

1 **Acetylation-mediated phase control of the nucleolus regulates cellular acetyl-CoA**
2 **responses**

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1 **Summary**

2 The metabolite acetyl-CoA serves as an essential element for a wide range of cellular functions
3 including ATP production, lipid synthesis and protein acetylation. Intracellular acetyl-CoA
4 concentrations are associated with nutrient availability, but the mechanisms by which a cell
5 responds to fluctuations in acetyl-CoA levels remain elusive. Here, we generate a cell system to
6 selectively manipulate the nucleo-cytoplasmic levels of acetyl-CoA using CRISPR-mediated gene
7 editing and acetate supplementation of the culture media. Using this system and quantitative
8 omics analyses, we demonstrate that acetyl-CoA depletion alters the integrity of the nucleolus,
9 impairing ribosomal RNA synthesis and evoking the ribosomal protein-dependent activation of
10 p53. This nucleolar remodeling appears to be mediated through the class IIa HDAC deacetylases
11 regulating the phase state of the nucleolus. Our findings highlight acetylation-mediated control of
12 the nucleolus as an important hub linking acetyl-CoA fluctuations to cellular stress responses.

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14

15 **Keywords**

16 acetyl-CoA, acetylation, nucleolus, stress response, p53, Class IIa HDAC family, ribosomal
17 protein, membraneless organelle, liquid-liquid phase separation

1 **Introduction**

2 Intracellular metabolites dynamically fluctuate in living organisms, in association with the
3 availability of their source nutrients, such as glucose, lipids and amino acids. A cell employs a
4 variety of molecular machineries to monitor the concentration of these metabolites. Upon
5 metabolic fluctuations, those machineries activate signaling pathways that modulate gene
6 expression and protein function to maintain metabolic homeostasis. Molecular links between the
7 metabolite sensing and cellular responses have been extensively studied and several dedicated
8 molecular networks have been described (Campbell and Wellen, 2018; Efeyan et al., 2015; Wang
9 and Lei, 2018). However, partly due to experimental difficulties to selectively manipulate target
10 metabolites among highly interconnected metabolic networks, the mechanisms for cellular
11 primary responses towards fluctuations for most metabolites are not understood.

12 Acetyl-coenzyme A (acetyl-CoA) is a central metabolite that integrates diverse nutritional inputs
13 into the biosynthesis of essential biomaterials including ATP, fatty acids and steroids, and
14 therefore can be viewed as a critical indicator of the cellular metabolic state (Pietrocola et al.,
15 2015; Shi and Tu, 2015). In addition, acetyl-CoA provides an acetyl donor for acetyltransferases
16 catalyzing protein acetylation that modulates biophysical properties of modified proteins, thereby
17 altering their protein stability, enzymatic activity, or interactions with other proteins. This
18 modification is reversed by deacetylases. Hence, in accordance with the nutrient availability and
19 the cellular metabolic state, protein acetylation can reversibly regulate a variety of biological
20 processes including gene expression, signal transduction pathways and metabolic flux
21 (Choudhary et al., 2014; Menzies et al., 2016).

22 In mammalian cells, acetyl-CoA is compartmentalized into a mitochondrial pool, and a
23 nuclear/cytoplasmic pool (Pietrocola et al., 2015; Sivanand et al., 2018). In the mitochondrial
24 matrix, acetyl-CoA is generated by the metabolism of nutrients including glucose, fatty acids and
25 amino acids. Mitochondrial acetyl-CoA can enter the tricarboxylic acid (TCA) cycle thereby
26 generating ATP and reducing equivalents (e.g. NADH) or it can be utilized for acetylation of

1 mitochondrial proteins. Since acetyl-CoA is membrane impermeant, there is no direct exchange
2 between mitochondrial acetyl-CoA and the acetyl-CoA in the cytosol. Rather, the TCA
3 intermediate citrate can be exported from the mitochondria to the cytosol where it can become
4 the predominant source for cytoplasmic and nuclear acetyl-CoA. The mitochondrial exported
5 citrate is converted to acetyl-CoA and oxaloacetate by the enzyme ATP citrate lyase (ACLY)
6 (Wellen et al., 2009). This nucleo-cytoplasmic acetyl-CoA pool serves as a building block for lipid
7 synthesis including fatty acids, cholesterol and steroids, as well as serving as an acetyl donor for
8 acetylation of cytosolic and nuclear proteins including histones. Another source of nucleo-
9 cytoplasmic acetyl-CoA comes from the metabolite acetate, which derives from various extra- and
10 intracellular sources such as food digestion, gut microbial metabolism, alcohol oxidation and
11 deacetylation of acetylated proteins (Schug et al., 2016). Acetate can be utilized for acetyl-CoA
12 synthesis through a reaction catalyzed by the enzyme acyl-CoA synthetase short chain family
13 member 2 (ACSS2). In contrast to whole body *ACLY* deficient mice that are embryonic lethal,
14 *ACSS2* deficient mice have no apparent phenotype under normal breeding conditions (Beigneux
15 et al., 2004; Comerford et al., 2014). This would support a predominant role of the citrate-*ACLY*
16 pathway for the nucleo-cytoplasmic acetyl-CoA production during development, and potentially
17 under other circumstances as well. However, it should be noted that in some mouse tumor models
18 or hypoxic tumor cells, or in other conditions where mitochondrial metabolism is dampened, the
19 acetate-*ACSS2* pathway can play a critical role in cell proliferation, lipid synthesis and histone
20 acetylation (Comerford et al., 2014; Gao et al., 2016; Mashimo et al., 2014; Schug et al., 2015;
21 Yoshii et al., 2009). Moreover, *ACLY* deficient mouse embryonic fibroblasts (MEFs) exhibit
22 upregulation of *ACSS2* and exogenously added acetate can be utilized for acetyl-CoA production
23 in these cells (Zhao et al., 2016). These observations indicate a coordinated relationship between
24 these two pathways in order to ensure the requisite supply of acetyl-CoA in the nucleo-
25 cytoplasmic compartment.

1 Intimate connections between nucleo-cytoplasmic acetyl-CoA levels, the status of protein
2 acetylation and cellular responses have been illustrated by multiple recent studies. In yeast cells
3 during continuous glucose-limited growth, oscillations of acetyl-CoA are observed in accordance
4 with distinct metabolic phases and an increase in acetyl-CoA is highly correlated with acetylation
5 of several proteins including transcriptional coactivators and histones (Cai et al., 2011). Also, in
6 various mammalian cell models, transcription is regulated either by acetyl-CoA abundance, or
7 acetyl-CoA producing enzymes, i.e. ACLY or ACSS2, or by their nutrient sources (Gao et al.,
8 2016; Huang et al., 2018; Lee et al., 2018; Lee et al., 2014; Li et al., 2017; Mews et al., 2017).
9 Moreover, nutrient deprivation or starvation causes a rapid decline in acetyl-CoA abundance in
10 cells in culture and in some mouse tissues, which is accompanied by deacetylation of proteins
11 (Marino et al., 2014). In mammalian cells, as well as in yeast, pharmacological or genetic
12 manipulations to deplete cytosolic acetyl-CoA have been reported to induce autophagy
13 (Eisenberg et al., 2014; Marino et al., 2014). Moreover, the acetylation status of autophagy
14 proteins, regulated by the p300 acetyltransferase and multiple deacetylases, plays a crucial role
15 for autophagy induction (Lee et al., 2008; Lee and Finkel, 2009; Marino et al., 2014). As such,
16 acetyl-CoA fluctuations appear to influence various biological responses through alterations in
17 protein acetylation.

18 Here, we have developed a cell line which lacks ACLY and whose nucleo-cytoplasmic acetyl-
19 CoA production is thereby solely dependent on exogenously supplemented acetate. Removal of
20 acetate from the culture media enables us to rapidly deplete intracellular acetyl-CoA. Using this
21 cell line, we provide quantitative data sets for alterations in mRNA levels, protein abundance and
22 acetylation status in cells experiencing acute acetyl-CoA depletion. These analyses uncovered
23 that in response to acetyl-CoA depletion, the integrity of the nucleolus, an organelle critical for
24 ribosomal biosynthesis, was morphologically, biophysically and functionally remodeled. This
25 nucleolus remodeling leads to the upregulation of the stress responsive transcription factor p53
26 in a ribosomal protein-dependent manner. Furthermore, these alterations were found to be

1 suppressed by class IIa HDAC inhibitors. We identified multiple nucleolar proteins whose
2 acetylation levels were potentially regulated by the class IIa HDACs, suggesting that class IIa
3 HDAC-dependent deacetylation of nucleolar proteins may play an important role in regulating the
4 integrity of the nucleolus and the induction of a nucleolus-dependent stress response when acetyl-
5 CoA levels decline.

1 Results

2

3 Acetate-dependent control of acetyl-CoA production in ACLY KO cells

4 In order to understand cellular responses to fluctuations in nucleo-cytoplasmic acetyl-CoA
5 abundances, we sought to devise a cell system where we could selectively and robustly control
6 acetyl-CoA levels. To achieve this, we focused on establishing a cell line whose acetyl-CoA in the
7 nucleo-cytoplasmic compartment was solely synthesized through the acetate-ACSS2 axis,
8 assuming that such a cell line would enable us to manipulate acetyl-CoA levels by simply
9 modulating the amount of acetate exogenously supplemented in the culture media (**Figure 1A**).
10 Hence, using the CRISPR-Cas9 system, we targeted the *ACLY* gene in HT1080 human
11 fibrosarcoma cells to make *ACLY* deficient cell lines. A plasmid coding an *ACLY*-targeting single
12 guide RNA (sgRNA) together with Cas9 was transfected into HT1080 cells and the transfected
13 cell clones were recovered either in the standard culture media (0 mM added acetate) or in the
14 same media but with excess amounts (2 or 20 mM) of sodium acetate (**Figure 1-figure**
15 **supplement 1A**). Among the cell clones screened, putative *ACLY* genome-edited clones were
16 found to only exist in the acetate supplemented media (**Figure 1-figure supplement 1B**),
17 suggesting that acetate supplementation increased the viability or growth of *ACLY* deficient cells.
18 This observation was consistent with the previously reported phenotype of *ACLY* deficient MEFs
19 (Zhao et al., 2016). We selected two independent *ACLY* deficient (KO) and *ACLY* expressing
20 (WT) clones, respectively, that henceforth were continuously cultured in the presence of 20 mM
21 acetate for further analyses (**Figure 1B and Figure 1-figure supplement 1C**). Hereafter, we refer
22 to these cells as ASA-KO and ASA-WT (acetate-supplemented *ACLY* KO and WT), respectively.
23 In this culture condition, ASA-KO cells did not exhibit any defect in viability when compared with
24 ASA-WT cells (**Figure 1C**, (+) Acetate, 10% FBS). However, removal of acetate from the culture
25 media for 4 days significantly impaired the viability of ASA-KO but not ASA-WT cells (**Figure 1C**).
26 Consistent with the observation that fetal bovine serum (FBS) contains submillimolar

1 concentration of acetate (Kamphorst et al., 2014; Zhao et al., 2016), either a decrease in FBS
2 concentration from 10% to 1% or the use of 10% dialyzed FBS (dFBS) enhanced the vulnerability
3 of ASA-KO cells, while these manipulations had no significant effect on viability in the acetate-
4 supplemented conditions (**Figure 1C**). Moreover, exogenous expression of ACLY in ASA-KO
5 cells rescued the sensitivity to acetate removal, indicating that the acetate dependence of these
6 cells was caused by *ACLY* deficiency (**Figure 1D**).

7 Using this cell system, we next examined whether acetate removal modulated intracellular
8 acetyl-CoA levels in ASA-KO cells. We used media containing 1% FBS during acetate removal
9 to maximize the effect of acetate withdrawal, although similar, albeit slightly milder effects were
10 seen following acetate withdrawal in the setting of 10% FBS. Quantifications of acetyl-CoA in the
11 whole cell lysates demonstrated that withdrawing acetate for 4 hours drastically decreased the
12 amount of acetyl-CoA in ASA-KO cells, while this intervention had only a marginal effect in ASA-
13 WT cells (**Figure 1E**). In contrast, the amount of Coenzyme A (CoA) was reciprocally increased
14 in ASA-KO cells in response to acetate removal (**Figure 1E**). Taken together, in ASA-KO cells,
15 even in the presence of other nutrient sources such as glucose and lipids, the nucleo-cytoplasmic
16 acetyl-CoA levels are seemingly tunable solely by adding or removing acetate in the culture media.

17

18 **Acetyl-CoA depletion modulates protein acetylation**

19 Using the ASA-KO cell system, we profiled global cellular responses following acute acetyl-CoA
20 depletion. As cytoplasmic acetyl-CoA is mainly utilized for lipid synthesis and protein acetylation,
21 we examined whether a rapid decline in acetyl-CoA levels affected these downstream events.
22 Quantifications of total cholesterol and a series of fatty acids revealed that although some fatty
23 acids including linoleic acid and Dihomo- γ -linolenic acid (DGLA) exhibited significant decreases,
24 overall alterations in total amounts of cholesterol and fatty acids after 4 hours of acetate removal
25 were modest when compared to the dramatic decline in acetyl-CoA levels at the same time point

1 **(Figure 2A and Figure 1E)**. Thus, at least at early time points, dramatic reductions in acetyl-CoA
2 are not fully reflected by marked alterations in total cholesterol or lipid amounts.

3 In order to examine global changes in the status of protein acetylation upon acetyl-CoA
4 depletion, we conducted an acetylome analysis using an acetyl-lysine motif antibody-based
5 immunoaffinity purification. Recovered acetylated lysine-containing peptides were then detected
6 through liquid chromatography mass-spectrometry. This approach identified 1307 acetylated
7 lysine-containing peptides in ASA-KO cells cultured in the presence of acetate (**Table S1**). Among
8 these, 658 peptides exhibited more than a 50% decrease after 90 minutes of acetate withdrawal
9 $\{\log_2 [\text{fold change (FC): (-) Acetate} / (+) \text{Acetate}] < -1.0\}$, suggesting that acetyl-CoA depletion
10 caused rapid deacetylation of nearly half of all acetylated proteins (**Figure 2B and Table S1**,
11 Column B). These peptides included a wide variety of proteins including molecules involved in
12 transcription, translation, ribosomal RNA (rRNA) processing, messenger RNA (mRNA) splicing,
13 nucleosome assembly and cell-cell adherence junctions (**Figure 2C**). By immunoblotting using
14 pan- and site specific- anti-acetyl-lysine antibodies, we detected immediate and robust decreases
15 in acetylation of both histone and non-histone proteins upon acetate withdrawal (**Figure 2D-2E**).
16 Collectively, these findings suggest that unlike the cholesterol or lipid pool, alterations in
17 intracellular acetyl-CoA levels are rapidly reflected in the status of protein acetylation.

18

19 **Acetyl-CoA depletion modulates gene expressions**

20 Since we observed marked alterations in protein acetylation including on histones, we performed
21 transcriptome analyses by RNA sequencing of ASA-KO cells cultured in the presence or absence
22 of acetate to examine the transcriptional alterations following acetyl-CoA depletion. Consistent
23 with a notion that histone deacetylation suppresses transcription, we identified a large number of
24 transcripts downregulated 4 hours after acetate withdrawal $\{526 \text{ and } 1096 \text{ transcripts in } 10\% \text{ FBS}$
25 $\text{and } 1\% \text{ FBS conditions, respectively, } \log_2 [\text{FC: (-) Acetate} / (+) \text{Acetate}] < -1.0, q < 0.05\}$ (**Figure**
26 **2F and Table S2**, sheet 1 and sheet 2 for 10% FBS and 1% FBS conditions, respectively).

1 Intriguingly, we also found many transcripts that were significantly upregulated in these conditions
2 {365 and 899 transcripts in 10% FBS and 1% FBS conditions, respectively, \log_2 [FC: (-) Acetate
3 / (+) Acetate] > 1.0, $q < 0.05$ }. These differentially expressed transcripts in the 10% and 1% FBS
4 conditions were highly overlapped (70.5% or 33.8% of downregulated transcripts in 10% or 1%
5 FBS conditions, respectively, and 65.2% or 26.4% of upregulated transcripts in 10% or 1% FBS
6 conditions, respectively, **Figure 2-figure supplement 1A**), suggesting that a similar but stronger
7 transcriptional response occurs in the 1% FBS condition. Gene ontology analyses for differentially
8 expressed genes in the 1% FBS condition uncovered that several clusters of genes were similarly
9 regulated (**Figure 2G**). For example, genes related to transcriptional regulation were highly
10 enriched in both up- and down-regulated transcripts. Of particular interest was that genes involved
11 in cellular stress responses such as apoptotic process, cell cycle arrest and oxidation-reduction
12 process were selectively upregulated in acetate-withdrawn cells, suggesting that stress signaling
13 pathways are activated in response to acetyl-CoA depletion.

