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Highlights:

- In hepatocytes, TRPM2 channels are mainly localised in intracellular organelles.
- Oxidative stress increases the number of TRPM2 channels in the plasma membrane.
- TRPM2 trafficking to the PM may contribute to positive Ca^{2+}-dependent feedback.

Oxidative stress promotes redistribution of TRPM2 channels to the plasma membrane in hepatocytes

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Abstract

Transient Receptor Potential Melastatin (TRPM) 2 is a non-selective Ca^{2+} permeable cation channel and a member of the Transient Receptor Potential (TRP) channel family. TRPM2 has unique gating properties; it is activated by intracellular ADP-ribose (ADPR), whereas Ca^{2+} plays a role of an important co-factor in channel activation, increasing TRPM2 sensitivity to ADPR. TRPM2 is highly expressed in rat and mouse hepatocytes, where it has been shown to contribute to oxidative stress-induced cell death and liver damage due to paracetamol-overdose. The mechanisms regulating the activity of TRPM2 channels in hepatocytes, however, are not well understood. In this paper, we investigate the localisation of TRPM2 protein in hepatocytes. The presented results demonstrate that in rat hepatocytes under normal conditions, most of the TRPM2 protein is localised intracellularly. This was determined by confocal microscopy using TRPM2- and plasma membrane (PM)-specific antibodies and immunofluorescence, and biotinylation studies followed by western blotting. Interestingly, in hepatocytes treated with either H_2O_2 or paracetamol, the amount of TRPM2 co-localised with PM is significantly increased, compared to the untreated cells. It is concluded that trafficking of TRPM2 to the PM could potentially contribute to a positive feedback mechanism mediating Ca^{2+} overload in hepatocytes under conditions of oxidative stress.

Keywords: TRP channels, TRPM2, oxidative stress, hepatocyte, Ca^{2+}, protein trafficking

Introduction

The Transient Receptor Potential Melastatin 2 (TRPM2) channel is a Ca^{2+} permeable, non-selective cation channel which belongs to the TRP superfamily of ion channels [1]. As all other TRP channels, TRPM2 is a tetramer with each monomer being composed of six transmembrane (TM) domains and cytoplasmic N- and C-termini. The main agonist of TRPM2 is intracellular ADP ribose (ADPR). ADPR binding to the TRPM2 NUDT9-H motif, which is homologous to Mg^{2+}-dependent mitochondrial pyrophosphatase NUDT9, in the C-terminus leads to opening of the channel pore [2–5]. Hydrogen peroxide (H_2O_2) and other oxidants activate TRPM2 by increasing the production of ADPR. ADPR generation in the cell mainly occurs through activation of poly ADP-ribose (pADPR) polymerase (PARP) in response to reactive oxygen species (ROS)-mediated DNA damage and subsequent activation of pADPR glycohydrolase (PARG) [2,6,7]. A direct mechanism of activation of TRPM2 through oxidation of cysteine residues has also been proposed, however, it remains controversial [5,8].

TRPM2 channels are expressed in a range of tissues including brain, cells of immune system, vasculature, liver, pancreas and prostate [9–11]. Previously, we identified functional TRPM2 channels expressed in hepatocytes and showed that these channels are responsible for liver damage induced by paracetamol or H_2O_2, generators of ROS [12]. TRPM2 currents recorded in hepatocytes in response to intracellular application of ADPR through a patch pipette exhibited a slower time course of activation compared to TRPM2 currents in TRPM2-transfected HEK293T cells [12]. The time course of cytosolic Ca^{2+} ($[Ca^{2+}]_c$) rise induced by H_2O_2 in hepatocytes was also significantly slower compared to that in HEK293T cells [12]. The reasons for this discrepancy are not clear; however, there is a possibility that TRPM2 localisiation differs between cell types. When expressed heterologously in HEK293T cells, TRPM2 channels localise primarily on the plasma membrane (PM),[13]. On the other hand, in primary cell types, where TRPM2 channels are expressed endogenously, their localisation varies [14]. In neuronal cells, TRPM2 is mostly expressed on the PM [8,13,15], whereas in monocytes, TRPM2 is localised on the membranes of intracellular

organelles, mainly lysosomes [16,17]. Our knowledge about TRPM2 localisation, regulation and gating in hepatocytes is incomplete.

In this work we investigated the localisation of TRPM2 channels in hepatocytes and the possibility of their redistribution between intracellular organelles and the PM in response to oxidative stress. Using immunofluorescence and confocal microscopy, we show that in control (untreated) hepatocytes TRPM2 is mostly localised intracellularly; however, after treatment with H_2O_2 or paracetamol, large proportion of TRPM2 is co-localised with the PM. Similar results are obtained using biotinylation of the surface proteins and western blotting with the TRPM2 antibody. Overall, the results suggest that during oxidative stress TRPM2 channels translocate from intracellular compartments to the PM in hepatocytes, increasing TRPM2-mediated Ca^{2+} entry which provides a positive feedback promoting further trafficking of TRPM2 to the PM and finally cell death.

Methods and Materials

Animals

Hooded Wistar rats were housed and bred in the controlled environment with a 12-h light–dark cycle about three weeks before the experiments. Male rats aged 8-12 weeks were used. All animal studies were approved by the Animal Ethics Committees of the University of Adelaide and Flinders University of South Australia.

