Univerzita Karlova v Praze
1. lékařská fakulta

Autoreferát dizertační práce

Epigenetika v genové regulaci a struktuře chromatinu

Epigenetics in gene regulation and chromatin structure

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1. Souhrn

Metylace histonů hraje důležitou roli téměř ve všech buněčných procesech a udržování její správné hladiny zajišťují histon metyltransferázy a histon demetylázy. Nesprávná regulace hladiny metylace histonů je spojena s narušením regulace genové exprese a následně také s různými vývojovými defekty a nemocemi. V této práci jsme se zaměřili na lyzin demetylázy KDM2A a KDM2B a na jejich KDM2A-SF a KDM2B-SF izoformy postrádající demetylační aktivitu. Lyzin demetylázy KDM2A a KDM2B byly studovány převážně pro svojí demetylační funkci, kterou vykonávají na promotorech obsahujících CpG ostrovy. Jejich alternativní izoformy KDM2A-SF a KDM2B-SF však nebyly doposud detailněji studovány. Hlavním úkolem této práce bylo proto podrobně charakterizovat KDM2A-SF a zaměřit se také na roli, kterou by KDM2A-SF a KDM2B-SF mohli potenciálně hrát v kanonické Wnt signalizaci. Zjistili jsme, že KDM2A-SF mRNA vzniká působením alternativního intronového promotoru a není tak produktem alternativního sestřihu. Ukázali jsme, že KDM2A-SF startovní kodón leží v exonu, který odpovídá KDM2A exonu 14 a určili jsme tak přesnou aminokyselinovou sekvenci KDM2A-SF proteinu. Pomocí knockdown experimentů specifických pro jednotlivé KDM2A izoformy jsme dale ukázali, že KDM2A-SF narozdíl od KDM2A-LF vytváří v jádře distiktní struktury na pericentromerickém heterochromatin a to v závislosi na proteinu HP1α. Tyto transkripčně umlčené pericentromerické oblasti vykazují vysoké hladiny H3K36me2. Jelikož H3K36me2 je substrátem demetylázové activity KDM2A, došli jsme k závěru, že tyto oblasti jsou vázány KDM2A-SF izoformou. Dále jsme ukázali, že KDM2A-SF a KDM2B-SF potlačují kanonickou Wnt signalizaci navzdory tomu, že jim chybí demetylázová doména. Tato práce zdůrazňuje důležitost rozlišovaní mezi různými proteinovými izoformami a jejich rozdílnými funkcemi, které mohou v některých případech být i antagonistické.

2. Abstract

Histone methylation plays an important role in almost all cellular processes and its homeostasis is maintained by histone methyltransferases and histone demethylases. Misregulation of histone methylation levels is associated with gene expression misregulation and consequently also with various developmental defects and diseases. In this thesis we focus on the lysine demethylases KDM2A and KDM2B and on their demethylation deficient isoforms KDM2A-SF and KDM2B-SF. The lysine specific demethylases KDM2A and KDM2B have been predominantly studied for their demethylation function on CpG island-rich gene promoters. However, KDM2A-SF and KDM2B-SF have not been studied in detail. Therefore, the main goal of this thesis was to characterize KDM2A-SF more in detail and to focus on the role that KDM2A/B-SF might potentially play in canonical Wnt signaling pathway. We found that the KDM2A-SF mRNA arises through the action of an alternative intronic promoter and not by alternative splicing. We showed that the KDM2A-SF start codon is located in the exon that corresponds to KDM2A exon 14 and we thus determined the exact amino acid sequence of the KDM2A-SF protein. Furthermore, using an isoform specific knockdown assay we showed that KDM2A-SF, unlike KDM2A-LF, forms distinct nuclear foci on pericentromeric heterochromatin dependingly on the heterochromatin protein HP1α. These transcriptionally silent pericentromeric regions exhibit high levels of H3K36me2. Since H3K36me2 is the substrate of the KDM2A demethylase activity, we concluded that these regions are occupied by the demethylase deficient KDM2A-SF isoform. Further, we demonstrated that KDM2A-SF and KDM2B-SF repress canonical Wnt signaling despite lacking the demethylase domain. This thesis highlights the importance of distinguishing between different protein isoforms and their different functions, which can be sometimes even antagonistic.

