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1 NatB-dependent acetylation protects procaspase-8 from UBR4-mediated degradation and is
2 required for full induction of the extrinsic apoptosis pathway

3

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25 **ABSTRACT**

26 N-terminal acetyltransferase B (NatB) is a major contributor to the N-terminal acetylome
27 and is implicated in several key cellular processes including apoptosis and proteostasis.
28 However, the molecular mechanisms linking NatB-mediated N-terminal acetylation to
29 apoptosis and its relationship with protein homeostasis remain elusive. In this study, we
30 generated mouse embryonic fibroblasts (MEFs) with an inactivated catalytic subunit of NatB
31 (*Naa20^{-/-}*) to investigate the impact of NatB deficiency on apoptosis regulation. Through
32 quantitative N-terminomics, label-free quantification, and targeted proteomics, we
33 demonstrated that NatB does not influence the proteostasis of all its substrates. Instead, our
34 focus on putative NatB-dependent apoptotic factors revealed that NatB-mediated acetylation
35 serves as a protective shield against UBR4 and UBR1 Arg/N-recognin-mediated degradation.
36 Notably, *Naa20^{-/-}* MEFs exhibited reduced responsiveness to extrinsic pro-apoptotic stimuli, a
37 phenotype that was partially reversible upon UBR4 Arg/N-recognin silencing and consequent
38 inhibition of procaspase-8 degradation. Collectively, our results shed light on how the interplay
39 between NatB-mediated acetylation and the Arg/N-degron pathway impacts apoptosis
40 regulation, providing new perspectives in the field including in therapeutic interventions.

41

42 **Keywords:** N-terminal acetylation, proteostasis, NatB, N-acetyltransferases, N-terminomics
43 E3 ubiquitin ligases, N-degrons, N-recognins, apoptosis, caspases

44

45 INTRODUCTION

46 N-terminal protein modifications (NPMs) are among the first and fastest responses to the
47 cellular environment, influencing not only the modified protein but also cell, tissue, and whole
48 organism phenotypes^{1, 2, 3, 4}. One major NPM is N-terminal (Nt-) acetylation, an essential
49 multitasking protein modification implicated in many diseases^{5, 6}, where an acetyl group is
50 transferred from acetyl coenzyme A to the α -amino group of a protein N-terminus catalyzed by
51 Nt-acetyltransferases (NATs)^{5, 7}. The eight currently known eukaryotic NATs have been
52 defined based on their subunits and substrate specificity^{6, 8}. Nt-acetyltransferase B (NatB)
53 comprises the catalytic subunit NAA20 and the auxiliary subunit NAA25. NAA25 binds to the
54 ribosome and ensures co-translational activity of NatB⁹, while the catalytic site of NAA20
55 anchors and acetylates the N-alpha amino group of the initiator methionine (iMet) of all proteins
56 immediately followed by an asparagine, glutamine, aspartic or glutamic acid residue¹⁰,
57 ¹¹. Together with NatA, which acetylates proteins that have lost their iMet, NatB is the major
58 contributor to Nt-acetylation. NatB substrates cover nearly 21% of human, *Caenorhabditis*
59 *elegans*, and *Arabidopsis thaliana* proteomes and 15% of yeast proteome. In all these
60 organisms, almost all NatB substrates are fully acetylated, unlike the substrates of other NATs
61 (especially NatA), many of which are only partially modified^{10, 12, 13}.

62 For a few well-studied proteins, NatB has been shown to be involved in promoting
63 resistance to aggregation, protein-protein interactions, protein sorting to distinct cellular
64 compartments, and regulating protein half-life^{6, 14}. However, the overall impact of NatB-
65 mediated Nt-acetylation on proteostasis is still poorly understood. Global protein stability does
66 not seem to be affected by NatB subunit depletion in yeast or human cells^{15, 16, 17}, suggesting
67 that NatB-mediated Nt-acetylation may not be a dominant factor in proteostasis. Conversely,
68 several studies have highlighted the importance of NATs-mediated Nt-acetylation on the
69 homeostasis of specific proteins in the context of N-degrons, which are N-terminal degradation
70 signals recognized by the N-degron pathways¹⁸. For instance, the N-terminal acetylated
71 variants of MX-Rgs2, a G-protein regulator, have been shown to be selectively targeted by the
72 Ac/N-end rule pathway when X is either Arg or Glu¹⁹.

73 Whatever the impact of NatB-mediated Nt-acetylation on proteostasis or protein half-
74 life, the inactivation or downregulation of any NatB subunit influences fundamental cellular
75 processes such as actin cytoskeleton organization^{10, 20, 21, 22, 23}, NAD⁺ homeostasis²⁴, influenza
76 virus protein PA-X²⁵, proliferation^{22, 23, 26}, and apoptosis^{23, 26, 27}. However, the exact molecular
77 mechanisms linking defective NatB or NatB-mediated Nt-acetylation to specific cellular
78 processes and their interdependence have not yet been elucidated.

79 Given the prevalence and importance of Nt-acetylation and apoptosis in numerous
80 human diseases^{6, 28}, we sought to establish the mechanistic role of NatB-dependent Nt-
81 acetylation on apoptosis regulation. Here, we show that NatB inactivation in mouse embryonic
82 fibroblasts (MEFs) leads to degradation of proapoptotic proteins, particularly of procaspase-8,
83 mediated by the UBR4 E3 ubiquitin ligase, thereby limiting activation of the extrinsic apoptotic
84 pathway. Our findings provide the first evidence implicating NatB-Arg/N-degron pathway in
85 apoptosis regulation.

86

87 **METHODS**

88 **Standard methods are reported in the Supplementary Information**

89 **Inactivation of *Naa20* in MEFs**

90 MEFs were obtained from *Naa20*^{tm1a(KOMP)Wtsi} transgenic mice as described in the
91 **Supplementary Methods**. *Naa20* was inactivated with a recombinant adenovirus expressing
92 CRE recombinase (Ad5CMVCre, MOI 2000). An empty adenovirus was used as a negative
93 control (AdEmpty, MOI 2000). Recombinant adenovirus was inoculated in DMEM
94 supplemented with 2% FBS and 1% penicillin/streptomycin and, 24 h later, regular DMEM was
95 added. Cells were trypsinized and re-plated, two or five days post-infection.

96

97 **Apoptosis assays**

98 Cells were seeded in six-well plates, infected, and six days post-infection treated with 50 μ M
99 etoposide, 100 ng/mL TNF- α plus 500 nM SMAC, 5 μ M of MG132, or 5 ng/ μ L of tunicamycin

100 for 0, 8, 12, and 24 h. At each time point, cells were harvested and collected for protein
101 extraction for further analysis by western blotting.

102

103 **N-terminal acetylation assays**

104 Eight biological replicates of WT or *Naa20^{-/-}* MEFs (10^6 cells) were harvested and six
105 days after adenovirus infection with AdEmpty and Ad5CMVCre, respectively, were centrifuged
106 at 6000 rpm for 5 min at 4°C, and pellets were washed with PBS 1× and centrifuged again.
107 Next, cell pellets were resuspended in 300 µL of protein extraction buffer (50 mM
108 HEPES/NaOH pH 7.2; 1.5 mM MgCl₂; 1 mM EGTA; 10% glycerol; 1% Triton X-100; 150 mM
109 NaCl; 2 mM PMSF; 1 protease inhibitor cocktail tablet (EDTA+) in 50 mL), subjected to lysis
110 by sonication, centrifuged at 17 000 rpm for 20 min at 4°C, and the supernatant recovered for
111 proteomic analysis.

112 For each sample, 1 mg of protein was used following the SILProNAQ protocol
113 previously described²⁹. Briefly, N-acetoxy-[²H₃] succinimide was used to label protein N-termini
114 and lysine ε-amino groups before being digested by trypsin. Peptides were then fractionated
115 by chromatography using a strong cation exchange (SCX) column to discriminate acetylated
116 and unacetylated internal peptides. Individual fractions 2-11 were analyzed by LC-MS using
117 55 min methods on the LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific,
118 Waltham, MA), and three pools of fractions (2-5, 6-8, and 9-11) were analyzed using 100 min
119 methods on the TIMS-ToF (Bruker, Billerica, MA). Data were searched against the *Mus*
120 *musculus* SwissProt database through Mascot Distiller then parsed using the in-house
121 eNcounter script to validate the detected N-termini and obtain the Nt-acetylation yield³⁰.

122

123 **Label-free proteome quantitation**

124 In parallel with the N-terminal acetylation assay, the same MEF samples were used for
125 full proteomic analysis. 30 µg of protein extract were loaded on an SDS-PAGE gel and digested
126 following an in-gel digestion protocol³¹. The resulting peptides were extracted, dried, and then

127 analyzed on the TIMS-ToF mass spectrometer using the same 100 min method as before.
128 Data were processed using MaxQuant to identify and quantify.

129

130 **siRNA silencing of E3 ubiquitin ligases**

131 WT or *Naa20*^{-/-} MEF cells were seeded in six-well plates and infected the day after with the
132 corresponding adenovirus as described above. Two days later, cells were trypsinized and re-
133 plated as described previously and, four days post-infection, cells were re-plated and
134 transfected with the corresponding siRNAs. 150 000 cells/ml were transfected in suspension
135 with the siRNAs (**Supplementary Methods**) and the Lipofectamine[®] RNAiMAX Transfection
136 Reagent (Invitrogen, Thermo Fisher Scientific 13778) according to manufacturer's instructions.
137 Transfected cells were collected 48 h post-transfection for western blot and real-time PCR
138 analysis.

139

140 **Statistical analysis**

141 Statistical analyses were performed using Prism 8 (GraphPad Software, La Jolla, CA).
142 Normality of groups was assessed with the Shapiro-Wilk test. The unpaired *t*-test was used to
143 compare parametric data the Mann-Whitney test for non-parametric data. Two-way ANOVA
144 was used to analyze proliferation data. The significance of enriched NatB substrates in different
145 MS quantifications was calculated using the built-in two-tailed *t*-test function for comparison of
146 two samples with equivalent variances in Microsoft Excel for the N-terminome analysis, while
147 a two sample Student *t*-test, with a threshold p-value of 0.05, was used in Perseus for the
148 label-free proteome analysis.

