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The Smyd Family of Methyltransferases: Role in Cardiac and Skeletal Muscle Physiology and Pathology

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Abstract

Protein methylation plays a pivotal role in the regulation of various cellular processes including chromatin remodeling and gene expression. **SET** and **MYND** domain-containing proteins (Smyd) are a special class of lysine methyltransferases whose catalytic SET domain is split by an MYND domain. The hallmark feature of this family was thought to be the methylation of histone H3 (on lysine 4). However, several studies suggest that the role of the Smyd family is dynamic, targeting unique histone residues associated with both transcriptional activation and repression. Smyd proteins also methylate several non-histone proteins to regulate various cellular processes. Although we are only beginning to understand their specific molecular functions and role in chromatin remodeling, recent studies have advanced our understanding of this relatively uncharacterized family, highlighting their involvement in development, cell growth and differentiation and during disease in various animal models. This review summarizes our current knowledge of the structure, function and methylation targets of the Smyd family and provides a compilation of data emphasizing their prominent role in cardiac and skeletal muscle physiology and pathology.

Keywords

Smyd; Smyd1; Smyd2; Smyd3; Smyd4; Smyd5; histone methyltransferase; epigenetics; heart

1. INTRODUCTION

The methylation of histones was first identified in 1964, however the link between this modification and gene expression was not discovered until decades later. In 1999, Strahl et

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al. reported that transcriptionally active macronuclei in tetrahymena were enriched in histone H3 methylated on lysine 4. Since that time, histone H3 in various cell types has been shown to be methylated on multiple lysine residues, with both gene activation and silencing observed depending on which residue is methylated. An additional level of complexity for this post translational modification arises from the capacity of each modified lysine to be mono-, di- or tri-methylated. The first mammalian histone methyltransferase, Suv39h1, was identified by Rea et al. who established that the SET domain of the molecule was responsible for its catalytic activity. Emerging evidence indicates that the control of lysine methylation is critical in maintaining genomic integrity and regulating gene expression, with defects in histone methylation contributing to the development of cardiac disease [1]. Indeed, a genome-wide histone methylation profile of the failing heart in rat and human revealed changes in global histone H3K4 and H3K9 methylation during the development of heart failure [2] with additional evidence implicating other histone methylation marks in cardiac remodeling [3]. Overall, histone methylation has been shown to play a significant role in cardiac development, as well as the pathogenesis of both congenital and adult heart disease [1]. However, the specific methyltransferases and demethylases involved in regulating these processes and their specific down-stream effects are only beginning to be identified.

The Smyd family of lysine methyltransferases is a family consisting of five proteins (Smyd1-5) whose name is derived from the presence of two conserved domains: the catalytic Su(var)3-9, Enhancer-of-zeste and Trithorax (SET) domain which, in this family, is split by a Myeloid-Nervy-DEAF1 (MYND) domain, containing a zinc finger motif, involved in protein-protein interactions (Figure 1). The founding member of this family, Smyd1, was shown to methylate histone H3 on lysine 4 [4], which was thought to be the hallmark feature of this family. However, studies have since shown that this family methylates several unique histone residues as well as multiple non-histone proteins (See Table 1) in various cellular locations, establishing their involvement in a much broader repertoire of cellular processes. With emerging evidence solidifying the involvement of epigenetic factors regulating cardiac physiology and pathology, as well as other diseases, this family has seen increased scientific interest and may represent potential drug targets, particularly in cardiovascular disease and cancer [5].

2. STRUCTURE AND EXPRESSION OF SMYD FAMILY

All five Smyd family members share a common domain structure consisting of an N-terminal SET domain (which often co-exists with post-SET and SET-I domains, both present in this family) split by a MYND domain and, with the exception of Smyd5, a C-terminal domain (CTD). The split SET domain consists of two sections: an S-sequence, which may play a role in cofactor binding and protein-protein interaction [5], and a core SET domain (ET), which functions as the primary catalytic domain. Despite being split by the MYND domain, the topology of the SET domain in Smyd proteins is relatively similar to other SET proteins [5, 6]. SET domains often exist with post-SET, SET-I and pre-SET domain, which also aid in cofactor and substrate binding as well as protein stability, though the pre-SET is absent in SMYD proteins [5]. All Smyd proteins maintain significant homology from fish to humans (Figure 1).

The MYND domain contains a zinc-finger motif that has been shown to bind proline-rich regions and facilitate protein-protein interaction. Specifically, Smyd1 has been shown to bind the PPLIP motif of skNAC [7], while Smyd2 has been shown to bind multiple proteins with a PXLXP motif [8]. Although the MYND domain splits the catalytic SET domain in this family, the MYND domain is not involved in substrate or cofactor binding [5, 6, 9, 10]. In addition, while the MYND domain is highly positively charged, which in some proteins contributes to DNA binding, only Smyd3 has been shown to directly bind DNA *in vitro*, with preference for a 6bp motif 5'-CCCTCC-3' [11, 12].

