

25 **Abstract**

26 NR4A is a nuclear receptor protein family whose members act as sensors of cellular
27 environment and regulate multiple processes such as metabolism, proliferation, migration,
28 apoptosis, and autophagy. Since the ligand binding domains of these receptors have no
29 cavity for ligand interaction, their function is most likely regulated by protein abundance
30 and post-translational modifications. In particular, NR4A1 is regulated by protein
31 abundance, phosphorylation, and subcellular distribution (nuclear-cytoplasmic
32 translocation), and acts both as a transcription factor and as a regulator of other
33 interacting proteins. SUMOylation is a post-translational modification that can affect protein
34 stability, transcriptional activity, alter protein-protein interactions and modify intracellular
35 localization of target proteins. In the present study we evaluated the role of SUMOylation
36 as a posttranslational modification that can regulate the activity of NR4A1 to induce
37 autophagy-dependent cell death. We focused on a model potentially relevant for neuronal
38 cell death and demonstrated that NR4A1 needs to be SUMOylated to induce autophagic
39 cell death. We observed that a triple mutant in SUMOylation sites has reduced
40 SUMOylation, increased transcriptional activity, altered intracellular distribution, and more
41 importantly, its ability to induce autophagic cell death is impaired.

42

43 **Summary Statement**

44 The modification of the nuclear receptor NR4A1 by SUMO regulates its transcriptional
45 activity, intracellular localization and is required to induce autophagic cell death.

46

47 **Short title:** SUMOylated NR4A1 induces autosis

48

49 **Keywords:**

50 NR4A1, SUMO, autophagy, cell death, Substance P, NK₁R

51

52 **Abbreviations list**

53	NR4A	Nuclear receptor group family A
54	NR4A1	Nuclear receptor group 4 family A member 1 (Nur77, NGF1B, TR3, etc.)
55	NR4A2	Nuclear receptor group 4 family A member 2 (Nurr1, NOT1, etc)
56	NR4A3	Nuclear receptor group 4 family A member 3 (Nor1, MINOR)
57	SUMO	small ubiquitin-like modifier
58	SP	Substance P
59	NK ₁ R	Neurokinin 1 Receptor
60	TAD	Trans-Activation Domain
61	DBD	DNA Binding Domain
62	LBD	Ligand Binding Domain
63	PTM	Post Translational Modifications

64 **Introduction**

65 Nuclear receptors are a superfamily of transcription factors involved in a vast number of
66 biological processes. They share three common structural domains: a N-terminal
67 transactivation domain (TAD), a central double zinc finger DNA binding domain (DBD) and
68 a C-terminal ligand binding domain (LBD). Among the known human nuclear receptors,
69 the ones belonging to the NR4A family act as sensors of the cellular environment and
70 contribute to cell fate decisions, such as cell proliferation, differentiation, migration, cell
71 death, etc. Physiologically, NR4A members influence the adaptive and innate immune
72 system, angiogenesis, metabolism and brain function. The NR4A family is comprised of
73 three members that bind the same DNA elements (1): NR4A1 (Nur77, TR3, NGF1B, etc.),
74 NR4A2 (Nurr1, NOT, TINUR, etc.) and NR4A3 (Nor1, MINOR, etc.). NR4A family
75 members are considered orphan nuclear receptors since endogenous ligands are
76 unknown and, although several natural and artificial compounds enhance their
77 transcriptional activity, they are able to activate transcription by solely up-regulating the
78 expression of their genes (1). Therefore, rather than regulation by ligand interactions, their
79 endogenous function is most likely regulated by NR4A family members protein abundance
80 and post-translational modifications (PTM). All NR4A family members are mainly modified
81 by phosphorylation by over 20 different kinases described so far, and at least NR4A1 is
82 also acetylated by CBP/p300 and deacetylated by HDAC1. Specific PTM affect their
83 interaction with either DNA or other proteins, as well as their intracellular localization (1).

84 A wide variety of stimuli induces the expression of *Nr4a* genes, both during
85 development and in adulthood. For example, they are induced in response to caloric
86 restriction (2), exercise (3) or during learning and long term memory (4, 5). NR4A family
87 proteins regulate the expression of several genes, some of which are involved in lipid and
88 glucose metabolism, insulin sensitivity and energy balance. Another important function of
89 NR4A family proteins is to prevent DNA damage and to promote DNA repair (6, 7). NR4A1

90 also has non-genomic activities both in the nucleus and in the cytoplasm, altering the
91 function of interacting proteins. For all the functions described above, understanding the
92 molecular regulation of NR4A activity is an active area of research.

93 Autophagy is mainly a catabolic process that allows cells to recycle
94 macromolecules when needed or to eliminate damaged proteins and organelles, among
95 other components, contributing to cell health (8); occasionally, however, it also contributes
96 to an alternative secretion mechanism (9) or even to cell death (10). When inhibition of
97 autophagy prevents cell death, it is referred to as autophagic cell death, although the
98 actual cause of cell death still needs to be understood. We found previously that NR4A1
99 plays an essential role in a form of cell death induced by several stimuli that is non-
100 apoptotic (in apoptosis-competent cells) and is dependent on autophagy. NR4A1
101 inactivation by either over-expression of dominant negative mutants or by RNAi prevents
102 cell death, and inhibition of autophagy either pharmacologically or by RNAi to reduce
103 autophagic gene expression also prevents cell death (11-13). Hence, NR4A1 mediates
104 autophagic cell death, a phenomenon that was subsequently confirmed by others. In the
105 case of melanoma cells treated with THPN, a chemical compound targeting NR4A1, cell
106 death occurs after induction of excessive mitophagy, due to NR4A1 translocation to the
107 inner membrane of the mitochondria, causing dissipation of mitochondrial membrane
108 potential by the permeability pore complex ANT1/VDAC (14). Furthermore, Dendrogenin
109 A, a mammalian cholesterol metabolite ligand of liver-X-receptors (LCRs) induces *Nr4a1*
110 expression in association with excessive autophagic cell death both *in vitro* and *in vivo*
111 (15, 16), displaying anticancer and chemopreventive properties in mice(17). Interestingly,
112 NR4A1 interaction with anti-apoptotic BCL2 family members outside the BH3 domain
113 induces autophagic cell death (18), which seems to be mediated by releasing BECN1 in a
114 model of cigarette smoke-induced autophagic cell death(19). Taken together, these
115 findings indicate that NR4A1 could function as a broad inducer of autophagic cell death.

116 Some authors have coined autophagic cell death as autosis (20); we will use this term
117 hereafter for simplicity, although whether NR4A1-induced autophagic cell death is
118 mediated by Na⁺,K⁺- ATPase pump, as has been documented for autosis, has not been
119 addressed.

120 In the present work we aimed to study whether specific PTM confer upon NR4A1
121 the ability to induce autosis, by using our already described model of NR4A1-mediated
122 autosis, induced upon Substance P (SP) binding to its NK₁R receptor (here referred to as
123 SP/NK₁R-induced autosis). SP is a neuropeptide involved in several physiological
124 functions and pathological situations, including emotional behavior, pain perception,
125 addiction, inflammation, neurodegeneration, etc. Accordingly, NK₁R is expressed all
126 around the body, including endothelial cells and in both central and peripheral nervous
127 system, and SP is present in all body fluids such as cerebrospinal fluid, blood, etc. (21).
128 We focused in this model for its relevance in neuronal cell death, as interfering with SP
129 signaling reduces infarct volume and neuronal cell death in *in vivo* models of ischaemia
130 (22) and of excitotoxin-induced seizures (23), both situations in which NR4A1 expression
131 is also induced (*i.e.* ischaemia (24) and kainic acid-triggered seizures (25)).

132 During SP/NK₁R-induced autosis, NR4A1 is regulated by both protein abundance
133 and phosphorylation (12), and undergoes nuclear-cytoplasmic translocation (13),
134 potentially having both genomic and non-genomic functions. A previous report showed
135 that NR4A1 undergoes sequential SUMOylation and ubiquitination, which together control
136 the degradation of NR4A1 after induced stress (26). SUMO modification can affect protein
137 stability, transcriptional activity, protein-protein interactions and intracellular localization of
138 target proteins (27). In addition, SUMO modification affects numerous cellular processes
139 overlapping with those described for NR4A1 function, both in development and adulthood.
140 Even though the SUMO machinery is mainly located in the nucleus, numerous cytoplasmic
141 proteins have also been identified to have their function modulated by SUMO modification

142 (28, 29). Therefore, we hypothesize that NR4A1 might be SUMOylated in response to
143 SP/NK₁R signaling, conferring upon it the ability to induce autosis.

