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Chemical Cryptology of Cancer's Histone Code

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Somatic mutations in non-Hodgkin's lymphoma frequently activate EZH2, a protein methyltransferase responsible for H3K27 trimethylation. In this issue of *Chemistry and Biology*, Bradley and coworkers describe a new set of EZH2 inhibitors amenable to probing the targetable role of H3K27 trimethylation in lymphoma.

The human genome provides a common text capable of encoding the hundreds of different cell types found in the human body. The malleable nature of genomic information arises from the careful orchestration of gene expression. Higher eukaryotes regulate gene expression by housing genes within chromatin, the nucleoprotein complex composed of histones and DNA. Enzymes known as chromatin modifiers add and remove covalent modifications to histones that correlate with changes in genomic accessibility, providing a context that can either permit or oppose transcription. Due to their central role in gene expression, mutation or dysregulation of chromatin modifiers can lead to the rapid rewiring of cell function, facilitating transformation and tumorigenesis.

One specific chromatin modifier subject to oncogenic mutation in many cancers is the histone methyltransferase EZH2. EZH2 is the catalytic domain of the polycomb repressive complex 2 (PRC2), an evolutionarily conserved multiprotein assembly responsible for keeping large swaths of the genome in a transcriptionally "off" state after differentiation (Di Croce and Helin, 2013). Biochemically, EZH2 is most proficient at catalyzing histone H3 lysine 27 (H3K27) monomethylation, more slowly catalyzes dimethylation, and only weakly promotes the trimethylated state. However, in a wide variety of cancers—most notably non-Hodgkin's lymphoma—EZH2 undergoes recurrent gain-of-function mutations in its catalytic domain that alter this substrate specificity, allowing it to establish H3K27 trimethylation (H3K27me3) at a greatly increased rate (Figure 1). By changing the substrate specificity of EZH2, these mutations harden a normally plastic epigenome, constitutively silencing genes that would ordinarily trigger senescence. This unique gain-of-function mechanism has spurred efforts to develop small molecule inhibitors of mutant EZH2, which could provide powerful tools to understand PRC2 biology and assess the therapeutic potential of drugging the mutant gene.

In late 2012, groups from GlaxoSmithKline, Epizyme, and Novartis published concurrent reports on the discovery of potent small molecule inhibitors of mutant EZH2 (Knutson et al., 2012; McCabe et al., 2012; Qi et al., 2012). Interestingly, each of these inhibitors was based on a 4,6-dimethyl 3-amidomethyl-pyridone core. These molecules inhibited EZH2-catalyed H3K27me3 in cells, promoted transcriptional de-repression of select genes, and displayed potent antiproliferative effects in cell and animal models of EZH2 mutant lymphoma. In this issue of *Chemistry and Biology*, Bradley et al. (2014) build on their previous efforts (Garapaty-Rao et al., 2013) and report two new pyridone EZH2 inhibitors, CPI-360 and CPI-169. A key early step in the characterization of CPI-360 was the finding that it inhibits mutant EZH2 ~50-fold more potently than EZH1, another PRC2-associated histone methyltransferase. This is nontrivial, because EZH1's catalytic domain is 96% identical to EZH2. Interestingly, several studies have suggested EZH1-associated PRC2 is responsible for cellular H3K27 monomethylation, while EZH2-associated PRC2 is necessary for di- and trimethylation. Identifying small molecules that selectively inhibit EZH2 enables dissection of the role these distinct modifications play in disease phenotypes. Consistent with studies of structurally related pyridones (Knutson et al., 2012), CPI-360 suppressed H3K27me2/me3 levels in lymphoma cells bearing an EZH2 mutation, but did not inhibit H3K27me1. Together with the selective biochemical inhibition shown for EZH2 over EZH1, these findings corroborate recent genetic data suggesting that EZH2 controls H3K27me3, while EZH1 may control bulk H3K27me1 levels (Hidalgo et al., 2012).

One common challenge in drug development is determining that a biochemical inhibitor actually engages its target in living cells. Bradley et al. (2014) address this issue using a label-free technology known as a cellular thermal shift assay (CETSA) (Martinez Molina et al., 2013). This method allows them to not only verify interaction of CPI-360/EZH2, but also to measure the duration of CPI-360 occupancy following drug removal. The verification of the small molecule-EZH2 interaction at an early stage provided a solid foundation from which to launch mechanistic studies. When compared with previously discovered pyridones (Figure 1, bottom) CPI-360 showed a similar ability to de-repress EZH2-silenced gene expression, arguing strongly for a conserved mechanism of action. To augment these cellular studies, Bradley et al. (2014) developed a structural analog, CPI-169, with pharmacokinetic properties amenable to animal studies. This molecule induced regression of EZH2 mutant tumor xenografts while affecting H3K27me3, but not H3K27me1, levels. Seeking to expand the applications of this molecule, they also tested its activity against a panel of 43 lymphoma cell lines comprising a range of EZH2 genotypes. Surprisingly, these studies found the effects of EZH2 inhibition were not restricted to EZH2 mutant lymphomas. This is not completely unprecedented, as previous studies of an EZH2 inhibitor GSK-126 had shown similar broad-spectrum antilymphoma activity at elevated concentrations (McCabe et al., 2012). In all cases, phenotypic response was preceded by potent suppression of H3K27me3, implying an EZH2-mediated mechanism.