With the exception of Smyd5, Smyd proteins also contain a C-terminal domain (CTD), which is structurally similar to tetratricopeptide repeats (TPR), a motif important for the binding of co-chaperones with heat shock protein-90 (Hsp90) [5]. Interestingly, it has been shown that Hsp90 enhances the methyltransferase activity of Smyd1, 2, and 3 [4, 12, 13]. Comparison of the Smyd1-3 crystal structures [6, 9–11, 14, 15] suggests that the CTD domain may also play an auto-inhibitory role, where hinge-like movement of the CTD may facilitate exposure of the substrate binding site, thereby regulating enzymatic activity [9, 10, 16]. In fact, truncation of the CTD increases activity in Smyd1 and Smyd2 [6]. Conversely, a similar truncation in Smyd3 completely abrogated methylation of histone H4, suggesting that the CTD actually stabilizes the active site of Smyd3 [17], which was supported by molecular dynamics studies [18]. Given these differing roles of the CTD among Smyd family members, additional investigation is needed to understand the functional significance of this domain, how it regulates the protein's methyltransferase activity and if it could be exploited to control Smyd activity.

Although directed studies of Smyd family members have only been carried out in a limited number of cell or tissue types, we compiled mass spectrometry data generated by Kim et al. to show the unique expression patterns of Smyd proteins across specific tissues and developmental states [19] (Figure 2). Interestingly, all five family members are predominantly expressed in cardiac muscle during development with Smyd1 maintaining the most restricted expression, whereas the other four members have a wider tissue distribution, with Smyd5 almost ubiquitously expressed (Figure 2). Although skeletal muscle was not assayed in this compilation, several studies have confirmed that Smyds are expressed in striated muscle [20–25]. Most studies of Smyd proteins in muscle have focused on myocytes with little data from other cell types, however, Smyd1 and Smyd3 have both been shown to play roles in angiogenesis through expression in endothelial cells [26, 27] and Smyd5 has been shown to be involved in regulating histone methylation in macrophages [28]. Nevertheless, a comprehensive expression profile of the Smyd family members in various cells and tissue has not been reported.

Given Smyd1's unique tissue-specific expression, its emerging importance in the fetal and adult heart, and the inconsistent nomenclature used to reference Smyd1, we have compiled recent data on this specific family member (Figure 3). The human *smyd1* gene produces a single transcript, which shares striated muscle specificity with two of the three murine *smyd1* transcripts, Smyd1a and b; the third, Smyd1c, is restricted to T-cells. However, in zebrafish, two Smyd1 genes are present, *smyd1a* and *smyd1b*, and are thought to be a product of whole-genome duplication occurring in teleost species [23]. Similar to human, rat

and mouse, both of these zebrafish variants are muscle specific, although here *smyd1b* generates two splice variants, with transcript variant 1 (b_tv1) containing a 13 amino acid segment in the SET domain which is lost in transcript variant 2 (b_tv2). The two Smyd1 splice variants (Smyd1a and Smyd1b in mouse, Smyd1b_tv1 and Smyd1b_tv2 in zebrafish) only differ by a 13aa deletion, making it difficult to distinguish between them biochemically. Consequently, only two studies have attempted to examine the functional difference between these two variants. The first was carried out in zebrafish, which showed that global knockdown of Smyd1b_tv1 and Smyd1b_tv2 together resulted in cardiac and skeletal muscle defects defined by disrupted myofibril organization and the absence of a heartbeat, with no functional difference revealed and no effect seen with individual knockdown [4, 29]. Smyd1a in zebrafish displays similar expression patterns and biological activity to Smyd1b, but knockdown had no significant effect on myofibril assembly or cardiac function [30]. The second study, in isolate rat cardiomyocytes, revealed that overexpression of Smyd1a was sufficient to inhibit phenylephrine-induced hypertrophy, however, Smyd1b had no effect on myocyte size [45]. Although these results are intriguing additional studies will be needed to further characterize these splice variants and determine their functional differences in mammals.