144 SUMOs are small ubiquitin-related peptides, approximately 11 KDa size, that
145 become conjugated to a variety of proteins and are deconjugated by SUMO-specific
146 proteases. Despite the name, SUMO shares less than 20% homology with ubiquitin. The
147 similarity is more significant in the biochemical mechanism of ligation, as it involves three
148 conjugating enzymes: E1, a dimer known as SAE1/SAE2; E2, a protein named UBC9; and
149 E3, which are a group of proteins conferring target specificity. There are four genes in
150 mammals coding for four SUMO peptides (SUMO1-4). SUMO2 and 3 share 95%
151 homology and there are no antibodies that distinguish between them, so they are referred
152 to as SUMO2/3; they are only 50% identical to SUMO1 (reviewed in (30)).

153 NR4A1 is an early response gene, whose expression is induced in minutes, and the
154 protein is degraded after a couple of hours. However, we observed that NR4A1 expression
155 is sustained during SP/NK₁R-induced autosis, suggesting that in this scenario NR4A1
156 protein would scape degradation. In this work, we confirmed that NR4A1 is indeed
157 SUMOylated during SP/NK₁R-induced autosis, and mutants with reduced SUMOylation
158 showed increased transcriptional activity and altered intracellular distribution. More
159 importantly, the ability to promote SP/NK₁R-induced autosis was impaired when
160 SUMOylation of NR4A1 was reduced.

161

162 **Experimental Methods**

163 *Cell culture, plasmid transfection and cell death evaluation.*

164 Human embryonic kidney 293 (HEK293) cells were grown in high glucose DMEM
165 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma, St. Louis,
166 MO) and penicillin/streptomycin 100 U/ml (Invitrogen, Carlsbad, CA). The cultures were
167 incubated at 37°C in 95% air and 5% carbon dioxide with 95% humidity. Transient

168 transfection was performed with Polyethylenimine (PEI, 25 kDa Polysciences Inc. # 23966-
169 2) mixed with DNA in a 3:1 ratio. Briefly, 2×10^5 cells/well were seeded into 35 mm wells
170 16-20 hr prior to transfection. Transfection solution: 1 μ g of DNA was diluted into 75 μ l
171 OPTIMEM; 3 μ g PEI was diluted into 75 μ l OPTIMEM; then mixed and incubated for 15
172 minutes at room temperature. 0.5 ml of medium was removed from each well and the
173 mixture was added dropwise to the cells. After 4 hr at 37 °C the medium was refresh
174 supplemented without antibiotics. After 24 hr, 100 nM SP (SIGMA) was added when
175 necessary. Expression of each construct in the transient transfections was determined by
176 Western blot or immunofluorescence. Transient transfection efficiencies were in all cases
177 >80%. The plasmid pcDNA3.1-NK₁R has previously been described (11, 31). Dr. Jacques
178 Drouin (Laboratoire de Génétique Moléculaire, Institut de Recherches Cliniques de
179 Montréal, Canada) kindly provided POMC-Luc, NBRE-POMC-Luc and NuRE-POMC-Luc
180 reporter plasmids. Site-directed mutagenesis was performed using QuickChange Kit
181 (Invitrogene). The alignment and prediction of consensus motifs was performed using
182 Clustal W2 software, using the sequence UniProtKB - P22736 (NR4A1_HUMAN). All
183 position information refers to this entry. Cell death was determined by Trypan blue
184 exclusion or LDH release. The software PRISM 6.0 (GraphPad Software, La Jolla, CA,
185 USA) was used for the one-way ANOVA statistical analysis, and the p values between
186 indicated treatments in figures were calculated by Bonferroni's Multiple Comparison Test.

187 **Western blot, immunoprecipitation and immunofluorescence analysis.**

188 For Western blotting, the transfected human embryonic kidney 293 cells were washed with
189 cold PBS and homogenized in lysis buffer (150 μ M NaCl, 1% Triton X-100, 50 μ M Tris HCl
190 pH 8.0, proteinases inhibitor cocktail cOmplete ULTRA and phosphatases inhibitor cocktail
191 PhosSTOP (Roche Diagnostic Corporation, Indianapolis, IN, USA)). Cytoplasmic extracts
192 were collected after 10 min. centrifugation at 14,000 rcf. Protein was quantified by
193 Bradford assay and electrophoresis of equal amounts of total protein was performed on

194 SDS-polyacrylamide gels. Separated proteins were transferred to polyvinylidene fluoride
195 membranes at 4° C. Membranes were probed with the following antibodies: SUMO1
196 (1:1000 #4930), SUMO2/3 (1:1000 #4971), phospho-Threonine (1:500 #9381), Myc (1:350
197 #2276) and NR4A1 (1:2000 #3960) from Cell Signaling Technology Inc., Danvers, MA,
198 USA; GAPDH (1:8000, Research Diagnostics, Flanders, NJ, USA); TUBULIN (1:7000,
199 Abcam, Cambridge, MA, USA); FLAG (1:1000, #F3165 SIGMA, San Luis, Missouri, USA);
200 UBC9 (1:250, #610748 BD Biosciences, San Jose, CA); UBIQUITIN (1:1000, #U5379
201 SIGMA, San Luis, Missouri, USA); HA (#H6908 SIGMA, San Luis, Missouri, USA). The
202 membranes were incubated in the appropriate horseradish peroxidase-coupled secondary
203 antibody for 1 hr followed by enhanced chemiluminescence detection (Amersham,
204 Arlington Heights, IL). Alternatively, appropriate infrared dye-coupled secondary antibodies
205 (1:10,000 dilution of anti-rabbit IRDye800 and anti-mouse IRDye700, Rockland,
206 Gilbertsville, PA, USA) were used and the blots were scanned in an Odyssey Imager (LI-
207 COR Biosciences, Lincoln, Nebraska, USA). The immunoprecipitations were carried out
208 using super-paramagnetic Microbeads conjugated to protein A or protein G, following the
209 manufacturer's instructions (MACS; Milteyi Biotec, Auburn, CA). For immunofluorescence,
210 cells were seeded into Lab-Tek CC2 treated slide chambers (Nalgene Nunc International,
211 Naperville, IL, USA); after washing with PBS cells were fixed with 4%
212 paraformaldehyde/PBS for 10 min; then washed with PBS and permeabilized with 0.2%
213 Triton X-100/PBS for 10 min. Afterwards cells were washed with PBS, pre-incubated 30
214 min with 4% BSA/PBS and 4% goat serum and washed again with PBS. Anti-NR4A1 (M-
215 210 Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted 1:200 in 2%BSA/PBS
216 and incubated over night at 4°C. After washing with PBS cells were incubated with anti-
217 rabbit coupled to Alexafluor 594 (Invitrogen, Carlsbad, CA) diluted 1:1000 in 2%BSA/PBS
218 for 30 min at room temperature. Then, cells were treated with 10mg/ml RNAse for 30 min
219 at 37°C and washed again with PBS. Cells were counterstained with 10 ng/ml DAPI and

220 mounted for microscope observation on an Axiovert 200M (Carl Zeiss) confocal
221 microscope.

222

223 **RNAi**

224 Four regions were targeted for 3'UTR hNR4A1 mRNA (GenBank NM_002135) starting at
225 positions: 2383 5'gcgccgugcuguaaaauaaguu3'; 2401 5' gccagugcugcuguaaaauuu3'; 2529
226 5'ccacauguacauaaacuguuu3'; 2535 5'guacauaaacugucacucuuu3'. The corresponding
227 siRNAs were simultaneously transfected. These siRNAs were purchased as SMARTpool
228 from Dharmacon (Lafayette, CO, USA). Control siRNA targeting a protein of Rotavirus
229 was a kind gift from Dr. Susana López (Instituto de Biotecnología, UNAM).

230 siRNA transfection: Human embryonic kidney 293 cells (10^5 cells per well in 12-well
231 plates) were grown in high-glucose DMEM supplemented with 10% fetal bovine serum
232 (Sigma, St. Louis, MO), with no antibiotics for 16 hr. The siRNA specific for each target
233 gene was transfected with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA)
234 according to the manufacturer's instructions, using 3 μ g siRNA: 6 μ l Lipofectamine 2000
235 ratio. After 4 hr of incubation, plasmids were transfected.

