Histone deacetylase inhibitors regulate the proteasomal degradation of oncoproteins

Dissertation

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Table of Contents

1 Zusammenfassung

Posttranslationale Proteinmodifikationen spielen eine beutende Rolle bei der eukaryotischen Entwicklung. Krebszellen weisen häufig eine abnormale Aktivität von Histondeazetylasen (HDACs) auf, die sowohl die Dynamik des Chromatins als auch die posttranslationale Azetylierung von Proteinen regulieren. Inhibitoren von Histondeazetylasen (HDACi) können die daraus resultierenden abnormalen Deazetylierungsmuster modifizieren und dadurch Onkogenese relevante Signalnetzwerke beeinflussen. Fundiertes Wissen über Substanzen und Enzyme, welche das zelluläre Proteom kontrollieren, ermöglicht weitere Erkenntnisse über molekularen Signalwege sowie die Entwicklung potentieller Strategien zum Korrigieren pathologischer Fehlfunktionen.

Die Leukämogenese von hämatopoetischen Zellen ist häufig verknüpft mit von chromosomalen Translokationsprodukten kodierten Fusionsproteinen. Beispiele hierfür sind AML1-ETO und PML-RARα, welche zur Pathogenese von akuter myeloischer Leukämie (AML) beitragen. Die hier präsentierte Arbeit zeigt, daß die Proteinstabilität von AML1-ETO und PML-RARα maßgeblich von der HDACi induzierbaren Ubiquitin Konjugase UBCH8 und der Ubiquitin Ligase SIAH1 abhängig ist. Darüber hinausgehend wird in dieser Arbeit dargelegt, daß die Ubiquitin Ligase RLIM gleichermaßen ein Substrat für SIAH1 ist und demzufolge eine bisher unbekannte hierarchische Ordnung von Ubiquitin Ligasen das Ubiquitin-Proteasom System beeinflussen kann.

Da die konstitutiv-aktiv mutierte FMS-ähnliche Tyrosinkinase 3 (FLT3-ITD) ebenfalls zur leukämischen Transformation beiträgt und häufig in Verbindung mit AML1-ETO und PML-RARα in AML Patienten gefunden wird untersuchte ich, ob HDACi auch die Stabilität von FLT3-ITD beeinflussen können. UBCH8 und SIAH1 interagieren Tyrosin-phosphorylierungsabhängig mit FLT3-ITD und vermitteln dessen proteasomalen Abbau. In Übereinstimmung hiermit ist die Stabilität von unstimulierten Wildtyp FLT3 durch HDACi nahezu unbeeinflusst. Folglich ist UBCH8, welches bisher hauptsächlich mit nukleären Prozessen in Verbindung gebracht wurde, ein neuer wichtiger HDACi induzierbarer Regulator der FLT3-ITD Stabilität und des Überlebens von Leukämiezellen.

Zusammenfassend konnte ich in verschiedenen AML Zelllinien und in einem heterologen Expressionssystem zeigen, daß UBCH8 und SIAH1 mit FLT3-ITD, AML1-ETO, PML-RARα und RLIM interagieren und diese für den proteasomalen Abbau markieren. Da HDACi sowohl UBCH8 induzieren, als auch abnormaler Genexpression und Signalgebung entgegenwirken, können diese Substanzen für die Therapie von AML von Nutzen sein.

Desweiteren vermittelt diese Arbeit ein tiefergehendes Verständnis, wie Enzyme des proteasomalen Abbauweges reguliert werden können und wie sie untereinander, als auch mit ihren für die Krebsentwicklung relevanten Substraten interagieren. Rückschlüsse aus der hier dargelegten Arbeit zeigen neue biochemische Mechanismen und molekulare Netzwerke auf.

2 Summary

Posttranslational protein modifications play an important role in the eukaryotic development. Aberrant activity of histone deacetylases (HDACs) which control the dynamics of chromatin and regulate reversible protein acetylation is commonly found in cancer cells. Inhibitors of HDACs (HDACi) correct aberrant deacetylation patterns and alter signaling networks relevant for oncogenesis. These drugs are pleiotropic effectors and it remains to be deciphered how they correct tumor-associated transcriptomes and proteomes. Knowledge on enzymes and parameters controlling the biochemistry of the cellular proteome allows insights into molecular pathways as well as potential strategies to correct pathological dysfunctions.

Leukemogenesis is often linked to fusion proteins generated by chromosomal translocation products. Examples are AML1-ETO and PML-RARα which contribute to the pathogenesis of acute myeloid leukemia (AML). The work presented here reveals the novel insight that the turnover of both, AML1-ETO and PML-RARα, depends on the HDACi-inducible ubiquitin conjugase UBCH8 and the ubiquitin ligase SIAH1. Beyond showing that HDACi promote the degradation of oncoproteins, this work reveals that the ubiquitin ligase RLIM equally is a substrate for SIAH1. Thus, a formerly unknown hierarchical order of ubiquitin ligases affects the ubiquitin-proteasome system.

Since constitutively activated mutant FMS-like tyrosine kinase 3 (FLT3-ITD) causally contributes to leukemic transformation and is frequently found in conjunction with AML1-ETO and PML-RARα in AML patients, it was also tested whether HDACi attenuate FLT3-ITD. Indeed, UBCH8 together with SIAH1 interact in a tyrosine phosphorylation-dependent way with FLT3-ITD and promote its proteasomal degradation. Accordingly, unstimulated wild-type FLT3 is hardly affected by HDACi. Thus, UBCH8, which has been implicated primarily in nuclear processes, could be identified as a novel important HDACi-inducible modulator of FLT3-ITD stability and leukemic cell survival.

In summary, I could demonstrate in various AML cell lines and heterologous expression systems that UBCH8 and SIAH1 physically interact with and target FLT3-ITD, AML1-ETO, PML-RARα, and RLIM for proteasomal degradation. As HDACi induce UBCH8 and counteract aberrant gene expression and unbalanced signaling, these agents could be beneficial for the treatment of AML. This work furthermore provides a deeper understanding on how enzymes promoting proteasomal degradation are regulated and how they interact with each other as well as with their cancer-relevant substrates. Conclusions presented here reveal novel biochemical mechanisms and molecular networks. In addition, they have implications for translational research.

3 Introduction

3.1 Acute myeloid leukemia

3.1.1 Classification and genetic characteristics of acute myeloid leukemia

Leukemia is a malignant transformation of hematopoietic cells, characterized by the uncontrolled high proliferation of immature, nonfunctional blood cells, the so called leukemic blasts. Leukemic diseases are manifested by suppression of normal hematopoiesis and blast infiltration of organs. They can be divided into four categories: Based on the affected hematopoietic cells in myelocytic or lymphocytic leukemia with either acute or chronic disease progression. Acute myeloid leukemia (AML) accounts for approximately 30% of all adult leukemias and is characterized by accumulation of immature, myeloid blasts that fail to differentiate and invade the bone marrow and peripheral blood [1]. The requisite blast percentage for the diagnosis of AML is the presence of at least 20% leukemic blasts in the patient blood and bone marrow [2]. The World Health Organization (WHO) classification of AML and myeodysplastic syndromes (MDS), a preleukemic disorder that can transform into AML, incorporates morphology, cytogenetic and molecular biology and immunologic markers of the leukemic blasts in an attempt to construct a classification that is universially applicable and prognostically valid. The separation of homogenous classes allows to distinguish prognostic parameters and to identify groups of patients sensitive to drugs or specific treatment. Four unique subgroups of AML are distinguished by the WHO system. 1. AML with recurrent cytogenetic abnormalities, 2. AML with multi-lineage dysplasia and 3. AML and MDS, related to therapy. Cases of AML that do not fulfill the criteria for any of these subgroups are classified in group 4. AML, not otherwise categorized. This is contrary to the older French-American-British (FAB) cooperative group classification, which is solely based on blast morphology as determined by the degree of differentiation along different cell lines and the extent of cell maturation [3].

In the WHO classification, the first group of “de novo” AML is characterized by recurring cytogenetic abnormalities, mainly encompassing balanced chromosomal translocations. These structural chromosomal rearrangements result in the formation of fusion genes encoding chimeric proteins contributing to the initiation and progression of leukemia. Among the most commonly identified genetic aberrations are reciprocal translocations, found in approximately 40% of AML patients [4]. These are e.g. t(8;21) (AML1-ETO), t(15;17) (PML-RARα); t(16;16) (CBFβ/MYH11) and translocations involving the 11q32 (MLL) breakpoint. With the exception of MLL gene rearrangements, the genetic chromosomal

translocations are frequently associated with high rates of remission during cytotoxic chemotherapy and favorable prognosis [5,6].

Mutations leading to the constitutive activation of the FMS-like tyrosine kinase 3 (FLT3) have been identified in 30% of patients diagnosed with AML. They were also found in smaller fractions of chronic myeloid leukemia, myelodysplasia as well as B- and T-cell acute lymphoblastic leukemia, making FLT3 the most frequently mutated gene in haematological malignancies [7-9]. Notably, approximately 40% of t(15;17) and t(18;21) AML patients harboring constitutively active FLT3 mutations and mutated FLT3 was shown to confer poor clinical prognosis [7,10,11].

3.1.2 PML-RARα in acute promyelocytic leukemia

Acute promyelocytic leukemia (APL), which accounts for 5-8% of all AML cases, is morphologically characterized by a differentiation block of the granulocytic lineage at the promyelocytic stage, leading to accumulation of promyelocytes. APL is almost always characterized by a rearrangement of the retinoic acid receptor alpha (RARα) gene as a consequence of a chromosomal translocation. In this genetic rearrangement, the promyelocytic leukemia (PML) gene on chromosome 15 is fused to the RARα gene on chromosome 17, generating the formation of the t(15;17) PML-RARα fusion protein. The treatment of APL with diagnosed t(15;17) translocation differs from the therapy of other subtypes of AML, because patients with this phenotype represent a unique group characterized by response to retinoic acid treatment and good prognosis [12,13].

In normal cells, PML was found to be part of sub-nuclear multi-protein complexes, the so-called PML bodies. Although their exact functions are still discussed controversially, these structures are implicated in normal cell maintenance, stress response and apoptosis [14-16]. PML has tumor growth suppressor properties, as it facilitates the acetylation and stabilization of p53. RARα is a member of the retinoid acid receptor nuclear receptor superfamily and acts as s ligand-activated transcription factor. In the absence of the ligand retinoic acid (RA), RARα forms heterodimers with the retinoid X receptor (RXR), and binds to specific RA response elements (RARE) [14,17]. The unliganded RARα/RXR heterodimer represses transcription by recruitingcorepressor complexes containing histone deacetylases. Stimulation with RA releases the nuclear corepressors from the RARα/RXR heterodimer and results in RA-dependent gene activation by recruitment of coactivator complexes. The fusion protein PML-RARα mediates constitutive gene silencing. This capacity depends on the strong PML dimerization domain and the formation of stable homodimers, recruiting corepressor complexes including HDACs and DNA methylating enzymes [18,19]. As a

consequence, cells expressing PML-RARα are resistant to physiological levels of RA and normally activated genes remain silent [18,20]. Since physiological concentrations of RA cannot induce the conformational change necessary to release the corepressors, oncogenic PML-RARα functions as a dominant negative receptor over wild-type RARα. However, pharmacological doses of RA have been shown to mediate dissociation of PML-RARα from the corepressor complex and induce proteasome- and caspase-dependent degradation of this fusion protein, thereby overcoming its repressive function and restoring normal cell differentiation [21-24]. Aside its role as a negative regulator of RARα target genes via direct binding to DNA, recent studies revealed further functions of PML-RARα in altering gene expression. Gene activation via specific interaction with other transcription factors or the capacity to promote HDAC-dependent deacetylation and subsequent proteasomal degradation of the tumor suppressor p53 can also account for its oncogenic transforming potential [25].

3.1.3 AML1-ETO translocation in AML

AML carrying the translocation t(18;21) accounts for 5-12% of cases of AML and is characterized by myelo-monocytic differentiation, increased eosinophils in leukemic bone marrow and good response to chemotherapy and a high remission rate with long-term disease-free survival [5,26-28]. AML1, also known as RUNX1, functions by association with the core binding factor beta (CBFβ) as the heterodimeric transcriptional activator CBF (AML1/CBFβ). CBF regulates a large number of target genes expressed in hematopoietic cells. The CBF heterodimer complex was shown to be a key regulator of hematopoiesis, and loss of either of these genes resulted in embryonic lethality [11]. The t(18;21) translocation replaces the C-terminus of transcriptional activator AML1, with almost the entire protein of the transcriptional repressor eight-twenty-one (ETO), but retain the central runt homology domain (RHD) of AML1, essential for the recognition of the AML1 target promoter and dimerization [29,30]. Similar to the PML part of the t(15;17) fusion protein, the constitutive repression of differentiation genes is mediated by the recruitment of corepressor complexes interacting with the ETO domain (Figure 1) and by interfering of AML1-ETO, as a dominant negative inhibitor, with normal activity of CBF [31-33]. Beside the AML1-ETO- mediated gene repression via recruitment of repressor complexes, several studies demonstrate that AML1-ETO physically interacts with and thus inhibits the function of other transcriptional regulators of hematopoiesis, such as C/EBPα, c-Jun, PLZF, SMAD3 and VDR.

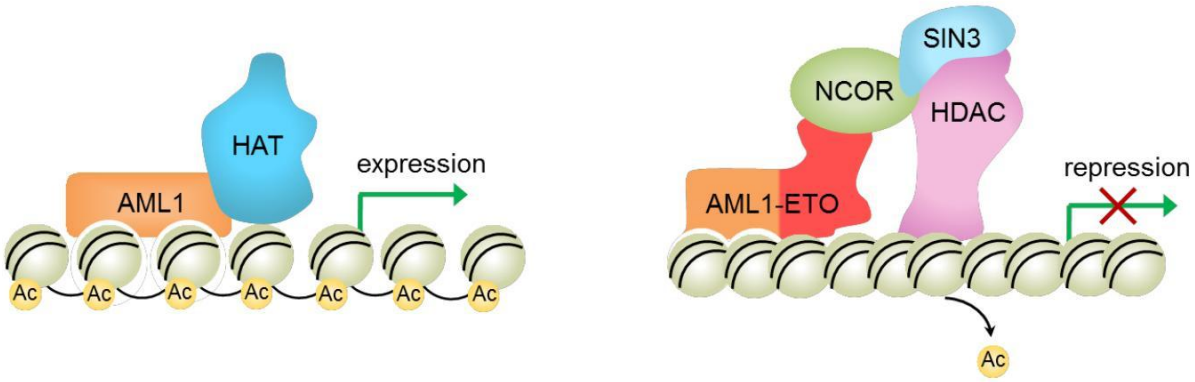

Fig. 1 Transcriptional repression of AML1 target genes by AML1-ETO. (**a**) Regulation in normal hematopoietic progenitors. AML1 binds to target genes and recruits co-activators. The histone acetyltransferase (HAT) activity of the co-activator causes an open chromatin structure by histone acetylation (Ac) and thereby induces AML1 target genes. (**b**) Regulation in cells expressing AML1-ETO. The ETO domain recruits co-repressors such as NCOR, SIN3 and histone deacetylases (HDAC) to AML1 binding sites in the DNA. In consequence, the DNA structure condensed and AML1 target genes are repressed. Figure is adapted from [34]

Moreover, it has been shown that AML1-ETO suppresses C/EBPα on the transcriptional level, a transcription factor crucial for normal differentiation of granulocytes [35]. Although the contribution of AML1-ETO to leukemogenesis is still under intense investigation, it is thought that suppression of CBF and C/EBPα-dependent gene activation is the key mechanism responsible for the development of myeloid diseases [11,35,36]. Further studies demonstrated that the t(18;21) translocation product not only induces a block of differentiation, but also enhances pro-survival signaling in AML cells via suppression of p53. Similar to the PML-RARα-mediated suppression of p53 signaling, AML1-ETO was shown to destabilize the tumor suppressor p53 indirectly, as it represses the expression of $p14^{ARF}$, which inhibits the ubiquitinylation and degradation of p53 by binding to its ubiquitin ligase HDM2 [34,37].

3.1.4 FLT3 Internal Tandem Duplication mutation in AML

The FLT3 receptor, which has an important function in hematopoiesis, belongs the type III receptor tyrosine kinases (RTK) subfamily that also includes c-KIT, c-FMS and PDGFRα/β. FLT3 plays an important role in regulating many cellular processes such as proliferation, differentiation and survival of multipotent hematopoietic stem cells. In normal bone marrow, FLT3 is mainly expressed by early $CD34^+$ myeloid and lymphoid progenitor cells and expression is lost as hematopoietic cells differentiate [38-40]. The FLT3 receptor is composed of an immunoglobulin-like extracellular ligand-binding domain, a trans-membrane domain, a juxta-membrane dimerization domain and an intracellular kinase domain, interrupted by a kinase insert [41]. Stimulation with FLT3 ligand induces receptor dimerization, auto-phosphorylation and downstream signaling, including RAS/MAPK, and PI3/AKT signal transduction pathways [4,34,38].

Remarkably, beyond its crucial role for normal hematopoiesis, wild-type FLT3 has been shown to be highly expressed in several hematopoietic malignancies, with 70-100% cases of AML [42]. Expression of high levels of FLT3 promotes constitutive receptor activation, linked to leukemic transformation and poor disease/survival prognosis of AML patients [34,43,44]. As already mentioned activating mutations of FLT3 also occur in several hematological malignancies and are present in up to 30% of patients suffering from AML [45]. The most common FLT3 mutations in AML are internal tandem duplications (ITDs) in the juxtamembrane region (JM) [46,47].

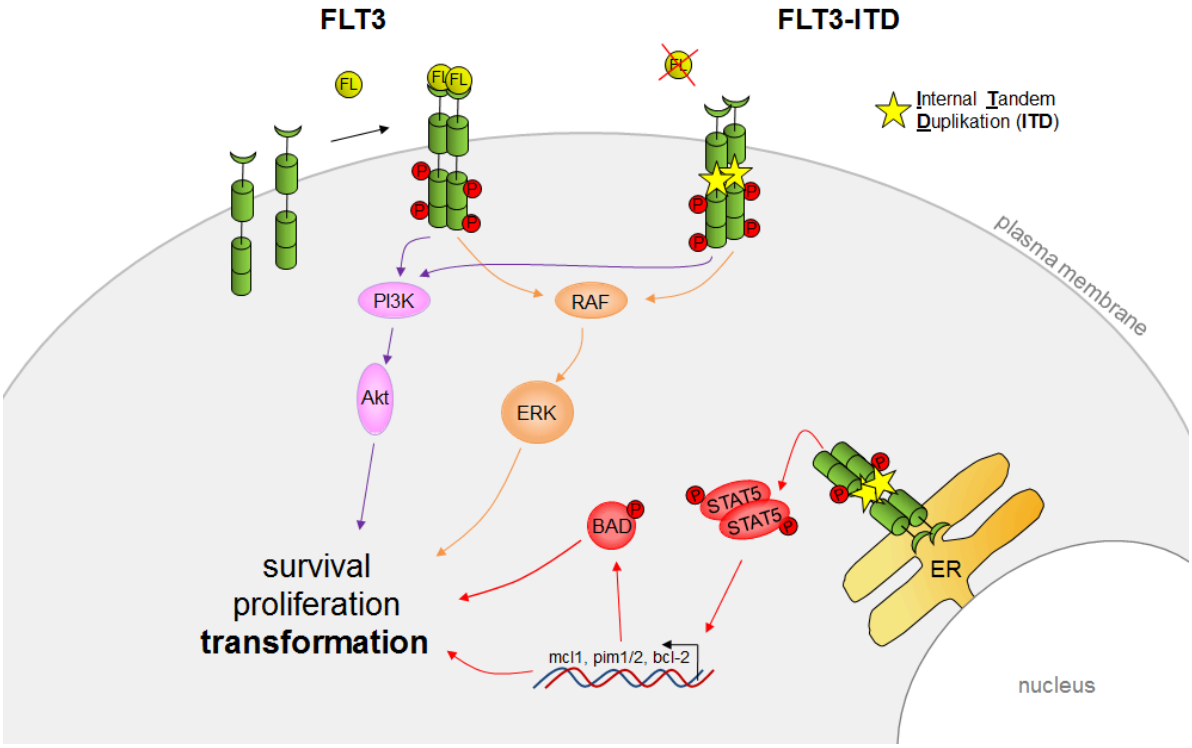

Fig. 2 Receptor signaling by wild-type and mutant FLT3. Ligand (FL) binding to the extracellular domain of FLT3 induces receptor dimerization and autophosphorylation, leading to activation of the MAPK and PI3-K signaling pathway. Constitutive receptor activation by mutations is often found in hematologic malignancies. Oncogenic mutation by insertion of an internal tandem duplication (ITD) in the juxtamembrane domain of the FLT3 receptor disrupts its autoinhibitory function resulting in constitutive receptor signaling. Constitutive activation partly blocks receptor maturation and trafficking to the plasma membrane via the biosynthesis route. Aberrant receptor localization to intracellular compartments, such as the endoplasmic reticulum (ER) results in an altered receptor phosphorylation pattern and induction of oncogenic signaling pathways. These are different from the pathways activated by cell-surface localized receptor. ER-retained FLT3-ITD strongly activates STAT5. STAT5 in turn regulates the activation and expression of downstream targets such as PIM-1/2, BAD and BCL-2, key regulators of cell-cycle progression and anti-apoptotic signaling. The figure is adapted from [51].

ITDs are a result of a segmental duplication of a fragment within the JM domain coding region. The JM region is thought to act as a negative regulatory domain for receptor activation by preventing access of ATP to the active center in the kinase domain of the enzyme. Insertion of an ITD in the JM disrupts the auto-inhibitory function by conformational

changes, leading to ligand-independent dimerization, resulting in constitutive receptor activation and cytokine independent cell proliferation [48]. Moreover, beyond the aberrant activation of FLT3-ITD, profound differences were found between the transforming capacity and signaling properties of ligand-activated wild-type FLT3 and constitutively activated FLT3-ITD. Constitutive receptor activation of FLT3-ITD has been shown to prevent receptor glycosylation and cell surface trafficking, leading to partial mislocalization of immature, underglycosylated FLT3-ITD to intracellular compartments [49,50]. It has been demonstrated that aberrant receptor localization alters the pattern of auto-phosphorylation and results in activation of oncogenic signaling pathways, different from the pathways activated by surface-localized FLT3 [51,52]. One of the most striking deviations of activated wild-type FLT3 and FLT3-ITD lies in the ability of the mutated FLT3 to induce enhanced oncogenic signaling via potent activation of STAT5, as illustrated in Figure 2 [34,51,53,54]. STAT5 in turn can activate specific downstream targets such as Pim-1, Pim-2, Cdc25A, Bad, and Bcl-2 which are key mediators of cell cycle progression and cell survival. Moreover, STAT5 also mediates repression of the myeloid transcription factors Pu.1 and C/EBPα, important regulators of cell differentiation. Aberrant STAT5 activation has been reported in several hematologic malignancies, linked to FLT3-ITD mutation and activation of STAT5 is therefore discussed as one of the key features of FLT3-ITD signaling for malignant cell transformation [4,55-57].

Large-scale analyses of AML patients revealed FLT3-ITD mutations as independent prognostic markers for poor clinical outcome in AML [7,45,58]. Moreover, several studies with murine models demonstrated the transforming potential of mutant FLT3. As a single mutation, FLT3-ITD is sufficient to induce a myeloproliferative phenotype and in some cases also lymphoid disease in FLT3-ITD transgenic or bone marrow transplanted mice. These findings link FLT3-ITD directly to the pathogenesis of myeloid and lymphoid leukemia [59-63].

3.1.5 Genetic model of AML: Cooperating gene mutations

It has been hypothesized that the development of AML is a multi-step process, requiring two classes of genetic mutations that independently disrupt at least two regulatory processes in hematopoietic cells [42]. Class one mutations are those activating signal transduction pathways such as in FLT3, thereby conferring proliferative and survival advantages on hematopoietic cells. The second class of mutations, including t(8;21) and t(15;17) chromosomal translocations, resulting in altered gene transcription and repression of hematopoietic cell differentiation. Hence, the combined existence of these mutations can generate the AML phenotype due to unregulated proliferation and impaired differentiation of hematopoietic cells. In support of this model, activating mutations of FLT3 are present in 30% of all AML cases and frequently found in conjunction with other genetic rearrangements: Up to 40% of

AML patients with activating FLT3 mutations also harboring t(15;17) and t(8;21) fusions [7,10,11]. Numerous murine *in vivo* models documented that PML-RARα as single mutation can induce a myeloproliferative disorder in transgenic or bone marrow transplanted mice [64-68]. Remarkably, analysis of further mice models and human APL phenotypes also indicate that PML-RARα is necessary but not always sufficient for the development of an APL-like disease. These studies revealed that PML-RARα initially exerted relatively modest effects on myelopoiesis *in vivo*. The PML-RARα transgenic mice develop a nonfatal myeloproliferative disorder but only in the minority of cases a leukemia-like phenotype after long disease latency [64-67,69]. In agreement with the findings that multiple mutations are prerequisite for the development of acute leukemia, PML-RARα was shown to initiate a more progressive APL in association with additional genetic changes [68,70-72].

Similar as for PML-RARα, AML1-ETO expression in transgenic mice generates a myeloproliferative syndrome [11,12], even though numerous murine models also documented that AML1-ETO *per se* does not induce an acute leukemic phenotype [11,73-76]. The importance of collaborating genetic events in the pathogenesis of AML has been shown in AML1-ETO transgenic mice. Only mice additionally treated with the mutagenic substance ETU (N-ethyl-N-nitrosourea), which induces further genetic lesions by inflicting DNA point mutations, developed AML or T-cell lymphoma [74,75].

Consistent with the hypothesis that AML develops as a consequence of multiple genetic alterations, several reports demonstrated that FLT3 mutations cooperate with fusion proteins in generating leukemia *in vivo* [11,77-79]. For example, the additional expression of FLT3-ITD in bone marrow cells of PML-RARα transgenic mice greatly enhances the progression of an APL-like disease, compared to the prolonged disease latency in PML-RARα transgenic mice, as shown in Figure 2 [70,71].

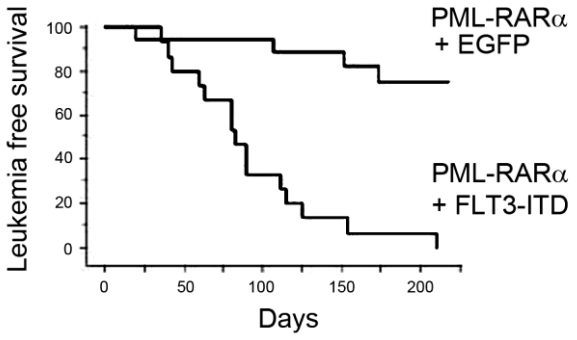

Fig. 2 PML-RARα cooperates with FLT3-ITD in the development of leukemia in mice. Kaplan-Meier analysis for leukemia-free survival. The percentage of surviving mice (y axis) is plotted with respect to time in days (x axis). Leukemia-free survival of $(B6xC3H)F_1$ animals receiving PML-RARα transgenic cells transduced with either EGFP or FLT3-ITD. This plot presents data from two independent experiments. Data and adapted figure are taken from [71].

Moreover, AML1-ETO and FLT3-ITD were found to collaborate in inducing acute leukemia in murine bone marrow transplantation models [11]. Congruent with the findings discussed in the preceding section, that single mutations were not sufficient to induce AML, FLT3-ITD cooperates with further aberrant chromosomal rearrangements, e.g. MLL-fusion proteins such as MLL-AF9 or MLL-SEPT6, in cell transformation *in vitro,* and the development of acute biphenotypic or myeloid leukemia *in vivo* [78,79]. These findings emphasize the role of FLT3-ITD for the development of hematologic malignancies and illustrate, that FLT3-ITD can serve as a second hit mutation in the multi-step pathway of acute leukemia. Further support for the need of cooperative mutations in AML comes from the finding that the occurrence of additional FLT3-ITD mutation reduces the favorable prognosis for AML patients with t(15:17) chromosomal rearrangement, indicated by a shorter disease-free survival and higher relapse rates [38,80,81].

These findings have important implications for novel therapies of AML, since the combined molecular targeting of mutant FLT3 and oncogenic fusion proteins may improve the outcome of AML patients harboring both types of mutations.

3.2 The ubiquitin-proteasome system

3.2.1 Protein polyubiquitinylation

Intracellular protein degradation plays an essential role in many physiological processes by removing short-lived regulatory or misfolded proteins. The selective removal of regulatory proteins such as transcription factors or signal transduction proteins represents an efficient and rapid strategy to control many vital cellular processes, including cell cycle, proliferation, differentiation or apoptosis [82]. Protein ubiquitinylation was discovered in the early 1980s as a posttranslational modification, a process of covalently attaching a 76 amino acid ubiquitin polypeptide to a protein substrate [83]. It has been demonstrated, that ubiquitinylation is an important modification for cellular trafficking and transcriptional activation, as well as proteasomal and lysosomal degradation of proteins, necessary for the regulation of normal cell function [84].