3. METHYLTRANSFERASE ACTIVITY OF SMYD FAMILY

All of the Smyd family members, with the exception of Smyd4, have been shown to methylate histones, either *in vivo* or *in vitro* (Table 1). Histone methylation is an emerging epigenetic mechanism for regulating gene transcription with some methyl marks, such as H3K4, H3K36, and H3K79, associated with gene activation, while others, such as H3K9, H3K27, and H4K20, are typically associated with gene silencing [31]. Disruption of histone methylation has also been implicated in various forms of congenital and adult heart disease and is summarized in a recent review [1], however the enzymes responsible for regulating these methyl marks in cardiac and skeletal muscle are only beginning to be identified. Indeed, several lysine methyltransferases and demethylases have led to the recent development of drugs to mitigate their function, the first of which are in early stages of clinical trials [31]. Additionally, one recent study demonstrated that the SU(VAR)3-9 methyltransferase inhibitor chaetocin preserved changes in histone methylation and improved survival in a rat model of high salt diet-induced heart failure [32], implying the therapeutic potential for methyltransferase inhibitors in treating heart disease. However, much work will still be necessary to comprehensively understand how these proteins function in various processes and ultimately how they may be utilized therapeutically.

Although methylation of H3K4 was thought to be the hallmark feature of the Smyd family, it is now established that Smyd proteins can methylate residues associated with both transcriptional activation and repression (Figure 4), with growth and differentiation being the most significantly affected biological process. These recent findings highlight the importance of context-specific chromatin modification, where this seemingly dichotomous behavior of Smyd family members, as both activator and repressor, may define two opposing functions of these enzymes (as has been seen with other proteins).

Smyd1, Smyd2, and Smyd3 have been shown to methylate histone H3 on lysine 4, a mark of gene activation, *in vitro* [4, 12, 13]. This transcriptional activation is consistent with Smyd2's ability to bind the TACC2 promoter and regulate gene activation [13], and Smyd3's activation of the androgen receptor via di- and tri-methylation of histone H3 lysine 4 and recruitment of histone acetyltransferases and Sp1 to the promoter region [33]. Smyd1 has also been implicated in activating transcription of several genes. For example, knockout of *smyd1* leads to downregulation of Hand2, a transcription factor essential for right ventricle formation [21]. Additionally *smyd1* silencing resulted in reduced transcription of MyoD, Myogenin, Mef2C, and Mef2D transcription factors, as well as MCK and MHC muscle markers [34]. However, it remains to be determined whether Smyd1 activates transcription of these genes directly through histone methylation, or indirectly, perhaps by methylating transcriptional machinery, transcription factors or other regulatory proteins.

Despite their roles in transcriptional activation, evidence exists showing all Smyd family members can also repress transcription. Specifically, Smyd1 has been shown to function as a histone deacetylase (HDAC)-dependent transcriptional repressor, which can associate with HDAC1-3, NCoR and SMART [7] and can subsequently be inhibited via trichostatin A, a potent HDAC inhibitor, in 10T1/2 cells [21]. In addition, a recent study demonstrated that Smyd1 binds directly to the promoters of *nppa* and *tgfb3* and inhibits their expression in cardiomyocytes [35]. Similarly, Smyd2 has been shown to repress transcription from an SV40-luciferase reporter [36], and dimethylation of histone H3 lysine 36 by Smyd2 is associated with its interaction of Sin3, a HDAC1-containing complex [36], again suggesting a role in HDAC-dependent transcriptional repression. Regarding Smyd3, Foreman *et al.* has shown that it can methylate multiple histone variants *in vitro* (H4>H2A>H3>H2B), although it preferentially tri-methylates histone H4 lysine 20 [14], a mark associated with transcriptional repression. Smyd4 has been shown to recruit class I HDACs [25] and reduce the expression of platelet-derived growth factor receptor alpha polypeptide (Pdgfra) in tumor cells through transcriptional repression [37]. Smyd5 has also been shown to tri-methylate histone H4 on lysine 20, which led to the repression of inflammatory response genes, including Tnf, Ccl4, Il1b [28], supporting its function as a transcriptional repressor. Although we are beginning to understand the repertoire of histone residues methylated by this family and their effect on gene activation or repression, very little data exists identifying the specific genes regulated by these individual family members, and to date, only a few targeted ChIP-PCR studies have been performed for some of the Smyd family members. ChIP-Seq analyses of each of the Smyd family members will be critical for identifying these unique gene targets.

In discussion of what residues are methylated by this unique family, a natural question arises about their specificity: how do they recognize which specific nucleosomes throughout the genome to methylate? One of the most well defined factors guiding protein binding to specific regions of the genome is primary DNA sequence. However, with the exception of Smyd3 [11, 12], no other Smyd protein has been shown to interact directly with DNA. Consequently, the factors influencing their specificity are difficult to identify, some of which could include unique histone variants, individual or combinatorial post-translational modifications or ancillary binding proteins. This level of regulation for the Smyd family is almost completely unknown and requires further investigation. In addition to questions about

how the Smyd family is directed to specific genes, many histone methyltransferases do not act independently; instead they carry out their enzymatic function as part of an active complex, although these regulator proteins are only beginning to be defined. Specifically, Hsp90 has been shown to enhance the activity of Smyd 1, 2, and 3 [4, 12, 13] and Smyd5 has been shown to be directed to TLR4-responsive promoters through its association with the NCoR complex [28]. Understanding the necessary complexes Smyd proteins require to carry out their enzymatic function represents a largely untapped and important area of focus.

