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SMYD2 is induced during cell differentiation and participates in early development

BORJA SESÉ[1], MARIA J. BARRERO[1], MARIA-CARME FABREGAT[1], VERONIKA SANDER[1] and JUAN CARLOS IZPISUA BELMONTE*[,1,2]

[1]*Center for Regenerative Medicine in Barcelona, Barcelona, Spain and*
[2]*Salk Institute for Biological Studies, La Jolla, CA, USA*

ABSTRACT Histone modifying enzymes play critical roles in cell differentiation and development. In this study, we report that SMYD2 (SET and MYND domain containing protein 2), a histone lysine methyltransferase, is induced during human embryonic stem (ES) cell differentiation and it is preferentially expressed in somatic cells versus pluripotent cells. Knockdown of SMYD2 in human ES cells promotes the induction of endodermal markers during differentiation, while overexpression has opposite effects. *In vivo* experiments in zebrafish revealed that knockdown of *smyd2a* (a homologue gene of human SMYD2) causes developmental delay and aberrant tail formation, which is coincident with low expression of *ntl* and over induction Nodal-related genes during gastrulation. Taken together, these findings suggest that SMYD2 plays a critical role at early stages of development and in human ES cell differentiation.

KEY WORDS: *SMYD2, differentiation, development, stem cell, methyltransferase*

Introduction

Embryonic stem (ES) cells are characterized by their ability to self-renew indefinitely *in vitro* maintaining their undifferentiated state, and the capacity to give rise to any cell type in the body (Thomson *et al.*, 1998). The molecular mechanisms underlying ES cells identity and their potential for differentiation are still poorly understood (Boyer *et al.*, 2005, Niwa, 2007). During the last years, post-translational covalent modifications of histone proteins have emerged as a crucial epigenetic event to regulate the pluripotent state of ES cells and to establish cell fate decisions (Bibikova *et al.*, 2008, Goldberg *et al.*, 2007, Meissner, 2010). Histone modifications help to define chromatin structure and can be associated with active marks, offering accessible DNA for transcription, and repressive marks, where DNA is more packaged and inaccessible (Cheung and Lau, 2005, Kouzarides, 2007). One particular property of ES cells is that many developmental genes present both active H3K4 and repressive H3K27 methylation on their regulatory regions, the so-called "bivalent domains" (Azuara *et al.*, 2006, Bernstein *et al.*, 2006). This bivalency keeps these genes silenced in ES cells, but "poised" to become activated by losing the repressive mark, or kept silenced by removal of active marks during differentiation (Spivakov and Fisher, 2007).

In order to select candidates of histone modifying enzymes involved in pluripotency and differentiation of human ES cells, we identified a group of histone lysine demethylases and methyltransferases differentially expressed between undifferentiated and differentiated human ES cells (data not shown). Among them, SMYD2 emerged as a potential candidate due to its barely expression in undifferentiated human ES cells and later up-regulation after 30 days of differentiation towards the three germ layers. The SMYD (SET and MYND domain) protein family presents methyltransferase activity provided by its SET domain, which is split into two segments by its MYND domain, responsible for protein-protein interactions (Brown *et al.*, 2006). In human, there are five members in the SMYD protein family (SMYD1-5) and have been shown to participate regulating gene transcription and cell proliferation. SMYD1 is a heart and muscle specific histone methyltransferase involved in cardiomyocyte and myogenic differentiation (Gottlieb *et al.*, 2002, Li *et al.*, 2009). The lack of Smyd1 in mice development results in embryonic death due to cardiac defects (Gottlieb *et al.*, 2002). Knockdown of *smyd1a/b* in zebrafish causes skeletal and cardiac muscle defects and presents a disrupted expression of myofibril organization (Tan *et al.*, 2006), SMYD3 has been mainly

Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; ES, embryonic stem (cell); MO, morpholino; SMYD, SET and MYND domain containing protein.

***Address correspondence to:** Juan-Carlos Izpisua-Belmonte. Salk Institute for Biological Studies, La Jolla, CA, USA
Fax: +1 858 453 2573. e-mail: belmonte@salk.edu - web: www.cmrb.eu

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related with cancer cell proliferation (Hamamoto *et al.*, 2004). Several findings indicate that endogenous expression of SMYD3 is present at very high levels in hepatocellular, colon and breast carcinoma, and silencing through siRNAs have an inhibitory effect in cell growth (Hamamoto *et al.*, 2004, Hamamoto *et al.*, 2006). Similarly to *smyd1a/b*, *smyd3* plays an important role in cardiac and skeletal muscle development in zebrafish (Fujii *et al.*, 2011). In the other hand, SMYD4 is significantly reduced in tumor cells and its re-expression dramatically decreases cancer cell growth (Hu *et al.*, 2009). Also, *Drosophila* SMYD4 homologue has been involved in muscle development (Thompson and Travers, 2008). Little is known so far about SMYD5. Unlike the rest of family members, SMYD5 does not present a C-terminal tetratrico peptide repeat (TPR) domain (Abu-Farha *et al.*, 2011).