In eukaryotic cells, the specific degradation of proteins is regulated by the ubiquitin-proteasome system (UPS). Posttranslational protein modification by poly-ubiquitinylation was found to regulate the half-life of proteins, ranging from minutes to several days. Poly-ubiquitinylated proteins undergo rapid degradation by the proteasome, a multiprotein complex mediating the proteolytic cleavage of proteins. Ubiquitinylation is usually carried out by the hierarchical action of three classes of enzymes, working consecutively to recognize and transfer ubiquitin molecules to substrate proteins [85]. See Figure 4. These are ubiquitin-

activating enzymes (E1s), ubiquitin conjugases (E2s), and ubiquitin ligases (E3s). The selectivity of ubiquitinylation represents the crucial step in substrate selection and is mainly determined by the ubiquitin ligase, which is responsible for

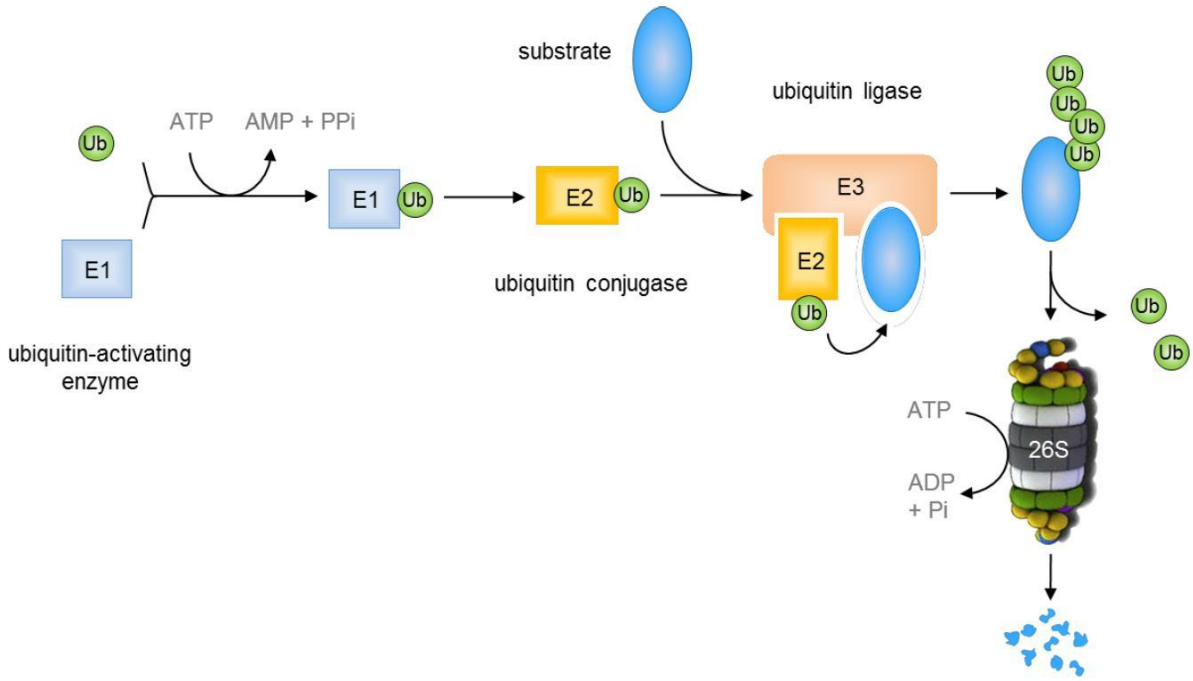

Fig. 4 The ubiquitin-proteasome pathway. The E1 ubiquitin-activating enzyme activates the ubiquitin (Ub) by ATP-dependent formation of a thiolester intermediate to an internal cysteine residue in the E1. The activated ubiquitin molecule is then transferred to an E2 ubiquitin-conjugating enzyme by binding to a cystein residue. Substrate specificity is delivered by E3 ubiquitin ligases, specific only for a defined number of substrate proteins. In the case of RING-finger E3 ligases, the E2 enzyme binds to the E3-substrate complex and directly transfers ubiquitin to an internal lysine residue in the substrate protein by covalent attachment. A poly-ubiquitin chain of at least four ubiquitin molecules serves as signal for binding to the 26S proteasome and subsequent degradation in an ATP-dependent manner. Ubiquitinylation is reversed by deubiquitinylating enzymes that remove substrate-conjugated ubiquitin and disassemble poly-ubiquitin chains.

substrate recognition. Based on their characteristic protein motifs, Ubiquitin ligases are divided into three general classes: HECT (homologues to the E6-associated protein C-terminus) E3 ligases, RING (really interesting new gene) E3 ligases, and U-box domain containing E3 ligases. While HECT E3s form a thioester bond with ubiquitin and directly ubiquitinylate a substrate, RING and U-box domain containing E3s have no intrinsic catalytic activity but coordinate the transfer of ubiquitin to the substrate by binding E2s, thereby functioning as a scaffold. While mammals have only two confirmed E1 enzymes, over thirty E2 conjugases and several hundreds of E3 ligases have been found [84,86]. The specific substrate recognition largely relies on the diversity of E3s, which themselves cooperate with a defined set of E2s, allowing the ubiquitin system to confer substrate specificity. Moreover, substrate recognition by the appropriate E3 ligase and the effect of ubiquitinylation on the substrate`s fate can also be influenced by association with adaptor proteins in different ways

[87]. Thus, the regulated abundance of interacting E2 and E3 enzymes, as well as accessory proteins may allow a large flexibility in the recognition and specificity of protein substrates [88].

3.2.2 The 26S proteasome

An elongated ubiquitin chain can be formed after the initial conjugation of an ubiquitin molecule. This process results in poly-ubiquitinylation of the substrate. It is generally accepted, that conjugation of a polymeric ubiquitin chain, in which the ubiquitin molecules are linked through the ubiquitin lysine residue at position 48 (K48), targets such modified proteins for degradation by the proteasome. A tetrameric chain of K48-linked ubiquitins is the minimal recognition motif for the proteasome. The 26S proteasome, the key enzyme of intracellular protein degradation in eukaryotic cells, is a cylindrically assembled multiprotein complex, composed of a core 20S complex capped by regulatory 19S complexes at both ends. Poly-ubiquitinylated proteins can bind either directly to ubiquitin recognition domains, the so-called Ub-receptors, in the 19S complex or via association with adaptor proteins, which bind to the ubiquitin modification and to the proteasome [87,89]. Binding of the substrate to the 19S complex of the proteasome is followed by removal of the poly-Ub chain by proteasome-associated deubiquitinylating enzymes and the ATP-driven unfolding and translocation of the substrate into the 20S core complex. The central 20S core complex contains the catalytic chamber, where substrate proteins are cleaved into small peptide fragments by specific sites, generally classified as trypsin-, chymotrypsin-, or caspase-like peptidases [84,89].

3.2.3 Diversity of ubiquitinylation signals

Beyond its crucial role for proteolytic protein degradation, ubiquitin has other functions. Unlike substrate degradation in the 26S proteasome, which requires a poly-ubiquitin chain, ubiquitinylation on several lysine residues (multimeric mono-ubiquitinylation) in cell-surface proteins, such as receptor tyrosine kinases (RTKs), promote their internalization and sorting to lysosomal degradation [86]. Beside K48, other lysine residues in the ubiquitin molecule participate in linkage of multiple ubiquitins. Interestingly, the different types of poly-ubiquitin chains determine effects on the substrate. Poly-ubiquitinylation via K63 of the ubiquitin molecule can promote internalization and recycling of cell-surface receptors, modulation of protein-protein interactions and similar to mono-ubiquitinylation, also in the control of gene transcription and DNA-repair. Recent studies of protein ubiquitinylation also demonstrated, that all seven lysine residues of the ubiquitin molecule can participate in the formation of poly-ubiquitin chains. Further complexity emerged from the discovery of heterotypic mixed linear or branched ubiquitin chains, starting from multiple lysines on a single ubiquitin [83]. It

still remains to be elucidated, whether these modifications govern preotein degradation or have other effects on cellular signaling.

3.3 Histone deacetylase inhibitors as anti-cancer substances

3.3.1 Aberrant epigenetic acetylation patterns in leukemia

As already explained above, AML is a heterogeneous, clonal disorder of hematopoietic progenitor cells that lost the ability to normally differentiate and to respond to regulators of proliferation [90,91] . In addition to genetic mutations, epigenetic dysfunction also play a critical role in the onset and progression of leukemia [92,93]. This key feature in the pathophysiology of AML results in aberrant transcription of genes involved in cell growth, proliferation, differentiation and apoptosis.

Reversible acetylation of nucleosomal histone tails is a major epigenetic mechanism for the regulation of gene expression and is balanced by the opposing activities of acetyltransferases and deacetylases. Since histones were the first identified targets of these enzymes, they were termed histone acetytransferases (HATs) and histone deacetylases (HADCs). Acetylation of histones provides a more open chromatin structure which correlates with gene activation, while histone deacetylation mediates transcriptional repression [94,95]. Aberrant activity of HATs and HDACs results in gene expression pattern commonly observed in cancer cells, and is particular a hallmark of leukemia and lymphoma. In leukemic cells, the normal recruitment of HATs and HDACs may be disrupted by mechanisms that include chromosomal translocations encoding the chimeric fusion oncoproteins PML-RARα or AML1-ETO. These repressive transcription factors promote oncogenesis by aberrant recruitment of HDACs, which results in the constitutive silencing of genes involved in cellular differentiation [96,97].

3.3.2 Non-histone protein acetylation

HATs and HDACs were found to be deregulated in cancer [98,99] and aberrant expression and unbalanced recruitment of HDACs has been observed in various tumor types [100-102]. Thus, an altered balance of protein acetylation can contribute to cell transformation and cancer development [103,104]. Remarkably, HDAC enzymes are not exclusively targeted towards histones. A steadily growing number of non-histone proteins have been described to be subject to reversible acetylation by HATs and HDACs. Acetylation of proteins, which are often collectively termed the ‘‘acetylome”, modulates a wide variety of cellular events that are involved in many biological processes such as cell proliferation, survival, and apoptosis

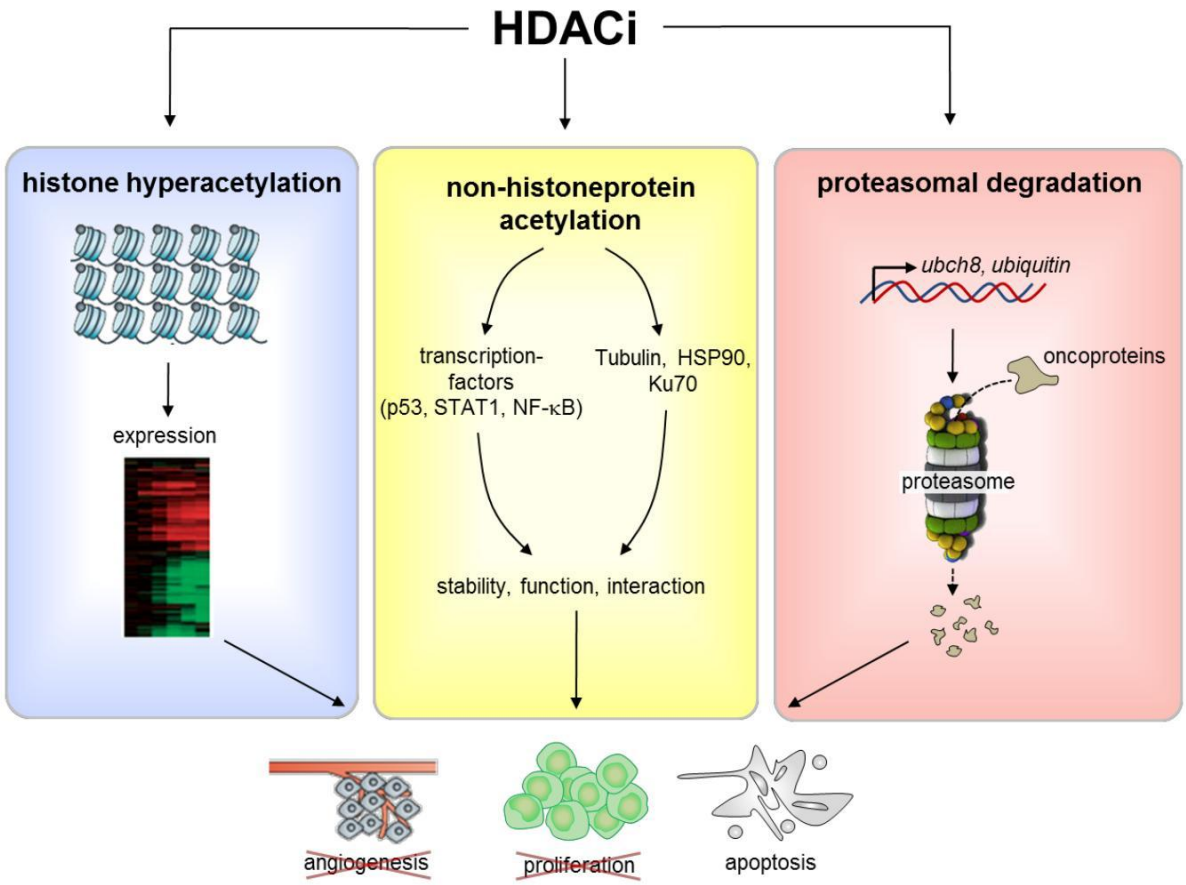

Fig. 5 Effects of HDACi on histone and non-histone proteins. In addition to regulating the acetylation state of histones, histone deacetylase (HDAC) can bind to, deacetylate and regulate the activity of many non-histone proteins, including transcription factors, such as p53, STAT1, or NF-κB, and proteins with diverse biological functions, e.g. the cytoskeleton protein Tubulin, the chaperone HSP90, and the DNA-repair enzyme Ku70. Hyperacetylation of transcription factors by HDAC-inhibitors (HDACi) can augment their gene-regulatory activities and contribute to the changes in gene expression observed following direct HDACi-mediated histone hyperacetylation. Hyperacetylation of proteins such as Ku70 or HSP90 by HDACi have no direct or indirect effect on gene expression but could be important for certain biological effects of HDACi, in particular in the induction of cell-cycle arrest, suppressed angiogenesis and apoptosis. HDACi not only induce the acetylation of proteins, they can also induce their turnover via indirect mechanisms. HDACi regulate the proteasomal degradation of (proto-) oncoproteins by modulating the amounts of limiting enzymes or regulators of the ubiquitin-proteasome system. For example HDACi can induce the expression of the ubiquitin conjugase UBCH8 or ubiquitin itself, thereby regulating the proteasomal degradation of cancer-relevant proteins like e.g. HDAC2 or Survivin. The figure is adapted from [98].

(Figure 5) [103,105]. Among the non-histone targets are transcription factors and signal transducers, such as p53, NF-κB, E2F1, c-Myc, and STATs.These factors play key-roles in tumorgenesis and anti-tumor response. In addition, proteins that do not directly regulate gene expression but instead regulate DNA repair (Ku70), the cellular cytoskeleton protein (Tubulin), and protein stability (HSP90) are also subjected to reversible acetylation [92].

3.3.3 Histone deacetylase inhibitors

An accepted approach in cancer therapy is the forced reexpression of repressed genes essential for growth inhibition and cell death, as well as the alteration of aberrant acetylation

pattern of non-histone proteins, as described above (Section 2.3.2) [106,107]. Recently, small compounds that inhibit HDAC activity have become available for cancer treatment. These inhibitors of HDACs (HDACi) are promising new cancer-therapeutic agents that can change patterns of gene expression and protein acetylation [108-110]. These substances not only induce protein acetylation by inhibiting deacetylase activity, but also alter the turnover of several (proto-) oncoproteins via indirect mechanisms. HDACi-induced abrogation of chaperone function correlates with enhanced degradation and depletion of client proteins. Moreover, HDACi can induce the turnover of cancer-relevant proteins by caspase-mediated proteolytic cleavage or via modulating the abundance of proteins regulating the ubiquitin-dependent proteasomal degradation (Figure 5).

Treatment with HDACi was demonstrated to induce growth arrest, differentiation or apoptosis in a wide spectrum of cancer cells *in vitro* and *in vivo* [98,106,111,112]. Furthermore, HDACi sensitize cancer cells to the cytotoxic effects of other therapeutic agents [113,114], and they display selective toxicity against tumor cells compared to untransformed cells [90,92,106,115]. Accordingly, HDACi are considered as candidate drugs for cancer therapy [98,116].

3.4 HDACi-regulated proteasomal degradation of oncoproteins

3.4.1 Acetylation-dependent abrogation of HSP90 chaperone function

One more very important action of HDACi is the induction of proteasomal protein degradation. HDACi-induced, acetylation-dependent protein degradation can follow indirect mechanisms modulating key regulatory proteins, such as chaperones, involved in the stability control of a variety of factors [88]. The non-histone HDAC substrate heat-shock protein 90 (HSP90) plays a major role as chaperone in the proper folding and stability of several major (onco-) proteins. Recently, several reports revealed, that HSP90`s protective role on client proteins is impaired by posttranslational modifications such as phosphorylation and acetylation [103]. Acetylation of HSP90 inhibits ATP-binding, affects association with co-chaperones and induces dissociation of client proteins, thereby directing them to subsequent poly-ubiquitination and proteasomal degradation [117,118,31]. HDACs can remove the impairing acetylation and therefore stabilize HSP90 client proteins. Proteins that are described to be downregulated after HDACi treatment are the leukemia fusion protein BCR-ABL, the receptor tyrosine kinase FLT3, mutant p53, the kinase c-Raf, cytoskeleton Tubulin, and the estrogen receptor ERα [119-121]. Depletion of these proteins is associated with reduced cellular motility, induction of cell growth inhibition, and cell death.

3.4.2 HDACi modulate the abundance of enzymes/regulators of the ubiquitin-proteasome system

Several groups have reported that HDACi also can induce the turn-over of certain cancer-relevant proteins by modulating the abundance of limiting amounts of proteins, which regulate protein ubiquitinylation and proteasomal degradation [122-126]. For example, the HDACi-mediated transcriptional up-regulation of ubiquitin conjugases and ubiquitin ligases of the ubiquitin-proteasome pathway acts in concert to specifically induce protein degradation. Increased protein degradation by HDACi treatment via such an indirect mechanism was first shown by our group. We could demonstrate that HDAC2, most interestingly an HDAC itself, is selectively degraded upon treatment with HDACi via up-regulation of the UBCH8 ubiquitin conjugase [122]. A similar mechanism is also reported for the degradation of the transcription factor WT1 [123]. Furthermore, HDACi-mediated upregulation of Skp1a and Skp2, subunits of a multiprotein ubiquitin ligase complex, and the ubiquitin conjugase UBCH5 can be crucial for HDACi-induced protein degradation [124-126]. Very recent findings further suggest that modulated proteasome activity contributes to the anti-cancer activity of HDACi. A genome-wide-loss-of-function screen identified HR23B, which shuttles ubiquitinylated cargo proteins to the proteasome, as a sensitivity determinant for HDACi-induced apoptosis [127]. Moreover, HDACi induce the activation and upregulation of cellular levels of ubiquitin, and ubiquitin was revealed as an essential regulator of HDACi-induced tumour-cell-selective apoptosis [128]. Of note, HDACi also evoke proteasomal depletion of the anti-apoptotic regulator Survivin and several (proto-) oncoproteins, e.g. DNMT1, RARβ and Aurora-A [129-131]. The ubiquitinylating-enzymes participating still remain to be elucidated.

4 Aim of the work

Analyzing how HDACi influence the fine-tuned abundance of enzymes of the proteasomal pathway and induce the turnover of cancer-relevant proteins provides further insights into the actions of these compounds. Given the pleiotropic effects HDACi have on multiple pathways, such data will aid in understanding how HDACi specifically affect different cancer cell types. In agreement with the important role that proteasomal degradation can have in the HDACi-evoked protein depletion and based on our finding that HDACi affect protein stability by upregulating the ubiquitin conjugase UBCH8, I investigated whether HDACi can induce the degradation of the leukemia-associated oncoproteins AML1-ETO, PML-RARα and FLT3-ITD.

5 Manuskript 1:

HDACi—Targets beyond chromatin

HDACs are involved in the remodelling of chromatin and have a key role in the epigenetic regulation of gene expression. In addition to histones, HDACs have many other protein substrates involved in gene expression, cell proliferation and cell death. In this review I summarize the findings how HDACi-mediated inhibition of HDACs alters oncogenic signaling networks and regulates the turnover of (proto-)oncoproteins by modulating the ubiquitin-proteasome system.

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Mini-review

HDACi – Targets beyond chromatin

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ABSTRACT

Histone deacetylases (HDACs) play an important role in gene regulation. Inhibitors of HDACs (HDACi) are novel anti-cancer drugs, which induce histone (hyper-) acetylation and counteract aberrant gene repression. On the other hand, HDACi treatment can also result in decreased gene expression, and targeting HDACs affects more than chromatin. Recently, HDACi were shown to evoke non-histone protein acetylation, which can alter signaling networks relevant for tumorgenesis. Furthermore, HDACi can promote the degradation of (proto-) oncoproteins. Here, we summarize these findings and discuss how these substances could be beneficial for the treatment and prevention of human ailments, such as cancer and unbalanced immune functions.

1. Introduction

The mammalian histone deacetylase (HDAC) family comprises 18 members. These enzymes are grouped into four classes, based on their homology to yeast deacetylase proteins. Class I HDACs (HDAC1, -2, -3, and -8), homologues of the yeast Rpd3 protein, are ubiquitously expressed and with exception of HDAC3 display a primarily nuclear localization. Class II HDACs share homology to yeast Hda1 and can be further subdivided into class IIa (HDAC4, -5, -7, and -9) and class IIb HDACs (Hdac6 and -10), which contain two catalytic sites. HDAC11 shares sequence conservation with both, Rpd3 and Hda1, and is therefore placed in class IV. Expression of class II and IV HDACs is restricted to certain tissues, where they shuttle between nucleus and cytoplasm. Classes I, II, and IV HDACs are Zn^+-dependent, while the class III HDACs (SIRT1 – 7), homologues of the yeast SIR2 protein, require NAD^+ for their activity.

Since histones were the first identified targets of deacetylases, these enzymes were termed histone deacetylases. Reversible acetylation of nucleosomal histone tails, an epigenetic modification involved in the regulation of gene expression, is balanced by the opposing activities of histone acetyltransferases (HAT) and deacetylases. Acetylation of histones provides a more open chromatin structure which correlates with gene activation, while histone deacetylation mediates transcriptional repression [1,2]. Inhibitors of HDACs can change the expression pattern of genes [3–5], and were shown to induce growth arrest, differentiation or apoptosis in cancer cells *in vitro* and *in vivo* [6–9]. Remarkably, HDAC enzymes are not exclusively targeted towards histones. A steadily growing number of non-histone proteins have been described to be subject to reversible acetylation by HATs and HDACs. Among these non-histone targets are transcription factors, hormone receptors, signal transducers, chaperones and proteins of

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Abbreviations: AML1-ETO, acute myeloid leukemia 1-eight-twenty-one; Bcr–Abl, breakpoint cluster region–abelson murine leukemia; CBP, CREB-binding protein; DNMT1, DNA methyltransferase 1; c-Raf, rat fibrosarcoma; FLT3, FMS-like tyrosine kinase 3; FoxO1, forkhead box 1; Hsp90, heat shock protein 90; HIF-1α, hypoxia-inducible factor 1 alpha; IFN-γ, interferon gamma; JAK, janus kinase; NF-κB, nuclear factor kappa B; PML–RARα, promyelocytic leukemia–retinoic acid receptor alpha; SAHA, suberoylanilide hydroxamic acid; SF-1, steroidogenic factor 1; Siah-1, seven in absentia homolog 1; STAT, signal transducer and activator of transcription; TNFα, tumor necrosis factor alpha; TSA, trichostatin A; Ubc8, ubiquitin conjugating enzyme 8; WT1, wilms tumor 1; VPA, valproic acid.

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the cytoskeleton [10]. Acetylation of these proteins modulates a wide variety of cellular events that are involved in many biological processes such as cell proliferation, cell survival or apoptosis [3,11,12]. Therefore, it is not surprising that aberrant protein acetylation can affect gene expression, cell signaling, enzymatic activity, protein localization, protein stability and protein–protein interactions [11]. Moreover, HATs and HDACs were found to be deregulated in cancer [6,13] and aberrant expression of HDACs has been observed in various tumor types [14–17]. Therefore, an altered balance of protein acetylation appears to contribute to cell transformation and cancer development [11,18]. Re-expression of down-regulated genes essential for growth inhibition and cell death, as well as the alteration of abnormal acetylation pattern of non-histone proteins, are considered as a viable approach in cancer therapy [9,19]. It has been demonstrated that HDACi equally sensitize cancer cells to the cytotoxic effects of other therapeutic agents [20,21], and they display selective toxicity as anti-cancer agents against tumor cells compared to untransformed cells [9,22]. Accordingly, HDACi are considered as candidate drugs for cancer therapy [6,23].

Acetylation of non-histone proteins has been shown to modulate their functions by altering their stability, cellular localization or interactions. A prominent target of non-histone acetylation is the tumor supressor p53, which can be acetylated at multiple lysines by distinct acetyltransferases [24,25]. This results in either increased DNA binding or loss of p53 transcriptional activity [26]. A similar complex acetylation-dependent regulation of gene expression is described for NF-κB p65, which can be positively or negatively affected by site-specific lysine acetylation [27,28]. Furthermore, lysine acetylation can create docking sites for transcriptional coactivators, as shown for binding of the HAT CBP to p53, or association of the HAT p300 with STAT3 [29]. Additionally, acetylation can affect STAT protein hetero- and homodimerization, cellular localization and crosstalk with NF-κB p65 and p52 signaling [3,30–32]. Furthermore, acetylation can interfere with other posttranslational modifications, like phosphorylation or ubiquitination. For example, acetylation of p53 can block ubiquitination of the same lysine residue, thus preventing p53 nuclear export and proteasomal degradation [25]. A similar mechanism was described for the transcription factor c-Myc, which is stabilized and protected from degradation upon acetylation [33]. In contrast to the positive effect, acetylation can have on protein stability, acetylation of proteins such as HIF-1α and FoxO1 can directly or indirectly facilitate their binding to enzymes of the ubiquitin-pathway, which promotes their enhanced proteasomal degradation [10,34]. Moreover, several studies suggested that HDACi-mediated anti-tumor effects are associated with hyperacetylation of the chaperone Hsp90. HDACi-induced hyperacetylation of Hsp90 disrupts the association with its client proteins, which correlates with their subsequent proteasomal degradation, as described for the leukemia fusion protein Bcr–Abl, the FLT3 kinase, and the c-Raf kinase [35–37].

Acetylation of proteins, which are often collectively termed the "acetylome", contributes to several processes which are crucial for cellular fate. Besides their ability to induce protein acetylation by inhibiting deacetylase activity, HDACi can also alter the turnover of several proteins via direct or indirect mechanisms. In this review, we focus on the HDACi-mediated indirect depletion of (proto-) oncoproteins via modulating the abundance of enzymes of the ubiquitin–proteasome pathway. In addition, we summarize the cytokine- and HDACi-stimulated acetylation-dependent crosstalks of STAT/NF-κB signaling pathways. We discuss the relevance of STAT protein acetylation for NF-κB signaling in carcinogenesis and inflammation. Extended knowledge on the cellular responses evoked via HDACi may contribute to further development of these drugs and expand their application range.

2. Acetylation-dependent crosstalk of signaling pathways

In mammals, the NF-κB family consists of five members: NF-κB p100/p52, NF-κB p105/p50, NF-κB p65 (RelA), RelB and c-Rel. These transcription factors regulate the expression of numerous genes controlling important cellular processes, such as differentiation, proliferation, inflammation, and cell death. Of note, increased NF-κB activity is linked to inflammatory diseases, malignant transformation and tumor progression [38,39]. Cytoplasmic p65/p50 heterodimers are kept in an inactive state by the inhibitors of NF-κB proteins (IκBs). Stimulus-induced activation of the IκB kinase (IKK) complex results in phosphorylation and degradation of IκBs, leading to nuclear translocation of NF-κB and NF-κB-dependent gene expression. Acetylation has been described to be crucial for NF-κB signaling. Acetylation of NF-κB proteins was shown to alter their activity and association with regulatory factors enhancing or repressing NF-κB signaling. Since the complex regulation and consequences of NF-κB modulation by reversible acetylation were extensively reviewed recently [11,40], we focus here on the relevance of STAT acetylation for NF-κB signaling.

The STAT proteins are a family of transcription factors comprising seven members. Basal and induced STAT activation regulates diverse biological processes, such as cell proliferation, survival, apoptosis, and differentiation. STATs show a predominantly cytoplasmic localization in unstimulated cells, and are activated by several stimuli, including cytokines and growth factors. Upon ligand binding to cell surface receptors, STAT homo- or heterodimers bind to phosphorylated receptor tyrosine residues and are activated via tyrosine phosphorylation by the JAKs. Activated STATs subsequently translocate to the nucleus, where they promote gene expression by binding to promotors containing GAS and ISRE sequences [41]. Remarkably, based on different signal transduction pathways and target genes, diverse STAT family members can inhibit or promote malignancies. STAT3 is suggested to act as an oncogenic transcription factor [42,43], and STAT3 activation has been detected in various tumor entities, for example in head and neck cancer, breast cancer, prostate cancer, and hematologic malignancies [44]. Furthermore, constitutively activated STAT3 is reported to induce cellular trans-

formation *in vitro* and tumor formation *in vivo* [45]. In contrast, cytokine-mediated apoptosis and inhibition of cell growth are linked to STAT1 signaling [46]. Thus, STAT1 is widely accepted as a negative regulator of cell transformation and proliferation [3,47]. Moreover, constitutive activation of STAT3 and reciprocal STAT1 repression has been found in tumors [42]. In contrast to the predominantly negative role of STAT1 activation on tumor growth and cell proliferation, recent reports show enhanced activation and pro-survival functions of STAT1 in tumor cells [47] pointing towards a more complex role of STAT1 in cancer development. On the other hand, enhanced STAT activation in tumors might also be a consequence of enhanced immunological tumor surveillance.

