Cristae undergo continuous cycles of fusion and fission in a MICOS-dependent manner

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Abstract

The mitochondrial inner membrane can reshape under different physiological conditions. How and at which frequency this occurs *in vivo* and what are the molecular players involved is unknown. Here we show using state-of-the-art live-cell stimulated emission depletion (STED) super-resolution nanoscopy that crista junctions (CJs) are dynamically fusing and dividing in a reversible and balanced manner at a timescale of seconds. CJ dynamics is strongly reduced in the absence of the MICOS subunit MIC13. Staining of the cristae membrane using different protein markers or two inner mitochondrial membrane-specific dyes revealed that cristae also undergo continuous cycles of fusion and fission. These processes are dependent on MIC13 and occur at a timescale of seconds, resembling CJ dynamics. Our data further suggest that MIC60 acts as a docking platform pioneering CJ formation. Overall, by employing a variety of advanced imaging techniques including FRAP (Fluorescence-Recovery-After-Photobleaching), SPT (Single-Particle-Tracking), live-cell STED and confocal Airyscan microscopy we demonstrate that cristae undergo continuous cycles of fusion and fission in a manner that is mechanistically linked to CJ formation and dynamics.

Introduction

Mitochondria are vital organelles with key roles in energetics and metabolism of the cell. The ultrastructural morphology of this double-membrane enclosed organelle is highly variable and altered in numerous human disorders (Zick et al, 2009). The internal mitochondrial structure is characterized by invagination of the inner membrane (IM) called cristae. The IM that closely remains apposed to the outer membrane (OM) is called the inner boundary membrane (IBM). The cristae membrane (CM) connects the IBM via a highly curved, circular or slit- or pore-like structures called crista junctions (CJs) (Acehan et al, 2007; Perkins et al, 1997; Perkins et al, 2003; Perkins et al, 2010; Rabl et al, 2009; Zick et al, 2009). CJs are highly conserved with a diameter of 12-40 nm and were proposed to act as diffusion barriers for proteins or metabolites (Frey et al, 2002; Mannella et al, 2013; Mannella et al, 1994; Mannella et al, 2001). Thus, the

52   presence of CJs could create several mitochondrial subcompartments by separating IBM from

53   CM and intermembrane space (IMS) from intracristal space (ICS). Indeed, the CM is enriched

54   in proteins involved in oxidative phosphorylation (OXPHOS), mitochondrial protein synthesis

55   or iron-sulfur cluster biogenesis, whereas the IBM mainly contains proteins involved in

56   mitochondrial fusion and protein import (Vogel et al, 2006). CJs could regulate bioenergetics

57   by limiting the diffusion of ADP/ATP and affect the pH gradient across the IM (Frey et al, 2002;

58   Mannella et al, 2013; Mannella et al, 1994; Mannella et al, 2001). As early as 1966, isolated

59   mitochondria are known to occur in different morphological states, condensed (matrix

60   condensed, high ADP, state III) or an orthodox (matrix expanded, low ADP, state IV) state,

61   depending on the bioenergetic status (Hackenbrock, 1966; Hackenbrock, 1968; Hackenbrock,

62   1972). Later, tomographic images of mitochondria undergoing this transition indicated that

63   remodelling of the IM occurs in isolated mitochondria (Mannella et al, 1994). Cristae exist in

64   different shapes and sizes depending on the physiological, bioenergetic or developmental cues

65   (Mannella et al, 1994; Nicastro et al, 2000; Zick et al, 2009). Moreover, the general ability of

66   cristae or CJs to dynamically remodel is well exemplified during apoptosis, where widening of

67   CJs is observed, promoting cytochrome *c* release from the intra-cristal space into the cytosol

68   (Frezza et al, 2006; Scorrano et al, 2002). However, molecular mechanisms for cristae and

69   CJs remodelling in response to metabolic and physiological adaptations are not known.

70   Aberrant and altered cristae are associated with several human diseases including

71   neurodegeneration, cancer, diabetes and cardiomyopathies (Vincent et al, 2016; Zick et al,

72   2009) but their relevance to disease progression is unclear.

73   The formation of a highly curved, membrane-embedded CJ structure is likely to require an

74   intricate partnership between phospholipids and scaffolding proteins (Koob & Reichert, 2014;

75   Quintana-Cabrera et al, 2017; Rampelt et al, 2016). We identified that Fcj1(*formation of crista*

76   *junction protein 1*)/Mic60 resides preferentially at CJs in yeast and its deletion leads to

77   complete loss of CJs with cristae arranged as concentric stacks separate from the IBM. In

78   addition, Fcj1/Mic60 and Su e/g (subunits of F_1F_o-ATP synthase) act antagonistically to control

79   F_1F_o-ATP synthase oligomerization and thereby modulate formation of CJs and cristae tips

80   (Rabl et al, 2009). Several groups have identified a large oligomeric complex termed MICOS

81   (Mitochondrial contact site and cristae organizing system) which is required for the formation

82   and maintenance of CJs and contact sites between IM and OM (Harner et al, 2011; Hoppins

83   et al, 2011; von der Malsburg et al, 2011). The MICOS complex contains at least seven

84   subunits in mammals: MIC10, MIC13, MIC19, MIC25, MIC26, MIC27 and MIC60 named after

85   a uniform nomenclature (Pfanner et al, 2014). MIC60 and MIC10 are considered to be the core

86   components of the MICOS complex in baker's yeast as their deletion causes complete loss of

87   CJs. MIC60 has a binding interface for a variety of proteins including TOM complex, OPA1,

88   SAM/TOB, Ugo1 (mammalian homolog SLC25A46), DnaJC11, CHCHD10, and DISC1

89   (Disrupted-in-Schizophrenia 1) and is proposed to provide the scaffold for MICOS as well as

90   contact between IM and OM (Abrams et al, 2015; Barrera et al, 2016; Genin et al, 2015;

91   Glytsou et al, 2016; Körner et al, 2012; Ott et al, 2012; Pinero-Martos et al, 2016; Xie et al,

92   2007). MIC10 contains conserved glycine motifs in its transmembrane domain that are crucial

93   for MIC10 self-oligomerization, and are required for the stability of CJs (Barbot et al, 2015;

94   Bohnert et al, 2015; Milenkovic & Larsson, 2015). MIC10 additionally interacts with the dimeric

95   F_1F_o-ATP synthase and promotes its oligomerization (Eydt et al, 2017; Rampelt et al, 2017).

96   Both MIC60 and MIC10 have the capability to bend membranes (Barbot et al, 2015;

97   Hessenberger et al, 2017; Tarasenko et al, 2017). Using complexome profiling, we identified

98   MIC26/APOO and MIC27/APOOL as *bona fide* subunits of the MICOS complex (Koob et al,

99   2015; Weber et al, 2013). Depletion or overexpression of MIC26 or MIC27 lead to altered

100   cristae morphology and reduced respiration. MIC27 binds to cardiolipin, the signature lipid in

101   mitochondria (Weber et al, 2013). The non-glycosylated form of MIC26 is a subunit of the

102   MICOS complex, but not the glycosylated form (Koob et al, 2015). Recently, another group

103   and we have discovered that MIC13/QIL1 is an essential component of the MICOS complex

104   and is responsible for the formation of CJs (Anand et al, 2016; Guarani et al, 2015a). Loss of

105   MIC13 resulted in reduced levels of MIC10, MIC26 and MIC27, accompanied by impaired

106   OXPHOS. The protein levels of MIC60, MIC19 and MIC25 remain unaltered, suggesting that

107   MICOS comprises two subcomplexes, MIC60/25/19 and MIC10/13/26/27 with MIC13 acting

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108 as a bridge between both subcomplexes (Anand et al, 2016; Guarani et al, 2015a). Altered

109 levels of MICOS components and their interactors are associated with many human diseases

110 such as epilepsy, Down syndrome, frontotemporal dementia-amyotrophic lateral sclerosis,

111 optic atrophy, Parkinson's disease, diabetes, and cardiomyopathy (Abrams et al, 2015; Penttila

112 et al, 2015; Zerbes et al, 2012). Mutations in *MIC60* have been found in Parkinson's disease

113 (Tsai et al, 2018). Mutations in *MIC13/QIL1* lead to mitochondrial encephalopathy and hepatic

114 dysfunction (Godiker et al, 2018; Guarani et al, 2016; Russell et al, 2019; Zeharia et al, 2016).

115 Here we studied cristae membrane remodelling in living cells and what role the MICOS

116 complex plays in this context. To study systematically intra-mitochondrial dynamics of CJs and

117 cristae, we devised a novel state-of-the-art method of live-cell STED super-resolution

118 nanoscopy using the C-terminally SNAP-tagged versions of distinct mitochondrial proteins

119 marking CJs and cristae. MIC10- and MIC60-SNAP marking CJs move dynamically and join

120 and split continuously at a time-scale of seconds in a MIC13-dependent manner. In

121 conjunction, we also observed that cristae marked by ATP5I-SNAP and COX8A-SNAP or by

122 IM-specific dyes undergo continuous fusion and fission cycles in a balanced manner in a

123 similar timescale of seconds. Overall, by improving temporal and spatial resolution using live-

124 cell STED super-resolution nanoscopy in combination with the SNAP-tag technology and use

125 of newly generated genetic models lacking MICOS subunits we achieved to resolve cristae

126 membrane dynamics. Based on these findings we propose a model rationalizing the

127 physiological importance of the MICOS complex and of cristae remodelling.

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Results

Mammalian MIC10 and MIC60 are required for cristae morphogenesis and cellular respiration

MIC60 and MIC10 are the core subunits of the MICOS complex that are also evolutionary well conserved (Huynen et al, 2016; Munoz-Gomez et al, 2015). As the mammalian MICOS complex is not well understood, we obtained human *MIC10* and *MIC60* knockout (KO) HAP1 cells. *MIC10* KO and *MIC60* KO have 29 bp deletion in exon 1 and 10 bp deletion in exon 8, respectively, leading to frameshift and subsequent loss of the respective proteins (**Fig** 1B). Analysis of electron micrographs from these cells revealed that CJs are virtually absent in *MIC10* and *MIC60* KO cells (**Fig** 1A, 1C). The cristae membrane (CM) appears as concentric rings detached from the IBM (**Fig** 1A), consistent with earlier observations in baker's yeast and mammalian cells (Callegari et al, 2019; Harner et al, 2011; Hoppins et al, 2011; John et al, 2005; Rabl et al, 2009; von der Malsburg et al, 2011). In addition, the abundance of cristae per mitochondrion is reduced (**Fig** 1D). While the knockout of *MIC10* primarily causes a selective destabilization of the MICOS subcomplex comprising MIC13, MIC26 and MIC27, loss of MIC60 results in a clear destabilization of all subunits of the MICOS complex (**Fig** 1B), confirming its role as a main scaffolding subunit of MICOS. The basal and the maximal oxygen consumption rates of *MIC10* and *MIC60* KOs are significantly decreased compared to controls (**Fig** 1E, IF), confirming the role of CJs in ensuring full bioenergetic capacity.

CJs are dispensable for regular arrangement of MIC60 in the IBM

MICOS is a large oligomeric complex present at CJs. Using STED (stimulated emission depletion) super-resolution images of fixed WT cells, we show a regularly spaced symmetrical arrangement of MIC10 and MIC60 punctae along the IBM (**Fig** 2A) consistent with earlier reports (Jans et al, 2013; Stoldt et al, 2019). We determined the distance between the two consecutive puncta of MIC10 and MIC60 in a mitochondrion and called it interpunctae distance

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156   (IPD). The IPD between two consecutive punctae was in the range of 280 nm both for MIC10

157   and MIC60 (**Fig** 2B) confirming that both subunits assemble into the MICOS complex in WT

158   cells. Since *MIC60* deletion lead to reduction of all proteins of MICOS, while loss of *MIC10* still

159   preserves MIC60 (**Fig** 1B), we asked whether MIC10 is required for the punctae-like

160   appearance of MIC60. Deletion of MIC10, albeit leading to a loss of CJs, did not disturb the

161   regular arrangement of MIC60 punctae along the mitochondrial length (**Fig** 2A). In line with

162   this, the IPD of MIC60 in control and *MIC10 KO* cells was not significantly different (**Fig** 2B).

163   Hence, lack of CJs and loss of the MICOS subcomplex of MIC10/13/26/27 did not alter the

164   symmetrical arrangement of MIC60, concluding that CJs are not necessary for regular spacing

165   of MIC60 in the IBM (**Fig** 2C). This supports the conclusion that MIC60 is positioned at uniform

166   distances in IBM representing a docking and scaffolding platform for other MICOS subunits

167   (**Fig** 2C) such as MIC10 for CJs formation, consistent with an earlier report in baker´s yeast

168   (Friedman et al, 2015).

