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Activation of long non-coding RNA NEAT1 leads to survival advantage of multiple myeloma cells by supporting a positive regulatory loop with DNA repair proteins

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1 **Activation of lncRNA NEAT1 leads to survival advantage of multiple myeloma cells by**
2 **supporting a positive regulatory loop with DNA repair proteins**

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26

27 **CONFLICT OF INTEREST**

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

29

30 **ABSTRACT**

31 Malignant plasma cells of patients affected by multiple myeloma (MM) express at high levels the
32 nuclear long non-coding RNAs NEAT1, which represents the core structural component of
33 paraspeckle organelles (PS). We previously demonstrated that NEAT1 silencing negatively impacts
34 proliferation and viability of MM cells, both *in vitro* and *in vivo*, highlighting its pivotal role in
35 regulating the Homologous Recombination pathway. This study suggested NEAT1 involvement in
36 virtually all DNA repair mechanisms, through the significant increase of PS number within cells
37 and the activation of a molecular axis including the two fundamental kinase proteins ATM and
38 DNA-PKcs, and the direct targets pRPA32 and pCHK2. Furthermore, we found that NEAT1
39 overexpression is associated with oncogenic and pro-survival advantages in MM cells exposed to
40 nutrient starvation or hypoxic microenvironment, which are stressful conditions often associated
41 with more aggressive disease phases. Overall, we provided novel important insights into NEAT1
42 role in supporting MM cells adaptation to stressful conditions by improving the maintenance of
43 DNA integrity. Taken together, our results suggest that NEAT1, and probably PS organelles, could
44 represent a potential therapeutic target for MM treatment.

45 INTRODUCTION

46 Multiple myeloma (MM) is a malignant proliferation of bone marrow plasma cells (PCs)
47 characterized by different clinical course and a highly heterogeneous genetic background with both
48 structural chromosomal alterations and specific genes mutations [1,2].

49 Over the past decade, a causal relationship between deregulation of long non-coding RNAs
50 (lncRNA) and the pathogenesis of human cancers, including MM, has emerged by different
51 functional studies [3-6]. LncRNAs participate in several biological processes, such as
52 transcriptional gene regulation, genomic integrity maintenance, cell differentiation and development
53 [7].

54 We have identified the nuclear paraspeckle assembly transcript 1 (NEAT1) as one of the
55 abundantly expressed lncRNAs in malignant PCs compared to its normal counterpart [6,8,9]
56 consistently with its high expression levels in many solid tumors [10]. NEAT1 is a mono-exonic
57 lncRNA, transcribed from the Multiple Endocrine Neoplasia (MEN) type I locus, localized on the
58 chromosome 11q13 [11]. Two different variants of NEAT1 exist and share identical 5' terminus:
59 NEAT1_1, a shorter polyadenylated isoform of 3.7 kb, and NEAT1_2, a longer isoform of 22.7 kb,
60 lacking the polyA tail [12]. NEAT1 has been demonstrated to be a fundamental structural
61 component of nuclear paraspeckles (PSs), which are irregularly shaped compartments of the nuclear
62 interchromatin space, considered as membraneless lncRNA-directed nuclear bodies involved in
63 stress response [9,13]. Consistently with this function, we highlighted a pivotal role for NEAT1 in
64 the maintenance of DNA integrity, through the regulation of the homologous recombination (HR)
65 pathway, by demonstrating that NEAT1 depletion led to a significant downregulation of genes and
66 active fractions of proteins involved in initial and crucial steps of the HR pathway [6]. Moreover,
67 our research group demonstrated that NEAT1 silencing negatively regulates proliferation and
68 viability of MM cells, both *in vitro* and *in vivo* [6].

69 Despite the growing amount of data obtained by loss-of-function approaches, concerning the
70 role of NEAT1 in the DNA damage repair (DDR) system and maintenance of genome integrity
71 [6,13-15], the biological scenario underlying MM cells following NEAT1 overexpression remains
72 virtually absent.

73 In recent years, different approaches of CRISPR/Cas9 system have been explored to induce
74 gene activation [16] or gene repression [17]. In particular, the CRISPR/Cas9 Synergistic Activation
75 Mediator (SAM) system is a cutting-edge technique that uses MS2 bacteriophage coat proteins
76 combined with p65 and HSF1 to para-physiologically induce the transcription of target genes
77 without altering the DNA sequence [18].

78 Using this approach, here, we investigated the functional role of NEAT1 transactivation in
79 human MM cell lines (HMCLs). In particular, we established a relevant role for NEAT1 in DNA
80 repair molecular mechanisms through the up-regulation of the two fundamental kinases, namely
81 ATM and DNA-PKcs. Furthermore, our data strongly indicate NEAT1 involvement in conferring
82 survival advantage to MM cells exposed to stressful conditions such as nutrient starvation or
83 hypoxic microenvironment, thus suggesting that the specific targeting of the PSs backbone could be
84 a promising novel strategy in MM treatment.

85

86 **MATERIALS AND METHODS**

87 Full details of lentivirus production and *in vitro* transduction, plasmid constructs and cloning
88 of sgRNAs, quantitative real-time PCR, colony-forming assay, cell cycle analysis and apoptosis,
89 immunofluorescence, RNA FISH, gymnotic delivery, inhibitors and antibiotics, proteomic assays
90 are provided in Supplementary Methods.

91 **MM Cell Lines and Drugs**

92 AMO-1 was kindly provided by Dr. C. Driessen (University of Tübingen, Germany). LP1,
93 MM1.S, OPM2, and NCI-H929 were purchased from DSMZ, which certified authentication
94 performed by short tandem repeat DNA typing. All HMCLs were immediately frozen and used
95 from the original stock within 6 months. HMCLs were cultured in RPMI-1640 medium (Gibco®,
96 Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100
97 U/ml penicillin, and 100 mg/ml streptomycin (Gibco®) at 37°C in 5% CO₂ atmosphere, and tested
98 for mycoplasma contamination.

99 **Hypoxia and HIF-1 α stabilization**

100 Hypoxia was induced by placing the cells for 24/48 h into a modular incubator chamber
101 (Billups Rothenberg Inc., Del Mar, CA, USA) flushed with a mixture of 1% O₂, 5% CO₂ and
102 94%N₂ at 37°C. To achieve oxygen-independent HIF-1 α stabilization, cells were exposed to 100
103 μ M CoCl₂ for 24 h [19].

104 **Statistical analysis**

105 Statistical significance of differences observed was determined by Student t test analysis;
106 differences were considered significant when P value was <.05 (*), <.01 (**), or <.001 (***). All
107 statistical analyses were performed using the Prism 5.0 software (GraphPad Software, Inc.)

108 **RESULTS**

109 **NEAT1 transactivation increases the amount of paraspeckles in MM cells**

110 To activate the endogenous expression of NEAT1 in MM cells, we took advantage of the
111 CRISPR/Cas9 SAM genome editing system. We first engineered the AMO-1 cell line to stably
112 express the components of the CRISPR activation system (dCas9/ MS2-p65-HSF1) (Supplementary
113 Fig. 1a). Then, AMO-1 cells were individually transduced with three sgRNAs targeting the NEAT1
114 promoter or a scramble sgRNA (Supplementary Table 1 and Supplementary Fig. 1b). RT-qPCR of
115 selected cells demonstrated that two out of three NEAT1 targeting sgRNAs induced significant
116 NEAT1 transactivation (AMO-1^{N#5} and AMO-1^{N#8} cells) compared to the scramble condition
117 (AMO-1^{SCR} cells) (Fig. 1a). Specific RNA FISH confirmed the sustained NEAT1 expression levels
118 obtained in both targeted cell lines (Supplementary Fig. 1c).

119 Since NEAT1 has been demonstrated to be the fundamental structural component of nuclear
120 PSs [9], we evaluated whether the increased NEAT1 transcription affects the expression of the
121 essential PS proteins (PSPs). Interestingly, NEAT1 overexpression was associated with a significant
122 increase of NONO and SFPQ protein expression in AMO-1^{N#5} and AMO-1^{N#8} cells (Fig. 1b).
123 Similarly, we detected also the overexpression of the FUS protein, whose prionlike low complexity
124 domain is required for the organization of a microscopically visible mature PS [20] (Fig. 1b).
125 However, PSPs mRNA expression levels were not significantly modulated in AMO-1^{N#5} and AMO-
126 1^{N#8} cells (Supplementary Fig. 1d). This prompted us to investigate the role of NEAT1 in the
127 regulation of PSPs stability. To address this issue, we monitored the time course of NONO and FUS
128 disappearance in the presence of the protein synthesis inhibitor cycloheximide (CHX). Our analysis
129 indicated that the degradation rate of NONO and FUS was significantly slower in NEAT1-
130 transactivated AMO-1 cells (Fig. 1c). Furthermore, neither NONO nor FUS completely disappeared
131 even after long CHX exposure (Supplementary Fig. 1e).

