

**Phosphorylation of the histone demethylase KDM5B and regulation of the phenotype
of triple negative breast cancer**

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SUPPLEMENTARY METHODS

Co-immunoprecipitations—MDA-MB-231 cells were lysed using RIPA buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Igepal, 1 mM EDTA and 0.1% SDS) and were incubated with Protein G beads that had been pre-adsorbed with antibodies against phosphoserine (catalog number AB1603, Millipore), KDM5B (catalog number A301-813A, Bethyl Laboratories), KDM4B (catalog number A301-477A, Bethyl Laboratories), FLAG (catalog number F1804, Sigma-Aldrich), CDK1, or non-specific Rabbit/Mouse IgG (as negative controls). The beads were washed with RIPA buffer and PBS twice. After the final wash, immunoprecipitated proteins were eluted using SDS sample buffer, and analyzed by SDS-PAGE electrophoresis. Western blot analyses were performed as described below.

Chromatin Immunoprecipitation and ChIP qRT-PCR—Cells grown in 150 mm diameter dishes were fixed with 1% formaldehyde and lysed in SDS-lysis buffer with protease inhibitors. Lysed cells were sonicated using a Branson 450 sonicator. Clarified sonicated chromatin was diluted 10-fold in ChIP dilution buffer and used for immunoprecipitation with antibody. The antibody–chromatin complexes were bound with Protein G beads and subjected to a series of washes. The antigen–DNA complexes were eluted, reverse cross-linked overnight at 65°C, and the DNA purified using phenol/chloroform extraction. Ethanol precipitated pellets were resuspended in water and used as a template for PCR analysis. qRT-PCR reactions were performed in duplicate with SYBR Green Master Mix (catalog number 170-8882, Bio-Rad) and 10 μM of forward and reverse primers. Products were subjected to melting curve analysis. The fold enrichment values for known KDM5B binding sites were determined by normalizing to enrichment values for the control site.

Western Blotting—Total protein was extracted using MPER Mammalian Protein Extraction reagent (Thermo Fisher Scientific). Proteins were detected using their respective primary antibodies and HRP (horseradish peroxidase)-conjugated secondary antibody. GAPDH or tubulin were used as loading controls. Signals were visualized using the LI-COR Odyssey System, and quantified using the Image J software. Signals in each case were normalized to their respective GAPDH or tubulin values to calculate the relative expression levels.

Cell Invasion Assay. Transwell inserts (8-mm-diameter pore size; Corning Costar) were coated with 10% Matrigel™ and placed inside the wells of a 24-well plate. MDA-MB-231 cells transfected with control vector, FLAG-KDM5B, shKDM5B, and/or shCDK1 suspended in Opti-MEM® (serum-free culture medium, Invitrogen) were aliquoted onto the upper chamber of the Transwell insert (50,000 cells/well). The lower chambers contained MEM (minimal essential medium) supplemented with 5% FBS. The cells were allowed to migrate to the lower chamber at 37°C for 48 h. After incubation, cells invading the Matrigel™ were fixed with 3% paraformaldehyde and stained with 0.5% crystal violet. Invading cells were counted from five random fields per well under a microscope.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Regulation of pluripotency genes by KDM5B in non-TNBC lines. MCF-7 and T47D cells were transduced with shKDM5B lentiviruses. Shown are western blotting analyses of endogenous and/or transfected KDM5B and Nanog relative to loading control (GAPDH). Figures are representative of at least 3 independent experiments.

Figure S2. Detection of the phosphorylation of KDM5B by an ELISA assay. Serially diluted products from an *in vitro* kinase assay were loaded onto GST coated wells and incubated with phosphoserine antibody and HRP-conjugated goat anti-rabbit. Signals were detected by the addition of chromogenic substrate (TMB) followed by quantification of absorbance at 450 nm wavelength. GST-WT is KDM5B^{WT} conjugated with GST, while GST-S1456A is KDM5B^{S1456A} conjugated with GST. GST-Control (GST-Ctl) served as negative control, while GST-HIF1 α as positive control. Figures are representative of at least 3 independent experiments.

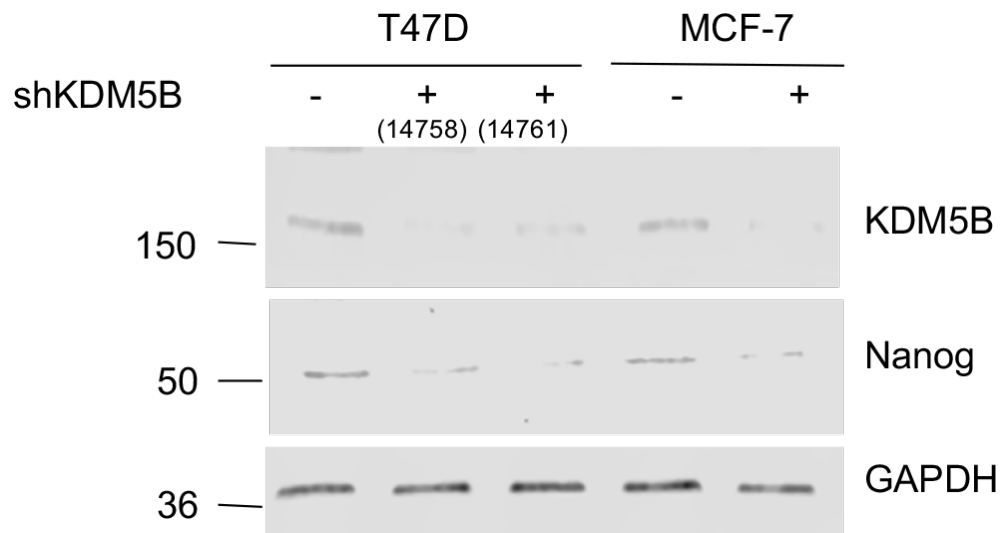
Figure S3. Mammosphere formation assay in MDA-MB-453 and HCC1937 cells. MDA-MB-453 and HCC1937 cells were transfected as indicated with expression vectors for FLAG-KDM5B^{WT} or shKDM5B respectively. Transfected cells were cultured in mammosphere media and colonies were quantified. Figures are representative of at least 3 independent experiments.

Figure S4. Regulation of cell invasion by KDM5B and CDK1. MDA-MB-231 cells were transfected with control vector (Ctl), or expression vectors for KDM5B^{WT}, shKDM5B, and/or shCDK1. Invasion of cells through MatrigelTM was assessed in the Transwell invasion assay. Figures are representative of at least 3 independent experiments.

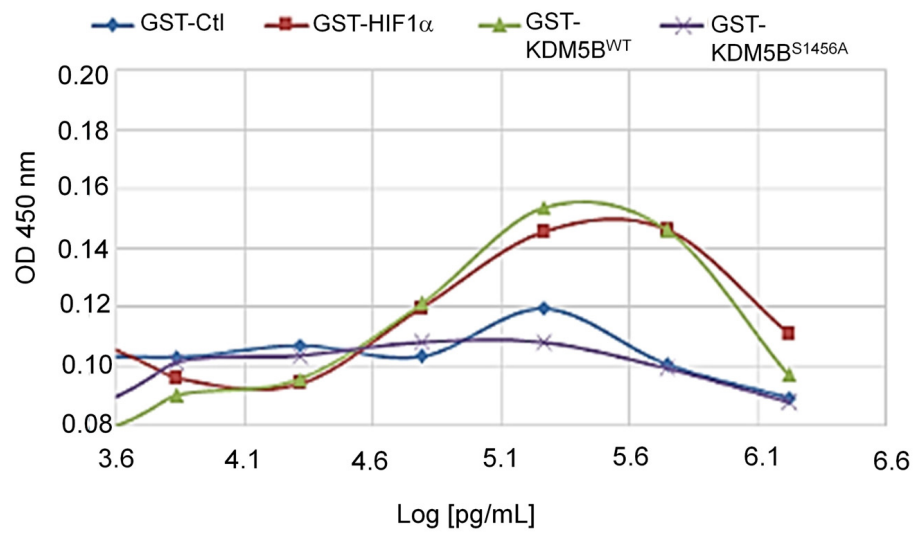
Figure S5. Involvement of another histone demethylase, KDM4B, in the regulation of Nanog by CDK1. (A) Lysates from MDA-MB-231 cells were immunoprecipitated using antibodies against KDM4B or CDK1 and analyzed for co-immunoprecipitation of CDK1 or KDM4B by Western blotting. Normal rabbit IgG was used as a negative control. Input lanes represent 25% of the total protein. MDA-MB-231 cells were transfected with control vector or expression vector for shCDK1. (B) ChIP analyses of lysates from MDA-MB-231 cells immunoprecipitated with antibodies against KDM4B, H3K36me3, or control non-specific rabbit immunoglobulin, followed by PCR amplification of the promoter region of *SOX2* and *NANOG*. Figures are representative of at least 3 independent experiments. (C) Western blotting analyses of endogenous KDM4B, Nanog, and Sox2 relative to loading control (GAPDH). (D) MDA-MB-231 cells were transfected with expression vector for shKDM4B. Transfected cells were cultured in mammosphere media and colonies were quantified. Figures are representative of at least 3 independent experiments

