Supplementary information

Activation of IGF-1 pathway and suppression of atrophy related genes are involved in *Epimedium* extract (icariin) promoted C2C12 myotube hypertrophy

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Supplementary Table

Supplementary Table S1. The list of antibodies

Antibodies	Company	Product #	Dilution WB (IF)	Host
p-AKT Ser473	Cell signaling	9271S	1:1000	Rabbit
AKT	Cell signaling	9272S	1:5000	Rabbit
p-AMPKα Thr172	Cell signaling	2531S	1:2000	Rabbit
AMPKα	Cell signaling	2532S	1:500	Rabbit
p-ERK1/2 Thr185/Tyr187	Millipore	04-797	1:2000	Rabbit
ERK1/2	Millipore	05-1152	1:5000	Mouse
p-mTOR Ser2448	Cell signaling	2971S	1:3000	Rabbit
mTOR	Cell signaling	2972S	1:5000	Rabbit
p-IGF-1Rβ Tyr1135/1136	Cell signaling	3024S	1:500	Rabbit
IGF-1Rβ	Cell signaling	3027S	1:2000	Rabbit
p-P70S6K Thr389	Cell signaling	9029S	1:2000	Rabbit
P70S6K	Cell signaling	9202S	1:5000	Rabbit
AR	Santa Cruz	SC-816	1:500	Rabbit
β-actin	Sigma Aldrich	A5441	1:10000	Mouse
GAPDH	GeneTex	GTX100118	1:10000	Rabbit
MSTN	GeneTex	GTX32624	1:1000	Rabbit
MYH (MyHC-T)	Santa Cruz	SC-20641	1:1000 (1:200)	Rabbit
MYH1/2/4 (MyHC-F)	Santa Cruz	SC-58797	1:250	Mouse
MYH7 (MyHC-S)	ABclonal	A7564	1:3000	Rabbit
Goat anti-rabbit IgG	Millipore	AP307P	1:5000	Goat
Goat anti-mouse IgG	Millipore	AP124P	1:5000	Goat
Alexa Flour 488 Goat-anti mouse IgG	Jackson Lab	111-545-003	1:5000	Goat

AR: androgen receptor; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; MSTN: myostatin; MyHC-F: myosin heavy chain-fast isoform; MyHC-S: myosin heavy chain-slow isoform; MyHC-T: myosin heavy chain-total isoform

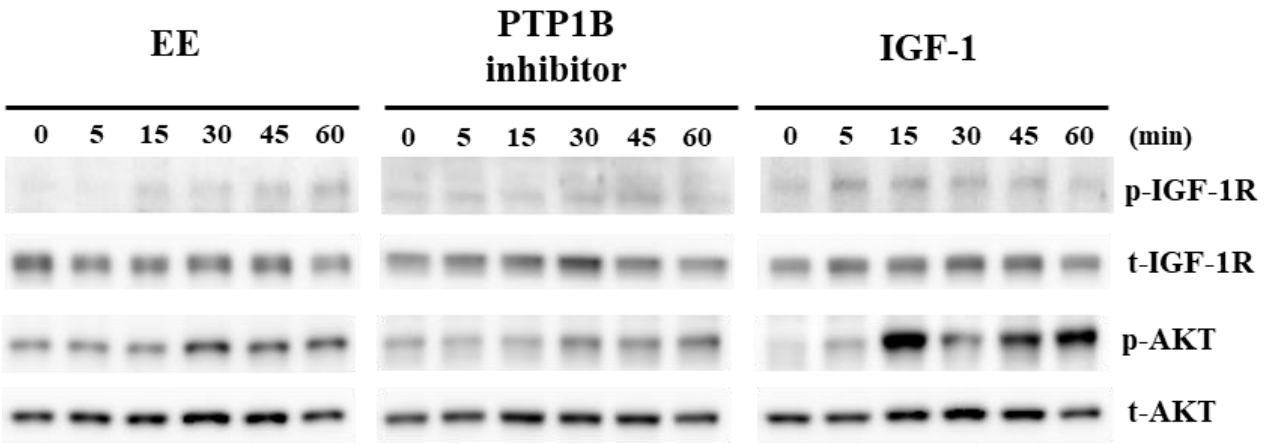
Supplementary Table

Supplementary Table S2. PCR primer sequences used for gene expression

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
MRF4	GTGGCCAAGTGTTTCGGATC	AAAGGCGCTGAAGACTGCTG
MSTN	GTCCAGAGGGATGACAGCAG	GTCTTGACGGGTCTGAGATA
MAFbx	AAGGAAGATGAACGCTGTCA	ATTGCCTCCCAGATAAAGTATGT
MuRF1	AGCCAAGACAATAGAGATGCCTACTTC	GGCCTTGAACTCATAGAGATCCAAC
GAPDH	TTCACCACCATGGAGAAGGC	GGCATGGACTGTGGTCATGA

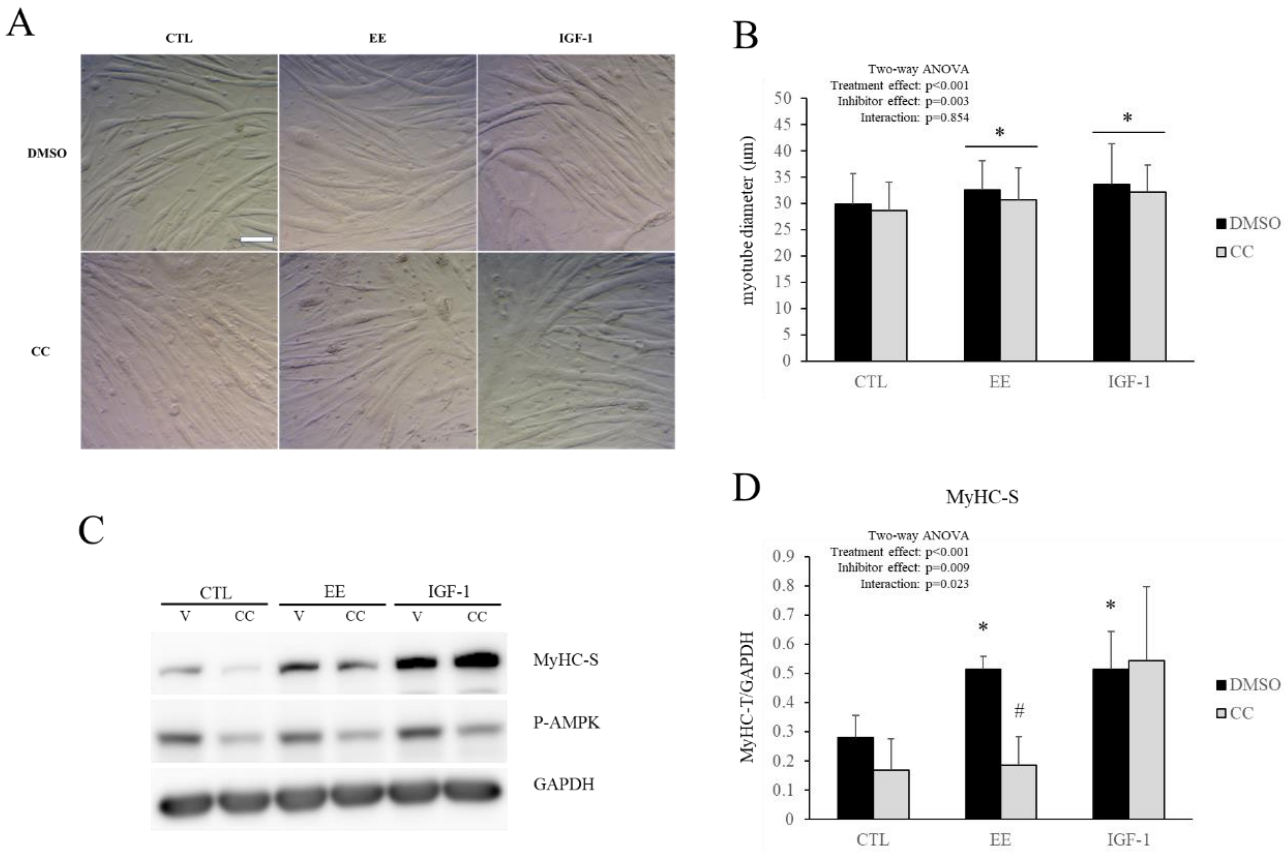
GAPDH: glyceraldehyde 3-phosphate dehydrogenase; MAFbx: muscle atrophy F-box; MRF4: myogenic regulatory factor 4; MSTN: myostatin; MuRF1: muscle RING finger 1;

Supplementary Figure



Supplementary Figure S1. The time-dependent effect of EE, PTP1B inhibitor, and IGF-1 on IGF-1R and AKT phosphorylation.

