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# Focused Ultrasound Stimulates ER Localized Mechanosensitive PANNEXIN-1 to Mediate Intracellular Calcium Release in Invasive Cancer Cells

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Focused ultrasound (FUS) is a rapidly developing stimulus technology with the potential to uncover novel mechanosensory dependent cellular processes. Since it is non-invasive, it holds great promise for future therapeutic applications in patients used either alone or as a complement to boost existing treatments. For example, FUS stimulation causes invasive but not non-invasive cancer cell lines to exhibit marked activation of calcium signaling pathways. Here, we identify the membrane channel PANNEXIN1 (PANX1) as a mediator for activation of calcium signaling in invasive cancer cells. Knockdown of PANX1 decreases calcium signaling in invasive cells, while PANX1 overexpression enhances calcium elevations in non-invasive cancer cells. We demonstrate that FUS may directly stimulate mechanosensory PANX1 localized in endoplasmic reticulum to evoke calcium release from internal stores. This process does not depend on mechanosensory stimulus transduction through an intact cytoskeleton and does not depend on plasma membrane localized PANX1. Plasma membrane localized PANX1, however, plays a different role in mediating the spread of intercellular calcium waves via ATP release. Additionally, we show that FUS stimulation evokes cytokine/chemokine release from invasive cancer cells, suggesting that FUS could be an important new adjuvant treatment to improve cancer immunotherapy.

**Keywords:** mechanotransduction, focused ultrasound (FUS), calcium signaling, pannexin 1 (Panx1), ATP, invasive cancer cells

## INTRODUCTION

Cancer cell invasion and tumor metastasis play a critical role in cancer mortality. The mechanisms by which malignant tumors leave the primary tumor site, invade, and metastasize to other organs are complex, interrelated and only partially understood. Calcium signaling, however is known to be critical in these processes. To develop a functional assay of cancer cell invasion potential, we recently used focused ultrasound (FUS) stimulation to probe the altered calcium signaling pathways exhibited by invasive cancer cells. FUS stimulation caused invasive, but not non-invasive cancer cell lines, to exhibit marked calcium signaling suggesting a novel means to determine the invasion potential. We validated this using a Matrigel invasion assay, demonstrating that the degree of invasion correlates well with the degree of FUS-dependent  $\text{Ca}^{2+}$  signaling (Hwang et al., 2013; Weitz et al., 2017). FUS stimuli evoke widespread  $\text{Ca}^{2+}$  oscillatory dynamics in several invasive cancer cell lines (breast MDA-MB-231, prostate PC-3 and bladder T24/83), but not in non-invasive cells of the same cancer type (MCF-7, BPH-1, and RT112/84) suggesting that this is a general property of invasive cells (Hwang et al., 2013; Weitz et al., 2017). Also, different FUS stimulation frequencies result in similar responses indicating that  $\text{Ca}^{2+}$  signaling is independent of stimulation frequency (3-, 38-, or 200-MHz).

Focused ultrasound stimulation of invasive cells also results in a time dependent propagation of an extracellular calcium wave spreading away from cells located at the transducer focus. The mechanism(s) for extracellular calcium wave propagation is unclear, it does not depend on ultrasonic surface waves or gap junctions (Weitz et al., 2017). Additional pharmacological studies in invasive cancer cells suggested the involvement of  $\text{IP}_3$  receptors ( $\text{IP}_3\text{Rs}$ ) or TRP channels (Weitz et al., 2017), as suggested by others (Diver et al., 2001; Bootman et al., 2002; Xu et al., 2005). Non-focused US also stimulates calcium signaling mediated by the Piezo1 mechanosensitive ion channel directly coupled to microbubbles (Pan et al., 2018). Other ER-localized mechanosensitive channels (i.e., *Msy1* and *Msy* in fission yeast) regulate intracellular  $\text{Ca}^{2+}$  and cell volume for survival upon hypo-osmotic shock (Nakayama et al., 2012).

Focused ultrasound technology has also been proposed for use in cancer therapy and particularly immunotherapy. High-intensity ( $>5 \text{ W/cm}^2$ ) continuous FUS generates a systemic immune stimulatory effect resulting in tumor ablation (Lu et al., 2009). Pulsed FUS (i.e., non-continuous stimulus to minimize heat generation) (Hersh et al., 2016) may induce a more refined cellular/molecular immune response (Ziadloo et al., 2012) by initiating inflammatory responses which boost cancer immunotherapy (Curley et al., 2017; Mauri et al., 2018). FUS may thus offer a new approach to overcome cancer immune-resistance, a well-known limitation preventing more wide-spread clinical adoption of successful immunotherapies such as CAR T cells (Caliendo et al., 2019; Tokarew et al., 2019). A detailed understanding of the mechanistic aspects of FUS-response mechanisms however is currently lacking.

In this study we establish a new role for the mechanosensitive PANX1 hemichannel (Bao et al., 2004) in mediating  $\text{Ca}^{2+}$

signaling in invasive cancer cells. PANX1 localizes to both plasma membrane (PM) as well as endoplasmic reticulum (ER) (Vanden Abeele et al., 2006). Mechano-sensitive responses have previously been described in neurons, including retinal ganglion cells (Xia et al., 2012) and other cell types. Our results suggest that FUS can directly stimulate ER localized PANX1 in invasive PC-3 cancer cells to generate  $\text{Ca}^{2+}$  release from intracellular ER stores, independently of extracellular  $\text{Ca}^{2+}$  entry. This is a newly described role of PANX1 as a regulator of calcium ion exchange between the ER and cytoplasm, suggesting a new working model of how FUS interacts with cancer cells to initiate and propagate  $\text{Ca}^{2+}$  signaling. In addition, our results suggest that continued development of FUS technology could provide not only a new way to probe mechanosensitive functions of signaling pathways located in specific intracellular compartments but also to harness the potential to regulate adjunct immune cell responses through the coupling of mechanosensory stimulus to chemokine/cytokine release profiles.

## MATERIALS AND METHODS

### Cell Lines

PC-3 prostate cancer lines and HEK 293T cells were used in this study. PC-3 cells were purchased from ATCC and HEK 293T cells obtained from Dr. Fabien Pinaud at University of Southern California. Both cells were cultured in DMEM. The medium was supplemented with 10% FBS and 2 mM L-glutamine, and Penicillin/Streptomycin. All cell lines were tested to be free of mycoplasma contamination using a mycoplasma PCR detection kit (Sigma). Cell lines were authenticated using short tandem repeat (STR) analysis by the University of Arizona Genetics Core. PC-3 cells are highly invasive cell lines, while HEK cells were used as non-invasive because of poor transfection efficiency of BPH-1.

### Cell Preparation and Transfection

Cells were plated on 35-mm culture dishes, or 24-well culture plates to a density of  $10^6$  or  $10^5$  cells per dish or well. All cells were stained with cell membrane permeant Fluo-4 AM (Thermo Fisher Scientific), a fluorescent reporter of intracellular calcium activity. Staining was performed by incubating dishes/wells with 1  $\mu\text{M}$  Fluo-4 AM for 30 min immediately prior to imaging. Following calcium dye loading, cells were washed with and maintained in external buffer solution consisting of 140 mM NaCl, 2.8 mM KCl, 1 mM  $\text{MgCl}_2$ , 2 mM  $\text{CaCl}_2$ , 10 mM HEPES, and 10 mM D-glucose, adjusted to pH 7.3 and 290–300 mOsm.

Lipofectamine 3000 (Invitrogen) was used for cDNA construct transfection experiments. cDNA constructs used are WT PANX1-EGFP and mt PANX1-mRFP that are provided by Dr. Tavazoie (Furlow et al., 2015). FL WT PANX1 with no EGFP fusion (WT PANX1) was made for Fluo-4 AM  $\text{Ca}^{2+}$  detection, after deletion of EGFP using a standard sub-cloning method. For siRNA transfection, we used DharmaFect (Thermo Scientific) according to the manufacturer's instructions. siRNA specifically designed to target FL PANX1 (si-PANX1) was purchased from Dharmacon (GE Healthcare) (D-018253-02) (**Supplementary**

**Figure S5).** Control siRNA (negative) was obtained from GE Healthcare (D-001810-10).

## Analysis of mRNA Expression

Expression of mRNA was quantified by qRT-PCR as described previously (Lee et al., 2015). Primers are the followings: for *PANX1*, forward 5'-agcccacggagcccaagtca and reverse 5'-gcgcgaaggccagcgaga, for *GAPDH* and *CyclophilinA*, they are described in our previous paper (Lee et al., 2015).

## Ultrasound Transducers

A single-element, lithium niobite (LiNbO<sub>3</sub>), press-focused 46 MHz (f-number = 2, focal length = 6 mm) transducer was fabricated in house as described previously (Lam et al., 2013) and used in most experiments. In addition, a PZT, pressed-focused 3-MHz transducer (f-number = 1.5, focal length = 4 mm) was also tested. To drive the transducers, sinusoidal bursts from a signal generator (SG382; Stanford Research Systems) were fed to a 50-dB power amplifier (525LA; Electronics & Innovation) whose output was used to excite the transducer. For the 46-MHz transducer, amplitude was tested at different input  $V_{p-p}$ , pulse repetition frequency (PRF) at 1 kHz, and duty cycle at 5%, in PC-3 and HEK cells (**Figures 1E–G, Supplementary Figure S2**). The acoustic output of the 46-MHz transducer was measured with a needle hydrophone (HGL-0085; Onda). **Figure 1C** shows the measured beam width of the focused ultrasound to be ~70  $\mu$ m, which may focus on ~6 cells. Using the standard cell stimulation parameters provided above [12  $V_{p-p}$  (40 mV) amplitude, 1 kHz PRF, and 5% duty cycle], the intensity and pressure at the focus were measured by the hydrophone.

