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Integrating Genetics and Epigenetics In MDS: Advances in Pathogenesis and Disease Evolution

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Abstract

The myelodysplastic syndromes (MDS) are a group of clonal diseases characterized by inefficient haematopoiesis, increased apoptosis and risk of evolution to acute myeloid leukaemia. Alterations in epigenetic processes, including DNA methylation, histone modifications, miRNA and splicing machinery, are well known pathogenical events in MDS. Although many advances have been made in determining the mutational frequency, distribution and association affecting these epigenomic regulators, functional integration to better understand pathogenesis of the disease is a challenging and expanding area. Recent studies are shedding light on the molecular basis of myelodysplasia and how mutations and epimutations can induce and promote this neoplastic process through aberrant transcription factor function (*RUNX1*, *ETV6*, *TP53*), kinase signalling (*FLT3*, *NRAS*, *KIT*, *CBL*) and epigenetic deregulation (*TET2*, *IDH1/2*, *DNMT3A*, *EZH2*, *ASXL1*, *SF3B1*, *U2AF1*, *SRSF2*, *ZRSR2*). In this review we will try to focus on the description of these mutations, their impact on prognosis, the functional connections between the different epigenetic pathways, and the existing and future therapies targeting these processes.

Keywords

Genetics; Epigenetics; MDS

Introduction

The myelodysplastic syndromes (MDS) are a group of clonal diseases characterized by inefficient haematopoiesis, increased apoptosis and risk of evolution to acute myeloid

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Conflict of Interest

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leukaemia (AML). Disease complexity is not only entailed by morphological diversity, which translates in an array of entities encompassed under the term MDS, but by the increasing number of molecular pathways and hallmarks that participate in disease initiation, evolution and progression to AML. Approximately 10–20% patients with MDS will ultimately develop a secondary AML. Efforts have been made to try to identify patients at risk of this final event. Solid evidence of the molecular events related to disease progression still remains elusive, with frequent contradictory or scarce data on the field. Understanding of the different molecular mechanisms responsible for initiation and progression of the disease have been an area of active research in the last decades.

The main biological hallmarks in myelodysplasia have been well described and include both genomic and epigenomic alterations in transcription factors, epigenetic modulators, miRNA, microenvironment and innate immunity (Bejar *et al*, 2011). DNA methylation remains the single most important mechanism of epigenetic regulation through the activation or repression of gene expression both at CpG-enriched promoters (CpG islands), and gene enhancers present outside of these genomic areas (Bird, 2002; Issa 2013). Altered methylation patterns have been shown to exist in myelodysplasia, with global hypermethylation of CpG islands inducing silencing of target genes (many of them being cell cycle regulators), to appear in initial stages of the disease and to be associated with disease progression (Figueroa *et al*, 2009; Jiang *et al*, 2009; Issa, 2013). Histone modifications represent another of the essential cellular mechanisms implicated in tissue-specific, transcription factor-dependent regulation of gene expression. Through methylation, acetylation and ubiquitination of different histones, specific changes to transcriptional activation and repression participate in cell fate, differentiation and regulation of proliferation. Aberrant expression of these processes due to alterations in histone regulators is one of the key pathogenic features in MDS (Issa, 2013).

The goal of this review is to focus on the known epigenetic alterations described in MDS in order to try and integrate the current mutational landscapes in these modulators with functional modelling of the disease and existing and potential therapies.

Epigenetic modifications in MDS

The epigenetics behind MDS remained a mystery until the advent of next-generation whole-genome and whole-exome sequencing techniques, allowing a more comprehensive evaluation of the epigenome in MDS. The majority of novel gene mutations that have been identified play a role in DNA methylation, histone modification and RNA splicing. Figure 1 summarizes the different epigenetic modifiers that are known to be mutated in MDS and their relation to overall prognosis or evolution to AML.

DNA methylation

In normal tissue, the CpG-rich promoters remain mainly unmethylated regardless of differentiation state (Bird 2002). In MDS, about 3–5% of these promoter-associated CpG islands become aberrantly hypermethylated (Figueroa *et al*, 2009; Jiang *et al*, 2009), often independent of cytogenetic changes. The hypermethylation may occur early in the disease and is found to be associated with more rapid progression to AML (Shen *et al*, 2010).

TET2—Loss-of-function mutations occur in about 19–26% of MDS patients (Guo *et al* 2011). Various studies have shown both hypermethylating and hypomethylating profiles related to *TET2* mutations (Figueroa *et al* 2010, Ko *et al* 2010), with *TET2* deletions leading to an increase in haematopoietic stem cell (HSC) compartment and self-renewal (Quivoron *et al*, 2011; Moran-Crusio *et al*, 2011; Ko *et al*, 2011; Li *et al*, 2011). The clinical implication of *TET2* mutations remains unclear. A recent study demonstrated that patients with *TET2* mutations had a higher overall response rate to azacytidinetreatment with no difference in OS (Itzykson *et al*, 2011).

DNMT3A—Mutations in *DNMT3A*, leading to a reduction in methyltransferase catalytic activity, are found in about 3–8% of MDS patients (Thol *et al*, 2011a; Walter *et al*, 2011), with nearly all bone marrow cells harbouring the mutation when present, therefore suggesting an early occurrence in the disease. It is associated with older age at diagnosis but not with other cytogenetic or clinical features (Walter *et al*, 2011). Some studies have demonstrated a worse clinical outcome with lower survival and rapid progression to AML (Walter *et al*, 2011); however this was not confirmed in further studies (Thol *et al*, 2011a; Bejar *et al*, 2012).

IDH1/IDH2—Isocitrate dehydrogenase 1 and 2 mutations lead to decreased α-ketoglutarate (α-KG), TET inhibition and widespread promoter hypermethylation of DNA (Xu *et al*, 2011). Other α-KG dependent enzymes have also been found to be inhibited by 2-hydroxyglutarate (2-HG) (Chowdhury *et al*, 2011; Lu *et al*, 2012). However, the impact on OS and evolution to AML remains unclear (Patnaik *et al*, 2012; Kosmider *et al*, 2010; Thol *et al*, 2010). About 4–12% of MDS cases show *IDH1/2* gain-of-function mutations (Patnaik *et al*, 2012; Kosmider *et al*, 2010; Thol *et al*, 2010). Functional effects of *IDH1* and *IDH2* mutations on haematopoiesis are unclear, with early studies demonstrating impaired HSC differentiation (Figueroa *et al*, 2010).

Histone modification

Post-translational modifications of histones are also an important part of epigenetic regulation. These proteins can be acetylated, methylated, and ubiquinated by a group of histone-modifying enzymes.

EZH2—*EZH2* is an important part of the Polycomb Repressive Complex 2 (PRC2), which trimethylates lysine 27 of histone 3 regulating stem cell differentiation and repression of gene transcription. Loss-of-function mutations are found in about 6.4–12% of patients (Bejar *et al*, 2012; Ernst *et al*, 2010; Bejar *et al*, 2011) and have been associated with poor overall survival (OS) independently of other prognostic factors and mutations.

ASXL1—Another gene that is a component of the PRC and plays a role in MDS is *ASXL1*. Loss-of-function mutation in this gene is the third most frequently mutated in MDS, occurring in about 11–21% of MDS patients (Rocquain *et al*, 2010; Gelsi-Boyer *et al*, 2009; Boultwood *et al*, 2010; Thol *et al*, 2011b) and in about 10–15% of patients with myeloproliferative neoplasm (MPN) syndromes (Rocquain *et al*, 2010; Gelsi-Boyer *et al*,

2009; Boultwood *et al*, 2010). Like the *EZH2* mutation, it is associated with poor prognosis with worse OS independent of other clinical factors.