Supplementary Table S1. Primer sequences

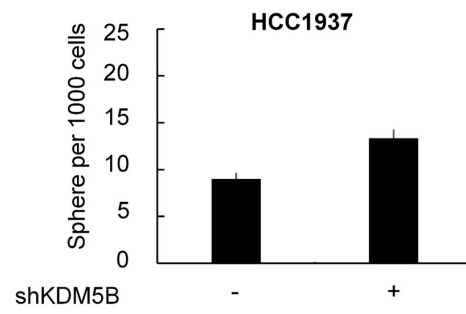
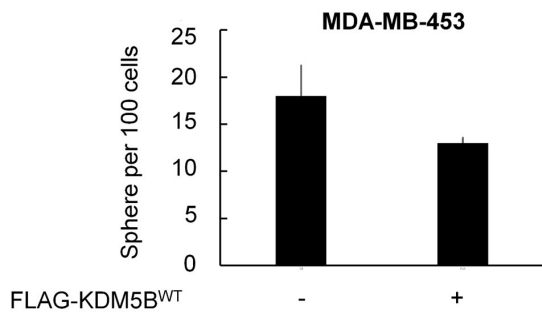
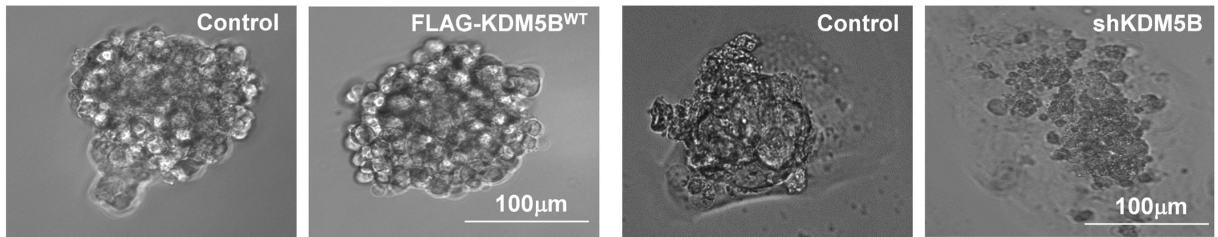
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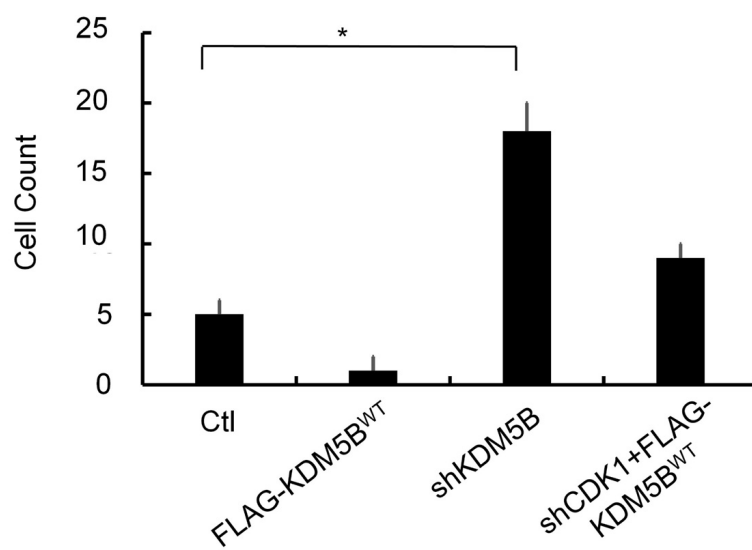
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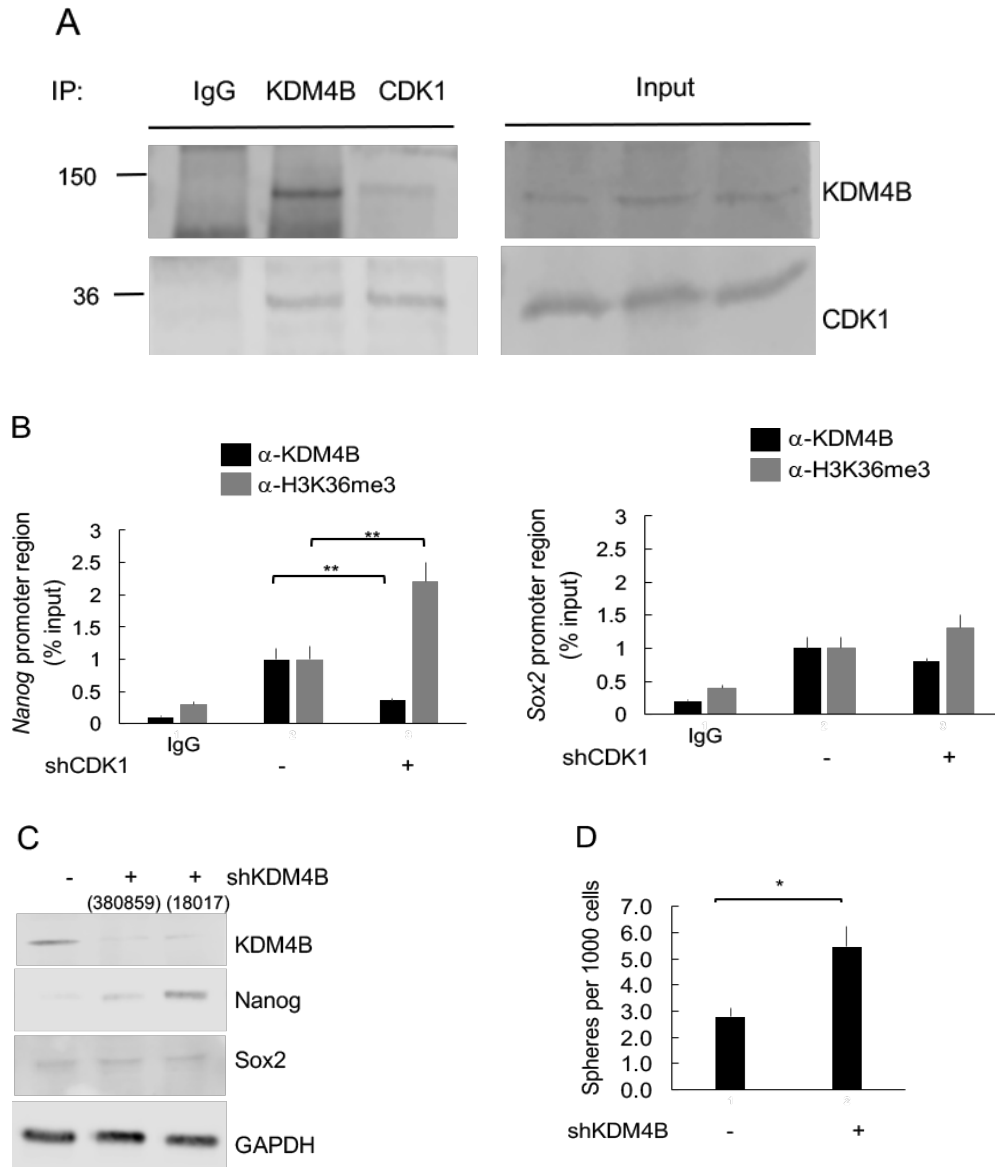
S3



S4



S5



Supplementary Table S1.