## Ultrasound Stimulation and Fluorescence Imaging

A custom microscope system was used to image cellular fluorescence while performing simultaneous ultrasonic stimulation as described previously (Hwang et al., 2013; Weitz et al., 2017). Petri dishes or plates containing cells were placed on the stage of an inverted epifluorescence microscope (Olympus IX70), and the ultrasound transducer was lowered into the external buffer solution. A motorized three-axis micromanipulator was used to position the transducer in focus with the cell monolayer. In each experiment, live-cell fluorescence imaging was performed for 240 s (and sometimes, 300 s), with the ultrasound stimulus being delivered continuously between  $t = 50$  and 200 s. Excitation light was provided by a mercury arc lamp and filtered through an excitation bandpass filter (488  $\pm$  20 nm). Fluorescence emitted from the calcium dye was filtered through an emission bandpass filter (530  $\pm$  20 nm) and recorded at 1 Hz (30% exposure duty cycle) with a digital CMOS camera (ORCA-Flash2.8; Hamamatsu). All imaging was performed at 4 $\times$  magnification in order to capture activity from hundreds or thousands of cells simultaneously. For each cell line, simulation and imaging experiments were replicated in at least two different dishes of cells, and over least three independent fields of view per dish. Experiments involving pharmacological blockers were limited to a single field of view

per dish. Figures show representative data obtained from one field of view.

## Data Processing

Data were post-processed to determine the calcium response of every imaged cell as described previously (Weitz et al., 2017). Cell locations were identified automatically with CellProfiler image analysis software (Carpenter et al., 2006) and used to extract the raw fluorescence intensities of each cell. These intensities were exported to MATLAB (MathWorks) in order to calculate each cell's normalized change in fluorescence ( $\Delta F/F$ ) during every imaging frame. Responding cells were defined as those that exhibited a  $\Delta F/F_{max}$  greater than 3.5 times the pre-stimulus root-mean-square noise level. Two types of plots were generated for each 240 s experiment: a histogram showing the percentage of responding cells over time and a scatter plot indicating the time at which each cell first responded to the stimulus. Responding cells in these plots were arranged with respect to their distance from the transducer focus. The cell response index (CRI) was obtained as described previously (Hwang et al., 2013).

## Pharmacology

To investigate the mechanism of ultrasound-induced calcium rise in invasive cancer cells, PC-3 cells were stimulated in the presence of various pharmacological agents. We tested several different blockers, each applied separately (**Supplementary Table S1**). Blockers were dissolved in the external buffer solution 15–30 min before performing imaging and ultrasound stimulation. Cellular responses were measured before adding the blockers and in the presence of blockers.

## ATP Release Assay

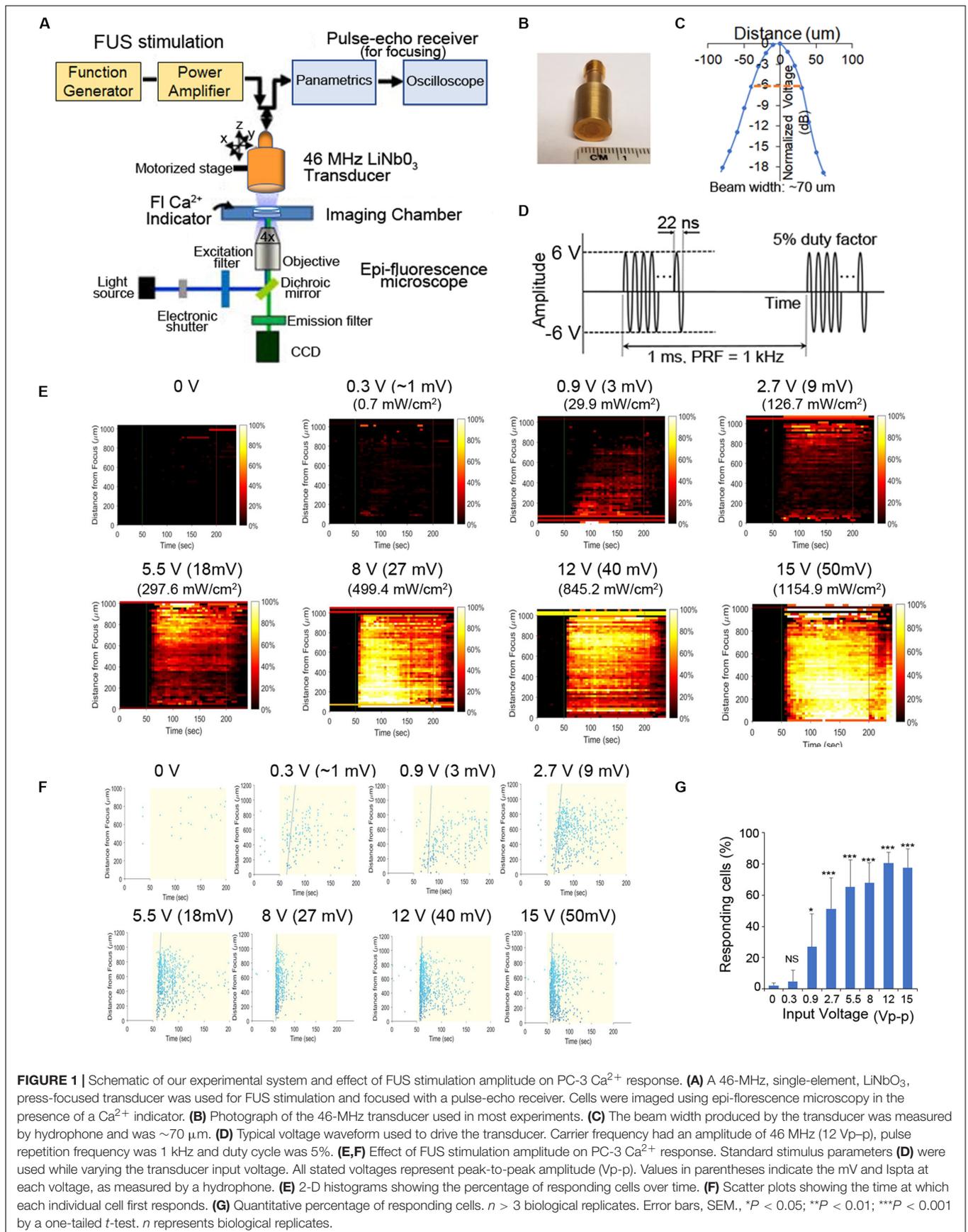
Cells were seeded in quadruplicate at 100,000–200,000 cells per well in 24-well plates and grown overnight. Each well was then washed with 1 ml external buffer solution (EBS). For PANX1 inhibition, cells were incubated at room temperature for 10 min in EBS supplemented with one of the following reagents: CBX (500  $\mu$ M), probenecid (2 mM; Life Technologies), <sup>10</sup>Panx1 (100  $\mu$ M) or an equivalent dose of the appropriate vehicle control (EBS or scrambled peptide). The wash or pretreatment solution was then aspirated, replaced with 1 ml EBS for 10 min, collected and transferred to microcentrifuge tubes, and then spun at 86 g for 2 min at room temperature. 50 or 100  $\mu$ l of supernatants was transferred to 96-well plates and ATP was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions.

## Immunofluorescence and Confocal Microscopy

Cells expressing fluorescently tagged proteins were fixed in 4% paraformaldehyde, stained with DAPI (Thermo Fisher) and mounted using ProLong Gold antifade reagent (Thermo Fisher), and imaged using Leica TCS microscope.

## TIRF Imaging and Immunocyto Staining (ICS)

TIRF microscopy images were acquired on an inverted Nikon Eclipse Ti-E microscope, equipped with a 100  $\times$  1.49 NA



**FIGURE 1** | Schematic of our experimental system and effect of FUS stimulation amplitude on PC-3 Ca<sup>2+</sup> response. **(A)** A 46-MHz, single-element, LiNbO<sub>3</sub>, press-focused transducer was used for FUS stimulation and focused with a pulse-echo receiver. Cells were imaged using epi-fluorescence microscopy in the presence of a Ca<sup>2+</sup> indicator. **(B)** Photograph of the 46-MHz transducer used in most experiments. **(C)** The beam width produced by the transducer was measured by hydrophone and was ~70 μm. **(D)** Typical voltage waveform used to drive the transducer. Carrier frequency had an amplitude of 46 MHz (12 Vp-p), pulse repetition frequency was 1 kHz and duty cycle was 5%. **(E,F)** Effect of FUS stimulation amplitude on PC-3 Ca<sup>2+</sup> response. Standard stimulus parameters **(D)** were used while varying the transducer input voltage. All stated voltages represent peak-to-peak amplitude (Vp-p). Values in parentheses indicate the mV and Ispta at each voltage, as measured by a hydrophone. **(E)** 2-D histograms showing the percentage of responding cells over time. **(F)** Scatter plots showing the time at which each individual cell first responds. **(G)** Quantitative percentage of responding cells. *n* > 3 biological replicates. Error bars, SEM, \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001 by a one-tailed *t*-test. *n* represents biological replicates.

objective (Nikon), an iXon EMCCD camera (Andor), laser lines at 405, 488, 561, and 647 nm (Agilent), a multiband pass ZET405/488/561/647× excitation filter (Chroma), a quad-band ZT405/488/561/647 dichroic mirror (Chroma), and appropriate emission filters for imaging of mRFP (600/50 nm, Chroma) and GFP (525/50 nm, Chroma). Illumination was performed by TIRF to ensure exclusive illumination of the plasma membrane.

For ICS, the cells are fixed with 100% methanol at  $-20^{\circ}\text{C}$  for 10 min. After washing with PBS, they were permeabilized with 1% Triton X-100 at  $37^{\circ}\text{C}$  for 30 min. After blocking, add primary C-terminus anti-PANX1 (Santa Cruz Biotech.) or N-terminus anti-PANX1 (Alomone Labs) was added and incubated at  $4^{\circ}\text{C}$  at 12 h followed by addition of second anti-mouse-PE (Santa Cruz) at room temperature for 30 min. After washing, the cover slips containing cells were mounted and observed using a confocal microscope (Leica).

## Human Cytokine Assay

One day after FUS stimulation, cell culture supernates were collected and centrifuged. Seven hundred  $\mu\text{l}$  of supernates are applied for human XL cytokine array (R&D systems) according to the manufacturer's instructions. Pixel densities on developed X-ray film were collected and analyzed using a transmission-mode scanner and image analysis software (Image Studio Lite).

## Statistical Analyses

In general results are expressed as mean  $\pm$  s.e.m. Statistical analysis of multiple groups used one-way ANOVA, and Dunnett's correction for multiple comparisons (GraphPad Prism, V8). Two group comparisons were tested using the Student's t-test (one- and two-tailed) in Excel (v2016).