1. Introduction

Chromatin is subject to various epigenetic modifications that affect almost all biological processes ranging from embryogenesis to aging (Sharma et al. 2010; Skinner 2011; Calvanese and Fraga 2012; Moosavi and Motevalizadeh Ardekani 2016; Ashapkin et al. 2017). Histone methylation is one of the important epigenetic modifications and its misregulation can lead to various developmental defects and diseases. This epigenetic modification is regulated by histone methyltransferases and histone demethylases. In this thesis we are focused on lysine demethylases KDM2A and KDM2B and their demethylation deficient isoforms KDM2A-SF and KDM2B-SF. KDM2A was originally identified as a H3K36me2 demethylase (Tsukada et al. 2006; Cheng et al. 2014) and KDM2B as H3K36me1/me2 and H3K4me3 demethylase (Frescas et al. 2007; He et al. 2008). These methylations are usually associated with transcriptionally active promotors, which makes KDM2A and KDM2B transcriptional repressors (D'Oto et al. 2016; Vacik et al. 2018). They bind to promoters using their CXXC zinc finger DNA binding domain and demethylate substrate, making it inaccessible for transcriptional machineries. Alternative promoters located in *KDM2A* and *KDM2B* gene introns drive the expression of the alternative mRNAs that lack the N-terminal JmjC enzymatic domain and therefore they are unable to function as demethylases (Inagaki et al. 2015; Lađinović et al. 2017). However, they share all the other functional domains (the CXXC, PHD, F-box, and LRR domain) with the full-length isoforms (KDM2A-LF and KDM2B-LF) (Inagaki et al. 2015; Liu et al. 2016; Lađinović et al. 2017). Recently KDM2A and KDM2B have been shown to repress transcription of Wnt signaling target genes (Lu et al. 2015). Wnt signaling is evolutionary conserved pathway, essential in cellular regulation, cellular differentiation, cell migration, so as in development and organogenesis (Sokol 2011; Steinhart and Angers 2018; Wang et al. 2018). Using their JmjC demethylation domain, they demethylate beta-catenin marking it for ubiquitination and degradation. As a

result of beta-catenin KDM2A/B-dependent degradation, transcription of Wnt signaling target genes is repressed.

2. Thesis goals

1. Characterization of the short isoform of the lysine demethylase KDM2A

Elevated levels of the epigenetic marks H3K4me3 and H3K27Ac are associated with transcriptionally active promoter regions and their presence suggests that the expression of the KDM2A-SF mRNA is driven by the alternative promoter located in an intron. This alternative promoter is located in KDM2A intron 13 in the region where the alternative first KDM2A exon was detected. Characterization of the KDM2A-SF coding region and of the transcription start site, was one of the thesis goals.

2. Characterization of chromatin regions bound by the short KDM2A-SF isoform

The structural differences between the KDM2A-SF and KDM2A-LF protein isoforms are likely to result in the two proteins assuming different conformations and consequently in KDM2A-SF being involved in different protein complexes than KDM2A-LF. Although both KDM2A isoforms should theoretically bind to the same DNA regions through their identical zinc finger CXXC DNA-binding domain, it is possible that KDM2A-SF, as a conformationally different protein, can complex differently from KDM2A-LF, and interacts with DNA regions different from those bound by KDM2A-LF.

3. Defining the function of KDM2A-SF and KDM2B-SF in Wnt signaling pathway

Despite that KDM2A-SF and KDM2B-SF lack JmjC demethylation domain, they contain zinc finger binding domains through which they are likely to bind beta-catenin, one of the key mediators of canonical Wnt signaling, and protect it from demethylation. By doing so, KDM2A-SF and KDM2B-SF should be able to affect Wnt signaling pathway and its target genes.

Considering the above-mentioned possible differences between protein isoforms and the lack of research on the KDM2A demethylation deficient isoform, this thesis was focused on the alternative demethylase-inactive KDM2A isoform, on its characterization and on the chromatin regions it is associated with.

