Anticancer and Chemosensitization Effects of Cannabidiol in 2D and 3D cultures of TNBC: Involvement of GADD45α, Integrin-α5, -β5, -β1 and autophagy

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Running Title: Cannabidiol induces apoptosis in TNBC by altering GADD45A

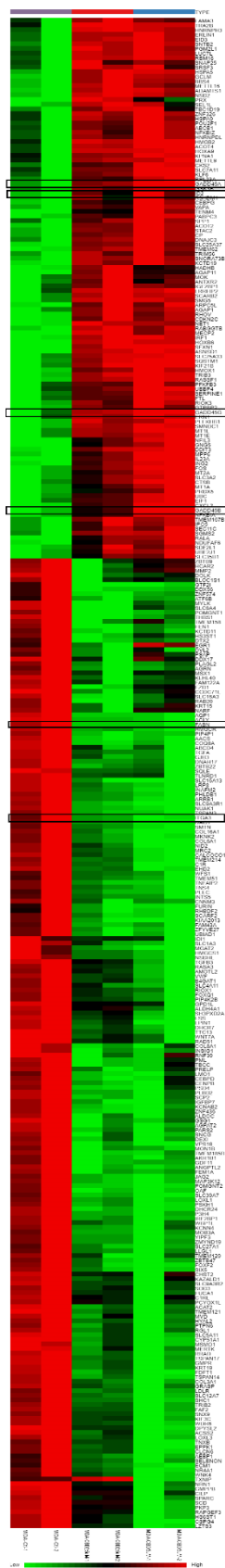**Journal name: Drug Delivery and Translational Research**

Acknowledgments
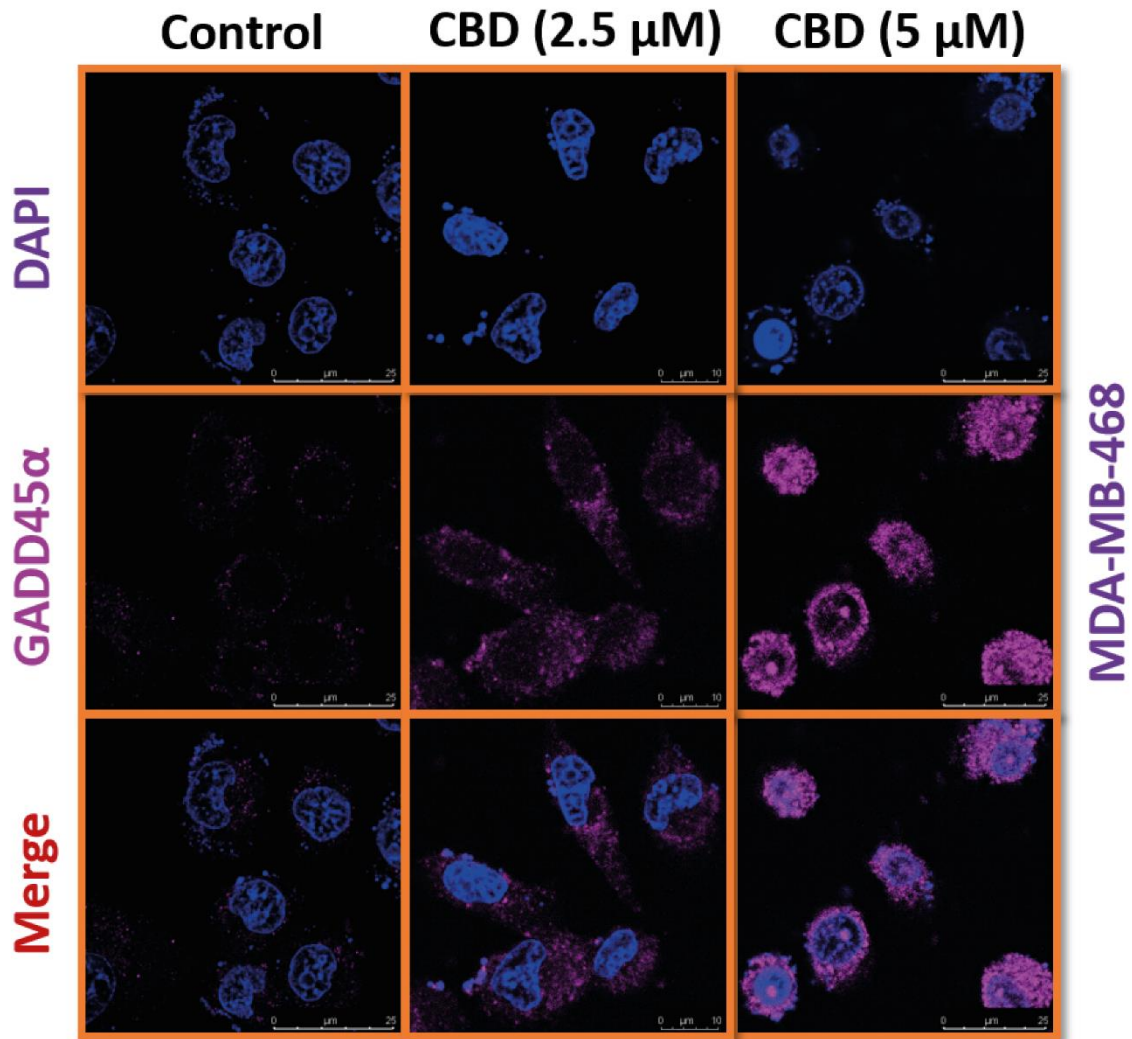
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3) Uncropped blots images
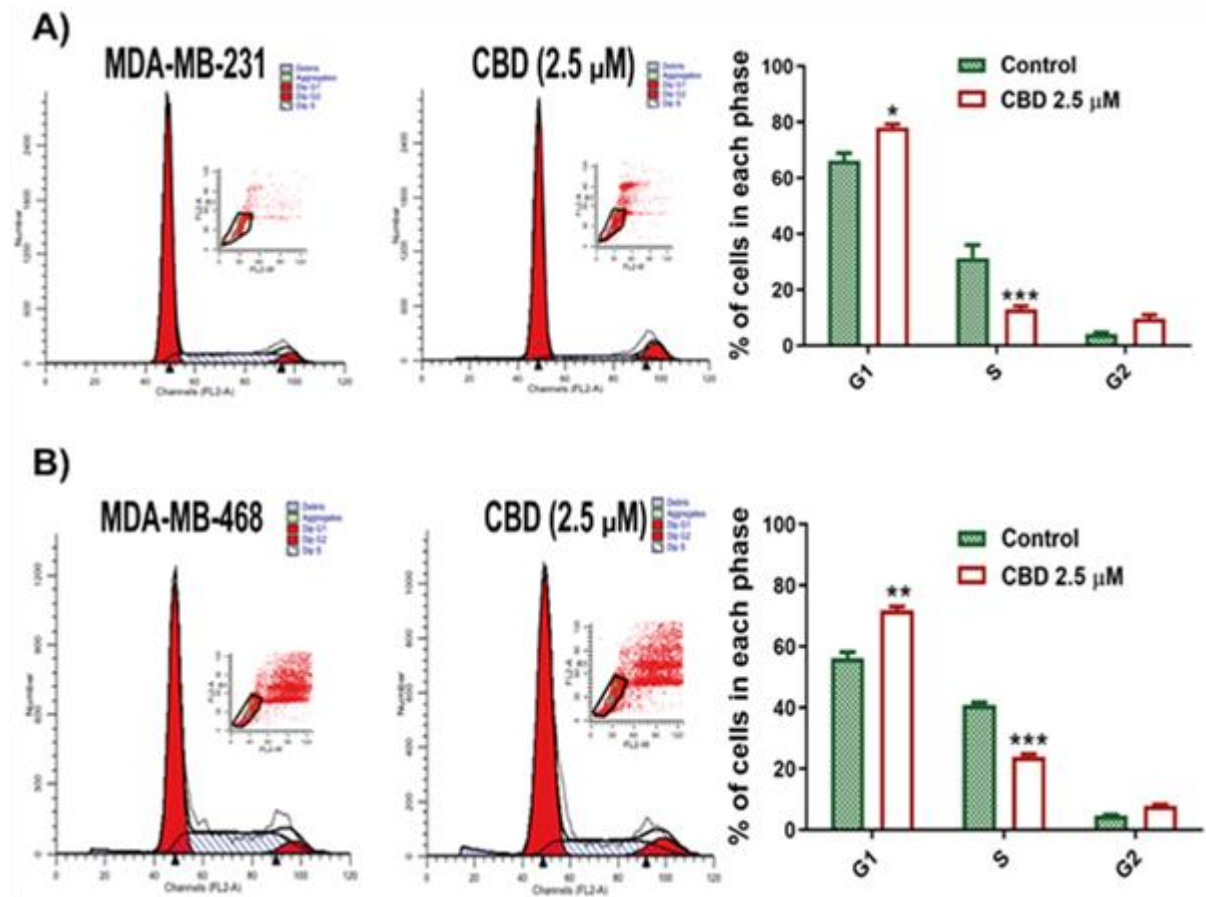
1) Supplementary Figures

Supplementary Fig. 1 High resolution heat map image of differentially expressed genes with CBD treatment in MDA-MB-231 cells

Supplementary Fig. 2 Effect of CBD on the expression of GADD45α in MDA-MB-468 cells


Supplementary Fig. 2 Immunocytochemical analysis of GADD45α in MDA-MB-468 cells treated with CBD for 48 h.

Supplementary Fig. 3 Effect of CBD on cell cycle progression of triple negative breast cancer cell lines


Supplementary Fig. 3 Flow cytometry of cell cycle analysis. A & B) Representation of the flow histograms and bar graphs showing cell cycle analysis after staining with propidium iodide (PI) in MDA-MB-231 and MDA-MB-468 cells treated with CBD (2.5 μM) and compared to control. All values are expressed as mean ± SEM (n=3). *p<0.05, ***p < 0.001 v.s Control.

