SUPPLEMENTARY INFORMATION

Inhibition of endothelial cell Ca^{2+} entry and Transient Receptor Potential channels by Sigma-1 receptor ligands

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Supporting data for effects of Sig1R ligands on Ca^{2+} signals in SVECs. Data are from intracellular Ca^{2+} measurements. (**a**) Mean data to accompany those in Figure 1d, showing analysis of the peak responses to histamine (n/N=3/12 for each experiment). (**b**, **c**) Example data showing effects of 10 μM histamine in the presence of 100 μM 4-IBP (**b**) or 100 μM SKF10047 (**c**) compared with vehicle controls (N=4 for each). The ligands were applied 30 min before testing histamine and maintained throughout the experiments. (**d**) Example data showing effects of 100 ng/ml VEGF in the presence of 100 μM BD1063 compared with the vehicle control. BD1063 was applied for 30 min before testing of VEGF and was maintained throughout the experiment. (**e**) Mean data for the effects of Sig1R ligands (100 μM) on the rate of rise of the VEGF response (n/N=3/12 for each), from the same experiments analysed in Figure 1e. (**f**) Dependence of the response to H_2O_2 on extracellular Ca^{2+}. Typical experiment showing 1 mM H_2O_2 responses in the presence (1.5 Ca^{2+}) or absence (0 Ca^{2+}) of 1.5 mM extracellular Ca^{2+} (N=4 for each). (**g**) Example data showing effects of 1 mM hydrogen peroxide (H_2O_2) in the presence of 100 μM BD1063 compared with the vehicle control.

Supplementary Figure 2. Lack of effect of Sig1R ligands on thapsigargin-evoked Ca^{2+}-release in SVECs. Data were generated by measurement of intracellular Ca^{2+} in SVECs. (**a-d**) Example data showing effects of 1 μM thapsigargin in the absence of extracellular Ca^{2} (0 Ca^{2+}) but the presence of 100 μM BD1047 (**a**), 100 μM BD1063 (**b**), 100 μM 4-IBP (**c**) or 100 μM SKF10047 (**d**) compared with vehicle controls (N=4 for each). The compounds were applied 30 min before testing of thapsigargin and were maintained throughout the experiments. (**e**) Mean data for the types of experiment illustrated in (**a-d**), showing analysis of the peak responses to thapsigargin (n/N=3/14). 4-IBP data are shown separately because of the use of a different vehicle control in these experiments.

Supplementary Figure 3. Effects of Sig1R ligands on TRPC5, TRPM2 and TRPM3 channels in HEK 293 cells. (**a**) Example Ca^{2+} measurement data for TRPC5 cells, showing responses to 10 μM LPC in the presence of 100 μM BD1047 or the vehicle control (N=6 for each). Also shown is the lack of effect of Gd^{3+} in cells lacking TRPC5 expression (Tet-). (**b**) Mean data of the type shown in Figure 2b but for maximum responses to 5 μM sphingosine-1-phosphate (S1P) (n/N=3/18 for each condition). (**c**) Example Ca^{2+} measurement data for TRPM2 cells, showing responses to 1 mM H_2O_2 in the presence of 100 μM BD1047 or the vehicle control (N=8 for each). (**d**) Whole-cell voltage-clamp data from TRPM3 cells showing current-voltage relationships (I-Vs) for the experiment shown in Figure 3d.

Supplementary Figure 4. Absence of stimulatory effects of Sig1R ligands on TRPC5 or TRPM3. (**a**) Data were generated by Ca^{2+} measurement from HEK 293 cells induced to express TRPC5 (Tet+) or not induced to express TRPC5 (Tet-). Cells were exposed to 100 μM Gd^{3+} (n/N=4/36) (a positive control), 100 μM SKF10047, 100 μM 4-IBP, 100 μM BD1063 or 100 μM BD1047 (n/N=3/18). (**b**) Data were generated by Ca^{2+} measurement from HEK 293 cells induced to express TRPM3 (Tet+) or not induced to express TRPM3 (Tet-). Cells were exposed to 5 μM PregS (n/N=4/32) (a positive control), 100 μM SKF10047, 100 μM 4-IBP, 100 μM BD1063 or 100 μM BD1047 (n/N=3/18).

