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1 **The RanBP2/RanGAP1-SUMO complex gates β -arrestin2**
2 **nuclear entry to regulate the Mdm2-p53 signalling axis**

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39
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42 **Abstract**

43

44 Mdm2 antagonizes the tumour suppressor p53. Targeting the Mdm2-p53
45 interaction represents an attractive approach for the treatment of cancers with
46 functional p53. Investigating mechanisms underlying Mdm2-p53 regulation is
47 therefore important. The scaffold protein β -arrestin2 (β -arr2) regulates tumour
48 suppressor p53 by counteracting Mdm2. β -arr2 nucleocytoplasmic shuttling displaces
49 Mdm2 from the nucleus to the cytoplasm resulting in enhanced p53 signalling. β -arr2
50 is constitutively exported from the nucleus, via a nuclear export signal, but
51 mechanisms regulating its nuclear entry are not completely elucidated. β -arr2 can be
52 SUMOylated, but no information is available on how SUMO may regulate β -arr2
53 nucleocytoplasmic shuttling. While we found β -arr2 SUMOylation to be dispensable
54 for nuclear import, we identified a non-covalent interaction between SUMO and β -
55 arr2, via a SUMO interaction motif (SIM), that is required for β -arr2 cytonuclear
56 trafficking. This SIM promotes association of β -arr2 with the multimolecular
57 RanBP2/RanGAP1-SUMO nucleocytoplasmic transport hub that resides on the
58 cytoplasmic filaments of the nuclear pore complex. Depletion of RanBP2/RanGAP1-
59 SUMO levels result in defective β -arr2 nuclear entry. Mutation of the SIM inhibits β -
60 arr2 nuclear import, its ability to delocalize Mdm2 from the nucleus to the cytoplasm
61 and enhanced p53 signalling in lung and breast tumour cell lines. Thus, a β -arr2-SIM
62 nuclear entry checkpoint, coupled with active β -arr2 nuclear export, regulate its
63 cytonuclear trafficking function to control the Mdm2-p53 signalling axis.

64

65 Abstract 217 words

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67 Main body text 4463 words

68 **Introduction**

69

70 Initially discovered for the roles they play in the regulation of G protein-coupled
71 receptors (GPCRs), the β -arrestins (β -arr1 and β -arr2) have now been shown to act
72 as scaffolding hubs that control multiple signalling pathways (1-3). Through their
73 scaffolding properties β -arrestins dynamically regulate the activity and/or subcellular
74 distribution of non-GPCR protein partners such as ERK1/2 (4, 5) JNK3 (6), Mdm2 (7-
75 9), PTEN (10-12) and FAK (13). While strong sequence homology exists between β -
76 arr1 and β -arr2 they display different subcellular distributions (9, 14). Whereas β -arr1
77 is found in both the nucleus and cytoplasm at steady state, β -arr2 shows an apparent
78 cytoplasmic distribution. We previously showed that β -arr2 is constitutively excluded
79 from the nucleus by a leptomycin B-sensitive pathway driven by a nuclear export
80 signal (NES) that is absent in β -arr1 (14). We also found that β -arr2 is actively
81 imported into the nucleus, indicating that it undergoes constitutive nucleocytoplasmic
82 shuttling (14). β -arr2 shuttling displaces nuclear partners, such as JNK3 and Mdm2,
83 from the nucleus to the cytoplasm (9, 14). In the case of Mdm2, its β -arr2-mediated
84 delocalization results in increased p53 signalling and cell cycle arrest (7). In contrast
85 to the well characterized nuclear export mechanism of β -arr2 the entry mechanism(s)
86 of β -arr2 into the nucleus are not completely elucidated.

87 β -arr2 can be SUMOylated (15, 16), but no information is available on how
88 SUMO may regulate β -arr2 nucleocytoplasmic trafficking. SUMOylation is a key
89 dynamic regulatory post-translational modification that impacts the activity and
90 localization of protein targets including into the nucleus (17-19). These functional
91 consequences are generally due to modification in protein-protein interactions
92 caused by SUMOylation. Akin to the situation with the ubiquitination pathway,
93 SUMOylation involves a series of sequential enzymatic reactions ultimately leading to
94 covalent conjugation of the 12kDa protein SUMO on a lysine residue contained within
95 a SUMOylation site (ψ -K-X-E; ψ , a bulky aliphatic residue, typically L, I or V) on a
96 target protein (17, 18, 20). There are four SUMO isoforms in mammals: SUMO1,
97 SUMO2 and SUMO3 are expressed ubiquitously, whereas SUMO4 displays tissue
98 restricted expression and it is not clear whether it is conjugated to cellular proteins
99 (21). SUMO2 and SUMO3 display 97% homology and contain internal SUMOylation
100 sites allowing them to form poly-SUMO chains. SUMO1 shares 45% homology with
101 SUMO2/3 (17). SUMOylation is reversible with SUMO-modified targets being subject

102 to cleavage of the isopeptide bond by SUMO-specific proteases that release SUMO
103 molecules that become available for further SUMOylation cycles. SUMOylated
104 proteins can establish non-covalent interaction with protein partners via SUMO
105 interaction motifs (SIMs). Identified via two-hybrid screening and biophysical studies
106 SIMs are composed of a short stretch of hydrophobic residues (generally V/I-V/I-X-
107 V/I) flanked N- or C-terminally by serine residues and/or several acidic residues (17,
108 22). When bound to SUMO, SIMs adopt a parallel or antiparallel β -strand
109 conformation that permits the hydrophobic side chains of the SIM to interact with a
110 hydrophobic pocket on the surface of SUMO. The acidic flanking residues in the SIM
111 form electrostatic interactions with a basic interface on SUMO, and thus also
112 contribute to the SUMO-SIM interaction. SIMs can be involved in *cis*-SUMOylation or
113 in recruitment/targeting of SIM-containing proteins to SUMOylated partners (17).

114 Using various *in vitro* and cell-based approaches we have characterized both
115 a SUMOylation site and SIM in human β -arr2. Whereas β -arr2 SUMOylation is not
116 required for nuclear import, mutation of the SIM prevents β -arr2 nuclear import. The
117 SIM promotes association of β -arr2 with the multimolecular RanBP2/RanGAP1-
118 SUMO nucleocytoplasmic transport hub and depletion of this complex inhibits β -arr2
119 nuclear import. The β -arr2 Δ SIM mutant loses its ability to delocalize Mdm2 from the
120 nucleus to the cytoplasm with functional consequences for p53 activity. Combined,
121 our data reveal that a SUMO-SIM nuclear entry checkpoint, coupled with the nuclear
122 export function of β -arr2, cooperate to regulate its cytonuclear trafficking function and
123 subsequent control of the Mdm2-p53 pathway.

124 Results

125

126 Human β -arr2 is SUMOylated at lysine 295

127 To investigate the potential role of SUMO in the nucleocytoplasmic trafficking
128 action of β -arr2 we first searched for potential SUMOylation sites within human β -
129 arr2. Ubc9 fusion-directed SUMOylation (UFDS) experiments (23), which permit
130 efficient and selective protein SUMOylation, were conducted by cloning the E2
131 conjugase Ubc9 coding sequence upstream of the open reading frame of β -arr2 with
132 a C-terminal FLAG-tag, generating a coding sequence for a Ubc9- β -arr2-FLAG
133 fusion protein (**Fig. 1a**). The fusion protein was expressed in HeLa cells in the
134 presence of HA-SUMO1 or HA-SUMO2. Immunoprecipitations of the protein extracts
135 using anti-FLAG antibodies followed by western blotting with anti-FLAG antibodies
136 revealed the presence of a molecular weight (MW) band of ~100kDa corresponding
137 to the Ubc9- β -arr2-FLAG fusion conjugated with HA-SUMO1 or HA-SUMO2 (**Fig. 1b**
138 **and c**, lanes 2). The fused Ubc9 catalysed SUMOylation of β -arr2, as the active site
139 mutant Ubc9C93S fused to β -arr2 (**Fig. 1a**) failed to promote β -arr2 SUMOylation in
140 the presence of either SUMO1 or SUMO2 (**Fig. 1b and c**, lanes 3). Similar results
141 were obtained with the fusions in β -arr1/2 knockout (KO)-mouse embryonic
142 fibroblasts (**Fig. S1**). The expected MW of native β -arr2 is 55kDa. Following highly
143 denaturing lysis, affinity pulldown with Ni-NTA resin greatly enriched SUMOylated
144 human β -arr2 with a MW of ~75kDa corresponding to β -arr2 covalently conjugated
145 with one molecule of SUMO2 in HEK-293 cells expressing exogenous native β -arr2,
146 Ubc9 and His-SUMO2, or FLAG-tagged β -arr2, Ubc9 and His-SUMO2 (**Fig. 1d and**
147 **Fig. 1e**, right lanes). SUMOylated β -arr2 was not observed in control cells lacking
148 His-SUMO2 (**Fig. 1d and Fig. 1e**, left lanes). Similar results were obtained using a
149 yellow fluorescent protein (YFP)-tagged form of β -arr2 (**Fig. 1f**, MW band at
150 ~100kDa).

