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The Role of Nuclear Receptor–Binding SET Domain Family Histone Lysine Methyltransferases in Cancer

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The nuclear receptor–binding SET Domain (NSD) family of histone H3 lysine 36 methyltransferases is comprised of NSD1, NSD2 (MMSET/WHSC1), and NSD3 (WHSC1L1). These enzymes recognize and catalyze methylation of histone lysine marks to regulate chromatin integrity and gene expression. The growing number of reports demonstrating that alterations or translocations of these genes fundamentally affect cell growth and differentiation leading to developmental defects illustrates the importance of this family. In addition, overexpression, gain of function somatic mutations, and translocations of NSDs are associated with human cancer and can trigger cellular transformation in model systems. Here we review the functions of NSD family members and the accumulating evidence that these proteins play key roles in tumorigenesis. Because epigenetic therapy is an important emerging anticancer strategy, understanding the function of NSD family members may lead to the development of novel therapies.

Histone lysine methyltransferases (HMTases) catalyze the transfer of up to three methyl groups to specific lysine (K) residues on the tails of histones H3 and H4 that are critical for chromatin maintenance and the fine regulation of gene expression. Histone marks created by lysine HMTases are associated with either active transcription (such as H3K4me or H3K36me2) or repressed transcription (such as H3K27me or H2K9me) (Lachner et al. 2003; Barski et al. 2007). The global activities of these enzymes keep genes in a state poised for rapid activation or repression. Methylated histones are sensed and linked to downstream biological functions by an array of methyllysine-binding proteins. Thus, lysine HMTases play important roles in many downstream cellular processes, such as DNA replication, DNA damage response, cell-cycle progression, cytokinesis, and transcriptional regulation of important developmental and tumor-suppressor genes. The human genome encodes more than 50 predicted lysine methyltransferases, and for many of these genes dysregulation has been reported to play a causative role in human disease (Kouzarides 2007; Morishita and di Luccio 2011). Here we review the most recently reported functions of nuclear receptor–binding SET domain (NSD) family

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members with particular attention paid to their roles in cancer.

NSD FAMILY MEMBERS ARE STRUCTURALLY CONSERVED HISTONE H3 LYSINE 36 MONO- AND DIMETHYLTRANSFERASES

The NSD family of HMTases are a phylogenetically distinct subfamily of lysine-HMTases comprised of: NSD1, NSD2 (MMSET/WHSC1), and NSD3 (WHSC1L1) (Morishita and di Luccio 2011). The full-length members of the NSD family are large multidomain proteins that contain the evolutionarily conserved catalytic SET [Su(var)3-9, Enhancer-of-zeste, Trithorax] domain that is further subdivided into pre-SET, SET, and post-SET domains (Fig. 1) (Dillon et al. 2005; Herz et al. 2013). In addition, full-length NSD family members have two PWWP (proline–tryptophan–tryptophan–proline) domains that are critical for binding to methylated histone H3 as well as DNA and plant homeo-

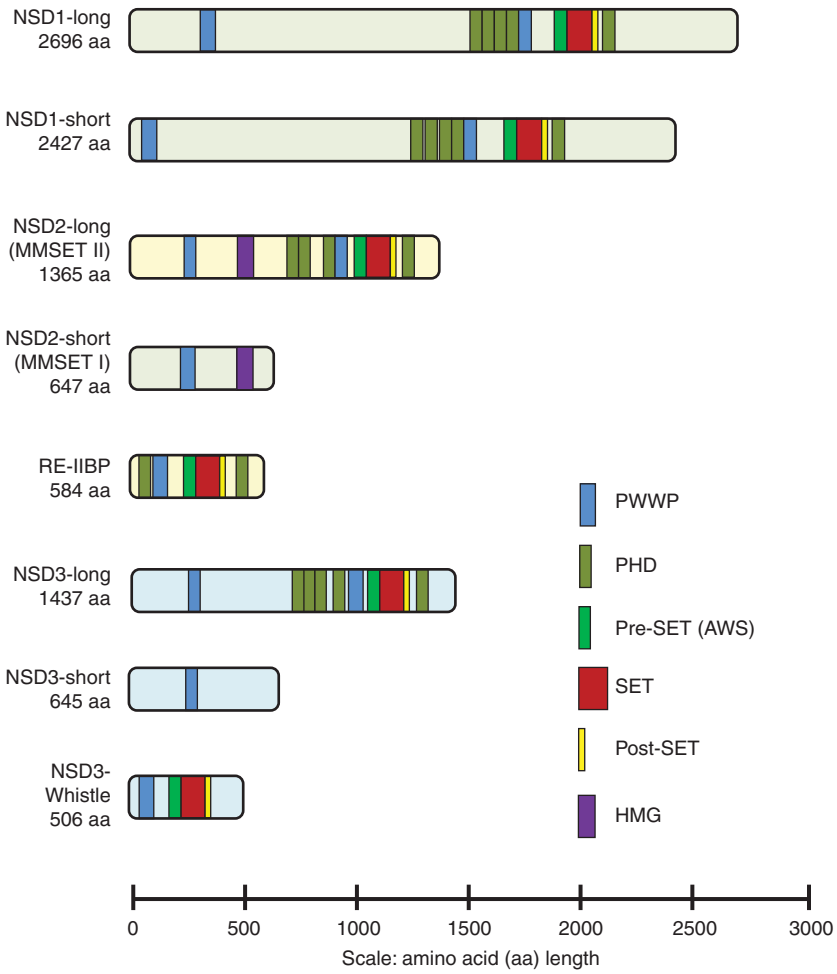

Figure 1. Conserved structural domains in the nuclear receptor–binding SET domain (NSD) family of histone lysine methyltransferases. Protein domain assignments were calculated using a simple modular architecture research tool (SMART) and the following UniProtKB entries: NSD1-long, Q96L73-1; NSD1-short, Q96L73-2; NSD2-long, O96028-1; NSD2-short, O96028-3; RE-IIBP, O96028-4; NSD3-long, Q9BZ95-1; and NSD3-short, Q9BZ95-3. NSD3-WHISTLE domain assignments are from data in Kim et al. (2006).

domain (PHD) zinc fingers important for interactions with other methylated histones (Fig. 1) (Baker et al. 2008; Pasillas et al. 2011; Sankaran et al. 2016).

