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Epigenetic mechanisms in tumorigenesis, tumor cell heterogeneity and drug resistance

Roel H. Wilting, Jan-Hermen Dannenberg*

Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital, Division of Gene Regulation, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

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ABSTRACT

Resistance of cancer cells to chemotherapeutics and emerging targeted drugs is a devastating problem in the treatment of cancer patients. Multiple mechanisms contribute to drug resistance such as increased drug efflux, altered drug metabolism, secondary mutations in drug targets, and activation of downstream or parallel signal transduction pathways. The rapid kinetics, the reversibility of acquired drug resistance and the absence of genetic mutations suggest an epigenetic basis for drug insensitivity. Similar to the cellular variance seen in the human body, epigenetic mechanisms, through reversible histone modifications and DNA methylation patterns, generate a variety of transcriptional states resulting in a dynamic heterogeneous tumor cell population. Consequently, epigenomes favoring survival in the presence of a drug by aberrant transcription of drug transporters, DNA-repair enzymes and pro-apoptotic factors render cytotoxic and targeted drugs ineffective and allow selection of rare drug-resistant tumor cells. Recent advances in charting cancer genomes indeed strongly indicate a role for epigenetic regulators in driving cancer, which may result in the acquisition of additional (epi)genetic modifications leading to drug resistance. These observations have important clinical consequences as they provide an opportunity for "epigenetic drugs" to change reversible drug-resistance-associated epigenomes to prevent or reverse non-responsiveness to anti-cancer drugs.

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1. Introduction

Resistance acquired upon drug treatment ("acquired drug resistance") is a major problem in the treatment of many diseases, including cancer. World-wide an estimated 7.5 million cancer patients die each year, many of them due to failed anti-cancer therapies as a consequence of acquired resistance to cytotoxic chemotherapeutics and targeted drugs (Boyle and Levin, 2008). Thus, understanding the mechanisms causing unresponsiveness to anti-cancer drugs will dramatically improve the design of therapies aimed at preventing the selection of drug-resistant tumor cells. Consequently, such therapies may significantly reduce cancer mortality rates. With the rise of targeted drug therapies it has become increasingly evident that genetic mutations are a critical component of acquired drug resistance. However, genetics are not sufficient in explaining the relatively rapid appearance or the reversibility of non-responsiveness to drug treatment. In addition, the lack of genetic mutations in drug targets and activated parallel pathways suggested a role for non-genetic mechanisms in acquired drug resistance (Glasspool et al., 2006). This review will focus on the role of chromatin biology in tumorigenesis and in non-genetic acquired drug resistance.

2. Tumor heterogeneity and epigenetics

Until now, several mechanisms underlying acquired drug resistance have been uncovered including increased drug efflux, enhanced drug metabolism, inactivation of apoptotic pathways, secondary mutations in drug targets, and activation of downstream or parallel pathways (Redmond et al., 2008). The basis for these mechanisms has been attributed to increased genetic instability and accelerated mutation rate in tumor cells providing genetic diversity that allows selection of cells with a survival advantage during drug treatment.

Although a genetic basis for acquired drug resistance contributes to anti-cancer therapy failure, a number of observations imply a non-mutational contribution to drug non-responsiveness. First, the high prevalence of drug resistance suggests that acquisition of mutations cannot solely account for this phenomenon. Secondly, the reversibility of acquired drug resistance has been described in patients receiving retreatment upon a drug-free period. Third, a substantial number of drug-resistant tumors do not harbor mutations in drug targets or activated pathways. Finally, despite the notion that tumors arise through clonal expansion of

* Corresponding author. Tel.: +31 205122024; fax: +31 2055121989.
E-mail address: j.dannenberg@nki.nl (J.-H. Dannenberg).

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cells that have acquired genetic alterations advantageous for proliferation, survival and metastasis (Hanahan and Weinberg, 2011), there is large variability in malignancy and drug resistance of individual cells (Gupta et al., 2011; Shipitsin et al., 2007). A basis for this variability may be found in the different transcriptional network states produced by the same cancer genome, in a similar fashion as transcriptional network states realize a variety of cell types in the body by one genome. It has been suggested that reversible transcriptional network states controlled by DNA- and chromatin modifications play a role in generating dynamic heterogeneity required for differentiation (Chang et al., 2008). Similarly, heterogeneity in a tumor cell population, based on dynamic variation in epigenome configurations, is thought to provide a non-genetic variance source for selection of drug-resistant cells (Brock et al., 2009; Gupta et al., 2011). Indeed, tumors exhibit extensive genetic and non-genetic heterogeneity within the tumor cell population (Fraga et al., 2005; Seligson et al., 2005).

A non-genetic basis for tumor cell heterogeneity and acquired drug resistance has long been neglected. With the discovery of "cancer stem cells" or "tumor-initiating cells", tumor heterogeneity is more appreciated and has raised new questions with respect to treatment outcome. Although many questions remain about the concept of tumor stem cells and their contribution to tumorigenesis it has become evident that these cells are intrinsically more resistant to various anti-cancer drugs, either by increased drug efflux, inability to execute apoptosis, enhanced DNA repair, different protein dynamics or by displaying a quiescent cell-cycle state (Cohen et al., 2008; Eyler and Rich, 2008; Frank et al., 2010). As epigenetics has a crucial role in cell fate determination (Ji et al., 2010; Mikkelsen et al., 2007; Sarmento et al., 2004), it has been proposed that DNA and histone modifications may drive non-genetic heterogeneity resulting in the establishment of tumor-initiating cells and/or drug-resistant cells (Feinberg et al., 2006).

Epigenetics, defined as changes in gene expression that are independent of changes in the DNA sequence and persist over many cell divisions, has been recognized as an important factor in generating non-genetic heterogeneity (Chang et al., 2008). Although gene expression is controlled by multiple mechanisms, covalent modifications of DNA and histones are at the heart of regulating gene transcription. Epigenetic changes affect gene transcription by modulating the packaging of chromatin, thereby regulating the accessibility of DNA to sequence-specific transcription factors (van Steensel, 2011). In addition, combinations of epigenetic marks are "read" by specialized protein modules present in transcription complexes, and are consequently "translated" into a biological output such as switching on or off transcription (Taverna et al., 2007). Ultimately, the readout of epigenome variation dictates the diversity in cellular phenotypes of cells harboring the same genome. In a similar fashion, a specific combination of genetic and epigenetic marks may produce a drug-resistant phenotype in a heterogeneous tumor cell population (Fig. 3A). In order to design therapies aimed at preventing or reversing drug resistance it will be important to identify epigenetic marks and their biological consequences in drug-resistant tumor cells. In the first part of this review we focus on the link between tumorigenesis and post-translational modifications of DNA and histones, in particular methylation and acetylation (Table 1). In the second part we will discuss the role of tumor cell epigenomes in mechanisms underlying drug resistance.

2.1. DNA methylation

DNA is predominantly methylated at cytosines (5-methyl-C; 5mC) by DNA methyltransferases (DNMT1, 3A, 3B and DNMT3L) (Fig. 1) (Cedar and Bergman, 2011). While methylation at transcribed regions or gene bodies is thought to facilitate transcription elongation, methylation of promoter regions is in general associated with gene silencing (Maunakea et al., 2010). Methylated cytosines can serve as docking sites for methyl-binding proteins which subsequently recruit co-repressor complexes resulting in gene silencing (Jones et al., 1998; Nan et al., 1998). Other functions besides gene regulation have been attributed to cytosine methylation such as preventing chromosomal instability by methylation of repetitive genomic sequences dispersed over the genome (Bird, 2002). Long-term silencing of genes is normally only associated with inactive X-linked genes, imprinted genes and germ-cell-specific genes. In particular, CpG islands, cytosine and guanine-rich DNA sequences, are frequently hypermethylated in tumor cells which can result in silencing of tumor suppressor genes (De Smet et al., 1999; Herman et al., 1994; Merlo et al., 1995).

The observation that indirect inhibition of DNA methyltransferases using the nucleoside analog 5-azacytidine (5-aza-CR) resulted in re-expression of silenced genes and inhibited tumor cell growth by inducing differentiation has resulted in the use of 5-aza-CR (Vidaza) and 5-aza-dCR (Decitabine) as therapeutic agents in the

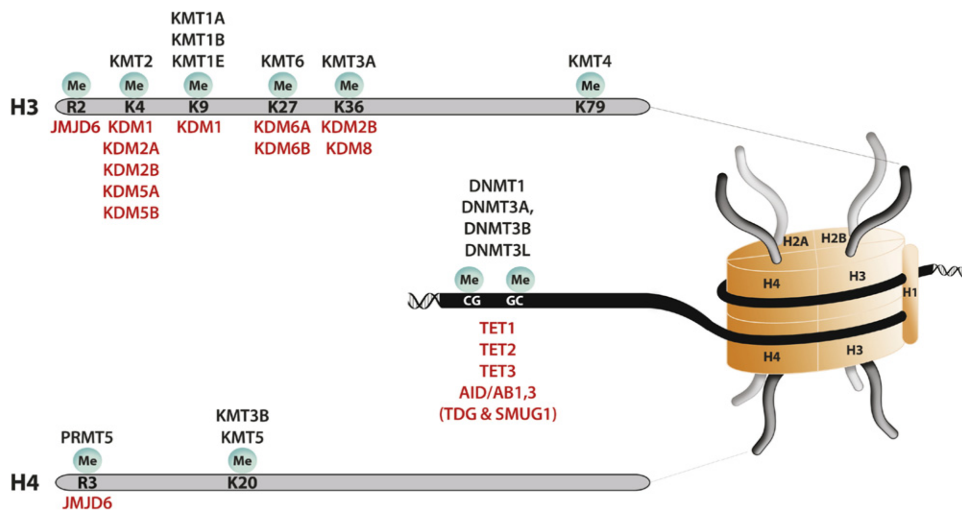

Fig. 1. Histone and DNA methylation enzymes involved in tumorigenesis and drug resistance. Histone H3 and H4 lysine (K) and arginine (R) residues present in the N-terminal tail, or histone core (H3K79) are targeted by methyltransferases (KMTs; in black) and demethylases (KDMs; in red). DNA methylation takes place at cytosine residues in GC-rich nucleotide stretches and is modulated by DNA methyltransferases (DNMTs) and enzymes that modify methylated cytosines (for interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

treatment of myelodysplastic syndromes (MDS). In addition, direct inhibition of DNMTs using small molecule inhibitors targeting specific DNMTs are being developed (Foulks et al., 2011). Despite their clinical efficacy the clinical relevant transcriptional targets of these compounds remain unclear.