132 Finally, we evaluated if the stabilization of PSPs induced by NEAT1 activation was
133 associated with a positive modulation of PSs size and distribution in AMO-1 cells. Our analyses of

134 AMO-1^{N#5} and AMO-1^{N#8} cells by confocal microscopy revealed that NEAT1 and NONO co-
135 localized in PS organelles, whose number and size were increased compared to AMO-1^{SCR} cells
136 (Supplementary Fig. 2).

137

138 **NEAT1 transactivation provides a survival advantage to MM cells cultured under stressful** 139 **conditions**

140 To evaluate whether MM cells could benefit from a survival advantage upon NEAT1
141 transactivation, we monitored the growth rate and viability of AMO-1^{N#5}, AMO-1^{N#8}, and AMO-
142 1^{SCR} cells in physiological and stressful conditions. In the first case, AMO-1^{N#5}, AMO-1^{N#8} cell
143 lines showed cell growth and viability similar to AMO-1^{SCR} cells (Supplementary Fig. 3a and 3b).
144 We confirmed the absence of significant modulation in the cell cycle phase distributions and in the
145 apoptotic rate by flow cytometric analysis (Supplementary Fig. 3c and 3d). Consistently, we did not
146 observe any change also in the clonogenic potential (Supplementary Fig. 3e).

147 Based on NEAT1 involvement during cellular stress response [9,13,21], we assessed the
148 relevance of NEAT1 transactivation in stressful conditions, such as FBS starvation or hypoxia.

149 First, we assessed NEAT1 expression modulation under stressful condition. Both total
150 NEAT1 and NEAT1_2 isoform expression levels of AMO-1^{N#5}, AMO-1^{N#8}, and AMO-1^{SCR} cells
151 cultured for 48 h at 1% FBS condition resulted significantly up-regulated in comparison with the
152 relative counterpart maintained in 10% FBS (Fig. 2a), confirming that both variants are induced
153 under stressful conditions. Interestingly, FBS starvation increased the fraction of the NEAT1_2
154 isoform (Fig. 2b), which is known to be the fundamental structural scaffold for the biogenesis of PS
155 organelles. Confocal microscopy analysis revealed that FBS starvation not only induces NEAT1
156 expression but also NONO protein expression, leading to an increase in the number of PSs
157 identifiable by co-localized signals (Fig. 2c). Similar results were obtained by growing MM cells in
158 hypoxic microenvironment, resulting in a significant up-regulation of both NEAT1 isoforms
159 (Supplementary Fig. 4a), particularly of the fraction of NEAT1_2 long variant (Supplementary Fig.

160 4b). Moreover, in line with results obtained with serum starvation, the up-regulation of NEAT1
161 upon hypoxia positively correlated with the increase of NONO expression and the number and size
162 of PS organelles (Supplementary Fig. 4c).

163 From a biological point of view, upon FBS starvation NEAT1-activated cells showed a
164 significant higher viability as compared to AMO-1^{SCR} cells (Fig. 3a). This finding is supported also
165 by a reduced plasmatic membrane integrity observed in AMO-1^{SCR} compared to AMO-1^{N#5} and
166 AMO-1^{N#8} (Supplementary Fig. 5a) and by a cell cycle analysis that showed a higher percentage of
167 cells distributed in sub G0/G1 phase (Fig. 3b and Supplementary Fig. 5b). Additionally, the
168 presence of a significant higher percentage of apoptotic cells (Fig. 3c) in AMO-1^{SCR} cells suggests
169 also an anti-apoptotic role of NEAT1. Moreover, NEAT1 transactivation significantly increased the
170 clonogenic potential of MM cells cultured in the same condition (Fig. 3d). In detail, sustained
171 NEAT1 expression increased the number of colonies that also resulted to be larger, more structured
172 and compact (Fig. 3e and Supplementary Fig. 5c).

173 In agreement with these biological data, in condition of serum starvation, the active fraction
174 of ERK1/2 and AKT proteins were up-regulated in NEAT1 transactivated cells, displaying a higher
175 increase in the more aggressive AMO-1^{N#8} compared to AMO-1^{SCR} cells (Fig. 3f).

176 Finally, we could observe a greater viability and modulation of cell cycle phase distribution
177 also in NEAT1 transactivated AMO-1 cells maintained in hypoxic condition (Supplementary Fig.
178 5d, 5e, and 5f).

179

180 **NEAT1 transactivation leads to the phosphorylation of RPA32 and CHK2 through a** 181 **molecular mechanism dependent on ATM and DNA-PKcs**

182 We previously demonstrated NEAT1 involvement in the DDR system [6]. With the aim of
183 dissecting the molecular mechanisms associating NEAT1 with DNA repair processes, we
184 investigated RPA32 expression following NEAT1 transactivation.

185 Specifically, WB and confocal microscopy analysis showed an up-regulation of pRPA32
186 levels in NEAT1 transactivated cells compared to AMO-1^{SCR} cells (Fig. 4a and Supplementary Fig.
187 6a), whereas RPA32 total form remained unchanged at both protein (Fig. 4a) and mRNA level
188 (Supplementary Fig. 6b). Furthermore, AMO-1^{N#5} and AMO-1^{N#8} cells displayed increased levels
189 of the active fraction of CHK2 (Fig. 4b), another important player of DDR system, whereas no
190 significant modulation of pCHK1 was detected (Fig. 4b).

191 To shed light on the possible mechanism by which NEAT1 leads to an increase of activated
192 RPA32, we evaluated the expression levels of the three main kinase proteins responsible for its
193 phosphorylation, i.e. ATM, ATR and DNA-PK [22]. Our results showed that NEAT1 induction did
194 not associate with significant modulations of ATM, ATR and PRKDC mRNA levels, ruling out a
195 possible role of NEAT1 in the transcriptional regulation of these proteins (Supplementary Fig. 6c).
196 We also confirmed at protein level that NEAT1 transactivation did not induce a significant
197 modulation of ATR and its activated form (Fig. 4c), in line with the absence of modulation of its
198 direct target pCHK1. Conversely, ATM protein levels increased in AMO-1^{N#5} and AMO-1^{N#8} cells
199 with respect to AMO-1^{SCR} cells (Fig. 4c), in agreement with the increased pCHK2 and pRPA32
200 expression levels. Furthermore, also the catalytic subunit of DNA-PK (DNA-PKcs) showed a
201 significant increase in NEAT1 activated cells compared to AMO1^{SCR} cells (Fig. 4c), data also
202 confirmed by immunofluorescence (Supplementary Fig. 6d).

203 Overall, our data are consistent with a signaling pathway downstream NEAT1 triggered
204 through the increase of ATM and DNA-PKcs protein levels, which in turn trigger RPA32 and
205 CHK2 activation via phosphorylation.

206

207 **NEAT1-mediated activation of RPA32 is enhanced upon FBS starvation and hypoxic**
208 **condition**

209 The evidence that FBS starvation positively modulates NEAT1 expression (Fig. 2) prompted
210 us to verify the outcome of FBS starvation on the molecular axis “NEAT1-pRPA32”. At this
211 purpose, we compared AMO-1^{SAM} cells cultured upon FBS starving and physiological conditions.

212 Interestingly, confocal microscopy analyses demonstrated increased levels of both DNA-
213 PKcs and its target pRPA32 in AMO-1^{SCR} cells cultured in FBS starving condition (Fig. 5a), in line
214 with a possible relationship between NEAT1/PSs expression and DNA-PKcs activity. Of note,
215 AMO-1^{N#5} and AMO-1^{N#8} cells exposed to 1% FBS further increase the expression levels of
216 pRPA32, DNA-PKcs and ATM compared to AMO-1^{SCR}, confirming the role of NEAT1 in
217 triggering the mechanism that brings to RPA32 phosphorylation (Fig. 5b).

218 Similarly, hypoxia, another condition that increases NEAT1 expression (Supplementary
219 Fig. 4), determines DNA-PKcs levels higher than those observed in normoxic condition, above all
220 in both AMO-1^{N#5} and AMO-1^{N#8} cells compared to AMO-1^{SCR} cells (Fig. 5c).

221 To further validate the effects of hypoxic stress, we investigated AMO-1^{SAM} cells upon CoCl₂
222 treatment that chemically stabilize HIF-1 transcription factor. Also in this case, we detected the
223 overexpression of NEAT1 and NONO in association with the increase of both DNA-PKcs and
224 pRPA32 protein levels (Supplementary Fig. 7a). In agreement with the effects of hypoxic and FBS
225 starving conditions, the up-regulation of DNA-PKcs upon CoCl₂ treatment further increased in
226 NEAT1 transactivated cells (Supplementary Fig. 7b), which, at the same time, showed a higher
227 viability and a lower percentage of apoptotic cells than AMO-1^{SCR} cells (Supplementary Fig. 7c and
228 7d).