RNA splicing

RNA splicing is an essential part of pre-mRNA maturing into mRNA for translation into proteins. More than 90% of human genes undergo splicing and translate into various protein isoforms; therefore, splicing is an integral process of gene expression diversity (Wahl *et al*, 2009; Chen & Manley, 2009). RNA splicing is the most commonly mutated pathway in MDS, and appears to occur early in disease evolution (Papaemmanuil *et al*, 2013). These mutations play a major role in dictating clinical features of the disease.

SF3B1—*SF3B1* is one of the most common mutations seen in MDS and is highly associated with the presence of ringed sideroblasts. It is seen in about 6–18% of non-ringed sideroblast MDS and in about 57–75% of ringed sideroblast MDS (Yoshida *et al*, 2011; Papaemmanuil *et al*, 2011). *SF3B1* encodes a component of U2 snRNP that recognizes 3' splice site at intron-exon junctions. Many studies have demonstrated improved OS, higher neutrophil and platelet counts, less bone marrow blasts, and low risk of leukemic evolution in patients with *SF3B1* mutations (Bejar *et al*, 2012; Malcovati *et al*, 2011).

U2AF1—*U2AF1* encodes the small subunit of U2 auxiliary factor complex, which is required for the binding of U2 snRNP to the pre-mRNA branch site. This mutation can be found in 5.4–8.7% of MDS patients (Graubert *et al*, 2012; Thol *et al*, 2012; Damm *et al*, 2012), especially in younger patients (Wu *et al*, 2013). Although it has been associated with *ASXL1* and *DNMT3A* (Thol *et al*, 2012; Damm *et al*, 2012) data from other groups have shown no significant co-occurrence of these mutations (Wu *et al*, 2013). The clinical impact of this mutation remains unclear, with contradictory data regarding the risk of progression to secondary AML (Graubert *et al*, 2012; Thol *et al*, 2012; Damm *et al*, 2012; Makishima *et al*, 2012). Recent data suggest that this mutation appears to be stable during disease progression, implying it probably plays a role in the development of MDS but not in the progression to AML (Wu *et al*, 2013).

SRSF2—*SRSF2* encodes a serine/arginine-rich splicing factor 2 that is important for selection of splice-site, assembly of spliceosome and both constitutive and alternative splicing (Long & Caceres, 2009; Wu *et al*, 2012). Mutation in this gene occurs in about 1.5–11.6% of patients (Bejar *et al*, 2012), is closely associated with the male gender and older age (Wu *et al*, 2012), poorer OS (Makishima *et al*, 2012; Wu *et al*, 2012) and other mutations such as *RUNX1*, *IDH2* and *ASXL1* (Wu *et al*, 2012; Nagata *et al*, 2011). Like *U2AF1*, it appears to be stable throughout disease progression, suggesting a role in disease initiation but not in the evolution to AML (Wu *et al*, 2012).

ZRSR2—Similar to *UA2F1*, *ZRSR2* also encodes a subunit of U2 auxiliary unit and interacts with *U2AF1* and *SF3B1* to bind to U2snRNP (Yoshida, *et al* 2011). The mutation in this gene occurs in about 1.4–8% of MDS patients (Damm *et al*, 2012). The biological effect of this mutation remains unclear and needs to be further studied.

Epigenetic Modifications in AML

Although it is beyond the scope of this review to describe the epigenetics of AML in a detailed manner, it still remains important to note that many of the previously described mutations are also found in a substantial number of AML cases (Abdel-Wahab & Levine, 2013). This data is shown in Figure 2. Additionally, recent data suggest different frequencies in mutations in epigenetic modifiers may exist between *de novo* and secondary AML. One example of this is the elegant work done by Fernandez-Mercado *et al* (2012), analysing mutation patterns of 16 genes in 84 cases of primary and secondary AML. Similar frequencies of mutations in *IDH1/2* were found in primary and secondary AML, with a higher frequency of *TET2* mutations in AML evolving from chronic monomyelocytic leukaemia (CMML) and a higher frequency of *ASXL1* mutations in secondary AML. Mutations in *DNMT3A* were found to be more frequent in primary AML. Further research analysing the frequency of these mutations in greater number of patients is nevertheless required to confirm and expand our knowledge in the field.

Several studies have shown increased overall methylation in both *de novo* and secondary AML, with higher mean overall methylation in cases with an antecedent haematological disorder, such as MDS, compared to primary AML (Figueroa *et al*, 2009; Wilop *et al*, 2011; Deneberg *et al*, 2010). Moreover, current data points to the existence of methylation patterns that lead to distinct gene-promoter signatures in primary AML compared to secondary AML and MDS, and that these patterns seem to impact evolution and clinical outcome in both diseases (Figueroa *et al*, 2009; Deneberg *et al*, 2010; Galm *et al*, 2005; Toyota *et al*, 2001). Available evidence also indicates an impact of hypomethylating agents in global and gene-specific methylation levels (Figueroa *et al*, 2009; Negrotto *et al*, 2012), allowing us to speculate if the therapeutic potential of these drugs may be related to these methylation changes. Unfortunately, most of this data still remains anecdotal and we are lacking large confirmatory studies trying to further explore methylation patterns in both MDS and AML along with their evolution with epigenetic therapies. With the development of current technologies we may yet be able to better answer these uncertainties in the following years.

Integrating the Different Epigenetic Alterations in MDS and AML Evolution

Translational and basic research in the past decades has been of utmost importance in advancing our knowledge regarding the biological markers and hallmarks of MDS. Despite the development of new technologies, which have allowed for more sophisticated methods of study in molecular oncology, the scientific community still has to face the enigma posed by the integration and interpretation of the data these advances have offered. Our ability to determine the presence of cytogenetic and molecular alterations (both genomic and epigenomic) in different settings has, unfortunately, developed at a far quicker pace than our ability to understand in a profound manner the impact and relevance of these alterations in the initiation, progression and development of oncogenesis.

Many somatic mutations in different biological markers have been described in MDS. Apart from the previously described affecting regulators of DNA methylation (Abdel-Wahab & Figueroa, 2012; Issa, 2013) (*TET2*, *IDH1*/*IDH2*, *DNMT3A*), histone modifiers (*ASXL1,*

EZH2) and splicesosome machinery (*SF3B1*, *U2AF1*, *SRSF2*, *ZRSR2*) there are others, such as transcription factors (*RUNX1*, *TP53*, *ETV6*) and signal transduction kinases (*NRAS*, *FLT3*, *JAK2*, *KIT*, *CBL*), whose detailed description is beyond the scope of this review.

In the current section of this review we will try to summarize the current evidence supporting the functional mechanisms by which these different mutations and epigenetic alterations interact in the development of MDS.

DNA methylation and its link to Histone Modification and Innate Immunity

Current understanding of the biology and functional regulation of the different DNA methylation regulators has shed light in the progressive integration of epigenetic regulation. TET2 participates in the promotion of DNA demethylation through the conversion of 5-methylcytosine (5mC) into 5-Hydroxymethylytosine (5hmC), and has recently been shown to modulate other epigenetic processes beside methylation changes due to its hydroxylase activity.

