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► To cite this version:

Isadora Pontes Cavalcante, Anna Vaczlavik, Ludivine Drougat, Claudimara Ferini, Karine Perlemoine, et al.. Cullin 3 targets the tumor suppressor gene ARMC5 for ubiquitination and degradation. *Endocrine-Related Cancer*, 2020. hal-03014005

HAL Id: hal-03014005

<https://cnrs.hal.science/hal-03014005>

Submitted on 19 Nov 2020

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1 **Title:** Cullin 3 targets the tumor suppressor gene ARMC5 for ubiquitination and degradation

2

3

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20

21 **Abstract**

22 *ARMC5* (Armadillo repeat containing 5) was identified as a new tumor suppressor gene responsible
23 for hereditary adrenocortical tumors and meningiomas. *ARMC5* is ubiquitously expressed and encodes
24 a protein which contains a N-terminal Armadillo repeat domain and a C-terminal BTB (Bric-a-Brac,
25 Tramtrack, Broad-complex) domain, both docking platforms for numerous proteins. At present,
26 expression regulation and mechanisms of action of *ARMC5* are almost unknown.

27 In this study, we showed that *ARMC5* interacts with *CUL3* requiring its BTB domain. This interaction
28 leads to *ARMC5* ubiquitination and further degradation by the proteasome. *ARMC5* alters cell cycle
29 (G1/S phases and Cyclin E accumulation) and this effect is blocked by *CUL3*. Moreover, missense
30 mutants in the BTB domain of *ARMC5*, identified in patients with multiple adrenocortical tumors, are
31 neither able to interact and be degraded by *CUL3*/proteasome nor alter cell cycle. These data show a
32 new mechanism of regulation of the *ARMC5* protein and open new perspectives in the understanding
33 of its tumor suppressor activity.

34

35 **Introduction**

36 Germline mutations of Armadillo repeat containing 5 gene (*ARMC5*) were identified in patients
37 diagnosed with multiple bilateral adrenocortical tumors (PBMAH or primary bilateral macronodular
38 adrenal hyperplasia) (Alencar, et al. 2014; Assie, et al. 2013; Bourdeau, et al. 2016; Elbelt, et al. 2015;
39 Espiard, et al. 2015; Faucz, et al. 2014; Gagliardi, et al. 2014) conducting to increased production of
40 cortisol. The excess of cortisol (Cushing's Syndrome) leads to central obesity, hypertension, diabetes
41 mellitus and osteoporosis. A subset of patients also develop meningiomas (Alencar et al. 2014; Elbelt
42 et al. 2015). These germline mutations are heterozygous and within each tumor a second alteration
43 leads to *ARMC5* biallelic inactivation in keeping with the Knudson's two-hit model of tumor suppressor
44 gene. The discovery of *ARMC5* alterations established the first direct genetic link to PBMAH and
45 several cases of familial PBMAH have been described (Alencar et al. 2014; Bourdeau et al. 2016;
46 Elbelt et al. 2015; Gagliardi et al. 2014). *In vitro* studies in cell lines show that wild type (WT)
47 *ARMC5* induces apoptosis and that *ARMC5* point mutants lose this ability (Assie et al. 2013;
48 Cavalcante, et al. 2018; Espiard et al. 2015). Moreover, in addition to its tumor suppressor gene role,
49 *ARMC5* modulates the adrenal steroid production (Assie et al. 2013; Espiard et al. 2015). *Armc5*
50 knockout mice suggest a role in embryogenic development and immune system (Berthon, et al. 2017b;
51 Hu, et al. 2017).

52 *ARMC5*, located in the chromosome 16p11.2, is ubiquitously expressed (Berthon, et al. 2017a) and
53 encodes a protein of 935 amino acids mostly and uniformly distributed in the cytoplasm (Assie et al.
54 2013; Espiard et al. 2015). The protein *ARMC5* contains a N-terminal Armadillo repeat domain and a
55 C-terminal BTB (Bric-a-Brac, Tramtrack, Broad-complex) domain, both docking platforms for
56 numerous proteins. To date, the expression regulation and mechanisms of action of *ARMC5* are
57 unknown. Two large-scale protein-protein interaction screenings (Bennett, et al. 2010; Huttlin, et al.
58 2017), a Yeast Two Hybrid assay (Hu et al. 2017) and co-immunoprecipitation (co-IP) followed by
59 mass spectrometry (MS) analysis (personal unpublished data), suggest that *ARMC5* interacts with
60 Cullin3 (*CUL3*).

61 *CUL3* is a protein involved in the ubiquitin-proteasome system (UPS), mediating the ubiquitination
62 process and leading to target proteins to the 26S proteasome complex (Ciechanover 2017; Dubiel, et

63 al. 2018). UPS regulates various important cellular processes, such as cell cycle regulation and cell
64 growth (Ciechanover 2017). Ubiquitination is a process that relies on transferring ubiquitin (Ub) to
65 specific substrates through complexes dependent on the action of an E1 ubiquitin-activating enzyme,
66 an E2 ubiquitin conjugating enzyme and an E3 ligase, which provides the specificity of substrate
67 degradation (Ciechanover 2017). E3 ubiquitin ligases are classified into three main groups:
68 homologous to the E6-AP carboxyl terminus domain (HECT)-type, really interesting new gene
69 (RING)-type and RING-in-between-RING (RBR)-type E3 ligases (Morreale and Walden 2016).
70 Cullins are scaffold proteins that organize the largest class of RING E3 ligases, known as the cullin-
71 RING ligase complexes (CRLs). CRLs typically catalyze the addition of poly-ubiquitin chains to
72 substrates and their subsequent degradation by the proteasome (Ciechanover 2017; Dubiel et al. 2018).
73 CRL3 is composed of the CUL3 protein, the RING protein, RBX1, which binds the E2-enzyme, and a
74 protein with a BTB domain acting as a substrate-specific adaptor (Dubiel et al. 2018). The substrate
75 adaptor proteins can also be ubiquitinated by the CRL complex which they are a part of, such as
76 Keap1, RhoBTB2 and SPOP (Wilkins, et al. 2004; Zhang, et al. 2004; Zhou, et al. 2015). The balance
77 that regulates the stability of E3 ligases and substrates are important to maintain physiological
78 homeostasis. CRL3s have been involved in various biological processes (cell cycle control, protein
79 trafficking, stress responses and apoptosis) and its alterations have been associated to pathologies
80 (metabolic disorders, muscle atrophy, neurodegeneration and cancer) (Ciechanover 2017; Dubiel et al.
81 2018).

82 In this study, we showed that ARMC5 interacts with CUL3 requiring its BTB domain. This interaction
83 leads to the ubiquitination of ARMC5 leading to its degradation by the proteasome. Interestingly,
84 ARMC5 silencing or overexpression alters cell cycle (G1/S phases and Cyclin E accumulation) and
85 this effect was blocked by CUL3 (in case of ARMC5 overexpression). Moreover, missense mutants in
86 the BTB domain of ARMC5, identified in patients diagnosed with PBMAH, are neither regulated by
87 the CUL3/proteasome system nor alter cell cycle. These data show a new mechanism of regulation of
88 the ARMC5 protein and open new perspectives in the understanding of the tumor suppressor action of
89 ARMC5 and its role in adrenal tumors development.