SMYD2 was first described as a histone lysine methyltransferase mainly expressed in heart and brain tissue, with specific catalytic activity for H3K36 dimethylation, a mark associated with actively transcribed genes (Brown *et al.*, 2006). Moreover, SMYD2 was associated to interact with HDAC1 and the Sin3A repression complex (Brown *et al.*, 2006). In yeast, there is evidence of a link between H3K36 methylation and the recruitment of a repressive Rpd3 (the prototypical yeast HDAC) complex (Carrozza *et al.*, 2005, Joshi and Struhl, 2005, Keogh *et al.*, 2005). However, SMYD2 was also described to specifically monomethylate H3K4 in the presence of HSP90α *in vitro*, with no activity for H3K36 (Abu-Farha *et al.*, 2008). Despite observing a weak activity of H3K36 methylation in the absence of HSP90α, *in vivo* experiments suggested that H3K4 is the predominant site of methylation for SMYD2 (Abu-Farha *et al.*, 2008). Regarding histone methylation, recent studies determined that SMYD2 also methylates histones H2B and H4 more efficiently than H3 *in vitro* (Wu *et al.*, 2011). Further, several non-histone proteins have been identified as substrates for SMYD2 methylation such as p53 and retinoblastoma (Rb) (Cho *et al.*, 2012, Huang *et al.*, 2006, Jiang *et al.*, 2011, Saddic *et al.*, 2010, Scoumanne and Chen, 2008). Monomethylation of p53 at K370 reduces its binding ability to promoter target genes like p21 and mdm2, resulting in a decreased expression of these genes (Huang *et al.*, 2006). Unexpectedly, adult hearts of Smyd2 conditional knockout mice showed no changes in p21 and mdm2 expression levels, and had no global effect in H3K36 or H3K4 methylation (Diehl *et al.*, 2010). Additionally, Smyd2 was found dispensable for proper heart development in mouse (Diehl *et al.*, 2010). Rb protein can be methylated by SMYD2 at K860 and facilitates its interaction with the methyl-binding protein L3MBTL1 (Saddic *et al.*, 2010). More recently, SMYD2 was also found to methylate Rb at K810, which increases phosphorylation of Rb protein, and promotes cell cycle progression (Cho *et al.*, 2012). In accordance with the effects observed on p53 and Rb tumour suppressors, a wide variety of human cancer showed high levels of SMYD2 expression (Cho *et al.*, 2012, Komatsu *et al.*, 2009). It was also reported that SMYD2 is involved in maintaining self-renewal activity of MLL-AF9-induced acute myeloid leukaemia (Zuber *et al.*, 2011). Another novel non-histone substrate for SMYD2, cytoplasmic HSP90, is methylated at K209 and K615 (Abu-Farha *et al.*, 2011). In mouse, Smyd2 methylates HSP90 to form a complex with the sarcomeric protein titin to protect myocyte organization (Donlin *et al.*, 2012, Voelkel *et al.*, 2012). Here we reported the characterization of SMYD2 during human ES cell differentiation. Our data suggest that SMYD2 plays an important role during early differentiation events.

Results

SMYD2 expression is strongly induced during human ES cell differentiation

We first set up to analyze the potential differential expression of the SMYD family members in pluripotent and somatic cells by qPCR. mRNA levels of all five SMYD family members were measured in human embryonic stem (ES) cells (ES[2] and ES]4]), induced pluripotent (iPS) cells derived from human keratinocytes (KiPS) and human fibroblasts (HF) and keratinocytes (HEK) (Fig. 1A). Among all family members, SMYD2 clearly showed higher mRNA levels in somatic cells compared to pluripotent cells. We next tested the expression profile of the SMYD family members during the differentiation of human ES cells in the form of embryoid bodies (Fig. 1B). As expected, SMYD2 showed the most remark-