In addition to methylating lysine residues on histone proteins, it is now established that the Smyd family plays a prominent role in methylating non-histone targets as well. A number of non-histone methylation-targets (both nuclear and cytosolic) have been identified for most of these members (Table 1, targets are defined as proteins that are directly methylated by a Smyd family member, not simply an interacting partner), with their transport between these two compartments facilitated by their association with other factors (in the absence of a nuclear localization sequence) [38]. Smyd2 and Smyd3 methylate several oncogenic proteins supporting a link for these members in cancer. Consequently, much of the current research on these two family members has focused primarily on their involvement in this pathology. For example, Smyd2 methylates p53 (on lysine 370) which reduces its DNA-binding affinity and represses p53-mediated transcriptional regulation [39]. Another Smyd2 methylation target is the Poly (ADP-ribose) polymerase-1 (PARP1) on lysine 528, which enhances PARP1 activity [40]. Smyd2 also methylates retinoblastoma tumor suppressor (RB) on lysine 860, during cell cycle progression, differentiation, and in response to DNA damage [41]. Similarly, Smyd3 has been shown to methylate both MAP3K2 [42] and VEGFR1 [27], augmenting kinase signaling pathways in various forms of cancer. One Smyd2 methylation target with more cardiac relevance is the cytosolic chaperone Hsp90. Smyd2 methylates Hsp90, which promotes the binding of Hsp90 complexes to titin, a giant protein of striated muscles, which plays a role in muscle elasticity and is integral to the myofibril structure [43, 44]. Large scale proteomic studies of Smyd2 have revealed several other methylation targets (Table 1), though their physiological relevance still needs to be explored.

In contrast to other Smyd proteins, less is known about Smyd1's non-histone targets. Smyd1 has been shown to methylate TRB3 at K16, activating it as a co-repressor to control further transcription of TRB3, as well as potentially regulating oxidative and ER stress responses in the developing heart [45]. With its sustained expression in the heart into adulthood, Smyd1 may play a role in regulating these pathways in normal and pathological states [45], but the full extent to which it regulates heart homeostasis remains largely unknown. Smyd1 has also been shown to methylate its well characterized binding partner skNAC [46]. Given Smyd1 and skNAC's roles in transcriptional regulation [7, 47] and skNAC's involvement in Smyd1 translocation [38, 48], verifying and understanding this methylation could help reveal mechanisms that govern Smyd1 function. Similarly, potential non-histone targets of the other Smyd proteins have yet to be determined and therefore warrant further investigation.

4. ROLE OF SMYD PROTEINS IN THE FETAL AND ADULT HEART

It is now well established that the Smyd family plays key roles in the regulation of growth and differentiation in both development and disease in the heart and other tissues.

Interestingly, in-line with their opposing roles in activating and repressing transcription, these family members also inhibit and promote growth in a context-specific manner. Smyd2 has been shown to have both oncogenic [39, 49, 50] and tumor suppressing capabilities [36]. Smyd3 promotes cancerous growth [17, 33, 51, 52], while Smyd4 functions as a tumor suppressor [37]. Interestingly, all five family members are expressed in the fetal heart and have been shown to regulate key aspects of muscle development (Table 2). Specifically, constitutive loss of Smyd1 in the mouse resulted in embryonic lethality at E9.5 due to heart defects, including disrupted maturation of ventricular cardiomyocytes and malformation of the right ventricle as well as truncation of the outflow tract [21, 45]. Conditional knockout of *smyd1* in cardiomyocytes and the outflow tract was also embryonic lethal, but at E12.5-15.5 and exhibited pericardial edema, thinned pericardium and decreased trabeculation in similar heart sections [45]. Similar to Smyd1 in mice, knockdown of Smyd3 in zebrafish embryos resulted in abnormal heart looping accompanied by pericardial edema and abnormal mRNA expression of three heart-chamber markers (i.e. *cmlc2*, *amhc* and *vmhc*) and myogenic regulatory factors (i.e. *myod*, *myog*) [24]. Interestingly, despite having cardiac defects and curved trunks in Smyd3 knockdown embryos, they showed normal cardiac and skeletal myogenesis at early stages, pointing to a role of Smyd3 in maturation and proliferation of myogenic cells rather than in early differentiation. Although Smyd4's role in the developing heart remains unknown, it is necessary for skeletal muscle development in *Drosophila* [25].

