A PERK-miR-211 axis suppresses circadian regulators and protein synthesis to promote cancer cell survival

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The unfolded protein response (UPR) is a stress-activated signalling pathway that regulates cell proliferation, metabolism and survival. The circadian clock coordinates metabolism and signal transduction with light/dark cycles. We explore how UPR signalling interfaces with the circadian clock. UPR activation induces a 10 h phase shift in circadian oscillations through induction of miR-211, a PERK-inducible microRNA that transiently suppresses both Bmal1 and Clock, core circadian regulators. Molecular investigation reveals that miR-211 directly regulates Bmal1 and Clock via distinct mechanisms. Suppression of Bmal1 and Clock has the anticipated impact on expression of select circadian genes, but we also find that repression of Bmal1 is essential for UPR-dependent inhibition of protein synthesis and cell adaptation to stresses that disrupt endoplasmic reticulum homeostasis. Our data demonstrate that c-Myc-dependent activation of the UPR inhibits Bmal1 in Burkitt’s lymphoma, thereby suppressing both circadian oscillation and ongoing protein synthesis to facilitate tumour progression.

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To assess UPR–circadian clock cross-talk in vivo, livers were collected from eight-week-old wild-type (PERK loxp/loxp) or knockout (PERK Δ/Δ) mice treated as above following 1 μM GSK2606414 pre-treatment for 1 h. Data are representative of n = 3 biologically independent experiments. Cell lysates from wild-type or IRE-1 knockout (IRE-1−/−) MEFs were treated with 0.5 mM Tg at the indicated hours were subjected to western analysis with the indicated antibodies. Data are representative of n as indicated. Representative western blots are provided from 3 biologically independent experiments. Source data are provided in Supplementary Table 3.

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Treatment of PERK Δ/Δ mice generated discoloured livers (Supplementary Fig. 1b), reflective of extensive hepatonecrosis (Supplementary Fig. 1c). Consistent with PERK mediating stress-dependent survival, PERK Δ/Δ mice had to be euthanized within 2 days of Tg treatment, compared to 6 days for PERK loxp/loxp mice.
PERK-inducible miR-211 suppresses Bmal1 and Clock. The kinetics of UPR-dependent Bmal1/Clock suppression is analogous to the induction of PERK-induced miR-211 (ref. 22) (Fig. 3a,b), suggesting miR-211 as a plausible link. Consistent with a regulatory interaction, a miR-211 inhibitor (A211) abolished UPR-dependent Bmal1 and Clock suppression (Fig. 3c) and restored their circadian oscillation (Fig. 3d). We also noted strong induction of miR-211 in mouse livers during entrainment to new light/dark cycles (Fig. 3c). Dicer−/− cells were refractory to ER stress-dependent repression of Bmal1 and Clock (Fig. 3e), supporting a role for miRNA-dependent regulation. The induction of miR-211 during entrainment to a new light/dark cycle correlated with increased PERK activity and with reduced CHOP expression, as might be expected from previous work; miR-211 induction also correlated with diminished Bmal1 and Clock expression.

To address the mechanism of miR-211 action, we performed a bioinformatic analysis for potential miR-211 seed matches and noted one for miR-211 in the Clock 3′ untranslated region (UTR) (Supplementary Fig. 2). A luciferase reporter was generated harbouring either the wild-type 3′UTR of Clock or one with mutated seed sequences. Expression of the Luciferase–Clock reporter was suppressed by miR-211 in a seed-dependent manner (Fig. 3f). Additionally, wild-type, but not the mutant Luciferase–Clock reporter, was responsive to ER stress, while A211 abrogated UPR-dependence (Fig. 3g).

No miR-211 seed sequences were identified in the 3′UTR of Bmal1; however, three high-relevance matches are present in the proximal promoter/5′UTR region (Supplementary Fig. 3a), suggesting the potential for miR-211 to regulate Bmal1 via RNA-induced transcriptional silencing (RITS)25. RITS, unlike canonical cytoplasmic RISC, is a nuclear micro-RNA complex that recruits EZH2 to target promoters through rare RNA transcripts with extended 5′ sequences; miRNA-dependent engagement of these transcripts promotes the generation of heterochromatic marks, facilitating suppression of transcription (Supplementary Fig. 3b). To assess this model of regulation, a Bmal1–luciferase reporter with either a wild-type Bmal1 promoter or one containing mutations in miR-211 seed sites was engineered. ER stress reduced expression of the wild-type promoter but not mutations in all potential miR-211 seed sequences (Fig. 4a). Mutation of individual sites reduced stress-dependent silencing, but to a lesser degree (Fig. 4a). Ectopic expression of miR-211 repressed wild-type but not mutant Bmal1–luciferase, consistent with direct regulation by miR-211 (Fig. 4b).

To address miR-211 regulation of endogenous Bmal1, we introduced biotinylated wild-type or mutant miR-211 (ref. 22) into cells. Nuclear extracts were subjected to streptavidin pulldown, and Bmal1 nascent mRNA was quantified by qPCR. Wild-type but not mutant miR-211 was enriched for Bmal1 nascent RNA with a preference for Seed1 and Seed2 (Fig. 4c–e), demonstrating the direct interaction between miR-211 and the Bmal1 promoter. Equivalent concentrations of wild-type and mutant miR-211 were present on beads (Supplementary Fig. 3c). Binding specificity was confirmed by assessing interactions with the neighbouring gene PTH (Supplementary Fig. 3d) and sequences 1,000 bp upstream from Seed3 sequences (Supplementary Fig. 3e). Consistent with current models of RITS, chromatin immunoprecipitation (ChIP) analysis revealed miR-211-dependent recruitment of Argonaut to Bmal1 (Fig. 3f). Enhanced H3K27me3 methylation (Fig. 4g), but no H3K9me2 methylation (Supplementary Fig. 3f), was observed. In addition, RNA polymerase II occupancy was reduced by ER stress and miR-211 induction (Fig. 4h). Collectively, these data suggest that the UPR modulates circadian oscillation via regulation of Clock 3′UTR and Bmal1 transcription through PERK-dependent induction of miR-211.

