Supplementary information

Estradiol/GPER affects the integrity of mammary duct-like structures *in vitro*

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Supplementary figure legends

Supplementary Fig. 1 A 3D model of the milk duct using MCF-10A cells. **a** Representative confocal images of MCF-10A acini. MCF-10A cells were cultured on Matrigel for 2 weeks and immunostained with an antibody against pan-cadherin (green) to detect cell junctions as well as an antibody against the centriole (red) to illustrate its apical orientation to the hollow lumen of the acini. Hoechst-stained (blue) cells represent the reconstructed image of the acini structure (first row); a continuous confocal section (1–3) through the acinus is shown in the middle. **b** Representative images of normal breast tissue stained with pan-cadherin (green), centriole (red), and Hoechst (blue). Scale bars = 10 μm. **c** Representative confocal images of MCF-10A cell ($p53^{-/-}$) acini treated with (up) or without (bottom) 32 nM estradiol for 14 days and immunostained with laminin V (red) to delineate basement membrane secretion and pan-cadherin (green) to show the cell junctions. Blue, Hoechst staining. Scale bars = 10 μm.

Supplementary Fig. 2 Inhibition of E2-dependent signaling by siRNA-GPER. **a** cAMP assay of cAMP levels (nM) in MCF-10A cells following treatment with 32 nM E2-Glow or 17a-estradiol for 30 min. Five independent experiments were performed. Bars

represent +/-SD. **b** Western blot analysis of MCF-10A cells showing GPER expression after transfection with Accell siRNA-GPER. **c** cAMP assay of cAMP levels (nM) in siRNA-GPER transfected-MCF-10A cells or siRNA-control transfected cells following treatment with 32 nM E2 for 30 min. Five independent experiments were performed. Bars represent +/-SD. DATA were analyzed using a Mann-Whitney *U* test. *p values less than 0.05 were considered statistically significant. **d** Western blot analysis of MCF-10A cells showing phospho-p38 (Thr180/Tyr182) and p38 after treated with 17a-estradiol (32nM) and E2-Glow (32nM). **e** Representative confocal images of MCF-10A cells in 3D culture through the middle acini, which were treated with 17α-estradiol (32 nM,) or E2-Glow (32 nM) for 7 days. Laminin V (red); pan-cadherin (green). Scale bars = 10 μm. **f** Western blot analysis of MCF-10A cells showing GPER expression after transfection with siRNA-GPER. **g** Western blotting of MCF-10A cells showing phospho-p38 (Thr180/Tyr182) and p38 following treatment with 32 nM E2 and transfection with siRNA-control (left panel) or with 32 nM E2 and transfection with siRNA-GPER (right panel). **h** Western blotting of MCF-10A cells showing phospho-JNK (Thr183/Tyr185) and JNK after treatment with 32 nM E2 and transfection with siRNA-control (left panel) or with 32 nM E2 and transfection with siRNA-GPER (right panel). **i** Western blotting of MCF-7 cells treated with 32 nM E2 for 0–60 min showing phospho-p38 (Thr180/Tyr182) and p38. **j** Western

blotting of MCF-7 cells showing JNK and phospho-JNK (Thr183/Tyr185) following treatment with 32 nM E2 for 0–60 min. The presented blots were cropped. Full-length blots are presented in Supplementary Fig. 6.

Supplementary Fig. 3 Induction of the time-dependent IL-1β expression and pyroptosis following E2 exposure. **a** Western blotting of siRNA-MMP-3-transfected MCF-10A cells showing pro-MMP-3 expression after treating with E2 (32 nM) for 24 h. **e** Representative images of si-RNA control-transfected or siRNA GPER-transfected MCF-10A cells treated with E2 (32 nM).Green staining, pan-cadherin; blue staining, Hoechst. Scale bars = 20 μm. **b** IL-1β ELISA of MCF-10A cells examined for the concentration of secreted IL-1β in the cell culture media after treating the cells with 32 nM E2 for 0–120 h. Four independent experiments were performed. Bars represent +/-SD. **c** The caspase-1 inflammasome assay was used to measure caspase-1 activity in MCF-10A cells after adding 2 nM E2 for 24 h. YVAD-CHO was used as a caspase-1 inhibitor. Three independent experiments were performed. Bars represent +/-SD. DATA were analyzed using a Mann-Whitney *U* test. *p values less than 0.05 were considered statistically significant. **d** Pyroptotic bodies were detected by phase-contrast microscopy 26 h after the addition of E2. The pyroptotic bodies are shown in the square. The presented blots

were cropped. Full-length blots are presented in Supplementary Fig. 7.

Supplementary Fig. 4 Detection of the interaction between GST-IL-1β and IL-1R by immunostaining. Representative confocal images of MCF-10A cells showing the colocalization of added GST-IL-1β and IL-1R by immunofluorescence staining using GST (green) and IL-1R (red) antibodies. The white line delineates the secretion of the cell nucleus. Yellow shows the colocalization of GST-IL-1β and IL-1R. Fluorescence intensity profiles along the lines were drawn in the staining patterns. GST-IL-1β and IL-1R were closely merged. Scale bars = 10 μm.