NSD1, NSD2, and NSD3 function to mono- and dimethylate histone H3 on lysine 36 (H3K36) (Kim et al. 2006, 2008; Marango et al. 2008; Li et al. 2009b; Nimura et al. 2009; Lucio-Eterovic et al. 2010; Kuo et al. 2011; Qiao et al. 2011; Rahman et al. 2011). Histone H3K36 is found in nonmethylated and mono-, di-, and trimethylated forms (me1, me2, me3, respectively), and methylation of H3K36 is associated with transcription of active euchromatin (Rao et al. 2005; Wagner and Carpenter 2012). To date, eight different mammalian H3K36 HMTases have been identified with distinct preferences for which methylation state of H3K36 they recognize and modify in vitro and/or in vivo (Wagner and Carpenter 2012). In addition, the various methylated forms of H3K36 may have different biological functions depending on the organism or cellular context. In worms and humans, K36me3 has been shown to link transcription with splicing (Kolasinska-Zwierz et al. 2009; Sims and Reinberg 2009; Spies et al. 2009). In flies, H3K36me3 functions in dosage compensation (Larschan et al. 2007). In yeast, H3K36me2 and H3K36me3 have been implicated in transcription frequency and elongation that is coupled to histone acetylation (Kaplan et al. 2003; Carrozza et al. 2005; Joshi and Struhl 2005; Kizer et al. 2005; Morris et al. 2005; Shilatifard 2006; Lee and Shilatifard 2007; Xu et al. 2008b; Li et al. 2009a). In *Arabidopsis* and chicken, H3K36me2 and H3K36me3 mark actively transcribed chromatin (Bannister et al. 2005; Xu et al. 2008a). In humans, there is a preference for K36me1 at active promoters, and this mark is detected in active regions of the β-globin locus (Kim et al. 2007). Furthermore, at the human globin genes, H3K36me3 is broadly associated with transcription (Kim et al. 2007).

Evidence suggests that NSD1, NSD2, and/or NSD3 normally play nonredundant roles during development because genetic deletion of either *NSD1* or *NSD2* is lethal in mice (Rayasam et al. 2003; Nimura et al. 2009). Thus, alterations or amplifications of *NSD1*, *NSD2*, and/or *NSD3* that dysregulate H3K36 methylation marks have profound effects on cell growth and differentiation and are linked to numerous developmental defects. In addition, overexpression, gain of function somatic mutations, and translocation of NSDs have been reported to frequently occur in a variety of cancers.

IDENTIFICATION AND FUNCTION OF NSD

The human nuclear receptor SET domain-containing 1 (*NSD1*, *KMT3B*) gene is comprised of 24 exons on chromosome 5q35. NSD1 has two protein isoforms: a predominant 2427 amino acid (aa) short form and a less abundant 2696 aa long form that occurs by retention of the intron between exons 2 and 3 (Fig. 1) (Lucio-Eterovic et al. 2010). NSD1 was originally identified to contain two nuclear steroid receptor interaction domains that regulate the function of retinoic acid, thyroid, retinoid X, and estrogen nuclear receptors (Huang et al. 1998). Subsequent studies revealed that the SET domain of NSD1 methylated H3K36 and H4K20 in vitro (Rayasam et al. 2003). However, more recent experiments suggest that the enzyme is specific for H3K36 (Bender et al. 2006; Bell et al. 2007; Stabell et al. 2007; Li et al. 2009b). Importantly, reports using defined nucleosome substrates with various forms of methylated histone H3 at lysine 36 show that NSD1 is a dimethylase specific for H3K36 (Li et al. 2009b; Qiao et al. 2011). Furthermore, depletion of NSD1 reduced the levels of H3K36me1, 2, and 3, suggesting that NSD1 is a mono/dimethylase and that this modification serves as a substrate for trimethylation by the HMTase SETD2 (Lucio-Eterovic et al. 2010). In addition, depletion of NSD1 reduced RNA polymerase II (RNAPII) promoter occupancy and inhibited the transition of RNAPII from an initiation to an elongation-competent state (Lucio-Eterovic et al. 2010).

The crystal structure for NSD1 has been solved and serves as a model for the other NSD family proteins (Qiao et al. 2011; Graham et al. 2016). This structure reveals that an autoinhibitory loop blocks binding of NSD1 to the histone peptide as well as the entrance to the

lysine-binding channel. As nucleosomal DNA contacts the NSD1 post-SET loop, the active conformation is stabilized. This may explain the preference of NSD family members for nucleosomal H3K36 substrates compared to octamers and why NSD family HMTases methylate histone lysine residues other than H3K36 when octamers, recombinant histones, or peptides are used as substrates (Li et al. 2009b; Kudithipudi et al. 2014; Morishita et al. 2014).