Hepatocyte Isolation and Culture

The rat hepatocytes were isolated and cultured as previously described [12,18].

Patch-Clamp Recording

Membrane currents were measured at room temperature (~23°C) using standard patch clamping in a whole-cell mode, and a computer-based EPC-9 patch-clamp amplifier run by PULSE software as described previously [12,18]. In order to monitor the development of membrane currents, voltage ramps between -120 to +120 mV were applied every two seconds following the achievement of whole-cell configuration. The data were analysed using PULSEFIT software (HEKA). The TRPM2 current in hepatocytes or HEK293T cells heterologously expressing TRPM2 was activated by adding 1 mM ADPR to the pipette solution. Patch pipettes were pulled from borosilicate glass and fire-polished to a resistance ranging 1.5-2.5 MΩ. The series resistance did not exceed 7.5 MΩ and was 50 to 70% compensated. The onset of ADPR-induced TRPM2 activation was determined by finding the earliest 5 consecutive current traces which had significantly larger average amplitude than the previous 5 consecutive traces, but smaller amplitude than the next 5 consecutive recordings.

Confocal Microscope Imaging

Isolated hepatocytes plated on glass coverslips were treated with 10 mM paracetamol for two hours, or 1 mM H_2O_2 for 45 min. The hepatocytes were immediately fixed with 100% methanol at -20^{o}C and washed in PBS. Following the blocking step with 20% FBS in PBS for 1 h, the cells were incubated with sheep anti-rat TRPM2 (ab63015, Abcam, UK) and mouse anti Pan-Cadherin antibodies, used to visualise plasma membrane, for two hours. After washing with PBS, the cells were separately incubated for 1 h with FITC anti-sheep followed by Cyan-5 anti-mouse antibodies. The coverslips were then mounted on slides using Gold Antifade and dried.

Images were captured using Leica Spectral SP5 confocal microscope at 100x magnification. Laser-generated excitation wavelengths of 496 and 633 nm were used to detect FITC (TRPM2 channel) and Cyan-5 (plasma membrane), respectively. After capturing more than 50 images in each group (control, H_2O_2-treated and paracetamol-treated hepatocytes), the images were processed by Image J using JACoP Plugin to calculate Pearson's and Manders' (M1, fraction of cellular plasma membrane merged with TRPM2 channel) coefficients. In addition, the scatter plots were created in MATLAB (Mathworks, Natick, MA).

Detection of TRPM2 Protein on the Plasma Membrane Using Cell Surface Biotinylation

The surface proteins were extracted using a Pierce Cell Surface Protein Isolation Kit as follows. Two hours post isolation, the hepatocytes were incubated in a KRH solution at 37°C for 45 min with or without 1 mM H_2O_2. After incubation, the hepatocytes were washed twice with cold PBS and pelleted by centrifugation at 500×g (2 min). Cells from each group (~2×10^6 cells) were then resuspended and incubated with 25 ml Sulfo-NHS-SS-Biotin containing solution for 30 min at 4ºC, with slow shaking. Following the incubation, cells were pelleted and lysed for 30 min on ice using 500 µl lysis buffer containing 50-mM Tris, 150-mM NaCl, 1% Trinton-X, 3-mM EDTA and 10-µl protease inhibitor. The lysate was centrifuged 15 min at 12,000×g, and 500 µl of the supernatant containing the total protein was collected. To separate the biotinylated cell surface proteins from the total protein isolated, 400 µl of the supernatant were incubated in NeutrAvidin Agarose column for

1 h at room temperature. The column was then centrifuged 1 min at 1000×g. The biotinylated protein bound to NeutrAvidin Agarose was eluted with 400 µl of SDS-Page solution containing 50-mM DTT. The concentrations of the total proteins, the remnant proteins passed through the column and the surface biotinylated proteins were determined using a Bicinchoninic acid (BCA) kit (Sigma-Aldrich, Australia). The same amounts of total protein and the remnant protein solutions and 10 times of that amount of the corresponding surface protein solution were loaded and separated on the SDS-PAGE gel using electrophoresis.

After protein transfer, the nitrocellulose membrane was blocked 1 h with 5% skim milk in TBST solution and incubated with primary antibody against TRPM2 protein at 1/750 dilution in TBST containing 1% skim milk overnight at 4°C. After washing, the membrane was incubated with HRP anti-sheep antibody (ab6747, Abcam, UK). The TRPM2 protein bands were visualised using H_2O_2 containing ECL western blotting substrate. GAPDH protein used as a standard control for intracellular protein loading was detected using mouse anti-GAPDH **(**ab8245, Abcam, UK) in TBST containing 1% skim milk overnight at 4°C overnight followed by washing in TBST and incubating in HRP anti-mouse **(**ab6728, Abcam, UK) for 1h. The experiment was repeated 3 times using different preparations of hepatocytes.

The autoradiographs were scanned using a GS-800 densitometer (Bio-Rad Laboratories, Hercules, California, US), and the band densities were quantified using Quantity One software version 4.3.1 (Bio-Rad Laboratories).