90

91 **Material and methods**

92 Cell culture and cell transfection

93 The HEK293 and the H295R cell lines authenticated by short tandem repeats analysis were obtained
94 from American Type Culture Collection (ATCC) and cultured as previously described (Ragazzon, et
95 al. 2009) for no more than 15 passages and regularly tested for mycoplasma contamination. Human
96 adrenals were obtained after informed consent from two patients undergoing surgery for PBMAH.
97 Adrenal tissue collection was approved by the ethics committee of the Institute of Biomedical
98 Sciences of the University of Sao Paulo. PBMAH cell dissociation and cell culture were performed as
99 previously described (Cavalcante et al. 2018). Cells were transfected with plasmids or/and small
100 interfering RNA (siRNA) with Jet Prime reagent (Poly Plus Transfection) according to the
101 manufacturer's instructions. When indicated, cells were treated or not with cycloheximide (CHX,
102 Sigma-Aldrich), MG132 (Sigma-Aldrich) and MLN4924 (Calbiochem), **Aphidicolin (Sigma-Aldrich)**
103 as described in each figure legends.

104

105 Plasmids and small interfering RNA (siRNA)

106 All information and origin concerning the plasmids and siRNA used are listed in Supplemental Table
107 1. Plasmids were sequenced to confirm the absence of undesirable mutations. More details of plasmids
108 are available on request.

109

110 RNA extraction, RT-qPCR and primer sequences

111 Total RNA was extracted from the cell lines using Promega RNA extraction kit (Promega) and the
112 expression levels of target genes were determined by means of real-time polymerase chain reaction
113 (PCR) using a LightCycler Fast Start SYBR Green kit (Roche Diagnostics) according to the
114 manufacturer's instructions. Relative quantification of target cDNA was determined by calculating the
115 difference in cross-threshold (C_T) values after normalization to *PPIA* (CYCLO) signals ($\Delta\Delta C_T$
116 method). Primer sequences and conditions for all target genes were described in Supplemental Table
117 1.

118

119 Protein extraction, immunoblotting and immunoprecipitation experiments

120 Cell were lysed in lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 5 mM EDTA, 30mM Sodium
121 pyrophosphate, 5mM NaF, 2% Triton, pH 7.5) supplemented with protease inhibitors and phosphatase
122 inhibitors (Roche), followed by centrifugation at 15,000g for 10 min at 4°C. Equal amount of proteins
123 were resolved by SDS–PAGE, transferred to nitrocellulose membranes, and incubated with primary
124 antibodies overnight at 4°C. After washing, the membranes were incubated with the secondary
125 antibody used at a dilution of 1:5.000 for 1 h at room temperature. Bound antibodies were revealed
126 using an ECL system (Pierce) and signal detected with a Pxi Camera (Ozyme). For co-
127 immunoprecipitation experiments, cells were lysed in same lysis buffer and immunoprecipitations
128 were performed on 800 µg of protein extracts with 2 µg of mouse anti-FLAG or mouse anti-HA
129 overnight at 4°C with gentle agitation and magnetic beads (Protein G Mag Sepharose #28-9440-08,
130 GE healthcare) were added for 2h. After washing, immunoprecipitates were eluted with 2x SDS
131 loading buffer. For ubiquitination assay, cells were lysed under denaturing conditions (lysis buffer
132 supplemented with 1% SDS, 1mM DTT and 1.25mg/ml N-Ethylmaleimide (Sigma-Aldrich)) and
133 boiled at 95°C for 5 minutes. For these assays, immunoprecipitations were performed with ANTI-
134 FLAG M2 Affinity Gel (Anti-FLAG®M2; Sigma-Aldrich) and immunoprecipitates were eluted with
135 3X FLAG peptide (F4799, Sigma-Aldrich). All antibodies used in this study are listed in Supplemental
136 Table 1.

137

138 Bioluminescence resonance energy transfer (BRET) assays

139 The apparent affinity of wild type and the different mutants of ARMC5 for CUL3 was evaluated by
140 BRET. In each experiment, a fixed amount of BRET donor plasmids (WT or mutants ARMC5- The
141 apparent affinity of wild type and the different mutants of ARMC5 for CUL3 was evaluated by BRET.
142 In each experiment, a fixed amount of BRET donor plasmids (WT or mutants ARMC5-luciferase
143 (Luc)) was transfected in HEK293 cells (6-well plates) in association with increasing amounts of the
144 BRET acceptor plasmid CUL3-Yellow Fluorescent Protein (CUL3-YFP plasmid) (10-300ng). For
145 each transfection point, luciferase, YFP, and BRET signals were measured using a Mithras Multimode
146 Microplate reader LB 940 multimode reader (Berthold). BRET results were expressed in milli-BRET

147 units (mBRET), plotted as a function of YFP/Rluc, in which YFP represents the actual amount of
148 expressed BRET acceptor and Rluc the amount of BRET donor in each sample.

149

150 Cell cycle analysis

151 For cell cycle experiments, cells were trypsinized, rinsed with PBS and fixed in 70% ethanol after
152 transfection. Fixed cells were centrifuged, washed with PBS and resuspended in Propidium Iodide (PI)
153 solution containing 50 μ g/mL of PI (Sigma Aldrich) and 100 μ g/mL RNase A (Sigma Aldrich) in PBS.
154 For each experiment, 20.000 events were acquired by flow cytometry using Novocyte Cytometer
155 (Ozyme). Data were analysed using the Novoexpress Software.

156

157 Statistical analysis

158 Data are presented as mean \pm standard deviation (SD). No statistical method was used to predetermine
159 sample size. Statistical analyses were performed by student t-test, by one-way ANOVA followed by
160 Tukey's test or by two-way ANOVA. Statistically significant differences are indicated as * $p < 0.05$,
161 ** $p < 0.01$, *** $p < 0.001$.

162

163 Results

164 *CUL3 interacts with ARMC5 through the BTB domain*

165 We tested the interaction of ARMC5 with CUL3 in human embryonic kidney (HEK293) cell protein
166 extracts. Overexpressed WT ARMC5-FLAG co-immunoprecipitated (IP) with HA-CUL3 (Fig. 1-A-
167 left panel). Reciprocal co-IP experiments confirmed the interaction between HA-CUL3 with ARMC5-
168 FLAG (Fig. 1-A-right panel). This interaction was further analyzed in living cells using a
169 bioluminescence resonance energy transfer (BRET) proximity assay. Specific close proximity
170 (<10nm) between ARMC5 and CUL3 was demonstrated by a hyperbolic BRET saturation curve upon
171 the expression of increasing concentrations of the BRET acceptor (CUL3-YFP) in the presence of
172 constant amounts of BRET donor (ARMC5-Luc) (Fig. 1-B). Then, we investigated if three *ARMC5*
173 missense mutations located in different parts of the protein (p.L548P, p.L754P and p.R898W) and
174 identified in patients diagnosed with PBMAH might alter its interaction with CUL3. ARMC5 p.L754P

175 mutant did not co-immunoprecipitate with HA-CUL3, contrary to ARMC5 WT and mutants p.L548P
176 and p.R898W (Fig. 1-C). Similarly, we observed a drastic loss of the BRET signal with
177 ARMC5.p.L754P-Luc as BRET donor and the acceptor CUL3-YFP, in contrast to ARMC5 WT or to
178 both other ARMC5 mutants (p.L548P and p.R898W) (Fig. 1.B). Interestingly, the amino acid 754 is
179 localized in the BTB domain (748-816aa) of ARMC5. Finally, for better characterizing the importance
180 of the BTB domain in this interaction, we used different fragments of ARMC5 protein and observed
181 that only the construction 442-935aa, containing the middle, the BTB and the C-terminal domains of
182 ARMC5, co-immunoprecipitated with HA-CUL3 (Fig. 1-D). However, CUL3 does not interact with
183 shorter protein fragments containing the BTB domain (442-818aa and 745-935aa). Altogether, these
184 complementary approaches demonstrate that ARMC5 and CUL3 form a complex and that the BTB
185 domain of ARMC5 is necessary but not sufficient for this interaction.

