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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Abstract

Long non-coding RNA NEAT1 is the core structural component of the nuclear paraspeckle (PS) organelles and it has been found to be deregulated in multiple myeloma (MM) patients. Experimental evidence indicated that NEAT1 silencing negatively impacts proliferation and viability of MM cells, both *in vitro* and *in vivo*, suggesting a role in DNA damage repair (DDR). In order to elucidate the biological and molecular relevance of NEAT1 upregulation in MM disease we exploited the CRISPR/Cas9 synergistic activation mediator genome editing system to engineer the AMO-1 MM cell line and generate two clones that para-physiologically transactivate NEAT1 at different levels. NEAT1 overexpression is associated with oncogenic and prosurvival advantages in MM cells exposed to nutrient starvation or a hypoxic microenvironment, which are stressful conditions often associated with more aggressive disease phases. Furthermore, we highlighted the NEAT1 involvement in virtually all DDR processes through, at least, two different mechanisms. On one side NEAT1 positively regulates the post-translational stabilization of essential PS proteins, which are involved in almost all DDR systems, thus increasing their availability within cells. On the other hand, NEAT1 plays a crucial role as a major regulator of a molecular axis that includes ATM and the catalytic subunit of DNA-PK kinase proteins, and their direct targets pRPA32 and pCHK2. Overall, we provided novel important insightsthe role of NEAT1 in supporting MM cells adaptation to stressful conditions by improving the maintenance of DNA integrity. Taken together, our results suggest that NEAT1, and probably PS organelles, could represent a potential therapeutic target for MM treatment.

Introduction

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

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

We have identified the nuclear paraspeckle assembly transcript 1 (NEAT1) as one of the abundantly expressed lncRNA in malignant PC compared to its normal counterpart,[6,8,9] consistently with its high expression levels in many solid

tumors.[10] NEAT1 is a mono-exonic lncRNA, transcribed from the multiple endocrine neoplasia (MEN) type I locus, localized on chromosome 11q13.[11] Two different variants of NEAT1 exist and share an identical 5' terminus: NEAT1_1, a shorter polyadenylated (polyA) isoform of 3.7 kb, and NEAT1_2, a longer isoform of 22.7 kb, lacking the polyA tail.[12] NEAT1 has been demonstrated to be a fundamental structural component of nuclear paraspeckles (PS), which are irregularly shaped compartments of the nuclear interchromatin space, considered as membraneless lncRNA-directed nuclear bodies involved in many different biological processes (see review[9]). Indeed, PS are able to regulated gene expression by editing and sequestering mRNA; in addition, PS can act as a molecular sponge for RNA binding proteins, not impacting their functionality, but affecting their ability to interact with their target genes. PS also contribute to promote microRNA (miRNA) processing; furthermore, the capability for NEAT1 to sponge many miRNA[13] adds a further level of complexity to PS biology. Finally, PS are also involved in stress response.[9,14] Consistently with this function, we highlighted a pivotal role for NEAT1 in the maintenance of DNA integrity, through the regulation of the homologous recombination (HR) pathway, by demonstrating that NEAT1 depletion leads to a significant downregulation of genes and actives fractions of proteins involved in initial and crucial steps of the HR pathway.[6] Moreover, our research group demonstrated that NEAT1 silencing negatively regulates proliferation and viability of MM cells, both *in vitro* and *in vivo*.[6] Despite the growing amount of data obtained by loss-of-function approaches, concerning the role of NEAT1 in the DNA damage repair (DDR) system and maintenance of genome integrity,[6,14-16] the biological scenario underlying MM cells following NEAT1 overexpression remains virtually absent.

In recent years, different approaches of CRISPR/Cas9 genome editing system have been explored to induce gene activation[17] or gene repression.[18] In particular, the CRISPR/Cas9 synergistic activation mediator (SAM) system is a cutting-edge technique that uses MS2 bacteriophage coat proteins combined with p65 and HSF1 to para-physiologically induce the transcription of target genes without altering the DNA sequence.[19]

Using this approach, we investigated the functional role of NEAT1 transactivation in human MM cell lines (HMCL). In particular, we established a relevant role for NEAT1 in DNA damage repair (DDR) molecular mechanisms through the upregulation of the two fundamental kinases, namely ATM and the catalytic subunit of DNA-PK (DNA-PKcs). Furthermore, our data strongly indicate NEAT1 involvement in conferring survival advantage to MM cells exposed to stressful conditions such as nutrient starvation or hypoxic microenvironment, thus suggesting that the specific targeting of the PS backbone could be a promising novel strategy in MM treatment.

Methods

Full details of lentivirus production and *in vitro* transduction, plasmid constructs and cloning of single-guide RNA (sgRNA), quantitative real-time polymerase chain reaction (qRT-PCR), colony-forming assay, cell cycle analysis and apoptosis, immunofluorescence, RNA fluorescence *in situ* hybridization (FISH), gymnotic delivery, inhibitors and antibiotics, proteomic assays are provided in the *Online Supplementary Appendix*. For all western blots, images of the whole membranes showing all bands, densitometry readings and molecular weight markers are included in *Online Supplementary Appendix*.

Multiple myeloma cell lines and drugs

AMO-1 was kindly provided by Dr C. Driessen (University of Tubingen, Germany). LP1, MM1.S, OPM2, and NCI-H929 were purchased from DSMZ, which certified authentication performed by short tandem repeat DNA typing. All HMCL were immediately frozen and used from the original stock within 6 months. HMCL were cultured in RPMI-1640 medium (Gibco®, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin (Gibco®) at 37°C in 5% CO_2 atmosphere, and routinely tested to rule out mycoplasma contamination.

Hypoxia and HIF-1α stabilization

Hypoxia was induced by placing the cells for 24/48 hours (h) in a modular incubator chamber (Billups Rothenberg Inc., Del Mar, CA, USA) flushed with a mixture of 1% O_2, 5% CO_2 and 94% N_2 at 37°C. In order to achieve oxygen-independent HIF-1α stabilization, cells were exposed to 100 μM $CoCl_2$ for 24 h.[20]

Statistical analysis

Statistical significance of differences observed was determined by Student's *t*-test analysis; differences were considered significant when *P* values were $*P<0.05$, $**P<0.01$ or $***P<0.001$. All statistical analyses were performed using the Prism 5.0 software (GraphPad Software, Inc.)

Ethics approval and consent to participate

Written informed consent was obtained from all patients in accordance with the declaration of Helsinki. The study was approved by the Ethical Committee of the University of Milan (N°24/15, May 06 2015).

Results

NEAT1 transactivation increases the amount of paraspeckles in multiple myeloma cells

In order to activate the endogenous expression of NEAT1 in

MM cells, we took advantage of the CRISPR/Cas9 SAM genome editing system. We first engineered the AMO-1 cell line to stably express the components of the CRISPR activation system (dCas9/ MS2-p65-HSF1) *(Online Supplementary Figure S1A*). Then, AMO-1 cells were individually transduced with three sgRNA targeting the NEAT1 promoter or a scramble sgRNA (*Online Supplementary Table S1*; *Online Supplementary Figure S1B*). qRT-PCR of selected cells demonstrated that two of three NEAT1 targeting sgRNA induced significant NEAT1 transactivation (AMO-$1^{N\#5}$ and AMO-$1^{N\#8}$ cells) compared to the scramble condition (AMO-1^{SCR} cells) (Figure 1A). Specific RNA FISH confirmed the sustained NEAT1 expression levels obtained in both targeted cell lines (*Online Supplementary Figure S1C*).

Since NEAT1 has been demonstrated to be the fundamental structural component of nuclear PS,[9] we evaluated whether the increased NEAT1 transcription affects the expression of the essential PS proteins (PSP). Interestingly, NEAT1 overexpression was associated with a significant increase of NONO and SFPQ protein expression in AMO-$1^{N\#5}$ and AMO-$1^{N\#8}$ cells (Figure 1B). Similarly, we detected also a moderate overexpression of the FUS protein, whose prion-like low complexity domain is required for the organization of a microscopically visible mature PS[21] (Figure 1B; *Online Supplementary Figure S2*). However, PSP mRNA expression levels were not significantly modulated in AMO-$1^{N\#5}$ and AMO-$1^{N\#8}$ cells (*Online Supplementary Figure S3A*). This prompted us to investigate the role of NEAT1 in the regulation of PSP stability. In order to address this issue, we monitored the time course of NONO and FUS disappearance in the presence of the protein synthesis inhibitor cycloheximide (CHX). Our analysis indicated that the degradation rate of NONO and FUS was significantly slower in NEAT1-transactivated AMO-1 cells (Figure 1C). Furthermore, neither NONO nor FUS completely disappeared even after long CHX exposure (*Online Supplementary Figure S3B*).

