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Article

Deacetylase Inhibitors Dissociate the Histone-Targeting ING2 Subunit from the Sin3 Complex

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SUMMARY

Histone deacetylase (HDAC) inhibitors are in clinical development for several diseases, including cancers and neurodegenerative disorders. HDACs 1 and 2 are among the targets of these inhibitors and are part of multisubunit protein complexes. HDAC inhibitors (HDACis) block the activity of HDACs by chelating a zinc molecule in their catalytic sites. It is not known if the inhibitors have any additional functional effects on the multisubunit HDAC complexes. Here, we find that suberoylanilide hydroxamic acid (SAHA), the first FDA-approved HDACi for cancer, causes the dissociation of the PHD-finger-containing ING2 subunit from the Sin3 deacetylase complex. Loss of ING2 disrupts the in vivo binding of the Sin3 complex to the *p21* promoter, an important target gene for cell growth inhibition by SAHA. Our findings reveal a molecular mechanism by which HDAC inhibitors disrupt deacetylase function.

INTRODUCTION

Histone deacetylases (HDACs) remove acetyl groups from histones as well as nonhistone proteins. Histone hyperacetylation is generally correlated with gene expression, and HDACs often work to repress gene expression. Inhibitors of HDACs (HDACis) show promise as anticancer agents as well as in therapies for neurodegenerative diseases (Khan and La Thangue, 2008; Wiech et al., 2009). The hydroxamic acid SAHA is currently used as a treatment for advanced and refractory cutaneous T cell lymphoma (CTCL) (Khan and La Thangue, 2008; Mann et al., 2007). A second HDACi, Istodax (also known as romidepsin, depsipeptide, and FK228), has also recently been approved for CTCL treatment (http://www.fda.gov/AboutFDA/CentersOffices/CDER/ucm189466.htm). HDACis can inhibit cancer progression through a number of mechanisms, including inducing apoptosis, arresting cells in G1/S or G2/M, and causing cells to differentiate (Frew et al., 2009; Marks and Xu, 2009; Smith and Workman, 2009). One of the mechanisms by which HDACis work is through modulation of gene expression by acetylation of histones, to produce a transcriptional program that is favorable for cell cycle arrest or apoptosis (Frew et al., 2009; Marks and Xu, 2009; Smith and Workman, 2009). Overall, HDACis cause a small percentage of genes to be misregulated transcriptionally, and, in this subset of genes, some are upregulated, whereas some are downregulated (Smith, 2008; Van Lint et al., 1996). In addition, HDACis mediate the acetylation of many nonhistone proteins, although this also appears to be a rather small subset of all possible acetylated proteins (Choudhary et al., 2009; Spange et al., 2009).

There are four classes of HDACs. Classes I, II, and IV are zinc-dependent hydrolases, whereas Class III HDACs are nicotinamide adenine dinucleotide (NAD^+)-dependent enzymes called sirtuins (Yang and Seto, 2008). There are 11 known zinc-dependent HDACs (Class I: HDACs 1–3 and 8; Class II: HDACs 4–7, 9, and 10; Class IV: HDAC 11) (Yang and Seto, 2008). Many inhibitors being tested as anticancer agents affect several of these enzymes. Crystal structures have been solved for a bacterial Class I homolog and for human HDACs 7 and 8 in complex with the hydroxamic acid inhibitors trichostatin A (TSA) and SAHA (Finnin et al., 1999; Schuetz et al., 2008; Vannini et al., 2004). These inhibitors work by chelating a zinc molecule in the active site of the HDACs through their hydroxamic acid moieties (Finnin et al., 1999; Schuetz et al., 2008; Vannini et al., 2004). Because these molecules contain aliphatic chains that extend out through the normal acetyl lysine-binding pockets in the HDACs, they also may inhibit binding of the HDAC to their normal acetyl lysine substrates (Finnin et al., 1999; Schuetz et al., 2008; Vannini et al., 2004). Many inhibitors in clinical development affect several HDACs; therefore, work has recently focused on understanding which HDACs are needed to mediate the anticancer effects of the inhibitors (Balasubramanian et al., 2009; Witt et al., 2009). The goal is to obtain cancer cell growth-inhibiting properties while maximizing the selectivity of the inhibitors. Studies suggest that, in vivo, HDACs 1 and 2 play a role in mediating cell growth arrest by these molecules (Glaser et al., 2003; Haberland et al., 2009).

However, HDACs1 and 2 do not work alone; rather, they reside in multisubunit chromatin modifying complexes, of which three have been characterized: Mi-2/NuRD, which contains HDAC, histone demethylase, and chromatin remodeling activities; CoREST, which can repress neuronal-specific genes in non-neuronal cells; and Sin3, which has been implicated in cell cycle control (Wang et al., 2009; Yang and Seto, 2008). Residency in these complexes is important for full activity and specificity of these HDACs in the cell (Alland et al., 2002; Denslow and Wade, 2007). However, it is not known if HDACis act directly on