NSD1 is critical for normal murine embryonic development. Homozygous loss-of-function embryos are able to gastrulate and initiate mesoderm formation but fail at embryonic day 6.5 (Rayasam et al. 2003). Defects of *NSD1* are linked to Sotos syndrome (aberrant *NSD1* expression is present in 80% of patients) as well as unique variants of Weaver or Beckwith–Wiedemann syndrome (Kurotaki et al. 2002; Douglas et al. 2003; Baujat et al. 2004; Gibson et al. 2012). Characterized as overgrowth disorders, these patients experience pre-/postnatal overgrowth, enhanced bone age, neurodevelopmental delay, and an enhanced risk for cancer (Rahman 2005). A genomic analysis of 435 Sotos patient samples revealed nonsense, deletion/insertion/duplication, splice site, and missense *NSD1* mutations (Waggoner et al. 2005). Although half of the point mutations (nonsense and missense) occurred at CpG sites, missense mutations were primarily in exons that code for functional domains (i.e., PHD, PWWP, and SET domains) (Waggoner et al. 2005). Although not experimentally confirmed, there is evidence of *NSD1* gene-dosage effects linked to significant phenotypic outcomes beyond Sotos syndrome. Microdeletions lead to bone overgrowth/macrocephaly, whereas microduplications are mirrored by stunted growth and microcephaly (Dikow et al. 2013; Rosenfeld et al. 2013). *NSD1* knockout mouse are embryonic lethal (Rayasam et al. 2003). However, mice carrying a heterozygous 1.5-Mb deletion of 36 genes on mouse chromosome 13 that corresponds to the human chromosome 5q35.2-q35.3 region where *NSD1* is located have been characterized (Migdalska et al. 2012). Although these mice did not show the phenotypic overgrowth observed in Sotos syndrome, they did display impaired long-term memory and renal abnormalities (Migdalska et al. 2012).

In addition to histone H3, nonhistone targets of NSD1 have been reported. The carboxyl-terminus of NSD1 contains a unique PHD finger region termed the PHDvC5HCH domain that interacts with the transcription repressor NIzp1, and mutations in the NSD1 PHDvC5HCH domain may interfere with NIsp1 transcription repression (Nielsen et al. 2004; Berardi et al. 2016). Also, on activation of NF-κB, NSD1 has been reported to coimmunoprecipitate with the NF-κB p65 subunit, and be required for inducible p65 methylation at lysine residues K218 and K221 (Lu et al. 2010). However, whether p65 is a direct substrate of NSD1 has yet to be confirmed in vitro. These functions may contribute to the overgrowth phenotype observed when *NSD1* is mutated in Sotos syndrome.

NSD1 ROLE IN CANCER

Aberrant *NSD1* expression has been associated with many cancer pathologies, and tumors occur in 3% of patients diagnosed with Sotos syndrome (Tatton-Brown et al. 2005). In addition, *NSD1* was inactivated via CpG island–promoter hypermethylation in neuroblastomas and gliomas (Berdasco et al. 2009). This transcriptional silencing was associated with diminished methylation of H3K36 and H4K20. In prostate tumors, enhanced *NSD1* expression was associated with metastases, whereas reduced expression was associated with cancers having biochemical recurrence (Bianco-Miotto et al. 2010). Also, *NSD1* expression was up-regulated in patient-derived metastatic melanoma cell lines compared to primary epidermal melanocytes. However, during the progression from nonmetastatic to metastatic melanoma, expression of *NSD1* was down-regulated (de Souza et al. 2012). Furthermore, a transposon screen for frequently occurring mutations in skin tumors of mice revealed that *NSD1* is among those genes with significantly decreased expression during tumor development (Quintana et al. 2013). Taken together, these studies suggest that increased *NSD1* expression may pro-

mote oncogenic initiation through enhanced methylation of H3K36, which activates genes normally silenced by H3K27me3 (e.g., *MEIS1* and *Hox*) (Wang et al. 2007; Berdasco et al. 2009). Subsequently, metastatic progression may activate negative feedback of NSD1 in a tissue and/or stage-specific manner. Further studies are required to elucidate these potential roles of NSD1 in cancer.

An NUP98-NSD1 fusion protein has been observed in childhood acute myeloid leukemia (AML) (Jaju et al. 2001; Cerveira et al. 2003; La Starza et al. 2004). This recurrent translocation at t(5;11)(q35;p15.5) contains the FG-repeat domain of NUP98, a nucleoporin protein family member that can interact with the histone acetyltransferase CBP/p300 and the carboxyl terminal of NSD1 that retains the five PHD fingers, the Cys-His-rich domain (C5HCH), one PWWP domain, and the catalytic SET domain. NUP98-NSD1 is the most frequent (4%–5% of cases) fusion reported in pediatric AML and is associated with poor prognosis (Shiba et al. 2013). Retroviral infection of NUP98-NSD1 enhanced expression of *HoxA7*, *HoxA7*, *HoxA9*, and *Meis1* proto-oncogenes (Wang et al. 2007). In addition, marrow progenitors transduced with NUP98-NSD1-induced AML when transplanted into lethally irradiated mice (Wang et al. 2007). Mechanistically, the NUP98-NSD1 fusion drives methylation of H3K36 and histone acetylation. This persistent methylation prevents transcriptional repression of the HoxA locus by EZH2 causing progenitor immortalization (Wang et al. 2007). Although the NUP98-NSD1 fusion protein occurs in a low percentage of patients, the incidence of concurrence between NUP98-NSD1 and FLT3 (FMS-like tyrosine kinase 3) internal tandem duplication (ITD) mutants is high (70%) in human AML cases (Thanasopoulou et al. 2014). Coinfection of progenitor cells expressing both NUP98-NSD1 and FLT3-ITD mutation resulted in a strikingly decreased latency of development of AML in mice compared to those reconstituted with NUP98-NSD1 alone (Thanasopoulou et al. 2014). Furthermore, experiments using immortalized bone marrow progenitor cells show that NUP98-NSD1 promotes the expression and activation of FLT3-ITD, suggesting a potent cooperation between NUP98-NSD1 and FLT3-ITD during leukemic transformation that may be therapeutically targeted with SET domain or FLT3 inhibitors (Thanasopoulou et al. 2014).

In summary, NSD1 is an H3K36-specific mono- and dimethyltransferase that promotes transcription and is critical for normal growth and development. Aberrant expression of *NSD1* drives the pathobiology of Sotos syndrome and tumorigenesis. Although much work has been performed regarding the structure and function of NSD1, questions remain. For instance, whereas normal and cancer cell lines endogenously express both the short and long form of NSD1 the specific functions of these protein isoforms have not been well characterized. In addition, work remains to determine what cellular context or mechanisms regulate the oncogenic properties of NSD1 and how transcription of *NSD1* may be altered during metastasis.

