



**HAL**  
open science

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

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

► **To cite this version:**

Meenakshi Basu-Shrivastava, Barbara Mojsa, Stéphan Mora, Ian Robbins, Guillaume Bossis, et al.. Trim39 regulates neuronal apoptosis by acting as a SUMO-targeted E3 ubiquitin-ligase for the transcription factor NFATc3. *Cell Death and Differentiation*, 2022, 10.1038/s41418-022-01002-2 . hal-03033907v2

**HAL Id: hal-03033907**

**<https://hal.science/hal-03033907v2>**

Submitted on 26 Apr 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 This preprint has not undergone peer review or any post-submission improvements or  
2 corrections. The Version of Record of this article is published in Cell Death and Differentiation,  
3 and is available online at [https://doi.org/\[10.1038/s41418-022-01002-2\]](https://doi.org/[10.1038/s41418-022-01002-2])

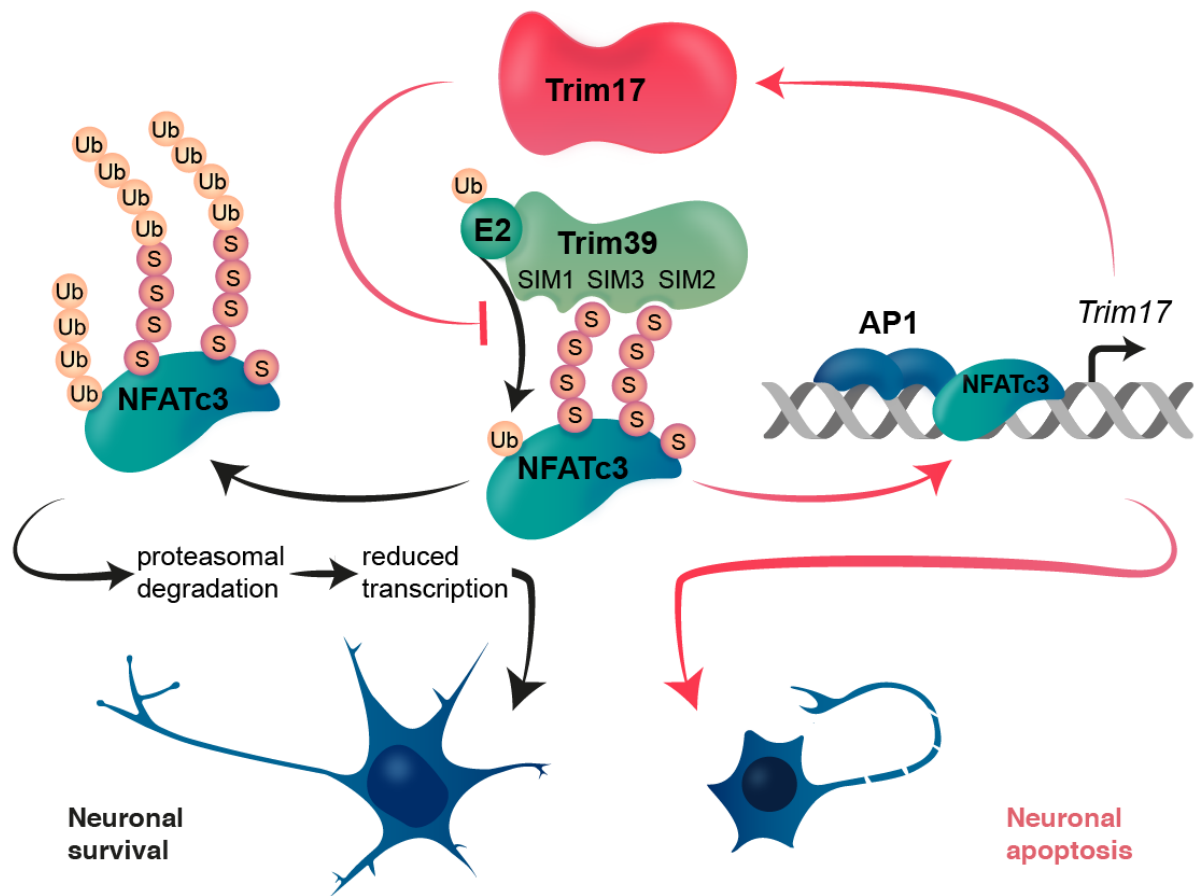
4  
5  
6  
7 Trim39 regulates neuronal apoptosis by acting as a SUMO-targeted  
8 E3 ubiquitin-ligase for the transcription factor NFATc3

9  
10  
11 Meenakshi Basu-Shrivastava<sup>1</sup>, Barbara Mojsa<sup>1,2</sup>, Stéphan Mora<sup>1</sup>, Ian Robbins<sup>1</sup>, Guillaume  
12 Bossis<sup>1</sup>, Iréna Lassot<sup>1</sup> and Solange Desagher<sup>1\*</sup>

13  
14 <sup>1</sup> IGMM, Univ Montpellier, CNRS, Montpellier, France;

15 <sup>2</sup> Centre for Gene Regulation and Expression, School of Life Science, University of Dundee,  
16 Dundee, UK

17 \* For correspondence: [solange.desagher@igmm.cnrs.fr](mailto:solange.desagher@igmm.cnrs.fr)



20

21

## 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  $\alpha$ -  
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 $\beta$  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- $\Delta$ 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- $\Delta$ 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- $\Delta$ 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 ( $\Delta$ RING). Moreover, even  
185 when NFATc3 was not transfected, Trim39- $\Delta$ 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- $\Delta$ 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 ( $\psi$ KXE with  $\psi$  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 $\kappa$ 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<sup>Cdh1</sup>-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 *Sall* 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  $\mu$ 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  $\mu$ 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  $\mu$ g/ml) and poly-D-lysine (33,3  $\mu$ 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 His<sub>6</sub>-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 Ca<sup>2+</sup> and Mg<sup>2+</sup> 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 Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, 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 Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, 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 Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, 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  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ , 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  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ , 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  $\mu\text{M}$  IPTG and was carried out at 20°C overnight  
790 in the presence of 100  $\mu\text{M}$   $\text{ZnCl}_2$  and 200  $\mu\text{M}$   $\text{MgSO}_4$ . 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  $\mu\text{g}/\text{ml}$ ). 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  $\mu\text{M}$   $\text{ZnCl}_2$  and 200  $\mu\text{M}$   $\text{MgSO}_4$ .  
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  $\text{MgSO}_4$ ) 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 MgCl<sub>2</sub>, 2 mM DTT, 10 mM phosphocreatine, 0.5 U creatine kinase, 20 µM ZnCl<sub>2</sub>), 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)<sub>2</sub>, 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  $\beta$ -mercaptoethanol, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml  
880 leupeptin, 1  $\mu$ 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  $\beta$ -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  $\mu$ g of each form of GST-Trim39 were incubated with  $\approx$  1  $\mu$ g SUMO-2 chains for 1 h at room  
891 temperature in 200  $\mu$ l shift assay buffer (20 mM HEPES [pH 7.3], 110 mM KOAc, 2 mM  
892 Mg(OAc)<sub>2</sub>, 0.5 mM EGTA, 1 mM DTT, 0.05% Tween 20, 0.2 mg/ml ovalbumin, 1  $\mu$ g/ml  
893 leupeptin, 1  $\mu$ g/ml aprotinin, 1  $\mu$ 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  $\mu$ 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](http://www.graphpad.com)). Unless stated, data are  
913 representative of at least three independent experiments.

914

915

916 **Acknowledgements:** This work was supported by the Centre National de la Recherche  
917 Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSERM),  
918 the Université de Montpellier, La Fondation de l'Association pour la Recherche contre le  
919 Cancer (ARC, grant number PJA 20141201882 to SD), La Ligue contre le Cancer (grant  
920 number TDUM13665 to BM) and La Fondation pour la Recherche Médicale (grant number  
921 FDT201904008340 to MBS). This article is based upon work from COST Action  
922 (PROTEOSTASIS BM1307), supported by COST (European Cooperation in Science and  
923 Technology). We would like to thank the staff of the Montpellier Genomic Collection platform  
924 for providing human TRIM39 and human TRIM17 cDNA clones. We acknowledge the imaging  
925 facility MRI (Montpellier Ressources Imagerie), member of the national infrastructure France-  
926 BioImaging infrastructure supported by the French National Research Agency (ANR-10-INBS-  
927 04, "Investments for the future"). We are grateful to Frédérique Brockly for the production and  
928 purification of recombinant proteins. We thank Drs Dimitris Liakopoulos and Manuel  
929 Rodriguez for interesting discussions.

930

931 **Competing interests:** The authors declare that no competing interests exist.

932

933

## 934 **References**

935 Abed, M., Barry, K. C., Kenyagin, D., Koltun, B., Phippen, T. M., Delrow, J. J., Parkhurst, S. M., &  
936 Orfan, A. (2011). Degringolade, a SUMO-targeted ubiquitin ligase, inhibits Hairy/Groucho-mediated