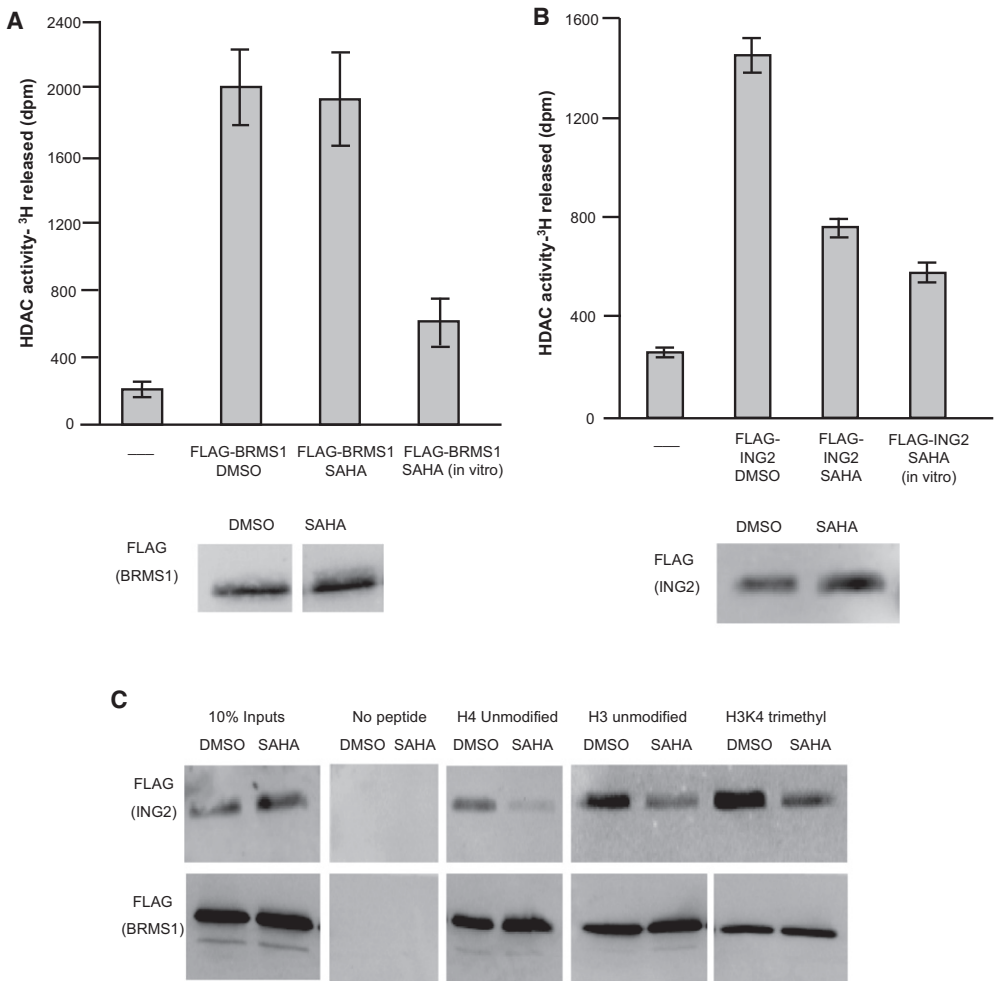

Figure 1. SAHA Alters the Biochemical Properties of the Sin3 Complex

(A and B) HDAC assays were performed on ^{3}H acetylated core histones with (A) FL-BRMS1-purified complexes (top panel) or (B) FL-ING2-purified complexes (top panel). DMSO and SAHA labels indicate that complexes were purified from 293T cells treated for 9 hr with these compounds. The SAHA (in vitro) label indicates that complexes were purified from untreated cells and that SAHA was added directly to the deacetylation reaction. Error bars in (A) and (B) represent ± standard deviation. Amounts of complex used in the assays were normalized to levels of (A) FL-BRMS1 (lower panel) or to levels of (B) FL-ING2 (lower panel).

(C) Western blot analysis of FLAG-tagged proteins bound to histone peptides.

these multisubunit complexes. The Sin3 complex is a 1.2 MDa complex implicated in cell cycle control through its interactions with the tumor suppressor protein Rb and can repress E2F-mediated transcription to prevent progression to S phase (Lai et al., 2001). The Sin3 complex is also implicated in controlling progression through the G2 phase of the cell cycle (David et al., 2003; Pile et al., 2002). Therefore, this complex is among the potential targets of HDACis that could mediate the growth arrest by these molecules. We set out to determine if HDACis had any effects on the multisubunit Sin3 complex, and if the complex was still intact after the HDACs were bound to the inhibitors.

RESULTS

ING2-Purified Complexes Are Altered by HDACis

To determine if HDACis alter the properties of the Sin3 complex, we purified the complex from 293T cells that stably expressed tagged subunits. We used two different known subunits of the Sin3 complex as baits for these purifications, inhibitor of growth 2 (ING2), which binds to H3K4 that is di- and trimethylated through its PHD finger, and breast cancer metastasis suppressor 1 (BRMS1), which has an unknown function in the complex (Doyon et al., 2006; Meehan et al., 2004; Shi et al., 2006). The *ING2* gene is deleted in some head and neck carcinomas, whereas BRMS1 is important for suppressing cancer metastasis, suggesting that their roles in the Sin3 complex could be related to cell growth and cancer progression (Seraj et al., 2000; Sironi et al., 2004). We performed purifications from cells treated with the HDAC inhibitor SAHA (7.5 μM) or DMSO and tested if there were differences in the HDAC activities of the complexes. Sin3 complexes purified through the BRMS1 subunit from SAHA-treated cells still had HDAC activity on acetylated core histones, suggesting that the inhibitor was lost during the purification (Figure 1A). This result is consistent with kinetic analyses of these competitive inhibitors (Sekhavat et al., 2007). These complexes were

still sensitive to SAHA, however, because adding inhibitor in vitro to the HDAC assay reduced catalytic activity (Figure 1A). By contrast, complexes purified through ING2 from SAHA-treated cells had reduced HDAC activity, similar to that observed when SAHA was added directly to the HDAC assay (Figure 1B). We wondered if this was due solely to a change in catalytic activity, or if it also affected the ability of the complex to bind directly to histones.

To ask if histone binding was disrupted, we tested the binding of Sin3 complexes purified from SAHA/DMSO-treated cells to histone peptides. The Sin3 complex preferentially binds to hypoacetylated histones through the RbAp46/48 subunits (Vermeulen et al., 2004; Yoon et al., 2005). It can also be recruited to chromatin through the H3K4-di/trimethyl mark by ING1/2 (Pena et al., 2008; Shi et al., 2006). Therefore, we tested the ability of the complexes to bind unmodified and H3K4 trimethylated peptides. We found that complexes purified through the ING2 subunit in the presence of SAHA were compromised in their ability to bind to histone peptides (Figure 1C). By contrast, complexes purified through BRMS1 retained the ability to bind to histone peptides after SAHA treatment (Figure 1C). Thus, Sin3 complexes purified through ING2 from cells treated with SAHA lost HDAC activity and histone-binding ability, whereas the BRMS1 purified complexes did not.

