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Trim39 regulates neuronal apoptosis by acting as a SUMO-targeted E3 ubiquitin-ligase for the transcription factor NFATc3

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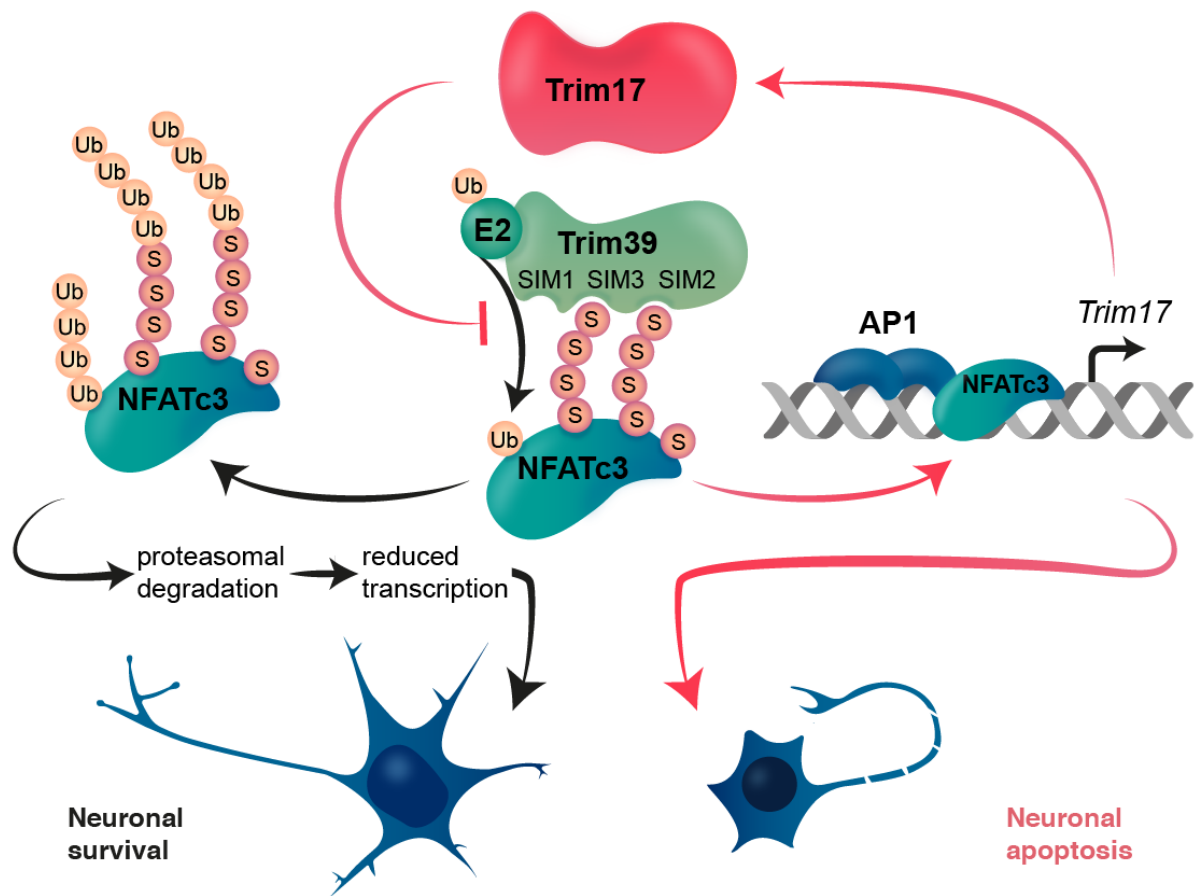
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7 Trim39 regulates neuronal apoptosis by acting as a SUMO-targeted
8 E3 ubiquitin-ligase for the transcription factor NFATc3

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22 Synopsis:

23 Trim39 acts as a SUMO-targeted E3 ubiquitin-ligase for the transcription factor NFATc3.
 24 Indeed, Trim39 preferentially binds and ubiquitinates the SUMOylated forms of NFATc3 by
 25 its SUMO interacting motifs (SIM), mostly its so-called SIM3. In the absence of Trim17 (black
 26 arrows), Trim39 ubiquitinates SUMOylated NFATc3, possibly on its SUMO chains or other
 27 sites. This induces the proteasomal degradation of NFATc3 and reduces the expression of its
 28 target genes, thereby promoting neuronal survival. In the presence of Trim17 (red arrows),
 29 Trim39-mediated ubiquitination and degradation of NFATc3 is inhibited, which stabilizes
 30 NFATc3 and increases the expression of its target genes, including Trim17 itself. This creates
 31 a positive feedback loop that favors neuronal apoptosis.

32

33 **Abstract** (175 words)

34 NFATc3 is the predominant member of the NFAT family of transcription factors in neurons,
35 where it plays a pro-apoptotic role. Mechanisms controlling NFAT protein stability are poorly
36 understood. Here we identify Trim39 as an E3 ubiquitin-ligase of NFATc3. Indeed, Trim39
37 binds and ubiquitinates NFATc3 *in vitro* and in cells where it reduces NFATc3 protein level
38 and transcriptional activity. In contrast, silencing of endogenous Trim39 decreases NFATc3
39 ubiquitination and increases its activity, thereby resulting in enhanced neuronal apoptosis. We
40 also show that Trim17 inhibits Trim39-mediated ubiquitination of NFATc3 by reducing both
41 the E3 ubiquitin-ligase activity of Trim39 and the NFATc3/Trim39 interaction. Moreover, we
42 identify Trim39 as a new SUMO-targeted E3 ubiquitin-ligase (STUbL). Indeed, mutation of
43 SUMOylation sites in NFATc3 or SUMO-interacting motifs in Trim39 reduces
44 NFATc3/Trim39 interaction and Trim39-induced ubiquitination of NFATc3. In addition,
45 Trim39 preferentially ubiquitinates SUMOylated forms of NFATc3 *in vitro*. As a consequence,
46 a SUMOylation-deficient mutant of NFATc3 exhibits increased stability and pro-apoptotic
47 activity in neurons. Taken together, these data indicate that Trim39 modulates neuronal
48 apoptosis by acting as a STUbL for NFATc3.

49

50

51 **Introduction**

52 The NFAT (Nuclear Factor of Activated T cells) family of transcription factors is a key player
53 in a wide range of physiological and pathological processes. Initially discovered in activated T
54 cells (Shaw et al., 1988), the different members of the NFAT family have been identified in
55 most tissues where they play both redundant and specific roles (Fric et al., 2012; Kipanyula et
56 al., 2016; Mognol et al., 2016; Wu et al., 2007). They are implicated in the development and
57 the function of the immune system, brain, cardiovascular system, skeletal muscles, bones and
58 other organs by regulating the expression of different target genes involved in cytokine
59 production but also in cell proliferation, differentiation and apoptosis. As a consequence, NFAT
60 deregulation is involved in many pathologies including auto-immune diseases, cancer and
61 neurodegenerative diseases (Kipanyula et al., 2016; J.-U. Lee et al., 2018; Müller & Rao, 2010).
62 A better understanding of NFAT regulation, in particular by post-translational modification and
63 degradation, is therefore of crucial importance.

64 The calcium-regulated, cytoplasmic-nuclear shuttling of NFATc1, NFATc2, NFATc3 and
65 NFATc4 has been extensively studied. These NFAT members are normally found in the
66 cytoplasm in a hyperphosphorylated and inactive state. Upon an increase in intracellular
67 calcium levels, they are dephosphorylated by the calcium-dependent phosphatase calcineurin,
68 which triggers their nuclear import and activation. Once in the nucleus, NFATs induce (or
69 repress) the transcription of specific target genes, usually in cooperation with partner
70 transcription factors such as AP-1 or co-activators (Hogan et al., 2003; Mognol et al., 2016;
71 Müller & Rao, 2010). In contrast, the regulation of NFAT stability by the ubiquitin-proteasome
72 system remains elusive. Only a few studies have addressed this issue. Nevertheless, NFATs are
73 relatively short-lived proteins and previous studies have shown that interfering with the
74 regulation of NFAT levels by the ubiquitin-proteasome system can have a marked impact on
75 the physiology of various cell types (Chao et al., 2019; X. Li et al., 2015; Narahara et al., 2019;
76 Singh et al., 2011; Yoeli-Lerner et al., 2005; Youn et al., 2012). In addition to phosphorylation
77 and ubiquitination, NFAT proteins have been shown to be regulated by SUMOylation. Several
78 studies have shown that covalent conjugation of SUMO to NFATs has an impact on their
79 cytoplasmic-nuclear shuttling, subnuclear localization and transcriptional activity (E. T. Kim
80 et al., 2019; Nayak et al., 2009; Terui et al., 2004; Vihma & Timmusk, 2017). In general,
81 SUMOylation can have many consequences on its substrate proteins, including modification of
82 their activity, interaction properties and subcellular localization (Henley et al., 2018; X. Zhao,
83 2018). In addition, SUMOylation of proteins can regulate their stability (Liebelt & Vertegaal,
84 2016). Indeed, a few E3 ubiquitin-ligases that specifically recognize and ubiquitinate
85 SUMOylated proteins have been described (M. C. Geoffroy & Hay, 2009; Prudden et al., 2007;
86 Sriramachandran & Dohmen, 2014). These SUMO-targeted E3 ubiquitin-ligases (STUBLs)
87 generally induce the degradation of their substrates by the proteasome, raising the possibility
88 that SUMO might also modulate NFAT ubiquitination and degradation.

89 NFATc3 is the predominant NFAT family member expressed in various neuronal types (M. S.
90 Kim & Usachev, 2009; Luo et al., 2014; Mojsa et al., 2015; Ulrich et al., 2012; Vashishta et al.,
91 2009). We have previously shown that NFATc3 is involved in the regulation of neuronal
92 apoptosis (Mojsa et al., 2015). Two independent studies have also implicated NFATc3 in α -
93 synuclein-induced degeneration of midbrain dopaminergic neurons in Parkinson's disease
94 (Caraveo et al., 2014; Luo et al., 2014). Following depolarization-induced elevations of
95 intracellular calcium in neurons, NFATc3 is rapidly and strongly activated (Ulrich et al., 2012).
96 Once in the nucleus, activation of pro-apoptotic protein kinases such as GSK3 β does not seem

97 to be sufficient to induce NFATc3 nuclear exclusion in neurons (Mojsa et al., 2015; Ulrich et
98 al., 2012). Proteasomal degradation could therefore be an alternative way to reduce its activity
99 in this case. However, only one study relating NFATc3 ubiquitination and degradation has been
100 reported so far, in the context of LPS-induced cardiac hypertrophy (Chao et al., 2019). In
101 previous work, we have shown that NFATc3 can be SUMOylated on three consensus sites
102 (Mojsa et al., 2015). We have also found that NFATc3 binds to Trim17 (Mojsa et al., 2015),
103 which belongs to a large family of RING-containing E3 ubiquitin-ligases (Basu-Shrivastava et
104 al., 2021). Although its E3 ubiquitin-ligase activity has been confirmed (I. Lassot et al., 2010;
105 Urano et al., 2009), Trim17 does not induce NFATc3 ubiquitination. On the contrary,
106 overexpression of Trim17 reduces the ubiquitination of NFATc3 and increases its steady-state
107 protein level (Mojsa et al., 2015). Since TRIM17 can prevent ubiquitination of some of its
108 binding partners by inhibiting other E3 ubiquitin-ligases from the TRIM family (Iréna Lassot
109 et al., 2018; Lionnard et al., 2019), we hypothesized that the stability of NFATc3 might be
110 regulated by a TRIM protein interacting with Trim17, such as Trim39.

111 In the present study, we demonstrate that Trim39 is a genuine E3 ubiquitin-ligase for NFATc3.
112 We also show that Trim39-mediated ubiquitination of NFATc3 is inhibited by Trim17.
113 Moreover, we found that mutation of NFATc3 SUMOylation sites both decreases its
114 ubiquitination by Trim39 and increases its stability. The same effects are reproduced by
115 mutation of one of the SUMO-interacting motifs (SIMs) in Trim39. These data indicate that
116 Trim39 acts as a STUbL for NFATc3. As a result, SUMOylation and Trim39 modulate the
117 transcriptional activity of NFATc3 and its pro-apoptotic effect in neurons. Therefore, our study
118 provides the identification of a new STUbL and a first insight into complex mechanisms
119 regulating the stability of NFATc3 in neurons.

120

121 **Results**

122 **Trim39 is an E3 ubiquitin-ligase for NFATc3**

123 Human TRIM39 and TRIM17 proteins have been found to interact with each other in three
124 independent proteome-scale yeast two-hybrid screens (Rolland et al., 2014; Rual et al., 2005;
125 Woodsmith et al., 2012). To determine whether mouse Trim39 and Trim17 proteins can also
126 bind to each other, and whether Trim39 can bind to NFATc3, co-immunoprecipitation
127 experiments were performed. Indeed, in cells co-transfected with Trim17-GFP and Flag-
128 Trim39, immunoprecipitation of Trim39 using an anti-Flag antibody co-precipitated Trim17,

129 likewise, immunoprecipitation of Trim17 using GFP-Trap beads co-precipitated Trim39 (Fig.
130 1A). In a similar way, in cells co-transfected with HA-NFATc3 and Flag-Trim39, the two
131 proteins were reciprocally co-immunoprecipitated by using either anti-Flag or anti-HA
132 antibodies (Fig. 1B). To confirm this interaction at the endogenous level, we first showed that
133 endogenous Trim39 is co-immunoprecipitated with endogenous NFATc3 (Fig. 1C). Second,
134 we performed an *in situ* proximity ligation assay (PLA) in Neuro2A cells, using anti-NFATc3
135 and anti-Trim39 antibodies. Close proximity was detected between endogenous NFATc3 and
136 endogenous Trim39 as assessed by a PLA signal (Fig. 1D) that was predominantly cytoplasmic
137 (Fig. 1D, endo 1 slice). Overexpression of Trim39 increased the PLA signal, as expected (Fig.
138 1D). To confirm the specificity of the assay, we used a specific shRNA against Trim39 which
139 effectively reduces the level of endogenous Trim39 protein as assessed by immunofluorescence
140 (Fig. S1). As expected, silencing of Trim39 using this shRNA strongly decreased the PLA
141 signal (Fig. 1E). Taken together, these data indicate that Trim39 interacts with both Trim17 and
142 NFATc3.

143 We next examined whether Trim39 could mediate the ubiquitination of NFATc3. In Neuro2A
144 cells co-transfected with His-tagged ubiquitin, the ubiquitination level of NFATc3 was
145 significantly increased by overexpressed Trim39 but not by an inactive mutant deleted of its
146 RING domain (Trim39- Δ RING; Fig. 2A). In contrast, silencing of Trim39 using three different
147 specific shRNAs, decreased the ubiquitination of NFATc3 (Fig. 2B). Interestingly, the decrease
148 in NFATc3 ubiquitination was directly correlated with the silencing efficiency of the different
149 shRNAs, with the best one (T39#3) strongly reducing both the expression of endogenous
150 Trim39 and the ubiquitination of NFATc3 (Fig. 2B). Therefore, these data suggest that Trim39
151 is the major E3 ubiquitin-ligase involved in the ubiquitination of NFATc3, at least in Neuro2A
152 cells. To further demonstrate that Trim39 is an E3 ubiquitin-ligase of NFATc3, we carried out
153 *in vitro* ubiquitination using *in vitro* translated/immuno-purified NFATc3 and purified
154 recombinant proteins. In these experiments, GST-Trim39 stimulated NFATc3 ubiquitination in
155 the presence of ubiquitin, E1 and E2 enzymes (Fig. 2C). In contrast, an inactive mutant of
156 Trim39, in which two Cys residues of the RING domain were mutated (GST-Trim39-
157 C49S/C52S), did not have any effect (Fig. 2C). The wheat germ extract that was used to produce
158 NFATc3 by *in vitro* transcription-translation (TnT) could contain contaminant proteins.
159 However, NFATc3 was immunopurified from the TnT mix prior to the ubiquitination reaction.
160 The probability that proteins which can modify NFATc3 were co-purified with NFATc3 from
161 a plant extract is low. Moreover, the fact that no modification of NFATc3 is visible when the

162 reaction is performed in the absence of ubiquitin or with an inactive mutant of Trim39, strongly
163 suggest that NFATc3 is a direct substrate for the E3 ubiquitin-ligase activity of Trim39.

164

165 **Trim39 induces the degradation of NFATc3 and decreases its transcriptional activity**

166 Because ubiquitination often targets proteins for proteasomal degradation, we examined
167 whether Trim39 could impact the protein level of NFATc3. Indeed, the level of NFATc3
168 progressively decreased when co-transfected with increasing amounts of Trim39 (Fig. 3A).
169 Interestingly, the inactive mutant Trim39- Δ RING did not decrease the protein level of NFATc3
170 but rather increased it, in a similar way to the proteasome inhibitor MG-132 (Fig. 3A).
171 Mutations of the RING domain of E3 ubiquitin-ligases generally induce a dominant-negative
172 effect (I. Lassot et al., 2010; Pickart, 2001). Therefore, this increase in NFATc3 protein may be
173 due to the inhibition of endogenous Trim39 by Trim39- Δ RING, as it has been previously
174 reported for the effect of Trim39 on the half-life of p53 (Zhang, Huang, et al., 2012).
175 Consistently, silencing of endogenous Trim39 using a specific siRNA also significantly
176 increased the protein level of endogenous NFATc3 in Neuro2A cells, without significantly
177 modifying its mRNA level (Fig. 3B). Taken together, these data strongly suggest that Trim39-
178 mediated ubiquitination is involved in the proteasomal degradation of NFATc3.

