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Supplemental Information

Down-Regulation of miR-301a-3p Reduces Burn-Induced Vascular Endothelial Apoptosis by potentiating hMSC-Secreted IGF-1 and PI3K/Akt/FOXO3a Pathway

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TRANSPARTENT METHODS

Cell culture

The human umbilical cord MSC (hMSC) was obtained according to the protocols as described previously (Liu et al., 2013). The human umbilical cord MSC were maintained in Mesenchymal Stem Cell Medium (MSCM, ScienCell, USA) and humidified at 37ºC with 5% CO_2.

First-passage human umbilical vascular endothelial cell (HUVEC) was purchased from ScienCell Research Laboratories (ScienCell, USA). HUVEC was cultured in Endothelial Cell Medium (ECM, ScienCell, USA), and humidified at 37ºC with 5% CO_2. HUVEC of passages 2-5 were used for the following experiments.

MicroRNA array analysis of severe burn induced-hMSC

The severe burn serum culture system *in vitro* was used to mimic organic microenvironment of the severe burn. Total RNAs was extracted from the hMSC, which were incubated in culture system of severe burn serum and sham serum (control) for 24h, using TRIzol (Invitrogen, USA), following the manufacturers' protocols. A miRNA array *(GeneChip miRNA Array; Affymetrix Inc., Santa Clara, CA, USA),* which contains 6153 probe sets, was used to detect the miRNA expression. miRNAs of intensities ≥30 were chosen for calculation. Unsupervised hierarchical clustering was analyzed by the Multi Experiment Viewer software, version 4.6 (The Institute for Genomic Research, Rockville, MD, USA). Dysregulated miRNAs were analyzed based on the following criteria: the fold change>2 and Q<0.05.

hMSC cotransfection with IGF-1 and miR-301a-3p

The hMSC (50000 cells/well) was seeded in 6-well plates with 3 mL antibiotics-free MSCM-sf. 1 day later, the cells were transfected with lentivirus-mediated overexpression vector and siRNA (negative control and IGF-1) or mimics and inhibitors (negative control and miR-301a-3p), which were synthesized by GeneChem (Shanghai, China), according to the corresponding protocol provided by manufacturers. In the first part, hMSC was divided into negative control group (un-transfected), mock group (Lenti-control transfected), IGF-1-o group (Lenti-IGF-1 transfected), mock group (Lenti-control-siRNA transfected), IGF-

1-i group (Lenti-IGF-1-siRNA transfected). In the second part, hMSC was divided to negative control group (un-transfected), mock group (Lenti-control-mimic transfected), miR-301a-3p-o group (Lenti-miR-301a-3p-mimic transfected), miR-301a-3p-o+IGF-1-o (Lenti-miR-301a-3p mimic and Lenti-IGF-1 co-transfected), mock group (Lenti-control-inhibitor transfected), miR-301a-3p-i group (Lenti-miR-301a-3p-inhibitor transfected), miR-301a-3p-i+IGF-1-i (Lenti-miR-301a-3p-inhibitor and Lenti-IGF-1-siRNA co-transfected). 12 hours later, the medium was replaced by antibiotics-free MSCM-sf. After culturing for 3-4 days, these cells were used for analysis and detection. To confirm the transfection efficiency, inverted fluorescence microscope (Leica, Germany) and flow cytometry analysis were used to detect GFP fluorescence intensity.

Dual-luciferase reporter gene assay

The binding sites of miR-301a-3p in IGF-1 was analyzed using Targetscan (*www.targetscan.org*) and miRanda (*www.microrna.org*). The wild-type (WT) and mutant (MUT) 3'-UTR of IGF-1 was coloned into luciferase expressing pMIR-REPORT vector (Ambion; Thermo Fisher Scientific, Inc.). In brief, Luciferase reporter vectors were co-transfected with mimics, antisense and corresponding mutant sequences of miR-301a-3p by Lipofectamine 2000 into 293 T cells. Luciferase activity was detected using the kit (cat. no: RG027; Beyotime Institute of Biotechnology), following the manufacturer's protocols.

Real time-PCR analysis

Total RNA was isolated from hMSC using TRIzol (Invitrogen). 2 μg of total RNA was reversely transcribed into cDNA using moloney murine leukemia virus reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Real time-PCR was performed with SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) on the Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific, Inc.). Relative level of mRNA and miRNAs was calculated using the $2^{-\Delta\Delta}$Cq method (Livak and Schmittgen, 2001) and normalized to GAPDH and U6, respectively. The primer sequences were as following: IGF-1: Forward 5'-ACACTCCAGCTGGGAAAAGCTG GGTTGAGA-3' and reverse

5'-ACACTCCAGCTGGGTCGCCCTC-3'; GAPDH: Forward 5'-TATCGGACGCCTGGTTAC-3' and reverse 5'-CGTTCAAGTTGCCGTGTC-3'. miR-301a-3p: Forward 5'-ACACTCCAGCTGGGCAGTGCAATAGTATTGTC-3' and reverse 5'-CTCAACTGGTGTCGTGGA-3'; U6: Forward 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'.

Western blotting analysis

Total proteins were extracted using RIPA buffer, supplied with Halt protease and phosphatase inhibitor cocktail (Servicebio, Wuhan, China). Proteins were separated on SDS-PAGE gel and transferred onto PVDF membranes. Then the membranes were blocked with 5% skim milk and incubated with primary antibodies at 4^{o}C overnight. After incubated with secondary antibodies at room temperature for 3 hours and washed by PBST for three time, the protein signal on the membranes was detected using ECL-Plus kit. Primary antibodies against IGF-1, p-PI3K, PI3K, p-Akt, Akt, p-FOXO3a, FOXO3, Cleaved Caspase 3, Caspase 3 (1:1000) and β-actin (1:20,000) were from R&D Systems (Minneapolis, MN). The quantitative densitometric image analysis of the western blot images were performed using Image J software.

Co-cultured system

Transwell co-cultured system was used to investigate the paracrine effect of hMSC on severe burn-induced HUVEC apoptosis. The HUVEC in ECM were seeded to the upper chamber (0.4 μm pore size polyester membrane from Corning, Inc.) in six-well plates (Corning, NY). The hMSC in MSCM were seeded to the lower chamber in other six-well plates (Corning, NY). They were cultured for 1 to 3 days to form a confluent monolayer. Next, the HUVEC on the upper chamber were placed to the hMSC on the lower chamber. They were simultaneously treated with DMEM-high glucose containing 20% severely burned rat serum for 24h. Finally, the hMSC and HUVEC were collected for apoptosis analysis.

Flow Cytometry assay

Apoptosis of HUVEC was analyzed using Annexin V-FITC/PI Apoptosis Detection kit I (BD, Biosciences). HUVEC and hMSC were seeded to the upper and lower chambers of co-culture system as described above. HUVEC were washed twice with PBS and

resuspended in 1×binding buffer. 1×10^5 cells in 100 uL 1×binding buffer were added with 5 uL Annexin V-FITC and 5uL propidium iodide. Flow cytometry (Becton Dikinson, USA) was used to assess the apoptotic cells.

Severely burned rat injected with hMSC that cotransfected with IGF-1and miR-301a-3p

Six-week-old male Wistar rats (180–220g) were purchased from SPF (Beijing) Biotechnology Co., Ltd. The animals were hosted under 22 °C, 55% humidity and 12-hour light-dark cycle, fed with standard food and water ad libitum. All experimental protocols were in according with the International Guiding Principles for Biomedical Research Involving Animals issued by the Council for the International Organizations of Medical Sciences (CIOMS.), and approved by the Institutional Animal Care and Use Committee at the Fourth Medical Center Affiliated to PLA General Hospital.