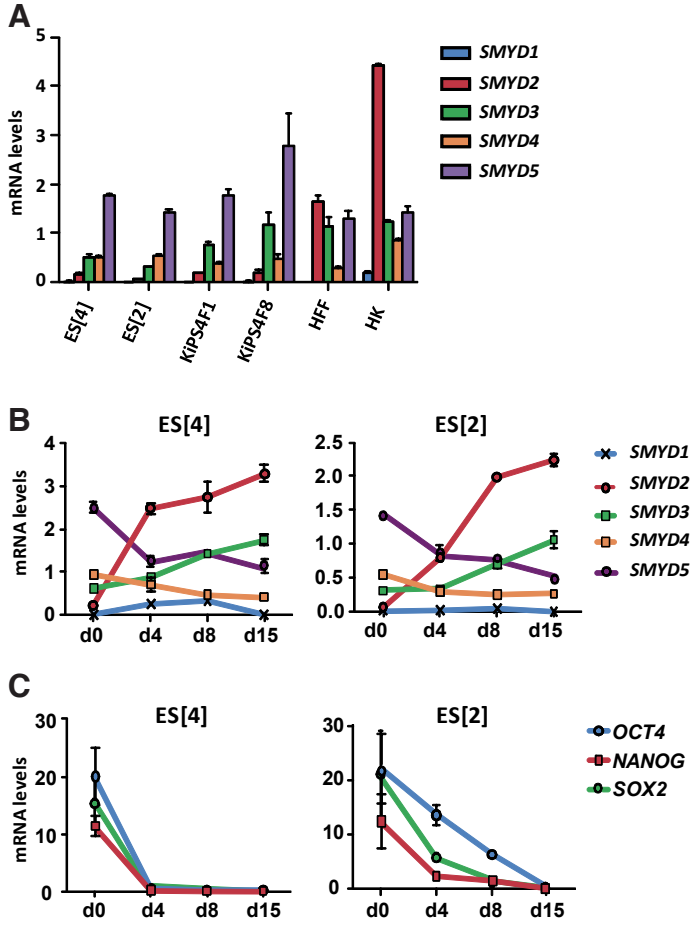


Fig. 1. Expression of SMYD family members during human embryonic stem (ES) cell differentiation. (A) *mRNA levels of* SMYD1-5 *family members in human embryonic stem cells (ES[4] and ES[2]), induced pluripotent stem cells from human keratinocytes (KiPS4F1 and KiPS4F8), human fibroblasts (HF) and human keratinocytes (HEK).* **(B)** *mRNA levels of* SMYD1-5 *in ES[4] and ES[2] undifferentiated (d0) and at days 4, 8 and 15 of differentiation* **(C)** *mRNA levels of pluripotency-related genes during. ES[4] and ES[2] differentiation. Three independent differentiations were performed and one representative experiment is shown. Levels were determined by qPCR and normalized to GAPDH. Mean and standard deviation of triplicates are shown.*

able induction among the SMYD family members, which is coincident with the dowregulation of the expression of pluripotency-related genes (Fig. 1C). Taken together, our results show that SMYD2 is preferentially expressed in human somatic cells and induced during the differentiation of human pluripotent cells.

SMYD2 promoter presents active chromatin marks in human ES cells

Since SMYD2 is expressed at low levels in human ES cells but rapidly induced during differentiation we considered the potential presence of bivalent domains on its promoter in pluripotent cells. We performed ChIP assay to detect the presence of histone marks related with transcriptional activation (H3K4me) and repression (H3K27me) in ES[4] and ES[2] (Fig. 2). As a control, we tested the presence of histone marks for the pluripotency gene *OCT4*, and two well described genes containing bivalent domains in human ES cells, *SOX17* and *FOXA2*. Despite being transcribed at low levels, *SMYD2* showed remarkable levels of H3K4me2/3 at its promoter. Levels of H3K27me3 showed variability between lines, being very low in ES[4] but significant in ES[2], while compared with the well known bivalent genes *SOX17* and *FOXA2*. The presence of H3K4me2/3 only at the *SMYD2* promoter in ES[4] might suggest the existence of post-transcriptional mechanisms responsible for blocking its expression in human ES cells.

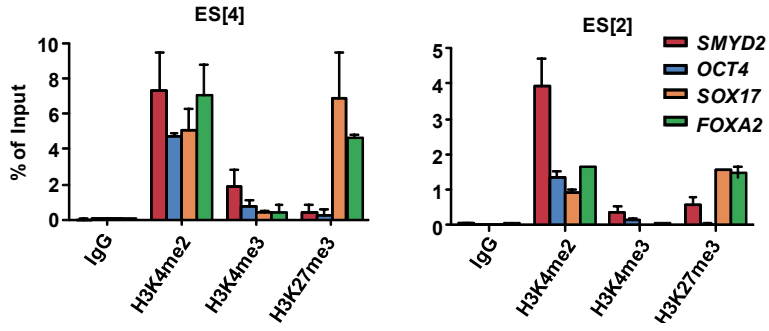


Fig. 2. Analysis of histone methylation marks in the *SMYD2* promoter. *ChIP assays using antibodies against H3K4me2, H3K4me3 and H3K27me3 in ES[4] and ES[2]. The presence of the indicated gene regulatory regions in the immunoprecipitated chromatin was analyzed by qPCR. Values are represented as percentage of input. Mean and standard deviation corresponding to three independent experiments are shown.*

The knockdown of SMYD2 promotes induction of endodermal genes during human ES cell differentiation

To test if SMYD2 is plays a role in the differentiation of human ES cells we performed loss-of-function experiments. We generated lentiviral-transduced stable ES[4] cell lines expressing a shRNA against *SMYD2* (shSMYD2) (Huang *et al.*, 2006) and a random non target shRNA (shControl). In self-renewing cells, the knockdown of SMYD2 did not show any morphological differences compared to the control, neither differences regarding the expression of selected pluripotency and differentiation genes (data not shown). However, during *in vitro* differentiation the SMYD2 knockdown line showed higher levels of endodermal genes (*HNF4*, *FOXA2*, *SOX17*) compared to the shControl line (Fig. 3A), but not significant differences in the induction of ectordermal (*PAX6*) or mesodermal (*BRACHYURY*) genes. The pluripotency-related genes *OCT4*, *NANOG* and *SOX2* were similarly downregulated in the shSMYD2 and shControl lines. Our results suggest that SMYD2 might act as a negative regulator of endodermal differentiation.