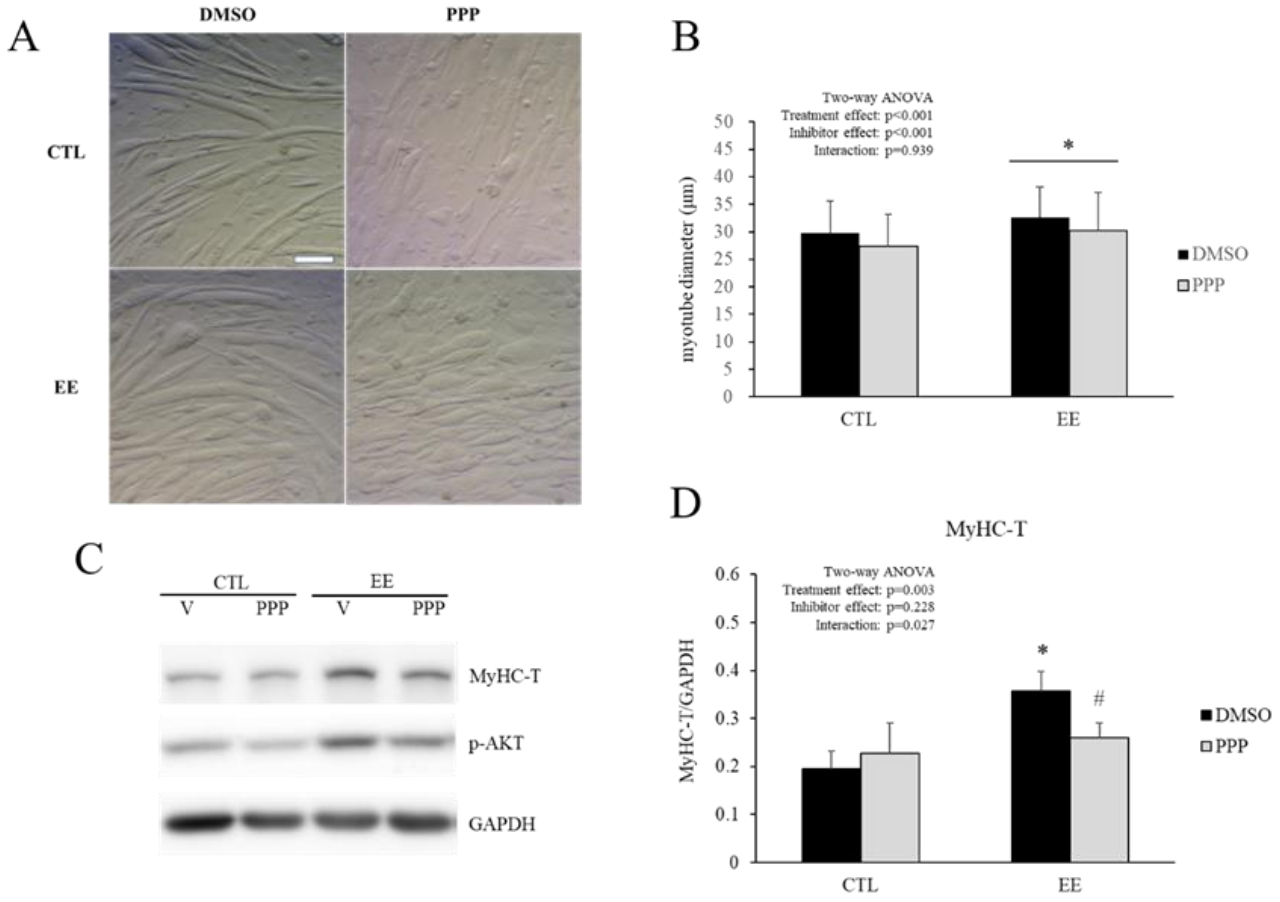
Differentiated C2C12 cells were starved overnight in serum-free medium before the indicated treatment. The levels of phosphorylated and total proteins before (0 min) and after treatments of EE (100 μg/ml), PTP1B inhibitor (10 μM), and IGF-1 (20 ng/ml) were evaluated by western blot for indicated time intervals (5-60 min) (N=3).



Supplementary Figure S2. The effect of AMPK inhibition on EE and IGF-1 induced MyHC-S expression.

AMPK inhibitor (compound C, CC; 10 μM) was applied 30 min before EE (100 μg/ml), IGF-1 (20 ng/ml) or control (CTL) treatment, where DMSO (0.1%) served as vehicle control (V). Differentiated C2C12 cells were maintained in serum-free medium overnight before pre-treated with CC or DMSO followed by indicated treatment for further 24h. (A) Representative images of differentiated C2C12 cells were taken at the end of the indicated treatment by a light microscope (scale bar, 100 μm). (B) The myotube diameter of each treatment group was determined by repetitive measurements of 90 myotubes in triplicate. Myotube hypertrophy was documented by the significant increment of myotube diameter as compared to CTL. Representative western blots (C) and densitometric analysis (D) showed both EE and IGF-1 induced AMPK phosphorylation and enhanced MyHC-S protein overexpression (N=3). CC treatment significantly abolished EE, but not IGF-1 induced MyHC-S overexpression. All data were expressed as mean ± SD. The symbol * stands for $p < 0.05$ as compared to the basal (CTL or CTL-DMSO); the symbol $^{\#}$ stands for $p < 0.05$ as compared to the intra-group DMSO treatment.

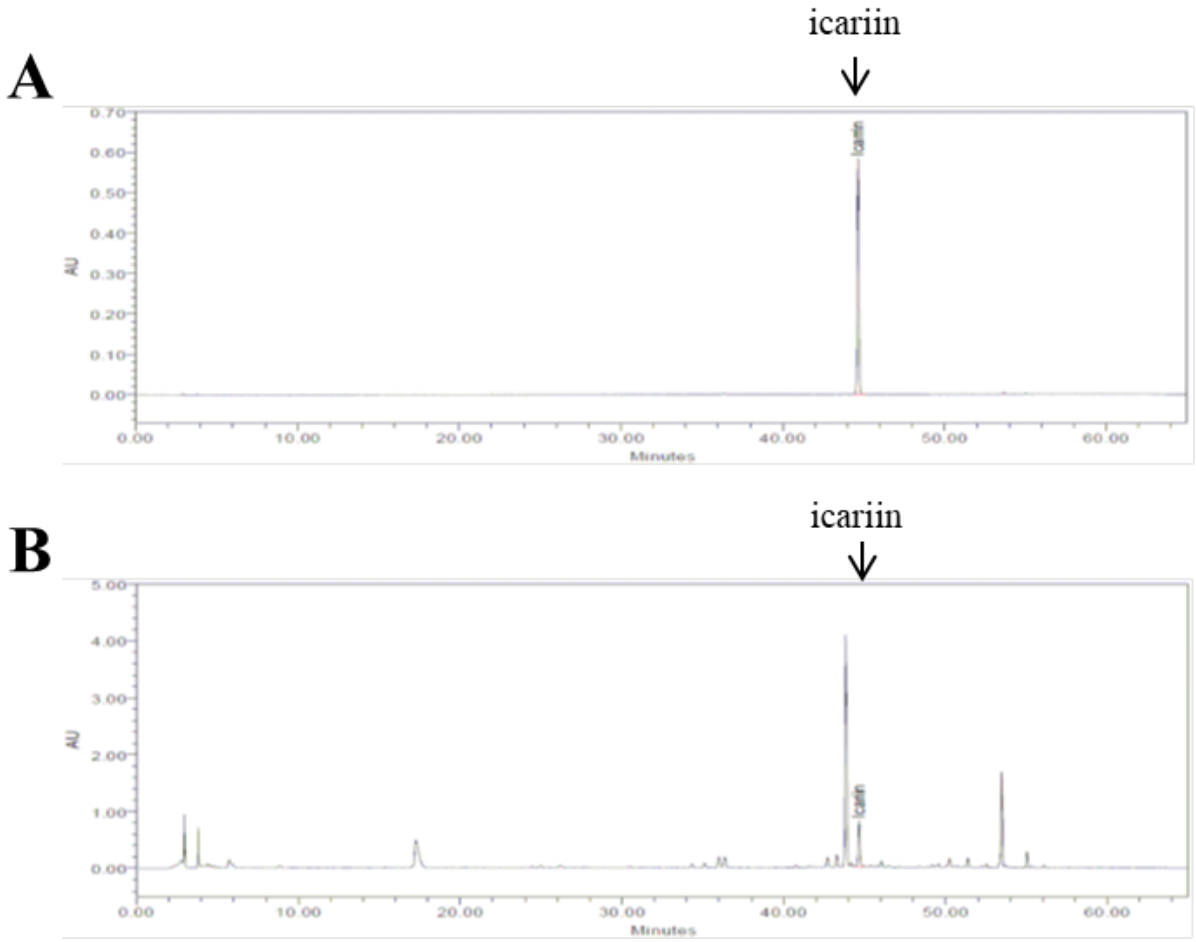
Supplementary Figure



Supplementary Figure S3. IGF-1R antagonist picropodophyllin blocked EE-induced myotube hypertrophy.

