Table 1:

Summary of H3 56Ac staining intensity and % of cells with H3 K56Ac staining from independent samples of normal brain and brain tumors, as determined by IHC. The calls on intensity and cell % were made by a certified Pathologist and head of the UC Pathology Core, Scott Lucia MD.

Grade	Pathology	Intensity	Cell%
Normal	-	+++ (glial cells, oligodendrocytes)	60
Normal	-	+++(glial cells, oligodendrocytes)	70
Normal	-	+++(glial cells, oligodendrocytes)	80
II	Astrocytoma	++	70
II	Astrocytoma	+++	90
II	Astrocytoma	++	100
II	Astrocytoma	+++	90
II	Astrocytoma	+++	100
II	Astrocytoma	++	50
II	Astrocytoma	+++	90
II	Astrocytoma	+	50
II	Astrocytoma	+++	90
II	Astrocytoma	+++	70
III	Astrocytoma	+++	100
III	Astrocytoma	+++	100
III	Astrocytoma	+++	90
III	Astrocytoma	+++	100
III	Astrocytoma	+	30
III	Astrocytoma	+++	90
III	Astrocytoma	+++	90
III	Astrocytoma	++	50
III	Astrocytoma	+++	70
III	Astrocytoma	+++	100
III	Astrocytoma	+	50
III	Astrocytoma	+++	100
III	Astrocytoma	+++	90
III	Astrocytoma	+++	60

III	Oligodendroglioma	+++	80
III	Oligodendroglioma	++	50
III	Oligodendroglioma	+++	90
III	Oligodendroglioma	+	30
III	Oligodendroglioma	+++	100
III	Oligodendroglioma	+++	90
III	Glioma	++	50
III	Glioma	+++	100
III	Glioma	++	80
III	Glioma	+++	100
III	Glioma	++	70
III	Glioma	++	80
III	Glioma	+++	70
III	Ependymoma	+++	90
IV	Astrocytoma	+++	100
IV	Astrocytoma	++	60
IV	Astrocytoma	+++	70
IV	Glioma	+	rare
IV	Glioma	+++	100
IV	Glioma	+++	80
IV	Glioma	+++	100
IV	Glioma	+++	100
IV	Glioma	+++	90
IV	Glioma	++	30
IV	Glioma	++	70
IV	Glioma	++	90
IV	Glioma	+	20
IV	Glioblastoma multiformae	+++	100
IV	Glioblastoma multiformae	+++	100
IV	Glioblastoma multiformae	+++	100
IV	Glioblastoma multiformae	+	30
IV	Medulloblastoma	+++	100

Supplemental Figure Legends

Suppl. Fig. 1. Model for role of H3 K56Ac in higher eukaryotes.

Drosophila CBP (Nejire) or human CBP/p300 acetylate histones on H3 K56. This acetylation presumably only occurs on free histones due to the inaccessible nature of the unacetylated lysine within nucleosomes and the fact that the yeast Rtt109 enzyme can only acetylate free non-nucleosomal histones. H3 K56Ac is recognized by CAF-1, which then deposits the histones onto newly-synthesized DNA – blockage of this process, either by preventing H3 K56 acetylation or inactivation of CAF-1, inhibits DNA replication. This inhibition of DNA replication may be a consequence of the resulting excess free histones that are sensed by the cell [1]. Following DNA repair, histones carrying H3 K56Ac are assembled onto chromatin in a localized manner. *Drosophila* Sir2 and human SirT2 /SirT1 then remove the acetyl group from H3 K56.

Suppl. Fig. 2. Demonstration of the highly specific nature of the antibodies used in this study. Chromatinized yeast histones were isolated from isogenic strains that either were WT or carried the H3 K56R mutation. Two different amounts of the yeast histones were loaded, indicated by "+" and "++", followed by western blotting with the Epitomics H3 K56Ac antibody, Upstate H3 K56Ac antibody or the Abcam H3 C-terminus antibody as a loading control.

Suppl. Fig. 3. H3 K56Ac levels on chromatin increase with increased dose of Hydroxyurea. Chromatinzed histones were extracted from *Drosophila* S2 cells following treatment with the indicated amounts of hydroxyurea and were analyzed by western analysis for H3 K56Ac and H3 levels.

Suppl. Fig. 4. Incorporation of H3 K56Ac into chromatin is dependent on the amount of DNA damage. A. Staining of histone H3 and H3 K56Ac in triton-X treated *Drosophila* S2 cells following exposure to UV, HU and MMS. This analysis demonstrates that the increase in H3 K56Ac on chromatin following DNA damage is not a consequence of increased levels of bulk H3 on chromatin following DNA damage. **B.** Dose response of H3 K56Ac staining on chromatin dependent on the amount of DNA damage. *Drosophila* S2 cells were treated with the indicated amounts of MMS, followed by triton-X extraction of loosely bound proteins and immunofluorescence for chromatin bound H3 K56Ac.

Suppl. Fig. 5. Treatment of S2 cells with DNA damaging agents does not lead to accumulation in S-phase. Samples of the *Drosophila* S2 cells from Figure 1A that had been treated with UV, MMS or HU were subject to flow cytometry analysis of their DNA content as determined by propidium iodide staining.

Suppl. Fig. 6. RNAi mediated depletion of *Drosophila* proteins. Following treatment of *Drosophila* S2 cells with long ds RNAs corresponding to **A**. Asf1, **B.**

CBP, **C.** Gcn5 or **D**. Sir2, total protein extracts were western blotted to demonstrate efficiency of protein knockdown. **E.** Gcn5 is not required for acetylation of H3 K56. Following treatment of *Drosophila* S2 cells with RNAi against Gcn5 or GFP, chromatin fractions were western blotted for H3 K56Ac, H3 K9Ac or total H3. The asterisk denotes a likely degradation product of H3.

Suppl. Fig. 7. Co-immunoprecipitation of CBP with Asf1 from *Drosophila* cells. *Drosophila* extracts were subjected to immunoprecipitation with antibodies to Asf1, the corresponding preimmune serum or no antibody, followed by western blotting for Asf1 and for CBP. Two independent experiments are shown for each.

Suppl. Fig. 8. Time dependence and dose dependence of Nicotinamide-induced increase in H3 K56Ac levels. *Drosophila* S2 were treated with 25mM Nicotinamide for the indicated lengths of time, or with 50mM Nicotinamide or the same amount of the related compound Nicotinic acid that does not inhibit NAD-dependent HDACs. Chromatin fractions were then western blotted for H3 K56Ac or total H3. The asterisk denotes a putative degradation product of H3.

Suppl. Fig. 9. Comparison of detection of *Drosophila* and human histones by the Epitomics and Upstate H3 K56Ac antibodies. Chromatinized histones were isolated from *Drosophila* S2 cells and HeLa cells. Two different amounts of the histones were loaded, indicated by "+" and "++", followed by western blotting

with the Epitomics H3 K56Ac antibody, Upstate H3 K56Ac antibody or the Abcam H3 C-terminus antibody as a loading control.

Suppl. Fig. 10. Detection and quantitation of H3 K56Ac in human cells by mass spectrometry. A. CID spectrum of the doubly charged precursor ion m/z 416.25 used to identify histone H3 peptide "STELLIR" (E<5.8E-6) and **B.** at m/z 646.86 to identify the acetylated histone H3 peptide "YQ-AcK-STELLIR" (E<7.5E-5) from HeLa cells following treatment with a cocktail of pan HDAC inhibitors. **C.** Extracted ion chromatogram of the precursor corresponding to the peptide "STELLIR" (m/z 416.25) and acetylated peptide "YQ-AcK-STELLIR" (m/z 646.86) from histones isolated from HeLa cells following treatment with a cocktail of pan HDAC inhibitors.

Suppl. Fig. 11. Human cells in all phases of the cell cycle have significant H3 K56Ac. HeLa cells were treated with triton-X to remove loosely bound proteins prior to these staining and flow cytometry analyses. **The left hand panels** show DNA content as determined by propidium iodide staining (on the x-axis) plotted against cell number (y-axis) for cells that were **A.** not exposed to DNA damage, **B**. not exposed to DNA damage but were incubated with the antibody to H3 K56Ac followed by an anti-rabbit FITC conjugate. **The middle panels** show the FITC staining (x-axis) plotted aginst cell number (y-axis) for the same samples, where the signal in **A**. indicates the background signal in the FITC channel in the absence of adding the primary antibody to H3 K56Ac. **The**

right hand panels show the DNA content plotted against the H3 K56Ac staining with FITC. The boxes indicate the cell cycle phases in regions with H3 K56Ac staining that is significantly above background. Note that cells in all phases of the cell cycle, not just S-phase cells, have significant H3 K56Ac staining in **B**. **C.** DNA content as determined by propidium iodide staining (on the x-axis) plotted against cell number (y-axis) for cells that were exposed to MMS, HU or UV, as indicated.

Suppl. Fig. 12. Acetylation and deacetylation of H3 K56Ac in human cells. **A.** Curcumin decreases the level of H3 K56Ac in HeLa cells. **B.** RNAi knockdown of p300 and CBP but not exposure to a scrambled (Sc) siRNA results in lowered H3 K56Ac in HeLa cells. **C.** Efficiency of knockdown of CBP. **D.** Efficiency of knockdown of p300. **E.** Efficiency of knockdown of Asf1a. **F.** Curcumin inhibits the in vitro HAT activity of p300. Purified *Drosophila* core histones were added to all HAT reactions, as indicated by the amido black staining of total proteins, followed by addition of CBP and curcumin as indicated. The histones were then western blotted to detect the extent of H3 K56 acetylation. **G.** Treatment of HeLa cells with sodium butyrate leads to greatly elevated levels of H3 K56Ac on chromatin.

Suppl. Fig. 13. Verification and quantitation of H3 K56Ac generated in vitro by CBP/p300. A. CID spectrum of the doubly charged precursor ion m/z 416.25 used to identify the histone H3 peptide “STELLIR” (E<0.00012) from in vitro

treated sample. **B.** CID spectrum of the doubly charged precursor ion m/z 646.86 used to identify the acetylated histone H3 peptide “YQ-AcK-STELLIR” (E<0.00061). **C.** Extracted Ion Chromatograms of the precursor corresponding to the peptide “STELLIR” (m/z 416.25) and acetylated peptide “YQ-AcK-STELLIR” (m/z 646.86) in a **C.** mock **D.** CBP and **E.** p300-mediated acetylation reactions of histone H3. **F.** Comparison of the abundance of different H3 acetylation levels in vitro and in vivo by Spectral count as detailed in the supplemental methods section. Specifically, the number of spectra assigned to a given acetylated lysine containing tryptic peptide were counted and reported according to the following criteria for semiquantitation of lysine acetylation: 0 identifications (-), 1-5 spectra (+), 6-20 spectra (++), 21-40 spectra (+++), and more than 40 spectra (++++). Experiment #1 and #2 indicate independent analyses performed for the in vivo samples from HeLa cells, with and without a combination of sodium butyrate, nicotinamide and TSA HDAC inhibitors (“HDACi”).

Suppl. Fig. 14. SirT1 and SirT2 can deacetylate CBP-acetylated H3 K56Ac in vitro. Purified *Drosophila* core histones that had been acetylated by CBP in vitro were used as substrates for HDAC reactions, as indicated by the amido black staining of total proteins. All HDAC reactions included NAD, curcumin to inhibit CBP and the indicated recombinant SirT1 or SirT2, and the reactions were allowed to continue for 1 hour. The histones were then western blotted to detect the extent of H3 K56 deacetylation.

