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The cancer COMPASS: navigating the functions of MLL complexes in cancer

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The mixed-lineage leukemia family of histone methyltransferases (*MLL1−4*, or *KMT2A−D*) were previously linked to cancer through the founding member, *MLL1/KMT2A*, which is often involved in translocation-associated gene fusion events in childhood leukemias. However, in recent years, a multitude of tumor exome sequencing studies have revealed that orthologues *MLL3/KMT2C* and *MLL2/KMT2D* are mutated in a significant percentage of a large variety of malignancies, particularly solid tumors. These unexpected findings necessitate a deeper inspection into the activities and functional differences between the *MLL/KMT2* family members. This review provides an overview of this protein family and its relation to cancers, focusing on the recent links between MLL3/KMT2C and MLL2/4/KMT2D and their potential roles as tumor suppressors in an assortment of cell types.

Keywords Epigenetics, enhancers, chromatin, lysine methyltransferase, COMPASS complex

Chromatin and epigenetic modifiers in cancer

During the processes of organismal development and cellular differentiation, somatic cells receive both internal and external cues that lead to the reprogramming of the genome with consequent coordinated changes in gene expression. When these signals are imposed on stem cells, the cells respond through a series of developmental steps to become progenitor cells that will continue on a path of lineage commitment and become fully differentiated. Disruptions of these pathways can lead to a block in differentiation and a concomitant loss of regulatory controls that normally impose constraints on growth. Over the past two decades, many oncogenes and tumor suppressor genes have been implicated in growth and differentiation control. Among these genes are regulators that control transcription initiation and elongation as well as DNA repair processes, mainly through effects on chromatin structure, either by enzymatically adding or removing covalent modifications on histones, through displacement of histones within chromatin or by facilitating interactions between transcription regulatory elements.

Chromatin is the main repository of genetic information in the eukaryotic nucleus, comprising DNA compacted into structures called *nucleosomes* by a set of small basic histones and nonhistone chromosomal proteins. As the basic building block of chromatin and the primary scaffold for chromosome protection and compaction, each nucleosome is made up of 147 bp of DNA wrapped around a core set of eight histones, including two each of histones H2A, H2B, H3, and H4. Nucleosomes also act as barriers to restrict access to DNA and impede translocation of replication and transcription complexes. Controlling access to genetic information embedded in the genome is essential for proper development and homeostasis; thus, it is of great importance to understand the cellular processes that guide proper gene regulation in vivo. Chromatin remodeling, in a broad sense, refers to any modification of the nucleosome that allows for changes in gene expression through effects on the structure of the chromatin by posttranslational covalent modifications of the histone proteins, disruptions in the DNA−histone contacts, or removal or replacement of histones with variant forms. Simple addition or removal of specific histone "marks" was elegantly described in 2000 by Strahl and Allis as the "histone code hypothesis," where specific covalent modifications of several histone N-terminal tails were associated with transcriptional outcome (1). Our current view of chromatin regulation has expanded greatly to include variant histones, the influence of larger chromatin domains,

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intermediate states of chromatin control of gene expression ("poised" enhancers and promoters), alternative transcripts, trans-tail regulation of histones, sequential chromatin remodeling, and histone modifications.

The unstructured N-terminal tail of histone 3 (H3) is the site of many posttranslational covalent modifications that influence the expression of genes and is perhaps the best studied (2). The methylation of H3 at the lysine 4 position (H3K4) is generally recognized as a mark of transcriptional activity at enhancers, at promoters, and throughout the lengths of coding genes. Lysine methylation does not appear to alter chromatin structure directly or alter residue charge. However, the addition of methyl residues to the terminal nitrogen atom of H3K4 serves to recruit the binding of transcription factors and coactivators, while blocking associations with proteins that repress transcription. Lysine residues can be mono-, di-, or trimethylated, and although all forms are broadly associated with gene expression, each type tends to localize to specific genomic regions. H3K4me3 is enriched at the promoter and transcription start sites (TSS) of active genes, with lower levels throughout the gene body. Less is known about the distinct localization of H3K4me2 modifications; although, they are commonly observed throughout many regions of active genes where they are enriched in the gene body downstream of the TSS, particularly at the 5′ end of the transcribed regions, and within regions near transcription factor binding sites (3). The significance of H3K4me2 is uncertain and it has been suggested some lysine di-methylation sites may represent incomplete trimethylation. Mono-methylation of H3K4 (H3K4me1) was initially found to be enriched within chromatin near the 3′ region of genes. However, whole genome analyses have revealed distinct H3K4me1 enrichment within intergenic regions. The sites of H3K4me1 enrichment are known as enhancers, comprising short stretches of 200−500 bp of DNA that harbor recognition sites for transcription factors and their associated coactivators that drive transcription from the promoters of nearby gene(s). The enrichment of H3K4me1 in the absence of H3K4me3 is now considered a principal mark of active enhancers (4).

Recent high-throughput sequencing of genomes, epigenomes, and transcriptomes and various other mass data screenings have provided a vast new reservoir of data linking cancers to dysregulated functions of genes that encode for epigenetic modifiers. These studies have not only confirmed the presence and central importance of well-described oncogenic and tumor-suppressor mutations in cancer development, but they have also revealed startling and often unanticipated correlations between mutations in genes encoding chromatin regulators and numerous cancers. Frequent among the more recently identified genes harboring cancer-associated mutations are those involved in epigenetic gene regulation, including DNA methylation and histone covalent modification (e.g., methylation, acetylation, phosphorylation) that provide epigenetic memory. Modifications of histone residues, particularly those found in nucleosomes within enhancer and promoter regulatory regions of genes, are believed to regulate gene expression, by being both stable enough to retain transcription status information through multiple cell divisions and dynamic enough to respond to developmental and hormonal signals to ultimately affect cell fate or activity (4).