Many groups have studied the implication of *TET* genes in myeloid malignancies, including MDS, with mutations in *TET2* having been shown to induce a loss of function leading to a predominance of 5mC in DNA (Abdel-Wahab *et al*, 2009; Delhommeau *et al*, 2009; Mohamedali *et al*, 2009; Smith *et al*, 2010). This modification in the normal methylation status of DNA, due to a disruption in the normal methylation/demethylation balance, is partially responsible for the MDS phenotyp,e as has been elegantly shown using murine models by several groups (Quivoron *et al*, 2011; Moran-Crusio *et al*, 2011; Li *et al*, 2011; Ko *et al*, 2011). Data from these experiments suggests loss of function of *Tet2* induces an increased self-renewal capacity and expansion with greater repopulating potential that could be linked to an impairment of the normal regulation of *Hoxa* genes in the affected haematopoietic compartment suggesting a tumour suppressor effect (through induction of differentiation and inhibition of proliferation/stem-cell expansion) of wild-type *Tet2*. Interestingly enough, *Tet2* mutation was enough to induce myeloid expansion with a clear predominance of the monocytic compartment in a similar manner to what has been shown with other molecular markers, such as *SETBP1* (Damm *et al*, 2013) and *NRAS*, in human samples. This functional finding is consistent with clinical observations regarding disease phenotype and mutational patterns, as is the case for CMML, a subtype of MDS exhibiting a high frequency of mutations in these three genes, and morphologically characterized by peripheral and bone marrow monocytic expansion (Cazzola *et al*, 2013). Although the exact mechanisms behind this specific monocyte differentiation still remain unclear, there is room for speculating a possible synergistic effect of different epigenetic mutations in the development of such a phenotype. In this sense, there is solid data supporting a clear relationship between CMML and the simultaneous occurrence of *TET2* and *SRSF2* mutations (Cazzola *et al*, 2013) possibly due to complex epigenetic and RNA-dependent deregulation of myeloid regulatory genes. Additional investigation is however required in order to determine mutational hierarchy, identify these candidate genes and elucidate the molecular mechanisms responsible for the specific phenotype.

TET2-mediated epigenetic gene activation is also regulated through the ability of TET to mediate histone modifications through the recruitment of OGT (O-linked β-D-N-Acetylglucosamine Transferase). Once OGT is recruited, it interacts with chromatin inducing O-GlcNAcylation of Histone 2B (Chen *et al*, 2013) and host cell factor 1 (HCF1), a key regulator of the H3K4 methyltransferase complex (SET1/COMPASS) (Solary *et al*, 2014). Histone 3 Lysine 4 trimethylation (H3K4me3) is a well-known histone marker with importance in transcriptional activation and lineage determination of haematopoietic stem cells and progenitors (Barski *et al*, 2007; Orford *et al*, 2008). Loss-of-function mutations in *TET2* are responsible for a block in this histone regulation leading to a decrease in H3K4me3 and, therefore, a decrease in transcription of essential regulators of haematopoiesis (Deplus *et al*, 2013). These data clearly exemplify the complexity of functional consequences of mutations in epigenetic modifiers, and opens the possibility of speculation to possible functional links to other pathogenic events in MDS, such as innate immunity modifications. Both TET (through OGT) and JMJD3, a JmjC domain protein involved in histone methylation, act as regulators of H3K4 trimethylation whose inhibition has been shown to be related to MDS pathogenesis. A recent publication (Wei *et al*, 2013a) suggests innate immunity signals mediated through Toll-Like Receptor (TLR) signalling via nuclear factor (NF)-κB induce activation of JMJD3 which, in turn, leads to the transcriptional activation of genes promoting NF-κB expression and innate immunity signalling regulated by H3K4me3, therefore inducing an activation loop of this pathway.

An interesting observation in the original mutational studies in MDS was the mutually exclusiveness of *TET2* and *IDH1/2* mutations, which was later shown to be the result of a link between metabolism and epigenetics. TET enzymes require α-KG in order to be able to be active. Somatic mutations in the *IDH* genes lead to a disruption of their catalytic activity inducing a product shift from α-KG to its homolog, 2-HG (Ward *et al*, 2010). This phenomenon leads to an inhibition of TET not only through α-KG depletion, but through direct inhibition by 2-HG leading to increased *HOXA* activity (Xu *et al*, 2011), and therefore represents a biological explanation to the fact that *TET* and *IDH1/2* mutations are not usually present in the same MDS clone. A similar α-KG dependency occurs with JmJC demethylases suggesting, again, new functional links between these epigenetic regulators (Chowdhury *et al*, 2011; Lu *et al*, 2012).

Consistent with the above epigenetic mechanisms, deregulation of *DNMT3A*, another DNA methylation regulator, has been described in MDS and AML (Walter *et al*, 2011; Bejar *et al*, 2011). The DNMTs (DNA methyltransferases) are a group of enzymes that catalyse the addition of a methyl group in carbon 5 of cytosine to produce 5mC, and can act as either *de novo* or as maintenance transferases regulating methylation, primarily in dinucleotides at CpG islands (Li *et al*, 2013). Constitute methylation is essential for HSCs self-renewal. Evidence of a fundamental impact on the normal homeostasis of these enzymes has not only been shown through the presence of point mutations in cases of MDS (as previously noted), but through functional essays using murine models, which prove the importance of DNMTs in haematopoiesis by modulation of tissue and context-specific gene expression (Challen *et al*, 2011). In this model, *Dnmt3a*–null mice HSCs were shown to exhibit an upregulation of multipotency genes (*Runx1*, *Pbx1*, *Cdkn1a*) and a downregulation of differentiation factors (*Flt3*, *Ikzf1*, *Spi1*, *Mef2c*) due to changes in the methylation signature (including substantial

hypermethylation at CpG islands along with a global hypomethylation), further supporting the role of loss-of-function mutations in *DNMT3A* in leukaemia and MDS. A recent interesting observation by Jost *et al* (2013) is the characterization of epigenetic modifications in *DNMT3A* in AML due to aberrant hypermethylation at an internal promoter region of *DNMT3A*. Interestingly, this methylation pattern, with a silencing effect on the methyltransferase, was particularly observed in samples without mutations at the *DNMT3A* gene, and was correlated with changes in gene expression including upregulation of *HOXA* and *HOXB* clusters in a similar manner as the $DNMT3_{mut}$ cases. Comparable epigenetic modulation of essential key regulators of haematopoeisis and MDS pathogenesis may yet be described in the near future and may well be one of the reasons for mutational patterns in MDS, including co-occurrence and mutual exclusiveness of different mutations. Genomic alterations in an epigenetic modulator may lead to aberrant epigenetic silencing of a second gene (as the above mentioned *DNMT3A* epimutation), result in a proliferation disadvantage in the presence of other mutations (such as the previously described case of *TET2* and *IDH1/2*) or induce a potentiation of its effect through cooperation of a mutation on another epigenetic regulator. An example of the latter is the cooperative effect of *Tet2* mutations in *Ezh2*-deleted mice, which showed a more accelerated and advanced myelodysplasia than $Ezh2^{-/-}$ $Tet2_{wt}$ mice (Muto *et al* 2013).

Similarly to TET2, DNMT3A not only regulates transcription through DNA methylation, but can also bind to methylated and non-methylated H3K4 through its PWWP and ADD domains respectively (Li *et al*, 2013), therefore modulating histones and chromatin compaction by the formation of a DNA-DNMT3A–DNMT3L complex. In this complex, DNMT3L acts as a co-regulatory methyltransferase-like protein that modulates the activity of DNMT3A (Li *et al* 2013; Neri *et al*, 2013). As we will describe latter, DNMT recruitment to H3K4 participates in the regulation of transcription through the PRC1 and PRC2 complexes, some of whose components (such as ASXL1 and EZH2) are important players in MDS pathogenesis. It appears then that separation of different mutational events in epigenetic modulators in MDS will ultimately lead to deregulation of common hallmarks of gene regulation. Current molecular biology is slowly closing the gap between the different observational discoveries the scientific community has made in the last years, allowing us to understand the intricate mechanisms of homeostasis and disease evolution. A graphical representation of the main interactions and functions of these epigenetic modulators can be found in Figure 3.

Histone Modifications in MDS: Polycomb Repressor Complexes and regulation of H3K27me3

Modification of histones through different biochemical processes, including methylation and acetylation, represent one of the main epigenetic mechanisms in gene expression regulation in a transcription factor-dependent manner. The Polycomb pathway is a key regulator of cell-fate decisions and differentiation through H3K27 trymethylation, and is composed of two protein complexes (Polycomb group proteins or PcG), PRC1 and PRC2, which are functionally related and integrated (Morey & Helin, 2010).

