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Lysine Methyltransferases Signaling: Histones are Just the Tip of the Iceberg

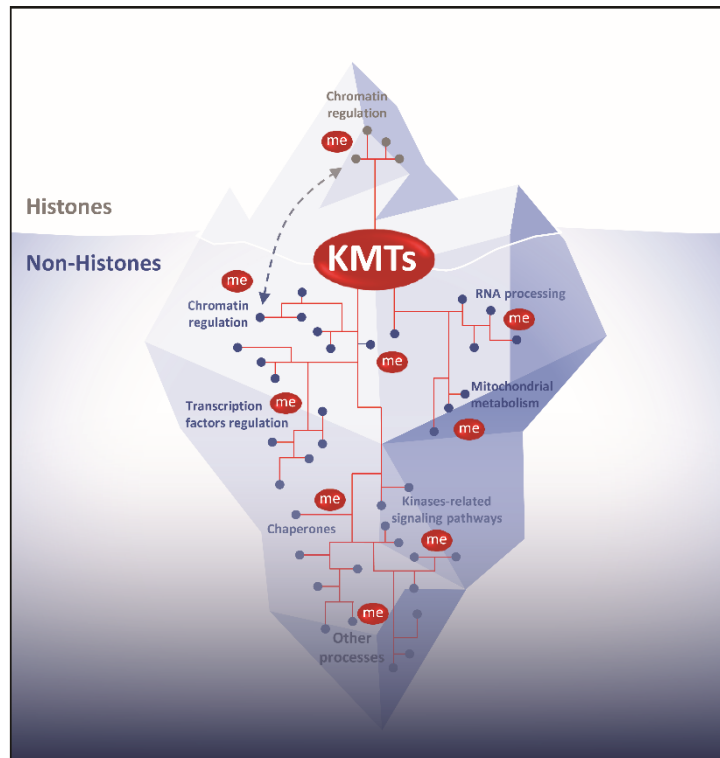
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Graphical abstract:



Abstract : Protein lysine methylation is a functionally diverse post-translational modification involved in various major cellular processes. Lysine methylation codes for an additional layer of information at the protein level and can modulate proteins stability, localization, interaction or activity, resulting in specific downstream signaling and biological outcomes. Lysine methylation is a dynamic and fine-tuned process, dysregulation of which often leads to human pathologies, and the lysine methylome and its signaling network catalyzed by methyltransferases can notably be linked to carcinogenesis and cancer progression.

Histone modifications and chromatin regulation is a major aspect of lysine methylation importance, but more and more evidence suggesting the high relevance and impact of non-histone lysine methylation signaling emerged in the recent years. In this review, we draw an updated picture of the current scientific knowledge regarding non-histone lysine methylation signaling and its implication in physiologic and pathologic processes. We aim to demonstrate the significance of lysine methylation as a major and yet underestimated posttranslational modification, and to raise the importance of this modification in both epigenetic and cellular signaling, by focusing on the observed activity of SET- and 7 β -strand- containing human lysine methyltransferases.

The lysine methyltransferases “methylome network”, together with evidence suggesting that only the tip of the iceberg has been observed so far regarding its implication in human pathologies, raise the possibility to use these enzymes and their signaling as promising new therapeutic targets for the development of future epigenetic and methyllysine signaling cancer treatments.

Keywords: Lysine methylation, protein lysine methyltransferases, KMT, cancer.

1. LYSINE METHYLATION SIGNALING

Post-translational modifications (PTMs) are covalent chemical modifications occurring on proteins after their synthesis [1]. Biological importance of PTMs is demonstrated through their involvement in regulation of various cellular processes, including dynamic intracellular signaling [1–3]. PTMs can occur on either single or multiple sites of a protein, and a specific residue can undergo multiple PTMs at times [2,3]. Phosphorylation, methylation, ubiquitination and acetylation are some of the PTMs well described in cell biology, dysregulation of which often leads to development of biological disorders, depending on the environmental context, the type of PTM and its substrates [4]. These modifications can modulate protein activity and subsequently control biological processes, making actors of PTM signaling interesting clinical targets. One of the examples of disease development caused by imbalanced PTMs is cancer. Cancer, along with genetic mutations, is triggered by changes or dysregulation of epigenetic control mechanisms [5]. Since cancer progression often involves deregulation of PTM signaling, there is a clear value in deeper understanding of PTMs as fine-tuning mechanisms of cell homeostasis.

Protein methylation is one of the functionally most diverse PTMs, and results from the transfer of one or several methyl groups to lysine, arginine (or more rarely other amino acids such as histidine and cysteine), as well as to the amino- and carboxy-termini of a protein. Protein methylation signaling is a fine-tuned, dynamic and complex biochemical process, with 3 different set of actors: writers (adding methyl group(s)), readers (detecting methylated residues) and erasers (removing methyl group(s)). The history of methylation, its discovery and first outbreaks in this field is in great detail described in the review by Murn *et al.*, who followed the progress in understanding this PTM throughout few decades [6]. At the beginning of era on methylation studies, a lot of gathered information involved describing the methylation events on histones, implying the importance of this PTM in epigenetic regulation. Several excellent reviews previously summarized the participation of histone lysine methylation in the establishment of the histone code and its implication in regulation at the epigenetic level [7–9]. Brief description of histone methylation with recent news in this field will be described in the section below. However, it is difficult nowadays to draw the line in classifying methyltransferase (MT) enzymes into exclusively histone or non-histone MTs, since many of them show chromatin-independent activity along with histone methylation capability. For example, Chen *et al.* summarized histone and non-histone methylation having an important role in DNA damage response, when following changes in histone methylation levels as well as methylation of p53, E2F transcription factor 1 (E2F1) and PARP1 [10].

In 2011, Petrossian and Clarke presented a classification of human MTs using available enzyme structures [11]. This subsequently enabled recognition of new family members, which was then further used for identification of potential substrates. Using bioinformatics analysis, they identified 208 SAM-dependent MTs, from which 30% were already linked to various disorders and cancers at that time [11]. The majority of human MTs harbors either one of three different catalytic domains: a seven- β -strand domain (131 MTs), a SET domain (57 MTs) or a SPOUT domain (8 MTs), with few other unrelated potential domains existing (13 MTs). Among those MTs, the SET-domain containing family is linked to protein lysine methylation. Enzymatic activity of SET domain proteins is largely described on histone proteins, while non-histone substrates have been identified more recently [12–15]. In their well-written review, Carlson and Gozani classified SET domain enzymes into three groups: enzymes methylating histones, enzymes modifying non-histone proteins and enzymes that do not have clear enzymatic activity yet, called “orphans” [13]. However, as it will be described further in this review, lysine methylation is not restricted to SET domain methyltransferases, and more and more seven- β -strand (7β -strand) MTs are described as capable to specifically methylate proteins' lysines.

Non-histone lysine methylation is now investigated in greater details and some of the first discoveries were related to the p53 protein and its regulation by dynamic protein modifications. Therefore, to describe the role and importance of lysine MTs in particular biological processes or in context of human health, the enzymes and their activity on specific substrates have to be carefully studied. Hence, building each lysine methyltransferase (KMT) “methylome” and signaling network may definitely demonstrate that lysine methylation is involved in various important cellular pathways and directly linked to diseases such as cancer. This is becoming plausible by great improvements in recent years in this research field, through development of proteomics analysis as well as the improvement of technologies for methylation sites prediction. One of the examples is presented by Biggar *et al.*

who developed a predictive approach for identification of methylated lysines using available sites identified through the literature and data sets, enabling use of targeted MS approaches and improving of further understanding of methylome [16].

Protein methylation can play different functions, either as independent PTM or through interplay with other PTMs [15]. Interestingly, methylation does not cause changes in protein structure due to its small steric hindrance, nor affect the charge of modified protein residue. However, since Arginine or Lysine residues can be modified up to two or three times, this event can potentially affect their hydrogen-bonding capacity [6]. Therefore, lysine methylation is a regulator of protein-protein interactions, often through the change in abovementioned bonding capacity. Any change in these interactions can have an effect on a protein stability, on its activity through specific complex, or on its subcellular localization. Furthermore, lysine methylation can compete with other PTMs affecting lysines, such as acetylation and ubiquitination. Based on existing literature, lysine methylation can influence in different ways diverse biological processes [10,13,15,17], and altogether MTs are identified as potential targets for inhibitor development. Indeed, with more than 60 potential KMTs and 30 lysine demethylases reported so far, as well as new substrates constantly emerging, there are constant indications for importance of these enzymes in disease development. Therefore, thanks to the specific, fine-tuned and dynamic aspects of lysine methylation signaling, all these indications lead to further possibility of implementing KMTs or their activity as prognostic markers and potential druggable targets [18–20], and inhibitors are already developed and tested in clinical trials for methyltransferases (DOT1L and EZH2) and demethylase (LSD1) [19].

This review will provide an updated knowledge on lysine methylation events, mainly focusing on non-histone lysine methylation in cancer and. Past and future uncovering of lysine methylation signaling will certainly offer new targets for further alternative treatments to cancer, and we are entering exciting times to tackle the importance of lysine methylation signaling and the development of new epigenetic and cell signaling-related new therapies.