151 Human β -arr2 is a 409 amino acid protein composed of N- and C- globular
152 domains linked together by a short hinge region, and a flexible regulatory C-terminal
153 tail (**Fig. 2a**). To screen for a SUMO acceptor site(s) in β -arr2, a library of overlapping
154 peptides (25-mers), each shifted by 5 amino acids across the entire sequence of β -
155 arr2 (**Fig. 2a**), was SPOT-synthesized on cellulose membranes. The peptide arrays
156 were subjected to *in vitro* SUMOylation assays using a SUMO assay mix containing

157 recombinant forms of E1-activase, E2-conjugase Ubc9, SUMO1, SUMO2 and
158 SUMO3, and Mg-ATP solution. Control arrays were performed using SUMO assay
159 mixtures that omitted Ubc9. The arrays were then probed with anti-SUMO antibodies
160 and SUMOylated peptides were identified as dark spots. Using this approach, three
161 consecutive SUMOylated peptides were identified comprising amino acids T276-
162 G310 (**Fig. 2b and Fig. S2a**) that was absent in the control arrays. These peptides
163 contained a consensus SUMOylation motif in β -arr2 294-L-K-H-E-297 that has
164 previously been documented (15, 16). Confirming that K295 is indeed SUMOylated in
165 the peptides, its mutation to alanine in a “progeny” 25-mer containing amino acids
166 276-300 inhibited SUMOylation (**Fig. 2c and Fig. S2b**). To confirm the SUMO
167 acceptor site in cells, HEK-293 cells were co-transfected with YFP- β -arr2 or mutant
168 YFP- β -arr2K295R in the presence of Myc-Ubc9 and His-SUMO2. While a ~100kDa
169 band of SUMOylated YFP- β -arr2 was observed in Ni-NTA experiments in cells
170 expressing wild-type β -arr2 (**Fig. 2d**, lane 1), this band was lost with the YFP- β -
171 arr2K295R mutant (**Fig. 2d**, lane 2), consistent with this being a major SUMOylation
172 site for β -arr2. Taken together, the above findings indicate that β -arr2 is SUMOylated
173 both *in vitro* and in cells, and that K295, located in the regulatory “ariat loop” of the
174 C-domain of β -arr2 (**Fig. 2e**), represents a major SUMO conjugation site contained
175 within a consensus SUMOylation site.

176

177 **Human β -arr2 contains a SIM in its N-domain**

178 In addition to SUMOylation sites for covalent SUMO conjugation on lysine
179 residues, SIMs exist that mediate non-covalent interaction between SUMO and SIM-
180 containing proteins. We used a Joined Advanced Sumoylation Site and SIM Analyzer
181 (JASSA) programme (24), which predicts SIMs, to analyse the primary sequence of
182 β -arr2. A potential SIM was predicted in the N domain of β -arr2 between amino acids
183 41 and 44: 41-V-V-L-V-44 (**Fig. 3a and b**). **Figure 3a** shows the alignment of the
184 potential SIM sequence in β -arr2 against other known SIMs found in Daxx, RanBP2,
185 PML-III and PIAS1. In addition, the sequence in β -arr2 also contains two aspartic
186 acid residues juxtaposed to the hydrophobic core (**Fig. 3a and b**), another important
187 feature of functional SIMs. To test whether the potential SIM in β -arr2 is capable of
188 non-covalent interaction with SUMO, we carried out pulldown experiments using
189 SUMO1 and SUMO2 proteins coupled to agarose beads. Whereas wild-type β -arr2
190 bound to both SUMO1 and SUMO2 beads, a β -arr2 Δ SIM mutant with the 41-V-V-L-

191 V-44 sequence mutated to 41-A-A-L-A-44 displayed greatly decreased binding (**Fig.**
192 **3c**). Thus, the SIM domain in β -arr2 is functional and provides non-covalent
193 interaction with both SUMO1 and SUMO2.

194

195 **β -arr2 SIM is required for nuclear import**

196 β -arr2 is actively imported into the nucleus and subsequently excluded from
197 the nuclear compartment via active nuclear export (14). As SUMOylation and non-
198 covalent SUMO interactions are both known to impact protein targeting to the
199 nucleus, we next investigated their possible involvement in the coordination of β -arr2
200 nucleocytoplasmic trafficking. HeLa cells were transfected with YFP-tagged forms of
201 wild-type β -arr2, SUMOylation mutant (β -arr2K295R) or SIM mutant (β -arr2 Δ SIM)
202 (**Fig 4a**). The cells were then incubated with vehicle or Leptomycin B (LMB), a drug
203 that specifically inhibits nuclear export of proteins containing leucine-rich nuclear
204 export signals (NES) through inactivation of CRM1/exportin1 (25). All fusions
205 demonstrated a cytoplasmic distribution at steady state (**Fig 4b**). As expected, a 1h
206 incubation with LMB elicited wild-type β -arr2 nuclear accumulation (**Fig 4b**); β -
207 arr2K295R also accumulated in the nucleus in the presence of LMB, demonstrating
208 that SUMOylation on K295 is not required for nuclear import. In contrast, the β -
209 arr2 Δ SIM mutant failed to accumulate in the nucleus suggesting a defect in its
210 nuclear import. Similar results were obtained with non-tagged β -arr2 Δ SIM, FLAG-
211 tagged β -arr2 Δ SIM and mCherry-tagged β -arr2 Δ SIM (**Fig. S3**). Live cell imaging in
212 cells transfected with YFP-tagged forms of β -arr2 corroborated results obtained in
213 fixed cells: β -arr2 progressively accumulated in the nucleus during LMB incubation,
214 whereas β -arr2 Δ SIM did not (**Fig. 4c and d**), confirming that this mutant presents a
215 defect in nuclear import. Despite this defect in nuclear import, control endocytosis
216 experiments showed that β -arr2 Δ SIM is still functional as it was able to promote
217 internalization of the GPCR V2 vasopressin receptor (**Fig. S4a and b**).