Supplementary Fig. 5

Original full length blot of figures 1d, 1g, and 2b-2g. Boxes indicated areas shown in the figures.

Supplementary Fig. 6

Original full length blot of figures supplementary 2b, 2d, and 2f-2j. Boxes indicated areas shown in the figures.

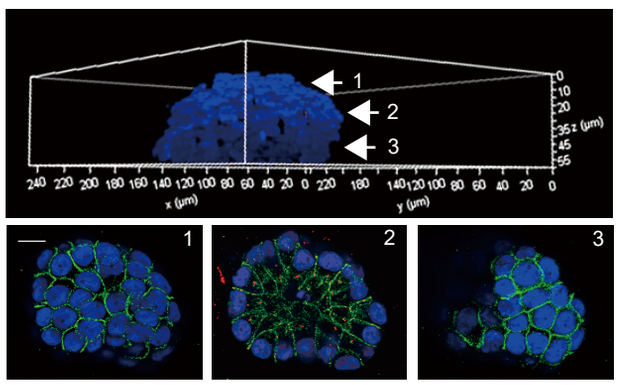
Supplementary Fig. 7

Original full length blot of figures 3a, 4f, 4g, 5b-5f, and supplementary 3a. Boxes indicated areas shown in the figures.

Supplementary Fig. 8

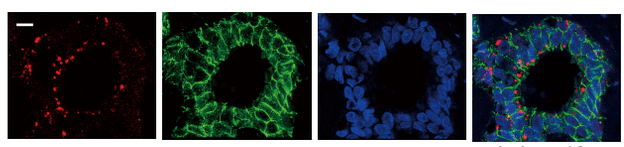
Original full length blot of figure 6c. Boxes indicated areas shown in the figure.