Suppl. Fig. 15. Elevated H3 K56Ac in cancerous tissue. Sections of human tissue were stained for nuclei (blue) and with the antisera to H3 K56Ac (brown). **A.** Normal liver and liver tumor. **B.** Normal rectum and rectal tumor. **C.** Normal testis and testis tumor.

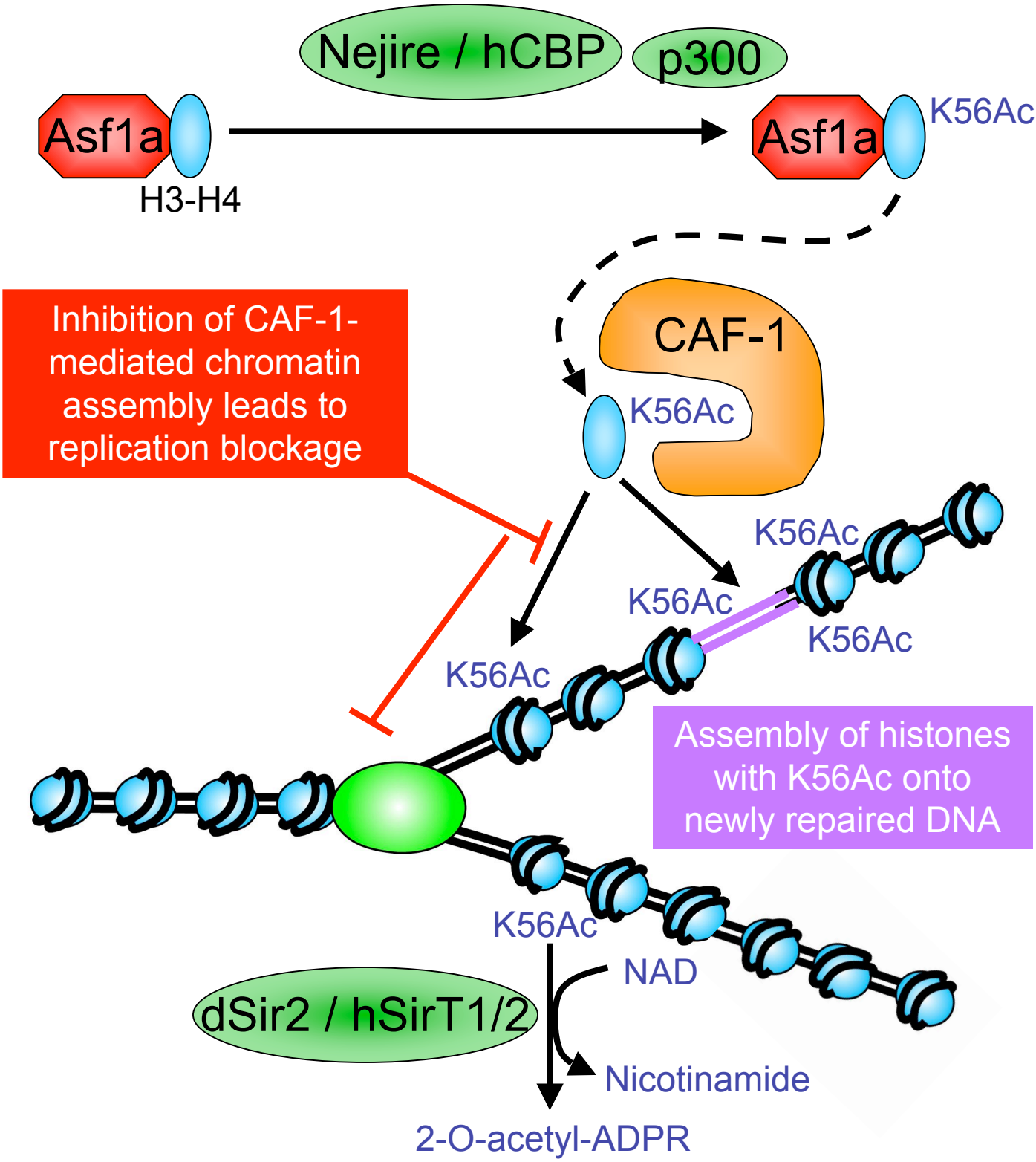
Suppl. Fig. 16. Immunohistochemistry of H3 K56Ac in normal and cancerous colon tissue. A. Normal colon crypts, showing staining of H3K56Ac in epithelial nuclei around the crypts, and in some epithelial nuclei between the crypts. **B.** Normal colon crypts with adjacent invading cancer, showing increased H3 K56Ac signal in cancer cells. **C**. Cancerous colon tissue, showing staining of cancer cells with H3 K56Ac. **D**. Cancerous colon tissue, showing staining of cancer cells with H3 K56Ac.

Suppl. Fig. 17. H3 K56Ac staining increases with brain tumor grade. H3 K56Ac staining (brown) and nuclear staining (blue) normal brain tissue and the indicated grade and type of brain tumor.

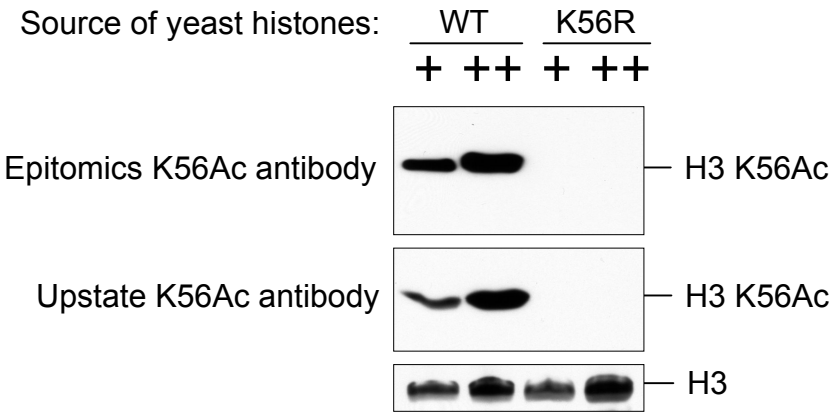
Suppl. Fig. 18. Confirmation of differentiation of SH-5YSY cells upon retinoic acid treatment. Aliquots of the same SH-5YSY cells analyzed in Fig. 4l were examined by phase contrast analysis to show the morphological changes typical of differentiation after 6 days of retinoic acid treatment.

1. Groth, A. et al. Regulation of replication fork progression through histone supply and demand. Science 318, 1928-31 (2007).

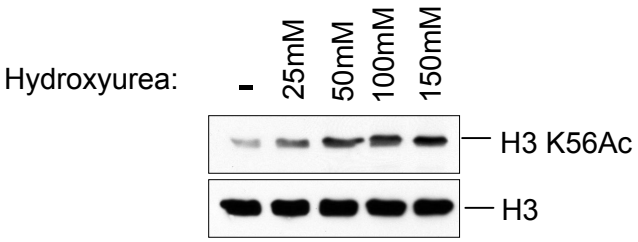
Sup. Figure 1


Nejire / hCBP
p300
Asf1a
H3-H4
Asf1a
K56Ac
CAF-1
K56Ac
Inhibition of CAF-1-mediated chromatin assembly leads to replication blockage
K56Ac
K56Ac
K56Ac
K56Ac
Assembly of histones with K56Ac onto newly repaired DNA
K56Ac
NAD
dSir2 / hSirT1/2
Nicotinamide
2-O-acetyl-ADPR

Sup. Figure 2


Source of yeast histones:
WT
K56R
+ ++ + ++
Epitomics K56Ac antibody
H3 K56Ac
Upstate K56Ac antibody
H3 K56Ac
H3

