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Class I HDACs Affect DNA Replication, Repair, and Chromatin Structure: Implications for Cancer Therapy

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Abstract

Significance: The contribution of epigenetic alterations to cancer development and progression is becoming increasingly clear, prompting the development of epigenetic therapies. Histone deacetylase inhibitors (HDIs) represent one of the first classes of such therapy. Two HDIs, Vorinostat and Romidepsin, are broad-spectrum inhibitors that target multiple histone deacetylases (HDACs) and are FDA approved for the treatment of cutaneous T-cell lymphoma. However, the mechanism of action and the basis for the cancer-selective effects of these inhibitors are still unclear. **Recent Advances:** While the anti-tumor effects of HDIs have traditionally been attributed to their ability to modify gene expression after the accumulation of histone acetylation, recent studies have identified the effects of HDACs on DNA replication, DNA repair, and genome stability. In addition, the HDIs available in the clinic target multiple HDACs, making it difficult to assign either their anti-tumor effects or their associated toxicities to the inhibition of a single protein. However, recent studies in mouse models provide insights into the tissue-specific functions of individual HDACs and their involvement in mediating the effects of HDI therapy. **Critical Issues:** Here, we describe how altered replication contributes to the efficacy of HDAC-targeted therapies as well as discuss what knowledge mouse models have provided to our understanding of the specific functions of class I HDACs, their potential involvement in tumorigenesis, and how their disruption may contribute to toxicities associated with HDI treatment. **Future Directions:** Impairment of DNA replication by HDIs has important therapeutic implications. Future studies should assess how best to exploit these findings for therapeutic gain. *Antioxid. Redox Signal.* 23, 51–65.

Introduction

ONE KEY CONSEQUENCE of the cellular production of reactive oxygen species (ROS) is DNA damage. There are multiple origins of this damage, especially in cancer cells, where ROS can affect the levels or activity of DNA replication factors, the levels of deoxyribonucleotide triphosphates (dNTPs) due to changes in metabolism, and cause the formation of DNA adducts. These effects are mutagenic and they also cause DNA replication stress that results in DNA double-strand breaks (DSBs) and genomic instability (17). There are many links between histone deacetylases (HDACs) and the production of ROS and/or the downstream repair of these lesions. For example, HDAC inhibition may induce the accumulation of ROS (98, 99, 102, 122, 130). Downstream of ROS, HDACs play a key role in the ability of cells to repair

these DNA lesions either by unfolding compacted chromatin, modification of lysine acetylation on DNA repair factors, the regulation of DNA replication, or by the regulation of gene expression. The role of HDAC1, HDAC2, and HDAC3 in these processes and what we have learned by studying deletions of these genes in mice will be the focus of this review.

HDACs are divided into four classes based on their homology to yeast proteins (Fig. 1). Class I HDACs (HDAC1, 2, 3, and 8) are homologous to the yeast protein Rpd3, are predominantly nuclear, and are typically found in association with transcriptional repressor complexes. Class II HDACs (HDAC4, 5, 6, 7, 9, and 10) are homologous to the yeast Hda1 protein and are both nuclear and cytoplasmic. HDAC6 contains two catalytic domains, the second of which is specific for tubulin rather than histones, providing one specific example of how HDACs often have nonhistone as well as

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FIG. 1. Classification of the nonsirtuin HDAC families. Eleven human HDACs are pictured (class III HDACs, the sirtuins, are not included). Class I HDACs are largely nuclear and are related to the yeast protein Rpd3 within the conserved, deacetylase domain (DAC). Class II HDACs are related to the yeast Hda1 protein. Class IIa proteins have a number of N-terminal serine phosphorylation sites (S), which facilitate cytoplasmic shuttling. Class IIb HDACs contain tandem deacetylase domains (only a partial domain in the case of HDAC10). In addition, HDAC6 contains an SE14 repeat domain, important for its cytoplasmic retention, and a ubiquitin-binding zinc finger domain (PAZ domain). HDAC11 is the sole member of the class IV HDACs and shows homology with both class I and class II HDACs. HDAC, histone deacetylase.

histone substrates. The third class of HDACs contains the sirtuins, which are homologous to the yeast Sir2. The lone member of class IV is HDAC11, which shares homology with both class I and II HDACs.

The enzymatic activity of HDACs 1, 2, and 3 requires their association with multi-subunit repression complexes (Fig. 2). For instance, the catalytic activity of HDAC3 requires its association with the nuclear hormone co-repressor 1 (NCOR1), or its homolog, silencing mediator of retinoid, or thyroid-hormone receptors (SMRT or NCOR2). Recently, the structure of the complex between HDAC3 and the SMRT “deacetylase activating domain” (DAD) was solved and found to contain an essential inositol tetraphosphate molecule (Ins(1,4,5,6)P₄), which mediates the interaction of these two proteins (127) (Fig. 3). A subsequent study demonstrated a similar requirement of Ins(1,4,5,6)P₄ in HDAC1-containing repression complexes and further concluded that the presence of Ins(1,4,5,6)P₄ dramatically enhanced HDAC activity (75). Given the apparent requirement of Ins(1,4,5,6)P₄ for class I HDAC function, targeting of the Ins(1,4,5,6)P₄–HDAC/co-repressor interaction could provide a novel and selective node for therapeutic intervention, enabling the more specific targeting of class I HDACs.