Upon various stimuli, the structurally related STAT1 and STAT3 proteins undergo acetylation. Their reversible lysine acetylation is mediated by HATs and HDACs which can associate with STAT proteins [29,31,48]. Cytokines not only induce STAT3 phosphorylation, but can equally evoke its acetylation. Two lysine residues, K48 and K87 within the N-terminal domain of STAT3, were demonstrated to play a critical role in STAT3 target gene expression [49]. Of note, N-terminal acetylation is necessary for the association of STAT3 with p300 and STAT3 transcriptional activity. Since both HDAC1 and p300 can interact with this region and acetylation was shown to be prerequisite for HDAC1 binding, Ray and co-workers [29] suggested that HDAC1-mediated repression of cytokine-induced gene activation relies on the deacetylase activity of HDAC1 and competitive binding with p300 to STAT3. Oncostatin M (interleukin-6 family member) evoked, p300-mediated acetylation of another lysine, K685 in the SH2-domain of STAT3, seems to be also critical for STAT3 dimerization, increased DNA binding, and target gene activation [31]. The inhibitory effect of class I HDAC overexpression on STAT3 activation could be abolished by the HDACi TSA, whereas STAT3 target gene expression was induced upon treatment with TSA. Furthermore, TSA-induced acetylation of STAT3 was accompanied by enhanced nuclear localization of STAT3 [50]. In summary, these data point to a positive role of cytokine and HDACi-induced acetylation in the regulation of STAT3 activity. Still it remains to be clarified, whether or not STAT3 can be acetylated on multiple lysine residues and how this may cooperatively affect its activity. Moreover, the relevance of STAT3 acetylation on K685 is controversially discussed in the literature [51] and these results contrast the well-established anti-proliferative functions of HDACi. Several genes under the control of STAT3 are indeed down-regulated by HDACi and inhibition of this pro-oncogenic factor may well contribute to the actions of HDACi [6,9,52].

Similar to STAT3, STAT1 has been reported to associate with HATs and HDACs [48,53]. In sharp contrast to STAT3 signaling, HDAC1, HDAC2 and HDAC3 are necessary for STAT1-dependent gene activation [48,54]. Silencing of these HDACs by siRNAs or structurally diverse HDACi, such as TSA, VPA, butyrate and SAHA, blocks the induction of IFN-stimulated STAT1 target gene expression [54,55]. Consistently, overexpression of HDACs enhanced STAT1-dependent gene expression. Although several studies have shown that HDACi can inhibit cytokine signaling via STAT1 and STAT2, no formal proof exists whether or not HDACi inhibit IFN signaling via acetylation of STAT1 or other mechanisms. We recently demonstrated, that a functional phospho-acetyl switch, regulated by an acetylation/deacetylation balance, modulates STAT1 signaling. IFN-induced STAT1 phosphorylation promotes STAT1 nuclear translocation, which enables CBP-mediated acetylation of STAT1. Acetylation of STAT1 via CBP forms a docking site for the phosphatase TCP45. This in turn leads to STAT1 dephosphorylation and persistent functional inactivation [56]. This phospho-acetyl switch provides an appealing model for the described negative role of acetylation on IFN-dependent signaling [48,54]. Moreover, these data demonstrate that CBP-mediated acetylation of STAT1 counteracts STAT1 activation and IFN-induced STAT1/STAT2 signaling mediated by the HAT GCN5 [57]. Remarkably, STAT1, haboring arginine exchanges in the STAT1 acetylation sites K410 and K413, is not inhibited by HDACi which demonstrates the functional importance of these residues in the acetylation-dependent STAT1 inactivation.

Besides the fact that acetylation affects STAT3-dependent biological processes, an acetylation-dependent crosstalk of STAT3 and NF-κB was described [32]. Overexpression of constitutively activated STAT3, but not of wild-type STAT3, induces proteolytic processing of the inhibitory NF-κB p100 precursor protein yielding active NF-κB p52. Further studies with activated endogenous STAT3, knockdown experiments and co-expression of dominant-negative STAT3 ($STAT3^{Y705F}$) indicate that STAT3 phosphorylation and activation are required for p100 processing in prostate cancer cells. Furthermore, acetylation of STAT3 might be required for STAT3-induced p100 processing. Overexpression of p300 resulted in STAT3 acetylation and enhanced p52 levels, whereas p300 depletion by siRNAs abolished p100 processing. Inhibition of deacetylase activity by TSA congruently enhanced p100 processing, whereas expression of an acetylation-defective STAT3 ($STAT3^{K685R}$) was unable to induce p100 processing. p100 has been discussed in the literature as a pro-apoptotic protein [32,58]. Overexpression of p100 increased apoptotic cell death in prostate cancer, which counteracts the anti-apoptotic, cytoprotective effect of p52. Therefore, processing of p100–p52, mediated by STAT3, may shift the balance between the pro-apoptotic p100 and the anti-apoptotic p52 towards p52. Since this NF-κB family member harbors oncogenic features, enhanced p100 processing may contribute to the transforming regulation of cell proliferation and survival via STAT3 [32]. Further studies have to reveal how this may inactivate anti-proliferative, pro-apoptotic actions of HDACi [6,9].

A potential crosstalk of STAT1 and NF-κB signaling, with a negative role of STAT1 on NF-κB, has been proposed [59,60]. In melanoma and fibrosarcoma cells, TSA, VPA, and the cytokine IFN-α induce STAT1 expression and acetylation, which correlates with the induction of apoptosis [3]. Since STAT1 has been directly linked to apoptosis induction, this observation is consistent with previous reports demonstrating enhanced sensitivity of STAT1-positive cell lines for apoptotic cell death upon different stimuli [61,62]. Gene expression analyses of melanoma cells treated with HDACi did not reveal changes in the expression of

STAT1 target genes pointing towards other signal pathways required for apoptosis induction via HDACi. We demonstrated that acetylated STAT1 interacts with NF-κB p65, which reduces nuclear p65 and modulates NF-κB signaling negatively [3]. Therefore, HDACi and IFN-α decrease NF-κB DNA binding and target gene expression via an acetylation-dependent STAT1/NF-κB interaction. The importance of this signaling crosstalk in the repression of NF-κB transcriptional activity is emphasized by the observation, that no significant differences in HDACi-induced histone hyperacetylation were detectable between HDACi-resistant and sensitive melanoma cell lines [3,63]. We identified lysines K410 and K413, located within the DNA binding domain of STAT1, as residues specifically acetylated by the HAT CBP in response to treatment with HDACi or IFN-α. Reconstitution of STAT1 null cells with pseudo-acetylated (K410Q, K413Q) and non-acetylated (K410R, K413R) STAT1 mutants further revealed that acetylation-mediated inhibition of NF-κB activity renders cells permissive to apoptosis induced by HDACi and IFN-α. Acetylation of STAT1 furthermore correlates with dissociation of HDACs from STAT1 and increased binding to CBP. Fig. 1.

Based on our observation that HDACi and IFN-α induce STAT1 acetylation and thereby modulate NF-κB activity [3], we have proposed a model in which acetylated STAT1 binds to and sequesters NF-κB in the cytoplasm, which interferes with NF-κB functions. Our results indicate that Stat1 acetylation acts as a molecular switch, which permits binding of STAT1 to NF-κB and thus reduces NF-κB signaling. These findings could contribute to the understanding of how HDACi-induced inhibition of HDAC activity can equally result in gene repression. Remarkably, acetylation of Stat1 is indispensable for its interaction with and inhibition of NF-κB p65, though not sufficient to mimic the HDACi-mediated attenuation of NF-κB. Hence, beside STAT1 acetylation, additional changes in signaling pathways or alterations in gene expression appear to be required for apoptosis induction via HDACi and IFNs.

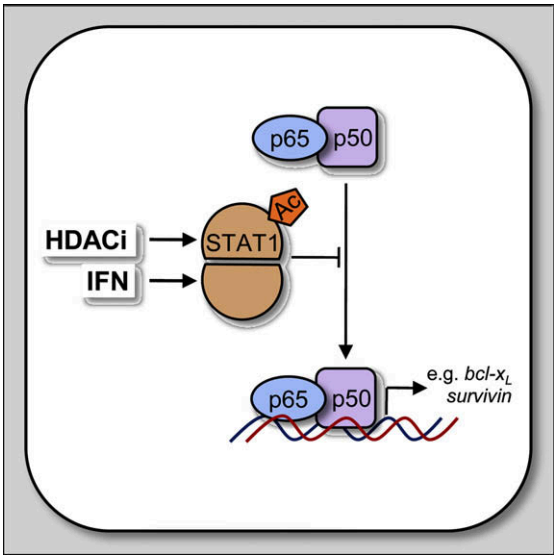

Fig. 1. STAT1/NF-κB signaling crosstalk. HDACi and IFN-mediated acetylation of STAT1 interferes with NF-κB signaling and inhibits the transcriptional activity of NF-κB p50/p65 heterodimers.

In support of these results, it was recently shown that STAT1 acetylation can repress pro-tumorgenic NF-κB activity and target gene expression *in vivo* [64]. Moreover, STAT1 acetylation was augmented in wild-type compared to IFN-γ deficient mice. Decreased acetylation of STAT1 in mice lacking IFN-γ correlates with enhanced NF-κB activity *in vivo*. In agreement with the observations in primary melanoma and fibrosarcoma cells, treatment with IFN-γ resulted in STAT1 acetylation and reduced nuclear NF-κB levels and NF-κB-dependent gene expression in LPS-treated primary murine neutrophils [64]. Worth mentioning, sustained NF-κB activation linked to inflammation can cause cancer [65].

In accordance with the negative effect of acetylated STAT1 on NF-κB signaling, Ganster and co-workers reported IFN-γ and STAT1-dependent inhibition of NF-κB signaling. These authors equally demonstrated that NF-κB and STAT1 interact on DNA [66], and STAT1 was shown to be required for IFN-α-mediated downregulation of TNFα-induced NF-κB activation [67]. On the other hand, synergistically induced gene transcription via STAT1/NF-κB upon co-stimulation with IFN-γ and TNFα was mediated by simultaneously recruitment of CBP. Since the acetyltransferase activity of CBP was dispensable for this process, STAT1 acetylation though appears unlikely to be involved in this process [68]. In light of these findings, it is plausible that inhibition of NF-κB via acetylation of STAT1 contributes to the anti-tumorgenic and anti-inflammators effects of these agents [69].

3. HDACi induce protein degradation by regulating the abundance of enzymes of the ubiquitin–proteasome pathway

Proteins are targeted by polyubiquitination to the 26S proteasome, a multiprotein complex mediating the proteolytic cleavage of proteins. Ubiquitination is usually carried out by the hierarchical action of three enzymes, a ubiquitin activating enzyme (E1), a ubiquitin-conjugase (E2), and a ubiquitin ligase (E3). E3 ligases are generally supposed to mediate substrate specificity.

Modulating the abundance of enzymes catalyzing ubiquitination can affect target protein turnover. Several groups have reported that HDACi induce degradation of certain (proto-) oncoproteins by modulating the expression of limiting amounts of enzymes catalyzing ubiquitination [70–73]. Increased protein degradation by HDACi treatment via such an indirect mechanism was first shown for HDAC2 [70]. Protein turnover of HDAC2 can be induced by the class I-selective HDACi valproic acid (VPA) and butyrate. The increased turnover of HDAC2 upon HDACi treatment is paralleled by enhanced expression of the E2 ubiquitin-conjugase Ubc8. Overexpression and siRNA knockdown studies demonstrated that Ubc8 and its associated E3 ligase RLIM are the enzymes required for polyubiquitination and degradation of HDAC2. Similar to VPA, the HDACi TSA, an inhibitor of class I and class II HDACs, induces Ubc8 expression but

simultaneously enhances degradation of RLIM. As a consequence, HDAC2 protein levels remain constant in the presence of this HDACi, indicating that the amount of both, the E3 RLIM and the HDACi-inducible E2 Ubc8, are critical for the turnover of HDAC2 [70]. Thus, HDACi triggered ubiquitination and turnover of HDAC2 are regulated by the abundance of the E2 conjugase Ubc8 and its associated E3 ligase RLIM. Abberant gene expression by HDAC recruitment contributes to malignant cell transformation, and HDAC2 expression is essential for proliferation and survival of colonic cancer cells [17,18]. Hence, one may speculate that depletion of HDAC2 protein via the Ubc8-RLIM axis induces apoptosis.

Recently, we also demonstrated that the abundance of two chimeric fusion proteins, AML1-ETO and PML–RARα, by HDACi is regulated via a mechanism similar to the one described above [71]. The chromosomal translocation products AML1-ETO and PML–RARα are crucial for the transformation of myeloid precursors, which contributes to the pathogenesis of leukemia [71,74,75]. Treatment of leukemic cell lines with HDACi increased polyubiquitination and subsequent proteasomal degradation of AML1-ETO and PML–RARα. Although a decline in protein levels was paralleled by reduced mRNA expression, the loss of protein due to HDACi was largely blocked by inhibition of the proteasome. Hence, an HDACi-induced proteasomal mechanism appears to be the major cause for the degradation of AML1-ETO and PML–RARα. Similar to the HDACi-mediated degradation of HDAC2 [70], VPA strongly induces the ubiquitin-conjugase Ubc8 in leukemic cells. Additionally, depletion of Ubc8 abolishes HDACi-induced apoptosis, indicating a fundamental role of Ubc8 and protein degradation in the induction of apoptosis by HDACi. Contrary to the formerly described role of RLIM in HDAC2 turnover [70], degradation of AML1-ETO and PML–RARα does not require RLIM. Instead, the turnover of these proteins critically depends on the E2 conjugase Ubc8 and the E3 ligase SIAH-1, which was previously identified as E3 ligase for PML–RARα [76]. Moreover, VPA-induced Ubc8 expression caused a loss of RLIM in the analyzed leukemic cell lines. In agreement with these findings, RLIM associates with Ubc8 and SIAH-1, and overexpression and knockdown studies revealed that Ubc8 and SIAH-1 cooperatively promote RLIM turnover. Leukemic fusion proteins can repress gene expression by recruitment of repressor complexes containing HDACs, and they compete with transcription factors of proper hematopoiesis [23]. Hence, anti-leukemic properties of HDACi [77] may depend on direct inhibition of fusion protein-associated HDACs and equally on proteasomal degradation of AML1-ETO and PML–RARα protein. Both events can ameliorate aberrant repression and restore proper gene expression (Fig. 2).

The transcription factor WT1 is a further protein downregulated by HDACi at the mRNA and protein levels in different cell lines. In addition to transcriptional repression of the WT1 gene, TSA mediates enhanced proteasomal degradation of the WT1 protein. TSA-induced WT1 degradation correlates with increased expression of the E2 Ubc8. Silencing of Ubc8 expression by siRNA blocks TSA-mediated WT1 degradation, emphasizing the relevance of proteasomal degradation for HDACi-modulated WT1 protein stability [72].

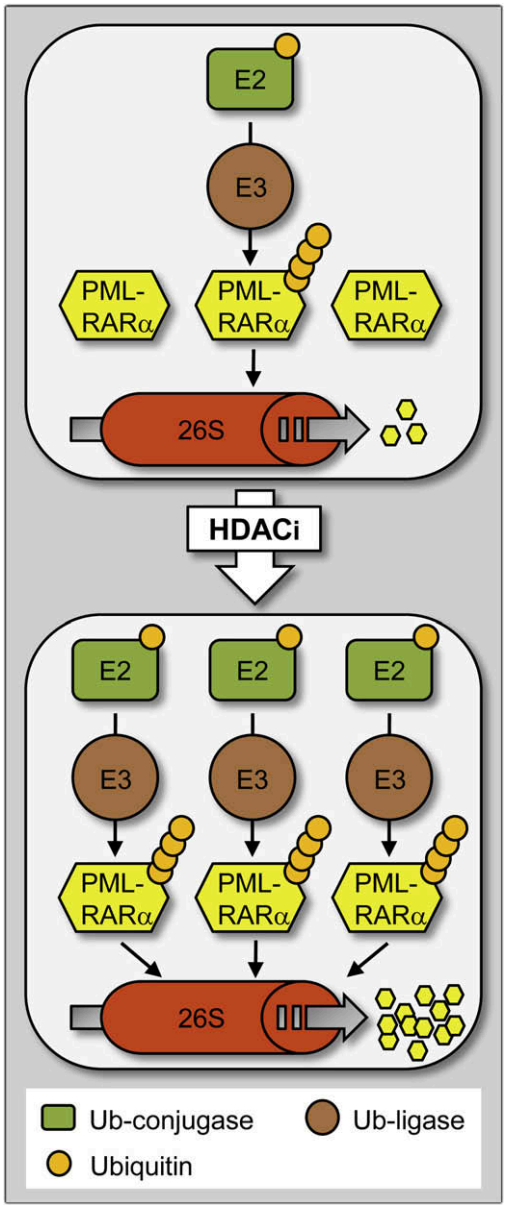

Fig. 2. HDACi-induced protein degradation. HDACi regulate the expression of E2 ubiquitin-conjugases and/or E3 ligases. Increased abundance of these limiting enzymes of the ubiquitin–proteasome pathway triggers polyubiquitination and protein degradation. Thus, HDACi treatment leads to reduced levels of substrate proteins, e.g., of HDAC2, AML1-ETO, and PML–RARα.

In agreement with the important role proteasomal degradation can have in the HDACi-evoked depletion of (proto-) oncoproteins, induced protein degradation via the ubiquitin–proteasome pathway can occur independent from inhibition of mRNA expression (e.g. for Aurora-A, mutated FLT3, DNMT1) [36,78,79]. Moreover, HDACi can for example reduce RARβ protein levels via the proteasomal pathway, although they upregulate expression of RARβ mRNA [80].

RLIM and SIAH are not the only E3 ligases reported to be involved in enhanced proteasomal degradation via HDACi treatment. In adrenocortical tumor cells, structurally diverse HDACi can enhance the ubiquitination and degradation of SF-1, a nuclear receptor superfamily member that controls the expression of genes involved in steroidogenesis [81,82]. HDACi reduce SF-1 protein levels without affecting mRNA levels, but cause increased expression of Skp1a. This protein represents a subunit of the Skp1-Cullin-F-box (SCF) complex, which belongs to the RING finger class of E3 ubiquitin ligases. Knockdown of Skp1a expression with siRNA abolished the HDACi-induced degradation of SF-1, which points to Skp1a as rate-limiting E3 in SF-1 protein turnover. Gene expression analyses also revealed that HDACi upregulate *Ube2D1*, which encodes for an E2 conjugating enzyme of the SCF-complex. Further investigations are required to prove whether Ube2D1 is the ubiquitin-conjugase responsible for SF-1 degradation caused by TSA. Another subunit of the SCF-complex, the Skp2 F-box protein (Skp2), was found to be induced in breast cancer cells by treatment with TSA [83]. Induced accumulation of Skp2 has been shown to be crucial for TSA-induced, ubiquitin-dependent degradation of cyclin D1, with a decline of cyclin D1 protein within six hours in human breast carcinoma cell lines [73]. Of note, this protein is a regulator of cell cycle transition and important for breast cancer development.

Several proteasome inhibitors such as bortezomib and MG-132, reveal anti-tumorgenic effects *in vitro* and *in vivo*. Inhibition of proteasome activity blocks NF-κB activity by cytoplasmic accumulation of the IκBα protein. Furthermore, these small-molecules generate reactive oxygen species (ROS), induce activation of caspases and accumulation of non-degraded cellular proteins, which in turn stimulates endoplasmatic reticulum stress and apoptosis [84]. Moreover, proteasome inhibitors in combination with HDACi further increase ROS generation and reduce NF-κB activity, compared to single agents alone, and were shown to synergize in induction of apoptotic cell death [85–87]. Thus, multiple anti-tumorgenic effects induced by proteasome inhibitors alone or in combination with HDACi may act in concert. Depending on the cellular context, these combined cytotoxic effects could readily overcome a reduced degradation of oncoproteins due to proteasome inhibition.

4. Summary

Protein acetylation and gene expression regulate protein stability, which can influence cellular signaling, cell proliferation and apoptosis. Acetylation of proteins can interfere with other posttranscriptional modifications such as ubiquitination and prevent or induce the proteasomal degradation of (proto-) oncoproteins, e.g. HIF-1α and c-Myc [11]. Moreover, HDACi-induced abrogation of chaperone function correlates with enhanced degradation of client proteins, such as FLT3, Bcr–Abl, mutated p53, or the estrogen receptor ERα. Analyzing how HDACi influence the fine-tuned abundance of enzymes of the proteasomal pathway, and induce the turnover of cancer-relevant proteins, such as AML1-ETO, PML–RARα, HDAC2, or cyclin D1, provides further insights into the actions of these compounds. Given the pleiotropic effects HDACi have on multiple pathways, such data will aid in understanding how HDACi specifically affect different cell types.

Conflicts of interest statement

The authors state that no conflicts of interest exist.

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6 Manuskript 2:

Mechanism for ubiquitylation of the leukemia fusion proteins AML1-ETO and PML-RARalpha

This study demonstrates that the expression of the oncogenic fusion proteins AML1-ETO and PML-RARα is regulated by the ubiquitin-proteasome system and that their turnover depends on the HDACi-inducible E2 ubiquitin conjugase UBCH8 and the E3 ubiquitin ligase SIAH1. Furthermore, this work reveals that UBCH8 is the ubiquitin conjugase mediating SIAH1 auto-ubiquitylation and turnover and I identified the UBCH8-interacting ubiquitin ligase RLIM as a target of SIAH1. These observations shed light on the interplay between E2 and E3 enzymes and provide a novel strategy for the downregulation of oncogenic leukemia fusion proteins.

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Mechanism for ubiquitylation of the leukemia fusion proteins AML1-ETO and PML-RARα

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ABSTRACT **The chromosomal translocation products AML1-ETO and PML-RARα contribute to the pathogenesis of leukemias. Here, we demonstrate that both AML1-ETO and PML-RARα are degraded by the ubiquitin-proteasome system and that their turnover critically depends on the E2-conjugase UbcH8 and the E3-ligase SIAH-1. Contrary to its role in HDAC2 degradation, the E3-ligase RLIM does not target AML1-ETO and PML-RARα for ubiquitin-dependent elimination. RLIM rather is a substrate of SIAH-1, which indicates that these E3-ligases operate in a hierarchical order. Remarkably, proteasomal degradation of leukemia fusion proteins, in addition to the block of histone deacetylase (HDAC) enzymatic activity is a consequence of HDAC-inhibitor treatment. The former requires the induction of UbcH8 expression and each of these processes might be beneficial for leukemia treatment. Our observations shed light on the mechanism determining the interplay between E2-conjugases, E3-ligases, and their substrates and suggest a strategy for utilizing the ubiquitylation machinery in a therapeutic setting.—Krämer, O. H., Müller, S., Buchwald, M., Reichardt, S., Heinzel, T. Mechanism for ubiquitylation of the leukemia fusion proteins AML1-ETO and PML-RARα. *FASEB J.* 22, 000–000 (2008)**

Key Words: proteasomal degradation · RLIM · Siah-1 · UbcH8 · HDAC-inhibitor VPA

Several diseases are linked to changes in gene expression caused by the conversion of transcriptional activators into repressors due to chromosomal translocations. Their occurrence correlates with certain leukemia subtypes, and fusion proteins likely are the initiating event in leukemia development. In ~10–15% of all *de novo* acute myeloid leukemia (AML) patients, and especially in the M2 subtype (FAB, French-American-British classification), the *AML1* (acute myeloid leukemia, *RUNX1*) gene located on chromosome 21 is fused to the *ETO* (eight-twenty-one, *MTG8*, *CBF2T1*) gene on chromosome 8 (1). This translocation t(8;21) replaces the transactivation domain of AML1, a crucial factor for definitive hematopoiesis, with almost the complete ETO protein. The chimeric AML1-ETO protein interferes with normal AML1-dependent transcription by constitutive repression of genes for hematopoietic differentiation *via* the ETO part, which recruits repressor complexes containing N-CoR, mSin3, and histone deacetylases (HDACs). Association of AML1-ETO with these proteins thereby blocks hematopoietic gene expression, differentiation, and apoptosis, and provides a reservoir of preleukemic cells (1–5).

Similar events occur in the M3 subtype (FAB) of AML, acute promyelocytic leukemia (APL). APL is predominantly associated with the chromosomal translocation t(15;17), resulting in the fusion protein PML-RARα (promyelocytic leukemia-retinoic acid receptor α). This oncoprotein binds to RAR target genes and recruits HDACs *via* N-CoR and mSin3. The recruitment of these factors is insensitive to physiological levels of retinoic acid and blocks differentiation and PML-dependent apoptosis of promyelocytes (6).

The roles of AML1-ETO and PML-RARα during leukemogenesis, the signaling pathways they affect, and factors determining their turnover are under intense investigation (1, 6). PML-RARα has been shown to undergo proteasomal degradation, which involves polyubiquitylation exerted by the hierarchical action of an E1-enzyme, an E2-conjugase and an E3-ligase (7). The E3-ligase SIAH-1 was identified as an enzyme mediating PML-RARα degradation (8). Moreover, turnover of PML-RARα can be increased by chemotherapy, which is appreciated as a mechanism accounting for its effectiveness in APL treatment (6). A small-molecule approach that would eliminate the initiating factor predisposing myeloid precursors for transformation could also be beneficial for patients with t(8;21) leukemia.

Inhibitors of HDACs (HDACi) might be a possible treatment option for cancer and have recently received appreciable attention. Considering the important role of HDACs in M2 AML and M3 APL, HDACi are promising therapeutic agents for leukemias. Recent findings show that these compounds not only affect HDAC activity, but also protein stability (reviewed in 9). Both effects may explain the molecular actions of HDACi *in vitro* as well as in experimental therapy (9–15). We previously showed that valproic acid (VPA)

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not only inhibits HDACs but also induces proteasomal degradation of the histone deacetylase HDAC2, which is regulated by limiting amounts of the E2-conjugase UbcH8 and the RING-finger E3-ligase RLIM (12). Selective VPA-induced attenuation of HDAC2 has been confirmed in murine tissues and in patient material (10, 12, 16). Analysis of the HDACi-induced protein turnover may hence be useful for molecular monitoring and for the development of therapeutic strategies.

MATERIALS AND METHODS

Cell culture, Western blot, immunoprecipitations, pull-down experiments, *in vivo* ubiquitylation analysis and real-time quantitative PCR analysis

Cell culture, lysate preparation, and transfections are described in refs. 12, 17. Cells were grown in Dulbecco modified Eagle medium (DMEM) (293T) or Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal calf serum (FCS), 2% L-glutamine, antibiotics and 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; SKNO-1). *N*-ethyl-maleimide (10 mM; Sigma, St. Louis, MO, USA) was added to lysates to preserve protein modifications and UbcH8 complex formation. MG-132 (1 μM) was added for 4–16 h to prevent the degradation of polyubiquitylated leukemia fusion proteins. For pull-down experiments, glutathione *S*-transferase (GST) -UbcH8 was expressed in *Escherichia coli* BL21-Gold (DE3) pLysS (Stratagene, La Jolla, CA, USA). Bacteria were lysed in PBS containing 0.5 mg/ml lysozyme, 1 mM dithiothreitol (DTT), 5 mM EGTA, 5 mM EDTA, and protease inhibitors. GST fusion proteins were immobilized and purified on glutathione-Sepharose 4B beads (Amersham, Piscataway, NJ, USA). Two hundred micrograms of cell lysate and 50 μg of the GST fusion protein were incubated in hypotonic lysis buffer (17). To detect the ubiquitylation of leukemia fusion proteins, lysates were prepared under stringent conditions in radioimmunoprecipitation assay (RIPA) buffer. All immunoprecipitations were done with 1 μg of antibody and 40 μl protein A/G beads (Amersham). After incubation for 16 h at 4°C, beads were washed, and bound proteins were eluted with Laemmli buffer and analyzed by immunoblotting. Antibodies for Western blot analysis were from Santa Cruz Biotechnology (Santa Cruz, CA, USA; AML1-ETO, sc-9797/-9737; caspase-3, sc-7272; Gal4, sc-510; GFP, sc-9996; GST, sc-138; HDAC2, sc-7899, sc-9959; HA, sc-7392; HSP90, sc-13119; Myc, sc-40; PML, sc-966; SIAH-1, sc-5506; antiacK, sc-8663), Sigma (actin, A2066/A5060; Flag, F3165; α-tubulin, T5168; ubiquitin, U5379), Abgent (San Diego, CA, USA; UbcH8, AP2118b), Chemicon (Temecula, CA, USA; ubiquitin, MAB1510), Upstate (Lake Placid, NY, USA; antiacK), New England Biolabs (Beverly, MA, USA; antiacK, 9441), Invitrogen (Carlsbad, CA, USA; V5-tag, 46–0705). Immune sera against RLIM and PML-RARα were described elsewhere (18, 19). Western blots were probed for actin to ensure equal sample loading. Real-time quantitative RT-PCR analyses determining delta-delta-C_t were carried out with SYBRGreen as described (20). 18S rRNA primer sequences were 5′-CGGCTACCACATCCAAGGA and 5′-CCAATTACAGGGCCTCGAAA.

Plasmids, transfections, and apoptosis assays

Plasmids for UbcH8, RLIM, SIAH-1, AML1-ETO, and PML-RARα are described elsewhere (4, 8, 12, 21, 22). 293T cells were transfected with lipofectamine or polyethylenimine (PEI) (10 mM; 2.7 μl/μg DNA). Empty vector pcDNA3.1 was used to obtain equal amounts of transfected DNA. Transfection efficiencies were around 80–100% for 293T cells, as measured by green fluorescent protein (GFP) expression. UbcH8 and SIAH-1 levels were lowered by lipofectamine-mediated transfection of siRNAs directed against the UbcH8 mRNA or the SIAH-1/2 mRNAs (sc-44102) (12) as specified by Invitrogen. Irrelevant siRNAs (sc-37007, (12) served as controls. Kasumi-1 cells were transfected with lipofectamine on seven consecutive days. 293T cells were transfected once, and lysates were prepared 48–72 h later. NB-4 cells were electroporated as recommended by Amaxa (Cologne, Germany). Dominant-negative SIAH-1 was generated by site-directed mutagenesis (Stratagene) and verified by sequencing. The primers (Thermo, San Jose, CA, USA) 5′-GCCCAAAGCTCACATGTTCTCCAACTTGCCGG and 5′-CCGGCAAGTTGGAGAACTGTGGC-TTGGGC exchange C^{72} to S^{72}. Apoptosis assays were performed as described (17). 10^5 cells were fixed in 1 ml 70% EtOH with 0.05% Tween-20 overnight at 4°C. The following day, cells were washed with 3 ml 38 mM sodium citrate, pH 7.4, and incubated for 20 min at 37°C in 0.5 ml 38 mM sodium citrate, pH 7.4, supplemented with 50 μg/ml propidium iodide and 10 μg/ml RNase-A. DNA contents of the cells were measured by fluorescence-activated cell sorter (FACS). This method allows detection of the apoptotic subG1-fraction, which has a DNA content below 2n due to apoptotic cleavage of DNA. Equally, the cell cycle profile can be assessed with this method. Nucleosomal laddering was performed according to the method described by Yeung (23).