While loss of Smyd1 and Smyd3 were deleterious [21, 24], Smyd2 and Smyd5 were dispensable for cardiac development in mice and zebrafish, respectively [53, 54]. However, Smyd2's role in zebrafish heart development has produced seemingly dichotomous results with two studies reporting that decreased Smyd2a or Smyd2a/b resulted in malformation in both the atrium and ventricle and 50% reduction in fractional shortening (FS) as well as tail deformation [43, 44]. Video analyses of Smyd2 knockdown hearts revealed additional cardiac defects, including pericardial edema, elongation and thinning of the hearts, inflow tract edema, and reduced heart rate. These same zebrafish also showed skeletal muscle defects evidenced by tail deformation, highlighting the role of Smyd2 in skeletal muscle. However, a separate study in zebrafish showed similar tail deformation, but no cardiac defects in Smyd2a knockdown embryos [55].

In the adult heart much less is known about the Smyd family; indeed only data for Smyd1 in mouse and humans has been reported. Specifically, Smyd1 is remarkably upregulated in a mouse model of pressure overload-induced hypertrophy and failure [35]. This differential regulation is consistent with Smyd1 expression in human heart failure patients [56, 57]. In addition, the phenotype of inducible, cardiac-specific Smyd1 knockout mice is quite striking with loss of Smyd1 in the adult heart resulting in chamber dilation in both right and left ventricles, hypertrophic growth, and progressive decline in ejection fraction. These results indicate that Smyd1 functions to suppress growth and maintain heart size under normal conditions and in response to pathologic stress [35]. Future work will be necessary to determine the role of the other Smyd family members, which are also expressed in the adult mouse and human heart.

Given Smyd1's striated muscle specificity and the significant effects of its knockout on the mammalian heart, a natural question is what regulates the expression of Smyd1, both in

cardiac development and under pathological conditions. The human Smyd1 promoter contains highly conserved binding sites of the muscle regulatory factors, serum response factor (SRF) and myogenin [58]. In fact, overexpression of SRF and myogenin significantly increased Smyd1 expression in C2C12 cells, while deletion of SRF in the mouse embryonic heart decreased the mRNA level of Smyd1, suggesting that Smyd1 is a downstream target of SRF and myogenin. Interestingly, cardiac-specific deletion of SRF blocked the appearance of rhythmic beating myocytes [59], consistent with the absence of a heartbeat after Smyd1 knockdown in zebrafish [4]. However, it still remains unclear whether the regulation of Smyd1 in myocardial differentiation and development is mediated by transcriptional control of SRF. Interestingly, cardiac-specific overexpression of Thioredoxin 1 (Trx1) in mice led to the upregulation of Smyd1 at the protein level, concomitant with the increased levels of lysine methylation in the heart [60]. This link is consistent with the fact that Trx1 is downregulated in the failing heart while overexpression shows protective effects on the development of heart failure [61]. Yet, what regulates the expression of Smyd1 under pathological conditions remains unknown.

Studies link the regulation of Smyd2 to the transcription factor CCAAT/enhancer binding protein α in hepatocellular carcinoma, where overexpression of the 30 kDa isoform resulted in a significant decrease in Smyd2 mRNA levels [62]. Other studies have implicated the RNA-binding proteins TDP-43 and FUS/TLS in the regulation of Smyd3 pre-mRNAs in neuronal cells [63]. A direct link to either of these regulations and the downstream functions of Smyd2 or 3 has not been identified yet. Furthermore, what regulates the expression of Smyd4 and Smyd5 has not been demonstrated.

5. ROLE OF SMYD PROTEINS IN SKELETAL MUSCLE

The Smyd family has also been shown to have prominent roles in skeletal muscle, with Smyd1, 2 and 3 displaying unique functionality, however, no data exists for Smyd4 and 5. Smyd1's role in cardiac and skeletal development is not limited to epigenetic regulation, but also plays a direct role in sarcomere organization. However, due to the embryonic lethality of Smyd1 knockout in mice, most studies on muscle development have been performed in zebrafish and have focused on skeletal muscle with some observations in cardiac effects. Loss of Smyd1 in zebrafish heart did not affect heart tube patterning, but led to complete disorganization of all key sarcomeric structures including thick, thin, and titin filaments, as well as M- and Z-lines in skeletal and cardiac muscles of early-stage zebrafish embryos [4, 29, 64]. Smyd1 is more critical for myofibrillogenesis in fast muscle than slow muscle, with studies showing either no disrupted myofibrillogenesis in slow muscle [64] or that it becomes dispensable in late-stage embryonic slow muscles [29]. Recent studies with skeletal muscle-specific conditional knockout mice support these findings. Deletion of Smyd1 impaired myoblast differentiation, resulted in fewer secondary myofibers and decreased expression of muscle-specific genes during the second wave of mammalian skeletal myogenesis [65]. Likewise, conditional knockout of Smyd1 in differentiated muscle specifically produced centralized nuclei and hypotrophied fibers in fast-twitch muscles, while slow-twitch muscle seemed unaffected [66]. These conditional knockout mice were also smaller with reduced strength. Additionally, they showed myofibrillar disorganization, but not to the same extent seen in zebrafish.