Histone Acetylation and Its Regulation

Chromatin is a dynamic structure that enables the packaging of more than 5 billion base pairs of DNA into the cell nucleus, while also allowing context-specific accessibility to

FIG. 2. Recruitment of HDAC-containing repression complexes by the BCL6 proto-oncogene. Gene rearrangements involving BCL6 are commonly associated with diffuse large B-cell lymphoma. BCL6 represses target gene expression (A) through the recruitment of both HDAC1- and HDAC2-containing Sin3a complexes (B), as well as through the recruitment of HDAC3-containing NCoR/SMRT complexes (C). In addition, BCL6 is itself regulated by acetylation. Acetylation of BCL6 has been ascribed to both Sirtuin and HDAC activities (10). NCoR, nuclear hormone co-repressor; SMRT, silencing mediator of retinoid or thyroid-hormone receptors.

FIG. 3. The HDAC co-repressor association is mediated by Ins(1,4,5,6)P₄. Crystallography studies have solved the structure of the interaction between the SMRT DAD (upper left) and human HDAC3. These studies have revealed the requirement of the inositol tetraphosphate molecule [Ins(1,4,5,6)P₄, arrow] for both the stabilization and activation of the HDAC-corepressor complex (127). DAD, deacetylase activating domain.

factors controlling cellular processes such as DNA replication, DNA damage repair, and gene transcription. The basic unit of chromatin is the nucleosome, which is composed of 147 bp of DNA wrapped around a core histone octamer (two copies each of H3, H4, H2A, and H2B) (69). Histones undergo a number of post-translational modifications, including acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation, which occur predominantly on the N-terminal histone tails (29). These modifications regulate the organization and relative compaction of the chromatin by altering inter- and intra-nucleosomal contacts and by serving as docking sites for other chromatin-modifying proteins (22).

In particular, lysine acetylation is associated with chromatin relaxation through disruption of nucleosomal contacts (69). In addition, acetylated lysine residues within the conserved amino-terminal histone tails serve as binding sites for bromodomain-containing proteins, which further regulate chromatin structure and DNA-templated processes (32). Thus, the appropriate levels of histone acetylation should be tightly regulated by the opposing activities of histone acetyltransferases and HDACs in order to appropriately regulate the many cellular processes that require access to DNA. For instance, histone acetylation opens chromatin, making DNA accessible for activation of gene expression or DNA replication, while deacetylation of those same histones renders DNA more compact, facilitating gene silencing (69). In many cases, these same lysines are subject to other forms of modification, including methylation, which may be associated with gene silencing (*e.g.*, H3K9me3). Therefore, in instances such as these, lysines should first be deacetylated before other repressive marks may be added, thus creating a multilayered regulatory system (4, 38, 89). Remarkably, HDACs 1–3 deacetylate many of the same residues on the

histone tails, including H3K4, H3K9, H3K14, H3K27, H4K5, H4K8, H4K12, and H4K16 (13, 131). The lone exception appears to be H3K56ac, which is affected by inactivation of Hdac1 and Hdac2, but not Hdac3 (76, 128). The acetylation of specific histone lysines may have unique functions. For instance, residues such as H3K9ac are most often associated with active transcription; whereas H4K5 and H4K12 are associated with histone deposition during DNA replication.

HDACs Regulate DNA Replication

The idea that HDACs play a role in the regulation of replication and S-phase progression is not a new one; however, in many ways, it has been overshadowed by studies examining the involvement of HDACs in the regulation of gene expression. Yeast cells lacking the ortholog of mammalian class I HDACs, Rpd3, exhibited a delay in S-phase progression, which is consistent with the idea that class I HDACs play a prominent role in S-phase regulation (125). Furthermore, studies in mouse embryonic fibroblasts (MEFs) demonstrated that disrupting Hdac3 function causes the accumulation of S-phase-dependent DNA damage (11). Similarly, acute treatment of cancer cell lines with suberoyl anilide hydroxamic acid (SAHA) induced DNA damage, which co-localized with sites of active DNA replication (25), further suggesting that HDAC inhibition may promote DNA damage and subsequent cell killing by first inducing replication stress. Mechanistically, it is possible that HDACs act at the replication fork or regulate global changes in chromatin structure to affect DNA polymerase functions to cause DNA damage (Fig. 4).

HDACs affect DNA replication fork functions

While it is obvious that the inhibition of HDAC function has profound effects on S-phase progression and DNA replication, how HDACs function in the regulation of these processes is less obvious. During the S phase, dynamic

FIG. 4. Changes in chromatin structure and histone acetylation are linked to DNA replication. HDACs 1, 2, and 3 localize to replication forks, and the disruption of HDAC function slows replication fork progression. This could be through direct effects on the replication machinery, as replication factors such as PCNA are regulated through acetylation. Alternatively, newly synthesized histones are acetylated on H4K5K12 before deposition onto nascent DNA. Histone chaperones, such as CAF-1, are important for histone deposition and associate with HDACs. Furthermore, HDACs 1, 2, and 3 have been implicated in the removal of histone deposition marks (H4K5 and H4K12), which is critical for chromatin maturation and the disruption of which can contribute to replication stress. CAF-1, chromatin assembly factor 1; PCNA, proliferating cell nuclear antigen.

changes in the chromatin landscape occur both as chromatin is disrupted to make way for the replication of DNA polymerase and as chromatin structure is re-established behind the moving fork. In addition, nucleosome positioning and histone modifications may play a role in the selection, licensing, and firing of replication origins (4, 74, 125). For instance, the origin recognition complex (ORC) tends to occupy nucleosome-free regions of chromatin, while histone H4 acetylation by the acetyltransferase HBO1 facilitates minichromosome maintenance loading/pre-replication complex assembly at origins (78). Additional studies in yeast and flies have revealed that the HDAC, Rpd3, localizes to replication origins (61), where it deacetylates histones to impair origin firing (2). Consistent with this idea, loss of Rpd3 is associated with redistribution of the ORC2 (2) and the early activation of late firing origins (8, 125).