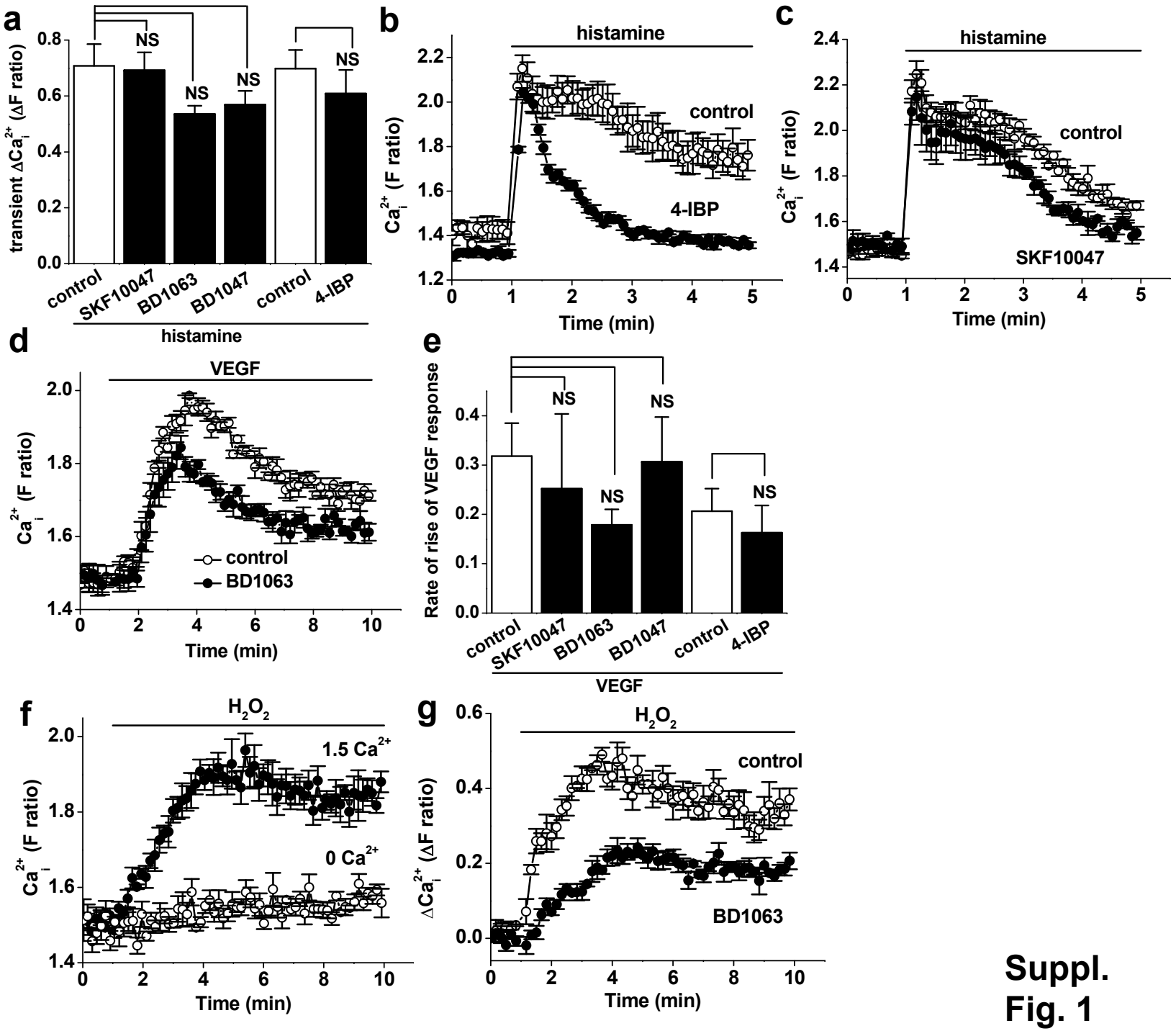
Supplementary Figure 5. Validation of Sig1R depletion. (**a**) PCR products from SVECs transfected with scrambled siRNA (sc.si) or Sig1R siRNA (Sig1R.si). Expected product sizes were 196 (β-actin, i) and 222 (Sig1R, ii) base pairs (bp). The 100 bp DNA marker ladder is on the left (M) and an example Sig1R PCR reaction without reverse transcriptase (-RT) is on the right. (**b**) As for (**a**) but mRNA was isolated from HEK 293 cells. (**c**) Western blot with anti-Sig1R antibody for HEK 293 cell proteins after transfection with scrambled (control) siRNA (sc.si) or Sig1R siRNA (Sig1R.si).

Supplementary Figure 6. Supporting data suggesting lack of functional role of the Sig1R. (**a**) Mean Ca^{2+} measurement data for effects of sc.si (control) and Sig1R siRNA (Sig1R.si) on 10 μM histamine-evoked signals (n/N=3/12). (**b**) Mean data for the effect of 50 μM BD1063 (30 min treatment) on 10 μM histamine-evoked Ca^{2+} signals in SVECs transfected with sc.si or Sig1R.si (n/N=3/12 for each data set). Data are shown for the peak response to histamine and the response 5 min after the application of histamine. (**c**, **d**) Example Ca^{2+} measurement data showing 10 μM LPC-evoked TRPC5 activity (**c**, N=5) and 5 μM PregS-evoked TRPM3 activity (**d**, N=4) in HEK 293 cells transfected with sc.si or Sig1R.si. (**e**) Mean data for the maximum responses to 5 μM PregS in TRPM3 cells treated with sc.si or Sig1R.si in the presence of 100 μM BD1047 or vehicle control (n/N=3/16 for each condition).