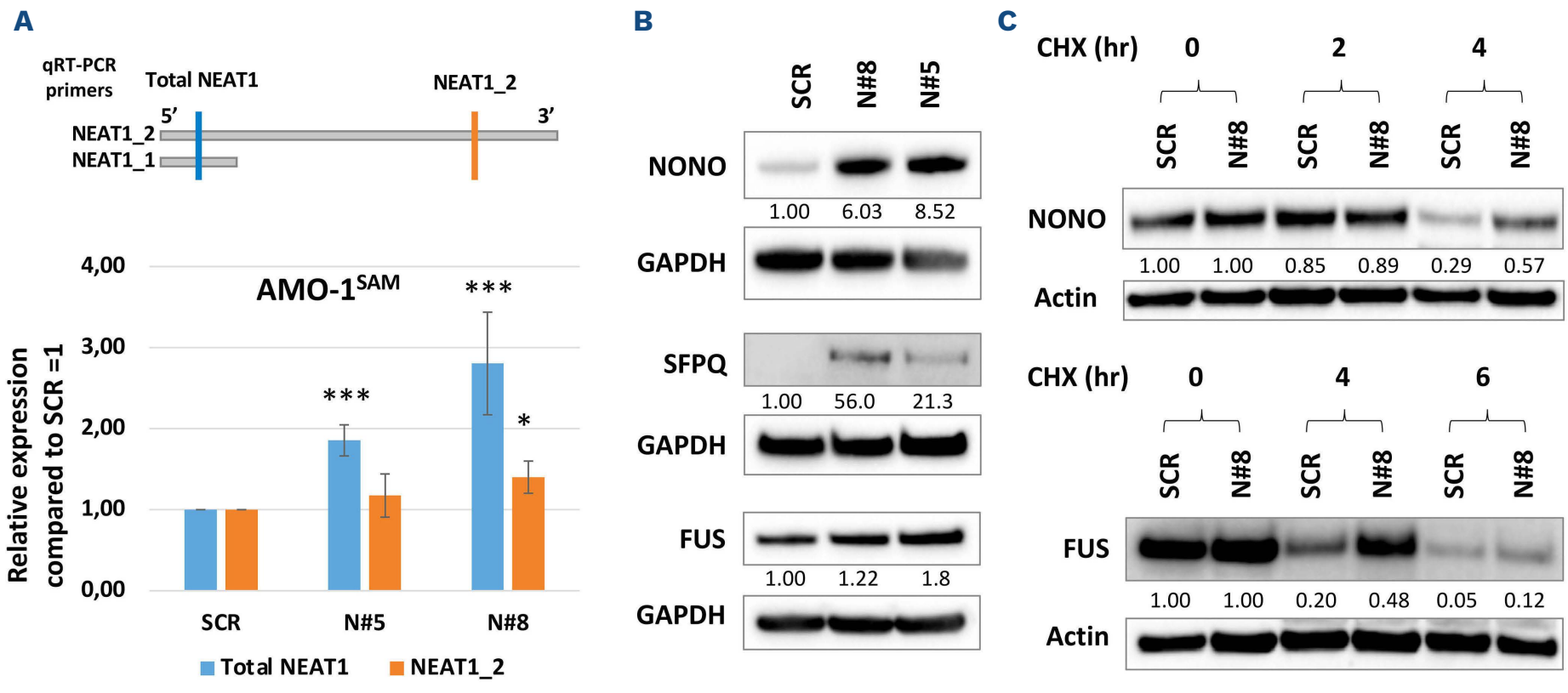
Finally, we evaluated if the stabilization of PSP induced by NEAT1 activation was associated with a positive modulation of PS size and distribution in AMO-1 cells. Our analyses of AMO-$1^{N\#5}$ and AMO-$1^{N\#8}$ cells by confocal microscopy revealed that NEAT1 and NONO co-localized in PS organelles, whose number and size were increased compared to AMO-1^{SCR} cells (Figure 1D).

NEAT1 transactivation provides a survival advantage to multiple myeloma cells cultured under stressful conditions

In order to evaluate whether MM cells could benefit from a survival advantage upon NEAT1 transactivation, we monitored the growth rate and viability of AMO-$1^{N\#5}$, AMO-$1^{N\#8}$, and AMO-1^{SCR} cells in physiological and stressful conditions. In the first case, AMO-$1^{N\#5}$, AMO-$1^{N\#8}$ cell lines showed cell growth and viability similar to AMO-1^{SCR} cells (*Online Supplementary Figure S4A* and *B*). We confirmed the absence of significant modulation in the cell cycle phase distributions and in the apoptotic rate by flow cytometric analysis (*Online Supplementary Figure S4C* and *D*). Consistently, we also did not observe any change in the clonogenic potential (*Online Supplementary Figure S4E*).

Based on NEAT1 involvement during cellular stress response,[9,14,22] we assessed the relevance of NEAT1 transactivation in stressful conditions, such as FBS starvation or hypoxia.

First, we assessed NEAT1 expression modulation under stressful conditions. Both total NEAT1 and NEAT1_2 iso-



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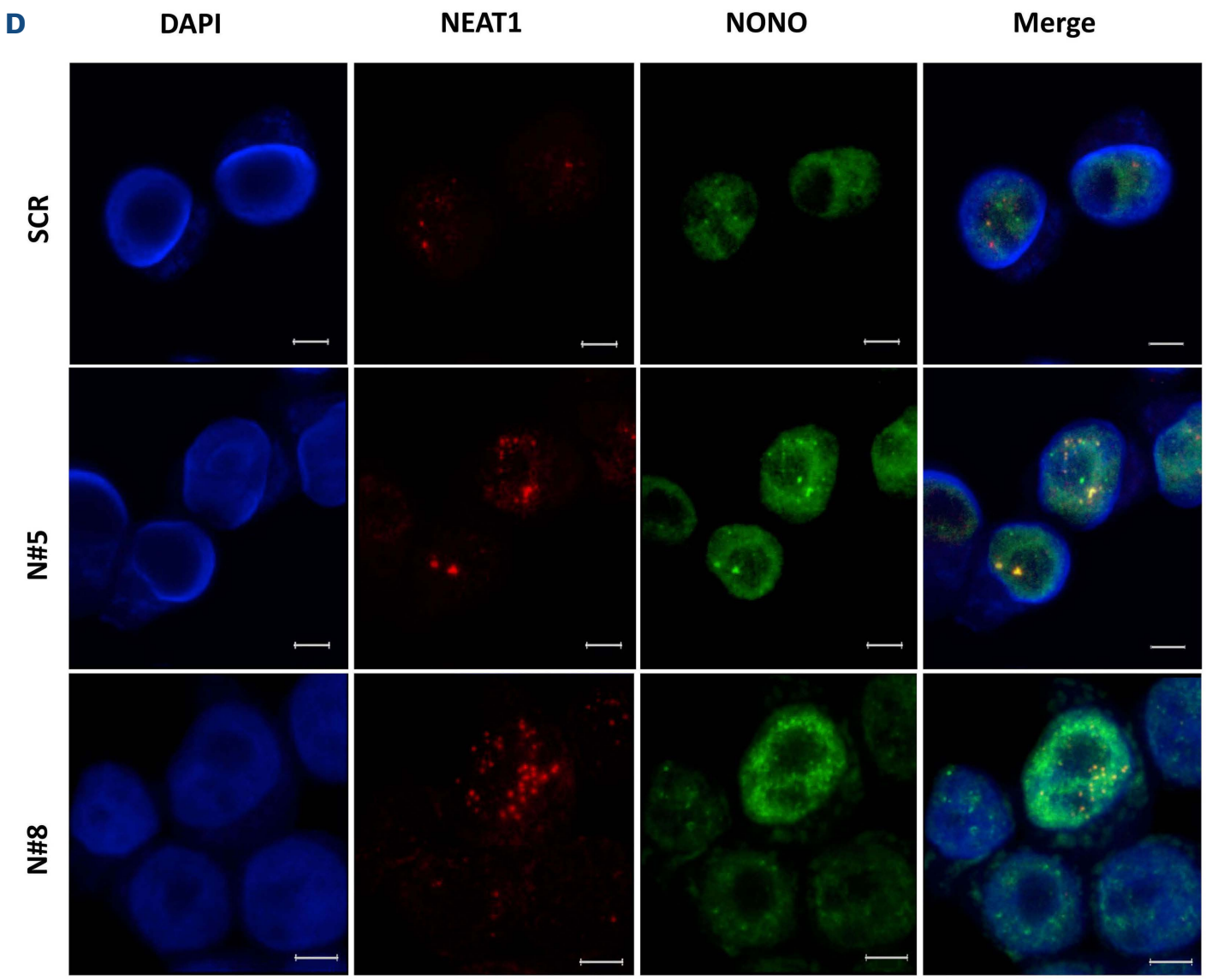


Figure 1. NEAT1 transactivation associates with increased paraspeckle protein expression levels. (A) Analyses of total NEAT1 and NEAT1_2 expression levels in NEAT1-transactivated AMO-1$^{N\#5}$ and AMO-1$^{N\#8}$ cell lines with respect to AMO-1^{SCR} cells, based on the real-time quantitative polymerase chain reaction (qRT-PCR) approach described in the schematic representation. NEAT1 expression was expressed as $2^{-\Delta\Delta Ct}$ relative to the scrambled (SRC) condition. **P*<0.05 *vs.* SCR; ****P*< 0.001 *vs.* SCR. (B) Western blot of NONO, SFPQ, and FUS in AMO-1^{SAM} cells. GAPDH protein expression was included for protein loading normalization. (C) Effect of NEAT1 transactivation in the presence of the protein synthesis inhibitor cycloheximide (CHX) (100 μM) on the decay of NONO and FUS protein levels in AMO-1^{SCR} and AMO-1$^{N\#8}$ cells at indicated time points. Actin protein expression was included for protein loading normalization. The densitometric analysis of immunoreactive bands is reported with respect to SCR condition in western blotting experiments. (D) Confocal microscopy results of NEAT1 specific RNA fluorescence *in situ* hybridization and NONO immunofluorescence in AMO-1^{SAM} cells (scale bar 5μm).

form expression levels of AMO-1$^{N\#5}$, AMO-1$^{N\#8}$, and AMO-1^{SCR} cells cultured for 48 h at 1% FBS conditions resulted in a significant upregulation in comparison with the relative counterpart maintained in 10% FBS (Figure 2A), confirming that both variants are induced under stressful conditions. Interestingly, FBS starvation increased the fraction of the NEAT1_2 isoform (Figure 2B), which is known to be the fundamental structural scaffold for the biogenesis of PS organelles. Confocal microscopy analysis revealed that FBS starvation not only induced NEAT1 expression but also NONO protein expression, leading to an increase in the number of PS identifiable by co-localized signals (Figure 2C). Similar results were obtained by growing MM cells in a hypoxic microenvironment, resulting in a significant upregulation of both NEAT1 isoforms (Figure 3A), particularly of the fraction of NEAT1_2 long variant (Figure 3B). Moreover, in line with results obtained with serum starvation, the upregulation of NEAT1 upon hypoxia positively correlated with the increase of NONO expression and the number and size of PS organelles (Figure 3C). Notably, the amount of PS and the fraction of the NEAT1_2 isoform returned to the levels observed in physiological conditions when normal FBS amounts and oxygen were restored (Figures 2C and 3D; *Online Supplementary Figure S5*).

