University of California
Santa Cruz

The role of HDAC complexes in cell-cycle gene repression

A dissertation submitted in partial satisfaction of the requirements for the degree of
DOCTOR OF PHILOSOPHY
in
CHEMISTRY

By

Alison Kate Barrett

March 2023

The dissertation of Alison Kate Barrett is approved:

_____
Dr. Seth Rubin, chair

_____
Dr. Carrie Partch

_____
Dr. Doug Kellogg

_____

Peter Biehl
Vice Provost and Dean of Graduate Studies

TABLE OF CONTENTS

LIST OF FIGURES

DEDICATION

This piece is dedicated to my parents Kathy and Tom Barrett. Everything I have, and have accomplished I owe to my parents. I couldn't have asked for a better upbringing and childhood where my scientific endeavors started. From the puzzles at age zero onward, to the shelves of science and engineering books (although I mostly looked at the pictures), to the amazing opportunity to always be outside poking at stuff, I had so much enrichment in my environment that no doubt fostered my curious nature. Thanks for putting up with me all these years. Love you guys more than all the things put together and more than that.

ACKNOWLEDGMENTS

I also have thanks to spread all around the Rubin Lab. In the Rubin Lab, we study the cell cycle, but we also have an amazing culture due to Seth. It has always felt like a big family with humor, teasing, and a ton of support. Thanks to Seth, who allowed me to be my whacky self, allowed me to fail (over and over), and helped me grow into the baller scientist I most certainly am. Jokes aside, I do feel quite competent and I wouldn't have had the same opportunities, independence, and top-notch mentorship anywhere else, I'm sure of it.

I want to thank Gerd Müller who has been a good friend, science-partner, and mentor. Some of the most fun I've had was assembly-lining it with Gerd and Manisha with an unreasonable amount of samples. There's something special about being able to work side-by-side with someone, without saying a single word. Thanks for supporting me through my ugly but functional science. I hope you manage to make magic coffee without me someday.

Lastly I want to thank my home-base: Carlos and Worg. From cooking hotdogs with nacho cheese for me in my stress-frenzy before quals, to setting up a luxury office environment for me to write this very thesis within, Carlos has helped me more than he knows. Thanks also for tolerating my financial deficiencies while I try to "cure cancer" for a meager morsal.

Thanks to Worg, who has been the most bestest boy his whole doggie life. His husky coat has undoubtedly made its way into most of my experiments, and his occasional presence in lab has brought most of the Chemistry Dept. joy and comfort over the years.

PREFACE

As our scientific community grows to welcome and include members with more diverse backgrounds, I believe we shall also allow for diverse styles in communicating science, so long as they remain accurate and respectful. If my writing appears to diverge from what one might expect in such a piece, I can assure the reader that the divergence is in voice alone, and not meant as a disservice to the institution, community, or to any other piece of scientific communication that has come before mine. I merely mean to communicate in the most genuine and accurate way I know: with my own voice, and my own thoughts as they are. With this in mind, please enjoy.

ABSTRACT

The role of HDAC complexes in cell-cycle gene repression

Alison Kate Barrett

It is difficult to find a more devastating illness packaged into a single word than “cancer”. Cancer is in fact a large collection of illnesses that share similar behaviors and outcomes. Cancers arise from lesions in cell-cycle regulation that ultimately allow aberrant cell proliferation which may result in tumor growth, angiogenesis, metastasis, and a destructive takeover of surrounding tissues which may inevitably lead to the death of the organism. The challenge to overcoming cancer, and why a “cure” for cancer is a misnomer, is that at the root of this family of diseases is an evolutionarily advantageous mechanism: cell survival and proliferation. Without these traits we ourselves would not survive. With these traits comes the risk of cancer. There is no cure for cancer because we cannot dissolve the very thing that allows us to live as the complex multi-cellular organisms that we are. We can however harness a better understanding of the pathways that regulate cell proliferation and cancer, and pursue therapeutic strategies that are intelligently created with this knowledge. While I touched on both of these areas throughout my years at UCSC, this piece focuses on my contributions toward understand the mechanism of a critical cell-cycle repressor. Chapter 2 describes my research toward understanding the importance of chromatin-modifying complexes in the transcriptional regulation of the cell-cycle.

CHAPTER 1: INTRODUCTION

Cell-cycle regulation overview

The cells making up a multi-cellular organism, such as a human, engage in different programs throughout their individual lifetimes. Cellular programs such as proliferation, differentiation, quiescence, senescence, and apoptosis employ both overlapping and unique molecular complexes to regulate the gene expression that results in the outcome that is each program's namesake. Core to each of these programs is the mitotic cell cycle, and regulators of the cell cycle are thus paramount to achieving a program's outcomes. Regulation of the cell cycle involves tides of gene expression that must be timed appropriately to ensure proper progression through the phases of the cell-cycle, namely G1, S (synthesis, alias DNA replication), G2, and M (mitosis). Two main phase-transitions occur that, in a healthy cell, have tightly regulated checkpoints: G1/S and G2/M. Genes that are involved in the G1/S transition are regulated by both the RB-E2F complex and the MuvB complex "DREAM" (DP, RB-like, E2F And MuvB), while the G2/M transition is regulated by MuvB complexes. These transcription factor complexes may also recruit chromatin modifiers, which has been the subject of my research [1,2].

RB:E2F complexes in repression and activation

The retinoblastoma tumor-suppressor protein RB owes its notoriety in cancer to its inhibitory function(s) of E2F-responsive gene transcription. The E2F family of transcription factors, along with their heterodimer counterparts DP1/2 bind the consensus DNA sequence 5'TTT(C/G)(C/G)CGC3' upstream of the genes they regulate. Although E2Fs appear capable of binding DNA on their own, it has been shown that DP1/2 greatly enhances the affinity[3]. The activator E2Fs (E2F1-3) to which RB preferentially binds include a nuclear localization

sequence (NLS) and are found localized in the nucleus. E2F1-3 are thus found to be in an active state unless sterically inhibited by RB[4], or after having been destabilized by phosphorylation and proteasomal degradation[5]. Freed activator E2Fs are required for S-phase progression, and overexpression of any one activator E2F is sufficient to drive cells out of quiescence and into the cell cycle[4,6].

S-phase genes are expressed through association of the activator E2Fs with their promotors[7]. During early G1, RB inhibits E2F through both direct and indirect methods. With no known enzymatic activity, the most direct method of RB repression is through multidomain interactions between RB and E2F-DP, which function to sterically inhibit E2F's transactivation domain from initiating gene expression[8,9]. Once this steric inhibition is relieved, E2F:DP is free to promote transcription of its target S-phase genes. The G1/S-phase transition typically occurs after a cascade of events, starting with extracellular cues, drives the cell out of its quiescent state[2]. Regardless of the pathway of origin (e.g. estrogen receptor activation, etc), key cyclin-dependent kinases (CDKs) must become activated by their affiliated cyclins for RB inactivation to occur. E2F-bound RB exists in a hypo-phosphorylated state until CDK4/6:CyclinD and CDK2:CyclinE accrue phosphorylation marks on RB across up to 15 sites, resulting in hyper-phosphorylated RB, which then lacks the ability to bind and inhibit E2F[10].

While the interaction between RB and E2F is required for S-phase repression, RB is thought to additionally aid in S-phase repression through recruitment of histone deacetylase complexes I/II (HDACs I/II)[11,12]. HDACs are canonically involved in gene repression through the removal of activating acetyl marks on histone lysines. Nucleosome architecture is influenced by the acetylation and methylation of histones, as well as their removal. Histones H3 and H4 host the bulk of these marks on their disordered "tails", and certain marks are consistently associated with specific transcriptional outcomes. Most notably are the H3K27ac, H3K9ac and H3K4me3 marks which are associated with gene activation, as well as H3K27me3 and H3K9me2/3 which typically mark repressed genes[13].

The interaction between HDACs and RB occurs through an LxCxE peptide motif and LxCxE-binding site, respectively[11]. While deletion of the LxCxE site in RB has been shown to have no phenotypic affect on growth, development, and viability in mouse models, it does seem to affect chromatin structure and lead to minor de-repression of cell-cycle genes[14–17]. This may indicate that while HDAC complexes seem to interact with RB and affect RB:E2F gene regulation, it is likely a fine-tuning mechanism rather than a core mechanism.

MuvB complexes in repression and activation

While RB exclusively regulates the G1/S set of genes, MuvB regulates both G1/S and G2/M genes[2]. Like RB, MuvB lacks any known enzymatic activity, yet it's presence at promotors is required for full cell-cycle gene repression and entry to quiescence It is comprised of several scaffolding proteins: LIN52, LIN9, LIN54, LIN37, and RBBP4 (alias P48)[18]. LIN9 is regarded as a core scaffold for the rest of the MuvB members, who themselves form the interfaces necessary for MuvB function as both a repressive and activating transcription factor[19,20]. LIN54 binds DNA at the "CHR" (Cell-cycle genes Homology Region) consensus motif 5'TTYRAA3' (where Y is a pyrimidine and R is a purine), which is specific to the G2/M gene-set[1,21–23]. LIN37 and RBBP4 make important contacts at nucleosomes[19,20], and RBBP4 is additionally found in chromatin-modifying complexes and is well-known as a histone-binder[24,25]. LIN52 acts as a bridge to the RB-like pocket proteins p107 and p130 in an LxCxE-dependent manner[2,26,27].

During G0 and early G1, MuvB is incorporated into the DREAM complex, repressing G1/S and G2/M genes[26]. Along with MuvB, DREAM hosts repressive E2F:DP heterodimers that bind to DNA through the same E2F sites as the activators. These repressive E2F:DPs are bound to p107 or p130 in a homologous manner to RB and the activator E2F:DP dimers, and the binding between p107/p130 and LIN52 in turn ties E2F:DP to MuvB[18]. Unlike the activator

E2Fs that are bound by RB, the repressor E2Fs within DREAM, E2F4/5, contain a nuclear export signal that drives them from the nucleus when not bound by p107/p130[4].

Due to the inclusion of E2F:DP and MuvB in DREAM, DREAM binds both the E2F and CHR sites, and thus represses a subset of G1/S genes, as well as the G2/M gene set[2]. When CDK4/6 and CDK2 become activated in G1, p107 and p130 accrue phosphorylation just as RB does, and release the repressor E2F:DP dimer. DREAM is disassembled at this point, and the MuvB subcomplex is free to interact with other partners. During the wave of G1/S transcription, transcription factors B-MYB and FOXM1 are expressed. These factors have been found to interact with MuvB at G2/M genes, which promotes the second wave of cell-cycle transcription during G2/M[2,28].

The mechanism by which DREAM represses cell-cycle genes has not yet been established. There have thus far been two models offered on the topic that are not mutually exclusive, but the degree to which either model accurately describes the mechanism of repression by DREAM has yet to be determined. The most recent model comes from structural studies of DREAM components binding to nucleosomes, as well as positioning data derived from ChIP-seq analysis. Here, it was determined that LIN37 and RBBP4 both play a role in nucleosome binding[19,20], this binding correlates to transcriptional repression, and that the +1 nucleosome appears to be stabilized positionally when bound by MuvB[19]. It is thus a matter of intrigue whether this mechanism is sufficient to account for the total gene repression seen though DREAM activity, or whether this mechanism is supplemented.

Indeed there has been evidence to support another model of DREAM repression, wherein HDAC complexes are recruited by DREAM to the promotor of cell-cycle genes, and the genes are then repressed through the removal of activating acetyl marks[29,30]. Members of HDAC complexes have been seen at cell-cycle promotors through ChIP-seq across may cell lines[31,32], and positional dependencies at promoters have been shown between E2F4:DP1 and a member of some HDAC complexes, SIN3B[33]. Most poignantly was a set of results in serum-starved T98G cells depicting a significant dependency for SIN3B in DREAM-mediated

repression, wherein SIN3B -/- lines lost repression of DREAM target-genes, and interactions between MuvB components and SIN3B were observed through co-immunoprecipitation[29]. Interestingly, Co-IPs persist in the context of a RB/p107/p130 triple knockout cell line, indicating that pocket proteins are indispensable to the observed interaction[29]. This would make sense, since the LxCxE binding site that RB interacts with HDAC complexes through is occupied by LIN52-binding in p107 and p130 in the context of DREAM[26,34]. If this interaction were to take place then, there is likely another interface of DREAM that binds HDAC complexes. One speculation is that this may be a missing role of LIN37.

LIN37 has been shown to be required for DREAM activity, despite DREAM being able to otherwise assemble and bind promotors[27,35]. One possibility is that LIN37 recruits chromatin modifiers, such as HDACs, to the nucleosome. If this were the case, however, the logic would follow that nucleosome positioning by DREAM is insufficient for repression, and that recruitment of other complexes is required for DREAM's repressive activity. With such substantial lingering questions and speculations regarding the mechanism of DREAM-mediated repression, the need for further investigation is clear. In Chapter 2, I explore the possibility of HDAC-recruitment as a general mechanism of DREAM-mediated repression.

SIN3 family in cell-cycle regulation

In mammals, the SIN3 family consists of two paralogues: SIN3A and SIN3B[36–38]. Having no DNA-binding domain or enzymatic activity themselves, SIN3A/B are thought to act as a scaffold to form larger complexes, aiding in the recruitment of HDACs to histones by bridging them to transcription factors[39–41]. HDAC/SIN3 complexes have also been seen to target non-histone proteins such as p53, where the deacetylase activity of HDAC1 stabilizes p53 through degradation avoidance[42]. With an amino acid identity of only 63% in humans, the two SIN3 family members have both overlapping and unique functions, but both have roles tied to development and the cell cycle[38,40,43–46]. SIN3B is typically seen in repressive contexts and is

particularly important for terminal differentiation and permanent gene silencing. SIN3A on the other hand is often seen at sites of active transcription but has also been shown to function as a co-repressor[30,33,38,39,47–49]. The genetic knockout of either isoform is embryonic lethal in mice but through differing pathways, again highlighting their independent roles in cell-cycle regulation. While Sin3A knockout leads to defects in cell survival, Sin3B knockout correlates with improper cell differentiation[47,48,50]. Despite these differences, both members are seen through chromatin-immunoprecipitation at many cell-cycle promoters across many cell types[31,32].