2. HISTONE METHYLATION SIGNALING

History of histones modification and dynamics of epigenetic regulations is presented in the review by Allis and Jenuwein, implying the intriguing nature of targeting these events based on the reversibility of lysine methylation [7]. Nowadays, it is evident that Su(var)3-9 E(z) Trithorax (SET) domain proteins have a role in epigenetic regulation, with their enzymatic activity on histone first shown by Jenuwein in 2000 on SUV39H1 [7]. This pioneer finding led further studies into describing SET domain proteins and identifying range of enzymes that catalyze methylation events on histones and either activate or repress gene expression. All of these subsequent discoveries and identified events promoted the theory of the “histone code”, where it is hypothesized that each modification on histones, the pattern of specific modifications and the precise crosstalk between them, lead to different information and interpretations promoting specific biological outcomes. This postulate originated from the identification of “reader” proteins that can bind and detect specific histone modifications through methyl-binding domains [7]. A lot of investigation was done in this direction, identifying different reader domains, such as the chromodomain, the Tudor domains and the PHD domain [7]. Besides “reader” proteins, another group of proteins, protein “erasers” or lysine demethylase enzymes (KDM), are contributing to dynamics of this PTM by reversing lysine methylation signaling and therefore participating in fine-tuned regulations of biological processes. The first KDM identified is LSD1 (lysine specific demethylase 1), able to demethylate mono and dimethyl-lysines. Afterwards, Jumonji domain-containing KDMs were discovered as capable to erase trimethylation on histone proteins [21]. For example, mono- and di- histone H3 lysine 4 (H3K4) methylated lysine marks are erased by LSD1, whereas tri-methylated H3K4 is removed by the Jumonji domain-containing protein KDM5B [21–23].

Modifications involved in the “histone code” include various methylation marks among other modifications and we have now evidence that such epigenetic landscapes can be time and context specific. Indeed, histone lysine methylation is more complex than simply promoting active or repressive chromatin states as exemplified by H3K4me3 (related to active gene expression) or H3K27me3 (related to repressed gene expression). Under specific context, poised or bivalent chromatin can present simultaneous contradictory histone modifications associated with both gene activation and repression such as H3K4me3 and H3K27me3. Such chromatin state is normally

associated with promoters of transcription factor genes that are expressed at low levels, and is also found to play a role in developmental regulation in pluripotent embryonic stem cells, as well as in gene imprinting [24]. Moreover, monomethylation of H3K4 is related to genomic elements that act as poised enhancers, meaning that upon certain type of cell stimuli, these enhancers change from poised to active, regulating transcription and consequently directing cell-type in human embryonic stem cell development [25,26]. Additionally, lysine methylation marks such as H3K36me2 can serve as memory state in differentiating cells through silencing the transcription [27].

Overall, due to the early discovery and thorough research of methylation on histone proteins, recent discoveries largely involve better characterization of already known marks, while rarely including characterization of new methylation events. However, with constant findings supporting cytoplasmic and soluble nuclear activity of various KMTs, recent discoveries unveil the importance and prevalence of non-histone lysine methylation and an interesting new field of cell signaling to explore.

3. HISTONE-KMTs THAT HAVE EVIDENCE OF NON-HISTONE SUBSTRATES

Due to the early interest on histone modifications, most of the KMTs were described as histone methyltransferases. However, primarily identified histone KMTs are not exclusively restricted to histones methylation, an assumption originating from their easier characterization thanks to high abundance of histone proteins and their enrichment for lysine residues. This biased candidate approach, together with the difficult development of adapted proteomic tools, delayed the discovery of broader capacity of KMTs to methylate non-histone substrates, including KMTs previously identified as histone MTs. Still, characterizations of non-histone MTs to date mostly rely on functions regarding chromatin and gene expression regulation. For example, KMTs such as EZH2 (KMT6) can alter gene expression either through histone modification (H3K27 as a repressive mark) or regulation of ROR α and STAT3 transcription factors [15,28]. Notably, a same KMT can have opposite effect on gene regulation, depending on the context and substrates affected. While SETD1A (KMT2F) is important for embryonic stem cell self-renewal and differentiation through regulation of homeotic genes by methylation of histone H3 [29], it can have oncogenic functions through the methylation of YAP protein and its sequestration into nucleus [30]. Importantly, several known histone methyltransferases can affect histone and non-histone substrate's activity using similar mechanism. For example, G9a/GLP (EHMT2/EHMT1) methylation of either H3K9 or ATF7IP (activating transcriptional factor 7-interacting protein) facilitates the recruitment of the MPP8 chromodomain protein to both substrates and represses specific genes expression [31]. However, enzymatic activity of KMTs can also be related to different processes. For example, Ferry *et al.* found that G9a/GLP, previously described to methylate H3K9 resulting in transcriptional repression, can also methylate histone mimic residues in DNA ligase 1 [10,32]. This non-histone methylation event promotes DNA ligase 1 interaction with UHRF1 and is important for its recruitment and engagement in DNA replication [32]. Moreover, while methylation of SET8 (KMT5A) on H4K20 is important in DNA replication and damage repair [33], SET8 methylation of p53 results in p53-dependant genes repression [33].

It is also important to note that among the various characterized KMTs to have histone-methylation activity, some remains mostly questionable. Indeed, because of initial *in vitro* biased candidate approach used to characterize KMTs and initial studies based on controversial specific methyl-antibodies, some of them have been tagged as histone MTs while such activity might be either false, aspecific or not physiologic. One of the best exemplified demonstration of potentially wrong assessment for KMT activity on histone concerns the SET subfamily of SMYD proteins. Indeed, SMYD2 and SMYD3 (KMT3C & KMT3E) have first been demonstrated to methylate H3K36me2 and H3K4me3 respectively [34,35], while several caveats suggest otherwise. Both enzymes are mostly cytoplasmic and only localized into nucleus under rare conditions. They also have strong evidence of cytoplasmic enzymatic activity, or activity on substrates either cytoplasmic or able to shuttle between nucleus and cytoplasm. Moreover, SMYD2 can methylate almost every lysine residues on free histone H3 *in vitro*, but not when H3 is incorporated into physiologic nucleosomes. Moreover, several other characterized SMYD2 substrates suggest a specific mono-methylation activity for SMYD2. Therefore, it is unlikely that SMYD2 can promote H3K36 dimethylation. One of the interesting recent findings supporting important activity of SMYD2 on non-histone substrates is brought by Olsen *et al.* in 2016, where the authors presented a list of potential SMYD2

substrates using combination of SILAC-based proteomics, Kme1-peptide enrichment and nano-LC-MS/MS analysis, in esophageal squamous cell carcinoma cell line [36]. Looking for the downregulation of methylated proteins after inhibition or knockdown of SMYD2, the authors confirmed changes in methylation levels of previously identified substrates and a great diversity of new ones. Interestingly, the authors did not observe significant changes in methylation levels of histone proteins, such as those observed in the non-histone monomethylated lysine sites, firmly cementing that SMYD2 is mainly a non-histone KMT [36]. Similarly, SMYD3 does not methylate H3 *in vitro*, but can in some extent methylate lysine 5 of histone H4, in either free or nucleosomal configuration. Original claim of H4K20 methylation activity can also be overrun thanks to *in vitro* methylation assay performed using SMYD3 and specific recombinant lysine-mutated version of histone H4, where H4K20>R mutant can still be methylated by SMYD3. Intriguingly, several studies regarding SMYD3 oncogenic activity observe a chromatin-bound fraction of SMYD3, and a SMYD3-dependant methylation of H3K4 or H4H20 at specific gene promoters [37–39]. Indeed, Sarris *et al.* found that SMYD3 is both nuclear and cytoplasmic in colorectal cancer (CRC) and hepatocellular carcinoma (HCC) and that SMYD3 is involved in the activation of transcriptional programs related to oncogenic pathways. However, the authors found that a catalytically dead SMYD3 still activates oncogenic targets such as Myc and Ctnnb1, arguing for a non-catalytic function of nuclear SMYD3 in these cancers. Furthermore, SMYD3 co-incidence with H3K4me3 can be indirect, as SMYD3 might be recruited to already active chromatin regions, might promote H3K4 methylation by other KMTs through recruitment of specific complex, or as proposed by Sarris *et al.*, can directly interact with H3K4me3.

To add another level of complexity in understanding KMTs and identifying related methylation events, a long list of other SET-containing MTs still miss a clear demonstration of a methylation activity so far, and can be classified as “orphans” [13]. It is highly possible that these enzymes are incapable to methylate histones, even non-specifically, leading to their poor characterization and little interest by the scientific community. Another possibility is that such enzymes need specific contexts to be active, being modified by PTMs or being part of a larger complex, which makes identification of their substrates extremely difficult. Moreover, some recent literature indicates new function of SET-domain containing enzymes, suggesting that lysine is not the single methylated residue by such domain. It was indeed found that SETD3 acts as a histidine methyltransferase, modifying the histidine 73 of actin [40]. Kwiatowski *et al.* identified this methylation event catalyzed by SETD3, giving a new role to SET domain enzymes, and showing that in human and drosophila this event is reflected on glycolytic activity through regulation of actin’s stability [40]. The authors also found that this methylation event was significantly higher than the H3K4 SETD3-methylation, when using recombinant proteins or peptides, which is supported by mainly cytoplasmic localization of SETD3. Not long after, Wilkinson *et al.* confirmed and characterized this event in further details, showing that the methylation of actin is not observed in *Setd3*^{-/-} mice, and demonstrated by quantitative proteomics studies that lack of SETD3 only affects actin methylation, presenting it as its main substrate [41]. Additionally, comparing mice models which lack SETD3, they observed that actin H73 methylation has a role in signal transition and actin polymerization in smooth muscles, which is giving altogether a completely new function to the SET domain enzyme SETD3 [41].