Sup. Figure 3



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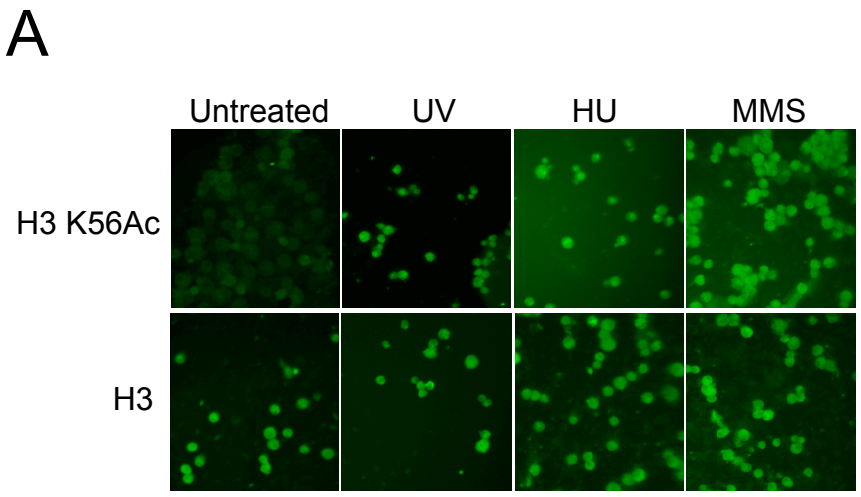
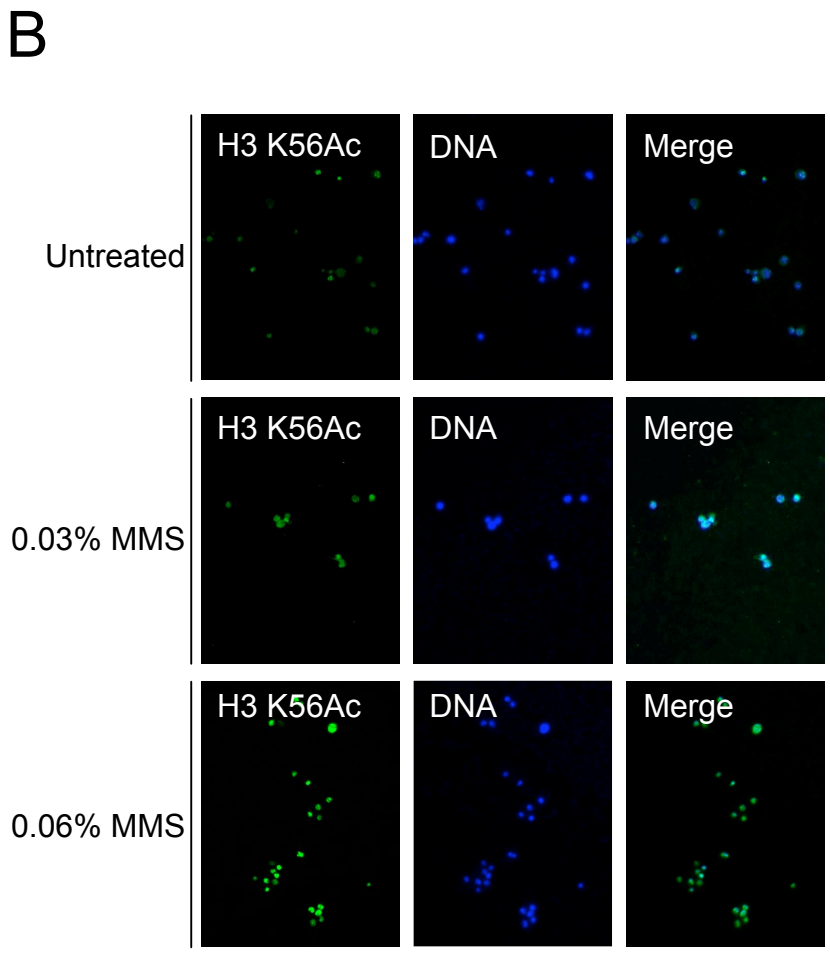
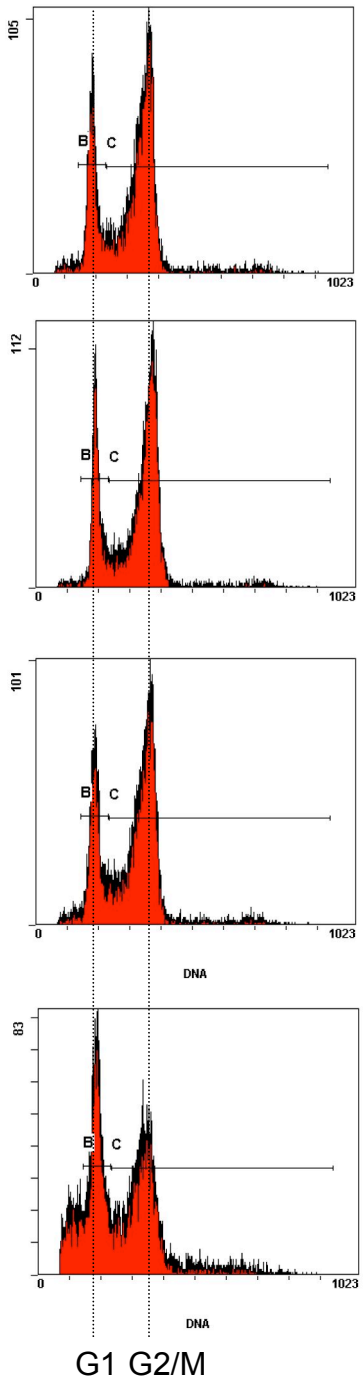
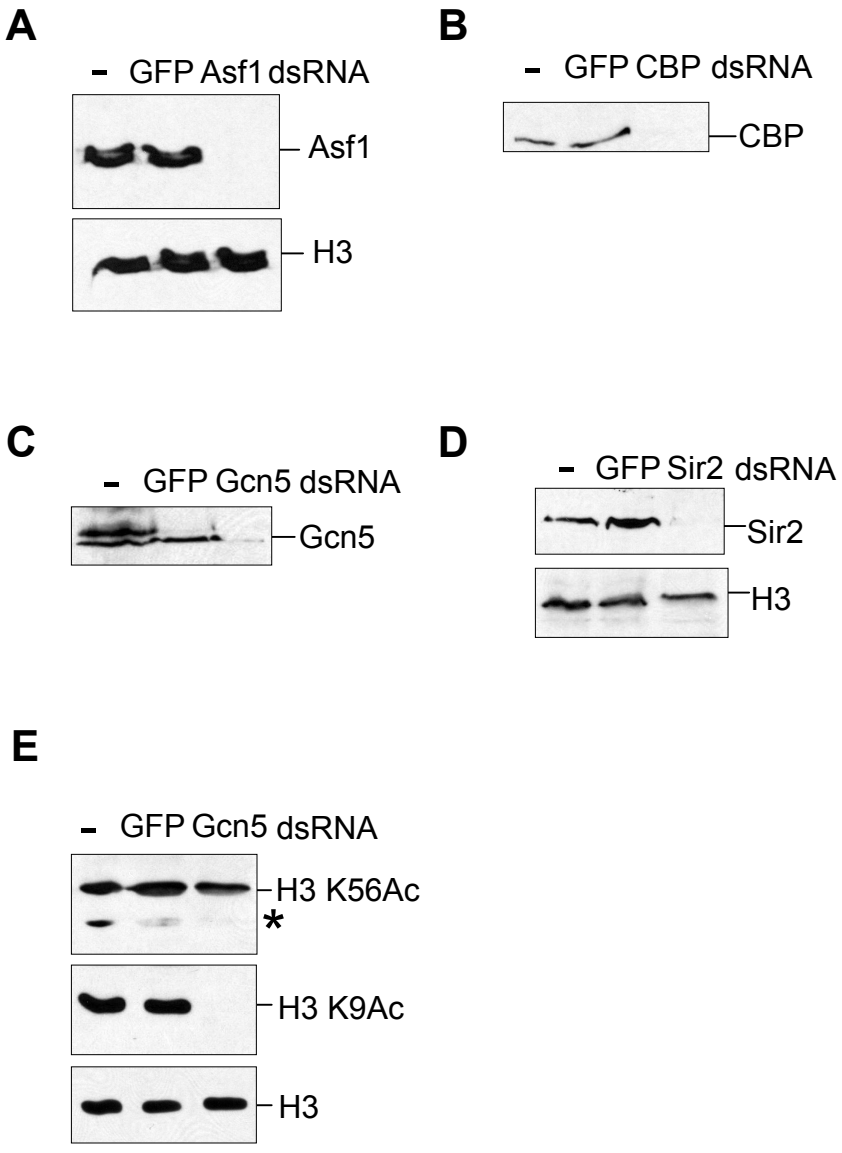

A
Untreated
UV
HU
MMS
H3 K56Ac
H3


B
H3 K56Ac
DNA
Merge
Untreated
0.03% MMS
0.06% MMS

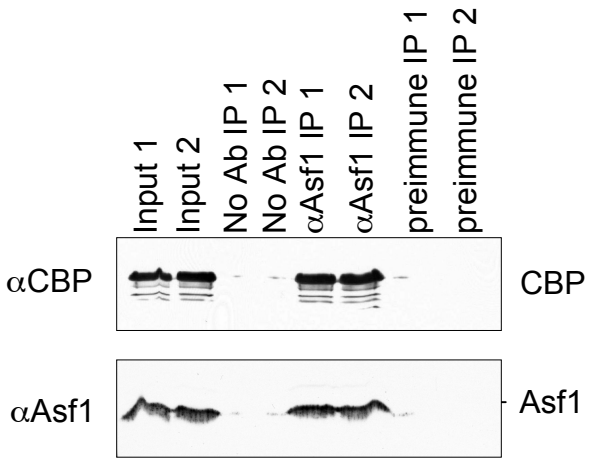
Sup. Figure 5


105
B
C
0
1023
No damage
112
B
C
0
1023
UV (50kJ/m2)
101
B
C
0
1023
HU (150mM)
DNA
83
B
C
0
1023
MMS (0.05%)
DNA
G1 G2/M

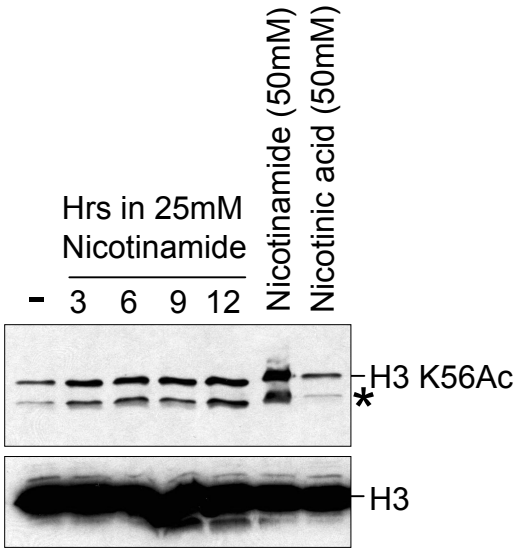
Sup. Figure 6


A
- GFP Asf1 dsRNA
Asf1
H3
B
- GFP CBP dsRNA
CBP
C
- GFP Gcn5 dsRNA
Gcn5
D
- GFP Sir2 dsRNA
Sir2
H3
E
- GFP Gcn5 dsRNA
H3 K56Ac
*
H3 K9Ac
H3

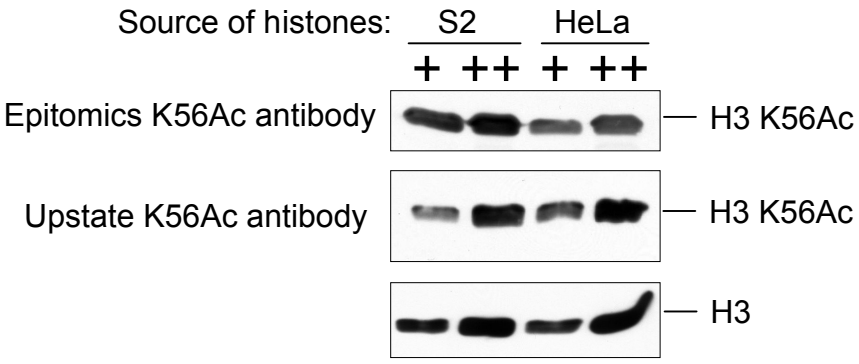
Sup. Figure 7


Input 1
Input 2
No Ab IP 1
No Ab IP 2
αAsf1 IP 1
αAsf1 IP 2
preimmune IP 1
preimmune IP 2
αCBP
CBP
αAsf1
Asf1