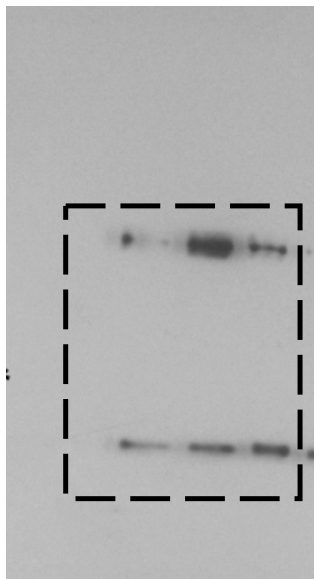
Primers for Chromatin-IP	
<i>SOX2</i> promoter	Fw. GGT ACC GGC CAA AGA GCT GAG TTG GA Rev. AAG CTT GAG GAC AAC TGG AAT CAG GAT C
<i>NANOG</i> promoter	Fw. GTT CTG TTG CTC GGT TTT CT Rev. TCC CGT CTA CCA GTC TCA CC

Primers for RT-PCR	
<i>KDM5B</i>	Fw. CAT CAC TGG CAT GTT GTT CAA ATT C Rev. GAA TGT AGT AAG CCA CAA GAA GC
<i>CDK1</i>	Fw. CCT AGT ACT GCA ATT CGG GAA ATT Rev. CCT GGA ATC CTG CAT AAG CAC
<i>SOX2</i>	Fw. AGC TCG CAG ACC TAC ATG AA Rev. CCG GGG AGA TAC ATG CTG AT
<i>NANOG</i>	Fw. ACC CAG CTG TGT GTA CTC AA Rev. GGA AGA GTA AAG GCT GGG GT
<i>GAPDH</i>	Fw. GTC ATC ATC TCT GCC CCC TCT GCT Rev. CTT CTT GAT GTC ATC ATA TTT G

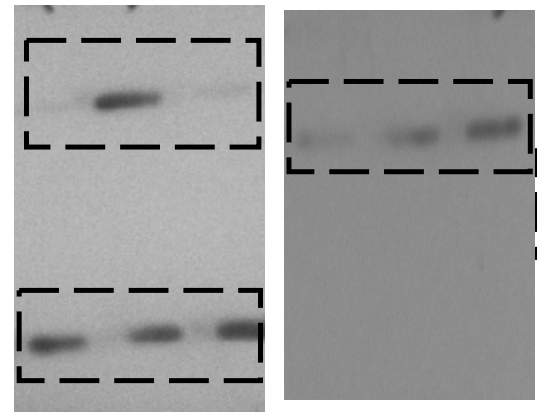
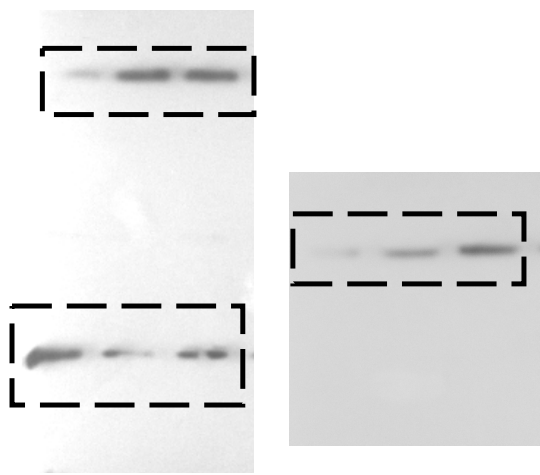
Primers for Subcloning	
<i>KDM5B</i> ^{S1384A}	Fw. GGG TCT CAC TGG TGC GCT TCG GTC AGT CT Rev. AGA CTG ACC GAA GCG CAC CAG TGA GAC CC
<i>GST-KDM5B</i> ^{S1456A}	Fw. GCA GAA CGA ACT AAT TCA TAG GCA CGC TCT CTC TCT AAC TTG AA Rev. TTC AAG TTA GAG AGA GAG CGT GCC TAT GAA TTA GTT CGT TCT GC
<i>GST-KDM5B</i> ^{WT}	Fw. CGC GGA TCC ATG CAG TCT CTC AGA Rev. CCG CTC GAG TTA CTT TCG GCT TGG T

Figure 1

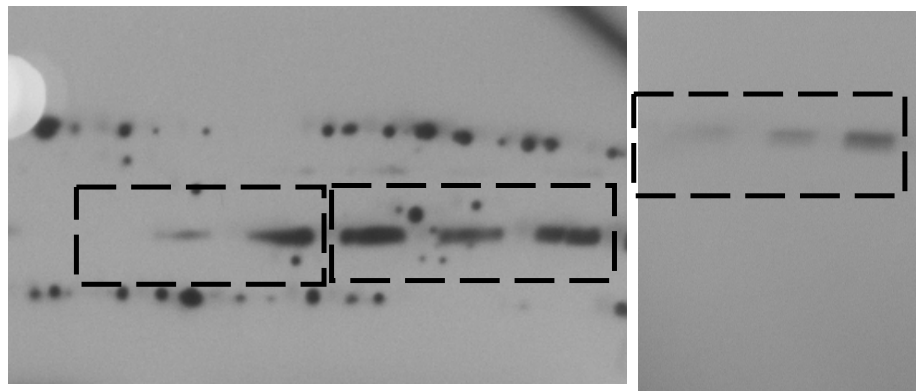
A



B



C



D

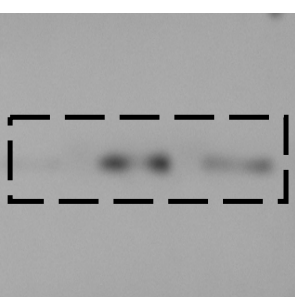
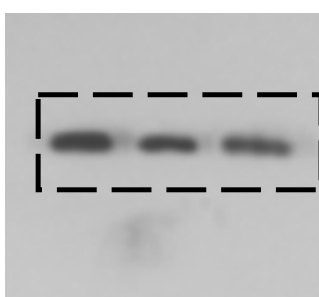
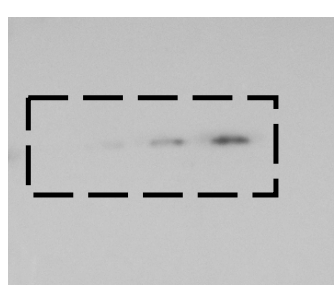
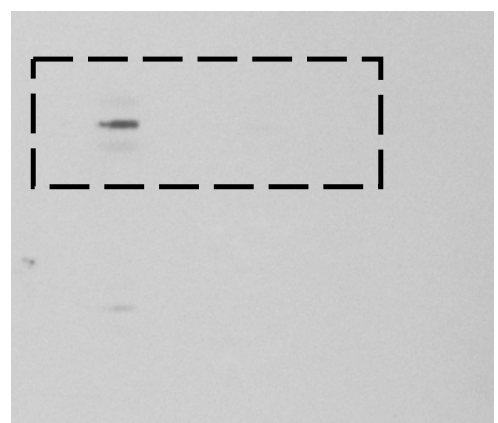


Figure 2

C

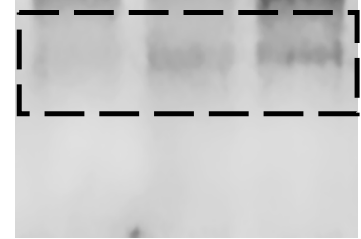
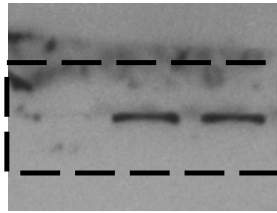
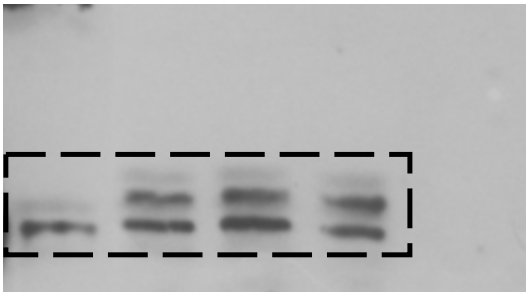
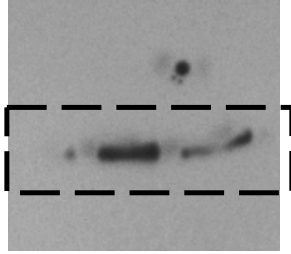
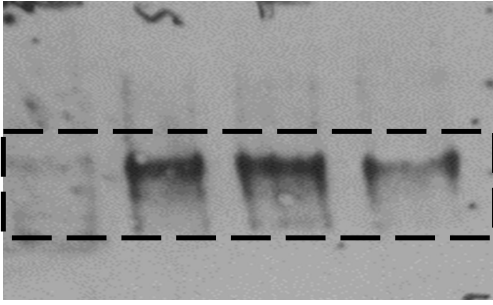


