

Electronic Supplementary Material (ESM)

Low-intensity pulsed ultrasound induces multifaced alterations in chromosome segregation, cytoskeletal filaments and cell junctions

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Section S1. Ultrasound exposure setup and calibration

For the experimental setup assembly and calibration, the following components and equipment were utilized:

- Agilent function waveform generator capable of delivering signals of variable intensity in both continuous and pulsed modes;
- Radiofrequency signal amplifier by Amplifier Research;
- Precision Acoustic Ltd immersion transducer with a diameter of 25 mm operating at a frequency of 1 MHz;
- Philips oscilloscope;
- Needle hydrophone (diameter 0.5 mm) with Precision Acoustic Ltd amplifier, utilizing a PVDF sensitive element.

To perform in vitro US exposures under known and reproducible conditions, the exposure setup depicted in Figure S1 was employed.

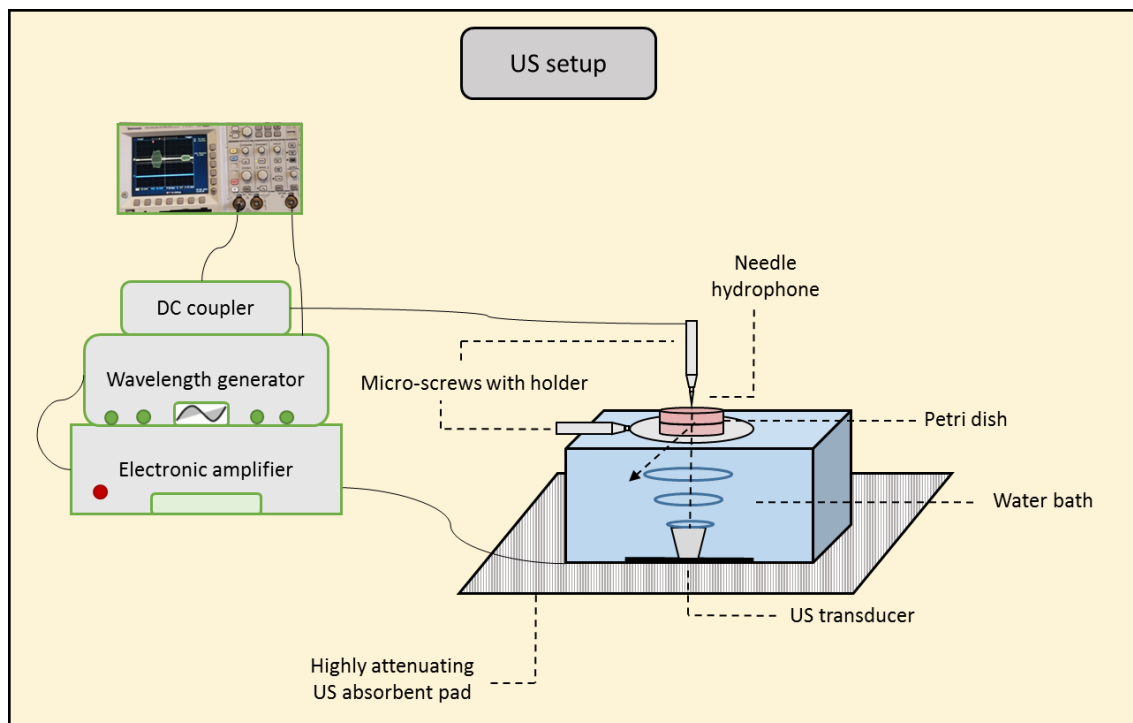


Figure S1. US irradiation setup in calibration mode consists of a radiofrequency amplifier and a sinusoidal waveform generator connected to a waterproof US piezoresistive transducer (affixed at the bottom of a water-filled plexiglass tank). The ultrasonic signal is translated into voltage by a needle hydrophone endowed with a micrometric positioning system and positioned within a Petri dish. For signal detection, the needle hydrophone is linked to a booster amplifier (DC coupler) and an oscilloscope. To avoid interferences from the reflected waves at the water/water interface US absorber pads are positioned under the water bath.