RESULTS

Proteasomal degradation of AML1-ETO is induced by VPA

There is increasing evidence that apart from inhibiting the catalytic activity of HDACs, HDACi can affect protein stability (9, 12). Because knowledge about leukemia fusion protein stability in the presence of HDACi is limited, we investigated whether the well-tolerated HDACi valproic acid affects the stability of endogenous AML1-ETO in the human t(8;21)-positive AML cell line Kasumi-1. **Figure 1*A*** shows a significant decrease of AML1-ETO at the protein level in Kasumi-1 cells treated with the therapeutically achievable concentration of 1.5 mM VPA (24, 25) for 24 h. Compared to the leukemia fusion protein, the intact proteins AML1 and ETO were clearly less susceptible to VPA-induced degradation (Supplemental Fig. S1).

Because VPA destabilizes HDAC2 *via* a proteasomal mechanism (9, 12), we investigated whether AML1-ETO degradation in Kasumi-1 cells equally depends on ubiquitin-dependent elimination by the 26 S proteasome. We found that the proteasomal inhibitors MG-132 or ALLN significantly increased the AML1-ETO level and abolished its VPA-induced degradation (Fig. 1*B* and data not shown). Real-time quantitative RT-PCR revealed that AML1-ETO mRNA transcripts also decreased in response to VPA (Fig. 1*C*). However, MG-132 treatment also reduced the mRNA levels of AML-ETO, although its protein stability was increased. Thus, a

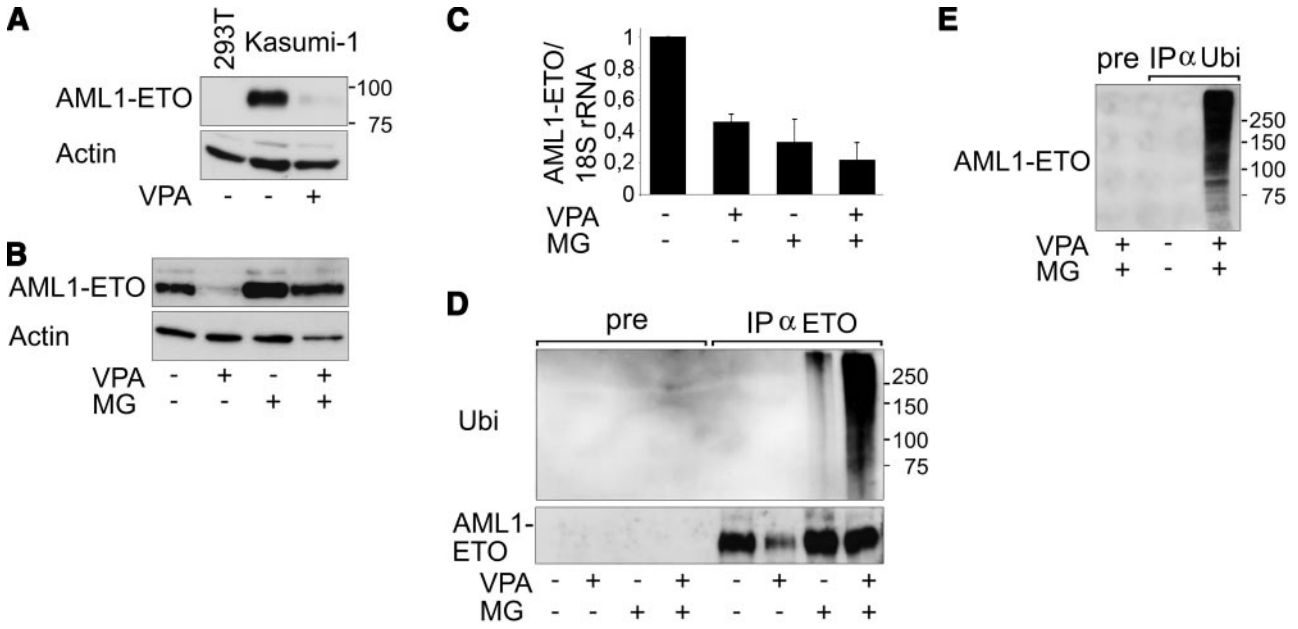

Figure 1. VPA reduces AML1-ETO protein levels *via* the ubiquitin-proteasome pathway. *A*) Kasumi-1 AML cells were left untreated (–) or treated for 24 h with the HDACi VPA (+; 1.5 mM). Untreated 293T cells served as a control for the specificity of AML1-ETO detection. Amounts of AML1-ETO and the loading control actin were determined by Western blot analysis. Molecular weight markers are indicated on the right. *B*) Kasumi-1 cells were treated for 24 h with 1.5 mM VPA (+) or left untreated (–). MG-132 (MG; 10 μM) was added during the last 4 h of incubation. AML1-ETO and actin protein levels were determined by Western blot analysis. *C*) Kasumi-1 cells were incubated for 24 h with 1.5 mM VPA or 1 μM MG-132 (MG), or left untreated. AML1-ETO and 18 S RNA levels were determined by real-time RT-PCR. Relative AML-ETO mRNA expression levels are shown. *D*) Precipitation of endogenous polyubiquitylated AML1-ETO with anti-AML1-ETO antiserum and Western blot analysis with an antibody against ubiquitin. Kasumi-1 cells were left untreated (–) or treated with 1.5 mM VPA (+) for 24 h. The proteasome inhibitor MG-132 (MG; 10 μM) was added 4 h before cell harvest. Nonimmune serum (pre) served as control. *E*) Same as in *D*, except that an antiubiquitin antibody was used for immunoprecipitation and high-molecular-weight AML1-ETO-reactive proteins were detected with an AML1-ETO antibody.

mechanism involving enhanced proteasomal turnover is the most likely explanation for AML1-ETO degradation after VPA treatment.

We obtained direct evidence for AML1-ETO ubiquitylation when AML1-ETO immunoprecipitates were probed with an antibody against ubiquitin. This experiment showed that Kasumi-1 cells contain ubiquitylated AML1-ETO, which can be detected on proteasomal inhibition (Fig. 1*D*). Thus, a basal level of AML1-ETO ubiquitylation occurs independently of VPA-treatment. Cotreatment with VPA and MG-132 led to the occurrence of dramatically increased amounts of polyubiquitylated AML1-ETO.

To confirm these observations, we probed antiubiquitin immunoprecipitates with an antibody against AML1-ETO. In this direction, AML1-ETO-reactive high-molecular-weight bands migrating more slowly than unmodified AML1-ETO were detected in Kasumi-1 cells treated with both VPA and MG-132 (Fig. 1*E*). We concluded that ubiquitylation of endogenous AML1-ETO occurs *in vivo* and can be increased by HDACi.

UbcH8 critically regulates the degradation of AML1-ETO

Polyubiquitylation of proteins requires the concerted action of an E2-conjugase and an E3-ligase (7). HDACi up-regulate the E2 UbcH8 at the mRNA and protein level, which results in the proteasomal degradation of HDAC2 (12). We hypothesized that UbcH8 also regulates the turnover of AML1-ETO. Because Kasumi-1 cells undergo apoptosis in response to HDACi (see below) and have a very low DNA transfection efficiency, we transiently expressed AML1-ETO and UbcH8 in 293T cells. This approach is commonly used to investigate protein turnover (8). Indeed, AML1-ETO expression was strongly reduced on cotransfection of UbcH8 (**Fig. 2*A***), whereas the ectopically expressed Gal4 protein under the control of the same promoter was unaffected (Supplemental Fig. S2). This showed that the abundance of UbcH8 determines the stability of AML1-ETO.

To obtain further proof for the role of UbcH8 in the turnover of AML1-ETO, AML1-ETO was transfected together with siRNAs specific for UbcH8 or murine Ubce8 as a nontargeting control in human 293T cells. Data obtained with this experiment confirmed that AML1-ETO is a UbcH8 target (Fig. 2*B*). Next, we transfected Kasumi-1 cells with these siRNA oligonucleotides. We found that UbcH8 siRNAs specifically decreased UbcH8 and augmented AML1-ETO levels in Kasumi-1 cells. Moreover, in VPA-treated Kasumi-1 cells, the siRNAs directed against human UbcH8 significantly reduced the degradation of the leukemic fusion

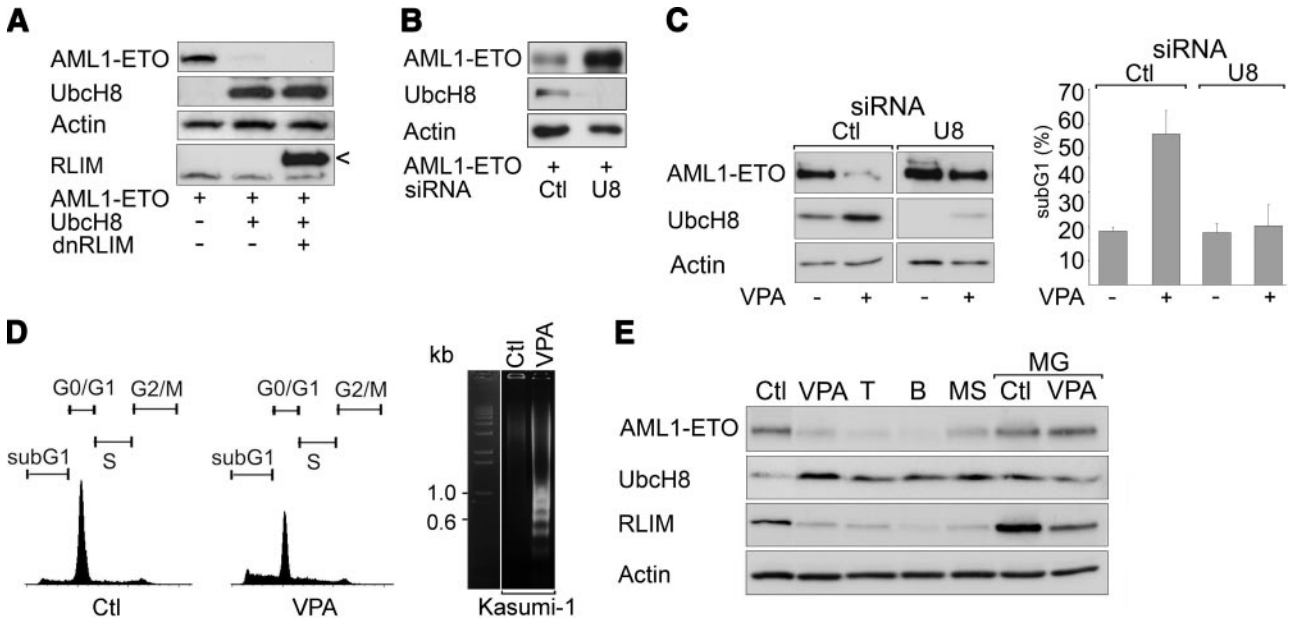

Figure 2. The E2-conjugase UbcH8 induces AML1-ETO degradation. *A*) 293T cells were transfected with 0.4 μg of Gal-AML1-ETO and 0.2 μg UbcH8 or 0.2 μg of a myc-tagged dominant-negative RING-finger mutant form of RLIM (dnRLIM). Empty vector pcDNA3.1 was added if appropriate to achieve 1 μg DNA per 12 wells. Expression of AML1-ETO, UbcH8, actin, and RLIM were detected by Western blot analysis 48 h after transfection; the lower band represents endogenous RLIM (arrowhead: overexpressed dnRLIM). Actin protein levels were determined to verify equal loading. *B*) 293T cells were transfected with Gal-AML1-ETO (1 μg) and two siRNAs directed against human UbcH8 (U8) or murine Ubce8 (Ctl) as a control (40 pmol). AML1-ETO, UbcH8, and actin levels were determined 48 h later by Western blot analysis. *C*) Endogenous UbcH8 levels were lowered in Kasumi-1 cells by transfection of two siRNAs directed against the UbcH8 mRNA (U8) or a nonrelated mRNA (Ctl). Cells were left untreated or were incubated with 1.5 mM VPA for 24 h. AML1-ETO, UbcH8, and actin levels were determined by Western blot analysis. Apoptosis rates were measured by PI-FACS-analysis (right; subG1, apoptotic cells with a DNA content <2n). *D*) Kasumi-1 cells were treated for 24 h with 1.5 mM VPA or left untreated (Ctl). The occurrence of apoptosis was determined by PI-FACS-analysis (left) and by assessment of nucleosomal laddering on a 2% agarose TAE-gel (right; kb, kilobase pairs). *E*) Kasumi-1 cells were treated with 1.5 mM VPA, 100 nM TSA (T), 1.5 mM butyrate (B), 5 μM MS-27–275 (MS), or MG-132 (MG; 1 μM) alone or with 1.5 mM VPA for 24 h. AML1-ETO, UbcH8, RLIM, and actin levels were determined by Western blot analysis.

protein (Fig. 2*C*, left). These data together with those depicted in Fig. 1*D, E* suggest a basal as well as a VPA-induced proteasomal degradation of AML1-ETO *via* UbcH8.

Similar to other HDACi, VPA can induce apoptosis and cell-cycle arrest in transformed hematopoietic progenitor cells and leukemic blasts from AML patients *in vitro* and *in vivo* (24, 26, 27). Kasumi-1 AML cells underwent apoptosis in response to VPA (Fig. 2*D* and Supplemental Fig. S3), which correlated with the induction of UbcH8 and the reduction of AML1-ETO (Fig. 2*C*, left, and Supplemental Fig. S3). Furthermore, depletion of UbcH8 by siRNA protected these cells from VPA-induced apoptosis (Fig. 2*C*, right), indicating a role of UbcH8 in the HDACi-mediated induction of apoptosis in this cell line.

UbcH8 serves as an E2 for the RLIM E3 and both act together on HDAC2 as a substrate. The HDACi trichostatin A (TSA) and MS-27–275, unlike VPA and butyrate, induce proteasomal degradation of RLIM and thus fail to induce HDAC2 degradation in 293T and F9 cells (12). In contrast, AML1-ETO levels decreased after application of each of these HDACi to Kasumi-1 cells (Fig. 2*E*). Therefore, we analyzed RLIM expression in these cells. We observed that UbcH8 levels increased and RLIM levels decreased after incubation with HDACi. The proteasomal inhibitor MG-132 prevented the reduction of RLIM, which indicates its HDACi-enhanced proteasomal degradation. Because all HDACi that we tested reduce RLIM (Fig. 2*E*), it appears unlikely that this E3 is crucial for the proteasomal degradation of AML1-ETO. To test this hypothesis, we transfected dominant-negative RLIM (19) together with AML1-ETO and UbcH8. This RLIM mutant was abundantly expressed but failed to stabilize AML1-ETO (Fig. 2*A*). Hence, RLIM is not critical for the degradation of AML1-ETO. Moreover, Kasumi-1 cells had stable HDAC2 levels in the presence of VPA (**Figs.** 2*E* and **3***A*), which confirms that VPA blocked RLIM functions.

Next, we investigated the time course of AML1-ETO degradation in Kasumi-1 cells. After a 7-h exposure to 1.5 mM VPA, AML1-ETO started to decrease (Fig. 3*A*). VPA concentrations required for AML1-ETO reduction are similar to those for the inhibition of HDAC enzymatic activity, and the delayed degradation of AML1-ETO before the onset of apoptosis argues against a direct destabilization by VPA. Moreover, unlike the apoptosis-inducing compound MG-132, the pancaspase inhibitor Z-VAD-FMK could not rescue AML1-

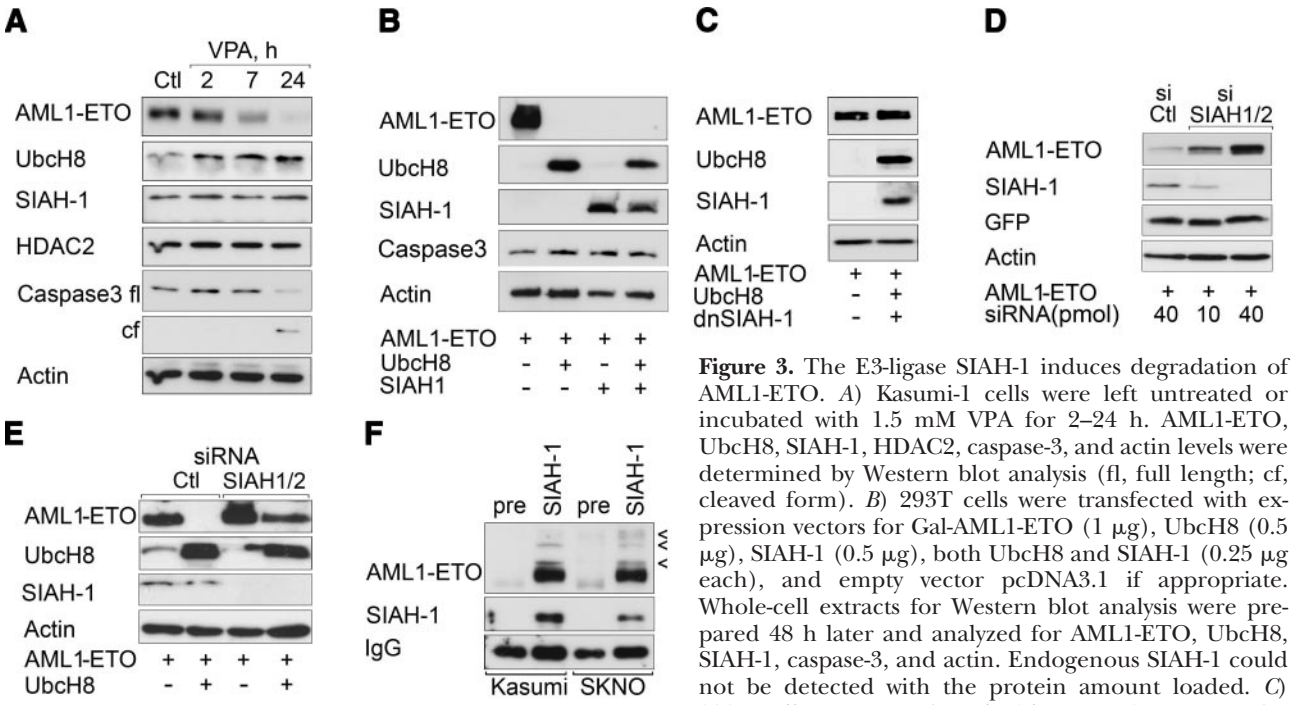

Figure 3. The E3-ligase SIAH-1 induces degradation of AML1-ETO. *A*) Kasumi-1 cells were left untreated or incubated with 1.5 mM VPA for 2–24 h. AML1-ETO, UbcH8, SIAH-1, HDAC2, caspase-3, and actin levels were determined by Western blot analysis (fl, full length; cf, cleaved form). *B*) 293T cells were transfected with expression vectors for Gal-AML1-ETO (1 μg), UbcH8 (0.5 μg), SIAH-1 (0.5 μg), both UbcH8 and SIAH-1 (0.25 μg each), and empty vector pcDNA3.1 if appropriate. Whole-cell extracts for Western blot analysis were prepared 48 h later and analyzed for AML1-ETO, UbcH8, SIAH-1, caspase-3, and actin. Endogenous SIAH-1 could not be detected with the protein amount loaded. *C*) 293T cells were transfected with expression vectors for Gal-AML1-ETO (0.4 μg), UbcH8 (0.2 μg), dominant-negative SIAH-1^{C72S} (dnSIAH-1; 0.05 μg), and pcDNA3.1 if appropriate. Lysates for Western blot analysis were prepared 48 h later and analyzed for the expression of AML1-ETO, UbcH8, SIAH-1, and actin. *D*) 293T cells were transfected with AML1-ETO (0.5 μg) and with either control siRNA (si Ctl, 40 pmol) or increasing amounts of siRNA against SIAH-1/2 (10–40 pmol). GFP expression from a cotransfected vector served as internal control. Forty-eight hours after transfection, AML1-ETO, SIAH-1, GFP and actin levels in whole-cell lysates were determined by Western blot analysis. *E*) 293T cells were transfected with control siRNA (Ctl) or siRNA against SIAH-1/2 (40 pmol) together with expression vectors for Gal-AML1-ETO and UbcH8. Forty-eight hours after transfection, AML1-ETO, UbcH8, SIAH-1, and actin levels were determined by Western blot analysis. *F*) Endogenous SIAH-1 interacts with endogenous AML1-ETO and induces polyubiquitylation *in vivo*. Interaction of SIAH-1 with AML1-ETO was tested by coimmunoprecipitation from Kasumi-1 and SKNO-1 cell lysates. AML1-ETO coprecipitated with the SIAH-1 antibody was detected by Western blot analysis. Control immunoprecipitations were performed with normal goat serum (pre; IgG, immunoglobulin heavy chain; arrowheads: ubiquitylated AML1-ETO).

ETO from HDACi-induced destabilization (Fig. 1*B* and data not shown). These observations indicate that the intermediary induction of proteins such as UbcH8 leads to the degradation of AML1-ETO (Figs. 2*E* and 3*A*).

SIAH-1 is a crucial factor for the degradation of AML1-ETO

Previous work showed that the E3-ligase SIAH-1 is involved in proteasomal degradation of nuclear proteins (8, 21, 28), and UbcH8 was identified as the E2-conjugase preferentially interacting with SIAH-1 (29). Therefore, we analyzed whether this E3 regulates AML1-ETO stability. First, we found that in Kasumi-1 cells SIAH-1, in contrast to RLIM, remained stable in the presence of HDACi (Figs. 2*E* and 3*A*). More important, SIAH-1 strongly reduced AML1-ETO in transient transfection experiments (Fig. 3*B*), and a dominant-negative SIAH-1 molecule (SIAH-1^{C72S}) prevented AML1-ETO degradation by UbcH8 (Fig. 3*C*). To challenge the role of SIAH-1 in the turnover of AML1-ETO, we transfected siRNA oligonucleotides against SIAH-1/2 together with AML1-ETO into 293T cells. We observed that a highly efficient knockdown of SIAH-1/2 blocked the degradation of this oncoprotein by UbcH8 and SIAH-1 (Fig. 3*D, E*; compare lane 2 and lane 4 in *E*). The expression of a GFP construct, which is like AML1-ETO under control of a CMV promoter, served as a control for specificity. These experiments clearly indicate that equally to a reduction of UbcH8 (Fig. 2*B, C*), the attenuation of SIAH-1 stabilizes AML1-ETO.

Since the transfer of ubiquitin to a target protein requires interaction with its E3-ligase, we next tested whether SIAH-1 interacts with AML1-ETO in coimmunoprecipitation experiments. Endogenous SIAH-1 was precipitated from lysates of t(8;21)-positive Kasumi-1 and SKNO-1 cells, and Western blots were probed against AML1-ETO. This approach provides clear evidence of a physical interaction between AML1-ETO and SIAH-1 (Fig. 3*F*). Remarkably, AML1-ETO coprecipitated with SIAH-1 shows slower migrating bands indicative of mono- and polyubiquitylation. *In vitro* experiments confirmed that SIAH-1 in conjunction with UbcH8 induces polyubiquitylation of AML1-ETO (Supplemental Fig. S4). We also precipitated SIAH-1 from lysates of 293T cells transfected with SIAH-1 and

AML1-ETO or PML-RARα and confirmed the association of both proteins with SIAH-1 (Supplemental Fig. S5).

HDACi, UbcH8, and SIAH-1 determine PML-RARα turnover

Similar to AML1-ETO in Kasumi-1 cells, PML-RARα was degraded in t(15;17)-positive NB-4 APL cells treated with various HDACi (**Fig. 4*A***). HDACi attenuated this oncoprotein, with clear effects detectable after ~16 h, and this correlated with UbcH8 induction (Fig. 4*B*). PML-RARα degradation induced by HDACi hence correlates with HDAC-inhibition and the intermediary induction of protein expression. Real-time RT-PCR revealed that as AML1-ETO, PML-RARα mRNA levels decreased in response to VPA (data not shown). In sharp contrast to PML-RARα, levels of RARα were unaffected, and PML was even induced by VPA (Fig. 4*A*).

To analyze whether UbcH8 affects PML-RARα stability, we transfected NB-4 cells with UbcH8. A strong decrease of endogenous PML-RARα occurred under these conditions (Fig. 4*C*). In an independent approach, we cotransfected 293T cells with PML-RARα and siRNAs against UbcH8. This approach showed that a knockdown of UbcH8 increased PML-RARα levels and confirmed that equally to AML1-ETO, PML-RARα is a target of UbcH8 (Fig. 4*D*). In agreement with this, PML-RARα was degraded by UbcH8 and SIAH-1 (Fig. 4*E*). This degradation mechanism does not appear to target another leukemia fusion protein, STAT5-RARα, since this protein remained stable in the presence of overexpressed UbcH8 and SIAH-1 (Supplemental Fig. S6). These observations indicate that SIAH-1 and UbcH8 selectively determine protein stability.

Equally to Kasumi-1 cells, VPA-treated and UbcH8-transfected NB-4 cells had stable levels of SIAH-1, though not of RLIM (Fig. 4*B, C*). Experiments with the dominant-negative RLIM construct showed that analogous to AML1-ETO, PML-RARα levels were decreased by UbcH8 and SIAH-1, but not by RLIM (Fig. 4*E*). Therefore, RLIM is unlikely to be an E3-ligase for both, AML1-ETO and PML-RARα. If RLIM is functionally inactivated in NB-4 cells treated with VPA, its target HDAC2 should remain stable in these cells (12). In-

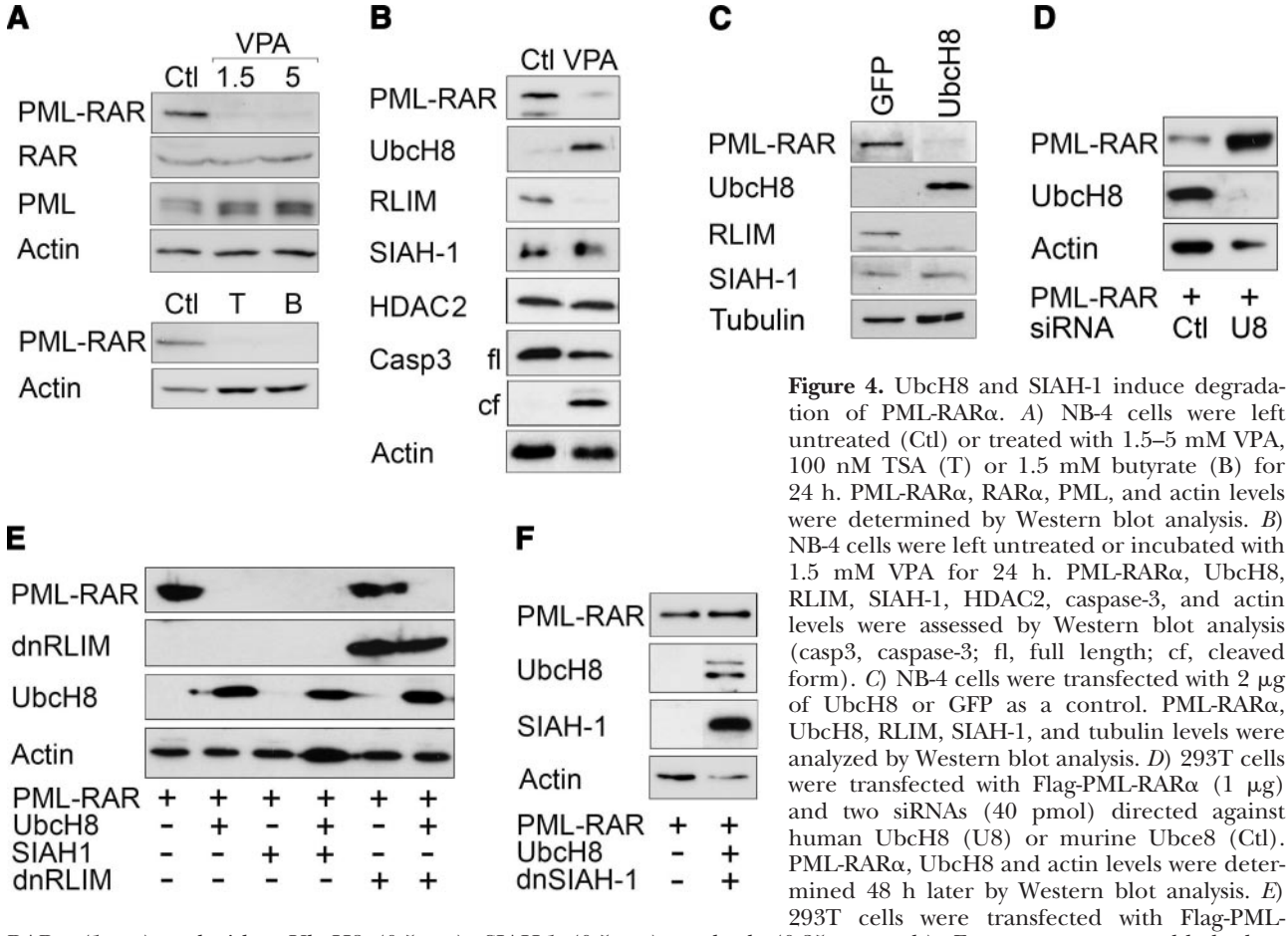

Figure 4. UbcH8 and SIAH-1 induce degradation of PML-RARα. *A*) NB-4 cells were left untreated (Ctl) or treated with 1.5–5 mM VPA, 100 nM TSA (T) or 1.5 mM butyrate (B) for 24 h. PML-RARα, RARα, PML, and actin levels were determined by Western blot analysis. *B*) NB-4 cells were left untreated or incubated with 1.5 mM VPA for 24 h. PML-RARα, UbcH8, RLIM, SIAH-1, HDAC2, caspase-3, and actin levels were assessed by Western blot analysis (casp3, caspase-3; fl, full length; cf, cleaved form). *C*) NB-4 cells were transfected with 2 μg of UbcH8 or GFP as a control. PML-RARα, UbcH8, RLIM, SIAH-1, and tubulin levels were analyzed by Western blot analysis. *D*) 293T cells were transfected with Flag-PML-RARα (1 μg) and two siRNAs (40 pmol) directed against human UbcH8 (U8) or murine Ubce8 (Ctl). PML-RARα, UbcH8 and actin levels were determined 48 h later by Western blot analysis. *E*) 293T cells were transfected with Flag-PML-RARα (1 μg) and either UbcH8 (0.5 μg), SIAH-1 (0.5 μg), or both (0.25 μg each). Empty vector was added when appropriate. Some dishes were also transfected with a myc-tagged dominant-negative RLIM mutant (dnRLIM; 0.5 μg). Expression of PML-RARα, dnRLIM, UbcH8, and actin were detected by Western blot analysis 48 h post-transfection. *F*) 293T cells were transfected with expression vectors for Flag-PML-RARα (0.4 μg), UbcH8 (0.2 μg), dominant-negative SIAH-1^{C72S} (0.05 μg), and pcDNA3.1 if appropriate. Extracts for Western blot analysis were prepared 48 h later and analyzed for PML-RARα, UbcH8, SIAH-1, and actin.

deed, HDAC2 was unaffected by VPA in NB-4 cells (Fig. 4*B*).