186

187 *ARMC5 is a direct substrate of the CUL3-based ubiquitin ligase complex*

188 Whereas *ARMC5* silencing had no effect on accumulation of CUL3 protein (data not shown), *CUL3*
189 silencing leads to increased endogenous ARMC5 protein level without changing *ARMC5* mRNA
190 levels in both HEK293 and human adrenocortical (H295R) cell lines (Fig. 2-A and 2-B). Moreover,
191 endogenous ARMC5 protein half-life is increased in CUL3-deficient cells compared to control cells
192 after inhibition of the *de novo* protein synthesis (Fig. 2-C). To determine whether the mutation in the
193 BTB domain might affect the stability of ARMC5, we compared protein's half-life of overexpressed
194 WT ARMC5-FLAG and mutated L754P ARMC5-FLAG. Half-life of L754P ARMC5-FLAG mutant
195 is higher than WT ARMC5-FLAG (Fig. 2-D). Moreover, CUL3 overexpression decreased the half-life
196 of WT ARMC5, while no significant effect was observed on ARMC5 L754P mutant (Fig. 2-D). These
197 results suggest that CUL3 and potentially the CRL3 complex control the accumulation of ARMC5
198 protein through a post translational modification.

199 Inhibition of cullin neddylation (required for CRL activity) with MLN4924 increased endogenous
200 ARMC5 protein level (Fig. 3-A). Moreover, in H295R cells and in cell cultures from PBMAH
201 inhibition of the UPS with MG132 increased accumulation of the endogenous and/or overexpressed
202 ARMC5 WT (Fig. 3-B, C-left panel and D-left panel) while no effect was observed in the

203 accumulation of BTB mutated ARMC5 proteins (p.L754P and p.H808P) (Fig. 3-C-right panel and D-
204 right panel). These results suggest that ARMC5 half life and degradation are dependent of its BTB
205 domain and likely regulated by CUL3 and UPS. In order to investigate if ARMC5 is a substrate of the
206 CUL3-based complex, we performed cell-based ubiquitination assays. We observed that CUL3
207 silencing decreases ubiquitination of overexpressed WT ARMC5 (Fig. 4-A). On the other hand,
208 overexpressed WT ARMC5 ubiquitination was increased when CUL3 was co-expressed in both
209 HEK293 and H295R cell lines (Fig. 4-B and C). As expected, ubiquitination of overexpressed
210 ARMC5 p.L754P mutant was not increased when CUL3 was co-expressed (Fig. 4-B and C). Finally,
211 we performed the same ubiquitination assays using two Ub mutants (K48R and K63R), the most
212 predominant forms of Ub chain linkages in the cell (Ciechanover 2017; Dubiel et al. 2018). Through
213 this approach, we observed that the ubiquitination level of ARMC5 was drastically decreased when
214 Ub-K48R was used, while Ub-K63R did not affect WT ARMC5 ubiquitination (Fig. 4-D). This result
215 is consistent with the above data (Fig. 2 and 3), given that K48-linked Ub chains are the main signal
216 for targeting substrates for degradation by the 26s proteasome (Ciechanover 2017; Dubiel et al. 2018).
217 Taken together, these data provide evidences for a model in which ARMC5 is a direct substrate of a
218 CUL3 ubiquitin ligase complex and is degraded by the UPS.

219

220 *ARMC5/CUL3 participates in the G1-S cell cycle progression*

221 We investigated cell cycle progression in cells depleted for ARMC5 compared to control cells.
222 Propidium iodide DNA staining analyzed by flow cytometry in asynchronized cells showed that
223 ARMC5 silencing decreases the percentage of cells in G1 phase and increases the percentage of cells
224 in S phase in H295R and HEK293 cells (Fig. 5A and S1A). Consistent with this cell cycle alteration,
225 Cyclin E mRNA and protein accumulation (essential for the cell cycle G1-S phase progression) were
226 increased in ARMC5-deficient cells compared to control cells (Fig. S2, Fig. 5B and S1B). Moreover,
227 ARMC5-deficient cells synchronized in late G1 phase with aphidicolin for 24h and then released,
228 progress in cell cycle faster than control cells (Fig S3A-F). Indeed, 12h after release ARMC5-depleted
229 cells returned to G1 phase more rapidly compared to control cells (Fig S3E). On the other hand, WT
230 ARMC5 overexpression increased the percentage of cells in G1 phase (Fig. 5C and S1C) and

231 decreased Cyclin E protein accumulation (Fig. 5D) suggesting a cell cycle arrest in G1 phase. These
232 latest effects were not observed in cells which co-overexpressed CUL3 with ARMC5 (Fig. 5C-D and
233 S1C) suggesting that ubiquitination of ARMC5 by CUL3 alter its functions. As expected, ARMC5
234 p.L754P mutant overexpression (alone or in combination with CUL3) had no effect on the cell cycle
235 phases or the accumulation of Cyclin E protein (Fig. 5C-D and S1C). These results show that ARMC5
236 is involved in cell cycle progression and the Cyclin E accumulation. Moreover, this ARMC5 feature
237 can be controlled by the CUL3/proteasome system.

238

239 Discussion

240 Our results demonstrate that ARMC5 is a direct substrate of the CUL3 ubiquitin complex and that the
241 BTB domain of ARMC5 is important for this interaction. ARMC5 degradation is prevented by
242 mutations in its BTB domain or by CUL3 silencing/inhibition leading to increased ARMC5 half-life.
243 Hence, ARMC5 stability is mainly regulated by CUL3, even though other mechanisms may be
244 involved. Moreover, ARMC5 is involved in cell cycle progression (G1/S phases and Cyclin E
245 accumulation) and this effect is regulated by CUL3/proteasome system. Interestingly, Cyclin E was
246 found to be overexpressed in other types of adrenocortical tumors (Bourcigaux, et al. 2000; Tissier, et
247 al. 2004) suggesting a general role in adrenocortical pathophysiology. ARMC5 might not be just a
248 target of CUL3 ubiquitin complex but also an adaptor protein for recruiting specific substrates.
249 ARMC5 alterations would lead to an increase of one or more proteins acting as oncogenes. Indeed,
250 several E3 ligases or adaptor proteins can be targeted for degradation in a self-ubiquitination manner
251 (Wilkins et al. 2004; Zhou et al. 2015). Moreover, it has been suggested that the CUL3 dimer complex
252 formation is mediated via the BTB domains of the substrate adaptors. In this way, a Yeast Two Hybrid
253 assay in which ARMC5 protein served as bait identified ARMC5 as prey, suggesting a ARMC5 self-
254 dimerization (Hu et al. 2017). In this context, some ARMC5 missense mutants (as p.L754P) fail to
255 bind CUL3 and would therefore not recruit target protein(s) for ubiquitination/degradation. Other
256 ARMC5 missense mutants (as p.L548P and p.R898W), which are still able to bind CUL3, would not
257 be able to recruit substrate(s) or to lead to their ubiquitination. As depletion of ARMC5 increases
258 Cyclin E accumulation and free full length Cyclin E is known to be targeted by CUL3 complex (Lu