Supplementary Fig. 4 Effect of CBD on the expression of integrin-α5 and integrin-β5 in 3D cultures of MDA-MB-231 cells.
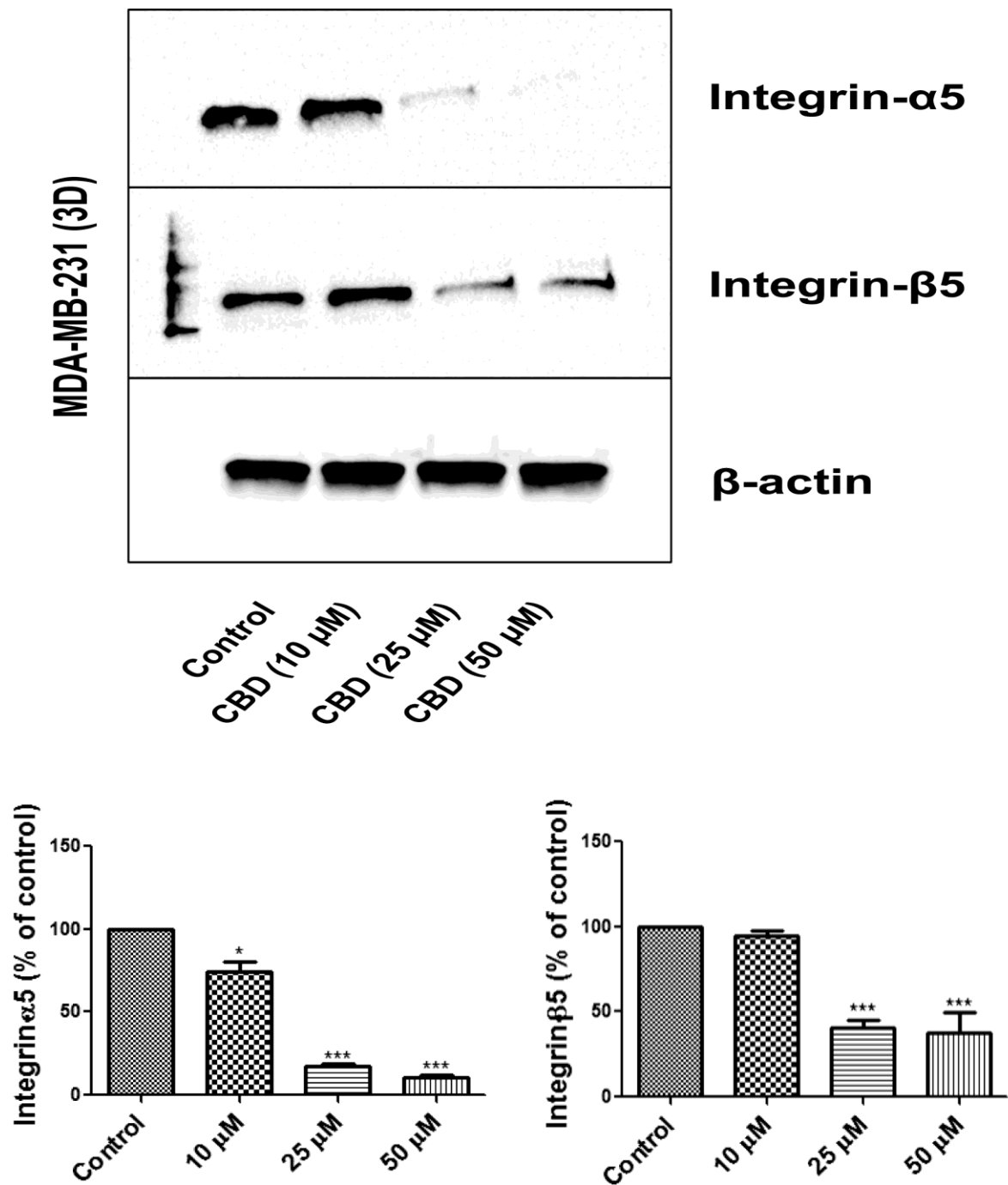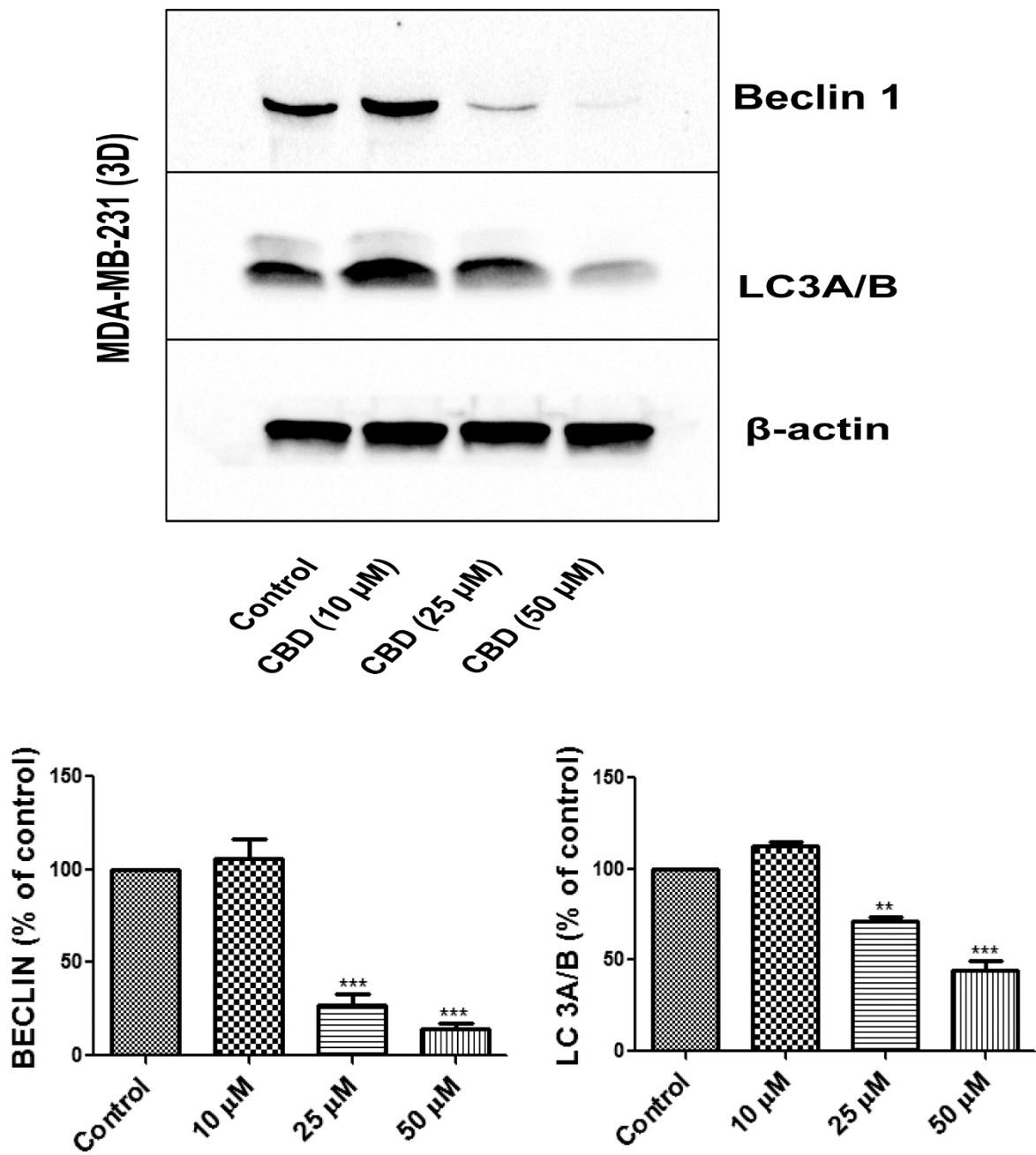

Supplementary Fig. 4 Immunoblots and densitometry data of integrin-α5, integrin -β5 and integrin-β1 in CBD treated 3D cultures of MDA-MB-231 cells for 48 h. After checking the expression of Integrin β5, we had performed stripping and reprobed the same blot with Integrin α5 antibody. Uncropped images of the blots have been shown in supplementary information. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ significant vs control.

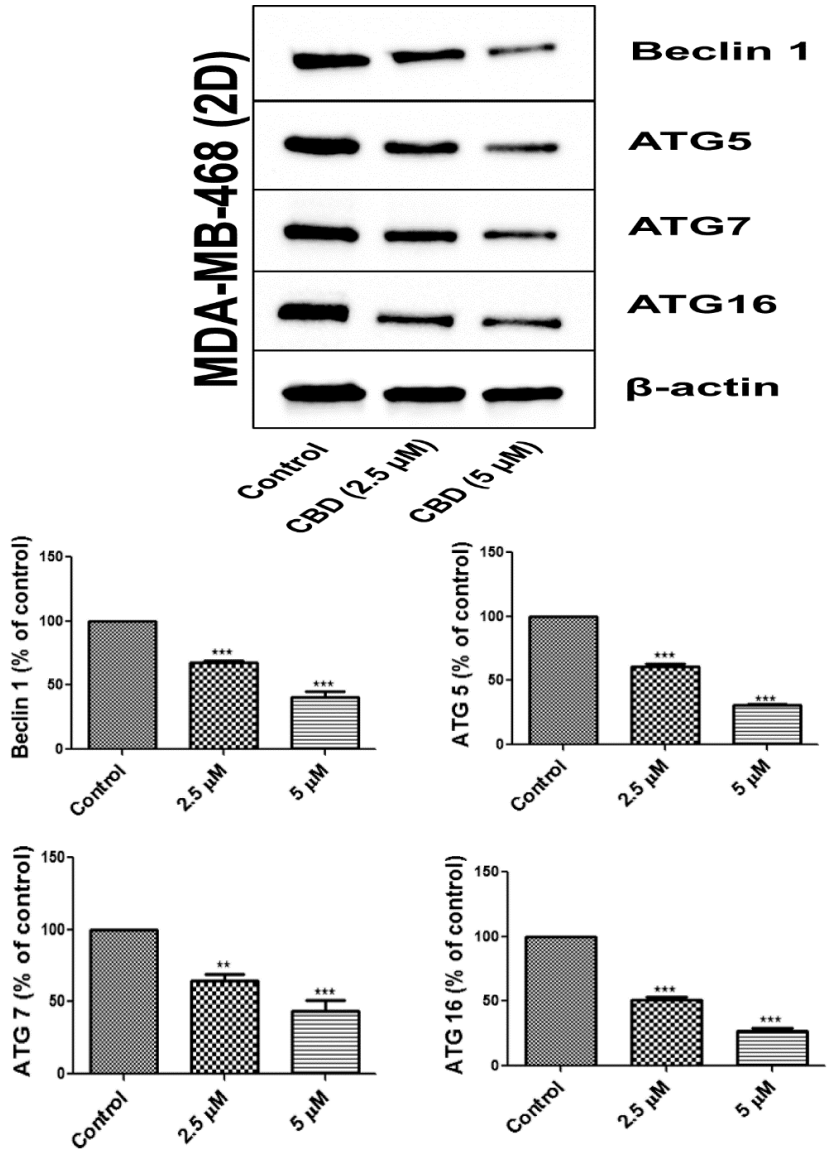**Supplementary Fig. 5** *Effect of CBD on the autophagy of MDA-MB-231 cells*
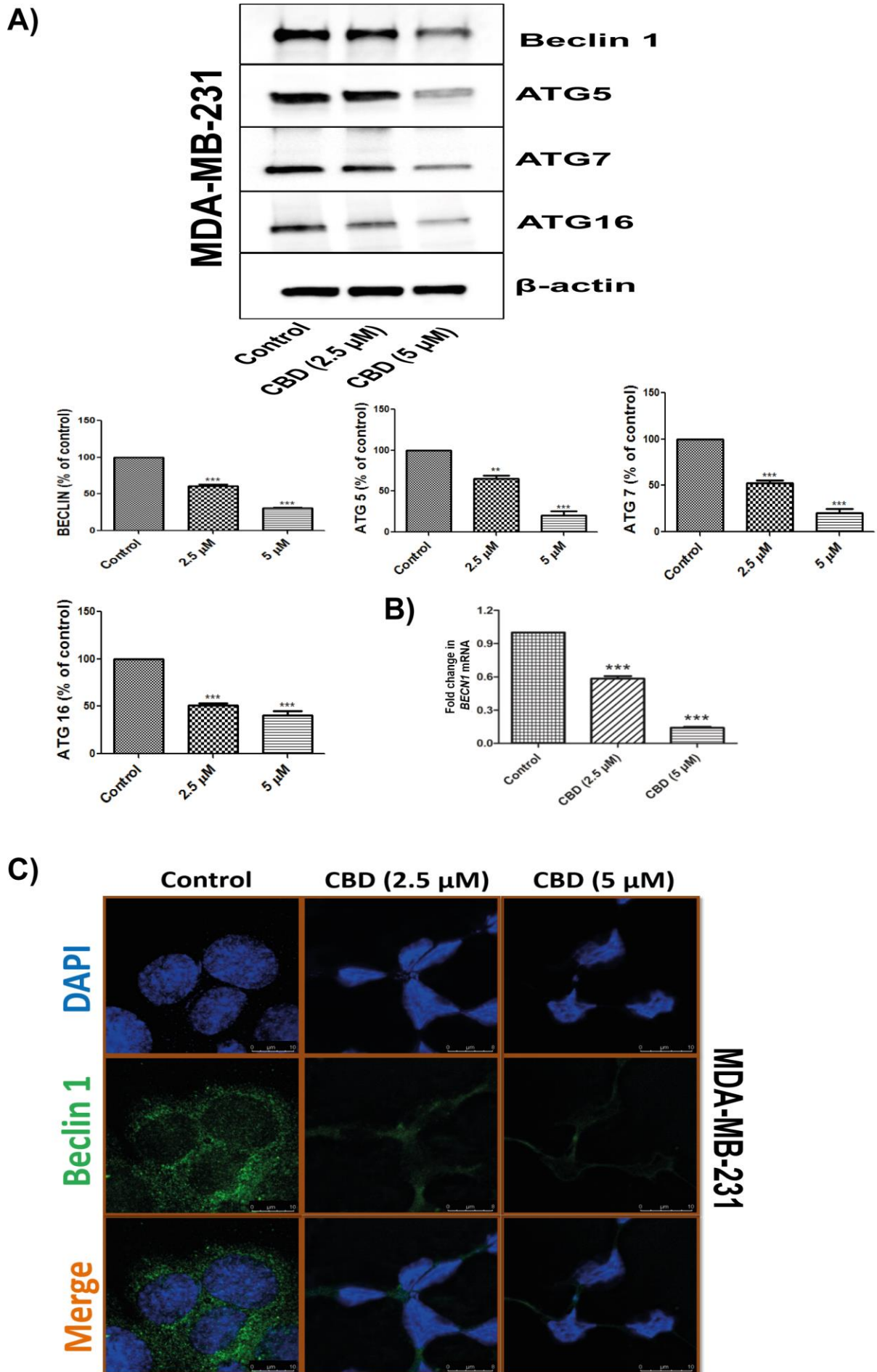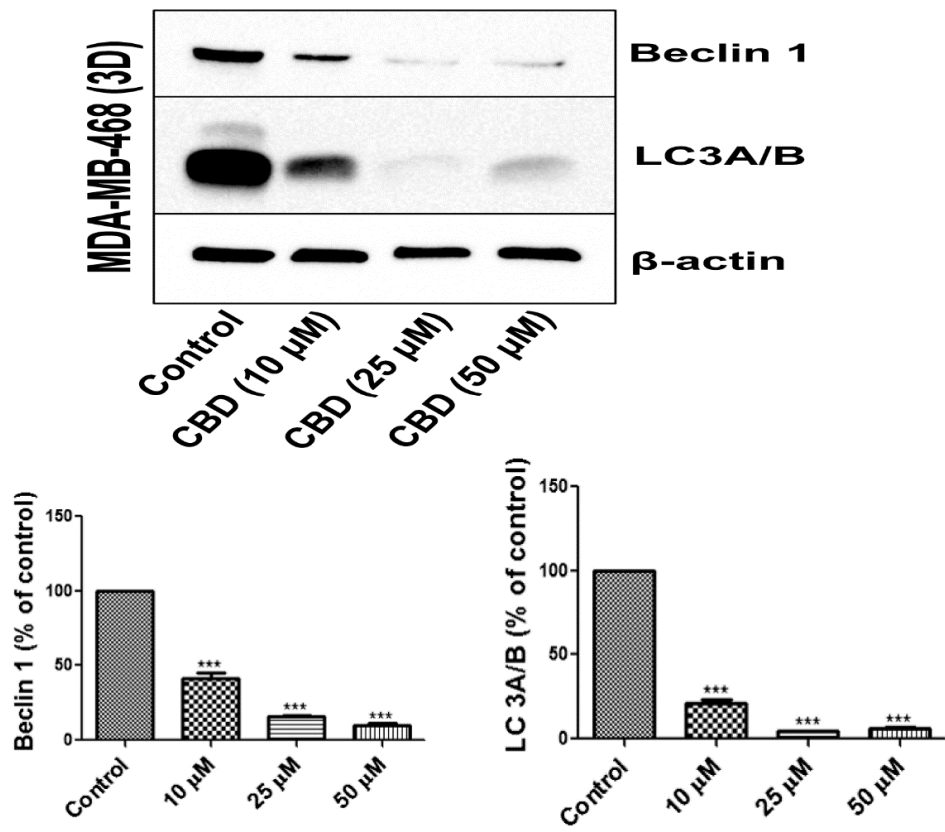
D)

