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Histone H3.3 G34 mutations alter histone H3K36 and H3K27 methylation *in cis*

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High-throughput sequencing of numerous patient samples has identified a myriad of frequent mutations of epigenetic regulators in human cancers, including recently discovered mutations in histone-encoding genes. Histone H3 genes, particularly *H3F3A* and *H3F3B*, the genes encoding the variant histone H3.3, are mutated at high frequency in pediatric brain and bone malignancies. These mutations are missense mutations that affect three amino acids on the N-terminus of H3.3, K27, G34 and K36. K27 and K36 are mutated to methionine (M) in pediatric diffuse intrinsic pontine glioma (DIPG) and chondroblastoma patients respectively [1, 2], and G34 mutated to various amino acids, including arginine (R) and valine (V) in pediatric glioblastoma (GBM) and tryptophan (W) and leucine (L) in giant cell tumors of the bone (GCTB) [3]. Several exciting studies have begun to uncover the molecular mechanisms by which the H3K27M and H3K36M mutations in altering chromatin dynamics and promoting tumorigenesis. Elegant biochemical studies revealed that the H3K27M mutant has a higher affinity for EZH2, the H3K27-specific lysine methyltransferase (KMT), than K27, thus the K27M mutant competes for binding to EZH2 and thus effectively sequesters EZH2 and the PRC2 complex to prevent it from further propagating the repressive H3K27 methylation mark [4, 5]. Similarly, the K36M mutant inhibits KMTs specific to H3K36, including NSD1, NSD2 and SETD2 *in vitro* [6], and reduces global H3K36 methylation *in vivo* [7, 8]. All together, these results suggest that lysine-to-methionine mutations share a common mechanism of inhibiting methylation pathways at the genome-wide level to promote tumorigenesis.

Unlike the K27M and K36M mutations, the mechanism underlying the molecular and cellular changes due to H3G34 alterations is yet to be determined. H3G34 itself is not post-translationally modified; however, G34 lies in close proximity to K36, which undergoes methylation during transcriptional elongation. Therefore, it is conceivable that G34 mutations may impact the accessibility of K36 to its KMTs, thus altering H3K36

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methylation on the same histone tail (*in cis*). Indeed, G34R and G34V-containing nucleosomes show reduced methylation of H3K36 by SETD2 *in vitro* [4, 5], suggesting that the G34R/V mutation may inhibit gene expression by attenuating SETD2 function in transcriptional elongation. However, in contrast to the *in vitro* data, in GBM cells G34 mutations lead to increased H3K36me3 levels and RNA Pol II occupancy on several key oncogenes including MYCN, thus resulting in an elevated expression of these potent tumorigenic initiators [9].

To solve the discrepancies between these *in vitro* and *in vivo* data, we sought to imitate disease-specific mutations of H3.3G34 and determine their effect on H3K36 and H3K27 methylation in culture cells and *in vitro* biochemical assays. We first generated HeLa cell lines stably expressing either wild-type (WT) H3.3 or the G34L or G34W mutants, the mutations frequently occur in GCBT patients. We also established an H3.3K36M stable cell line as a positive control for global reduction of histone H3K36 methylation. We tagged the ectopic H3.3 proteins with a C-terminal 3xFlag tag to distinguish them from the endogenous WT counterpart; and we selected the stable cells with the ectopic H3.3 proteins expressed at a level comparable to the endogenous H3.3 (Fig. 1a). Western blot analysis of the whole cell extract revealed that as expected, ectopic K36M leads to global reduction of H3K36 methylation, whereas G34L/W mutations have no effect on global levels of methylation on H3K36, H3K27, or other major methylation sites on endogenous histone H3, which include both H3.3 and the canonical H3.1/H3.2 proteins (Fig. 1a, left panels). However, long exposures of the blots revealed that methylation on the ectopic Flag-H3.3 proteins are affected by G34 mutations: with di- and trimethylation on H3K36 reduced whereas H3K27me3 increased in the G34L and G34W mutated H3.3 compared to the WT H3.3 (Fig. 1a, right panels). Methylation on H3K4 and H3K79 is largely not affected by G34 mutations; but the repressive H3K9me3 is slightly higher in the G34 mutants. Concomitant with the increase of the repressive H3K27me3, the active mark H3K27ac is slightly reduced in the G34 mutants.