With so many disparate pieces of evidence supporting a model wherein the recruitment of chromatin-modifying complexes such as HDACs is a mechanism of repression for both RB:E2F and DREAM targets, the question begged at this point is whether this is a core mechanism that can be generalized, or whether HDAC involvement is circumstantial and fine-tuning. This chapter describes the research undertook to answer this question. Given that loss of LIN37 completely abrogates DREAM activity[27,35], if SIN3B/HDAC are required for repression by DREAM, I would expect loss of SIN3B to phenocopy a loss of LIN37. It has additionally been shown that double genetic knockout of LIN37 and RB results in a total loss of cell-cycle repression and entry to quiescence is barred[35]. I would further expect that a SIN3B/RB double knockout would phenocopy a LIN37/RB double knockout if SIN3B is involved in a core mechanism of DREAM-mediated repression. I start this chapter, by testing this hypothesis. I expand the research to several cell lines to establish whether certain outcomes are context-specific, as previous literature has been limited to one or two cell-lines per study at best.

Conclusion

The ultimate importance of the work we do in this field is the foundational knowledge that eventually provides the basis for intelligently design strategies to better manage human

health and disease. In the field of cell-cycle genetic regulation, there are two main avenues towards gaining control of dysregulation in cancer: de-stabilizing activators, and stabilizing repressors. The chapter that follows touches on the area of repressors. Before any strategic approach could be considered toward stabilizing the repressive DREAM complex, we must understand the mechanism of repression. To date this not understood, and in Chapter 2 I show how we can put to rest the hypothesis of a mechanism that relies on HDAC-recruitment to cell-cycle genes.

CHAPTER 2: The mechanism of DREAM repression is independent of HDAC activity

The work in this chapter was in collaboration with Gerd Müller who took on a mentorship role throughout this time. I use "I" when any element was exclusively intellectually or physically produced from myself, and use "we" when any element had a collaborative nature, whether the work stemmed from a discussion, or whether the science performed was a joint effort.

SIN3B is not essential for p53-dependent cell-cycle gene repression in HCT116 cells

It has previously been reported that the repression of cell-cycle genes by the DREAM complex in serum-starved T98G cells depends on an interaction between DREAM with SIN3B/HDAC[29]. Based on these results, we aimed to address whether DREAM-dependent gene repression generally relies on recruiting SIN3/HDAC complexes in additional cell lines and when cell-cycle arrest or exit is induced by other treatments than serum deprivation. Furthermore, we were interested to analyze whether the loss of DREAM complex repressor function upon knockout of LIN37[27,35] can be phenocopied by loss of SIN3B. To this end, we utilized wild-type HCT116 cells and several isogenic knockout lines (LIN37-/-, RB-/-) that had been previously created[35] to generate cells negative for SIN3B and combinations of SIN3B/LIN37 or SIN3B/RB. To minimize off-target effects, we chose a Cas9-double-nickase approach[51] and targeted regions in exon 3 or exon 4 of the SIN3B gene. By probing SIN3B protein expression in clonal cell lines with two independent antibodies, we confirmed the generation of SIN3B-/-, SIN3B-/-;LIN37-/-, and SIN3B-/-;RB-/- HCT116 cells (Fig. 1A).

To stimulate DREAM formation and cell-cycle gene repression, we activated the p53 pathway by treating wild-type and knockout lines with doxorubicin or with the MDM2 inhibitor Idasanutlin in two independent clones of each knockout line (Fig. 1B, C). As expected, we observed a strong repression of G1/S and G2/M gene mRNA expression in the wild-type HCT116 cells. This effect was impaired in LIN37-/- and RB-/- cells. We observed a stronger

de-repression of G2/M genes in LIN37-/- cells, and G1/S genes in RB-/- cells, consistent with previously published data[27,35]. In contrast, loss of SIN3B did not have any effect on cell-cycle gene repression as a double knockout compared to the respective parental knockout line, and single knockout of SIN3B had either no, or even more repressive effects compared to wild-type. Upon doxorubicin treatment, repression of all analyzed cell-cycle genes did not change significantly, or repression was even slightly, but significantly stronger in SIN3B-/- than in wild-type HCT116 cells. Furthermore, we did not observe any additive effects in cells negative for LIN37 or RB together with SIN3B (Fig. 1B). Cell-cycle gene expression was slightly elevated in SIN3B negative cells after Idasanutlin treatment, however loss of LIN37 and RB resulted in a more pronounced de-repression (Fig. 1C). Importantly, combined loss of SIN3B and RB did not reflect the almost complete loss of repression observed in LIN37-/-;RB-/- cells. To test if there were any measurable changes in the protein level, I analyzed several G2/M and G1/S expressed proteins in untreated and Idasanutlin-treated cells by Western blot (Fig. 1D). p53-dependent repression of G2/M expressed proteins was exclusively lost in LIN37-/-;RB-/- cells. Deficiency for SIN3B did not result in an upregulation of G2/M protein expression – neither when it was knocked out alone or in combination with LIN37 or RB. Comparable effects were observed for the G1/S proteins CDC6 and MCM5, however MCM5 expression after Idasanutlin treatment was upregulated in all cell lines that do not express RB (Fig. 1D). Thus, loss of SIN3B does not influence p53-induced repression of DREAM target genes in HCT116 cells.

Since it was previously reported that SIN3B serves as an adapter protein to recruit HDAC1/2 to the DREAM complex in T98G cells (Bainor et al., 2018), we wondered whether immunoprecipitated DREAM from Idasanutlin-treated HCT116 cells contains HDAC activity. I immunoprecipitated HDAC1, SIN3B, LIN37, and the histone-binding protein RBBP4, which is a component of MuvB as well as several chromatin-modifying complexes including the SIN3/HDAC complex, from extracts of HCT116 wild-type and knockout cells. With a luciferase-based HDAC-activity assay, I measured robust HDAC activity in the eluates from

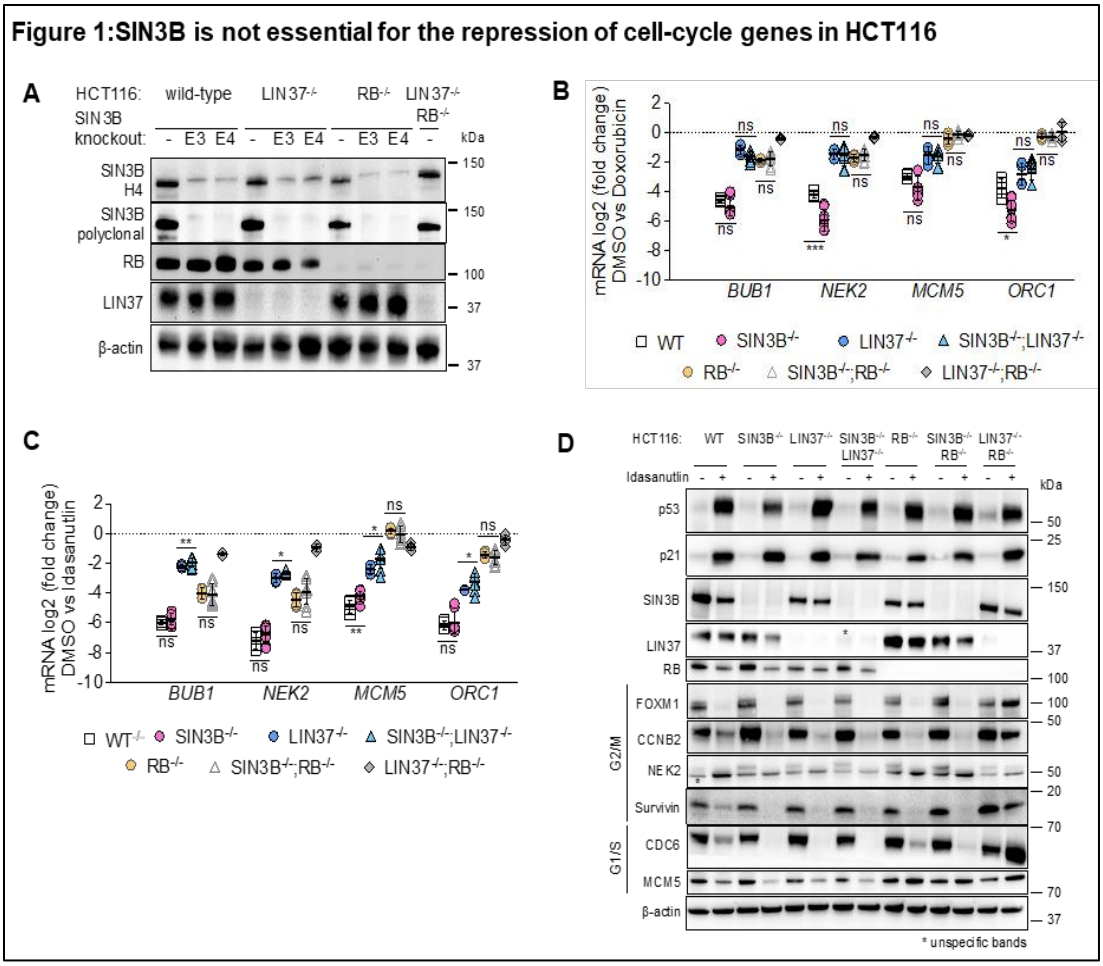


Figure 1: (A) A CRISPR/Cas9-nickase approach was applied to generate cell lines negative for SIN3B. Two pairs of guide RNAs targeting exon 3 (E3) or exon 4 (E4) were selected. Knockout clones were confirmed with antibodies targeting amino acids 172-228 (H4) or amino acids 668-758 (polyclonal). Cells negative for LIN37 or RB were generated from single knockout clones we have described earlier[33]. (B-C) mRNA expression of cell cycle genes was analyzed in wild-type and knockout lines after 48hr treatment with 0.5μM doxorubicin (B), or 5uM Idasanutlin (C). The log2 fold change between untreated and treated cells is shown. Two independent SIN3B-/-; SIN3B-/-;LIN37-/-, and SIN3B-/-;RB-/- clones were compared with wild-type cells, with one LIN37-/-, RB-/-, and LIN37-/-;RB-/- clone. The data set contains two biological replicates, each one measured with two technical replicates. (D) Protein levels in wild-type and knockout clones were probed via Western blot after 48hr Idasanutlin-treatment

HDAC1, SIN3B, and RBBP4 IPs (Fig. 2A). As expected, eluates immunoprecipitated with the SIN3B antibody from extracts of SIN3B-/- cells showed only background activity. The activity of samples immunoprecipitated with the LIN37 antibody from wild-type extracts was higher,

however, HDAC activity did not change in samples precipitated from LIN37-/- or SIN3B-/- cells which shows that the antibody nonspecifically precipitates some HDAC activity independent of LIN37. These results were confirmed by Western blot analyses of the eluates (Fig. 2B). HDAC1 co-precipitated with SIN3B and RBBP4, but not with LIN37. In the LIN37 immunoprecipitations, I detected the MuvB component LIN9, but not HDAC1 or SIN3B. Thus, we fail to observe endogenous DREAM and SIN3B/HDAC interaction in arrested HCT116 cells, contrary to what was shown for serum-starved T98G cells (Bainor et al., 2018).

Several other publications also failed to show an interaction between SIN3B and MuvB complex components in immunoprecipitated samples[18,33,38], yet binding of SIN3 and HDAC proteins to cell-cycle gene promoters has been shown in several cell lines by chromatin-immunoprecipitations[31,32], I wondered whether I could observe a dependence between RB or LIN37 and SIN3B/HDAC for binding at cell-cycle promoters. I thus performed ChIP-qPCR on cross-linked samples from Idasanutlin-arrested wild-type and knockout HCT116 cell lines. The cross-linked sampled were MNase-digested to around 300bp fragments, a size that has previously been shown to capture SIN3B at cell-cycle promoters. Members of DREAM and SIN3/HDAC were chromatin-immunoprecipitated in all samples, and qPCR of both G1/S and G2/M genes was performed (Fig. 2C). While replicates for this experiment are still being performed at the time of this writing, I so far show that SIN3B, SIN3A, and HDAC1 do indeed bind to at least a subset of cell-cycle gene promotors. Aside from the cell-cycle genes measures, a primer set for an unspecific region of chromosome 4 was used as a negative control for cell-cycle related promotor binding. In all cell-cycle genes measured, SIN3B co-immunoprecipitated DNA from promotor regions in wild-type, but not SIN3B-/- cells. LIN37 was consistently bound to the indicated promotors in both wild-type and SIN3B-/- cells. HDAC1 and SIN3A were found to co-immunoprecipitate cell-cycle gene promotors as well, to varying degrees. Additional ChIP experiments are in progress, which include IPs with p130 and several histone-marker specific antibodies. Additionally, another set of ChIP experiments

are in progress which utilize NIH-3T3 cells that have a hetero-allele mutation of the CHR in the CCNB2 (Cyclin B2) promotor[35]. This experiment shall be carried out in the same manner as the HCT-116 ChIP experiment, and I expect these results to further elucidate whether binding of HDACs or SIN3A/B at this promotor depends on the CHR element at all.

With the above results all taken together, we did not find evidence that SIN3B plays a role in cell-cycle gene repression by the DREAM complex in HCT116 cells when cell-cycle arrest is induced by activation of the p53 pathway.

