8-2015

NEW INSIGHTS ON THE ROLE OF JMJD2A IN CANCER PROGRESSION

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A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
MD Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

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Houston, Texas

August 2015

Dedication

I dedicate my dissertation to my most beloved parents. Thank you for believing in me and loving me.

Acknowledgements

This dissertation would not have been possible without the help and support of so many people in so many ways. First and foremost, I would like to thank my dearest parents for their unconditional love for me. They have supported me in all my pursuits and have always believed in me. Their positive encouragement and relentless love have shaped me into the person I am today. I understand all the sacrifices that they have made to give me the best, to put my needs in front of theirs, and for that, I cannot imagine completing this major stage in my life without my parents. Mom and Dad, I only hope to spend the rest of my life loving you guys as much as you love me.

For all the achievements I have made in my Ph.D pursuit, I owe it all to my advisor Dr. Hui-Kuan Lin. It has been an honor for me to study in his lab. During the past six years, he has set up a great role model for me as a successful and motivational scientist, and he is my constant inspiration to do better. I appreciate all his guidance, encouragement and support. Thank you Dr. Lin, you have made my Ph.D. experience educational, exciting and unforgettable, and because of that, I believe I am ready to take on any challenges I will face later in life.

I would also like to extend my gratitude to my committee members, Dr. Xin Lin, Dr. Angabin Matin, Dr. Min Gyu Lee, and Dr. Shiaw-Yih Lin. They have also contributed tremendously to my Ph.D. study by attending my committee meetings, providing constructive comments to improve my thesis and supporting my career choice.

I also would like to extend my gratitude to our collaborator Dr. Yuan-Soon Ho from Taipei Medical University, Taiwan for his help on generating the FRET, FLIM and Split-luciferase activity results, which further solidified our findings.

Of course, I cannot forget my lab members. They have made my life in lab so much easier and entertaining. It has been such a privilege for me to work together with such an intelligent, kind and amazing group of people. The friendship we have built over the years means everything to me. I cannot express how much I will miss our Friday girls' night out, BBQ, dinner parties and our common obsession in PAD. To the postdocs, Guoxiang Jin, Zhen Cai, Chi-Yun Wang, Ben Chou and Hossein Rezaeian, thank you all for providing guidance for me and answering my questions when I needed help desperately. To my fellow classmates, Fei Han, Xian Zhang, thank you for being my best friends, for listening to my complaints, for sharing all the good and bad memories that we had in graduate school. To all the visiting scientists and students Jie Long, Xiaohong Xu, Peter Pan, Chunfang Liu, Anmei Zhang, and Chuan Xu, thank you for being my "personal" physicians and helping me with my experiments in lab. I would also like to thank our previous lab members Chia-Hsin Chan, Szu-Wei Lee, Wei-Lei Yang, and YunSeong Jeong. They were the ones who taught me all the lab techniques when I was just a clueless rotation student in Dr. Lin's lab. They also showed me how to be a resilient scientist and how to be a stronger person.

Another person I must mention is my dearest Lao Lao (grandma). She has been there to take care of me since the day I was born, and has contributed so

much to my personal growth. Also, I would like to thank my grandpa. Although he is no longer with us, but he was the first one to instill the importance of education and career in me, and the first one to tell me that I should always believe in myself, no matter how rough the time is. I know he would have been very proud of all my achievements.

Last but not least, I would like to thank my boyfriend Brian Lee. He has been nothing but supportive, patient and caring throughout this long and difficult journey. Thank you for everything!

NEW INSIGHTS ON THE ROLE OF JMJD2A IN CANCER PROGRESSION

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Changes in chromatin architecture are known to be one of the underlying causes of cancer because of its ability to alter gene transcription. Histone methylation is one of the most intricate epigenetic marks because it adds multiple layers of modification on the targeted sites. Therefore, many studies have brought histone methylases and demethylases into focus, hoping to decipher their roles in cancer progression. Among these enzymes, JMJD2A is the first to shown to have demethylation activity against trimethylation, and to regulate gene expression, development, and cancer progression. While many studies have mainly focused on its role in transcriptional regulation, only recently, its non-enzymatic function has started to unveil, but the studies are scarce and inconclusive.

In our study, we showed that JMJD2A is essential in mediating activation of the canonical Wnt/β-catenin pathway, a highly conserved and complex signaling cascade that ultimately leads to nuclear accumulation of β-catenin. Nuclear β-catenin serves as a transcriptional coactivator by forming a transcriptional complex with TCF to activate Wnt target gene expressions. The presence of JMJD2A keeps β-catenin from interacting with the destruction

complex components, which functions in subsequent phosphorylation and proteasomal degradation of β-catenin. JMJD2A, through maintaining the protein stability of β-catenin, affects the transcriptional activity of β-catenin and the expression of its target genes, and this is independent of the demethylase activity of JMJD2A. Surprisingly, in resting cells without Wnt ligand stimulation, JMJD2A, a supposedly nucleus-localized histone modifying protein, is mainly resided in the cytosol, and its nuclear translocation can be enhanced by active Wnt signaling.

Moreover, JMJD2A interacts with β-catenin endogenously and is required for the nuclear translocation of β-catenin upon Wnt ligand stimulation. Finally, we showed that JMJD2A has indispensable roles in cell proliferation, tumorigenesis, metastasis, and cancer stem cell traits through stabilizing β-catenin protein. Our study highlights a non-histone and non-enzymatic function of JMJD2A in the Wnt signaling pathway, and also provides cues to design inhibitors that target not only the enzyme activity but also the protein-protein interactive ability of JMJD2A.

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ALDH	Aldehyde dehydrogenase
APC	Adenomatous Polyposis Coli
AR	Androgen Receptor
β-TrCP	β-transducing repeats-containing proteins
CHX	Cycloheximide
CK1	Casein Kinase 1
Co-IP	Co-immunoprecipitation
DMEM	Dulbecco's Modified Eagle's Medium
DPC	Days Post Coitum
Dvl	Disheveled
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EMT	Epithelial to Mesenchymal Transition
ER	Endoplasmic Reticulum
FBS	Fetal bovine serum
FRET	Fluorescence Resonance Energy Transfer
Fz	Frizzled
GSK3β	Glycogen Synthase Kinase 3β
HBSS	Hank's Balanced Salt Solution
KDM4A	Lysine (K)-specific demethylase 4A
LEF	Lymphoid Enhancing Factor
MEF	Mouse embryonic fibroblast

mRNA	messenger RNA
MSCV	Murine Stem Cell Virus
NLS	Nuclear Localization Signal
PCR	Polymerase chain reaction
PHD	Plant Homeodomain
shRNA	Short hairpin RNA
TCF	T-cell Factor
WNT	Wingless-type MMTV integration site family
WT	Wild-type

Chapter 1

Introduction

1.1 Histone Demethylases: JMJD2 family

Changes in chromatin structure are closely linked to tumor since they largely affect gene expression, which suggests that abnormal epigenetic regulation may be an underlying cause for cancer. Chromatin modulation can be regulated through several mechanisms, with histone posttranslational modification being one of them. These modifications (acetylation, methylation, phosphorylation, ADP-ribosylation, ubiquitination, SUMOlyation) occur on histone tails to either promote or repress gene transcription, depending on their effects on chromatin architecture and compaction (Berger, 2007; Luger and Hansen, 2005).
Unlike other modifications, histone methylation was long thought to be permanent until the identification of histone demethylases, which make this mark reversible and dynamic (Byvoet et al., 1972; Shi et al., 2004; Thomas et al., 1972). Different from the other posttranslational modifications, methylation has multiple layers and states of complexity, and each layer is capable of employing different effect on overall chromatin biology (Lachner et al., 2001; Rea et al., 2000). Although more labile than previously assumed, histone methylation pattern must be correctly established for correct embryonic development and prevention of diseases such as cancer. This buttresses the idea that histone modifiers such as methylases and demethylases serve pivotal roles in maintaining normal biological processes and must be tightly regulated (Dawson and Kouzarides, 2012).