The impact of HDAC function on higher-order spatial reorganization of chromatin may also be important for DNA replication (26). For example, cohesin complexes are regulated by acetylation (109), are enriched at origins, and may facilitate the organization of origins into looping replication domains, which contain multiple origins firing almost simultaneously (41). Deregulation of chromatin structure throughout the process of DNA replication is associated with anomalies such as uncontrolled origin firing and replication fork collapse, each of which can promote DNA damage and genomic instability, thereby having catastrophic and potentially oncogenic consequences for the cell (4). Therefore, the ability to modulate chromatin structure is crucial for successful DNA replication, and it is possible that HDAC-targeted therapies impact replication through the deregulation of these chromatin structures.

While such general changes in chromatin architecture may play a role in the generation of replication stress after HDAC inhibition, disruption of HDAC functions may also directly affect replication, as very short treatment with histone deacetylase inhibitors (HDIs) still generated robust reductions in the rates of replication fork progression (112, 128). In cutaneous T-cell lymphoma (CTCL) cell lines treated with both broad-spectrum and HDAC3-selective inhibitors, the effects of HDI treatment on replication fork velocity were observed before the accumulation of global increases in histone acetylation (128). These results suggest that HDACs function to regulate replication (or local chromatin structure) on or around the replication fork. Since HDACs also have a multitude of nonhistone substrates, one possibility is that HDACs act directly on components of the replication machinery. In fact, replication factors themselves are subject to regulatory post-translational modifications, including acetylation. For instance, the ability of the replication processivity factor, proliferating cell nuclear antigen (PCNA) to associate with DNA polymerase is regulated by acetylation (86). Moreover, PCNA co-purified with HDAC1 (77, 86), suggesting that this enzyme may regulate PCNA acetylation status to modulate its function.

Some of the most compelling data implicating HDAC function in DNA replication come from a powerful technique called isolation of proteins on nascent DNA (iPOND). iPOND enables the isolation of newly synthesized DNA (*i.e.*, the replication fork) and the associated proteins (107). Consistent with the idea that HDACs function at or around the replication fork, this technique detected HDAC1, 2 and 3 at

sites of active DNA replication. Interestingly, while HDAC1 appeared to move with the replication fork, HDAC2 and HDAC3 more stably associated with nascent chromatin (108). This suggests that HDAC1 moves with the fork, while the other HDACs are associated with chromatin before and after the fork. While the disparate kinetics of nascent chromatin association implies that these HDACs may have different functions at the replication fork, a more detailed understanding of their activities there awaits further study.

HDACs function in histone deposition

Since DNA is replicated, chromatin structure should be re-established behind the replication fork. This requires the incorporation of both old and new histones into the newly generated chromatin fiber in a process known as histone deposition. Importantly, the disruption of histone deposition can have dramatic effects on the stability of the departing replication fork. In yeast, impaired nucleosome deposition due to depletion of H4 resulted in replication fork collapse, DNA DSBs, and even large chromosomal rearrangements (24). The deposition of newly synthesized histones onto nascent DNA is mediated by histone chaperone proteins such as chromatin assembly factor 1 (CAF-1). Disruption of CAF-1 function in U2OS cells resulted in the accumulation of DNA damage and S-phase cell cycle arrest, suggesting that histone chaperone function and proper histone deposition is critical for chromatin maturation and maintenance of genomic stability (133). Consistent with a role in histone deposition, HDACs 1, 2, and 3 associate with histone chaperones, including CAF-1 (3, 87, 121); so, histone deacetylation likely occurs soon after histone deposition.

New histones are marked by acetylation (*e.g.*, H4K5ac and H4K12ac) for deposition onto newly synthesized DNA. Treatment of cells with Depsiptide (FK228) prevented the removal of H4K5 and H4K12 acetylation marks from nascent chromatin (108), suggesting that HDACs are required for deacetylation of newly deposited histones. Genetic studies confirmed these results, as deletion of *Hdacs1/2* resulted in the accumulation of histone deposition marks in MEF models (131). In addition, *Hdac3*^{-/-} MEFs displayed an accumulation of histone deposition marks as well as DNA damage, which is consistent with the idea that defects in chromatin maturation behind the replication fork can affect genome stability (11). Therefore, HDAC1, 2, and 3 remove acetylation marks after the deposition of newly synthesized histones onto nascent DNA. Furthermore, the disruption of this chromatin maturation process on HDAC inhibition may contribute to the accumulation of DNA damage observed after HDI treatment.