- 937 repression. *The EMBO Journal*, 30(7), 1289–1301. <https://doi.org/10.1038/emboj.2011.42>
- 938 Bailey, D., & O'Hare, P. (2005). Comparison of the SUMO1 and ubiquitin conjugation pathways  
939 during the inhibition of proteasome activity with evidence of SUMO1 recycling. *The Biochemical*  
940 *Journal*, 392(Pt 2), 271–281. <https://doi.org/10.1042/BJ20050873>
- 941 Basu-Shrivastava, M., Kozoriz, A., Desagher, S., & Lassot, I. (2021). To Ubiquitinate or Not to  
942 Ubiquitinate: TRIM17 in Cell Life and Death. *Cells*, 10(5). <https://doi.org/10.3390/cells10051235>
- 943 Beauclair, G., Bridier-Nahmias, A., Zagury, J.-F., Saïb, A., & Zamborlini, A. (2015). JASSA: A  
944 comprehensive tool for prediction of SUMOylation sites and SIMs. *Bioinformatics (Oxford, England)*,  
945 31(21), 3483–3491. <https://doi.org/10.1093/bioinformatics/btv403>
- 946 Bossis, G., Chmielarska, K., Gartner, U., Pichler, A., Stieger, E., & Melchior, F. (2005). A  
947 fluorescence resonance energy transfer-based assay to study SUMO modification in solution. *Methods*  
948 *in Enzymology*, 398, 20–32. [https://doi.org/10.1016/S0076-6879\(05\)98003-8](https://doi.org/10.1016/S0076-6879(05)98003-8)
- 949 Boutell, C., Cuchet-Lourenço, D., Vanni, E., Orr, A., Glass, M., McFarlane, S., & Everett, R. D.  
950 (2011). A viral ubiquitin ligase has substrate preferential SUMO targeted ubiquitin ligase activity that  
951 counteracts intrinsic antiviral defence. *PLoS Pathogens*, 7(9), e1002245.  
952 <https://doi.org/10.1371/journal.ppat.1002245>
- 953 Brockly, F., Piechaczyk, M., & Bossis, G. (2016). Production and Purification of Recombinant  
954 SUMOylated Proteins Using Engineered Bacteria. *Methods in Molecular Biology (Clifton, N.J.)*, 1475,  
955 55–65. [https://doi.org/10.1007/978-1-4939-6358-4\\_4](https://doi.org/10.1007/978-1-4939-6358-4_4)
- 956 Caraveo, G., Auluck, P. K., Whitesell, L., Chung, C. Y., Baru, V., Mosharov, E. V., Yan, X., Ben-  
957 Johny, M., Soste, M., Picotti, P., Kim, H., Caldwell, K. A., Caldwell, G. A., Sulzer, D., Yue, D. T., &  
958 Lindquist, S. (2014). Calcineurin determines toxic versus beneficial responses to  $\alpha$ -synuclein.  
959 *Proceedings of the National Academy of Sciences*, 111(34), E3544–E3552.  
960 <https://doi.org/10.1073/pnas.1413201111>
- 961 Chao, C.-N., Lai, C.-H., Badrealam, K. F., Lo, J.-F., Shen, C.-Y., Chen, C.-H., Chen, R.-J.,  
962 Viswanadha, V. P., Kuo, W.-W., & Huang, C.-Y. (2019). CHIP attenuates lipopolysaccharide-induced  
963 cardiac hypertrophy and apoptosis by promoting NFATc3 proteasomal degradation. *Journal of*  
964 *Cellular Physiology*, 234(11), 20128–20138. <https://doi.org/10.1002/jcp.28614>
- 965 Contestabile, A. (2002). Cerebellar granule cells as a model to study mechanisms of neuronal  
966 apoptosis or survival in vivo and in vitro. *Cerebellum (London, England)*, 1(1), 41–55.  
967 <https://doi.org/10.1080/147342202753203087>
- 968 D'Mello, S. R., Galli, C., Ciotti, T., & Calissano, P. (1993). Induction of apoptosis in cerebellar  
969 granule neurons by low potassium: Inhibition of death by insulin-like growth factor I and cAMP.  
970 *Proceedings of the National Academy of Sciences of the United States of America*, 90(23), 10989–  
971 10993. <https://doi.org/10.1073/pnas.90.23.10989>
- 972 Erker, Y., Neyret-Kahn, H., Seeler, J. S., Dejean, A., Atfi, A., & Levy, L. (2013). Arkadia, a novel  
973 SUMO-targeted ubiquitin ligase involved in PML degradation. *Molecular and Cellular Biology*,  
974 33(11), 2163–2177. <https://doi.org/10.1128/MCB.01019-12>
- 975 Esposito, D., Koliopoulos, M. G., & Rittinger, K. (2017). Structural determinants of TRIM protein  
976 function. *Biochemical Society Transactions*, 45(1), 183–191. <https://doi.org/10.1042/BST20160325>
- 977 Fric, J., Zelante, T., Wong, A. Y. W., Mertes, A., Yu, H.-B., & Ricciardi-Castagnoli, P. (2012). NFAT  
978 control of innate immunity. *Blood*, 120(7), 1380–1389. <https://doi.org/10.1182/blood-2012-02-404475>
- 979 Geoffroy, M. C., & Hay, R. T. (2009). An additional role for SUMO in ubiquitin-mediated  
980 proteolysis. *Nat Rev Mol Cell Biol*, 10(8), 564–568. <https://doi.org/10.1038/nrm2707>
- 981 Geoffroy, M.-C., & Hay, R. T. (2009). An additional role for SUMO in ubiquitin-mediated  
982 proteolysis. *Nature Reviews. Molecular Cell Biology*, 10(8), 564–568.



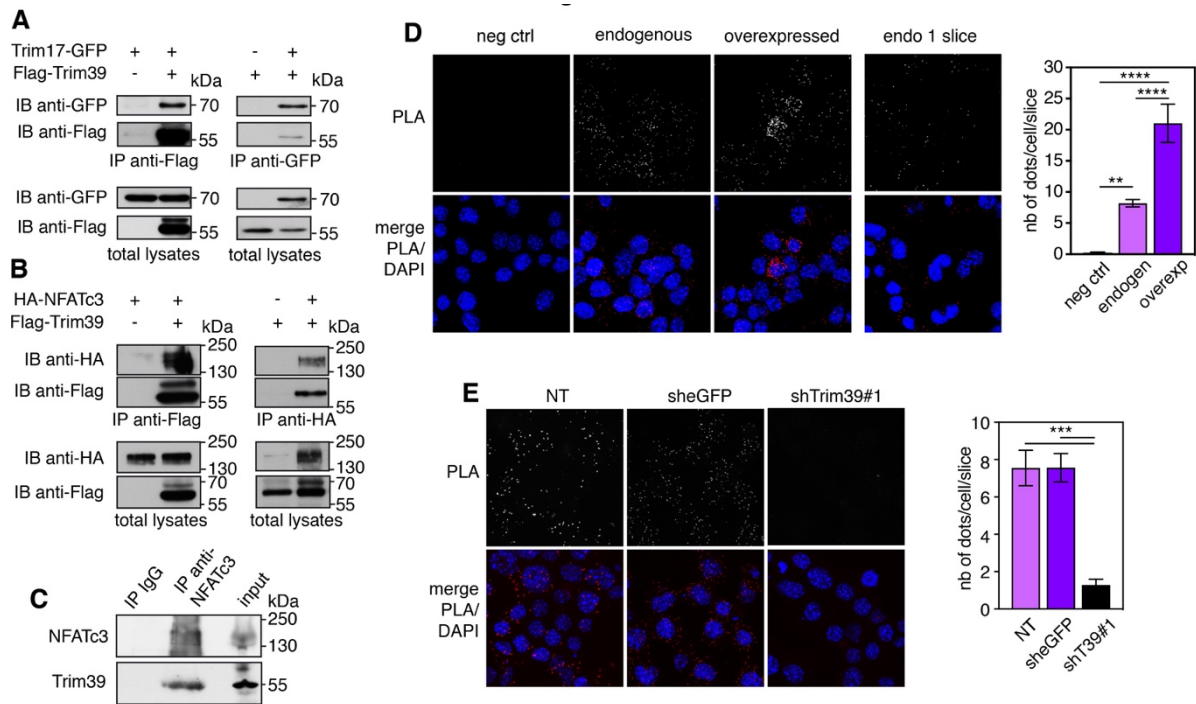
- 983 <https://doi.org/10.1038/nrm2707>
- 984 Hao, Y.-H., Fountain, M. D., Fon Tacer, K., Xia, F., Bi, W., Kang, S.-H. L., Patel, A., Rosenfeld, J.  
 985 A., Le Caignec, C., Isidor, B., Krantz, I. D., Noon, S. E., Pfotenhauer, J. P., Morgan, T. M., Moran, R.,  
 986 Pedersen, R. C., Saenz, M. S., Schaaf, C. P., & Potts, P. R. (2015). USP7 Acts as a Molecular Rheostat  
 987 to Promote WASH-Dependent Endosomal Protein Recycling and Is Mutated in a Human  
 988 Neurodevelopmental Disorder. *Molecular Cell*, *59*(6), 956–969.  
 989 <https://doi.org/10.1016/j.molcel.2015.07.033>
- 990 He, X., Riceberg, J., Soucy, T., Koenig, E., Minissale, J., Gallery, M., Bernard, H., Yang, X., Liao, H.,  
 991 Rabino, C., Shah, P., Xega, K., Yan, Z.-H., Sintchak, M., Bradley, J., Xu, H., Duffey, M., England, D.,  
 992 Mizutani, H., ... Pulukuri, S. M. (2017). Probing the roles of SUMOylation in cancer cell biology by  
 993 using a selective SAE inhibitor. *Nature Chemical Biology*, *13*(11), 1164–1171.  
 994 <https://doi.org/10.1038/nchembio.2463>
- 995 Henley, J. M., Carmichael, R. E., & Wilkinson, K. A. (2018). Extranuclear SUMOylation in Neurons.  
 996 *Trends in Neurosciences*, *41*(4), 198–210. <https://doi.org/10.1016/j.tins.2018.02.004>
- 997 Hogan, P. G., Chen, L., Nardone, J., & Rao, A. (2003). Transcriptional regulation by calcium,  
 998 calcineurin, and NFAT. *Genes and Development*, *17*(18), 2205–2232.  
 999 <https://doi.org/10.1101/gad.1102703> 17/18/2205 [pii]
- 1000 Hu, J., Ding, X., Tian, S., Chu, Y., Liu, Z., Li, Y., Li, X., Wang, G., Wang, L., & Wang, Z. (2021).  
 1001 TRIM39 deficiency inhibits tumor progression and autophagic flux in colorectal cancer via  
 1002 suppressing the activity of Rab7. *Cell Death & Disease*, *12*(4), 391. <https://doi.org/10.1038/s41419-021-03670-3>
- 1004 Huang, N. J., Zhang, L., Tang, W., Chen, C., Yang, C. S., & Kornbluth, S. (2012). The Trim39  
 1005 ubiquitin ligase inhibits APC/CCdh1-mediated degradation of the Bax activator MOAP-1. *Journal of*  
 1006 *Cell Biology*, *197*(3), 361–367. <https://doi.org/10.1083/jcb.201108062> 10.1083/jcb.201111141
- 1007 Ikonomidou, C., Bosch, F., Miksa, M., Bittigau, P., Vöckler, J., Dikranian, K., Tenkova, T. I.,  
 1008 Stefovskaja, V., Turski, L., & Olney, J. W. (1999). Blockade of NMDA receptors and apoptotic  
 1009 neurodegeneration in the developing brain. *Science (New York, N.Y.)*, *283*(5398), 70–74.  
 1010 <https://doi.org/10.1126/science.283.5398.70>
- 1011 Jansen, N. S., & Vertegaal, A. C. O. (2021). A Chain of Events: Regulating Target Proteins by SUMO  
 1012 Polymers. *Trends in Biochemical Sciences*, *46*(2), 113–123. <https://doi.org/10.1016/j.tibs.2020.09.002>
- 1013 Kerscher, O. (2007). SUMO junction-what's your function? New insights through SUMO-interacting  
 1014 motifs. *EMBO Reports*, *8*(6), 550–555. <https://doi.org/10.1038/sj.embor.7400980>
- 1015 Kim, E. T., Kwon, K. M., Lee, M. K., Park, J., & Ahn, J.-H. (2019). Sumoylation of a small isoform  
 1016 of NFATc1 is promoted by PIAS proteins and inhibits transactivation activity. *Biochemical and*  
 1017 *Biophysical Research Communications*, *513*(1), 172–178. <https://doi.org/10.1016/j.bbrc.2019.03.171>
- 1018 Kim, J. H., Kim, K., Jin, H. M., Song, I., Youn, B. U., Lee, S. H., Choi, Y., & Kim, N. (2010).  
 1019 Negative feedback control of osteoclast formation through ubiquitin-mediated down-regulation of  
 1020 NFATc1. *Journal of Biological Chemistry*, *285*(8), 5224–5231.  
 1021 <https://doi.org/10.1074/jbc.M109.042812>
- 1022 Kim, M. S., & Usachev, Y. M. (2009). Mitochondrial Ca<sup>2+</sup> cycling facilitates activation of the  
 1023 transcription factor NFAT in sensory neurons. *Journal of Neuroscience*, *29*(39), 12101–12114.  
 1024 <https://doi.org/10.1523/JNEUROSCI.3384-09.2009>
- 1025 Kipanyula, M. J., Kimaro, W. H., & Seke Etet, P. F. (2016). The Emerging Roles of the Calcineurin-  
 1026 Nuclear Factor of Activated T-Lymphocytes Pathway in Nervous System Functions and Diseases.  
 1027 *Journal of Aging Research*, *2016*, 5081021. <https://doi.org/10.1155/2016/5081021>
- 1028 Koliopoulos, M. G., Esposito, D., Christodoulou, E., Taylor, I. A., & Rittinger, K. (2016). Functional  
 1029 role of TRIM E3 ligase oligomerization and regulation of catalytic activity. *EMBO Journal*.