Sup. Figure 8

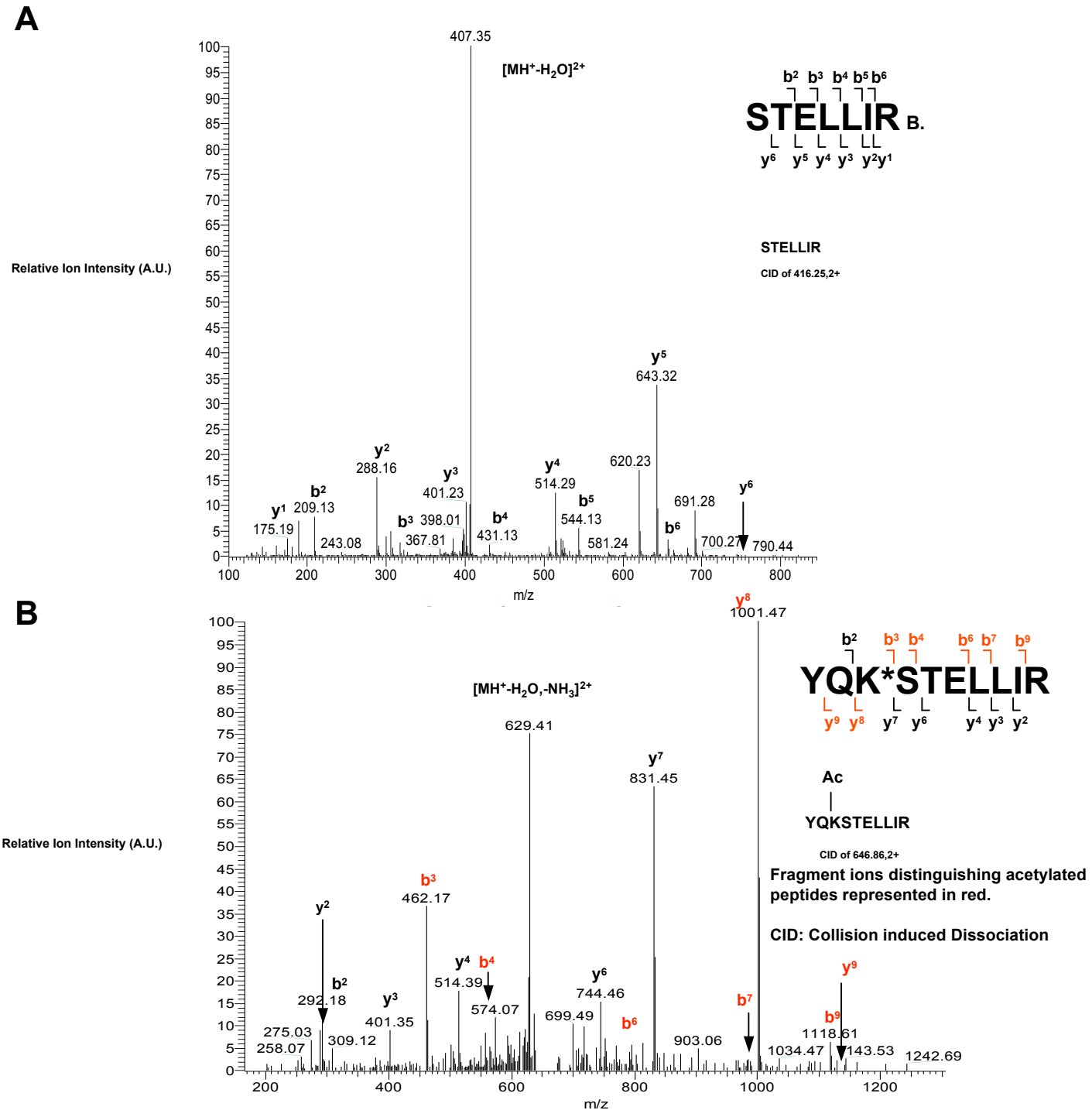

Hrs in 25mM Nicotinamide
-
3
6
9
12
Nicotinamide (50mM)
Nicotinic acid (50mM)
H3 K56Ac
*
H3

Sup. Figure 9


Source of histones:
S2
HeLa
+ ++ + ++
Epitomics K56Ac antibody
H3 K56Ac
Upstate K56Ac antibody
H3 K56Ac
H3

