

1 **Title: Live Imaging Reveals the Cellular Events Downstream of SARM1 Activation**

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17 **Abstract**

18

19 SARM1 is an inducible NAD⁺ hydrolase that triggers axon loss and neuronal cell death
20 in the injured and diseased nervous system. While SARM1 activation and enzyme
21 function are well defined, the cellular events downstream of SARM1 activity but prior to
22 axonal demise are much less well understood. Defects in calcium, mitochondria, ATP,
23 and membrane homeostasis occur in injured axons, but the relationships among these
24 events have been difficult to disentangle because prior studies analyzed large
25 collections of axons in which cellular events occur asynchronously. Here we used live
26 imaging with single axon resolution to investigate the cellular events downstream of
27 SARM1 activity. Our studies support a model in which SARM1 NADase activity leads to
28 an ordered sequence of events from loss of cellular ATP, to defects in mitochondrial
29 movement and depolarization, followed by calcium influx, externalization of
30 phosphatidylserine, and loss of membrane permeability prior to catastrophic axonal self-
31 destruction.

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36 Introduction

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38 SARM1 is the central executioner of pathological axon degeneration, an early feature of
39 many neurodegenerative diseases (Figley and DiAntonio, 2020; Krauss et al., 2020).

40 SARM1 is the founding member of the TIR-domain family of NAD⁺ hydrolases
41 (Essuman et al., 2018, 2017), and a metabolic sensor activated by disrupted NAD⁺
42 homeostasis (Figley et al., 2021; Gilley et al., 2015; Sasaki et al., 2016). Activation of
43 the SARM1 NADase depletes cellular NAD⁺ and initiates a metabolic crisis that
44 ultimately leads to axon degeneration and/or neuronal cell death (Essuman et al., 2017;
45 Gerdts et al., 2015). SARM1 is a compelling target for therapeutic intervention, as loss
46 of SARM1 is profoundly protective in animal models of multiple neurodegenerative
47 diseases including nerve injury, peripheral neuropathies, traumatic brain injury,
48 glaucoma, retinitis pigmentosa, and Leber congenital amaurosis (Geisler et al., 2016;
49 Gerdts et al., 2013; Henninger et al., 2016; Ko et al., 2020; Osterloh et al., 2012; Ozaki
50 et al., 2020; Sasaki et al., 2020b; Turkiew et al., 2017). Moreover, as an enzyme,
51 SARM1 is a druggable target, and both small molecule inhibitors and gene therapeutics
52 effectively block axon degeneration (Bosanac et al., 2021; Geisler et al., 2019; Hughes
53 et al., 2021). Recently, there has been tremendous progress in dissecting the structure
54 of SARM1 (Bratkowski et al., 2020; Jiang et al., 2020; Sporny et al., 2020), the
55 mechanism by which SARM1 is autoinhibited in healthy neurons (Shen et al., 2021) and
56 activated in diseased neurons (Figley et al., 2021), and its role as an NAD⁺ hydrolase
57 (Essuman et al., 2017; Horsefield et al., 2019; Zhao et al., 2019). However, the events
58 downstream of NAD⁺ loss but prior to catastrophic axon fragmentation are much less
59 well understood.

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61 SARM1 and its NADase activity are essential for injury-induced axon degeneration, and
62 the SARM1 enzyme is activated within 1-2 hours after injury in cultured DRG neurons
63 (Sasaki et al., 2020a). Axonal fragmentation occurs much later in this system, with
64 axons fragmenting approximately 4-6 hours after injury. Numerous molecular and
65 cellular events occur in the time between SARM1 activation and axon loss, including
66 calcium influx, mitochondrial stalling and depolarization, loss of ATP, and disrupted

67 membrane integrity. We reasoned that temporally ordering these events would give
68 insights into causal relationships among these degenerative mechanisms. Unfortunately,
69 studies in bulk culture are not appropriate for assessing the temporal sequence
70 because axon loss is asynchronous, and so this effort requires live imaging. Prior live
71 imaging studies have demonstrated that calcium influx precedes axonal fragmentation
72 (Adalbert et al., 2012; Loreto et al., 2015; Vargas et al., 2015), however no
73 comprehensive live imaging analysis of the cellular and molecular events underlying
74 axon degeneration has been reported.

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76 Here, we explore the cellular events that occur in injured axons following SARM1
77 activation. First, we investigate whether changes to mitochondria and calcium require
78 SARM1 NADase activity, as prior studies used a complete knockout and so left open
79 the possibility of NADase-independent functions. Indeed, such an NADase-independent
80 function was recently described in *Drosophila* for organelle stalling after injury (Hsu et
81 al., 2020). Next, we revisit the role of both intracellular and extracellular calcium in axon
82 degeneration. We confirm that blocking extracellular calcium influx blocks axon
83 fragmentation (Vargas et al., 2015; Villegas et al., 2014; Witte et al., 2019), however,
84 these apparently morphologically intact axons are not metabolically active, as
85 mitochondria are immobile and depolarized. We then develop a live imaging approach
86 in cultured DRG neurons with single axon resolution and assess dynamic changes to
87 calcium, mitochondria, ATP, and the plasma membrane. Our findings describe an
88 ordered series of events in which 1) ATP is lost, 2) mitochondria stop moving and
89 subsequently depolarize, 3) extracellular calcium enters the axons, 4)
90 phosphatidylserine is exposed on the outer leaflet of the plasma membrane, and 5) the
91 membrane loses integrity. This work identifies a stereotyped cascade of dysfunction
92 following SARM1 activation, and highlights ATP loss as the likely key intermediate
93 between NAD⁺ cleavage and widespread dysfunction in injured axons.

94 **Results**

95

96 **SARM1 NADase activity promotes mitochondrial stalling and calcium influx in**
97 **injured axons**

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99 SARM1 is an injury-activated NAD⁺ hydrolase, and this enzymatic activity is required for
100 injury-induced axon degeneration (Essuman et al., 2017). However, it is unclear
101 whether all SARM1-dependent processes require this enzymatic function. Recently,
102 Hsu et al. working in *Drosophila* demonstrated that injury-dependent organelle stalling
103 can be SARM1-dependent but NADase-independent (Hsu et al., 2020). Previously,
104 Loreto et. al presented the even more surprising finding that injury-dependent
105 mitochondrial stalling in superior cervical ganglion axons did not depend on SARM1,
106 although loss of mitochondrial potential did depend on SARM1 (Loreto et al., 2015).
107 Here we test the applicability of these findings to mammalian sensory neurons,
108 assessing mitochondrial movement in cultured mouse dorsal root ganglion (DRG)
109 neurons and assaying the requirement for SARM1 NADase activity. This is of particular
110 interest because SARM1 is a mitochondrial associated protein and so alternate
111 mechanisms of action are plausible. We cultured DRG neurons from SARM1 knockout
112 (KO) embryos and used lentivirus to express either GFP, SARM1, or catalytically
113 inactive SARM1(E642A) together with MitoDsRed (MitoDR) in order to track
114 mitochondria. After seven days, axons were severed and mitochondrial movement was
115 imaged 0, 2 and 4 hours after injury. In SARM1 KO neurons expressing GFP,
116 mitochondrial movement was unchanged four hours after injury. In contrast, the number
117 of motile mitochondria declines precipitously between 2 and 4 hours after injury in
118 SARM1 KO axons re-expressing SARM1. Catalytically-inactive SARM1(E642A) is
119 expressed at similar levels to wild-type (WT) SARM1 (Supplemental Fig. 1), but did not
120 result in loss of mobile mitochondria after injury (Fig. 1A and 1B). Hence, the loss of
121 mitochondrial mobility in injured axons is not only SARM1-dependent, but also SARM1
122 NADase-dependent. Injury-induced loss of mitochondrial potential is also SARM1-
123 dependent (Loreto et al., 2015; Geisler et al., 2019), and so here we investigated
124 whether or not this effect also requires a functional SARM1 NADase. The mitochondrial

125 potential is the driving force for ATP production and can be measured with the
126 fluorescent dye TMRM (tetramethylrhodamine methy ester). As with mitochondrial
127 dynamics, expression of WT SARM1, but not SARM1(E642A), leads to a dramatic loss
128 of mitochondrial membrane potential after injury (Fig. 2C and 2D). The finding that
129 SARM1 NADase activity is required for loss of mitochondrial membrane potential
130 suggests that the SARM1-induced decline in cytosolic NAD⁺ levels either directly or
131 indirectly influences bioenergetics inside the mitochondria.

132

133 In addition to mitochondrial dysfunction, calcium homeostasis is also disrupted in injured
134 axons (Adalbert et al., 2012; George et al., 1995; Ma et al., 2013; Yang et al., 2013).
135 Here, we test if this calcium influx requires SARM1 enzymatic activity. We used Fluo-4,
136 a calcium-sensitive fluorescent dye, to assess axonal calcium four hours after injury, the
137 time point by which mitochondrial mobility and potential are disrupted. Axons show no
138 calcium rise in SARM1 KO neurons expressing either GFP or SARM1(E642A), but have
139 a significant increase in calcium when expressing WT SARM1 (Fig. 1E and 1F). Hence,
140 the loss of calcium homeostasis in injured axons also requires SARM1 NADase activity.
141 Taken together, these findings support the view that SARM1 enzyme activity is
142 essential for not only axon degeneration (Essuman et al., 2017), but also for the major
143 proximate events that occur in injured mammalian axons.

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146 **Manipulating either intracellular or extracellular calcium is ineffective in** 147 **preserving injured axons**

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149 Having demonstrated that calcium influx into injured axons requires SARM1 NADase
150 activity, we next explored the role of calcium influx in axonal demise. Prior studies
151 argued that calcium release through the mitochondrial permeability transition pore
152 (MPTP) (Barrientos et al., 2011; Villegas et al., 2014) or extracellular calcium influx
153 (Vargas et al., 2015; Witte et al., 2019) are key drivers of axon degeneration. To test
154 whether the MPTP has a role in axon degeneration, we incubated embryonic DRGs
155 neurons with the MPTP inhibitor (Cyclosporin A, CsA), axotomized, and assessed the

156 progression of axon degeneration and the rise in intracellular calcium. In contrast to
157 prior findings, we observed no delay in the timing of axon degeneration with CsA
158 treatment (Fig. 2A). We also assayed the increase in intracellular calcium after injury
159 and again found no significant effect of CsA (Fig. 2B). To further explore the role of
160 intracellular calcium, we incubated DRG neurons with 10 μ M BAPTA to chelate
161 intracellular calcium for 30 minutes prior to axotomy. This treatment had no influence on
162 the progression of axon degeneration (Fig. 2C). These results indicate that internal
163 calcium is not a major determinant of injury-induced axon degeneration in this system,
164 however it may play a role in scenarios where SARM1 is less potently activated (Li et al.,
165 2021).