HDACis Cause ING2 to Dissociate from the Sin3 Complex

To elucidate the molecular basis for the altered properties of ING2-purified Sin3 complexes from cells treated with SAHA, we examined the composition of the complexes. Silver-stained gels of the purified complexes revealed that ING2 purified from SAHA-treated cells had reduced levels of other Sin3 complex subunits associated with it (Figure 2A). Multidimensional protein identification technology (MudPIT) (Paoletti et al., 2006) and western blot analysis confirmed that this loss included reductions in HDAC1, Sin3a, SDS3, and other known subunits (Figure 2B; see Table S1 available online). This effect was not limited to SAHA, because other HDACis, including TSA and a cyclic tetrapeptide, apicidin, also dissociated FLAG-ING2 from Sin3 complex subunits; however, sodium butyrate and valproic acid did not (Figures S1A and S1B). Therefore, specific deacetylase inhibitors can mediate the dissociation of ING2 from the Sin3 deacetylase complex.

The tagged bait protein ING2 was overexpressed in these initial experiments; therefore, we tested if the association of endogenous ING2 with the Sin3 complex was changed after SAHA treatment of 293T cells. We analyzed Sin3 complexes purified through BRMS1 and an additional subunit, BRMS1-LIKE (Nikolaev et al., 2004), by SDS-PAGE. BRMS1 purifications from cells treated with SAHA for 9 hr showed reduced silver staining of a band at ~33 kDa, which is the predicted size of ING2 (Figure 2C). Western blots and MudPIT analysis confirmed a reduction of endogenous ING2, but not ING1, in the BRMS1 and BRMS1-LIKE purifications (Figures 2D and 2F; Table S2), and this occurred as early as 3 hr postdrug treatment (Figure S1C). Total nuclear levels of ING2 and HDAC1 were not changed by SAHA treatment (Figure 2E). Thus, the ING2 protein was still intact in SAHA-treated cells, and SAHA was causing its dissociation from the Sin3 complex.

SAHA and other HDACis have been shown to effectively halt the growth of many cell types, including breast cancer cells (Huang and Pardee, 2000). Therefore, we wanted to know if the dissociation of ING2 also occurred in cancer cells. We treated MDA-MB-231 breast carcinoma cells with DMSO or SAHA and performed immunoprecipitations with antibodies to endogenous ING2 or HDAC1. After treatment of these cells with SAHA, the amount of ING2 that coprecipitated with HDAC1 was reduced (Figure 2G). Conversely, the amount of HDAC1 coprecipitated with ING2 was also reduced (Figure 2G). Thus, ING2 and HDAC1 were also dissociated after SAHA treatment of breast carcinoma cells, suggesting that this effect is a consequence of HDACi treatment in diverse cell types.

Dissociation of ING2 Occurs through a Direct Mechanism

HDACis cause an accumulation of acetylated proteins in the cell. This hyperacetylation raised the possibility that a subunit of the Sin3 complex became acetylated during SAHA treatment, which could be responsible for ING2 dissociation. We tested if acetylated proteins were detected in the SAHA-treated purifications; however, we did not detect acetylated lysines in any Sin3 complex component after SAHA treatment (Figures S2A and S2B) This finding is consistent with a recently published study on the acetylome (Choudhary et al., 2009) that did not find large increases in the acetylation of Sin3 subunits after HDACi treatment. Next, we asked if the dissociation between ING2 and the Sin3 complex could occur in vitro. We treated 293T whole-cell extract with a panel of HDACis and again observed the dissociation between ING2 and the Sin3 complex with SAHA, TSA, and apicidin (Figures 3A and 3B; Figure S1D). The addition of Acetyl-CoA in the presence of SAHA to the whole-cell extract did not enhance the dissociation of ING2 (Figure S2C). Finally, we tested if histone acetyltransferase (HAT) inhibitors could prevent ING2 dissociation from the Sin3 complex. However, we found that SAHA and TSA still caused dissociation of ING2 from the complex in a whole-cell extract in the presence of a HAT inhibitor (Figure S2D). Together, the results suggest that acetylation is not needed for ING2 dissociation to occur.

Because ING2 can tether the Sin3 complex to chromatin, we wondered if chromatin binding by ING2's PHD finger was necessary for SAHA to mediate the dissociation. We expressed an ING2 lacking its PHD finger in 293T cells. This deletion mutant was previously shown to maintain association with the Sin3 complex (Shi et al., 2006). We found that ING2 lacking its PHD finger still dissociated from the Sin3 complex, after cells were treated with SAHA (Figure S2E). Consistent with this result, we also found that TSA did not prevent recombinant ING2 from binding to an H3K4 trimethylated peptide (Figure S2F). Together, the data suggest that neither acetylation nor chromatin binding by ING2 are necessary for HDACi-mediated dissociation from the Sin3 complex.

To determine if ING2 dissociation from the Sin3 complex might be a direct consequence of the HDAC binding to the inhibitor, we asked if the dissociation could occur with purified Sin3 complex. To test this hypothesis, we immobilized the Sin3 complex purified through the BRMS1 subunit to FLAG beads and incubated the complex with increasing amounts of TSA. We found that the addition of TSA to the purified complex resulted in dissociation of