Selective IGF-1R antagonist picropodophyllin (PPP, 5 μM) was applied 2h before EE (100 μg/ml) or control (CTL) treatment, where DMSO (0.1%) served as vehicle control (V). Differentiated C2C12 cells were maintained in serum-free medium overnight before pre-treated with PPP or DMSO followed by indicated treatment for further 24h. (A) Representative images of differentiated C2C12 cells were taken at the end of the indicated treatment by a light microscope (scale bar, 100 μm). (B) The myotube diameter of each treatment group was determined by repetitive measurements of 90 myotubes in triplicate. Myotube hypertrophy was documented by the significant increment of myotube diameter as compared to CTL. Representative western blots (C) and densitometric analysis (D) showed EE significantly induced AKT phosphorylation and MyHC-T overexpression (N=3). PPP suppressed EE induced myotube hypertrophy and MyHC-T overexpression. All data were expressed as mean ± SD. The symbol * stands for $p < 0.05$ as compared to the basal (CTL or CTL-DMSO); the symbol $^{\#}$ stands for $p < 0.05$ as compared to the intra-group DMSO treatment.

Supplementary Figure



Supplementary Figure S4. Icariin (ICA) determination by HPLC.

Represented images showed typical chromatograms for ICA standard solution (A) and *Epimedium* extraction (EE) solution (B).

Unprocessed WB images

Fig. 1G

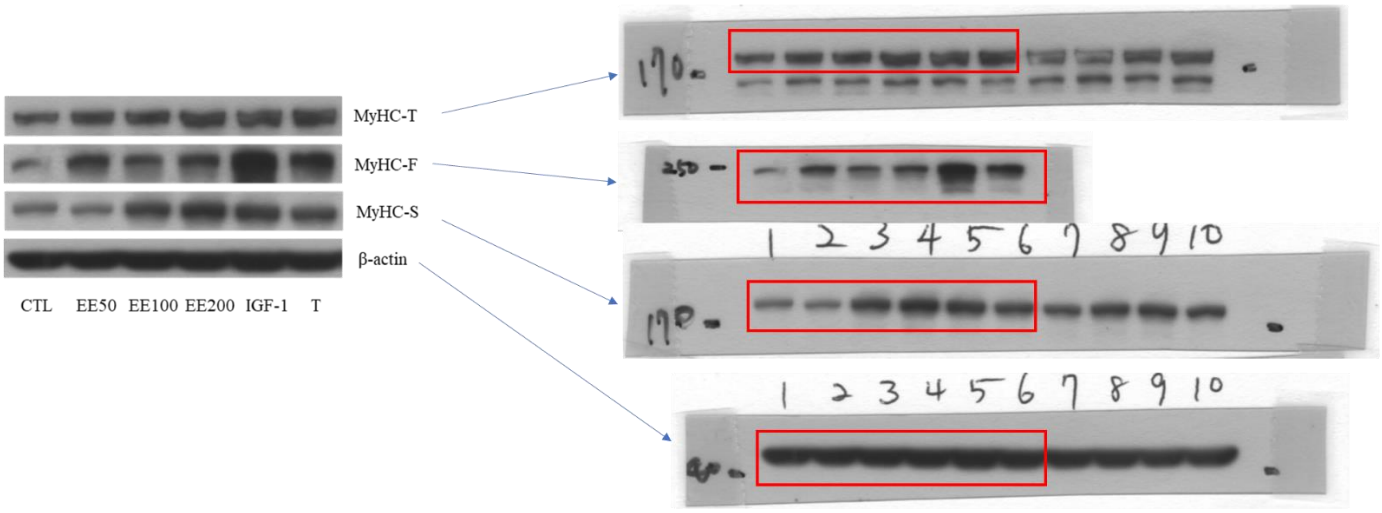
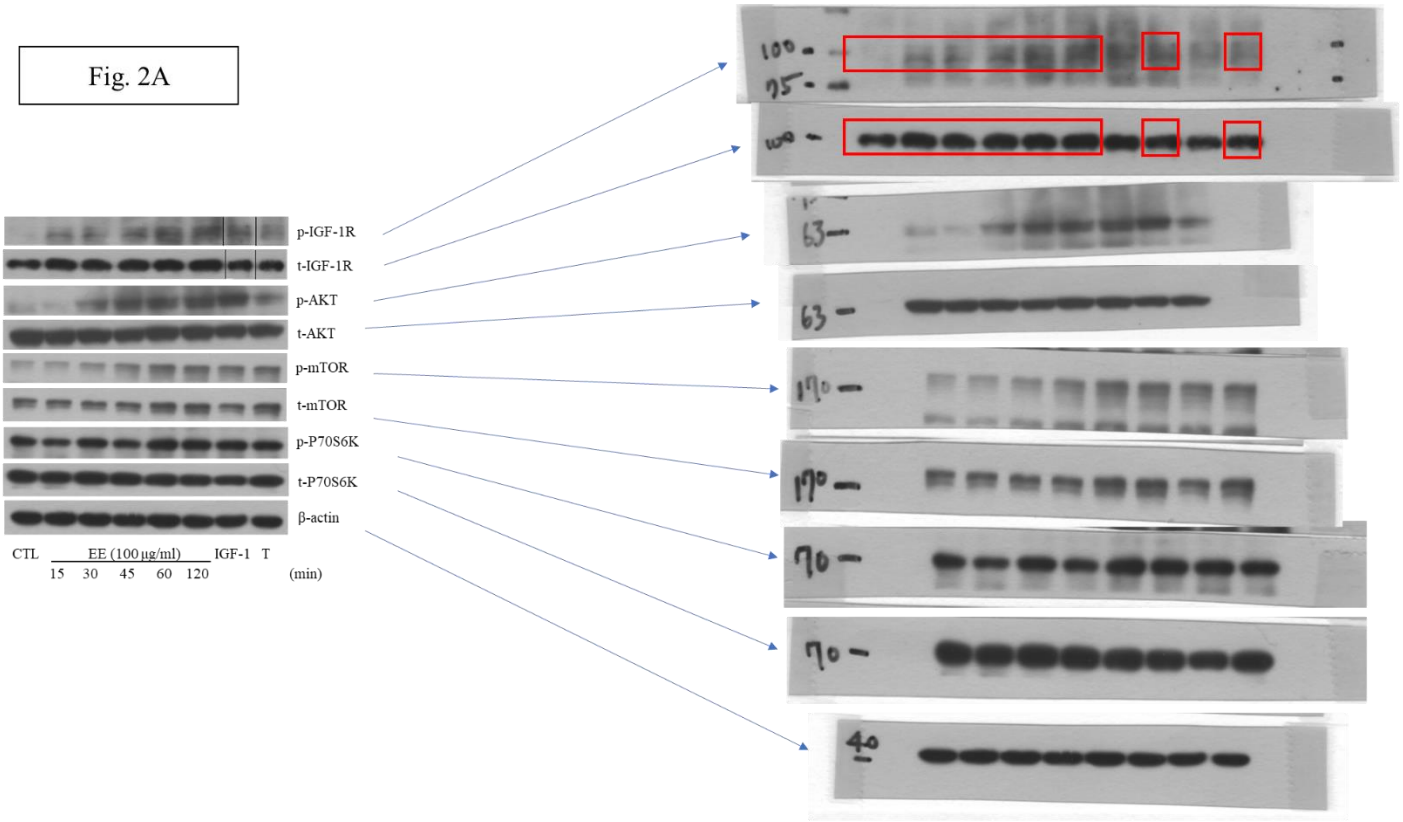