IDENTIFICATION AND FUNCTION OF NSD2

NSD2 (also known as WHSC1 and MMSET) was identified by its presence in a chromosomal region found to be deleted in the Wolf–Hirschhorn syndrome (WHS) (Stec et al. 1998), and separately by its rearrangement with the immunoglobulin locus in a subset of cases of the plasma cell malignancy, multiple myeloma (Chesi et al. 1998). Since then, *NSD2* has been found to play a role in many facets of development and malignancy. This 90-kb, 25-exon gene on chromosome 4p16.3 encodes two main isoforms, *NSD2*-short (MMSET-I) and NSD2-long (MMSET-II), and an intronic transcript that encodes response element II–binding protein (RE-IIBP). The 1365 amino acid full-length species is composed of two PWWP domains, a high-mobility group (HMG) DNA-binding domain, four PHD zinc fingers, and a SET domain (Fig. 1), whereas the 647 amino acid short species lacks all but the initial PWWP and HMG domains (Stec et al. 1998).

NSD2 catalyzes the mono- and dimethylation of H3K36 (Martinez-Garcia et al. 2011; Morishita et al. 2014). The first amino-terminal

PWWP domain of NSD2 specifically binds to H3K36me2 to stabilize NSD2 at chromatin, and the catalytic SET domain of NSD2 propagates this gene-activating mark to adjacent nucleosomes (Waggoner et al. 2005; Kuo et al. 2011; Martinez-Garcia et al. 2011; Morishita et al. 2014; Sankaran et al. 2016). Similar to NSD1, the NSD2 post-SET domain is attached to the catalytic SET domain via an autoinhibitory loop region and inhibition is relieved on nucleosome binding (Qiao et al. 2011; Poulin et al. 2016a). Furthermore, NSD2 has been reported to preferentially catalyze H3K36 dimethylation compared to H3K36 monomethylation (Li et al. 2009b; Kuo et al. 2011; Poulin et al. 2016b).

NSD2 is broadly expressed and its importance in development is highlighted by its involvement in Wolf–Hirschhorn malformation syndrome (WHS). The full syndrome is characterized by brain defects associated with developmental delay and epilepsy as well as craniofacial anomalies, growth delay, heart defects, and midline fusion abnormalities (Stec et al. 1998; Bergemann et al. 2005). Variable deletions in the short arm of chromosome 4 (4p16.3) are typical of WHS and *NSD2* is the only gene in this region that is deleted in almost every case. Partial or full hemizygosity of *NSD2* appears to be necessary but not sufficient for the development of WHS, as the deletion of other genes nearby contributes to the constellation of abnormalities that make up the syndrome (Bergemann et al. 2005; Andersen et al. 2013). Mice with a homozygous NSD2 SET domain deletion do not survive past 10 days of age, and heterozygous mice develop significant developmental defects that imitate WHS (Nimura et al. 2009). Furthermore, chromatin immunoprecipitation (ChIP) experiments on embryonic stem (ES) cells of these mice revealed that NSD2 binds to several genes associated with development including *Sall1*, *Sall4*, and *Nanog*. Additionally, WHS patients often have antibody deficiencies, suggesting a role for NSD2 in B-cell development. This theory is supported by the ability of NSD2 to recruit the DNA-damage-responsive, p53-binding protein 1 (53BP1), which is critical for class switch recombination and also implicates NSD2 in DNA repair (Hajdu et al. 2011; Pei et al. 2011). Indeed, it was recently reported that NSD2 regulates the expression of DNA repair genes and may play a critical function in multiple myeloma chemoresistance (Shah et al. 2016). Cancer cells expressing high levels of NSD2 repaired DNA damage at a much faster rate than cells with low levels of NSD2, allowing them to survive and proliferate even while being treated with DNA-damaging chemotherapy.

NSD2 ROLE IN CANCER

NSD2 was initially described as a gene rearranged and linked to regulatory sequences of the immunoglobulin heavy chain gene in t(4;14) multiple myeloma (Chesi et al. 1998; Stec et al. 1998); *NSD2* and its translocations, amplifications, and mutations were subsequently identified in a wide spectrum of malignancies. In multiple myeloma, the t(4;14) translocation is present in 15%–20% of cases, resulting in overexpression of *NSD2* and *FGFR3* (Chesi et al. 1998; Stec et al. 1998; Finelli et al. 1999). However, NSD2 is purported to be the primary oncogenic driver, as approximately 30% of cases harboring this translocation have normal expression of *FGFR3* alongside *NSD2* overexpression (Santra et al. 2003). Furthermore, knockdown of *NSD2* expression in $t(4;14)^{+}$ multiple myeloma cell lines reduces proliferation, cell-cycle progression, and DNA repair, while increasing apoptosis and adhesion (Lauring et al. 2008; Brito et al. 2009; Martinez-Garcia et al. 2011; Huang et al. 2013; Shah et al. 2016). These phenotypic changes are driven by redistribution of activating and repressive chromatin marks that, in turn, affect gene expression. Overexpression of *NSD2* globally increases levels of H3K36 dimethylation (Martinez-Garcia et al. 2011; Zheng et al. 2012; Popovic et al. 2014; Sankaran et al. 2016). Interestingly, NSD2 also affects enhancer of zeste homolog 2 (EZH2), which is responsible for the reciprocal repressive chromatin mark, histone 3 lysine 27 trimethylation (H3K27me3). The spread of H3K36me2 from *NSD2* overexpression leads to global reduction in H3K27me3 and restricts EZH2 to small islands of chroma-

