

Mitochondrial dynamics regulate genome stability via control of caspase-dependent DNA damage

Kai Cao, Joel Riley, Rosalie Heilig, Alfredo Montes-Gómez, Esmee Vringer, Kevin Berthenet, Catherine Cloix, Yassmin Elmasry, David Spiller, Gabriel Ichim, et al.

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28 Summary

29 Mitochondrial dysfunction is interconnected with cancer. Nevertheless, how defective 30 mitochondria promote cancer is poorly understood. We find that mitochondrial dysfunction 31 promotes DNA damage under conditions of increased apoptotic priming. Underlying this 32 process, we reveal a key role for mitochondrial dynamics in the regulation of DNA damage 33 and genome instability. The ability of mitochondrial dynamics to regulate oncogenic DNA 34 damage centres upon the control of minority MOMP, a process that enables non-lethal 35 caspase activation leading to DNA damage. Mitochondrial fusion suppresses minority MOMP, 36 and its associated DNA damage, by enabling homogenous mitochondrial expression of anti-37 apoptotic BCL-2 proteins. Finally, we find that mitochondrial dysfunction inhibits pro-apoptotic 38 BAX retrotranslocation, causing BAX mitochondrial localization thereby promoting minority 39 MOMP. Unexpectedly, these data reveal oncogenic effects of mitochondrial dysfunction that 40 are mediated via mitochondrial dynamics and caspase-dependent DNA damage. 41

42 Introduction

Mitochondrial dysfunction has pleiotropic impact on cancer (Giampazolias and Tait,
2016). For instance, mitochondrial respiratory complex proteins and TCA enzymes bearing
tumour associated mutations, generate oncometabolites (Isaacs et al., 2005; Pollard et al.,
2007; Sciacovelli et al., 2016; Selak et al., 2005). Moreover, loss of function mutations in
mitochondrial DNA (mtDNA) are common in cancer and have been shown to accelerate
tumorigenesis (Gorelick et al., 2021; Smith et al., 2020). Nonetheless, how dysfunctional
mitochondria promote cancer largely remains an open question.

50

51 While inhibition of mitochondrial apoptosis has well established oncogenic effects, 52 through increased apoptotic priming, tumour cells are often sensitized to cell killing cancer 53 therapies (Certo et al., 2006; Singh et al., 2019). Mitochondria regulate apoptosis via 54 mitochondrial outer membrane permeabilization or MOMP (Bock and Tait, 2020). This key 55 event releases soluble mitochondrial intermembrane space proteins into the cytoplasm, 56 notably cytochrome c, that activate caspases proteases causing rapid cellular demise. 57 Because it dictates cell fate, mitochondrial outer membrane integrity is tightly regulated by 58 BCL-2 protein family members (Campbell and Tait, 2018).

59

60 MOMP is usually considered a lethal point-of-no-return due to its extensive nature, 61 often occurring in all mitochondria, coupled to an invariable loss of mitochondrial function 62 (Goldstein et al., 2000; Lartigue et al., 2009; Rehm et al., 2003). However, we have previously 63 described conditions whereby MOMP can be heterogenous permitting cell survival (Ichim et 64 al., 2015; Tait et al., 2010). Following a sub-lethal stress, a limited mitochondrial cohort selectively permeabilizes, which we termed minority MOMP (Ichim et al., 2015). Strikingly, 65 66 minority MOMP can engage sub-lethal caspase activity promoting DNA damage that is 67 dependent upon caspase-activated DNAse (CAD) (Ichim et al., 2015). By causing DNA 68 damage, minority MOMP may contribute to the paradoxical oncogenic effects of apoptotic 69 signaling reported in different studies (Ichim and Tait, 2016). Moreover, minority MOMP has been recently implicated in an expanding array of functions including increased cancer 70 71 aggressiveness, innate immunity and inflammation triggered by mtDNA double-strand breaks 72 (Berthenet et al., 2020; Brokatzky et al., 2019; Tigano et al., 2021).

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Here, we investigated the relationship between mitochondrial dysfunction and DNA damage. Surprisingly, we uncovered a key role for mitochondrial dynamics in the regulation of DNA damage. Mitochondrial fission, a consequence of mitochondrial dysfunction, promotes minority MOMP causing caspase-dependent DNA damage and genome instability. Secondly, we find reduced retrotranslocation of pro-apoptotic BAX on dysfunctional mitochondria, thus facilitating minority MOMP. These data reveal an unanticipated link between mitochondrial
 dysfunction and oncogenic DNA damage that is mediated through minority MOMP and
 caspase activity.

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84 Results

85 Mitochondrial dynamics regulate DNA damage

86 We aimed to understand how mitochondrial dysfunction can be oncogenic. Given the tumor 87 promoting roles of DNA damage, we initially investigated its interconnection with mitochondrial 88 function. To cause mitochondrial dysfunction, U2OS and HeLa cells were treated with the 89 uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). In order to phenocopy 90 increased apoptotic priming that is found in pre-malignant and tumour cells, we co-treated 91 cells with ABT-737, a BH3-mimetic compound that selectively neutralises anti-apoptotic BCL-92 2, BCL-xL and BCL-w. The response to DNA damage was measured by yH2AX staining and 93 flow cytometry. In both HeLa and U2OS cells, BH3-mimetic treatment led to an increase in 94 yH2AX positive cells that was significantly enhanced by combined treatment with CCCP, 95 consistent with mitochondrial dysfunction promoting DNA damage (Figure 1A). Given the low 96 level of yH2AX-positive cells observed by flow cytometry, we visualised the degree of DNA 97 damage in U2OS and HeLa cells treated with ABT-737 by indirect immunofluorescence and 98 western blot. In agreement with the flow cytometry data, we observed a low, but detectable, 99 level of yH2AX and pATM foci in cells treated with ABT-737 (Figure 1B, Supplementary 100 Figure 1A, B). Mitochondrial dynamics and function are tightly interconnected such that 101 mitochondrial dysfunction causes mitochondrial fission (Oltersdorf et al., 2005). We therefore 102 investigated whether mitochondrial dynamics affected DNA damage triggered by BH3-mimetic treatment. To disrupt mitochondrial fusion, we used *Mfn1/2^{-/-}* murine embryonic fibroblasts 103 (*Mfn1/2^{-/-}* MEF) and, as control, reconstituted these cells with MFN2 (*Mfn1/2^{-/-}* + Mfn2 MEF). 104 As expected, *Mfn1/2^{-/-}* MEF displayed a hyper-fragmented mitochondrial network whereas 105 MFN2 reconstitution of these cells ($Mfn1/2^{-1} + Mfn2$) restored mitochondrial fusion, resulting 106 107 in a filamentous mitochondrial network, but did not affect growth following treatment with ABT-108 737 (Figure 1C, D, Supplementary Figure 3A, B). Of note, although Mfn2 protein expression 109 is higher in *Mfn1/2-/-* MEF reconstituted with Mfn2 compared to Wt MEF, mitochondrial fusion was not enhanced in $Mfn1/2^{-1}$ + Mfn2 MEF and mitochondrial fragmentation occurred with the 110 111 same kinetics when exposed to CCCP, relative to Wt MEF (Supplementary Figure 1C, D). 112 Furthermore, expression of mitochondrial fusion proteins Mfn1 and Mfn2 and the 113 mitochondrial fission protein Drp1 were not changed following ABT-737 treatment 114 (Supplementary Figure 1E). *Mfn1/2^{-/-}* and *Mfn1/2^{-/-}* + Mfn2 MEF were treated with ABT-737 115 (10 µM, 3 hours) and the DNA damage response was assessed by analyzing vH2AX levels