229 With the aim of validating our data, we extended the study to other three HMCLs (OPM2,
230 MM1.S and LP1), based on the hypothesis that FBS starving condition could induce NEAT1
231 expression. As expected, serum starvation induced NEAT1 expression in all HMCLs tested
232 (Supplementary Fig. 8a), and we detected an increase of the NEAT1_2 variant contribution with
233 respect to total NEAT1 expression (Supplementary Fig. 8b). Moreover, WB analysis demonstrated
234 an increase in the pRPA32 amount in FBS starved cells compared to cells maintained at normal

235 FBS condition (Supplementary Fig. 8c). In agreement with our model, DNA-PKcs and ATM
236 protein levels increased in FBS starved cells (Supplementary Fig. 8c). Finally, also FUS protein
237 resulted overexpressed, likely to enhance PSs assembling (Supplementary Fig. 8c).

238

239 **The long NEAT1_2 variant is essential for the regulation of the DNA-PK, ATM/pRPA32 axis**

240 To confirm NEAT1 fundamental role in the regulation of DNA-PKcs and ATM and the
241 consequent RPA32 activation, we evaluated the expression levels of both kinase proteins in AMO-1
242 and LP1 cells specifically silenced for NEAT1. As expected, the down-regulation of both NEAT1
243 isoforms, obtained through the gymnotic delivery of the antisense LNA-gapmeR g#N1_E (Fig. 6a),
244 results in the reduction of the pRPA32 protein levels (Fig. 6b). In addition, g#N1_E-silenced cells
245 showed a significant reduction of both DNA-PKcs and ATM protein levels, thus confirming a
246 pivotal role of NEAT1 in the regulation of pRPA32 expression through a mechanism DNA-PK and
247 ATM-mediated (Fig. 6b).

248 Finally, to evaluate if the molecular effect of NEAT1 silencing was orchestrated mainly by
249 the short and more abundant isoform of NEAT1 or by the long NEAT1_2 variant, we silenced
250 AMO-1 and LP1 cells with an LNA-gapmeR specific for the NEAT1_2 isoform (g#N1_G). Of
251 note, the silencing of NEAT1_2 was sufficient to determine the down-regulation of pRPA32 and of
252 both DNA-PKcs, and ATM (Fig. 6b).

253

254 **The NEAT1/ pRPA32 positive axis is responsible for survival advantages of MM cells**

255 Finally, we investigated whether the pro-survival effect observed in NEAT1 transactivated
256 cells cultured in non-physiological conditions, such as FBS starvation, was supported, at least in
257 part, by the novel identified NEAT1-orchestrated molecular axis.

258 Hence, we chemically inhibited ATM and DNA-PK activity in AMO-1^{SAM} cells cultured in
259 FBS starving conditions, and evaluated the biological impact. As expected, in FBS starvation we
260 found that pRPA32 levels in AMO-1^{N#8} were higher than in AMO-1^{SCR} cells; however, the

261 inhibition of both ATM and DNA-PK kinases led to the impairment of RPA32 phosphorylation in
262 both cell lines (Supplementary Fig. 9a). In line with our hypothesis, both ATM and DNA-PK
263 inhibition resulted in a significant reduction of cell viability in AMO-1^{SCR} and AMO-1^{N#8} starved
264 cells, even more evident upon simultaneous inhibition of ATM and DNA-PK activities (Fig. 7a).
265 Furthermore, FBS starved cells clearly showed alteration of cell membrane integrity and membrane
266 blebbing in AMO-1^{SCR} cells compared to NEAT1 transactivated cells. These characteristics are
267 typical of apoptotic cells, in line with the higher level of mortality (Fig. 7b and Supplementary Fig.
268 9b). Interestingly, the same morphological changes were detectable in FBS starved-AMO-1^{SAM} cells
269 upon the simultaneous inhibition of ATM and DNA-PK (Fig. 7b and Supplementary Fig. 9b), in
270 agreement with the significant decrease of cell viability. Moreover, the inhibition of ATM and
271 DNA-PK was associated with a massive cytoplasmic vacuolization, more pronounced upon
272 simultaneous inhibition of both protein kinases, suggesting a suffering cell phenotype (Fig. 7b and
273 Supplementary Fig. 9b).

274 Notably, NEAT1 silencing in NCI-H929 cell line and in CD138+ purified primary PCs also
275 led to cytoplasmic vacuolization, even if at a lesser extent (Fig. 7c), suggesting that the molecular
276 and biological effect observed upon NEAT1 silencing could be the result, at least in part, of the
277 reduced activity of the NEAT1/ pRPA32 axis.

278

279 **DISCUSSION**

280 We previously reported that NEAT1 expression in purified PCs from MM patients is
281 significantly higher than in normal counterpart and that NEAT1 silencing negatively regulates
282 proliferation and viability of MM cells, both *in vitro* and *in vivo*, by affecting the DNA damage
283 cellular response [6].

284 PSs are dynamic compartment in the nuclear interchromatin space. They respond to different
285 stimuli among which DNA damage. These pieces of evidence together with the function of NEAT1
286 in PSs assembly, prompted us to investigate the mechanism underlying the pro-tumoral effect of

287 NEAT1 by studying how NEAT1 overexpression observed in MM can influence the activity of PSs
288 during DDR.

289 To mimic the effect of NEAT1 overexpression in MM, we get advantage of two NEAT1
290 transactivated MM clones obtained by using the innovative CRISPR-Cas9 SAM gain of function
291 approach [17].

292 First, we demonstrated that the para-physiological activation of NEAT1 is associated with a
293 significant increase of the levels of essential PSPs such as NONO, SFPQ, and FUS. In details,
294 NEAT1 extends the half-life of these three PSPs, thereby rising their availability within MM cells.
295 Moreover, confocal microscopy analysis clearly showed that overexpressed NEAT1 and NONO co-
296 localize in PS organelles, whose number significantly augmented in NEAT1 transactivated cells,
297 confirming previous data reporting that NEAT1 transcription was coupled with PSs assembling
298 [23]. Importantly, the three PSPs are involved in virtually all DNA repair mechanisms (see review
299 [9]). In particular, NONO/SFPQ creates a heterodimer that can associate with the major proteins of
300 the non-homologous end-joining (NHEJ) thus promoting their activity; SFPQ was reported to be
301 essential for HR pathway activation, by promoting the formation of D-loops during the homologous
302 pairing recombination. Moreover, SFPQ can act as both activator and inhibitor of RAD51, exerting
303 a tight regulation of HR pathway. FUS plays a fundamental role in both double strand breaks
304 (DSBs), where its depletion leads to HR and NHEJ impairment, and single strand breaks (SSBs)
305 repair, since it is recruited by PARP1 to promote the recruitment of base excision repair (BER)
306 proteins at the DNA damage site. Overall, this evidence, together with the fact that NEAT1 can be a
307 direct target of the TP53 transcription factor [14], support the hypothesis of a functional
308 contribution of NEAT1, and likely of PSs, in DDR pathway.

309 Since PSs are important in the cell response to different stimuli including stressful conditions,
310 such as nutrient starvation and hypoxia, we investigated the role of NEAT1 in stressful culturing
311 conditions. Our investigation established that NEAT1 overexpression is crucial to sustain the
312 growth and the survival potential of MM cells when maintained in non-physiological culturing

313 conditions. Indeed, hypoxia and nutrient starvation further induce NEAT1 expression in AMO-1^{SAM}
314 clones, which positively correlates with an increase of both PSs number within cells and cell
315 survival. In agreement with our data, NEAT1 up-regulation under hypoxic condition has been
316 already reported in hepatocellular carcinoma and non-small cell lung cancer, where NEAT1
317 represents a target of the HIF-1 or HIF-2 transcription factors, respectively; also in this context,
318 NEAT1 overexpression provides the cell with pro-survival and oncogenic properties [24,25]. In
319 MM cells as well, we found that the transactivation of NEAT1 not only guarantees pro-survival
320 advantages, but also increases their oncogenic potential, as demonstrated by the higher colony-
321 forming potential obtained upon FBS starving conditions, as well as the more structured colonies
322 organization.

323 In line with the role of NEAT1 in supporting MM cell survival during FBS starvation, we
324 observed the activation of ERK and AKT-mediated cell survival pathways in NEAT1 transactivated
325 cells cultured.

326 Overall, these findings suggest that NEAT1 targeting may have great translational relevance
327 since both serum starvation and hypoxia are typical stressful conditions for tumor cells *in vivo* and
328 are often associated with more aggressive tumor stages and mechanisms of chemo-resistance.

