Mitofusin-2 modulates the epithelial to mesenchymal transition in thyroid cancer progression

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Running title: Mitofusin-2 deficiency elicits EMT in thyroid cancer

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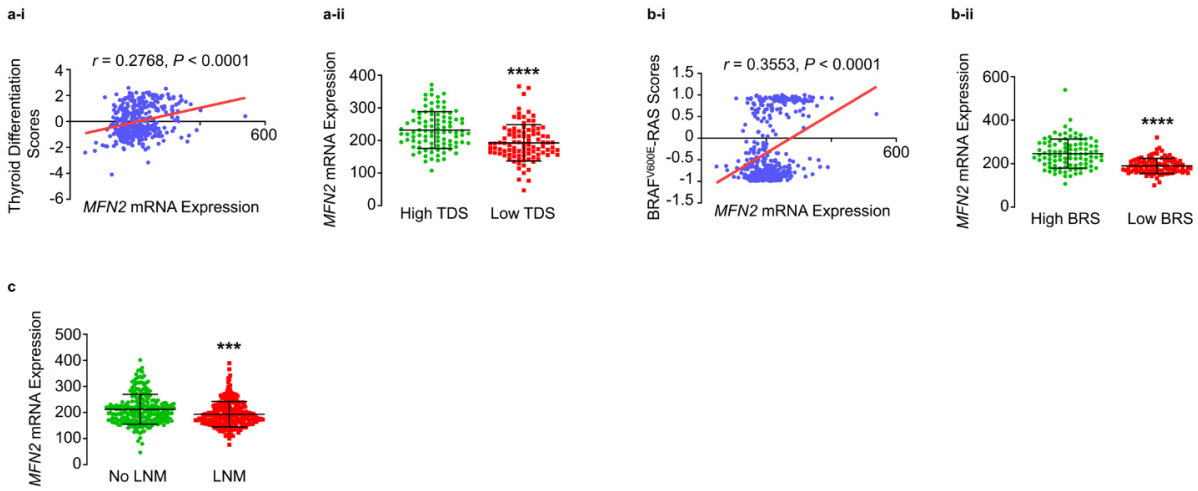
Supplementary Figure 1

MFN2 expression is a prognostic marker in human thyroid cancer.

(A-i) Relationship of *MFN2* expression with TDS. Correlation analysis using Pearson correlation coefficient and (A-ii) comparison of *MFN2* expression between high and low TDS tumors.

(B-i) Relationship of *MFN2* expression with BRS. Correlation analysis using Pearson correlation coefficient and (B-ii) comparison of *MFN2* expression between high and low BRS tumors.

(C) Comparison of MFN2 expression between tumors without and with LNM. Data represent the mean ± SD. Asterisks (P < 0.001 [***], P < 0.0001 [****]) indicate significant differences from the statistical analyses. Abbreviations: TDS, thyroid differentiation scores; BRS, BRAFV600E-RAS scores; LNM, lymph node metastasis.



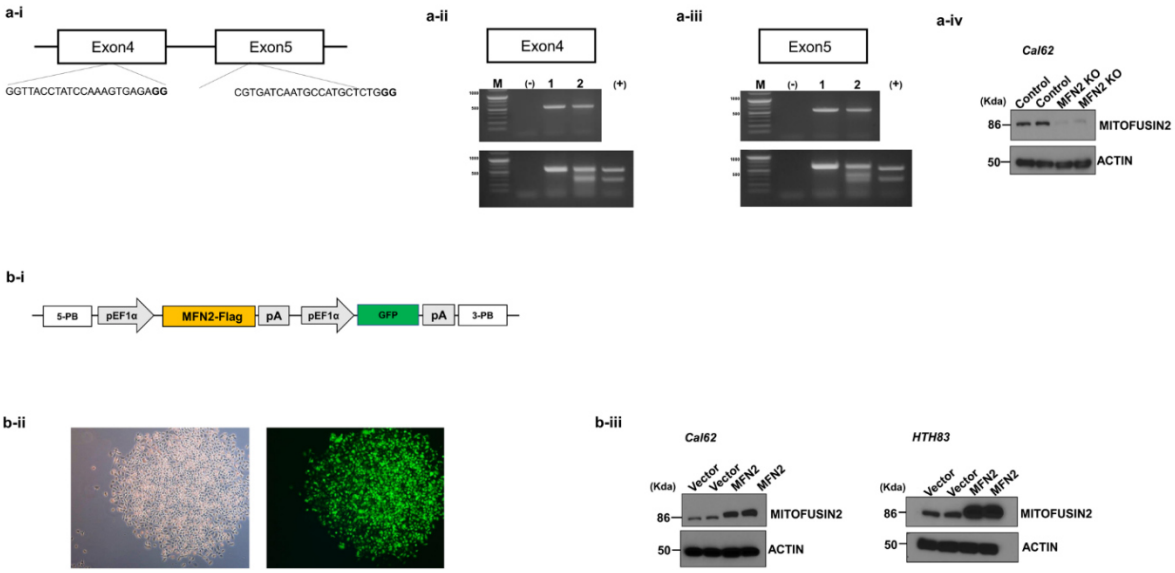
Supplementary Figure 2

(A) Knockout of MFN2 in the Cal 62 cell line. (A-i) Information on two guide RNAs on exon 4 and exon 5. Mutation assay with the T7E1 enzyme on exon 4 (A-ii) and exon 5 (A-iii); M: marker DNA ladder, (1): negative control, 1: wild-type genome, 2: transfected cells, (+): positive control. (A-iv) MFN2 knockout in the Cal62 cell line confirmed by western blotting.

(B-i) Illustration of the MFN2 and GFP expression vector.

(B-ii) Representative GFP-positive colony (left: brightness, right: fluorescence).

(B-iii) MFN2 overexpression in the Cal62 and HTH83 cell lines confirmed by western blotting.



Supplementary Figure 3

(A) Mitofusin-2 was not associated with the proliferation ability in Cal62 cells.

(A-i) Assay for comparing the proliferation ability of *MFN2* KO and control Cal62 cells (n.s; not significant).

(A-ii) Assay for comparing colony formation between *MFN2* KO and control Cal62 cells on day 7. (A-iii) Quantification of the colony forming assay (n.s; no significant difference).

(A-iv) Assay for comparing the proliferation ability of Cal62 Vector and *MFN2* groups.

(A-v) Image representing the colony formation by the Cal62 Vector and *MFN2* groups on day 7. (A-vi) Quantification of (A-v).

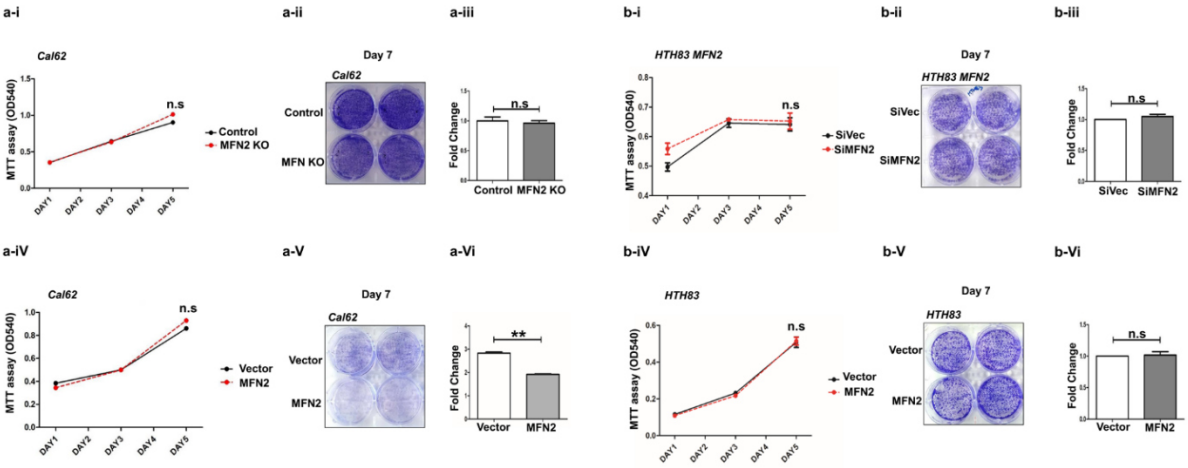
(B) Mitofusin-2 was also not associated with cell proliferation ability in HTH83 cells.

(B-i) Assay for comparing the proliferation ability of HTH83 *MFN2*-SiVector and SiMFN2 groups (n.s; not significant).

(B-ii) Image representing colony formation by the HTH83 *MFN2*-SiVector and SiMFN2 groups. (B-iii) Quantification of (B-ii) (n.s; no significant difference).

(B-iv) Assay for comparing the proliferation ability of the HTH83 Vector and *MFN2* groups. (B-v) Image representing colony formation by the HTH83 Vector and *MFN2* groups.