To further evaluate the changes of histone modifications at a higher resolution, we carried out chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) to determine genome-wide occupancies of H3K36me3, H3K36me2, H3K27me3 and H3K27ac. To specifically identify G34 mutants' binding sites, we performed ChIP-seq of the ectopic Flag-H3.3 using the M2 anti-Flag antibody. We identified 60,006 Flag-H3.3 bound peaks in the WT Flag-H3.3 expressing cells; and H3.3 occupancy on these regions was further corroborated by ChIP-seq experiments using the H3.3-specific antibody, which also identified additional 80,112 peaks that have no Flag signals (Fig. 1b and Supplementary Table 1). Because the G34 mutations only affect modifications on H3K36 and H3K27 on the same histone tail, we focused on the overlapped Flag-H3.3 peaks for further analysis. The Flag-H3.3 peaks share significant co-occupancy with active histone marks (36% with H3K36me2, 41% with H3K36me3 and 56% with H3K27ac), but not with the repressive mark H3K27me3 (Supplementary Fig. 1). The ChIP-seq peaks of H3.3, Flag-H3.3 and the histone modifications we've tested are largely consistent among the WT H3.3 and G34 mutants-expressing cells (Supplementary Fig. 2 and Supplementary Table 1), but the binding strength of histone modifications is different between cell lines. Compared to the WT H3.3-expressing cells, the levels of H3K36me2 and H3K36me3 are reduced, whereas H3K27me3

increased on the Flag-H3.3 binding sites in the G34 mutated cells (Fig. 1c, d). The increase of H3K27me3 is more obvious in G34L cells than G34W cells; and changes in H3K27ac levels are relatively subtle. Together, these results indicate that mutations of H3.3G34 affect methylation on H3K36 and H3K27 *in cis*.

To determine whether the effects on H3K36 and H3K27 methylation by G34 mutations are direct or indirect, we carried out *in vitro* methylation assays using recombinant histone proteins and purified SETD2 SET domain, p300 HAT domain and full-length EZH2 in reconstituted PRC2 complex. Consistent with previous *in vitro* studies using the G34R/V mutants [4, 5], G34L/W mutants abolish SETD2 methylation of H3K36. In contrast, the enzymatic activities of both EZH2 and p300 on H3K27 are not affected by G34 mutations (Supplementary Fig. 3), suggesting that the changes in H3K27me3 in cells are secondary to changes in H3K36 methylation.

Methylation on histones functions as molecular signals to reader proteins that recognize methylation in site-specific manner. To determine whether changes in H3K36 and H3K27 methylation affect the binding of reader proteins, we performed Flag-IP in the stable cells to purify nucleosomes containing WT or mutant H3.3, which were then subjected to mass spectrometric analysis to identify proteins associated with these nucleosomes. Consistent with changes in H3K27me3 and H3K36me3 levels, we found increased binding of PRC2 (EZH2, SUZ12 and EED) and PRC1 complex components (CBX8 and RING2) and reduced binding of H3.3K36me3 reader such as ZMYND11 to the G34 mutants (Fig. 1e and Supplementary Table 2). Interestingly, to our surprise, the H3K36 KMTs (NSD1, NSD2 and NSD3), which are defective in K36 methylation on the G34 mutant histones, bind stronger to the G34 mutants than the WT nucleosomes.

In summary, our study revealed that histone H3.3 G34 mutations alter histone K36 and K27 methylation *in cis*, and affect the binding of readers specific to K36 or K27 methylation. Interestingly, we found that G34 mutations also inhibit the H3.3 chaperone HIRA binding to histones. The HIRA complex is known to bind to the A₈₇AIG₉₀ segment that is far from the N-terminal G34 region [10]. It is worth noting that almost all the H3.3 G34R/V GBM tumors are also mutated in *ATRX* or *DAXX*, another H3.3-specific chaperone [1]. It is interestingly to determine in the future how G34 onco-mutations affect the function of H3.3 chaperones and possibly higher order chromatin structure.

Supplementary Material

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Acknowledgements.

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Figure 1. H3.3G34L/W mutations affect H3K36me2/3 and H3K27me3 level in cis.

a, Western blots of whole-cell extracts from HeLa cells expressing WT H3.3, G34L, G34W or K36M. Cells expressing an empty vector (Ctrl) or H3.1 are used as controls. The left panels show the short exposure and the right panels show to long exposure. Arrows indicate the Flag-tagged ectopic H3 proteins and the asterisks indicate the endogenous H3 proteins.

b, Heatmaps profiles showing the overlap of Flag- and H3.3 ChIP-seq peaks and non-Flag H3.3 ChIP-seq peaks in HeLa cells expressing WT H3.3. Full list of the ChIP-seq peaks is included in Supplementary Table 1.

c, Heatmap profiles of H3K36me2, H3K36me3, H3K27ac, and H3K27me3 levels. The heatmaps show the levels of these marks across the distance to the Flag-H3.3 Peak Center (kb). The color scale ranges from -5 (blue) to 5 (red).

H3K27ac and H3K27me3 peaks in cells expressing WT H3.3 and G34L (top panels), or WT H3.3 and G34W (bottom panels), and the difference between them (, G34L/W-WT). Gray indicates a decrease and orange indicates an increase in G34L/W cells compared to the WT H3.3 cells. **d**, Genome browser views of the ChIP-seq peaks on the indicated genes in cells as in (c). **e**, Heatmap of the indicated proteins differentially bound to nucleosomes purified from HeLa cells expressing WT or G34L/W-containing nucleosomes. The colors are mapped to the log2 value of fold change in H3.3G34L/W samples compared to WT H3.3 samples. Full list of proteins differentially associated with the G34L or G34W nucleosomes is included in Supplementary Table 2.