Up to date, two evolutionarily conserved classes of histone demethylases, LSD and JMJC, have been identified. Compared to LSD, which was the first

direct histone demethylase class to be discovered, JMJC class is much bigger and utilizes a different demethylation mechanism from LSD1 (Black et al., 2012; Shi et al., 2004). The name JMJC originated from the fact that these enzymes all contain the Jumonji C catalytic domain (JmjC). The JMJC demethylase class can further be defined into seven evolutionarily conserved groups based on the homology of their JmjC domains (Klose et al., 2006). Among these groups, the JMJD2 family has drawn much attention due to their substrate specificity and prominent role in cancer. The JMJD2 family contains six members of demethylase, JMJD2A, JMJD2B, JMJD2C, JMJD2D, JMJD2E and JMJD2F (Figure 1-1) (Katoh, 2004). While JMJD2A, JMJD2B and JMJD2C are structurally similar and share the same target specificity, JMJD2B shows much lower catalytic activity than the other members of demethylase, and its knockout mice did not display any phenotypes. Interestingly, JMJD2A-C proteins seem to share similar physiologic functions in cancer, which suggests that the JMJD2 family may execute overlapping roles and may compensate each other for their functions (Kawazu et al., 2011). Unlike JMJD2A-C, JMJD2D lacks both the PHD and Tudor domain. It also has different substrate specificity. Although JMJD2D seems to have some functions in cancer, the *Jmjd2d* knockout mice have no obvious phenotypes as well (Krishnan and Trievel, 2013). The remaining two members of JMJD2 families, JMJD2E and JMJD2F do not possess promoters and are intronless, thus, are considered pseudogenes. So far, it remains unclear whether they are functional genes (Whetstine et al., 2006).

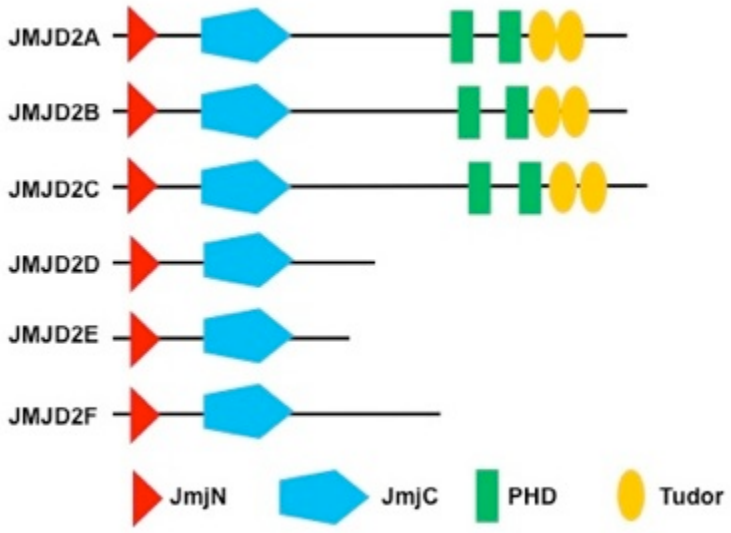


Figure 1-1: Schematic representation of the JMJD2 family demethylases.

The JMJD2 family demethylase architectures all contain one JmjN domain, which interacts and stabilizes the catalytic domain JmjC. JmjC is the most evolutionarily conserved domain among all the other ones. The PHD and Tudor domains are only present in the JMJD2A-C demethylases, which are presumably important for substrate site recognition and binding.

1.2 The protein structure of JMJD2A

Compared to the other members of the family, JMJD2A, also known as KDM4A or JHDM3A (JmjC domain-containing histone demethylase 3A), is more well-recognized and studied. It is evolutionarily conserved from *Caenorhabditis elegans (C. elegans)* to human (Clissold and Ponting, 2001). JMJD2A is composed of several domains to make up for its structure (Figure 1-2A). JMJD2A has a Jumonji C (JmjC) and a Jumonji N (JmjN) domain, in which JmjC shows the strictest sequence conservation, while JmjN is less conserved. In addition, JMJD2A is also endowed with a double plant homeodomain (PHD) and a double Tudor domain, whereas they exhibit minimal conservation. Intriguingly, the degree of the sequence conservation presumably correlates with their importance for the demethylation activity of JMJD2A. The JmjC domain, shown by the crystal structure, is folded into eight β-sheets, forming an enzymatically active pocket, which directly participates in the demethylation reaction. The JmjN domain forms extensive interactions with and provide structure integrity and stability for JmjC domain (Chen et al., 2006; Shi and Whetstine, 2007). The PHD and Tudor domains are believed to be mainly responsible for binding to methylated marks for site recognition. However, more studies are needed to understand the functional significance of these two domains since JMJD2D, while lacking these domains, can still exert its demethylation function on the appropriate histone sites. Thus, there must be other mechanisms in which these demethylases recognize and bind to their substrates.

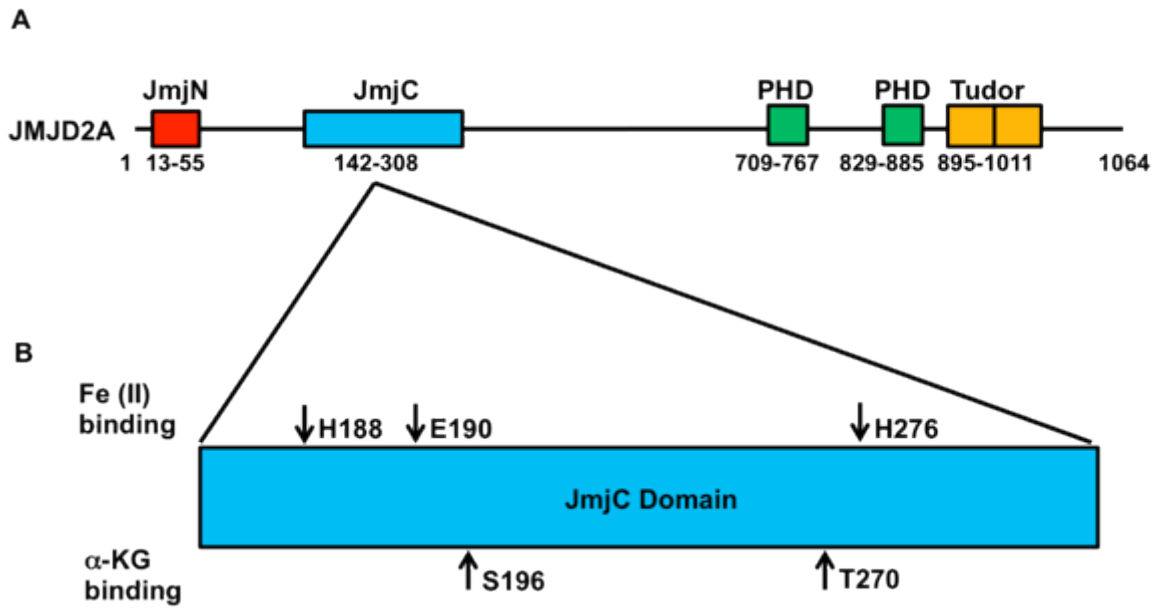


Figure 1-2: Functional domains of JMJD2A

(A) A schematic representation of the structure ofJMJD2A. Its architecture is consisted of one JmjN and one JmjC domain, which are important for its catalytic activity. The two PHD domains and hybrid Tudor domains are important for substrate site recognition.