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170   **Crista junction proteins show a markedly reduced mobility in the inner membrane and**

171   **loss of *MIC13* affects the mobility of distinct inner membrane proteins.**

172   We asked how mobile the two core MICOS subunits MIC60 and MIC10 are compared to other

173   membrane proteins localized to different mitochondrial subcompartments. We constructed

174   GFP-tagged versions of MIC60/MIC10, TOMM20, TIMM23 and ATP5I, established markers of

175   CJs, OM, IBM and CM respectively (**Fig** 3A; EV4)**,** and performed Fluorescence-Recovery

176   After-Photobleaching (FRAP) experiments. For TOMM20, TIMM23 and ATP5I we observed

177   substantially shorter $T^{1/2}$ recovery times and higher mobile fractions than for MIC10 and MIC60,

178   demonstrating that CJ proteins are much more restricted in movement compared to membrane

179   proteins of other mitochondrial complexes present in various subcompartments (**Fig** 3B, 3D,

180   EV1). In line with this, MIC60 was reported to show restricted diffusion in the inner membrane

181   compared to outer membrane proteins in another study (Appelhans & Busch, 2017). To know

182   whether the mobility of these proteins depends on the presence of a fully assembled MICOS

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183   complex, which is essential for formation of CJs, we generated *MIC13* KO Hela

184   cells as well suited for microscopy of mitochondria. Consistent with prior reports (Anand et al,

185   2016; Guarani et al, 2015a), we observed a loss of MIC10, MIC26, MIC27, altered cristae

186   morphology, and loss of CJs in *MIC13* KO Hela cells (**Fig** EV2). Upon deletion of *MIC13,* in

187   particular the mobility of TIMM23 and MIC10 was altered but not of MIC60 (**Fig** 3C, 3D, EV1B,

188   EV1D, EV1F, EV1G). The latter is consistent with our finding that MIC60 can arrange in

189   regularly spaced punctae in the absence of other MICOS subunits. Interestingly, the loss of

190   MIC13 decreases the mobile fraction of TIMM23 considerably. As in baker's yeast Tim23 was

191   shown to dynamically redistribute between the CM and the IBM in a manner dependent on

192   mitochondrial protein import (Vogel et al, 2006), we propose that the decreased TIMM23

193   mobile fraction in cells lacking CJs is due to trapping of a fraction of TIMM23 in the CM

194   providing experimental support for the role of CJs as 'gates' between the CM and the IBM and

195   acting as diffusion barriers. Moreover, the mobility of MIC10 increases drastically in *MIC13* KO

196   Hela cells compared to WT cells, in line with the view that MIC13 is required for specifically

197   stabilizing MIC10 at the MICOS complex (Anand et al, 2016; Guarani et al, 2015a).

198   To study the mobility of MIC10 and MIC60 by a different approach we used single particle

199   tracking (**Fig** EV3). For this, we constructed the SNAP-tagged versions of MIC60 and MIC10

200   and confirmed their functionality as revealed by coimmunoprecipitation using anti-MIC13

201   antibodies (**Fig** EV4AB), BN-PAGE analysis (**Fig** EV4D), or restoration of MIC13 levels upon

202   MIC10 expression (**Fig** EV4C). This confirmed that the tagging of MIC10 and MIC60 with

203   SNAP or GFP does not interfere with their function and proper incorporation into MICOS

204   complex. Using single particle tracking data, we observed that on average MIC60-SNAP

205   showed a smaller instantaneous diffusion coefficient (insD) compared to MIC10-SNAP (**Fig**

206   EV3E), consistent with the view that MIC60 represents regularly arranged nascent sites

207   priming CJ formation which has to be steadily positioned. Deletion of *MIC13* led to a significant

208   shift of tracks towards long-range movements (increased instantaneous diffusion coefficient;

209   insD) of both MIC10 and MIC60 suggesting an increased mobility in both cases (**Fig** EV3E).

210   This is fully in line with the FRAP data and the interpretation that the MICOS complex is not

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211   fully assembled in *MIC13 KO* cells. Further, in order to obtain a better understanding of the

212   dynamic behaviour of individual single particles, each track was further subdivided into

213   subtracks based on confinement in increasing order of directionality as confined diffusion,

214   subdiffusion, normal diffusion, or directed motion (**Fig** EV3F-N). The percentage of subtracks

215   showing directed motion is significantly higher for MIC10 than for MIC60 in WT Hela cells

216   suggesting a considerably higher directionality for MIC10 (**Fig** EV3FG). Additionally, directed

217   motion of both MIC10 & MIC60 is significantly higher in *MIC13* KO cells compared to WT (**Fig**

218   EV3FG), indicating that both subunits, when present in the fully assembled MICOS complex

219   have lower directed motion compared to the situation when the MICOS complex exists in

220   separate subcomplexes. Intriguingly, MIC10 directed motion in *MIC60* KOs is significantly

221   reduced compared to control cells indicating that the presence of MIC60 is specifically required

222   for the enhanced directed motion of MIC10 (**Fig** EV3F). This suggests that MIC10 is recruited

223   to the MICOS complex via MIC60. We propose that MIC60 acts as a docking platform

224   pioneering CJ formation, consistent with earlier findings in baker's yeast showing that MIC60-

225   GFP assembles into discrete spots in a strain lacking all MICOS subunits (Friedman et al,

226   2015).

227

228   **Crista junctions move dynamically within seconds**

229   FRAP and SPT data provided insights about the mobility of MIC10 and MIC60 as a group of

230   molecules or individual MIC10 and MIC60 molecules within the IBM, respectively. As we

231   observe regularly arranged CJ punctae using immunostaining of MIC10 and MIC60 and STED

232   nanoscopy (**Fig** 2A), we next asked how and at what timescale do individual CJs move. For

233   this, we devised a novel method to perform live-cell STED super-resolution nanoscopy using

234   MIC60-SNAP and MIC10-SNAP as markers of CJs. SNAP-tag, a well characterized protein

235   tag binds covalently to silicone-rhodamine (Gautier et al, 2008; Keppler et al, 2003) and allows

236   high-resolution imaging with minimal fluorophore bleaching (Lukinavicius et al, 2013). The

237   functionality and the proper incorporation of MIC10- and MIC60-SNAP into the MICOS

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238   complex was verified as described above (**Fig** EV4). The MIC10-SNAP and MIC60-SNAP

239   fusion proteins were arranged in the same regularly spaced punctae-like pattern across the

240   IBM (**Fig** 4A, 4C) as shown for the endogenous proteins using immunostaining (**Fig** 2A),

241   providing further evidence for proper localization of the tagged constructs. We were able to

242   image MIC10- and MIC60-SNAP for a total of ~15s at least every 2.6 seconds. While

243   mitochondria as a whole stay relatively static during this observation time, we observed rapid

244   movements of MIC10/MIC60-SNAP punctae during this short time period **(Movie 1, Movie 2)**,

245   demonstrating that CJs show an unexpectedly pronounced dynamic behaviour at a timescale

246   of seconds. We repeatedly visualized these movies to observe any regular pattern in

247   movement of MIC10 or MIC60 within a mitochondrion. Firstly, we focused on MIC10-SNAP

248   and manually tracked the individual punctae of MIC10 in the subsequent frames (**Fig** 4A,

249   **Movie 3).** We observed numerous instances where two MIC10-SNAP punctae rapidly joined

250   each other and split and re-joined at a timescale of seconds (**Fig** 4B, **Movie 3**). We quantified

251   the occurrence of joining/splitting events per unit length of mitochondria and found that the

252   number of joining and splitting events were similar in a given time, indicating that these events

253   are balanced and possibly coupled (**Fig** 5F). MIC60-SNAP showed a very similar pattern of

254   joining and splitting (**Fig** 4D, 5E, **Movie 4**) that occurred at similar temporal frequency as

255   MIC10-SNAP. We conclude that fully assembled MICOS in WT cells representing CJs undergo

256   continuous cycles of joining and splitting in a rapid and balanced manner.

257

258   **CJs undergo joining and splitting event in a MIC13-dependent manner**

259   To test whether the dynamic behaviour of MIC60-SNAP punctae depends on the presence of

260   a fully assembled MICOS complex, we analysed dynamics of MIC60-SNAP in *MIC13* KO cells.

261   We again observed the regular arrangement of MIC60-SNAP punctae in the IBM of

262   mitochondria in *MIC13* KO cells (**Fig** 5A) consistent with our observation that MIC60 forms

263   regularly arranged punctae also in the absence of MIC10 (**Fig** 2). MIC60-SNAP punctae

264   showed dynamic movements in *MIC13* KO cells (**Fig** 5B, **Movie 5**), however, this occurred at

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265   a markedly reduced frequency when compared to WT Hela cells (**Fig** 5B, 5E, **Movie 5**). This

266   indicates that, although the regular arrangement of MIC60 in principle is unaltered, the

267   movement of MIC60-positive punctae reflected normally by balanced joining and splitting

268   events is drastically impaired in the absence of CJs in *MIC13 KO* cells. We also overexpressed

269   MIC10-SNAP in *MIC13* KOs and found that MIC10-positive punctae are dynamic, yet that

270   joining and splitting events were markedly reduced as well (**Fig** 5C, 5D, 5F). These

271   observations indicate that the frequency of regular joining and splitting of CJs depends on a

272   functional MIC13-containing MICOS complex, which would be needed for cristae to emerge

273   from CJs (**Fig** 5G).

274   To further corroborate our findings on CJ dynamics, we decided to analyse the movement of

275   MIC13-SNAP, another marker for CJs, in WT Hela cells. Different from MIC60 or MIC10, we

276   observed that the staining pattern of MIC13-SNAP not only marked regularly arranged punctae

277   in the IBM but also appeared to label transverse bridges across mitochondria resembling

278   cristae membranes (**Fig** 6A). Apparently, MIC13-SNAP is dually localized to both CJs and

279   cristae. We cannot rule out that the partial localization of MIC13 to cristae is due to excess of

280   MIC13-SNAP in the IM. Nevertheless, in this context this would even beneficial as it can help

281   us to visualize cristae and CJs at the same time. We analysed the movement of MIC13-SNAP

282   in live-cell STED nanoscopy and observed rapid dynamics of both CJs and cristae membranes

283   at a timescale of seconds **(Movie 6).** We not only observed joining and splitting events of CJs

284   as shown by MIC10-SNAP and MIC60-SNAP, but also rapid movement of cristae attached to

285   these CJs (**Fig** 6B, 6D). Upon careful analysis we observed several instances showing that,

286   when two CJs join, they bring adjoining cristae close by to fuse along near the IBM (**Fig** 6B,

287   from 0s to 2.5s, **Movie 7**). Moreover, we observe other occasions where cristae were

288   apparently fusing along the length of other cristae, sometimes forming structures resembling

289   the letters X and Y (**Fig** 6B, at 5 s and 14.9 s, respectively). In many instances, fusion events

290   are coupled to a subsequent apparent fission event at or near the site of the prior fusion event

291   (**Fig** 6B, arrow pointing outwards). To better visualize these events, we acquired images every

292   1.3 s and also found numerous examples of fusion and fission of cristae (**Fig** 6C, 6D, **Movie**

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293 **8, Movie 9**). We propose that cristae undergo continuous remodelling in form of cycles of

294 fusion and fission in a manner that is linked to CJ dynamics.

295

Cristae undergo dynamic remodelling by continuous cycles of fusion and fission

297 To substantiate our observation of cristae fission and fusion using MIC13-SNAP, we obtained

298 SNAP tagged versions of *bona fide* cristae markers, ATP5I and COX8A of F_1F_O ATP synthase

299 and complex IV, respectively. Hela cells expressing ATP5I-SNAP and COX8A-SNAP were

300 imaged by live-cell STED super-resolution nanoscopy. The STED images of both ATP5I and

301 COX8A showed transversely spanning bridges across the mitochondria (**Fig** 7A, 7C),

302 consistent with electron micrographs of cristae in Hela cells. Cristae were rapidly changing

303 their position in consecutive timeframes confirming that cristae are dynamic within

304 mitochondria at a timescale of seconds (**Fig** 7B, 7D, **Movie 10**). Upon careful visualization, we

305 observed several instances where cristae came close together and apparently fused either

306 across the IBM (transverse type-fusion) or along their length (X- or Y- type fusion) (**Fig** 7B, 7D,

307 **Movie 11, Movie 12**), consistent with our observations in MIC13-SNAP. Again, in most of the

308 instances fusion events were accompanied by a subsequent fission event (**Fig** 7B, 7D). We

309 quantified the rate of fusion or fission events across the unit length of mitochondria (µm) and

310 determined that they occur in a balanced manner with ~2 events per µm per min (**Fig** 7G). As

311 opposed to the standard biochemistry textbook view, this makes it unlikely that cristae are

312 constantly attached to CJs but rather can pinch-off transiently. Subsequently, they can re-fuse

313 with the IBM or with other cristae or they can fuse across their length to make connections or

314 even a branched network. Moreover, we checked whether the presence of a fully assembled

315 MICOS is required for these continuous fusion and fission cycles using *MIC13* KO cells. We

316 overexpressed COX8A-SNAP in *MIC13* KO cells and found that fusion and fission cycles are

317 significantly reduced in cells lacking MIC13 (**Fig** 7E, 7G, **Movie 13**). Hence, a functional

318 MICOS complex is required for these dynamic processes further supporting the view that CJ

319 formation and dynamics are linked to cristae membrane dynamics**.**

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Cristae membrane dynamics allows redistribution of membrane potential indicating merging and content mixing of membranes

The timescales of intra-mitochondrial membrane dynamics, the balanced occurrence of these events, and the morphological features observed were very similar despite using a variety of protein markers (MIC10-, MIC60-, MIC13-, ATP5I- and COX8A-SNAP) for both CJs and cristae. This makes it unlikely that the observed cristae remodelling events are merely due to diffusion of different membrane protein complexes within a rather static IM. Still, we decided to use mitochondria-specific membrane dyes not labelling specific protein complexes. We further rationalized that cristae fusion events may result in an immediate change in the membrane potential $\Delta\Psi$ of the two fusion mates, similar to the situation previously described for fusion between two mitochondria (Twig et al, 2008). Thus, we used TMRM, a dye labelling the inner membrane in a membrane-potential manner. Performing STED with TMRM, as opposed to SNAP-tagged proteins, is challenging due to its high sensitivity to photo-bleaching. Nevertheless, we could record movies for a similar time period as we did with SNAP-tagged protein markers using STED nanoscopy (**Fig** 8A, 8B). TMRM labelling showed transversely arranged cristae that were highly dynamic, consistent with our results for SNAP-tagged inner membrane proteins (**Fig** 8A, 8B). Moreover, we observed instances of immediate distribution of a TMRM signal from one crista (e.g. high-intensity signal at 1.5s in **Fig.** 8B) to another crista and IBM (lower-intensity signal at 3s), coinciding with the time of a cristae fusion event. Our observations strongly suggest that the location of the TMRM label within one lipid bilayer is rapidly altered in a short time period, implying a true membrane fusion event accompanied with content mixing. We also stained mitochondria with a non-potential specific dye, nonyl acridine orange (NAO), and employed another microscopy high-resolution technique, namely confocal laser scanning using a Zeiss Airyscan module. We observed that the cristae membrane was stained as transverse bridges in mitochondria and found instances of cristae dynamics at similar timescales (**Fig** 8C). We thus confirmed by two dyes and by a different

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347   imaging technique that cristae and CJs are highly dynamic and undergo continuous cycles of

348   fusion and fission. Overall, we propose a model termed _Cri_stae-_F_ission-_F_usion' (CriFF)**.** In this

349   unified model, we illustrate the observed fusion and fission cycles of cristae and how they are

350   apparently linked to the observed dynamics of CJs (**Fig** 8D).