Statistical Analysis

Data are presented as means ± SEM. Statistical significance was assessed using one-way ANOVA with multiple comparisons or unpaired t-test with Welch's correction as stated.

Results

It has been previously demonstrated that rat hepatocytes express functional TRPM2 channels [12]. Consistent with the previous findings, intracellular perfusion of rat hepatocytes with the pipette solution containing 1 mM ADPR caused activation of a non-selective cation current mediated by TRPM2 channels. The time course of TRPM2 current activation in hepatocytes was relatively slow; the onset of current development occurred at 120±28 s and the time from the onset to the maximum current activation was 208±17 s (Fig 1A; see also [12]). In comparison, the onset of current development in HEK293T cells heterologously expressing TRPM2 occurred at 17.2±1.9 s, whereas the average time to full activation was 78±10 s (Fig 1A). Both, the onset of the current development (indicated by arrows in Fig. 1A) and the time required to reach maximum amplitude were significantly longer in hepatocytes compared to TRPM2-HEK293T cells ($p<0.01$, Fig. 1B and 1C). Since the activation of several other TRP channels involves trafficking of the TRP protein from intracellular organelles to the PM [19,20], one of the reasons that could account for such differences in the time course of activation between cell types, is the difference in trafficking of TRPM2 channels during current activation from intracellular compartments to the plasma membrane.

To investigate localisation of TRPM2 channels in intracellular compartments and PM, we used confocal microscopy in conjunction with TRPM2- and PM-specific immunofluorescence staining. In control untreated hepatocytes plated on glass coverslips for 24h after isolation, TRPM2-specific staining (Fig. 2, green (FITC)) was mainly intracellular and did not appreciably overlap with red (Cyan-5) staining of the PM (Fig. 2). Merging images together confirmed lack of co-localisation of green and red fluorescence (Fig. 2). We have shown previously that oxidative stress induced by H_2O_2 or paracetamol leads to production of ADPR and activation of TRPM2 current in hepatocytes [12]. Therefore, to activate TRPM2 channels, hepatocytes were incubated with either H_2O_2 or paracetamol as previously described [12]. TRPM2-specific immunostaining of H_2O_2- and paracetamol-treated hepatocytes exhibited a clear outline of the cell shapes which coincided with the red PM stain (Fig. 2). Merged pseudocoloured confocal images of PM and TRPM2 suggests a significant overlap of TRPM2- with the PM- specific fluorescence in treated hepatocytes,

represented by yellow coloured pixels (Fig. 2). The overlap of green and red was further analysed by employing a custom-made MATLAB code to build scatter plots of the TRPM2 (green) and the PM (red) immunofluorescence shown in merged and zoomed area panels in Fig. 2. We measured the fluorescence values at every pixel and normalised it to the maximum florescence intensity. As expected, in control hepatocytes there were two separate groups of red and green pixels, which indicated that there was no or a little co-localisation between the TRPM2 and the PM (Supplemental Fig. 1). In contrast, immunofluorescence data obtained from hepatocytes treated with H_2O_2 or paracetamol produced scatter plots with clear overlap of red and green colours to the extent where red pixels can no longer be seen on the images (Supplemental Fig. 1).

To quantify the co-localisation of TRPM2 and PM observed in the scatter plots, we used ImageJ software to determine the Pearson's and Manders' coefficients. These tests were performed using the confocal images of hepatocytes from control (n=48), H_2O_2-treated (n=48) and paracetamol-treated (n=54) groups. The Pearson's coefficient values confirmed that there was a significant increase in the overlap of TRPM2- and PM-specific fluorescence in hepatocytes treated with H_2O_2 and paracetamol, compared to the untreated control group (One way ANOVA with multiple comparisons, $p<0.0001$, Fig. 3A). Manders' coefficients indicated that the proportion of the PM-specific fluorescence co-localised with TRPM2 increased 6-7 folds in hepatocytes treated with H_2O_2 or paracetamol, compared to control cells ($p < 0.0001$, Fig. 3B & 3C). However, there was no significant change in the proportion of TRPM2 overlapped with PM between the control and the treated groups ($p > 0.05$); most likely due to high intracellular expression of TRPM2.

To further confirm the findings of the immunofluorescence co-localisation of TRPM2 with PM, we next determined the amount of TRPM2 located at the PM as a fraction of the total TRPM2 protein expressed in rat hepatocytes using Thermo Scientific™ Pierce™ Cell Surface Protein Isolation Kit. This utilises Sulfo-NHS-SS-Biotin for labelling PM surface proteins for further analysis by western blotting using TRPM2-specific antibody, as described in the Methods. Freshly isolated hepatocytes were incubated in KHR solution either with or without 1 mM H_2O_2 for 45 min and then used for

surface protein extraction. Suspension of hepatocytes used in these experiments contained at least 90% of viable cells, however, inevitably, some damaged or dying cells were also present [21]. It is likely that the number of damaged cells increased after hepatocytes were treated with H_2O_2. To exclude the confounding effect of biotin labelling of intracellular TRPM2 in damaged hepatocytes, we also measured the amount of GAPDH, an intracellular protein, in extracellular fraction and normalised the TRPM2 expression accordingly. The weak staining of GAPDH in the surface protein fraction indicates most of these proteins are from the extracellular surface. Measurements of the densities of the TRPM2-corresponding bands on the western blots suggested that TRPM2 channel expression on the PM increased in hepatocytes treated with H_2O_2 (Fig. 4). In the control group only 3.57±0.47% of the total TRPM2 protein was detected at the PM, whereas in hepatocytes treated with H_2O_2, this increased to 16.17±0.73% (n=3; p=0.0077; Fig. 4).