(B) A schematic representation of the JmjC domain, showing the position and amino acid residues that are important for Fe (II) binding (top) and α–KG binding (bottom)

1.3 The function of JMJD2A and its substrate specificity

The function of JMJD2A is to mainly demethylate histone 3 lysine 9 (K9) and lysine 36 (K36), albeit its ability to demethylate H3K9me_3 is about 5-fold more efficient than H3K36me_3. In particular, H3K9me_3 is linked to heterochromatic regions of the chromatin and usually leads to transcription repression. Meanwhile, its other substrate site H3K36me_3 inhibits gene transcription at the start site by preventing unwanted transcription initiation within the body of the gene. JMJD2A overexpression, thus, relieves chromatin from its compact structure, increases chromatin accessibility, and promotes gene transcription and aberrant transcription initiation (Loh et al., 2007; Wagner and Carpenter, 2012). The demethylation function of JMJD2A acts through a five-step dioxygenase reaction mechanism that requires Fe (II) in the catalytic center and α-ketoglutarate to demethylate its targets (Hausinger, 2004). Within the JmjC catalytic domain, three amino acids bind to Fe (II), while two amino acids bind to α-ketoglutarate (Figure 1-2B). To catalyze the reaction, the cofactor-bound JmjC will produce a very reactive specie oxoferryl species that could hydroxylate the methylated site, allowing the lost methyl group to become formaldehyde (Clifton et al., 2006; Klose et al., 2006). More specifically, its demethylase activity against trimethylation is much higher compared to dimethylation, and essentially has no activity against monomethylated residues. The proposed reason, as revealed by X-ray crystallographic analysis, is that the space and the electrostatic environment in the methyl group-binding pocket of JMJD2A only allows tri- and di- but not monomethylated lysine to position a methyl group efficiently toward

the iron-containing catalytic center (Chen et al., 2007; Chen et al., 2006; Couture et al., 2007; Ng et al., 2007).

1.4 JMJD2A and its role in cancer progression

Only recently, the role of JMJD2A in different types of cancer has started to emerge (Table 1-1). A major emphasis on the role of JMJD2A has been transcription regulation. When JMJD2A is expressed aberrantly, the downstream effects may either to activate oncogene or repress tumor suppressor expressions. One of the first discoveries that linked JMJD2A to cancer is its overexpression in prostate cancer. In this study, JMJD2A can form a complex with androgen receptor (AR) through its C-terminal ligand binding domain upon ligand binding. JMJD2A is considered a coactivator of AR to stimulate its transcriptional activity, such as activating prostate-specific antigen (PSA), an AR target gene and pivotal marker in screening prostate cancer (Shin and Janknecht, 2007). More importantly, in this event, the catalytic activity of JMJD2A is essential. Similarly, in breast cancer, JMJDA can also interact with estrogen receptor (ER) to enhance its transcriptional activity. Hence, deletion of JMJD2A in ER-positive cells leads to decreased ER target genes such as c-Jun and cyclinD1, which in turn causes reduced cell proliferation. Interestingly, in endometrial cancer, JMJD2A also promotes cell migration and invasion by acting as a transcriptional cofactor by forming complexes with ER to promote its activity (Wang et al., 2014). In addition to activating these hormonal receptors, as seen in squamous cell carcinoma and lymph node metastases, JMJD2A also enhances the transcription factor activating protein 1 (AP-1), which is essential

for metastatic and tumorigenic potentials. Even though AP-1 can be transcriptionally activated by JUN and FOS, but through a positive feedback loop, JUN and FOS can also be activated by recruiting AP-1 to their own promoters. However, H3K9me$_3$, being the transcriptional repressive mark, inhibits AP-1 recruitment, and JMJD2A serves the opposite by relieving the region from repressive histone mark to allow AP-1 recruitment. To further expand its transcriptional role, in colon cancer, JMJD2A interacts with p53 on the promoter of p21, and serves as a transcriptional repressor for p53-mediated p21 transcription. The depletion of JMJD2A leads to increases in p53, p21, proapoptotic protein PUMA to induce apoptosis in colon cancer cells. JMJD2A is also overexpressed in human lung cancer cell lines. In one study, JMJD2A transcriptionally represses chromodomain helicase DNA binding protein 5 (CHD5), which is involved in the p53-dependent senescence. This repression of the CHD5 tumor suppressor leads to transformation of primary cells in lung adenocarcinoma (Mallette and Richard, 2012). Another study suggested that JMJD2A may have additional target genes in lung cancer cell line, through demethylating H3K9me$_3$ to promote their transcription and expression. These target genes include ADAM12, CXCL5 and JAG1, which are cancer related genes that are known to enhance cell proliferation, tumor growth, angiogenesis in various cancer signaling pathways such as Notch (Kogure et al., 2013; Simon et al., 2012).

Table1-1. The role of JMJD2A in different cancer types.

Cancer type	JMJD2A expression	Associated with	References
Prostate	Overexpression	AR, PSA	(Shin and Janknecht, 2007)
Colorectal	Overexpression	p53, p21	(Kim et al., 2012)
Lung	Overexpression	CHD, Ras, JAG1, ADAM12, CXL5	(Kogure et al., 2013; Mallette and Richard, 2012)
Squamous cell carcinoma, lymph node metastasis	Overexpression	FOS1, JUN, AP-1	(Ding et al., 2013)
Breast	Overexpression	ER, c-Jun, Cyclin D1, SP1	(Berry et al., 2012; Li et al., 2014)
Gastric	Overexpression	miR-34a	(Hu et al., 2014)
Endometrial	Overexpression	ERα	(Wang et al., 2014)

Table is adopted from review article (Guerra-Calderas et al., 2014), with additional information supplemented from the listed references.

1.5 The implications of JMJD2A in development

The history of Jumonji family proteins originated from studies in examining mouse development. The name “Jumonji” means “cruciform” in Japanese, and the name was given because Jumonji mutant mice developed abnormal grooves on their neural plates, and this phenotype resembled a cross (Takeuchi et al., 1995). Thus, it is not surprising to see that JMJD2A also has roles in body development. One study demonstrates that JMJD2A induces myogenesis. It is responsible for activating myogenin (*Myog*), a skeletal system gene that is responsible for skeletal muscle differentiation from myoblasts into myotubes, by removing the H3K9me$_3$ methylation on the promoter of *Myog* (Verrier et al., 2011). Another study linked JMJD2A to embryonic development and neural crest specification. By removing H3K9me$_3$ and H3K36me$_3$, JMJD2A, expressed in the forming neural folds, controls the expression of developmental genes such as Snail and Sox10 at the appropriate developmental time (Strobl-Mazzulla et al., 2010). Moreover, mice with homozygous deletion of JMJD2A exhibit embryonic lethality due to a spectrum of heart developmental defects and heart failure phenotypes. This genetic evidence further supports the role of JMJD2A in embryonic development and also in cardiac development (Takeuchi et al., 2006; Zhang et al., 2011). Finally, by using the *C. elegans* model, depletion of JMJD2A increases germ cell apoptosis, and it is also responsible for maintaining low levels of H3K36me$_3$ on the X chromosome for low gene expression during germline development (Whetstine et al., 2006).

1.6 The functional relevance of the enzymatic domain in JMJD2A

One of the unresolved questions in the field regarding JMJD2A is whether its enzyme activity is absolutely required for its oncogenic role in cancer. To date, very few studies have focused on the non-enzymatic function for JMJD2A. Among them, one study showed that JMJD2A, independent of its enzymatic activity, can impair DNA damage response and induce genomic instability. In this study, the Tudor domain of JMJD2A and p53-binding protein 1 (53BP1), which is a DNA damage factor, compete for binding at dimethylated histone H4 lysine 20 ($H4K20me_2$). Upon DNA damage response, JMJD2A undergoes K48-linked ubiquitination and proteasomal degradation induced by E3 ligases RNF8/RNF168 to allow the recruitment of 53BP1 to DNA damage foci for repair. Nevertheless, overexpression of JMJD2A can suppress the recruitment of 53BP1 to DNA damaged sites for efficient DNA repair (Mallette et al., 2012b). Furthermore, in *Drosophila melanogaster*, genes repressed or activated by JMJD2A were observed to not rely on its catalytic activity, suggesting that JMJD2A may have a role on gene expression independent of its enzymatic and transcriptional activity (Crona et al., 2013). Other studies show that in the ER-negative breast cancer cells, depletion of JMJD2A still exhibits reduced cell growth and migration *in vitro*, and overexpression of JMJD2A is observed in about 60% of breast tumors, regardless of the ER status. This means that other than serving as a coactivator to stimulate the ER activity, there must be other ways for JMJD2A to induce breast tumor in the ER-negative breast cancer cells (Berry et al., 2012; Li et al., 2011). However, the mechanisms remain to be

uncovered. One study may have provided some clue that in MDA-MB-231 triple negative breast cancer cells, JMJD2A negatively regulates the transcriptional activity of Sp1, a tumor suppressor that predicts late stage breast cancer. Intriguingly, this inhibition is not affected by the enzyme-dead mutant H188A, but is significantly affected by JMJD2A protein-interacting truncation mutant (Li et al., 2014). This finding provides some solid evidence that protein-protein interactive activity may be one of the mechanisms important for the oncogenic function of JMJD2A. Therefore, more effort should be dedicated to studying the non-enzymatic function of JMJD2A.