a



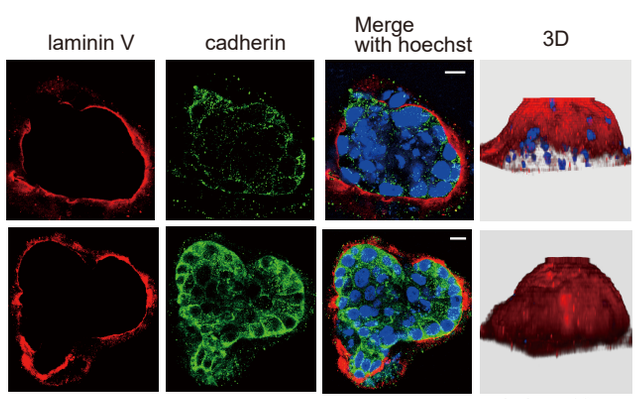
scale bar: 10 µm

b



pan-cadherin centriole hoechst scale bar: 10 µm

c



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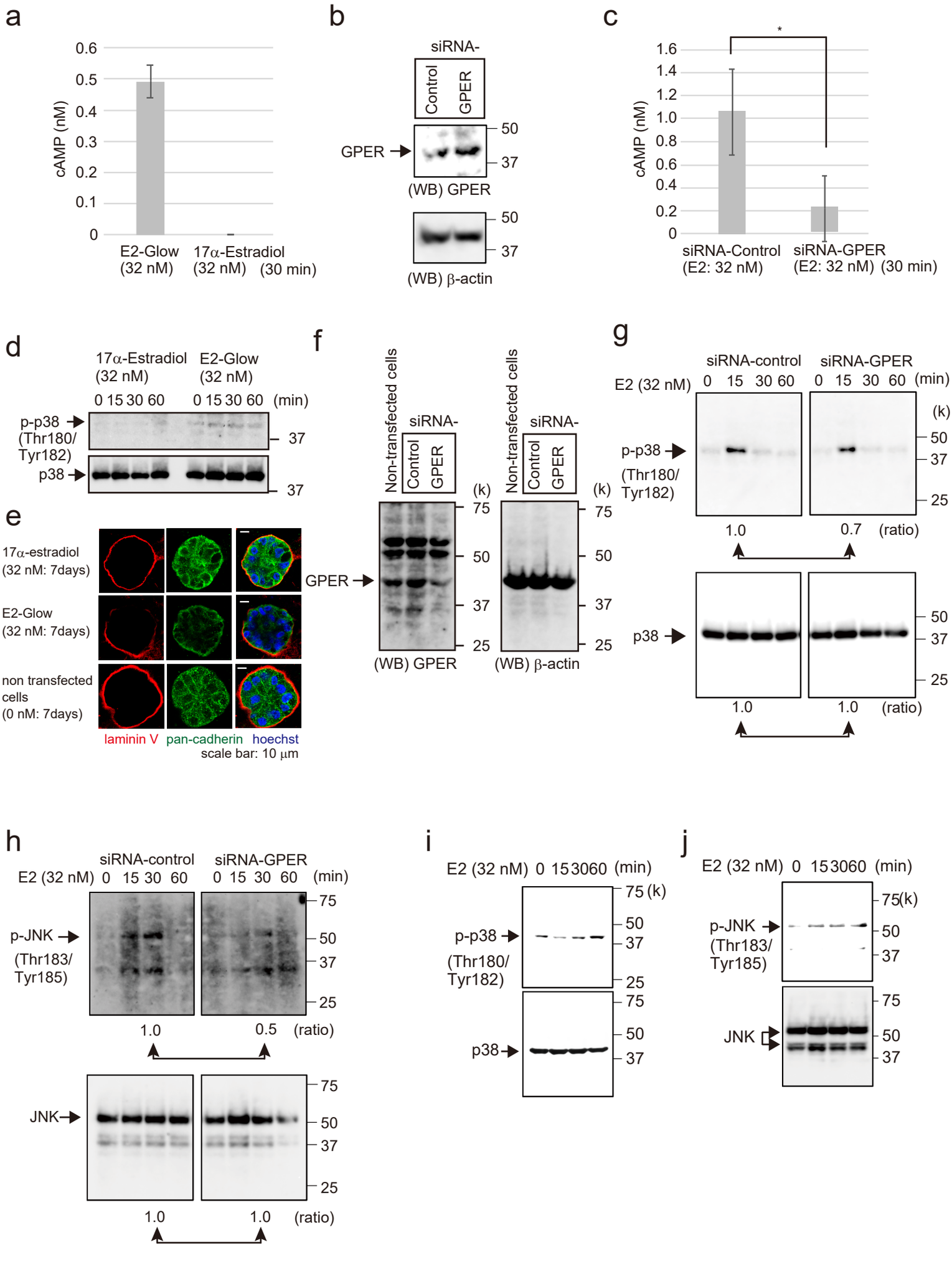
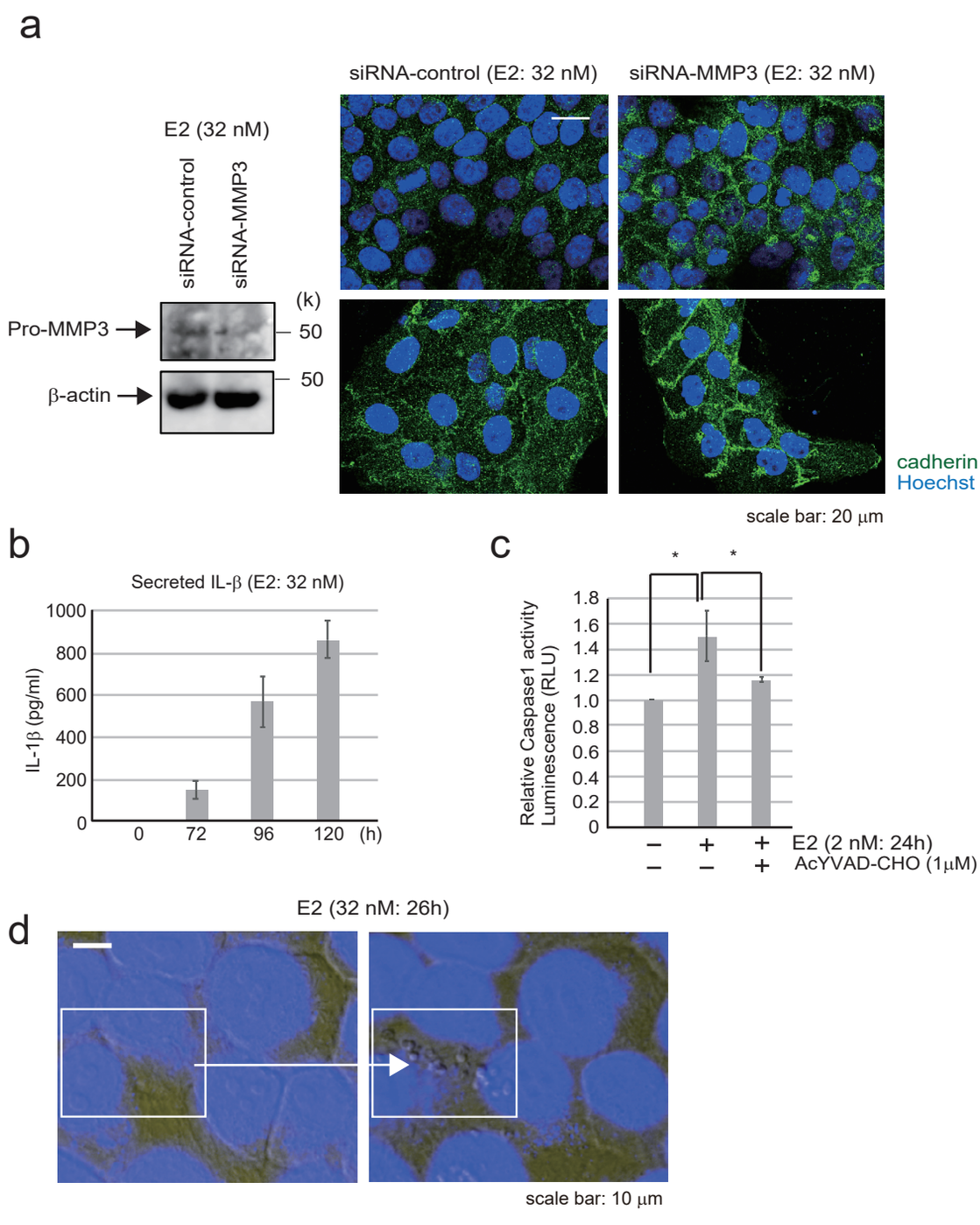