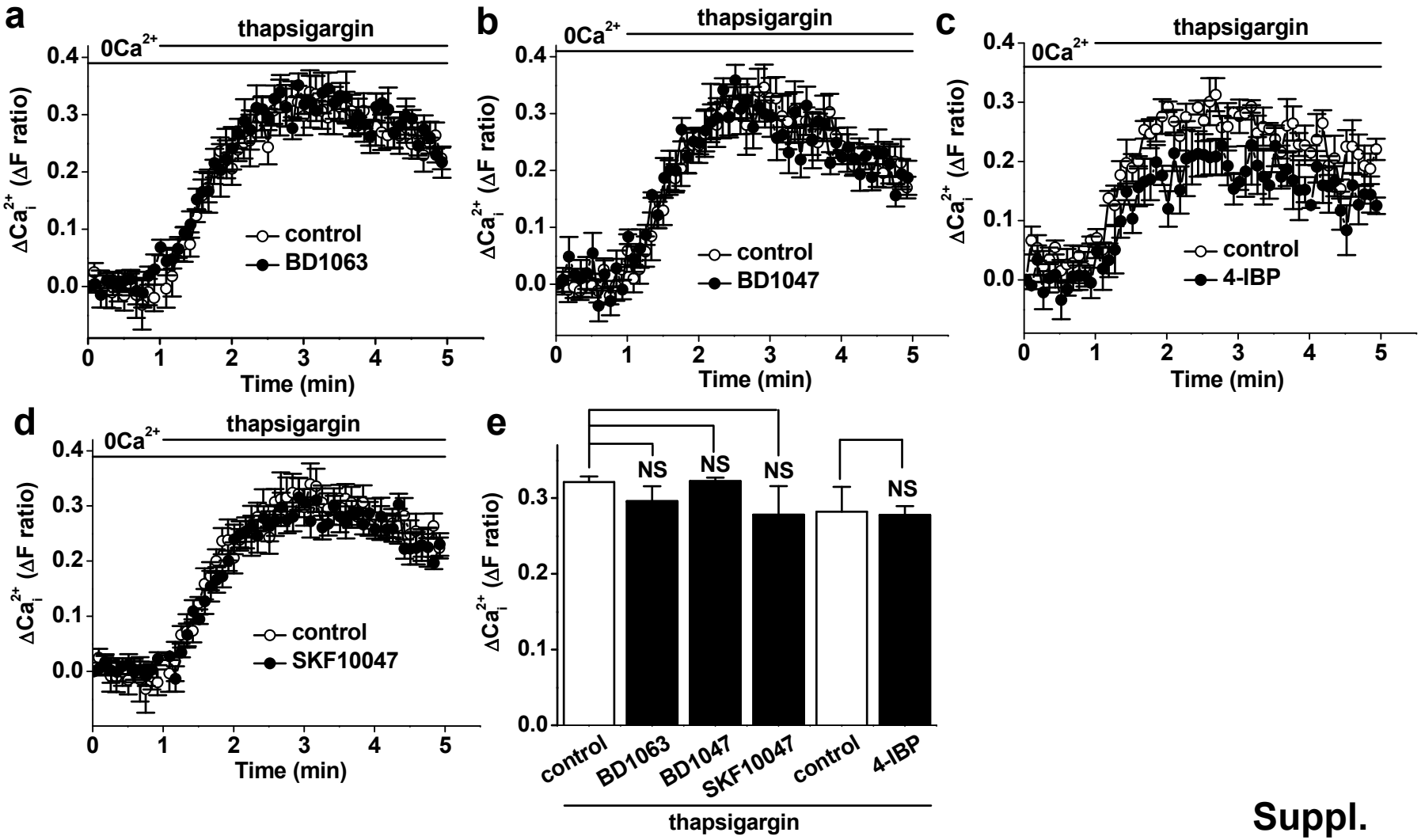
Supplementary Figure 7. Supporting data for Sig1R staining and localisation of TRPC5-GFP and TRPM3-YFP channels. (**a**) Peptide-specificity of endogenous Sig1R detection by anti-Sig1R antibody. Example images are shown of HEK 293 cells exposed to anti-Sig1R antibody (upper image) or anti-Sig1R antibody preadsorbed to its antigenic peptide (lower image). Positive labeling is shown by the red of the secondary antibody. The blue is from DAPI, the counter nuclear stain. (**b**, **c**) Images show typical non-induced HEK 293 cells transiently expressing TRPC5-GFP (**b**) or TRPM3-YFP (**c**). Fluorescence from the GFP or YFP is shown in

green. Cells were stained with anti-Sig1R antibody (red). Cells were pre-treated with 100 μM SKF10047 (**b**) or BD1047 (**c**).