Supplementary Fig. 5 Effect of CBD on the autophagy markers in MDA-MB-231 cells. A) Immunoblots and densitometry data of Beclin 1, ATG5, ATG7, and ATG16 in CBD treated MDA-MB-231 cells for 48 h. After checking the expression of Beclin 1 and ATG16, we had performed stripping and reprobed the same blots with ATG5, and β-actin antibodies respectively. Uncropped images of the blots have been shown in supplementary information. B) Quantitative real-time PCR of *BECN1* gene after treatment with CBD for 48 h in MDA-MB-231 cells. C) Immunocytochemical analysis of Beclin 1 in MDA-MB-231 cells treated with CBD for 48 h. D) Immunoblots and densitometry data of Beclin 1 and LC3A/B in 3D cultures of CBD treated MDA-MB-231 cells for 48 h. After checking the expression of Beclin 1, we performed stripping and reprobed the same blot with β-actin antibody. Uncropped images of the blots have been shown in supplementary information. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ significant vs control.

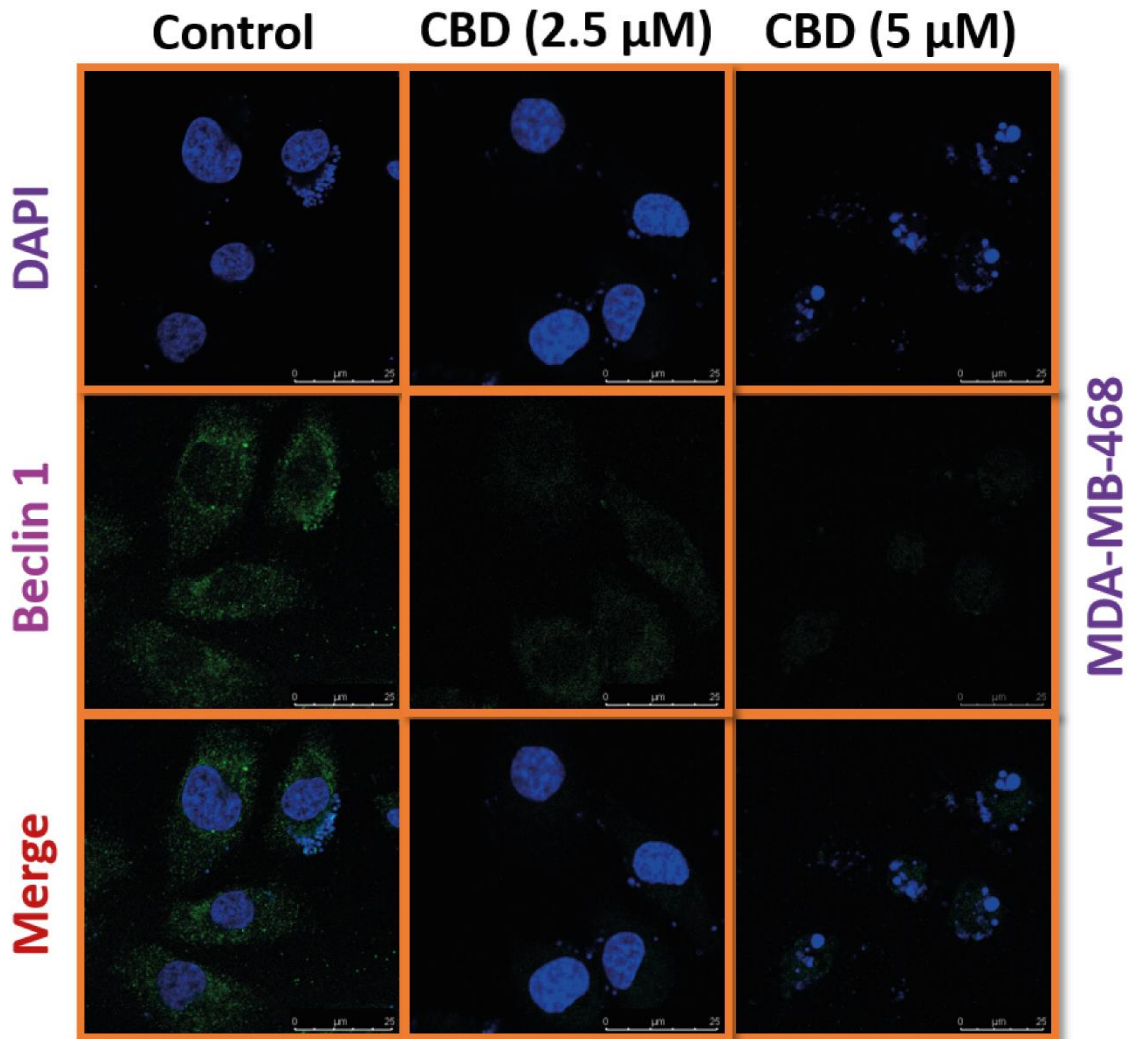Supplementary Fig. 6 *Effect of CBD on the autophagy of MDA-MB-468 cells*

A)

B)

C)

Supplementary Fig. 6 Effect of CBD on the autophagy markers in MDA-MB-468 cells. A) Immunoblots and densitometry data of Beclin 1, ATG5, ATG7, and ATG16 in 2D cultures of CBD treated MDA-MB-468 cells for 48 h. After checking the expression of ATG7, we performed stripping and reprobed the same blots with ATG5, Beclin1, ATG16, and β-actin antibodies respectively. B) Immunoblots and densitometry data of Beclin 1 and LC3A/B in 3D cultures of CBD treated MDA-MB-468 cells for 48 h. After checking the expression of Beclin 1, we performed stripping and reprobed the same blot with β-actin antibody. Uncropped images of the blots have been shown in supplementary information. ***p < 0.001 significant vs control. C) Immunocytochemical analysis of Beclin 1 in MDA-MB-468 cells treated with CBD for 48 h.

Supplementary Fig. 7 *Effect of CBD on the expression of LOX protein in both MDA-MB-231 and MDA-MB-468 cells*
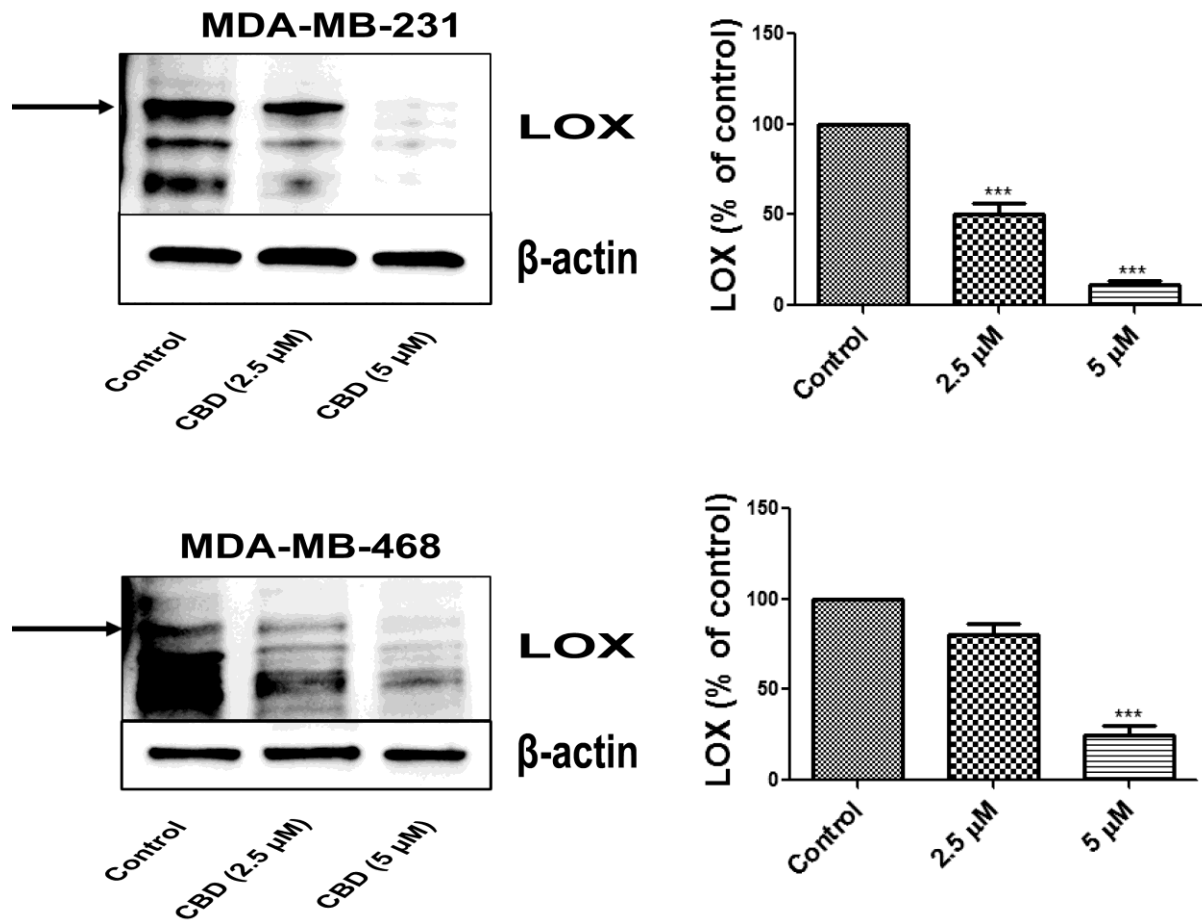
Supplementary Fig. 7 Immunoblots and densitometry data of LOX in 2D cultures of CBD treated MDA-MB-231 and MDA-MB-468 cells for 48 h. β After checking the expression of LOX, we performed stripping and reprobed the same blot with β-actin antibody. Uncropped images of the blots have been shown in supplementary information. ***p < 0.001 significant vs control.