by western blot or by flow cytometry (Figures 1E, F). Mfn1/2^{-/-} MEF exhibited increased 116 117 yH2AX, consistent with mitochondrial fission promoting DNA damage. Because DNA damage 118 can be oncogenic, we investigated if cells with extensive mitochondrial fission were more 119 prone to transformation. Mfn1/2-/- and Mfn1/2-/- + Mfn2 MEF were passaged repeatedly in ABT-120 737. Following treatment, cells were assayed for transformation in vitro by determining 121 anchorage-independent growth in soft agar. Specifically following culture in ABT-737, Mfn1/2 122 ^{/-} MEF formed colonies more readily than *Mfn1*^{-/-} + Mfn2 MEF (**Figure 1G, H**). MFN2 can have 123 functions independent of mitochondrial fusion, for instance mitochondrial-endoplasmic 124 reticulum (ER) tethering (de Brito and Scorrano, 2008). Therefore, we investigated whether 125 mitochondrial dynamics regulate DNA-damage through an alternative approach by inhibiting 126 mitochondrial fission. DRP1 plays a central role in mitochondrial fission (Ishihara et al., 2009; 127 Wakabayashi et al., 2009). To inhibit mitochondrial fission we used *Drp1^{t/fl}* MEF, which when 128 infected with adenoviral Cre efficiently delete Drp1, causing a hyper-fused mitochondrial 129 network (Figure 1I, Supplementary Figure 1F, 3A). Infection with adenoviral Cre did not 130 cause DNA damage, nor impact ABT-737-induced DNA damage or growth following ABT-737 treatment (Supplementary Figure 1G, 3B). Drp1^{##} and Drp1^{-/-} MEF were treated with ABT-131 132 737 and yH2AX was measured by flow cytometry, as before. MEF expressing Drp1 have 133 elevated levels of yH2AX after exposure to ABT-737, but this was completely abolished in 134 Drp1-deficient cells (Figure 1J). These data suggest that mitochondrial dysfunction and 135 fission promote oncogenic DNA damage and transformation.

136

Mitochondrial dynamics regulate DNA damage and genome-instability in a caspase and CAD dependent manner

139 We next sought to understand how mitochondrial dynamics regulate DNA damage. 140 Because we had found that pro-apoptotic BH3-mimetic treatment potentiated DNA damage, 141 we investigated a role for apoptotic caspase function. Wild type MEF or MEF overexpressing 142 DRP1 were treated with the pan-caspase inhibitor qVD-OPh and γ H2AX was measured by 143 flow cytometry and western blot. MEF cells overexpressing DRP1 displayed a more 144 fragmented mitochondrial network and had higher levels of γ H2AX compared to their empty 145 vector counterparts, consistent with our earlier data, but showed similar proliferation rates 146 following treatment with ABT-737 (Figure 2A, B, Supplementary Figure 2A, B, 3A, B). 147 Crucially, yH2AX was prevented by treatment with the pan-caspase inhibitor qVD-OPh, 148 demonstrating a key role for caspase activation in DNA damage (Figure 2B). We next set out 149 to establish whether loss of Drp1 impacted oncogenic transformation following repeated 150 culture in ABT-737; however, loss of Drp1 alone was sufficient to render cells resistant to 151 Myc/Hras-induced transformation (Supplementary Figure 2C), consistent with the results of 152 Serasinghe and colleagues (Serasinghe et al., 2015). Given these findings, we investigated a 153 possible correlation between expression of the mitochondrial fission protein DRP1 and 154 mutational burden in cancer. TCGA PanCancer Atlas studies were investigated through 155 cBioportal. Of these, a significant association between increased mutational count 156 in DNM1L mRNA high quartile versus DNM1L mRNA low quartile was found in invasive 157 breast carcinoma and lung adenocarcinoma (out of 22 studies) with the inverse relationship 158 not observed in any cancer type (Figures 2C, 2D, Supplemental Table 1 and data not 159 shown). In both invasive breast cancer and lung adenocarcinoma, DNA damage response 160 pathways were enriched in the DNM1L mRNA high quartile consistent with engagement of 161 DNA damage (Supplementary Figures 2D, E, F). Moreover, high DNM1L mRNA expression 162 correlates with poorer survival in a cohort of lung adenocarcinoma (Supplementary Figure 163 2G). To further investigate the role of caspase activity, we investigated the impact of 164 mitochondrial dynamics upon genome instability. To this end, we used the PALA assay, in 165 which gene amplification of CAD (carbamyl phosphate synthetase/aspartate 166 transcarbamylase/dihydro-orotase, note that this is distinct from caspase-activated Dnase 167 described later) enables resistance to PALA (N-phosphonoacetyl-L-aspartate) (Wahl et al., 168 1979). To determine if alterations in mitochondrial dynamics, also affect genome instability dependent upon caspase activity, we passaged *Mfn1/2^{-/-}* and *Mfn1/2^{-/-}* + Mfn2 MEF with sub-169 170 lethal doses of ABT-737 in the presence or absence of QVD-OPh. Following treatment, cells 171 were grown in the presence of PALA and clonogenic survival was measured (Figure 2E). Importantly, ABT-737 treated *Mfn1/2^{-/-}* MEF gave significantly more colonies than *Mfn1/2^{-/-}* + 172 173 Mfn2 following PALA treatment, in a caspase-dependent manner (Figure 2E, F). In line with 174 increased survival following PALA treatment, gPCR revealed amplification of the Cad locus 175 only in *Mfn1/2^{-/-}* MEF repeatedly treated with ABT-737 (Figure 2G). We and others have 176 previously found that non-lethal caspase activity can cause DNA damage and genome 177 instability dependent upon caspase-activated DNAse (CAD) (Ichim et al., 2015; Lovric and 178 Hawkins, 2010). To examine the role of CAD in genomic instability we used the *Mfn1/2^{-/-}* and 179 *Mfn1/2^{-/-}* + Mfn2 MEF in which we deleted the *Dff40* gene (encoding CAD) using CRISPR-180 Cas9 genome editing (Supplementary Figure 3C). As before, *Mfn1/2^{-/-}* cells resisted PALA 181 treatment and efficiently grew as colonies following ABT-737 treatment, whereas Mfn1/2-/- + 182 Mfn2 cells did not (Figure 2H, I). However, deletion of CAD completely abrogated clonogenic 183 potential. Cad DNA expression and anchorage-independent growth were also diminished 184 following ABT-737 treatment in CAD/Dff40 deleted cells as compared to their controls (Figure 185 2J, K, Supplementary Figure 3D). Together, these data show that mitochondrial fission 186 promotes genome instability in a caspase and CAD dependent manner.