## RESULTS

### FUS Stimulation Evokes $\text{Ca}^{2+}$ Signaling in Invasive PC-3 Cells

To further clarify the mechanism(s) of FUS-dependent  $\text{Ca}^{2+}$  response, our usual stimulus protocol used a 46-MHz, single-element, LiNbO<sub>3</sub>, press-focused transducer focused via a pulse-echo receiver coupled with epi-fluorescence microscopy to assess both intra- and intercellular changes in  $\text{Ca}^{2+}$  dynamics (Figures 1A–D, see also section “Materials and Methods” for additional details). In our previous work we demonstrated that the magnitude of the FUS-induced  $\text{Ca}^{2+}$  response did not depend on the frequency of stimulation (Weitz et al., 2017). Here we examine its dependence on stimulus amplitude (i.e., intensity). Increasing voltage during FUS stimulation of PC-3 cells results in a larger  $\text{Ca}^{2+}$  response (i.e., a dose–response relationship as shown in Figures 1E–G). Standard stimulus parameters (see section “Materials and Methods,” Figure 1) were used while varying the transducer input voltage (Figures 1E,F). Stimulation at  $\sim 1$  Vp-p, 2.7 Vp-p, and 12 Vp-p evoked calcium activity in  $\sim 20\%$ ,  $\sim 50\%$ , and  $> 80\%$  of cells, respectively (Figure 1G). We stimulated at 12 Vp-p for the remainder of this study, while keeping pulse repetition frequency (PRF) at

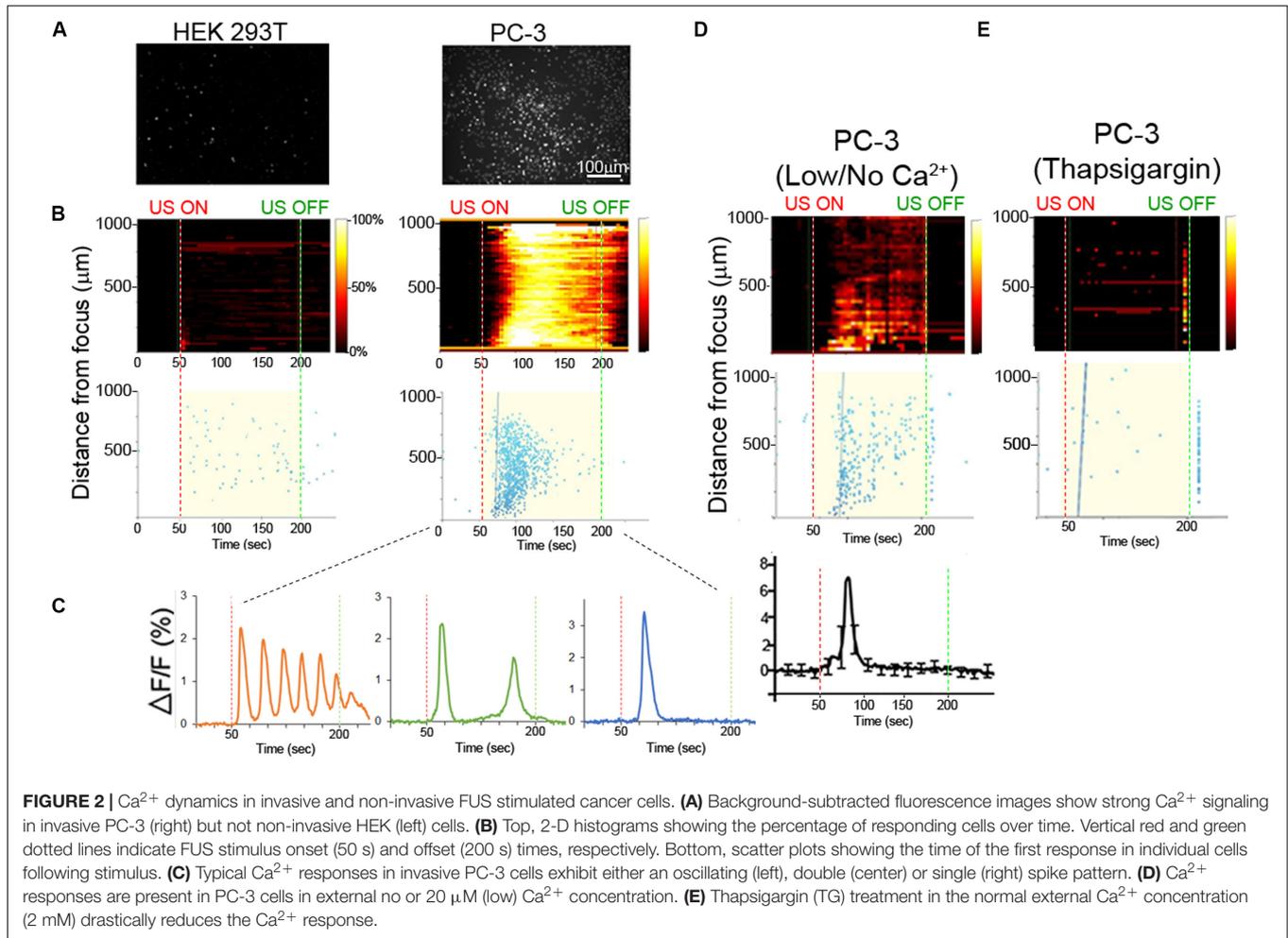
1 kHz and duty cycle at 5%. A 3-MHz stimulus was also effective in PC-3 cells (Supplementary Figure S1), reconfirming the independence of stimulus frequency in eliciting  $\text{Ca}^{2+}$  responses (Weitz et al., 2017).

Our previous studies (Weitz et al., 2017) suggested ER localized IP<sub>3</sub> receptors or PM localized TRP channels were involved in mediating invasive cancer cell FUS-dependent  $\text{Ca}^{2+}$  responses.

Here we use PC-3 cells as a model of an invasive cancer cell type and compared FUS-dependent  $\text{Ca}^{2+}$  responses to those in a non-invasive HEK293 cell line. Non-responsive HEK cells were chosen as an appropriate control line in this study, rather than previously used BPH-1 cells, since they were much easier to transfect than BPH-1 ( $\sim 90\%$  vs.  $< 5\%$  transfection efficiency). As expected, FUS stimulation evoked strong  $\text{Ca}^{2+}$  responses in PC-3 cells but not in non-invasive HEK cells (Figures 2A,B, Supplementary Figure S2, and Supplementary Videos S1, S2). In PC-3 cells, three distinct stimulus-dependent  $\text{Ca}^{2+}$  patterns are observed in individual cells in the presence of normal external  $\text{Ca}^{2+}$ :  $\text{Ca}^{2+}$  oscillation, double  $\text{Ca}^{2+}$  spikes or a single spike (Figure 2C). We tested if PC-3 responses were mediated by  $\text{Ca}^{2+}$  influx by severely reducing or eliminating extracellular  $\text{Ca}^{2+}$  (0 or 20  $\mu\text{M}$  vs. normal 2 mM). FUS stimulation in low or no external  $\text{Ca}^{2+}$  still exhibited  $\text{Ca}^{2+}$  response, but only a single spike pattern was observed, in PC-3 cells (Figure 2D, Bottom). We additionally investigated  $\text{Ca}^{2+}$  influx blockers to assess their effects on FUS-dependent  $\text{Ca}^{2+}$  dynamics. Surprisingly, treatment of PC-3 cells with two different  $\text{Ca}^{2+}$  influx blockers (BTP2 or SKF96365) still showed all three patterns of  $\text{Ca}^{2+}$  response (Supplementary Figure S3 and Table 1) rather than the single spike when external  $\text{Ca}^{2+}$  is absent or low. This suggests that the specific route of  $\text{Ca}^{2+}$  entry may determine the specificity of subsequent response patterns. Notably, FUS stimulation in normal external  $\text{Ca}^{2+}$  (i.e.,  $\text{Ca}^{2+}$  influx) following thapsigargin (TG) treatment completely abolished all  $\text{Ca}^{2+}$  responses (Figure 2E). TG is an agent that depletes intracellular  $\text{Ca}^{2+}$  stores. Our results indicate that external  $\text{Ca}^{2+}$  influx is not necessary for a FUS induced single spike  $\text{Ca}^{2+}$  response in PC-3 cells, suggest that this response is likely due to release from an internal storage site and differs from pharmacologically blocking 2 different PM  $\text{Ca}^{2+}$  channels. The mechanism of  $\text{Ca}^{2+}$  entry may thus play an important role in mediating complex  $\text{Ca}^{2+}$  dynamics following mechanosensory stimulation.

### PANX1 Mediates Intracellular $\text{Ca}^{2+}$ Release in PC-3 Cells

To further investigate the complex pattern of  $\text{Ca}^{2+}$  signaling following FUS stimulation, we tested  $\text{Ca}^{2+}$  release from an internal storage site. IP<sub>3</sub> Receptors are known to mediate  $\text{Ca}^{2+}$  release from ER or sarcoplasmic reticulum (SR) stores (Mery et al., 2005; Sasse et al., 2007; Rizaner et al., 2016). Treatment of cells with Xestospongin C (an IP<sub>3</sub>R inhibitor) partly inhibits the FUS-induced  $\text{Ca}^{2+}$  response (Figure 3). This suggests that other  $\text{Ca}^{2+}$  channels may also mediate release from internal stores. One potential candidate for such a role is PANX1. PM localized