14

15 **Acetyl-CoA depletion alters the integrity of the nucleolus.**

16 To further understand the complete cellular response after acetyl-CoA depletion, we also
17 performed quantitative proteomics analyses using a tandem mass tag (TMT) system with the
18 same experimental conditions used for our previous experiments (i.e. analysis 4 hours after
19 acetate removal in 1% FBS containing media). Although overall alterations in protein levels
20 appeared to be less drastic, we observed that the expression of 51 proteins were significantly
21 increased more than 1.2-fold and 135 proteins were decreased less than 0.8-fold in response to
22 acetate removal (**Table S3**). Intriguingly, we found that many proteins that are known to be
23 localized to the nucleolus were significantly altered (**Figure 3A**, shown in red). Changes in these
24 nucleolar proteins were not evident on the RNA seq analysis (**Figure 3A**, y-axis), implying that
25 these proteins were likely regulated at a post-transcriptional level. By immunoblotting, we
26 confirmed that ribosome biogenesis factors RRP1 and BOP1 were increased while PICT1 (also

1 known as NOP53 or GLTSCR2) was decreased after acetate removal in ASA-KO cells but not in
2 ASA-WT cells (**Figure 3-figure supplement 1A and 1B**). These observations indicate that
3 alterations of the nucleolus might occur upon acetyl-CoA depletion. Hence, we next addressed
4 whether acetyl-CoA depletion affected nucleolar structures and functions. The nucleolus is the
5 organelle primarily responsible for ribosomal biosynthesis, where rRNA synthesis, rRNA
6 processing and assembly of rRNA and ribosomal proteins takes place (Mangan et al., 2017;
7 Nemeth and Grummt, 2018). Using a 5-Fluorouridine (FUrd) incorporation assay, we monitored
8 newly synthesized rRNA in the nucleolus and found that acetate removal rapidly reduced rRNA
9 synthesis in ASA-KO but not ASA-WT cells (**Figure 3B-3C**). We also utilized a rRNA specific dye
10 to stain cells in conjunction with immunostaining for nucleolar proteins. In the presence of acetate,
11 the nucleolar transcription factor UBF localized within the nucleolar region as evident by its
12 overlap with rRNA (**Figure 3D**). Consistent with observed the impairment of rRNA synthesis
13 (**Figure 3B-3C**), fluorescent intensities for nuclear rRNA were significantly decreased in ASA-KO
14 cells upon acetate removal (**Figure 3D-3E**). Interestingly, residual rRNA signals detected in the
15 acetate-deprived ASA-KO cells seemingly segregated from UBF (**Figure 3D**, magnified nuclear
16 images with the line profiles). The nucleolar structure can be divided into three distinct functional
17 subcompartments including the UBF containing compartment for rRNA synthesis, as well as
18 compartments for rRNA splicing or for rRNA-ribosomal protein assembly (**Figure 3-figure**
19 **supplement 1C**) (Thiry and Lafontaine, 2005). We therefore performed a co-staining of rRNA
20 with marker proteins for the latter two compartments; fibrilalin (FBL) or nucleolin (NCL),
21 respectively. Interestingly, while both proteins colocalized with rRNA in the acetate-supplemented
22 cells, neither FBL nor NCL fully merged with the segregated rRNA signals in the acetate-deprived
23 ASA-KO cells (**Figure 3-figure supplement 1D-1E**). These observations imply that acetyl-CoA
24 depletion redistributes the rRNA containing compartment within the nucleolus. Furthermore, we
25 found that in ASA-KO cells, the nucleolar localization of a nucleolar scaffolding protein,
26 nucleophosmin (NPM1), became dispersed following acetate removal (**Figure 3F**). We also noted

1 that ASA-WT cells did not exhibit any of these morphological alterations in the nucleolus in
2 response to acetate removal (**Figure 3-figure supplement 1F-1G**). Altogether, these
3 observations suggest that acetyl-CoA depletion markedly influences nucleolar components
4 including changes in levels and in localization, thereby structurally and functionally remodeling the
5 nucleolus.

6

7 **Acetyl-CoA depletion induces ribosomal protein-dependent p53 activation**

8 In addition to the nucleolar alterations, we observed an increase in the level of p53 protein in the
9 quantitative proteomics of acetyl-CoA-depleted cells (**Figure 3A**, shown in green). We also found
10 upregulation of the p53 target genes, including *MDM2*, *CDKN1A*, *BBC3* and *PLK3*, in our RNA
11 sequencing analysis (**Figure 4-figure supplement 1A**). We therefore next demonstrated by
12 immunoblotting that removing acetate from the media increased p53 expression only in ASA-KO
13 cells (**Figure 4A**). A number of studies have demonstrated that so called “nucleolar stress”, which
14 is caused by aberrations in ribosome biogenesis, induces p53 stabilization in a ribosomal protein-
15 dependent manner (Boulon et al., 2010; Deisenroth et al., 2016). Mechanistically, the most well
16 studied ribosomal proteins in this context are RPL11 and RPL5. These components are
17 incorporated into a large ribosomal subunit by forming a complex with 5S rRNA in the unstressed
18 nucleolus. Once the nucleolus is stressed by, for instance, the RNA pol I inhibitor actinomycin D
19 (ActD), the 5S-rRNA-RPL11-RPL5 complex is known to translocate to the nucleoplasm, where it
20 interacts and inhibits MDM2, an E3 ubiquitin ligase for p53, thereby stabilizing p53 (**Figure 4B**)
21 (Donati et al., 2013; Sloan et al., 2013). Moreover, it has been reported that PICT1 is degraded
22 by nucleolar stressors and that depletion of PICT1 is sufficient for p53 induction in a ribosomal
23 protein-dependent manner (Maehama et al., 2014; Sasaki et al., 2011). Since we observed that
24 acetyl-CoA depletion induced nucleolar alterations and reciprocal alterations in p53 and PICT1
25 levels, we next assessed whether these responses were mediated through a nucleolar stress
26 condition. Upon acetate removal, interactions between endogenous MDM2 and FLAG-tagged

1 RPL11 were found to increase in a time-dependent manner in ASA-KO cells (**Figure 4C**).
2 Moreover, small interfering (si) RNA-mediated knockdown of RPL11 or RPL5 diminished acetate-
3 induced p53 upregulation, as well as PICT1 degradation, suggesting that RPL11 and RPL5 are
4 required for these processes (**Figure 4D**). These observations were consistent with other
5 nucleolar stress-dependent p53 activation (Donati et al., 2013; Sloan et al., 2013). DNA damage
6 is a potent inducer of p53, but neither acetate removal nor ActD treatment induce phosphorylation
7 of Serine 139 in Histone H2A.X (γ H2A.X), a marker for the DNA double strand breaks, suggesting
8 DNA damage-independent mechanisms for the p53 upregulation observed in acetyl-CoA-
9 depleted cells (**Figure 4-figure supplement 1B-1C**). Collectively, these observations suggest
10 that acetyl-CoA depletion-induced p53 activation is mediated through ribosomal proteins
11 associated with the nucleolar stress response.

12

13 **Class IIa HDAC family mediates acetyl-CoA-dependent nucleolar alterations**

14 We then addressed what mediates these nucleolus-dependent responses induced by acetyl-CoA
15 depletion. As described above, we found drastic alterations in protein acetylation in acetyl-CoA-
16 depleted ASA-KO cells (**Figure 2B-2E and Table S1**). Therefore, we tested a possible role for
17 lysine deacetylation in the acetyl-CoA depletion-dependent nucleolar alterations and subsequent
18 p53 activation. The histone deacetylase (HDAC) family members are the predominant
19 deacetylases in mammalian cells, which can be grouped into zinc ion-dependent (class I, II and
20 IV) and nicotinamide adenine dinucleotide (NAD⁺)-dependent (class III, also known as Sirtuin
21 family) families (**Figure 5A**) (Haberland et al., 2009). We utilized chemical inhibitors for HDACs as
22 this way enabled us to transiently inhibit HDACs while depleting acetate. We demonstrated that
23 general inhibitors for zinc-dependent HDACs, Tricostatin A and Vorinostat (also known as SAHA)
24 completely inhibited the acetyl-CoA depletion-induced reciprocal regulation of p53 and PICT1
25 (**Figure 5B**), suggesting that zinc-dependent HDACs are likely required for this process. In
26 contrast, EX-527, an inhibitor for the NAD⁺-dependent deacetylase Sirtuin 1, did not alter this

1 response (**Figure 5-figure supplement 1A**). Using selective inhibitors for each HDAC class, we
2 sought to further address which HDAC is responsible for the acetyl-CoA depletion-induced p53
3 and PICT1 regulation (**Figure 5A**). While the class I inhibitor Entinostat (also known as MS-275)
4 and the class IIa inhibitor TMP195 exhibited differential inhibitory effect on deacetylation of
5 Histone H3 K9 and α Tubulin K40, we found that TMP195 but not Entinostat blocked the reciprocal
6 regulation of p53 and PICT1 (**Figure 5B**) (Lobera et al., 2013; Saito et al., 1999). TMP195 also
7 suppressed the acetate-dependent induction of p21(CDKN1A), a downstream target of p53
8 (**Figure 5C**). Another class IIa inhibitor LMK235 also exhibited the same effect, while other class
9 I inhibitors including Mocetinostat and RGFP966 (HDAC3 selective) or the class IIb HDAC6
10 inhibitor Tubastatin A did not (**Figure 5C** and **Figure 5-figure supplement 1B**) (Butler et al.,
11 2010; Fournel et al., 2008; Malvaez et al., 2013). These observations let us hypothesize that the
12 class IIa HDACs-mediated deacetylation may modulate the nucleolus. Thus, we investigated
13 whether the inhibition of class IIa HDACs also maintained nucleolar integrity and rRNA synthesis
14 using immunostaining with the rRNA dye and FURd assay, respectively. We noted that the class
15 IIa HDAC inhibitors TMP195 and LMK235 both restored the intensity of rRNA and its co-
16 localization with NCL in acetyl-CoA-depleted ASA-KO cells (**Figure 5-figure supplement 5C-5D**).
17 Furthermore, TMP195 and LMK235 recovered FURd incorporation while the class I inhibitor
18 Entinostat was ineffective (**Figure 5D-5E**). These findings suggest that the class IIa HDACs are
19 selectively required for acetyl-CoA depletion-induced nucleolar remodeling and p53 activation.

20

21 **Class IIa HDACs regulate deacetylation of nucleolar proteins and nucleolar dynamics**

22 To investigate whether the effect of class IIa HDAC inhibitors on the nucleolus is mediated through
23 inhibition of deacetylation, we conducted an acetylome analysis for acetyl-CoA-depleted cells with
24 and without the class IIa inhibitor TMP195 and identified 365 acetylated peptides whose
25 acetylation levels were maintained at a level two-fold or greater by TMP195 treatment in the
26 setting of acetate removal {**Table S1**, Column C, Log₂ [FC: (-) Acetate + TMP195 / (-) Acetate]}

1 >1.0}. Importantly, among these TMP195 sensitive proteins, we identified multiple nucleolus-
2 resident proteins including ribosomal proteins and NCL (**Figure 5-figure supplement 2A**). Using
3 the acetyl-lysine motif antibody immunoprecipitation assay, we demonstrated that acetylation of
4 RPL11 decreased upon acetate removal, and that this was suppressed by TMP195 but not by
5 the class I inhibitor Entinostat (**Figure 5F**). Deacetylation of NCL by acetate removal was also
6 partially recovered by TMP195 (**Figure 5-figure supplement 2B**). These results suggest that the
7 class IIa HDACs mediate deacetylation of at least certain nucleolar proteins including RPL11 and
8 NCL.

9 The nucleolus is known to be formed through a process called liquid-liquid phase separation
10 (LLPS), a central mechanism for facilitating the formation of biomolecular condensates that exhibit
11 liquid droplet-like or other related material properties (Banani et al., 2017; Shin and Brangwynne,
12 2017; Snead and Gladfelter, 2019). Because LLPS is mediated through multivalent weak
13 interactions among the component proteins and nucleotides, post translational modifications
14 (PTMs) of proteins including phosphorylation, methylation and acetylation can shift the assembly
15 and material properties of condensates by modulating their interactions (Hofweber and Dormann,
16 2019; Snead and Gladfelter, 2019). Therefore, we sought to examine the effect of acetylation on
17 the LLPS state of the nucleolus. As the molecular dynamics in a condensate can be seen as an
18 indicator for its material property, we measured the mobility of NCL-mGFP by using fluorescence
19 recovery after photobleaching (FRAP) (**Figure 5G**). We set a photobleaching area within the
20 nucleolar region marked by NCL-mGFP to monitor its mobility within the nucleolus. This
21 experiment revealed that 90 minutes of acetate removal, where we observed massive
22 deacetylation (**Figure 2B-2D**), accelerated the half recovery times of NCL-mGFP (**Figure 5G-**
23 **5H**). This result suggests that the mobility of NCL-mGFP within the nucleolus was facilitated by
24 acetyl-CoA depletion. Moreover, the presence of the class IIa inhibitor LMK235 but not the class
25 I inhibitor Entinostat restored the half recovery times in acetate-depleted cells to similar levels as
26 in acetate-supplemented cells (**Figure 5G-5H**). We note that, when compared with LMK235,

1 TMP195 exhibited only partial effects on the FRAP of NCL-mGFP. As also seen in the effect on
2 acetylation of NCL (**Figure 5-figure supplement 2B**), this suggests that TMP195 might be less
3 efficient in inhibiting the particular HDAC responsible for deacetylation of NCL. Overall, these
4 observations demonstrate that class IIa HDACs-mediated deacetylation alters the dynamics of
5 nucleolar proteins, which are likely associated with changes in the phase state of the nucleolus.

7 **Acetylation-dependent regulation of the nucleolus in HCT116 cells**

8 To extend our observations to additional cell types, we generated an HCT116 human colorectal
9 carcinoma cell line-based ASA-KO cell line using the same CRISPR-mediated strategy as for
10 HT1080 cells (**Figure 1-figure supplement 1A**). Again, viable *ACL*YKO cells were only achieved
11 by supplementation of acetate in the culture media (**Figure 6-supplement figure 1A**). Acetate
12 removal upregulated p53 in HCT116 ASA-KO but not ASA-WT cells and this was inhibited by the
13 class IIa HDAC inhibitors (**Figure 6-supplement figure 1A-1B**). Our HCT116 cells harbor p53-YFP
14 stably expressing at low levels under non stress conditions (Stewart-Ornstein and Lahav, 2017).
15 Upon acetate withdrawal, p53-YFP expression was similarly induced as endogenous p53, adding
16 further evidence for the post-transcriptional regulation of p53 upon acetyl-CoA depletion (**Figure**
17 **6-supplement figure 1A-1B**). Moreover, class IIa HDAC-dependent nucleolar remodelings were
18 also observed in HCT116 ASA-KO cells (**Figure 6-supplement figure 1C**). These observations
19 extend the generality of our findings regarding acetylation-mediated regulation of the nucleolar
20 stress responses.

21 22 **Discussion**

23 **The nucleolus as an acetyl-CoA responder**

24 In this study, we demonstrated that the nucleolus rapidly responds to acetyl-CoA depletion. We
25 propose a model for this cellular response to a decline in acetyl-CoA nucleolar-cytoplasmic levels
26 (**Figure 6**). In cells that contain high levels of acetyl-CoA, multiple nucleolar proteins are highly

1 acetylated and these post-translational modifications play a crucial role in maintaining the
2 nucleolar integrity, a phase separation state allowing for efficient rRNA synthesis. Once acetyl-
3 CoA levels fall, class IIa HDACs deacetylate a significant number of nucleolar proteins. These
4 reactions remodel the properties of the LLPS of the nucleolus, resulting in impairment of rRNA
5 synthesis and induction of the p53-mediated stress responses. This model highlights an important
6 role of the nucleolus as an organelle sensor for coupling acetyl-CoA fluctuations to cellular stress
7 responses.