Figure 3A

Figure 3B

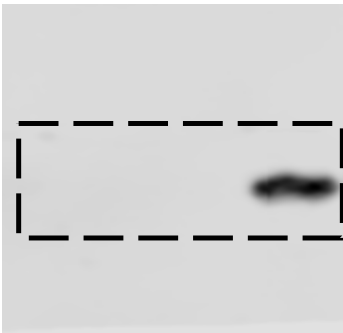
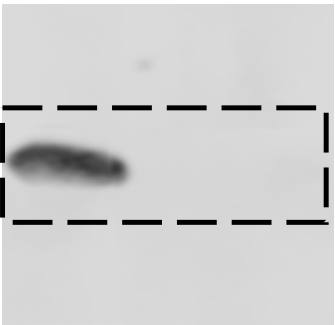
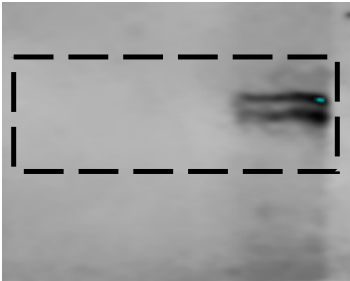
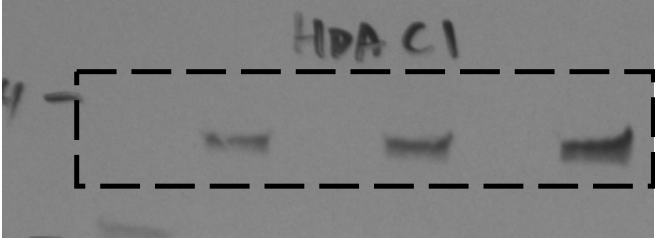
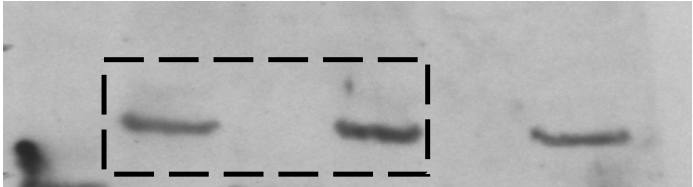
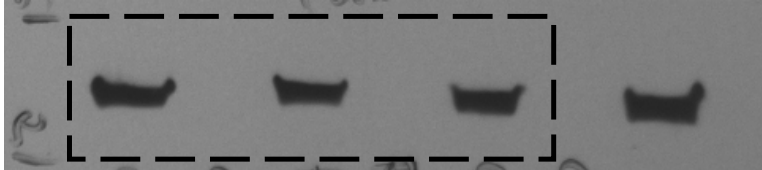
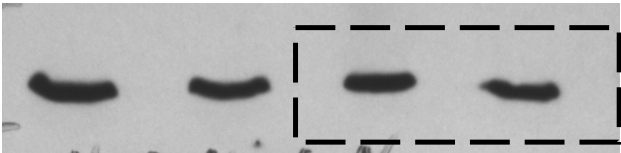
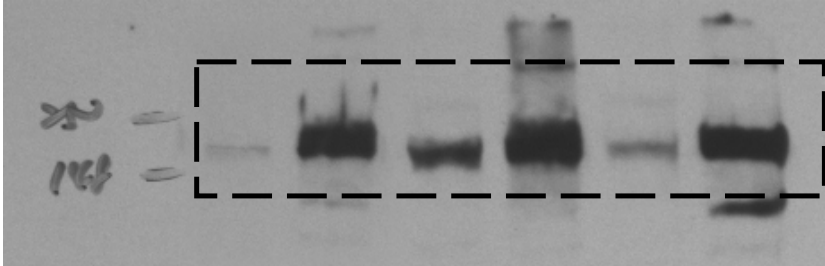
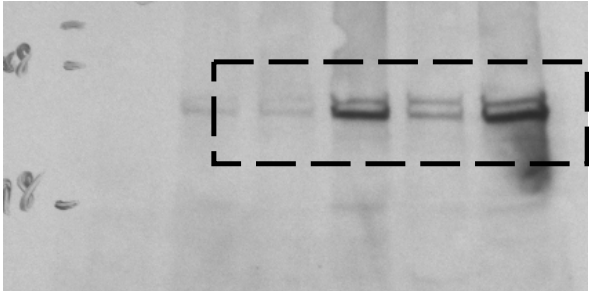
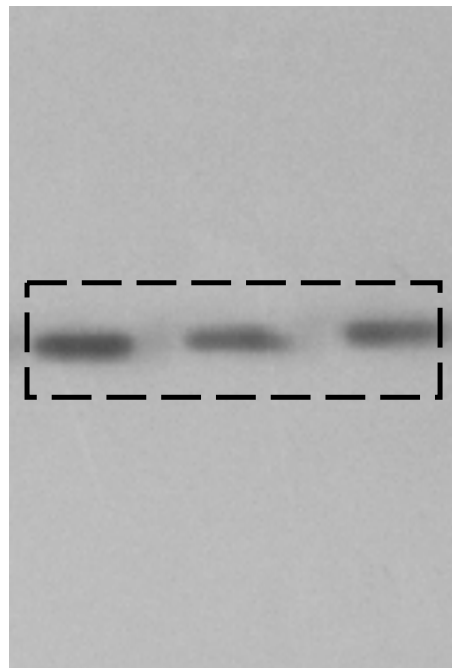
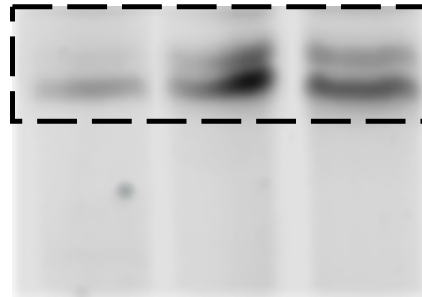
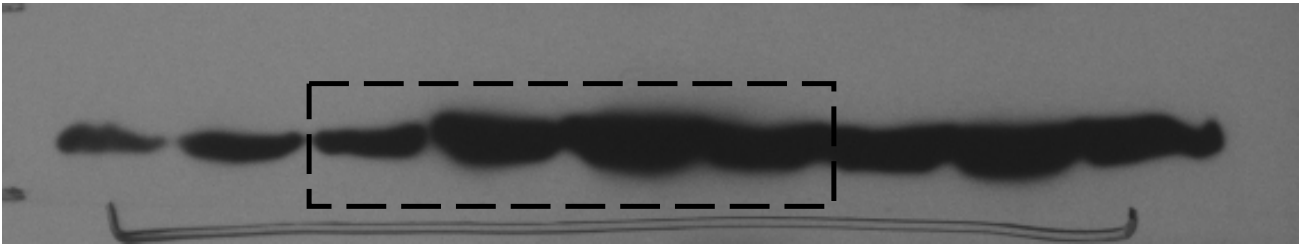
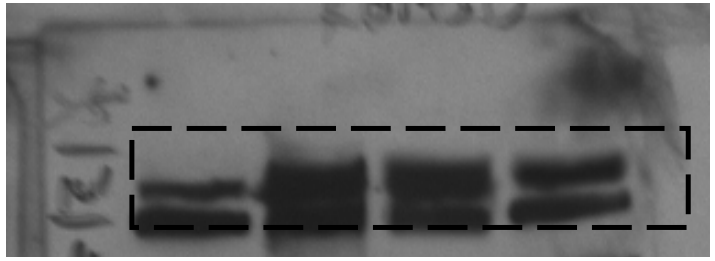
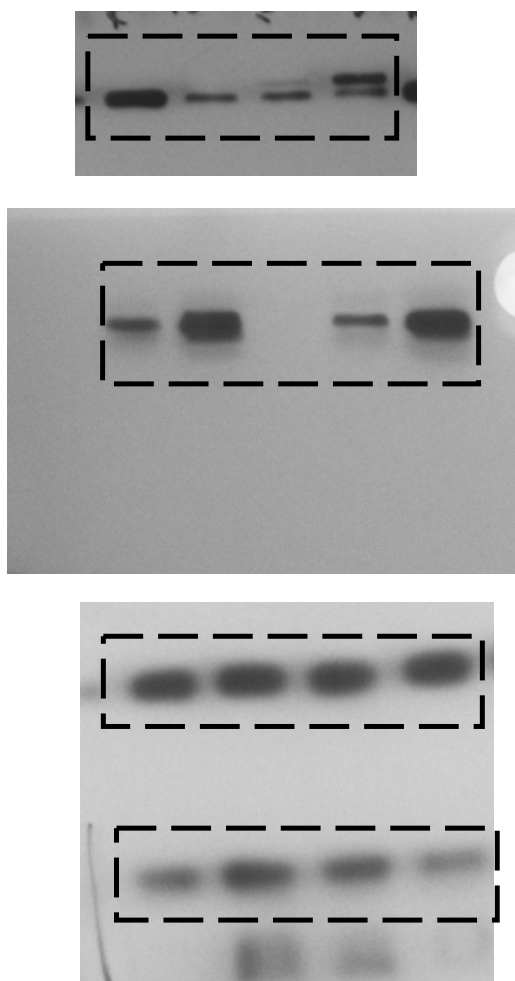