Figure 2: SIN3B and HDAC do not bind to DREAM but are both found at cell cycle promotors

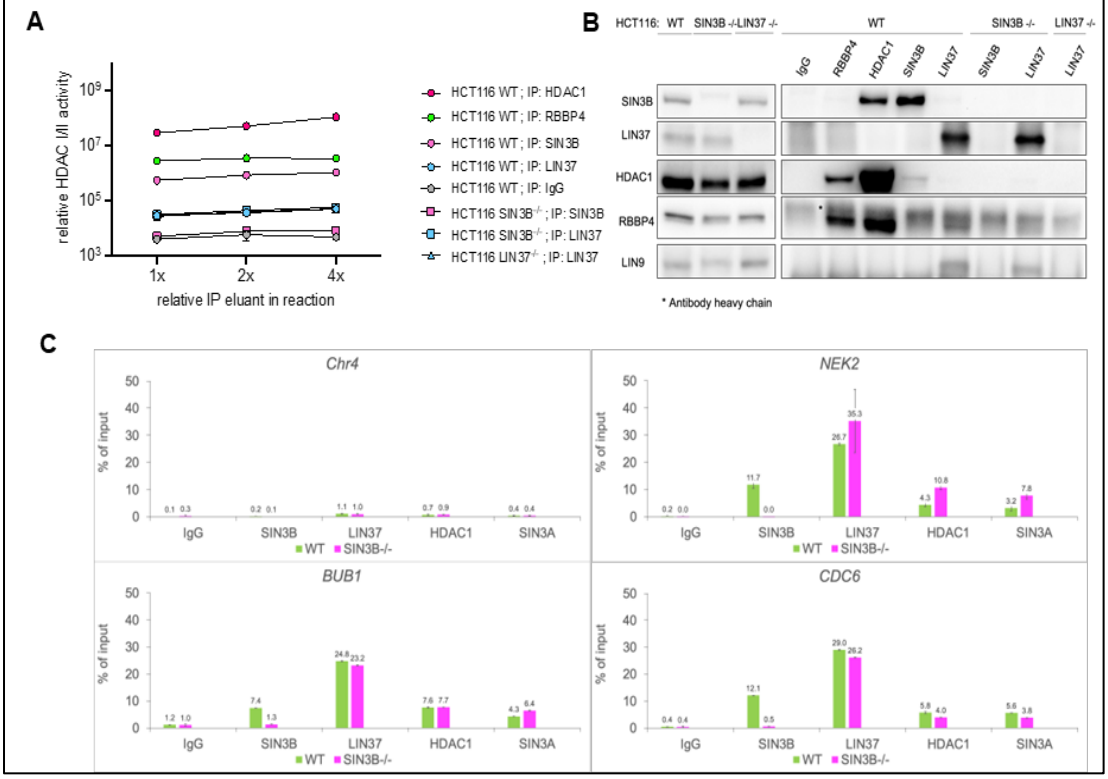


Figure 2. (A) Co-immunoprecipitation was performed in the indicated clones after 48hr Idasanutlin-treatment, with the indicated antibodies. On-bead HDAC activity was assess using HDAC Glo (Promega). Eluates were further assessed for protein content via Western blot (B). (C) Crosslinked cells were subject to chromatin-immunoprecipitation following Mnase digestion and evaluated for precipitated DNA content pertaining to the indicated genes via qPCR.

Loss of SIN3B effects DREAM targets in serum-starved but not Palbociclib-treated T98G

Since we did not observe an influence of SIN3B on the repression of DREAM target genes in HCT116 cells, we next asked whether the reduced DREAM target gene repression in SIN3B-/- T98G cells[29] is phenocopied by the loss of LIN37 in the same cellular system. Our CRISPR-double-nickase approach for generating SIN3B and LIN37 knockouts T98G cells was less efficient than in other lines. This is likely because T98G is a hyperpentaploid cell line averaging 128 chromosomes and multiple copies of chromosome 19 (on average 6) that encodes for both SIN3B and LIN37 and must be targeted to achieve a complete knockout. However, we successfully identified clones that did not express detectable protein levels of SIN3B, LIN37, or both (Fig. 3A). Two clones of each knockout type were serum-starved, and mRNA expression of G1/S and G2/M genes was measured at several time-points after serum deprivation and compared to proliferating cells (Fig. 3C). mRNA levels of all analyzed genes were strongly reduced in starved wild-type cells. In contrast, gene expression was de-repressed in all knockout lines. The upregulation of cell-cycle gene expression in serum-starved SIN3B-/- cells corroborates the results published by Bainor et al [29]. However, these effects were less pronounced than in LIN37-/- or SIN3B-/-;LIN37-/- cells. The upregulation of cell-cycle gene expression in cells negative for SIN3B or LIN37 was also clearly detectable on the protein level (Fig. 2B).

To analyze cell-cycle gene repression in a setting other than serum starvation, we treated the T98G lines with Palbociclib. We chose Palbociclib (as opposed to Idasanutlin) to directly inhibit CDK4/6, since T98G cells do not express wild-type p53. Surprisingly, even though the repression of cell-cycle genes was delayed in SIN3B-/- in comparison to wild-type cells, we did not observe a robust loss of mRNA repression after 48h of Palbociclib treatment. In contrast, loss of LIN37 resulted in a highly significant loss of repression of all measured cell-cycle genes (Fig. 2E). Palbociclib treatment led to comparable repression of DREAM targets

in wild-type and SIN3B-/- cells on the protein level, while increased expression could be observed in LIN37-/- and SIN3B-/-;LIN37-/- cells (Fig. 2D).

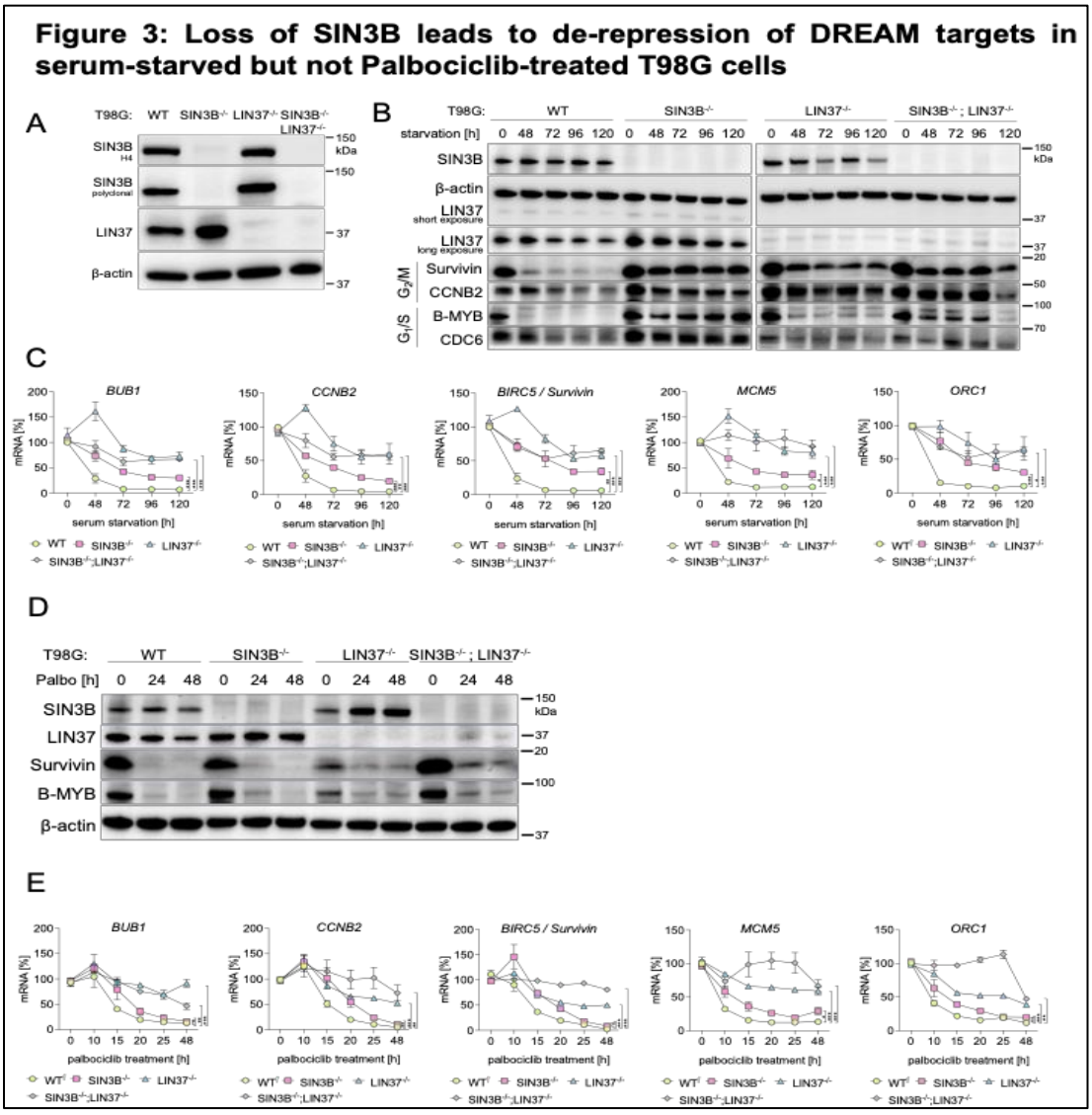


Figure 3. (A) A CRISPR/Cas9-nickase approach to introduce mutations in exon 4 of SIN3B and exon 6 of LIN37 was applied to generate cell lines negative for SIN3B, LIN37, or both. SIN3B knockout clones were confirmed with antibodies targeting amino acids 172-228 (SIN3B-H4) or amino acids 668-758 (SIN3B polyclonal). LIN37 knockout was confirmed with a polyclonal antibody raised against full-length LIN37. (B) protein levels and mRNA expression (C) of cell cycle genes was analyzed in wild-type and knockout lines arrested by serum-starvation over 120hrs. Two independent SIN3B-/-, LIN37-/-, and SIN3B-/-;LIN37-/- clones were compared with two wild-type clones measured with two technical replicates. Similarly, (D) protein levels and mRNA expression (E) of cell cycle genes was analyzed in wild-type and knockout lines arrested by Palbociclib over 48hrs.

I next asked whether addition of Palbociclib could reinforce cell-cycle gene repression in serum-starved T98G knockout lines. To this end, I compared cell-cycle gene expression on mRNA and protein levels in cells that were either serum-starved for 96h or starved for the same period of time but with the addition of Palbociclib for the final 48 hours. As observed before, loss of SIN3B or LIN37 resulted in a loss of repression of cell-cycle genes in starved cells compared to the parental line (Fig. 4A). The addition of Palbociclib increased the repression in the wild-type cells, and the measured genes were repressed to the same or even stronger extent in the SIN3B knockouts. In contrast, addition of Palbociclib to LIN37-negative cells led to only minimal changes in cell-cycle gene expression. Comparable effects were also observed on the protein level (Fig. 4B). I interpreted this to mean that the observed reduction in cell-cycle gene repression in serum-starved SIN3B-/- T98G cells is not caused by a loss of DREAM repressor function, but by upstream mechanisms that result in an impaired CDK inhibition, which prevents the formation of DREAM and RB/E2F complexes. These defects can be bypassed by directly inhibiting CDKs, but only in LIN37-positive cells that can assemble a functional DREAM complex.

To analyze whether endogenous DREAM contains HDAC activity in T98G cells, I immunoprecipitated HDAC1, SIN3B, and LIN37 from serum-starved T98G cells and performed HDAC1/2 activity assays with the eluates. As expected, I detected strong HDAC activity in the samples containing immunoprecipitatedHDAC1 and SIN3B (Fig. 4C). The HDAC activities in eluates precipitated with the LIN37 antibody were comparable between samples obtained from LIN37 positive and negative cell lines, suggesting that the signals are nonspecific. The data obtained from HDAC assays are in line with Western blot results that show a coprecipitation of HDAC1-SIN3B and LIN37-LIN9, but no interaction of MuvB components with SIN3B or HDAC1 (Fig. 4D).

With the possibility that HDAC activity could contribute to the repression of cell-cycle genes independently of DREAM, we asked whether HDAC inhibition in Palbociclib-treated cells would result in a de-repression of cell-cycle genes. We chose the HDAC inhibitors