Initial studies in zebrafish demonstrated that the methyltransferase activity of Smyd1 is essential for muscle cell differentiation and myofiber maturation [4], though it was unclear whether this was due to methylation at histones or another possible target. Later, it was demonstrated that Smyd1b localizes to myosin at the A-band and M-line in sarcomere, but that the methyltransferase activity was not required for Smyd1b function in myofibrillogenesis in zebrafish [64], yet no change in methylation was assessed. However, Li *et al.* later demonstrated that myosin methylation is decreased in Smyd1b knockdown embryos, suggesting Smyd1 methylates myosin as part of its role in myofibrillogenesis [29]. One possible explanation to this discrepancy is that zebrafish contain two distinct Smyd1 genes. Smyd1a has some redundancy in function and can compensate for defective Smyd1b. In fact, ectopic expression of Smyd1a rescues the muscle defects in Smyd1b knockdowns [30]. Moreover, it was reported that Smyd1 (Smyd1b-tv1/tv2) undergoes a nucleus-to-cytoplasm translocation during myoblast differentiation into myotubes and that Smyd1b-tv1 was specifically localized on the M-line [67], while Smyd1b_tv2 and Smyd1a show weaker or no sarcomeric localization. Furthermore, Smyd1b_tv1 specifically interacts with Hsp90 and Unc45, both known to play a role in myosin folding and sarcomere assembly [29]. Taken together, these studies suggest that Smyd1 plays a critical role in myofibril assembly during myofibrillogenesis, possibly in complex with Hsp90 and Unc45, by increasing the stability of the assembling complexes through myosin methylation (presumably by preventing ubiquitination of myosin). However, it cannot be excluded that Smyd1 may regulate myosin organization with its dual functions by controlling transcription of myosin-associated genes in the nucleus [34] and assembling myosin complexes in the cytoplasm.

Similar to Smyd1, Smyd2 plays a role in sarcomeric organization and maintenance. Smyd2 methylates Hsp90 at K616 to promote a ternary complex between Smyd2, Hsp90 and Titin at the N2A domain [43, 44]. This interaction promotes structural integrity of the N2A domain, thereby stabilizing the I-band and Z-discs, but not the M-band, of the sarcomere [43]. A Smyd4 homologue has also been implicated in muscle development in flies. Mesodermal knockdown of a dSmyd4 in *Drosophila* resulted in pupal lethality due to flies being unable to escape the pupal case [25].

Smyd3 controls skeletal muscle development and maintenance through transcriptional regulation. Smyd3 zebrafish morphants developed curved trunks, which was associated with sustained expression of *myod* and *myog* at a late developmental stage [24]. Also, reduction of Smyd3 in C2C12 skeletal muscle cells reduced expression of *myostatin* and *c-Met* genes. This transcriptional activation occurs through Smyd3's recruitment of a complex containing the acetyl-lysine recognition domain bromodomain BRD4 protein and pause-release factor pTEFb, thereby promoting PolII Ser2P chromatin engagement at the corresponding promoter sites. Smyd3 reduction resulted in hypertrophic C2C12 myotubes, as well as prevented Dex-induced atrophy in adult mouse skeletal muscle, implicating Smyd3 in skeletal muscle mass maintenance and glucocorticoid-induced skeletal muscle atrophy [68].

6. CONCLUSIONS AND FUTURE DIRECTIONS

Emerging evidence implicates the important role of the Smyd family in cardiac and skeletal muscle development, homeostasis, and pathology (Figure 5). However, their specific

molecular function on the cellular level is only beginning to be defined. This is, in part, due to their dual roles in transcriptional regulation (both gene activation and repression) and sarcomere assembly. In terms of epigenetic regulation, a genomic-based systematic approach, such as ChIP-sequencing and genome-wide histone profiling, is needed to identify the direct target genes and histone methylation sites of Smyd proteins, respectively. The hypertrophic and failing heart undergoes extensive gene reprogramming and these proteins are likely key regulators of this pathophysiology. Indeed, besides Smyd4, all Smyd proteins show sustained protein levels in the adult heart, suggesting a role in cardiac and muscle homeostasis. Currently, only Smyd1 has been examined in the adult heart where it acts as a key regulator of cardiac hypertrophy and failure, and as a transcriptional repressor of the genes involved in cellular hypertrophy [35]. However, further studies are needed to investigate how other Smyd family members modulate the histone code of their target genes and what regulates the expression of this family under pathological stress. Given the upregulation of chromatin-bound Smyd1 in cardiac hypertrophy in mice, it is possible that Smyd proteins are also involved in gene expression changes in physiological hypertrophy. In this regard, the relationship with exercise might provide a new physiological role for Smyd proteins in the heart and skeletal muscle.