- 1030 <https://doi.org/10.15252/embj.201593741>
- 1031 Lassot, I., Robbins, I., Kristiansen, M., Rahmeh, R., Jaudon, F., Magiera, M. M., Mora, S., Vanhille,  
1032 L., Lipkin, A., Pettmann, B., Ham, J., & Desagher, S. (2010). Trim17, a novel E3 ubiquitin-ligase,  
1033 initiates neuronal apoptosis. *Cell Death and Differentiation*, 17(12), 1928–1941.  
1034 <https://doi.org/10.1038/cdd.2010.73>
- 1035 Lassot, Iréna, Mora, S., Lesage, S., Zieba, B. A., Coque, E., Condroyer, C., Bossowski, J. P., Mojsa,  
1036 B., Marelli, C., Soulet, C., Tesson, C., Carballo-Carbajal, I., Laguna, A., Mangone, G., Vila, M.,  
1037 Brice, A., & Desagher, S. (2018). The E3 Ubiquitin Ligases TRIM17 and TRIM41 Modulate  $\alpha$ -  
1038 Synuclein Expression by Regulating ZSCAN21. *Cell Reports*, 25(9), 2484–2496.e9.  
1039 <https://doi.org/10.1016/j.celrep.2018.11.002>
- 1040 Lee, J.-U., Kim, L.-K., & Choi, J.-M. (2018). Revisiting the Concept of Targeting NFAT to Control T  
1041 Cell Immunity and Autoimmune Diseases. *Frontiers in Immunology*, 9, 2747.  
1042 <https://doi.org/10.3389/fimmu.2018.02747>
- 1043 Lee, S. S., Fu, N. Y., Sukumaran, S. K., Wan, K. F., Wan, Q., & Yu, V. C. (2009). TRIM39 is a  
1044 MOAP-1-binding protein that stabilizes MOAP-1 through inhibition of its poly-ubiquitination process.  
1045 *Experimental Cell Research*, 315(7), 1313–1325. [https://doi.org/S0014-4827\(08\)00511-9](https://doi.org/S0014-4827(08)00511-9) [pii]  
1046 10.1016/j.yexcr.2008.11.021
- 1047 Li, X., Wei, W., Huynh, H., Zuo, H., Wang, X., & Wan, Y. (2015). Nur77 prevents excessive  
1048 osteoclastogenesis by inducing ubiquitin ligase Cbl-b to mediate NFATc1 self-limitation. *ELife*, 4,  
1049 e07217. <https://doi.org/10.7554/eLife.07217>
- 1050 Li, Y., Wu, H., Wu, W., Zhuo, W., Liu, W., Zhang, Y., Cheng, M., Chen, Y. G., Gao, N., Yu, H.,  
1051 Wang, L., Li, W., & Yang, M. (2014). Structural insights into the TRIM family of ubiquitin E3  
1052 ligases. *Cell Research*, 24(6), 762–765. <https://doi.org/10.1038/cr.2014.46>
- 1053 Liebelt, F., & Vertegaal, A. C. O. (2016). Ubiquitin-dependent and independent roles of SUMO in  
1054 proteostasis. *American Journal of Physiology. Cell Physiology*, 311(2), C284–296.  
1055 <https://doi.org/10.1152/ajpcell.00091.2016>
- 1056 Lionnard, L., Duc, P., Brennan, M. S., Kueh, A. J., Pal, M., Guardia, F., Mojsa, B., Damiano, M.-A.,  
1057 Mora, S., Lassot, I., Ravichandran, R., Cochet, C., Aouacheria, A., Potts, P. R., Herold, M. J.,  
1058 Desagher, S., & Kucharczak, J. (2019). TRIM17 and TRIM28 antagonistically regulate the  
1059 ubiquitination and anti-apoptotic activity of BCL2A1. *Cell Death and Differentiation*, 26(5), 902–917.  
1060 <https://doi.org/10.1038/s41418-018-0169-5>
- 1061 Luo, J., Sun, L., Lin, X., Liu, G., Yu, J., Parisiadou, L., Xie, C., Ding, J., & Cai, H. (2014). A  
1062 calcineurin- and NFAT-dependent pathway is involved in  $\alpha$ -synuclein-induced degeneration of  
1063 midbrain dopaminergic neurons. *Human Molecular Genetics*, 23(24), 6567–6574.  
1064 <https://doi.org/10.1093/hmg/ddu377>
- 1065 Lutfalla, G., & Uze, G. (2006). Performing quantitative reverse-transcribed polymerase chain reaction  
1066 experiments. *Methods in Enzymology*, 410, 386–400. [https://doi.org/10.1016/S0076-6879\(06\)10019-1](https://doi.org/10.1016/S0076-6879(06)10019-1)
- 1067 Magiera, M. M., Mora, S., Mojsa, B., Robbins, I., Lassot, I., & Desagher, S. (2013). Trim17-mediated  
1068 ubiquitination and degradation of Mcl-1 initiate apoptosis in neurons. *Cell Death and Differentiation*,  
1069 20(2), 281–292. <https://doi.org/10.1038/cdd.2012.124>
- 1070 Meroni, G., & Diez-Roux, G. (2005). TRIM/RBCC, a novel class of “single protein RING finger” E3  
1071 ubiquitin ligases. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology*,  
1072 27(11), 1147–1157. <https://doi.org/10.1002/bies.20304>
- 1073 Mognol, G. P., Carneiro, F. R. G., Robbs, B. K., Faget, D. V., & Viola, J. P. B. (2016). Cell cycle and  
1074 apoptosis regulation by NFAT transcription factors: New roles for an old player. *Cell Death &*  
1075 *Disease*, 7, e2199. <https://doi.org/10.1038/cddis.2016.97>
- 1076 Mojsa, B., Mora, S., Bossowski, J. P., Lassot, I., & Desagher, S. (2015). Control of neuronal apoptosis

- 1077 by reciprocal regulation of NFATc3 and Trim17. *Cell Death and Differentiation*, 22(2), 274–286.  
 1078 <https://doi.org/10.1038/cdd.2014.141>
- 1079 Müller, M. R., & Rao, A. (2010). NFAT, immunity and cancer: A transcription factor comes of age.  
 1080 *Nature Reviews. Immunology*, 10(9), 645–656. <https://doi.org/10.1038/nri2818>
- 1081 Napolitano, L. M., & Meroni, G. (2012). TRIM family: Pleiotropy and diversification through  
 1082 homomultimer and heteromultimer formation. *IUBMB Life*, 64(1), 64–71.  
 1083 <https://doi.org/10.1002/iub.580>
- 1084 Narahara, S., Sakai, E., Kadowaki, T., Yamaguchi, Y., Narahara, H., Okamoto, K., Asahina, I., &  
 1085 Tsukuba, T. (2019). KBTBD11, a novel BTB-Kelch protein, is a negative regulator of  
 1086 osteoclastogenesis through controlling Cullin3-mediated ubiquitination of NFATc1. *Scientific*  
 1087 *Reports*, 9(1), 3523. <https://doi.org/10.1038/s41598-019-40240-2>
- 1088 Nayak, A., Glockner-Pagel, J., Vaeth, M., Schumann, J. E., Buttmann, M., Bopp, T., Schmitt, E.,  
 1089 Serfling, E., & Berberich-Siebelt, F. (2009). Sumoylation of the transcription factor NFATc1 leads to  
 1090 its subnuclear relocalization and interleukin-2 repression by histone deacetylase. *Journal of Biological*  
 1091 *Chemistry*, 284(16), 10935–10946. <https://doi.org/M900465200> [pii] 10.1074/jbc.M900465200
- 1092 Nicklas, S., Hillje, A.-L., Okawa, S., Rudolph, I.-M., Collmann, F. M., van Wuellen, T., Del Sol, A.,  
 1093 & Schwamborn, J. C. (2019). A complex of the ubiquitin ligase TRIM32 and the deubiquitinase USP7  
 1094 balances the level of c-Myc ubiquitination and thereby determines neural stem cell fate specification.  
 1095 *Cell Death and Differentiation*, 26(4), 728–740. <https://doi.org/10.1038/s41418-018-0144-1>
- 1096 Parker, J. L., & Ulrich, H. D. (2012). A SUMO-interacting motif activates budding yeast ubiquitin  
 1097 ligase Rad18 towards SUMO-modified PCNA. *Nucleic Acids Research*, 40(22), 11380–11388.  
 1098 <https://doi.org/10.1093/nar/gks892>
- 1099 Pichler, A., Fatouros, C., Lee, H., & Eisenhardt, N. (2017). SUMO conjugation—A mechanistic view.  
 1100 *Biomolecular Concepts*, 8(1), 13–36. <https://doi.org/10.1515/bmc-2016-0030>
- 1101 Pickart, C. M. (2001). Mechanisms underlying ubiquitination. *Annual Review of Biochemistry*, 70,  
 1102 503–533. <https://doi.org/10.1146/annurev.biochem.70.1.503>
- 1103 Prudden, J., Pebernard, S., Raffa, G., Slavin, D. A., Perry, J. J. P., Tainer, J. A., McGowan, C. H., &  
 1104 Boddy, M. N. (2007). SUMO-targeted ubiquitin ligases in genome stability. *The EMBO Journal*,  
 1105 26(18), 4089–4101. <https://doi.org/10.1038/sj.emboj.7601838>
- 1106 Qiu, S., Liu, H., Jian, Z., Fan, Z., Liu, S., Xing, J., & Li, J. (2020). Characterization of the primate  
 1107 TRIM gene family reveals the recent evolution in primates. *Molecular Genetics and Genomics: MGG*,  
 1108 295(5), 1281–1294. <https://doi.org/10.1007/s00438-020-01698-2>
- 1109 Reymond, A., Meroni, G., Fantozzi, A., Merla, G., Cairo, S., Luzi, L., Riganelli, D., Zanaria, E.,  
 1110 Messali, S., Cainarca, S., Guffanti, A., Minucci, S., Pelicci, P. G., & Ballabio, A. (2001). The tripartite  
 1111 motif family identifies cell compartments. *The EMBO Journal*, 20(9), 2140–2151.  
 1112 <https://doi.org/10.1093/emboj/20.9.2140>
- 1113 Ristic, M., Brockly, F., Piechaczyk, M., & Bossis, G. (2016). Detection of Protein-Protein Interactions  
 1114 and Posttranslational Modifications Using the Proximity Ligation Assay: Application to the Study of  
 1115 the SUMO Pathway. *Methods in Molecular Biology (Clifton, N.J.)*, 1449, 279–290.  
 1116 [https://doi.org/10.1007/978-1-4939-3756-1\\_17](https://doi.org/10.1007/978-1-4939-3756-1_17)
- 1117 Rodriguez, M. S., Dargemont, C., & Hay, R. T. (2001). SUMO-1 conjugation in vivo requires both a  
 1118 consensus modification motif and nuclear targeting. *The Journal of Biological Chemistry*, 276(16),  
 1119 12654–12659. <https://doi.org/10.1074/jbc.M009476200>
- 1120 Rolland, T., Taşan, M., Charlotiaux, B., Pevzner, S. J., Zhong, Q., Sahni, N., Yi, S., Lemmens, I.,  
 1121 Fontanillo, C., Mosca, R., Kamburov, A., Ghiassian, S. D., Yang, X., Ghamsari, L., Balcha, D., Begg,  
 1122 B. E., Braun, P., Brehme, M., Broly, M. P., ... Vidal, M. (2014). A proteome-scale map of the human  
 1123 interactome network. *Cell*, 159(5), 1212–1226. <https://doi.org/10.1016/j.cell.2014.10.050>