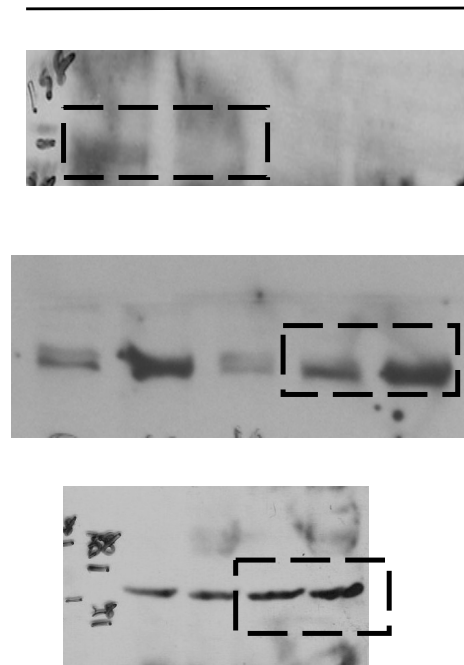
Figure 4D



MDA-MB-231 +/- shKDM5B or FLAG-KDM5B



MDA-MB-231 +/- shKDM5B



MDA-MB-231 +/- shKDM5B

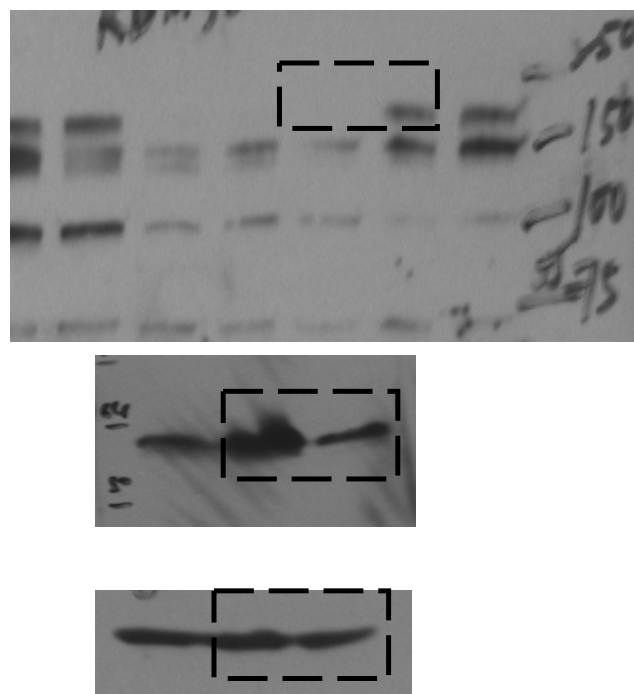
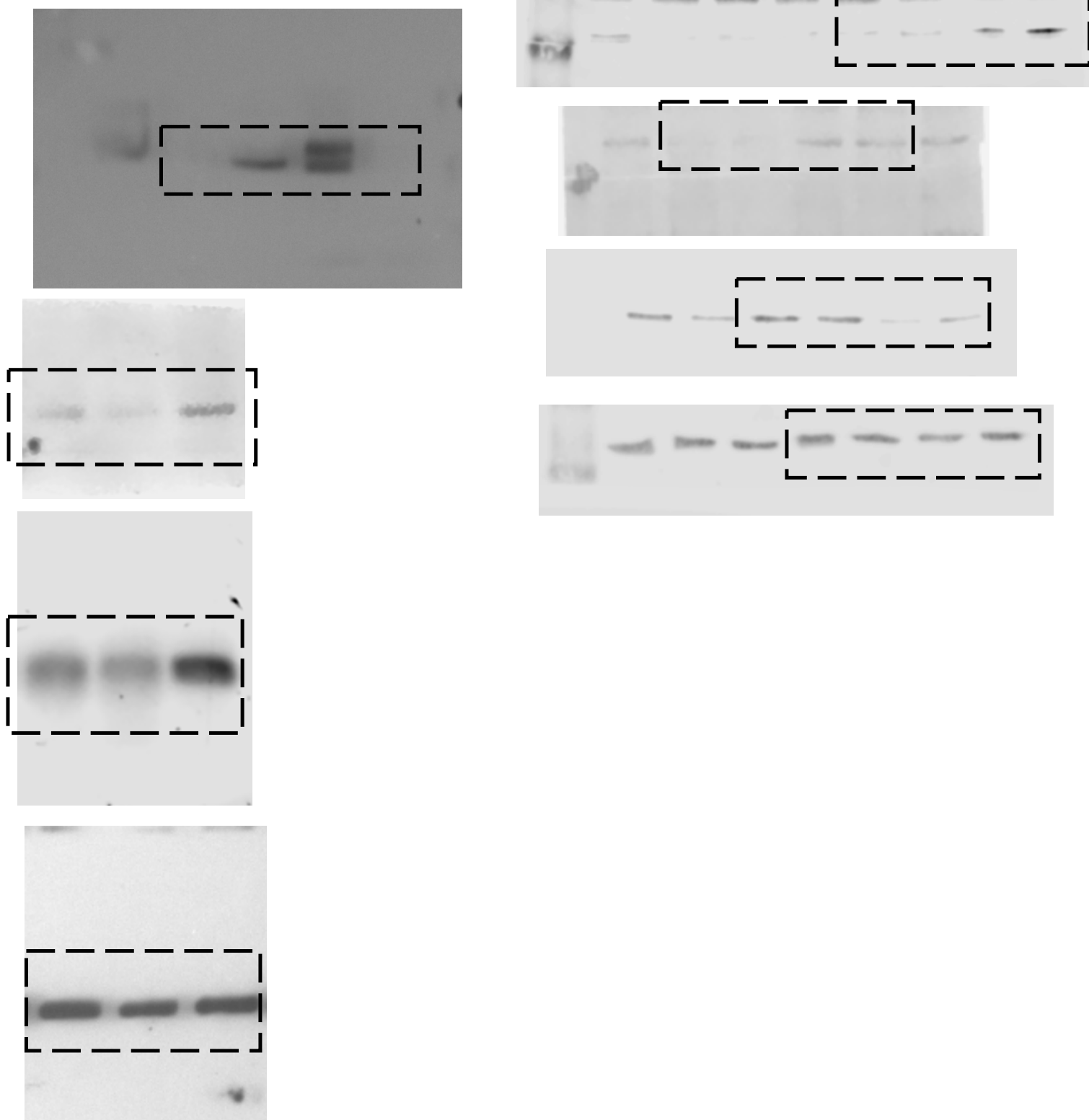


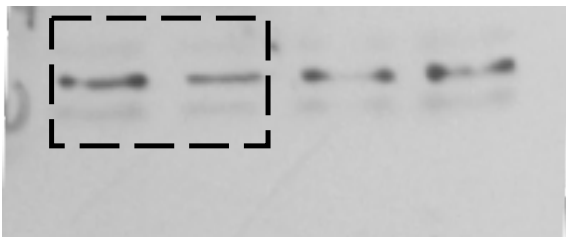
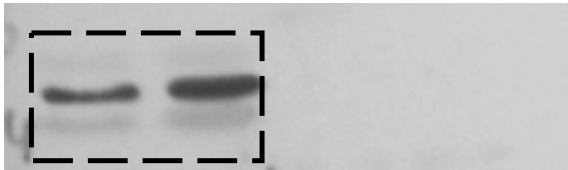
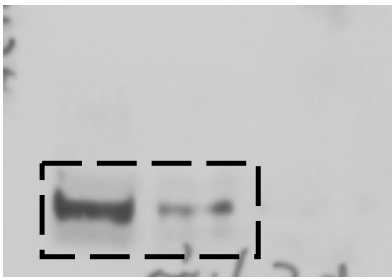
Figure 5C