166
167 While we found no clear role for intracellular calcium, there are numerous studies
168 highlighting the importance of extracellular calcium for injury-induced axon degeneration
169 (Gerdtts et al., 2011; Mishra et al., 2013; Ribas et al., 2017; Vargas et al., 2015; Yang et
170 al., 2013). As such, we explored the impact of chelating extracellular calcium on the
171 progression of axon degeneration. In agreement with prior studies, we find that pre-
172 incubation with 3 mM EGTA maintained morphologically intact axons for up to 48 hours
173 after axotomy (Fig. 2D and 2E). To explore when the influx of extracellular calcium is
174 required, we treated with EGTA at the time of axotomy and then performed washout
175 after 2 hours, or added the EGTA two hours after axotomy. The presence of EGTA from
176 0-2 hours after axotomy had no impact on the timing of axon fragmentation, while
177 addition of EGTA two hours post-axotomy was as protective as treatment at the time of
178 axotomy (Fig. 2D and 2E). Hence, late influx of extracellular calcium is critical for axon
179 degeneration in DRG axons, a finding consistent with previous reports (Loreto et al.,
180 2015; Vargas et al., 2015; Witte et al., 2019).

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182 While these results are consistent with prior work highlighting the importance of
183 extracellular calcium for axon degeneration, we did observe that severed axons
184 developed prominent axonal swellings following extracellular calcium chelation (Fig. 2D,
185 red arrowheads; (George et al., 1995)). Moreover, previous work showed that SARM1 is
186 activated within two hours after injury and leads to NAD⁺ depletion and metabolic

187 catastrophe (Sasaki et al., 2020a), raising the question of whether blocking the later
188 influx of calcium maintains axons in a healthy state. To investigate this question, we
189 assessed mitochondrial membrane potential and mobility in injured axons treated with
190 EGTA. We find that mitochondria potential is lost by four hours post-axotomy whether or
191 not EGTA is present (Fig. 2F and 2G). Similarly, EGTA treatment fails to maintain
192 mitochondrial mobility after axotomy (Fig. 2H and 2I). Indeed, treatment with EGTA
193 halts mitochondrial movement after four hours even in the absence of injury,
194 demonstrating that chelating extracellular calcium is not an effective method to maintain
195 healthy axons, and instead disrupts normal axonal physiology. These findings are in
196 contrast to loss of SARM1, which maintains both mitochondrial potential and mobility
197 after axotomy (Fig. 1). Taken together, these findings suggest that prior studies showing
198 protection of axons by blocking calcium influx were likely maintaining axonal structure
199 but not axonal physiology, and suggest that the key role for calcium influx may be to
200 trigger fragmentation of metabolically non-functional axons.

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202

203 **Live single axon imaging defines the temporal relationship between calcium** 204 **influx and axonal fragmentation**

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206 To quantitatively assess the relationship between calcium influx and axonal
207 fragmentation, we developed a live imaging system with single axon resolution
208 (Supplemental Fig. 2). This allows us to assess temporal relationships of the
209 asynchronous axon degeneration process that is not possible in mass cultures. We
210 used lentivirus to co-express GCaMP6 and mRuby3 in cultured DRG neurons to
211 monitor calcium influx and axon morphology simultaneously. After seven days in culture,
212 when both proteins were strongly expressed, we performed axotomy by focusing laser
213 light on a 1 x 1 μm region containing a single axon (Fig. 3A). Images of the distal axon
214 were repeatedly acquired until the injured axon degenerated. Figure 3B and
215 supplemental video 1 show the progression of calcium influx and axon degeneration for
216 a single axon, features that are representative of all the injured axons analyzed.
217 Immediately after axotomy, a first peak of calcium bidirectionally propagates along the

218 axon from the injury site (white triangle). This elevated intracellular calcium is rapidly
219 cleared, demonstrating that calcium homeostasis functions normally at this time, and is
220 consistent with the findings in Fig 2E that the initial influx of calcium does not contribute
221 to axon fragmentation (Adalbert et al., 2012; Loreto et al., 2015; Vargas et al., 2015).
222 After nearly four hours, there is a large second influx of calcium that precedes any
223 obvious change in axonal morphology. Soon thereafter, the axon thins and small
224 swellings appear, then the axon swellings enlarge, and finally the axon fragments (Fig.
225 3B and 3C). From analysis of 22 single axons, we found that the time to the appearance
226 of the second calcium peak varied dramatically, from less than four hours to nearly ten
227 hours after axotomy (Fig. 3D). However, once the second peak of calcium appeared,
228 the axon fragmented soon thereafter. The correlation between the initiation of the 2nd
229 peak of calcium and axon degeneration was very strong (Fig. 3D), with degeneration
230 occurring ~100 minutes after initiation of calcium influx. In contrast, neither the duration
231 nor intensity of the 2nd calcium peak was well correlated with the timing of axon
232 degeneration (Fig. 3E and 3F). The very tight correlation between the initiation of the 2nd
233 influx of calcium and axonal fragmentation is consistent with the hypothesis that this
234 calcium triggers the final disintegration of the axon.

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236

237 **Mitochondrial dysfunction precedes calcium influx in injured axons.**

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239 With a method established for live imaging of single axons, we next explored the
240 temporal relationship between calcium influx and mitochondrial stalling and loss of
241 potential. Calcium influx is a potent mechanism for stopping mitochondria (Wang and
242 Schwarz, 2009), and so we predicted that calcium influx would occur before
243 mitochondrial stalling. We used lentivirus to express MitoDR and GCaMP6 in DRG
244 neurons and performed laser axotomy. We imaged mitochondrial movement with
245 MitoDR every 5 seconds for 300 seconds followed by calcium measurements. Images
246 from MitoDR and GCaMP6 were acquired until mitochondria stopped, at which point
247 only GCaMP6 imaging continued until its level increased more than 2-fold (Fig. 4A). As
248 shown in Fig. 4A and in contrast to expectations, mitochondrial movement stops before

249 the influx of calcium. Analysis of this single axon shows that fewer mitochondria are
250 moving three hours after axotomy (Fig 4B), and that all mitochondrial are stalled by 4.4
251 hours after injury (Fig. 4B, inset). At this point, the GCaMP6 signal is unchanged from
252 baseline, but begins to rise soon thereafter. Quantitative analysis of single injured
253 axons demonstrates that loss of mitochondrial mobility precedes calcium influx in each
254 case, and this occurs ~25 minutes after mitochondria stop (Fig. 4B and 4C). Hence, loss
255 of mitochondrial mobility in injured axons cannot be due to calcium influx.

256

257 Next, we addressed the relationship between loss of mitochondrial potential and
258 calcium influx. Calcium overload can induce loss of mitochondrial potential (Abramov
259 2008)—if this occurs in injured axons, then we expect calcium influx to precede
260 mitochondrial depolarization. We expressed GCaMP6 in DRG neurons, incubated the
261 neurons with TMRM to assess mitochondrial potential, and imaged single axons in both
262 channels every ten minutes until axons degenerated (Fig. 4D). This imaging frequency
263 was chosen to avoid photobleaching. The images and analysis from a single axon (Fig.
264 4D and 4E) demonstrate that the fluorescent intensity of TMRM is fairly stable at
265 baseline, then has an initial drastic drop (dark red dot in Fig. 4E) followed by a steady
266 decline (Fig. 4E and 4F, red dots). In contrast, calcium has an abrupt rise (dark green
267 dot), quickly reaching a higher steady-state level (Fig. 4E and 4F, green dots). In the
268 example shown, the abrupt drop in mitochondrial potential occurs one frame prior to the
269 large increase in calcium. To assess this across axons, we identified the time at which
270 the TMRM and GCaMP6 signal showed the largest frame to frame variation (Fig. 4F
271 and 4G and Supplemental Fig 3). We found that the drastic drop of TMRM intensity
272 occurs one frame prior to the large increase in calcium in 5 out of 7 axons (71.43%),
273 while these changes occurred during the same frame in 2 out of 7 axons. The images
274 are taken ten minutes apart, and so these data indicate that in injured axons
275 mitochondria begin depolarizing prior to the calcium influx (Fig. 4G). Moreover, these
276 data, in conjunction with the analysis of mitochondrial mobility and calcium influx above,
277 demonstrate a sequence of events in which first mitochondria stop, then begin losing
278 their potential, and after that calcium enters the axon which subsequently degenerates.

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281 **ATP loss precedes mitochondrial stalling**

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283 Calcium influx cannot explain the loss of mitochondrial mobility, so we considered other
284 potential mechanisms. Once the SARM1 NADase is activated, ATP is lost soon
285 thereafter (Gerdt et al., 2015) because NAD^+ is required for ATP synthesis via both
286 glycolysis and oxidative phosphorylation. In addition, mitochondria are transported by
287 ATP-dependent molecular motors. Therefore, we hypothesized that loss of ATP may
288 cause loss of mitochondrial mobility, and so explored the relationship between ATP loss
289 and mitochondrial stalling. We used lentivirus to express PercevalHR, a sensor of
290 relative ATP levels (Tantama et al., 2013), and MitoDR in DRG neurons and performed
291 laser axotomy. We found that ATP loss and loss of mitochondrial mobility temporally
292 overlap, and so it was not possible to define a window after which one process was
293 complete and the other had yet to start, as we did with calcium and mitochondrial
294 dynamics above. Instead, we quantitatively assessed the relationship between the
295 degree of ATP loss as defined by loss of the PercevalHR signal and the fraction of
296 mitochondria that stop moving in injured axons. Based on ATP measurements from bulk
297 injured axons, we knew that most ATP loss occurred between 3 and 4 hours after injury
298 (Sasaki et al., 2016). Therefore, we imaged baseline mitochondrial mobility and relative
299 ATP levels before injury, and then re-imaged the PercevalHR every 5 minutes for 3.5
300 hours after single axon injury. We then calculated the percent change in PercevalHR
301 intensity from baseline. Immediately after the final imaging of PercevalHR at 3.5 hours,
302 we imaged mitochondrial mobility by acquiring images every 5 seconds for 300 seconds
303 and calculated the percent drop in the fraction of motile mitochondria compared to
304 baseline (Fig. 5A and 5B). In every axon the percent drop in the ATP sensor was larger
305 than the percent drop in the fraction of mobile mitochondria (Fig. 5B left), and there was
306 a strong correlation between the extent of ATP loss and mitochondrial stalling (Fig. 5B
307 right; $R^2 = 0.61$, $n=9$). We continued to measure mitochondrial mobility from 3.5 hours
308 after injury until mitochondrial movement ended. We found a strong inverse correlation
309 between the extent of ATP loss at 3.5 hours and the remaining time until the complete
310 loss of mitochondrial mobility (Fig. 5C; $R^2 = 0.76$, $n=9$). In other words, the extent of

311 ATP loss by 3.5 hours after injury is a strong predictor of when mitochondrial will
312 ultimately stop moving in an injured axon. All of these results are consistent with the
313 model that ATP loss causes loss of mitochondrial mobility.