236

237 **Transcription Assays**

238 Human embryonic kidney 293 cells were seeded in 24-well plates (1×10^5 cells/well).
239 Transient transfections were performed with 2.1 μ g PEI plus 700 ng DNA [225 ng NK₁R;
240 225 ng reporter plasmid (POMC minimal promoter-Luciferase that lacks responsive
241 elements, NBRE-Luciferase or NurRe-Luciferase); 225 ng NR4A1 (or mutants) and 25 ng
242 of a plasmid encoding Renilla luciferase to normalize transfections]. Twenty-four hours
243 after transfection, the cells were incubated or not with 100 nM SP for 3 h. Luciferase and
244 Renilla activities were determined using the Dual-Luciferase Reporter Assay (Promega

245 #E1980), according to manufacturer's instructions and a FLUOstar OMEGA luminometer
246 (BMG LABTECH).

247

248 **Results**

249 **NR4A1 is SUMOylated during SP/NK₁R-induced autosis upon previous**
250 **phosphorylation.**

251 SUMO peptides are conjugated to a Lys residue, frequently within a consensus motif
252 Ψ KXE, where Ψ represents any hydrophobic residue and X any amino acid(30). It has
253 been shown that NR4A2 is SUMOylated in two motifs conserved among the family
254 members that lead to SUMO ligation to lysines K91 (32) or K558 (33), depending on the
255 cell context. In order to find additional potential SUMOylation sites also conserved in the
256 three members of the family, we aligned NR4A protein sequences and searched for the
257 SUMO consensus motif Ψ KXE. We identified K102 and K577 within a SUMO motifs, which
258 are indeed SUMOylated (26), and observed that K558 is conserved in NR4A1 and NR4A2,
259 and hence it is also a potential site for SUMOylation in NR4A1 (positions numbers refer to
260 the human canonic sequence UniProtKB P22736) (Figure 1A).

261 To analyze NR4A1 SUMOylation during SP/NK₁R-induced autosis, we
262 immunoprecipitated NR4A1 at different time points after SP exposure and looked for
263 SUMO1 or SUMO2/3 conjugation by Western blot. Starting at 3 hr after SP induction, a
264 fraction of NR4A1 was SUMOylated by SUMO1 and the modification lasted up to 12 hr, a
265 time point at which SUMO2/3 ligation was also observed (Figure 1B). We verified that
266 SUMO peptide conjugation of NR4A1 occurs during SP/NK₁R-induced autosis by over-
267 expressing Myc-tagged SUMO1 (Figure 1C) or HA-tagged SUMO2 (Figure 1D). To study
268 the role of SUMOylated NR4A1 we substituted the three lysine residues expected to be
269 modified by SUMO (K102, K558 and K577) for arginine. First, we evaluated whether the
270 triple NR4A1_K102,558,577R mutant (named TriMut) had reduced SUMOylation in

271 comparison to the level determined in NR4A1 wild type. We immunoprecipitated FLAG-
272 NR4A1 wild type or FLAG-NR4A1_TriMut and looked for the presence of SUMO2 by
273 Western blot. Indeed, NR4A1_TriMut showed reduced SUMOylation (Figure 1D), although
274 additional Lys residues seem to be also modified by SUMO, as a weak signal was still
275 detected.

276 There are several examples of crosstalk between phosphorylation and
277 SUMOylation, having phosphorylation both positive and negative regulation over SUMO
278 modification (34). During SP/NK₁R-induced autosis NR4A1 is phosphorylated by ERK2,
279 which is necessary for autosis induction, as the inhibition of MEK2 and ERK2 (but not the
280 inhibition of MEK1 or ERK1) prevents NR4A1 phosphorylation and SP/NK₁R-induced
281 autosis (12). Accordingly, NR4A1 is phosphorylated *in vitro* by ERK2 but not by ERK1 in
282 threonine 143 (35). Therefore, we mutated T143 to alanine, expecting to abolish its
283 phosphorylation. Indeed, T143 is phosphorylated during SP/NK₁R-induced autosis, since
284 mutant NR4A1_T143A had reduced phosphorylation level as compared to NR4A1 wild
285 type (Figure 2A). Nevertheless, a weak signal was still observed, indicative of an additional
286 phosphorylated threonine. We found two additional ERK consensus motifs in the NR4A1
287 sequence (Figure 1A), although additional phosphorylation sites could be targeted by
288 other kinases.

289 To analyze whether SUMO conjugation influences phosphorylation, we studied the
290 level of phosphorylation when SUMOylation is reduced. As shown in Figure 2A, the level
291 of phosphorylation on threonine residues was not affected in NR4A1_TriMut. This result
292 indicates that phosphorylation is not affected by SUMOylation in this case. On the other
293 hand, SUMOylation resulted dependent on previous phosphorylation in T143, as
294 NR4A1_T143A was barely SUMOylated (Figure 2B). Therefore, NR4A1 SUMOylation,
295 subsequent to phosphorylation, could alter NR4A1 stability and/or intracellular distribution
296 and, consequently, the ability to induce autosis.

297 In previous work we observed that *Nr4a1* expression is induced by SP signaling
298 (12), but NR4A1 protein abundance cannot be explained by transcriptional regulation
299 alone. We noticed that NR4A1 produced by expression of its cDNA (lacking both 5' and 3'
300 UTR) from a viral promoter (CMV) that lacks endogenous regulatory regions, also
301 accumulates in response to SP/NK₁R (for example, look at Figure 2A, Input). We
302 rationalized then that NR4A1 stability should be post-translationally regulated. MAPK
303 signaling activated by SP/NK₁R does not affect NR4A1 abundance, as it still accumulates
304 when ERK2 signaling is inhibited (12). SUMO modification commonly enhances protein
305 stability by binding to lysine residues that otherwise would be ubiquitinated, targeting the
306 protein for proteasome degradation; nevertheless, it has also been observed that some E3
307 ubiquitin ligases, such as RNF4, which have both SIM (SUMO interacting motif) and RING
308 domains, attach ubiquitin to SUMO-modified proteins (30), and this mechanism has been
309 described to occur to NR4A1(26). Therefore, we asked whether NR4A1 SUMOylation
310 regulated NR4A1 stability during SP/NK₁R-induced autosis. First, we determined the
311 endogenous NR4A1 half-life. NR4A1 reached a maximum level of expression 3 hr after SP
312 exposure and by 9 hr it was still detected but clearly reduced (Figure 2C). We then
313 inhibited new protein synthesis (with cycloheximide) after 3 hr of induction with SP (to start
314 with the highest amount of NR4A1), and observed that NR4A1 was degraded very rapidly,
315 as 3 hr after cycloheximide addition it was no longer detected (Figure 2C).

316 To compare the half-life of wild type NR4A1 with NR4A1_TriMut with reduced
317 SUMOylation, we tagged it with FLAG, and compared FLAG-NR4A1 wild-type half-life with
318 FLAG-NR4A1_TriMut (which allowed to distinguish NR4A1_TriMut from endogenously
319 induced NR4A1). Since these constructs are expressed from a strong constitutive
320 promoter (CMV), FLAG-NR4A1 started in these experiments with a higher amount of
321 protein than the endogenously-induced NR4A1, therefore its expression could be
322 observed it for a longer period of time. We inhibited new protein synthesis 24 hr after

323 transfection. Notably, the stability imposed by SP signaling still occurred in FLAG-tagged
324 NR4A1 (Figure 2D, upper panel). Under basal expression SUMOylation seemed to indeed
325 contribute to NR4A1 stability, as the half-life of TriMut (which was SUMOylated to a lesser
326 degree) was clearly reduced compared to wild type. Surprisingly, however, TriMut
327 abundance still increased in response to SP (Figure 2D, bottom panel) reducing its
328 degradation rate. Other reports describe that NR4A1 stability can be modulated by
329 acetylation, which also occurs at Lys residues. We looked for acetylation during SP/NK₁R-
330 induced autosis, but no difference was found in the level of acetyl-Lysine content in
331 immunoprecipitated NR4A1 from non-treated cells compared with SP treated cells (data
332 not shown). We do not know at this point which mechanism mediates the increase in
333 NR4A1 stability in response to SP signaling but, possibly, the remaining SUMOylation
334 sites observed in TriMut (Figure 1D) could be involved.