149

150 **RESULTS**

151 **Complete *Naa20* inactivation induces cytoskeletal abnormalities and decreases**
152 **proliferation**

153 Based on our previous studies that showed increased sensitivity of *Naa25^{-/-}* MEFs²⁷ and
154 *Naa20^{-/-}* HeLa cells²⁶ to the apoptotic inducer MG132, we aimed to further investigate the role
155 of NatB acetylation on apoptosis by inactivating the NatB catalytic subunit in MEFs.
156 Six days of infection with Ad5CMVCre (*Naa20^{-/-}* MEFs) resulted in a 99.6% reduction of *Naa20*
157 transcript compared with AdEmpty-infected MEFs (WT) (**Fig. 1A**), and NAA20 protein was no
158 longer detectable in *Naa20^{-/-}* MEFs (**Fig. 1B**). *Naa20* inactivation was associated with a
159 significant reduction in *Naa20^{-/-}* MEF proliferation four days after infection (**Fig. 1C**). Moreover,
160 six days after AdCre infection, actin fibers became disorganized in *Naa20^{-/-}* MEFs compared
161 to WT (**Fig. 1D**), and, in keeping with this actin disorganization, focal adhesions decreased, a
162 phenotype commonly observed upon NatB complex inactivation or downregulated in other cell
163 types. These results confirmed that *Naa20* is inactivated in *Naa20^{-/-}* MEF cells and induces
164 phenotypes similar to those previously observed in other NatB-downregulated cellular
165 contexts^{10, 20, 21, 23}.

166

167 **Quantitative N-terminomics of *Naa20^{-/-}* MEFs reveals reduction in Nt-acetylation of only** 168 **NatB substrates**

169 We next performed N-terminomics analysis of WT and *Naa20^{-/-}* MEFs using the 'Stable Isotope
170 Labeling Protein N-terminal Acetylation Quantification' method to identify and quantify N-
171 termini (SILProNAQ²⁹). Analysis of WT and *Naa20^{-/-}* MEF cells identified 25,048 protein N-
172 termini corresponding to 1988 non-redundant proteoforms (**Supplementary Dataset 1 and**
173 **Supplementary Table 1**). The acetylation yields of 1191 of them (1073 in the WT and 945 in
174 the NatB mutant background) could be quantified. The analysis of these unique proteoforms
175 unravelled that 769 underwent removal of the initial Methionine (iMet) in agreement with known
176 N-terminal methionine excision (NME) rules in eukaryotes³², whereas 258 proteoforms
177 retained their iMet (**Supplementary Table 1**). The remaining 164 underwent larger cleavages
178 in keeping with leader peptide removal. The quasi-totality of the iMet starting N-termini featured
179 at position two an amino acid with a large lateral chain (**Supplementary Dataset 1**).

180 In *Naa20^{-/-}* MEFs, there was an overall reduction in Nt-acetylation, primarily in proteins
181 retaining their iMet (**Fig. 2A, B**), with a relative decrease in the number of fully acetylated N-
182 termini to only partially acetylated N-termini (**Supplementary Dataset 1** and **Supplementary**
183 **Table 1**). By contrast, acetylation of N-termini devoid of iMet or N-termini processed
184 downstream were unaffected (**Fig. 2C, D**). We compared our N-terminomics data to those
185 previously reported for human cells in which NatB was downregulated¹⁰. Common identified
186 and quantified N-termini of the two datasets revealed mostly decreases of the acetylation yields
187 of NatB substrates (**Supplementary Dataset 2**). This effect was significantly stronger in
188 *Naa20^{-/-}* MEFs (**Supplementary Dataset 2**). N-termini with the highest decreased Nt-
189 acetylation in *Naa20^{-/-}* MEFs (26, **Table 1** and **Fig. 2E, F**) corresponded to NatB substrates
190 (23), of which 18 displayed Asp and Glu at position two and, to a minor extent (5), Asn and Gln
191 (**Table 1** and **Supplementary Dataset 1**). In *Naa20^{-/-}* MEFs, only three non-NatB substrates
192 also showed strongly decreased Nt-acetylation (**Table 1**), but for which no information is
193 available for the human counterparts (**Supplementary Dataset 2**).

194 We observed that the functions of many retrieved proteins with reduced Nt-acetylation
195 in *Naa20^{-/-}* MEFs were apoptosis-related (**Supplementary Dataset 1** and **Table 1**). Among
196 them, we found TMEM258, a central mediator of ER quality control via apoptosis³³; importin
197 7, which is involved via the Hippo pathway in the expression of genes important in apoptosis³⁴;
198 and IFM3, a member of the Interferon-induced transmembrane proteins (IFITMs) family³⁵. This
199 intriguing observation led us to note that procaspases-3, -8, -9, BAX, APAF1, and BID, which
200 are major contributors to extrinsic and intrinsic apoptosis (**Supplementary Fig.1**),
201 systematically displayed Asp or Glu at position 2. These proteins are also inferred as NatB
202 substrates based on sequence similarity between mouse and human (100% conservation of
203 the first amino acids) and a combination of experimental and computational evidence^{10, 36, 37},
204 ³⁸. Unfortunately, our SilProNAQ approach did not allow us to retrieve and analyze these N-
205 termini due to the distal position of Arg residues required for trypsin cleavage and peptide
206 identification, combined with low protein expression (**Table 1**).

207 To further explore the relationship between NatB-dependent Nt-acetylation and
208 proteostasis, we next examined the NatB-dependent proteome, particularly that of caspases.
209 Label-free quantification (LFQ protein ratio) analysis showed that only 36 of 1235 statistically
210 relevant proteins, among which 189 are NatB-substrates, were drastically deregulated in
211 *Naa20^{-/-}* MEFs (**Supplementary Dataset 1, Table 2, and Fig. 2G**). Almost all upregulated
212 proteins were NatA substrates and, where Nt-acetylation could be measured, no variation was
213 observed (**Table 2**). By contrast, the amino terminal sequence of 10 of 21 strongly
214 downregulated proteins indicated that they were predicted as NatB substrates, including
215 ANP32B (Q9EST5), a multifunction protein directly cleaved by caspase-3 and a negative
216 regulator of caspase-3-dependent apoptosis (**Supplementary Dataset 1 and Table 2**).
217 Although, Nt-acetylation of procaspase-3, -8, and -9 was not quantifiable, caspase-3 and the
218 UBR4 E3-ligase were also found to be reduced in *Naa20^{-/-}* MEFs to some extent
219 (**Supplementary Dataset 1 and Table 1**). We obtained information of the Nt-acetylation yield
220 of 12 out of the 36 most drastically deregulated proteins. Of 9 downregulated proteins, Nt-
221 acetylation yields were unmodified for five of them (% Nt-acetylation difference <1.3), while
222 four including three NatB substrates - spermidine synthase, caveolae-associated protein 1,
223 and reticulon-4 - displayed a parallel reduction in Nt-acetylation and protein accumulation
224 (**Table 2**).

225 Taken together, our proteomic investigation suggest that NatB is not likely involved in
226 the proteostasis of all of its substrates, but rather indicates that NatB-mediated Nt-acetylation
227 may still protect a specific set of proteins from degradation, such as components of the
228 apoptotic pathways.

229

230 ***Naa20* inactivation reduces the expression of several components of the apoptotic**
231 **pathways decreasing activation of procaspases in response to TNF- α plus SMAC**
232 **mimetic and etoposide**

233 Although a significant number of key components of the apoptotic pathway are NatB substrates
234 (see above and **Supplementary Fig. 1**), our proteomic analysis did not allow us to
235 simultaneously quantify the Nt-acetylation yield and protein accumulation of none of them. As
236 it has been shown that Cre recombinase expression could be cytotoxic through unspecific
237 activities³⁹, we first confirmed that after 6 days infection it did not affect the expression levels
238 of intrinsic and extrinsic apoptotic components, including the NatB substrates BID and
239 procaspase-8,-9 and -3, independently of *Naa20* inactivation (**Supplementary Fig. 2**). We
240 then performed a targeted time-course assay after *Naa20* inactivation to analyze their
241 accumulation. *Naa20*^{-/-} MEFs showed no variation in apoptotic NatA substrates such as
242 procaspase-6 and SMAC (or DIABLO) (**Fig. 3A**), whose N-termini start with a Thr and Ala,
243 respectively. However, the levels of procaspases-8, -9, -3 and BID significantly decreased in
244 *Naa20*^{-/-} MEFs (**Fig. 3A** and **Supplementary Fig. 3A**), while no differences in *procaspase-8*, -
245 *9*, -3 and *Bid* transcripts were observed six days after infection (**Fig. 3B**). Protein levels of BAX
246 and APAF1, two other NatB substrates, were unaffected and accumulated in the absence of
247 NAA20 (**Fig. 3A**). Interestingly, both *Bax* and *Apaf1* mRNA levels were markedly upregulated
248 six days post-infection in the absence of NAA20 (**Fig. 3B**), suggesting that BAX protein may
249 be less stable in the *Naa20*^{-/-} background, and that transcriptional upregulation compensates
250 for its decreased stability. Interestingly, we observed the presence of cleaved PARP in the
251 absence of NAA20, despite not detecting active caspase-3 (**Fig. 3A** and **Supplementary Fig.**
252 **3A**). This suggests the presence of apoptotic cells in the context of NAA20 deficiency. These
253 data clearly show a strong negative effect on procaspase-3, -8, and -9 and BID expression of
254 NatB inactivation as a result of a reduced Nt-acetylation.

255 The decrease in procaspases accumulation prompted us to assess their activation in *Naa20*^{-/-}
256 MEFs in response to apoptotic inducers like etoposide and TNF- α plus SMAC mimetic. For
257 both stimuli, procaspase-8, -9, and -3 levels slightly decreased in WT cells through proteolytic
258 processing (**Fig. 3C, D**, and **Supplementary Fig. 3B, C**). However, procaspases and BID
259 protein levels in *Naa20*^{-/-} MEFs - while lower at time 0 compared with WT MEFs - remained
260 constant throughout the experiment and, as expected, this decrease was associated with lower

261 levels of cleaved caspases and PARP. BID protein levels in *Naa20^{-/-}* MEFs followed a similar
262 pattern to procaspases.

263 We next questioned whether the observed blockade of procaspase activation on *Naa20*
264 inactivation could be extended to other apoptosis inducers including tunicamycin or MG132,
265 which induce apoptosis by activating caspase-9 and caspase-8, respectively⁴⁰. Throughout the
266 experiment, the differences in protein levels of cleaved caspase-8, -9, -3, and PARP between
267 *Naa20^{-/-}* MEF and WT cells were diminished for both treatments, in contrast to the significant
268 blockade observed when cells were treated with etoposide and TNF- α plus SMAC mimetic
269 (**Supplementary Fig. 4A, B, 5A and B**).

270 As *Naa20* inactivation in MEFs decreased procaspase activation in response to the
271 intrinsic apoptosis inducer etoposide, we addressed whether blockage of apoptosis could be
272 due to the impairment of BAX activation. There were no differences in mitochondrial BAX and
273 no detectable cytosolic cytochrome *c* (cyt *c*) and SMAC in WT and *Naa20^{-/-}* MEFs under basal
274 conditions (**Fig. 4A and Supplementary Fig. 6A**). Curiously, *Naa20^{-/-}* and WT MEFs, 12 h
275 after etoposide treatment, showed similar BAX translocation from the cytosol to mitochondria.
276 However, mitochondrial release of cyt *c* and SMAC was lower in *Naa20^{-/-}* than WT MEFs
277 despite increases in cellular cyt *c* and SMAC after etoposide treatment, as previously
278 described⁴¹ (**Fig. 4B and Supplementary Fig. 6B**). On the other hand, similar cyt *c* and SMAC
279 release from mitochondria to the cytosol, and consequently of procaspase-3 activation were
280 observed in WT and *Naa20^{-/-}* MEFs 8 h after MG132 treatment (**Fig. 4C and Supplementary**
281 **Fig. 6C**).