218 SIMs can promote *in cis* SUMOylation of protein targets and this can occur on
219 lysine residues that do not lie in strict consensus SUMOylation sequences (17). To
220 rule out that potential additional minor SUMOylation on a secondary lysine in β -arr2
221 could contribute to nuclear import the β -arr2 primary sequence was analysed using
222 the JASSA programme (24). In addition to the major K295 site, K11, K25, K53, K158,
223 K293 and K400 were predicted as potential SUMOylation conjugation sites (**Fig. 5a**).
224 We mutated these lysine residues to arginine either individually, or in combination

225 with the K295R mutation, and tested their impact on nuclear import by incubating
226 HeLa cells expressing the mutant forms of β -arr2 with LMB for 1h. All mutants
227 accumulated in the nucleus, like K295R, ruling out the possibility that they are
228 implicated in nuclear import (**Fig. 5a** and **b**, and **Fig. S5**). Finally, we also created a
229 SUMO1- β -arr2 Δ SIM fusion tagged with YFP, to determine if SUMO fused to β -
230 arr2 Δ SIM could promote nuclear import. In the presence of LMB this fusion also
231 failed to accumulate in the nucleus, in contrast to a fusion of β -arr2 with the nuclear
232 localization signal (NLS) of SV40, which was able to rescue β -arr2 Δ SIM nuclear
233 import (**Fig. 5c**). We next tested the effect of the β -arr2 Δ SIM mutation in a context
234 where nuclear export of β -arr2 is inhibited through mutation of its NES (L394A). As
235 expected YFP- β -arr2 Δ NES strongly accumulated in the nucleus, however the
236 Δ SIM Δ NES mutant did not, again confirming the importance of the β -arr2 SIM in
237 nuclear import (**Fig. 5d**). Similar results were obtained with fractionation experiments,
238 showing marked enrichment of YFP- β -arr2 Δ NES in the nuclear fraction, whereas
239 YFP- β -arr2 Δ SIM Δ NES, like wild-type β -arr2, was not (**Fig. 5e**). We also used
240 enhanced bystander bioluminescence resonance energy transfer (ebBRET), based
241 on energy transfer between the naturally occurring chromophores luciferase (Rluc)
242 and green fluorescent protein (rGFP) from Renilla (26) to monitor nuclear
243 accumulation of β -arr2. For this, rGFP was targeted to the nucleus through fusion of
244 an NLS (rGFP-NLS) and relative nuclear residency of Rluc- β -arr2 fusions were
245 assessed by their ability to generate BRET signals (**Fig. 5f**). Rluc- β -arr2 Δ NES
246 generated a stronger BRET signal compared to wild-type in agreement with its
247 expected nuclear localization (**Fig. 5f**). The Δ SIM Δ NES mutant displayed a marked
248 decrease in BRET compared to Δ NES indicating reduced nuclear accumulation.

249

250 **The RanBP2/RanGAP1-SUMO complex gates β -arr2 nuclear entry**

251 The above results indicate that whereas SUMOylation is not required for β -
252 arr2 nuclear import, a non-covalent interaction of the β -arr2 SIM with a SUMOylated
253 protein partner may contribute to its nuclear import. Interestingly, we found that β -
254 arr2 associates with the nucleoporin RanBP2 and RanGAP1-SUMO1 (**Fig. 6a**),
255 components of a multimolecular SUMO E3 ligase complex that resides on the
256 cytoplasmic filaments of the nuclear pore complex. This complex is involved in
257 substrate SUMOylation and acts as a hub for nucleocytoplasmic transport (27, 28).
258 RanBP2 is a 358-kDa protein that contains multiple domains including several FG

259 repeats, four Ran-binding domains and a region that interacts with RanGAP1 (29)
260 (30) (31) (32). The interaction of RanGAP1 with RanBP2 requires RanGAP1
261 SUMOylation (29). When the SIM in β -arr2 is mutated there is a marked reduction in
262 coimmunoprecipitation of both RanGAP1-SUMO1 and RanBP2 with β -arr2, indicating
263 the SIM is important for RanBP2 and RanGAP1-SUMO1 association with β -arr2 (**Fig.**
264 **6a**). To determine if RanBP2 may play a role in β -arr2 nuclear import we subjected
265 HeLa cells to RanBP2 siRNA treatment and subsequently transfected the cells with
266 mCherry- β -arr2 Δ NES. Both RanBP2 and RanGAP1-SUMO1 levels were significantly
267 reduced compared to control cells (**Fig. 6b**) as previously observed (33). Under these
268 conditions Cherry- β -arr2 Δ NES shifted from a nuclear to cytoplasmic distribution
269 demonstrating that the RanBP2/RanGAP1-SUMO1 complex plays an important role
270 in promoting β -arr2 nuclear import. These results indicate that the β -arr2 SIM targets
271 β -arr2 to the RanBP2/RanGAP1-SUMO1 complex, which is involved in stimulation of
272 β -arr2 nuclear entry.

273

274 **Coordinated nuclear import and export of β -arr2 regulates Mdm2 subcellular** 275 **localization and p53 activity**

276 The nucleocytoplasmic shuttling function of β -arr2 displaces nuclear binding
277 cargoes from the nucleus to the cytoplasm. It was previously demonstrated that
278 displacement of Mdm2, the major negative regulator of p53, from the nucleus to the
279 cytoplasm results in increased p53 activity (7). We anticipated that a functional SIM
280 would be essential for Mdm2 displacement by β -arr2. In HEK 293 cells transfected
281 with wild-type β -arr2, GFP-Mdm2 was displaced from the nucleus to the cytoplasm,
282 unlike control cells, where GFP-Mdm2 remained nuclear (**Fig. 7a** and **b**).
283 Quantification of Mdm2 displacement by β -arr2 demonstrated ~60% of Mdm2
284 cytoplasmic relocalization: in ~10% of cells Mdm2 was predominantly cytoplasmic
285 and in ~50% partly cytoplasmic, in agreement with previous studies (7) (**Fig. 7c**). In
286 the presence of β -arr2 Δ SIM, the distribution of Mdm2 was similar to control
287 Cherry/Mdm2 expressing cells (**Fig. 7a-c**). This indicates that due to defective
288 nuclear import, β -arr2 Δ SIM fails to displace Mdm2 from the nucleus to the cytosol.
289 From a functional viewpoint with regard to Mdm2 displacement, the β -arr2 Δ SIM
290 therefore behaves like the β -arr2 Δ NES mutant, which enters in the nucleus but
291 cannot displace Mdm2 due to its defective nuclear export (**Fig. 7a-c**). The

292 SUMOylation K295R mutant of β -arr2 was still able to displace Mdm2 in line with its
293 normal capacity to shuttle through the nucleus (**Fig. 7a-c**). Therefore, the SIM in β -
294 arr2, coupled with its nuclear export function, combine to regulate β -arr2 cytonuclear
295 trafficking function with consequences for Mdm2 subcellular localization.

296 To determine functional consequences of the defect in cytonuclear shuttling
297 found with β -arr2 Δ SIM on p53 signalling, we first used a H1299 non-small cell lung
298 carcinoma cell line (p53-null) engineered to express p53 using the TETON system
299 (34). We confirmed that the β -arr2 Δ SIM defect in nuclear import was also found in
300 these H1299-p53-TETON cells (**Fig. 8a**). We next transfected H1299-p53-TETON
301 cells with a plasmid coding for luciferase under the control of multiple p53 response
302 elements. As expected, incubation of the cells with doxycycline, to induce p53
303 expression comparative to endogenous levels found in MCF-7 breast cancer cells
304 carrying *TP53* (**Fig. S6a**), robustly stimulated the luciferase signal, which was
305 markedly reduced by exogenous Mdm2 (**Fig. 8b**). When β -arr2 was co-transfected
306 with Mdm2, there was a significant increase in p53-dependent luciferase activity
307 compared to Mdm2 alone. However, with the β -arr2 Δ SIM mutant, which is defective
308 in displacing Mdm2 to the cytoplasm (**Fig. 7a-c**), no significant increase in p53-
309 dependent activity was observed. This indicates that β -arr2 Δ SIM failed to rescue
310 Mdm2-mediated inhibition of p53 activity.

311 As an additional cancer cell model, we used MCF-7 cells with endogenous
312 levels of β -arr2, SUMO1, SUMO2, Ubc9, RanBP2, SUMO-RanGAP, Mdm2 and p53
313 (**Fig. S6b**) to investigate the effect of β -arr2 Δ SIM versus wild-type β -arr2 on p53
314 signalling. We observed a similar defect in β -arr2 Δ SIM nuclear import in MCF-7 cells
315 (**Fig. 8c**), to that documented in HeLa, HEK and H1299-p53-TETON cells. Finally, we
316 performed p53-dependent gene reporter experiments in MCF-7 cells. An enhancing
317 effect on p53 signalling by wild-type Rluc-tagged β -arr2, expressed at comparative
318 levels to endogenous β -arr2 (**Fig. S6c**), was observed (**Fig. 8d**) and this effect was
319 lost with β -arr2 Δ SIM. Taken together, the above data therefore demonstrate the
320 importance of the SIM in β -arr2 for enhancing p53 function in different cancer cell
321 types.