259 and Pfeffer 2013), we could speculate that ARMC5 could be an adaptor of the CUL3 complex leading
260 to ubiquitination of Cyclin E. However, *ARMC5* silencing leads to increased *CCNE1* mRNA
261 accumulation (Fig. S2), accordingly to previously described in PBMAH cell cultures (Cavalcante et al.
262 2018). Moreover, ARMC5 regulates preferentially low molecular Cyclin E protein (Fig. 5B,D and
263 S1B), which have been shown to not be ubiquitinated by CUL3 (Davidge, et al. 2019; Singer, et al.
264 1999). These data suggest that ARMC5 regulates Cyclin E by underlying mechanisms that remain to
265 be elucidated. In this study, we show a novel mechanism of ARMC5 protein regulation, opening up
266 new perspectives for the study of adrenal tumors. Consistently with this finding, recent studies
267 highlight the growing evidences of the ubiquitination/proteasome system alterations in endocrine and
268 more specifically adrenal and pituitary tumors causing Cushing syndrome development. While the
269 ubiquitin-specific protease 8 (USP8) gene is frequently altered in pituitary corticotroph adenomas
270 (Ma, et al. 2015; Reincke, et al. 2015), the ubiquitin ligase SIAH1 is involved in the adrenal cortex
271 organization (Scortegagna, et al. 2017). Furthermore, the most frequently altered gene in aggressive
272 adrenal tumors is an E3 ubiquitin ligase, *ZNRF3* (Zinc and ring finger protein 3) (Assie, et al. 2014;
273 Zheng, et al. 2016). Beyond being a substrate of the CUL3-based ubiquitin complex, we cannot
274 exclude that ARMC5 might also be a substrate adaptor protein that recruits specific substrates for
275 degradation. If confirmed, this mechanism could participate to the tumor suppressor function of
276 ARMC5. Further investigations are needed to identify other ARMC5 binding proteins and potential
277 substrates for the CUL3-ARMC5 complex, ultimately leading to the identification of pathways
278 regulated by ARMC5.

279

280 Acknowledgments

281 This research was supported in part by FAPESP-ANR to MCBVF and to JB (number 2015/50192-9),
282 by the Fondation pour la Recherche Médicale (FRM) grant (EQU201903007864), by the ANR grant
283 18-CE14-008-01 and by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).
284 IPC is recipient of a post-doctoral fellowship of the FRM (SPF201809007096).

285

286 Competing interests

287 The authors declare no potential conflicts of interest.

288

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371

372 **Figure legends**

373 **Figure 1** - Identification of ARMC5 as a partner of Cullin3.

374 (A) HEK293 cells were transfected with HA-CUL3 and WT ARMC5-FLAG. Cell extracts were
 375 immunoprecipitated with HA (left) or FLAG antibodies (right), followed by immunoblotting. (B)
 376 Cells were transfected with the indicated constructs, followed by BRET proximity assays. Hyperbolic
 377 saturation curves were obtained between CUL3-YFP, WT ARMC5-Luc, R898W ARMC5-Luc and
 378 L548P-Luc, but between CUL3-YFP and L754P ARMC5-Luc. (C) Cells were transfected with HA-
 379 CUL3, WT ARMC5-FLAG, R898W ARMC5-FLAG, L548P ARMC5-FLAG and L754P ARMC5-
 380 FLAG, followed by immunoprecipitation with HA antibody. The interaction between ARMC5 and
 381 CUL3 is disrupted by a mutation in the BTB domain of ARMC5 (L754P). (D) Cell extracts were
 382 obtained from cells transfected with the five indicated fragments of the ARMC5 protein-FLAG tagged
 383 and HA-CUL3, and immunoprecipitated with HA antibody. Images are representative of at least three
 384 independent experiments.

385

386 **Figure 2** - CUL3 regulates ARMC5 protein stability.

387 *CUL3* silencing by 3 different siRNA increases accumulation of ARMC5 protein (middle panel)
 388 without modifying *ARMC5* mRNA level (bottom panel) in (A) HEK293 and (B) H295R cells
 389 compared to control cells (siCTR). (C) Protein stabilization assays with 10 μ M cycloheximide (CHX)
 390 treatment for 4, 15 and 24 hours were performed, demonstrating that CUL3 knockdown stabilizes
 391 ARMC5 protein in HEK293 cells. (D) HEK293 cells were transfected with equal amounts of WT
 392 ARMC5-FLAG, L754P ARMC5-FLAG and HA-CUL3 and submitted to 10 μ M cycloheximide
 393 treatment of 4 and 8 hours. Co-expression of HA-CUL3 accentuates WT ARMC5 degradation, while
 394 no effect is observed in the L754P ARMC5 degradation. Images are representative of at least three
 395 independent experiments. To simplify the representation of the results, we only display on the graph D
 396 the most informative comparisons. All values are provided hereafter: at 4h of treatment with CHX, the

397 percentage of protein was $74\% \pm 12$ (WT + CUL3) vs 97 ± 1 (L754P) ($p < 0.01$) ; $74\% \pm 12$ (WT +
 398 CUL3) vs $97\% \pm 4$ (L754P + CUL3) ($p < 0.01$); at 8h, the percentage of protein was $77\% \pm 6$ (WT) vs
 399 $97\% \pm 2$ (L754P) ($p < 0.05$); $42\% \pm 12$ (WT + CUL3) vs $87\% \pm 3$ (L754P + CUL3) ($p < 0.001$) and 77%
 400 ± 6 (WT) vs $42\% \pm 12$ (WT + CUL3) ($p < .001$). Student's t-test was used to analyze experiments A
 401 and B, and two-way ANOVA followed by Bonferroni post-test was used for experiments C and D.

402

403 **Figure 3** - ARMC5 is regulated by the ubiquitin/proteasome system *in vitro*.

404 (A) Endogenous ARMC5 is stabilized by inhibition of cullin neddylation (NEDD8) with $2\mu\text{M}$
 405 MLN4924 and (B) by proteasome inhibition with $20\mu\text{M}$ MG132 in H295R cells. (C) WT ARMC5 is
 406 less stable than the L754P ARMC5 mutant. The degradation of WT ARMC5 is blocked by
 407 proteasomal inhibition with $20\mu\text{M}$ MG132 during 4h, while no effect is observed in the L754P
 408 ARMC5 mutated protein. (D) Endogenous WT ARMC5 is stabilized by proteasomal inhibition with
 409 $20\mu\text{M}$ MG132 during 4h, while no effect is observed in H808P ARMC5 mutated protein in PBMAH
 410 cell cultures. Images are representative of at least three independent experiments. Significance was
 411 assessed by student's t-test for experiments shown in A and B, while two-way ANOVA followed by
 412 Bonferroni post-test was used for experiment shown in C and D.