4. SET-KMTs NON-HISTONE LYSINE METHYLATION SIGNALING

While methylated modifications of various proteins have been observed long ago, the scientific community almost exclusively focused during the last decade on the characterization of histone methylation dynamics and epigenetics signaling. The scientific interest shifts towards non-histone methylation only recently, with growing evidence of lysine MTs deregulation in human pathologies that could not be simply explained by histone methylation. Indeed, SMYD3 was one of the first KMTs linked to cancer, while this enzyme is mostly cytoplasmic in cancer cells and does not seem to harbor a clear activity on histones. Similarly, the frequent cytoplasmic localization of a large quantity of KMTs definitely suggests some non-histone related signaling. Naturally, the first thorough characterization of non-histone protein methylation heavily focused on well-studied proteins such as p53. As will be detailed later in this review, p53 is massively modified by various PTMs crosstalk, regulating its activity in a manner reminiscent to the “histone code”. Indeed, the C-Terminal Domain (CTD) domain of p53 harboring a lysine and arginine-rich stretch is prone to acetylation, methylation, phosphorylation, ubiquitination and can resemble to histone tails configuration. At least five KMTs modify p53 activity through lysine

methylation. However, other lysine MTs are clearly linked to cancer with either oncogenic or tumor suppressor activity and abnormal expression in specific pathologies. Among frequently deregulated KMTs in human tumorigenesis with non-histone substrates are EZH2 and SMYD proteins. EZH2 is part of Polycomb-repressive complex which is overexpressed in many cancers, and also related to drug resistance, but because the enzyme's major function is on histone and chromatin regulation, its potential non-histone signaling and its related oncogenic implication is difficult to decipher. The major hint of such signaling is that EZH2 can have opposite effect on cancer progression given a specific context, such as being either a transcriptional suppressor or a co-activator. On the other hand, involvement of SMYDs in cancer is more straightforward as their histone-related methylation is controversial, while stronger evidence of non-histone substrates exists. Notably, proteins SMYD2 and SMYD3 are linked to carcinogenesis and cell proliferation through histone-independent signaling. From the above-mentioned arguments, it is clear that KMTs have a regulatory role in various pathological signaling pathways and are related to cell proliferation and cell fate in general, either through histone or non-histone methylation [14]. In this section, we will go through several important cellular pathways that are either regulated or influenced by non-histone methylation events and that might explain some of KMTs' implication in cancer.

4.1. Major cellular signaling regulated by SET-KMTs

Chromatin regulation by histone lysine methylation events and the so-called "histone code" relies on posttranslational modifications crosstalk, promoting specific epigenetic landscapes adaptation to a given biological context. Although still in its early characterization, lysine methylation on non-histone proteins is also clearly involved in the regulation of various important cellular processes. Not surprisingly, similar PTM crosstalk occurs on non-histone proteins and lysine methylation is actively participating in this regulatory process, either directly, as for example p53 activity differently regulated by methylation/acetylation competition at its CTD domain, or indirectly, for example by regulating phosphorylation cascade signaling through the alteration of kinases activity.

4.1.1. Kinase-related signaling pathways

MAPKinase pathway

The MAPKinase pathway promotes extremely fast adaptation to environment stimuli through a cascade of phosphorylation signaling. The downstream activation of transcription factors relies on high regulation of involved kinases such as ERK1/2. One of the early identified examples of lysine methylation signaling affecting the MAPKinase pathway was observed in 2014, with the identification of MAP3K2 methylation by SMYD3 [42]. Interestingly, the trimethylated lysine 260 of MAP3K2 is not located within the kinase domain of the protein, therefore not directly affecting its activity. However, this methylation repels the interaction of the phosphatase PP2a, leading to the incapacity to inactivate phosphorylated MAP3K2, and to the constitutive stimulation of downstream kinases [42]. This observation explains the oncogenic activity of SMYD3 in RAS-induced lung and pancreatic adenocarcinoma. The methyltransferase SMYD2 is also involved in RAS-induced cancer progression, this time affecting another facet of the MAPKinase pathway. SMYD2 monomethylates lysine 355 of MAPKAPK3, a kinase downstream of stress-related p38 kinase pathway [43]. This methylation event was demonstrated to influence inflammatory response and cytokines production of cancer cells, and repressing SMYD2 and MAPKAPK3 cooperates with standard chemotherapy to treat pancreatic ductal adenocarcinoma [43].

AKT/mTOR signaling pathway

Lysine methylation regulation of AKT/mTOR pathway is another example of PTM crosstalk linked to cancer progression. Yoshioka *et al.* found that methylation of AKT1 on its lysine 14 by SMYD3 is important for its activity [44]. Indeed, this methylation event is located within the PH domain of AKT1 important for its proper localization to membrane. Logically, SMYD3 inactivation prevents plasma membrane accumulation of AKT1 with a decrease of T308 phosphorylation level required for AKT1 activation. Finally, this study suggests that SMYD3 methylation of AKT1 is important for the phosphorylation of mTOR, a substrate of AKT1 and inhibiting this methylation event prevent cancer cells proliferation [44]. Recently, an interplay between the methyltransferase SETDB1 (KMT1E) and the demethylase KDM4B, regulating the dynamic lysines 140 and 142 trimethylation of

AKT, also linked aberrant AKT methylation to its activation and oncogenic functions [45]. SETDB1 is amplified in several cancers and its inhibition could be a potential therapeutic strategy against AKT-driven cancers. Finally, Nakakido *et al.* found that SMYD2 also plays an indirect role in AKT pathway through the regulation of phosphatase and tensin homologue (PTEN) by methylating its lysine 313 [46]. PTEN is a tumor suppressor counteracting AKT signaling and authors observed that knockdown of SMYD2 decreases AKT phosphorylation, suggesting that SMYD2-mediated methylation negatively regulates PTEN tumor suppressor activity and results in activation of AKT pathway [46].

ALK signaling pathway

An example of a receptor kinase regulated by lysine methylation is ALK (anaplastic lymphoma kinase). Wang *et al.* discovered that SMYD2 methylates *in vitro* lysines 1451, 1455 and 1610 of ALK [47]. Although they do not demonstrate that these methylation events are physiologic and occur *in vivo*, they observed that SMYD2 repression in non-small cell lung cancer cells (NSCLC) with ELM4-ALK fusion results in decrease of ELM4-ALK phosphorylation level. Moreover, they observed that ectopic expression of ELM4-ALK K1610A mutant leads to the decrease of AKT phosphorylation, a downstream factors relevant for oncogenic ELM4-ALK signaling [47]. Interestingly, combining SMYD2 and ALK inhibition additively suppressed the growth of NSCLC cell. However, the exact mechanism and whether this methylation signaling can regulate normal ALK kinase activity remains elusive.

TGF- β /BMP signaling pathway

Bone morphogenic protein (BMP) and transforming growth factor beta (TGF- β) are cytokines involved in embryonic development and differentiation, and binding to their respective receptors (BMPR and TGF- β R) activate specific signaling transduced by SMAD proteins. Activated SMAD proteins then regulate expression of subset of genes related to cell growth, proliferation and differentiation. SMYD2 methylates BMP type II receptor within its kinase domain, although the exact lysine site is unknown, and this modification has a positive regulatory role in BMP-induced pathway. Indeed, SMYD2 repression impairs phosphorylation of signal-transducing protein SMAD1/5 as well as their nuclear localization and interaction with SMAD4 [48]. On the other hand, SETD7 (KMT7) methylates SMAD7, a downstream inhibitor of TGF- β signaling [49]. The methylation of its lysine 70 promotes its ubiquitination and its degradation by E3 ligase Arkadia. Therefore, SETD7 repression results in SMAD7 increased stability and inhibits TGF- β -dependent genes expression [49]. Finally, SMYD3 is important for epithelial to mesenchymal phenotype transition in breast cancer and acts as a SMAD3 cofactor to promote TGF- β -dependent mesenchymal gene expression and cell migration [50]. However, in this context, SMYD3 enzymatic activity is not required and the exact molecular mechanism underlying SMYD3 function remains unknown [50].

Other kinase receptors regulation

Membrane receptor proteins receive and transduce specific extra cellular signals and their activation is *per se* the first important step of signaling pathways to allow proper cell response. SMYD3 is known to methylate membrane receptors VEGFR1 and HER2. VEGFR1 plays important function in angiogenesis through interaction with specific ligands, and SMYD3 methylation at lysine 831 increases VEGFR1 autophosphorylation. However, VEGFR1 kinase activity is extremely weak and the biological significance of its slight increased activity remains unknown [51]. Enzymatic SMYD3 activity on lysine 175 of HER2 is related to the protein homodimerization and autophosphorylation, critical for its activation and subsequent stimulation of associated pathways involved with cancer development and progression, such as PI3K-AKT, RAS-MAPK, PLC γ -PKC pathways [52].