1.7 The history of Wnt and its biogenesis

The *Wnt1* gene, also known as *int-1*, was initially identified in 1982, and encodes a secreted cysteine-rich glycolipoprotein. The highly conserved Int-1 homolog was also extensively studied in Drosophila and designated the name *Wingless* “Wg”. The combination of *int-1* and *Wingless* led to the name Wnt. Wnt was found as an oncogene that could be activated by a proviral insertion of the Mouse Mammary Tumor Virus (MMTV) at the *Wnt1* locus in murine breast cancer, and *Wnt1* transgenic mice also developed mammary tumors. Hence, these pioneering studies linked WNT1 to mammary tumorigenesis and established its role in cancer (Nusse and Varmus, 1982; Nusslein-Volhard and Wieschaus, 1980). Since these findings, 19 Wnt genes were identified in the human genome, and they all have crucial roles in cell proliferation, survival, migration, cell polarity, cell fate determination during embryonic development and tissue homeostasis (Logan and Nusse, 2004; Willert et al., 2003).

Wnt proteins are cysteine-rich of roughly around 40 kDa in size that harbor an N-terminal signal peptide for secretion (Tanaka et al., 2002). Wnt proteins are lipid modified, and this is important for stimulating efficient signaling and may also be essential for Wnt secretion. The biogenesis of Wnt proteins starts in the endoplasmic reticulum (ER), where it gets palmitoylated by Porcupine, a multipass transmembrane ER protein that contains an O-acyl transferase domain (Banziger et al., 2006; Hofmann, 2000). The lipid modified Wnt proteins are then transported by Wntless (Wls) from the Golgi to the plasma membrane for secretion and signaling facilitation (Banziger et al., 2006; Hofmann, 2000; Kadowaki et al., 1996).

1.8 The canonical Wnt signaling pathway

Once matured and secreted, Wnt proteins can act as stimulant to activate the Wnt signaling pathway. The Wnt signaling pathway is mainly divided into two: the canonical and non-canonical pathway. The canonical pathway involves β-catenin (CTNNB1), and is also called the Wnt/β-catenin pathway (Figure 1-3). The non-canonical pathway is independent of β-catenin and is related to cell polarity. Although the two pathways have been well characterized, the precise mechanism by which Wnt stimulates one pathway versus the other is currently unclear. Some studies suggest that this pathway preference may depend on specific Wnt receptors (van Amerongen et al., 2008). For the topic of this thesis, we mainly focus on the canonical Wnt signaling pathway.

The hallmark of the canonical Wnt signaling pathway is the nuclear accumulation of β-catenin, an important coactivator that binds to TCF

transcriptional factor to promote gene transcription, which is the ultimate outcome of the activated Wnt signal. This nuclear localization of β-catenin contributes to cancerous phenotypes (Valenta et al., 2012). In the absence of Wnt protein stimulation, the surface receptor Frizzled (Fz) and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) are not engaged (He et al., 2004). The destruction complex, which is consisted of APC, GSK3β, Axin1 and CK1, is intact, and holds β-catenin within the formation of this complex. CK1 phosphorylates the Axin1-bound β-catenin first at serine 45 (Ser45), which primes it for sequential phosphorylation by GSK3β at threonine 41 (Thr41), serine 37 (Ser37) and serine 33 (Ser33). β-catenin phosphorylation at Ser33 and Ser37 forms a "degron" motif that can be recognized by the F-box/WD repeat E3 ubiquitin ligase β-TrCP. Consequently, β-TrCP targets the phosphorylated β-catenin for K48-linked ubiquitination and proteasomal degradation (Kimelman and Xu, 2006). The degraded β-catenin can no longer translocate to the nucleus to serve as a coactivator for transcription factor TCF. This causes TCF to interact with corepressor TLE1, which recruits histone deacetylases (HDACs) to promote chromatin compaction and consequently turns off gene transcription (Cavallo et al., 1998; Roose et al., 1998).

On the other hand, once Wnt protein stimulates the signaling pathway, it forms a complex with Fz and LRP5/6. The Fz intracellular domain recruits Dvl to the plasma membrane, which directly results in the relocation of Axin1 and GSK3β to the plasma membrane as well. GSK3β, then, is in the proximity to phosphorylate LRP5/6, which is a key step in this cascade. The phosphorylated

receptor serves as a docking site for Axin1 to recruit more GSK3β for phosphorylation (Davidson et al., 2005; Mao et al., 2001; Tamai et al., 2004; Zeng et al., 2005). This cellular membrane translocation of the destruction complex releases β-catenin from CK1 and GSK3β phosphorylation, thus is in the stabilized form to translocate and accumulate in the nucleus. In the nucleus, β-catenin binds to TCF to displace TLE1, and recruits other coactivator to eventually turn on gene transcription (MacDonald et al., 2009).

Although nuclear accumulation of β-catenin by Wnt pathway activation has long been demonstrated by many studies, but the exact nuclear route and molecular mechanism that β-catenin takes for nuclear translocation remains unclear. Some studies suggest that post-translational modifications on β-catenin are nuclear localization drivers while other studies suggest that binding partners are essential (Jamieson et al., 2014). Thus, the β-catenin nuclear and cytoplasmic distribution and dynamic most likely involves multiple complex mechanisms that require further research.

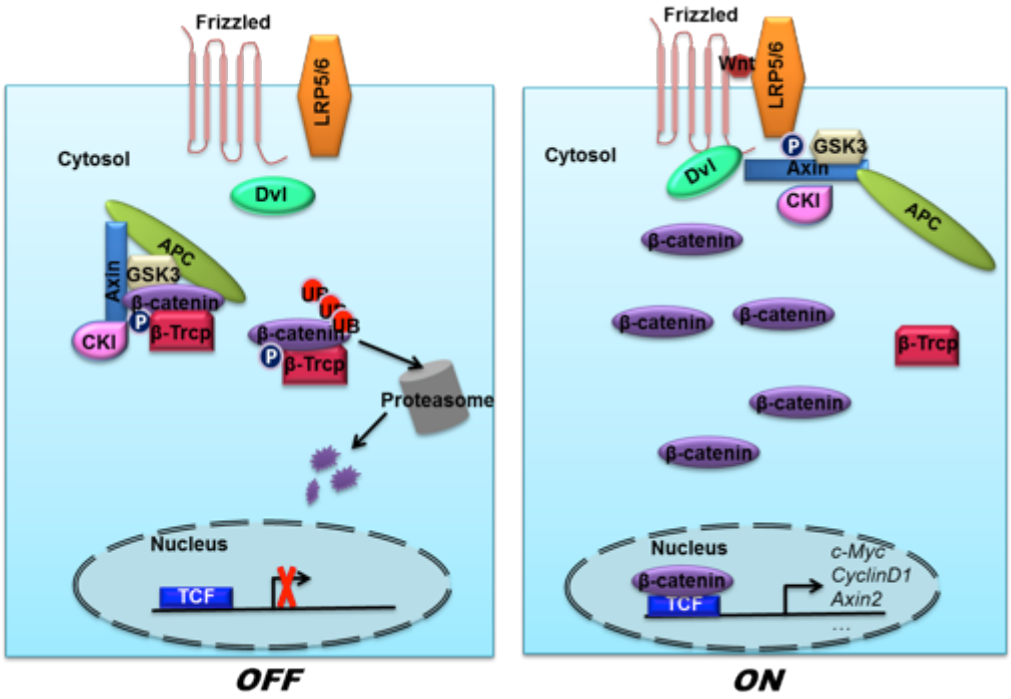


Figure 1-3. The canonical pathway of Wnt signaling.