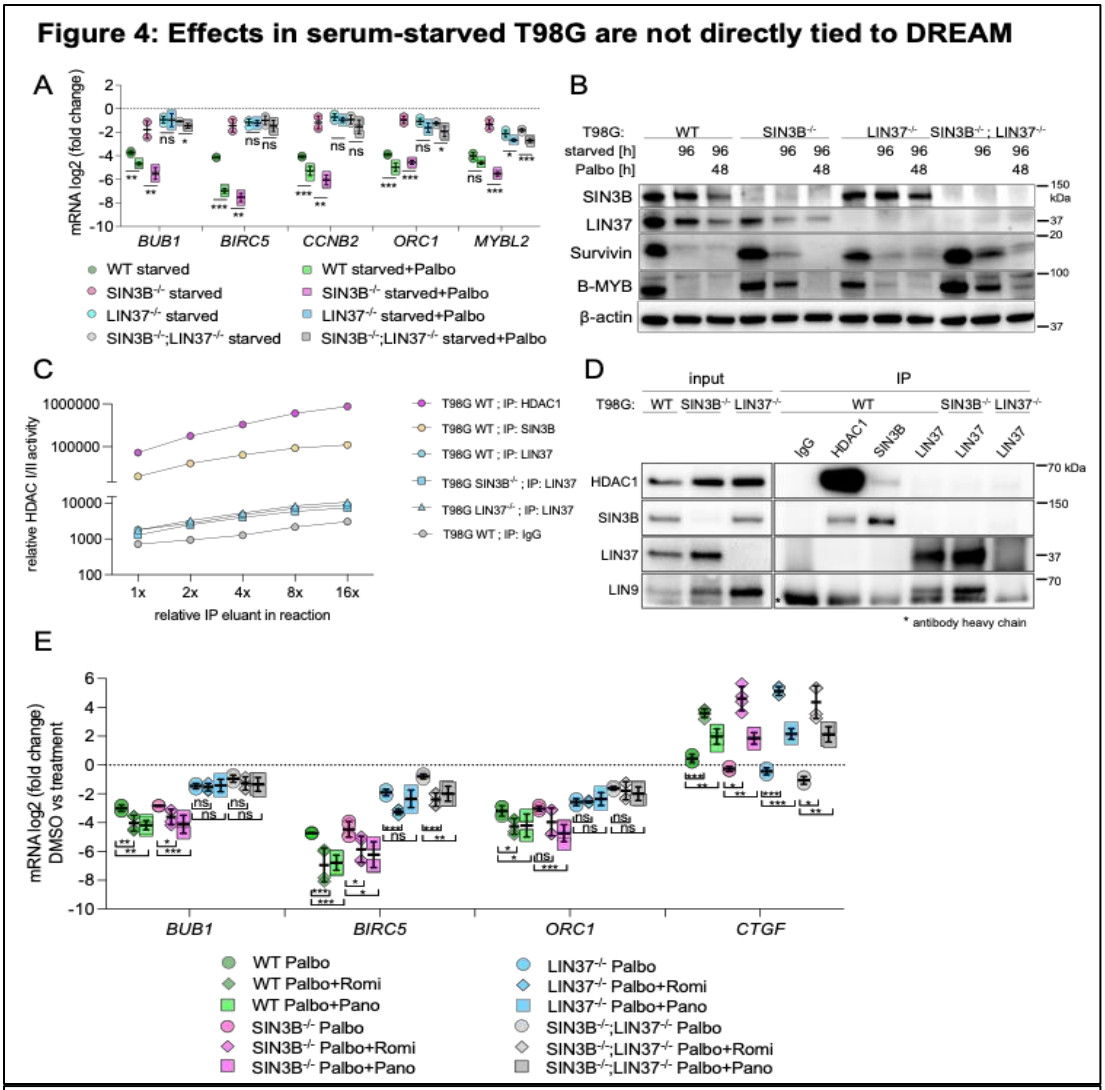


Figure 4. (A) Indicated clones were serum-starved for 96hrs with or without 10uM Palbociclib for the final 48hrs. mRNA was measured with qPCR and compared with untreated wild-type mRNA levels. Samples from Fig. 2F were additionally measured on the protein level (B). (C) Co-immunoprecipitation was performed in the indicated clones after 96hr serum-starvation, with the indicated antibodies. On-bead HDAC activity was assess using HDAC Glo (Promega). Eluates were further assessed for protein content via Western blot (D). (E) The indicated clones were treated with 10uM Palbociclib for 48hrs, and 4nM Romidepson or 20nM Panobinostat for the final 24hrs when indicated. mRNA levels were evaluated through qPCR and compared to untreated wild-type cells.

Romidepsin[52,53], which specifically inhibits HDAC1/2, and the pan-HDAC inhibitor Panobinostat[54]. However, instead of an upregulation, we observed an additional decrease in cell-cycle gene expression in wild-type and SIN3B-/- cells treated with Palbociclib and HDAC inhibitors (Fig. 4E). This is not terribly surprising since HDAC inhibition can result in

upregulation of p21. In LIN37-/- and SIN3B-/-;LIN37-/- cells, HDAC inhibition had either no impact on gene repression, or like the wild-type and SIN3B-/- resulted in even further repression than Palbociclib-only treated cells. Furthermore, we again do not see significant de-repression of cell-cycle genes in SIN3B-/- compared to wild-type, where LIN37-/- and SIN3B-/-;LIN37-/- cells show significant de-repression upon Palbociclib treatment to similar degrees as each other. Lastly, as a positive control we see increased expression of CTGF upon HDAC inhibitor treatment in all T98G cell lines, a gene previously reported to be regulated by HDAC activity[55].

Gene de-repression in serum-starved T98G SIN3B-/- does not generalize to C2C12:

Given that we and others[29] observed a loss of cell-cycle gene repression in serum-starved T98G cells, I wondered whether loss of SIN3B generally inhibits cell-cycle repression in response to serum starvation. Since HCT116 cells cannot efficiently be arrested by serum deprivation, I created Sin3b-negative mouse C2C12 cells (Fig. 5A) and compared cell-cycle gene repression after serum-starvation with wild-type and previously established Lin37-/- cells[27,35]. Starvation for 48 or 72 hours led to repression of G1/S and G2/M genes in the wild-type cells. Loss of Sin3b did not result in de-repression of these genes, and appeared to have slightly stronger repression than wild-type cells. In contrast, all measured genes were significantly de-repressed in serum-starved Lin37-/- C2C12 cells (Fig. 5B). Comparable effects were observed in Idasanutlin-treated C2C12 lines on mRNA (Fig. 5C) and protein (Fig. 5D) levels, in agreement with what we saw in Idasanutlin-treated HCT-116. We conclude that the impaired cell-cycle gene repression observed in serum-starved SIN3B-/- T98G cells is specific to this cell line, and that loss of SIN3B does not generally influence the response of cells to the withdrawal of mitogenic stimuli. Furthermore, we do not have any indication that loss of Sin3b impairs cell-cycle gene repression during reversible cell-cycle arrest in C2C12 cells.

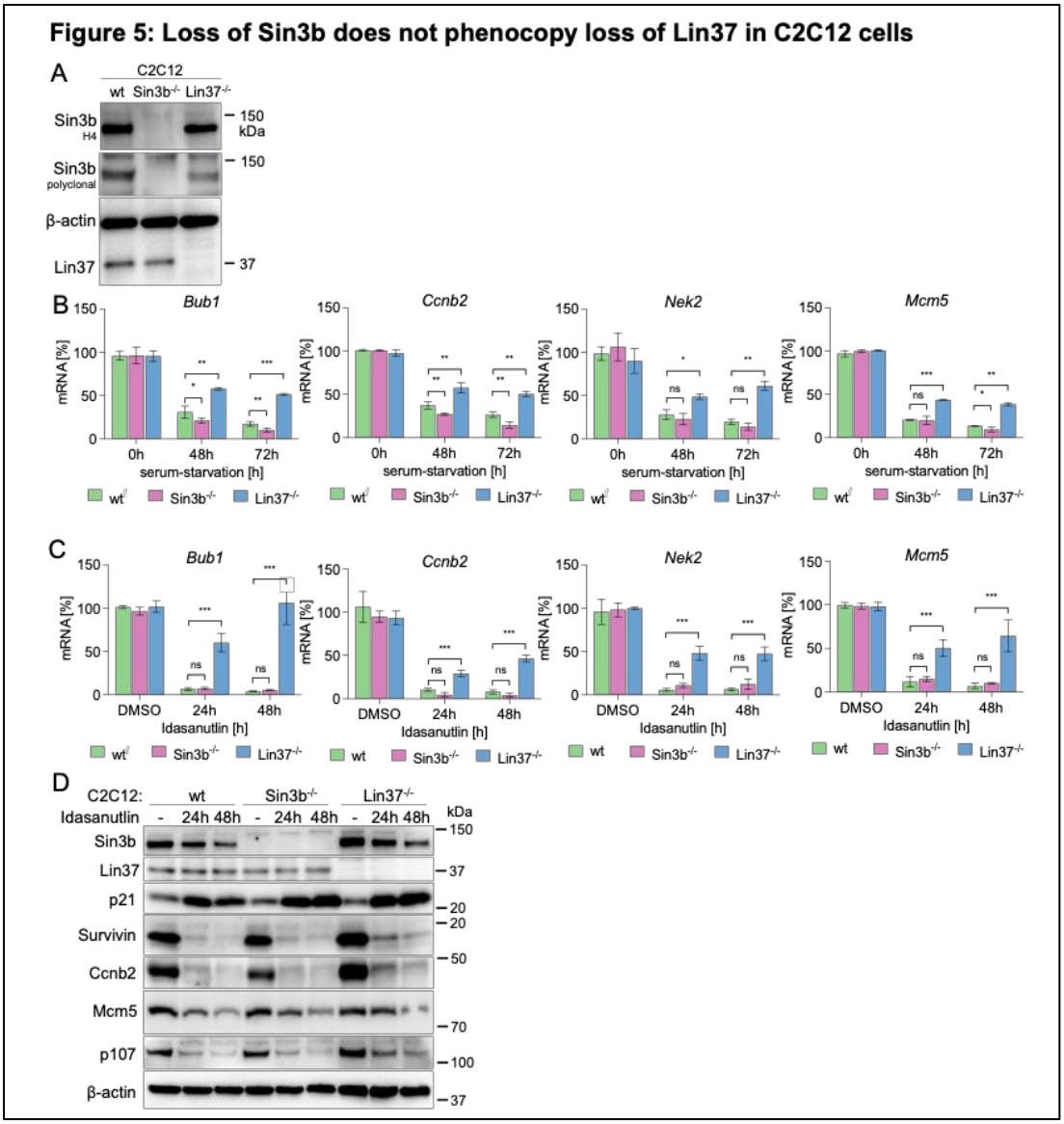


Figure 5. (A) A CRISPR/Cas9 approach was applied to generate cell lines negative for SIN3B, or LIN37. SIN3B knockout clones were confirmed with SIN3B-H4 or SIN3B polyclonal. LIN37 knockout was confirmed with a polyclonal antibody raised against full-length LIN37. (B) mRNA expression of cell cycle genes was analyzed in wild-type and knockout lines arrested by serum-starvation over 48 and 72hrs. Wild-type, SIN3B-/- and LIN37-/- clones were compared with the proliferating wild-type average. Two wild-type, one Lin37-/-, and three SIN3B-/- clones were measures, with two technical replicates each. Similarly, (C) mRNA expression, and protein levels (D) of cell cycle genes was analyzed in wild-type and knockout lines arrested by Idasanutlin for 24 and 48hrs.

Since it has been previously shown that Sin3b occupies cell-cycle gene promoters in differentiated C2C12 cells[33] and has further been shown to contribute to cellular senescence[56–58], we next turned to this context to evaluate whether cell-cycle gene repression is dependent on Sin3b. These experiments are ongoing but preliminary results thus far indicate that the expression of cell-cycle genes is not influenced by HDAC inhibition. Interestingly, when cells are treated with Romidepsin before initiation of differentiation as opposed to during and after, that SIN3B-/- C2C12 cells exclusively lose the ability to differentiate. Tied together, our findings corroborate past research showing the importance of Sin3b for cellular senescence , but this is not directly related to cell-cycle gene regulation.

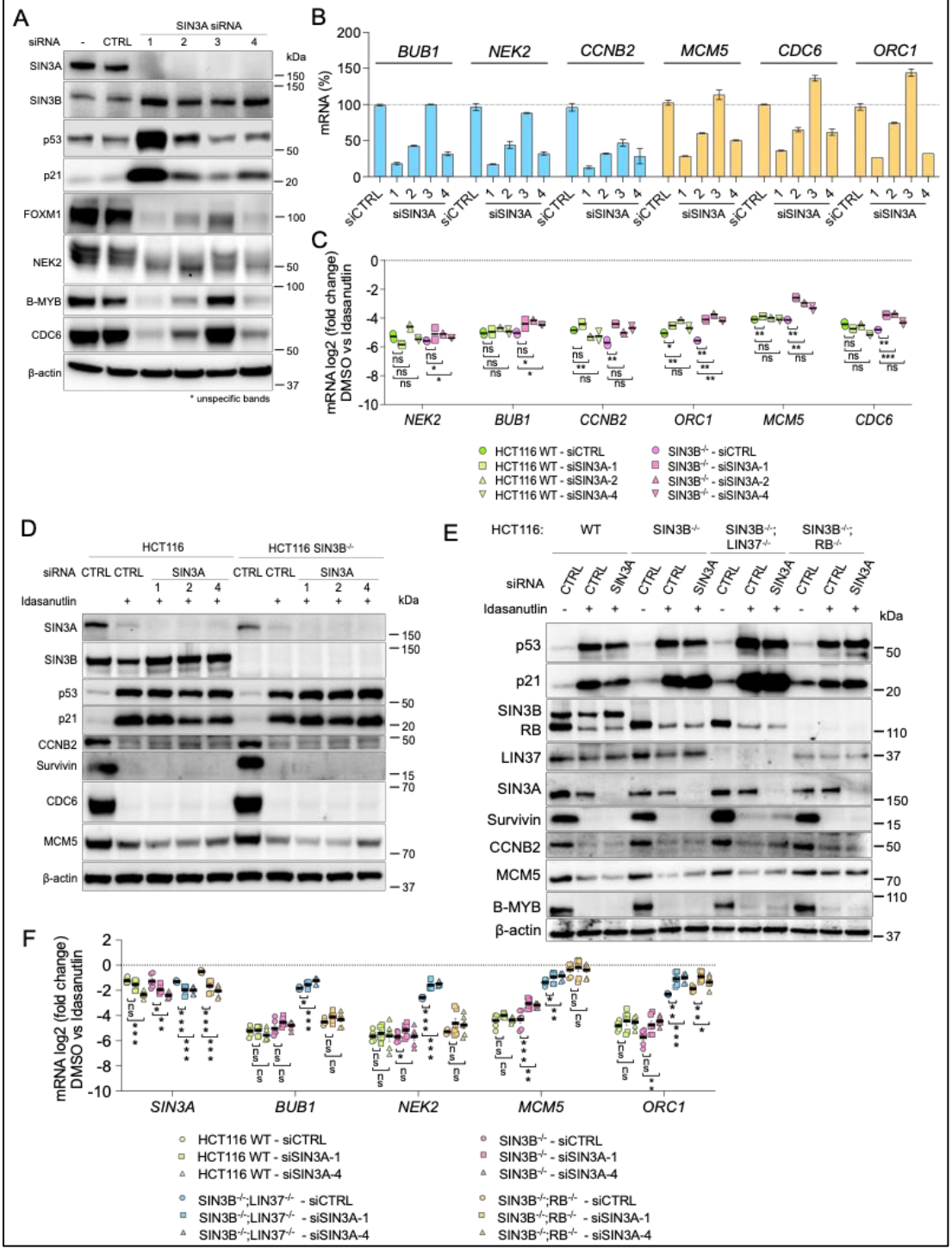
Combined loss of SIN3A/SIN3B de-represses genes independently of DREAM/RB:

Since we did not find a deregulation of DREAM targets in arrested HCT116, we asked whether SIN3A can compensate for the loss of SIN3B in these cells. It has been published before that SIN3A is essential for mouse embryogenesis[47,50] and that loss of SIN3A results in cell-cycle arrest and apoptosis through activation of CDKN1A/p21 in a p53-dependent and -independent manner. Based on data, we refrained from trying to knockout SIN3A and chose an siRNA-based approach instead to reduce the expression of SIN3A. First, we tested the knockdown efficiency of four independent SIN3A siRNAs in proliferating HCT116 cells. All four siRNAs drastically reduced the protein expression of SIN3A, while SIN3B levels, interestingly, were increased (Fig. 6A). Expression of CDKN1A (alias p21) followed the accumulation of p53 and was induced after treatment with all SIN3A siRNAs. A particularly strong expression was observed with siRNAs 1, 2, and 4, while siRNA 3 lead only to a minor increase of p53 and p21 proteins. As expected, cell-cycle protein expression behaved inversely to p21 expression: mitotic and S-phase regulators were repressed upon transfection of SIN3A siRNAs 1, 2, and 4, while we observed only minor differences between cells treated with a non-silencing siRNA or SIN3A siRNA 3. These trends were on the mRNA level as well

(Fig. 6B). We then analyzed whether SIN3A knockdown in Idasanutlin-treated SIN3B+/+ and SIN3B-/- cells influenced repression of cell-cycle genes. Interestingly, SIN3A protein expression was reduced in arrested HCT116 cells without siRNA treatment, indicating already that a compensation mechanism of SIN3A for SIN3B in this context is unlikely. Additionally, siRNA-knockdown decreased SIN3A protein levels still further (Fig. 6D). Protein expression of G2/M and G1/S cell-cycle regulators was strongly repressed upon Idasanutlin treatment in both wild-type and SIN3B-/- cells, and knockdown of SIN3A did not result in a detectable de-repression. Knockdown of SIN3A in wild-type HCT116 cells lead to minor effects regarding the mRNA expression of the analyzed cell-cycle genes, while a combined loss of SIN3B and SIN3A resulted in an upregulation, particularly of G1/S genes (Fig. 6C). To analyze these effects in more detail, we repeated the experiment with the addition of SIN3B-/-;LIN37-/- and SIN3B-/-;RB-/- cells. Here, the increase in cell-cycle gene mRNA expression upon knockdown of SIN3A in SIN3B-/- cells was reproduced, and interestingly, comparable effects were measured in SIN3B-/-;LIN37-/- and SIN3B-/-;RB-/- cells (Fig. 6F). I conclude that these effects are independent of DREAM and RB. On the protein level, I did not detect an increase of cell-cycle gene expression after knockdown or knockout of SIN3A and SIN3B respectively, but I did see de-repression in cells negative for LIN37 or RB (Fig. 6E).

Figure 6. Four SIN3A-targeting siRNAs were tested in HCT116 and analyzed for protein (A) or mRNA expression (B) wild-type and SIN3B -/- HCT116 were transfected with SIN3A siRNA for 48hrs and 5uM Idasanutlin for the final 28hrs. mRNA expression was evaluated through qPCR (C) The log2-fold change is shown compared to untreated wildtype HCT116. Resulting protein levels were analyzed through Western blot (D). (E) This experiment was repeated with the addition of SIN3B-/-;LIN37-/- and SIN3B-/-;RB-/- double knockouts, with protein (E) and mRNA (F) levels evaluated.

Figure 6: Combined loss of SIN3A/SIN3B upregulates cell cycle gene expression independently of DREAM or RB



Taken together, loss of SIN3B or SIN3A did not result in an upregulation of cell-cycle gene expression in arrested HCT116 cells, but a combined loss of SIN3A/SIN3B moderately increased gene expression independently of DREAM and RB. However, these effects were still relatively minor compared to the de-repression observed after loss of LIN37 or RB. To obtain a fuller picture of cell-cycle gene expression in the context of individual vs. combined loss of SIN3A and SIN3B, we prepared Idasanutlin-arrested HCT116 wild-type, SIN3B-/-, SIN3A siRNA-treated, or a combination of the two for RNAseq. The RNAseq has been completed and is currently being processed at the time of this writing. With these results, we will determine if the upregulation of cell-cycle genes is a general trend or specific to the genes we chose to analyze with qPCR. It will also be interesting to see if an upstream regulator can be identified that may contribute to the DREAM/RB-independent nature of this phenotype.

HDAC activity has no general effect on cell-cycle gene repression

It has been shown that the repression of some RB-regulated genes depends on HDAC activity[59], but only limited data are available for DREAM-regulated G2/M genes. Thus, we analyzed whether the repression of G2/M and G1/S genes in arrested cell lines is reduced when HDAC activity is inhibited. Since HDAC1/2 inhibition itself results in the upregulation of cell-cycle inhibitors like p21 and induces cell-cycle arrest[60–62], we arrested HCT116 cells with Idasanutlin first and then added the HDAC1/2 inhibitor Romidepsin[52,53]. We measured the expression of 13 G2/M and G1/S genes and compared their repression in cells treated exclusively with Idasanutlin or with Idasanutlin and Romidepsin (Fig. 7A). For both groups of genes, we did not observe a significant loss of repression upon HDAC1/2 inhibition, although several genes like NEK2, E2F8, RBL1, and ORC1 were slightly, but significantly de-repressed. In contrast, a set of genes that had been previously reported to be upregulated in

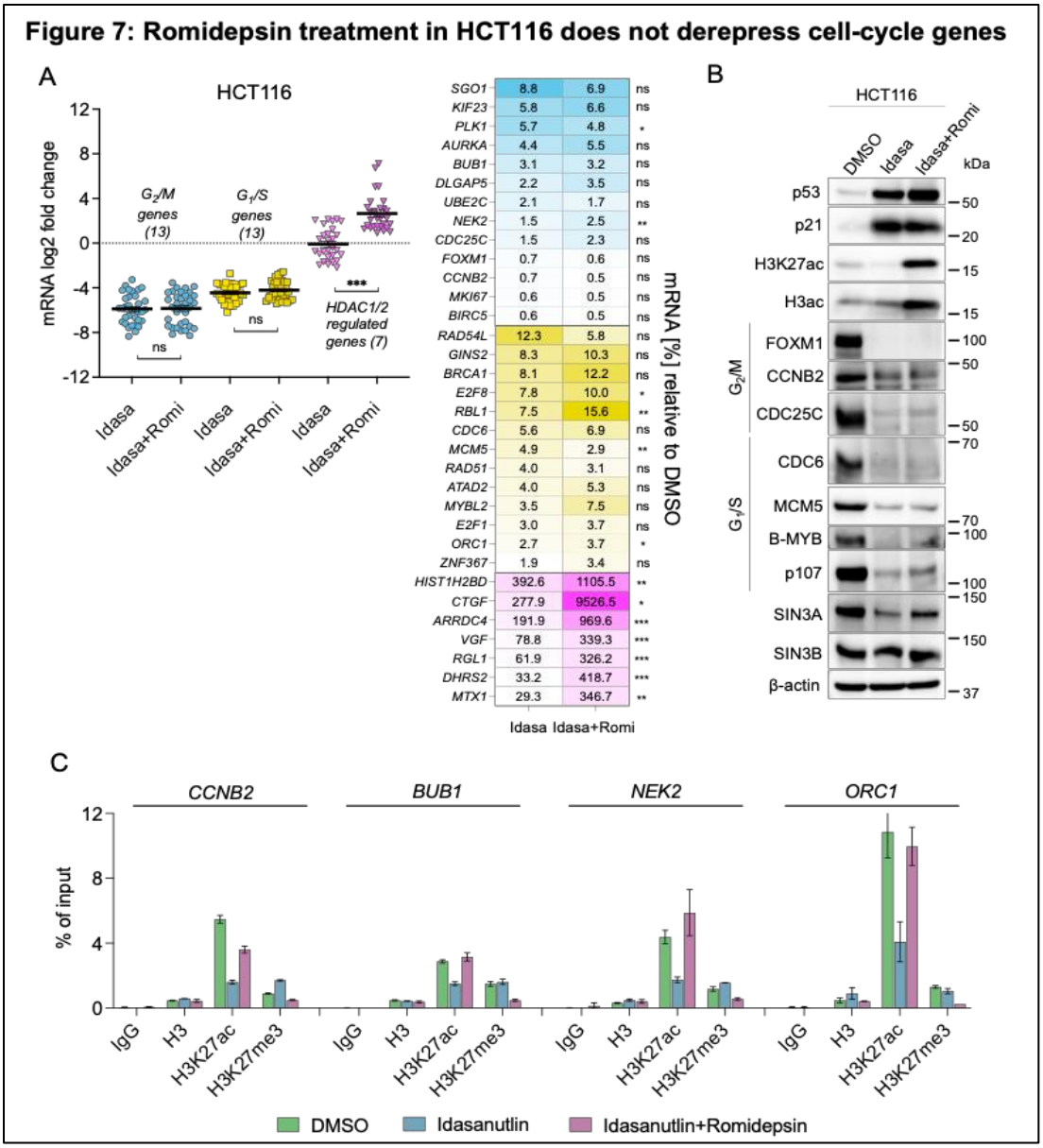


Figure 7. For all the following, wild-type cells were treated with 5uM Idasanutlin for 48hrs and 4nM for the final 24hrs as indicated. mRNA expression was evaluated via qPCR and compared to untreated wild-type cells for each respective line and is shown as gene-set clusters (left) and individual genes (right) in HCT116 (A). Protein expression was evaluated via Western blot with the indicated antibodies (B). (C) Wild-type HCT116 were further evaluated for the indicated histone mark enrichments via cross-linking, MNase digestion, then ChIP-qPCR. The antibodies specific to each histone mark were immunoprecipitated on protein A/G magnetic beads (Pierce), and the linked DNA was purified and assessed with qPCR for the indicated gene promotors.

proliferating HCT116 cells after HDAC inhibition[55] showed a highly significant increase in expression (Fig. 7A). Furthermore, Western blot analysis confirmed upregulation of p53 and p21 in response to Idasanutlin treatment and a strong increase of acetylated histone H3 upon Romidepsin treatment (Fig. 7B). Expression of G2/M and G1/S proteins was strongly repressed in Idasanutlin-treated cells, and addition of Romidepsin did not increase protein levels. Interestingly, ChIP-qPCR revealed that H3K27 acetylation at the promoters of several MuvB target genes was reduced upon Nutlin treatment, and addition of Romidepsin reversed this effect (Fig. 7C). In contrast, H3K27 trimethylation was strongly reduced in Romidepsin-treated cells. While the reduction in H3K27ac levels correlates with Idasanutlin-induced gene repression, the increase that follows HDAC inhibition does not result in an upregulation of gene expression. These data indicate that cell-cycle gene repression can be maintained even when the chromatin at the promoters shows hallmarks of actively expressed genes.

To test the effect of HDAC inhibition in an additional cell lines, we treated A549 lung carcinoma cells with Idasanutlin and Romidepsin. In these cells, multiple G2/M and G1/S genes were significantly de-repressed in Idasanutlin-treated cells after Romidepsin treatment (Fig. 8A). However, this upregulation of mRNA level did not lead to a detectable increase in protein expression (Fig. 8B). In another cell line, non-transformed BJ-hTERT, I saw no significant de-repression of G2/M genes, but did calculate significant de-repression in the G1/S gene set (Fig. 8C). Like the A549, mRNA de-repression in BJ-hTERT also did not translate to the protein level (Fig. 8D). Further, the de-repressed gene sets in A549 and BJ-hTERT are dissimilar. To analyze whether additional HDACs that are not inhibited by Romidepsin influence the repression of DREAM target genes, we repeated the experiment in HCT116 with the pan-HDAC inhibitor Panobinostat[54] and obtained comparable results (Fig. 9A, 9B). Treatment of Idasanutlin-arrested A549 cells with the pan-HDAC inhibitor Panobinostat led to some minor but predominantly non-significant changes in mRNA expression of G2/M and G1/S genes (Fig. 9C), and these minor effects did not translate to detectable changes in protein expression (Fig. 9D). We have additionally administered

Figure 8: Romidepsin treatment does not generally derepress genes across cell lines