In addition, we currently do not understand how Smyd proteins target specific nucleosomes through the genome to regulate unique gene targets. Only Smyd3 has been shown to bind DNA directly, but the conserved positive charge across the MYND domain of Smyd1, 2, and 3 suggest that other Smyd family members may have the ability to bind DNA [5] and warrants further investigation. Smyd proteins lack a nuclear localization signal [69], yet obviously play significant roles in regulating transcription, therefore, extensive binding partner studies in physiologically relevant models could help elucidate the complexes that likely exist to translocate, target, and activate Smyd proteins in their methyltransferase functions.

Both Smyd1 and Smyd2 have been shown to play important roles in sarcomere assembly, but the complete mechanisms by which they function have yet to be demonstrated. For example, it has been suggested that Smyd1 methylates myosin, potentially preventing ubiquitination and subsequent degradation. However, there are no studies identifying which myosin residues are methylated by Smyd1, which could suggest a common motif for Smyd1 targets. Studies on Smyd3 highlight a role in transcriptional regulation in zebrafish heart, however, this protein has yet to be analyzed in mammalian heart models both in development and adult disease. Relatively little is known about Smyd4 and Smyd5 and increased focus on these family members could reveal general mechanisms across all Smyd proteins. Additionally, we have very limited data in the adult human heart for any of the Smyd family members, which would provide beneficial for verifying zebrafish and mouse studies, as well as assist in direct future research towards therapeutic relevance.

Methylation of non-histone proteins plays vital roles in cellular homeostasis, including the regulation of molecular interactions, trans-localization, and protein turnover. Non-histone targets have been identified for several Smyd proteins; however, the Smyd family interactome in cardiomyocytes is largely unknown. A global analysis of these non-histone

binding partners for the Smyd family would be very informative in not only identifying substrates, but also revealing patterns consistent among the Smyd family members.

With their emerging roles in cardiac disease and cancers, the Smyd family holds great therapeutic interest. The CTD appears to inhibit the methyltransferase activity of both Smyd1 and 2, but activates Smyd3 enzymatic activity. It is possible that controlling the conformation of the CTD relative to the SET and MYND domains may regulate the activity of Smyd proteins, and could represent a potential therapeutic target to either enhance or prevent Smyd enzymatic activity. Because of their cancer relevance, inhibitors targeting the active site already exist for Smyd2 and Smyd3. Currently there are no inhibitors for Smyd1, Smyd4, and Smyd5, however more cardiac or muscle relevant disease studies for all Smyd proteins are still needed to determine their potential benefit in cardiac or skeletal muscle disorders. Additionally, structure studies of Smyd proteins bound to substrates would be needed to help guide the design. Understanding what role Smyd family members play in maintaining cellular homeostasis and responding to cardiac insults will be key to defining their impact on adult health and determining their therapeutic potential in the treatment of cardiovascular diseases.

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ABBREVIATIONS

HDAC	histone deacetylases
CTD	C-terminal domain
TPR	tetratricopeptide repeats

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HIGHLIGHTS

- The first comprehensive compilation of Smyd proteins critical role in cardiac and skeletal muscle.
- Demonstrates the Smyd family's unique ability to regulate transcription through activation and repression.
- Highlights gaps in our knowledge regarding the molecular function of the Smyd family.
- Smyd family holds therapeutic potential in treatment of cardiac pathologies.

Figure 1. Linear representation of the structural domains of the Smyd proteins
The split SET (shown as S and ET, red), MYND (blue), SET-I (orange), Post-SET (brown) and CTD or TPR (green) domains are indicated. The numbers represent the final residue number in each domain. Percent homology is reported showing a comparison of human, mouse, and rat (HMR) sequences as well as a comparison for human, mouse, rat, and zebrafish (HMRZ).

Figure 2. Expression of Smyd proteins in human tissue and cells
The relative expression levels of the five Smyd proteins across various tissues are shown as measured by Kim et al via mass spectrometry [19]. Each protein is normalized individually with the scale bar indicating the degree of expression from low to high. All Smyd proteins are expressed in both fetal and adult heart, with the exception of Smyd4.