8 Accumulating evidence has illustrated that the nucleolus serves as a stress responsive organelle
9 towards a variety of stresses including genotoxic stress, oxidative stress, heat shock, nutrient
10 deprivations, virus infections and oncogene activations (Boulon et al., 2010; Deisenroth et al.,
11 2016; Rubbi and Milner, 2003). These nucleolar stresses, as well as chemical or genetic
12 interventions of ribosomal biogenesis, are known to induce morphological and functional
13 alterations in the nucleolus, which are coupled with the activation of p53 or other signaling
14 pathways in a stimulus- and cell context-dependent manner (Boulon et al., 2010; Chen and Stark,
15 2019). mRNA translation is known to be among the most energy demanding processes in
16 mammalian cells and efficient ribosome biogenesis is necessary for cell growth and proliferation
17 (Buttgereit and Brand, 1995). Therefore, it is likely that a fall in acetyl-CoA levels signals a state
18 of energetic stress and that cells acutely reduce ribosomal biogenesis in the nucleolus in order to
19 limit energy consumption. Similar couplings of translational control and stress responses, but at
20 different translational steps, are also observed in the integrated stress response (Pakos-Zebrucka
21 et al., 2016).

22 This study uncovered that the metabolite acetyl-CoA can be a novel modulator of the nucleolus
23 through protein acetylation. It has been reported that rRNA synthesis and rRNA processing are
24 regulated by multiple mechanisms through nutrient signaling pathways to meet the cellular
25 energy demands (Grummt, 2013). Together with these regulations, the cell likely employs this

1 acetylation-mediated nucleolar stress response in order to help cope with physiological or
2 pathological fluctuations in the cellular nutrient status.

3

4 **Nucleolus, acetylation and phase separation**

5 Recent studies have revealed that PTMs can promote or oppose phase separation of
6 biomolecular condensates through diverse mechanisms (Hofweber and Dormann, 2019; Snead
7 and Gladfelter, 2019). Rapid and reversible alterations of PTMs that are induced by various
8 stimuli is capable of tuning the state of phase separation in a context-dependent manner. It has
9 been recently reported that the LLPS-mediated formation of stress granules (SGs),
10 membraneless organelles forming in response to stress, is modulated by acetylation of its
11 component RNA helicase DDX3X (Saito et al., 2019). In this case, deacetylation of DDX3X by
12 HDAC6 is required for SGs maturation. Acetylation is also known to regulate the phase separation
13 of pathogenic proteins which are involved in neurodegenerative disorders, including Tau and TDP-
14 43 (Carlomagno et al., 2017; Cohen et al., 2015; Ferreon et al., 2018). Hence, it is tempting to
15 speculate that the acetylation status of the nucleolar components may also participate in
16 regulating the nucleolar phase separation and thereby affect its functional state. We indeed
17 observed enhanced mobility of GFP-NCL upon acetyl-CoA depletion (**Figure 5G-5H**), indicating
18 alterations in the phase material property of the nucleolus, which is reported to be tightly linked to
19 the nucleolar function (Zhu et al., 2019). In addition, our observations that acetyl-CoA depletion
20 triggered redistribution of rRNA and NPM1, known contributors to the LLPS of the nucleolus,
21 indicate changes in the phase state of the nucleolus (**Figure 3D-3F**) (Feric et al., 2016; Mitrea et
22 al., 2016; Mitrea et al., 2018). Our acetylome analyses identified multiple acetylated lysine
23 residues in various nucleolar protein (**Table S1 and Figure 5-supplement figure 1E**). The
24 diversity of this list made it difficult to point out particular acetylation sites that are critical for the
25 nucleolar integrity. It is likely that the entire balance of nucleolar protein acetylation may determine
26 the propensity for LLPS. In this regard, we do not exclude a possible involvement of acetylation

1 of non-nucleolar proteins that reside in the environment surrounding the nucleolus, including the
2 nuclear lamina, actin cytoskeleton or chromatin, as these components are also known to influence
3 the nucleolar structures and functions (Buchwalter and Hetzer, 2017; Feric and Brangwynne,
4 2013; Martin et al., 2009; Murayama et al., 2008; Sen Gupta and Sengupta, 2017).

5

6 **Class IIa HDACs regulate acetylation of nucleolar proteins**

7 Compared with the class I HDACs or the class IIb HDAC6, substrates of the class IIa HDACs
8 have been less characterized (Haberland et al., 2009; Parra, 2015). It has been previously shown
9 that the class IIa HDACs have only minimal deacetylase activity on acetylated histones in vitro
10 (Fischle et al., 2002; Lahm et al., 2007). Also, the transcriptional repressor activity by the class
11 IIa HDACs expressed in cells or the enzymatic activity associated with immunoprecipitated class
12 IIa HDACs are due to the deacetylase activity of the class I HDACs endogenously forming a
13 complex with the class IIa HDACs (Fischle et al., 2002; Lahm et al., 2007). A catalytic tyrosine
14 residue within the HDAC domain conserved in all other HDACs was found to be replaced with
15 histidine in the class IIa HDACs, which can explain their weak catalytic activity towards canonical
16 HDAC substrates (Lahm et al., 2007). Therefore, the importance of the catalytic activity of the
17 class IIa HDACs for their biological functions has been questioned. Our HDAC inhibitor profiling
18 however demonstrated that the acetyl-CoA depletion-dependent nucleolar alterations and p53
19 induction was selectively sensitive to class IIa HDAC inhibitors, indicating that a class IIa HDAC
20 catalytic domain-dependent reactions exist in this context. Our acetylome analyses with TMP195
21 provide a list of potential substrates for class IIa HDACs (**Table S1**). This list highlights that various
22 non-histone proteins including nucleolar proteins and ribosomal proteins are potential targets for
23 this family of enzymes. Comparison with acetylome analyses using other class-selective HDAC
24 inhibitors and in vitro reconstitution study will define bona fide substrates for the class IIa HDACs.

25

1 In conclusion, we demonstrated that our ASA-KO cell system can be a useful tool for analyzing
2 acute responses to acetyl-CoA depletion. Our findings indicate that acetylation-mediated control
3 of the nucleolar phase separation serves as an important hub linking acetyl-CoA metabolism to
4 cellular stress responses. Furthermore, our findings highlight a potential application of the class
5 Ila HDAC inhibitors for modulating nucleolar functions, which may provide novel insights for
6 therapeutic interventions in a range of human diseases and in normal aging, where alterations or
7 malfunctions of the nucleolus have been increasingly identified to play a role (Orsolich et al., 2016;
8 Tiku and Antebi, 2018).

9
10

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20

21 **Author Contributions**

22 Y.S. and T.F. conceived and led the project. Y.S., R.H. and S.S. performed experiments and
23 analyzed data. H.K. contributed to developing the method for quantification of acetyl-CoA and
24 CoA. S.G.W. conducted the lipid quantification and the data analysis. A.J.N. and M.P.S.
25 conducted the acetylome experiments and the data analysis. Y.L. supervised the RNA
26 sequencing experiments. F.S. and M.P. computationally analyzed the RNA sequencing data. G.W.

1 and M.G. conducted the TMT proteomics and the data analysis. D.A.M. conducted the microscopy
2 image data analysis. S.C.W supervised and M.J.C. conducted the FRAP experiments. J.S.-O.
3 contributed to developing the HCT116 p53-YFP cell system. Y.S. and S.S. visualized data and
4 schemes. Y.S. wrote the initial draft with input of other authors. T.F. and all other authors reviewed
5 and edited the manuscript.

6

7 **Declaration of Interest**

8 The authors declare no competing interests.

1 **Materials and Methods**

2

3 **Cell culture**

4 HT1080 human fibrosarcoma cells (ATCC, CCL-121) were cultured at 37°C with 5% CO₂ in
5 Minimum Essential Medium Eagle (with Earle's salts, L-glutamine, and sodium bicarbonate)
6 (Sigma-Aldrich, St. Louis, MO), supplemented with 10% heat inactivated Fetal Bovine Serum
7 (Thermo Fisher Scientific, Asheville, NC, Gibco or VWR Life Science, Randor, PA), 1x Penicillin
8 Streptomycin Solution (Corning Incorporated, Corning, NY) and 1x MEM Non-essential Amino
9 Acid Solution (Sigma). Sodium Acetate (Sigma) was supplemented in the culture medium for the
10 ACLY sgRNA-targeted cells. Dialyzed FBS was purchased from Thermo Fisher Scientific (Gibco).
11 293T cells (Lenti-X, Takara, San Francisco, CA) and HCT116 cells (Stewart-Ornstein and Lahav,
12 2017) were cultured in the same condition as HT1080 cells but in Dulbeccos's Modified Eagle
13 Medium (Gibco) supplemented with 10% FBS, 1 mM Sodium pyruvate (Gibco), non-essential
14 amino acids (Gibco) and GlutaMAX (Gibco).

15

16 **Plasmid constructions and lentivirus productions**

17 Oligo duplexes containing a single guide (sg)RNA sequence for ACLY (as shown in Figure 1-
18 figure supplement 1A) were inserted into pCas9(BB)-2A-GFP (a gift from Feng Zhang, Addgene
19 #48138) following the published procedures (Ran et al., 2013) to generate genome-edited cells
20 using the CRISPR-Cas9 system. For lentivirus-mediated gene expression, a coding sequence
21 (CDS) of human ACLY was PCR amplified from a HeLa cDNA pool and inserted into the *EcoRI*-
22 digested pLVX-puro vector (Takara) using the In-Fusion cloning system (Takara). A CDS of
23 RPL11 was amplified from an HT1080 cDNA pool and inserted into the *EcoRI/BglII*-digested
24 p3xFLAG-CMV-7.1 (Sigma) to attach the 3xFLAG tag at its N-terminus. The 3xFLAG-RPL11
25 sequence was then inserted into the *EcoRI*-digested pLVX-puro vector. The NCL CDS was
26 amplified from the GFP-Nucleolin plasmid (a gift from Michael Kastan, Addgene #28176)(Takagi

1 et al., 2005). The NCL-3xFLAG fragment was amplified using primers that attach the 3xFLAG at
2 NCL's C-terminus and was inserted into the *EcoRI*-digested pLVX-puro vector. For the NCL-
3 mGFP expressing construct, the NCL-CDS was amplified from the GFP-Nucleolin plasmid and
4 monomeric GFP (mGFP) was amplified from the LAMP1-mGFP plasmid (a gift from Esteban
5 Dell'Angelica, Addgene #34831)(Falcon-Perez et al., 2005) and these fragments were inserted
6 into the *EcoRI*-digested pLVX-puro vector. Lentivirus was produced in 293T cells by transfecting
7 the pLVX-puro vectors, psPAX2 (a gift from Didier Trono, Addgene #12260) and pCMV-VSV-G
8 (a gift from Bob Weinberg, Addgene #8454) (Stewart et al., 2003).

9

10 **Generation of the *ACLY* genome-edited cell lines by CRISPR-Cas9**

11 The pCas9(BB)-2A-GFP plasmids containing the sgRNA sequence targeting *ACLY* were
12 transfected into HT1080 cells or HCT116 p53-YFP cells using Lipofectamine LTX (Thermo Fisher
13 Scientific) or X-treamGENE (Sigma) according to the manufacturer's instruction. The next day,
14 GFP positive cells were collected using the FACS Fusion sorter (BD Biosciences) and cultured in
15 three different medium conditons; standard culture media or the same media with 2 or 20 mM
16 sodium acetate as illustrated in Figure 1-figure supplement 1A. Cell clones expanded from each
17 condition were screened by genotyping PCR (the forward primer; gtggtgaagagctatgtccag and
18 the reverse primer; cctctgctgtgcacatctgtc) combined with *XhoI* digestion (as illustrated in Figure
19 1-figure supplement 1B) and by immunoblotting.

20

21 **Immunoblotting**

22 ASA cells were plated at a density of 8×10^4 cells per well in a 6 well plate and cultured in the 20
23 mM sodium acetate-supplemented culture medium (10% FBS). The next day, the cells were
24 washed with PBS twice and 1% FBS culture medium once, and then cultured in the media
25 containing 10% or 1% FBS with or without 20 mM sodium acetate, or with indicated chemicals
26 according to experiments. After indicated time periods, the cells were washed with PBS or 1%FBS

1 culture medium once and lysed in 150 μ l of 1x NuPAGE LDS sample buffer (Thermo Fisher
2 Scientific) supplemented with 10 mM DTT. The samples were shaken at max speed in a table top
3 incubator at room temperature for 10 min, boiled at 98°C for 5 min, and centrifuged at 15,000 rpm
4 for 5 min. The supernatant containing approximately 15-30 μ g protein was separated by SDS-
5 PAGE using a 4-20% gradient Mini-PROTEAN TGX Precast Gel (Bio-Rad, Hercules, CA) and
6 then transferred to a 0.2 μ m nitrocellulose membrane. The membrane was blocked with Odyssey
7 Blocking Buffer (LI-COR, Lincoln, NE) and incubated with the indicated primary antibodies at 4°C
8 overnight. After washing with PBS-T (PBS + 0.05% Tween-20), the membrane was incubated
9 with near-infrared fluorescent IRDye secondary antibodies (LI-COR) or HRP-conjugated
10 secondary antibodies (Thermo Fisher Scientific) and washed again with PBS-T. Detection was
11 performed with either the ODYSSEY CLx Infrared Imaging System together with Image Studio
12 Lite version 5.2 Software (LI-COR), or iBright CL1000 Imaging System (Thermo Fisher Scientific).
13 For immunoblotting of BOP1 and RRP1, the cells were lysed with 1% Triton buffer [1% Triton-
14 X100, 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, Phosphatase inhibitors (PhosSTOP,
15 Sigma) and protease inhibitors (cOmplete, Sigma)]. After centrifugation, the supernatant was
16 mixed with 4x NuPAGE LDS sample buffer and boiled. For siRNA-mediated knockdown
17 experiments, silencer siRNAs (5 nM) for indicated genes or Negative Control siRNA #1 (Ambion,
18 AM4611) were transfected with Lipofectamin RNAi MAX transfection reagent (Thermo Fisher
19 Scientific, 13778075) into 4×10^4 cells in a 6 well plate. The next day, the culture media were
20 replaced with fresh media, and the day after that the cells were subjected to immunoblotting
21 experiments. Following Silencer Select Pre-designed siRNAs were used; RPL5 (ID s56731),
22 RPL11 (ID s12168).

23

24 **Immunoprecipitation**

25 ASA-KO17 cells and the ASA-KO17 cells stably expressing 3xFLAG-RPL11 were plated at a
26 density of 7×10^5 cells per 10 cm culture dish and cultured in the 20 mM sodium acetate-

1 supplemented culture medium (10% FBS). The next day, the cells were washed with PBS twice
2 and 1% FBS culture medium once, and then cultured in 1% FBS culture medium with or without
3 20 mM sodium acetate (two 10 cm dishes were prepared for each condition). After indicated time
4 periods, the cells were washed with PBS and lysed in 500 μ l of 1% Triton buffer [1% Triton-X100,
5 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, Phosphatase inhibitors (PhosSTOP, Sigma)
6 and protease inhibitors (cOmplete, Sigma)]. After centrifugation, 30 μ l of the supernatant was
7 taken for “Lysate” sample and the rest of the supernatant was mixed with 10 μ l of Anti-FLAG M2
8 Magnetic beads (Sigma). After 30 min of incubation at 4°C, the beads were washed with 1%
9 Triton buffer three times and then incubated in 30 μ l of 1% Triton buffer with 0.2 μ g/ μ l of 3xFLAG
10 peptide (Sigma) at room temperature for 20 min. Then the supernatant was mixed with 4x
11 NuPAGE LDS sample buffer (Thermo Fisher Scientific) supplemented with 10 mM DTT and boiled.
12 The immunoprecipitation assay was performed using an acetyl-Lysine motif antibody to detect
13 acetylated protein. The ASA-KO17 cells stably expressing 3xFLAG-RPL11 or NCL-3xFLAG were
14 plated, stimulated, and lysed as the FLAG immunoprecipitation assay described above. The
15 supernatant was mixed with either 0.8 μ g Rabbit IgG (Santa Cruz, Dallas, TX, sc-2027) or 4 μ l of
16 the acetyl-lysine motif antibody (Cell Signaling Technology, Danvers, MA, #9814) and incubated
17 over night at 4°C. Then the supernatant was mixed with 10 μ l of Protein A/G Magnetic Beads
18 (Thermo Fisher Scientific) and incubated for 1 hour at 4°C. The beads were washed with 1%
19 Triton buffer three times and mixed with 35 μ l of 1x NuPAGE LDS sample buffer supplemented
20 with 10 mM DTT and shaken at room temperature for 10 min. Then the supernatant was subjected
21 to SDS-PAGE.