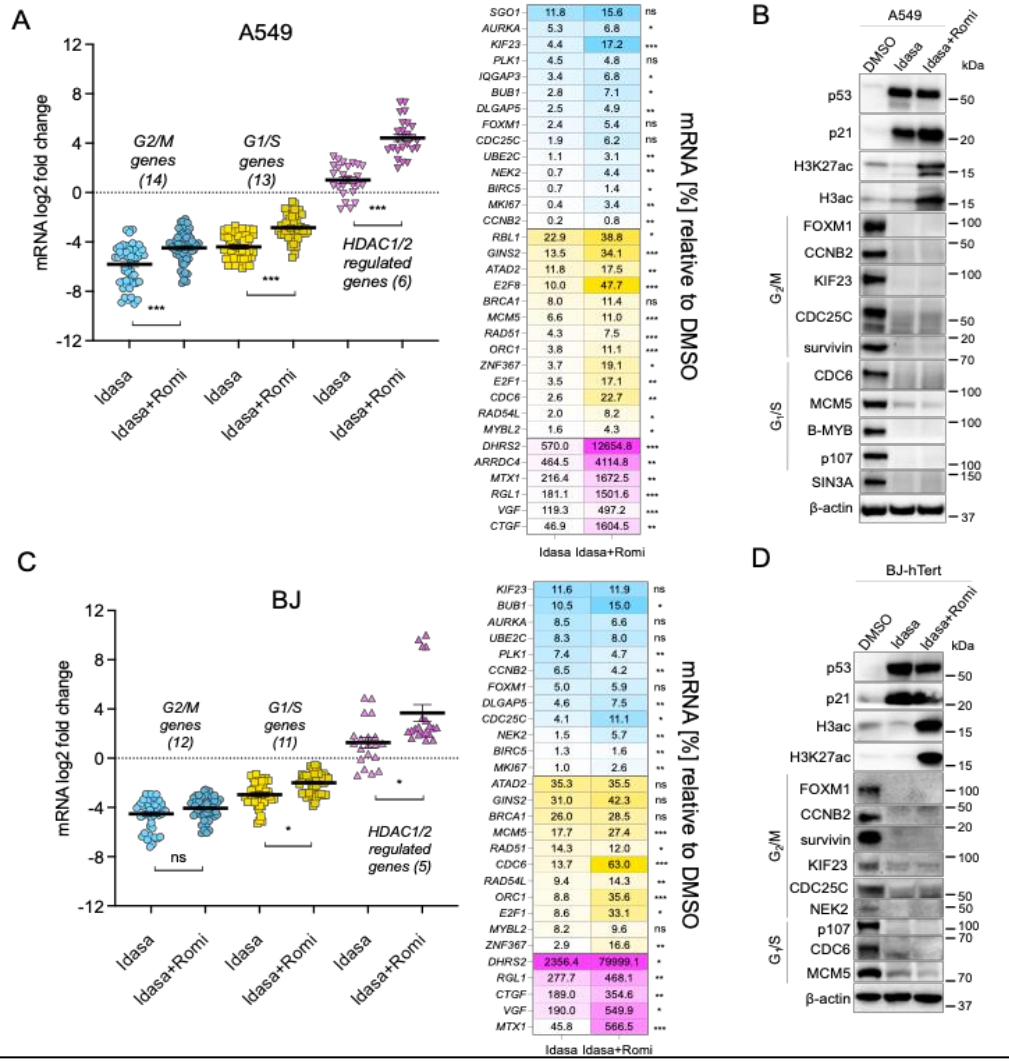


Figure 8. For all the following, wild-type cells were treated with 5uM Idasanutlin for 48hrs and 4nM Romidepsin for the final 24hrs as indicated. mRNA expression was evaluated via qPCR and compared to untreated wild-type cells for each respective line and is shown as gene-set clusters (left) and individual genes (right) in A549 (A) or BJ-hTERT (C). Protein expression was evaluated via Western blot with the indicated antibodies in A549 (B) or BJ-hTERT (D).

Figure 9: Panobinostat treatment does not generally derepress genes across cell lines

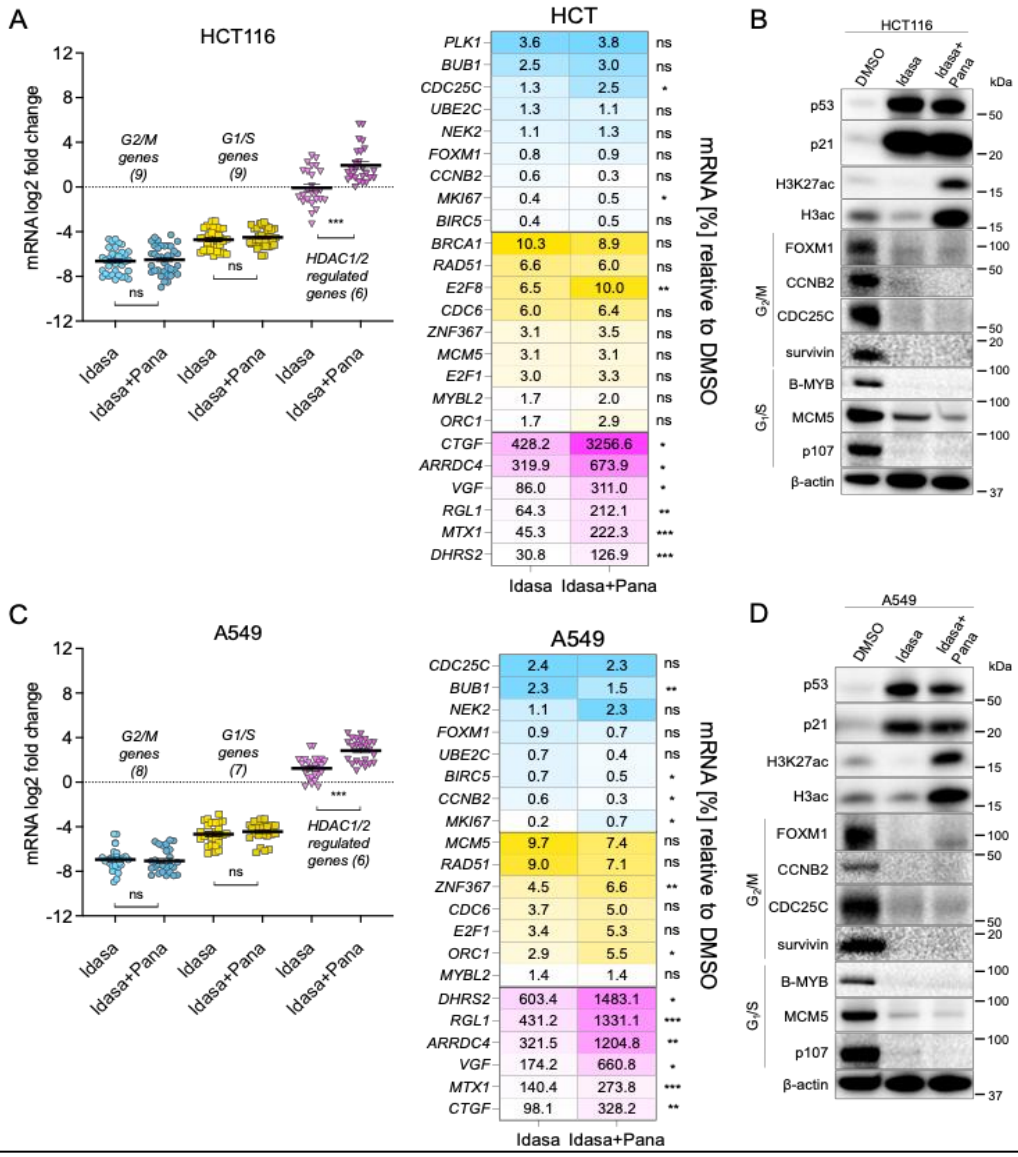


Figure 9. For all the following, wild-type cells were treated with 5uM Idasanutlin for 48hrs and 20nM Panobinostat for the final 24hrs as indicated. mRNA expression was evaluated via qPCR and compared to untreated wild-type cells for each respective line and is shown as gene-set clusters (left) and individual genes (right) in HCT116 (A) or A549 (C). Protein expression was evaluated via Western blot with the indicated antibodies in HCT116 (B) or A549 (D).

Panobinostat in BJ-hTert cells and are currently completing the qPCR and Western blots for this experiment at the time of this writing.

All in all, our results provide evidence that HDAC inhibition in Idasanutlin-treated HCT116 predictably leads to the upregulation of known HDAC-dependent non-cell-cycle genes but does not lead to a general de-repression of DREAM target genes. Interestingly, we see significant de-repression in some cell-cycle genes in other cell-lines, although the affected gene sets between cell lines is variable. I conclude that HDAC involvement in cell-cycle gene regulation is neither a general mechanism of repression nor essential.

Discussion

Prior to this study, the relationship between HDAC complexes and cell-cycle gene regulation had remained unclear despite many reports on the matter. Even among equally credible and convincing reports are contrary findings, likely due to context-specific phenotypes. Within the scope of my own research herein, I observed varying but reproduceable effects on cell-cycle gene repression based on cell line or arresting conditions used. Furthermore, LIN37 and RB remained a consistent requirement for cell-cycle gene repression, but SIN3 and HDAC were generally found to be disposable. While the role of HDAC activity in the context of cell-cycle gene repression has nuance that remains in need of elucidation, I can conclude with this study that HDACs are not an essential element of transcriptional repression of cell-cycle genes, and moderate effects seen in certain contexts are not generalizable.

Although RB has been shown to directly interact with HDAC1 through an LxCxE-dependent interface[11,12,34], others have reported an indirect interaction in H1299 cells wherein SAP30:SIN3B act as a bridge between the two complexes[63]. Regardless of the mode of interaction, several studies indicate that the LxCxE-binding motif of RB is dispensable, depending on the context, for overall cell-cycle-gene repression, cell-cycle arrest, and induction of carcinogenesis[14–17,64]. In the contexts we explored, HDAC and SIN3 are not

essential to cell-cycle gene repression, however I did notice a trend toward more highly significant changes in G1/S gene repression, opposed to G2/M, in the cases where any effects were seen.

The involvement of HDAC complexes with DREAM target genes is certainly perplexing. Although loss of SIN3B leads to significant de-repression of DREAM target genes in serum-starved T98Gs, DREAM components have been inconsistently seen to interact with HDAC complex members in the same context. While Bainor et al were able to detect clear interactions between the complexes both through co-immunoprecipitation and IP-tandem mass-spec[29], we and others have not [18,33,38,65]. For instance a previous study performed a MudPIT proteomics study in T98G cells and neither LIN9, LIN37, LIN54, nor p130 were found with chromosome-modifying partners, including HDAC1/2[18]. It is not presently clear why these discrepancies exist, but one possibility may be genetic drift between specific T98G lines. It would be interesting to exchange material between the above-mentioned parties to assess reproducibility.

A striking discovery of this study is that, although we and others have reported binding of HDAC and SIN3 members at cell-cycle promotors[29,33], we find that HDAC inhibition is not sufficient to illicit a change in mRNA or protein expression despite clear differences in acetyl marks on histones at the same genes. One explanation, that would corroborate a model recently suggested by Mitra et al, is that HDAC complexes operate to fine-tune cell-cycle gene repression[66]. It may be the case that on a cell-to-cell basis HDAC activity has a more significant effect on cell-cycle gene regulation as per the requirements of that specific cell, but with population-based techniques these very small differences average-out or are in the noise. Single-cell studies using techniques such as next generation sequencing (DNA and mRNA) and bioluminescence resonance energy transfer (protein interactions) would be a great contribution in probing this model. An alternative explanation could be a model in which HDAC activity at cell-cycle promoters acts as a fail-safe mechanism for contexts where lesions in cell-cycle regulation exist. If so, perhaps this is an artifact of what was once an

evolutionary advantage but is no longer essential to mammalian cell-cycle gene repression. Indeed, we see that at least in the context of LIN37-/- cells, HDAC activity does not compensate in a repressive manner. It is important to note that MuvB retains its ability to assemble at cell-cycle promotors in the absence of LIN37, but its activity is lost[19,27,35]. An exciting prospect and motivation for pursuing the current research that came from this detail was the potential role of HDAC-recruitment through LIN37, which would have been an elegant explanation for this phenomenon. I find however that individual loss of HDAC activity, SIN3B, SIN3A, or combinations thereof, do not phenocopy loss of LIN37.

While a loss of LIN37 was not mimicked, a combined loss of SIN3A and SIN3Bin HCT116 cells did show significant de-repression of cell-cycle genes in HCT116. However, this outcome persisted in SIN3B-/-;LIN37-/- and SIN3B-/-;RB-/- knockouts, revealing a DREAM and RB-independent mechanism. Additionally, I found that the de-repression seen in serum-starved SIN3B-/- T98Gs was lost with the addition of Palbociclib, while levels of de-repression were unchanged in LIN37-/- T98Gs. I interpreted these results to mean that in serum-starved conditions in T98G cells, SIN3B has an effect somewhere upstream in the pathway that promotes CDK activity. When CDKs are directly inhibited by Palbociclib, this effect is bypassed and DREAM repression is fully re-engaged in cells with intact DREAM components. The LIN37-/- T98Gs were therefore unable to reconstitute repression in this way. Taken together, the above results suggest a context-dependent role for SIN3A/B in cell-cycle gene regulation that is independent of RB or DREAM.

The seemingly fickle activity of HDAC complexes is not a new phenomenon. While HDAC complexes are shown to have repressive roles in cell-cycle regulation and senescence, they are also associated with oncogenesis, and HDAC1 has been reported to be overexpressed in 33% of breast cancers[67]. To this end, several HDAC inhibitors are used as cancer therapeutics, such as the Romidepsin and Panobinostat used in our studies, as well as Trichostatin, Belinostat, and suberoylanilide hydroxamic acid (SAHA)[68]. A pointed example of HDAC activity in this context is the direct deacetylation of p53 by HDAC1. Here, HDAC1

destabilizes the p53 protein through removal of activating acetyl groups, preventing p53-dependent cell-cycle arrest, and apoptosis[42]. However, even within an oncogenic setting, HDAC activity appears to be context dependent. This nuance is highlighted in a study from Zhang et al, wherein they found that HDAC1 substrate-selection and binding partners were cell-type specific between triple-negative breast cancer lines[69].

In conclusion, I find that HDAC activity is not essential for cell-cycle gene regulation. I corroborate past research by others – that SIN3B and HDAC bind to and have activity at least a subset of cell-cycle gene promotors, with the novel addition that this activity does not translate to mRNA or protein expression. I additionally support the previous finding by Bainor et al – that genetic loss of SIN3B results in significant de-repression of cell-cycle genes in serum-starved T98G, but I provide evidence that this effect is due to upstream mechanisms independent of DREAM and that it does not generalize to other serum-starved cell lines. I show that a combined loss of SIN3A and SIN3B affect cell-cycle gene repression in HCT116, but the data also point to a mechanism that is DREAM and RB-independent. Lastly, I find that HDAC inhibition de-represses a selection of cell-cycle genes, particularly from the G1/S-phase gene set, but that this selection is not constant across cell-lines. What contributes to these differences is yet unknown. Aside from this larger question, several other lingering questions remain: (1) RBBP4 is a known member of both HDAC complexes as well as MuvB, for the identical role of histone-binding. Is RBBP4-binding mutually exclusive or permissible between these complexes? (2) Is nucleosome positioning the primary mechanism of repression by DREAM? One thought on determining this is looking at whether tail-less histone mutants can remain repressed by DREAM ex vivo. This would take some clever experiment design, but I have no doubt a whacky enough scientist can pull it off. Best of luck (mic drop).