PRC1 (Polycomb Repressor Complex 1) catalyses the ubiquitylation of lysine 119 in histone H2A (H2AK119Ub1), which is a repressor mark, and is dependent on PRC2 for recruitment to target genes and subsequent silencing (Müller & Verrijzer, 2009). PRC1 is composed of several proteins, including ASXL1. ASXL1 assembles various proteins to form several complexes with different functional effects, including ASXL1-BAP1, ASXL1-NHR and ASXL1-PRC2 (Katoh, 2013; Abdel-Wahab & Dey, 2013). Through its binding to PRC2, ASXL1 regulates H3K27, inducing its trimethylation through the SET domain in EZH2, inhibiting transcription of target genes (including the *HOXA* cluster of genes) by blocking transcription factors access to DNA (due to chromatin compaction), and further recruiting PRC1 for maintained gene repression. *In vitro* models assessing the effect of *ASXL1* loss have shown a marked global loss of H3K27me3 independent of the expression levels of PRC2 members, which was reversible with re-expression of *ASXL1* (Abdel-Wahab *et al*, 2012). Various murine models further support the driver effect of this mutation, with *Asxl1*+/− mice developing an MDS-like and MDS/MPN disease with increased apoptosis and proliferation in bone marrow (similarly to what is found in human MDS) (Wang *et al*, 2014). In this model, *Asxl1*-deficient mice showed upregulation of *Hoxa* genes that, again, represent a genomic hallmark of the disease common to other mutations such as *TET2*, *IDH1/2*, *EZH2* and *DNMT3A*. Without ASXL1, there is an upregulation of microRNA 125a (MIR125A) and subsequent suppression of C-type lectin domain family 5, member a (CLEC5A), leading to impairment of myeloid differentiation and development of MDS (Inoue *et al*, 2013). Interestingly, despite the presence of *ASXL1* mutations in different myeloid disorders, CMML shows a higher frequency of mutations than other MDS subtypes or AML (Cazzola *et al*, 2013) suggesting an essential role of this gene in granulomonocytic development. In fact, a recent study by Davies *et al* (2013) has demonstrated an impairment of granulomonocytic differentiation in *ASXL1*-deficient CD34+ cells due to downregulation of genes involved in myeloid and monocyte-macrophage development (*MSR1*, *IL6*, *APOC1*, *CCL2*, *CCL* and *CD14*), further supporting a possible connection between this gain of function-mutation and a monocytic phenotype, as in CMML.

BAP1 is a nuclear-localized deubiquitinating enzyme that binds to ASXL1 participating in H2AUb deubiquitination, thereby regulating myelopoiesis, mutated in 3% MDS cases. Interestingly, there is data to support the activity of BAP1 as a positive regulator of HCF-1 and OGT (Dey *et al*, 2012), the latter being related to TET2-mediated histone modulation as we previously described. All this data highlights the intertwined alterations in the epigenetic transcriptional regulation in MDS pathogenesis.

PRC2 catalyses the trimethylation of H3K27 in a direct manner, and indirectly regulates the ubiquitination of H2AK119 by recruitment of PRC1 (Morey & Helin, 2010; Müller & Verrijzer, 2009; Lund *et al*, 2014), inducing transcriptional repression. Components of PRC2 include EZH2, EED, SUZ12 and RBBP4. This assembled complex can bind to H3K27, inducing its trimethylation via the SET domain of EZH2 and, as a result, chromatin compaction and inhibition of transcription factor access to DNA. EZH2 can also induce DNA methylation by recruiting DNMT, further repressing transcription of target genes through gene promoter silencing (Vire *et al*, 2006). Through these mechanisms *EZH2* is an important regulator of cell fate controlling the balance between self-renewal and differentiation, having both the ability to stabilize chromatin structure to maintain long-term

self-renewal potential of HSCs (Kamminga *et al*, 2006), and to induce a shift toward proliferation by increased cell cycle genes (Bracken et al, 2003) via regulation of PI3K pathway, *MYC* expression and several miRNA. Deregulation of this balance and the functions of *EZH2* are responsible for its contribution to oncogenesis through different mechanisms. *EZH2* has been described to be altered in MDS through missense, frameshift and nonsense loss-of-function mutations in the SET, CXC and D2 domains (Ernst *et al*, 2010; Nikoloski *et al*, 2010), as well as in MDS cases with −7/del(7q) and MDS/MPN with uniparental disomy of 7q (which harboured concomitant inactivating mutations on *EZH2* in 71% of cases) (Jerez *et al*, 2012). These findings suggest a tumour suppressor effect of *EZH2* through loss of H3K27 trimethylation, in a similar manner as seen with *ASXL1*-inactivating mutations, leading to de-repression of proliferation and stem-cell expansion genes such as *HOXA* and *HOXB*.

Deregulation of histone modifications represents a key player in myelodysplasia initiation and progression to AML, with mutations and epigenetic alterations in different regulators being associated with different stages of the disease. Understanding of the molecular integration of these processes (including TET, DNMTs, PRC1 and PRC2) will allow us to develop new therapeutic strategies to target epigenetic deregulation in a much more effective manner. Representation of these molecular communications can be seen in Figure 4.

Impact of Splicesosome mutations on MDS Pathogenesis and Evolution: Connection to other Epigenetic regulators, Transcription factors and Innate immunity

As described earlier in this review, mutations in the different components of the splicing machinery have become a common finding along the different stages of MDS, suggesting their role as founder mutations of the disease (Mian *et al* 2013). Recent data suggest most cases of MDS (up to 85%) (Yoshida *et al*, 2011) harbour a mutation in one of the members of the RNA-splicing machinery, and that these mutations tend to be mutually exclusive. These mutations can alter the normal function of splicesosome machinery inducing inappropriate inclusion of introns or exons into RNA, disrupting the normal function of target proteins and, therefore, the ability of the cell to differentiate, hence generating dysplasia (Abrahamsson *et al*, 2009). *SF3B1* is common in RARS and RARS-T as described by several groups (Thol *et al*, 2010; Yoshida *et al*, 2011; Papaemmanuil *et al*, 2011; Mian *et al*, 2013) and recent data using murine models with $Sf3b1^{+/-}$ mice has shown its ability to induce a phenotype similar to RARS (Rogers *et al*, 2013). Interestingly enough, expression levels of *Ezh2*, as well as *Npm1* and *Tp53*, were found to be significantly lower in *Sf3b1*-deficient mice, suggesting a regulatory effect over PRC2 and histone modification. This is consistent with other findings of the ability of Sf3b1 to physically interact with PRC1 proteins in $Sf3b1^{+/-}$ mice models.

Other murine models exploring the effects of *Srsf2* in HSCs showed that both wild-type and P95H (the most common *SRSF2* mutation in MDS) *Srsf2* induced distinct changes in alternative splicing of more than 100 genes, with several changes in genes with known roles in haematopoietic malignancies being uniquely induced by the P95H mutant (Qiu *et al*, 2013). This finding suggests a possible gain of function effect on *SRSF2* mutations, which may induce modifications in key regulators of MDS pathogenesis, further supporting the

idea of splicesosome mutations as initiators of myelodysplasia by induction of additional mutations in epigenetic modulators, transcription factors and signal transductors as described by Mian *et al* (2013).