2) Supplementary Tables

Supplementary Table 1: Combinatorial effects of CBD and DOX in MDA-MB-231 cells

Combinations (µM)	Cytotoxicity (%; Mean)	DRI (CBD; DOX)	CI value	Conclusion
CBD (1) + DOX (0.39)	41.8	2.16; 5.77	0.635	Synergism
CBD (1) + DOX (0.78)	48.3	2.62; 3.55	0.662	Synergism
CBD (1) + DOX (1.56)	53.1	3.02; 2.06	0.814	Synergism
CBD (1) + DOX (3.12)	62.6	4.02; 1.40	0.959	Synergism
CBD (1) + DOX (6.25)	67.9	4.78; 0.84	1.394	Antagonism
CBD (1) + DOX (12.5)	70.8	5.28; 0.46	2.318	Antagonism
CBD (1) + DOX (25)	74.2	5.99; 0.26	3.889	Antagonism
CBD (2.5) + DOX (0.39)	44.3	0.93; 6.26	1.232	Antagonism
CBD (2.5) + DOX (0.78)	45.2	0.95; 3.22	1.354	Antagonism
CBD (2.5) + DOX (1.56)	57.8	1.39; 2.40	1.135	Antagonism
CBD (2.5) + DOX (3.12)	60.7	1.51; 1.31	1.416	Antagonism
CBD (2.5) + DOX (6.25)	69.7	2.03; 0.90	1.601	Antagonism
CBD (2.5) + DOX (12.5)	71.6	2.17; 0.48	2.524	Antagonism
CBD (2.5) + DOX (25)	76.4	2.61; 0.29	3.774	Antagonism

Supplementary Table 1: Combinatorial effects of CBD and DOX in MDA-MB-231 cells. CI and DRI values were determined by using CI equation algorithms and DRI equation algorithms through CompuSyn software. CI=1, <1, and >1 represents an additive effect, synergism, and antagonism, respectively. DRI=1, >1, and <1 represent no dose reduction, favourable dose reduction, and unfavourable dose reduction respectively, for each drug in the combination. CI: combination index; DRI: dose-reduction index
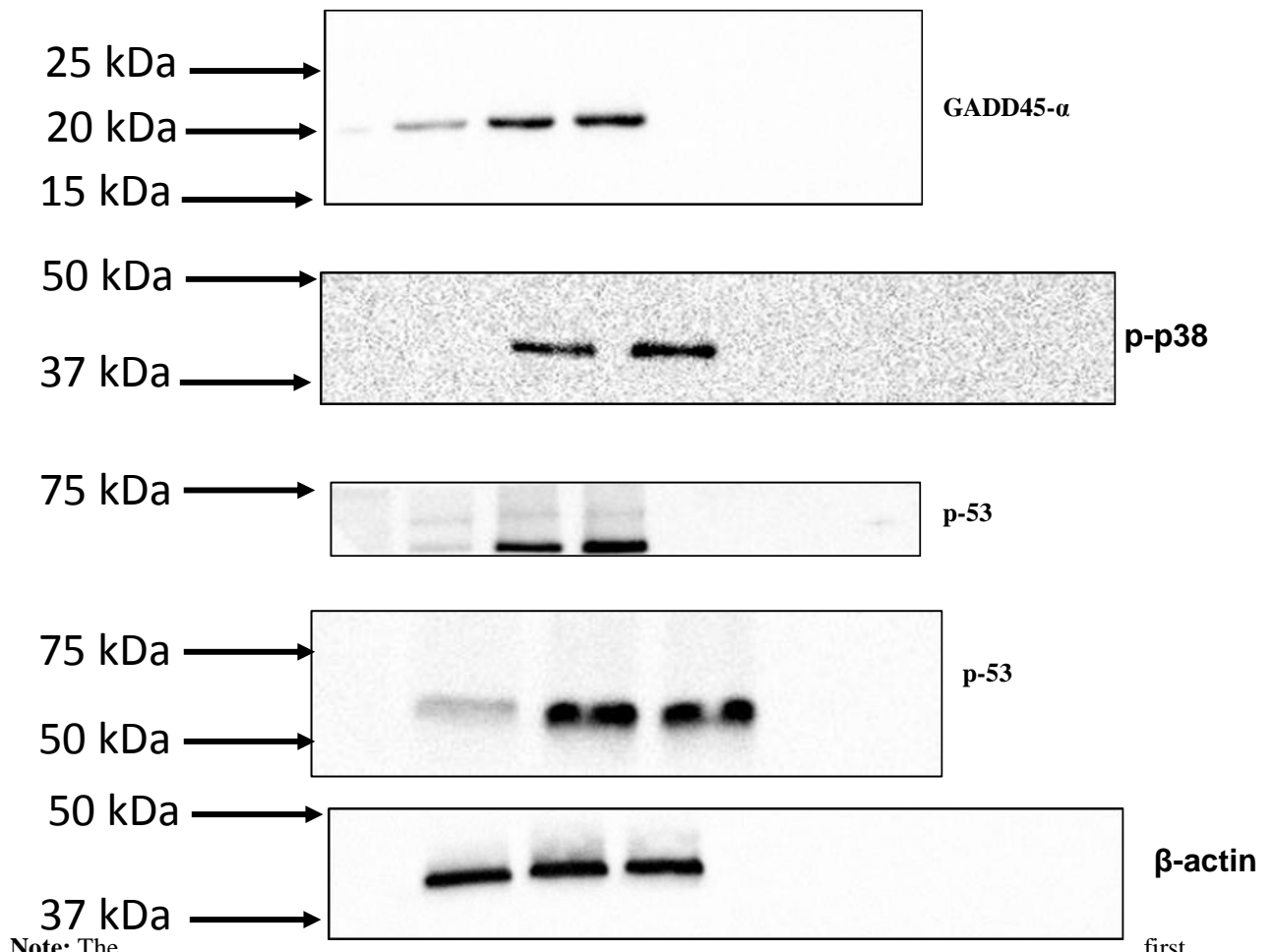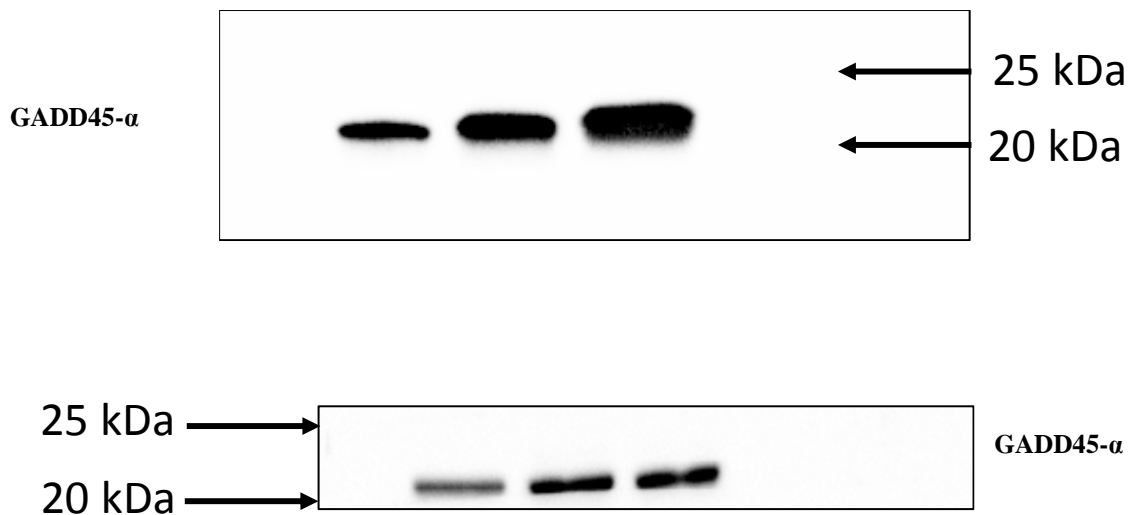
Supplementary Table 2: Combinatorial effects of CBD and DOX in MDA-MB-468 cells

Combinations (µM)	Cytotoxicity (%; Mean)	DRI (CBD; DOX)	CI value	Conclusion
CBD (1) + DOX (0.39)	38.3	2.15; 3.33	0.764	Synergism
CBD (1) + DOX (0.78)	39	2.21; 1.72	1.032	Additive
CBD (1) + DOX (1.56)	43.9	2.72; 1.06	1.302	Antagonism
CBD (1) + DOX (3.12)	56	4.48; 0.90	1.332	Antagonism
CBD (1) + DOX (6.25)	67.9	7.54; 0.77	1.421	Antagonism
CBD (1) + DOX (12.5)	73.2	9.79; 0.51	2.061	Antagonism
CBD (1) + DOX (25)	76.9	11.99; 0.31	3.251	Antagonism
CBD (2.5) + DOX (0.39)	41.8	0.99; 3.89	1.257	Antagonism
CBD (2.5) + DOX (0.78)	46.9	1.23; 2.43	1.220	Antagonism
CBD (2.5) + DOX (1.56)	53.1	1.59; 1.58	1.257	Antagonism
CBD (2.5) + DOX (3.12)	62.6	2.37; 1.20	1.248	Antagonism
CBD (2.5) + DOX (6.25)	67.9	3.01; 0.77	1.620	Antagonism
CBD (2.5) + DOX (12.5)	70.8	3.46; 0.44	2.514	Antagonism
CBD (2.5) + DOX (25)	74.5	4.19; 0.27	3.883	Antagonism