187

188 Minority MOMP occurs on fragmented mitochondria and is regulated by mitochondrial

189 dynamics

190 We have previously found that permeabilization of a small number mitochondria in a 191 cell – called minority MOMP – can engage non-lethal caspase activity causing CAD activation 192 and DNA damage (Ichim et al., 2015). This knowledge, coupled to our previous data, led us 193 to investigate a role for mitochondrial dynamics in the regulation of minority MOMP. To 194 address this, we combined super-resolution Airyscan confocal microscopy together with our 195 fluorescent reporter that allows detection of minority MOMP (Ichim et al., 2015). This reporter 196 comprises cytosolic FKBP-GFP (cytoGFP) and mitochondrial inner membrane targeted FRB-197 mCherry (mito-mCherry). Upon loss of mitochondrial outer membrane integrity, and in the 198 presence of chemical heterodimeriser (AP21967), these two proteins bind one another, 199 recruiting cytoGFP to the permeabilized mitochondria (Figure 3A). HeLa or U2OS were 200 treated with a non-lethal dose of BH3-mimetic ABT-737 (10 µM) for 3 hours. Consistent with 201 our previous data, this treatment was sufficient to engage minority MOMP, as evidenced by 202 localization of cytoGFP to specific mitochondria (Figure 3B). Super-resolution analysis of 203 these mitochondria revealed that selectively permeabilised mitochondria were separate from 204 the mitochondria network, suggesting that minority MOMP preferentially occurs on fragmented 205 mitochondria (Figure 3B, C). Extensive mitochondrial fission is a well-established 206 consequence of MOMP (Bhola et al., 2009; Frank et al., 2001). Therefore, to approach 207 whether mitochondria fragmentation was a cause or consequence of minority MOMP, U2OS 208 cells expressing cytoGFP and mito-mCherry were imaged by live-cell microscopy. Treatment 209 with ABT-737 (10 µM) led to minority MOMP, apparent by the translocation of cytoGFP into 210 mitochondria after 124 minutes. Importantly, these mitochondria were fragmented from the 211 mitochondrial network prior to cytoGFP translocation at 120 minutes (Figure 3D, Movie 1). 212 This suggests that minority MOMP preferentially occurs on fragmented mitochondria. We next 213 used these cells to investigate a role for mitochondrial fusion in regulating minority MOMP. Mfn1/2^{-/-} and Mfn1/2^{-/-} + Mfn2 MEF expressing the MOMP reporter, were treated with a sub-214 215 lethal dose of ABT-737. Strikingly, increased levels of minority MOMP were observed in 216 $Mfn1/2^{-/-}$ MEF when compared to $Mfn1/2^{-/-}$ + Mfn2 MEF (Figure 3E, Supplementary Figure 217 **4A**). This is consistent with minority MOMP occurring primarily on fragmented mitochondria, 218 with mitochondrial fusion having an inhibitory effect. To further address this, we investigated 219 the impact of inhibiting mitochondrial fission upon minority MOMP following treatment of 220 *Drp1*^{t/fl} and *Drp1*^{-/-} MEF with ABT-737. MEF expressing Drp1 undergo minority MOMP after 221 exposure to ABT-737, but this was completely abolished in Drp1-deleted cells (Figure 3F, 222 **Supplementary Figure 4B**). Together, these data demonstrate that mitochondrial dynamics 223 regulate minority MOMP; mitochondrial fusion appears to be inhibitory whereas fission 224 promotes minority MOMP.

225

226 **Pro-survival BCL-2 proteins display inter-mitochondrial heterogeneity in localisation**

227 Our data demonstrate that mitochondrial fission promotes minority MOMP enabling 228 caspase-dependent DNA damage. Nevertheless, how mitochondrial dynamics regulate 229 minority MOMP is not known. Mitochondrial outer membrane integrity is regulated by the 230 balance of pro- and anti-apoptotic BCL-2 family proteins (Campbell and Tait, 2018). We 231 hypothesised that inter-mitochondrial variation in BCL-2 family localisation may underlie 232 minority MOMP. To investigate this hypothesis, we set out to visualise endogenous levels of 233 BCL-2 family proteins on individual mitochondria. CRISPR-Cas9 genome editing can be used 234 to knock-in fluorescent proteins at defined genomic loci to enable endogenous tagging of 235 proteins (Bukhari and Muller, 2019). Using this approach, we generated clonal knock-in HeLa 236 cell lines where the red fluorescent protein Scarlet was fused to the N-termini of BCL-2, BCL-237 xL and MCL-1. As verification, western blotting using antibodies specific BCL-2, BCL-xL, MCL-238 1 and Scarlet confirmed that these cell lines expressed these fusion proteins at similar levels 239 to their endogenous counterparts (Figure 4A, B). Secondly, Airyscan super-resolution 240 microscopy demonstrated mitochondrial localisation of Scarlet-BCL-2, BCL-xL and MCL-1, as 241 expected (Figure 4C) and is comparable to mitochondrial localisation of untagged 242 endogenous BCL-2 proteins in parental cells (Supplementary Figure 5A, B). Finally, we 243 monitored cell viability using SYTOX Green exclusion and IncuCyte real-time imaging in 244 response to BH3-mimetic treatment (ABT-737 and S63845). This demonstrated that all knock-245 in cell lines underwent cell death in response to BH3-mimetic treatment (Supplementary 246 Figure 5C). Using these knock-in cells, we next acquired super-resolution microscopy images of Scarlet-tagged BCL-2, BCL-xL and MCL-1 then applied a colour grading lookup table (LUT) 247 248 such that the brighter the Scarlet signal, the more bright the image. This revealed 249 heterogeneity of Scarlet BCL-2, BCL-xL and MCL-1 across the mitochondrial network, which 250 is also evident in parental cells stained with BCL-2 family antibodies (Figure 4D, 251 **Supplementary Figure 5A**). Given our previous data, we hypothesised that BCL-2 family 252 protein heterogeneity is regulated by mitochondrial dynamics. To test this, we inhibited 253 mitochondrial fission through CRISPR-Cas9 deletion of DRP1. Western blot confirmed DRP1 254 deletion, resulting in extensive mitochondrial hyperfusion (Supplementary Figure 5D, E). 255 Strikingly, cells with hyperfused mitochondria displayed much reduced inter-mitochondrial heterogeneity of MCL-1, BCL-2 or BCL-xL (Figure 4E, F, Supplementary Figure 5F). 256 257 Combined, these data show that within a cell extensive inter-mitochondrial heterogeneity in 258 BCL-2 localisation exists that is impacted by mitochondrial dynamics.