(B-vi) Quantification of (B-v) (n.s; no significant difference). Asterisks (P < 0.01 [**]) indicate significant differences from the statistical analyses. Each data point represents the mean±standard error of three independent experiments.

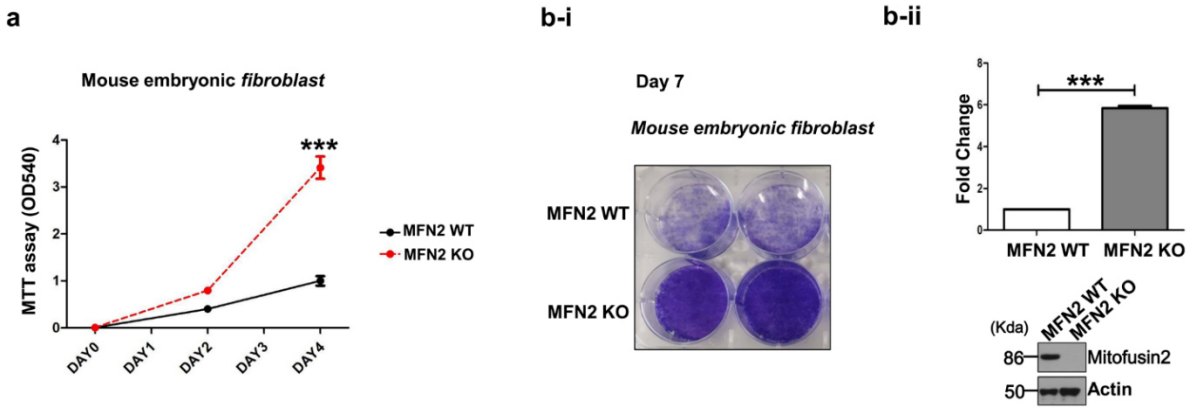


Supplementary Figure 4

(A) Mitofusin-2 was associated with proliferation ability of MEF cells. Assay for comparing the proliferation ability of the *MFN2* KO and MEF WT cells.

(B-i) Colony forming assay of *MFN2* KO and WT MEF cells on day 7.

(B-ii) Quantification of the colony forming assay. Asterisks ($P < 0.05$ [*], $P < 0.01$ [**], $P < 0.001$ [***]) indicate significant differences from the statistical analyses. Each data point represents the mean±standard error of three independent experiments.

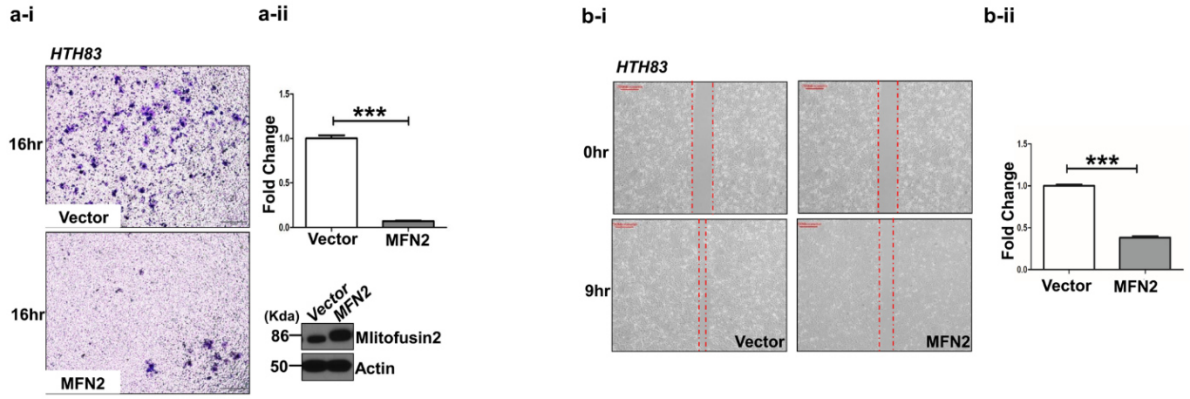


Supplementary Figure 5

(A-i) Transwell assays were performed in the HTH83 Vector and *MFN2* overexpression groups. A representative image was taken 12 h after the initial seeding (pore size 0.8 μm). (A-ii) Graph of the quantified results of (A-i).

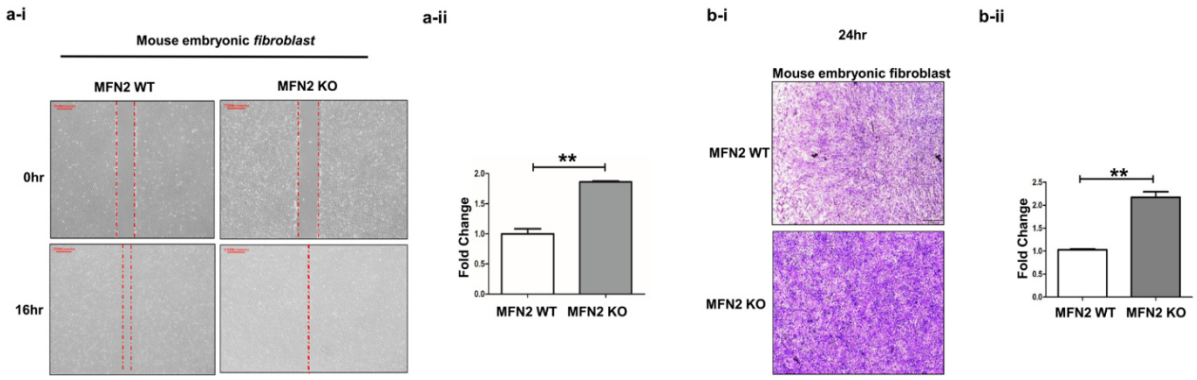
(B-i) Wound-healing assays were performed to investigate the differences between the HTH83 Vector and HTH83 *MFN2* OE groups. We measured the gap distance 9 h after the initial scratching. (B-ii) Graph of the quantified results of (B-i). Asterisks ($P < 0.05$ [*], $P < 0.01$ [**], $P < 0.001$ [***]) indicate significant differences from the statistical analyses. Each data point represents the mean

±standard error of three independent experiments.



Supplementary Figure 6

(A-i) Transwell assays were performed in *MFN2* WT and KO MEF cells. A representative image was taken 24 h after the initial seeding (pore size 0.8 μm). (A-ii) Graph of the quantified results of (A-i). Significantly more cells migrated from *MFN2* KO MEFs than from the *MFN2* WT MEFs. (B-i) Wound-healing assays were performed to investigate the differences between *MFN2* WT and KO MEFs. We measured the gap distance 16 h after the initial scratching. (B-ii) Quantification of (B-i) compared to the initial scratch size of the first gap distance after 16 h. Asterisks ($P < 0.05$ [*], $P < 0.01$ [**], $P < 0.001$ [***]) indicate significant differences from the statistical analyses. Each data point represents the mean±standard error of three independent experiments.



Supplementary Figure 7

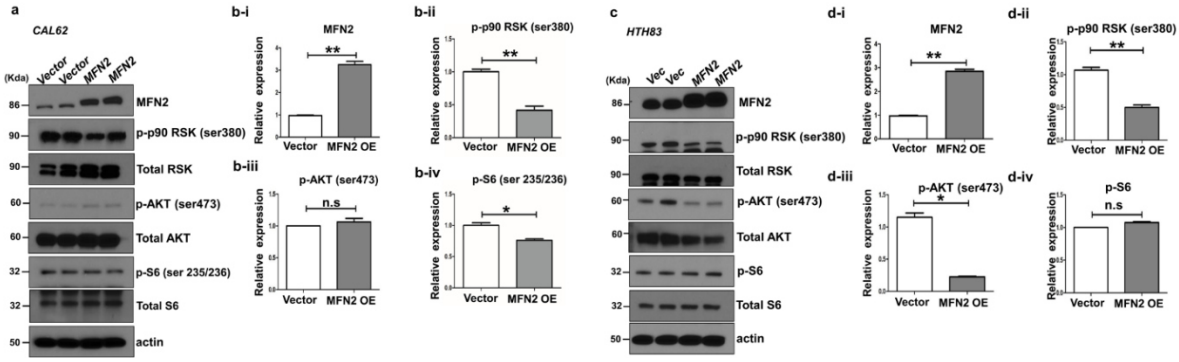
MFN2 overexpression leads to pERK signal suppression.

(A) Immunoblotting of MFN2, *p-p*90 RSK (Ser380), *p*AKT (Ser473), and *p*-S6 (Ser235/236) was conducted using lysates from Cal62 C and Cal62 MFN2 OE cells.

(B) Quantification of (B-i) MFN, (B-ii) p-p90 RSK (Ser380), (B-iii) p-AKT (Ser473), and (B-iv) p-S6 (Ser235/236) levels.

