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

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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**

43 Mitochondrial dysfunction has pleiotropic impact on cancer (Giampazolias and Tait,  
44 2016). For instance, mitochondrial respiratory complex proteins and TCA enzymes bearing  
45 tumour associated mutations, generate oncometabolites (Isaacs et al., 2005; Pollard et al.,  
46 2007; Sciacovelli et al., 2016; Selak et al., 2005). Moreover, loss of function mutations in  
47 mitochondrial DNA (mtDNA) are common in cancer and have been shown to accelerate  
48 tumorigenesis (Gorelick et al., 2021; Smith et al., 2020). Nonetheless, how dysfunctional  
49 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  
65 selectively permeabilizes, which we termed minority MOMP (Ichim et al., 2015). Strikingly,  
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  
70 been recently implicated in an expanding array of functions including increased cancer  
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).

73

74 Here, we investigated the relationship between mitochondrial dysfunction and DNA  
75 damage. Surprisingly, we uncovered a key role for mitochondrial dynamics in the regulation  
76 of DNA damage. Mitochondrial fission, a consequence of mitochondrial dysfunction, promotes  
77 minority MOMP causing caspase-dependent DNA damage and genome instability. Secondly,  
78 we find reduced retrotranslocation of pro-apoptotic BAX on dysfunctional mitochondria, thus



79 facilitating minority MOMP. These data reveal an unanticipated link between mitochondrial  
80 dysfunction and oncogenic DNA damage that is mediated through minority MOMP and  
81 caspase activity.

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83

## 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  $\gamma$ H2AX staining and  
93 flow cytometry. In both HeLa and U2OS cells, BH3-mimetic treatment led to an increase in  
94  $\gamma$ H2AX 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  $\gamma$ H2AX-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  $\gamma$ H2AX 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  
103 treatment. To disrupt mitochondrial fusion, we used *Mfn1/2*<sup>-/-</sup> murine embryonic fibroblasts  
104 (*Mfn1/2*<sup>-/-</sup> MEF) and, as control, reconstituted these cells with MFN2 (*Mfn1/2*<sup>-/-</sup> + Mfn2 MEF).  
105 As expected, *Mfn1/2*<sup>-/-</sup> MEF displayed a hyper-fragmented mitochondrial network whereas  
106 MFN2 reconstitution of these cells (*Mfn1/2*<sup>-/-</sup> + Mfn2) restored mitochondrial fusion, resulting  
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*<sup>-/-</sup> MEF reconstituted with Mfn2 compared to Wt MEF, mitochondrial fusion  
110 was not enhanced in *Mfn1/2*<sup>-/-</sup> + Mfn2 MEF and mitochondrial fragmentation occurred with the  
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*<sup>-/-</sup> and *Mfn1/2*<sup>-/-</sup> + Mfn2 MEF were treated with ABT-737  
115 (10  $\mu$ M, 3 hours) and the DNA damage response was assessed by analyzing  $\gamma$ H2AX levels

116 by western blot or by flow cytometry (**Figures 1E, F**). *Mfn1/2<sup>-/-</sup>* MEF exhibited increased  
117  $\gamma$ H2AX, 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<sup>-/-</sup>* and *Mfn1/2<sup>-/-</sup> + 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<sup>-/-</sup>*  
122 MEF formed colonies more readily than *Mfn1<sup>-/-</sup> + 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<sup>fl/fl</sup>* 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  
131 treatment (**Supplementary Figure 1G, 3B**). *Drp1<sup>fl/fl</sup>* and *Drp1<sup>-/-</sup>* MEF were treated with ABT-  
132 737 and  $\gamma$ H2AX was measured by flow cytometry, as before. MEF expressing *Drp1* have  
133 elevated levels of  $\gamma$ H2AX 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

### 137 **Mitochondrial dynamics regulate DNA damage and genome-instability in a caspase and** 138 **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  $\gamma$ 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  $\gamma$ 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,  $\gamma$ H2AX 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 (carbonyl phosphate synthetase/aspartate  
166 transcarbamylase/dihydro-ototase, 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  
169 dependent upon caspase activity, we passaged *Mfn1/2<sup>-/-</sup>* and *Mfn1/2<sup>-/-</sup> + Mfn2* MEF with sub-  
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**).  
172 Importantly, ABT-737 treated *Mfn1/2<sup>-/-</sup>* MEF gave significantly more colonies than *Mfn1/2<sup>-/-</sup> +*  
173 *Mfn2* following PALA treatment, in a caspase-dependent manner (**Figure 2E, F**). In line with  
174 increased survival following PALA treatment, qPCR revealed amplification of the *Cad* locus  
175 only in *Mfn1/2<sup>-/-</sup>* 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<sup>-/-</sup>* and  
179 *Mfn1/2<sup>-/-</sup> + Mfn2* MEF in which we deleted the *Dff40* gene (encoding CAD) using CRISPR-  
180 Cas9 genome editing (**Supplementary Figure 3C**). As before, *Mfn1/2<sup>-/-</sup>* cells resisted PALA  
181 treatment and efficiently grew as colonies following ABT-737 treatment, whereas *Mfn1/2<sup>-/-</sup> +*  
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  $\mu$ M) 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  $\mu$ M) 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.  
214 *Mfn1/2<sup>-/-</sup>* and *Mfn1/2<sup>-/-</sup> + Mfn2* MEF expressing the MOMP reporter, were treated with a sub-  
215 lethal dose of ABT-737. Strikingly, increased levels of minority MOMP were observed in  
216 *Mfn1/2<sup>-/-</sup>* MEF when compared to *Mfn1/2<sup>-/-</sup> + 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<sup>fl/fl</sup>* and *Drp1<sup>-/-</sup>* 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.

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

Our data demonstrate that mitochondrial fission promotes minority MOMP enabling caspase-dependent DNA damage. Nevertheless, how mitochondrial dynamics regulate minority MOMP is not known. Mitochondrial outer membrane integrity is regulated by the balance of pro- and anti-apoptotic BCL-2 family proteins (Campbell and Tait, 2018). We hypothesised that inter-mitochondrial variation in BCL-2 family localisation may underlie minority MOMP. To investigate this hypothesis, we set out to visualise endogenous levels of BCL-2 family proteins on individual mitochondria. CRISPR-Cas9 genome editing can be used to knock-in fluorescent proteins at defined genomic loci to enable endogenous tagging of proteins (Bukhari and Muller, 2019). Using this approach, we generated clonal knock-in HeLa cell lines where the red fluorescent protein Scarlet was fused to the N-termini of BCL-2, BCL-xL and MCL-1. As verification, western blotting using antibodies specific BCL-2, BCL-xL, MCL-1 and Scarlet confirmed that these cell lines expressed these fusion proteins at similar levels to their endogenous counterparts (**Figure 4A, B**). Secondly, Airyscan super-resolution microscopy demonstrated mitochondrial localisation of Scarlet-BCL-2, BCL-xL and MCL-1, as expected (**Figure 4C**) and is comparable to mitochondrial localisation of untagged endogenous BCL-2 proteins in parental cells (**Supplementary Figure 5A, B**). Finally, we monitored cell viability using SYTOX Green exclusion and IncuCyte real-time imaging in response to BH3-mimetic treatment (ABT-737 and S63845). This demonstrated that all knock-in cell lines underwent cell death in response to BH3-mimetic treatment (**Supplementary 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) such that the brighter the Scarlet signal, the more bright the image. This revealed heterogeneity of Scarlet BCL-2, BCL-xL and MCL-1 across the mitochondrial network, which is also evident in parental cells stained with BCL-2 family antibodies (**Figure 4D, Supplementary Figure 5A**). Given our previous data, we hypothesised that BCL-2 family protein heterogeneity is regulated by mitochondrial dynamics. To test this, we inhibited mitochondrial fission through CRISPR-Cas9 deletion of DRP1. Western blot confirmed DRP1 deletion, resulting in extensive mitochondrial hyperfusion (**Supplementary Figure 5D, E**). 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**). Combined, these data show that within a cell extensive inter-mitochondrial heterogeneity in BCL-2 localisation exists that is impacted by mitochondrial dynamics.

