT and C10 G58R to 12.31 and 11.12 times endogenous levels, respectively ($p = 0.99$ for WT vs. G58R comparison). A dose responsive increase in OMA1 activation was observed with both WT C10 and C10 G58R overexpression; however, OMA1 activation was significantly greater for C10 G58R at each level of expression (Figure 3F and G). Thus, the C10 G58R mutation leads to greater OMA1 activation at a similar concentration as C10 WT.

Intense C2/C10 foci accumulate on blocking L-OPA1 cleavage by OMA1

We observed that wildtype C10-Flag formed foci within OMA1 KO cells, rather than the typical smooth appearance throughout the mitochondrial network of overexpressed C10 protein by light microscopy (Figure 3C, arrows in right panels). To investigate whether the pattern of endogenous C2 and C10 distribution also changes in the absence of OMA1, we used antisera specific for these proteins in OMA1 KO HeLa cells (Figure 4A – C and Supplemental Figure 5C and D). We previously reported that endogenous C2 and C10 form foci in the intermembrane space that extend into the mitochondrial cristae in WT HeLa cells (Huang et al., 2018). Strikingly, in the absence of OMA1, C2 and C10 foci exhibited substantially increased intensity (Figure 4A – C and Supplemental 5C and D). As the pattern was similar for both C2 and C10, we focused on C2 in subsequent analyses. High intensity foci were present in the majority of OMA1 KO cells and absent in OMA1 KO cells re-expressing WT but not protease-dead OMA1 (Figure 4A and B). The appearance of these foci did not appear to be driven by a global change in bioenergetics, as membrane potential in OMA1 KO HeLa cells was similar to WT HeLa cells (Supplemental Figure 5E). Thus, the pattern of C2 and C10 distribution is greatly altered in the absence of OMA1 with intensification of C2/C10 foci in the mitochondrial network.

Given OPA1 processing is altered in OMA1 KO cells, we next investigated the dependence of high intensity C2 foci on OPA1 and its processing. To assess whether C2 foci formation depends on OPA1 processing by OMA1, we used genomic editing to delete the canonical OMA1 cleavage site within the endogenous OPA1 locus (HeLa^{OPA1 Δ S1}). This blocked formation of the canonical OMA1 cleaved products *c* and *e* — although residual degradation of OPA1 was observed following mitochondrial depolarization by CCCP, possibly due to limited cleavage by OMA1 at a non-canonical site (Supplemental Figure 5F). Importantly, C2 foci were observed also in HeLa^{OPA1 Δ S1} cells, indicating that partially blocking cleavage of OPA1 by OMA1 leads to formation of high intensity C2 foci (Figure 4D and Supplemental Figure 5G). To further assess the dependence of C2/C10 formation on OPA1, we depleted OPA1 using siRNA (Figure 4E). Interestingly, C10 levels increased following OPA1 knockdown in HeLa cells ($302 \pm 75\%$ in HeLa^{WT} and $325 \pm 85\%$ in HeLa^{OMA1KO}, $p = 0.0018$). Despite an increase in total C2 protein levels, C2 foci were found to be significantly decreased following OPA1 KD in OMA1 KO cells (Figure 4F and G). Together these findings demonstrate that C2 foci formation is a consequence of L-OPA1 stabilization.

The C2/C10 foci resembled those that we previously observed to form spontaneously with transient expression of the C10 mutant S59L (Huang et al., 2018). To further explore the relationship between these foci, we co-expressed C2(WT)-HA with C10(WT)-Flag or C10(S59L)-Flag. Only C10(S59L)-Flag formed foci in wildtype HeLa cells; but, notably, failed to recruit C2(WT)-HA into the foci (Figure 4H and I). By contrast, both C10(S59L)-Flag and C10(WT)-Flag formed foci in similar numbers in HeLa OMA1 KO cells. The majority of these foci also contained C2-HA. Although not conclusive, we hypothesize these intense foci may represent stalled intermediates that can form either from failure of C10(S59L) to disperse, due to increased hydrophobicity in the N-terminal α H, or from blocked OPA1 cleavage in the absence of OMA1.

A pool of C2/C10 is degraded following activation of OMA1 by mitochondrial stressors

We and others previously demonstrated that C2 and C10 exhibit short half-lives in cultured cells (Burststein et al., 2018; Huang et al., 2018). To test whether OMA1 or another intermembrane space facing protease such as YME1 or PARL might be the protease responsible for basal C2/C10 degradation, we assessed C2/C10 levels in knockout HeLa cell lines both at steady-state and following inhibition of translation with CHX. CHX treatment times were chosen based on our prior work (Huang et al., 2018). Similar to our findings in HEK293 cells, we found that C2/C10 steady-state levels are slightly increased following loss of OMA1 (Supplemental Figure 5H). Likewise, C2/C10 protein levels were slightly increased following knockout of YME1 and slightly decreased following loss of Parl (Supplemental Figure 6A and B). However, C2/C10 half-lives were similar to WT cells following knockout of OMA1, YME1, or PARL, demonstrating that these proteases are not solely responsible for basal turnover of C2/C10 (Figure 5A and Supplemental Figure 6A and B) (Wai et al., 2016). Thus, another protease or combination of proteases may be responsible for basal turnover of C2/C10.

We next tested cells subject to mitochondrial stressors known to activate OMA1. Actinonin treatment for 3 hrs has been previously shown to accumulate aberrant mtDNA-encoded polypeptides in the inner membrane and potentially activate OMA1 prior to collapse of the mitochondrial membrane potential (Richter et al., 2015). Unexpectedly, we found that C2/C10 levels are reduced following 3 hr treatment with actinonin (Figure 5B - D). This reduction was dependent on OMA1, as it was partially blocked in OMA1 KO cells. Consistently, degradation was restored in OMA1 KO cells stably re-expressing WT but not catalytically inactive OMA1. Together these findings suggest that OMA1 is dispensable for basal turnover of C2/C10, but, when activated by inner membrane proteostatic stress, degrades C2/C10.

OMA1 is also potentially activated by mitochondrial uncoupling with drugs such as CCCP (Ehse et al., 2009; Head et al., 2009). As we reported previously and in contrast to treatment with actinonin, C2/C10 levels increased in response to loss of membrane potential (Huang et al., 2018). This increase in

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C2/C10 abundance with uncoupling was independent of OMA1, as it occurred in both WT and OMA1 KO HeLa cells (Figure 5A and B). Co-treatment with CCCP and actinonin resulted in accumulation of C2/C10 similar to treatment with CCCP alone (Figure 5B). Thus, although C2/C10 is degraded following OMA1 activation in the presence of a mitochondrial membrane potential, the pool of C2/C10 that accumulates with loss of membrane potential is protected from OMA1 and other proteases.

Together these findings suggest that OMA1 degrades C2/C10 upon activation but is not responsible for their basal turnover. This also implies that the formation of C2/C10 intense foci in the absence of OMA1 is not a direct consequence of failed C2/C10 degradation by OMA1, but rather reflects loss of OMA1 activity against another substrate (e.g., L-OPA1).

In summary, these findings suggest that the functional interaction between C2/10 and OPA1/OMA1 is bidirectional. C2/10 loss or C10 mutation leads to OMA1 activation and OPA1 processing. Conversely, OMA1 loss blocks the stress-induced degradation of C2/10; and C2/10 collect in intense foci in OMA1-deficient mitochondria.

Pathogenic C10 mutation activates OPA1 cleavage by OMA1 in vivo

Having identified a functional interaction between pathogenic C10 mutants and OMA1 activation in cell culture, we next asked whether mutant C10 activates OMA1 *in vivo*.

To model the pathogenic S59L mutation in human C10, we generated a transgenic mouse with the equivalent substitution in mouse C10 (S55L in mouse; but, for clarity, numbering corresponding to human C10 is used throughout) (Figure 6A). Consistent with two recent reports on independent lines (Genin et al., 2019; Anderson et al., 2019), we found that C10^{S59L/+} mice developed cardiomyopathy by early adulthood (17 – 42 weeks). % ejection fraction (%EF) in C10^{S59L/+} animals was significantly lower than WT littermates by echocardiography (39.6% vs 59.7%, $p = 0.003$) (Figure 6B – D and Table S2). Similarly, peak left ventricular outflow tract (LVOT) and pulmonary artery (PA) velocities were significantly reduced, indicating, respectively, left and right ventricular dysfunction (624.1 vs. 1204

mm/s, $p < 0.0001$; and 424.5 vs. 686.5 mm/s, $p < 0.0001$, respectively). Additionally, the anterior and posterior left ventricular walls were significantly thickened in diastole by echocardiography (Supplemental Figure 7A and Table S2). Vacuole formation in cardiac tissue and fibrosis were apparent on histology with H&E and Masson trichrome staining, respectively (Figure 6E). There was also an increase in apoptotic nuclei detected in C10^{S59L/+} knock-in mice compared to control mice detected by the TUNNEL assay (Supplemental Figure 7B). Primary fibroblasts from WT and C10^{S59L/+} KI mice were similarly susceptible to cytochrome *c* release in response to H₂O₂, suggesting that tissue context is important for apoptotic cell death due to mutant C10 (Supplemental Figure 7C).