Sup. Figure 10

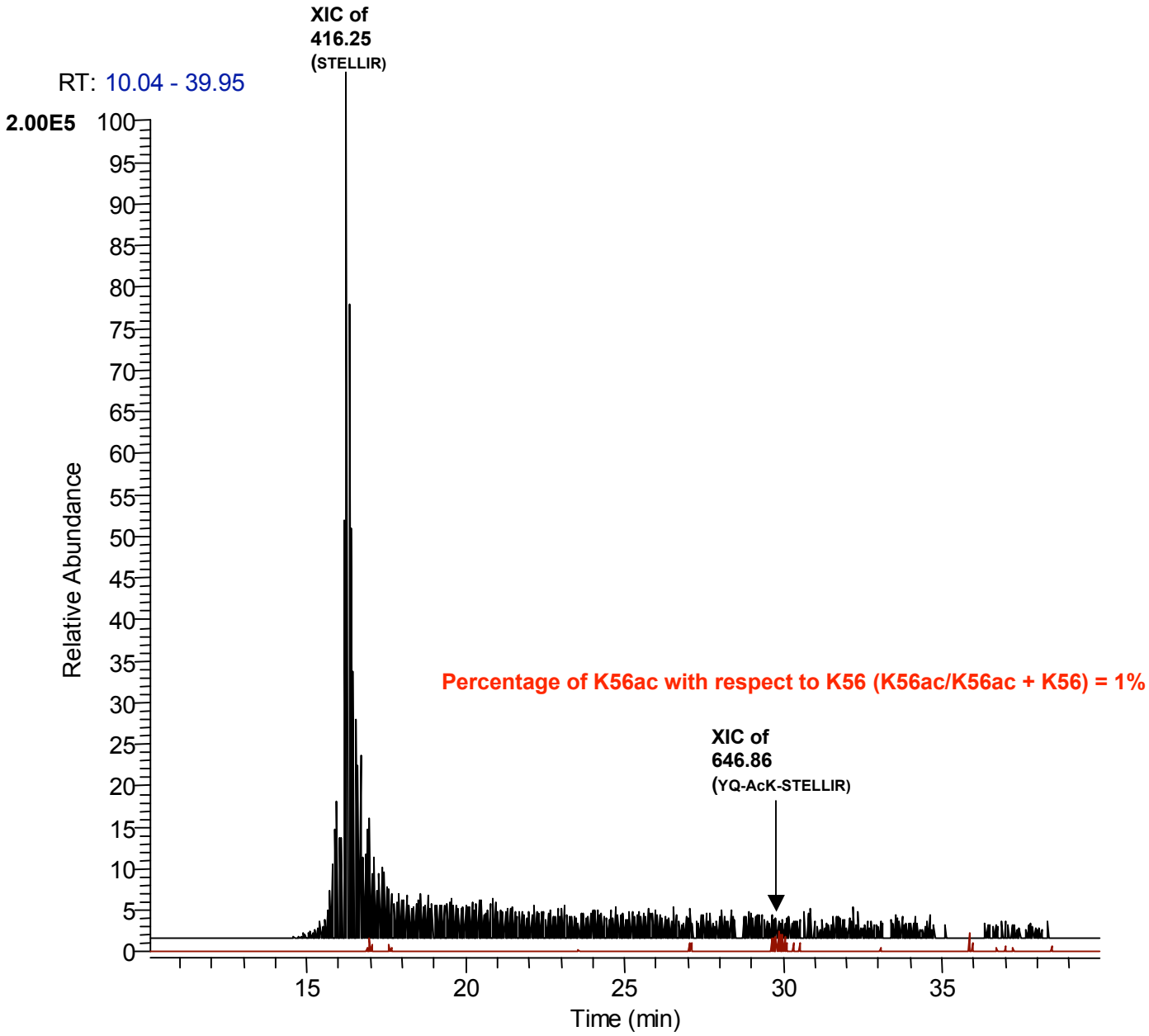
MS/MS for in vivo assignment



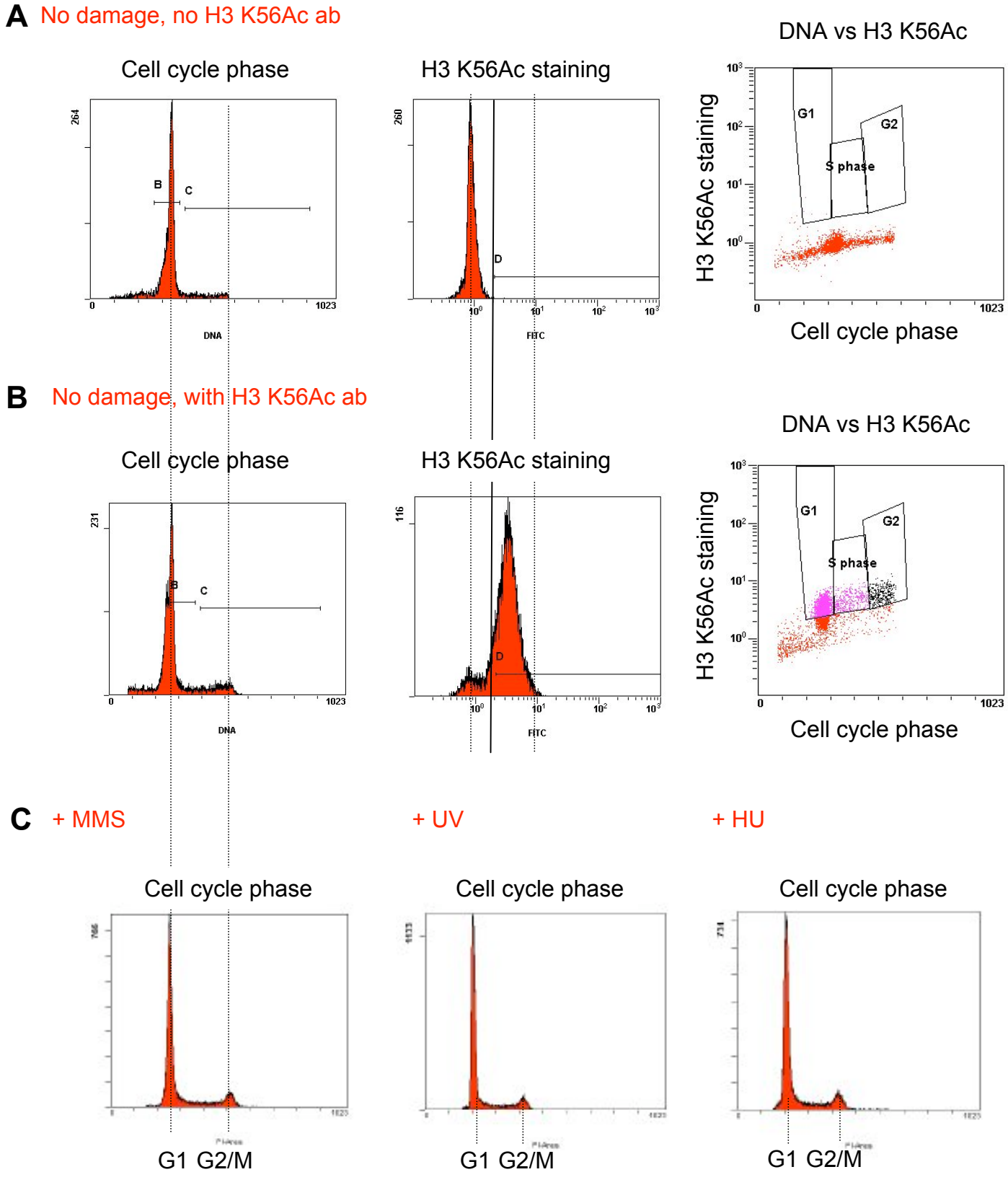
Sup. Figure 10

C

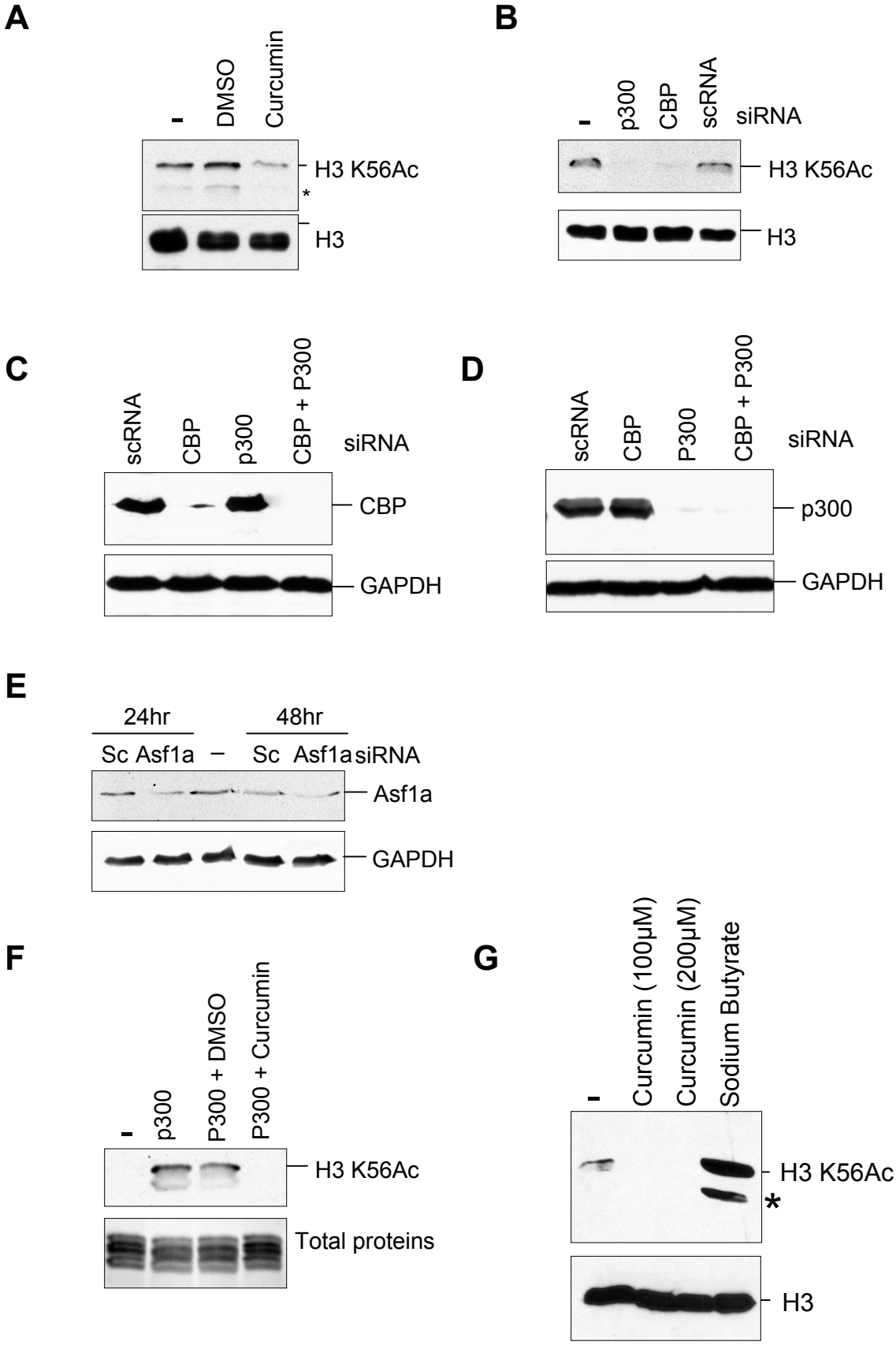
Extracted Ion Chromatography (XIC) of in vivo histones following HDAC inhibitor treatment



Sup. Figure 11



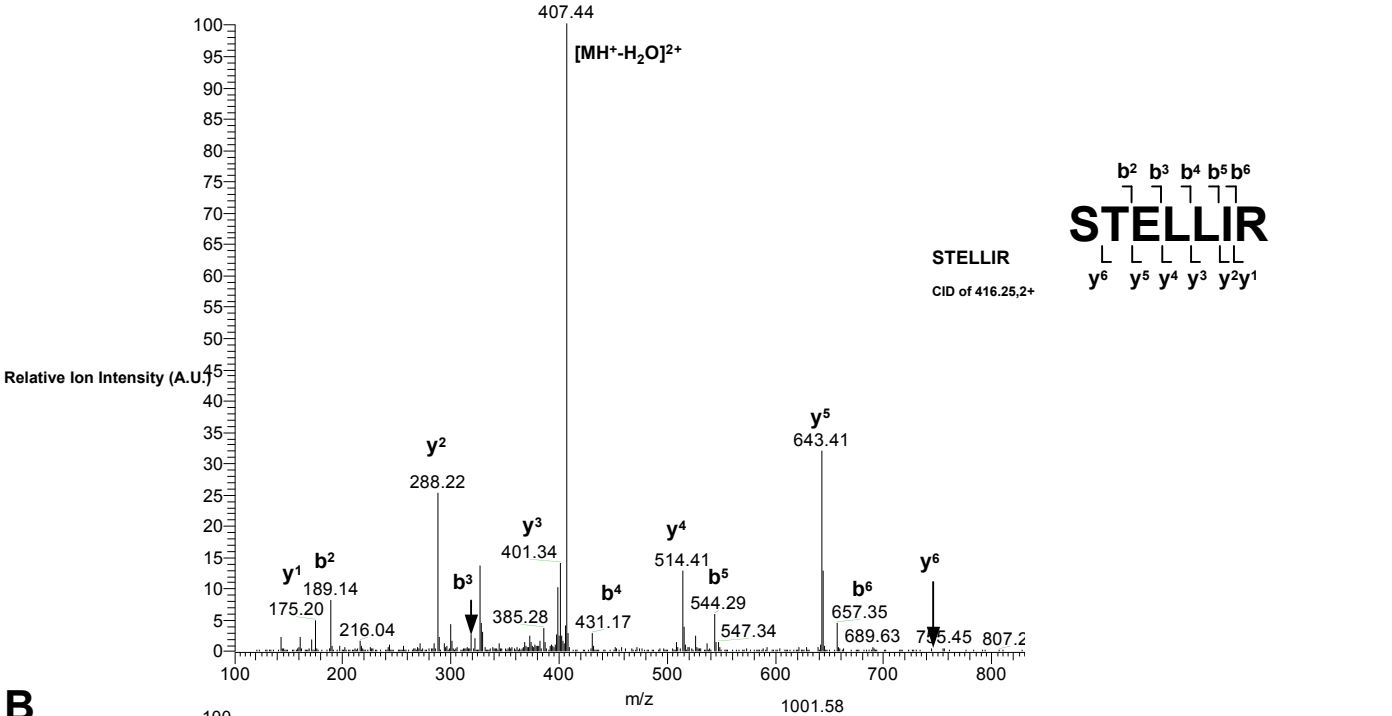
Sup. Figure 12



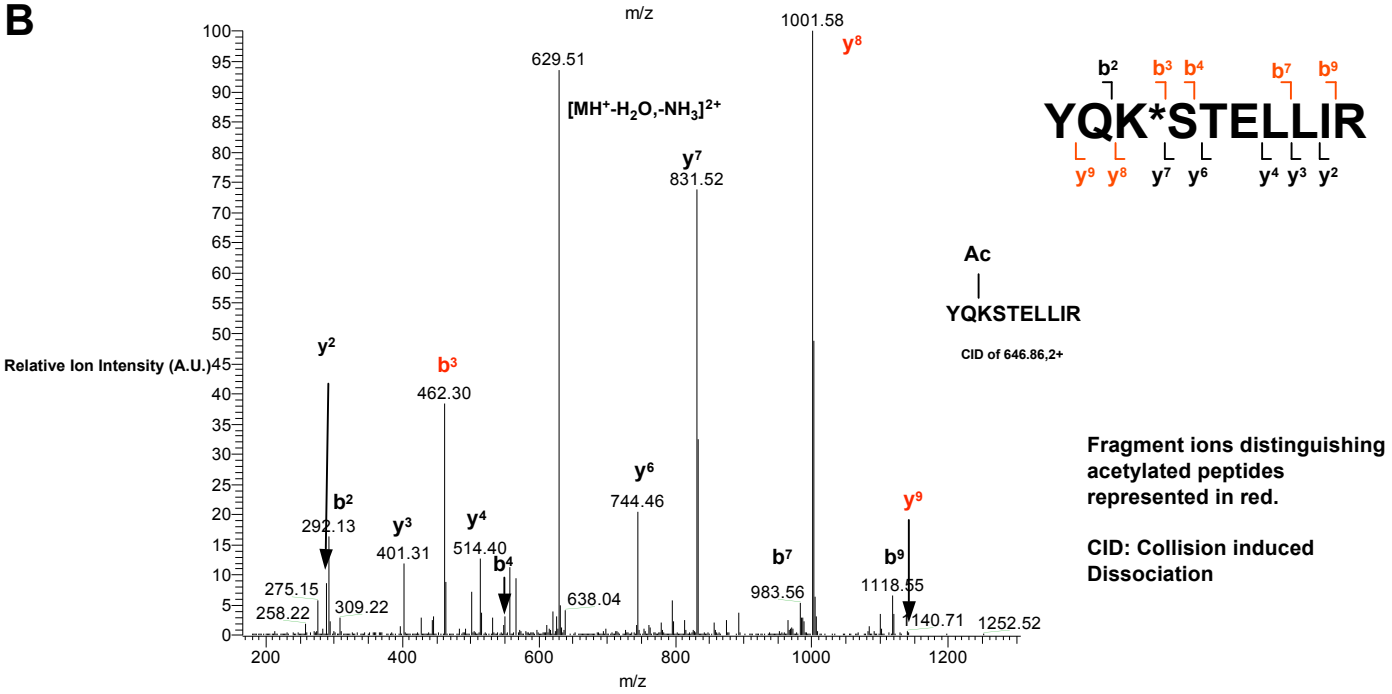
Sup. Figure 13

A

MS/MS for in vitro assignment



B

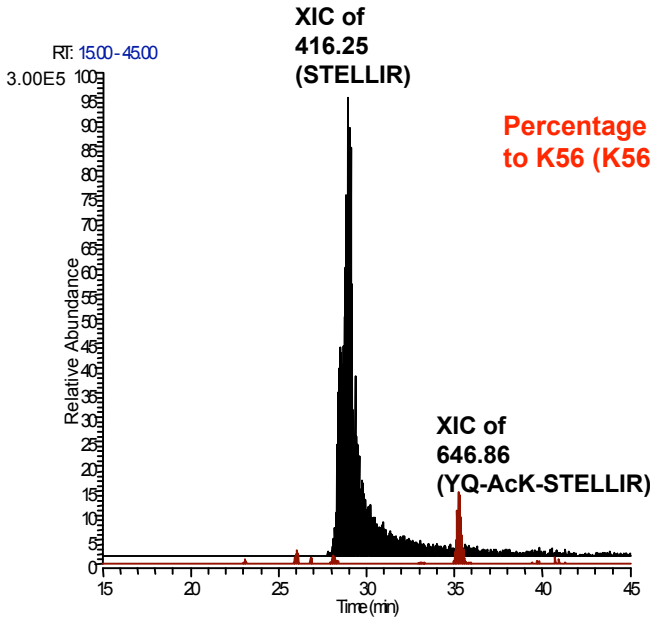


Fragment ions distinguishing acetylated peptides represented in red.

CID: Collision induced Dissociation

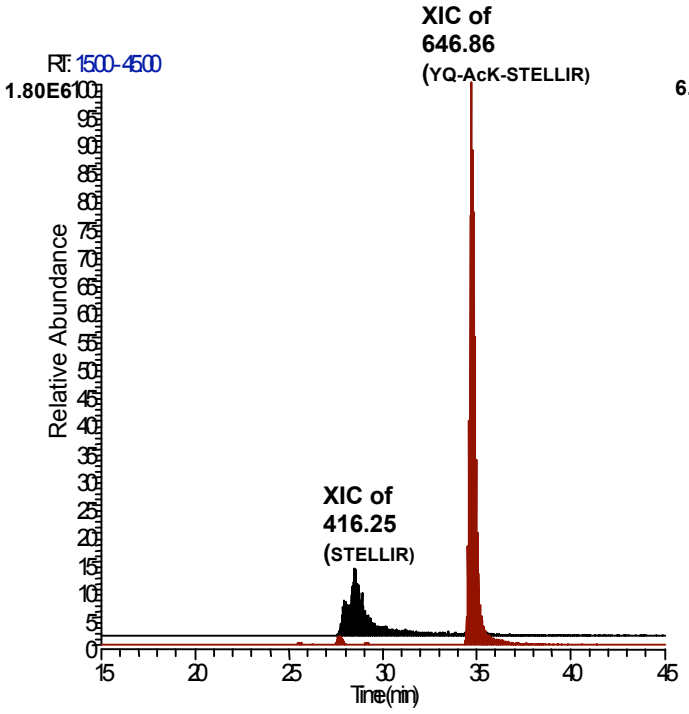
Sup. Figure 13 cont.