4.1.2. Transcription factor regulation

p53

p53 is one of the first non-histone substrate observed prone to multiple lysine methylation and other PTMs events, indicating that complex posttranslational regulation goes beyond the histone code. p53 is a great example of transcription factor and tumor suppressor regulated by PTMs. Lysines of the p53 C-terminal domain (CTD) are

critical for controlling p53 activation because they are the sites of competitive PTMs by acetylation, ubiquitination and methylation, resulting in different biological outcomes. Lysine methylation of p53 results from the activity of several KMTs: SMYD2, SET8, G9a, SETDB1 and SETD7, and regulation of p53 by lysine methylation was reviewed several years ago by West and Gozani [53]. The two best studied methylations of p53 (K370me1 by SMYD2 & K382me1 by SET8) are linked to transcriptional repression of its classic target-gene P21/WAF1, through different mechanisms. SET8-dependent K382me1 recruits L3MBTL1 to repress p53-target genes [54]. In addition to this, SET8 also methylates the protein Numb which forms a complex with p53, leading to dissociation of the complex and degradation of p53 [55]. Similarly, p53 repression by Smyd2 K371me1 methylation acts by decreasing p53 occupancy at p21 and mdm2 gene promoters, and SMYD2 depletion facilitates p53-mediated apoptosis in response to various types of DNA damage [56]. Conversely, SETDB1 and SETD7 p53 methylation (respectively K370me2 and K372me) are linked to p53 activation. While K370me1 by SMYD2 represses p53, SETDB1-mediated K370me2 recruits the co-activator TP53BP1 and facilitates p53-target gene expression [57]. The dynamic of K370 methylation is even complexified by the fact that the KDM LSD1 can demethylate both activating p53K370me2 and repressing K370me1 marks [58]. p53K372me1 by SETD7 leads to increased p53 promoter occupancy and transcriptional activation of target genes, and the underlying mechanism relies on the recruitment of the acetyltransferases TIP60 that acetylates surrounding lysines, activating p53 [59]. Moreover, SETD7 K372 methylation prevents SMYD2 interaction and repressing methylation of p53 K370. Finally, studies of methylation event by G9a on K373 are contradictory, and it is still not clear if this methylation inhibits or activates p53 or even if G9a methyltransferase activity is required for p53 regulation [60,61].

Rb

Rb1 is another transcription factor finely regulated by different PTMs, especially acetylation, phosphorylation and methylation. K860 methylation by SMYD2 provides a direct interaction site for the methyl-binding domain of the transcriptional repressor L3MBTL1, and inactivates Rb target genes expression [62]. Rb is also methylated by SETD7 at lysine 810, an event recruiting the heterochromatin protein HP1 and required for pRb-dependent cell cycle arrest and transcriptional repression [63]. Finally, SETD7 also indirectly regulates Rb, as the enzyme can methylate lysine 441 of MYPT1, a known regulator of Rb [64].

GATA4

EZH2, known to be part of the PRC2 repressive complex and to silence gene expression by histone H3K27 methylation can also promote gene silencing through a histone-independent mechanism. EZH2 methylates lysine 299 of the transcription factor GATA4 and represses GATA4 transcriptional activity by altering its association with the acetyltransferase p300 which normally activates GATA4 by acetylation [65].

STAT3

STAT3 is involved in the JAK/STAT pathway, important for signal transduction and maintenance of cell homeostasis. A balanced signaling is important for cell proliferation and differentiation, and dysregulation often results in abnormal activation of transcription factor STAT3, linked to human pathologies. Upon specific AKT phosphorylation of EZH2, the enzyme is able to bind and methylate STAT3 on its lysine 180, leading to its activation and increased gene expression [66]. Moreover, another methylation event catalyzed by EZH2 on STAT3 was identified on its lysine 49, which promotes the expression of a major subset of IL-6-induced STAT3-dependent genes [67]. These EZH2-promoted positive gene transcription are surprising knowing its PRC2-related gene silencing, and exemplifies the complexity of lysine methylation signaling and the importance of specific biological context. On the other hand, STAT3 is also methylated by SETD7 on the lysine 140 when it is promoter-bound, leading this time to repression of its transcriptional activity [68]. Finally, although no mechanistic characterization was performed, downregulation of SMYD2 in breast cancer cells leads to a global STAT3 methylation level decrease. It is unclear if SMYD2 promotes direct methylation of STAT3 under triple negative breast cancer context, or if the methylation signal results from other KMTs which activity are regulated by SMYD2 [69]. Similarly to this, Liu *et al.* found that pSTAT3 and SMYD3 can be used as prognostic indicators for gastric cancer as well as potential treatment targets [70].

NF- κ B

NF- κ B is a transcription factor playing important role in various biological processes often related to immunity, inflammation and apoptosis. It consists of several subunits regulated by PTMs often initiated by specific extracellular stimuli, causing an adapted regulation of NF- κ B and expression of its target genes. Constitutive activation of the complex is often linked to human pathologies. The NF- κ B subunit RelA ubiquitination and degradation is one possible process to stop NF- κ B activation. RelA degradation process is notably triggered after lysines 314/315 monomethylation by SETD7 [71]. On the other hand, RelA methylation on lysine 218/221 by NSD1 activates NF- κ B signaling [72,73]. This transcription factor is further regulated by the activity of SETD6, as methylation of RelA at lysine 310 decreases NF- κ B transcriptional programs, by recruiting the methyltransferase GLP and indirectly promoting a repressed chromatin state at RelA target genes [74].

HIF1

HIF-1 (hypoxia-inducible factor-1) is another transcription factor heavily modified and regulated by PTMs. HIF-1 is involved in transcriptional regulation and cell adjustment to the conditions of low oxygen concentration. It is methylated by SETD7 under hypoxia conditions when localized in the nucleus, and promotes its degradation through increased protein ubiquitination [75]. Importantly, HIF-1 α mutant that cannot be demethylated results in increased tumor growth, demonstrating the importance of its methylation for regulation of angiogenesis and vascularization. HIF-1 is also methylated by G9a/GLP, and the mono- and di-methylation of lysine 674 suppressed its transcriptional activity and expression of its downstream target genes. However, G9a/GLP methylation does not induce HIF-1 degradation like for SETD7, but rather reduces its transactivation domain activity [76].

4.1.3. Histone-independent chromatin regulation

DNA methylation signaling

DNA methyltransferases (DNMT) are enzymes catalyzing the methylation of cytosine nucleotides at CpG sites and can lead to gene silencing. DNA methylation patterns can be affected by local methylation events on histones and reciprocally, but DNMTs activity can also be regulated by direct lysine methylation. Indeed, DNMT1, important for DNA maintenance activity, is methylated by SETD7 at lysine 142 and by G9a at lysine 70. While the biological significance of methylation by G9a is yet to be determined [77], the result of SETD7 enzymatic activity on DNMT1 is important for its stability [78]. Interestingly, DNMT1 methylation at lysine 142 can have opposite effects depending on the availability of methyl-sensitive interactors. The recognition by PHF20L1 inhibits DNMT1 ubiquitination and degradation, while recruitment of L3MBTL3 lead to its degradation after ubiquitination by E3 ligase CRL4. Furthermore, the methylation of DNA Ligase 1 (LIG1) by G9a/GLP recruits the UHRF1 protein to replication foci where in turn it recruits DNMT1 to maintain DNA methylation [32]. Additionally, the methyltransferases G9a/GLP are important for the regulation of *de novo* methyltransferase DNMT3a. This event is nicely representing the PTMs dynamic and crosstalk at chromatin, both on DNA and histones, where the G9a/GLP-methylated DNMT3a and automethylated G9a/GLP are both recognized by dimeric MMP8 proteins. This sort of reinforcing loop mechanism ensure a strong dual chromatin repression both at the histone level (G9a/GLP H3K9 methylation) and DNA level (DNMT3 DNA methylation) [79].

DNA replication and repair

Another identified substrate of SET8, besides p53 and Numb mentioned earlier, is PCNA (proliferating cell nuclear antigen), and SET8-dependent lysine 248 methylation of PCNA stabilizes the protein through inhibition of its ubiquitination and degradation [80]. Moreover, PCNA methylation also promotes specific interaction with the endonuclease FEN1, important during DNA replication, a process where SET8 H4K20me is also known to play a major role during S-phase progression through regulation of heterochromatin replication [81]. Aberrant SET8 methylation of PCNA therefore results in altered DNA replication and DNA damages [80]. Interestingly, FEN1 itself was identified as a new substrate of SETD7. Lysine 377 monomethylation of FEN1 is not affecting its enzymatic activity, but seems to be important for proper cellular response to replicative stress through unknown mechanism [82]. As previously mentioned, G9a/GLP can also methylate histone mimic residues in DNA ligase 1, a methylation event important to promote LIG1 interaction with UHRF1 and for its recruitment and engagement

in DNA replication [32]. The authors observed that after inhibition of G9a/GLP, LIG1 still localizes to DNA replication sites but UHRF1 is not, leading to subsequent reduction of DNA methylation. This finding changes the proposed mechanism of DNA replication and UHRF1 mechanism of enrollment to DNA methylation. Another protein involved in many important nuclear processes is PARP1, important for DNA repair, transcription and chromatin remodeling. The functions of PARP1 are largely regulated by PTMs and notably, SMYD2 methylation at lysine 528 increases by unknown mechanism the enzymatic activity of PARP1 after oxidative stress [83]. Finally, PTEN is a tumor suppressor phosphatase known to negatively regulate AKT signaling pathway and often repressed in cancer. A recent study from Zhang *et al.* demonstrates how nuclear PTEN methylation by NSD2 increases efficient DNA double-strand break repair and affects cell resistance to DNA damage. Following DNA double-strand break, NSD2 dimethylates lysine 349 of PTEN, which is specifically recognized by TP53BP1 and in turn recruits PTEN to DNA damage sites where it can govern efficient DNA repair.

4.1.4. Other regulations by non-histone lysine methylation events

WNT/ β -catenin signaling pathway

SETD6 is another SET-KMT methylating several proteins involved in important pathways related to carcinogenesis. Notably, a recent description of new methylation event on p21-activated kinase 4 (PAK4) by SETD6 linked its activity to the activation of WNT/ β -catenin pathway in cancer progression, as PAK4 methylation strengthen its interaction with β -catenin [84]. PAK4 phosphorylates β -catenin, and both PAK4 and SETD6 depletions are lethal for embryonic development. Finally, the WNT/ β -catenin signaling pathway is important for normal embryonic development, and is also directly regulated by the SMYD2-dependent methylation of β -catenin lysine 133, modification that regulate nuclear translocation and further signal transduction and gene expression promoted by β -catenin [85].