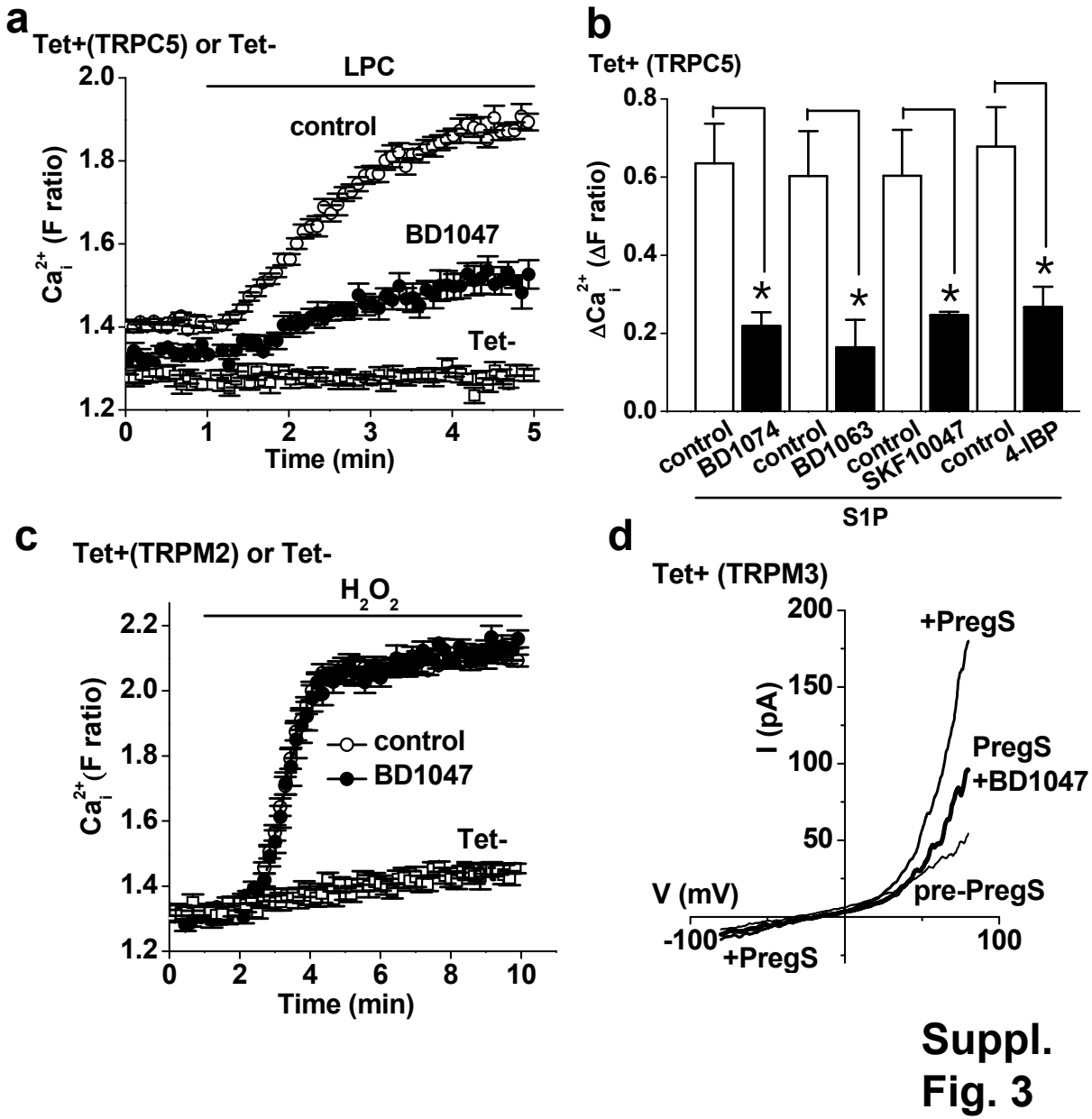
Supplementary Figure 8. Comparison of the chemical structures of BD1063, 4-IBP, BD1047, resveratrol, and diethylstilbestrol. Basic nitrogens are highlighted in red. (**a**) The lowest energy conformations of BD1063 (1) and 4-IBP (2) (chair, all equatorial) show a common structural motif, which is represented by preliminary pharmacophore model **3**. (**b**) BD1047 (4) can be considered a ring-opened analogue of BD1063 (1) with significant conformational freedom, and one of its possible conformations is highly similar to that of BD1063. Being an analogue of the common metal chelator tetramethylethylenediamine (TMEDA, 6), BD1047 might also act as a ligand for metal ions (M^{n+}) such as Mg^{2+}, Zn^{2+}, Cu^{2+} and Ca^{2+} (5) in the same way as TMEDA (7). (**c**) In its lowest energy conformation, resveratrol (8) is flat because of conjugation of all π bonds. The ethyl groups of diethylstilbestrol (9) force rotation of its aromatic groups by ca. 90°, thereby preventing full conjugation. Thus, despite sharing a hydroxylated stilbene scaffold, resveratrol and diethylstilbestrol have markedly different 3D structures in their lowest energy conformations.


a
transient ΔCa$_i^{2+}$ (ΔF ratio)
NS
control
SKF10047
BD1063
BD1047
control
4-IBP
histamine
b
histamine
control
4-IBP
Ca$_i^{2+}$ (F ratio)
Time (min)
c
histamine
control
SKF10047
Ca$_i^{2+}$ (F ratio)
Time (min)
d
VEGF
control
BD1063
Ca$_i^{2+}$ (F ratio)
Time (min)
e
Rate of rise of VEGF response
NS
control
SKF10047
BD1063
BD1047
control
4-IBP
VEGF
f
H$_2$O$_2$
1.5 Ca^{2+}
0 Ca^{2+}
Ca$_i^{2+}$ (F ratio)
Time (min)
g
H$_2$O$_2$
control
BD1063
ΔCa$_i^{2+}$ (ΔF ratio)
Time (min)

