

Mitochondria-derived nuclear ATP surge protects against confinement-induced proliferation defects

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The physical tissue microenvironment regulates cell state and behaviour. How mechanical confinement rewires the subcellular localisation of organelles and affects cellular metabolism is largely unknown. In this study, proteomics analysis revealed that cellular confinement induced a strong enrichment of mitochondrial proteins in the nuclear fraction. Quantitative live cell microscopy confirmed that mechanical cell confinement leads to a rapid re-localisation of mitochondria to the nuclear periphery in vitro, reflecting a physiologically relevant phenomenon in patient-derived tumours. This nucleus-mitochondria proximity is mediated by an endoplasmic reticulum-based net that entraps the mitochondria in an actin-dependent manner. Functionally, the nucleus-mitochondria proximity results in a nuclear ATP surge, which can be regulated by the genetic and pharmacological modulation of mitochondrial ATP production or via alterations of the actin cytoskeleton. The confinement-induced nuclear ATP surge has physiologically significant long-term effects on cell fitness, driven by changes in chromatin state, enhanced DNA damage repair, and cell cycle progression during mechanical cell deformation. Together, our data describe a confinement-induced metabolic adaptation that is required to enable prompt DNA damage repair and cell proliferation under mechanical confinement stress by facilitating chromatin state transitions.

Actively proliferating cells, such as cancer cells within the tumour microenvironment or in circulation, undergo physically constraining processes like intravasation, extravasation, and tissue infiltration¹. Similarly, developmental processes such as gastrulation² and immune cell migration³ expose cells to physically confining environments, leading to acute mechanical stress. While long-term confined tissue

environments may exist as a result of unchecked growth, acute short-term spatial constraints can also occur transiently in vivo, such as when cells encounter constrictions in the extracellular matrix or during invasive processes⁴. This can eventually induce alterations to the cytoskeleton organisation^{5–7}, nuclear structure⁸, and chromatin organisation⁹, potentially leading to DNA damage and mitotic

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defects^{10–12}. Despite these challenges, cell proliferation persists, suggesting that cells employ adaptive mechanisms to maintain fitness and survival under such conditions. Mechanical signals from the micro-environment could trigger both immediate and long-term adaptive responses, involving physical remodelling and functional reprogramming. Depending on the context, time scales of cell deformation can also vary widely from rapid cell deformations during fast amoeboid migration and acute mechanical impacts¹³, to slower cell shape deformations during tissue morphogenesis and tissue growth^{14,15}. For instance, mechanical remodelling has been documented for intracellular organelles like the nucleus⁸ and the mitochondria^{16–19}. However, whether these changes are required for an immediate and rapid metabolic rewiring to mechanical stress or driven by active reorganisation of subcellular structures and inter-organelle crosstalk remains unclear.

Here, we studied how the localisation of cellular organelles and molecular components changes upon short-term acute mechanical confinement and how they can trigger rapid metabolic cell changes relevant for mechano-adaptive cell responses involved in stress resistance and cell fitness. We performed a proteomics-based subcellular fractionation of acutely confined cells, which allowed us to identify the redistribution of proteins within the nucleus and cytoplasmic compartments. We discovered that upon mechanical cell deformation from acute confinement, mitochondria accumulate at the nuclear periphery *in vitro* and *in vivo*. Using an experimentally tractable model of acute mechanical confinement, we further demonstrate that this nucleus-mitochondria proximity is orchestrated through a complex coordinated regulation between the endoplasmic reticulum (ER), actin cytoskeleton and mitochondrial morphology dynamics. This leads to a multi-organelle architectural reorganisation, involving mitochondrial accumulation at the nuclear periphery that deforms the nucleus and leads to characteristic nucleus shape changes. The proximity between mitochondria and the nucleus is further responsible for mechanical stress-induced metabolic changes within the nucleus in the form of increased ATP levels of mitochondrial origin. We functionally show that these elevated nuclear ATP levels facilitate changes in chromatin accessibility, which are required for an efficient DNA damage repair signalling and proper cell cycle progression of cells in the S-phase. Thus, cell proliferation and fitness is safeguarded via a mechano-metabolic adaptation mechanism during acute confinement stress. Altogether, these findings reveal a mechano-metabolic adaptation mechanism that preserves cell proliferation and genomic integrity despite acute confinement stress.

Results

Cell confinement induces a mitochondrial enrichment at the nucleus

To explore cellular adaptation mechanisms to mechanical stress in specific cell compartments and organelles, we performed a subcellular fractionation of HeLa cells acutely confined for 15 min (henceforth referred to as acute confinement). HeLa cells were considered confined when compressed to a height of 3 μm , resulting in cytoskeleton reorganisation and bleb formation²⁰. To achieve cell confinement, we adopted a previously established agarose-based confiner^{21,22}, where an agarose disc applies pressure onto cells, simulating a physically constrained environment (Fig. 1A). Using confocal microscopy of confined HeLa cells expressing a whole-cell green fluorescent protein (GFP) marker, we validated the cell confinement height and mechano-sensitive bleb formation (Fig. S1A), as previously documented^{5,20}. To investigate relative changes in the nucleus versus cytoplasmic cell compartments following acute confinement (Fig. 1B), we performed a nucleo-cytoplasmic fractionation by injecting a cytoplasmic lysis buffer through the agarose pad. The lysate was then centrifuged to obtain a nuclear protein fraction and a cytoplasmic protein supernatant.

Unconfined HeLa cells in suspension (henceforth referred to as suspension cells) were lysed to obtain reference cytoplasmic and nuclear fractions. The two fractions were validated using Western blot analysis, where Vinculin, a cytoskeletal protein, and nuclear histone H3 were detected within either the cytoplasmic or nuclear fractions, respectively (Fig. S1B).

A principal component analysis of mass spectrometry (MS) data showed a clear separation between the nucleus and the cytoplasm, as well as between confinement and suspension, suggesting an altered proteomic distribution within these subcellular compartments upon acute confinement (Fig. S1C). As expected, proteins known to be localised to the nucleus²³ were enriched in the nuclear fraction of suspension and acutely confined cells (Fig. S1D, E). We then performed a Gene Set Enrichment Analysis (GSEA) for differentially detected proteins in the nuclear fraction versus the cytoplasmic fraction, in suspension (Fig. S1F) and in acutely confined cells (Fig. S1G). We observed a clear enrichment of nuclear and chromatin-related gene ontologies (GOs), and a depletion of cytoplasm-related GOs, in the nuclear fraction, thus validating our fractionation approach (Fig. S1F, G).

While the comparison of cytoplasmic fractions did not reveal a pronounced protein redistribution (Fig. S1H, I), comparing the two nuclear fractions notably revealed a marked enrichment of mitochondrial proteins in the confined nuclear fraction (Fig. 1C), which was much more pronounced than the enrichment of other cellular organelles (Fig. 1D). GSEA of these enriched proteins also showed a clear enrichment of mitochondria-related GO terms (Fig. 1E).

Altogether, our subcellular proteomics approach revealed that acute confinement induces an enrichment of mitochondria in the nuclear fraction.

Mitochondrial accumulation drives nuclear shape changes in acute confinement

The presence of mitochondria-related terms in the nuclear fraction of acutely confined cells may be attributed to a more robust association between mitochondria and the nuclear surface, or alternatively, may be a consequence of mitochondria being situated within the nucleus. To test which of these hypotheses could explain the mitochondrial enrichment in our proteomics data, we performed high-resolution confocal microscopy of suspension and acutely confined HeLa cells and monitored mitochondrial localisation with respect to the nucleus, using MitoTracker and Hoechst staining. We used a pressurised dynamic cell confiner with PDMS-based microspacers (3 μm height), which allowed for the precise control of cell confinement height (Fig. S2A). Notably, suspension and acutely confined cells showed a markedly different mitochondrial localisation. In contrast to suspension cells, where the nuclear region was devoid of mitochondria, acutely confined cells exhibited a pronounced increase in mitochondrial signal (MitoTracker) around the nucleus (Fig. 2A). Three-dimensional (3D) image reconstructions showed that, while in suspension cells mitochondria were homogeneously distributed in the cytoplasm, in acutely confined cells mitochondria were enriched at the nuclear periphery, and notably above and below the nucleus (Fig. 2B). We then used an *in silico* 3D image reconstruction approach which revealed distinctive nuclear shape alterations in the confined cells, characterised by indentations at the superior, inferior, or both superior and inferior surfaces of the nucleus, within which mitochondria were enriched (Fig. 2C). A transversal section image analysis of confined HeLa cells, transiently overexpressing the inner nuclear membrane marker LAP2 β -GFP, also confirmed that during acute confinement, mitochondria were redistributed and accumulated at nuclear indentations (Fig. 2D). Notably, mitochondria did not cross the nuclear membrane and remained at the outer nuclear periphery, indicating that mitochondria were not physically inside the nucleus (Fig. 2D). Together, our observations corroborated the presence of

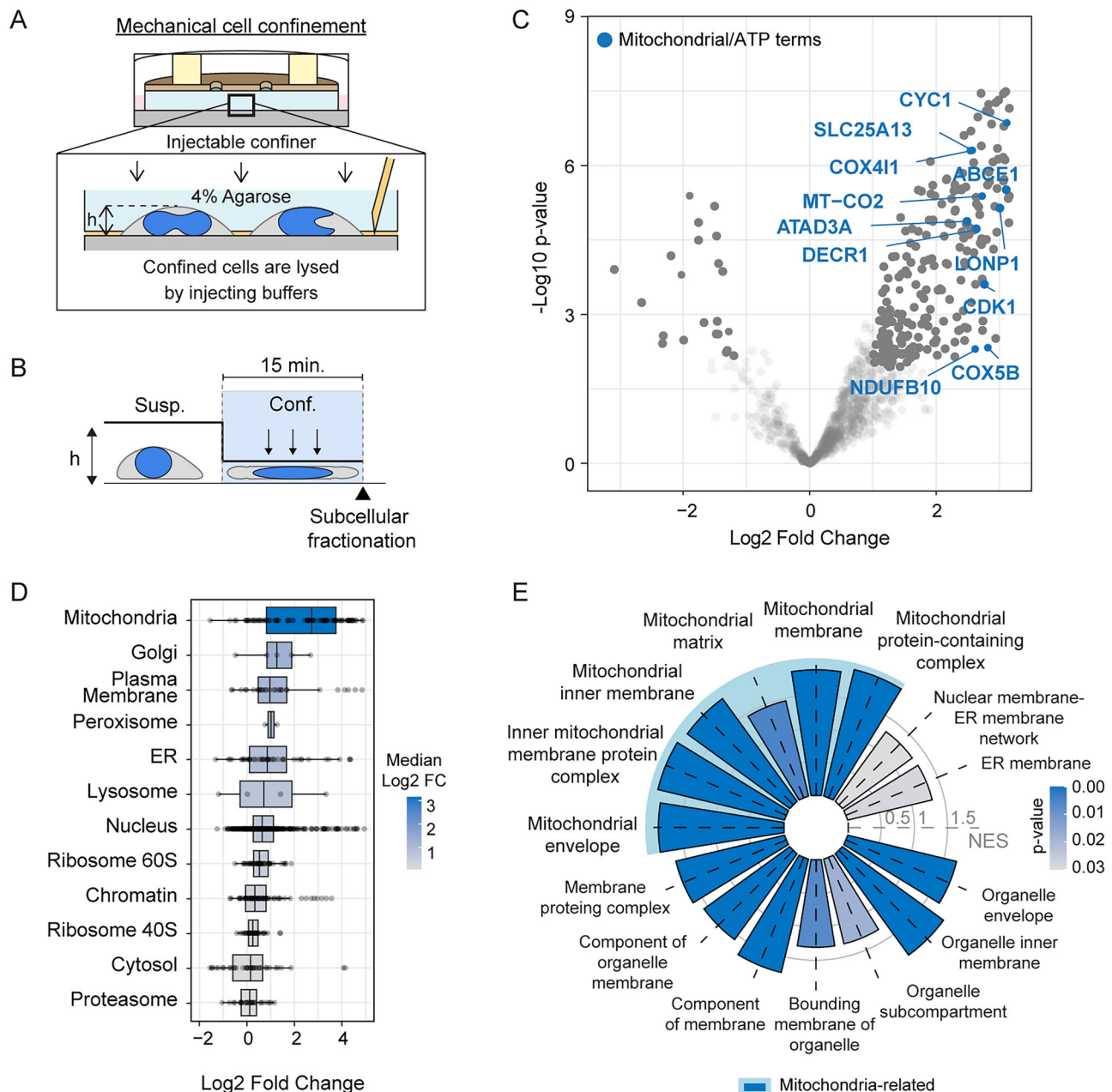


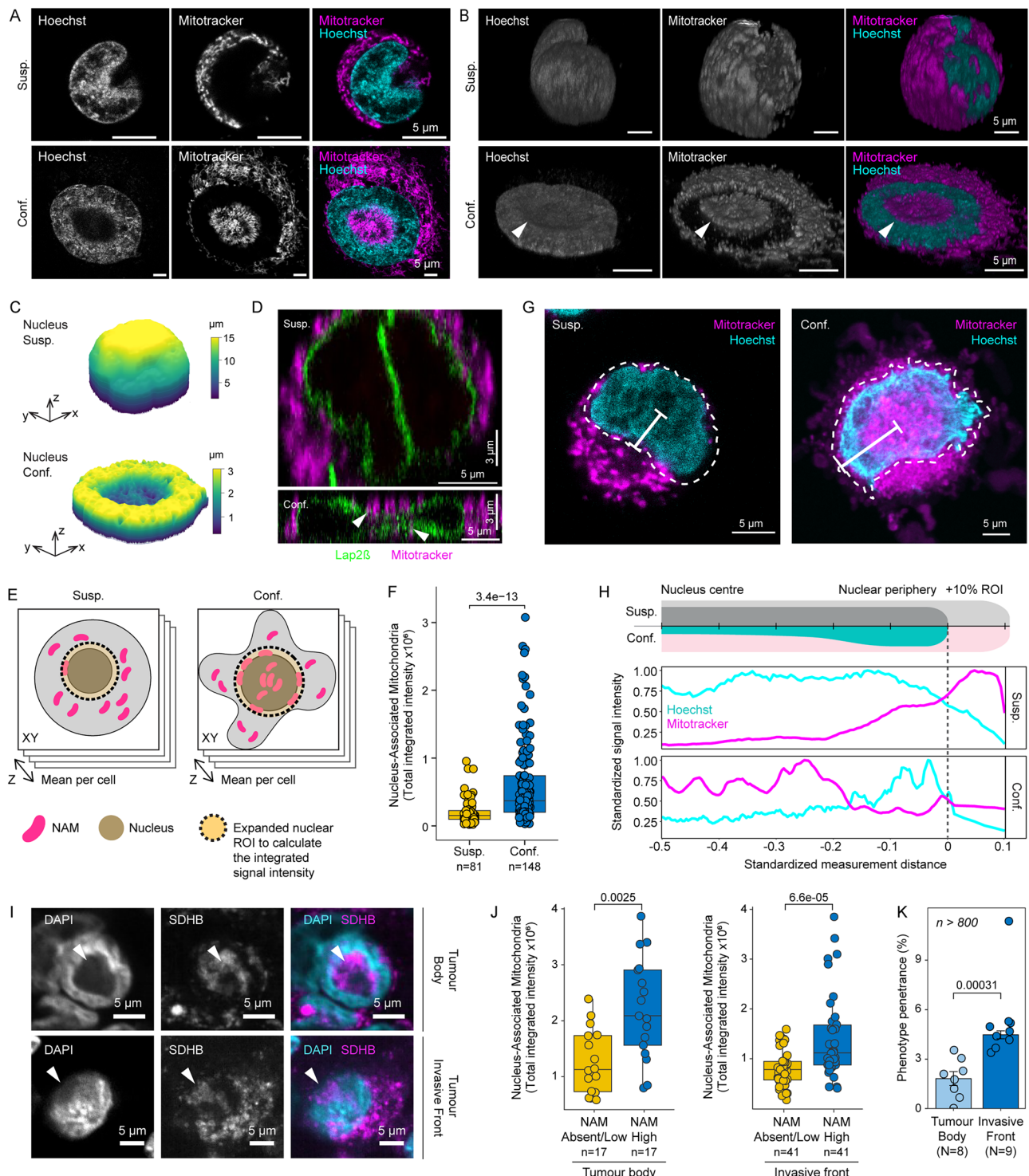
Fig. 1 | Proteomic analyses reveal mitochondrial enrichment within the nuclear fraction. **A** Schematic of mechanical cell confinement for proteomics analysis, which is based on a 4% agarose pad for confinement through which reagents can be injected. **B** Schematic of the experimental strategy to apply acute confinement prior to subcellular fractionation. **C** Enriched and depleted terms when comparing the nuclear fraction of HeLa cells in mechanical confinement versus control conditions. **D** Comparison of the enriched terms from the nuclear fractions in (C), mapped to organelles based on their known localisation from the hyperLOPIT

dataset²³. Data is obtained from three biological replicates. **E** Cellular components—GSEA results performed on the nuclear fractions in (C). Light blue background highlights terms related to mitochondria. Concentric circles represent normalised enrichment scores (NES). Box plots represent the lower quartile, median and upper quartile. Whiskers represent the minimum and maximum. *P* values are FDR adjusted. See also Fig. S1 for the quality control of subcellular fractionation and proteomics results.

mitochondria-enriched nuclear indentations upon acute mechanical cell confinement.