329 Based on the functional impact of NEAT1 both in the DDR pathways and in the growth and
330 survival of MM cells when cultured in non-physiological conditions, we hypothesized that NEAT1
331 transactivation may induce survival advantage in MM cells under stressful conditions by improving
332 DNA repair mechanisms. In agreement with this theory, we demonstrated that, along with the
333 stabilization of NONO, SFPQ, and FUS, NEAT1 transactivation is associated with the
334 overexpression of other two crucial proteins of the DDR system, DNA-PKcs and ATM, thus
335 leading to the activation of their target proteins RPA32 and CHK2 (Fig 4 and Supplementary Fig.
336 6). We validated the positive association between NEAT1 transcription levels and the expression of
337 the DDR proteins in NEAT1 transactivated cells under stressful condition; in fact, we showed that,
338 similarly to NEAT1 expression levels, also DDR protein levels further increase in non-

339 physiological culture conditions (Fig 5 and Supplementary Fig. 7). Finally, transactivated NEAT1
340 cells in stressful conditions also exhibit significantly greater viability (Supplementary Fig. 7),
341 indicating a pro-survival and pro-oncogenic role of NEAT1-mediated axis in MM.

342 The molecular circuit driven by NEAT1 and its crucial role for MM cell survival under
343 stressful condition was further validated by different approaches. First, we demonstrated that the
344 chemical inhibition of ATM and/or DNA-PK in NEAT1 transactivated AMO-1^{SAM} cells under
345 stressful conditions significantly decreases cell viability and leads to cell morphological changes
346 characterized by a massive cytoplasmic vacuolization (Fig. 7 and supplementary Fig 9). In
347 agreement with our observations, this peculiar phenotype preceding cell death has been reported in
348 osteosarcoma cells, upon ATR inhibition [26].

349 To validate the obtained results, we verified NEAT1-mediated molecular axis in other
350 HMCLs, thus excluding a cell line specific effect. Indeed, serum starving conditions induce the up-
351 regulation of NEAT1, DNA-PK, ATM, and pRPA32 in OPM2, LP1, and MM1.S HMCLs
352 (Supplementary Fig. 8). Furthermore, NEAT1 silencing in AMO-1 and LP1 negatively affects the
353 expression levels of both DNA-PKcs and ATM protein kinases, and of the active amount of RPA32
354 (Fig. 6). Of note, NEAT1 silencing in NCI-H929 cell line and in CD138+ purified primary PCs
355 leads to a pattern of cytoplasmic vacuolization similar to the phenotype obtained with the chemical
356 inhibition of ATM and/or DNA-PK (Fig. 7c), suggesting that the molecular and biological effect
357 highlighted upon NEAT1 silencing could be the result, at least in part, of the reduced activity of
358 DNA-PK and ATM on pRPA32.

359 Taken together, our data indicated that the up-regulation of ATM, DNA-PKcs, and pRPA32
360 could be considered a general molecular response of MM cells to NEAT1 induction, which, in turn,
361 could represent a survival advantage for MM cell facing adverse conditions.

362 Interestingly, our results pointed out an important role for the long NEAT1_2 variant under
363 stressful conditions; in fact, both in AMO-1^{SAM} clones and in all HMCLs tested, FBS starvation or
364 hypoxia significantly increase the NEAT1_2 percentage respect to total NEAT1 expression. This

365 finding suggests that non-physiological conditions may shift NEAT1 transcription towards the long
366 NEAT1_2 isoform, likely to assemble the PSs needed to counteract the stressful condition. In
367 accordance with this evidence, the specific targeting of the long NEAT1_2 variant negatively
368 affects the expression levels of both DNA-PKcs and ATM protein kinases, and of the active amount
369 of RPA32, suggesting that the structural scaffold of PSs, and maybe PSs themselves, should be
370 considered master regulators of DNA damage response in MM cells.

371 In conclusion, our study attributes a crucial role to NEAT1 overexpression in MM;
372 pathological NEAT1 expression and the consequent deregulation of PS organelles availability,
373 could represent an Achilles' heel for MM PCs survival and strongly suggest that NEAT1 and PSs
374 targeting could be considered a novel promising strategy for innovative anti-MM therapies.

375

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386

387 **AUTHOR CONTRIBUTIONS**

388 ET, CB, VKF, IS, NP, and KT performed experiments and analyzed the data; ET, DG and IS
389 performed cytofluorimetric experiments; ET and SE performed confocal analysis; NB, NA, AC,
390 RC, YT, and RP provided critical evaluation of experimental data and of the manuscript. ET, AN,
391 and DR conceived the study and wrote the manuscript.

392

393 **COMPETING INTERESTS**

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

395

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470

471

472 **LEGEND TO FIGURES**

473 **Figure 1. NEAT1 transactivation associates with PSPs increased expression levels.** **a** Analyses
474 of total NEAT1 and NEAT1_2 expression levels in NEAT1 transactivated AMO-1^{N#5} and AMO-
475 1^{N#8} cell lines with respect to AMO-1^{SCR} cells, based on the qRT-PCR approach described in the
476 schematic representation. NEAT1 expression was expressed as $2^{-\Delta\Delta Ct}$ relative to the scramble
477 condition. * $p < 0.05$ vs. SCR; *** $p < 0.001$ vs. SCR. **b** WB of NONO, SFPQ, and FUS in AMO-
478 1^{SAM} cells. GAPDH protein expression was included for protein loading normalization. **c** Effect of
479 NEAT1 transactivation in the presence of the protein synthesis inhibitor CHX (100 μ M) on the
480 decay of NONO and FUS protein levels in AMO-1^{SCR} and AMO-1^{N#8} cells at indicated time point.
481 Actin protein expression was included for protein loading normalization. The densitometric analysis
482 of immunoreactive bands is reported with respect to SCR condition in WB experiments.

483 **Figure 2. FBS starvation up-regulates NEAT1 and increases PSs number.** **a** qRT-PCR analyses
484 of total NEAT1 and NEAT1_2 expression in AMO-1^{SAM} cells maintained for 48 h in physiological
485 FBS culturing condition (10% FBS) and in FBS starving medium. NEAT1 expression was
486 expressed as $2^{-\Delta Ct}$. **b** Percentage of NEAT1_2 variant contribution respect to total NEAT1
487 expression in AMO-1^{SAM} cells maintained for 48 h in physiological FBS culturing condition (10%
488 FBS) and in FBS starving medium. **c** Confocal microscopy results of NEAT1 specific RNA-FISH
489 and NONO IF in AMO-1 cells cultured for 48 h in physiological FBS culturing condition (10%
490 FBS) and in FBS starving medium (scale bar 5 μ m).

491 **Figure 3. NEAT1 transactivation improves MM cells survival and oncogenic potential in non-**
492 **physiological culturing conditions.** **a** Growth curve and viability of AMO-1^{SAM} cells cultured for
493 72 h in FBS starving condition. * $p < 0.05$ vs. SCR. **b** Cell cycle analysis by PI staining performed in
494 AMO-1^{SAM} cells after 48 h of culture in FBS starving condition; specific histograms representing
495 the percentage of cells in sub G0/G1 are also shown. * $p < 0.05$ vs. SCR. **c** Flow cytometric analysis
496 of apoptosis in AMO-1^{SCR}, AMO-1^{N#5} and AMO-1^{N#8} cultured for 72 h in FBS starving condition.

497 **d** Colony formation assay performed on AMO-1^{SAM} cultured for 31 days in FBS starving condition;
498 representative pictures of colonies distribution at day 31 are also shown. **e** Representative pictures
499 of colonies formed in AMO-1^{SCR}, AMO-1^{N#5} and AMO-1^{N#8} 24 days after seeding (10x
500 magnification). **f** WB of pERK 1/2, ERK 1/2, pAKT, and AKT in AMO-1^{SAM} cells after 48 h of
501 culture in FBS starving conditions. GAPDH protein expression was included for protein loading
502 normalization. Percentage of pERK1/2 and pAKT with respect to total ERK1/2 and AKT (both
503 normalized for GAPDH expression) is also shown.