Additionally, we found C10 distribution to be greatly altered in cardiac tissue from C10^{S59L/+} knock-in mice, forming aggregates, similar to what was reported previously (Anderson et al., 2019) (Figure 6F). Unlike the foci that formed in cell culture with transient overexpression of C10 S59L, however, C10 aggregates in cardiac tissue did not co-localize with mitochondria (Figure 4H, Figure 6F, and Supplemental Figure 7D). Together these findings confirmed development of hypertrophic cardiomyopathy in C10^{S59L/+} knock-in mice by early adulthood, previously reported in two independent mouse models (Genin et al., 2019; Anderson et al., 2019).

Examining cardiac tissue lysates from C10^{S59L/+} mice, we observed a substantial elevation in C2 and C10, consistent with a recent report *in vivo* and our prior observation that C2 and C10 increase in response to mitochondrial distress in cell culture (Figure 7A) (Huang et al., 2018; Anderson et al., 2019). Interestingly, C2 and C10 protein levels decreased slightly with aging in wildtype mice. MTHFD2 and MTHFD1L, which are sensitive markers of the mitochondrial integrated stress response (mt-ISR) in muscle (Kühl et al., 2017; Khan et al., 2017), also progressively increased from younger (9 - 13 weeks) to older (22 – 37 weeks) C10^{S59L/+} animals (Figure 7A). Consistent with activation of the mt-ISR in C10^{S59L/+} mice, expression of transcription factors reported to mediate the response in cardiac tissue, ATF4, ATF5, CHOP, and Myc (Kühl et al., 2017), were significantly elevated relative to younger wildtype mice (Figure 7B). Altogether, in the younger C10^{S59L/+} vs. younger WT comparison, altered expression reached gene-level significance for 15 of 22 pre-specified mt-ISR-associated genes, recently identified to be

differentially regulated genes (DEGs) in the heart of an independent C10^{S59L/+} mouse line (Supplemental Figure 8 and Table S3) (Anderson et al., 2019).

We next assessed OPA1 processing in C10^{S59L/+} mice. Notably, in the heart, L-OPA1 (isoforms *a* and *b*) was excessively processed to OMA1 cleavage products (isoforms *c* and *e*) in younger C10^{S59L/+} mice and to an even greater extent in older C10^{S59L/+} mice (Figure 7A and D). OMA1 levels were likewise decreased in C10^{S59L/+} hearts, as expected given OMA1 is degraded shortly after its activation (Head et al., 2009). Sampling lysates from the atria and ventricles, separately, demonstrated that the myocardium is similarly affected throughout (Supplemental Figure 9A and B). OMA1 activation was absent in the brain and liver but present in the muscle (Supplemental Figure 10A). C2 and C10 protein levels were also normal in the liver and brain but slightly elevated in skeletal muscle; thus, OMA1 activation correlates with elevated C2 and C10 levels *in vivo*. Notably, OMA1 was not activated in either C10^{S59L/+} or C10^{S59L/S59L} primary fibroblast lines, in contrast to C2/C10 DKO cell lines, suggesting that the pathogenic effect of mutant C10 may require its high expression in the heart (Supplemental Figure 10B). Together these findings demonstrate that the pathogenic C10 mutation activates OPA1 cleavage by OMA1 *in vivo*, possibly following its accumulation with C2 in response to an inciting mitochondrial stress.

C2/C10 double knockout activates OPA1 cleavage by OMA1 and mt-ISR in vivo

Guided by parallel results in DKO fibroblasts and C10^{S59L/+} mutant hearts, we assayed OPA1 processing in lysates from younger C2/C10 single and double knockouts mice (9 – 13 weeks). Whereas processing of OPA1 to OMA1-cleaved forms was mildly increased in hearts from C2/C10 single knockout mice, loss of both C2 and C10 had a synergistic effect on L-OPA1 cleavage (Figure 7C and D; Figure 1F and G). OMA1 levels were likewise decreased in DKO hearts, as expected given OMA1 is degraded shortly after its activation. OMA1 cleavage of L-OPA1 was also apparent in liver, brain, and skeletal muscle at this age, albeit to a lesser extent (Supplemental Figure 10A and B). Comparing OMA1 activation in the younger C10^{S59L} model to the younger knockout models, showed OMA1 activation in

C10^{S59L} to be intermediate between C10 single KO and C2/C10 DKO (Figure 7D). Additionally, whereas OMA1 activation was observed in all tissues examined in C2/C10 DKO mice, it was only observed in skeletal muscle and heart of C10 S59L mice, coincident with the elevated C2 and C10 levels in those tissues. Together these findings demonstrated that OMA1 is activated by C2 and C10 loss *in vivo*, phenocopying OMA1 activation by C10^{S59L/+}.

It was recently reported that C10 KO does not activate the mt-ISR in the mouse heart, in contrast to C10^{S59L/+} KI. Consistently, we did not see an increase in MTHFD2 or MTHFD1L in hearts of either C10 or C2 single KO mice (Figure 7C). By contrast, double knockout of C2 and C10 led to a substantial elevation in MTHFD2 and a more modest elevation in MTHFD1L protein levels. MTHFD2 was similarly activated in skeletal muscle and brain (Supplemental Figure 11A and B). Consistently, expression of transcription factors associated with the mt-ISR (namely, ATF4, ATF5, and Myc) were significantly elevated in younger DKO mice compared to younger WT mice with CHOP reaching nominal significance (Figure 7B and Table S3). Altogether, 6/22 genes in a pre-specified set of mt-ISR associated genes previously identified as DEGs in the C10^{S59L/+} KI model reached gene-level significance in the overall DKO vs. WT comparison (Figure 7B and Supplemental Figure 8). With the exception of ATF5 expression, generally the mt-ISR was less robust in C2/C10 DKO mice compared to C10^{S59L/+} KI mice examined at a similar age. Comparison of total DEGs between DKO vs. WT and C10^{S59L/+} vs. WT also underscored the similarity in overall transcriptional response and the greater severity in the younger C10^{S59L/+} mice compared to younger DKO mice. The C10^{S59L/+} vs. WT comparison revealed 1,386 DEGs, whereas the C2/C10 DKO vs. WT comparison produced 548 DEGs, 290 (52.9%) of which were shared. Thus, a similar transcriptional response occurs in both C2/C10 DKO and C10^{S59L/+} KI hearts but involves a more extensive set of genes in C10^{S59L/+} KI hearts.

Given early biochemical changes in the heart, we next assessed our younger DKO mice for early signs of cardiomyopathy. Although fibrosis was not apparent by 13 weeks, numerous vacuoles similar to those seen in C10^{S59L/+} KI were observed in tissues from DKO mice with both H&E and Masson Trichrome staining (Figure 7E). These changes were not apparent in single C10 KO litter mates. In an

exploratory analysis, there was also a slight increase in apoptotic nuclei detected in C2/10 DKO mice by the TUNNEL assay compared to their C10 KO littermates (Supplemental Figure 7B). This trend did not reach significance but was underpowered as limited samples were available for analysis. Primary fibroblasts from WT and C2/C10 DKO mice were similarly susceptible to cytochrome *c* release in response to H₂O₂ treatment, suggesting that tissue context is important for apoptotic cell death due to loss of C2/C10 (Supplemental Figure 7C). Consistent with TEM findings in C10 KO and C2/10 DKO primary fibroblasts and L-OPA1 cleavage patterns in the heart, TEM cristae structure was abnormal in the majority of mitochondria from a C2/10 DKO heart but normal in most mitochondria from a litter-matched C10 KO heart (96/116 [82.8%] abnormal vs. 11/143 [7.6%] abnormal) (Figure 7F). To assess for cardiomyopathy, we evaluated our oldest cohorts of C2/C10 DKO and C10 KO littermates by echocardiography. By 29 - 56 weeks, significantly decreased % ejection fraction was apparent in DKO mice when compared to both their C10 KO littermates and unrelated WT mice of the same strain (43.70% vs. 59.96% and 43.70% vs. 59.68%, p-value < 0.0001 and p = 0.0002, respectively) (Figure 7G and Table S2). Similarly, LVOT peak velocity and PA peak velocity were decreased in DKO mice compared to their C10 KO littermates and unrelated WT mice, consistent with left and right ventricular dysfunction, respectively (Figure 7G, and Table S2). Overall, cardiac dysfunction in C2/C10 DKO mice was milder than in unrelated C10^{S59L/+} KI mice. Whereas all C10^{S59L/+} KI mice had succumbed to cardiomyopathy by 14 months, the two oldest C2/C10 DKO mice in our colony were still alive at 14 months. Thus, similar to C10^{S59L/+} KI mice, C2/C10 DKO mice develop cardiomyopathy, but to a milder degree. Together these findings demonstrate that C2/C10 DKO partially phenocopies C10^{S59L/+} KI mice in vivo with the development of cardiomyopathy and activation of mt-ISR and OMA1 in affected tissues.