322

323 Discussion

324

325 Mdm2 is the principal negative regulator of p53. Investigating mechanisms
326 underlying Mdm2 regulation is therefore important in understanding p53 biology. β -
327 arr2 nucleocytoplasmic shuttling serves to titrate Mdm2 from the nucleus to the
328 cytoplasm to enhance p53 signalling (7). Under steady-state conditions this is a
329 receptor-independent signalling mode of β -arr2. While the active nuclear export
330 mechanism of β -arr2, due to the presence of a NES in its C-terminal tail, has been
331 well characterized (9, 14), the mechanism(s) involved in its nuclear import are not
332 completely elucidated. Previous studies have suggested that the N-domain is likely to
333 play an important role in β -arr2 nuclear import (9, 14). Here, we demonstrate that
334 SUMO orchestrates β -arr2 cytonuclear traffic. Whereas β -arr2 SUMOylation is not
335 required for β -arr2 nuclear import, a SIM in the N-terminus of β -arr2 is involved in its
336 nuclear import. Mutation of the SIM inhibits β -arr2 association with the
337 RanBP2/RanGAP1-SUMO1 nucleocytoplasmic transport hub, and β -arr2 nuclear
338 import. As a consequence, the ability of β -arr2 to titrate Mdm2 from the nucleus to
339 the cytoplasm and effect on p53 signalling is impaired. Our data therefore unveil that
340 a SUMO-SIM nuclear entry checkpoint, coupled with the nuclear export function of β -
341 arr2, regulates its cytonuclear trafficking function to control the Mdm2-p53 loop.

342 We confirmed that K295 is a major SUMOylation site in human β -arr2.
343 Previous studies of β -arr2 SUMOylation have shown that bovine β -arr2 is
344 SUMOylated on both lysines K295 and K400 but that K400 represents the main
345 SUMOylation site in this species (15). Inhibition of bovine β -arr2 SUMOylation,
346 decreased its association with the endocytic partner β 2-adaptin and attenuated β 2-
347 AR endocytosis (15). These data suggest that β -arr2 SUMOylation enhances its
348 binding to β 2-adaptin to promote the canonical function of β -arr2 in GPCR
349 endocytosis. Alignment of the bovine and human sequences around K400, however,
350 demonstrates that the human sequence does not fit a strict consensus SUMOylation
351 site here. A subsequent study using human β -arr2 demonstrated that, in contrast to
352 bovine β -arr2, the main SUMOylation site resides at K295 (16). SUMOylation on this
353 site was found to inhibit β -arr2 binding to TRAF6, permitting enhanced TRAF6
354 oligomerization and autoubiquitination, and promoting TRAF6-mediated NF- κ B
355 signalling (16). Our results therefore agree with the study on human β -arr2 and
356 confirm K295 as a main SUMOylation site. We found that SUMOylation of β -arr2,

357 however, was not essential for its nuclear import. This finding does not, however, rule
358 out potential intranuclear roles for β -arr2 SUMOylation.

359 We also identified and characterized a SIM in the N-terminal domain of β -arr2
360 that we found was required for nuclear delivery. Similar to the role for the β -arr2 SIM
361 in promoting nuclear delivery, SIMs have also been proposed to participate in the
362 nuclear import/accumulation of several other proteins to date. These include the
363 vaccinia virus protein E3 (35), the epstein-barr virus protein kinase BGLF4 (36), the
364 viral restriction factor TRIM5 α (37), and the MAPK p38 (38). This suggests that SIM-
365 SUMO mediated transport may be a wider phenomenon involved in nuclear delivery
366 of protein cargoes. SIMs promote recruitment/targeting of SIM-containing proteins to
367 SUMOylated partners. For example, the transcriptional corepressor Daxx contains a
368 SIM that is crucial for subnuclear targeting of Daxx to PML oncogenic domains and
369 for the transrepression of several SUMOylated transcription factors (17, 39). Our
370 results point towards a targeting role of the SIM in β -arr2 to RanBP2/RanGAP-
371 SUMO1, which forms part of a SUMO E3 ligase complex, localized at the cytoplasmic
372 nuclear pore complex (40). It is involved in the SUMOylation of certain substrates
373 including Ran-GDP (27) and provides a hub for nucleocytoplasmic transport (28). A
374 study investigating a nuclear import enhancement role by RanBP2 analyzed the
375 distribution of approximately 200 nuclear proteins following RanBP2 depletion (28).
376 The vast majority did not change subcellular distribution upon RanBP2 depletion, but
377 around 5% were clearly affected, demonstrating cytoplasmic accumulation due to
378 defective nuclear import. RanBP2 can therefore act as a platform for nuclear import
379 for a subset of import cargos (28). Our results demonstrating cytoplasmic
380 accumulation of β -arr2 Δ NES following RanBP2 depletion clearly indicate that this
381 nucleoporin is required for β -arr2 nuclear entry. It was proposed that RanBP2 can
382 enhance nuclear import by at least two mechanisms. First, it reduces the active
383 concentration of import receptors required for efficient transport (41, 42) and second
384 import receptor-independent interaction of selected cargos with RanBP2 can
385 increase efficiency of nuclear import (28). This suggests that β -arr2 nuclear import
386 probably involves multiple steps coordinated by RanBP2. Indeed, a recent study
387 identified a nuclear localization signal in β -arr2 and importin β 1-dependent nuclear
388 import (43). This suggests that β -arr2 has dual nuclear entry signals similar to what
389 has been documented with the nuclear protease Calpain 5 (44). RanBP2 serves as a
390 binding site for importin β 1 keeping the transport receptor in association with the

391 nuclear pore complex. RanBP2 therefore likely acts as a hub to coordinate
392 spatiotemporal regulation of β -arr2 nuclear import occurring through dual nuclear
393 entry motifs. In summary, our findings demonstrate that the β -arr2 SIM targets it to
394 the RanBP2/RanGAP-SUMO1 complex, which gates β -arr2 nuclear entry.

395 As mutation of the SIM inhibits β -arr2 nuclear import, its ability to delocalize
396 Mdm2 from the nucleus to the cytoplasm and effect on enhanced p53 signalling is
397 impaired. The Δ SIM mutant therefore gives rise to the same impaired p53 signalling
398 effect as the Δ NES mutant, which also fails to displace Mdm2 from the nucleus (7).
399 Our results uncovering the critical role of a β -arr2 SIM in its nuclear entry, coupled
400 with the previously characterized export mechanism, generate an emerging picture of
401 regulatory nodes that impact receptor-independent β -arr2-mediated control of the
402 Mdm2/p53 axis. Future studies will be required to determine if β -arr2 cytonuclear
403 shuttling function is altered in cancer settings.

404

405 **Materials and Methods**

406

407 **Reagents, plasmids and antibodies.**

408 A full list of reagents, plasmids and antibodies is provided in the
409 Supplementary Materials and Methods section.

410

411 **Cell culture and transfection**

412 Cell culture and transfection conditions are provided in the Supplementary
413 Materials and Methods section.

414

415 **His-tagged protein purification using Ni-NTA beads**

416 Assay conditions for His-tagged protein purification are provided in the
417 Supplementary Materials and Methods section

418 **SUMO beads pulldown assay**

419 For isolation and enrichment of SUMO interacting proteins, SUMO agarose
420 beads from ENZO were used. Assay conditions are provided in the Supplementary
421 Materials and Methods section.

422 **SPOT synthesis of peptides**

423 A peptide library of overlapping 25-mers, that scan the entire human β -arr2
424 sequence, was produced by automatic SPOT synthesis and synthesized on
425 continuous cellulose membrane supports on Whatman 50 cellulose membranes
426 using Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry with the AutoSpot-Robot ASS
427 222 (Intavis Bioanalytical Instruments), as described previously (45).

