**Nuclear compression-mediated DNA damage drives ATR-dependent Lamin expression and mouse ESC differentiation**

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**Abstract:**

Embryonic stem cells (ESCs) which are susceptible to DNA damage depend on a robust and highly efficient DNA damage response (DDR) mechanism for their survival. However, the implications of physical force-mediated DNA damage on ESC fate remains unclear. We show that stiffness-dependent spreading of mouse ESCs (mESCs) induces DNA damage through nuclear compression, with DNA damage causing differentiation through early induction of Lamin A/C expression. Interestingly, differentiation is associated with rescue of DNA damage and activation of the DDR factor ATR. While ATR is typically known to play roles in DDR pathway, its role during stiffness-mediated nuclear compression and mESC differentiation is unknown. Nuclear enrichment of activated ATR on stiff substrates and reduction of Lamin A/C expression upon ATR inhibition suggests that mESC differentiation is driven by nuclear compression-mediated DNA damage and involves ATR-dependent modulation of Lamin A/C.

**Keywords:** ESCs, stiffness, Lamin A/C, ATR, nuclear compression, DNA damage, differentiation.

## Introduction

Embryonic stem cells (ESCs) isolated from the inner cell mass of a late blastocyst of a developing embryo [1], serve as an important tool for studying pluripotency and development apart from stem cell-based therapies and genetic engineering [2]. Due to their high rate of proliferation and a shorter G1 phase [3,4], ESCs are particularly susceptible to DNA damage, and depend on a robust and highly efficient DNA damage response (DDR) mechanism to maintain their self-renewability and pluripotency [5]. ATM (Ataxia telangiectasia mutated) and ATR (ATM and Rad3-related) are key players entailed during DDR which are not only activated by DNA damage signals but also implicated in oxidative stresses [6,7], and metabolic processes [8,9].

The dynamic stem cell microenvironment or the stem cell niche regulates stem cell fate through chemical (secretory) as well as physical cues necessary for survival and proliferation [10–15]. Force transmission to the nucleus through the linker of nucleoskeleton and cytoskeleton (LINC) complex modulates expression of mechanosensitive genes through chromatin reorganization [16–18] that directs stem cell differentiation [19] through extracellular matrix (ECM) stiffness [14] and topography [15] [NO_PRINTED_FORM]. Lamin A/C – a critical component of the LINC complex, not only plays an important role in maintenance of genome integrity [20–23] but also controls and contributes to nuclear mechanotransduction and stem cell fate determination [24–27].

Extreme nuclear deformation has been shown to cause nuclear membrane rupture leading to genomic instability [28–30] . Beyond their canonical DNA repair functions in somatic cells, recent studies have documented ATM/ATR activation in cells subjected to mechanical stresses [31–34]. While alterations in ATR can cause nuclear envelope breakdown and aberrant chromatin organization [31,32], loss in ATM triggers nuclear deformation and reduced Lamin A levels thus regulating nuclear stiffness and mechanics [35]. Given the association between extracellular matrix (ECM) stiffness and chromosomal instability in mouse ESCs (mESCs) [36], in this study, we have probed the inter-relationships between ECM stiffness, DNA damage, DNA repair and mESC fate. By culturing mESCs on substrates of increasing stiffness, we show stiffness-dependent mechanoadaptation induces DNA damage through increased nuclear compression. This DNA damage in turn triggers stiffness dependent induction of Lamin A/C expression and early differentiation. Similar induction of DNA damage and Lamin A/C expression during chemical factor induced differentiation suggests DNA damage as an early event in the differentiation cascade. We further identify ATR as the regulator of Lamin A/C expression. Collectively, our study establishes stiffness-induced nuclear compression that mediates early ESC differentiation and ATR-dependent modulation of Lamin A/C expression.

## Materials & Methods

**Cell Culture & Reagents:** Tg2a mouse embryonic stem cells (mESCs) were cultured on 0.1% porcine gelatin (Sigma) coated dishes in presence of KO-DMEM (Gibco) and supplemented with 10% KO-Serum replacement (Gibco), 1X Glutamax (Gibco), IX non-essential amino acids (NEAA) (Gibco) and 1X antibiotic-antimycotic (Gibco). These cells were supplemented with 10ng/ml leukemia inhibitory factor (LIF) and 0.1mM 2-mercaptoethanol (Gibco) to maintains pluripotency. Mouse embryonic fibroblasts were cultured in DMEM (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) of South American origin (Gibco) and 1X antibiotic-antimycotic (Gibco). Both mESCs and MEFs were maintained at $37^0$C and 5% $CO_2$ concentration (NUAIRE incubator). For experiments, cells were dislodged with 1X TrypLE Express (Gibco) and seeded on glass or MEFDMs or polyacrylamide hydrogels (PA gels) coated with either 5µg/cm fibronectin (Merck) or 10µg/$cm^2$collagen-I (Sigma) without LIF. mESCs were treated with etoposide (ETO) (2µM, Sigma), Blebbistatin (Blebb) (5µM, Sigma), $MnCl_2$ (0.5 mM), N-acetyl cysteine (NAC) (5 mM, Sigma), Retinoic acid (RA) (1µM, Sigma), Ascorbic acid (AA) (50µM, Sigma), KU-55933 (10µM, Sigma) and VE-821 (5µM, Sigma) [37] for a period of 24 hours.

**Fabrication of Polyacrylamide (PA) gels:** PA hydrogels of varying stiffness of 0.6 kPa, 4 kPa and 33 kPa were fabricated by mixing varying ratios of 40% Acrylamide (Bio-Rad) and 2% Bis-Acrylamide

(Bio-Rad) with milliQ water as described elsewhere [38,39]. Gels were polymerized on either 12mm,18mm or 60mm glass coverslips for immunostaining, comet assay and western blotting/flow cytometry experiments respectively. Gels were coated with either 10µg/cm² rat-tail collagen-I (Sigma) or 5µg/cm$^2$ human-plasma fibronectin (Merck) overnight in 4°C, post functionalization using Sulfo-SANPAH (Pierce).

**Fabrication of MEF derived matrix (MEFDM):** MEFDMs were generated by seeding WT MEFs (on 1% gelatin coated dishes (Porcine gelatin, Sigma) at a density of 4.6 x $10^5$/cm$^2$ as described elsewhere in [36].

**Immunostaining and Microscopy:** For immunostaining, cells were fixed after 3 hours, 6 hours, 24 hours or 72 hours of culture using 4% PFA (Sigma) in PBS for 15 minutes, permeabilized with 0.1% TritonX-100 (Sigma) in PBS for 10 minutes, and then incubated with one or more primary antibodies overnight at 4°C post blocking with 5% FBS for 1 hr in room temperature. Primary antibodies used for immunostaining were against pMLC2-Ser19 (1:250, Cell Signaling Technologies), γ-H2AX (1:500, Cell signaling Technologies), Lamin A/C (1:400, Cell Signaling Technologies), Lamin B1 (1:500, Abcam), 53BP1 (1:2500, Invitrogen), pATM-Ser1981 (1:500, Invitrogen) and pATR-Thr1989 (1:500, Invitrogen). The following day, cells were washed with PBS and then incubated with one or more secondary antibodies at room temperature for 2 hours. Secondary antibodies used were Alexa-fluor 488 anti-mouse IgG (1:1000, Invitrogen), Alexa-fluor 555 anti-rabbit IgG (1:1000, Invitrogen). Cells were then stained with fluorescently labeled wheat-germ agglutinin (1:1000, Invitrogen) to stain lectin and nuclei were labeled with DAPI (1:2000, Sigma). Cells were imaged at 120X magnification using either an inverted fluorescent microscope (Olympus, IX83) or at 63X magnification using Spinning Disc Confocal Microscope (Zeiss, CSU-X1) for greater resolution. Cell spreading area, DNA damage foci, mean intensities, nuclear enrichment and nuclear height were quantified using Fiji ImageJ software while nuclear volumes were quantified using Imaris X64 8.3.1. Stiffness and time-dependent Lamin A/C distributions were generated based by normalizing raw intensity values with the maximum value across all conditions and time-points. The normalized intensities ($I_N$) on a given stiffness gel ($g$) were categorized as low (Lo), Moderate (Mo) and High (Hi) based on the following criteria: Lo: $I_N \leq \mu_g - \sigma_g$, Mo: $\mu_g - \sigma_g < I_N \leq \mu_g + \sigma_g$, Hi: $I_N > \mu_g + \sigma_g$, where $\mu_g$ and $\mu_g$ correspond to the mean and the standard deviation of normalized intensity observed at the 3 hr time-point. The frequency curves were generated using Origin 2021. The percentage of Lo, Mo and Hi cells were calculated thereafter.

**Alkaline Comet Assay:** To quantify the level of DNA damage in mESCs, alkaline comet assay was performed. For this, mESCs were cultured on PA gels of varying stiffnesses. Post 24 hours, these cells were isolated from the gels using TrypLE Express (Gibco). $10^5$ cells per ml were suspended in PBS and were mixed with 1% low melting agarose (GeneI) cooled to 37°C at a 1:10 v/v cell solution to agarose ratio. Approximately 50µl of the cell-agarose mixtures were evenly spread and incubated on comet slides (Trevigen) at 37°C for 5-10 minutes, and then transferred to 4°C in dark for 30 minutes until the agarose polymerized. The slides were then dipped in ice cold lysis solution (Trevigen) kept in a coplin jar and incubated overnight at 4°C. The next day, the lysis solution was removed and the slides dipped in freshly prepared alkaline unwinding solution (300mM NaOH, 1mM EDTA, pH>13) for 1 hour at 4°C in dark. Slides were then placed in a horizontal electrophoresis apparatus and run at 330 mA for 30 minutes in the presence of ice-cold alkaline electrophoresis solution (300mM NaOH, 1mM EDTA, pH>13). Slides were removed from the electrophoresis solution and washed twice with distilled water for 5 minutes prior to dipping in 70% ethanol for 5 minutes. Samples were air dried for 20-30 minutes at RT. 50-100 µl of SYBR green dye (1:10,000, Invitrogen) diluted in TE buffer (100mM tris-HCl, 1mM EDTA, pH 7.5) was added on top of the sample containing cells and incubated at RT in dark for 30 minutes. Excess SYBR green solution was removed by tapping the slides and rinsed briefly in distilled water. Slides were allowed to dry completely at 37°C before visualizing them under the microscope at 10X magnification. Comets were analyzed by feeding tif images into the OpenComet plugin in ImageJ Fiji software. Extent of DNA damage was quantified by comparison of the olive tail moment of mESCs on varying substrate stiffnesses.