Nuclear indentations could be classified into different shapes as ‘doughnuts’, ‘beans’ or more complex shapes based on the Hoechst signal and localisation of mitochondria (Fig. S2B). Doughnut-shaped nuclei contained indentations either on the top, bottom, or both the top and bottom; bean-shaped nuclei contained indentations on one side; complex shapes contained multiple indentations and could not be classified into either doughnuts or beans. In HeLa cells, doughnut-shaped nuclei were the most abundant (75%), followed by beans (24%)

and complex nuclei (2%; Fig. S2B). To define a quantitative metric for mitochondria-nucleus association, we quantified Nucleus-Associated Mitochondria (NAM) as mitochondria positioned within a peripheral nuclear region, determined by expanding the nuclear circumference by 10% (Fig. 2E). Our results revealed a significantly elevated nucleus-mitochondria association in cells subjected to acute confinement, in comparison to suspension cells (Fig. 2F). We also used an orthogonal analysis approach to quantify NAM, where the mitochondria at the nuclear periphery was quantified as a percentage of the total mitochondria in the cell. As before, we observed a significant increase in the



fraction of mitochondria at the nuclear periphery in cells under confinement, validating our findings (Fig. S2C). To further quantify the localisation of the mitochondria relative to the nucleus, we used line profiles of MitoTracker and Hoechst signal intensities from the centre of the nucleus to the cell periphery, in both suspension and acutely confined cells (Fig. 2G). Confinement showed a clear overlap between the signals of mitochondria and the nucleus, as opposed to the mutually exclusive signals in suspension cells (Fig. 2H). The different categories of nuclear indentations contributed equally to the increase in NAM (Fig. S2D). Time-lapse imaging performed while confining showed that the confinement-induced enrichment of mitochondria at the nuclear periphery and within nuclear indentations occurred within

seconds of applying mechanical stress and persisted over the measurement period of 35 min under confinement ($R^2 < 0.1$, Fig. S2E, Supplementary Movies S1 and S2). This indicates that the mitochondrial redistribution within nuclear indentations is a product of the rapid and dynamic remodelling of organelle localisation, which promotes a mitochondrial accumulation within the nuclear region.

To assess the penetrance of the NAM phenotype within the HeLa cell population, we classified confined cells as 'NAM-high' or 'NAM-absent/low' and found 84% of the population displaying a strong NAM phenotype (Fig. S2F), indicating that the vast majority of cells underwent a physical remodelling of nucleus-mitochondrial organisation upon acute mechanical cell confinement. We next assessed the

Fig. 2 | Confinement induces nuclear indentations and an accumulation of mitochondria at the nuclear periphery. **A** Cross-section of HeLa cells showing Hoechst (nuclei) and MitoTracker (mitochondria) in suspension and acutely confined cells. **B** 3D representation of (A). White arrows indicate nuclear indentations where mitochondria localise. **C** In silico reconstruction of nuclei based on Hoechst in cells from (A, B). Colour bar indicates z-height (μm). **D** X-Z view of the inner nuclear membrane (LAP2 β -GFP) and mitochondria (MitoTracker) of HeLa cells in [top] suspension and [bottom] confinement. White arrows highlight indentations of the nucleus where mitochondria localise. **E** Schematic of the data analysis to quantify Nucleus-Associated Mitochondria (NAM). Nuclear Regions Of Interest (ROIs) were enlarged by 10%, and NAM formation was quantified as integrated signal intensity within the ROI, averaged across z-slices per cell. **F** NAM quantification in suspension and confined HeLa cells as outlined in (E). Each point represents a cell, n indicates the number of cells analysed. **G** Representative images of HeLa cells in suspension and confinement. Solid white line depicts the line profiles

used in (H); dashed line indicates the 10% expanded nuclear ROI. **H** Hoechst and MitoTracker line profiles from the nuclear centre to the 10% expanded ROIs in representative (based on $n = 20$) suspension and confined HeLa cells as in (G). **I** Single cells from breast cancer tissue microarrays (TMA) from the tumour body or invasive front were stained for the nucleus (DAPI) and mitochondria (SDHB). White arrows show nuclear indentations and mitochondrial accumulation. Additionally, see Fig. S2N. **J** NAM quantification (SDHB signal within the nuclear ROI) in NAM-High or NAM-Absent/Low cells in the tumour body or tumour invasive front. Each point represents a cell; n indicates the number of cells analysed. **K** Penetrance of the NAM phenotype in the tumour body or invasive front. Tumours from 17 individuals were analysed. Barplot represents mean $\pm$ SEM. n indicates the number of analysed cells. Statistics in (F, J, K) were performed using the Wilcoxon–Mann–Whitney test. Box plots show the lower quartile, median and upper quartile. Whiskers represent the minimum and maximum. See Fig. S2 for further characterisation and validation in other cell lines.

presence of this phenotype in different cell lines and applied an acute 3 μm confinement to the osteosarcoma cell line U2OS and the triple negative breast cancer cell line MDA-MB-231. With a variable penetrance, both U2OS and MDA-MB-231 cells showed a consistent increase in NAM formation upon confinement (Fig. S2F). To evaluate if the observed nuclear indentations were a direct result of NAM accumulation, we quantified the percentage of NAM-negative cells displaying nuclear indentations. This analysis showed that in the absence of NAM, almost no cells exhibited nuclear indentations (Fig. S2G), confirming that the observed nuclear shape changes are a direct result of NAM. NAM quantification in U2OS, MDA-MB-231 and pancreatic cancer cell line MIA PaCa-2 showed a significant increase upon mechanical confinement (Fig. S2H–M), suggesting that the NAM phenotype is a conserved response in different cancer cell lines.

Next, to evaluate the physiological relevance of our in vitro findings, we used breast cancer tissue microarrays (Tissue Array, BR725), stained with DAPI and mitochondrial protein SDHB, to evaluate the presence of NAMs. We evaluated tumours from 17 different individuals, and categorised the tumour regions into either the tumour body or the tumour invasive front. Tumour invasive fronts were characterised by dispersed tumour cells interspersed with abundant stromal cells, whereas tumour core regions exhibited densely packed tumour cells with minimal stromal infiltration. (Fig. S2N). We hypothesised that the dynamic mechanical stresses encountered by invading tumour cells^{1,4} would result in a higher penetrance of the NAM phenotype at the invasive front. As before, we classified each tumour cell as either ‘NAM-high’ or ‘NAM-absent/low’. Although tumour cells containing NAMs were observed in both the tumour core and invasive front (Figs. 2I, J and S2N), our analysis revealed a penetrance of 1.8% in the tumour body, compared to a significantly higher penetrance of 5.4% at the invasive front (Fig. 2K). Overall, our results revealed that in acutely confined cells, mitochondria accumulate at the nuclear periphery and within nuclear indentations. Importantly, our findings in vitro reflect a physiologically relevant phenomenon, as seen in patient-derived tumours.

The actin cytoskeleton underlies NAM formation

The actin cytoskeleton plays a major role in regulating mitochondrial²⁴ and nuclear positioning²⁵, cellular response to mechanical stress, and works in conjunction with various mechano-sensitive signalling cascades such as those regulated by intracellular calcium levels^{26,27}. In addition, polymerisation of actin filaments is often required for the formation of mitochondria–ER contacts (MERCs)^{28–30}, while Ca²⁺ channelling across MERCs regulates mitochondrial activity^{29,31}.

To assess whether actin plays a role in NAM formation upon acute confinement, we pre-treated HeLa cells with pharmacological agents targeting the actin cytoskeleton (Fig. S3A). First, we interfered with actin polymerisation by treating HeLa cells with Latrunculin A, which

led to a significant reduction in NAM formation upon acute confinement (Fig. 3A, B). We further evaluated NAM accumulation in HeLa cells treated with the Actin-Related Protein 2/3 (Arp2/3) inhibitor CK-666, which impairs actin branching³², or the Formin inhibitor SMIFH2, which interferes with actin elongation³³. While treatment with CK-666 did not lead to a reduction in NAM formation compared to control confined cells (Fig. S3B, C), treatment with SMIFH2 was able to reproduce the reduction in NAM accumulation observed with Latrunculin A treatment (Fig. 3A, B), suggesting that actin elongation, but not branching, plays a role in regulating NAM formation. To confirm whether de novo actin polymerisation was required for dynamic NAM formation upon confinement, we used Jasplakinolide, which stabilises actin polymers³⁴. Cells treated with Jasplakinolide were unable to establish NAM in response to confinement (Fig. 3A, B), confirming the need for dynamic actin polymerisation in NAM formation. The absence of NAM formation in cells with a stabilised actin cytoskeleton via Jasplakinolide treatment further suggests that a passive frictional trapping of mitochondria in the actin cytoskeleton is not sufficient to drive the accumulation of mitochondria around nuclei under mechanical confinement stress. We also asked whether interfering with intracellular calcium signalling, which is a key regulator of the actin cytoskeleton³⁵, might influence actin-mediated NAM formation. Treatment with BAPTA-AM, a cell-permeable calcium chelator³⁶, was not sufficient to significantly reduce NAM formation (Fig. S3B, C). Finally, given that mitochondrial translocation has been strongly linked to microtubules^{37,38}, we wondered if depolymerisation of microtubules using Nocodazole might inhibit NAM formation. While treatment with Nocodazole alone showed no effect on mitochondria in the suspension cells, it produced a significant increase in NAM formation together with mechanical confinement compared to control confined cells (Fig. 3C, D). All pharmacological treatments were validated by immunofluorescence microscopy of actin and α -Tubulin structures (Fig. S3D, E). Notably, treatment with Latrunculin A, which significantly reduced the presence of mitochondria within nuclear indentations, also prevented the formation of nuclear shape change and nuclear indentations (Fig. 3E–G).

Given this direct role of actin in NAM accumulation, we next quantified perinuclear actin levels by measuring actin signal intensity within a defined boundary region along the nucleus. Live cell imaging of actin in HeLa cells showed a significant increase in perinuclear actin from an adherent to non-adherent state in suspension, and from suspension to acute cell confinement (Fig. 3H, I). Treatment with Latrunculin A entirely abolished the increase in perinuclear actin between adhesion and suspension conditions, and significantly limited the increase between suspension and confinement (Fig. 3H, I).

Together, these results suggest a de novo actin polymerisation-mediated mechanism by which NAM accumulation leads to nuclear shape changes in confined environments.

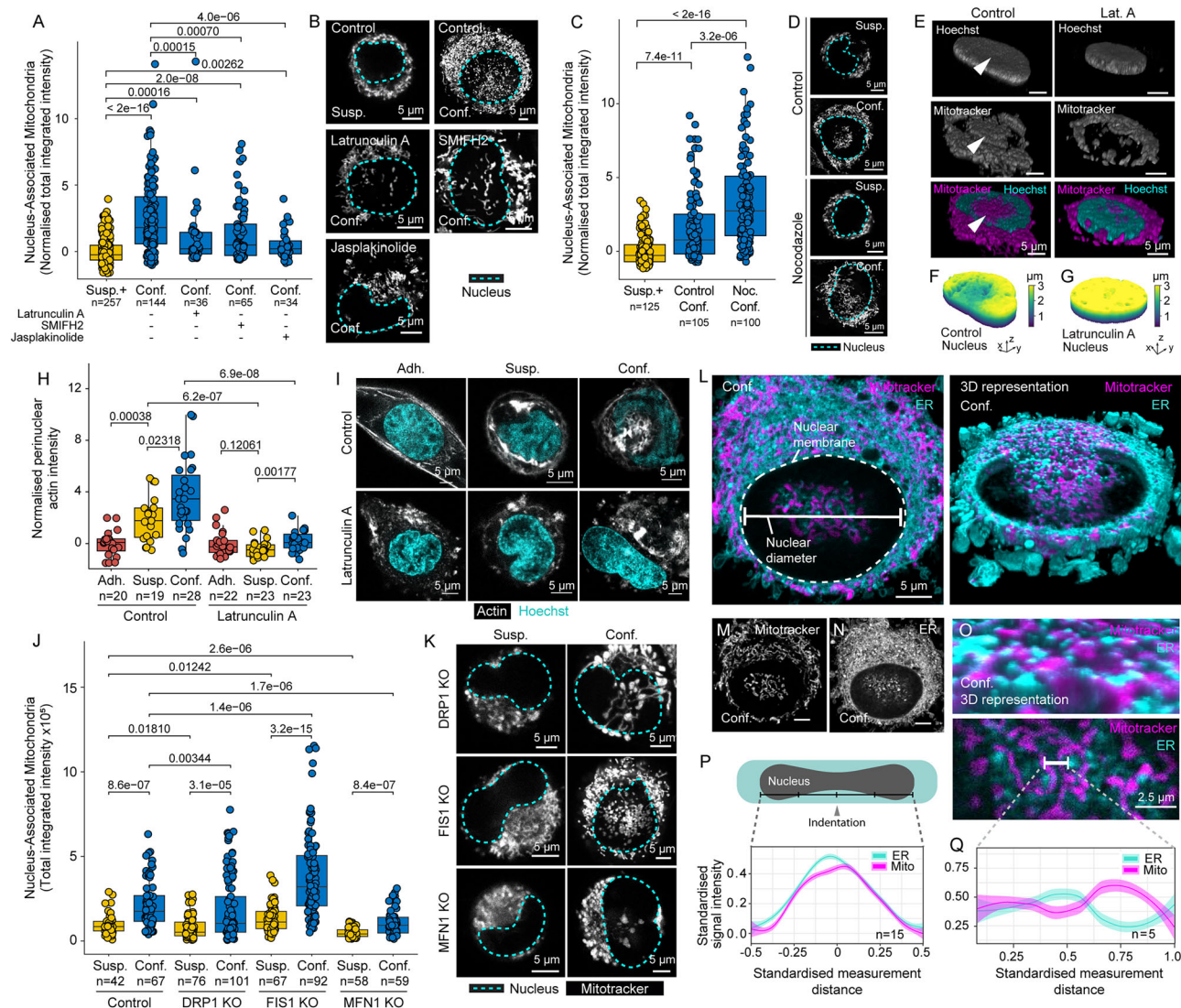


Fig. 3 | Actin, mitochondrial morphology and ER play a role in regulating mitochondrial accumulation at the nuclear periphery. **A** NAM levels in HeLa cells treated with Latrunculin A (500 nM), SMIFH2 (100 μM), or Jasplakinolide (500 nM), zero mean normalised with Fig. S3B (see ‘Methods’). Each point represents one cell; n is the number of cells analysed. **B** Corresponding images of control suspension, control confinement and drug-treated confined cells as in (A). Mitochondria are (MitoTracker) in grey; nuclei (Hoechst) are outlined in cyan. **C** NAM levels in HeLa cells treated with Nocodazole (10 μM), zero mean normalised. Each point is a cell; n is the number of cells analysed. **D** Corresponding images of control and Nocodazole-treated HeLa cells under suspension or confinement. Mitochondria are (MitoTracker) in grey; nuclei (Hoechst) are outlined in cyan. **E** 3D views of confined HeLa cells, untreated or Latrunculin A-treated, stained with Hoechst and MitoTracker. Arrows indicate mitochondria-containing nuclear indentations. In silico reconstructions of **F** untreated or **G** Latrunculin A-treated nuclei from (E). **H** Perinuclear actin (SiR-Actin) quantification in HeLa cells under adhesion, suspension, or confinement, untreated or Latrunculin A-treated. Intensity averaged across z-slices per cell. Suspension and confinement were normalised to respective adhesion. Each point

