Sonoepigenetics: Spatiotemporal Calcium Dynamics and cAMP Signaling Driven by High Frequency Nanomechanostimulation Regulates Transient Epigenetic Modifications and Early Osteogenic Commitment in Mesenchymal Stem Cells

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Abstract

Cells effectively balance and integrate numerous pathways to adapt to external signals in an attempt to regain homeostasis, although the complex nuclear mechanotransduction mechanism through which this occurs is not as yet fully understood. Contrary to prevalent thought that the relay of extracellular cues to the nucleus to effect its fate and function predominantly relies on direct transmission through the cytoskeletal structure, we demonstrate, through the use of a unique form of high frequency (10 MHz) nanomechanostimulation, that manipulations to the cells' nuclear chromatin response are primarily influenced by the spatiotemporal dynamics associated with the bidirectional crosstalk between two key second messengers, namely calcium (Ca^{2+}) and cyclic adenosine monophosphate (cAMP). In stem cells, we show this conditioning, as an adaptive response to the mechanostimuli, to correlate with a 'mechanopriming' effect that is responsible for their early induction towards an osteogenic lineage in as little as three days with just brief (10 mins) daily bursts of mechanostimulation, without the need for osteogenic factors.

keywords: nuclear mechanotransduction; calcium signaling; cAMP; RNA polymerase II; histone modification

Introduction

There is growing recognition that the interplay between physical cues and their impact on cell homeostasis, mediated by nuclear mechanics and chromatin regulation, are central to virtually all physiological and developmental processes [1, 2, 3]. It has been observed, for example, that mechanical perturbations can induce changes in the distribution of lamin A/C at the nuclear periphery and wrinkling of the nuclear membrane, implying the mechanotransductive role of the cytoskeletal structure in relaying signals from the extracellular environment to the nuclear membrane through the LINC (linker of nucleoskeleton and cytoskeleton) complex [4, 5, 6, 7]. Additionally, mechanical forces have also been found to drive chromatin changes by reorganizing the epigenomic landscape through histone modifications [3, 8, 9], enabling cells to finely tune gene expression in response to external cues to maintain homeostasis [10, 11]. The specific molecular mechanisms by which extracellular cues from the cells' periphery are transmitted to the nucleus to influence chromatin organisation, and how these aspects affects cell fate, however, remain poorly understood [3].

The discrepancy in the timescales associated with the nuclear mechanical responses—with nuclear deformation occurring over millisecond timescales [12, 13], and chromatin reorganization taking seconds to minutes [14, 15, 16]—nevertheless hints at a more complex mechanotransduction picture. The involvement of Ca^{2+}-responsive kinases and phosphatases that influence numerous epigenomic and chromatin regulators, for instance, suggests that other, and possibly multiple, mechanisms could be involved in the regulation of chromatin and epigenomic landscapes in response to the extracellular cues [17, 18, 19, 20, 21, 22]. That Ca^{2+}-induced signalling pathways activated by external stressors to the cell are typically counterbalanced by other signalling cascades involving another second messenger cyclic adenosine monophosphate (cAMP) during the restoration of the cell to its homeostatic state, suggests that cAMP may also

play a pivotal role in the dynamics of epigenetic regulation associated with the recovery process [23, 24].

The way by which transmission of mechanical forces through the cytoskeleton dynamically interplays with these biochemical signals, such as Ca^{2+}[20, 21, 25, 26, 27, 28, 29] and cAMP [23, 24], over different time scales to facilitate latent recovery of the cell towards homeostasis (and to ultimately influence transcriptional outcomes and cell fates [30], including those related to stem cell differentiation and function [31, 32, 33]) remains to be explicated. To this end, we have found it expedient to utilize high frequency (10 MHz) nanomechanostimulation in the form of nanometer-amplitude hybrid surface and bulk sound waves (surface reflected bulk waves; SRBW [34]) to examine the nuclear mechanotransduction process, given its ability to spatiotemporally modulate Ca^{2+} and cAMP within the cell [35, 36]. In particular, we show that such spatiotemporal Ca^{2+} dynamics initiates a biphasic nuclear response in the cells associated with the initial challenge and subsequently their recovery, thereby offering a window by which insight can be gleaned into the multi-timescale role and bidirectional crosstalk between Ca^{2+} and cAMP in the dynamics of nuclear and epigenetic regulation.

More specifically, we unravel the role of the cytoskeletal structure and second messenger signalling in the nuclear mechanotransduction process. Tension triggered by the actin cytoskeletal rearrangement in response to the mechanostimulation was observed to drive transient modifications in nuclear morphology and lamin dynamics; on the other hand, the histone modification response was seen to arise as a consequence of the dynamic interplay between Ca^{2+} and cAMP signaling—interestingly, without direct involvement of actin cytoskeletal dynamics. Further, we note that, together with the induced fluctuation of H3K27me3, H3K9me3 and H3K27ac, the mechanostimuli promoted clustering of RNA polymerase II and Cajal bodies (CBs), thereby revealing for the first time, the mechanoresponsive behavior of these nuclear bodies as a result of the second messenger dynamics.

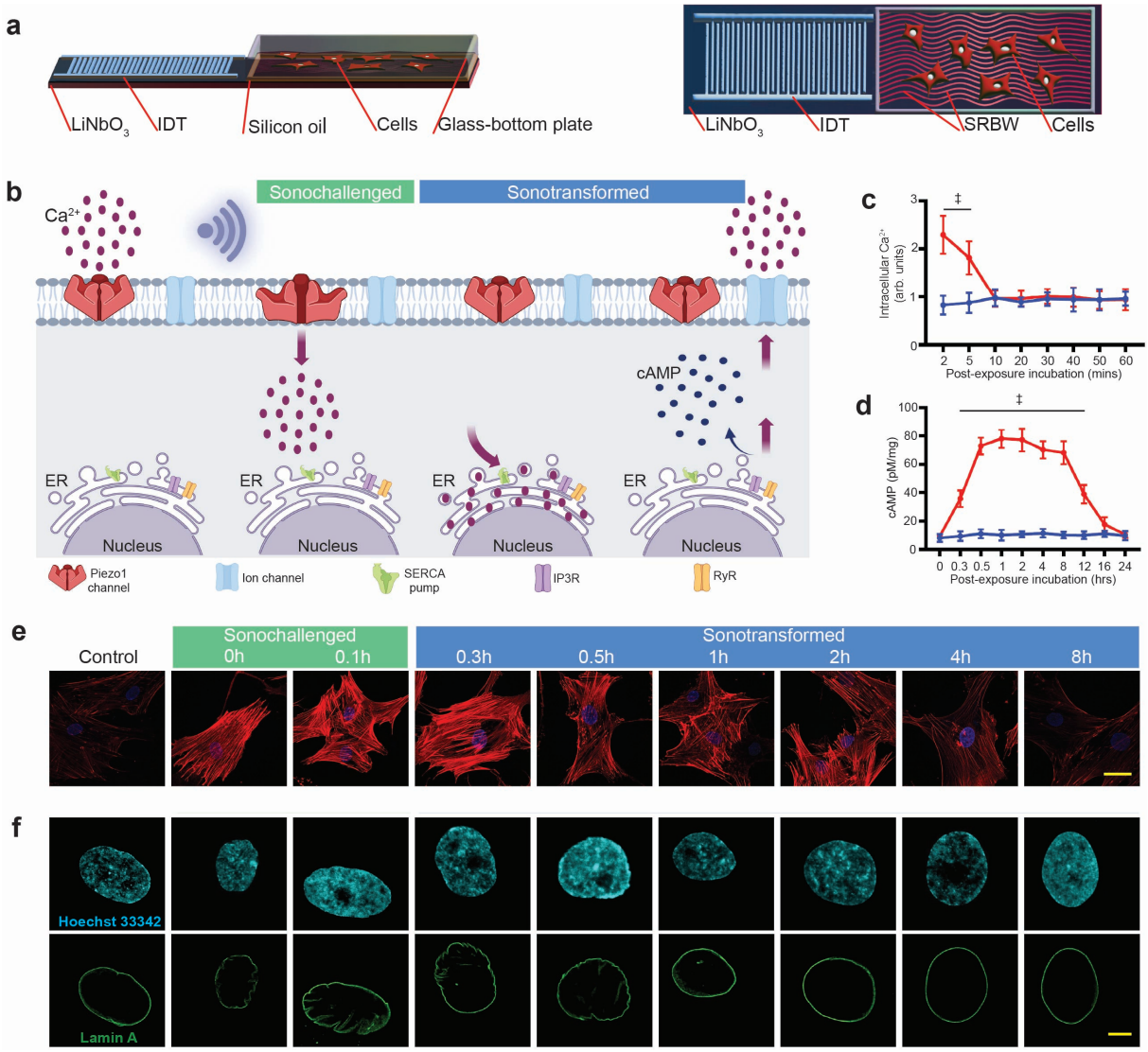
Altogether, we elucidate how such epigenetic regulation dynamics, influenced by spatiotemporal changes in intracellular Ca^{2+} and cAMP levels to recover cellular homeostasis, are intricately related to the process by which mesenchymal stem cells are mechanically primed for early commitment towards osteogenic differentiation that was observed with the SRBW nanomechanostimulation [37]. Importantly, inhibition of such second messenger dynamics was observed to suppress the early osteogenic lineage induction of the cells.