MLL/KMT2 family of histone lysine methyltransferases

One class of histone-modifier genes implicated in an increasingly large number and variety of cancers is the MLL/Set1 class of lysine methyltransferases (KMTs) (5,6). The Set1 family of KMTs are embedded in large multimeric complexes referred to as Set1/COMPASS-like complexes (7), which often serve as coactivators of nuclear receptor transcription factors. The COMPASS (COMplex of Proteins ASsociated with Set1) family of KMTs carries out the methylation of histone H3 lysine 4 (H3K4) (8). Whereas there is a single yeast COMPASS complex, in *Drosophila* there are three and in vertebrates six closely related complexes, often referred to as COMPASS or COMPASS-like complexes (9). The related and conserved COMPASS complexes share a common set of four core subunits and a unique set of complex-specific subunits with important regulatory functions, for a total of 7−10 distinct proteins within the complexes (Figure 1) (10). The Set1/COMPASS complex or complexes carry out the bulk of H3K4 di- and trimethylation (11), whereas the COMPASS-like complexes are associated with development-specific genes and principally contribute to mono- and dimethylation of H3K4 (12−14). In mammals, the COMPASS-like complex KMTs are members of the mixed-lineage leukemia (MLL) family of proteins that contain the KMTase (also known as SET) domain, clustered chromatin-binding PHD zinc fingers, FY-rich regions, and domains involved in DNA recognition (HMG, AT-hooks, CXXC) (Figure 2). These MLL proteins are large (2700−5800 amino acids) and are collectively referred to as the KMT2 family. The MLL1/KMT2A and MLL4/KMT2B subfamily proteins include several conserved domains (bromodomain, CXXC, AT-hook) and they are cleaved by specific proteases to produce separate N- and C-terminal portions. The MLL2/KMT2D and MLL3/KMT2C proteins contain a second PHD finger cluster and interact directly with nuclear receptors through multiple conserved LXXLL motifs.

The KMT2A/B subfamily comprises human paralogues MLL1/KMT2A and MLL4/KMT2B and their *Drosophila* orthologue, Trithorax (Trx). The mammalian KMT2A/B family is required for homeotic (*HOX*) gene transcription (15−17), possibly through regulation of bivalent promoters containing both active (H3K4me3) and inactive (H3K27me3) histone marks often associated with lineage-specific gene expression programs (13,18,19). Members of the KMT2C/D subfamily are orthologues of *Drosophila* trr (Trithorax-related), including the homologous and perhaps functionally redundant MLL2/ALR/KMT2D (HGNC-7133) and MLL3/HALR/KMT2C (HGNC-13726). The KMT2C/D subfamily catalyzes the monomethylation of H3K4 in collaboration with hormone receptors and transcription factors involved in developmental signaling (20,21), and are generally thought to be responsible for most H3K4 monomethylation at transcription enhancer regions (12,13,21,22). To circumvent the confusion regarding the nomenclature of the MLL2/ALR protein (variably referred to as ALR, MLL2, or MLL4 in publications), for simplicity we will refer to the mammalian protein as MLL2/KMT2D. In humans, the Set1A and Set1B COMPASS complexes (Figure 1) are responsible for the vast majority of H3K4 di- and trimethylation. They appear to work in a nonredundant manner, localizing to distinct regions within

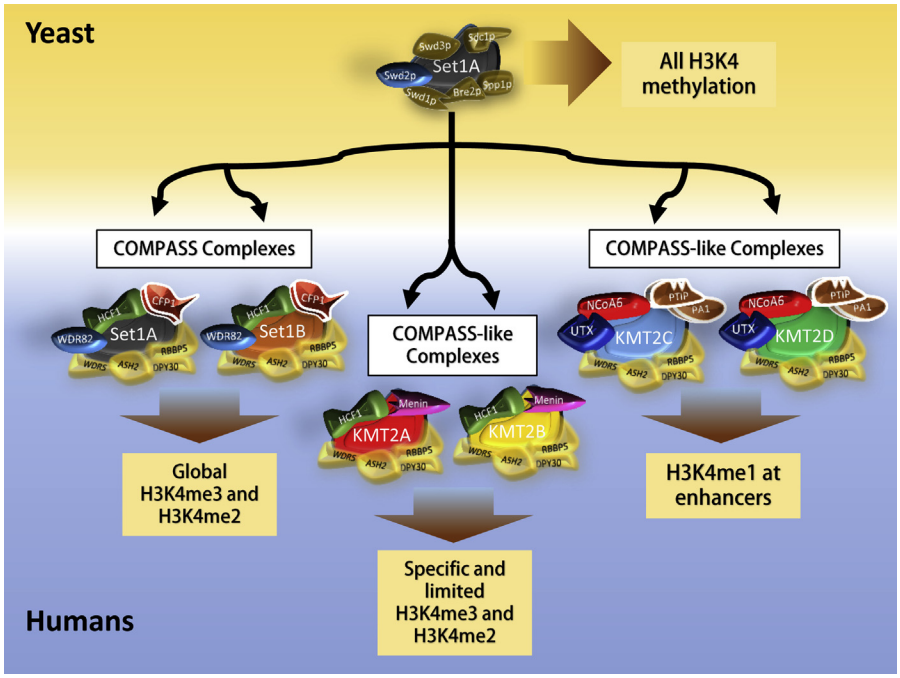

Figure 1 The orthology of COMPASS and COMPASS-like complexes in yeast and humans and their functions. Vertebrates contain six related complexes consisting of one Set1-paralogue methyltransferase unique to each complex, a common set of four core subunits, and complex-specific proteins for additional activity and/or targeting. Each complex harbors 7–10 distinct subunits.

A

	Synonyms	*Chromosomal Location*	*HGNC ID*	*Peptide Length*
KMT2A	***MLL***, *MLL1, HRX*	11q23	HGNC:7132	3969 aa
KMT2B	***MLL4***, *MLL2, HRX2*	19q13.1	HGNC:15840	2715 aa
KMT2C	***MLL3***, *HALR*	7q36.1	HGNC:13726	4911 aa
KMT2D	***MLL2***, *MLL4, ALR*	12q13.12	HGNC:7133	5537 aa