335

336 **SUMOylation and phosphorylation alter NR4A1 transcriptional activity and**
337 **intracellular distribution.**

338 SUMO modification of transcription factors usually inhibits their transcriptional activity,
339 either by recruiting corepressors to promoter regions or by sequestering the SUMOylated
340 form in nuclear bodies (36). NR4A1 transcriptional activity could also be negatively
341 regulated by SUMOylation, since NR4A1 interacts with HDAC1, potentially recruiting
342 corepressor complexes (1), and with PML, being potentially recruited into nuclear bodies
343 (37). In previous studies we found that NR4A1 is transcriptionally active during SP/NK₁R-
344 induced autosis (13). Therefore we analyzed whether the transcriptional activity of NR4A1
345 is increased in the triple mutant in which predicted Lys targets of SUMOylation were
346 replaced by arginine. NR4A receptors bind as monomers to the DNA element NBRE or as
347 homo- or heterodimers to the DNA element NuRE. The experiments shown were
348 performed with NuRE. First, we verified that SUMO NR4A1 mutant maintains basal

349 transcriptional activity, and then we compared its transcriptional activity with that of NR4A1
350 WT in response to SP. As can be seen in Figure 3A, NR4A1_TriMut showed enhanced
351 transcriptional activity in response to SP signaling. Therefore, SUMOylation appears to be
352 a relevant negative regulator of NR4A1 transcriptional activity.

353 NR4A1 can have both nuclear and cytoplasmic functions(15); during SP/NK₁R-
354 induced autosis around 15% of the cells showed NR4A1 also in the cytoplasm (13). To
355 assess whether SUMOylation affects NR4A1 localization, we analyzed the intracellular
356 distribution of the TriMut. In accordance with the higher level of transcriptional activity we
357 observed, the TriMut was more frequently retained in the nucleus. We also tested the
358 mutant in the target of phosphorylation T143, as it has been previously reported that
359 phosphorylation of NR4A1 also modulates its localization, although in response to other
360 kinases and phosphorylated in other sites (38). We compared NR4A1_T143A and
361 NR4A1_T143D, phosphorylation-defective and threonine phosphorylation-mimic (D mimics
362 the negative charge provided by phosphorylation) mutants, respectively. While most of the
363 cells have NR4A1 restricted to the nucleus, again we observed a small proportion (18%) of
364 cells with NR4A1 also in the cytoplasm (Figure 3B). It appears then than SUMOylation
365 regulates the exportation of NR4A1 out of the nucleus, while phosphorylation might be
366 necessary for retaining NR4A1 in the nucleus. A fine regulation of combined PTMs could
367 determine the intracellular distribution of NR4A1.

368

369 **NR4A1 SUMOylation is necessary for SP-induced autosis**

370 We hypothesized that the signaling pathway activated upon NK₁R activation by SP binding
371 triggers SUMOylation of NR4A1, conferring upon it the ability to induce autosis instead of
372 apoptosis or proliferation, among other processes. To inhibit NR4A1 SUMOylation we
373 expressed GAM1, a viral protein that inhibits SUMOylation by promoting SAE1/SAE2 and
374 UBC9 degradation (39). As shown in Figure 4A, in the presence of GAM1, but not of an

375 inactive GAM1 mutant, the amount of SUMOylated NR4A1 was reduced. Supporting the
376 notion that SUMOylation increases its basal stability, the total amount of NR4A1 was also
377 reduced in the presence of GAM1. More significantly, *Gam1* expression completely
378 prevented SP/NK₁R-induced autosis (Figure 4B).

379 Finally, to confirm whether SUMOylation of NR4A1 is indeed necessary to mediate
380 SP-induced autosis, we tested the ability of TriMut to induce autosis in response to SP. To
381 eliminate endogenous expression of NR4A1 that could mask the mutant phenotype, we
382 silenced it by targeting small interfering RNAs to the 3' untranslated region of NR4A1
383 mRNA, which is absent in the NR4A1 expression vector. As expected, silencing the
384 expression of endogenous NR4A1 reduced cell death in response to SP, which was
385 restored by the expression of NR4A1 WT but not by the expression of TriMut (Figure 4C).
386 Therefore, SUMOylation of NR4A1 is necessary for SP/NK₁R-induced autosis.

387

388 Discussion

389 NR4A receptors regulate multiple processes such as metabolism, proliferation, migration,
390 apoptosis, DNA repair and autophagy. Accordingly, NR4A receptors are involved in
391 several pathological processes like cardiovascular diseases, diabetes, atherosclerosis and
392 neurodegeneration (40). Interestingly, NR4A1 expression is also induced by caloric
393 restriction, and a reduction in expression of at least NR4A2 accompanies human aging (2).
394 Since DNA damage accumulates with aging, perhaps the reduced expression of NR4A is
395 a contributory factor. Intriguingly, in cancers from different origins, such as melanoma,
396 breast, colon and pancreas, both pro- and anti-tumorigenic activities have been described
397 for NR4A family proteins (7, 41). Indeed, pharmacological regulation of NR4A activity has
398 been proposed to not only counteract aging, including cognitive decline (40), but also
399 cancer and metabolic diseases. Therefore, understanding the mechanisms that regulate
400 NR4A function is an active area of research.

401 SUMO modification affects numerous cellular processes overlapping with those
402 described for NR4A1 function. Disruption of SUMOylation affects differentiation of cells
403 representative of the three germ layers: endoderm, ectoderm and mesoderm (42).
404 SUMOylation also affects DNA repair and stability, as it acts as a master organizer of
405 protein complexes with functions in chromatin remodeling, double-strand break repair and
406 ribosome biogenesis (36).

407 In the present work, we demonstrated that NR4A1 is SUMOylated during SP-
408 induced autosis. Specific mutants of NR4A1 with reduced SUMOylation have decreased
409 basal stability but yet responded to SP signaling increasing its stability, as well as its
410 transcriptional activity. This latter effect could be due to additional SUMOylation sites of
411 NR4A1 since there is still a weak SUMO signal in the triple mutant. A recent more
412 comprehensive analysis including many SUMO substrates have shown that the E residue
413 is preferred over D in the consensus motif (43), so there could be additional SUMOylation
414 motifs in the NR4A1 sequence. Even though RNF4 can attach ubiquitin to SUMO-modified
415 NR4A1 in response to signals such as PMA and target it for proteasomal degradation (26),
416 the ubiquitination sites for basal NR4A1 degradation seem to be different to Lys 102, 558
417 and 577, since when those residues were substituted by arginine the stability of TriMut
418 was reduced. Clearly NR4A1 can have different ubiquitination sites in different cellular
419 contexts. For example, during inflammation, ubiquitinated NR4A1 is not send to
420 proteasomal degradation but functions as a label of damaged mitochondria, interacting
421 with the autophagic receptor p62/SQSTM1 for their engulfment and elimination by
422 mitophagy (44). Further experiments will be needed to understand the combinations of
423 PTMs that regulate NR4A1 half-life, intracellular localization and interactors, which render
424 specific functions in different contexts.

425 Finally, we show that the ability of NR4A1 to induce autosis is impaired when
426 SUMOylation is reduced. Autosis could be triggered by specific molecular interactions of

427 SUMOylated NR4A1 in the cytoplasm or in the nucleus. Interestingly, NR4A1_TriMut
428 (NR4A1_K102,558,577R) showed a nuclear localization in 98% of the cells. It is possible
429 that SUMOylation in K558 is required for cytoplasmic NR4A1 localization, as K558 is part
430 of the third nuclear export signal (LLGKLPELRTL) located in the LBD (45). Therefore,
431 NR4A1_TriMut's inability to induce autosis might relate to a reduction in a cytoplasmic
432 function or an alteration in a NR4A1 transcriptionally-regulated pathway that induces
433 autophagy. The present work encourage further experiments to underscore the molecular
434 mechanisms by which NR4A1 and SUMOylation influence the induction of autosis, or by
435 the repression of a target gene. This knowledge is particularly relevant, since NR4A1
436 expression is also induced in response to ischemia (24) and kainic acid-triggered seizures
437 (25), situations where Substance P mediates neuronal cell death. Screening for small
438 molecules able to inhibit NR4A1 SUMOylation, or inducing its deSUMOylation, would
439 potentially contribute to treatments that prevent or reduce neuronal cell death. From a
440 different point of view, since both NR4A(4, 5) and SUMO (46) regulated pathways have
441 functions in memory, SUMOylation of NR4A1 could have a role in cognitive activities.
442 Altogether, understanding the molecular regulation of NR4A1 function has a potential
443 impact in biomedicine.