SMYD2 overexpression impairs proper differentiation of human ES cells

To further confirm the involvement of SMYD2 in differentiation we generated stable ES[4] cell lines stably overexpressing SMYD2 (SMYD2) or GFP as a control (GFP). The overexpression of SMYD2 in self-renewing cells did not cause significant morphological differences neither differences in the expression of selected pluripotency and differentiation genes compared to the control (Fig. 3B). Contrary to the knockdown, cells overexpressing SMYD2 showed a reduced induction of endodermal and mesodermal genes compared to the control line during differentiation (Fig. 3B). However, the ectodermal gene *PAX6* was more induced in SMYD2 overexpressing cells than control cells. No differences were detected between the SMYD2 and GFP overexpressing cell lines regarding the silencing of pluripotency genes during differentiation. These results confirm that SMYD2 acts as a repressor of endodermal genes and has no effect in the downregulation of pluripotency genes during human ES differentiation.

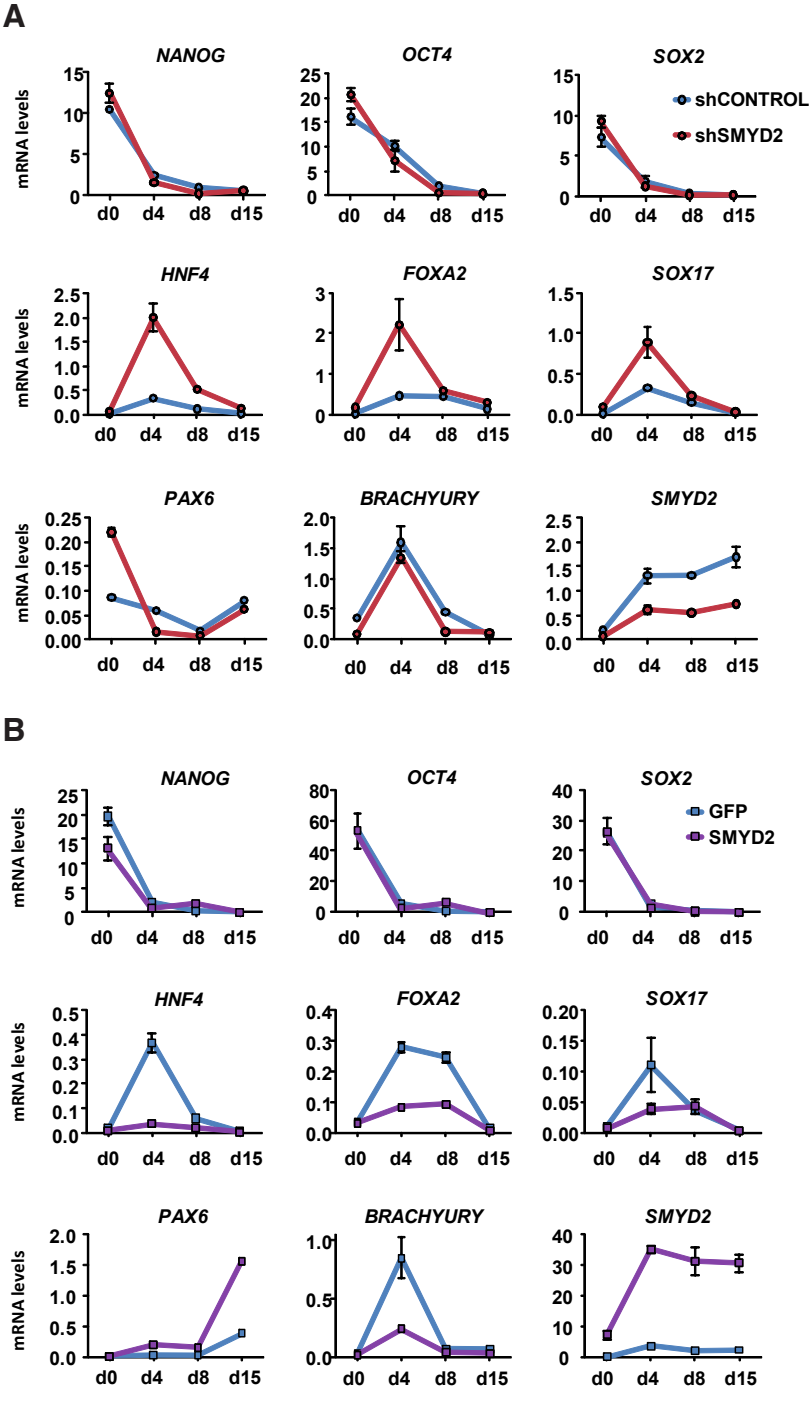
The knockdown of smyd2a *in zebrafish results in tail formation defects*