**AFM:** Cell stiffness was measured using an Atomic Force Microscope (MFP3D, Asylum). Cells were probed with 10 kHz soft, pyramidal silicon nitride probes (Olympus) of nominal stiffness 20 pN/nm. The exact cantilever stiffness was determined using the thermal calibration method. Estimates of cell stiffness were obtained by fitting the first 500 nm of experimental force-indentation curves with Hertz model [40]. Similarly, the stiffness of gels was estimated by fitting the first 1000 nm of the experimental force-indentation curves as described elsewhere [39].

**Western Blotting:** mESC protein samples were isolated from mESC pellets using ice-cold RIPA buffer (Sigma) in the presence of freshly added 1% protease and phosphatase inhibitor cocktail (Invitrogen). Cell lysates were cleared by centrifugation at 12,000 rpm for 15 minutes at 4°C. Protein concentration was determined by Lowry method. 60 µg of total protein sample was loaded into each well and resolved by SDS-PAGE on gradient acrylamide-bisacrylamide gels (6% to 15%). Samples were transferred on to 0.2 µm PVDF membranes (Bio-Rad). Membranes were blocked with 5% BSA in TBST for 1 hour at RT and then incubated one or more of the following primary antibodies overnight at 4°C: Lamin A/C (1:1000, CST), Lamin B1 (1:1000, Abcam), γH2AX (1:1000, CST), H2AX (1:500, CST), pMLC2-Thr18/Ser19 (1:700, CST), MLC2 (1:700, Abcam), Oct3/4 (1:1000, Abcam), Nanog (1:1000, CST), pATM-Ser1981 (1:1000, Invitrogen), pATR-Thr1989 (1:1000, Invitrogen), ATM (1:1000, CST), ATR (1:1000, CST), RAD51 (1:1000, Abcam), GATA4 (1:1000, Invitrogen), Brachyury (1:1000, Invitrogen), Otx2 (1:1000, Invitrogen) and GAPDH (1:1000, CST). The following day, after washing with TBST, membranes were incubated with HRP conjugated secondary antibody (1:10,000, Invitrogen) for 2 hours at RT. Subsequently, membranes were washed again with TBST and developed using ECL detection kit (Advansta) and ultra-sensitive chemiluminescent substrate (Cynagen). Images were captured using a chemidoc imaging system (Bio-Rad).

**RNA Sequencing (RNASeq) and heat map generation:** Total RNA from bulk cells was isolated using TRIzol (Ambion) according to instructions from the manufacturer. The concentration and purity of samples was checked using the Nanodrop 2000 spectrophotometer. Ribo-Zero Plus stranded library preparation kit (Illumina) was used to generate libraries from a total of 1$\mu$g RNA for each sample. Library concentration was estimated using Qubit dsDNA High Sensitivity Assay Kit (Invitrogen). The libraries were sequenced on the Illumina NovaSeq 6000 platform to a depth of 50 million paired-end reads. The quality assessment of sequenced reads was evaluated using FASTQC (v0.12.0) followed by mapping them to the Mus_musculus.GRCm39.cdna reference cDNA transcriptome using Kallisto (v0.45.0) for estimating transcript abundance. Data annotation was carried out using AnnotationHub and genes exhibiting a Counts Per Million (CPM) value of less than 1 in at least three samples were excluded from further analysis. The resulting filtered count matrix was then normalized by Trimmed Mean of M-values (TMM) method using edgeR package, and was subsequently log2-transformed to mitigate data heteroskedasticity. Principal Component Analysis (PCA) was performed using the Scikit-learn library of python and a 3D PCA plot generated. Heatmaps were generated using Count matrix values followed by normalizing the data against +LIF control and then applying z-score normalization across each row to get relative gene expression values. The representative matrices were sorted based on gene expression value of average of 72-hour samples.

**Gene Ontology Analysis:** Differential gene expression analysis conducted with the PyDESeq2 package identified upregulated and downregulated genes across stiffness conditions at 72 hours, using +LIF as a reference and applying a log2 fold change threshold of $\geq 1$ and an adjusted p-value of $\leq 0.01$. Following gene identification, gene ontology analysis via the gprofiler2 library was utilized to enrich transcription factor pathways. Subsequently, common transcription factor pathways upregulated across three stiffness conditions were identified. Prevalence of genes associated with endoderm, mesoderm, ectoderm and extraembryonic ectoderm lineage specification within each identified transcription factor pathway was quantified and sorted based on this occurrence frequency. The top 20 pathways were ranked based on their p-values and visually represented in a grouped bar chart format.

**Flow cytometry:** For cell-cycle analysis, mESCs were pelleted down using TrypLE Express (Gibco) and fixed using 0.25% PFA for 15 minutes on ice followed by permeabilization with 70% methanol in 4°C for at least 1 hour. Cell pellets were washed with PBS to remove residual methanol and then 50µg/ml Propidium Iodide (Himedia) was added to each condition before acquisition for cell cycle measurements on BD FACS Aria Fusion. Gating was done with respect to pluripotent mESCs using FlowJo software. For apoptosis assay, mESC pellets were incubated with FITC conjugated Annexin V and Propidium Iodide in 1X binding buffer as per manufacturer's protocol (BD Pharmingen). 10,000 events were acquired per sample. Gating was done with respect to unstained control using FlowJo software.

**Pharmacological inhibition assays using zebrafish embryos:** *Tg(cmlc2):mCherry* embryos were raised in embryo medium at $28^{O}$C till 24 hours post fertilization (hpf). At 24 hpf, embryo medium was replaced with embryo medium containing 10µM KU55933 or 10µM VE821. Embryos were kept in the inhibitors for 24 hours at $28^{O}$C. The next day, the medium was replaced with fresh embryo medium without inhibitors and embryos were left to grow for another day. Heart phenotypes were quantitated and imaged at 48 hpf (24 hours of exposure to inhibitor) and at 72 hpf (24 hours of exposure to inhibitor, followed by 24 hours of recovery). Live images were obtained using Olympus MVX10 and processed using CellSens and ImageJ softwares.

**Statistical Analysis:** For statistical analysis, raw data were processed in Origin 2021. The normality of the data was first checked using the Kolmogorov-Smirnov normality test. Based on these results, either parametric or nonparametric tests were subsequently performed. Statistical significance was assessed using unpaired t-tests for comparing two parametric datasets. One-way or two-way ANOVA tests were performed for parametric datasets with multiple conditions, followed by Tukey's post-hoc test to compare the means. For multiple non-parametric datasets, Kruskal-Wallis ANOVA followed by Dunn's post-hoc test while Mann-Whitney test for comparing means between two non-parametric datasets were performed. $p < 0.05$ being considered as statistically significant.

## Results

### Stiffness-mediated mechanoadaptation is associated with DNA damage in mESCs

Long-term culture of mESCs on stiff gelatin-coated coverslips have been shown to accumulate greater aneuploidy compared to that on mouse embryonic fibroblast (MEF) derived matrices, which are much softer [36]. To probe the link between stiffness-mediated mechanoadaptation and genomic integrity, we cultured mESCs on collagen (Col) and fibronectin (FN) coated 0.6 kPa, 4 kPa and 33 kPa PA gels that span a physiologically relevant stiffness range (Fig. S1). As mESCs exhibited greater spreading and focal adhesion intensities on FN coated gels compared to that on Col coated gels, indicative of a more robust mechanoadaptation response on FN coated gels (Fig. S 1A, B), all subsequent experiments were performed on FN coated substrates up to 72 hrs in culture. While mESCs spread minimally on the softest 0.6 kPa gels, spreading increased substantially on the 4 and 33 kPa gels (Fig. 1Ai, ii). Increased spreading on 4 and 33 kPa gels was associated with increased cytoskeletal organization as assessed by active actomyosin contractility (phosphorylated myosin light chain, pMLC) (Fig. 1Bi, ii, Fig. S 2Ai) and cortical stiffness (Fig. 1Ci, ii). Interestingly stiffness-mediated mechanoadaptation was associated with stiffness-dependent increase in the extent of DNA damage assessed by counting the number of γH2AX foci in individual cells (Fig. 1Di, ii), total protein levels assessed using westerns (Fig. 1Diii, Fig. S 2Aii), as well as in comet assay where longer comet tails were observed in cells cultured on stiffer gels (Fig. 1Ei, ii). Together, these results establish a direct relationship between stiffness-dependent mechanoadaptation and DNA damage in mESCs.

### DNA damage is induced by mechanoadaptation-mediated nuclear compression

To test if stiffness-induced DNA damage was ROS dependent, DNA damage was assessed by quantifying γH2AX foci in cells cultured in the presence and absence of the ROS scavenger N-acetyl cysteine (NAC). Insensitivity of DNA damage to NAC suggests that mechanoadaptation-mediated DNA damage in mESCs is ROS independent (Fig. 2Ai, ii). Contractility-mediated nuclear compression has been shown to induce nuclear envelope rupture and DNA damage in cancer cells cultured on rigid

glass coverslips [29]. To check the link between DNA damage and nuclear compression, we captured transverse sections of Lamin B1-stained nuclei of mESCs cultured on different gels and quantified nuclear height. Increased DNA damage on stiffer substrates was associated with increased nuclear compression observed as early as 3 hrs (Fig. 2Bi, ii). In comparison, nuclear volume increased marginally with time, but remained unchanged across different gels (Fig. 2Biii).

Based on these results, we propose that increased spreading and cytoskeletal organization on stiffer substrates induces DNA damage by nuclear compression through increased nucleo-cytoplasmic coupling. To test this, the extent of nuclear compression was perturbed by modulating cell spreading, and its impact on DNA damage assessed. While cells were treated with $MnCl_2$ on 0.6 kPa gels to increase cell spreading via increased integrin association [41–43], on 33 kPa gels, cells were treated with the contractility inhibitor blebbistatin (Blebb) (Fig. 2Ci). On 0.6 kPa gels, $MnCl_2$ treatment led to increased spreading (Fig. 2 Cii, iii), decreased nuclear height (Fig. 2 Cii,iv) and increased γH2AX foci (Fig. 2 Di, ii). Conversely, on the stiff 33 kPa gels, treatment with Blebb led to decreased cell spreading (Fig. 2 Cii, iii), increase in nuclear height (Fig. 2 Civ) and reduction in the extent of DNA damage (Fig. 2 Di, ii). Western blotting of total cell lysates revealed increased pMLC and decreased γH2AX levels in $MnCl_2$-treated cells, and the opposite in Blebb-treated cells (Fig. 2 E, Fig. S 2 B). Collectively, these results demonstrate that mechanoadaptation-mediated DNA damage in mESCs is a consequence of nucleo-cytoplasmic coupling-induced nuclear compression.