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317 **Calcium is required for loss of membrane integrity during axon fragmentation**

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319 Having shown that calcium influx is a late event in the axon degeneration process, we
320 assessed the temporal relationship between calcium influx and two other late events,
321 loss of membrane lipid asymmetry and loss of membrane integrity. In healthy
322 membranes, phosphatidylserine is preferentially found in the inner leaflet of the plasma
323 membrane. In cells undergoing apoptosis and in degenerating axons,
324 phosphatidylserine is exposed on the outer leaflet where it serves as an “eat-me” signal
325 to phagocytic cells (Segawa and Nagata, 2015). To assess the temporal relationship
326 among calcium influx, loss of membrane asymmetry, and axon fragmentation, we
327 expressed GCaMP6 and mRuby3 in DRG neurons, laser axotomized, and incubated
328 with Alex Fluor 647-conjugated Annexin-V, which binds extracellular phosphatidylserine
329 (Sievers et al., 2003). In the example shown, calcium rises first, followed by staining
330 with Annexin-V, and soon thereafter the mRuby3 signal declines indicative of axon
331 fragmentation (Fig. 6A). Indeed, in all cells analyzed calcium influx preceded
332 phosphatidylserine exposure, and occurred $\sim 0.51 \pm 0.04$ (hr) prior to Annexin-V
333 staining (Fig. 6B). Calcium can inhibit the ATP-dependent flippase that maintains
334 phosphatidylserine on the inner leaflet (Bitbol et al., 1987; Soupene, 2008), and so the
335 influx of calcium and/or the decline in ATP likely triggers the loss of membrane
336 asymmetry during axon degeneration. We wished to test the role of calcium by blocking
337 influx with EGTA, however this experiment is not possible because Annexin-V binding to
338 phosphatidylserine requires extracellular calcium.

339

340 Next, we assessed the relationship among SARM1, calcium influx, and the loss of
341 membrane integrity in injured axons. To assess membrane integrity, we applied

342 fluorescently labeled macromolecules (3kDa dextran) to neurons expressing cytosolic
343 GFP. In uninjured neurons, cytosolic GFP fills the axon while the dextran is excluded
344 (Fig. 6C). After injury, axon swelling is apparent, and mitochondria localize to these
345 swellings (Supplemental Fig. 4). The swellings retain GFP and still exclude dextran.
346 Later, discrete puncta of dextran appear in axonal fragments, and such fragments
347 contain no visible GFP. We interpret this as axon swellings that burst, releasing soluble
348 GFP and allowing entry to the high molecular weight dextran (Fig. 6C). Next, we
349 compared dextran uptake in injured axons from wild type and SARM1 KO neurons, as
350 well as wild type neurons treated with EGTA. By four hours after axotomy of wild type
351 neurons, dextran is present throughout the axons, indicative of a loss of membrane
352 integrity. In axotomized SARM1 KO neurons, dextran is excluded from axons for at least
353 48 hours. Interestingly, when wild type neurons are incubated with EGTA, injured axons
354 still swell (arrowheads, Fig. 6D), but dextran is excluded (Fig. 6D and 6E). Therefore,
355 we conclude that calcium is necessary for the loss of membrane integrity and the
356 morphological transition from axonal swelling to fragmentation.

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359

360 **Discussion**

361

362 In injured axons, the molecular function of SARM1 is well understood, but the
363 ensuing molecular and cellular changes leading to axonal demise are much more poorly
364 defined. SARM1 NADase activity is critical for the ultimate demise of injured axons
365 (Essuman et al., 2017), and here we show that this enzymatic activity is also required
366 for intermediate phenotypes such as disrupted mitochondrial and calcium homeostasis.
367 To explore the events that occur after NAD⁺ cleavage, we used live imaging with single
368 axon resolution to investigate dynamic changes to ATP, mitochondria, calcium, and
369 membranes. The data describe an ordered series of events beginning with loss of ATP,
370 and followed by mitochondrial dysfunction, calcium influx, exposure of
371 phosphatidylserine and loss of membrane permeability ultimately resulting in
372 catastrophic axon fragmentation.

373

374 In this study, we used live imaging of single axons to monitor structural and
375 physiological changes during axon degeneration. While the absolute timing of cellular
376 events varied dramatically from axon to axon, the relative timing was quite consistent.
377 Hence, this method allowed us to order cellular events, which is not possible when
378 averaging responses from many axons that are responding asynchronously. Our
379 findings in conjunction with prior studies lead to a simple model of axon degeneration
380 (Fig. 7). Following activation of SARM1, NAD⁺ is cleaved and its levels drop rapidly.
381 Upon robust NAD⁺ depletion, both glycolysis and oxidative phosphorylation will be
382 impaired, and so ATP production will decline. This loss of ATP will impact molecular
383 motors, leading to the observed halting of mitochondria. Soon after mitochondria halt,
384 they lose their membrane potential. Since this occurs before calcium increases, this
385 cannot be due to calcium overload. Instead, the loss of NAD⁺ and ATP likely disrupts
386 mitochondrial homeostasis. We next observed influx of extracellular calcium. The loss of
387 ATP is a likely culprit, as ionic pumps require ATP and their loss will lead to disrupted
388 calcium extrusion, membrane depolarization, and calcium influx. The subsequent
389 exposure of phosphatidylserine to the outer leaflet of the membrane is likely due to the
390 failure of lipid flippases to maintain asymmetry. Since these flippases are ATP-
391 dependent and can be inhibited by calcium (Bitbol et al., 1987; Pomorski and Menon,
392 2006; Soupene, 2008), loss of ATP and calcium influx may both contribute to the
393 externalization of phosphatidylserine. The ultimate loss of membrane integrity, however,
394 requires influx of extracellular calcium, as EGTA blocks fragmentation of injured axons.
395 Such axons have extensive swellings and completely dysfunctional mitochondria. While
396 calcium influx has long been assumed to be the essential final step in axon loss, our
397 findings suggest instead that calcium influx is merely leading to the morphological
398 destruction of axons that are already physiologically dead. Instead, our findings highlight
399 ATP loss as the likely point of no return for an injured axon, disrupting mitochondrial,
400 calcium, and membrane homeostasis and thereby triggering axonal demise.

401

402

403

404 **Methods**

405

406 **Animals**

407 All procedures were performed in accordance with guidelines mandated in the National
408 Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by
409 the Washington University School of Medicine in St. Louis Institutional Animal Care and
410 Use committee. CD1 mice (gestation day 11.5) for sensory neuron cultures were
411 purchased from Charles River Laboratories, and SARM1KO mice were a gift from M.
412 Colonna at Washington University in St. Louis (Szretter et al., 2009).

413

414 **Western blot analysis**

415 Lysate buffers (60 mM Tri-HCl, pH 6.8; 50% glycerol; 2% SDS; 0.1% bromophenol blue)
416 contain protease cocktail (cOmplete™, mini, EDTA-free protease inhibitor; 1183617001,
417 Millipore Sigma) and phosphatase inhibitor cocktail (P0044, Millipore Sigma). The
418 lysates were precleared of debris by centrifugation at 10,000 g in a refrigerated
419 microcentrifuge for 10 mins. Supernatants were mixed with 5% 2-mercaptoethanol
420 (Millipore Sigma) and then boiled for 10 mins. Antibodies used: Rabbit anti- β -Tubulin III
421 (1:4,000, Millipore Sigma); HRP conjugated anti-rabbit antibody (1:10,000, #111-035-
422 045, Jackson ImmunoResearch); Rabbit-anti-SARM1 (1:1,000, #13022, Cell signaling)
423 and (1:5,000); mouse anti-GFP (1:1,000, #2955S, Cell signaling); HRP-conjugated anti-
424 mouse antibody (1:5,000, 115-035-003, Jackson ImmunoResearch).

425

426 **TMRM / Fluo-4**

427 50 nM TMRM (T668, Thermo Fisher Scientific) and 1 μ M Fluo-4 (F14201, Thermo
428 Fisher Scientific) were pre-incubated for 30 minutes prior to image acquisition. When
429 Fluo-4 was incubated more than 2 hours, we found that the intensity of Fluo-4 suddenly
430 increased even in the absence of injury, and then axons degenerated. So, we only used
431 Fluo-4 to check the current status of calcium and finished the imaging session within 1
432 hour.

433

434 **Lentivirus construction/ production**

435 FUGW-PercevalHR (Addgene #49083) GCaMP6 and mRuby3 (Ko et al., 2020), human
436 SARM1.WT and human SARM1.E642A (Essuman et al., 2017) and MitoDsRed
437 (Summers et al., 2014) were transfected into HEK 293 cells for lentivirus production.
438 Briefly, the cells were seeded at 70~80% confluency per 35 mm well the day before
439 transfection. The constructs (1.2 µg) were cotransfected with vesicular stomatitis virus G
440 (600 ng) and pSPAX2 (800 ng) using FuGENE 6 (Promega). The lentiviral supernatants
441 were collected 2 days after transfection, and then the cleared supernatant was
442 concentrated with Lenti-X Concentrator (Clontech) to a final concentration of $1 \sim 10 \times$
443 10^7 particles / ml. Lentivirus transduction efficiency was monitored with tagged
444 fluorophore and western blot analysis and is routinely ~100% in DRG neurons.