3. Material and metods

Cells: HEK293T, U2OS, HeLa, MCF-7, NIH3T3.

Transfections: Silencer Select siRNAs (Life Technologies), Lipofeectamine 3000, RNAiMAX (Thermo Fisher Scientific 56532), Fugene 6 (Promega E2691).

RNA analysis: TRIzol, Superscript III kit system, GeneElute mRNA Mini prep Kit, NorthernMax kit, Chemiluminiscent Detection Module, T7 RNA polymerase, Biotin RNA labeling mix, Rneasy Min elute Cleanup Kit.

Quantitative PCR and Q-ChIP PCR: CFX96 Touch Real Time PCR Detection System, iQ, SYBR Green Supermix.

Recombinant proteins: pCS2 (+)- Flag expression construct; TNT Sp6 Quick Coupled Transcription Translation system.

Western analysis: anti-KDM2A and anti-FLAG antibodies.

Chromatin immunoprecipitation: Magnify ChIP system, anti-pan-KDM2A, anti-H3K36me2, rabbit control IgG.

Microscopy: Confocal microscopy, Leica TCS SP5 confocal microscope; anti-pan-KDM2A, anti-HP1a, anti-H3K9me3, anti-CENP-A, Fiji software.

Luciferase assay: TCF/LEF luciferase reporter plasmids pNL1.1-TOP5/FOP5 and pGL 4.53 [luc2/PGK]; Nano-Glo Dual-Luciferase Reporter Assay System (Promega; N1610).

4. Results

4.1. KDM2A mRNA isoforms

To confirm that KDM2A-SF is naturally occurring isoform we performed 5'RACE using primers specific for the *KDM2A* exon 13 (Figure 1A) and Northern blot analysis using 3 isoform specific antisense RNA probes. These results confirmed that the *KDM2A-SF* mRNA is not a product of alternative splicing, but that it is an alternative variant of mRNA that originates in the *KDM2A* intron 12 (Figure 1B).

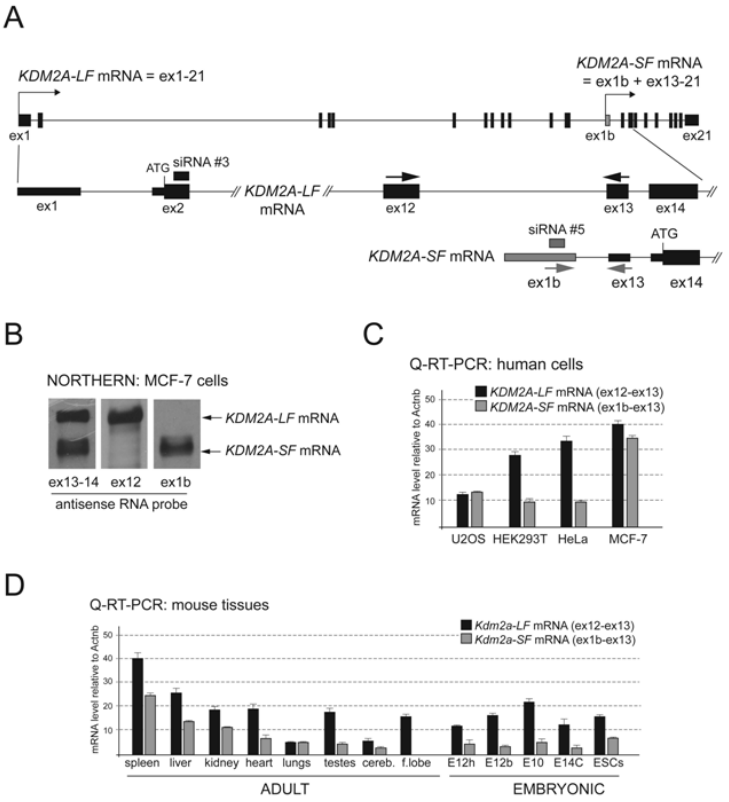

Figure 1. *The KDM2A mRNA isoforms.* ***(A)*** *The KDM2A-SF is encoded by alternative mRNA that originates in KDM2A intron 12* ***(B)*** *The human KDM2A mRNA isoforms detected in the MCF-7 cells by northern blot* ***(C)*** *Q-RT-PCR of the human KDM2A-SF and KDM2A-*