Results and Discussion

Nuclear Mechanoresponse

Subjecting bone-marrow-derived human mesenchymal stem cells (hMSCs) to 10 mins of 2.5 W SRBW nanomechanostimulation (optimized to achieve the observed effect in the cells whilst maximizing their viability ($\approx 95\%$)), as shown in the experimental setup described in the Methods section and depicted in Fig. 1a, can be seen to induce a biphasic response (Fig. 1b). First, we observe an immediate surge in Ca^{2+} within the cell by approximately 2.3 times (Fig. 1b,c), enabled by activation of mechanosensitive Piezo1 channels as a consequence of the mechanostimulation (i.e., the *sonochallenge* phase) [38]. Following this spike, the intracellular Ca^{2+} levels returned to their baseline values within 10 mins of post-exposure incubation. As a secondary latent response to the mechanostimulation, we also observed a steady increase in intracellular cAMP levels at around 15 mins post-exposure, reaching a peak within an hour, at which they remained elevated before gradually returning to baseline levels after 8 hrs (i.e., the *sonotransformation* phase; Figs. 1b,d).

Over this time course, a rapid, pronounced increase in actin stress fibers over the nuclear region is apparent (Figs. 1e and S2 (Supporting Information)). This characteristic cap can be seen to persist up to 0.3 hrs following the SRBW nanomechanostimulation, commensurate with the timescale of the initial Ca^{2+} spike transient associated with the sonochallenge. At 0.5 hrs,,


a
LiNbO3
IDT
Silicon oil
Cells
Glass-bottom plate
LiNbO3
IDT
SRBW
Cells
b
Sonochallenged
Sonotransformed
Ca2+
cAMP
ER
Nucleus
Piezo1 channel
Ion channel
SERCA pump
IP3R
RyR
c
Intracellular Ca2+ (arb. units)
Post-exposure incubation (mins)
d
cAMP (pM/mg)
Post-exposure incubation (hrs)
e
Control
Sonochallenged
0h
0.1h
Sonotransformed
0.3h
0.5h
1h
2h
4h
8h
f
Hoechst 33342
Lamin A

the actin organization is already focused toward the periphery of the cell, and after 8 hrs, the cell gradually returns to its homeostatic state, similar to the unstimulated control (see Fig. 1e). Morphometric measurements of the nucleus with lamin A staining (Fig. 1f) showed that while no appreciable changes were observed in the total nuclear volume (Fig. S3a (Supporting Information)), the SRBW nanomechanostimulation nevertheless induced significant alterations to the nucleus' general shape, as highlighted by the decrease in its transverse area (Fig. S3b (Supporting Information)) and an increase in its height (Fig. S3c (Supporting Information)), with consequent changes in its circularity, solidity and eccentricity (Figs. S3d–S3f (Supporting Information)). Additionally, we observe the lamin A in the nucleus to transition from its initially smooth, oval shape to an undulated and wrinkled appearance (Fig. 1f).

The time-course analysis of the images indicates the alignment of the observed evolution in morphological transformation with the biphasic Ca^{2+} and cAMP time scales in Figs. 1c and

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Figure 1 *(preceding page)*: **SRBW nanomechanostimulation induces nuclear deformation and lamin A wrinkling in hMSCs**. (a) Side (left) and top (right) view schematic representations of the experimental setup in which the SRBW (not to scale), produced by applying a sinusoidal electrical signal at its resonant frequency (10 MHz) to an interdigitated transducer (IDT) that is photolithographically patterned on a single crystal piezoelectric substrate ($LiNbO_3$), is transmitted through a thin layer of silicon oil into a glass-bottom culture plate containing adherent bone-marrow-derived mesenchymal stem cells (hMSCs). (b) Schematic illustrating the pathways associated with the dynamics of the Ca^{2+} mobilization within the cell throughout the time frame associated with the biphasic response of the cell to the SRBW nanomechanostimulation (see Fig. S1 (Supporting Information)): from left to right: control or pre-stimulation condition, the sonochallenge phase following stimulation, and the sonotransformation phase associated with the recovery stage of the cell towards homeostasis. (c) Intracellular Ca^{2+} ($n = 4$) and (d) cAMP ($n = 4$) concentration in the hMSCs as a function of post-exposure incubation time for both control (untreated) and SRBW nanomechanostimulated cells; data are represented in terms of the mean value $\pm$ the standard error over multiple runs, and, ‡ indicates statistically significant differences with $p < 0.0001$. (e,f) Representative confocal microscopy images illustrating (e) the reorganization of the actin cytoskeleton ($60\times$ magnification; scale bar: 50 μm), and, (f) the nucleus ($100\times$ magnification; scale bar: 5 μm) in hMSCs subjected to the SRBW nanomechanostimulation at different post-exposure incubation times compared to the control (untreated) group. Cell nuclei were stained with Hoechst 33342 (displayed in cyan) and lamin A immunofluorescence (displayed in green), whereas F-actin was labeled using ActinRed™ 555 (red).

1d: Frist, rapid and sharp morphological changes over the timescale (minutes) over which Ca^{2+} levels are elevated following the initial sonochallenge phase—characterized by stress-fiber formation and reduction in nuclear size. This was then followed by their gradual recovery (size, area, volume, and shape descriptors) over the timescale (hours) associated with the subsequent sonotransformation phase during which the cell relaxes back to its ground state (Fig. S1 (Supporting Information)).

Alterations in nuclear morphology, particularly those involving nuclear lamins, have been reported to affect chromatin organization, leading to modifications in chromatin condensation and accessibility [6, 8, 10, 39, 40]. Considering the effects of the SRBW nanomechanostimulation on the cells, we assessed the distribution of H3K27me3 (Fig. 2a), a facultative heterochromatin marker mediated by the Polycomb repressive complex EZH2, and the constitutive heterochromatin marker H3K9me3 (Fig. 2b). The results show an initial slight downregulation of H3K27me3, which precede significant upregulation upon normalization of cytosolic Ca^{2+} levels and elevation of cAMP during the sonotransformation phase at 0.5 hrs. More specifically, H3K27me3 increases by approximately fivefold before gradually decreasing back to baseline levels with the recovery of the cell over 4–8 hrs (Fig. 2c). Interestingly, the immunofluorescence for H3K9me3 shows a different dynamic and is somehow anticorrelated with the H3K27me3 nuclear distribution, exhibiting a decrease to approximately 0.2-fold at 0.5 hrs before gradually regaining its signal to baseline levels with the recovery of the cell over 4–8 hrs (Fig. 2d). Examination of a chromatin marker for active enhancers H3K27ac [41] in Fig. 3a, on the other hand, showed its significant upregulation upon normalization of cytosolic Ca^{2+} levels and elevation of cAMP during the sonotransformation phase at 0.5 hrs by approximately threefold, followed by a return to the baseline levels over 4–8 hrs (Fig. 3d).