Discussion

The main findings of this study are that, in untreated rat hepatocytes, TRPM2 channels are principally localised intracellularly and oxidative stress induced by H_2O_2 or high concentrations of paracetamol increases TRPM2 numbers in the PM. This is likely due to trafficking of TRPM2 from intracellular compartments to the PM. It is probable that this trafficking of TRPM2 channels to the PM contributes to the sustained $[Ca^{2+}]_c$ rise and hepatocellular death previously observed in paracetamol overdose-induced liver damage [12].

TRPM2 is one of the TRP channels superfamily members that are activated during oxidative stress [6,8,22]. TRPM2 channels are responsible for the rise of $[Ca^{2+}]_c$ which activates apoptotic and necrotic pathways in many cell types, leading to cell death [12,23–25]. Initially TRPM2 channels were characterised as non-selective Ca^{2+} permeable cation channel located in the PM [8]. However, later it became apparent that TRPM2 are also expressed in intracellular compartments including lysosomes and are involved in Ca^{2+} release from these organelles [14].

TRPM2 is not the only TRP family member located in the intracellular organelles. Other TRP channels, including TRPML1, TRPML2, TRPML3, TRPM7, TRPM8, TRPV1, TRPV2, TRPV4, TRPA1 and TRPC3-6 were found in lysosomes, intracellular vesicles, ER and Golgi [26]. Physiological roles of some of these channels are quite clear, however, function of the majority of intracellular TRP channels remains to be identified. TRPMLs, for example, are the best candidates for lysosomal Ca^{2+} release channels, which play a critical role in lysosomal and endosomal trafficking, membrane fusion and fission [27–30]. The likely function of TRPM8 channels expressed in the ER of prostate cancer cells is to provide a pathway for an agonist-induced Ca^{2+} release from the ER Ca^{2+} stores in store-operated Ca^{2+} entry pathway [31]. Physiological roles of other intracellular TRP channels, however, are far less clear.

A feasible mechanism that could be proposed to explain increase of TRPM2 numbers in hepatocyte PM is lysosomal/endosomal trafficking to PM under the oxidative stress. It has been shown previously that TRPM2 is expressed in lysosomes of β-cells [14], and also in endolysosomal

vesicles in dendritic cells [16], where it plays a role of Ca^{2+} release channel. In these cells, oxidative stress that causes activation of TRPM2 channels has multiple effects including lysosomal trafficking towards the PM. It has been previously shown that, oxidative stress alters the expression of several proteins on the plasma membrane, including acid sphingomyelinase, which is normally located in the lysosomal membrane [32–35]. This suggests that oxidative stress triggers lysosomal exocytosis [32]. Therefore, it is possible that during oxidative-stress induced by H_2O_2 or paracetamol, TRPM2 is trafficked to hepatocyte PM through lysosomal exocytosis.

Insertion into PM through exocytosis, as a step in the activation mechanism, has been proposed for other TRP channels. It has been shown that TRPC5 channels localised in intracellular vesicles in neurites of hippocampal neurons are rapidly inserted into the PM in response to stimulation with a growth factor [20]. Similarly, vesicular TRPC3 channels are trafficked to the PM in response to G-protein coupled receptor stimulation [19]. An important step in endolysosomal trafficking and fusion with PM is elevation in $[Ca^{2+}]_c$. It is likely that the local Ca^{2+} rise which plays a critical role in this process is provided by Ca^{2+} release from endolysosomes themselves [36,37].

In conclusion, the data presented in this work shows that, in rat hepatocytes, TRPM2 is mainly localised in intracellular organelles and oxidative stress-mediated damage to the cell results in increased expression of TRPM2 channels on the cell surface, most likely due to lysosomal trafficking and fusion with the PM. However, the exact mechanism of this process and whether TRPM2 mediated release of Ca^{2+} from lysosomes plays any role in this requires further investigation.

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Figure Legends

Fig. 1. Time course of TRPM2 activation in hepatocytes and TRPM2-expressing HEK293T cells. A. Representative traces of the time course of TRPM2 activation in hepatocytes and TRPM2-expressing HEK293T cells. Each point represents current amplitude at -100 mV, obtained from currents in response to 100 ms voltage ramps between -120 and 120 mV, applied every 2 s. The onset of current activation is marked by black (hepatocytes) and red (HEK293T cells) arrows. 140 mM NMDGCl instead of 140 mM NaCl, and 30 μM ACA were used in the bath to confirm that the current is mediated by TRPM2. **B.** The times of onset of TRPM2 activation and **C.** The times required for a maximum TRPM2 activation in hepatocytes (black symbols) and in TRPM2-expressing HEK293T cells (red symbols) ($p<0.01$, t-test with Welch's correction).