179 To examine whether the effect of Trim39 on the protein level of NFATc3 could have an impact
180 on its activity as a transcription factor, we measured the mRNA level of one of its target genes:
181 *Trim17*. Indeed, we have previously shown that *Trim17* is transcriptionally induced by NFATc3
182 (Mojsa et al., 2015). Consistently, in the present study, *Trim17* mRNA level was increased
183 when NFATc3 was overexpressed (Fig. 3C). Interestingly, this induction was completely
184 abrogated by co-expression of wild type but not inactive Trim39 (Δ RING). Moreover, even
185 when NFATc3 was not transfected, Trim39- Δ RING significantly increased the expression level
186 of *Trim17* (Fig. 3C), suggesting that the inhibition of endogenous Trim39 through a dominant
187 negative effect of TRIM39- Δ RING (Fig. 3A) is sufficient to increase the activity of endogenous
188 NFATc3. To confirm these data, Neuro2A cells were treated with the calcium ionophore
189 A23187 and phorbol 12-myristate 13-acetate (PMA) to activate both endogenous NFATc3
190 (through calcium-induced nuclear translocation) and its transcriptional partner AP-1. As
191 previously reported (Mojsa et al., 2015), *Trim17* mRNA level was increased following
192 treatment with A23187 and PMA (Fig. 3D, left panel). Although an increase in intracellular
193 calcium should activate other members of the NFAT family, this induction of *Trim17* is

194 probably due to NFATc3 as it is the NFAT transcription factor predominantly expressed in
195 Neuro2A cells (Mojsa et al., 2015). Again, this Trim17 induction was completely abrogated by
196 overexpression of wild type Trim39 but not inactive Trim39 (Fig. 3D, left panel). Notably, the
197 mRNA level of NFATc3 was not significantly altered by these different treatments (Fig. 3D
198 right panel). Taken together, these data suggest that exogenous Trim39 reduces the
199 transcriptional activity of both overexpressed and endogenous NFATc3. To determine the
200 impact of endogenous Trim39, Neuro2A cells were transfected with two different specific
201 siRNAs that efficiently decreased the mRNA level of Trim39 (Fig. 3E, left panel). Silencing of
202 Trim39 resulted in Trim17 induction, notably following treatment with A23187 and PMA,
203 which activates endogenous NFATc3 (Fig. 3E, middle panel). The mRNA levels of NFATc3
204 were slightly increased by silencing of Trim39, but this modest induction is unlikely to have
205 significantly contributed to the increase in NFATc3 activity (Fig. 3E, right panel, see legend).
206 These data therefore indicate that endogenous Trim39 also regulates the endogenous NFATc3
207 protein. As we have previously shown that Trim17 can bind and inhibit the activity of NFATc3
208 by preventing its nuclear translocation (Mojsa et al., 2015), we examined whether Trim39 could
209 have the same effect on NFATc3. Indeed, under conditions where Trim17 decreased the nuclear
210 translocation of NFATc3 by more than twofold, Trim39 had no impact on the subcellular
211 localization of NFATc3 (Fig. S2). Taken together, these data strongly suggest that Trim39
212 inhibits the transcription factor activity of NFATc3 by ubiquitinating it and by inducing its
213 proteasomal degradation, but not by preventing its nuclear translocation.

214

215 **Trim17 inhibits the ubiquitination of NFATc3 mediated by Trim39**

216 As we initially observed that Trim17 decreases the ubiquitination level of NFATc3 (Mojsa et
217 al., 2015), we tested whether Trim17 could affect Trim39-mediated ubiquitination of NFATc3.
218 Indeed, the increase in NFATc3 ubiquitination induced by Trim39 overexpression was
219 abolished by the co-transfection of Trim17 in cells (Fig. 4A). This effect was confirmed *in vitro*.
220 Indeed, the ubiquitination of *in vitro* translated/immunopurified NFATc3 by recombinant His-
221 TRIM39 was completely prevented by recombinant MBP-TRIM17 (Fig. 4B). As only purified
222 proteins were used in a completely acellular medium for this assay, these results indicate that
223 TRIM17 directly inhibits the ubiquitination of NFATc3 induced by TRIM39. Interestingly, the
224 ubiquitination level of Trim39 was strongly decreased in the presence of Trim17 both in cells
225 (Fig. 4A) and *in vitro* (Fig. 4B), excluding the possibility that Trim17 acts by ubiquitinating
226 Trim39. Moreover, as *in vitro* auto-ubiquitination gives a measure of E3 ubiquitin-ligase

227 activity (Pickart, 2001), this also suggests that TRIM17 can directly inhibit the E3 ubiquitin-
228 ligase activity of TRIM39. Interestingly, in these experiments, the *in vitro* auto-ubiquitination
229 of TRIM17 was also decreased by TRIM39 (Fig. 4B) and the ubiquitination level of Trim17 in
230 cells was also reduced in the presence of Trim39 (Fig. S3), suggesting a reciprocal inhibition
231 of the two TRIM proteins.

232 To further investigate the mechanisms underlying the inhibitory effect of Trim17, the impact
233 of Trim17 on the interaction between NFATc3 and Trim39 was assessed. Strikingly, when co-
234 transfected with HA-NFATc3 and Flag-Trim39, Trim17-GFP almost completely prevented the
235 co-immunoprecipitation of Flag-Trim39 with HA-NFATc3 (Fig. 5A) or the co-
236 immunoprecipitation of HA-NFATc3 with Flag-Trim39 (Fig. 5B). Moreover, in PLA
237 experiments, the close proximity signal between endogenous NFATc3 and Trim39 proteins was
238 significantly reduced by the overexpression of Trim17-GFP compared to GFP (Fig. 5C,D).

239 Taken together, these data strongly suggest that Trim17 inhibits the ubiquitination of NFATc3
240 mediated by Trim39, by inhibiting both the E3 ubiquitin-ligase activity of Trim39 and the
241 interaction between NFATc3 and Trim39.

242

243 **SUMOylation of NFATc3 modulates its ubiquitination and stability**

244 In a previous study, we have identified three consensus SUMOylation sites in NFATc3 (Mojsa
245 et al., 2015). As SUMOylation can modify the stability of proteins (Liebelt & Vertegaal, 2016),
246 we tested whether alteration of the SUMOylation of NFATc3 can have an impact on its
247 ubiquitination and half-life. We had previously used NFATc3 K/R mutants in which the
248 acceptor Lys residues of the SUMOylation consensus motifs were replaced by Arg (Mojsa et
249 al., 2015). However, large-scale mass spectrometry studies have shown that a quarter of SUMO
250 acceptors lysine residues are also used for ubiquitin modification (Liebelt & Vertegaal, 2016).
251 Therefore, additional NFATc3 mutants were generated in order to prevent SUMOylation
252 without affecting a possible ubiquitination at these sites. For this purpose, the Glu residues of
253 the NFATc3 SUMOylation consensus motifs (ψ KXE with ψ representing a large hydrophobic
254 residue and X any amino acid (Pichler et al., 2017; Rodriguez et al., 2001) were substituted for
255 Ala to generate NFATc3 E/A mutants. As expected, *in vitro* SUMOylation of the NFATc3-
256 EallA mutant (in which the Glu residues of the three consensus motifs were replaced by Ala)
257 and the NFATc3-KallR mutant (in which the Lys residues of the three consensus motifs were
258 replaced by Arg), was almost completely abrogated (Fig. 6A). The slow-migrating bands

259 detected in this assay were confirmed to be SUMOylated forms of NFATc3 as they were not
260 produced when the reaction mix did not contain SUMO-1 (Fig. S4A). Interestingly, the
261 ubiquitination level of NFATc3 in Neuro2A cells was not significantly decreased by single
262 mutations or the double E437A/E706A mutation whereas it was decreased by the
263 E706A/E1015A mutation and strongly decreased by the triple mutation (Fig. 6B,C). These data
264 are consistent with our previous observation that the second and third consensus sites play a
265 major role in NFATc3 SUMOylation (Mojsa et al., 2015). These data also suggest that
266 SUMOylation of at least one of the three consensus motifs is necessary to favour the
267 ubiquitination of NFATc3. To further confirm the SUMOylation-dependent ubiquitination of
268 NFATc3, we assessed the ubiquitination level of NFATc3 following treatment of the cells with
269 the SUMO-activating enzyme inhibitor ML-792 (He et al., 2017). Indeed, treatment of
270 Neuro2A cells with ML-792 for 8 h abrogated global SUMOylation without any toxicity (Fig.
271 S4B) and deeply decreased NFATc3 ubiquitination (Fig. 6D). Consistently, the half-life of the
272 NFATc3-EallA mutant, measured after inhibition of protein synthesis with cycloheximide, was
273 significantly increased compared to WT NFATc3 (Fig. 6E,F). Taken as a whole, these data
274 suggest that SUMOylation of NFATc3 favours its ubiquitination and subsequent degradation.

275

276 **Trim39 acts as a SUMO-targeted E3 ubiquitin-ligase for NFATc3**

277 To better understand the mechanisms underlying the regulation of NFATc3 by SUMO, we
278 examined whether mutation of its three consensus SUMOylation sites could affect its
279 ubiquitination by Trim39. Indeed, the ubiquitination level of the NFATc3-EallA mutant was
280 decreased compared to WT NFATc3 when co-expressed with Trim39 in Neuro2A cells (Fig.
281 7A). Moreover, inhibition of global SUMOylation by ML-792 (Fig. S4B) strongly reduced the
282 increase of NFATc3 ubiquitination mediated by Trim39 (Fig. 7B). To determine whether this
283 inhibition of ubiquitination could be due to a reduced interaction between NFATc3 and Trim39,
284 co-immunoprecipitation experiments were performed. The amount of Trim39 co-precipitated
285 with NFATc3-EallA was decreased compared to the amount of Trim39 co-precipitated with
286 WT NFATc3 (Fig. 7C left panel). Consistently, the amount of NFATc3 co-precipitated with
287 Trim39 was decreased when its three SUMOylation sites were mutated (Fig. 7C right panel).
288 Therefore, these data strongly suggest that Trim39 binds and ubiquitinates preferentially
289 SUMOylated forms of NFATc3.

290 Proteins interacting non-covalently with SUMO generally harbor SUMO-interacting motifs.
291 (SIMs). These motifs typically consist of three hydrophobic residues in a sequence of four

292 amino acids, sometimes flanked by acidic or phosphorylated residues (Kerscher, 2007). Using
293 the web-based tool GPS-SUMO (Q. Zhao et al., 2014), we identified three putative SIMs in the
294 Trim39 sequence, which are conserved from mouse to human. We named these motifs SIM1
295 (39-PVII-42, located in the RING domain), SIM2 (125-VCLI-128, in the B-Box domain) and
296 SIM3 (211-LLSRL-215, in the coiled-coil domain) (Fig. 7D). These three putative SIMs exhibit
297 the highest predictive scores with GPS-SUMO. Two of them, SIM1 and SIM2, are also
298 predicted with a high score by the JASSA bioinformatics tool (Beauchair et al., 2015). Trim39
299 constructs were generated in which most residues of the three SIMs were mutated to Ala
300 (respectively into mSIM1: 39-PAAA-42, mSIM2: 125-AAAA-128 and mSIM3: 211-AAARA-
301 215). To confirm the ability of Trim39 to bind SUMO and to determine the impact of these
302 mutations, we conducted GST pull-down experiments using purified recombinant proteins.
303 Interestingly, GST-Trim39 could bind di-, tri-, tetra- and higher-order SUMO-2 chains but not
304 free SUMO-2 whereas GST alone showed no interaction (Fig. 7E). Single mutations of SIM1
305 and SIM2 had no significant effect, either individually (Fig. 7E) or together (Fig. S5). In
306 contrast, mutation of SIM3 strongly reduced the SUMO-binding ability of Trim39 (Fig. 7E),
307 an effect which was not significantly modified by combination with single SIM1 mutation, and
308 only slightly increased by combination with single SIM2 and double SIM1/SIM2 mutations
309 (Fig. S5). This is reminiscent of what has been previously described for the SUMO-targeted E3
310 ubiquitin-ligase (STUbL) Arkadia/RNF11 (Erker et al., 2013). Our data suggest that SIM3
311 plays a pivotal role in the binding of Trim39 to SUMO chains. Consistently, mutation of SIM3
312 reduced the ability of Trim39 to interact with NFATc3 in co-transfection/co-
313 immunoprecipitation experiments (Fig. 7F). As for SUMO-2 chain binding (Fig. 7E), the
314 concomitant mutation of SIM1, SIM2 or both, together with SIM3, did not significantly modify
315 the binding of Trim39 to NFATc3 compared to the single mutation of SIM3 (Fig. 7F).
316 Moreover, SIM3 mutation reduced the ability of Trim39 to induce the ubiquitination of
317 NFATc3 in Neuro2A cells (Fig. 7G). To further determine whether Trim39 is a STUbL for
318 NFATc3, *in vitro* experiments were performed. *In vitro* translated HA-NFATc3 was first
319 subjected (or not) to *in vitro* SUMOylation, then immunopurified using anti-HA antibody and
320 finally subjected to *in vitro* ubiquitination using purified recombinant WT Trim39 or mSIM3-
321 Trim39. In some samples, the reaction was treated with the SUMO-specific protease SENP1 to
322 remove SUMO chains from NFATc3 and better visualize ubiquitinated forms. Importantly,
323 ubiquitination of NFATc3 by WT Trim39 was strongly increased by its prior SUMOylation
324 and the resulting higher molecular weight forms of NFATc3 were not drastically changed by
325 SENP1 (Fig. 7H). In contrast, mSIM3-Trim39 did not significantly modify NFATc3 and the

326 higher molecular bands resulting from SUMOylation disappeared following treatment with
327 SENP1 (Fig. 7H). Yet, the E3 ubiquitin-ligase activity of mSIM3-Trim39 assessed by its auto-
328 ubiquitination was not significantly different from that of WT Trim39 (Fig. 7H). Furthermore,
329 as described for other STUbLs (M.-C. Geoffroy & Hay, 2009), recombinant purified WT
330 Trim39 was able to ubiquitinate SUMO chains *in vitro*, whereas its mSIM3 mutant was not
331 (Fig. 7I). Collectively, these data indicate that Trim39 acts as a SUMO-targeted E3 ubiquitin-
332 ligase for NFATc3 by preferentially binding the SUMOylated forms of NFATc3 through its
333 SIM, in order to mediate their ubiquitination.

334

335 **SUMOylation and Trim39 modulate the pro-apoptotic effect of NFATc3 in neurons**

336 We have previously shown that overexpression of NFATc3 in primary cultures of cerebellar
337 granule neurons (CGNs) aggravates apoptosis induced by KCl deprivation (Mojsa et al., 2015).
338 Primary CGNs represent one of the best characterized *in vitro* models of neuronal apoptosis
339 (Contestabile, 2002). These neurons survive in the presence of serum and depolarizing
340 concentrations of KCl (25 mM) that mimic the neuronal activity required for their survival *in*
341 *vivo* (Ikonomidou et al., 1999). They undergo apoptosis following withdrawal of serum and
342 lowering of KCl to 5 mM (K5) (D’Mello et al., 1993), which recapitulates the programmed cell
343 death naturally occurring in the cerebellum during post-natal development (Wood et al., 1993).
344 We used this model to examine whether mutation of the SUMOylation sites of NFATc3, which
345 increases its stability (Fig. 6E,F) by reducing its interaction with Trim39 (Fig. 7C) and its
346 ubiquitination (Fig. 7A), could have an impact on its pro-apoptotic effect in CGNs. As shown
347 previously (Mojsa et al., 2015), we found that serum and KCl deprivation-induced apoptosis is
348 significantly increased in CGNs transfected with WT GFP-NFATc3 compared to GFP, as
349 shown by the increased number of apoptotic/condensed nuclei (Fig. 8A,B). Interestingly,
350 neuronal apoptosis was further increased in neurons overexpressing GFP-NFATc3-Ealla
351 compared with WT GFP-NFATc3 (Fig. 8A,B), suggesting that stabilization of NFATc3 by
352 preventing its SUMOylation increases its pro-apoptotic effect. Consistently, efficient silencing
353 of endogenous Trim39 using a lentivirus expressing a specific shRNA (Fig. S6A) aggravated
354 neuronal apoptosis. Indeed, the number of apoptotic nuclei (Fig. 8C,D) and the level of active
355 caspase 3 (Fig. 8E,F) were significantly increased in neurons transduced with the shRNA
356 against Trim39 compared to neurons transduced with an unrelated control shRNA. Importantly,
357 efficient knock-down of endogenous NFATc3 using two different shRNAs (Fig. S6B)
358 completely abrogated the increase in apoptosis induced by the shRNA Trim39 (Fig. 8E,F),

359 indicating that the pro-apoptotic effect of Trim39 silencing depends on NFATc3. Our data
360 therefore strongly suggest that SUMOylation and Trim39 negatively regulate the pro-apoptotic
361 function of NFATc3, most likely by reducing its stability and thereby its activity as a
362 transcription factor.

363 Since Trim17 expression is highly induced in CGN following serum and KCl deprivation (I.
364 Lassot et al., 2010) and Trim17 inhibits Trim39 (Fig. 4), Trim39-mediated degradation of
365 NFATc3 is expected to be decreased by Trim17 following apoptosis induction. Indeed, in serum
366 and KCl-deprived neurons, the protein level of NFATc3 was increased although its mRNA
367 level was unchanged, suggesting a stabilization of the protein (Fig. 8G). To further study the
368 mechanisms underlying this effect, we estimated the level of NFATc3 SUMOylation by PLA,
369 using antibodies against NFATc3 and SUMO-2. This type of assay has been set up to measure
370 the SUMOylation level of a given protein (Ristic et al., 2016). The SUMOylation inhibitor ML-
371 792 led to a strong decrease of the PLA signal, confirming its specificity (Fig. 8H).
372 Interestingly, the SUMOylation of endogenous NFATc3 was strongly decreased following
373 serum and KCl withdrawal (Fig. 8H), which should also participate in the stabilization of
374 NFATc3.

375

376 **Discussion**

377 In contrast to NFAT nuclear translocation mediated by calcium/calcineurin, the regulation of
378 NFAT protein stability by the ubiquitin-proteasome system has been poorly studied.
379 Independent studies have suggested that certain E3 ubiquitin-ligases may be responsible for
380 ubiquitination and proteasomal degradation of different NFAT members: HDM2 for NFATc2
381 in breast and pancreatic cancers (Singh et al., 2011; Yoeli-Lerner et al., 2005); Cbl-b, c-Cbl,
382 VHL or KBTBD11/Cullin3 for NFATc1 during osteoclastogenesis (J. H. Kim et al., 2010; X.
383 Li et al., 2015; Narahara et al., 2019; Youn et al., 2012); CHIP for NFATc3 in LPS-induced
384 cardiomyopathies (Chao et al., 2019). However, no demonstration has been made to establish
385 that these proteins are genuine NFAT E3 ubiquitin-ligases, with the exception of HDM2 for
386 NFATc2 (Yoeli-Lerner et al., 2005). In the present study, we provide the first formal
387 identification of an NFATc3 E3 ubiquitin-ligase by showing several lines of evidence
388 demonstrating that Trim39 is an E3 ubiquitin-ligase for NFATc3. First, we found a physical
389 interaction between endogenous or overexpressed Trim39 and NFATc3 proteins. Second,
390 Trim39 ubiquitinated NFATc3 *in vitro*. Third, overexpression of WT Trim39, but not of its

391 inactive RING mutant, increased the ubiquitination level of NFATc3 in cells. In contrast,
392 silencing of Trim39 decreased NFATc3 ubiquitination. Finally, Trim39 overexpression
393 decreased the protein level of NFATc3 whereas the silencing of endogenous Trim39 increased
394 it, suggesting that Trim39-mediated ubiquitination of NFATc3 targets it for proteasomal
395 degradation. As a physiological consequence, overexpressed Trim39 resulted in reduced
396 transcriptional activity of NFATc3 without affecting its nuclear translocation. Conversely,
397 silencing of endogenous Trim39 increased both the expression of an NFATc3 target gene and
398 its pro-apoptotic effect in neurons. Taken together, these data strongly suggest that Trim39
399 modulates neuronal apoptosis by acting as a physiological E3 ubiquitin-ligase for NFATc3.