## **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

### 294 **Mitochondrial dysfunction promotes BAX accumulation promoting minority MOMP**

295 We aimed to define the underlying mechanism of mitochondrial intrinsic apoptotic  
296 priming. In healthy cells, BAX undergoes mitochondrial retrotranslocation and inhibiting this  
297 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  
299 with the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) to induce  
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  
306 treatment) but not following CCCP treatment (**Supplementary Figure 7B**). Since  
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  
310 MOMP. We imaged *Mfn1/2<sup>-/-</sup>* and *Mfn1/2<sup>-/-</sup>* + *Mfn2* MEF with MitoTracker Red, a potentiometric  
311 dye, that reports mitochondrial  $\Delta\psi^m$  as a measure of mitochondrial function. Consistent with  
312 defective mitochondrial function, mitochondria in fusion defective cells (*Mfn1/2<sup>-/-</sup>*) displayed  
313 heterogenous MitoTracker Red signal and lower total signal than fusion competent *Mfn1/2<sup>-/-</sup>* +  
314 *Mfn2* MEF (**Figures 6B-D**). We next analysed GFP-BAX localisation in *Mfn1/2<sup>-/-</sup>* and *Mfn1/2<sup>-/-</sup>*  
315 + *Mfn2* MEF, using fluorescence loss in photobleaching (FLIP) to help visualise mitochondrial  
316 localised GFP-BAX. FLIP analysis of GFP-BAX revealed slower mitochondrial  
317 retrotranslocation in *Mfn1/2<sup>-/-</sup>* cells compared to *Mfn1/2<sup>-/-</sup>* + *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

352 We sought to define how mitochondrial dynamics might control minority MOMP.  
353 Surprisingly, we found inter-mitochondrial heterogeneity in anti-apoptotic BCL-2 localisation.  
354 This heterogeneity was suppressed 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  
361 cell death. For instance, during apoptosis, extensive mitochondrial fragmentation occurs  
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

366 We have previously found that ectopic expression of BCL-2 can lead to incomplete  
367 MOMP, consistent with BCL-2 anti-apoptotic function (Tait et al., 2010). In the current study,  
368 we find that increased local mitochondrial levels of anti-apoptotic BCL-2 family proteins  
369 correlates with selective mitochondrial permeabilisation. While this may seem initially counter-  
370 intuitive, precedence for increased apoptotic priming, correlating with high anti-apoptotic BCL-  
371 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

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

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

424

## 425 **Methods**

426

### 427 **Cell Lines**

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

430

431 *Mfn1/2*<sup>-/-</sup> MEF were provided by David Chan, Caltech and reconstituted with LZRS-MFN2 in  
432 our laboratory. *Drp1*<sup>fl/fl</sup> MEF were provided by Hiromi Sesaki, Johns Hopkins University School  
433 of Medicine. MEF *Wt* and MEF loxP-STOP-loxP EYFP were a kind gift from Douglas Green,  
434 St Jude's Research Children's Hospital. All cell lines were cultured in DMEM high-glucose  
435 medium supplemented with 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, penicillin  
436 (10,000 units/ml) and streptomycin (10,000 units/ml).

437

438 To delete Drp1 from *Drp1*<sup>fl/fl</sup> MEF, 2 x 10<sup>6</sup> 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**

445 We used a modified version of the knock-in strategy described in (Stewart-Ornstein and  
446 Lahav, 2016). Two vectors were used: the first vector comprises 500bp homology arm before  
447 and after the start codon of BCL-2, in between which is the Scarlet coding sequence, cloned  
448 into pUC-SP. The second vector, pSpCas9(BB)-2A-Puro (Addgene #48139) comprises Cas9  
449 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

454 To generate the knock-in cells, HeLa cells were transfected with 1 µg of homology arm vector  
455 and 1 µg of pSpCas9(BB)-2A-Puro with Lipofectamine 2000, according to the manufacturer's  
456 instructions. Media was removed 5 h later, and replaced with media containing 1 µM SCR7  
457 for 2 days. Cells were selected with 1 µg/mL puromycin for a further two days before selecting  
458 Scarlet positive clones by FACS. Cells which expressed Scarlet signal which co-localised with  
459 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 µg plasmid was transfected into 293FT along with 1.86 µg psPAX2 (Addgene #12260) and  
473 1 µg 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 were  
478 cloned into LentiCRISPRv2-puro (Addgene #52961)

479 Human Drp1:            5'- AAATCAGAGAGCTCATTCTT – 3'

480 Mouse Dff40:           5'- ACATGGAGCCAAGGACTCGC -3'

481

482 **Plasmids**

483 LZRS-Drp1 was generated by cloning the Drp1 coding sequence from pcDNA3.1(+) Drp1  
484 (Addgene #34706) into LZRS backbone using Gibson Assembly. pBABE iRFP-Omp25 was  
485 cloned by Gibson Assembly using fragments derived from pLJM2 SNAP-Omp25 (Addgene  
486 #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  $\gamma$ H2AX, 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  $\mu$ L and 700  $\mu$ L 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- $\gamma$ H2AX  
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<sup>-/-</sup>* and *Mfn1/2<sup>-/-</sup> + 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  $\alpha$ -MEM medium supplemented with 10% dialysed FBS. PALA was added

518 at the LD<sub>50</sub> 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**

526 A 1% base low melting temperature agarose solution (Sigma-Aldrich) was added to 6 well  
527 plates and allowed to set. 7,500 cells were suspended in 0.6% agarose in a 1:1: ratio to  
528 achieve a final concentration of 0.3% agarose., which was added on top of base agarose.  
529 When set, the cell/agarose mix was overlaid with complete DMEM media and colonies  
530 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<sup>-ΔΔCt</sup> 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           CCGGAATCGAACCCCTGATT

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

555 heterodimerizer (Clontech). Minority MOMP was scored based on co-localisation of CytoGFP  
556 with mito-mCherry. A minimum of 100 cells were analysed per condition, and we defined  
557 minority MOMP as a cell which has 1 or more instances of cytoGFP/mito-mCherry co-  
558 localisation.

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-recommended slice size was used. Live-cell experiments  
567 were performed in an environmental chamber at 37°C and 5% CO<sub>2</sub>. Airyscan processing was  
568 performed using the Airyscan processing function in the ZEN software, and to maintain clarity  
569 some images have been pseudocoloured 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% CO<sub>2</sub>. Images were minimally processed in FIJI (ImageJ v2.0.0) to adjust  
578 brightness and contrast.

579

### 580 ***3D rendering and image analysis***

581 Z-stacks acquired on the Zeiss LSM 880 with Airyscan microscope were imported into Imaris  
582 (Bitplane, Switzerland). To segment Omi and BCL-2, a surface was created using the Omi-  
583 GFP pixel information. Masks were applied to differentiate between BCL-2 inside and outside  
584 the Omi surface. From these masks, spots were created from the BCL-2 channel and  
585 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

592 in Resolution vs. Signal mode using a combination of LP500 and BP485-550 filters with a  
593 zoom of 3.6. A letterbox bleach region covering approximately a third of the cell area was  
594 achieved using combination of 405 and 488nm wavelengths of light at 100% laser power and  
595 100 iterations which took approximately 14 seconds.

596

### 597 ***Digitonin permeabilisation***

598 Prior to digitonin permeabilisation, cells were incubated in FluoroBrite DMEM without FBS. To  
599 permeabilise the plasma membrane, 20  $\mu$ M digitonin (Sigma) was added and cells imaged  
600 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 quartile versus *DNM1L* mRNA lowest quartile 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 quartile versus *DNM1L* mRNA lowest quartile (mRNA expression z-scores relative to  
630 diploid samples (RNA Seq 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