413

414 **Figure 4** - ARMC5 turnover is regulated by the CUL3-based ubiquitin complex.

415 HEK293 cells were transfected with UB-WT, UB-K48R, UB-K63R, WT ARMC5-FLAG and HA-
 416 CUL3 as indicated in each experiment, followed by cell-based ubiquitination assays. (A) Followed by
 417 endogenous CUL3 knockdown, ubiquitination assays were performed. WT ARMC5 ubiquitination
 418 level was decreased in the absence of CUL3. Ubiquitination of WT ARMC5 was increased by the co-
 419 expression of HA-CUL3 while no effect was observed in the L754P mutant protein in HEK293 and
 420 H295R (B and C, respectively) cells. (D) Ubiquitination of WT ARMC5 was decreased by a mutation
 421 in the K48 ubiquitin chains, responsible for proteasome degradation, while no effect was observed by
 422 K63 ubiquitin mutated chains. Images are representative of at least three independent experiments.

423

424 **Figure 5** – ARMC5 regulates cell cycle and cyclin E turnover in H295R cells

425 Propidium iodide was used to determine DNA content. (A) Flow cytometry analysis after ARMC5
426 depletion revealed a decrease in the percentage of cells in G1 phase and an increase in S phase. (B)
427 Depletion of ARMC5 led to an increase in full length (FL) and low molecular weight (LMW) cyclin
428 E. (C) Overexpression of WT ARMC5 increases the number of cells in G1 phase and (D) inhibits
429 cyclin E protein accumulation compared to control cells. However, co-expression of WT ARMC5 and
430 CUL3, as well as overexpression of p.L754P mutated ARMC5 have no longer an effect in cell cycle
431 and (D) in cyclin E inhibition. Images are representative of at least three independent experiments.
432 Significance was assessed by using two-way ANOVA, followed by Bonferroni post-test.

433

434 **Figure S1** - ARMC5 regulates cell cycle and cyclin E turnover in HEK293 cells

435 Propidium iodide was used to determine DNA content. (A) Flow cytometry analysis after ARMC5
436 depletion revealed a decrease in the percentage of cells in G1 phase and an increase in S phase. (B)
437 Depletion of ARMC5 led to an increase in full length (FL) and low molecular weight (LMW) cyclin
438 E. (C) Overexpression of WT ARMC5 increases the number of cells in G1 phase. However, co-
439 expression of WT ARMC5 and CUL3, as well as overexpression of p.L754P mutated ARMC5 have
440 no longer an effect in cell cycle. Images are representative of at least three independent experiments.
441 Significance was assessed by using two-way ANOVA, followed by Bonferroni post-test.

442

443 **Figure S2** - ARMC5 depletion increases *CCNE1* mRNA transcription in both (A) H295R and (B)
444 HEK293 cells. Significance was assessed by using student's t-test.

445

446 **Figure S3** - ARMC5 depletion favours cell cycle progression in H295R cells

447 Propidium iodide was used to determine DNA content. (A) Flow cytometry analysis after ARMC5
448 depletion. (B) Synchronization of cells in late G1 phase with aphidicolin (10 μ M) for 24h. (C) Flow
449 cytometry analysis 4h (C), 8h (D), 12h (E) and 24h (F) after release from aphidicolin. Significance
450 was assessed by using two-way ANOVA, followed by Bonferroni post-test.