Studies in mice and men suggested that reduction or mutational inactivation of DNMTs may have adverse effects. Mice expressing reduced levels of Dnmt1 developed with high incidence thymic lymphomas due to hypomethylation of pericentric heterochromatin, suggesting that reduced Dnmt1 levels may result in chromosome missegregation and consequently genomic instability (Gaudet et al., 2003). Although no *DNMT1* mutations have been identified in human malignancies, *DNTM3B* mutations are associated with the immune deficiency, centromere instability and facial anomalies (ICF) syndrome. In addition, elevated levels of DNMT3B have been observed in tumors, in part due to amplification of *DNMT3B* (Simó-Riudalbas et al., 2011). Moreover, the discovery of highly recurrent, heterozygous somatic *DNMT3A* mutations in MDS and acute myeloid leukemia (AML), which are correlated with poor prognosis, provides another cautionary note on inhibiting DNMTs in anti-cancer therapy (Ley et al., 2010). Acquired drug resistance has been associated with selection of cells displaying hypomethylation of drug efflux gene promoters, hypermethylation of promoter regions of pro-apoptotic genes, or altered promoter methylation patterns of DNA-repair genes (see below). In addition, the poor prognosis profile of MDS and AML patients carrying somatic *DNMT3A* mutations may attest to increased acquired drug resistance due to DNA methylation changes that generate cells with a drug resistance favorable epigenome. Therefore, characterization of a drug-resistance-associated DNA methylome may guide the design of therapeutics aimed at targeting DNA methylation either through 5mC nucleoside analogs or direct and indirect inhibitors of DNMTs.

2.2. *DNA demethylation*

Until now, DNA demethylases have not been identified and DNA demethylation is thought to occur through passive dilution during replication. However, active mechanisms have been discovered which prime 5mCs by hydroxylation, deamination and/or oxidation, which are subsequently removed by DNA-repair mechanisms. Three enzyme families are known to be involved in active demethylation (Fig. 1). Priming of the methylated cytosines is conducted by ten-eleven translocation (TET1, 2 and 3) enzymes to form 5-hydroxy methyl cytosine (5hmC), the function of which has not been fully characterized (Bhutani et al., 2011). Intriguingly, TET2 is a frequent target of mutations in hematopoietic malignancies suggesting that one TET family member has a tumor suppressor function possibly by affecting 5hmC modifications and generating a (locally) hypermethylated cancer epigenome (Delhommeau et al., 2009; Langemeijer et al., 2009). Cytidine deaminases (AID/APOBEC) are a second class of enzymes able to modify 5mC or 5hmC into 5-methyluracil or 5-hydroxymethyluracil. Eventually, replacement of the intermediate forms of methylated cytosines into unmethylated cytosines is initiated by the UDG family of base excision repair (BER) glycosylases (TDG/SMUG1). Since hyper- and hypomethylation of promoter regions is involved in drug resistance, studies aimed at correlating modified methylation of cytosine and prognosis upon treatment may provide a hint whether the enzymes involved in generating modified forms of 5mC are involved in unresponsiveness to drugs.

3. Histone modifications

A vast variety of biological processes ranging from gene transcription to DNA repair are regulated at the nucleosomes, the basic unit of chromatin consisting of DNA and an octamere of small basic histone proteins (H2A, H2B, H3 and H4). The interaction between DNA and histone proteins is tightly regulated by histone modifications predominantly at the N-terminal tails of histone proteins extruding from the nucleosome core (Figs. 1 and 2). Post-translational modifications (PTMs) of histones are diverse, reversible and delicately balanced by an expanding set of histone-modifying enzymes. Currently known histone modifications exist in many forms and include acetylation, methylation, phosphorylation, propionylation, butyrylation, N-formylation, ubiquitylation, sumoylation, citrullination, proline isomerization, and ADP ribosylation (Cohen et al., 2011). The recent identification of 67 previously unknown PTM sites in histones as well tyrosine hydroxylation and lysine crotonylation indicates the vast complexity that can be achieved by combinations of histone PTMs (Tan et al., 2011b).

3.1. *Lysine acetylation*

Acetylation of lysine residues at histone tails is catalyzed by lysine acetyl transferases (KATs), also known as histone acetyl transferases (HATs; for nomenclature see Allis et al., 2007). Acetyl groups neutralize the positive charge of lysines, resulting in a loose DNA-nucleosome association that increases DNA accessibility for transcription factors and subsequent transcriptional activity. KATs are divided in three major families based on sequence similarities: GNAT-, p300/CBP- and the MYST family. Additional KATs have been identified and are classified as a separate group of enzymes due to their sequence divergence from other KATs (Sadoul et al., 2011). Although KATs are predominantly located in the nucleus, an increasing number of studies report nucleocytoplasmic transport of these enzymes suggestive of cytoplasmic substrates and functions for KATs (Sadoul et al., 2011). Indeed, the identification of numerous acetylation sites in approximately 2000 proteins supports the notion that KATs regulate also the acetylation status of a large number of non-histone proteins (Choudhary et al., 2009; Zhao, 2010).

Several KATs have been implicated in tumorigenesis. KAT2 (GCN5) activated the BRCA1 and TP53 tumor suppressors as well as the oncogenic activity of c-MYC (Gamper and Roeder, 2008; McMahon et al., 2000; Oishi et al., 2006). KAT8 (MOF) is known to acetylate TP53 at lysine residue 120 (K120) together with KAT5 (TIP60) (Sykes et al., 2006; Tang et al., 2006). Furthermore, loss of histone H4 lysine 16 acetylation (H4K16ac), which is specifically regulated by KAT8, is a common hallmark of human cancer (Fraga et al., 2005; Taipale et al., 2005) (Fig. 2). KAT3A (CREBBP) and KAT3B (EP300) regulate the function of TP53, RB, E2F and MYB proteins, which play an important role in tumorigenesis (Chan et al., 2001; Gu and Roeder, 1997; Martínez-Balbás et al., 2000; Tomita et al., 2000). These findings were further corroborated by studies in *KAT3A* and *KAT3B* mutant mice which show that KAT3A and -3B can act as tumor suppressors of hematological malignancies (Kung et al., 2000). Moreover, *KAT3A* and *-3B* loss-of-function mutations were identified in various human cancers (Bryan et al., 2002; Kishimoto et al., 2005; Miller and Rubinstein, 1995; Muraoka et al., 1996). In addition, oncogenic translocations involving *KAT3A* and *KAT3B* have been observed in hematological malignancies such as AML, myeloid/lymphoid or mixed lineage leukemia (MLL) (Iyer et al., 2004). *KAT3A* point mutations were also identified in acute lymphoblastic leukemia (ALL) and bladder cancer (Gui et al., 2011; Pasqualucci et al., 2011). Interestingly, analysis of relapsed ALL found *KAT3A* mutations in 18.3% of non-responders, which were either present at diagnosis or acquired at relapse. Several mutations acquired at relapse were detected in sub-clones at diagnosis, suggesting that the mutations may confer resistance to therapy (Mullighan et al., 2011). These results suggest that inactivation of KAT3A and KAT3B

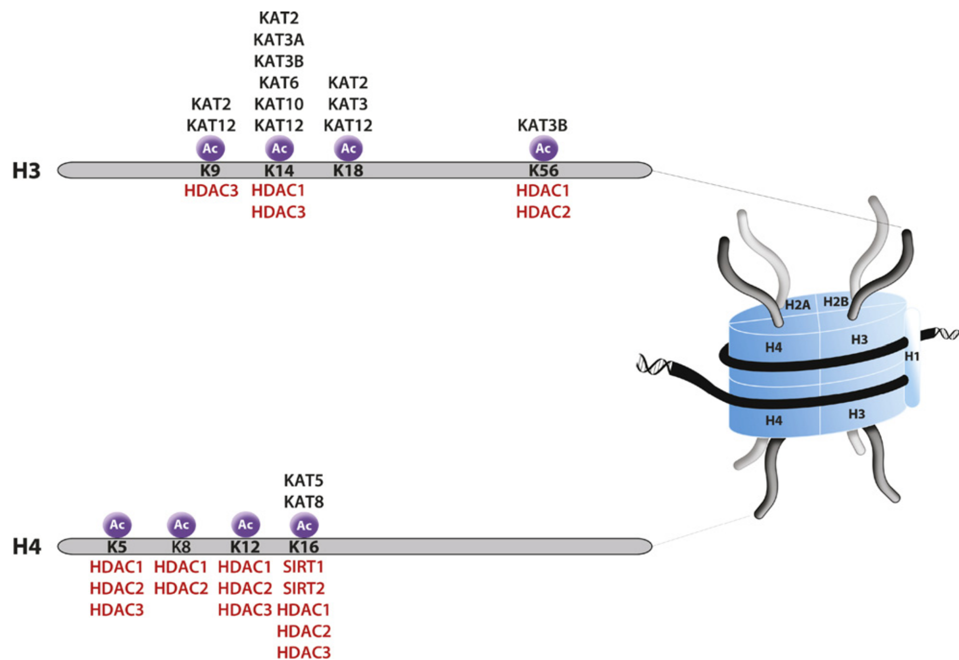

Fig. 2. Histone acetylation sites and enzymes linked to tumorigenesis and drug resistance. Lysine acetyl transferases (KATs; in black) target various lysine residues at the histone H3 and H4 N-terminal tail or histone core (H3K56). Acetylated lysines are deacetylated by histone deacetylases (HDACs and SIRTs; in red) (for interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

may change the epigenomic landscape of cancer cells thereby promoting tumorigenesis and resistance in ALL. Indeed, acetylation of lysine 18 on histone H3 (H3K18ac) was impaired in cells expressing tumor-associated mutant KAT3A (Fig. 2). Interestingly, loss of H3K18ac is correlated with tumor grade and poor prognosis in patients with prostate, pancreatic, lung, breast and kidney cancer (Elsheikh et al., 2009; Manuyakorn et al., 2010; Seligson et al., 2009; Seligson et al., 2005). Moreover, H3K18 hypoacetylation is associated with an increased risk of tumor recurrence in patients with low-grade prostate cancer (Seligson et al., 2005), suggesting that this histone modification may be involved in acquired drug resistance. In contrast, studies in esophageal squamous cell carcinoma and glioblastoma patients correlated H3K18 hypoacetylation with better prognosis, suggesting that H3K18ac status may effect tumorigenesis or drug sensitivity in a cell-type-dependent manner (Liu et al., 2010; Tzao et al., 2009).