B

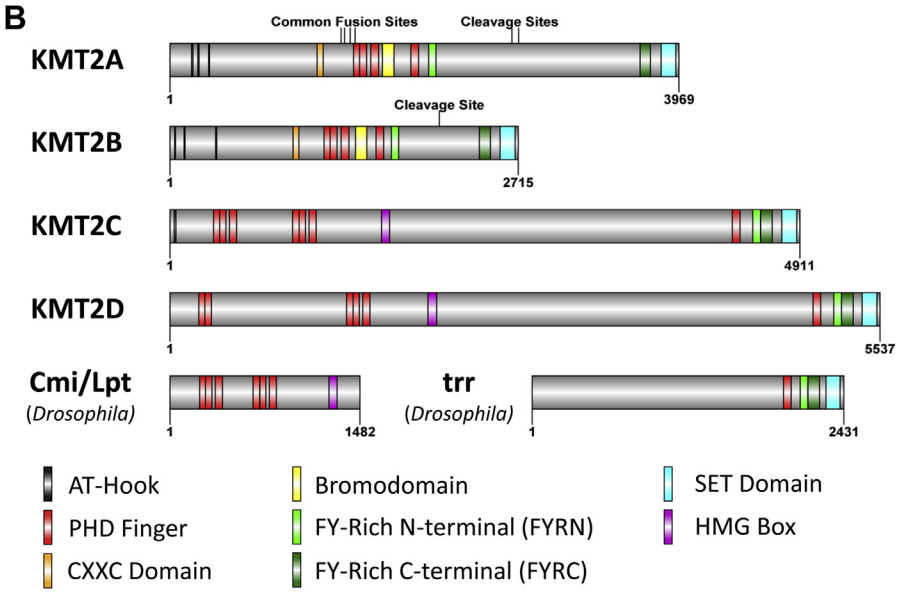

Figure 2 KMT2A–D identification and domains. (A) Gene and peptide details are listed to distinguish *KMT2A–D*. HGNC-designated MLL nomenclature is indicated in bold. (B) Crucial known domains in KMT2A–D family methyltransferases are illustrated, including the catalytic SET domain, sites of proteolytic cleavage in KMT2A/B, and common gene fusion sites in KMT2A resulting in chimeric proteins in leukemia. Peptides and domains depicted using Domain Graph (DOG) software (177).

euchromatin (23). MLL1/KMT2A and MLL4/KMT2B may regulate these same forms of lysine methylation in a more reduced and specific capacity. MLL1/KMT2A genome localization studies using chromatin immunoprecipitation (ChIP) have demonstrated significantly fewer enriched sites compared with those in Set1A/B and only approximately 5% of global H3K4me3 is MLL1/KMT2A-dependent, including the *HOX* genes that are known targets of MLL1/KMT2A activity (17). *Mll1/Kmt2A* and *Mll4/Kmt2B* mutation and knockout studies in mice suggest that these KMTase activities are essential for viability in the early embryo, and they have important functions in normal hematopoiesis and neuronal differentiation in adult tissues (24–28).

The MLL2/KMT2D and MLL3/KMT2C proteins were first found to be in nuclear receptor coactivator complexes initially termed ASCOM, required for the activation of estrogen receptor targets (29,30). Studies in both *Drosophila* and mammals have demonstrated that MLL2/KMT2D and MLL3/KMT2C are encoded by essential genes. These proteins are the principal monomethyltransferases of H3K4, and they generally appear to act in a nonredundant manner, although there may be significant overlap on enhancers in various cells and tissues. The COMPASS-like complexes are recruited to enhancer regions through the binding of nuclear receptors (ER, PPARγ, GR, RAR, LXR, etc.) and other DNA-binding transcription factors, including p53 (31,32). Through this mechanism, cells can dynamically regulate their expression based on transcription factor availability, responding to developmental, hormonal, and other signals. Active enhancers will come in contact with a related promoter or promoters through a mediator complex and cohesin proteins. This interaction activates RNApolII activity and facilitates transcription. Enhancer regions are enriched with modifications on H3K4 and H3K27. Active enhancers contain both H3K4me1 and H3K27ac (acetylation) marks, whereas inactive enhancers are enriched with H3K4me1 and H3K27me3, where the presence of both methylation marks denote bivalent or "primed" enhancers, common in undifferentiated cells and thought to prepare the enhancer for rapid activation (reviewed in Calo et al. (4)).

MLL1 (KMT2A) involvement in leukemias

Mixed-lineage leukemia (MLL or MLL1, now known as KMT2A) is the founding member of the MLL family and was initially discovered more than 20 years ago (33) as being frequently associated with a subpopulation of aggressive lymphoid and myeloid leukemias (reviewed recently in Bellabio et al. (34) and Somervaille et al. (35)). These malignancies are known to be acute, with poor prognosis, and more common in young children than adults. Chromosome translocations that break within the *MLL1* gene and fuse the N-terminal region of MLL1 with a variety of different partner proteins to create fusion proteins account for all of the *MLL1*-associated leukemias and 5–10% of human acute leukemias. These fusion proteins are responsible for initiating leukemogenesis in mouse and xenograft studies and serve as an important model for the role of epigenetic modifiers in human disease. In all of the MLL1/KMT2A leukemogenic translocations the C-terminal SET domain containing the H3K4 methyltransferase activity is lost, yet the DNA-binding N-terminal domains remain as part of the fusion protein. There are over 50 known in-frame chimeric partners of MLL1 involved in these oncogenic fusions, yet the most common (AF4, AF9, and ENL) belong to a family of nuclear proteins shown to recruit the DOT1L complex to histones. DOT1L catalyzes H3K79 methylation, a covalent histone modification often associated with active transcription. Several theories have been put forth regarding the function of MLL1 fusions and DOT1L activity in leukemias. First, the abnormal juxtaposition of MLL1 gene-targeting function with DOT1L histone methylation activity could be the driving mechanism of transformation in a subset of fusion proteins. It has also been well established that MLL1 regulates self-renewal genes, including *HOXA9* and *MEIS1,* in hematopoietic stem cells (HSC), possibly in concert with DOT1L activity (36). In cells harboring an MLL1 fusion protein, these genes remain constitutively expressed, contributing to driving leukemogenesis. Another theory is that oligomerization of the MLL1 fusion partners and/or recruitment of transcription activating proteins (including RNApolII) drive the oncogenic transformation. It seems likely that a combination of these mechanisms may be at work in recombinant MLL1/KMT2A-driven mixed-lineage leukemias (37).

In addition to the relationship between MLL1/KMT2A fusions and DOT1L, there are other protein–protein interactions shown to be important for the leukemia phenotype. One such interaction is between the MLL1 fusion protein and the wild-type MLL1 protein encoded on the homologous chromosome. Cells require the wild-type *MLL1* gene for viability in the presence of a heterozygous *MLL1* fusion gene, as complete knock-down of wild-type *MLL1* reduces tumor growth and angiogenesis in vivo (38). The mechanism is uncertain, but it may involve physical associations between the normal MLL1 and the MLL1 fusion proteins or possibly another functional interaction. This explanation suggests that cells harboring the MLL1 translocations become malignant due to the MLL1 fusion protein driving aberrant gene transcription and the wild-type MLL1 function maintaining viability. Many of these studies have been covered in recent reviews (37,39,40).