Fig. 2A



Unprocessed WB images

Fig. 2F

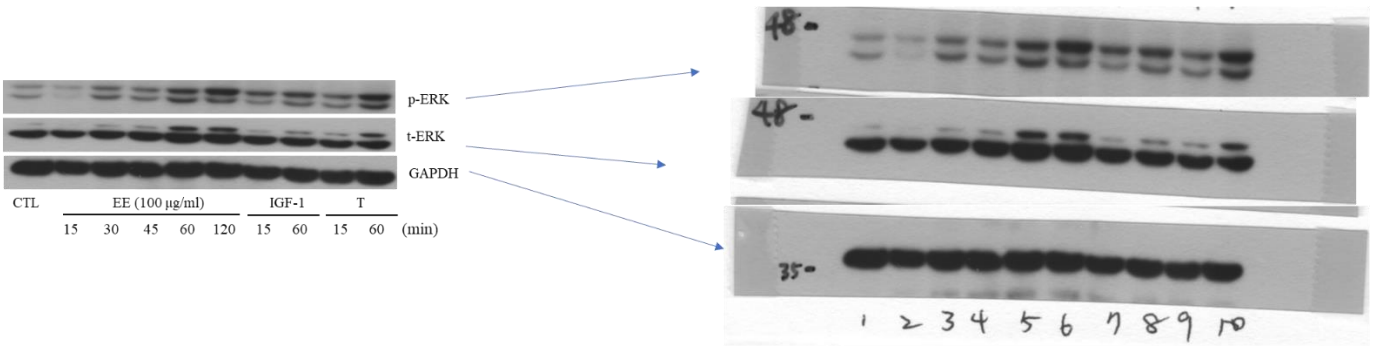


Fig. 2G

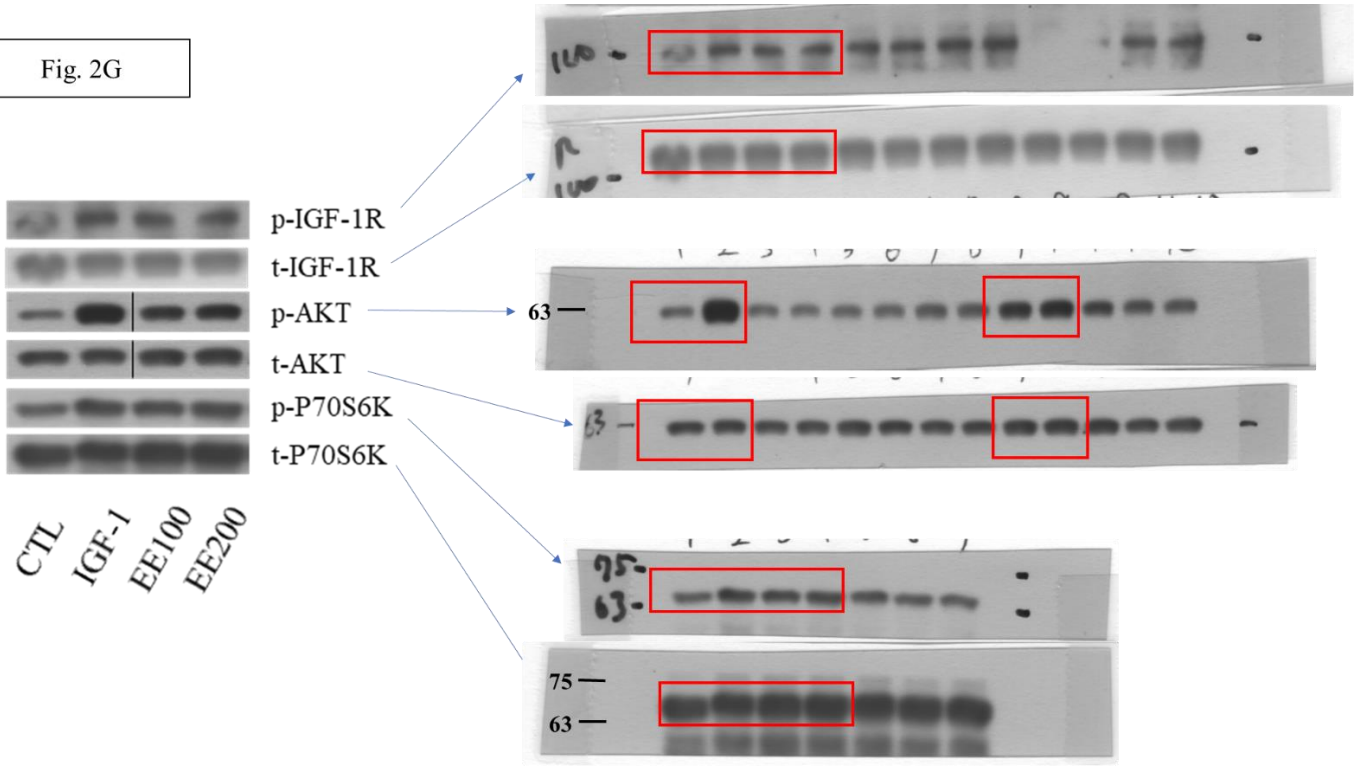
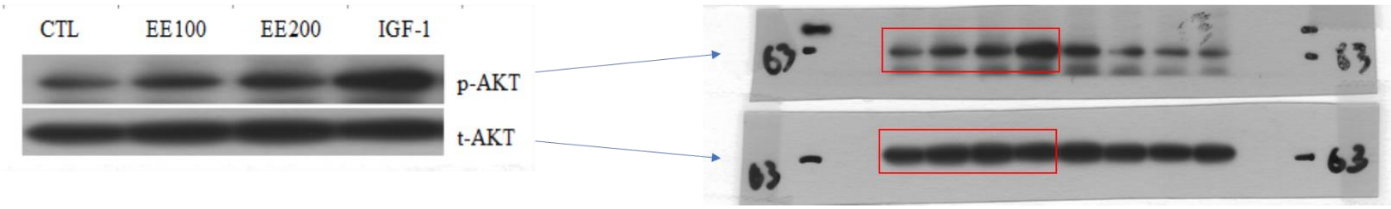