351

352   **Discussion**

353   The idea that cristae must be able to undergo remodelling under certain physiological

354   conditions has been around for half a decade, yet studying the dynamic behaviour of cristae

355   membrane in real time was technically challenging due to limitations in optical resolution of

356   diffraction-limited conventional light microscopy and due to the fact that electron microscopy

357   can only capture fixed samples. In addition, the majority of studies using super-resolution

358   techniques to study mitochondria have used fixed samples (Jans et al, 2013; Kukat et al, 2015;

359   Stoldt et al, 2019). STED super-resolution microscopy of living cells has been utilized so far to

360   visualize other cellular organelles, such as ER or Golgi (Schroeder et al, 2018; Shin et al,

361   2018). To date, only one study illustrated the power of Hessian structured illumination

362   microscopy by giving an example in a single mitochondrion indicating cristae dynamics (Huang

363   et al, 2018). Here we provide several lines of evidence demonstrating for the first time that CJs

364   and cristae undergo fission and fusion cycles in a MICOS-dependent manner. These findings

365   are based on the application of different advanced imaging techniques such as live-cell STED,

366   SPT, FRAP and confocal Airyscan microscopy, which were applied to a wide variety of protein

367   markers to visualize distinct mitochondrial substructures (MIC60-SNAP, MIC10-SNAP, MIC13-

368   SNAP, ATP5I-SNAP, COX8A-SNAP), as well as two dyes labelling the inner membrane. We

369   further obtained three mammalian knockout cell lines lacking _MIC10_, _MIC60_ and _MIC13_,

370   respectively, and expressed the various constructs of SNAP- and GFP- tagged mitochondrial

371   IM proteins in those and wild type cells to reveal the functional role of the MICOS complex in

372   this regard. Our improved tagging and imaging conditions allowed us to achieve a time

14

373   resolution of up to 1.2 - 2.6 s per frame revealing that CJs and cristae dynamics occurs at very

374   similar timescales (seconds) and both depend on the MICOS-complex.

375   The idea of MIC60 as a central pioneer of the MICOS complex was proposed from experiments

376   in baker's yeast as reintroducing MIC60-GFP into a strain lacking all MICOS subunits, shows

377   MIC60-GFP to be assembled into discrete spots (Friedman et al, 2015). This in addition to our

378   results corroborates that MIC60 is the pioneering subunit of the MICOS complex positioning

379   MICOS assembly and CJs formation. Overall, we propose the '*Cri*stae-*F*ission-*F*usion' (CriFF)

380   model which integrates our observations as follows (**Fig** 8D). (1) MIC60 serves as a docking

381   site for the formation of CJs via recruitment of other subunits of the MICOS complex. (2) Once

382   the MICOS complex is fully assembled, CJs are the sites for cristae to grow and emerge from

383   the IBM. This does not exclude that the MICOS complex can dynamically change its

384   composition or assembly-state. (3) CJs can dynamically join and split. (4) Cristae are mainly,

385   but not necessarily, attached to CJs. (5) Cristae can undergo transient pinching-off from CJs

386   and rejoining at the same CJ or another one (transverse or neighbouring CJ; **Fig** 8Di). The

387   formation of separated cristae subcompartments is consistent with another study showing that,

388   within the same mitochondrion, individual cristae can maintain a stable membrane potential

389   over time and that neighbouring cristae can maintain disparate $\Delta\Psi$ (preprint: Wolf et al, 2019).

390   This is also consistent with our FRAP data showing that TIMM23-GFP shows a reduced

391   mobility upon loss of CJs, presumably by enhanced trapping in the CM. (6) Formation of Y-

392   type cristae fusion may be preceded by CJ joining and subsequent cristae membrane

393   remodelling (**Fig** 8Dii). This would represent one possibility to link CJ dynamics and cristae

394   remodelling. (7) X-type cristae fusion could represent preceding CJ splitting from a Y-type like

395   cristae morphology or cristae fusion between cristae distant from CJs (**Fig** 8Diii). This clearly

396   is a working model and will require further testing in future studies.

397   What could be the physiological advantage for cristae to undergo fusion and fission and why

398   could impairment thereof lead to severe human diseases including mitochondrial

399   encephalopathy with liver dysfunction as reported for mutations in *MIC13* (Godiker et al, 2018;

15

400   Guarani et al, 2016; Russell et al, 2019; Zeharia et al, 2016)? Although future experiments will

401   have to provide the answer to these questions, from the results shown here and in other

402   studies, it is very clear that OXPHOS is impaired in cells lacking MICOS subunits such as

403   MIC10, MIC60, MIC26, MIC27, or MIC13 (Anand et al, 2016; Guarani et al, 2015b; Koob et al,

404   2015; Weber et al, 2013). How can we explain this and what could comprise possible functions

405   of highly dynamic cristae membranes? To answer this, it is worth to consider that cristae fission

406   would result in transient cristae subcompartments, which are physically isolated from the rest

407   of the inner membrane. This goes along with a reduced surface of the inner membrane that is

408   directly accessible to the cytosol via passing the intermembrane space and the outer

409   membrane. On the other hand, cristae fusion reverts this and leads to an increase in accessible

410   IM surface. The former situation is expected to favour trapping of metabolites and protons

411   within the intracristal space, whereas the latter would increase the accessible surface, which

412   is expected to favour metabolite exchange. As both processes, decreasing vs. increasing IM

413   surface, are critical for mitochondrial function we propose that cristae dynamics is a way to

414   adapt between these opposing necessities. Moreover, cristae fission and fusion may help to

415   distribute and mix proteins, lipids, and important metabolites in the mitochondrion. In summary,

416   we propose that cristae fission and fusion (CriFF) has major implications for the following

417   processes: (1) Proton trapping; fission of cristae at CJs, resulting in transient intramitochondrial

418   vesicles, could prevent proton leakage from the intercristal space to the cytosol, thereby

419   promoting ATP synthesis. This does not exclude other ways of proton trapping proposed

420   earlier, including proton buffering via cardiolipin (Kates et al, 1993) and preferential

421   electrostatic local concentration at highly curved cristae tips (Strauss et al, 2008). Interestingly,

422   pH differences within the intermembrane space have been reported, as the pH near ATP5I-

423   SEcGFP (Superecliptic GFP, pH sensitive version of GFP), residing at cristae tips, was shown

424   to be 0.3 higher compared to COX8A-SEcGFP (Rieger et al, 2014) located in the cristae. This

425   was proposed to reflect a concentration gradient of protons, which dilutes at the cristae tips,

426   which appear to act as a sink. (2) Content mixing; cristae fission and fusion may promote

427   metabolite exchange including ADP, which was proposed to be limiting under certain

16

428 conditions for OXPHOS (Mannella et al, 2013). (3) Intra-mitochondrial quality control by protein

429 content mixing; ongoing fusion and fission dynamics of cristae may serve to mix partially

430 damaged protein complexes, such as ETC complexes, in the CM to allow intra-mitochondrial

431 complementation and protein quality control by proteases. (4) Biogenesis of membrane protein

432 complexes; continuous mixing of newly imported protein subunits (nuclear as well as mtDNA

433 encoded) may promote efficient assembly of membrane protein complexes, in particular when

434 considering that the cristae membrane is very densely packed with proteins. This view is

435 consistent with hybrid cristae, which were reported to be formed upon mitochondrial fusion,

436 following PEG fusion of cells expressing mitochondria with different tags to respiratory chain

437 complexes (Wilkens et al, 2013). (5) Lipid remodeling, mixing, and exchange; cristae dynamics

438 could promote exchange of lipids between organelles and OM and IM and optimize cardiolipin

439 synthesis. Contact sites between the IM and the OM, defined by the MICOS complex, were

440 shown to coordinate the synthesis of phosphotidylethanolamine by Psd1 to dictate lipid

441 remodeling in mitochondrial membranes (Aaltonen et al, 2016). (6) Other processes likely to

442 be affected by altered cristae dynamics could include redox homeostasis, thermogenesis, Ca^{2+}

443 buffering, and iron sulfur biogenesis.

444 What are the molecular machineries required for these processes? We show that these

445 processes are dependent on fully assembled MICOS complex in a MIC13-dependent manner.

446 This and the fact that both, CJs and cristae dynamics, occur at the similar timescales in a

447 balanced manner, strongly suggest that they are mechanistically linked. Indeed, the MIC13-

448 SNAP live-cell STED movies allow the simulatenous visualization of CJs and cristae

449 supporting this view. Still, we cannot fully exclude that the MICOS complex has two

450 independent functions explaining our observation equally well. Further experiments will provide

451 insights into the bioenergetic parameters and the molecular mechanisms of these fascinatingly

452 fast and continuously occurring dynamics of CJs and cristae.

453 In conclusion, live-cell STED super-resolution nanoscopy in combination with biochemical and

454 genetic methods has expanded our view on the dynamics of IM remodelling at a nanoscopic

455 level in real-time. Future research will provide further insights into the regulation, physiology,

456 and pathophysiology of these newly revealed dynamic processes occurring within

457 mitochondria.

458

459 **Materials and Methods**

460 **Cell culture, transfection and generation of knockout cell lines.** Hela and HEK293 cells

461 were maintained in DMEM (Sigma-Aldrich) supplemented with 10 % fetal bovine serum (PAN

462 Biotech), 2 mM glutaMAX (Gibco), 1 mM sodium pyruvate (Gibco) and penstrep (Sigma-

463 Aldrich, penicillin 100 units/ml and streptomycin 100 µg/ml) whereas HAP1 cells were cultured

464 using Isocove´s modified DMEM media (IMDM) supplemented with 20 % fetal bovine serum

465 (PAN Biotech), 2 mM glutaMAX (Gibco) and penstrep (Sigma-Aldrich, penicillin 100 units/ml

466 and streptomycin 100 µg/ml). Cells were grown in incubator with 37°C and 5% CO_2. All cell

467 lines were tested negative for possible mycoplasma contamination. Hela and HAP1 cells were

468 transfected with 1 µg of corresponding plasmid using GeneJuice® (Novagen) according to the

469 manufacturer's instructions. In case of SNAP-tag constructs, 0.2 µg of MitoGFP (matrix-

470 targeted) and 1 µg of corresponding SNAP-tag were co-transfected. HEK293 cells were grown

471 on a large-scale in 10 cm dishes and transfected with 10 µg of respective plasmids were used

472 for biochemical experiments. *MIC13* KO Hela cells were generated using CRISPR/Cas method

473 as described before (Anand et al, 2016). *MIC10* and *MIC60* KO HAP1 cells along with WT cells

474 were custom-made by Horizon (UK).

475 **Molecular cloning.** Human MIC60, MIC10, MIC13, ATP5I and TOMM20 were cloned into

476 pSNAPf vector (NEB) using Gibson assembly cloning kit (NEB). COX8A-SNAP vector was

477 commercially obtained from NEB. MIC60 and MIC10 were cloned into pEGFPN1 using

478 restriction digestion by *Xho*I and *Bam*H1 followed by ligation. Human TOMM20, TIMM23,

479 ATP5I were cloned into pEGFPN1 vector using Gibson assembly cloning kit (NEB) to acquire

480 their respective GFP-tagged versions.

18

481   **SDS electrophoresis and western blotting.** For preparing samples for western blotting,

482   corresponding cells were collected and proteins were extracted using RIPA lysis buffer. The

483   amount of solubilized proteins in all the samples were determined using the Lowry method

484   (Biorad). 15% SDS-PAGE was performed, separated proteins were subsequently blotted onto

485   a PVDF membrane, and probed with indicated antibodies: MIC10 from Abcam (84969), MIC13

486   (custom-made by Pineda (Berlin) against human MIC13 peptide

487   CKAREYSKEGWEYVKARTK), MIC19 (Proteintech, 25625-1-AP), MIC25 (Proteintech,

488   20639-1-AP), MIC26 (Thermo-Fisher, MA5-15493), MIC27 (Atlas Antibodies, HPA000612),

489   MIC60 (custom-made, Pineda (Berlin) against human IMMT using the peptide

490   CTDHPEIGEGKPTPALSEEAS), SNAP-tag (P9310S, NEB), and β-tubulin (abcam, ab6046).

491   Goat anti-mouse IgG HRP conjugated (ab97023) and goat anti-rabbit IgG HRP conjugated

492   antibody (Dianova, 111-035-144) were used as secondary antibodies. Chemiluminescence

493   was captured using a VILBER LOURMAT Fusion SL (Peqlab).

494   **Coimmunoprecipitation**

495   For coimmunoprecipitation, isolated mitochondria from HEK293 cells overexpressing either

496   MIC10-SNAP, MIC10-GFP, MIC60-SNAP or MIC60-GFP were used. Mitochondrial isolation

497   was done according as described before (Anand et al, 2016). The coimmunoprecipiation

498   experiment was performed using the protocol described in (Barrera et al, 2016) with following

499   modification. The beads were incubated with 4 µg of MIC13 antibody (custom-made by Pineda

500   (Berlin) against human MIC13 peptide CKAREYSKEGWEYVKARTK). During lysis of the

501   mitochondria a detergent/protein ratio of 2 g/g was used.

502   **Isolation of macromolecular complexes by blue native gels**

503   Mitochondria from HEK293 cells overexpressing MIC60-SNAP or MIC60-GFP were isolated

504   as previously shown by (Anand et al, 2016). The experiment was performed as shown by

505   (Anand et al, 2016) with the use of a detergent/protein ration of 2 g/g during solubilization.