282

283 **The Arg/N-degron pathway is involved in the reduction of procaspase-8, -9 and BID** 284 **levels and defective procaspase activation in *Naa20^{-/-}* MEFs**

285 As BID and procaspase-8, -9, and -3 protein levels decreased in the absence of *Naa20* (**Fig.**
286 **3A**), we next assessed if the possible lack of the N-terminal acetyl group in NatB substrates
287 was sensed as an Arg/N-degron and consequently targeted for degradation. To this end, we
288 used siRNAs to silence E3 ubiquitin ligases of the Arg/N-degron pathway (*Ubr1*, *Ubr2* and

289 *Ubr4*). As it has been reported a functional complementarity between Arg/N-degron and Ac/N-
290 degron pathways, we also used siRNAs to silence the Ac/N-recognins *Cnot4*, *March6* in
291 *Naa20^{-/-}* and WT MEFs, and evaluated procaspase and BID protein levels (**Fig. 5A** and
292 **Supplementary Fig. 7A**).

293 While procaspase-3 levels were not positively affected by silencing any of the tested E3
294 ubiquitin ligases, *Ubr4* silencing increased procaspase-8 levels almost comparable to the level
295 in WT MEFs, while *Ubr1* silencing slightly increased procaspase-9 in *Naa20^{-/-}* MEFs. Moreover,
296 silencing *Ubr4* or *Ubr1* also increased BID protein levels. Interestingly, downregulation of *Ubr2*
297 negatively affected procaspase-8 and procaspase-3 expression in WT MEFs to similar levels
298 as in *Naa20^{-/-}* MEFs. Furthermore, silencing of *March6* and *Cnot4* did not affect the expression
299 levels of procaspases and BID.

300

301 ***Ubr4* silencing in *Naa20^{-/-}* MEFs partially reverts the decrease of procaspase-8** 302 **expression and its activation in response to TNF- α plus SMAC mimetic**

303 Our data suggest that the procaspase-8, -9 and BID N-termini may be recognized as
304 degradation signals when *Naa20* is inactivated. We, then, wanted to explore the physiological
305 relevance of these NAA20-dependent degradation signals on the induction of the two major
306 apoptotic pathways (e.g., extrinsic and intrinsic ones). We, therefore, investigated whether the
307 procaspase-8 and BID increase observed after silencing of *Ubr4*, and the increase in
308 procaspase-9 and BID after silencing of *Ubr1*, could rescue procaspase activation in *Naa20^{-/-}*
309 MEFs treated with TNF- α plus SMAC mimetic or etoposide, respectively. In addition to
310 restoring procaspase-8 accumulation in *Naa20^{-/-}* MEFs, *Ubr4* silencing also activated caspase-
311 8 12 hours after TNF- α plus SMAC treatment, as it recovered and increased caspase-8 p43
312 polypeptide production and caspase-8 p18 polypeptide accumulation, respectively (**Fig. 5B**
313 **and Supplementary Fig. 7B**). *Ubr4* downregulation also promoted slight caspase-3 activation
314 in *Naa20^{-/-}* MEFs. Nonetheless, procaspase-3 activation was less pronounced than
315 procaspase-8 activation. Therefore, the decrease in procaspase and caspase-8 in response
316 to TNF- α plus SMAC mimetic caused by *Naa20* inactivation can be partially reverted by

317 silencing the *Ubr4* E3 ubiquitin protein ligase. Conversely, although *Ubr1* silencing restored
318 procaspase-9 accumulation in *Naa20*^{-/-} MEFs (**Fig. 5A**), etoposide treatment barely induced
319 procaspase-9 activation in the double mutant (**Supplementary Fig. 8A**). Of note, no caspase-
320 9 activation was observed when silencing *Ubr4* in response to etoposide (**Supplementary Fig.**
321 **8B**).

322

323 **DISCUSSION**

324 By targeting around 21% of the proteins, NatB is the second major contributor to the Nt-
325 acetylome and its inactivation or downregulation affects several cellular processes such as
326 actin cytoskeleton function^{10, 20, 21, 42}, stress responses^{13, 43}, proliferation^{22, 23, 26}, and apoptosis^{26,}
327 ²⁷. Although NatB is known to regulate apoptosis in MEFs and tumor cells, the association
328 between NatB and apoptosis regulation and its interdependence with N-degron pathways have
329 not previously been investigated. Curiously, several main components of intrinsic and extrinsic
330 apoptosis pathways are NatB substrates based on their second amino acid identity
331 (**Supplementary Fig. 1**). Accordingly, BAX and procaspase-3 are Nt-acetylated by NatB in
332 human cells¹⁶. In addition, we recently showed that human BAX acetylation by NatB is
333 essential to maintain BAX in an inactive conformation in the cytosol of MEFs²⁷. Thus, to further
334 reveal how apoptosis is modulated by NatB-mediated Nt-acetylation, we explored the effect of
335 *Naa20* inactivation on protein and gene expression of several apoptotic pathway components.
336 We observed a strong and selective decrease in the NatB substrates procaspase-8, -9, and -
337 3 and BID but not procaspase-6 and SMAC, both of which are NatA substrates. Nevertheless,
338 when *Naa20* was inactivated, *Apaf1* and to lesser extent *Bax* mRNAs were upregulated while
339 *procaspase-8*, *-9*, and *-3* and *Bid* were not affected. In *D. melanogaster*, *Naa20* knockdown
340 decreased protein expression of NatB substrate Drk, while accumulation of its mRNA was
341 unaffected⁴⁴. In sum, decreases in procaspase-8, -9, and -3 and BID proteins in *Naa20*^{-/-} MEFs
342 is not caused by transcriptional repression of their genes or through destabilized mRNA,
343 suggesting a mechanism of selective translational repression or instability of these proteins.

344 Our proteomic analysis is consistent with NatB not being involved in the overall control
345 of its substrates stability, with the accumulation of most of these proteins being not affected by
346 *Naa20* inactivation. Instead, NatB-dependent NTA of specific proteins - particularly of the pro-
347 apoptotic factors procaspase-8, -9, -3 and BID - prevents these proteins from degradation.
348 Therefore, we addressed whether their reduced levels affected *Naa20*^{-/-} MEFs susceptibility to
349 different apoptotic stimuli. Loss of apical initiators caspase-8 and -9 is known to block extrinsic
350 and intrinsic apoptosis, respectively^{45, 46}. Our results revealed a decrease in procaspase-8, -9,
351 and -3 activation in response to etoposide- and TNF- α plus SMAC mimetic-induced apoptosis
352 in *Naa20*^{-/-} but not in WT MEFs, reflecting a negative impact of *Naa20* inactivation on both
353 pathways. Intriguingly, *Naa20*^{-/-} MEFs display basal levels of cleaved PARP (Asp214), which
354 suggests the activation of caspase-3, even though the cleaved caspase-3 peptide remains
355 undetectable. However, this particular state does not promote the induction of apoptosis upon
356 cell exposure to etoposide and TNF- α plus SMAC mimetic treatment suggesting the
357 dysfunction or inhibition of apoptotic signaling components.

358 To understand whether this apoptosis blockade was related to impaired BAX activation,
359 we assessed its subcellular localization and cyt *c* and SMAC mitochondrial release. In *Naa20*
360 ^{-/-} MEFs, 12 h after etoposide treatment, BAX mitochondrial translocation was similar to WT
361 MEFs; however, cyt *c* and SMAC release was attenuated, indicating a potential inability to
362 activate procaspase-9 in response to etoposide. As etoposide is a typical caspase-9-
363 dependent drug^{47, 48}, the reduction in native and cleaved procaspase-9, cytosolic cyt *c* and
364 SMAC mediated by *Naa20* inactivation likely affect etoposide's ability to elicit apoptosis.
365 Conversely, TNF- α , a pleiotropic ligand of TNFR1 and 2, promotes cell survival by activating
366 NF- κ B or cell death by activating procaspase-8⁴⁹. The observed decrease in procaspase-8
367 protein levels in the absence of NatB-mediated Nt-acetylation limits caspase-8 activation which
368 ultimately hampers the apoptotic cascade⁵⁰.

369 An early study investigating the biological function of the catalytic subunit NAA20 in
370 human revealed increased susceptibility of HeLa cells to MG132 after *Naa20* knockdown²⁶.
371 Consistently, our previous study with *Naa25*^{-/-} MEFs also revealed increased susceptibility to

372 MG132²⁷. Moreover, another study performed in *caspase-9*^{-/-} MEFs revealed efficient MG132-
373 induced but weak etoposide-induced apoptosis, which was restored by synergizing with active
374 cytosolic SMAC⁴⁷. Interestingly, the reduced initial protein levels of procaspase-8, -9 and -3
375 and their active forms in *Naa20*^{-/-} compared with WT MEFs did not promote significant
376 differential procaspase activation and PARP1 cleavage in response to MG132 or tunicamycin,
377 neither affected BAX activation nor cyt c and SMAC release when proteasome was inhibited.
378 It is possible that inhibition of the proteasome by MG132 could counteract the degradation of
379 the proapoptotic factors in the absence of NatB-mediated acetylation. Furthermore,
380 proteasome inhibition and ER stress induction are two biological processes that can promote
381 apoptosis through diverse molecular pathways. Importantly, these mechanisms can operate
382 independently of apoptosome formation and can utilize either the extrinsic or intrinsic pathway.
383 Together, these data indicate that the effect of *Naa20* inactivation on apoptosis induction, likely
384 due to reductions in procaspase-8, -9 and -3 and BID protein levels, is stimulus-dependent.

385 Since Nt-acetylation prevents protein degradation and plays a role in maintaining partial
386 proteome stability in MEFs, we sought to identify the N-recognins involved in *Naa20*-mediated
387 degradation of apoptosis effectors. Partial reversion of the decrease in procaspase-8, -9 and
388 BID protein expression levels through *Ubr1* or *Ubr4* downregulation confirmed that their
389 unacetylated N-terminus may be sensed as an N-degron and targeted for degradation.
390 Interestingly, *Ubr4* silencing in *Naa20*^{-/-} MEFs restored procaspase-8 activation after TNF- α
391 plus SMAC mimetic treatment. In response to these stimuli, caspase-3 and -9 activation was
392 less pronounced restored when the *Ubr4* N-recognin was downregulated in *Naa20*^{-/-} MEFs,
393 reflecting that cleavage of BID and procaspase-3 by caspase-8 was insufficient to activate
394 properly the initiator and effector caspases of the intrinsic pathway. Similarly, we show that
395 UBR1 recognizes procaspase-9 as an N-degron, as its decreased protein level in *Naa20*^{-/-}
396 MEFs was also partially restored after *Ubr1* silencing. However, *Ubr1* downregulation in
397 *Naa20*^{-/-} MEFs did not promote procaspase-9 activation in response to etoposide, suggesting
398 that it was insufficient to restore activation of the intrinsic pathway.