(C) Immunoblotting for investigating the levels MFN2, *p-p*90 RSK (Ser380), *p*AKT (Ser473), and *p*- S6 (Ser235/236) was performed using cell lysates from the HTH83 Vector and HTH83 MFN2 overexpression groups.

(D) Quantification of (D-i) *MFN2*, (D-ii) *p-p*90 RSK (Ser380), (D-iii) *p-AKT* (Ser473), and (D-iv) *p*-S6 (Ser235/236). Asterisks ($P < 0.05$ [*], $P < 0.01$ [**], $P < 0.001$ [***]) indicate significant differences from the statistical analyses. Each data point represents the mean±standard error of three independent experiments.



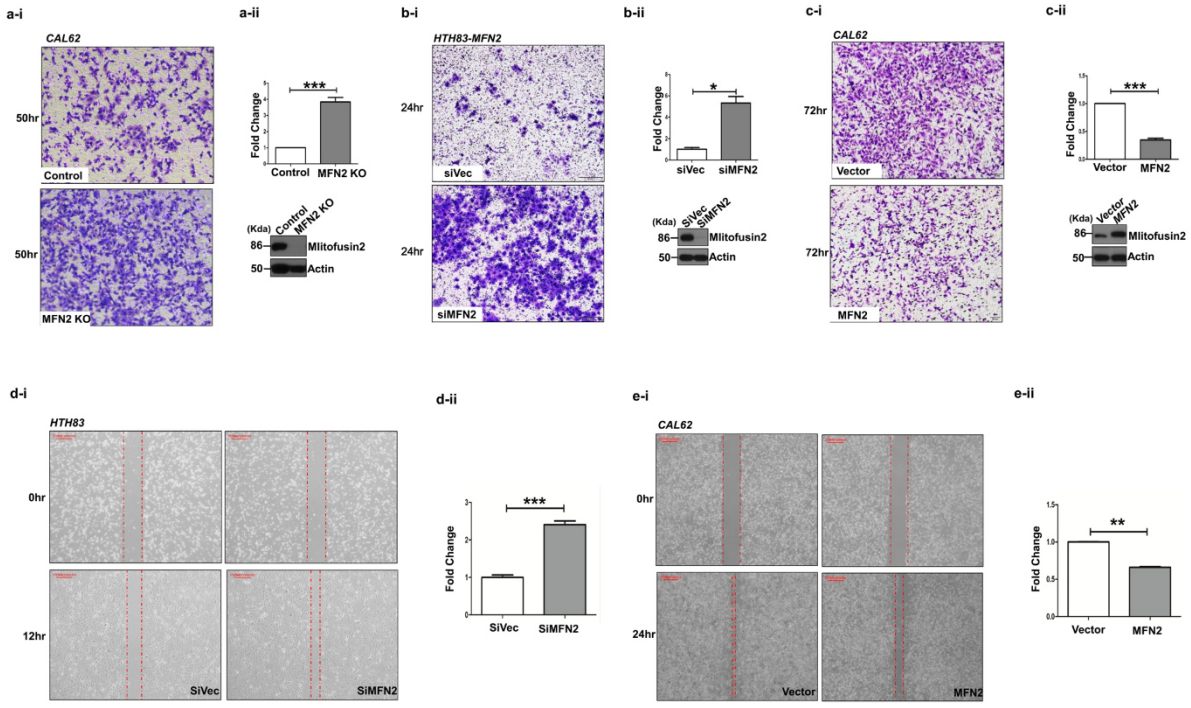
Supplementary Table 1. Real-time q-RT-PCR primer sequences

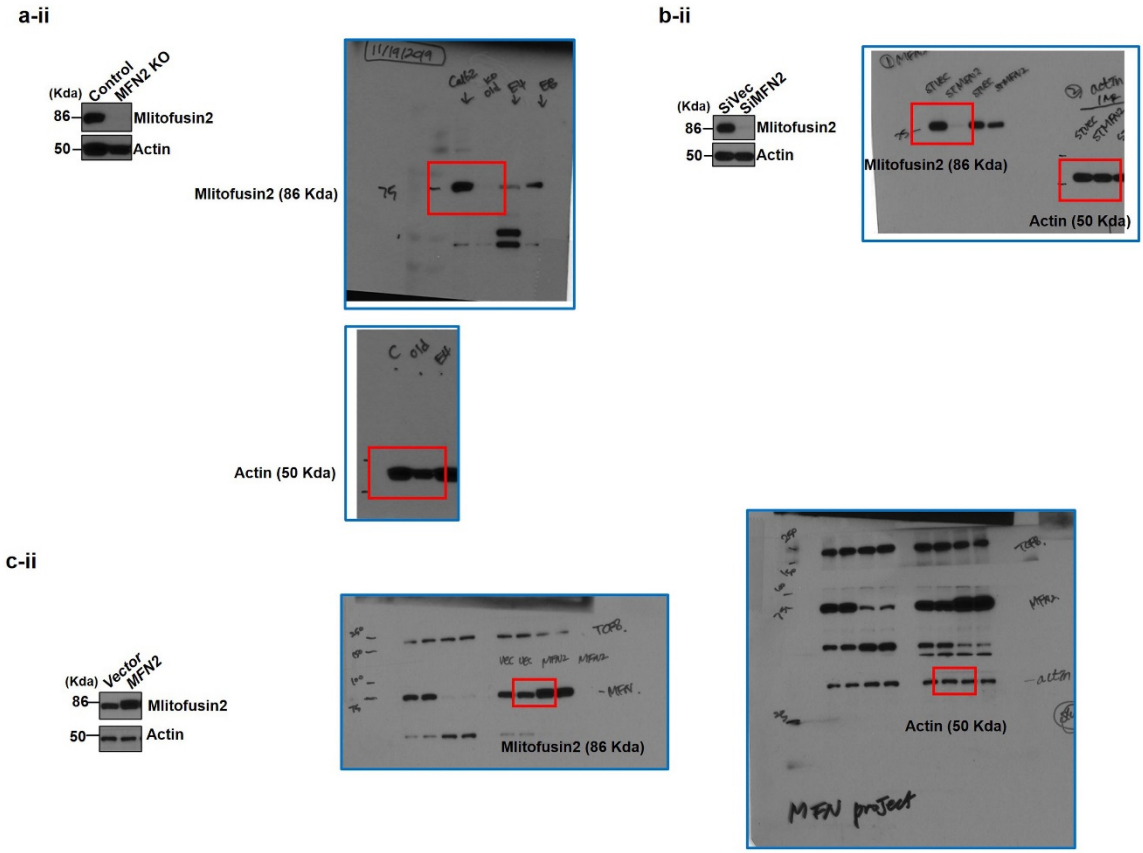
Supplementary Table 1. Primers used for mRNA expression analysis

Gene	Primer	5'→ 3' sequence
E-Cadherin	Forward	GAACGCATTGCCACATACAC
	Reverse	GAATTCGGGCTTGTTGTCAT
N-Cadherin	Forward	CCTGAGGGATCAAAGCCTGGAAC
	Reverse	TTGGAGCCTGAGACACGATTCTG
Snail	Forward	CTTCCAGCAGCCCTACGAC
	Reverse	CGGTGGGGTTGAGGATCT