506 **Electron microscopy.** HAP1 WT, *MIC10* KO and *MIC60* KO cells were grown on petri dishes,

507 cells were washed with PBS, and fixed using 3% glutaraldehyde in 0.1M sodium cacodylate

508 buffer, pH 7.2. After fixation, cells were collected in a small tube using a cell scraper and

509 pelleted. These cell pellets were washed with 0.1M sodium cacodylate, pH 7.2 and

510 subsequently embedded in 2% agarose. The pellets were stained using 1% osmium tetroxide

511 for 50 min and 1% uranyl acetate/ 1% phosphotungstic acid for 1 h. The samples were

512 dehydrated using graded acetone series and embedded in spur epoxy resin for polymerization

513 at 65°C for 24 h. The ultrathin sections were prepared using microtome and the images were

514 acquired using transmission electron microscope (Hitachi, H600) at 75V equipped with

515 Bioscan model 792 camera (Gatan) and analysed with ImageJ software.

516 **Cellular respiration measurements.** All respiration measurements were performed using

517 Seahorse XFe96 Analyzer (Agilent). The HAP1 cells were seeded in Seahorse XF96 cell

518 culture plate (Agilent) at a density of 30,000 cells per well overnight. Next day, cells were

519 washed and incubated in basic DMEM media (Sigma, D5030) supplemented with glucose,

520 glutamine and pyruvate at 37°C in non-CO_2 incubator 1 h prior to the assay. Mitochondrial

521 respiration function was measured using Seahorse XF Cell Mito Stress Test kit (Agilent)

522 according to the manufacturer's instructions. Briefly, the delivery chambers of the sensor

523 cartridge were loaded with oligomycin (F_1F_O-ATPase synthase inhibitor) or FCCP (uncoupler)

524 or rotenone and antimycin (Complex I and Complex III inhibitors, respectively) to measure

525 basal, proton leak, maximum and residual respiration. Cell number was normalized after the

526 run using Hoechst staining. Data was analysed using wave software (Agilent).

527 **Immunofluorescence staining.** HAP1 cells were fixed with pre-warmed (37°C) 3.7 %

528 paraformaldehyde for 15 min. After fixation, cells were washed 3 times with PBS,

529 permeabilized with 0.15 % Triton-X 100 for 15 min, blocked using 10 % goat serum for 15 min

530 followed by incubation with appropriate dilution of primary antibodies for 3 h at room

531 temperature or overnight at 4°C. After washing thrice with PBS, samples were incubated at

532 room temperature with appropriate secondary antibody for 1 h and washed three times with

20

533   PBS before proceeding for microscopy. For STED super-resolution imaging primary antibodies

534   used were against MIC60 (custom-made, Pineda (Berlin)), MIC10 (abcam, 84969). Goat anti-

535   rabbit Abberior STAR 635P (abberior) was used as secondary antibody.

536   **Quantification of mitochondrial interpunctae distance (IPD).** The longitudinal distance

537   along the mitochondrial length between two successive MIC60 or MIC10 punctae is termed as

538   interpunctae distance (IPD). The IPD between MIC60 and MIC10 punctae was calculated

539   using the Image J software by manually drawing lines between two spots from the centre of

540   the punctae and measured using the 'Analyze' function to calculate the length of that particular

541   line. In order to avoid repetition of measuring the IPD, the length between the punctae was

542   measured in a clockwise direction. If the edges of mitochondria containing the MIC60 or MIC10

543   spots were curved, a segmented line tool was used to measure the IPD. In rare cases where

544   MIC60 spots were replaced by longitudinal bridges, the centre of the line was taken into

545   consideration for defining the spot. The distance between MIC60 or MIC10 punctae per

546   mitochondrion was calculated (from an average of 36-55 spots/mitochondrion) and a median

547   interpunctae distance was obtained for that individual mitochondrion and plotted as boxplots.

548   **2D Live-STED (stimulated emission depletion) super-resolution nanoscopy.** Cells

549   transfected with corresponding SNAP-tags and stained with silicone-rhodamine dye (SNAP-

550   cell 647-SiR (NEB) (3 μM)) were imaged at 37°C and 5 % CO_2 mimicking cell culture incubator

551   conditions in fluoroBrite DMEM media supplemented with 10 % fetal bovine serum (PAN

552   Biotech), 2 mM glutaMAX (Gibco), 1 mM sodium pyruvate (Gibco) and penstrep (Sigma-

553   Aldrich, penicillin 100 units/ml and streptomycin 100 μg/ml). For TMRM imaging, Hela cells

554   were stained with 50 nM TMRM dye (Invitrogen) for 30 mins. Live-STED super-resolution

555   nanoscopy was performed on Leica SP8 laser scanning confocal microscope fitted with a

556   STED module. Before imaging the alignment of excitation and depletion laser was checked in

557   reflection mode using colloidal 80 nm gold particles (BBI Solutions). Cells expressing the

558   SNAP-tag and stained with SNAP-cell 647-SiR (NEB) were excited with a white light laser at

559   an excitation wavelength of 633 nm. Images were collected using a hybrid detector (HyD) at

21

560   an emission range from 640 nm to 730 nm using a 93X glycerol (N.A = 1.3) or 100X oil objective

561   (N.A = 1.4) while using a pulsed STED depletion laser beam at 775 nm emission wavelength.

562   The images were obtained at a zoom to acquire 9.7 X 9.7 µm area (12X magnification for 100X

563   or 12.9 X magnification for 93X objective). Movies were obtained at a frame rate of ~2.5

564   s/frame. Gating STED was used from 1 ns onwards in order to increase the specificity of the

565   signal in experiments where silicone-rhodamine was used. Image processing post-image

566   acquisition of live-cell SNAP movies was performed with Huygens Deconvolution software.

567   Due to the strong depletion and the drastic decrease of emitting fluorophores in STED

568   microscopy, the resulting images tend to have a lower signal to noise ratio. The increase of

569   axial resolution, and by that reduction of out of focus blur, is not the only benefit of

570   deconvolution. The other major advantages are increase in signal to noise ratio and resolution,

571   which enhance image quality for 2D datasets as well, as shown before (Schoonderwoert et al,

572   2013).

573   **Quantification of crista junction and cristae dynamics.** Intramitochondrial joining/splitting

574   events in case of MIC10- and MIC60-SNAP marking CJs and fusion/fission events in case of

575   COX8A-SNAP marking cristae were quantified manually by observing the corresponding live-

576   STED movies for a time span of ~15 s obtained at a frame rate of ~2.5 s/frame. The number

577   of joining/splitting or fusion/fission events were   divided by the mitochondrial length to yield

578   corresponding number of events/ unit length (µm) of mitochondrion. Events/µm length/min of

579   mitochondria was subsequently calculated. All quantifications were performed with 3

580   independent experiments using 5 to 10 mitochondria, which were all chosen from separate

581   cells.

582   **FRAP and associated quantification.** FRAP experiments were performed on Leica SP8

583   using the FRAP module with Fly mode function switched on. Images were acquired with 40 X

584   water objective (N.A = 1.1) using 25 X zoom. In order to avoid acquisition photobleaching, only

585   1-1.5% laser power of the Argon laser line at 488 nm was used to acquire the images using a

586   PMT in green emission range. A square region of 0.7 X 0.7 µm was bleached using 100 %

587   laser power at 488 nm. 10 pre-bleach images were acquired while 10 images were acquired

588   during bleaching. 200 post-bleach images were acquired at a maximal possible frame rate of

589   88-89 ms/frame to monitor the recovery of fluorescence. After the images were acquired,

590   quantification of the FRAP experiment was done in the following way: three different ROIs

591   (Regions of Interest) were taken into consideration: 1) ROI^1, an area where no mitochondria

592   were found in the image, was used to perform background subtraction. 2) ROI^2 was the area

593   of mitochondria where the photobleaching was performed. 3) ROI^3, another region of a

594   separate mitochondria not subjected to FRAP, was used to obtain correction factor for

595   acquisition photobeaching. $ROI^2(P)$ was the average of 10 pre-bleach measurements of ROI^2

596   whereas $ROI^3(P)$ was the average of 10 pre-bleach measurements of ROI^3. Hence,

597   photobleach correction was performed by using the formula: ROI^2-ROI^1/ROI^3-ROI^1 and

598   normalization was performed by using the formula: ROI^2-ROI^1/ROI^3-ROI^1 X $ROI^3(P)$-

599    $ROI^1/ROI^2(P)-ROI^1$. All the mitochondria belonging to a particular condition from independent

600   experiments were pooled and averaged for their FRAP curves. Standard error of mean (SEM)

601   was plotted for all the pooled mitochondria for each condition.

602   Once the FRAP recovery values were obtained, they were fitted, using Graphpad Prism 7.04,

603   by nonlinear regression two phase association model for all molecules. First and second phase

604   association $T^{1/2}$ recovery values were obtained for each condition when the curves were fitted

605   by nonlinear regression two phase association model. Mobile fraction was calculated by using

606   the formula: $F_m = F_p-F_0/F_{initial}-F_0$ where F_m denotes the mobile fraction, F_p denotes the fraction

607   of fluorescence when the plateau is reached, $F_{initial}$ is 1 and F_0 is the fraction of initial

608   fluorescence after the last pulse of photobleaching for all cases except MIC60-GFP, MIC10-

609   GFP and TOMM20-GFP (in *MIC13* KO cells), where the Fp was calculated from the average

610   of last 5 intensity values. Diffusion coefficients (D) were calculated using the formula D=0.25

611   X $r^2/T^{1/2}$, where r is the radius of the bleached ROI and $T^{1/2}$ is the recovery time in seconds, as

612   suggested before (Kang et al, 2012). We bleached a square ROI region of 0.49 μm^2 in the

613   mitochondria for our experiments. Hence, we used 0.49 μm^2 as the area of the circle to

614   calculate the radius.

23

615   **Single particle tracking imaging and quantification.** Cells transfected with the SNAP-tag

616   and stained with low concentrations of SNAP-cell 647-SiR (NEB) (15 nM for MIC60-SNAP and

617   0.225 nM for MIC10-SNAP) were imaged in fluoroBrite DMEM media supplemented with 10 %

618   fetal bovine serum (PAN Biotech), 2 mM glutaMAX (Gibco), 1 mM sodium pyruvate (Gibco)

619   and penstrep (Sigma-Aldrich, penicillin 100 units/ml and streptomycin 100 µg/ml). Lower

620   concentrations of silicone-rhodamine were used here compared to STED super-resolution

621   imaging to allow for selective labelling of only few molecules in a mitochondrion. Movies were

622   acquired on a Zeiss Elyra PS.1 microscope equipped with a 63x (NA = 1.46) objective lens in

623   total internal reflection fluorescence (TIRF) mode, where highly inclined and laminated optical

624   sheet (HILO) illumination was used, at a frame rate of 33 ms/frame for 1000 frames. The angle

625   of the illuminating laser, EMCCD gain and laser intensity were manually adjusted to acquire

626   the best signal to noise ratio (SNR). The first 50-100 frames of every movie were not used for

627   analyses so that acquisition bleaching will additionally provide a sufficiently low concentration

628   of single particles. For analyses of movies, a ROI was set around each cell and single particle

629   tracking (SPT) was performed using the Fiji / ImageJ (Schindelin et al, 2012) plugin TrackMate

630   (Tinevez et al, 2017) where the following TrackMate settings were used: Detector: Laplacian

631   of Gaussian, Estimated blob diameter: 0.5 µm, Do sub-pixel localisation: Yes, Initial

632   thresholding: No, Filter on spots: SNR above 0.4. For tracking spots, the LAP (Linear

633   Assignment Problem) tracker with following settings worked well: Frame to frame linking: 0.3

634   µm, Gap-closing maximal distance: 0.5 µm, Gap-closing maximal frame gap: 5 frames. The

635   filter on tracks function were used to only analyse tracks with more than 20 spots within a track.

636   After analysis, the calculated mean square displacement (MSDs) and instantaneous diffusion

637   coefficients (insD) of all tracks were loaded into the Fiji plugin "Trajectory Classifier" (Wagner

638   et al, 2017). MSD analysis allows to determine the mode of displacement of particles over time.

639   For the calculation of the MSD for every track and time point, the MATLAB class

640   @msdanalyzer was used (Tarantino et al, 2014). Additionally the insD were calculated using

641   insD=mean MSD / 4*dt (Giannone et al, 2010) where mean MSD were calculated using the

642   first 4 MSD points of every track. Comparison of our results of insD of well characterized GPI-

24

643 anchored proteins in our hands matched well with literature validating our SPT method and its

644 quantification (data not shown). Beside for the calculation of the MSD and insD, tracks were

645 analysed using the Fiji plugin Trajectory classifier (Wagner et al, 2017). The plugin classifies

646 tracks generated with TrackMate into confined diffusion, subdiffusion, normal diffusion and

647 directed motion in increasing order of directionality. As most tracks were relatively short,

648 following settings for the classification were used: Minimal track length: 20, Window size: 10,

649 Minimal segment length: 10. SPT data was obtained for each condition in 2 independent

650 experiments from 2-5 cells in each experiment.

651 **Airyscan microscopy**

652 Hela cells were stained with 100nM nonyl acridine orange for 1 h and imaged using Plan-

653 Apochromat 100X/1.46 Oil DIC M27 objective on the Zeiss LSM 880 with Airyscan. Raw.czi

654 files were processed into deconvoluted images using the Zen software automatically.

655 **Statistics.** Statistical analysis for different experiments were done in Graphpad Prism 7.04

656 using unpaired Student's t-test except for comparison in Seahorse experiments where

657 differences in basal oxygen consumption were compared by one sample t-test. Mean ± SEM

658 was used for different experiments and sample size was not predetermined using any

659 statistical methods.