Fig. 2H



Unprocessed WB images

Fig. 3D

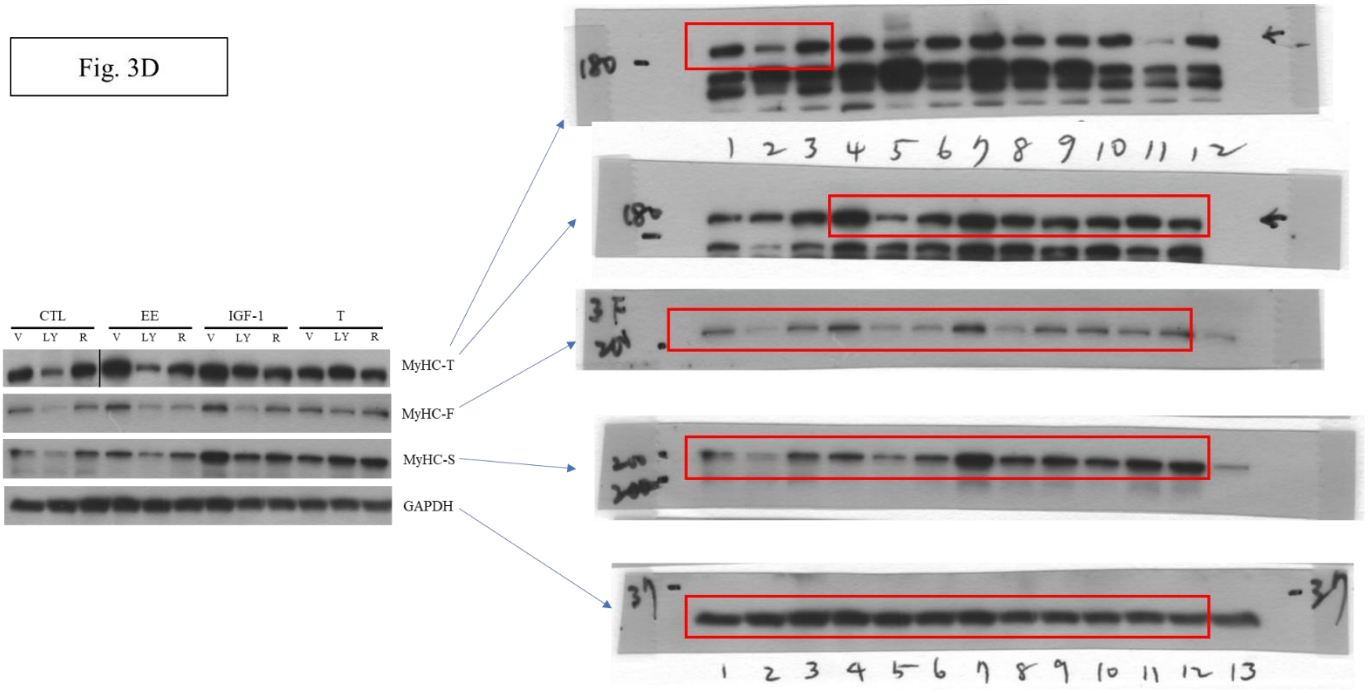


Fig. 3H

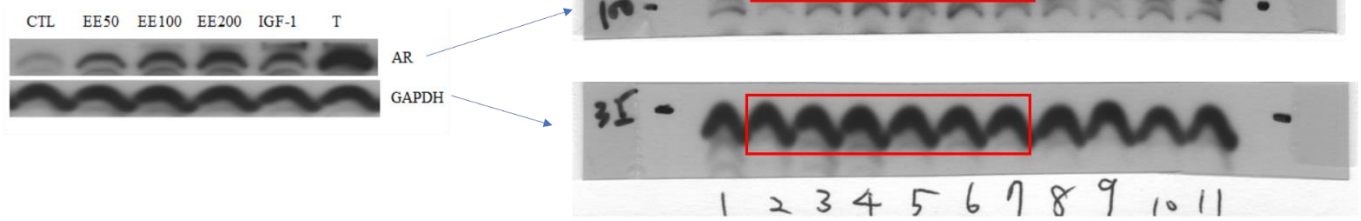
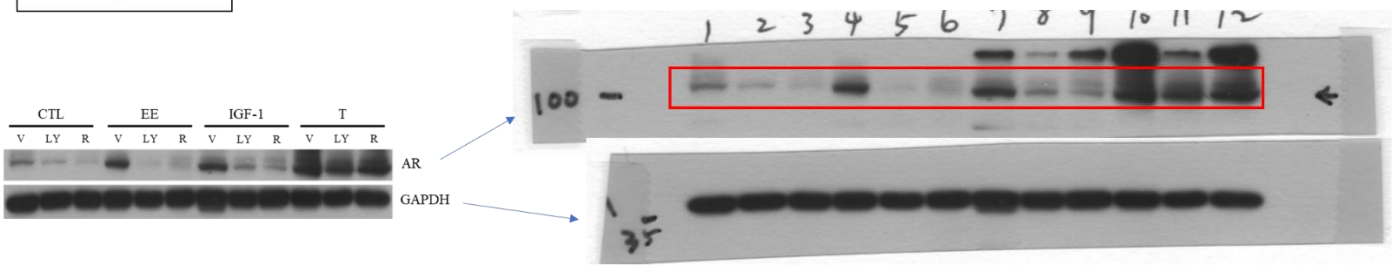


Fig. 3I



Unprocessed WB images

Fig. 3J

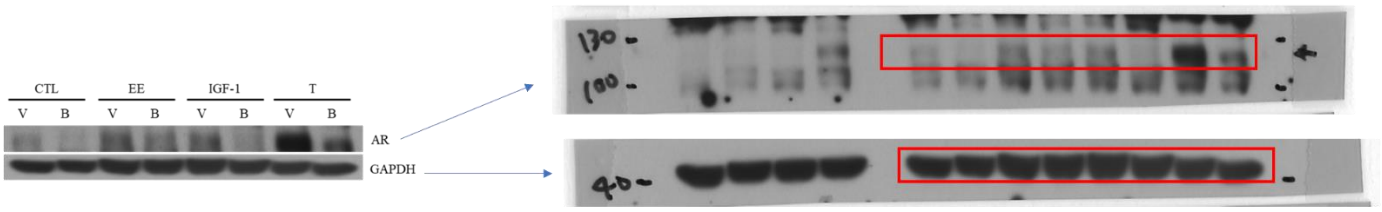


Fig. 3K

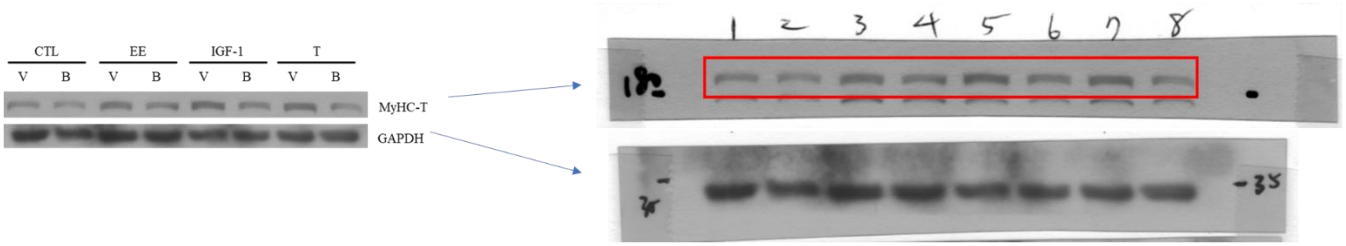


Fig. 4A

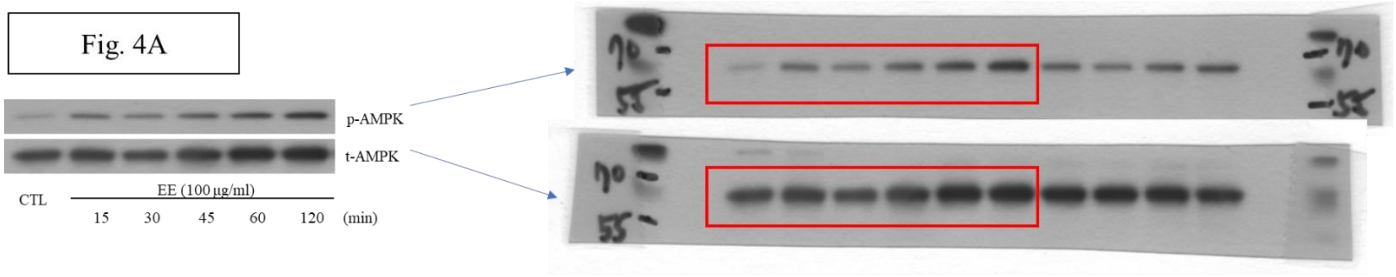
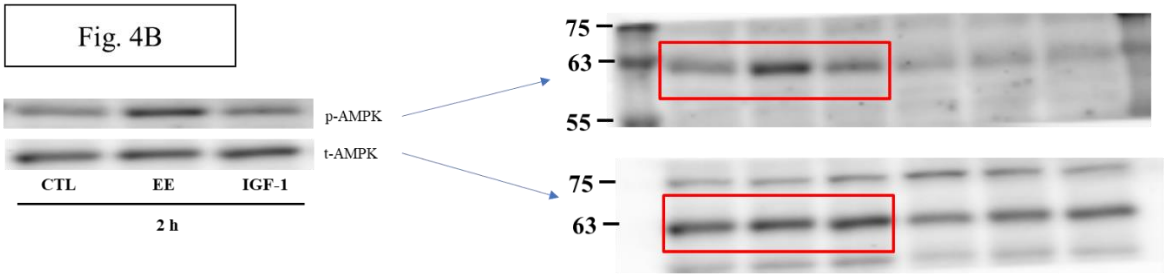


Fig. 4B



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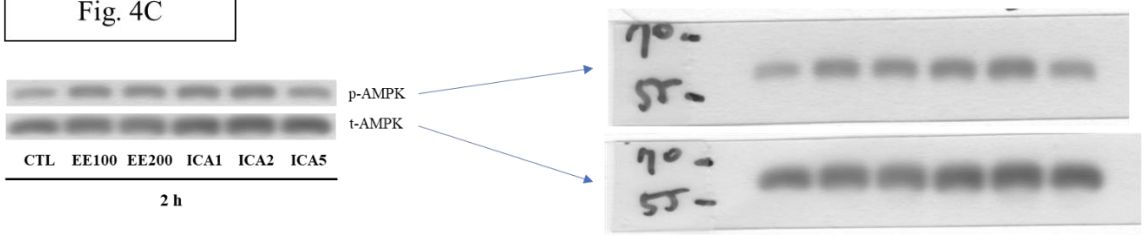