Supplementary Table 2: Combinatorial effects of CBD and DOX in MDA-MB-468 cells. CI and DRI values were determined by using CI equation algorithms and DRI equation algorithms through CompuSyn software. CI=1, <1, and >1 represents an additive effect, synergism, and antagonism, respectively. DRI=1, >1, and <1 represent no dose reduction, favourable dose reduction, and unfavourable dose reduction respectively, for each drug in the combination. CI: combination index; DRI: dose-reduction index.
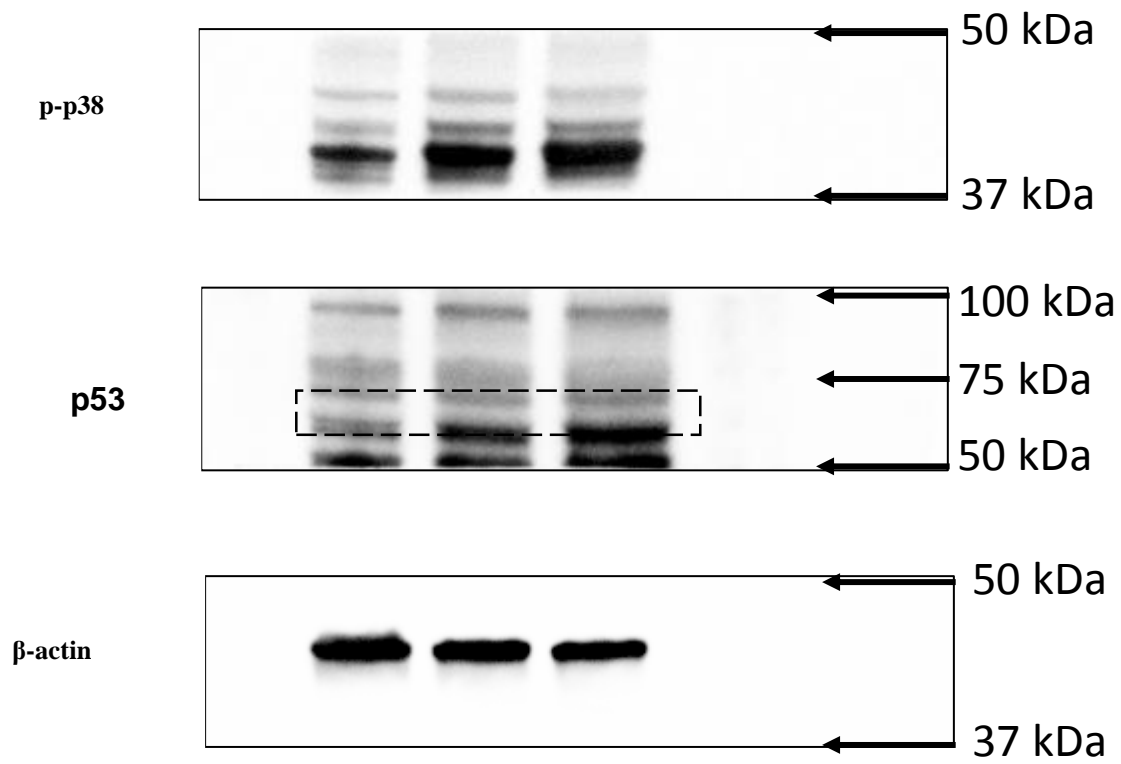
3) Uncropped western blot images

1) Fig. 3A (MDA-MB-231 2D)

25 kDa ——→

20 kDa ——→ **GADD45-α**

15 kDa ——→

50 kDa ——→

p-p38

37 kDa ——→

75 kDa ——→

p-53

75 kDa ——→

p-53

50 kDa ——→

50 kDa ——→

β-actin

37 kDa ——→

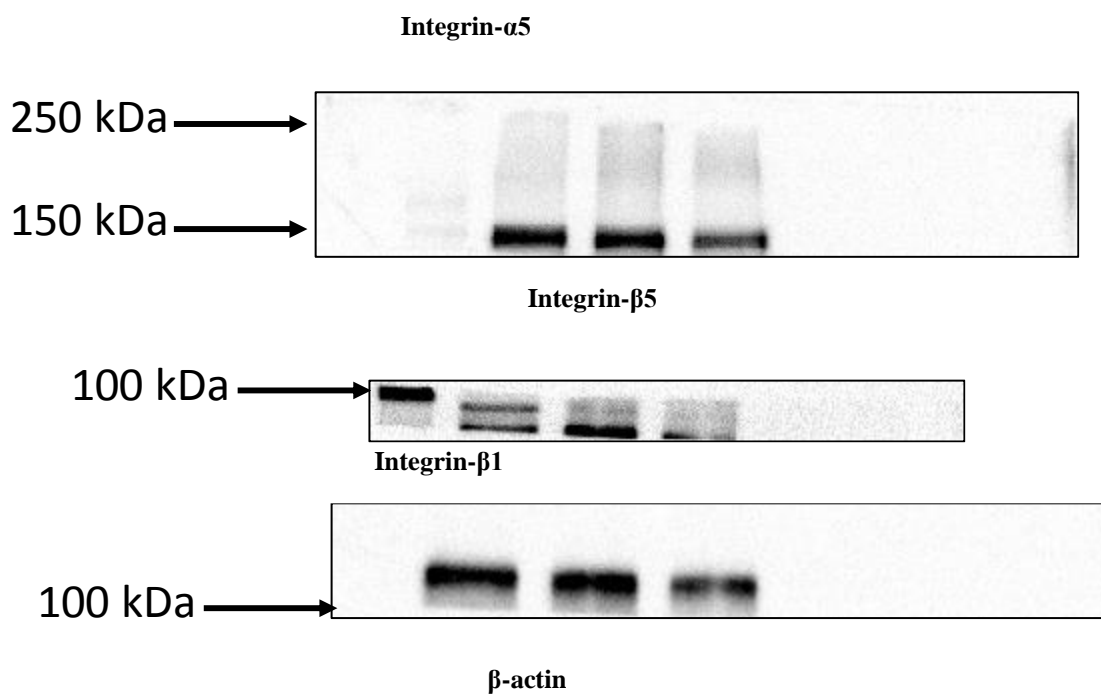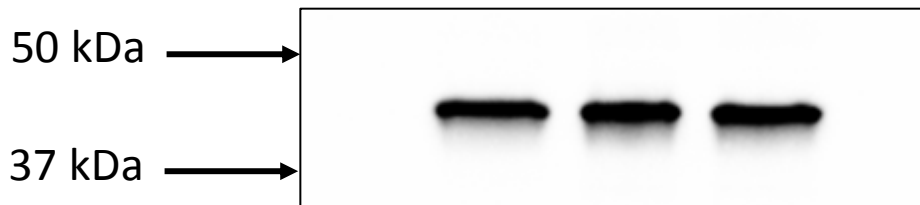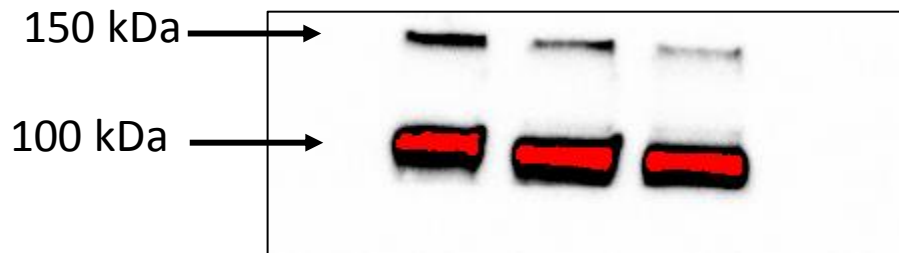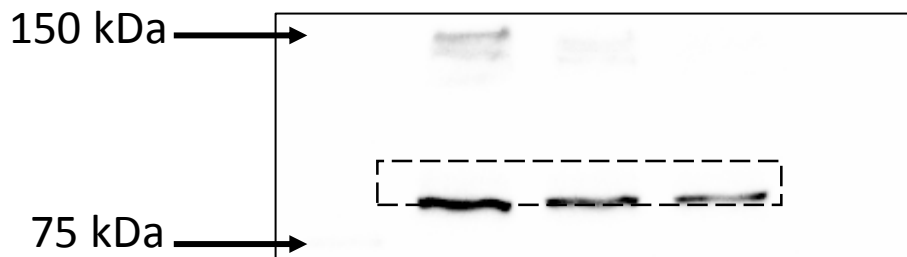**Note:** The   first
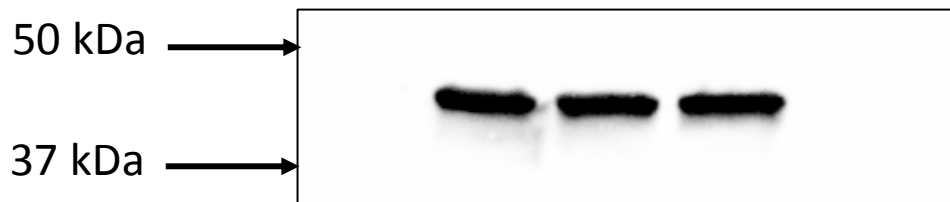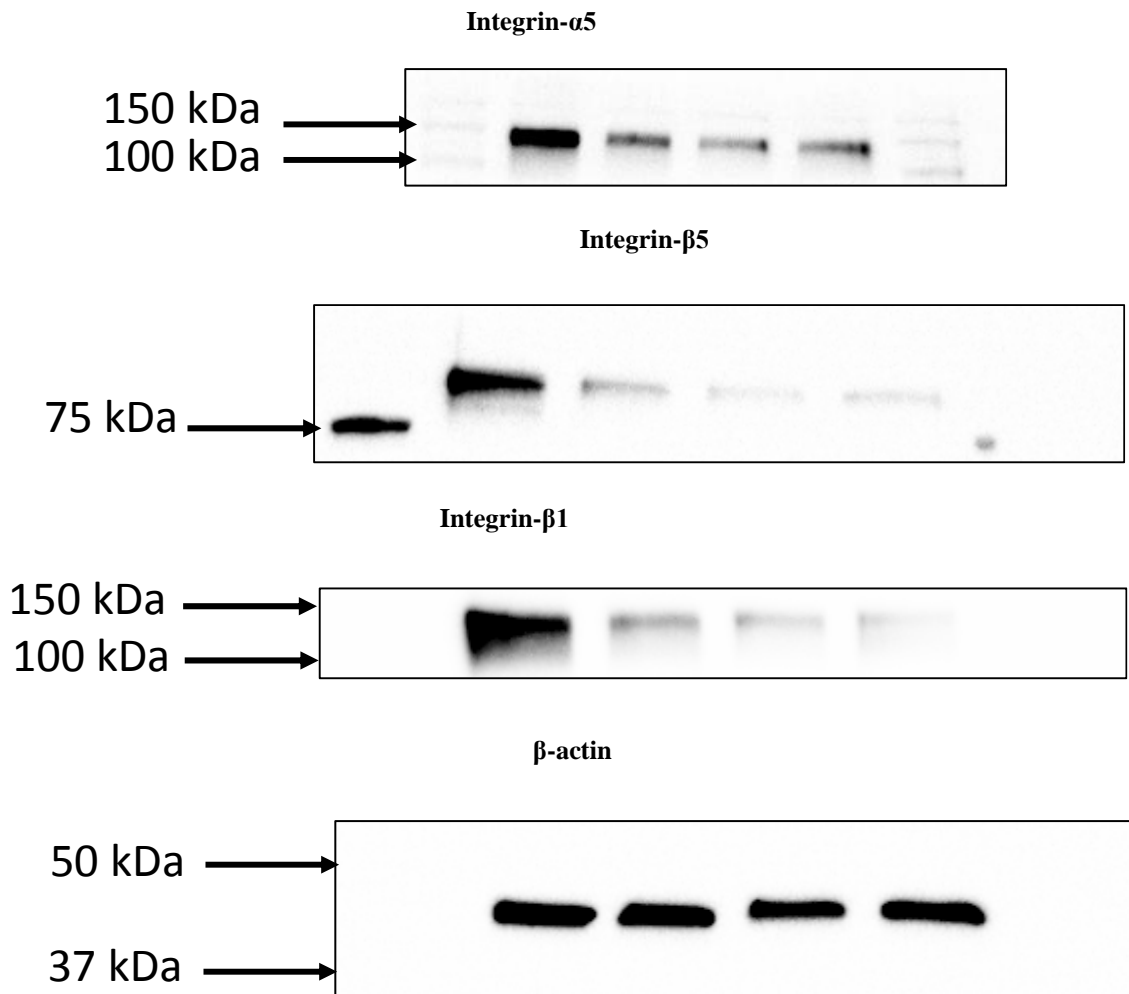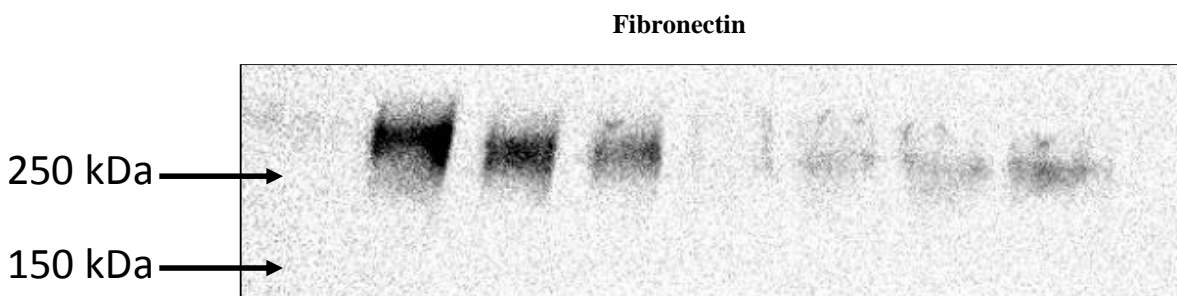lane, second lane and the third lane indicate Control, CBD (2.5 µM) and CBD (5 µM) treated groups
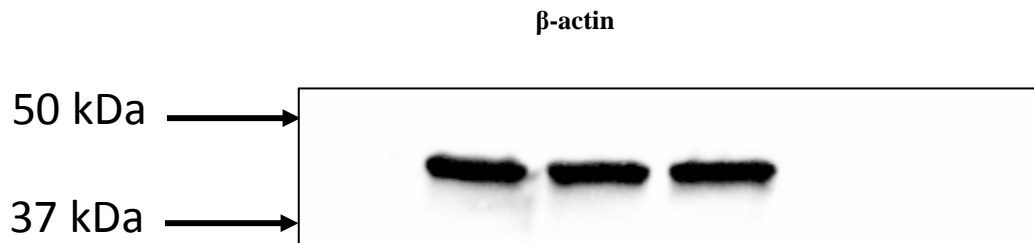respectively in the above represented blots.