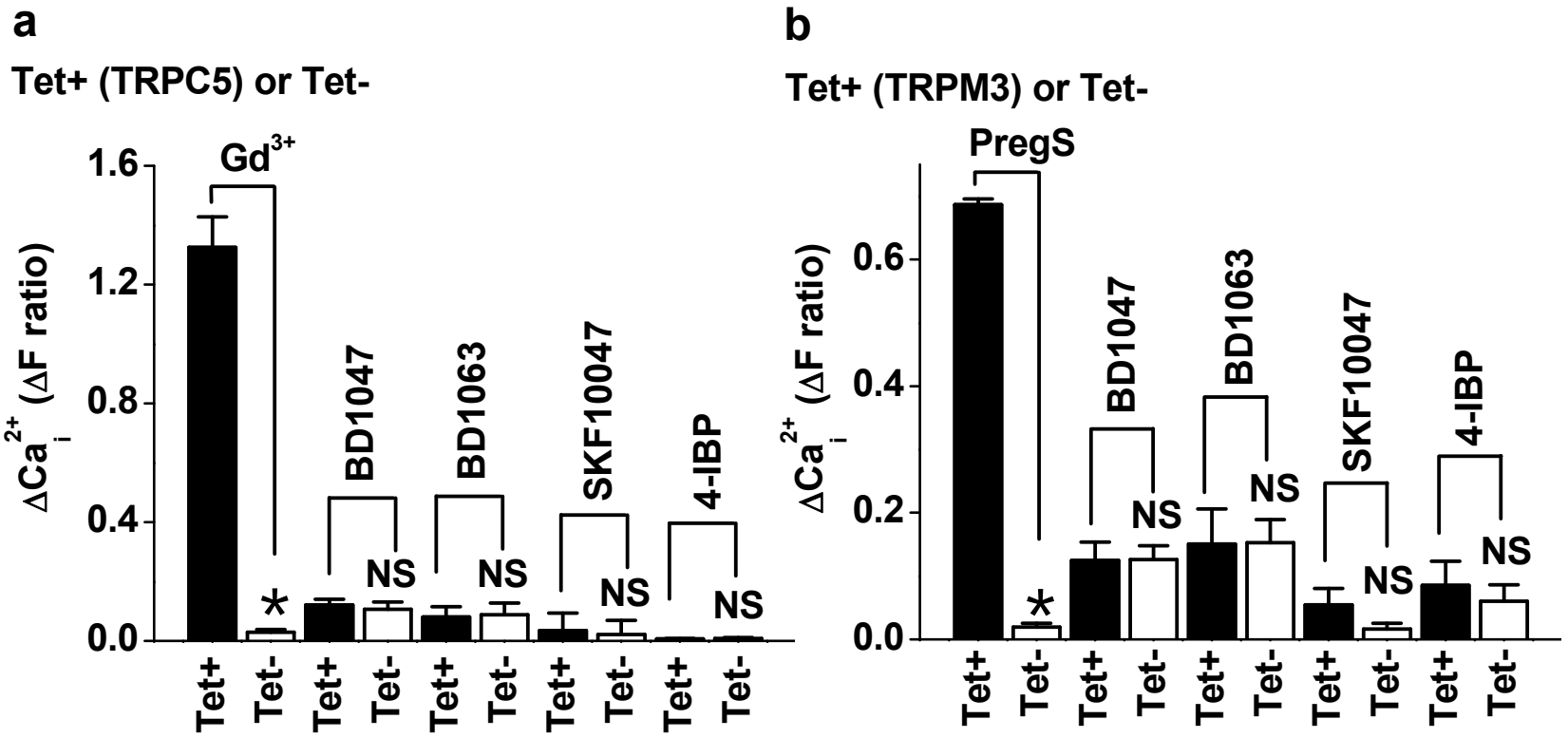
Suppl. Fig. 1


a
0Ca2+
thapsigargin
ΔCai2+ (ΔF ratio)
Time (min)
control
BD1063
b
BD1047
c
4-IBP
d
SKF10047
e
NS
control
BD1063
BD1047
SKF10047
4-IBP