- 1124 Rual, J.-F., Venkatesan, K., Hao, T., Hirozane-Kishikawa, T., Dricot, A., Li, N., Berriz, G. F.,  
 1125 Gibbons, F. D., Dreze, M., Ayivi-Guedehoussou, N., Klitgord, N., Simon, C., Boxem, M., Milstein,  
 1126 S., Rosenberg, J., Goldberg, D. S., Zhang, L. V., Wong, S. L., Franklin, G., ... Vidal, M. (2005).  
 1127 Towards a proteome-scale map of the human protein-protein interaction network. *Nature*, *437*(7062),  
 1128 1173–1178. <https://doi.org/10.1038/nature04209>
- 1129 Sanchez, J. G., Okreglicka, K., Chandrasekaran, V., Welker, J. M., Sundquist, W. I., & Pornillos, O.  
 1130 (2014). The tripartite motif coiled-coil is an elongated antiparallel hairpin dimer. *Proceedings of the*  
 1131 *National Academy of Sciences of the United States of America*, *111*(7), 2494–2499.  
 1132 <https://doi.org/10.1073/pnas.1318962111>
- 1133 Shaw, J. P., Utz, P. J., Durand, D. B., Toole, J. J., Emmel, E. A., & Crabtree, G. R. (1988).  
 1134 Identification of a putative regulator of early T cell activation genes. *Science (New York, N.Y.)*,  
 1135 *241*(4862), 202–205. <https://doi.org/10.1126/science.3260404>
- 1136 Short, K. M., & Cox, T. C. (2006). Subclassification of the RBCC/TRIM superfamily reveals a novel  
 1137 motif necessary for microtubule binding. *The Journal of Biological Chemistry*, *281*(13), 8970–8980.  
 1138 <https://doi.org/10.1074/jbc.M512755200>
- 1139 Singh, S. K., Baumgart, S., Singh, G., Konig, A. O., Reutlinger, K., Hofbauer, L. C., Barth, P., Gress,  
 1140 T. M., Lomber, G., Urrutia, R., Fernandez-Zapico, M. E., & Ellenrieder, V. (2011). Disruption of a  
 1141 nuclear NFATc2 protein stabilization loop confers breast and pancreatic cancer growth suppression by  
 1142 zoledronic acid. *Journal of Biological Chemistry*, *286*(33), 28761–28771.  
 1143 <https://doi.org/10.1074/jbc.M110.197533>
- 1144 Song, K.-H., Choi, C. H., Lee, H.-J., Oh, S. J., Woo, S. R., Hong, S.-O., Noh, K. H., Cho, H., Chung,  
 1145 E. J., Kim, J.-H., Chung, J.-Y., Hewitt, S. M., Baek, S., Lee, K.-M., Yee, C., Son, M., Mao, C.-P., Wu,  
 1146 T. C., & Kim, T. W. (2017). HDAC1 Upregulation by NANOG Promotes Multidrug Resistance and a  
 1147 Stem-like Phenotype in Immune Edited Tumor Cells. *Cancer Research*, *77*(18), 5039–5053.  
 1148 <https://doi.org/10.1158/0008-5472.CAN-17-0072>
- 1149 Sriramachandran, A. M., & Dohmen, R. J. (2014). SUMO-targeted ubiquitin ligases. *Biochimica et*  
 1150 *Biophysica Acta*, *1843*(1), 75–85. <https://doi.org/10.1016/j.bbamcr.2013.08.022>
- 1151 Streich, F. C., Ronchi, V. P., Connick, J. P., & Haas, A. L. (2013). Tripartite motif ligases catalyze  
 1152 polyubiquitin chain formation through a cooperative allosteric mechanism. *The Journal of Biological*  
 1153 *Chemistry*, *288*(12), 8209–8221. <https://doi.org/10.1074/jbc.M113.451567>
- 1154 Suzuki, M., Watanabe, M., Nakamaru, Y., Takagi, D., Takahashi, H., Fukuda, S., & Hatakeyama, S.  
 1155 (2016). TRIM39 negatively regulates the NFκB-mediated signaling pathway through stabilization of  
 1156 Cactin. *Cellular and Molecular Life Sciences: CMLS*, *73*(5), 1085–1101.  
 1157 <https://doi.org/10.1007/s00018-015-2040-x>
- 1158 Terui, Y., Saad, N., Jia, S., McKeon, F., & Yuan, J. (2004). Dual role of sumoylation in the nuclear  
 1159 localization and transcriptional activation of NFAT1. *Journal of Biological Chemistry*, *279*(27),  
 1160 28257–28265. <https://doi.org/10.1074/jbc.M403153200> M403153200 [pii]
- 1161 Ulrich, J. D., Kim, M. S., Houlihan, P. R., Shutov, L. P., Mohapatra, D. P., Strack, S., & Usachev, Y.  
 1162 M. (2012). Distinct activation properties of the nuclear factor of activated T-cells (NFAT) isoforms  
 1163 NFATc3 and NFATc4 in neurons. *Journal of Biological Chemistry*, *287*(45), 37594–37609.  
 1164 <https://doi.org/10.1074/jbc.M112.365197>
- 1165 Urano, T., Usui, T., Takeda, S., Ikeda, K., Okada, A., Ishida, Y., Iwayanagi, T., Otomo, J., Ouchi, Y.,  
 1166 & Inoue, S. (2009). TRIM44 interacts with and stabilizes terf, a TRIM ubiquitin E3 ligase.  
 1167 *Biochemical and Biophysical Research Communications*, *383*(2), 263–268. [https://doi.org/S0006-](https://doi.org/S0006-291X(09)00685-8)  
 1168 [291X\(09\)00685-8](https://doi.org/S0006-291X(09)00685-8) [pii] 10.1016/j.bbrc.2009.04.010
- 1169 Uzunova, K., Götsche, K., Miteva, M., Weisshaar, S. R., Glanemann, C., Schnellhardt, M., Niessen,  
 1170 M., Scheel, H., Hofmann, K., Johnson, E. S., Praefcke, G. J. K., & Dohmen, R. J. (2007). Ubiquitin-  
 1171 dependent proteolytic control of SUMO conjugates. *The Journal of Biological Chemistry*, *282*(47),