Discussion

In this study, we identified a functional interaction between C2/C10 and stress-induced processing of L-OPA1 by OMA1 that is shared by C2/C10 loss of function and mutant C10 gain-of-function. This suggests a model in which the normal function of C2/C10 may be related to the mechanism of C10 mutant pathogenesis (Figure 8). Together our findings suggest that WT C2 and C10 are required within a narrow range of expression to maintain normal L-OPA1/S-OPA1 balance, as either too little or too much WT C2 or C10 activates OMA1 (Figure 8A). This may account for dramatic changes in C2/C10 protein expression levels in response to, e.g., bioenergetic stress (Huang et al., 2018).

In this context, we propose that mutant C2 or C10 may cause aberrant OMA1 activation by one of two mechanisms. With the first mechanism, the mutation may cause C2 or C10 to activate OMA1 at a lower concentration than its WT counterpart (Figure 8A). If a given cell has a concentration of mutant C2 or C10 above that threshold, then OMA1 may be activated at the physiologic level of C2 and C10 in the absence of aggregation. We suggest that this is the mechanism by which the myopathy-causing mutation C10 G58R leads to OMA1 activation (Figure 8A). This is based on the observations that: (1) C10 G58R is not prone to insolubility (Huang et al., 2018); and (2) C10 G58R activates OMA1 at a lower concentration than C10 WT in cellular models (Figure 3C – G). We observed similar behavior C10 with the middle α -helical region deleted, suggesting that G58R may induce a large structural change that leads to OMA1 activation at a lower concentration than WT C10.

In a second mechanism, mutation in C2 or C10 may render the protein prone to aggregate with WT C2 and C10, leading to increased total C2/C10 and decreased soluble C2/C10 (Figure 8B). In this scenario OMA1 may be activated due to decreased soluble C2/C10, phenocopying C2/C10 DKO by a dominant negative mechanism. Alternatively, or in addition, increased insoluble C2/C10 may trigger activation of OMA1 by a toxic gain-of-function mechanism. We propose that this is the mechanism by which the ALS/FTD/myopathy causing mutation C10 S59L activates OMA1 based on the observations that: (1) C10 S59L forms aggregates in cells and affected tissues, (2) C10 S59L leads to decreased solubility of C2 and C10 in affected tissues, and (3) increased tissue C2/C10 levels coincides with OMA1 activation, both of which are observed in heart and muscle but not liver and brain (Huang et al., 2019;

Anderson et al., 2019). Notably, the PD-causing mutation C2 T61I is also prone to insolubility in cell culture and in autopsy tissue, and the SMAJ-causing mutation C10 G66V is prone to insolubility in cell culture, suggesting that this mechanism may also be relevant to the pathogenesis of these disorders (Huang et al., 2018; Cornelissen et al., 2020; Ikeda et al., 2019).

Examining primary cells from the first C2/C10 DKO mouse we found cristae to be decreased and occasionally circular and detached from the boundary membrane, phenocopying cristae abnormalities observed in fibroblasts and skeletal muscle from patients with C10 mutations (S59L and G58R, respectively) (Ajroud-Driss et al., 2015; Bannwarth et al., 2014). These findings are also consistent with swirled cristae recently reported in *Drosophila* muscle lacking the predominate C2/C10 ortholog in that tissue, CG5010 (Meng et al., 2017). As an explanation for cristae abnormalities, we identified L-OPA1 cleavage by activated OMA1. This was observed in HEK293 cells, primary fibroblasts, and heart and skeletal muscle *in vivo*. OMA1 activation was independent of the mild decrease in MICOS subunits observed in C2/C10 DKO cells, as disruption of the MICOS complex with MIC60 KD was insufficient to activate OMA1. However, we did not rule out the possibility that the observed decrease in MICOS subunits with C2/C10 loss may also contribute to the cristae abnormalities. We extended these findings to mutant C10, identifying OMA1 processing of OPA1 as a key event in affected organs, such as the heart and skeletal muscle. Thus, we identify L-OPA1 cleavage by OMA1 as a novel mechanism for cristae abnormalities resulting from dominant C10 mutation or C2/C10 loss.

C2 and C10 are 58% identical and have a single ortholog in yeast and *Drosophila*, suggesting that they are paralogs produced by gene duplication (Cavallaro, 2010). They have also been shown to directly interact (Burstein et al., 2018; Straub et al., 2018; Huang et al., 2018). Whereas these observations imply that C2 and C10 may be functionally redundant, our results provide the strongest evidence that this is indeed the case. Cardiomyopathy with strong activation of OMA1 and the mt-ISR was observed in C2/C10 DKO but not single C2 or C10 KO mice. In cell culture, loss of C2 as well as C10 had a synergistic effect on L-OPA1 processing, and enforced expression of either C2 or C10 in C2/C10 DKO

cells partially rescued L-OPA1 processing. Together these findings demonstrate that C2 and C10 are partially functionally redundant paralogs.

We found L-OPA1 processing also provided a useful functional assay for C2 and C10 activity more generally. Notably, the ALS/FTD/myopathy causing mutation C10 S59L failed to rescue C2/C10 processing, suggesting that it does not retain function. This was not due solely to its propensity for insolubility, as two other disease-causing mutations C2 T61I and C10 G66V, which are more insoluble in cell culture, retained function (Huang et al., 2018). Together with the observation that the C10 G58R mutation strongly activates OMA1 at a low concentration, these observations suggests that mutations in C2/C10 may form at least three distinct classes, namely, those with (i) decreased solubility and retained function (C2 T61I and C10 G66V), (ii) decreased solubility and loss of function (C10 S59L), and (iii) retained solubility and potent OMA1 activation (C10 G58R). We speculate that these differences in C2/C10 mutant behavior may account for phenotypic differences clinically, as the mutant protein interacts with distinct cellular environments, including myocytes (with C10 G58R and C10 S59L), lower motor neurons (with C10 S59L and C10 G66V), peripheral nerves (C10 G66V), upper motor neurons (C10 S59L), cortical neurons (C10 S59L), and dopaminergic neurons (C2 T61I) (Bannwarth et al., 2014; Ajroud-Driss et al., 2015; Penttilä et al., 2015; Auranen et al., 2015; Funayama et al., 2015).

The precise trigger for OMA1 activation in the absence of C2 and C10 is not clear at present. However, there are at least three plausible models: (1) C2/C10 loss or mutation causes bioenergetic stress that indirectly activates OMA1, (2) C2/C10 loss or mutation causes proteostatic stress that indirectly activates OMA1, and (3) C2/C10 directly modulates L-OPA1 processing by OMA1.

Our data seem to rule out the first model: that OMA1 is activated indirectly due to bioenergetic collapse. Under growth conditions in which OMA1 was activated in C2/C10 DKO cells, we found that oxygen consumption and membrane potential were minimally affected. Additionally, inducing a moderate bioenergetic stress in HEK293 cell (by inhibiting mtDNA translation with chloramphenicol) resulted in a reduced membrane potential and a more severe reduction in COX2 expression compared to HEK293

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C2/C10 DKO cells but failed to activate OMA1. Together these data suggest that loss of C2/C10 does not cause bioenergetic stress substantial enough to activate OMA1 on its own.

The second model, that loss of C2/C10 triggers OMA1 activation by increasing proteostatic stress, is intriguing and has some circumstantial evidence in support of it (although direct evidence is lacking). First, we observe that insoluble protein increases in the mitochondrial fraction following C2/C10 loss. Additionally, previous studies have identified interactions between either C2 or C10 and the proteases YME1 and PARL, which are components of the intermembrane space facing SPY complex that also contains OMA1 (Floyd et al., 2016; Wai et al., 2016). We were able to replicate an interaction between C2 and YME1 in this study (although interactions with PARL and SLP-2 were not observed under our study conditions). These findings are at least consistent with a transient interaction between C2/C10 and components of this complex of quality control proteases and a possible role for C2/C10 in maintaining proteostasis. Notably, other twin CX₉C proteins in the intermembrane space have chaperoning functions in the intermembrane space mediated by the CX₉C motif, including Mdm35/TRIAP1, CHCHD4/Mia40, and Cox11 (Longen et al., 2009; Potting et al., 2013). Together these findings suggest that it is at least plausible that C2/C10 may activate OMA1 through decreased proteostasis perhaps due to loss of a chaperoning function. Direct evidence for such a function, however, is not clear at present and will require further work.

Finally, there is some circumstantial evidence in favor of the third model: that C2/C10 directly modulates processing of L-OPA1 by OMA1. First, we found that both low and high levels of C2/C10 activate OPA1 processing by OMA1. This suggests that the proteins are required in a narrow range of expression to prevent OMA1 activation. Additionally, we found that C2/C10 distribution dramatically changes in the absence of OMA1, and C2/C10 protein levels increase in response to OPA1 or OMA1 reduction. This suggests a bidirectional functional interaction between C2/C10 and OPA1/OMA1. Finally, we find that C2/C10 are possible substrates of activated OMA1, suggesting that OMA1 and C2/C10 may be in close proximity. Against the model, we failed to detect direct physical interactions between C2 and OPA1 or OMA1 stable enough for immunocapture, and at least the predominate pool of

C2/C10 appears in complexes that are of distinct in size from the predominate OPA1- and OMA1-containing complexes by BN-PAGE (Frezza et al., 2006; Wai et al., 2016; Straub et al., 2018). However, this does not rule out more transient interactions between C2/C10 and either OPA1 or OMA1. Thus, while the direct model has circumstantial evidence in favor of it, a direct link between C2/C10 and OPA1/OMA1 has not been identified and if present will require further work to establish.