399 Taken together, our data reveal that NatB-dependent acetylation of several apoptotic
400 factors protects them from specific UBR E3 ligases-mediated degradation. This further
401 highlights the impact of NatB and Arg/N-degron pathway interdependence on apoptosis. Most
402 interestingly, absence of NatB-dependent acetylation of procaspase-8 exposed the
403 unacetylated N-terminus to UBR4-mediated degradation, thereby limiting caspase-8 activation
404 upon extrinsic stimuli (**Fig. 6**). Our study supports the relevance of NatB-dependent NTA for
405 cellular proteostasis of key pro-apoptotic factors and consequent apoptosis, paving the way
406 for new therapeutic strategies.

407

408 **Data availability**

409 Mass-spectrometry based proteomics data are deposited in ProteomeXchange Consortium
410 (<https://proteomecentral.proteomeexchange.org>) via the PRIDE repository
411 (<https://www.ebi.ac.uk/pride/>) with the data set identifier PXD029641, username:
412 reviewer_pxd029641@ebi.ac.uk, **Password:** O8uvsKWw.

413

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426 **Author Contributions**

427 Conceptualization, R.A, C.G. and M.C.R.; Methodology design, C.G., R.A., M.C.R., T.M.,
428 J.B.B., J.P.G., J.E., and B.C.; Formal analysis, C.G., R.A., M.C.R., T.M., J.B.B., J.P.G., J.E.,
429 B.C., V.R. and L.S.; Investigation, C.G., R.A., M.C.R., T.M., J.B.B., J.P.G., J.E., B.C., V.R. and
430 L.S.; Writing original draft, R.A, C.G. M.C.R. and J.P.G.; Writing-Review and Editing, C.G.,
431 R.A., M.C.R., J.P.G., T.M., and J.B.B. ; Visualization, C.G., R.A., M.C.R., J.P.G., T.M., and
432 J.B.B.; Supervision, R.A, C.G. and M.C.R; Project administration, R.A, C.G. and M.C.R;
433 Funding acquisition, R.A, C.G., and T.M.

434

435 **Competing interests**

436 The authors declare no competing interests.

437

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441

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583

584 **Tables**

585 **Table 1. Most affected N-terminal acetylated substrates resulting from *Naa20* knockout**
586 **in MEFs and their correlation with differences in protein expression when label-free**

587 **quantitation was possible in WT and mutant samples.** Proteins with a significant decrease
 588 in Nt-acetylation (NTA) according to a FDR <5%. NTA Pos.: NTA position; Nt-Seq.: N-terminal
 589 sequence; n.i.: protein not identified in the experiment; #: the first eight most statistically
 590 significant NTA downregulated proteins shown in Fig. 2F are highlighted in grey.

Plot#	Entry Name	Protein Description	NTA Pos.	Nt- Seq.	%NTA (WT)	%NTA (Naa20 ^{-/-})	NTA Difference (Naa20 ^{-/-} - WT)	NTA Ratio (Naa20 ^{-/-})/(WT)	LFQ Protein Ratio (Naa20 ^{-/-} /WT)
1	RS24_MOUSE	40S ribosomal protein S24	1	MNDTVTIRTR	99.4 ± 0.6	33.9 ± 3.4	-65.5	0.341	0.693
2	TM258_MOUSE	Oligosaccharyl transferase subunit TMEM258	1	MELEAMSRYT	93.3 ± 4.2	13.4 ± 2.1	-79.9	0.144	-
3	PRUN1_MOUSE	Exopolyphosphatase PRUNE1	1	MEDYLQDCRA	99.8 ± 0.3	44.9 ± 1.1	-54.9	0.450	2.075
4	IPO7_MOUSE	Importin-7	1	MDPNTIIEAL	99.5 ± 0.8	44.3 ± 3.5	-55.1	0.446	0.516
5	IFM3_MOUSE	Interferon-induced transmembrane protein 3	1	MNHTSQAFIT	97.9 ± 0.6	32.8 ± 1.8	-65.2	0.335	-
6	KEAP1_MOUSE	Kelch-like ECH-associated protein 1	1	MQPEPKLSGA	99.1 ± 0.7	41.5 ± 1.4	-57.6	0.419	-
7	TEBP_MOUSE	Prostaglandin E synthase 3	1	MQPASAKWYD	86.2 ± 7.3	40.7 ± 0.3	-45.5	0.472	0.624
8	AAAS_MOUSE	Aladin	2	CSLGLFPPPP	99.9 ± 0.0	5.9 ± 5.5	-94.1	0.059	-
9	INT3_MOUSE	Integrator complex subunit 3	1	MELQKKGKTV	98.6 ± 0.9	26.1	-72.5	0.265	-
10	WDR46_MOUSE	WD repeat-containing protein 46	1	METAPKPGRG	100.0	50.3 ± 0.7	-49.7	0.503	-
11	PRAF3_MOUSE	PRA1 family protein 3	1	MDVNLAPLRA	99.7 ± 0.3	52.8 ± 3.8	-46.9	0.529	-
12	BABA2_MOUSE	BRISCA and BRCA1-A complex member 2	1	MSPEIALNRI	99.2 ± 0.2	57.8	-41.4	0.583	-
13	RFA3_MOUSE	Replication protein A 14 kDa subunit	1	MEDIMQLPKA	99.6 ± 0.4	58.5 ± 3.1	-41.1	0.588	-
14	ILK_MOUSE	Integrin-linked protein kinase	1	MDDIFTQCRE	99.9 ± 0.1	63.4 ± 0.9	-36.5	0.635	-
15	GTR1_MOUSE	Solute carrier family 2	1	MDPSSKKVTG	99.3 ± 0.7	63.3 ± 2.3	-36.0	0.638	0.623
16	P4K2A_MOUSE	Phosphatidylinositol 4-kinase type 2-alpha	1	MDETSPLVSP	99.0 ± 0.8	66.7	-32.3	0.674	-
17	FBX22_MOUSE	F-box only protein 22	1	MEPAGGGGV	99.9 ± 0.0	67.9 ± 2.9	-32.0	0.680	-
18	TTL12_MOUSE	Tubulin--tyrosine ligase-like protein 12	1	MEIQSGPQPG	99.9 ± 0.1	70.7	-29.2	0.708	0.748
19	KT3K_MOUSE	Ketosamine-3-kinase	1	METLLKRELG	99.9 ± 0.0	70.9 ± 0.1	-29.1	0.709	-
20	BCD1_MOUSE	Box C/D snoRNA protein 1	1	MESAAEKEGT	99.5 ± 0.5	71.9 ± 1.8	-27.5	0.723	-
21	NDUAC_MOUSE	NADH dehydrogenase 1 alpha subcomplex subunit 12	1	MELVEVLKRG	99.9 ± 0.0	73.3 ± 2.0	-26.7	0.733	-
22	FARP1_MOUSE	FERM, RhoGEF and pleckstrin domain-containing protein 1	2	GEIEQKPTPA	96.9 ± 2.7	70.4 ± 2.6	-26.6	0.726	0.936
23	TCPA_MOUSE	T-complex protein 1 subunit alpha	1	MEGPLSVFGD	99.6 ± 0.5	74.0 ± 4.3	-25.5	0.743	0.944
24	AN32E_MOUSE	Acidic leucine-rich nuclear phosphoprotein 32 family member E	1	MEMKKKINME	99.9 ± 0.0	75.8 ± 2.6	-24.2	0.758	0.990
25	THIC_MOUSE	Acetyl-CoA acetyltransferase	1	MNAGSDPVVI	99.6 ± 0.3	76.9 ± 4.4	-22.8	0.772	0.921
26	SYRC_MOUSE	Arginine--tRNA ligase	1	MDGLVAQCSA	99.5 ± 0.5	79.5 ± 4.7	-20.0	0.799	1.009
	CASP3_MOUSE	Procaspase-3	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	0.649
	CASP8_MOUSE	Procaspase-8	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
	CASP9_MOUSE	Procaspase-9	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
	MARH6_MOUSE	E3 ubiquitin-protein ligase MARCHF6	1	MDTAEEDICR	NTA	NTA	-	-	n.i.
	UBR4_MOUSE	E3 ubiquitin-protein ligase UBR4	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.	0.699

591

592

593 **Table 2. Differentially accumulated proteins when the NatB catalytic subunit is**
 594 **inactivated in MEFs.** Most affected proteins ($2 < \text{LFQ protein ratio} < 0.5$) are presented.
 595 For an exhaustive list, see Supplementary Dataset1. Blue line divides the
 596 downregulated proteins from the upregulated proteins. See also Volcano plot, Fig. 2F.
 597

Plot#	Entry Name	Protein Description	LFQ Protein Ratio <i>Naa20^{-/-}</i> / WT	%NTA Difference <i>Naa20^{-/-}</i> - WT 600
1	TAGL_MOUSE	Transgelin	0.331	-1.3 (Ala2)
2	SERC_MOUSE	Phosphoserine aminotransferase	0.492	-0.4 (iMet)
3	LYOX_MOUSE	Protein-lysine 6-oxidase	0.228	-
4	USO1_MOUSE	General vesicular transport factor p115	0.367	-
5	SPEE_MOUSE	Spermidine synthase	0.444	-3.3 (iMet)
6	AN32B_MOUSE	Acidic leucine-rich nuclear phosphoprotein 32 family member B	0.404	-
7	SET_MOUSE	Protein SET	0.314	-
8	CAVN1_MOUSE	Caveolae-associated protein 1	0.425	-15.9 (iMet)
9	FACR1_MOUSE	Fatty acyl-CoA reductase 1	0.474	-
10	HNRL2_MOUSE	Heterogeneous nuclear ribonucleoprotein U-like protein 2	0.282	-
11	LOXL1_MOUSE	Lysyl oxidase homolog 1	0.423	-
12	RTN4_MOUSE	Reticulon-4	0.462	-4.0 (iMet)
13	RAB18_MOUSE	Ras-related protein Rab-18	0.468	-
14	RS30_MOUSE	40S ribosomal protein S30	0.372	0.0 (Lys1)
15	IMA7_MOUSE	Importin subunit alpha-7	0.416	-0.4 (iMet)
16	EF1B_MOUSE	Elongation factor 1-beta	0.405	-
17	U2AF1_MOUSE	Splicing factor U2AF 35 kDa subunit	0.450	-
18	RLA1_MOUSE	60S acidic ribosomal protein P1	0.406	-0.1 (Ala2)
19	YTHD2_MOUSE	YTH domain-containing family protein 2	0.438	-0.2 (Ser1)
20	QKI_MOUSE	Protein quaking	0.460	-
21	LYAR_MOUSE	Cell growth-regulating nucleolar protein	0.466	-
22	KCD12_MOUSE	BTB/POZ domain-containing protein KCTD12	5.143	-
23	BGLR_MOUSE	Beta-glucuronidase	2.274	-
24	ASAH1_MOUSE	Acid ceramidase	2.084	-
25	NEP_MOUSE	Nephrilysin	3.020	-
26	HYEP_MOUSE	Epoxide hydrolase 1	3.681	-
27	HXK1_MOUSE	Hexokinase-1	3.404	-
28	SCOT1_MOUSE	Succinyl-CoA:3-ketoacid coenzyme A transferase 1	2.209	-
29	TPP1_MOUSE	Tripeptidyl-peptidase 1	2.099	-
30	INF2_MOUSE	Inverted formin-2	2.108	-0.2 (Ser2)
31	CSN3_MOUSE	COP9 signalosome complex subunit 3	2.273	0.0 (Ala2)
32	S61A1_MOUSE	Protein transport protein Sec61 subunit alpha isoform 1	2.887	-0.3 (Ala2)
33	H32_MOUSE	Histone H3.2	3.601	-
34	DDX3Y_MOUSE	ATP-dependent RNA helicase DDX3Y	2.001	-
35	HEXB_MOUSE	Beta-hexosaminidase subunit beta	2.052	-
36	BACH_MOUSE	Cytosolic acyl coenzyme A thioester hydrolase	2.098	-