MLL4(2)/KMT2B role in carcinoma

The involvement of *MLL1/KMT2A* translocations in acute leukemias is well established; however, its close paralogue *MLL4/KMT2B* was only recently directly implicated in cancer. The *MLL4/KMT2B* gene at chromosomal position 19q13 was found to be inactivated by *Hepatitis B virus* insertions in seven out of 25 cases of hepatocellular carcinoma (HCC) (41,42). Intriguingly, some of these insertions resulted in HBV-MLL4 fusions that were involved in gene repression in HepG2 cells. The *MLL4/KMT2B* gene has also been found to be associated with translocations to chromosome 17p11.2 in 22 out of 32 HCC patients (41). More recently, *MLL4/KMT2B* translocations to the *GPS2* gene on chromosome 17 were found to be involved in undifferentiated spindle cell sarcomas (43).

MLL3/KMT2C and *MLL2/KMT2D* mutations in cancer

The genomic regions harboring the *MLL3/KMT2C* and *MLL2/KMT2D* genes were first associated with cancer through

chromosome aberration mapping studies. These early experiments identified amplifications of the *MLL2/KMT2D*-containing region in certain pancreatic cancer cell lines and as a common site of translocations in glioblastomas (44). Deletion of the region harboring the *MLL3/KMT2C* gene was noted to be the most frequently recurrent chromosomal abnormality in acute myeloid leukemias (45). Early attention to this family of methyltransferases was focused mainly on their roles in hematological malignancies. However, advances in sequencing technologies over the past decade have made it possible to analyze the entire exome of many primary tumors and comprehensive sequencing of cancer cell lines to enable the identification of frequently mutated genes (Table 1). These studies revealed a significant and unexpected role of *MLL3/KMT2C*- and *MLL2/KMT2D*-inactivating mutations in solid tumors. *MLL3/KMT2C* haploinsufficiency in acute myeloid leukemia has been corroborated by these "next-gen" techniques (46–48). Similar exome sequencing studies have also revealed *MLL3/KMT2C* (without *MLL2/KMT2D*)–inactivating mutations in pancreatic ductal carcinoma and bile duct carcinoma, aggressive cutaneous squamous cell carcinoma, hepatocellular carcinoma, and gastric adenocarcinoma (49–56). *MLL2/KMT2D* (without *MLL3/KMT2C*) mutations have been statistically linked with renal carcinoma and non-Hodgkin lymphoma that includes both diffuse large B-cell (DLBCL) and follicular lymphoma, whereas mutations in *MLL2/KMT2D* were found to be early drivers of oncogenesis in mantle cell lymphoma, as well as squamous cell carcinomas of the head and neck (57–64). *MLL2/KMT2D* is among the most frequently mutated genes in a variety of pediatric tumors (65). A recent study of Chinese patients with non–small cell lung cancers revealed that as many as 11.4% of cases were associated with mutations in *MLL2/KMT2D* (66), whereas another study found nearly 17% of small cell lung cancers associated with mutations in *MLL2/KMT2D* (67).

Several cancer exome studies have identified frequent mutations in both *MLL3/KMT2C* and *MLL2/KMT2D* in the same cancer type. For example, both genes are found to be inactivated to similar extents in breast cancers, where it is thought that they cooperate with the tumor suppressor p53 or estrogen receptor (ER) (68–73). However, there are numerous reports indicating mutations in only one of the two paralogues being associated with certain cancer types, raising the possibility that despite their overall extended protein similarities, the two lysine methyltransferases are not completely redundant and that each COMPASS-like complex may have distinct functions in different cell types or developmental and metabolic pathways. Exceptions include some studies of medulloblastoma, urothelial carcinomas, endometrial carcinomas, esophageal carcinomas, thymic carcinomas, head and neck squamous cell carcinoma, and lung squamous cell and adeno cancers that identified both paralogues as frequently inactivated to different extents (74–80). Whereas many reports identify one or the other gene mutation in various cancers, it is difficult and likely premature to draw conclusions based on these differences. For example, one prostate cancer sequencing study determined *MLL3/KMT2C* to be a significant mutation target (81), whereas a second focusing on castration-resistant prostate tumors identified *MLL2/KMT2D* as significant and *MLL3/KMT2C* as a background mutation (82). Similarly, although *MLL3/KMT2C* has been identified as significantly downregulated in radiation-resistant esophageal squamous cell cancer cell lines and in primary tumors (83), a separate study of primary tumors found both *MLL3/KMT2C* and *MLL2/KMT2D* as mutation targets (76). It cannot be concluded on this information alone that the loss of *MLL3/KMT2C* corresponds to radiation resistance in tumors. In another example, germline truncating mutations in *MLL3/KMT2C* were identified in a large study of ovarian cancers, but *MLL2/KMT2D* mutations were not identified (84). Sequenced glioblastomas were found to harbor a significant number of mutations in *MLL3/KMT2C* but not in *MLL2/KMT2D* (49). One study found both *MLL3/KMT2C* and *MLL2/KMT2D* to be inactivated in hypermutated (MSI-H) colorectal tumors, whereas two other studies identified *MLL3/KMT2C* alone, at the highest frequency in MSI-H and among the highest in general colorectal cancer (85–87). These examples serve to emphasize that whereas high-throughput cancer sequencing is able to identify an abundance of statistically significant new targets of mutation in cancers, without any mechanistic evidence these targets must be verified for their importance in the cancer phenotype through other means.

It becomes clear across the many high-throughput cancer studies that *MLL3/KMT2C* and *MLL2/KMT2D* can be categorized as haploinsufficient tumor suppressors in a wide variety of malignancies. A substantial number of mutations in these two genes are heterozygous and inactivating in somatic tissues. These include frame-shifting mutations, insertions/deletions, and truncations removing the C-terminal SET methyltransferase domain. Also, epigenetic aberrations, including restrictive heterochromatin and silencing associated with promoter DNA hypermethylation, in these genes have been linked with cancer (58,88). The dearth of homozygous inactivating mutations in *MLL3/KMT2C* and *MLL2/KMT2D* in the sequenced malignancies suggests that the cancerous cells still require a heterozygous wild-type homologue to sustain viability and drive cell proliferation. This suggestion is interesting considering that homozygous inactivation of these proteins in adult tissues led to largely deleterious effects in only certain cell types.