Identifying OMA1 activation as a key event in C10 mutant pathogenesis, also nominates OMA1 as a potential therapeutic target for C2/C10-related disorders. Although OMA1 is required for OPA1 processing in response to mitochondrial stress, it is not essential for mammalian life (Quirós et al., 2012). The OMA1 KO mouse is viable and loss of function mutations occur in human population at close to the rate predicted by chance, including two “knockout” adult individuals with homozygous frameshift mutations (observed SNV/expected SNV = 0.78 (0.55 – 1.13); gnomAD v.2.1.1 database) (Karczewski et al., 2019). Indeed, in the setting of a high proteostatic load, such as that resulting from loss of YME1 or the scaffolding protein prohibitin-2, its excessive activation may be detrimental, leading to heart failure and neurodegeneration, respectively (Wai et al., 2015; Korwitz et al., 2016).

In this scenario, OMA1 cleavage of OPA1 and the resulting cristae abnormalities may be responsible for the observed cardiomyopathy, multiple mtDNA deletions, and mt-ISR following C2/C10 loss or dominant mutation. L-OPA1 is known to be necessary for mtDNA stability, raising the possibility that OPA1 cleavage by OMA1 may drive mtDNA instability in patients and the C10^{S59L/+} mouse (Bannwarth et al., 2014; Genin et al., 2019). Similarly, OMA1 activation was recently suggested to cause mt-ISR activation, by cleaving the inner mitochondrial membrane protein DELE1 (Fessler et al., 2020, 1; Guo et al., 2020). DELE1 subsequently retro-translocates to the cytosol and activates the eIF2 α kinase HRI, which in turn, activates ATF-4. It is interesting to speculate that this may be the mechanism for mt-ISR activation in response to C2/C10 loss and dominant C10 mutation, given strong OMA1 activation observed in this context. However, we cannot rule out the alternative explanation that a mitochondrial stress resulting from C2/C10 loss or dominant mutation may independently cause OMA1 activation and drive cardiomyopathy, mtDNA mutations, and the mt-ISR. In this case, OMA1 activation would be

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biomarker of C2/C10 related toxicity but would not cause the subsequent tissue degeneration. Future studies of mutant C10 (and C2) on an OMA1 knockout background will be important for evaluating OMA1 as a therapeutic target, as well as determining which aspects of the mutant phenotype (e.g., cardiomyopathy, mt-ISR activation, multiple mtDNA deletion, cytosolic TDP-43 accumulation) are dependent on L-OPA1 processing by OMA1.

Methods

Generation of transgenic mice

C2^{-/-}, C10^{-/-}, and C10^{S59L/+} transgenic lines were produced using CRISPR/Cas9 endonuclease-mediated genome editing on the C57Bl6J background. CRISPR guide RNAs targeted exon 2 and 4 in mC10 and exon 1 and 4 in mC2 to generate breaks in DNA. Animals were screened for large deletions lacking the aforementioned exons by Sanger sequencing. C10 KI mice were generated with a guide RNA targeting near the S55 position (equivalent of S59 in human C10) in mC10 co-injected with Cas9 and an ssODN containing a TCA → CTG substitution at the S55L codon in the C57Bl6J background. All animal studies were approved by the Animal Care Use Committee at the NINDS intramural program. Both genders were used in all studies.

Cell Culture

WT, C2, C10 and DKO fibroblast cells were generated from 1 day old newborn pups. Pups were removed from litter and placed in DMEM containing 1% L-glutamine, 15% FBS, MEM-NEAA, 1% sodium pyruvate, 1X pen/strep, 1X gentamicin, and 1X amphotericin B. Sterile scalpels were used to cut the skin from the animals into thin slices around 1 - 2mm squares. The tissues were transferred to a 1% gelatin-coated plate containing the dissection media and covered with sterile cover slips. High glucose

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DMEM with sodium pyruvate and supplemented with 10% FBS and pen/strep was used for all other tissue culture and for primary fibroblasts after passage 1.

OPA1 null Mouse Embryonic Fibroblasts were obtained from ATCC (ATCC CRL-2995) and transformed MEF WT cells with SV40 were obtained as a kind gift from the laboratory of David Chan (Caltech). Generation of the HEK293 C2/C10 DKO cell line was described previously (Huang et al., 2018). HeLa^{PARL} KO, HeLa^{OMA1 KO}, HeLa^{OMA1 KO} stably expressing OMA1 WT or OMA1 E328Q and matched HeLa^{WT} cell lines were a kind from Richard Youle (NIH) and have been described previously (Sekine et al., 2019). HeLa^{YME1 KO} and matched HeLa^{WT} cell lines were a kind gift from Thomas Langer (Cologne) and have been described previously (Hartmann et al., 2016).

HEK293 OMA1 KO and HEK293 C2/C10/OMA1 TKO lines were produced by CRISPR/Cas9, using the pSpCas9(BB)-2A-GFP (PX458) plasmid with the guide sequences 5'

CACCGAGTGCAATCGAGACGTCCCG 3' and 5' CACCGAGATCGCACACGCAGTCCTG 3'

targeting exons 4 and 5, respectively. Knockout was verified by immunoblotting for OMA1 and

characteristic change in OPA1 processing. The HeLa^{OPA1 Δ s1} was produced using the pSpCas9(BB)-2A-GFP (PX458) plasmid with the guide sequence 5' CACCGCGTTTAGAGCAACAGATCG 3' and the ssODN sequence: 5'

gggttgcatatttatctttaagGTTCTCCGGAAGATCGTGGATCTGAAAGTGACAAGCATTTTAGAAAGg 3'.

pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid # 48138;

<http://n2t.net/addgene:48138>; RRID:Addgene_48138). HeLa cells stably expressing C2-His6 or C2-Flag

were generated by lentiviral transduction, using the pCIG3 vector containing the C2-His6 or C2-Flag

sequence. pCIG3 (pCMV-IRES-GFP version 3) was a gift from Felicia Goodrum (Addgene plasmid #

78264 ; <http://n2t.net/addgene:78264> ; RRID:Addgene_78264). The fibroblasts were incubated at 37°C

with 5% CO₂ and 5% O₂. All other cell lines were incubated at 37°C with 5% CO₂ and 21% O₂.

Antibodies

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The following antibodies were used: anti-FLAG M2 (Sigma, cat# F1804-1MG), anti-Tom20 F-10 (Santa Cruz, cat# sc-17764), anti-C2 C-term (Sigma, cat#HPA027407), anti-cytochrome C 6H2.B4 (bd pharmingen, cat# 556432), anti-OPA1 (BD Bioscience, cat# 612606), anti- β -Tubulin (Sigma, cat# T8328), OMA1 (ProteinTech, cat# 17116-1-AP), anti-C2 (ProteinTech, cat# 19424-1-AP), anti-C2 (ProteinTech, cat# 6602-1-Ig), anti-C10 (Sigma, cat# HPA003440), anti-MIC60 (ProteinTech, cat#430909), anti-MIC19 (Sigma, cat# HPA042935), anti-MIC27 AntI-MIC27 (Sigma, cat# HPA000612), anti-GAPDH (ProteinTech, cat# 60004), anti-HSP90 (ProteinTech, cat# 13171-1-AP), anti-MTHFD2 (ProteinTech, cat# 12270-1-AP), and anti-MTHFD1L (ProteinTech, cat# 16113-1-AP).

Transmission Electron Microscopy

Mouse fibroblasts were fixed with 4% glutaraldehyde (Electron Microscopy Services) in EM buffer (0.1 N sodium cacodylate at pH 7.4 with 2 mM calcium chloride) for 30 minutes at room temperature and then at 4°C for at least 24 hours. Samples were washed with buffer and treated with 1% osmium tetroxide in 0.1 N cacodylate buffer at pH 7.4 for 1 h on ice, washed and *en bloc* stained with 0.25–1% uranyl acetate in 0.1 N acetate buffer at pH 5.0 overnight at 4°C, dehydrated with a series of graded ethanol and finally embedded in epoxy resins. Ultrathin sections (70 nm) were stained with lead citrate and imaged with a JEOL 1200 EXII Transmission Electron Microscope. Images were evaluated and scored by a blinded experimenter. Mitochondrial were scored as abnormal if they displayed cristae that were ring shaped and detached from the boundary membrane, had a total course of the cristae turned more than 90°, and/or there was extreme variability in mitochondrial diameter in which the region with the thinnest caliber was less than half of the region with greater caliber. Cristae were only scored when the outer and boundary inner membrane surrounding the cristae were sharply in view.