22

23 **Cell viability analysis (WST-1 assay)**

24 Cells were plated at a density of 5,000 cells per well in a 6 well plate and cultured in the culture
25 medium (10% FBS) with or without 20 mM sodium acetate. The next day, the cells were washed
26 with PBS twice and 1% FBS culture medium once, and then cultured in the media containing 10%

1 FBS, 1% FBS or 10% dialyzed FBS with or without 20 mM sodium acetate. 24 hours later, the
2 culture media were replaced with 10% FBS culture medium with or without 20 mM sodium acetate
3 and the cells were cultured for another 2 days. Then, the media were replaced with 10% FBS
4 medium containing 50 μ M WST-1 (Dojindo, Gaithersburg, MD) and 20 μ M 1-methoxy PMS
5 (Dojindo), and the cells were incubated for 2 hours at 37°C in the cell culture incubator before the
6 absorbance at 440 nm in the culture media (100 μ l aliquots transferred in a 96 well plate) was
7 measured using Spectra Max Plus 384 microplate Reader (Molecular Devices, Sunnyvale, CA).

8

9 **Quantification of Acetyl-CoA and Coenzyme A in cell lysate by HPLC**

10 ASA-WT5 and ASA-KO17 cells were plated at a density of 4×10^5 cells per 10 cm culture dish
11 and cultured in the 20 mM sodium acetate-supplemented culture medium (10% FBS) for 1 day
12 (10 dishes were prepared for each cell line). The cells were then washed with PBS twice and
13 cultured in 1 % FBS culture media with or without 20 mM sodium acetate (5 dishes were prepared
14 for each condition). After 4 hours, cells were washed with PBS, detached by trypsin, and
15 resuspended in the culture media (approximately $1.5\text{-}2.0 \times 10^6$ cells). Cell pellet was collected by
16 centrifugation, washed with PBS twice, and resuspended in 100 μ l of 5% 5-Sulfosalicylic acid
17 (Sigma) solution. To permeabilize the cells, samples were frozen in liquid nitrogen and thawed on
18 ice. This freeze-thaw cycle was repeated twice. After centrifugation, the supernatant was filtered
19 using Ultrafree-MC LH Centrifugal Filter (Millipore, Billerica, MA, UFC30LH25). Then, the samples
20 were transferred into SUN-SRi Glass Microsampling Vials (Thermo Fisher Scientific, 14-823-359)
21 with SUN-SRi 11mm Snap Caps (Thermo Fisher Scientific, 14-823-379) and 80 μ l of each sample
22 was separated using an Agilent 1100 HPLC (Agilent Technologies, Wilmington, DE) equipped
23 with a reverse phase column, Luna 3 μ m C18(2) 100 Å, 50 x 4.6mm, 3 μ m (Phenomenex, Los
24 Angeles, CA). The HPLC-reverse phase column was calibrated with acetyl-CoA (Sigma, A2056)
25 and coenzyme A (Sigma, C3144). The mobile phase was described previously (Shibata et al.,
26 2012) and consisted of two eluents: 75 mM sodium acetate and 100 mM NaH_2PO_4 pH 4.6 (buffer

1 A). Methanol was added to the buffer A at a ratio of 70:30 (v/v) = buffer A:methanol (buffer B).
2 The gradient started with 10% of buffer B and was run under following conditions; 10 min at up to
3 40% of buffer B, 13 min at up to 68% of buffer B, 23 min at up to 72% of buffer B, 28 min at up to
4 100% of buffer B, and held for an additional 5 min. The initial condition was restored after 10 min
5 with 10% of buffer B. The flow rate was 0.6 ml/min and the detection was performed at 259 nm.
6 UV chromatograms were analyzed using the software ChemStation version A.10.01 (Agilent
7 Technologies).

8

9 **Quantification of Cholesterol and fatty acids**

10 ASA-KO17 cells were plated and stimulated and the cell pellet (approximately $1.1\text{-}2.8 \times 10^6$ cells)
11 was collected as described in the section for quantification of acetyl-CoA. For measurement of
12 cholesterol and fatty acids, 500 μL of aqueous cell lysate was extracted with 2 mL of
13 chloroform:methanol (2:1) after the addition of 10 μL of a deuterated fatty acid internal standard
14 mix (50 ppm) and 20 μL of cholesterol- d_7 (10 ppm). The samples were vortexed and centrifuged
15 at 2500 rpm for 10 min at 4°C and the organic layer was transferred into two new glass vials in
16 equal volumes for fatty acid derivatization and cholesterol analysis. Both vials were dried under
17 N_2 . Samples for cholesterol analysis were reconstituted in 100 μL of chloroform:methanol (2:1)
18 and 10 μL was injected into a Shimadzu LC with CTC PAL autosampler coupled to a Sciex 5000
19 triple quadrupole mass analyzer. Analytes were separated on a C18(2) Luna column (2.1 X 100
20 mm, Phenomenex) at a flow rate of 0.63 mL/min using 50:50 H_2O :ACN with 0.1% formic acid for
21 solvent A and 40:60 ACN:IPA with 0.1% formic acid for solvent B. The gradient started at 50% B
22 and increased to 100% B over 6 minutes before returning to initial conditions for column
23 equilibration. Cholesterol and cholesterol- d_7 were detected by selective reaction monitoring using
24 369 \rightarrow 147 and 376 \rightarrow 147 transitions, respectively. Peak area ratios of cholesterol to cholesterol-
25 d_7 were normalized to cell number and are reported as relative amount.

1 Fatty acids were derivatized for LC-MS analysis using a Vanquish UPLC coupled to a Q Exactive
2 mass spectrometer (Thermo Fisher Scientific). Derivatization of the samples was conducted as
3 described (Li and Franke, 2011). To each sample, 200 μ L of oxalyl chloride (2 M in
4 dichloromethane) was added and the mixture was incubated for 5 min at 65°C. Samples were
5 dried under N₂ and 150 μ L of 3-picolylamine (1% v/v in ACN) was added for a 5 min incubation
6 at room temperature to form the 3-picolylamide (PA) ester. Samples were dried a final time under
7 N₂ and reconstituted in 1 mL MeOH and 5 μ L was injected onto the Vanquish-QE system. Fatty
8 acid-PA esters were separated on a Phenomenex C8 column (2.1 X 150 mm, 5 μ pore size) using
9 H₂O + 0.1% acetic acid for solvent A and ACN + 0.1% acetic acid for solvent B. The gradient
10 started at 65% B and increased linearly to 85% B at 10 min and was held for 1 min before ramping
11 to 100% B for 2min. Finally, the gradient returned to 65% B for a 2 min equilibration. Samples
12 were analyzed using full scan accurate mass at a resolution of 70K. Peak area ratios of fatty acid-
13 PA ester to corresponding internal standard were normalized to cell number and reported as
14 relative amount.

15

16 **Acetylome analysis**

17 ASA-KO17 cells were plated at a density of 6×10^6 cells per 15 cm culture dish and cultured in
18 the 20 mM sodium acetate-supplemented culture media (10% FBS) for 1 day. The cells were then
19 washed with PBS twice and 1% FBS culture medium once, and cultured in 1% FBS culture media
20 with or without 20 mM sodium acetate, or without acetate but with 50 μ M TMP195 (21 dishes
21 were prepared for each condition). After 90 min, the cells were washed with 10 ml of cold PBS
22 once and lysed in 10 ml of PTMScan Urea Lysis Buffer [20 mM HEPES (pH 8.0), 9.0 M urea, 1
23 mM sodium orthovanadate (activated), 2.5 mM sodium pyrophosphate, 1 mM β -glycerol-
24 phosphate] (Cell Signaling Technology). Samples were frozen in dry ice/ethanol and then
25 analyzed using the PTMScan method (Cell Signaling Technology) as previously described (Rush

1 et al., 2005; Stokes et al., 2015). Briefly, the lysates were sonicated, centrifuged, reduced with
2 DTT, and alkylated with iodoacetamide. 15 mg of total protein for each sample was digested with
3 trypsin and purified over C18 columns for enrichment with the Acetyl-Lysine Motif Antibody
4 (#13416). Enriched peptides were purified over C18 STAGE tips. Enriched peptides were
5 subjected to secondary digest with trypsin and second STAGE tip prior to LC-MS/MS analysis.
6 Replicate injections of each sample were run non-sequentially on the instrument. Peptides were
7 eluted using a 90-minute linear gradient of acetonitrile in 0.125% formic acid delivered at 280
8 nL/min. Tandem mass spectra were collected in a data-dependent manner with a Thermo
9 Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer using a top-twenty MS/MS method, a
10 dynamic repeat count of one, and a repeat duration of 30 sec. Real time recalibration of mass
11 error was performed using lock mass with a singly charged polysiloxane ion $m/z = 371.101237$.
12 MS/MS spectra were evaluated using SEQUEST and the Core platform from Harvard University.
13 Files were searched against the SwissProt *Homo sapiens* FASTA database. A mass accuracy
14 of +/-5 ppm was used for precursor ions and 0.02 Da for product ions. Enzyme specificity was
15 limited to trypsin, with at least one tryptic (K- or R-containing) terminus required per peptide and
16 up to four mis-cleavages allowed. Cysteine carboxamidomethylation was specified as a static
17 modification, oxidation of methionine and acetylation on lysine residues were allowed as variable
18 modifications. Reverse decoy databases were included for all searches to estimate false
19 discovery rates, and filtered using a 2.5% FDR in the Linear Discriminant module of Core.
20 Peptides were also manually filtered using a +/- 5ppm mass error range and presence of an
21 acetylated lysine residue. All quantitative results were generated using Skyline to extract the
22 integrated peak area of the corresponding peptide assignments. Accuracy of quantitative data
23 was ensured by manual review in Skyline or in the ion chromatogram files. GO analysis was done
24 using functional annotation in DAVID Bioinformatics Resources (david.ncifcrf.gov).

25

26 **RNA sequencing analysis**

1 ASA-KO17 cells were plated at a density of 4×10^5 cells per 10 cm culture dish and cultured in
2 the 20 mM sodium acetate-supplemented FBS culture media (10% FBS) for 1 day. The cells were
3 then washed with PBS twice, and cultured in the culture media containing 10% or 1% FBS with
4 or without 20 mM sodium acetate (2 dishes were prepared for each condition). After 4 hours, cells
5 were washed with PBS, collected in 1 mM EDTA in PBS and total RNAs were isolated using
6 RNeasy Mini Kit (Qiagen, 74106) according to the manufacturer's instruction. The sequencing
7 libraries were constructed from 100 ng – 500 ng of total RNA using the Illumina's TruSeq Stranded
8 Total RNA kit with Ribo-Zero following the manufacturer's instruction. The fragment size of
9 RNAseq libraries was verified using the Agilent 2100 Bioanalyzer (Agilent) and the concentrations
10 were determined using Qubit instrument (LifeTech). The libraries were loaded onto the Illumina
11 HiSeq 3000 for 2x75 bp paired end read sequencing. The fastq files were generated using the
12 bcl2fastq software for further data analysis. Data analysis: After quality assessment, adapter and
13 low-quality bases trimming, reads were aligned to the reference genome using the latest version
14 of HISAT2 (Kim et al., 2015), which sequentially aligns reads to the known transcriptome and
15 genome using the splice-aware aligner built upon HISAT2. Only uniquely mapped paired-end
16 reads were then used for subsequent analyses. FeatureCounts (Liao et al., 2014) was used for
17 gene level abundance estimation. Differential expression analysis were then carried out using
18 open source Limma R package (Ritchie et al., 2015). Limma-voom was employed to implement
19 a gene-wise linear modelling which processes the read counts into \log_2 counts per million
20 (logCPM) with associated precision weights. The logCPM values were normalized between
21 samples using trimmed mean of M-values (TMM). We adjust for multiple testing by reporting the
22 FDR q-values for each feature. Features with $q < 5\%$ were declared as genome-wide significant. .
23 GO analysis was done using functional annotation in DAVID Bioinformatics Resources
24 (david.ncifcrf.gov).

25

26 **Tandem mass spectrometry analysis**

1 ASA-KO17 cells were plated at a density of 4×10^5 cells per 10 cm culture dish and cultured in
2 the 20 mM sodium acetate-supplemented culture media (10% FBS) for 1 day. The cells were then
3 washed with PBS twice, and cultured in 1% FBS culture media with or without 20 mM sodium
4 acetate (2 or 3 dishes were prepared for each condition). After 4 hours, cells were washed with
5 ice-cold PBS three times, harvested with 1 mM EDTA in PBS, collected by centrifugation, and
6 lysed with RIPA buffer [1% NP-40, 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 0.1% SDS, 0.5% Na-
7 DOC, Phosphatase inhibitor (PhosSTOP, Sigma) and protease inhibitor (cOmplete, Sigma)]. After
8 centrifugation, the supernatant containing 100 μ g protein was used for the tandem mass
9 spectrometry analysis. The samples were reduced, alkylated, digested, and labeled according to
10 instructions for the TMT 10plex kit (Thermo Fisher Scientific). TMT-labeled peptides were mixed,
11 desalted, and fractionated on an Agilent 1200 HPLC system to 24 fractions using basic reversed-
12 phase chromatography. LC-MS/MS was performed on a Dionex UltiMate 3000 rapid separation
13 nano UHPLC system (Thermo Fisher Scientific) coupled online to an Orbitrap Fusion Lumos
14 tribrid mass spectrometer (Thermo Fisher Scientific). Peptides were first loaded onto a nano trap
15 column (Acclaim PepMap100 C18, 3 μ m, 100 \AA , 75 μ m i.d. x 2 cm, Thermo Fisher Scientific), and
16 then separated on a reversed-phase EASY-Spray analytical column (PepMap RSLC C18, 2 μ m,
17 75 μ m i.d. x 50 cm, Thermo Fisher Scientific) using a linear gradient of 4-32% B (buffer A: 0.1%
18 formic acid in water; buffer B: 0.1% formic acid in acetonitrile) for 100 min. The mass spectrometer
19 was equipped with a nano EASY-Spray ionization source, and eluted peptides were brought into
20 gas-phase ions by electrospray ionization and analyzed in the orbitrap. High-resolution survey
21 MS scans and HCD fragment MS/MS spectra were acquired in a data-dependent manner with a
22 cycle time of 3 s. Dynamic exclusion was enabled. Raw data files generated from LC-MS/MS
23 were analyzed using a Proteome Discoverer v2.2 software package (Thermo Fisher Scientific)
24 and the Mascot search engine (Matrix Science). The following database search criteria were set
25 to: database, SwissProt human; enzyme, trypsin; max miscleavages, 2; variable modifications,
26 oxidation (M), deamidation (NQ); fixed modifications, TMT (K, N-term), carbamidomethylation (C);

1 peptide precursor mass tolerance, 20 ppm; MS/MS fragment mass tolerance, 0.03 Da. Peptide-
2 spectrum matches (PSMs) were filtered to achieve an estimated false discovery rate (FDR) of 1%
3 based on a target-decoy database search strategy. The relative abundance of a protein or peptide
4 in different samples was estimated by TMT reporter ion intensities.

5

6 **Light Microscopy analysis**

7 Cells were plated at a density of 4×10^4 cells per well in a 4 well chamber slide (Nunc Lab-Tek II
8 Chamber slide system, Thermo Fisher Scientific), and cultured in the 20 mM sodium acetate-
9 supplemented culture medium (10% FBS). The next day, the cells were washed with PBS twice
10 and 1% FBS culture medium once, and cultured in 1% FBS culture media with or without 20 mM
11 sodium acetate, or with or without indicated compounds. After indicated periods, the cells were
12 washed with PBS, fixed with 4% formaldehyde/PBS for 10 min, permeabilized with 1% Triton-
13 X/PBS for 15 min and blocked with 2% BSA/PBS for 30 min at room temperature. Primary
14 antibodies in 2% BSA/PBS were incubated overnight at 4°C and secondary antibodies (Thermo
15 Fisher Scientific, Alexa Fluor) were incubated for 2 hours at room temperature and washed with
16 PBS-T. 1 μ M Nucleolus bright red (Dojindo) was treated in PBS for 10 min and washed with PBS
17 just before the observation. For FUrd incorporation assay, 5-Fluorouridine (1 mM) (Sigma) was
18 added in the culture media 15 min before fixation. The FUrd signal was detected by staining with
19 anti-BrdU [BU1/75 (ICR1)] Rat mAb (abcam, Boston, MA, ab6326) and Alexa Fluor 488 anti-Rat
20 IgG (Jackson Labratory, Bar Harbor, ME, 712-547-003). Fluorescent images were acquired using
21 Leica SP8 LIGHTNING Confocal Microscope (Leica, Buffalo Grove, IL) equipped with a 63 \times /1.4
22 NA oil immersion objective and driven by LAS X software. Image quantifications were performed
23 with LAS X software. Fluorescent images in Figure 4S were acquired using a Zeiss LSM 780
24 Confocal Microscope (Carl Zeiss, Dublin, CA) equipped with a 63 \times /1.4 NA oil immersion
25 objective and driven by ZEN software.