Fig. 3B (MDA-MB-468 2D)

←—— 25 kDa

GADD45-α

←—— 20 kDa

25 kDa ——→

GADD45-α

20 kDa ——→

p-p38

50 kDa

37 kDa

p53

100 kDa

75 kDa

50 kDa

β-actin

50 kDa

37 kDa

Note: The first lane, second lane and the third lane indicate Control, CBD (2.5 μM) and CBD (5 μM) treated groups respectively in the above represented blots.

2) Fig. 4A (MDA-MB-231 2D)

Integrin-α5

250 kDa

150 kDa

Integrin-β5

100 kDa

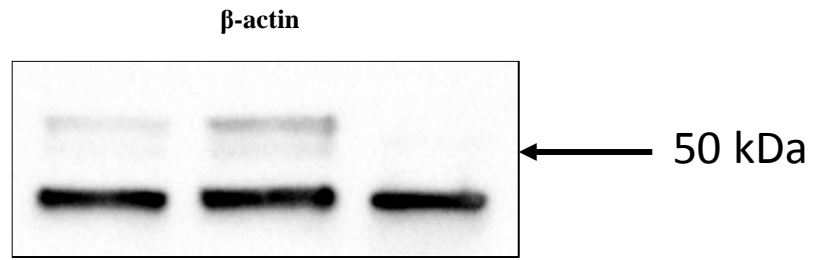**Integrin-β1**

100 kDa

β-actin

Note: The first lane, second lane and the third lane indicate Control, CBD (2.5 μM) and CBD (5 μM) treated groups respectively in the above represented blots.

Fig. 4B (MDA-MB-468 2D)

Integrin-α5
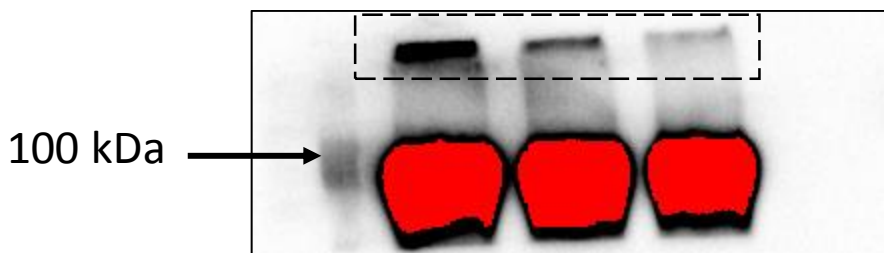
Integrin-β5

Integrin-β1

β-actin

50 kDa

37 kDa

Note: The first lane, second lane and the third lane indicate Control, CBD (2.5 µM) and CBD (5 µM) treated groups respectively in the above represented blots.

Fig. 4C (MDA-MB-468 3D)

Integrin-α5

150 kDa
100 kDa

Integrin-β5

75 kDa

Integrin-β1

150 kDa
100 kDa

β-actin

50 kDa

37 kDa

Note: The first lane, second lane, third lane and the fourth lane indicate Control, CBD (10 µM), CBD (25 µM) and CBD (50 µM) treated groups respectively in the above represented blots.

3) Fig. 5B (MDA-MB-231 2D)
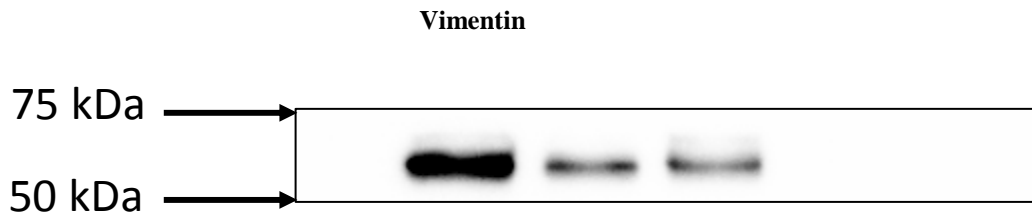
Fibronectin

250 kDa

150 kDa

Vimentin

75 kDa ➞
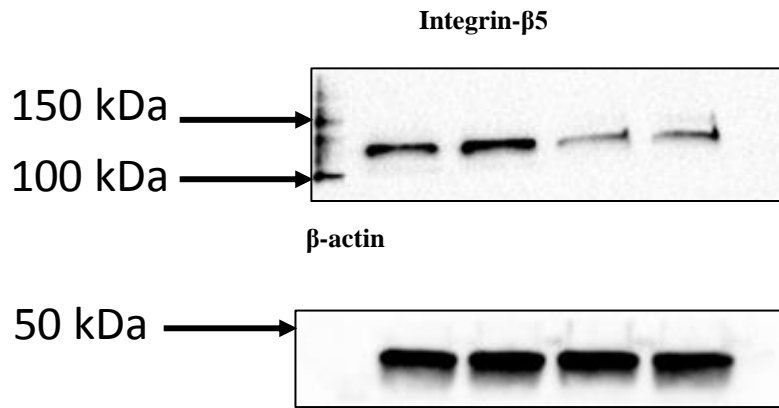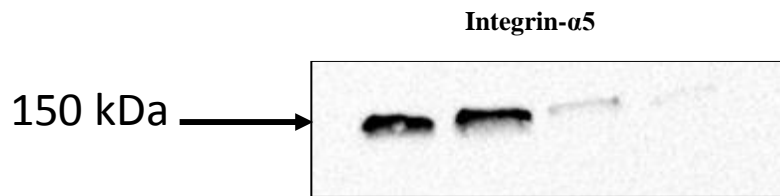
50 kDa ➞

β-actin

50 kDa ➞

37 kDa ➞

Note: The first lane, second lane and the third lane indicate Control, CBD (2.5 µM) and CBD (5 µM) treated groups respectively in the above represented blots.