Oxygen Consumption Rate

Seahorse Extracellular Flux Analyzer XF (Aglient) was used to measure the oxygen consumption rates (OCR) of mouse primary fibroblasts. Fibroblast cells were seeded at 20,000 cells/well in XF96 Cell Culture Microplates coated with 1% gelatin and incubated for 24 hrs at 37°C with 5% CO₂ in DMEM containing glucose. An hour before the assay, the cells were placed in DMEM lacking bicarbonate and culture plates were moved to a 37°C incubator with atmospheric CO₂. The assay measures OCR at basal levels, after addition of 1 µM oligomycin, 2 µM FCCP and 0.5 µM of rotenone and antimycin. Data are represented as means ± standard error of at least 6 biological replicates, normalized by protein concentration as determined by the BCA assay.

Mass Spectrometry

HEK293^{WT} cells were labelled to >95% incorporation of ¹²C₆, ¹⁵N₂ L-Lysine and ¹³C₆, ¹⁵N₄ L-Arginine (Cambridge Isotope Laboratories) in SILAC DMEM media lacking L-Lysine and L-Arginine (Thermo Fischer). Mitochondria were isolated from both heavy labeled HEK293^{WT} and light labeled HEK293^{DKO} cells. Protein concentration was determined by BCA assay and heavy and light mitochondria were mixed 1:1. The mixed mitochondria were solubilized in 1% digitonin and complexes were separated on a BN-PAGE gels with bovine heart mitochondria used as a molecular weight standard. Lanes were cut into ten gel slices and digested with trypsin. Extracted peptides were desalted and used for LC-MS/MS data acquisition on an Orbitrap Luminos mass spectrometer (Thermo Fisher Scientific) coupled with a 3000 Ultimate high-pressure liquid chromatography instrument (Thermo Fisher Scientific). Peptides were separated on an ES802 column (Thermo Fisher Scientific) with mobile phase B increasing from 2 to 27% over 60 min. The LC-MS/MS data were acquired in data-dependent mode. The resolution of the survey scan (300–1600 m/z) was set at 60k at m/z 400 with a target value of 10 × 10⁶ ions. Collision-induced dissociation was performed on the top ten most abundant precursor ions with an isolation window of 2.0 Da. Database search and H/L ratio calculation were performed using MaxQuant against Sprout Human database (Tyanova et al., 2016). Oxidation (M), Label:13C(6)15N(2) (K), and Label:13C(6)15N(4) (R)

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were included as variable modifications in the database search. Identified protein groups tagged as reverse, contaminants, or identified only by modified peptide were filtered out using the MaxQuant companion program, Perseus. For the HEK293^{WT} condition intensities were normalized for each protein group across the gel slices by dividing protein intensities in each gel slice by the sum of the intensities from all of the gel slices. Intensities for the HEK293^{DKO} values were calculated by multiplying the relative wildtype value for each protein group in each gel slice by the L/H ratio quantified for each slice. When a ratio was missing the WT value was multiplied by 1.

Transfection, Immunocytochemistry, and Confocal Microscopy

Cells were plated on 8-well chambered slides (Ibidi) at a seeding density of 25,000 cells/well overnight. Where indicated cells were transfected with plasmids using FugeneHD (Promega). C10 (del Δ H) was produced by cloning a gene block (IDT) comprising a human codon optimized C10 Δ (45 - 69) into a YFP-N1 vector (Clontech) digested with NotI and BamHI to replace the YFP insert, using HiFi NEB-Builder (NEB). Other plasmids were cloned as described previously (Huang et al., 2018). For siRNA knockdown experiments, cells were plated on day 1, transfected with siRNA on day 2, transfected again on day 4 and fixed on day 6. OPA1 was targeted using SMARTpool: On-TARGETplus (Dharmacon, Cat# L-005273-00-0005) and Mic60/IMMT was targeted using siGENOME Human IMMT (10989) siRNA – SMARTpool (Dharmacon, Cat# M-019832-01-0005), using RNAiMAX (Thermo Fischer). Cells were fixed for 10 min with 4% paraformaldehyde (Electron Microscopy Services) in PBS 1X. Cells were then permeabilized with 0.25% Triton X-100, blocked with 1% BSA for at least 30 minutes, and then incubated in primary antibody for 2 hrs to overnight in PBS at 4°C. Cells were washed with PBS three times and then incubated at room temperature for 1 hour with Alexa Fluro goat secondary antibodies (488, 555, 594 or 647) at 1:1000 dilution in 1% BSA. Confocal microscopy was performed on Fluoview3000 (Olympus) or Airy LSM 880 (Zeiss). The antibodies used for immunocytochemistry were anti-FLAG M2 (Sigma cat# F1804-1MG), anti-Tom20 F-10 (Santa Cruz, cat# sc-17764), anti-C2 C-term

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(Sigma, Cat#HPA027407), anti-HA (3F10) rat monoclonal (Sigma, 11867423001), and anti-cytochrome C 6H2.B4 (BD Pharmingen, cat# 556432).

Fragmentation of the mitochondria in HeLa cells stained with Mitotracker Red (Thermo Fischer) or immunostained for cytochrome *c* or Tom20 was scored to fall into one of three categories: tubular, short-tubes, and fragmented mitochondrial network. Cells with and without transfection were determined through anti-Flag M2 (Sigma) immunocytochemistry staining. To determine number of cells with greater than 2 foci, HeLa cells stained with Tom20 (Santa Cruz) and C2 (Sigma) were imaged with confocal microscopy. Experimenter counted number of cells in each condition that contained greater than 2 foci visible by wide-field fluorescent microscopy using a 60X objective. To determine C2 foci count and intensity in HeLa cells, Imaris software was used to identify and calculate fluorescent intensity from z-stack confocal images. The “Spots” detection tool in Imaris was used to identify foci from C2 (Sigma) immunostaining. For colocalization of C10 and C2 foci, z-stack confocal images were analyzed in Imaris. The “Spots” tool was used to detect C2 and C10 foci. Then, the total number of C10 foci that colocalized with any C2 focus was calculated. Two foci are considered colocalized if their centers were within 0.01 mm.

Echocardiography

Mice were lightly anesthetized with isoflurane during exams and placed in the supine position over a heated platform with electrocardiography (ECG) leads and a rectal temperature probe. Heart images were acquired using the Vevo2100 ultrasound system (VisualSonics, Toronto, Canada) with a 30 MHz ultrasound probe (VisualSonics, MS-400 transducer). Measurements were made from standard 2D and M-mode images from the parasternal long axis and mid-papillary short axis views of the LV.

Histology Analysis

Hearts were harvested from mice anesthetized with isoflurane. Harvested hearts were cut into three segments with a razor blade. The apex and base were snap frozen and a mid-section was fixed in 4% paraformaldehyde (Electron Microscopy Services) for 24 hrs at 4°C, washed with PBS, dehydrated, and embedded in paraffin (through HistoServ), sliced into 5 µm sections and stained with H&E and Masson trichrome (through HistoServ). Sections were imaged with a wide-field microscope (Zeiss).

For immunohistochemistry, deparaffinized cardiac sections were treated in blocking buffer (5% goat serum in 0.3% Triton X-100, 0.02% NaN₂). The sections were then incubated in primary antibody anti-C10 (1:1000, Sigma-Aldrich, HPA003440) and anti-Tom20 (Santa Cruz sc-17764 1:100 dilution) in PBS overnight at 4°C. After washes in PBS, the sections were incubated with secondary antibodies (Alexa Fluro-labelled goat antibodies) in blocking buffer for 1hr at room temperature. After immunostaining, the tissue was mounted with mounting agent (KPL 71-00-16) and imaged with constant microscopy settings on Fluoview3000 confocal microscope (Olympus) with 40X silicon oil immersion lens. The antibodies used are anti-Tom20 F-10 (Santa Cruz, cat# sc-17764) and anti-C10 (Sigma, cat# HPA003440).

Immunoblotting

Lysates from HEK293, HeLa, and fibroblast cells were processed as described previously (Huang et al., 2018). Mouse heart and skeletal muscle were lysed in RIPA buffer and a buffer contains 20mM Tris pH 7.8, 137mM NaCl, 2.7mM KCl, 1mM MgCl₂, 1% TX-100, 10% glycerol, 1mM EDTA and 1mM DTT respectively with 1% proteinase inhibitor. Lysates were sonicated by a Vibra-Cell Ultrasonic Disruptor for 15s, 4 times at an output level of 20. Protein concentration was determined by the BCA assay. Lysates were separated on SDS-PAGE gels and analyzed by immunoblotting. OMA1 produced S-OPA1 cleavage products were calculated by measuring the maximum intensity of each of the five bands in a linescan of the optical density of the five OPA1 bands on the blot using the software program FIJI.

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The peaks of c and e bands were summed and divided by sum of the five bands (a - e) to obtain percentage OMA1-generated S-OPA1 of total OPA1.