is a cell; n is the number of cells analysed. **I** Representative images quantified in (H) of HeLa cells stained with SiR-Actin and Hoechst, untreated or Latrunculin A-treated. **J** NAM levels in wild-type, DRP1 KO, FIS1 KO, or MFN1 KO HeLa cells under suspension or confinement. Each point is one cell; n is the number of cells analysed. **K** Corresponding images of KO cells as in (J), showing mitochondria (grey) and nuclei (cyan). **L** Confined HeLa cells co-stained with MitoTracker (see M) and ER marker Turquoise-KDEL (see N) shown as (left) cross-sections and (right) 3D views. See also Fig. S4A for suspension. Single-plane images of the cell in (L) stained with MitoTracker (M) and Turquoise-KDEL (N). **O** Zoomed 3D (top) and cross-sectional (bottom) views showing mitochondria-ER interaction within a nuclear indentation. Line profiles of ER and mitochondrial signals across **P** nuclear diameter, or **Q** 1.5 μm long lines starting from mitochondrial peaks. Generalised additive models are shown with 95% confidence intervals. n is the number of cells analysed. Nuclei were centred, and the measurement distances were standardised. See also Fig. S4 for late endosomes, lysosomes and peroxisomes analyses. Statistics in (A, C, H, J) were performed using the Wilcoxon–Mann–Whitney test. Box plots represent the lower quartile, median and upper quartile. Whiskers represent the minimum and maximum.

Mitochondrial dynamics, the actin network, and the ER regulate NAM accumulation during mechanical confinement

Actin plays a crucial role in the regulation of mitochondrial fission and fusion processes, which are essential for maintaining optimal mitochondrial morphology and functionality. Actin filaments assemble on the outer membrane of elongated mitochondria, thereby promoting fission events. This assembly is transient and

occurs in a cyclical manner on a minute time scale³⁹. Moreover, actin and Myosin II are localised at MERCs, where they initiate the recruitment of Dynamin-related Protein (DRP1), a key protein in mitochondrial fission^{40,41}. Following fission events, actin disassembles from the fragmented mitochondria, thereby allowing them to rapidly re-fuse and reintegrate into the mitochondrial network^{42,43}.

This tight relationship between actin and mitochondrial fission and fusion led us to hypothesise that mitochondrial dynamics may control NAM formation during acute confinement.

Additionally, the ER is anchored to both the nuclear membrane³¹, and mitochondria, and plays an important role in mediating the regulation between the nuclear membrane and the actin cytoskeleton^{44–46}. Moreover, the ER can regulate nuclear envelope dynamics, mediated by the Linker of the Nucleoskeleton to the Cytoskeleton (LINC) complexes^{45,46}. Hence, we hypothesised that the ER may also serve as a structural scaffold that can entrap mitochondria and retain them in the perinuclear region upon confinement.

To first delineate the role of mitochondrial fission and fusion in regulating NAMs, we postulated that fused mitochondria, which behave as a network, would demonstrate an elevated propensity for NAM formation in comparison to rounder, fragmented mitochondria, which behave more like individual physical entities. To assess the functional relevance of mitochondrial architecture, we produced knock-out (KO) HeLa cell lines^{47,48} for master regulators of mitochondrial fission and fusion proteins: Fission 1 (FIS1), Mitofusin-1 (MFN1) or DRP1 (Fig. S3F). To validate that each of these KO lines exhibits the expected mitochondrial alterations in confinement, we used high-resolution confocal microscopy images and validated mitochondrial shape using three parameters—branch junction count, total branch length and sphericity (Fig. S3G–I). Compared to control cells, DRP1 KO cells did not show significant differences in terms of branch junction count or total branch length but showed a significantly lower sphericity compared to control HeLa cells (Fig. S3I), in agreement with the fact that DRP1 is involved in the regulation of mitochondrial fission to preserve mitochondrial homeostasis^{41,49}. FIS1 KO, on the other hand, displayed a much stronger phenotype with significantly increased branch junctions, total branch length, and a significantly decreased sphericity compared to control cells, confirming almost complete inhibition of mitochondrial fission (Fig. S3I). In contrast, MFN1 KO, which inhibits fusion of mitochondria, showed significantly decreased branch junction counts, total branch length, and significantly increased sphericity compared to control cells, confirming a fragmented mitochondrial morphology (Fig. S3I).

Using these cell lines, we next performed a quantification of NAM formation under acute mechanical confinement stress. We found that DRP1 KO cells showed similar results as in control cells, where acute confinement led to a significant increase in NAMs (Fig. 3J, K). FIS1 KO cells, compared to control cells, showed a much higher level of NAM in acutely confined cells compared to their respective suspension cells. In contrast, although acute confinement of MFN1 KO cells also showed an increase in NAM compared to the suspension cells, the absolute levels of NAM compared to control confined cells remained significantly lower (Fig. 3J, K).

Given the divergent NAM phenotypes specifically observed in FIS1 KO and MFN1 KO cells, we sought to determine whether it was associated with alterations in actin levels in these cell lines. Evaluation of total and perinuclear actin, by live cell imaging of actin, revealed no differences between the two cell lines in adhesion or suspension conditions (Fig. S3J, K). In confinement conditions, we observed a higher increase of perinuclear actin in MFN1 KO cells compared to FIS1 KO cells (Fig. S3J, K), indicating that mitochondrial fission and fusion impact on NAM formation independently of actin.

Next, we investigated whether the ER may also serve as a scaffold that can entrap mitochondria and retain them at the nuclear periphery upon confinement. Acutely confined HeLa cells expressing a Turquoise-tagged ER-localising KDEL sequence, when compared to their suspension control, showed an ER enrichment within the perinuclear region and within nuclear indentations, which co-localised with the MitoTracker signal (Figs. 3L–N and S4A). Closer analysis, however, showed that the two signals were not completely overlapping, but rather behaved as two intertwined meshes (Fig. 3O). Line

profiles of MitoTracker and KDEL-Turquoise signals spanning either the diameter of the nucleus (Fig. 3L, P), or shorter distances within the nuclear indentations (Fig. 3O, Q), confirmed the presence of mitochondria and ER within nuclear indentations, organised as a network. The ER–mitochondria network dynamics regulates intracellular organelle remodelling and shape^{46,50,51}. We have also previously shown that the ER network, which is a highly dynamic structure in suspension cells, gets immobilised at the nucleus-plasma membrane interface in confined cells⁵. Although the effect of cellular confinement on regulating ER immobilisation in human cells is not known, it could physically explain the anchorage of mitochondria within the nuclear region. Kymographs generated from line profiles showed a high dynamicity of ER signals, which was strongly reduced in confinement (Fig. S4B, C, Supplementary Movie S3). This observation supports our hypothesis of ER immobilisation in the nucleus-plasma membrane proximal region that can promote a nucleus-mitochondria association.

To understand whether the confinement-induced enrichment of mitochondria was specific to the mitochondria–ER network, we also stained late endosomes, lysosomes and peroxisomes in confined cells. In suspension, late endosomes were present only within the cytoplasm but showed an enrichment within the nuclear region in acutely confined cells (Fig. S4D, E). However, unlike the ER, late endosomes did not form a network-like structure with mitochondria in confinement (Fig. S4E). Lysosomes also exhibited similar properties as late endosomes (Fig. S4F, G). Peroxisomes showed similar properties in suspension cells, but appeared to be more dispersed in confinement, showing very little association with mitochondria (Fig. S4H, I).

Together, our data support that, mitochondrial morphology, perinuclear actin, and the ER network, each play a vital role in regulating NAM formation. The close coordination between actin polymerisation, ER–mitochondria contact sites and mitochondrial dynamics strongly suggests that it is their integrated interaction that drives NAM formation under mechanical confinement.

NAM fuels a nuclear ATP surge upon cell confinement

Functional analysis of proteins enriched in the nuclear fraction after confinement (Fig. 1C) revealed biological processes that regulate ATP synthesis (Fig. 4A). We hypothesised that mitochondria within nuclear indentations may actively produce energy to facilitate cellular adaptation to mechanical confinement stress. To test whether confinement-induced NAM formation could affect nuclear ATP levels, we performed Förster resonance energy transfer (FRET) imaging using cells transiently expressing a nuclear ATP sensor⁵². We found a significant and rapid increase in nuclear ATP levels in HeLa cells upon acute confinement (Fig. 4B, C), which was not dependent on the duration of the mechanical stress (Fig. S5A).

To further confirm the biological relevance of the nuclear ATP surge during acute confinement, we inhibited the ATP synthase enzyme (Complex V) with Oligomycin A, in the absence of glucose to prevent ATP synthesis from glycolysis and tricarboxylic acid cycle activity. A short 30-min Oligomycin A treatment (Fig. S3A) was sufficient to prevent the nuclear ATP surge observed in acutely confined HeLa cells (Fig. 4B, C), without impacting the actin cytoskeleton or NAM accumulation (Fig. S5B, D), thus, indicating that mitochondrial ATP synthesis is required for the nuclear ATP surge upon acute confinement. To investigate whether the upregulation of glycolysis could circumvent the inhibition of NAM-dependent ATP synthesis during confinement, Oligomycin A treatment was repeated in the presence of glucose. As observed in the absence of glucose, control cells also demonstrated a significant increase in nuclear ATP levels in cells exposed to acute confinement when glucose was present in the media (Fig. 4B, C). Furthermore, the inhibitory effect of Oligomycin A was also preserved in the presence of glucose, indicating that glycolysis is unable to compensate for the ATP production necessary to facilitate the confinement-induced ATP surge (Fig. 4B, C). In the absence of

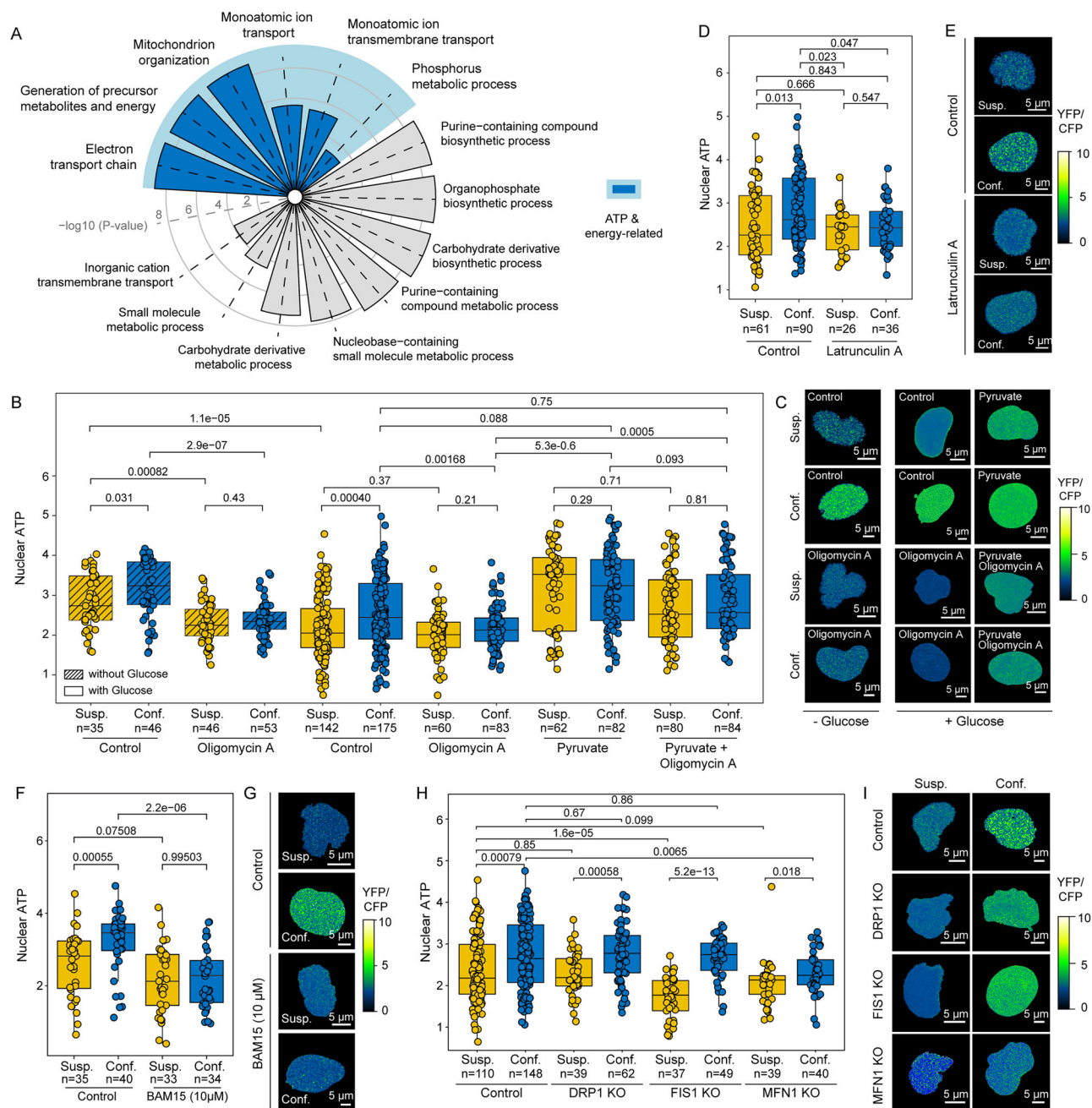


Fig. 4 | Confinement induces a NAM-fuelled nuclear ATP surge. **A** Biological processes—GSEA of proteins enriched in the confined nuclear fraction. Concentric circles represent $-\log_{10}(P\text{-value})$. P values are FDR adjusted. **B** Quantification of nuclear ATP levels in HeLa cells treated with Oligomycin A (1 μM), Pyruvate (3 mM) or combination. Cells were cultured in glucose-containing or glucose-free media as indicated by the hatched boxplot. Each data point represents a cell, and n indicates the total number of cells analysed. **C** Representative images of nuclear ATP levels in HeLa cells quantified in (B). Nuclear ATP is shown as the YFP/CFP ratio using a pseudo-colour scale to indicate ratio intensity and is applicable throughout this figure for ATP analysis. Brighter colours correspond to higher YFP/CFP ratios. **D** Nuclear ATP levels in HeLa cells, either untreated or treated with Latrunculin A (500 nM) in suspension and confinement. Each point represents a cell, and n indicates the total number of cells analysed. **E** Representative images of nuclear

ATP in HeLa cells corresponding to the YFP/CFP ratio as quantified in (D). **F** Nuclear ATP levels in HeLa cells, either untreated or treated with BAM15 (10 μM). Quantification was performed per z-plane and averaged per cell. Each point represents a cell, and n indicates the total number of cells analysed. **G** Representative images of nuclear ATP levels in HeLa cells corresponding to the YFP/CFP ratio as quantified in (F). **H** Nuclear ATP quantification in HeLa wild-type, DRP1 KO, FIS1 KO, and MFN1 KO cells. Each point represents a cell, and n indicates the total number of cells analysed. **I** Representative images of nuclear ATP in HeLa wild-type or KO cells corresponding to the YFP/CFP ratio as quantified in (H). Statistics in (B, D, F, H) were performed using the Wilcoxon–Mann–Whitney test to compare means. Box plots represent the lower quartile, median and upper quartile. Whiskers represent the minimum and maximum. All nuclear ATP was quantified using a FRET-based nuclear ATP sensor⁵².

glucose, cells exhibited a higher baseline level of nuclear ATP. This is likely a product of the higher oxidative phosphorylation rate in the absence of glucose⁵³ (Fig. 4B). The nuclear volume did not change between suspension and confined cells (Fig. S5E), supporting that

observed changes in nuclear ATP levels do not occur due to nuclear size changes.