Wistar rats (n=216) were divided into 9 groups, including Sham, Burn, Burn+hMSC, Burn+mock, Burn+IGF-1-o, Burn+IGF-1-i, Burn+miR-301a-3p-o, Burn+miR-301a-3p-i and miR-301a-3p-i+IGF-1-i. Each group was divided equally into three subgroups of 8 rats at 12h, 24h, 48h after severe burn. Rats were anesthetized by intraperitoneal injection of 300 mg/kg Avertin (20 mg/ml) (2,2,2-tribromoethanol, Sigma, USA). Both whole backside and abdomen were placed in hot water (94°C) for 12s and 6s, respectively, which caused 50% TBSA with a full-thickness burn. Intraperitoneal injections of balanced salt solution (40 ml/kg) were immediately administered to prevent shock.

The rats in the Burn+mock, Burn+IGF-1-o, Burn+IGF-1-i, Burn+miR-301a-3p-o, Burn+miR-301a-3p-i and miR-301a-3p-i+IGF-1-i groups immediately received a tail vein injection of 5×10^6 hMSC, which were transfected with Lenti-GFP-vehicle, Lenti-IGF-1-overexpression, Lenti-IGF-1-siRNA, Lenti-miR-301a-3p mimic, Lenti-miR-301a-3p inhibitor and Lenti-miR-301a-3p-i inhibitor combined Lenti-IGF-1-i-siRNA. The rats in the Burn+hMSC group immediately received 5×10^6 hMSC by tail vein injection. And the rats in sham and burn groups received PBS by tail vein injection. The burn wound was treated with 1% tincture of iodine and kept dry to avoid infection.

Pulmonary function test (PFT)

Pulmonary function test (PFT) can provide a simple noninvasive method of assessing airway compromise. At 24h after severe burn, pulmonary function was measured by AniRes2005 animals lung funtion detector, then data were assayed using AniRes2005 software (Beijing Bestlab High-Tech Co.,Ltd, China).

Specimen collection and detection

The blood samples were collected from aortaventralis at 12h, 24h and 48h post burn injury or sham injury, and serum were used for the ELISA assay and blood biochemical detection, as well as serum at 24h post burn for building the severe burn serum culture system *in vitro*. Meanwhile, organs including lung, liver, kidney and heart samples were collected for immunofluorescence staining and permeability detection, and their tissues supernatant were used for the ELISA assay. The 24 hours urine volume in each group was collected using metabolic cage and recorded. The metabolic cages (Nalgene, Thermo Fisher) consist of a circular upper portion, which houses the rat; a wire-grid floor (diameter, 21.5 cm; approximate surface area, 363 cm^2; opening, 1×3.1 cm); and a lower collection chamber with a specialized funnel that separates fecal pellets and urine that fall through the grid floor for their collection into 2 separate tubes (diameter, 4 cm; Nalgene, Thermo Fisher).

Blood biochemical detection

The serum biochemical parameters, including alanine transaminase (ALT), aspartate transaminase (AST), urea (Urea) and creatinine (Crea), troponin, creatine kinase (CK), MB isoform of CK (CK-MB), lactate dehydrogenase (LDH), a-hydroxybutyrate dehydrogenase (HBDH)were measured at the Central Institute of Clinical Chemistry and Laboratory Medicine of the Fourth Medical Center Affiliated to PLA General Hospital.

Dual immunofluorescence staining

The lung, liver, kidney and heart samples were fixed with 4% paraformaldehyde and embedded in paraffin. 5 um sections were deparaffinized, rehydrated, and stained with immunofluorescence according to standard procedures. In brief, the sections were labeled with 1:50 monoclonal mouse against human MSC FITC-CD105 antibody (Abcam, USA) and monoclonal rabbit against rat endothelial CD31 antibody (Abcam, USA) at 4°C. Next, 1:500 Alexa Fluor 594 AffiniPure Goat Anti-Rabbit IgG secondary antibody was used. In

addition, other sections were stained by One Step TUNEL Apoptosis Assay Kit (Beyotime, China) and monoclonal rabbit against rat endothelial CD31 antibody (Abcam, USA), followed by incubation with the CD31 secondary antibody. Finally, 500μL 4,6-diamidino-2-phenylindole (DAPI) was used for nuclear staining (green-TUNEL+red-CD31-VEC+blue-DAPI-nuclear). Cover sheets were glued on the glass slides with anti-fluorescence quenching sealant and observed with a laser scanning confocal microscope(Guo et al., 2018).

Vascular permeability assay

Evans blue (EB) was applied to detect the vascular permeability of tissues or organs. 2% EB (2 ml/kg, Sigma, St. Louis, MO, USA) was intravenously administrated into the rats. 1h later, the rats were anesthetized and transcardially perfused with 0.9% NaCl. The organs were removed and immersed in formamide (10 ml/kg, Sigma, St. Louis, MO, USA) at 60℃ for 24h. The organs were ground and centrifuged at 4℃. OD632 was measured on an automatic microplate reader (BioTek, USA). Organs were collected for wet weight (WW) and dry weight (DW) measurment. Water content ratio of vital organ tissues was calculated as (WW-DW)/WW×100% (Chai et al., 2013).

Enzyme-linked immunosorbent assay (ELISA)

The IGF-1 level in cultural supernatant of hMSC and in serum, organ tissues of severely burned rats was examined with a double-antibody sandwich ELISA kit (eBioscience, USA) following the manufacturer's instructions. The OD value was detected on a multi-detection microplate reader.

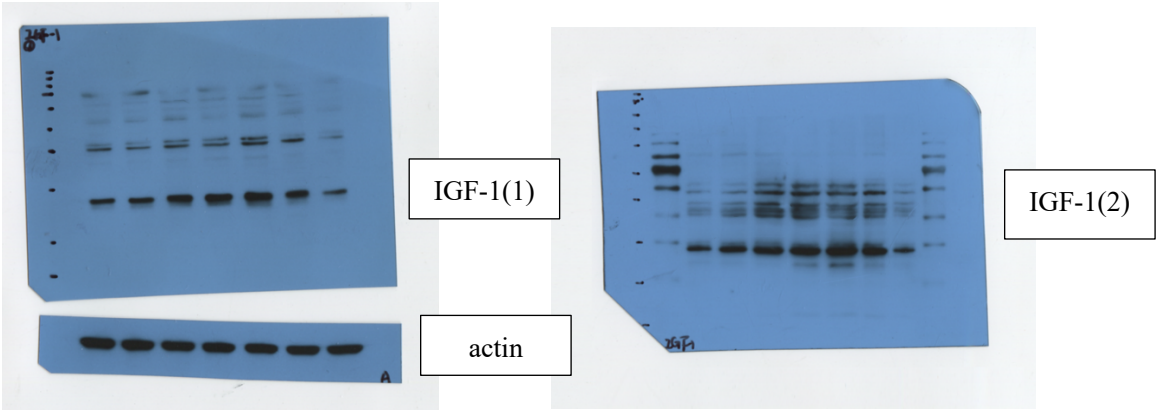
Statistical analysis

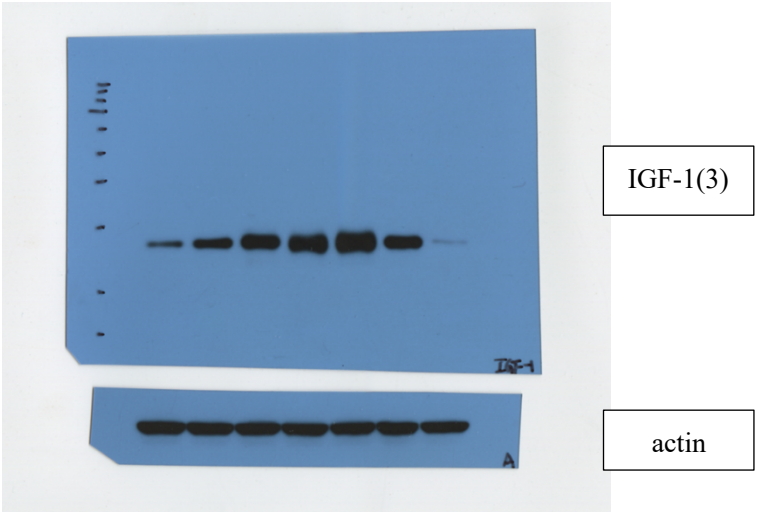
The data shown as the mean ± SD ($\bar{x}$ ± s) were analyzed by SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Statistical difference was analyzed using one-way ANOVA and multiple comparisons were performed using Student-Newman-Keuls tests. Statistical difference was considered significant when $p<0.05$.