A recent publication (Khan *et al* 2013) details the effect of *EZH2* mutations in H3k27 trimethylation and the expression of different known gene targets in 469 cases of different myeloid malignancies. Of special significance was the fact that cases with $EZH2_{wt}$ that harboured splicesosome mutations (including *U2AF1, SRSF2* and *U2AF26*) were often related to a similar phenotype as those with *EZH2* mutations. This was derived from a reduction of spliced compared to unspliced *EZH2* pre-mRNA leading to decreased levels of the protein and, therefore, H3K27me3. Increased expression levels of *IRF4* (IFN Regulatory Factor 4), *HOXA9*, *SPI1* and *CEBPA* were found in these *EZH2*-deficient cases, suggesting there not only is an upregulation of genes that participate in haematopoiesis, stem-cell renewal and proliferation, but in innate immunity as well. This is represented in Figure 5. We can therefore hypothesize that these different hallmarks of MDS development (epigenetic regulation, splicing, proliferation deregulation and innate immunity) may well be connected in a more direct manner than could be originally anticipated. With the increasing complexity of MDS pathogenesis, further exploration and understanding of the extent of these molecular relationships is of paramount importance. Several innate immunity alterations in a variety of pathways are being discovered in MDS, including overexpression of *TLR2* and *TLR6* (Wei *et al*, 2013b), mutations in *TLR2* (*TLR2* F217S) (Wei *et al*, 2013b), upregulation of NF-κB (Wei *et al*, 2013a; Wei *et al*, 2013b), TNF signalling, PD-L1 (CD274)/ PD-L2 (PDCD1LG2)/ PD-1 (PDCD1)/CTLA4 (Yang *et al*, 2013) and others, with evidence of impact of these at an epigenetic level. If a stronger connection can be established between these microenvironment dependent factors and different epigenetic alterations, a new potential area of research and treatment development will arise.

Closing the gap with the Clinic: Therapeutic Implications

Several decades ago therapeutic options for patients with MDS were scarce. Fortunately, during recent years, research has enabled the development of several drugs with both proven and potential therapeutic activity in myelodysplasia. Although lower risk MDS are still mainly treated with growth factor support when needed, agents modulating epigenetic changes in the disease have become the standard of therapy in higher risk disease, with potential in lower risk MDS. DNA hypomethylating- and histone deacetylase-inhibiting agents have been largely studied in the last decade in patients with MDS and AML with promising results. Additional molecules directed at the epigenetic alterations of the disease are being developed to further enhance the therapeutic armamentarium of MDS and AML and are summarized in Table I.

Hypomethylating agents (HMAs)

Azacytidine and decitabine are cytidine analogues that exert potent inhibition of DNA methylation both *in vitro* and *in vivo* by inducing a depletion of methyltransferase levels (Borthakur *et al*, 2008). Additionally, azacytidine also induces disruption of nucleic acid and protein metabolism by incorporation into RNA, and inhibits ribonucleotide reductase, an

essential enzyme for DNA synthesis (Aimiuwu *et al*, 2012). However, the specific mechanism of action of these agents still remains controversial. Data suggests that DNMT1 depletion leading to hypomethylation may exert a transcriptional change, inducing cellular differentiation and subsequent TP53 independent apoptosis (Hollenbach *et al*, 2013). This may be the biological reasoning behind the responses observed to these therapies in patients with high-risk MDS with del(17p) and *TP53* loss-of function mutations.

HMAs induce responses in patients with MDS and AML and reduce risk of transformation to AML compared to best supportive care (Fenaux *et al*, 2009), with azacytidine showing a slight advantage in survival compared with decitabine (Lee *et al*, 2013). Currently, both agents are approved for the treatment of MDS, and are considered the standard of care for patients with high risk MDS or AML not eligible for intensive chemotherapy or stem cell transplantation.

Some current studies are trying to elucidate if low doses of azacytidine or decitabine are clinically active and safe in low-risk disease patients with poor prognostic features.

Ultimate loss of response to therapy unfortunately remains a near constant in the history of the disease. Efficacy of one of these agents in the setting of failure to the other has been reported (Borthakur *et al*, 2008), however responses remain underwhelming with short OS (Jabbour *et al*, 2010). This has lead to increasing efforts in the development of additional therapies such as SGI-110, a new DNMT inhibitor with a longer half-life than decitabine due to decreased *in vivo* deamination. A phase I/II trial in patients with AML recently presented at the 2013 American Society of Hematology (ASH) annual meeting has shown promising results (Jabbour *et al*, 2013). Identification of the mechanisms behind failure to therapy still remains elusive, with increasing efforts having been made in order to determine the existence of possible predictive factors of response to therapy. Contradictory results have been obtained when analysing baseline methylation or reduction of methylation levels after therapy as predictors of response (Shen *et al*, 2010).

Efforts to identify molecular markers of response to therapy have also been made, with *TET2* and *DNMT3A* mutations having been associated with better response to azacytidine, albeit with contradictory data regarding their impact on OS (Traina *et al*, 2014). Polycomb complex gene mutations have also been associated with longer survival after hypomethylating therapy (Kulasekararaj *et al*, 2010).

Despite these important advances, the identification of clear subgroups of patients at a potential risk of failure to HMAs remains a challenge.

Histone Deacetylase (HDAC) Inhibitors

Despite the revolution of HMA in MDS therapy, there is still much room for improvement in order to significantly reduce leukaemic transformation, increase OS and induce long-term haematological and cytogenetic responses in the majority of patients. To this end, development of additional therapeutic targets has been sought in recent decades. HDAC inhibitors represent the most promising and explored group of drugs in this context and have therefore been tested in MDS and AML both as single agents and in combination with HMA

and other therapies, with promising results. Initial trials with valproic acid combined with azacytidine demonstrated a modest advantage in response rate compared to azacytidine alone (Garcia-Manero *et al*, 2006). Vorinostat has been extensively studied, with *in vitro* data suggesting its ability to promote cell cycle arrest and growth inhibition, and induce apoptosis and differentiation of bone marrow cells from patients with MDS and AML (Silva *et al*, 2013). Results from a Phase II trial in combination with azacytidine in patients with MDS have been recently presented at the 2013 ASH annual meeting, with very promising results (Verma *et al*, 2013).

Apart from vorinostat, other HDAC inhibitors have been developed. Entinostat showed increased rate of haematological responses when combined with azacytidine in patients with AML and MDS (Prebet *et al*, 2010). Pracinostat has shown extremely promising results in a preliminary Phase II study in combination with azacytidine in 9 patients with high-risk MDS (Quintas-Cardama *et al*, 2012). In this trial an overall response rate of 89% was observed, with 56% patients showing a complete cytogenetic response. Despite the small study population these encouraging results have led to further trials in different disease settings. Finally, Panobinostat is currently being evaluated in older patients with MDS and AML in combination with decitabine.

Future perspectives: Targeting EZH2, IDH and the splicing machinery

As has been described throughout this review, the progressive discovery of additional alterations on epigenetic modulators is opening the door to potential future therapies.

EZH2—Although *EZH2* mutations in MDS are associated with loss of function and suggest a tumour suppressor function of the PRC2 component, there is solid biological data supporting the potential effect of *EZH2* as an inducer of proliferation, stem-cell renewal and inhibitor of differentiation. Although in the early stages of development, several EZH2 inhibitors are currently being explored in lymphoma with promising preclinical data (Lund *et al*, 2014). 3-Deazaneplanocin, or DZNep, is a S-adenosylhomocysteine hydrolase inhibitor that seems to induce EZH2 depletion by degradation of PRC2 complex and upregulate MIR29, leading to decreased lymphoma growth. A more specific EZH2 inhibitor in development is EI1. This drug inhibits cell growth and induces apoptosis and differentiation in lymphoma cells by reduction of H3K27me3 levels. Further research is required in order to determine if a subset of patients with MDS in which *EZH2* could be a potential pro-oncogenic player could be candidates to therapy with these new therapies.

IDH1/2—As has been previously stated, *IDH* mutations lead to an important metabolic and epigenetic deregulation. Recent studies in AML have elucidated the possible different mechanisms responsible for the metabolic chaos and epigenetic disruption. The presence of mutant *IDH* genes, especially in heterozygous cases, induces *HOXA9* genes, promotes cell-cycle transition through *CDKN2A* and *CDKN2B* epigenetic silencing, and activates different signalling pathways, such as MAPK (Chaturvedi *et al*, 2013). This opens an area of potential therapies that could directly or indirectly target AML and, presumably, MDS cases harbouring *IDH* mutations. Targeting MAPK through drugs such as ARRY-614 (a dual p38/MAPK and Tie2 inhibitor) could be considered. Additionally, direct inhibition of mutant

IDH is being explored in AML. Such is the case for HMS-101, a drug that has shown *in vitro* activity with reduction of 2-HG levels, induction of apoptosis and inhibition of cell division in mouse and human IDH_{mut} AML cells (Chaturverdi *et al*, 2013). Evaluating the possible efficacy of this molecule in MDS could be an interesting future prospect with potential to impact the treatment of a subset of patients with both AML and MDS.