MYC suppresses Bmal1 and Clock through engagement of the UPR and miR-211. Burkitt's lymphoma is associated with chromosomal translocation 14q32, resulting in c-Myc over-expression. High c-Myc triggers increased protein synthesis26,27, robust UPR and accumulation of miR-211 (ref. 22). Because c-Myc potently triggers PERK-dependent miR-211 accumulation and can also intersect with circadian machinery28, we assessed the impact of c-Myc and oncogenic ER stress on Bmal1 and Clock. Comparison of normal human germinal centre B (GC B) cells isolated from human tonsils with Burkitt's lymphoma cell lines Raji, Ramos and CA46 revealed high levels of active PERK and undetectable Bmal1 and Clock expression in lymphoma cells (Fig. 5a). Reduced Bmal1/Clock was reflective of increased miR-211 expression (Fig. 5b). Downstream circadian targets of Bmal1/Clock (Per1, Per2, Cry1 and Cry2) were also reduced (Supplementary Fig. 4). Expression of A211 in lymphoma cells lines restored expression of Bmal1 and Clock (Fig. 5c,d), as did treatment of cells with a PERK small-molecule inhibitor GS2606414 (Fig. 5e,f). A211 expression or PERK inhibition also restored circadian oscillation of Bmal1 and Clock in lymphoma cells (Fig. 5g–j). Metabolic genes, including NAMPT (ref. 29), ODC1 (ref. 30) and UPP2, exhibit BMAL1-dependent circadian oscillations31. The rescue of Bmal1/Clock by A211 or through PERK inhibitor increased expression of these metabolic genes (Fig. 5k,l). These data collectively provide support for the role of PERK–miR-211 in mediating the impact of Myc and the UPR on circadian oscillations through regulation of Bmal1 and Clock.

To further address the interconnection of miR-211, circadian gene expression and oncogenic stress, we analysed murine lymphomas driven by c-Myc32. Reduced expression of Bmal1 and Clock was noted in all tumours (Fig. 6a and Supplementary Fig. 5); this correlated with PERK-dependent phosphorylation of eIF2α (Fig. 6a) and
Fig. 2 | Light/dark reversal triggers the UPR. a, UPR components are expressed in a circadian oscillating manner in mouse livers. Data are presented as mean ± s.d. for n = 5 mice in each group. b, c, Eight-week-old male wild-type C57BL/6 mice were randomly divided into two groups. Control mice followed the regular 12:12 h light/dark cycles and had free access to food (control group). Darkness groups were placed in darkness for 48 h. Mouse livers were collected every 6 h for 36 h. The corresponding Zeitgeber hours (ZT hours) are indicated at the bottom. Light and dark bars represent light and dark, respectively. Protein was isolated for western blot (b). The image is representative for n = 3 mice per group. RNA was isolated for qPCR in c. Data are presented as mean ± s.d. from n = 3 mice per time point per group. d, e, Wild-type C57BL/6 mice were randomly divided into two groups. Control mice followed the regular 12:12 h light/dark cycles (control group). In light/dark reversed mice (DL reversed), light/dark cycles were shifted by 12 h. Mouse livers were collected every 6 h. 0 h indicates 6 h after the initial light/dark shift. Protein was isolated for western blot (d). RNA was isolated for qPCR (e). Data are presented as mean ± s.d. for n = 3 mice per time point per group. Source data are provided in Supplementary Table 3.
**Fig. 3 | miR-211 directly regulates Clock.**

**a.** miR-211 accumulation in U2OS cells treated with 500 nM Tg. Data are presented as mean ± s.d. for n = 3 biologically independent experiments, comparing PERK+/+ and PERK−/− at the same time point (two-tailed Student’s t-test). **b.** miR-211 was assessed by qPCR for n = 5 mice at each time point per group; liver samples are from Fig. 1d. **c.** Cell lysates prepared from U2OS cells transfected with scrambled microRNA or A211 for 48 h and treated with Tg for 5 h were analysed by immunoblot. Blots are representative of n = 3 biologically independent experiments. **d.** U2OS cells transfected with scrambled control or A211 were synchronized with 0.1 μM dexamethasone and treated with or without 500 nM Tg. RNA was collected every 4 h for 48 h for qPCR. Data are presented as mean ± s.d. for n = 3 biologically independent experiments. **e.** Wild-type and Dicer−/− MEFs were treated with 500 nM Tg for the indicated hours. Cell lysates were collected for western blot analysis. Blots are representative of n = 3 biologically independent experiments. **f.** U2OS cells were transfected with luciferase–Clock 3′UTR wild-type or miR-211 site mutant reporter constructs along with Renilla–Luc as internal control. Luciferase activity was measured 48 h post transfection. Data are presented as mean ± s.d. for n = 3 biologically independent experiments (two-tailed Student’s t-test). *P = 0.002: control mimic versus miR-211 mimic in Clock 3′UTR wild-type constructs. **g.** Luciferase–Clock 3′UTR wild-type or mutant were introduced into U2OS cells along with scrambled microRNA or A211. Cells were challenged with Tg for 5 h and luciferase activity was measured and normalized to Renilla. Data are presented as mean ± s.d. for n = 3 biologically independent experiments (two-tailed Student’s t-test). Source data are provided in Supplementary Table 3.
miR-211 accumulation (Fig. 6b). We subsequently turned to human Burkitt's lymphoma, a tumour in which Bmal1 expression is significantly reduced\(^1\) (Fig. 6c). We assessed miR-211 versus Bmal1/Clock expression in five available cases of human primary lymphoma with a documented c-Myc translocation\(^1\). Bmal1 expression was reduced relative to normal, and this correlated negatively with miR-211 (miR-211 expression previously reported in ref. \(^2\)) in these tumours (Fig. 6d)\(^1\). To address whether the miR-211/Bmal1/Clock relationship is lymphoma-specific, we turned to a model of Her2/Neu-driven mammary carcinoma. miR-211 expression in murine MMTV-Neu tumours is dependent on the PERK\(^2\). As with lymphoma, Bmal and Clock expression inversely correlates with miR-211 (Fig. 6e).