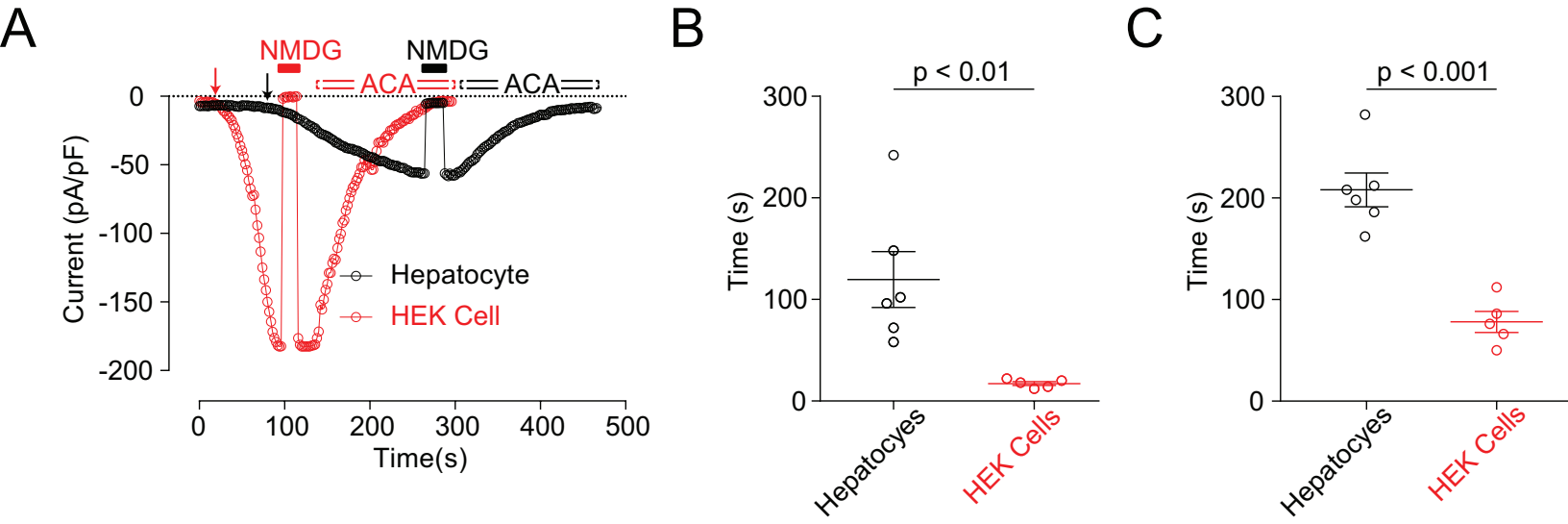
Fig. 2. Effect of H_2O_2 and paracetamol on TRPM2 channel distribution in hepatocytes. Confocal images of untreated control, 1mM H_2O_2- and 10mM paracetamol-treated hepatocytes. Green fluorescence corresponds to the TRPM2- and red fluorescence to the cadherin- (PM) specific

immunostaining. Yellow colour indicates the overlap between green and red fluorescence (TRPM2 and plasma membrane, correspondingly).

Fig. 3. Co-localisation analysis of PM- and TRPM2-specific immunofluorescence in hepatocytes treated with H_2O_2 or paracetamol. A. Pearson's coefficients of TRPM2 and plasma membrane co-localisation in untreated control (0.35±0.01, n=48), H_2O_2- (0.75±0.01, n=48) and paracetamol-treated (0.73±0.01, n=54) hepatocytes. There was a significant difference between the untreated control and 1mM H_2O_2- or 10 mM paracetamol-treated hepatocytes. (*, $p < 0.0001$, One way ANOVA with multiple comparisons). **B.** Manders' coefficient values for the fraction of the plasma membrane merged with TRPM2 protein (X-axis) plotted against the fraction of the TRPM2 protein merged with plasma membrane (Y-axis) for untreated control (black circles), 1mM H_2O_2-treated (red circles) and 10mM paracetamol-treated (green circles) hepatocytes. **C.** Manders' coefficient values for the fraction of the plasma membrane merged with TRPM2 immunofluorescence (X-axis) in untreated control (6.63±1.17, n=48), 1mM H_2O_2- (45.14±2.06, n=48) and 10mM paracetamol-treated (41.5±2.65, n=54) hepatocytes. There was a significant difference in Manders' coefficients between the untreated control and H_2O_2- or paracetamol-treated hepatocytes (*, $p < 0.0001$).

Fig. 4. The effect of H_2O_2 on TRPM2 channel expression in hepatocyte plasma membrane. A. Detection of TRPM2 expression in the plasma membrane. Total, remnant (total, minus surface) and surface protein extracts of untreated control and 1mM H_2O_2-treated hepatocytes were obtained as described in Methods. GAPDH protein was used as a loading control. **B.** The corrected plasma membrane/total TRPM2 channel fraction ratio (surface/total TRPM2 expression) in untreated and 1mM H_2O_2-treated hepatocytes from three different experiments.