26

1 **FRAP analysis**

2 ASA-KO17 cells were plated at a density of 2×10^5 cells per well in a 35 mm glass bottom dish
3 (No. 1.5 Coverslip, 14 mm glass diameter, uncoated, MatTek), and cultured in the 20 mM sodium
4 acetate-supplemented culture medium (10% FBS). The next day, the cells were washed with PBS
5 twice and 1% FBS culture medium once, and cultured in 1% FBS culture media with or without
6 20 mM sodium acetate, or with or without indicated compounds. 90 minutes later, confocal images
7 were taken on a Leica SP8 confocal microscope with a HC PL APO 1.30 NA 93x glycerine
8 objective using a Acousto-Optic Beam Splitter (AOBS) emission system on LASX software (Leica,
9 v3.5.2.18963). Images were acquired at 1.54 frames per second, with 132nm pixels using a galvo
10 scanner at 400 Hz. Marker mobility was measured using the FRAP module, bleaching within the
11 ROI with three passes of high power 405nm laser. Immediately post-bleach, samples were
12 imaged continuously for approximately 50 sec. T1/2 was measured using Elements (Nikon,
13 Melville, NY, v5.210) time measurement tool.

14

15 **Reagents and antibodies**

16 Reagents were sourced as indicated: Tricostatin A (Sigma, T1952), Vorinostat (SAHA, MK0683)
17 (Selleck Chemicals, Houston, TX, S1047), Entinostat (MS-275) (Selleck Chemicals, S1053),
18 Mocetinostat (Cayman Chemical, Ann Arbor, MI, 18287), RGFP966 (Cayman Chemical, 16917),
19 TMP195 (Cayman Chemical, 23242), LMK235 (Cayman Chemical, 14969), Tubastatin A (Apex
20 Biomedical, Clackamas, OR, A4101), Camptothecin (Selleck Chemicals, S1288), Actinomycin D
21 (Sigma, A1410).

22 Antibodies were sourced as indicated:

23 ATP-Citrate Lyase Rabbit polyAb (1:1000, Cell Signaling Technology, #4332)

24 Histone H3 (D1H2) XP[®] Rabbit mAb (1:1000, Cell Signaling Technology, #4499)

25 Acetyl-Histone H3 (Lys9) (C5B11) Rabbit mAb (1:1000, Cell Signaling Technology, #9649)

26 Acetyl-Histone H3 (Lys27) (D5E4) XP[®] Rabbit mAb (1:1000, Cell Signaling Technology, #8173)

- 1 Acetyl-Histone H4 (Lys8) Rabbit polyAb (1:1000, Cell Signaling Technology, #2594)
- 2 Phospho-Histone H2A.X (Ser139) (20E3) Rabbit mAb (1:1000 or 1:200, Cell Signaling
- 3 Technology, #9718)
- 4 Histone H2A (D6O3A) Rabbit mAb (1:1000, Cell Signaling Technology, #12349)
- 5 Acetylated-Lysine Antibody Rabbit polyAb (1:1000, Cell Signaling Technology, #9814)
- 6 α -Tubulin (clone DM1A) Mouse mAb (1:5000, Sigma, T6199)
- 7 Acetyl- α -Tubulin (Lys40) (D20G3) XP[®] Rabbit mAb (1:1000, Cell Signaling Technology, #5335)
- 8 p53 (DO-1) Mouse mAb (1:5000, Santa Cruz, sc-126)
- 9 GLTSCR2/PICT1 Rabbit polyAb (1:1000, Cell Signaling Technology, 73225)
- 10 MDM2 (SMP14) Mouse mAb (1:1000, Santa Cruz, sc-965)
- 11 RPL11 (D1P5N) Rabbit polyAb (1:1000, Cell Signaling Technology, 18163)
- 12 RPL5 Rabbit polyAb (1:1000, Cell Signaling Technology, 14568)
- 13 anti-BrdU [BU1/75 (ICR1)] Rat mAb (1:500, abcam, ab6326)
- 14 BOP1 (E-1) Mouse mAb (1:250, Santa Cruz, sc-390672)
- 15 RRP1 Rabbit polyAb (1:200, GeneTex, Irvine, CA, GTX115107)
- 16 UBF (F-9) Mouse mAb (1:500, Santa Cruz, sc-13125)
- 17 FBL (G-8) Mouse mAb (1:500, Santa Cruz, sc-374022)
- 18 FBL (B-1) Mouse mAb (1:500, Santa Cruz, sc-166001)
- 19 NCL/C23 (H-6) Mouse mAb (1:500, Santa Cruz, sc-55486)
- 20 NCL (D4C7O) Rabbit mAb (1:1000, Cell Signaling Technology, #14574)
- 21 NPM1 Rabbit polyAb (1:200, , Cell Signaling Technology, #3542)
- 22 Anti-FLAG M2 Mouse mAb (1:1000, Sigma, F3165)
- 23

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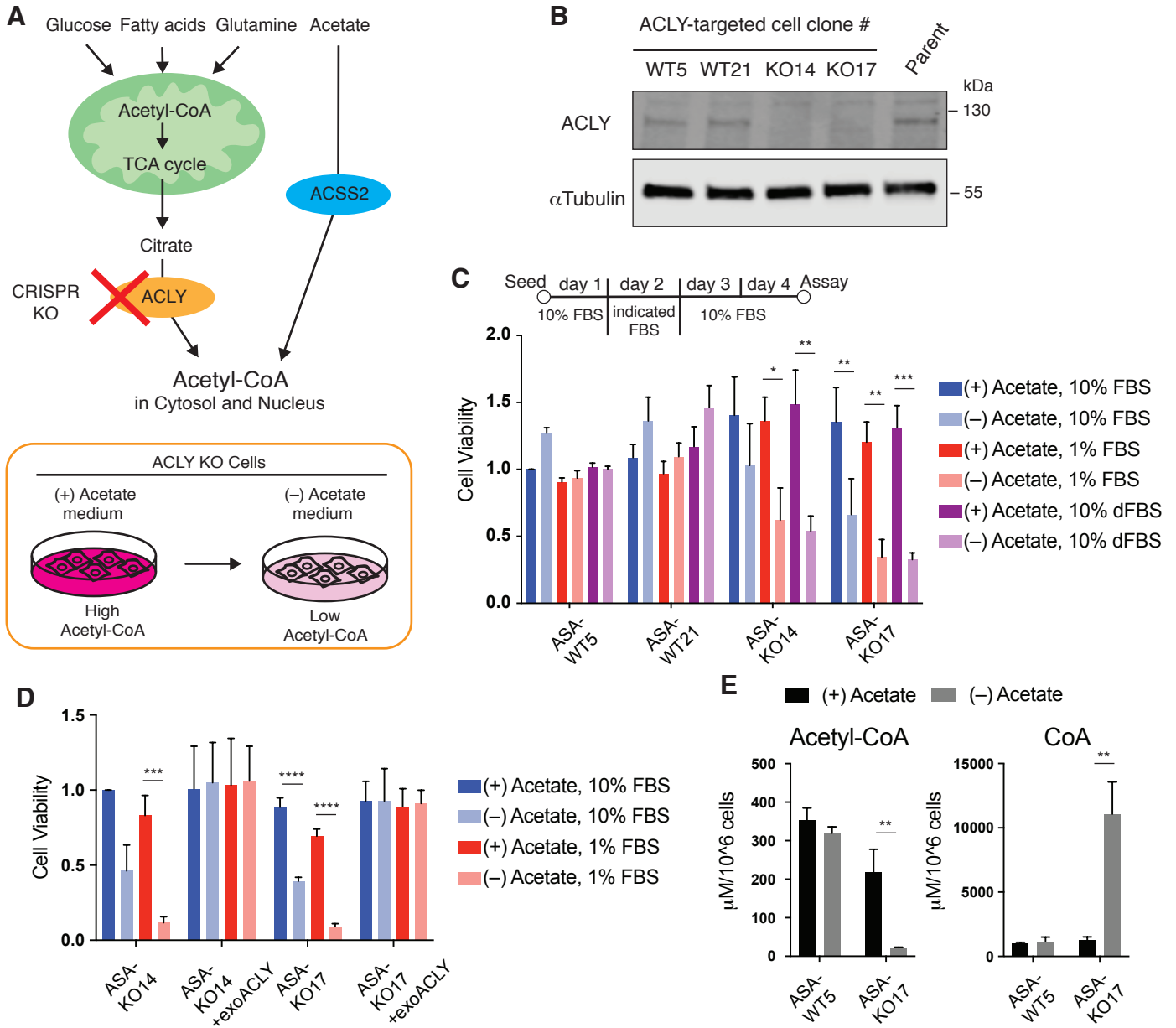
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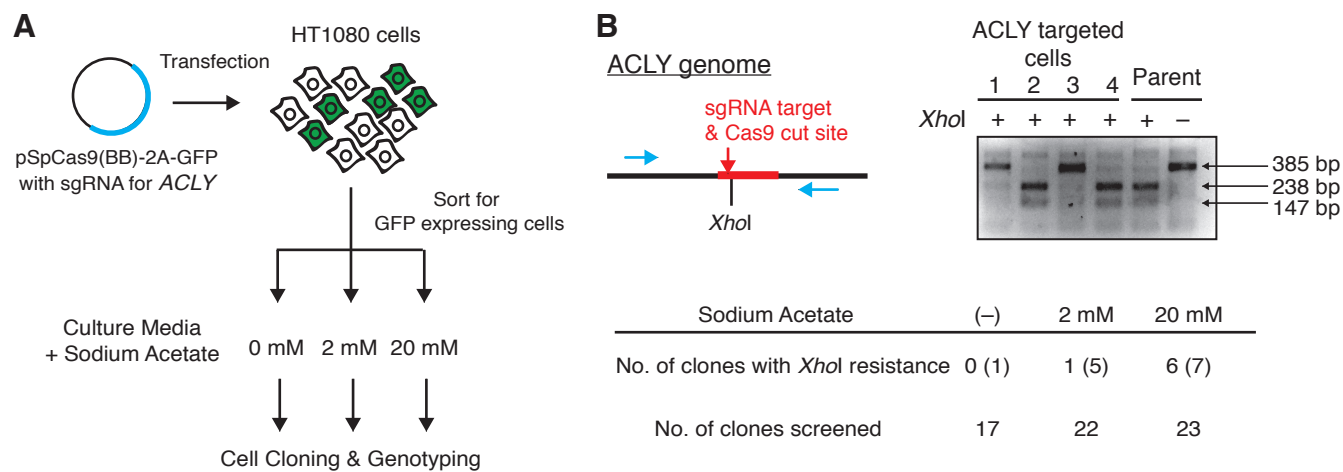
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14
15





C

ACLY Exon 6

aattctggccagttttatctccggccttcaatttctacgaggacttgacttcacctacctcgagatcaatccccttg
 Cas9
 ↓
 XhoI

ACLY KO14

aattctggccagttttatctccggccttcaatttctacgaggacttgacttcacctacctcgagatcaatccccttg
 141 bp insertion

ACLY KO17

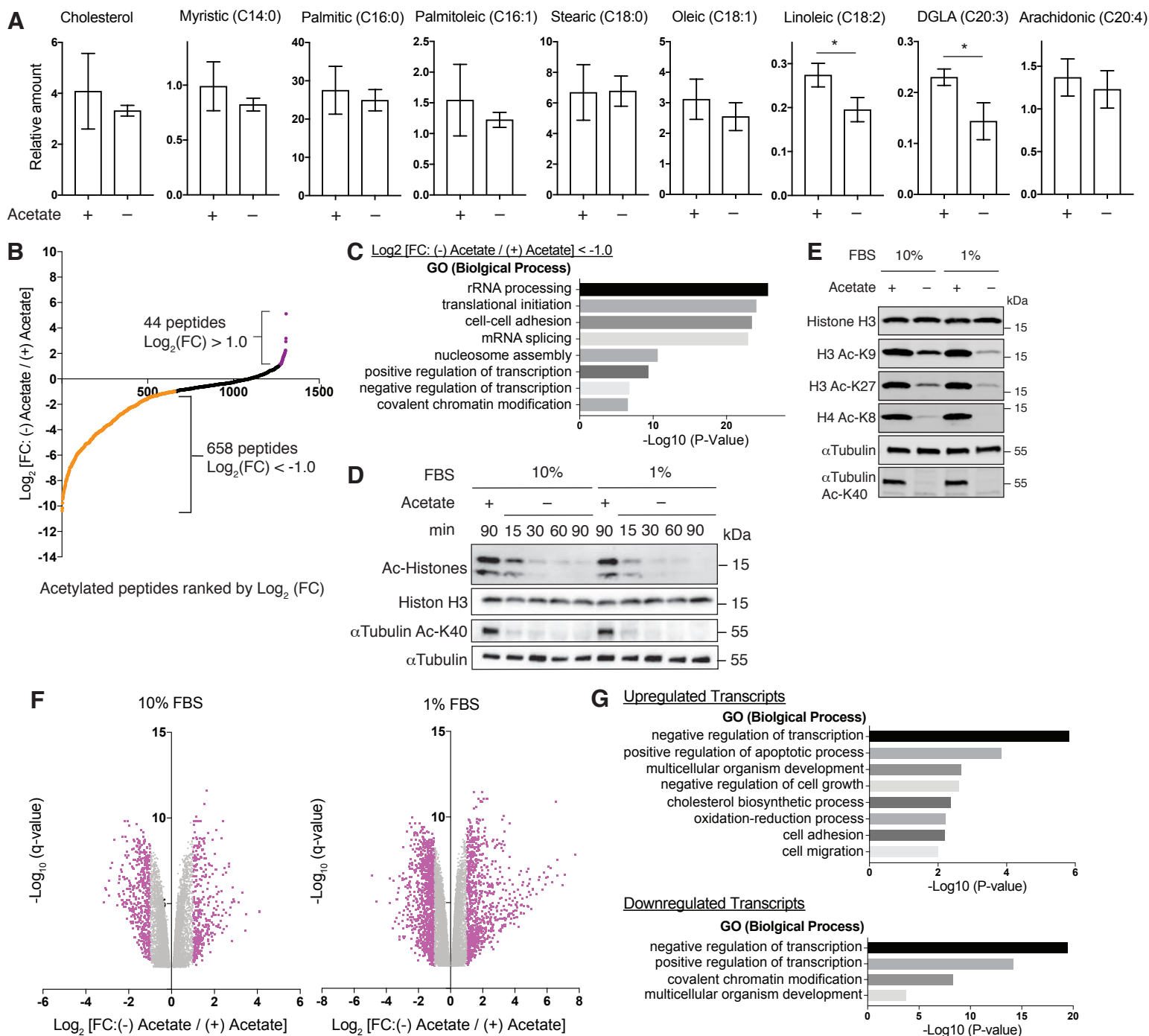
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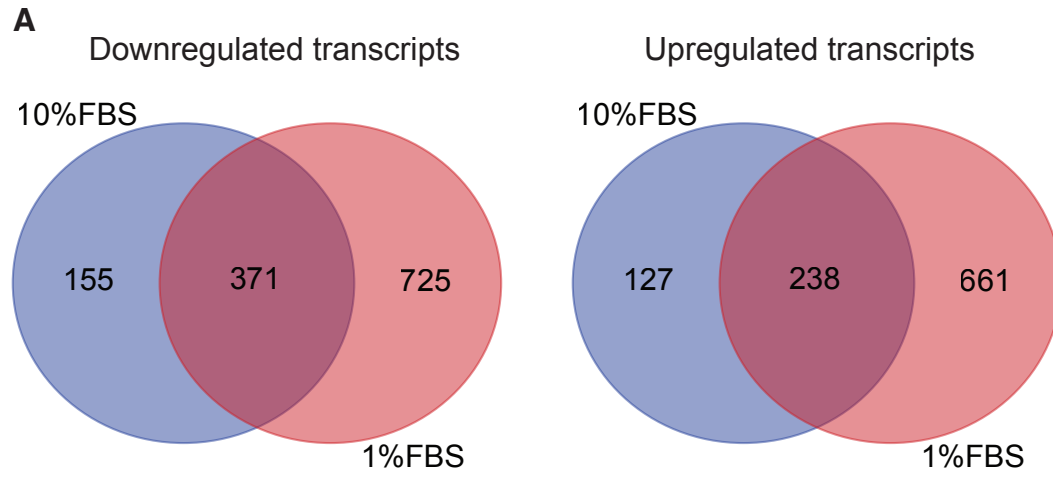
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 31 bp deletion