MDA-MB-453 +/- fIKDM5B or shKDM5B

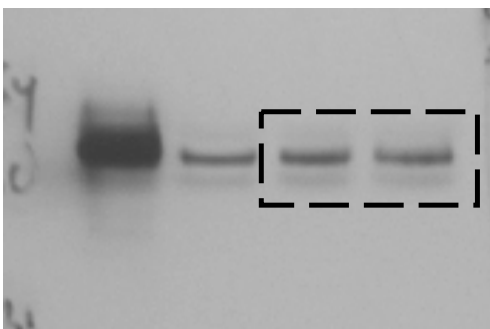
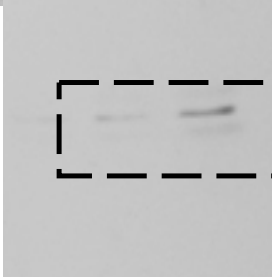
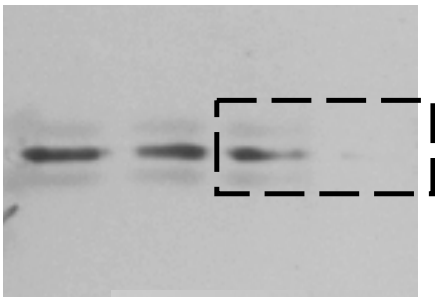
MDA-MB-231 +/- shCDK1



HCC70 +/- shKDM5B



HCC1937 +/- shKDM5B



MDA-MB-231

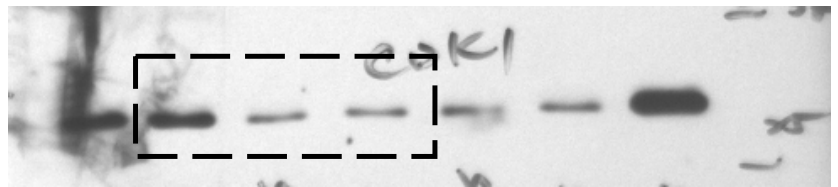
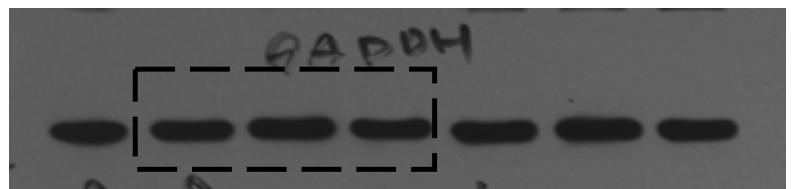
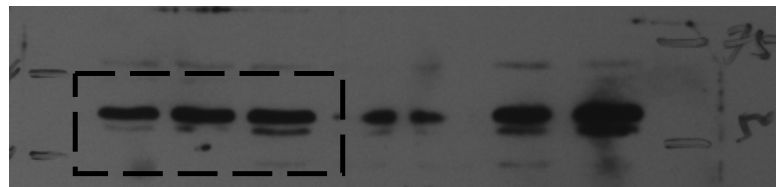
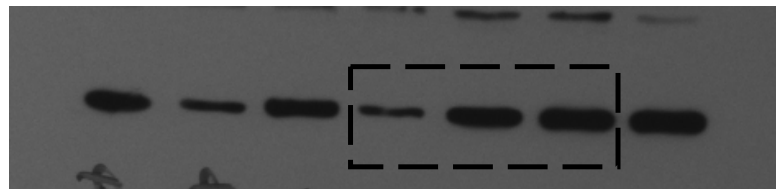
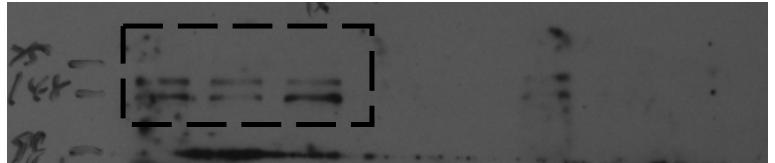
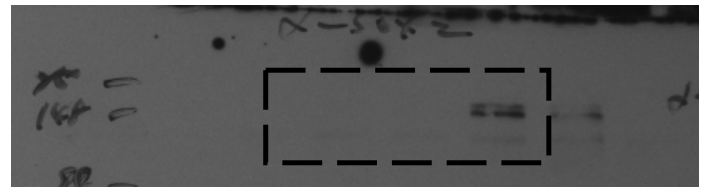
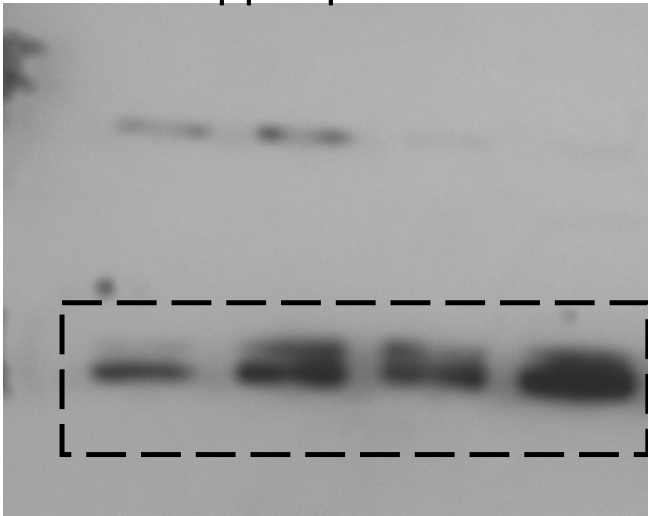


Figure 5D

Upper panel



lower panel

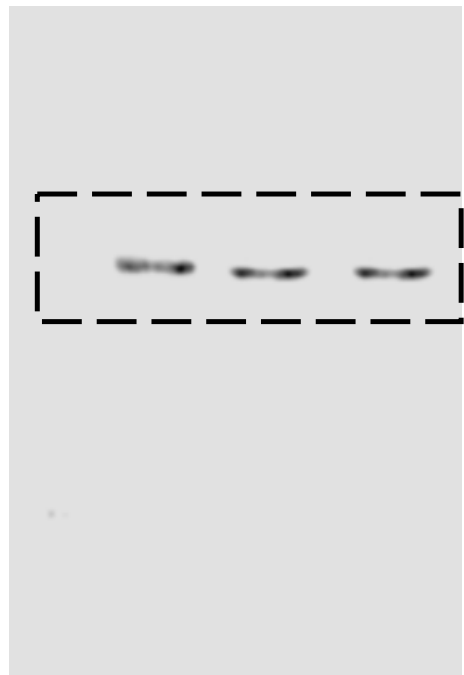
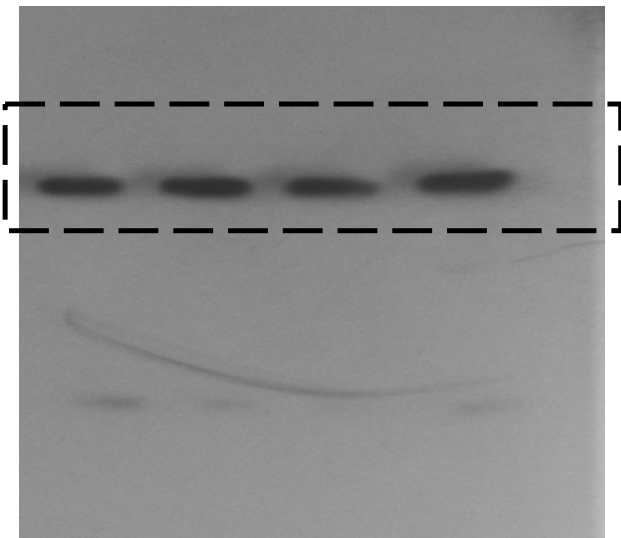
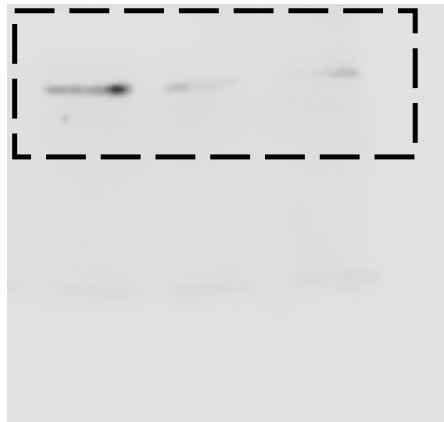
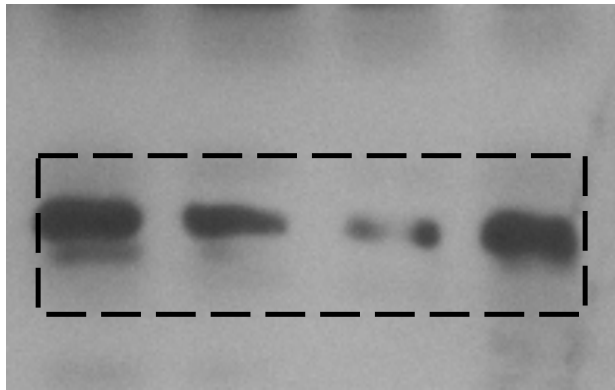
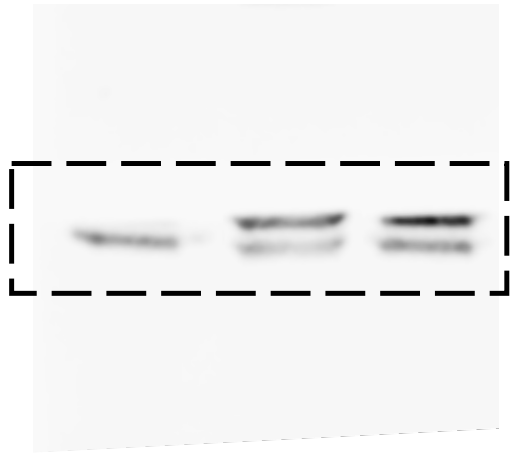