3.2. Lysine deacetylation

Acetylation of lysine residues is counteracted by histone deacetylases (HDACs), which restore the positive charge of lysine residues resulting in a tightly packed chromatin configuration and subsequently transcriptionally inactive gene regions. Based on sequence homology HDACs can be divided in four classes: class I HDACs (HDAC1, 2, 3, and 8) are homologous to *Sacchromyces cerevisae* Rpd3, class II HDACs (HDAC4, 5, 6, 7, 9, 10) share high homology with yeast Hda1, while HDAC11 is the sole member of class IV HDACs and shares homology with both classes (Gao et al., 2002; Gregoretti et al., 2004; Haberland et al., 2009; Yang and Seto, 2008). Class III HDACs are homologous to yeast Sir2 and require NAD^+ as a cofactor for deacetylation, in contrast to class I, II and IV HDACs, which hydrolyze acetyl groups using Zn^{2+} as a cofactor (Haigis and Guarente, 2006). HDACs are present in distinct protein complexes associated with gene repression such as NuRD, SIN3A, Co-REST (HDAC1 and 2) and SMRT/N-CoR (HDAC3) (Yang and Seto, 2008). Class I HDAC complexes also harbor other histone-modifying enzymes such as histone demethylases KDM1, KDM4A and KDM5A (Klose et al., 2007; Shi et al., 2004; Tong et al., 1998; You et al., 2001; Zhang et al., 2005). Although no HDAC mutations have been identified in human cancers, hypoacetylation is a hallmark of various human malignancies. Global loss of H4K16ac occurs in various primary tumors simultaneously with loss of trimethylation at histone H4 lysine 20 (H4K20me3) and DNA hypomethylation (Figs. 1 and 2) (Fraga et al., 2005; Seligson et al., 2005). Furthermore, H4K16ac loss correlates with tumor progression and it is associated with chemotherapy resistance (Elsheikh et al., 2009; Fraga et al., 2005; Hajji et al., 2010). The prognostic value of elevated levels of SIRT1, a H4K16ac targeting deacetylase, in various cancers may therefore relate to the induction of drug resistance (Chen et al., 2005; Hida et al., 2007; Huffman et al., 2007) (Fig. 2).

Class I HDACs are recruited by a variety of oncogenic fusion proteins involved in hematological malignancies, including PML-RAR and AML-ETO (Amann et al., 2001; Gelmetti et al., 1998; Grignani et al., 1998). The observation that many proteins involved in tumorigenesis, such as TP53, RB, E2F1 and oncogenic fusion proteins are modified by HDAC-controlled acetylation underscores the role of HDACs as regulatory proteins during tumorigenesis. Moreover, increased levels of HDAC2, responsible for deacetylation of histone H3 lysine 56, and histone H4 lysines K5, K8, K12 and K16, were observed upon loss of the adenomatosis polyposis coli (APC) tumor suppressor gene in a mouse colon cancer model (Fig. 2). Inhibition of HDACs using valproic acid (VPA) reduced the number of adenomas in these mice, suggesting a tumor-initiating role for HDAC2 in colon cancer. HDAC3, which deacetylates histone H3 lysines K9 and K14, and histone H4 lysines K5, K12 and K16, is implicated in tumorigenesis by virtue of its binding to the transcriptional corepressor NCOR which in turn binds BCL6, a protein frequently mutated in diffuse B-cell lymphoma (DBCL) (Ahmad et al., 2003; Bhaskara et al., 2008; Bi and Ye, 2010) (Fig. 2). Besides recruitment of HDAC3 by oncogenic fusion proteins, HDAC3 is frequently deleted in breast cancer and MDS (Ebert, 2009; Johannsdottir et al., 2006). Moreover, liver-specific deletion

of HDAC3 in mice increased the incidence of hepatocellular carcinoma (HCC), suggesting a tumor suppressive function for HDAC3, probably by inducing genomic instability (Bhaskara et al., 2008).

Targeting HDACs using small molecule inhibitors such as trichostatin A (TSA), sodium butyrate (NaBu) and valproic acid (VPA) was shown to induce differentiation and apoptosis of a variety of tumor cell lines (Richon et al., 1998). Ultimately, these observations let to the FDA approval of the HDAC inhibitor (HDACi) SAHA (Vorinostat/Zolinza) for the treatment of cutaneous T-cell lymphoma (CTCL) and romidepsin (Istodax) for the treatment of CTCL and peripheral T-cell lymphoma (PTCL) (Coiffier et al., 2012; Prince et al., 2009). In addition, a variety of HDACi are currently being tested as a monotherapy or in combination with existing anti-cancer therapies of various human malignancies.

3.3. *Histone methylation*

While acetylation is found exclusively at lysine residues, histone methylation occurs at lysine and arginine residues of the histone tails. In contrast to acetylation and phosphorylation, histone methylation does not substantially change the amino acid charge, but does increase their hydrophobicity. Histone methylation leads to activation or repression depending on which residues are modified and the number of methyl groups present. These histone methylation states exhibit a genome-wide distribution pattern in which combinations of marks are linked to transcriptionally active regions, for instance monomethylation of H3K9 (H3K9me) and trimethylation of H3K4 (H3K4me3), while others associate with repression of transcription, such as trimethylation of H3K9 (H3K9me3) and H3K27 (H3K27me3) (Fig. 1) (Barski et al., 2007). In embryonic stem cells bivalent modification patterns exist, consisting of large areas of repressive H3K27 methylation harboring smaller regions of activating H3K4 methylation, which repress gene transcription, but keep these genes poised for action. These bivalent domains are seen as a mechanism in which the chromatin state controls cellular plasticity and thereby determines cell fate (Bernstein et al., 2006; Mikkelsen et al., 2007). This suggests that global histone modification patterns could be involved in tumorigenesis and therefore may play a role in specific characteristics of tumor cells, such as drug resistance (Feinberg et al., 2006).

3.3.1. *Arginine methylation*

Methylation of histones is performed by protein arginine methyltransferases (PRMTs) and lysine methyltransferases (KMTs). The family of PRMTs consists of 11 enzymes, of which PRMT1, 4, 5 and 6 are known histone methyltransferases. Compared to lysine methylation, arginine methylation is under-explored and therefore little is known about the influence of histone arginine methylation on cancer and drug resistance (Bedford and Clarke, 2009). Nevertheless, PRMT1 was identified as an essential component of the MLL-oncogenic fusion proteins, highlighting the role of arginine methyltransferases in tumorigenesis (Cheung et al., 2007). Others have identified PRMT5 as an arginine methyltransferase affecting the target gene specificity of *TP53* and apoptosis (Jansson et al., 2008). In addition, PRMT5 seems to act as a SNAIL co-repressor of E-cadherin thereby inducing epithelial–mesenchymal transition, a hallmark of metastasis (Hou et al., 2008). Indeed, overexpression of PRMT5 induces anchorage-independent growth and elevated PRMT5 levels are found in gastric and hematological malignancies (Kim et al., 2005; Wang et al., 2008). Moreover, high levels of dimethylated H4R3, a mark for active transcription, correlated with poorly differentiated prostate cancer and were associated, in combination with other histone marks, with poor patient prognosis and tumor recurrence (Seligson et al., 2005). In contrast, oncogenic JAK2 kinases were shown to phosphorylate and inactivate PRMT5 resulting in global reduction of H2A/H4R3 methylation suggesting a context-dependent function of PRMT5 in tumorigenesis (Liu et al., 2011) (Fig. 1).

3.3.2. *Lysine methylation*

Lysine methylation, predominantly at the N-terminus of histone tails, is regulated by a large number of histone/lysine methyltransferases (HMTs/KMTs) and lysine demethylases (KDMs). An increasing number of these proteins is found mutated, overexpressed or mislocated in human malignancies.

Although no mutations so far have been identified in *KMT1A* or *KMT1B* (*SUV39H1*/2), which specifically trimethylate H3K9 (Fig. 1), both proteins have been linked to tumorigenesis by their ability to physically interact with the retinoblastoma tumor suppressor (RB) and repress promoters by recruiting heterochromatin protein 1 (HP1) (Nielsen et al., 2001). Association of KMT1A and the oncogenic pro-myeloid leukemia/retinoic acid receptor fusion protein suggests a tumor promoting function in leukemia (Carbone et al., 2006). In mice, simultaneous inactivation of KMT1A and KMT1B resulted in profound chromosomal instability and consequently B-cell lymphomagenesis (Peters et al., 2001). A tumor suppressive function for KMT1A was further supported by the observation that KMT1A plays a critical role in providing thymocytes with an H3K9me3-dependent senescence fail-safe mechanism to inhibit oncogenic transformation upon oncogenic insults. Furthermore, KMT1A seems to determine the cellular response upon treatment with chemotherapeutics; loss of KMT1A prevents activation of a senescence checkpoint, suggesting that H3K9 methylation may be a critical determinant of acquired drug resistance (Braig et al., 2005).

Recurrent amplifications of genomic regions harboring the KMT1E (SETDB1) H3K9 methyltransferase (Fig. 1) have been identified in melanomas carrying $BRAF^{V600E}$ mutations. Using a zebrafish melanoma model it was shown that KMT1E collaborates with oncogenic BRAF in driving melanomagenesis probably by regulating HOX gene expression (Ceol et al., 2011). Another member of the SET methyltransferase gene family, KMT3A (SET2D) a histone H3 lysine 36 (H3K36) methyltransferase (Fig. 1), was recently found to be inactivated in clear cell renal cell carcinoma (ccRCC) (Dalgliesh et al., 2010; Sun et al., 2005).