626 **Figure Legends**

627 **Fig. 1 In *Naa20*^{-/-} MEFs lacking the catalytic subunit of NatB, *Naa20* mRNA and NAA20**
628 **protein are not expressed, and cellular proliferation and the actin cytoskeleton are**
629 **perturbed. (A)** *Naa20* mRNA quantification analysis in MEFs infected with the AdEmpty (WT)
630 and Ad5CMVCre (*Naa20*^{-/-}) by RT-PCR. Data are the mean ± SEM of six independent
631 experiments and are normalized to a housekeeping gene (Histone 3) and analyzed by
632 Student's *t*-test. **(B)** NAA20 protein levels were detected by western blotting in WT and *Naa20*^{-/-}
633 cells using antibodies targeting NAA20. **(C)** Cell proliferation was visualized as the number of
634 cells per well along post-infection days. Values represent the average number of WT or *Naa20*^{-/-}
635 MEFs from three independent replicates as mean ± SEM (Student's *t*-test). **(D)**
636 Representative confocal microscopy images of actin (phalloidin) and focal adhesions (vinculin)
637 in WT or *Naa20*^{-/-} MEFs.

638

639 **Fig. 2 N-terminomic and proteomic analyses of *Naa20*^{-/-} MEFs reveal reduced N-terminal**
640 **(Nt-) acetylation, specifically of NatB substrates, and deregulated protein levels.**
641 Accumulated distribution representing the percentage of Nt-acetylated **(A)** total proteome, **(B)**
642 peptides with the initial methionine (iMet) present, **(C)** peptides with Nt-methionine excised,
643 and **(D)** peptides with processed N-termini identified in normal (WT) and *Naa20*^{-/-} MEFs. **(E)**
644 IceLogo representation of the N-terminal sequences with a minimum 20% decrease in NTA
645 yield to those with <5% variation, when comparing the *Naa20*^{-/-} to the WT MEFs. Volcano plot
646 representing **(F)** proteins with an Nt-acetylation reduction of 40% or more (green spots). The
647 dashed horizontal line shows the p-value cut off, and the eight points highlighted in green
648 indicate the affected proteins with the highest statistical significance. **(G)** Proteins with a label-
649 free quantification (LFQ protein ratio) either <0.5 (green spots) or >2.0 (red spots) in the *Naa20*^{-/-}
650 compared with WT samples. The dashed horizontal line shows the p-value cut off, and the
651 points highlighted in green and red represent the downregulated and upregulated proteins,
652 respectively. All plots contain only the N-termini that have been quantified at least once in each
653 condition (WT or *Naa20*^{-/-}). FDR - false discovery rate; N – number of samples.

654

655 **Fig. 3 Inactivation of *Naa20* decreases the protein expression of procaspase-8, -9, and -**

656 **3, and BID, which block the extrinsic and intrinsic apoptotic pathways. (A)**

657 Representative western blot images of the NatB substrates procaspase-8, -9, and -3 and of

658 BAX, BID, APAF1 and non-NatB substrate procaspase-6, SMAC and cleaved PARP. MEFs

659 were harvested 4, 5, and 6 days after infection with AdEmpty (WT) or Ad5CMVCre (^{-/-}) inducing

660 *Naa20* inactivation. Procaspase-6 and SMAC, which are not NatB substrates, were used as

661 controls. GAPDH was used a loading control. **(B)** Relative quantification by RT-PCR of the

662 same NatB substrates as in (A), six days post-infection. Data are the mean ± SEM of six

663 independent experiments and are normalized to a housekeeping gene (Histone 3). Student's

664 *t*-test was used to evaluate differences between groups with the obtained values indicated

665 when statistical significance was achieved (*p*<0.05). **(C)** Representative western blot images

666 of procaspase-8, -9, and -3 and of respective cleaved caspases, cleaved PARP and BID in

667 *Naa20*^{-/-} MEFs six days after infection with AdEmpty (WT) or Ad5CMVCre (^{-/-}) 0, 8, 12, and 24

668 h after treatment with 100 ng/mL TNF-α plus 500 nM SMAC mimetic. **(D)** As in (C) but after

669 treatment with 50 μM etoposide.

670

671 **Fig. 4 Inactivation of *Naa20* has no impact on BAX localization in response to etoposide,**

672 **TNF-α + SMAC mimetic, and MG132 treatments but decreases the release of cytochrome**

673 **c and SMAC in response to etoposide. (A)** Representative western blot images of BAX,

674 cytochrome c, COX IV, and SMAC in total extracts, the cytosolic fraction, and mitochondrial

675 fraction of *Naa20*^{-/-} MEFs six days after infection with AdEmpty (WT) or Ad5CMVCre (^{-/-}), under

676 basal conditions. **(B)** As in (A) 12 h after 50 μM etoposide treatment. **(C)** As in (A) 8 h after 5

677 μM MG132 treatment. WT and *Naa20*^{-/-} MEF cells were fractionated 6 days after adenovirus

678 inoculation. Cytosolic GAPDH and mitochondrial COX IV were used as loading controls of

679 cytosolic and mitochondrial fractions, respectively.

680

681 **Fig. 5 Silencing the ubiquitin ligases *Ubr4* and *Ubr1* in *Naa20*^{-/-} MEFs rescues the**
682 **decrease in procaspase-8 and -9 protein expression, respectively, as well as BID and**
683 **promotes the activation of procaspase-8 and procaspase-3 in response to TNF- α plus**
684 **SMAC mimetic. (A)** Representative western blot images of procaspase-8, -9, and -3 and BID
685 protein levels after silencing *March6*, *Ubr4*, *Cnot4*, *Ubr1*, and *Ubr2* ubiquitin ligases in *Naa20*
686 ^{-/-} MEFs six days after infection with AdEmpty (WT) or Ad5CMVCre (^{-/-}). A specific siRNA
687 (siControl) was used as control. **(B)** Representative western blot images of the procaspases
688 and cleaved caspase-8, -9, and -3 protein levels after silencing the *Ubr4* ubiquitin ligase in
689 NAA20 MEFs six days after infection with AdEmpty (WT) or Ad5CMVCre (^{-/-}), before (basal
690 conditions) and 12 h after treatment with 100 ng/ml TNF- α plus 500 mM SMAC mimetic. PBS
691 plus DMSO was used as a negative control of TNF- α plus SMAC mimetic. A non-specific
692 siRNA (siControl) was used for control.

693

694 **Fig. 6 Model depicting the role of NatB-dependent Nt-acetylation in the control of**
695 **extrinsic apoptosis induction.** Procaspase-8 and procaspase-3 are NatB substrates Nt-
696 acetylated in normal cells. TNF- α binding to its receptor in normal cells promotes procaspase-
697 8 activation, which activates procaspase-3 and promotes apoptosis. Inactivation of the NatB
698 catalytic subunit (*Naa20* KO) reduces procaspase-8 Nt-acetylation, and therefore E3-ubiquitin
699 ligase UBR4 recognizes procaspase-8 N-terminus as an N-degron, marks, and sends
700 procaspase-8 for proteasomal degradation. Procaspase-3 is also less stable when *Naa20* is
701 inactivated, but the E3-ubiquitin ligase has not been identified. Therefore, binding of TNF- α to
702 its receptor when NatB is inactivated limits apoptosis induction as a consequence of reduced
703 procaspase-8 and -3 in these cells. Image created with Biorender.