Figure 1

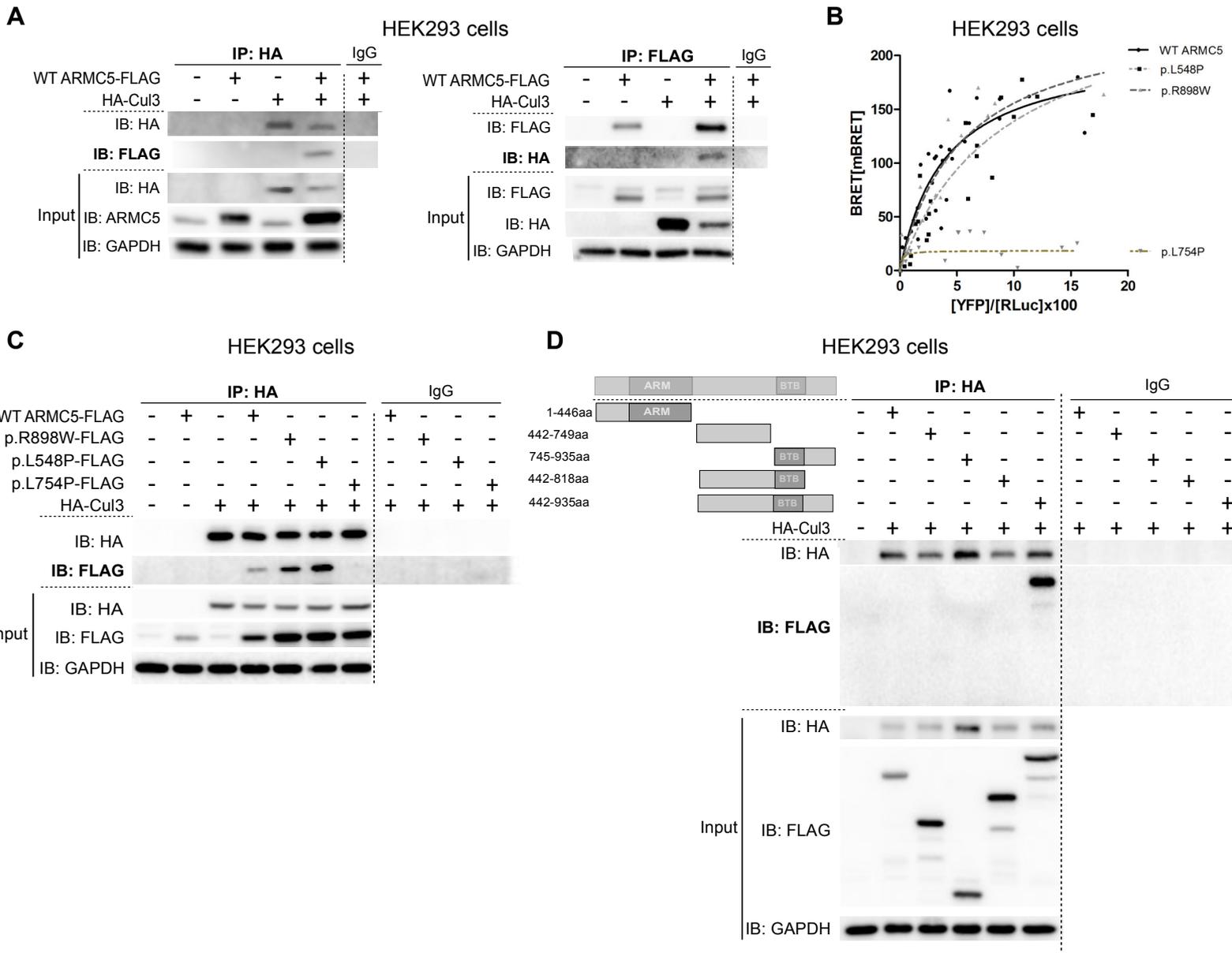


Figure 2

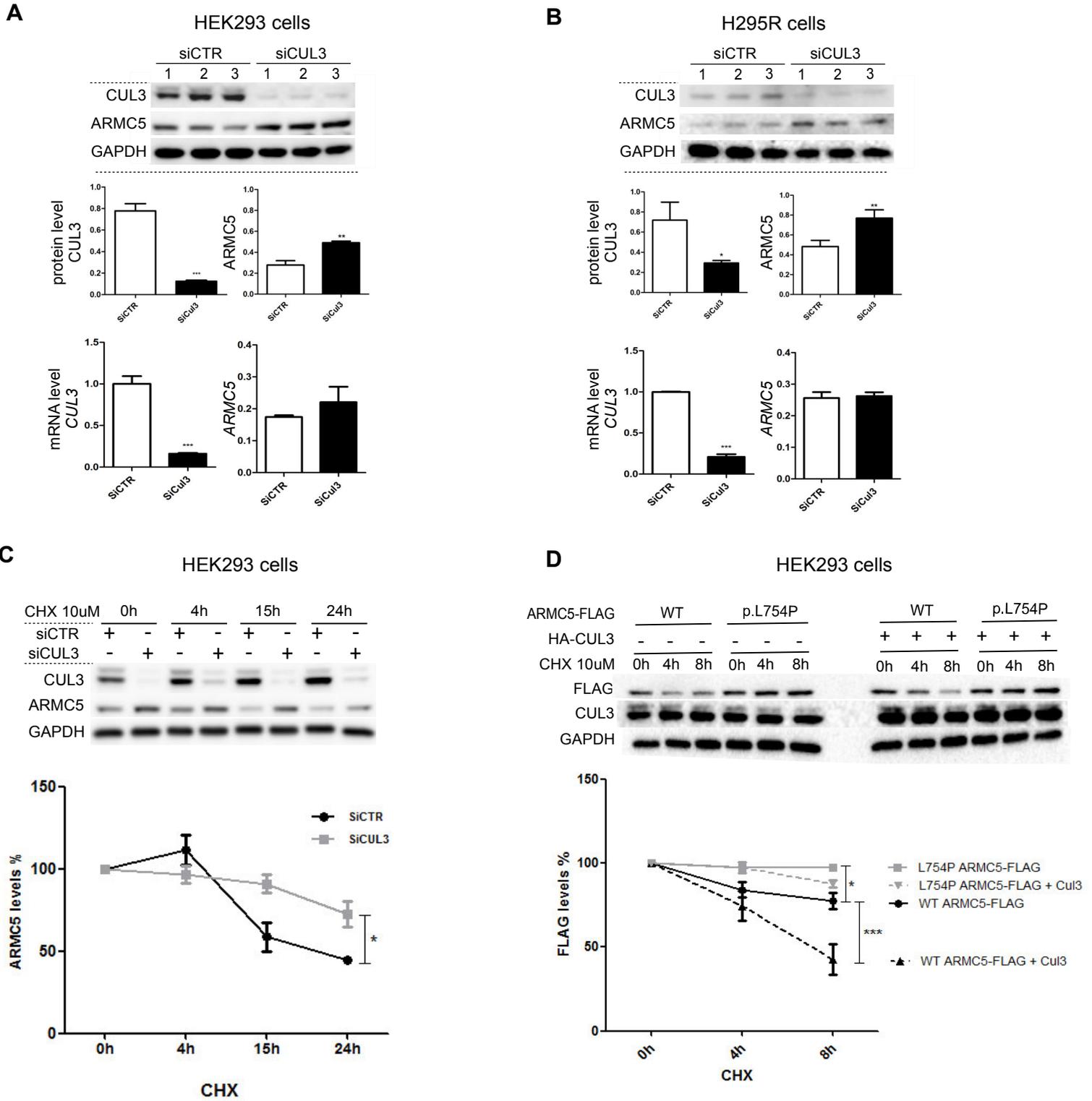


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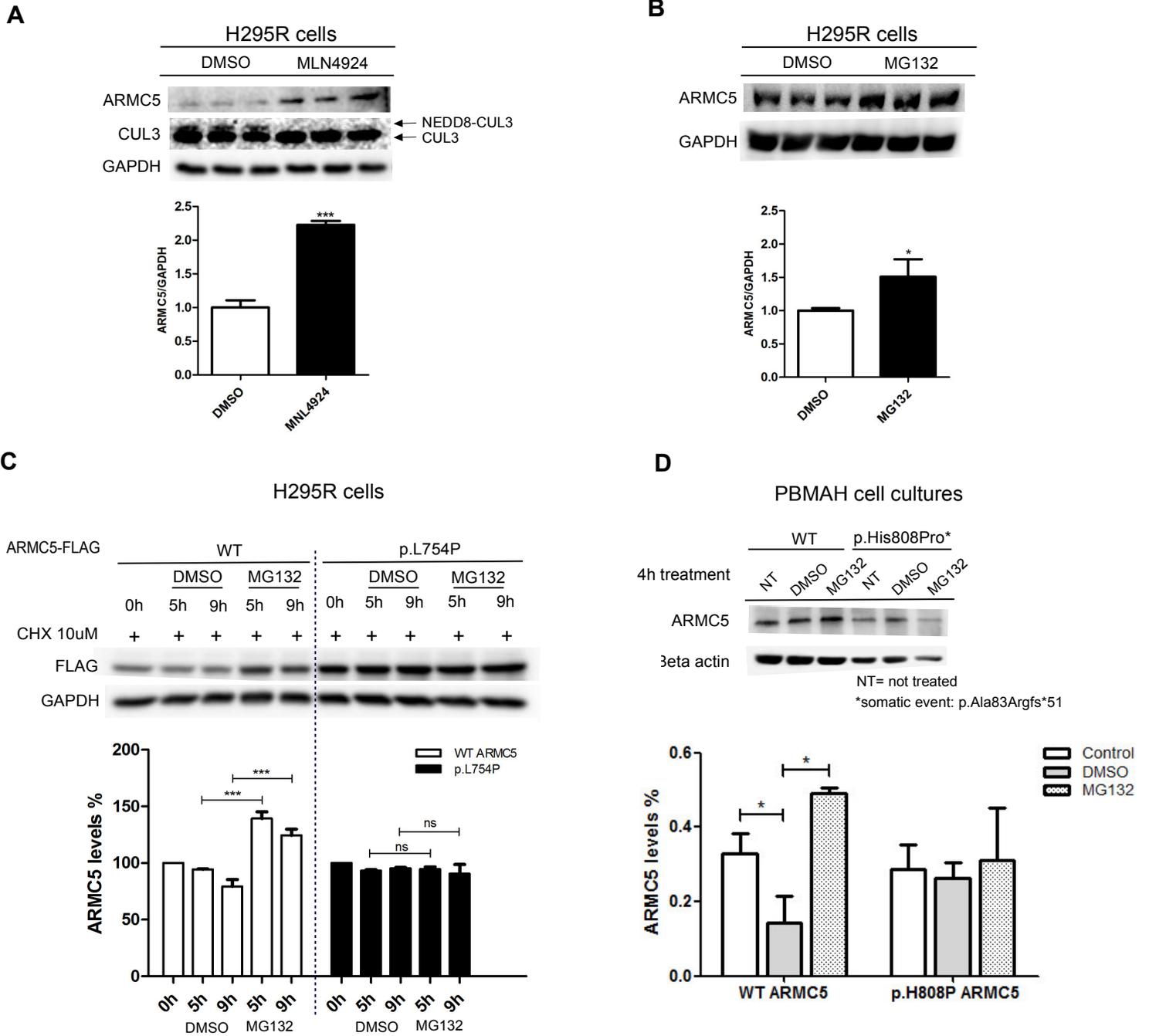