444

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449

450 **Declaration of interest**

451 Authors declare no conflict of interest

452

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458

459 **Author contribution**

460 GZG, GMH, MRSC, WVG, APPR and CA contributed to the investigation and formal
461 analysis; MEA and LC contributed with resources and supervision; CW contributed to
462 visualization; SCO contributed with conceptualization, funding acquisition, project
463 administration, resources, supervision and writing original draft. All authors reviewed and
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467

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579

580 **Figure Legends**

581 **Figure 1. NR4A1 is SUMOylated. A, Protein sequence alignment of NR4A family**
582 *members.* Two conserved SUMOylation consensus motifs among members (green
583 squares) and three potential ERK phosphorylation consensus sites in NR4A1 (blue
584 squares) are shown. K558 is conserved in NR4A2 and NR4A1. **B, NR4A1 is SUMOylated**
585 *in response to SP.* Immunoprecipitation (IP) of NR4A1 from cells transfected with NK₁R
586 expression vector and exposed to SP for the indicated times, to detect by Western blot
587 either SUMO1, SUMO2/3 or NR4A1. IgG from IP was detected by the secondary antibody.
588 **C, Over-expression of myc-SUMO1 enhances the amount of NR4A1 conjugation to**
589 *SUMO1 in response to SP.* Cells were transfected with NK₁R and MYC-tagged SUMO1
590 expression vectors (Myc-SUMO1) and exposed (+) or not (-) to SP for 3 hr. NR4A1 was
591 immunoprecipitated and developed by Western blot to detect Myc tag or NR4A1. Tubulin
592 was detected in whole extracts as a loading reference (Input). **D, Triple mutant**
593 *NR4A1_K102,558,577R (TriMut) has reduced SUMOylation.* Cells were transfected with
594 expression vectors for HA tagged SUMO2, FLAG-NR4A1 wild type or FLAG-
595 NR4A1_TriMut, and NK₁R to allow post-translational modifications in response to SP (+).
596 Immunoprecipitation was performed with FLAG antibody (antiFLAG) or IgG as a control of
597 irrelevant antibody. WB was developed with antibodies against indicated proteins.
598 TUBULIN was detected as a loading reference (Input).

599

600 **Figure 2. NR4A1 SUMOylation depends on former phosphorylation. A, SUMOylation**
601 *in K102, K558 and K577 is not necessary for NR4A1 phosphorylation in response to SP.*
602 Cells were transfected with expression vectors for FLAG-NR4A1 wild type or FLAG-

603 NR4A1_TriMut and NK₁R, and exposed (+) or not (-) to SP for 3 hr. Total protein extracts
604 were immunoprecipitated with anti-FLAG or IgG and the level of threonine phosphorylation
605 was estimated by WB. TUBULIN was detected for comparison of total protein extraction
606 for IP, and FLAG to show the level of expression of each construct. **B**, *Phosphorylation in*
607 *T143 is necessary for NR4A1 SUMOylation in response to SP*. Cells were transfected with
608 expression vectors for FLAG-NR4A1 wild type or FLAG-NR4A1_T143A, HA-SUMO2 and
609 NK₁R, and exposed (+) or not (-) to SP for 3 hr. Total protein extracts were
610 immunoprecipitated with anti-FLAG and the level of SUMOylation was estimated by WB
611 detecting HA. FLAG was detected to show the level of expression of NR4A1 constructs. **C**,
612 *NR4A1 peak of synthesis is at 3 hr after SP addition and has a half-life of less than three*
613 *hours*. Lanes 1-6, total protein extracts were obtained from cells transfected with NK₁R
614 and exposed to SP for the indicated times. Lanes 7-12, total protein extracts were
615 obtained from cells transfected with NK₁R expression vector, treated for 3 hr with SP to
616 reach maximum expression of NR4A1 (considered time 0 for cycloheximide treatment),
617 and then exposed to cycloheximide (CHX) for the indicated time to inhibit new protein
618 synthesis. Three hr after CHX less than half of the initial amount of NR4A1 protein
619 remains. **D**, *NR4A1 basal stability is enhanced by SUMOylation, but TriMut still becomes*
620 *stabilized in response to SP signaling*. To compare stability of NR4A1 wild type with
621 NR4A1_TriMut, cells were transfected with expression vectors for NK₁R and either FLAG-
622 NR4A1 or FLAG-NR4A1_TriMut. 24 hr after of transfection cells were treated with CHX for
623 the indicated times, or treated for 3 hr with SP and then with CHX for the indicated time.
624 Clearly, the amount of FLAG-NR4A1_TriMut was reduced and was degraded faster than
625 wild type, but yet responded to SP induction. **E**. Quantitative comparison of the
626 degradation rate of NR4A1 WT and TriMut, with or without SP, from densitometric analysis
627 of at least three independent experiments described in **D**. Each blot was normalized with
628 their corresponding TUBULIN. The measurement obtained for each NR4A1 (WT or TriMut)

629 before CHX treatment (time zero) was arbitrary considered 100 units. Notice that the faster
630 degradation rate of TriMut is overcome in response to SP. Each dot represents the mean
631 and bars represent standard deviation.

632

633 **Figure 3. SUMOylation regulates NR4A1 transcriptional activity and its intracellular**
634 **distribution. A, NR4A1 mutant in Lys residues located in SUMOylation motifs (TriMut)**
635 *have increased transcriptional activity compared to wild type in response to SP.* Cells were
636 transfected with a reporter containing Luciferase cDNA under the control of NuRE/POMC
637 and the indicated plasmids to assess basal transcriptional activity (left panel). Then cells
638 were co- transfected with NK₁R and treated or not with SP for 3 hr. TriMut showed
639 enhanced transcriptional activity in response to SP and at a higher level than NR4A1 WT.
640 A mean of 5 independent experiments (each with duplicate wells) is plotted. Error bars
641 represent the standard error. ** p<0.01; n=5. A representative Western blot showing the
642 level of expression of each construct, with or without SP, is shown below. Both anti-FLAG
643 and anti-Tubulin were incubated simultaneously. **B, SUMOylation and phosphorylation**
644 *regulate NR4A1 intracellular distribution.* Cells were transfected with indicated plasmids
645 and the intracellular localization of the proteins was determined by immunofluorescence to
646 detect NR4A1 (green). Two-hundred cells from two independent experiments for each
647 construct were counted. The percentage of cells with only nuclear, or nuclear-cytoplasmic
648 localization of NR4A1, is plotted. Representative confocal microscopy images are shown
649 below; nuclei were stained with DAPI (blue).

650

651 **Figure 4. NR4A1 SUMOylation is necessary for SP-induced autosis. A, The viral**
652 *protein Gam1 reduced SUMOylation of NR4A1.* Cells were transfected with NK₁R
653 expression vector and the indicated plasmids, and were treated or not with SP for 3 hr.
654 Then, NR4A1 was immunoprecipitated and developed with anti-SUMO2/3 or antiNR4A1.

655 Notice that only in the presence of GAM1, and not with inactive mutant GAM1, there was a
656 reduction in SUMOylated NR4A1. GAPDH was detected in total extract (input) as a
657 reference of initial similar amount of protein. **B**, *Inhibiting SUMOylation by the expression*
658 *of the viral protein GAM1 prevents SP-induced autosis*. Cells were transfected with NK₁R
659 expression vector and an empty vector or *Gam1* expression vector and treated or not with
660 SP for 24 hr. Cell death was estimated by Trypan blue exclusion. A mean of three
661 independent experiments is plotted. Error bars represent the standard deviation. ***
662 $p < 0.0001$ 2way ANOVA. The Western blot below shows the expression of *Myc-Gam1* or
663 *Myc-Gam mutant*. **C**, *SUMOylation defective mutant (TriMut) is not able to induce autosis*
664 *in response to SP*. Cells were co-transfected with NK1R expression vector and either
665 NR4A1 WT, TriMut or empty vector as indicated. Cells were also transfected with a control
666 siRNA targeting a viral sequence not present in mammals or a siRNA targeting the 3' UTR,
667 only present in endogenous mRNA. Cells were exposed or not to SP for 24 hr and cell
668 death was estimated by Trypan blue exclusion or LDH activity released. Every experiment
669 was performed in triplicate and averaged. The mean of three independent experiments is
670 plotted. Error bars represent the standard deviation. ***, $p < 0.0001$. Total protein extracts
671 were obtained from replica wells taken at 3hr after SP addition to estimate the content of
672 NR4A1 by WB. Tubulin was detected as a loading reference.