a
cAMP (nM)
0.6
0.5
0.4
0.3
0.2
0.1
0
E2-Glow (32 nM)
17α-Estradiol (32 nM)
(30 min)
b
siRNA-
Control
GPER
GPER
50
37
(WB) GPER
50
37
(WB) β-actin
c
*
cAMP (nM)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
siRNA-Control (E2: 32 nM)
siRNA-GPER (E2: 32 nM)
(30 min)
d
17α-Estradiol (32 nM)
E2-Glow (32 nM)
0 15 30 60
0 15 30 60
(min)
p-p38 (Thr180/ Tyr182)
p38
37
37
e
17α-estradiol (32 nM: 7days)
E2-Glow (32 nM: 7days)
non transfected cells (0 nM: 7days)
laminin V
pan-cadherin
hoechst
scale bar: 10 μm
f
Non-transfected cells
siRNA-
Control
GPER
(k)
75
50
37
25
GPER
(WB) GPER
(WB) β-actin
g
siRNA-control
siRNA-GPER
E2 (32 nM)
0 15 30 60
0 15 30 60
(min)
(k)
p-p38 (Thr180/ Tyr182)
50
37
25
1.0
0.7
(ratio)
p38
50
37
25
1.0
1.0
(ratio)
h
siRNA-control
siRNA-GPER
E2 (32 nM) 0 15 30 60
0 15 30 60
(min)
p-JNK (Thr183/ Tyr185)
75
50
37
25
1.0
0.5
(ratio)
JNK
75
50
37
25
1.0
1.0
(ratio)
i
E2 (32 nM) 0 15 30 60 (min)
75 (k)
p-p38 (Thr180/ Tyr182)
50
37
25
p38
75
50
37
j
E2 (32 nM) 0 15 30 60 (min)
75(k)
p-JNK (Thr183/ Tyr185)
50
37
JNK
75
50
37


a
siRNA-control (E2: 32 nM)
siRNA-MMP3 (E2: 32 nM)
E2 (32 nM)
siRNA-control
siRNA-MMP3
(k)
Pro-MMP3
50
β-actin
50
cadherin
Hoechst
scale bar: 20 μm
b
Secreted IL-β (E2: 32 nM)
IL-1β (pg/ml)
1000
800
600
400
200
0
0
72
96
120
(h)
c
Relative Caspase1 activity
Luminescence (RLU)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
E2 (2 nM: 24h)
AcYVAD-CHO (1μM)
d
E2 (32 nM: 26h)
scale bar: 10 μm