LF mRNAs ***(D)*** *Q-RT-PCR of the mouse KDM2A-SF and KDM2A-LF mRNAs. cereb.: cerebellum, f.lobe: front lobe, E12h: embryonic day 12 head, E12b: embryonic day 12 body, E10: embryonic day 10 whole embryo, E14c: embryonic day 14 cortex, ESCs: ebmryonic stem cells.*

Further, we analyzed the levels of the *KDM2A-LF* and *KDM2A-SF* mRNAs in the U2OS, HEK293T, MCF-7 and HeLa cells. We detected very high levels of the *KDM2A-LF* and *KDM2A-SF* mRNAs in the human breast cancer cells, MCF-7 (Figure 1C). We also analyzed the levels of the mouse KDM2A-LF and KDM2A-SF mRNAs in various adult and embryonic mouse tissues (Figure 1D). The results showed that KDM2A-LF, as well as KDM2A-SF are expressed in most of the tested mouse tissues, as well as during mouse embryonic development. Furthermore, the *KDM2A-LF* and *KDM2A-SF* mRNAs are both expressed even in mouse embryonic stem cells (mESCs) (Figure 1D). These data, together with the data in the USCS genome browser imply that KDM2A-SF is a naturally occurring and evolutionary conserved isoform.

4.2. KDM2A-SF protein isoforms

To analyze KDM2A-SF and KDM2A-LF on the protein level, we designed siRNA to knock down specifically just the *KDM2A-LF* mRNA or just the *KDM2A-SF* mRNA. We confirmed the knockdown down regulation in the MCF-7 cells first by Q-RT-PCR (Figure 2A) and then also by a western blot analysis (Figure 2B). We analyzed the sequence of the *KDM2A-SF* mRNA (RefSeq: NM_001256405.1) for potential start codons and found two potential start codons, one in alternative first exon 1b and one in exon 14. To distinguish between the two potential start sites, we *in vitro* translated two different proteins: one starting with the start codon in *KDM2A* exon 1b (the first *KDM2A-SF* exon) and the other one starting with the start codon in exon 14. We then analyzed these *in vitro* translated proteins by western together with the nuclear protein extract from the MCF-7 cells using the anti-pan-KDM2A antibody that recognizes both the long and the short KDM2A isoform (Figures 2C and 2D).

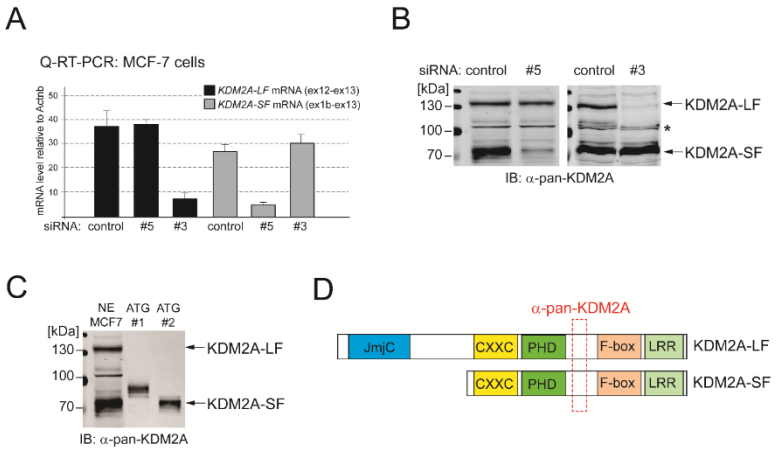

Figure 2. *KDM2A isoform-specific knockdown.* ***(A)*** *Q-RT-PCR of knocked down KDM2A-LF and KDM2A-SF* ***(B)*** *Western blot of downregulated of KDM2A-SF and KDM2A-LF in MCF-7 cells* ***(C)*** *In vitro translation - ATG in KDM2A exon 14 is the start codon for translation of KDM2A-SF* ***(D)*** *The KDM2A-LF and KDM2A-SF protein functional domains.*

This western blot analysis showed that the protein translated from the start codon in *KDM2A* exon 14 corresponds to the endogeneous 75 kDa protein that is strongly downregulated after knocking down the short KDM2A-SF mRNA (Figures 2B and 2C). This conclusion is further supported by a comparison of the two potential start codons with the Kozak consensus sequence. The sequence around the start codon in exon 14 showed a higher similarity to the Kozak consensus sequence then that in exon 1b (Figure 3).