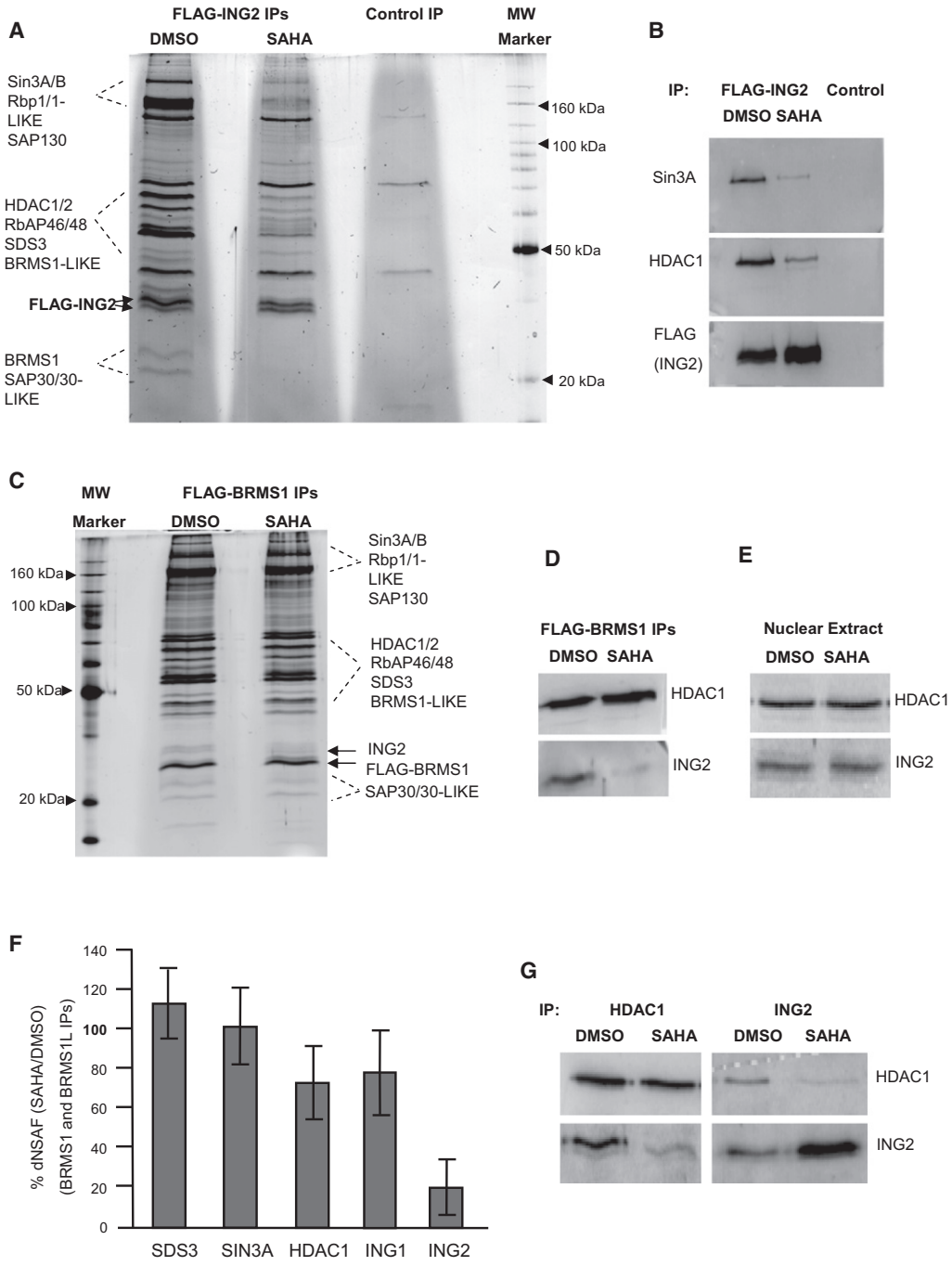

Figure 2. HDAC Inhibitors Dissociate ING2 from the Sin3 Complex

(A) Silver stain analysis of FL-ING2 complexes purified from DMSO or SAHA-treated 293T cells (7.5 μM SAHA or an equal volume of DMSO was added to the cells for 9 hr).

(B) Western blot analysis of FL-ING2 immunoprecipitations from (A) in the presence of SAHA.

(C) Silver-stained gels of FL-BRMS1 complexes purified from DMSO- or SAHA-treated 293T cells (7.5 μM SAHA or an equal volume of DMSO was added to the cells for 9 hr). The arrows indicate the locations of endogenous ING2 and FLAG-BRMS1.

(D) Western blots of FLAG-BRMS1 purifications from (C).

(E) Western blots of nuclear extracts from DMSO- or SAHA-treated 293T cells.

(F) Relative percent dNSAF (distributed normalized spectral abundance factor) of proteins in SAHA versus DMSO purifications as determined by MudPIT analysis. Error bars represent ±1 standard deviation.

(G) Western blots of immunoprecipitated proteins from MDA-MB-231 breast carcinoma cells treated with 7.5 μM SAHA or DMSO for 9 hr.

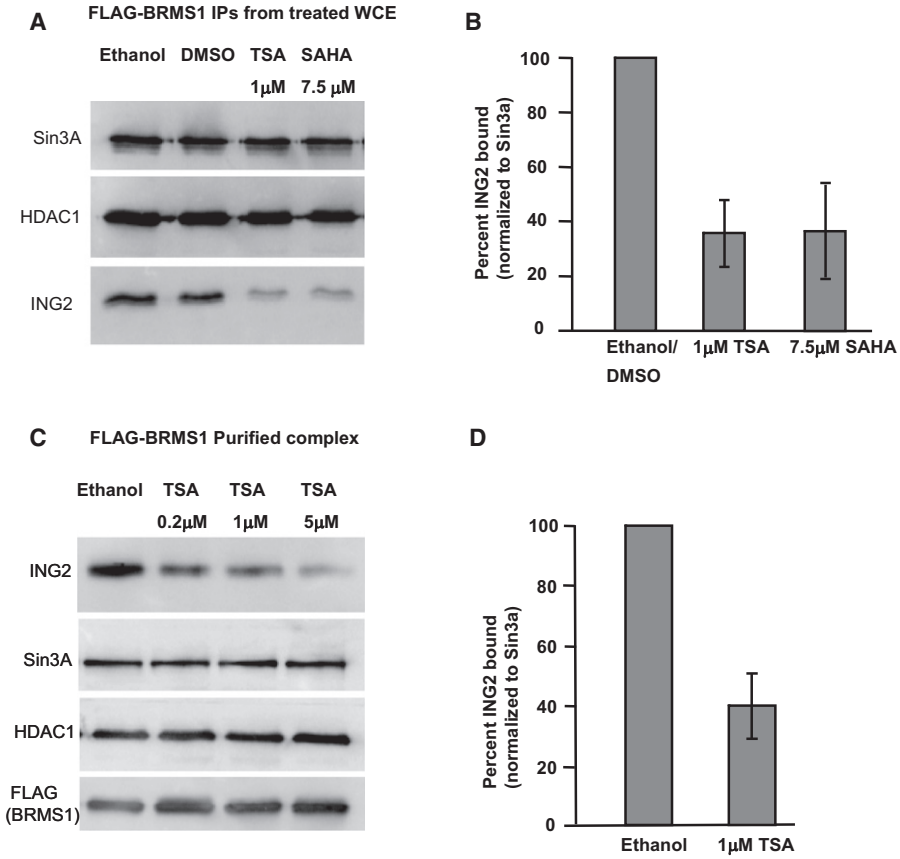

Figure 3. ING2 Dissociates through a Direct Mechanism

(A) HDACis or control compounds were added to whole-cell extracts from FLAG-BRMS1-expressing 293T cells, purified with FLAG affinity beads, and then probed by western blot for the indicated proteins.