400 Our present data show that Trim17 inhibits the ubiquitination of NFATc3 mediated by Trim39.
401 Indeed, the increase in the ubiquitination level of NFATc3 due to overexpression of Trim39 is
402 abolished by the co-transfection of Trim17. Both Trim39 and Trim17 belong to the family of
403 TRIM proteins which forms one of the largest classes of RING-containing E3 ubiquitin-ligases
404 (Meroni & Diez-Roux, 2005), comprising 82 members in humans (Qiu et al., 2020). TRIM
405 proteins are characterized by their N-terminal tripartite motif that consists of a RING domain,
406 one or two B-box domains and a coiled-coil domain (Esposito et al., 2017; Reymond et al.,
407 2001). In addition to this common motif, TRIM proteins generally exhibit a C-terminal domain
408 that varies from one member to another and categorizes them into different subtypes (Short &
409 Cox, 2006). This C-terminal domain, which is a PRY-SPRY domain for both Trim17 and
410 Trim39, generally mediates target recognition (Esposito et al., 2017; Y. Li et al., 2014). While
411 the RING domain confers an E3 ubiquitin-ligase activity by binding ubiquitin-loaded E2
412 ubiquitin-conjugating enzymes, the B-box and especially the coiled-coil domain are involved
413 in the formation of homo- or hetero-dimers or multimers (Koliopoulos et al., 2016; Y. Li et al.,
414 2014; Napolitano & Meroni, 2012; Sanchez et al., 2014). As homo-multimerization seems to
415 be necessary for the E3 ubiquitin-ligase activity of TRIM proteins (Koliopoulos et al., 2016;
416 Streich et al., 2013; Yudina et al., 2015), Trim17 may inhibit Trim39-mediated ubiquitination
417 of NFATc3 by forming inactive hetero-multimers with Trim39 at the expense of Trim39 homo-
418 multimers. Indeed, we show here that Trim17 and Trim39 physically interact with each other.
419 In a similar way, we have previously shown that TRIM17 inhibits the activity of two other
420 TRIM proteins to which it is able to bind: TRIM41 (Ir ena Lassot et al., 2018) and TRIM28
421 (Lionnard et al., 2019). The inhibitory effect of Trim17 might result from two mechanisms that
422 are not mutually exclusive. First, the formation of hetero-dimers or hetero-multimers with
423 Trim17 may inhibit the E3 ubiquitin-ligase activity of its TRIM partner, possibly by preventing

424 the binding of the E2 enzyme. Indeed, we show here that TRIM17 prevents the auto-
425 ubiquitination of TRIM39 *in vitro*, similarly to what we have previously shown for the
426 inhibition of TRIM41 by TRIM17 (Iréna Lassot et al., 2018). Second, Trim17 may prevent the
427 binding of the substrate to its TRIM partner. Indeed, Trim17 reduces the interaction between
428 Trim39 and NFATc3, as determined by both co-IP of overexpressed proteins and proximity
429 ligation of endogenous proteins (PLA). These results are reminiscent of the effect of TRIM17
430 on the interaction between TRIM41 or TRIM28 and their respective substrates (Basu-
431 Shrivastava et al., 2021). Further experiments are required to identify the structural
432 determinants of the inhibitory effect of Trim17 on other TRIM proteins. Nevertheless, it is
433 unlikely that Trim17 inhibits Trim39-mediated ubiquitination of NFATc3 by associating with
434 a deubiquitinating enzyme (DUB), as shown for other TRIM proteins (Hao et al., 2015; Nicklas
435 et al., 2019). Indeed, TRIM17 is able to inhibit the *in vitro* ubiquitination of NFATc3 mediated
436 by TRIM39, in a completely acellular medium and therefore in the absence of any DUB. It is
437 also clear that Trim17 does not inhibit Trim39 by inducing its ubiquitination and subsequent
438 degradation. Indeed, Trim17 rather decreases the ubiquitination level of Trim39 both *in vitro*
439 and in cells. Moreover, it is interesting to note that TRIM39 reciprocally decreases the *in vitro*
440 auto-ubiquitination of TRIM17, further suggesting that Trim17 and Trim39 form inactive
441 hetero-dimers or hetero-multimers, in which the E3 ubiquitin-ligase activity of the two partners
442 is inhibited.

443 SUMOylation has recently emerged as an important regulator of protein stability (Liebelt &
444 Vertegaal, 2016). Consistently, our data clearly indicate that SUMOylation of NFATc3
445 promotes its ubiquitination and subsequent degradation. Indeed, mutation of three
446 SUMOylation consensus sites of NFATc3 decreased its ubiquitination level, increased its half-
447 life and aggravated its pro-apoptotic effect in neurons. As the SUMOylation sites were modified
448 in order to specifically prevent SUMOylation without affecting a putative ubiquitination of the
449 acceptor Lys residues, our results unambiguously designate SUMOylation as the post-
450 translational modification implicated in these effects. This mechanism could be conserved in
451 other members of the NFAT family. Indeed, effective SUMOylation of NFAT proteins has been
452 described, notably for NFATc1 and NFATc2 (Nayak et al., 2009; Terui et al., 2004). Although
453 the functional consequences of NFAT SUMOylation that have been reported so far are rather
454 related to their nuclear translocation and transactivation activity (E. T. Kim et al., 2019; Nayak
455 et al., 2009; Terui et al., 2004; Vihma & Timmusk, 2017), it might also influence the stability
456 of these proteins. Indeed, Singh et al. reported that the double mutation of Lys684 and Lys897

457 in murine NFATc2 prevents its ubiquitination and degradation induced by zoledronic acid
458 (Singh et al., 2011). One possible conclusion is that NFATc2 is normally ubiquitinated on these
459 Lys residues (Singh et al., 2011). However, as these two Lys residues have been shown to be
460 SUMOylated (Terui et al., 2004), an alternative and plausible explanation is that SUMOylation
461 of NFATc2 on these consensus sites might be necessary for the recognition by its E3 ubiquitin-
462 ligase. In line with this hypothesis, the protein levels of different NFATc1 and NFATc2
463 isoforms have been reported to be increased by the double K/R mutation (lysine to arginine
464 substitution) of their C-terminal SUMOylation sites (Vihma & Timmusk, 2017). However,
465 further investigation is required to demonstrate that SUMOylation indeed plays a role in these
466 effects.

467 A few SUMO-targeted E3 ubiquitin-ligases (STUbLs) have been described (M. C. Geoffroy &
468 Hay, 2009; Prudden et al., 2007; Sriramachandran & Dohmen, 2014). These proteins generally
469 combine two features: a RING domain that confers them an E3 ubiquitin-ligase activity and
470 SUMO interacting motifs (SIMs) that mediate their preference for SUMOylated substrates.
471 Inhibition of the proteasome leads to an important accumulation of high molecular weight
472 SUMO-modified proteins in yeast and human cells (Bailey & O'Hare, 2005; Uzunova et al.,
473 2007), suggesting that ubiquitination and degradation of SUMOylated proteins mediated by
474 STUbLs play an important role in proteostasis. However, only two STUbLs have been
475 identified so far in mammals: RNF4 and Arkadia/RNF111 (Jansen & Vertegaal, 2021), which
476 may not be sufficient to account for the regulation of all SUMOylated proteins. Therefore,
477 additional STUbLs probably remain to be discovered. Here we provide a series of arguments
478 indicating that Trim39 acts as a STUbL for NFATc3. First, Trim39 is able to bind and
479 ubiquitinate SUMO-2 chains *in vitro*. Second, we identified three SIMs in the sequence of
480 Trim39 and mutation of one these motifs (SIM3) strongly decreases its capacity to bind and
481 ubiquitinate SUMO-2 chains *in vitro*. Moreover, the binding and the ubiquitination of NFATc3
482 mediated by Trim39 in cells is reduced by mutation of SIM3 in Trim39. Third, *in vitro*
483 ubiquitination of NFATc3 by Trim39 is increased by prior *in vitro* SUMOylation of NFATc3.
484 Consistently, Trim39 binds and ubiquitinates preferentially the SUMOylated forms of NFATc3
485 in cells. Indeed, mutation of SUMOylation consensus sites in NFATc3 reduces its co-
486 immunoprecipitation with Trim39 and its ubiquitination by Trim39. Furthermore, treatment of
487 the cells with the SUMOylation inhibitor ML-792 strongly decreases the ubiquitination of
488 NFATc3 mediated by Trim39. We cannot exclude that RNF4 and/or Arkadia/RNF111
489 contribute to the SUMO-targeted ubiquitination of NFATc3. However, partial silencing of

490 Trim39 is enough to deeply decrease NFATc3 ubiquitination in Neuro2A cells, suggesting that
491 Trim39 is the major STUbL involved in this process, at least in these cells.

492 Most of the STUbLs studied so far bear multiple SIMs, which mediate cooperative binding to
493 multiple SUMO units, thereby providing a preference for substrates with SUMO chains
494 (Sriramachandran & Dohmen, 2014). Of the three SIMs predicted in the Trim39 sequence with
495 a high score, only one is instrumental for the binding of Trim39 to purified SUMO chains *in*
496 *vitro* and SUMOylated NFATc3 in cells. SIMs are characterized by a loose consensus sequence
497 and some non-canonical SIMs have been described (Kerscher, 2007; Sriramachandran &
498 Dohmen, 2014). Therefore, it is possible that another SIM in Trim39, that was not identified or
499 was not credited with a high predictive score by GPS-SUMO or JASSA, may participate in the
500 binding of Trim39 to SUMO. It is also possible that a single SIM is sufficient to fulfill this
501 function, as reported for other STUbLs (Erker et al., 2013; Parker & Ulrich, 2012). For example,
502 of the three SIMs identified in Arkadia/RNF111, only one has been shown to be essential for
503 its interaction with SUMO-2 chains, similar to SIM3 in Trim39 (Erker et al., 2013). Moreover,
504 as TRIM proteins generally homo-multimerize (Koliopoulos et al., 2016; Streich et al., 2013;
505 Yudina et al., 2015), it is possible that in its multimerized form, Trim39 harbors several SIMs
506 in close proximity. Indeed, SIM3 is located in the coiled-coil domain of Trim39 that is expected
507 to form antiparallel dimers or higher-order multimers, as reported in other TRIM proteins
508 (Koliopoulos et al., 2016; Y. Li et al., 2014; Napolitano & Meroni, 2012; Sanchez et al., 2014).
509 Therefore, the unique active SIM of one molecule of Trim39 may cooperate with the active
510 SIM of other Trim39 molecules, in multimers, for binding SUMO chains. Alternatively, another
511 binding site, such as the RING domain, may cooperate with the SUMO-SIM interaction to bind
512 the substrate, as reported for the *Drosophila* STUbL Degringolade (Abed et al., 2011). Trim39
513 may also bind NFATc3 by a dual mechanism, involving both SUMO-dependent and SUMO-
514 independent recognition, as shown for viral STUbLs (Boutell et al., 2011; Wang et al., 2011).
515 Indeed, we found that the NFATc3/Trim39 interaction is decreased but not completely
516 abrogated in co-immunoprecipitation experiments, by mutation of either the SUMOylation sites
517 in NFATc3 or SIM3 in Trim39. Further studies are therefore required to fully characterize the
518 mechanisms mediating the SUMO-dependent interaction of NFATc3 with Trim39.

519 As with other TRIM proteins, Trim39 is certainly involved in a wide variety of cellular
520 processes and human diseases. To date, Trim39 has been shown to regulate cell cycle
521 progression by directly mediating the ubiquitination of p53 (Zhang, Huang, et al., 2012) and by
522 indirectly modulating the protein level of p21 (Zhang, Mei, et al., 2012). Trim39 has also been

523 implicated in the negative regulation of NF κ B signaling (Suzuki et al., 2016), as well as in
524 tumor progression and autophagic flux in colorectal cancer (Hu et al., 2021). In the present
525 study, we show that silencing of Trim39 enhances apoptosis triggered by survival-factor
526 deprivation in primary cultures of neurons. As Trim39 induces NFATc3 degradation and as
527 NFATc3 aggravates neuronal apoptosis, these data suggest that silencing of Trim39 favors
528 apoptosis in neurons by stabilizing NFATc3. Additional effects of Trim39 on apoptosis
529 regulation cannot be excluded. For example, silencing of Trim39 has been reported to aggravate
530 apoptosis following a genotoxic stress in HCT116 cells (Zhang, Mei, et al., 2012) and to
531 increase nutlin3a-induced apoptosis in several p53-positive cancer cell lines, presumably by
532 stabilizing the pro-apoptotic factor p53 (Zhang, Huang, et al., 2012). Conversely, in p53 null
533 cell lines, silencing of Trim39 reduces DNA damage-induced apoptosis (S. S. Lee et al., 2009;
534 Zhang, Huang, et al., 2012), probably by directly inhibiting APC/C^{Cdh1}-mediated degradation
535 of the pro-apoptotic protein MOAP-1 (Huang et al., 2012; S. S. Lee et al., 2009). However, in
536 the present study, the increase in neuronal apoptosis triggered by Trim39 knock-down is
537 completely abrogated by NFATc3 silencing. This strongly suggests that, in these conditions,
538 Trim39 regulates apoptosis by controlling the stability of NFATc3. Therefore, Trim39 appears
539 to be a key factor in several processes regulating apoptosis, the final outcome depending on its
540 targets or binding partners present in the cell and the nature of the cellular stress. Similarly, we
541 and others have reported that Trim17 plays an important role in apoptosis regulation (I. Lassot
542 et al., 2010; Lionnard et al., 2019; Magiera et al., 2013; Mojsa et al., 2015; Song et al., 2017).
543 Notably, we have shown that Trim17 is highly induced in CGNs following serum and KCl
544 deprivation, and that it is both sufficient and necessary to trigger neuronal apoptosis (I. Lassot
545 et al., 2010). This pro-apoptotic effect is in part mediated by the ubiquitination and subsequent
546 degradation of the anti-apoptotic protein Mcl-1 (Magiera et al., 2013). In addition, Trim17 can
547 modulate neuronal apoptosis by acting on NFATc3 through antagonistic mechanisms. On one
548 hand, we have previously shown that Trim17 can prevent the nuclear translocation and
549 transcriptional activity of NFATc3 (Mojsa et al., 2015) and should therefore inhibit its pro-
550 apoptotic effect. On the other hand, we show here that Trim17 can inhibit Trim39-mediated
551 ubiquitination and degradation of NFATc3 and should therefore aggravate its pro-apoptotic
552 effect by increasing its protein level. Moreover, we have identified *Trim17* as a target gene of
553 NFATc3 (Mojsa et al., 2015). The effects of Trim17 on the protein level and activity of
554 NFATc3 should therefore influence its own expression, creating both a negative and a positive
555 feedback loop and eventually resulting in fine tuning of neuronal apoptosis. Moreover, we show
556 here that NFATc3 SUMOylation is reduced in neurons following apoptosis induction. This

557 should also contribute to the inhibition of Trim39-mediated NFATc3 degradation, resulting in
558 the NFATc3 increase that we observe in KCl-deprived neurons. We have previously shown that
559 knock-down of NFATc3 in CGNs reduces apoptosis, indicating that NFATc3 is involved in
560 neuronal apoptosis (Mojsa et al., 2015). The combination of Trim17 induction and decreased
561 SUMOylation of NFATc3 after serum and KCl deprivation should therefore contribute to the
562 induction of neuronal apoptosis by stabilizing NFATc3. Further investigation will determine
563 whether these mechanisms can be manipulated for therapeutic purposes to prevent neuronal
564 loss in neurodegenerative diseases.

565

566

567 **Materials and Methods**

568 **Materials**

569 Culture media were from Thermo Fisher Scientific. Fetal calf serum, other culture reagents,
570 protease inhibitor cocktail, DAPI, PMA, A23187, cycloheximide, N-ethylmaleimide (NEM),
571 MG-132, puromycin, anti-Flag M2 affinity gel beads (#A2220), ML-792 (#HY-108702) and
572 other chemicals were from Sigma-Aldrich. Protein G-agarose and protein A-agarose beads
573 were from Roche. GFP-Trap®-A beads were from Chromotek (Planegg-Martinsried,
574 Germany). Rat monoclonal anti-HA antibody (clone 3F10; #11867432001), mouse monoclonal
575 anti-Flag antibody (clone M2, #F3165), mouse monoclonal anti-tubulin antibody (clone
576 DM1A, #T6199), rabbit anti-TRIM17 antibody (#AV34547) and rabbit IgG (#I5006) were
577 from Sigma-Aldrich. Rabbit anti-GFP antibody (#TP401) was from Torrey Pines Biolabs Inc.
578 (Houston, TX USA). Mouse monoclonal antibody against actin (clone C4) was from Millipore
579 (#MAB1501). Rabbit polyclonal antibody against NFATc3 was from Proteintech (18222-1-
580 AP). Mouse monoclonal antibody against Trim39 was from Origene (#TA505761). Rabbit
581 polyclonal antibody against Trim39 was from Proteintech (#12757-1-AP). Monoclonal mouse
582 antibody against SUMO-2 (clone #8A2) was purified from hybridomas obtained from the
583 Developmental Studies Hybridoma Bank. Fluorescent and horseradish peroxidase-conjugated
584 goat anti-rabbit, anti-rat and anti-mouse secondary antibodies were from Thermo Fisher
585 Scientific and Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA),
586 respectively.

587

588 **Cell culture and transient transfection**

589 Lenti-X 293 T (Clontech), Neuro2A (mouse neuroblastoma) and Baby Hamster Kidney (BHK)
590 cell lines were grown in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose
591 supplemented with 10% fetal bovine serum and penicillin-streptomycin 100 IU/ml-100 µg/ml.
592 Cells were transfected with plasmids using GenJet™ *in vitro* transfection reagent (Ver. II) pre-
593 optimized and conditioned for transfecting Neuro2A and BHK-21 cells respectively (SignaGen
594 laboratories, Ijamsville, MD) according to the manufacturer's instructions. Neuro2A cells were
595 transfected with siRNAs using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher
596 Scientific) following the manufacturer's instruction. For one 35 mm dish, 2.5 µl of transfection
597 reagent was used with 25 pmoles of siRNA. The sequences of the siRNAs used were as follows:
598 siTrim39#1 5' CCAAGCGGGTAGGCATATT 3'; siTrim39#2
599 5' GCGTCAAGTTTGTGGAGACAA3'; siRNA ctrl (targeting Luciferase gene):
600 5' CGTACGCGGAATACTTCGA 3'.

601 Primary cultures of CGNs were prepared from 7-day-old murine pups (C57Bl/6 J mice) as
602 described previously (I. Lassot et al., 2010). Briefly, freshly dissected cerebella were
603 dissociated by trypsinization and mechanical disruption, and plated in Basal Medium Eagle
604 (BME) medium supplemented with 10% fetal bovine serum, 2 mM L-Gln, 10 mM HEPES,
605 penicillin-streptomycin 100 IU/ml-100 µg/ml and 20 mM KCl. Primary CGNs, grown on glass
606 coverslips in 24-well plates, were transfected at DIV 5 with 2 µg of plasmids using a calcium
607 phosphate protocol optimized for neuronal cultures as previously described (I. Lassot et al.,
608 2010).