Additionally, in the presence of glucose, cells were supplemented with 3 mM pyruvate for 30 min prior to confinement. This allowed us

to assess whether accelerating the tricarboxylic acid cycle could effectively enhance ATP production and provide sufficient ATP for the observed nuclear ATP surge during confinement in the absence of mitochondrial ATP synthesis. As expected, pyruvate treatment increased the levels of nuclear ATP in suspension cells to the level observed in control confined cells (Fig. 4B, C). The confinement of pyruvate-treated cells did not result in a further increase in nuclear ATP. Additionally, co-treatment of cells with pyruvate and Oligomycin A did not result in a significant reduction in ATP levels compared to pyruvate treatment alone (Fig. 4B, C). These data support that the lack of mitochondrial ATP production could be rescued by a surplus of pyruvate. Additionally, treatment of HeLa cells with Latrunculin A, which interfered with the confinement-induced increase in NAM (Fig. 3A, B), also abolished the increase in nuclear ATP in acutely confined cells (Fig. 4D, E), indicating that the proximity between the nucleus and the mitochondria is required for the observed nuclear ATP surge.

We also briefly pre-treated cells with BAM15, a mitochondrial uncoupler that inhibits ATP synthesis via dissipation of the proton gradient necessary for ATP production without directly inhibiting the ATP synthase itself⁵⁴. Two distinct concentrations of BAM15 (10 and 50 μ M) were assessed. While the higher concentration resulted in mitochondrial degradation as has been previously observed with prolonged BAM15 treatment⁵⁵, the lower concentration did not alter mitochondrial integrity and NAM formation (Fig. S5F, G). To assess the impact of BAM15, we quantified nuclear ATP levels in cells treated with 10 μ M of BAM15. We found that, like Oligomycin A, treatment with BAM15 could inhibit the confinement-induced nuclear ATP surge (Fig. 4F, G), suggesting that mitochondrial ATP synthesis is necessary for observing this nuclear metabolic adaptation to confinement.

Given that alterations in respiration or in the electron transport chain can influence reactive oxygen species (ROS) production^{56,57}, we quantified nuclear and mitochondrial ROS in control and Oligomycin A-treated suspension and confined cells. Confinement led to an increase in nuclear and mitochondrial ROS levels⁵⁸ in both control and Oligomycin A-treated cells (Fig. S5H, I). Given the difference in nuclear ATP levels in confined control and confined Oligomycin A-treated cells, the confinement-dependent increase in ROS, regardless of treatment, suggests that the nuclear ATP surge is not driven by ROS production.

To verify whether confinement induces a nuclear ATP surge in a cell line-independent manner, we measured confinement-dependent nuclear ATP levels in U2OS cells in glucose-free conditions. Consistent with our observations in HeLa cells, mechanical confinement induced a nuclear ATP surge in U2OS cells that could be blocked by inhibiting mitochondrial ATP synthesis through Oligomycin A treatment (Fig. S5J, K). Finally, we asked whether this increase in nuclear ATP was due to increased mitochondrial ATP production upon confinement. Using a FRET-based mitochondrial ATP sensor⁵² transfected into HeLa cells, we did not observe any confinement-dependent differences in mitochondrial ATP production when analysing either total mitochondria or only NAM (Fig. S5L–N). Thus, suggesting that the nuclear-mitochondrial proximity favours the ATP surge during confinement rather than the localised upregulation of mitochondrial ATP synthesis at the nuclear periphery.

Previously, we showed that FIS1 KO cells exhibited increased mitochondrial networks and consequently increased NAM formation, whereas MFN1 KO cells, which exhibited fragmented mitochondria, showed lower levels of NAM (Fig. 3J, K). Therefore, we hypothesised that differences in NAM levels in FIS1 KO and MFN1 KO cells should be associated with changes in nuclear ATP levels observed upon confinement. To that end, we quantified the levels of nuclear ATP in cells lacking DRP1, FIS1, or MFN1. As expected, DRP1 KO cells did not exhibit any alteration in ATP levels in response to confinement. However, in line with our hypothesis, FIS1 KO cells exhibited a markedly higher increase in nuclear ATP levels under confinement compared to their

respective suspension cells, surpassing the relative nuclear ATP increase observed in control cells (Fig. 4H, I). In contrast, while MFN1 KO cells showed a mild increase in ATP under confinement relative to their suspension cells, the overall ATP levels remained significantly lower than the surge observed in control confined cells (Fig. 4H, I).

In conclusion, these findings suggest that NAM accumulation plays a direct role in driving the nuclear ATP surge induced by mechanical confinement. The close physical proximity of NAM to the nucleus, at nuclear indentations, facilitates the observed ATP increase in confined cells, generated through mitochondrial oxidative phosphorylation.

Nuclear ATP regulates chromatin states under confinement

ATP plays an important role as a biological hydrotrope⁵⁹. Nuclear ATP has previously been hypothesised to regulate chromatin condensation by influencing free Mg^{2+} ion levels⁶⁰. Upon hydrolysis of ATP, Mg^{2+} ions are transiently released, leading to chromatin condensation, for instance, during cell division⁶¹. Conversely, high ATP levels promote ATP- Mg^{2+} complexes, which may result in increased chromatin accessibility and solubility⁶⁰. In the context of mechanical stress, this increased accessibility, mediated by the loss of heterochromatin, results in nuclear softening, thereby insulating genetic material against mechanical force⁶².

To evaluate spatial chromatin density fluctuations between suspension and acutely confined HeLa cells, we analysed the Hoechst signal to assess the nuclear coefficient of variation. Using principles of Image Pattern Analysis, we divided the fluorescence image into smaller sub-areas, or 'bins'. For each bin size, we calculated the intensity variation within these sub-areas. As confirmed by simulations, spatial patterns of specific length scales lead to a characteristic intensity variation that steadily decreases as the bin size increases, eventually reaching a constant level (Fig. S6A). Applying this approach on control suspension, control confined, and Oligomycin A-treated suspension cells showed a sharp decline in their respective coefficient of variation with increased binning sizes. Conversely, the Oligomycin A-treated confined cells exhibited a persistently elevated nuclear coefficient of variation (Fig. S6B, C), indicative of higher chromatin compaction that leads to a more pronounced spatial heterogeneity of fluorescent signal intensities in the nucleus.

We also performed Western blot analysis of H3K9me3 and H3K27me3, which respectively decorate constitutive and facultative heterochromatin. Acute confinement resulted in an increase in constitutive heterochromatin (Fig. 5A). Notably, treatment with Oligomycin A during acute confinement specifically increased the facultative heterochromatin mark H3K27me3, indicating that chromatin is less accessible in the absence of an ATP surge during confinement (Fig. 5A). This finding corroborates our spatial image analysis of nuclear chromatin density variation. As a complementary metric for chromatin rearrangements, we next examined the number of nucleoli and the positioning of centromeres at their periphery. Centromeres, which are heterochromatin-packed genomic regions, localise around nucleoli, which are hubs for heterochromatin organisation in the nucleus^{63–65}. We hypothesised that if heterochromatin is disrupted, this should also be reflected at the level of centromere distribution around nucleoli, which has previously been shown to be a proxy for chromatin organisation. Using CENPA-GFP tagged HeLa cells, in combination with an image segmentation approach to detect nucleoli, we observed that the combination of acute confinement and Oligomycin A treatment strongly reduced the number of nucleoli and completely disrupted the association between centromeres and the nucleolar periphery. Confinement or Oligomycin A treatment alone caused only a mild effect compared to untreated control cells (Fig. 5B–D).

To further assess the dependence of the chromatin state during acute confinement on the mechano-sensitive nuclear ATP surge, we performed an assay for transposase-accessible chromatin with

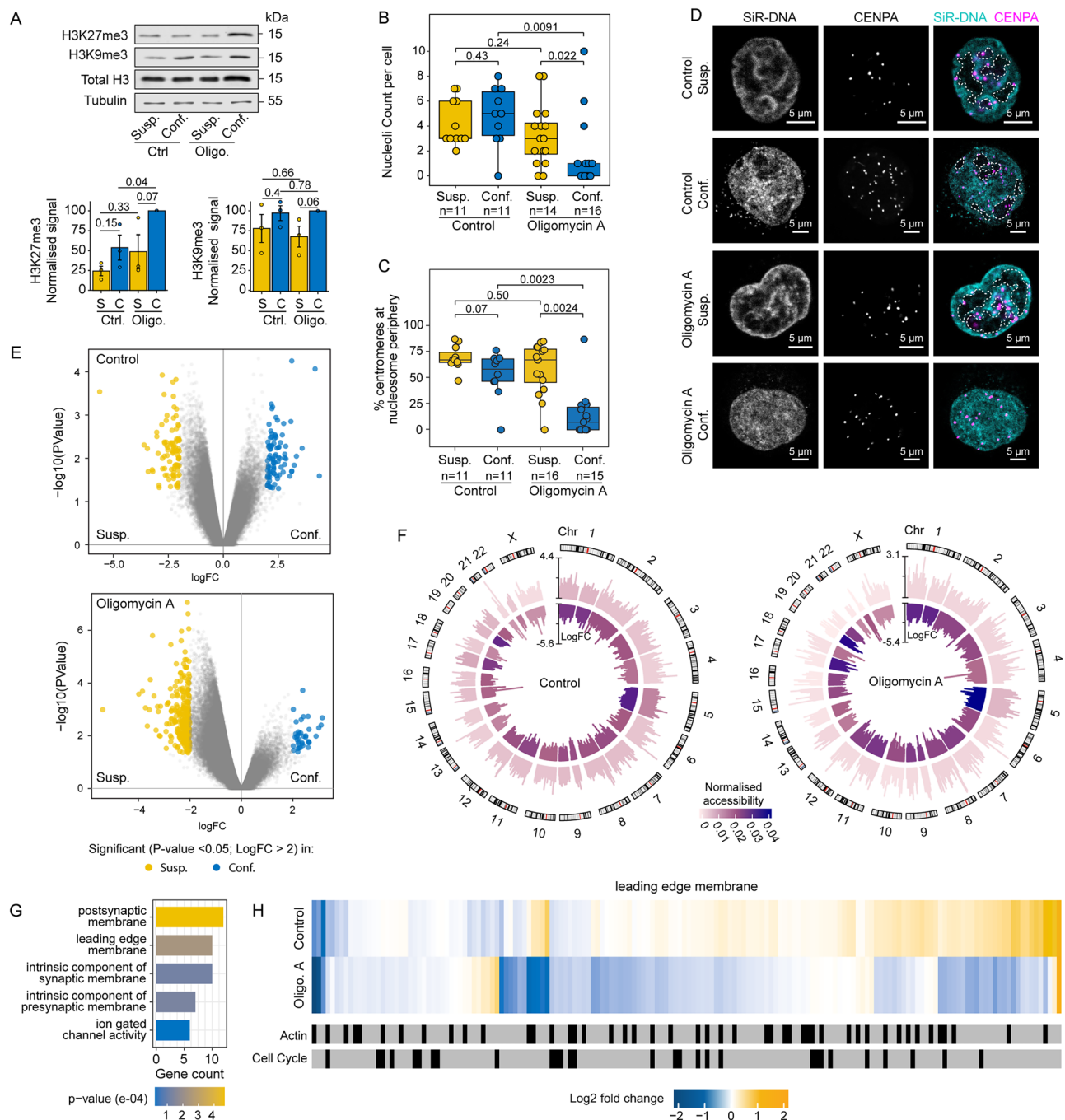


Fig. 5 | Nuclear ATP regulates chromatin state depending on mechanical confinement. **A** Western blot of H3K27me3 (facultative heterochromatin mark) and H3K9me3 (constitutive heterochromatin mark), and total H3 in control or Oligomycin A-treated suspension and acutely confined HeLa cells. α -Tubulin was used as a loading control. Quantification of H3K27me3 and H3K9me3 was normalised to their respective loading controls, and fold change was calculated relative to the Oligomycin A-treated confined condition, for three biological replicates. S indicates suspension cells and C indicates confined cells. Individual points show replicates, and bar charts show mean $\pm$ S.E. Quantification of the number of nucleoli per cell (**B**) and the percentage of centromeres (CENPA) at the nucleolar periphery (**C**) in control or Oligomycin A-treated, suspension or acutely confined HeLa cells.

D Representative images of control or Oligomycin A-treated, suspension or acutely confined HeLa cells expressing a CENPA-GFP reporter, stained with SiR-DNA.

E ATAC-seq accessible peaks in control or Oligomycin A-treated HeLa cells. LogFC was calculated for the comparison of acute confinement vs. suspension. Positive LogFC corresponds to differentially more accessible regions in confinement, while

negative LogFC corresponds to differentially more accessible regions in suspension. Significant genes are highlighted. P values are FDR adjusted. **F** Chromosome-wide ATAC-Seq peaks in control or Oligomycin A-treated HeLa cells. The colour scale is based on normalised accessibility per chromosome, which was calculated as the total accessible region (in base pairs) for each chromosome divided by the respective chromosome length. **G** Over Representation Analysis (ORA) of significantly altered differentially accessible regions (in suspension and confinement) in Oligomycin A-treated HeLa cells. P values are FDR adjusted. **H** Log2-fold change of promoters of genes detected in the ATAC-Seq dataset corresponding to the gene ontology GO:0031256 leading edge membrane, enriched in (**G**). Annotations in black highlight genes belonging to either actin cytoskeleton organisation (GO:0030036) or cell cycle (GO:0007049). Statistics in (**A**) were performed using a Two-way ANOVA followed by the Student's t -test for specific interactions. Statistics in (**B**, **C**) were performed using the Wilcoxon–Mann–Whitney test to compare means. Box plots represent the lower quartile, median and upper quartile. Whiskers represent the minimum and maximum.

sequencing (ATAC-Seq) on control or Oligomycin A-treated, suspension and confined cells. A principal component analysis (PCA) showed no significant separation between suspension and confined cells either in the control or the Oligomycin A-treated conditions (Fig. S6D), likely due to the short duration of the Oligomycin A (30 min) and confinement (15 min). The evaluation of differential accessibility between suspension and confined conditions, in both control and Oligomycin A-treated cells, revealed that acute confinement induced a mild increase in chromatin compaction, while the lack of an ATP surge during confinement considerably reduced chromatin accessibility (Fig. 5E). The differential impact of confinement on chromatin compaction, in the presence or absence of the mechano-sensitive nuclear ATP surge, was evident at the whole-genome level (Fig. 5F). This aligns with the observed changes in the nuclear coefficient of variation as well as alterations in H3K9me3 and H3K27me3 following acute confinement in the presence or absence of Oligomycin A treatment. Interestingly, the reduction in chromatin accessibility observed in acutely confined cells treated with Oligomycin A occurred in smaller, gene-dense chromosomes (Fig. S6E). Overrepresentation analysis (ORA) of differentially accessible genomic regions in control conditions did not reveal any pathway enrichment upon confinement. On the contrary, prevention of the nuclear ATP surge upon acute confinement with Oligomycin A treatment induced a decrease in chromatin accessibility at genomic regions associated with genes involved in membrane remodelling (Fig. 5G), related to the actin cytoskeleton and the cell cycle (Figs. 5H and S6F). This suggests a feedback loop between the actin cytoskeleton and nuclear metabolism, potentially crucial for cellular resilience under mechanical stress.