We then tested whether the dominant-negative SIAH-1^{C72S} protein affects PML-RARα and its degradation by UbcH8. This SIAH-1 mutant completely abolished the degradation of PML-RARα by UbcH8. Similar results were obtained with siRNA against SIAH-1/2 (Fig. 4*F* and data not shown). We concluded that UbcH8 and SIAH-1 abundance modulate PML-RARα turnover.

Functional and physical interaction between AML1-ETO, PML-RARα, UbcH8, SIAH-1 and RLIM

We speculated that SIAH-1 not only targets leukemia fusion proteins but also RLIM for proteasomal degradation. To test this, 293T cells were transfected with SIAH-1 and assessed for endogenous RLIM levels. It became clear that the E3 RLIM, which is required for proper embryonic development (19), is subject to degradation induced by SIAH-1 (**Fig. 5*A***). Transfection of HA-RLIM and SIAH-1 confirmed this result (Fig. 5*B*). On the other hand, ectopic expression of RLIM or a dominant-negative RLIM did not alter SIAH-1 stability (data not shown). These results suggest that SIAH-1 is the E3 for RLIM, though not *vice versa*. Since E3-ligases have to interact with substrates to induce their ubiquitylation, immunoprecipitates of endogenous SIAH-1 were probed for the presence of RLIM. RLIM was readily detectable in these precipitates (Fig. 5*C*), consistently indicating that SIAH-1 acts as an E3 for RLIM.

In contrast to Kasumi-1 and NB-4 cells, 293T cells have stable RLIM levels in the presence of VPA and when UbcH8 is overexpressed (Figs. 2*E* and 4*B* and ref. 12). We analyzed whether this is due to a differential expression of RLIM in these cells and observed far higher RLIM amounts in 293T cells compared to hematopoietic cells (Fig. 5*D*). Subsequently, we analyzed whether our previous observation that TSA, though not VPA, induces RLIM degradation correlates with a differential induction of SIAH-1 by these compounds. Indeed, a 24-h incubation with TSA but not VPA induced SIAH-1 and decreased RLIM in 293T cells (Supplemental Fig. S7 and ref. 12). Apparently, RLIM levels in 293T cells are too high to be significantly lowered by VPA.

SIAH-1 is highly unstable *in vivo* because of autoubiquitylation followed by proteasomal degradation (30, 31). In agreement with these findings, a SIAH-1 antibody detected unmodified SIAH-1 and incremental increases of molecular weight by 8 kDa, indicative of ubiquitylation (Fig. 5*E*). As SIAH-1 preferentially interacts with UbcH8 (29), UbcH8 may well be the E2-conjugase responsible for this process. To test this, we lowered UbcH8 levels with siRNAs and analyzed SIAH-1 expression in 293T cells. This experiment showed that the specific reduction of UbcH8 increased the amount of endogenous SIAH-1 (Fig. 5*F*).

Finally, we analyzed whether leukemia fusions and proteins of the ubiquitylation machinery occur in a complex. Since UbcH8 and SIAH-1 critically determine leukemia fusion protein stability in a concerted action, these proteins should be in close proximity. We performed subcellular fractionation and immunocytochemical analysis of Kasumi-1, NB-4, and AML1-ETO- or PML-RARα-transfected 293T cells. Our data show that in cells harboring these fusion proteins, SIAH-1 predominantly resides in the nucleus and UbcH8 is

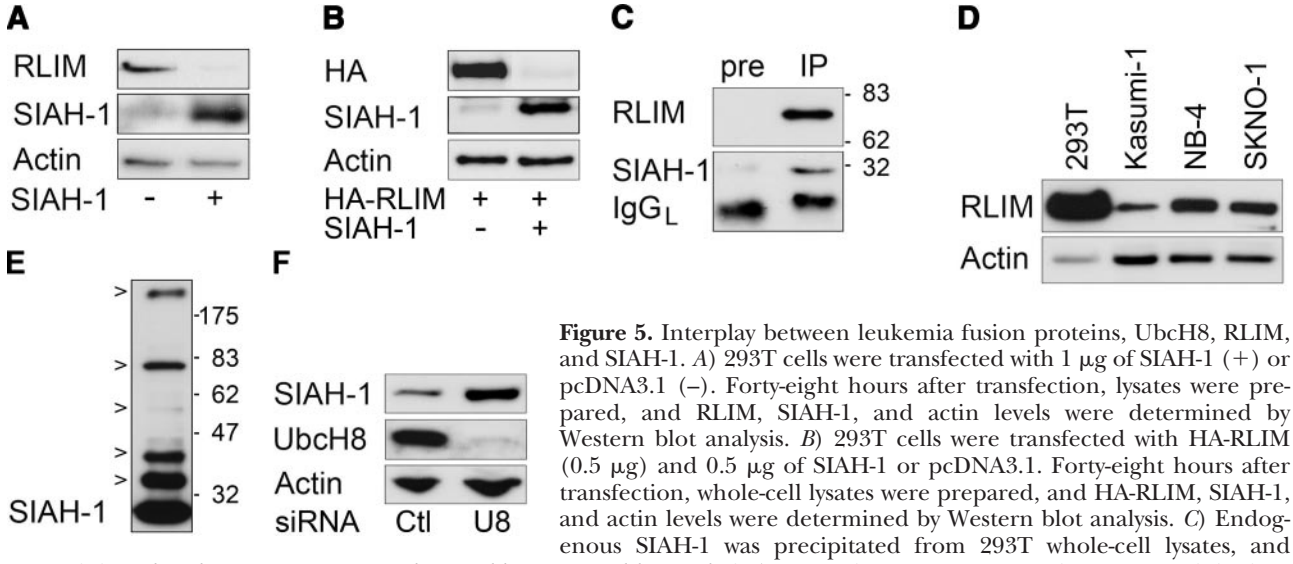

Figure 5. Interplay between leukemia fusion proteins, UbcH8, RLIM, and SIAH-1. *A*) 293T cells were transfected with 1 μg of SIAH-1 (+) or pcDNA3.1 (–). Forty-eight hours after transfection, lysates were prepared, and RLIM, SIAH-1, and actin levels were determined by Western blot analysis. *B*) 293T cells were transfected with HA-RLIM (0.5 μg) and 0.5 μg of SIAH-1 or pcDNA3.1. Forty-eight hours after transfection, whole-cell lysates were prepared, and HA-RLIM, SIAH-1, and actin levels were determined by Western blot analysis. *C*) Endogenous SIAH-1 was precipitated from 293T whole-cell lysates, and coprecipitated endogenous RLIM was detected by Western blot analysis (pre, nonimmune serum; IP, immunoprecipitation; IgG$_L$, immunoglobulin light chain). Molecular weight standards are indicated on the right. *D*) Comparison of the expression of RLIM in untreated 293T, Kasumi-1, SKNO-1 and NB-4 cells. Actin serves as a loading control. *E*) 293T cells were transfected with SIAH-1 (1 μg) and treated with 1 μM MG-132 (16 h). NEM-treated cell lysates were analyzed with a SIAH-1 antibody (arrowheads: ubiquitylated SIAH-1). *F*) 293T cells were transfected with siRNAs against mRNAs for human UbcH8 (U8) or murine Ubce8 (Ctl) as a control (40 pmol). Forty-eight hours after transfection, SIAH-1, UbcH8, and actin levels were determined by Western blot analysis.

distributed between the cytoplasmic and nuclear compartment. Hence, these proteins of the ubiquitylation machinery can potentially interact with the strictly nuclear proteins AML1-ETO and PML-RARα (**Fig. 6*A*** and data not shown). To further prove this, recombinant GST-UbcH8 was incubated with cellular lysates and Western blots were probed for SIAH-1 and the leukemia fusion proteins. This assay clearly showed the physical interaction of these proteins with UbcH8 (Fig. 6*B*). Next, UbcH8 immunoprecipitates were probed for SIAH-1, AML1-ETO, and PML-RARα. Results obtained with this experiment indicate the presence of a complex between UbcH8, SIAH-1, and the leukemia fusion proteins *in vivo* (Fig. 6*C*). As expected (12), HDAC2 and RLIM were also found in these immunoprecipitates.

DISCUSSION

The chimeric fusion proteins AML1-ETO and PML-RARα are crucial for the transformation of myeloid precursors (1, 6). Our study shows that the polyubiquitylation and subsequent proteasomal degradation of AML1-ETO and PML-RARα can be increased by HDACi. Several independent lines of evidence obtained from ectopic expression, dominant-negative molecules, siRNA experiments, and coimmunoprecipitation approaches coherently indicate that this process is mediated by the E2-conjugase UbcH8 and the E3-ligase SIAH-1. Hence, the leukemia fusion proteins AML1-ETO and PML-RARα represent key targets of the E2 UbcH8, the E3 SIAH-1 and HDACi. Moreover, we also identified UbcH8 as an E2 mediating autoubiquitylation of SIAH-1, and SIAH-1 as the E3 for RLIM in a previously undescribed hierarchical order (Fig. 6*D*).

HDACi appear to act as inhibitors of leukemia fusion proteins indirectly *via* the induction of their degradation and directly by inhibiting the catalytic activity of their associated HDACs. The HDACi-induced proteasomal degradation of AML1-ETO and PML-RARα may be particularly relevant, since they compete as oligomers with the remaining intact transcription factors for proper hematopoiesis (1, 6, 32), which prevents the expression of their target genes in response to appropriate stimuli. Furthermore, AML1-ETO directly binds, sequesters, and competes with further transcriptional regulators of hematopoietic differentiation, *e.g.*, c-Jun, PLZF, C/EBP, SMAD3, and VDR (1); PML-RARα interferes with C/EBP transcription factors and cell cycle restricting proteins (6). Since the expression of leuke-

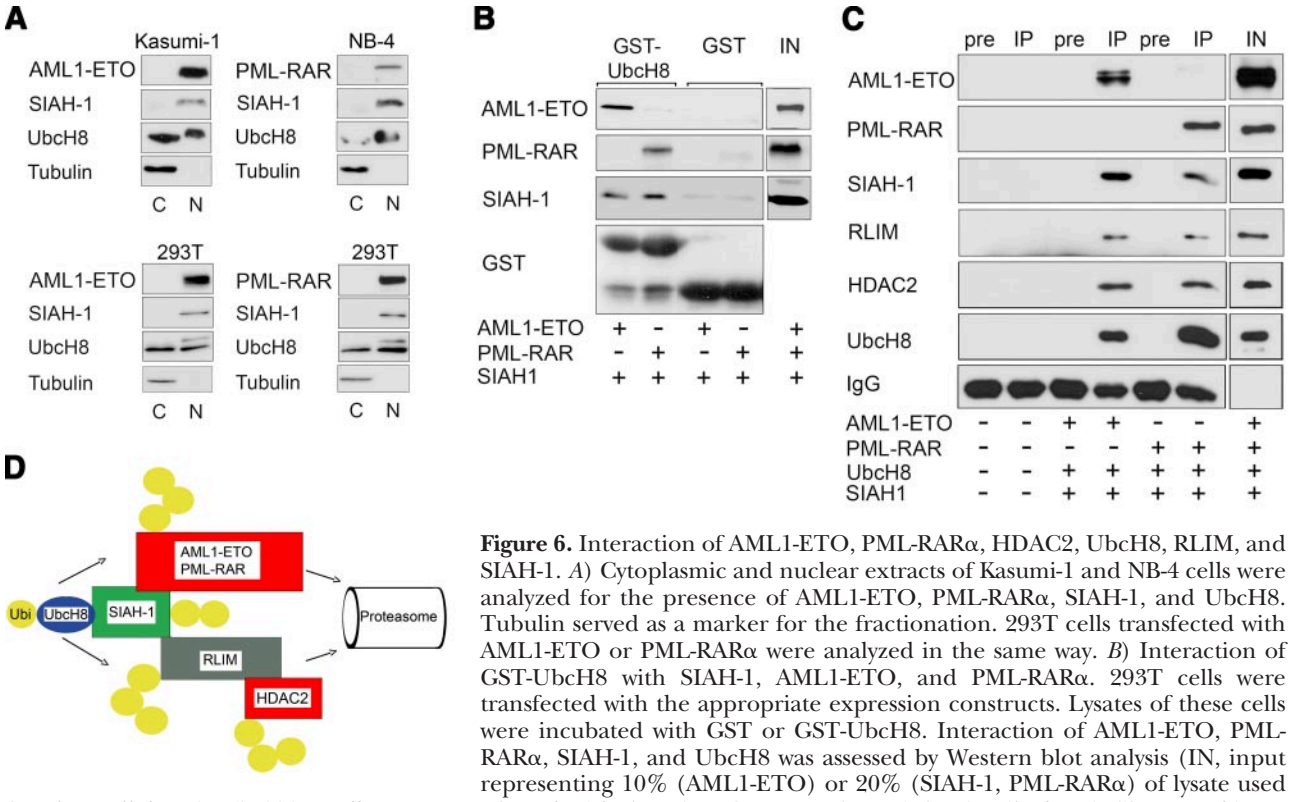

Figure 6. Interaction of AML1-ETO, PML-RARα, HDAC2, UbcH8, RLIM, and SIAH-1. *A*) Cytoplasmic and nuclear extracts of Kasumi-1 and NB-4 cells were analyzed for the presence of AML1-ETO, PML-RARα, SIAH-1, and UbcH8. Tubulin served as a marker for the fractionation. 293T cells transfected with AML1-ETO or PML-RARα were analyzed in the same way. *B*) Interaction of GST-UbcH8 with SIAH-1, AML1-ETO, and PML-RARα. 293T cells were transfected with the appropriate expression constructs. Lysates of these cells were incubated with GST or GST-UbcH8. Interaction of AML1-ETO, PML-RARα, SIAH-1, and UbcH8 was assessed by Western blot analysis (IN, input representing 10% (AML1-ETO) or 20% (SIAH-1, PML-RARα) of lysate used for the pull-down). *C*) 293T cells were transfected with AML1-ETO, PML-RARα, SIAH-1, V5-UbcH8 (5 μg each), or pcDNA3.1. UbcH8 was precipitated with a V5 antibody. Presence of AML1-ETO, PML-RARα, SIAH-1, RLIM, HDAC2, and UbcH8 in these immunoprecipitates was analyzed by Western blot analysis (pre, nonimmune serum; IP, immunoprecipitation; IN, 10% of IP input, IgG, immunoglobulin heavy chain). *D*) Schematic representation of the mechanism of AML1-ETO, PML-RARα, SIAH-1, and RLIM degradation mediated by UbcH8. Proteasomal degradation of these proteins involves the E3-ligase SIAH-1, and limiting amounts of the E2-conjugase UbcH8. UbcH8 is induced in HDACi-treated leukemia cells and can mediate oncoprotein depletion. HDAC2 degradation occurs depending on the basal abundance and stability of the ubiquitin ligase RLIM.

mia fusion proteins is highly restricted to leukemic cells, the degradation of such proteins may become a molecularly defined, selective intervention strategy.

Our results show that the well-tolerated drug VPA (24) induces leukemia fusion protein degradation. This HDACi might therefore be particularly suited for leukemia therapy. However, the screening of additional parameters such as the degradation of HDAC2 and RLIM or the induction of Stat1 (10, 12–14, 17) is required to identify patients with cancer traits susceptible to HDACi. Greater benefits may equally be achieved when VPA is used in conjunction with drugs targeting DNA-replication (33, 34). Moreover, several newly developed HDACi currently undergo clinical trials. Remarkably, Depsipeptide has been shown to be beneficial for patients with t(8;21) leukemia, and this HDACi induces AML1-ETO degradation (35). These reports confirm and support our results and conclusions. Our data additionally extend these observations to PML-RARα and provide detailed information on the enzymology of these processes.

Since HDACi also induce nonhistone protein acetylation, the stability of leukemia fusion proteins may also be influenced by their acetylation status or *via* Hsp90 acetylation (36). However, VPA is a class 1-selective HDACi, which does not inhibit HDAC6 (24, 37), the class 2 deacetylase targeting Hsp90 (38, 39). It is therefore not surprising that several attempts failed to detect Hsp90 acetylation with three antiacetyl-lysine antibodies in VPA-treated cells at various time points and concentrations. Similarly, AML1-ETO was not detectable with antiacetyl-lysine antibodies (ref. 35 and data not shown). Furthermore, the siRNA-mediated knockdown of Ubc8 prevented the degradation of endogenous AML1-ETO, even in the presence of HDACi (Fig. 2*C*). On the other hand, leukemia fusion proteins were degraded under conditions that do not inhibit HDAC activity, such as UbcH8 or SIAH-1 overexpression (Figs. 2*A*; 3*B*, *E*; 4*C*, *E* and ref. 8). Still, Hsp90 acetylation may be below the detection limit and could be masked by its HDACi-induced cleavage (Supplemental Fig. S8). Nevertheless, acetylation of Hsp90 is unlikely to be a prerequisite for the degradation of AML1-ETO and PML-RARα in the context of our experiments. Consistent with this assumption, we observed that Hsp90-bound AML1-ETO is not protected but also degraded on HDAC-inhibition (Supplemental Fig. S8). Moreover, a knockdown of HDAC6 induces Hsp90 acetylation and impairs its function but hardly affects the stability of the leukemia fusion protein Bcr-Abl (38). This study also implies that limiting amounts of enzymes of the ubiquitylation machinery determine oncoprotein degradation.

As VPA induces apoptosis in Kasumi-1 and NB-4 cells (Figs. 2*C*, *D*; 3*A*; 4*B* and Supplemental Figs. S3 and S10), caspases could equally cleave AML1-ETO and PML-RARα. However, HDACi trigger the degradation of these proteins before the onset of caspase cleavage, a pan-caspase inhibitor did not block the HDACi-induced decrease of AML1-ETO levels, and no cleavage products typical for caspase-induced degradation were observed. In contrast, MG-132 is a strong proapoptotic stimulus, which stabilized the AML1-ETO protein (Figs. 1*B* and 2*E* and data not shown). Furthermore, UbcH8 and SIAH-1 did not induce apoptosis in 293T cells, although they destabilized AML1-ETO and PML-RARα (Figs. 3*A*, *B* and 4*E*), and MG-132 blocked this degradation (data not shown). Nevertheless, it is possible that caspases cleave leukemia fusion proteins at late stages of HDACi treatment.

HDACi counteract transcriptional repression and also selectively affect protein stability, but only limited knowledge exists about the E2 and E3 enzymes involved in HDACi-induced ubiquitylation (9). UbcH8 and RLIM appear to be crucial for at least a subset of these processes, and we could confirm our initial observation that HDACi induce UbcH8 expression in several different cell lines (Figs. 2*E* and 4*B,* data not shown, and ref. 12). The E3 RLIM together with UbcH8 induces proteasomal degradation of HDAC2 (12) and high HDAC2 levels correlate with certain neoplastic malignancies. Nevertheless, RLIM does not contribute to AML1-ETO and PML-RARα degradation (Figs. 2*A*, *E*; 3*A*; and 4*B*, *E*), and RLIM is expressed at much lower levels in Kasumi-1, NB-4, and SKNO-1 cells compared to 293T cells (Fig. 5*D*). Apparently, an increased expression of UbcH8 on VPA treatment suffices to trigger the proteasomal degradation of such low RLIM levels in hematopoietic cells *via* the E3 SIAH-1. As expected from these results, VPA does not induce proteasomal degradation of the RLIM target HDAC2 in Kasumi-1 and NB-4 cells (Figs. 3*A* and 4*B*) (12). Hence, the molecular mechanism through which UbcH8 controls protein degradation depends on the abundance of its E3-ligases and not on HDACi treatment *per se.* On the basis of these data, we propose that a hierarchical ubiquitylation system modulates E3-ligases *via* cross-regulation. This, in turn, leads to the degradation of different substrates, including E3 enzymes themselves.

E3 enzymes typically target multiple substrates, and therefore proteins in addition to RLIM, HDAC2, PML-RARα, and AML-ETO could be subject to VPA-induced proteasomal degradation in a cell-type-specific manner. Furthermore, RLIM and SIAH-1 may not be the only E3-ligases downstream of the E2-conjugase Ubc8, and such pleiotropic effects of an E2 may explain why a knockdown of UbcH8 protects Kasumi-1 cells from VPA-induced apoptosis (Fig. 2*C*). Such complex interplays between ubiquitin E2-conjugases and different E3-ligases may control multiple regulatory networks in health, disease, and development.

In contrast to RLIM, SIAH-1 appears to bind and directly control the proteasomal turnover of AML1-ETO and PML-RARα (Figs. 3 and 4*E, F*). This is in agreement with the presence of consensus motifs for SIAH-1 binding in these proteins (8, 40). Four of these motifs are clustered in the AML1-ETO fusion protein. This fact could explain why the intact AML1 and ETO proteins, each containing only two SIAH-1 binding sites, are far less susceptible to VPA-induced degradation (Supplemental Fig. S1). A similar situation occurs

in PML-RARα (Fig. 4*A*), where the SIAH-1 binding sites of RARα are brought into close proximity of the PML coiled coil, which also recruits this E3-ligase (12). Neither the clustering of SIAH-1 binding sites nor a PML-type coiled coil exist in STAT5-RARα. Accordingly, this fusion protein is not degraded *via* UbcH8 and SIAH-1 (Supplemental Fig. S6).

All of the cell lines that we used endogenously express UbcH8 and SIAH-1, which are detectable on loading of high amounts of protein, immunoprecipitation, or subcellular fractionation (Figs. 3*D, E*; 5; and 6*A* and Supplemental Fig. S9 for comparison). Previously published data indicating that certain cell lines are SIAH-1 negative may be due to lower detection sensitivities of earlier antibody generations. Difficulties in detecting SIAH-1 also reflect its high turnover (30, 31). Our experiments with UbcH8 siRNAs indicate that SIAH-1 stability depends on its E2 UbcH8 (Fig. 5*E, F*). Nevertheless, UbcH8 induction was not sufficient to cause SIAH-1 degradation (Figs. 2*E*, 3*A*, and 4*B, C* and Supplemental Fig. S7). This discrepancy could be explained by a model in which the stability of SIAH-1 depends on the abundance of substrates to which ubiquitin can be transferred. Such a model of an E3 autoinactivation has been proposed before and is consistent with much higher expression rates of enzymatically inactive SIAH-1 RING-finger mutants (28, 30, 41).

Considering our results on the role of the UbcH8-SIAH-1 axis in leukemia fusion protein degradation, this pathway could be shared by the basal and the HDACi-induced proteasomal degradation of oncoproteins (30, 36, 42). Future studies are required to determine whether UbcH8 is a tumor suppressor similar to SIAH-1 (43). The dependence of transformed cells on oncoproteins and their HDACi-induced degradation could be one reason why these compounds are more toxic to leukemic cells than to normal cells (24). Further analyses are also required to clarify whether HDACi impose post-translational modifications other than ubiquitylation on leukemia fusion proteins and whether they affect protein stability. Remarkably, it was reported that a truncated variant of AML1-ETO that does not interact with corepressors promotes leukemia development (44). Similarly, transformation by PML-RARα depends on its cleavage by neutrophil elastase (45). One could thus speculate that the benefits of HDACi in leukemia treatment rely on the elimination of leukemia fusion proteins as well as on HDAC inhibition. Regulation of such processes by HDACi is likely to be relevant for the activity of these drugs in cultured cells, animal models, and patients (10, 12, 14, 16, 42). FJ

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Supplementary Figures

(**S1**) Kasumi-1 cells were treated with 1.5 mM VPA for 24 h (+). Protein levels of AML1-ETO, AML1 and ETO were detected by Western blot of nuclear extracts. Antibodies used are reactive against AML1 or ETO. Expression of mSin3 was analyzed as loading control. Band densities were estimated with Adobe Photoshop. The intensity of the bands was divided by the mSin3 signal and this value was arbitrarily set as 1 for untreated control cells (-). Values given for treated cells are in relation to this normalized signal.

(**S2**) 293T cells were transfected with 0.4 µg of Gal and 0.2 µg UbcH8 or empty vector pcDNA3.1. Expression of Gal and UbcH8 were detected by Western blot 48 h after transfection. Actin protein levels were determined as loading control.

(**S3**) Kasumi-1 cells were treated with 1.5-5 mM VPA, TSA (100 nM), butyrate (B, 1.5 mM) or MS-27−275 (MS; 5 µM) for 24 h. Cells were fixed and analyzed for apoptosis by PI-staining and FACS-analysis (Ctl, untreated; subG1, apoptotic cells). Kasumi-1 and NB-4 cells treated with 1.5 mM VPA for 24 h were also analyzed for nucleosomal laddering on a TAE 2%-agarose gel (right panel; Ctl, untreated, kb, kilo base pairs).

(**S4**) TNT-translated UbcH8, SIAH-1 and [^{35}S]methionine-labeled Gal-AML1-ETO, were subjected to *in vitro* ubiquitylation for 0-120 h as described in (14). For control (0), the reaction was stopped immediately. Molecular weight standards, loss of input Gal-AML1-ETO and high molecular weight radiolabeled proteins (>) were determined by SDS–PAGE and autoradiography.

(**S5**) Interaction of AML1-ETO and PML-RARα with SIAH-1 was tested by co-IP from extracts of 293T cells transfected with SIAH-1 and AML1-ETO or PML-RARα. AML1-ETO and PML-RARα co-precipitated with the SIAH-1 antibody were detected by Western blot.

(**S6**) 293T cells were transfected for 48 h with expression vectors for STAT5-RARα (0.5 µg) and UbcH8 (0.5 µg) or SIAH-1 (0.5 µg). Expression of the corresponding proteins was detected by Western blot. Tubulin levels were determined as loading control.

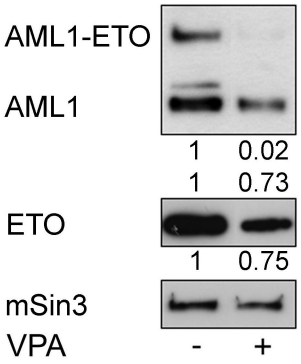
(**S7**) 293T cells were left untreated (Ctl) or were treated with 1.5 mM VPA or 100 nM TSA for 24 h. Expression of SIAH-1 and actin were determined by Western blot.

(**S8**) The interaction of Hsp90 with AML1-ETO was tested by co-IP. Hsp90 was immunoprecipitated from Kasumi-1 lysates and Western blots were probed for AML1-ETO. Cells remained untreated (Ctl) or were incubated with 1.5 mM VPA for 24 h (IgG, immunoglobulin heavy chain). The lower panel shows 5% of the IP-input and the cleavage of full-length Hsp90 (fl; cf, cleaved form).

(**S9**) 293T cells were transfected with SIAH-1 (+) or pcDNA3.1 (-). Western blot was analyzed with the SIAH-1 antibody.