The apparatus consists of a radiofrequency amplifier paired with a sinusoidal waveform oscillator connected to waterproof US piezoresistive transducers. The transducer is affixed to the bottom of a plexiglass tank filled with water, oriented vertically for propagation. The transducers are designed to produce US waves in the aqueous medium in both cavitation and subcavitation regimes, in both pulsed and continuous modes. The plexiglass tank measures 30×30×30 cm³, with an acoustically absorbent coating applied to the bottom wall to minimize the effects of wave reflections.

Characterization of the exposure setup was performed by measuring the ultrasonic pressure inside the water tank using a needle hydrophone. A transducer-to-hydrophone distance of 7 cm was chosen to minimize near-field effects and thus achieve stable ultrasonic field intensity relative to small variations in sample position.

The characterization of the acoustic field at the utilized frequency of 1 MHz was conducted using the needle hydrophone. Exposure intensity measurements are provided in terms of spatial peak temporal average intensity (I_{spta}). I_{spta} for a perfectly sinusoidal wave can be expressed in terms of experimental parameters through the formula:

$$I_{spta} = \frac{n_p}{vT_B} \cdot \frac{(PNP)^2}{2\rho c}$$

where $\rho = 996.8 \text{ kg/m}^3$ is the density of water, $c = 1499.3 \text{ m/s}$ is the speed of acoustic wave propagation in water, $TB = 300 \text{ } \mu\text{s}$ is the period of the pulsed regime, ν is the frequency of the acoustic wave, n_p is the number of pulses of the acoustic field in each period of the pulsed regime, and PNP is the negative peak pressure measured with the hydrophone. Figure S2 shows the calibration curve obtained at 1 MHz with a duty cycle of 10%, considering the effects of temperature increase (approximately 2°C in 1 hour) monitored with an immersion thermometer (Hanna Instruments Checktemp4, sensitivity $\pm 0.1^\circ\text{C}$). Each point represents the result of the average of 4 independent measurements.

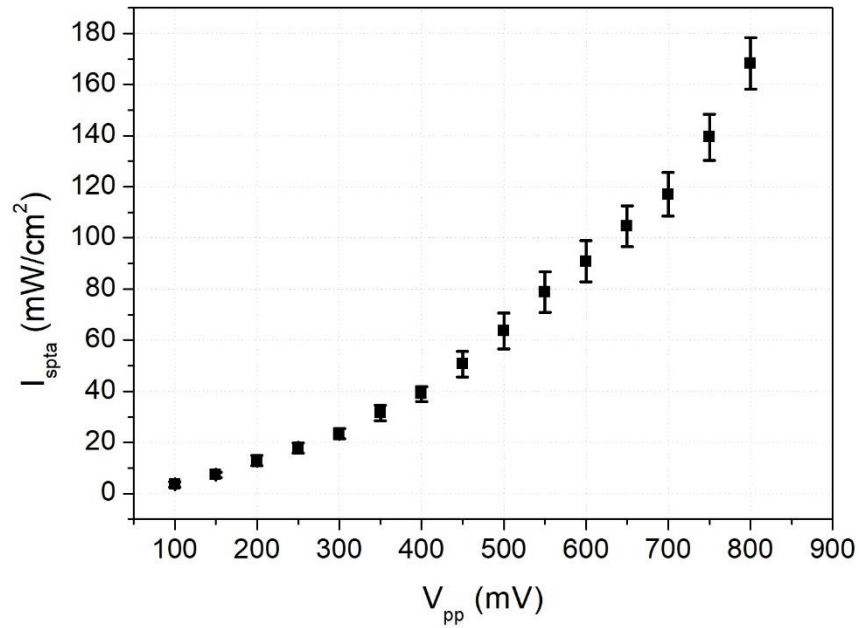


Figure S2. Calibration curve of the US field utilized in the experiments, obtained in a Petri dish at 1 MHz and duty cycle (DC) of 10%.