C Extracted Ion Chromatography (XIC) of in vitro mock acetylation



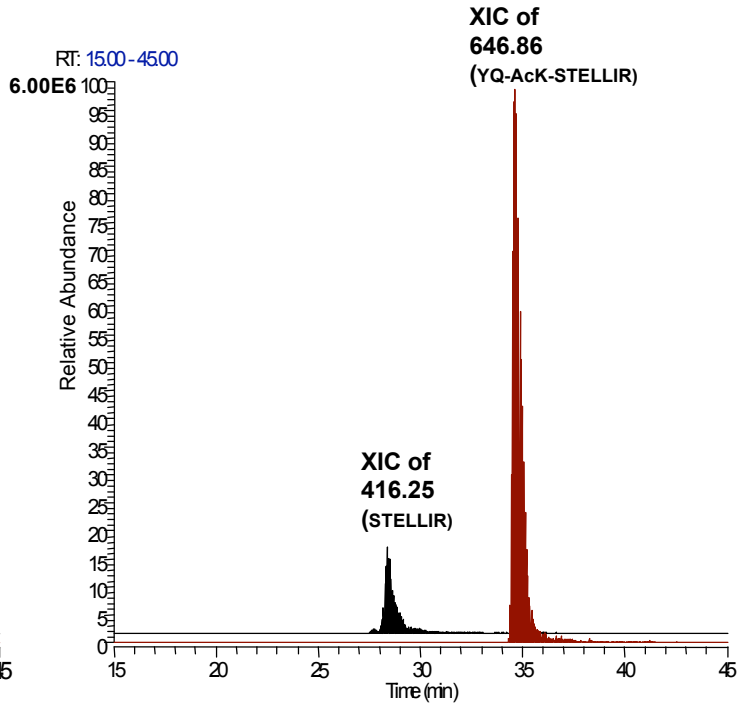
Percentage of K56ac with respect to K56 (K56ac/K56ac + K56) = 6%

D CBP-mediated K56 acetylation



Percentage of K56ac with respect to K56 (K56ac/K56ac + K56) = 75%

E p300-mediated K56 acetylation



Percentage of K56ac with respect to K56 (K56ac/K56ac + K56) = 85%

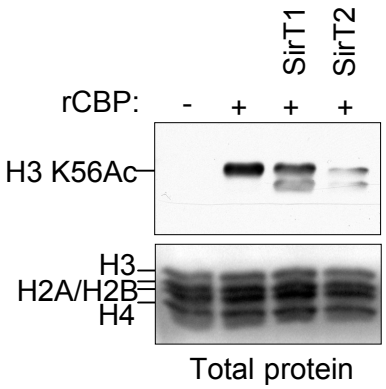
Sup. Figure 13 cont.

F **Comparing the different histone H3 acetylation levels in vitro and in vivo by Spectral Count**

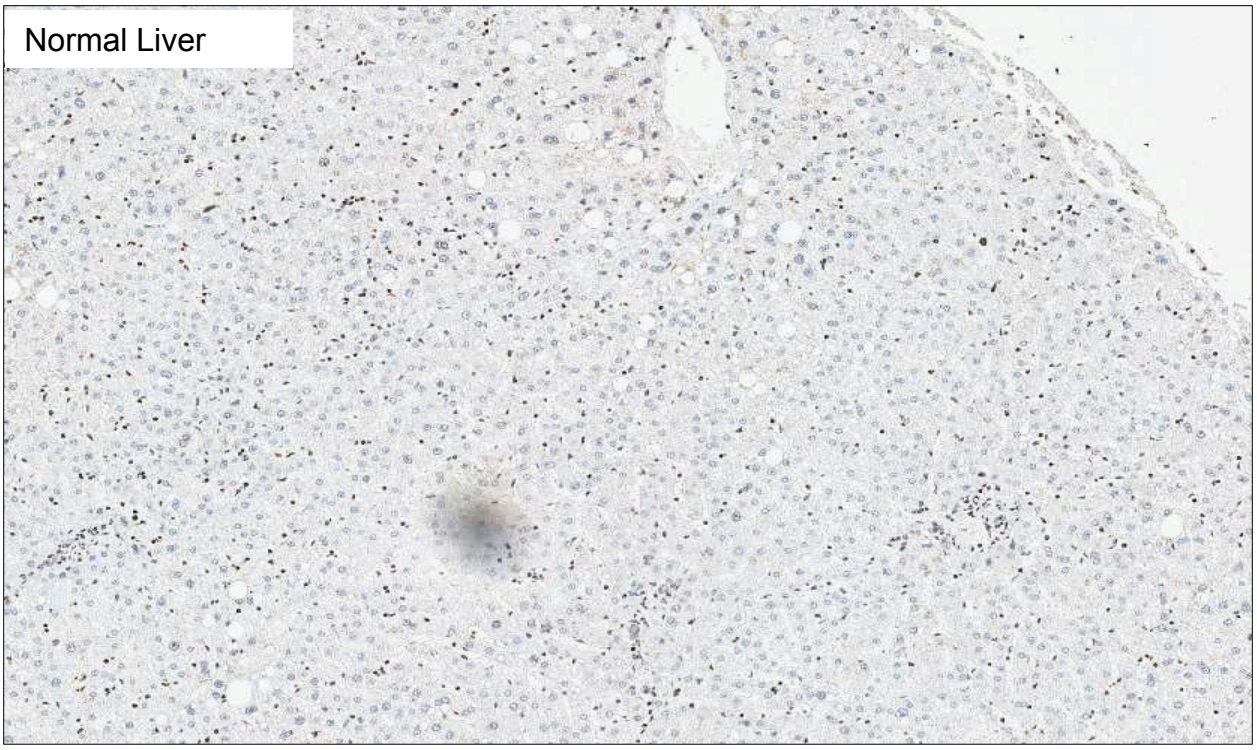
	In vitro			In vivo		In vivo	
K Residues	Mock	CBP	p300	Experiment #1	Experiment #2	with HDACI #1	with HDACI #2
K4	-	-	-				
K9	+	+	++				
K14	-	++	+++				
K18	-	++	++	-	-	+	-
K23	-	++	++	++	++	++	+++
K27	-	++	+++				
K36	-	++	+++				
K37	-	+	++				
K56	+	++	+++	-	-	+	+
K64	-	-	+				
K79	+	+	+				
K115	-	+	-	++	++	++	++
K122	+	+++	++++	++	+++	-	+

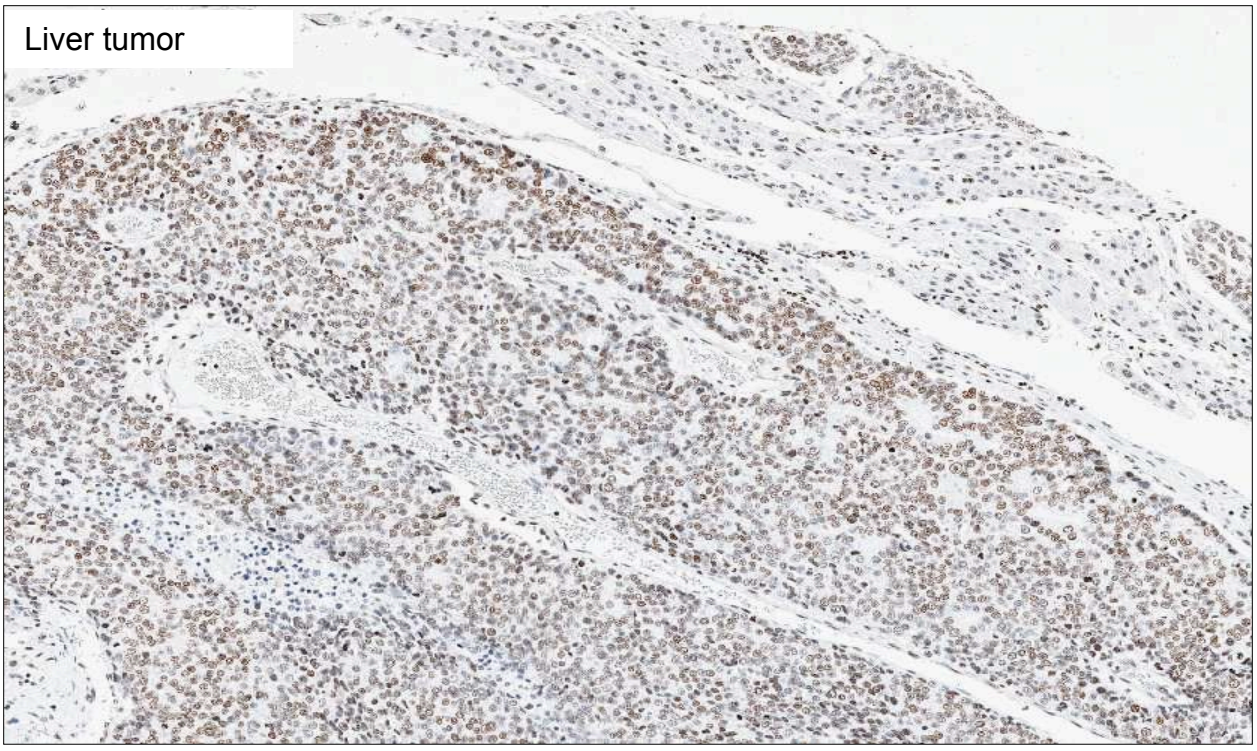
-	0 spectra
+	1 to 5 spectra
++	6 to 20 spectra
+++	21 to 40 spectra
++++	over 40 spectra