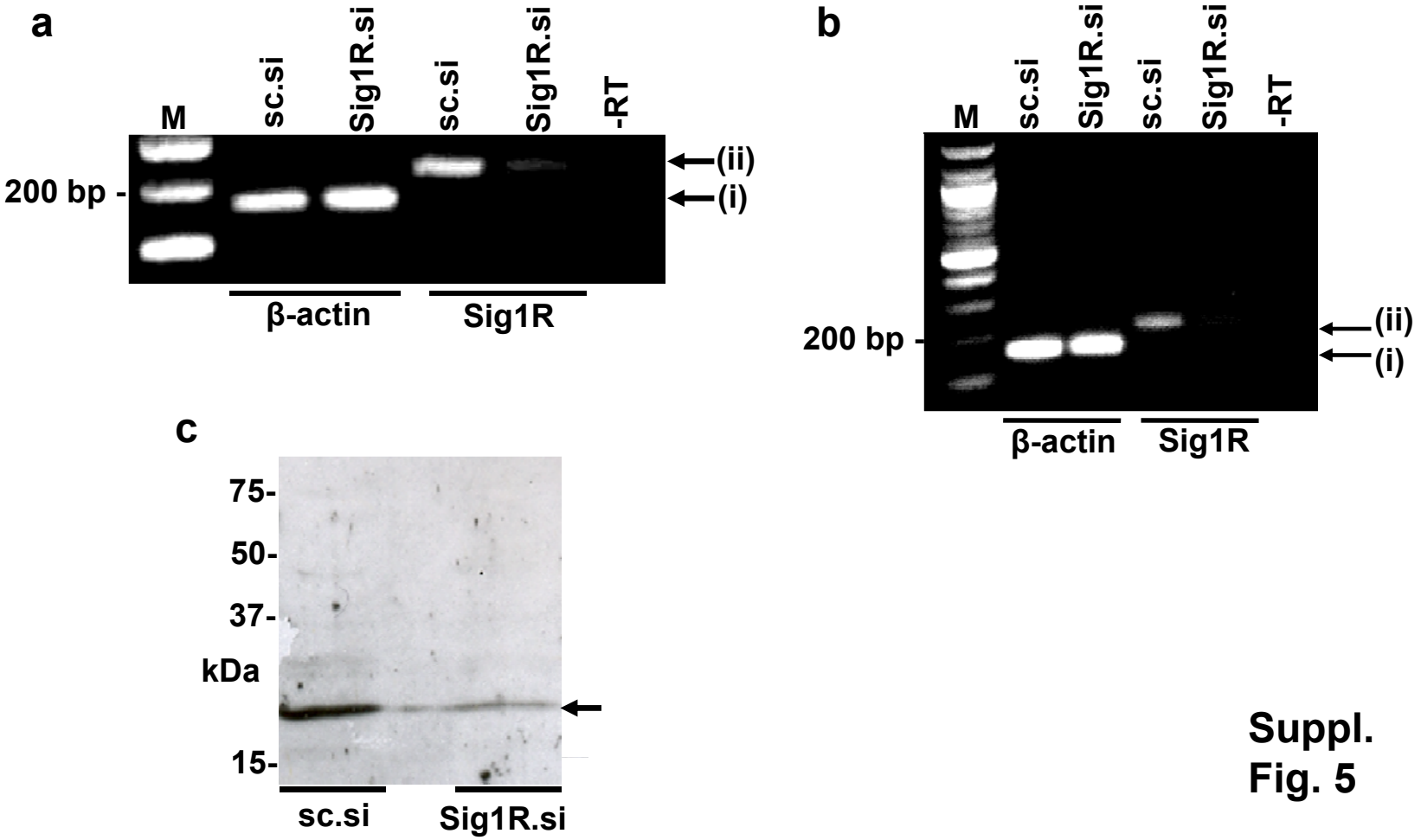
Suppl. Fig. 2


a
Tet+(TRPC5) or Tet-
LPC
control
BD1047
Tet-
Ca_i^{2+} (F ratio)
Time (min)
b
Tet+ (TRPC5)
ΔCa_i^{2+} (ΔF ratio)
control
BD1074
control
BD1063
control
SKF10047
control
4-IBP
S1P
c
Tet+(TRPM2) or Tet-
H_2O_2
control
BD1047
Tet-
Ca_i^{2+} (F ratio)
Time (min)
d
Tet+ (TRPM3)
+PregS
PregS
+BD1047
pre-PregS
I (pA)
V (mV)
-100
100
+PregS