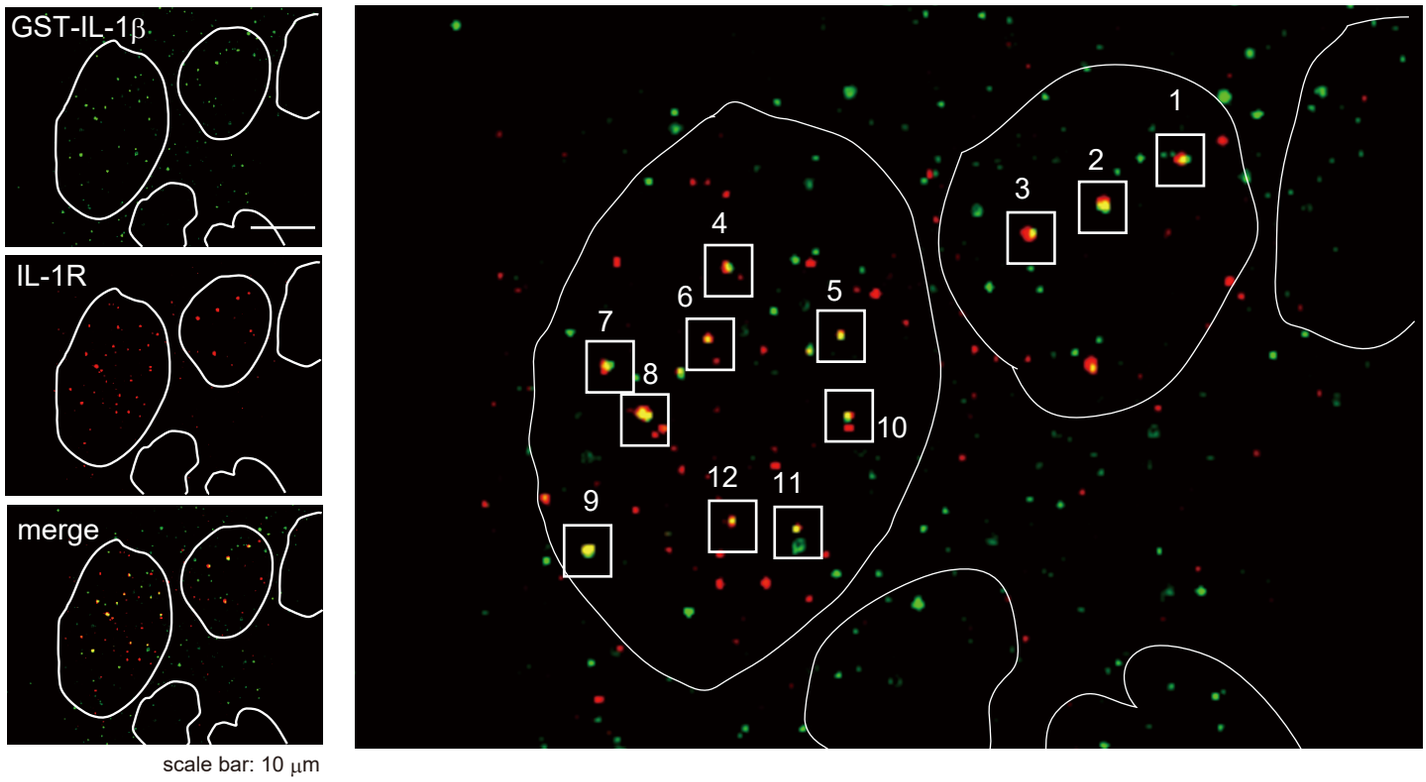

GST-IL-1β
IL-1R
merge
1
2
3
4
5
6
7
8
9
10
11
12
scale bar: 10 μm
a
b
300
800
350
500
700
600
0

Fig. 1d

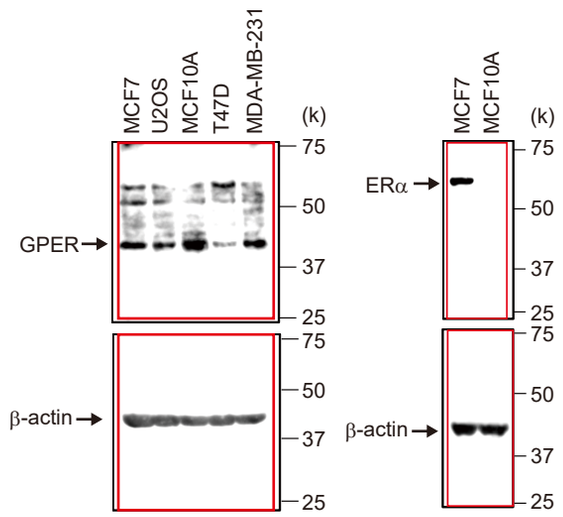


Fig. 1g

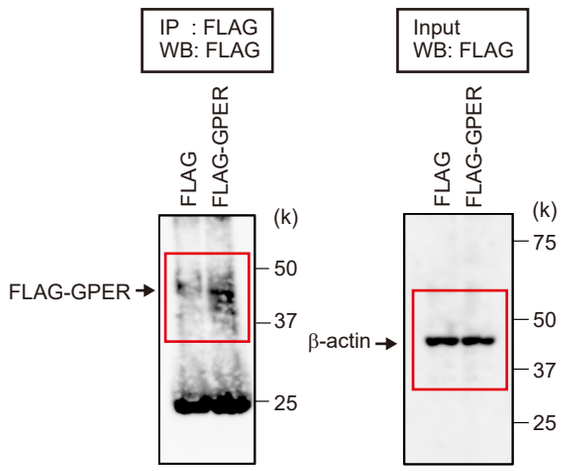
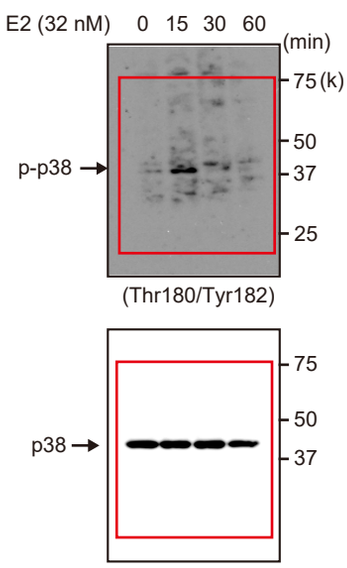


Fig. 2b



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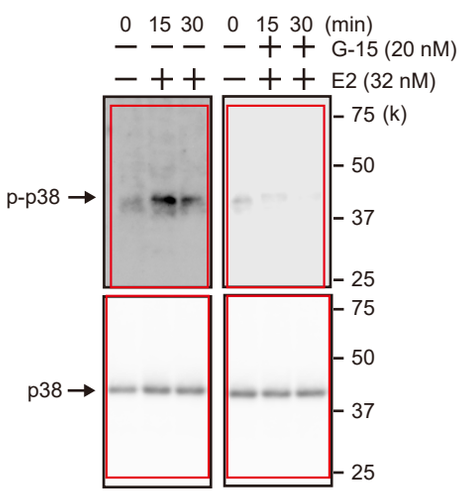
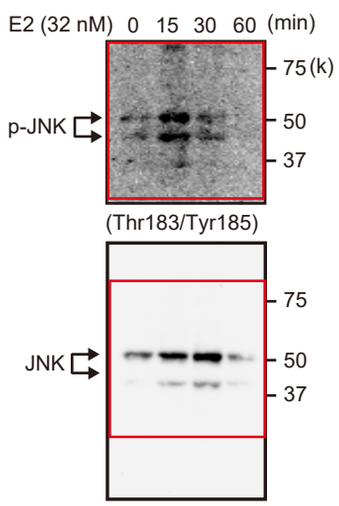