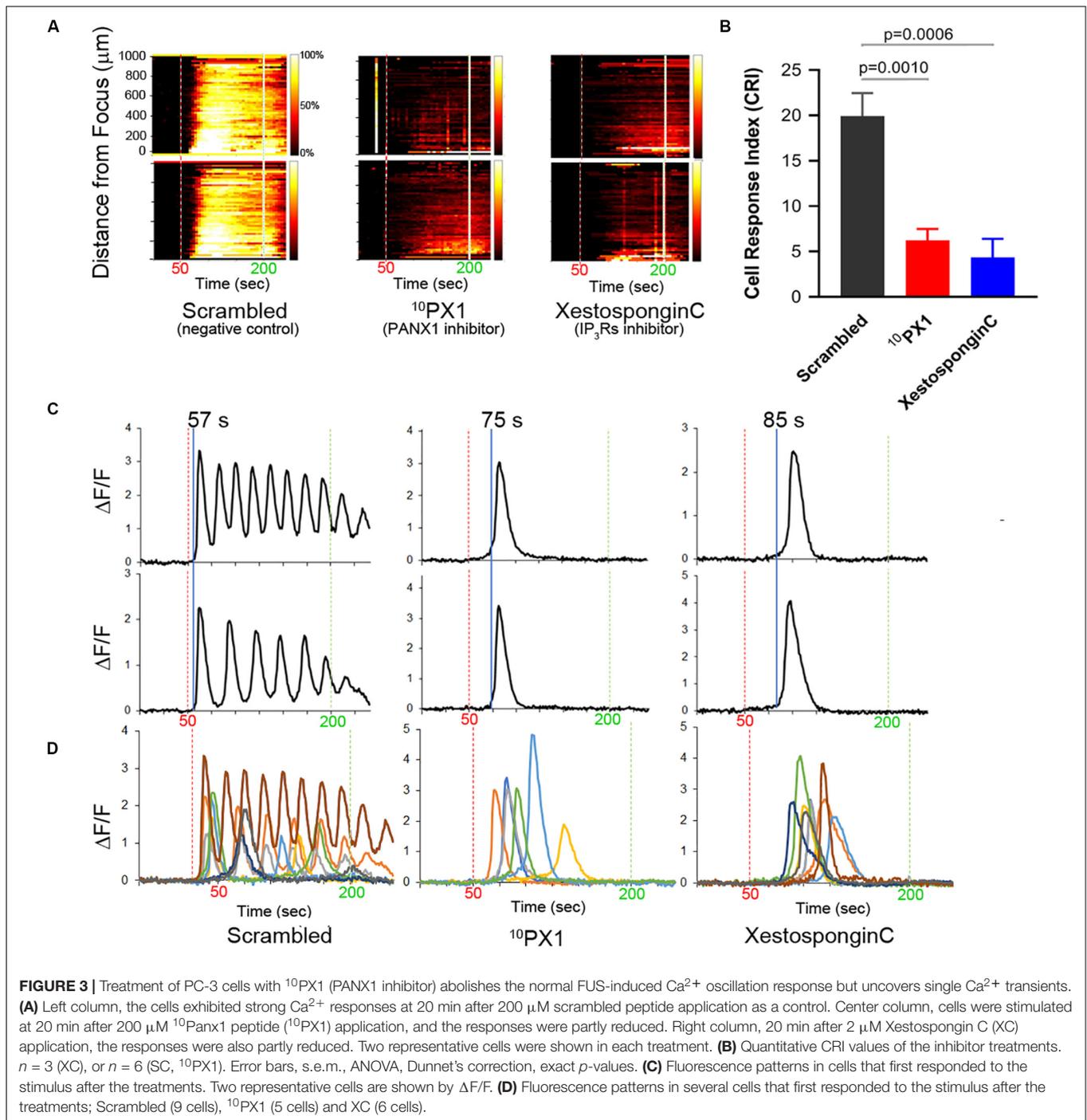


PANX1 is well studied for its role in ATP release but PANX1 is also localized to the ER where its function(s) is unknown (except for involvement in  $\text{Ca}^{2+}$  leaks, Vanden Abeele et al., 2006).

We treated PC-3 cells with  $^{10}\text{Panx1}$  peptide, a PANX1 inhibitor (Furlow et al., 2015). This results in a decrease in the FUS stimulated  $\text{Ca}^{2+}$  response (Figure 3).  $\text{Ca}^{2+}$  oscillations and

**TABLE 1** | Pharmacological effects of agents on FUS-stimulated  $\text{Ca}^{2+}$  dynamics in PC-3 cells.

Agents	Target effect	$\text{Ca}^{2+}$ Response
<b>External <math>\text{Ca}^{2+}</math> influx</b>		
BTP2	CRAC inhibitor, Blocks $\text{Ca}^{2+}$ Influx	Normal, <b>Supplementary Figure S3</b>
SKF96365	TRP antagonist, Blocks $\text{Ca}^{2+}$ Influx	Normal, <b>Supplementary Figure S3</b>
<b>Intracellular <math>\text{Ca}^{2+}</math> release</b>		
Carbenoxolone	PANX1 inhibitor	Blocked, <b>Supplementary Figure S4</b>
Flufenamic acid	PANX1, CX43 inhibitor	Blocked, <b>Supplementary Figure S4</b>
Probenecid	PANX1 inhibitor	Blocked, <b>Supplementary Figure S4</b>
$^{10}\text{Panx1}$	PANX1 inhibitor	Partly Blocked, <b>Figure 3</b>
Xestospongion C	$\text{IP}_3\text{Rs}$ inhibitor	Partly Blocked, <b>Figure 3</b>
<b>Intercellular <math>\text{Ca}^{2+}</math> wave propagation</b>		
Apyrase	Extracellular ATPase	Blocked, <b>Figure 7</b>
Suramin	P2 purinergic receptor antagonist	Blocked, <b>Figure 7</b>
PPADS	P2 purinergic receptor antagonist	Blocked, <b>Figure 7</b>
AZ11645373	Selective P2X7 inhibitor	Normal, <b>Supplementary Figure S3</b>
MRS2179	Selective P2Y1 antagonist	Normal, <b>Supplementary Figure S3</b>



double transients were eliminated but not the single transients (Figures 3C,D). This result is remarkably similar to Xestosponginc C treatment (Figures 3A–D; see also Supplementary Videos S3–S5). The primary difference between  $^{10}\text{Panx1}$  and Xestosponginc C-treated cells was in the timing of the single  $\text{Ca}^{2+}$  transients.  $^{10}\text{Panx1}$ -treated cells had a  $\sim 20$  s delay after stimulation to onset (relative to the control) (Figures 3C,D, middle) while Xestosponginc C had a slightly longer  $\sim 30$  s delay. In both cases the response was maintained for  $\sim 30$ – $40$  s (Figures 3C,D,

right). Treatment with a scrambled version of  $^{10}\text{Panx1}$  exhibited the normal  $\text{Ca}^{2+}$  response (three patterns). Treatment with 2 additional PANX1 inhibitors probenecid and carbenoxolone (CBX) completely eliminated  $\text{Ca}^{2+}$  response (Supplementary Figure S4 and Table 1). These data indicate that both PANX1 and IP<sub>3</sub>Rs likely initiate and maintain FUS-induced  $\text{Ca}^{2+}$  oscillatory responses. Simultaneous addition of  $^{10}\text{Panx1}$  and Xestosponginc C did not further reduce the  $\text{Ca}^{2+}$  response suggesting that the underlying mechanisms are complementary rather than

independent (**Supplementary Figure S4**), suggesting that they may be part of the same response pathway.

We next investigated a role for ER (or SR) localized PANX1 as a mediator of  $\text{Ca}^{2+}$  release by reducing PANX1 expression in PC-3 cells using si-PANX1RNA knockdown. Following treatment FUS-induced  $\text{Ca}^{2+}$  oscillations were variably reduced ~50–70% (**Figures 4A,B**). Single or double calcium transients were delayed by ~20 s compared to the control response (**Figure 4C** and **Supplementary Videos S6, S7**). We additionally transfected non-FUS responsive HEK cells (see **Figures 1E,F**) with WT PANX1 (i.e., a full-length WT PANX1<sup>1–425</sup> sequence). This construct had no EGFP fusion since this interferes with the  $\text{Ca}^{2+}$  imaging assay (see section Materials and Methods). The WT PANX1 transfection converts HEK cells to robust FUS stimulation dependent  $\text{Ca}^{2+}$  responsiveness (**Figures 4D,E**). We also transfected HEK cells with a mutant form of PANX1<sup>1–89</sup> lacking the normal C-terminal amino acids which was fused to mRFP (mt PANX1-mRFP). Interestingly, mt PANX1-mRFP transfection resulted in spontaneous  $\text{Ca}^{2+}$  activity even before FUS stimulation as well as exhibiting robust FUS-induced  $\text{Ca}^{2+}$  responsiveness (**Figures 4D,E** and **Supplementary Videos S8–S10**). The FUS-induced response in mt PANX1-mRFP transfected cells however was reduced relative to WT PANX1-transfected HEK cells (**Figure 4E**). Taken together these results indicate that PANX1 appears to be both necessary to generate FUS dependent  $\text{Ca}^{2+}$  responsiveness in PC-3 cells and sufficient to convert non-responsive HEK cells to a responsive state as well as generating non-FUS  $\text{Ca}^{2+}$  internal release dependent (i.e.,  $\text{Ca}^{2+}$  leaks, Vanden Abeele et al., 2006).

## PANX1 Localizes to ER and PM in PC-3 Cells

We next established the cellular localization of PANX1 in PC-3 and HEK cells used in this study. HEK cells transfected with either a WT PANX1-EGFP fusion construct (WT PANX1-EGFP; Furlow et al., 2015) or mt PANX1-mRFP construct (**Figure 5A**) were imaged with wide field fluorescence microscopy. The mt PANX1-mRFP fluorescence is detected preferentially in perinuclear regions, consistent with expected ER localization (Furlow et al., 2015) while WT PANX1-EGFP fluorescence localized primarily to PM with a reduced signal localized to putative ER (**Figure 5C**). A similar result was obtained after staining PC-3 cells with anti-PANX1 antibodies that specifically recognize the N and C terminal located epitopes (**Figure 5C**). These observations, together with other studies (Vanden Abeele et al., 2006; Furlow et al., 2015), suggest that the C-terminus of PANX1 is important for PM localization and its absence results in mt PANX1-mRFP accumulating in ER. To further confirm this differential localization, we used high resolution total internal reflectance fluorescence (TIRF) microscopy of transfected HEK cells. WT PANX1-EGFP fluorescence is clearly detected by TIRF at PM while cells expressing mt PANX1-mRFP display little or no PM fluorescence (**Figure 5E**, the first column). Imaging the same cells using wide-field microscopy, both WT PANX1-EGFP and mt PANX1-mRFP signals are observed in the ER (**Figure 5E**, the middle columns). We conclude that WT PANX1-EGFP localizes to both ER and the PM, while mt PANX1-mRFP localization