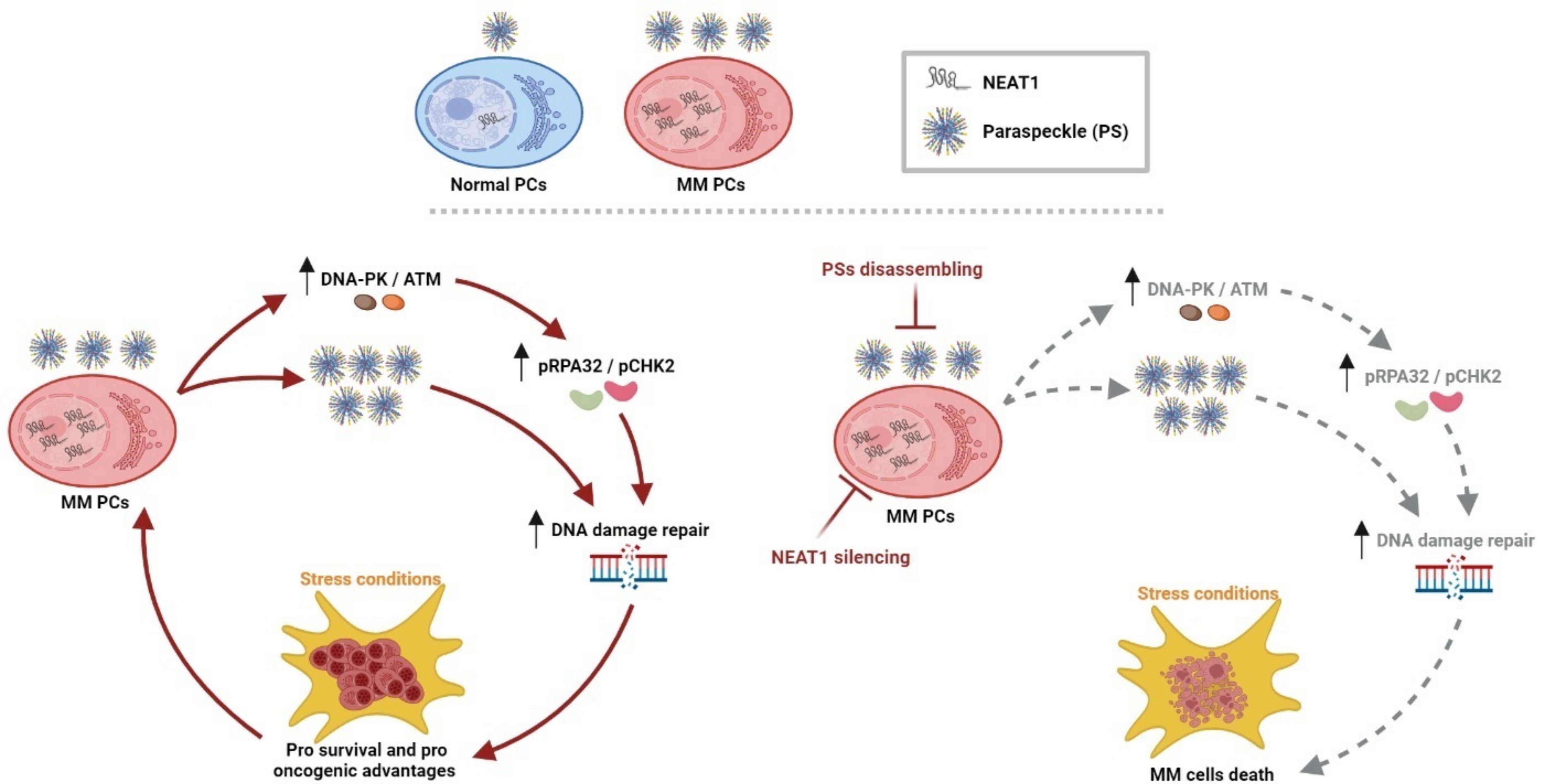
504 **Figure 4. NEAT1 transactivation up-regulates proteins involved in DNA repair process. a** WB
505 of pRPA32 and RPA32 in AMO-1^{SAM} cells. **b** WB of pCHK2, CHK2, pCHK1, and CHK1 in
506 AMO-1^{SAM} cells **c** WB of pATR, ATR, pATM, ATM, and DNA-PKcs in AMO-1^{SAM}. The
507 densitometric analysis of DNA-PKcs immunoreactive bands is reported with respect to SCR
508 condition. Furthermore, the percentage of activated fraction of all proteins with respect to relative
509 total amount (both normalized for GAPDH expression) is reported.

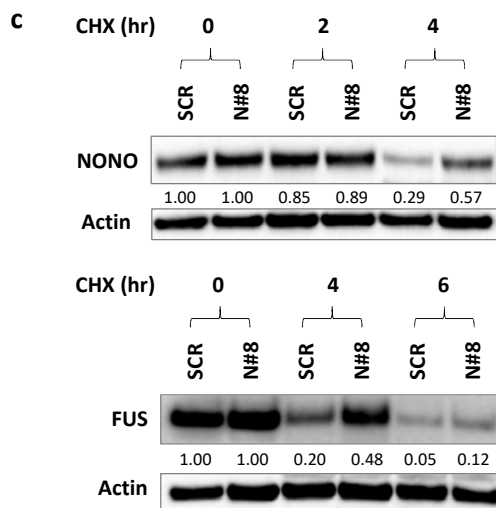
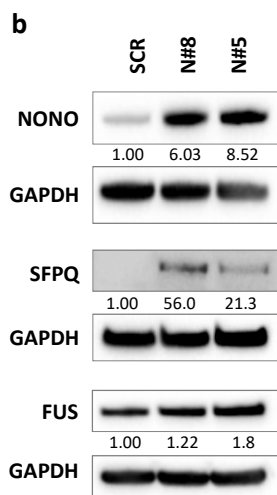
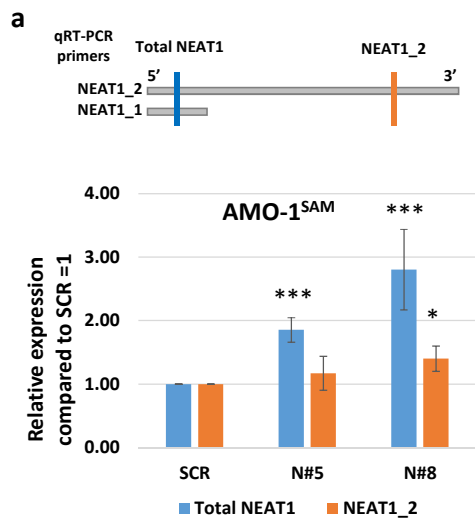
510 **Figure 5. MM cells under stressful conditions overexpress NEAT1 and other proteins involved**
511 **in DNA repair process. a** Confocal microscopy results of DNA-PKcs and pRPA32 specific IF in
512 AMO-1 cells cultured in physiological FBS culturing condition (10% FBS) and in FBS starving
513 medium (scale bar 20µm). **b** WB analysis of pRPA32, RPA32, DNA-PKcs and ATM in AMO-1^{SAM}
514 cells after 48 h of culture in FBS starving condition. GAPDH protein expression was included for
515 protein loading normalization. Percentage of pRPA32 with respect to total RPA32 (both normalized
516 for GAPDH expression) is also shown. **c** WB analysis of DNA-PKcs in AMO-1^{SAM} cells after 48 h
517 of culture in normoxic and hypoxic conditions. Actin protein expression was included for protein
518 loading normalization. The densitometric analysis of immunoreactive bands is reported with respect
519 to SCR condition.