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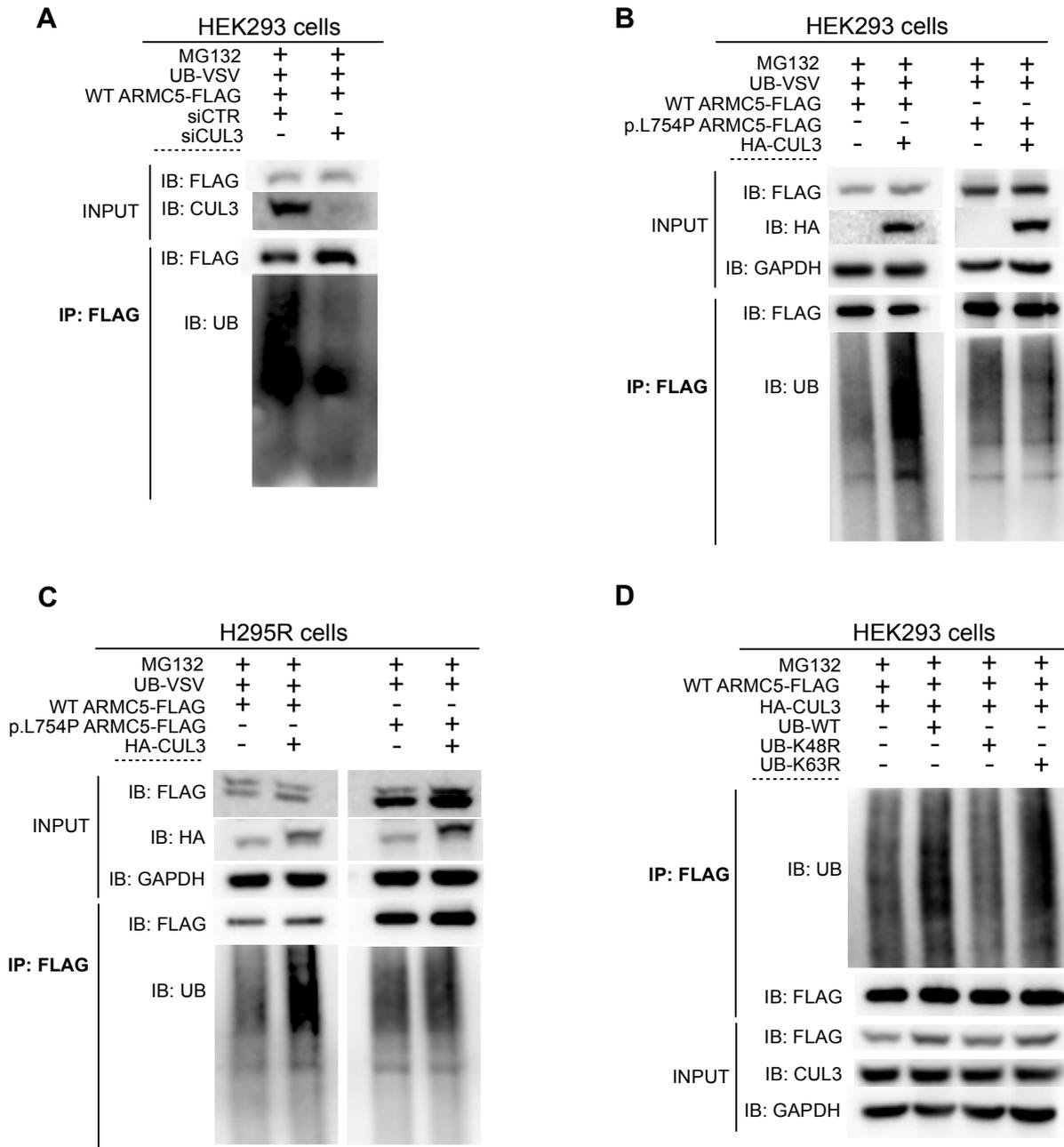


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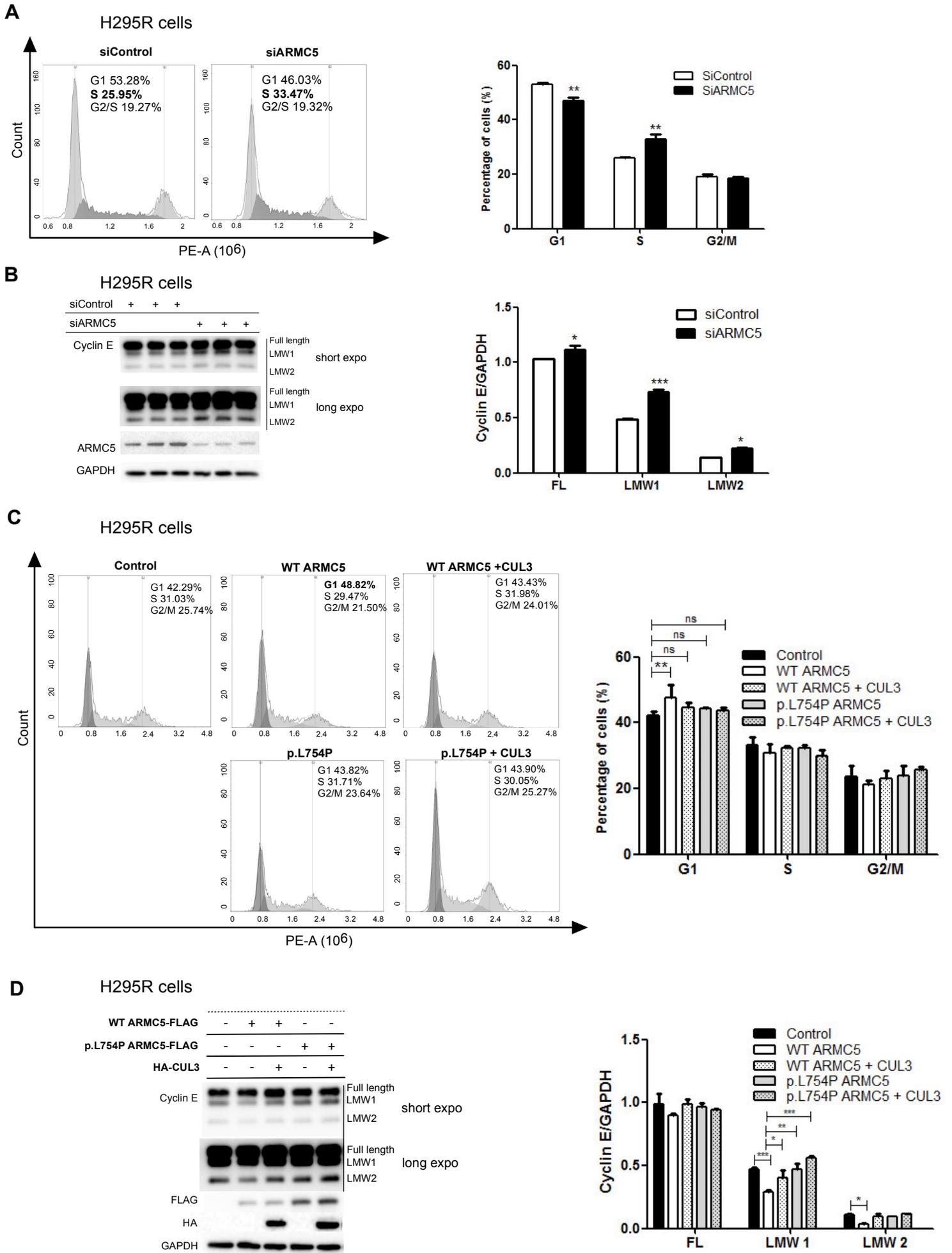