660

25

668   (ASR), Research Committee of the Medical faculty of Heinrich Heine University Düsseldorf

669   FoKo-37/2015 (AKK) and Foko-02/2015 (RA & ASR), SFB 1208 Project Z02 (SWP) and grant

670   WE 5343/1-1 (SWP).

671   **Author Contributions**

672   AKK, RA and ASR developed the underlying concept of the study, designed the experiments

673   and wrote the manuscript with comments from all other authors. AKK performed all

674   experiments of STED super-resolution imaging with help from SH and SWP. RA performed all

675   the biochemical experiments, and electron microscopy. FRAP experiments were done by AKK

676   with help from SWP. TZ and AKK performed the SPT experiments while TZ performed

677   quantification of SPT data using Image J with a MATLAB interface. JU characterized the *MIC13*

678   KO cells and performed co-IP and BN-PAGE experiments. DMW, MS performed all

679   experiments using Zeiss Airyscan imaging and analysis. ML and OSS contributed with

680   scientific and critical inputs to the manuscript.

681   **Conflict of interest**

682   The authors declare that they have no conflict of interest.

683

26

Figure Legends

Figure 1 - *MIC10* and *MIC60* KO HAP1 cells show loss of crista junctions and impaired cellular respiration.

A Representative electron micrographs of WT, *MIC10* KO and *MIC60* KO HAP1 cells. Scale bar 0.5 µm.

B Western blot analysis of lysates from WT and *MIC10* KO or *MIC60* KO HAP1 cells. *MIC10* KO show a drastic reduction of MIC13, MIC26 and MIC27 protein levels while protein levels of other MICOS components remain unchanged. *MIC60* KO cells show a drastic reduction of protein levels of all MICOS components.

C Quantification of CJs per mitochondrion in WT, *MIC10* and *MIC60* KO HAP1 cells using EM represented as boxplot (2 independent experiments, 24-40 mitochondria for each experiment). ****$P < 0.0001$ (using unpaired Student's *t*-test).

D Quantification of cristae per mitochondrion in WT, *MIC10* and *MIC60* KO HAP1 cells using EM represented as boxplot (2 independent experiments, 24-40 mitochondria for each experiment). ****$P < 0.0001$ (using unpaired Student's *t*-test).

E Comparison of oxygen consumption rates of basal, proton leak, maximum and non-mitochondrial respiration in WT and *MIC10* KO cells obtained from 3 independent experiments. *$P = 0.034$ for basal (using one sample *t*-test) and *$P = 0.018$ for maximum respiration (using unpaired Student's *t*-test).

F Comparison of oxygen consumption rates of basal, proton leak, maximum and non-mitochondrial respiration in WT and *MIC60* KO cells obtained from 3 independent experiments. *$P = 0.01$ for basal (using one sample *t*-test), ***$P = 0.0004$ for maximum respiration and *$P = 0.012$ for non-mitochondrial respiration (using unpaired Student's *t*-test).

27

709 **Figure 2 - MIC60 assembled as regularly spaced punctae in absence of CJs**

710 **A** Representative STED super-resolution images of WT HAP1 or *MIC10* KO HAP1 cells

711 immunostained with indicated antibodies (MIC60 or MIC10). Scale bar 1 μm.

712 **B** Quantification of mitochondrial interpunctae distances (IPD) between MIC60 or MIC10

713 punctae (2 independent experiments, 5-10 mitochondria for each experiment). ns, not

714 significant, unpaired Student's *t*-test.

715 **C** Scheme representing MIC60 marking the nascent sites of CJs formation independent of

716 MIC10.

717 **Figure 3 - Mobility of crista junction proteins is restricted compared to proteins of other**

718 **mitochondrial subcompartments and loss of MIC13 affects the mobility of distinct inner**

719 **membrane proteins.**

720 **A** Scheme of investigated marker proteins, subjected to FRAP, located at different

721 mitochondrial subcompartments.

722 **B, C** FRAP curves (curve fitted) of WT **(B)** and *MIC13* KO Hela cells **(C)** expressing GFP-

723 tagged fusion proteins of MIC60 (CJs), MIC10 (CJs), TOMM20 (OM), TIMM23 (IBM) and

724 ATP5I (CM). Average FRAP curves (intensities in arbitrary units) for each marker were

725 obtained (3 independent experiments, 6-10 mitochondria for each experiment). Error bars for

726 each time point representing SEM are shown.

727 **D** Histogram showing average percentage of mobile fractions, obtained from curve fits of all

728 mitochondria expressing of GFP-tagged versions of MIC60 (CJs), MIC10 (CJs), TOMM20

729 (OM), TIMM23 (IBM) and ATP5I (CM) in WT and *MIC13* KO Hela cells.

730 **Figure 4 - Crista junctions marked by MIC60-SNAP and MIC10-SNAP dynamically join**

731 **and split within a mitochondrion**

28

A, C Representative live-cell STED super-resolution image (t=0 s) showing WT Hela cells expressing MIC10-SNAP **(A)** and MIC60-SNAP **(C)** stained with silicone-rhodamine. Box in **A** and **C** mark selection shown as a zoom in panel **B** and **D** respectively. Scale bar 0.5 µm.

B, D Time-lapse image series of a mitochondrion expressing MIC10-SNAP **(B)** or MIC60-SNAP **(D)** (2.6 s/frame). Green and magenta asterisks show joining and splitting events of MIC10-SNAP and MIC60-SNAP, respectively. Green arrows pointing inward connected by solid line and magenta arrows pointing outward connected by dotted line show sites of imminent joining and splitting events, respectively. Scale bar 0.5 µm.

Figure 5 - Joining and splitting of crista junctions occur in a MIC13-dependent manner

A, C Representative live-cell STED super-resolution image (t=0 s) showing *MIC13* KO Hela cells expressing MIC10-SNAP **(A)** or MIC60-SNAP **(C)** stained with silicone-rhodamine. Box in **A** and **C** mark selection shown as a zoom in panel **B** and **D** respectively. Scale bar 0.5 µm.

B, D Time-lapse image series of a mitochondrion expressing MIC10-SNAP **(B)** and MIC60-SNAP in *MIC13* KO cells **(D)** (2.6 s/frame). Green and magenta asterisks show joining and splitting events of MIC10-SNAP and MIC60-SNAP, respectively. Green arrows pointing inward connected by solid line and magenta arrows pointing outward connected by dotted line show sites of imminent joining and splitting events, respectively. Scale bar 0.5 µm.

E Quantification of joining and splitting events of CJs in WT Hela and *MIC13* KO cells expressing MIC60-SNAP (3 independent experiments, 5-7 mitochondria for each experiment). ***P = 0.0004 (joining events WT vs. *MIC13* KO) and *P =0.019 (splitting events WT vs. *MIC13* KO), unpaired Student's *t*-test.

F Quantification of joining and splitting events of CJs in WT Hela and *MIC13* KO cells expressing MIC10-SNAP (3 independent experiments, 5-8 mitochondria for each experiment). ***P = 0.0002 (joining events WT vs. *MIC13* KO) and ****P < 0.0001 (splitting events WT vs. *MIC13* KO), unpaired Student's *t*-test.

G Scheme representing the dynamic nature of CJs in WT and *MIC13* KOs cells.

29

758   **Figure 6 - MIC13-SNAP shows that CJs and cristae undergo remodelling at a timescale**

759   **of seconds**

760   **A, C** Representative live-cell STED super-resolution image (t=0 s), showing WT Hela cells

761   expressing MIC13-SNAP, from a time-series of images acquired at a time interval of 2.5 s **(A)**

762   or 1.3 s **(C)** stained with silicone-rhodamine. Box in **A** and **C** mark selection shown as a zoom

763   in panel **B** and **D** respectively. Scale bar 0.5 µm.

764   **B, D** Time-lapse image series of a mitochondrion expressing MIC13-SNAP imaged at a time

765   interval of 2.5 s/ frame **(B)** or 1.3 s/ frame **(D)**. Green and magenta asterisks show fusion and

766   fission cycles of cristae marked by MIC13-SNAP. Green arrows pointing inward connected by

767   solid line and magenta arrows pointing outward connected by dotted line show sites of

768   imminent joining/fusion and splitting/fission events, respectively. A cristae fusion event that

769   forms a structure resembling letter `X´ is marked in B at 5 s.

770

771   **Figure 7 - Cristae undergo balanced fusion and fission events in a MICOS-dependent**

772   **manner.**

773   **A, C** Representative live-cell STED super-resolution image (t=0 s) showing WT Hela cells

774   expressing ATP5I-SNAP **(A)** or COX8A-SNAP **(C)** stained with silicone-rhodamine. Box in **A**

775   and **C** mark selection shown as a zoom in panel **B** and **D** respectively. Scale bar 0.5 µm.

776   **B, D** Time-lapse image series of a mitochondrion expressing ATP5I-SNAP **(B)** or COX8A-

777   SNAP **(D)** (~2.5 s/frame). Green and magenta asterisks show fusion and fission events of

778   cristae marked by ATP5I-SNAP or COX8A-SNAP, respectively. Green arrows pointing inward

779   connected by solid line and magenta arrows pointing outward connected by dotted line show

780   sites of imminent joining and splitting events, respectively.

781   **E** Representative live-cell STED super-resolution image (t=0 s) showing *MIC13 KO* Hela cells

782   expressing COX8A-SNAP stained with silicone-rhodamine. Box in **E** mark selection shown as

783   a zoom in panel **F** respectively. Scale bar 0.5 µm.

F Time-lapse image series of a mitochondrion expressing COX8A-SNAP in *MIC13 KO* **(D)** (2.6 s/frame). Rare fusion and fission events of cristae occurring in *MIC13* KO are marked by green and magenta asterisks respectively. Green arrows pointing inward connected by solid line and magenta arrows pointing outward connected by dotted line show sites of imminent fusion and fission respectively.

G Quantification of cristae fission and fusion events in WT and *MIC13* KO Hela cells expressing COX8A-SNAP (3 independent experiments, 5-10 mitochondria for each experiment; ****$P < 0.0001$, unpaired Student's *t*-test).

Figure 8 - Cristae fission and fusion is corroborated using mitochondrial inner membrane-specific dyes

A Representative live-cell STED super-resolution image (t=0 s) showing WT Hela cells stained with TMRM. Box in **A** marks selection shown as a zoom in panel **B**. Scale bar 0.5 µm.

B Time-lapse image series of a mitochondrion stained with TMRM (1.5 s/frame). Green asterisks show fusion events and redistribution of TMRM intensity from a high-intensity cristae to a low-intensity cristae (two such instances are shown at 3 and 7.4 s). Green arrows pointing inward connected by solid line at 1.5 and 5.9 s indicate imminent fusion events

C Representative time-lapse images of WT Hela cells stained with NAO, and imaged using Airyscan microscope, indicate cristae fusion and fission dynamics. Yellow and magenta asterisks show fusion and fission events of cristae respectively. Yellow arrows pointing inward connected by solid line and magenta arrows pointing outward connected by dotted line show sites of imminent fusion and fission events, respectively. Scale bar 0.5 µm.

D Schematic illustration of the 'Cristae fission and fusion' (CriFF) model and its link to CJ dynamics.

Figure EV1 - Detailed analysis of FRAP measurements of fusion proteins localizing to different mitochondrial subcompartments in WT and *MIC13* KO Hela cells.

809 **A-E** FRAP curves (curve-fitted) of **A,** MIC60-GFP **B,** MIC10-GFP **C,** TOMM20-GFP **D,**

810 TIMM23-GFP **E,** ATP5I-GFP in WT and *MIC13* KO Hela cells. Average FRAP curves

811 (intensities in arbitrary units) for each protein were obtained (3 independent experiments, 6-10

812 mitochondria for each experiment). Error bars for each time point representing SEM are shown.

813 **F, G** $T^{1/2}$ recovery time **(F)** and diffusion coefficients **(G)** of indicated proteins.

814 **Figure EV2 - Deletion of *MIC13* in Hela cells leads to a loss of crista junctions.**

815 **A** Western blot analysis of WT and *MIC13* KO Hela cells showing a reduction of MIC10, MIC26

816 and MIC27 protein levels in *MIC13* KOs.

817 **B** Representative electron micrographs of WT and *MIC13* KO Hela cells show loss of CJs in

818 *MIC13* KOs. Scale bar 0.5 µm.

819 **C** Boxplot showing quantification of CJs per mitochondrion in WT and *MIC13* KO Hela cells (2

820 independent experiments, 24-49 mitochondria for each experiment). ****$P < 0.0001$ (WT vs.

821 *MIC13* KO), unpaired Student's *t*-test.

822 **D** Boxplot showing quantification of cristae per mitochondrion in WT and *MIC13* KO Hela cells

823 (2 independent experiments, 24-49 mitochondria for each experiment). ****$P < 0.0001$,

824 unpaired Student's *t*-test.

825 **Figure EV3 - Single particle tracking reveals requirement of MIC60 for high directionality**

826 **of MIC10.**

827 **A-D** Representative single particle tracks of cells expressing MIC60- **(A)** and MIC10-SNAP **(C)**

828 in WT Hela cells and MIC60- **(B)** and MIC10-SNAP **(D)** in *MIC13* KO Hela cells, stained with

829 silicone-rhodamine dye, and imaged at a rate of 33 ms/frame. Single tracks were color-coded

830 according to temporal appearance. Insets in each image **(A-D)** show zoomed-in images of few

831 representative tracks.

832 **E** Cumulative frequency of tracks having corresponding values of instantaneous diffusion

833 coefficients (insD) in WT and *MIC13* KO Hela cells expressing MIC60- and MIC10-SNAP

32

834   stained with silicone-rhodamine. Number of tracks analysed: 2541, 2540, 3560 and 1441 in

835   WT Hela cells expressing MIC60-SNAP and MIC10-SNAP, *MIC13* KO Hela cells expressing

836   MIC60-SNAP and MIC10-SNAP, respectively. (****$P \le 0.0001$ for all possible comparisons,

837   unpaired Student's *t*-test).

838   **F** Percentage of subtracks having directed motion in WT and *MIC13* KO Hela cells expressing

839   MIC60- and MIC10-SNAP and WT and *MIC60* KO HAP1 cells expressing MIC10-SNAP.

840   Number of corresponding subtracks analysed: 2561, 2443, 3402, 1360, 561 and 1110. Data

841   are mean ± SEM. *$P \le 0.05$, **$P \le 0.01$, ***$P \le 0.001$, ****$P \le 0.0001$, unpaired Student's *t*-test.