**DNA damage induces loss of pluripotency and Lamin A/C expression at early time-points**

Lamin A/C is an integral part of the nuclear membrane that plays a key role in nuclear mechanics as well as in genome protection. ECM stiffness has been shown to drive differentiation of mesenchymal stem cells (MSCs) with induction of stiffness-dependent Lamin A/C expression [14,15]and affect ESC differentiation [44,45]. However, the importance of stiffness-induced DNA damage in this context remains unclear. Interestingly, increase in Lamin A/C levels on 4 kPa and 33 kPa gels and drop in pluripotency markers Oct3/4 and Nanog suggests that DNA damage and early differentiation may be closely related (Fig. 3Ai, Aii, Fig. S 2 C). To test this, experiments were performed wherein DNA damage was induced with low doses of the topoisomerase II inhibitor – etoposide (ETO) and its impact on pluripotency and Lamin A/C expression assessed. Remarkably, ETO treatment led to robust increase in Lamin A/C levels across all the gels with corresponding loss in Oct3/4 and Nanog expression, suggesting that low doses of DNA damage also induce loss in pluripotency and induction of Lamin A/C expression (Fig. 3 Bi, Bii, Fig. S 2 D).

To next probe if chemical factor induced differentiation also involves DNA damage, mESCs were cultured on the softest 0.6 kPa gels—where mechanoadaptation and DNA damage were lowest—in the presence of the neuronal differentiation factor retinoic acid (RA) and the osteogenic differentiation factor ascorbic acid (AA) while tracking DNA damage and Lamin A/C levels at 3 and 24 hr time-points. RA and AA treatments led to increased levels of the neuronal marker, β-III tubulin at the 24 hr time-point (Fig. S 2 E). Strikingly, both RA and AA treatment led to increase in γH2AX and Lamin A/C levels, and loss in Oct3/4 levels at the early 3 hr time-point (Fig. 3 Ci, ii, Fig. S 2 F). Though Nanog was unchanged at the 3 hr time-point, near complete loss in its expression was observed at 24 hrs indicative of differentiation. Surprisingly, at this timepoint both γH2AX and Lamin A/C levels dropped to baseline levels. In comparison, no change in Lamin B1 levels was observed.

To further probe the occurrence of DNA damage during mESC differentiation on more *in-vivo* mimetic conditions, we cultured mESCs on mouse embryonic fibroblast (MEF) derived matrices (MEFDMs) which induce mESC differentiation [46] (Fig. 3Di, ii). In comparison to mESCs cultured on gelatin-coated glass coverslips in the absence of LIF (serving as controls, CTL), in mESCs cultured on MEFDMs for 24 hrs, increase in γH2AX, loss in Oct3/4 levels and induction of Lamin A/C was observed (Fig. 3 Diii, iv, Fig. S 2 G). While Nanog levels remained unchanged, surprisingly, loss in Lamin B1 was observed. In MEFs which possess high levels of Lamin A/C, no DNA damage was observed. Collectively, our results establish DNA damage as an early event in mESC differentiation that induces loss of pluripotency and Lamin A/C expression.

**Temporal loss of pluripotency is associated with mechanoadaptation mediated differentiation in mESCs**

Stimulation by differentiation factors cause pluripotent mESCs to differentiate into either endoderm, ectoderm or mesoderm layers. To probe how mechanoadaptation mediated loss in pluripotency and induction of Lamin A/C expression influences mESC fate, these cells were cultured up to 72 hrs on the PA gels and RNA sequencing (RNAseq) performed at 3, 6, 24 and 72 hr time-points. Principal Component Analysis (PCA) plot shows a clear time dependent clustering of mESCs, with no prominent stiffness dependence (Fig. 4 A). Though DNA damage and Lamin A/C induction was also seen in ETO treated cells, this point did not co-cluster with any of the other time-points. Compared to pluripotent controls (+LIF), temporal RNAseq profiling revealed stemness genes including *Pou5f1* [47,48], *Sox2* [49], *Nanog* [50,51] and *Klf4* [52] were maintained till 6 hrs, but were lost completely at the 24 hr time-point (Fig. 4 B). Similar trend was also observed in western blots of Oct3/4 and Nanog (Fig. 4 C, Fig. S 4 A). Concomitant with loss in pluripotency, several known lineage-specific markers were upregulated at the 72 hr time point. These included endoderm markers such as *Cldn6* [53,54], *Gata4* [55], *Sox17* [56] and *Foxa2* [57,58], mesoderm markers such as *Tbx6* [59,60], *Twist1*[61–63] and *Snai1* [64,65], and ectoderm markers such as *Nes* [66] and *RunX2* [67] (Fig. 4 B, Fig. S 3 A). Intriguingly, expression of extraembryonic ectodermal markers such as *Krt8* [68], *Wnt7b* [69] and *Gata3* [70] was also observed. While these results are indicative of heterogeneous differentiation, quantification of the average Z-score values across the three stiffnesses revealed most prominent induction of mesoderm lineage (0.4), and weaker induction of endoderm (0.14) and ectoderm (0.1) lineages, indicating a shift towards mesodermal lineage. Protein level expression of one marker from each lineage – Gata4 for endoderm, Brachyury (T) for mesoderm and Otx2 for ectoderm – showed similar trend with RNAseq profiles (Fig. 4 D). In addition, gene ontology analysis revealed lineage specification is mediated by activation of several common transcription factors such as *Zf5*, *E2F*, *SP3*, *Kaiso*, *Pax4*, *Foxn1* (Fig. S 3 B).

Temporal RNAseq profiling of Lamin genes across the 4 time-points revealed an increase in *Lmna* gene but drop in *Lmnb1* and *Lmnb2* genes as expected in differentiating stem cells (Fig. 4 Ei). Although Lamin A/C is known to enhance differentiation of MSCs [26], surprisingly however, protein level analysis shows a gradual decline in both Lamin A/C and Lamin B1 across all the gels, prominently at the 72 hr time-point (Fig. 4 Eii, Fig. S 4 Bi) with considerable heterogeneity in Lamin A/C expression in each condition (Fig. 4 Fi, Red arrowhead: Lamin A/C high; Yellow arrowhead: Lamin A/C low). Lamin A/C intensity distribution revealed an increase in the fraction of Lamin A/C low (Lo) cells at 72 hrs across all the gels (Fig. 4 Fii, iii), with ~60%, 80% and 40% Lamin A/C low cells at 72 hrs on 0.6 kPa, 4 kPa and 33 kPa gels respectively (Fig. 4 Fiii). However, long term culture of mESCs upto 7 days on the stiff 33 kPa gels, exhibited a recovery of Lamin A/C levels after its initial decline at 72 hrs (3 days) (Fig. S 4 Bii), consistent with previous findings that shows that Lamin A/C loss primes mESCs towards cardiac-specific gene expression [27,71].

Since, *LMNA* deficient embryonic hearts have been shown to exhibit cell cycle perturbations during DNA damage [72], we next went on to evaluate the cell cycle distribution of mESCs on the PA gels at different time-points. Quantitative analysis showed that sub-G1 phase significantly increased with time – from <20% cells in +LIF mESCs to ~40% cells at 72 hrs and ~80% upon ETO treatment (Fig. S 5 Ai, ii). The Sub-G1 cells at 72 hrs may correspond to that of low Lamin A/C expressing mESCs at the same time-point on 33 kPa gels (Fig. 4 F i-iii), suggesting that transient Lamin A/C loss affects mESC cell cycle distribution on PA gels. Furthermore, quantification of cell survival using Annexin V-PI staining revealed ~60% live mESCs at 72 hr time-points on all the gels compared to ~85% live cells in presence of LIF (Fig. S 5 Bi, ii). While temporal RNASeq profiling revealed activation of few apoptosis genes (*Casp8, Casp6, Casp7*) at 72 hrs time-point (Fig. S 5 C), Annexin-PI staining shows no significant increase in apoptotic cells but % necrotic cells were dramatically increased at 72 hr time-point and upon ETO treatment (Fig. S 5 Bi, ii).

Since embryogenesis is mediated by cell-matrix adhesions and mechanical forces [73] that control complex mechano-signaling cascades via integrins [74], we examined the temporal profile of several genes involved in mechanoadaptation. RNAseq profiles show clear upregulation of several such genes at 72 hrs such as integrin subunits – *ItgaV, Itga3, Itga11, Itgb1, Itgb3, Itgb5, Itgb8,* integrin like kinase

(*Ilk*), genes involved in integrin mediated cell adhesion – *Tln1, Vcl, Gsn, Tsn2, Parva, Parvb, Acta1, Acta2, Actg1*, myosin genes – *Mylk, Myl6, Myl9, Myl12a, Myl12b*, along with *Cdh1* and *Cdh2* (Fig. S 4 C). Few LINC complex genes were also upregulated at 72 hrs such as *Sun2, Syne2* and *Lmna* (Supp. Fig. 4 C), thus implicating an integrin dependent mechanoadaptation response that mediates mESC differentiation at 72 hrs. Together, these results suggest that loss in pluripotency in mESCs is associated with transient loss of Lamin A/C protein expression leading to early differentiation (72 hrs) into all three germ layers (with a prominent shift towards mesodermal lineage) through activation of mechano-signaling pathways.

**mESC differentiation is associated with ATR activation**

While mESCs have a robust DNA damage response (DDR) mechanism to counter intrinsic and extrinsic assaults to the genome, compared to their differentiated counterparts [75], accumulation of DNA damage can cause apoptosis and differentiation to eradicate them from the stem cell pool [76,77]. We propose that differentiation is triggered via activation of DDR pathways and DNA repair. Consistent with this notion, temporal immunostaining and quantification of γH2AX foci on mESCs displays a steady level upto 24 hrs and then a sharp decline at the 72 hr time-point (Fig. 5 Ai, ii). Similarly, 53BP1 quantification shows a significant increase at 6 hrs which then notably diminished at the 72 hr time-point (Fig. 5 Bi, ii), indicative of a temporal resolution of DNA damage. To further confirm our observations, immunoblotting of mESCs also revealed a significant drop in γH2AX as well as pRPA2 (Single strand break marker) levels at the 72 hr time-point. (Fig. 5 C, Fig. S 6 Ai, ii).