Supplemental figures

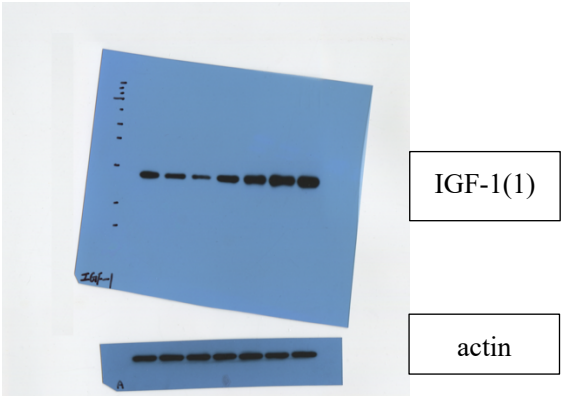
Data S1: Full unedited bolts for Fig1E, Fig 3E, Fig 6A and Fig 6B, each experiment was repeated for 3 times.

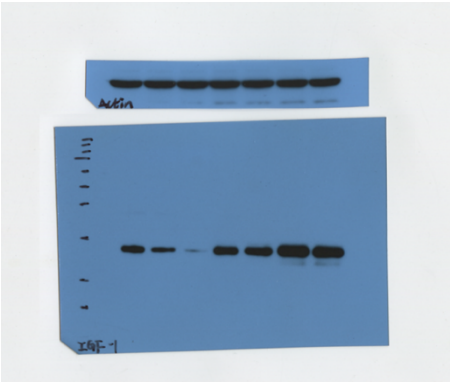
Full unedited blots for Fig 1E

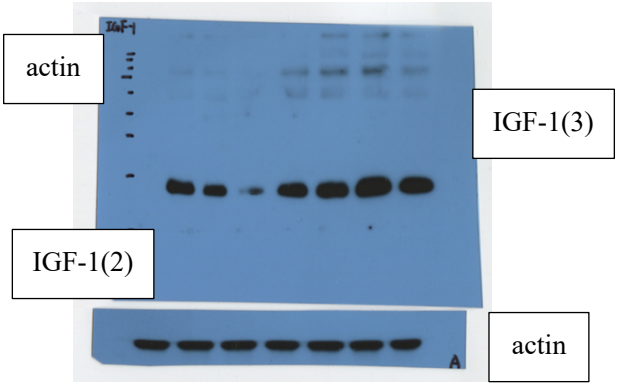




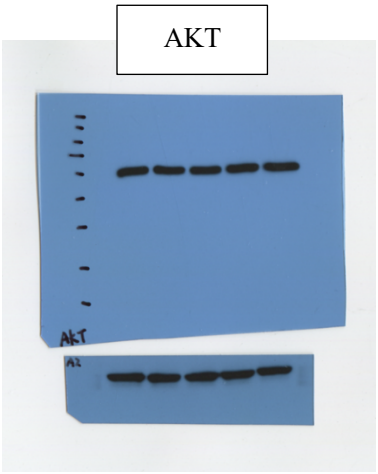
Full unedited blots for Fig 3E

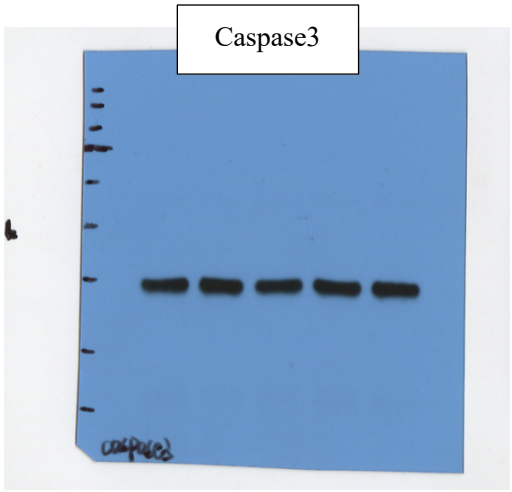


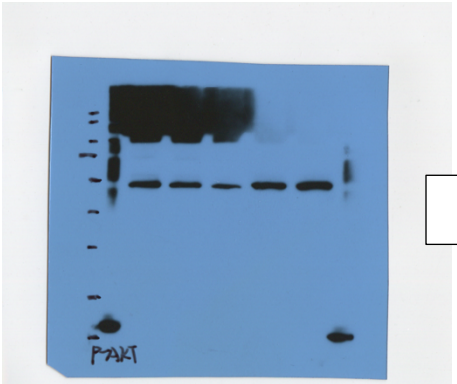


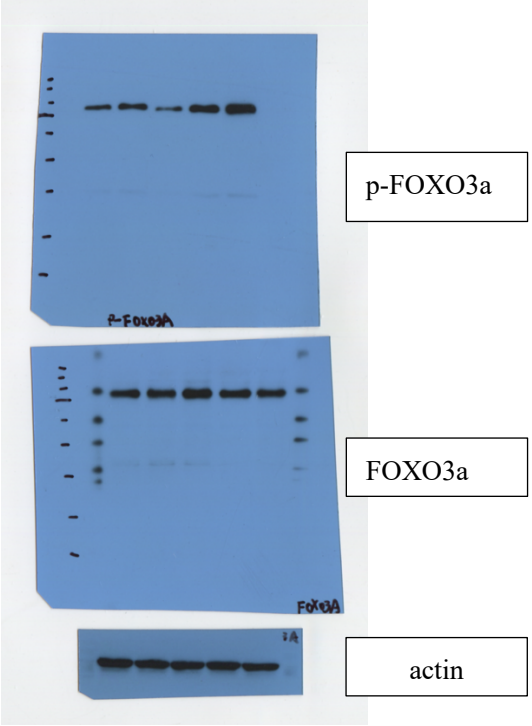


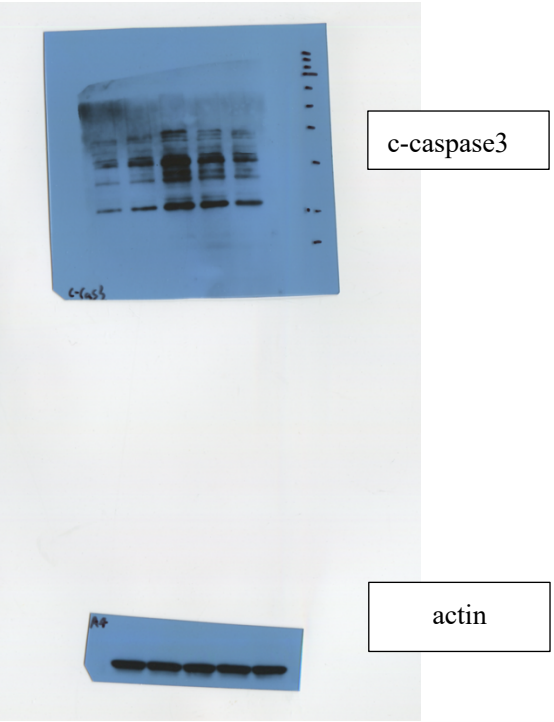
Full unedited blots for Fig 6A PI3K signal (1)