609

610 **Silencing of Trim39 using shRNA-expressing lentiviruses**

611 The HIV-derived lentiviral vectors pLKO.1 containing control shRNAs respectively against
612 eGFP and Luciferase (SHC005, SHC007), shRNAs against Trim39: TRCN0000037281
613 (shRNA Trim39#1), TRCN0000037282 (shRNA Trim39#2) and TRCN0000438509 (shRNA
614 Trim39#3), and shRNAs against NFATc3: TRCN0000377122 (shRNA NFATC3#1) and
615 TRCN0000097129 (shRNA NFATC3#2) were from Sigma-Aldrich. Lentiviral particles were
616 produced as previously described (Iréna Lassot et al., 2018). Neuro2A cells and CGNs were
617 transduced one day after plating. The lentiviral preparations were added directly to the culture
618 medium for 8 h (approximately 500 ng p24 per million neurons, approximately 100 ng p24 per
619 million Neuro2A cells). Cells were then replaced in fresh medium. Culture was continued until

620 6 days in vitro for CGNs. Neuro2A cells were maintained in culture for 24 h after transduction
621 and then selected using 2 µg/ml puromycin for an additional 48 h.

622

623 **Expression vectors and site directed mutagenesis**

624 The following plasmids were described previously: pCI-GFP, pCS2-3×HA-NFATc3, pCS2-
625 3×HA-NFATc3-KallR, pCS2-GFP-NFATc3 and pCI-Trim17-GFP (Mojsa et al., 2015). All the
626 primers used to generate the constructs described below are listed in supplementary Table1.
627 The sequences of all the constructs were confirmed by automatic sequencing. Single point
628 mutations in the SUMOylation-consensus sites of NFATc3 (E437A, E706A and E1015A) or
629 their double and triple combinations (E437A/E706A, E706A/E1015A and
630 E437A/E706A/E1015A=Ealla) were obtained by site-directed mutagenesis of pCS2-3×HA-
631 NFATc3 using the QuickChange® II XL kit (Agilent Technologies) using the indicated
632 primers. To increase the expression of NFATc3, HA-NFATc3 and HA-NFATc3-Ealla from
633 respective pCS2-3×HA expression vectors were first sub-cloned between *XhoI* and *NheI* sites
634 of the pCDNA3.1 plasmid, and then sub-cloned between *SallI* and *NheI* sites of the pCI plasmid,
635 to generate pCI-3×HA-NFATc3 and pCI-3×HA-NFATc3-Ealla. The plasmid pCS2-GFP-
636 NFATc3-Ealla was obtained by removing the WT NFATc3 cDNA from the plasmid pCS2-
637 GFP-NFATc3 and by replacing it with NFATc3-Ealla between *EcoRI* and *XhoI* sites of the
638 plasmid. The cDNA of mouse Trim39 (GenBank: NM_024468) was amplified, from primary
639 CGN cDNAs, by using PCR with the indicated primers. Amplicons were then cloned into pCI-
640 3×Flag plasmid between *EcoRI* and *XbaI* sites to obtain mouse pCI-3×Flag-Trim39. pGEX-
641 4T1-Trim39-ΔRING mutant was generated by PCR amplification of Trim39 coding region
642 using pCI-3×Flag-Trim39 as template and the indicated primers. Then, the amplicons were
643 cloned between *EcoRI* and *XhoI* sites of the plasmid pGEX4T1. To obtain pCI-3×Flag-Trim39-
644 ΔRING, the insert was released from the plasmid pGEX4T1-Trim39-ΔRING and sub-cloned
645 between *EcoRI* and *NotI* sites of the plasmid pCI-3×Flag. The following Trim39 SIM mutants
646 were obtained by site-directed mutagenesis using pCI-3×Flag-Trim39 as a template and the
647 indicated primers: single mutants mSIM1 (PVII→PAAA), mSIM2 (VCLI→ACAA) and
648 mSIM3 (LLSRL→AAARA); double mutants mSIM12, mSIM13 and mSIM23; and triple
649 mutant mSIM123. The expression vector pmCherry-C1 was purchased from Takara Bio Inc.
650 (#632524). The plasmid mouse pCI-Trim39-mCherry was obtained by recombinant PCR. The
651 first PCR was performed with the indicated primers using pCI-3×Flag-Trim39 as a template.

652 The second PCR was performed with the indicated primers using pmCherry-C1 as a template.
653 The amplicons from both PCRs were purified, mixed and used as template for the recombinant
654 PCR (third PCR) with the indicated primers. The resulting amplicon was cloned between *EcoRI*
655 and *XbaI* sites of the empty pCI plasmid to obtain pCI-Trim39-mCherry.

656 In order to produce recombinant N-terminal GST-tagged Trim39 protein in *Escherichia coli*,
657 the pGEX-4T1-Trim39 construct was produced by PCR amplification of the Trim39 coding
658 region using pCI-3×Flag-Trim39 as a template and the indicated primers. The PCR products
659 were cloned between the *EcoRI* and *XhoI* sites of the pGEX-4T1 expression vector (GE-
660 Healthcare). The mutant GST-Trim39-C49S/C52S was generated by site-directed mutagenesis
661 using GST-Trim39 as a template and the indicated primers. SIM1, SIM2 and SIM3 GST-
662 Trim39 mutants were generated by site-directed mutagenesis using GST-Trim39 as a template
663 and the same primers as for pCI-3×Flag-Trim39 SIM mutants. The cDNA of human TRIM39
664 (GenBank: NM_172016.2), N-Terminally fused to a histidine tag in the plasmid pET-15, and
665 the cDNA of human TRIM17 (GenBank: NM_016102), C-terminally fused to GFP in the
666 pEGFP plasmid were obtained from the ORFeome library of the Montpellier Genomic
667 Collections facility. Human TRIM17 was first amplified by PCR using p-TRIM17-EGFP as a
668 template and indicated primers, and the amplicons were cloned between the *EcoRI* and *Sall*
669 sites of the pGEX-4T1 expression vector. In order to produce recombinant N-terminally MBP-
670 tagged TRIM17 protein in *Escherichia coli*, the pMAL-c2-TRIM17 plasmid was generated by
671 subcloning. The insert was released from the plasmid pGEX-4T1-TRIM17 and sub-cloned
672 between the *EcoRI* and *Sall* sites of the plasmid pMAL-c2 to obtain pMAL-c2-TRIM17. To
673 produce N-terminally His-tagged recombinant WT mouse Trim39 and its mutant mSIM3 in
674 *Escherichia coli*, the pET-15-Trim39 constructs were generated by PCR amplification of the
675 Trim39 coding region using pCI-3×Flag-Trim39 as a template and the indicated primers. The
676 PCR products were cloned between the *NdeI* and *HindIII* sites of the pET-15 expression vector.

677

678 **Protein extraction and western blot analysis**

679 Cells were harvested in lysis buffer A (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM NaF,
680 5 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 5 mM EDTA, 10 mM NEM, 20 μM
681 MG-132, and protease inhibitor cocktail) supplemented with 1% NP-40 and homogenized by
682 thorough vortexing. Cell debris were removed by centrifugation at 1000 × g for 5 min at 4°C
683 and the protein concentration of the resulting supernatant was estimated using the BCA protein

684 assay kit (Thermo Fisher Scientific) with bovine serum albumin as the standard. Total lysates
685 were diluted in 3 × Laemmli sample buffer and incubated at 95°C for 5 min. Proteins were
686 separated by 8% to 12% SDS-PAGE and transferred to Immobilon-P PVDF membrane
687 (Millipore). Blocking and probing with antibodies were performed as previously described
688 (Iréna Lassot et al., 2018). Visualization of immunoreactive proteins was performed using
689 horseradish peroxidase-linked secondary antibodies and Covalight enhanced chemiluminescent
690 substrate (Covalab, Bron, France) or Immobilon® Western (Millipore). Membranes were
691 revealed using films or Amersham Imager 680 (GE Healthcare). When necessary, membranes
692 were stripped using Restore™ PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific)
693 and re-probed with additional antibodies.

694

695 **Co-immunoprecipitation**

696 Following transfection with the indicated plasmids for 24 h, Neuro2A or BHK cells were
697 incubated for 5 h with 10-20 µM MG-132. They were then homogenized in lysis buffer A,
698 supplemented with 1% NP-40 for immunoprecipitation with anti-HA, 0.5% NP-40 for
699 immunoprecipitation with GFP-Trap-A and 1% Triton X-100 for immunoprecipitation with
700 anti-Flag. For anti-HA and anti-Flag immunoprecipitation, cell lysates (500 µl) were
701 centrifuged at 300 × g for 5 min at 4°C. The resulting supernatants were pre-cleared by rotation
702 for 1-3 h at 4°C with 15 µl protein G-agarose beads and then rotated overnight at 4°C with 25 µl
703 protein G agarose beads together with 7 µl anti-HA antibody or with 30 µl anti-Flag M2 affinity
704 gel beads. The beads were recovered by centrifugation and washed four times with 1 ml of lysis
705 buffer A without detergent and containing 0,3 M NaCl for anti-HA or 0,5M NaCl for anti-Flag
706 (instead of 150 mM NaCl). For GFP-Trap precipitation, cell lysates (200 µl) were diluted with
707 300 µl dilution buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM NaF, 5 mM sodium
708 pyrophosphate, 25 mM β-glycerophosphate, 0.5 mM EDTA, 20 µM MG-132, 10 mM NEM
709 and protease inhibitor cocktail) and cell debris were removed by centrifugation. Resulting
710 supernatants were rotated for 2 h at 4°C with 10-25 µl GFP-Trap®-A beads to
711 immunoprecipitate proteins fused to GFP. Beads were recovered by centrifugation and washed
712 four times with dilution buffer. Material bound to the protein G agarose, anti-Flag M2 affinity
713 gel beads or GFP-Trap beads was eluted by the addition of 3 × Laemmli sample buffer and
714 incubation at 95°C for 5 min. Precipitated proteins were analyzed by western blot.

715

716 **In situ proximity ligation assay**

717 Neuro2A cells seeded onto glass coverslips, were left untreated or transfected with pCI-Flag-
718 Trim39, pCI-GFP or pCI-Trim17-GFP for 24 h, and then treated with 10 μ M MG-132 for 4 h.
719 Cell were fixed with 4% paraformaldehyde for 20 min at room temperature, washed with 0.1
720 M Gly (pH = 7.11), permeabilized with 0.2 % Triton X-100 in PBS for 10 min and washed with
721 PBS. The interaction between endogenous Trim39 and endogenous NFATc3, in the presence
722 or absence of GFP or Trim17-GFP, was detected using the Duolink® In Situ kit (Olink®
723 Bioscience, Uppsala, Sweden), according to the manufacturer's instructions, as described
724 previously (Mojsa et al., 2015). Briefly, cells were successively incubated with blocking
725 solution for 60 min at 37°C, with primary antibodies against Trim39 (Origene, 1:400) and
726 NFATc3 (Proteintech, 1:200) overnight at 4°C and with secondary antibodies conjugated with
727 oligonucleotides (PLA probe MINUS and PLA probe PLUS) for 1 h at 37°C. The cells were
728 then incubated with two connector oligonucleotides together with DNA ligase for 30 min at
729 37°C. If the two secondary antibodies are in close proximity, this step allows the connector
730 oligonucleotides to hybridize to the PLA probes and form a circular DNA strand after ligation.
731 Incubation, for 100 min at 37°C, with DNA polymerase consequently leads to rolling circle
732 amplification (RCA), the products of which are detected using fluorescently-labeled
733 complementary oligonucleotides. Cells were washed with Duolink In Situ wash buffers
734 following each of these steps. In the last wash, 1 μ g/ml DAPI was added to the cells for 5 min
735 at room temperature in the dark to stain the nuclei and coverslips were set in Mowiol (polyvinyl
736 alcohol 4-88, Fluka), on glass slides. Cells were analyzed using a confocal Leica SP5-SMD
737 microscope, with a LEICA 63x/1.4 OIL HCX PL APO CS objectives. Images were acquired
738 by the Confocal head TCS SP5 II using the Leica Application Suite X software. Images were
739 processed using Image J. When indicated, z-stacks of images were submitted to maximum
740 intensity projection. The number of dots per transfected cell was estimated in one slice, in
741 around 100 cells in each condition, with an automated procedure using plugins from the Image
742 J software.

743

744 **Immunofluorescence**

745 BHK and Neuro2A cells were seeded onto glass coverslips. Primary CGNs were cultured on
746 coverslips previously coated with laminin (16,67 μ g/ml) and poly-D-lysine (33,3 μ g/ml). Cells
747 and neurons were treated as described in the figure legends and then fixed with 4%

748 paraformaldehyde. Overexpressed HA-NFATc3 and endogenous Trim39 were detected using
749 anti-HA (1:500) and anti-Trim39 (from Proteintech 1:200, from Origene 1:400) antibodies
750 respectively, as described previously (Iréna Lassot et al., 2018). GFP and mCherry-fused
751 proteins were visualized by fluorescence and nuclei were stained with DAPI. Coverslips were
752 analyzed by conventional or confocal microscopy, as mentioned in the figure legends. Image
753 acquisition and analysis were performed on work stations of the Montpellier imaging facility
754 (Leica DM600 for conventional microscopy, Leica SP5-SMD for confocal microscopy). For
755 quantification of NFATc3 nuclear localization, BHK cells with predominant cytoplasmic or
756 nuclear localization of NFATc3 were counted, in a blinded manner, among double GFP/HA
757 positive cells. At least 100 double positive cells were scored for each experiment and each
758 condition.

759

760 **In vivo ubiquitination of NFATc3**

761 Neuro2A or BHK cells cultured in 60 mm or 100 mm dishes were transfected with pCI-HA-
762 NFATc3 together with a plasmid expressing eight His₆-tagged ubiquitin (His-Ub), or empty
763 pCI, in the presence or the absence of pCI-Flag-Trim39, pCI-Flag-Trim39-ΔRING, pCI-
764 Trim17-GFP or a combination of these plasmids. Following 24 h transfection, the medium was
765 supplemented with 20 μM MG-132 for 6 h. Then, cells were harvested in 1 ml PBS without
766 Ca²⁺ and Mg²⁺ supplemented with 10 μM MG-132. In some experiments, a 100 μl sample of
767 the homogenous cell suspension was taken for input analysis and transfection efficiency
768 control. After centrifugation, the pellet from the remaining 900 μl cell suspension was
769 homogenized in 1 ml lysis buffer B (6 M guanidinium-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄, 10 mM
770 Tris-HCl [pH 8.0]) supplemented with 5 mM imidazole, 510 mM β-mercaptoethanol, 0.5 M
771 NaCl and 10 mM NEM. The lysate was sonicated, cleared by centrifugation at 1,500 × g for
772 5 min at room temperature. In some experiments, input analysis was made at this stage by
773 precipitating 50 μl of the resulting supernatants with TCA. The remainder of each extract was
774 added to 40 μl magnetic nickel beads (MagneHis™ Ni-Particles, Promega). Beads were rotated
775 for 2 h at room temperature to purify ubiquitinated proteins and washed once with lysis buffer
776 B supplemented with 20 mM imidazole, 0.5 M NaCl and 10 mM NEM, once with 8 M urea,
777 0.1 M Na₂HPO₄/NaH₂PO₄, 10 mM Tris-HCl (pH 8.0), 20 mM imidazole, 0.5 M NaCl and 10
778 mM NEM, three times with 8 M urea, 0.1 M Na₂HPO₄/NaH₂PO₄, 10 mM Tris-HCl (pH 6.3),
779 20 mM imidazole, 0.5 M NaCl, 10 mM NEM, 0.2% Triton X-100, once with 8 M urea, 0.1 M

780 Na₂HPO₄/NaH₂PO₄, 10 mM Tris-HCl (pH 6.3), 20 mM imidazole, 0.5 M NaCl, 10 mM NEM,
781 0.1% Triton X-100 and once with 8 M urea, 0.1 M Na₂HPO₄/NaH₂PO₄, 10 mM Tris-HCl (pH
782 6.3), 10 mM imidazole, 10 mM NEM. Materials bound to the beads were eluted by the addition
783 of 3 × Laemmli sample buffer and boiling for 5 min. These purification products and initial
784 total lysates were resolved by SDS-PAGE and analyzed by immunoblotting.

785

786 **Production of recombinant TRIM proteins**

787 BL21-CodonPlus®(DE3)-RP competent cells (Agilent) were transformed with the expression
788 vectors pGEX-4T1 expressing GST-Trim39 fusion proteins (WT and mutants). Protein
789 expression was induced by the addition of 500 μM IPTG and was carried out at 20°C overnight
790 in the presence of 100 μM ZnCl₂ and 200 μM MgSO₄. Bacteria were harvested by
791 centrifugation and resuspended in BugBuster® Protein Extraction Reagent (Millipore #70584-
792 4) supplemented with protease inhibitor cocktail (cOmplete EDTA-free, Sigma-Aldrich).
793 Bacterial suspensions were incubated for 30 min at room temperature with 1 mg/ml lysozyme
794 (Fluka) and further lysed by sonication. The soluble protein fraction was recovered by
795 centrifugation. GST fusion proteins were isolated by binding to glutathione magnetic beads
796 (MagneGST™ Glutathione Particles, Promega) for 30 min at room temperature on a rotating
797 wheel. The beads were then washed three times with PBS.

798 ArcticExpress (DE3) competent cells (Agilent) were transformed with the expression vector
799 pET-15-HIS-TRIM39 and pMAL-c2-TRIM17 (expressing MBP-TRIM17 fusion protein).
800 Bacteria were grown overnight in LB medium supplemented with Ampicillin and Gentamycin
801 (20 μg/ml). Recombinant protein expression was induced by the addition of 1 mM IPTG and
802 was carried out at 12°C overnight in the presence of 100 μM ZnCl₂ and 200 μM MgSO₄.
803 Bacteria were harvested by centrifugation. Pellets were resuspended in bacterial lysis buffer
804 (50 mM Tris-HCl [pH 8,6], 0.5 M NaCl, 50 mM MgSO₄) supplemented with lysozyme and
805 protease inhibitors, and frozen in liquid nitrogen to lyse bacteria. Lysates were then cleared by
806 centrifugation. MBP-TRIM17 proteins were purified on amylose resin (New England BioLabs,
807 #E8021L) and then eluted in a buffer containing 20 mM maltose before dialysis in PBS. HIS-
808 TRIM39 proteins were purified on Ni-NTA agarose beads (Qiagen, #1018244) and then eluted
809 in a buffer containing 0.5 M imidazole before dialysis in PBS.

810

811

812 ***In vitro* ubiquitination assay**

813 NFATc3 was first transcribed and translated *in vitro*. For this, 1 µg of pCS2-HA-NFATc3 was
814 incubated for 2 h at 30°C in 50 µl of the TNT® SP6 coupled wheat germ extract system
815 (Promega, #L5030), according to the instructions of the manufacturer. For each ubiquitination
816 condition, the equivalent of 3 µl of the *in vitro* translation reaction was immunopurified using
817 1 µl rat anti-HA antibody and 10 µl protein G-agarose beads in a buffer containing 50 mM Tris-
818 HCl (pH 7.5) and 50 mM NaCl buffer for 1 h. Beads were washed 3 times in the same buffer,
819 as described above for co-immunoprecipitation. Then, beads carrying NFATc3 were incubated
820 in 20 µl of ubiquitination reaction buffer (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 4 mM ATP,
821 4 mM MgCl₂, 2 mM DTT, 10 mM phosphocreatine, 0.5 U creatine kinase, 20 µM ZnCl₂), in
822 the presence of 50 ng human recombinant His-tagged ubiquitin-activating enzyme E1 (from
823 BostonBiochem, #E-304), 500 ng human recombinant His-tagged ubiquitin-conjugating
824 enzyme (E2) Ube2d3 (from BIOMOL International, #U0880), in the presence or the absence
825 of 10 µg N-terminal-His-tagged ubiquitin (Sigma-Aldrich, #U5507), and ≈ 2 µg of purified
826 recombinant mouse GST-Trim39 or His-Trim39 (WT) or GST-Trim39-C49S/C52S or mSIM3
827 His-Trim39, or ≈ 2 µg of purified recombinant human His-TRIM39 in the presence or the
828 absence of ≈ 2 µg MBP-TRIM17. Reactions were incubated at 37°C for 1 h. Beads were washed
829 once and the reaction was stopped by adding 10 µl of 3 × Laemmli sample buffer and by heating
830 at 95°C for 5 min. The samples were analyzed by SDS-PAGE and immunoblotting with anti-
831 NFATc3, anti-Trim39 and anti-TRIM17 antibodies.