842   **G-N,** Percentage of subtracks having directed motion **(G & K)**, normal diffusion **(H & L)**,

843   subdiffusion **(I & M)** and confined diffusion **(J & N)** in WT, *MIC13* KO Hela cells expressing

844   MIC60- and MIC10-SNAP and in WT HAP1 cells expressing MIC60- and MIC10-SNAP, *MIC60*

845   KO HAP1 cells expressing MIC10-SNAP and *MIC10* KO HAP1 cells expressing MIC60-SNAP,

846   respectively. Data are mean ± SEM. *$P \le 0.05$, **$P \le 0.01$, ***$P \le 0.001$, ****$P \le 0.0001$,

847   unpaired Student's *t*-test.

848   **Figure EV4 - Functionality and localization of MIC10 and MIC60 is not impaired by C-**

849   **terminal protein tags.**

850   **A, B** HEK293 cells expressing either MIC10-SNAP/GFP **(A)** or MIC60-SNAP/GFP **(B)** were

851   used for coimmunoprecipitation using anti-MIC13. Endogenous MIC13 could pull down MIC10-

852   SNAP or MIC10-GFP (A) as well as MIC60-SNAP or MIC60-GFP (B).

853   **C** *MIC10* KO HAP1 cells expressing an empty vector (ev), 1 and 3 µg of MIC10-SNAP/GFP

854   and probed with antibodies against SNAP/GFP, MIC10 and MIC13. MIC13 protein levels in

855   *MIC10* KO HAP1 cells are rescued by expression of MIC10-SNAP (3 µg) and MIC10-GFP (1

856   and 3 µg) showing that the GFP and SNAP tags do not interfere with the MIC10 function.

857   **D** The isolated mitochondria expressing MIC60-SNAP or MIC60-GFP were probed for their

858   incorporation into the native MICOS complex. The complex containing MIC60-GFP or MIC60-

859   SNAP runs at similar molecular weight as MICOS complex in native state marked by MIC60

860   or MIC10 antibody, confirming their incorporation into native complex.

861

862 **Movies Legends**

863 **Movie 1** - **MIC10-SNAP punctae marking CJs move dynamically within seconds**

864 Representative STED super-resolution time-lapse movie of Hela cell expressing MIC10-SNAP

865 shows rapid movement of MIC10-SNAP punctae marking CJs. Box marks selection shown as

866 a zoom in Movie 3.

867 **Movie 2 - MIC60-SNAP punctae marking CJs moves dynamically within seconds**

868 Representative STED super-resolution time-lapse movie of Hela cell expressing MIC60-SNAP

869 shows rapid movement of MIC60-SNAP punctae marking CJs. Box marks selection shown as

870 a zoom in Movie 4.

871 **Movie 3 - CJs marked by MIC10-SNAP undergo joining and splitting events**

872 Representative STED super-resolution time-lapse movie showing a magnified view of a

873 mitochondrion expressing MIC10-SNAP marking CJs. Two MIC10 punctae rhythmically join

874 and split as also shown in in Fig 4B. Green and magenta asterisks mark joining and splitting

875 events respectively.

876 **Movie 4 - CJs marked by MIC60-SNAP undergo joining and spliting events**

877 Representative STED super-resolution time-lapse movie showing a magnified view of a

878 mitochondrion expressing MIC60-SNAP, marking CJs. This movie is represented in Fig 4D.

879 Green and magenta asterisks mark joining and splitting events respectively.

880 **Movie 5 - Joining and splitting of MIC60-SNAP punctae occurs in a MIC13-dependent**

881 **manner**

882 Representative STED super-resolution time-lapse movie showing a magnified view of a

883 mitochondrion from *MIC13* KO cells expressing MIC60-SNAP. This movie is represented in

884 Fig 5B. Number of joining and splitting events are drastically reduced. Green asterisks mark a

885 rare joining event.

886 **Movie 6 - MIC13-SNAP show that CJs and cristae undergo remodelling within seconds**

887 Representative STED super-resolution time-lapse movie, (2.5 s/frame) of Hela cell expressing

888 MIC13-SNAP, shows rapid movement of CJs as well as cristae marked by MIC13-SNAP. Box

889 marks selection shown as a zoom in Movie 7.

35

890   **Movie 7 - MIC13-SNAP show continuous cristae remodelling in form of fusion and**

891   **fission cycles linked to CJs dynamics**

892   Representative STED super-resolution time-lapse movie showing a magnified view of a

893   mitochondrion expressing MIC13-SNAP at a framerate of 2.5 s/frame. This movie is

894   represented in Fig 6B. Various cristae remodelling events are marked by green and magenta

895   asterisks representing fusion and fission of cristae respectively. Cristae remodelling event that

896   forms a structure resembling letter `X´ is also marked.

897   **Movie 8 - MIC13-SNAP show that CJs and cristae undergo remodelling within seconds**

898   Representative STED super-resolution time-lapse movie (1.3 s/frame) of Hela cell expressing

899   MIC13-SNAP shows rapid movement of CJs as well as cristae marked by MIC13-SNAP. Box

900   marks selection shown as a zoom in Movie 9.

901   **Movie 9 - MIC13-SNAP show continuous cristae remodelling in form of fusion and**

902   **fission cycles linked to CJs dynamics**

903   Representative STED super-resolution time-lapse movie showing a magnified view of a

904   mitochondrion expressing MIC13-SNAP at a frame rate of 1.3 s/frame. This movie is

905   represented in Fig 6D. Various cristae remodelling events are marked by green and magenta

906   asterisks representing fusion and fission of cristae respectively.

907   **Movie 10 - ATP5I-SNAP marking cristae show cristae remodelling within seconds**

908   Representative STED super-resolution time-lapse movie (2.5 s/frame) of Hela cell expressing

909   ATP5I-SNAP, marking cristae as transverse bridge across mitochondria, shows rapid

910   movement of cristae. Box marks selection shown as a zoom in Movie 11.

911   **Movie 11 - ATP5I-SNAP marking cristae show continuous cristae remodelling in form of**

912   **fusion and fission cycles**

913   Representative STED super-resolution time-lapse movie showing a magnified view of a

914   mitochondrion expressing ATP5I-SNAP at a frame rate of 2.5 s/frame. This movie is

915   represented in Fig 7B. Various cristae remodelling events are marked by green and magenta

916   asterisks representing fusion and fission of cristae. A cristae remodelling event that forms a

917   structure resembling letter `Y´ is also marked.

918 **Movie 12 - COX8A-SNAP marking cristae show continuous cristae remodelling in form**

919 **of fusion and fission cycles**

920 Representative STED super-resolution time-lapse movie showing a magnified view of a

921 mitochondrion expressing COX8A-SNAP at the frame rate of 2.6 s/frame. This movie is

922 represented in Fig 7D. Various cristae remodelling events are marked by green and magenta

923 asterisks representing fusion and fission of cristae.

924 **Movie 13 - Fusion and fission cycles of cristae marked by COX8A-SNAP are reduced in**

925 ***MIC13* KO cells**

926 Representative STED super-resolution time-lapse movie showing a magnified view of a

927 mitochondrion expressing COX8A-SNAP in *MIC13* KO cells at a frame rate of 2.6 s/frame.

928 This movie is represented in Fig 7F. The number of fusion and fission events of cristae are

929 reduced. Some rare events are marked by green and magenta asterisks representing fusion

930 and fission of cristae.

931 **Movie 14 - Cristae dynamics helps redistribute membrane potential, marked by TMRM,**

932 **between cristae**

933 Representative STED super-resolution time-lapse movie showing a magnified view of a

934 mitochondrion stained with membrane potential specific dye TMRM at a frame rate of 1.5

935 s/frame. This movie is represented in Fig 8B. The green asterisks show the cristae which

936 undergo fusion accompanied by immediate distribution of TMRM signal.