259

260 Heterogeneity in apoptotic priming underpins minority MOMP

261 We next investigated whether there was a relationship between localisation of anti-262 apoptotic BCL-2 proteins and minority MOMP. To determine this, we acquired super-263 resolution images of HeLa cells expressing endogenous Scarlet-BCL-2, BCL-xL or MCL-1, 264 together with Omi-GFP and MitoTracker Deep Red. During MOMP, soluble intermembrane 265 space proteins, including Omi, are released from mitochondria (Bock and Tait, 2020). 266 Mitochondria retain MitoTracker Deep Red even after loss of mitochondrial integrity; thus, 267 mitochondria that have undergone MOMP are identifiable by loss of Omi and MitoTracker 268 retention. Surprisingly, live-cell imaging of BCL-2 family protein knock-in cells following 269 treatment with ABT-737 revealed that mitochondria (determined by MitoTracker positivity) that 270 release Omi-GFP have higher levels of BCL-2, BCL-xL or MCL-1 local mitochondrial levels 271 prior to MOMP (Figure 5A-C). Computational segmentation allowed us to distinguish BCL-2 272 family protein mitochondrial localisation, on MitoTracker positive structures, which lack Omi 273 expression, confirming that these mitochondria have indeed undergone minority MOMP. 274 Quantification across a number of cells shows that mitochondria which undergo minority 275 MOMP have increased BCL-2 family protein mitochondrial localisation (Figure 5D-F). 276 Furthermore, line scans revealed regions of the mitochondrial network with high BCL-2 family 277 residency, but low Omi expression, that is mitochondria which have likely undergone minority 278 MOMP (Supplementary Figure 6A, B). Unexpectedly, these data reveal a correlation 279 between increased anti-apoptotic BCL-2 mitochondrial localisation and selective 280 mitochondrial permeabilisation. We reasoned that this may be analogous to increased 281 apoptotic priming at the cellular level, where high anti-apoptotic BCL-2 mitochondrial 282 localisation can correlate with apoptotic sensitivity in some cell types. Mitochondrial 283 association of pro-apoptotic BAX is indicative of increasing apoptotic priming (Edlich et al., 284 2011; Reichenbach et al., 2017; Schellenberg et al., 2013). To investigate whether 285 mitochondria with high BCL-2 local mitochondrial localisation may also display high BAX 286 localisation (indicative of selective, increased apoptotic priming), we generated GFP-BAX 287 expressing BCL-2 family knock-in HeLa cells and imaged them by super-resolution 288 microscopy. In line with the notion that mitochondria with higher BCL-2 family localisation also 289 have elevated BAX localisation, we observed BAX co-localising with high BCL-2 mitochondria, 290 indicative of increased apoptotic priming (Figure 5G-I). These data demonstrate that inter-291 mitochondrial heterogeneity in anti-apoptotic BCL-2 mitochondrial localisation and apoptotic 292 priming underlies minority MOMP.

293

Mitochondrial dysfunction promotes BAX accumulation promoting minority MOMP
 We aimed to define the underlying mechanism of mitochondrial intrinsic apoptotic
 priming. In healthy cells, BAX undergoes mitochondrial retrotranslocation and inhibiting this
 process causes BAX mitochondrial accumulation, sensitising to MOMP (Edlich et al., 2011;

298 Schellenberg et al., 2013). HeLa cells expressing GFP-BAX and iRFP-Omp25 were treated with the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) to induce 299 300 mitochondrial dysfunction. To facilitate visualisation of mitochondrial localised GFP-BAX, cells 301 were treated with digitonin to selectively permeabilise the plasma membrane, as described 302 previously (Bender et al., 2012). Inducing mitochondrial dysfunction by CCCP treatment led 303 to robust mitochondrial recruitment of GFP-BAX (Figure 6A, Supplemental Figure 7A, 304 Movies 2-5). Immunostaining of HeLa cells with the activation specific BAX antibody 6A7 305 revealed BAX activation, as expected, under conditions of apoptosis (combined BH3-mimetic treatment) but not following CCCP treatment (Supplementary Figure 7B). Since 306 307 mitochondrial fusion promotes efficient oxidative phosphorylation, reducing heterogeneity in 308 mitochondrial function (Chen et al., 2003), we hypothesised that by impacting mitochondrial 309 function, mitochondrial fission may promote BAX recruitment thereby facilitating minority MOMP. We imaged $Mfn1/2^{-/-}$ and $Mfn1/2^{-/-}$ + Mfn2 MEF with MitoTracker Red, a potentiometric 310 dye, that reports mitochondrial $\Delta \psi^m$ as a measure of mitochondrial function. Consistent with 311 312 defective mitochondrial function, mitochondria in fusion defective cells ($Mfn1/2^{-1}$) displayed heterogenous MitoTracker Red signal and lower total signal than fusion competent Mfn1/2^{-/-} + 313 314 Mfn2 MEF (Figures 6B-D). We next analysed GFP-BAX localisation in Mfn1/2^{-/-} and Mfn1/2^{-/-} 315 + Mfn2 MEF, using fluorescence loss in photobleaching (FLIP) to help visualise mitochondrial localised GFP-BAX. FLIP analysis of GFP-BAX revealed slower mitochondrial 316 317 retrotranslocation in *Mfn1/2^{-/-}* cells compared to *Mfn1/2^{-/-}* + Mfn2 cells (Figure 6E-H, Movies 318 6, 7). This suggests that mitochondrial dysfunction, a consequence of defective mitochondrial 319 dynamics, can promote GFP-BAX mitochondrial accumulation, serving as an mitochondrial 320 intrinsic priming mechanism that facilitates minority MOMP.