To assess the effect of SMYD2 at early stages of development we performed knockdown experiments in zebrafish as *in vivo* model. The zebrafish presents two homologous genes of *smyd2a* and *smyd2b*. We first quantified the mRNA levels of both genes during zebrafish development from 0.2 to 48 hours post-fertilization (hpf) (Fig. 4A). Interestingly, *smyd2a* was maternally expressed, rapidly degraded after fertilization and induced again during gastrulation (5 to 10 hpf). *smyd2b* expression levels remained low until 10 hpf and was dramatically induced after gastrulation. The induction of *smyd2a* during gastrulation suggests a potential role in germ layer specification. To confirm our hypothesis we designed a splice-blocking morpholino (smyd2a-MO) at the exon 1-intron 1 junction, to block proper splicing of the zygotic transcripts without affecting the maternal mRNA (Fig. 4B, upper panel). Zebrafish embryos were injected at one-cell stage with smyd2a-MO and a standard-control morpholino (Control-MO). As expected, the mature mRNA of *smyd2a* was undetectable in embryos injected with smyd2a-MO at 24 hpf compared to embryos injected with Control-MO, while *smyd2b* expression was unaffected by the injection of smyd2a-MO (Fig. 4B, lower panel). Injected embryos were examined morphologically up to 6 days after injection (Fig. 4C). At 5 hpf, smyd2a-MO injected embryos could not be morphologically distinguished from the controls. At 24 hpf, we could detect some *smyd2a* morphant embryos with a tail defect (mild), and some others with a strong delay in development (severe). At 48 hpf, we could observe several degrees of tail defects, including a morphant phenotype with a complete absence of the tail (very severe). Later on, at 6 days after injection, all three *smyd2a* morphant phenotypes were distinguishable. To further confirm the specificity of smyd2a-MO effect, we performed a rescue experiment by co-injecting with *in vitro*-transcribed human *SMYD2* mRNA (smyd2a-MO + *SMYD2* mRNA) and evaluated the percentage embryos with different phenotypes at 24h post-injection (Fig. 4D). Table 1 shows that

co-injection of smyd2a-MO with human *SMYD2* mRNA rescues both mortality (from 57% to 36%) and morphology (from 25% mild and 60% severe phenotype to 11% mild and 18% severe phenotype). Co-injection of human *SMYD2* mRNA and Control-MO had no effects on mortality or phenotype compared to the injection of Control-MO alone. Therefore, the effects of *smyd2a* knockdown in zebrafish development appear specific.

smyd2a *knockdown in zebrafish affects the expression of genes involved in the Nodal pathway*

To gain insights into the potential pathways affected by the *smyd2a* knockdown we investigated the expression pattern of the T-box gene *no tail* (*ntl*), a zebrafish Brachyury ortholog required for the formation of mesoderm and essential for tail development (Halpern *et al.*, 1993, Schulte-Merker *et al.*, 1994), by whole-mount *in situ* hybridization (ISH) on embryos at 6 hpf (Fig. 5A). As expected, all control embryos showed *ntl* expression at the margin cells as gastrulation proceeds. In contrast, most smyd2a-MO injected embryos showed altered pattern of *ntl* expression characterized by either a reduction (35% of embryos) or complete absence of *ntl* expression (53% of embryos). Moreover, the ratio of the three different *ntl* expression patters in *smyd2a* morphants is similar to the percentages of different phenotypes at 24 hpf (Fig. 4D, Table 1). To further identify specific transcripts affected by *smyd2a* knockdown, we analyzed the expression profile of a set of genes involved in gastrulation from 4 hpf to 10 hpf by qPCR (Fig. 5B). Nodal-related genes (*chd*, *gsc*, *bon* and *cas*) were up-regulated in the smyd2a-MO compared to the Control-MO. In addition to their strong induction, *gsc* and *bon* presented also a different profile of expression as they were still present at high levels even at 8 hpf, while the control-MO injected embryos had already reduced their expression levels. *bmp2a* and β-*catenin-1* are unaffected by *smyd2a* knockdown, suggesting that genes of the Bmp2 and Wnt pathways remain unaffected. These results indicate that *smyd2a* knockdown causes an induction of Nodal-target genes *chd*, *gsc*, *bon* and *cas* during gastrulation events in zebrafish.



Discussion

In this work we show that SMYD2 is involved in human ES cell differentiation. SMYD2 is expressed at high levels in somatic cells compared with pluripotent cells, whereas the rest of family members are not so differentially expressed. During human ES cell differentiation, SMYD2 is also the most induced family member. SMYD1 and 3, which share the highest degree of sequence homology with SMYD2 (Abu-Farha *et al.*, 2011), are also induced but not so remarkably. SMYD3 present a progressive induction but only around 3-fold change compared with undifferentiated cells. Even at low levels, SMYD1 was induced during differentiation reaching its peak of expression at day 8. This indicates that SMYD1 might be involved also in early development, consistent with previous studies involving Smyd1 in mouse developing heart

Fig 3. Gain-and-loss of function of *SMYD2* during human embryonic stem (ES) cell differentiation. (A) *mRNA levels of the indicated genes during the* in vitro *differentiation of SMYD2 knockdown (shSMYD2) and control (shControl) ES[4] cell lines.* **(B)** *mRNA levels of the indicated genes during the* in vitro *differentiation of SMYD2 overexpressing (SMYD2) and control (GFP) ES[4] cell lines. mRNA levels were measured by qPCR at the indicated days of differentiation and normalized to GAPDH. One representative experiment out of three independent experiments is shown. Mean and standard deviation from triplicates are shown.*

TABLE 1

SURVIVAL RATES AND PHENOTYPES OF MORPHOLINO INJECTIONS AND RESCUE EXPERIMENT AT 24 HPF

	n.	Dead (%)	No. Surviving	wild type (%)	mild (%)	severe (%)
Control-MO	339	9 ± 3	307	94 ± 3	6 ± 3	0 ± 0
smyd2a-MO	545	57 ± 3	238	15 ± 7	25 ± 5	60 ± 12
smyd2a-MO + *SMYD2* RNA	665	36 ± 9	436	71 ± 16	11 ± 4	18 ± 16
Control-MO + *SMYD2* RNA	512	7 ± 2	475	97 ± 3	3 ± 2	0 ± 0

Morpholino injections: Control-MO and smyd2a-MO;

Rescue experiments: smyd2a-MO + SMYD2 RNA and Control-MO + SMYD2 RNA

Mean and standard deviation from three independent experiments are shown.

as a direct target of MEF2C (Phan *et al.*, 2005).