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

650

651

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659

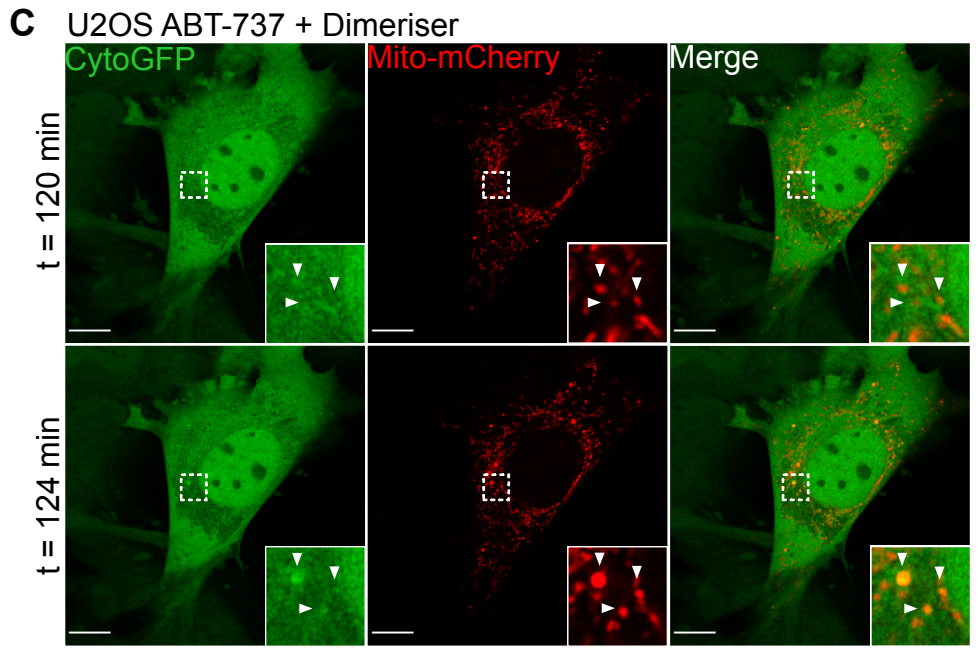
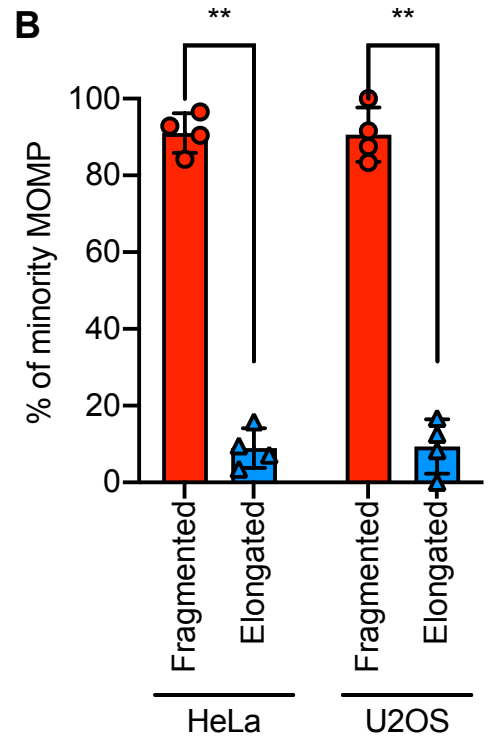
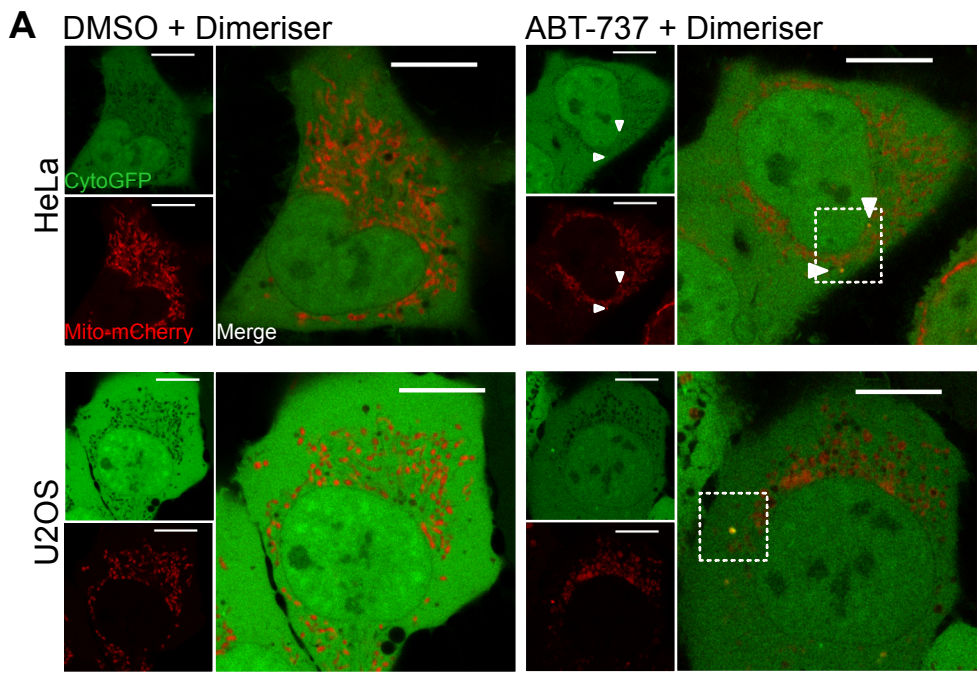
## 660 **Author contributions**