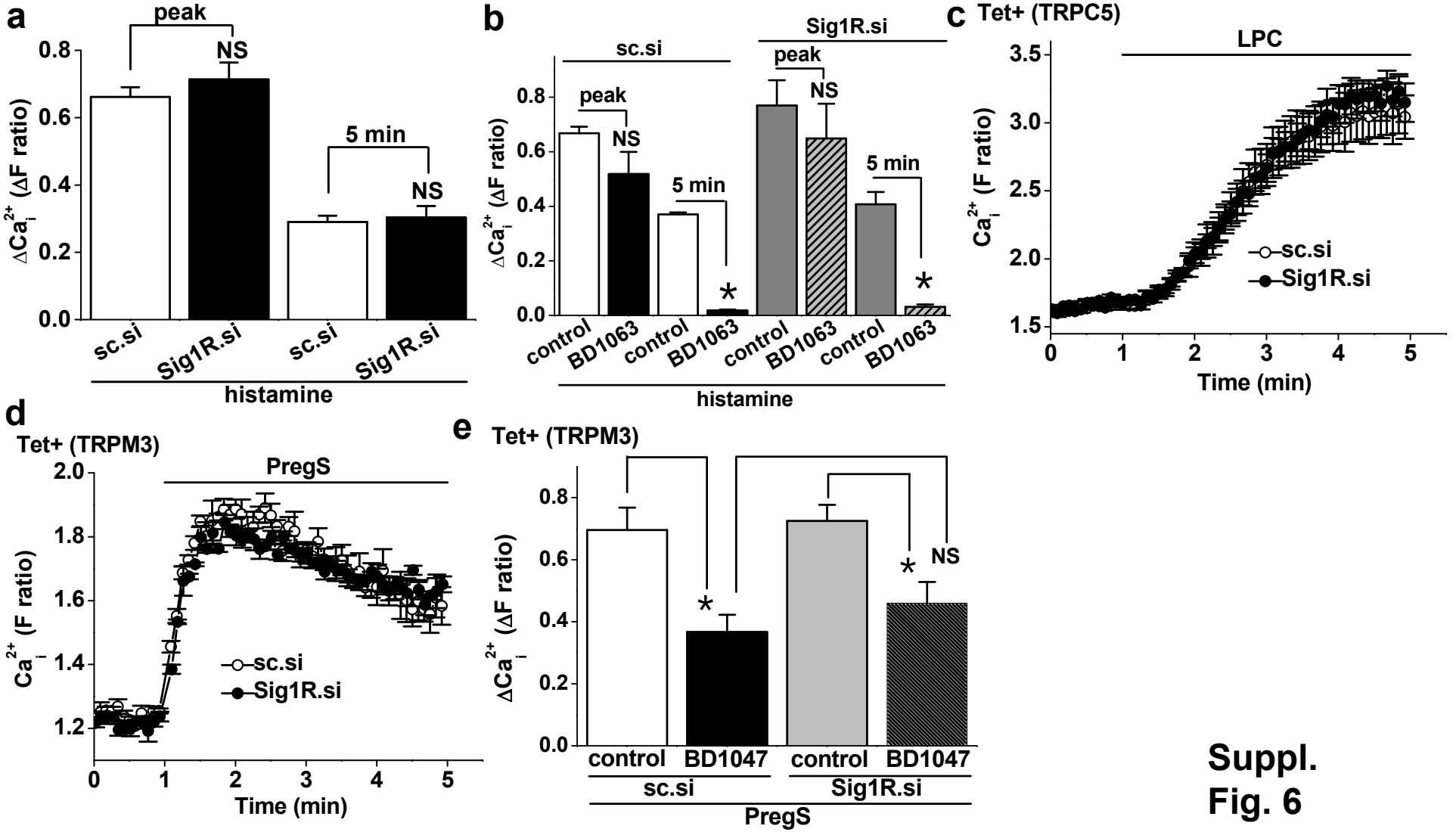
Suppl. Fig. 3


a
Tet+ (TRPC5) or Tet-
ΔCa2+i (ΔF ratio)
1.6
1.2
0.8
0.4
0.0
Gd3+
*
BD1047
BD1063
SKF10047
4-IBP
NS
Tet+
Tet-
b
Tet+ (TRPM3) or Tet-
PregS
0.6
0.4
0.2
0.0

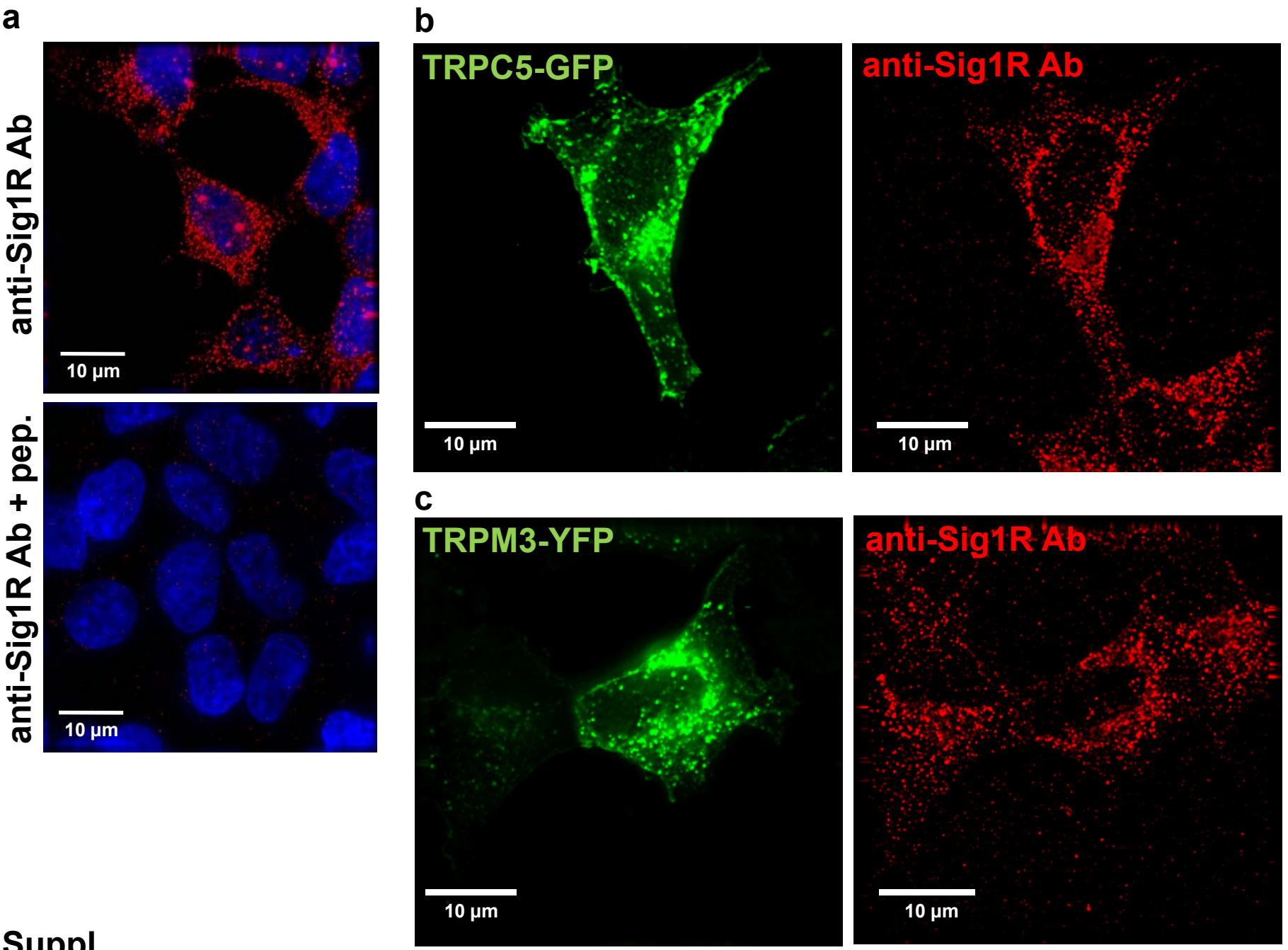
Suppl. Fig. 4


a
M
sc.si
Sig1R.si
sc.si
Sig1R.si
-RT
200 bp
(ii)
(i)
β-actin
Sig1R
b
M
sc.si
Sig1R.si
sc.si
Sig1R.si
-RT
200 bp
(ii)
(i)
β-actin
Sig1R
c
75-
50-
37-
kDa
15-
sc.si
Sig1R.si