445

446 **DRG neurons culture / Experimental timeline**

447 All plates for DRG cultures are coated with 0.1 mg/ml poly-D-lysine (Millipore Sigma)
448 followed by laminin (3 µg/ml; Invitrogen). CD1 mouse and SARM1 KO DRG neurons
449 were dissected from embryonic day 13.5 or 14.5. They were incubated with 0.05%
450 trypsin containing 0.05% EDTA at 37 °C for 20 mins and then washed 3 times with DRG
451 growth medium (neurobasal media from Gibco) containing 2% B27 (Invitrogen), 50
452 ng/ml nerve growth factor (Harlan Laboratories), 1 µM 5-fluoro-2'-deoxyuridine (Millipore
453 Sigma), 1 µM uridine (Millipore Sigma), and penicillin/ streptomycin (Thermo Fisher
454 Scientific). The cell density of these suspensions was adjusted to $\sim 7 \times 10^6$ cells/ml. 2 µl
455 suspensions were placed in 24-well plates (Corning) for western blots and axon
456 degeneration assays, Chamber slides (Nunc™ Lab-Tek™, Thermo Fisher Scientific)
457 were used for immunocytochemistry and FluoroDish (FD35-100, World Precision
458 Instruments) were used for live single axon imaging. Lentivirus was transduced at 1 or 2
459 days in vitro (DIV). At DIV 7, assays for axon degeneration and/ or live axon imaging
460 were performed.

461

462 **DRG neuron culture for single axon imaging**

463 For single axon imaging, conventional 2 µl suspensions ($\sim 7 \times 10^6$ DRG cells / ml) lead
464 to extensive overlap of DRG axons making it difficult to distinguish individual axons.
465 Moreover, DRG neurons did not survive well in low density culture ($\sim 7 \times 10^4$ cell / ml).

466 To circumvent these problems, we plated two different densities of DRG neurons in one
467 FluoroDish (Supple. 3). Briefly, 2 μ l suspensions ($\sim 7 \times 10^6$ cells/ml) were plated on one
468 side of FluoroDish, and then 2 μ l suspensions ($\sim 7 \times 10^4$ cells/ml) were thinly spread with
469 a pipette tip on the other side of FluoroDish. This method provides the robust health of a
470 high-density culture with the capacity to identify and image single axons.

471

472 **Live single axon imaging**

473 DRG neurons were cultured in a glass bottom FluoroDish, enabling use of an immersion
474 oil objective for calcium (GCaMP6), mitochondrial movement (MitoDR) and potential
475 (TMRM), ATP (PercevalHR), and axon morphology (GFP or mRuby3). At DIV 2,
476 lentivirus was transduced to cultured DRG neurons. Chemical dyes such as Annexin-V
477 (#A23204, Thermo Fisher Scientific) and 3 kDa Dextran-Texas Red (#D3328, Thermo
478 Fisher Scientific) were applied according to product instructions. Chamlide TCTM (Live
479 Cell Instrument, South Korea) was used to maintain 37 °C temperature, 100 ml/min 5%
480 CO₂/ 95% airflow rate. A Leica DMI4000B microscope under confocal setting using 20x
481 oil immersion objective (NA 0.6) and Leica DFC7000 T 2.8 MP color microscope
482 camera at RT was used under the control of the Leica Application Suite X software
483 platform to acquire and analyze images. Optical sectioning and laser settings were kept
484 constant across all image data acquisition sessions.

485

486 **Laser Axotomy**

487 Using a standard confocal microscope equipped with a 405-nm laser, a UV ablation
488 method was utilized to selectively induce axonal injury of cultured DRG neurons in real-
489 time (Fig. 3A). To effectively induce laser axotomy of culture DRG neurons, a glass
490 bottom (<0.17 mm) culture dish such as FluoroDish is necessary. 405-nm laser with 100%
491 intensity was used to induce laser axotomy with the FRAP (Fluorescence recovery after
492 photobleaching) wizard in Leica application Suite X software. The injury site should be
493 carefully chosen around the middle between the soma and axon terminal. If the injury
494 site is close to soma, cell body death was often observed. If the injury site is too close to
495 the distal axon, then the immediate retraction of the injured axon results in too little
496 residual axon for imaging.

497

498 **Mitochondria movement / kymograph analysis**

499 For consecutive real-time imaging capture of mitochondria, images of MitoDR were
500 recorded at 5 seconds intervals for a total of 60 frames by 558 (ex) / 583 (em)-nm laser
501 at the designated time before and after laser axotomy. The mitochondria are considered
502 mobile if the net displacement is more than 5 μm . Otherwise, they are defined as
503 stationary.

504

505 **Data analysis**

506 *Axon degeneration*

507 Axon degeneration is quantified based on axon morphology as the axon degeneration
508 index (DI) using an ImageJ-based javascript (Sasaki et al., 2009). Axons should have
509 less than 0.2 DI at baseline, otherwise, they were not used for the experiment. We
510 define axon degeneration as an axon DI >0.4 .

511

512 *Calcium influx*

513 The relative intensity of GCaMP6 from baseline was calculated as a measure of calcium
514 influx. Given the interval of 5 ~ 10 minutes between images, the intensity change of
515 GCaMP6 is dramatic. We defined a 2-fold increase or greater of GCaMP6 intensity from
516 the baseline as calcium influx.

517

518 *Axon continuity*

519 The intensity of mRuby3 was used to monitor the intactness of axons. Because mRuby3
520 is a cytosolic protein, as the integrity and thickness of axonal membrane is narrowed
521 and lost, the intensity of mRuby3 decreases. So, we defined more than 50% intensity
522 reduction of mRuby3 signal as the beginning of axon degeneration. When axonal
523 fragmentation is observed, it is defined as a degenerated axon regardless of mRuby3
524 intensity.

525

526 *TMRM*

527 After single axon injury, the intensity of TMRM were measured every 10 minutes (Fig. 4).
528 We found that there was a less 10% fluctuation of fluorescent intensity between image
529 frames. We calculated the percentage change of fluorescent intensity from the previous
530 image (Diff_TMRM in Supple. 2), and then defined a more than 30% reduction as a
531 significant loss of mitochondrial membrane potential.

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558 **Figures**

559

560 **Fig. 1. SARM1 enzymatic activity regulates mitochondrial movement and calcium**
561 **homeostasis in injured axons**

562

563 A. Representative kymograph of injured SARM1 KO axons expressing either GFP,
564 SARM1, or SARM1.E642A (E642A). For imaging mitochondria movement, MitoDsRed
565 lentivirus was transduced in all experimental conditions. Live cell imaging was
566 performed at different times (0, 2, or 4 hr) after axon injury. Scale bar = 20 μm

567

568 B. Quantification of mobile mitochondria for the neurons in (A). Data represent the mean
569 \pm SEM; n = 5 ~ 13 for each condition; one-way ANOVA with post hoc Tukey test,
570 $F(11,99) = 12.28$, $P < 0.0001$; NS, not significant; *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$

571

572

573 C. Representative images of mitochondrial potential imaged with 50 nM TMRM
574 fluorescent dye in SARM1 KO axons expressing either of GFP, SARM1, or
575 SARM1.E642A. Live cell imaging was performed at the indicated times (0, 4, or 24 hr)
576 after axon injury. Scale bar = 30 μm

577

578 D. Quantification of TMRM intensity from the experiment in (C). Injured SARM1 KO
579 axons expressing the enzymatically disabled SARM1 mutant (E642A) maintained
580 TMRM signal without significant loss. Data represent the mean \pm SEM; n = 5 ~ 6 for
581 each condition; one-way ANOVA with post hoc Tukey test, $F(4,23) = 53.11$, $P < 0.0001$;
582 NS, not significant; *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$

583

584 E. Representative images of calcium influx imaged with 1 μM Fluo-4 fluorescent dye in
585 SARM1 KO axons expressing either of GFP, SARM1, or SARM1.E642A. Live cell
586 imaging was performed at different times (0, or 4 hr) after axon injury. Scale bar = 30
587 μm

588

589 F. Quantification of Fluo-4 intensity from the experiment in (E). Injured SARM1 KO
590 axons expressing the enzymatically disabled SARM1 mutant (E642A) completely
591 prevent calcium influx. Data represent the mean \pm SEM; n = 5 ~ 8 for each condition;
592 one-way ANOVA with post hoc Tukey test, $F(3,22) = 23.05$, $P < 0.0001$; NS, not
593 significant; *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$

594

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596

597 **Fig. 2. The role of calcium in axon degeneration.**

598

599 A. Pre-incubation of MPTP inhibitor (1, 10, or 100 μ M CsA) did not significantly prevent
600 the degeneration of wild-type axons after axon injury. Axon degeneration is defined as a
601 degeneration index > 0.4 (dashed line). n = 3

602

603 B. (Left) Representative images of calcium influx acquired with 1 μ M Fluo-4 dye. Scale
604 bar = 30 μ m (Right) The degree of calcium influx in CsA pre-incubated injured axons is
605 not significantly different from DMSO pre-incubated injured axons. Fold increment of
606 injured axons at 4 hr after axotomy is calculated from uninjured axons. Data represent
607 the mean \pm SEM; n = 5 for each condition; two-tailed unpaired t test, $p = 0.39$; NS, not
608 significant; *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$

609

610 C. Internal calcium chelator (pre-incubation with 10 μ M BAPTA) did not delay axon
611 degeneration after axonal injury.

612

613 D. (Top) Experimental design. Extracellular calcium chelator, 3 mM ETGA, was included
614 in the culture medium at different time points (2 ~ 48 hr vs 0 ~ 2 hr vs 0 ~ 48 hr). For
615 addition of EGTA from 0 ~ 2 hr, culture medium was replaced at 2 hr. (Bottom, left)
616 Representative bright-field images of axons.