321

322

323 Discussion

324 We describe that mitochondrial dysfunction, inducing mitochondrial fission, promotes 325 DNA damage and genome instability. This process requires caspase activity, that is engaged 326 by minority MOMP, in order to trigger DNA damage. Investigating the underlying mechanism, 327 we find that mitochondrial dynamics affect inter-mitochondrial heterogeneity of anti-apoptotic 328 BCL-2, permitting increased apoptotic priming of fragmented mitochondria. Mitochondrial 329 dysfunction acts as a mitochondrial intrinsic priming signal by inhibiting pro-apoptotic BAX 330 retrotranslocation, promoting minority MOMP. Unexpectedly, by affecting mitochondrial BCL-331 2 heterogeneity and apoptotic priming, our data reveal crucial roles for mitochondrial 332 dysfunction and dynamics in the regulation of minority MOMP leading to caspase dependent 333 DNA damage and genome instability.

334

335 Our study highlights that mitochondrial dynamics are integral to minority MOMP, 336 whereby mitochondrial fusion inhibits, and fission promotes this process. Consistent with this 337 finding, the ability of sub-lethal apoptotic stress to engage oncogenic caspase-dependent DNA 338 damage and genome instability was regulated in a similar manner. Moreover, we found in 339 some cancer types, a correlation between the expression of the mitochondrial fission protein 340 DRP1, DNA damage and mutational burden. These data support an oncogenic role for 341 mitochondrial fission, through its capacity to promote minority MOMP and associated sub-342 lethal caspase activity. This also suggests that the multitude of cellular signalling pathways 343 and stresses that impact mitochondrial dynamics, for instance as hypoxia or high glycolytic 344 rates, might facilitate minority MOMP induced transformation (Chen and Chan, 2017; Wu et 345 al., 2016). Indeed, we found that enforced mitochondrial fission (through MFN1/2 deletion), 346 promoted minority MOMP induced transformation. Supporting previous findings, we also 347 report that DRP1 contributes to oncogene induced transformation - potentially, minority 348 contributory role Serasinghe et al. Our study underscores extensive interplay exists between 349 mitochondrial dynamics and cancer (Chen and Chan, 2017; Gao et al., 2017; Kashatus et al., 350 2015; Serasinghe et al., 2015; Zhao et al., 2013).

351

We sought to define how mitochondrial dynamics might control minority MOMP. 352 353 Surprisingly, we found inter-mitochondrial heterogeneity in anti-apoptotic BCL-2 localisation. 354 This heterogeneity was supressed by mitochondrial fusion, most likely because mitochondrial 355 fusion enables homogenous distribution of BCL-2 proteins across the mitochondrial network. 356 As we further discuss, heterogeneity in anti-apoptotic BCL-2 localisation enables differences 357 in apoptotic priming of specific mitochondria. Interestingly, during cell death, mitochondrial 358 variation in pro-apoptotic BAK levels have previously been found to influence the kinetics of 359 MOMP (Weaver et al., 2014). Though myriad interconnections between mitochondrial 360 dynamics and apoptosis exist, mitochondrial fission is largely considered a consequence of cell death. For instance, during apoptosis, extensive mitochondrial fragmentation occurs 361 362 subsequent to MOMP (Bhola et al., 2009). By promoting homogenous BCL-2 localisation 363 across the mitochondrial network, our data reveal an indirect role for mitochondrial fusion in 364 preventing minority MOMP.

365

We have previously found that ectopic expression of BCL-2 can lead to incomplete MOMP, consistent with BCL-2 anti-apoptotic function (Tait et al., 2010). In the current study, we find that increased local mitochondrial levels of anti-apoptotic BCL-2 family proteins correlates with selective mitochondrial permeabilisation. While this may seem initially counterintuitive, precedence for increased apoptotic priming, correlating with high anti-apoptotic BCL-2 levels is evident in various cancers (Certo et al., 2006; Singh et al., 2019). This is perhaps

372 best demonstrated in high-BCL-2 expressing chronic lymphocytic leukaemia (CLL) that is 373 often highly sensitive to the BCL-2 selective BH3-mimetic, venetoclax (Roberts et al., 2016). 374 In healthy cells, BAX mitochondrial localisation is indicative of increased apoptotic priming 375 (Edlich et al., 2011; Kuwana et al., 2020; Reichenbach et al., 2017; Schellenberg et al., 2013). 376 Indeed, further investigation revealed that high pro-apoptotic BAX localisation correlated with 377 high-BCL-2 localisation on mitochondria. Our data argue that heterogeneity in apoptotic 378 priming exists not only between cell types, but also intracellularly, at the level of individual 379 mitochondria.

380

381 Finally, we sought to understand how inter-mitochondrial heterogeneity in apoptotic 382 priming might occur. Pro-apoptotic BAX is subject to constant mitochondrial retrotranslocation; 383 inhibition of BAX retrotranslocation leads to mitochondrial accumulation, sensitising to 384 apoptosis (Edlich et al., 2011; Schellenberg et al., 2013). We find that reduction of 385 mitochondrial inner membrane potential ($\Delta \psi_m$) promotes BAX mitochondrial localisation. 386 Importantly, reduction of $\Delta \psi_m$, provides a mitochondrial-intrinsic signal to increase apoptotic 387 priming. Under conditions of mitochondrial dysfunction imposed by loss of mitochondrial 388 fusion, we find decreased rates of BAX retro translocation, enabling its mitochondrial 389 accumulation. In essence, BAX retrotranslocation may serve as a barometer of cellular 390 metabolic health. Because loss of mitochondrial function causes mitochondrial fission, it 391 promotes minority MOMP in a two-fold manner, segregating dysfunctional mitochondria and 392 promoting BAX accumulation (Twig et al., 2008). Further investigation will be required to 393 mechanistically delineate how mitochondrial function regulates BAX retrotranslocation. We 394 consider it likely that additional mechanisms of mitochondrial-intrinsic priming also exist, for 395 instance levels of anti-apoptotic BCL-2 mitochondrial localisation will dictate a given cell's 396 propensity to engage minority MOMP. Moreover, degradation of dysfunctional mitochondria 397 through mitophagy, may also impact the occurrence of minority MOMP. Indeed, others have 398 reported that permeabilised mitochondria are targeted for mitophagy - this likely affects the 399 steady-state detection of minority MOMP (Lindqvist et al., 2018).