Strikingly, staining for RNAP II CTD Serin5 phosphorylation (RNAPII-S5p)—a specific post-translational modification that characterizes transcriptionally engaged RNAP II (active

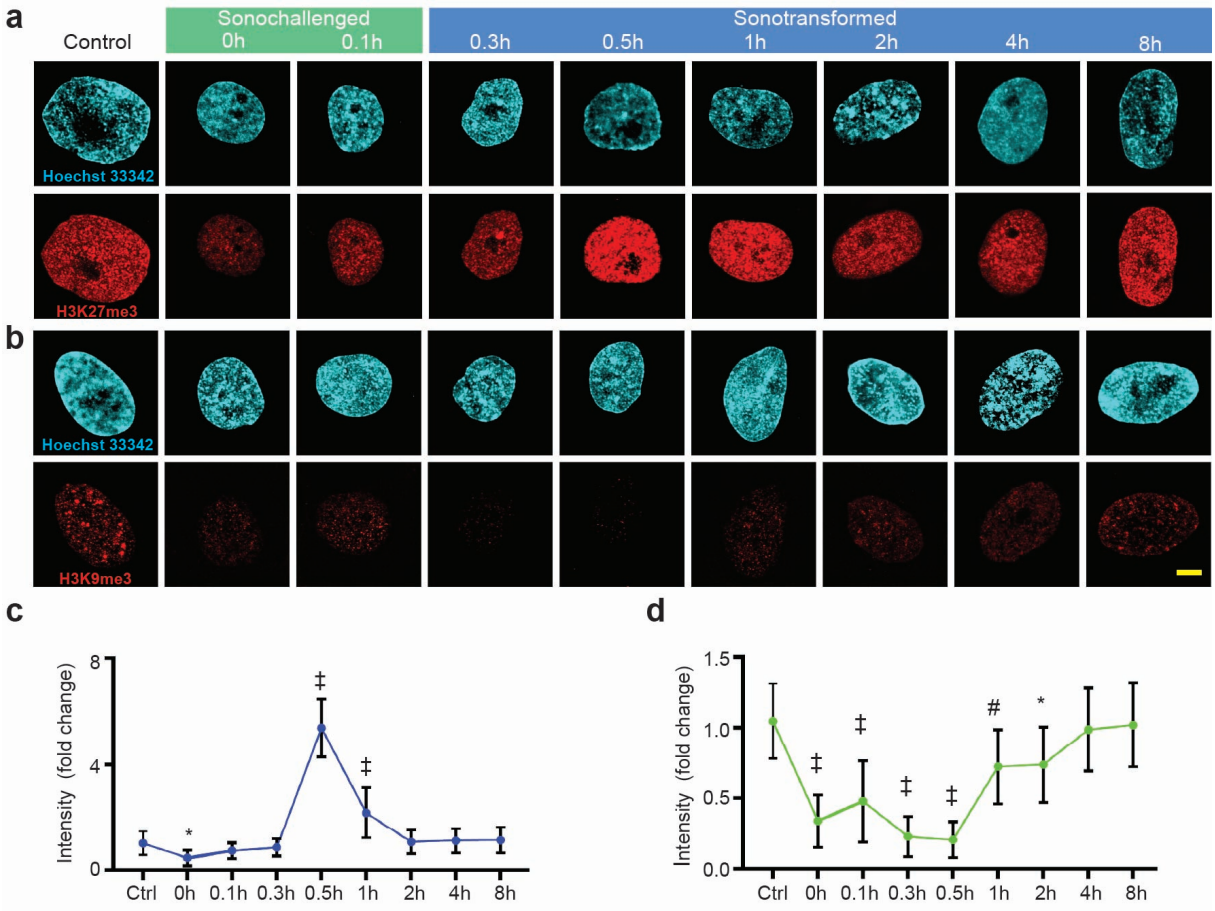
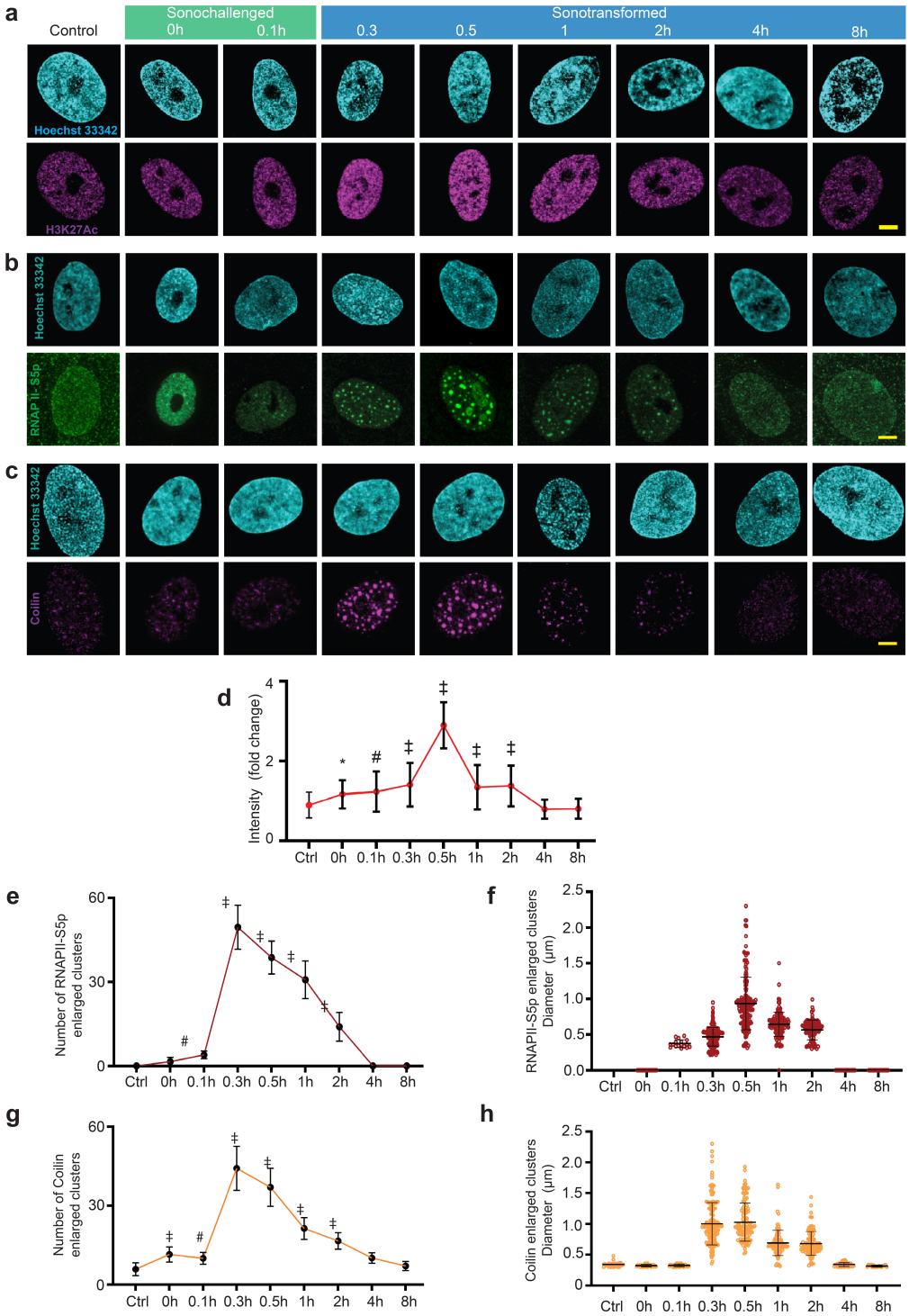


Figure 2: **hMSCs show fluctuations in chromatin repressive marks in response to the SRBW nanomechanostimulation**. Representative confocal microscopy images of the nucleus (stained with Hoechst 33342 and displayed in cyan) together with (a) H3K27me3 and (b) H3K9me3 (displayed in red) immunofluorescence following SRBW nanomechanostimulation of the hMSCs at different post-exposure incubation times, compared to those of the control (untreated) cells; all images were acquired at $100\times$ magnification and the scale bars denote lengths of 5 µm. Also shown are the relative immunofluorescence of (c) H3K27me3 and (d) H3K9me3 in the SRBW nanomechanostimulated cells at different post-exposure incubation times with respect to that in the control (untreated) cells. Data are represented in terms of the mean value $\pm$ the standard error over multiple runs ($n > 50$ cells/condition, visualized across four independent experiments); $*$, $\#$ and $\ddagger$ indicate statistically significant differences with $p < 0.05$, $p < 0.01$ and $p < 0.0001$, respectively.

and poised) [42, 43]—revealed a dramatic pattern distribution accompanying the spike in intracellular Ca^{2+} during the initial sonochallenge phase (Fig. 3b). In contrast to active RNAP II molecules which are typically localized within distinct but adjacently-placed spatial compartments with approximate lengthscales on the order 100 nm [44, 45], the SRBW nanomechanostimulation can instead be observed by 0.3 hrs to induce an increase in the number of the RNAPII-S5p larger clusters (Fig. 3e), which progressively grew into bigger condensates (Fig. 3f). That these condensates could have resulted from a block in transcription due to DNA damage can be ruled out since we have not found evidence of any such damage due to the SRBW nanomechanostimulation. This is demonstrated by the absence of histone H2AX phosphorylation—a marker for early cellular responses to DNA damage [46]—in the treated cells (Fig. S4 (Supporting Information)).