RNA Expression Studies

For each for the following groups, RNA was extracted from hearts of four mice: WT younger, WT older, C10^{S59L/+} younger, C10^{S59L/+} older, and C2/C10 DKO mice. RNA expression was measured using the Clariom_S_Mouse microarray (Affymetrix). Data was analyzed using the Transcriptome Analysis Console (TAC) Software (version 4.0) on the default settings (Affymetrix). Specifically, data was summarized using the Gene Level – SST-RMA model. Differentially expressed genes (DEGs) were required to have gene-level fold change < -2 or > 2 and gene level P-Value < 0.05 measured using the ebayes Anova method. Additionally, expression levels of a pre-specified list of 24 individual genes consisting of C2, myc, and 22 genes previously identified as mt-ISR related DEGs in an independent C10^{S59L/+} mouse line were extracted for each sample and analyzed nominally using one-way ANOVA with Sidak's multiple comparison test applied for each gene.

Statistical Analysis

For all statistical analyses with 2 samples, Student's *t*-tests (2-tailed) were performed in Excel (Microsoft) or Prism (Graphpad). For analyses comparing more than 2 samples one-way ANOVA with Sidak's multiple comparison test was performed in Prism (Graphpad). Statistical analysis for RNA expression studies is discussed above. The animal and cellular experiments were performed as exploratory studies and explicit power analysis was not performed prior to experimentation. For initial animal studies of C10^{S59L/+} KI mice groups of echocardiography groups N > 12 were chosen based on review of the literature and prior experience. Based on the large effect size observed for the C10^{S59L/+} KI mice and availability of animals old enough for analysis groups of 6 litter matched C2/10 DKO and C10 KO mice were tested by echocardiography. For RNA expression studies groups of 4 were used based on

review of the literature and prior experience. For all cellular studies with the exception of electron microscopy at least three replicates on two occasions were performed. For electron microscopy studies of cells, three biological replicates were performed with the following exceptions: one biological replicate was performed for electron microscopy analysis of mouse heart tissue and one replicate for analysis of OPA1 KO mouse fibroblasts. No randomization was used in the animal experiments as a treatment was not being tested. Experiments were performed unblinded.

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Conflict of Interest Statement

None declared.

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Figure 1. Cristae abnormalities in C2/C10 DKO primary fibroblasts associated with OPA1 processing by OMA1 activation. (A) C2 and C10 KO mice were generated by CRISPR/Cas9 genomic editing with CRISPR cut sites (scissor icon) and guide RNA sequence (red text) and PAM sequence (underlined) indicated. Sanger sequencing across the deletions verifies loss of the intervening exons. (B - D) Analysis of TEM images from WT and C2/C10 DKO fibroblast cells. Representative images are shown in (B). Arrowhead indicates detached cristae and arrow points to convoluted cristae. (C) Quantification of abnormal mitochondria. (D) The number of cristae per mitochondrion area (μm^2) was calculated. For (C) and (D) ≥ 267 mitochondria were quantified per condition from ≥ 21 cells. 2 independent WT cell lines, 3 independent C10 KO cell lines (2 litter matched to DKO and 1 to WT), and 2 independent C2/C10 DKO cell lines were evaluated. Data from cell lines were pooled by genotype. A total of three biological replicates were performed per genotype on two occasions. (E) Representative TEM images of transformed WT and OPA1 KO MEF cells. Arrowhead indicates detached cristae. (F and G) Immunoblot from lysates of WT, C2^{-/-}, C10^{-/-}, C2^{+/-};C10^{-/-}, and C2^{-/-};C10^{-/-} (DKO) primary mouse fibroblasts. Immunoblot is representative of 3 biological replicates. HSP90 serves as the loading control. OPA1 isoforms (a – e) depicted in (F) are indicated. (H) Model depicts processing of the two membrane-bound long forms of OPA1 (L-OPA1) at protease sites s1 and s2 into the three short forms (S-OPA1) by the proteases OMA1 (at the s1 site) and YME1 (at the s2 site). Red arrows depict cleavage by OMA1 at the s1 site to generate isoform c from a and e from b. The green error depicts cleavage at s2 by YME1 to generate d from a (I) Oxygen consumption rates (OCR) of WT, C2^{-/-}, C10^{-/-}, C2^{-/-};C10^{-/-} (DKO) primary fibroblasts measured by the Seahorse instrument. Plot is representative of at least 6 biological replicates performed on at least two occasions. Scale bars in all images = 600 nm.

Figure 2: Cristae abnormalities in C2/C10 DKO cells are due at least in part to OMA1 activation. (A) Representative TEM images of WT, C2/C10 DKO, and C2/C10/OMA1 triple KO (TKO) HEK293 cells. Scale bar = 800 nm. Arrows indicate abnormal mitochondria. (B) The number of cristae per mitochondrion area (μm^2) was calculated. For each condition ≥ 275 mitochondria were measured from \geq

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22 cells in 3 biological replicates performed on two occasions. “****” indicates p-value < 0.001 after correction for multiple comparisons. Error bars represent standard error of the mean. (C) Immunoblot from lysates of WT, C2/C10 DKO, OMA1 KO, and TKO HEK293 cells. Tubulin served as the loading control. Blot is representative of at least 3 biological replicates performed on at least two occasions. (D) Immunoblot of WT and C2/C10 DKO HEK293 cells following knockdown of MIC60 or left untransfected (NT). Immunoblot is representative of 3 biological replicates obtained on 2 different occasions. Lysates from 2 replicates (r1 and r2) are shown. (E) Heat map depicts relative abundance of MICOS complex subunits in SILAC labelled WT and DKO HEK293 mitochondria, solubilized in 1% digitonin and separated by BN-PAGE prior to detection by quantitative mass spectrometry. Columns in the heat map represent gel slices at the indicated position. Rows indicate proteins detected. Data represents 2 biological replicates performed on 1 occasion. (F) Total protein was stained from soluble and insoluble mitochondrial fractions of HEK293 WT and C2/C10 DKO separated on SDS-PAGE gels and transferred to a membrane. Relative intensity of total protein in soluble or insoluble HEK293 DKO fractions relative to WT was quantified for N = 3 biological replicates performed on at least 2 occasions. Mean +/- SE is represented on the bottom on the membrane.

Figure 3: Mitochondrial fragmentation induced by C10 mutant depends on OMA1 processing of OPA1.

(A and B) HEK293^{WT} and HEK293^{C2/C10 DKO} were not transduced (NT) or stably transduced with C10 or C2 containing the disease-causing mutations indicated. Lysates were separated on SDS-PAGE gels and OPA1 processing was accessed by immunoblotting. (B) OMA1 activity was measured as the abundance of OMA1 cleaved S-OPA1 isoforms (c and e) divided by the sum of all five isoforms in 3 biological replicates performed on at least 2 occasions. (C) Representative Airyscan confocal images of WT and OMA1 KO HeLa cells transiently transfected with C10 WT-Flag (C10 WT) or C10 G58R-Flag (C10 G58R). Anti-Flag immunostaining in green and anti-Tom20 in red. (D) Quantification of mitochondrial morphology depicted in (A) for > 150 cells in 3 biological replicates per condition. Untransfected cells (NT) were identified by the absence of Flag staining in each of the samples. Counts were pooled as they

did not differ among the samples. (E) Immunoblot from lysates of WT and OMA1 KO HeLa cells transduced with vector (V), C10 WT (C10 WT), or C10 G58R (C10 G58R). Tubulin and HSP90 serve as the loading controls in the respective blots. Immunoblot is representative of 2 biological replicates. (F and G) HeLa^{WT} cells stably expressing tetracycline inducible C10 WT and C10 G58R constructs were treated with 100 or 1000 µg doxycycline for 48 hrs and whole cell lysates were immunoblotted for OPA1, C10, and HSP90, which served as a loading control. OMA1 cleaved L-OPA1 was quantified as percent of the OMA1 specific isoforms (*c* and *e*) divided by the sum of all 5 isoforms. Experiment was performed in 3 biological replicates on 2 occasions. In all graphs. “*” indicates a p-value <0.05, “***” indicates a p-value <0.001, “*****” indicates p-value < 0.001 after correction for multiple comparisons. Error bars represent standard error of the mean.

Figure 4: Altered C2/C10 localization and processing in the absence of OMA1. (A) Airyscan confocal images from of WT and OMA1 KO HeLa Cells immunostained for Tom20 (red) and C2 (green). Where indicated OMA1 KO HeLa cells stably expressed OMA1 WT or the protease-dead OMA1 E328Q mutation. Scale bar = 20 µm. (B) Quantification of cells with greater than 2 intense foci in (A). Greater than 150 cells were scored in 3 biological replicates. (C) Graph depicts intensities of individual foci from representative WT and OMA1 KO HeLa cells. (D) Quantification of cells with greater than 2 intense C2 foci in WT or two independent OPA1Δs1 HeLa cell lines (#10 and #15). >150 cells were scored in three biological replications for each condition. (E) Immunoblot of WT and OMA1 KO HeLa cells treated with control or OPA1 siRNA as in (A). HSP90 served as the loading control. Immunoblot is representative of 3 biological replicates. (F) Representative Airyscan confocal images of OMA1 KO HeLa cells treated with control or OPA1 siRNA and stained for C2 (green), Mitotracker Red (MTR) (red), and Tom20 (blue). (G) Quantification of intense C2 foci in OMA1 KO HeLa cells following treatment with either control or OPA1 siRNA. >150 cells were scored in three biological replications for each condition. “*****” indicates “p-value < 0.0001”. (H) Representative confocal images of WT or OMA1 KO HeLa

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cells transient co-transfected with CHCHD2-HA and either CHCHD10(WT)-Flag or CHCHD10(S59L)-Flag, immunostained for Flag and HA. (I) Quantification of (H) for CHCHD10 foci that overlap with CHCHD2 foci. > 20 cells in each condition were analyzed. Scale bars in all images = 10 μ m. All confocal images were acquired as a z-stack and represented as a maximum projection image.