Uncropped Gels shown as representative blots in Main& Supplementary Figures

Figure2


a-i
CAL62
50hr
Control
50hr
MFN2 KO
a-ii
Fold Change
***
Control
MFN2 KO
(Kda)
Control
MFN2 KO
86
Mlitofusin2
50
Actin
b-i
HTH83-MFN2
24hr
siVec
24hr
siMFN2
b-ii
Fold Change
*
siVec
siMFN2
(Kda)
SiVec
SiMFN2
86
Mlitofusin2
50
Actin
c-i
CAL62
72hr
Vector
72hr
MFN2
c-ii
Fold Change
***
Vector
MFN2
(Kda)
Vector
MFN2
86
Mlitofusin2
50
Actin
d-i
HTH83
0hr
12hr
SiVec
SiMFN2
d-ii
Fold Change
***
SiVec
SiMFN2
e-i
CAL62
0hr
24hr
Vector
MFN2
e-ii
Fold Change
**
Vector
MFN2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(Kda)
Control
MFN2 KO
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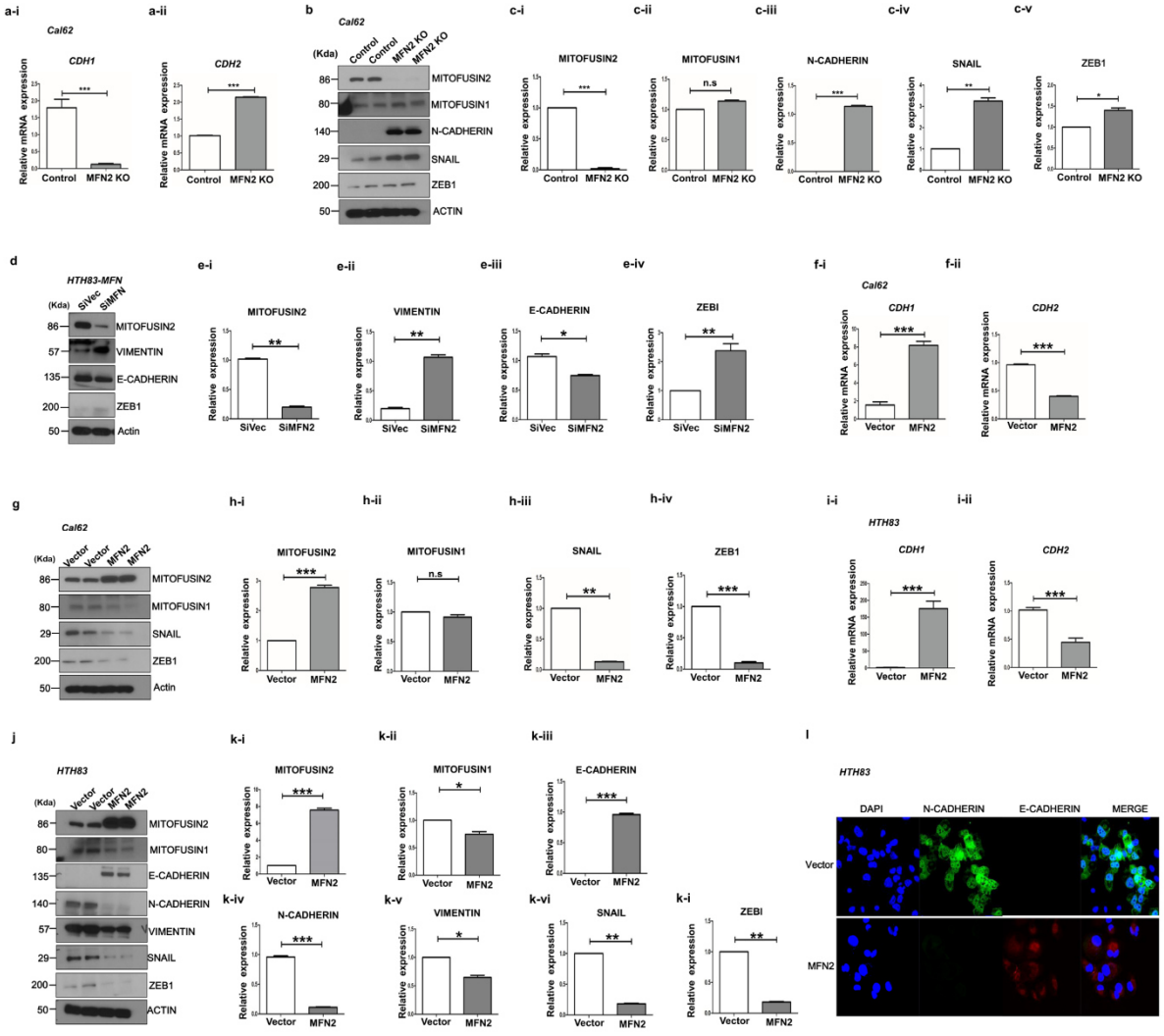
Mlitofusin2
50
Actin
Mlitofusin2 (86 Kda)
Actin (50 Kda)
b-ii
(Kda)
SiVec
SiMFN2
86
Mlitofusin2
50
Actin
Mlitofusin2 (86 Kda)
Actin (50 Kda)
c-ii
(Kda)
Vector
MFN2
86
Mlitofusin2
50
Actin
Mlitofusin2 (86 Kda)
Actin (50 Kda)
MFN project

Figure3


a-i
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CDH1
Relative mRNA expression
***
Control
MFN2 KO
a-ii
CDH2
Relative mRNA expression
***
Control
MFN2 KO
b
Cal62
(Kda)
Control
Control
MFN2 KO
MFN2 KO
86
80
140
29
200
50
MITOFUSIN2
MITOFUSIN1
N-CADHERIN
SNAIL
ZEB1
ACTIN
c-i
MITOFUSIN2
Relative expression
***
Control
MFN2 KO
c-ii
MITOFUSIN1
n.s
Control
MFN2 KO
c-iii
N-CADHERIN
***
Control
MFN2 KO
c-iv
SNAIL
**
Control
MFN2 KO
c-v
ZEB1
*
Control
MFN2 KO
d
HTH83-MFN
(Kda)
SiVec
SiMFN
86
57
135
200
50
MITOFUSIN2
VIMENTIN
E-CADHERIN
ZEB1
Actin
e-i
MITOFUSIN2
Relative expression
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SiVec
SiMFN2
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VIMENTIN
**
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E-CADHERIN
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e-iv
ZEBI
**
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SiMFN2
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Cal62
CDH1
Relative mRNA expression
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f-ii
CDH2
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MITOFUSIN2
MITOFUSIN1
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Relative expression
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MFN2
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Vector
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HTH83
CDH1
Relative mRNA expression
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N-CADHERIN
VIMENTIN
SNAIL
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k-i
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Relative expression
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k-ii
MITOFUSIN1
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MFN2
k-iii
E-CADHERIN
***
Vector
MFN2
k-iv
N-CADHERIN
***
Vector
MFN2
k-v
VIMENTIN
*
Vector
MFN2
k-vi
SNAIL
**
Vector
MFN2
k-i
ZEBI
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Vector
MFN2
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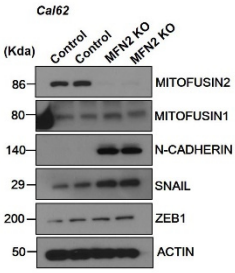
N-CADHERIN
E-CADHERIN
MERGE
Vector
MFN2

b



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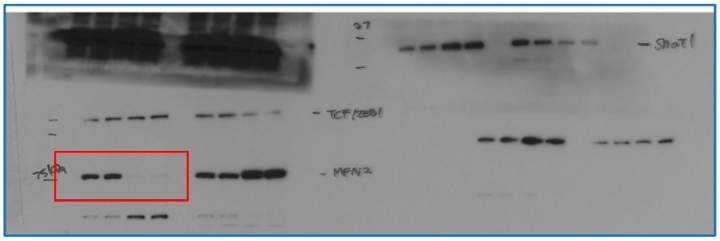

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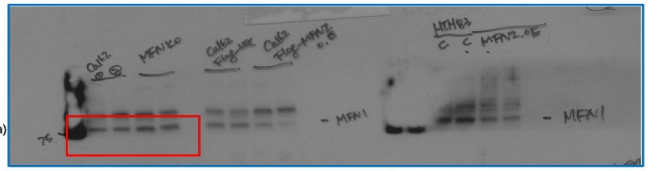

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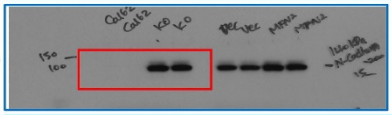

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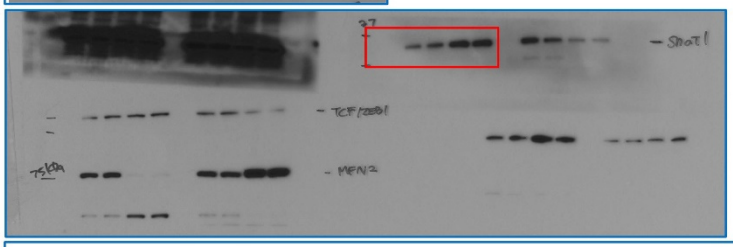
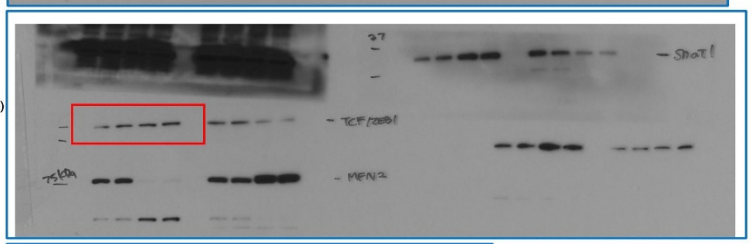


ZEB1 (200 Kda)



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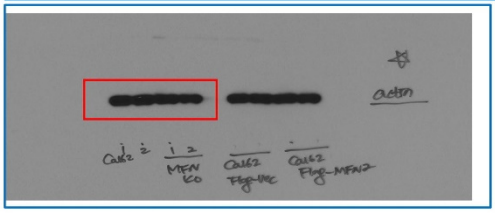