832

833 ***In vitro* SUMOylation assay**

834 WT NFATc3 and its KallR and EallA mutants were first transcribed and translated *in vitro* as
835 described above. Equivalent amounts of the different forms of NFATc3 (between 1,5 and 6 µl
836 of the *in vitro* translation reaction) were incubated for 1 h to 1 h 30 min at 37°C in the presence
837 of 3 µg recombinant SUMO-1, 150 ng recombinant His-tagged Aos1/Uba2 (E1 enzyme), 100
838 ng recombinant Ubc9 (E2 enzyme) and 300 ng recombinant GST-PIASxα (E3 enzyme) in 20
839 µl shift-assay buffer (20 mM Hepes [pH 7.3], 110 mM KOAc, 2 mM Mg(OAc)₂, 0.5 mM
840 EGTA, 1 mM DTT, 0.05% Tween 20, 0.2 mg/ml ovalbumin, 1 µg/ml leupeptin, 1 µg/ml
841 aprotinin, 1 µg/ml pepstatin) supplemented with 1 mM ATP. Negative controls (time 0) were
842 obtained by mixing all reagents directly into loading buffer. In some cases, when SUMOylation

843 was followed by *in vitro* ubiquitination, the SUMO-specific protease GST-SEN1 was added
844 at the end of the reaction, for 15 min at 37 °C, to remove SUMO chains from NFATc3.
845 Recombinant proteins used in this assay were produced and purified as previously described
846 (Bossis et al., 2005). Reaction products were separated by SDS-PAGE (Tris-acetate gels,
847 Invitrogen), transferred to PVDF membranes and analyzed by western blot using anti-NFATc3
848 or anti-HA antibodies.

849

850 **RNA preparation and quantitative RT-PCR**

851 Total RNA was extracted using the RNeasy kit (Qiagen) and treated with the DNase I from the
852 DNA-free™ kit (Thermo Fisher Scientific) according to manufacturer's instructions. RNA was
853 used to perform a two-step reverse-transcription polymerase chain reaction (RT-PCR). In brief
854 1 µg of total RNA was reverse-transcribed using 200 U reverse transcriptase Superscript II
855 (Thermo Fisher Scientific) in the presence of 2.5 µM N6 random primers and 0.5 mM dNTP.
856 The equivalent of 6 ng of resulting cDNA was used as a template for real time PCR using a
857 Mx3000P thermocycler (Agilent) with a home-made SYBR Green qPCR master mix (Lutfalla
858 & Uze, 2006). PCR reactions were performed in 10 µl in the presence of 200 nM primers.
859 Thermal cycling parameters were 10 min at 95°C, followed by 40 cycles of 95°C for 30 s, 64°C
860 for 30 s and 72°C for 30 s. Specific primers used to amplify mouse *Trim39* and mouse *Trim17*
861 are listed in supplementary Table 1. Data were analysed and relative amounts of specifically
862 amplified cDNA were calculated with MxPro software (Agilent), using the mouse *Gapdh*
863 amplicon as a reference.

864

865 **Protein sequence analysis**

866 The sequence of mouse Trim39 (GenBank: NM_024468) was analyzed by using the prediction
867 web-based tools JASSA (Joined Advanced SUMOylation site and SIM analyzer,
868 (<http://www.jassa.fr/>) and GPS SUMO (group-based prediction system,
869 <http://sumosp.biocuckoo.org/online.php>) to identify putative SUMO-interacting motifs.

870

871 **Production of SUMO chains and GST-pull down**

872 Recombinant free SUMO-2 and poly-SUMO-2 chains were produced in bacteria co-expressing
873 His-SUMO-2, Aos1/Uba2 (SUMO conjugating E1 enzyme) and Ubc9 (SUMO E2 conjugating

874 enzyme). For this, BL21 competent cells were transformed with the plasmid pE1-E2-His-Su2
875 (described in (Brockly et al., 2016)). The transformed bacteria were grown with strong agitation
876 (210 rpm) at 37°C until the OD reaches 0.4-0.6. Protein expression was induced by adding 1
877 mM IPTG for 6 h at 25°C. The bacteria were resuspended in 30 ml of bacterial lysis buffer,
878 frozen in liquid nitrogen and stored at -80°C. The resuspended bacteria were thawed and
879 supplemented with lysozyme (1 mg/ml), 8 mM β -mercaptoethanol, 1 μ g/ml aprotinin, 1 μ g/ml
880 leupeptin, 1 μ g/ml pepstatin and incubated for 1 h on ice before centrifugation at 100,000 \times g
881 for 1 h at 4°C. The supernatant was loaded on a Ni-NTA column equilibrated in bacterial lysis
882 buffer supplemented with 8 mM β -mercaptoethanol, 0.5% Triton X-100, 10 mM imidazole and
883 protease inhibitors. The column was washed 3 times with 10 ml of the same buffer and eluted
884 with 15 ml of Ni-NTA elution buffer (bacterial lysis buffer supplemented with 250 mM
885 imidazole and protease inhibitors).

886 For GST-pull down, GST-Trim39 and its different SIM mutants were produced in bacteria and
887 purified as described above. The quantity and the quality of the different forms of GST-Trim39
888 bound to glutathione magnetic beads was first estimated on a poly-acrylamide gel using
889 Coomassie blue staining, by reference to known amounts of BSA. Beads binding approximately
890 1 μ g of each form of GST-Trim39 were incubated with \approx 1 μ g SUMO-2 chains for 1 h at room
891 temperature in 200 μ l shift assay buffer (20 mM HEPES [pH 7.3], 110 mM KOAc, 2 mM
892 Mg(OAc)₂, 0.5 mM EGTA, 1 mM DTT, 0.05% Tween 20, 0.2 mg/ml ovalbumin, 1 μ g/ml
893 leupeptin, 1 μ g/ml aprotinin, 1 μ g/ml pepstatin). Beads were recovered by centrifugation and
894 washed 4 times with PBS. Material bound to the beads was eluted by the addition of 3 \times
895 Laemmli sample buffer and incubation at 95°C for 5 min. Both GST-Trim39 proteins and bound
896 SUMO-2 chains were analyzed by immunoblotting.

897

898 **Assessment of neuronal apoptosis**

899 After 6 days *in vitro* (DIV), transfected or transduced CGNs were maintained in initial culture
900 medium (control) or were washed once and incubated in serum-free BME supplemented with
901 L-Gln, HEPES, antibiotics and 1 μ M (+)-MK-801, and containing 5 mM KCl (K5 medium) for
902 the indicated times. Neurons were then stained with DAPI and mounted on glass slides in
903 Mowiol. In experiments in which the CGNs were transfected with GFP, GFP-NFATc3 or GFP-
904 NFATc3-Ealla, apoptosis was assessed among GFP-positive neurons, by examining neuronal
905 morphology and nuclear condensation. For each experiment and each condition, at least 100

906 GFP-positive neurons were scored in a blinded manner. In experiments in which CGNs were
907 transduced with shRNA-expressing lentiviruses, apoptosis was estimated by counting the
908 percentage of condensed nuclei in five random fields for each condition (more than 500 cells).

909

910 **Statistics**

911 Statistical analyses were performed using GraphPad Prism version 7.0c for Mac OS X
912 (GraphPad Software, San Diego California USA, www.graphpad.com). Unless stated, data are
913 representative of at least three independent experiments.

914

915

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932

933

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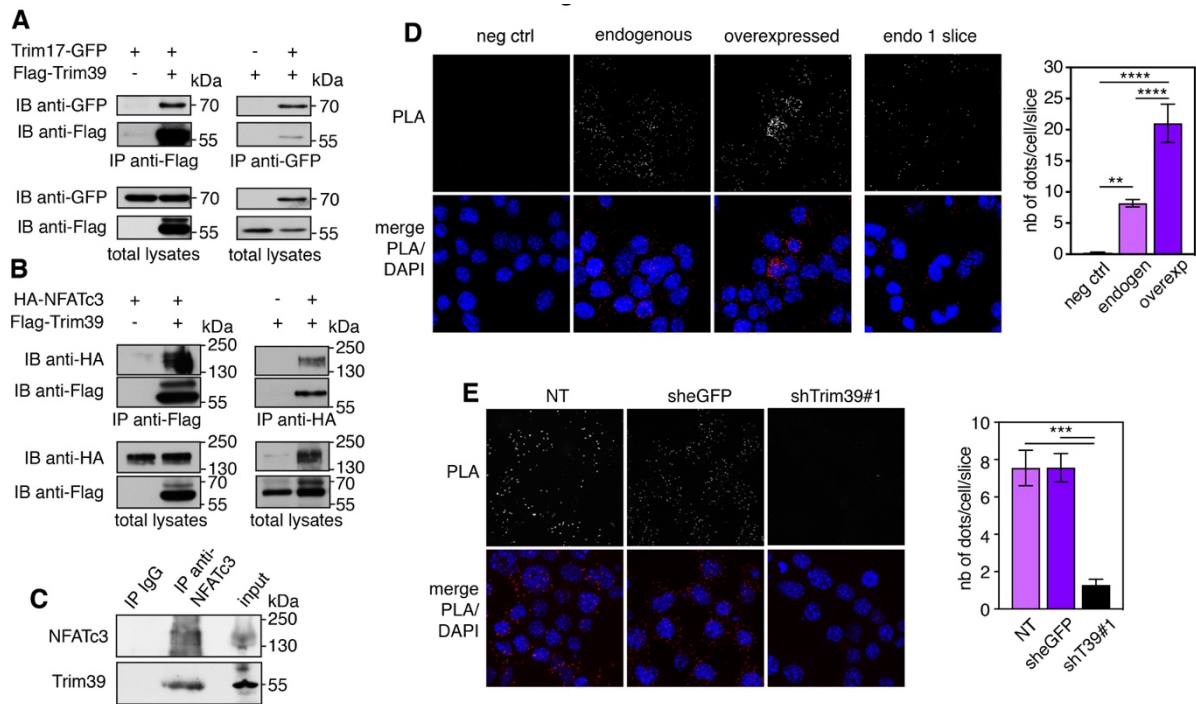
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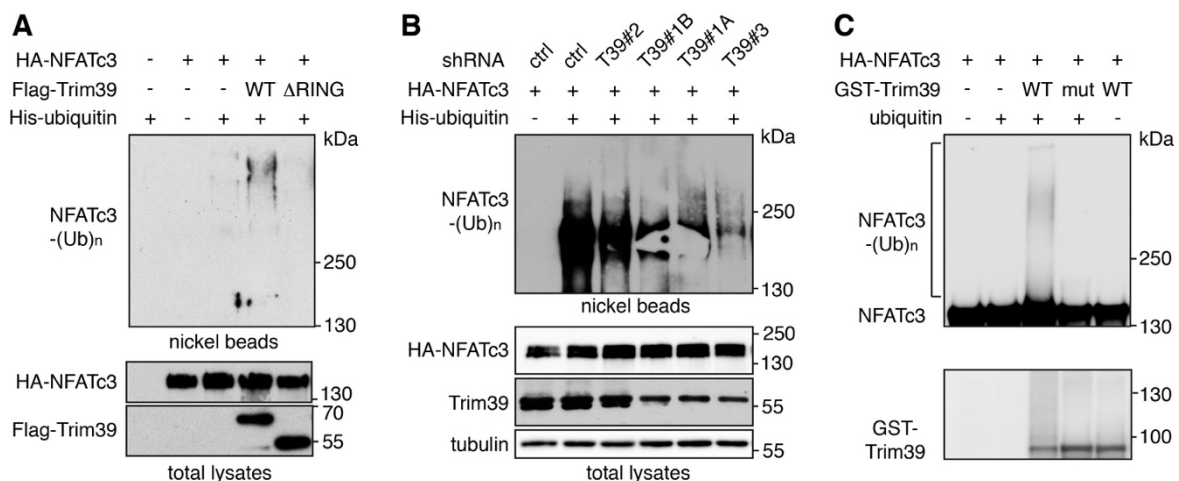
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1221 **Figure 1. Trim39 interacts with both Trim17 and NFATc3.** **A.** BHK cells were transfected
 1222 with Trim17-GFP together with Flag-Trim39 or empty plasmid (as a negative control) for 24 h.
 1223 Cells were then treated with 20 μ M MG-132 for 5 h. The cells were subsequently harvested
 1224 and lysates were subjected to immunoprecipitation using anti-Flag agarose beads (left panel) or
 1225 GFP-Trap beads (right panel). Immunoprecipitates and total lysates were analyzed by western
 1226 blot using anti-GFP and anti-Flag antibodies. **B.** Neuro2A cells were transfected with HA-
 1227 NFATc3 together with Flag-Trim39 or empty plasmid for 24 h. Cells were then treated as in A
 1228 and lysates were subjected to immunoprecipitation using anti-Flag (left panel) or anti-HA (right
 1229 panel) antibodies. Immunoprecipitates and total lysates were analyzed by western blot using
 1230 anti-HA and anti-Flag antibodies. **C.** Total lysate from Neuro2A cells was subjected to
 1231 immunoprecipitation using normal rabbit IgG (as a negative control) or anti-NFATc3 antibody.
 1232 Immunoprecipitates and input total lysates were analyzed by western blot using anti-NFATc3
 1233 and anti-Trim39 antibodies. **D.** Neuro2A cells were treated with 10 μ M MG-132 for 4 h and
 1234 then fixed and subjected to *in situ* PLA using rabbit anti-NFATc3 and mouse anti-Trim39
 1235 antibodies. Each bright red spot indicates that the two proteins are in close proximity. Negative
 1236 control was obtained by omitting anti-Trim39 antibody. When indicated, cells were previously
 1237 transfected with Trim39 for 24 h (overexpressed). Images were analyzed by confocal
 1238 microscopy. To better visualize the differences in PLA intensity, maximum intensity projection
 1239 was applied to the z-stacks of images on the left panel. To better determine the subcellular

1240 location of the NFATc3/Trim39 interaction, a single slice of the z-stack is presented on the
 1241 right panel (endo 1 slice). Nuclear staining was performed using DAPI and visualized in merged
 1242 pictures with PLA signal (merge). The number of dots per cell was determined in one slice of
 1243 each image using Fiji. The graph shows the mean \pm SEM of more than 12 images per condition,
 1244 including a total of more than 140 cells, from one experiment representative of two independent
 1245 experiments. ** $P < 0.01$, **** $P < 0.0001$ significantly different (one way ANOVA followed by
 1246 Tukey's multiple comparison test). **E.** Neuro2A cells were transduced with lentiviral particles
 1247 expressing a control shRNA (sheGFP) or a specific shRNA against Trim39 (shTrim39#1) for
 1248 24 h. Transduced cells were selected using puromycin for two additional days and plated onto
 1249 coverslips. The day after plating, cells were treated, analyzed by PLA and presented as in D.
 1250 The graph shows the mean \pm SEM of 6 images per condition, including more than 75 cells,
 1251 from one experiment representative of two independent experiments. *** $P < 0.001$ significantly
 1252 different (one way ANOVA followed by Tukey's multiple comparison test).

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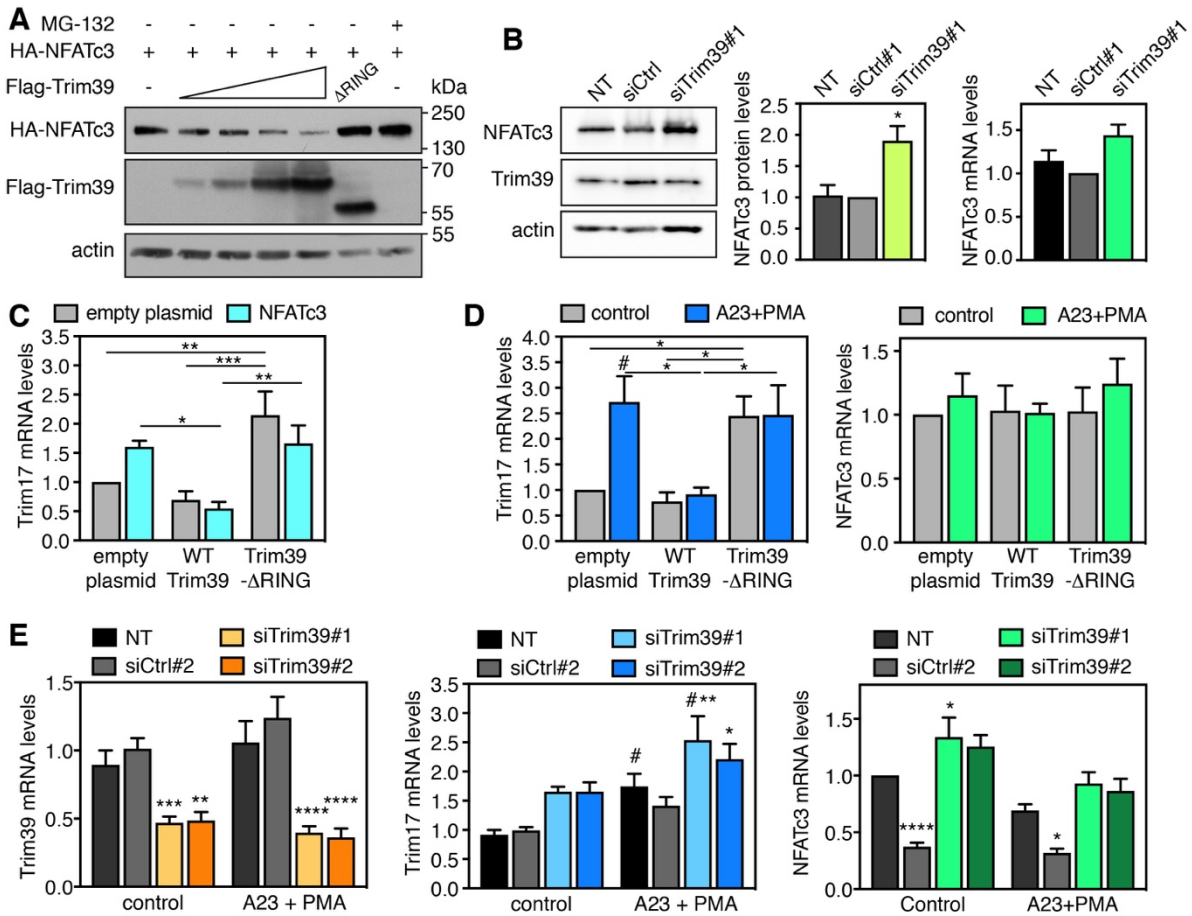


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1256 **Figure 2. Trim39 is an E3 ubiquitin-ligase of NFATc3.** **A.** Neuro2A cells were transfected
 1257 with HA-NFATc3 together with empty plasmid or His-tagged ubiquitin, in the presence or
 1258 absence of Flag-Trim39 or the inactive mutant Flag-Trim39-ΔRING for 24 h. Cells were then
 1259 incubated with 20 μ M MG-132 for 6 h before harvesting. The ubiquitinated proteins were
 1260 purified using nickel beads and analyzed by western blotting using anti-HA antibody to detect
 1261 ubiquitin-conjugated HA-NFATc3. In a separate SDS-PAGE, samples of the input lysates used
 1262 for the purification were analyzed with antibodies against HA and Flag. **B.** Neuro2A cells were
 1263 transduced with lentiviruses expressing a control shRNA (directed against eGFP) or three
 1264 different shRNAs targeting Trim39 (two different preparations of lentiviruses expressing

1265 shRNA-Trim39#1 were used: A and B). Following 48 h transduction and 48 h selection of
 1266 transduced cells using puromycin, cells were kept in culture for one week and plated in new
 1267 dishes. Then, cells were transfected with HA-NFATc3 or His-tagged ubiquitin or both for 24
 1268 h, and treated as in A. C. *In vitro* translated HA-NFATc3 was first immunopurified from wheat
 1269 germ extract using anti-HA antibody. Then, beads used for immunopurification of NFATc3
 1270 were incubated for 1 h at 37°C in the *in vitro* ubiquitination reaction mix (containing E1 and
 1271 E2 enzymes) with purified recombinant GST-Trim39 (WT) or its inactive mutant GST-Trim39-
 1272 C49S/C52S (mut) in the presence or the absence of ubiquitin as indicated. Poly-ubiquitinated
 1273 forms of NFATc3 were detected by immunoblotting using an anti-NFATc3 antibody. The same
 1274 membrane was immunoblotted with an anti-TRIM39 antibody to verify that similar amounts of
 1275 recombinant WT GST-Trim39 and GST-Trim39-C49S/C52S were used in the assay. Note that
 1276 in the presence of ubiquitin the levels of unmodified form of WT GST-Trim39 are lower due
 1277 to high Trim39 ubiquitination.