Nuclear receptors regulation

Nuclear receptors are involved in regulation of many cellular processes, such as cell differentiation, development and proliferation, and can act as transcription factors. Estrogen receptor alpha (ER α) and its response to hormone stimulation is largely regulated by fine-tuning of several PTMs. The stability of this receptor is regulated by SETD7 on lysine 302, event that further enhances the chromatin recruitment of ER α to its target genes [86]. Interestingly, SMYD2 can also methylates ER α , this time on its lysine 266, with an opposite repressive effect on estrogen signaling [87]. SMYD2 methylation indeed acts in opposition with another modification on ER α lysine 266, as estrogen stimulation induces p300/CBP-dependent acetylation of residue K266, promoting ER α transactivation activity. Finally, G9a also methylates ER α on lysine 235 and participates in an elegant crosstalk between different PTMs signaling on both non-histone and histone proteins. The Tudor domain of PHF20 interacts with G9a-dependent dimethylation of ER α K235, and later on recruits the MOF histone acetyltransferase (HAT) complex to ER α target genes promoters leading to surrounding histone H4K16 acetylation, therefore enabling a stable active transcription [88]. ROR α (Retinoid-related orphan receptor alpha) is another modified nuclear receptor, methylated this time by EZH2. This methylation is recognized by the chromo domain of DCAF1, an adapter protein bound to DDB1/CUL4 E3 ubiquitin ligase promoting ubiquitination and substrate degradation. Authors of this study suggest that such methylation-dependent ubiquitination mechanism might regulate stability of various proteins [89].

mRNA translation and splicing

Over the last few years, several proteomics studies observed that proteins components of the mRNA splicing machinery are frequently methylated [90–92]. While the responsible enzymes and the consequences of these lysine methylation events remains unclear, it is interesting to imagine that lysine methylation signaling could alter the splicing machinery and actively participate in alternative proteins isoforms translation upon specific stimuli. Notably, the methyltransferase SETMAR is known to methylate snRNP70 at lysine 130, a key early regulator of 5' splice site selection, suggesting that such regulatory is probable.

Chaperones regulation

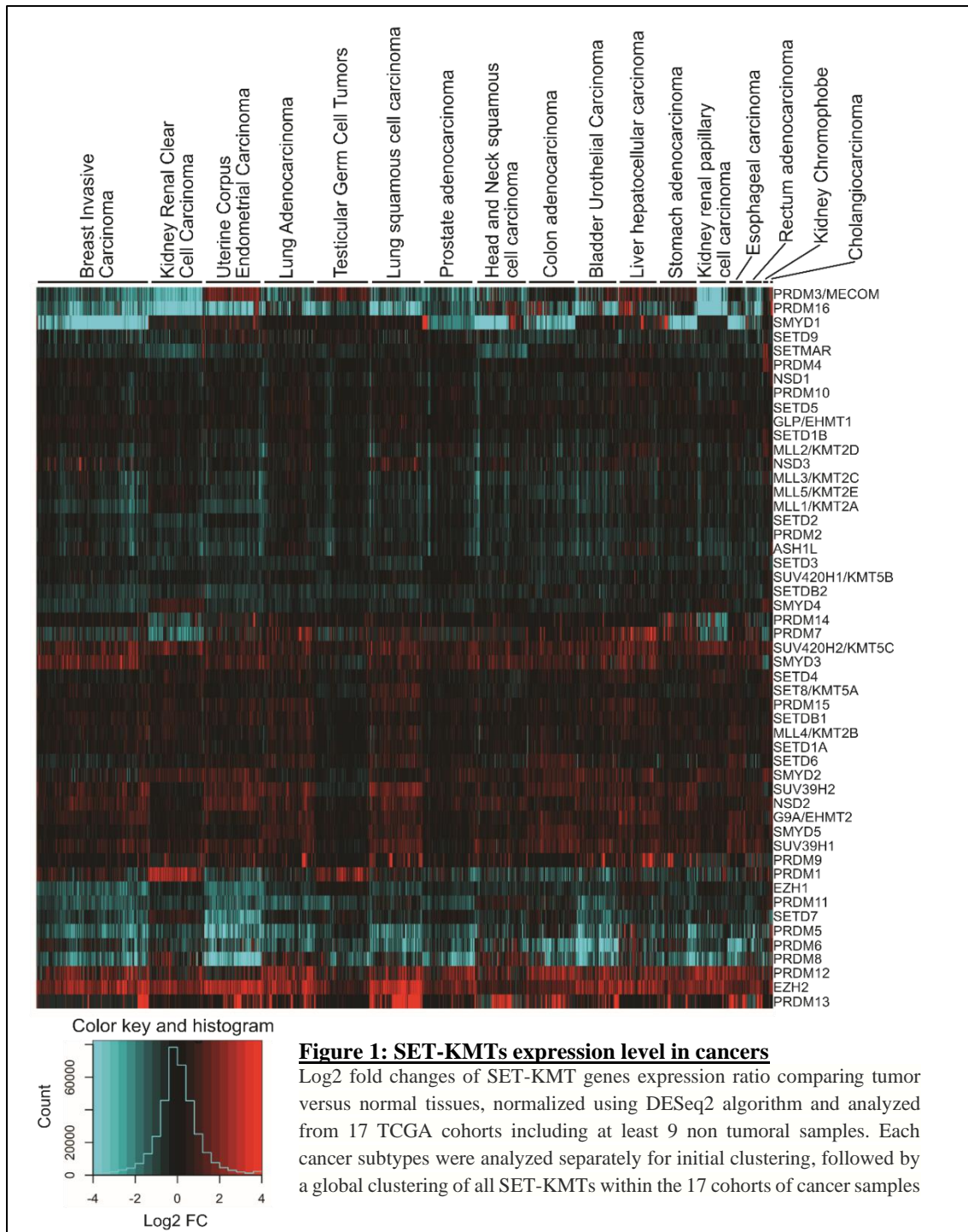
Two important chaperones regulated by lysine methylation are evolutionarily conserved heat-shock proteins (HSP) 70 and 90. From immunohistochemistry analysis it was found that HSP70 is heavily methylated in cancer tissues. Cho *et al.* observed that HSP70 dimethylation by SETD1A leads to cytoplasmic to nuclear change in protein localization, which enables this chaperon to interact with protein Aurora kinase B and thereby influences cell cycle [93]. However, this methylation characterization is controversial as it was also demonstrated that the 7 β -strand enzyme METTL21A methylates the same lysine residue and that METTL21 depletion leads to complete loss of HSP70 methylation [94]. The second chaperone HSP90 interacts with several SMYD proteins, and is found to be methylated by SMYD2. This modification is important in cancer, by promoting HSP90 dimerization and allowing chaperone complex formation, as well as in muscle development through myofilament organization [95,96].

Muscle development

Although SMYD2 and SMYD3 deregulations in cancer suggest their high oncogenic importance, the entire SMYD family members seem to play a role in development of skeletal muscles [97–99]. SMYD1 is specifically expressed in muscle cells and is linked to cardiac differentiation as histone methyltransferase, but this activity remains controversial. Additionally, Warren *et al.* recently described its relevance in heart development, by regulating mitochondrial respiration through interaction with PGC-1 α (proliferator-activated receptor gamma coactivator 1-alpha) [100]. Other members of the SMYD family seem less specific to muscle cells but all seems related somehow to muscle physiology such as myofilament organization, sarcomere assembly, heart development and adult muscle functions, as already well documented in the review by Du *et al.* [99].

4.2. SET-KMTs and cancer

Most of the above mentioned KMTs functions can be directly or indirectly linked to cancer, and their dynamic, reversible and specific methylation signaling suggest they could be interesting prognosis biomarkers or therapeutic targets. Protein methylation and other PTM signaling therefore led to the development of “epigenetic therapy”, however mainly focusing on histones PTMs and chromatin regulation. Unfortunately, targeting lysine methylation signaling for therapy is just at its beginning compared to other PTMs, because of the highly specific and context-dependent signaling of lysine methylation that could promote different outcomes in specific cancers. Therefore, different tumors should be classified for epigenetic therapy based on their methylation signaling pattern [101]. This becomes especially important on the example of EZH2 which can have a dual function, as oncogene taking part in cell proliferation and survival and even drug resistance, or on the other hand, acting as tumor suppressor [102]. Therefore, the therapy targeting this KMT should be adjusted depending on its function [102–104]. For instance, EZH2 is considered an interesting target for different types of leukemia with several inhibitors already in clinical trials, and is additionally in another clinical trial as part of a combination therapy for metastatic castration resistant prostate cancer [101]. In addition, our actual knowledge on lysine methylation events demonstrates that many modifications occur in combination with other PTMs, crosstalk of which often results in different biological consequences. These interactions are therefore adding another layer of complexity in understanding the methylation network in human diseases and cancer. From different examples listed in the section above, it is clear that a great number of non-histone methylation signaling are also important for cancer development, such as p53. Interestingly, while TP53 is one of the most mutated gene in cancer because of its multiple facets of tumor suppressor activity, some cancers are quite notorious for being rarely mutated for TP53. It is highly likely that these cancers still need to downregulate p53 functions, and one possibility of such alternative repressive mechanism is lysine methylation by SMYD2 and SET8. Other examples of KMTs strongly correlated with cancer development are SMYD2 and SMYD3. While both seems to strengthen RAS-dependent oncogenic signaling already induced by multiple pathways in particular cancers, they might not be highly efficient targets for therapy because of alternative oncogenic pathways in these cancers. However, because of their low ubiquitous expression, limiting potential side-effects, they might be advantageous for combinatorial therapeutic strategy to increase efficiency of RAS signaling inhibitors or to decrease the required dosage of such treatment for similar efficiency [105]. Interestingly but not surprisingly, the abnormal expression of SET-KMT is a frequent event in human tumors, with a large spectrum of potential deregulated lysine methylation signaling therefrom (Figure 1). Here, we analyzed the expression ratio of each SET-KMT in a given cancer sample compared to its basal