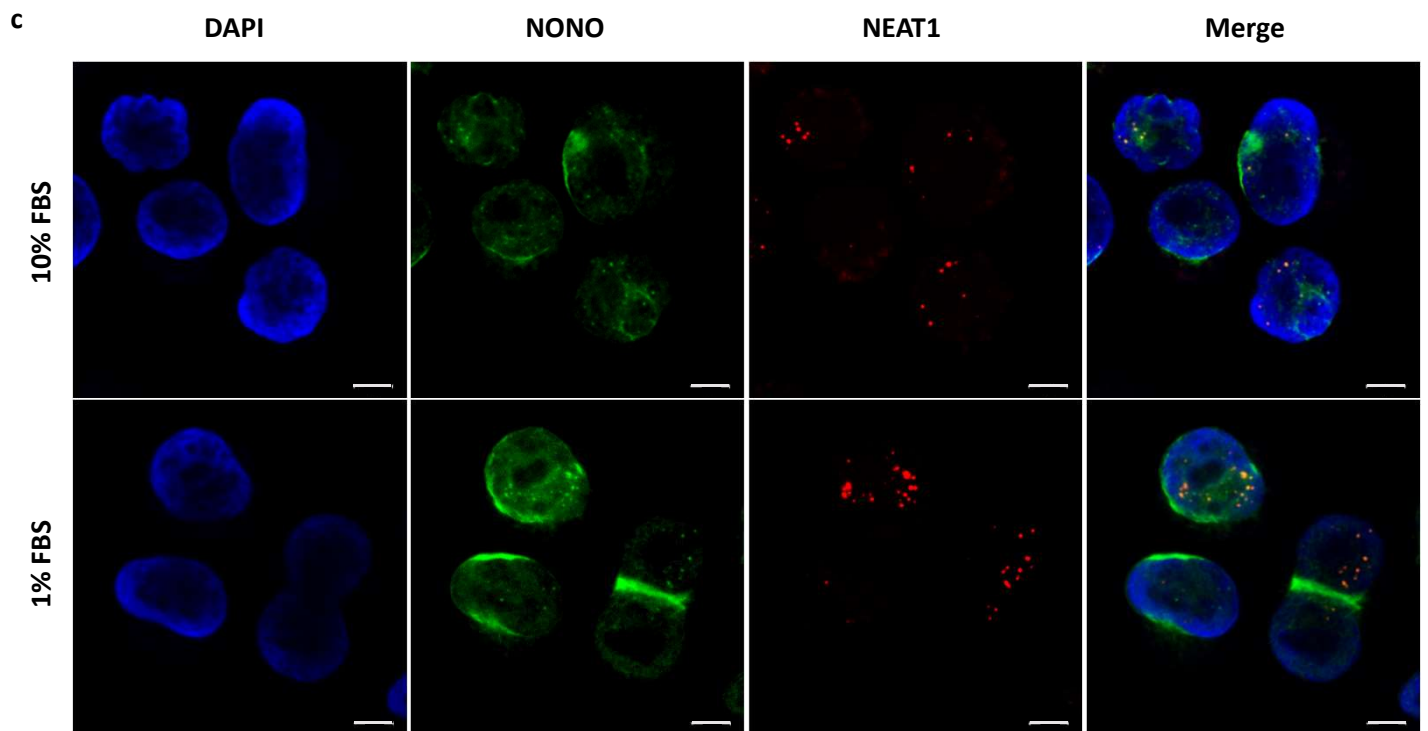
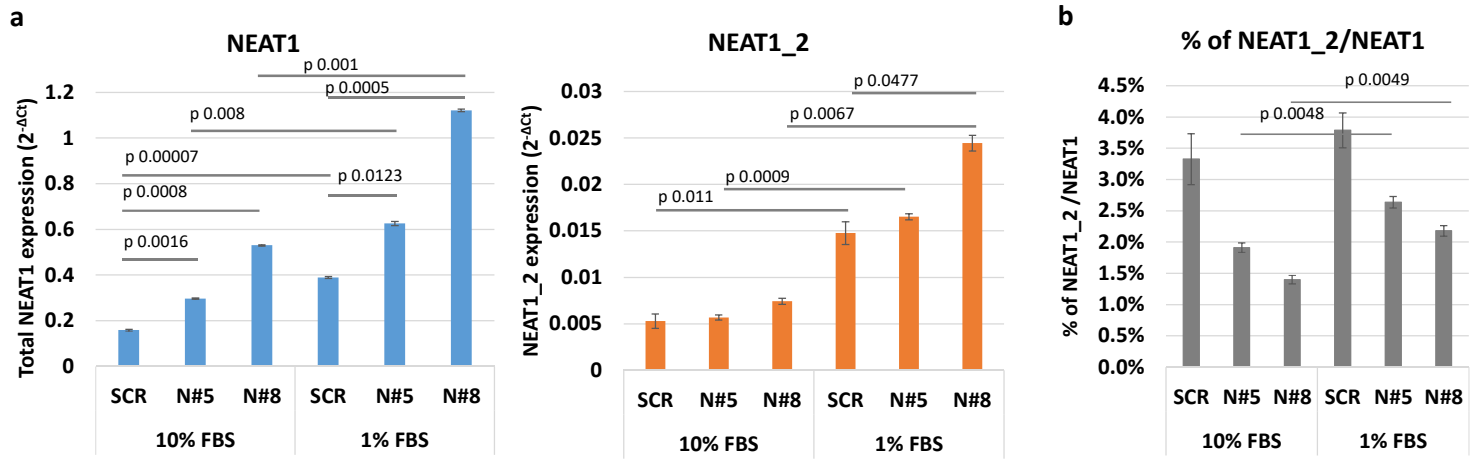
520 **Figure 6. NEAT1 silencing down-regulates proteins involved in DNA repair process. a** Scheme
521 of LNA-gapmeR localization on NEAT1 transcript; qRT-PCR analyses of NEAT1 expression

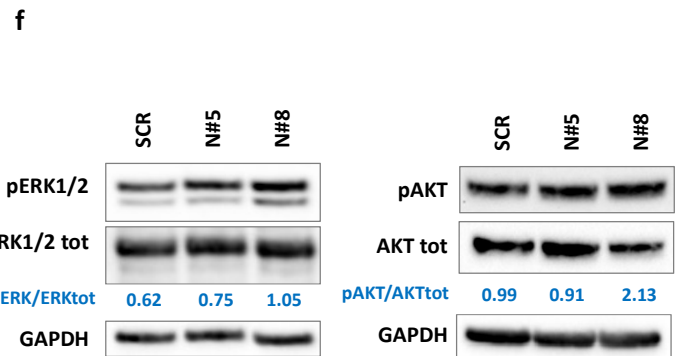
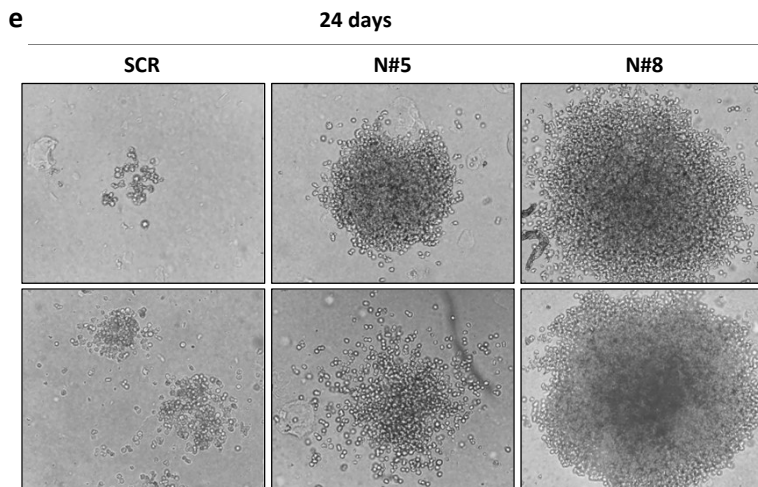
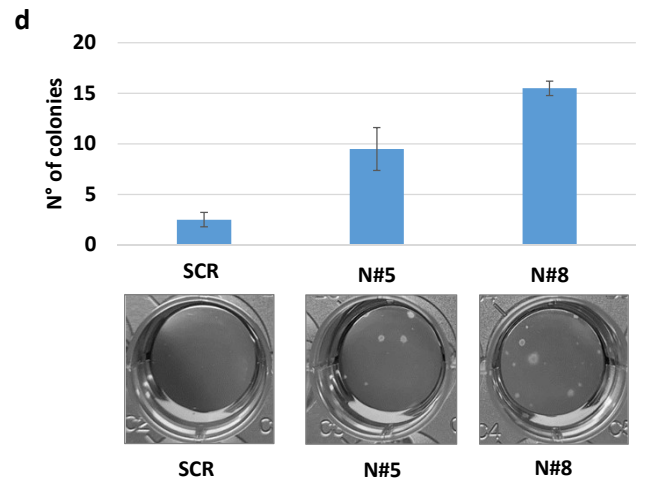
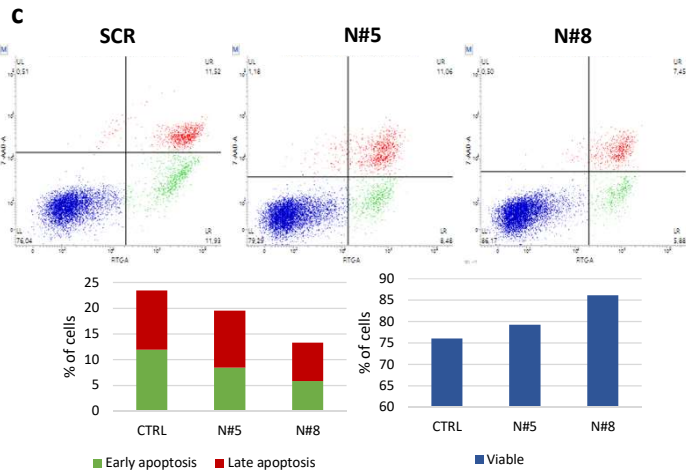
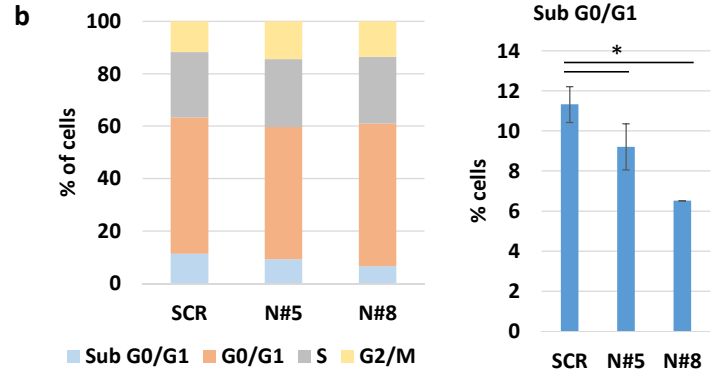
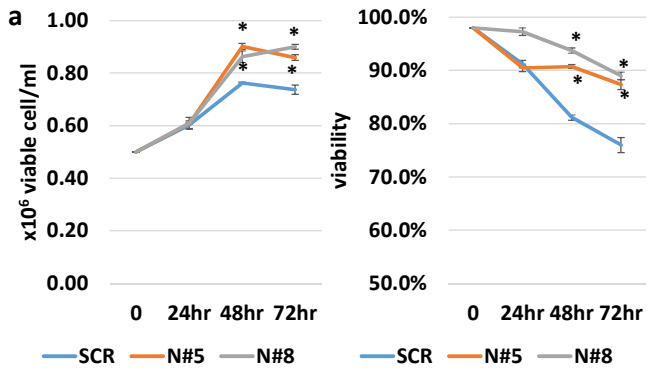
522 levels in AMO-1 and LP1 MM cell lines upon gymnotic delivery of g#N1_E or g#N1_G LNA-
523 gapmeR. NEAT1 expression was expressed as $2^{-\Delta Ct}$. **b** WB analysis of pRPA32, RPA32, DNA-
524 PKcs, and ATM in AMO-1 and LP1 cells after gymnotic delivery of NEAT1-targeting gapmeR (5
525 μ M). GAPDH protein expression was included for protein loading normalization. The densitometric
526 analysis of DNA-PKcs and ATM immunoreactive bands is reported with respect to SCR condition.
527 Furthermore, the percentage of pRPA32 with respect to total RPA32 (both normalized for GAPDH
528 expression) is also shown.