From a biological point of view, upon FBS starvation NEAT1-activated cells showed a significantly higher viability as compared to AMO-1^{SCR} cells (Figure 4A). This finding is supported also by a reduced plasmatic membrane integrity observed in AMO-1^{SCR} compared to AMO-1$^{N\#5}$ and AMO-1$^{N\#8}$ (*Online Supplementary Figure S6A*) and by a cell cycle analysis that showed a higher percentage of cells distributed in sub G0/G1 phase (Figure 4B; *Online Supplementary Figure S6B*). Additionally, the presence of a significantly higher percentage of apoptotic cells (Figure 4C) in AMO-1^{SCR} cells suggests also an anti-apoptotic role of

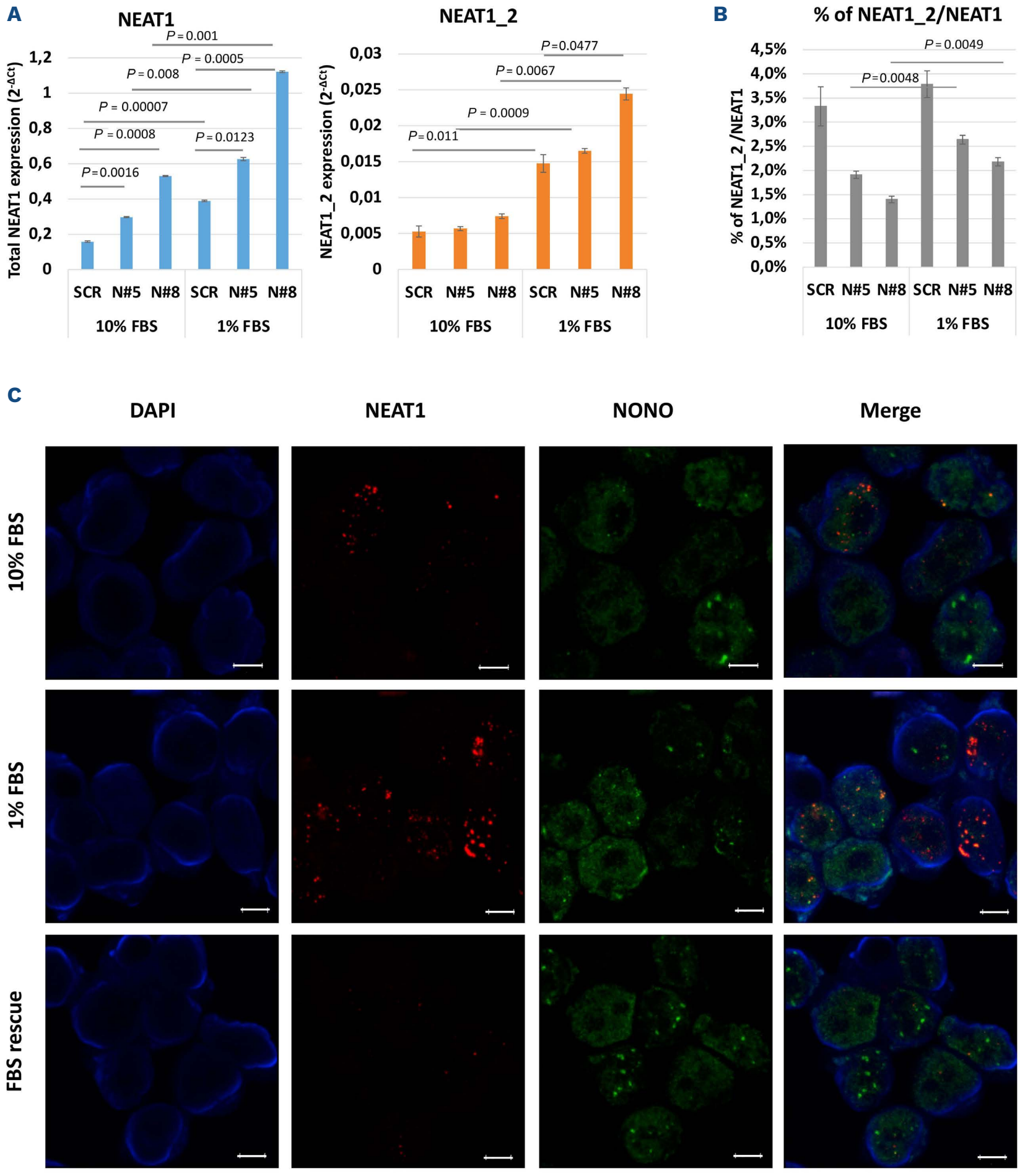


Figure 2. Fetal bovine serum starvation upregulates NEAT1 and increases paraspeckle numbers. (A) Real-time quantitative polymerase chain reaction (qRT-PCR) analyses of total NEAT1 and NEAT1_2 expression in AMO-1^{SAM} cells maintained for 48 hours (h) in physiological fetal bovine serum (FBS)-culturing conditions (10% FBS) and in FBS-starving medium. NEAT1 expression was expressed as $2^{-\Delta Ct}$. (B) Percentage of NEAT1_2 variant contribution respect to total NEAT1 expression in AMO-1^{SAM} cells maintained for 48 h in 10% FBS and in FBS-starving medium. (C) Confocal microscopy results of NEAT1-specific RNA fluorescence *in situ* hybridization and NONO immunofluorescense in AMO-1 cells cultured for 48 h in 10% FBS, in FBS-starving medium, and upon restoring physiological 10% FBS-culturing conditions (scale bar 5µm).

NEAT1. Moreover, NEAT1 transactivation significantly increased the clonogenic potential of MM cells cultured in the same conditions (Figure 4D). In detail, sustained NEAT1 expression increased the number of colonies that also resulted to be larger, more structured and compact (Figure 4E; *Online Supplementary Figure S6C*).