HDAC function is required for maintenance of heterochromatin

Failure to remove histone deposition marks in a timely fashion has been associated with the loss of silencing at particular loci as well as disruption of pericentric heterochromatin (31, 116). HDACs 1, 2, and 3 have been implicated in the maintenance of heterochromatin domains (13, 46, 106, 136), and deletion of *Hdac3* in the murine liver caused increases in H3K9ac, a reduction in H3K9me3 (which is bound by heterochromatin proteins), and widespread losses of heterochromatin (13). These disruptions of histone acetylation

and heterochromatin structure in the absence of HDACs or after treatment with HDIs have been linked to aberrant mitoses, which can be associated with mitotic catastrophe and cell killing (54, 72, 115, 116). Likewise, treatment of *Drosophila melanogaster* embryos with an HDI in the absence of *de novo* transcription generated aberrant mitoses, suggesting that mitotic defects observed on HDAC inhibition occurred independently of HDI-mediated changes in gene expression (126). These effects could be due to loss of heterochromatin or loss of checkpoint control, resulting in a loss of Aurora B kinase activity (11, 67). In all, these studies suggest that aberrations in replication, histone deposition, and chromatin maturation behind the replication fork after HDAC inhibition may be associated with replication fork stalling, accumulation of DNA damage, disruption of heterochromatin, and genomic instability.

Replication Defects Likely Contribute to Cancer-Selective Cell Killing by HDIs

The idea that HDIs exert their anti-cancer effects by targeting replication is a tempting hypothesis, as the therapeutic window of traditional chemotherapies has been attributed to their ability to cause DNA damage after induction of replication stress. Consistent with this idea, the treatment of CTCL cell lines with Depsipeptide and the treatment of breast cancer cells with SAHA demonstrated an effect of HDAC inhibition on replication rates (25, 128). Furthermore, these reductions in replication rates were associated with the induction of DNA damage, and in the case of CTCL cells, an accumulation of cells in the S phase (128). Therefore, it is likely that the ability to hinder replication is an important mechanism contributing to the efficacy of HDAC inhibition.

In particular, specific targeting of HDAC3 through either a selective inhibitor or siRNAs impaired replication in two different cancer models (25, 128). These data are consistent with *Hdac3* gene deletion models, which demonstrated that apoptosis in the absence of *Hdac3* was associated with impaired S-phase progression and the accumulation of DNA DSBs rather than dramatic changes in gene transcription (11). Given that *Hdac3* is targeted by HDIs currently being used in cancer treatment (*i.e.*, SAHA, Depsipeptide), it is possible that some of the anti-tumor effects associated with these drugs are related to their ability to target DNA replication on HDAC3 inhibition in tumor cells. Furthermore, deletion of *HDAC1* and *HDAC2* caused dramatic genomic instability in transformed cells, suggesting replication defects (12, 42). This is consistent with the presence of these HDACs at replication forks and their established role in histone deposition (108).

Studies implicating HDACs in replication control further hint at a mechanism for the cancer-selective effects of HDAC inhibition. Targeting of replication and S phase would spare the largely noncycling normal cells, while causing the accumulation of DNA damage and, ultimately, cell death in the rapidly dividing cancer cell populations. In addition, while primary cells have intact cell cycle checkpoints, which may enable them to cope with the stress imposed by HDIs, the abrogation of these checkpoints that characterizes cancer cells may further sensitize them to the effects of HDAC inhibition. For instance, while SAHA treatment resulted in the accumulation of DNA damage in both normal and transformed cells, normal cells were able to efficiently repair that

damage, while cancer cell lines struggled to achieve efficient repair, ultimately resulting in cancer cell-specific cell death (65). Likewise, HDI-induced G₁ arrest after p21 induction (40, 49, 97) might protect normal cells from accumulation of S-phase-dependent DNA damage, while the disruption of either the G₁/S or intra-S-phase checkpoints frequently observed in cancer cells could result in either the failure to maximally induce p21 expression or the failure to arrest in its presence. This idea is supported by multiple reports suggesting that p21 induction by HDIs hampers their ability to induce apoptosis (16, 93, 100). Finally, inactivation of *Hdac3* in immortal cells caused a loss of Aurora B kinase activity that was not observed in normal cells (11, 67). These effects might explain why cancer cells often exhibit a G₂/M arrest after HDI treatment (54, 91, 101, 111).

In all, these studies suggest that altered cell cycle checkpoints found in cancer cells impair their ability to respond to HDI-induced genomic instability, and may account for the cancer-specific toxicity observed with these drugs. Furthermore, these studies suggest that replication stress and the resulting genomic instability likely contribute to the efficacy of HDAC-targeted therapies. However, other cellular processes, such as DNA repair, are also impacted by HDIs and may cooperate with HDI-induced replication stress to promote cell killing.

Loss of HDAC Function Impairs DNA Repair

Altered DNA repair has also been observed after HDI treatment, and it may be expected to cooperate with HDI-induced replication stress to further promote genomic instability and cancer cell killing. Furthermore, multiple lines of evidence suggest that HDACs directly participate in the repair process. In particular, HDAC1 and HDAC2 rapidly localize to DSBs, where they deacetylate H3K56 and H4K16 (27, 35, 76). This initial deacetylation has been proposed to facilitate DNA repair by maintaining the broken DNA ends in close proximity, as well as by inhibiting transcription in the area of the DSB (30). In addition, H4K16 deacetylation by HDAC1/2 favored repair by nonhomologous end-joining (NHEJ) compared with homologous recombination repair (HRR) by promoting 53BP1 binding (35, 47, 117). Consistent with a role in NHEJ, knockdown of HDAC1 and HDAC2 resulted in impaired DSB repair by NHEJ, increased γ H2AX, and hypersensitivity to ionizing radiation (IR) (76). *Hdac3*-deficient cells also exhibited defects in DNA repair. *Hdac3*-deleted fibroblasts showed increased and persistent γ H2AX foci after IR, suggesting a role for *Hdac3* in DSB repair (11). However, unlike knockdown of *HDAC1* and *HDAC2*, which seemed to affect NHEJ in particular, knockdown of *HDAC3* impaired both HR- and NHEJ-mediated repair (73). In addition, repair defects in *Hdac3*-deficient cells were characterized by an increase in H3K9/K14ac and a concomitant decrease in H3K9me3. This loss of H3K9me3 may hamper repair by preventing the recruitment of Tip60, which functions as a critical mediator of chromatin remodeling at DSBs, enabling repair factors to access the damaged DNA (113).