RNAseq profiles of early responders of DNA damage revealed upregulation of *ATR* and *RAD50*, downregulation of *ATM, Mre11a, MDC1, Trp53bp1, RPA1, RPA2, Atrip,* and upregulation of downstream DNA repair genes including *Brca2* and *Rbbp8* at 72 hr time-point. (Fig. 5 D). These observations motivated us to investigate the functional roles of ATM and ATR during stiffness mediated mechanoadaptation in regulating lamin levels. No phosphorylated ATM (pATM, Ser1981) bands were detected on 33 kPa gels across the different time-points, notwithstanding the fact that ATM levels remained unchanged across these conditions when compared to +LIF condition (Fig. 5 E, Fig. S 6 B). However, pATM was detected in immunostained images of mESCs cultured on 33 kPa gels (Fig. 5 Fi, ii), and exhibited uniform spatial distribution (Fig. 5 Fiii). In contrast to ATM, phosphorylated ATR (pATR, Thr1989) was detected from 3 hr onwards (Fig. 5 E, Supp. Fig. 6 B). Furthermore, quantification of nuclear to cytosolic (N/C) ratio revealed increased nuclear localization of pATR across all time-points (Fig. 5 Fiii) with greater nuclear enrichment on the stiffer 33 kPa gels (Fig. S 6 C). Near complete loss in RAD51 levels at the 72 hr time-point (Fig. 5 D, E, Supp. Fig. 5 B) may correspond to resolution of DNA damage and mESC differentiation (Fig. 5 A, B and C). Taken together, these results suggest that DNA damage-induced differentiation is associated with ATR mediated DDR activation followed by DNA repair.

Furthermore, since RNAseq analysis of mESCs revealed an upregulation of mesodermal genes, we analyzed the effect of inhibiting DNA repair on development of the heart, a mesodermal organ, which is also mechanically active during its formation. The zebrafish heart forms as a tube and develops into a two-chambered contractile organ by 24 hours post fertilization (hpf). The heart then undergoes morphogenetic movements resulting in a left-ward ventricle and right-ward atrium and chamber ballooning by ~72 hpf [78]. Zebrafish embryos exposed to ATM and ATR inhibitors (KU55933 and VE821 respectively) for 24 hours showed mild body curvature defects at 48 hpf (Fig. S 6 Di, ii, n=71 for KU and n=73 embryos for VE) and defects in chamber size and ballooning (Fig. S 6 Dii). Following 24 hours exposure to inhibitors, larvae were allowed to recover in inhibitor-free medium for another 24 hours. However, the defects in heart morphogenesis worsened by 72 hpf, wherein the ventricle and atrium failed to attain their characteristic ballooning and left-right positioning in the larvae (Fig. S 6 Diii, n=35 for KU and n=44 embryos for VE, Supp. movies S1 to S3). Interestingly, in several embryos cardiac contractility was absent at 72 hpf (7/35 for KU and 2/44 for VE). These results suggest that ATM and ATR function is required for normal morphogenesis of the developing zebrafish heart and inhibition of ATM/ATR during cardiac morphogenesis causes inability of embryos to recover from the damage.

**ATR modulates Lamin expression**

Since both ATM and ATR have been linked to regulation of nuclear envelope integrity and lamin levels [31,35,79], we probed the impact of ATM and ATR perturbations on lamin levels. To check whether downstream DDR pathway is blocked post KU and VE treatments, we assessed the γH2AX foci post 24 hrs of treatment and observed significant drop in γH2AX foci counts upon both ATM and ATR inhibition as compared to untreated controls (Fig. 6 A and B), suggesting that early response to mechanoadaptation mediated DNA damage in mESCs is activated by both ATM and ATR. However, while ATM inhibition led to no significant reduction in Lamin A/C and Lamin B1, both the lamins were significantly reduced upon ATR inhibition (Fig. 6 A, C, Fig. S 6 E). Taken together, our observations suggests that during mechanoadaptation, lamin levels in mESCs are modulated through activation and nuclear localization of ATR and not ATM.

**Discussion**

Genome integrity has been associated with transmission of physical forces to the nucleus leading to nuclear envelope deformation, blebbing and rupture in cancer cells [28,29]. Lamin A/C is a crucial component of the nuclear envelope with Lamin A/C deficiency causing laminopathies and [80], increased DNA damage [23,81,82], and defective mechanotransduction [25]. Here, using FN coated PA gels of varying stiffness, we report stiffer matrices trigger greater DNA damage in mESCs through induction of nucleo-cytoplasmic coupling and nuclear compression. We show that DNA damage brings about loss in pluripotency and Lamin A/C induction at early time-points, thus functioning as a mediator of early differentiation. Differentiation of mESCs at the 72 hr time-point was associated with early induction of DNA damage response factors (γH2AX, 53BP1 and pRPA2) and downstream DNA repair factors (RAD51). Of the two DNA damage response (DDR) factors ATM and ATR, we establish a role for ATR in modulating Lamin A/C and Lamin B1 expression. Collectively, our results demonstrate how stiffness-dependent DNA damage drives differentiation through ATR-dependent Lamin regulation (Fig. 6 E).

Stem cell maintenance and differentiation are regulated by several components of the stem cell niche. Cell-ECM interactions are known to regulate stem cell self-renewability and differentiation [83]. While collagen-I (Col-I) has been reported to maintain mESC self-renewability [84], fibronectin (FN) has been associated with self-renewability as well as differentiation [85–87]. MEFDMs of different composition, organization and stiffness have been shown to induce distinct mESC germ layer commitment [46]. In addition to its association with stem cell self-renewability and lineage commitment [14,26,45,88], matrix stiffness has also been implicated in regulating genomic integrity (ploidy) of mESCs [36]. Our findings establish a direct link between stiffness mediated mechanoadaptation and DNA damage in mESCs on FN coated PA gels. While MSCs and cancer cells undergo nuclear compression, DNA damage and nuclear envelop rupture during confined migration [28,29,89,90], we show for the first time that DNA damage in mESCs is attributed to stiffness-dependent nuclear compression. Increase in nuclear compression and DNA damage upon $MnCl_2$ treatment on the softest gels and decrease in nuclear compression and DNA damage upon Blebb treatment on the stiffest gels suggests that despite lacking a prominent acto-myosin network [91,92], nucleocytoplasmic coupling is sufficient to induce DNA damage in mESCs.

Consistent with a previous study where Lamin A/C was shown to have a direct correlation with tissue stiffness and MSC differentiation [26], our results show a matrix stiffness dependent increase in Lamin A/C expression which is triggered by stiffness dependent DNA damage induction at 24 hr time-point. Similar loss of pluripotency factors and induction of Lamin A/C was observed in cells treated with ETO at 24 hrs. Remarkably, induction of DNA damage and Lamin A/C as early as 3 hrs in cells cultured in the presence of differentiating agents RA and AA suggests that DNA damage and induction of Lamin A/C expression are early events in mESC differentiation. Even in mESCs cultured on ~2 kPa MEFDMs wherein cells lose pluripotency within 24 hrs, DNA damage and higher Lamin A/C levels were observed. Drop in both DNA damage and Lamin A/C in RA/AA treated cells at 24 hrs suggests that Lamin A/C induction is DNA damage-dependent and temporal.

Literature reports have documented opposite effects of DNA damage in inducing differentiation. While genotoxic DNA damaging agents have been shown to disrupt myogenic differentiation [93], physiological

DNA damage induced by caspase 3 has been shown to promote differentiation [94]. Moreover, confined migration has been reported to cause impaired cell cycle and delayed differentiation in myoblasts [95]. LIF maintains *in-vitro* mESC self-renewability through the STAT3 pathway [96], and has been implicated as a mechano-inhibitor [36]. In mESCs, LIF withdrawal triggers an exit from pluripotency and induction of apoptosis [97]. However, mESCs on soft substrates maintain pluripotency even in absence of LIF by downregulation of traction forces [45]. Using RNA sequencing analysis and western blotting we show temporal downregulation of most stemness markers accompanied by upregulation of differentiation genes involved in germ layer commitment, in the absence of LIF. In line with previous observations [97,98], temporal cell survivability assay on mESCs show ~ 40% dead cells at 72 hr time-point. These observations suggests that a proportion of mESCs undergo cell death which could be triggered due to LIF withdrawal for longer duration. Since we also observed time dependent impairment of cell cycle and loss in mESC survivability with ~60% Sub-G1 cells, the cells undergoing mixed lineage differentiation at the 72 hr time-point likely correspond to the surviving cells where DNA damage in resolved. Consistent with this, expression of the DNA repair protein RAD51 reduced at 72 hrs.

Though there was loss of Lamin A/C at Day 3, Lamin A/C levels were subsequently recovered on Day 7. This is consistent with previous findings [27,71] illustrating the role of Lamin A/C oscillations in mESC differentiation. Interestingly, immuno-staining images revealed heterogenous Lamin A/C expression across all time-points. Analysis of Lamin A/C heterogeneity revealed 40% Lamin A/C low cells at 72 hr time-point that correlates with the % dead cells from flow cytometric analysis of Annexin-PI. We speculate that restoration of lamin A/C levels also restores cell viability and constitutes the differentiating mESC population. Thus, matrix stiffness mediated DNA damage induces early Lamin A/C expression and loss in pluripotency at 24 hrs but a time-dependent Lamin A/C loss and restoration is required for non-aberrant mESC fate and differentiation.

Early DNA damage response (DDR) factors – ATM and ATR – have been extensively linked to nuclear envelope integrity, regulation of lamins and mechanosensitivity [31–33,35,79]. Our experiments in zebrafish embryos also show that ATM and ATR function is required for normal morphogenesis of the mechanically contractile zebrafish heart during critical stages of cardiac development. In mESCs, while pATM Ser1981 is uniformly present in nucleus and cytosol, here we show a temporal ATR activation and localization to the nucleus on stiff gels and its selective inhibition leading to lamin loss, thus illustrating the spatio-temporal role of pATR Thr1989 in modulation of both Lamin A/C and Lamin B1 in mESCs. However, apart from their canonical roles in regulation of DNA damage response, the DDR kinases, ATM and ATR have been reported to play roles in nuclear mechanotransduction and nuclear envelope (NE) integrity during cancer migration and invasion [31,32,34,35,79]. While ATM has been claimed to be activated by cell stretching [34], ATR is activated by nuclear compression during interstitial migration [31]. Here, we report that the ATR kinase is activated upon matrix stiffness induced nuclear compression-mediated DNA damage that leads to modulation of lamin levels in mESCs. Taken together, our results indicate a novel role of matrix stiffness induced nuclear compression that triggers DNA damage in mediating ESC differentiation and ATR activation to the nucleus.