expression in a corresponding normal tissue. One important observation that can be drawn from this bioinformatics analysis using cancer cohorts from TCGA is the heterogeneity of SET-KMTs deregulation. About half of all SET-KMTs presents significant altered expression level in cancer. Some are clearly upregulated in few specific cancers while otherwise unaltered (see PRDM1 for example), some are frequently but lightly upregulated in cancers (see SUV420H2 for example) while other are strongly expressed or repressed in various cancers (EZH2 and reversely PRDM16). Interestingly, apart from already known clear oncogenic enzymes such as EZH2 being massively upregulated in almost every cancer samples, some poorly characterized ones like PRDM12 are also found to be significantly deregulated, claiming for a strongest scientific interest. It is also worth noting that specific patterns of SET-KMTs deregulation can be observed in specific cancers (for example, kidney renal clear cell carcinoma with specific SMYD4, PRDM1, SETD7 upregulation together with specific PRDM7 and PRDM16 downregulation). One example of MT without clear function but linked to breast cancer is SETD4. Breast cancer

cell proliferation is correlated with high expression of SETD4 and lack of ER, with potential involvement of SETD4 in cell cycle regulation [106]. Finally, while there is evidence of SET-KMTs still without clear functions being deregulated in various cancers, there is also evidence arising from recent proteomics analysis suggesting the existence of a great number of cancer-related proteins being methylated without known responsible MTs. For example, several proteins of the splicing machinery are methylated without clear functional consequences, but cancer cell can be highly dependent to abnormal splicing.

5. 7 β -STRAND KMTs: A NEW SPECTRUM OF LYSINE METHYLATION SIGNALING

Until recently, conserved histone H3 methyltransferase DOT1L was the only described histone KMT not containing a SET domain [107]. It belongs to the largest class of SAM-dependent methyltransferases, not structurally related to SET family, called 7 β -strand MTs (representing 60% of MTs in human) [11]. In the recent years, several new atypical MT enzymes of this group were classified as lysine MTs, but the vast majority of them are still uncharacterized. An interesting review by Falnes and colleagues summarizes in 2016 the atypical protein lysine methylation from 7 β -strand enzymes [108]. However, recent identifications of new lysine methylated events related to these class of enzymes prompted us to update this original review (Table 1).

Compared to SET-domain MTs that specifically targets lysine residue (with the recent exception of SETD3 methylating histidine 73 on actin), 7 β -strand enzymes encompass methylation activity for a broad range of substrates such as DNA, RNA and proteins, and are structurally more related to arginine MTs than SET-KMTs. Therefore, it was not thought at first that such enzymes could methylate lysine residues similar to SET MTs, and DOT1L, methylating lysine 79 on histone H3, was perceived as an exception. Interestingly, a striking difference between SET and 7 β -strand lysine MTs is that SET-KMTs mainly target easily accessible N-terminal end of histones, whereas DOT1L methylates H3K79, a residue within the nucleosome core and supposedly less accessible [107]. This suggests a different mode of substrate recognition and indeed, 7 β -strand enzymes seems to have different recognition patterns for their non-histone substrates, as SET-KMTs tend to methylate lysines within unfolded and disordered region of a protein while 7 β -strand enzymes need to recognize their substrates within three-dimensional folded domain in order to methylate their targeted lysine [108]. Moreover, 7 β -strand enzymes seem to target a single substrate, or a unique group of structurally similar proteins, and to massively methylate such substrate, compared to SET-KMT more inclined to modify only a small subset of their substrate's relative population. This high ratio of methylation among a substrate's population, suggests that 7 β -strand KMTs signaling are less dynamic and rather a required modification for a substrate's function rather than a reversible and fine-tuned regulation system promoted by SET-KMTs. Finally, also different from the processive mode of action of SET enzymes, 7 β -strand MTs have a distributive mode of action, meaning that the enzymes have to dissociate from the substrate in order to process with next methylation step [108]. Interestingly, beside DOT1L and recently N6AMT1, all lysine methylation events described so far for this class of MTs are affecting non-histone proteins. Even though there is a growing evidence of wide variety of methylation events for 7 β -strand MTs, including lysine methylation as will be further detailed here, the vast majority of them still needs a better characterization together with a better understanding of the biological significance for each of their related methylation events.

Table 1: 7 β -strand lysine methyltransferases in human

Listing of human 7 β -strand-containing enzymes validated as lysine methyltransferases, with their identified substrates, the indication targeted lysine residues and the importance of these methylation events in different signaling regulations.

Enzyme	Alternative names	Substrate	Lysine residue	Signaling regulation	Refs
DOT1L	KMT4	Histone H3	K79me1/2/3	Chromatin regulation	[107]
FAM86A	EEF2-KMT	eEF2	K525me3	mRNA translation	[118]
FAM173A	ANT-KMT	ANT1/2	K52me3	Mitochondrial metabolism	[140]

FAM173B	ATPSC-KMT	ATP synthase c-subunit	K43me3	Mitochondrial metabolism	[133]
CAMKMT	C2orf34	Calmodulin	K115me3	Neural development	[136]
ECE2	EEF1A-KMT4	eEF1A	K36me2/3	mRNA translation	[115]
METTL10	EEF1A-KMT2	eEF1A	K318me3	mRNA translation	[114]
METTL12	CS-KMT	Citrate synthase	K368me1/2/3 or K395me1/2/3	Mitochondrial metabolism	[131, 132]
METTL13	EEF1A-KMT5	eEF1A	K55me2	mRNA translation	[116, 117]
METTL20	ETFB-KMT	ETF β	K200me2/3 and K203me2/3	Mitochondrial metabolism	[128– 130]
METTL21A	HSPA-KMT	HSPA1 HSPA5 HSPA8	K561me3 K585me3 K565me3	Chaperones / protein stability	[94]
METTL21B	EEF1A-KMT3	eEF1A	K165me1/2/3	mRNA translation	[111]
METTL21C	C13orf39	HSPA8 VCP/p97	K561me3 K315me3	Chaperones / protein stability	[121]
METTL21D	VCP-KMT	VCP/p97	K315me3	Chaperones / protein stability	[119– 121]
METTL22	C16orf68	KIN17	K135me3	Chromatin regulation	[119]
N6AMT1	KMT9	Histone H4	K12me1	Chromatin regulation	[137]
N6AMT2	EEF1AKMT1	eEF1A	K79me3	mRNA translation	[113]

5.1. Major cellular signaling regulated by 7 β -strand KMTs

5.1.1. mRNA translation

Proteins are synthesized from a messenger RNA after translation by the ribosomal machinery. This event is divided in three phases, the initiation where ribosome assembles around the mRNA, the elongation where amino acids are transferred from tRNA corresponding to their codon counterpart, and the termination triggered by a stop codon. Such process is highly regulated and requires various specific proteins, such as elongation factors facilitating translational elongation. Currently, the most described methylated substrate by 7 β -strand MTs is the eukaryotic elongation factor 1 α (eEF1A), a protein involved in the delivery of tRNA to ribosomes during translation. Other suggested roles of eEF1A are cytoskeleton modulation, protein degradation and apoptosis [109], and this protein is known to be heavily modified by posttranslational modification. Phosphorylation events are largely described [110], but multiple lysine residues can also be methylated, as discovered by mass spectrometry analysis [109]. However, only recently enzymes catalyzing these methylation events were identified as 7 β -strand MTs [110–112]. These multiple methylation events are described and summarized in the review by Hamey and Wilkins, showing the process in identification of methylation sites using mass spectrometry analysis, *in vitro* methylation assays and gene knockouts [110]. eEF1a is methylated at lysine 79 by N6AMT2/eEF1A-KMT1, an enzyme previously thought to be a N(6)-adenine-specific DNA methyltransferase [113]. eEF1a is also methylated at lysine 318 by METTL10/eEF1A-KMT2, the ortholog of yeast SEE1, previously known for methylating yeast eEF1a [114]. However, in both cases, the molecular and biological consequences of eEF1a methylation remains

unclarified. Hamey *et al.* found in 2017 using CRISPR/Cas9 technology that METTL21B (eEF1A-KMT3) is a new KMT modifying eEF1A on lysine 165 [111]. Here again, the molecular consequences of this PTM are unknown, but it has been shown by ribosomal profiling that METTL21B repression affects mRNA translation. Interestingly, eEF1A K318 methylation level is tissue and cell dependent, and can be induced by stress signals also affecting METTL21B expression [112]. Another methylation event characterized on lysine 36 of eEF1A and catalyzed by ECE2 (EEF1A-KMT4) also affects mRNA translation and its dynamics by changing translation speed of distinct codons [115]. Finally, an additional eEF1a methylation event was identified in 2018, induced on lysine 55 by METTL13 [116]. Initial studies observed that once again this modification affects mRNA translation efficiency, and in 2019, the work of Liu and colleagues demonstrated the first oncogenic impact of a 7 β -strand KMT. Indeed, METTL13-dependent methylation increases eEF1A's intrinsic GTPase activity *in vitro* and protein production in cells, and RAS-driven cancer highjacks this signaling to meet their elevated protein synthesis requirement [117]. To conclude this section, the related eEF2 protein is also regulated by PTMs and its methylation on lysine 525 by FAM86A might also be important for protein translation, as suggested by the study on its respective yeast homolog [118].