**Bmal1 repression contributes to tumour cell survival.** The inhibition of Bmal1/Clock by the UPR implies that reduction and the
Fig. 5 | Antagonizing miR-211 restores circadian oscillation. a,b. Representative western blot images (a) and qPCR (b) of GCB cells (GCB: Raji, Ramos and CA46). Data are presented as mean ± s.d. for n = 3 biologically independent measurements. Statistical analysis was performed by two-tailed Student’s t-test, comparing to GCB cells. c,d. Lysates from Raji (c) and CA46 (d) cells transfected with scrambled microRNA or A211 were analysed by western blot. Blots are representative of n = 3 biologically independent experiments. e,f. Raji (e) and CA46 (f) cells were treated with the PERK inhibitor (PERKi; GSK2606414) and subjected to western blot or qPCR. Blots are representative of n = 3 biologically independent experiments. qPCR data are presented as mean ± s.d. for n = 3 biologically independent measurements. Significance was determined by two-tailed Student’s t-test (95% confidence interval).

g,h. Raji (g) and CA46 (h) cells were transfected with scrambled or A211, synchronized by serum shock and then collected at 4 h intervals. Bmal1 and Clock mRNAs were assessed by qPCR. Data are presented as mean ± s.d. for n = 3 biologically independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 compared to scramble groups at each time point. Significance was determined by two-tailed Student’s t-test (95% confidence interval). i,j. Raji (i) and CA46 (j) cells were treated with 1μM PERKi (GSK2606414) and cells were collected for qPCR. Data are presented as mean ± s.d. for n = 3 biologically independent experiments. Statistical analysis was calculated by two-tailed Student’s t-test. ***P < 0.001, **P < 0.01. Source data and precise P values are provided in Supplementary Table 3. k,l. PERK inhibition (PERKi) or anti-miR-211 (A211) restores circadian and metabolic gene expression in Burkitt’s lymphoma cell lines. Data are presented as mean ± s.d. for n = 3 biologically independent experiments. Statistical analysis was calculated by two-tailed Student’s t-test. ***P < 0.001, *P < 0.05. Source data and precise P values are provided in Supplementary Table 3.

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Fig. 6 | Bmal1 is lost in Myc-driven tumours. a, b, Lymphomas21 generated by c-Myc expression were analysed by western blot (a) and qPCR (b). Representative blots are taken from n = 3 biological independent measures. P = 0.03 for Myc-driven tumour compared to normal samples (95% confidence interval). Statistical analysis was performed by a two-sided two-sample t-test. c, Bmal1 mRNA levels are exclusively low in Burkitt’s lymphoma. Data and statistical methods are available from www.Oncomine.org (reporter ID: 36896_s_at)10. Individual plots for each sample are provided in Supplementary Table 3. The GEO accession is GSE2350. 0: control (n = 41); 1: Burkitt’s lymphoma (n = 127); 2: centroblastic lymphoma (n = 12); 3: chronic lymphocytic leukaemia (n = 34); 4: diffuse large B-cell lymphoma (n = 41); 5: embryonal rhabdomyosarcoma (n = 11); 6: follicular lymphoma (n = 6); 7: hairy cell leukaemia (n = 16); 8: Hodgkin’s lymphoma (n = 4); 9: mantle cell lymphoma (n = 8); 10: multiple myeloma (n = 1); 11: plasma cell leukaemia (n = 3); 12: primary effusion lymphoma (n = 9). *P < 0.05 compared to the control samples. d, qPCR analysis of primary human B lymphocytes isolated from normal donors (n = 2) and lymphomas from patients with confirmed translocation of c-Myc (n = 5). miR-211 levels in each sample have been reported previously10. Data are presented as mean ± s.d. P value compares Tumour versus Normal by two-tailed Student’s t-test (95% confidence interval). The correlation between Bmal1 and miR-211 rho = −0.29, P = 0.56. The correlation between Clock and miR-211 rho = −0.53, P = 0.22, calculated by Spearman’s correlation using R. e, miR-211 induction and circadian gene expression are dependent on PERK in mouse MMTV-neu driven breast cancer mouse model. MMTV-neu PERK+/– and MMTV-neu PERK−/− tumours were collected and analysed by qPCR for the circadian gene mRNA and miR-211. P values indicate gene level comparing PERK+/– and PERK−/− by two-sample t-test. Spearman correlation between miR-211 and Bmal1: Rho = −0.75, P = 0.0001; Spearman’s correlation between miR-211 and Clock in those tumours: Rho = −0.59, P = 0.006.
resulting loss of Bmal1 and/or Clock regulatory functions are important for cell viability and ultimately for tumour cell survival. We focused on Bmal1 under ER stress, because the level of Bmal1 repression in tumours was more striking than that of Clock. We addressed Bmal1 contributions to survival through annexin V staining (acute stress) or clonogenic assay (transient stress and long-term survival) in U2OS cells where Bmal1 expression is driven by a heterologous promoter. Enforced Bmal1 expression conferred high sensitivity to ER stress (Fig. 7a and Supplementary Fig. 6a). Importantly, enforced Bmal1 expression also suppressed anchorage-independent growth of Burkitt’s lymphoma cells (Supplementary Fig. 6b,c), conditions that induce ER stress44. In vivo, Bmal1 overexpression or A211 significantly suppressed CA46 xenograft tumour growth (Fig. 7b,c and Supplementary Fig. 6d,e). Tumours overexpressing wild-type Bmal1 or A211 exhibited high rates of apoptosis and decreased proliferative indices (Fig. 7d,e and Supplementary Fig. 6f). We also examined additional human cancer aetiologies by mining online databases to assess the impact of Bmal1 expression on tumour progression. Critically, breast, lung and gastric cancer patients who have higher Bmal1 overexpression showed better overall survival (Fig. 7f)35.