Similar to RNAPII-S5p staining, we also observed the immunofluorescence signal for Coilin—a marker of Cajal Bodies (CBs)—to also transiently cluster towards bigger condensates by 0.3 hrs (Fig. 3c,h), suggesting the broader effect of the SRBW nanomechanostimulation on inducing alterations to other nuclear condensates. Quantitative analysis of the CB clusters shows that they increased in their dimension from 0.3 μm at 0.1 hrs before reaching a peak of 1.1 μm at 0.5 hrs (Fig. 3h), albeit with decreasing number of condensates (Fig. 3g). Quite interestingly, the results reveal a lack of coincidence between the total numbers and dimensions of RNAPII-S5p and the CBs, suggesting that the two aggregates could represent independent functions despite a certain level of interaction. Akin to the other chromatin features, it can also be seen that the RNAPII-S5p and CB expression gradually returned to their original size upon recovery of the cell during the sonotransformation phase over which intracellular cAMP levels were elevated.


a
Control
Sonochallenged
0h
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0.3
0.5
1
2h
4h
8h
Hoechst 33342
H3K27Ac
b
Hoechst 33342
RNAP II- S5p
c
Hoechst 33342
Coilin
d
Intensity (fold change)
Ctrl 0h 0.1h 0.3h 0.5h 1h 2h 4h 8h
e
Number of RNAPII-S5p enlarged clusters
f
RNAPII-S5p enlarged clusters Diameter (µm)
g
Number of Coilin enlarged clusters
h
Coilin enlarged clusters Diameter (µm)

Role of Spatiotemporal Ca^{2+} and cAMP Dynamics

The aforementioned results highlight the biphasic response of the cells toward the SRBW nanomechanostimulation, through which the spatiotemporal dynamic interplay between Ca^{2+} and cAMP in modulating nuclear morphology and the chromatin fluctuations becomes evident. (1) An initial phase where the Piezo1-mediated influx of Ca^{2+} into the cell, upon being *sonochallenged* by the SRBW nanomechanostimulation, triggers various signaling pathways involving the small guanosine triphosphate hydrolase (GTPase) Rho (Ras homolog gene family) and its downstream effector ROCK (Rho-associated protein kinase), which are responsible for the formation of the stress fibers observed in Figs. 1e and S2 (Supporting Information) [47, 48, 49] up to around 0.3 hrs post-exposure. (2) A subsequent *sonotransformation* phase after approximately 0.5 hrs in which the stress fibers are dissolved upon activation of the cAMP-activated guanine nucleotide exchange protein/Ras-related protein 1 (Epac/Rap1) signaling cascade driven by the elevated cAMP concentration [35, 50, 51, 52, 53]. This biphasic behavior (Fig. 1b), which is a typical with the SRBW nanomechanostimulation [35], starkly contrasts with the classical monophasic response of Ca^{2+}-induced stress fiber formation following challenges to the cell either with chemical or conventional (low frequency (<1 MHz)) mechanical stimulation [54, 55, 56]. This contrasting behavior is likely due to the distinctive capacity of

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Figure 3 *(preceding page)*: **Nuclear bodies exhibit mechanoresponsive behavior to SRBW nanomechanostimulation**. (a–c) Representative confocal microscopy images of of the nucleus (stained with Hoechst 33342 and displayed in cyan) together with (a) H3K27Ac (displayed in purple), (b) RNAPII-S5p (displayed in green), and, (c) Coilin (displayed in purple) immunofluorescence following SRBW nanomechanostimulation of the hMSCs at different post-exposure incubation times, compared to those of the control (untreated) cells; all images were acquired at $100\times$ magnification and the scale bars denote lengths of 5 μm. Also shown are (d) the relative immunofluorescence of H3K27Ac, (e) the number, and, (f) the size of the RNAPII-S5p condensates, respectively, and, those of (g,h) the Coilin clusters, respectively, in the SRBW nanomechanostimulated cells at different post-exposure incubation times. Data are represented in terms of the mean value $\pm$ the standard error over multiple runs ($n > 50$ cells/condition, visualized across four independent experiments); $*$, $\#$ and ‡ indicate statistically significant differences with $p < 0.05$, $p < 0.01$ and $p < 0.0001$, respectively.

the SRBW to facilitate bidirectional crosstalk between the spatiotemporal Ca^{2+} dynamics and opposing cAMP signaling, given its ability to modulate sufficient levels of Ca^{2+} into the cell without driving the cells toward apoptosis. This allows excess Ca^{2+} to be sequestered into the endoplasmic reticulum (ER) such that its subsequent latent release back into the cytosol through ryanodine (RyR) channels and inositol trisphosphate (IP3R) receptors (calcium-induced calcium release; CICR [57]) over considerably longer time periods (typically hours) enables cAMP signaling and hence Epac/Rap1 counteraction as part of the recovery process by which the cells return to their homeostatic state [35].

Specific inhibitors were adopted to glean further insight into the mechanotransduction mechanism giving rise to the changes in nuclear morphology and chromatin organization that were observed, and the role of the interactions between Ca^{2+} and cAMP in the process (Fig. 4a). Notably, the nuclear morphology of SRBW nanomechanostimulated cells that were prior treated with cytochalasin D—an inhibitor of actin polymerization—remained predominantly unaltered (Fig. 4b and Figs. S5 and S6 (Supporting Information)). A similar observation was noted when intracellular Ca^{2+} was chelated using BAPTA-AM (Fig. 4c and Fig. S6 and S7a (Supporting Information)). Together, these allude to the critical role of actin in modulating the organization and morphology of nuclear lamin: the interruption of the signal relay to the nucleus in the absence of the actin stress fibers, either due to disruption of actin polymerization or due to a lack of elevated intracellular Ca^{2+} levels, hints at the primary function of the actin cytoskeleton in transmitting extracellular signals from the plasma membrane to the nucleus via nesprin proteins of the LINC complex to the nuclear lamina, consistent with that previously reported [4, 17, 40, 58, 59, 60, 61, 62].

In contrast, the nuclear morphological changes induced by the SRBW nanomechanostimulation were unaffected when elevation in intracellular cAMP levels was obstructed, either through inhibition of the sarco/endoplasmic reticulum calcium ATPase (SERCA) pump with