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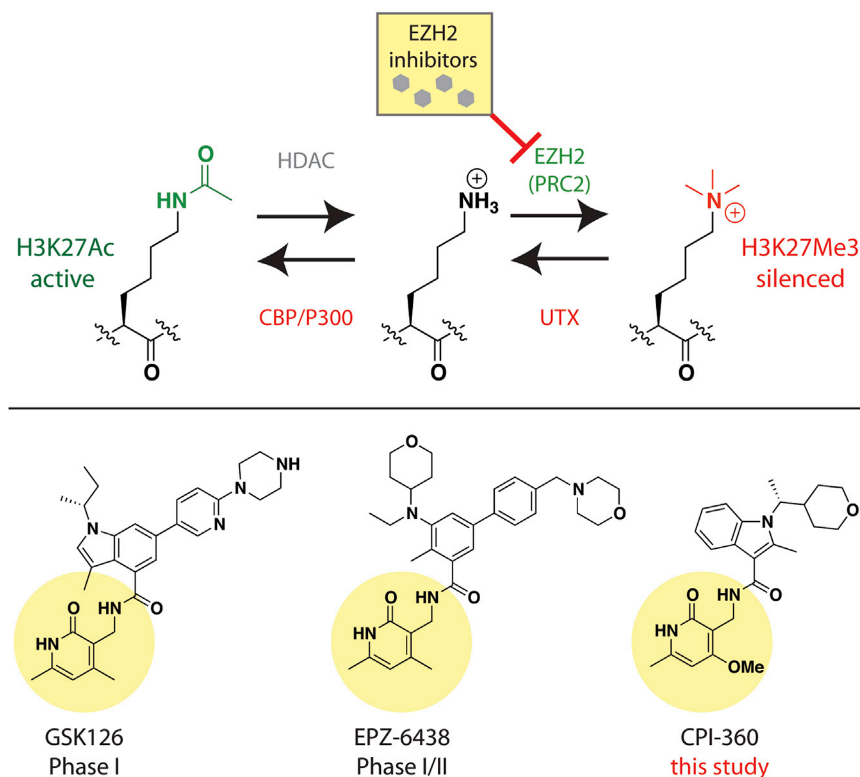

Figure 1. Histone H3K27 Modification as a Central Hub for Oncogenic Signaling and Therapeutic Intervention in Lymphoma

Activating (green) and inactivating (red) mutations in the histone lysine modification machinery drive the equilibrium of H3K27 modification toward trimethylation, a state associated with transcriptional repression. Transcriptional repression can be relieved by targeting the methyltransferase activity of EZH2, a member of polycomb repressive complex 2 (PRC2). Bottom: structurally-related 3-amidomethyl pyridone inhibitors of EZH2. All three inhibitors inhibit EZH2-containing PRC2 selectively compared to EZH1-containing complexes, resulting in specific effects on H3K27me2/3 in cells.

Although this suggests overactive wild-type EZH2 may drive growth in these cell lines, phenotypic responses did not correlate with H3K27me3 or EZH2 protein levels, arguing against this simple interpretation. Bradley et al. (2014) also showed in some cell lines their EZH2 inhibitor synergized with a selective inhibitor of BCL-2, an antiapoptotic protein whose expression is commonly amplified in non-Hodgkin's lymphoma. Exploiting such synergies may provide a way to expand the reach of EZH2-targeted therapies even further.

This latest chapter in the EZH2 inhibitor story contains illustrative lessons for chromatin chemical biology. For example, while EZH2 mutant lymphomas remain the best starting point for clinical applications of EZH2 inhibitors, the finding that the therapeutic effects of these molecules outstrip their initially defined genetic context makes the case that chemical probes for chromatin modifiers are likely to have unanticipated applications in disease research. This is because, despite much work, our knowledge of how different signaling cascades propagate through chromatin remains far from perfect. Chromatin modifiers may represent critical dependencies for signal transduction, regardless of mutational status. For example, loss-of-function mutations in the SWI/SNF chromatin remodeling complex cause malignant rhabdoid tumors to become reliant on EZH2 activity for growth (Knutson et al., 2013). Small molecules such as CPI-169 provide powerful tools for defining these dependencies and assessing their targetable role in therapy.