(B) Quantification of (A).

(C) FL-BRMS1-purified complex was immobilized on FLAG beads and treated with TSA or control (ethanol) and then probed by western blot for indicated proteins.

(D) Quantification of (C). Bars in (B) and (D) represent the average of three experiments expressed as percent band intensity of ING2 in SAHA- or TSA-treated extract or complex compared to the intensity in the control treatment, normalized to intensity of Sin3a. Error bars represent ±1 standard deviation.

endogenous ING2 (Figures 3C and 3D). This finding suggests that the dissociation of ING2 could be mediated by a direct/physical disruption that does not require additional factors in cell extracts or living cells. Thus, it appears that binding of the small-molecule inhibitors to the catalytic sites of the HDAC enzymes leads to physical dissociation of ING2 from the Sin3 complex.

SAHA Disrupts Sin3 Complex Binding at p21 through Dissociation of ING2

The Sin3 complex contains several proteins that can recruit or retain it at chromatin. These include RbAp46/48, SAP30 and SAP30-LIKE, Sin3, and ING1 and ING2 (Shi et al., 2006; Vermeulen et al., 2004; Viiri et al., 2009; Yoon et al., 2005). Therefore, ING2 is one of the many subunits through which the Sin3 complex can be tethered to chromatin. Because HDACis were effective at dissociating ING2 from the Sin3 complex, we hypothesized that SAHA could cause changes in occupancy of the Sin3 complex at promoters where binding was dependent on ING2. The cyclin-dependent kinase inhibitor p21 is a tumor suppressor that is transcriptionally induced in response to HDACis (Richon et al., 2000; Smith and Workman, 2009). Previous studies have shown that the occupancies of HDAC1 and HDAC2 were reduced at the *p21* TATA after HDAC inhibitor treatment (Gui et al., 2004; Lin et al., 2008). To test if this might be due to ING2 dissociation, we performed chromatin immunoprecipation (ChIP) in 293T cells. We found that binding of ING2 and the Sin3 complex were enriched at the *p21* TATA region compared to intron 1 in control-treated cells (Figure 4A). SAHA treatment for 10 hr caused a reduction in ING2, HDAC1, and SAP30 occupancies (Figure 4A), but did not affect the levels of H3K4 trimethylation (Figure 4B) or H3 occupancy (Figure 4C) at the *p21* TATA. These results are consistent with our biochemical studies indicating that ING2 is dissociated from the Sin3 complex in the presence of SAHA.

Because the Sin3 complex can be recruited and tethered to chromatin through multiple subunits, we tested if occupancy of the Sin3 complex was dependent on ING2 at *p21*. To do this experiment, we assessed the occupancy of the Sin3 complex in ING2 knockdown cells and compared this to control cells expressing noneffective shRNA. As expected, the occupancy