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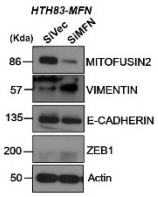
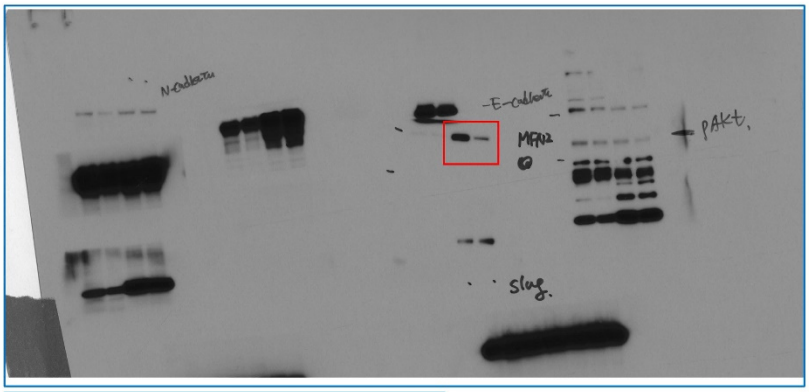
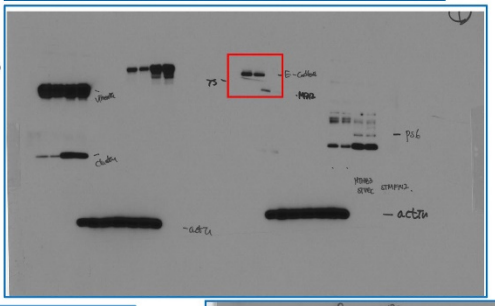


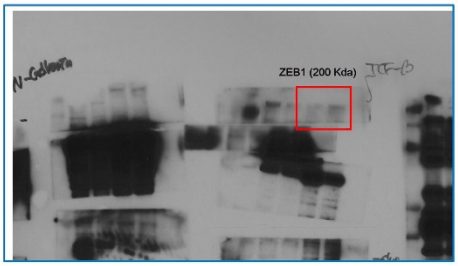
MITOFUSIN2 (86 Kda)



VIMENTIN (57 Kda)

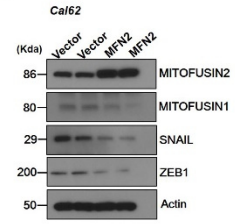
E-CADHERIN (135 Kda)

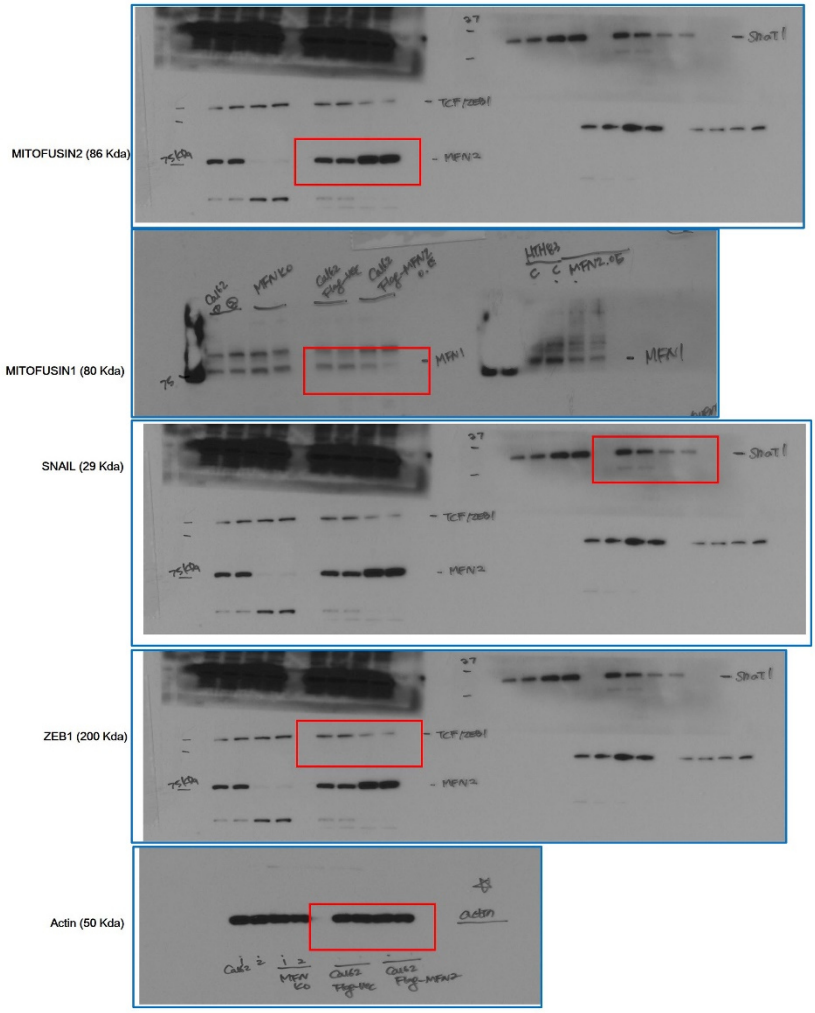




Actin (50 Kda)

g


Cal62
(Kda)
Vector
Vector
MFN2
MFN2
86
MITOFUSIN2
80
MITOFUSIN1
29
SNAIL
200
ZEB1
50
Actin


MITOFUSIN2 (86 Kda)
MITOFUSIN1 (80 Kda)
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Short
TCF/ZEB1
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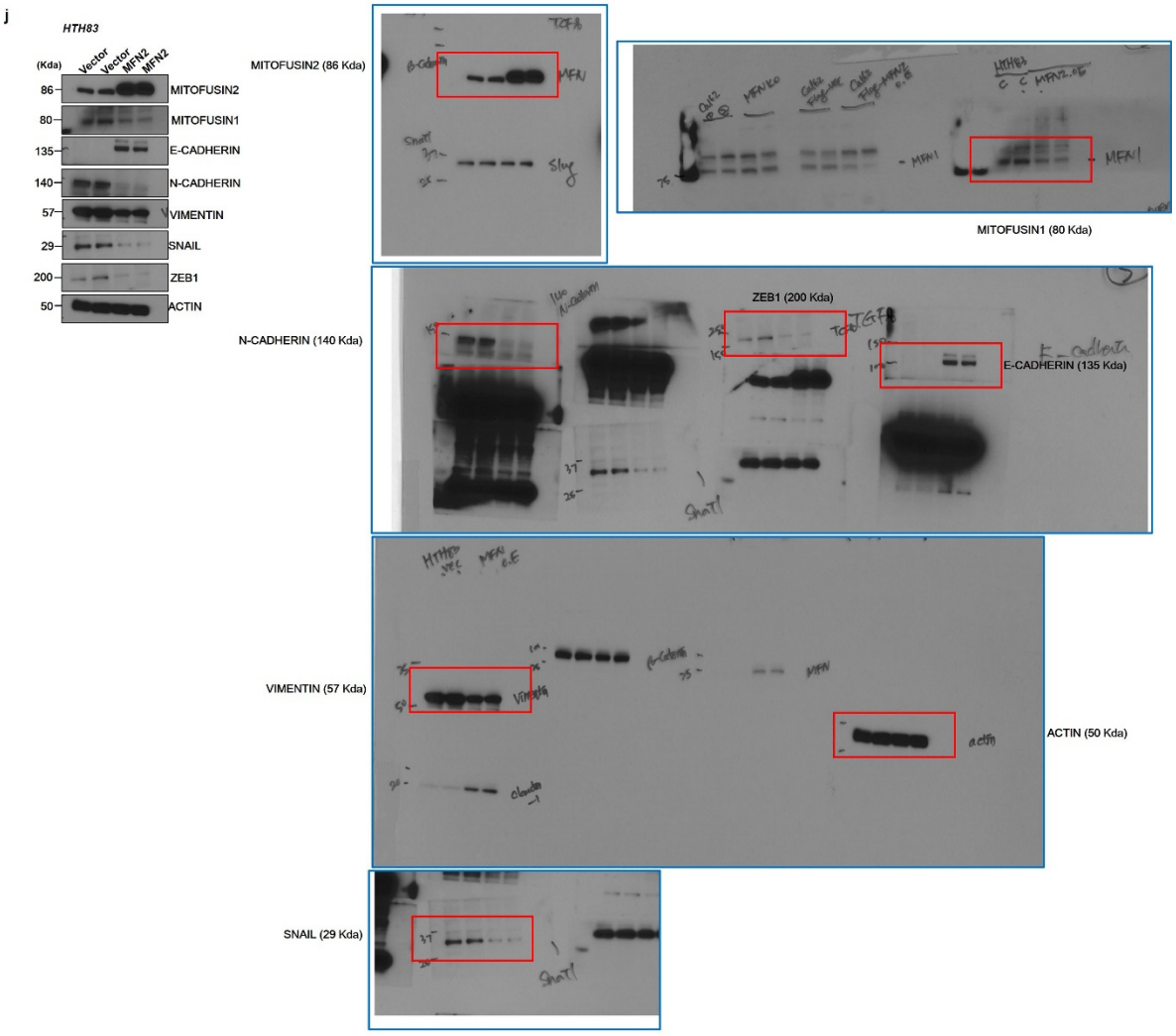
MFN1
Actin