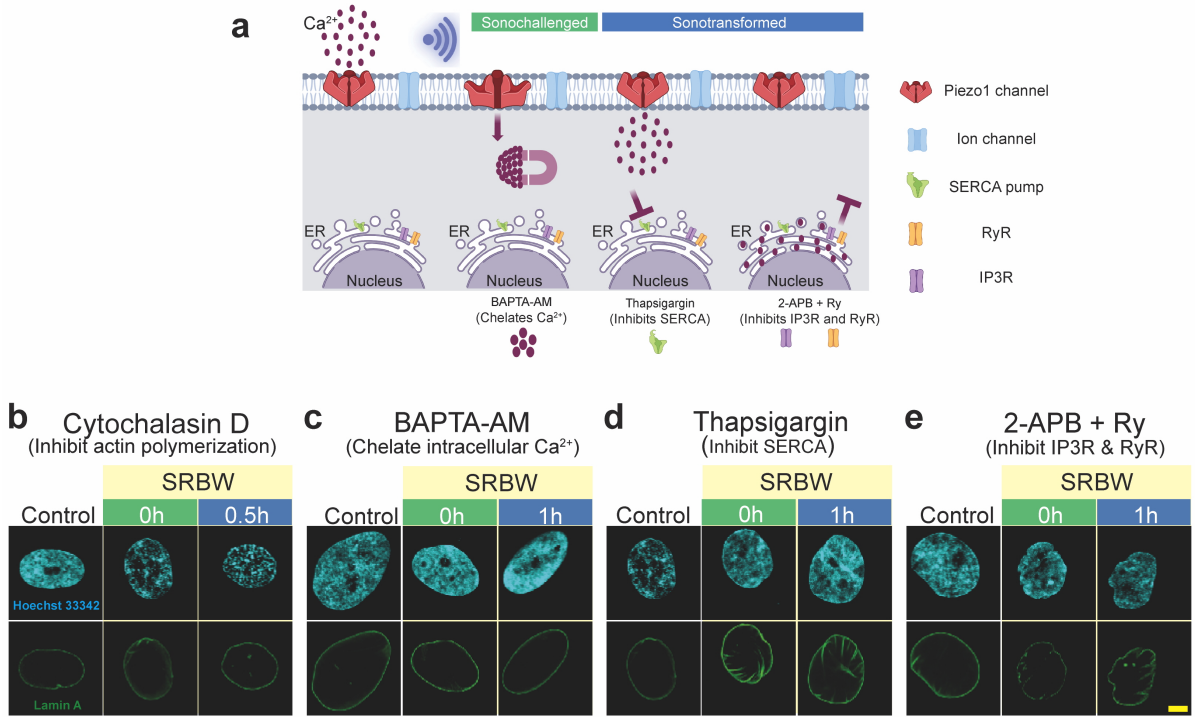
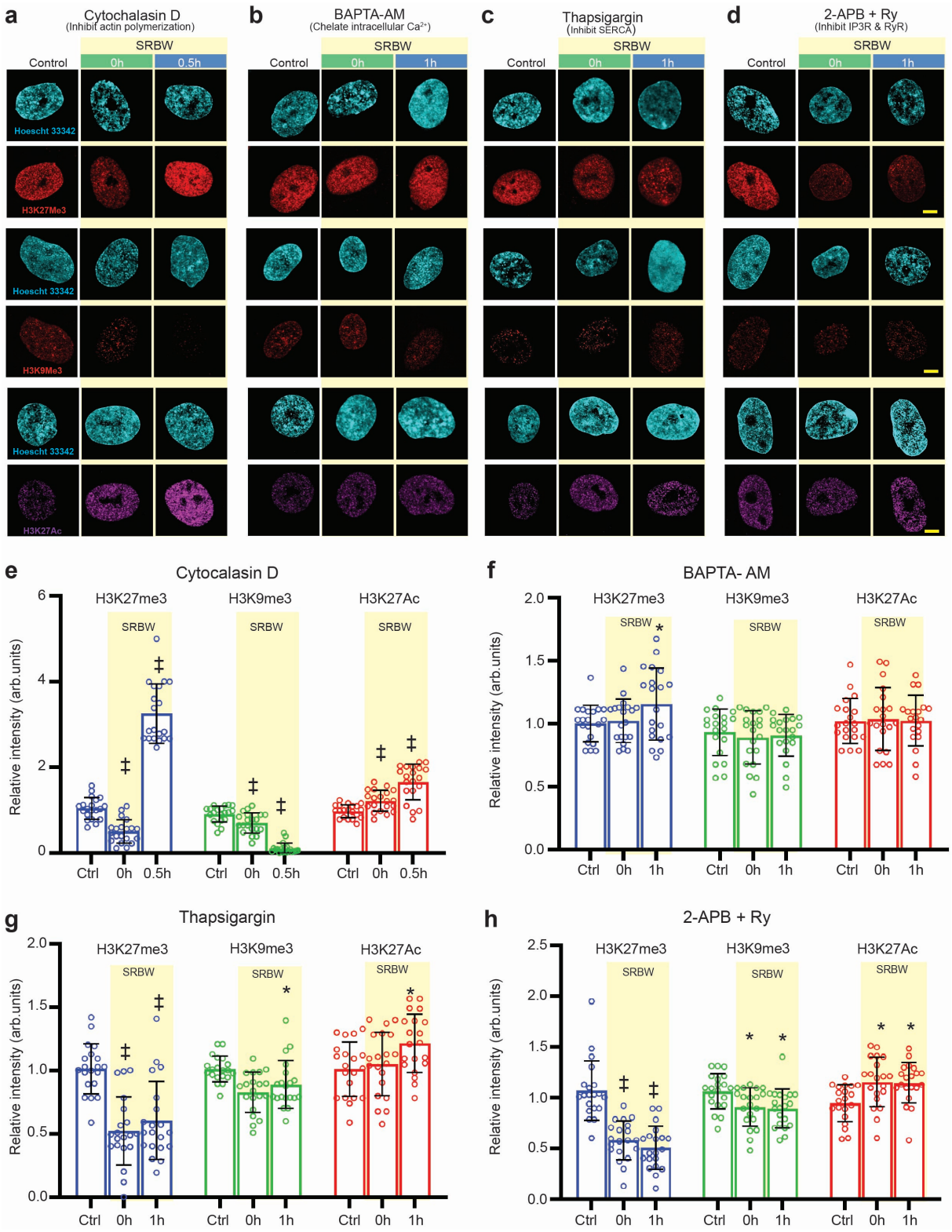


Figure 4: **Actin polymerization regulates nuclear morphology upon SRBW nanomechanostimulation**. (a) Schematic illustration showing the Ca^{2+} mobilization pathway in the presence of the various Ca^{2+} chelators and inhibitors: BAPTA-AM, a chelator of cytosolic Ca^{2+}, thapsigargin, which blocks the SERCA pump to prevent Ca^{2+} influx into the ER, and, 2-APB and ryanodine (Ry), which impedes the efflux of Ca^{2+} from the ER back into the cytosol. (b–e) Representative confocal images of lamin A immunofluorescence (displayed in green) in the presence of (b) cytochalasin D, (c) BAPTA-AM, (d) thapsigargin, and, (e) 2-APB and ryanodine (Ry), at different post-exposure incubation compared to that for control (untreated) cells; all images were acquired at $100\times$ magnification and the scale bars denote lengths of 5 μm.

thapsigargin to prevent influx of cytosolic Ca^{2+} into the ER (Fig. 4d and Figs. S6 and S7b (Supporting Information)), or when the ER Ca^{2+} channels—IP3R and RyR—were blocked with 2-aminoethyl diphenylborinate (2-APB) and ryanodine (Fig. 4e and Figs. S6 and S7b (Supporting Information)). We also note in these cases that the nuclear membrane maintained its wrinkled appearance up to 1 hr post-exposure, beyond the 0.5 hr time point at which recovery began to occur in the uninhibited case. Both effects—be it the accumulation of cytosolic Ca^{2+} by blocking its influx into the ER through SERCA, or arresting the efflux of Ca^{2+} from the ER to trigger cAMP signalling—led to the sustained presence of the actin stress fibers in the cytoskeletal network, thus delaying recovery of the nucleus from its deformation.

These findings highlight the centrality of the Ca^{2+} spatiotemporal dynamics (which, in turn, dictates the regulation of cAMP) in determining the nuclear morphological response to extracellular cues. The initial elevation of cytosolic Ca^{2+} during the sonochallenge phase activates the Rho/ROCK pathway in the cytoskeletal network to induce changes in nuclear morphology, mediated by the actin stress fibers. Mobilization of cytosolic Ca^{2+} into the ER, and the subsequent efflux of ER Ca^{2+} back into the cytosol through the CICR mechanism, to trigger an increase in cytosolic cAMP levels in the sonotransformation phase then initiates Epac/Rap1 signalling to counteract the Ca^{2+}-triggered Rho/ROCK pathway and hence accommodate relaxation of the nuclear morphology back to its ground state. This bidirectional crosstalk between Ca^{2+} and cAMP therefore serves as a feedback mechanism from the nucleus as a means for maintaining cellular homeostasis [24, 35, 50, 51, 52, 53, 63, 64, 65, 66].

Unlike the nuclear morphological changes described above, the reorganization of the chromatin marks appear to remain unaffected when actin polymerization was disrupted by cytochalasin D (Fig. 5a,e). Notably, this seems to strongly allude to the decoupling of the influence of the actin cytoskeleton—which we observed previously to play a central role in the nuclear morphological response to the SRBW nanomechanostimulation—from the regulatory aspects of


a
Cytochalasin D
(Inhibit actin polymerization)
SRBW
Control
0h
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b
BAPTA-AM
(Chelate intracellular Ca2+)
SRBW
Control
0h
1h
c
Thapsigargin
(Inhibit SERCA)
SRBW
Control
0h
1h
d
2-APB + Ry
(Inhibit IP3R & RyR)
SRBW
Control
0h
1h
Hoescht 33342
H3K27Me3
Hoescht 33342
H3K9Me3
Hoescht 33342
H3K27Ac
e
Cytocalasin D
H3K27me3
H3K9me3
H3K27Ac
SRBW
Relative intensity (arb.units)
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0h
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BAPTA- AM
H3K27me3
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H3K27Ac
SRBW
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H3K27me3
H3K9me3
H3K27Ac
SRBW
Relative intensity (arb.units)
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0h
1h
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2-APB + Ry
H3K27me3
H3K9me3
H3K27Ac
SRBW
Relative intensity (arb.units)
Ctrl
0h
1h

the epigenome. This therefore suggests that force transduction through the LINC complex that anchors the cytoskeletal network to the nuclear membrane is only one of the players relaying the extracellular cues to the nucleus. Instead, we observe the primary role of the spatiotemporal intracellular Ca^{2+} dynamics to be the dominant mechanism in driving histone modifications, as confirmed by the suppression of the histone chromatin marks observed (i.e., H3K27me3, H3K27Ac and H3K9me3) upon removal of cytosolic Ca^{2+} with BAPTA-AM (Fig. 5b,f) or with inhibition of RyR and IP3R (Figs. 5d,h). Inhibiting Ca^{2+} influx into the ER with thapisgargin, on the other hand, can be seen to delay normalization of these histone markers (Fig. 5c,g).