617

618 E. Quantification of axon degeneration for the experiment in (D). Although there are
619 axonal swellings (red triangle) in the presence of EGTA, injured axons remain intact
620 when the EGTA is present 2 hours after axotomy. $n = 3$

621
622 F. Representative images of mitochondria potential (TMRM fluorescent dye) and axon
623 morphology (GFP lentivirus) in uncut axons and cut axons +/- EGTA. Scale bar = 100
624 μm

625
626 G. Quantification of the TMRM staining for the experiment in (F). EGTA incubation in
627 injured axons does not maintain mitochondrial hyperpolarization. Data represent the
628 mean \pm SEM; $n = 6 \sim 7$ for each condition; one-way ANOVA with post hoc Tukey test,
629 $F(2,16) = 98.27$, $P < 0.0001$; NS, not significant; *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$

630
631
632 H. Representative kymograph of uncut and cut axons +/- EGTA as indicated.
633
634 I. Quantification of total number of mitochondria (left) and mobile mitochondria (right) for
635 the experiment in (H). Data represent the mean \pm SEM; $n = 8 \sim 12$ for each condition;
636 one-way ANOVA with post hoc Tukey test, for mobile mitochondria $F(3,24) = 16.34$,
637 $P < 0.0001$; for number of mitochondria, $F(3,34) = 0.8787$, $P = 0.46$; NS, not significant; *,
638 $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$

639
640
641
642 **Figure 3. Live single axon imaging enables temporal dissection of cellular events**
643 **in injured axons.**

644
645 A. Schematic diagram of laser axotomy in cultured embryonic DRG neurons. GCaMP6
646 and mRuby3 were expressed to observe calcium fluctuations and axonal morphology.
647

648 B. Snapshots of an injured wild-type axon. Also see supplemental video 1. Progression
649 of axon degeneration is described at the bottom of the schematic. Note that there is
650 both an early and late phase of calcium influx. The 1st peak of calcium occurs at the
651 injury site (white triangle) before calcium levels return to normal. The 2nd calcium peak
652 persists until the axon degenerates. Scale bar = 100 μm

653

654 C. Representative analysis of a single injured axon. The calcium response (left y-axis)
655 and measure of axon continuity (right y-axis) for a single axon is plotted over time after
656 axonal injury. Note the two distinct calcium peaks.

657

658 D-F. Grouped analysis from single axons for the (D) initiation, (E) duration, and (F)
659 intensity of the 2nd peak of calcium compared to the time at which each axon fragments.
660 The initiation of the second calcium peak occurs ~ 1.4 hrs before and is strongly
661 correlated with axon fragmentation, while the duration of the second peak is weakly
662 correlated and the intensity of the second peak is not correlated with axon
663 fragmentation. $n=22$.

664

665

666

667 **Figure 4. Mitochondrial dysfunction precedes calcium influx in injured axons.**

668

669 A. (Top) Experimental design for observing calcium influx (GCaMP6) and mitochondria
670 movement (MitoDR) after axon injury. MitoDR images were acquired every 5 seconds
671 (for 300 second, 60 frames) followed by GCaMP6 imaging. Once the mitochondria in
672 that axon stopped, GCaMP6 images were then acquired once/minute. (Bottom)
673 Representative images of GCaMP6 and kymograph at the indicated times. Note that
674 mitochondria stop prior to calcium influx. Scale bar = 30 μm

675

676 B. (Top) Single axon analysis after injury. The percentage of mobile mitochondria (red,
677 left y-axis) and the fold change in calcium (green, right y-axis) for a single axon were

678 plotted over time after axonal injury. (Bottom) Inset from graph highlights that
679 mitochondria stop moving before calcium levels rise.

680

681 C. Group data from single axons show that the time difference (ΔT) between cessation
682 of mitochondrial mobility and calcium influx, defined as a 2-fold increase from baseline,
683 is $\sim 0.42 \pm 0.02$ hr, indicating that mitochondria stop before calcium influx in injured
684 axons. The grey line ($\Delta T=0$) shows expected results if mitochondria stopped and
685 calcium influx occurred simultaneously. $n=9$

686

687 D. (Top) Experimental design to observe calcium influx (GCaMP6) and loss of
688 mitochondrial potential (TMRM) after axonal injury. GCaMP6 and TMRM were imaged
689 every 10 minutes until axon fragmentation. (Bottom) Representative images shown at
690 the indicated times. The TMRM signal declines by 2.83 hr after axonal injury, while
691 calcium influx does not occur until 3.0 hr after injury. Scale bar = 30 μm

692

693 E. (Left) Analysis of the single axon in D. The ratio of TMRM signal from baseline (left y-
694 axis) and the fold increment of calcium (right y-axis) were plotted over time after axonal
695 injury. (Right) The enlarged insight highlights the point at which there is a dramatic
696 change in the mitochondria potential (brighter red dot) and calcium levels (brighter
697 green dot). Note that the change in TMRM from baseline precedes the change in
698 calcium. This was observed in 5 out of 7 axons, while in 2 out of 7 axons the change
699 occurred in the same 10 minute imaging bout.

700

701 **Figure 5. ATP levels drop before mitochondria stop in injured axons.**

702

703 A. (Left) Experimental design for imaging changes to ATP (PercevalHR) and
704 mitochondrial movement (MitoDR) after axonal injury. Prior to axotomy, baseline
705 PercevalHR intensity and mitochondrial movement were measured. PercevalHR was
706 imaged every 5 minutes until 3.5 hr after axonal injury, while mitochondria were imaged
707 every 5 minutes starting 3.5 hr after axotomy until movement ceased. (Right)

708 Representative images for PercevalHR and kymographs of moving mitochondria at the
709 indicated times. Scale bar = 30 μm

710

711 B. (Left) Percentage decline from baseline at 3.5 hr post-axotomy for PercevalHR
712 intensity and for the fraction of motile mitochondria. Lines connect data for individual
713 cells. (Right) Linear regression plot of group data. $n=9$

714

715 C. The percentage decline of PercevalHR intensity at 3.5 hr after axonal injury is plotted
716 against the subsequent time until mitochondria stop moving for that axon. Linear
717 regression plot of group data. $n=9$

718

719

720 **Figure 6. Calcium influx disrupts membrane integrity**

721

722 A. (Left) Snapshots of representative live axon images for GCaMP6, mRuby3, and
723 Alex647-conjugated Annexin-V at baseline, and 4.78 and 6.28 hr after axotomy. (Right)
724 Representative single axon analysis. Y-axis (left) is plotted by the fold increase of
725 fluorescent intensity (F / F_{base}) of either GCaMP6 (green color dots) or Annexin-V (cyan
726 color dots) from the baseline after axon injury. Axon integrity (y-axis, right) is calculated
727 by the relative mRuby3 intensity from the baseline. Note that calcium influx precedes
728 Annexin-V exposure in an injured axon. Scale bar = 50 μm

729

730 B. After axotomy, the time until calcium influx is plotted vs the time until the rise in
731 Annexin-V. Dashed line ($\Delta T=0$) represents the values if phosphatidylserine exposure
732 (Annexin-V staining) and calcium influx occurred simultaneously. Calcium influx
733 precedes phosphatidylserine exposure by an average of 0.51 ± 0.04 hr. $n=10$.

734

735 C. (Top) Representative images of intact, swollen, and fragmented axons during the
736 process of axon degeneration. The axonal morphology is labelled with GFP that was
737 transduced through GFP-lentivirus. Texas Red conjugated Dextran-3kDa was pre-

738 incubated 30 minutes prior to image acquisition. Note that Dextran-3kDa is only
739 observed in the fragmented axons, not in swollen axons. Scale bar = 10 μ m

740

741 D. Representative images of GFP-expressing axotomized wild-type and SARM1 KO
742 axons. The membrane impermeable Dextran-3k enters injured wild-type axons, but after
743 injury is excluded from both EGTA-treated wild-type axons and SARM1 KO axons (GFP
744 labels axons). Scale bar = 100 μ m

745

746 E. Group data. Quantification of dextran-3k staining intensity in the indicated genotypes
747 and times. Data represent the mean \pm SEM; n = 6 for each condition; one-way ANOVA
748 with post hoc Tukey test, $F(6,35) = 46.16$, $P < 0.0001$; NS, not significant; *, $P < 0.05$; **,
749 $P < 0.01$ and ***, $P < 0.001$

750

751

752 **Figure 7. Model of Axon degeneration**

753 The model depicts the ordered series of events that occur in an injured axon following
754 SARM1 activation. These begin with NAD^+ loss, followed by ATP decline, loss of
755 mitochondrial mobility, loss of mitochondrial polarization, influx of calcium,
756 externalization of phosphatidylserine to the outer leaflet of the plasma membrane, and
757 finally fragmentation of the axon allowing for influx of large molecular weight dextrans.
758 Mitochondria localize to axonal swellings (see supplemental figure 4).

759

760 **Supplemental Figure 1. Expression level of SARM1.WT and SARM1.E642A**

761

762 Western blot analysis demonstrates that the lentiviral mediated expression of
763 SARM1.WT and SARM1.E642A is very similar.

764

765 **Supplemental Figure 2. Assessing mitochondrial potential and calcium influx in** 766 **injured axons**

767

768 (Left) Differentiation of TMRM and GCaMP6 measurements in Fig. 4E. The
769 differentiation is defined as the percentage change from the previous intensity. The
770 percentage change (y-axis) of both mitochondrial potential and calcium influx over the
771 time after axon injury. (Right) This graph highlights the time window when calcium level
772 (brighter green dot) increases at least 2-fold and mitochondria potential (brighter red dot)
773 are maximally changed.

774

775 **Supplemental Figure 3. DRG neuron culture for single axon imaging**

776

777 For single axon imaging, 2 μ l high-density cell suspensions ($\sim 7 \times 10^6$ cells / ml) were
778 plated on the one side of a FluoroDish and 2 μ l low-density cell suspensions ($\sim 7 \times 10^4$
779 cells / ml) were thinly spread on the opposite side. The high-density culture was
780 required for maintenance of neurons in the low-density culture, and the low-density
781 culture enabled imaging of single axons.

782

783 **Supplemental Figure 4. Mitochondria accumulate in axonal swellings in injured** 784 **axons**

785

786 (Top) Representative images of injured (a) and uninjured (b) axons labelled with GFP
787 and MitoDR. (Bottom) Pixel intensity of each axon is plotted. Note that most axonal
788 swellings overlap with mitochondria (Asterisks).

789

790 **Supplemental Video 1. Time lapse imaging of injured single axon**

791 Total 45 second video. (0 ~ 20 seconds) Brief demonstration of experimental design for
792 single axon imaging. (21 ~ 45 seconds) Example of Figure 3. Briefly, image the baseline
793 activity at distal axon, followed by axon injury with a 405 nm laser (blue square). Note
794 that there is an increase of GCaMP6 intensity at the injury site, which is the first peak of
795 calcium. Massive calcium influx enters the injured distal axon and then later axon starts
796 degenerate. Image acquisition of both GCaMP6 and mRuby3 continues until the axon
797 degenerates.