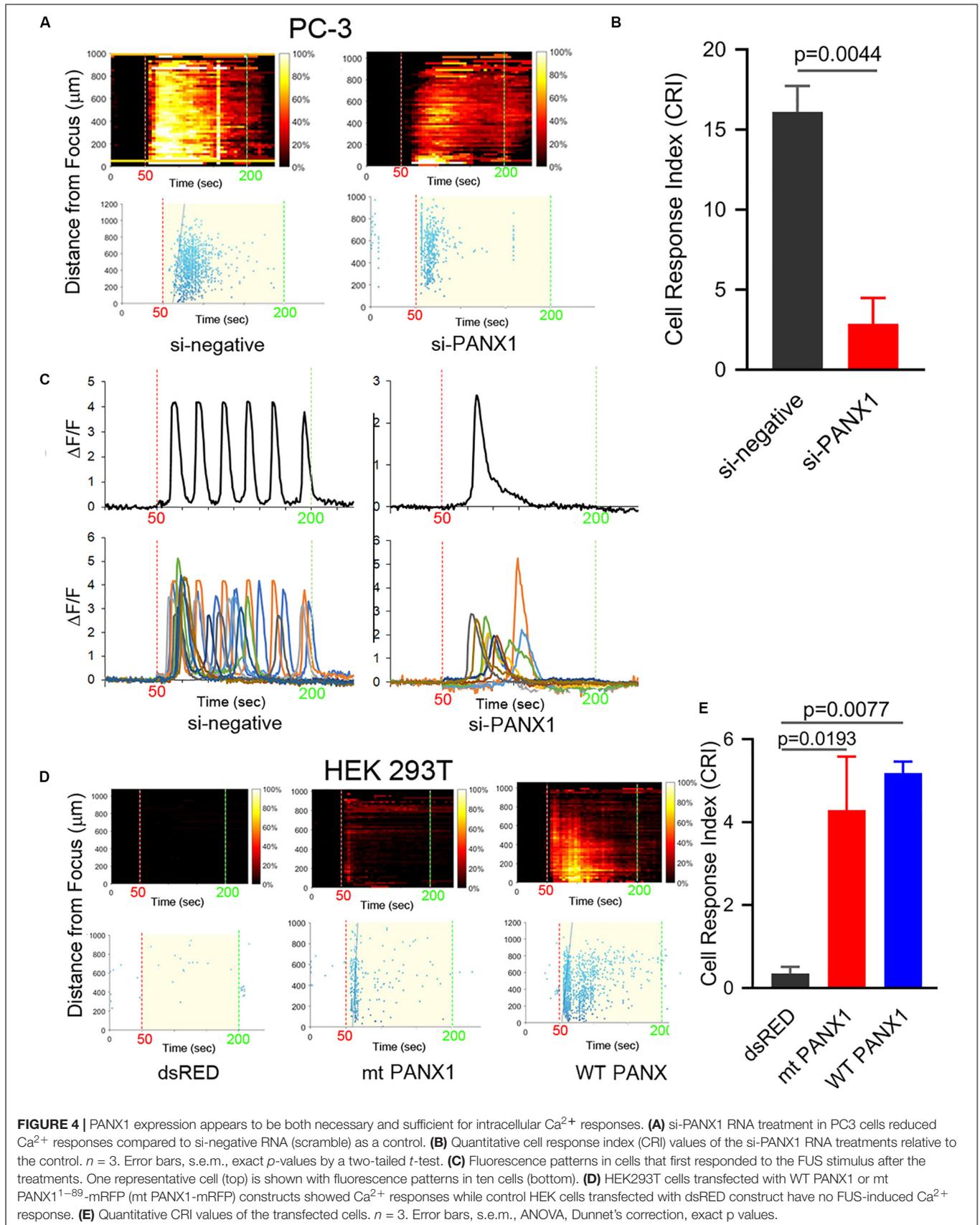
is restricted to ER. Combining this localization data with FUS-dependent stimulus data suggests that FUS may be capable of directly or indirectly stimulating ER-localized PANX1 to evoke the internal  $\text{Ca}^{2+}$  oscillations.

## FUS-Dependent $\text{Ca}^{2+}$ Oscillatory Response Does Not Depend on Cytoskeletal Integrity

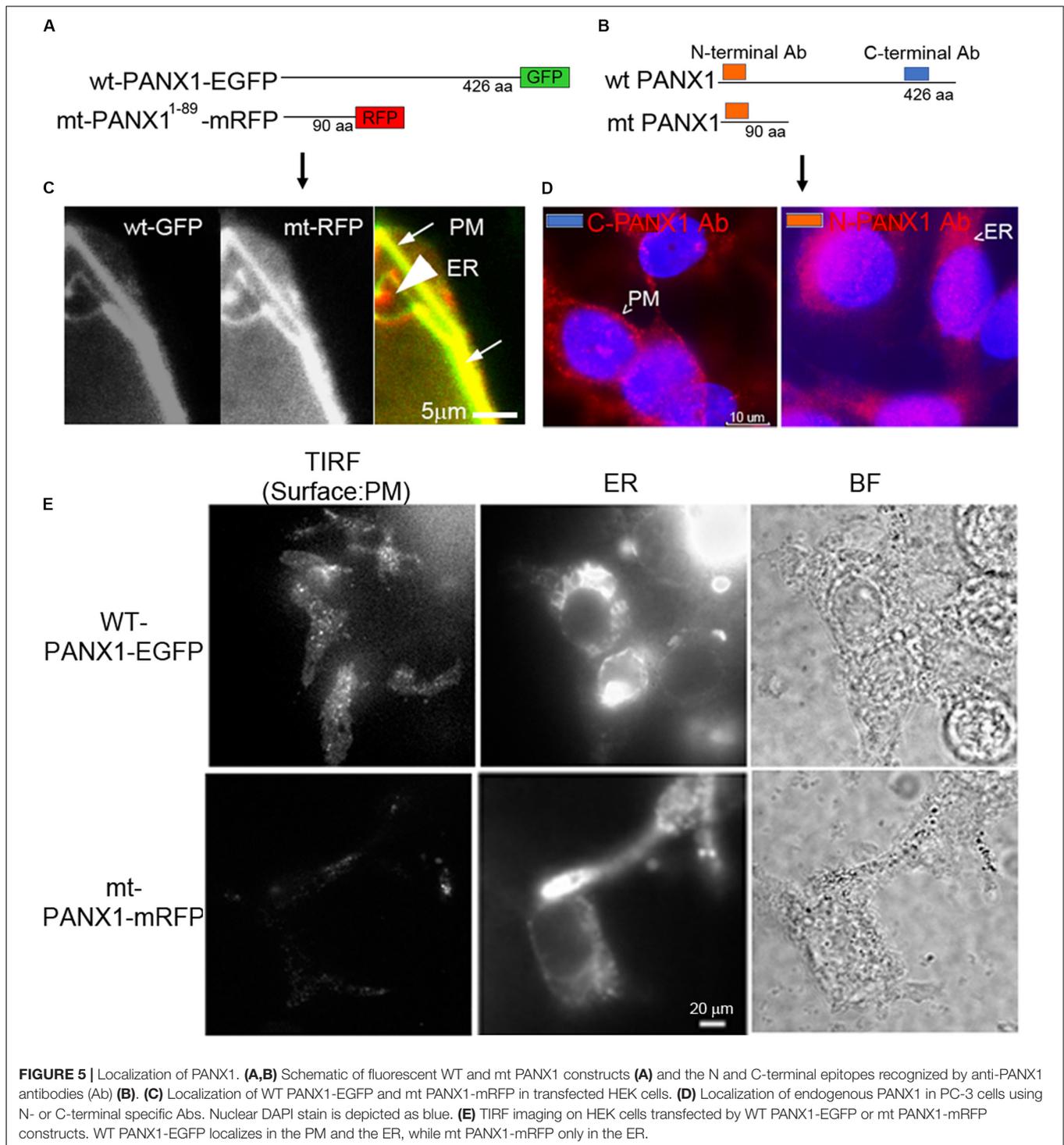
The cytoskeleton is believed to be important for the transmission of mechanical forces to internal cellular structures (Fletcher and Mullins, 2010; Cox et al., 2016) following US stimulation of mechanosensitive PM channels. PANX1 channels are mechanosensitive (Bao et al., 2004). We tested the role of cytoskeletal integrity in FUS stimulation by addition of cytoskeletal protein/process disrupters, including CytochalasinD (actin filaments), Nocodazole (microtubules), ML-7 and Blebbistatin (actomyosin contractility). FUS-evoked  $\text{Ca}^{2+}$  oscillatory responses appeared essentially normal in PC-3 cells when any of these disruptors were present (**Figures 6A–C**) and are thus not dependent on intact functional cytoskeletal proteins in invasive PC-3 cells. Additionally, this result suggests that FUS may be able to directly mechanostimulate ER localized PANX1. This result is in contrast with previous studies that identified an important role for cytoskeletal networks in transducing US stimuli (De Cock et al., 2015). An important difference between our high frequency non-contact focused US stimulus and most other studies is that the latter used low frequency US and required physical contact with the PM through microbubbles (Clapham, 2007; Carreras-Sureda et al., 2018; Burks et al., 2019). This could explain why an intact cytoskeleton appeared necessary for internal  $\text{Ca}^{2+}$  release in these other studies. We conclude from our results that FUS stimulation appears to be sufficient to result in internal mechanosensory activation of ER localized PANX1 and this coupling results in  $\text{Ca}^{2+}$  release from internal stores.

## FUS Stimulation Induces Propagation of Intercellular $\text{Ca}^{2+}$ Waves Mediated via ATP Release and PANX1

We previously demonstrated that FUS-induced calcium waves were not caused by ultrasonic surface waves or gap junction-mediated paracrine signaling (Weitz et al., 2017). However, they may depend on paracrine as well as autocrine signaling via the release of extra-cellular messengers, such as ATP. To test this possibility, we performed FUS stimulation of PC-3 cells in the presence of extracellular apyrase (an ATP degrading enzyme) or in the presence of Suramin or PPADS (2 purinergic receptor blockers). These treatments completely abolished FUS-stimulated  $\text{Ca}^{2+}$  responses (**Figures 7A–D**). These data indicate that extracellular ATP can induce  $\text{Ca}^{2+}$  waves, and that FUS stimulation might evoke ATP release into the extracellular space where it activates PM-bound purinergic receptors on the same or nearby cells (e.g., P2X or P2Y). It is unlikely, however that P2 $\times$ 7 or P2Y1 receptors are involved in this process due to pharmacological studies summarized in **Table 1** and **Supplementary Figure S3**.