j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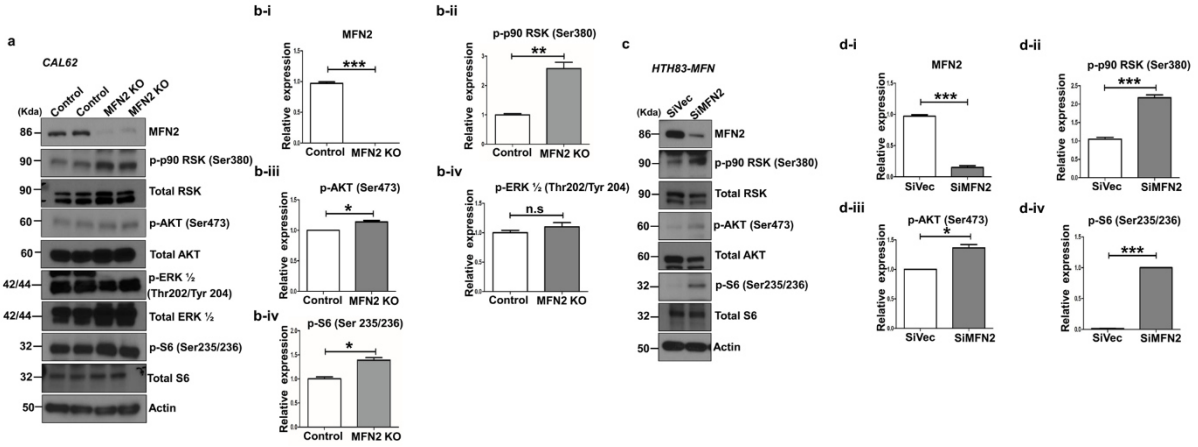
140
N-CADHERIN
57
VIMENTIN
29
SNAIL
200
ZEB1
50
ACTIN
MITOFUSIN2 (86 Kda)
MITOFUSIN1 (80 Kda)
ZEB1 (200 Kda)
N-CADHERIN (140 Kda)
E-CADHERIN (135 Kda)
VIMENTIN (57 Kda)
ACTIN (50 Kda)
SNAIL (29 Kda)

Figure 4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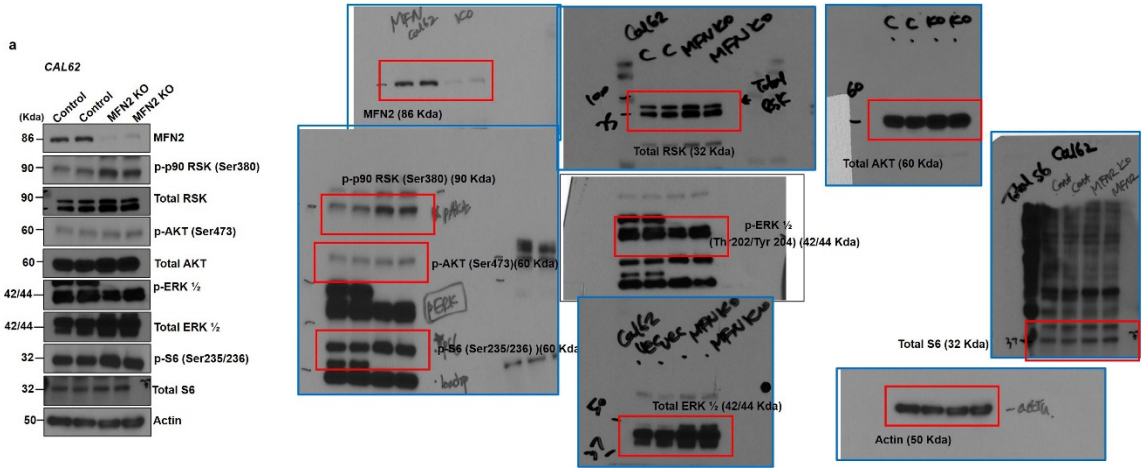
CAL62
(Kda)
Control
Control
MFN2 KO
MFN2 KO
86
MFN2
90
p-p90 RSK (Ser380)
90
Total RSK
60
p-AKT (Ser473)
60
Total AKT
42/44
p-ERK ½ (Thr202/Tyr 204)
42/44
Total ERK ½
32
p-S6 (Ser235/236)
32
Total S6
50
Actin
b-i
MFN2
Relative expression
***
Control
MFN2 KO
b-ii
p-p90 RSK (Ser380)
**
b-iii
p-AKT (Ser473)
*
b-iv
p-ERK ½ (Thr202/Tyr 204)
n.s
p-S6 (Ser 235/236)
c
HTH83-MFN
SiVec
SiMFN2
d-i
MFN2
SiVec
SiMFN2
d-ii
p-p90 RSK (Ser380)
d-iii
p-AKT (Ser473)
d-iv
p-S6 (Ser235/236)


a
CAL62
(Kda)
Control
Control
MFN2 KO
MFN2 KO
MFN2
p-p90 RSK (Ser380)
Total RSK
p-AKT (Ser473)
Total AKT
p-ERK ½
Total ERK ½
p-S6 (Ser235/236)
Total S6
Actin
MFN2 (86 Kda)
Total RSK (32 Kda)
Total AKT (60 Kda)
p-p90 RSK (Ser380) (90 Kda)
p-AKT (Ser473)(60 Kda)
p-ERK ½ (Thr202/Tyr 204) (42/44 Kda)
Total S6 (32 Kda)
Total ERK ½ (42/44 Kda)
Actin (50 Kda)