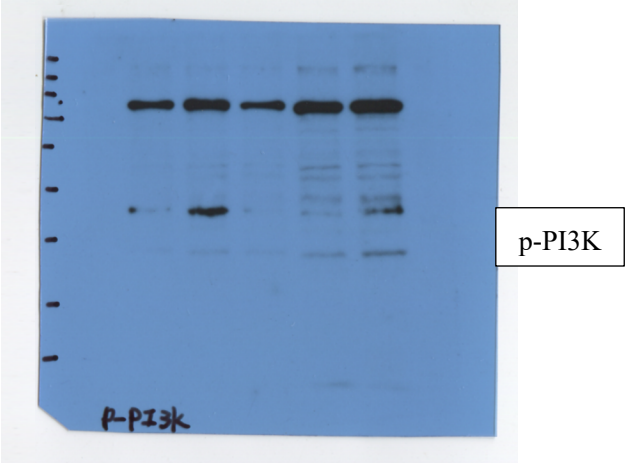







p-FOXO3a
FOXO3a
actin


c-caspase3
actin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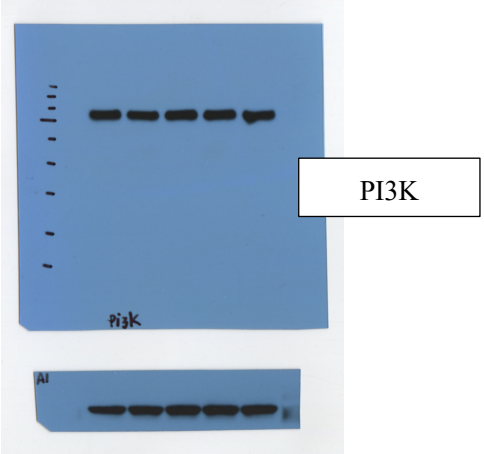
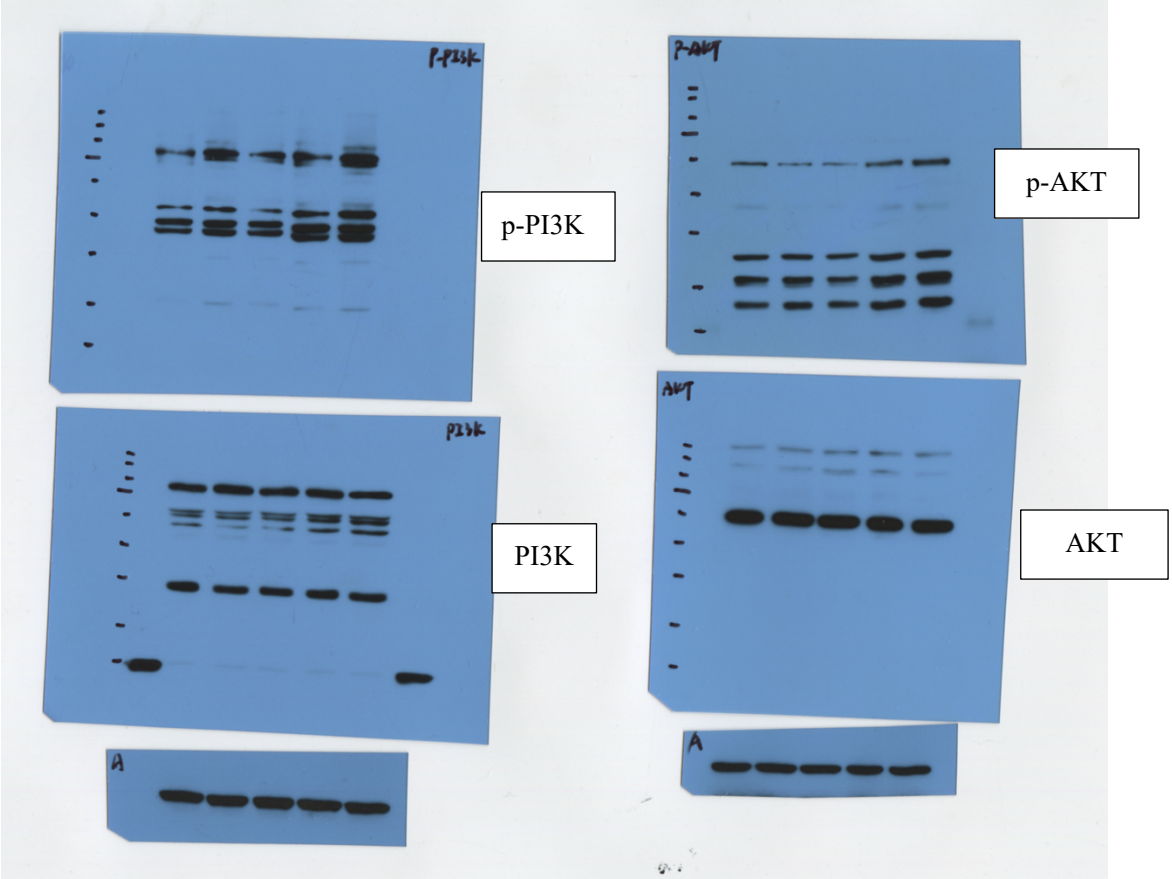
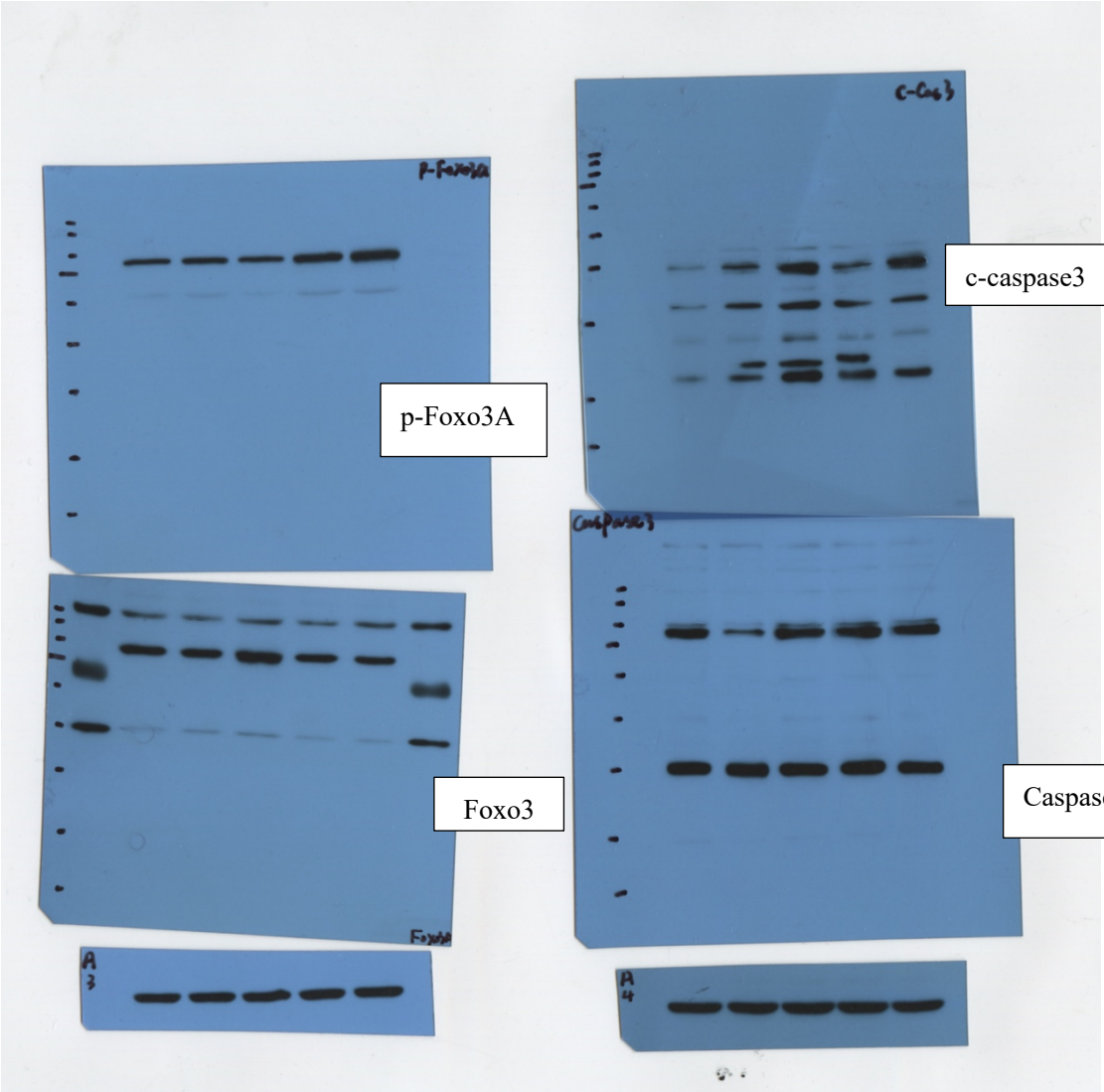