Figure S1

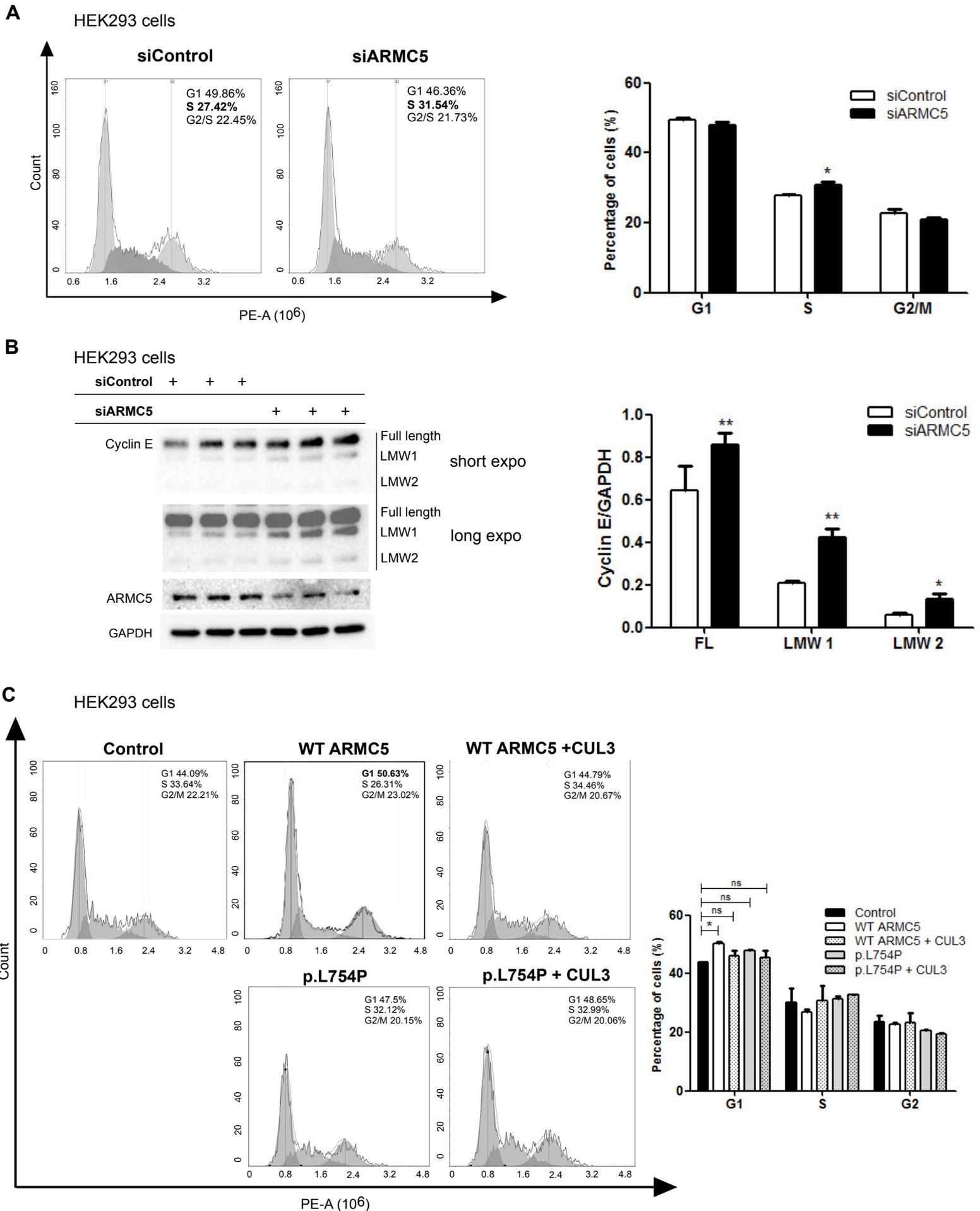
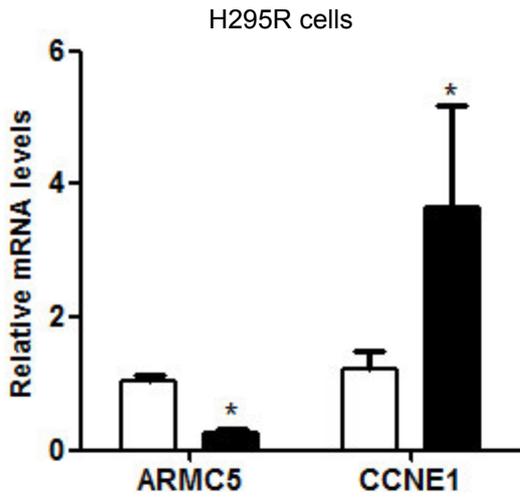


Figure S2

A



B

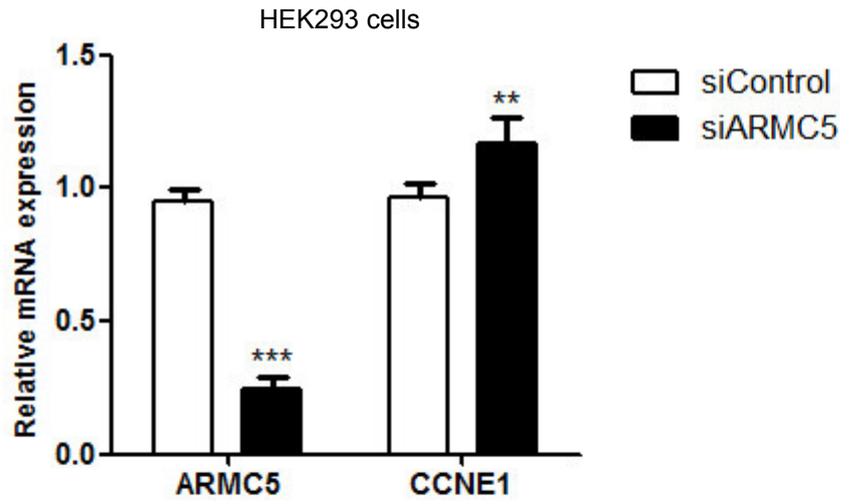


Figure S3

H295R cells