5.1.2. Chaperones

Chaperone proteins allow for covalent folding or unfolding of other proteins and the assembly or disassembly of macromolecular structures. Chaperones are therefore involved in folding of newly made proteins as they are extruded from the ribosome, as well as maintaining folded proteins during specific cellular contexts such as elevated temperatures. In 2013, a pioneer work by Cloutier and colleagues observed that various 7 β -strand lysine MTs interact with chaperone proteins. Using TAP-tag purification, they demonstrated that METTL18, CAMKMT, METTL20, METTL21A, METTL21B, METTL21C, METTL21D, METTL22 and METTL23 interact with single or several chaperones such as HSP70 and HSP90-related proteins, chaperonins HSPD1 and CCT or the valosin-containing protein (VCP or p97) [119]. As expected, several of these partners proved to be substrates. The same authors found that METTL21D methylates VCP, confirming previous finding by Kernstock *et al.* about the methylation event on lysine 315 [119,120]. Another study suggests that METTL21C also methylates VCP and regulates its activity in skeletal muscles and mitochondrial activity [121]. However, the consequences of VCP methylation are still not clear and further studies need to relate this PTM signaling to the regulation of VCP functions in cell cycle, autophagy, protein degradation, or its ability to act as ATP-dependent chaperone [120]. Another well documented methylation of chaperone is the trimethylation of the respective lysines 561, 585 and 565 of HSP70 isoforms HSPA1, HSPA5, and HSPA8. The discovery of HSP70 methylation date to 1982 [122] but was only recently linked to METTL21A activity [94]. Moreover, it was recently observed that METTL21C may also methylate the same HSP70 residue, but the veracity of such event needs further confirmation and seems less robust than METTL21A activity. HSP70 methylation seems to alter the chaperone affinity for the Parkinson disease-associated protein α -synuclein, leading to a reduced ability to sequester the protein. However, it is likely that HSP70 methylation plays other important functions as stress-inducible HSP70 is overexpressed in tumor. It was found that dimethylated HSP70 by SETD1A predominantly localizes to the nucleus of cancer cells and that nuclear HSP70 directly interacts with Aurora kinase B (AURKB) in a methylation-dependent manner and promotes AURKB activity [123]. However, this potential oncogenic signaling is controversial [124] and further studies are required to truly establish a link between HSP70 methylation and cancer, whether through SETD1A, METTL21A or METTL21C activity.

5.1.3. Mitochondria respiratory - metabolism

Mitochondria plays a central role in cell metabolism and in providing cell's energy, therefore any dysregulation might result in drastic development of disorders and pathologies, including cancer [125]. Cancer cells are dependent on high level of ATP and metabolites because of their fast division rate, and often become addicted to mitochondria's alterations promoting energy metabolism advantages. Interestingly, one protein involved in mitochondrial homeostasis is p53, previously mentioned as largely regulated by PTMs. Mutations in p53 genes lead to deregulation of its target genes, in part resulting in a change in mitochondrial activity from energy metabolism to glycolysis, which can be reversed by restoring proper p53 function [126]. However, p53 methylation by SET-KMTs have yet to be linked to mitochondria homeostasis. While few evidence suggest that

SET-KMTs may play a role in mitochondria metabolism, such as PGC-1 α methylation by SMYD1 [100] or SETD7/9's implication in ROS production [127], 7 β -strand KMTs appear to methylate and regulate various important mitochondrial proteins. In 2014, two independent works classified METTL20 as the first mitochondrial 7 β -strand KMT. Logically, they identified a mitochondrial protein as its substrate, the electron carrier ETF β [128,129]. METTL20 trimethylates two residues of ETF β , K200 and K203, located near the "recognition loop", a region of the protein involved in the electron transfer to dehydrogenases. It is proposed that the two close-by methylations result in a bulky group in this loop that influence ETF β interaction with dehydrogenases, indirectly influencing the oxidation of fatty acids. Therefore, METTL20 biological functional significance is linked to mitochondria metabolism, and potential METTL20 mutations could have an effect on metabolic diseases. Confirming this hypothesis, mutating lysines 200 and 203 of ETF β abolished metabolic phenotypes observed, demonstrating the regulatory importance of this methylation signaling [129]. Further study by Shimazu *et al.* using METTL20 KO mice confirmed *in vivo* that the methylation of ETF β is important in oxidation of fatty acids when they are the main energy source [130]. However, METTL20 KO mice develop normally, suggesting METTL20 is not essential for general mitochondria metabolism but rather specific to special contexts requiring a proper level of energy production. In 2017, a second mitochondrial human lysine methyltransferase was discovered, METTL12. This enzyme catalyzes lysine methylation of citrate synthase (CS) on its lysine 395, located near its active site, thereby potentially affecting the enzyme activity consisting of the condensation of acetyl-CoA and oxaloacetate to form citrate [131]. Interestingly, some sort of negative feedback loop was observed as it was demonstrated that METTL12 is inhibited by the CS's substrate oxaloacetate. However, a second methylation event on lysine 368 of CS, also catalyzed by METTL12, was identified by Rhein *et al.* at the same time, with a potential impact on CS interaction with other enzymes of the tricarboxylic acid cycle (Krebs cycle) [132]. It is not clear if both methylation events exist concomitantly, independently or if one site has been mislabeled, but the biological impact of METTL12 on mitochondria metabolism seems definitely significant. A third mitochondrial human KMT was identified when FAM173B (family with sequence similarity 173 member B) was found by bioinformatics analyses to harbor an atypical mitochondrial targeting sequence responsible for its localization to mitochondria. FAM173B catalyzes the trimethylation of lysine 43 of ATPSc, the ATP synthase c-subunit, and lack of ATPSc methylation following FAM173B repression leads to aberrant incorporation of ATPSc into the ATP synthase complex and decreased mitochondrial respiration [133]. Recently, Malecki *et al.* observed that the paralogue FAM173A shares the mitochondrial localization sequence of FAM173B, while being known as a membrane protein [133]. Consistent with its localization to mitochondria membrane, the authors found that FAM173A methylates ANT2/3, (adenine nuclear transporters 2 and 3) on their lysine 52, two proteins important for ATP/ADP exchange. The exclusive trimethylation of ANT2/3 was confirmed both in human cells and in rat organs, suggesting the modification to be constitutive. Authors observed that FAM173A-deficient cells present an increased mitochondrial respiration, suggesting the enzyme to have a repressive impact on mitochondrial metabolism. Altogether, these different evidences demonstrate that the 7 β -strand family members have an important role in establishing the equilibrium in mitochondrial activity and overall cell metabolism.

5.1.4. Other signaling

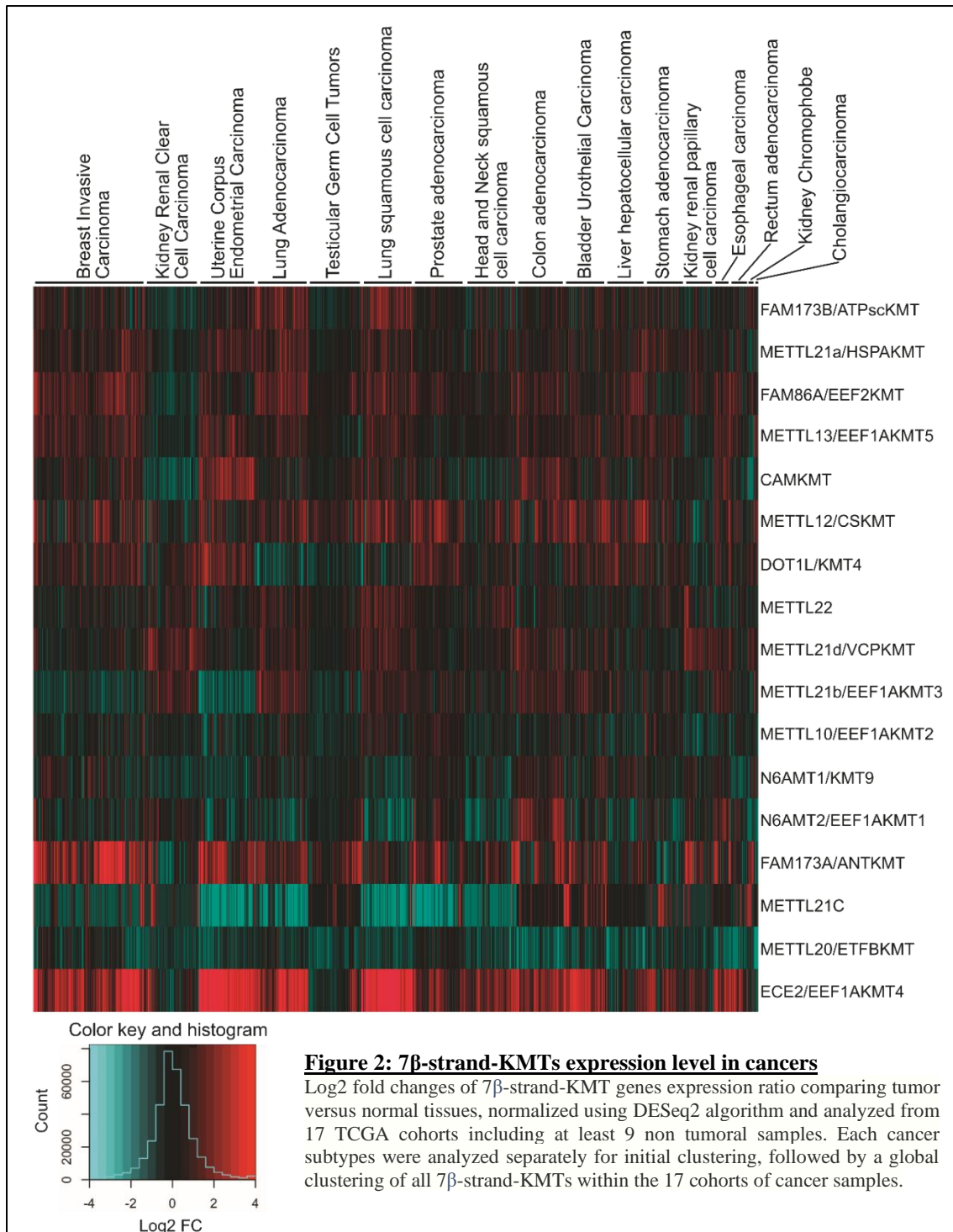
One of the first discovery related to 7 β -strand KMTs and non-histone lysine methylation is related to C2orf34 (CAM-KMT), identified as an enzyme catalyzing the methylation of Calmodulin (CAM) on lysine 115, a protein crucial for calcium-dependent signaling largely known to be regulated by PTMs [134]. Interestingly, this methylation signaling seems highly conserved as CAM-KMT and CAM homologs are present in human, rat, frog, insect and plants. Plant homolog CAM-KMT functions has been linked with root length and radial developmental [135]. It was found only several years after this original discovery that CAM methyltransferase in mouse and human is related to growth and muscle strength as well as brain function. Knock-out mice for CAM-KMT show developmental alterations and age-dependent mitochondrial defects that interestingly reflects human 2P21 deletion syndrome associated with mental disorder from patients harboring hypomethylation for protein CAM and mutations in CAM-KMT gene located on locus P21 on chromosome 2 [136].