Fig. 7 | Bmal1 loss is critical for cell survival following ER stress. a, Parental U2OS–vector or U2OS–Bmal1 cells were treated with 500 nM Tg for 72 h, and apoptosis was quantified by annexin V. Representative images from n = 3 biologically independent experiments and FACS analysis (top) are shown. Data in bar charts represent mean ± s.d. for n = 3 biologically independent experiments (lower panel) (two-tailed Student’s t-test). b, CA46 cells were infected with vector, Bmal1 wild-type, Bmal1 S42G or A211 and transplanted into female SCID mice. Data are presented as mean ± s.d. for n = 10 tumours in each group. Tumour size was recorded every other day. *P < 0.05 for Bmal1 versus vector, **P < 0.005 for Bmal1 versus Bmal1 S42G, #P < 0.05 for A211 versus vector. Statistical analysis was performed by two-tailed Student’s t-test. Source data and precise P values are provided in Supplementary Table 3. c, Representative tumour images from each group. d,e, Quantification of immunohistological analysis of the tumour sections: Ki-67 (d) and TUNEL assay (e). Quantification was made for n = 3 different tumour slides (two-tailed Student’s t-test). NS, not significant. f, High Bmal1 expression confers better survival on breast cancer, lung cancer and gastric cancer patients. Kaplan–Meier plots assess the impact of Bmal1 expression on survival of breast, lung and gastric cancer patients. Log-rank P values and hazard ratios (HRs; 95% confidence interval in parentheses) are shown at the bottom. Plots were generated by the online tool in kmplot.com. Source data are provided in Supplementary Table 3.
Fig. 8 | Bmal1 loss is critical for PERK-dependent inhibition of protein synthesis. a, $^{35}$S incorporation in U2OS–vector or U2OS–Bmal1 cells following Tg. Autoradiograms were normalized to total protein. Representative images were from n = 3 independent experiments and are provided. Bar chart data are presented as mean ± s.d. for n = 3 independent experiments. Statistical analysis was performed by two-tailed Student’s t-test (95% confidence interval). b, Lysates from U2OS–vector or U2OS–Bmal1 cells treated with Tg. Western blots are representative of n = 3 independent experiments. c, Lysates from U2OS–vector or U2OS–Bmal1 cells treated with Tg were subjected to m$^2$GTP pulldown. V = vector only; B = Bmal1 overexpressing cells. Representative blots are provided for n = 3 independent experiments. d, $^{35}$S incorporation in control or U2OS–mir-211 cells as indicated. Autoradiograms were normalized to total protein. Representative images are provided for n = 3 biologically independent experiments. Bar chart data are provided as mean ± s.d. for n = 3 independently performed experiments, and statistical analysis was performed by two-tailed Student’s t-test (95% confidence interval). e, U2OS vector or U2OS Bmal1 cells were treated with Tg (300 nM) alone or Tg + cycloheximide for 3 h, re-fed with fresh medium, and colonies were quantified at 2 weeks. The image is representative of three independent experiments (upper panel). Values are presented as mean ± s.d. from n = 3 independent experiments (lower panel). Statistical analysis was performed by two-tailed Student’s t-test, comparing vector and Bmal1 cells in each group. NS, not significant. f, Bmal1 was knocked down, cells were treated with Tg, and apoptosis was quantified. Data are presented as mean ± s.d. for n = 3 independent observations. g, U2OS cells expressing vector, Bmal1, Clock, Bmal1 + Clock, Bmal1S42G or Bmal1ΔHLH were treated with Tg for 0 or 24 h. Apoptosis was assessed by annexin V staining (top). Bmal1 and Clock expression was assessed by immunoblot (bottom). Representative data are for n = 3 independent experiments. h, Cells were treated with vehicle or 4-PBA (5 mM) for 24 h, stained with annexin V, and apoptosis quantified by FACS (top, representative of three independent experiments; lower panel, mean ± s.d. from three independent experiments). P values indicate the comparison of vehicle and 4-PBA treated cells (95% confidence interval). Statistical analysis was performed by two-tailed Student’s t-test. Source data are provided in Supplementary Table 3.
We next determined whether N-myc, like C-Myc, regulates Bmal1 through the use of N-myc amplified neuroblastoma cell lines (Kelly and NLF). SHEP and SKNAS do not contain N-myc amplicons and served as controls34. Kelly and NLF have significantly higher miR-211 expression, p-eIF2α activation and correspondingly low Bmal1 expression (Supplementary Fig. 7a). Use of a small-molecule bromodomain inhibitor JQ1 (ref. 35) to suppress myc transcription reduced p-eIF2α and miR-211 and increased Bmal1 expression (Supplementary Fig. 7b). In contrast, increasing N-myc expression in SHEP and SKNAS cells induced miR-211, with a concomitant decrease in Bmal1 (Supplementary Fig. 7c), demonstrating that N-myc also regulates the miR-211/Bmal1 axis. In colon cancer cell line HCT116, cervical cancer cell line HeLa and breast cancer cell line MCF7, we observed that myc knockdown decreased miR-211 levels and rescued Bmal1 expression, while no rescue was observed in H1299 (Supplementary Fig. 7d).