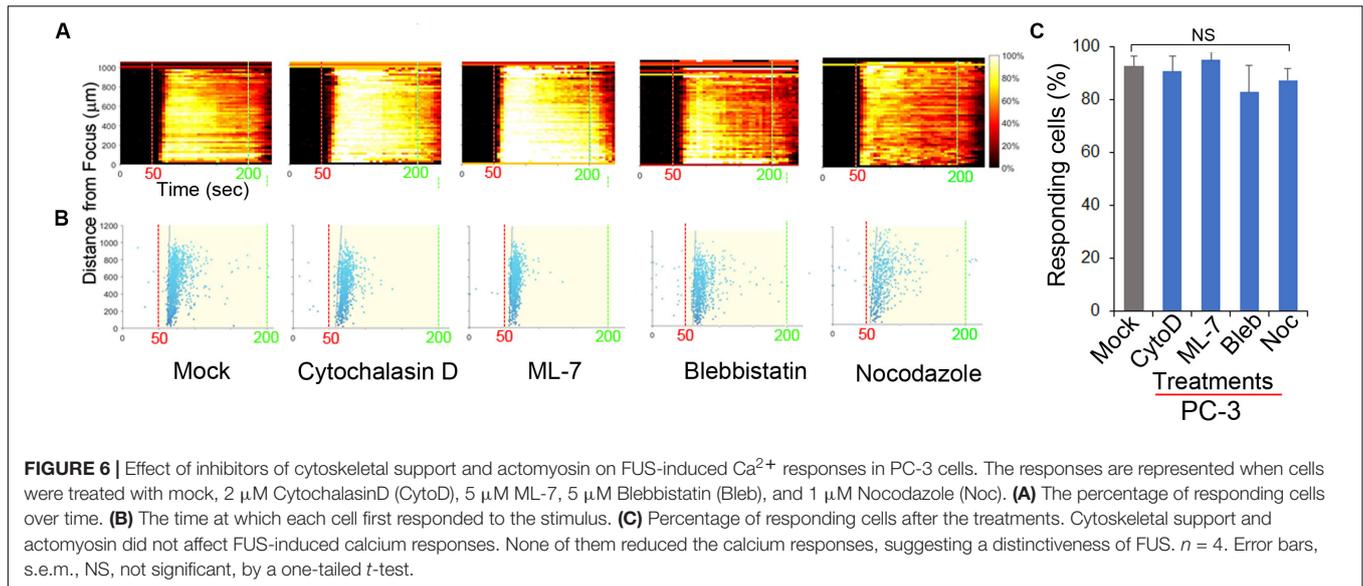
**FIGURE 4 |** PANX1 expression appears to be both necessary and sufficient for intracellular  $\text{Ca}^{2+}$  responses. **(A)** si-PANX1 RNA treatment in PC3 cells reduced  $\text{Ca}^{2+}$  responses compared to si-negative RNA (scramble) as a control. **(B)** Quantitative cell response index (CRI) values of the si-PANX1 RNA treatments relative to the control.  $n = 3$ . Error bars, s.e.m., exact  $p$ -values by a two-tailed  $t$ -test. **(C)** Fluorescence patterns in cells that first responded to the FUS stimulus after the treatments. One representative cell (top) is shown with fluorescence patterns in ten cells (bottom). **(D)** HEK293T cells transfected with WT PANX1 or mt PANX1<sup>1-89</sup>-mRFP (mt PANX1-mRFP) constructs showed  $\text{Ca}^{2+}$  responses while control HEK cells transfected with dsRED construct have no FUS-induced  $\text{Ca}^{2+}$  response. **(E)** Quantitative CRI values of the transfected cells.  $n = 3$ . Error bars, s.e.m., ANOVA, Dunnett's correction, exact  $p$  values.



In our previous study we showed that FUS stimulation and subsequent  $\text{Ca}^{2+}$  responses did not depend on formation of gap junctions (Weitz et al., 2017). This previous finding supports that PANX1 forms ATP permeant hemichannels which mediate extracellular  $\text{Ca}^{2+}$  wave propagation. To evaluate this, we treated PC-3 cells with  $^{10}\text{PX1}$  or si-PANX1 and measured ATP levels. Treated cells have significantly reduced extracellular ATP release

(Figures 7E,F), indicating that PC-3 cells mediate substantial ATP release through PANX1 channels.

To determine whether mt PANX1-mRFP or WT PANX1-EGFP alters extracellular ATP release through PANX1 channels, we measured CBX-sensitive extracellular ATP release from HEK cells expressing mt PANX1-mRFP or WT PANX1-EGFP. PANX1-mediated ATP release was quantified by measuring the



reduction in ATP release in the presence of CBX (Thompson et al., 2008; Chekeni et al., 2010; Gulbransen et al., 2012). When WT PANX1-EGFP was expressed in HEK cells, CBX-sensitive ATP release was enhanced (Figure 7G). However, CBX-sensitive ATP release was not enhanced when mt PANX1-mRFP was expressed (Figure 7G). This suggests that mt PANX1-mRFP, localized to ER is capable of mediating intracellular  $\text{Ca}^{2+}$  release (see Figures 4D,E) but that it may operate differently than PM WT PANX1. Perhaps mt PANX1 lacking the C-terminal amino acids cannot form homo-oligomers which are necessary to form functional PM ATP release channels (Romanov et al., 2012; Wang et al., 2014; Wang and Dahl, 2018). Additional work will be necessary since the construct we used also contains an additional RFP fusion which may interfere with oligomer formation and we also cannot completely rule out the possibility of mt-PANX1 artifacts.

## FUS Stimulation Induces Cytokine/Chemokine Secretion From PC-3 Cells

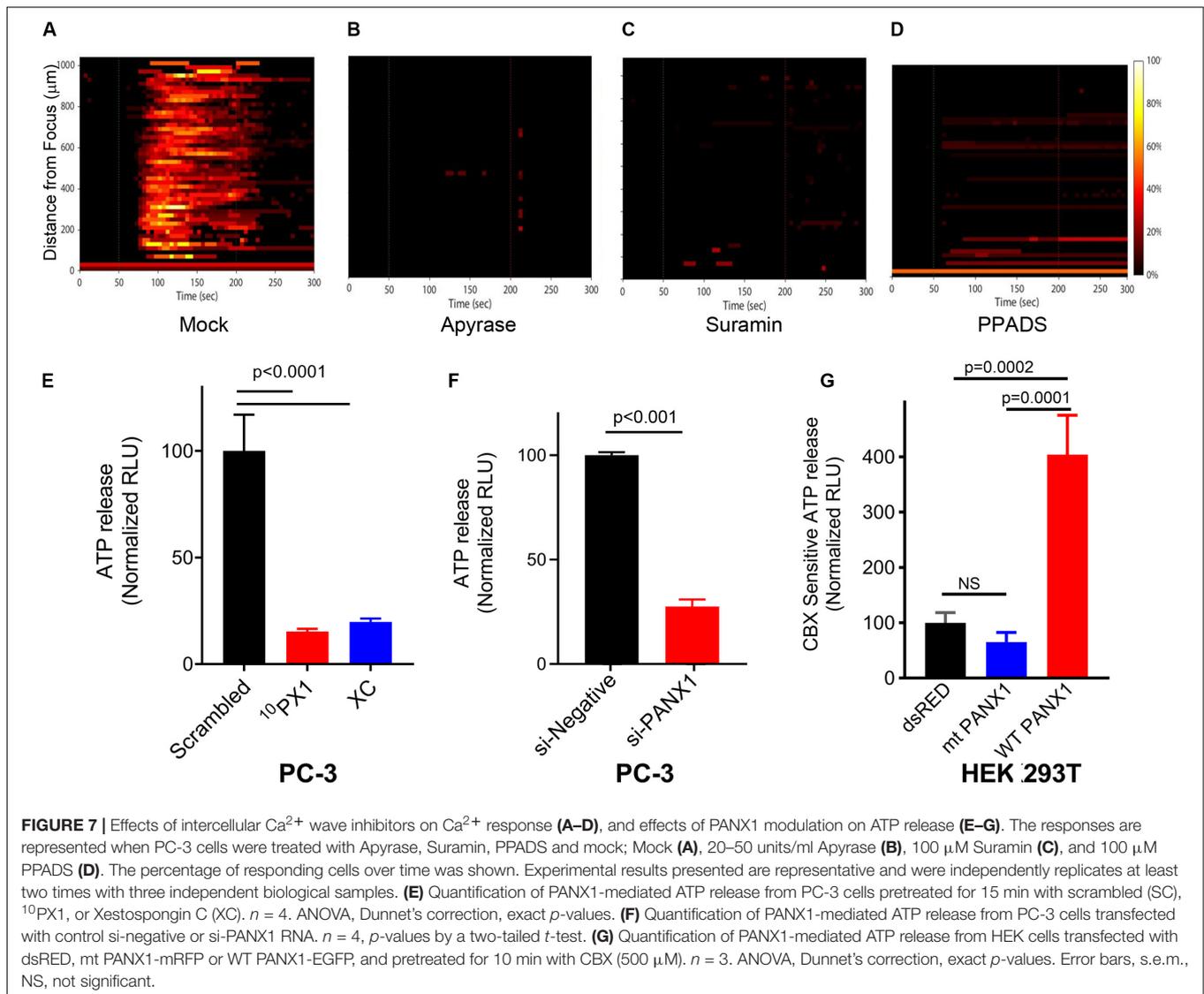
PANX1 is important for inflammasome activation (Silverman et al., 2009). Using a human cytokine array, we examined whether FUS effectively triggers PC-3 cells to secrete specific cytokines and chemokines. The assay was performed on the supernatants of cells grown for 1 day in media supplemented with charcoal-stripped fetal bovine serum after 46-MHz FUS stimulation repeated five times under several conditions (Figure 8A). PC-3 cells exhibited  $\text{Ca}^{2+}$  responses over a range of ultrasound intensities ( $\sim 300$ – $1,155 \text{ mW/cm}^2$ ) (Figures 1E–G), while non-invasive BPH-1 cells showed no response at this range of intensities (Weitz et al., 2017). Notably, FUS stimulation showed both qualitative and quantitative differences in the levels of cytokine and chemokine secretion from PC-3 cells as the intensity of stimulation was varied (Figure 8B). This suggests that FUS stimulation may be fine-tuned to control release of

specific cytokine/chemokine profiles, an exciting possibility with potentially important therapeutic applications.