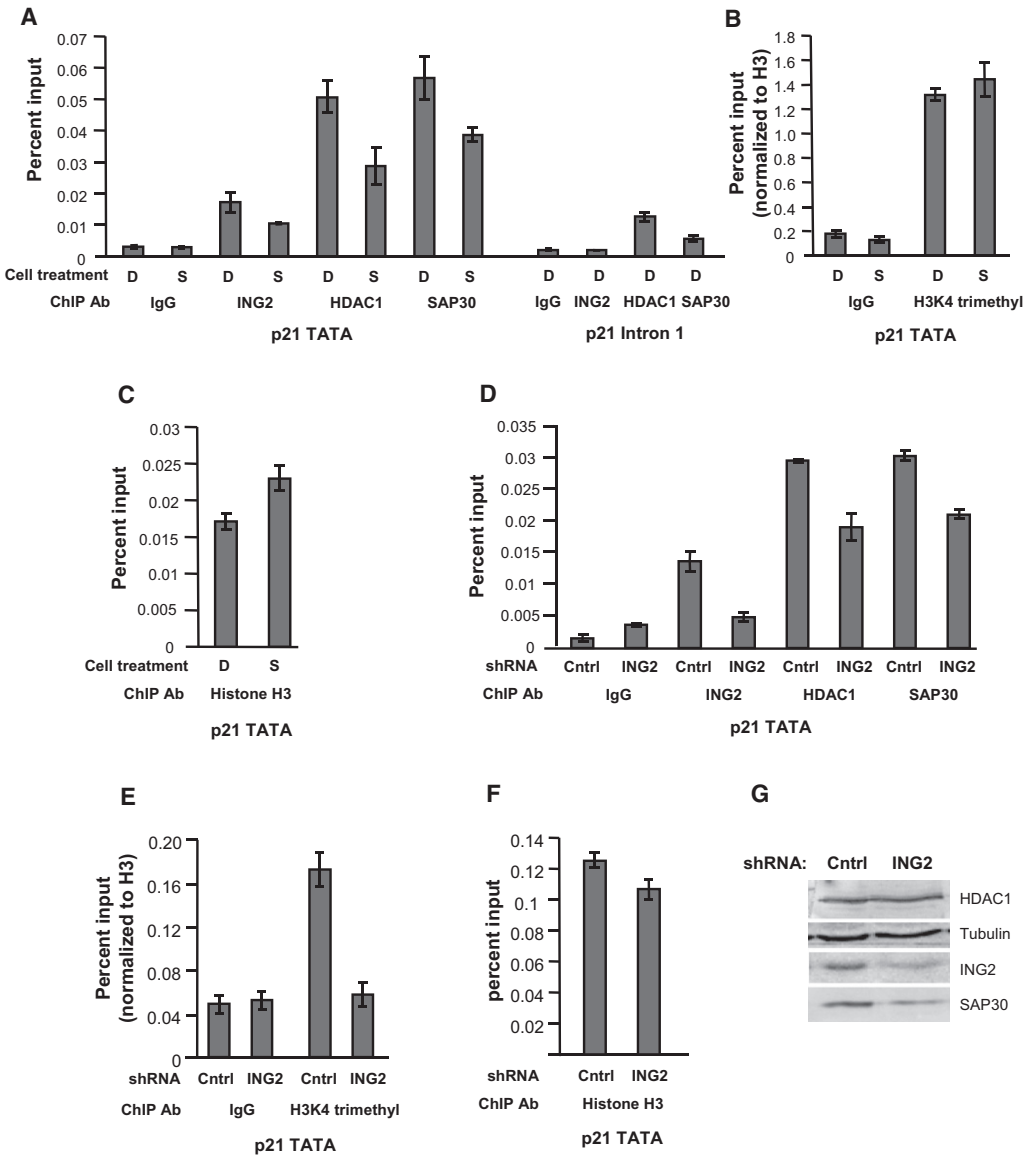

Figure 4. ING2 Dissociation Causes the Loss of the Sin3 Complex from the p21 Promoter

(A–F) (A–C) ChIP was performed in 293T cells treated for 10 hr with DMSO (D) or SAHA (S). (D–F) ChIP was performed in ING2 shRNA knockdown or noneffective control (cntrl) shRNA knockdown 293T cells. Bars in (A)–(F) show the average from a representative experiment with primers adjacent to the *p21* TATA or in intron 1 of p21. As a control in (B) and (E), percent inputs were normalized to the amount of H3 immunoprecipitated for each treatment condition or cell line. Error bars represent ±1 standard deviation of triplicate or quadruplicate real-time PCR reactions.

(G) Total nuclear extracts from the ING2 knockdown cells or GFP shRNA control cells were probed for the indicated proteins.

of ING2 at the *p21* TATA was reduced in ING2 knockdown cells (Figure 4D). Interestingly, the level of H3K4 trimethylation at this region was also reduced in the ING2 knockdown line (Figure 4E), but occupancy of H3 was not altered (Figure 4F), suggesting that ING2 binding could protect this region from demethylation. The occupancies of HDAC1 and SAP30 were also reduced at *p21* in the ING2 knockdown cells compared to the control cell line, showing that they were dependent on ING2 (Figure 4D). Total levels of HDAC1 were not changed in the ING2 knockdown cells; however, SAP30 protein levels were reduced in the ING2 knockdown line, but not in cells treated with SAHA (Figure 4G; data not shown). Thus, SAHA treatment promotes the loss of the Sin3 complex from the *p21* promoter in vivo through the dissociation of the ING2 subunit (Figures 5A and 5B).

DISCUSSION

Previous studies showed that the catalytic activities of HDACs are important for their association with other proteins (Hassig et al., 1998; Matsuoka et al., 2007). Catalytically inactive mutants of HDAC1 do not associate with RbAp48 or Sin3 (Hassig et al., 1998). However, here, we find that SAHA, the first FDA-approved

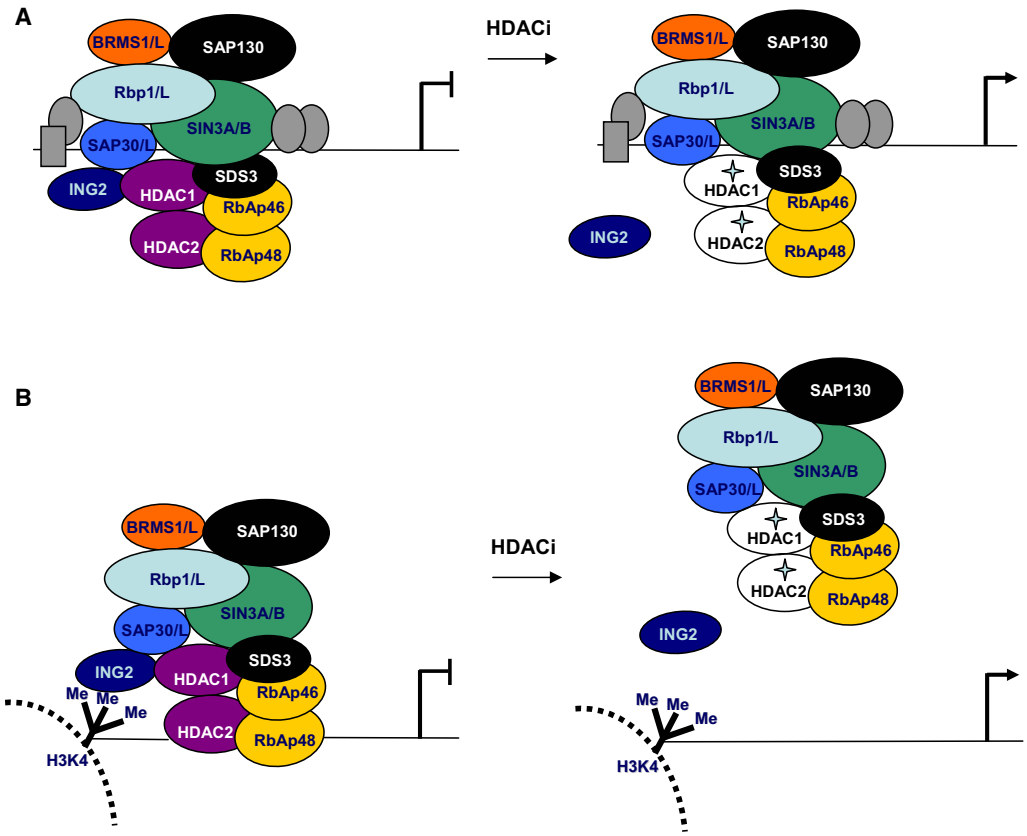

Figure 5. Models for Gene Activation after HDAC Inhibitor Treatment

(A) The Sin3 complex can be tethered to chromatin through interactions of Rbp1 or Sin3 subunits with transcription factors (gray ovals and rectangle) and through SAP30/SAP30-LIKE and RbAp46/48 subunits. At these promoters, HDACis cause inactivation of the HDACs and dissociation of ING2 from the complex.