Synergy studies such as those explored by Bradley et al. (2014) likely represent a major focus of future cancer research. Just as cancer requires multiple cooperating mutations to cross the proliferation threshold, multiple fronts of attack may be necessary to stably repress oncogenic phenotypes. Interestingly, in addition to EZH2 mutations, many diffuse large B cell lymphoma (DLBCL) patient samples demonstrate loss-of-function mutations in the chromatin modifiers *EP300*, *CBP*, and *UTX*, all of which are involved in the modification of H3K27 (Shaknovich and Melnick, 2011). *EP300/CBP* are H3K27 acetyltransferases whose loss would potentially clear the road for H3K27me3 establishment by mutant PRC2. *UTX* encodes a demethylase that normally removes H3K27me3 and is found in a subset of DLBCL cells known to be sensitive to EZH2 inhibition. Defining how cooperating epi-mutations impact the efficacy of EZH2 inhibitors may help maximize the effects of these agents. The flipside of this is that EZH2 inhibitors also have the potential to cooperate with existing biology to exert deleterious phenotypes. For example, loss of SUZ12, a noncatalytic member of the PRC complex, appears to cooperate with Ras-activating *NF1* mutations to facilitate growth in familial cancers, glioblastomas, neuroblastomas, and lung cancers (De Raedt et al., 2014). Thus, it has been proposed that screening patients for *NF1* mutations prior to administering EZH2 inhibitors may be a sensible step to avoid activating latent protumorigenic *NF1* mutations. Understanding the balance of positive and negative synergies will be important to future applications of epigenetic-targeted therapies.

One final question arising from these studies is how precisely EZH2 inhibitors exert their effects on gene expression. Changes in histone modifications are associated with altered gene expression, but evidence for the direct causative nature of these changes can be difficult to come by. In line with this, expression profiling has so far been unable to identify a common set of genes activated by EZH2 inhibitors that underlie their antiproliferative effects (McCabe et al., 2012). This may be partially explained by the recent proposal that PRC2 is required for the maintenance, rather than the initiation, of transcriptionally repressive chromatin states (Riising et al., 2014). Notably, the data of Bradley et al. (2014) indicate that H3K27me3 removal and subsequent gene activation require cell division, consistent with EZH2′s role in preserving, rather than directly driving, transcriptional repression (Bradley et al., 2014). While the role of chromatin modifications as "cause" or "cog" in gene expression has been hotly debated, both functions

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present opportunities for drug discovery. The ability of enzymes such as EZH2 to reflect and amplify the underlying genomic causes of disease suggests chromatin modifiers will remain important therapeutic targets for the foreseeable future.

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Now Playing: Farnesol in the Biofilm

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The evolutionary pathway of specialized metabolism often takes unexpected, perplexing turns. In this issue of *Chemistry & Biology*, Feng and coworkers provide evidence for a unique phosphatase whose enzymatic product plays a critical role in biofilm formation in *Bacillus subtilis*.

Microbial biofilms have intrigued the scientific community for centuries. Their peculiarities have been influential in prompting some of the earliest microscopic studies as well as the more modern, cutting edge molecular analyses (Høiby, 2014). Although not given full appreciation until the late 20th century, biofilms have forged an expanding scientific frontier that spans disciplines ranging from healthcare to agriculture (Lappin-Scott et al., 2014). Their effects, good and bad, have continued to draw attention from researchers in many fields who hope to identify and/or exploit interesting and unique aspects of this "slimy" part of the microbial world.

Despite the consensus that most microbes generate biofilms at some point during their lifecycle, considerable variations exist in the mechanisms through which biofilm formation is supported, even among the most extensively studied bacterial systems (López et al., 2010). One of these systems, the Gram-positive bacterium *Bacillus subtilis*, utilizes a common biofilm theme whereby a polysaccharide matrix infused with proteins anchors cells to one another and a surface (Vlamakis et al., 2013). While this and other areas of biofilm formation tend to follow common trends, other aspects of the process seem to be species specific, such as the signaling molecules that elicit the biofilm response (López et al., 2010).

In this issue of *Chemistry & Biology*, Feng et al. (2014) add a very interesting new layer to the requirements for biofilm assembly in *B. subtilis*. These authors provide evidence that not only does the *B. subtilis* squalene synthase-like enzyme (YisP) catalyze the formation of farnesol (FOH) from farnesyl diphosphate (FPP), but also that its product plays an important role in biofilm assembly. Earlier work by López and Kolter (2010) originally proposed YisP to be a squalene synthase without truly identifying its reaction product. Additionally, López and Kolter (2010) demonstrated that when YisP was knocked out in *B. subtilis*, the ability to assemble biofilm was lost and therefore surmised that squalene played an important role in *B. subtilis* biofilm formation. However, close inspection of the protein sequence by Hu et al. (2013) during the elucidation of the crystal structure for YisP revealed that one of the canonical aspartate-rich motifs found in all squalene synthases was out of register in YisP.

Thus, with the YisP crystal structures in hand (additional YisP structures were solved for this work) and the discrepancy found by Hu et al. (2013) in mind, the current authors calculated the volume of the YisP active site pocket and compared it to the active site pocket volume of the *Staphylococcus aureus* dehydrosqualene synthase (CrtM) and human squalene synthase (HsSS) enzymes. In comparing these measurements, Feng et al. (2014) realized that the YisP active site was likely

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