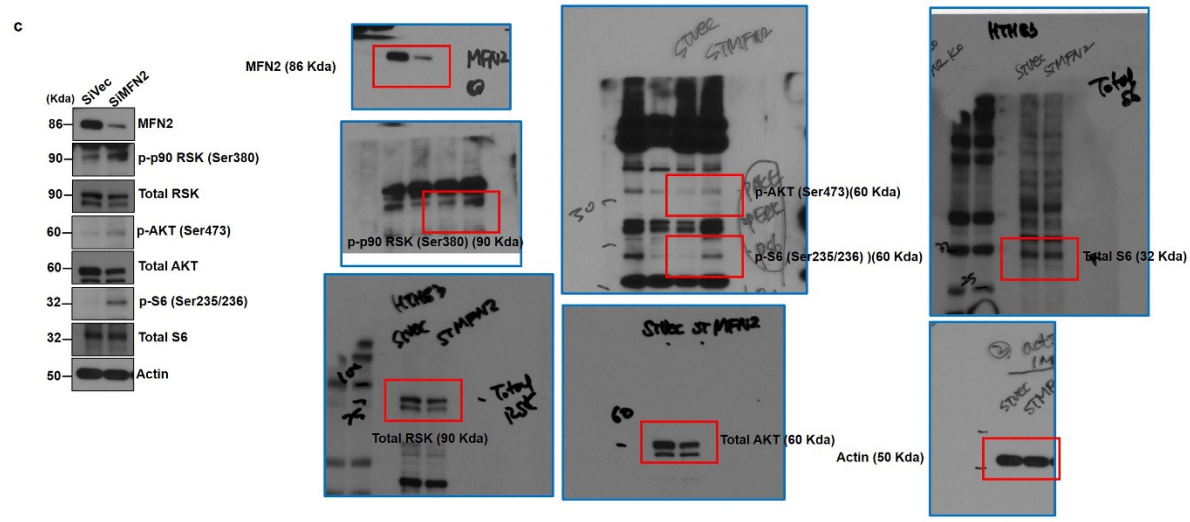
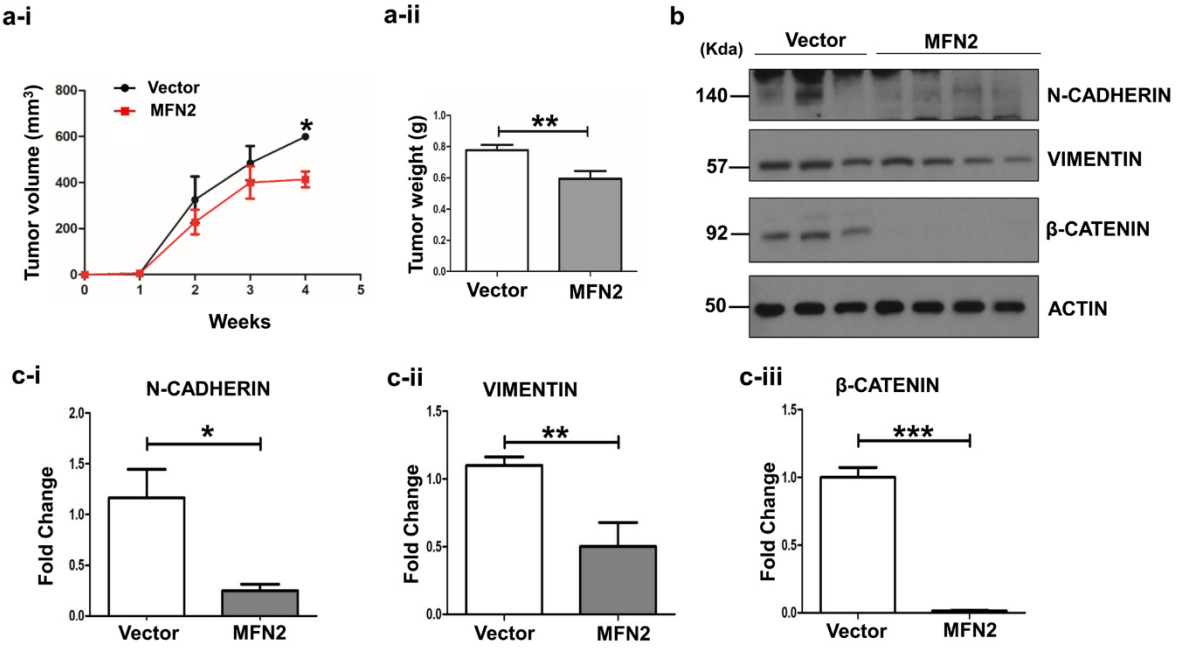
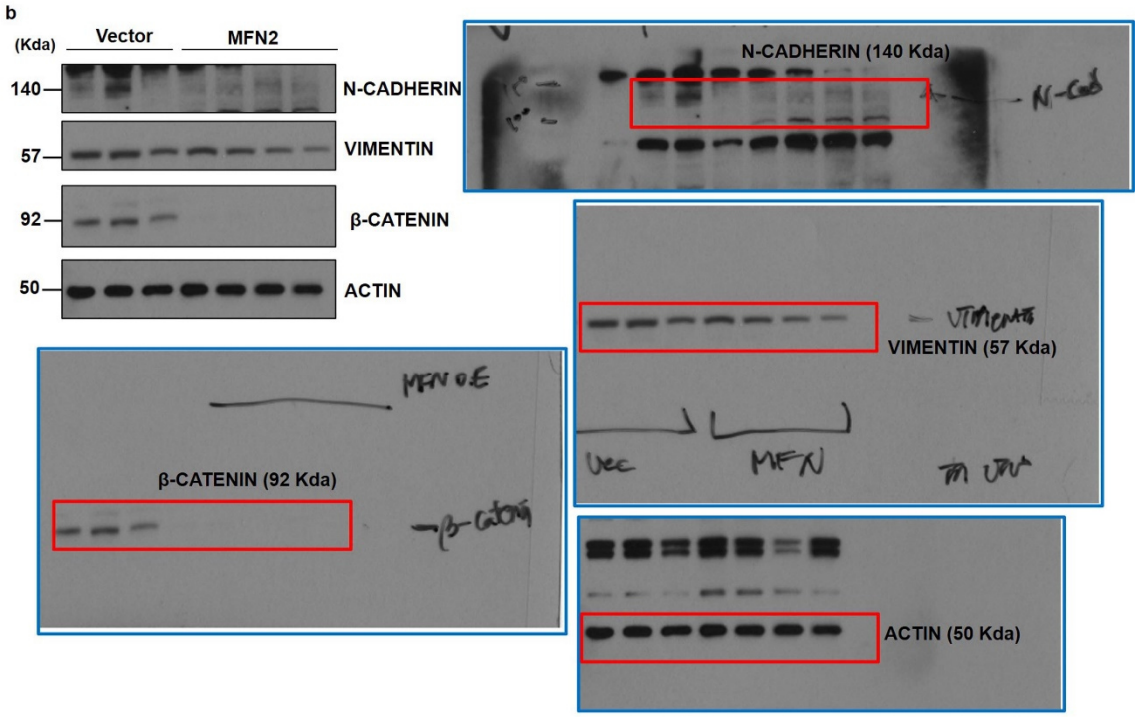
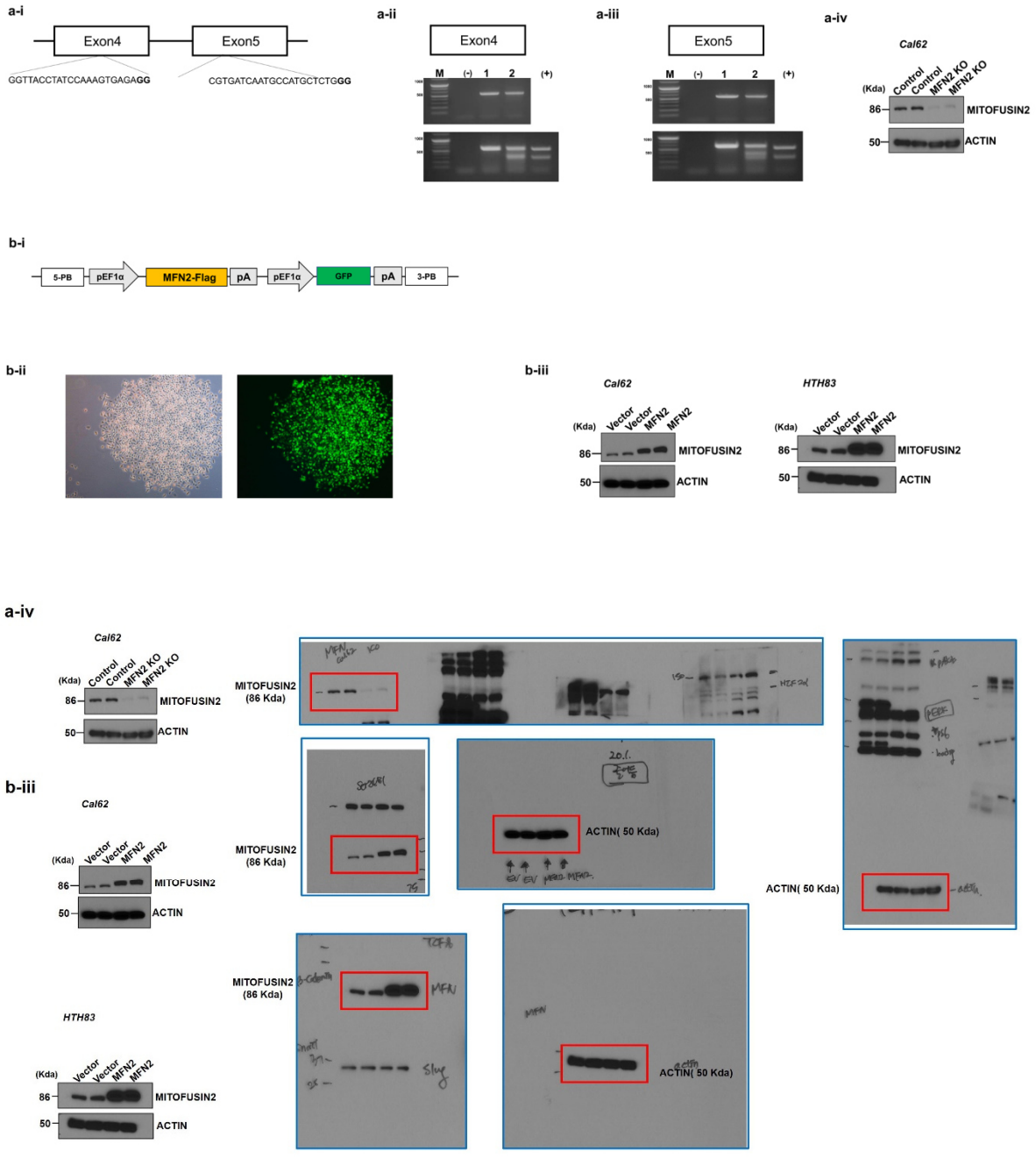

c
(Kda)
SiVec
SiMFN2
MFN2
p-p90 RSK (Ser380)
Total RSK
p-AKT (Ser473)
Total AKT
p-S6 (Ser235/236)
Total S6
Actin
MFN2 (86 Kda)
p-p90 RSK (Ser380) (90 Kda)
p-AKT (Ser473)(60 Kda)
p-S6 (Ser235/236))(60 Kda)
Total S6 (32 Kda)
Total RSK (90 Kda)
Total AKT (60 Kda)
Actin (50 Kda)