(B) ING2 is required for tethering the Sin3 complex at some promoters where H3K4 is di-/trimethylated. At these regions, HDACi treatment causes inactivation of the HDACs as well as dissociation of the Sin3 complex from chromatin.

HDACi for cancer treatment, does not alter HDAC1 or HDAC2 interactions with the Sin3 complex, consistent with findings from a previous study (Sekhavat et al., 2007). Instead, we find that HDACis affect the association of a nonenzymatic subunit important for chromatin targeting, ING2.

The inhibitor of growth family of proteins contains five known members in humans. ING1 and ING2 reside in Sin3 deacetylase complexes, whereas ING3, ING4, and ING5 are in HAT complexes (Doyon et al., 2006). It is interesting that all of these proteins are thought to bind the same methylated histone mark, H3K4 that is di- or trimethylated. It is unclear what controls which ING protein will be bound to this epigenetic mark at a given time, and, therefore, whether a HAT or HDAC complex will be recruited.

ING family members are of potential therapeutic interest (Unoki et al., 2009). ING2 is of interest because of its role in modulating p53 activity (Menendez et al., 2009; Nagashima et al., 2001). In addition, ING2 can recruit the repressive Sin3 complex to the *cyclin D1* promoter after DNA damage (Shi et al., 2006). Here, we find that ING2 is also functionally targeted by HDACis, and that HDACis cause ING2's dissociation from the Sin3 HDAC1/2 complex. ING2 is likely required for Sin3 complex occupancy at a specific subset of Sin3 target genes. Therefore, the dissociation of ING2 is one possible mechanism that contributes to gene expression alterations after HDAC inhibitor treatment (Figure 5).

We have tested several possibilities to explain the mechanism by which ING2 dissociates from the Sin3 complex. We found that TSA can cause ING2 to dissociate from purified Sin3 complex in vitro and does not require additional factors in the cell. In addition, we also showed that the dissociation does not require acetylation of specific proteins, nor does this dissociation involve the PHD finger of ING2 directly. We also showed that not all inhibitors are effective at dissociating ING2, suggesting that dissociation is not simply due to the inhibition of catalytic activity of the HDACs. Interestingly, the inhibitors that do mediate the dissociation (TSA, SAHA, and apicidin) are larger and bulkier than those that do not (Na butyrate, valproic acid), suggesting that the ability to dissociate ING2 may involve the structure of the inhibitors themselves. All of these results together suggest that ING2 dissociates through a direct effect of the HDACs binding to the inhibitors in the context of the complex.

Our studies leave open the possibility that a conformational change occurs in the complex after inhibitor binding. Showing this change will require further structural studies of the HDACs

in the contexts of their complexes. To date, structural studies have only been carried out on isolated deacetylases in complex with inhibitors (Finnin et al., 1999; Schuetz et al., 2008; Vannini et al., 2004). Future studies will have to address several questions. First, it is unclear how the multisubunit complexes are assembled. Second, it is not known if the HDACs undergo structural changes when they are assembled in the complexes. Third, it is also unclear if inhibitor binding alters HDAC conformation in the context of their complexes. Our results suggest that the HDACs bind to the inhibitors, and that this causes a conformational change in at least part of the Sin3 complex, which is sufficient for dissociation of ING2. Because the Sin3 complex largely stays intact and is active without ING2, this suggests that ING2 is not needed for the integrity of the complex as a whole or for catalytic activity. The data also suggest that ING2 resides on an outer surface of the complex and support a main role for ING2 in targeting the Sin3 complex to chromatin.

ING2 is considered a stable subunit of the Sin3 complex; however, we show here that dynamic interactions can occur between ING2 and the Sin3 complex, and that this association can be perturbed by HDAC inhibitors. ING2 has the ability to dynamically respond to several cellular signals, including phosphoinositides and DNA damage (Gozani et al., 2003; Shi et al., 2006). The model proposed by Shi et al. (2006) suggests that ING2 can recruit the Sin3 complex for immediate gene repression when needed. Our results suggest that HDACis cause the physical release of the Sin3 complex from promoters through dissociation of ING2. This may be an important step that allows recruitment of other factors and gene activation. Overall, our results implicate ING2 and the Sin3 complex in mediating the cellular response to HDAC inhibitors.

Disrupting just the ING2/Sin3 complex interaction should lead to a smaller subset of gene expression changes than treatment with pan-HDACis. Our results raise the question of whether modulating the ING2/Sin3 interaction may be sufficient to have any anticancer growth effects. It will be interesting to test if reduction of ING2 protein levels is sufficient for induction of *p21* and other genes associated with SAHA-mediated growth inhibition. If so, disrupting this interaction may be of therapeutic value, either alone or in combination with other chemotherapeutic agents. Together, our findings reveal that the Sin3 complex is among the in vivo targets for HDACis and describe a new mechanism by which HDACis can alter HDAC function.