Figure 3. Linear representation of the structural domains of the Smyd1 isoform/variants across human, mouse, rat and zebrafish

Smyd1 genes for human, mouse, rat, and zebrafish are represented in purple and splice variants of the same gene are grouped in brackets. The split SET (shown as S and ET, in red), MYND (blue), SET-I (orange), Post-SET (brown) and CTD (green) domains are indicated. The 13 amino acid insert is indicated in pink. The numbers represent the final residue number in each domain. Percent homology is reported compared to human for each species variant. Other published names for variants are also indicated with their corresponding references (aka-also known as) (m-Bop, m-Bop1 [21], skm-Bop [20], BOP [70], m-Bop2 [21], t-Bop [20]; ZF: (a) Smyd1a [23, 30], (b_tv1) Smyd1a [64], SmyD1a [4, 22], Smyd1b [23], (b_tv2) Smyd1b [64], SmyD1b [4]⁴⁸).

Figure 4. Smyd proteins in chromatin remodeling and transcriptional control

Smyd proteins can modify chromatin structure by methylating histone tails. For instance, trimethylation of histone 3 lysine 4 (H3K4, represented by red methylation marks) by Smyd1 is thought to unpack chromatin structure and render the DNA accessible to transcription factors to facilitate gene expression. In contrast, trimethylation of histone H4 lysine20 (represented by purple methylation marks) by Smyd5 compacts chromatin to repress transcriptional expression.

Figure 5. Smyd proteins in cardiac and skeletal muscle physiology

Smyd proteins can methylate 1) histones to both activate and repress transcription and 2) non-histone proteins to promote protein:protein interactions and modulate enzymatic activity. Through these two mechanisms, Smyd proteins regulate a number of processes in cardiac and skeletal muscle in development and disease.

Table 1**Smyd Family Methylation Targets and Function**

	Methylates Histone H3K4	Unique Histone Targets	Activation/ Repression	Unique Non-Histone Targets*	Regulates Growth
Smyd1	Yes[4, 48]	Unknown	Activation[21, 34] Repression[48]	skNAC[46], TRB3[45]	Inhibit[35, 71]
Smyd2	Yes[13]	H3K36[36]	Activation[13], Repression[13, 36, 55]	Hsp90α[43], p53[39], RB[41], PARP1[40], MAPKAPK3[50], PTEN[72], ERα[73], BTF3, PDAP1, AHNAK, AHNAK2[74], MAP1, CCAR2, eEF2, NCOA3, STUB1, UTP14A[75], Six1, Six2, SIN3B, DHX15[76]	Inhibit[36] Promote[50]
Smyd3	Yes[12]	H4K5[77], H4K20[14]	Activation[12, 52, 68, 78] Repression[14]	MAP3K2[42], VEGFR1[27]	Promote [12] Inhibit[68]
Smyd4	Unknown	Unknown	Repression[25, 37]	Unknown	Inhibit[25, 37]
Smyd5	Unknown	H4K20[28]	Repression[28]	Unknown	Unknown

*"Targets" are defined as being directly methylated by Smyd family members, not simply interacting partners

Table 2**Role of Smyd Family in Cardiac and Skeletal Muscle**

	Role in Fetal Heart	Role in Adult Heart	Cardiac Phenotype of KO/KD*	Role in Skeletal Muscle
Smyd1	Morphogenesis [21, 45], Myofibrillogenesis [4, 29, 64, 67], Cell proliferation [45]	Inhibit hypertrophic growth [35]	<u>Mice</u> : Disrupted right ventricle formation and cardiomyocyte maturation [21, 45] <u>Zebrafish</u> : Impaired myofibril formation [4] and no heartbeat [4, 64]	Myofibrillogenesis [4, 29, 64, 67] Myoblast differentiation [65] Myofibril integrity [66]
Smyd2	Dispensable [53] Myofibrillogenesis [44]	No Phenotype [53]	<u>Mice</u> : Normal heart development [53] <u>Zebrafish</u> : Impaired myofibril formation [44]	Sarcomeric Organization/maintenance [43, 44],
Smyd3	Morphogenesis [24] Maturation/proliferation of differentiated myocytes [24]	Unknown	<u>Zebrafish</u> : Abnormal looping of heart tube, pericardial edema [24]	Myogenic Maturation [24], Maintenance & Atrophy [68]
Smyd4	Unknown	Unknown	Unknown	Muscle Development/Function [25]
Smyd5	Dispensable [54]	Unknown	<u>Mice</u> : Normal heart development [54]	Dispensable [54]

* Knockout/Knockdown