Sup. Figure 14

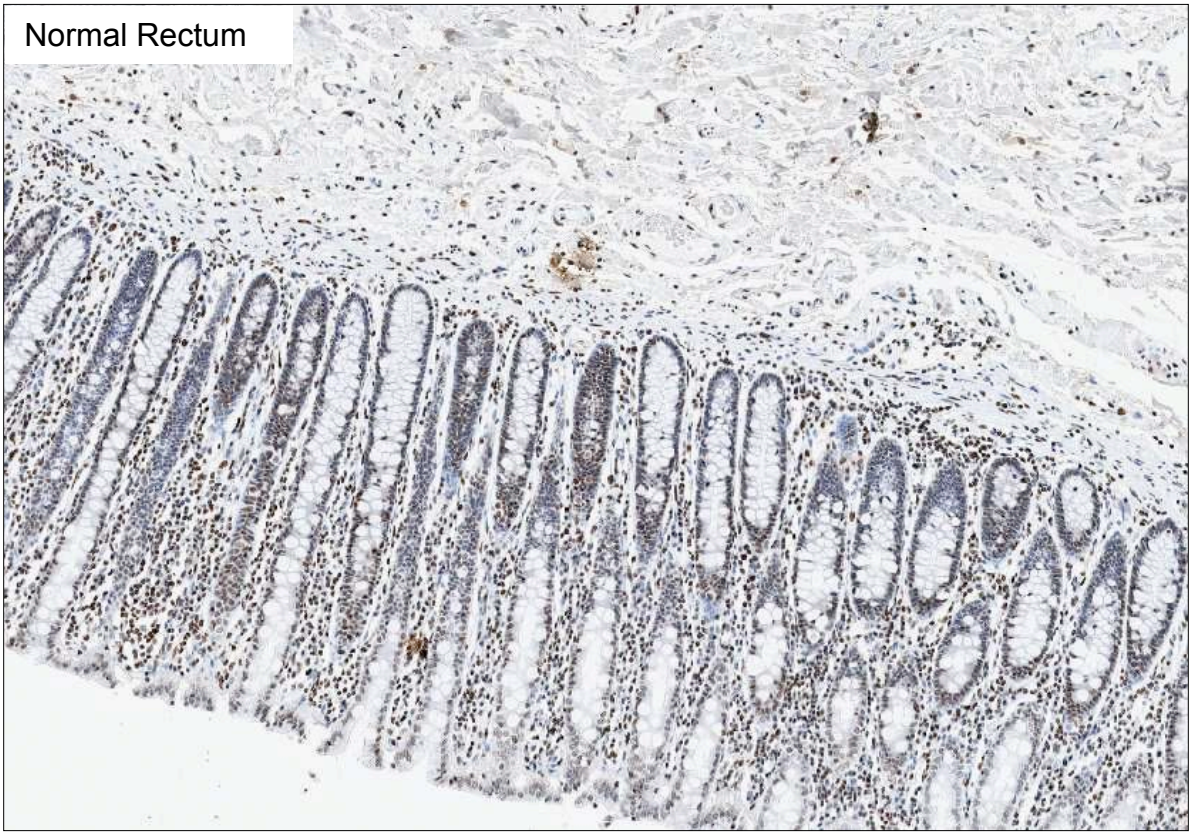


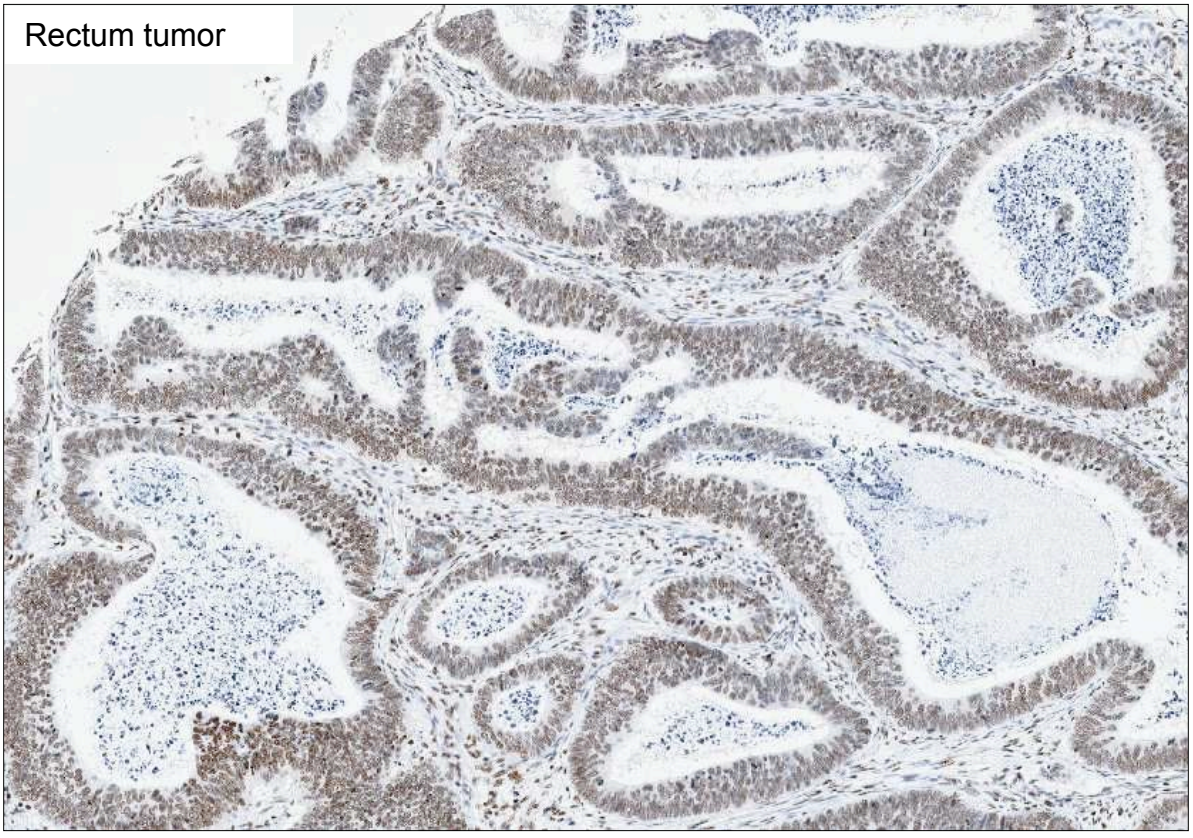
Sup. Figure 15A



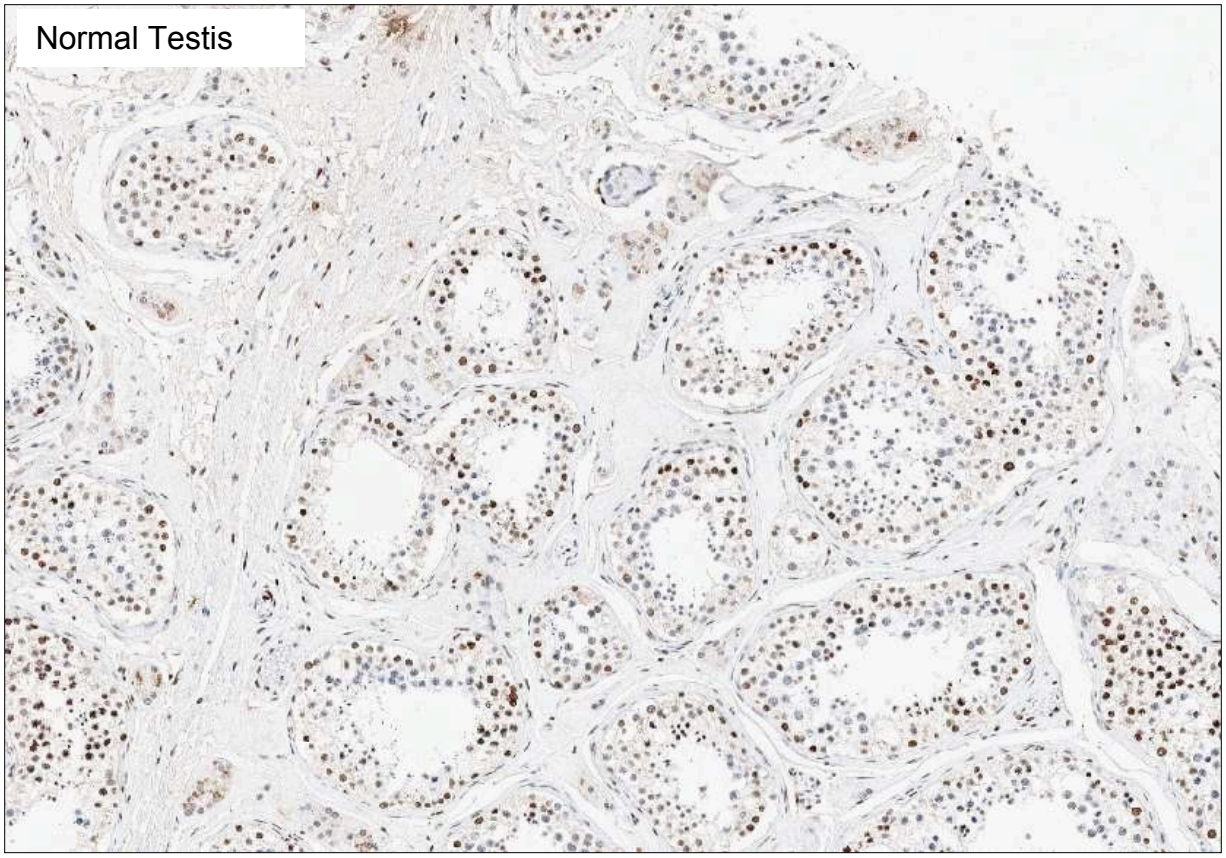


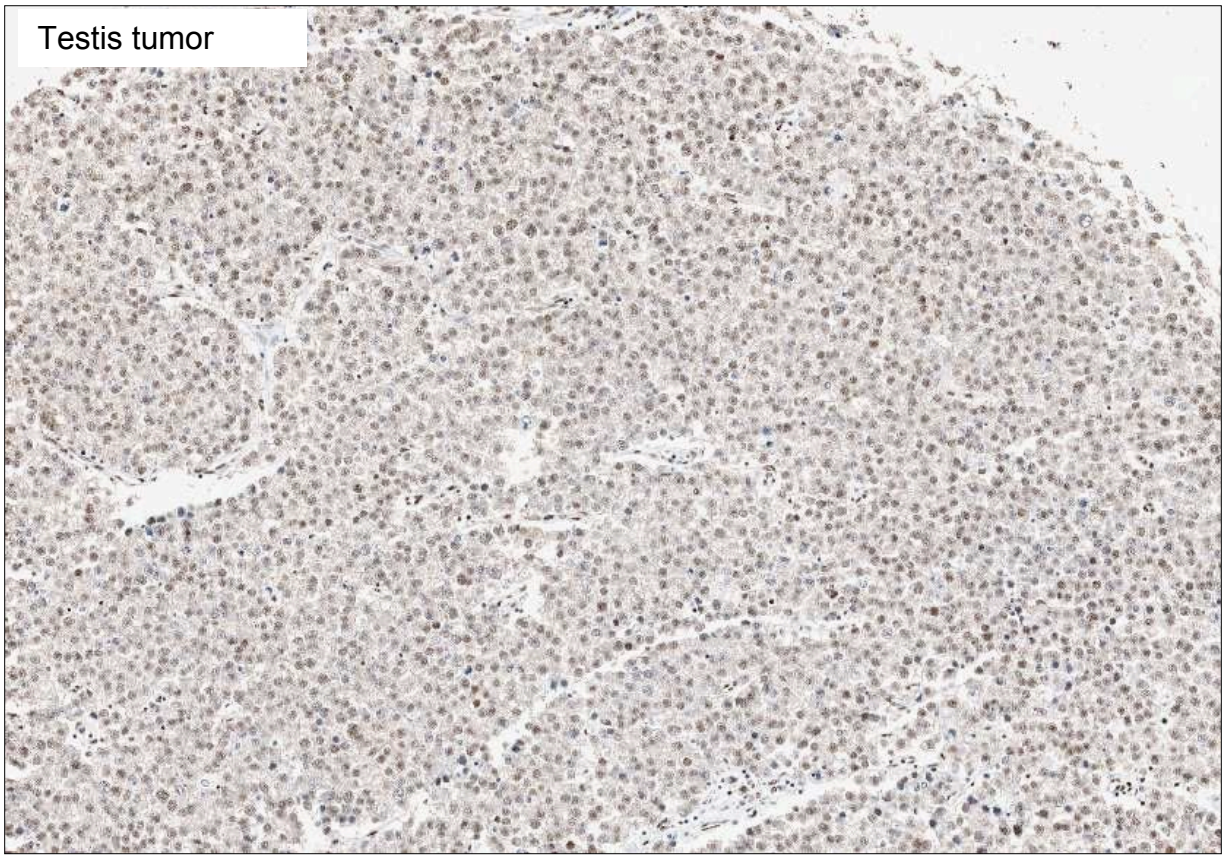
Sup. Figure 15B



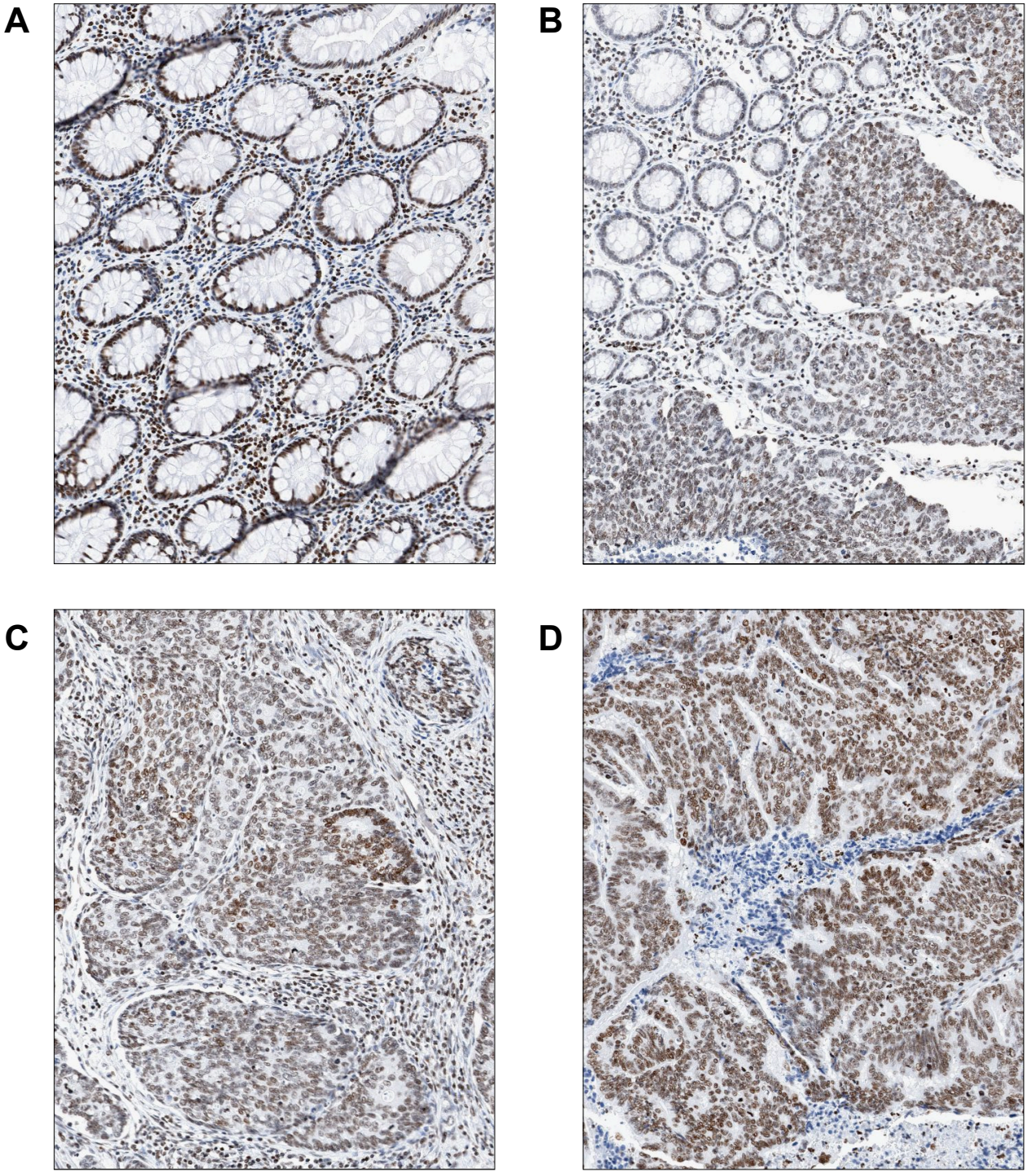