Fig. 2d



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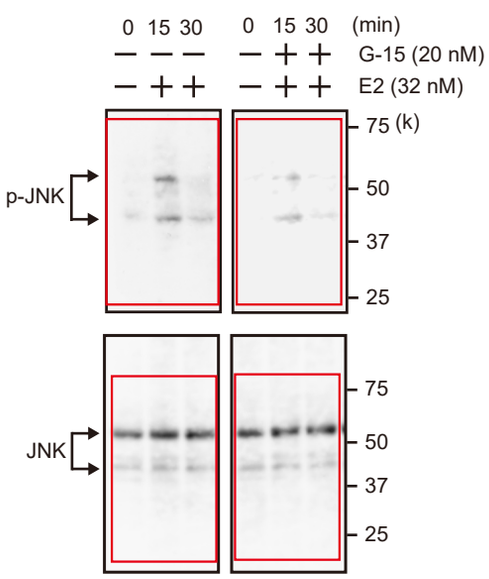

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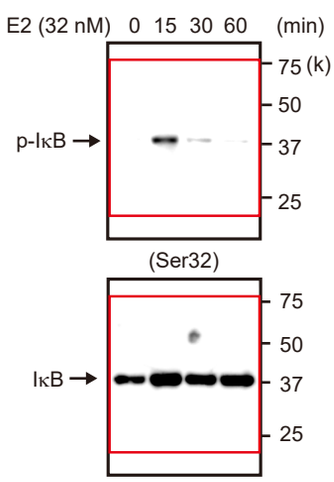
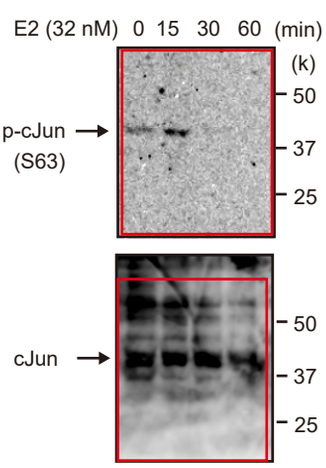
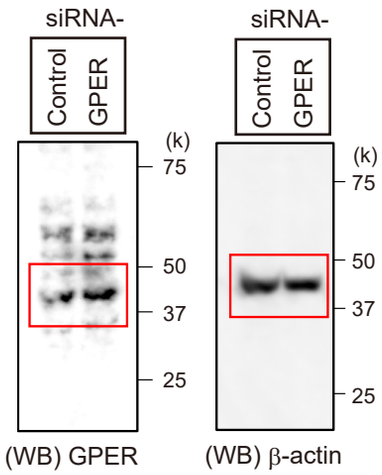


Fig. 2g



Supplemetary Fig. 2b



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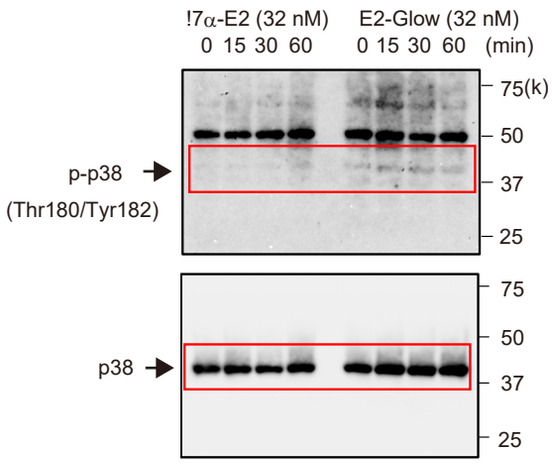

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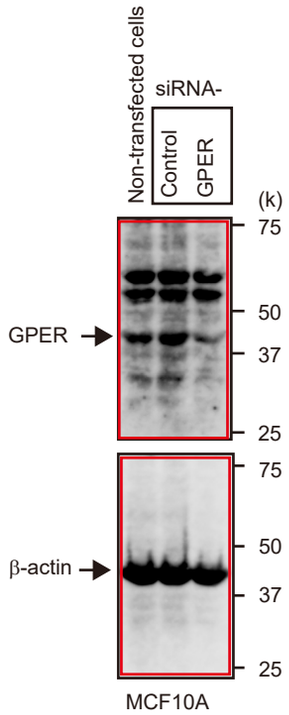

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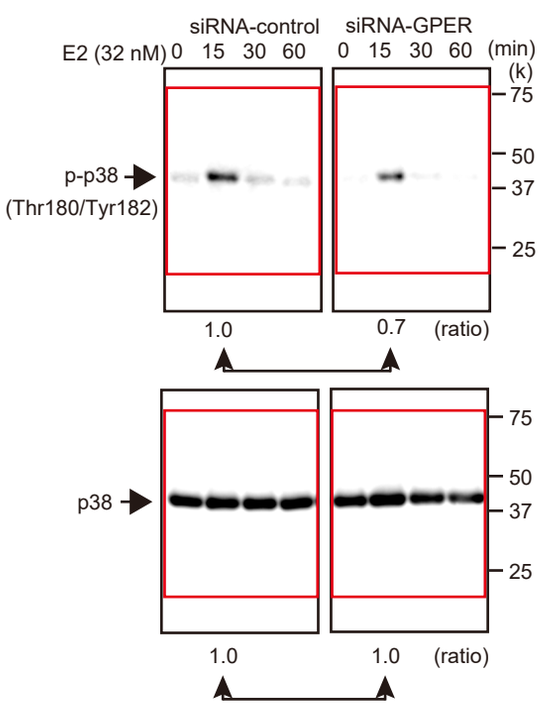