The effects on specific types of repair suggest that HDACs also impact repair by regulating the acetylation of key checkpoint and repair proteins. Treatment of cancer cell lines with SAHA or MS275 followed by immunoaffinity purification with anti-acetyl lysine and mass spectrophotometry identified changes in acetylation of a number of DNA

damage response (DDR) proteins, including p53, Ku70, FEN1, DNA-PK, and ataxia telangiectasia mutated (ATM) (92). Furthermore, HDAC1 associates with ATM and this interaction was increased on exposure to IR (60), again suggesting that HDACs may alter DNA repair by directly regulating the activity of repair proteins. In addition to direct regulation of repair protein activity, other studies have proposed that HDI treatment impairs DNA repair by down-regulating the expression of critical repair factors (98, 120). In all, the disrupted DDR observed on HDAC inhibition likely results from changes in histone acetylation as well as changes in repair factor recruitment, activity, and/or levels. In addition, attenuation of DNA repair after HDI treatment likely cooperates with HDI-induced replication stress to increase DNA damage accumulation and, ultimately, cancer cell death.

HDIs Alter Gene Expression, but Is It Important?

While the preceding sections focused on impaired replication as a potential mechanism of action for HDIs, the disruption of other chromatin-associated processes, including transcription, also likely contribute to the anti-tumor effects associated with the disruption of HDAC function (1, 43, 44, 58). In fact, in some cases, it seems likely that the HDIs exert their effects through altering transcriptional programs. For instance, in the case of acute promyelocytic leukemia containing the PLZF-RAR α fusion protein, it appears that combination therapy with all-trans retinoic acid (ATRA) and HDIs is required to restore normal retinoic acid receptor (RAR)-regulated transcriptional programs and, subsequently, normal promyelocyte differentiation and cell death (44). It remains possible, however, that such cases are the exception rather than the rule. For instance, multiple groups reported relatively modest changes in gene expression (5%–22% of genes) after HDI treatment, and while many transcriptional studies have been performed, no common gene expression signature has been identified that would trigger cell death (34, 48, 68, 79, 90, 114, 123).

In most gene expression studies, the cyclin-dependent kinase inhibitor p21 is commonly upregulated and has been associated with an HDI-induced G₁ arrest (40, 49, 97). While p21 expression is directly regulated by HDAC1 (40, 97, 137), p21 induction on HDAC inhibition is severely impaired in the absence of the critical DNA damage-sensing kinase, ATM (55). The idea that p21 induction on HDI treatment results entirely from a direct transcriptional effect is based largely on the fact that HDIs induce p21 expression even in the absence of p53 (49, 85, 110), but multiple mechanisms exist for the p53-independent induction of p21 in response to a variety of cellular stresses (6, 23, 103). Such observations support the idea that induction of p21 expression by HDIs, in addition to direct changes in acetylation at the p21 locus, could be related to DNA damage and/or DNA replication stress.

While histone acetylation is associated with active transcription, in most of these gene expression studies, just as many genes were downregulated as were up-regulated, suggesting that much of the altered transcription observed was not a direct result of gene-specific increases in histone acetylation after HDI treatment. Moreover, while HDIs display a good degree of selectivity for cancer cells, the treatment of normal cells with HDIs results in comparable numbers of transcrip-

tional changes though the specific genes altered may be different. However, two observations point toward a defect in replication rather than altered transcription as the mechanism of action for HDI therapies. First, the overexpression of p16^{INK4a} and associated G₁-phase cell cycle arrest in leukemia cells prevented SAHA-induced apoptosis without dramatically altering SAHA-induced changes in gene expression profiles (90). Second, serum starvation to arrest cells in G₀ also protected cells from HDI-mediated cell death, again supporting the idea that transcriptional changes which would be expected to occur in noncycling or G₁-phase cells may not be the primary mechanism of HDI-mediated cell killing (11). These relatively simple cell cycle studies support the hypothesis that HDIs induce cell death by targeting replication, as induction of G₁ arrest would prevent HDI-induced replication stress, the accumulation of DNA damage and/or loss of heterochromatin marks, and subsequent apoptosis. Finally, it is notable that *in vivo* mutagenesis of Hdac3 indicated that the deacetylase activity was not required for regulating transcription, suggesting that HDIs act by other means (109).