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



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Movie X

Figure 1

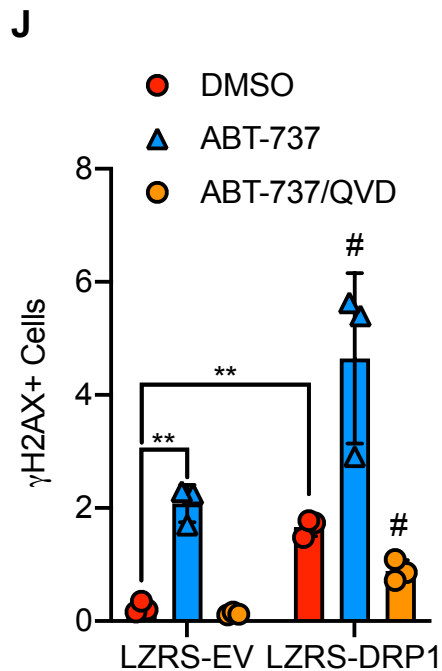
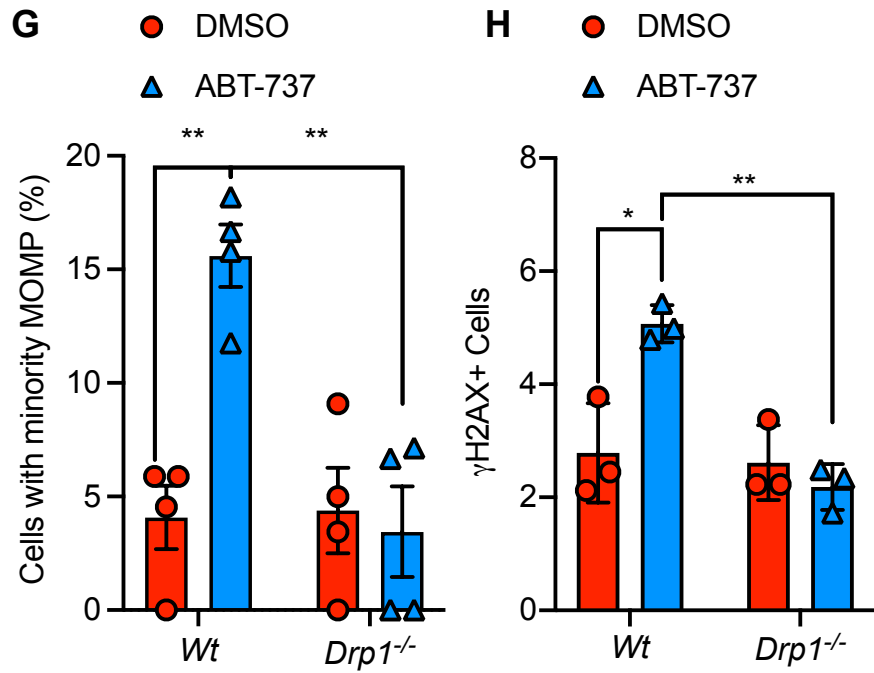
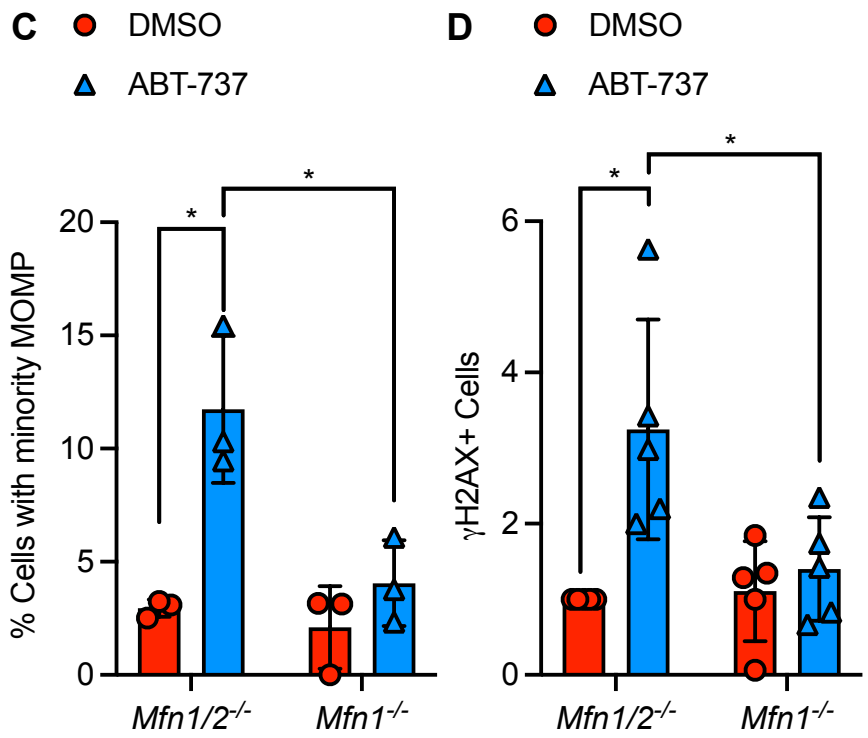