Given the low levels of expression of SMYD2 in pluripotent cells, we hypothesized the presence of bivalent domains on its promoter to keep it repressed. Unexpectedly, we found variable levels of H3K27me3 between cell lines. In ES[4] the *SMYD2* promoter was marked with H3K4 methylation marks only, suggesting that SMYD2 expression could be blocked by an alternative mechanism. Recently, Lipchina *et al.,* (Lipchina *et al.*, 2011) identified SMYD2, together with other 145 genes, as a high-confidence target of the miR-302/367 cluster in human ES cells. miR-302/367 is expressed in undifferentiated conditions and is downregulated upon neuronal differentiation, releasing the expression of its target genes (Lipchina *et al.*, 2011). Thus, a possible miR-302/367 regulation of SMYD2 might explain the low levels of expression in pluripotent cells.

The knockdown of SMYD2 promoted the induction of endodermal genes during human ES cell differentiation, whereas overexpression had opposite effects. We further investigated the effect of SMYD2 depletion *in vivo* using the zebrafish as a model. Our results show that both *smyd2a* and *smyd2b* were induced during development, and surprisingly, *smyd2a* was found to be maternally expressed in zebrafish embryos. Knockdown of *smyd2a* causes a delay in zebrafish development and tail deformities in more than 80% of surviving embryos at 24 hpf, whereas rescue experiments reduced the morphant phenotype to 30%. The fact that we were able to rescue *smyd2a* morphant phenotype by co-injecting human *SMYD2* mRNA suggests a very well conserved function of SMYD2 among species. Intriguingly, it was recently reported that *smyd2a* knockdown generates severe skeletal and cardiac muscle defects in zebrafish (Donlin *et al.*, 2012, Voelkel *et al.*, 2012). Regarding the cardiac phenotype, we did not appreciate any abnormalities, even in the very severe morphant phenotype at 6 dpf, consistent with mice conditional-knockout experiments where Smyd2 was dispensable for heart development (Diehl *et al.*, 2010).

The *smyd2a* knockdown phenotype that we observe is characteristic of embryonic dorsalization and resembles the phenotype of *ntl* mutant embryos (Odenthal *et al.*, 1996). Accordingly, the observed