Krämer et al., Supplementary Figures

S1



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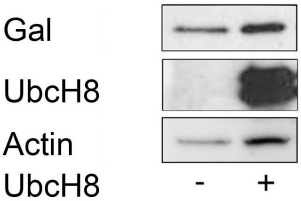

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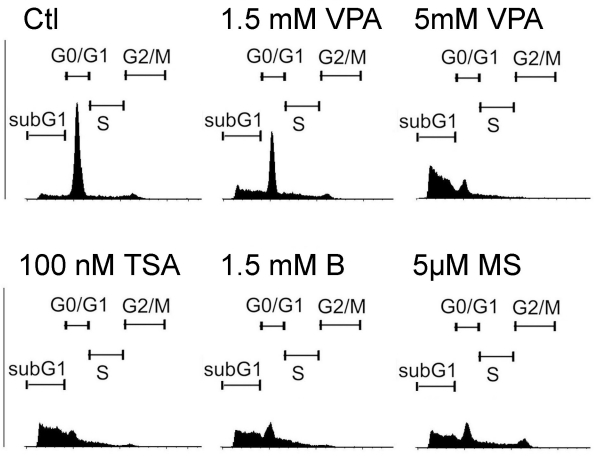

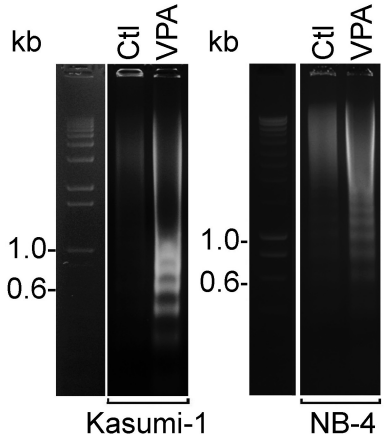

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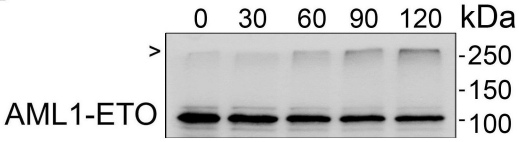

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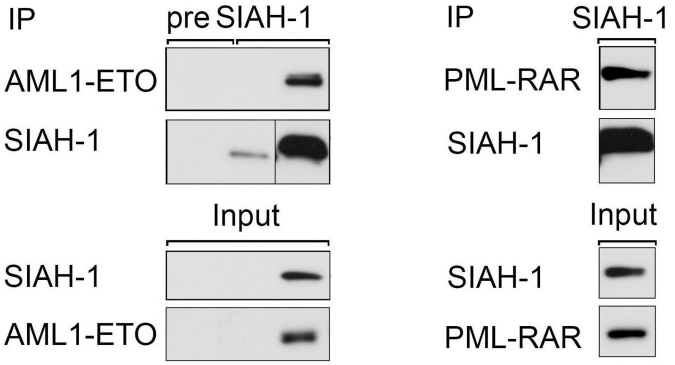

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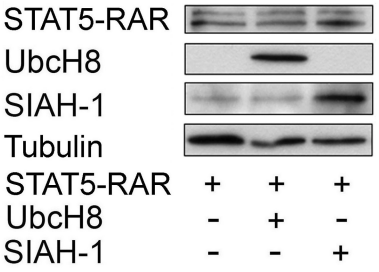

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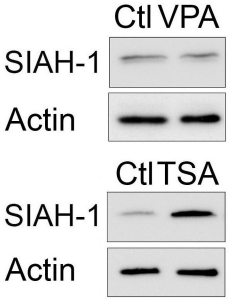

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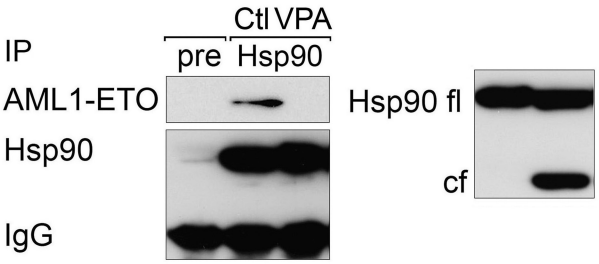

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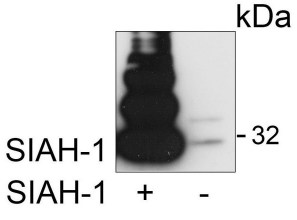

7 Manuskript 3:

Ubiquitin conjugase UBCH8 targets active FMS-like receptor tyrosine kinase 3 for proteasomal degradation

The data presented in this manuscript provide insights into the molecular mechanism regulating the turnover of a tyrosine kinase associated with high-risk leukemia. I demonstrate that compared to wild-type FLT3, mutant FLT3 is much more susceptible to HDACi-mediated degradation in AML cells. Studies carried out with pharmacological and physiological modulators and with kinase-dead FLT3-ITD reveal that tyrosine phosphorylation triggers UBCH8/SIAH1-dependent degradation of activated FLT3 and FLT3-ITD

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ORIGINAL ARTICLE

Ubiquitin conjugase UBCH8 targets active FMS-like tyrosine kinase 3 for proteasomal degradation

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The class III receptor tyrosine kinase FMS-like tyrosine kinase 3 (FLT3) regulates normal hematopoiesis and immunological functions. Nonetheless, constitutively active mutant FLT3 (FLT3-ITD) causally contributes to transformation and is associated with poor prognosis of acute myeloid leukemia (AML) patients. Histone deacetylase inhibitors (HDACi) can counteract deregulated gene expression profiles and decrease oncoprotein stability, which renders them candidate drugs for AML treatment. However, these drugs have pleiotropic effects and it is often unclear how they correct oncogenic transcriptomes and proteomes. We report here that treatment of AML cells with the HDACi LBH589 induces the ubiquitin-conjugating enzyme UBCH8 and degradation of FLT3-ITD. Gain- and loss-of-function approaches show that UBCH8 and the ubiquitin-ligase SIAH1 physically interact with and target FLT3-ITD for proteasomal degradation. These ubiquitinylating enzymes though have a significantly lesser effect on wild-type FLT3. Furthermore, physiological and pharmacological stimulation of FLT3 phosphorylation, inhibition of FLT3-ITD autophosphorylation and analysis of kinase-inactive FLT3-ITD revealed that tyrosine phosphorylation determines degradation of FLT3 and FLT3-ITD by the proteasome. These results provide novel insights into antileukemic activities of HDACi and position UBCH8, which have been implicated primarily in processes in the nucleus, as a previously unrecognized important modulator of FLT3-ITD stability and leukemic cell survival.
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Keywords: FLT3; FLT3-ITD; AML; UBCH8; SIAH

Introduction

Class III receptor tyrosine kinases (RTKs) control the development of hematopoietic progenitor cells.[1,2] Wild-type FMS-like tyrosine kinase 3 (FLT3) has been shown to be highly expressed in several hematopoietic malignancies including 70–100% cases of acute myeloid leukemia (AML).[3–6] Overexpression of FLT3 promotes receptor autophosphorylation linked to leukemic transformation.[4,5,7] Activating mutations of FLT3 occur in up to 30% of patients having AML. The most common FLT3 mutation in AML represents an internal tandem duplication (ITD) in the juxtamembrane region.[8] This FLT3-ITD mutation has also been found in smaller fractions of chronic myeloid leukemia, myelodysplasia as well as B- and T-cell acute lymphoblastic leukemia.[9–11] FLT3-ITD exists partially in an immature, underglycosylated, constitutively phosphorylated form. Aberrant intracellular localization and activity of FLT3-ITD generate oncogenic phosphorylation patterns.[12–17] Large-scale analyses of AML patients revealed FLT3-ITD as an independent prognostic marker for disease relapse and poor prognosis.[9,18,19] Point mutations in the tyrosine kinase domain (TKD) are a second type of FLT3 mutation found in AML, acute lymphoblastic leukemia and myelodysplasia.[7,20] Although both types of mutations result in constitutive receptor activation, they are associated with distinct signaling properties and disease phenotypes. In contrast to FLT3-ITD, the prognostic impact of TKD mutations of FLT3 is discussed rather controversially.[14,21–24]

Several studies with murine models show the transforming potential of mutant FLT3. As single mutation, FLT3-ITD is sufficient to induce a myeloproliferative phenotype. Moreover, similar to FLT3-TKD, FLT3-ITD causes also lymphoid disease in transgenic or bone-marrow-transplanted mice.[6,21,25–27] These findings link FLT3-ITD to the pathogenesis of myeloid and lymphoid leukemias. Consistent with the development of AML being a consequence of multiple genetic alterations, FLT3-ITD cooperates with further lesions generating leukemogenesis, manifested by development of acute leukemia with short disease latency *in vivo*.[28–31] For example, FLT3-ITD and the chromosomal translocation t(15;17) encoding PML/RARα are found in combination in approximately 40% of patients with t(15;17) AML.[32] The fact that expression of FLT3-ITD induces murine acute promyelocytic leukemia-like disease in PML/RARα transgenic mice corroborates their oncogenic cooperation.[33,34] Such observations argue for a critical role for mutated FLT3 in the pathogenesis of acute leukemia. Consequently, detailed analyses of the functions, properties, and turnover of FLT3 and its mutants are warranted.

Because targeted deletion of FLT3 or FLT3-ligand (FL) does not compromise the viability of mice, intervention with deregulated FLT3 functions appears as a potential treatment option for AML.[35–37] The proteasome, a multiprotease complex, limits the stability of activated FLT3 and mutant FLT3-ITD.[38–41] Therefore, proteasomal degradation provides a pharmacological avenue for interference with aberrant FLT3. Targeting of a protein to the proteasome requires polyubiquitinylation carried out by the hierarchical action of ubiquitin-activating enzymes (E1s), ubiquitin conjugases (E2s) and ubiquitin ligases (E3s) conferring substrate specificity.[42,43] A complex containing the E3 ubiquitin ligase CBL contributes to FL-induced ubiquitinylation and degradation of ectopically expressed human wild-type FLT3 in COS7 and 32D cells.[38] Enzymes and molecular mechanisms regulating FLT3-ITD expression and stabilities are defined sparsely.

Histone deacetylase inhibitors (HDACi) are promising epigenetic cancer drugs, which affect gene expression and signaling by inducing acetylation of histones and nonhistone proteins.[42,44–47] The HDACi NVP-LBH589 (LBH589) and

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NVP-LAQ-824 target FLT3 and FLT3-ITD for degradation. Acetylation-dependent HSP90 chaperone complex disassembly has been proposed as a mechanism underlying this process.[39,40,48,49] Nevertheless, these compounds may equally destabilize these kinases independent of HSP90. Moreover, it is unclear which E2 and E3 enzymes catalyze the degradation of mutant FLT3 in AML cells treated with HDACi.

We have previously shown that HDACi induce ubiquitinylation and proteasomal degradation of the nuclear leukemia fusion proteins AML1/ETO and PML/RARα. Upregulation of the E2 ubiquitin conjugase UBCH8 acting in concert with the E3 ubiquitin ligase SIAH1 accounts for this process.[50] These and other reports suggest that HDACi can induce degradation of (proto-)oncoproteins by modulating the expression of enzymes catalyzing polyubiquitinylation.[42,51–53] Therefore, we analyzed whether HDACi alter the expression of UBCH8 and SIAH1 in AML cells. We further determined if these enzymes catalyze degradation of FLT3-ITD by the ubiquitin–proteasome system (UPS). In addition, we assessed the role of tyrosine phosphorylation for basal and HDACi-induced turnover of FLT3 and FLT3-ITD in AML cells and in a heterologous expression system. Our findings emphasize a critical role for UBCH8 in basal and HDACi-mediated turnover of FLT3-ITD. Induction of UBCH8 by HDACi and subsequently enhanced proteasomal degradation of its targets might contribute to antileukemic effects of HDACi.

Materials and methods

Drugs and chemicals

NVP-LBH589 (LBH589) was a gift from Novartis; FLT3-specific inhibitor bis(1H-indol-2-yl)methanones cpd. 102 (cpd.102) has been already described,[54] recombinant human FLT3 ligand (FL) was purchased from PeproTech GmbH (Hamburg, Germany); the pan-caspase inhibitor Z-VAD-FMK (Z-VAD) from Bachem (Weil am Rhein, Germany); Amaxa Nucleofector kit from Lonza (Köln, Germany); Lipofectamine 2000 from Invitrogen (Karlsruhe, Germany); the proteasome inhibitor Z-Leu-Leu-Leu-al (MG132), *N*-ethyl-maleimide, sodium orthovanadate (Na_3VO_4), cycloheximide (CHX) and polyethylenimine were purchased from Sigma-Aldrich (Steinheim, Germany).

Cell lines

HEK293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% penicillin/streptamycin and 2% L-glutamine. MV4-11 (expressing FLT3 with ITD, FLT3-ITD) and RS4-11 (expressing wild-type FLT3) cell lines were grown in Rosewell Park Memorial Institute (RPMI) medium containing same additives. All cell lines were cultured at 37 °C in a 5% CO_2 atmosphere.

Plasmids, siRNAs, transfection assays and FACS analyses

The following plasmids have been described previously: UBCH8-V5;[51] SIAH1, SIAH2;[55] dominant-negative SIAH1,[50] FLT3-HA, FLT3-ITD-HA, FLT3-ITD(KA)-HA, EGFP-FLT3-ITD and[13] CBL-HA.[56] HEK293T cells were transfected with polyethylenimine as described.[50,57] Unless otherwise stated, transient protein expression in HEK293T cells was carried out for 48 h by using the following amounts of DNA for cell transfection: FLT3-HA, FLT3-ITD-HA and FLT3-ITD(KA)-HA (0.5 μg); SIAH1 and SIAH2 (0.2 μg); UBCH8-V5 (0.5 μg); CBL-HA (0.2 μg). The Empty vector pcDNA3.1 was used to obtain equal amounts of transfected DNA (total of 1 μg per 5×10^5 cells). For fluorescence microscopy, HeLa cells were transfected with Lipofectamine 2000. Antibodies directed against the HA- or V5-Tag were used in immunoprecipitations or western blots to detect exogenously expressed wild-type or mutant FLT3(-ITD)-HA, CBL-HA and UBCH8-V5. MV4-11 cells were transfected with Amaxa Nucleofector kit as recommended by the manufacturer using programs A-30 and Solution V (Lonza). Cells were incubated with the pan-caspase inhibitor Z-VAD-FMK (20 μM) immediately after nucleofection to lower basal caspase activity. For UBCH8-V5 and SIAH1 protein expression in MV4-11 cells, we transfected 2 μg of plasmids (1 μg of each construct). Lowering of UBCH8 protein level by mRNA knockdown was carried out by nucleofection of specific siRNAs (40 pmol) directed against the mRNA of human UBC8 (UBCH8) or mouse UBC8 (as Control).[50] Fluorescence-activated cell sorting (FACS) analyses for detection of apoptotic cell death were performed as described.[50]

Western blot, immunoprecipitation, GST pull-down and antibodies

Cells were collected and lysed in NET-N buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl (pH 7.8), 0.5% Nonidet P-40 (NP-40), 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin and 0.5 μg/ml pepstatin) followed by mild sonification on ice. Protein lysate (50–100 μg) was boiled in Laemmli buffer and separated on SDS–polyacrylamide gel electrophoresis for western blotting with specific antibodies. All lysates were assessed by Bradford assay for protein concentrations. Tubulin served as loading control for all western blots. For immunoprecipitations, HEK293T cells were transfected with a total of 10 μg DNA per 5×10^6 cells and lysates were prepared 48 h later. MG132 (1–2 μM) was added 16 h before cell lysis to prevent RTK degradation. Cell lysis and precipitations were performed in NET-N buffer (additionally supplemented with *N*-ethyl-maleimide 10 mM, NaF 1 mM, Na_3OV_4 1 mM). For precipitation, 1 μg of antibody and 10 μl of protein G Sepharose beads (GE Healthcare, München, Germany) were added to 500 μg of lysate protein. The immunoprecipitation volume was adjusted to a total volume of 600 μl with NET-N. Precipitation was performed under continuous rotation for 16 h at 4 °C. Beads were washed and bound proteins were eluted with Laemmli buffer and analyzed by western blotting. As a control, immunoprecipitations were performed in parallel with normal IgG (pre, preimmune serum) from the same hosts. For pull-down experiments, UBCH8 and SIAH1 were expressed as glutathione *S*-transferase (GST) fusion proteins as described.[50] FLT3-ITD was expressed *in vitro* in a cell-free reticulocyte lysate system (Promega, Mannheim, Germany). Pull-down experiments were performed with 5 μg heterologously expressed GST-UBCH8 or GST-SIAH1 (GST as control) and 10 μl of reticulocyte lysate. GST pull-downs experiments were performed as stated for immunoprecipitation studies. Preparation of detergent-soluble and -insoluble fractions was performed as described.[40] Cell extracts from different fractions were boiled in Laemmli buffer and analyzed by western blot. Densitometric analyses of protein bands in western blots were performed with Adobe Photoshop_CS4. To determine the relative protein band density in each sample, we set the average gray value of the specific protein band in relation to the tubulin signal. Relative protein levels were calculated by normalization against mock sample from the same experiment.

Antibodies used in western blot or immunoprecipitation experiments were purchased from Santa Cruz Biotechnology

(Heidelberg, Germany; Caspase 3, catalog no. sc-7272; FLT3, catalog no. sc-480; HSP90α/β, catalog no. sc-13119; SIAH1, catalog no. sc-5506; SIAH2, catalog no. sc-5507; GFP, catalog no. sc-9996; normal mouse IgG, catalog no. sc-2025; normal goat IgG, catalog no. sc-2028), Sigma-Aldrich (tubulin, catalog no. T5168), Invitrogen (V5-Tag, catalog no. 46-0705), BioCat (Heidelberg, Germany; UBCH8, catalog no. AP2118b), New England Biolabs (Frankfurt am Main, Germany; cleaved Caspase 3, catalog no. 9664; Phospho-FLT3 Tyr591, catalog no. 3461), Covance (Freiburg im Breisgau, Germany; HA-Tag HA.11, catalog no. MMs-101P).

Results

HDACi selectively target FLT3-ITD for degradation

Recent studies showed that the HDACi LAQ-824 and LBH589 attenuated FLT3 and FLT3-ITD in AML cell lines.[39,40] First, we investigated whether HDACi affect RTK levels in such cells to the same extent. We found that LBH589 and valproic acid reduced FLT3-ITD time dependently in MV4-11 cells. In contrast, the same treatment conditions decreased FLT3 levels significantly lesser in RS4-11 cells (Figure 1a; data not shown).

We and others previously showed that HDACi induce the E2 enzyme UBCH8, which in conjunction with the ubiquitin E3-ligase SIAH1, evokes proteasomal degradation of nuclear proteins involved in tumorigenesis.[50–52,58] We found that LBH589 induced UBCH8 in MV4-11 cells in a time-dependent manner. Remarkably, increased expression of UBCH8 correlated inversely with the levels of FLT3-ITD. In contrast to UBCH8, SIAH1 protein levels were unaffected by LBH589 (Figure 1b). Analyzing the mRNA transcript levels of FLT3-ITD by quantitative real-time PCR analysis revealed that LBH589 did not decrease FLT3-ITD mRNA expression (data not shown), which suggests that enhanced UBCH8 levels promote the turnover of FLT3-ITD.

Because HDACi potently induce apoptosis of leukemic cells, we next analyzed the possibility of caspase-mediated proteolysis of FLT3-ITD. MV4-11 cells were treated with LBH589 in the presence of the cell-permeable pan-caspase inhibitor Z-VAD-FMK. Incubation of MV4-11 cells with LBH589 for 24 h strongly decreased FLT3-ITD levels. This process could be partially restored by Z-VAD-FMK, indicating that degradation of FLT3-ITD is partly mediated by a caspase-dependent process. Attenuation of FLT3-ITD also correlated with the processing of its chaperone HSP90 and of caspase 3 (Figure 1c).

Fluorescence-activated cell sorting analysis quantifying sub-G_1 DNA content revealed that co-incubation with Z-VAD-FMK completely blocked LBH589-induced apoptosis in MV4-11 cells. Hence, caspase-dependent cell death is responsible for the for the growth inhibitory effect of LBH589 (Figure 1c, right). Because even relatively high concentrations, up to 50 μM Z-VAD-FMK, could only partially abolish the LBH589-induced decrease of FLT3-ITD levels (data not shown), a caspase-independent pathway must also contribute to the loss of FLT3-ITD in LBH589-treated MV4-11 cells. An obvious candidate is proteasomal degradation.

To test the putative role of the proteasomal pathway for FLT3-ITD degradation, we co-incubated MV4-11 cells with LBH589 and the proteasomal inhibitor MG132 (Figure 1d). Cells were pretreated with LBH589 for 16 h, which evoked FLT3-ITD degradation and upregulation of UBCH8 (Figure 1b). Afterwards, MG132 was added and cells were incubated with CHX to prevent new protein synthesis. Exposure to MG132 prolonged the half-life of FLT3-ITD, which indicates basal degradation of this RTK through the proteasome. Furthermore, LBH589 accelerated the turnover of FLT3-ITD, whereas MG132 attenuated LBH589-induced destabilization of FLT3-ITD (Figure 1d; Supplementary Figure S1). This experiment confirmed that the proteasome mediates the basal turnover of this oncoprotein and that its proteasomal degradation is accelerated by LBH589.

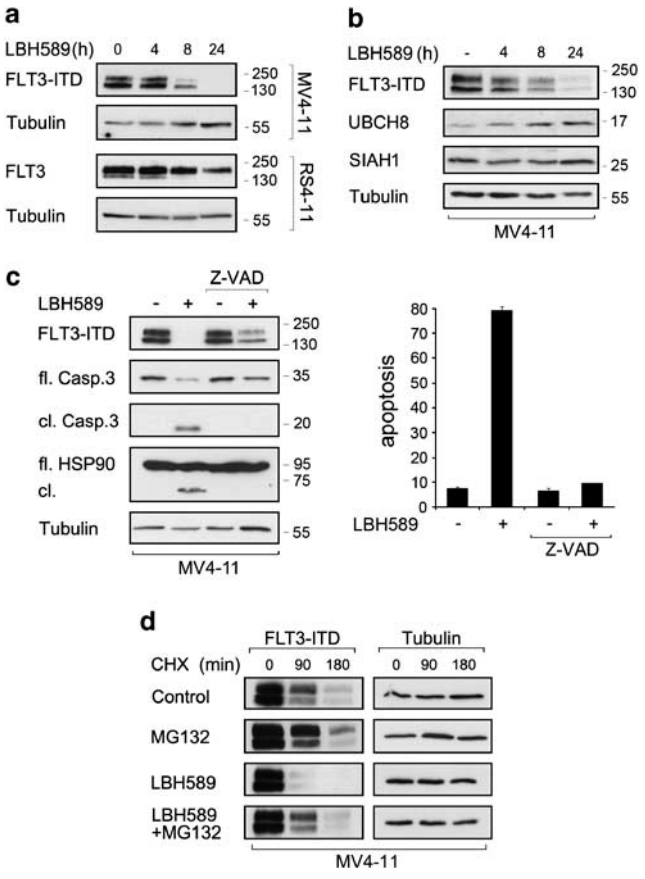

Figure 1 LBH589 induces FLT3 degradation by induction of caspase- and proteasome-dependent pathways. (**a**) MV4-11 (expressing FLT3-ITD) and RS4-11 (expressing wild-type FLT3) cells were incubated with LBH589 (30 nM) for the indicated time periods. FLT3 protein levels of cell lysates were analyzed by western blot. Tubulin was used as loading control. (**b**) MV4-11 cells were treated as described in (**a**). Protein expression of FLT3-ITD, UBCH8 and SIAH1 were detected by western blot analysis. (**c**) MV4-11 cells were either co-treated with LBH589 (30 nM) and Z-VAD-FMK (20 μM) or with these agents alone for 24 h. Protein levels were analyzed by western blot (fl., full-length; cl., cleaved). Apoptosis rates were measured by propidium iodide (PI)–FACS analysis (sub-G_1 fraction, apoptotic cells with a DNA content $<2n$, means ± s.d., $n=3$). (**d**) MV4-11 cells were left untreated (Control) or incubated with LBH589 (30 nM) for 15 h. MG132 (1 μM) was then added 1 h before protein synthesis was blocked with cycloheximide (CHX, 100 μg/ml, 90–180 min). FLT3-ITD level was detected by western blot analysis. Equal signal intensities of the blots for FLT3-ITD in CHX-untreated cells were chosen to compare the turnover rates of FLT-ITD.

Our study data show that the HDACi LBH589 evokes apoptosis and induction of UBCH8 correlating with degradation of FLT3-ITD in AML cells. Both, proteasome- and caspase-dependent mechanisms account for this proteolytic process.

UBCH8 mediates degradation of mutant FLT3 in AML cells

These observations prompted us to test whether increased UBCH8 expression triggered proteasomal degradation of

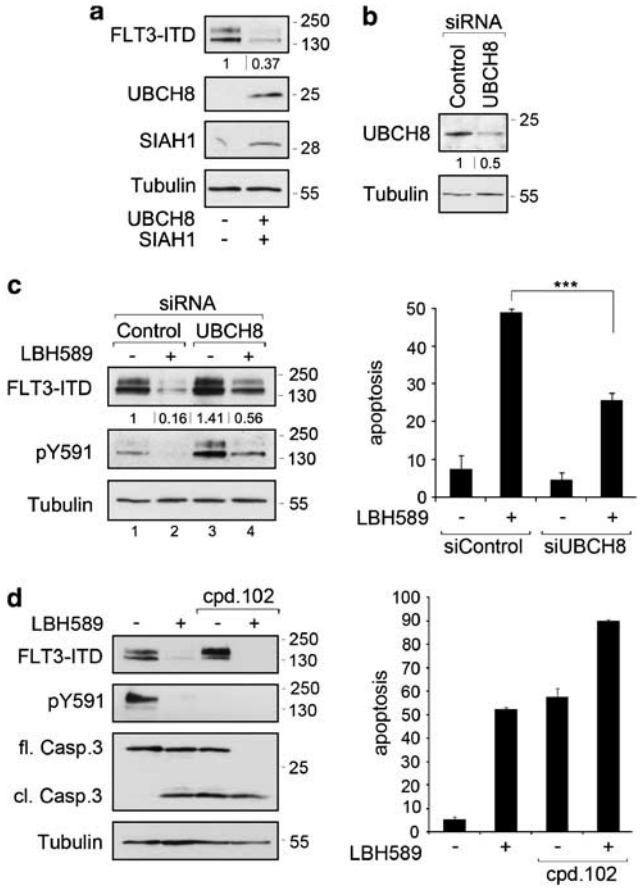

Figure 2 UBCH8 and SIAH1 expression triggers FLT3-ITD degradation in AML cells. (**a**) Ectopic expression of UBCH8 and SIAH1 in MV4-11 was carried out by nucleofection. Cell lysates were subjected to western blotting. Data below the upper panel show the densitometric analysis of the FLT3-ITD protein bands. (**b**) Knockdown of UBCH8 expression was achieved by transfection of siRNAs directed against the UBCH8 mRNA or unspecific siRNAs (Control). Lowering of basal UBCH8 protein expression was analyzed by western blot. (**c**) MV4-11 cells were left untreated or incubated 8 h after transfection with LBH589 (30 nM) for 16 h. Protein levels were analyzed by western blot. Number values are from densitometry measurement of UBCH8 or FLT3-ITD protein bands. Apoptosis rates were measured by PI–FACS analysis (means ± s.d., $n=3$, $^{***}P<0.001$). (**d**) Cells were treated for 16 h with the FLT3-specific kinase inhibitor cpd.102 (1 μM) and LBH589 (30 nM). Caspase 3 activation and FLT3-ITD protein levels were determined by western blot. Induction of apoptosis was measured by PI–FACS analysis (means ± s.d., $n=3$).

FLT3-ITD. To address the role of UBCH8, which operates in conjunction with the E3 ligase SIAH1, we ectopically expressed these proteins in MV4-11 cells. To exclude putative apoptotic degradation of FLT3-ITD, we also added Z-VAD-FMK. Indeed, enhanced levels of UBCH8 and SIAH1 markedly destabilized FLT3-ITD (Figure 2a). To further investigate the role of UBCH8 in the LBH589-induced FLT3-ITD degradation, we lowered endogenous UBCH8 levels with siRNAs that were previously shown to target this E2 ubiquitin conjugase efficiently.[50,51] Our transfection protocol allowed at least 50% reduction of UBCH8 at the protein and mRNA levels (Figure 2b; data not shown) and this sufficed to increase FLT3-ITD levels by 40% in MV4-11 cells (Figure 2c, lane 1 versus lane 3). Transfection of UBCH8 siRNAs also reduced the LBH589-induced degradation of FLT3-ITD. FLT3-ITD showed a more than threefold enhanced stability in cells transfected with UBCH8 siRNAs compared with cells transfected with a nontargeting control (Figure 2c, lane 2 versus lane 4).

Together with the approximately 50% reduction of UBCH8, we found that LBH589-induced apoptosis was decreased by 50%. These data suggest a causal role for UBCH8-mediated degradation of FLT3-ITD and apoptosis induction by HDACi. To analyze the relevance of FLT3-ITD activity for cell survival, we incubated MV4-11 cells with the FLT3-ITD-specific inhibitor cpd.102, which has previously been shown to inhibit FLT3-ITD autophosphorylation and receptor signaling efficiently in these cells.[54,59] Treatment with cpd.102 inhibited FLT3-ITD phosphorylation at Y591 (pY591), a site indicating receptor activation,[60] and this agent induced apoptosis to a similar extent as LBH589 did. Hence, both FLT3-ITD inactivation and elimination are linked to leukemia cell death confirming oncogene addiction. Co-incubation of MV4-11 cells with both substances resulted in complete cleavage of caspase 3 and even enhanced apoptosis (Figure 2d).

These data emphasize the role of UBCH8 for the basal stability and LBH589-evoked proteasomal degradation of mutant FLT3 and indicate that FLT3-ITD and UBCH8 critically regulate cell survival and HDACi-mediated apoptosis in MV4-11 AML cells.

UBCH8 preferentially induces proteasomal degradation of FLT3-ITD

To further analyze the mechanism of UBCH8-mediated degradation of FLT3-ITD, we ectopically expressed FLT3-ITD together with UBCH8 in HEK293T cells. Because HEK293T cells do not undergo apoptosis, or any other form of cell death, when UBCH8 or SIAH proteins are overexpressed,[51,61] apoptotic processes are unlikely to contribute to FLT3-ITD degradation in this setting. As shown in Figure 3a, co-expression of UBCH8 strongly decreased the levels of FLT3-ITD.

Next, we analyzed whether this effect of UBCH8 depends on the proteasome. When we treated UBCH8- and FLT3-ITD-expressing HEK293T cells with the proteasomal inhibitor MG132, we noted accumulation of basal FLT3-ITD levels and that the UBCH8-induced turnover of FLT3-ITD was blocked. Polyubiquitinylated FLT3-ITD was shown to accumulate in the detergent-insoluble lysate fraction.[40] Therefore, we probed cell fractions representing detergent-insoluble proteins from HEK293T cells co-expressing UBCH8 and FLT3-ITD for FLT3. This analysis revealed a smear of species with higher molecular mass, which most likely represent polyubiquitinylated receptors (Figure 3a). These findings indicate that UBCH8 targets FLT3-ITD to the UPS.