In conclusion, our results illustrate the role of matrix stiffness in mediating nucleo-cytoplasmic coupling induced nuclear compression in mESCs leading to DNA damage. Our results also show that such DNA damage not only drives early differentiation and Lamin A/C induction but also activates and localizes ATR to the nucleus, indicative of the role of ATR in determination of lamin expression in mESCs during mechanoadaptation.

## Author contributions

Conceptualization: T.R., S.K., S.N., C.S.P., S.S.; Methodology: T.R., N.P., S.D., N.T., C.W.K., S.S.P., P.S., S.G., L.K.S., D.T.S., S.N., C.S.P., S.K, S.S.; Software: T.R., S.D., N.T., C.W.K., P.S., D.T.S., C.S.P., S.N., S.K., S.S.; Validation: S.S.; Formal analysis: T.R., S.D., N.T., C.S.P., S.K., S.S.; Investigation: T.R., S.K., S.N., S.S.; Resources: S.S.; Data curation: T.R., N.P., S.D., N.T.; S.S.P.;

C.S.P.; S.K., S.S.; Writing - original draft: T.R., S.S.; Writing - review & editing: T.R., N.P., S.D., C.W.K., N.T., C.S.P., S.N, S.K., S.S.; Visualization: T.R., S.S.; Supervision: S.S.; Project administration: S.S.; Funding acquisition: S.S.

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**Declaration of Interests**

The authors declare no competing interests.

**Figure Legends**

**Figure 1: Association of mechanoadaptation and DNA damage in mouse embryonic stem cells (mESCs): (Ai, ii)** Representative lectin-stained images of mESCs on fibronectin (FN)-coated polyacrylamide (PA) gels of varying stiffness post 3, 6, 12, 24 hours and 72 hours of seeding and quantification of cell spreading ($n \geq 60$ cells per condition across $N$ = 3 independent experiments). Scale Bar = 10$\mu m$. Error bars represent SD. Two- way ANOVA with Tukey's test was used for comparing means (**$p$-value ≤0.01, **** $p$-value ≤0.0001). **(Bi, ii)** Assessment of pMLC expression in mESCs cultured on PA gels for 24 hours based on immunofluorescence and western blotting. GAPDH served as loading control. Scale Bar = 10$\mu m$. **(Ci-iii)** Measurement of mESC cortical stiffness using atomic force microscopy (AFM) after 24 hrs in culture. Cells were probed with a soft pyramidal probe and first 500 nm of the raw force-indentation curves were fitted with Hertz model to estimate cortical stiffness ($n \geq 60$ cells per condition across $N$ = 3 independent experiments). One- way ANOVA with Tukey's test was used for comparing means (**** $p$-value ≤0.0001). **(Di-iii)** Quantification of DNA damage in mESCs cultured on PA gels for 24 hrs. DNA damage was assessed by co-staining cells with DAPI and γH2AX and counting γH2AX foci per nucleus ($n \geq 50$ nuclei per condition across $N$ = 3 independent experiments) and western blotting of whole cell lysates. Error bars represent SEM. One- way ANOVA with Tukey's test was used for comparing means (**** $p$-value ≤0.0001). **(Ei, ii)** Representative images of mESC comet assay on PA gels (Insets show single comets) and quantitative analysis of olive tail moment ($n \geq 30$ cells per condition across $N$ = 3 independent experiments). One- way ANOVA with Tukey's test was used for comparing means (*$p$-value ≤0.05, ** $p$-value ≤0.01, ns = non-significant $p$-value>0.05).
See also Figure S1 and Figure S 2 A.

**Figure 2: Mechanoadaptation mediated nuclear compression induces DNA damage in mESCs**: **(Ai, Aii)** Representative images of mESC nuclei co-stained with γH2AX on PA gels of varying stiffnesses after 24 hrs in culture in presence and absence of the ROS inhibitor, N-acetyl cysteine (NAC) and quantification of DNA damage ($n \geq 60$ cells per condition across $N$ = 3 independent experiments). Scale Bar = 5$\mu m$. Error bars represent SEM. Student t-test was used for comparing means between control and NAC treated samples on each PA gel (*ns* = non-significant $p$-value>0.05). **(Bi-Biii)** Representative images showing transverse sections of mESC nuclei immunostained with Lamin B1 on PA gels of varying stiffness post 3, 6, 12 and 24 hours of seeding and quantification of nuclear height and nuclear volume ($n \geq 60$ cells per condition across $N$ = 3 independent experiments). Scale Bar = 5$\mu m$. Error bars represent SD. Two- way ANOVA with Tukey's test was used for comparing means (**** $p$-value ≤0.0001, ns = non-significant $p$-value>0.05). **(Ci-Civ)** Schematic and representative lectin-stained images of mESCs on PA gels of 0.6 kPa in presence and absence of $MnCl_2$ and 33 kPa in presence and absence of Blebbistatin and quantification of cell spreading post 24 hrs of treatment. Representative images showing transverse sections of mESC nuclei stained with Lamin B1 on PA gels of 0.6 kPa in presence and absence of $MnCl_2$ and 33 kPa in presence and absence of Blebbistatin and quantification of nuclear height ($n \geq 50$ cells per condition across $N$ = 3 independent experiments). Scale Bar = 5$\mu m$. Student t-test was used for comparing means between control and treated samples on each PA gel (**$p$-value ≤0.01, **** $p$-value≤ 0.0001). **(Di, Dii)** Assessment of extent of DNA damage of mESCs on PA gels of 0.6 kPa in presence and absence of $MnCl_2$ and 33 kPa in presence and absence of Blebbistatin. Quantification of γH2AX foci co-stained with DAPI (n ≥ 40 cells per condition across $N$ = 3 independent experiments). Scale Bar = 5$\mu m$. Error bars represent SEM. Student t-test was used for comparing means between control and treated samples on each PA gel (***$p$-value ≤0.001, **** $p$-value≤ 0.0001). **(E)** Representative immunoblots showing pMLC and γH2AX levels in ESCs cultured on 0.6 kPa gels in presence and absence of $MnCl_2$ and 33 kPa gels in presence and absence of Blebbistatin. GAPDH served as loading control.
See also Figure S 2 B.

**Figure 3: DNA damage induces Lamin A/C expression in a temporal manner: (Ai, Aii)** Representative images of mESC nuclei immunostained with Lamin A/C on PA gels of varying stiffnesses post 24 hrs of culture. Scale Bar = 5$\mu m$. Representative immunoblots of Lamin A/C

expression and pluripotency (Oct3/4 and Nanog) of mESCs on varying stiffnesses post 24 hrs culture. **(Bi, Bii)** Representative images of Lamin A/C expression and DNA damage in presence of 2μM etoposide (ETO) post 24 hrs treatment on mESCs by immunofluorescence. Scale Bar = 5$\mu m$. Representative immunoblots of Lamin A/C expression, pluripotency levels and γH2AX of whole cell mESC lysates in presence and absence of ETO post 24 hrs treatment. **(Ci, Cii)** Representative immunofluorescence images of Lamin A/C and γH2AX on mESCs post 3 hrs and 24 hrs of retinoic acid (RA) and ascorbic acid (AA) treatment on 0.6 kPa PA gels. Scale Bar = 5$\mu m$. Representative immunoblots of Lamin A/C, Lamin B1, Oct3/4, Nanog and γH2AX of whole cell mESC lysates post treatment of RA and AA on 0.6 kPa PA gels. **(Di-iii)** Schematic representation of MEF derived matrices (MEFDMs) preparation from MEFs and mESC seeding. Representative immunofluorescence image of MEFDM stained with NHS-ester (Scale Bar = 50$\mu m$). Representative immunofluorescence images of Lamin A/C and γH2AX of mESCs on gelatin coated glass coverslips and on MEFDMs compared to differentiated MEFs. Scale Bar = 5$\mu m$. Representative immunoblots of Lamin A/C, Lamin B1, Oct3/4, Nanog, γH2AX and H2AX of whole cell mESC lysates isolated from gelatin coated dishes (CTL) and from MEFDM compared to MEF whole cell lysates. For all blots, GAPDH served as loading control.
See also Figure S 2 C-G.

**Figure 4: Germ layer differentiation is associated with loss in Lamin A/C: (A)** 3-D PCA plot representing clusters of mESCs in +LIF condition, Etoposide (ETO) and varying stiffness post 3 hrs, 6 hrs, 24 hrs and 72 hrs of culture based on RNAseq profiles. **(B)** Heatmaps showing temporal evolution of mESC RNAseq profiles of genes involved in pluripotency, endodermal, mesodermal and ectodermal lineage across different stiffnesses and in ETO-treated cells on 0.6 kPa gels. Normalization was carried out with respect to +LIF condition. **(C)** Representative immunoblots of Oct3/4 and Nanog in mESCs cultured in the presence of LIF and in the absence of LIF on PA gels across different time-points. **(D)** Representative immunoblots of endodermal marker (GATA4), mesodermal marker (Brachyury, T) and ectodermal marker (Otx2) in mESCs cultured on PA gels across different time-points. **(Ei)** Heatmaps showing RNAseq profiles of Lamin genes (*LMNA, LMNB1 and LMNB2*) in mESCs cultured on PA gels across different time-points as well as in ETO treated cells on 0.6 kPa gels. Normalization was carried out with respect to +LIF condition. **(Eii)** Representative immunoblots of Lamin A/C and Lamin B1 in mESCs cultured on PA gels across different time-points. **(Fi-Fiii)** Representative immunofluorescence images of Lamin A/C co-stained with DAPI in mESCs cultured on PA gels across different time-points showing heterogenous expression of Lamin A/C (red arrowhead: high expression; yellow arrowhead: low expression) ($n \geq 50$ cells per condition across $N = 3$ independent experiments). Scale Bar = 20$\mu m$. Lamin A/C heterogeneity was captured by plotting the normalized Lamin A/C distribution across different time-points. Normalization was done wrt the maximum intensity observed across all conditions and timepoints ($n \geq 50$ cells per condition across $N = 3$ independent experiments). Assessment of percentage of low (Lo), moderate (Mo) and high (Hi) Lamin A/C expression across different conditions (see Materials & Methods for details). For all blots, GAPDH served as loading control.
See also Figure S 3- S 5.