Left: Without Wnt ligand activation, β-catenin is held in the destruction complex and is phosphorylated by CKI and GSK3β. E3 ubiquitin ligase β-TrCP recognizes the phosphorylated β-catenin and consequently targets it for proteasomal degradation. Degraded β-catenin can no longer be translocated to the nucleus to turn on gene transcription of its target genes; right: with Wnt ligand activation, it binds to Fz receptor and LRP5/6 coreceptors, which recruits Dvl, inactivates the destruction complex, and releases β-catenin from protein degradation. β-catenin accumulates in the nucleus and activates target gene transcription.

1.9 The Wnt signaling in cancer

Since the initial finding of the oncogenic function of WNT1 in mouse models, much effort was invested in understanding the compound role of the Wnt signaling pathway in cancer (Table 1-2). Just like any other cancer signaling pathway, the suppressing components are usually mutated or experience loss of function under oncogenic condition, while the activating components are constitutively active (Polakis, 2012). The most well studied negative regulator of the Wnt signaling pathway is APC, and it is also one of the most frequently mutated genes in human cancers. The truncating mutation of APC can no longer bind to Axin1, which causes the disassembly of the destruction complex, causing β-catenin to translocate the nucleus to activate gene transcription. Genetic defects of APC are the origins of familial adenomatous polyposis, a condition that could ultimately lead to colorectal cancer in those who inherit it (Kinzler and Vogelstein, 1996; Korinek et al., 1997; Morin et al., 1997; Salahshor and Woodgett, 2005; Segditsas and Tomlinson, 2006). Likewise, Axin1, also a tumor suppressor, is susceptible to deletions and truncations in hepatocellular carcinoma and colorectal cancer, and these mutations prevent Axin1 from acting as a scaffold protein to eventually lead to disruption in the destruction complex and inability to degrade β-catenin (Salahshor and Woodgett, 2005;(Satoh et al., 2000). Another group recently uncovered that an in-frame splice deletion has a negative effect on the kinase domain of GSK3β in chronic mylogenous leukemia (Abrahamsson et al., 2009). This causes failure of GSK3β to phosphorylate β-catenin, leading to its nuclear translocation. At the level of cellular membrane,

Wnt signaling pathway receptor LRP5 is also subjected to oncogenic mutations. The mutation deletes the extracellular domain and transforms LRP5 into its constitutively active form, where it binds and destabilizes Axin1 (Mao et al., 2001). This, once again, releases β-catenin from its destruction complex for nuclear translocation. In addition to mutations of the Wnt signaling components, overexpression of WNT ligands also has a crucial role in the proliferation and survival of cancer cells by aberrantly "turning-on" the Wnt signaling pathway. Even though it is unclear so far how different cancers respond to various WNT ligands stimulation to activate the Wnt signaling pathway, it is clear that the expression patterns of WNT ligands can serve as cancer markers and prognostic factors for patient outcomes (Aguilera et al., 2006; Chim et al., 2007; Kansara et al., 2009; Klarmann et al., 2008).

The ultimate oncogenic outcome of an active Wnt signaling pathway is mainly the nuclear accumulation of β-catenin. The presence of nuclear β-catenin is resulted from even a small mutation from the Wnt signaling pathway. In addition to serving as the outcome of the Wnt signaling pathway activity, β-catenin itself is also disposed to oncogenic mutations in different types of cancer. These mutations usually occur on the serine and threonine sites, which are essential sites for GSK3β-mediated phosphorylation. The mutations then abrogate the interaction between β-catenin and β-TrCP, making β-catenin refractory to degradation, and eventually nuclear accumulation. These mutations have been found in many cancer tissues such as liver, kidney, ovary, endometrium and soft tissues (Giles et al., 2003). Many studies have pinpointed

β-catenin as a key modulator in cancer cell proliferation and survival. This oncogenic effect results from activating a variety of oncogenes by serving as a coactivator from binding to TCF/LEF transcription activating complex. The plethora genes that β-catenin is capable of activating include ones that drive cell transformation, cell proliferation and survival, migration, invasion, EMT, angiogenesis and cancer stem cell (Table 1-2 with references).

Table 1-2. Wnt signaling proteins and their involvement in different types of cancer.

Protein	Cancer Type	Relevance in Cancer	References
APC	Breast	Expression is increased in grade 3 tumors compared with normal breast tissues	(Wong et al., 2002a)
	Colorectcal	Truncation mutation occurs in the vast majority colorectal cancer	(Clements et al., 2003; Liu et al., 2011)
β-catenin	Breast	Nuclear β-catenin expression is associated with reduced metastasis and overall survival in breast cancer	(Geyer et al., 2011; Lin et al., 2000)
	Colorectal	Overexpression of nuclear β-catenin is associated with invasive tumors and patient death	(Cheah et al., 2002)
	Glioblastoma	High nuclear β-catenin is associated with poor survival in glioblastoma	(Liu et al., 2011)
	Lymph node metastases	Overexpression of nuclear β-catenin is significantly associated with metastatic lymph nodes	(Cheng et al., 2011; Kageshita et al., 2001)
	Prostate	Overexpression of nuclear β-catenin is associated with bone metastatic prostate cancer	(Wan et al., 2012)
	Melanoma	Nuclear accumulation of β-catenin in primary tumors predict patient deaths	(Kageshita et al., 2001)
WNT1	Breast	WNT1 protein expression is increased in breast tumor tissues compared to non-cancerous tissue	(Wong et al., 2002b)
	Gliobastoma	Increased WNT1 expression is associated with poor survival in glioma	(Yin et al., 2012)
WNT2	Esophageal	Overexpression of WNT1 is correlated with poor survival in esophageal squamous cell carcinoma	(Fu et al., 2011)
TCF	Lung	Increased TCF activity is associated with metastatic lung adenocarcinoma	(Nguyen et al., 2009)

Table is mainly adopted from (Anastas and Moon, 2013) supplemented with additional information from the listed references.

Table 1-3. Major Wnt signaling target genes and their functions in cancer.

Gene Name	Function	Reference
cyclinD1, c-Myc, c-Jun, survivin, fra-1, Hath1, Met, FGF9, FGF18	Cell growth, cell proliferation, survival	(Boon et al., 2002; Hendrix et al., 2006; Leow et al., 2004; Mann et al., 1999; Shimokawa et al., 2003; Shtutman et al., 1999; Tetsu and McCormick, 1999; Zhang et al., 2001)
JAG1, PPARdelta, c-Myc binding protein,	Cell transformation	(Jung and Kim, 2005; Kim et al., 2005; Rodilla et al., 2009)
CD44, VEGF, Ephb, BMP4, EDN1, GREM1	Angiogenesis	(Batlle et al., 2002; Goncalves et al., 2008; He et al., 1999; Kim et al., 2002; Kim et al., 2005; Zhang et al., 2001)
MMP2, MMP9, Tiam1, MMP-7, Twist, uPAR, Snail, MMP26	Migration, Invasion, EMT	(Brabletz et al., 1999; Hendrix et al., 2006; Li and Zhou, 2011; Mann et al., 1999; Marchenko et al., 2002; Wu et al., 2007; Yook et al., 2006)
CD44, Oct4, Nanog	Cancer stem cell	(Cole et al., 2008; Goncalves et al., 2008; Hoffmeyer et al., 2012)

Table is mainly adopted from (Thakur and Mishra, 2013) supplemented with additional information from the listed references.