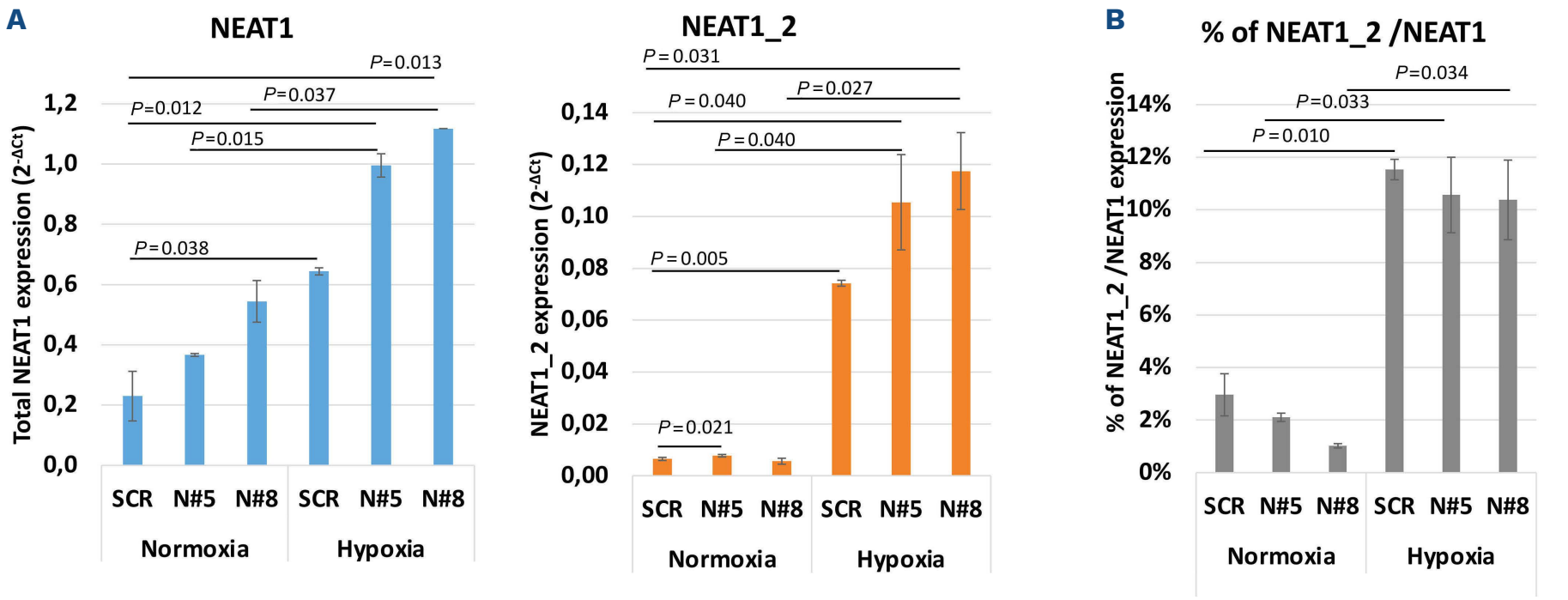


Figure 3. Hypoxia upregulates NEAT1 and increases paraspeckle numbers. (A) Real-time quantitative polymerase chain reaction (qRT-PCR) analyses of total NEAT1 and NEAT1_2 expression in AMO-1^{SAM} cells maintained for 48 hours (h) under normoxic and hypoxic microenvironment. NEAT1 expression was expressed as $2^{-\Delta Ct}$. (B) Percentage contribution of NEAT1_2 respect to total NEAT1 expression in AMO-1^{SAM} cells maintained for 48 h under normoxic and hypoxic microenvironment. (C) Confocal microscopy results of NEAT1-specific RNA fluorescence *in situ* hybridization (RNA-FISH) and NONO immunofluorescence (IF) in AMO-1^{SAM} cells cultured for 48 h upon normoxic or hypoxic conditions (scale bar 5µm). (D) Confocal microscopy results of NEAT1-specific RNA-FISH and NONO IF in AMO-1 cells cultured for 48 h upon normoxic, hypoxic conditions, and upon restoring physiological normoxic conditions (scale bar 5µm).

In agreement with these biological data, in conditions of serum starvation, the active fraction of ERK1/2 and AKT proteins were upregulated in NEAT1-transactivated cells, displaying a higher increase in the more aggressive AMO-$1^{N\#8}$ compared to AMO-1^{SCR} cells (Figure 4F).

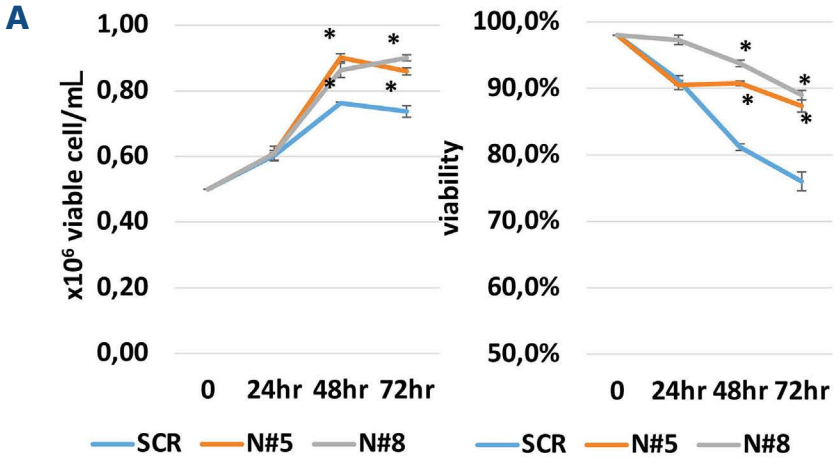
Finally, we could observe a greater viability and modulation of cell cycle phase distribution also in NEAT1-transactivated AMO-1 cells maintained in hypoxic conditions (*Online Supplementary Figure S6D* to *F*).

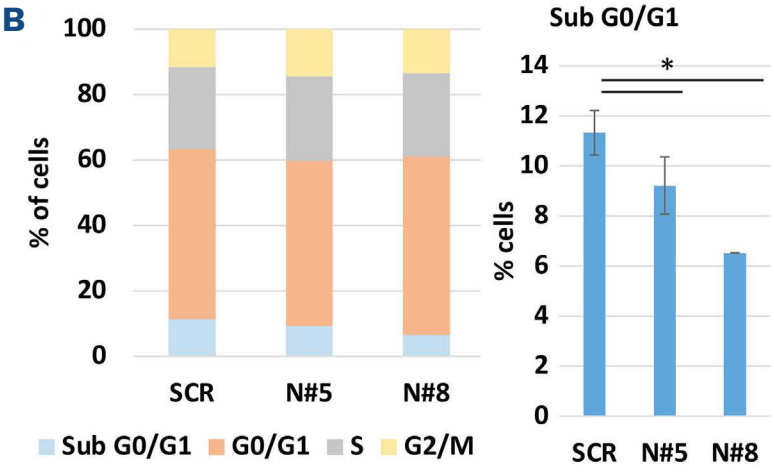
NEAT1 transactivation leads to the phosphorylation of RPA32 and CHK2 through a molecular mechanism dependent on ATM and DNA-PKcs

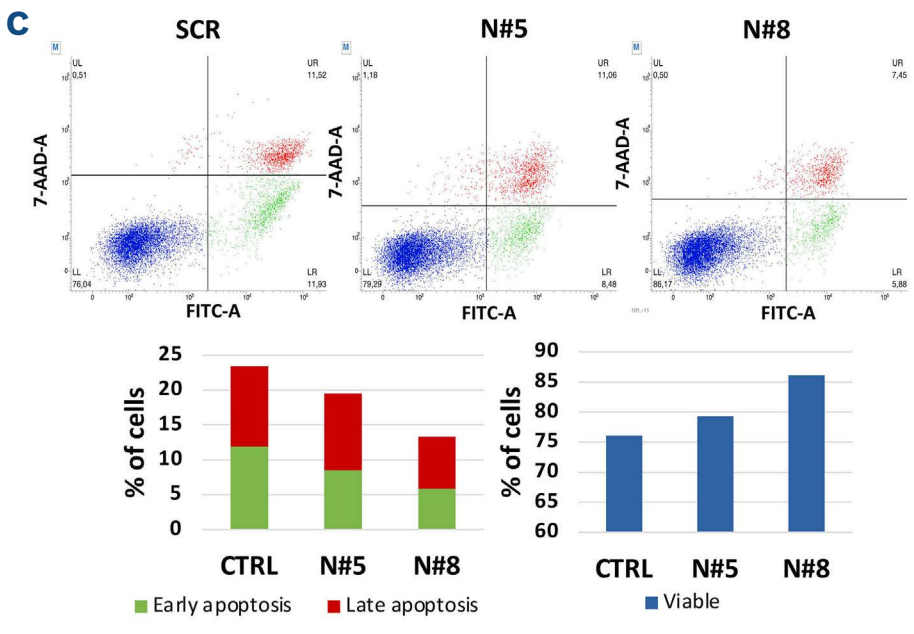
We previously demonstrated NEAT1 involvement in the DDR system.[6] With the aim of dissecting the molecular mechanisms associating NEAT1 with DNA repair processes, we investigated RPA32 expression following NEAT1 transactivation.

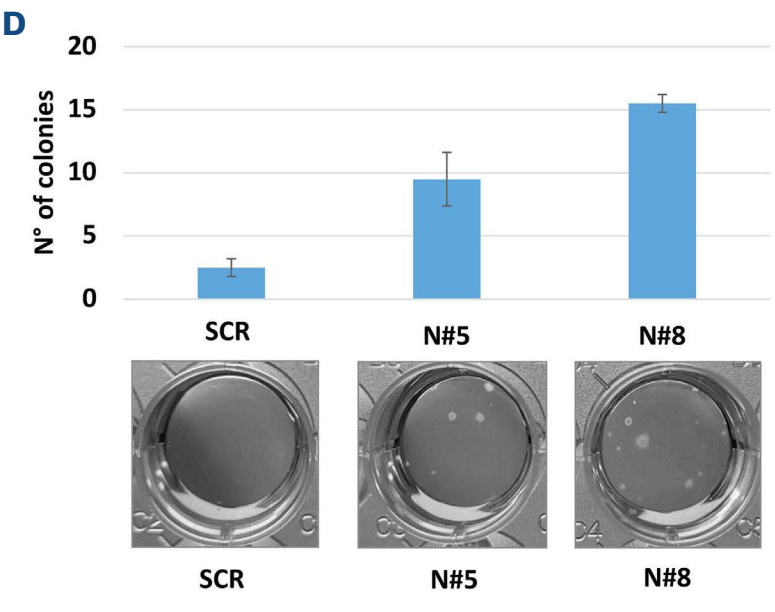
Specifically, western blot and confocal microscopy analysis showed an upregulation of pRPA32 levels in NEAT1 transactivated cells compared to AMO-1^{SCR} cells (Figure 5A and B; *Online Supplementary Figure S7A*), whereas the RPA32 total form remained unchanged at both protein (Figure 5A) and mRNA level (*Online Supplementary Figure S7B*). Furthermore, AMO-$1^{N\#5}$ and AMO-$1^{N\#8}$ cells displayed increased levels of the active fraction of CHK2, another important player of the DDR system, whereas no significant modulation of pCHK1 was detected (Figure 5C).