## DISCUSSION

Our previous work demonstrated that mechanosensory FUS-stimulation generates a robust  $\text{Ca}^{2+}$  signaling response which can be used to distinguish invasive from non-invasive cancer cells (Hwang et al., 2013; Weitz et al., 2017). Others have also demonstrated mechanosensory  $\text{Ca}^{2+}$  signaling responses using non-focused US-stimulation in other contexts (Wood and Sehgal, 2015; Carina et al., 2018; Pan et al., 2018) as well as more general studies to clarify the physiological, cell biological and molecular mechanisms underlying mechanosensory dependent  $\text{Ca}^{2+}$  signaling responses (Tyler et al., 2008; Castellanos et al., 2016; Liu et al., 2017; Maresca et al., 2018). Our results include several new findings that further clarify the mechanically responsive  $\text{Ca}^{2+}$  signaling pathways and identify a new role for PANX1 in mediating the FUS-dependent responses. However, further studies would be needed to include more cancer and non-cancer prostate and other types of cancer cell lines to draw the general conclusion.

Removing or lowering external  $\text{Ca}^{2+}$  from culture medium did not eliminate FUS dependent  $\text{Ca}^{2+}$  signaling (see Figure 1D). This raised the possibility that an internal mechanosensory event is present and coupled to  $\text{Ca}^{2+}$  release from an internal storage site. Other work has also identified an ER dependent  $\text{Ca}^{2+}$  response mechanism using more conventional US stimulation involving  $\text{IP}_3\text{R}$  activation (Burks et al., 2019). This response required an intact cytoskeleton believed to be important for the mechanotransduction of the stimulus to the ER membrane localized  $\text{IP}_3\text{R}$  (Kim et al., 2015). However, in our study, FUS-dependent internal  $\text{Ca}^{2+}$  release is present even when cytoskeletal integrity has been disrupted (see Figure 6). This raises the interesting possibility that FUS mechanostimulation



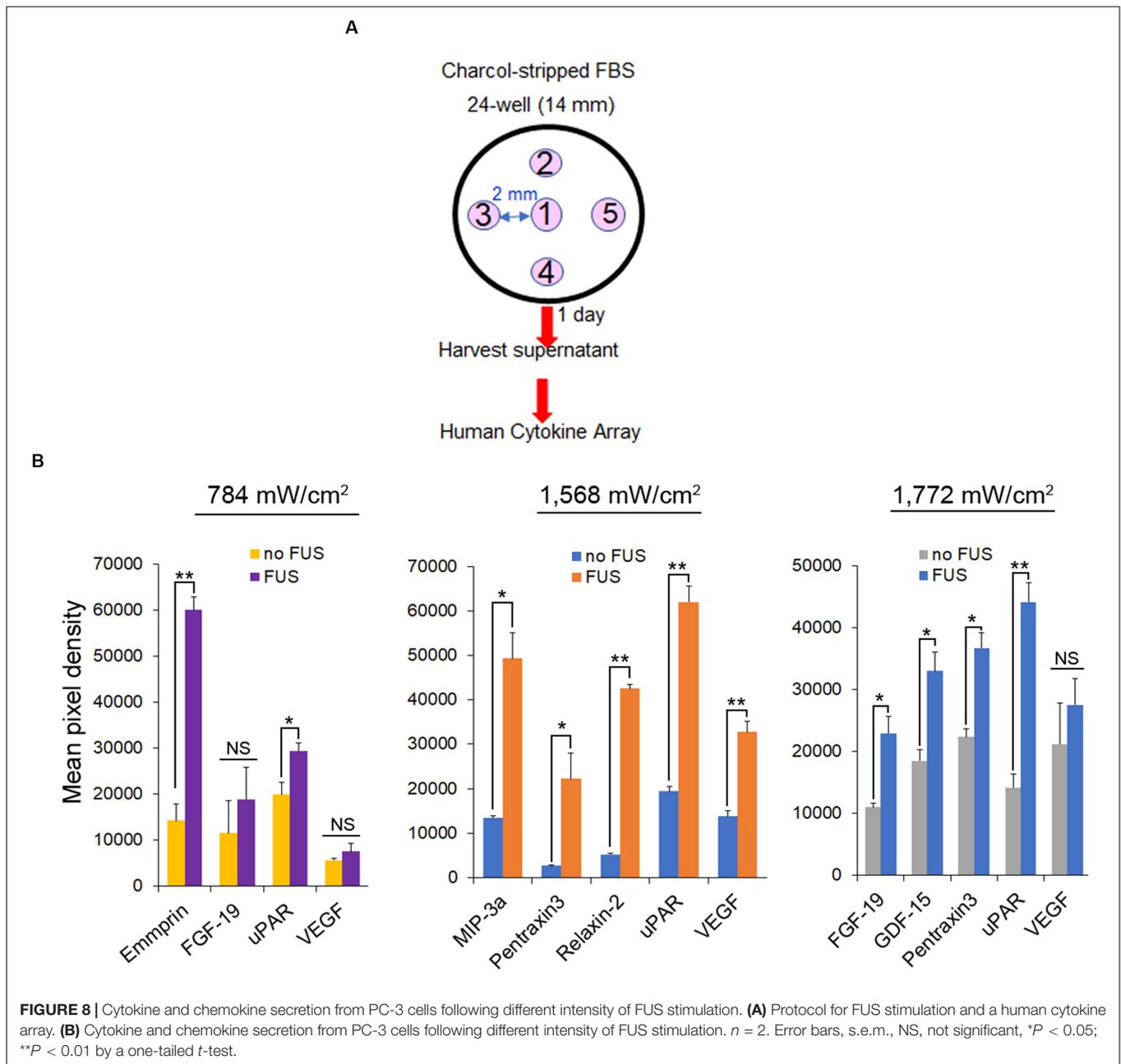
may be able to directly activate internal  $\text{Ca}^{2+}$  release and we identified mechanosensitive PANX1, partially localized to ER, as the potential internal target for this process (see Figures 3–5). However, the  $\text{Ca}^{2+}$  dynamics following mechanosensory stimulation is complex and needs further investigation before drawing specific conclusions regarding initiation of FUS-induced calcium signaling in cancer cells.

PANX1 is localized to the PM where it functions as an ATP release channel involved in intercellular signaling events (Wang and Dahl, 2018). We also confirm this for PC-3 cells (see Figures 7E–G). PANX1 channels respond to different types of chemical and mechanical stimuli with distinct channel open conformations ('large' and 'small') (Dahl, 2018). CBX and PB inhibit both PANX1 conformations (Dahl, 2018). In our study  $\text{Ca}^{2+}$  responses are eliminated in PC-3 cells treated with CBX or PB (Table 1 and Supplementary Figure S4) suggesting that these cells contain both PANX1 conformers.

The "small" conformer of PANX1 channel is reported to be impermeant to ATP (Romanov et al., 2012; Wang

et al., 2014; Wang and Dahl, 2018). We show that HEK cells transfected with mt PANX1-mRFP also do not release ATP but can confer the internal  $\text{Ca}^{2+}$  response (see Figure 4). Perhaps the mt PANX1-mRFP construct we used is functionally similar to the "small" form of PANX1. Interestingly, a mutant truncated PANX1 channel (PANX1<sup>1–89</sup>) has also been associated with highly metastatic breast cancer cells (Furlow et al., 2015). Taken together these results suggest that FUS-induced ER calcium release mediated through mt PANX1 may play a key role in cancer cell invasion and tumor metastasis. Further studies will be required to determine the mechanistic significance of this correlation.

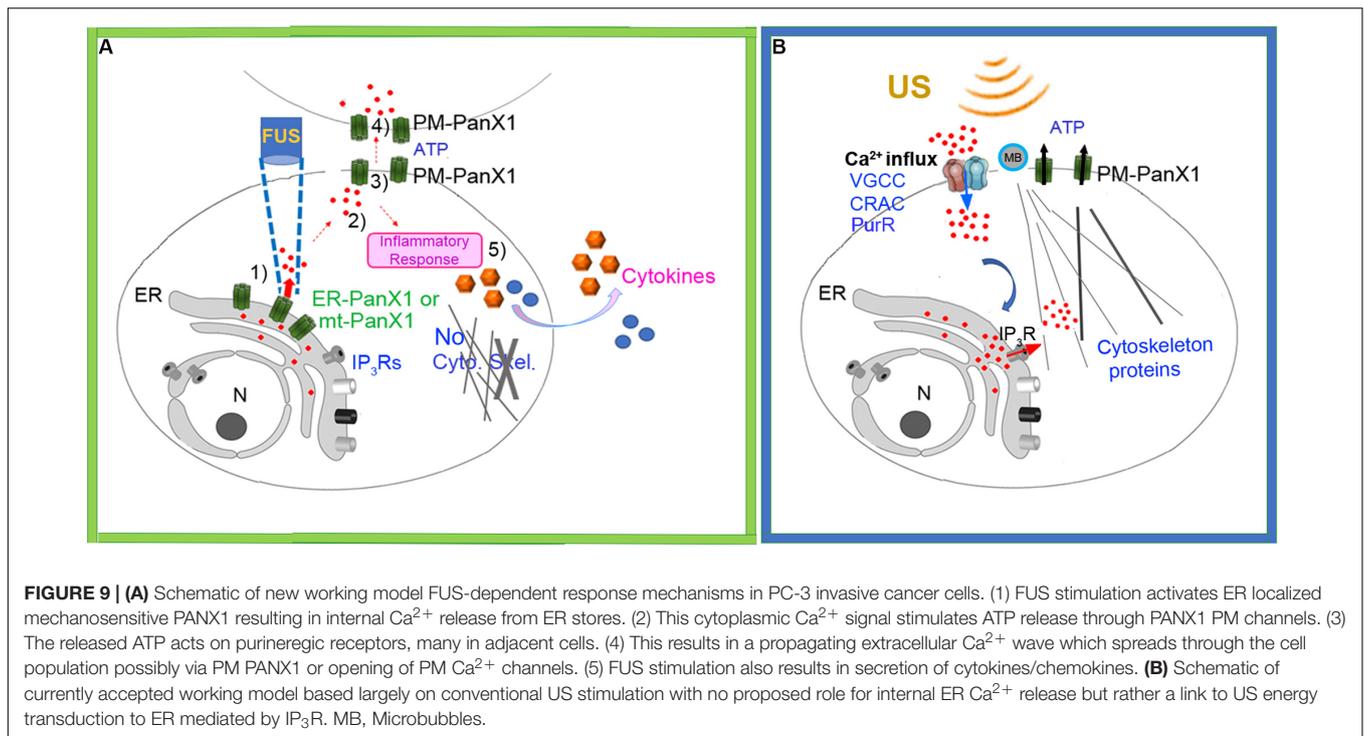
While PANX1 has clearly been localized to ER (Vanden Abeele et al., 2006; Furlow et al., 2015, see also Figure 5) its functional significance is largely unknown. In addition to demonstrating its potential role in  $\text{Ca}^{2+}$  release from ER, we show a remarkable similarity with some aspects of previously established IP<sub>3</sub>R function in this regard (Mery et al., 2005;



Sasse et al., 2007; Rizaner et al., 2016) (see **Figure 3**). Since Xestospongine C also inhibits SERCA  $\text{Ca}^{2+}$  pumps (Kume et al., 1997), the partial inhibition of the  $\text{Ca}^{2+}$  signal in **Figure 3** by using Xestospongine C (**Figure 3**) might occur. Alternatively, the partial inhibition of the  $\text{Ca}^{2+}$  signal might be due to the relatively low concentration of Xestospongine C (2  $\mu\text{M}$ ). Effective  $\text{IP}_3\text{R}$  inhibition monitoring at higher concentrations of Xestospongine C application, or agonist ( $\text{IP}_3$ )-induced calcium rises, would be needed to validate the experiments. Since the simultaneous inhibition of  $\text{IP}_3\text{R}$  and PANX1 exhibit similar patterns of FUS-dependent internal  $\text{Ca}^{2+}$  release they may be part of the same  $\text{Ca}^{2+}$  signaling pathway and

provide a mechanism to transduce various stimuli into similar cellular responses.