A.

```

NR4A2 1 MPCVQAQYGGSSPQGASPASQSYSHSSGEYSSDFLTPEFVKFSMDLTNTEITAT--TSLPSFSTFMDNYSTGYD-----VKPPCLYQMP-----LS 84
NR4A3 1 MPCVQAQYSPSPPGSSYAAQTYS----SEYTTTEIMNPDYTKLTMDLGSTEITATATTSLSISTFVEGYSSNYE-----LKPSCVYQMQRPLIKVEE 88
NR4A1 1 MPCIQAQYGTAPSPGPR-----DHLASDPLTPEFIKPTMDLASPEAAPAAPTALPSFSTFMDGYTGEFDTFLYQLPGTVQPCSSASSASSTSSS 91

NR4A2    GQQS----SIKVEDIQMHNYQQHSHLPPQSEEMMPHSG-----SVYYKPSPTPTTPGFQVQHSPMWDD--PGSLHNFHQNYVAT---THMIEQR 166
NR4A3    GRAP----SYHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH 181
NR4A1    SATSPASASFKFEDFQVYGCYPGPLSGPVDEALSSSGS-----DYYGSPCSAPSPSTPSFQPPQLSPWDG----SFGHFSPSQTYEGLRAWTEQLP 178
          K102

NR4A2    KTPVSRLSLFSFKQSPPGTPVSS----CQMRFD----GPLHVPMNPEPAGSHHVVDGQTFVAVPNPIRKPAS-MGFPGQLQIG---HASQLLDT--QVPSPP 252
NR4A3    TVAGARFPLFHFVKPSPPHPPAPSPAGGHHLGYDPTAAAALSLPLGAAAAAGSQAAALESHPYGLPLAKRAAPLAFPPPLGLTPSPTASSLLGESPSLPSPP 281
NR4A1    KASGPPQPPAFFSFSPTGSPS-----LAQSPLKLFPSQAT-HQLGEGESYSMPTAFPGLAP----TSEHLE----GSGILDTPVTSTKAR 256

          ↓
NR4A2    548 RPNYLSKLLGKLPPELRTLCTQGLQRIFYLKLEDLVPPPAIIDKLFDLTLPF 597
NR4A3    576 LEPTESKVLGALVELRKICTLGLQRIFYLKLEDLVSPPSIIDKLFDLTLPF 575
NR4A1    548 PASCLSRLLGKLPPELRTLCTQGLQRIFYLKLEDLVPPPPPIIDKIFMDTLPF 597
          ↑
          K558
          K577
  
```

ERK phosphorylation consensus (Px(S7T)P)
SUMOylation consensus motif (ΨKXE)

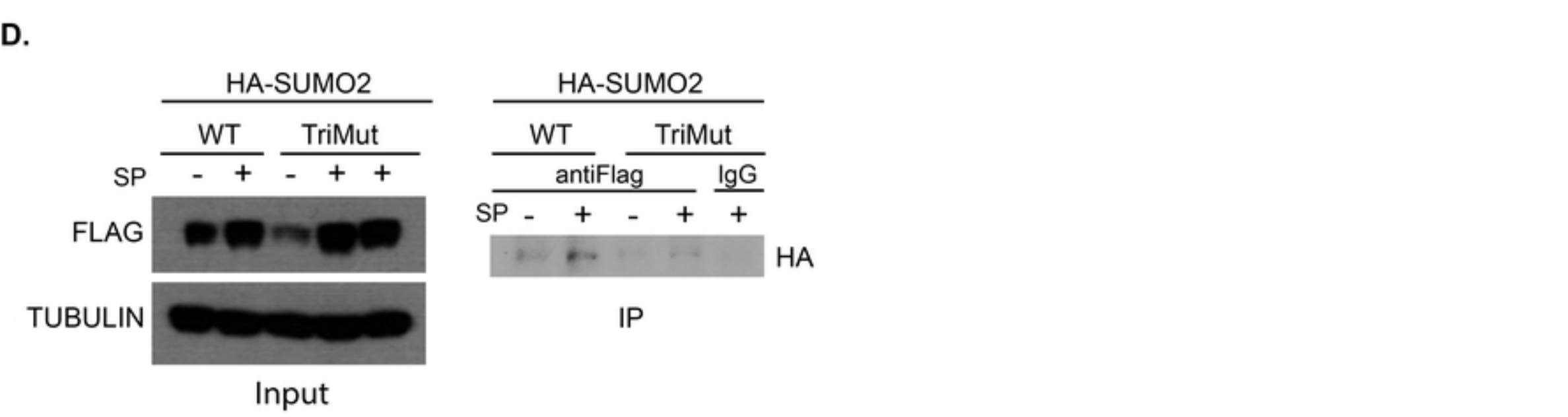
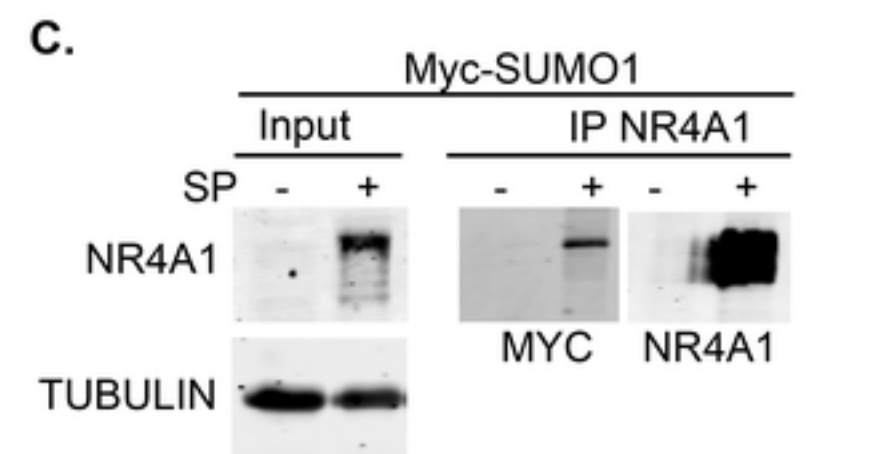
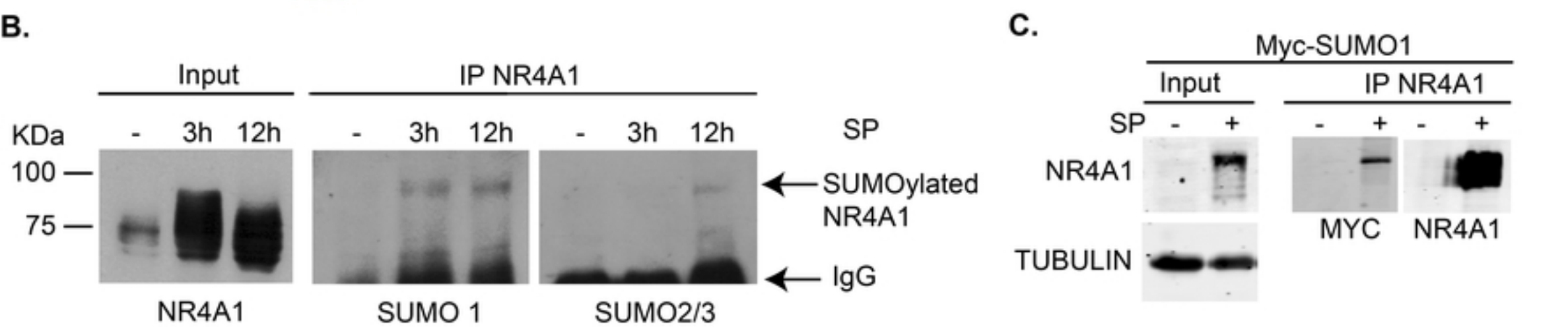