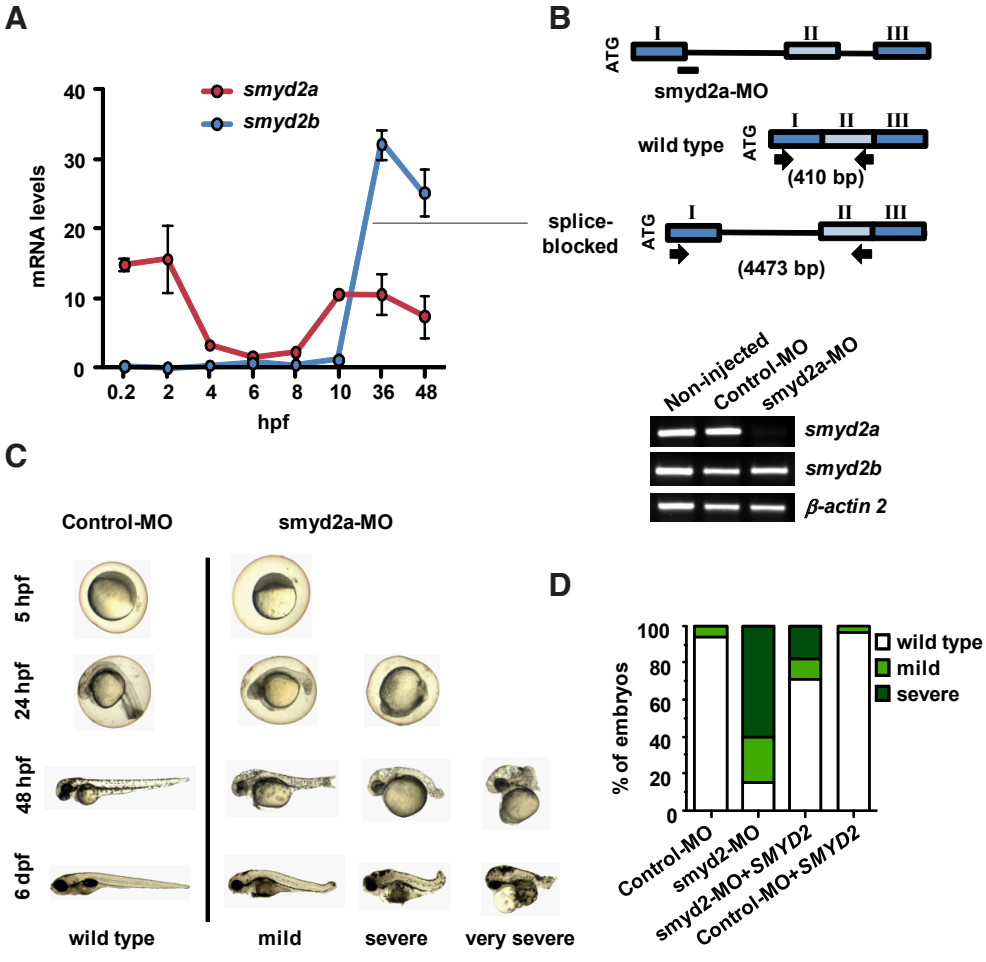


Fig 4. *smyd2a* knockdown in zebrafish development. (A) smyd2a *and* smyd2b *mRNA levels at different hours post fertilization (hpf). Levels were determined by qPCR and normalized to 18S. Two independent experiments were carried out and one representative experiment is shown. Mean and standard deviation from two independent quantifications are shown.* **(B)** *The upper panel shows a schematic representation of the splice-blocking morpholino smyd2a-MO at the exon1-intron 1 junction. Specific primers for* smyd2a *were designed at each side of exon 1 and intron 1 flanking a region of 410 bp in wild type mRNA, and 4473 bp in unspliced mRNA. Lower panel shows the absence of mature* smyd2a *mRNA at 24 hpf in smyd2a-MO compared to Control-MO and non-injected embryos. Expression of* smyd2b *was unaffected by the smyd2a-MO injection. β-*actin 2 *was used as control.* **(C)** *Different phenotypes at 5, 24, 48 hpf and 6 dpf of embryos injected with Control-MO and smyd2a-MO.* **(D)** *Percentage of phenotypes of morpholino injection (Control-MO and smyd2a-MO) and rescue experiment (smyd2a-MO +* SMYD2 *mRNA and Control-MO +* SMYD2 *mRNA) at 24 hpf. Means from three independent experiments are shown.*

dorsalization effects were consistent with reduced or absent levels of *ntl* transcript at 6 hpf. On the contrary, knockdown of SMYD2 in human ES cell differentiation did not affect the expression of the *ntl* ortholog, *BRACHYURY* (Fig. 3A). This discrepancy might be caused by the differences between the two model systems or to the lower efficiency of SMYD2 knockdown in ES cells. We also observed increased expression levels of Nodal-related genes, such as *bon* and *cas,* described to promote the formation of endoderm in zebrafish (Reiter *et al.*, 2001, Stainier, 2002). Also, in *Xenopus, goosecoid* and *Mix.1*, the homologous genes of *gsc* and *bon* respectively, act together to promote endodermal differentiation and suppress expression of mesodermal *Xbra*, the *ntl* homologous gene (Latinkic and Smith, 1999). Eventually, high levels of *gsc* and *bon* at 6 and 8 hpf in smyd2a-MO embryos might have repressive effects over mesoderm induction. Overall, the zebrafish phenotype seems consistent with the induction of endodermal genes in the knockdown of SMYD2 during human ES cell differentiation.

Since, SMYD2 has been previously involved in transcriptional activation through monomethylation of H3K4 we speculate that it might participate in the induction of repressors of the endodermal fate. Alternatively, it might act as a transcriptional repressor itself through its association with HDACI and Sin3a repression complexes (Brown *et al.*, 2006). However, methylation of non-histone targets might also account for these effects. For example, recent findings indicate that p53 knockdown causes a delay in differentiation of human ES cells, and ectopic expression of p53R175H, a mutated inactive form of p53, failed to induce differentiation (Jain *et al.*, 2012).

In summary, our study shows that SMYD2 is expressed at low levels in pluripotent cells but is strongly induced during differentiation. Knockdown of SMYD2 induced expression of endodermal genes, whereas overexpression leads to a blockade of differentiation by impairing induction of most of differentiation genes. Moreover, *in vivo* experiments in the zebrafish showed that *smyd2a* is involved in mesoderm formation and has a critical role from very early stages during development. In conclusion, our work suggests that SMYD2 plays an early decisive role in embryonic differentiation.