SIGNIFICANCE

HDACis, such as SAHA, are being tested as treatments for cancers and neurodegenerative diseases. Despite the therapeutic successes of these molecules, we are just beginning to understand how they work. The recognized mode of action for these drugs is via binding to their target enzymes, HDACs, and catalytically inhibiting their activity. This inhibition, in turn, leads to changes in the expression of specific genes and produces a gene expression program that is overall favorable for cell cycle arrest, apoptosis, or differentiation. Unexpectedly, our findings here show that HDACis can also disrupt critical protein-protein interactions between HDACs 1 and 2 and ING2, a protein responsible for recruitment of the Sin3/HDAC1/2 complex to chromatin. Abrogation of this interaction disrupts the targeting of the Sin3/HDAC complex to chromatin. Therefore, HDACis disrupt HDAC function not only by inhibiting HDAC catalytic activity, but also by disrupting HDAC complex subunit composition and chromatin targeting. These findings reveal that these small-molecule inhibitors exert their effects through multiple molecular modes of action.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture

293T cells stably expressing FLAG-tagged subunits of the Sin3 complex were made by using the Flp-In system (Invitrogen). 293T FRT cells were a gift from Drs. Joan and Ron Conaway. MDA-MB-231 cells were obtained from American Type Culture Collection. All cell lines were maintained in DMEM (GIBCO) supplemented with 10% FBS, Pen/Strep, and Glutamax (GIBCO) in a humidified atmosphere at 37°C. For experiments involving drug treatment, HDACis or appropriate vehicles (ethanol or DMSO) were added directly to the culture media, and cells were collected at the indicated times. TSA and SAHA were purchased from Cayman Chemical or BioVision, Inc., and apicidin, valproic acid, and sodium butyrate were purchased from Sigma.

Purifications

293T whole-cell extracts were made by using a high-salt extraction method (Mahrour et al., 2008). Anti-FLAG M2 agarose resin was added overnight to the soluble protein fraction with rotation according to manufacturer's instructions (Sigma). Complexes were eluted with 3× FLAG peptide and then analyzed or used for MudPIT analysis or in biochemical assays. In purifications with drug treatment, HDACis were included at all steps of the purifications, except the elution step.

HDAC Assays

HDAC assays were performed with purified HDAC complexes, essentially as described (Meehan et al., 2004), except HeLa core histones acetylated by yeast SAGA were used as a substrate, and reactions were incubated for 1 hr at 37°C. DMSO or SAHA (7.5 μM) were included in the HDAC assays where indicated.

Peptide-Binding Assays

Biotinylated histone peptides were either purchased from Upstate Biotechnology or were a gift from Dr. Matthias Mann (Max-Planck Institute). Binding reactions were performed in 150 mM NaCl in the presence of 0.1% Triton X-100 as described (Shi et al., 2006). For each assay, the entire bound sample was run on SDS-PAGE.

In Vitro Dissociation Experiments

HDACis and Garcinol (where indicated) were added to whole-cell extracts from 293T cells stably expressing FLAG-BRMS1. FLAG resin beads were added concurrently with the HDAC/HAT inhibitors. In experiments with Acetyl-CoA, HDACis and Acetyl-CoA were added at the same time with FLAG resin to whole-cell extracts from 293T cells stably expressing FLAG-ING2. In both experiments, the immunoprecipitations were incubated at 4°C overnight with rotation. The next day, unbound protein was removed, and beads were washed four times with Buffer A (10 mM HEPES [pH 7.5], 0.2% Triton X-100, 300 mM NaCl, 10 mM KCl, and 1.5 mM $MgCl_2$). For dissociation with purified complex, FL-BRMS1 complex was purified from untreated whole-cell extracts, immobilized on FLAG beads, and washed four times with Buffer A. Beads were then incubated in Buffer B (10 mM HEPES [pH 7.5], 0.05% Triton X-100, 150 mM NaCl, and 1.5 mM $MgCl_2$) with ethanol or TSA for 2 hr at 30°C with rotation. Unbound protein was removed, and beads were washed four times with Buffer A.

Plasmids

Full-length cDNAs encoding human BRMS1, BRMS1L, or ING2, or ING2 lacking amino acids 208–280 (ING2ΔPHD) were cloned into pcDNA5/FRT

(a gift from Drs. Joan and Ron Conaway) with a single N-terminal (BRMS1, ING2, ING2ΔPHD) or C-terminal (BRMS1L) FLAG tag. HuSH 29-mer shRNA constructs against ING2 (cat# TG312145) or noneffective shRNA (cat# TR30008) were purchased from Origene Technologies and stably expressed in 293T cells.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described by Upstate Biotechnology, Inc., except antibodies were first bound to preblocked Protein A Sepharose (Sigma) or Protein G Sepharose (Amersham) in binding buffer (5 mM Tris [pH 7.5], 250 mM NaCl, 1 mM EDTA, 0.05% NP-40). ChIPs were performed at least three independent times.

Antibodies

Antibodies used for ChIP, western blot, and immunoprecipitations are as follows: anti-acetyl H4 (Upstate, 06-866), HDAC1 (Abcam-Ab7028), ING2 antibody (a gift from Dr. Or Gozani [Stanford] or purchased from ProteinTech Group, Inc.; cat# 11560-1-AP), H3 antibody (Abcam-Ab1791), H3K4 trimethyl (Abcam-Ab8580), Sin3a (Abcam-Ab3479), Sap30 (Upstate-06-875), acetylated-lysine antibodies (Cell Signaling Technology, cat# 9681, cat# 9441), FLAG-HRP (Sigma).

Real-Time PCR

PCR was performed with a BioRad iCycler machine with SYBR green. Cycling conditions are as follows: 3 min at 95°C, then 41 cycles of: 10 s at 95°, 30 s at 55° or 60°, and 30 s at 72°, then followed by a melt curve. A standard curve of input DNA was used to determine relative ChIP sample abundance. Each sample was run in triplicate or quadruplicate. Primers adjacent to the p21 TATA or in intron 1 (negative control) were used for real-time PCR of ChIP samples. Sequences of primers are as follows: +85p21TATA-F: 5′-GATTCG CCGAGGCACCGAGGCA-3′, +218p21TATA-R: 5′-GAACACGCATCCTCGCG GACAC-3′, p21-IN1-F: 5′-GTGCCTGCCTAGATCCTAGTCCT-3′, p21-IN1-R: 5′-GGAGACACACTGGTATGTTTGAA-3′.

Quantification of Signal Intensity on Western Blots

Western blots were developed with ECL-Plus (GE Healthcare) and then scanned on a Typhoon 9400 imaging system. Image Quant V 5.2 was used to quantify bands by the volume integration method. Background was normalized by using a local average, and a volume report was generated. ING2 band intensity was normalized to Sin3a band intensity per lane.

SUPPLEMENTAL INFORMATION

Supplemental Information include two figures, two tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.chembiol.2009.12.010.

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