KMT6 (EZH2), a H3K27 methyltransferase (Fig. 1), which is part of the polycomb repressive complex 2 (PRC2) is considered an oncogene as it is overexpressed in various solid tumors and is associated with invasion and progression of tumors (Kleer et al., 2003; Min et al., 2010; Varambally et al., 2002; Wagener et al., 2010). Indeed, primary prostate cancer samples displayed a tumor-specific H3K27me3 genome-wide pattern, which correlated with repression of genes involved in embryonic stem cell biology and with poor prognosis (Yu et al., 2007). Notably, also loss of H3K27me3 has been observed in various human malignancies such as prostate, ovarian, breast and pancreatic cancer and correlated with poor prognosis (Wei et al., 2008). Although these results may seem contradictive, increased or reduced H3K27me3 may have context-dependent consequences. Moreover, other substrates besides H3K27me3 may play a significant role in specific tumor types. Nevertheless, in general, aberrant H3K27 trimethylation correlated with poor prognosis of patients. A possible mechanism underlying the role of KMT6 and H3K27me3 in tumorigenesis could be silencing of tumor suppressor genes. Indeed, overexpression of KMT6 resulted in H3K27me3-mediated repression of tumor suppressors including CDKN2A, CDKN2B, CDKN1B, CDKN1C, CDH1, DAB2IP, BRCA1 and ADRB2 (Bracken and Helin, 2009; Cao et al., 2008; Gonzalez et al., 2009; Min et al., 2010; Ougolkov et al., 2008; Yang et al., 2009). Surprisingly, massive parallel DNA sequencing of follicular lymphoma (FL) and the germinal center subtype of diffuse large B-cell lymphoma (DLBCL) revealed somatic KMT6 mutations (Morin et al., 2010). Although initially

considered loss-of-function, a recent study indicated a dominant role for tumor-associated KMT6 mutations. $KMT6^{Y641N,F}$ in the presence of wild-type KMT6 resulted in decreased affinity for un- and monomethylated H3K27 and enhanced conversion of dimethylated into transcription repressive trimethylated H3K27. In line with these results, increased H3K27 trimethylation was found in tumor samples carrying tumorigenic KMT6 mutations (Yap et al., 2011). These findings are another demonstration of genetic mutations in chromatin-modifying enzymes generating altered epigenomes that will affect a variety of biological processes including those involved in drug sensitivity.

KMT2 family enzymes (SET1A-B, MLL and ASH1) are responsible for the methylation of histone H3 at lysine 4 (H3K4) (Fig. 1). Trimethylation of H3K4 is associated with active gene transcription. Gene rearrangement of MLL (KMT2A-E) is one of the most common chromosomal abnormalities in human leukemia (Chi et al., 2010; Krivtsov and Armstrong, 2007). As a result of in frame chromosomal translocations, the MLL N-terminus is fused to one of more than 50 partners, including members of the AF and ENL family of proteins, such as AF4, AF9, AF10, and ENL (Krivtsov and Armstrong, 2007). Multiple studies have shown a tumor-initiating role of these fusion proteins by regulating the expression of HOX genes, which results in blocking differentiation and stimulating oncogenic transformation (Chi et al., 2010). MLL-fusion proteins interact directly or indirectly with KMT4 (DOT1L), a histone H3 lysine 79 (H3K79) methyltransferase and drive acute myeloid leukemia in a KMT4 methyltransferase activity dependent manner (Okada et al., 2005; van Leeuwen et al., 2002) (Fig. 1). Interaction of oncogenic MLL-fusion proteins and KMT4 resulted in an increase of H3K79 methylation at promoters and concomitant upregulation of transcription of leukemia-relevant genes including *HOXA9* (Okada et al., 2005). Recently, a KMT4 small molecule inhibitor (EPZ004777) was shown to selectively eradicate leukemic cells bearing the *MLL* gene translocation while having little effect on non-*MLL*-translocated cells (Daigle et al., 2011). Furthermore, administration of the KMT4 inhibitor increased to some extent the survival of mice xenografted with a MLL tumor cell line, indicating that inhibition of the histone modifier KMT4 may serve as a targeted therapeutic against MLL. The possibility of treating leukemia harboring oncogenic MLL-fusion proteins with epigenetic drugs took an exiting turn with the development of small molecule inhibitors targeting members of the bromodomain and extraterminal (BET) subfamily of human bromodomain proteins (BRD2, BRD3 and BRD4) (Filippakopoulos et al., 2010). Bromodomain-containing proteins associate with acetylated chromatin and facilitate transcriptional activation by recruiting transcriptional activators including MLL-fusion partners (Dawson et al., 2011). BET-inhibitors (BET-i) were shown to be effective against mouse and human MLL-fusion leukemias by inducing cell-cycle arrest and apoptosis. Mechanistically, BET-i inhibited transcription of cancer relevant genes such as *BCL2*, *C-MYC* and *CDK6*, through displacement of BET proteins from chromatin (Dawson et al., 2011; Delmore et al., 2011; Zuber et al., 2011). The notion that many human malignancies require the oncoprotein C-MYC for tumor maintenance provides opportunities for BET-inhibitors as epigenetic drugs in the treatment of a variety of tumors (Delmore et al., 2011; Soucek et al., 2008).

3.4. Lysine demethylation

Histone methylation is counteracted by lysine-specific demethylases (KDMs). Two classes of histone demethylases have thus far been identified. KDM1A (LSD1) and KDM1B (LSD2) comprise the KDM1 class, which are FAD-dependent amine oxidases and demethylate only mono- and dimethylated lysines. The Jumonji C (JmjC) domain is a signature motif for the second class of demethylases, which consists of 30 Fe(II) and 2-oxoglutarate-dependent enzymes. Proteins in this class are subdivided based on JmjC domain sequence homology and the overall architecture of additional motifs (Pedersen and Helin, 2010). Demethylase activity has been observed for several of these proteins, of which the majority targets methylated lysines, although JMJD6 demethylates histone H3 arginine 2 (H3R2) and histone H4 arginine 3(H4R3) (Chang et al., 2007) (Fig. 1).

Similar to histone methyltransferases, aberrant function of histone demethylases is associated with cancer, indicating that a balanced control of chromatin configuration is imperative for normal cell growth. For example, downregulation of KDM1 (LSD1), a subunit of the NuRD transcriptional repressive complex, was observed in breast carcinomas and may correlate with metastatic potential of these tumors (Wang et al., 2009b). However, in specific tissues, such as in the prostate and testis, KDM1 is present in a chromatin-associated complex together with the androgen receptor (AR) and demethylates the repressing histone marks mono- and dimethyl H3K9 and thereby promotes gene activation of AR target genes (Metzger et al., 2005) (Fig. 1). These observations suggest a role for KDM1 in androgen responsive tissues such as brain, prostate and testis. Indeed, KDM1 expression correlated with aggressive prostate cancer possibly by constitutive activation of AR-mediated growth signals (Kahl et al., 2006). Furthermore, others found strong expression of KDM1 in poorly differentiated neuroblastoma (Schulte et al., 2009). Inhibition of KDM1 using small molecule inhibitors or *KDM1* shRNAs increased H3K4 methylation and inhibited tumor growth (Metzger et al., 2005) (Fig. 1). In addition, KDM1 was found to suppress TP53 activity by demethylation of dimethylated TP53 lysine 370, thereby preventing interaction between TP53 and 53BP1 (Huang et al., 2007). These results suggest that KDM1 can either repress or activate gene transcription, depending on the proteins it interacts with, underlining the ambiguous nature of KDMs.

While KDM1 removes mono- and dimethyl groups, jumonji domain containing demethylases remove mono-, di- and trimethyl groups (Klose and Zhang, 2007). Mislocalization of jumonji domain demethylases has been observed in various cancer types. A subset of AML patients presented with chromosomal translocations involving nucleoporin 98 (NUP98) and the H3K4me2/3 recognizing PHD motif of KDM5A (JARID1A/RBP2) or PHF23 (Wang et al., 2009a) (Fig. 1). The oncogenic potential of such fusion proteins relies on the PHD-motif-mediated H3K4me2/3 recognition and the inability to remove this histone mark as well as concomitant inhibition of KMT6-mediated H3K27 trimethylation (H3K27me3) (Fig. 1). The increase in H3K4me2/3 and reduction of H3K27me3 subsequently enforces the expression of genes critical for hematopoietic development leading to leukemia (Cui et al., 2009; Wang et al., 2009a). Recently, direct evidence was provided showing that increased KDM5A levels were sufficient to acquire resistance to cytotoxic and targeted drugs (Sharma et al., 2010).

KDM5B (JARID1B/PLU-1) overexpression was found in advanced stages of prostate cancer and breast cancer where it induced the removal of H3K4me2/3 leading to repression of several tumor suppressor genes, including *BRCA1* (Xiang et al., 2007b; Yamane et al., 2007) (Fig. 1). Repression of tumor suppressor genes by histone demethylases seems to be a recurrent theme, since KDM2B (JHDM1B/FBXL11) erased dimethylation of H3K36me2 and/or H3K4me3, leading to repression of the $p14^{ARF}$, $p15^{INK4B}$ and $p16^{INK4A}$ cell inhibitory protein encoding *CDKN2A/B* locus in T-cell lymphomas (He et al., 2008; Tzatsos et al., 2009) (Fig. 1). In agreement with the role of this locus as an anti-tumorigenic fail-safe mechanism, expression of KDM2B and the related KDM2A (JHDM1A/FBXL10) inhibit replicative- and oncogene-induced senescence, thereby promoting tumorigenesis.

In addition to misregulation of KDMs by overexpression and chromosomal translocation, somatic inactivating mutations of

Table 1
Histone modifications and related modifying enzymes associated with tumorigenesis, patient prognosis and drug resistance.