Figure 1

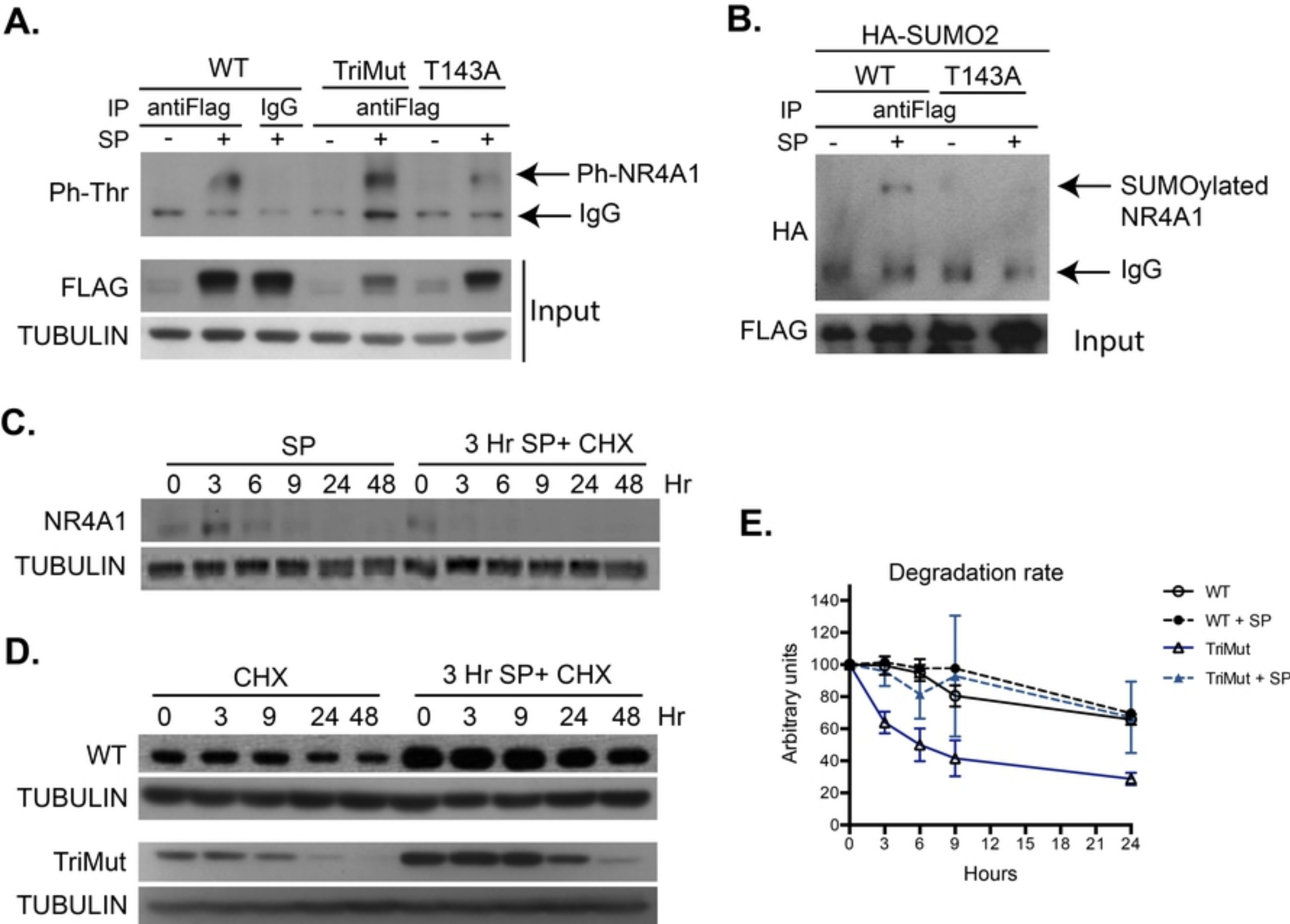


Figure 2

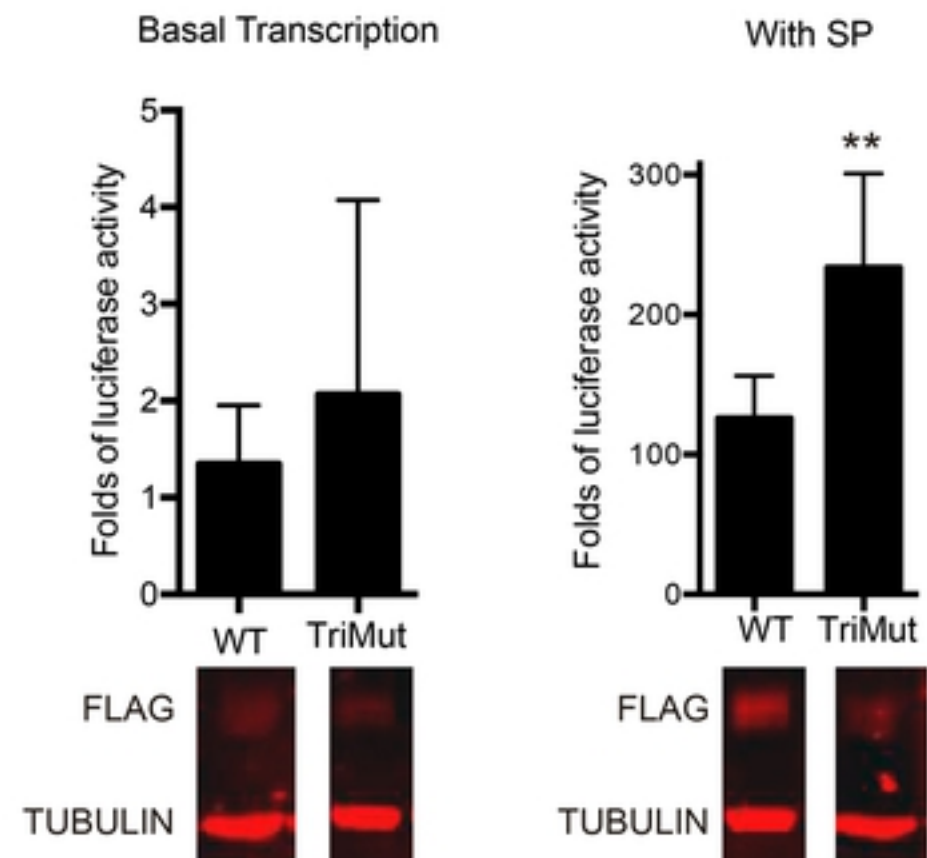
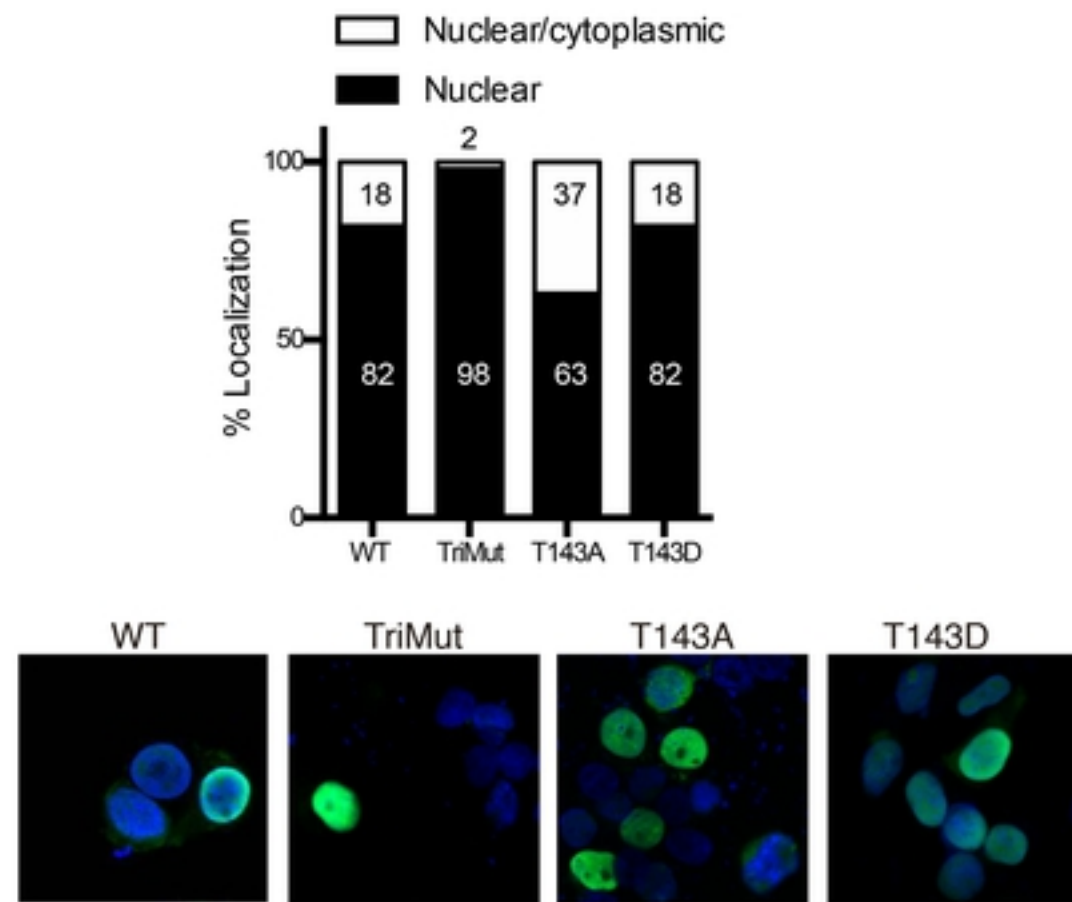
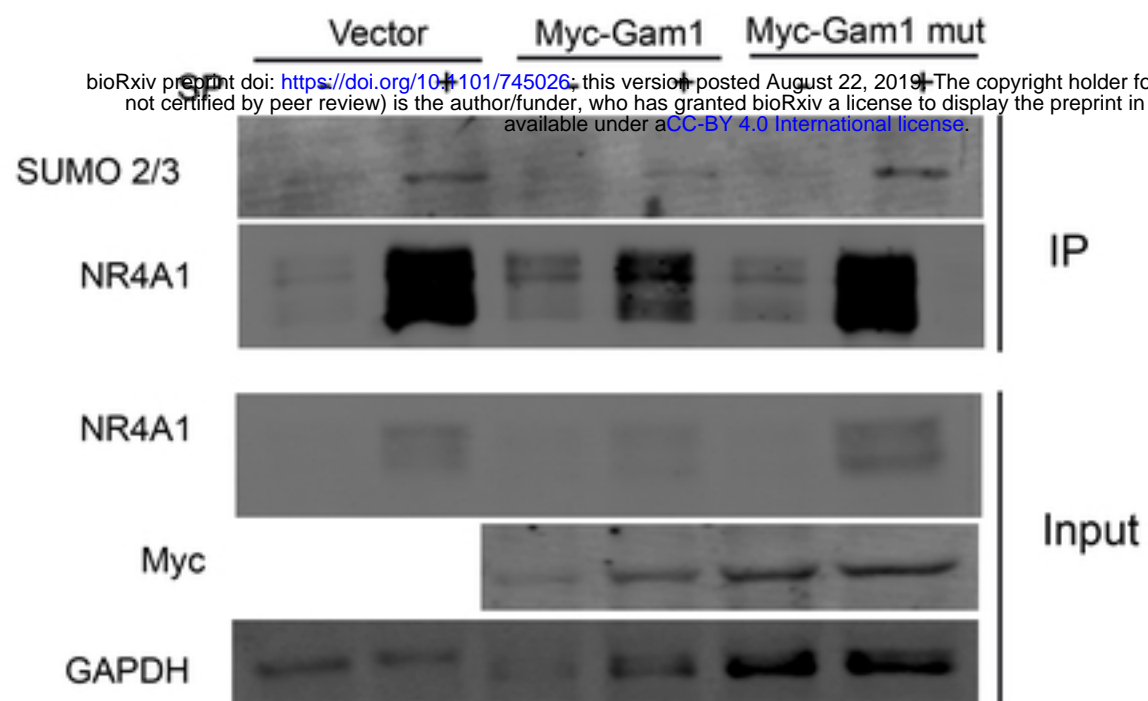