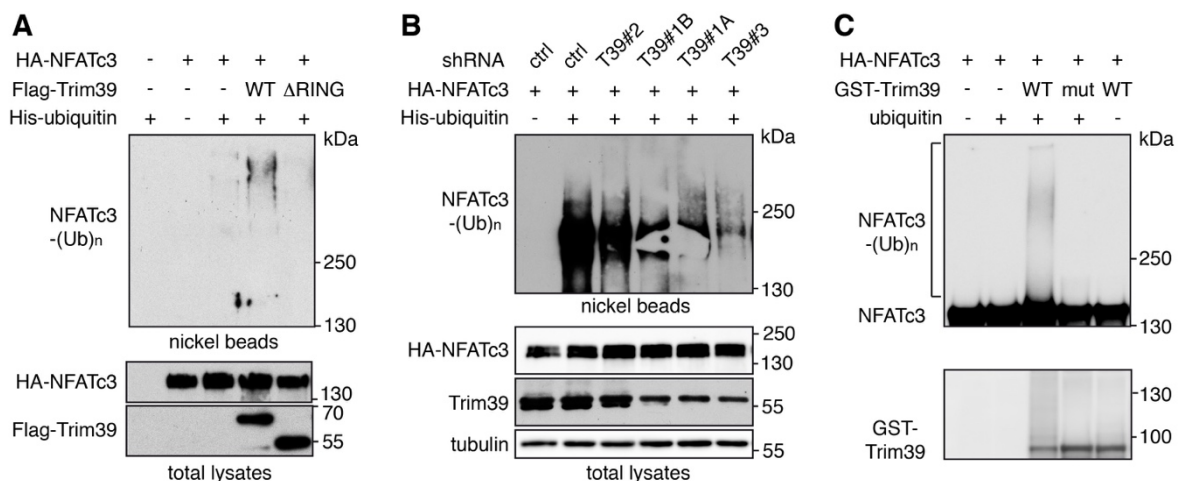
- 1172 34167–34175. <https://doi.org/10.1074/jbc.M706505200>
- 1173 Vashishta, A., Habas, A., Pruunsild, P., Zheng, J. J., Timmusk, T., & Hetman, M. (2009). Nuclear  
1174 factor of activated T-cells isoform c4 (NFATc4/NFAT3) as a mediator of antiapoptotic transcription in  
1175 NMDA receptor-stimulated cortical neurons. *Journal of Neuroscience*, *29*(48), 15331–15340.  
1176 <https://doi.org/29/48/15331> [pii] 10.1523/JNEUROSCI.4873-09.2009
- 1177 Vihma, H., & Timmusk, T. (2017). Sumoylation regulates the transcriptional activity of different  
1178 human NFAT isoforms in neurons. *Neuroscience Letters*, *653*, 302–307.  
1179 <https://doi.org/10.1016/j.neulet.2017.05.074>
- 1180 Wang, L., Oliver, S. L., Sommer, M., Rajamani, J., Reichelt, M., & Arvin, A. M. (2011). Disruption of  
1181 PML nuclear bodies is mediated by ORF61 SUMO-interacting motifs and required for varicella-zoster  
1182 virus pathogenesis in skin. *PLoS Pathogens*, *7*(8), e1002157.  
1183 <https://doi.org/10.1371/journal.ppat.1002157>
- 1184 Wood, K. A., Dipasquale, B., & Youle, R. J. (1993). In situ labeling of granule cells for apoptosis-  
1185 associated DNA fragmentation reveals different mechanisms of cell loss in developing cerebellum.  
1186 *Neuron*, *11*(4), 621–632. [https://doi.org/10.1016/0896-6273\(93\)90074-2](https://doi.org/10.1016/0896-6273(93)90074-2)
- 1187 Woodsmith, J., Jenn, R. C., & Sanderson, C. M. (2012). Systematic analysis of dimeric E3-RING  
1188 interactions reveals increased combinatorial complexity in human ubiquitination networks. *Molecular  
1189 & Cellular Proteomics: MCP*, *11*(7), M111.016162. <https://doi.org/10.1074/mcp.M111.016162>
- 1190 Wu, H., Peisley, A., Graef, I. A., & Crabtree, G. R. (2007). NFAT signaling and the invention of  
1191 vertebrates. *Trends in Cell Biology*, *17*(6), 251–260. [https://doi.org/S0962-8924\(07\)00103-1](https://doi.org/S0962-8924(07)00103-1) [pii]  
1192 10.1016/j.tcb.2007.04.006
- 1193 Yau, T.-Y., Molina, O., & Courey, A. J. (2020). SUMOylation in development and neurodegeneration.  
1194 *Development (Cambridge, England)*, *147*(6). <https://doi.org/10.1242/dev.175703>
- 1195 Yoeli-Lerner, M., Yiu, G. K., Rabinovitz, I., Erhardt, P., Jauliac, S., & Toker, A. (2005). Akt blocks  
1196 breast cancer cell motility and invasion through the transcription factor NFAT. *Molecular Cell*, *20*(4),  
1197 539–550. <https://doi.org/10.1016/j.molcel.2005.10.033>
- 1198 Youn, M.-Y., Yokoyama, A., Fujiyama-Nakamura, S., Ohtake, F., Minehata, K., Yasuda, H., Suzuki,  
1199 T., Kato, S., & Imai, Y. (2012). JMJD5, a Jumonji C (JmjC) domain-containing protein, negatively  
1200 regulates osteoclastogenesis by facilitating NFATc1 protein degradation. *The Journal of Biological  
1201 Chemistry*, *287*(16), 12994–13004. <https://doi.org/10.1074/jbc.M111.323105>
- 1202 Yudina, Z., Roa, A., Johnson, R., Biris, N., de Souza Aranha Vieira, D. A., Tshiperson, V., Reszka, N.,  
1203 Taylor, A. B., Hart, P. J., Demeler, B., Diaz-Griffero, F., & Ivanov, D. N. (2015). RING Dimerization  
1204 Links Higher-Order Assembly of TRIM5 $\alpha$  to Synthesis of K63-Linked Polyubiquitin. *Cell Reports*,  
1205 *12*(5), 788–797. <https://doi.org/10.1016/j.celrep.2015.06.072>
- 1206 Zhang, L., Huang, N. J., Chen, C., Tang, W., & Kornbluth, S. (2012). Ubiquitylation of p53 by the  
1207 APC/C inhibitor Trim39. *Proceedings of the National Academy of Sciences of the United States of  
1208 America*, *109*(51), 20931–20936. <https://doi.org/10.1073/pnas.1212047110>
- 1209 Zhang, L., Mei, Y., Fu, N. Y., Guan, L., Xie, W., Liu, H. H., Yu, C. D., Yin, Z., Yu, V. C., & You, H.  
1210 (2012). TRIM39 regulates cell cycle progression and DNA damage responses via stabilizing p21.  
1211 *Proceedings of the National Academy of Sciences of the United States of America*, *109*(51), 20937–  
1212 20942. <https://doi.org/10.1073/pnas.1214156110>
- 1213 Zhao, Q., Xie, Y., Zheng, Y., Jiang, S., Liu, W., Mu, W., Liu, Z., Zhao, Y., Xue, Y., & Ren, J. (2014).  
1214 GPS-SUMO: A tool for the prediction of sumoylation sites and SUMO-interaction motifs. *Nucleic  
1215 Acids Research*, *42*(Web Server issue), W325–330. <https://doi.org/10.1093/nar/gku383>
- 1216 Zhao, X. (2018). SUMO-Mediated Regulation of Nuclear Functions and Signaling Processes.  
1217 *Molecular Cell*, *71*(3), 409–418. <https://doi.org/10.1016/j.molcel.2018.07.027>

1219  
1220

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  $\mu$ 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  $\mu$ 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).

1253

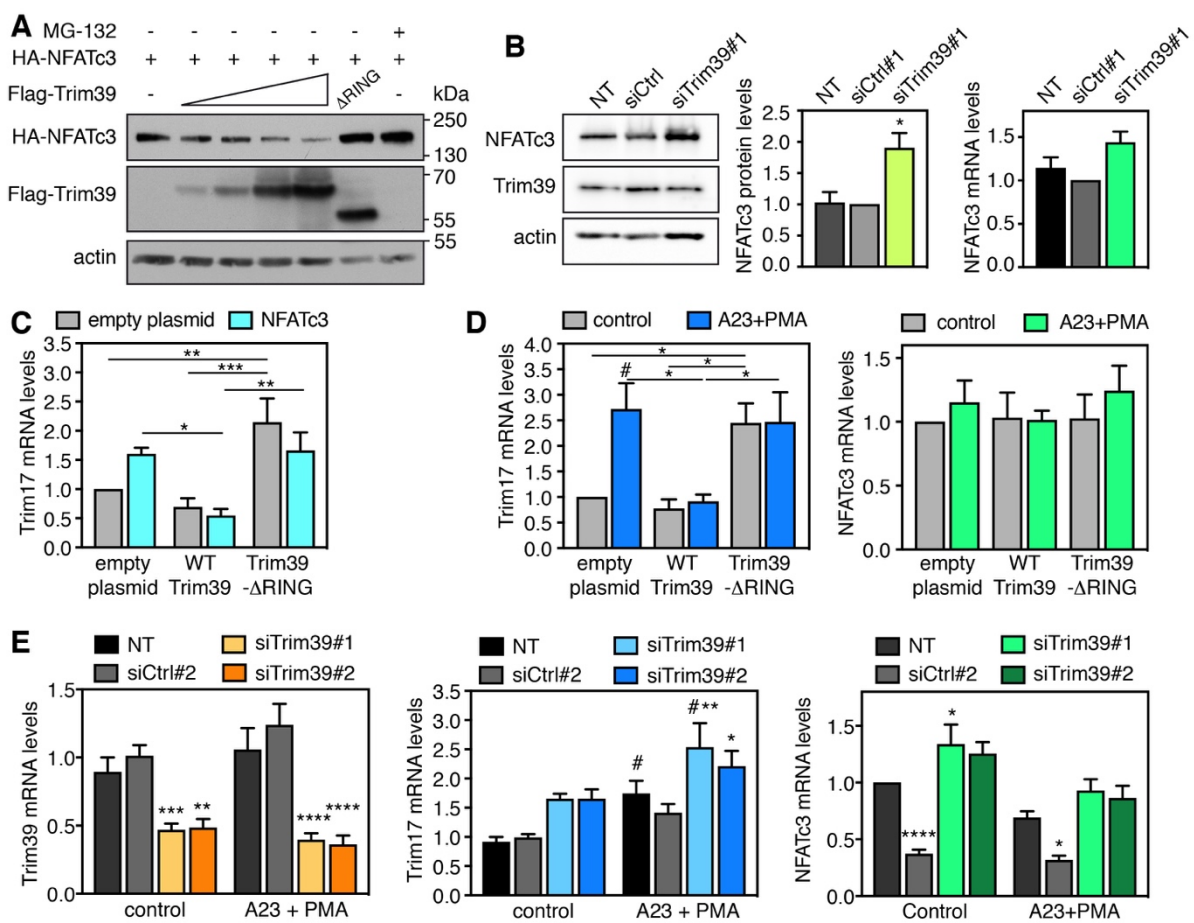


1254  
 1255

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- $\Delta$ RING for 24 h. Cells were then  
 1259 incubated with 20  $\mu$ 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.  
 1278



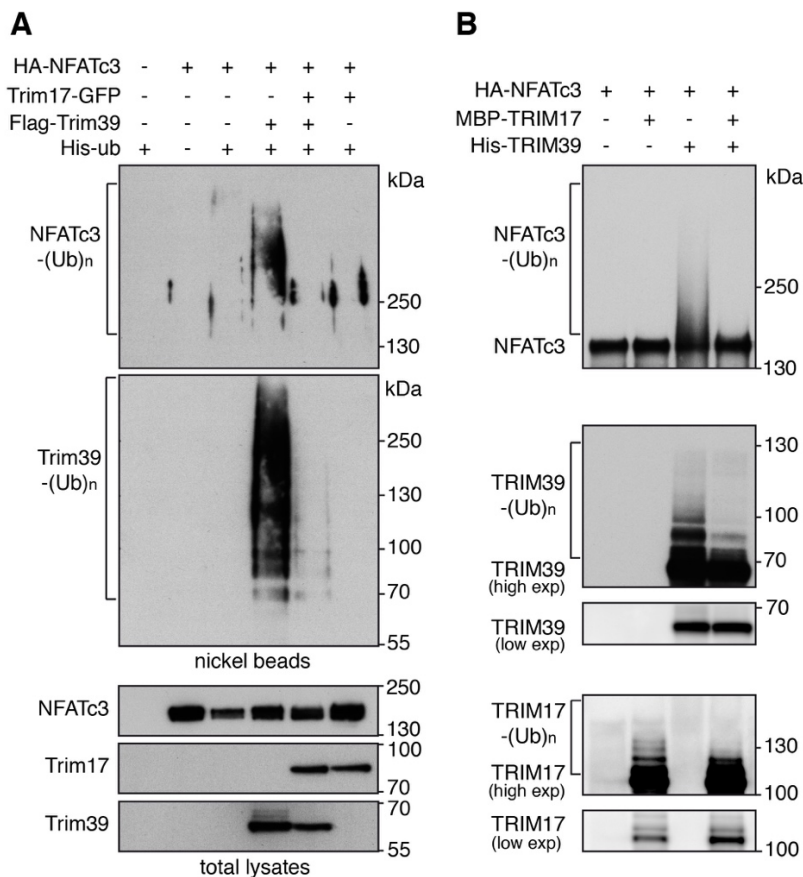
1279  
 1280



1281 **Figure 3. Trim39 mediates NFATc3 degradation.** **A.** BHK cells were transfected with a fixed  
1282 amount of a HA-NFATc3 expressing vector (1  $\mu$ g) together with empty plasmid (-) or  
1283 increasing amounts of Flag-Trim39 expressing vector (0.1, 0.2, 0.5 and 1  $\mu$ g) or 0.2  $\mu$ g of a  
1284 vector expressing the inactive mutant Flag-Trim39- $\Delta$ RING. When indicated, the cells were  
1285 treated with 10  $\mu$ 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- $\Delta$ 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- $\Delta$ RING for 24 h. Then, cells were left untreated (control) or were  
1301 deprived of serum for 3 h and subsequently treated with 1  $\mu$ 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  $\mu$ 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.