798

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803

804 **Author contributions**

805 K.W.K. and A.D. designed research. K.W.K performed all research and data analysis.
806 K.W.K., J.M., and A.D. wrote the manuscript. J.M. and A.D. supervised experiments.

807

808 **Competing of interests**

809 A. DiAntonio and J. Milbrandt are cofounders, scientific advisory board members, and
810 shareholders of Disarm Therapeutics, a wholly owned subsidiary of Eli Lilly and
811 Company. The authors declare no additional competing financial interests.

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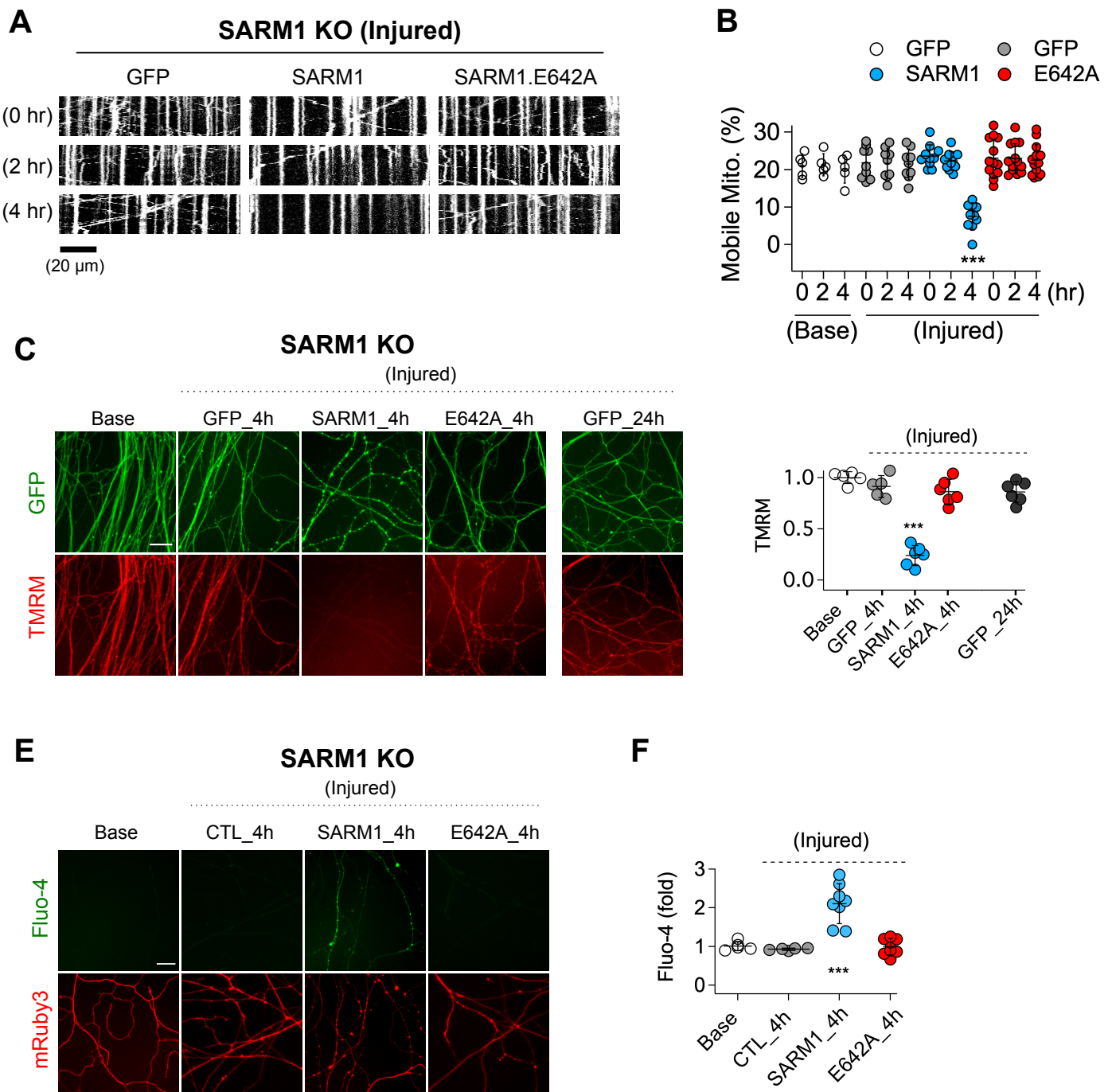
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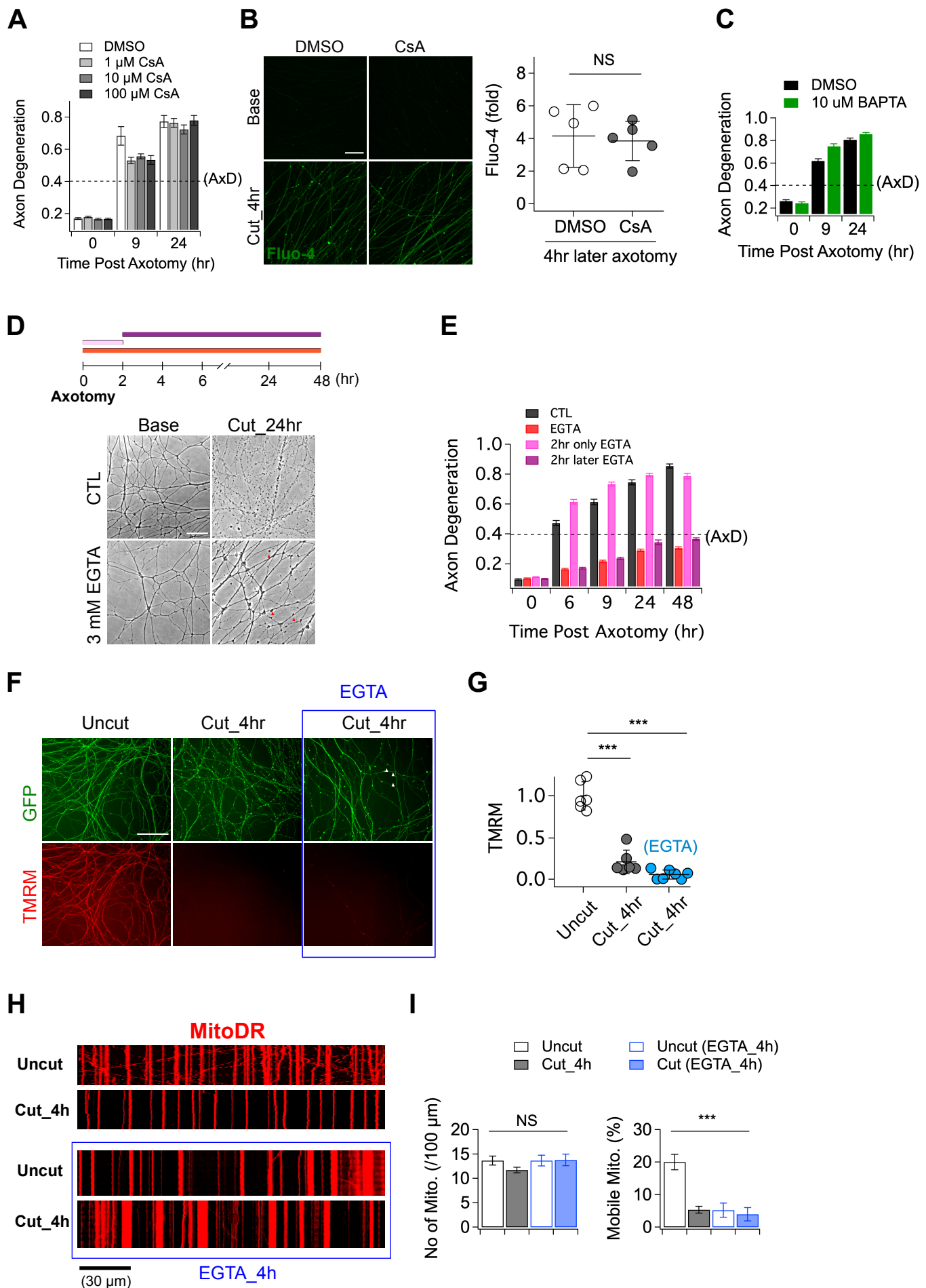
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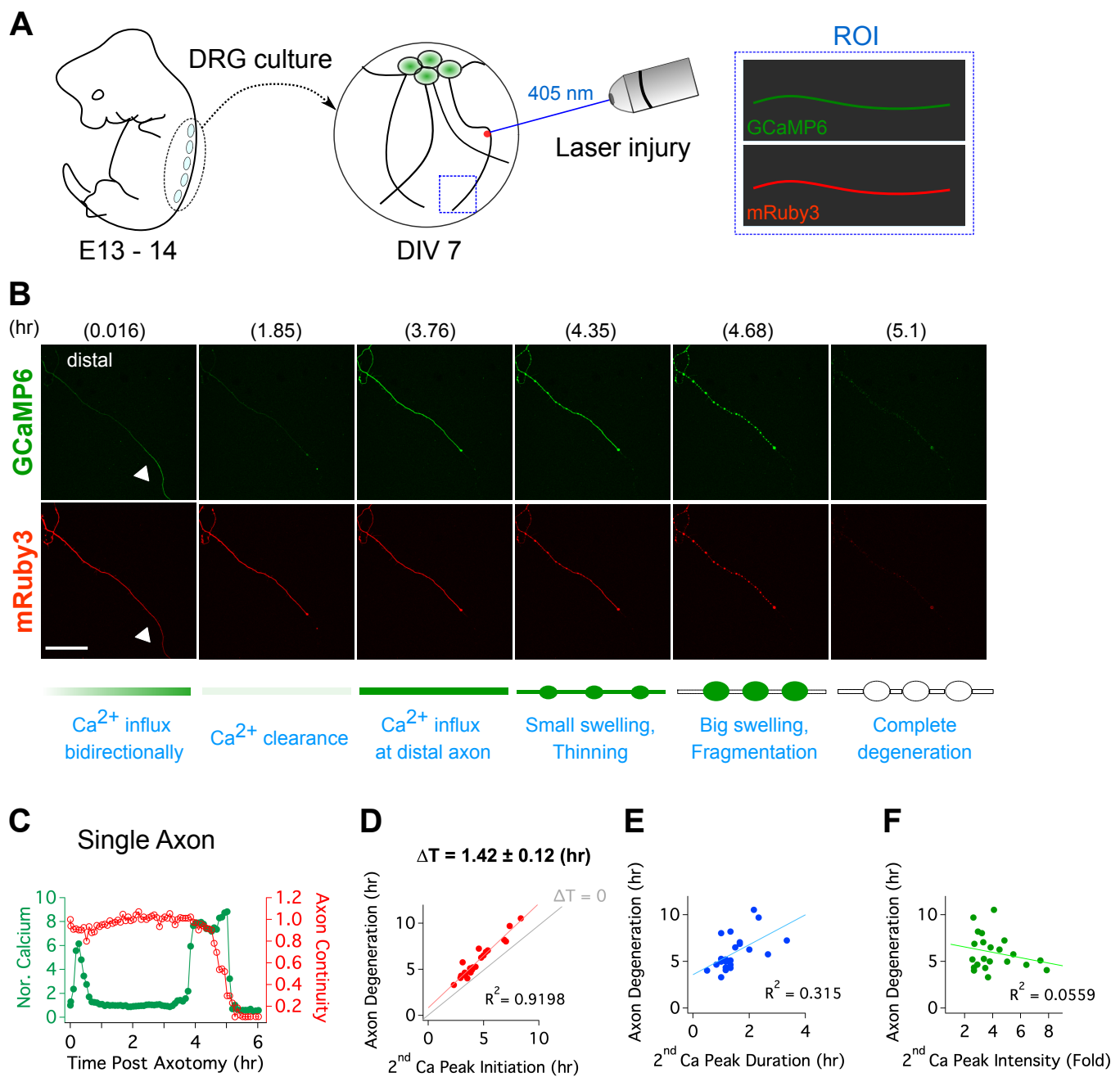
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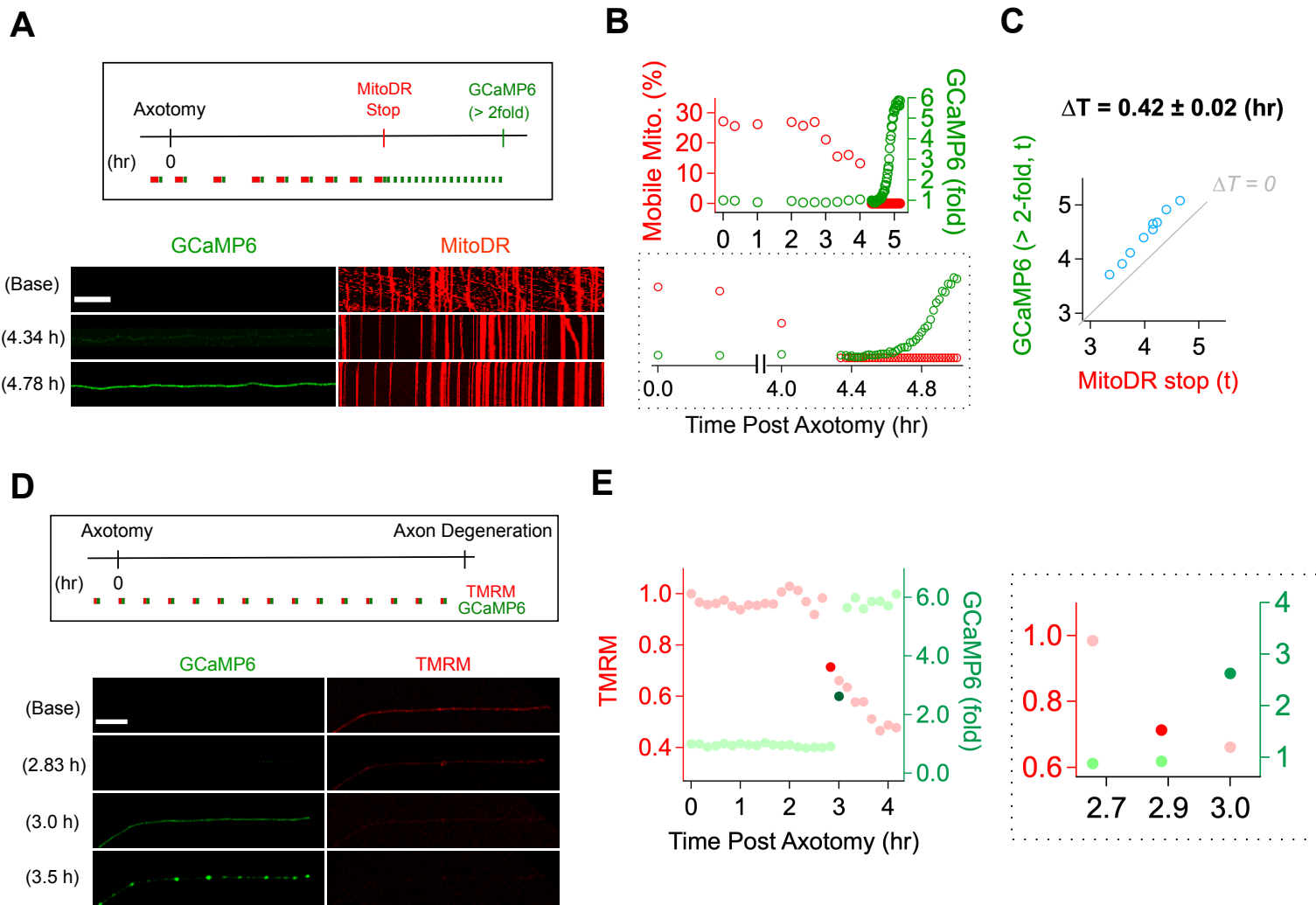
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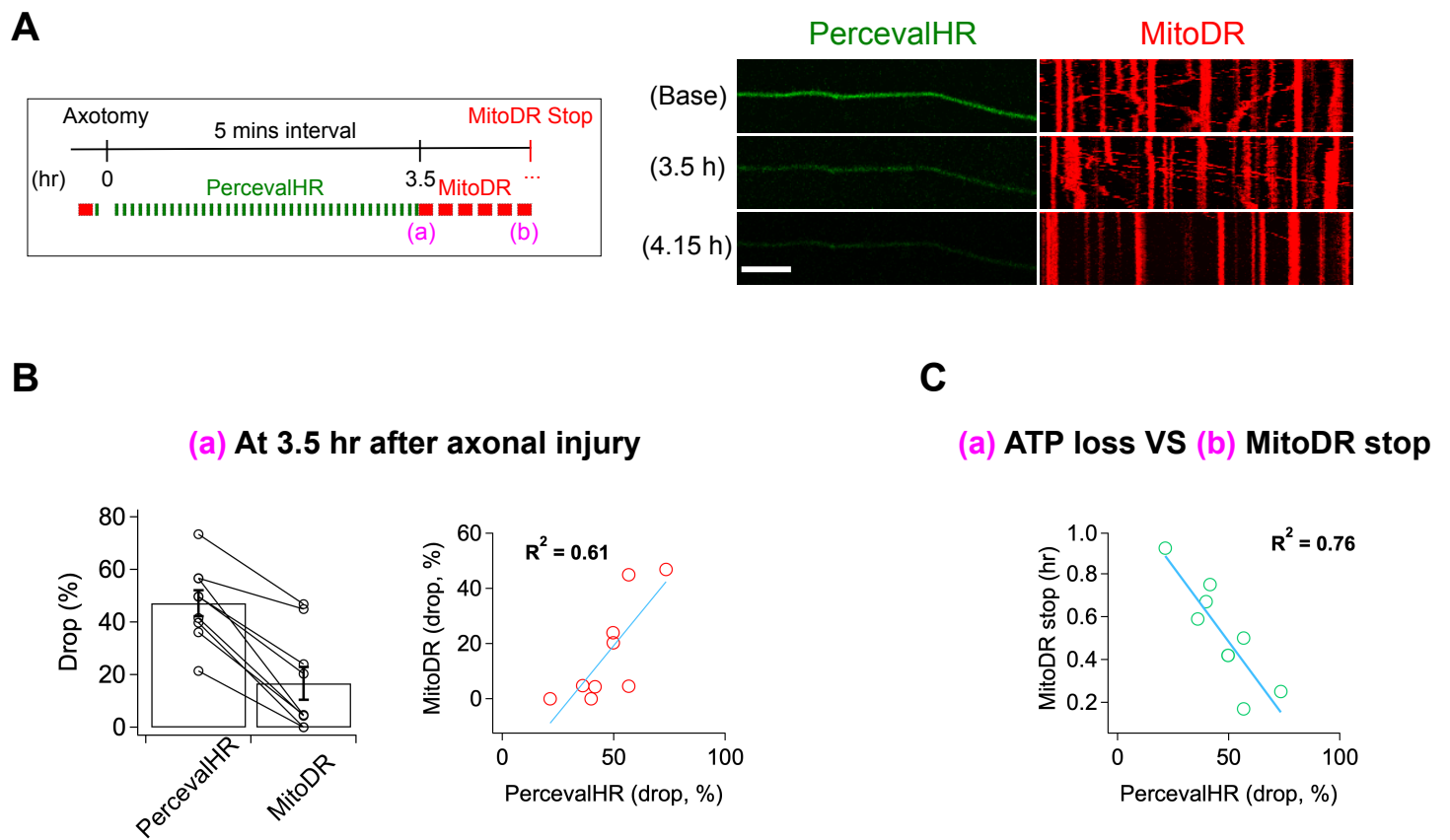
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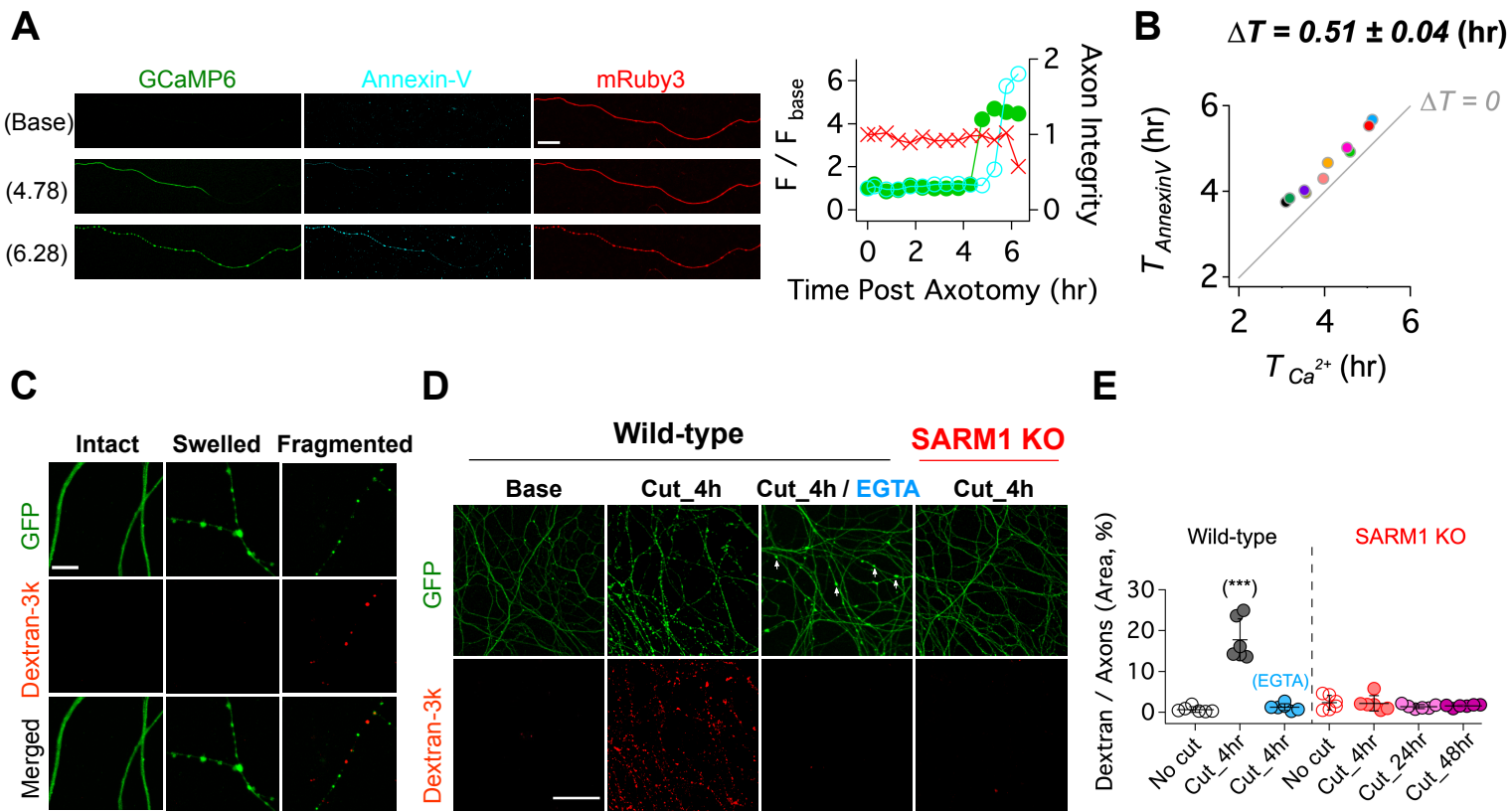
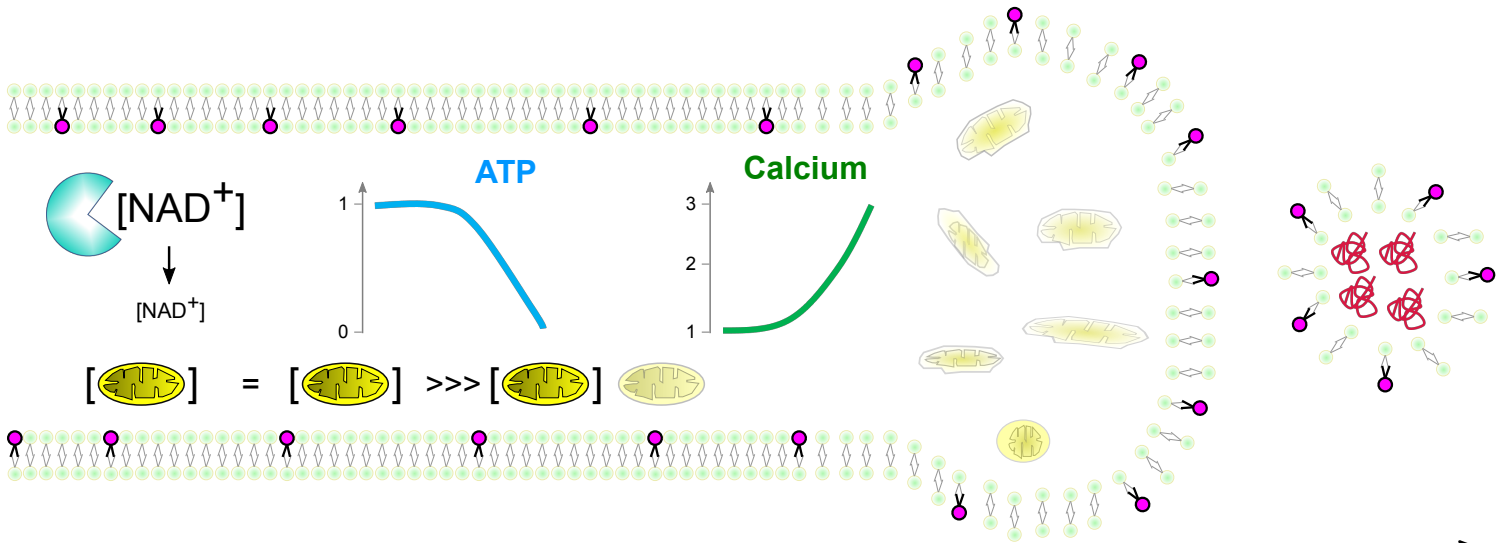
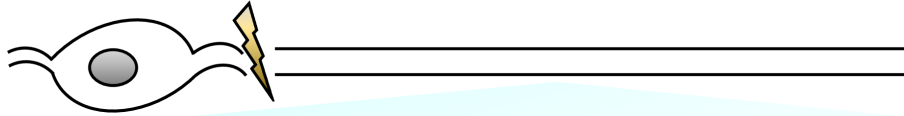