In order to shed light on the possible mechanism by which NEAT1 leads to an increase of activated RPA32, we evaluated the expression levels of the three main kinase proteins responsible for its phosphorylation, i.e., ATM, ATR and DNA-PK.[23] Our results showed that NEAT1 induction did not associate with significant modulations of *ATM*, *ATR* and *PRKDC* mRNA levels, ruling out a possible role of NEAT1 in the transcriptional regulation of these proteins (*Online Supplementary Figure S7C*). We also confirmed at protein level that NEAT1 transactivation did not induce a significant modulation of ATR and its activated form (Figure 5D), in line with the absence of modulation of its direct target pCHK1. Conversely, ATM protein levels increased in AMO-$1^{N\#5}$ and AMO-$1^{N\#8}$ cells with respect to AMO-1^{SCR} cells (Figure 5D), in agreement with the increased pCHK2 and pRPA32 expression levels. Furthermore, also DNA-PKcs showed a significant increase in NEAT1 activated cells compared to AMO1^{SCR} cells (Figure 5D), data also confirmed by immunofluorescence (Figure 5E; *Online Supplementary Figure S7D*).









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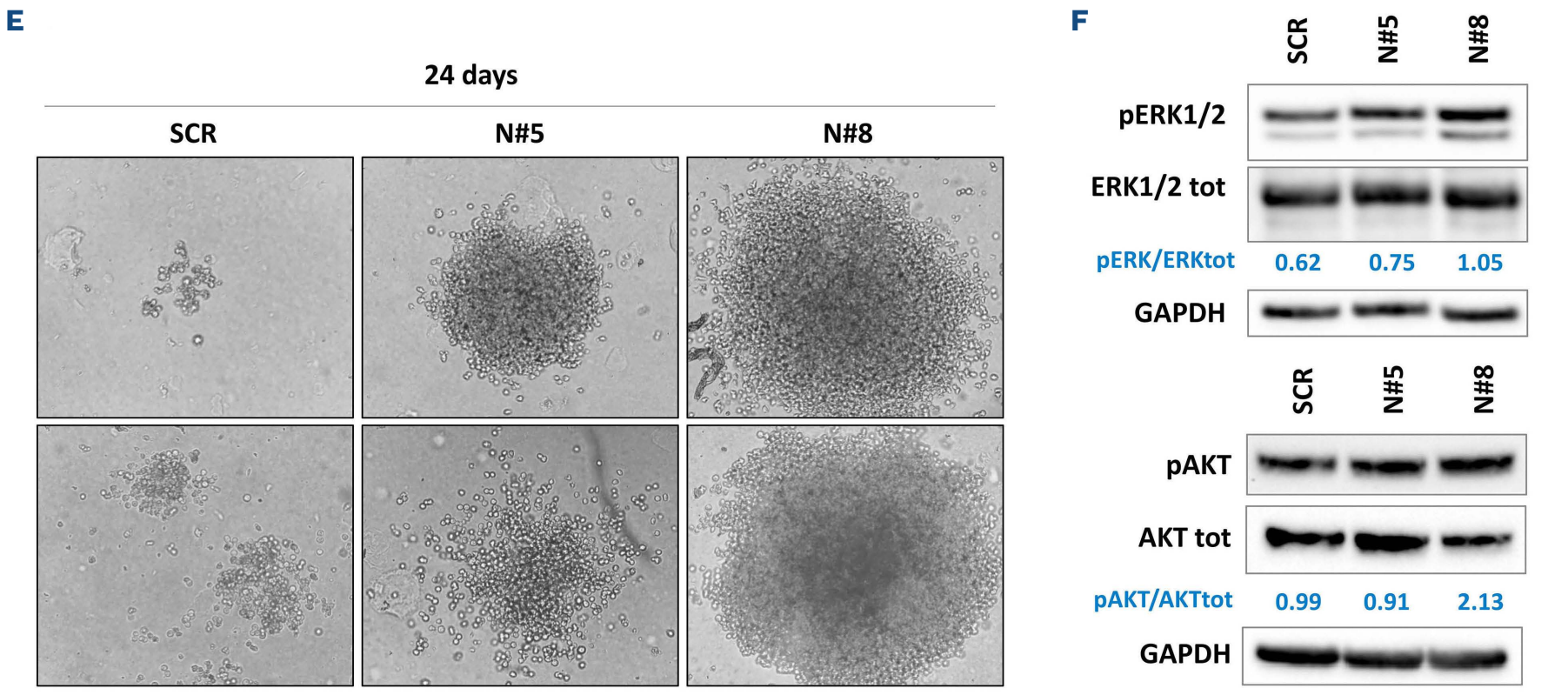

Figure 4. NEAT1 transactivation improves multiple myeloma cells survival and oncogenic potential in non-physiological culturing conditions. (A) Growth curve and viability of AMO-1^{SAM} cells cultured for 72 hours (h) in fetal bovine serum (FBS)-starving conditions. *P<0.05 *vs.* scrambled (SCR). (B) Cell cycle analysis by propidium iodide (PI) staining performed in AMO-1^{SAM} cells after 48 h of culture in FBS-starving conditions; specific histograms representing the percentage of cells in sub G0/G1 are also shown. *P<0.05 *vs.* SCR. (C) Flow cytometric analysis of apoptosis in AMO-1^{SCR}, AMO-1$^{N\#5}$ and AMO-1$^{N\#8}$ cultured for 72 h in FBS-starving conditions. (D) Colony formation assay performed on AMO-1^{SAM} cultured for 31 days in FBS-starving conditions; representative pictures of colonies distribution at day 31 are also shown. (E) Representative pictures of colonies formed in AMO-1^{SCR}, AMO-1$^{N\#5}$ and AMO-1$^{N\#8}$ 24 days after seeding (10x magnification). (F) Werstern blot of pERK 1/2, ERK 1/2, pAKT, and AKT in AMO-1^{SAM} cells after 48 h of culture in FBS-starving conditions. GAPDH protein expression was included for protein loading normalization. Percentage of pERK1/2 and pAKT with respect to total ERK1/2 and AKT (both normalized for GAPDH expression) is also shown.

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

NEAT1-mediated activation of RPA32 is enhanced upon fetal bovine serum starvation in hypoxic conditions

The evidence that FBS starvation positively modulates NEAT1 expression (Figure 2) prompted us to verify the outcome of FBS starvation on the molecular axis "NEAT1-pRPA32". For this purpose, we compared AMO-1^{SAM} cells cultured in FBS-starving and physiological conditions.

Interestingly, confocal microscopy analyses demonstrated increased levels of both DNA-PKcs and its target pRPA32 in AMO-1^{SCR} cells cultured in FBS-starving conditions (Figure 6A), in line with a possible relationship between NEAT1/PS expression and DNA-PKcs activity. Of note, AMO-1$^{N\#5}$ and AMO-1$^{N\#8}$ cells exposed to 1% FBS further increased the expression levels of pRPA32, DNA-PKcs and ATM compared to AMO-1^{SCR}, confirming the role of NEAT1 in triggering the mechanism that brings to RPA32 phosphorylation (Figure 6B).

Similarly, hypoxia, another condition that increases NEAT1 expression (Figure 3), resulted in DNA-PKcs levels higher than those observed in normoxic conditions, above all in both AMO-1$^{N\#5}$ and AMO-1$^{N\#8}$ cells compared to AMO-1^{SCR} cells (Figure 6C).

In order to further validate the effects of hypoxic stress, we investigated AMO-1^{SAM} cells upon $CoCl_2$ a treatment that chemically stabilizes HIF-1 transcription factor. Also in this case, we detected the overexpression of NEAT1 and NONO in association with the increase of both DNA-PKcs and pRPA32 protein levels (*Online Supplementary Figure S8A*). In agreement with the effects of hypoxic and FBS-starving conditions, the upregulation of DNA-PKcs upon $CoCl_2$ treatment further increased in NEAT1-transactivated cells (*Online Supplementary Figure S8B*), which, at the same time, showed a higher viability and a lower percentage of apoptotic cells than AMO-1^{SCR} cells (*Online Supplementary Figure S8C* and *D*).

With the aim of validating our data, we extended the study to other three HMCL (OPM2, MM1.S and LP1), based on the hypothesis that FBS-starving conditions could induce NEAT1 expression. As expected, serum starvation induced NEAT1 expression in all HMCL tested (*Online Supplementary Figure S9A*), and we detected an increase of the NEAT1_2 variant contribution with respect to total NEAT1 expression (*Online Supplementary Figure S9B*). Moreover, western blot analysis demonstrated an increase in the pRPA32 amount in FBS-starved cells compared to cells maintained at normal FBS conditions (*Online Supplementary Figure S9C)*. In agreement with our