Toxicities Associated with Hdac1, Hdac2, and Hdac3 Inhibition

Just as multiple cellular processes are targeted by HDIs to promote cancer cell killing, multiple HDAC proteins are also targeted by these inhibitors, making it difficult to ascribe the effects of these drugs to the inhibition of a single protein. Simultaneously, while it is obvious that HDIs selectively target cancer cells compared with normal cells (134), their use is not devoid of toxicity, suggesting that normal tissues are also affected by these drugs. In particular, HDI treatment has been associated with neurological, cardiac, and a number of blood-related toxicities (14, 15, 92, 96). Gene-specific and tissue-specific mouse models have begun to shed light onto which HDACs may be responsible for both the efficacy and the toxicities associated with broad-spectrum HDAC inhibition (21, 36, 39, 52, 53, 63, 81, 95, 119, 132) (Table 1). In addition, while clinical trials are currently underway to determine the effectiveness of HDIs in the treatment of a number of tumor types, hematologic malignancies remain the only cancers for which these drugs have obtained FDA approval (94). Therefore, it is important to gain an understanding of the function of these proteins within the hematopoietic compartment, which is an ideal model system to study the function of the class I HDACs in stem cell biology and cellular differentiation, as well as to predict the contribution of these proteins to HDI-associated hematological toxicities. Finally, it is likely that HDAC functions in replication control and maintenance of genome stability contributes to the phenotypes observed in mouse deletion models. For instance, replication stress was recently observed in a model of *Hdac3* deletion in hematopoietic stem cells (112).

Although germ line deletion in mice yields a much more severe phenotype than transient inhibition using HDIs, these genetic experiments have provided key insights. Biochemically, HDAC1 and HDAC2 often have the same activity and co-purify. However, while deletion of *Hdac1* was lethal by embryonic day 9.5, *Hdac2*-deficient mice exhibited perinatal lethality associated with cardiac defects, suggesting differences in Hdac1/2 function during development. Interestingly, cardiac-specific *Hdac2*-deletion did not result in a phenotype.

TABLE 1. TISSUE-SPECIFIC EFFECTS OF HISTONE DEACETYLASE TARGETING

	<i>Tissue</i>	<i>Cre-driver</i>	<i>Phenotype</i>	<i>Ref.</i>
Hdac1	Germline T cells	N/A CD4-Cre	Embryonic lethal E9.5–10.5; proliferative defects Increased inflammation and proliferation in allergy model	(51, 66) (29)
Hdac2	Germline Neuronal Brain	N/A Nestin-Cre GLAST::CreER T2/ZEG	Perinatal lethality; cardiac defects Enhanced memory formation Apoptosis during adult neurogenesis	(66, 107) (32) (44)
Hdac1 and Hdac2	CNS	GFAP-Cre	Increased proliferation of neural progenitors and differentiation block	(67)
	Oligodendrocytes	Olig1-Cre	Neonatal lethal, required for proper oligodendrocyte specification and differentiation	(109)
	PNS	DHH-Cre	Schwann cell apoptosis and associated loss of myelination	(16, 43)
	Heart	α MHC-cre	Postnatal lethal by day 14—severe ventricular dilation, arrhythmia, and increased apoptosis.	(66)
	Epidermis	KRT14-Cre	Perinatal lethal, cell cycle block, and impaired development of both epidermis and hair follicle	(52)
	Blood	Mx-Cre	Hypocellular marrow, megakaryocyte dysfunction, and reduction in erythrocytes/thrombocytes	(106)
	B cells	Mb1-cre, CD23-cre	Impaired differentiation and response to antigen	(107)
Hdac3	Germline Liver	N/A Alb-Cre	Embryonic lethal before E9.5 Altered metabolism, fatty liver, genomic instability, and HCC development	(50, 68) (10, 50)
	Blood	Vav-Cre	Loss of lymphoid populations, accumulation of multipotent progenitor cells, and impaired DNA replication	(93)
	Heart	α MHC-Cre	Lethal by 3–4 months. Cardiac hypertrophy, altered metabolism	(68)
	Bone	Osx-Cre	Runted animals, reduced bone density, and increased bone marrow adipogenesis	(79)
	T cells	CD4-Cre	Block in invariant natural killer T-cell development	(97)
	Macrophages	LysM-Cre; Mx-Cre	Skewing of macrophages toward alternative activation; loss of LPS-induced inflammatory gene expression	(15, 69)

Hdac, histone deacetylase.

However, α MHC-cre-mediated deletion of both *Hdac1* and *Hdac2* in the heart resulted in postnatal lethality associated with arrhythmia, severe ventricular dilation, and a three-fold increase in apoptosis in the heart (80). Importantly, the use of broad-spectrum HDIs, particularly Depsipeptide, has been associated with cardiac toxicities (59, 92, 105). These studies suggest that both HDAC1 and HDAC2 may significantly contribute to these adverse events.

It is notable that dramatic cardiac phenotypes did not manifest until both *Hdac1* and *Hdac2* were deleted. *In vitro* studies in MEF models revealed a compensatory upregulation of Hdac1 on *Hdac2* loss and a similar upregulation of Hdac2 on acute *Hdac1* deletion. However, deletion of both *Hdac1* and *Hdac2* resulted in a G₁-phase cell cycle arrest, which may be partially attributed to the upregulation of the cyclin-dependent kinase inhibitors, p21 and p57 (129, 131). A similar compensatory upregulation of these family members is observed in numerous cell types (64, 71, 80, 129, 131).

Are HDAC1 and HDAC2 tumor suppressors?