**Bmal1 loss is necessary for UPR-dependent repression of protein synthesis.** In addition to transcription, Bmal1 also regulates translation initiation through direct association with eIF4F (ref. 17). Because PERK plays an essential role in reducing protein overload during ER stress and myc-driven lymphoma3 through inhibition of protein synthesis, we investigated whether PERK–miR-211–triggered Bmal1 loss during UPR contributes to global protein translation inhibition. Pulse-label analysis of U2OS cells in which Bmal1 expression was enforced demonstrated Bmal1 overrides UPR-dependent protein translation inhibition in response to ER stress (Fig. 8a). Cells that retained Bmal1 did not exhibit significant alterations in S6 ribosomal protein phosphorylation, but maintained hyperphosphorylated 4E-BP1 relative to control cells (Fig. 8b). Consistently, although eIF4E and eIF4A binding to the m7GTP cap was reduced in control cells, enforced Bmal1 expression resulted in modestly increased binding (Fig. 8c). Because miR-211 targets Bmal1, we addressed the role of miR-211 in regulating protein translation regulation under ER stress. Expression of A211, which prevents Bmal1 loss (Fig. 3c), prevented UPR-dependent protein translation inhibition (Fig. 8d). PERK signalling regulates cell viability under ER stress via modulation of protein translation14. We determined whether increased sensitivity to ER stress is a reflection of Bmal1-dependent protein synthesis. Transient exposure to Tg in the presence of cycloheximide (CHX) reduced cell death in U2OS Bmal1 cells (Fig. 8e). Previous work demonstrated increased sensitivity of PERK−/− cells to ER stress due to the lacking of protein translational control via the p-eIF2α-ATF4 axis37. Bmal1 knockdown abrogated ER stress toxicity of PERK−/− cells, demonstrating the importance of PERK/miR211 suppression (Fig. 8f and Supplementary Fig. 7e).

Because Bmal1 regulates gene transcription and protein synthesis (Fig. 8a), we addressed whether Bmal1–dependent regulation of gene transcription or its impact on protein translation drives differential cell survival under ER stress. We introduced Bmal1S42G (defect in protein translation control38) or Bmal1ΔHIL1 (impaired DNA binding) into U2OS cells. Annexin V staining revealed that only Bmal1 S42G reduced the ER stress sensitivity (Fig. 8g), consistent with Bmal1-mediated protein translation regulation contributing to cell survival under conditions of ER stress. We also noted that the impact of Bmal1S42G expression on growth of CA46 xenografts was much less than wild-type Bmal1 expression (Fig. 7b). The capacity of 4-phenylbutyrate (4-PBA) treatment to reduce apoptosis in CA46 cells engineered to express Bmal1 is consistent with the model wherein enforced Bmal1 expression triggers ER stress (Fig. 8h).

**Discussion**

The UPR was initially identified and characterized as a response to pharmaceutical challenges that perturb the oxidative, pro-folding environment of the ER. Identification of a signalling pathway that coordinates the cellular response to such a challenge has facilitated the investigation of the physiological challenges that engage the UPR, challenges that include low oxygen, glucose restriction, metabolic stress and alterations in protein synthesis rates1. Although our understanding of how the UPR regulates cell fate has improved, there remains a gap in our understanding regarding the cross-talk between the UPR and distinct signalling pathways that respond to or regulate common pathways.

There is a growing appreciation of the capacity of the UPR and circadian machinery to contribute to cell growth and metabolism. Yet, the notion of a coordinated impact through UPR and circadian cross-talk has not been evaluated. This work demonstrates that the UPR directly regulates the core circadian clock. UPR activation in a cell culture triggers an 8–10 h shift in circadian phase oscillation, and this shift is exquisitely dependent upon PERK–eIF2α-ATF4 signalling. Mechanistically, the ATF4-inducible micro-RNA, miR-211, directly suppresses both Bmal1 and Clock. Because miR-211 is a labile miRNA under normal conditions27, Bmal1 and Clock suppression is transient. Cross-talk is not limited to cell culture, as activation of the UPR in mice by pharmacological treatment with tunicamycin also triggers PERK-dependent shifts in circadian oscillation. Importantly, we also note that alterations in the normal light/dark cycle trigger UPR activation, hinting that the UPR may contribute to clock re-entrainment.

The transient loss of Bmal1 has a direct impact on downstream circadian and metabolic gene expression, but also has a direct impact on protein translation. This unanticipated role for Bmal1 in the regulation of protein synthesis has a profound effect on cell survival following pharmacological ER stress and in tumour cells where Bmal1 is silenced as a consequence of PERK signalling.

The initial delay in circadian transcript oscillation will undoubtedly contribute to ultimate tumour progression by virtue of alterations in the coordination of metabolic gene expression. However, the necessity of Bmal1 suppression to reduce protein synthesis will have a more immediate impact. Targeted deletion of PERK in c-Myc-driven lymphoma results in ER protein overload and tumour cell death11. The results described herein suggest that PERK–miR-211-dependent suppression of Bmal1 is an essential aspect of this pathway necessary for progression of c-Myc-positive lymphoma. As tumours continue to progress and experience ongoing metabolic challenge, alterations in circadian gene expression and metabolic regulation will probably have a more direct contribution to tumour cell survival. Ultimately, the repression of Bmal1 by PERK-induced miR-211 has the remarkable capacity to robustly contribute to cell survival and tumour progression by limiting protein overload.

**Methods**

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41556-017-0006-y.

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**References**

1. Dhih, J. A., Fuchs, S. Y. & Koumenis, C. The cell biology of the unfolded protein response. *Gastroenterology* **141**, 38–41.e2 (2011).

2. Tiraparaphorn, W., Welhinda, A. A. & Kaufman, R. J. A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endoribonuclease (Ire1p) in mammalian cells. *Genes Dev.* **12**, 1812–1824 (1998).

3. Yoshida, H., Haze, K., Yanagi, H., Yura, T. & Mori, K. Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. *Involvement of basic leucine zipper transcription factors. J. Biol. Chem.* **273**, 33741–33749 (1998).