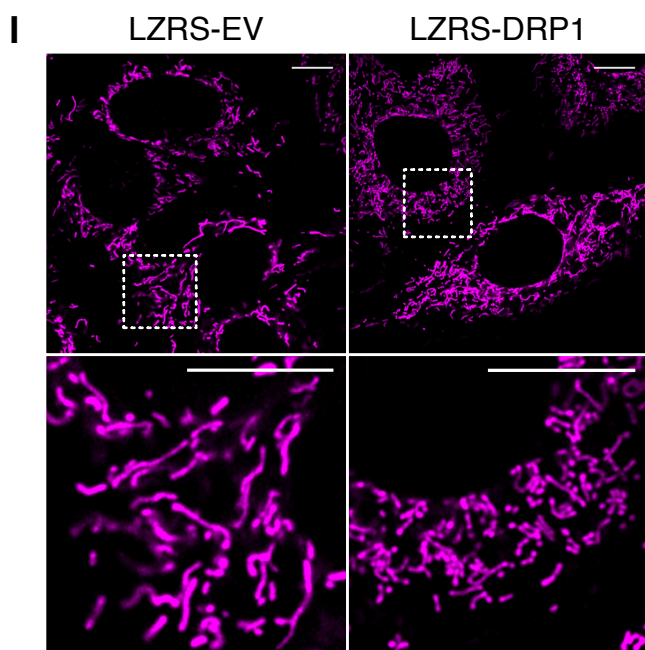
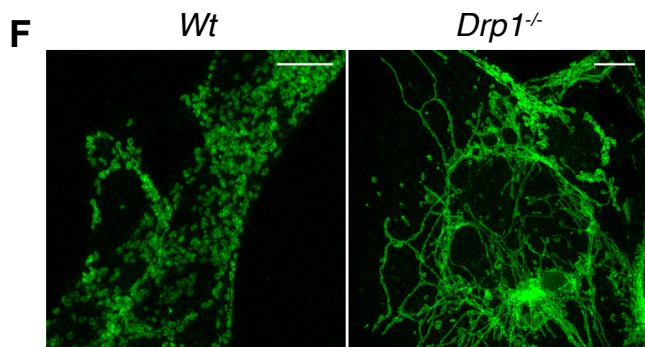
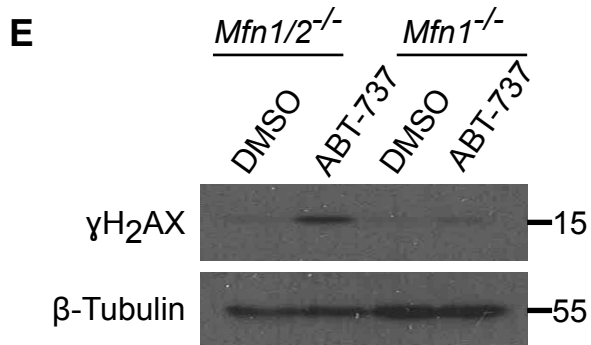
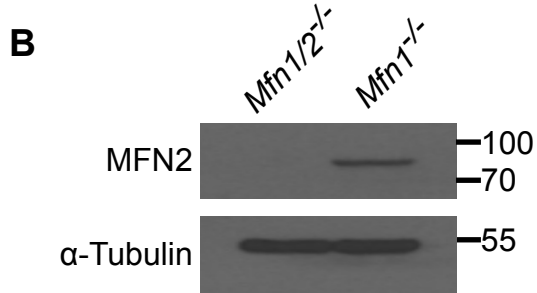
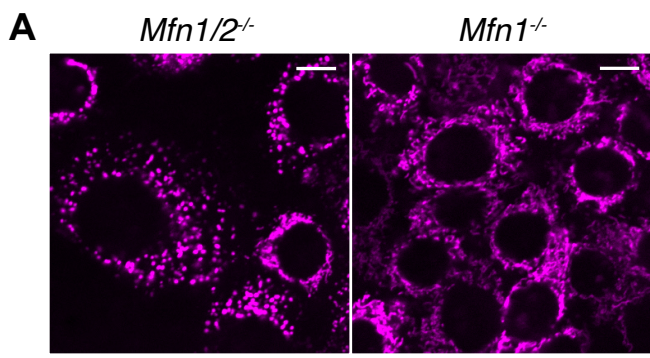
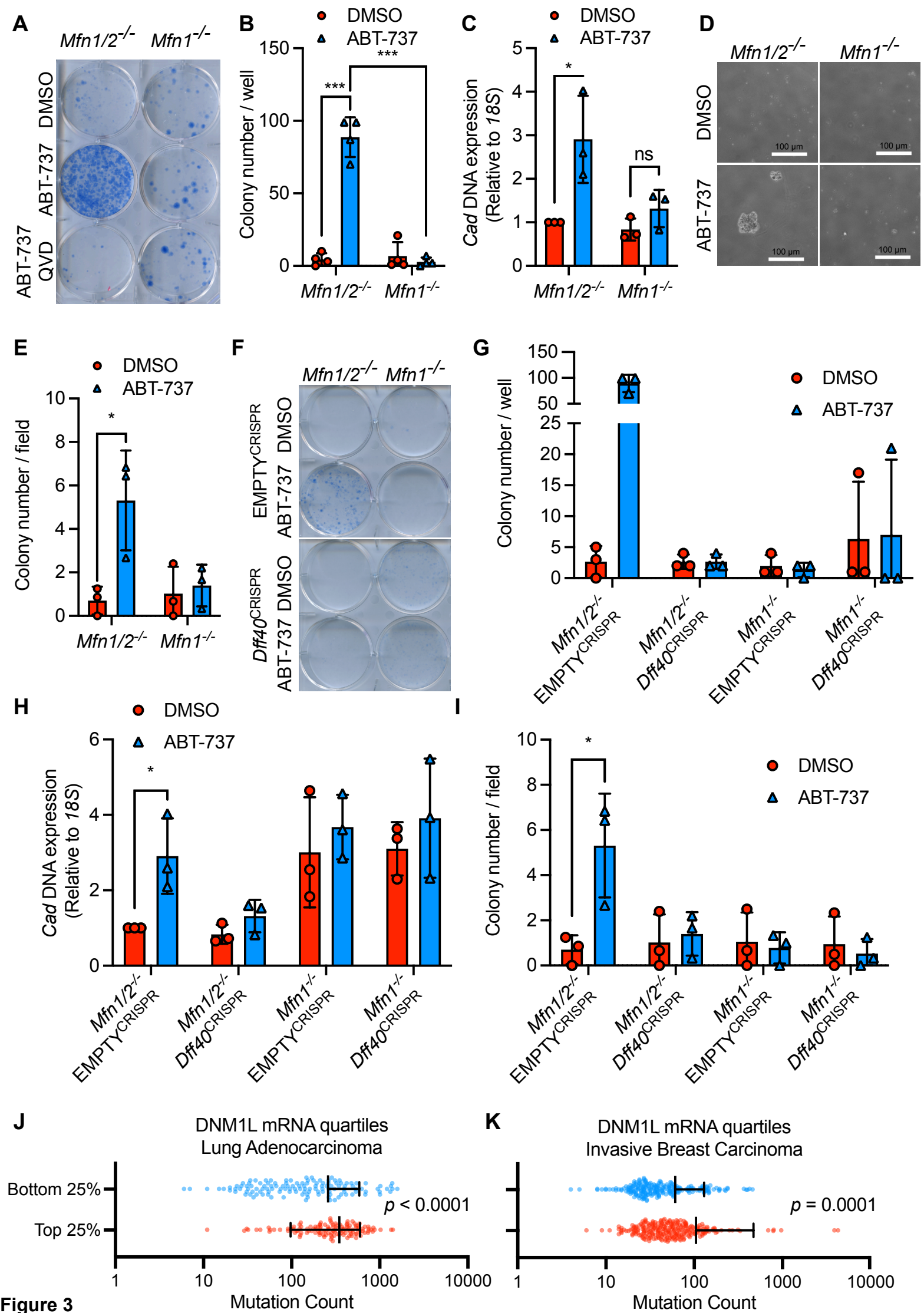
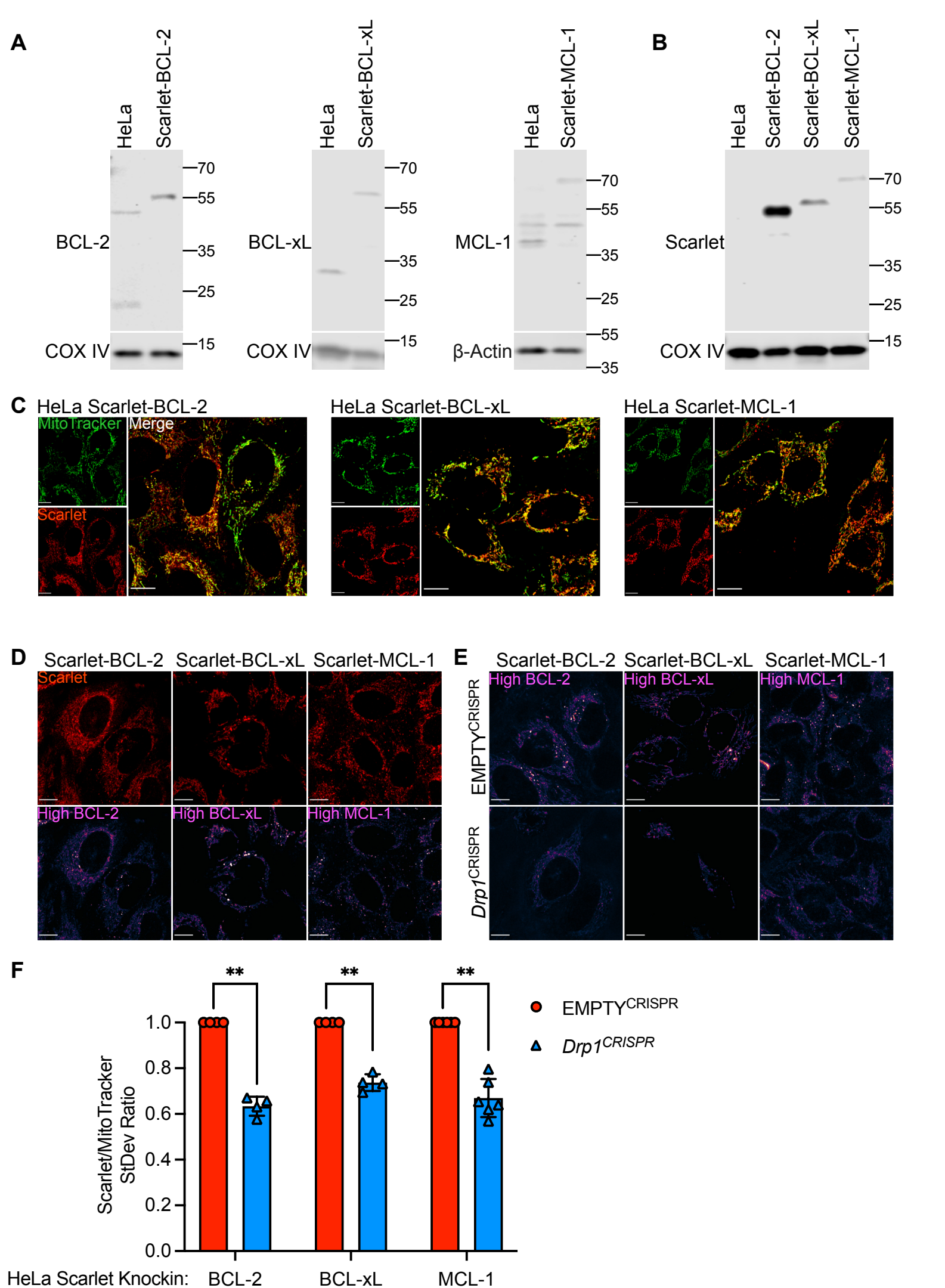