Overall, these findings underscore the importance of the nuclear ATP surge during acute confinement as a regulator of chromatin state in response to mechanical stress.

Nuclear ATP is required for timely DNA damage repair

We have shown that acute confinement induces nuclear ROS increase (Fig. S5H, I), which in turn is linked to DNA damage induction^{66,67}. Additionally, it has been shown that confinement induces DNA damage⁶⁸, which, if not repaired, can impair DNA replication and cell cycle progression^{69,70}. ATP-dependent chromatin remodelling facilitates efficient DNA damage repair through dynamic restructuring of the chromatin landscape, thereby enabling repair factors to access and repair damaged DNA⁷¹. To demonstrate that DNA damage repair requires a constant synthesis of ATP, we perturbed U2OS cells with a low dose of etoposide, a Topoisomerase 2 inhibitor that provokes the accumulation of double-strand breaks⁷². Following this, the cells were released in etoposide-free media to allow the DNA damage repair to occur⁷³. Metabolomic analysis indicated that monophosphate nucleosides exhibited a marked increase upon etoposide withdrawal, suggesting that they are readily produced for the purpose of repairing damaged DNA (Fig. S7A). In contrast, triphosphate nucleoside levels markedly declined upon withdrawal, indicating that the available pool was promptly utilised for DNA repair (Fig. S7A). Contrastingly, however, ATP exhibited a distinct pattern, demonstrating a sustained increase over time during the release period (Fig. S7A). This validates our hypothesis, suggesting a constant requirement for ATP production during DNA damage repair.

Using U2OS cells expressing a truncated-53BP1 reporter⁷⁴, we confirmed that acute confinement induces an immediate increase in DNA damage foci. However, independently blocking the confinement-induced nuclear ATP surge with either Latrunculin A (Fig. 6A, B) or Oligomycin A (Fig. 6C, D) prevented the formation of 53BP1 foci. To further validate the hypothesis that repressing ATP levels in the context of DNA damage prevents the formation of 53BP1 foci, we treated U2OS cells with etoposide or dimethyl sulfoxide (DMSO) for 3 h, in the presence or absence of Oligomycin A. Etoposide treatment alone resulted in a significantly increased number of 53BP1 foci, which was

lost upon co-treatment with Oligomycin A (Fig. S7B). Additionally, U2OS cells were also subjected to γ -irradiation treatment in the presence or absence of Oligomycin A. Here, too, 53BP1 foci were significantly increased upon DNA damage induction, but not when cells were irradiated in the presence of Oligomycin A (Fig. S7C). To corroborate our findings with the truncated-53BP1 reporter, we conducted a Western blot analysis of γ H2AX, a classical marker of double-strand breaks⁶⁷, in acutely confined cells and observed that mechanical confinement stress resulted in an increase in γ H2AX, which was reduced upon treatment with Oligomycin A (Fig. S7D).

Moreover, we sought to determine whether orthogonal modulations of nuclear ATP levels in acutely confined cells would result in a corresponding alteration in DNA damage levels. For this, we treated cells with BAM15, which also resulted in the suppression of the confinement-induced increase in 53BP1 foci (Fig. S7E, F). As expected, pyruvate treatment alone did not affect the dynamics of 53BP1 foci compared to the untreated control cells. However, the increased nuclear ATP level resulting from the pyruvate treatment was sufficient to partially rescue the suppression of 53BP1 foci induced by Oligomycin A (Fig. S7E, F).

DNA damage repair signalling relies on ATP-dependent chromatin remodelling, which facilitates the formation of 53BP1 foci and initiates subsequent downstream cascades^{71,75,76}. We therefore suspected that the lack of 53BP1 foci detected in confined Oligomycin A-treated cells may be due to the inability of cells to activate DNA damage repair signalling as a result of restricted chromatin accessibility, rather than the prevention of DNA damage itself. To test this hypothesis, we released cells from confinement and Oligomycin A treatment and quantified the accumulation of 53BP1 foci over time, following cell adhesion (Fig. 6E). Three hours after confinement release, U2OS cells which had been subjected to acute confinement, were fully adhered and showed trends of higher levels of 53BP1 foci (Fig. 6F). These foci returned to baseline levels 1 h later, in agreement with the reported dynamics of 53BP1⁷⁷. In contrast, despite the absence of DNA damage in Oligomycin A-treated cells during confinement, 3 h after confinement and Oligomycin A release, cells showed significantly increased levels of 53BP1 foci. These foci persisted for up to 7 h, exhibiting a 3-h delay in DNA damage repair compared to untreated confined cells (Fig. 6F).

Finally, we asked if DNA damage in general, independent of confinement, would also lead to an accumulation of mitochondria at the nuclear periphery. To evaluate this, we treated adherent HeLa cells with DMSO or etoposide for 3 h and quantified mitochondrial levels around the nucleus by using two separate mitochondrial markers, SHMT2 and MTHFD2. Etoposide treatment led to a strong increase in DNA damage as quantified from γ H2AX foci (Fig. S7G–I), but this did not alter levels of either SHMT2 (Fig. S7G, I) or MTHFD2 (Fig. S7H, I) compared to control cells. This suggests that the rapid accumulation of NAM is a specific response to acute confinement-induced DNA damage, but is not induced by DNA damage alone.

Together, our data confirm that the lack of a confinement-induced nuclear ATP surge prevents timely DNA damage signalling and repair.

Nuclear ATP is required for post-confinement S-phase progression and cell proliferation

The absence of DNA damage repair results in cell cycle arrest⁷⁸. Hence, we wondered whether the observed delay in DNA damage repair in confined and Oligomycin A-treated cells led to defects in cell proliferation. To assess this, U2OS cells were acutely confined for 15 min, with or without a short 30-min pre-treatment with Oligomycin A, and then returned to standard culture conditions where they were tracked for 36 h (Fig. 6E). Exposure to this brief mechanical confinement by itself did not significantly alter proliferation of U2OS cells when compared to unconfined control cells over 36 h (Fig. 6G). In contrast, the

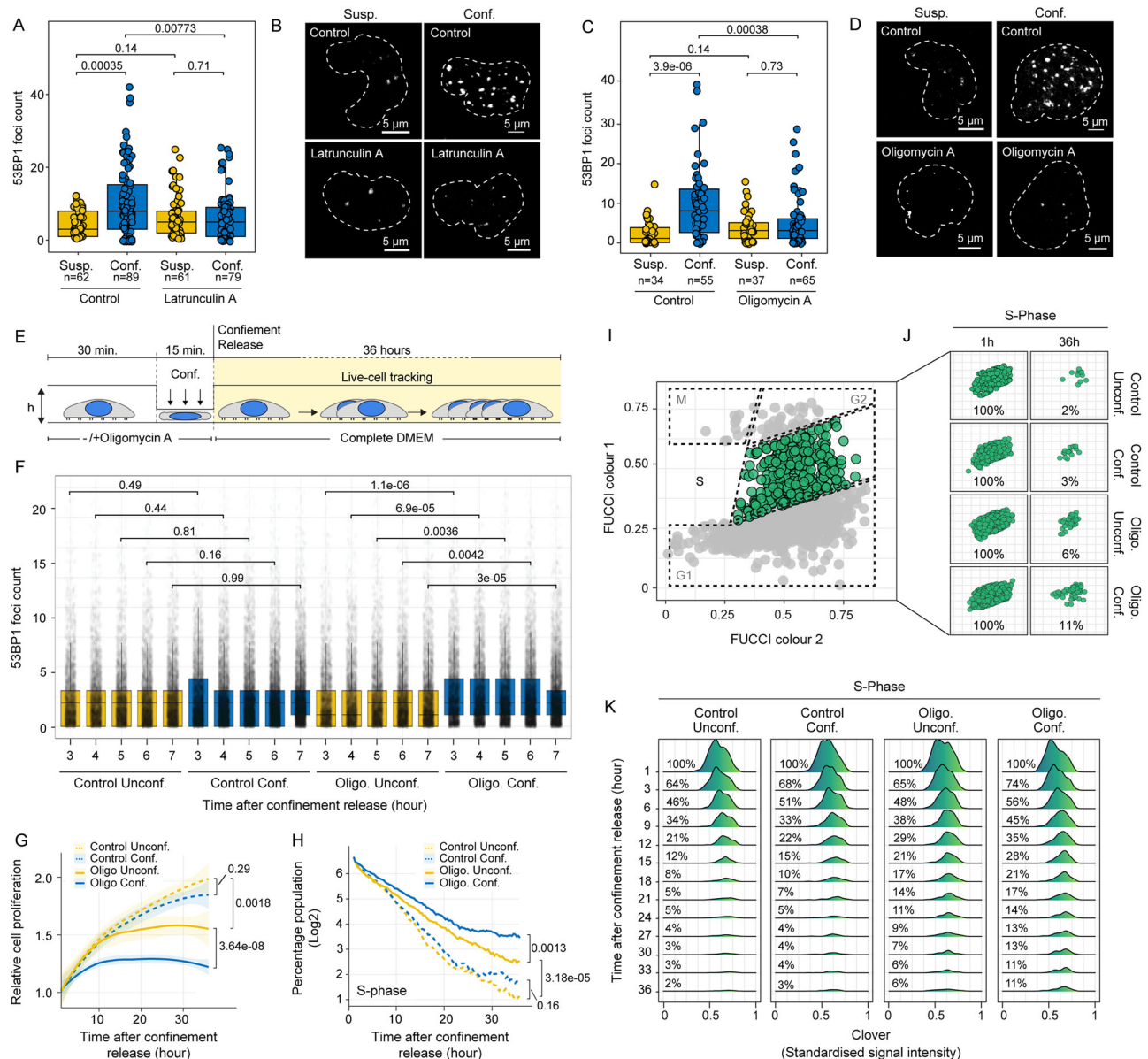


Fig. 6 | Nuclear ATP during confinement is required for efficient DNA damage response signalling and safeguards S-phase progression after confinement release. **A–D** Visualisation and quantification of 53BP1 in U2OS cells expressing a truncated-53BP1-Maroon reporter. Images are maximum intensity projections with nuclear outlines based on transmitted light. Quantification was performed on maximal projections for cells treated with **A**, **B** Latrunculin A (500 nM) or **C**, **D** Oligomycin A (1 μ M) versus controls. Each point represents a cell; n indicates the number of cells analysed. **E** Protocol to study the effect of acute confinement on cell dynamics and DNA damage repair upon confinement release. U2OS-FUCCI cells were given a 30-min treatment of Oligomycin A, followed by acute confinement for 15 min. Confinement and Oligomycin A treatment were then released, cells allowed to attach under normal growth conditions and single cells were tracked for 36 h. **F** Quantification of 53BP1 foci in U2OS cells post-confinement release. Data is from 3 biological replicates (number of cells analysed $n > 3500$). **G** Relative cell proliferation of unconfined or confined U2OS cells under control or Oligomycin A-treated

(1 μ M) conditions. Dark lines are best fit lines based on a generalised additive model, and shaded areas are 95% confidence intervals. At timepoint 1, $n > 2000$ for three biological replicates. **H** Progression of cells detected in the S-phase at the beginning of tracking, monitored for 36 h after confinement release. Data is shown as the mean of three biological replicates. **I** Single-cell analysis of FUCCI expression was performed by spatially distributing cells based on standardised signal intensity (see ‘Methods’). S-phase is shown in green. **J** Single cell representation of cells first detected in the S-phase after confinement release at the first (1st hour) and last (36th hour) time points. **K** Progression of the S-phase population at 3-h intervals. Statistics in **(A, C, F)** were performed using the Wilcoxon–Mann–Whitney test to compare means. Statistics in **(G, H)** were performed using the Wilcoxon test to compare the distribution/ranks of the groups. Box plots represent the lower quartile, median and upper quartile. Whiskers represent the minimum and maximum. See also Fig. S7 for FUCCI, G1-phase and G2 phase.

short 30-min pre-treatment with Oligomycin A alone significantly reduced cell proliferation, suggesting a metabolic stress memory that affects cellular fitness. Notably, this short 30-min Oligomycin A treatment, when followed by an acute 15-min confinement, almost entirely abolished cell proliferation (Fig. 6G). This defect was partially rescued in cells co-treated with pyruvate and Oligomycin A (Fig. S7J), in alignment with the elevated nuclear ATP levels observed upon pyruvate

treatment (Fig. 4B). Pyruvate treatment alone did not alter proliferation compared to untreated cells (Fig. S7J). This suggests that metabolic stress, in addition to acute mechanical confinement stress, sensitises cells towards acquiring a strong mechano-metabolic memory that arrests cell proliferation.

To further explore the effect of transient confinement on cell proliferation, we used an adapted FUCCI system (Fig. S7K)^{73,79}, which

facilitates live cell tracking of the different cell cycle phases (Fig. S7L). For each condition, we grouped the cell populations into the different phases—G1, S and G2—in which they were first detected upon confinement release and analysed their progression into the next cell cycle phases. While confinement or Oligomycin A treatment alone had a significant impact on the progression of G1 (Fig. S7M) and G2 (Fig. S7N) phases, there was little to no additional effect when combining the two treatments. These results suggest that confinement-induced nuclear ATP surge is not critical for G1 and G2 phase progression. Cells confined during the S-phase did not exhibit any delay in progressing to the next cell cycle phase. In contrast, treatment with Oligomycin A resulted in a significant delay in S-phase progression. When confinement was combined with Oligomycin A treatment, this delay in S-phase progression was further exacerbated (Fig. 6H), as evidenced by the increased duration of the S-phase (Fig. S7O). By the end of the 36-h tracking, control and acutely confined cells showed only a small population remaining of the initial S-phase (<2–3%; Fig. 6I–K). In contrast, while Oligomycin A treatment alone increased this remaining population by ~2-fold, the combination of a short Oligomycin A treatment and acute confinement together led to a 4-fold increase in the remaining S-phase population (Fig. 6J, K).

These data support that ATP depletion in mechanical confinement impacts on cell fitness by reducing cell proliferation, with a specific effect on S-phase progression, likely as a consequence of impaired DNA damage repair.

Discussion

Recent work has highlighted the importance of the nucleus as a hub for mechanotransduction, regulating cellular adaptation dynamics to mechanical stress^{20,62,80,81}. However, the effect of mechanical stress in the form of confinement on the subcellular distribution of proteins and metabolites remains largely unknown. Intracellular mitochondrial re-localisation under stress conditions enables energetically intensive processes such as cancer cell migration through crowded tumour microenvironments^{82,83}, and cellular adaptation through mitochondria-nucleus contact sites⁸⁴.