Methods

Cell culture and drug treatment

HCT116 (37), T98G, A549, BJ-hTert, and C2C12 wild-type and knockout cells were grown in Dulbecco's modified Eagle's medium (Lonza) supplemented with 10 % fetal calf serum (Biochrom) and penicillin/streptomycin (Sigma-Aldrich) and maintained at 37 °C and 10 % CO2.

For induction of p53, cells were treated with Nutlin-3a (5 μM; Cayman Chemicals) or doxorubicin (0.2 μg/ml; Medac GmbH) for 24 or 48 hours. Serum-starvation was induced with unsupplemented Dulbecco's modified Eagle's medium (Lonza) for up to 120 hours. Romidepsin was administered at 4nM for all cell lines, except for C2C12 which received 10nM, for 24 or 48 hours. Panobinostat was administered at 20nM for all cell lines, except for C2C12 which received 50nM, for 24 or 48 hours. Palbociclib was administered at 10uM for all cell lines for up to 48 hours.

Generation of knockout cell lines by CRISPR/Cas9 nickase

SIN3B-/-, SIN3B-/-;LIN37-/-, SIN3B-/-RB-/- HCT116 cells, SIN3B-/-, LIN37-/-, SIN3B-/-;LIN37-/- T98G, and SIN3B-/- C2C12 cells were created by CRISPR/Cas9 nickase applying the pX335-U6-Chimeric_BB-CBh-hSpCas9n(D10A) vector as described earlier[51]. InDel mutations were introduced in exons 3 and 4 of the SIN3B gene, and exon 6 of the LIN37 gene encoding for the LIN37-MUVB complex interaction domain[35]. RB-/- parental HCT116 cells were previously generated by introducing mutations to exon 13 of the RB gene encoding for the pocket domain which is essential for the interaction with E2F proteins[35]. Single-colony knockout clones were determined through Western-blot.

SDS-PAGE and western blot

SDS-PAGE and western blot were performed following standard protocols as described earlier[35]. The following antibodies were applied for protein detection: RB (C-2, Santa Cruz Biotechnology), LIN54 (A303-799A, Bethyl Laboratories), LIN9 (ab62329, Abcam), β-Actin (A5441, Sigma-Aldrich), RBBP4 (A301-206A, Bethyl Laboratories), LIN37 (custom-made at Pineda Antikörper-Service, Berlin, Germany), p107 (C-18, sc-318, Santa Cruz Biotechnology), p130 (RBL2 D9T7M, Cell Signaling Technologies), FOXM1 (D12D5, Cell Signaling Technologies,) Aurora Kinase A (A300-071A, Bethyl Laboratories), cyclin B2 (A-2, sc-28303, Santa Cruz Biotechnology), KIF23 (MKLP-1, sc-136473, Santa Cruz Biotechnology), CDC25C (H-6, sc-13138, Santa Cruz Biotechnology), CDC6 (180.2, sc-9964, Santa Cruz Biotechnology), CCNE2 (EP454Y, ab40890, Abcam), p18 (118.2, sc-9965, Santa Cruz Biotechnology), PEG10 (4C10A7, Novus Biologicals), Survivin (71G4B7, Cell Signaling Technologies), p53 (Ab-6 DO-1, Merck/Calbiochem), p21 (Ab-1 EA10, Merck/Calbiochem), HDAC1 (10E2, sc-81598, Santa Cruz Biotechnology), HDAC2 (C-8, sc-9959, Santa Cruz Biotechnology), SIN3B (H-4, sc-13145, Santa Cruz Biotechnology), SIN3B (H-5, sc-55516, Santa Cruz Biotechnology), SIN3B (NBP2-13309, Novus), SIN3A (G-11, sc-5299, Santa Cruz Biotechnology), NEK2 (D-8, sc-55601, Santa Cruz Biotechnology), MCM5 (E-10, sc-165994 Santa Cruz Biotechnology), p107/RBL1 (D3P3C, Cell Signaling Technologies) , H3K27ac (D5E4, Cell Signaling Technologies), H3ac (Histone H3 pan-ac, 61637, Active Motif), H3 (D1H2, Cell Signaling Technologies), H3K27me3 (C36B11, Cell Signaling Technologies).The monoclonal B-Myb LX015.1 antibody (hybridoma media 1:5) was a kind gift from Roger Watson.

RNA extraction, reverse transcription and semi-quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer's protocol. One-step reverse transcription and quantitative real-time PCR were performed with

an ABI 7300 Real-Time PCR System (Applied Biosystems) using QuantiTect SYBRGreen PCR Kit (Qiagen).

Chromatin immunoprecipitation – qPCR

Cells were treated with Nutlin-3a (5 μM; Cayman Chemicals) for 48 hours to induce formation of repressive complexes. The cells were harvested, cross-linked with PBS (Gibco) supplemented with 1% paraformaldehyde (Electron Microscopy Sciences), and quenched with 125mM Glycine (Fisher Scientific). Cells were lysed and prepared for treatment with MNase using Buffer A (Cell Signaling #7006S) and Buffer B (Cell Signaling #7007S). MNase enzyme was prepared in-house. Nuclei were MNase-treated on ice for 30 minutes followed by 15 minutes at 37°C, and further sonicated at 20% amplitude, to create ~300bp fragments. Nuclear extracts were immunoprecipitated with the indicated antibodies overnight at 4°C, and bound to protein A/G magnetic beads (Pierce). Eluants were treated with RNaseA for 30 minutes 37°C, then simultaneous Proteinase K-treated for 1 hour 55°C, and reverse cross-linked at 95°C for 20 minutes. DNA was purified using Zymo DNA Clean & Concentrator-5 kits and methods. qPCR was performed with purified DNA with an ABI 7300 Real-Time PCR System (Applied Biosystems).

HDAC activity assay

HDAC activity assays were performed first by immunoprecipitation with the indicated anti-bodies on protein A/G magnetic beads (Pierce), and activity was measured on-bead in 384-well format, white, flat-bottom plates using HDAC-Glo™I/II (Promega) reagents and protocol.

Acknowledgements

Thanks always to Seth Rubin. Special thanks to Gerd Müller for his mentorship and side-by-side support throughout this project. Thanks also to Manisha Shingara who helped in a big way to expedite the progress of this research. The three of us made for a great assembly line that was both productive and fun. Thanks to Tilini Wijeratne for assistance in performing some of the ChIP experiments, and to Kelsie Rodriguez and Miles Membreño for help in executing Edu/FACS experiments. Thanks to Andreas Rechtstiner for bioinformatics contributions for RNAseq analysis. Lastly, thanks to Worg Barrett for his intellectual contributions.

References

1. Fischer, M., and Müller, G.A. (2017). Cell cycle transcription control: DREAM/MuvB and RB-E2F complexes. Crit Rev Biochem Mol Biol *52*, 638–662. 10.1080/10409238.2017.1360836.

2. Fischer, M., Schade, A.E., Branigan, T.B., Müller, G.A., and DeCaprio, J.A. (2022). Coordinating gene expression during the cell cycle. Trends in Biochemical Sciences *47*, 1009–1022. 10.1016/j.tibs.2022.06.007.

3. Zheng, N., Fraenkel, E., Pabo, C.O., and Pavletich, N.P. (1999). Structural basis of DNA recognition by the heterodimeric cell cycle transcription factor E2F–DP. Genes Dev *13*, 666–674.

4. Trimarchi, J.M., and Lees, J.A. (2002). Sibling rivalry in the E2F family. Nat Rev Mol Cell Biol *3*, 11–20. 10.1038/nrm714.

5. Campanero, M.R., and Flemington, E.K. (1997). Regulation of E2F through ubiquitin–proteasome-dependent degradation: Stabilization by the pRB tumor suppressor protein. Proc Natl Acad Sci U S A *94*, 2221–2226.

6. Adams, P.D., and Kaelin, W.G. (1996). The cellular effects of E2F overexpression. Curr Top Microbiol Immunol *208*, 79–93. 10.1007/978-3-642-79910-5_4.

7. Takahashi, Y., Rayman, J.B., and Dynlacht, B.D. (2000). Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. Genes Dev *14*, 804–816.

8. Helin, K., Harlow, E., and Fattaey, A. (1993). Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. Mol Cell Biol *13*, 6501–6508. 10.1128/mcb.13.10.6501-6508.1993.

9. Hiebert, S.W., Chellappan, S.P., Horowitz, J.M., and Nevins, J.R. (1992). The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes Dev *6*, 177–185. 10.1101/gad.6.2.177.

10. Rubin, S.M. (2013). Deciphering the Rb phosphorylation code. Trends Biochem Sci *38*, 12–19. 10.1016/j.tibs.2012.10.007.

11. Brehm, A., Miska, E.A., McCance, D.J., Reid, J.L., Bannister, A.J., and Kouzarides, T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature *391*, 597–601. 10.1038/35404.

12. Luo, R.X., Postigo, A.A., and Dean, D.C. (1998). Rb interacts with histone deacetylase to repress transcription. Cell *92*, 463–473. 10.1016/s0092-8674(00)80940-x.

13. Bannister, A.J., and Kouzarides, T. (2011). Regulation of chromatin by histone modifications. Cell Res *21*, 381–395. 10.1038/cr.2011.22.

14. Andrusiak, M.G., Vandenbosch, R., Dick, F.A., Park, D.S., and Slack, R.S. (2013). LXCXE-independent chromatin remodeling by Rb/E2f mediates neuronal quiescence. Cell Cycle *12*, 1416–1423. 10.4161/cc.24527.

15. Bourgo, R.J., Thangavel, C., Ertel, A., Bergseid, J., McClendon, A.K., Wilkens, L., Witkiewicz, A.K., Wang, J.Y.J., and Knudsen, E.S. (2011). RB restricts DNA damage-initiated tumorigenesis through an LXCXE-dependent mechanism of transcriptional control. Mol Cell *43*, 663–672. 10.1016/j.molcel.2011.06.029.

16. Talluri, S., Francis, S.M., and Dick, F.A. (2013). Mutation of the LXCXE Binding Cleft of pRb Facilitates Transformation by ras In Vitro but Does Not Promote Tumorigenesis In Vivo. PLOS ONE *8*, e72236. 10.1371/journal.pone.0072236.

17. Vormer, T.L., Wojciechowicz, K., Dekker, M., de Vries, S., van der Wal, A., Delzenne-Goette, E., Naik, S.H., Song, J.-Y., Dannenberg, J.-H., Hansen, J.B., et al. (2014). RB family tumor suppressor activity may not relate to active silencing of E2F target genes. Cancer Res *74*, 5266–5276. 10.1158/0008-5472.CAN-13-3706.

18. Litovchick, L., Sadasivam, S., Florens, L., Zhu, X., Swanson, S.K., Velmurugan, S., Chen, R., Washburn, M.P., Liu, X.S., and DeCaprio, J.A. (2007). Evolutionarily Conserved Multisubunit RBL2/p130 and E2F4 Protein Complex Represses Human Cell Cycle-Dependent Genes in Quiescence. Molecular Cell *26*, 539–551. 10.1016/j.molcel.2007.04.015.

19. Asthana, A., Ramanan, P., Hirschi, A., Guiley, K.Z., Wijeratne, T.U., Shelansky, R., Doody, M.J., Narasimhan, H., Boeger, H., Tripathi, S., et al. (2022). The MuvB complex binds and stabilizes nucleosomes downstream of the transcription start site of cell-cycle dependent genes. Nat Commun *13*, 526. 10.1038/s41467-022-28094-1.

20. Koliopoulos, M.G., Muhammad, R., Roumeliotis, T.I., Beuron, F., Choudhary, J.S., and Alfieri, C. (2022). Structure of a nucleosome-bound MuvB transcription factor complex reveals DNA remodelling. Nat Commun *13*, 5075. 10.1038/s41467-022-32798-9.

21. Marceau, A.H., Felthousen, J.G., Goetsch, P.D., Iness, A.N., Lee, H.-W., Tripathi, S.M., Strome, S., Litovchick, L., and Rubin, S.M. (2016). Structural basis for LIN54 recognition of CHR elements in cell cycle-regulated promoters. Nat Commun *7*, 12301. 10.1038/ncomms12301.

22. Müller, G.A., Quaas, M., Schümann, M., Krause, E., Padi, M., Fischer, M., Litovchick, L., DeCaprio, J.A., and Engeland, K. (2012). The CHR promoter element controls cell cycle-dependent gene transcription and binds the DREAM and MMB complexes. Nucleic Acids Res *40*, 1561–1578. 10.1093/nar/gkr793.

23. Müller, G.A., Wintsche, A., Stangner, K., Prohaska, S.J., Stadler, P.F., and Engeland, K. (2014). The CHR site: definition and genome-wide identification of a cell cycle transcriptional element. Nucleic Acids Res *42*, 10331–10350. 10.1093/nar/gku696.

24. Verreault, A., Kaufman, P.D., Kobayashi, R., and Stillman, B. (1998). Nucleosomal DNA regulates the core-histone-binding subunit of the human Hat1 acetyltransferase. Curr Biol *8*, 96–108. 10.1016/s0960-9822(98)70040-5.

25. Verreault, A., Kaufman, P.D., Kobayashi, R., and Stillman, B. (1996). Nucleosome assembly by a complex of CAF-1 and acetylated histones H3/H4. Cell *87*, 95–104. 10.1016/s0092-8674(00)81326-4.

26. Guiley, K.Z., Liban, T.J., Felthousen, J.G., Ramanan, P., Litovchick, L., and Rubin, S.M. (2015). Structural mechanisms of DREAM complex assembly and regulation. Genes Dev *29*, 961–974. 10.1101/gad.257568.114.