Histone modification	Enzymes	Relative level in cancer	Role in cancer	References
H3K9ac	KAT2, KAT12, SIRT6	Reduction	Tumor progression	Seligson et al. (2005)
			Poor patient prognosis	Park et al. (2008)
				Zhen et al. (2010)
			Good patient prognosis	Barlési et al. (2007)
H3K14ac	KAT2, KAT3, KAT6, KAT7, KAT10, KAT12	Reduction	Drug resistance	Sharma et al. (2010)
H3K18ac	KAT2, KAT3, KAT12	Reduction	Tumor progression	Seligson et al. (2005)
			Tumor recurrence	Seligson et al. (2005)
			Poor patient prognosis	Manuyakorn et al. (2010)
			Good patient prognosis	Tzao et al. (2009)
H3K56ac	KAT3B	Gain	Tumorigenesis	Liu et al. (2010)
H4K16ac	KAT5, KAT8, SIRT1, SIRT2	Reduction	Drug resistance	Hajji et al. (2010)
			Transformation	Fraga et al. (2005)
H2A/H4R3me2	PRMT1, PRMT5	Gain	Tumor recurrence	Seligson et al. (2005)
			Poor patient prognosis	Seligson et al. (2005)
		Reduction	Tumorigenesis	Barlési et al. (2007)
H3K4me2/3	PRMT6, KMT2, KMT7, KDM1, KDM2, KDM5	Reduction	Drug resistance	Sharma et al. (2010)
			Tumor recurrence	Seligson et al. (2005)
			Poor patient survival	Manuyakorn et al. (2010)
H3K9me2/3	KMT1, KMT8, KDM1, KDM3, KDM4	Gain	Invasion, metastasis	Bracken et al. (2007)
			Poor patient prognosis	Chen et al. (2010)
			Drug resistance	Braig et al. (2005)
		Reduction	Poor patient survival	Manuyakorn et al. (2010)
H3K9me3	KMT1, KMT8, KDM3, KDM4	Gain	Tumor progression	Bachman et al. (2003)
			Tumor recurrence	Nguyen et al. (2002)
			Poor patient prognosis	Nguyen et al. (2002)
			Good patient prognosis	Ye et al. (2007)
H3K27me3	KMT6, KDM6	Reduction	Senescence	Shen et al. (2007)
			Poor patient prognosis	Wei et al. (2008)
		Gain	Apoptosis	Müller-Tidow et al. (2010)
			Pluripotency	Wu et al. (2010b)
			Poor patient prognosis	Yu et al. (2007)
H4K20me3	KMT3B, KMT5	Reduction	Transformation	Fraga et al. (2005)

KDM6A (UTX) have been found in various tumors, particularly in multiple myeloma and ccRCC (Dalgliesh et al., 2010; van Haaften et al., 2009). Both KDM6A and KDM6B (JMJD3) were shown to have H3K27me3 demethylating activity (Agger et al., 2007) (Fig. 1). Therefore, the tumorigenic potential of KDM6A mutations may relate to the inability to remove the repressive H3K27me3 mark at distinct genomic loci (van Haaften et al., 2009). In addition, KDM6B contributed to the activation of the *CDKN2A* locus (expressing p16^{Ink4a} and p14^{Arf}/p19^{Arf}) upon oncogenic BRAF and RAS expression, suggesting that inactivation of H3K27me3 demethylases may inactivate a tumor protective fail-safe mechanism (Agger et al., 2009). Indeed, downregulation of KDM6B expression levels was found in various hematological malignancies. In contrast, others have reported overexpression of KDM6B in Hodgkin's lymphoma and prostate cancer progression (Anderton et al., 2011; Xiang et al., 2007a) suggesting that KDM6B may have cell-type-specific substrates, which require either gain- or loss-of-function to provide a proliferative selective advantage during tumorigenesis.

Inactivating somatic KDM5C (JARID1C/SMCX) mutations were discovered in primary ccRCC. In these tumors, KDM5C mutations predominantly co-occurred with mutations in the Von Hippel–Lindau (VHL) tumor suppressor, and correlated with the transcriptional alteration of a specific ccRCC gene signature (Dalgliesh et al., 2010).

Finally, the jumonji-only histone demethylase KDM8 (JMJD5), an H3K36me2 demethylase was shown to be overexpressed in breast, thyroid, adrenal, bladder, uterine, and liver related cancers (Hsia et al., 2010) (Fig. 1). Inhibition of KDM8 in breast cancer cell lines resulted in a cell-cycle arrest, suggesting that KDM8 is essential for tumor cell proliferation. Mechanistically, KDM8 was shown to compete with class I HDACs in transcriptional regulation. Demethylation of H3K36me2, a repressive mark, resulted in loss of HDAC1 chromatin binding and consequently increased histone acetylation and active transcription of proliferation genes such as cyclin A1 (Hsia et al., 2010). While these results suggest a tumor-promoting function for KDM8, others identified KDM8 loss-of-function retroviral integrations in murine B-cell lymphomas. Consistent with studies in *C. elegans* (Pothof et al., 2003), downregulation of KDM8 in fibroblasts resulted in an increased mutation frequency, suggesting that KDM8, at least in this setting, suppresses genome instability and can be considered a tumor suppressor (Suzuki et al., 2006).

4. Epigenetics and drug resistance

Histone PTMs are recognized by proteins harboring one or more specialized structures, such as chromo-(methylation), bromo-(acetylation), BCRT-(phosphorylation) and PHD-domains (methylation) (Chi et al., 2010). Among these proteins are SWI/SNF, ISWI, CHD and INO80 chromatin remodeling proteins, which in turn move, destabilize, eject or restructure nucleosomes, thereby changing chromatin structure and adding an extra layer of regulation (Clapier and Cairns, 2009). The combination of DNA and histone modifications provides an enormous non-genetic diversity allowing a level of specificity that is required for the execution of a variety of biological processes including cell-type-specific transcription, replication and DNA repair (Baylin and Jones, 2011). Intriguingly,

recent efforts in mapping genetic alterations by whole genome or exome sequencing of a variety of human malignancies yielded evidence that DNA- and histone-modifying enzymes as well as nucleosome remodelers are frequently mutated and contribute to tumorigenesis (Delhommeau et al., 2009; Gui et al., 2004; Jiao et al., 2011; Jones et al., 2010; Langemeijer et al., 2009; Ley et al., 2010; Nikoloski et al., 2010; Pasqualucci et al., 2011; van Haaften et al., 2009; Varela et al., 2011; Versteege et al., 1998; Wang et al., 2009a). These studies not only establish a critical role for epigenetics as a driving force in tumorigenesis but also provide a rational for epigenomic changes and non-genetic heterogeneity observed in tumor cells (Table 1). The establishment of tumor epigenomes not only initiates tumorigenesis but also allows the acquisition of additional genetic and epigenetic changes that deregulate many biological processes including those favoring survival in the presence of a particular drug. Moreover, "misinterpretation" of chromatin states by mutated PTM readers will contribute to transcriptional states that induce non-genetic drug resistance. The dependency of such transcriptional states on reversible histone modifications encourages the design and development of epigenetic drugs to reverse tumor epigenomes to a state that is incompatible with cellular life or a drug responsive state.

Mechanisms involved in acquired drug resistance are increased drug efflux, inactivation of pro-apoptotic genes, perturbed DNA repair, activation of parallel or downstream signal transduction pathways and secondary mutations in drug targets. Although these mechanisms are associated with genetic alterations, several studies indicated a role for DNA and histone modifications in driving increased drug efflux, silencing of apoptotic genes and perturbed DNA repair (Table 1; Fig. 3B).

5. Multidrug resistance by epigenetic regulation of ABCB1

Increased expression of P-glycoprotein (Pgp; ABCB1) is a well-known mechanism involved in acquired drug resistance (Borst and Elferink, 2002; Sarkadi et al., 2006). Pgp belongs to the ATP binding cassette (ABC) transporter superfamily, which facilitates increased efflux of chemotherapeutic drugs from tumor cells resulting in drug insensitivity to various agents (Senior et al., 1995). Tumors with intrinsically low expression levels of Pgp generally respond well to chemotherapy. Expression of the Pgp encoding gene *ABCB1* or Multi Drug Resistance 1 (*MDR1*) is often induced upon chemotherapy, followed by subsequent upregulation of ABCB1 protein expression (Baker et al., 2005; Hu et al., 1999). Treatment of murine *Brca1*$^{-/-}$; *p53*$^{-/-}$ breast cancer with doxorubicin, docetaxel or the poly (ADP-ribose) polymerase inhibitor olaparib, resulted in acquired drug resistance correlating with upregulation of *Abcb1* RNA levels (Pajic et al., 2009; Rottenberg et al., 2007). Additional treatment of this murine breast cancer model with the topoisomerase inhibitor topotecan resulted in acquired drug resistance due to upregulation of the *Bcrp/Abcg2* drug transporter. Genetic deletion of *Abcg2/Bcrp* in the *Brca1*$^{-/-}$; *p53*$^{-/-}$ mouse breast cancer indeed increased the overall survival of topotecan treated animals, but did not eradicate tumors indicating that additional mechanisms are involved in acquired drug resistance (Zander et al., 2010). Studies in patients with AML revealed that drug treatment induced ABCB1 expression was observed already 4 h upon the start of chemotherapy (Hu et al., 1999). Moreover, in four out of five patients with unresectable pulmonary metastases, increased *ABCB1* mRNA levels were detected only 50 min after doxorubicin treatment (Abolhoda et al., 1999). In addition, progressive drug resistance correlated with the increase of ABCB1 expression and the intensity of the chemotherapy in neuroblastoma (Keshelava et al., 1998). In conclusion, the kinetics and dose-dependent induction of ABCB1 upon drug treatment favors a role for immediate transcriptional activation of *ABCB1* over selection of a tumor subpopulation expressing high *ABCB1* levels.

The question remains, which factors cause the induction of ABCB1 expression and in particular which chromatin changes at the promoter region affect *ABCB1* expression upon drug treatment? Hypo- and hypermethylated DNA has been found at the *ABCB1* promoter in different (tumorigenic) contexts. While the *ABCB1* promoter in normal bladder cells is hypomethylated, tumor cells of this tissue usually contain a hypermethylated *ABCB1* promoter. Interestingly, upon chemotherapy, methylation of the *ABCB1* promoter reverted to its hypomethylated state and correlated with overexpression of the *ABCB1* gene in tumors of the bladder and similar observations were made in AML patient samples (Nakayama et al., 1998; Tada et al., 2000). Hypomethylation of the promoter of the *ABCG2/BCRP* drug transporter also increased expression in response to chemotherapy, suggesting that this is a general mechanism to regulate ABC transporter expression in response to drug treatment (Bram et al., 2009).

How chemotherapeutic drugs induce hypomethylation of the *ABCB1* promoter is still not fully understood and different mechanisms have been postulated (Baker and El-Osta, 2004). For instance, chemotherapeutic drugs could induce active DNA demethylation by yet unknown DNA demethylases. Alternatively, chemotherapeutics could select for a small tumor subpopulation harboring hypomethylated *ABCB1* promoters. A model involving passive demethylation has been proposed in which tethering of methylases involved in DNA methylation (such as DNMT1) to the DNA is inhibited, resulting in a lack of methylation. Finally, changes in key chromatin remodeling complex activities could have an impact on the methylation status of the *ABCB1* promoter, or lead to alleviated repression at the *ABCB1* promoter, resulting in induced *ABCB1* expression. There are indications that histone-modifying complexes are recruited to the methylated *ABCB1* promoter serving its transcriptional repression. For instance, MeCP2, a well-known Methyl-CpG-binding protein (MBP) (Nan et al., 1997), binds to hypermethylated DNA at the *ABCB1* promoter (El-Osta et al., 2002) and may serve as a docking platform for nucleosome modifiers and remodelers, such as SWI/SNF, HDAC1, HDAC2 and mSIN3, thereby altering the chromatin state of gene promoters and subsequently transcription (Harikrishnan et al., 2005; Jones et al., 1998; Nan et al., 1998). Although a direct interaction of these co-repressors with the *ABCB1*-promoter-bound MeCP2 has not been described, *ABCB1* expression is induced upon inhibition of HDAC activity or by overexpression of the p300/CREB lysine acetyl transferase (KAT3B) (Baker et al., 2005; Jin and Scotto, 1998; Tabe et al., 2006). While HDACi treatment had a relative small effect on *ABCB1* expression in human T-cell acute lymphoblastic leukemia cells, combined inhibition of DNA methylation and HDAC activity did induce *ABCB1* expression, supporting a repressive role of the MeCP2/HDAC complex in regulation of *ABCB1* expression (El-Osta et al., 2002). Removal of HDAC inhibition dramatically reduces ABCB1 protein level, albeit with slow kinetics over a time period of 6 days, suggesting that other factors are involved in regulation of ABCB1 expression (Xiao et al., 2005).