tin where it then hypermethylates H3K27me3 (Zheng et al. 2012; Popovic et al. 2014). The transcriptional disturbance that results from *NSD2* overexpression primarily involves inappropriate activation of genes, but there are some genes that are inappropriately repressed because of these pockets of EZH2. This effect appears to be important for the survival of cells overexpressing *NSD2*, as they are sensitive to inhibition of EZH2. One contribution to this sensitivity is that inhibition of EZH2 decreases c-MYC protein levels, a fundamentally up-regulated gene in multiple myeloma (Popovic et al. 2014). In $t(4;14)^+$, multiple myeloma cells EZH2 represses miR-126, a microRNA that targets the MYC transcript (Min et al. 2012). Therefore, EZH2 inhibitors derepress miR-126, allowing it to reduce c-MYC protein levels and slow proliferation. However, c-MYC is not the sole cause of high NSD2-mediated oncogenesis. Transcriptional profiling of $t(4;14)^+$ multiple myeloma indicates that NSD2 regulates the expression of genes in apoptosis, DNA repair, cell-cycle control, and cell motility.

In addition to multiple myeloma, *NSD2* overexpression is detected in gastric, colon, lung, and skin cancer (Hudlebusch et al. 2011a). It may play a major role in neuroblastoma and breast, bladder, and prostate tumors, where overexpression is associated with worse prognosis (Hudlebusch et al. 2011b). In many of these cancers, *NSD2* expression is positively correlated with *EZH2* expression, and as opposed to the situation in $t(4;14)^+$ multiple myeloma, global levels of H3K27me3 and H3K36me2 are both increased (Asangani et al. 2013). In prostate cancer, EZH2 functions upstream of NSD2, repressing several microRNAs, including miR-203, miR-26, and miR-31, that target NSD2 (Asangani et al. 2013). However, it is the knockdown of NSD2 that abrogates the ability of prostate cancer cells to proliferate, form colonies, migrate, and invade (Ezponda et al. 2012). Furthermore, enforced expression of *NSD2* in nontransformed prostate epithelial cells promotes migration and invasion and leads to epithelial–mesenchymal transition (EMT). NSD2 directly binds the *TWIST1* locus and up-regulates expression of this EMT factor, which plays a key role in the aggressive biological behavior of advanced prostate cancer.

THE NSD2 E1099K POINT MUTATION IN LYMPHOID MALIGNANCIES

In cases of $t(4;14)^+$ multiple myeloma and some acute lymphoblastic leukemia (ALL) in which *TWIST1* is also overexpressed, *NSD2* harbors a recurrent point mutation in the SET domain (Oyer et al. 2014). The significance of the guanine to alanine substitution that results in a glutamic acid to lysine switch at amino acid 1099 (E1099K) of NSD2 was first noted following examination of the Broad Institute's Cancer Cell Line Encyclopedia (CCLE) that revealed numerous ALL cell lines with the mutation, which has since been identified in 10%–20% of relapsed pediatric ALL, 10% of mantle cell lymphoma (MCL), and 10% of chronic lymphocytic leukemia (CLL) (Fabbri et al. 2011; Barretina et al. 2012; Beà et al. 2013; Loh et al. 2013; Oyer et al. 2014). Rare mutations are found in glioblastoma, lung cancer, and multiple myeloma. Although the mechanism remains to be determined, the E1099K mutation was revealed to result in hyperactive NSD2, leading to increased H3K36me2 and decreased H3K27me3, similar to the epigenetic profile of $t(4;14)^+$ multiple myeloma (Oyer et al. 2014). Because of the common occurrence of this mutation in relapsed pediatric ALL, especially cases harboring other oncogenic lesions, such as the TEL-AML1 and E2A-PBX1 fusions, E1099K appears to be an important factor in progression of these malignancies rather than initiation (Jaffe et al. 2013; Loh et al. 2013).

In summary, NSD2 plays a significant role in normal development and malignancy. Haploinsufficiency of *NSD2* leads to severe developmental defects, such as cardiac lesions and midline abnormalities associated with WHS. In malignancy, NSD2 has prolific effects resulting from translocation, overexpression, and activating mutations of the gene. The t(4;14) translocation in multiple myeloma has been extensively characterized and indicates that high levels of NSD2 drive oncogenic phenotypes by spreading H3K36me2 throughout the genome and alter-

ing gene-expression profiles. The relationship between NSD2 and EZH2 is crucial in myeloma as the reciprocal H3K27me3 mark deposited by EZH2 is reduced and restricted to certain regions by *NSD2* overexpression. In other malignancies, such as prostate cancer, this relationship is different as EZH2 appears to function upstream of NSD2, although it is no less important. Inhibition or knockdown of NSD2 or EZH2 in both situations abrogates critical oncogenic pathways and phenotypes. However, there is still much to learn about NSD2 in these malignancies. Although NSD2 itself is already an attractive therapeutic candidate, thoroughly characterizing its binding partners will tell us how NSD2 functions both endogenously and in malignancy and provide more targets for designing therapies for NSD2-misregulated cancers. The recurrent E1099K mutation that results in a hyperactive NSD2 is also not fully understood. Its global effects on chromatin mimic *NSD2* overexpression, but it must be investigated further to identify its local effects on chromatin, gene expression, and oncogenicity. Additionally, as it appears that this mutation is more likely to contribute to relapse after therapy, it will be important to understand its role in drug resistance. Finally, we must understand the mechanism by which E1099K and other activating mutations alter the function of NSD2 to design directed therapies.

IDENTIFICATION AND FUNCTION OF NSD3

Using database searches for sequences similar to *NSD1* and *NSD2*, *NSD3* (also named Wolf–Hirschhorn syndrome candidate 1–like 1, WHSC1L1) was independently identified by two groups in 2001 (Angrand et al. 2001; Stec et al. 2001). The *NSD3* gene contains 24 exons that span approximately 112 kb of genomic DNA on chromosome 8p11.2 and is predicted to have 12 transcript variants. Three protein products of *NSD3* have been reported and characterized to date: Long, Short, and WHISTLE.