PI3K

Full unedited blots for Fig 6A PI3K signal (2)

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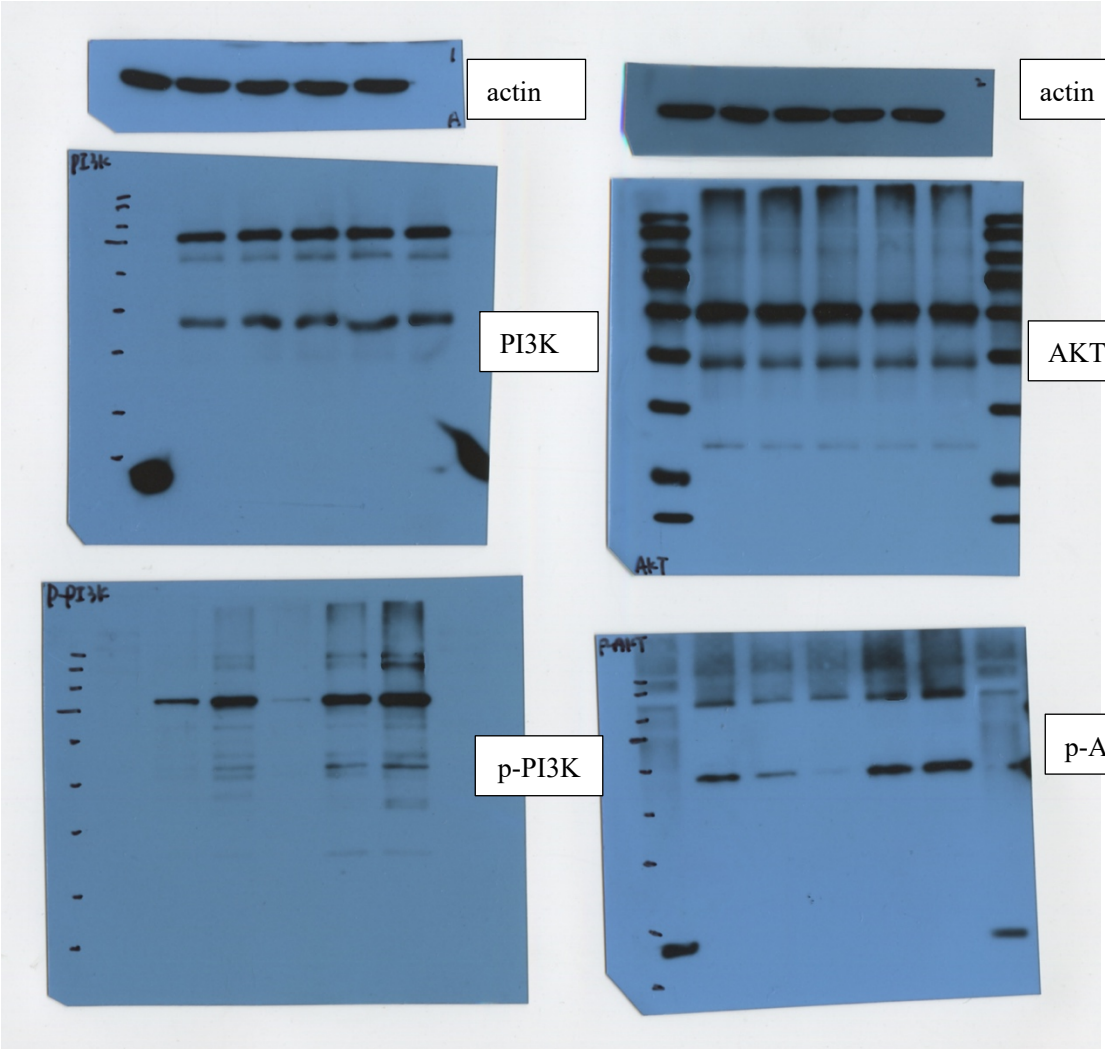
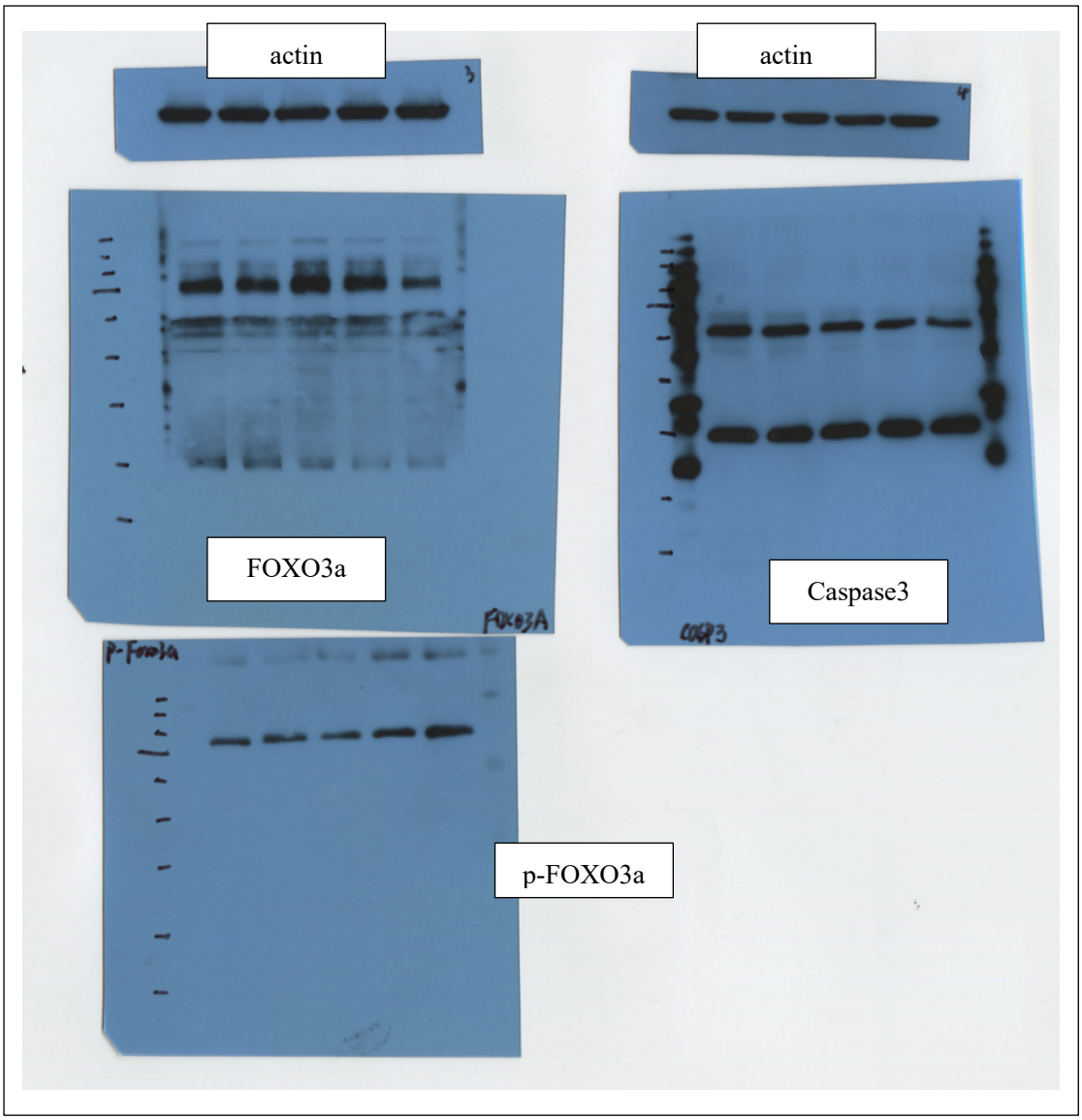
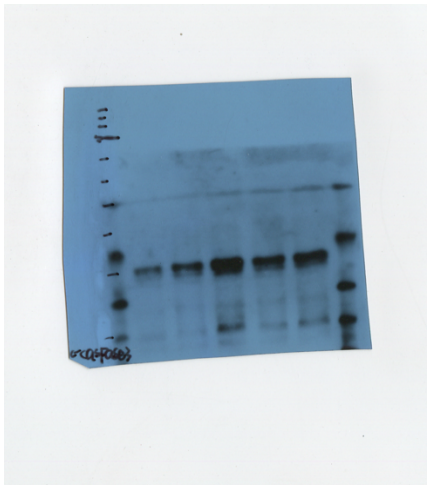
c-caspase3
Foxo3
Caspase3