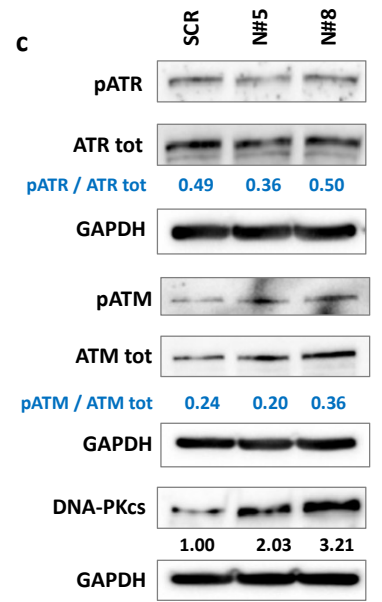
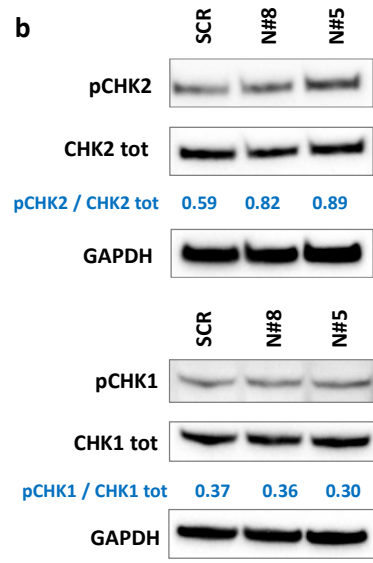
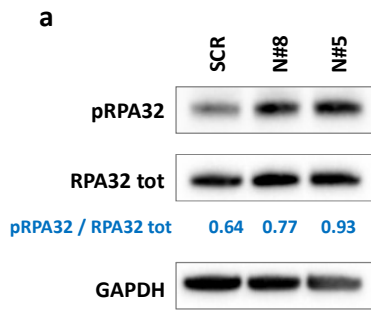
529 **Figure 7. ATM and DNA-PK inhibitions abrogate NEAT1 pro survival advantages. a** Viability
530 of AMO-1^{SCR} and AMO-1^{N#8} cells after three days of culture in FBS starving condition, in the
531 presence for the last 24 h of ATM and DNA-PK inhibitors, respectively KU-60019 and NU7026. **b**
532 Optical microscopy results of May Grunwald-Giemsa (MGG) staining obtained in AMO-1^{SCR} and
533 AMO-1^{N#8} cells after three days of culture in FBS starving condition, in the presence for the last 24
534 h of ATM and DNA-PK inhibitors. (100x magnification). **c** Optical microscopy results of May
535 Grunwald-Giemsa (MGG) staining obtained in NCI-H929 and CD138+ MM primary cells after
536 three days from the gymnotic delivery of NEAT1-targeting gapmeR (5 μ M). (100x magnification).

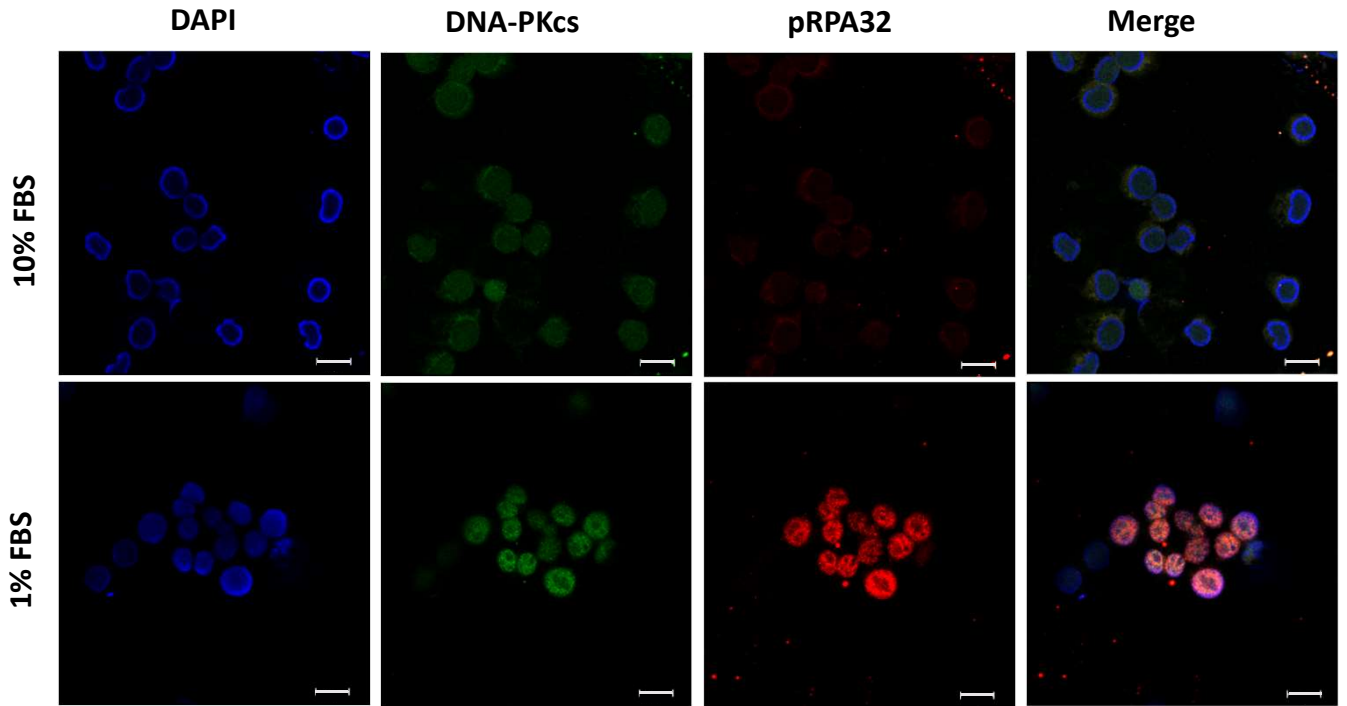
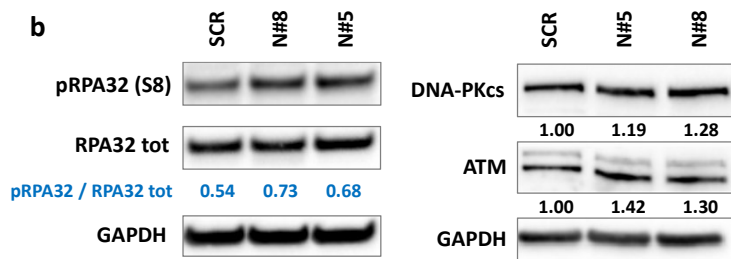
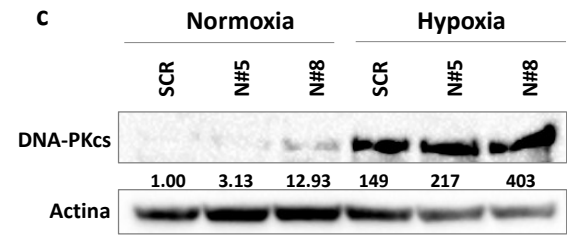