Section S2. Spatial distribution of F-actin in HaCaT and SK-MEL-28 cells upon LIPUS exposure

Immunofluorescence images of HaCaT and SK-MEL-28 cells exposed to 85 mW/cm^2 for 1 hour. Phalloidin (green) was used to detect the alteration of F-actin spatial distribution upon LIPUS irradiation. Specifically, in HaCaT cells we observed a thickening of F-actin in the perimembrane region. Moreover, in this system, cellular morphology was modified by US. Indeed, after treatment cells appeared rounder than control cells (Figure S3A). Figure S3B shows phalloidin staining in SK-MEL-28 after irradiation where it is possible to appreciate that F-actin is more delocalized compared to the control where it is mainly found in the perinuclear area. The fluorescence signal analysis reported in Figure S3C and D, respectively for HaCaT and SK-MEL-28 cells, demonstrated that Phalloidin fluorescence intensity of US-exposed cells was comparable to that of control cells.

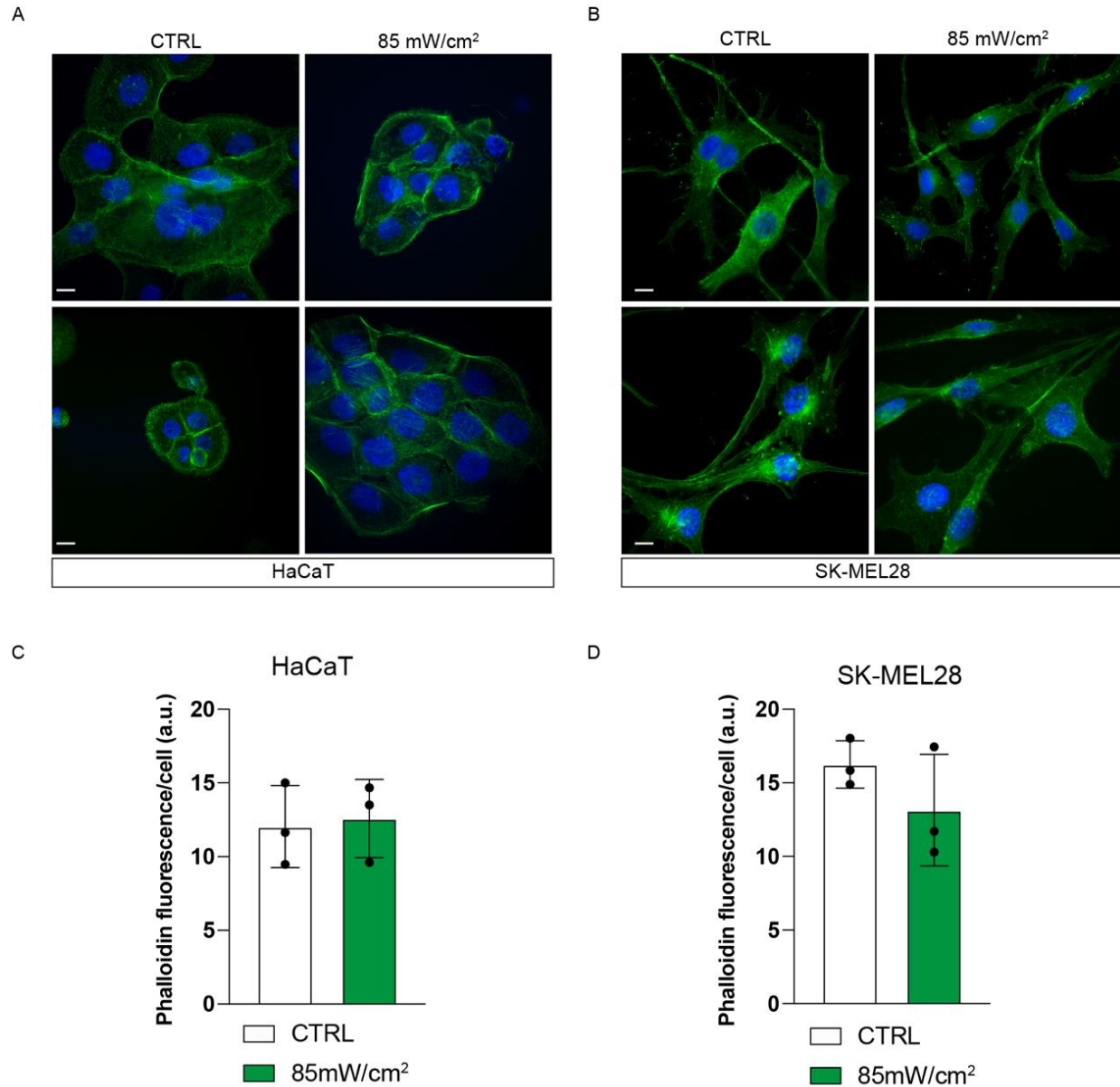


Figure S3. Phalloidin immunofluorescence in HaCaT (A) and SK-MEL- 28 cells (B) and its corresponding signal analysis (C and D) upon exposure to 85 mW/cm². Phalloidin (green) was used to label F-actin whilst nuclei were labeled with DAPI (blue). Data are presented as a mean $\pm$ SD. Scale bars correspond to 100 μ m; 60 $\times$ magnification.

Section S3. FE-SEM microscopy

S3.1 Images of HaCaT cells exposed to LIPUS irradiation

Supplementary FE-SEM images are reported to show alterations in plasma membrane integrity after LIPUS exposure. In the upper lane of Figure S4.1 are shown low magnification images of HaCaT cells after irradiation in which is highlighted the flattening of membrane protrusions. The lower lane of Figure S4.1 reports the details of plasma membrane roughness with the presence of breaks in treated cells (as also shown in Figure 3d of the manuscript).