The NSD3-long transcript encodes a 1437–amino acid protein containing two PWWP domains, five PHD-type zinc-finger motifs, a SET-associated Cys-rich (SAC) domain, and a SET domain (Fig. 1) (Angrand et al. 2001; Stec et al. 2001). NSD3-long is highly expressed in brain, heart, and skeletal muscle and to a lesser degree in liver and lung (Angrand et al. 2001). The sequence of NSD3-long from amino acids 703 to 1409 is highly conserved between NSD1 (68% identical) and NSD2 (55% identical) and contains the PHD, second PWWP, and SET domain (Angrand et al. 2001). Similar to NSD1 and NSD2, the carboxy-terminal region of NSD3-long that contains the catalytic pre-SET, SET, and post-SET domains is able to recognize and methylate histone H3 and H4 targets in vitro (Allali-Hassani et al. 2014; Morishita et al. 2014). However, unlike NSD2, a clear HMG box is missing from NSD3-long and the fourth PHD-type zinc finger contains an insertion of 49 amino acids (Stec et al. 2001). In addition, evidence suggests that the conserved PHD5 domain of NSD3-long is functionally distinct in its histone-binding properties from the PHD5 domain of NSD1 or 2 and targets NSD3 to specific genomic regions in vivo (He et al. 2013). The NSD3-short transcript encodes a protein of 645 amino acids (Stec et al. 2001). NSD3-long and NSD3-short have an identical amino-terminal 620–amino acid sequence, but NSD3-short lacks a catalytic SET domain and only contains the amino-terminal PWWP domain that binds to histone H3 when it is methylated on lysine 36 (Stec et al. 2001; Vermeulen et al. 2010; Wu et al. 2011). The NSD3-long and NSD3-short transcripts are coexpressed in many tissues (Stec et al. 2001).

WHISTLE (WHSC1-like 1 isoform 9 with methyltransferase activity to lysine) was identified by homology searching and functional assay to be the shortest isoform of NSD3 to retain a SET domain and methyltransferase activity (Kim et al. 2006). WHISTLE consists of 506 amino acids and was found expressed in testis and in bone marrow mononuclear cells of AML and ALL patients (Kim et al. 2006). It contains the PWWP, SET, and post-SET domains and was reported to facilitate transcription repression by promoting methylation of histone H3K4 and H3K27 (Kim et al. 2006; Allali-Hassani et al. 2014).

NSD3 has been identified as an essential methyltransferase for neural crest gene expression during specification (Jacques-Fricke and Gammill 2014). NSD3 is expressed in premigratory and migratory neural crest cells and is necessary for expression of the neural plate border gene *Msx1*, as well as the key neural crest transcription factors Sox10, Snail2, Sox9, and FoxD3 (Jacques-Fricke and Gammill 2014). In addition, recent reports indicate that neuronal ten-eleven translocation (TET) hydroxylase 3 interacts with and activates NSD3 to stimulate H3K36 trimethylation and transcription of neuronal genes in retinal cells (Perera et al. 2015). Thus, NSD3 may promote context-specific chromatin remodeling and gene activation that is necessary for neural crest migration and retinal network formation during development.

NSD3 ROLE IN CANCER

Since its discovery, amplification and overexpression of *NSD3* has been a consistently identified feature in many cancer types (Angrand et al. 2001; Mahmood et al. 2013). For example, integrated DNA-RNA analyses of regional amplifications and deletions coupled with gene-expression profiling and have identified the 8p11-12 *NSD3* locus as an amplicon commonly expressed in both pancreatic ductal adenocarcinoma and non-small-cell lung cancer (Tonon et al. 2005). In addition, a bioinformatics screen to identify putative cancer driver genes amplified across TCGA datasets discovered frequent *NSD3* amplifications in bladder, breast, liver, lung, ovarian, head and neck, and colorectal cancer samples (Chen et al. 2014). Amplification of the 8p11-12 region has also been reported to be present in about 15% of primary human breast cancer samples, which correlates with histological grade and is associated with poor prognosis (Angrand et al. 2001; Yang et al. 2010; Chen et al. 2014). Furthermore, NSD3 expression and protein level are increased in breast, lung, pancreatic, and colorectal cell lines, and immunohistochemical analysis indicates that NSD3 is increased in primary breast carcinoma, bladder cancer, lung cancer, and liver cancer (Kang et al. 2013; Mahmood et al. 2013; Chen et al. 2014).

Mounting evidence suggests that NSD3 activates signaling pathways that promote cell-cycle progression and proliferation. Although an *NSD3* knockout mouse has not yet been reported, NSD3 has been identified to cooperate with oncogenic KRAS to drive tumorigenesis in a pancreatic cancer model mouse (Mann et al. 2012). Furthermore, transduction of either NSD3-short or NSD3-long into the mammary epithelial cell line MCF10A cells has been reported to increase proliferation, soft agar colony formation, and cause abnormal acini formation (Yang et al. 2010). In contrast, knockdown of *NSD3* reduced proliferation and promoted cell death in 8p11-12-amplified breast cancer cells and significantly decreased anchorage-dependent and anchorage-independent growth of pancreatic adenocarcinoma and small-cell lung cancer–derived cell lines (Yang et al. 2010; Mahmood et al. 2013). In addition, knockdown of *NSD3*-expression induced G_2/M cell-cycle arrest and suppressed proliferation of breast, bladder, and lung cancer cell lines (Zhou et al. 2010; Kang et al. 2013). Expression profile analysis showed that *NSD3* affected the expression of a number of genes known to play crucial roles in cell-cycle progression (Kang et al. 2013).