Unprocessed WB images

Fig. 5A

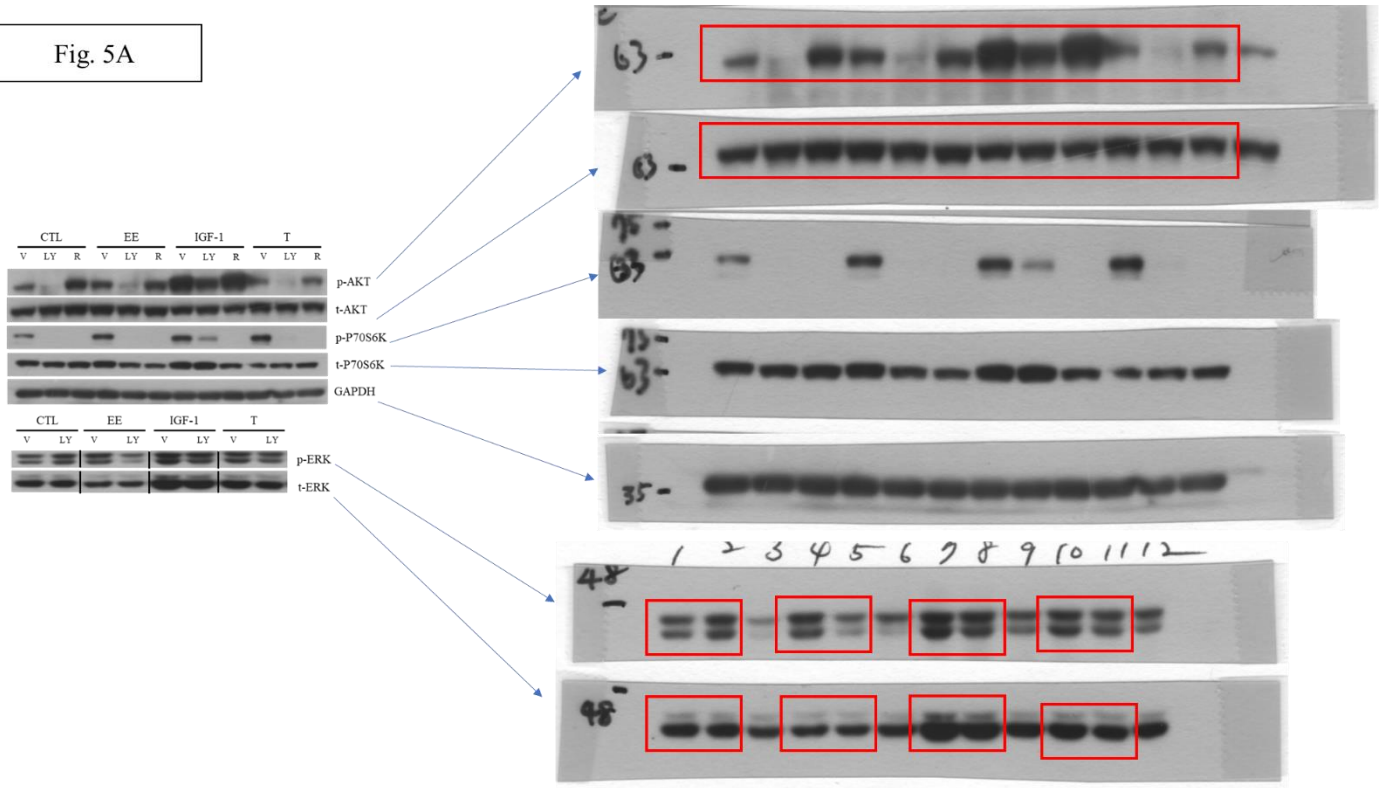


Fig. 5E

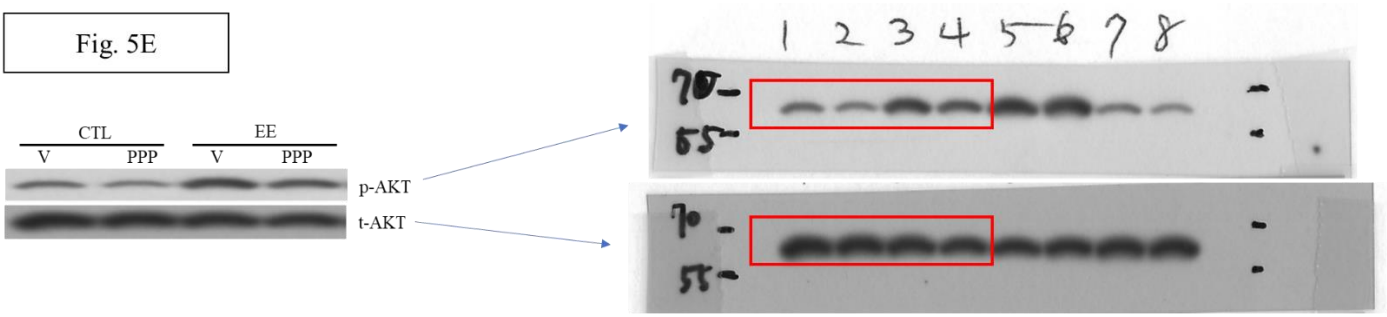
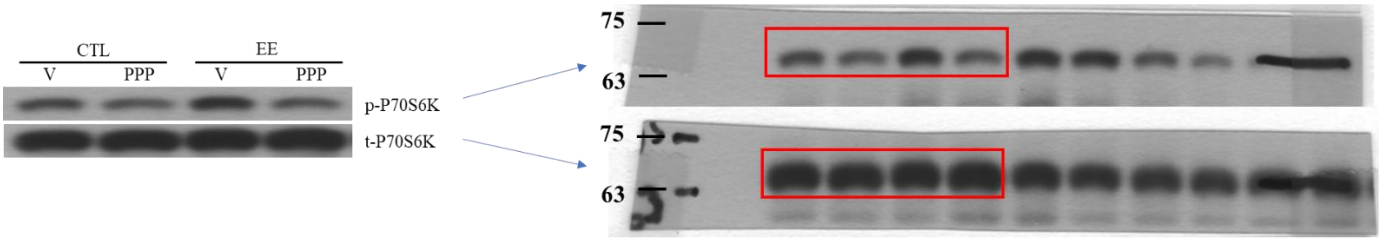
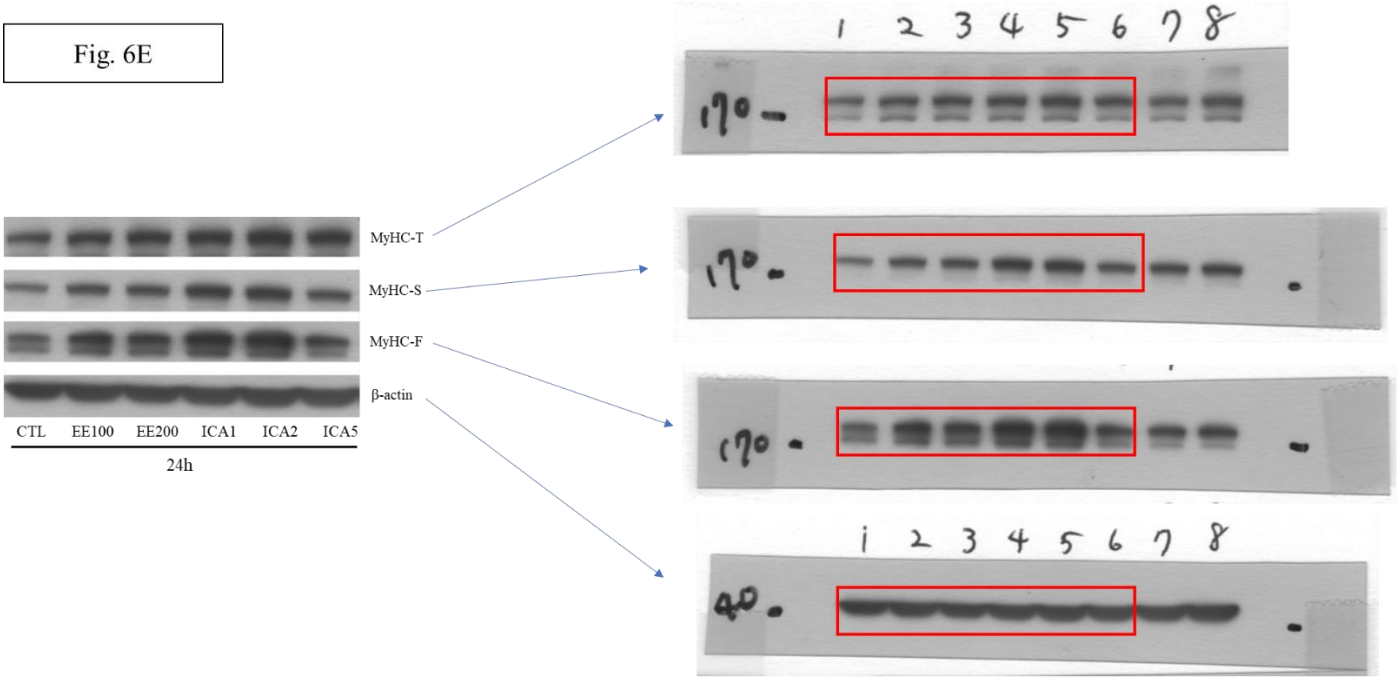