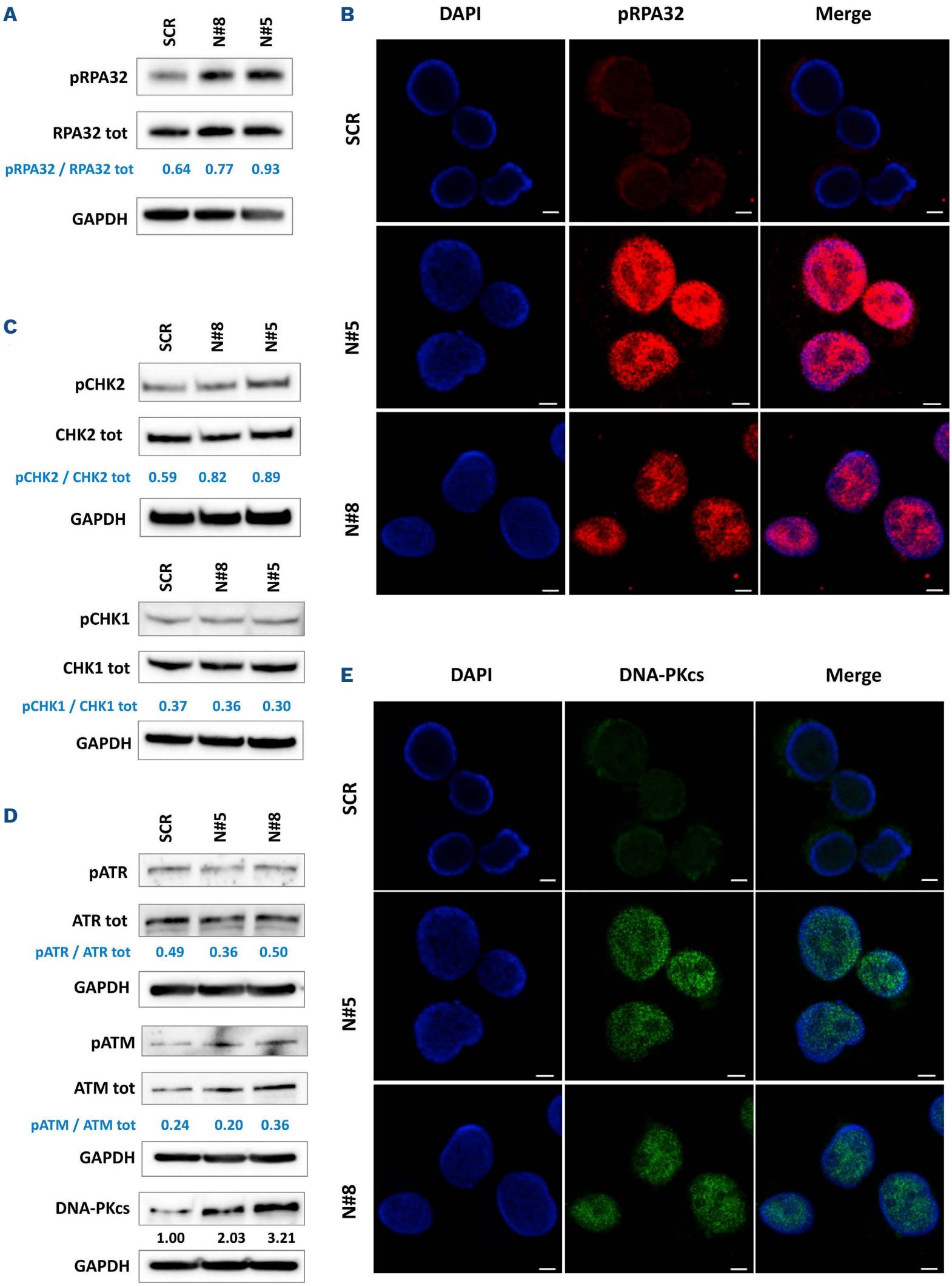

Figure 5. NEAT1 transactivation upregulates proteins involved in the DNA repair process. (A) Western blot (WB) of pRPA32 and RPA32 in AMO-1^{SAM} cells. (B) Confocal microscopy results of pRPA32 specific immunofluorecense (IF) in AMO-1^{SAM} cells cultured under physiological culturing conditions (scale bar 5μm). (C) WB of pCHK2, CHK2, pCHK1, and CHK1 in AMO-1^{SAM} cells. (D) WB of pATR, ATR, pATM, ATM, and DNA-PKcs in AMO-1^{SAM}. The densitometric analysis of DNA-PKcs immunoreactive bands is reported with respect to the scrambled (SCR) condition. Furthermore, the percentage of the activated fraction of all proteins with respect to the relative total (tot) amount (both normalized for GAPDH expression) is reported. (E) Confocal microscopy results of DNA-PKcs specific IF in AMO-1^{SAM} cells cultured under physiological culturing conditions (scale bar 5μm).

model, DNA-PKcs and ATM protein levels increased in FBS-starved cells (*Online Supplementary Figure S9C*). Finally, also FUS protein was found to be overexpressed, likely enhancing PS assembling (*Online Supplementary Figure S9C*).

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

In order to confirm the fundamental role of NEAT1 in the regulation of DNA-PKcs and ATM and the consequent RPA32 activation, we evaluated the expression levels of both kinase proteins in AMO-1 and LP1 cells specifically silenced for NEAT1. As expected, the downregulation of both NEAT1 isoforms, obtained through the gymnotic delivery of the antisense LNA-gapmeR g#N1_E (Figure 7A), resulted in the reduction of the pRPA32 protein levels (Figure 7B). In addition, g#N1_E-silenced cells showed a significant reduction of both DNA-PKcs and ATM protein levels, thus confirming a pivotal role of NEAT1 in the regulation of pRPA32 expression through a mechanism me-

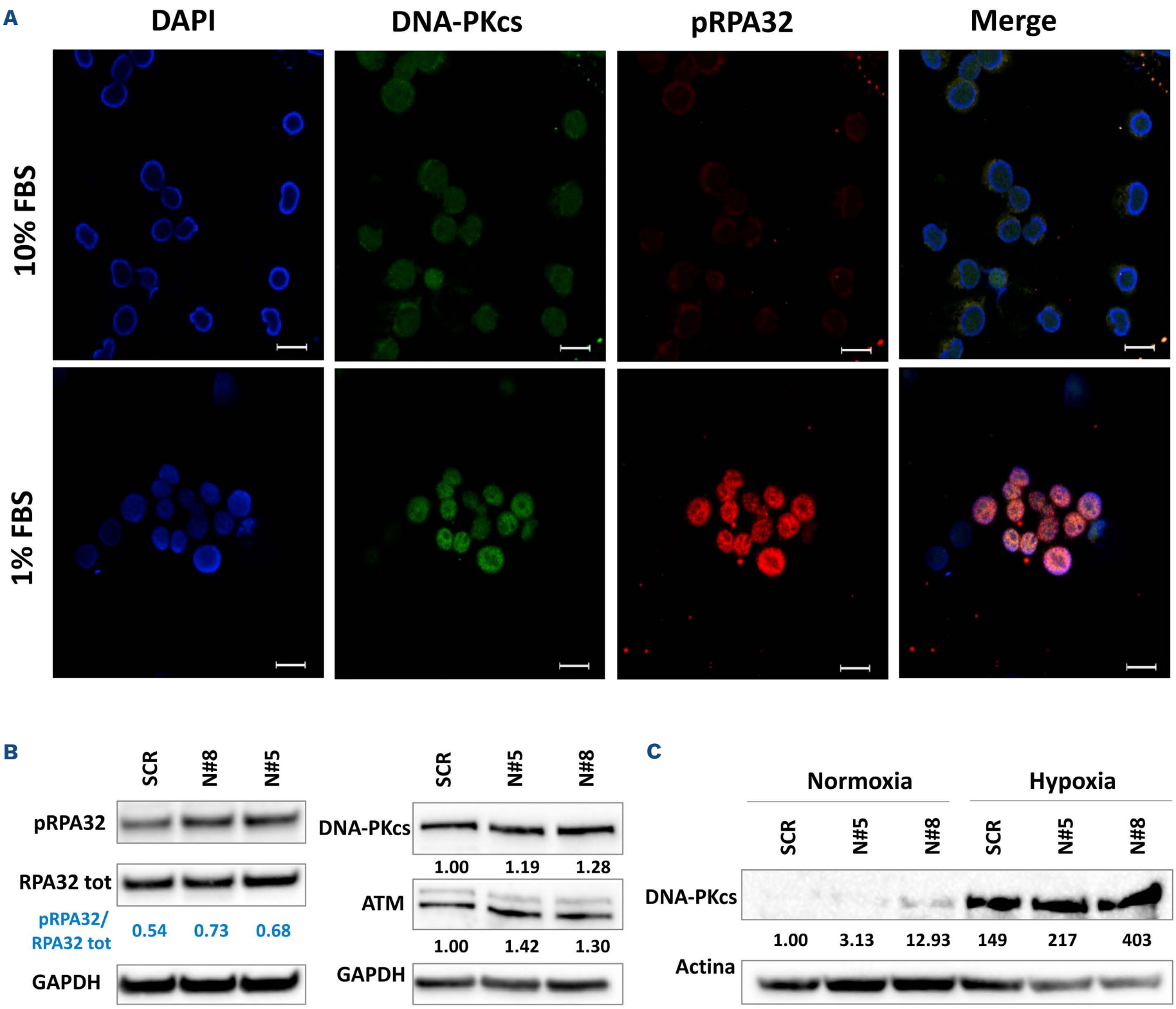

Figure 6. Multiple myeloma cells under stressful conditions overexpress NEAT1 and other proteins involved in the DNA repair process. (A) Confocal microscopy results of DNA-PKcs and pRPA32 specific immunofluorescence (IF) in AMO-1 cells cultured in physiological fetal bovine serum (FBS)-culturing condition (10% FBS) and in FBS-starving medium (scale bar 20µm). (B) Western blot (WB) analysis of pRPA32, RPA32, DNA-PKcs and ATM in AMO-1^{SAM} cells after 48 hours (h) of culture in FBS- starving condition. GAPDH protein expression was included for protein loading normalization. Percentage of pRPA32 with respect to total (tot) RPA32 (both normalized for GAPDH expression) is also shown. (C) WB analysis of DNA-PKcs in AMO-1^{SAM} cells after 48 h of culture in normoxic and hypoxic conditions. Actin protein expression was included for protein loading normalization. The densitometric analysis of immunoreactive bands is reported with respect to the scrambled (SCR) condition.

diated by DNA-PK and ATM (Figure 7B).