704

705

706

707

Fig. 1

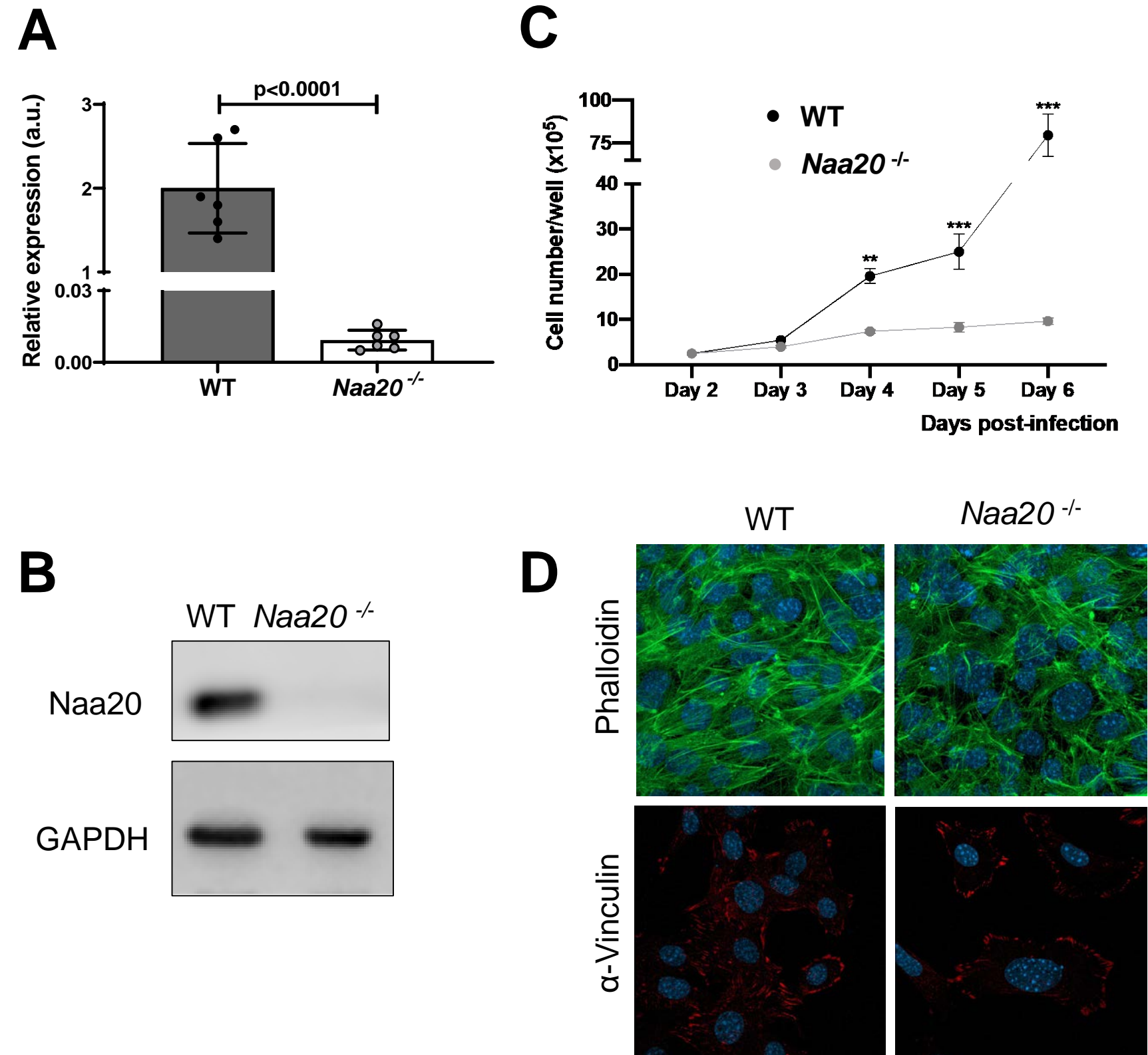


Fig. 2

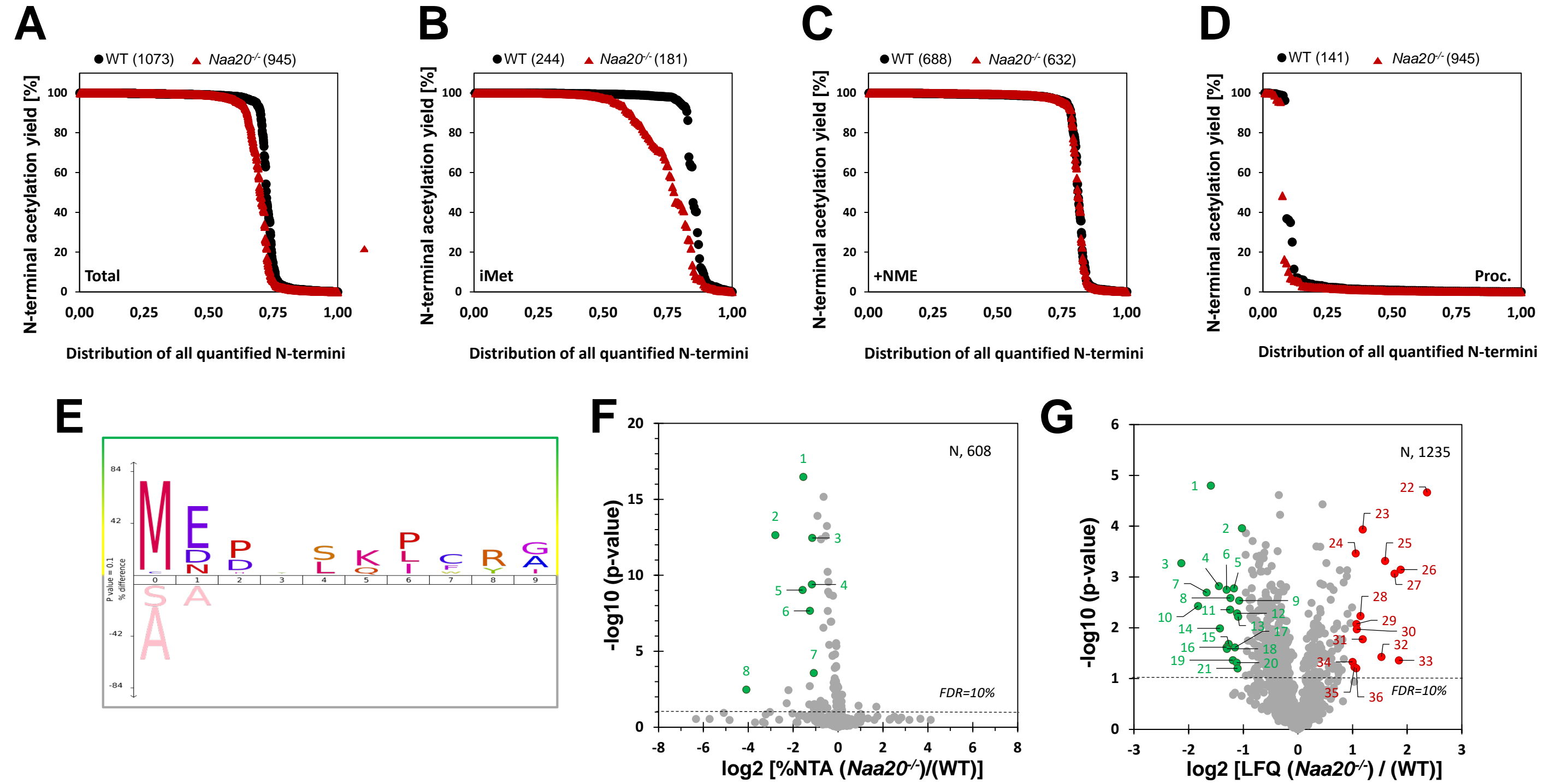
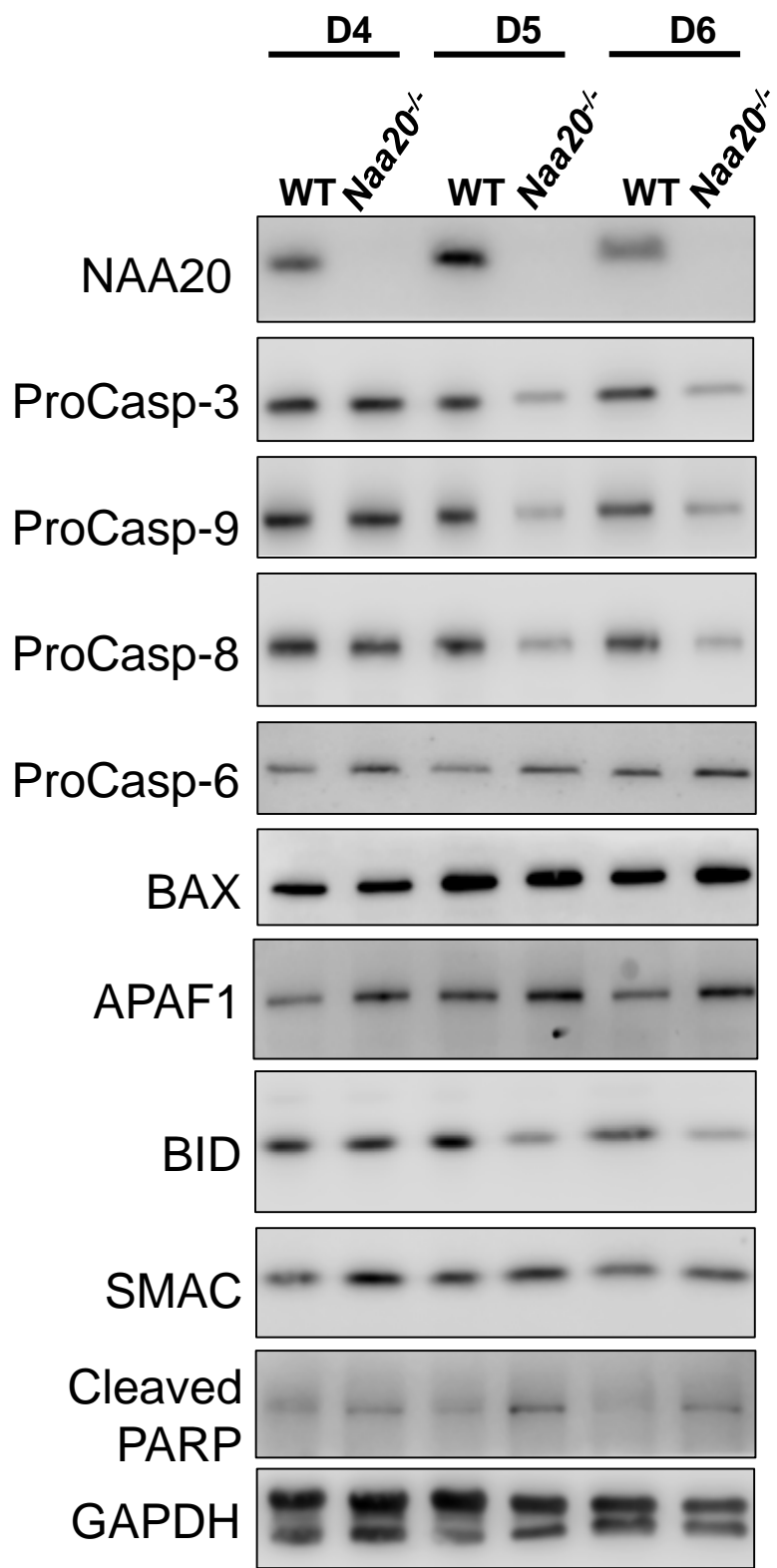
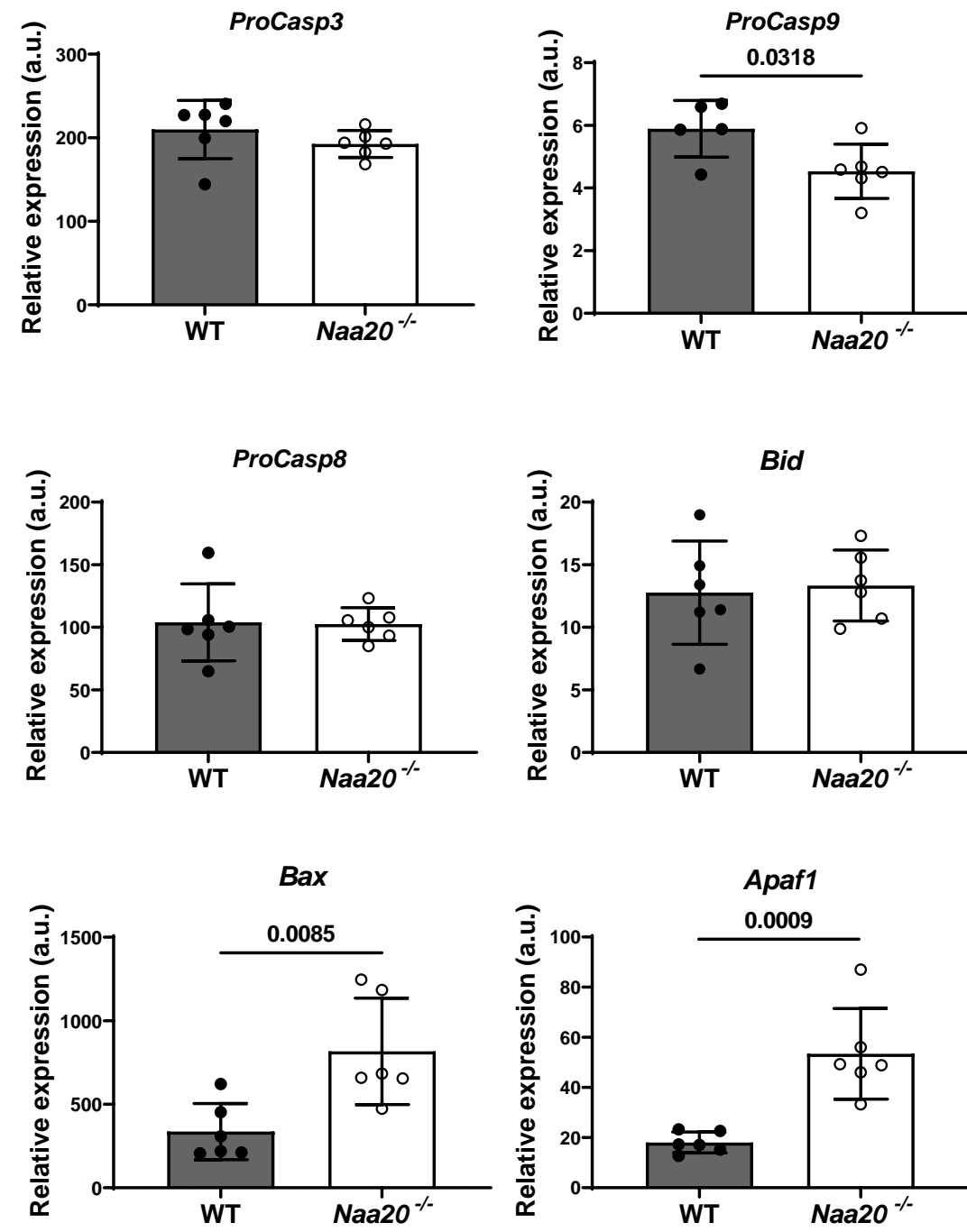