Figure 3. Alignment of the KDM2A-LF and KDM2A-SF start codons with the Kozak consensus sequence. The sequences around the ATGs in *KDM2A* exon1, exon 1b and exon14 were aligned with the Kozak sequence and the bases corresponding to one of the two most common bases in the Kozak sequence are highlighted in pink.

4.3. Nuclear localization of KDM2A

To further characterize these KDM2A structures we performed a series of immunofluorescence experiments. Our results show that the KDM2A foci partially co-localize with HP1α, as well as with H3K9me3 - an epigenetic mark of transcriptionally silent heterochromatin (Figures 4B and 4C). Our immunofluorescent data revealed that KDM2A structures localize next to CENP-A, a histone variant associated with centromeric heterochromatin (Sullivan et al., 2004) (Figure 4D). After knocking down specifically just KDM2A-LF the KDM2A structures remained present (Figure 4F), while the KDM2A structures were not detected after knocking down specifically just KDM2A-SF (Figure 5E). These results show that the KDM2A structures are formed at pericentromeric heterochromatin by KDM2A-SF and not by KDM2A-LF. We hypothesized that KDM2A-SF is forming specific structures on pericentromeric heterochromatin through interaction with HP1α. To confirm this hypothesis, we then analyzed the KDM2A nuclear pattern in the HP1a knockdown cells by immunofluorescence and found out that KDM2A-SF structures do not form in the absence of HP1α. Our results show that KDM2A-SF forms specific foci on pericentromeric heterochromatin in an HP1α dependent manner (Figure 5G).

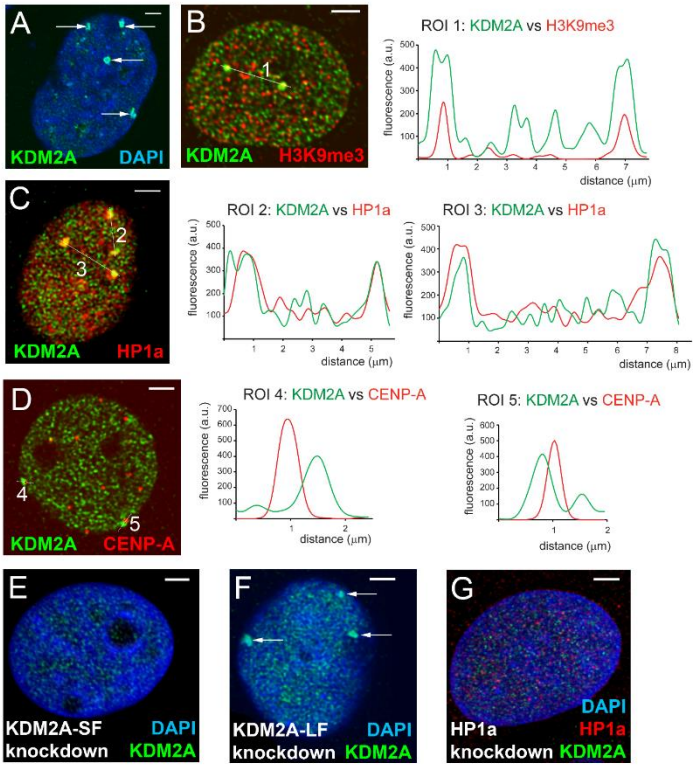

Figure 4. *KDM2A-SF forms distinct heterochromatic structures.* ***(A)*** *KDM2A nuclear bodies detected by the anti-pan-KDM2A antibody* ***(B)*** *KDM2A foci partially co-localize with H3K9me3 and* ***(C)*** *with HP1α and are formed just next to the centromeric heterochromatin marked by CENP-A* ***(D)****.* ***(E)*** *KDM2A bodies are not formed in the absence of KDM2A-SF* ***(F)*** *KDM2A structures remain intact in the MCF-7 cells lacking KDM2A-LF.* ***(G)*** *KDM2A-SF bodies are not formed in the absence of HP1α. Scale bar = 3μm.*