In response to HDACi treatment acetylation levels of histones H3 and H4, and methylation levels of H3K4 were increased at the *ABCB1* promoter (Baker et al., 2005; Xiao et al., 2005). In analogy, H3 acetylation, but not H4 acetylation and H3K4 methylation, was increased at the *ABCB1* promoter upon chemotherapy and preceded the *ABCB1* expression, suggesting that H3 promoter acetylation is involved in drug-treatment-induced *ABCB1* expression (Baker et al., 2005). In contrast, trimethylation of H3K4 at the *ABCB1* promoter was found to be dependent on the methyltransferase KMT5A (MLL1). Knockdown of KMT5A decreased the constitutive expression of *ABCB1* and sensitized cancer cells to chemotherapeutic agents (Huo et al., 2010).

In addition to *ABCB1*, breast cancer resistance protein (BCRP) and multidrug-resistance-associated protein 8 (MRP8) expression levels are also found upregulated upon HDACi treatment (Hauswald et al., 2009). These findings suggest that HDACs are recruited to the methylated promoter and mediate transcriptional repression of drug transporters, including *ABCB1*, *BCRP* and *MRP8*.

Collectively these data indicate that drug-treatment-induced ABCB1 expression occurs at the transcriptional level, which is mediated by DNA methylation and histone acetylation at the *ABCB1* promoter and can contribute to acquired resistance to anti-cancer therapies (Fig. 3B).

6. Epigenetic silencing of pro-apoptotic genes in drug tolerance

Drug treatment in anti-cancer therapy often leads to tumor cell death, i.e. apoptosis. Genetic or epigenetic perturbations resulting in a defective execution of an apoptotic response could potentially result in drug-tolerant tumor cells. Indeed, genetic mutations as well as epigenetic changes in pro-apoptotic genes are a hallmark of human malignancies (Hanahan and Weinberg, 2011). Although it remains controversial whether these mutations contribute to drug resistance (Borst et al., 2001), stochastic epigenetic silencing of pro-apoptotic genes in a fraction of the total tumor cell population may allow selection of these cells upon drug treatment. Indeed, several pro-apoptotic genes were found to be silenced by promoter methylation upon drug treatment including death associated protein kinase 1 (*DAPK1*) and apoptotic peptidase activating factor 1 (*APAF-1)*.

Cell-cycle arrest or apoptosis are promoted by tumor suppressor gene *TP53* in response to chemotherapy treatment, and as a consequence *TP53* loss can induce drug resistance. Caspase-9 and its cofactor Apaf-1 are essential downstream effectors of *p53* during Myc-induced apoptosis. Consistently, inactivation of caspase-9 and *Apaf-1* can substitute for the loss of *p53* in Myc-driven oncogenic transformation (Soengas et al., 1999). Indeed, loss of *APAF-1* expression is frequently observed in metastatic melanomas retaining TP53. *APAF-1* negative melanoma cells were unable to execute an apoptotic program in response to *TP53* activation, resulting in chemotherapy resistance. Loss of APAF-1 in metastatic melanoma occurred through genomic loss or epigenetic silencing. In the latter case *APAF-1* expression was restored by the DNA-methylation inhibitor 5-aza-dCR resulting in enhanced chemosensitivity and rescue of the apoptotic defects associated with APAF-1 loss (Soengas et al., 2001). In addition, others have reported increased APAF-1 expression in ovarian cancer cell lines treated with HDACi, suggesting a contribution in APAF-1 silencing by HDACs (Tan et al., 2011a). Surprisingly, APAF-1 negative melanoma cells did not display hypermethylation of the *APAF-1* promoter indicating that *APAF-1* expression is regulated by an indirect DNA-methylation-dependent mechanism (Soengas et al., 2001). While *APAF-1* promoter methylation was not apparent in multiple myeloma (Chim et al., 2007), transitional cell carcinomas of the bladder and ccRCC harbored hypermethylated *APAF-1* promoters which correlated with tumor stage and tumor grade (Christoph et al., 2006a; Christoph et al., 2006b). In conclusion, downregulation of pro-apoptotic *APAF-1* expression is correlated with resistance to chemotherapeutics, yet the mechanism underlying *APAF-1*-mediated drug resistance seems to be dependent on cell type and *TP53* status (Fig. 3B).

Reduced expression or inactivation of the pro-apoptotic *DAPK1* by genetic or epigenetic mechanisms is found in human malignancies (Michie et al., 2010; Raval et al., 2007). DAPK kinases induce apoptosis by phosphorylating serine and threonine residues in substrates contributing to apoptosis induced by interferon-γ, FAS, tumor necrosis factor-α (TNFα), TNF-related apoptosis-inducing ligand (TRAIL) and anoikis (Michie et al., 2010). In addition, DAPK1 suppresses MYC- and E2F-induced oncogenic transformation by activating $p19^{ARF}$-p53-dependent apoptosis (Raveh et al., 2001). Furthermore, DAPKs play a critical role in inhibiting cell migration and invasion of cancer cells (Kuo et al., 2006). Reintroduction of *DAPK* expression suppressed the metastatic potential of lung cancer in a mouse model underlining the tumor suppressing capabilities of the DAPKs (Inbal et al., 1997).

Loss of DAPK1 expression in tumors is caused by either homozygous gene deletion, loss of heterozygosity or hypermethylation of a CpG region in the 5′ UTR (Bialik and Kimchi, 2004). Epigenetic silencing of DAPK was found in the majority of sporadic chronic lymphocytic leukemia (CLL) (Raval et al., 2007). These results were supported by *DAPK1* promoter methylation in a wide range of human tumor types in which *DAPK* silencing correlated with tumor progression, histo-pathological staging, increased metastasis and high tumor recurrence (Bialik and Kimchi, 2004; Chaopatchayakul et al., 2010; Christoph et al., 2006b; Raval et al., 2007; Sugita et al., 2011; Tada et al., 2002; Toyooka et al., 2003; Voso et al., 2010). However, in AML and MDS the extent of *DAPK* promoter methylation is a matter of debate since some studies identified this as a very rare event, contradicting previous studies (Claus et al., 2011; Voso et al., 2010). DAPK expression could be restored in tumor cells by demethylating agent 5-aza-dCR or HDACi, indicating that *DAPK* silencing involves DNA-methylation and histone acetylation (Christoph et al., 2006b; Toyooka et al., 2003; Wu et al., 2010a; Zhang et al., 2006). A higher prevalence of *DAPK, FAS* and *TRAILR1* promoter hypermethylation in cervical carcinomas non-responsive to drug treatment suggested that epigenetic downregulation of *DAPK* expression in part may contribute to drug resistance (Chaopatchayakul et al., 2010). Indeed, HDACi-mediated restoration of DAPK expression in human gastric and lung cancer cell lines increased chemosensitivity to anti-cancer drugs (Wu et al., 2010a; Zhang et al., 2006). In conclusion, the use of epigenetic drugs capable of restoring *DAPK* expression, in combination with DNA-damaging agents, is an exciting approach in the treatment of DAPK negative, chemoresistant cancers (Fig. 3B and C).

7. Epigenetic regulation of DNA-repair genes in drug tolerance

Inactivation of mismatch repair genes (MMR) leads to unrepaired deletions in mono- and dinucleotide repeats resulting in variable repeat lengths, referred to as microsatellite instability (MSI), a hallmark of tumors with inactivated MMR genes (Aaltonen et al., 1993). Germline mutations of mismatch repair genes such as *MSH2, MLH1* and *PMS2* are frequently found in hereditary non-polyposis colon cancer (HNPCC) (Lynch et al., 2009). MMR deficient human cancer cell lines tolerated alkylating agents, suggesting that loss of MMR could cause chemotherapy resistance (Anthoney et al., 1996). MSI in tumors can only in part be explained by mutations in MMR genes such as *MLH1*, *MSH2*, *MSH3* and *PMS2* (Peltomäki, 2003). In less than 10% of all sporadic uterine endometrial carcinomas (UEC) MSI was associated with mutations in MMR genes, suggesting that mutations in genes regulating MMR proteins or epigenetics could contribute to inactivation of MMR gene expression. Indeed mutations affecting MSH2 protein degradation were identified in a variety of cancers including ALL and colon cancer (Diouf et al., 2011). Epigenetic-driven inactivation of MMR was illustrated by the identification of *MLH1* promoter hypermethylation in various tumors and as an early event in endometrial tumorigenesis, which correlated with drug resistance and

predicted poor overall survival (Esteller et al., 1998; Strathdee et al., 1999). However, an association between clinical response and MMR status in primary tumors was not observed (Helleman et al., 2006), even though *MLH1* silencing was correlated with tumor relapse during chemotherapy (Gifford et al., 2004).

The ability to measure *MLH1* promoter methylation status in plasma DNA from ovarian cancer patients provided a non-invasive method to monitor the potential acquisition of drug resistance during drug treatment (Gifford et al., 2004). Examination of plasma DNA of patients with epithelial ovarian cancer for methylation of the *MLH1* promoter before chemotherapy and at relapse revealed increased *MLH1* promoter methylation at relapse. Moreover, 25% of the samples from patients with a relapse had *MLH1* methylation that was not detected in matched prechemotherapy plasma samples. Acquisition of *MLH1* methylation in plasma DNA at relapse predicted poor overall survival of patients (Gifford et al., 2004). These results strongly suggest that selection favors tumor cells with repressed *MLH1* during chemotherapy resulting in a drug-resistant tumor, which provides a rational for the use of epigenetic drugs to restore *MLH1* expression and abolish drug resistance (Fig. 3B and C).