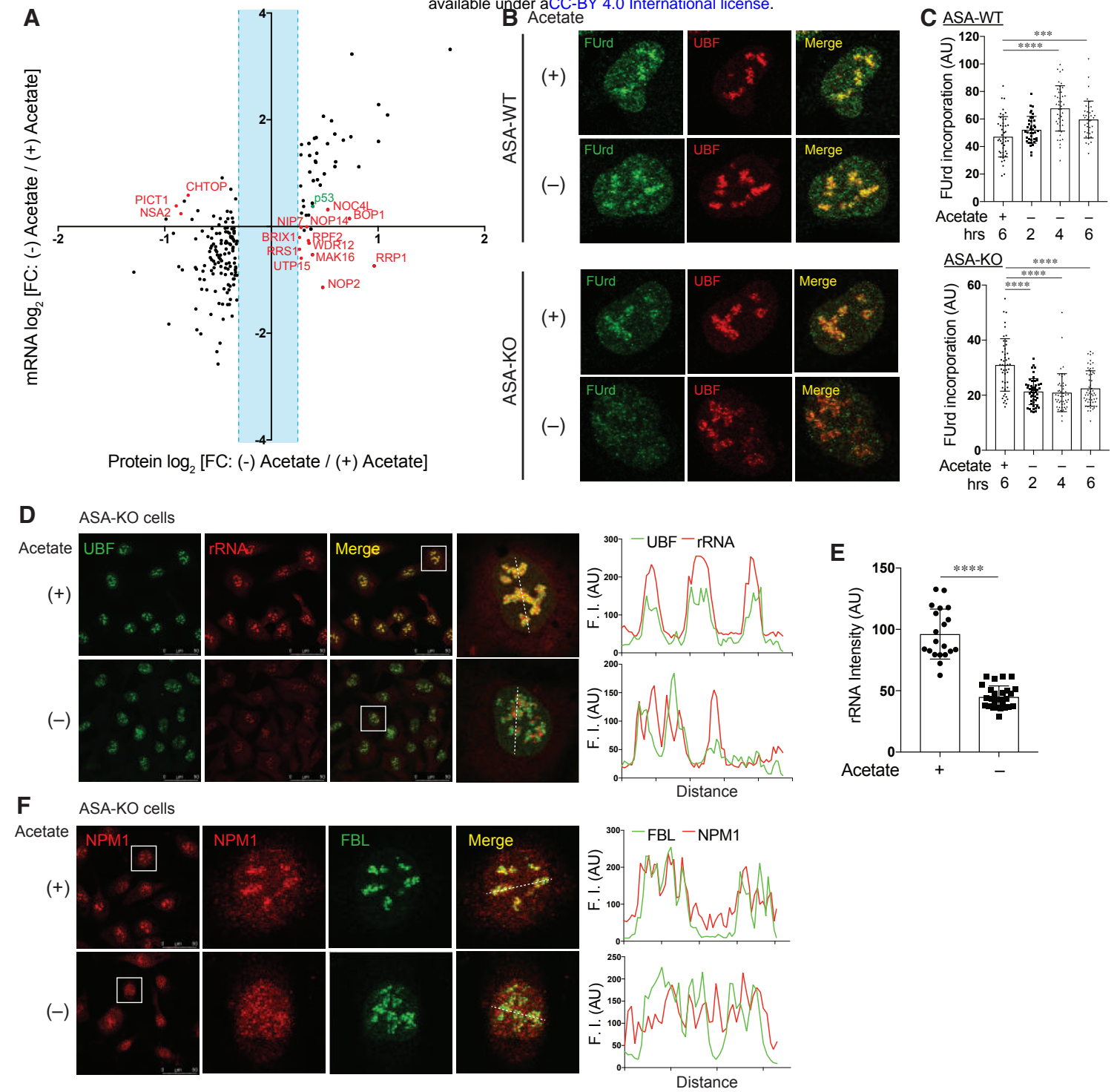
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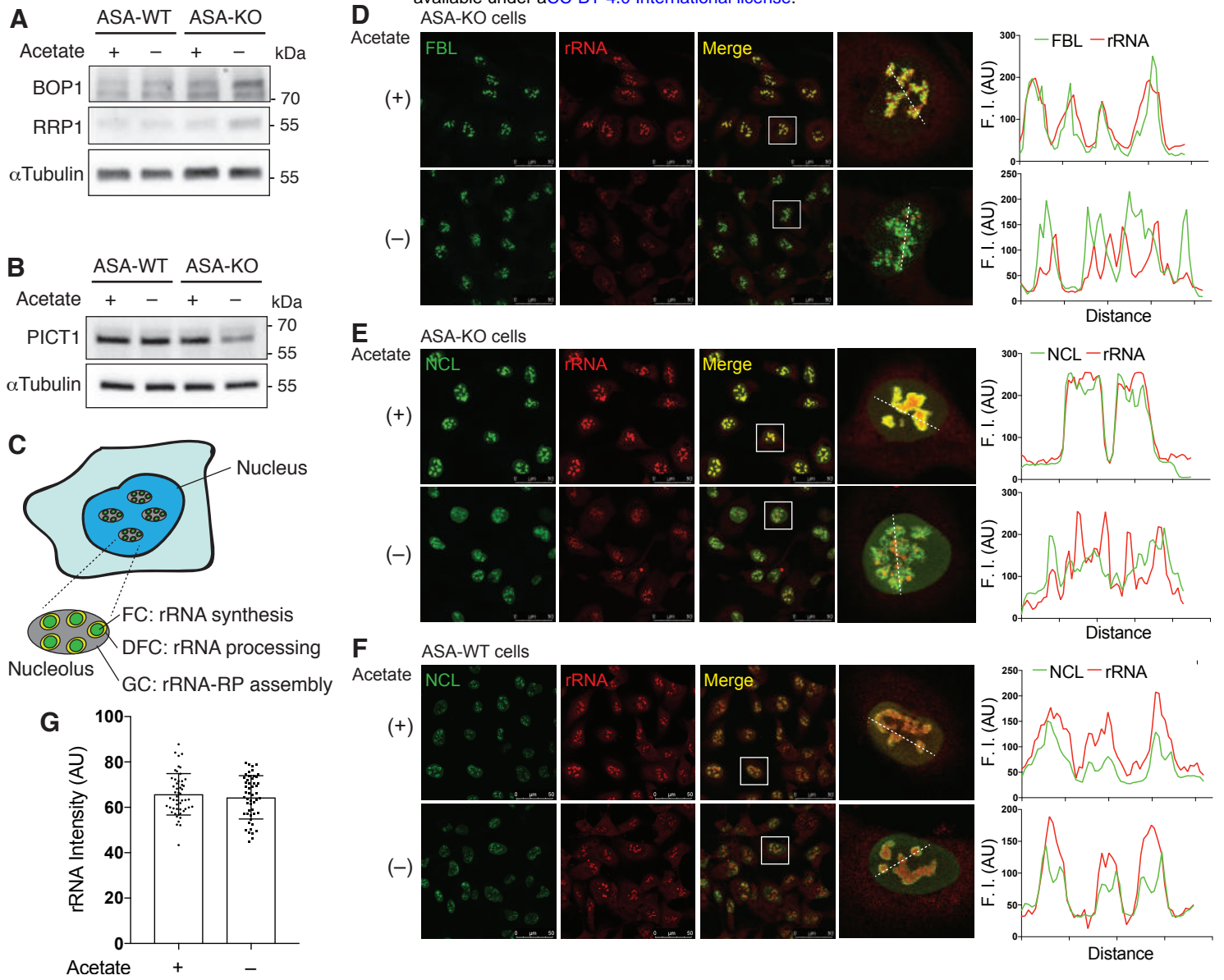
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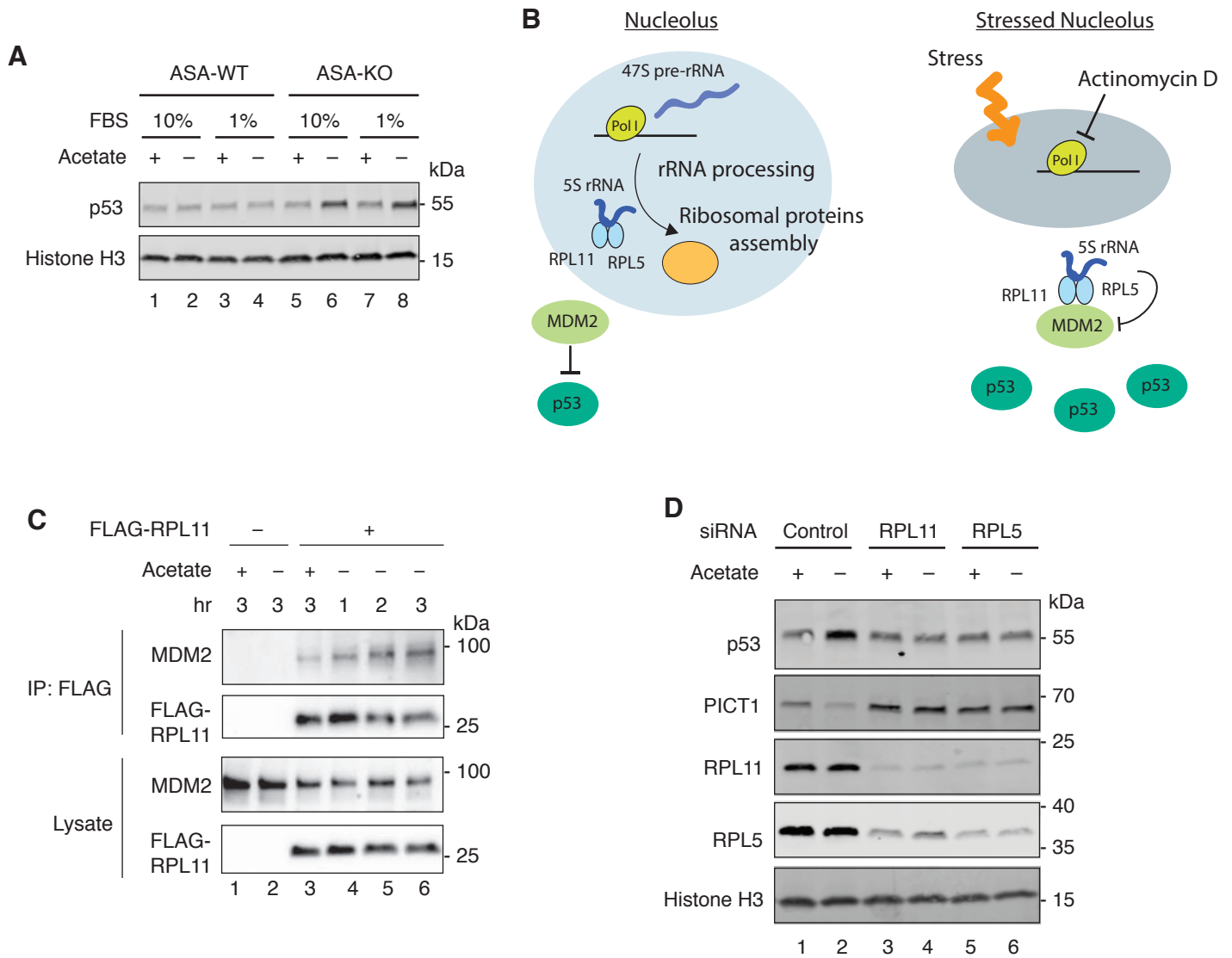
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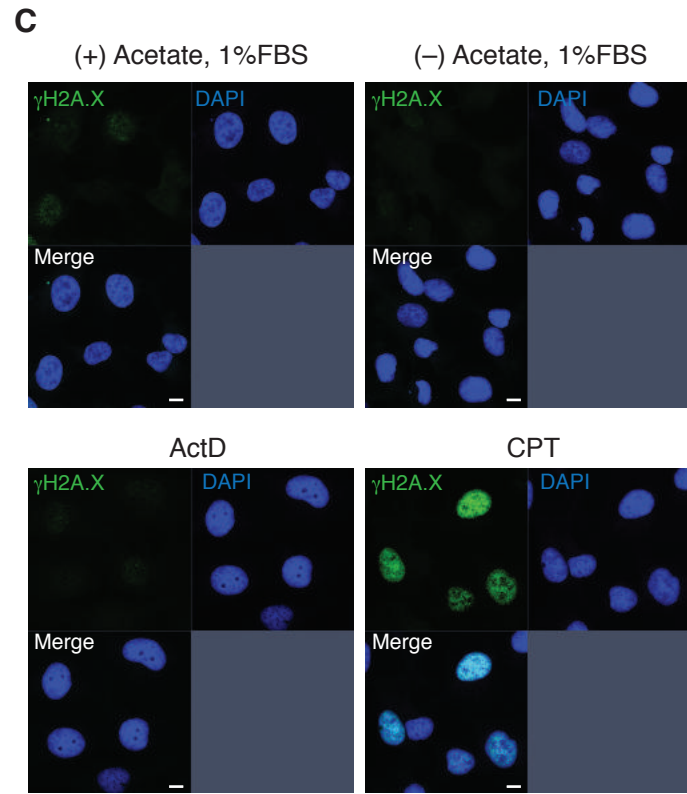
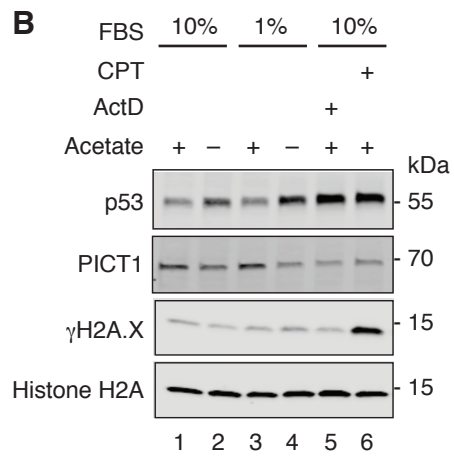
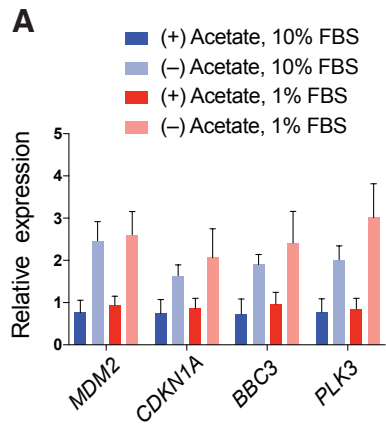