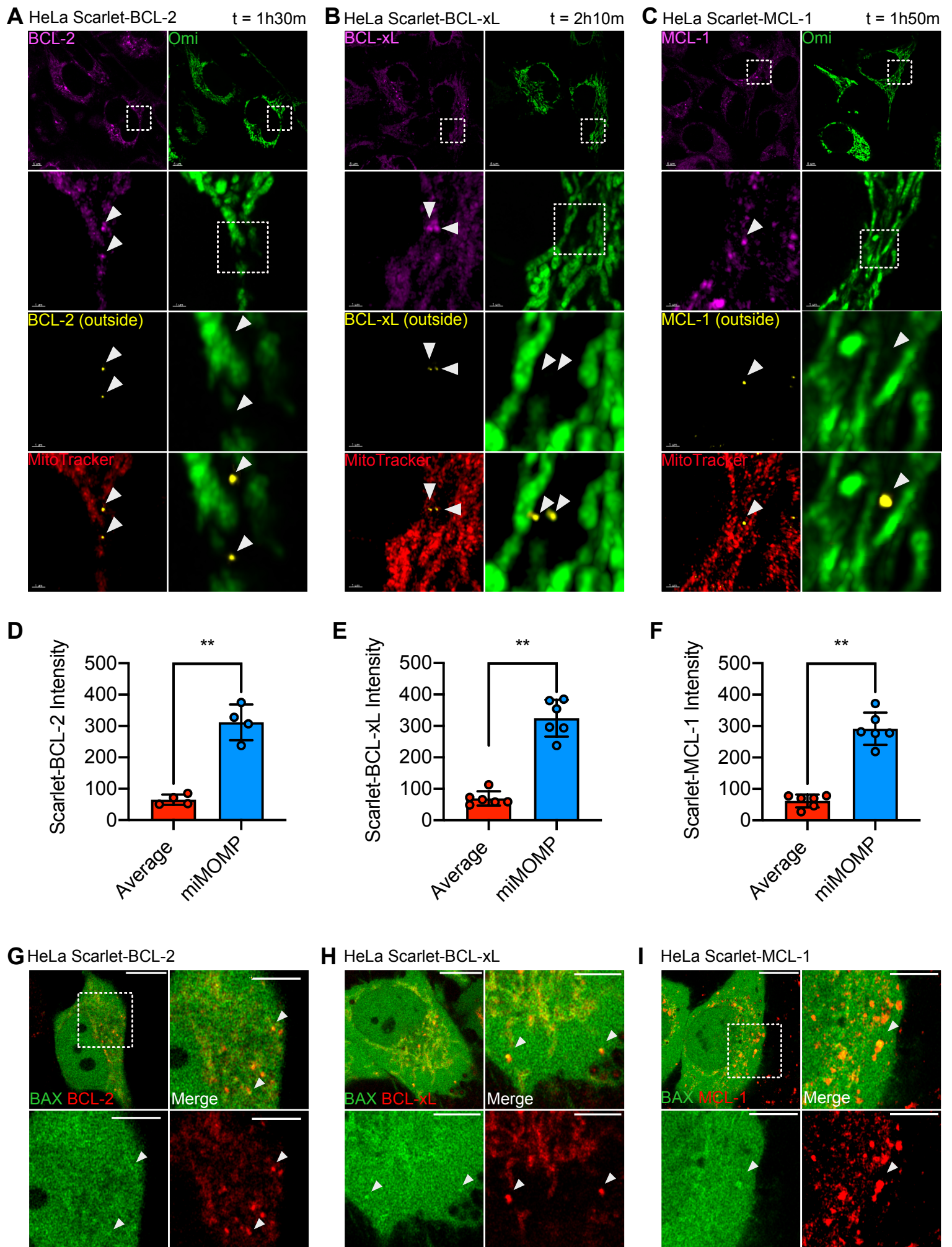
Figure 2





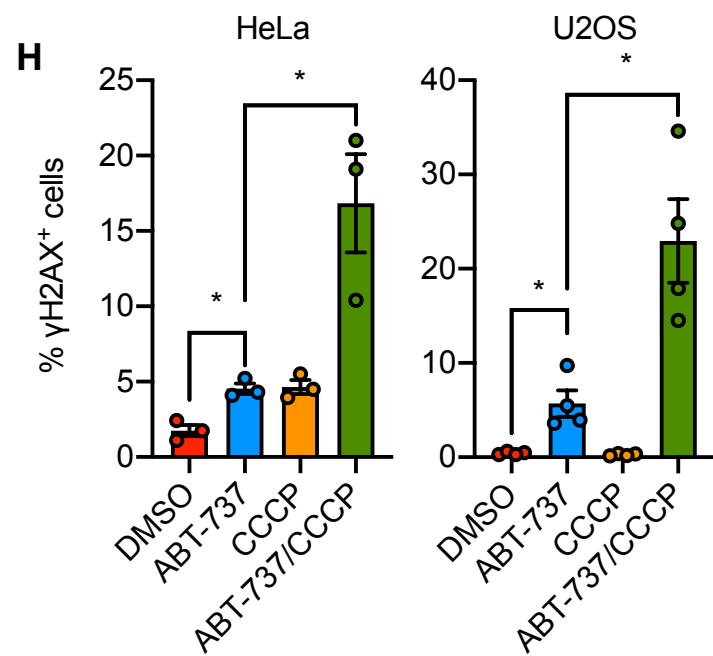
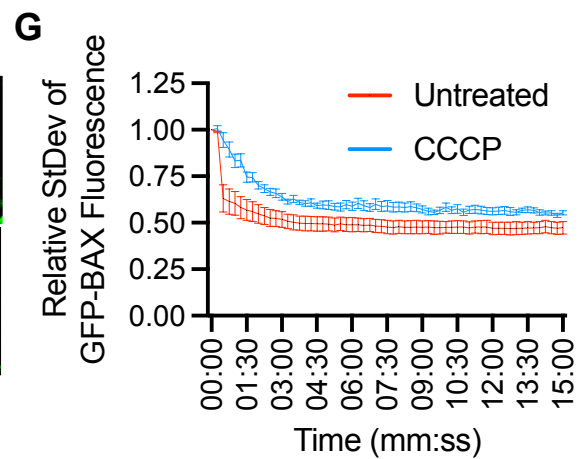
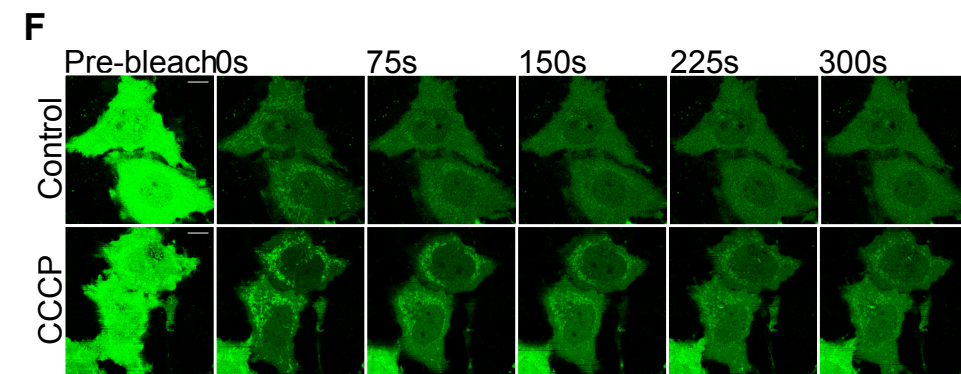
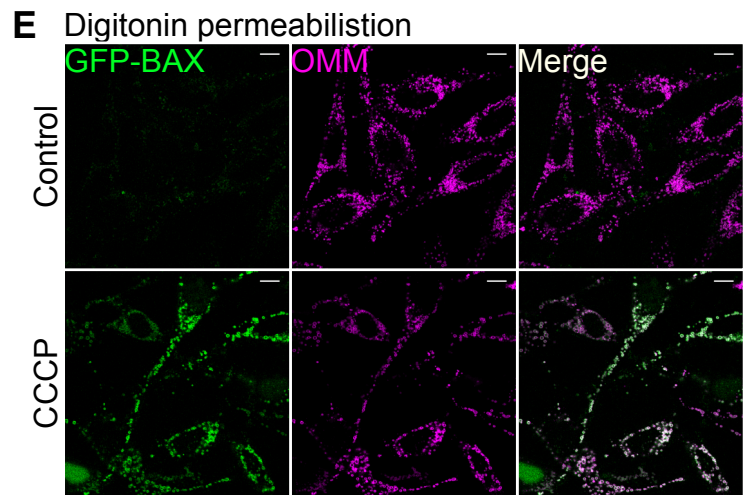
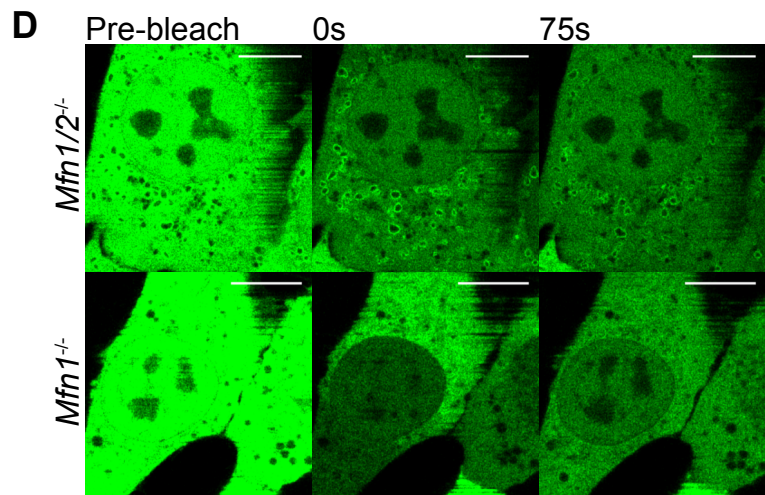
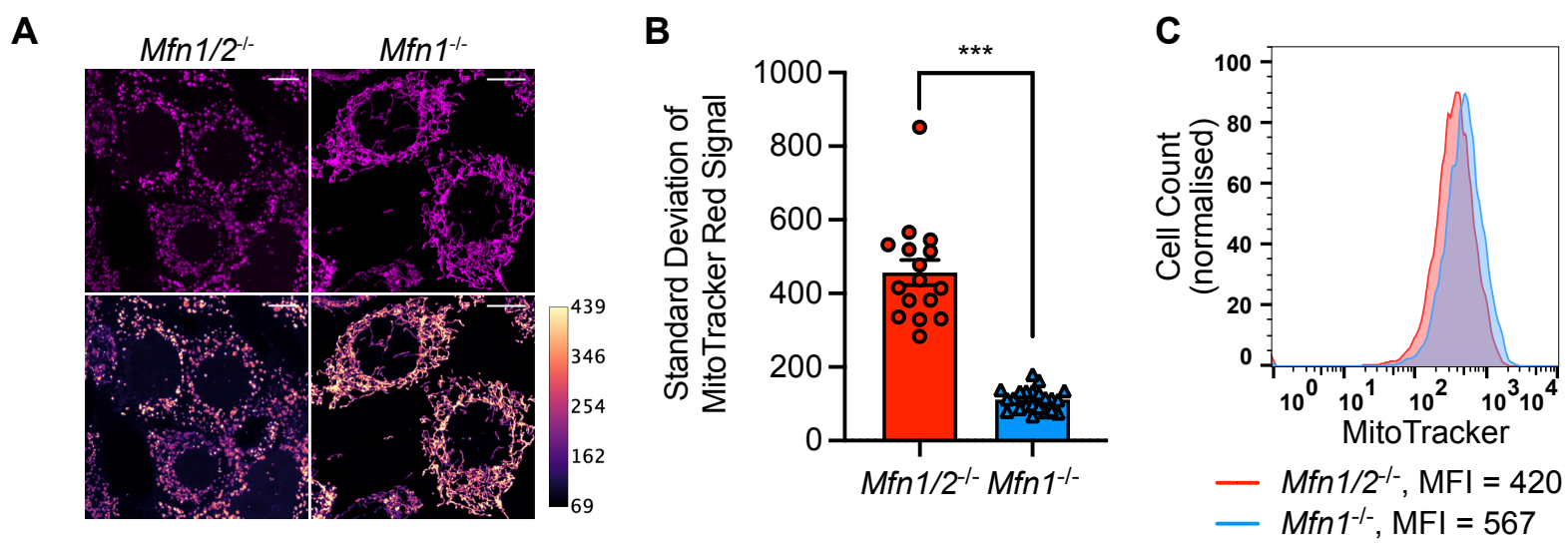