Although the mechanism by which NSD3 regulates the mechanism governing the cell cycle and proliferation is not well characterized, evidence suggests that *NSD3* amplification may promote transcription of a subset of genes involved in proliferation pathways. NSD3 can form complexes with H3K4-specific demethylase LSD2 and H3K9-specific methyltransferase G9a and may cooperate with these enzymes to coordinate the dynamics of H3K4 and H3K36 methylation to promote transcription elongation for a subset of genes (Fang et al. 2010). NSD3 promoted expression of transcription factors Iroquois homeobox 3 (IRX3) and TBIL1X known to regulate WNT signaling (Yang et al. 2010). Furthermore, knockdown of *NSD3* in MCF10A cells or breast cancer cell lines with amplified 8p11-12 resulted in increased expression of *SRFp1*, a negative regulator of WNT-signaling, and decreased expression of *TGFBI* leading to profound loss of growth and survival of these cells (Yang et al. 2010).

Taken together, these results suggest that *NSD3* is a putative cancer driver gene.

In addition to amplification, chromosomal translocations resulting in NSD3 fusion products have been described in myelodysplastic syndrome (MDS), AML, and nuclear protein in testis (NUT) carcinoma. NUP98-NSD3 fusion transcripts associated with t(8;11)(p11.2;p15) have been reported in one patient with AML, one patient with therapy-related MDS, and one patient with radiation-associated MDS, and both NSD3-long and NSD3-short isoforms have been detected to be fused to NUP98 in leukemic cell lines (Rosati et al. 2002; Romana et al. 2006; Taketani et al. 2009). In addition, NSD3 was detected as a fusion oncoprotein with the *NUT* gene in the rare and aggressive NUT midline carcinoma (NMC) (French et al. 2014; Harms et al. 2015; Kuroda et al. 2015; Suzuki et al. 2015). A t(8;15)(p12;q15) translocation was identified as responsible for the NSD3-NUT fusion in a patient-derived NMC cell line, and knockdown of the NSD3-NUT fusion revealed that this fusion protein functioned to block differentiation and promote proliferation (French et al. 2014).

NSD3 AS AN EFFECTOR OF BRD4 IN CANCER

The bromodomain and extraterminal domain (BET) family of transcriptional activators are promising therapeutic targets for cancers, particularly AML, because of their role in maintaining the expression of key oncogenes (Dawson et al. 2011; Mertz et al. 2011; Zuber et al. 2011). NSD3 can bind the ET domain of BET proteins and associates with BRD4 in nuclear lysates (Rahman et al. 2011; French et al. 2014; Crowe et al. 2016). The ET domain of BRD4 interacts with amino acids 100–263 of NSD3, a region immediately before the amino-terminal PWWP domain located at amino acids 270–333 (Fig. 2) (Shen et al. 2015). The corresponding regions in NSD1 and NSD2 have significant homology with NSD3, suggesting this may be a common element responsible for BRD4 interaction with NSD family members (Fig. 2). In addition, the NSD3-NUT fusion binds to BRD4 and BRD bromodomain inhibitors induce differentiation and arrest proliferation of t(8;15)(p12;q15) cells (French et al. 2014). Recent evidence suggests that interaction with NSD3-short may be required for the AML maintenance function of BRD4 (Shen et al. 2015). The NSD3-short isoform has been reported to be an adaptor protein that links BRD4 to the CHD8 chromatin remodeler. BRD4, NSD3, and CHD8 colocalize across the AML genome, and each is released from super-enhancer regions on treatment with BET inhibitors such as JQ1 (Shen et al. 2015). Furthermore, genetic targeting of *NSD3* or *CHD8* mimics the phenotypic and transcriptional effects of pharmacological BRD4 inhibition (Shen et al. 2015). Thus, BET inhibitors may function by evicting BRD4–NSD3–CHD8 complexes from chromatin to suppress transcription.

CONCLUDING REMARKS

The NSD HMTases are key regulators of development. Mutations, amplifications, or translocations of these genes lead to developmental defects and cancer. Differences between the function and tissue specificity of the many isoforms of each NSD family member are poorly understood, and additional research remains critical to improve our understanding of how dysregulation of NSD family members may lead to cancer. For instance, NSD family members have no known sequence-specific DNA-binding properties, and the precise mechanism that directs these lysine-HMTs to specific chromatin loci is unclear. Thus, identification of partner proteins that may guide NSD family members to specific promoter/enhancer regions is important to understanding how these proteins may be directed to specific functional contexts. The recent report that BRD4 may direct NSD3 to chromatin as well as other reports indicating NSD2 associates with BRD4 raises the intriguing possibility that bromodomains (acetyllysine side chains) on histone H3 may recruit BET family members such as BRD4 to enhancer and promoter regions that, in turn, recruit NSD family proteins to direct H3 K36 methylation, inhibit EZH2-mediated H3 K27