Fig. 3

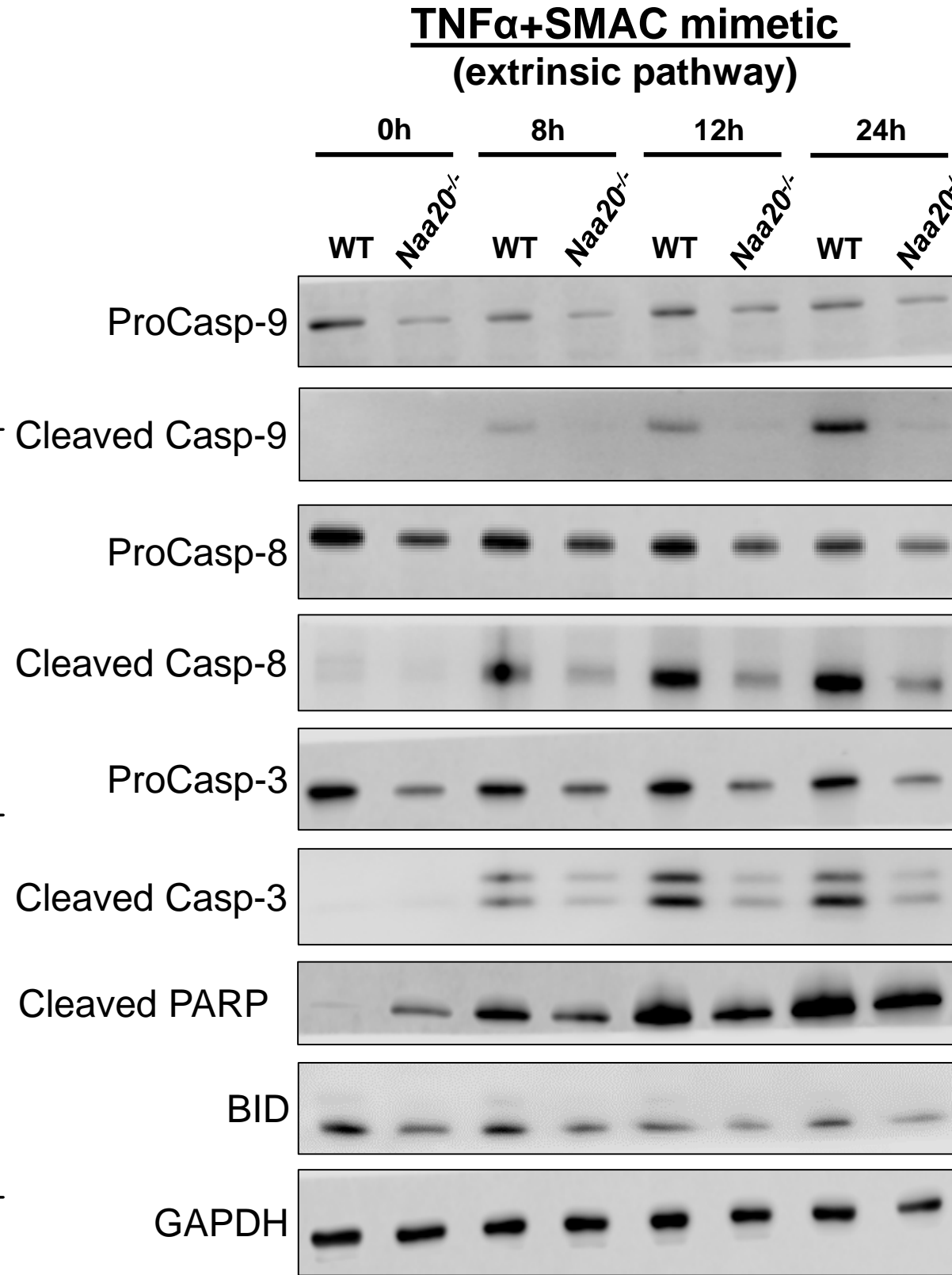
A



B



C



D

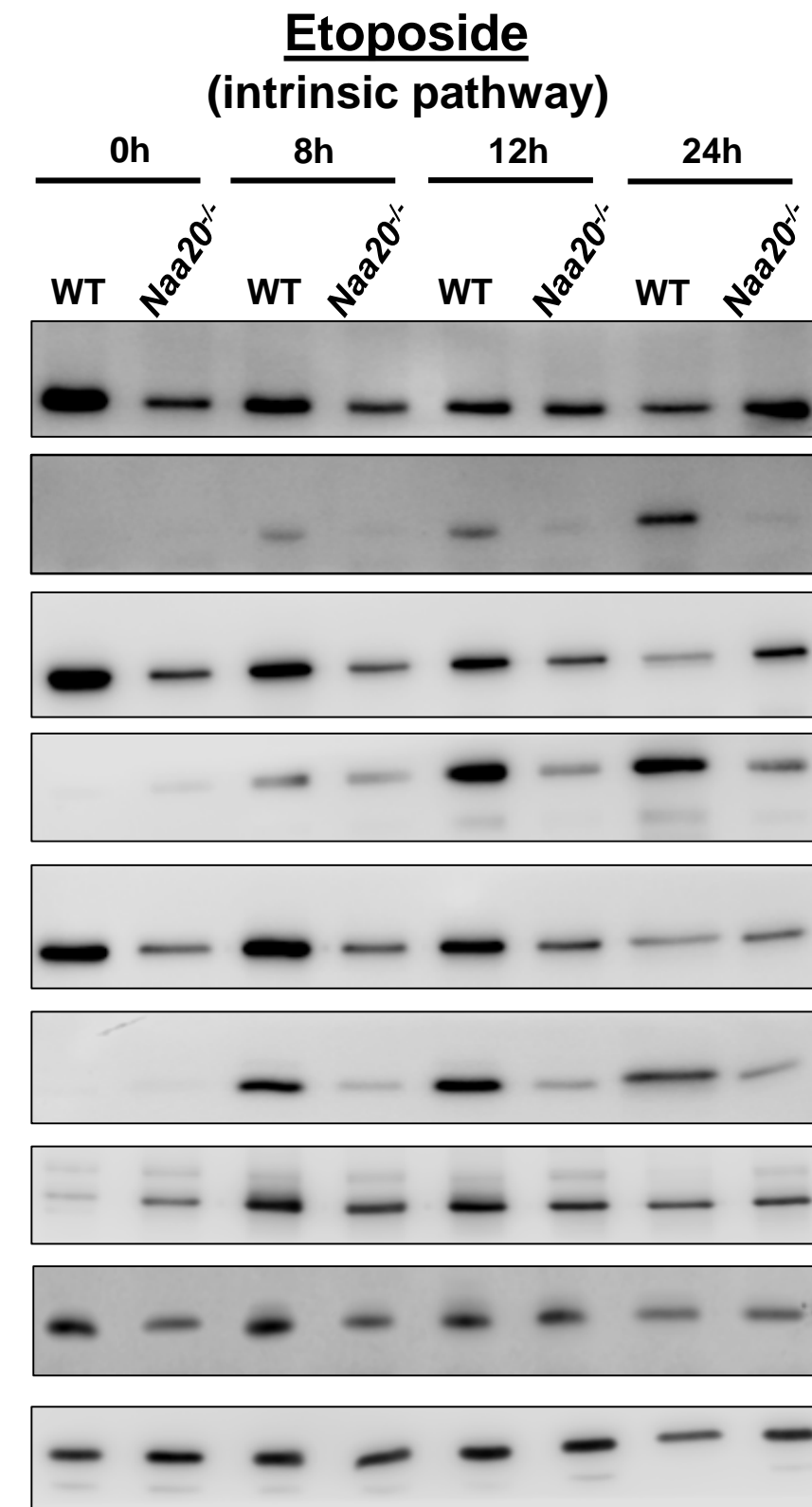


Fig. 4