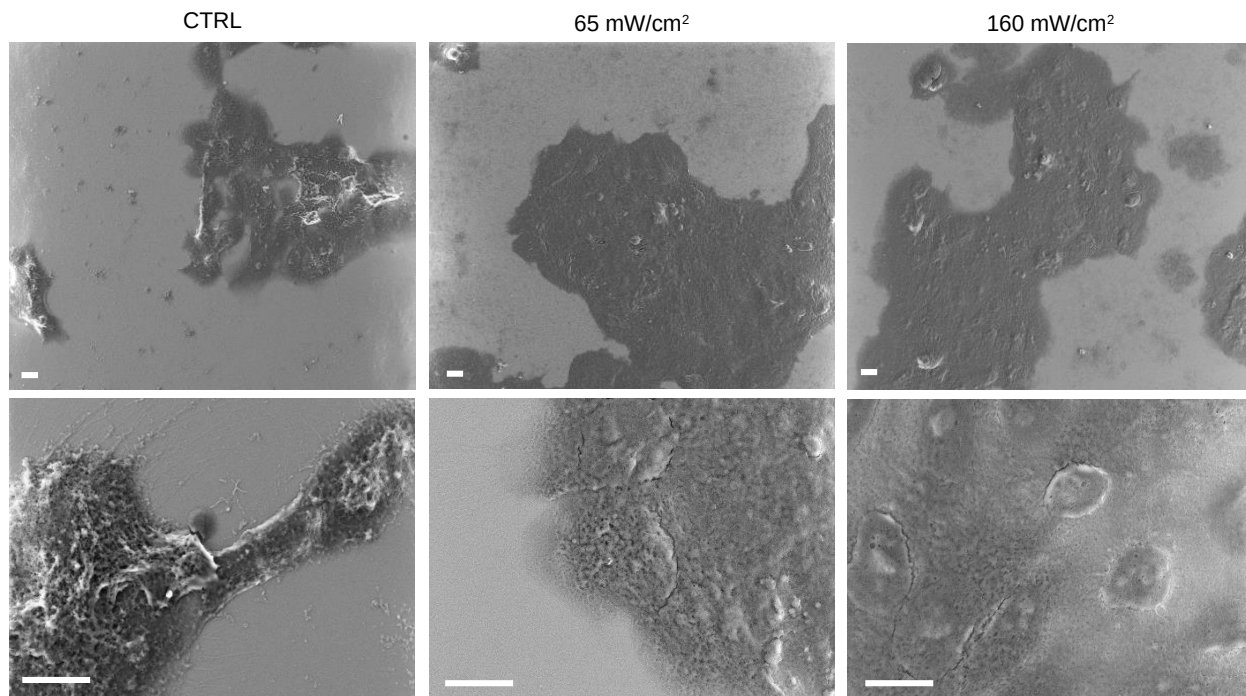


Figure S4. FE-SEM images of HaCaT cells exposed to 65 mW/cm² and 160 mW/cm² for 1 h (upper lane magnification 1.00 KX). Lower lane shows details of the cell membrane roughness upon US irradiation (Magnification 5.00 KX). Scale bar corresponds to 10 μ m.

S3.2 Fe-SEM details about cell membrane porosity after ultrasound irradiation

Using the Gwyddion software, we calculated the size of cell membrane fractures and the average of cell porosity and roughness upon US irradiation. The results are reported in Table S1. Specifically, an average length of 7.2 ± 0.3 and a width of 0.3 ± 0.1 μm were observed in samples irradiated at $I_{\text{spta}} = 65$ mW/cm^2 , while for those irradiated at $I_{\text{spta}} = 160$ mW/cm^2 we observed an average length of 9.7 ± 0.3 and an average width of 0.5 ± 0.1 μm . Then we analyzed the average plasma membrane roughness, S_q , which is the mean squared roughness of height calculated in all images. The analysis revealed an S_q of 28.1 a.u. for samples irradiated at $I_{\text{spta}} = 65$ mW/cm^2 and 35.3 a.u. for those irradiated at $I_{\text{spta}} = 160$ mW/cm^2 , as compared to