O^6-methylguanine DNA-methyltransferase (*MGMT*) encodes the DNA-repair O^6-alkylguanine-DNA-alkyltransferase (AGT), which removes alkylating lesions at position O^6 of guanine (Verbeek et al., 2008). DNA damage induced *MGMT* expression facilitates DNA repair and is a possible mechanism to acquire resistance against alkylating agents. Overexpression of MGMT in various mouse model studies suppressed tumor development due to enhanced DNA damage repair (Gerson, 2004). In contrast, MGMT knockout mice displayed increased sensitivity toward alkylating chemotherapeutics providing genetic evidence for a role of MGMT in drug responsiveness (Glassner et al., 1999). Although the precise mechanism of DNA-damage-induced *MGMT* expression is still unclear (Gerson, 2004; Verbeek et al., 2008), the *MGMT* promoter region contains transcription factor recognition sequences that explain induction of *MGMT* expression by glucocorticoids, cyclic AMP and protein kinase C activators. Hypermethylation of specific CpG regions in the *MGMT* promoter resulting in the silencing of *MGMT* expression has been described in primary human tumors, such as glioma, lymphoma, retinoblastoma, breast and prostate tumors (Esteller et al., 1999; Kang et al., 2002; Watts et al., 1997). Remarkably, a reduction of *MGMT* promoter methylation was acquired upon chemotherapy treatment resulting in *MGMT* expression, and increased AGT activity rendering the cytotoxic drugs ineffective (Chan et al., 1992; Fritz and Kaina, 1992). In line with these results, studies in patients with glioma, glioblastoma, non-Hodgkin lymphoma or male germ cell tumors identified *MGMT* promoter methylation as a marker for chemotherapy sensitivity: tumor cells with hypermethylated *MGMT* promoters responded better to chemotherapy, and these patients showed improved survival compared to patients with hypomethylated *MGMT* promoters (Esteller et al., 2000; Koul et al., 2004; Weller et al., 2010). As a result, *MGMT* promoter methylation status is an established molecular marker in clinical trials, which is predictive for the response to alkylating chemotherapy and radiotherapy (Kesari et al., 2009; Riemenschneider et al., 2010; Weller et al., 2010; Wick et al., 2009). In conclusion, elevated MGMT expression, caused by *MGMT* promoter hypomethylation is a strong mediator of acquired drug resistance, suggesting that *MGMT* silencing or AGT inhibition has the potential to sensitize tumors for chemotherapy (Fig. 3B and C).

Another DNA-repair pathway that is linked to chemotherapy resistance involves the Fanconi anemia (FA) group of proteins. FA germline mutations result in chromosomal instability leading to congenital defects, bone marrow failure and increased cancer susceptibility (D'Andrea, 2010). The Fanconi-BRCA1/2 molecular pathway plays an important role in DNA repair and is necessary for the normal cellular response to interstrand DNA cross-linking agents like cisplatin, mitomycin C and diepoxybutane (D'Andrea, 2010). Until now, 15 FA proteins have been identified, of which eight assemble into a core complex upon DNA damage (Kim et al., 2011; Stoepker et al., 2011). Monoubiquitination of FANCD2 and FANCI results in chromatin recruitment into nuclear DNA-repair foci. In addition, recruitment of the core complex by FANCM to the site of DNA damage results in BRCA1/2- (BRCA2 is also known as FANCD1) mediated DNA repair via homologous recombination (Deans and West, 2011).

Somatic mutations or epigenetic silencing of the FA pathway is observed in a variety of tumors (D'Andrea, 2010; Tischkowitz et al., 2003). Fancf deficiency causes increased ovarian tumor incidence in mice and in mouse embryonic fibroblasts it resulted in an aberrant response to DNA cross-linking agents (Bakker et al., 2012). Hypermethylation of the *FANCF* promoter was found in primary ovarian adenocarcinomas, ovarian granulosa-cell tumors, cervical cancer, non-small cell lung cancers (NSCLC) and squamous-cell head and neck cancers (Dhillon et al., 2004; Marsit et al., 2004; Narayan et al., 2004; Taniguchi et al., 2003). In ovarian, glioma and pancreatic cancer cell lines, *FANCF* promoter methylation resulted in decreased gene expression and was associated with increased sensitivity to cisplatin and other DNA cross-linking agents (Chen et al., 2007; Taniguchi et al., 2003). Conversely, restoration of FANCF expression by 5-aza-dCR-mediated promoter hypomethylation resulted in cisplatin resistance (Taniguchi et al., 2003). In conclusion, treatment of cancer patients with DNA cross-linking agents, such as cisplatin and alkylating agents may result in tumor relapse by selection for FANCF expressing tumors cells with hypomethylated *FANCF* promoters (Fig. 3B). Measuring promoter methylation status of FA genes during chemotherapy may therefore be a suitable approach to monitor drug resistance (D'Andrea, 2010).

Evidently, epigenetic regulation of genes involved in drug efflux, apoptosis and DNA repair contributes to acquired drug resistance. However, the chromatin biology underlying the epigenetic regulation remains largely unknown. Consequently, manipulating the expression of such genes to improve drug sensitivity or reverse drug resistance is restricted. Future studies aimed at decoding drug-resistance-associated histone marks, corresponding histone modifiers and nucleosome remodelers will increase our knowledge of critical players in epigenetic-driven drug resistance.

8. Chromatin-mediated drug resistance

Although the non-genetic transcriptional changes seen in drug-resistant tumors, as described above, are clearly linked to altered epigenetic regulation of genes, the underlying epigenetic mechanisms are largely unknown (Glasspool et al., 2006). While the treatment of chronic myeloid leukemia (CML), harboring the BCR–ABL fusion oncogene, with the targeted drug imatinib (Gleevec) shows unprecedented clinical efficacy and increased survival, a third of the patients require alternate therapy due to non-responsiveness of the tumor cells (Roychowdhury and Talpaz, 2011; Sellers, 2011). Secondary mutations in *BCR–ABL*, which render imatinib ineffective (Gorre et al., 2001), can only explain drug resistance to imatinib in 30% of the cases. The relative short time in which drug resistance to imatinib occurs (<1 year) combined with upregulation of relevant ERK and AKT pathways suggest a role for non-genetic heterogeneity driving drug resistance.

A similar pattern is observed in the treatment of melanomas harboring $BRAF^{V600E}$ mutations with BRAF inhibitors (PLX4032). Despite an initial response, virtually all patients develop resistance due to reactivation of the RAF–MEK pathway, activation of alternative signaling pathways or chromatin regulating events (Bollag

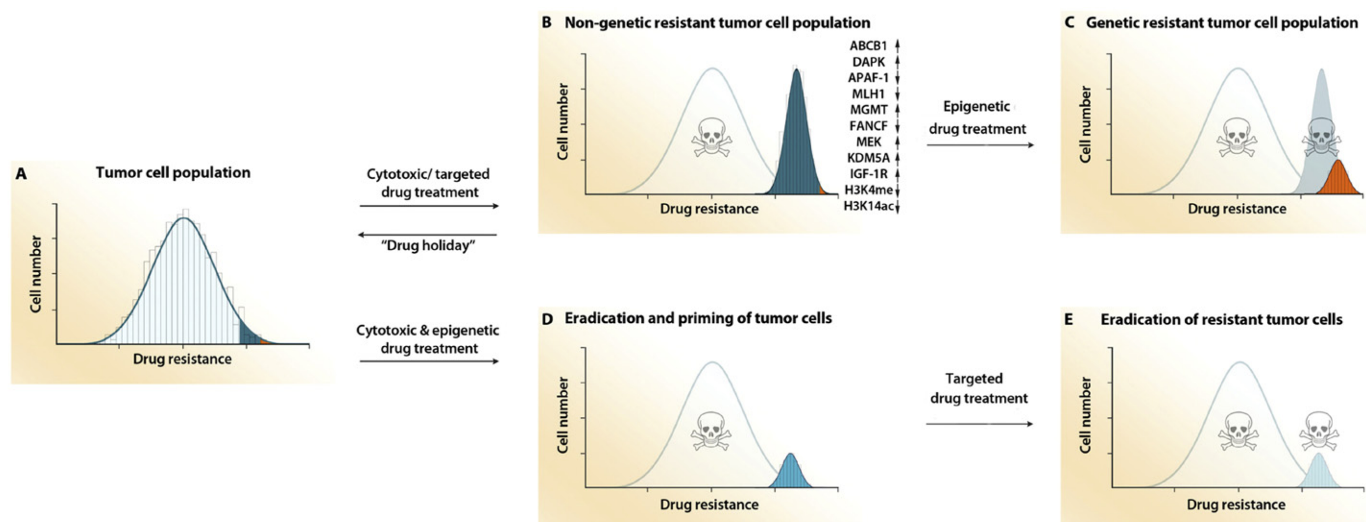

Fig. 3. Reversible non-genetic tumor heterogeneity as a source for drug resistance. (A) Tumors consist of a heterogeneous cell population, which display different drug sensitivities. Whereas the bulk of the tumor cell population is sensitive toward drug treatment (light shaded population) rare populations of cells exhibiting either non-reversible, genetic drug resistance (orange population) or reversible, non-genetic drug resistance (dark green population) are present before treatment. (B) Treatment of a heterogeneous tumor cell population with cytotoxic or targeted drugs will eradicate the majority of tumor cells (light shaded). A rare subpopulation of tumor cells (dark green in A and B) will survive due to non-genetic drug resistance. Mechanisms implicated in non-genetic drug resistance include epigenetic changes resulting in gene transcription of drug transporters (*ABCB1*) pro-apoptotic genes (*DAPK*, *APAF-1*), DNA-repair proteins (*MLH1*, *MGMT*, *FANCF*) and histone modifiers (*KDM5A*). In addition, a reduction in H3K4me and H3K14ac histone marks has been observed in drug-resistant cells, which may be linked to MEK and IGF-1R signal transduction pathway activation. In addition, cells harboring (secondary) genetic changes in drug targets or parallel pathways (orange population) will be maintained in the drug-resistant population. Withdrawal of the drug treatment resulting in a drug-free period ("drug holiday") may restore susceptibility for the primary cytotoxic/targeted therapy by reversal of drug-resistance-associated epigenetic marks. Alternatively, in the absence of the selective pressure of the drug, cells harboring an epigenome that enforces a proliferative advantage and drug sensitivity will grow out. (C) Treatment of non-genetic drug-resistant tumor cell populations with cytotoxic or targeted drugs in combination with epigenetic drugs, such as inhibitors of histone deacetylases (HDACi), DNA methyl transferases (DNMTi), histone methyltransferases (EPZ004777) and bromodomain proteins (BET-i) may reverse a drug-resistant epigenome into a drug sensitive epigenome thereby rendering tumor cells sensitive to the cytotoxic/targeted drug. Still, hard-wired genetic resistant tumor cells may arise (orange cell population). (D) In order to prevent the latter cells to arise upon treatment, a drug regimen may be used in which a cytotoxic drug is combined with an epigenetic drug. The rational for using this treatment plan is to debulk the tumor by cytotoxic drugs, killing the majority of the tumor cell population. Moreover, using this approach will avoid selection for genetic drug resistance toward targeted therapy. Simultaneous treatment with an epigenetic drug may sensitize surviving cells to a targeted drug, which is subsequently administered (E), thereby eradicating eventually all tumor cells including genetic resistant cells (for interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

et al., 2010; Flaherty, 2010; Johannessen et al., 2010; Sharma et al., 2010; Smalley, 2010). Similar observations have been made in the treatment of NSCLC carrying epidermal growth factor receptor (EGFR) activating mutations, with EGFR tyrosine kinase inhibitors (TKI). Remarkably, NSCLC patients who experienced a "drug holiday" upon developing EGFR TKI resistance responded well to retreatment with EGFR TKI (Becker et al., 2011; Kurata et al., 2004; Yano et al., 2005). These observations suggested a reversible, epigenetic basis for the EGFR TKI resistance phenotype (Fig. 3A and B).