428

429 ***In vitro* SUMOylation on β -arr2 peptide arrays**

430 A SUMOylation kit (Biomol) was used according to the manufacturer's
431 instructions for the SUMOylation of putative SUMO sites contained within the β -arr2
432 peptide array (46). The SUMO assay mix was incubated with array membranes at
433 30°C with shaking. Membranes were washed with TBS-T (Tris-buffered saline with
434 Tween 20: 137mM NaCl, 20mM Tris/HCl, pH7.6, and 0.1% Tween 20) followed by
435 probing the SUMOylated moities on the peptide array using an anti-SUMO antibody.

436 Control arrays were performed using SUMO assay mixtures that omitted the E2
437 conjugase Ubc9.

438

439 **Coimmunoprecipitation**

440 HEK cells were transiently transfected with plasmids as indicated in the figure
441 legends and following lysis subjected to coimmunoprecipitation as detailed in the
442 Supplementary Materials and Methods section.

443

444 **Live cell imaging and Immunofluorescence**

445 Live cell imaging and immunofluorescence experiments were conducted as
446 previously described (47). Details are provided in the Supplementary Materials and
447 Methods section.

448

449 **Flow Cytometry**

450 The details of the flow cytometry assay used to monitor β -arr2-dependent
451 endocytosis of HA-V2R-vYFP have been described previously (48). Details are
452 provided in the Supplementary Materials and Methods section.

453

454 **Cytoplasmic and nuclear fractionation**

455 Cytoplasmic and nuclear extracts were prepared from HeLa cells using the
456 Invent Biotechnologies MinuteTM fractionation kit according to the manufacturer's
457 instructions.

458

459 **Enhanced bystander bioluminescence resonance energy transfer (ebBRET)**

460 Endocytosis and nuclear localization ebBRET assays were performed in HEK
461 cells and are described in the Supplementary Materials and Methods section.

462

463 **Gene reporter experiments**

464 H1299-p53-TETON cells and MCF-7 cells were co-transfected with p53-luc
465 (Stratagene), containing 14xp53-response elements, and either pRL.TK (H1299
466 cells) or Rluc-tagged forms of β -arr2 (MCF-7 cells) using Lipo3000 in 12-well plates.
467 Cells were lysed using passive lysis buffer (Promega) and both firefly and Renilla
468 luciferase activities detected using the Dual-Luciferase Reporter Assay System
469 (Promega).

470

471 **Molecular Modelling**

472 The 3D structure used for β -arr2 is PDB:3P2D (Zhan et al., 2011). Figures
473 were prepared with PyMol Molecular Graphics System, Version 2.0 Schrödinger,
474 LLC.

475

476 **Data analysis and statistics**

477 Data are represented as mean \pm s.e.m. Statistical analysis was performed
478 using GraphPad Prism using unpaired *t*-tests or one-way analysis of variance with
479 Tukey's *post-hoc* test.

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481

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493

494 **Author contributions**

495

496 EBT, ML, BS, JSP, GSB, HE, MB, SA, SM and MGHS designed research. EBT, ML,
497 JSP, BS, MK, KS, JF, AP and MGHS performed research. EBT, ML, BS, JSP, CA,
498 ELF, AZ, LG, GSB, HE, MB, SA, SM and MGHS analyzed data. MGHS supervised
499 the project. EBT and MGHS wrote the manuscript, which was subsequently reviewed
500 by all other authors.

501

502 The authors declare that they have no conflict of interest.

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- 646

647 **Figure Legends**

648

649 **Figure 1. β -arr2 is SUMOylated in cells.** (a) Schematic diagram details the Ubc9- β -
650 arr2-FLAG and catalytically dead Ubc9C93S- β -arr2-FLAG UFDS fusions. (b and c)
651 SUMOylation of β -arr2 assessed by UFDS. Ubc9- β -arr2 or Ubc9C93S- β -arr2 were
652 coexpressed with HA-SUMO1 (b) or HA-SUMO2 (c) in HeLa cells as indicated. The
653 protein extracts were subject to immunoprecipitation using EZview Red ANTI-FLAG®
654 M2 Affinity Gel, and fusions and SUMOylated fusions were detected by western blot
655 using anti-FLAG antibodies. The top set of arrows indicate SUMOylated forms of
656 Ubc9- β -arr2. HEK-293T cells were co-transfected with plasmids encoding Ubc9, His-
657 tagged SUMO2 or empty vector, and either (d) β -arr2, (e) β -arr2-FLAG or (f) YFP- β -
658 arr2. 48h later, cells were lysed in denaturing conditions followed by purification on
659 Ni-NTA beads. Cell lysates and His-SUMO conjugated proteins purified with Ni-NTA
660 agarose beads were analyzed by western blot with the indicated antibodies. The
661 arrow indicates SUMO2-conjugated β -arr2.

662

663 **Figure 2. β -arr2 is SUMOylated on lysine 295.** (a) Schematic diagram showing the
664 library of overlapping 25-mer peptides that cover the entire β -arr2 sequence. (b) The
665 peptide library was overlaid with SUMO conjugation assay mixture (Ubc9+) including
666 recombinant His-SUMO1, His-SUMO2 and His-SUMO3. Dark spots represent a run
667 of peptides that displayed successful conjugation of recombinant SUMO to
668 immobilized peptides that are absent in the control array (Ubc9-). (c) Alanine
669 substitution at K295 inhibits SUMO conjugation on the immobilized 276-300 peptide.
670 (d) K295R mutation inhibits SUMOylation in cells following lysis in denaturing
671 conditions and purification on Ni-NTA beads. (e) Structure of β -arr2 in cartoon
672 representation, with SUMO site in red sticks representation (left), zoom on K295
673 residue (right).

674

675 **Figure 3. β -arr2 contains a SIM in its N-domain** (a) Alignment of the putative SIM
676 sequence in the N-domain of β -arr2 against other known characterized SIMs in Daxx,
677 RanBP2, PML-III and PIAS1. (b) Structure of β -arr2 in cartoon representation, with
678 SIM site in orange sticks representation (left), zoom on the SIM residues, also
679 highlighting the DPD loop in green sticks (right). (c) Pulldown of wild-type β -arr2 but
680 not β -arr2 Δ SIM mutant by SUMO1- or SUMO2-agarose beads.

681 **Figure 4. The SIM but not SUMOylation on lysine 295 is required for β -arr2**
682 **nuclear entry. (a)** Schematic diagram showing the SIM and K295 SUMOylation site
683 in β -arr2. The SIM was mutated from 41-VVLV-44 to 41-AALA-44 and the
684 SUMOylation site changed from 294-LKHE-297 to 294-LRHE-297. **(b)** HeLa cells
685 transfected with YFP-tagged β -arr2 wild-type, β -arr2-K295R, or β -arr2 Δ SIM were
686 incubated with methanol control (CTL) or 20 nM LMB for 60 min at 37°C, then fixed
687 and processed for confocal fluorescence microscopy. Representative images are
688 shown. **(c)** Direct visualization of YFP- β -arr2 WT or YFP- β -arr2 Δ SIM nuclear
689 accumulation in live HeLa cells in the presence of 20 nM LMB for 60 min. Images
690 were acquired using a spinning disk confocal microscope equipped with a 37°C
691 heated control chamber. Images acquired every 15 min are displayed. **(d)**
692 Quantification of β -arr2 WT or β -arr2 Δ SIM nuclear accumulation in the presence of
693 LMB in live cells. Fluorescence intensity was quantified using the previously
694 described ImageJ plugin (Blondel et al., 2019) and values were plotted as a
695 percentage of maximal WT response. Data represent mean \pm s.e.m (n=10;
696 ***P<0.001). Scale bars, 10 μ m.