Fig. 5F



Unprocessed WB images

Fig. 6E



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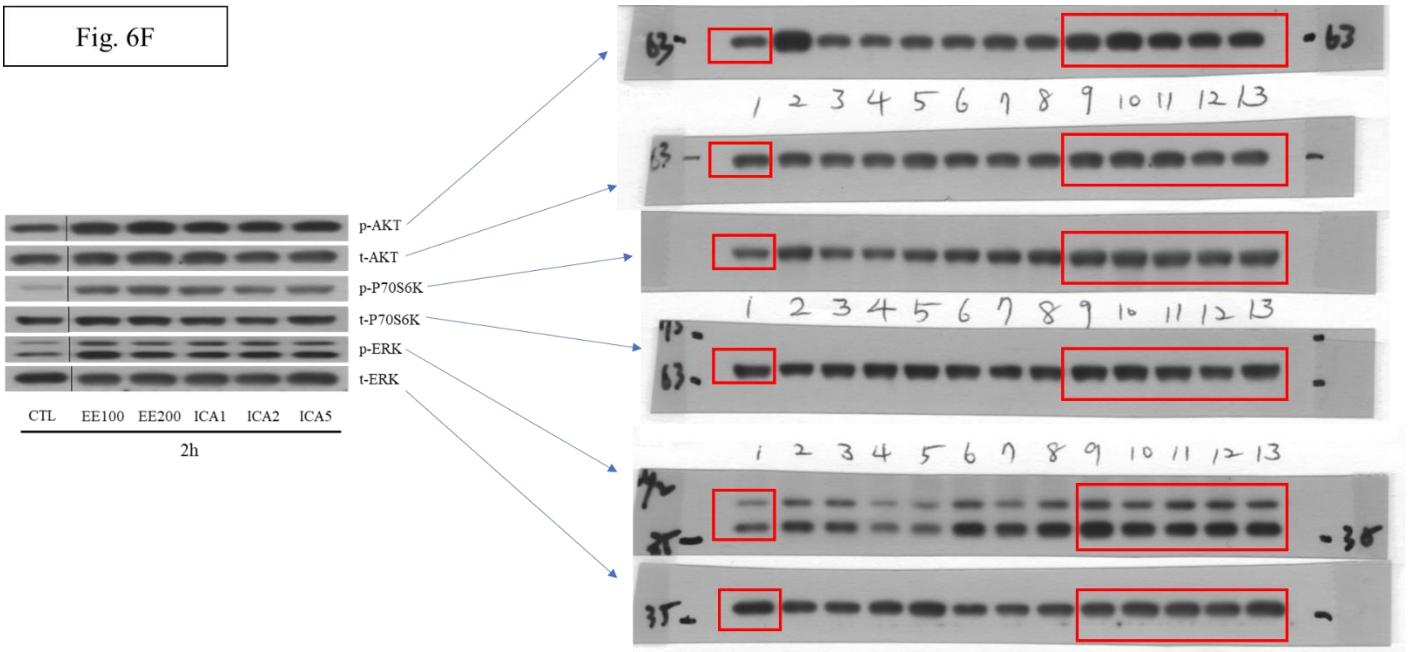


Unprocessed WB images

Fig. 6G

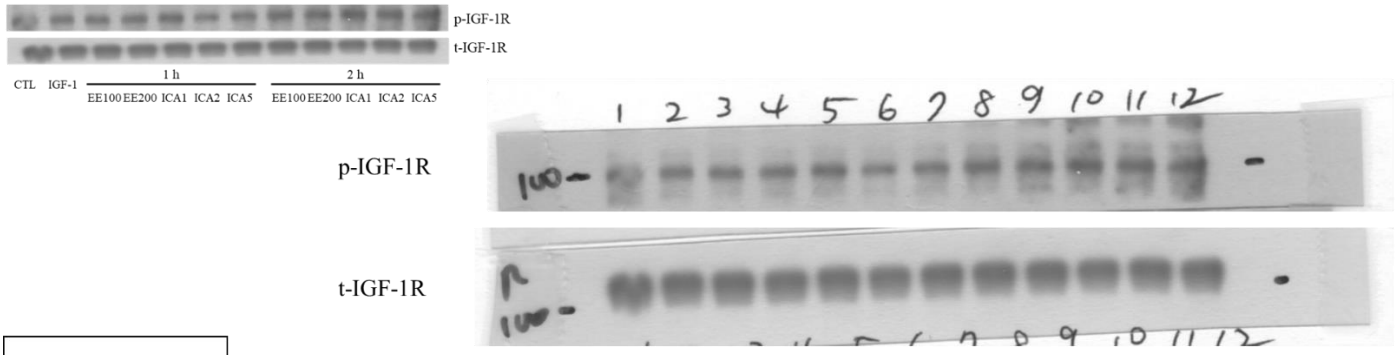
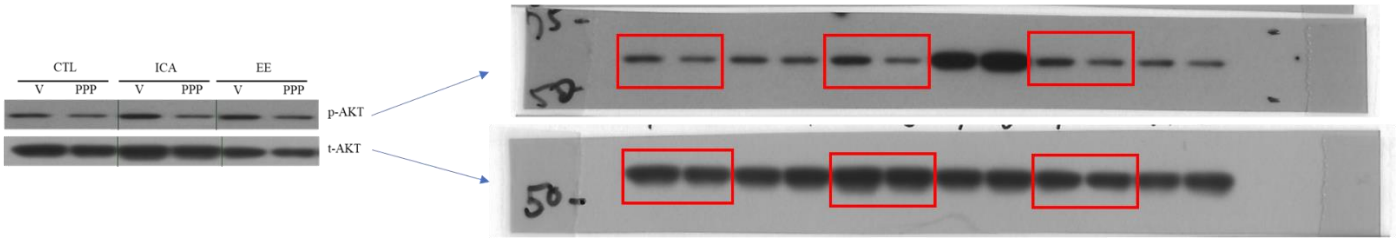