Full unedited blots for Fig 6A PI3K signal (3)







c-caspase3

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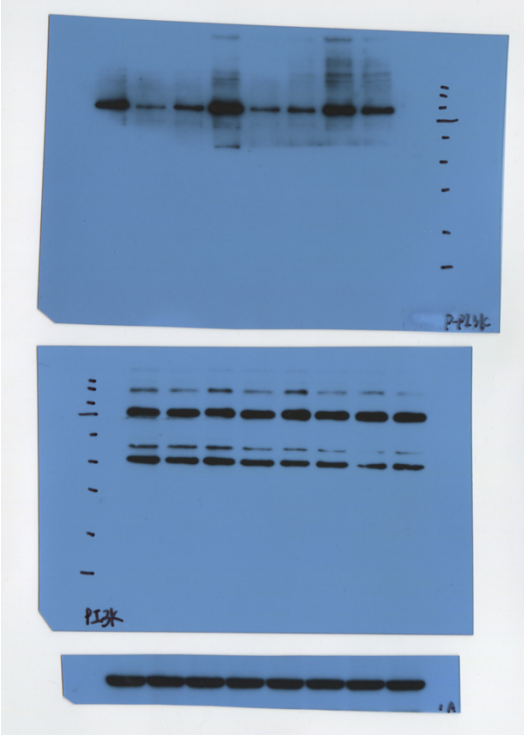
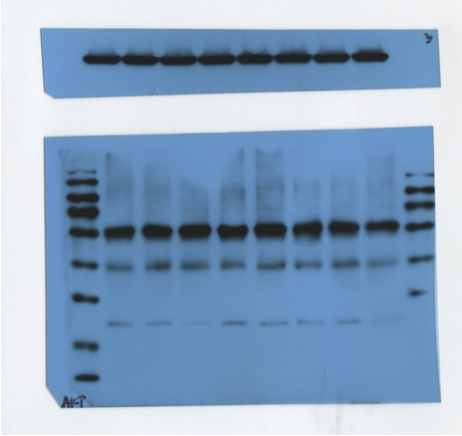