697

698 **Figure 5. SUMO1 fusion to β -arr2 Δ SIM does not rescue nuclear import but an**
699 **NLS fusion does. (a)** Schematic diagram showing other potential SUMOylation sites
700 predicted by JASSA. **(b)** HeLa cells were transfected with the indicated constructs
701 and incubated with 20 nM LMB for 60 min. Accumulation of the various fusions in the
702 nucleus is indicated. **(c)** HeLa cells transfected with plasmids encoding YFP-SUMO1-
703 β -arr2 Δ SIM or YFP-NLS- β -arr2 Δ SIM were incubated with methanol (vehicle control)
704 or 20 nM LMB during 1 hour at 37 °C, then fixed and processed for fluorescence
705 microscopy. **(d)** HeLa cells transfected with plasmids encoding YFP- β -arr2, YFP- β -
706 arr2 Δ NES, or YFP- β -arr2 Δ SIM Δ NES were subsequently fixed and processed for
707 fluorescence microscopy. All cells were visualized on a confocal microscope.
708 Representative images of all conditions are shown. Scale bars, 10 μ m. **(e)** Nuclear
709 and cytoplasmic fractions of HeLa cells, transfected with plasmids encoding YFP- β -
710 arr2, YFP- β -arr2 Δ NES, or YFP- β -arr2 Δ SIM Δ NES, were prepared. The fractions were
711 analyzed by western blot. GAPDH was used as a cytoplasmic marker and p75 as a
712 nuclear marker. **(f)** Schematic representation of the ebBRET system. The RlucII
713 donor is fused to β -arr2, and the rGFP acceptor to a nuclear localization signal
714 (NLS), to target it to the nucleus (see fluorescence panels). Changes in BRET signals

715 indicate changes in nuclear accumulation. HEK293 cells transfected with plasmids
716 encoding β -arr2-RlucII, β -arr2- Δ NES-RlucII or β -arr2- Δ SIM Δ NES-RlucII and rGFP-
717 NLS were used to monitor relative β -arrestin2 nuclear localization by ebBRET. The
718 inset shows equivalent expression of the different forms of β -arr2-RucII. Data
719 represent mean \pm s.e.m (n=5; **P<0.01, ns: non-significant).

720

721 **Figure 6. The β -arr2 SIM enhances association with the RanBP2/RanGAP1-**
722 **SUMO complex and RanBP2/RanGAP1-SUMO depletion inhibits β -arr2 nuclear**
723 **entry. (a)** Western blot of FLAG immunoprecipitates from HEK cells expressing β -
724 arr2- FLAG or β -arr2 Δ SIM-FLAG showing reduced association of β -arr2 Δ SIM with
725 RanGAP1-SUMO1 and RanBP2. Data shown represent the mean \pm s.e.m. of five
726 independent experiments (**P<0.001). **(b)** HeLa cells were transfected with control
727 siRNA or siRNA targeting RanBP2. Quantification of western blots demonstrated
728 knockdown of 85 \pm 1.6% for RanBP2, and 85 \pm 7.8% for RanGAP-SUMO1 (mean \pm
729 s.e.m., n=4). The cells were subsequently transfected with Cherry- β -arr2 Δ NES and
730 cytonuclear distribution visualized directly in live HeLa cells. Representative images
731 are shown. Scale bars, 10 μ m.

732

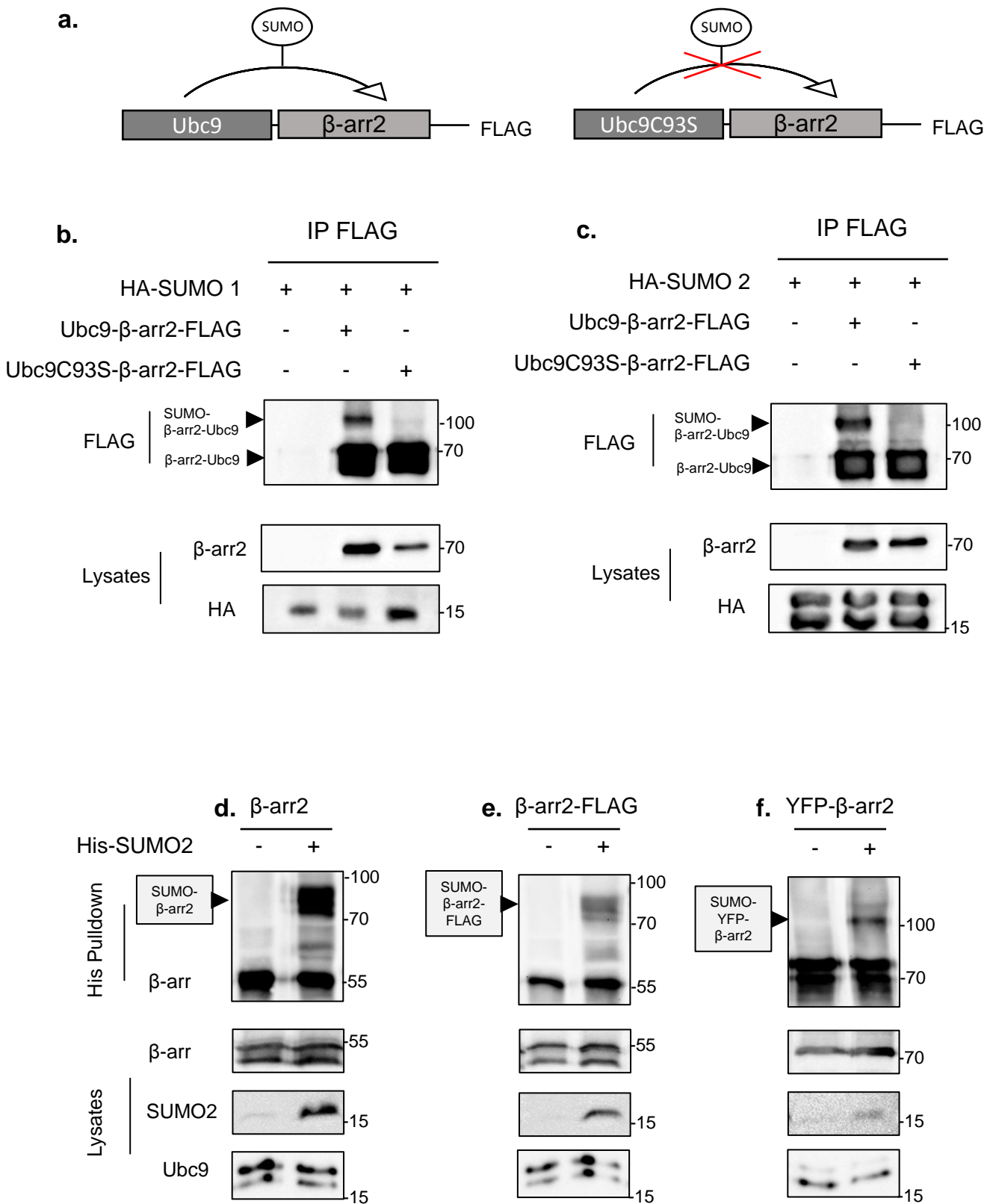
733 **Figure 7. A functional SIM domain is required for β -arr2-mediated cytoplasmic**
734 **delocalization of Mdm2. (a)** HEK cells expressing mCherry, mCherry- β -arr2,
735 mCherry- β -arr2 Δ SIM, mCherry- β -arr2-K295R or mCherry- β -arr2 Δ NES (left column)
736 and GFP-Mdm2 constructs (middle column) were imaged using a spinning disk
737 confocal microscope. Merged images are shown in the right column. Scale bar, 10
738 μ m. **(b)** Line traces generated in ImageJ of the corresponding traces (white dotted
739 lines) in the merged images shown in **(a)** with Cherry and GFP intensities displayed
740 in red and green respectively. The blue area defines the nuclear area. **(c)** Manual
741 quantification of nuclear (N), cytoplasmic (C), or partially displaced Mdm2 localization
742 (N/C). Bars indicate the percentage of cells in each category. Over 100 cells were
743 quantified for each experimental condition (**P<0.001, **P<0.01, ns: non-significant).