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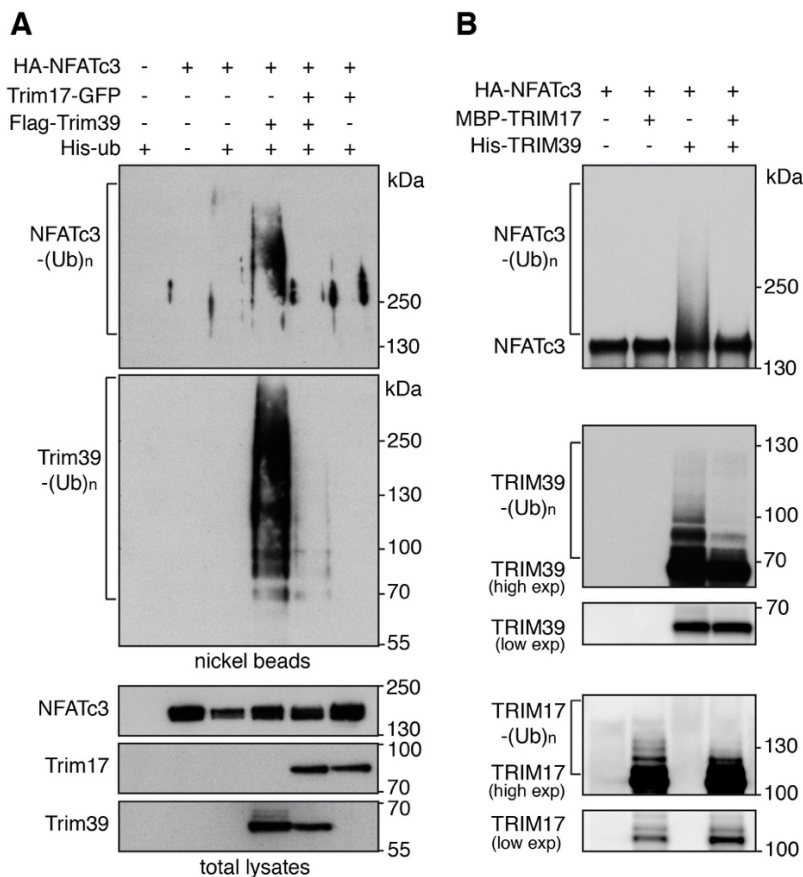


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1281 **Figure 3. Trim39 mediates NFATc3 degradation.** **A.** BHK cells were transfected with a fixed
1282 amount of a HA-NFATc3 expressing vector (1 μ g) together with empty plasmid (-) or
1283 increasing amounts of Flag-Trim39 expressing vector (0.1, 0.2, 0.5 and 1 μ g) or 0.2 μ g of a
1284 vector expressing the inactive mutant Flag-Trim39- Δ RING. When indicated, the cells were
1285 treated with 10 μ M MG-132 for 6 h before harvesting. Total lysates were analyzed by western
1286 blot using antibodies against HA, Flag and actin. **B.** Neuro2A cells were left untreated (NT), or
1287 transfected twice with an siRNA targeting specifically Trim39 (siTrim39#1) or with a negative
1288 control siRNA (siCtrl) for 48 h. Total lysates were analyzed by western blot using antibodies
1289 against NFATc3, Trim39 and actin. The intensity of the NFATc3 bands on the western blots
1290 was quantified, normalized by the intensity of the actin bands and expressed relative to the
1291 values obtained with the control shRNA. In the same experiments, the mRNA level of NFATc3
1292 was measured by quantitative PCR. The graphs show mean \pm SEM from three independent
1293 experiments. * P <0.05 significantly different from NT cells (one-way ANOVA followed by
1294 Dunnett's multiple comparisons test). **C.** Neuro2A cells were co-transfected with empty
1295 plasmid or HA-NFATc3, together with empty plasmid, Flag-Trim39 or the inactive mutant
1296 Flag-Trim39- Δ RING for 24 h. Then, total RNA was extracted and the mRNA level of Trim17
1297 was estimated by quantitative PCR. Data are the means \pm SEM of four independent
1298 experiments. * P <0.05, ** P <0.01, P <0.001 significantly different (two-way ANOVA followed
1299 by Tukey's multiple comparisons test). **D.** Neuro2A cells were transfected with empty plasmid,
1300 Flag-Trim39 or Flag-Trim39- Δ RING for 24 h. Then, cells were left untreated (control) or were
1301 deprived of serum for 3 h and subsequently treated with 1 μ M A23187 and 100 nM PMA in
1302 serum-free medium for 1 h (A23+PMA). Total RNA was extracted and the mRNA level of
1303 Trim17 was estimated by quantitative PCR. Data are the means \pm SEM of three independent
1304 experiments. * P <0.05; ** P <0.01 significantly different (two-way ANOVA followed by
1305 Tukey's multiple comparisons test). # P <0.05 significantly different with A23+PMA compared
1306 to the corresponding ctrl (two-way ANOVA followed by Sidak's multiple comparisons test).
1307 **E.** Neuro2A cells were transfected twice with two different siRNAs targeting specifically
1308 Trim39 or with a negative control siRNA for 48 h. Then, cells were left untreated (control) or
1309 were deprived of serum for 3h and subsequently treated with 1 μ M A23187 and 100 nM PMA
1310 in serum-free medium for 30 min (A23+PMA). Total RNA was extracted and the mRNA level
1311 of Trim39 (left panel), Trim17 (middle panel) or NFATc3 (right panel) was estimated by
1312 quantitative PCR (NT = non transfected). Data are the means \pm SEM of six independent
1313 experiments. * P <0.05; ** P <0,005; *** P <0.001; **** P <0.0001 significantly different from

1314 cells transfected with control siRNA or non-transfected cells in the same condition (two-way
 1315 ANOVA followed by Dunnet's or Sidak's multiple comparisons tests). #P<0.05 significantly
 1316 difference between A23+PMA and control for the indicated condition (two-way ANOVA
 1317 followed by Sidak's multiple comparisons test). For unknown reason the control siRNA
 1318 strongly decreased the mRNA levels of NFATc3. Note that this did not have a significant
 1319 impact on the expression of Trim17 taken as a target gene of NFATc3, probably because the
 1320 protein level of NFATc3 was not significantly reduced over the time course of the experiment
 1321 despite the decrease in its mRNA levels. It is therefore unlikely that the slight increase in
 1322 NFATc3 mRNA levels induced by siRNAs against Trim39 contributed significantly to the
 1323 increase in NFATc3 activity.

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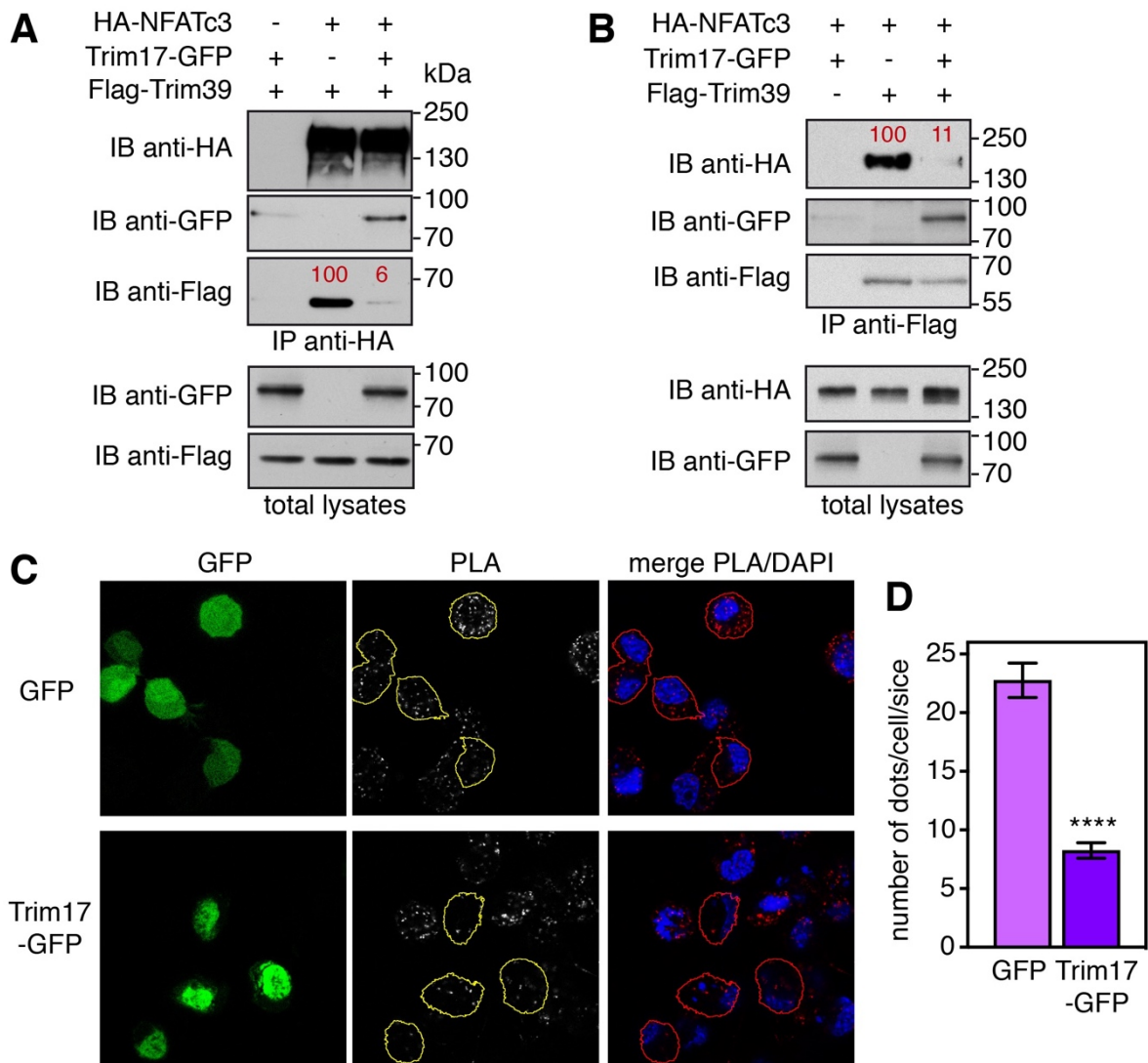


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1327 **Figure 4. Trim17 inhibits TRIM39-mediated ubiquitination of NFATc3.** A. BHK cells
 1328 were transfected with HA-NFATc3 together with His-tagged ubiquitin, in the presence or the
 1329 absence of Flag-Trim39, Trim17-GFP or both, as indicated, for 24 h. Then, cells were incubated
 1330 with 20 μ M MG-132 for 6 h before harvesting. The ubiquitinated proteins were purified using
 1331 nickel beads and analyzed by western blotting using anti-HA and anti-Flag antibodies to detect

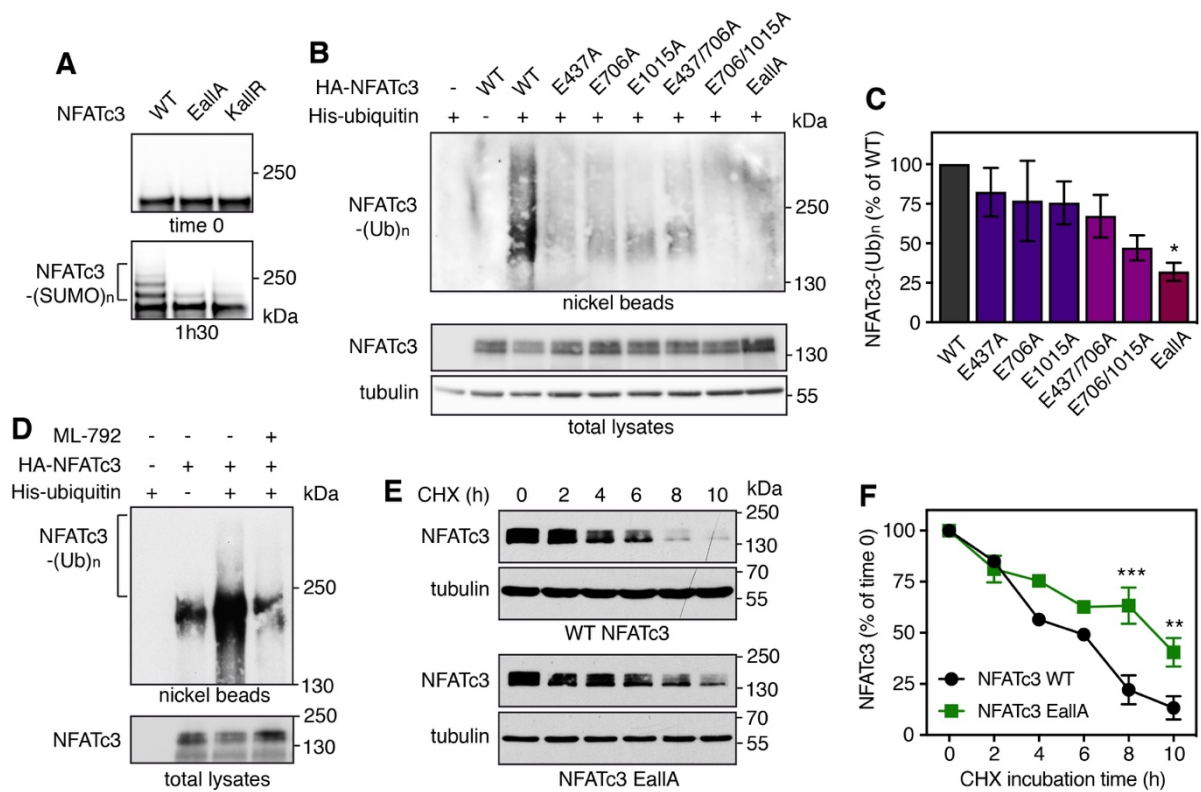
1332 poly-ubiquitinated forms of NFATc3 and Trim39. In a separate SDS-PAGE, samples of the
 1333 input lysates used for the purification were analyzed with antibodies against HA, Flag and GFP.
 1334 **B.** *In vitro* translated HA-NFATc3 was first immunopurified from wheat germ extract using
 1335 anti-HA antibody. Then, beads used for immunopurification of NFATc3 were incubated for 1
 1336 h at 37°C in the *in vitro* ubiquitination reaction mix (containing ubiquitin and E1 and E2
 1337 enzymes) with purified recombinant His-TRIM39 or MBP-TRIM17 as indicated. Poly-
 1338 ubiquitinated forms of NFATc3, TRIM39 and TRIM17 were detected by immunoblotting using
 1339 anti-NFATc3, anti-TRIM39 and anti-TRIM17 antibodies revealed using high exposure times.
 1340 Low exposure times were used to compare the level of TRIM39 and TRIM17 in the different
 1341 conditions.