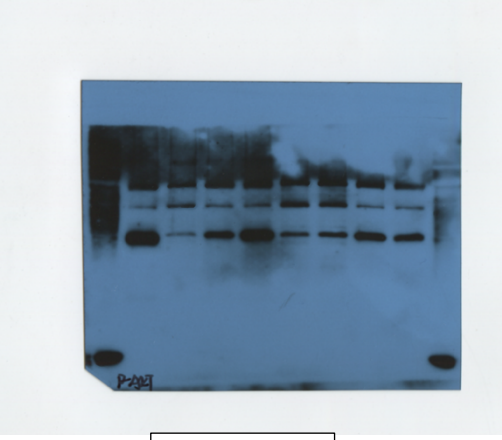
p-PI3K

PI3K

actin

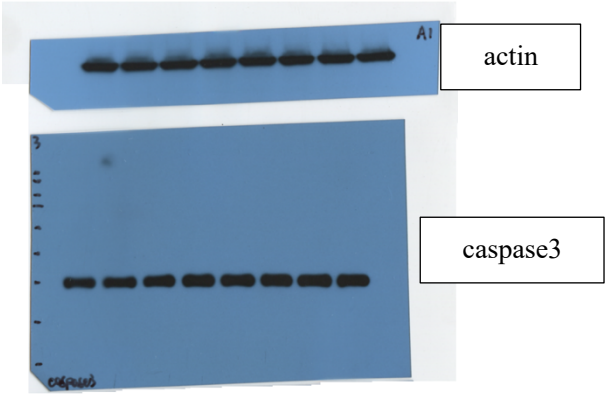


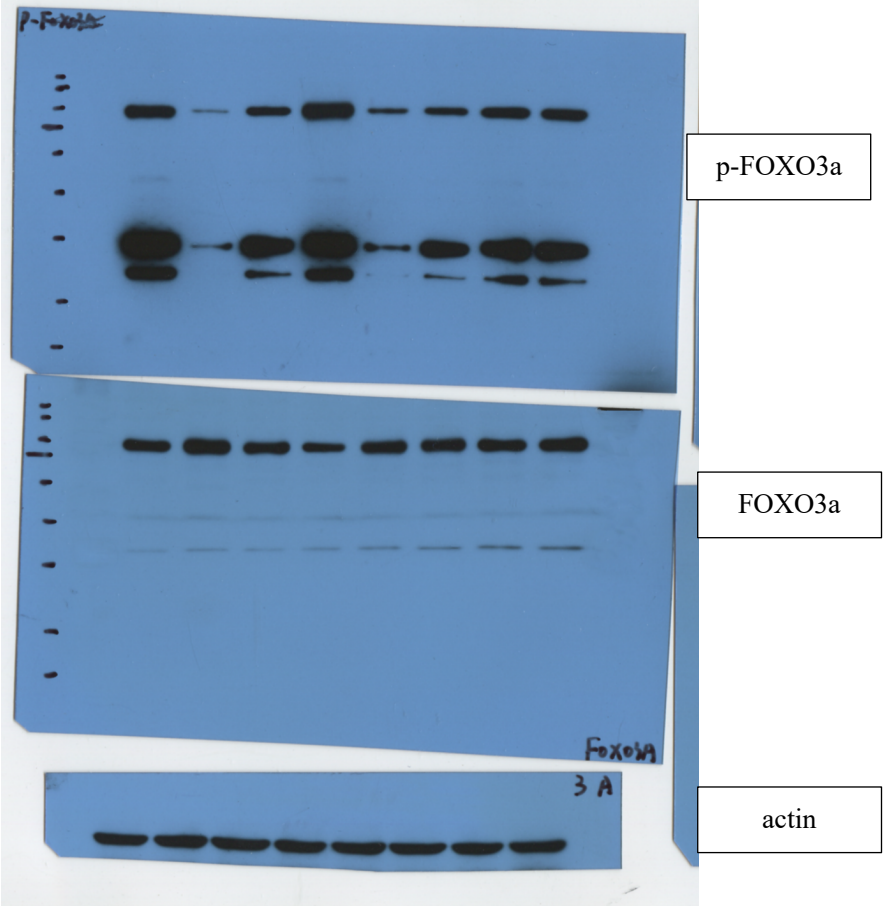
AKT



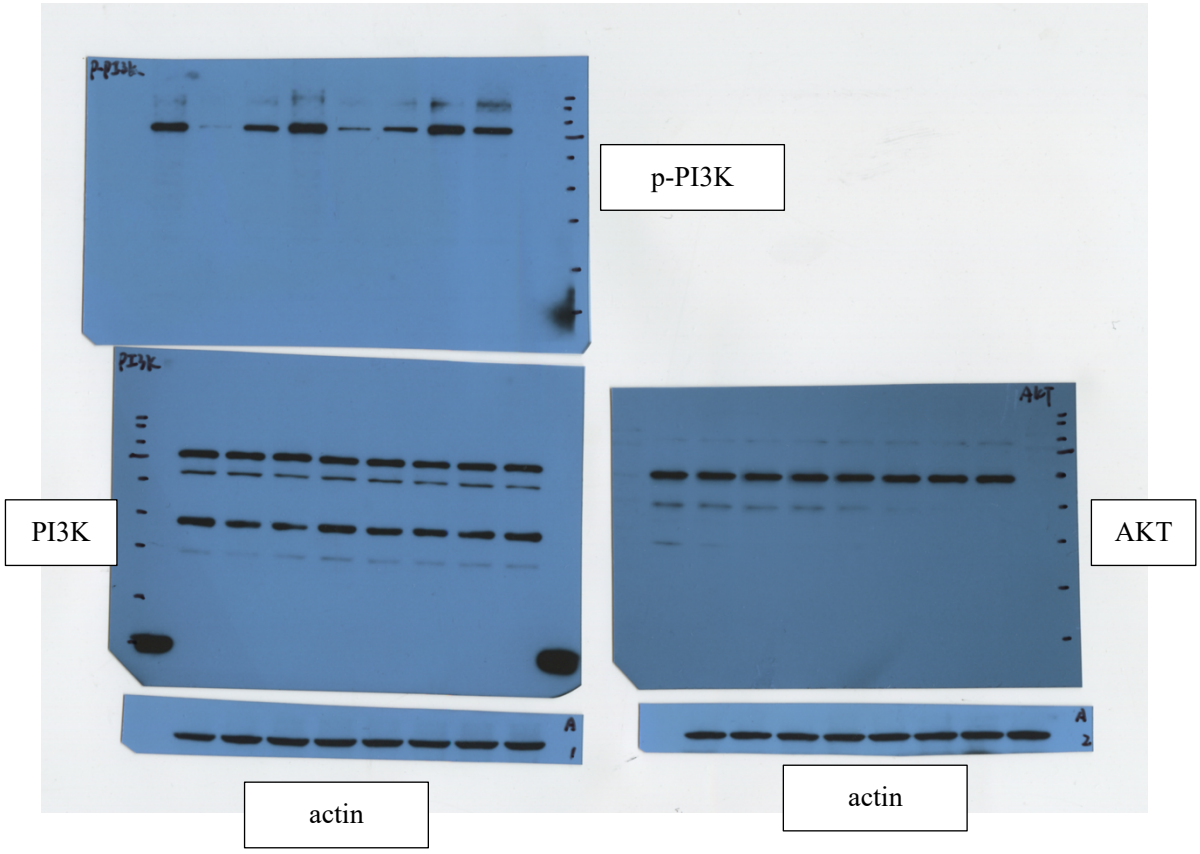
p-AKT

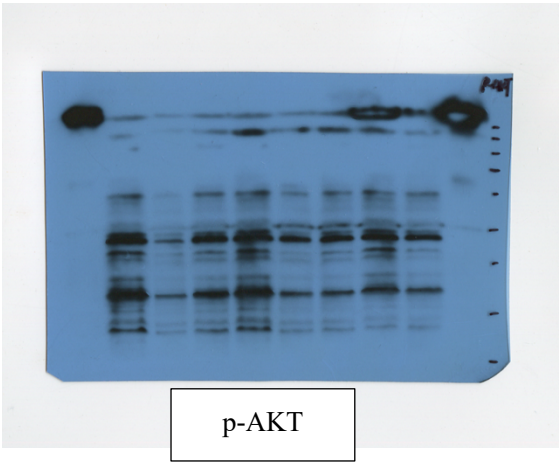

c-caspase3

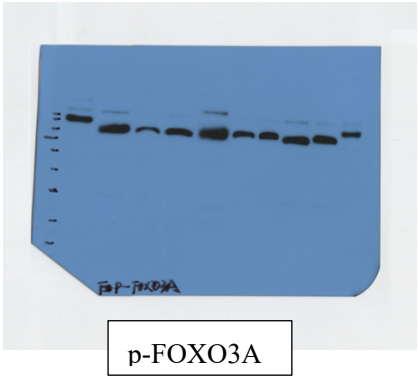

actin
caspase3

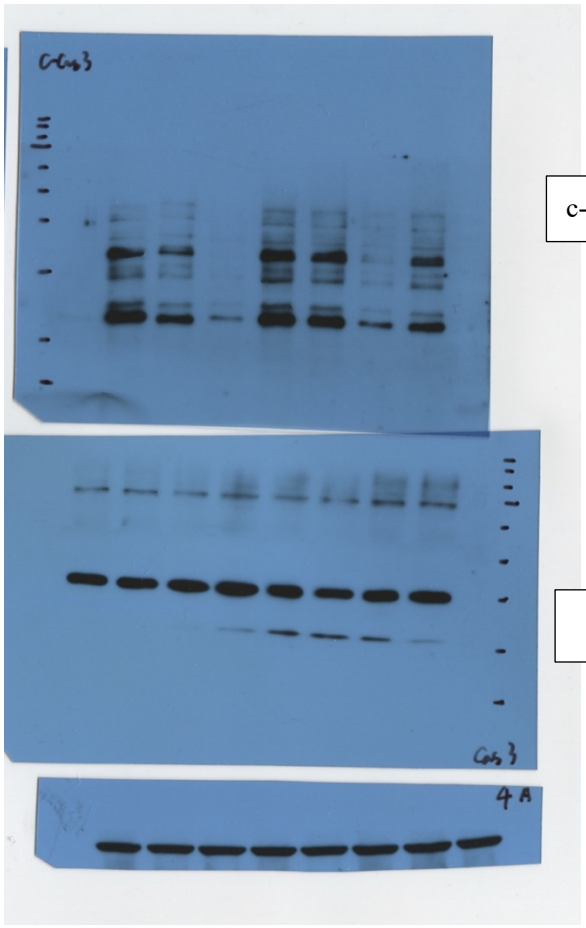

p-FOXO3a
FOXO3a
actin

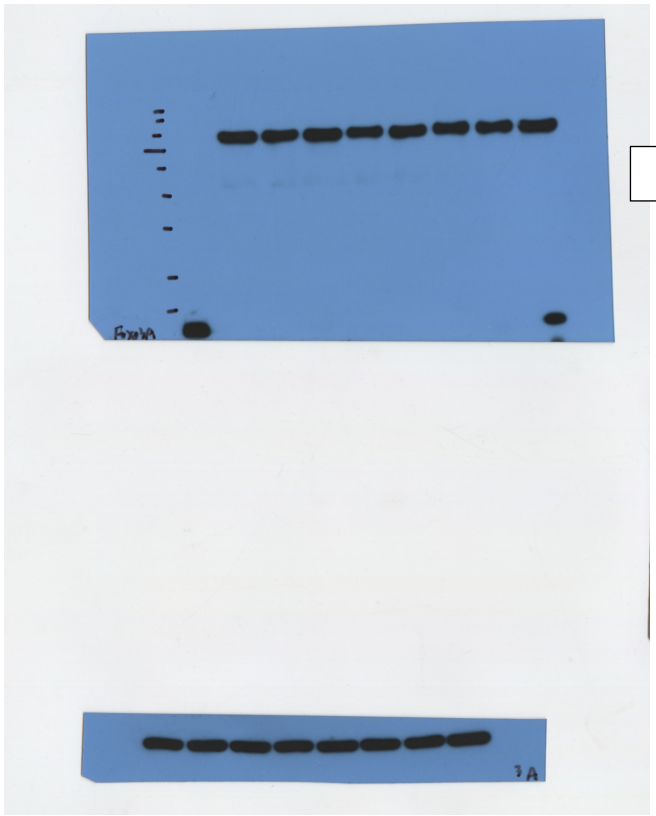
Full unedited blots for Figure 6B (2)