Fig. 5C (MDA-MB-468 2D)
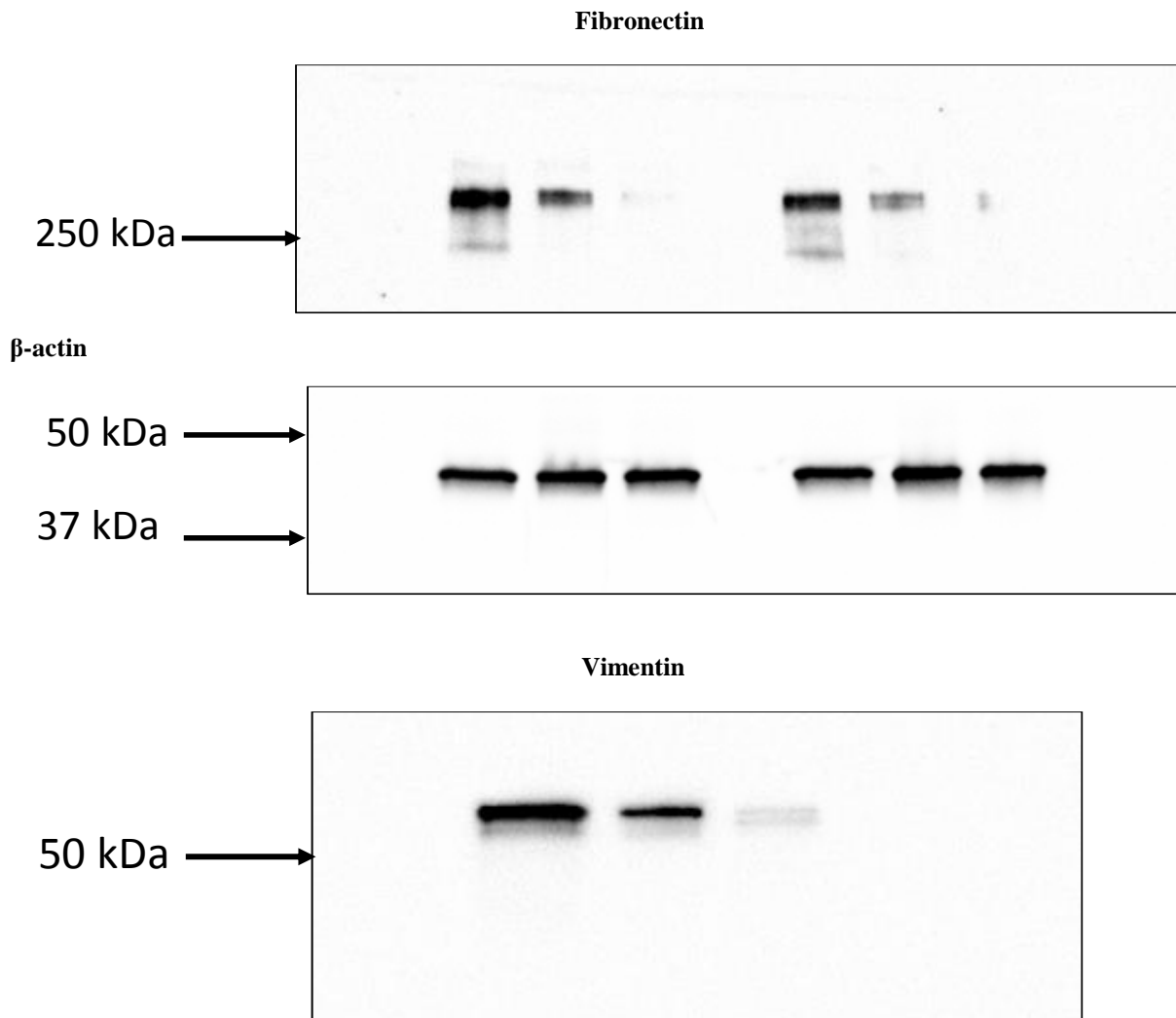
Fibronectin

250 kDa ➞

β-actin

50 kDa ➞

37 kDa ➞

Vimentin

50 kDa ➞

β-actin

⟵ 50 kDa

Note: The first lane, second lane and the third lane indicate Control, CBD (2.5 μM) and CBD (5 μM) treated groups respectively in the above represented blots.
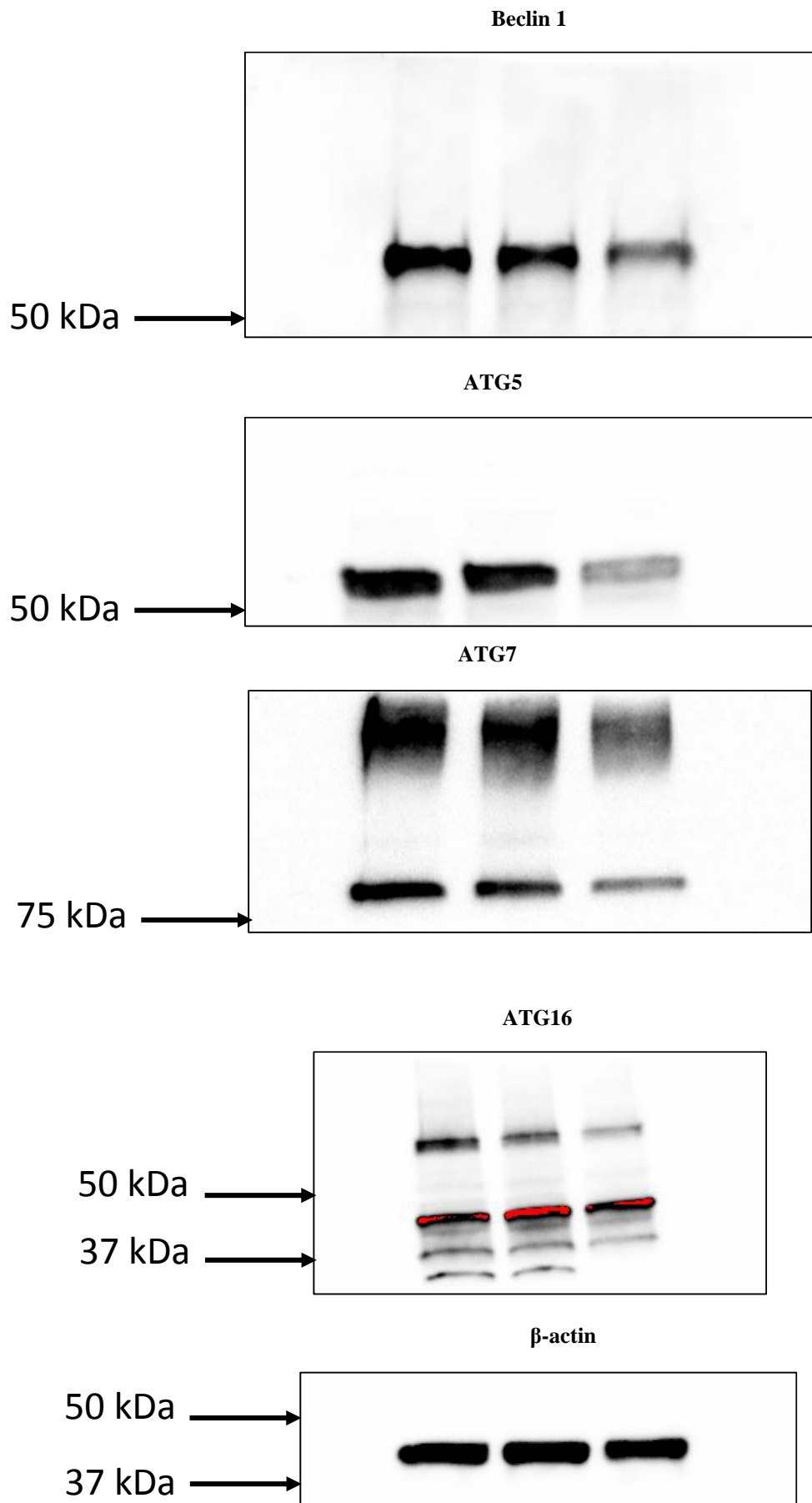
4) Fig. 6F (MDA-MB-468 2D)

LOX

50 kDa ⟶

Caspase-9

37 kDa ⟶

Integrin-α5

250 kDa ⟶

150 kDa ⟶

β-actin

50 kDa ⟶
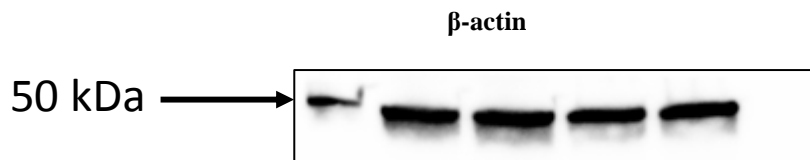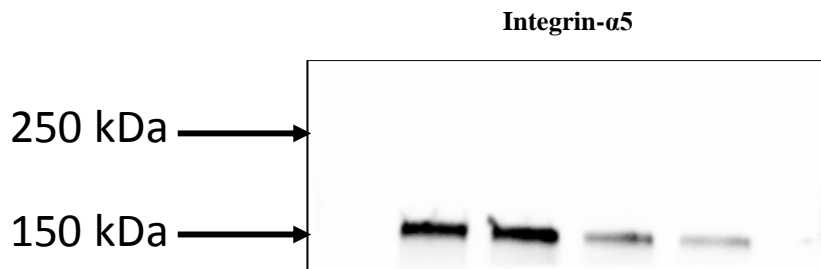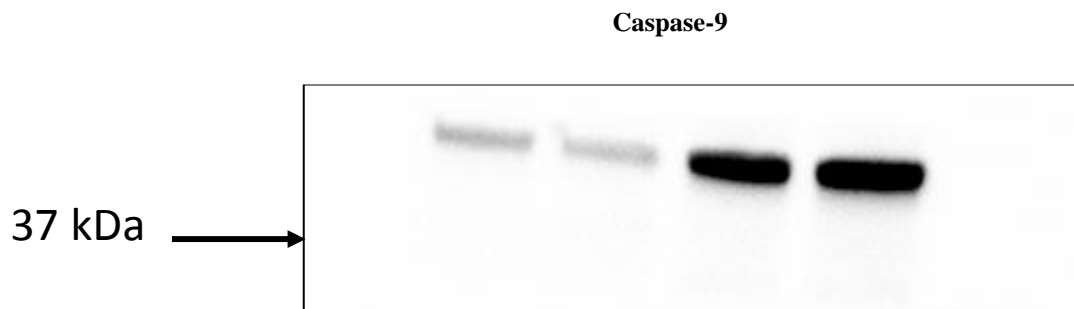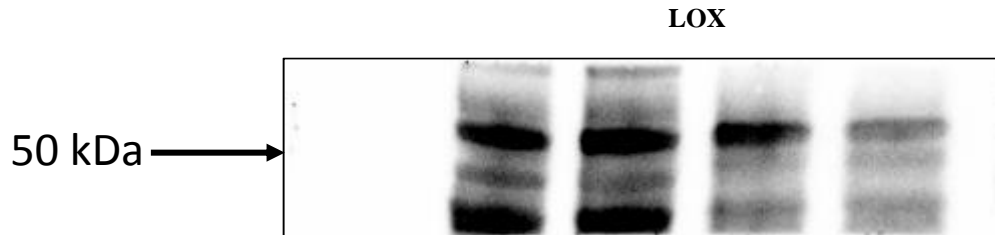
Note: The first lane, second lane, third lane and the fourth lane indicate Control, CBD (1 μM), DOX (1 μM) and CBD (1 μM) + DOX (1 μM) treated groups respectively in the above represented blots.
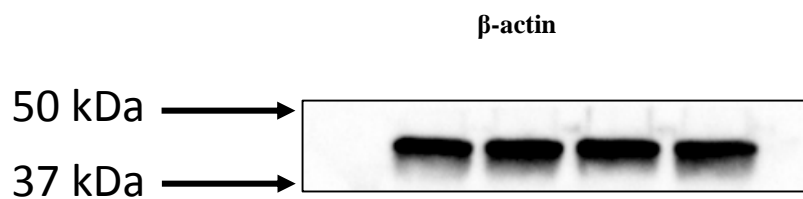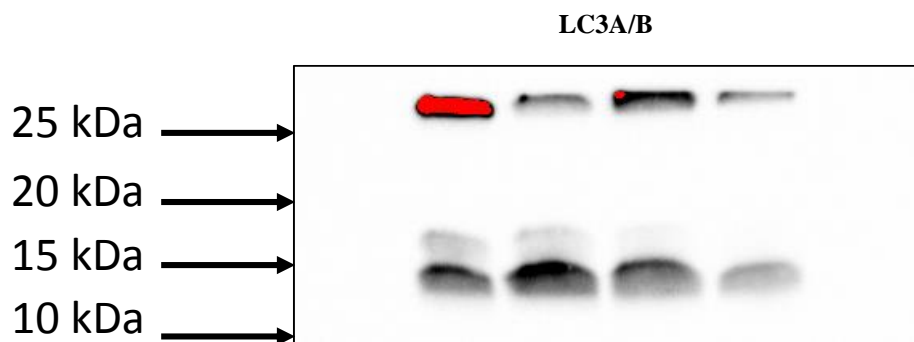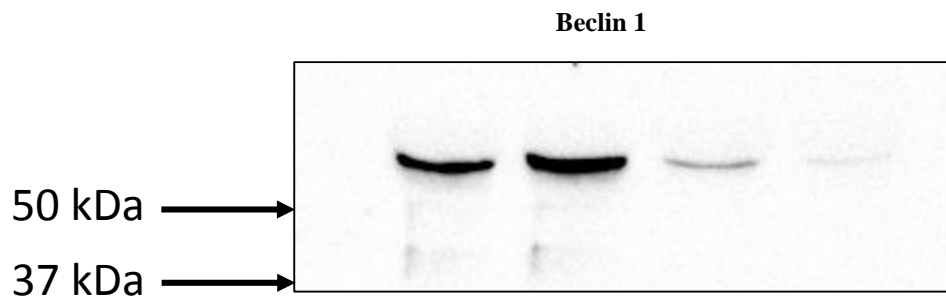
5) Supplementary Fig. 4 (MDA-MB-231 3D)