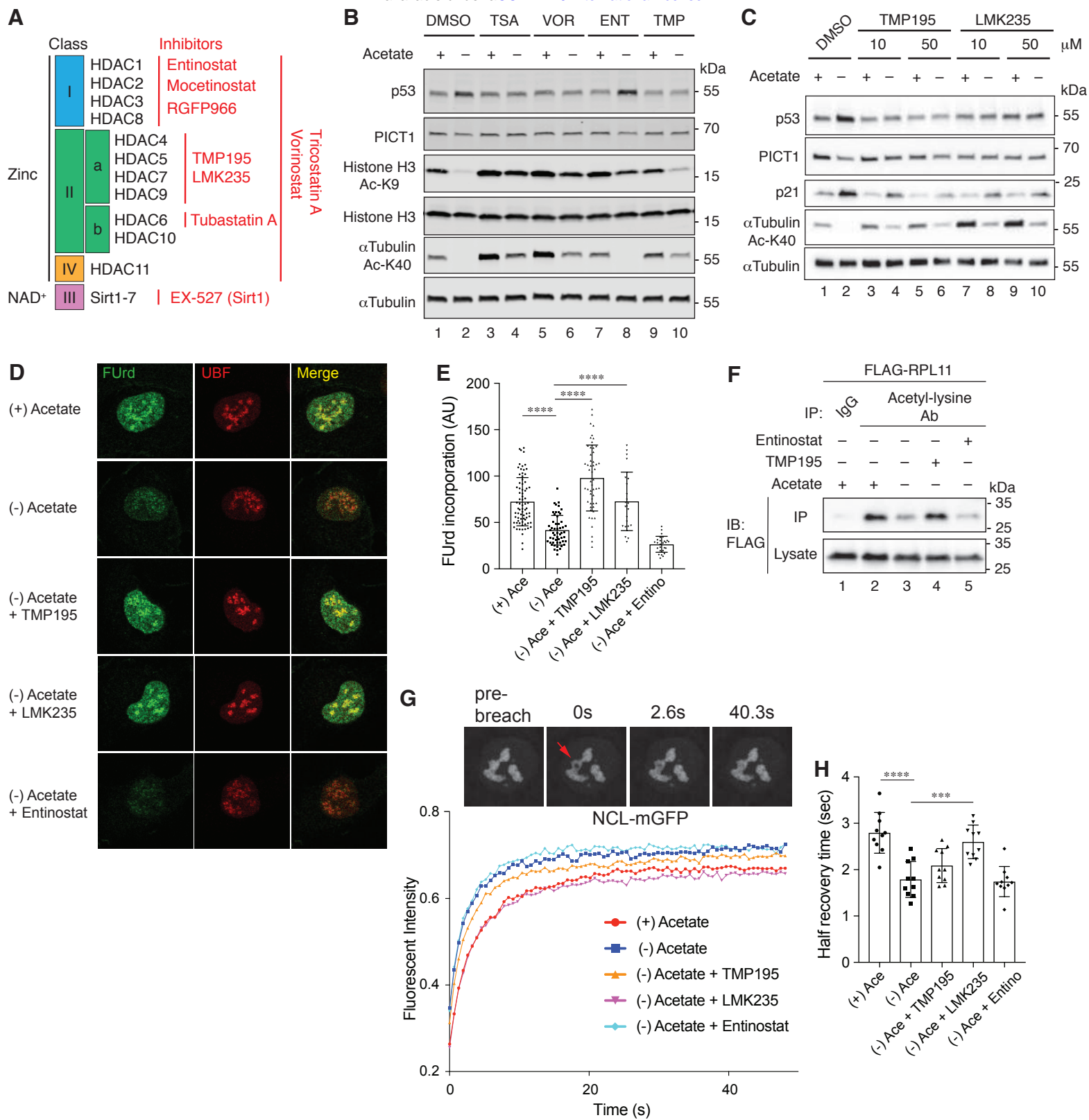


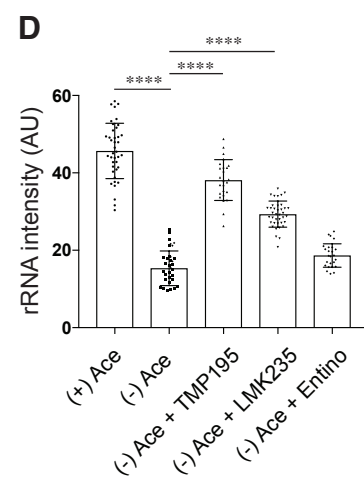
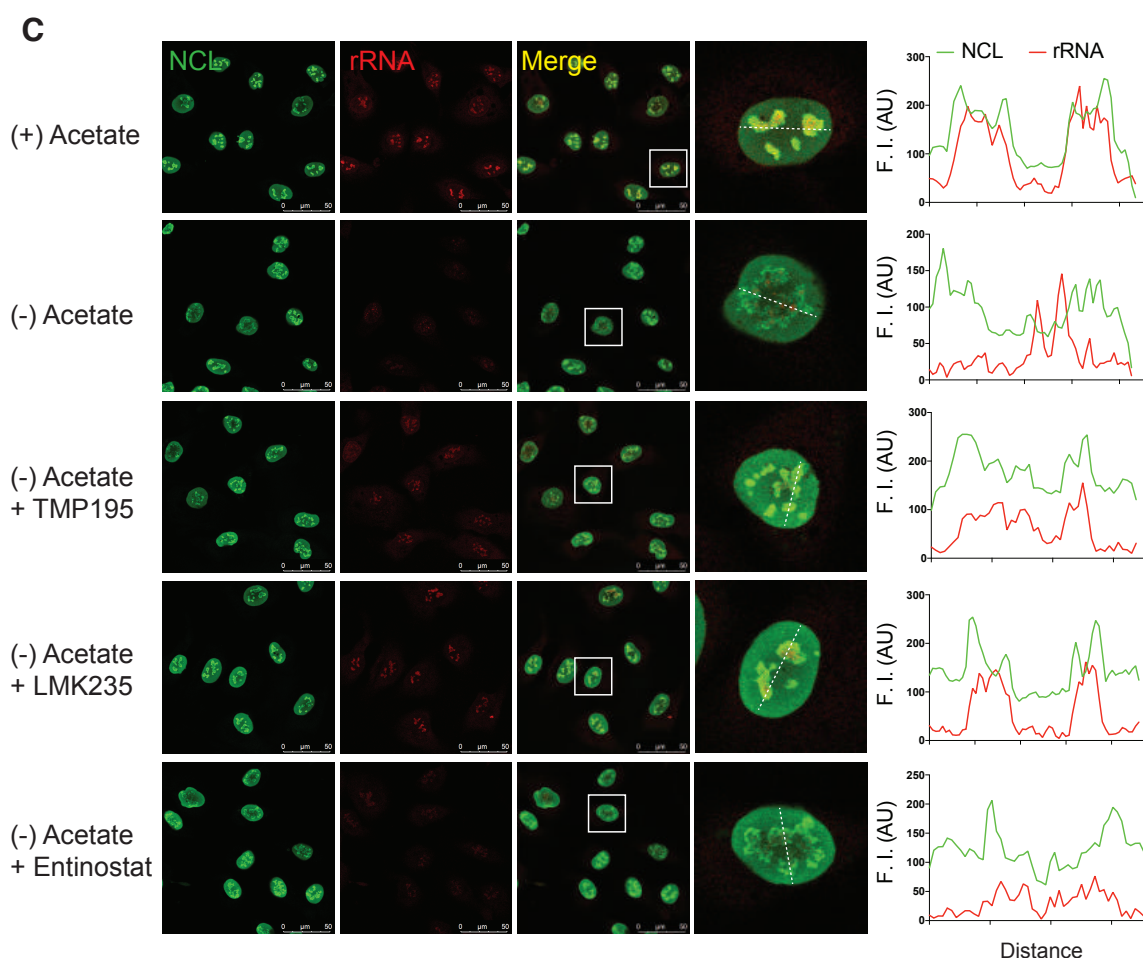
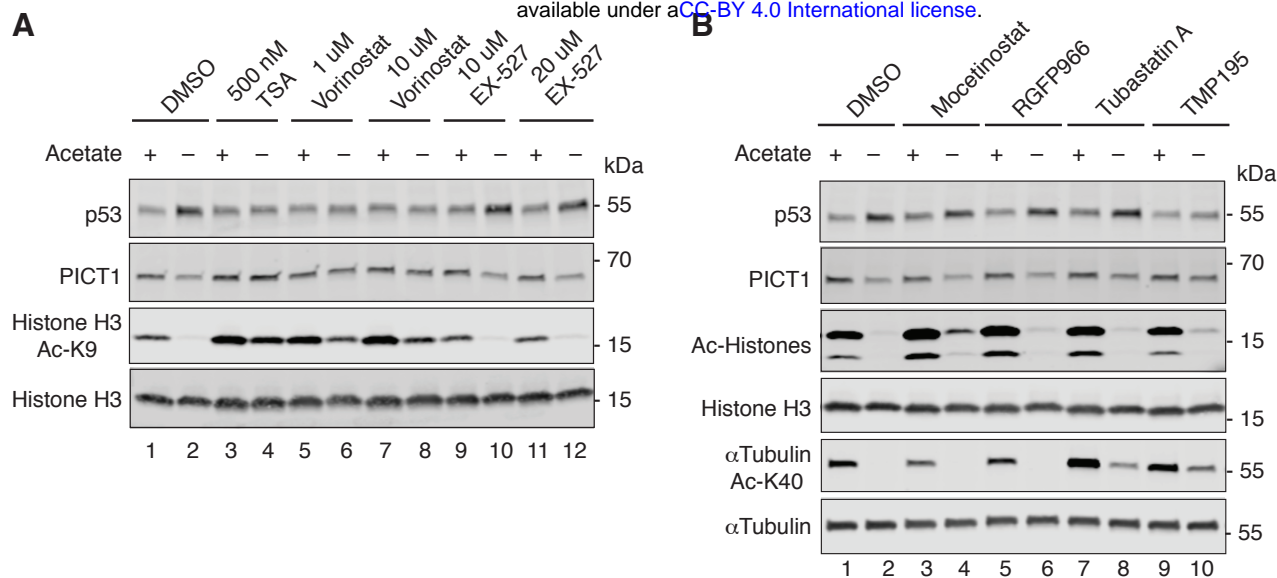








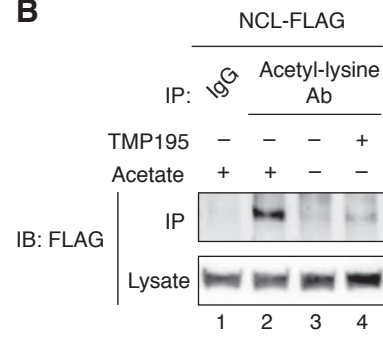


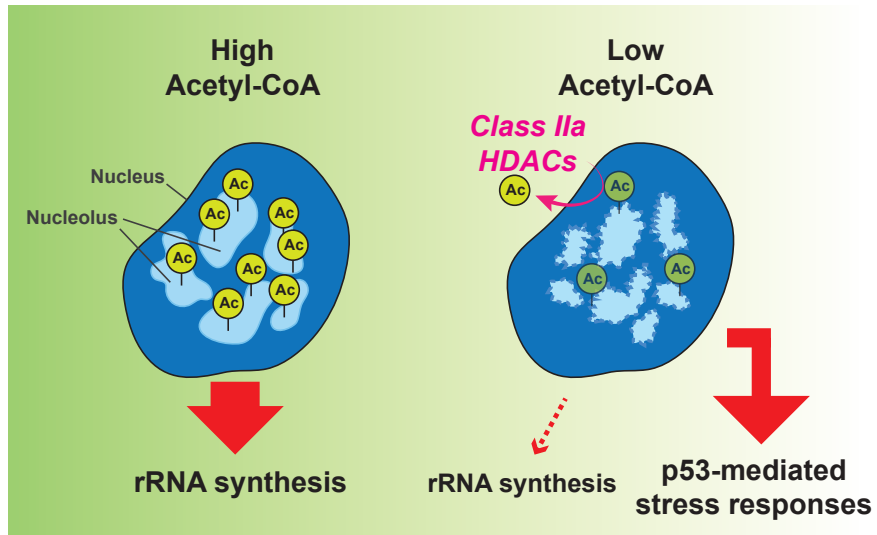


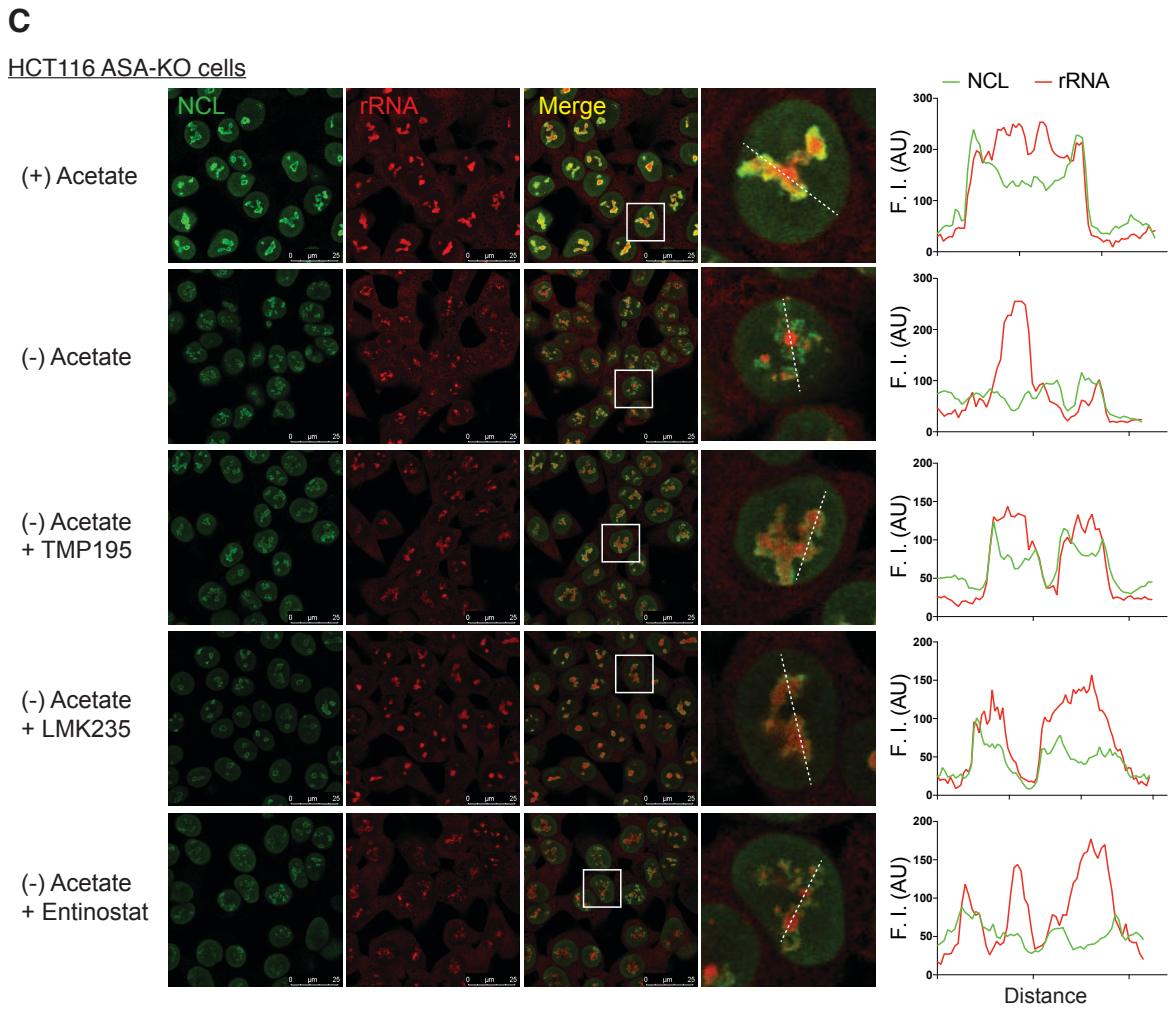
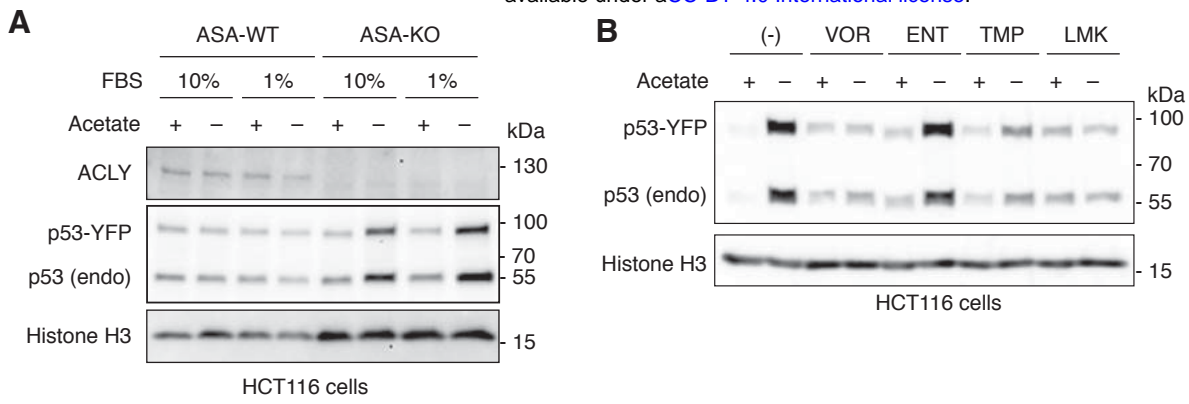
A

Nucleolar Proteins	Acetylation sites affected by Class IIa HDAC inhibitor TMP195
BOP1	K19
MYBBP1A	K1247
NCL	K70, K79, K87, K95, K102, K110, K116, K124, K132
NOLC1	K110, K197, K251
RPL11	K8, K67
RPL14	K187
RPL18A	K136
RPL24	K131
RPL27	K3
RPL29	K63
RPL36A	K97
RPL38	K67
RPL6	K26
RPS3	K62
RRS1	K341
TCOF1	K74, K400, K637, K655, K1273

B







1 **Figure 1 Acetate dependency for acetyl-CoA production in ASA-KO cells**

2 (A) Schematic representation of the pathways for nucleo-cytoplasmic acetyl-CoA production in
3 mammalian cells (Top) and of the acetate-dependent control of acetyl-CoA in ACLY KO cells
4 (bottom).