control cells which had an S_q value of 25.3 a.u. The relative variation in roughness (S_q^{rel}) was also calculated at $I_{\text{spta}} = 160$ mW/cm^2 and $I_{\text{spta}} = 65$ mW/cm^2 , with a modulation of 40% and 10% respectively, compared to the control (Table S1). Next, we analyzed pore diameters counting around 40 pores in three images for each experimental condition and the obtained data are reported in Table S1. The results revealed that pore diameters increased upon US irradiation, with the highest effect at $I_{\text{spta}} = 160$ mW/cm^2 . Relative variations of the average diameters (d_q^{rel}) highlighted a difference of 90% and 5 % at $I_{\text{spta}} = 160$ mW/cm^2 and $I_{\text{spta}} = 65$ mW/cm^2 respectively.

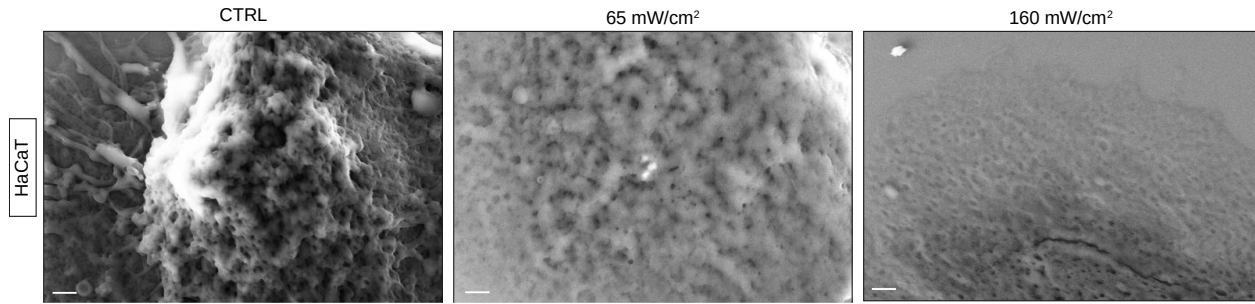


Figure S5. Detail of the cell membrane porosity upon 1 h LIPUS exposure. Images of HaCaT cells were obtained by FE-SEM microscopy and one representative image is shown for each experimental condition: (A) control, (B) sample treated at $I_{\text{spta}} = (65 \pm 3) \text{ mW/cm}^2$, (C) treated sample a $I_{\text{spta}} = (160 \pm 5) \text{ mW/cm}^2$. Scale bar corresponds to 1 μm .