Suppl. Fig. 5

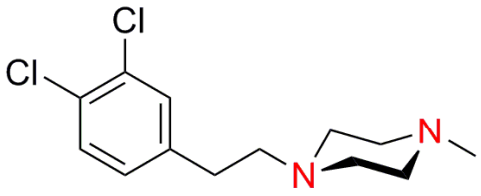

a
peak
NS
5 min
NS
0.8
0.6
0.4
0.2
0.0
ΔCa$_i^{2+}$ (ΔF ratio)
sc.si
Sig1R.si
sc.si
Sig1R.si
histamine
b
sc.si
Sig1R.si
peak
NS
5 min
peak
NS
5 min
*
*
ΔCa$_i^{2+}$ (ΔF ratio)
control
BD1063
control
BD1063
control
BD1063
control
BD1063
histamine
c
Tet+ (TRPC5)
LPC
3.5
3.0
2.5
2.0
1.5
Ca$_i^{2+}$ (F ratio)
sc.si
Sig1R.si
0
1
2
3
4
5
Time (min)
d
Tet+ (TRPM3)
PregS
2.0
1.8
1.6
1.4
1.2
Ca$_i^{2+}$ (F ratio)
sc.si
Sig1R.si
0
1
2
3
4
5
Time (min)
e
Tet+ (TRPM3)
0.8
0.6
0.4
0.2
0.0
ΔCa$_i^{2+}$ (ΔF ratio)
*
*
NS
control
BD1047
control
BD1047
sc.si
Sig1R.si
PregS

Suppl. Fig. 6


a
anti-Sig1R Ab
10 µm
anti-Sig1R Ab + pep.
10 µm
b
TRPC5-GFP
10 µm
anti-Sig1R Ab
10 µm
c
TRPM3-YFP
10 µm
anti-Sig1R Ab
10 µm

Suppl. Fig. 7

a



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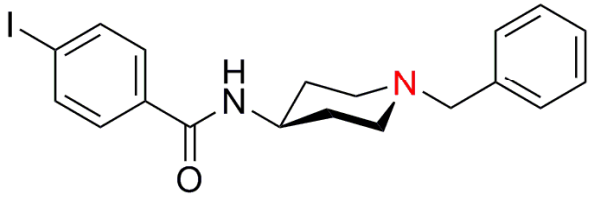

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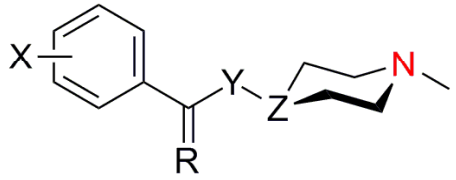
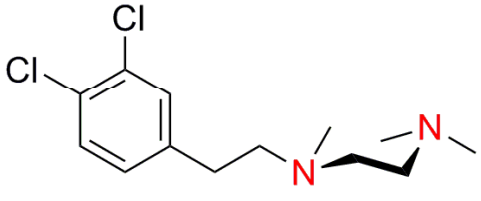


3 X = halogen; Y = C or N;
Z = N or C; R = H_2 or O

b



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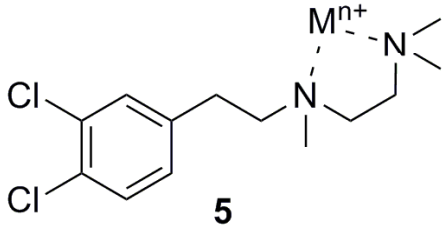

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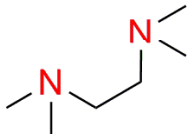

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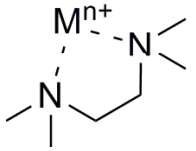
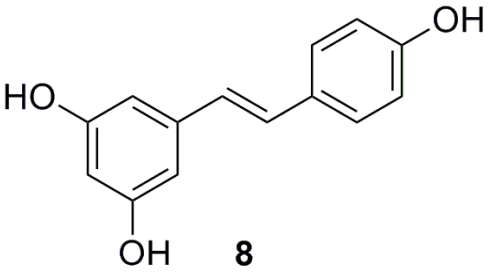


7

c



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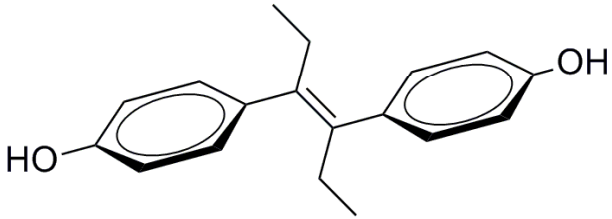

9

Suppl. Fig. 8