4.4. KDM2A on pericentromeric heterochromatin

To complement our immunofluorescence data (Figure 4), we analyzed the levels of KDM2A on the selected pericentromeric regions using chromatin immunoprecipitation (ChIP). Our ChIP experiments showed that the tested pericentromeric regions

exhibited even higher levels of KDM2A then the ones detected on the several selected CpG island promoters (Figure 5A). Using ChIP we also tested the levels of H3K36me2, and our experiment revealed that the pericentromeric regions exhibit extremely high levels of H3K36me2, higher even than those of the tested KDM2A bound CpG island promoter regions (Figure 5B). These results imply that the KDM2A isoform bound to the tested pericentromeric regions is KDM2A-SF and not the KDM2A-LF, which supports our immunofluorescence data (Figure 4).

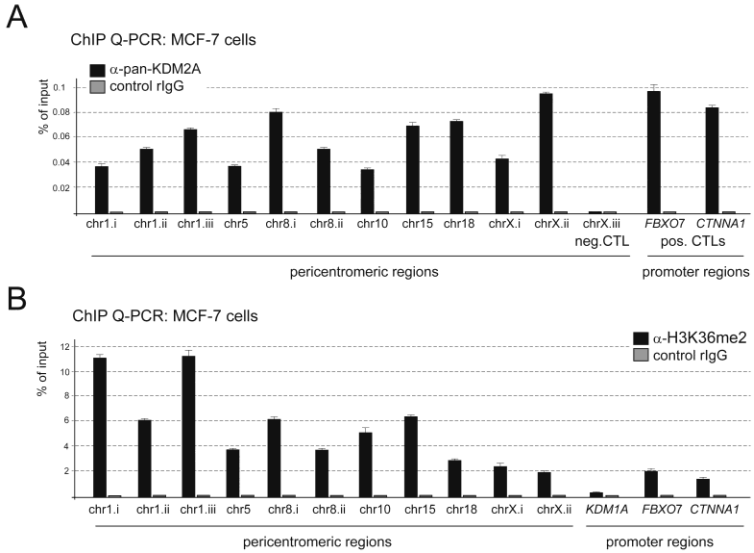

Figure 5. *KDM2A bound regions and their H3K36me2 levels.* ***(A)*** *ChIP Q-PCR of KDM2A on the tested pericentromeric regions. Chromosome X (chrX.iii) region serves as the negative control, whereas the two promotor regions (FBXO7, CTNNA1) serve as the positive controls.* ***(B)*** *ChIP Q-PCR of H3K36me2 ont the tested pericentromeric regions.*

4.5. KDM2A-SF and KDM2B-SF are repressing Wnt signaling target genes

To show activity of KDM2A-SF and KDM2B-SF we specifically knocked down (Figure 6A) or overexpressed (Figure 7) each isoform

and by qRT-PCR measured levels of Wnt signaling target genes. Obtained results are showing that knock down of both KDM2A isoforms is inducing Axin 2 transcription in MCF-7 cells and Cyclin D1 in NIH3T3 cells (Figure 6B).