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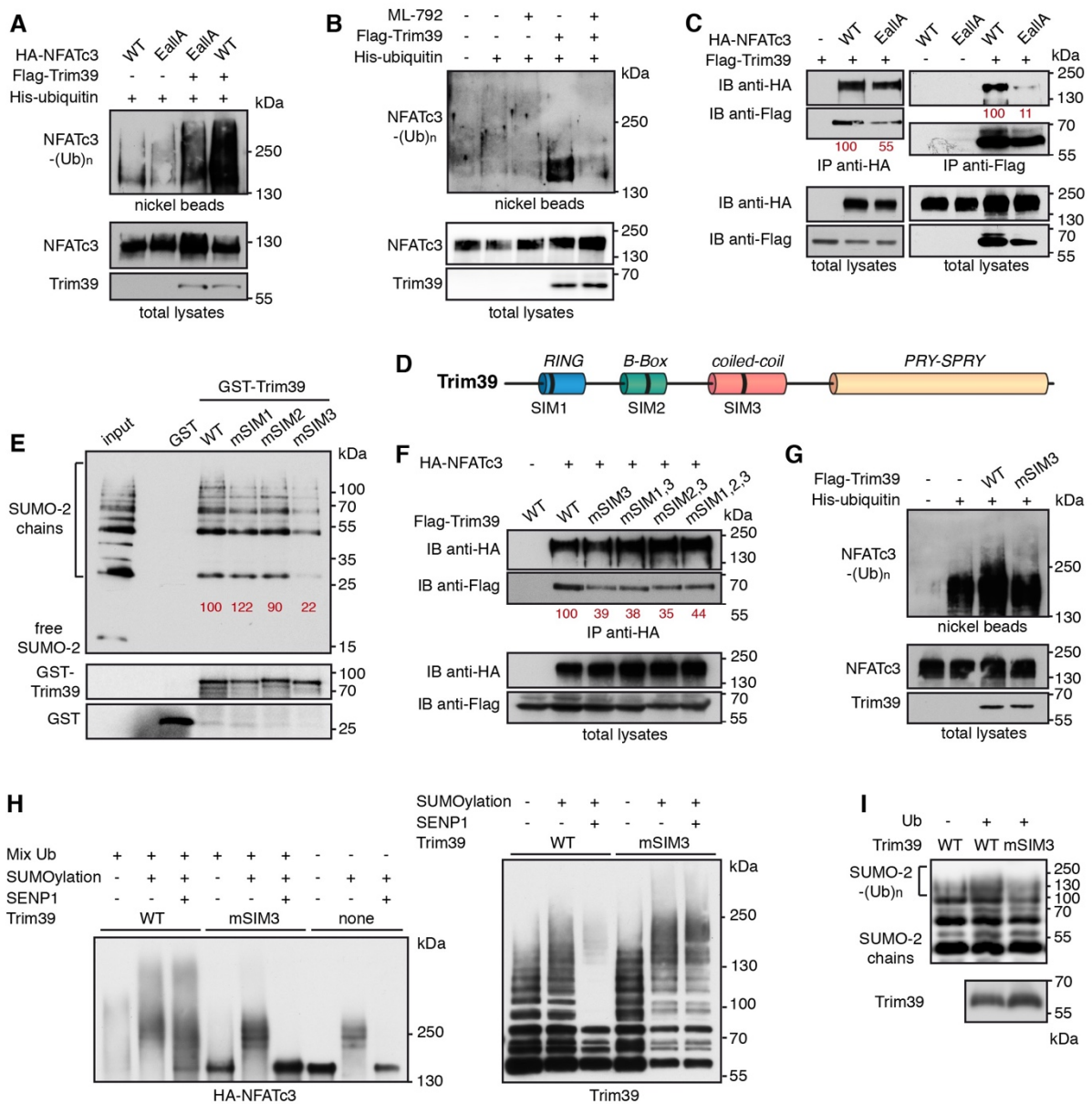
1345 **Figure 5. Trim17 reduces the interaction between endogenous Trim39 and NFATc3. A,B.**
1346 Neuro2A cells were transfected with HA-NFATc3 in the presence or the absence of Flag-
1347 Trim39, Trim17-GFP or both, as indicated, for 24 h. Cells were then treated with 20 μ M MG-
1348 132 for 7 h. The cells were subsequently harvested and lysates were subjected to
1349 immunoprecipitation using anti-HA (A) or anti-Flag (B) antibodies. Immunoprecipitates and
1350 total lysates were analyzed by western blot using anti-HA, anti-GFP and anti-Flag antibodies.
1351 The intensity of the bands containing Flag-Trim39 co-immunoprecipitated with HA-NFATc3
1352 was normalized by the intensity of the bands corresponding to immunoprecipitated HA-
1353 NFATc3 (A). The intensity of the bands containing HA-NFATc3 co-immunoprecipitated with
1354 Flag-Trim39 was normalized by the intensity of the bands corresponding to
1355 immunoprecipitated Flag-Trim39 (B). Relative values, indicated in red, correspond to the
1356 experiment shown in the figure, which is representative of three independent experiments. **C.**
1357 Neuro2A cells were transfected with GFP or Trim17-GFP for 24 h. Then cells were treated
1358 with 10 μ M MG-132 for 4 h, fixed and subjected to *in situ* PLA using rabbit anti-NFATc3 and
1359 mouse anti-Trim39 antibodies. Each bright red spot indicates that the two proteins are in close
1360 proximity. Images were analyzed by confocal microscopy and a single slice of the z-stacks is
1361 presented for each condition. Nuclear staining was performed using DAPI and merged with the
1362 PLA signal. Note that, in the Trim17-GFP condition, transfected cells (delineated by a yellow
1363 or red line) show less dots than neighboring non transfected cells, which is not the case in the
1364 GFP condition. **D.** The number of dots was determined on one slice for individual cells
1365 transfected with either GFP or Trim17-GFP using Fiji. Data represent one experiment,
1366 including 68 transfected cells for each condition, representative of two independent
1367 experiments. **** $p < 0.0001$, significantly different from GFP transfected cells (unpaired t test).
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1371 **Figure 6. SUMOylation of NFATc3 favors its ubiquitination and stability.** **A.** *In vitro*
 1372 translated HA-NFATc3 was incubated with *in vitro* SUMOylation reaction mix (containing
 1373 SUMO1, E1, E2 and E3 enzymes) for 1 h 30 or directly added to sample loading buffer together
 1374 with reaction mix (time 0). Multi-SUMOylated forms of NFATc3 were detected by
 1375 immunoblotting using anti-NFATc3 antibody. **B.** Neuro2A cells were transfected with His-
 1376 tagged ubiquitin or empty plasmid, together with WT HA-NFATc3 or the different HA-
 1377 NFATc3 E/A mutant constructs for 24 h. Then, cells were incubated with 20 μ M MG-132 for
 1378 7 h before harvesting. The ubiquitinated proteins were purified using nickel beads and analyzed
 1379 by western blotting using anti-HA antibody to detect ubiquitin-conjugated HA-NFATc3. In a
 1380 separate SDS-PAGE, samples of the input cell suspension used for the purification were
 1381 analyzed with antibodies against HA and tubulin. **C.** The intensity of the NFATc3 ladders from
 1382 the nickel bead purification was quantified in different experiments performed as in B. Data are
 1383 the mean \pm SEM of four independent experiments. * $p < 0.05$, significantly different from WT
 1384 NFATc3 (one-way ANOVA followed by Dunnett's multiple comparisons test). **D.** Neuro2A
 1385 cells were transfected with His-tagged ubiquitin or empty plasmid, together with HA-NFATc3
 1386 for 24 h. Then, cells were incubated with 20 μ M MG-132 in the presence or the absence of 0.3
 1387 μ M ML-792 for 8 h, as indicated. The ubiquitination level of NFATc3 was assessed as in B. **E.**
 1388 Neuro2A cells were transfected with WT HA-NFATc3 or NFATc3-EallA for 48 h. Then, cells

1389 were incubated with 20 $\mu\text{g/ml}$ cycloheximide (CHX) for increasing times before harvesting.
 1390 Proteins were analyzed by western blot using antibodies against HA tag and tubulin. **F.** The
 1391 intensity of the bands on the western blots of different experiments performed as in C was
 1392 quantified. For each experiment, the amount of NFATc3 was normalized by the level of tubulin
 1393 in each condition and plotted against CHX incubation time. Data are the mean \pm SEM of three
 1394 independent experiments. *** $p < 0.0001$, ** $p < 0.005$ significantly different from WT NFATc3
 1395 at the same incubation time (two-way ANOVA followed by Sidak's multiple comparisons test).
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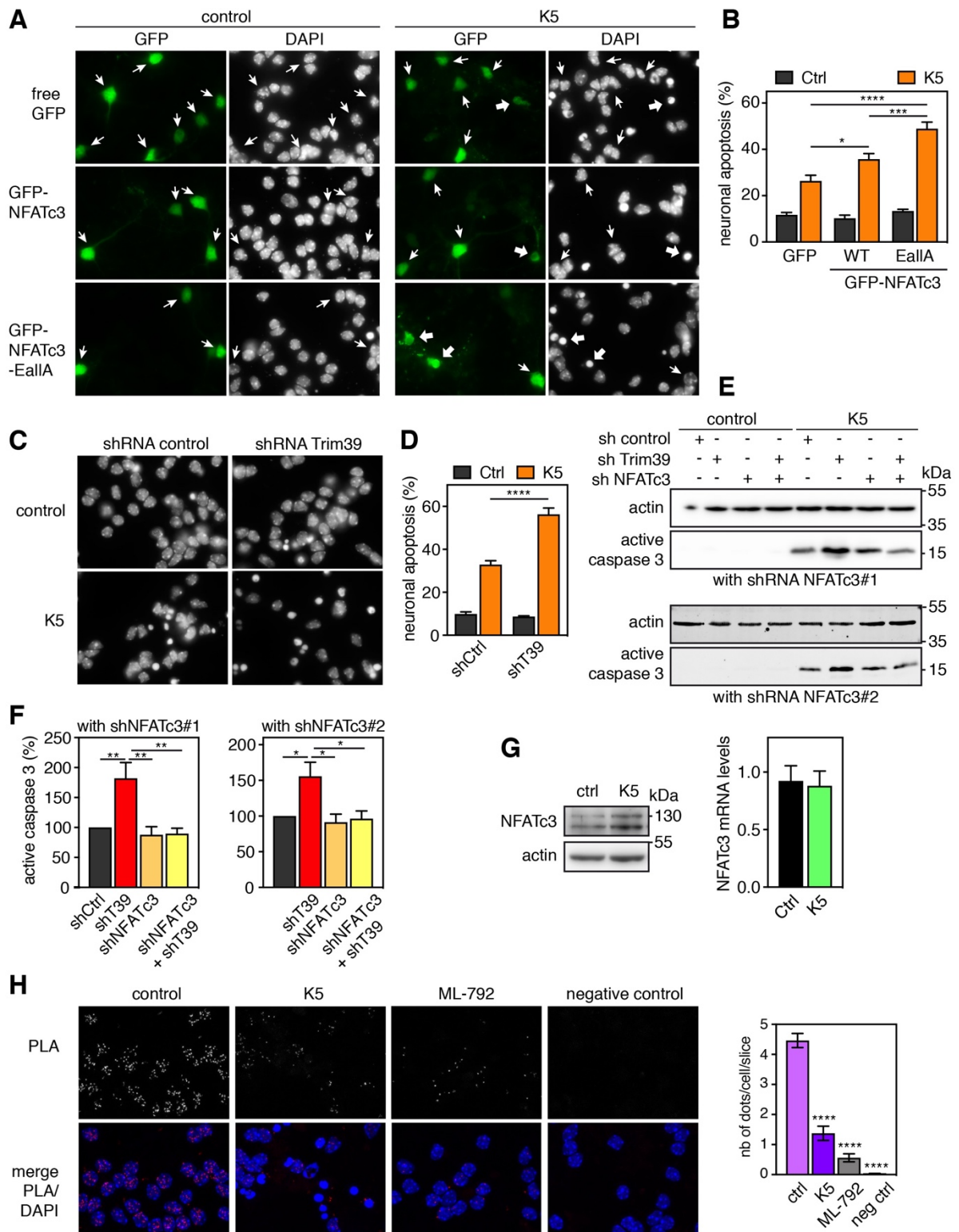
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1399 **Figure 7. Trim39 is a SUMO-targeted E3 ubiquitin-ligase for NFATc3.** **A.** Neuro2A cells
 1400 were transfected with His-tagged ubiquitin together with WT HA-NFATc3 or HA-NFATc3

1401 EallA, in the presence or the absence of Flag-Trim39, for 24 h. Then, cells were incubated with
1402 20 μ M MG-132 for 6 h before harvesting. The ubiquitinated proteins were purified using nickel
1403 beads and analyzed by western blotting using anti-HA antibody to detect ubiquitin-conjugated
1404 HA-NFATc3. In a separate SDS-PAGE, samples of the input lysates used for the purification
1405 were analyzed with antibodies against HA and Flag. **B.** Neuro2A cells were transfected with
1406 HA-NFATc3, in the presence or the absence of His-tagged ubiquitin and Flag-Trim39, as
1407 indicated, for 24 h. Then, cells were incubated with 20 μ M MG-132 in the presence or the
1408 absence of 0.3 μ M ML-792 for 8 h before harvesting. The ubiquitination level of NFATc3 was
1409 assessed as in A. **C.** Neuro2A cells were transfected with Flag-Trim39 together with WT HA-
1410 NFATc3, HA-NFATc3-EallA or empty plasmid for 24 h. Cells were then treated with 10 μ M
1411 MG-132 for 8 h. The cells were subsequently harvested and lysates were subjected to
1412 immunoprecipitation using anti-HA antibody (left panel) or anti-Flag beads (right panel).
1413 Immunoprecipitates and total lysates were analyzed by western blot using anti-HA and anti-
1414 Flag antibodies. Band intensities of co-immunoprecipitated Flag-Trim39 were quantified and
1415 normalized by band intensities of immunoprecipitated HA-NFATc3. Band intensities of co-
1416 immunoprecipitated HA-NFATc3 were normalized by band intensities of immunoprecipitated
1417 Flag-Trim39. Relative values, indicated in red, correspond to the experiment shown in the
1418 figure, which is representative of three independent experiments. **D.** Schematic representation
1419 of mouse Trim39 depicting its different domains and its three putative SIMs. **E.** Recombinant
1420 GST, GST-Trim39 and its different SIM mutants were purified using glutathione beads and
1421 subsequently incubated with purified recombinant SUMO-2 and SUMO-2 chains. Material
1422 bound to the beads was eluted and analyzed by western blot using anti-SUMO and anti-GST
1423 antibodies. A small fraction of the SUMO-2 chains was also loaded on the gel (input) for
1424 comparison. The intensity of bound SUMO-chain bands was quantified and normalized by the
1425 intensity of corresponding GST-Trim39 bands. Relative values, indicated in red, correspond to
1426 the experiment shown in the figure, which is representative of three independent experiments.
1427 Note that SUMO bands are multiple of \approx 15 kDa corresponding to mono-, di-, tri-, tetra-SUMO
1428 etc... **F.** Neuro2A cells were transfected with HA-NFATc3 or empty plasmid together with WT
1429 Flag-Trim39 or its SIM3 mutant for 24 h. Cells were treated as in B and lysates were subjected
1430 to immunoprecipitation using anti-HA antibody. Immunoprecipitates and total lysates were
1431 analyzed as in B. Band intensities of co-immunoprecipitated Flag-Trim39 were quantified and
1432 normalized by band intensities of immunoprecipitated HA-NFATc3. Relative values, indicated
1433 in red, correspond to the experiment shown in the figure, which is representative of three

1434 independent experiments. **G.** Neuro2A cells were transfected with His-tagged ubiquitin (or
1435 empty plasmid) together with HA-NFATc3 in the presence or the absence of Flag-Trim39 or
1436 its SIM3 mutant, for 24 h. Then, cells were treated as in A. Ubiquitinated proteins and input
1437 lysates were analyzed as in A. **H.** *In vitro* translated HA-NFATc3 was first incubated (+) in
1438 SUMOylation reaction mix (containing SUMO-1, E1, E2 and E3 enzymes) or not (-) for 1 h at
1439 37 °C. Then, NFATc3 was immunopurified from the reaction mix using anti-HA antibody.
1440 Beads used for immunopurification of NFATc3 were then incubated for 1 h at 37°C in the *in*
1441 *vitro* ubiquitination reaction mix (containing E1 and E2 enzymes and ubiquitin) with purified
1442 recombinant His-Trim39 (WT) or its SIM3 mutant (mSIM3 His-Trim39) as indicated. The
1443 reaction samples were then treated or not with the SUMO-specific protease SENP1 for 15 min
1444 at 37°C. The three last samples were not subjected to *in vitro* ubiquitination to assess the results
1445 of SUMOylation and SENP1 reactions. Poly-ubiquitinated and multi-SUMOylated forms of
1446 NFATc3 were detected by immunoblotting using an anti-HA antibody. The same membrane
1447 was immunoblotted with an anti-TRIM39 antibody to assess the auto-ubiquitination of the two
1448 forms of Trim39 and thereby their intrinsic E3 ubiquitin-ligase activity. **I.** Purified recombinant
1449 SUMO-2 chains were incubated in the *in vitro* ubiquitination reaction mix, in the absence or
1450 the presence of ubiquitin, with purified recombinant His-Trim39 (WT) or its SIM3 mutant
1451 (mSIM3 His-Trim39) as indicated. The reaction samples were analyzed by immunoblotting
1452 with an antibody against SUMO-2. The poly-ubiquitinated forms of SUMO-2 chains appear as
1453 a more intense smear above 100 kDa. In a separate SDS-PAGE, the same volumes of WT and
1454 mSIM3 His-Trim39 as used in the *in vitro* ubiquitination experiments were analyzed with
1455 antibodies against Trim39.

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1459 **Figure 8. SUMOylation and Trim39 attenuate NFATc3 pro-apoptotic effect in neurons.**
 1460 **A.** CGN primary cultures were transfected after 5 days *in vitro* (DIV 5) with GFP (as a negative
 1461 control), WT GFP-NFATc3 or GFP-NFATc3-Ealla for 16 h. Then, neurons were switched to
 1462 serum-free medium containing 5 mM KCl (K5) for 7 h or were left untreated (control).

1463 Following fixation, nuclei were visualized by DAPI staining and proteins fused to GFP were
1464 detected by fluorescent microscopy. Arrows indicate GFP-positive neurons with thick arrows
1465 for neurons undergoing apoptosis and thin arrows for healthy neurons. **B.** The percentage of
1466 transfected, GFP-positive neurons undergoing apoptosis was assessed by examining cell
1467 morphology and nuclear condensation. Data are the means \pm S.E.M. of four independent
1468 experiments performed as in A. * $P < 0.05$; *** $P < 0.001$ significantly different (two-way
1469 ANOVA followed by Sidak's multiple comparisons test). Apoptosis was highly significantly
1470 different in K5 *versus* Ctrl in all conditions (not shown). **C.** CGNs were transduced with
1471 lentiviral particles expressing a non-targeting control (directed against Luciferase) or an shRNA
1472 specifically targeting Trim39 one day after plating. At DIV 6 they were incubated for 8 h in K5
1473 medium, fixed and stained with DAPI. **D.** The percentage of apoptotic neurons was estimated
1474 by examining nuclear condensation. Data are the means \pm S.E.M. of four independent
1475 experiments performed as in C. **** $P < 0.0001$ significantly different (two-way ANOVA
1476 followed by Sidak's multiple comparisons test). Apoptosis was highly significantly different in
1477 K5 *versus* Ctrl in both conditions (not shown). **E.** CGNs were transduced with lentiviral
1478 particles expressing a non-targeting control, or an shRNA specifically targeting Trim39, or one
1479 of two different shRNAs against NFATc3, or a combination of shRNA Trim39 and shRNA
1480 NFATc3, one day after plating. At DIV 6 transduced neurons were incubated for 8 h in K5
1481 medium, and total cell extracts were analyzed by western blot using antibodies against the
1482 cleaved (active) form of caspase 3 and actin. **F.** The intensity of the bands of different
1483 experiments performed as in E was quantified. For each experiment, the amount of active
1484 caspase 3 was normalized by the level of actin, only in K5 conditions, and expressed as the
1485 percentage of shRNA control. Data are the mean \pm SEM of four independent experiments.
1486 * $P < 0.05$, ** $P < 0.01$ significantly different (one-way ANOVA followed by Tukey's multiple
1487 comparisons test). **G.** CGNs were left untreated (ctrl), or incubated in K5 medium for 6 h and
1488 total cell extracts were analyzed by western blot using antibodies against NFATc3 and actin. In
1489 parallel, total RNA was extracted and the mRNA level of NFATc3 was estimated by
1490 quantitative PCR. **H.** CGNs were left untreated (control), switched to K5 medium for 8 h or
1491 treated with 0.3 μ M ML-792 for 8 h. Then neurons were fixed and subjected to *in situ* PLA
1492 using rabbit anti-NFATc3 and mouse anti-SUMO-2 antibodies. Each bright red spot indicates
1493 that the two proteins are in close proximity and therefore that NFATc3 is SUMOylated.
1494 Negative control was obtained by omitting anti-NFATc3 antibody. Images were analyzed by
1495 confocal microscopy. To better visualize the differences in PLA intensity, maximum intensity
1496 projection was applied to the z-stacks of images. Nuclear staining was performed using DAPI

1497 and visualized in merged pictures with PLA signal (merge). The number of dots per cell was
1498 determined in one slice of each image using Fiji. The graph shows the mean \pm SEM of 5 images
1499 per condition, including a total of more than 100 cells, from one experiment representative of
1500 three independent experiments. ****P<0.0001 significantly different from control (one way
1501 ANOVA followed by Dunnet's multiple comparison test).