400

401 Our study investigates the impact of mitochondrial dynamics and dysfunction upon 402 BH3-mimetic induced minority MOMP - when might this occur physiologically? One possibility 403 is during intracellular bacterial infection (*Salmonella* Typhimurium and *Chlamydia trachomatis*) 404 where minority MOMP has been shown to cause inflammation as an innate immune response 405 (Brokatzky et al., 2019). Interestingly, while *C. trachomatis* actively promotes mitochondrial 406 fusion during early infection, late-stage infection is associated with mitochondrial 407 fragmentation, providing an ideal setting to engage minority MOMP induced inflammation

12

408 (Kurihara et al., 2019; Liang et al., 2018). More recently, mitochondrial DNA (mtDNA) damage 409 has been shown to promote minority MOMP, enabling mitochondrial-nuclear retrograde 410 signalling (Tigano et al., 2021). In this setting, mitochondrial fragmentation most likely enables 411 some mitochondria to selectively permeabilise in response to mtDNA-damage. A final 412 example may relate to the minority MOMP that we have previously observed under 413 homeostatic conditions (Ichim et al., 2015). Indeed, others have found basal levels of 414 caspase/CAD-dependent DNA-damage in cycling cells (Liu et al., 2017). Given extensive 415 mitochondrial fission occurs during mitosis, this may serve to underpin basal levels of minority 416 MOMP and caspase-dependent DNA-damage.

417

In summary, our findings that reveal that mitochondrial dynamics regulate DNA damage and genome instability via minority MOMP induced caspase-activity. This provides a mechanism linking mitochondrial dysfunction to pro-oncogenic DNA damage. Beyond protumourigenic effects, minority MOMP has also been shown to have roles in innate immunity and inflammation, as such, our findings suggest new approaches to modulate minority MOMP and its downstream functions.

424

425 Methods

426

427 Cell Lines

HeLa and U2OS cells were purchased from ATCC (LGC Standards). 293FT cells werepurchased from Thermo Fisher Scientific.

430

Mfn1/2^{-/-} MEF were provided by David Chan, Caltech and reconstituted with LZRS-MFN2 in
our laboratory. *Drp1^{fl/fl}* MEF were provided by Hiromi Sesaki, Johns Hopkins University School
of Medicine. MEF *Wt* and MEF loxP-STOP-loxP EYFP were a kind gift from Douglas Green,
St Jude's Research Children's Hospital. All cell lines were cultured in DMEM high-glucose
medium supplemented with 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, penicillin
(10,000 units/ml) and streptomycin (10,000 units/ml).

437

438 To delete Drp1 from $Drp1^{fl/fl}$ MEF, 2 x 10⁶ cells were seeded and infected with 200 MOI 439 Ad5CMVCre (Viral Vector Core, University of Iowa) for 8 h, after which the media was 440 replaced. Cells were used for experiments from the following day.

441

442 METHOD DETAILS

443

444 Generation of Scarlet-BCL-2 knock-in cell lines

- We used a modified version of the knock-in strategy described in (Stewart-Ornstein and Lahav, 2016). Two vectors were used: the first vector comprises 500bp homology arm before and after the start codon of BCL-2, in between which is the Scarlet coding sequence, cloned into pUC-SP. The second vector, pSpCas9(BB)-2A-Puro (Addgene #48139) comprises Cas9 and the sgRNA targeting sequence. The following sgRNA sequences were used
- 450 Human BCL-2 5'- ATGGCGCACGCTGGGAGAAC -3'
- 451 Human BCL-xL 5- AAAAATGTCTCAGAGCAACC -3'
- 452 Human MCL-1 5'- CGGCGGCGACTGGCAATGTT -3'
- 453

To generate the knock-in cells, HeLa cells were transfected with 1 μ g of homology arm vector and 1 μ g of pSpCas9(BB)-2A-Puro with Lipofectamine 2000, according to the manufacturer's instructions. Media was removed 5 h later, and replaced with media containing 1 μ M SCR7 for 2 days. Cells were selected with 1 μ g/mL puromycin for a further two days before selecting Scarlet positive clones by FACS. Cells which expressed Scarlet signal which co-localised with mitochondria we used for further experiments.

460

461 Generation of stable overexpressing cell lines

462 For retroviral transduction, 293FT cells were transfected with 5 µg of plasmid, together with 463 1.2 µg gag/pol (Addgene #14887) and 2.4 µg VSVG (Addgene #8454) using Lipofectamine 464 2000. Media was changed after 6 hours and collected, filtered and used to infect cells 24 and 465 48 h post-transfection in the presence of 1 µg/ml Polybrene. 24 h following infection, cells 466 were allowed to recover in fresh medium and incubated with selection antibiotic 24 h after. 467 Cells were selected with appropriate antibiotic or FACS sorted to isolate a high-expressing 468 population. Concentrations used for antibiotic selection were 200 µg/ml zeocin (Invivogen) or 469 1 μg/ml puromycin (Sigma).

470

471 For lentiviral transduction, the procedure was the same as for retroviral transduction, except

- 472 5 μ g plasmid was transfected into 293FT along with 1.86 μ g psPAX2 (Addgene #12260) and
- 473 1 μ g VSVG (Addgene #8454) using Lipofectamine 2000.
- 474

475 Generation of CRISPR knock-out cell lines

476 Human Drp1 and mouse Dff40 knock-out cell lines were generated by CRISPR-Cas9 gene

- 477 deletion, using the lentiviral transduction protocol above. The following sequences were478 cloned into LentiCRISPRv2-puro (Addgene #52961)
- 479 Human Drp1: 5'- AAATCAGAGAGCTCATTCTT 3'
- 480 Mouse Dff40: 5'- ACATGGAGCCAAGGACTCGC -3'
- 481

482 Plasmids

LZRS-Drp1 was generated by cloning the Drp1 coding sequence from pcDNA3.1(+) Drp1 (Addgene #34706) into LZRS backbone using Gibson Assembly. pBABE iRFP-Omp25 was cloned by Gibson Assembly using fragments derived from pLJM2 SNAP-Omp25 (Addgene #69599) and pMito-iRFP670 (Addgene #45462). Omi-GFP (in eGFPN2) was a kind gift from

- 487 Douglas Green, St Jude's Children's Research Hospital.
- 488

489 Western Blotting

490 Cells were collected and lysed in NP-40 lysis buffer (1% NP-40, 1 mM EDTA, 150 mM NaCl, 491 50 mM Tris-Cl pH 7.4) supplemented with complete protease inhibitor (Roche). Protein 492 concentration of cleared lysates was determined by Bradford assay (Bio-Rad). Equal amounts 493 of protein lysates were subjected to electrophoresis through 10 or 12% SDS-PAGE gels and 494 transferred onto nitrocellulose membranes, which were blocked for 1 h in 5% milk/PBS-Tween 495 at room temperature. Membranes were incubated with primary antibody overnight at 4°C 496 overnight. After washing, membranes were incubated with either goat-anti-rabbit Alexa Fluor 497 800, goat-anti-mouse Alexa Fluor 680 or goat-anti-rat DyLight 800 for 1 h at room temperature 498 before detection using a Li-Cor Odyssey CLx (Li-Cor).