Figure 6A

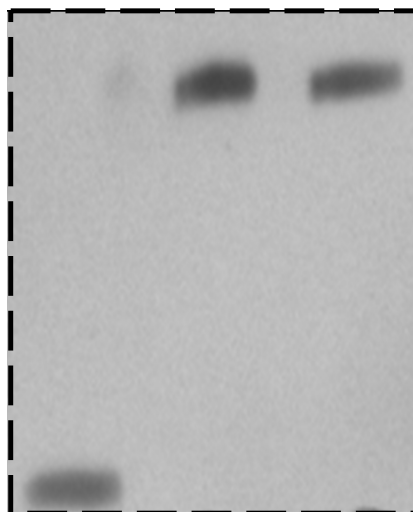
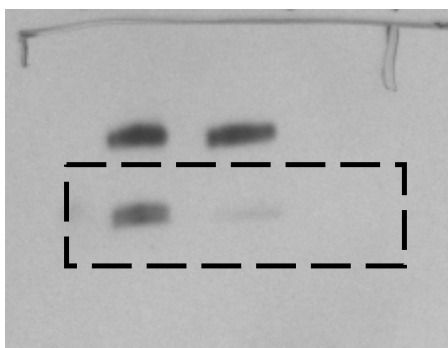


Figure 6B

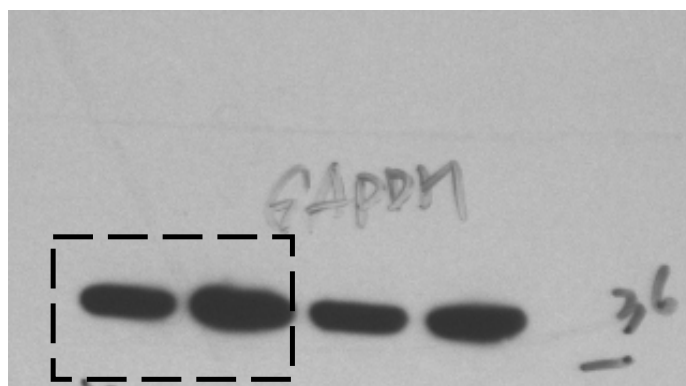
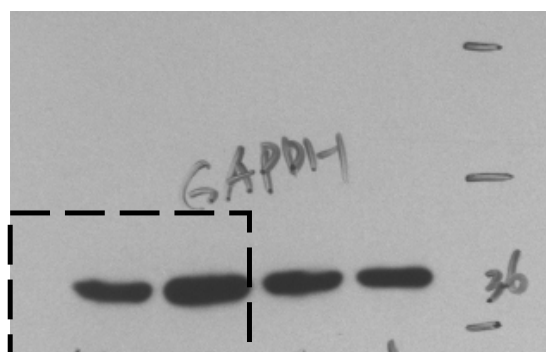
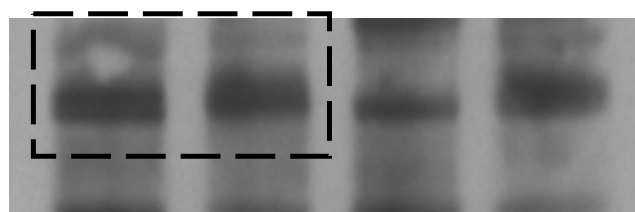
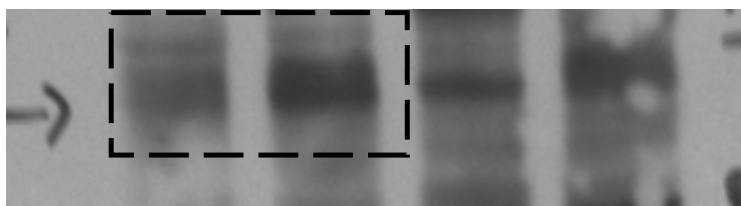
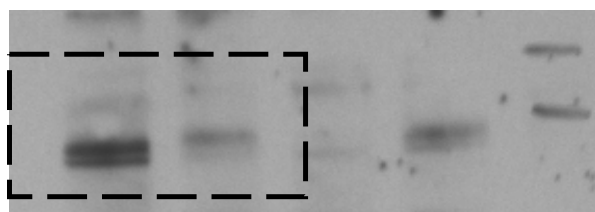
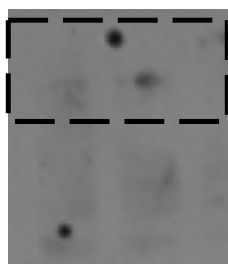


Figure 6C

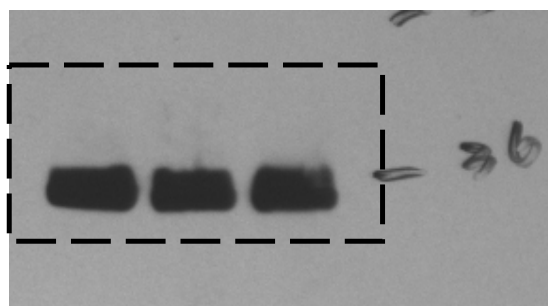
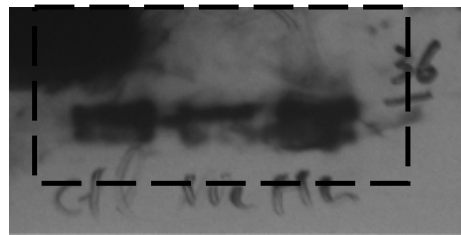
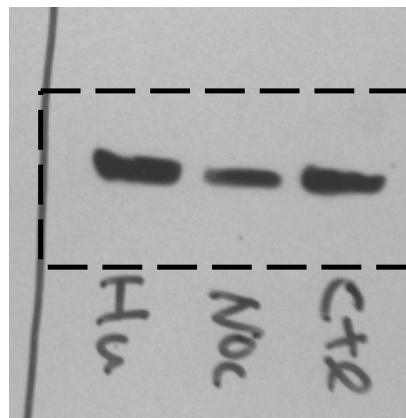
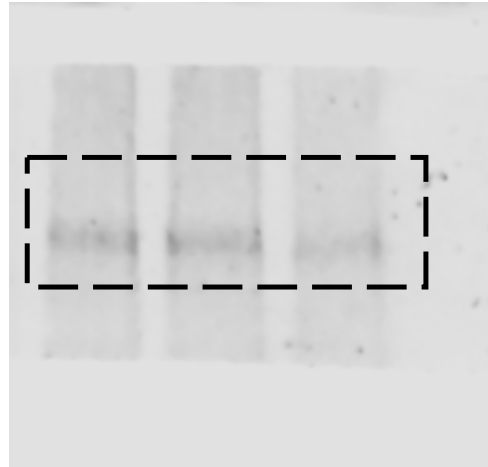
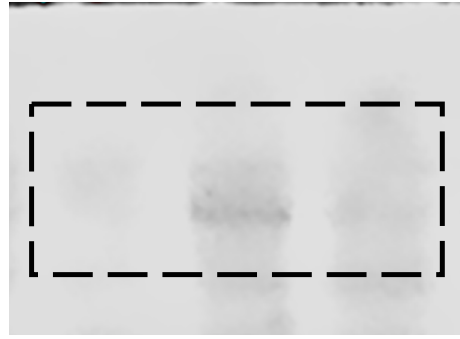