Disrupting actin polymerisation with cytochalasin D also had little effect on the SRBW-induced dynamics of RNAPII-S5p and the CBs, as can be seen in Fig. 6a. Chelating cytosolic Ca^{2+} with BAPTA-AM can be observed to prevent changes to the RNAPII-S5p and CB morphologies induced by the SRBW nanomechanostimulation (Fig. 6b). Also, maintaining elevated cytosolic Ca^{2+} levels while inhibiting intracellular cAMP by blocking the SERCA pump with thapsigargin led to the suppression of both RNAPII-S5p and CB cluster formation (Fig. 6c). We further noticed that inhibiting cAMP by blocking the RyR and IP3R channels prevented the formation of CB clusters and RNAPII-S5p condensation (Fig. 6d). Taken together, these results indicate that it is the Ca^{2+} spatiotemporal dynamics and the resulting cAMP signaling, rather

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Figure 5 *(preceding page)*: **Ca^{2+} and cAMP dynamics regulate mechanoresponsivbe histone fluctuations in hMSCs**. (a–d) Representative confocal microscopy images of the nucleus (stained with Hoechst 33342 and displayed in cyan) together with H3K27me3 (displayed in red), H3K9me3 (displayed in red) and H3K27Ac (displayed in purple) immunofluorescence in the presence of (a) cytochalasin D, (b) BAPTA-AM, (c) thapsigargin, and, (d) 2-APB and ryanodine (Ry), at 0 and 0.5 hrs or 1 hr post-exposure incubation following SRBW nanomechanostimulation, compared to that in the control (untreated) cells; all images were acquired at $100\times$ magnification and the scale bars denote lengths of 5 µm. (e–h) Relative immunofluorescence of H3K27me3, H3K9me3 and H3K27Ac in the SRBW mechanostimulated cells at 0 and 0.5 hrs or 1 hr post-exposure incubation compared to that in the control (untreated) cells (Ctrl). Data are represented in terms of the mean value $\pm$ the standard error over multiple runs ($n = 20$ cells/condition, visualized from four independent experiments); $*$, † and ‡ indicate statistically significant differences with $p < 0.05$, $p < 0.001$ and $p < 0.0001$, respectively.

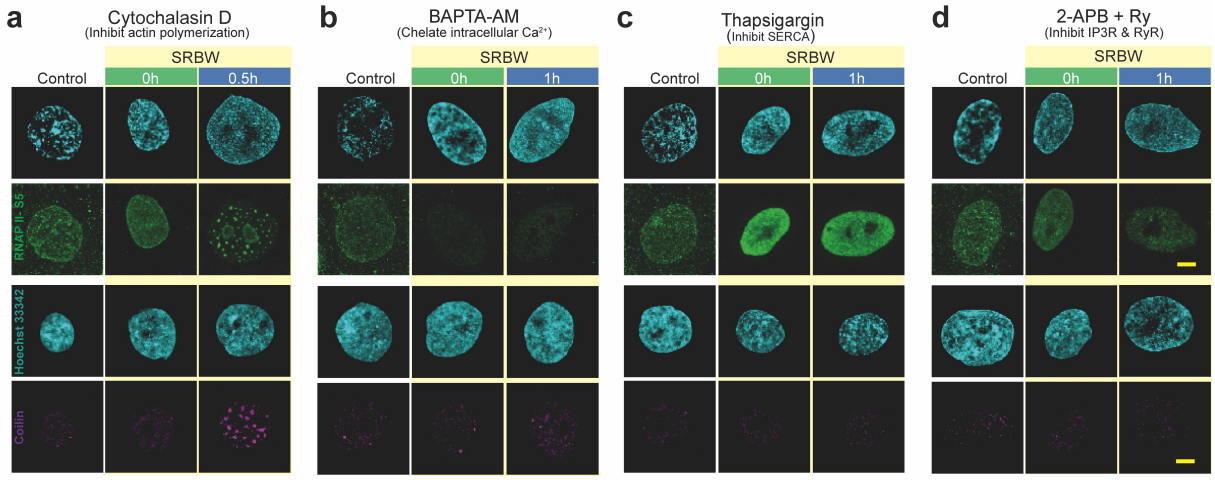

Figure 6: **Nuclear body inhibition studies**. Representative confocal microscopy images of the nucleus (stained with Hoechst 33342 and displayed in cyan) together with RNAPII Ser5 (displayed in green) and Coilin (displayed in purple) in the presence of (a) cytochalasin D, (b) BAPTA-AM, (c) thapsigargin, and, (d) 2-APB and ryanodine (Ry), at 0 and 0.5 hrs or 1 hr post-exposure incubation following SRBW nanomechanostimulation, compared to that in the control (untreated) cells. All images were acquired at $100\times$ magnification and the scale bars denote lengths of 5 μm.

than the actin cytoskeleton, that are the key mediators that predominantly guide the epigenetic modifications. Of particular interest is the paramount significance of the dynamics of second messenger Ca^{2+} and cAMP in regulating histone modifications and, consequently, the transcription processes associated with nuclear mechanotransduction. This process, particularly its feedback response, constitutes a multifaceted molecular switch that influences the downstream fate of the cell, such as a return to homeostasis or a transition towards differentiation [67, 68].

Downstream Cell Fate Regulation

We have previously reported that subjecting hMSCs to SRBW nanomechanostimulation 10 mins a day for 5 days led to the onset in osteogenic lineage commitment as early as 3 days [37]—without the need for osteogenic factors, in distinct contrast, for example, with continuous mechanostimulation at kHz frequencies over 21 days, where commitment towards osteogenic differentiation was only observed after 5–7 days [69, 70]. The mechanism that enables such