4. Lee, A. H., Iwakoshi, N. N. & Glimcher, L. H. XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. *Mol. Cell Biol.* **23**, 7448–7459 (2003).
Methods

Cell culture. Human osteosarcoma U2OS cells (ATCC HTB-96) were purchased in 2014 from the American Type Culture Collection (ATCC) and maintained in McCoy’s 5a medium (Mediatech) supplemented with 10% FBS (BenchMark) and 1% penicillin-streptomycin (293T cells (ATCC CRL-3216) were purchased from ATCC in 2014 and were maintained in DMEM medium (Mediatech) with 10% FBS. Burkitt’s lymphoma cell lines CAC6 (ATCC CRL-1648), Raji (ATCC CCL-86) and Ramos (ATCC CRL-1596) were purchased from ATCC in 2015 and maintained in RPMI-1640 medium (Mediatech) with 10% FBS and 1% penicillin-streptomycin (Gibco). Wild-type and PERK-Knockout (KO), IRE-1-knockout (KO) mouse embryo fibroblasts (MEFs) were maintained in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, 2 mM glutamine (Gibco), 55 μM β-mercaptoethanol (Gibco) and MEM non-essential amino acids (Gibco). U2OS Bmal1−/−:Luc cells were provided by J. Hegnesch (University of Cincinnati). SKNAS-NmycER cells were provided by L.J. Velt欣n, University of Amsterdam, Netherlands. In SKNAS-NmycER (ref. 19) and SHEP-NmycER cells39, N-myc transcript efficiency. miRNA was isolated using the mirEasy mini kit (Qiagen), amplified, and reverse transcribed using the Taqman reverse transcription kit (Applied Biosystems). U6 mRNA and snoRNA202 served as internal controls for human and murine samples, respectively.

Animal husbandry. All animal experiments were conducted within the guidelines with the Animal Care and Use Committee of Medical University of South Carolina. For circadian experiments, PERKΔm and PERK−/− mice were generated as previously described. The PERKΔm genotype was confirmed by PCR using the following primers: forward 5′-CACCTCTGCGTTCTACCTCCACAG-3′ and reverse 5′-GCTTCTAACAAAAAGGAGAGTGGAA. The CreERT2 genotype was confirmed by PCR using the following primers: forward 5′-TACACCAGAATTGTGCTGATCCGG-3′ and reverse 5′-TTTCCAAAGATGGAACGACTGCTGT-3′. PERKΔm mice were generated by intercrossing mice with PERKΔm mice23. For PERK deletion, tamoxifen (T5648, Sigma-Aldrich) was administered via oral gavage at a dose of 0.2 mg per g bodyweight per day for five consecutive days. The PERK excision was confirmed by genotype PCR using primers F1 5′-CATCCCCCATCAGCTGGTTCG-3′ and R1 5′-CTCCGTGTTGCTGCTGATGGA-3′. Retroviral vectors and helper plasmids were co-transfected into 293T cells by lipofectamine (ThermoFisher Scientific). Retroviral supernatants from Addgene. Retroviral vectors and helper plasmids were co-transfected into Clontech, cat. no. 011614). Human Bmal1 S42G was mutated from PCKPC4 Clock (mouse) plasmid was purchased from Addgene (no. 31366).

Protein was induced with 0.5 μM doxycycline and deprived of methionine for 30 min, and re-fed with medium supplemented with 10% FBS, 1% penicillin-streptomycin, 2 mM glutamine (Gibco), MEM non-essential amino acids (Gibco). U2OS Bmal1−/−:Luc cells were purchased from Addgene (no. 43370). Plenti-Bmal1 (human) and Plenti-Clock (mouse) plasmids was purchased from Clontech (cat. no. 011614). Human Bmal1 S42G was mutated from PCKPC4 Clock (mouse) plasmid was purchased from Addgene. Retroviral vectors and helper plasmids were co-transfected into 293T cells by lipofectamine (ThermoFisher Scientific). Retroviral supernatants were collected 48–72 h post transfection. Retroviral infection was mediated by polyclonal antibodies in IHC. CD10 was visualized using a biotinylated antibody against human CD10 (130-114-691) according to the manufacturer’s instructions. Magnetically labelled CD10 positive cells were isolated by passing the cells through an Anti-PE MultiSort Kit (130-090-151). Flow-through B cells were then labelled with anti-CD27-PE (130-091-151). Tissue was snap-frozen on dry ice. Lysis buffer (3.5 wt/vol) was prepared at room temperature and stored at −80°C. Tissue was sectioned in the range 6–8 μm, placed on positively charged slides, and air-dried on the bench before fixing. The tissue was fixed in acetone at −20°C for 20 min, washed with PBS and incubated with 0.3% H2O2 for 10 min. The tissue was blocked by normal goat serum and incubated with appropriate primary antibody overnight and secondary antibody for 2 h. Sections were visualized using an Apo-200 fluorescence microscope (Nikon). Representative fields from each section are presented in the figures. The Ki-67 antibody (ab15580) was purchased from Abcam. Apoptosis was detected using an APO-BRDU-IHC (TUNEL) apoptosis kit (Novus Biologicals). Methyl green was used for nuclear counterstain.

Western blot. Tissue was snap-frozen on dry ice. Lysis buffer (3.5 wt/vol) was added and homogenized for 30 s or until completely homogenized. Lysates were sonicated and put on ice for 30 min with vortexing every 5 min. The lysate was cleared twice by centrifugation at 25,000 r.p.m. for 30 min. Equal concentrations of total protein were subjected to western blot as described in ref. 42. Bmal1 antibody (A302-616A) was purchased from Milleniy Biotech.

Flow cytometric analysis. Apoptosis was quantified using the Annexin V apoptosis detection kit (BD Biosciences). FACS (fluorescence-activated cell sorting) was performed on a BD LSRFortessa (BD Biosciences) and analysed by FlowJo (TreeStar). The procedure was performed according to the manufacturer’s instructions. Briefly, single cell suspensions were prepared and washed with cold PBS then suspended in 1× binding buffer at a concentration of 1× 106 cells ml−1. Next, 100 μl of the solution was transferred to a tube and 5 μl APC annexin V was added. The cells were gently vortexed and incubated for 15 min at room temperature in the dark. A 400 μl volume of 1× binding buffer was added to each tube and subjected to FACS analysis within 1 h.