In this study, we described the response of physically confined cells to acute, transient compressive stress that mimics spatial constraints encountered during invasive processes. Given the complexity of *in vivo* mechanical stress dynamics, we focused on a defined, experimentally tractable aspect: how acute mechanical deformation alters nucleus-organelle organisation and triggers adaptive metabolic responses over short and long time scales. Our experimental approach evaluated the response to mechanical stress on a passivated surface, allowing us to isolate the effects of mechanical forces and spatial constraints on cellular behaviour, whilst eliminating the confounding influence of cell-substrate adhesion. We coupled nucleo-cytoplasmic subcellular fractionation with MS to identify an enrichment of mitochondrial proteins in the nuclear proteomic fraction during confinement. High-resolution microscopy confirmed the accumulation of mitochondria at the nuclear periphery (NAM), resulting in nuclear shape changes in the form of indentations. This NAM formation depends on *de novo* actin polymerisation and is associated with an immobilised ER network. The ER and mitochondria have been shown to co-regulate stress responses where actin-mediated MERCs are critical to maintain cellular homeostasis^{29,30}. Moreover, the LINC complexes are central to the mediation of a dynamic stress response between the nucleus, actin cytoskeleton and the ER^{45,46}. Our data also demonstrate that the impairment of actin polymerisation alters the formation of NAM during acute confinement, indicating that the interplay between mitochondria, ER and actin dynamics regulates the localisation of the mitochondria during mechanical stress. Interestingly, we also observed that while depolymerisation of microtubules using Nocodazole did not affect mitochondria in control suspension cells, it led to an increase in the level of NAM formation under

acute confinement. This may suggest that the actin-microtubule co-regulation of NAM formation is important for maintaining a balance between nucleus-associated versus cytoplasmic mitochondria for optimal cellular metabolism. While Nocodazole may indirectly impact actin, given its contrasting effect to actin perturbations in our study, the increase in NAM is most likely a result of microtubule depolymerisation, and not indirect effects on actin. In addition, mitochondrial fusion showed increased levels of NAM compared to mitochondrial fission. Microtubules have been shown to facilitate mitochondrial fission under stress^{37,85}. Therefore, their depolymerisation by Nocodazole likely impairs fission, thereby favouring mitochondrial fusion—an effect that aligns with observations in fission-deficient FIS1 KO cells, in which NAM formation is enhanced. Our findings using either Nocodazole or fission and fusion mutants orthogonally validate the relevance of mitochondrial morphology in NAM formation. The presence of large cell deformations is endogenously observed in various biological contexts during development, tissue homeostasis and cancer, and are commonly caused by mechanical constraints of the 3D tissue environment^{14,15}. Notably, large cell deformations influence the subcellular organisation of cytoskeletal structures and organelles, and particularly the nucleus is influenced by mechanical stress as the largest organelle of the cell^{5,20}. While our findings suggest a structurally adaptive mechanism involving ER and actin cytoskeletal networks facilitates rapid mitochondrial relocation, our study leaves scope for future work to delineate the upstream mechanosensing pathways initiating this response.

At the functional level, the redistribution of the mitochondria in nuclear indentations leads to a nuclear ATP surge during acute confinement. Interestingly, and in agreement with our observation, in germ cell development, recent work has highlighted the ability of mechanical stimuli from surrounding tissues to activate mitochondrial metabolism^{18,86}. We were also able to interfere with the mechanical stress-induced increase of nuclear ATP levels by independently inhibiting mitochondrial ATP synthesis, uncoupling cellular respiration from energy production, arresting actin depolymerisation and perturbing MERCs formation, all of which emphasise the relevance of nucleus-mitochondria proximity for the transfer of energy to the nucleus during mechanical confinement. Moreover, mitochondrial morphology also played an important role in NAM formation. Fused mitochondrial networks favoured a more robust NAM formation and subsequently a bolder confinement-induced nuclear ATP surge, aligning well with the higher mitochondrial energy production capacity in fused mitochondria⁸⁷. Interestingly, we also observed that an excess of pyruvate could rescue the nuclear ATP surge when mitochondrial ATP synthesis is blocked. This may suggest that cells are able to maintain a mechano-metabolic adaptive flexibility independent of their glycolytic or aerobic state. For instance, mechanical stress on tumour cells in crowded environments may be circumvented by lactate uptake and conversion to pyruvate, thus facilitating cell survival and cancer progression.

Our results indicate that actin plays a critical role in the formation of NAM and the associated increase in nuclear ATP under confinement. These data reveal another important role for actin in controlling cellular energy homeostasis. Indeed, actin has previously been shown to play a pivotal role in the regulation of mitochondrial fission⁵¹, cellular homeostasis through mitochondrial turnover and mitophagy⁸⁸, and mitochondrial trafficking through association with mitochondrial myosins⁸⁹. Moreover, in the absence of the nuclear ATP surge, chromatin accessibility decreases, particularly at genomic regions associated with actin remodelling. This suggests a feedback loop between the actin cytoskeleton and nuclear metabolism that may be essential for cells to withstand mechanical stress.

Among the many instances of mechanical confinement-induced stress in development and diseases^{90–92}, cancer cells must overcome important mechanically confining processes that challenge cell fitness

and survival, calling for buffering mechanisms against mechanical stress. For instance, compressive mechanical stress has been shown to regulate cancer cell spheroid growth, by inducing a G1 arrest due to volume limitation⁹³, or causing cell proliferation arrest and apoptosis⁹⁴. Previous work has also highlighted that nuclear ATP levels regulate DNA duplication^{95,96}, chromatin reorganisation and DNA damage repair⁶⁰, all of which are critical processes for S-phase progression. Our study, notably, highlights the short- and long-term response of cancer cells to acute mechanical stress. Confinement, within a matter of seconds, induces a rapid NAM formation and consequently nuclear shape changes and increases nuclear ATP, which is required for chromatin rearrangements⁶⁰ and DNA damage repair⁶². Pharmacological inhibition of the nuclear ATP surge significantly reduced chromatin accessibility, thus delaying DNA damage repair and affecting cell cycle progression. Nevertheless, our results also suggest that DNA damage alone does not induce NAM accumulation and that it is only in the context of a physical confinement stimulus that the nucleus-mitochondria association is required to develop a significant nuclear ATP pool, assuming a prepared state to combat stress. It is noteworthy that disrupting this metabolic nuclear adaptation during confinement markedly affects chromatin accessibility at genes involved in actin remodelling. This finding supports the notion that actin plays a crucial role in maintaining the physical and functional connection between mechano-stress adaptations and metabolic responses. Moreover, it suggests that the absence of a nuclear ATP surge during confinement may potentially establish a negative feedback loop that affects actin dynamics, thereby impairing cell mobility and, consequently, survival.

In essence, our results identify an adaptive mechano-metabolic response of cells to acute confinement stress and reveal how just a short (minute scale) confinement stress is sufficient to affect post-confinement cell fitness on the long term (hours to days). Nuclear ATP in this context serves as a mechano-responsive effector regulated by subcellular mitochondrial-nucleus remodelling under mechanical stress, with physiological relevance for effective DNA damage repair and cell proliferation in challenging physical tissue environments.

Methods

Cell culture

Unless otherwise mentioned, HeLa (ATCC), U2OS (ATCC), MIA PaCa-2 (ATCC) and MDA-MB-231 (ATCC) cells were maintained in complete Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% Fetal Bovine Serum (FBS, Gibco) at 37 °C and 5% CO₂. Cell washes were performed with phosphate-buffered saline and detachment using Trypsin (Gibco).

Generation of Mfn1, Drp1 and Fis1 KO HeLa cells

KOs for Mfn1, Drp1 and Fis1 were produced in HeLa cells^{47,48}. Briefly, 25 × 10⁴ HeLa cells were seeded on 6-well plates. Transfection solution mix was prepared with Lipofectamine 2000 (11668019, Invitrogen™) and plasmids for Mfn1 (Mfn1 CRISPR/Cas9 KO Plasmid, sc-400637), Drp1 (DRP1 CRISPR/Cas9 KO Plasmid, sc-400459), and Fis1 (FIS1 CRISPR/Cas9 KO Plasmid, sc-401107). All CRISPR/Cas9 KO plasmids were purchased from Santa Cruz Biotechnology. Forty-eight hours after transfection GFP-positive cells were single-cell sorted on 96-well plates. KO validation of clones was performed by Western blot analysis using an antibody against Fis1 (GTX111010; GeneTex), Drp1 (8570 (D6C7), Cell Signaling) or Mfn1 (14739 (D6E2S), Cell Signaling).

Subcellular fractionation

Subcellular fractionation was performed using a soft lysis buffer (1% CHAPS (cholamidopropyl dimethylammonium 1 propane sulphonate), PBS) and a harsh lysis buffer (0.1% IGEPAL, 20 mM Hepes pH 7.5, 300 mM NaCl, 0.2 mM EDTA). Both buffers contained 'complete'

proteinase inhibitors (Roche) according to the manufacturer's directions. Suspension or confined cells (using the static confiner) were left in the soft lysis buffer for 5 min, followed by centrifugation at 1 × 10³ relative centrifugal force to obtain the cytoplasmic fraction as the supernatant, and intact nuclei as the pellet. The pellet was then resuspended in the harsh lysis buffer to obtain the nuclear fraction. Fractionation was validated through Western blot.

Western blot

Following acquisition and lysis of samples using respective buffers, samples were homogenised using the ICOPUS Insulin Syringe (Twister Medical, N14080) or sonicated (30 s ON, 30 s OFF, 10 cycles) using the Bioruptor Pico (Diagenode). Samples were quantified using the BCA protein assay kit (Thermo Scientific PIER23225) and mixed with 4× Laemmli sample buffer (Bio-Rad) and boiled at 95 °C for 5 min. Proteins were separated by gel electrophoresis on homemade 4–20% SDS-polyacrylamide gels and detected with the following antibodies: β-actin (A1978, Sigma-Aldrich), Vinculin (Santa Cruz Biotechnology sc-25336, or Cell Signalling Technology 13901S), Tubulin (Sigma-Aldrich, T9026), H3 (Cell Signalling Technology 14269), H3K27me3 (Diagenode, C15410195), H3K9me3 (Diagenode C15410193), γH2AX (Sigma-Aldrich, 05-636), Fis1 (GTX111010; GeneTex), Drp1 (8570 (D6C7), Cell Signalling) or Mfn1 (14739 (D6E2S), Cell Signalling) antibodies. Uncropped blots are presented in Supplementary Information.

Proteomics

Sample preparation for LC-MS/MS analysis. For MS analysis, a total of 12 samples were analysed, including 3 biological replicates. Samples were processed using adapted Single-Pot solid-phase-enhanced sample preparation (SP3) methodology⁹⁷. Briefly, equal volumes (125 µl containing 6250 µg) of two different kinds of paramagnetic carboxylate modified particles (SpeedBeads 45152105050250 and 65152105050250; GE Healthcare) were mixed, and washed three times with 250 µl water and reconstituted to a final concentration of 50 µg/µl with LC-MS grade water (LiChrosolv; MERCK KGaA). Samples (~100 µL) were filled up to 200 µL with LC-MS grade water and mixed with 2× sample buffer (4% SDS, 100 mM HEPES, pH 8.0) to a final concentration of 2% SDS, and proteins were reduced with a final concentration of 10 mM DTT and incubated at 56 °C for 1 h. After cooling down to room temperature, reduced cysteines were alkylated with iodoacetamide at a final concentration of 55 mM for 30 min in the dark. For tryptic digestion, 400 µg of mixed beads were added to reduced and alkylated samples, vortexed gently and incubated for 5 min at room temperature. The formed particles-protein complexes were precipitated by the addition of acetonitrile to a final concentration of 70% [V/V], mixed briefly before incubating for 18 min at room temperature. Particles were then immobilised using a magnetic rack (DynaMag-2 Magnet; Thermo Fisher Scientific) and the supernatant was discarded. SDS was removed by washing two times with 200 µl 70% ethanol and one time with 180 µl 100% acetonitrile. After removal of the organic solvent, particles were resuspended in 100 µl of 50 mM NH₄HCO₃ and samples were digested by incubating with 2 µg of Trypsin overnight at 37 °C. Samples were acidified to a final concentration of 1% Trifluoroacetic acid (Uvasol; Merck KGaA) prior to immobilising the beads on the magnetic rack. The whole digest was desalted and concentrated using stage tips with two stacked C18 plugs (Empore; MERCK KGaA). Stage tips were activated with three times 100 µl acetonitrile and equilibrated with three times 100 µl of 0.4% formic acid, 2% TFA in water before loading the samples. Salts were cleaned up with 100 µl of 0.1% TFA and peptides were eluted using two times 50 µl 90% acetonitrile, and 0.4% formic acid. Finally, eluates were dried in a vacuum concentrator and reconstituted in 10 µl of 0.1% TFA. After solvent removal in a vacuum concentrator, samples were reconstituted in 5% formic acid for LC-MS/MS analysis.

Liquid chromatography coupled to tandem mass spectrometry LC-MS/MS. MS was performed on an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled to a Dionex U3000 RSLC nano system (Thermo Fisher Scientific, San Jose, CA) via a nanoflex source interface. Tryptic peptides were loaded onto a trap column (Acclaim™ PepMap™ 100 C18, 3 μ m, 5 $\times$ 0.3 mm, Fisher Scientific, San Jose, CA) at a flow rate of 10 μ L/min using 2% acetonitrile in 0.1% TFA as loading buffer. After loading, the trap column was switched in line with a 50 cm, 75 μ m inner diameter analytical column (packed in-house with ReproSil-Pur 120 C18-AQ, 3 μ m, Dr. Maisch, Ammerbuch-Entringen, Germany). Mobile-phase A consisted of 0.4% formic acid in water and mobile-phase B of 0.4% formic acid in a mix of 90% acetonitrile and 10% water. The flow rate was set to 230 nL/min and a 90 min gradient used (4 to 24% solvent B within 82 min, 24 to 36% solvent B within 8 min and, 36 to 100% solvent B within 1 min, 100% solvent B for 6 min before re-equilibrating at 4% solvent B for 18 min). Analysis was performed in a data-dependent acquisition mode. MS⁹⁸ spectra were acquired with a mass range of 375–1650 m/z in the Orbitrap at a resolution of 120,000 (at m/z 200). Automatic gain control (AGC) was set to a target of 2×10^5 and a maximum injection time of 50 ms. Precursor isolation width was set to 1.6 Da and HCD normalised collision energy to 30%. MS2 scans were acquired in the linear ion trap (LIT) in rapid scan mode using higher energy collision-induced dissociation (HCD) and a normalised collision energy of 30%. The intensity threshold for precursor selection was set to 1×10^3 with a quadrupole isolation window of 1.6 Da. AGC was set to a target of 1×10^4 and a maximum injection time of 50 ms. Additional parameters were MIPS enabled for peptide selection, charge state inclusion of 2–6 and dynamic exclusion for selected ions set to 60 s. A single lock mass at m/z 445.120024 for MS1 mass correction was employed⁹⁹. XCalibur version 4.3.73.11 and Tune 3.3.2782.28 were used to operate the instrument. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE¹⁰⁰ partner repository with the dataset identifier PXD043524.