Components of the human KMT2C/KMT2D COMPASS-like complexes

As previously mentioned, KMT2A–D as well as Set1A–B are the SET-domain lysine methyltransferases in the COMPASS complexes that globally regulate H3K4 methylation. The other subunits function to enhance methyltransferase functions, remove repressive marks, and aid in signal response and localization. Subunits ASH2, DPY30, RBBP5, and WDR5 are the most highly conserved across all of the COMPASS-related complexes in humans and within orthologous complexes in the vast majority of the animal kingdom (7). The fundamental SET domain–containing proteins are able to catalyze the monomethylation of lysine alone, but these other four crucial subunits allow for di- and trimethylation activity. Together, these five proteins compose the minimal core subunits necessary for targeted and accurate H3K4 methylation.

Conserved only within the KMT2C/D COMPASS-like complexes and their orthologues, are the subunits UTX, NCOA6, PA1, and PTIP (Figure 2). UTX (ubiquitously

Table 1 Mutation frequencies of *KMT2C/D* in compiled tumor exome sequencing[a]

Cancer type	Cases with mutated *KMT2C*[b], %	Cases with mutated *KMT2D*[b], %	Reference(s)
Follicular lymphoma	0	50–89	(59,62)
Colorectal adenocarcinoma	0–80	0–15	(77,86,87,145,146) TCGA–Prov[c]
Large B–cell lymphoma	0	7–32	(59,61) TCGA–Prov[c]
Bladder urothelial carcinoma	1–24	1–27	(61,77,147–149) TCGA–Prov[c]
Gynaecologic carcinosarcoma	27	4	(150)
Skin cutaneous melanoma	9–25	0–24	(142,151) TCGA–Prov[c]
Cervical or endocervical adenocarcinoma	22	17	TCGA–Prov[c]
Lung adenocarcinoma	15–21	4–9	(61,77,152,153)
Lung squamous cell carcinoma	15–17	20	(61,77,153,154)
Esophageal squamous cell carcinoma	6	19	(80)
Stomach adenocarcinoma	13–17	0–18	(53) TCGA–Prov[c]
Head and neck squamous cell carcinoma	3–9	0–18	(60,61,77,155) TCGA–Prov[c]
Kidney renal clear cell carcinoma	0–5	2–17	(57,77,156,157)
Bile duct cancer (liver fluke––associated)	15	0	(51)
Kidney renal papillary cell carcinoma	11	14	TCGA–Prov[c]
Uterine corpus endometrial carcinoma	5–11	8–14	(77,158)
Kidney chromophobic cancer	14	6	TCGA–Prov[c]
Medulloblastoma	0–4	3–14	(74,75,159–161)
Ovarian serous cystadenocarcinoma	2–12	0–2	(77,162) TCGA–Prov[c]
Adrenocortical carcinoma	10	11	TCGA–Prov[c]
Liver hepatocellular carcinoma	2–11	0–5	(52,163) TCGA–Prov[c]
Breast invasive carcinoma	6–11	0–3	(61,70,73,77,141,164,165) TCGA–Prov[c]
Thymic carcinoma	10	10	(78)
Prostate adenocarcinoma	1–9	0–10	(81,82,166–168) TCGA–Prov[c]
Small cell lung cancer	0–10	0–7	(143,169)
Glioblastoma	0–8	1–4	(77,170,171) TCGA–Prov[c]

[a] Partial list is presented. Additional unlisted cancer types with a mutation rate in *KMT2C/D* $\leq$ 6% include pancreatic adenocarcinoma (50), transitional cell carcinoma (98), esophageal adenocarcinoma (144), acute myeloid leukemia (46,172), multiple myeloma (173), and adenoid cystic carcinoma (174).

[b] Ranges represent the minimum and maximum values of the aggregated results of the cited studies.

[c] "TCGA Prov" refers to unfinalized data made available through The Cancer Genome Atlas cBioPortal database of sequencing studies (175,176).

transcribed tetratricopeptide repeat on chromosome X, also known as KDM6A) is a histone lysine demethylase (KDM) specifically targeting H3K27me3 and responsible for maintaining the steady-state level of this important modification. H3K27me3 is a repressive mark common in both inactive promoters and inactive or poised enhancers. In mammals, the H3K27me3 mark is deposited by the Polycomb repressive complex 2 (PRC2), which works in conjunction with PRC1 to compact silenced chromatin regions and retain that silent state over multiple cell generations. When a poised enhancer becomes activated, UTX/KDM6A removes the inhibitory H3K27me3 methyl marks at gene enhancers and promoters catalyzed by the repressive EZH2 component of the Polycomb/PRC2 complex (89,90). The histone acetyltransferase p300/CBP coactivators are then recruited to enhancer chromatin to acetylate H3K27 that together with H3K4me1 mark the region as an active enhancer. Mammalian UTX/KDM6A is essential for normal development (89). *Drosophila* UTX has been shown to be a critical regulator of *Hox* gene expression (91) and JAK-STAT signaling (92) and is important for functions of COMPASS-like complexes (93). Inactivating mutations in human *UTX* have been shown to be prevalent in a number of cancers, as well as in certain developmental disorders. Loss of UTX/KDM6A is linked to leukemias (94–97), bladder cancer (98,99), breast cancer (71,100), and renal carcinoma (101). In tumors, the loss of

UTX demethylase activity leads to aberrant H3K27 methylation and the silencing of genes that function as tumor suppressors (89). Significantly, EZH2 is overexpressed in non-Hodgkin lymphoma (102) and a variety of other cancers (103–106), where expression levels are often correlated with tumor grade and poor prognosis, suggesting it is a driver of oncogenesis. Inactivation of EZH2 in these tumors restores normal growth, suggesting an important balance normally exists between H3K27 methylation and demethylation in cellular homeostasis.