Integrin-α5

150 kDa ⟶

Integrin-β5

150 kDa ⟶

100 kDa ⟶

β-actin

50 kDa ⟶

Note: The first lane, second lane, third lane and the fourth lane indicate Control, CBD (10 µM), CBD (25 µM) and CBD (50 µM) treated groups respectively in the above represented blots.
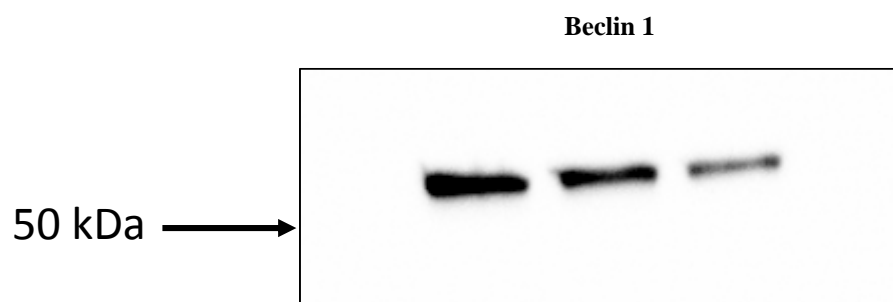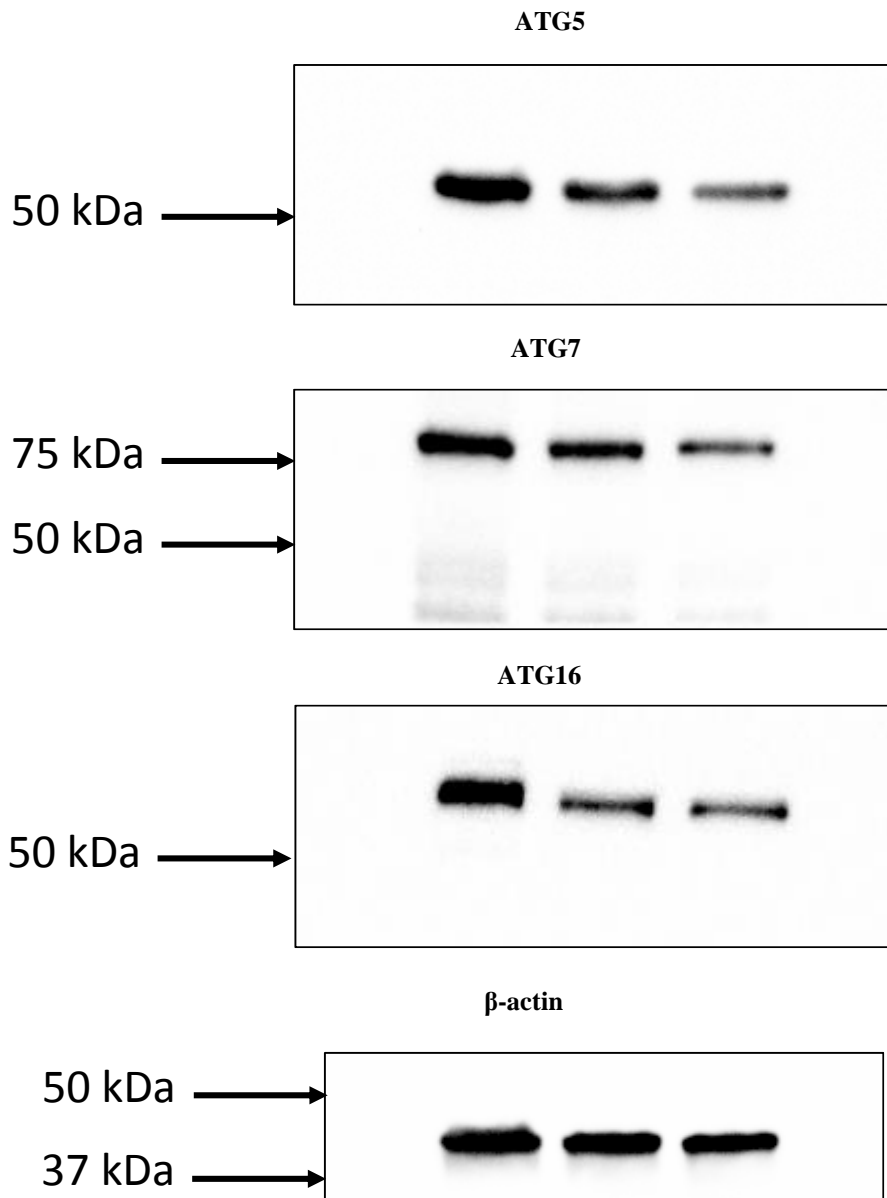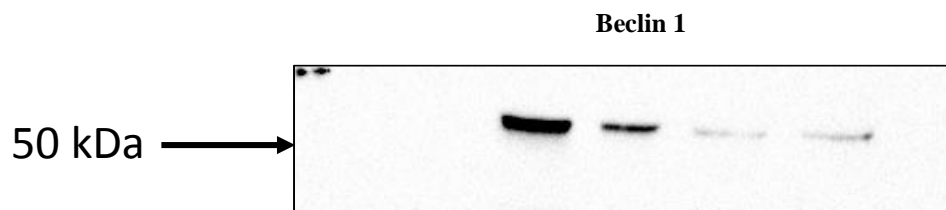
6) Supplementary Fig. 5A (MDA-MB-231 2D)

Beclin 1

50 kDa ⟶

ATG5

50 kDa ⟶

ATG7

75 kDa ⟶

ATG16

50 kDa ⟶

37 kDa ⟶

β-actin

50 kDa ⟶

37 kDa ⟶

Supplementary Fig. 5D (MDA-MB-231 3D)

Beclin 1

50 kDa ⟶

37 kDa ⟶

LC3A/B

25 kDa ⟶

20 kDa ⟶

15 kDa ⟶

10 kDa ⟶

β-actin

50 kDa ⟶

37 kDa ⟶

7) Supplementary Fig. 6A (MDA-MB-468 2D)

Beclin 1

50 kDa ⟶

ATG5

50 kDa →

ATG7

75 kDa →
50 kDa →

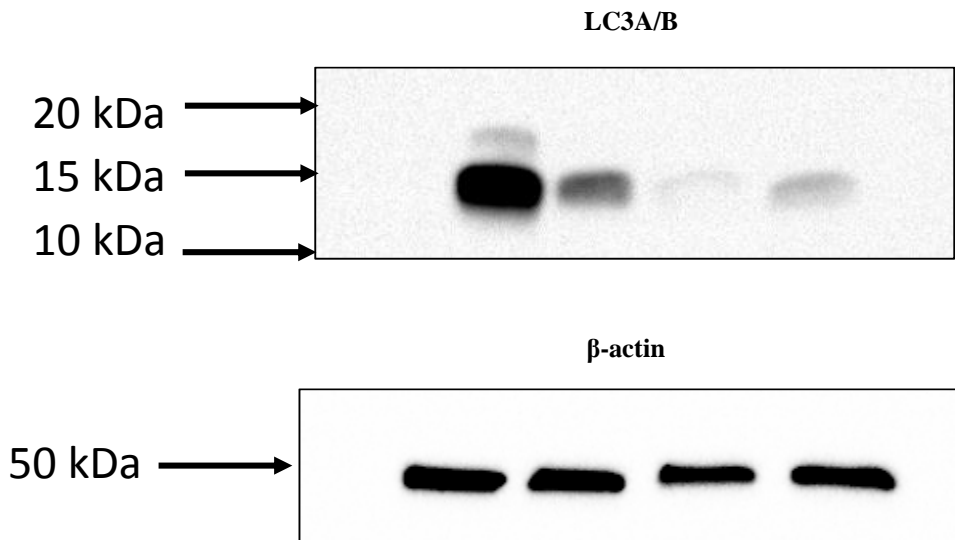**ATG16**

50 kDa →

β-actin

50 kDa →
37 kDa →

Note: The first lane, second lane and the third lane indicate Control, CBD (2.5 μM) and CBD (5 μM) treated groups respectively in the above represented blots.
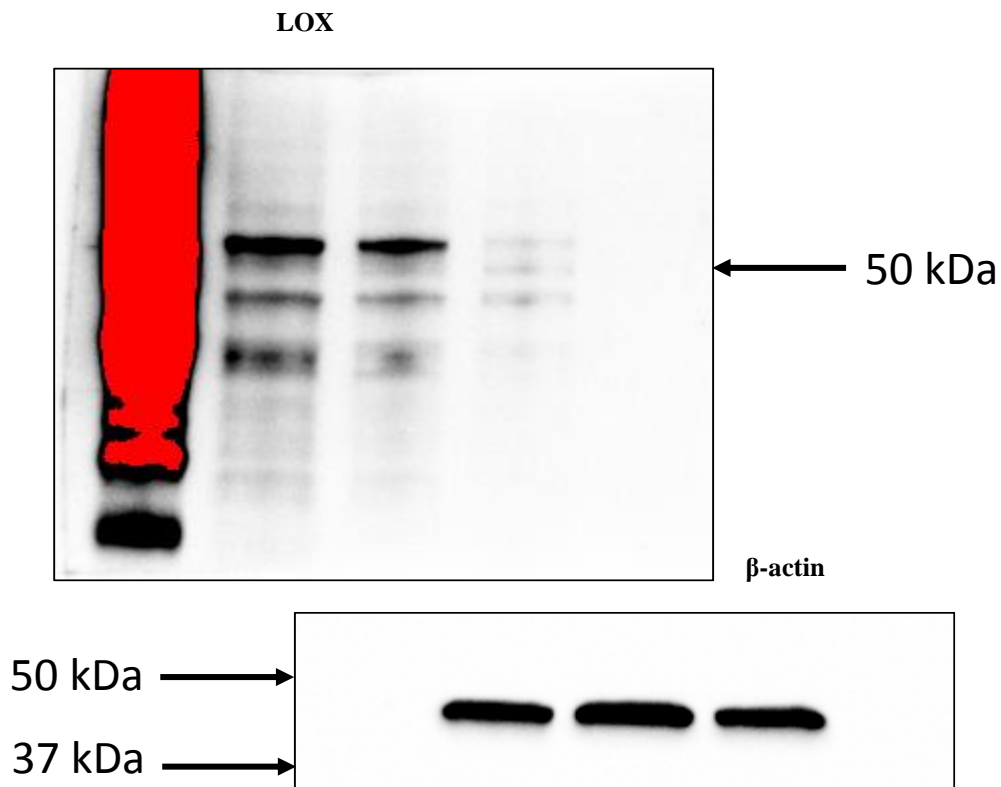
Supplementary Fig. 6B (MDA-MB-468 3D)

Beclin 1

50 kDa →

LC3A/B

20 kDa

15 kDa

10 kDa

β-actin

50 kDa

Note: The first lane, second lane, third lane and the fourth lane indicate Control, CBD (10 μM), CBD (25 μM) and CBD (50 μM) treated groups respectively in the above represented blots.
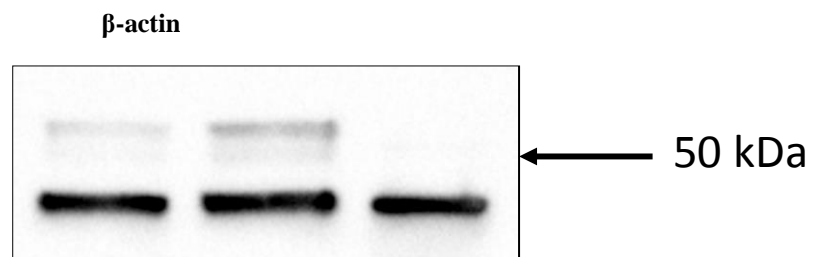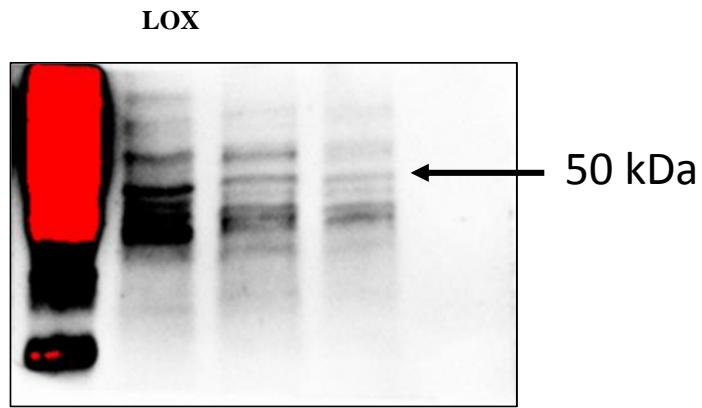
8) Supplementary Fig. 7 (MDA-MB-231 and MDA-MB-468 2D)

MDA-MB-231 2D

LOX

50 kDa

β-actin

50 kDa

37 kDa

Note: The first lane, second lane and the third lane indicate Control, CBD (2.5 μM) and CBD (5 μM) treated groups respectively in the above represented blots.

MDA-MB-468 2D

LOX

50 kDa

β-actin

50 kDa

Note: The first lane, second lane and the third lane indicate Control, CBD (2.5 µM) and CBD (5 µM) treated groups respectively in the above represented blots.