Size of cell membrane ruptures	Length (μm)	Width (μm)	Average porosity and roughness	S_q (nm)	S_q^{rel} (%)	d_m (nm)	d_q^{rel} (%)
			Control	25.3	-	145 ± 50	-
$I_{\text{spta}} = (65 \pm 3) \text{ mW/cm}^2$	7.2 ± 0.3	0.3 ± 0.1	$I_{\text{spta}} = (65 \pm 3) \text{ mW/cm}^2$	28.1	10	150 ± 50	5
$I_{\text{spta}} = (160 \pm 5) \text{ mW/cm}^2$	9.7 ± 0.3	0.5 ± 0.1	$I_{\text{spta}} = (160 \pm 5) \text{ mW/cm}^2$	35.3	40	270 ± 80	90

Table S1. Summary table about the average dimensions of cell roughness, membrane porosity and the size of membrane ruptures. Cells were irradiated at $I_{\text{spta}} = (65 \pm 3) \text{ mW/cm}^2$ and $I_{\text{spta}} = (160 \pm 5) \text{ mW/cm}^2$ for 1 h and then compared to the control.

Section S4. Full-length/uncropped blots

Full-length blots are reported in this section. The red boxes indicate the cropped parts.

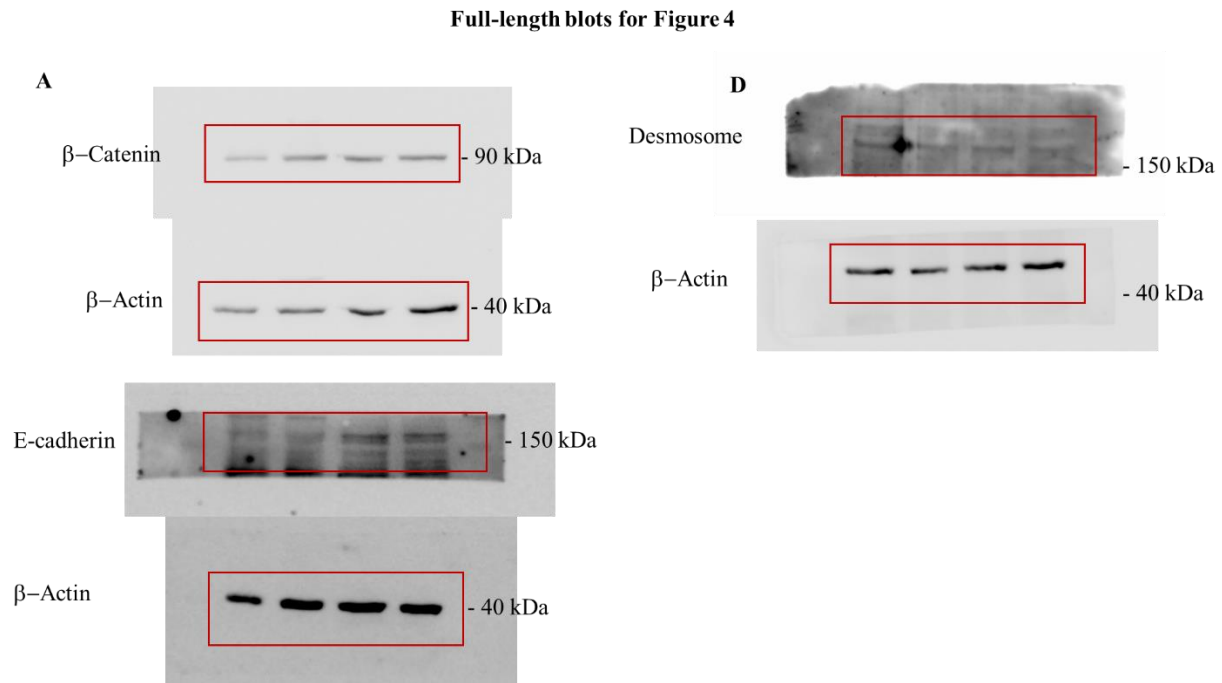


Figure S6. Full-length blots related to Figure 4 panel A and D. β -Catenin and E-cadherin were analyzed on different blots, both are reported with their respective β -Actin. In Figure 4 panel A a representative β -Actin was shown for both proteins. Original blots were cut to probe separately all proteins shown in Figure 4.