499

500 Flow Cytometry

501 For measuring levels of yH2AX, cells were trypsinised and washed once with PBS and fixed 502 in 4% PFA for 15 minutes at room temperature. After washing once in PBS, cells were 503 resuspended in 300 µL and 700 µL cold ethanol added dropwise while slowly vortexing. 504 Samples were frozen at -20°C overnight. The following day, samples were washed with PBS 505 and blocked in 2% BSA in PBS for 1 h at room temperature and incubated with anti-yH2AX 506 antibody conjugated to Alexa Fluor 647 (Biolegend) for 30 minutes protected from light. 507 Samples were analysed on the BD LSRFortessa flow cytometer (BD Biosciences) using 508 standard protocols.

509

510 To measure mitochondrial potential in $Mfn1/2^{-1}$ and $Mfn1/2^{-1}$ + Mfn2 MEF, cells were incubated

- 511 with 50nM MitoTracker CMXRos (Thermo Fisher Scientific) for 15 mins before collection. Cells
- 512 were analysed on a Attune NxT flow cytometer (Thermo Fisher Scientific) using standard
- 513 protocols, and analysed in FlowJo (BD).
- 514

515 PALA Assay and Cad Genomic Amplification

516 Cells were seeded in triplicate in 6 well plates at a density of 2500 cells per well and cultured

517 in nucleoside-free a-MEM medium supplemented with 10% dialysed FBS. PALA was added

- at the LD_{50} dose and cells maintained until visible colonies formed. Colonies were fixed and
- 519 stained in methylene blue (1% methylene blue in 50:50 methanol:water).
- 520
- 521 To assay Cad genomic amplification, DNA was extracted from PALA resistant colonies, or, in
- 522 the case of control treated cells where no colonies were viable, DNA was extracted from cells
- 523 passaged twenty times in DMSO, but not subjected to PALA treatment.
- 524

525 Anchorage-independent growth assay

A 1% base low melting temperature agarose solution (Sigma-Aldrich) was added to 6 well plates and allowed to set. 7,500 cells were suspended in 0.6% agarose in a 1:1: ratio to achieve a final concentration of 0.3% agarose., which was added on top of base agarose. When set, the cell/agarose mix was overlaid with complete DMEM media and colonies counted 14 days later from 15 fields of view per cell line.

531

532 **qPCR**

533 Genomic DNA was isolated from cells using the GeneJET DNA Extraction Kit (Thermo Fisher 534 Scientific). PCR was performed on a Bio-Rad C1000 Thermal Cycler using the following 535 conditions: 3 min at 95°C, 40 cycles of 20 s at 95°C, 30 s at 57°C, 30 s at 72°C and a final 5 536 min at 72°C using Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent 537 Technologies). Relative DNA quantification was analysed by the $2^{-\Delta\Delta Ct}$ method. Primer 538 sequences used are as follows:

- 539 Mouse CAD-F AAGCTCAGATCCTAGTGCTAACG
- 540 Mouse CAD-R CCGTAGTTGCCGATGAGAGG
- 541 Mouse 18S-F ATGGTAGTCGCCGTGCCTAC
- 542 Mouse 18S-R CCGGAATCGAACCCTGATT
- 543
- 544 Microscopy

545 *Fixed cell imaging*

546 Cells were grown on coverslips and fixed in 4% PFA/PBS for 10 min, followed by 547 permeabilization in 0.2% Triton-X-100/PBS for 15 min. Cells were blocked for 1 h in 2% 548 BSA/PBS and incubated with primary antibodies overnight at 4°C in a humidified chamber. 549 The following day, cells were washed in PBS and secondary antibodies added for 1 h at room 550 temperature, before final wash steps and mounting in Vectashield antifade mounting media.

551

552 MOMP assay

553 Cells were transfected with 250ng CytoGFP and 250ng mito-mCherry for 16 h with either 554 Lipofectamine 2000 or GeneJuice before treatment in combination with 50 nM A/C heterodimizer (Clontech). Minority MOMP was scored based on co-localisation of CytoGFP
with mito-mCherry. A minimum of 100 cells were analysed per condition, and we defined
minority MOMP as a cell which has 1 or more instances of cytoGFP/mito-mCherry colocalisation.

559

560 Airyscan super-resolution imaging

561 Super-resolution Airyscan images were acquired on a Zeiss LSM 880 with Airyscan 562 microscope (Carl Zeiss). Data were collected using a 63 x 1.4 NA objective for the majority of 563 experiments, although some were acquired using a 40 x 1.3 NA objective. 405nm, 561nm and 564 640 nm laser lines were used, in addition to a multi-line argon laser (488nm) and images 565 acquired sequentially using the optimal resolution determined by the Zeiss ZEN software. 566 When acquiring z-stacks, the software-recommend slice size was used. Live-cell experiments 567 were performed in an environmental chamber at 37°C and 5% CO₂. Airyscan processing was 568 performed using the Airyscan processing function in the ZEN software, and to maintain clarity 569 some images have been pseudocloured and brightness and contrast altered in FIJI (ImageJ 570 v2.0.0).

571

572 Nikon A1R imaging

573 Confocal images were acquired on a Nikon A1R microscope (Nikon). Data were collected 574 using a 60 x Plan Apo VC Oil DIC N2 objective. 405nm, 561nm, 638nm laser lines were used, 575 in addition to a multi-line argon laser (488nm). Images were acquired sequentially to avoid 576 bleedthrough. For live-cell imaging, cells were imaged in a humidified environmental chamber 577 at 37°C and 5% CO₂. Images were minimally processed in FIJI (ImageJ v2.0.0) to adjust 578 brightness and contrast.

579

580 **3D rendering and image analysis**

Z-stacks acquired on the Zeiss LSM 880 with Airyscan microscope were imported into Imaris (Bitplane, Switzerland). To segment Omi and BCL-2, a surface was created using the Omi-GFP pixel information. Masks were applied to differentiate between BCL-2 inside and outside the Omi surface. From these masks, spots were created from the BCL-2 channel and quantified based on intensity of BCL-2 on mitochondria undergoing minority MOMP.