c-caspase3
caspase3
4A


Foxo3a
3A

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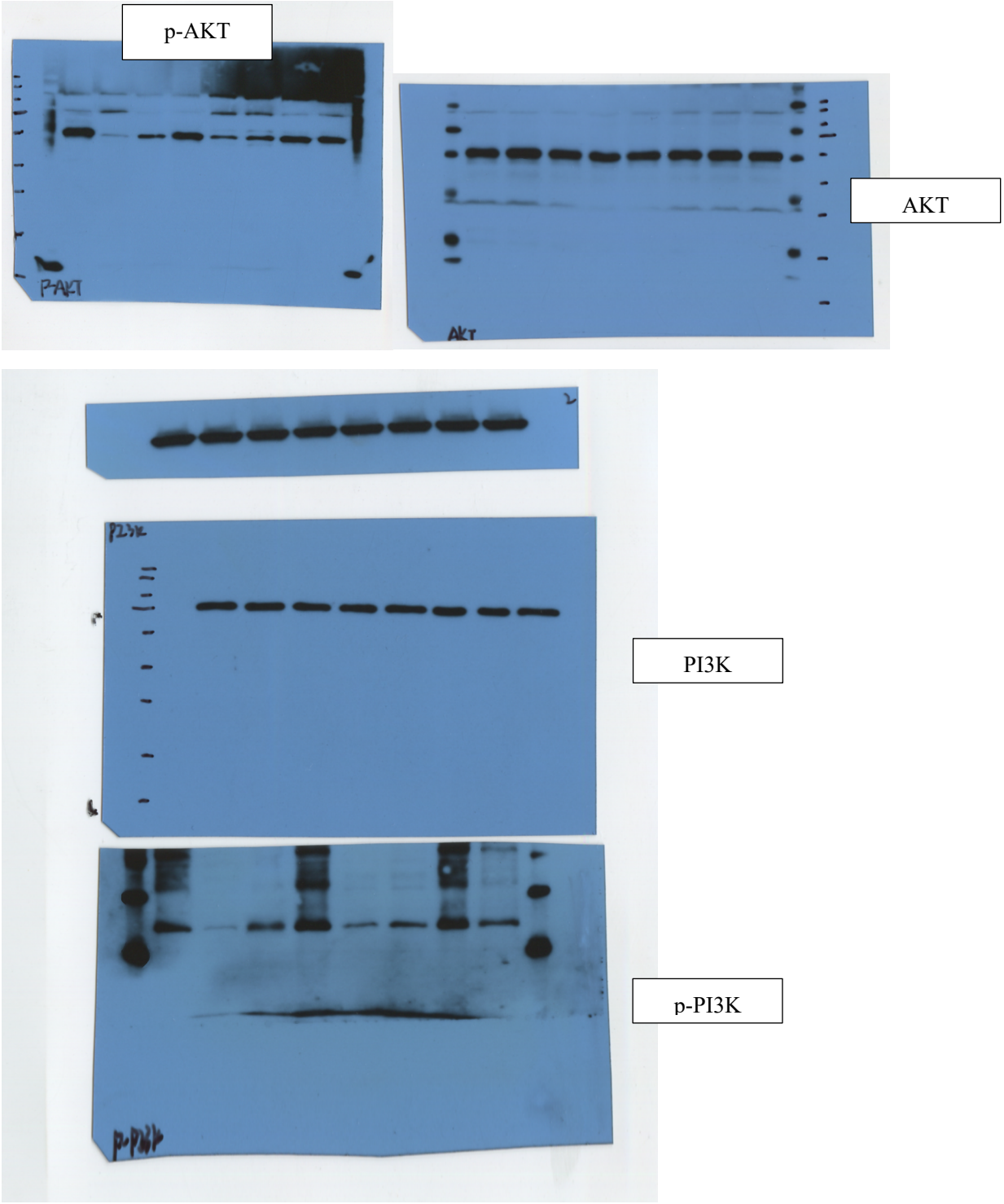

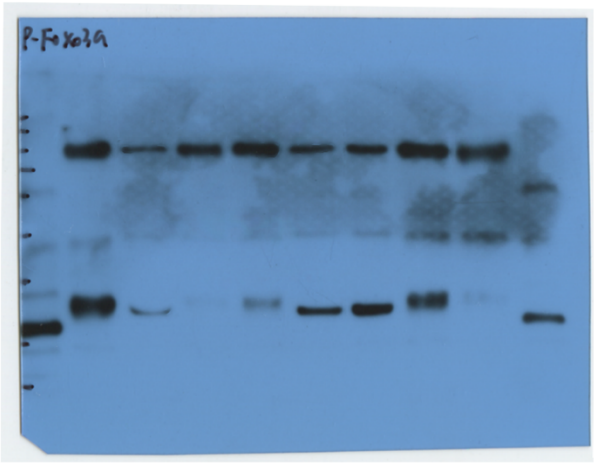
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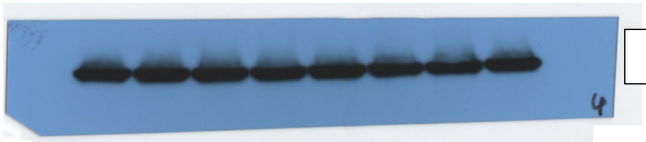
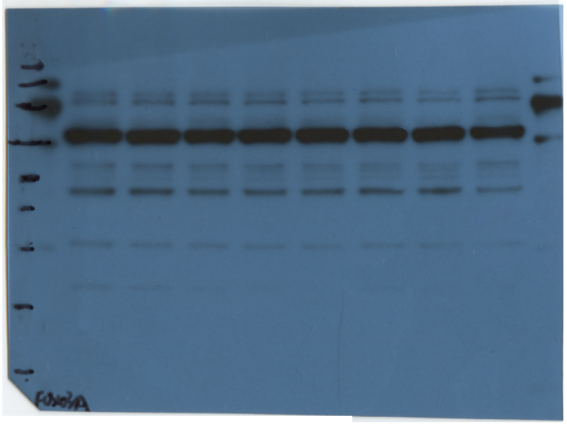

actin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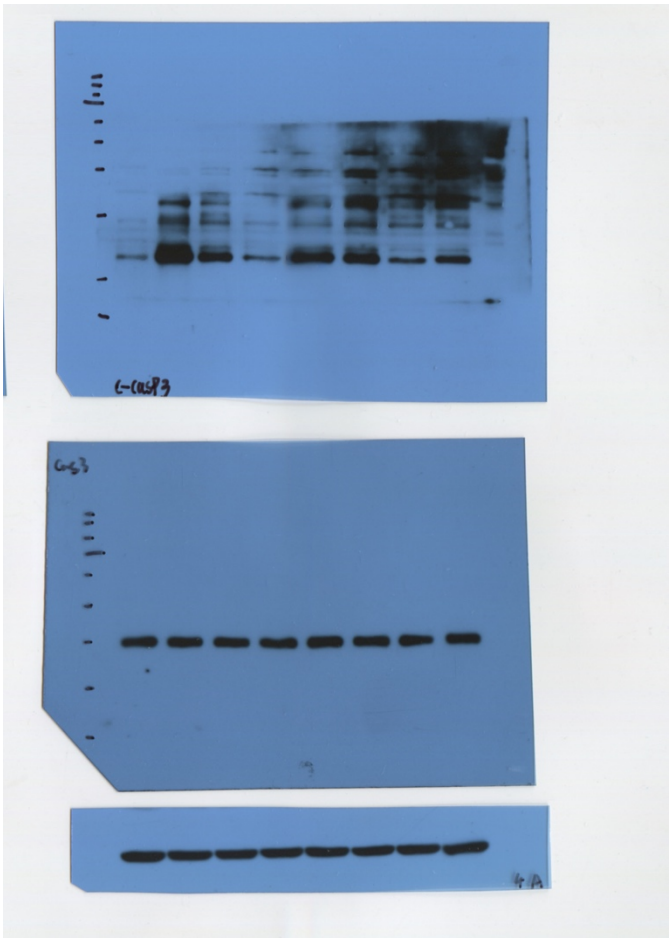

c-caspase3

caspase3