1502

Supplemental data to:

Trim39 regulates neuronal apoptosis by acting as a SUMO-targeted E3 ubiquitin-ligase for the transcription factor NFATc3

by Meenakshi Basu-Shrivastava, Barbara Mojsa, Stéphan Mora, Ian Robbins, Guillaume Bossis, Iréna Lassot and Solange Desagher

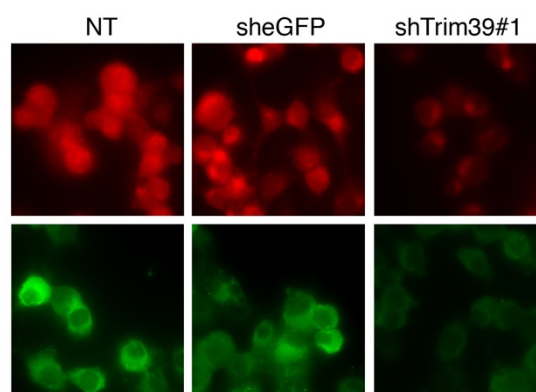


Figure S1. The specific shRNA shTrim39#1 efficiently reduces the protein level of Trim39. A. Neuro2A cells were transduced with lentiviral particles expressing a control shRNA (sheGFP) or a specific shRNA against Trim39 (shTrim39#1) for 24 h. Transduced cells were selected using puromycin for two additional days and plated onto coverslips. The day after plating, cells were analyzed by immunofluorescence using two different antibodies against Trim39; in red: antibody from Origene, in green: antibody from Proteintech. Images were set to the same minimum and maximum intensity to allow signal intensity comparison.

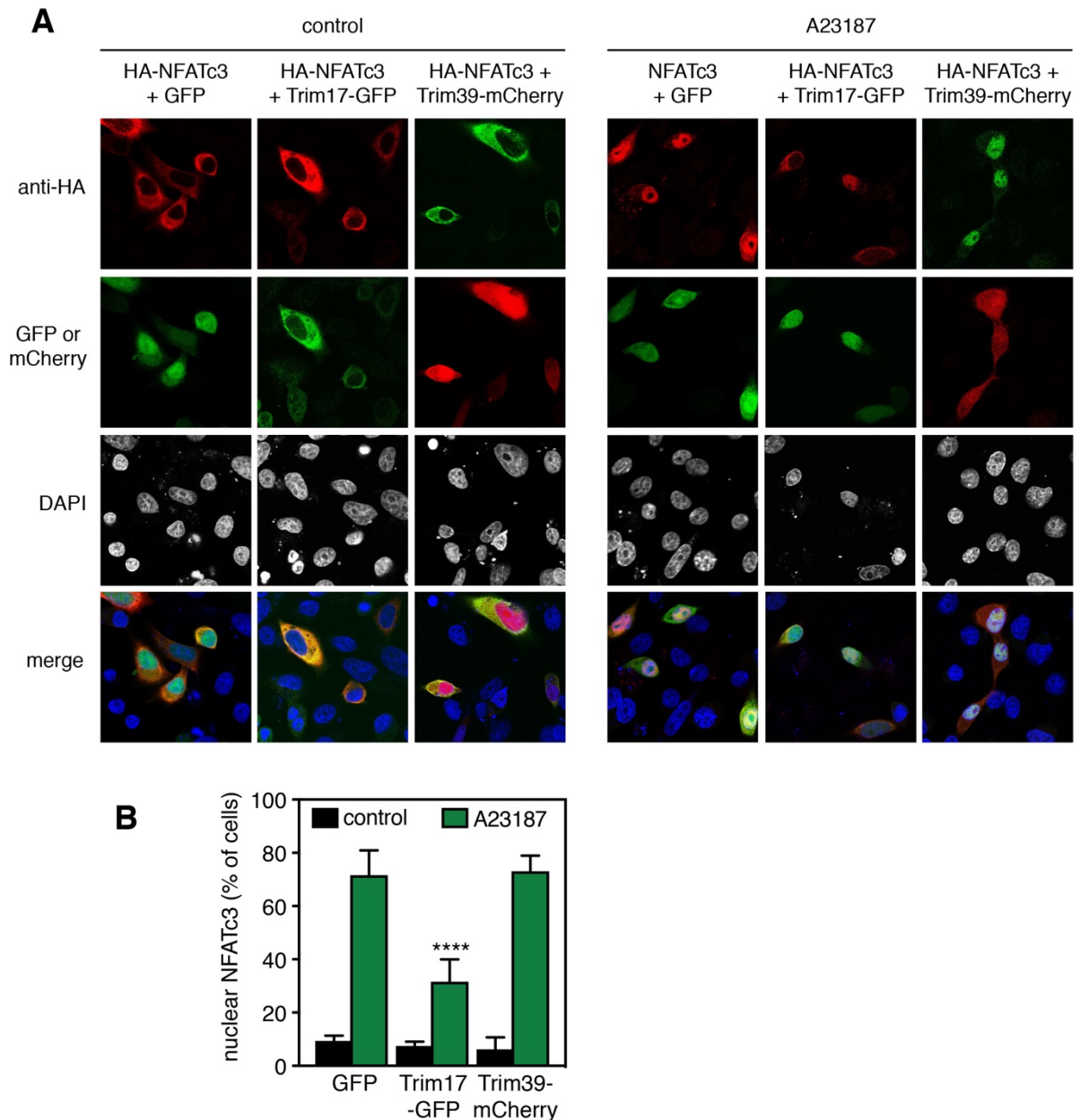


Figure S2. Trim39 does not alter calcium-induced nuclear translocation of NFATc3. A. BHK cells were transfected with HA-NFATc3 together with GFP (negative control) or Trim17-GFP, or Trim39-mCherry for 24 h. Then, cells were left untreated (control) or deprived of serum for 3h and treated with 1 μ M of the calcium ionophore A23187 in serum-free medium for an additional 30 min before fixation. NFATc3 was detected using an anti-HA antibody and visualized by confocal microscopy. GFP, Trim17-GFP and Trim39-mCherry were visualized by GFP or mCherry fluorescence and nuclei were stained with DAPI. **B.** Quantification of the nuclear localization of NFATc3 in experiments conducted as in A. The percentage of cells showing NFATc3 mainly in the nucleus was determined among the population of cells expressing both HA-NFATc3 and either GFP, Trim17-GFP or Trim39-mCherry. Data are the means \pm SD of three independent experiments. **** $P < 0.0001$ significantly different from the corresponding value obtained in cells transfected with GFP and NFATc3 (two way ANOVA followed by Dunnett's multiple comparisons test).

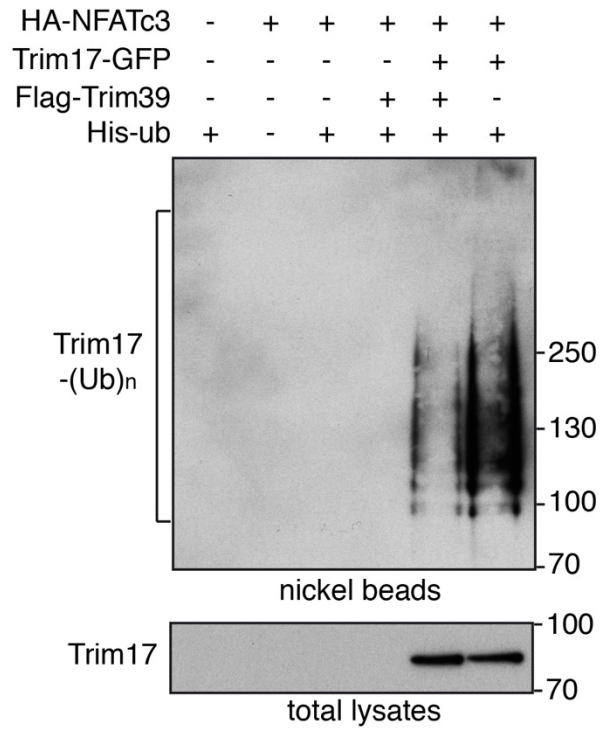


Figure S3. Trim39 decreases the ubiquitination of Trim17. The PVDF membrane presented in Figure 4A was stripped and blotted with an anti-GFP antibody.

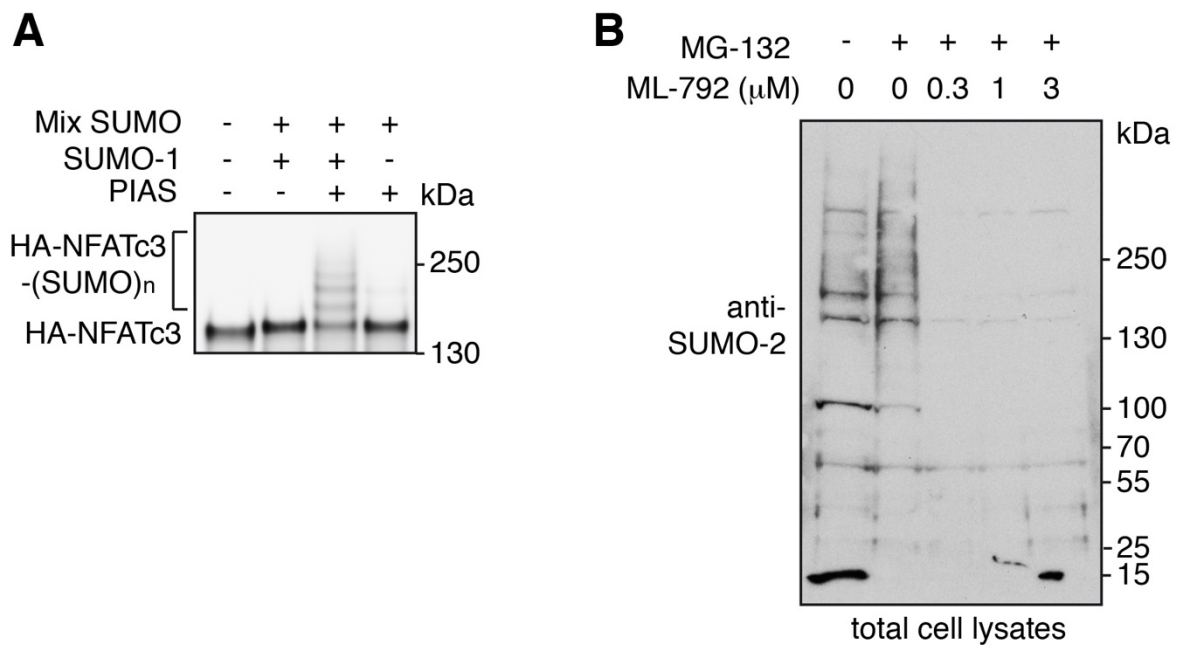


Figure S4. Role of SUMOylation in NFATc3 regulation. **A.** *In vitro* translated HA-NFATc3 was incubated with *in vitro* SUMOylation reaction mix (containing E1 and E2 enzymes) for 1 h in the presence or the absence of SUMO-1 and/or the E3 SUMO ligase PIAS. Multi-SUMOylated forms of NFATc3 were detected by immunoblotting using anti-HA antibody. **B.** Neuro2A cells were treated with increasing concentrations of the SUMO-activating enzyme inhibitor ML-792 for 7.5 h in the presence of 20 μM of the proteasome inhibitor MG-132. Total protein extracts were analyzed by Western blot using anti-SUMO-2 antibody to assess the level of global SUMOylation in cells. No toxicity was detected in any of these conditions.

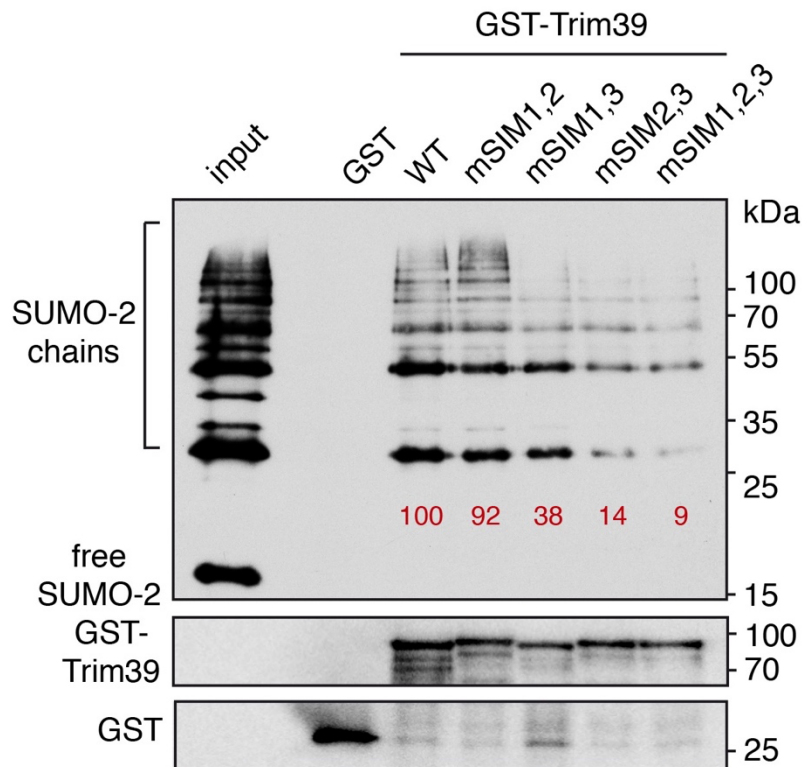


Figure S5. SIM3 is the predominant SIM involved in the interaction of Trim39 with SUMO-2 chains. Recombinant GST, GST-Trim39 and its different double or triple SIM mutants were purified using glutathione beads and subsequently incubated with purified recombinant SUMO-2 and SUMO-2 chains. Material bound to the beads was eluted and analyzed by western blot using anti-SUMO and anti-GST antibodies. A small fraction of the SUMO-2 chains was also loaded on the gel (input) for comparison. The intensity of bound SUMO-chain bands was quantified and normalized by the intensity of corresponding GST-Trim39 bands. Relative values are indicated in red.

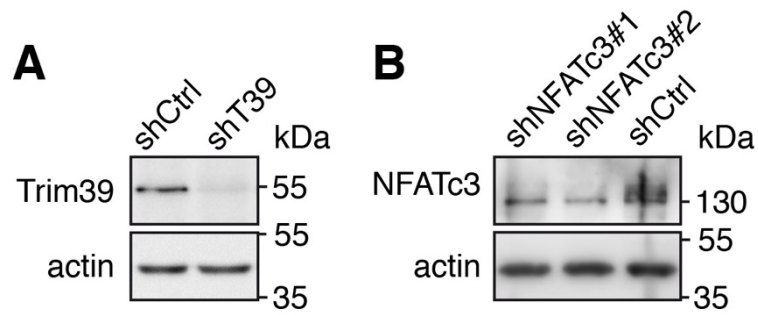


Figure S6: A. Primary cultures of CGNs were transduced with lentiviral particles expressing a non-targeting control shRNA (directed against Luciferase) or an shRNA specifically targeting Trim39 one day after plating. At DIV 6, total cell extracts from KCl-deprived neurons were analyzed by western blot using anti-Trim39 antibody (Origene). **B.** CGNs were transduced with lentiviral particles expressing the control shRNA or two different shRNAs targeting NFATc3 one day after plating. At DIV 6, total cell extracts from neurons were analyzed by western blot using anti-NFATc3 antibody.

Supplementary Table 1: list of the primers used to generate described constructs

PCR primer pairs used for constructs:		
Construct	Forward (5' – 3')	Reverse (5' – 3')
NFATc3-E437A	GAATTGAAAATAGCAGTGCAACCTAAAAC	GTTTGTAGGTTGCACTGCTATTTCAATTC
NFATc3-E706A	GATGAAGCAAGCACAAAGAGAAGAC	GTCTTCTCTTTGTGCTTGCTTCATC
NFATc3-E1015	CATTAAACCTGCACCTGAAGATCAAG	CTTGATCTTCAGGTGCAGGTTTAATG
Trim39	ATAGAATTCATGGCAGAGACAAGTCTG	TTATCTAGATCATTCCCAATCTGTTGG
Trim39-ΔRING	CGAGAATTCTCCCGATACCGG	CGACTCGAGTCATTCCCAATC
Trim39-mSIM1	GAGTATCTGAAGGAGCCAGCTGCTGCTGAATGTGGGCACAAC	GTTGTGCCACATTGAGCAGCAGCGCTGGCTCCTCAGATACTC
Trim39-mSIM2	GAAGACCAGGAGGCTGCATGTGCGCCTGTGCAATTTCTCATAACC	GGGTATGAGAAATTGCACAGGCGGCACATGCAGCCTCCTGGTCTTC
Trim39-mSIM3	CGAAGAGCAGCAGACAGCAGCAGCGCGAGCAGAGGAAGAAGAACAG	CTGTTCTTCTTCTCTGCTGCCGCTGCTGCTGTCTGCTGCTCTTCG
Trim39-mCherry 1 st PCR	ATAGAATTCATGGCAGAGACAAGTCTG	CTCCTCGCCCTTGCTCACTTCCCAA TCTGTTGGG
Trim39-mCherry 2 nd PCR	CCCAACAGATTGGGAAGTGAGCAAGGGCGAGGAG	ATATCTAGATTATCTAGATCCGGTGGATC
Trim39-mCherry 3 rd PCR	ATAGAATTCATGGCAGAGACAAGTCTG	ATATCTAGATTATCTAGATCCGGTGGATC
GST-Trim39	CGAGAATTCGCAGAGACAAGTC	CGACTCGAGTCATTCCCAATC
Trim39-C49S/C52S	GGCACAACTTCTCCAAAGCGTCCATTACCGCGTGG	CCAGCGGGTAATGGACGCTTTGGA GAAGTTGTGCC
TRIM17	CGAGAATTCGAGGCTGTGGAATCGCC	CGAGTCGACCTATCCTTTCACCCACATGGTCAC
His-Trim39	CGACATATGGCAGAGACAAGTCTGTTAGAGG	CGAAAGCTTTCATTCCCAATCTGTTGGG
Primers used for RT-qPCR		
cDNA	Forward (5' – 3')	Reverse (5' – 3')
Trim39	TGGAGGTGACTTCAGTATCCAT	TCACATCCGCAATTAGCTGTT
Trim17	ACTGAGTGGCAGGAGAGAGTGAA	CCAGAAACAAGACCACCTTCTGA
NFATc3	TTGAGTGCTCTCAGCGATCT	GACAATGTGAGCCCCTTGAC
GAPDH	AAATGGTGAAGGTCGGTGTG	CCTTGACTGTGCCGTTGAAT