5 (B) Immunoblotting for ACLY in representative ACLY-targeted cell clones maintained in 20 mM
6 acetate-supplemented media and in parental HT1080 cells. α Tubulin is shown as a loading
7 control.

8 (C) Cell viability assay for ASA-WT and KO cells cultured in the presence or absence of 20 mM
9 acetate in the culture media with indicated FBS conditions over 4 days. On day 2, the original
10 medium containing 10% FBS was replaced for 24 hours with media containing either 10% FBS,
11 1% FBS, or 10% dialyzed FBS (dFBS). Data are shown as mean \pm S.D. (n=3 independent
12 experiments). *P<0.05, **P<0.01, ***P<0.001 (One-way ANOVA followed by Tukey multiple
13 comparisons test).

14 (D) Cell viability assay for ASA-KO cells with or without exogenous expression of ACLY
15 (exoACLY) using the same experimental procedures as in "C". Data are shown as mean \pm S.D.
16 (n=3 independent experiments). ***P<0.001, ****P<0.0001 (One-way ANOVA followed by Tukey
17 multiple comparisons test).

18 (E) Quantification of acetyl-CoA and Coenzyme A (CoA) in cell lysate from ASA-WT or KO cells
19 cultured for 4 hours in media containing 1% FBS with or without 20 mM acetate. Data are shown
20 as mean \pm S.D. (n=3 biological replicates). **P<0.01 (Unpaired t test).

21

22 **Figure 1 -figure supplement 1**

23 (A) Schema of genome editing for *ACLY*. A GFP-tagged sgRNA/Cas9 plasmid targeting *ACLY*
24 was transfected into HT1080 cells. Following cell sorting by flow cytometry for GFP positive
25 transfected cells, cells were recovered in either standard medium (no added acetate) or 2 or 20
26 mM sodium acetate-supplemented media. Recovered cell clones were genotyped to identify

1 genome editing for *ACLY* as in “B”. (B) Schema of genotyping for *ACLY* genome-edited cell clones
2 and representative genotyping results (top left). The sgRNA-targeted and Cas9 cleavage site in
3 *ACLY* exon 6, which contains a single *XhoI* site overlapped with a Cas9 cleavage site, was PCR
4 amplified by flanked primers (blue). *XhoI* digestion of the PCR product (385 bp) provided 238 bp
5 and 147 bp fragments when the *XhoI* site was intact (top right). *XhoI* resistance was indicative of
6 insertion or deletion (in/del) by CRISPR-mediated non-homologous end joining (NHEJ). Numbers
7 of clones which exhibited complete (or partial) *XhoI* resistance and numbers of clones screened
8 were shown for each culture media condition (bottom). (C) The sequence of exon 6 in wildtype
9 *ACLY* and the edited sequences in ASA-KO14 and KO17 cells. The sgRNA target site (red), PAM
10 motif (green), Cas9 cut site (arrow head), *XhoI* site, and detected in/del were indicated. We
11 detected only a single pattern of insertion in ASA-KO14 cells. Note that one allele of ASA-KO17
12 possesses an indel which causes a frame shift mutation replacing amino acids “T-Y-I-E” with “Q”.
13 This might explain the detected *ACLY* peptides in the TMT proteomics using ASA-KO17 cells
14 (Table S3).
15

1 **Figure 2 Alterations in lipid synthesis, acetylation and gene expression following acetyl-**

2 **CoA depletion**

3 (A) Quantification of total cholesterol and fatty acids in ASA-KO cells cultured for 4 hours in 1%
4 FBS containing media with or without 20 mM acetate. Data are shown as mean of 3 biological
5 replicates. *P<0.05 (Unpaired t test).

6 (B) Plot of acetylated peptides ranked by log₂ fold changes 90 minutes after acetate removal in
7 ASA-KO cells. Values are from Table S1, Column B (mean of two independent technical
8 replicates).

9 (C) Gene ontology (GO) analysis for putative deacetylated peptides identified in the acetylome
10 analysis. Enriched representative Biological Process are shown.

11 (D) Immunoblotting for the indicated acetylated proteins in ASA-KO cells cultured in 10% or 1%
12 FBS containing media with or without 20 mM acetate for the indicated times. Acetylated (Ac)
13 histones were detected using an anti-acetyl-lysine antibody.

14 (E) Immunoblotting for the designated acetylated and total protein level in ASA-KO cells cultured
15 in 10% or 1% FBS containing media with or without acetate for 4 hours.

16 (F) Volcano plots for mRNA expression changes 4 hours after acetate removal in ASA-KO cells
17 cultured in 10% or 1% FBS containing media. Data shown represents the mean of 3 biological
18 replicates. Values are from Table S2 and genes with P<0.05 are shown.

19 (G) Gene ontology (GO) analysis for downregulated transcripts (log₂ FC [(-) Acetate / (+) Acetate]
20 < -1.0. q<0.05) and upregulated transcripts (log₂ FC [(-) Acetate / (+) Acetate] >1.0. q<0.05) in the
21 RNA sequencing with the 1% FBS condition. Enriched representative Biological Process are
22 shown.

23

24 **Figure 2 -figure supplement 1**

1 (A) Venn diagram for downregulated transcripts (\log_2 FC [(-) Acetate / (+) Acetate] < -1.0. $q < 0.05$)
2 and upregulated transcripts (\log_2 FC [(-) Acetate / (+) Acetate] > -1.0. $q < 0.05$) in the RNA
3 sequencing with the 10% and 1% FBS conditions.

4

5

1 **Figure 3 Functional and structural remodeling of the nucleolus by acetyl-CoA depletion**

2 (A) Scatter plot for log₂ fold changes (FC) in proteins (X-axis) and in mRNA (Y-axis) 4 hours after
3 acetate removal in ASA-KO cells. Proteins with log₂ fold changes more than 0.264 or less than -
4 0.322 are shown. Proteins that are known to localize in the nucleolus are highlighted in red and
5 p53 shown in green.

6 (B) FUrd incorporation assay in ASA-WT or ASA-KO cells cultured in 1% FBS containing media
7 with or without 20 mM acetate over 6 hours. Representative nuclear images immunostained for
8 FUrd and UBF are shown.

9 (C) Quantification of the mean fluorescent intensity of the FUrd signal per nucleus. The
10 microscopic images of the FUrd incorporation assay performed as in (B) were utilized for the
11 quantification. Data are shown as mean ± S.D. (n=39-55 cells per condition). ***P<0.001,
12 ****P<0.0001 (One-way ANOVA followed by Tukey multiple comparisons test). AU, arbitrary unit.

13 (D) Immunostaining for UBF along with rRNA dye staining in ASA-KO cells cultured in 1% FBS
14 containing media with or without acetate for 4 hours. Magnified nuclear images (surrounded by a
15 white square) are shown. Line profiles for indicated fluorescent intensities (F. I) determined along
16 the white dashed lines are shown to the right.

17 (E) Quantification of the mean fluorescent intensity of the rRNA signal per nucleus in (D). Data
18 are shown as mean ± S.D. [n=20 or 27 cells for (+) Acetate or (-) Acetate, respectively].
19 ****P<0.0001 (Unpaired t test).

20 (F) Immunostaining for NPM1 and FBL in ASA-KO cells cultured in 1% FBS containing media
21 with or without acetate for 4 hours. Magnified nuclear images (surrounded by a white square) are
22 shown. Line profiles for indicated fluorescent intensities (F. I) determined along the white dashed
23 lines are shown to the right.

24

25 **Figure 3 -figure supplement 1**

1 (A-B) Immunoblotting for nucleolar protein BOP1 and RRP1 (A) and PICT1 (B) in ASA-WT and
2 ASA-KO cells cultured in 10% or 1% FBS containing media with or without acetate for 4 hours.
3 α Tubulin was used as a loading control.
4 (C) Schematic representation of the nucleolus consisting of fibrillar center (FC; green), dense
5 fibrillar component (DFC; yellow) and granular component (GC; gray).
6 (D-F) Immunostaining for FBL (D) and NCL (E and F) along with rRNA staining in ASA-KO (E and
7 E) and ASK-WT (F) cells cultured in 1% FBS containing media with or without acetate for 4 hours.
8 Magnified nuclear images (surrounded by a white square) are shown. Line profiles for indicated
9 fluorescent intensities (F. I) determined along the white dashed lines are shown to the right. AU,
10 arbitrary unit.
11 (G) Quantification of the mean fluorescent intensity of the rRNA signal per nucleus in (F). Data
12 are shown as mean \pm S.D. (n=51 cells per condition).
13
14

1 **Figure 4 Ribosomal protein-dependent p53 activation by acetyl-CoA depletion**

2 (A) Immunoblotting for p53 in ASA-WT and ASA-KO cells cultured in 10% or 1% FBS containing
3 media with or without acetate for 4 hours. Histone H3 is shown as a loading control.

4 (B) Schematic representation of the nucleolus as an organelle for ribosomal biogenesis (left) and
5 the stressed nucleolus that induces p53 stabilization through the inhibitory interaction between
6 the 5S rRNA-RPL11-RPL5 and MDM2.

7 (C) Immunoprecipitation with FLAG-RPL11 and Immunoblotting for MDM2 and FLAG-RPL11 in
8 ASA-KO cells cultured in 1% FBS containing media with or without acetate for the indicated hours.

9 (D) Immunoblotting for p53 levels in ASA-KO cells pretreated with indicated siRNAs and cultured
10 in 1% FBS containing media with or without acetate for 4 hours. Histone H3 is shown as a loading
11 control.

12

13 **Figure 4-figure supplement 1**

14 (A) Relative mRNA expressions for p53 target genes selected from the RNA sequencing in Table
15 S2. Data are shown as mean \pm S.D. (n=3 biological replicates).

16 (B) Immunoblotting for p53, PICT1, γ H2A.X and Histone H2A in ASA-KO cells cultured in 10% or
17 1% FBS containing media with or without acetate for 4 hours, or treated with 5 nM ActD or 1 μ M
18 Camptothecin (CPT), a DNA damage inducer, for 4 hours.

19 (C) Immunostaining for γ H2A.X in ASA-KO cells cultured in the same conditions as in (A).

20

21

1 **Figure 5 Class IIa HDAC activity is required for acetyl-CoA-induced nucleolar alterations.**
2 (A) Schematic representation of mammalian HDAC family members and their inhibitors (red).
3 (B) Immunoblotting for the indicated proteins in ASA-KO cells cultured in 1% FBS containing
4 media with or without acetate, and in the presence or absence of indicated HDAC inhibitors for 4
5 hours. Following concentrations of HDAC inhibitors were used: 500 nM Triconstatin A, 10 μ M
6 Vorinostat, 70 μ M Entinostat, and 50 μ M TMP195.
7 (C) Immunoblotting for the indicated proteins in ASA-KO cells cultured in 1% FBS containing
8 media with or without acetate, and in the presence or absence of indicated class IIa HDAC
9 inhibitors for 4 hours.
10 (D) FUrd incorporation assay in ASA-KO cells cultured in 1% FBS conditions with or without
11 acetate and in the presence or absence of indicated HDAC inhibitors for 4 hours.
12 Representative nuclear images (surrounded by a white square) immunostained for FUrd and
13 UBF are shown.
14 (E) Quantification of the mean fluorescent intensity of the FUrd signal per nucleus in (E). Data are
15 shown as mean \pm S.D. (n=23-74 cells per condition). ****P<0.0001 (One-way ANOVA followed
16 by Tukey multiple comparisons test). AU, arbitrary unit.
17 (F) Immunoprecipitation with an acetyl-lysine motif antibody and immunoblotting for FLAG-RPL11
18 in ASA-KO cells cultured in 1% FBS containing media with or without acetate, and in the presence
19 or absence of 50 μ M TMP195 or 50 μ M M Entinostat for 90 minutes.
20 (G) FRAP of NCL-mGFP in ASA-KO cells cultured in 1% FBS containing media with or without
21 acetate, and in the presence or absence of 50 μ M TMP195, 10 μ M LMK235 or 50 μ M Entinostat
22 for 90 minutes. Data are shown as mean (n=10 independent cells from two independent
23 experiments). Representative nucleolar images expressing NCL-mGFP before and after
24 photobleaching are shown on the top. The red allow indicates the ROI for photobleaching.

1 (H) Half recovery times (sec) obtained from the FRAP curves in “G”. Data are shown as mean ±
2 S.D. (n=10 independent cells). ***P<0.001 ****P<0.0001 (One-way ANOVA followed by Tukey
3 multiple comparisons test).

4

5 **Figure 5-figure supplement 1**

6 (A) Immunoblotting for the indicated proteins in ASA-KO cells cultured in 1% FBS containing
7 media with or without acetate, and in the presence or absence of indicated HDAC inhibitors for 4
8 hours.

9 (B) Immunoblotting for indicated proteins in ASA-KO cells cultured in 1% FBS containing media
10 with or without acetate, and in the presence or absence of indicated HDAC inhibitors (10 μM) for
11 4 hours.

12 (C) Immunostaining for NCL along with rRNA staining in ASA-KO cells cultured in 1% FBS
13 containing media with or without acetate and in the presence or absence of indicated HDAC
14 inhibitors (50 μM TMP195, 10 μM LMK or 50 μM Entinostat) for 4 hours. Representative nuclear
15 images (surrounded by a white square) are shown. Line profiles for indicated fluorescent
16 intensities (F. I) determined along the white dashed lines are shown to the right. AU, arbitrary unit.

17 (D) Quantification of the mean fluorescent intensity of the RNA signal per nucleus in (C). Data are
18 shown as mean ± S.D. (n=29-53 cells per condition). ****P<0.0001 (One-way ANOVA followed
19 by Tukey multiple comparisons test). AU, arbitrary unit.

20

21 **Figure 5-figure supplement 2**

22 (A) Selected putative acetylated nucleolar proteins from Table S1 and their acetylation sites that
23 are sensitive to TMP195 treatment.

- 1 (B) Immunoprecipitation with anti-acetylated-lysine antibody and immunoblotting for NCL-
- 2 3xFLAG in ASA-KO cells cultured in 1% FBS containing media with or without acetate and in the
- 3 presence or absence of 50 μ M TMP195 for 90 minutes.

1 **Figure 6**

2 Model for the nucleolar responses upon nucleo-cytoplasmic acetyl-CoA fluctuations. See the main
3 text for detail.

4

5 **Figure 6-figure supplement 1**

6 (A) Immunoblotting for p53 in HCT116 p53-YFP ASA-WT and ASA-KO cells cultured in 10% or
7 1% FBS containing media with or without acetate for 4 hours. Histone H3 is shown as a loading
8 control.

9 (B) Immunoblotting for the indicated proteins in HCT116 p53-YFP ASA-KO cells cultured in 1%
10 FBS containing media with or without acetate, and in the presence or absence of indicated HDAC
11 inhibitors for 4 hours. Following concentrations of HDAC inhibitors were used: 10 μ M Vorinostat,
12 70 μ M Entinostat, 50 μ M TMP195 and 10 μ M LMK235.

13 (C) Immunostaining for NCL along with rRNA dye staining in ASA-KO cells cultured in 1% FBS
14 containing media with or without acetate and in the presence or absence of indicated HDAC
15 inhibitors (50 μ M TMP195, 10 μ M LMK or 50 μ M Entinostat) for 4 hours. Magnified nuclear images
16 (surrounded by a white square) are shown. Line profiles for indicated fluorescent intensities (F. I)
17 determined along the white dashed lines are shown to the right.