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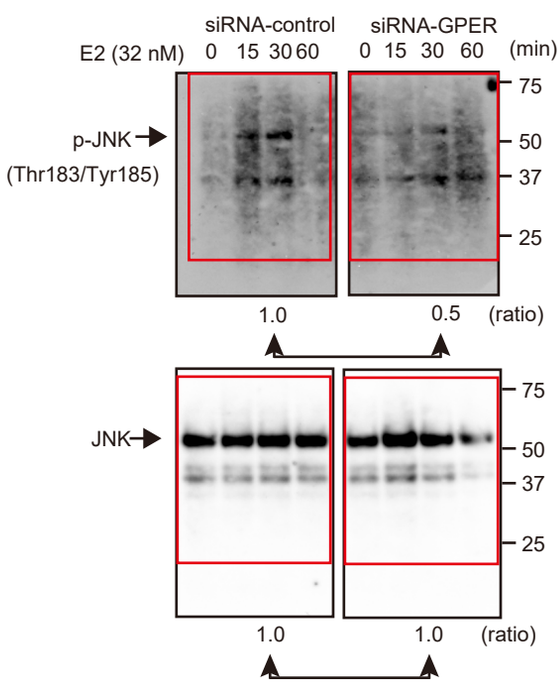

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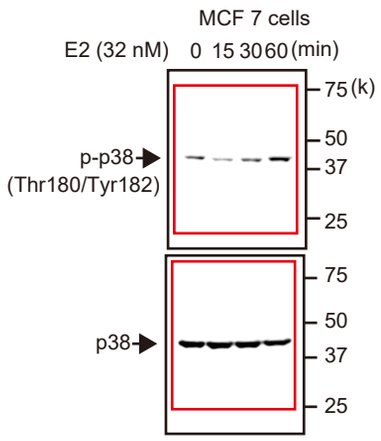

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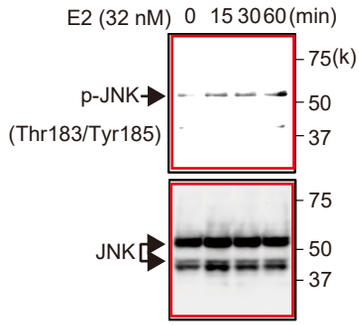
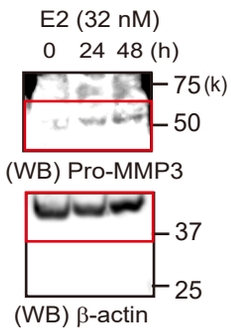


Fig. 3a



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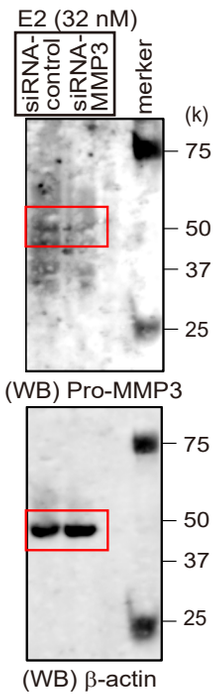