Figure 5: C2/10 are degraded by OMA1 activated by mitochondrial stress. (A) Immunoblot of OMA1 KO and WT HeLa cells treated with DMSO or 10 μ M CCCP overnight (O/N) and then treated with 100 μ M cycloheximide (CHX) for the indicated number of hours. GAPDH or Tubulin served as controls where indicated. (B) Immunoblot of WT HeLa cells treated with DMSO or 10 μ M CCCP overnight (O/N), followed by the addition of actinonin, CCCP, or 125 μ M actinonin + 10 μ M CCCP for the indicated number of hours. Tubulin served as a loading control. N = 3 biological replicates per sample. (C) Immunoblot of WT and OMA1 KO HeLa cell lines, which stably express OMA1 WT or OMA1 E328Q mutation where indicated, treated with either vehicle or 125 μ M actinonin for 3 hours. HSP90 served as the loading control. (D) Quantification of the C2 and C10 levels from (B). N = 3 biological replicates per sample. The levels of C2 of each cell type following DMSO (D) or actinonin (A) treatment are normalized to those from WT HeLa with DMSO treatment. Three biological replicates were quantified.

Figure 6: C10^{S59L/+} KI mice develop cardiomyopathy. (A) C10^{S59L/+} KI mice were generated using CRISPR/Cas9 genomic editing. The targeted CRISPR cut site (scissors), guide RNA sequence (red), and PAM sequence (underlined) are indicated. The donor ssODN used for repair is shown above. Sanger sequencing demonstrates the TCA -> CTG codon change, as well as a silent GGG -> GGC mutation at G58 introduced to disrupt the PAM sequence. (B) Representative echocardiogram in long axis view from WT and C10^{S59L/+} mice demonstrating thickening of the ventricular wall in C10^{S59L/+} mice. (C) Motion (M)-Mode view from the short axis of echocardiogram, demonstrating decreased left ventricular

contractility of the C10^{S59L/+} heart. (D) Graph representing % ejection fraction (%EF), left ventricular outflow tract velocities (LVOT), and pulmonary artery velocities (PA) of litter matched WT (N = 13) and C10^{S59L/+} (N = 12) mice between the ages of 17 - 42 weeks. Velocities were measured by pulsed-wave Doppler echocardiography. “****” denotes p-value < 0.001 and “*****” indicates p-value < 0.0001 after correcting for multiple comparisons. (E) Representative images of H&E stain (left) and Masson trichrome stain (right) from adult WT and C10^{S59L/+} mice. Arrows indicate vacuoles. Images are representative of at least 3 animals per genotype evaluated. (F) Airy scan confocal images of C10 immunostaining of heart slices from WT and C10^{S59L/+} mice. Scale bar = 10 μ m. All confocal images were acquired as a z-stack and represented as a maximum projection image. Images are representative of at least 2 animals per genotype evaluated.

Figure 7: OMA1 activation co-incident with cardiomyopathy and mt-ISR phenocopied in C2/C10 DKO and C10^{S59L/+} KI mice. (A) Representative immunoblot of heart lysates from younger (9 - 13 weeks) and older (36 – 37 weeks) WT and C10^{S59L/+} KI mice. Lysates from two different mice (m1 and m2) are shown for each condition. (B) Relative transcript levels of Atf4, CHOP, Atf5, and Myc in heart extracts of younger WT, C10^{S59L/+} KI, and C2/C10 DKO mice (9 - 13 weeks), and older WT and C10^{S59L/+} KI (22 - 37 weeks) mice measured by microarray and normalized to average of younger WT mice. N = 4 for each group. (C) Representative immunoblot of heart lysates from WT, C2 KO, C10 KO, and C2/C10 DKO mice (age 9 - 13 weeks). Lysates from two different mice (m1 and m2) are shown for each condition. (D) Quantification of OMA1-cleaved S-OPA1 relative to total OPA1 from heart lysates as in (A and C). Note: WT comparison group is same as in the right and left graphs. (E) Representative images of H&E stain and Masson trichrome stain from sibling-matched C10 KO and C2/C10 DKO mice (9 - 13 weeks). (F) Representative TEM images of C10 KO and C2/C10 DKO hearts. (G) Graph representing echography measurements of % ejection fraction (%EF) and left ventricular outflow tract (LVOT) and pulmonary artery (PA) peak velocities measured for sibling matched C10 KO and C2/C10 DKO mice and WT mice in (Fig. 5D). C10 KO and C2/C10 DKO mice were 29 - 56 weeks old at the time of echocardiography. At

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least 5 animals per genotype were assessed. In all graphs, “n.s.” indicates “not significant,” “*” corresponds to p-value < 0.05, “**” represents p-value < 0.01, “***” denotes p-value < 0.001 and “****” indicates p < 0.0001 after correction for multiple comparisons.

Figure 8. Model of OMA1 activation due to loss of C2/C10 and mutant C10. (A) WT C2/C10 are required in a narrow range of expression to prevent L-OPA1 processing by the stress-induced peptidase OMA1. Too low or too high expression of WT C2 or C10 activates OMA1. Activated OMA1 disrupts mitochondrial cristae by cleaving L-OPA1. Some C10 mutations such as G58R appear to lower the threshold concentration for activation of OMA1 by C10. Dotted line represents the threshold concentration for OMA1 activation. (B) Aggregation prone mutants of C2 or C10, such as C10 S59L, may activate OMA1 by a different mechanism. Aggregation prone C10 S59L may co-aggregate with soluble C2 and C10 pulling them into an insoluble fraction. In this setting OMA1 may be activated by decreased soluble C2/C10 thereby mimicking C2/C10 DKO in a dominant negative mechanism. Alternatively (or in addition), increased insoluble C2/C10 may activate OMA1 by a toxic gain-of-function mechanism.

Supplemental Methods

Antibodies

The following antibodies were used: MTHFD2 (Proteintech, cat# 122701-1-AP), MTHFD2 (Abcam, # ab1151447), Mic25 (Proteintech, cat# 20639-1-AP, Drp1 (BD Biosciences, cat# 611113), MFN2 (kind gift from Richard Youle [NINDS, Bethesda]), PGAM5 (Santa Cruz, cat# SC-515880, YME1L1 (Proteintech, cat# 11510-1-AP, SLP2 (Proteintech, cat# 10348-1-AP, PARL (Proteintech, cat# 26679-1-AP).

Measurement of membrane potential in primary fibroblasts by TMRM

Membrane potential of primary fibroblasts was measured using TMRM using an automated high content imaging system as described previously (Ashley et al., 2005). For live cell staining, 500 μ M PicoGreen and 500 μ M TMRM stocks were diluted in cell culture media 1:333 (3 μ l/ml) and 1:2000 respectively. 100 μ l of staining solution was added to each well of a 96-well plate. Fibroblasts were incubated in the staining solution for 30 mins at 37°C in a humidified incubator with 5% CO₂. The staining solution was removed and replaced with 150 μ l pre-warmed reduced serum Opti-MEM media for imaging. Image stacks were analysed by an in-house written protocol using the IN Cell Developer software (GE Healthcare) (Diot et al., 2015). A series of target sets were created by the segmentation of cellular components. These target sets were then linked together in order to create a set of cells with all the labelled organelles in which various measurements were carried out on a cell-by-cell bases. The cell body, the nucleus and mtDNA were segmented in the green channel (PicoGreen). Mitochondria were segmented in the red channel (TMRM).

Supplemental Figure Legends

Supplemental Figure 1. MICOS complex subunits levels in primary C2/C10 DKO fibroblasts. (A)

Representative TEM images of C10 KO primary mouse fibroblasts. 3 independent C10 KO primary mouse fibroblast lines were evaluated in 1 technical/biological replicate each. Scale bars in image = 600 nm. (B) Graph of mitochondrial area of primary mouse fibroblasts with the indicated genotype. At least 13,922 mitochondria from at least 49 cells in three biological replicates were analyzed per genotype. (C - D) Representative immunoblot of MICOS subunits in primary fibroblasts (C) and quantification (D). Data are from 9 biological replicates obtained on at least 2 occasions.