744

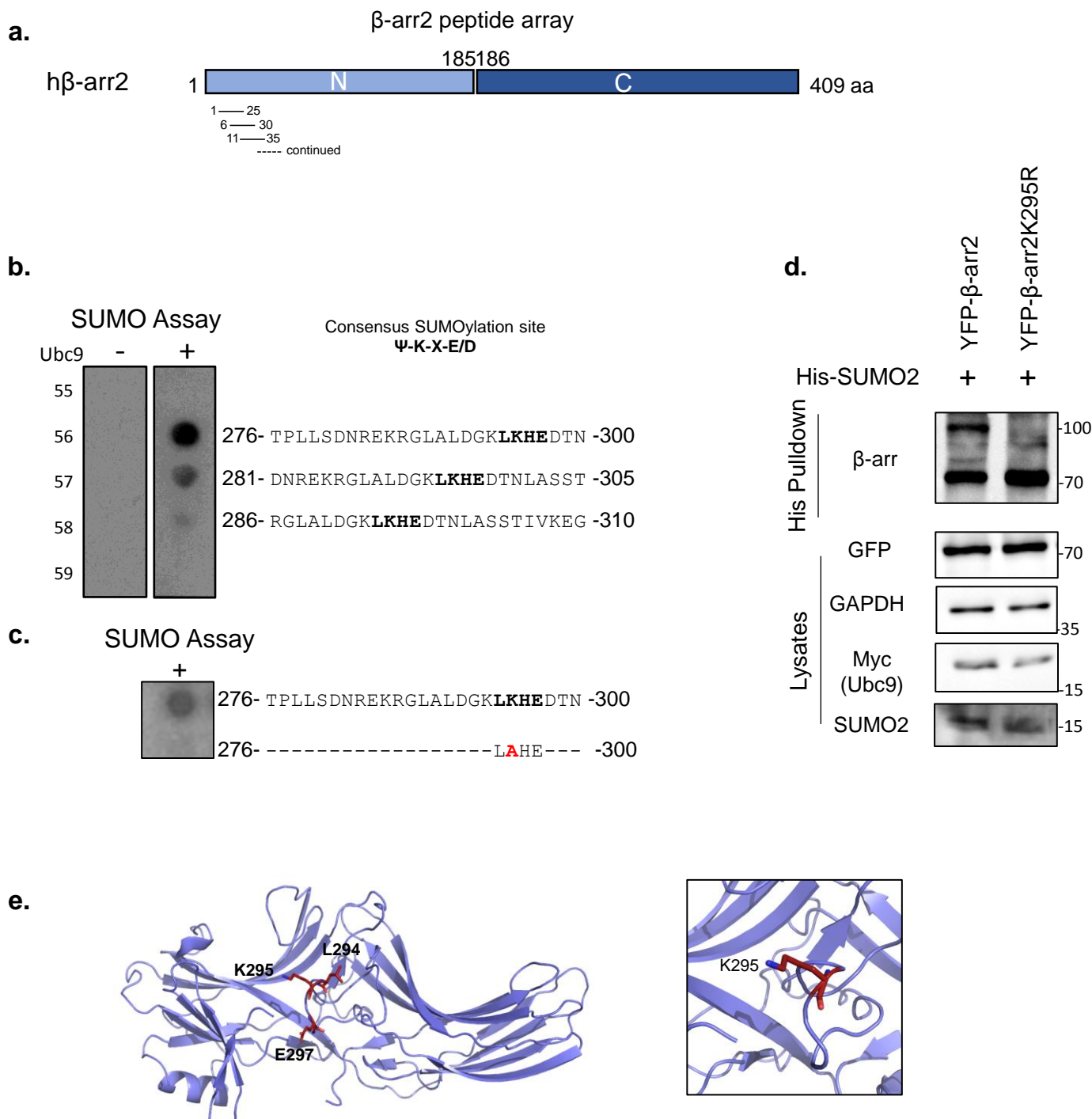
745 **Figure 8. The β -arr2 SIM domain is required for increased p53 signalling**

746 **(a)** H1299 cells expressing YFP- β -arr2 or YFP- β -arr2 Δ SIM were treated with
747 methanol control (CTL) or 20nM LMB for 60 minutes and live cells were imaged

748 directly using a spinning disk confocal microscope. Scale bar 10 μ m. **(b)** H1299-p53-
749 TETON cells were stimulated with 100ng/ml doxycycline to induce p53 expression
750 (+p53) and subsequently transfected with 14xp53-RE-luc and control pRL.TK, in
751 addition to the indicated combinations of empty vector (-), Mdm2 and β -arr2/ β -
752 arr2 Δ SIM plasmids. Promoter activity driven by p53 is expressed as a ratio of firefly
753 luciferase:Renilla luciferase activity. Equivalent expression of β -arr2 and β -arr2- Δ SIM
754 is shown in the western blot inset. Data are expressed as mean \pm s.e.m., n=3;
755 **P<0.01. **(c)** MCF-7 cells expressing Cherry- β -arr2 or Cherry- β -arr2 Δ SIM were
756 treated with methanol control (CTL) or 20nM LMB for 60 minutes and live cells were
757 directly imaged using a spinning disk confocal microscope. Scale bar 10 μ m. **(d)**
758 MCF-7 cells were transfected with 14xp53-RE-luc and either empty vector, Rluc- β -
759 arr2 or Rluc- β -arr2 Δ SIM. Equivalent luciferase expression of Rluc- β -arr2 or Rluc- β -
760 arr2 Δ SIM is shown in the inset. Data are expressed as mean \pm s.e.m., n=4; **P<0.01.



Blondel-Tepaz et al. Fig. 1

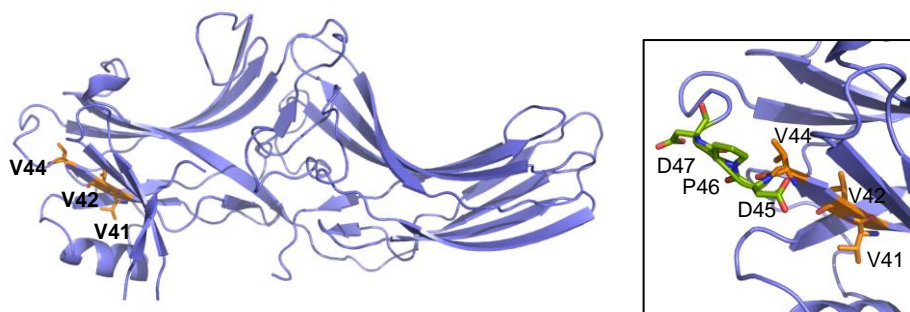


Blondel-Tepaz et al. Fig. 2

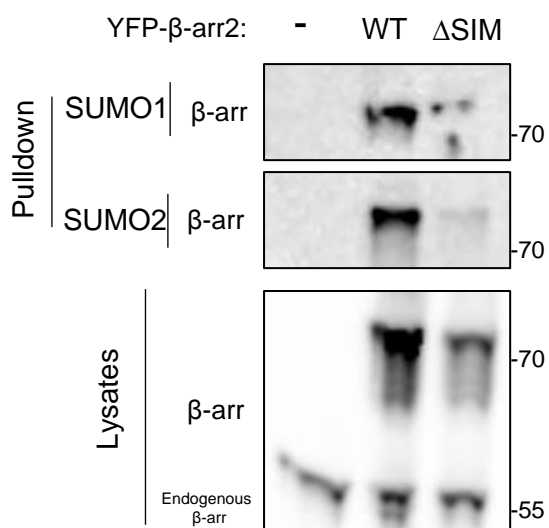
a.