**Figure 5: Differentiation involves DNA repair through ATR activation and nuclear enrichment: (Ai, Aii)** Representative images of temporal evolution of mESCs immunostained for γH2AX co-stained with DAPI on varying stiffness and quantification of γH2AX foci count per nucleus ($n \geq 60$ cells per condition across $N = 3$ independent experiments). Scale Bar = 5$\mu m$. Error bars represent SD. Two- way ANOVA with Tukey's test was used for comparing means (**$p$-value ≤0.01, **** $p$-value≤ 0.0001). **(Bi, Bii)** Representative images of temporal evolution of mESCs immunostained for DDR factor, 53BP1, co-stained with DAPI on varying stiffness and quantification of 53BP1 foci count per nucleus ($n \geq 60$ cells per condition across $N = 3$ independent experiments). Scale Bar = 5$\mu m$. Error bars represent SD. (*** p-value≤0.001, **** $p$-value≤0.0001, *ns* = non-significant $p$-value>0.05). **(C)** Immunoblots representing temporal expression of DDR factors, γH2AX/H2AX and pRPA2 of mESCs on varying stiffness. GAPDH served as loading control. **(D)** Heatmaps showing temporal evolution of mESC RNAseq profiles of genes involved in DNA damage response (DDR) factors and DNA repair across different stiffnesses and in ETO-treated cells on 0.6 kPa gels. Normalization was carried out with

respect to +LIF condition. **(E)** Immunoblots representing temporal expression of DDR factors (pATM/ATM, pATR/ATR) and DNA repair factor (RAD 51) in the presence of LIF and in the absence of LIF on 33 kPa PA gels. **(Fi, ii)** Representative pATM-Ser1981/pATR-Thr1989/DAPI stained images of mESCs across different timepoints on 33 kPa gels. Scale Bar = 5$\mu m$**.** Representative intensity profiles along white dotted lines depict nuclear/cytoplasmic localization of pATM (green) and pATR (red). **(Fiii)** Quantification of nuclear to cytoplasmic (N/C) ratio of pATM (green) and pATR (red) ($n \geq 50$ cells per condition across $N$ = 3 independent experiments). Error bars represent SD. One- way ANOVA with Tukey's test was used for comparing means (*ns* = non-significant $p$-value>0.05).
See also Figure S 6 A-D and Movies S 1-3.

**Figure 6: ATR regulates Lamin expression: (A, B)** Representative immunostained images of γH2AX co-stained with DAPI and Lamin A/C on mESCs cultured in the presence and absence of KU-55933 and VE-821 on 33 kPa gels. Bar plots show quantification of γH2AX foci per nucleus in these conditions ($n \geq 30$ cells per condition across $N$ = 3 independent experiments). Scale Bar = 5$\mu m$. Error bars represent SEM. Student t-test was used for comparing means between control and treated samples (****$p$-value ≤0.0001). **(C)** Representative immunoblots showing Lamin A/C and Lamin B1 expression of mESCs on 33 kPa gels in the presence and absence of KU-55933 and VE-821. For all blots, GAPDH served as loading control.
See also Figure S 6 E.

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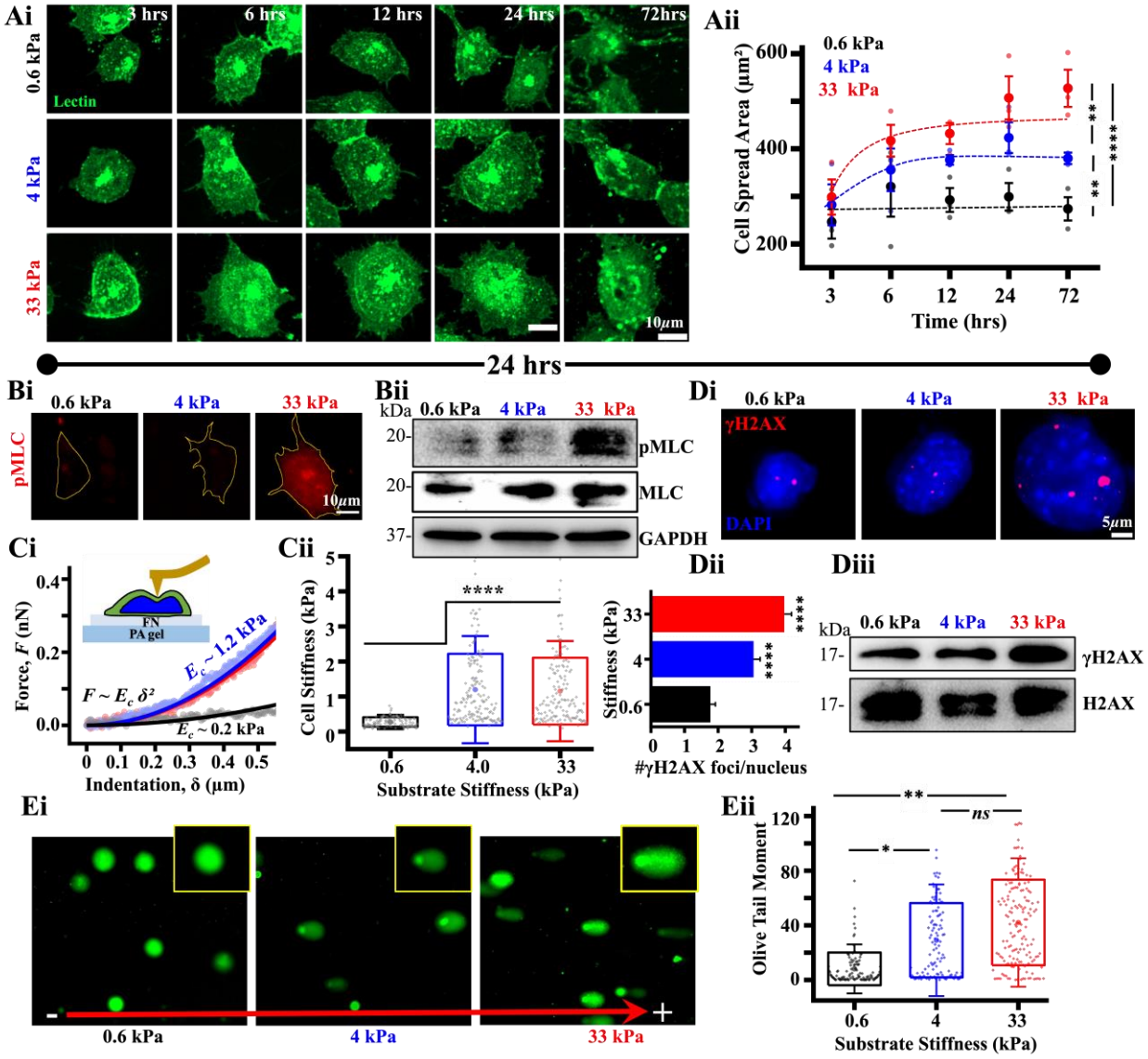
**Figure 1**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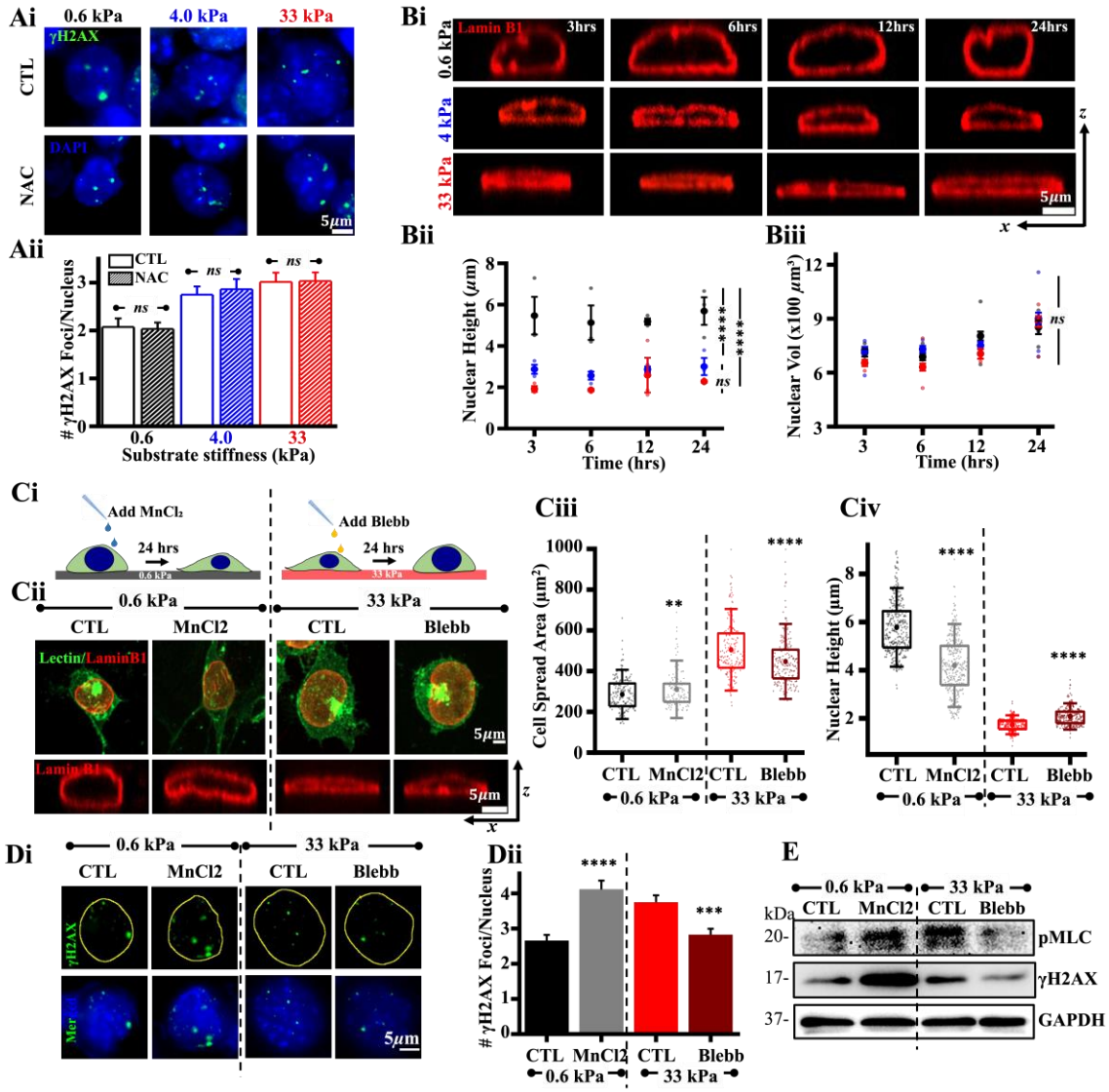
Substrate Stiffness (kPa)

**Figure 2**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Substrate stiffness (kPa)
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24 hrs
Add Blebb
Cii
0.6 kPa
33 kPa
CTL
MnCl2
Blebb
Lectin/LaminB1
Lamin B1
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Cell Spread Area (μm²)
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GAPDH