Supplemental Figure 2. Increased bioenergetic stress does not account for OMA1 activation in the absence of C2 and C10. (A) Immunoblot of WT and C2/C10 DKO cells treated with DMSO or 10 μ M CCCP overnight. No cleavage of endogenous Mic19 is observed. Immunoblot is representative of at least 3 biological replicates performed on at least 2 occasions. (B) TMRM intensity measured by automated high content imaging under high and low glucose conditions. $N > 100$ mitochondria per condition in 1 technical/biological replicate. Lines from litter matched C2^{+/+}C10^{-/-}, C2^{+/-}C10^{-/-}, and C2^{-/-}C10^{-/-} mice are indicated. (C) Relative TMRE fluorescence in HEK293 WT and DKO cells measured by flow cytometry. $N = 3$ biological replicates performed on at least 2 occasions. “***” indicates p-value < 0.01 . Error bars represent standard error of the mean. (D and E) HEK293 cells were treated with 2 μ M chloramphenicol or not treated for 5 – 7 days. (D) Mitochondrial membrane potential was assessed by TMRE fluorescence measured by flow cytometry. $N = 3$ biological replicates performed on at least 2 occasions. “***” indicates p-value < 0.01 . Error bars represent standard error of the mean. (E) Cell lysates were evaluated for expression of the Complex IV subunit, COX2, and OMA1 by immunoblotting. Quantification represents $N = 9$ tested on at least two separate occasions.

Supplemental Figure 3. MICOS complex expression in HEK293 C2/C10 DKO cells. (A) Quantification of OPA1, C2, and C10 protein levels in HEK293 WT and C2/C10 DKO cells from experiment depicted in (Figure 2B). $N = 9$ biological replicates performed on at least 2 occasions. (B) Immunoblot of PGAM5 processing in HEK293 WT and C2/C10 DKO cells. Immunoblot is representative of 2 biological replicates performed on 1 occasion. (C) Quantification of MICOS complex subunit levels in HEK293 WT and C2/C10 DKO. $N = 9$ biological replicates performed on at least 2 occasions. (D) Heat map depicts relative abundance of SPY and PHB complex subunits in SILAC labelled WT and DKO HEK293 mitochondria, solubilized in 1% digitonin and separated by BN-PAGE prior to detection by quantitative mass spectrometry. Columns in the heat map represent gel slices at the indicated position. Rows indicate proteins detected. Data are representative of 2 biological replicates performed on 1 occasion.

Supplemental Figure 4. Interactions between C2/C10 and OPA1, OMA1, and SPY complexes. (A)

Immunoblot of unbound and bound fractions following immunoprecipitation with anti-Flag antibodies from HeLa cells stably expressing C2-His6 (as a control) or C2-Flag. (B, left) Immunoblot of unbound and bound fractions following immunoprecipitation with anti-Flag antibodies from HeLa^{PARL KO} cells transiently expressing PARL-Flag or left untransfected. (B, right) Immunoblot of unbound and bound fractions following immunoprecipitation with anti-Flag antibodies from HeLa cells stably expressing C2-His6 (as a control) or C2-Flag. Immunoblots are representative of at least 3 biological replicates performed on at least 2 occasions. (C) Lysates from HeLa^{WT}, HeLa^{OMA1 KO}, HEK293^{WT}, and HEK293^{C2/C10 DKO} were separated on SDS-PAGE gels and immunoblotted for YME1 substrates, PRELID and TIM23, which were quantified in (D). Immunoblots are representative of at least 3 biological replicates. “*” indicates p-value < 0.05, “****” indicates p-value < 0.001.

Supplemental Figure 5. (A) Representative Airyscan confocal images of WT and OMA1 KO HeLa cells transiently transfected with C10 WT-Flag (C10 WT) or C10-Flag with the N-terminal α -helix containing G58 deleted (C10 $\Delta\alpha$ H). Anti-Flag immunostaining in green and anti-Tom20 in red. (B) Quantification of mitochondrial morphology in (A) for > 150 cells in 3 biological replicates per condition. Scale bars in all images = 10 μ m. Except where indicated all confocal images were acquired as a z-stack and represented as a maximum projection image. (C) Airyscan confocal image of WT or OMA1 KO HeLa cells immunostained for C10 or Tom20. Scale bar = 10 μ m. Experiment was performed in 3 biological replicates on 2 occasions with similar results. (D) Airyscan confocal images of WT and OMA1 KO HeLa cells immunostained for CHCHD2 acquired at settings optimized for intense foci in OMA1 KO HeLa cells (low exposure) and WT HeLa cells (high exposure). Experiment was performed in 3 biological replicates on 2 occasions with similar results. Scale bar = 5 μ m. (E) Representative Airyscan confocal images of WT or OPA1 Δ s1 HeLa cells immunostained for CHCHD2 (green) and Tom20 (red). (F)

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Immunoblot of HeLa^{WT} and two HeLa^{OPA1 Δ s1} clones (#10 and #15) treated with DMSO or 10 μ M CCCP for 3 hrs. HSP90 served as a loading control. Experiment was performed in 3 biological replicates on 2 occasions with similar results. (G) Representative Airyscan confocal images of WT or OPA1 Δ s1 HeLa cells immunostained for CHCHD2 (green) and Tom20 (red). Experiment was performed in 3 biological replicates on 2 occasions with similar results. (H) Quantification of C2 and C10 protein levels in HeLa^{WT} and HeLa^{OMA1 KO} cells from immunoblots. N = 10 biological replicates.

Supplemental Figure 6. C2/C10 degraded following OMA1 activation by actinonin and basal degradation of C2/C10 is independent of YME1 and PARL. (A and B) Immunoblot of YME1 KO, PARL KO, and matched WT HeLa cells treated with DMSO or 10 μ M CCCP overnight (O/N) and then treated with 100 μ M cycloheximide (CHX) for the indicated number of hours. GAPDH or Tubulin served as controls where indicated. Data represent at least 3 independent biological replicates performed on at least 2 occasions.

Supplemental Figure 7. C10^{S59L/+} KI mice develop cardiomyopathy. (A) Echocardiographic measurements of anterior and posterior left ventricular wall widths in diastole (LVWA;d and LVWA;d) and systole (LVWA;s and LVWA;s) of WT (N = 13) and C10^{S59L/+} (N = 12) mice between the ages of 17 - 42 weeks. (B) Assessment of apoptosis TUNNEL staining of cardiac tissue. Tissue from younger and older C10^{S59L/+} KI and WT mice were combined due to limited samples for analysis. These groups were also shown separate to facilitate exploratory comparison with younger C10 KO and litter matched C2/C10 DKO animals. “*” indicates $p < 0.05$ after correcting for multiple comparisons. (C) Cytochrome *c* release was assessed in primary mouse fibroblasts with the indicated phenotype with or without exposure to 1 mM H₂O₂ for 4 hrs in the presence of the caspase inhibitor zVAD. No significance difference was present among the genotypes in the 4 hrs treated group. N = 3 biological replicates were performed on 2

occasions. (D) Confocal images of heart sections from a C10^{S59L/+} KI mouse, depicting lack of co-localization between the mitochondrial marker pyruvate dehydrogenase (PDH) and C10 aggregates.

Supplemental Figure 8. Expression of pre-specified genes associated with mitochondrial integrated stress response and previously reported to be differentially regulated in C10^{S59L/+} mouse hearts. Relative transcript levels pre-specified genes associated with mitochondrial integrated stress response in heart extracts of younger WT, C10^{S59L/+} KI, and C2/C10 DKO mice (9 - 13 weeks), and older WT and C10^{S59L/+} KI (22 -37 weeks) mice measured by microarray and normalized to average of younger WT mice.

Supplemental Figure 9. OPA1 processing from C10^{S59L/+} KI mice is similar in apex and base of the heart. (A and B) Immunoblot of lysates from apex (A) or base (B) of hearts of wildtype or C10^{S59L/+} knock-in mice. Tubulin served as a loading control. (B) quantification of immunoblot depicted in (A). At least 3 animals of each genotype were evaluated.

Supplemental Figure 10. OPA1 processing in heart, skeletal muscle, liver, brain, and primary fibroblasts of C10^{S59L/+} KI mice. (A) Immunoblot of lysates from the heart, skeletal muscle, or liver of wildtype or C10^{S59L/+} knock-in mice. GAPDH served as a loading control. Lysates from individual mice in each condition are indicated (m1 – m3). At least 3 animals of each genotype were evaluated. (B) Immunoblot of primary fibroblasts lysates from WT, C10 KO (C10 KO), C10^{S59L/+}, C10^{S59L/S59L}, C2 KO (C2 KO), and C2/C10 DKO (C2/C10 DKO) mice. Immunoblot is representative of least 3 biological replicates performed on at least 2 occasions. GAPDH served as a loading control.

Supplemental Figure 11. OPA1 processing in tissues from C2/C10 DKO mice. (A and B) Immunoblot of lysates from the heart and skeletal muscle (A) and brain and liver (B) of wildtype or C2/C10 single or double KO mice. HSP90 served as a loading control. Lysates from individual mice in each condition are indicated (m1 – m2).

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Table S1. LC-MS BN-PAGE complexomic profiling.

Table S2. Mutant C10 and C2/C10 DKO echocardiography.

Table S3. Mutant C10 and C2/C10 RNA expression studies.