NCOA6 is an essential component of the KMT2C/D COMPASS-like complexes, required for hormone-dependent coactivation of enhancers. NCOA6 is a common coactivator (107) and has been demonstrated to be essential for embryo development and survival through knock-out studies in mice (108–110). The *Drosophila NcoA6* gene has also been shown to be important for viability and proper tissue development through interaction with the Yorkie/YAP transcriptional activator involved in the Hippo signaling pathway (111,112). NCOA6 has been shown to associate with a large variety of nuclear hormone receptors, including prostanoid, retinoid, vitamin D3, thyroid hormone, and steroid receptors in humans, enhancing the activating ability of these transcription factors. NCOA6 may also be linked with basal transcription as it can interact with core RNAPolII-associated transcription factors as well. NCOA6 is commonly amplified and overexpressed in a variety of cancers, including gastric (113), breast, colon, and lung cancers (107).

The PTIP and PA1 proteins are implicated in cellular response to DNA damage and repair, although this function has been shown to be independent of KMT2C/D. It has been demonstrated that PTIP is necessary to interact with transcription factor PAX2 and form COMPASS-like complexes at PAX2 targets (114). The tandem BRCT domains within PTIP that bind phosphopeptides are hypothesized to function by facilitating other protein–protein interactions (115). There is no currently known function of PA1 within the COMPASS complexes, besides the fact that it interacts with and possibly stabilizes PTIP.

KMT2C/D mutation and developmental disorders

Heterozygous germline inactivating mutations of both *MLL2/KMT2D* and *MLL3/KMT2C* have been associated with specific developmental disorders. Kleefstra syndrome is an inherited intellectual disorder presenting with distinct facial features and hypotonia (116). The majority of Kleefstra patients harbor inactivating mutations in *EHMT1* that encodes a histone methyltransferase responsible for H3K9 dimethylation, a repressive histone modification. However, de novo mutations in chromatin modifiers, including *MLL3/KMT2C* and *SMARCB1*, may cause a Kleefstra phenotype even in the presence of wild-type *EHMT1*. Genetic interaction tests using a *Drosophila* model involving RNA interference (RNAi) depletion of *trr* (a homologue of *MLL3/KMT2C*) and overexpression of *EHMT1* confirmed a functional cooperation between the genes (116). Interestingly, SMARCB1 encodes a component of the SWI/SNF chromatin remodeling complex, and it interacts directly with MLL3/KMT2C in regulating nuclear receptor–responsive enhancers (117).

Kabuki syndrome is a rare developmental disorder affecting approximately one in 32,000 individuals, involving distinct craniofacial malformations and susceptibility to infection (reviewed in Bokinni (118)). The vast majority of Kabuki Syndrome patients harbor de novo mutations in *MLL2/KMT2D* (reviewed in Bogershausen et al. (119)). Many of these mutations are missense and are found to occur throughout the gene, with some bias toward the 3′ end. Many mutations are located within or truncating the catalytic SET domain; however, a distinct subset of mutations localize to a conserved nonenzymatic region of the SET domain (120). Approximately 5% of Kabuki patients lack an *MLL2/KMT2D* mutation but harbor inactivating mutations in *UTX/KDM6A,* further suggesting essential roles for the COMPASS-like complex in normal development and control of cell proliferation.

Pathway analysis of *MLL3/KMT2C* and *MLL2/KMT2D* deficiency

There are relatively few *MLL3/KMT2C* and *MLL2/KMT2D* knock-out or RNAi depletion studies in genetic model systems that examine broad genetic and phenotypic implications of the deficiencies. Those that have been undertaken provide a complex but promising look into the regulatory roles of these factors, including specific targeted developmental and oncogenic pathways. The murine *Mll2/Kmt2D* and *Mll3/Kmt2C* genes are essential for embryonic development (22). Mice harboring homozygous deletions of the Mll3/Kmt2C SET domain *(Mll3/Kmt2C$^{\Delta SET/\Delta SET}$)* exhibit partial embryonic lethality, stunted growth, and decreased white adipose tissue (121,122). Ubiquitous knockout of murine *Mll3/Kmt2C* resulted in lethality around the time of birth, whereas *Mll2/Kmt2D* knock-out mice showed early embryonic lethality around E9.5 (22). Conditional deletion of *Mll2/Kmt2D* in adipocyte and myocyte progenitor cells results in impairment of adipogenesis and myogenesis, leaving the viability and proliferative ability of the progenitors themselves intact. These defects included reduction in key cellular regulators and markers of differentiation. Although impaired adipogenesis was observed in *Mll2/Kmt2D* but not in *Mll3/Kmt2C* deleted cells, a double knock-out of both resulted in near-complete penetrance of the defects, virtually preventing all differentiation. Indeed, in cells, *Mll2/Kmt2D* loss led to temporary upregulation of *Mll3/Kmt2C* during differentiation, likely as a compensatory mechanism. ChIP-sequencing studies of these cells demonstrated stage- and cell-type–specific Mll2/Kmt2D binding in differentiating preadipocytes and premyocytes, mainly colocalizing with lineage-specific transcription factors at active enhancer regions (22). A potential complication in single-gene deletion studies is the possibility of partial functional redundancy between *Mll2/Kmt2D* and *Mll3/Kmt2C* (22,121,122). Conditional knock-outs of *Mll2/Kmt2D* with or without simultaneous knock-out of *Mll3/Kmt2C* revealed that *Mll3/Kmt2C* could partially compensate for loss of *Mll2/Kmt2D* in adipogenesis. A partial genetic redundancy between *Mll3/Kmt2C* and *Mll2/Kmt2D* was confirmed in a recent study comparing both single and compound *Mll3/Kmt2C*$^{+/-}$ and *Mll2/Kmt2D*$^{+/-}$ heterozygous deletion mice (123). Gene expression profiling in these mice revealed that both *Mll3/Kmt2C* and *Mll2/Kmt2D* were important for circadian-clock control and similar metabolic pathways in hepatic cells, including bile acid and lipid synthesis. Knock-outs of either *Mll3/Kmt2C* or *Mll2/Kmt2D*

resulted in a strong reduction of global enhancer H3K4 monomethylation and H3K27 acetylation that are hallmarks of active enhancers (4); however, some H3K4me1,2 remained, suggesting that other methyltransferases (MLL1, Set7) were capable of those methylation reactions on some enhancers (22). These data suggest that transcription factor–dependent recruitment of the COMPASS-like coactivator complexes to gene enhancers is likely to be necessary for the targeted activation of these regulatory elements.