Considering the differential sensitivities of FLT3 and FLT3-ITD to HDACi in AML cells, we investigated whether these kinases show divergent susceptibility to UBCH8 in our heterologous expression system. Indeed, FLT3-ITD was effectively degraded upon co-expression of UBCH8 in HEK293T cells, whereas FLT3 was hardly affected (Figure 3b). We further noticed that despite comparable efficiencies of transfection (analyzed with fluorescence microscopy and western blot for co-expressed GFP), FLT3-ITD was expressed in HEK293T cells at lower levels than FLT3. Most likely, this reflected a higher turnover rate, which is consistent with a basal expression of UBCH8 in these cells (Figure 3b; data not shown).[50,51]

Prompted by these considerable differences in the sensitivity of FLT3 and its ITD mutant to UBCH8, we tested whether these kinases associate differentially with UBCH8. FLT3 or FLT3-ITD was co-expressed with UBCH8 in HEK293T cells. Probing of UBCH8 immunoprecipitates with an FLT3-specific antibody

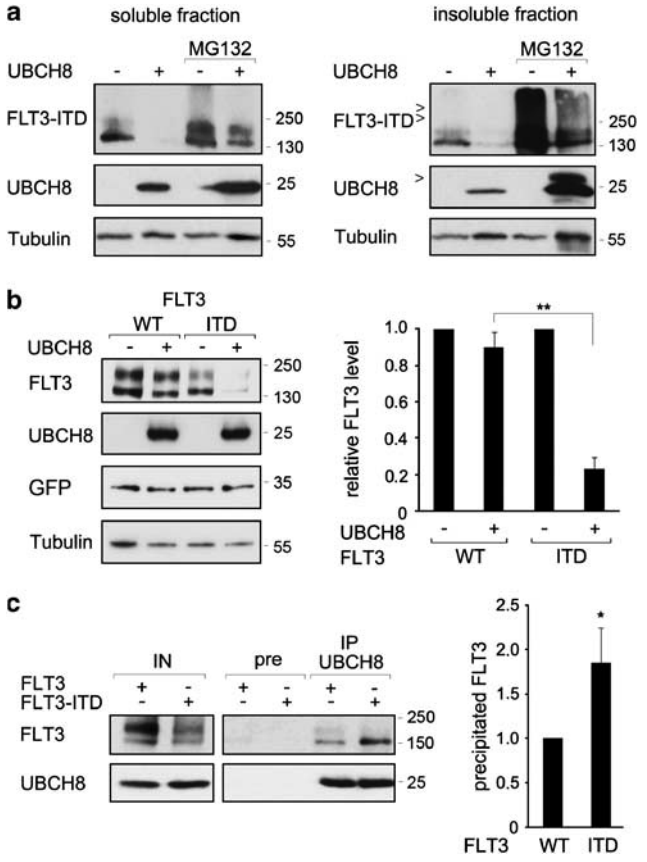

Figure 3 FLT3-ITD is more susceptible than FLT3 to UBCH8-induced degradation. (**a**) HEK293T cells were transfected with expression vectors for FLT3-ITD and UBCH8. Cells were incubated with MG132 (10 μM) 16 h before lysis to block proteasomal degradation. To detect ubiquitinylated proteins, we analyzed detergent-soluble and -insoluble fractions of cell extracts by western blotting (single arrowhead indicates monoubiquitinylated UBCH8, double arrowhead polyubiquitinylated FLT3-ITD). (**b**) FLT3 wild-type (WT) or FLT3-ITD (ITD) were expressed with UBCH8 followed by western blot for protein detection. The right panel shows the densitometric analysis of WT or ITD protein bands (means ± s.d., $n=7$, $^{**}P<0.01$). (**c**) FLT3 or FLT3-ITD was co-expressed with UBCH8. UBCH8 was immunoprecipitated in cell lysates. The presence of FLT3, FLT3-ITD and UBCH8 in the precipitates was analyzed by western blot (pre, preimmune serum; IP, immunoprecipitation; IN, 10% of IP input). Relative binding of FLT3 (WT) compared with FLT3-ITD (ITD) with UBCH8 was quantified by densitometry (means ± s.d., $n=4$, $^{*}P<0.05$).

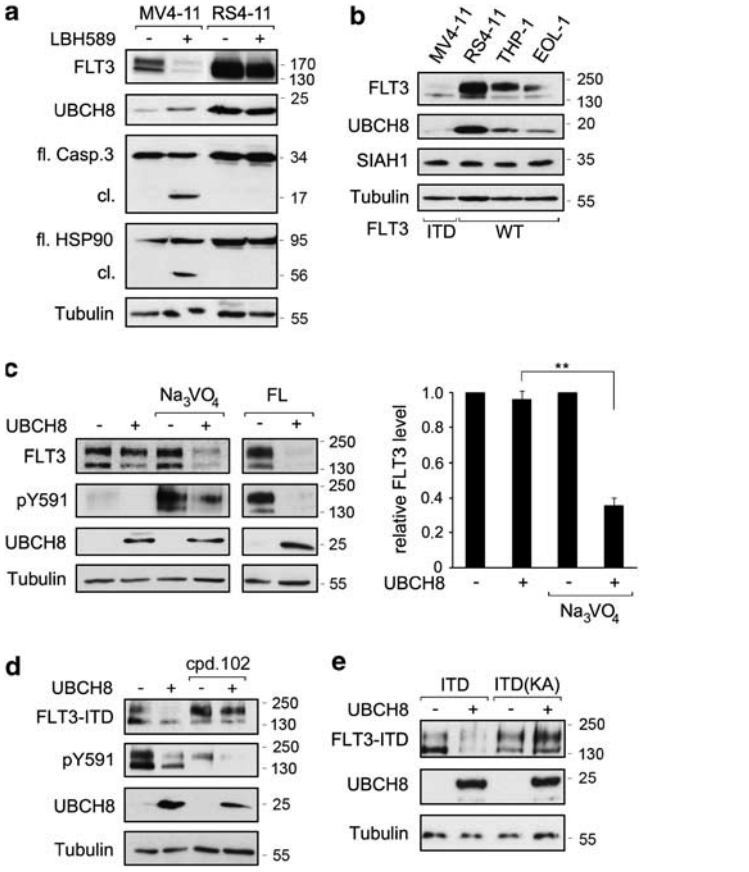

Figure 4 Kinase activation sensitizes FLT3 and FLT3-ITD for UBCH8-mediated degradation. (**a**) MV4-11 (FLT-ITD) and RS4-11 (wild-type FLT3) cells were treated with LBH589 (30 nM, 24 h) and cell lysates were subjected to western blot analysis. In MV4-11 cells, LBH589 induces UBCH8 expression, conversion of full-length Caspase 3 (fl. Casp.3) to the active p17/19 (cleaved, cl.) subunits and proteolytic cleavage of HSP90 (fl., full length; cl., cleaved). (**b**) To compare basal FLT3, UBCH8 and SIAH1 protein levels in different leukemic cell lines, we analyzed equal amounts of protein lysates of MV4-11 (FLT3-ITD) or wild-type FLT3-expressing RS4-11, THP-1 and EOL-1 cell lines by western blotting. (**c**) FLT3 and UBCH8 were expressed in HEK293T cells. At 4 h before lysate preparation, cells were incubated with orthovanadate (Na_3VO_4, 1 mM) to block phosphatase activity. FLT3, UBCH8 and pY591 levels were analyzed by western blot and quantified by densitometric analysis (means ± s.d., $n=3$, $^{**}P<0.01$). UBCH8 and FLT3 expressing cells were incubated with recombinant FLT3 ligand (FL, 10 ng/ml) for 16 h to stimulate FLT3 kinase activation. Western blot for detection of FLT3 and UBCH8 was performed. (**d**) The constitutively active FLT3-ITD (ITD) was expressed with UBCH8. Cells were incubated with the FLT3 kinase-specific inhibitor cpd.102 (1 μM, 16 h) followed by western blot for FLT3-ITD, UBCH8 and pY591. (**e**) Transient expression of FLT3-ITD (ITD) or its kinase-inactive mutant FLT3-ITD(KA) (ITD-(KA)) with UBCH8. Proteins were detected by western blot.

detected significantly more FLT3-ITD than FLT3 in immunocomplexes with UBCH8 (Figure 3c). In agreement with this finding, *in situ* immunofluorescence analysis showed that UBCH8 exhibited an overlapping localization with FLT3-ITD. Apparently, there was lesser colocalization detectable with the cell-surface-bound fraction of the wild-type receptor (Supplementary Figure S3).

Taken together, these results show that UBCH8 induces FLT3-ITD degradation by the UPS in HEK293T cells. Remarkably, UBCH8 associates with and degrades mutant FLT3-ITD more strongly than wild-type FLT3.

Tyrosine phosphorylation governs UBCH8-triggered degradation of FLT3-ITD

MV4-11 cells expressing FLT3-ITD were found to be more sensitive to HDACi-mediated induction of apoptosis than RS4-11 cells expressing FLT3.[39,62] We could confirm a higher sensitivity of MV4-11 cells to HDACi treatment. Cleavage of HSP90 and induction of apoptosis, indicated by processing of the precursor caspase 3 into the cleaved active form, could only be detected in MV4-11 cells. Surprisingly, UBCH8 protein was only induced in MV4-11 cells but not in RS4-11 cells, which express appreciable levels of this enzyme independent of HDAC inhibition (Figure 4a). Moreover, real-time PCR analyses revealed that RS4-11 cells have an approximately 10-fold higher number of transcripts coding for UBCH8 than MV4-11 cells (data not shown). Analysis of UBCH8 levels in further human

AML cell lines with wild-type FLT3 expression (RS4-11, THP-1 and EOL-1) revealed higher UBCH8 levels in these cell lines compared with MV4-11 cells. In contrast, SIAH1 was expressed at equal levels in these cells (Figure 4b).

On the basis of these observations and the fact that UBCH8 preferentially degraded constitutively active FLT3-ITD, we investigated whether tyrosine phosphorylation regulates this process. To enhance phosphorylation of FLT3, we treated HEK293T cells expressing FLT3 and UBCH8 with doses of the general protein-tyrosine phosphatase inhibitor orthovanadate not impairing cell viability.[57] Orthovanadate caused equal phosphorylation of FLT3 and FLT3-ITD at pY591 (Supplementary Figure S2). Consistent with our previous data, FLT3 protein level was nearly unaffected by exogenously expressed UBCH8. However, induction of FLT3 phosphorylation by orthovanadate caused its efficient degradation in the presence of UBCH8 (Figure 4c). Moreover, activation of FLT3 with its physiological ligand FL induced receptor autophosphorylation (Supplementary Figure S2) and UBCH8-mediated degradation to an extent similar to that observed for mutant FLT3 (compare Figures 3a, b and 4c).

To further substantiate the role of tyrosine phosphorylation for FLT3 degradation, we incubated HEK293T cells expressing UBCH8 and FLT3-ITD with its specific inhibitor cpd.102. In addition, we analyzed whether UBCH8 induced degradation of a catalytically inactive FLT3-ITD mutant harboring a lysine to alanine exchange in its catalytic cleft (ITD-KA).[13] Inhibition of tyrosine phosphorylation, with this chemical inhibitor or by mutagenesis, rendered FLT3-ITD insensitive to UBCH8-mediated degradation (Figures 4d and e). Of note, the effects of cpd.102 and the ITD-KA mutation on the UBCH8-mediated depletion of FLT3-ITD were as prominent as those achieved with an inhibitor of the proteasome (Figure 3a). Accordingly, inhibition of FLT3-ITD phosphorylation by cpd.102 stabilized its protein level in MV4-11 cells (Figure 2d, lane 3).

These data suggest that degradation of FLT3 and FLT3-ITD through UBCH8 depends on tyrosine phosphorylation.

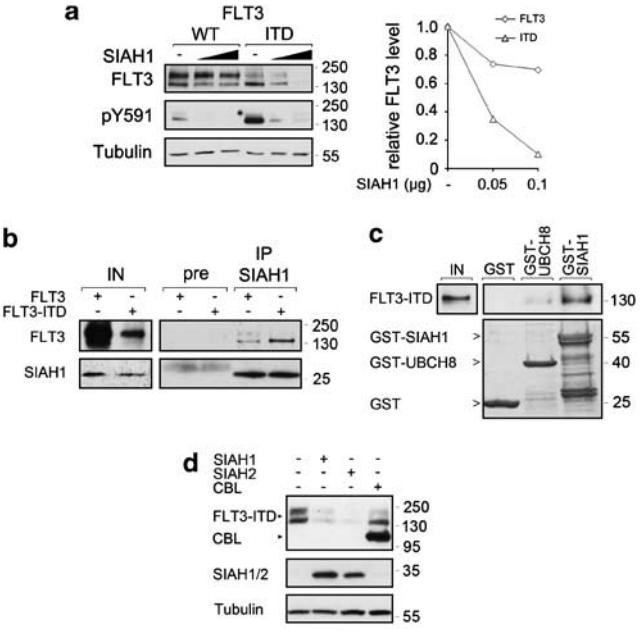

Figure 5 SIAH E3 ligases target FLT-ITD for degradation. (**a**) Increased degradation of mutant FLT3 in an SIAH1 concentration-dependent manner. HEK293T cells were co-transfected with FLT3 (WT), FLT3-ITD (ITD) and different amounts of SIAH1 DNA (0.05 µg, 0.1 µg). Western Blot was performed to detect FLT3 protein level. The right panel illustrates the densitometric values of FLT3 and FLT3-ITD protein bands. (**b**) Ectopic expression of SIAH1 and FLT3 or FLT3-ITD in HEK293T cells. SIAH1 was immunoprecipitated from cell extracts (IP, immunoprecipitation; pre, preimmune serum; IN, 10% of IP input). Western blot analysis was performed to detect SIAH1 and co-precipitated FLT3 or FLT3-ITD in the immunoprecipitates. (**c**) Detection of *in vitro* expressed FLT3-ITD bound to GST-UBCH8, GST-SIAH1 or GST (as control) in pull-down experiments (IN, 20% of input). Levels of GST fusion proteins were analyzed by gel staining with Coomassie brilliant blue. (**d**) FLT3-ITD was expressed with SIAH1, SIAH2 or CBL in HEK293T cells. Cell lysates were analyzed by western blot.

SIAH E3 ubiquitin ligases targets FLT3-ITD for proteasomal degradation

It is known that UBCH8 and the RING E3 ligase SIAH1 functionally cooperate in various cell types.[50,63,64] To test their potential interaction in the degradation of FLT3 and FLT3-ITD, we expressed these kinases together with the E3 ligase SIAH1 in HEK293T cells. Similar to overexpression of UBCH8, increasing amounts of SIAH1 lowered FLT3-ITD levels far more strongly than those of FLT3 (Figure 5a). In the latter case, a minor pool of autoactivated FLT3 (weak activation is caused by overexpression) was the primary target of degradation (Figure 5a).

We further investigated whether the increased degradation of FLT3-ITD on SIAH1 co-expression is reflected by binding to SIAH1. SIAH1 was precipitated with a specific antibody and immunocomplexes from HEK293T cell lysates were analyzed for the presence of FLT3. This experiment showed that a larger fraction of FLT3-ITD than FLT3 was found in precipitates formed under physiological conditions with an SIAH1 antibody (Figure 5b). This was similar to the complex formation detected between UBCH8 and FLT3-ITD (Figure 3c).

To address whether UBCH8 or SIAH1 is the direct binding partner of FLT3-ITD, we performed *in vitro* binding assays. FLT3-ITD was expressed in a cell-free system and pull-down experiments were performed with GST fusions of UBCH8 or SIAH1. FLT3-ITD preferentially bound to SIAH1. Although this agrees with RING E3 ligase-mediated substrate recognition, a very minor binding of UBCH8 to FLT3-ITD was also detectable (Figure 5c).

Finally, we tested whether overexpression of further ubiquitin-ligases, such as TRIAD1, RLIM or MDM2, had an effect on FLT3-ITD levels in HEK293T cells. However, these E3 enzymes had no effect on FLT3-ITD stability (data not shown). CBL, an SH2 domain-containing multiadapter protein with RING finger E3 ligase activity, regulates RTK endocytosis and sorting to the lysosomal degradation machinery.[65–69] Because CBL targets wild-type FLT3 for ubiquitinylation,[38] we investigated how expression of this ubiquitin ligase, in comparison to SIAH1 and the related SIAH2,[55] affects stability of the FLT3-ITD mutant in HEK293T cells. We found that expression of SIAH proteins resulted in complete loss of FLT3-ITD, whereas CBL overexpression selectively reduced the level of the higher migrating band of FLT3-ITD (Figure 5c). This band corresponds to the mature, glycosylated receptor.[13] These data may indicate a specificity of CBL for mature FLT3 at the cell-surface and that SIAH proteins potently target FLT3-ITD.

Discussion

Histone deacetylase inhibitors are promising anticancer drugs for the treatment of leukemia.[70] Detailed analyses of specific

and pleiotropic actions of these compounds are required to predict and ensure therapeutic benefits. Particularly less information is available on enzymes and molecular pathways participating in HDACi-induced proteasomal degradation. Here, we show with inhibitor studies, ectopic expression, and siRNA approaches that HDACi treatment leads to degradation of oncogenic FLT3-ITD through the HDACi-inducible E2 ubiquitin conjugase UBCH8 and the E3 ligase SIAH1 in AML cells.

Congruent with these findings, we and others already showed that a specific cooperation of SIAH1 with UBCH8 induces protein degradation.[50,63,64] In addition, we previously discovered that the leukemia-associated transcription factors AML1/ETO and PML/RARα, which are often found in conjunction with mutated tyrosine kinases,[60] are also degraded by UBCH8 and SIAH1.[50] Thus, targeting of both, nuclear leukemia fusion proteins and oncogenic RTK signaling, by induction of UBCH8, may contribute to the frequently observed antileukemic actions of HDACi.[42,44–46,71]

Remarkably, depletion of UBCH8 by siRNA reduces the sensitivity of MV4-11 cells toward LBH589-mediated induction of apoptosis, emphasizing the role of UBCH8 for the HDACi treatment of AML.[50] In line with previous reports, revealing FLT3-ITD as important factor for cell proliferation and survival of AML cells *in vitro* and in murine models,[7,72–74] we could show that specific kinase inhibition of FLT3-ITD not only induces apoptosis in AML cells *per se*, but also renders them more sensitive to HDACi-induced cell death. These data confirm that phosphorylation of FLT3-ITD and subsequent oncogenic signaling ensure cell survival and that this oncoprotein is a valid target for leukemia treatment.[59,72–75]

In agreement with these findings, combinatorial treatment of AML cells with HDACi and FLT3 inhibitor results in enhanced caspase activation and strong induction of apoptosis. Thus, caspases can function in a feedforward mechanism, which promotes degradation of FLT3-ITD and compensates reduced proteasomal degradation of inactivated receptor. Because targeting of FLT3-ITD stability appears as a crucial effect of HDACi, combining HDACi and FLT3-specific kinase inhibitors may gives beneficial effects *in vivo*. Such a strategy impairs both expression and activity of the oncoprotein.

It is generally accepted that RING E3 ligases selectively mediate the transfer of E2-bound ubiquitin to a substrate. Previous observations and our biochemical data presented here show that SIAH1 can function as E3 for UBCH8 in the HDACi-induced depletion of FLT3-ITD in AML cells.[50,63,64] In contrast to mutant FLT3, wild-type FLT3 remains stable in the presence of already high levels of basal UBCH8 in AML cells. According to our data, stimuli inducing phosphorylation of FLT3 and constitutive autophosphorylation of FLT3-ITD direct them for UBCH8/SIAH1-mediated degradation. Consistently, inhibition of FLT3-ITD phosphorylation prevents proteasomal degradation, which argues for preferential elimination of the active kinase. Because limited expression of UBCH8 might be a prerequisite for stable levels of FLT3-ITD and other (onco-) proteins,[50–52] it is tempting to speculate that deregulation of this E2 might be a step in cellular transformation processes.

CBL is an additional E3 ligase regulating RTK degradation, and it also facilitates ubiquitinylation of wild-type FLT3.[38,68,76,77] The results of our study suggest an alternative pathway, involving UBCH8 and SIAH1, for the degradation of activated wild-type and mutant FLT3. Consistently, CBL functionally interacts with the E2 enzymes UBCH4/5/7, though not with UBCH8,[65,76] whereas SIAH1 preferentially cooperates with the HDACi-inducible UBCH8.[50,63] Moreover, several reports show that CBL cannot catalyze ubiquitinylation or degradation of mutant forms of RTKs, such as mutant EGFR, FGFR3, TPR-MET or hybrid receptors of PDGFRα/β. Receptor mislocalization, aberrant phosphorylation and a lack of CBL recruitment account for the reduced efficiency of CBL-mediated degradation of these aberrant proteins.[53,78–81] FLT3-ITD has a defect in glycoprotein maturation and gives rise to relatively large amounts of intracellularly retained, albeit active protein. Our study data indicate that this pool of FLT3-ITD is resistant to CBL-mediated degradation and suggest a model in which SIAH proteins and CBL predominantly target either FLT3-ITD or FLT3 to the UPS. Congruent with our model, CBL has recently been shown to interact predominantly with the mature, cell-surface form of FLT3.[82] Further analyses are required to clarify another possibility, that these RTKs regulate activity and target selectivity of E2 and E3 enzymes by direct phosphorylation.

Although the results of our study imply a critical role of UBCH8 and SIAH proteins mediating FLT3-ITD degradation in cells treated with HDACi, additional mechanisms can contribute to its turnover. Several studies showed that HDACi evoke acetylation of HSP90, which affects co-chaperone and client protein binding.[40,49,83] It is possible that UBCH8 mediates receptor degradation downstream of HSP90 acetylation. However, inhibition of HSP90 impairs oncogenic signaling through FLT3-ITD but not its stability *per se*.[84] In agreement, our gain- and loss-of-function approaches indicated that UBCH8 and SIAH1 can evoke FLT3-ITD degradation bypassing the need for HDACi-induced HSP90 hyperacetylation in AML and HEK293T cells. Moreover, depletion of UBCH8 in MV4-11 cells as well as chemical inhibition of the proteasome diminished the basal and the HDACi-accelerated FLT3-ITD turnover.

In addition, our study results collected with the caspase inhibitor Z-VAD-FMK suggested that LBH589-induced caspase activity contributes to the degradation of FLT3-ITD. Caspase-dependent cleavage of HSP90 or its co-chaperone p23 is coupled to endoplasmatic reticulum/oxidative stress and apoptotic cell death.[85–87] Because caspases can cleave RTKs directly,[88–90] and HDACi are described as strong inducer of apoptosis in MV4-11 cells,[39,40] FLT3-ITD might also be a direct target of caspases. Apparently, caspase- and proteasome-catalyzed mechanisms cooperatively function on FLT3-ITD in HDACi-treated cells.

It is accepted that HDACi affect protein stability by modulation of the UPS. However, the enzymes catalyzing ubiquitinylation and subsequent degradation under such conditions have only been identified for about a handful of proteins. Even less is known about molecular mechanisms regulating substrate discrimination in this setting. Here, we report for the first time that the E2 ubiquitin conjugase UBCH8 and the E3 ubiquitin ligase SIAH1 phosphorylation dependently catalyze proteasomal degradation of an oncogenic class III RTK.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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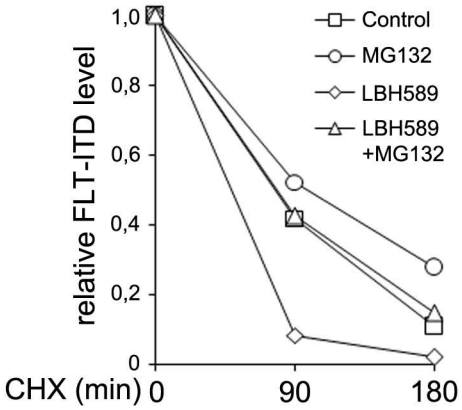
Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

Supplementary Figures

(**S1**) Densitometry-based analyses of FLT3-ITD protein bands revealed by Western blot analyses shown in Figure 1 (d). (**S2**) Test for drug potencies on FLT3 (WT) and FLT3-ITD (ITD) auto-phosphorylation. HEK293T cells were treated with indicated drugs as described in Figure 4 (c). Detection of tyrosine 591 phosphorylation (pY591) by Western blot analyses was used to define the degree of receptor-activation. (**S3**) EGFP tagged FLT3 (WT) or FLT3-ITD (ITD) was coexpressed with UBCH8-V5 and dominant-negative SIAH1 in Hela cells. The expression of FLT3 was monitored by EGFP fluorescence. UBCH8-V5 was detected by immunostaining with anti-V5 antibody and Cy3-labeld secondary antibody (arrows indicate regions of minor colocalization between UBCH8 and FLT3).

Supplemental Figures

S1



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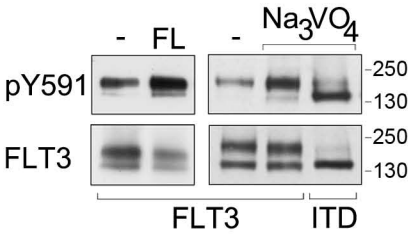
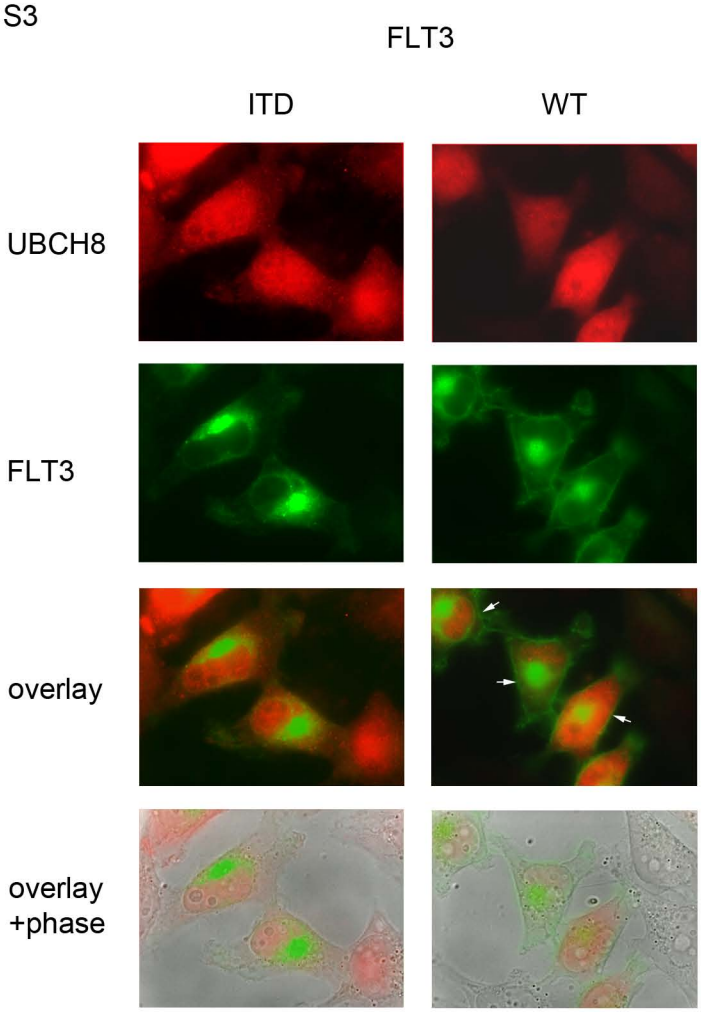


S3



8 Discussion

HDACi are promising anti-cancer drugs for the treatment of leukemia. Detailed analyses of specific and pleiotropic actions of these compounds are required to predict and ensure therapeutic benefits. HDACi have been shown to counteract transcriptional repression and they are known to selectively affect protein stability via modulation of the UPS. On enzymes and molecular pathways participating in the HDACi-regulated proteasomal degradation, particularly sparse information is available. The enzymes catalyzing ubiquitinylation and subsequent degradation upon HDACi treatment have only been identified for about a handful of proteins. Even less is known about molecular mechanisms regulating substrate discrimination in this setting. This work shows for the first time that the HDACi-inducible ubiquitin conjugase UBCH8 in cooperation with its ubiquitin ligase SIAH1 catalyze proteasomal degradation of the activated receptor tyrosine kinase FLT3 and the chimeric fusion proteins PML-RARα and AML1-ETO.

8.1 HDACi selectively target mutant FLT3 for degradation via the UBCH8-SIAH1-axis

Recent publications showed that HDACi induce apoptosis of AML cells, which correlates with the reduction of FLT3-ITD protein. However, these agents generate pleiotropic effects and the enzymes catalyzing the degradation of FLT3-ITD in HDACi-treated cells have not been identified at all. In addition, an explanation for the different stability of wild-type FLT3 and FLT3-ITD in leukemic cells has not been provided. In my work I show with inhibitor studies, ectopic expression, and siRNA-approaches that HDACi treatment leads to the degradation of activated FLT3 and FLT3-ITD via the HDACi-inducible E2 ubiquitin conjugase UBCH8 and the E3 ligase SIAH1 in AML cells. Compared to the unliganded wild-type receptor, both enzymes preferentially bind to and target mutant FLT3 for proteasomal degradation. Furthermore, depletion of UBCH8 by siRNA reduces the sensitivity of MV4-11 cells towards LBH589-mediated induction of apoptosis, emphasizing the role of UBCH8 for the HDACi-treatment of AML [132].

Previous reports revealed FLT3-ITD as an important factor for cell proliferation and survival of AML cells *in vitro* and in murine models [43,133-135]. In line with this, I could show that specific kinase-inhibition of FLT3-ITD not only induces apoptosis in AML cells *per se*, but also renders them more sensitive to HDACi-induced cell death. It is known that UBCH8 and SIAH1 functionally cooperate in protein degradation in various cell types. Pursuant to these previous observations, the biochemical data presented here show that SIAH1 can function as E3 ligase for UBCH8 in the HDACi-induced depletion of FLT3-ITD in AML cells [132,136,137]. I demonstrate that compared to FLT3, FLT3-ITD is much more susceptible to HDACi-

mediated degradation in AML cells. Moreover, by analyzing the endogenous protein expression levels of UBCH8, wild-type FLT3, and FLT3-ITD in diverse AML cell lines, I found that in contrast to mutant FLT3, wild-type FLT3 remains stable in the presence of already high levels of basal UBCH8. My data suggest that the UBCH8/SIAH1-mediated degradation of FLT3 is dependent on the phosphorylation status of the protein. Consequently, the activated wild-type FLT3 and the constitutively active mutant FLT3-ITD are likewise targeted. Consistently, inhibition of FLT3-ITD phosphorylation prevents its proteasomal degradation. Since limited expression of UBCH8 might be a prerequisite for stable levels of FLT3-ITD and other (onco-) proteins [122,123], it is tempting to speculate that deregulation of this E2 might be one step in cellular transformation processes. These data confirm that phosphorylation of FLT3-ITD and subsequent oncogenic signaling ensures cell survival and that this oncoprotein is a valid target for leukemia treatment [133-135,138,139].