27. Mages, C.F., Wintsche, A., Bernhart, S.H., and Müller, G.A. (2017). The DREAM complex through its subunit Lin37 cooperates with Rb to initiate quiescence. eLife *6*, e26876. 10.7554/eLife.26876.

28. Sadasivam, S., Duan, S., and DeCaprio, J.A. (2012). The MuvB complex sequentially recruits B-Myb and FoxM1 to promote mitotic gene expression. Genes Dev *26*, 474–489. 10.1101/gad.181933.111.

29. Bainor, A.J., Saini, S., Calderon, A., Casado-Polanco, R., Giner-Ramirez, B., Moncada, C., Cantor, D.J., Ernlund, A., Litovchick, L., and David, G. (2018). The HDAC-Associated Sin3B Protein Represses DREAM Complex Targets and Cooperates with APC/C to Promote Quiescence. Cell Rep *25*, 2797-2807.e8. 10.1016/j.celrep.2018.11.024.

30. Pilkinton, M., Sandoval, R., and Colamonici, O.R. (2007). Mammalian Mip/LIN-9 interacts with either the p107, p130/E2F4 repressor complex or B-Myb in a cell cycle-phase-dependent context distinct from the Drosophila dREAM complex. Oncogene *26*, 7535–7543. 10.1038/sj.onc.1210562.

31. Luo, Y., Hitz, B.C., Gabdank, I., Hilton, J.A., Kagda, M.S., Lam, B., Myers, Z., Sud, P., Jou, J., Lin, K., et al. (2020). New developments on the Encyclopedia of DNA Elements (ENCODE) data portal. Nucleic Acids Res *48*, D882–D889. 10.1093/nar/gkz1062.

32. Sloan, C.A., Chan, E.T., Davidson, J.M., Malladi, V.S., Strattan, J.S., Hitz, B.C., Gabdank, I., Narayanan, A.K., Ho, M., Lee, B.T., et al. (2016). ENCODE data at the ENCODE portal. Nucleic Acids Res *44*, D726-732. 10.1093/nar/gkv1160.

33. van Oevelen, C., Wang, J., Asp, P., Yan, Q., Kaelin, W.G., Kluger, Y., and Dynlacht, B.D. (2008). A role for mammalian Sin3 in permanent gene silencing. Mol Cell *32*, 359–370. 10.1016/j.molcel.2008.10.015.

34. Ferreira, R., Magnaghi-Jaulin, L., Robin, P., Harel-Bellan, A., and Trouche, D. (1998). The three members of the pocket proteins family share the ability to repress E2F activity through recruitment of a histone deacetylase. Proc Natl Acad Sci U S A *95*, 10493–10498. 10.1073/pnas.95.18.10493.

35. Uxa, S., Bernhart, S.H., Mages, C.F.S., Fischer, M., Kohler, R., Hoffmann, S., Stadler, P.F., Engeland, K., and Müller, G.A. (2019). DREAM and RB cooperate to induce gene repression and cell-cycle arrest in response to p53 activation. Nucleic Acids Res *47*, 9087–9103. 10.1093/nar/gkz635.

36. Ayer, D.E., Lawrence, Q.A., and Eisenman, R.N. (1995). Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. Cell *80*, 767–776. 10.1016/0092-8674(95)90355-0.

37. Schreiber-Agus, N., Chin, L., Chen, K., Torres, R., Rao, G., Guida, P., Skoultchi, A.I., and DePinho, R.A. (1995). An amino-terminal domain of Mxi1 mediates anti-Myc oncogenic activity and interacts with a homolog of the yeast transcriptional repressor SIN3. Cell *80*, 777–786. 10.1016/0092-8674(95)90356-9.

38. Adams, M.K., Banks, C.A.S., Thornton, J.L., Kempf, C.G., Zhang, Y., Miah, S., Hao, Y., Sardiu, M.E., Killer, M., Hattem, G.L., et al. (2020). Differential Complex Formation via Paralogs in the Human Sin3 Protein Interaction Network. Molecular & Cellular Proteomics *19*, 1468–1484. 10.1074/mcp.RA120.002078.

39. Adams, G.E., Chandru, A., and Cowley, S.M. (2018). Co-repressor, co-activator and general transcription factor: the many faces of the Sin3 histone deacetylase (HDAC) complex. Biochemical Journal *475*, 3921–3932. 10.1042/BCJ20170314.

40. Chaubal, A., and Pile, L.A. (2018). Same agent, different messages: insight into transcriptional regulation by SIN3 isoforms. Epigenetics & Chromatin *11*, 17. 10.1186/s13072-018-0188-y.

41. Zhang, Y., Iratni, R., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (1997). Histone deacetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex. Cell *89*, 357–364. 10.1016/s0092-8674(00)80216-0.

42. Luo, J., Su, F., Chen, D., Shiloh, A., and Gu, W. (2000). Deacetylation of p53 modulates its effect on cell growth and apoptosis. Nature *408*, 377–381. 10.1038/35042612.

43. Jelinic, P., Pellegrino, J., and David, G. (2011). A novel mammalian complex containing Sin3B mitigates histone acetylation and RNA polymerase II progression within transcribed loci. Mol Cell Biol *31*, 54–62. 10.1128/MCB.00840-10.

44. Li, B., Gogol, M., Carey, M., Lee, D., Seidel, C., and Workman, J.L. (2007). Combined Action of PHD and Chromo Domains Directs the Rpd3S HDAC to Transcribed Chromatin. Science *316*, 1050–1054. 10.1126/science.1139004.

45. Lewis, M.J., Liu, J., Libby, E.F., Lee, M., Crawford, N.P.S., and Hurst, D.R. (2016). SIN3A and SIN3B differentially regulate breast cancer metastasis. Oncotarget *7*, 78713–78725. 10.18632/oncotarget.12805.

46. Saha, N., Liu, M., Gajan, A., and Pile, L.A. (2016). Genome-wide studies reveal novel and distinct biological pathways regulated by SIN3 isoforms. BMC Genomics *17*, 111. 10.1186/s12864-016-2428-5.

47. Dannenberg, J.-H., David, G., Zhong, S., van der Torre, J., Wong, W.H., and Depinho, R.A. (2005). mSin3A corepressor regulates diverse transcriptional networks governing normal and neoplastic growth and survival. Genes Dev *19*, 1581–1595. 10.1101/gad.1286905.

48. David, G., Grandinetti, K.B., Finnerty, P.M., Simpson, N., Chu, G.C., and DePinho, R.A. (2008). Specific requirement of the chromatin modifier mSin3B in cell cycle exit and cellular differentiation. Proc Natl Acad Sci U S A *105*, 4168–4172. 10.1073/pnas.0710285105.

49. van Oevelen, C., Bowman, C., Pellegrino, J., Asp, P., Cheng, J., Parisi, F., Micsinai, M., Kluger, Y., Chu, A., Blais, A., et al. (2010). The Mammalian Sin3 Proteins Are Required for Muscle Development and Sarcomere Specification. Mol Cell Biol *30*, 5686–5697. 10.1128/MCB.00975-10.

50. Cowley, S.M., Iritani, B.M., Mendrysa, S.M., Xu, T., Cheng, P.F., Yada, J., Liggitt, H.D., and Eisenman, R.N. (2005). The mSin3A chromatin-modifying complex is essential for embryogenesis and T-cell development. Mol Cell Biol *25*, 6990–7004. 10.1128/MCB.25.16.6990-7004.2005.

51. Ran, F.A., Hsu, P.D., Lin, C.-Y., Gootenberg, J.S., Konermann, S., Trevino, A.E., Scott, D.A., Inoue, A., Matoba, S., Zhang, Y., et al. (2013). Double Nicking by RNA-Guided CRISPR Cas9 for Enhanced Genome Editing Specificity. Cell *154*, 1380–1389. 10.1016/j.cell.2013.08.021.

52. Nakajima, H., Kim, Y.B., Terano, H., Yoshida, M., and Horinouchi, S. (1998). FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. Exp Cell Res *241*, 126–133. 10.1006/excr.1998.4027.

53. Ueda, H., Nakajima, H., Hori, Y., Fujita, T., Nishimura, M., Goto, T., and Okuhara, M. (1994). FR901228, a novel antitumor bicyclic depsipeptide produced by Chromobacterium violaceum No. 968. I. Taxonomy, fermentation, isolation, physico-chemical and biological properties, and antitumor activity. J Antibiot (Tokyo) *47*, 301–310. 10.7164/antibiotics.47.301.

54. George, P., Bali, P., Annavarapu, S., Scuto, A., Fiskus, W., Guo, F., Sigua, C., Sondarva, G., Moscinski, L., Atadja, P., et al. (2005). Combination of the histone deacetylase inhibitor LBH589 and the hsp90 inhibitor 17-AAG is highly active against human CML-BC cells and

AML cells with activating mutation of FLT-3. Blood *105*, 1768–1776. 10.1182/blood-2004-09-3413.

55. LaBonte, M.J., Wilson, P.M., Fazzone, W., Groshen, S., Lenz, H.-J., and Ladner, R.D. (2009). DNA microarray profiling of genes differentially regulated by the histone deacetylase inhibitors vorinostat and LBH589 in colon cancer cell lines. BMC Medical Genomics *2*, 67. 10.1186/1755-8794-2-67.

56. Bainor, A.J., Deng, F.-M., Wang, Y., Lee, P., Cantor, D.J., Logan, S.K., and David, G. (2017). Chromatin-associated protein SIN3B prevents prostate cancer progression by inducing senescence. Cancer Res *77*, 5339–5348. 10.1158/0008-5472.CAN-16-3410.

57. Grandinetti, K.B., Jelinic, P., DiMauro, T., Pellegrino, J., Rodriguez, R.F., Finnerty, P.M., Ruoff, R., Bardeesy, N., Logan, S.K., and David, G. (2009). Sin3B expression is required for cellular senescence and is upregulated upon oncogenic stress. Cancer Res *69*, 6430–6437. 10.1158/0008-5472.CAN-09-0537.

58. Rielland, M., Cantor, D.J., Graveline, R., Hajdu, C., Mara, L., de Diego Diaz, B., Miller, G., and David, G. (2014). Senescence-associated SIN3B promotes inflammation and pancreatic cancer progression. J Clin Invest *124*, 2125–2135. 10.1172/JCI72619.

59. Siddiqui, H., Solomon, D.A., Gunawardena, R.W., Wang, Y., and Knudsen, E.S. (2003). Histone deacetylation of RB-responsive promoters: requisite for specific gene repression but dispensable for cell cycle inhibition. Mol Cell Biol *23*, 7719–7731. 10.1128/MCB.23.21.7719-7731.2003.

60. Vinodhkumar, R., Song, Y.-S., and Devaki, T. (2008). Romidepsin (depsipeptide) induced cell cycle arrest, apoptosis and histone hyperacetylation in lung carcinoma cells (A549) are associated with increase in p21 and hypophosphorylated retinoblastoma proteins expression. Biomed Pharmacother *62*, 85–93. 10.1016/j.biopha.2007.06.002.

61. Yamaguchi, T., Cubizolles, F., Zhang, Y., Reichert, N., Kohler, H., Seiser, C., and Matthias, P. (2010). Histone deacetylases 1 and 2 act in concert to promote the G1-to-S progression. Genes Dev *24*, 455–469. 10.1101/gad.552310.

62. Zupkovitz, G., Grausenburger, R., Brunmeir, R., Senese, S., Tischler, J., Jurkin, J., Rembold, M., Meunier, D., Egger, G., Lagger, S., et al. (2010). The cyclin-dependent kinase inhibitor p21 is a crucial target for histone deacetylase 1 as a regulator of cellular proliferation. Mol Cell Biol *30*, 1171–1181. 10.1128/MCB.01500-09.

63. Lai, A., Kennedy, B.K., Barbie, D.A., Bertos, N.R., Yang, X.J., Theberge, M.-C., Tsai, S.-C., Seto, E., Zhang, Y., Kuzmichev, A., et al. (2001). RBP1 Recruits the mSIN3-Histone Deacetylase Complex to the Pocket of Retinoblastoma Tumor Suppressor Family Proteins Found in Limited Discrete Regions of the Nucleus at Growth Arrest. Mol Cell Biol *21*, 2918–2932. 10.1128/MCB.21.8.2918-2932.2001.

64. Dahiya, A., Gavin, M.R., Luo, R.X., and Dean, D.C. (2000). Role of the LXCXE Binding Site in Rb Function. Mol Cell Biol *20*, 6799–6805.

65. Silverstein, R.A., and Ekwall, K. (2005). Sin3: a flexible regulator of global gene expression and genome stability. Curr Genet *47*, 1–17. 10.1007/s00294-004-0541-5.

66. Mitra, A., Raicu, A.-M., Hickey, S.L., Pile, L.A., and Arnosti, D.N. (2021). Soft repression: Subtle transcriptional regulation with global impact. Bioessays *43*, e2000231. 10.1002/bies.202000231.

67. Müller, B.M., Jana, L., Kasajima, A., Lehmann, A., Prinzler, J., Budczies, J., Winzer, K.-J., Dietel, M., Weichert, W., and Denkert, C. (2013). Differential expression of histone deacetylases HDAC1, 2 and 3 in human breast cancer - overexpression of HDAC2 and HDAC3 is associated with clinicopathological indicators of disease progression. BMC Cancer *13*, 215. 10.1186/1471-2407-13-215.

68. Yoon, S., and Eom, G.H. (2016). HDAC and HDAC Inhibitor: From Cancer to Cardiovascular Diseases. Chonnam Med J *52*, 1–11. 10.4068/cmj.2016.52.1.1.

69. Zhang, Y., Nalawansha, D.A., Herath, K.E., Andrade, R., and Pflum, M.K.H. (2021). Differential profiles of HDAC1 substrates and associated proteins in breast cancer cells revealed by trapping. Mol. Omics *17*, 544–553. 10.1039/D0MO00047G.