Broad-scale metabolic and gene set pathway analyses associated with RNAi depletion of *MLL2/KMT2D* have been undertaken using HeLa cells and human embryonic kidney and human colon carcinoma cell lines (31,121,124). Phenotypically, HeLa cells lacking *MLL2/KMT2D* demonstrated aberrant cytoskeletal organization, reduced proliferation (possibly because of increased apoptosis), and considerably reduced tumorigenicity after subcutaneous injection (124). Significantly downregulated gene ontology groups in these cells included cell adhesion, polarization, and motility, as would be expected with a disorganized cytoskeletal phenotype. *MLL2/KMT2D* knockdown resulted in decreased expression of genes related to signaling responses, such as those related to the cytoskeleton, as well as those involved in metabolism and matrix formation and propagators in signal transduction. Interestingly, those genes that were downregulated were significantly enriched for muscle cell–specific genes (124). In a human embryonic kidney cell line, both MLL3/KMT2C and MLL4(2)/KMT2D were demonstrated to be coactivators for a retinoic acid receptor (RAR) isoform, yet transcription in response to signaling was reduced only with a double deficiency in both methyltransferases, again underscoring a likely redundancy between the two (121). The association between *MLL2/KMT2D* deficiency and signaling pathway defects continued in the colon carcinoma study, where factors integral to cAMP-, HER2-, and RXR-mediated signaling were downregulated, as were many genes involved in differentiation and lineage development (31). Additionally, the p53 pathway was found to be significantly downregulated in the carcinoma cells deficient in *MLL2/KMT2D*.

Although RNAi depletion or knock-out studies in vivo and in vitro are excellent tools for distinguishing MLL3/KMT2C and MLL2/KMT2D targets and functions, they do not accurately represent the state of malignant cells harboring mutations found in human cancers. This limitation is perhaps because nearly all analyzed cases of inactivating mutations are heterozygous, truncating or inactivating the SET methyltransferase activity on one allele. In one study, inactivation of the methyltransferase activity of *Mll3/Kmt2C* in mice (accomplished with in-frame deletions in the SET domain, *Mll3/Kmt2C*$^{\Delta SET/\Delta SET}$) retained the structure of the protein and presumably its ability to form a COMPASS-like complex (121). These mice presented substantially different phenotypes compared with those of *Mll3/Kmt2C* knock-out animals (22). Foremost, *Mll3/Kmt2C*$^{\Delta SET/\Delta SET}$ mice showed incomplete embryonic lethality, but escapers lived to adulthood and exhibited retarded development and hypofertility and were largely depleted of white fat, while harvested embryonic fibroblasts proliferated at a strongly reduced rate. Around 4 months of age, approximately 50% of the *Mll3/Kmt2C*-deleted mice exhibited cellular hyperproliferation and urothelial tumors (32). These phenotypes were 100% penetrant in the presence of a heterozygous loss of p53. These phenotypes closely correlated with *NcoA6* deficiencies studied in parallel (121), suggesting that the loss of methyltransferase activity in *Mll3/Kmt2C* mice and the loss of transcription factor targeting by NcoA6 lead to similar outcomes.

Other genetic model systems have provided important insight into the normal functions of the COMPASS-like complex during development and have elucidated critical signaling pathways that were disrupted because of the loss of the complex. In *Caenorhabditis elegans*, the Set16 protein is the only homologue of both MLL3/KMT2C and MLL2/KMT2D and is required in the germline for normal development and H3K4 methylation in early embryos (125). In *Drosophila,* there is a single set of orthologous proteins related to both MLL3/KMT2C and MLL2/KMT2D, representing a likely genetic split in a common ancestor of some insects and mammals (93,126). The trr protein is homologous to the C-terminal halves of its mammalian orthologues, containing the SET methyltransferase domain, whereas Cmi/Lpt corresponds to the N-termini of MLL3/KMT2C and MLL2/KMT2D, harboring the PHD finger domains that provide chromatin recognition and binding functions (Figure 2). Together, these separate proteins form into a COMPASS-like complex with highly conserved structure and function to KMT2C/D COMPASS-like complexes (7,9). The partition of these central elements on separate genes while retaining the same function makes *Drosophila* a useful and insightful model for studying activities of the complex. Genetic studies using RNAi knockdown and null mutants have determined that both *trr* and *Cmi* are essential genes, with critical roles in hormone-dependent gene activation (93,127). Genetically, somatic mosaic loss of *trr* in the developing *Drosophila* eye leads to a growth advantage over neighboring wild-type cells, which is similar to a situation that might exist in some cancers (21). However, widespread loss of trr was found to result in reduced cell proliferation and tissue growth, possibly related to aberrant regulation of the highly conserved Notch signaling pathway. Two recent studies determined that the COMPASS-like complex subunits trr and NcoA6 are required for the activation of Hippo pathway target genes, another important signaling pathway in cell growth and apoptosis (111,112). Tissue-specific *Cmi* depletion using RNAi or overexpression of the wild-type Cmi protein produced strong patterning-defect phenotypes consistent with altered Tgf-β/Dpp signaling, and Cmi protein localization experiments suggested possible direct regulation of *dpp* transcription enhancer regions (20). Thus, genetic studies of the normal functions of the COMPASS-like complexes using model organisms have revealed that the complex is important for the proper elaboration of critical developmental signaling pathways (including Notch, Hippo, and Tgf-β/Dpp), and they provide evidence for direct control of growth and apotosis pathways. These pathways have direct counterparts in mammals, where they are known to be crucial for development and are dysregulated in human cancer (128–130).

Mechanisms of cellular dysfunction associated with loss of MLL2/KMT2D and MLL3/KMT2C

Despite the rapidly growing correlations between loss of the COMPASS-like complex components and cancers, there is actually little understanding of the epigenetic mechanism of oncogenesis. The KMT2C/D-associated COMPASS-like

complexes are thought to be the primary coactivator enzymes recruited to enhancers via a number of nuclear receptor and other transcription factors, where the complex then enzymatically modifies histone lysine residues, both adding (methyltransferase) and removing (demethylase) methyl groups to regulate enhancer activities. Since global reductions in mono- and dimethylation of H3K4 and acetylated H3K27 are observed upon loss of COMPASS-like−complex functions and many of these marks are found within transcriptional enhancers, the diminished ability of enhancers to promote gene expression can lead to dramatic changes in both the execution of developmental programs and disruptions of critical metabolic pathways. Therefore, loss (or reduction) of the COMPASS-like complex functions in cells would likely lead to dysregulation of enhancers and to abnormal gene regulation, as has been suggested (131).