Figure 5

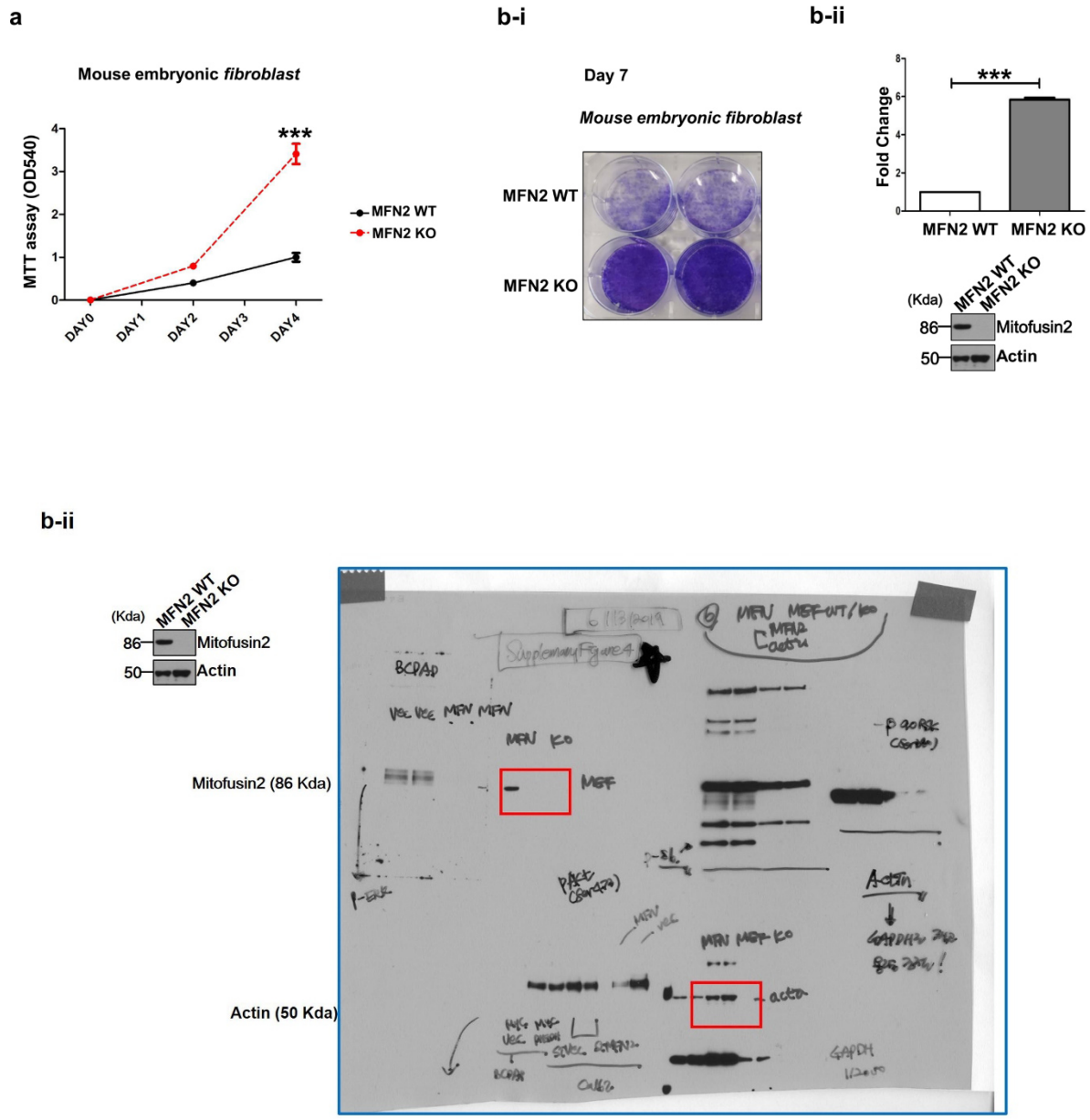

a-i
Tumor volume (mm³)
Vector
MFN2
Weeks
a-ii
Tumor weight (g)
Vector
MFN2
b
(Kda)
Vector
MFN2
140
N-CADHERIN
57
VIMENTIN
92
β-CATENIN
50
ACTIN
c-i
N-CADHERIN
Fold Change
c-ii
VIMENTIN
Fold Change
c-iii
β-CATENIN
Fold Change


b
(Kda)
Vector
MFN2
140
N-CADHERIN
57
VIMENTIN
92
β-CATENIN
50
ACTIN
N-CADHERIN (140 Kda)
VIMENTIN (57 Kda)
β-CATENIN (92 Kda)
ACTIN (50 Kda)

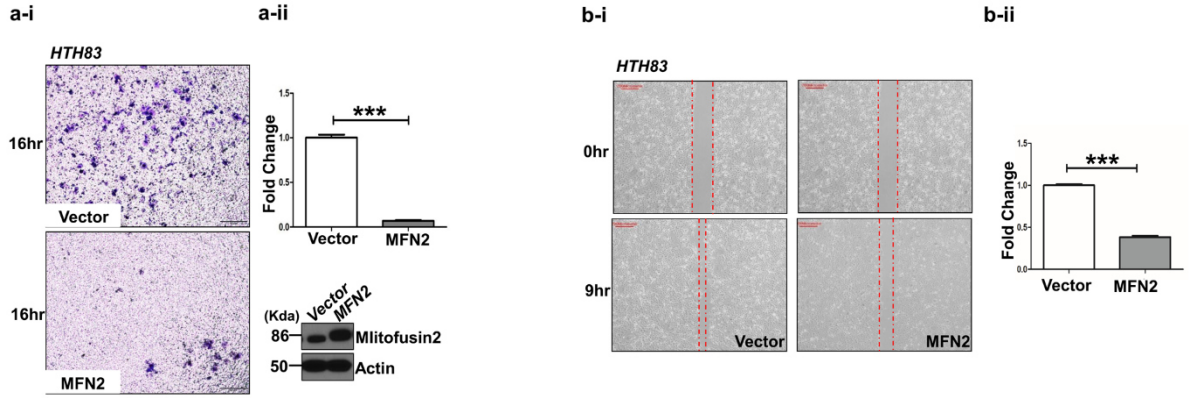
Supplementary Figure 2

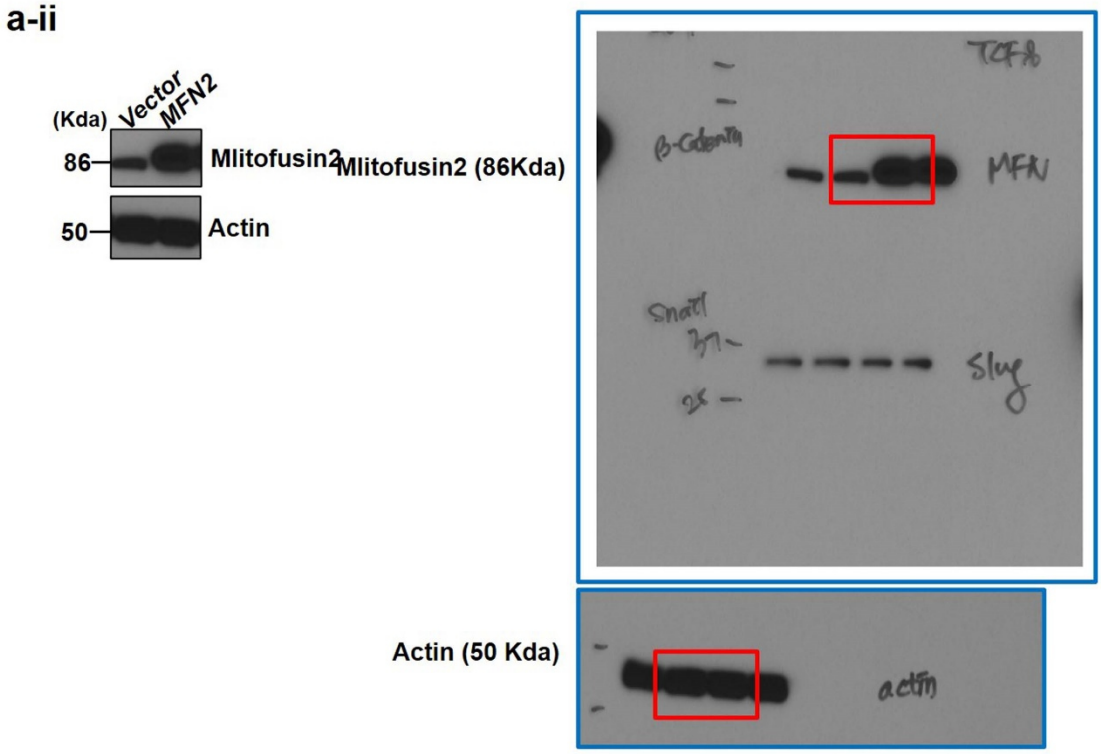


Supplementary Figure 4



Supplementary Figure 5





Supplementary Figure 7