Data analysis. Acquired raw data files were processed using Proteome Discoverer 2.4.1.15 SP1. Database search within PD 2.4 was done using the Sequest HT algorithm and Percolator validation software node (V3.04) to remove false positives with strict filtering at a false discovery rate (FDR) of 1% on peptide spectrum match (PSM), peptide and protein levels. Searches were performed with full tryptic digestion against the human SwissProt database V2017.06 including a common contamination list with up to two miscleavage sites. Oxidation (+15.9949 Da) of methionine was set as variable modification, whilst carbamidomethylation (+57.0214 Da) of cysteine residues was set as fixed modification. Data was searched with mass tolerances of ± 10 ppm and 0.6 Da on the precursor and fragment ions, respectively. Results were filtered to include PSMs with Sequest HT cross-correlation factor (Xcorr) scores of ≥ 1 and high peptide confidence. For analysis, chromatin data were normalised using the `normalize_vsn` and `median_normalisation` functions from the DEP¹⁰¹ and proDA¹⁰² packages, respectively. The rest of the pipeline was followed according to the DEP package, with the inclusion of `impute.mi()` function for protein-imputation from the `imp4p` package¹⁰³. Known subcellular localisations for proteins were obtained from the pRoloc package¹⁰⁴. For the enrichment, the clusterProfiler R package was used^{105,106} while CORREP was used for the correlations and clustering¹⁰⁷. REVIGO¹⁰⁸ was used to prune semantically similar GO terms.

Cell confinement

Two different confinement methods were used in this study.

Static cell confinement. Cells were confined in a custom-built device under PBS-based 4% (w/v) low melting point agarose (Agarose Ultra-pure Low Melting Point, Invitrogen) pads with a thickness of 6 mm

(Fig. 1A). Three pistons were used to press down on a plastic disk placed on top of the agarose pad to ensure a homogenous pressure distribution. Treatment buffers or reagents were injected through holes in the disk and through the agarose pads, directly to the layer of confined cells. After treatment, the agarose confinement was released, and samples were processed as described. This confinement approach was used to perform protocols that required the introduction of buffers or reagents whilst under confinement as for proteomics and western blot.

Dynamic cell confiner. Cells were confined using a pressurised dynamic confiner (4DCell) like the previously established planar microconfinement methods (Fig. S2A)¹⁰⁹. To confine cells at 3 μ m height, PDMS microspacers were made from a Si mould that was produced by photolithography in a clean room (Nanofabrication Laboratory, ICFO) by depositing an SU-8 resin on a silicon wafer. Using the mould, 10-mm confinement coverslips were prepared with polydimethylsiloxane (PDMS). Coverslips and dishes were plasma cleaned, coated with 0.5 mg/ml PLL(20)-g[3,5]-PEG(2) (SuSoS), and equilibrated in respective cell culture media before each experiment. Confinement chambers were assembled using glass-bottom dishes (#1.5, MatTek Corporation) and a pressure generator (LineUp™ Flow EZ™ Fluigent) connected to a suction cup to change the pressure for tuning the confinement heights. Validation of the correct height was performed by measuring the cell height in an x-z reslices. This confinement approach was used to perform microscopy-based protocols.

Cell staining and treatments

For the visualisation of mitochondria and lysosomes, live cells were incubated using 50 nM MitoTracker Deep Red (Thermo Fisher, M22426) or 75 nM LysoTracker Red DND-99 (Thermo Fisher, L7528), respectively, for 30 min. For the visualisation of late endosomes or peroxisomes, live cells were incubated for 16 h with respective Cell-Light BacMam 2.0 probes (Thermo Fisher, Peroxisome: C10604, Late Endosome: C10588). To visualise nuclei, 1 mg/ml DNA-Hoechst was incubated for 5 min. For imaging with the mitochondrial FRET sensor and centromeres, 1 μ M SiR-DNA (Spirochrome, SC007) incubation for 5 h together with 5 μ M Verapamil (Spirochrome, CY-SC001) was used to visualise nuclei. Post incubation, cells were trypsinized and resuspended in complete DMEM. To visualise the actin or microtubule cytoskeleton in live cells, cells were incubated using 100 nM SiR-Actin (Spirochrome, CY-SC001) or 500 nM SiR-Tubulin (Spirochrome, CY-SC002), respectively, for 16 h or overnight, together with 5 μ M Verapamil (Spirochrome, CY-SC001).

For imaging experiments using transient expression, live cells were nucleofected using the Lonza Cell Line Nucleofector Kit V (VCA-1003) 48 h prior to the experiment. Whole-cell visualisation was done using pmaxGFP (included in the Lonza kit). Inner nuclear membrane visualisation was done using a pCS2 LAP2 β -eGFP plasmid⁵. ER visualisation was done using a pmTurquoise2-KDEL plasmid (Addgene #36204), a gift from Dorus Gadella¹¹⁰. Nuclear and mitochondrial ATP were visualised using the previously published FRET-based ATeam ATP sensors⁵².

For treatment with pharmacological agents, cells were incubated in media containing Oligomycin A (1 μ M, MedChem Express HY-16589), CK-666 (100 μ M, Tocris #3950), Latrunculin A (500 nM, Sigma L5163), Jasplakinolide (500 nM, Tocris #2792), Nocodazole (10 μ M, Sigma M1404), BAM15 (10 μ M or 50 μ M, Merck Life Sciences #SML1760) or BAPTA-AM (10 μ M, Cayman #15551.5) for 30 min; and SMIFH2 (100 μ M, Sigma S4826) for 1 h. Experiments involving Oligomycin A treatment were either carried out in glucose-free DMEM or in complete DMEM. Where glucose-free DMEM was used, this was kept consistent for both treatment and control conditions. During imaging, cells were maintained in media containing the respective drugs at the indicated concentrations.

Treatment with pyruvate was performed by the addition of 3 mM pyruvate (Gibco) to the culture media for 30 min prior to imaging. Treatment was done either by itself or in combination with Oligomycin A.

For CellROX treatment, in the case of the suspension condition, cells were resuspended in PBS containing 5 μ M CellROX (Thermo Scientific, C10444). These cells were plated on plasma-cleaned dishes, coated with 0.5 mg/ml PLL(20)-g[3,5]-PEG(2) (SuSoS) for 30 min. Following this, imaging was done. In the case of confinement, cells were resuspended in PBS containing 5 μ M CellROX and incubation was performed under 3 μ m confinement using the dynamic confiner for 30 min, following which imaging was performed.

For immunofluorescence, cells were seeded in a tissue culture treated, black, 96-well optical bottom plates (Perkin Elmer, 6055308) and, following indicated drug treatments, fixed using 4% PFA (Thermo Fisher Scientific, 28908) for 20 min at room temperature. Fixed cells were then permeabilised using 0.25% Triton X-100 in PBS for 15 min at room temperature and blocked using 1% BSA in PBST (PBS+ 0.1% Tween 20) for 30 min at room temperature. α -Tubulin staining was performed using 1:1000 anti- α -Tubulin primary antibody (Sigma-Aldrich, T6199) for 1 h at room temperature. Actin staining was performed using 1:40 Phalloidin (Thermo Fisher Scientific, A12379) together with 1:250 Alexa Fluor 633 secondary antibody (Thermo Fisher Scientific, A-21050) for 1 h at room temperature. 1:1000 DAPI (Sigma-Aldrich, MBD0015) staining was performed for 5 min at room temperature. All steps were separated by 2–3 PBS washes. Fixed and stained cells were stored in PBS at 4 °C until imaged.

Tissue microarray (TMA) analysis

Breast cancer tissue microarrays (Tissue Array, BR725) were processed for immunofluorescence staining. Sections were deparaffinized and subjected to antigen retrieval by boiling in pH 9 buffer (Vector Laboratories, H-3301-250) for 40 min. After cooling, slides were blocked in 10% goat serum for 1 h at room temperature. The primary antibody anti-SDHB (mouse, Abcam ab14714, 1:25) was applied overnight at 4 °C in a humidified chamber. Following incubation, slides were washed three times for 10 min each in PBS containing 0.05% Tween 20. Alexa Fluor 555-conjugated anti-mouse secondary antibody was applied at 1:200 for 1 h at room temperature, followed by an identical wash procedure. Slides were mounted with Fluoromount-G containing DAPI (Invitrogen, 00-4959-52).

Imaging was performed using the Operetta High Content Screening System (PerkinElmer), and nuclei were segmented using the in-built Harmony analysis software. Nuclei were classified based on size, shape, and DAPI intensity to distinguish epithelial-like from stromal-like nuclei. To quantify nuclear-associated mitochondria (NAM), nuclear regions of interest (ROIs) were defined in each z-plane. Due to the lower resolution of TMA imaging compared to single-cell microscopy, only the nuclear ROI was used without an additional 10% expansion. For each z-slice, the raw integrated density (total pixel intensity) within the nuclear ROI was measured. The average intensity per nucleus was calculated by averaging values across all z-slices, with each mean intensity value representing a single data point.

ATAC-sequencing sample preparation

HeLa cells were treated with or without Oligomycin A (1 μ M) for 30 min in glucose-free complete media. Cells were then trypsinized and either left in suspension (controls) or confined using a hybrid confiner. Briefly, cells were confined in glass-bottom 6-well plates (adapted from 4D Cell, CSOW 610) under coverslips with microspacers of 3 μ m height. Both coverslips and the glass-bottom plate were plasma-cleaned and treated with 0.5 mg/ml PLL(20)-g[3,5]-PEG(2) (SuSoS) to prevent attachment. Following an acute 15-min confinement, 0.5×10^5 cells for three biological replicates, for each treated condition, were collected and treated with transposase Tn5 (Nextera Tn5 Transposase; Illumina

Cat #FC-121-1030). DNA was purified using AMPure XP beads to remove large fragments ($0.5 \times$ beads; >1 kb) and small fragments ($1.5 \times$ beads; < 100 bp). Samples were then amplified using NEBNext high-fidelity 2x PCR Master Mix (New England Labs #M0541) with primers containing a barcode to generate the libraries, as previously described¹¹¹. Each condition was amplified using a combination of the P7 forward primer and a P5 reverse primer containing multiplexing adaptors (Supplementary Table 1) and sequenced accordingly to allow for segregation of reads. The number of cycles of library amplification was calculated as previously described¹¹². DNA was purified using a MinElute PCR Purification Kit (Qiagen), and samples were sequenced using an Illumina NextSeq2000 at the CRG Genomics Core Facility.

ATAC-sequencing analysis

Fifty base-pairs paired-end reads were subjected to alignment against the hg19 human genome using the Bowtie2 aligner (v 2.5.1)¹¹³ after removal of adaptors with TrimGalore (v. 0.6.10). The alignment was carried out with default parameters, except for the utilisation of the ‘very-sensitive 2000’ option. To ensure the quality of aligned reads, those with a quality score below 30 were filtered out using Samtools 1.16^{114,115}. Mitochondrial reads were excluded from further analysis followed by removal of duplicated reads by using Picard (v 2.23.8). The alignment of reads was adjusted to account for Tn5 transposase insertions with deepTools (v 3.5.1). Peaks were identified using MACS2 (v 2.2.8) with a FDR threshold of less than 0.01. Regions designated as blacklisted were filtered out from further analysis (blacklist v2). Peak intersection between replicates and union between conditions was done using the GenomicRanges package in R (V4.4). Differentially accessible (DA) regions were quantified utilising the csaw R package¹¹⁶ with Trimmed Mean of M-values (TMM) normalisation on binned counts, followed by edgeR¹¹⁷ for DA analysis, employing a significance threshold of log-fold change > 0.5 and FDR < 0.1.

Volcano plots to visualise differentially accessible chromosome regions were plotted using the log-fold change and *p* value computed as above. Significance was *P* value < 0.05 and log-fold change greater than 2 in either positive or negative directions. To further analyse significant changes, peaks were assigned to genes and ORA was performed using clusterProfiler (version 3.0.4)^{105,106} R package. All genes significantly enriched or depleted in either control or Oligomycin A treatment were pooled to identify general pathways showing differential changes upon confinement. To produce heatmaps specific to the enriched GOs, genes related to GO Terms of interest were retrieved from the AnnotationDbi R package. Raw read counts for each gene obtained from csaw were aggregated according to peak identity as annotated by ChIPSeeker^{118,119} package, batch-corrected, and processed using variance stabilised transformation (vst) with DESeq2¹²⁰ function and sample-wise normalisation. Heatmaps were visualised using the log2-fold change associated with the promoter region of all genes in the GO that were identified in our ATAC-Seq dataset.

ATAC-seq peaks across the genome were visualised using circos plots, generated with the Circize R package¹²¹. Chromatin accessibility was normalised by chromosome length, and the colour scale in the circos plot represents this normalised accessibility. This was calculated by dividing the total accessible region (in base pairs) for each chromosome by the respective chromosome length. Peaks are displayed according to their chromosomal location, and the colours of the chromosomes reflect their normalised accessibility levels.

Changes in accessibility in confinement with respect to suspension, at the chromosome level, were visualised using a heatmap of confinement/suspension ratio of normalised accessibility. The heatmap was produced using the ComplexHeatmap package¹²², and heatmap annotations for chromosome size and chromosome gene density were shown based on previously published data¹²³. Ratio values greater than 1 represent chromosomes that are on average more accessible in

confinement, while ratio values less than 1 represent chromosomes that are on average more accessible in suspension.

Metabolomics

Metabolomics data were previously published⁷³. Briefly, U2OS cells were treated with 1 μ M etoposide for 3 h at staggered intervals. At the final time point, the medium was refreshed in all wells for consistency. Each condition was performed in triplicates. Cells were washed with 75 mM ammonium carbonate buffer (pH 7.4), and placed on ice, and metabolites were extracted with 80:20 ice-cold methanol. Samples were scraped, snap-frozen in liquid nitrogen, thawed, and centrifuged at 4 °C, and supernatants were stored at –80 °C.

Metabolites were analysed on an Agilent 1290 Infinity II UHPLC coupled to a 6470 triple quadrupole mass spectrometer, using a ZORBAX RRHD Extend-C18 column (2.1 $\times$ 150 mm, 1.8 μ m) with gradient elution. MS was operated in negative ESI mode, and metabolites were detected in dynamic MRM mode.

Absolute quantification was achieved with ten-point calibration curves and internal standardisation in MassHunter software (Agilent Technologies). Differential analysis was conducted using the DEP R package, and pathway analysis was performed with decoupleR's run_mean function. Raw metabolomics data are available on the Metabolomics Workbench (ID: ST002234). Heatmap generation was done by obtaining a z-score for each metabolite group, across the different time points or conditions.

Centromeres and nucleoli

Analysis of nucleoli and centromeres was performed using HeLa cells expressing a CENPA-GFP reporter as previously described¹²⁴, a gift from Patrick Meraldi. Cells were stained using 1 μ M SiR-DNA (Spirochrome, SC007) for 5 h together with 5 μ M Verapamil (Spirochrome, CY-SC001) to visualise nuclei.

Images were analysed using a custom-written ImageJ macro. The DNA and CENPA-GFP channels were split, and the nucleus was segmented from the DNA channel, excluding edge-touching objects. To detect nucleoli, the DNA signal was inverted, subjected to Gaussian blur ($\sigma = 2$), and an intensity threshold was applied. Nucleoli were detected as particles larger than 1 μ m² within the nuclear region.

For each nucleolus, a boundary ROI (ring region) was generated by computing the XOR between its dilated (+0.25 μ m) and eroded (–0.25 μ m) forms. These rings were used to identify nucleoli-associated CENPA foci. The percentage of centromeres at the nucleolar periphery was then calculated as the number of CENPA foci within the nucleolar boundary ROI (ring region), divided by the total number of CENPA foci in the nucleus.