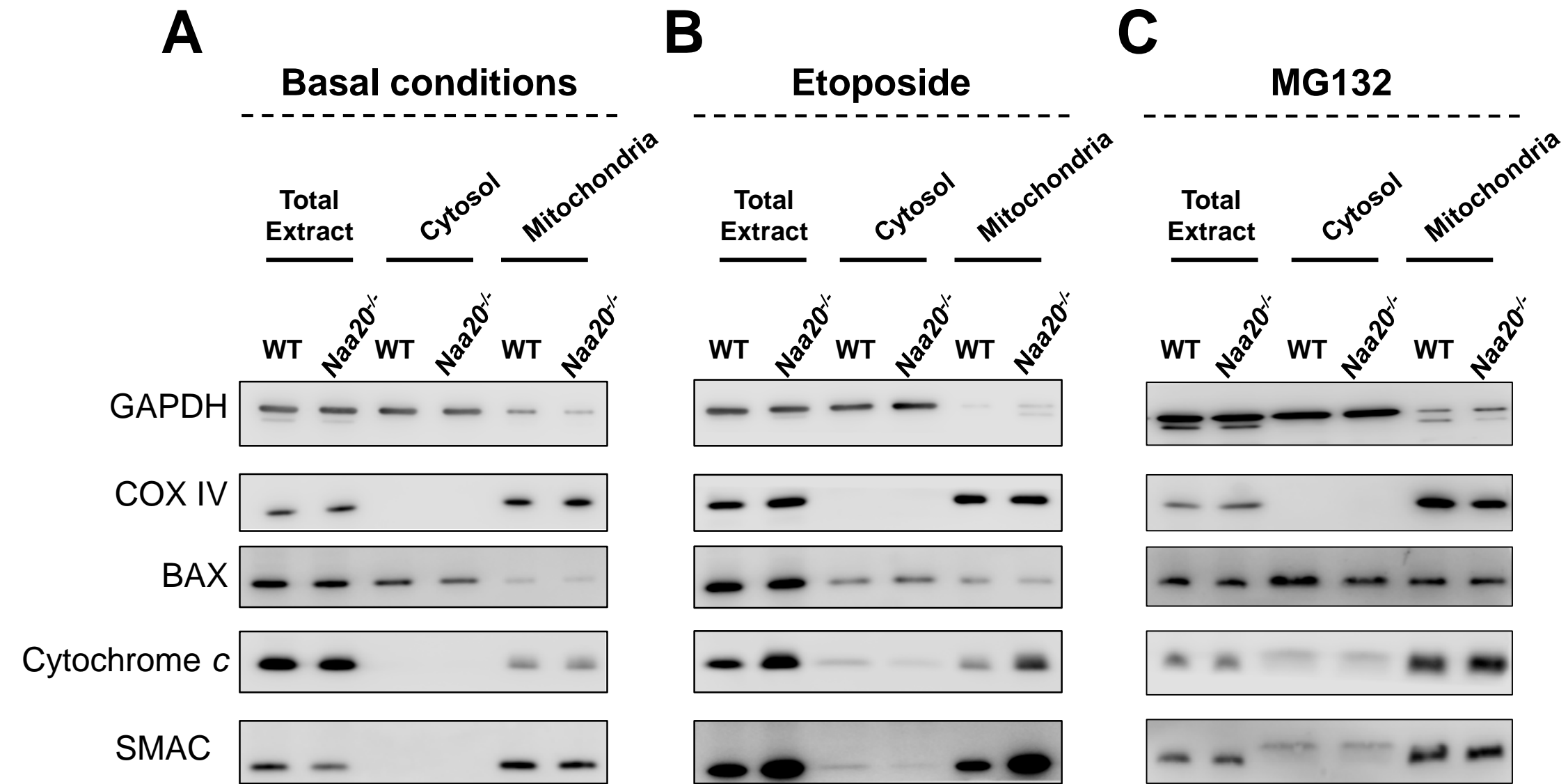


Fig. 5

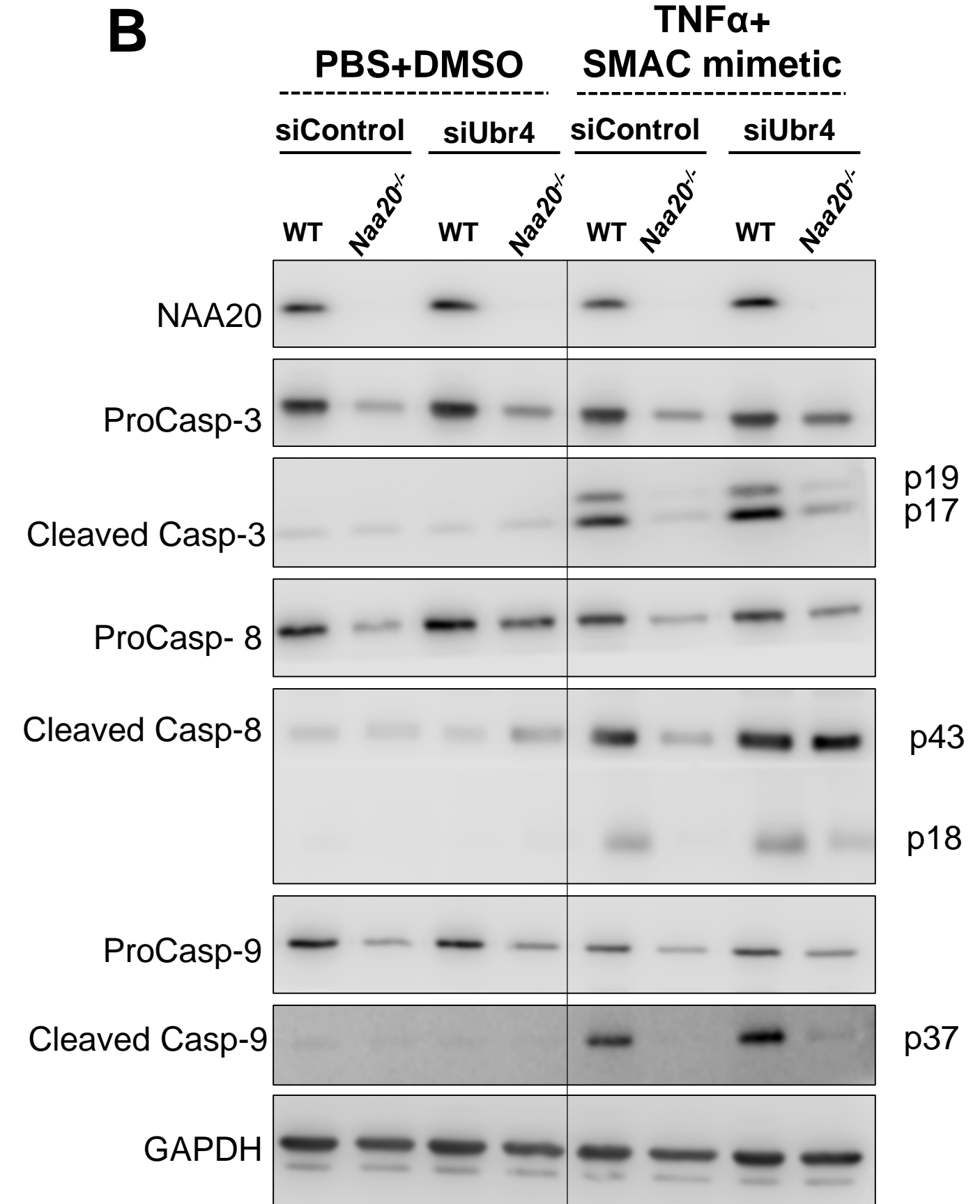
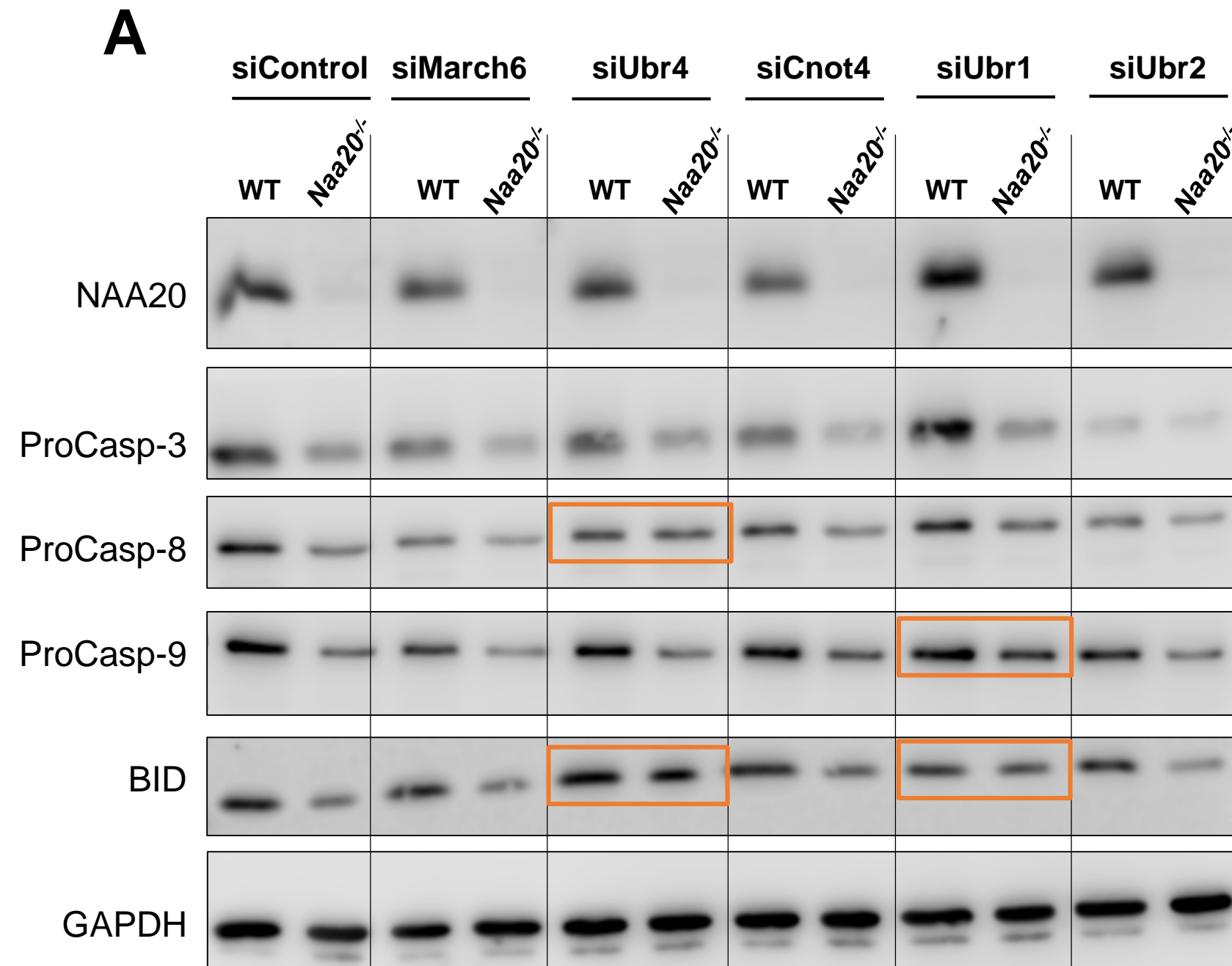


Fig. 6