The propensity for compensation after deletion of either *Hdac1* or 2 forced the conditional deletion of both *Hdac1* and

Hdac2 genes, and this analysis has changed the paradigm for how these proteins act during tumorigenesis. *Mx1-Cre*-mediated deletion of *Hdac1/2* in the blood resulted in dramatic reductions in erythrocyte and thrombocyte levels after increases in megakaryocyte apoptosis (129). These results suggest that inhibition of HDAC1/2 functions may contribute to the thrombocytopenia and anemia associated with broad-spectrum HDIs currently in the clinic. Hdac1 and 2 also play important roles in lymphocyte development and function. Deletion of *Hdac1/2* early in B-cell development resulted in a complete loss of B220⁺ cells in the spleen as well as dramatic reductions in immature B-cell populations in the marrow that were associated with a G₁-cell cycle arrest and elevated apoptosis. Similarly, *Hdac1/2*-deficient mature B cells failed to proliferate and undergo apoptosis in response to mitogenic stimulation (131). Finally, *Hdac1/2* deletion from T cells resulted in a reduction in total thymocytes associated with an increase in apoptosis and accumulation of cells at the double negative stage 3 (DN3) of development (45).

The fact that HDAC1 and 2 are found in complexes with both tumor suppressors [*e.g.*, p53 (56, 84) and retinoblastoma protein (RB) (70, 73)] and oncogenes (*e.g.*, BCL6, Fig. 2) highlights the complex roles of these proteins in cancer

development as well as their response to therapy. Combinations of thymocyte-specific deletion of *Hdac1* and/or *Hdac2* alleles led to the spontaneous development of thymic lymphoma with penetrance increasing as Hdac activity was further decreased. Interestingly, while reductions in Hdac1 and 2 activity promoted tumorigenesis, suggesting a tumor suppressive role for these proteins, complete loss of Hdac1 and 2 function was not compatible with tumor development, suggesting that a minimal level of Hdac1/2 activity is required for lymphomagenesis (45). Similarly, knockdown of *Hdac1* or *Hdac2* in PML-RAR α expressing pre-leukemic cells accelerated leukemogenesis, while knockdown of *Hdac1* in fully leukemic cells had anti-leukemic effects and resulted in a significant increase in animal survival (104). Thus, in the early stages of leukemogenesis, Hdacs 1 and 2 may play tumor suppressive roles but remain a therapeutic target for established disease. These studies highlight the complex roles of Hdac1 and 2 in cancer development and maintenance, as well as identify a potential concern with the use of broad-spectrum HDIs, suggesting that prolonged reductions in HDAC1 and HDAC2 activity may promote therapy-associated secondary cancers.

It will be interesting to determine whether these complex roles for HDAC1 and HDAC2 in tumor development and maintenance can be attributed to the effects of HDAC1/2 function in the maintenance of genome stability. It is easy to envision a scenario where disruption of HDAC1/2 function in normal or premalignant cells could be associated with enhanced replication stress and DNA damage, resulting in the acquisition of mutations and genomic instability, and, ultimately, accelerating tumor formation. In contrast, fully transformed cells may be unable to cope with the replication stress and genomic instability associated with the loss of HDAC1/2 functions, therefore resulting in cancer cell killing in their absence.

Hdac3 and tumorigenesis

The active NCOR (or SMRT), inositol phosphate, Hdac3 complex is recruited by various sequence-specific transcription factors, including nuclear hormone receptors, to repress transcription. Consistent with a role for nuclear hormone signaling in metabolism, both cardiac- and liver-specific deletion of *Hdac3* is associated with an altered metabolic phenotype resulting from deregulated gene expression (62, 82). Since mice with *Hdac3*-deleted livers were aged, signs of fibrosis were found and these mice, ultimately, died of hepatocellular carcinoma (13). Similar to the data with *Hdac1/2* deletion in T lymphocytes, these data support a role for class I HDACs in tumor suppression. This could be due to a loss of heterochromatin (13), genomic instability (11), or deregulation of gene expression patterns that both control metabolic enzymes and regulate circadian rhythms (5, 62, 82).

Nuclear hormone receptors such as the RAR also play critical roles in the regulation of hematopoiesis, and the hematopoietic-specific *Hdac3* deletion confirmed critical roles for Hdac3 in blood development. *Vav-Cre*-mediated deletion of *Hdac3* in the hematopoietic stem cell resulted in a dramatic loss of lymphopoiesis coupled with bone marrow hypocellularity and mild anemia. *Hdac3*-deficient stem cells were severely defective in competitive bone marrow transplant assays, and reduced long-term culture initiating capacity

further suggested a dramatic disruption of stem cell functions. *Hdac3*-deficiency also hindered normal differentiation, with cells accumulating at the multipotent progenitor stage of development. This block in differentiation was associated with severe reductions in both B and T cells as well as erythroid lineages (112). Given that both anemia and lymphopenia have been reported in patients treated with broad-spectrum HDIs, it is likely that abrogation of HDAC3 function contributes to these toxicities.

Mechanistically, a large percentage of these *Hdac3*^{-/-} hematopoietic stem and progenitors incorporated BrdU into their DNA, even when injected into the mice, suggesting an accumulation of cells in the S phase. In addition, cultures of *Hdac3*^{-/-} stem and progenitor cells failed to proliferate, suggesting defects in cell cycle control. DNA fiber labeling, which measures the movement of individual replication forks, demonstrated that *Hdac3* deficiency reduced replication fork velocity, suggesting that the loss of *Hdac3* hampered S-phase progression by directly affecting DNA replication rates. Moreover, even acute treatments of stem and progenitor cells with Depsipeptide resulted in a similar slowing of replication forks, suggesting that alteration of replication rates may be an immediate effect of HDAC inhibition (112). This rapid effect suggests that the defects are not due to altered transcriptional programs, but rather that Hdac3 has direct functions in replication. Combined, these data suggest that stem and progenitor cells lacking *Hdac3* were impaired in their transit through the S phase, which further supports the idea that HDAC proteins play important and physiologically relevant roles in replication control.