Full-length blot for Figure 5

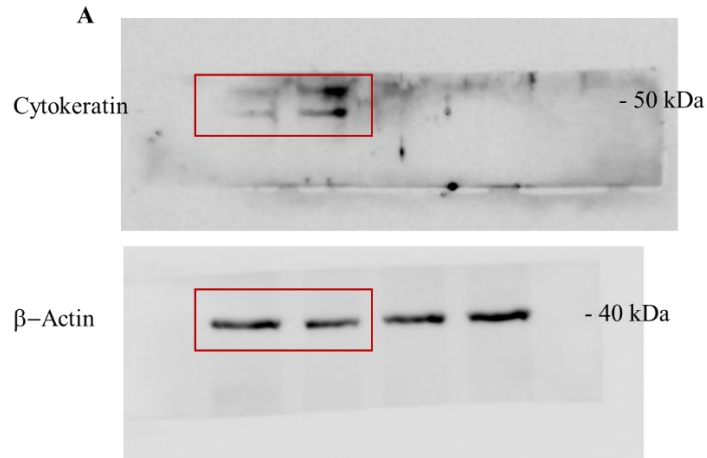


Figure S7. Full-length blot related to Figure 5 panel A. Original blot was cut to probe separately Cytokeratin and β -Actin.

Full-length blots for Figure 8

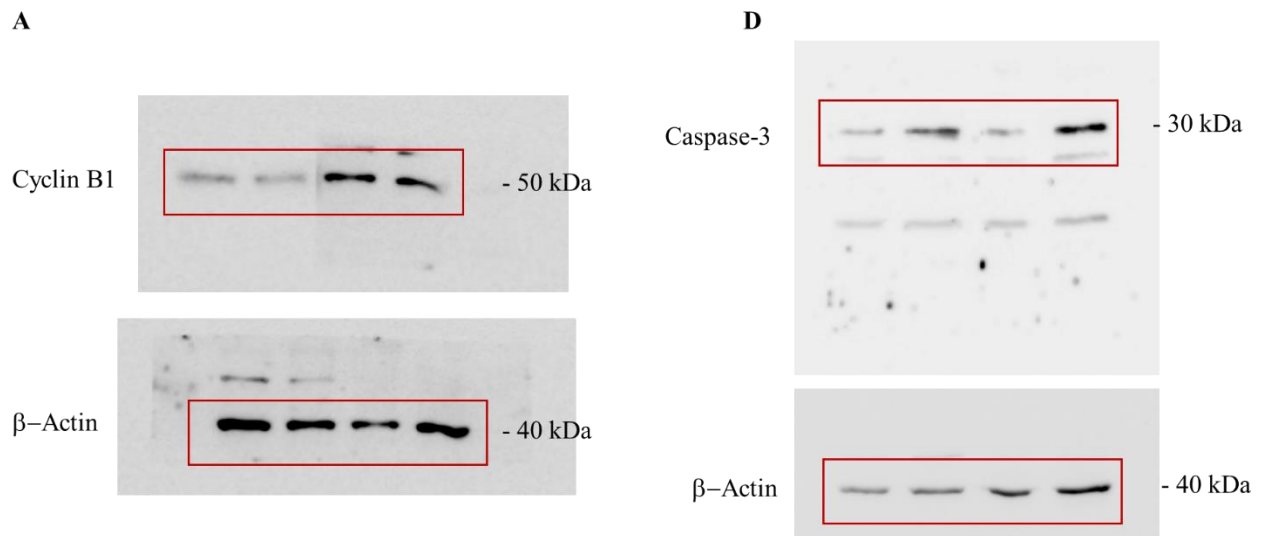


Figure S8. Full-length blots related to Figure 8 panel A and D. Original blots were cut to probe separately Cyclin B1 and Caspase-3.