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938

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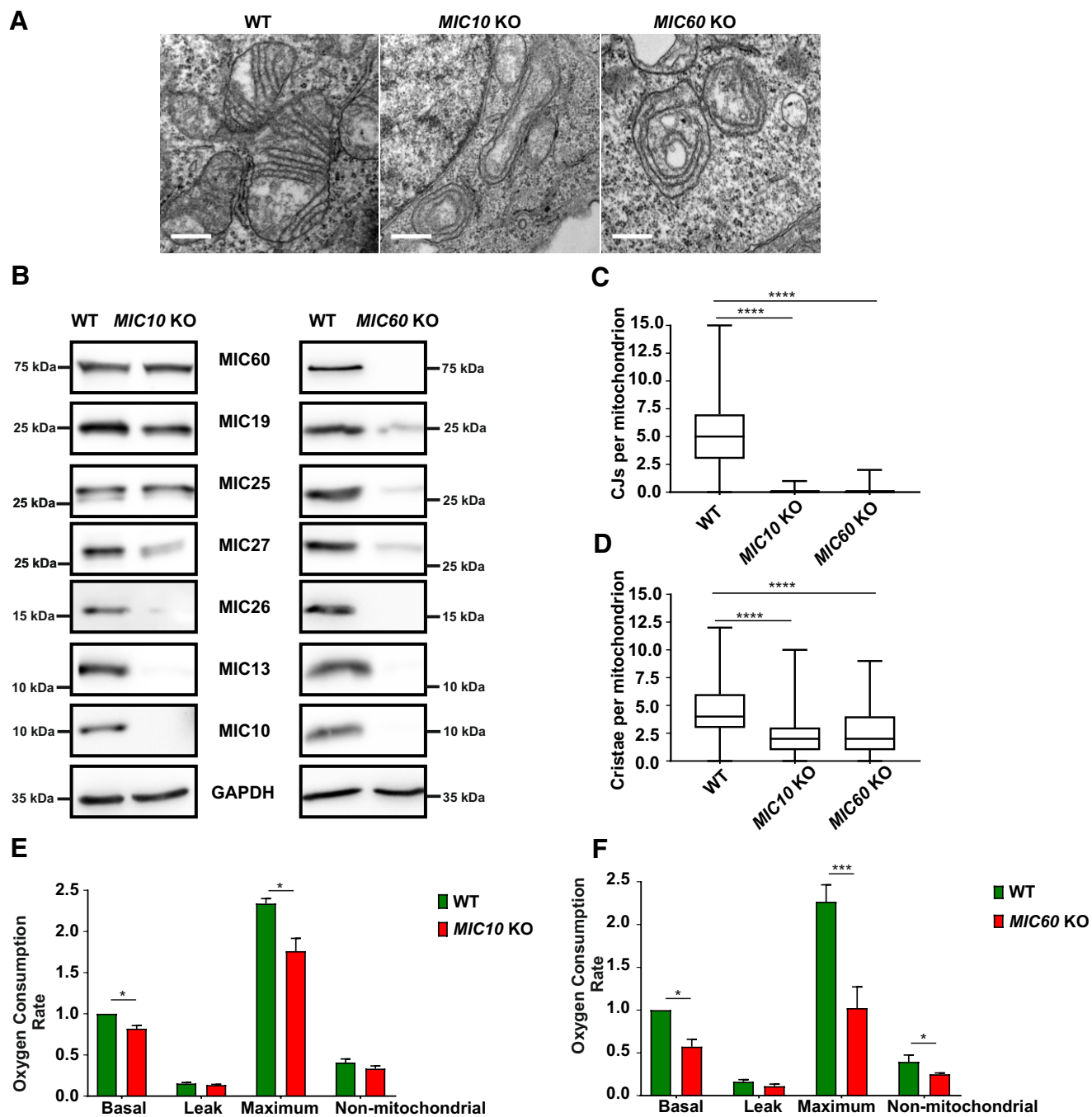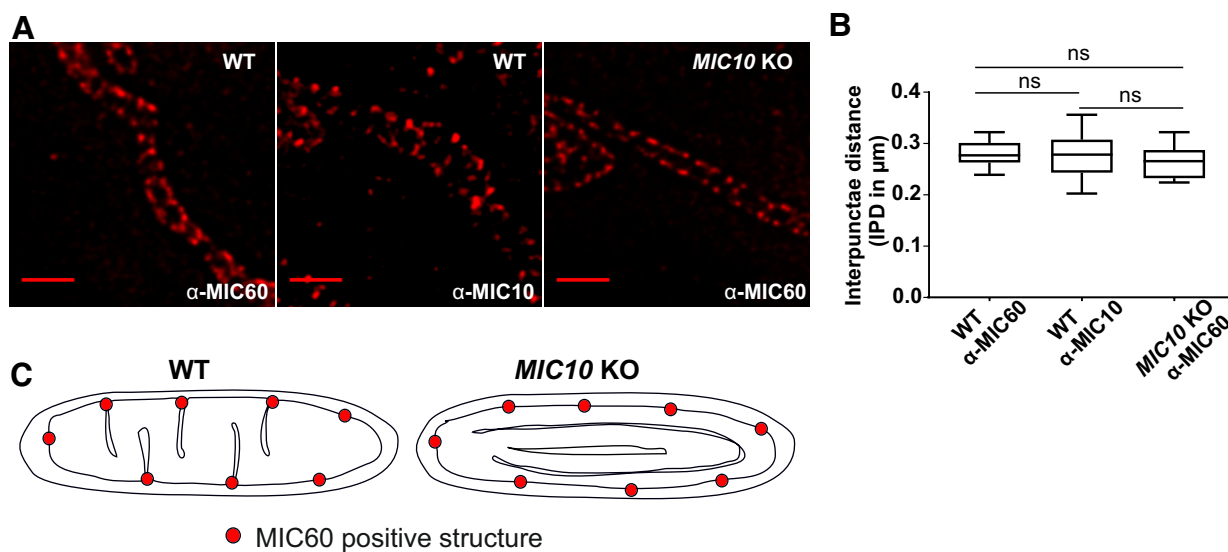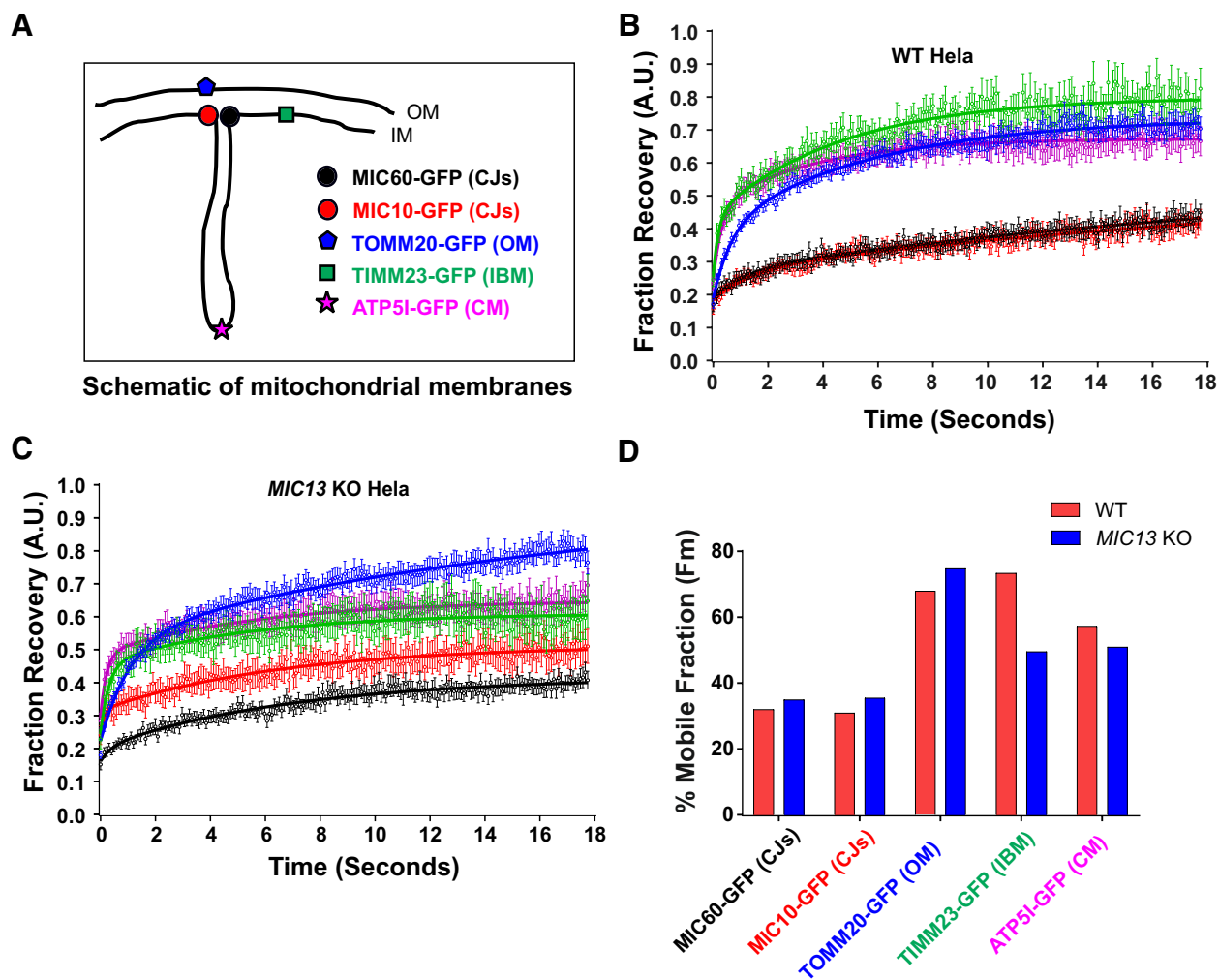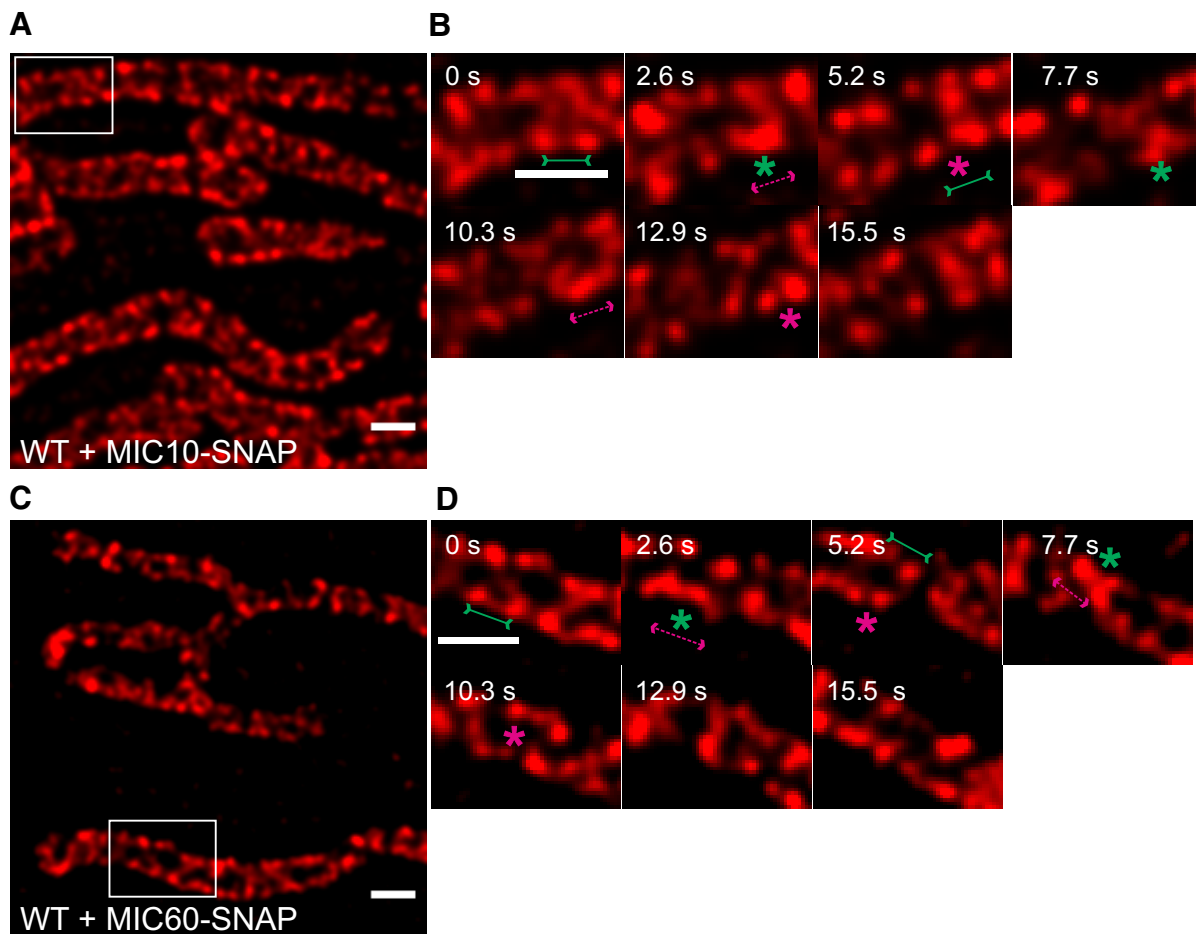
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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A

WT + MIC13-SNAP

B

0 s 2.5 s 5 s 7.5 s 10 s 12.5 s 14.9 s

C

WT + MIC13-SNAP

D

0 s 1.3 s 2.5 s 3.8 s 5 s 6.3 s 7.6 s
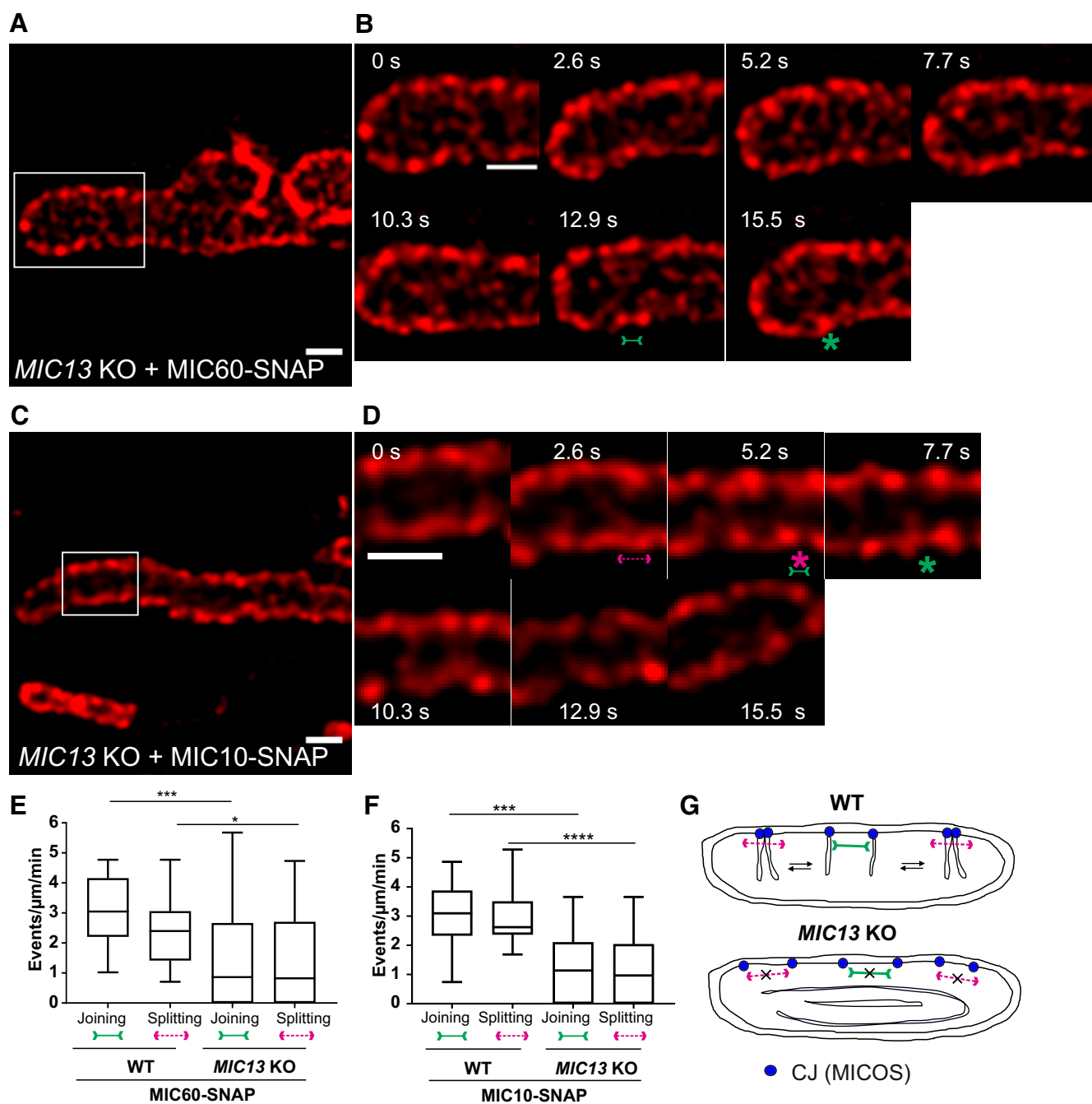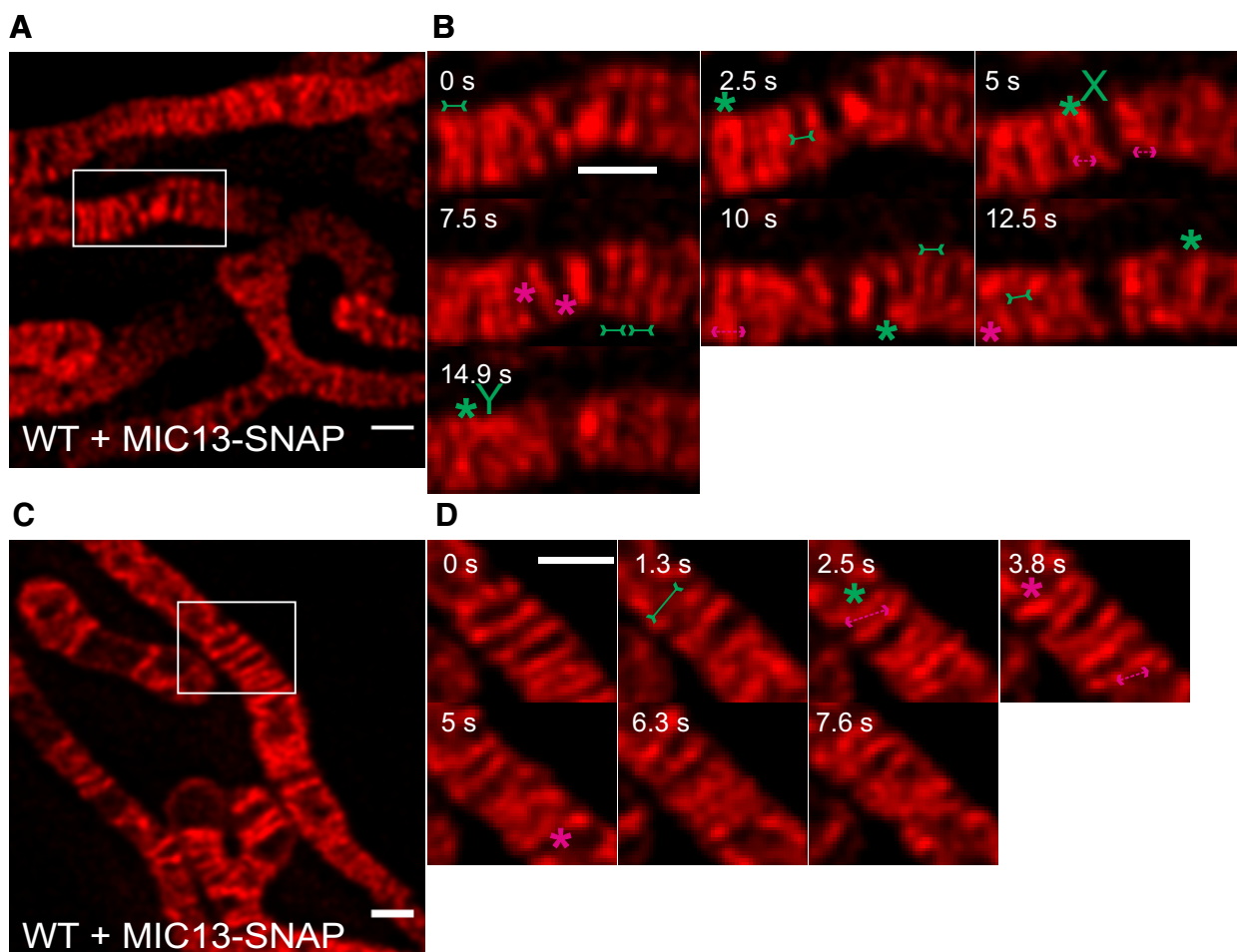
Figure 7