1324

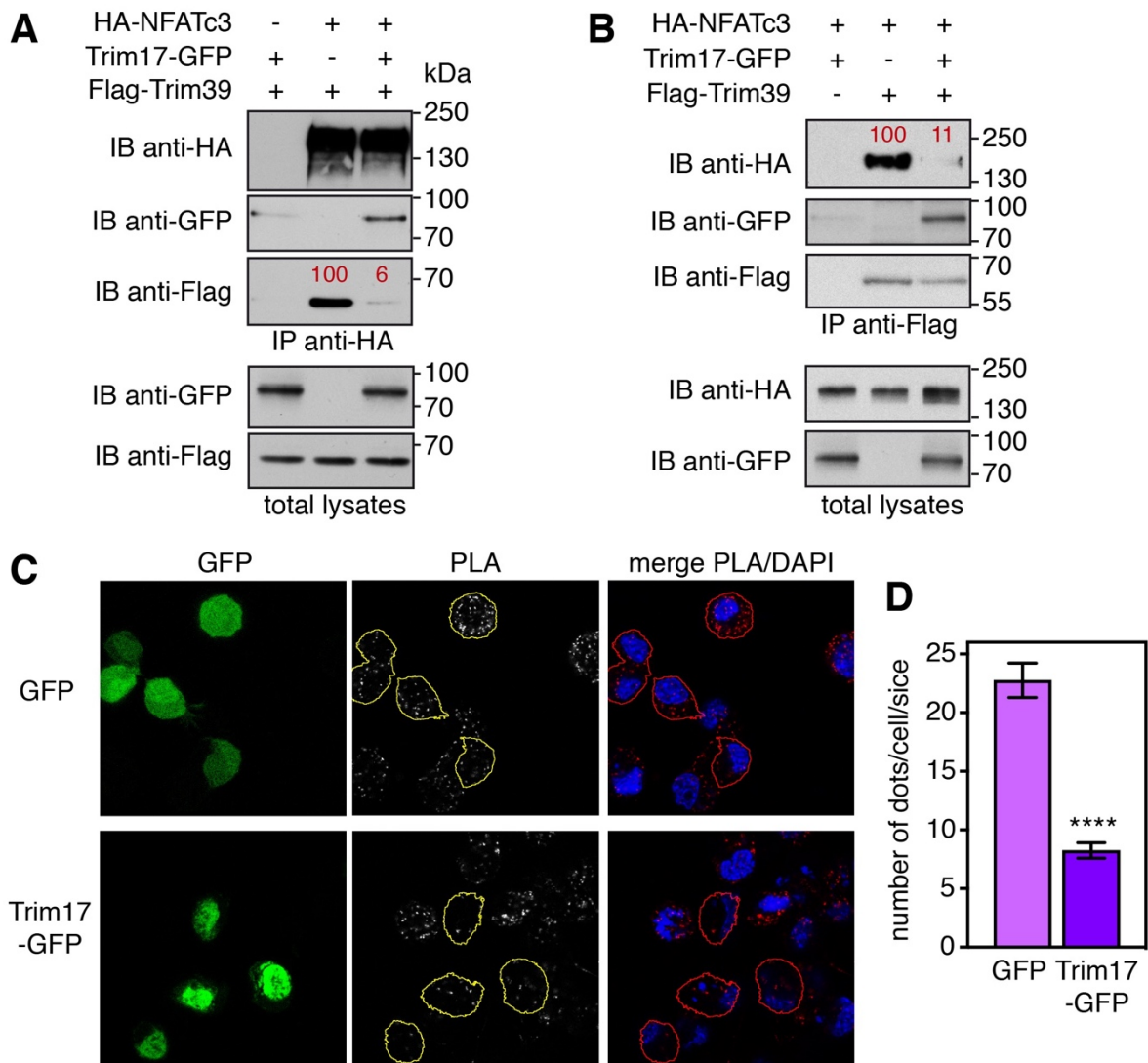


1325  
 1326

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  $\mu$ 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

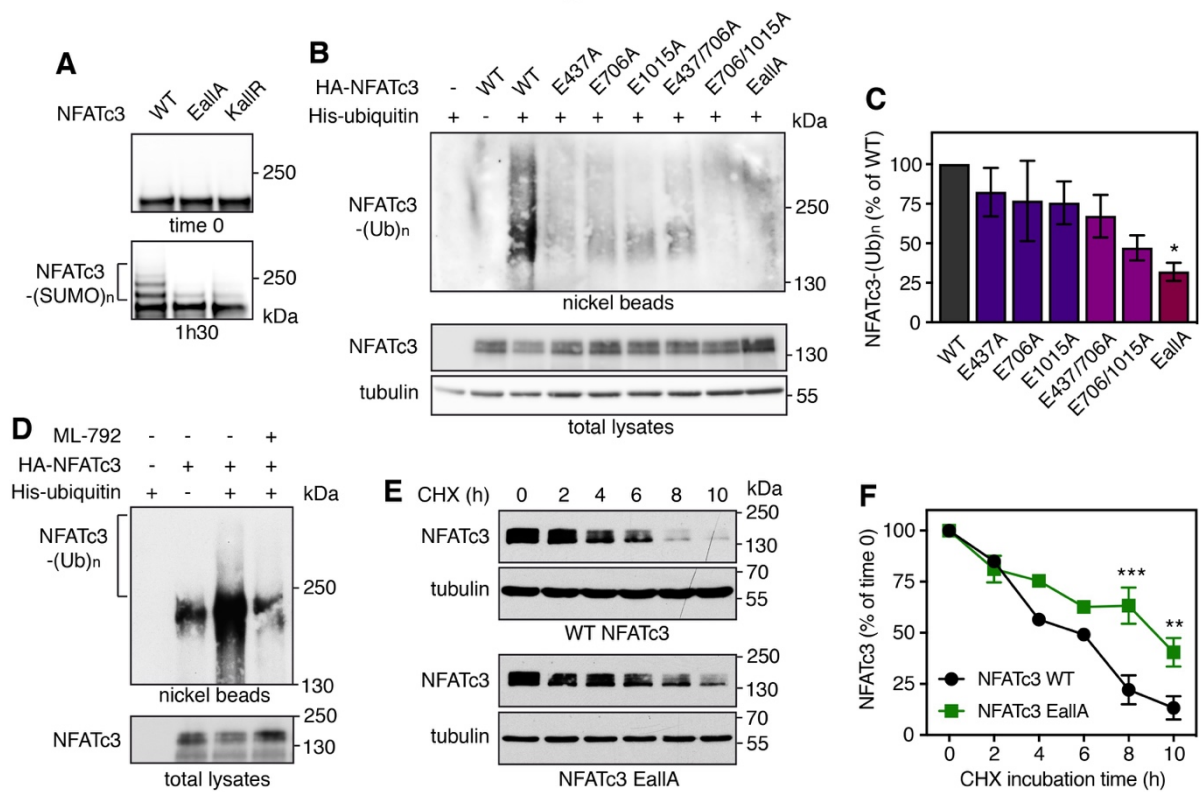
1332 poly-ubiquitinated forms of NFATc3 and Trim39. In a separate SDS-PAGE, samples of the  
 1333 input lysates used for the purification were analyzed with antibodies against HA, Flag and GFP.  
 1334 **B.** *In vitro* translated HA-NFATc3 was first immunopurified from wheat germ extract using  
 1335 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.

1342



1343  
 1344

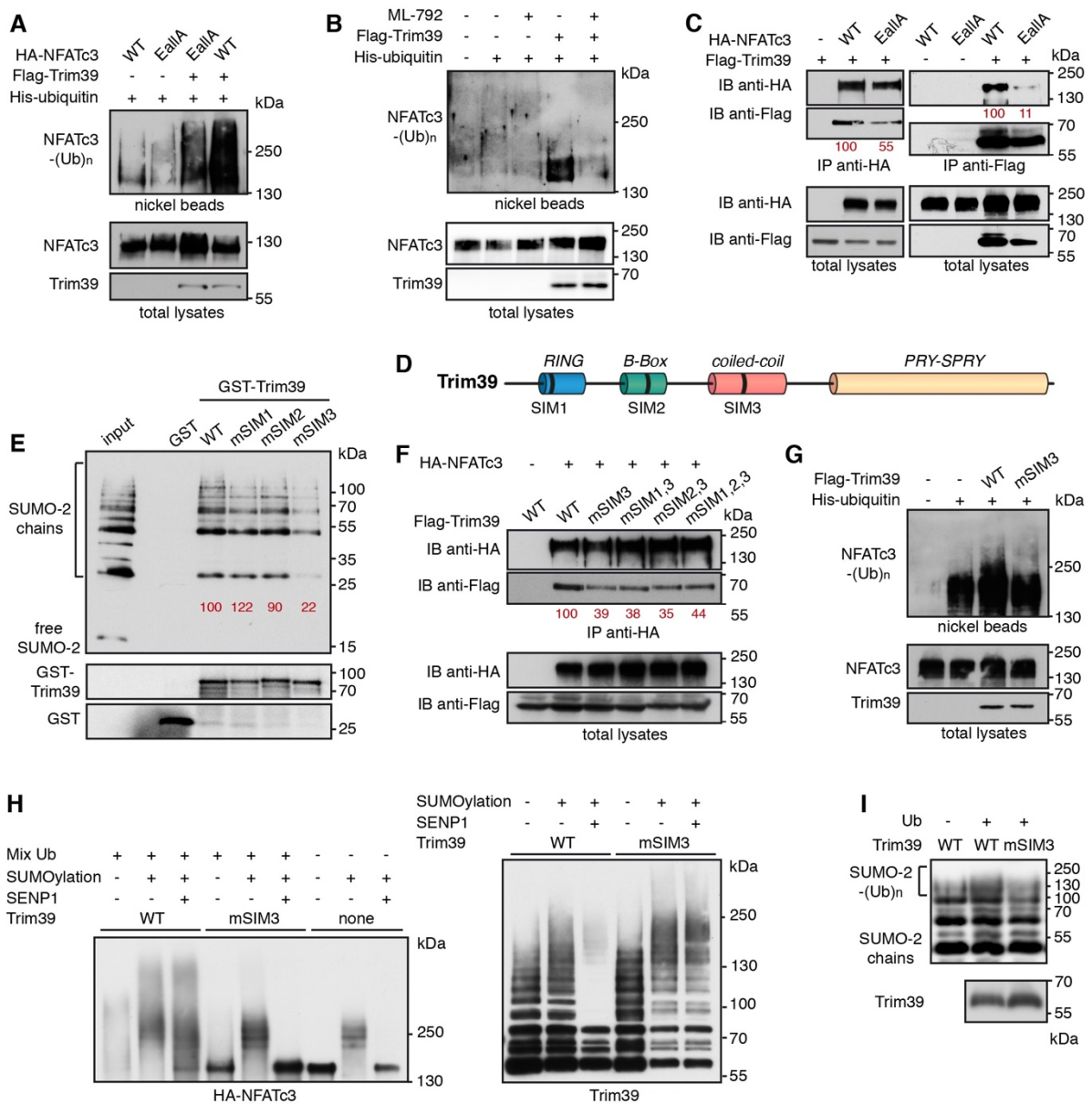
1345 **Figure 5. Trim17 reduces the interaction between endogenous Trim39 and NFATc3. A,B.**  
1346 Neuro2A cells were transfected with HA-NFATc3 in the presence or the absence of Flag-  
1347 Trim39, Trim17-GFP or both, as indicated, for 24 h. Cells were then treated with 20  $\mu$ M MG-  
1348 132 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  $\mu$ 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).  
1368