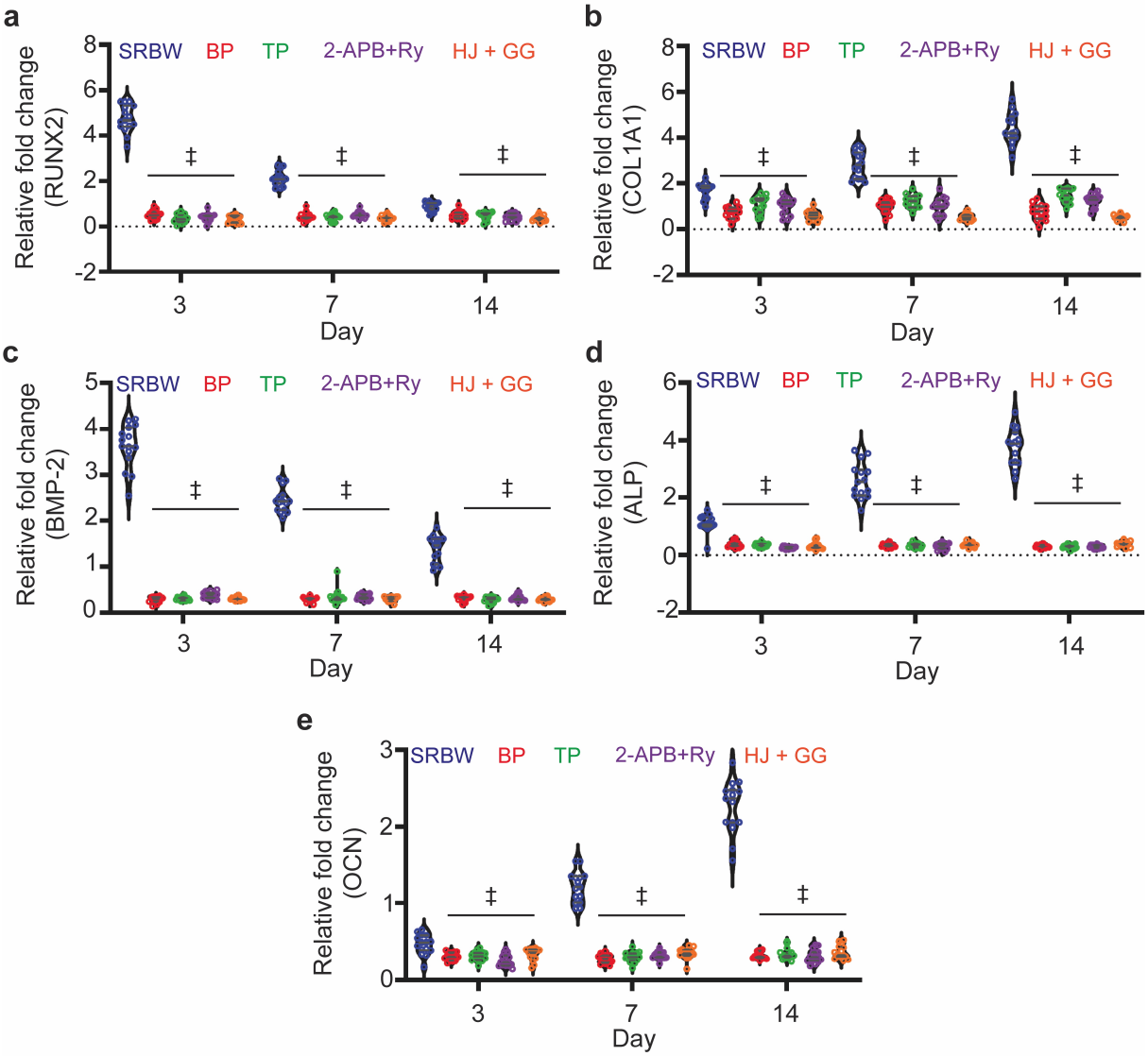

Figure 7: **Early osteogenic lineage commitment in SRBW nanomechanostimulated hMSCs**. mRNA transcriptomic profiling of (a) RUNX2, (b) COL1A1, (c) BMP-2, (d) ALP, and, (e) OCN, showing the relative fold change (against that of untreated control cells in osteogenic media) at different time points obtained for the SRBW-treated hMSCs in basal media, in the presence of the intracellular Ca^{2+} chelator BAPTA-AM (BP), 2-APB + Ry inhibiting increases in intracellular cAMP, an Epac1 inhibitor HJCO197 (HJ), and, a Rap1 inhibitor GGTI-298 (GG). The data represent the mean value $\pm$ the standard error over $n = 15$ experiments in triplicate runs; ‡ indicates statistically significant differences with $p < 0.0001$.

early osteogenic induction, however, has never been explicated to date.

The initial cellular and nuclear responses observed when the hMSCs are subjected to a *single 10 min burst* of SRBW nanomechanostimulation can therefore be seen to effectively provide a strong 'mechanopriming' effect that can initiate downstream signalling cascades that drive the cells toward a specific fate. This is as long as the multiple stimulations to the cell do not result in cellular or nuclear damage [71], as is the case with the SRBW nanomechanostimulation (Fig. S6 (Supporting Information)). This is confirmed by the results in Fig. 7a–e: inhibition of the Ca^{2+} and cAMP spatiotemporal dynamics responsible for the nuclear mechanotransduction and histone modification can be seen to downregulate RUNX2 ((runt homology domain transcription factor 2) and COL1A1 (collagen type I alpha 1) expression, as well as that of the other osteogenic markers, e.g., BMP-2 (bone morphogenetic protein-2), alkaline phosphatase (ALP), and osteocalcin (OCN). These observations can be taken to underscore the importance of the synergistic effects of the Ca^{2+} and cAMP second messenger signaling pathways in mechanopriming the cells for early commitment towards an osteogenic lineage through the SRBW nanomechanostimulation—possibly by inducing changes at the level of the chromatin folding.

Conclusions

Cells navigate a complex network of extracellular signals using precise signaling and regulatory mechanisms. To maintain cellular homeostasis and ensure proper function in both health and disease, it is essential to balance activation and inhibition, integrate multiple pathways, and adapt to external cues. The complex mechanotransduction pathways by which cells respond to persistent extracellular signals that alter their fate and function, however, has yet to be fully understood.

Through the use of high frequency nanomechanostimulation that allowed for spatiotem-

poral modulation of intracellular Ca^{2+} within the cell, we show that the cellular response to such extracellular mechanical cues, in its attempt to regain homeostasis, are dictated by the intricate spatiotemporal dynamics of these second messengers. Unlike the prevalent view that suggests the actin cytoskeleton as the direct and sole intermediary in relaying extracellular mechanical cues from the membrane to the nucleus via the LINC complex to exact epigenomic changes in the cell, we have found only changes in nuclear morphology to depend on such cytoskeleton transmission, and that this, in turn, is influenced by the biochemical signaling associated with the intracellular Ca^{2+} and cAMP dynamics. On the contrary, we have found the cells' epigenomic response to be decoupled, at least directly, from the cytoskeletal/nuclear tether, and that it is instead mediated predominantly by the Ca^{2+} dynamics and the secondary cAMP signaling it activates. In particular, the intricate bidirectional balance between these two second messengers—the latent cAMP-triggered Epac/Rap1 pathway following Ca^{2+}-induced Rho/ROCK activation upon the mechanostimulation challenge—is central to driving the resultant modifications in the chromatin landscape and the activation of the relevant transcription machinery in the process of its recovery back towards a homeostatic state. In stem cells, we show this conditioning in response to persistent insults gives rise to a mechanopriming effect that explains why they were observed to commit to an osteogenic lineage much earlier than has been typically observed.

Materials and Methods

Materials

Human mesenchymal stem cells, mesenchymal stem cell basal medium (MSCBM™), mesenchymal stem cell growth BulletKit™ medium (MSCGM™) and osteogenic differentiation BulletKit™ medium were procured from Lonza Pty. Ltd. (Mount Waverley, VIC, Australia). Sodium chloride, potassium chloride, magnesium chloride, disodium hydrogen

phosphate, sodium bicarbonate, ethanol, isopropanol, glucose, silicon oil, RNAse-free water, Triton™ X-100, phosphate buffered saline (PBS), Gibco penicillin–streptomycin, trypsin, ethylenediaminetetraacetic acid (EDTA), 3-isobutyl-1-methylxanthine (IBMX), formaldehyde, dimethylsulfoxide (DMSO), β-mercaptoethanol, trypan blue and ruthenium red was acquired from Sigma-Aldrich Pty. Ltd. (Bayswater VIC, Australia). 2-aminoethyl diphenylborinate (2-APB), ryanodine (Ry), 4-cyclopentyl-2-[[(2,5-dimethylphenyl)methyl]thio]-1,6-dihydro-6-oxo-5-pyrimidinecarbonitrile (HJC0197), N-[[4-(2-(R)-amino-3-mercaptopropyl)amino]-2-naphthylbenzoyl]leucine methyl ester trifluoroacetate salt hydrate (GGTI-298) from R&D systems (Minneapolis, MN, USA); and, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), TRiZOL™ reagent, bovine serum albumin (BSA), fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), 3,3',5,5''-tetramethylbenzidine (TMB), Fura-2 acetoxymethyl ester (Fura-2AM),1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM), thapsigargin, cytochalasin D, ActinRed™ 555 ReadyProbes™, NucBlue™ Live ReadyProbes™, bicinchoninic acid (BCA) protein assay kit, LunaScript® RT SuperMix kit, Luna® Universal qPCR Master Mix, T25 cell culture flasks, Nunc™ 8-well plate, and, Nunc™ Lab-Tek™ II Chamber Slide and Chambered Coverglass were obtained from Thermo Fischer Scientific Pty. Ltd. (Scoresby, VIC, Australia). FluoroDish cell culture plates were purchased from World Precision Instruments (WPI) Ltd. (Hertfordshire, England). Cyclic AMP XP™ assay, anti-lamin A antibody, anti-H3K9Me3 antibody, anti-H3K27Me3 antibody, anti-H3K27Ac antibody, anti-phospho-H2A.X antibody, anti-Coilin antibody, anti-RNAP II Ser5 antibody, anti-rabbit and anti-mouse IgG (H+L) F(ab')2 Fragment (Alexa Fluor 488 conjugate), anti-rabbit IgG (H+L) F(ab')2 Fragment (Alexa Fluor® 555 Conjugate) and F(ab')2 Fragment (Alexa Fluor® 647 Conjugate), on the other hand, were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA).

The following primers used for RT-qPCR analysis were acquired from Integrated DNA

Technologies Inc. (Coralville, IA, USA):

β-actin (forward): 5'-TGACGGGGTCACCCACACTGTGCCCAT-3',

β-actin (reverse): 5'-CTAGAAGCATTTGCGGTGGACGATGGA GGG-3',

ALP (forward): 5'-ATGAAGGAAAAGCCAAGCAG-3',

ALP (reverse): 5'-CCACCAAATGTGAAGACGTG-3',

COL1A1 (forward): 5'-ACATGTTCAGCTTTGTGGACC-3',

COL1A1 (reverse): 5'-TGATTGGTGGGATGTCTTCGT-3',

BMP-2 (forward): 5'-ATGGATTCGTGGTGGAAGTG-3',

BMP-2 (reverse): 5'-GTGGAGTTCAGATGATCAGCG-3',

OCN (forward): 5'-GACTGTGACGAGTTGGCTGA-3',

OCN (reverse): 5'-CTGGAGAGGAGCAGAACTGG-3',

RUNX2 (forward): 5'-AAGTGCGGTGCAAACTTTCTT-3',

RUNX2 (reverse): 5'-TCTCGGTGGCTGGTAGTGA-3',

PP1A (forward): 5'-CGGGTCCTGGCATCTTGT-3',

PP1A (reverse): 5'-CAGTCTTGGCAGTGCAGATGA-3'.