HDI therapies that are approved for the treatment of cancer also possess anti-inflammatory effects resulting from reductions in inflammatory cytokine production (66). Macrophage-specific *Hdac3* deletion models suggest that HDAC3 is a key target for the anti-inflammatory effects of HDIs. Activated macrophages are generally classified as either classically activated (M1) with a pro-inflammatory phenotype or alternatively activated (M2) and anti-inflammatory. In the absence of *Hdac3* expression, macrophage polarization was shifted toward alternative activation. Furthermore, *Hdac3* loss was protective in an inflammatory disease model (83). Consistent with these findings, *in vitro* stimulation of *Hdac3*^{-/-} macrophages revealed a significantly impaired induction of pro-inflammatory transcriptional programs, which was associated with an impaired INF- β response (20). Finally, HDAC3 contributed to induction of inflammatory gene expression after IL-1 stimulation through its ability to remove inhibitory acetylation marks on NF- κ B (135). These results are again consistent with the idea that inhibition of HDAC3 mediates the anti-inflammatory effects of broad-spectrum HDIs which are already in the clinic.

Conclusions: Targeting Replication, Transcription, or Inducing Synthetic Lethality?

The use of HDIs as nontargeted therapeutics continues to be tested in the clinic, but to date, the results have been modest. For instance, HDACs are recruited by oncogenic proteins such as BCL6 (10, 50, 51), PML-RAR α (37), and AML1-ETO (7). While it appears that HDIs have efficacy in leukemia and lymphoma carrying these oncogenes, the responses were less dramatic than observed with targeted

In contrast to the clinical data, inhibition of HDACs *in vitro* has dramatic consequences and kills nearly every cell type tested. It is likely that the requirements for HDACs 1, 2, and 3 in DNA replication and DNA repair make these drugs potent tumor cell killers. Indeed, multiple model systems have demonstrated that either genetic deletion of HDACs or HDAC inhibition results in replication stress, impaired S-phase progression, and the accumulation of DNA damage. While these studies do not preclude the contribution of other

Early-phase clinical trials are limited to patients for whom standard chemotherapy that targets DNA replication has already failed. If the broad spectrum of cell killing *in vitro* is primarily due to defects in DNA replication, it seems possible that the broad-spectrum effects *in vitro* might have led to clinical trials in the wrong types of cancers. Recent revelations might suggest that a more focused clinical approach is worth considering. For example, an inhibitor targeting the NCOR1:BCL6 interface has striking effects in preclinical models of BCL6-dependent lymphoma (Fig. 2) (19). While

broad-spectrum HDIs had less of an effect, an HDAC3-selective inhibitor might synergize with the NCOR1 inhibitor in these lymphomas. This “pseudo targeted” approach is gaining traction for these diffuse large B-cell lymphoma, but wider application to t(8;21)-containing acute myeloid leukemia seems appropriate based on biochemical and early clinical data (88).

A second therapeutic avenue which has come from the class I HDAC gene deletion studies is that a minimal level of class I HDAC activity is required for cell survival (45). Thus, inactivating mutations of class I HDACs or their activating partners (NCOR1 or NCOR2) might not only contribute to tumorigenesis by inducing genomic instability, but would also sensitize the tumor cells to HDIs, causing synthetic lethality (45, 104). With the ever-growing genomic sequencing data emerging from The Cancer Genome Atlas and other sources, it is becoming obvious that inactivating mutations of class I HDACs occur at only moderate rates (Fig. 5), but their associated cofactors are mutated at much higher frequencies. In fact, *NCOR1* and *SIN3a* were identified as frequently mutated genes, and these mutations were predicted to be inactivating mutations that “drive” tumorigenesis (28, 57). These tumors with reduced class I HDAC activity may be considered ideal clinical targets for HDIs with the synthetic lethality creating a wide therapeutic window. The incorporation of deep DNA sequencing of these genes into clinical trials and the development of more selective class I HDIs (9) will enable this synthetic lethal hypothesis to be tested in future genome-driven clinical trials.

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Author Disclosure Statement

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Abbreviations Used

ATM = ataxia telangiectasia mutated
 ATRA = all-trans retinoic acid
 CAF-1 = chromatin assembly factor 1
 CTCL = cutaneous T cell lymphoma
 DAD = deacetylase activating domain
 DDR = DNA damage response
 DN3 = double negative stage 3
 DSB = double strand break
 HDAC = histone deacetylase
 HDI = histone deacetylase inhibitor
 HRR = homologous recombination repair
 iPOND = isolation of proteins on nascent DNA
 IR = ionizing radiation
 MEF = mouse embryonic fibroblast
 NCOR1 = nuclear hormone co-repressor 1
 NHEJ = nonhomologous end-joining
 ORC = origin recognition complex
 PCNA = proliferating cell nuclear antigen
 RAR = retinoic acid receptor
 RB = retinoblastoma protein
 ROS = reactive oxygen species
 SAHA = suberoyl anilide hydroxamic acid
 SMRT = silencing mediator of retinoid or thyroid-hormone receptors