Figure 8A

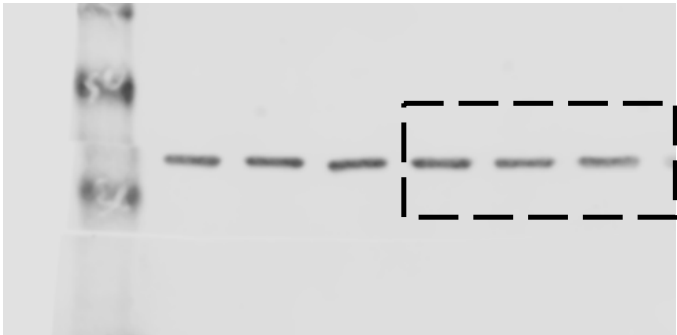
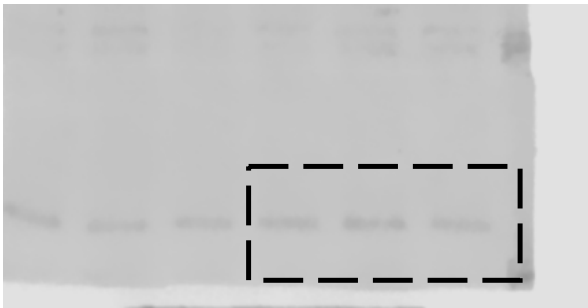
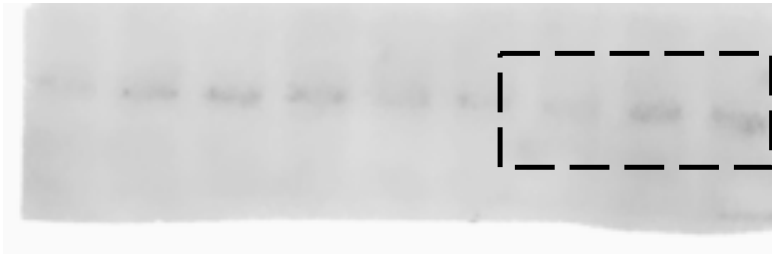


Figure 8B

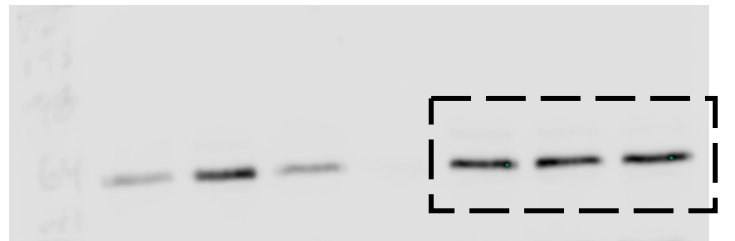
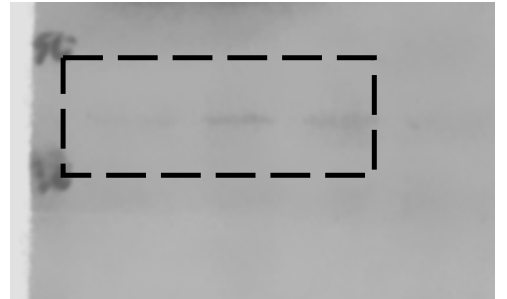


Figure S1

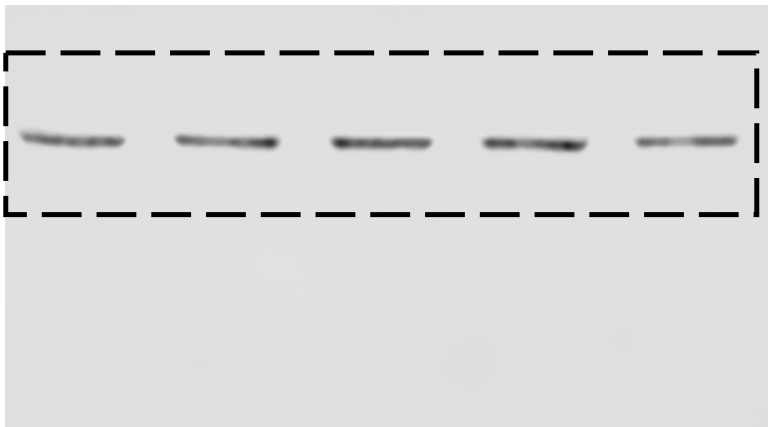
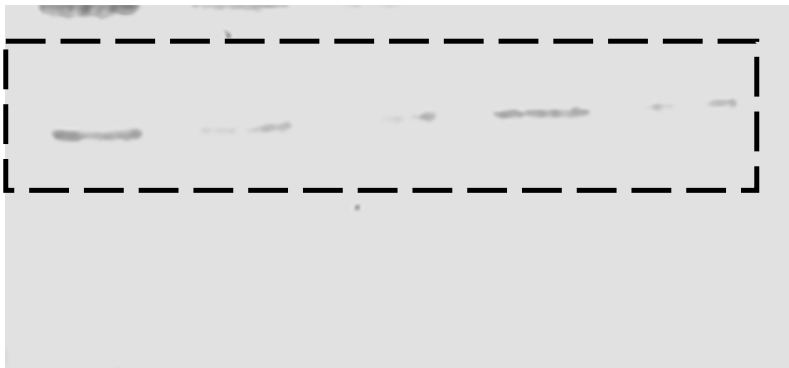
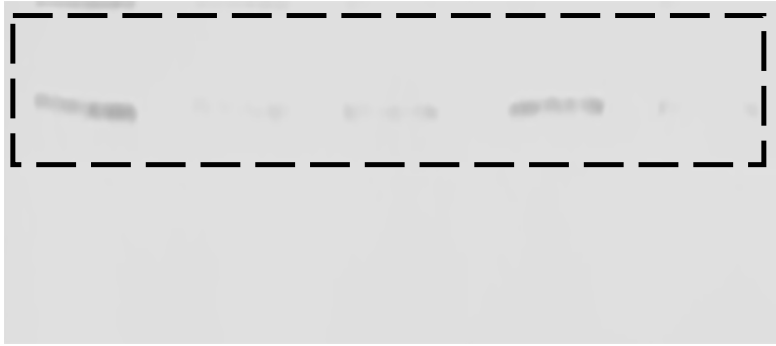


Figure S5A

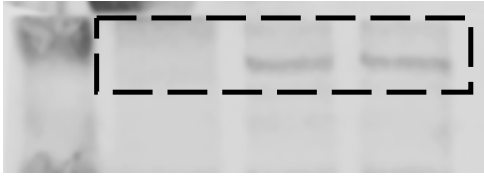
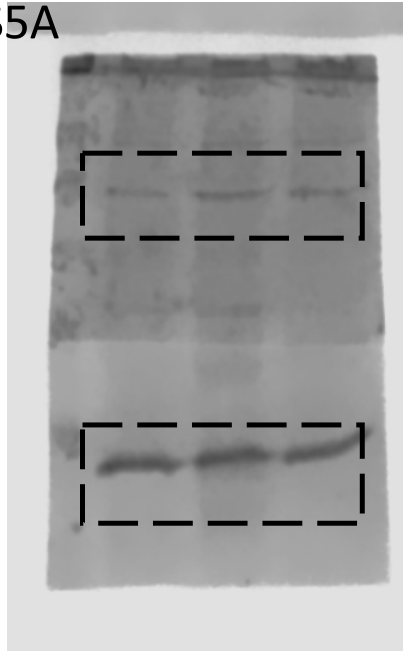
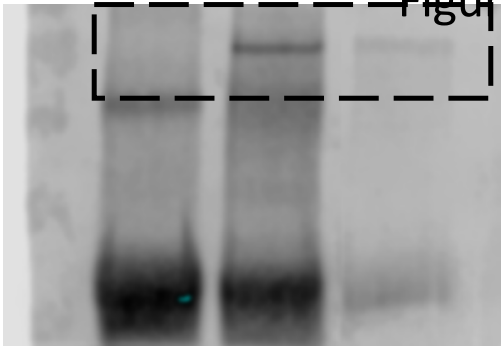


Figure S5C