Figure 1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NMDG
NMDG
ACA
ACA
0
-50
-100
-150
-200
Current (pA/pF)
Hepatocyte
HEK Cell
0
100
200
300
400
500
Time(s)
B
p < 0.01
300
200
100
0
Time (s)
Hepatocyes
HEK Cells
C
p < 0.001
300
200
100
0
Time (s)
Hepatocyes
HEK Cells

Figure 2

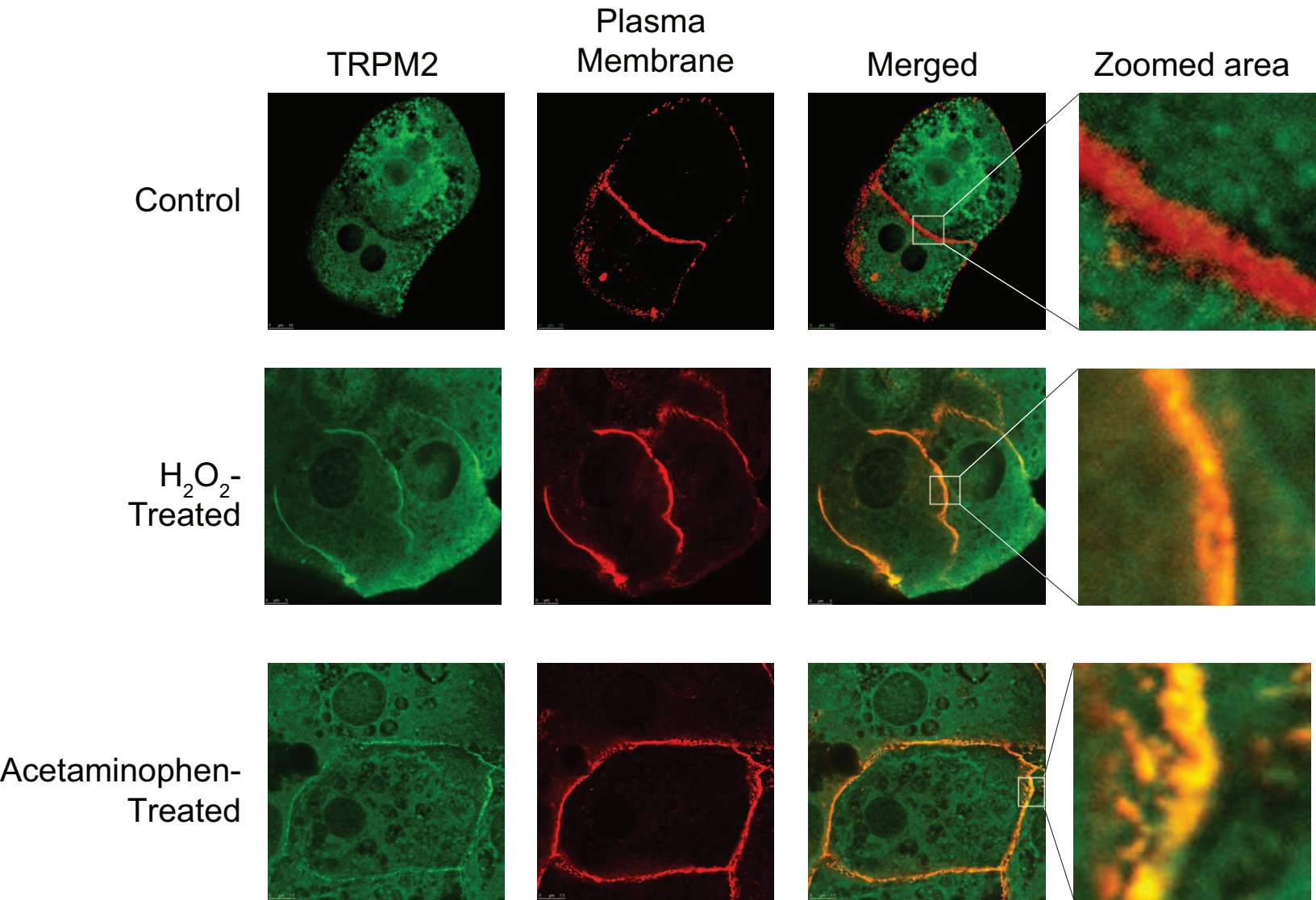

TRPM2
Plasma Membrane
Merged
Zoomed area
Control
H_2O_2-Treated
Acetaminophen-Treated