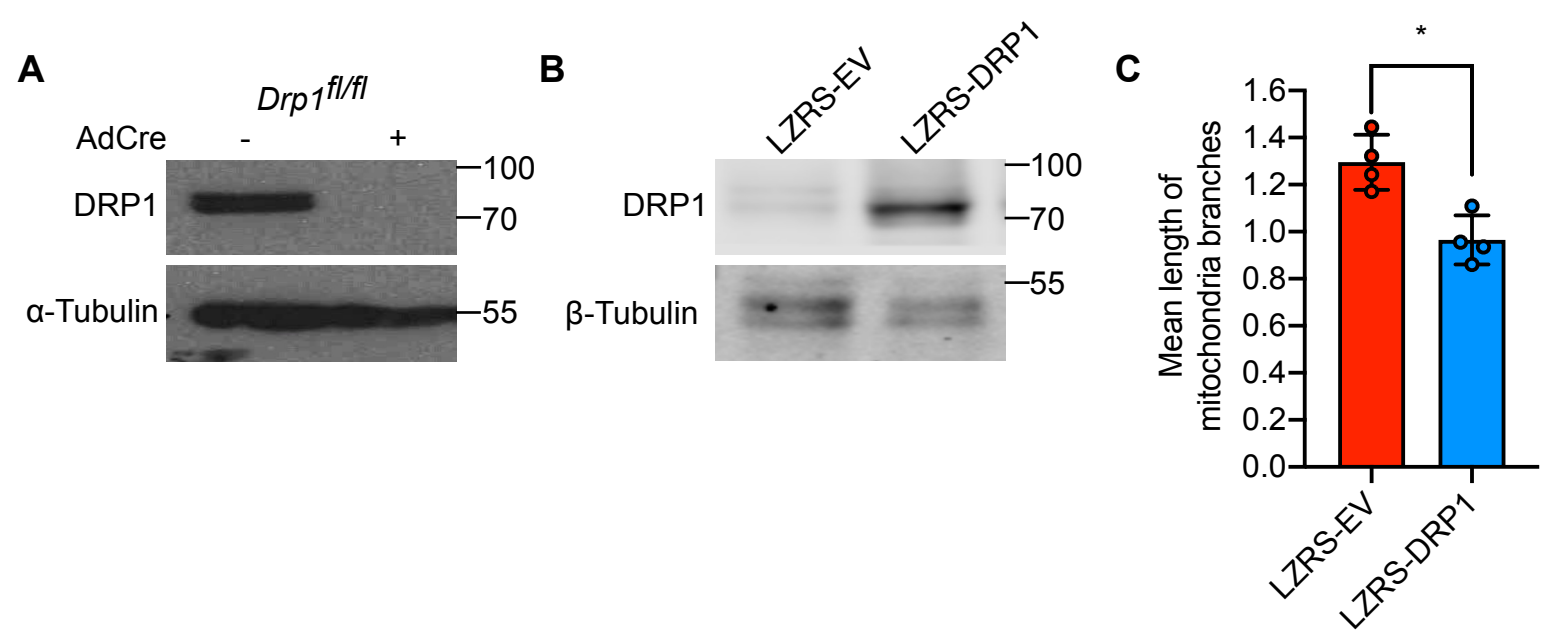
**Figure 4**



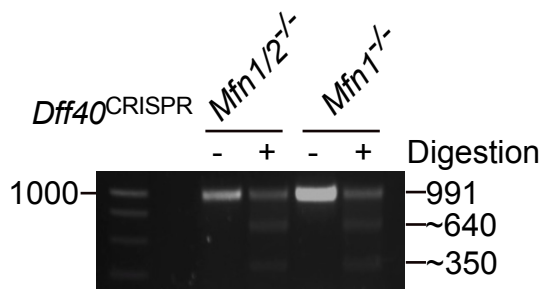
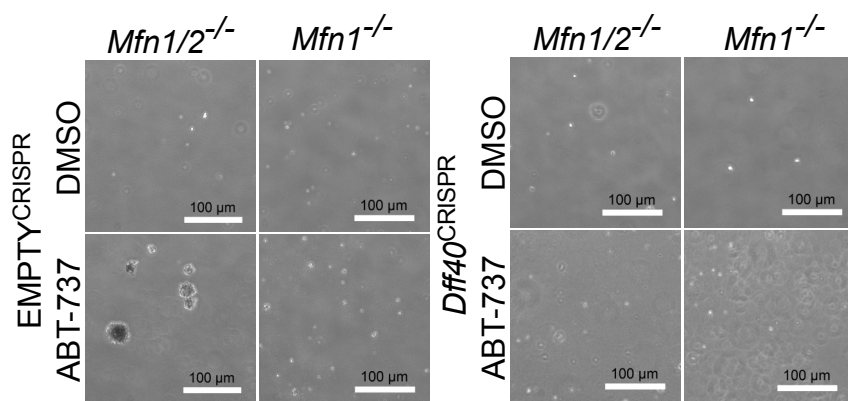
**Figure 5**



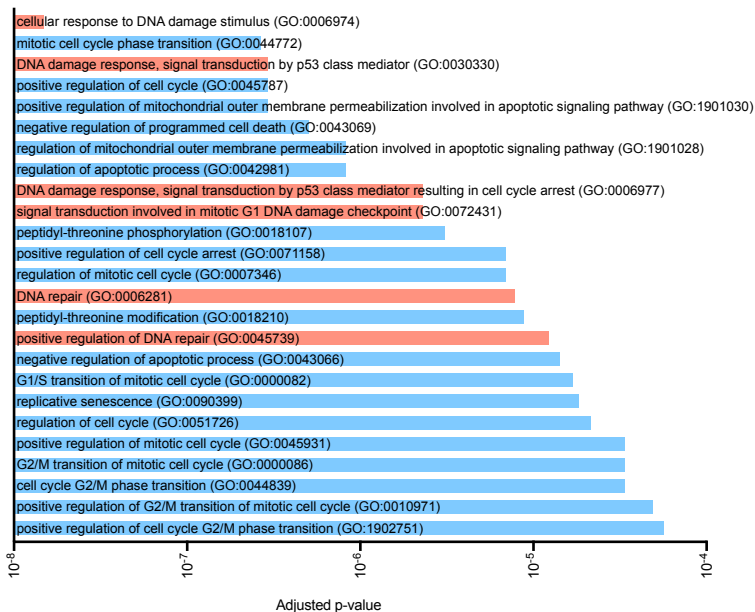




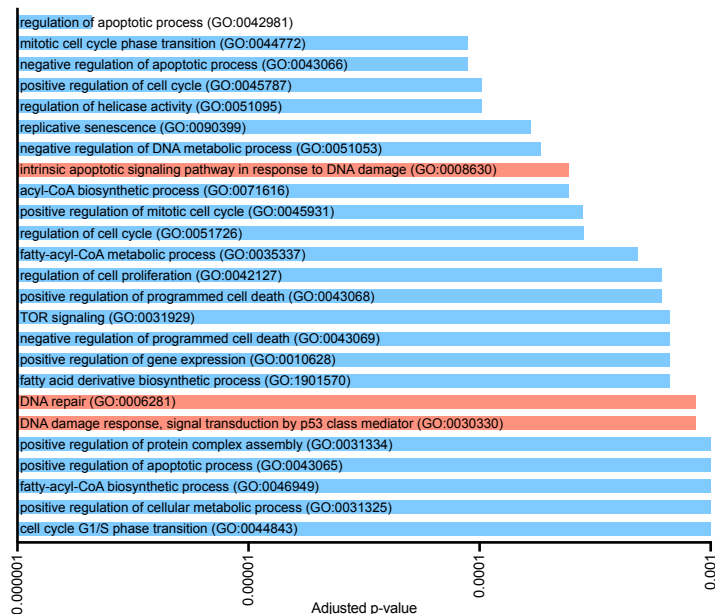


**A****B****C**

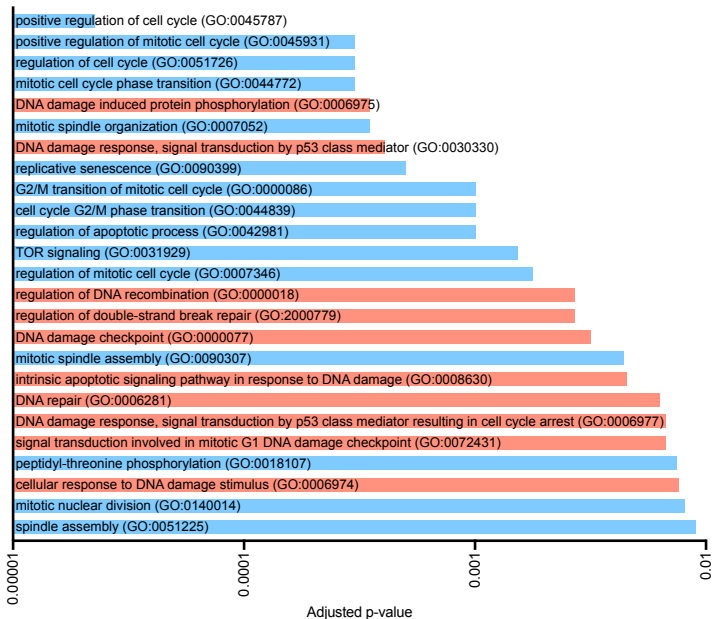
Lung Adenocarcinoma  
GO Biological Processes Enriched in DNMI1L High