Sup. Figure 15C

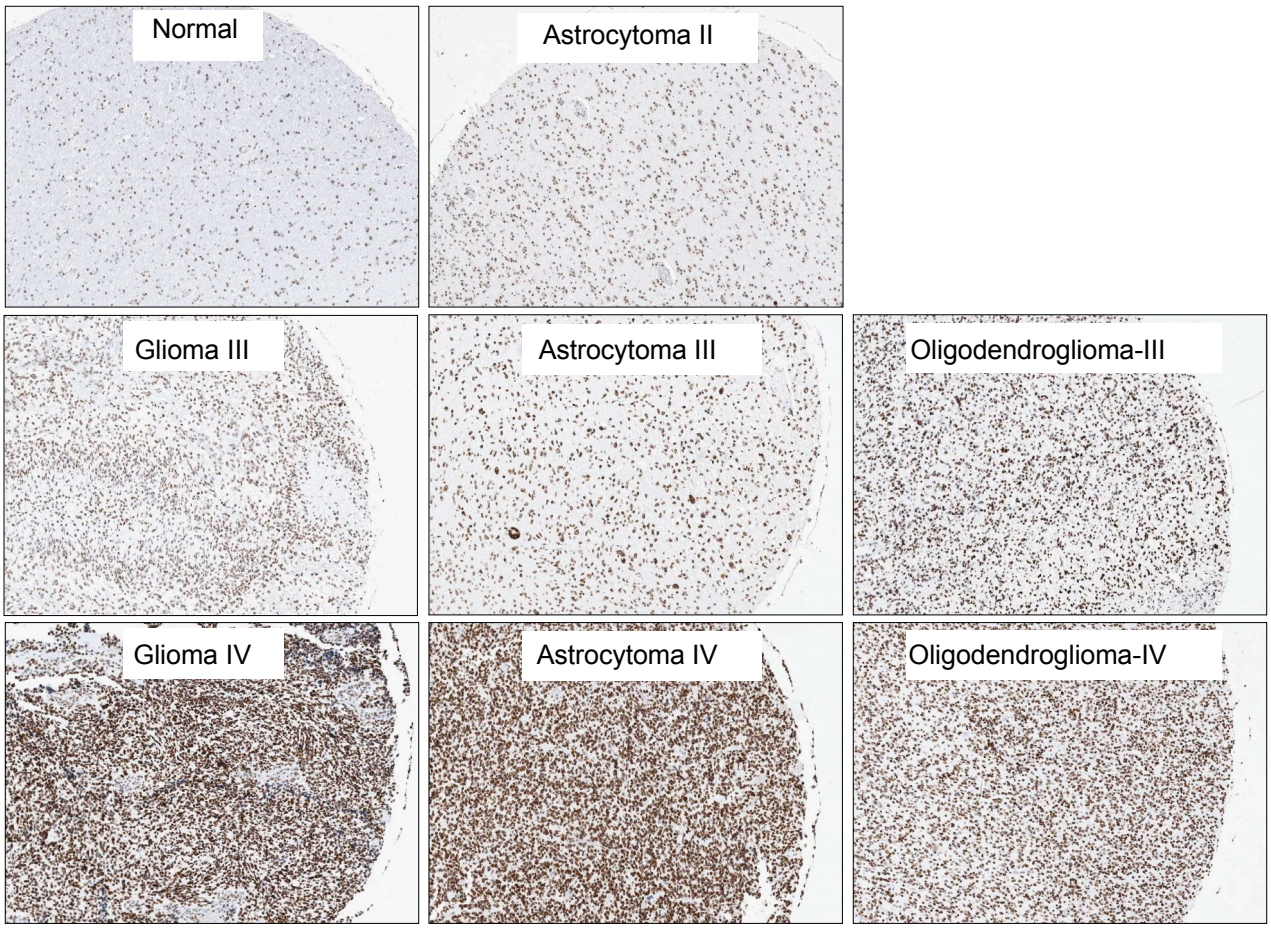




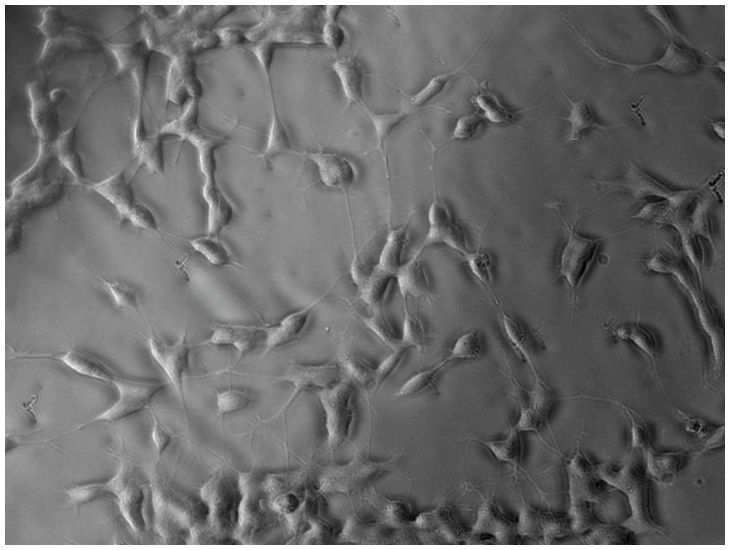
Sup. Figure 16



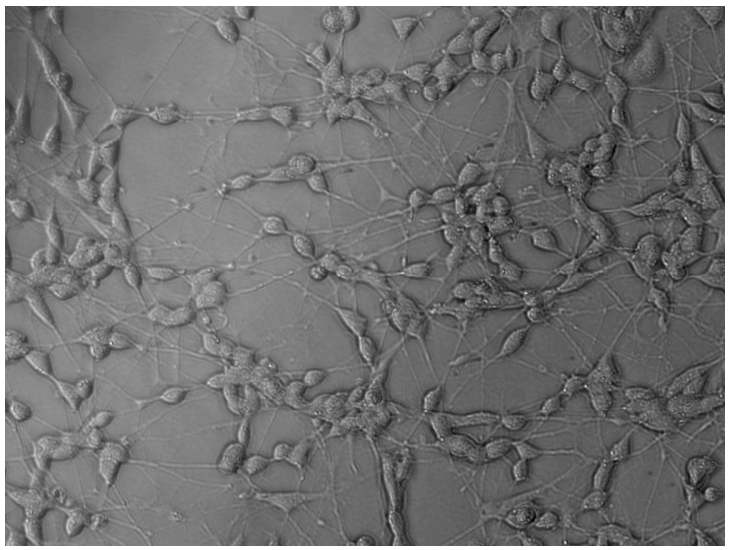
Sup. Figure 17



Sup. Figure 18



Untreated



RA-induced differentiation