Splicesosome—Mutations in the splicesosome complex leading to alterations in normal splicing of important genes may be another potential target of therapy in MDS, however additional understanding of the molecular mechanisms leading to myelodysplasia is required in order to identify potential therapeutic strategies and to develop specific drugs that target this group of genes.

Concluding Remarks

Epigenetic regulation represents an essential biological process leading to cell fate and differentiation programmes that are paramount in normal tissue development and homeostasis. Aberrant modulation of the different epigenetic processes has been consistently demonstrated to be a primordial pathogenic event in MDS, and although increasing understanding has been attained during the last decades, there are still many unknown mechanisms leading to disease promotion and progression. New molecular techniques have allowed us to start subdividing MDS into different mutational patterns associated with distinct prognosis, response to therapy and phenotypes. Although available data is still lacking a thorough integration with the different disease processes and its correlation with the clinic, much of this epigenetic data may be the basis for developing new classifications and prognostic models of the disease that expand on the current World Health Organization classification and Revised International Prognostic Scoring System. The development of an array of epigenetic modulators, such as DNMT and HDAC inhibitors, has had an impact on the treatment of patients with MDS and may contribute to future improvements in OS, progression-free survival and a potential to change the course of the disease and the need of bone marrow transplantation. The field of epigenetic modulation certainly represents a fascinating area of research that may lead to important changes in the understanding of molecular oncology and molecular biology and revolutionize the field of clinical oncology.

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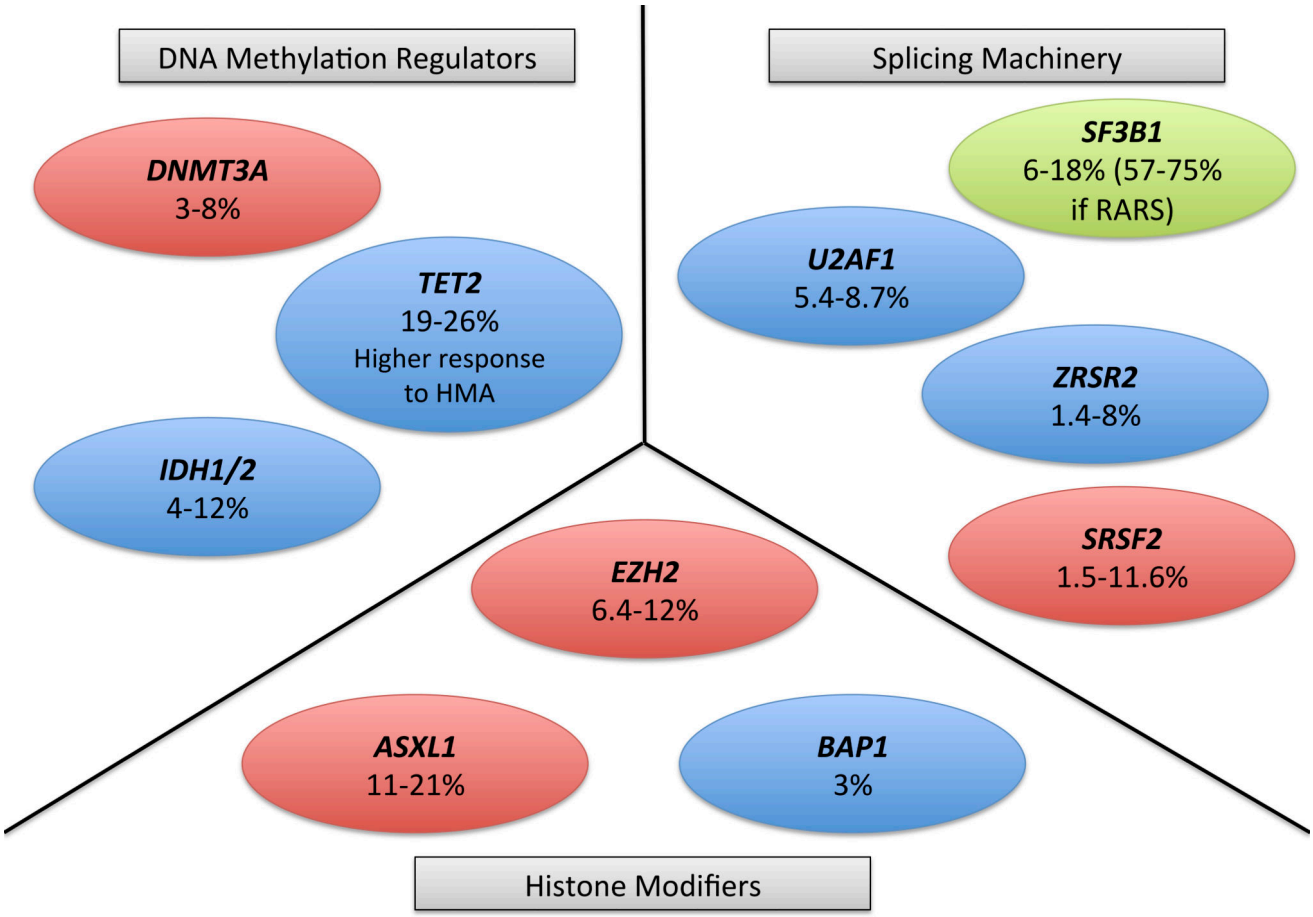

Figure 1.

Main mutated epigenetic regulators in myelodysplastic syndrome (MDS). Genes represented in red are known to be associated with worse prognosis. Genes in green are associated with better prognosis and those in blue have an unclear prognostic impact. Percentages represent the known prevalence of the mutation in MDS.