Cell cycle analysis and proliferation

A previously established stable U2OS cell line with a FUCCI system was used⁷³. Briefly, our FUCCI system is an adaptation of FUCCI4⁷⁹, showing 3 cell cycle-regulated fusion proteins: Clover-Geminin, SLBP-Turquoise2, and Cdt1-mKO2 (Fig. S6H). Fluorescence from tracked live cells was measured using the Operetta High Content Screening System (Perkin Elmer), using 20 $\times$ magnification (see ‘Microscopy’ methods for more details). Control or treated cells were confined for a duration of 15 min, following which they were released and seeded in cell culture plates. Fluorescence of FUCCI probes was then tracked using live cell imaging for 36 h. Quantification of the cell cycle was based on fluorescence from the FUCCI proteins and was performed using a custom R script. Briefly, fluorescence values were standardised between 0 and 1, and cell cycle status visualised as XYZ plots (x-axis = SLBP-Turquoise2, y-axis = Clover-Geminin, z axis plotted as colour = Cdt1-mKO2) (Fig. S6I). Cell cycle phase gates—G1, S, G2 and M—were dynamically assigned based on their distribution. Further analysis to calculate the percentage population abundance in various phases, cell cycle progression through various phases, and

time spent in a specific phase were all based on the above-mentioned gating strategy.

For cell proliferation, the same U2OS-FUCCI cell line was used. Cells were either left untreated or treated with Oligomycin A (1 μ M, in glucose-free media), pyruvate (3 mM), or pyruvate and Oligomycin A both, for a period of 30 min. Confinement was applied for 15 min in respective treatment conditions. Following confinement release, cells were seeded in complete DMEM without any treatment, and cell count was quantified using the Operetta High Content Screening System (Perkin Elmer), over a period of either 36 or 24 h. Relative cell proliferation was analysed by normalising the total cell count to that of the first time point.

DNA damage

DNA damage foci were quantified using a stable U2OS cell line expressing an mMaroon1-53BP1trunc reporter. Briefly, the reporter cell line was created by replacing the mApple fluorophore in the Apple-53BP1trunc Addgene vector #69531, a gift from Ralph Weissleder⁷⁴, with a mMaroon1 fluorophore derived from the Addgene vector #83842, a gift from Michael Lin⁷⁹. Using this vector, a stable U2OS cell line was created using lentivirus transduction. Cells were either treated or left untreated with Oligomycin A (1 μ M, in glucose-free media) or Latrunculin A (500 nM) or pyruvate (3 mM) or BAM15 (10 μ M) or in combination, as described above. Confinement was performed using the dynamic confiner and confocal microscopy to quantify foci during confinement. Quantification was done using Fiji¹²⁵ and was based on maximum projections to detect individual foci using thresholding and analyse particle features in Fiji. To quantify foci post-confinement release, confinement was released, and cells were seeded in 96-well plates under normal growth conditions and fluorescence from tracked live cells was measured using the Operetta High Content Screening System (Perkin Elmer).

DNA damage analysis using etoposide treatment and irradiation treatment was also done in adherent U2OS cells expressing an mMaroon1-53BP1trunc reporter or with adherent HeLa cells. For etoposide treatment, cells were either left untreated or treated with Oligomycin A (1 μ M) for 45 min. Following this pre-treatment, cells were either treated with DMSO or etoposide (1 μ M) for 3 h in the presence or absence of Oligomycin A. In the case of using untagged HeLa cells, primary antibody staining was performed using 1:500 γ H2AX (Sigma-Aldrich, 05-636), 1:600 SHMT2 (Proteintech 11099-1-AP) and/or 1:500 MTHFD2 (Abcam; #ab151447) using the immunofluorescence protocol described above in ‘Cell staining and treatments’. For irradiation treatment, cells were either left untreated or pre-treated with oligomycin A (1 μ M) for 45 min, following which 10 Gy gamma-irradiation was applied using an IBL-437C irradiator (CIS Biointernational). Imaging and quantification of 53BP1 foci were performed using the Operetta High Content Screening System (Perkin Elmer).

Microscopy

Image acquisition was performed on different microscopes as indicated below. All live cell microscopy was performed at 37 °C and 5% CO₂. Confocal fluorescence images were acquired using one of two systems.

The Leica STELLARIS microscope was equipped with 405, 448, 488 nm semiconductor lasers and a white light laser source (Leica Microsystems, Wetzlar, Germany). Fluorescence was detected using HyD detectors in photon counting mode for quantification. Transmission light was collected using a forward PMT. Image acquisition was performed using a 63 $\times$ NA 1.4 oil objective. For FRET measurements, a 448 nm laser was used for CFP excitation, and emitted photons were collected using a HyD detector in photon counting mode in the 458–504 nm range (EX_{CFP}/Em_{CFP}) and in the 524–600 nm range (EX_{CFP}/Em_{YFP}, which would correspond to the FRET signal). A 514 nm laser was used for YFP excitation, and emitted photons were collected using a HyD detector in photon counting mode in the 524–600 nm range (EX_{YFP}/Em_{YFP}).

The Leica TCS SP8 microscope was equipped with 405, 458, 488, 561 and 633 nm semiconductor lasers. Fluorescence was detected using two PMT detectors and/or a HyD detector. For FRET measurements, the CFP was excited using the 458 nm and emitted photons were collected using the PMTs in 468–504 nm and 524–600 nm ranges and the YFP with the 514 nm laser and emitted photons were collected using the PMTs in the 524–600 nm range. Image acquisition was performed using a 63× NA 1.4 oil objective.

Live cell time-lapse imaging was performed using the Operetta High Content Screening System (Perkin Elmer) at 37 °C and 5% CO₂, equipped with a 20× air high NA objective and 8 LEDs (excitation at 365, 405, 440, 475, 475, 510, 550, 630, 660 nm). Experiments were performed using excitation/emission filters 390-420/430-500 for Turquoise2, 460-490/500-550 for Clover, 530-560/570-650 for mKO2 and 615-645/655-760 for Maroon1.

Image analysis

Raw image data were analysed and quantified using the Fiji software¹²⁵ or MATLAB¹²⁶, unless otherwise mentioned.

Nucleus-associated mitochondria (NAM) quantification. Nuclear ROIs were defined for individual cells based on the nuclear Hoechst signal at every z-plane. For NAM quantification, ROIs were then enlarged by 10% to measure the mitochondria that were within the enlarged nuclear ROI. For each z-slice, the total pixel intensity (raw integrated density) within the ROI was quantified. The average intensity per cell was then calculated as the mean of all z-slices per cell and plotted as a single data point.

Where the same cell line was used, zero mean normalisation was performed to compare across treatments, as indicated. To maintain the relative changes between suspension and confinement conditions, zero mean normalisation for a particular treatment was performed by subtracting the mean of the suspension cells from both, suspension and confinement values, and then dividing by the standard deviation (SD) of the suspension. Following this, for representation, suspension conditions, which now all have a mean of 0 and a SD of 1, were plotted together, and confined conditions were shown separately.

In silico reconstruction. Representative 3D-stack images of nuclei were processed using built-in Fiji functions—threshold, dilate, erode, fill holes, and Gaussian blur—and saved as individual slice masks. The raster package in R¹²⁷ was then used to extract binary pixel values along a predefined grid of X and Y coordinates. The slice number multiplied by the Z height was used to determine the Z coordinate. The extracted data were compiled into a spatial data frame, where the maximum Z value for each XY coordinate was recorded where the signal was detected. This data was smoothed using a Gaussian blur with a kernel size of 10 and an SD of 0.5. Z-values below 0.1 were set to NA to remove low-intensity noise. Visualisation was performed as a 3D surface plot using the Plotly library¹²⁸.

Nucleus-mitochondria co-localisation analysis. Representative cell line profiles (Fig. 2E, F) were measured along defined distances across the cell with a 10-pixel thickness on a sum projection image. (i) A line was drawn from the centre of the nucleus to the 10% enlarged nuclear ROI; (ii) Quantification for the two signals (Hoechst and MitoTracker) was done; (iii) Line graphs of signal intensities were depicted as standardised signal intensity using MATLAB.

Mitochondria shape analysis. Mitochondria shape was evaluated using the MitochondriaAnalyzer¹²⁹. Analysis was performed as described in the manual. Briefly, 3D-stack images of confined HeLa cells, stained with MitoTracker, were used to first perform thresholding using the in-built ‘3D Threshold’ function. Thresholding was

empirically optimised for each cell line. On average, block size, which determines the size of the object, ranged between 1.5 and 2.5 μm, and the C-Value, which is used to regulate noise in the image, ranged between 2 and 3. Following thresholding, the ‘3D Analysis’ function was used to measure the total branch length, number of branch junctions and sphericity of the mitochondria.

Perinuclear actin. Cells were imaged with SiR-Actin and Hoechst and analysed using Fiji and cell image analysis software Cell Profiler¹³⁰. To perform the analysis, first using Fiji, the Hoechst signal was used to create a nuclear mask by thresholding. Next, a Cell Profiler was used to create a ring region ROI around the nucleus which was defined as 10-pixel larger than the nuclear circumference to 10-pixel smaller than the nuclear circumference. Mean actin intensity was then measured per slice within this perinuclear ROI and averaged per cell. Analysis was performed on adhesion, suspension and confined cells, and data were normalised using a modified zero mean normalisation. Briefly, values for adhesion, suspension and confinement were normalised using the SD and mean of the adhesion condition per cell line.

Organelle co-localisation analysis. Signal intensities were measured on the cell basal side along lines of defined distances with a 10-pixel thickness. (i) NAM-ER, NAM-late endosome, NAM-lysosome and NAM-peroxisome general co-localisation (wide zoom, Fig. 3L, Nuclear Diameter in Fig. S4D, F, H) were performed with 3 measurements per cell, for 5 cells, using lines spanning the longest nuclear diameter. (iii) Detailed NAM-ER, NAM-late endosome, NAM-lysosome, and NAM-peroxisome localisation (Fig. 3O, Fine distance in Fig. S4D, F–H) were analysed using lines of length 1.5 μm starting at a point of high MitoTracker signal. Line graphs of signal intensities were depicted as mean standardised signal intensity along with their 95% confidence interval. Measurement distances (either the nuclear diameters or the fine distance 1.5 μm lines) were standardised between –0.5 and 0.5, where 0 depicts the central point of the measurement. This analysis was performed using a generalised additive model available within the ggplot package¹³¹.

Kymograph and micro-dispersion analysis. Line profiles of ER signal across the diameter of the cell were measured for 70 s. Kymographs are line profiles (x-axis) across time (y-axis) (see Fig. S4B). To represent fluctuations in the ER signal which depict the mobility or immobility of the ER, we first standardised the measurement distance between 0 and 1 for all cells analysed. Following this, for each standardised pixel on the x-axis, we quantified the SD of the ER signal. Signal dispersion was depicted as mean SD across standardised distances along with their 95% confidence interval. This analysis was performed using a generalised additive model available within the ggplot package¹³¹.

FRET analysis. ATP levels were analysed using an adaptation of previously published protocols¹³². Briefly, a nuclear ROI per z-slice was selected (thresholding) based on the YFP signal (Ex_{YFP}/Em_{YFP}). Using this nuclear ROI, the ratio of the FRET signal (Ex_{CFP}/Em_{YFP}) to the CFP signal (Ex_{CFP}/Em_{CFP}) was calculated using the Image Calculator plugin from Fiji. Nuclear ATP was then represented as the mean of the YFP/CFP ratio per cell.

Nuclear volume. To estimate the volume of the nuclei, we utilised a z-stack imaging approach. Samples were imaged across multiple z-slices. For each slice, the cross-sectional area of the nucleus was measured using Fiji. The total volume of each nucleus was then calculated by summing the products of the area of each slice and the slice thickness. Specifically, the formula used was $\text{Volume} = \sum (\text{z}_{\text{height}} \times \text{Area}_i)$, where i denotes each individual slice in the z-stack, and z_{height} is the slice thickness.

ROS analysis. Nuclear and mitochondrial ROS levels were analysed by creating a custom Fiji script. Briefly, ROIs were drawn to select the entire cell and ROS levels were quantified as integrated density per z-slice. Finally, the mean integrated density per cell was calculated as the mean of integrated density across the z-slices. For plotting, a zero mean normalisation was performed for suspension and confined conditions within each treatment group, using the mean and SD of the respective suspension populations.

Simulations and data analysis for pixel binning and coefficient of variation. To simulate chromatin structure with controlled heterochromatin and euchromatin regions, we developed a computational model to simulate the spatial distribution of chromatin within a region representing the nucleus, focusing on the differential organisation of heterochromatin and euchromatin. The simulation was performed on a square grid representing a 2000×2000 pixel image, with each pixel corresponding to a specific intensity value. The image was initialised with a background noise level set to a mean intensity of 30, with a SD of 10, to mimic the inherent variability observed in biological imaging. The heterochromatin regions were modelled as elliptical clusters, occupying 30% of the total image area. The semi-major and semi-minor axes of these ellipses were sampled from an exponential distribution, with a mean ellipse size of 5, 8 and 11 pixels. To ensure realistic biological shapes, the ratio between the major and minor axes was constrained to be no greater than 3:1. Ellipses were randomly oriented and positioned within the image, and the process was repeated until the target heterochromatin coverage was achieved. Euchromatin was assigned to the remaining image area. The intensity values for heterochromatin and euchromatin regions were set to mean values of 70 and 50, respectively, with both exhibiting variabilities characterised by a normal distribution (SD of 30). To simulate the optical properties of microscopy, the image was convolved with a Gaussian Point Spread Function with a SD of 2 pixels. This blurring step mimicked the resolution limitations inherent in imaging systems. The simulated chromatin structure was displayed as a grayscale image, with additional processing to produce a colour-enhanced version where heterochromatin regions were highlighted in purple while maintaining the grayscale intensity representation of euchromatin. This model provided a controlled environment for analysing the spatial relationships between heterochromatin and euchromatin, facilitating subsequent image processing and analysis tasks. Binning analysis was then performed on the simulation data.

For analysis of HeLa cells stained with Hoechst, 3 central planes of nuclei were selected and used to produce maximum projection images. Hoechst signal (Ex 405/Em Hoechst) was quantified for mean signal intensity and signal SD. The coefficient of variation was calculated as the ratio of signal SD to mean signal intensity for each nucleus. This process was repeated for the same images using pixel binning at $1 \times 1 = 1\text{px}$; $2 \times 2 = 4\text{px}$; $3 \times 3 = 9\text{px}$; $4 \times 4 = 16\text{px}$; $5 \times 5 = 25\text{px}$; $6 \times 6 = 36\text{px}$; $7 \times 7 = 49\text{px}$; and $8 \times 8 = 64\text{px}$. Results are represented as mean coefficient of variation with 95% confidence intervals.

Statistics and reproducibility

All the relevant experiments have been conducted at least three times with biological replicates, and data were collected from distinct samples. Whenever indicated, n marks the number of analysed cells. Statistical tests and results have been presented in the figure or at the end of the figure legends.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data relevant for the conclusions of this work have been made available through the main text or the supplementary information. The

mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE¹⁰⁰ partner repository with the dataset identifier [PXD043524](https://doi.org/10.1038/s41467-025-61787-x). The ATAC-Seq data have been deposited to the GEO Repository (GSE248846 [<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE248846>]). Additional source data for plots presented in this work are available as Source Data 1 through 7. Due to size limitations, Larger Source Data L1 and L2 can be downloaded from Zenodo (<https://zenodo.org/records/15641870>). Please refer to the description of source data files and our code availability statement for further information. Source data are provided with this paper.

Code availability

All code to produce the figures relevant to proteomics analysis in this paper can be found at https://github.com/Skourtis/Rito_Fabio. All other code to produce figures from Source Data used in this manuscript can be found at <https://github.com/SdelciLab/CINAPS>.

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Competing interests

The authors declare no competing interests.

Additional information

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