**Figure 3**

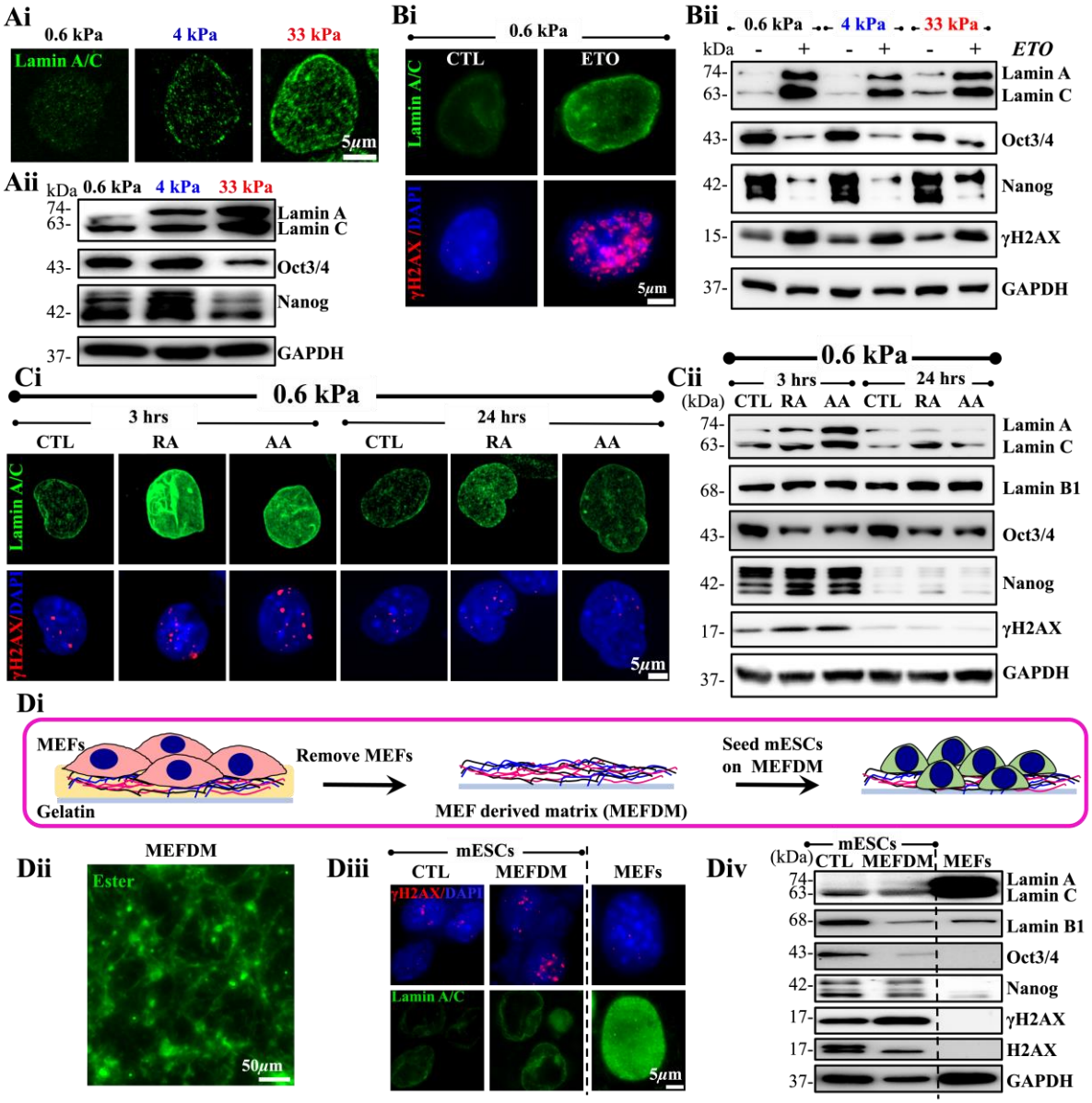

Ai
0.6 kPa
4 kPa
33 kPa
Lamin A/C
5μm
Aii
kDa
0.6 kPa
4 kPa
33 kPa
74-
63-
Lamin A
Lamin C
43-
Oct3/4
42-
Nanog
37-
GAPDH
Bi
0.6 kPa
CTL
ETO
Lamin A/C
γH2AX/DAPI
5μm
Bii
0.6 kPa
4 kPa
33 kPa
kDa
ETO
74-
63-
Lamin A
Lamin C
43-
Oct3/4
42-
Nanog
15-
γH2AX
37-
GAPDH
Ci
0.6 kPa
3 hrs
24 hrs
CTL
RA
AA
CTL
RA
AA
Lamin A/C
γH2AX/DAPI
5μm
Cii
0.6 kPa
3 hrs
24 hrs
(kDa)
CTL
RA
AA
CTL
RA
AA
74-
63-
Lamin A
Lamin C
68-
Lamin B1
43-
Oct3/4
42-
Nanog
17-
γH2AX
37-
GAPDH
Di
MEFs
Gelatin
Remove MEFs
MEF derived matrix (MEFDM)
Seed mESCs
on MEFDM
Dii
MEFDM
Ester
50μm
Diii
mESCs
CTL
MEFDM
MEFs
γH2AX/DAPI
Lamin A/C
5μm
Div
mESCs
(kDa)
CTL
MEFDM
MEFs
74-
63-
Lamin A
Lamin C
68-
Lamin B1
43-
Oct3/4
42-
Nanog
17-
γH2AX
17-
H2AX
37-
GAPDH

**Figure 4**

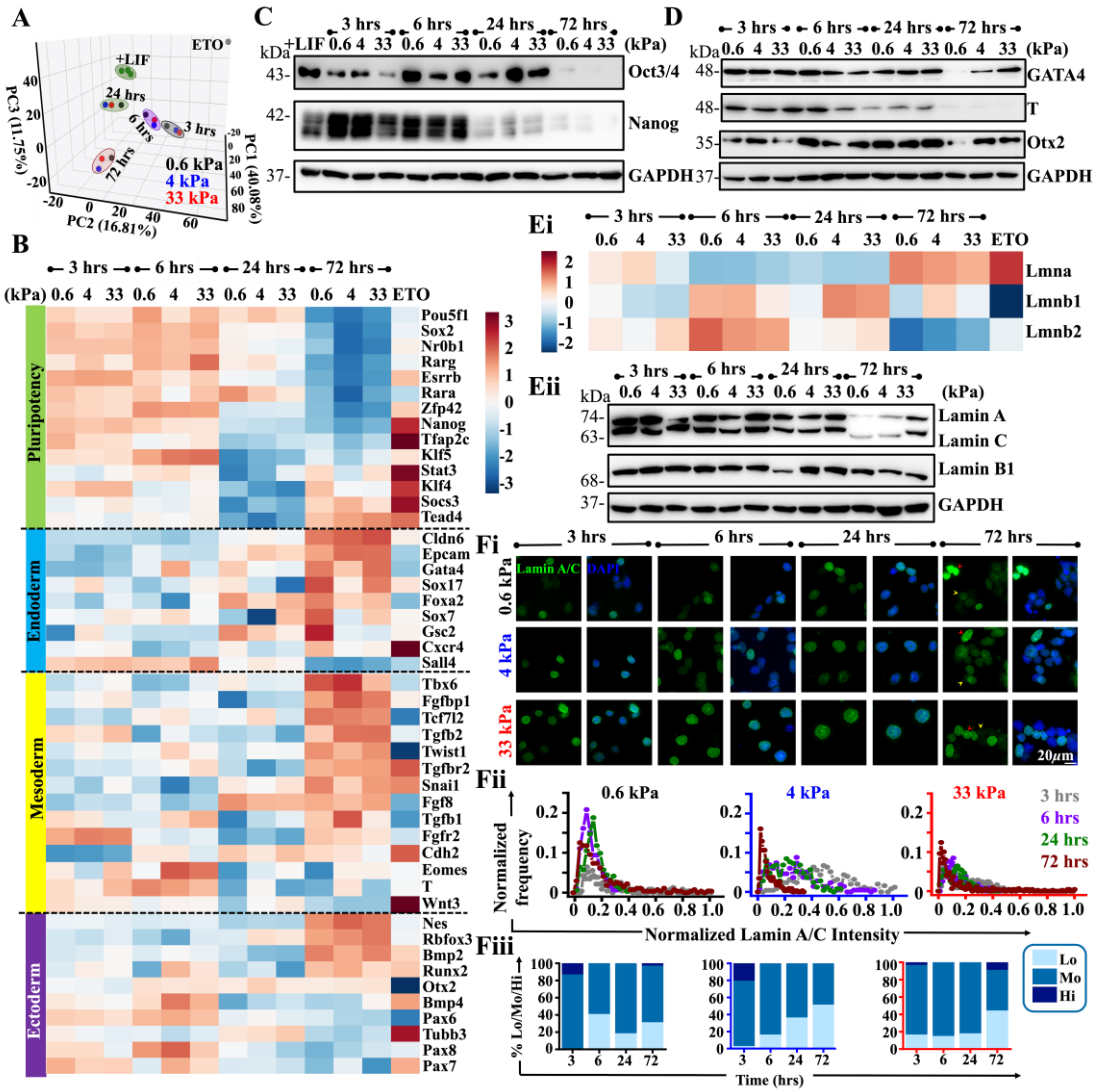

A
ETO
+LIF
24 hrs
6 hrs
3 hrs
72 hrs
0.6 kPa
4 kPa
33 kPa
PC3 (11.75%)
PC2 (16.81%)
PC1 (40.08%)
B
3 hrs
6 hrs
24 hrs
72 hrs
(kPa) 0.6 4 33 0.6 4 33 0.6 4 33 0.6 4 33 ETO
Pluripotency
Endoderm
Mesoderm
Ectoderm
Pou5f1
Sox2
Nr0b1
Rarg
Esrrb
Rara
Zfp42
Nanog
Tfap2c
Klf5
Stat3
Klf4
Socs3
Tead4
Cldn6
Epcam
Gata4
Sox17
Foxa2
Sox7
Gsc2
Cxcr4
Sall4
Tbx6
Fgfbp1
Tcf7l2
Tgfb2
Twist1
Tgfbr2
Snai1
Fgf8
Tgfb1
Fgfr2
Cdh2
Eomes
T
Wnt3
Nes
Rbfox3
Bmp2
Runx2
Otx2
Bmp4
Pax6
Tubb3
Pax8
Pax7
C
kDa +LIF 0.6 4 33 0.6 4 33 0.6 4 33 0.6 4 33 (kPa)
43-
42-
37-
Oct3/4
Nanog
GAPDH
D
kDa 0.6 4 33 0.6 4 33 0.6 4 33 0.6 4 33 (kPa)
48-
48-
35-
37-
GATA4
T
Otx2
GAPDH
Ei
0.6 4 33 0.6 4 33 0.6 4 33 0.6 4 33 ETO
Lmna
Lmnb1
Lmnb2
Eii
kDa 0.6 4 33 0.6 4 33 0.6 4 33 0.6 4 33 (kPa)
74-
63-
68-
37-
Lamin A
Lamin C
Lamin B1
GAPDH
Fi
Lamin A/C
DAPI
0.6 kPa
4 kPa
33 kPa
20μm
Fii
0.6 kPa
4 kPa
33 kPa
3 hrs
6 hrs
24 hrs
72 hrs
Normalized frequency
Normalized Lamin A/C Intensity
Fiii
% Lo/Mo/Hi
Time (hrs)
Lo
Mo
Hi