Since HDACi are used as drugs for the treatment of leukemia, the finding that such compounds attenuate FLT3-ITD appears particularly interesting. Targeting of FLT3-ITD stability by the proteasome appears to determine efficacy of HDACi treatment. The preferential degradation of mutant, constitutively active FLT3-ITD upon HDACi treatment could account for the observed specificity of HDACi against malignant cells compared to untransformed cells. Since AML cells expressing FLT3-ITD are more sensitive to HDACi-induced apoptosis, UBCH8, SIAH, and FLT3-ITD levels might also serve as pharmacodynamic markers for therapeutic efficacy. Moreover, since wild-type FLT3, an important regulator of normal hematopoiesis, was shown to be far less accessible for HDACi-induced degradation, this could explain the well tolerability of HDACi as anti-cancer drugs.

8.2 UBCH8 and SIAH1 regulate the turnover of PML-RARα and AML1-ETO

Furthermore, I could show here that the leukemia-associated transcription factors AML1-ETO and PML-RARα, which are often found in conjunction with oncogenic FLT3-ITD in AML [4], are also degraded by UBCH8 and SIAH1 [132]. The study shows that the poly-ubiquitinylation and subsequent proteasomal degradation of AML1-ETO and PML-RARα is increased by HDACi. Several independent lines of evidence obtained from ectopic expression, dominant-negative molecules, siRNA experiments and protein-protein-interaction approaches coherently indicate that this process, similar to the HDACi-mediated degradation of mutant FLT3, is mediated by HDACi-inducible UBCH8 in cooperation with SIAH1. Congruent with these novel findings, other groups already showed that a specific cooperation of SIAH1 with UBCH8 induces protein degradation [132,136,137].

HDACi can act as inhibitors of leukemia fusion proteins indirectly via the induction of their

degradation and directly by inhibiting the catalytic activity of co-repressor complex-associated HDACs [140]. The mutant fusion proteins AML1-ETO and PML-RARα compete with the remaining intact transcription factors for binding sites and therefore prevent the expression of target genes responsible for proper hematopoiesis. Furthermore, AML1-ETO was shown to bind directly, sequester and compete with further transcriptional regulators of hematopoietic differentiation, e.g. c-Jun, PLZF, C/EBP, SMAD3 and VDR [31] and PML-RARα interferes with C/EBP transcription factors and cell cycle restricting proteins [141]. In this context, the HDACi-induced proteasomal degradation of AML1-ETO and PML-RARα may be particularly relevant, since it improves the ratio of wild-type protein to fusion oncoprotein [31,141,142]. Therefore, HDACi are expected to restore normal function of transcriptional regulators, such as wild-type AML1 or RARα, and thereby affect the growth and differentiation ability of leukemic cells expressing the chimera. Since the expression of fusion proteins is highly restricted to transformed leukemic cells which are dependent on such oncoproteins, the HDACi-induced degradation of those could be one reason why these compounds are more toxic to leukemic cells than to normal cells [115]. Hence, the degradation of such proteins may become a molecularly defined, selective intervention strategy.

Further analyses are also required to clarify whether HDACi impose posttranslational modifications other than ubiquitinylation of leukemia fusion proteins and whether or not these modifications affect protein stability. Remarkably, it was reported that a truncated variant of AML1-ETO which does not interact with corepressors promotes leukemia development [143]. Similarly, transformation by PML-RARα depends on its cleavage by neutrophil elastase [144]. One could thus speculate that the benefits of HDACi in leukemia treatment rely on the destabilization of leukemia fusion proteins as well as on HDAC-inhibition. Regulation of such processes by HDACi is likely to be relevant for the activity of these drugs in cultured cells, animal models and patients [122,145-147].

In summary, the data presented here implicate that targeting of both, nuclear leukemia fusion proteins and oncogenic RTK signaling, via induction of UBCH8, may contribute to the frequently observed anti-leukemic actions of HDACi [98,103,105,116,148].

8.3 HDACi induce protein degradation independent of HSP90 acetylation

Although the results imply a critical role of UBCH8 and SIAH proteins in mediating oncoprotein degradation in cells treated with HDACi, additional mechanisms may contribute to the induced protein turnover. Several studies demonstrated that HDACi evoke acetylation of HSP90, which affects co-chaperone and client protein binding [119,130,149]. It is therefore possible that UBCH8 and SIAH1 mediate oncoprotein degradation downstream of HSP90

acetylation. However, inhibition of HSP90 by acetylation impairs oncogenic signaling via FLT3-ITD but not its stability *per se* [150]. In agreement, the gain- and loss-of-function approaches indicated that UBCH8 and SIAH1 can evoke FLT3-ITD degradation bypassing the need for HDACi-induced HSP90 hyperacetylation in AML cell lines and HEK293T cells. Moreover, depletion of UBCH8 in AML cells as well as chemical inhibition of the proteasome diminished the basal and the HDACi-accelerated FLT3-ITD turnover, pointing towards UBCH8 as limiting factor for the HDACi-induced proteasomal degradation.

Similar to FLT3-ITD, AML1-ETO and PML-RARα are also described as clients of HSP90 [119]. Thus, the stability of these leukemia fusion proteins may also be influenced by HSP90 acetylation. However, to investigate the HDACi-induced turnover of AML1-ETO and PML-RARα the HDACi VPA was used, which does not inhibit HDAC6 [115,151], the deacetylase targeting HSP90 [152,153]. It is therefore not surprising that acetylated HSP90 was not detectable upon VPA-treatment, indicating on HSP90 acetylation-independent degradation processes. Furthermore, the depletion of UBCH8 protein prevented the degradation of endogenous AML1-ETO even in the presence of HDACi, and leukemia fusion proteins were degraded under conditions that do not inhibit HDAC activity, such as UBCH8 or SIAH1 overexpression. Still, HSP90 acetylation may be below the detection limit and could be masked by its HDACi-induced cleavage. Nevertheless, acetylation of HSP90 is unlikely to be a mandatory prerequisite for degradation of AML1-ETO, PML-RARα or FLT3-ITD in the context of my experiments. Consistent with this assumption, I observed that HSP90-bound AML1-ETO is not protected but also degraded upon HDAC-inhibition. Of note, it was recently shown that knockdown of HDAC6 induces HSP90 acetylation and impairs its chaperone function, but hardly affects the stability of the leukemia fusion protein BCR-ABL [152]. In agreement with my findings, this study also implies that limiting amounts of enzymes of the ubiquitylation machinery determine oncoprotein degradation.

8.4 Caspase- and proteasome-catalyzed mechanisms cooperate in the HDACi-induced protein degradation

The results obtained with the caspase inhibitor Z-VAD-FMK revealed that HDACi-induced caspase activity contributes to the degradation of FLT3-ITD. Caspase-dependent cleavage of HSP90 or its co-chaperone p23 is coupled to endoplasmatic reticulum/oxidative-stress and apoptotic cell death [154-156]. Because caspases can cleave receptor tyrosine kinases directly [157-159] and HDACi are described as strong inducers of apoptosis in MV4-11 cells [119,120], FLT3-ITD might also be a direct target of caspases. Apparently, caspase- and proteasome-catalyzed mechanisms cooperatively act on FLT3-ITD in HDACi-treated cells. In agreement

with the above mentioned findings, combined treatment of AML cells with HDACi and FLT3-inhibitor results in enhanced caspase activation and strong induction of apoptosis. As a consequence, caspases may act in a feed-forward mechanism, further promoting the degradation of FLT3-ITD, thereby compensating reduced proteasomal degradation due to kinase inhibition.

Caspase-mediated proteolytical cleavage of AML1-ETO and PML-RARα is also possible, since HDACi induce apoptosis in AML cells harbouring the encoding chimeric genes. HDACi trigger the degradation of these proteins before the onset of caspase cleavage and chemical caspase-inhibition did not block the HDACi-induced decrease of AML1-ETO levels. Of note, no cleavage products typical for caspase-induced degradation were observed. In contrast, the proteasome inhibitor MG132 is a strong pro-apoptotic stimulus, which stabilized the AML1-ETO protein. Furthermore, UBCH8 and SIAH1 did not induce apoptosis in HEK293T cells, although they destabilized ectopically expressed AML1-ETO and PML-RARα in these cells, which could be blocked by inhibition of the proteasome. As discussed for the HDACi-induced reduction of FLT3-ITD protein, it is possible that in addition to the proteasomal degradation, caspases may cleave leukemia fusion proteins at late stages of HDACi treatment.

8.5 CBL and SIAH1 alternatively target either wild-type or mutant FLT3 for degradation

CBL is an additional E3 ligase regulating receptor tyrosine kinase degradation, and CBL also facilitates ubiquitinylation of wild-type FLT3 [160-163]. My results suggest an alternative pathway, involving UBCH8 and SIAH1, for the selective degradation of activated wild-type and constitutively active FLT3-ITD. CBL functionally interacts with the E2 enzymes UBCH4/5/7, though not with UBCH8 [161,164], whereas SIAH1 preferentially cooperates with the HDACi-inducible UBCH8 [132,136]. Moreover, several reports demonstrate that CBL cannot catalyze ubiquitinylation or degradation of mutant forms of RTKs, such as mutant EGFR, FGFR3, TPR-MET or hybrid receptors of PDGFRα/β. Receptor mislocalization, aberrant phosphorylation and a lack of CBL recruitment account for the reduced efficiency of CBL-mediated degradation of these aberrant proteins [124,143,165-167]. FLT3-ITD has a defect in glycoprotein maturation and gives rise to relatively large amounts of intracellularly retained, albeit active protein. The data presented in this work indicate that this pool of FLT3-ITD is resistant to CBL-mediated degradation and suggest a model in which SIAH proteins and CBL predominantly target either FLT3-ITD or FLT3 to the UPS. Congruent with my model, CBL

has recently been shown to predominantly interact with the mature, cell-surface form of FLT3 [168].

8.6 Substrate recognition by the SIAH1 ubiquitin ligase

SIAH1 appears to bind and directly control the proteasomal turnover of AML1-ETO and PML-RARα. This is in agreement with the presence of consensus motifs for SIAH1 binding in these proteins [169,170]. Four of these motifs are clustered in the AML1-ETO fusion protein. This fact could explain why the intact AML1 and ETO proteins, each containing only two SIAH1 binding sites, are far less susceptible to VPA-induced degradation. A similar situation occurs in PML-RARα, where the SIAH1 binding sites of RARα are brought into close proximity of the PML coiled coil domain, which also recruits this E3 ligase [122]. Neither the clustering of SIAH1 binding sites nor a PML-type coiled coil domain exists in STAT5-RARα, another fusion protein that I tested as possible substrate for SIAH1. Accordingly, this fusion protein is not degraded via UBCH8 and SIAH1.

Such a consensus motif that is found in the majority of SIAH-interacting proteins is not present in wild-type or mutant FLT3. Since its presence is not an essenial prerequisite for the interaction with SIAH proteins, other mechanisms may regulate the binding of active FLT3 to SIAH1. It is known that phosphorylation of RTKs can regulate their ubiquitinylation by the specific E3 ligase. Unlike the ubiquitin ligase CBL, SIAH1 does not possess an SH2 domain, which may recognize phosphorylated tyrosine residues in the FLT3 RTK. Thus, pointing towards a more indirect recruitment of SIAH1 to the activated receptor. Previous reports demonstrated that SIAH proteins are phosphorylated at multiple residues in different environments. Therefore it is conceivable, that the active RTK regulates activity and targets selectivity of the E2 and E3 enzymes by direct phosphorylation. Congruent with this consideration, SIAH phosphorylation is discussed as an important regulatory mode, mediating association with substrate molecules or adaptor proteins [86,171-174]. Accordingly, further studies have to clarify, whether phosphorylation of SIAH1 or other components of the ubiquitinylation machinery, can be responsible for the degradation of active FLT3. Such a model, in which SIAH1 is either activated by direct phosphorylation or recruited to active FLT3 via phosphorylation-dependent binding of adaptor-proteins, recognizing FLT3 phosphorylation patterns and recruiting the ubiquitinylating enzymes, would explain the preferential degradation of activated FLT3 by the UBCH8-SIAH1-axis.

8.7 Interplay between UBCH8 and its interacting E3 ligases

HDACi counteract transcriptional repression and also selectively affect protein stability, but only limited knowledge exists about the E2 and E3 enzymes involved in HDACi-induced ubiquitinylation [88]. In a previous work we found that high levels of HDAC2 correlate with certain neoplastic malignancies and that the E3 ubiquitin ligase RLIM in cooperation with UBCH8 mediates HDACi-induced proteasomal degradation of HDAC2 [122]. Therefore, UBCH8 and RLIM appear to be crucial for at least a subset of these processes. In the study presented here, I could confirm the initial observation that HDACi induce UBCH8 expression in several cell lines. Nevertheless, we could show that RLIM does not contribute to AML1-ETO and PML-RARα degradation via UBCH8. Of note, RLIM is expressed at much lower levels in AML cell lines expressing these fusion proteins. An increased expression of UBCH8 upon HDACi-treatment is sufficient to trigger the proteasomal degradation of such low RLIM levels in hematopoietic cells via the E3 SIAH1. As expected from these results, I found that HDACi does not induce proteasomal degradation of the RLIM target HDAC2 in AML cells [122]. Hence, the molecular mechanism through which UBCH8 controls protein degradation depends on the abundance of its E3 ligase(s) and not on HDACi treatment *per se*. Based on these data we propose that a hierarchical ubiquitinylation system modulates E3 ligases via cross-regulation. This in turn leads to the degradation of different substrates including E3 enzymes themselves.

In addition, the UBCH8 knockdown experiments indicate that SIAH1 stability depends on its E2 conjugase UBCH8. Nevertheless, UBCH8 induction was not sufficient to cause SIAH1 degradation. This discrepancy could be explained by a model, in which the stability of SIAH1 depends on the abundance of substrates to which ubiquitin can be transferred. Such a model of an E3 auto-inactivation has been proposed before and is consistent with much higher expression rates of enzymatically inactive SIAH1 RING-finger mutants [175-177].

Ubiquitin ligases typically target multiple substrates and therefore proteins in addition to HDAC2, PML-RARα, AML-ETO or FLT3-ITD could be subject to HDACi-induced proteasomal degradation in a cell type-specific manner. Furthermore, RLIM and SIAH1 may not be the only E3 ligases downstream of the E2 conjugase UBCH8 and such pleiotropic effects of an E2 may explain why depletion of UBCH8 protein protects AML cells from HDACi-induced apoptosis. Such complex interplays between E2 conjugases and different E3 ligases may control many regulatory networks in health, disease and development.

8.8 Rationales for HDACi in combination therapies of AML

My results show that the well-tolerated drug VPA and the newly designed HDACi LBH589 induce the degradation of leukemia fusion proteins and mutant FLT3 receptor. These HDACi are therefore particularly suited for leukemia therapy. Although HDACi show promising results if used as single-agent anti-cancer drug, given the range of molecular and biological responses that these agents can elicit and minimal toxicity to normal cells, their use in combination with other agents could prove to be their most useful application [98]. In this context it has already been shown that HDACi can collaborate with diverse pharmacological and biological anti-cancer agents in mediating synergistic apoptosis in leukemic cells.

Pronounced benefits may be achieved when these HDACi are used in conjunction with drugs targeting DNA- replication, considering the interplay between DNA-methyltransferases and histone deacetylases in the epigenetic silencing of genes necessary for cellular differentiation and survival [178]. This combination therapy has been investigated in several clinical studies of patients with MDS and AML, which showed a partial or complete response to the treatment with the HDACi VPA or phenyl butyrate in combination with the DNA-demethylating agent 5-azacytidine [180-182].

Moreover, some HDACi were shown to inhibit the activity of HDAC6 which results in the hyperacetylation of the chaperone HSP90 and subsequent proteasomal degradation of client proteins, such as CML-associated BCR-ABL and FLT3 in AML. In this work here I could demonstrate that HDACi also induce the degradation of HSP90 client proteins independent of HSP90 acetylation via upregulation of limiting amounts of enzymes of the UPS. Nevertheless, enhanced efficacy in oncogenic protein degradation and synergy in apoptosis induction in AML cells has been reported when the HDACi LBH589 was used in combination with the HSP90 inhibitor 17-AAG [119]. One explanation for the synergistic effect could be the further increased release of client proteins from the chaperone-complex due to consequent inactivation of HSP90 by acetylation and direct chemical inhibition, followed by increased downstream protein degradation, very probably via the HDACi-regulated UBCH8-SIAH1-axis. These findings give raise to the consideration to apply 17-AAG and HDACi in combination as anti-cancer approach in future clinical studies.

Since FLT3 is the most commonly found mutated gene in AML, inhibition of mutated FLT3 kinases by specific inhibitors is considered as a promising strategy for the treatment of AML. A number of structurally different inhibitors have been developed, including LS104, CEP701, and PKC412 which are currently being tested in clinical trials [133,183,184]. However, only few patients achieved complete or long-term remissions upon kinase-inhibitor single-agent therapy [134]. The frequent development of secondary resistance to these small molecule

inhibitors due to acquired FLT3 mutations limits the application of these substances as monotherapy for leukemia [185,186]. Furthermore, combination therapy of standard chemotherapeutics with the kinase-inhibitor sorafenib was shown to be effective in reducing mutant clones in AML patients with FLT3 mutations but was not able to completely eradicate them. In the study presented here, I demonstrate that combinatorial treatment of AML cells

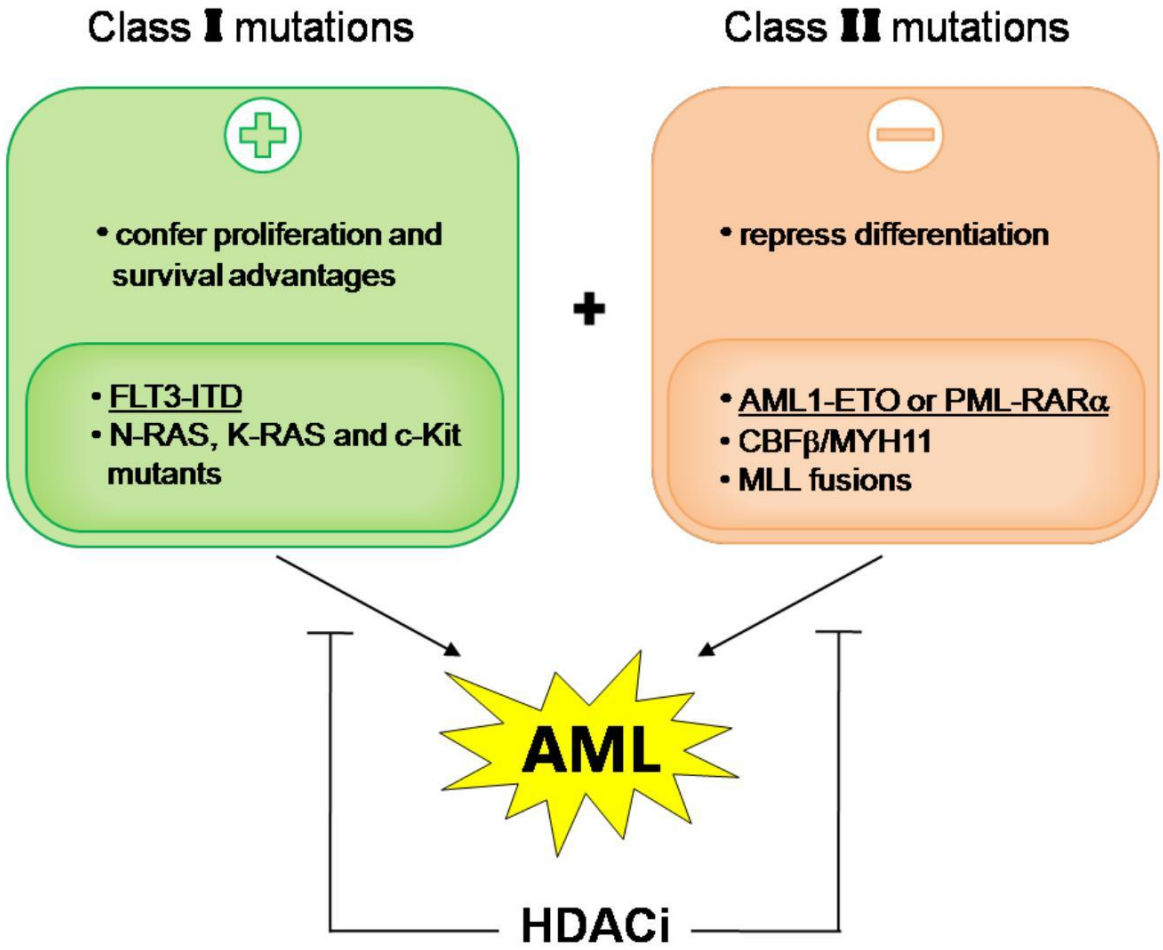


Fig. 6 HDACi as therapy for AML. It has been suggested that the development of acute myeloid leukemia is a consequence of the collaboration of two classes of mutations that independently disrupt two regulatory processes in hematopoietic cells. Class I mutations, exemplified by mutations in receptor tyrosine kinases or downstream signaling molecules such as FLT3-ITD, c-Kit, N-RAS or K-RAS, activate signaling pathways conferring proliferation and survival advantages. Class II mutations results in the loss-of-function of transcription factors that are important for normal hematopoietic cell differentiation and include chromosomal rearrangement products, such as PML-RARα, AML1-ETO, CBFβ/MYH11 or MLL fusions. The collaboration of both classes of mutations results in an acute leukemic phenotype characterized by abnormal proliferation and impaired differentiation of myeloid progenitor cells. Since multiple genetic lesions are required to induce AML, the combined targeting by therapeutic substances could restore cell differentiation and reverse the survival advantage. Therefore, HDACi-induced proteasomal degradation of these oncoproteins illustrates a promising anti-cancer strategy [38]. The figure is adapted from [179].

with HDACi and FLT3 inhibitor results in enhanced caspase activation and strong induction of apoptosis compared to single agent treatment. Thus, caspases can function in a feedforward mechanism, which promotes degradation and complete loss of FLT3-ITD

protein. Because targeting of FLT3-ITD stability appears as a crucial effect of HDACi, combining HDACi and FLT3-specific kinase inhibitors may gives beneficial effects *in vivo*. Such a strategy impairs both expression and activity of the oncoprotein. Moreover, combined or consecutive treatment of AML patients with FLT3-inhibitor and HDACi, primarily in combination with already approved chemotherapeutics, may overcome a possible resistance to kinase inhibitors and efficiently eliminate oncogenic FLT3-ITD signaling.

In the APL subtype of AML, in which aberrant recruitment of HDAC-containing repressor-complexes by PML-RARα contributes to cellular transformation, pharmacological doses of retinoic acid have been shown to mediate dissociation of the fusion protein from the corepressor-complex, thereby restoring normal gene expression. Thus, there is a logical consideration for using HDACi in combination with retinoic acid. The efficacy of this combination has been successfully demonstrated in *in vitro* and murine models, but needs to proof its benefit for AML patients in further clinical patient studies [98,187-189]. Moreover, based on the observation that one third of APL patients relapses or are refractory to retinoic acid alone or in combination with other chemotherapeutics, the HDACi-induced depletion of leukemia fusion proteins by proteasomal degradation might be a therapeutic option for these refractory patients. Congruent with this consideration, additional treatment with HDACi has been shown to overcome PML-RARα mutation-dependent resistance to retinoid acid therapy in multiple-relapsed APL patients [190,191].

Quite recently it has been shown, that corticosteroids diminished AML1-ETO protein in AML cells in a proteasome- and glucocorticoid receptor–dependent manner [192]. Based on my observations that AML1-ETO is degraded by UbcH8/SIAH1 and the finding that corticosteroids upregulate genes required for activity of the ubiquitin-proteasome pathway such as ubiquitin itself [193], the combinational treatment of AML patients with HDACi and corticosteroids could be a further therapeutical approach to efficiently target the AML1-ETO oncoprotein for proteasomal degradation and thereby improving disease prognosis.

The FLT3-ITD mutation is often found in conjunction with PML/RARα and AML1-ETO in AML patients and was shown to cooperate with these fusion oncoproteins in the pathogenesis of AML. Based on my findings, which reveal both classes of mutations as targets for the HDACi-induced proteasomal degradation, the application of HDACi as mono- or combinational anti-cancer therapy could be of pronounced benefit for AML patients, which were positively screened for the presence of either or both of these genetic lesions.

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10 Contribution to manuscripts

Manuscript 1: The manuscript was designed and written by myself as first author. The work was supervised by T.Heinzel and O.H. Krämer.

Manuscript 2: I contributed with quantitative Real-time PCR experiments, analyzing the effect of HDACi and MG132 on AML1-ETO and PML-RARα RNA-levels (*Figure 1C* and *data not shown*). I performed the DNA-laddering assays of HDACi-treated NB4 and Kasumi cell lines (illustrated in *Figure 2D* and *S3)*. I also analyzed the HDACi-regulated expression of UBCH8 and PML-RARα protein in NB4 cells (*Figure 2B)*. I established a DNA-transfection protocol for NB4 cells. Furthermore, I performed transient expression of ectopic UBCH8 in NB4 cells to analyze the effect of increased UBCH8 level on endogenous PML-RARα protein (*Figure 2C*). I expressed GST-UBCH8 in bacteria and I was responsible for the planning and realization of the GST-pull-down experiments, demonstrating interaction of UBCH8 with AML1-ETO and PML-RARα (*Figure 6B*). I established and performed transient co-expression of STAT5-RAR together with UBCH8 and SIAH1 in HEK293T cells, analyzing the stability of STAT5-RAR protein (*Figure S6*). Moreover, I did cell fractionation-assays of HDACi-treated NB4 cells to investigate cellular distribution of RAR, PML and PML-RARα protein. I additionally performed cell viability and Caspase-3/7 activation assays, to analyze the cytotoxic effects of HDACi on APL cells. The latter experiments were excluded from the manuscript due to space limitations. I contributed with proposals and ideas to the design of the manuscript, which was written by O.H. Krämer and T. Heinzel.

Manuscript 3: Kristin Pietschmann performed quantitative Real-time PCR experiments for analyzing the RNA levels of endogenous UBCH8 and FLT3/-ITD in diverse AML cell lines (*data not shown*). Immunofluorescence microscopy for detection of co-localization of UBCH8 with FLT3 or FLT3-ITD was carried out by O.H. Krämer and S. Knauer (*Supplemental Figure S3*). With exception of the above listed, all experiments were performed by myself. The experiments were designed and the manuscript was written by myself in collaboration with F.D. Böhmer, J. Müller, and O.H. Krämer.

11 Acknowledgement

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I specially would like to thank my parents and my girlfriend who always supported me and helped me to come this far.

12 Declaration of Independent Assignment

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of the Friedrich-Schiller University Jena that the submitted thesis was written only with the assistance and literature cited in the text.

People who assisted in the experiments, data analysis and writing of the manuscripts are listed as co-authors of the respective manuscripts. I was not assisted by a consultant for doctorate theses.

The thesis has not been previously submitted whether to the Friedrich-Schiller University Jena or to any other University.

Jena, June 2th 2010 _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _

Marc Buchwald

13 Curriculum Vitae

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1999 - 2004 **Diploma student**
Goethe University , Frankfurt am Main
Biology (Diploma)
Majors:
Cell and Development Biology
Biochemistry
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Diploma thesis:
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14 Publications

2010 **Buchwald, M.**, Pietschmann, K., Müller, J. P., Böhmer, F. D., Heinzel, T., Krämer, O. H.
Ubiquitin conjugase UBCH8 targets active FMS-like tyrosine kinase 3 for proteasomal degradation.
Leukemia 2010 May 27 [Epub ahead of print]

2009 **Buchwald, M.**, Krämer, O.H., Heinzel, T.
HDACi –Targets beyond chromatin.
Cancer Lett. 2009 Aug 8; 280(2):160-7. Epub 2009 Apr 1

2008 Kramer, O.H., Müller, S., **Buchwald, M.**, Reichardt, S., Heinzel, T.
Mechanism for ubiquitylation of the leukemia fusion proteins AML1-ETO and PML-RARalpha.
FASEB J. 2008 May; 22(5):1369-79. Epub 2007 Dec 11

Further manuscripts which are not included in this cumulative dissertation:

Müller, S., **Buchwald, M.**, Greiner, G., Heinzel, T., Krämer, O.H.
The ubiquitinylating enzymes UBCH8 and SIAH catalyze proteasomal turnover of the tyrosine kinases JAK2 and TYK2.
Submitted for publication at the journal *Cancer Research*

Pietschmann, K., **Buchwald, M.**, Müller, S., Knauer, S., Kögl, M., Heinzel, T., Krämer, O.H.
Regulation of ubiquitinylating enzymes in PML-RARα-positive leukemia cells sensitive to histone deacetylase inhibitors and all-trans retinoic acid.
Submitted for publication at the journal *Biochemical Pharmacology*

Buchwald, M., Pietschmann, K., Mahajan, N., Heinzel, T., Krämer, O.H.
Degradation of the non-receptor tyrosine kinase ACK1 is mediated by association with SIAH ubiquitin ligases.
In preparation for publication

Gross, A., Bartsch, I., **Buchwald, M.**, Brill, B., Heinzel, T.
The combination of VPA and dexamethasone leads to synergistic induction of apoptosis and improves disease outcome in an ALL mouse model.
In preparation for publication