Quantitative RT–PCR analysis. Total RNA and microRNA were extracted using the miReasy mini kit from Qiagen. mRNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s instructions.

Plasmids and retroviruses. pBMPC3-Bmal1(mouse) plasmid (no. 31367) was purchased from Addgene. Bmal1 was subcloned into MSCP-IRE5-GFP (MigR1). PCKPC4 Clock (mouse) plasmid was purchased from Clontech (cat. no. 011614). Human Bmal1 S42G was mutated from PCKPC4 Clock (mouse) plasmid was purchased from Addgene. Retroviral vectors and helper plasmids were co-transfected into 293T cells by lipofectamine (ThermoFisher Scientific). Retroviral supernatants were collected 48–72 h post transfection. Retroviral infection was mediated by polyclonal antibodies in IHC. CD10 was visualized using a biotinylated antibody against human CD10 (130-114-691) according to the manufacturer’s instructions. Magnetically labelled CD10 positive cells were isolated by passing the cells through an Anti-PE MultiSort Kit (130-090-151). Flow-through B cells were then labelled with anti-CD27-PE (130-091-151). Tissue was snap-frozen on dry ice. Lysis buffer (3.5 wt/vol) was prepared at room temperature and stored at −80°C. Tissue was sectioned in the range 6–8 μm, placed on positively charged slides, and air-dried on the bench before fixing. The tissue was fixed in acetone at −20°C for 20 min, washed with PBS and incubated with 0.3% H2O2 for 10 min. The tissue was blocked by normal goat serum and incubated with appropriate primary antibody overnight and secondary antibody for 2 h. Sections were visualized using an Apo-200 fluorescence microscope (Nikon). Representative fields from each section are presented in the figures. The Ki-67 antibody (ab15580) was purchased from Abcam. Apoptosis was detected using an APO-BRDU-IHC (TUNEL) apoptosis kit (Novus Biologicals). Methyl green was used for nuclear counterstain.

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Quantitative RT–PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and the data were normalized by GAPDH. The primer sequence for qPCR used in this study is provided in Supplementary Table 1.

**ChIP assays and biotin-211 chromatin precipitation.** Biotin-labelled miR-211 duplexes were generated as previously reported25. Wild-type miR-211 (5′-UUCCCCAUUGCUCCUUUGCCU3′ biotin) and mutant miR-211 (5′-UUUGUCAGAUCUCUUUGCCU3′ biotin) oligos were synthesized by Integrated DNA Technologies and transfected into U2OS cells with lipofectamine 2000 (ThermoFisher Scientific). Chromatin was prepared using the truChIP low cell chromatin shearing kit (Covaris) and sheared 200–700 bp fragments. Immunoprecipitation was performed using IgG, pan-Ago clone 2A8 (MABE56 Millipore), H3K27me3 (ab6002 Abcam), H3K9me2 (ab1220, Abcam) and RNA polII (ab817 Abcam, ChIP grade CTD repeats) antibodies with a Quick ChIP Kit (Imgenex)22,32. Primers used for ChIP assays are listed in Supplementary Table 2.

**m7GTP immunoprecipitation.** Cells were lysed in EBC buffer (50 mmol l−1 Tris, pH 8.0, 120 mmol l−1 NaCl, 1 mmol l−1 EDTA, 0.5% NP40) containing complete and PhosStop (1 tablet/10 ml, Roche). 1 mg lysates were incubated with 40 μl of pre-washed m7GTP beads (Creative Biolabs) overnight at 4°C. Bound proteins were resolved by SDS–PAGE and visualized by western blot.

**Statistics and reproducibility.** All western blot and 35S cooperation assays shown here were successfully repeated at least three times. qPCR was performed three independent times. Data are presented as mean ± s.d. Statistical significance was determined by the two-tailed Student’s t-test using Prism Graphpad or Excel. P < 0.05 was considered statistically significant. The correlation of Bmal1/miR-211 and Clock/miR-211 in human tumours and mice tumours was performed by Spearman’s correlation in R.

**Life Sciences Reporting Summary.** Further information on experimental design is available in the Life Sciences Reporting Summary.

**Data availability.** The previously published Burkitt’s lymphoma data that have been reanalysed here are available under GEO accession code GSE2350 (ref. 33). Data and statistical methods are available from www.Oncomine.org (reporter ID: 36896_s_at). Source data for Figs. 1–8 and Supplementary Figs. 1, 3, 6 and 7 are provided in Supplementary Table 3. All other data supporting the findings of this study are available from the corresponding author on reasonable request.

**References**

38. Valentijn, L. J. et al. Inhibition of a new differentiation pathway in neuroblastoma by copy number defects of N-myc, Cdc42, and nm23 genes. *Cancer Res.* 65, 3136–3145 (2005).

39. Ushmorov, A. et al. N-myc augments death and attenuates protective effects of Bcl-2 in trophically stressed neuroblastoma cells. *Oncogene* 27, 3424–3434 (2008).

40. Yoshida, A., Lee, E. K. & Diehl, J. A. Induction of therapeutic senescence in vemurafenib-resistant melanoma by extended inhibition of CDK4/6. *Cancer Res.* 76, 2990–3002 (2016).

41. Xu, Z. et al. miR-216b regulation of c-Jun mediates GADD153/CHOP-dependent apoptosis. *Nat. Commun.* 7, 11422 (2016).

42. Aggarwal, P. et al. Nuclear cyclin D1/CDK4 kinase regulates CUL4 expression and triggers neoplastic growth via activation of the PRMT5 methyltransferase. *Cancer Cell* 18, 329–340 (2010).
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Experimental design

1. Sample size
   Describe how sample size was determined. Unless explicitly stated, 3 biological independent experiments were performed to achieve Student’s t-test analysis. Animal study fig 1d related experiment has n=5; Fig 7b, S7e, n=10. For human samples, sample size was not predetermined due to the availability.