At high intensities, FUS has been used clinically to thermally ablate tumor cells (Fus and Cancer Immunotherapy Workshop, 2019). Perhaps more importantly at lower intensities FUS has been shown to stimulate an inflammatory response in cancer models which can boost the efficacy of immunotherapy (Curley et al., 2017; Li et al., 2018; Mauri et al., 2018; Bonaventura et al., 2019). Low-intensity FUS, has not yet been used in cancer therapy, partly due to our limited understanding of its effects and mechanism of action. In our study we clearly show the potential to use “tuned” FUS to specifically control release



of different cytokine/chemokine profiles from invasive cancer cells since this varies as the amplitude of the stimulation was changed (see **Figure 8B**). We are now attempting to extend this exciting observation to better understand FUS-induced anti-tumor immune response modulation by linking it to specific signaling pathways/molecules and epigenetic dynamics in simple cellular cancer models. This proof of principle work is critical before proceeding to *in vivo* experimentation or clinical utility.

For example, FUS applied to tumors could potentially modulate immune responses such as the ability to enhance infiltration of tumor targeting CAR T cells. Immunologically “cold” tumors are cancers that contain few infiltrating T cells thus making them impervious to current immunotherapy treatments (Li et al., 2018; Bonaventura et al., 2019). Classically immunologically “cold” cancers include glioblastomas, ovarian, prostate, pancreatic, and most breast cancers, all extremely resistant to current therapies. FUS can be potentially used as an adjunct therapy to induce secretion of cytokines/chemokines from ‘cold’ cancer cells and mediate conversion into a ‘hot’ tumor responsive to immunotherapy (Curley et al., 2017; Mauri et al., 2018). Of course, more work will be required in a well-controlled cellular model to understand the critical signaling pathways/molecules and mechanisms necessary for successful clinical translation of this technology.

FUS produces a focused beam of acoustic energy that precisely and accurately reaches large targets in the body without damaging surrounding normal cells (Mittelstein et al., 2020). One of the most striking findings of our study is the suggestion that FUS may also directly stimulate intracellular mechanosensory proteins located on particular membrane limited organelles such as ER. This raises the possibility that future studies could be designed

to prove this by designing appropriate reporter constructs, i.e., sensors and bioswitches (Kim et al., 2015; Piraner et al., 2017), and optimizing stimulus parameters (e.g., amplitude, frequency, duty factor, and duration) and thus provide a new tool to study mechanosensitive intracellular processes.

In summary, we demonstrate that non-contact mediated FUS stimulates ER localized PANX1 to initiate an intracellular  $\text{Ca}^{2+}$  release. This process does not require an intact cytoskeleton and is independent of external  $\text{Ca}^{2+}$  entry. PM localized PANX1, however, does appear necessary to mediate the intercellular spreading of  $\text{Ca}^{2+}$  waves likely through ATP release. In addition, FUS stimulation results in the release of specific chemokine/cytokine profiles from invasive PC-3 cancer cells. The specific cytokine/chemokine profile can be modified by varying FUS stimulus intensity.

Taken together our results suggest a new mechanistic working model for FUS-stimulation dependent  $\text{Ca}^{2+}$  signaling in cells which is shown schematically in **Figure 9A**. The initial cytoplasmic  $\text{Ca}^{2+}$  signal subsequently results in extracellular ATP release, possibly mediated by PM-PANX1 action and/or direct FUS stimulation. The ATP acts on purinergic receptors in nearby cells, thus propagating the spread of intercellular  $\text{Ca}^{2+}$  waves. Overall, these processes are not dependent on cytoskeletal integrity or other types of  $\text{Ca}^{2+}$  channels present in ER. The initial ER  $\text{Ca}^{2+}$  release, however, is not strictly related to mechanosensory stimulation of ER localized PANX1 but may also be influenced by ER localized  $\text{IP}_3\text{R}$ s as reported by others (Diver et al., 2001; Bootman et al., 2002; Xu et al., 2005). An additional response of FUS stimulation of PC-3 invasive cancer cells is the coupled release of specific cytokines/chemokines release from PC-3 cells. This new model can be compared to current working

model largely derived from conventional US stimulation for comparison (**Figure 9B**) (Clapham, 2007; Carreras-Sureda et al., 2018; Burks et al., 2019).

## DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

## AUTHOR CONTRIBUTIONS

NL conceived and designed experiments. NL and PS wrote the manuscript. CY, QW, SM, and KK executed the Ca<sup>2+</sup> imaging. CY and QW analyzed the Ca<sup>2+</sup> imaging. NL executed the all molecular and cell biological experiments and assays with CY and QW. HJ (46-MHz) and GL (3-MHz) characterized transducers with a needle hydrophone. RC and LJ fabricated transducers in house. AF and FP performed the TIRF imaging. AW helped develop calcium imaging program. PS, AW, RHC, and KS contributed helpful discussions/advice. CY, PS, AW, FP, and KS edited the manuscript with helpful suggestions. All authors reviewed the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2020.00504/full#supplementary-material>

**FIGURE S1** | Effect of FUS stimulation amplitude on PC-3 cell calcium response using 3-MHz transducer. All stated voltages represent peak-to-peak amplitude

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(Vp-p). Values in parentheses indicate the mV at each voltage, as measured by a hydrophone. **(A)** 2-D histograms showing the percentage of responding cells over time. **(B)** Scatter plots showing the time at which each cell first responded to the stimulus (each dot represents a responding cell). **(C)** Quantitative percentage of responding cells. *n* = 3. Error bars, s.e.m., *n* represents biological replicates.

**FIGURE S2** | Effect of FUS stimulation amplitude on HEK cell calcium response using 46-MHz transducer. All stated voltages represent peak-to-peak amplitude (Vp-p). Values in parentheses indicate the mV at each voltage, as measured by a hydrophone. Quantitative percentage of responding cells. *n* = 3. Error bars, s.e.m., *n* represents biological replicates. Some spontaneous response background was occasionally shown, so the percentages of responding cells are ~10%. However, the calcium response in HEK cells was not altered by different FUS stimulation amplitude, which is different from PC-3 cells.

**FIGURE S3** | Effect of treatment of inhibitors on PC-3 cell calcium response. 2-D histograms showing the percentage of responding cells over time. **(A)** Effect of treatments of P2 receptor inhibitors on PC-3 cell calcium response. **(B)** Effect of treatments of Ca<sup>2+</sup> influx inhibitors on PC-3 cell calcium response. These did not change the calcium response.

**FIGURE S4** | Effect of both treatment of <sup>10</sup>PX1 and XC on PC-3 cell calcium response using 46-MHz transducer. **(A)** 2-D histograms showing the percentage of responding cells over time. **(B)** Scatter plots showing the time at which each cell first responded to the stimulus (each dot represents a responding cell). **(C)** Effect of treatments of CBX, PB and FFA. The histograms showed the percentage of responding cells over time. Treatment of CBX, PB or FFA in PC-3 cells abolished Ca<sup>2+</sup> responses.

**FIGURE S5** | Quantitative RT-PCR analysis of WT PANX1 transcript expression in PC3 cells transfected two independent siRNAs that specifically target FL PANX1, as described previously; *n* = 2. Error bars, s.e.m., si-PANX1-N1 (L-018253-00) showed <35% reduction **(A)** so we did not use it. Another si-PANX1-N2 (D-018253-02) was used in most experiments and called as 'si-PANX1' **(B)**. The variations of reduction occurred because of cell heterogeneity.

**TABLE S1** | Pharmacological agents used to investigate the mechanism of FUS-induced calcium rise in PC-3 cells.

**VIDEO S1** | Calcium responses of strongly invasive PC-3 prostate cancer cells to stimulation with 46-MHz low-intensity FUS. The FUS stimulus onset and offset times are 50 and 200 s, respectively.

**VIDEO S2** | Calcium responses of non-invasive HEK 293T cells to stimulation with 46-MHz low-intensity FUS. The FUS stimulus onset and offset times are 50 and 200 s, respectively.

**VIDEOS S3–S5** | Calcium responses of strongly invasive PC-3 prostate cancer cells after scrambled peptide (SC, **S3**), <sup>10</sup>PX1 peptide (**S4**) and Xestospongine C (XC, **S5**) application for 20 min, to stimulation with 46-MHz low-intensity FUS. The FUS stimulus onset and offset times are 50 and 200 s, respectively.

**VIDEOS S6, S7** | Calcium responses of strongly invasive PC-3 prostate cancer cells after si-negative (**S6**) or si-PANX1 (**S7**) treatments for 2 days, with 46-MHz low-intensity FUS. The FUS stimulus onset and offset times are 50 and 200 s, respectively.

**VIDEOS S8–S10** | Calcium responses of non-invasive HEK 293T cells, after transfection of dsRED (**S8**), mt PANX1-mRFP (**S9**) or WT PANX1 (**S10**) constructs, to stimulation with 46-MHz low-intensity FUS. The FUS stimulus onset and offset times are 50 and 200 s, respectively.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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