1369  
1370

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  $\mu$ 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  $\mu$ M MG-132 in the presence or the absence of 0.3  
 1387  $\mu$ 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).  
 1396



1397  
 1398

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

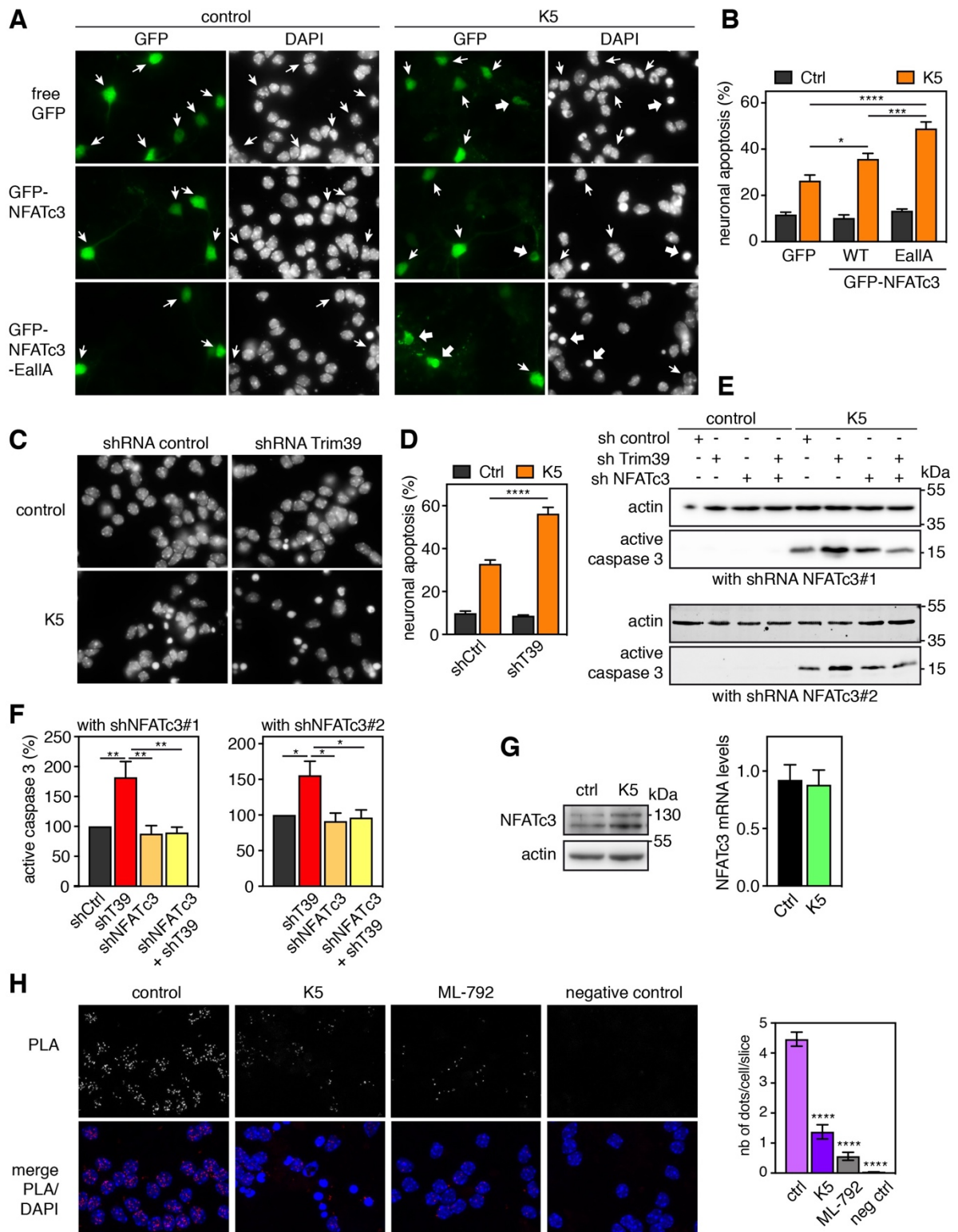


1401 EallA, in the presence or the absence of Flag-Trim39, for 24 h. Then, cells were incubated with  
1402 20  $\mu$ M MG-132 for 6 h before harvesting. The ubiquitinated proteins were purified using nickel  
1403 beads and analyzed by western blotting using anti-HA antibody to detect ubiquitin-conjugated  
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  $\mu$ M MG-132 in the presence or the  
1408 absence of 0.3  $\mu$ 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  $\mu$ 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.

1456





1457  
1458

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  $\mu$ 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

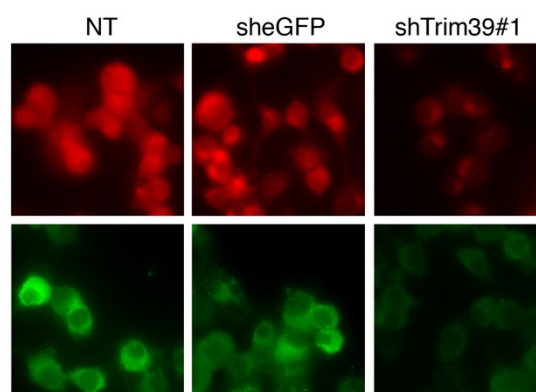
1497 and visualized in merged pictures with PLA signal (merge). The number of dots per cell was  
1498 determined in one slice of each image using Fiji. The graph shows the mean  $\pm$  SEM of 5 images  
1499 per condition, including a total of more than 100 cells, from one experiment representative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

1502

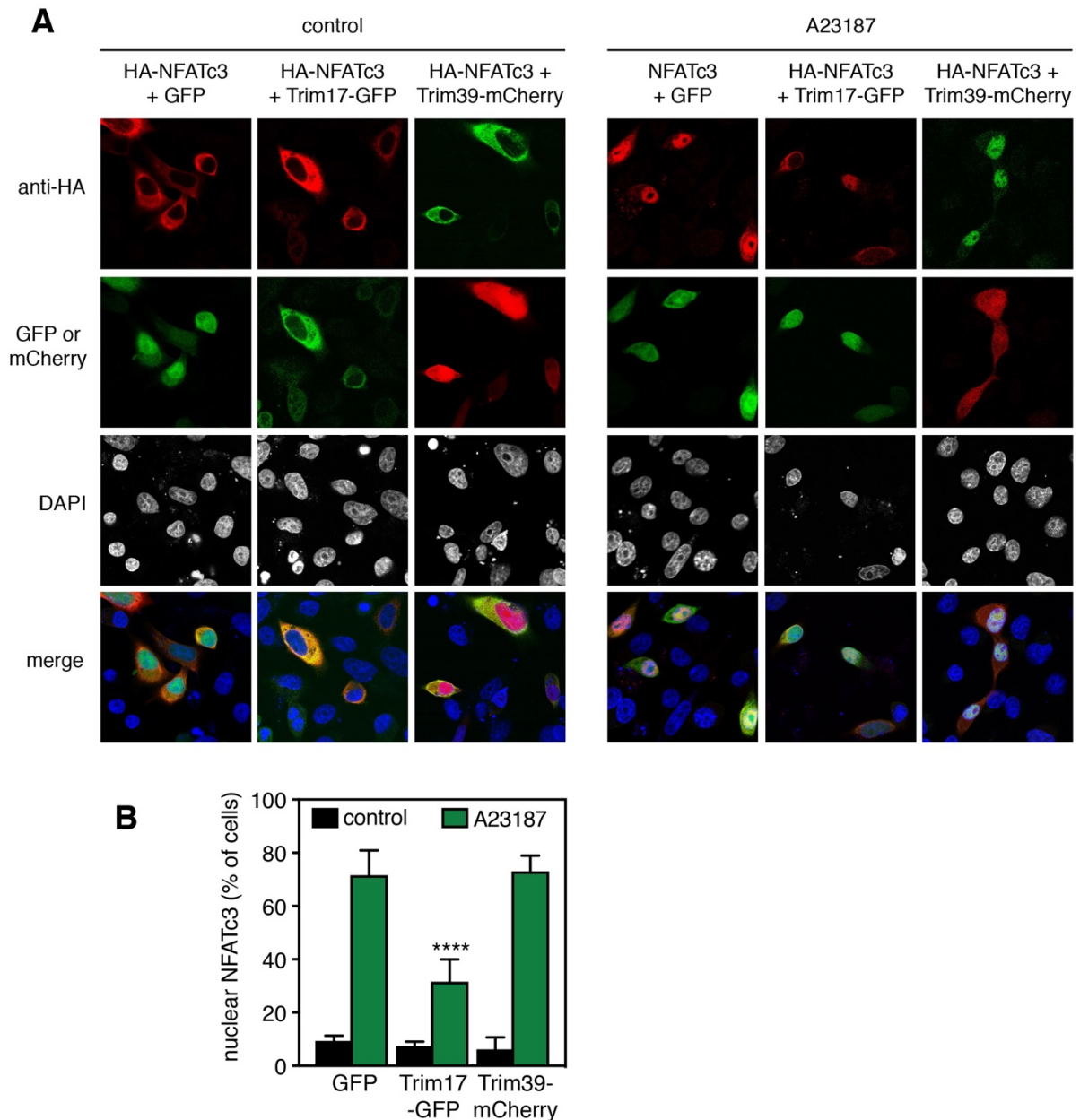
Supplemental data to:

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

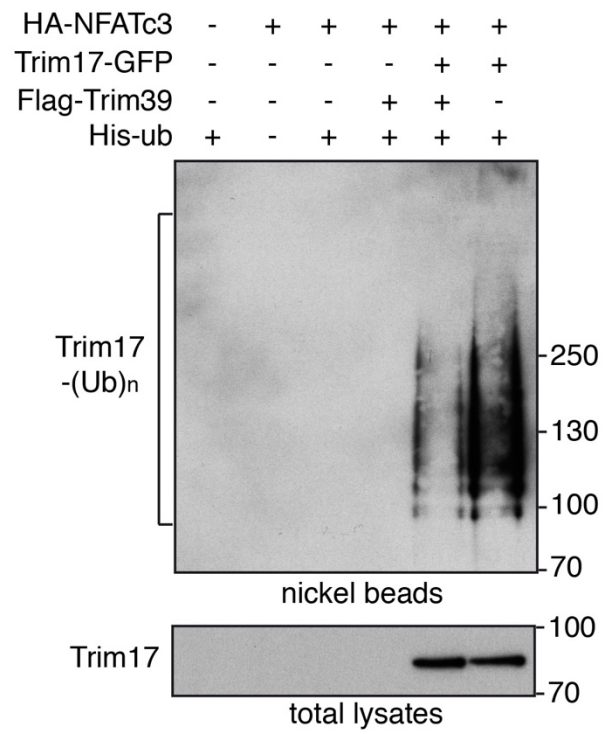
by Meenakshi Basu-Shrivastava, Barbara Mojsa, Stéphan Mora, Ian Robbins, Guillaume Bossis, Iréna Lassot and Solange Desagher



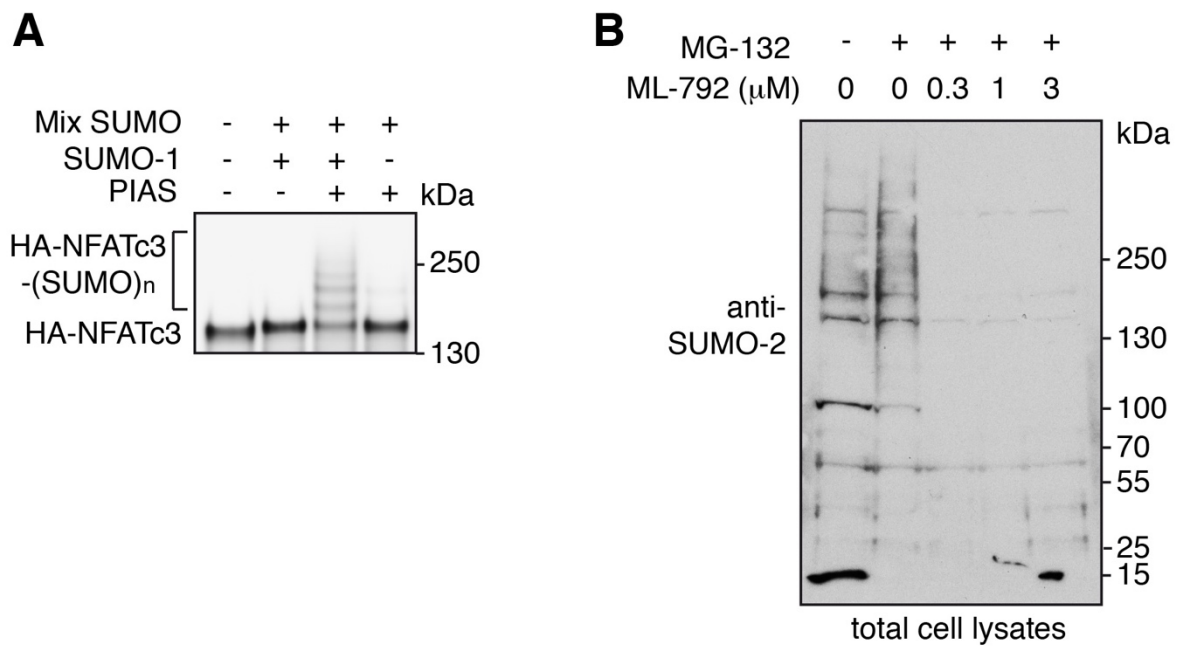
**Figure S1. The specific shRNA shTrim39#1 efficiently reduces the protein level of Trim39. A.** Neuro2A cells were transduced with lentiviral particles expressing a control shRNA (sheGFP) or a specific shRNA against Trim39 (shTrim39#1) for 24 h. Transduced cells were selected using puromycin for two 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.



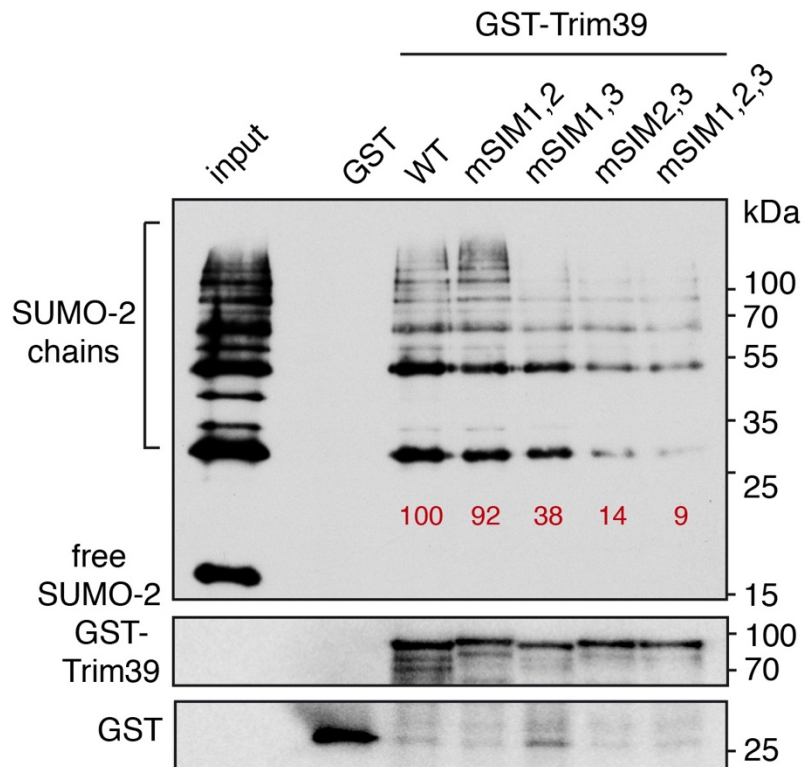
**Figure S2. Trim39 does not alter calcium-induced nuclear translocation of NFATc3. A.** BHK cells were transfected with HA-NFATc3 together with GFP (negative control) or Trim17-GFP, or Trim39-mCherry for 24 h. Then, cells were left untreated (control) or deprived of serum for 3h and treated with 1  $\mu$ 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.

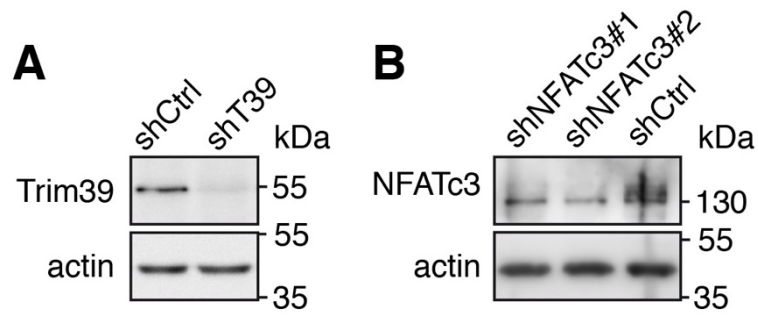


**Figure S4. Role of SUMOylation in NFATc3 regulation.** **A.** *In vitro* translated HA-NFATc3 was incubated with *in vitro* SUMOylation reaction mix (containing E1 and E2 enzymes) for 1 h in the presence or the absence of SUMO-1 and/or the E3 SUMO ligase PIAS. Multi-SUMOylated forms of NFATc3 were 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	GAAGACCAGGAGGCTGCATGTGCGCCTGTGCAATTTCTCATAACCC	GGGTATGAGAAATTGCACAGGCGGCACATGCAGCCTCCTGGTCTTC
Trim39-mSIM3	CGAAGAGCAGCAGACAGCAGCAGCGCGAGCAGAGGAAGAAGAACAG	CTGTTCTTCTTCTCTGCTGCCGCTGCTGCTGTCTGCTGCTCTTCG
Trim39-mCherry 1 <sup>st</sup> PCR	ATAGAATTCATGGCAGAGACAAGTCTG	CTCCTCGCCCTTGCTCACTTCCCAA TCTGTTGGG
Trim39-mCherry 2 <sup>nd</sup> PCR	CCCAACAGATTGGGAAGTGAGCAAGGGCGAGGAG	ATATCTAGATTATCTAGATCCGGTGGATC
Trim39-mCherry 3 <sup>rd</sup> PCR	ATAGAATTCATGGCAGAGACAAGTCTG	ATATCTAGATTATCTAGATCCGGTGGATC
GST-Trim39	CGAGAATTCGCAGAGACAAGTC	CGACTCGAGTCATTCCCAATC
Trim39-C49S/C52S	GGCACAACTTCTCCAAAGCGTCCATTACCGCGTGG	CCAGCGGGTAATGGACGCTTTGGA GAAGTTGTGCC
TRIM17	CGAGAATTCGAGGCTGTGGAAC TCGCC	CGAGTCGACCTATCCTTTCACCCAC ATGGTCAC
His-Trim39	CGACATATGGCAGAGACAAGTCTGTTAGAGG	CGAAAGCTTTCATTCCCAATCTGTTGGGG
Primers used for RT-qPCR		
cDNA	Forward (5' – 3')	Reverse (5' – 3')
Trim39	TGGAGGTGACTTCAGTATCCAT	TCACATCCGCAATTAGCTGTT
Trim17	ACTGAGTGGCAGGAGAGAGTGAA	CCAGAAACAAGACCACCTTCTGA
NFATc3	TTGAGTGCTCTCAGCGATCT	GACAATGTGAGCCCCTTGAC
GAPDH	AAATGGTGAAGGTCGGTGTG	CCTTGACTGTGCCGTTGAAT