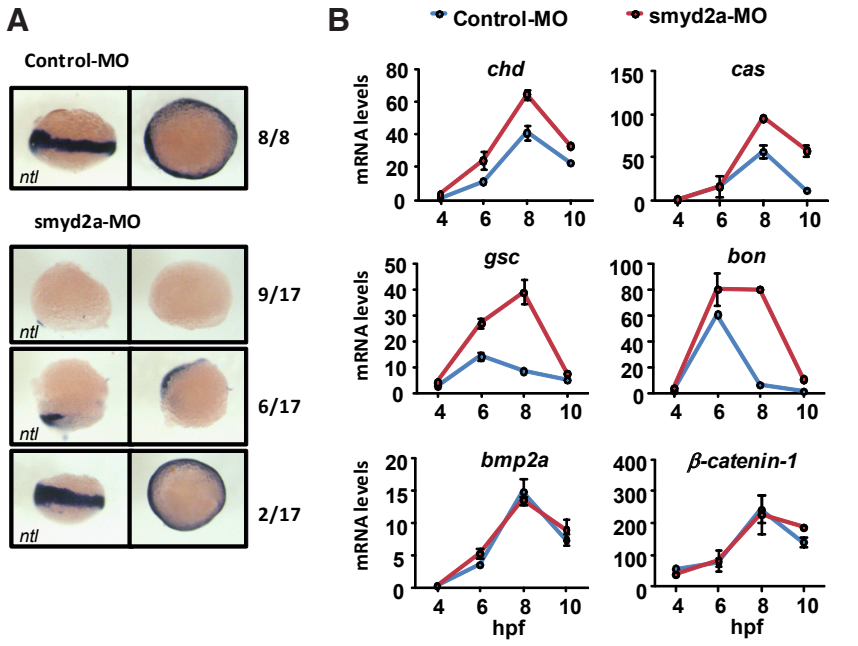


Fig. 5. Effects of *smyd2a* knockdown on gene expression during zebrafish gastrulation. (A) In situ *hybridization of ntl in Control-MO and smyd2a-MO injected embryos at 6 hpf. Embryos are shown in lateral views (left), and animal pole views (right).* **(B)** *mRNA levels of indicated genes in Control-MO and smyd2a-MO embryos at 4, 6, 8, and 10 hpf. 50 embryos were collected at each point. Mean and standard deviation of triplicate quantifications are shown.*

Materials and Methods

Cell culture and differentiation

Human embryonic stem cell lines ES[4] and ES[2] (Raya *et al.*, 2008), were grown on matrigel-coated plates in irradiated mouse embryonic fibroblasts conditioned HES media (Knock out DMEM, 20% Knock out serum replacement, nonessential amino acids, 2mM L-glutamine and 50μM β-mercaptoethanol) and supplemented with 10ng/ml FGF.

For *in vitro* differentiation, cells were trypsinized into a single cell suspension and resuspended in MEF-conditioned HES media. Embryoid body (EB) formation was induced by seeding 100,000 cells in each well of 96-well v-bottom, low attachment plates and centrifuging the plates at 950 g for 5 min to aggregate the cells. After 3 days the embryoid bodies were transferred to 0.1% gelatin-coated dishes and cultured in differentiation medium (Knock out DMEM, 20% fetal bovine serum, nonessential amino acids, 2mM L-glutamine and 50μM β-mercaptoethanol) up to 15 days.

RNA extraction and qPCR

RNA was extracted using TRIZOL (Invitrogen) and cDNA synthesis was performed using the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen). Quantification of mRNA levels was carried out by real time PCR using SYBER Green. qPCR levels were normalized to *GAPDH* in human cells and to *18S* in zebrafish.

ChIP assays

Chromatin immunoprecipitation (ChIP) assays were performed according to Adamo *et al.,* (Adamo *et al.*, 2011). The antibodies used were anti-H3K4me2 (07030 from Millipore), H3K4me3 (07-473 from Millipore), and H3K27me3 (07-449 from Millipore). Purified chromatin was quantified using qPCR. The sequences of the oligonucleotides are available on request.

Lentiviral production and infection

Previously described short hairpin against *SMYD2* (Huang *et al.*, 2006) and a non target short hairpin were cloned into the lentiviral pLVTHM vector (Addgene plasmid 12247). Virus production was performed as described (Wiznerowicz and Trono, 2003). After infection, GFP positive cells were selected by FACS sorting.

Vector construction and transfection

Flag-tagged SMYD2 cDNA was cloned into vector pTP6 (Pratt *et al.*, 2000). ES[4] were transfected with linearized pTP6-SMYD2 and pTP6 empty vector and clones were selected with 2ug/ml puromycin and pooled. SMYD2 expression was confirmed by qPCR and western blot.

Zebrafish microinjection of morpholino and SMYD2 mRNA

Antisense morpholino-oligonucleotides (MO) were supplied by Gene Tools LCC (Philomath, OR). The sequence of *smyd2a* splice-blocking MO (smyd2a-MO) is 5' TTATAAGGAGCGCTGACCTGGTAAA 3' and was designed to block *smyd2a* proper splicing by binding to the splice site located between exon 1 and intron 1. The sequence of the standard control MO (Control-MO) was 5' CCTCTTACCTCAGTTACAATTTATA 3' and was

used as an injection control. MOs were diluted in Danieau buffer to a final concentration of 0.35 mM and were injected at 1-2 cell stage of fertilized wild-type zebrafish (AB strain) eggs using microinjector. Flag-tagged SMYD2 cDNA was cloned into pCS2 vector for mRNA synthesis using the mMESSAGE mMACHINE kit (Ambion, AMI344, Life Technologies, USA) following the manufacturer's instructions. Rescue experiments were performed by co-injecting 30 pg of synthesized *SMYD2* mRNA with 0.35mM of smyd2a-MO or Control-MO.

Whole mount in situ hybridization

Antisense ntl RNA probe was synthesized using the DIG RNA Labeling Kit SP6/T7 (Roche). Embryos were collected and fixed at 6 hpf. Whole mount *in situ* hybridization was performed as previously described (Jopling *et al*., 2010).

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