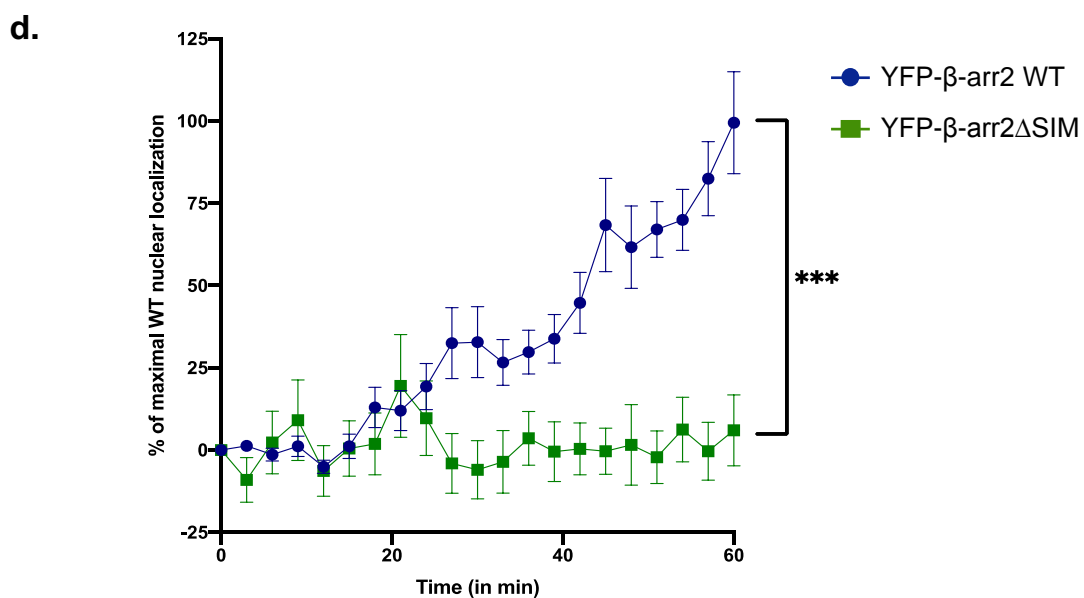
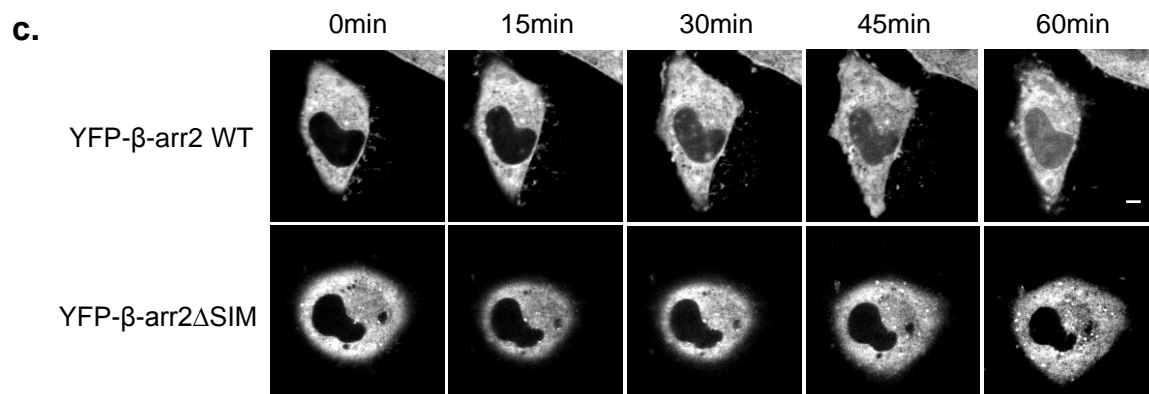
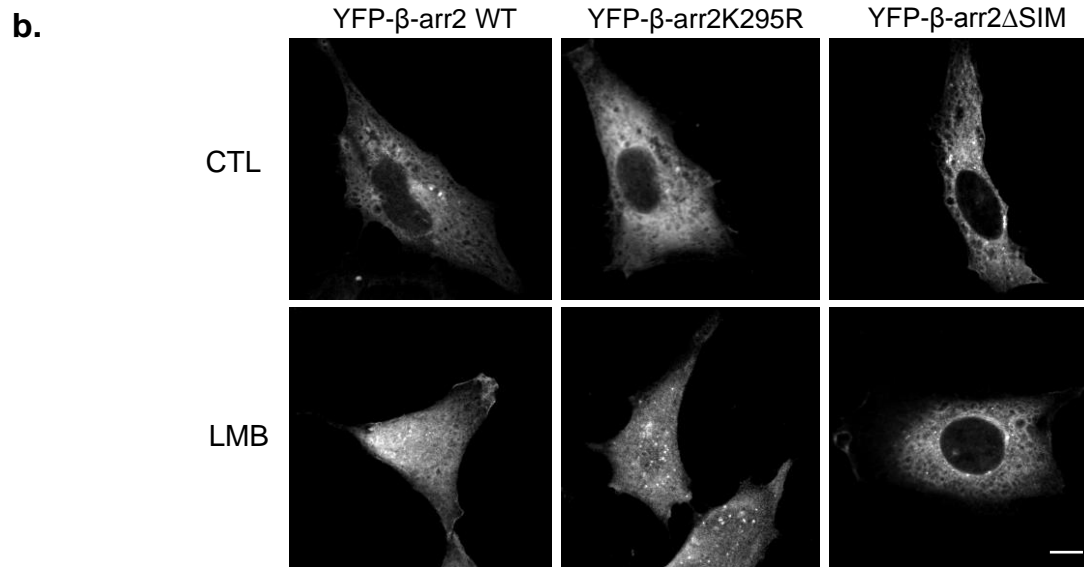
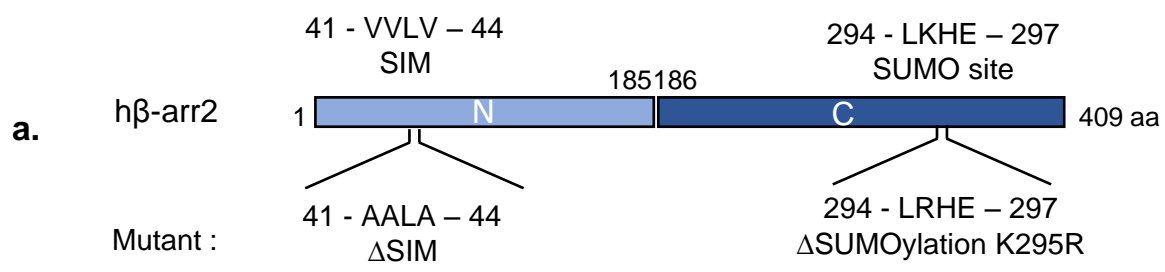
Consensus SIM	:	(V/I)-(V/I)-X-(V/I)	
hβ-arr2	37	- PVDG VVLV DPDY	- 48
Daxx	3	- TANS IIVL DDDDDEDE	- 17
	729	- DP EEIIVL SSDSD	- 740
Ran-BP2	2628	- SDD DVLIV YELTPTAE	- 2643
PML-III	552	- AEER IIVL SSSESDSD	- 566
PIAS1	455	- KKVE VIDL TIDSSSDEEEEE	- 474

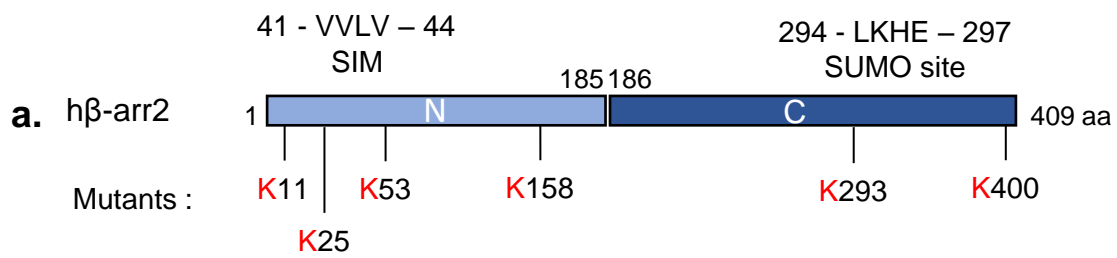
b.



c.







b.

Flag constructs	Nuclear distribution with LMB
h β -arr2K11R	+
h β -arr2K25R	+
h β -arr2K53R	+
h β -arr2K158R	+
h β -arr2K293R	+
h β -arr2K295R	+
h β -arr2K400R	+
h β -arr2K11R-K295R	+
h β -arr2K25R-K295R	+
h β -arr2K53R-K295R	+
h β -arr2K158R-K295R	+
h β -arr2K293R-K295R	+
h β -arr2K295R-K400R	+