Indeed, a recent study provided intriguing mechanistic insight into the epigenetics underlying acquired resistance for EGFR TKI, such as erlotinib and gefitinib in NSCLC (Sharma et al., 2010). Although EGFR TKI or chemotherapeutics were very effective in eradicating the majority of NSCLC tumor cells carrying an activating EGFR mutation, a very small percentage (0.3%) of rare, quiescent cells survived TKI treatment even at drug concentrations 100 times the IC_{50}. Interestingly, this rare drug-resistant cell population was observed in a variety of tumor cell lines upon cytotoxic and targeted drug treatment ranging from 0.3 to 5.5% of the total population, suggesting that non-genetic-mediated drug resistance may be a widespread mechanism across various malignancies and treatment modalities.

Although the resistant cells, dubbed "drug-tolerant persisters" (DTPs) were enriched for CD133 and CD24 stem cell markers, uniformity of expression was lost as soon as DTPs resumed proliferation in the presence of TKI. Proliferating DTPs (referred to as DTEPs, "drug-resistant expanded persisters") displayed a stem cell marker expression profile, which reflected the heterogeneity of the parental tumor cell line. Whether DTPs can be considered cancer stem cells or have functional cancer stem cell properties remains unclear. Using a genetic mouse mammary tumor model, it was shown that cisplatin-treated tumor remnants that allow regrowth of the mammary tumors were not enrichment for tumor-initiating cells (TICs; defined by CD24 and CD49f mammary stem cell markers), suggesting that TICs do not provide a mechanism for drug resistance (Pajic et al., 2010). Prolonged low dose cisplatin treatment of mouse mammary tumors in another mouse model did result in the enrichment of CD29; CD24 double positive TICs in cisplatin resistant tumors. Although these opposite results may relate to experimental differences it is also conceivable that rapid and dynamic epigenetic variations may drive the transition of TICs into more differentiated tumor cells and vice versa (Gupta et al., 2011).

A non-mutational basis for EGFR TKI drug resistance was suggested by the frequency at which DTPs exist in a heterogeneous tumor cell population (0.3–5.5%), the absence of secondary mutations in EGFR (like T790M), ERBB3 activation or MET amplification, and de novo appearance of DTPs in single cell derived tumor cell line clones. Moreover, prolonged passaging of DTEPs in drug-free medium restored TKI sensitivity and therefore implied a reversible, possibly epigenetic-based drug resistance. Comparative gene expression analysis of the parental tumor cell line and DTEPs revealed a non-random distribution of differential expressed genes, suggesting global chromatin changes in the resistant cells. Sharma et al. (Sharma et al., 2010) identified *KMD5A* (also known as *JARID1A* and *RBP2*) among the genes specifically upregulated in DTEPs. KMD5A is a histone demethylase that demethylates H3K4me2/3, which is a histone modification that marks open chromatin and active genes (Christensen et al., 2007; Iwase et al., 2007;

Klose and Zhang, 2007; Secombe et al., 2007) (Fig. 1). In concordance, EGFR TKI resistant cells displayed reduced H3K4me2/3, a histone mark correlated with poor prognosis (Seligson et al., 2005).

Notably, others identified enrichment of KDM5B (JARID1B) expressing, slow cycling cells upon treatment of melanoma cells with cytotoxic- or $BRAF^{V600E}$-targeted drugs. Interestingly, KDM5B expression marked a dynamic, temporarily distinct tumor subpopulation of slow-cycling cells, which were essential for tumor growth (Roesch et al., 2010). These results suggest that the H3K4 demethylases KDM5A and KDM5B both contribute to non-genetic tumor heterogeneity and drug resistance, which may suggest interplay between these enzymes.

Since KDM5A is found in complexes harboring HDACs, also reduced levels of acetylated histone H3 lysine 14 (H3K14ac) were found in TKI resistant cells. In line with these observations, drug-resistant cells of various tumor cell lines were extremely sensitive to RNA-interference-mediated inactivation of *KDM5A* or pharmacological inhibition of KDM5A-associated HDACs, while the parental tumor cell lines remained unaffected. Consequently, treatment of these cell lines with a combination of TKI and HDACi prevented the development of drug resistance. Although it remains unclear what drives the expression of KDM5A in EGFR TKI resistant cells, these results provided important molecular insight into the epigenetic mechanisms underlying drug resistance and simultaneously yield an exiting therapeutic approach to counteract acquired drug resistance (Fig. 3B and C).

The difference in HDACi sensitivity between the parental and drug-resistant cells could be explained by a differential DNA-damage response. In contrast to the parental cell population, HDACi induced γ-H2AX, a marker of DNA damage, only in drug-resistant cells in a checkpoint-dependent manner. How reduced H3K4 methylation and acetylation cause drug resistance and sensitize drug-resistant cells for HDACi-induced DNA damage remains unclear. Treatment of tumor cells with HDACi prior to incubation with EGFR TKI did not reduce drug resistance, probably because non-resistant cells constantly generate drug-resistant cells. These observations illustrate the dynamic nature of a tumor population and are in line with other studies addressing the plasticity of tumor cell populations (Gupta et al., 2011).

Remarkably, inhibitors of the insulin growth factor 1 receptor (IGF-1R) severely suppressed drug-resistant outgrowth in combination with EGFR TKI, cisplatin and BRAF inhibitor treatment. Drug-resistant cells displayed increased IGF binding protein 3 (IGFBP3), as well as increased IGF-1R phosphorylation providing a rational for the inhibitory effects of IGF-1R inhibitors. Even more interesting, IGF-1R inhibitors reduced *KDM5A* levels and concomitantly increased H3K4me2 levels, suggesting a direct link between the IGF signal transduction pathway and KDM5A. Although the cause and direction of the relationship between KDM5A and IGF signaling in drug-resistant cells is unclear, the IGF-1R pathway has been linked to drug resistance and poor prognosis in various malignancies (Casa et al., 2008; Pollak, 2008). The rise of drug-resistant cells in the presence of EGFR TKI and an IGF-1R inhibitor harboring secondary genetic mutations in EGFR indicated that drug resistance through genetic means is still selected for (Sharma et al., 2010) (Fig. 3C). Therefore, the design of treatment modalities that circumvent selection pressure for second site mutations in the drug target will be important to prevent genetic-based drug resistance. In general, combining chemotherapy or targeted drugs to eradicate the majority of the tumor cells ("debulking") with epigenetic drugs such as HDACi, DNMTi, BETi, or inhibitors of pathways affected by a drug-resistant epigenome (inhibitors of IGF-1R, MGMT, MLH1) may inform future anti-cancer treatment regimens to prevent tumor relapse (Fig. 3D and E).

Nuclear cloning has taught us that differentiated cells can be reprogrammed into pluripotent stem cells by erasing the epigenetic memory that drives a particular phenotype. In a similar fashion the studies mentioned above teach us that drug-resistant cells can be reprogrammed into drug-sensitive cells by erasing the epigenetic memory that dictates a drug-resistant phenotype using epigenetic drugs.

9. Future perspectives

To increase the survival rates of cancer patients will be impossible without improving the efficacy of cytotoxic and targeted anti-cancer drugs. Since drug resistance is the major cause of treatment failure it will be vital to avoid non-responsiveness toward current and future treatment modalities. The important contribution of DNA- and histone modifications in drug resistance implicates the possibility to prevent or abolish drug resistance by reverting the cancer epigenome of non-responsive cells to a drug responsive state. The development of various "epigenetic" drugs will provide us with the tools for studying the reversibility of drug resistance *in vitro*, in mouse models and clinical trials. Still, our understanding of the epigenetic mechanisms that drive cells into a drug-resistant state is in its infancy and requires additional research (Table 1). Efforts in charting the epigenome of normal and diseased cells will provide a platform to obtain insight into the drug resistance epigenome (Abbott, 2011; American Association for Cancer Research, 2008). However, the epigenome of a tumor biopsy or primary tumor cell line will only provide the average of a heterogeneous tumor cell population, which does not reflect a drug-resistant epigenome. Therefore, it will be imperative to determine the epigenetic landscape of the rare population of drug-resistant cells in order to identify the histone modifiers and nucleosome remodelers that render anti-cancer drugs ineffective. The results obtained from these studies will allow the design of new and enhanced drugs that change the epigenetic landscape of non-genetic drug-resistant cells to a drug sensitive state.

Furthermore, it will be key to identify the transcriptional targets or non-histone substrates sufficient to sustain the drug-resistant state. The use of loss-of-function or gain-of-function genetic screens has shown the ability to uncover factors underlying acquired drug resistance (Huang et al., 2009; Mullenders and Bernards, 2009). Finally, the design of anti-cancer therapies that combine tumor debulking strategies using cytotoxic or targeted drugs with drug sensitivity inducing "epigenetic drugs" (Fig. 3) will allow us to attack a heterogeneous tumor cell population from multiple angles, thereby minimizing the chance for drug-resistant cells to escape and generate a drug-resistant tumor.

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