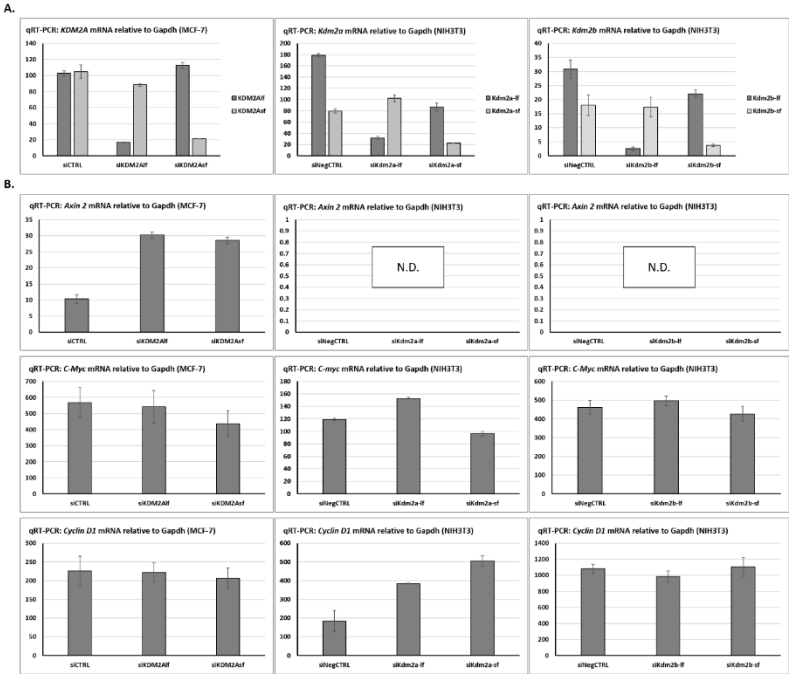

Figure 6. KDM2A and KDM2B knock down affects Wnt target genes transcription. (A) The KDM2A-LF and KDM2B-LF and KDM2A-SF and KDM2B-SF mRNAs were knocked down with isoform specific siRNAs. (B) *Axin2, C-Myc* and *Cyclin D1* mRNA levels in KDM2A-LF/SF or KDM2B-LF/SF knock down cells.

Overexpression results confirm that KDM2A both isoforms and KDM2B-SF are repressing Axin 2 and Cyclin D1 (Figure 7A and 7B). These results were complemented with Luciferase assay analysis where KDM2A-LF, KDM2A-SF and KDM2B-SF repressed stimulated Topflash, the Wnt/beta-catenin responsive reporter (Figure 8A and 8B).

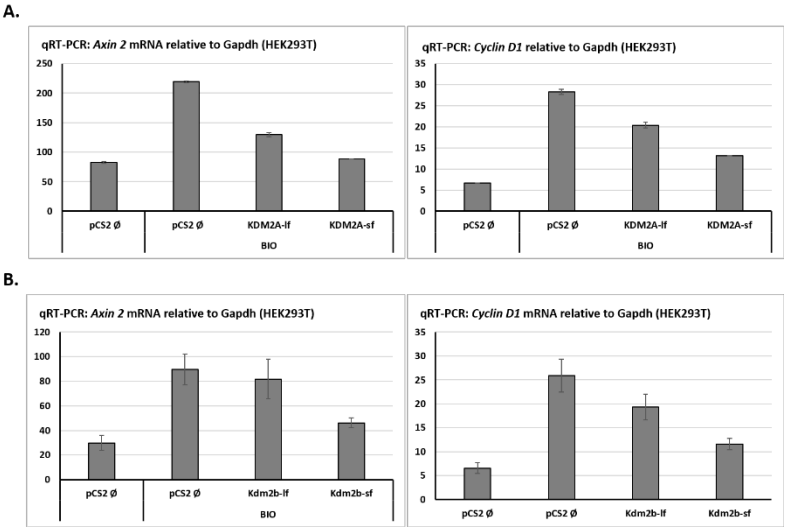

Figure 7. KDM2A-LF/SF and KDM2B-LF/SF overexpression (A) *Axin 2* and *Cyclin D1* mRNA levels in HEK293T cells overexpressed with KDM2A-LF or KDM2A-SF. (B) *Axin 2* and *Cyclin D1* mRNA levels in HEK293T cells overexpressed with Kdm2b-LF or Kdm2b-SF.