Figure 8

Figure EV1

A

MIC60-GFP

WT $T^{1/2}$ =0.8, 19.2 Sec
KO $T^{1/2}$ = 0.28, 4.89 Sec

B

MIC10-GFP

WT $T^{1/2}$ =0.95, 19.31 Sec
KO $T^{1/2}$ = 0.086, 4.57 Sec

C

TOMM20-GFP

WT $T^{1/2}$=0.44, 3.92 Sec
KO $T^{1/2}$ = 0.74, 12.05 Sec

D

TIMM23-GFP

WT $T^{1/2}$ =0.12, 3.22 Sec
KO $T^{1/2}$ = 0.20, 3.64 Sec

E

ATP5I-GFP

WT $T^{1/2}$ =0.21, 2.53 Sec
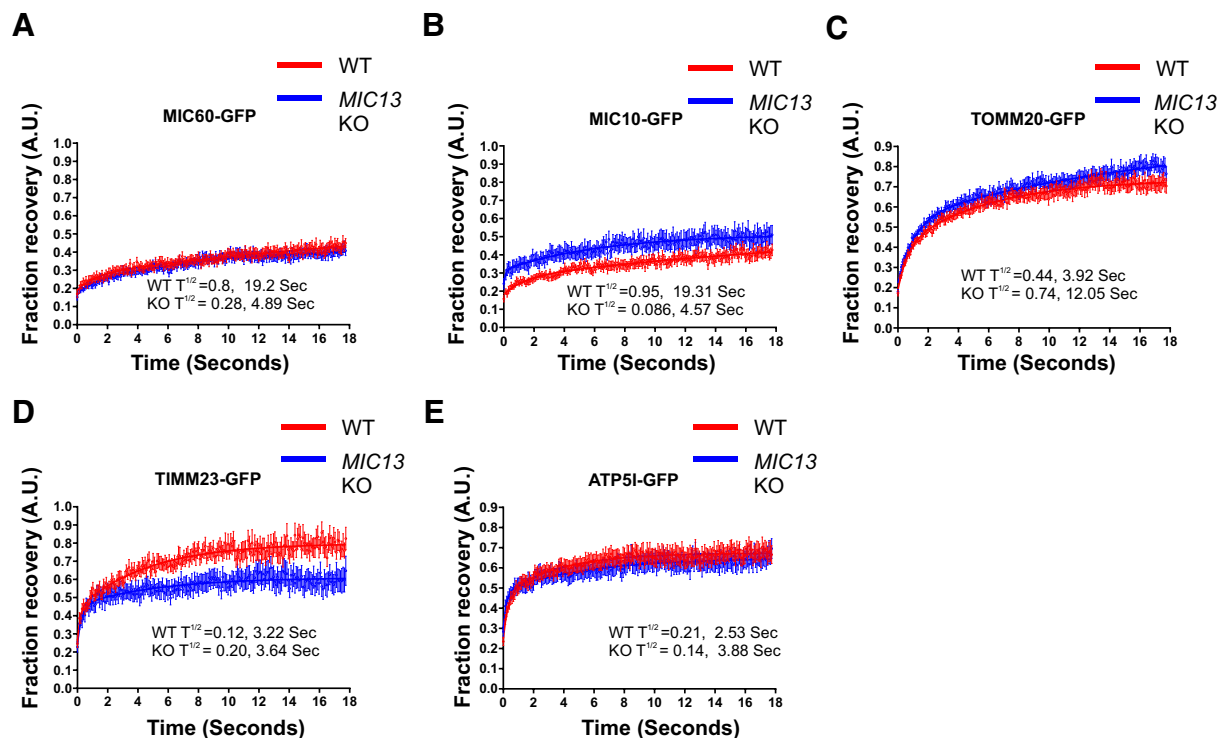KO $T^{1/2}$ = 0.14, 3.88 Sec
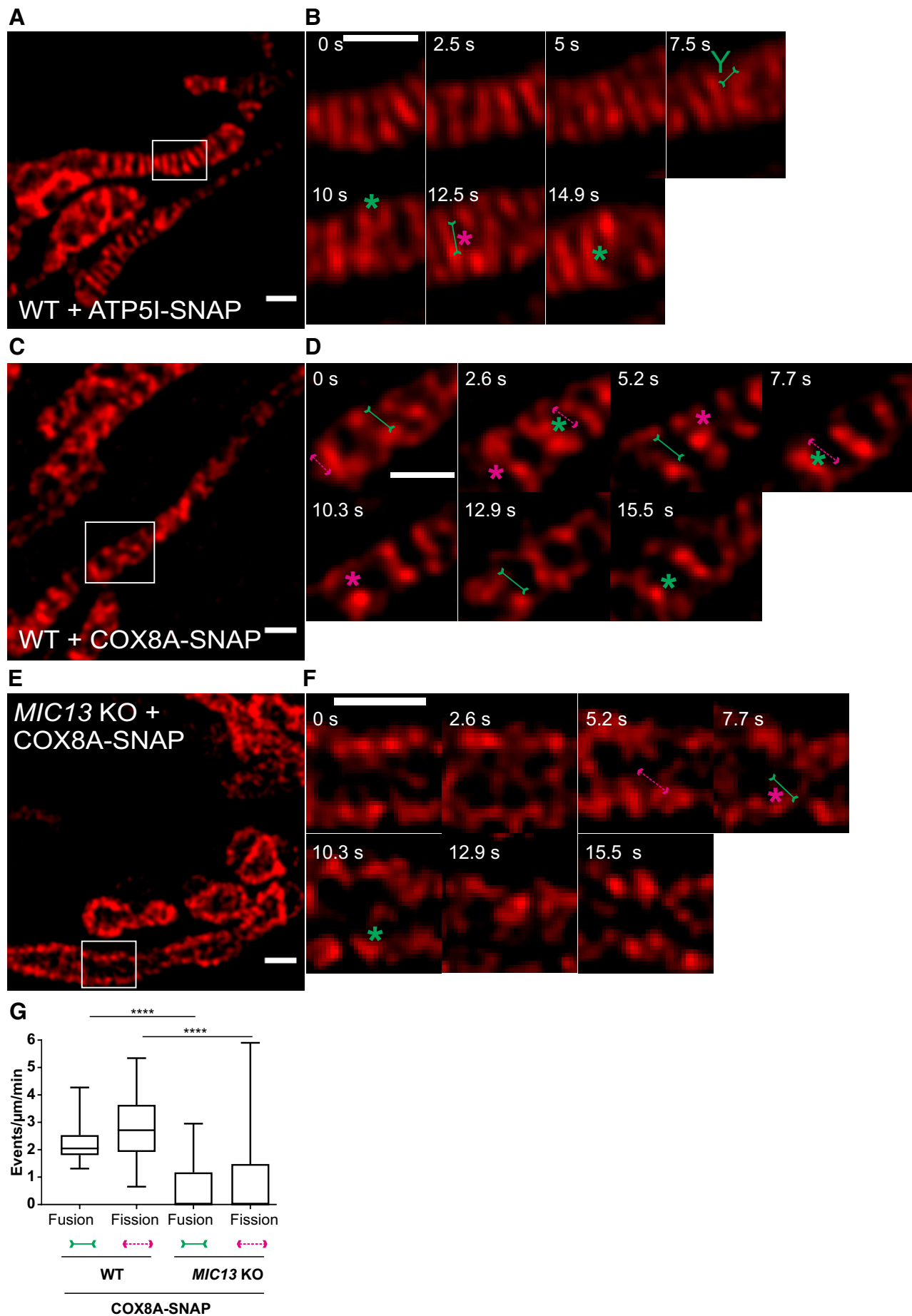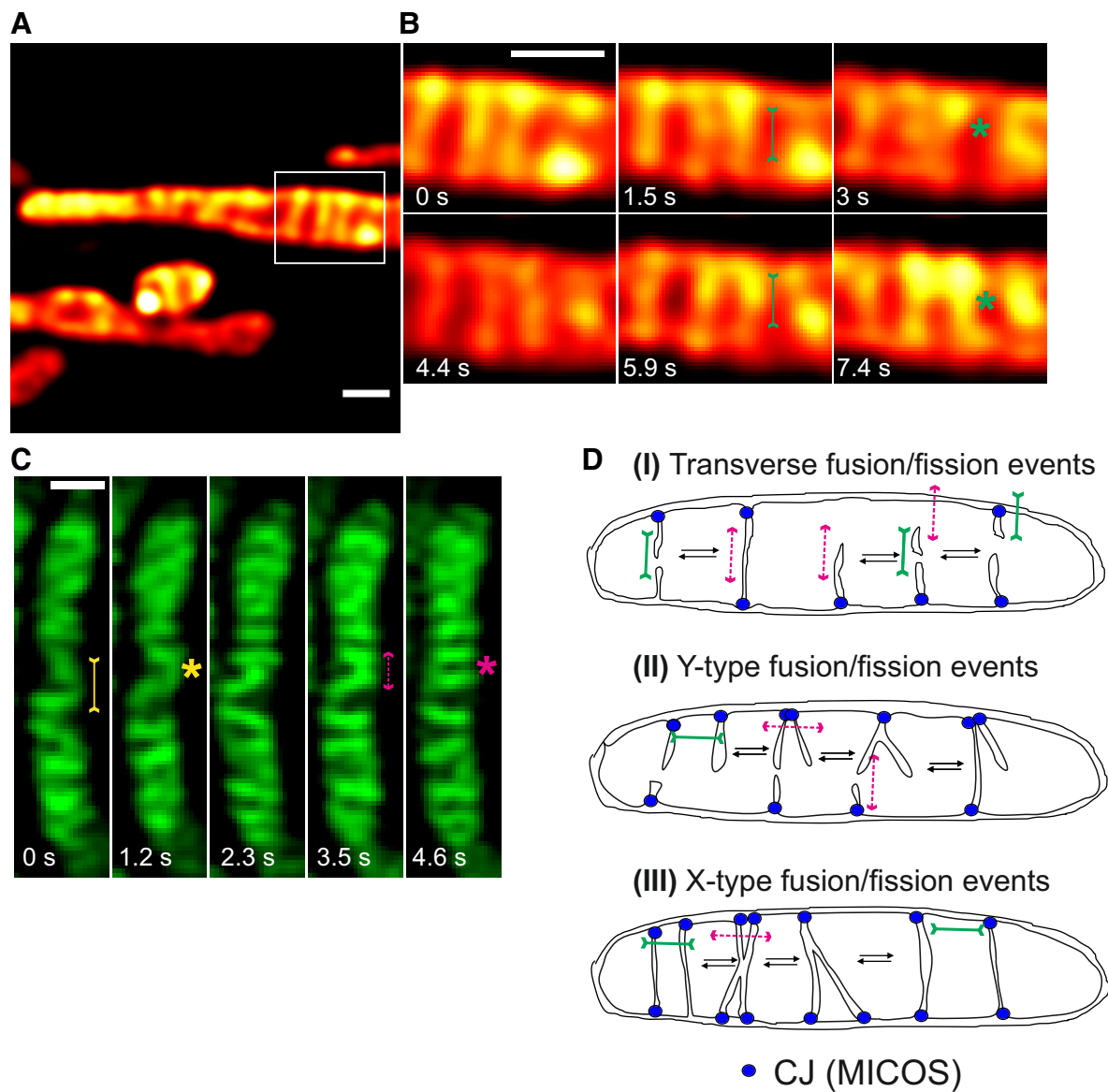
F $T^{1/2}$ recovery time (seconds) of different molecules

	Mitochondrial Compartment	Molecule	WT $T^{1/2}$ First Phase (seconds)	*Mic13* KO $T^{1/2}$ First Phase (seconds)	WT $T^{1/2}$ Second Phase (seconds)	*Mic13* KO $T^{1/2}$ Second Phase (seconds)
1	**CJs**	MIC60-GFP	0.8	0.28	19.26	4.89
2	**CJs**	MIC10-GFP	0.95	0.086	19.31	4.57
3	**OM**	TOMM20-GFP	0.44	0.74	3.92	12.05
4	**IBM**	TIMM23- GFP	0.12	0.20	3.22	3.64
5	**Cristae**	ATP5I-GFP	0.21	0.14	2.53	3.88

G   Diffusion coefficient (µm^2/second) of different molecules

	Mitochondrial Compartment	Molecule	WT (Fast)	*Mic13* KO (Fast)	WT (Slow)	*Mic13* KO (Slow)
1	**CJs**	MIC60-GFP	0.048	0.139	0.002	0.008
2	**CJs**	MIC10-GFP	0.041	0.453	0.002	0.008
3	**OM**	TOMM20-GFP	0.088	0.052	0.010	0.003
4	**IBM**	TIMM23-GFP	0.325	0.195	0.012	0.010
5	**Cristae**	ATP5I-GFP	0.185	0.278	0.015	0.010

Figure EV2

A

B

C

D

Figure EV3

Figure EV4