**Figure 5**

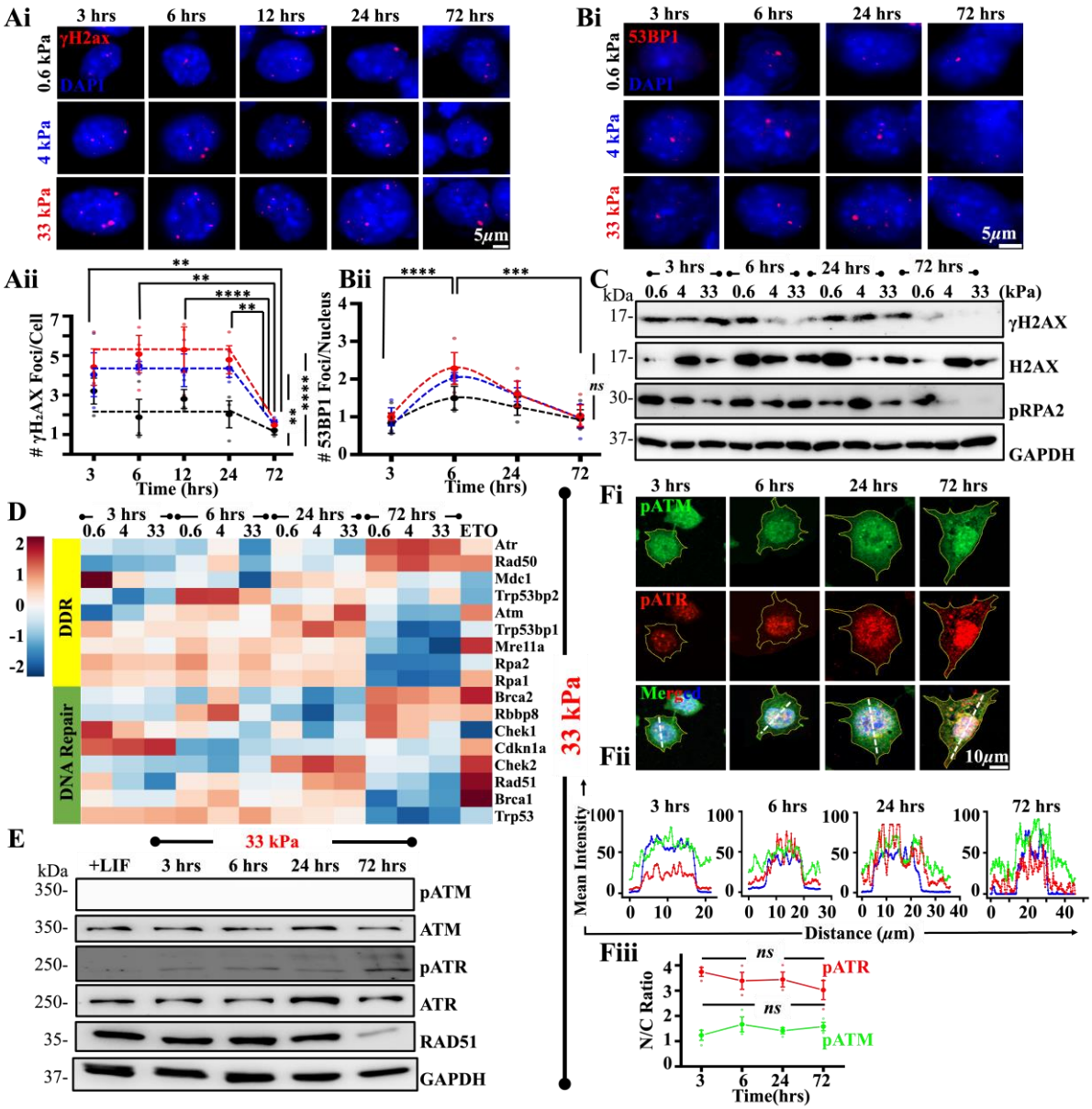
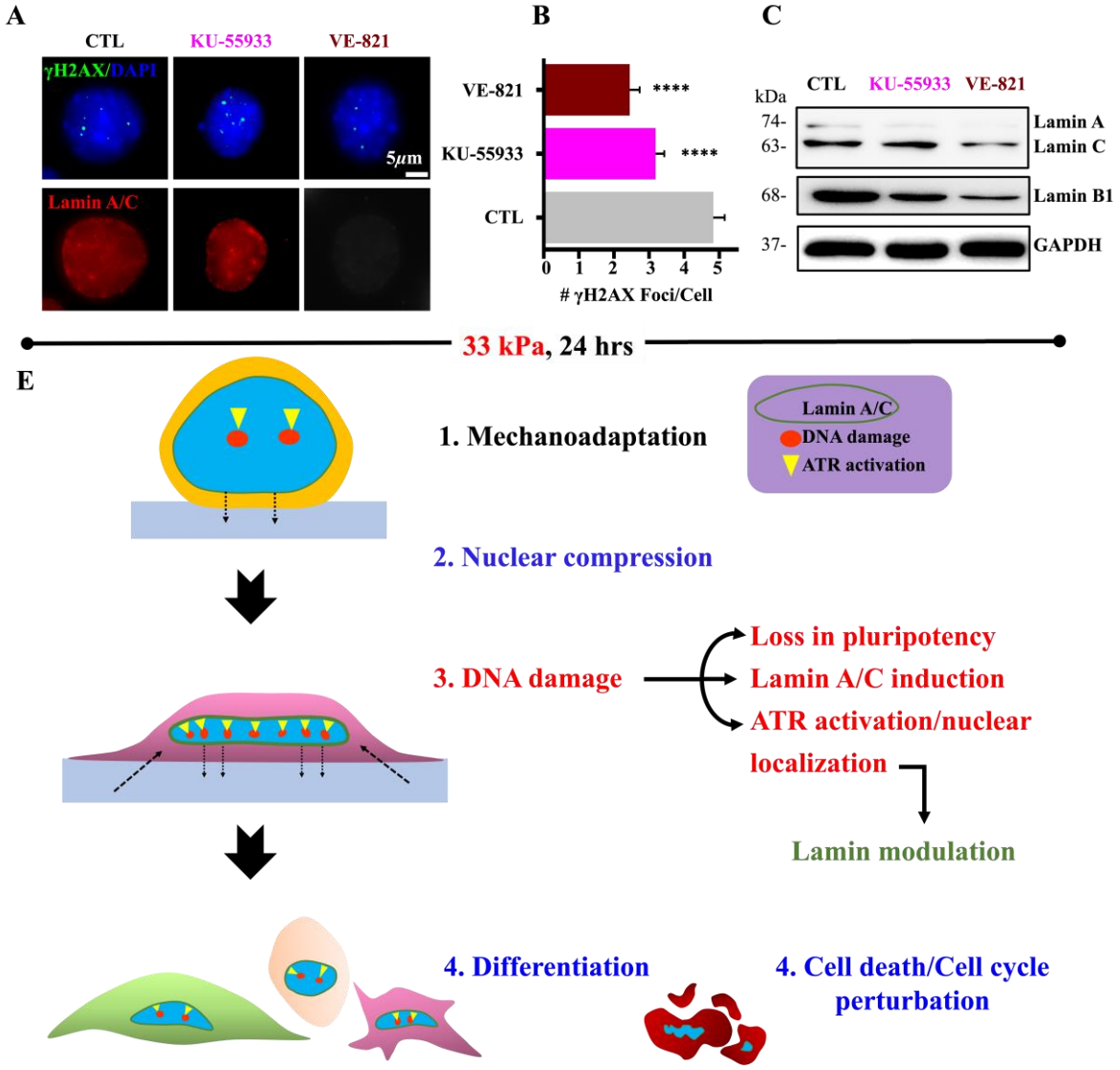

Ai
3 hrs
6 hrs
12 hrs
24 hrs
72 hrs
γH2ax
DAPI
0.6 kPa
4 kPa
33 kPa
5μm
Bi
53BP1
DAPI
5μm
Aii
# γH2AX Foci/Cell
Time (hrs)
Bii
# 53BP1 Foci/Nucleus
Time (hrs)
ns
C
kDa
0.6 4 33 0.6 4 33 0.6 4 33 0.6 4 33 (kPa)
γH2AX
H2AX
pRPA2
GAPDH
D
0.6 4 33 0.6 4 33 0.6 4 33 0.6 4 33 ETO
DDR
DNA Repair
Atr
Rad50
Mdc1
Trp53bp2
Atm
Trp53bp1
Mre11a
Rpa2
Rpa1
Brca2
Rbbp8
Chek1
Cdkn1a
Chek2
Rad51
Brca1
Trp53
33 kPa
E
kDa
+LIF
3 hrs
6 hrs
24 hrs
72 hrs
pATM
ATM
pATR
ATR
RAD51
GAPDH
Fi
pATM
pATR
Merged
10μm
Fii
Mean Intensity
Distance (μm)
Fiii
N/C Ratio
pATR
pATM
Time(hrs)

**Figure 6**


A
CTL
KU-55933
VE-821
γH2AX/DAPI
5μm
Lamin A/C
B
VE-821
KU-55933
CTL
****
****
0 1 2 3 4 5
# γH2AX Foci/Cell
C
kDa
CTL KU-55933 VE-821
74-
63-
68-
37-
Lamin A
Lamin C
Lamin B1
GAPDH
33 kPa, 24 hrs
E
1. Mechanoadaptation
Lamin A/C
DNA damage
ATR activation
2. Nuclear compression
3. DNA damage
Loss in pluripotency
Lamin A/C induction
ATR activation/nuclear localization
Lamin modulation
4. Differentiation
4. Cell death/Cell cycle perturbation