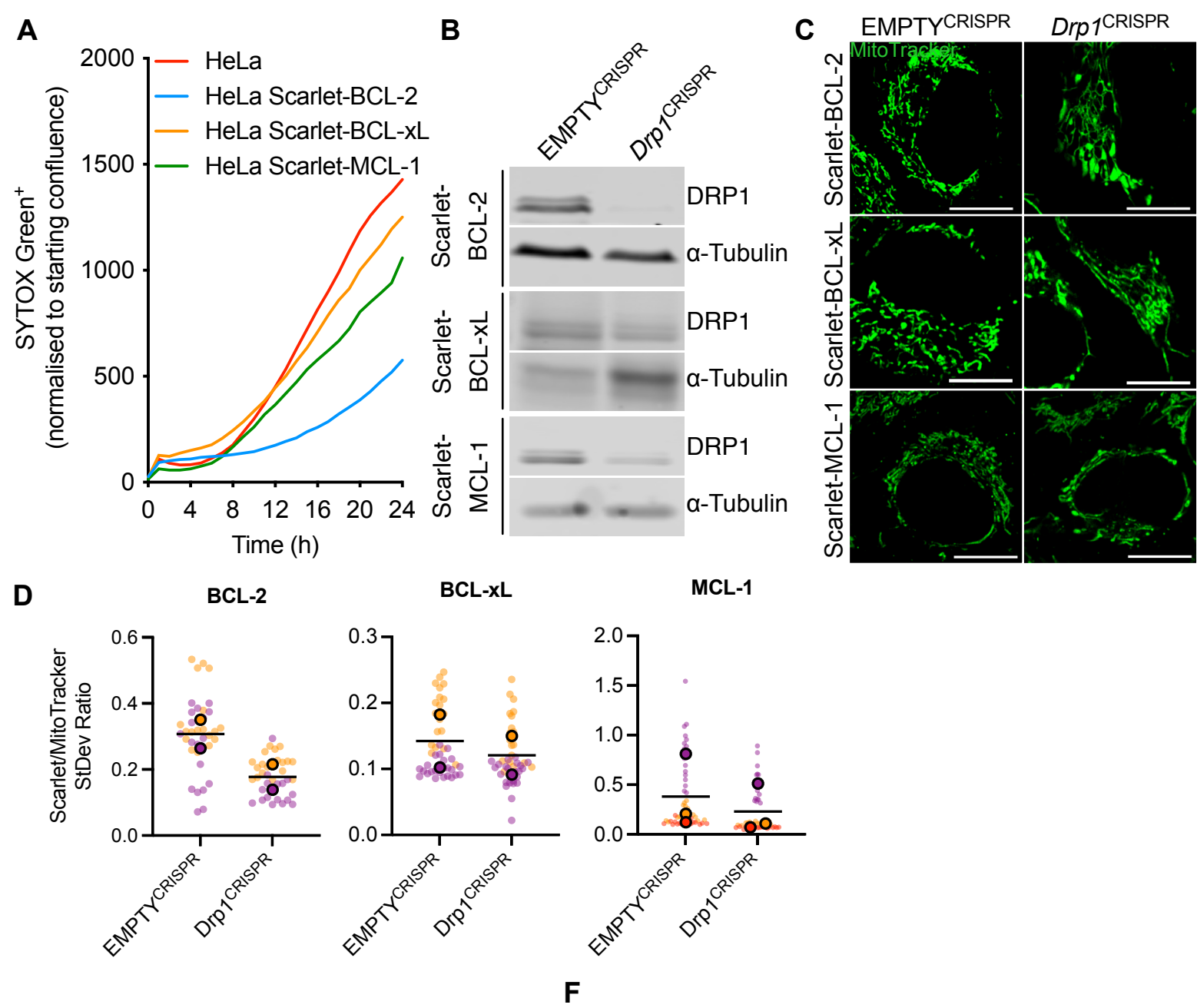
**D**

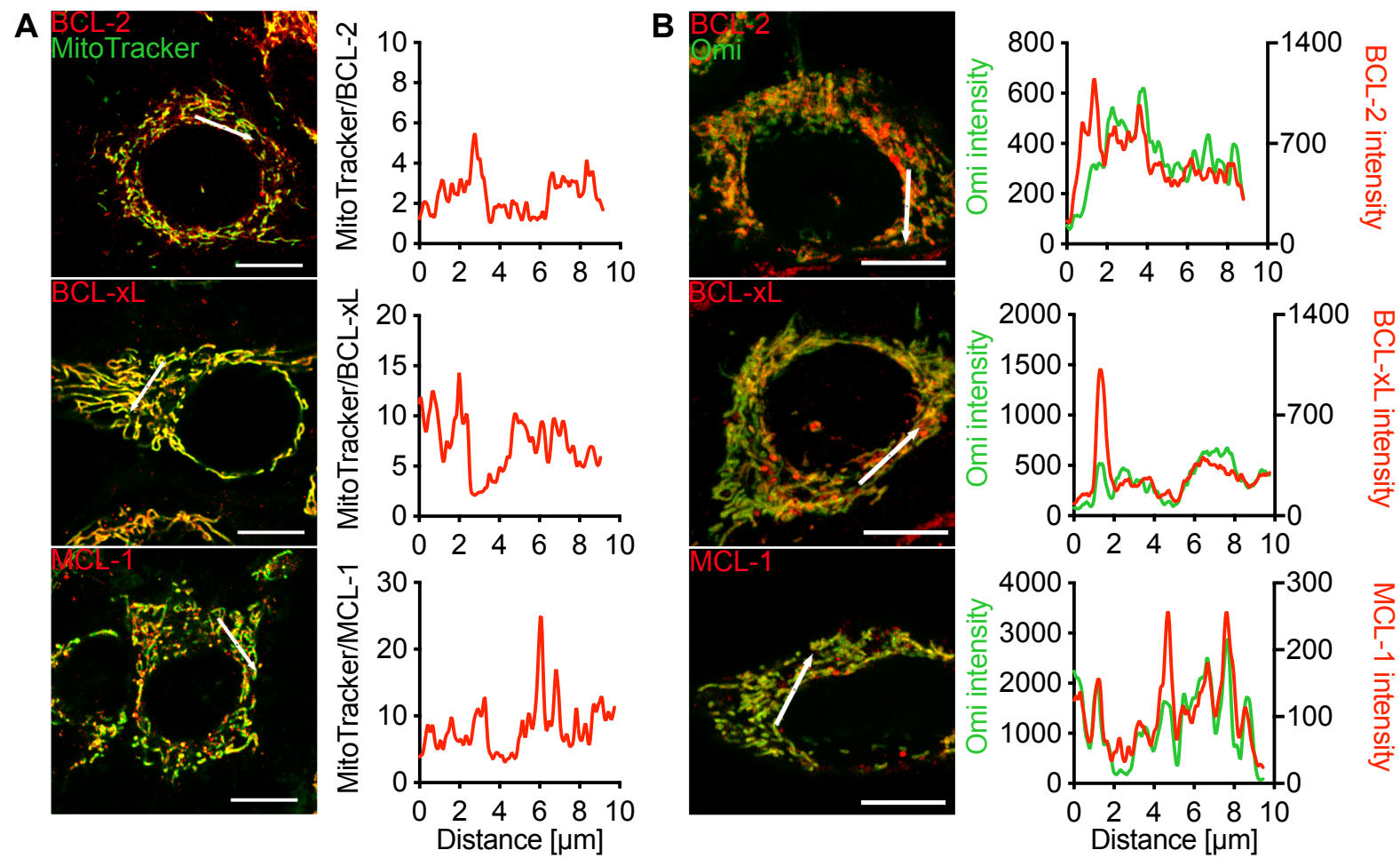
Breast Invasive Carcinoma  
GO Biological Processes Enriched in DNMI1L High

**E**

Breast Invasive Carcinoma and Lung Adenocarcinoma  
GO Biological Processes Enriched in DNMI1L High







Supplementary Figure 4