2. Data exclusions
   Describe any data exclusions. There is no inclusion/exclusion criteria for animal or human study.

3. Replication
   Describe whether the experimental findings were reliably reproduced. Unless explicitly stated, all data shown were obtained from 3 biological independent experiments with technical replicates.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups. All the animals were randomly grouped for experiments (figure 1d, 1e, Fig 7b, 7c, 7d, Fig S6 and correlated figures).

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis. The animal study and human sample data were not blinded assessed.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a | Confirmed
   --- | ---
   □ | X The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   □ | X A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   □ | X A statement indicating how many times each experiment was replicated
   □ | X The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   □ | X A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   □ | X The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
   □ | X A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   □ | X Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

FlowJo for flow cytometry analysis.
ImageLab (Biorad) for western blot band quantification.
Prism (Graphpad Software).
Office Excel (Microsoft).
R for statistics.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

Materials commercial available are indicated in the Methods section. In-house antibody used in this study is p-PERK Rabbit Ab, available on request. Plasmids used in this study are available for reasonable request.

9. Antibodies

Bmal1 antibody (A302-616A) was purchased from Bethyl Laboratories Inc. Clock (D45B10) Rabbit mAb #5157, PERK (C33E10) Rabbit mAb #5683, Chop (L63F7) Mouse mAb #2895 1:2000 dilution, ATF-4 (D4B8) Rabbit mAb #11815 1:2000 dilution, phospho-eIF2α (Ser51) (119A11) Rabbit mAb #3597, Phospho-4E-BP1 (Thr37/46) (236B4) Rabbit mAb #2855, eIF4E Antibody #9742, IRE1α (14C10) Rabbit mAb #3294, eIF4A (F52) Antibody #2425, S6 Ribosomal Protein (5G10) Rabbit mAb #2217, Phospho-p70 S6 Kinase (Thr389) (1A5) Mouse mAb #9206, and c-Myc Antibody #9402, BCL6(D412V) Rabbit mAb #14895 were from Cell Signaling Technology. eIF2α antibody was purchased from ThermoFisher Scientific, 1:2000 dilution. The Ki-67 antibody (ab15580), H3K27me3 (ab6002), H3K9me2 (ab1220), RNA polII (ab817 ChIP grade CTD repeats) antibodies were purchased from Abcam. anti-CD27-PE antibody (130-097-926) and anti-CD10-Biotin antibody (130-114-691) were purchased from Miltenyi Biotec. pan-Ago clone 2A8 (MABE56) was purchased from Millipore. β-actin Mouse mAb (AC-74) #5316 was purchased from Sigma (1:20000). p-PERK Rabbit Ab was generated inside of the lab, available on reasonable request. (Also see Methods section.) Unless indicated, all the antibodies were used in 1:1000 dilution.

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10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used. U2OS, CA46, Raji, Ramos, HCT116, Hela, MCF7, H1299, Kelly, NLF and HEK293T were purchased from ATCC. MEFs (WT, PERK⁻/⁻, IRE1⁻/⁻, Dicer fl/fl) were maintained in the lab in low passages (see previous publications). Dr. John Hogenesch (University of Cincinnati) provided the U2OS BMAL1:Luc cell line. Dr. Linda J. Valentijn (University of Amsterdam) provided SKNAS-NmycER cell line. Dr. Chi V. Dang (University of Pennsylvania) provided us the SHEP-NmycER cell line.

b. Describe the method of cell line authentication used. U2OS, CA46, Raji, Ramos, HCT116, Hela, MCF7, H1299, Kelly, NLF and HEK293T were purchased from ATCC directly. Other cell lines were authenticated using the relevant stimulation and is mentioned in the Methods section.

c. Report whether the cell lines were tested for mycoplasma contamination. Yes. They have been tested before conducting the experiments. There is no mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample.

Animals and human research participants

Policy information about studies involving animals: when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study. All animal experiments were conducted within the guidelines with the Animal Care and Use Committee of Medical University of South Carolina. C57BL/6j mice (male, eight-week old, male) were purchased from Jackson Laboratory for circadian study. For xenograft experiments, 6-week old female C.B-17 SCID mice were purchased from Taconic Biosciences. PERK fl/fl and CreERT2 PERK fl/fl were maintained in DLAR of Medical University of South Carolina.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants. All the human samples were obtained with informed consent and IRB approval. Human tonsils were obtained from routine human tonsillectomies performed in Philadelphia Children’s Hospital (CHOP). Informed consent was obtained from patients. Tissue collection was approved by the hospital ethical committee.
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data presentation
For all flow cytometry data, confirm that:

- 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- 3. All plots are contour plots with outliers or pseudocolor plots.
- 4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation.

U2OS cells (Fig 4a and 4k) and MEFs (Fig 4j) were used in this study for flow cytometry analysis. For Annexin V analysis, single cell suspensions were prepared and washed with cold PBS then suspend in 1 × Binding buffer at a concentration of 1 × 10^6 cells/ml. Transfer 100 μl of the solution to a tube and add 5 μl APC Annexin V. Gently rortex the cells incubate for 15 minutes at Room temperature in the dark. Add 400 μl of 1× Binding buffer to each tube and subject to FACS analysis within 1 hr. Germinal center B cells were magnetic isolated by B cell isolation kits, and enriched with CD10 and CD 27 surface marker (See Methods section on page 15).

6. Identify the instrument used for data collection.

BD LSRFortessa (BD Biosciences) in MUSC flow core facility and University of Pennsylvania flow cytometry core facility.

7. Describe the software used to collect and analyze the flow cytometry data.

FlowJo (TreeStar).

8. Describe the abundance of the relevant cell populations within post-sort fractions.

Purity was determined by relevant staining using flow cytometry.

9. Describe the gating strategy used.

All samples are FSC-A and SSC-A gated, followed by FSC-A/FSC-W gating to select singlet cells. Unstained and negative control (untreated cells) were used for control and gating.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ✗