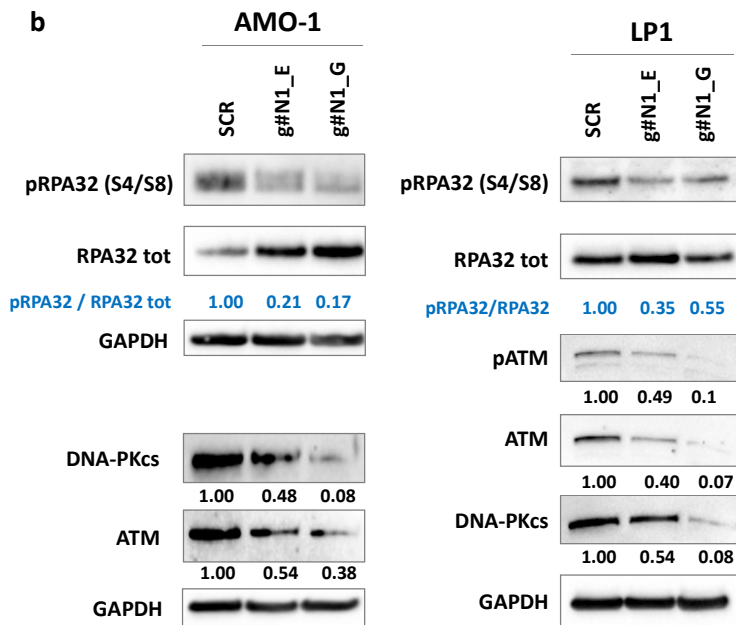
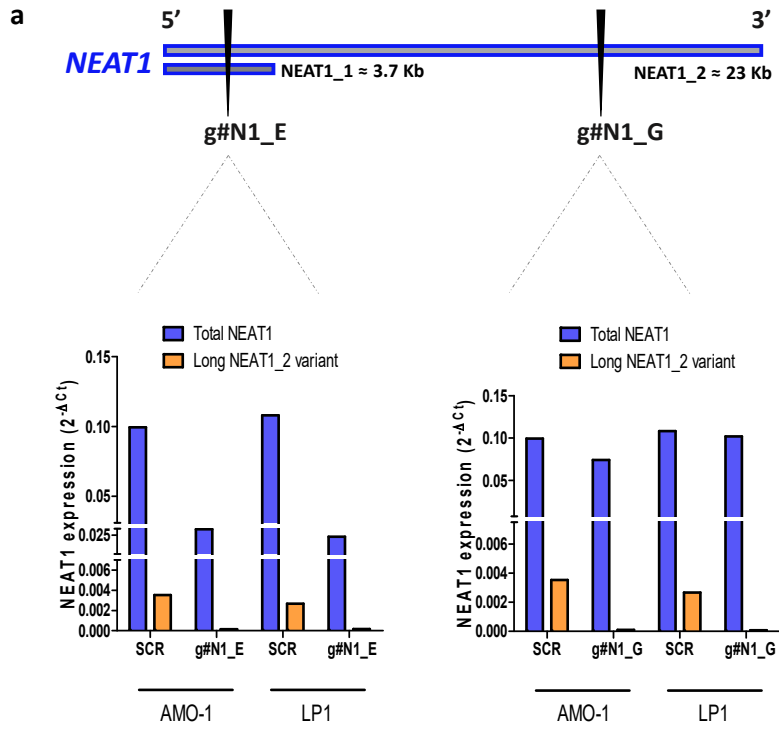




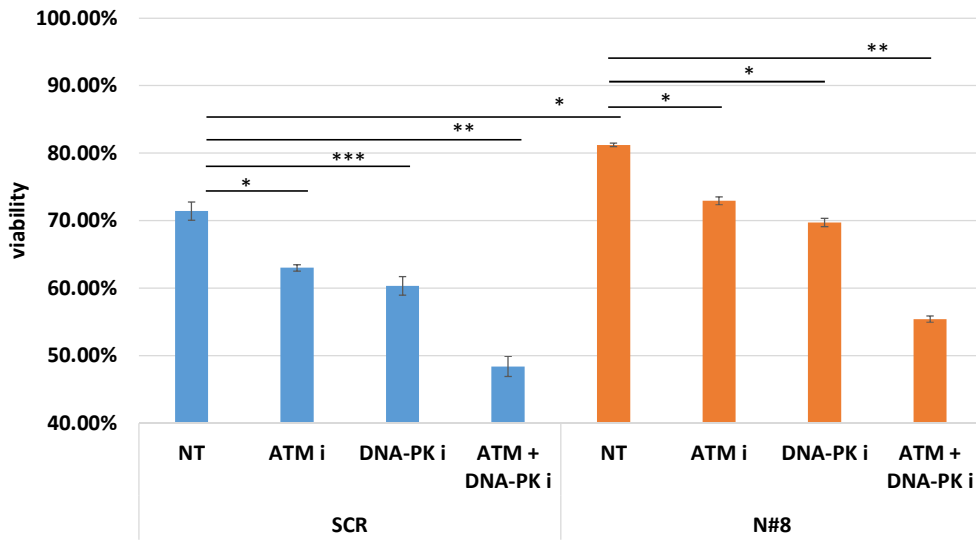




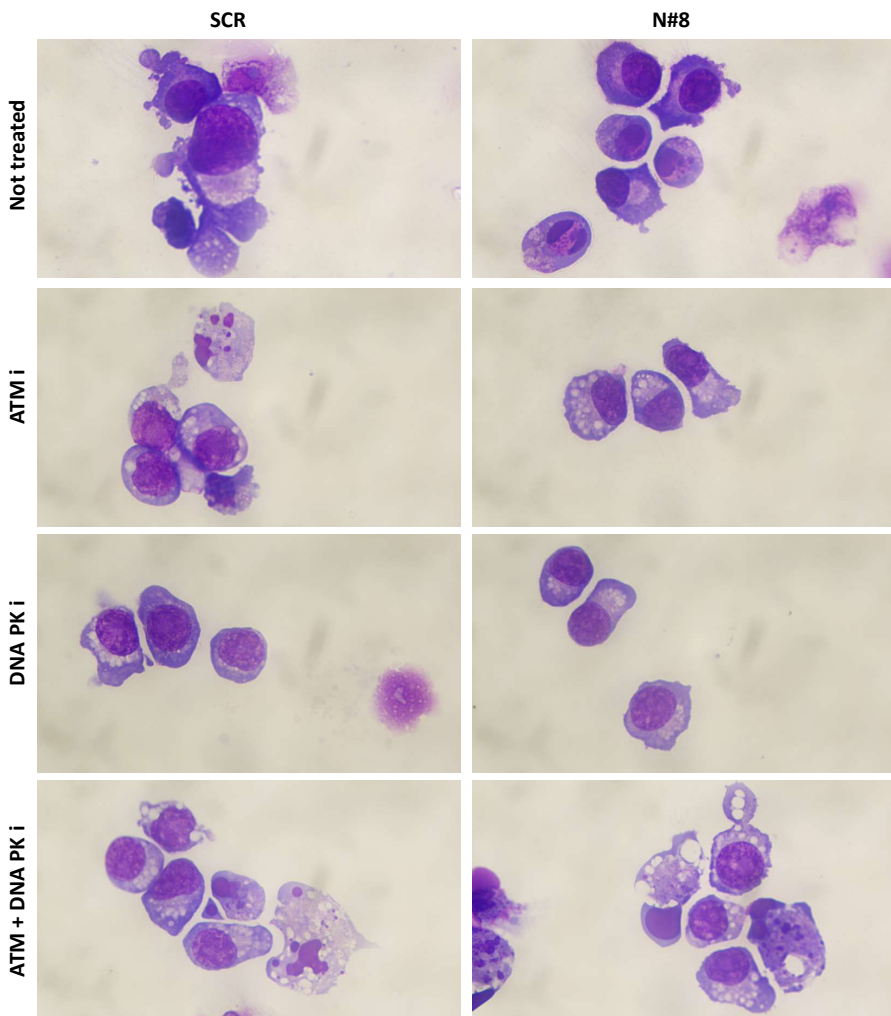
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