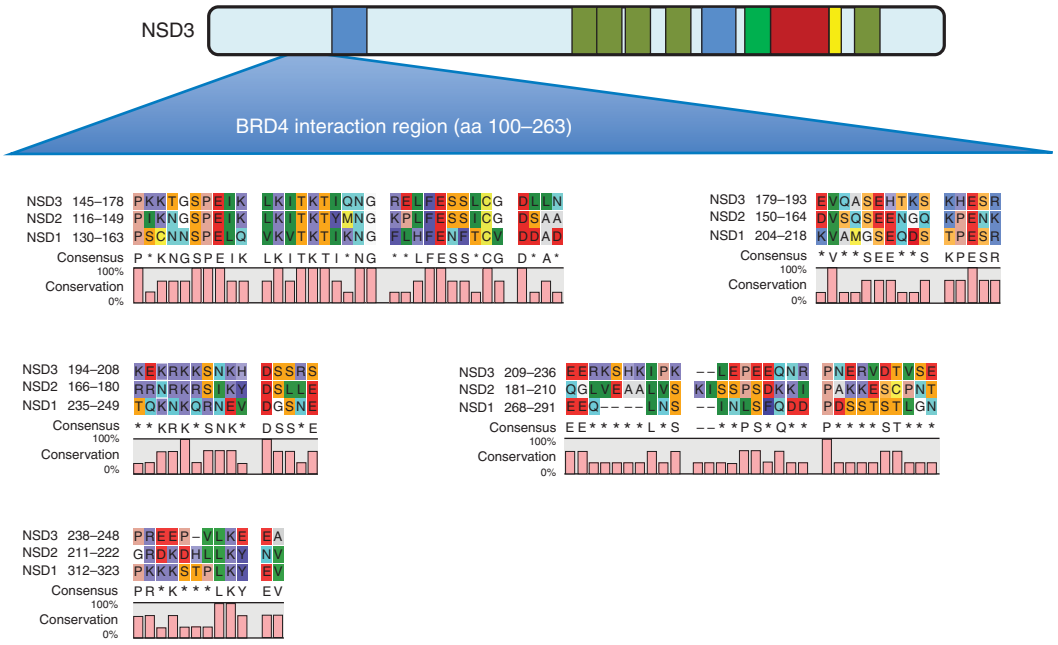

Figure 2. Sequence alignment of the region of nuclear receptor–binding SET domain 3 (NSD3) required for BRD4 interaction with NSD1 and NSD2 reveals conserved regions amino terminal to the first PWWP domain. The region in NSD3 identified as being required for interaction with BRD4 (amino acids 100-263) was found to have significant homology with the corresponding region in NSD1 and NSD2. Sequence analysis was performed using CLC Sequence Viewer (Qiagen).

methylation, and promote transcription initiation and elongation (Fig. 3) (Min et al. 2012; Shen et al. 2015). In addition, identifying how activating mutations such as E1099K in NSD2 may alter enzymatic activity to drive increased H3K36 dimethylation is critical to understanding the biological effect of this mutation in oncogenesis. Preliminary epigenetic and transcriptional profiling of E1099K in ALL cells indicates there are both similarities and differences between E1099K NSD2 and overexpression of *NSD2* (unpublished data). Elucidating the downstream effects of E1099K may provide new insight into the mechanism by which aberrant lysine HMTase activity promotes tumorigenesis.

The prominent role of translocations, upregulation, and activating mutations of NSD family members in driving tumor progression and aggressiveness, suggests that specific lysine-HMTase inhibitors are a promising therapeutic approach to suppress cancer growth. In support of this, abolition of the methyltransferase activity of NUP98-NSD1 by point mutation suppresses Hox-A gene activation and myeloid progenitor immortalization (Wang et al. 2007). In addition, knockdown of *NSD2* in several model systems has indicated the potential utility of NSD2 inhibitors for down-regulating critical tumor cell phenotypes. Targeting the SET domain is an attractive strategy for development of NSD family inhibitors, because the lysine HMTase activity is considered most likely to drive oncogenic reprogramming (Kuo et al. 2011; Martinez-Garcia et al. 2011). Recently, a high throughput luminescence-based assay using the NSD1 SET domain was used to screen for specific inhibitors of NSD1 (Drake et al. 2014). The HMTase inhibitor suramin was identified in this screen but had minimal selectivity for NSD1 over other histone methyltransferases and other compounds identified by this screen inactivated

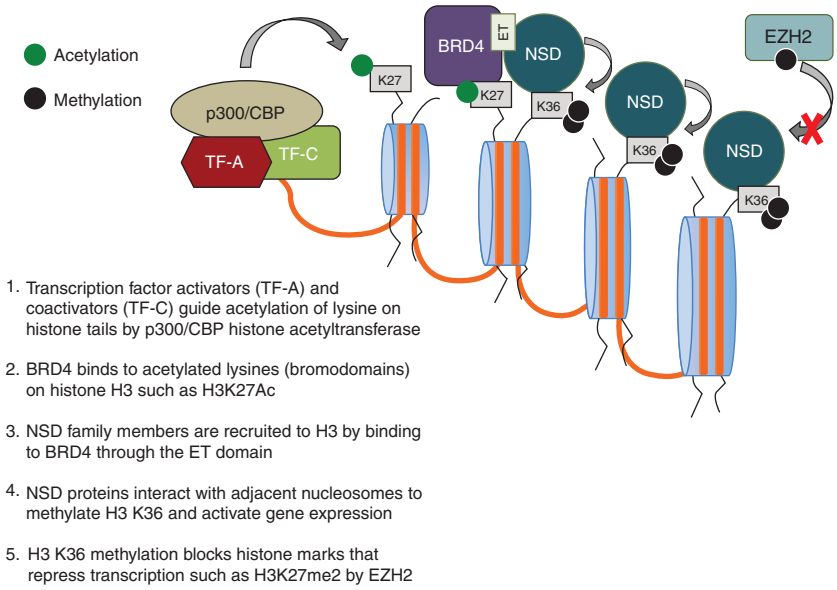

Figure 3. Proposed mechanism for targeting of nuclear receptor–binding SET domain (NSD) family proteins to specific chromatin loci.

NSD1 through nonspecific mechanisms. Alternatively, targeting domains outside of the catalytic SET domain may be a useful strategy for developing NSD inhibitors. The PHD zinc fingers 2, 3, and 4, as well as PWWP domain 2 of NSD2 are critical for the H3K36me2 up-regulation in $t(4;14)^{+}$ multiple myeloma (Popovic et al. 2014). The fourth PHD finger plays a role in the NSD2/EZH2 interplay, as its deletion abrogates the reduction of H3K27me3 typically observed with *NSD2* overexpression in myeloma. Furthermore, recent reports that BET inhibitors evict NSD3-BRD4 from chromatin raise the attractive hypothesis that these compounds may also inhibit interactions between other BRD and NSD family members to suppress aberrant gene activation and limit tumor growth. Although evidence suggests that genetic and epigenetic alterations cooperate in the stepwise initiation and progression of cancers, only epigenetic aberrations can be reversed. Thus, as we work to further our understanding of NSD family members, we are hopeful that inhibition of these epigenetic regulators will play a central role in the next generation of cancer therapy.

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