Finally, in order to evaluate if the molecular effect of NEAT1 silencing was orchestrated mainly by the short and more abundant isoform of NEAT1 or by the long NEAT1_2 variant, we silenced AMO-1 and LP1 cells with an LNA-gapmeR specific for the NEAT1_2 isoform (g#N1_G). Of note, the silencing of NEAT1_2 was sufficient to determine the downregulation of pRPA32 and of both DNA-PKcs, and ATM (Figure 7B).

The NEAT1/ pRPA32 positive axis is responsible for survival advantages of multiple myeloma cells

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

Hence, we chemically inhibited ATM and DNA-PK activity in AMO-1^{SAM} cells cultured in FBS-starving conditions, and evaluated the biological impact. As expected, in FBS starvation we found that pRPA32 levels in AMO-1$^{N\#8}$ were higher than in AMO-1^{SCR} cells; however, the inhibition of both ATM and DNA-PK kinases led to the impairment of RPA32 phosphorylation in both cell lines (*Online Supplementary Figure S10A*). In line with our hypothesis, the inhibition of both ATM and DNA-PK resulted in a significant reduction of cell viability in AMO-1^{SCR} and AMO-1$^{N\#8}$ starved cells, even more evident upon simultaneous inhibition of ATM and DNA-PK activities (*Online Supplementary Figure S10B*). Furthermore, FBS-starved cells clearly showed alterations of cell membrane integrity and membrane blebbing in AMO-1^{SCR} cells compared to NEAT1-transactivated cells. These characteristics are typical of apoptotic cells, in line with the higher level of mortality (Figure 8B; *Online Supplementary Figure S10C*). Interestingly, the same morphological changes were detectable in FBS-starved AMO-1^{SAM} cells upon the simultaneous inhibition of ATM and DNA-PK (Figure 8A; *Online Supplementary Figure S10C*), in agreement with the significant decrease of cell viability. Moreover, the inhibition of ATM and DNA-PK was associated with a massive cytoplasmic vacuolization, more pronounced upon simultaneous inhibition of both protein kinases, suggesting a suffering cell phenotype (Figure 8A; *Online Supplementary Figure S10C*).

Notably, NEAT1 silencing in the NCI-H929 cell line and in CD138+ purified primary PC also led to cytoplasmic vacuolization, even if to a lesser extent (Figure 8B), suggesting that the molecular and biological effect observed upon NEAT1 silencing could be the result, at least in part, of the reduced activity of the NEAT1/ pRPA32 axis.

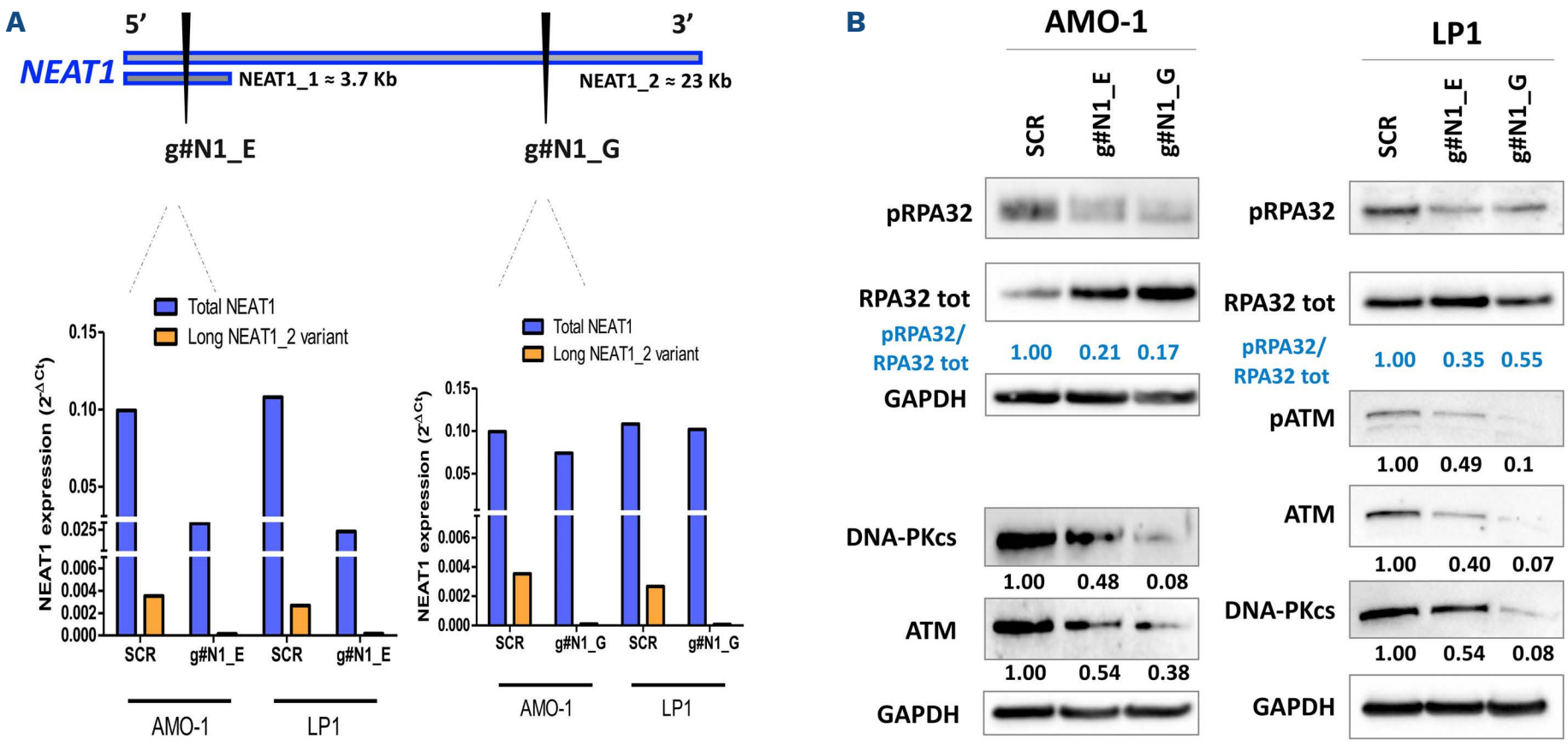

Figure 7. NEAT1 silencing downregulates proteins involved in the DNA repair process. (A) Scheme of LNA-gapmeR localization on NEAT1 transcript; real-time quantitative polymerase chain reaction (qRT-PCR) analyses of NEAT1 expression levels in AMO-1 and LP1 MM cell lines upon gymnotic delivery of g#N1_E or g#N1_G LNA-gapmeR. NEAT1 expression was expressed as $2^{-\Delta Ct}$. (B) Western blot analysis of pRPA32, RPA32, DNA-PKcs, and ATM in AMO-1 and LP1 cells after gymnotic delivery of NEAT1-targeting gapmeR (5 □M). GAPDH protein expression was included for protein loading normalization. The densitometric analysis of DNA-PKcs and ATM immunoreactive bands is reported with respect to the scrambled (SCR) condition. Furthermore, the percentage of pRPA32 with respect to total RPA32 (both normalized for GAPDH expression) is also shown. tot: total.

Discussion

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

PS are dynamic compartments in the nuclear interchromatin space. They respond to different stimuli including DNA damage. These pieces of evidence together with the function of NEAT1 in PS assembly, prompted us to investigate the mechanism underlying the protumoral effect of NEAT1 by studying how NEAT1 overexpression, observed in MM, can influence the activity of PS during DDR.

In order to mimic the effect of NEAT1 overexpression in MM, we took advantage of two NEAT1-transactivated MM clones obtained by using the innovative CRISPR-Cas9 SAM gain of function approach.[18]

First, we demonstrated that the paraphysiological activation of NEAT1 is associated with a significant increase of the levels of essential PSP such as NONO, SFPQ, and FUS. In details, NEAT1 extends the half-life of these three PSP, thereby rising their availability within MM cells. Moreover, confocal microscopy analysis clearly showed that overexpressed NEAT1 and NONO co-localize in PS organelles, whose number significantly augmented in NEAT1-transactivated cells, confirming previous data reporting that NEAT1 transcription is coupled with PS assembling.[24] Importantly, the three PSP are involved in virtually all DNA repair mechanisms (see review[9]). In particular, NONO/SFPQ creates a heterodimer that can associate with the major proteins of the non-homologous end-joining (NHEJ) thus promoting their activity; SFPQ was reported to be essential for HR pathway activation, by promoting the formation of D-loops during the homologous pairing recombination. Moreover, SFPQ can act as both activator and inhibitor of RAD51, exerting a tight regulation of HR pathway. FUS plays a fundamental role in both double-strand breaks (DSB), where its depletion leads to HR and NHEJ impairment, and single-strand brakes (SSB) repair, since it is recruited by PARP1 to promote the recruitment of base excision repair (BER) proteins at the DNA damage site. Overall, this evidence, together with the fact that NEAT1 can be a direct target of the TP53 transcription factor,[15] support the hypothesis of a functional contribution of NEAT1, and likely of PS, in the DDR pathway (Figure 8C).