Device fabrication

The SRBW devices illustrated in Figure 1a consisted of 40 alternating fingers, each 11 mm wide and 66 nm thick, arranged as a straight interdigitated aluminum transducer (IDT) electrode in a basic full-width interleaved design on 500-μm-thick $127.86°$ Y–X lithium niobate ($LiNbO_3$) single crystal piezoelectric substrates (Roditi Ltd., London, UK). The IDT was patterned on a 33-nm-thick chromium adhesion layer using sputter deposition and standard ultraviolet (UV) photolithography. The width and separation of the IDT fingers (λ/4) corresponds to the SRBW wavelength λ, which is set to be 398 μm, which, in turn, determines the resonant frequency of the device $f = c/\lambda = 10$ MHz, wherein c is the phase speed in $LiNbO_3$. To produce the SRBW,

an alternating electrical signal at the resonant frequency was applied to the IDT using a signal generator (SML01, Rhode & Schwarz Pty. Ltd., North Ryde, NSW, Australia) and amplifier (10W1000C, Amplifier Research, Souderton, PA, USA). The acoustic wave energy was then transmitted to a glass-bottom square-well chamber slide (0.15 mm thickness) containing the adherent cells through a thin layer of silicon oil with viscosity and density of 45–55 cP and 0.963 g/mL at $25°$C, respectively.

Cell culture and mechanostimulation

The hMSCs were grown in DMEM with 1% penicillin–streptomycin and 10% FBS in a humidified incubator at $37°$C and 5% CO_2 until they covered 80–90% of a standard T25 flask. They were then detached using 0.05% trypsin–EDTA and transferred to 8-well plates or FluoroDish cell culture plates at a seeding density of 3,000 cells per well before being incubated for 48 hrs in DMEM to ensure proper adhesion. The cells in the well-plate were subsequently exposed to the 10 MHz SRBW irradiation at an input power of 2.5 W for 10 mins. Control samples consisted of cells seeded in DMEM with 1% penicillin–streptomycin and 10% FBS at the same density and incubated for the same time period, but without any vibrational excitation. The cells were then fixed at 0, 0.1, 0.3, 0.5, 1, 2, 4 and 8 hrs post-exposure incubation.

To facilitate osteogenic differentiation, the cells were cultured in a standard T25 flask until they achieved 80–90% confluency. At this point, they were detached using 0.05% trypsin–EDTA and replated into 8-well plates at a density of 3,000 cells per well, allowing for a 48-hr incubation in basal media (BM; α–MEM) to ensure adequate adhesion. After this incubation, the media was refreshed with new basal media. The cells in the well plates were then subjected to irradiation with the 10 MHz SRBW at an input power of 2.5 W for a duration of 10 mins, which represented a single treatment regimen ($1\times$). Once the vibrational excitation ceased, the cells were left to incubate overnight at $37°$C. The $1\times$ treatment regimen was applied contin-

uously for the following four days. Following this five-day regimen, the cells underwent an additional incubation period of 14 days. Control samples for real-time quantitative polymerase chain reaction (RT-qPCR), consisted of cells plated in osteogenic media (OM) at the same density, which were incubated over the same timeframe but without any form of vibrational excitation.

The following inhibitor concentrations were used: actin polymerisation inhibitor (cytochalasin D): 5 μM; intracellular Ca^{2+} chelator (BAPTA-AM): 20 μM; SERCA ion channel blocker (thapsigargin): 300 nM; IP3R inhibitor (2-APB): 50 μM; RyR inhibitor (ryanodine): 15 μM.

Immunofluorescence staining

The cells requiring fixation were washed three times in PBS and then incubated in 4% formaldehyde for 20 mins at room temperature. Following this, they were washed three more times in PBS. To block non-specific binding, the cells were then incubated in 1X 5% normal serum in Triton™ X-100 for 60 minutes. They were subsequently incubated overnight at 4°C with primary antibodies: anti-lamin A (1:2000), anti-H3K27Me3 (1:800), anti-H3K27Ac (1:200), anti-H3K9Me3 (1:400), and either anti-RNAP II Ser5 or anti-Coilin (1:800) diluted using 1X PBS/0.3% Triton™ X-100. After three washes in PBS, the cells were incubated for 1 hr in the dark at room temperature with the secondary antibodies (anti-rabbit and anti-mouse IgG (H+L) F(ab')2 Fragment (Alexa Fluor 488 conjugate); 1:1000), followed by three additional washes in PBS. Nuclei were counterstained using NucBlue™ Live ReadyProbes™ and actin filaments were stained with ActinRed™ 555 ReadyProbes™ before imaging, which was performed on an inverted confocal laser scanning microscope (FV3000; Olympus, Tokyo, Japan).

Intracellular Ca^{2+}

The fluorescent calcium indicator Fura-2AM was utilized to measure free cytosolic Ca^{2+} levels. The cells were incubated in 5 μmol/l Fura-2AM mixed with Opti-MEM™ reduced serum medium containing 2% (vol/vol) heat-inactivated FBS at 37°C in the absence of light. Following a 1 hr incubation period, the medium containing extracellular Fura-2AM was replaced with fresh medium, and the cells were incubated for an additional 20 mins before subjecting the samples to irradiation with the SRBW. Fluorescence emission intensity was then measured at 510 nm using a spectrophotometric plate reader (CLARIOstar, BMG LabTech, Mornington, VIC, Australia) in individual wells, employing excitation wavelengths of 340 nm and 380 nm. The ratio of Fura-2AM fluorescence emission in response to the 340 nm and 380 nm excitation (340/380) was calculated and expressed as the fold change relative to the respective control (unexcited) cells.

cAMP

cAMP levels were assessed using a cyclic AMP XP™ assay. Initially, the cell underwent washing with Tyrode's buffer (137 mM sodium chloride, 12 mM sodium bicarbonate, 5.5 mM glucose, 2 mM potassium chloride, 1 mM magnesium chloride, and 0.3 mM disodium hydrogen phosphate) at pH 7.4, which was supplemented with 100 μmol/l IBMX—a phosphodiesterase inhibitor—to prevent the breakdown of cAMP. The cells were subsequently treated with the SRBW and incubated at various time intervals, following which they were lysed in 100 μl of the lysis buffer provided by the kit. A total of 25 μl of the cell lysate and HRP-linked cAMP solution was placed into the designated cAMP assay microtiter plates and incubated at room temperature for 3 hrs. After incubation, the plate underwent four washing cycles, and the excess liquid was discarded. Then, 100 μl of 3,3',5,5"-tetramethylbenzidine (TMB) was added, and the solution was kept in the dark for 15 mins. The reaction was terminated by adding 100 μl

of the provided STOP solution, and its absorbance was assessed at 450 nm with a spectrophotometric plate reader (CLARIOstar, BMG LabTech, Mornington, VIC, Australia). The average of three measurements for each treatment group was calculated from cells derived from each individual culture, allowing for the determination of cAMP concentrations based on a standard curve.

RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR)

RNA was extracted from control and SRBW-treated cells using TRiZOL™. Initially, the cells were homogenized in TRiZOL™, and chloroform was added to the mixture. After centrifugation, the RNA-containing aqueous layer was separated. The RNA was then precipitated with isopropanol, washed with ethanol, and resuspended in RNase-free water containing 0.1 μM EDTA. The RNA concentration was measured using a UV spectrophotometer (NanoDrop™ One; Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, cDNA synthesis was carried out using the LunaScript® RT SuperMix kit, and RT-qPCR was conducted with the Luna® Universal qPCR Master Mix along with the aforementioned designated primers.

Image analysis

Nuclear morphometry (volume, area, thickness), fluorescent intensity and transcription hub characteristics (number and size in diameter) were measured using ImageJ (v1.54p; National Institutes of Health, Bethesda, MD, USA), whereas the nuclear shape descriptors were measured using the measurement module in the CellProfiler tool (v4.2.8; Broad Institute, Cambridge, MA, USA).

Statistics

The data provided in this study were represented as the average $\pm$ the standard error. Where applicable, the data were assessed using ordinary one-way or two-way analysis of variance (ANOVA) or with a two-tailed, unpaired Student's t-test.

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Supplementary Materials

Figs. S1–S7