586

587 Fluorescence Loss in Photobleaching

588 The time lapse images were taken at one frame per second on a Zeiss LSM880 microscope 589 in a 37°C chamber, with a Fluor 40x/1.30 NA oil immersion lens. Cells were in HEPES 590 containing media. Fluorescence was excited using the 488nm line of an argon ion laser at 591 5.4% power through the AOTF and the emitted signal was captured with an Airyscan detector

- in Resolution vs. Signal mode using a combination of LP500 and BP485-550 filters with a
 zoom of 3.6. A letterbox bleach region covering approximately a third of the cell area was
 achieved using combination of 405 and 488nm wavelengths of light at 100% laser power and
 100 iterations which took approximately 14 seconds.
- 596

597 Digitionin permeabilisation

Prior to digitonin permeabilisation, cells were incubated in FluoroBrite DMEM without FBS. To
permeabilise the plasma membrane, 20 µM digitonin (Sigma) was added and cells imaged
immediately.

601

602 *Mitochondrial analysis*

603 Cells stained with TOM20 and imaged on the Zeiss LSM 880 with Airyscan. These images 604 were analysed by dividing mitochondrial length by width (mitochondria aspect ratio) in ImageJ.

605 Heterogeneity of BCL-2 localisation was measured by calculating the standard deviation of

606 Scarlet and mitochondrial signals in mitochondrial regions in FIJI (ImageJ v2.0.0).

607

608 Live-cell viability assays

609 Cell viability was assayed using either an IncuCyte ZOOM or IncuCyte S3 imaging system 610 (Sartorius). Cells were seeded overnight and drugged in the presence of 30 nM SYTOX Green 611 (Thermo Fisher Scientific), which is a non-cell-permeable nuclear stain. Data were analysed 612 in the IncuCyte software, and where different cell lines are compared the data are normalised 613 to starting confluency.

614

615 Bioinformatic Analysis

616 Relationship between DRP1 (DNM1L) expression and mutational count were investigated in 617 TCGA PanCancer Atlas studies through cBioportal (Cerami et al., 2012; Gao et al., 2013). 618 Studies with greater than 100 samples were analysed and samples divided into quartiles of 619 DNM1L: mRNA expression z-scores relative to diploid samples (RNA Seq V2 RSEM). Of 620 these, a significant association between increased mutational count in DNM1L mRNA highest 621 guartile versus DNM1L mRNA lowest guartile was found in 2 out of 22 studies with the inverse 622 relationship not observed in any cancer type. Mutation count in DNM1L quartiles was viewed 623 in the Clinical Tab, statistical analysis of mutation count was performed by cBioportal, 624 Wilcoxon test, q-value <0.05 was considered significant. As the relationship between DNM1L 625 and mutational count was highly significant in Invasive Breast Carcinoma and Non-Small Cell 626 Lung Cancer, we used these studies for further interrogation with cases of Lung 627 adenocarcinoma selected from Non-Small Cell Lung Cancer dataset (not Lung squamous cell 628 carcinoma). Data were downloaded from cBioportal and mutational count in DNM1L mRNA

629 highest guartile versus DNM1L mRNA lowest guartile (mRNA expression z-scores relative to 630 diploid samples (RNA Seg V2 RSEM)) plotted in GraphPad Prism Version 9.0.0 and statistical 631 significance between groups calculated by Mann-Whitney test. Data points represent 632 individual patient samples, bar is mean (SD). DNM1L quartiles each contain 128 samples 633 (Lung Adenocarcinoma TCGA PanCancer Atlas dataset) or 271 samples (Breast Invasive 634 Carcinoma TCGA PanCancer Atlas dataset). Differentially expressed proteins in DNM1L 635 highest versus lowest quartiles were also determined in cBioportal (measured by reverse-636 phase protein array, Z-scores) where significant differences are determined by Student's t-637 test (p value) and Benjamini-Hochberg procedure (q value). Pathway analysis was performed 638 using gene names of proteins identified with significantly higher expression in DNM1L high 639 versus DNM1L low quartiles (excluding phospho-specific proteins, see lists in Supplementary 640 Table 1) in GO Biological Process 2018 through Enrichr (Chen et al., 2013; Kuleshov et al., 641 2016).

642

Relationship between DRP1 (DNM1L) expression and patient survival were investigated in
TCGA PanCancer Atlas studies through cBioportal (Cerami et al., 2012; Gao et al., 2013).
Lung Adenocarcinoma TCGA PanCancer Atlas and Breast Invasive Carcinoma TCGA
PanCancer Atlas datasets were downloaded from cBioportal, samples divided into quartiles
of DNM1L: mRNA expression (z-scores relative to normal samples log RNA Seq V2 RSEM),
with survival of highest and lowest quartiles plotted in GraphPad Prism Version 9.0.0 and
statistical significance between groups calculated by Log-rank (Mantel-Cox) test.

650 651

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659

660 Author contributions

KC, JSR and SWGT conceived the study and designed the workplan. Experimental
work: KC, JSR, RH, AEM-G, EV, KB, CC, YE, DGS, GI, KJC, APG. Development and
contribution of reagents: KC, JSR, CC, GI. Data analysis: KC, JSR, YE, KJC, APG, SWGT
Intellectual input: KC, JSR, KJC, APG, SWGT. Manuscript writing: JSR and SWGT.

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- 701 the cytosol. Cell 145, 104-116.





С



Movie X



Figure 2





C HeLa Scarlet-BCL-2 MitoTracker Merge



HeLa Scarlet-BCL-xL

HeLa Scarlet-MCL-1



Ε Scarlet-BCL-2 Scarlet-BCL-xL Scarlet-MCL-1 D Scarlet-BCL-2 Scarlet-BCL-xL Scarlet-MCL-1 High BCL-2 High BCL-xL High MCL-1 EMPTYCRISPR High BCL-2 High MCL-1 High BCL-x Drp1CRISPR F ** ** ** EMPTYCRISPR 1.0 Drp1^{CRISPR} Δ Scarlet/MitoTracker 0.8 StDev Ratio 0.6 0.4 0.2 0.0 HeLa Scarlet Knockin: BCL-2 BCL-xL MCL-1

Figure 4







Figure 5







Supplementary Figure 2 - related to Figure 3









Α	Digitonin per	meabilisatio				
	0s	75s	150s	225s	300s	375s
Untreated	GFP BAX					13
	BCL-2					
CP						
00	Kipt			Kim		
		a da	Alter -		- Ale	