Fig. 7 bioRxiv preprint doi: <https://doi.org/10.1101/2021.06.14.448297>; this version posted June 15, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.



SARM1 activation

NAD⁺ depletion

ATP loss

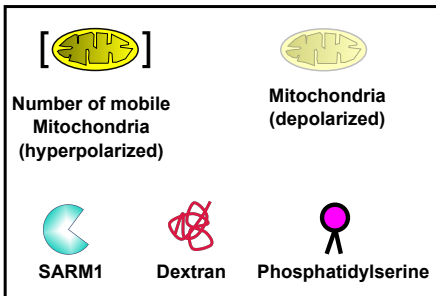
Time Post Axotomy

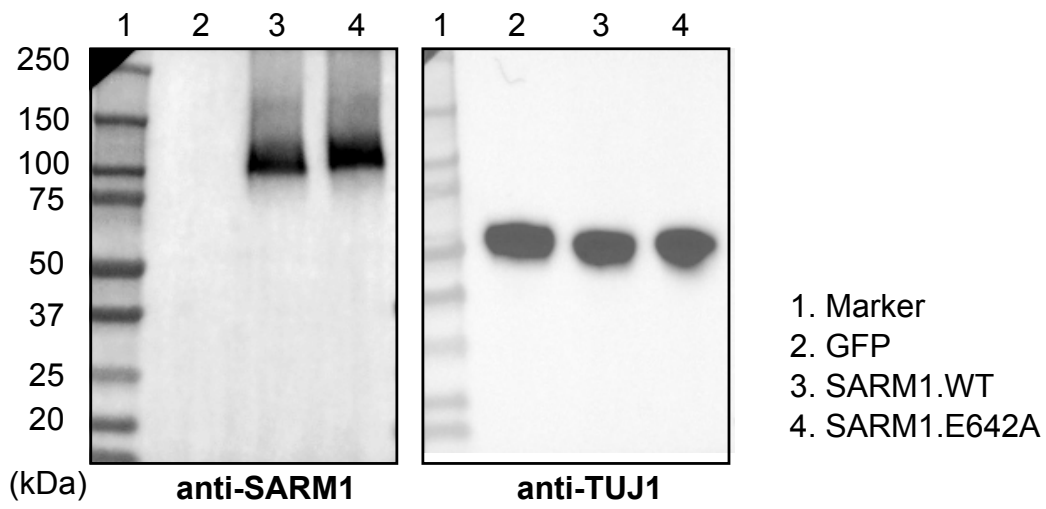
Mitochondria
- fewer mobile
- depolarized

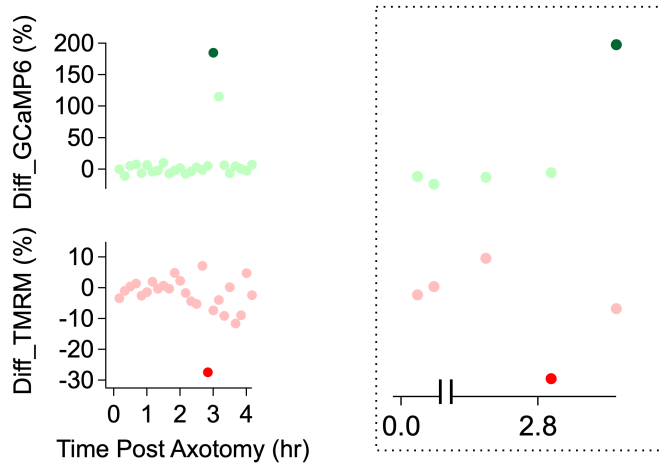
Calcium influx

Phosphatidylserine
(Outer leaflet)

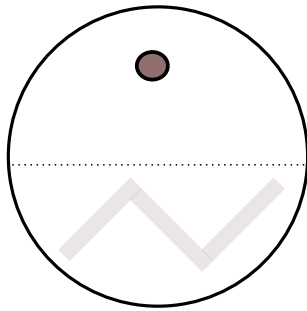
Dextran influx &
Axon fragmentation







Supple 3



FluoroDish



2 μ l suspension ($\sim 7 \times 10^6$ cells/ml)



2 μ l suspension ($\sim 7 \times 10^4$ cells/ml)

Single axon imaging area