1.10 The Wnt signaling in development and stem cell maintenance

To study the role of Wnt signaling in mammalian development, genetic engineered mouse models have given the field more insights and details on how Wnt affects the multiples stages of mammalian development and maintaining homeostatic tissue function. The knockout of 9 out of 18 mouse Wnt gene exhibited severe defective phenotypes in embryonic development. *Wnt3a* $^{-/-}$, which is one of the Wnt ligands that if frequently overexpressed in cancer, exhibited failure in primitive streak and gastrulation. Double knockout of *Lrp5*$^{-/-}$ */Lrp6* $^{-/-}$ mice also fail to form primitive streak and die during gastrulation (Kelly et al., 2004). Consistent with the notion that β-catenin is the keystone in the Wnt signaling pathway, *β-catenin* knockout mice recapitulated the gastrulation failure observed in both *Lrp 5/6* and *Wnt3* knockout mice. Further, *β-catenin*$^{-/-}$ mutant mice showed additional defect in body anterior-posterior (A-P) axis formation and die 7 days post coitum (dpc). Double knockout of *Wnt1* and *Wnt3a* displayed defects in neural crest development and somite patterning. Table 1-4 shows the knockout phenotype of some of the central players in the Wnt signaling pathway as well as important Wnt ligands and receptors. Because these defects are critical to the embryonic development, mice deficient for these core components of the Wnt signaling pathway are often embryonic lethal (van Amerongen and Berns, 2006).

Other than embryogenesis, Wnt signaling also plays multiple roles in regulating stem cell niches. In particular, inhibiting the Wnt signaling eliminates hair follicles and other skin appendages such as mammary glands (DasGupta

and Fuchs, 1999). Mice deficient of *Lef1* exhibit a significantly reduction in hair follicles while transgenic mice bearing overexpression of *Lef1* had increased *de novo* hair follicle formation. Consistently, transgenic overexpression of stable form of β-catenin expanded the hair follicle formation. In the hematopoietic system, treatment of Wnt3a increases self-renewal, as measured by clonogenic assays and long-term reconstitution in irradiated mice (Willert et al., 2003). Activated Wnt can also mediate maintenance of pluripotency in mouse embryonic stem cells (ten Berge et al., 2011).

Table 1-4. Phenotypes of Wnt signaling components knockout mice.

Gene knockout	Phenotype	Reference
β-catenin	No primitive streak, defects in gastrulation, embryonic ectoderm,	(Haegel et al., 1995; Huelsken et al., 2000)
Axin1	Neuroectodermal and cardiac abnormalities	(Zeng et al., 1997)
Lef1	Defects in hair follicle formation, brain abnormalities	(van Genderen et al., 1994)
Tcf3	A-P axis formation failure	(Merrill et al., 2004)
Lrp6	Failure in neural tube closure and brain development, limb patterning	(Pinson et al., 2000)
Wnt1	Mid- and hindbrain deficiencies	(Thomas and Capecchi, 1990)
Wnt3a	Defects in primary body axis formation,	(Takada et al., 1994)
Wnt5a	Truncated A-P axis, mesoderm formation defect	(Yamaguchi et al., 1999)
Wnt7a	A-P defects, reproductive system defects	(Miller et al., 1998; Parr and McMahon, 1995)

Table summarizes phenotypes observed in some conventional knockout mice of Wnt signaling components. *Gsk3β* -/- mice were not included because they suffer defects from NF-κB signaling, independent from Wnt signaling.

1.11 Rationale and Hypothesis

The Jumonji family proteins are mainly known to carry out their functions on histone post-translational modifications, thereby regulating gene expression. Among the Jumonji family proteins, JMJD2A, also known as KDM4A, is critical for demethylating H3K9me$_3$ and H3K36me$_3$. Because of its ability to regulate chromatin dynamics, JMJD2A is shown to govern embryonic, neural, muscle and cardiac development, and its knockout mice are embryonic lethal. Only in the recent years, the role of JMJD2A in cancer has started to unveil. In these studies, JMJD2A has indispensable functions in proliferation and metastasis in many *in vitro* cancer cell models, and its overexpression is seen in a variety of cancer tissues. This may be due to its ability to associate and modulate different oncogenic genes. Interestingly, these JMJD2A-modulated genes are also major target genes in the Wnt/β-catenin signaling pathway, such as cyclinD1, Jagged, and c-Jun. Wnt/β-catenin signaling pathway is a vital and complex regulatory system in developmental biology and cancer, which controls gene expressions via the transcriptional coactivator β-catenin. In particularly, JMJD2A can cooperate with oncogenic Ras to promote cellular transformation of normal lung fibroblast, while the co-expression of Ras and β-catenin induces lung tumor in mouse models. Furthermore, transgenic knockout mice of Wnt signaling components revealed defects in embryonic, neural crest, and cardiac development, and also exhibit embryonic lethality, which are all observed in JMJD2A deficient conditions. All these serve as indicators to provoke a rational question that whether JMJD2A may be associated to the Wnt/β-catenin signaling

pathway. ***Therefore, our goal in this study was to explore the potential interplay between JMJD2A and the Wnt/β-catenin signaling pathway.***

Chapter 2

Materials and Methods

2.1 Cell Culture and Reagents

MDA-MB-231, Hep3B, PC-3, HEK293, HEK293T cells were obtained from American Type Culture Collection. All cells lines were cultured in DMEM (Hyclone) supplemented with 10% fetal bovine serum (Sigma Aldrich), 1% penicillin/streptomycin solution and 1% L-glutamine (Hyclone). *Jmjd2a* $^{flox/flox}$ mouse embryonic fibroblasts (MEFs) were prepared from mice as previously described. In short, female pregnant mice were sacrificed, and embryos were isolated at 13.5 days after mating. Embryos were digested and the resulting cells were cultured in complete DMEM. All procedures were conducted under the approved protocol of Institutional Animal Care and Use Committee (IACUC). *Jmjd2a*$^{flox/flox}$ mice were provided by Dr. Zhi-Ping Liu (The University of Texas Southwestern). Wnt3a ligand (R&D System) was treated according to the experimental time points (100ng/ml). MG132 (Sigma) was treated up to 6 hours (20ng/ml). Cycloheximide (CHX) was treated according to the time points at 20μg/ml. Cultured cells were kept at 37°C in a standard 5% CO_2 incubator.

2.2 Antibodies and Constructs

Antibodies used in this study include: anti-JMJD2A from Active Motif (Western blot); anti-JMJD2A from Abcam (IP), Cell Signaling (immunofluorescence); anti-β-catenin from BD Laboratories (Western blot); anti-phosphorylated-β-catenin (Ser33/Ser37Thr41), anti-Axin1, anti-Axin2, anti-GSK3β and anti- β-TrCP from Cell Signaling; anti-c-Myc, anti-cyclinD1 and anti-ubiquitin from Santa Cruz Biotechnology; anti-Flag, anti-α-tubulin and anti-β-actin from Sigma Aldrich; anti-Lamin B1 from Abcam.

Mammalian expression vector pEV3S-Flag-JMJD2A was a gift from Dr. Ralf Janknecht from Oklahoma University. pBabe-JMJD2A was subcloned from Flag-JMJD2A using blunt end cloning method. Enzyme dead mutants (H188A) of JMJD2A were mutated using a PCR-based site-directed mutagenesis with the following primers:

forward, 5'-CCTTTGCTTGGGCCACTGAAGAC

reverse, 5'-CATGTCTTCAGTGGCCCAAGCAA

JMJD2A truncation delC domain was constructed using the following primers:

forward, 5'-GTGAACACCCCATATACCCGTCGGTGGAT

reverse, 5'-ATCCACCGACGGGTATATGGGGTGTTCAC

pcDNA3-Flag-β-catenin and pcDNA3-Flag-S33Yβ-catenin were purchased from Addgene. TOP-FLASH and FOP-FLASH plasmids were gifts from Dr. Mong-Hong Lee's lab, MD Anderson Cancer Center.

2.3 Immunoblotting and Immunoprecipitation

For immunoblotting (Western blots) analysis, cells were lysed in RIPA lysis buffer (50mM Tris-HCl [pH of 8.0], 150mM NaCl, 5mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40, protease inhibitor cocktail [Roche, Inc]). The lysed cell samples were then normalized based on protein concentration by using the Bio-Rad protein assay dye reagent. The normalized protein samples were then subjected to SDS-PAGE analysis. For immunoprecipitation, cell pellets were lysed in E1A buffer (50mM HEPES [pH of 7.5], 250mM NaCl, 5mM EDTA, 0.1% NP-40, protease inhibitor cocktail) followed by 60-70 strokes of sonication. The lysed cell samples were then normalized using the same method described

previously. Normalized cell samples were incubated on the rotator with the corresponding antibodies overnight at 4°C. Protein A/G beads (Santa Cruz Biotechnology) were further incubated with the lysates for 3 hours on the rotator at 4°C. Beads were washed 4-5 times with E1A buffer. Proteins were finally eluted from the beads using 2X SDS-sample buffer. Samples were then subjected Western blot analysis.