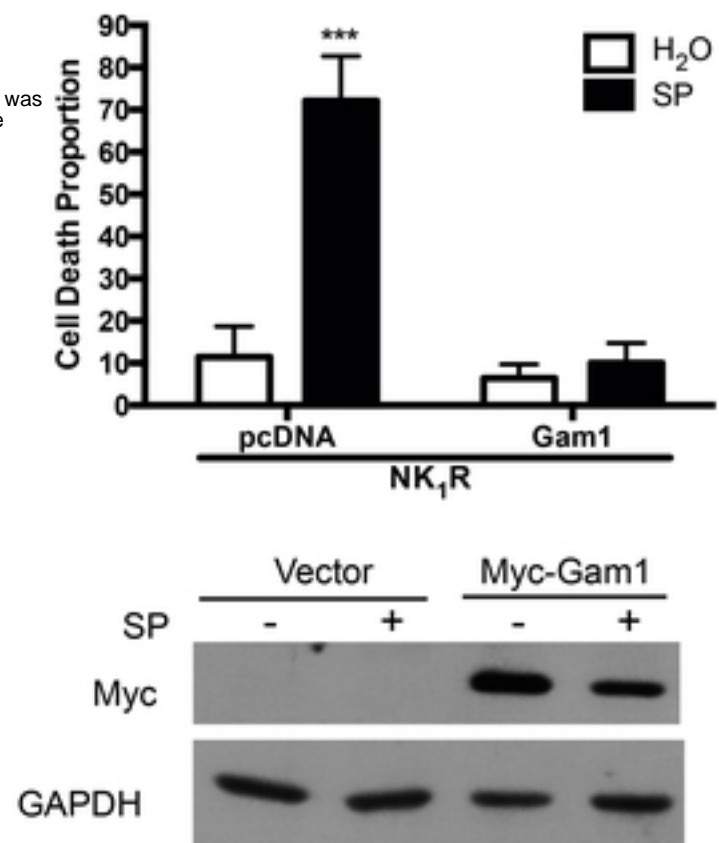
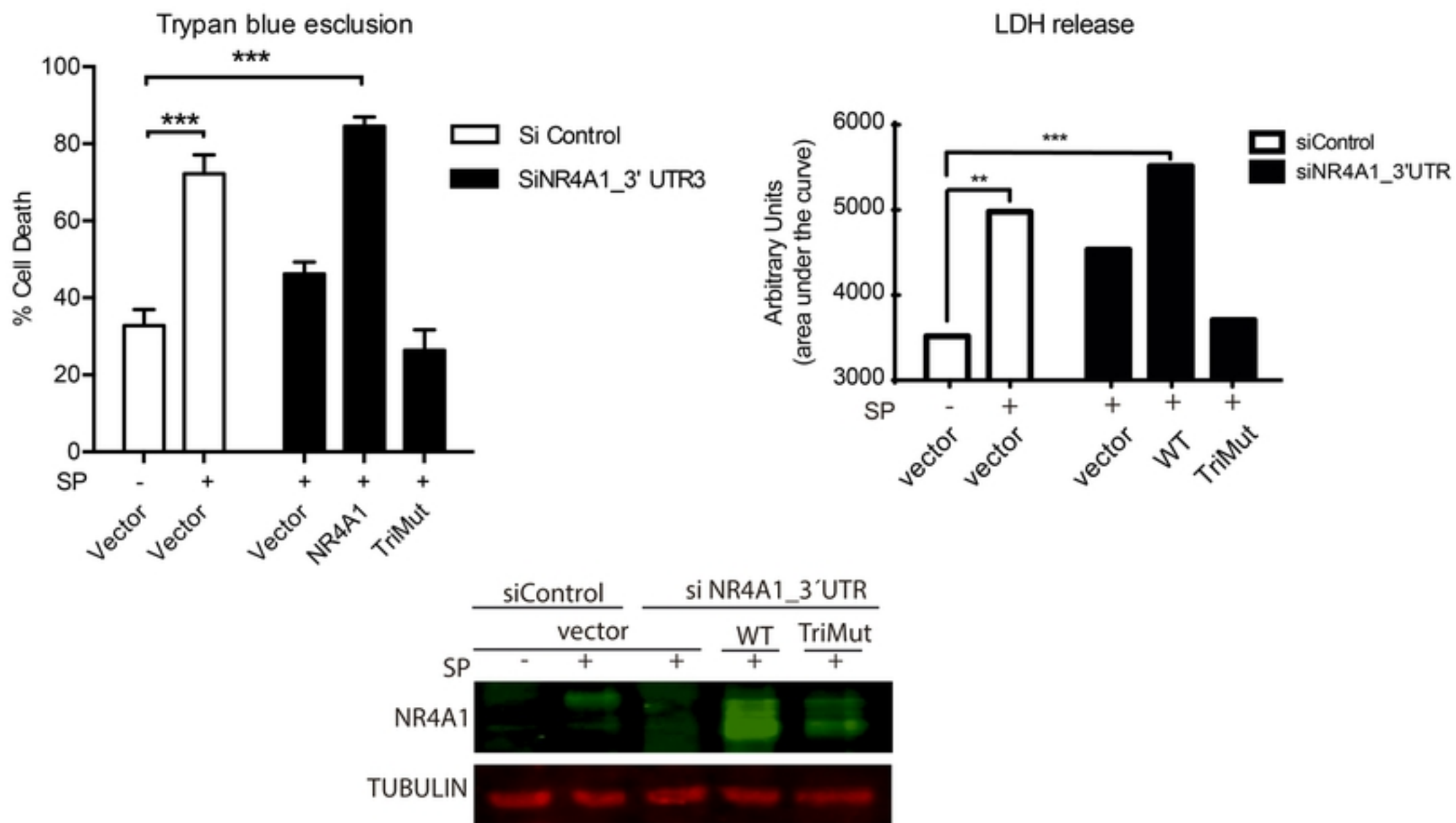
A.**B.**

Figure 3

A.**Gam1 Inhibits NR4A1 SUMOylation****B.****Gam1 Inhibits Cell Death****C.****Figure 4**