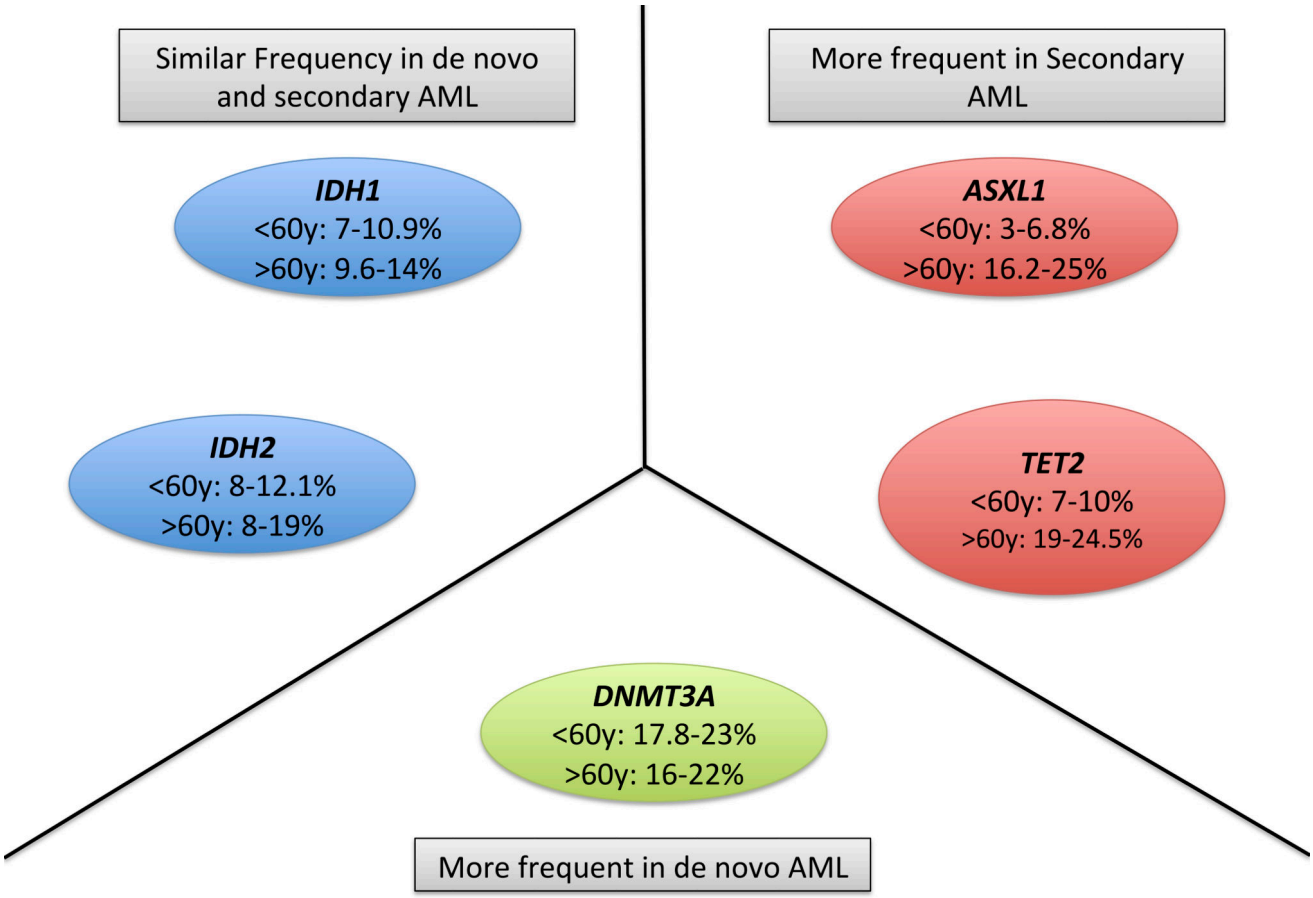

Figure 2.

Main mutated epigenetic regulators in acute myeloid leukaemia (AML). Frequencies of mutations include all AML cases (both *de novo* and secondary) in patients aged 60 years or younger (<60y) and older than 60 years (>60y).

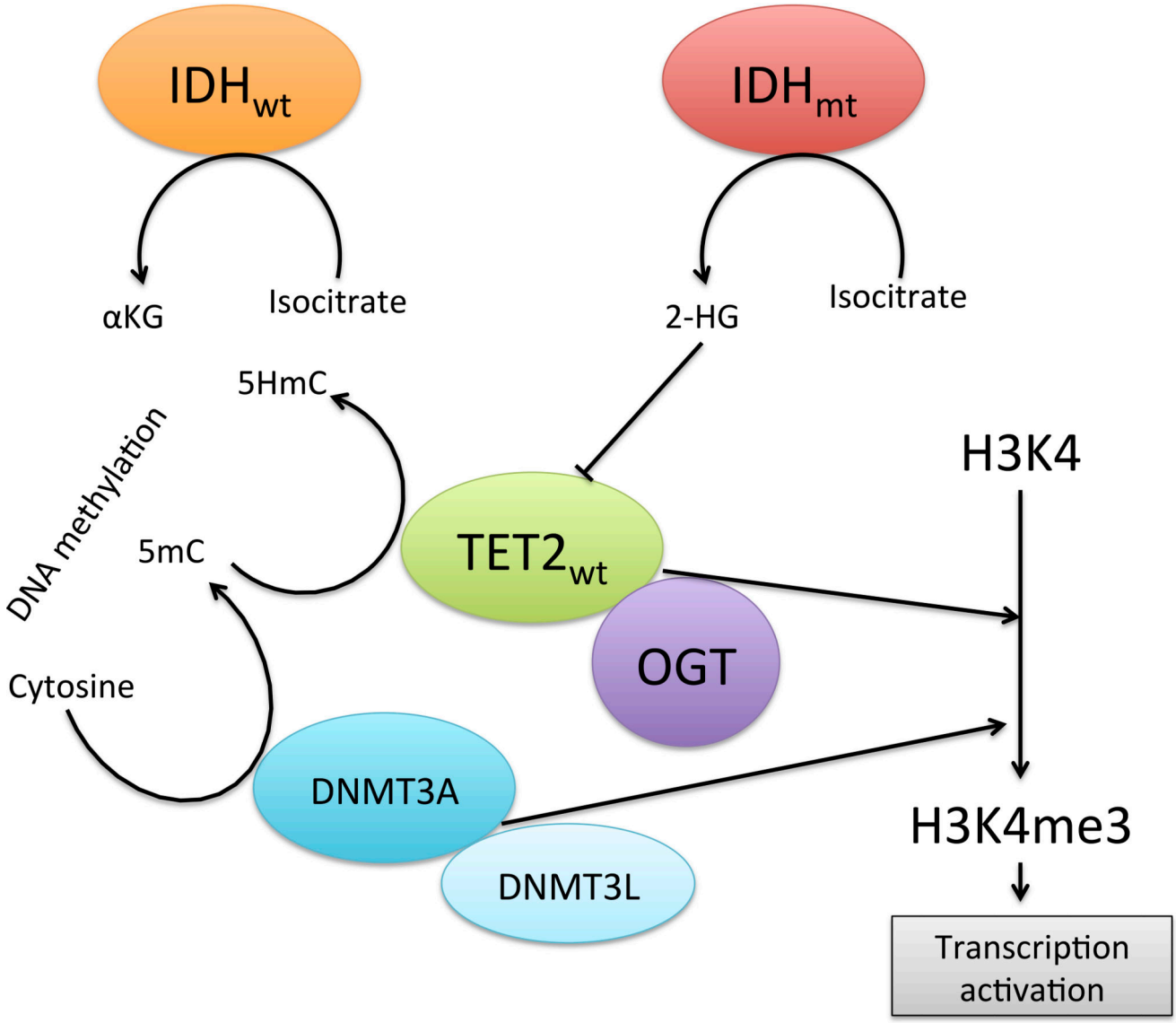

Figure 3.
DNA methylation and histone modifications by TET2 and DNMT3A.