2.4 Viral infection and Transfection

For lentiviral short hairpin RNA (shRNA) infection, 293T cells were prepared 50%-60% confluency) and cotransfected with either luciferase (shLuc) or target gene shRNA with packaging plasmid (pHelper) and envelop plasmid (pEnv) by using the calcium phosphate transfection method. Medium was changed 6 hours later. After 48 hours, the virus particles were harvested and used to infect parental cells for another 48 hours. The stably infected cells were then selected by 2 μg/ml puromycin for 5-7 days. The shRNA sequences used for this project are listed below (Sigma Aldrich).

Table 2-1. Lentiviral shRNA sequences

Name	Sequence
shJMJD2A 1	CCGGGCTGCAGTATTGAGATGCTAACTCGAGTTAGCATCTCAATACTGCAGCTTTTT
shJMJD2A 2	CCGGGCACCGAGTTTGTCTTGAAATCTCGAGATTTCAAGACAAACTCGGTGCTTTTT
shJMJD2A 3	CCGGCCGAAACTTCAGTAGATACATCTCGAGATGTATCTACTGAAGTTTCGGTTTTT
shJMJD2A 4	CCGGGCCTTGGATCTTTCTGTGAATCTCGAGATTCACAGAAAGATCCAAGGCTTTTT

For retroviral infection, MSCV and MSCV-Cre were transfected with VSV-G and Gag-Pol retroviral packaged plasmids into the HEK293T cells. Medium was replaced 6 hours later. After 48 hours, the virus particles were collected to infect MEFs for another 48 hours. The cells were selected by 1 μg/ml puromycin for 4-5 days. For transient transfection, plasmids were transfected by using either the calcium phosphate method or Lipofectamine 2000 (Invitrogen) reagent following the manufacturer's instructions. For calcium phosphate transfection, cells were plated around 60-70% confluency. Indicated plasmids were mixed with sterile H_2O, 2M $CaCl_2$, 2X HBSS (drop by drop). Mixed solutions were incubated for 30 minutes at room temperature, and added to the cells in DMEM high glucose medium. After 6-8 hours, medium was replaced with fresh regular medium. Transfected cells were harvested 48 hours after transfection.

2.5 Reverse Transcription and Real-Time PCR

For reverse transcription, RNA was extracted using TRIzol® (Invitrogen) following the company protocol. All apparatus and the autoclavable equipment were sterilized to be RNAse free. After RNA extraction, the purified RNA were normalized (5 μg) and used for reverse transcription using the SuperScript® III First-Strand Synthesis System (Invitrogen) to obtain cDNA for each sample. cDNAs were then used for real-time PCR analysis by using SYBR® Green PCR Master Mix (Invitrogen), following the standard protocol. The relative mRNA levels were calculated using the comparative Ct method: $2^{(\Delta Ct\ sample - \Delta Ct\ reference)}$ Primers for real-time PCR are listed in Table 2-2.

Table 2-2. Real-Time PCR Primer sequences used

Name	Sequence
GAPDH F	5'-GAGCCAAAAGGGTCATC
GAPDH R	5'-GTGGTCATGAGTCCTTC
β-Catenin F	5'-AAAATGGCAGTGCGTTTAG
β-Catenin R	5'-TTTGAAGGCAGTCTGTCGTA
Cyclin D1 F	5'-AAATGCCAGAGGCGGAGGAGAACAAAC
Cyclin D1 R	5'-GGAAGTTGTTGGGGCTCCTCAGGTT
c-Myc F	5′-TCAAGAGGCGAACACACAAC
c-Myc R	5′-GGCCTTTTCATTGTTTTCCA
Axin2 F	5'- GCAAACTTTCGCCAACCGTG
Axin2 R	5'- CTCTGGAGCTGTTTCTTACTGCCC
LEF-1 F	5'-ACAGCGGAGCGGAGATTACAGAGC
LEF-1 R	5'-TCAGATGTAGGCAGCTGTCATTCTGGGA

2.6 TOP/FOP Luciferase Reporter Assay

TOP-FLASH and FOP-FLASH reporter plasmids were transfected along with *Renilla* luciferase reporter plasmid in MDA-MB-231 shLuc and shJMJD2A cells. TOP-FLASH reporter plasmid contains three wild-type optimal copies of TCF/LEF binding sites for β-catenin while FOP-FLASH reporter plasmid contains three mutant copies of TCF/LEF binding sites in which β-catenin is unable to bind to drive transcription. 48 hours after transfection, cells were harvested and both firefly and *Renilla* luciferase activity was measured in triplicates using the Dual-Glo™ Luciferase Assay System (Promega). The firefly activity was normalized against the *Renilla* luciferase activity and fold increase in TOP-FLASH activity was compared to FOP-FLASH activity.

2.7 Cytosolic and Nuclear Fractions

After cell harvesting, cell pellets were resuspended in hypotonic buffer (10mM Tris-HCl [pH of 7.6], 10mM $MgCl_2$, 0.1% NP-40, protease inhibitor cocktail tablet)

and homogenized using dounce homogenizer (pestle B for tighter fit). The cell suspensions were centrifuged at 1,300 x g for 5 minutes at 4°C. The supernatant (cytosolic) was carefully separated from the pellets (nuclear). The supernatant was processed for cytosolic fractionation: centrifugation at 13,500 rpm for 15 minutes at 4°C. The nuclear pellets were washed once with hypotonic buffer and were centrifuged again. The pellets were then resuspended in RIPA buffer followed by brief sonication and centrifugation. For input analysis, the fractionation samples were normalized for immunoblotting. For protein-protein interaction analysis, follow the immunoprecipitation protocol.

2.8 Immunofluorescence Assay

Prior to immunostaining, cells were seeded in chamber slides (Nunc™ Lab-Tek™ II) and allowed to attach. For analysis, cells were fixed with 4% formaldehyde for 30 minutes, and permeabilized with 3% Triton x-100 PBS for 10 minutes. After blocking (2% BSA) and incubating with the indicated primary and corresponding secondary antibodies, cells were rinsed with 1X PBS and mounted with Antifade Reagent with DAPI.

2.9 Photobleaching fluorescence resonance energy transfer (FRET)

MDA-MB-231 cells were treated with Wnt3a ligand according to the time points. After washing with PBS, the cells were fixed for 15 min with 4% formaldehyde in PBS and incubated in blocking solution (PBS containing 10% fetal bovine serum) for 30 min at room temperature. This procedure was followed by incubation with primary monoclonal anti-β-catenin and anti-JMJD2A antibodies at 1:200 dilution in blocking solution for 2 hours at room temperature. Following incubation with

the primary antibodies, the cells were washed in PBS (0.5% Tween 20) and incubated with FITC- and Rhodamine-labeled anti-rabbit secondary antibody (Jackson ImmunoResearch Lab Co.). The samples were washed three times with PBS (0.5% Tween 20), mounted with Gel Mount (Sigma), and images were acquired using a Leica TCS SP5 Confocal Spectral Microscope Imaging System (Leica Microsystems, Wetzlar, Germany), as described previously (Chen et al., 2012). The acceptor bleaching FRET method (Xia and Liu, 2001) was used according to the manufacturer's instructions (FRET Wizards in the Leica Application suite). Briefly, the initial donor (FITC 488) image represents donor fluorescence in the presence of the acceptor, rhodamine. After complete photobleaching of the acceptor, a second donor image was collected. Quantitative analysis of the D/D_A values was performed using the method of Xia and Liu (Xia and Liu, 2001) after image acquisition with the Leica Application Suite-Advanced Fluorescence software (Leica FRET AB). The total FRET activity folds values of the nuclear and cytosolic parts were counted from the 500 randomly selected cells. The fold ratios of the Wnt-3a-treated cells (2, 6, and 10 hours) were obtained by comparison to the control-treated (0 hour) group.