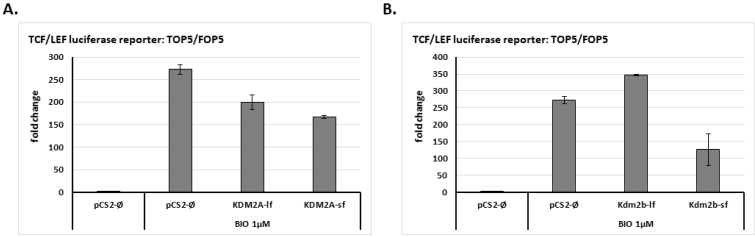

Figure 8. Wnt/beta-catenin responsive *Luciferase assay. (A) Overexpressed KDM2A-LF and KDM2A-SF are repressing luciferase reporter. (B) Overexpressed KDM2B-LF is not repressing luciferase reporter, while KDM2B-SF does.*

5. Conclusions

KDM2A is a lysine demethylase that binds directly to CpG islands through its CXXC zinc-finger DNA binding domain and demethylates H3K36 using its Jumonji C demethylation domain.

The same gene locus encodes a shorter KDM2A isoform that contains the DNA binding CXXC zinc finger, protein binding PHD zinc finger, F-box, and six leucine-rich repeats domain, but lacks the JmjC demethylation domain. One of the goals of this thesis was to characterize this demethylation deficient KDM2A isoform more in detail and to focus on the chromatin regions it is associated with. KDM2A-SF is produced by the action of an alternative intronic promotor characterized by elevated levels of the epigenetic marks H3K4me3 and H3K27Ac. This promotor and the alternative first exon of KDM2A are located in KDM2A intron 12. As opposed to the starting hypothesis, we showed that the first coding exon of KDM2A-SF is its third exon, which corresponds to KDM2A exon 14 and that the alternative first exon 1b is not coding. Despite lacking the Jumonji C demethylase domain KDM2A-SF retains its ability to bind CpG islands through its CXXC zinc finger DNA binding domain, which makes it a competitor of KDM2A-LF for binding to the same CpG islands. Considering that H3K36me2 is mostly associated with active gene promoters and its demethylation leads to transcriptional repression, KDM2A-SF could function as an activator by preventing KDM2A-LF from binding to and demethylating a CpG island containing promoter. However, KDM2A-LF and KDM2A-SF have been found to directly interact with HP1α which is involved in transcriptional silencing of pericentromeric heterochromatin. Even though pericentromeric heterochromatin is not transcriptionally active, it has been found that KDM2A bound pericentromeric regions exhibit high levels of H3K36me2 (Lađinović, D. et al., 2017). Considering that H3K36me2 is the KDM2A-LF demethylation substrate, these results suggests that pericentromeric regions are occupied by KDM2A-SF which lacks the demethylation function and not by KDM2A-LF. Since we failed to detect any transcriptional activity of the KDM2A-bound pericentromeric regions after knocking down both KDM2A isoforms, it can be hypothesized that H3K36me2 does not have a role in transcriptional regulation of these regions. It is possible, however, that H3K36me2 functions as a repression mark in pericentromeric heterochromatin and therefore it should not be removed by the demethylase domain of KDM2A-LF. But, because

some of the other KDM2A domains are necessary here in pericentromeric chromatin for currently unknown reason (e.g. structural integrity, transcriptional co-repressors), KDM2A-SF binds here. The exact function and mechanism of KDM2A-SF in the formation of pericentromeric heterochromatin remains poorly defined and, in the future, it should be studied more in detail. In this research we also discovered that KDM2A-SF is a naturally occurring isoform that is expressed in various adult mouse tissues, in developing mouse embryo and in mouse ES cells. Based on the publicly available GenBank EST and RNA-seq data, KDM2A-SF is likely to be expressed also in healthy human tissues including human ES cells. Another goal of this thesis was to show how despite KDM2A-SF and KDM2B-SF lack demethylation domain, they still have impact on various metabolic processes. We showed that KDM2A-SF and KDM2B-SF are repressing Wnt signaling target genes Axin 2 and Cyclin D1, although their mechanism of action is still unknown. This research highlighted the possible functional difference between KDM2A/B-LF and KDM2A/B-SF and opened new questions about KDM2A/B-SF, their functions and the role in pericentromeric heterochromatin, as well as its involvement in transcriptional regulation and signaling pathways.

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