Since PS are important in the cell response to different stimuli including stressful conditions, such as nutrient starvation and hypoxia, we investigated the role of NEAT1 in stressful culturing conditions. Our investigation established that NEAT1 overexpression is crucial to sustain the growth and the survival potential of MM cells when maintained in non-physiological culturing conditions. Indeed, hypoxia and nutrient starvation further induce NEAT1 expression in AMO-1^{SAM} clones, which positively correlates with an increase of both PS numbers within cells and cell survival. In agreement with our data, NEAT1 upregulation under hypoxic condition has been already reported in hepatocellular carcinoma and non-small cell lung cancer, where NEAT1 represents a target of the HIF-1 or HIF-2 transcription factors, respectively; also in this context, NEAT1 overexpression provides the cell with prosurvival and oncogenic properties.[26,27] In MM cells as well, we found that the transactivation of NEAT1 not only guarantees prosurvival advantages, but also increases their oncogenic potential, as demonstrated by the higher colony-forming potential obtained upon FBS-starving conditions, as well as the more structured colonies organization.

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

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

Based on the functional impact of NEAT1 both in the DDR pathways and in the growth and survival of MM cells when cultured in non-physiological conditions, we hypothesized that NEAT1 transactivation may induce survival advantage in MM cells under stressful conditions by improving DNA repair mechanisms. In agreement with this theory, we demonstrated that, along with the stabilization of NONO, SFPQ, and FUS, NEAT1 transactivation is associated with the overexpression of other two crucial proteins of the DDR system, DNA-PKcs and ATM, thus leading to the activation of their target proteins RPA32 and CHK2 (Figure 5; *Online Supplementary Figure S7*). We validated the positive association between NEAT1 transcription levels and the expression of the DDR proteins in NEAT1-transactivated cells under stressful conditions; in fact, we showed that, similarly to NEAT1 expression levels, also DDR protein levels further increase in non-physiological culture conditions (Figure 6; *Online Supplementary Figure S8*). Finally, transactivated NEAT1 cells in stressful conditions also exhibit significantly greater viability (*Online Supplementary Figure S8*), indicating a prosurvival and pro-oncogenic role of NEAT1-mediated axis in MM.

The molecular circuit driven by NEAT1 and its crucial role for MM cell survival under stressful conditions was further validated by different approaches. First, we demonstrated that the chemical inhibition of ATM and/or DNA-PK in NEAT1-transactivated AMO-1^{SAM} cells under stressful con-

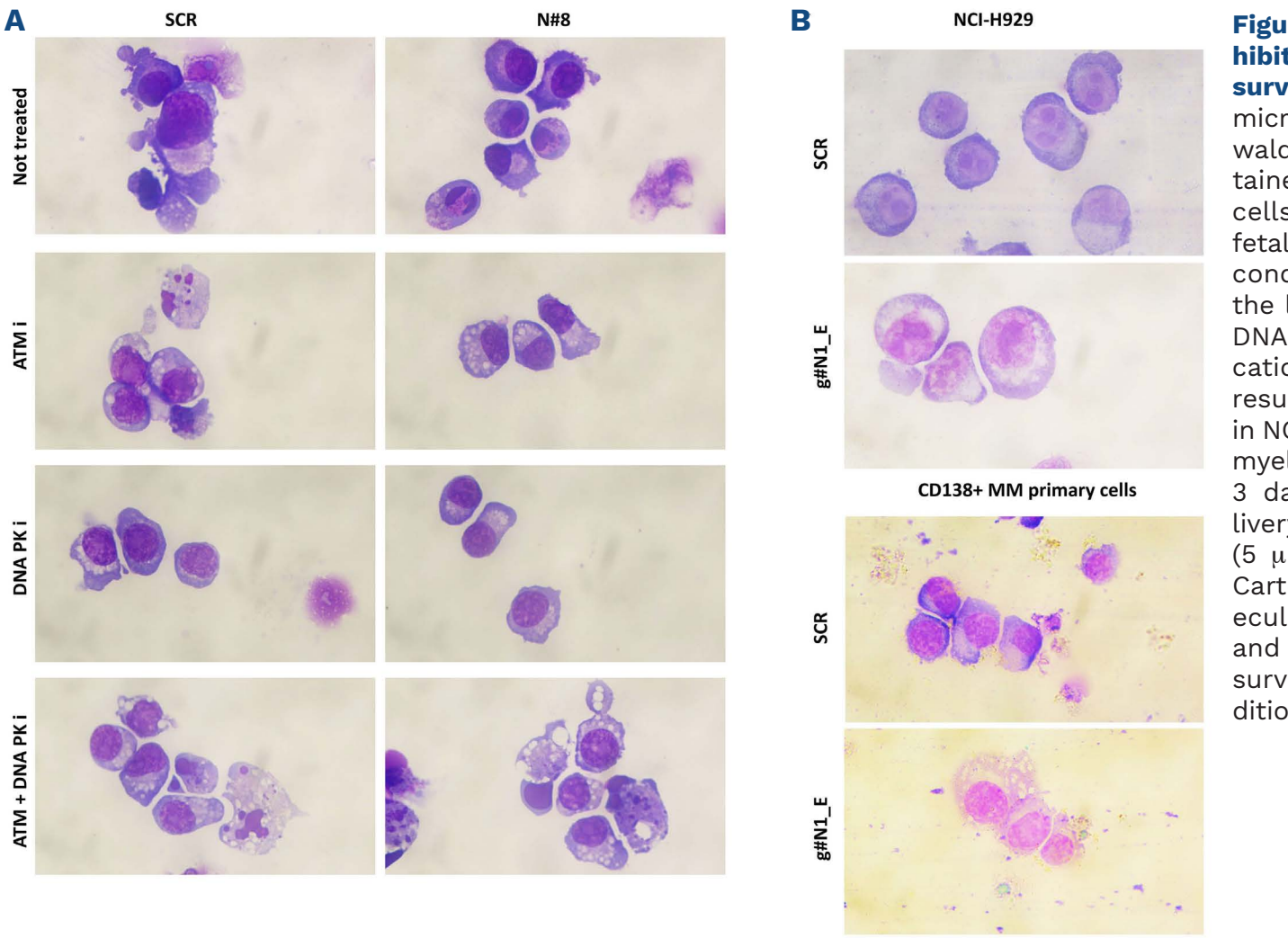

Figure 8. ATM and DNA-PK inhibitions abrogate NEAT1 pro-survival advantages. (A) Optical microscopy results of May Grunwald-Giemsa (MGG) staining obtained in AMO-1^{SCR} and AMO-1$^{N\#8}$ cells after 3 days of culture in fetal bovine serum (FBS)-starving conditions, in the presence for the last 24 hours (h) of ATM and DNA-PK inhibitors (100x magnification). (B) Optical microscopy results of MGG staining obtained in NCI-H929 and CD138+ multiple myeloma (MM) primary cells after 3 days from the gymnotic delivery of NEAT1-targeting gapmeR (5 μM) (100x magnification). (C) Cartoon summarizing the molecular circuit driven by NEAT1 and its crucial role for MM cell survival under stressful conditions. PC: plasma cells.

ditions significantly decreases cell viability and leads to cell morphological changes characterized by a massive cytoplasmic vacuolization (Figure 8; *Online Supplementary Figure S10*). In agreement with our observations, this peculiar phenotype preceding cell death has been reported in osteosarcoma cells, upon ATR inhibition.[27]

In order to validate the obtained results, we verified NEAT1-mediated molecular axis in other HMCL, thus excluding a cell line specific effect. Indeed, serum-starving conditions induce the upregulation of NEAT1, DNA-PK, ATM, and pRPA32 in OPM2, LP1, and MM1.S HMCL (*Online Supplementary Figure S8*). Furthermore, NEAT1 silencing in AMO-1 and LP1 negatively affects the expression levels of both DNA-PKcs and ATM protein kinases, and of the active amount of RPA32 (Figure 7). Of note, NEAT1 silencing in the NCI-H929 cell line and in CD138+ purified primary PC leads to a pattern of cytoplasmic vacuolization similar to the phenotype obtained with the chemical inhibition of

ATM and/or DNA-PK (Figure 8B), suggesting that the molecular and biological effect highlighted upon NEAT1 silencing could be the result, at least in part, of the reduced activity of DNA-PK and ATM on pRPA32.

Taken together, our data indicate that the upregulation of ATM, DNA-PKcs, and pRPA32 could be considered a general molecular response of MM cells to NEAT1 induction, which, in turn, could represent a survival advantage for MM cell facing adverse conditions (Figure 8C).

Interestingly, our results point out an important role for the long NEAT1_2 variant under stressful conditions; in fact, both in AMO-1^{SAM} clones and in all HMCL tested, FBS starvation or hypoxia significantly increase the NEAT1_2 percentage with respect to total NEAT1 expression. This finding suggests that non-physiological conditions may shift NEAT1 transcription towards the long NEAT1_2 isoform, likely to assemble the PS needed to counteract the stressful conditions. In accordance with this evidence, the specific targeting of the long NEAT1_2 variant negatively affects the expression levels of both DNA-PKcs and ATM protein kinases, and of the active amount of RPA32, suggesting that the structural scaffold of PS, and maybe PS themselves, should be considered master regulators of DNA damage response in MM cells.

In conclusion, our study attributes a crucial role to NEAT1 overexpression in MM; pathological NEAT1 expression and the consequent deregulation of PS organelles availability, could represent an Achilles' heel for MM PC survival. Importantly, our data suggest that NEAT1 overexpression could be considered a generalized rescue mechanism for MM plasma cells under stressful conditions and strongly suggest that NEAT1 and PS targeting could be considered a novel promising strategy for innovative anti-MM therapies for all subsets of MM patients.

Disclosures

No conflicts of interest to disclose.

Contributions

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

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Data-sharing statement

The materials used and analyzed during the current study are available from the corresponding author on reasonable request.

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