Fig. 4f

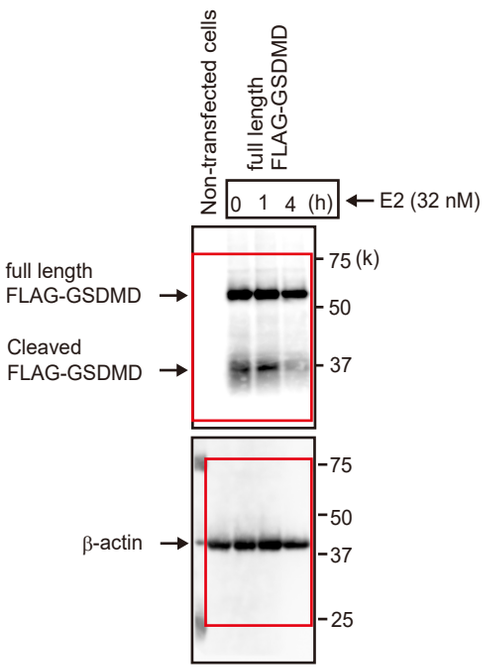


Fig. 4g

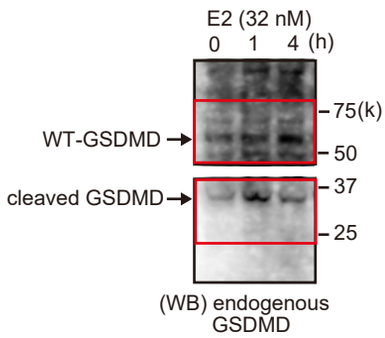


Fig. 5a

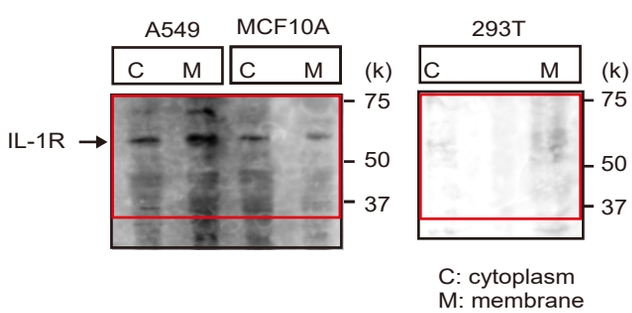


Fig. 5b

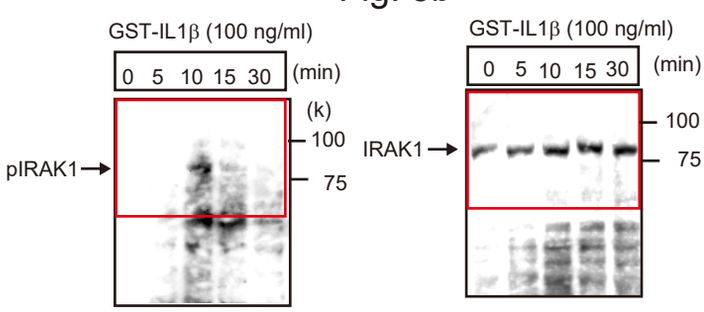


Fig. 5c

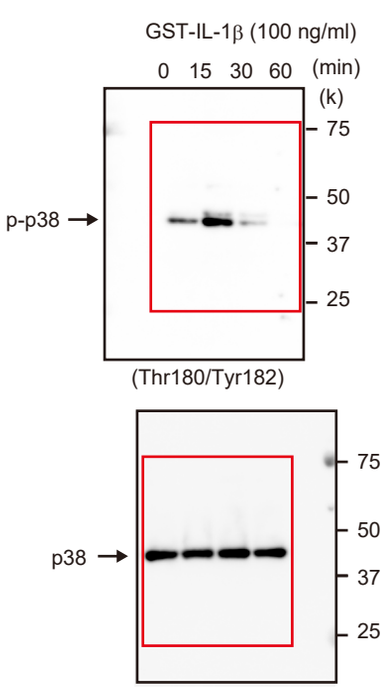


Fig. 5d

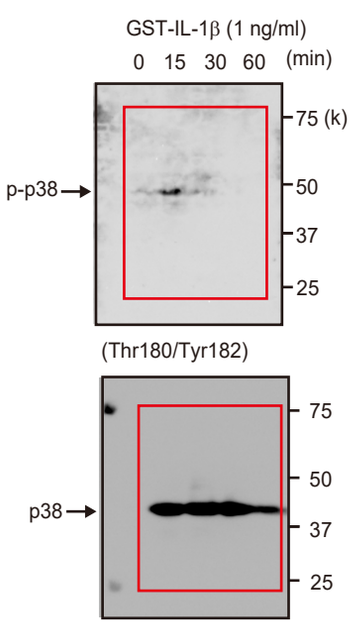


Fig. 5e

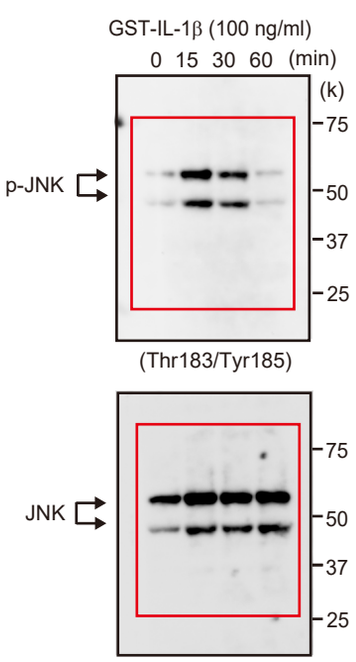


Fig. 5f

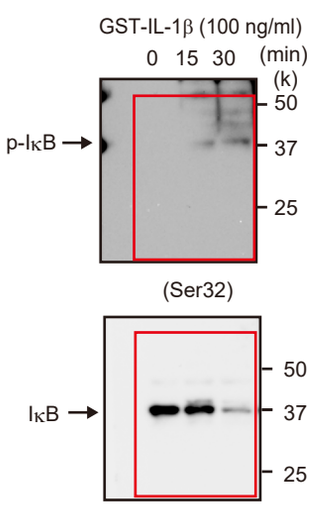
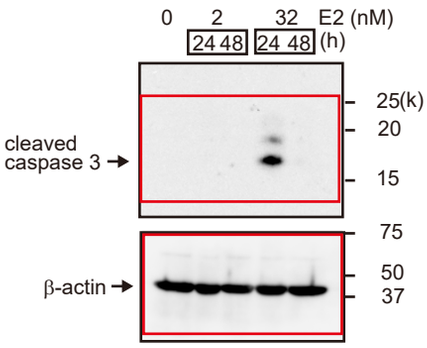


Fig. 6c


0
2
32
E2 (nM)
24 48
24 48
(h)
cleaved
caspase 3
β-actin
25(k)
20
15
75
50
37