Fig. 6H



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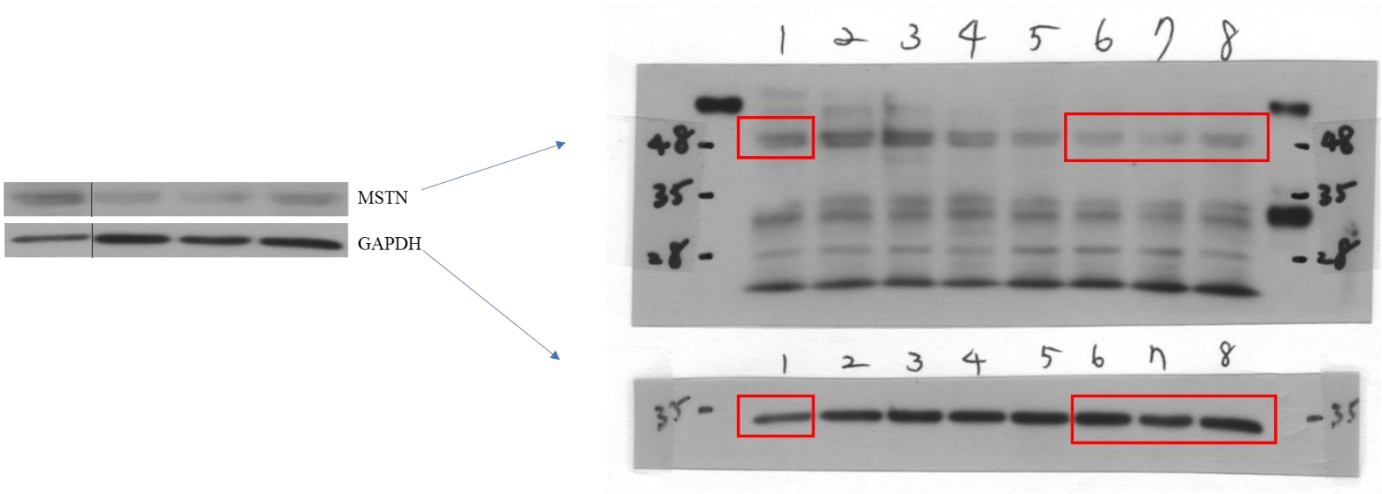
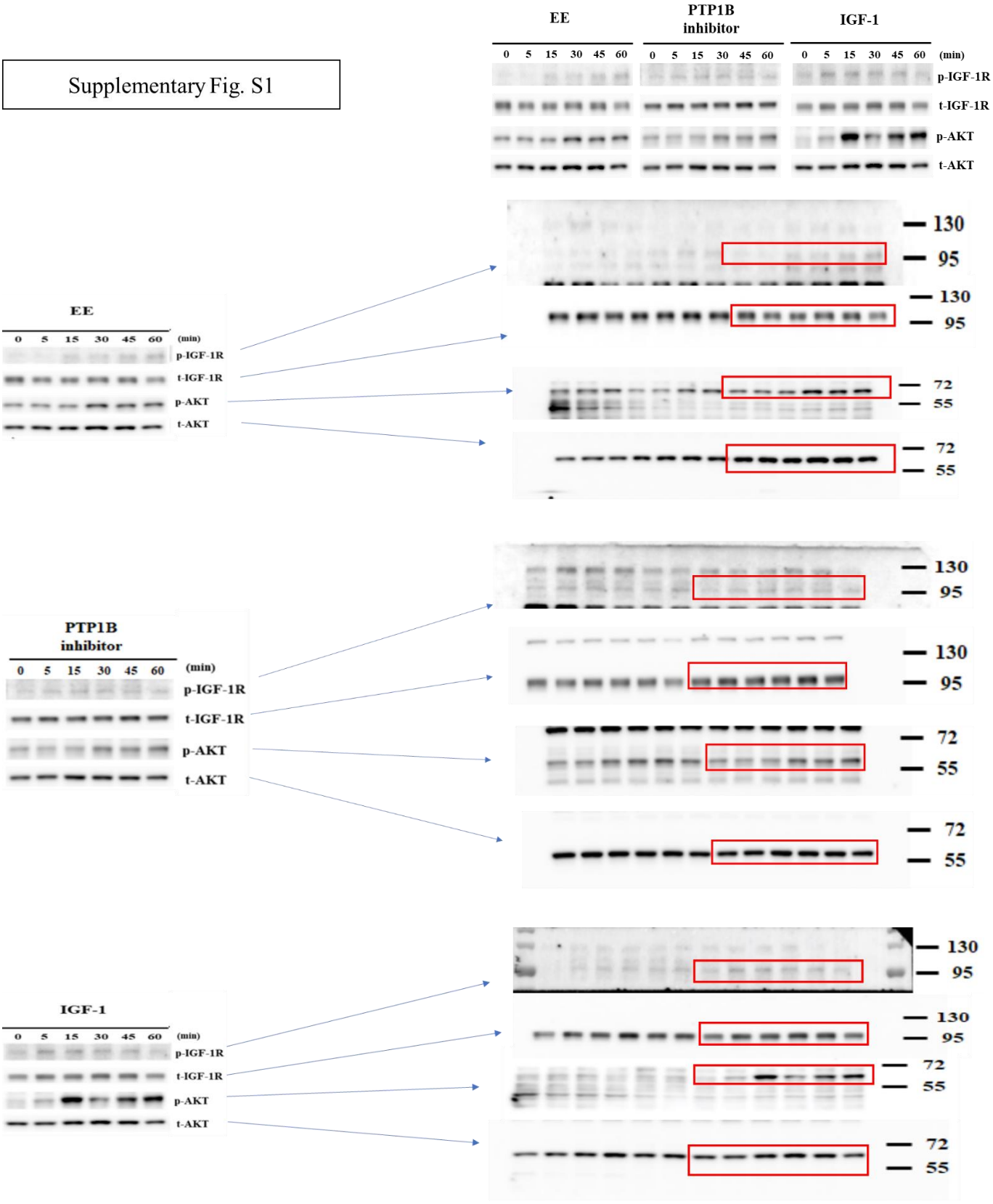


Unprocessed WB images

Supplementary Fig. S1



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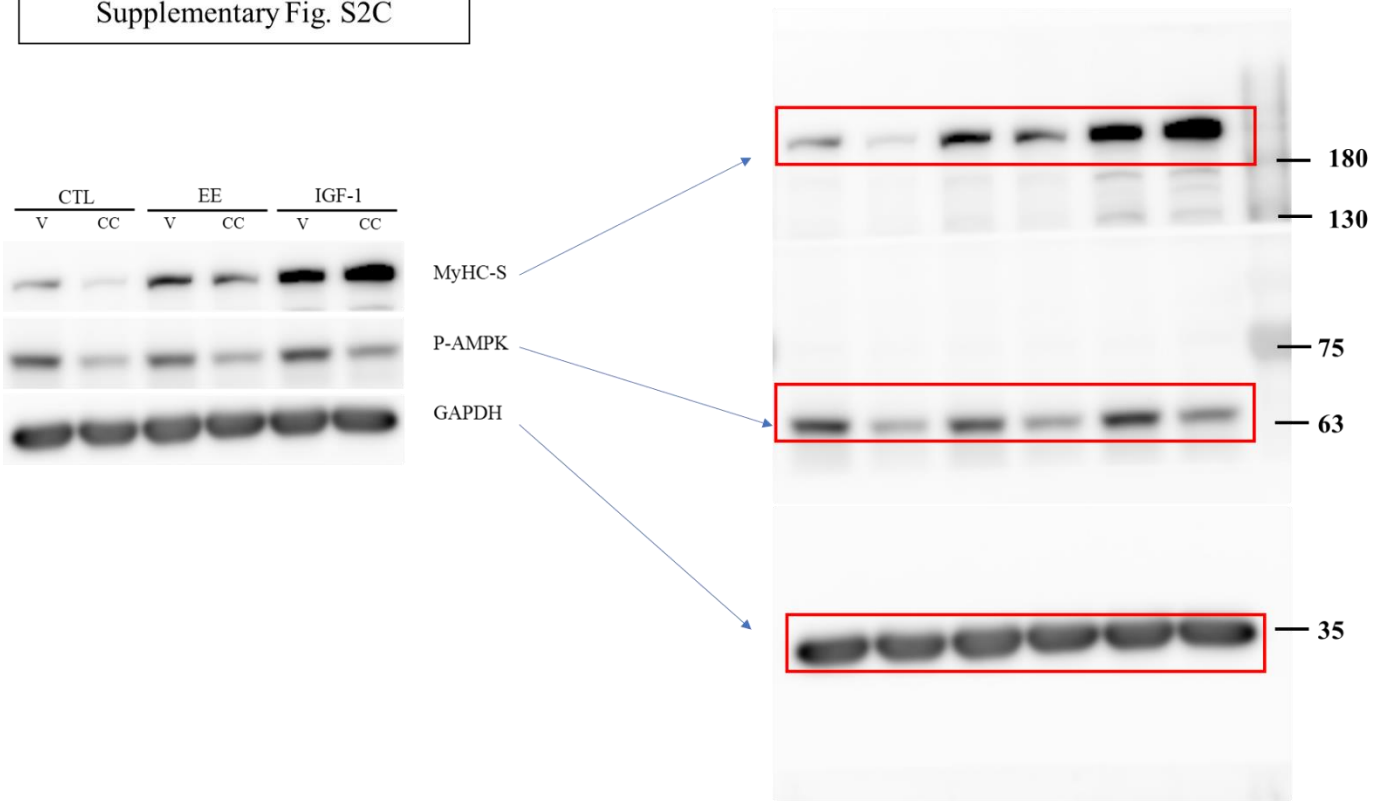
Supplementary Fig. S2C



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Supplementary Fig. S3C