Figure 3

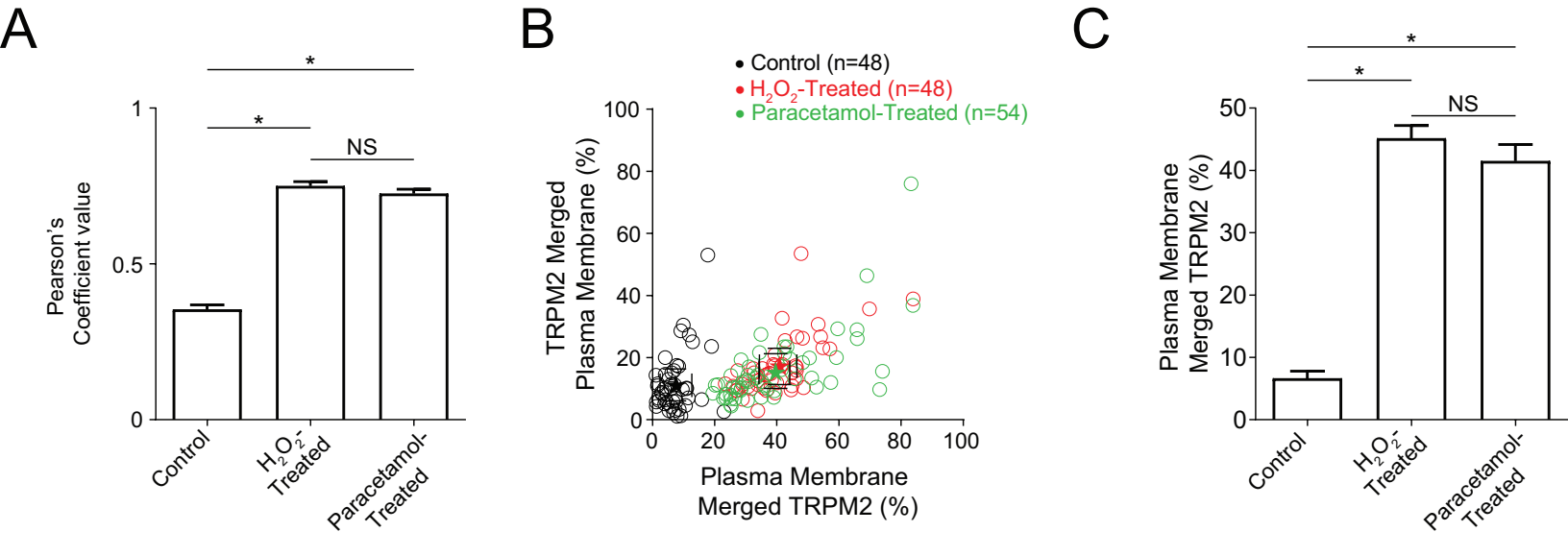
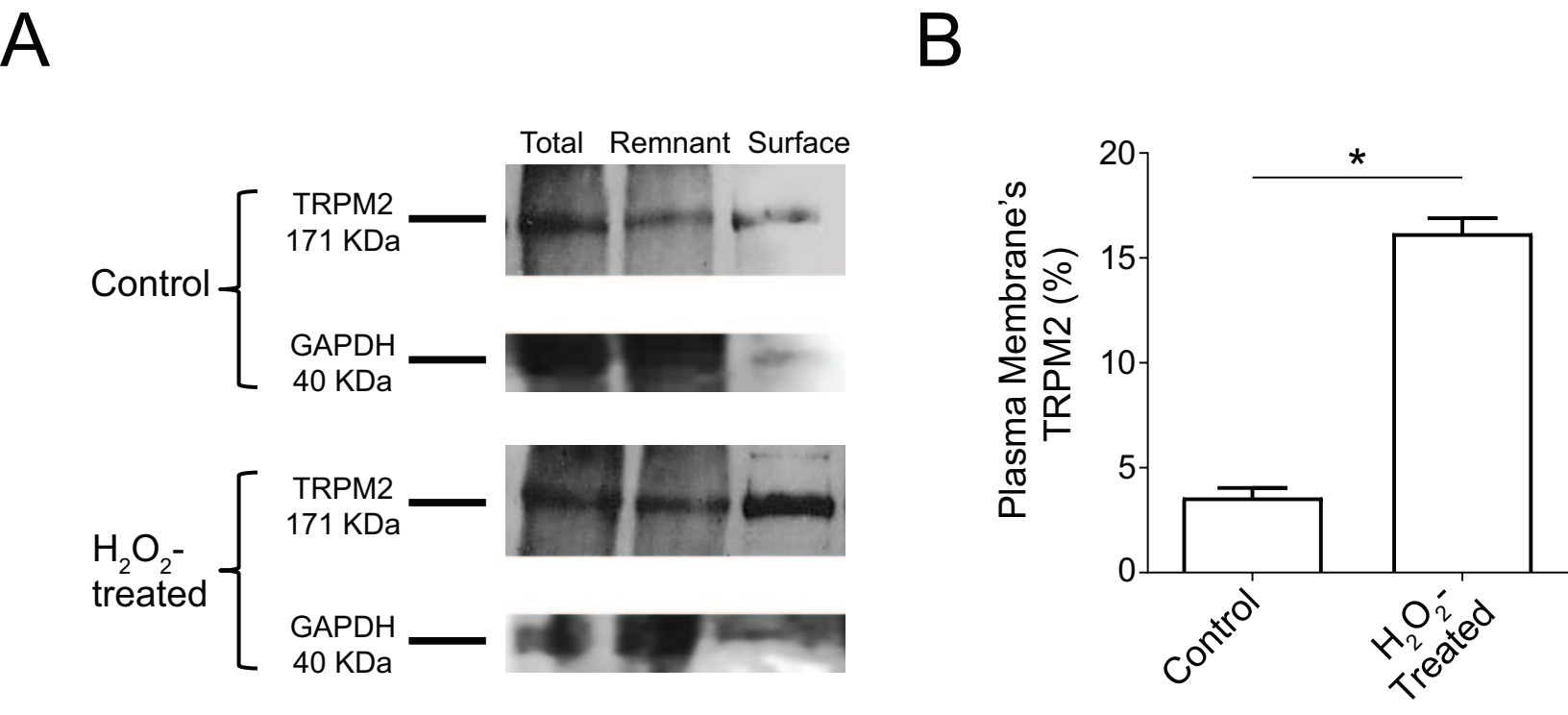
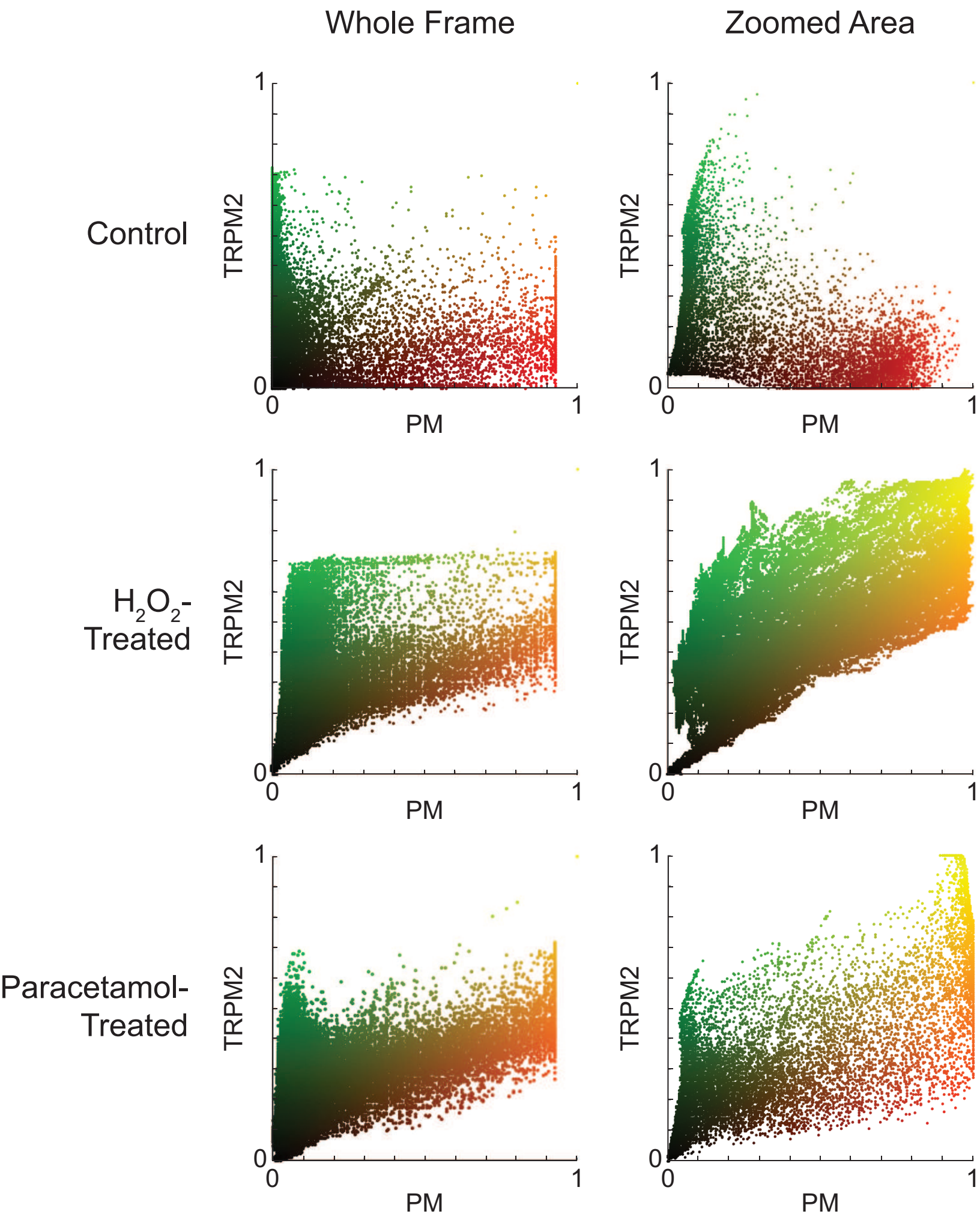

A
*
*
NS
1
0.5
0
Pearson's Coefficient value
Control
H2O2-Treated
Paracetamol-Treated
B
Control (n=48)
H2O2-Treated (n=48)
Paracetamol-Treated (n=54)
100
80
60
40
20
0
TRPM2 Merged Plasma Membrane (%)
0
20
40
60
80
100
Plasma Membrane Merged TRPM2 (%)
C
*
*
NS
50
40
30
20
10
0
Plasma Membrane Merged TRPM2 (%)
Control
H2O2-Treated
Paracetamol-Treated

Figure 4


A
Total
Remnant
Surface
Control
TRPM2
171 KDa
GAPDH
40 KDa
H2O2-
treated
TRPM2
171 KDa
GAPDH
40 KDa
B
Plasma Membrane's
TRPM2 (%)
20
15
10
5
0
*
Control
H2O2-
Treated

Supplimental Figure 1



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***Conflict of Interest**

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