Finally, few 7 β -strand MTs have been linked to chromatin regulation. METTL22 catalyzes KIN17's methylation on its lysine 315, with indication that this signaling influences the association of KIN17 with chromatin [119]. This methylation event might have important biological consequences as KIN17 is known to be important for

DNA double-strand break repair and to be upregulated in various cancers. Recently, a second 7 β -strand enzyme besides DOT1L was accredited with histone methylation capability, as N6AMT1 (KMT9) in complex with TRMT112 was linked to prostate cancer through its methylation of Histone H4 on lysine 12. This study demonstrated that KMT9 and H4K12me1 is enriched at the promoters of numerous genes encoding cell cycle regulators and control of cell cycle progression and that KMT9 depletion impairs proliferation of both androgen receptor-dependent and castration- and enzalutamide-resistant prostate cancer cells [137].

5.2. 7 β -strand KMTs and cancer

In regard to 7 β -strand lysine MTs functions in cell homeostasis through the regulation of metabolic, translation and protein folding processes, there is little questioning that their activity and their deregulation can be linked to



human pathologies such as cancer. However, only relatively few demonstrations of their oncogenic capability exist so far. One clear example is DOT11 and H3K79me-dependent chromatin regulation, playing a crucial role in cell transformation and notably in leukemia progression. Indeed, DOT11 repression is one of the very first epigenetic therapy under clinical trial among lysine methyltransferases. More recently, the KMT9 activity on H4K12 has been linked to prostate cancer cell proliferation, by facilitating cell cycle progression through the alteration of cell cycle related genes expression [137]. However, the first non-histone methylation event related to carcinogenesis and attributed to a 7 β -strand KMT is eEF1A methylation by METTL13. Upregulation of METTL13 in lung and pancreatic adenocarcinoma, two major RAS-induced cancers, promotes tumorigenesis and is associated with poor patient prognosis [117]. METTL13 knockout results in reduced RAS-driven tumorigenesis in mouse, as cancer cells become dependent on the METTL13-eEF1AK55me₂ signaling to reach their high protein synthesis requirement, offering a novel therapeutic direction for targeting these lethal malignancies [117]. Moreover, as shown in figure 2, a large number of 7 β -strand KMTs are deregulated in different cancers, and future works in the field will hopefully help to demonstrate their oncogenic or tumor suppressor capabilities, while providing important new prognostic markers or therapeutic options to clinicians. For example, FAM173A is strongly upregulated in a subset of breast cancer samples, and further analysis is required to demonstrate if these samples correspond to a specific type of breast cancer were FAM173A oncogenic activity could be related to mitochondrial metabolism deregulation through its methylation of ANT1/2. Moreover, ECE2 is massively upregulated in various cancers, especially in uterine corpus endometrial carcinoma and lung squamous cell carcinoma and it will be interesting to challenge if this oncogenic activity can be linked to its function on mRNA translation and the methylation of EEF1A. Reversely, METTL21C is clearly repressed in both uterine corpus endometrial carcinoma and lung squamous cell carcinoma as well as in prostate adenocarcinoma, suggesting a tumor suppressor function in these cancers. Finally, further studies will allow to demonstrate if expression level of 7 β -strand KMTs correlates with cancer prognosis and can be used as biomarkers for specific cancers along with the interest to target their signaling for therapy.

6. PERSPECTIVES AND FUTURE DIRECTIONS

The importance of histone lysine methylation in regulation of biological events led to development of different experimental approaches to further understand this PTM. The development of comprehensive proteomics approaches recently enabled the identification and description of larger sets of methylation events, which often occur in carcinogenesis related pathways [13]. Detection of methylation events presents a challenge because of the difficulty to enrich methylated proteins, the reason why this PTM is not as investigated as phosphorylation or acetylation. Indeed, the intrinsic mechanism of this PTM, by mostly regulating specific methyl-sensitive interactions, prevents the development of efficient pan-methyl antibodies or pan-methyl binding domains to identify a methylated lysine, not to mention considering the different methylation state of a same lysine. First approaches to detect methylation events were implementing candidate-based approach biased towards histones or well-studied proteins, and were using partial protein degradation (Edman degradation), radioactively labelled methyl donors or immunoblotting with methyllysine specific antibodies. Even though these techniques are useful, they do not allow for comprehensive identification of each KMT's specific substrates and the lysine "methylome", which led to recent development of high-throughput methods [17]. These methods largely involve using quantitative mass spectrometry for identification of methylated residues in proteins, after attempts to enrich methylated proteins, either by methyl-binding domains or methyl-lysine antibodies. Other techniques rely on *in vitro* methylation assay using protein arrays as template, or using degenerated peptide arrays to identify consensus sequence preferentially targeted by a KMT. An alternative approach consists to identify a KMT responsible for a well described methylated lysines. For example, Pinheiro *et al.* identified PRDM3 and PRDM16 as KMTs responsible for H3K9 monomethylation by carefully purifying the active complex after a series of chromatographies [138]. More recently, Liu *et al.* identified METTL13 as the KMT responsible for the dimethylation of lysine 55 of eEF1A (eEF1AK55me₂) by monitoring this methylation event in different cells engineered by CRISPR to repress each KMT individually [117]. Additionally, Scheer *et al.* recently designed chemical biology toolbox to study methyltransferases and epigenetic signaling, using various chemical probes to see effects of inhibition of different MTs on T cell differentiation. This approach may help to decipher biological functions/roles of yet poorly characterized MTs [139]. Combination of these techniques is significantly

contributing in building a lysine methylation network, allowing for a better envision of the importance of lysine methylation in cell biology. Lysine methylation signaling on non-histone proteins is an important field of research, and its thorough characterization will finalize the demonstration of its implication in the regulation of important cellular processes such as cell proliferation, growth or differentiation, either through direct modification or through interplay with other PTMs [14].

With novel methylation events regularly emerging in the literature, there is clearly a great potential and interest to study lysine methylation. In this review we summarized the available information of “writer enzymes”, and thereby present just one side of the puzzle. However, the other side related to “eraser enzymes” or demethylases, has to be considered when trying to understand the dynamic lysine methylome network. A challenging aspect related to this is the difficulty to detect a methyl group removal and overall understanding of demethylation events. However, in recent years emerged a number of active demethylation characterization, both on histone and non-histone substrates, leading to the development of several inhibitors [22]. Besides incorporating demethylases to decipher the dynamics of lysine methylation signaling, the actual snapshot of our knowledge still lacks large aspects of the lysine methylome. Already observed methylation events on non-histone substrates without identification of the responsible KMTs should be considered, as well as the role and potential activity of orphan KMTs enzymes. Investigating the uncharacterized KMTs and their activity can further facilitate the identification of potential new mechanisms of important cellular signaling regulation. In addition, it is important to take into account the large set of methyl-sensitive binders that can interplay fine-tuned lysine methylation signaling, as well as to embrace a broader vision of the importance of PTMs crosstalk allowing for a unique pattern of modifications. This “protein PTM code”, not restricted to histones, participates actively in the proper cell adaptation to a given context at a given time. Finally, a deeper understanding of the complex lysine methylome network can lead to more specific and successful targeting of KMTs and lysine methylation signaling, thereby improving epigenetics and PTM-based therapy. Indeed, based on the reversibility and specific mechanisms underlying lysine methylation signaling, actors of such signaling will probably turn out to be highly valuable therapeutic targets.

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Finally, the authors would like to apologize for potential omissions of specific works and articles. As exhaustive as this review was aimed to be, not all publications related to non-histone lysine methylation signaling could be presented and discussed properly.

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