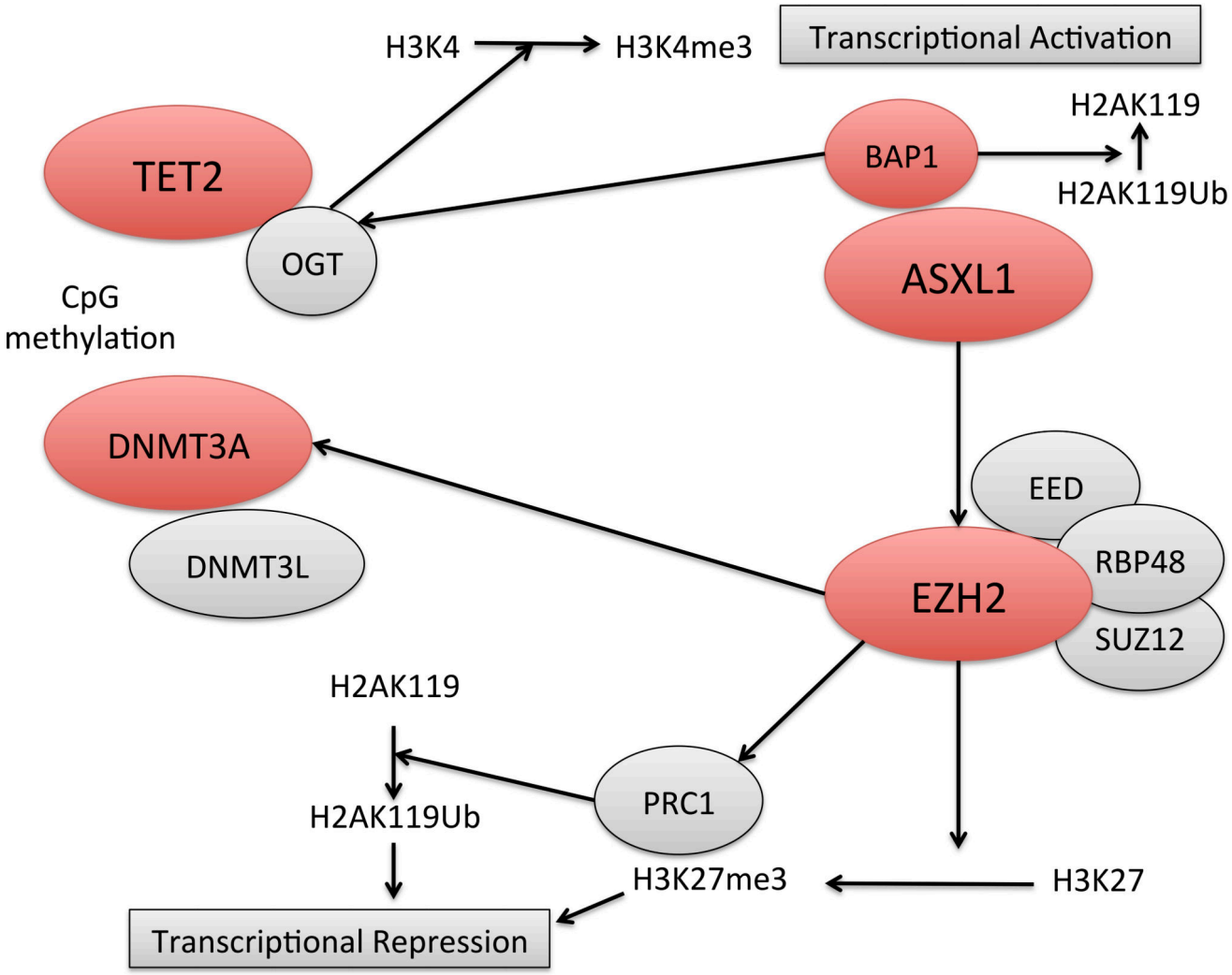

Figure 4.

Transcriptional regulation through PRC1, PRC2, TET2 and DNMT3A. Figures in red represent proteins known to harbour loss-of-function mutations in myelodysplastic syndrome.

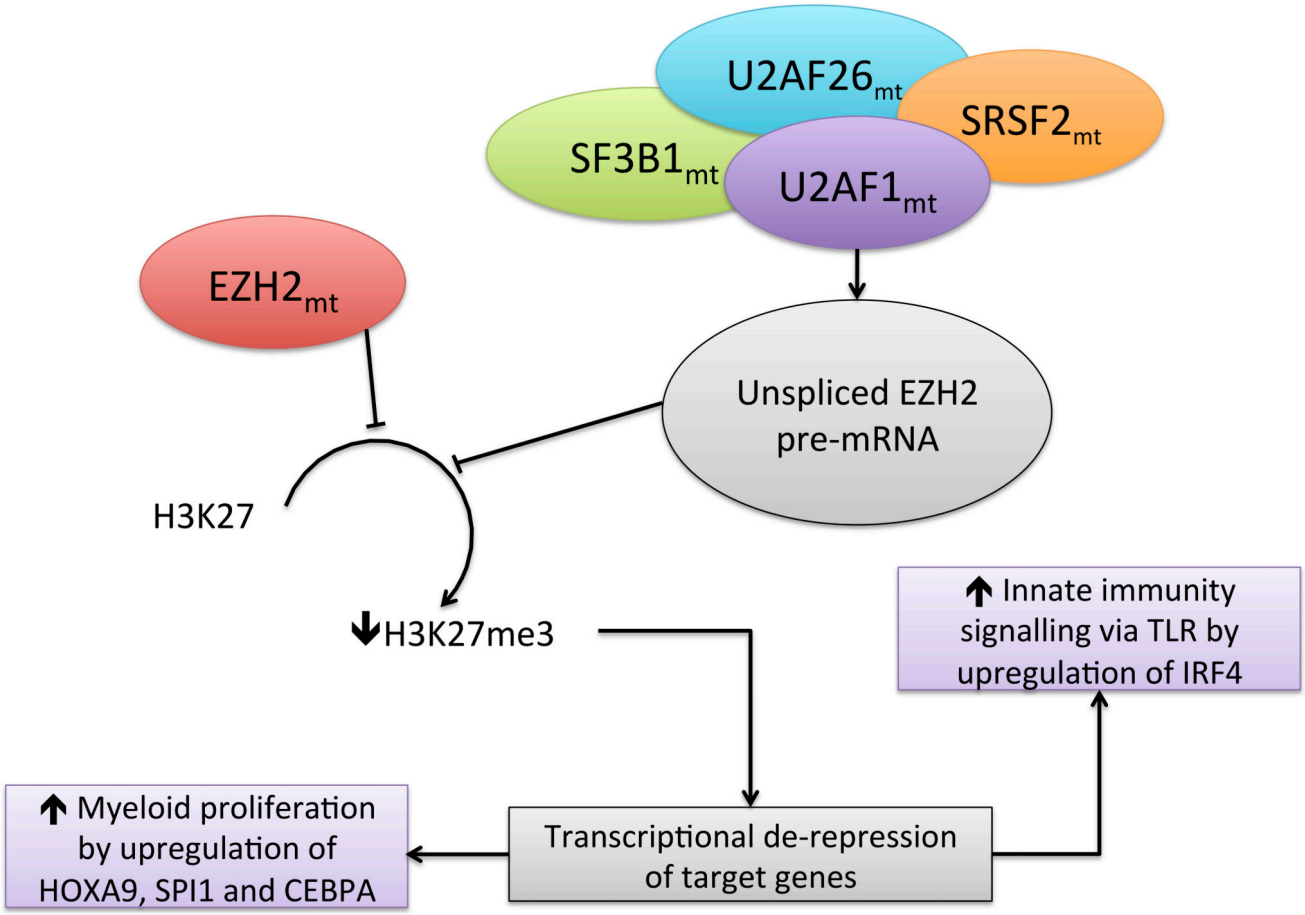

Figure 5.

EZH2 downregulation effects and splicing mutations in MDS. This figure represents the connection between splicing mutations, EZH2 loss of function and deregulation of proliferataion and innate immunity signalling through IRF4. Adapted from Rogers *et al* (2013) and Khan *et al* (2013). mt, mutated.

Table I

Approved and under-development drugs directed at epigenetic Regulation in MDS.

Drug	Mechanism of Action	Development Stage	Clinical Outcomes
Azacytidine	DNMT1 inhibition	Approved by FDA Phase II Oral formulation trial Phase II Low-dose for low risk disease	Increased OS, TFS and ORR (including CR, CCyR, PR and HI) Reduced transfusion dependency
Decitabine		Approved by FDA Phase II Low-dose for low risk disease	Increased OS, PFS and ORR (including CR, CCyR, PR and HI) Reduced transfusion dependency
SGI-110		Phase I/II	27% ORR in AML
Vorinostat	HDAC inhibition	Phase II in combination with Azacytidine	Increased ORR and improved OS
Panobinostat		Phase I/II	ORR of 50% with median OS of 18 months
Enzatiostat		Phase II in combination with Azacytidine	Increased ORR
Pracinostat		Phase II in combination with Azacytidine	CCyR in patients with HMA failure

DNMT1: DNA (cytosine-5-)-methyltransferase 1; FDA: US Food and Drug Administration; OS: Overall survival; TFS: transformation-free survival; ORR: Overall response rate; CR: complete response; PR: partial response; CCyR: Complete cytogenetic response; HI: Haematological improvement; AML, acute myeloid leukaemia; HMA, hypomethylating agent.