Based on our current knowledge of KMT2C/D COMPASS-like complex functions, there are several possible and likely overlapping mechanisms of cancer development associated with loss of the COMPASS-like complexes, all based on enhancer malfunctions in specific cell populations. First, decreased activation (or repression) of nuclear receptor−dependent gene transcription and developmental programs would result in an inability for cells or tissues to appropriately differentiate, because of a loss of genome reprogramming. An extension of this scenario would be that certain stem cell states might be maintained, thereby preventing or blocking progenitor cell gene expression programs. As an example, *Mll2/Kmt2D* and *Mll3/Kmt2C* deficiency studies in mice support a role for these methyltransferases in adipogenesis or myogenesis and demonstrate their necessity for the differentiation of certain lineages, through the signal-mediated activation of lineage-specific factors (22,122). Genetic studies in worms and *Drosophila* confirm that developmental pathways are blocked or misregulated when components of the COMPASS-like complexes are mutated or missing. The identification of MLL2/KMT2D target genes through genome-wide chromatin immunoprecipitation studies revealed an enrichment of genes important in signaling pathways and signal-response genes (31,124). In this fashion, complexes containing NCOA6 or other signal-directed coactivators may lack H3K4 methyltransferase activity or may simply not form, preventing the activation of regulatory elements (most likely enhancers) intended by the signaling pathways. This suggests that in cancers, some inactivating mutations may block terminal differentiation of cells, promoting a malignant fate. Recent biochemical analyses of *Mll3/Kmt2C*-deficient mice have also demonstrated the need for the methyltransferase in common daily signal-mediated activities, including circadian rhythm and liver metabolism (123,132). It is likely that an inability to respond to ligand-dependent activation signals (e.g., hormones or other secreted molecules) may underlie some cancer therapy failures.

Second, as both MLL2/KMT2D and MLL3/KMT2C were found to be coactivators of p53 (31,32), a reduced function of p53 could lead to an accumulation of additional DNA damage as a result of reduced apoptosis or failure of cell cycle checkpoints. Inhibited tumor suppressor genes or overly activated oncogenes may then be a root cause of the transformative ability of *MLL2/KMT2D* and *MLL3/KMT2C* mutations.

A third possible mechanism is that reduced formation or functions of COMPASS-like complexes may indirectly lead to reduced acetylation of H3K27 as the result of accumulation of repressive H3K27me3 histone marks catalyzed by EZH2. Since known tumor suppressor genes, such as $p16^{Ink4a}$, are known to be controlled by EZH2 activity and stem cell programs are maintained by Polycomb complexes (133), an inability to remove H3K27me3 repressive marks might lead to hyperactivated stem cell programs and reduced activation of differentiation pathways (134−136).

These scenarios, all involving loss of COMPASS-like complex functions, may seem to contradict data from whole-animal genetic and cultured cell studies demonstrating that *MLL2/KMT2D* and *MLL3/KMT2C* deficiencies or RNA depletion are detrimental to the proliferation and motility of cancerous cells (31,124,137). However, the associated malignancies are most often the result of heterozygous inactivating or truncating mutations, reducing but not eliminating functional COMPASS-like complexes. The remaining complexes (or limited redundancy of other Set1-related complexes) might allow for cell viability and proliferation in the absence of properly activated differentiation pathways, thereby promoting the malignant state.

MLL-family mutations in cancer therapy

The compound EPZ-5676, which was developed to specifically inhibit DOT1L activity in MLL1/KMT2A-fusion leukemias, has already demonstrated significant promise as a selective cancer drug (138) and is currently in phase 1 clinical trials. Successful clinical outcomes of trials of this compound would provide solid proof of principle for future investment in the development of drugs targeting the activity of the MLL family of lysine methyltransferases. From a cancer therapeutics standpoint, it is crucial to target the driving mutations in a tumor. Although the significant mutation frequencies of *MLL3/KMT2C* and *MLL2/KMT2D* in a plethora of malignancies suggest important roles for these genes in the early stages of oncogenesis, there is still dispute regarding whether these mutations represent driving events or passenger mutations in certain cancer types. The identification of a type of mutation as a driver of malignant transformation typically involves detecting those that occur early and remain in the rapidly mutating tumor population as well as selecting for nonsilent mutations that cluster in specific domains. Several recent exome sequencing studies have used these criteria to identify *MLL3/KMT2C* and *MLL2/KMT2D* mutations as probable drivers in multiple cancer types (139), including pediatric acute lymphoblastic leukemia (140) and breast cancer (141). However, many other groups have determined that mutations in these chromatin modifiers fail to significantly meet the criteria for a driving event (142−144). In fact, two separate studies disagree on the role of *MLL2/KMT2D* in the hierarchy of follicular lymphoma transformation, one identifying its mutation as a significant and potent driving event (63) and the other defining it as a later accelerating mutation (62). As methods for identifying critical transformative events in oncogenesis continue to improve, so will the therapeutic focus in developing anticancer drugs.

Conclusions and perspectives

Since the discovery of *MLL1/KMT2A* translocations in acute leukemia in 1991, we have acquired valuable insights into the important roles that histone modifications play in controlling the expression of genes found to be dysregulated in cancer, and into epigenetics in general. Within the past several years, the availability of fully sequenced genomes, exomes, epigenomes, and transcriptomes has allowed us a greatly expanded view of the range of gene mutations associated with many cancers. The emergence of highly conserved epigenetic regulators among the most frequently mutated genes in cancer has elevated our understanding of how chromatin remodeling factors, such as those associated with the conserved COMPASS-related complexes, affect normal developmental signaling pathways and cell proliferation. A rapidly growing list of sequenced cancer exomes will continue to provide even greater insights and opportunities to delve deeper into the mechanisms of epigenetic gene regulation in oncogenesis. Further identification of global targets of MLL3/KMT2C and MLL2/KMT2D, and those of their orthologues in genetic model organisms, will clarify how mutational loss of these conserved complexes can lead to malignant transformation in human cells and provide new avenues of investigation into novel therapies.

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