2.10 Cell Proliferation, In Vitro Migration and Invasion Assays

Approximately 5000 cells were seeded per well in 12-well plates in triplicates. Cells were then stained with trypan blue, and viable cells counted on the indicated days by using the hemocytometer under the microscope. For cell migration, 500 µL of complete medium was dispensed per well in the 12-well plates. 10^5 cells were seeded with serum free medium in the transwell inserts

situated in the wells. Cells were allowed to migrate toward FBS in the incubator for the indicated time period. Medium was removed, and inserts were washed with 1X PBS. 4% paraformaldehyde was then added to the lower chamber (between inserts and the bottom of the well) to fix the cells for 20 minutes at room temperature. The unmigrated cells in the transwell inserts were removed by Q-tips before staining. Cells were stained by Hematoxylin for 20 minutes at room temperature. Following staining, the inserts were washed with 1X PBS until all excess stain was removed. Invasion assay was executed similar to that of migration assay except transwell inserts were coated with Matrigel for 20 minutes in the incubator prior to cell seeding, and cells were allowed to invade for 24 hours. Migrated and invaded cells were counted, and three fields per group were recorded for statistical analysis.

2.11 Stem Cell Sphere Culture

1:1 ratio of DME/F12 and MEGM medium was used to culture stem cell sphere with the following ingredients: Insulin (Sigma), human EGF (Sigma), 4%BSA, hydrocortisone (Sigma), B27 supplement (Invitrogen), and1% penicillin and streptomycin. Before seeding, cells were washed twice with 1X PBS to remove residuals of FBS. 5000 cells were seeded per cell in triplicates in 12 well Ultra-low Attachment Plate (Corning), and let grow in the incubator for up to 14 days. Stem cell spheres with diameters bigger than 100 μm were counted with cell counter.

2.12 Stem Cell Aldefluor Assay

For each sample, $1X10^6$ cells were counted and diluted with 1 mL of the ALDEFLUOR™ (Stemcell Technologies) buffer. After labeling one “test” tube and one “control” tube, 1 mL of the diluted cell suspension was dispensed into the “test” tube. 5 µL of DEAB reagent was added to the “control” tube, and 5 µL of the activated reagent was added to the 1 mL cell suspension in the “test” tube. After adding the active reagent, the cell suspension was mix immediately and 0.5 mL of the suspension was dispensed into the DEAB “control” tube. After both of the tubes were ready, both samples were incubated for 30 minutes at 37°C. Following incubation, the tubes were then centrifuged for 5 minutes and resuspended each sample with 0.5 mL Assay Buffer. Cell mixtures were then stored on ice at 4°C. The samples were then place on the flow cytometer for data requisition. DEAB group served as the control group.

2.13 In Vivo Tumorigenesis and Metastasis Assay

For tumorigenesis, 3 million stable MDA-MB-231 cells with control and JMJD2A knockdown expressions were subcutaneously injected into the flanks of nude mice (Taconic, Inc.). Tumor size was measured by the caliper weekly, and tumor volume (mm^3) was calculated based on the equation: volume = $(width)^2$ x length/2. Approximately 5 mice were used for each group. All animal experiments were performed under the Institutional Animal Care and Use Committee approved protocol. Tumors were isolated, and pictures were taken to demonstrate the tumor size. For *in vivo* metastasis analysis, 2 million stable MDA-MB-231 cells with depletion of control and JMJD2A expressions were

injected through tail vein. Three mice were used for each group. After 3 months, the lungs of the mice were isolated and counted for nodule numbers. Pictures were taken to visually demonstrate *in vivo* metastasis to the lungs.

2.14 Statistical Analysis

The statistical significance of the data in this project was determined by unpaired two tailed Student's t-tests, unless indicated otherwise in the figure legends. P-values less than 0.05 were considered statistically significant.

Chapter 3

Results

3.1 JMJD2A affects Wnt target gene expressions

Recent studies have revealed that the expression of JMJD2A is aberrantly high in many different types of cancer, and it is exceptionally overexpressed in breast cancer (Berry et al., 2012). To confirm this observation, we used the ONCOMINE microarray database platform to examine the expression of JMJD2A in normal breast tissue and invasive ductal breast carcinoma. The box-plot indicates that the mRNA expression level of JMJD2A is significantly higher in the breast cancer tissue (lane 2) compared to the normal (lane 1) (Figure 3-1). Thus, for the remaining of this project, we mainly focused using breast cancer cells to perform the mechanistic studies of JMJD2A. First, to test the hypothesis that JMJD2A is linked to Wnt/β-catenin signaling pathway, we used the TCGA Cancer Genomics Browser platform and analyzed the TCGA exon datasets for JMJD2A and common Wnt/β-catenin target genes from microarray. The heat map shows that in invasive breast carcinoma patient tissues, JMJD2A (KDM4A) mRNA expression correlates significantly ($p<0.05$) with most of the Wnt/β-catenin target genes (Figure 3-2). To verify this data, we treated MDA-MB-231 control and JMJD2A knockdown cells with Wnt3a ligand to see whether the deficiency of JMJD2A would affect the major target genes of Wnt/β-catenin pathway. As shown by the real-time PCR results, mRNA levels of well-established Wnt signaling pathway target genes CCND1 (cyclinD1), MYC (c-Myc), AXIN2, and LEF1, were all expectedly induced upon Wnt ligand treatment in MDA-MB-231 control knockdown cells (Figure 3-3). However, in the JMJD2A knockdown cells, the mRNA expressions of these target genes were decreased, compared to the

control cells. Surprisingly, even after the treatment of Wnt3a ligand to activate the Wnt-signaling pathway, the mRNA expression levels of these target genes were much lower compared to the control knockdown cells (Figure 3-3). This data provided some cues for us that the manifestation of JMJD2A is critical for the activation of Wnt/β-catenin signaling pathway. To follow up on this finding, we revealed that the Wnt3a-induced transcriptional activity of β-catenin was significantly blocked by the knockdown of JMJD2A, as demonstrated by the TOPFlash luciferase assay (Figure 3-4). It is worth mentioning that while the mRNA levels of β-catenin target gene were reduced in the JMJD2A knockdown cells, the mRNA level of β-catenin itself did not change upon JMJD2A depletion (Figure 3-3). To validate this notion, we examined the protein level of these target genes in four different JMJD2A shRNA stable knockdowns, as well as *Jmjd2a*$^{flox/flox}$ MEF infected with MSCV-Cre cells. Consistent with their mRNA expression levels, the protein levels of these target genes also decreased under the ablation of JMJD2A, compared to their control counterparts (Figures 3-5 A-C). However, unlike its mRNA expression level, the protein level of β-catenin was decreased significantly upon deficiency of JMJD2A (Figures 3-5 A-C). Moreover, after treatment with Wnt3a ligand, while control cells nicely exhibited increased protein levels of expression in β-catenin and its target gene cyclinD1, the protein levels of Wnt target genes as well as β-catenin still failed to be induced in the JMJD2A knockdown cells (Figures 3-5D). These results collectively indicate that JMJD2A is needed to stimulate Wnt ligand-induced β-catenin transcriptional activity and to activate its target gene expressions.

Since JMJD2A is a histone protein that functions as a demethylase for histone tail post-translational modifications, we then asked the question whether this effect is through modulating the histone methylation status of these target genes, thereby affecting their expressions. We restored vector, WT JMJD2A and enzyme dead mutant H188A back into the MDA-MB-231 JMJD2A knockdown cells, and examined the protein level of the β-catenin and its target genes. Just like its WT counterpart, enzyme-dead mutant H188A still successfully restored the expressions of β-catenin target genes (Figure 3-6), indicating that the histone demethylase activity of JMJD2A is not required to exert its effect on the Wnt/β-catenin signaling pathway.

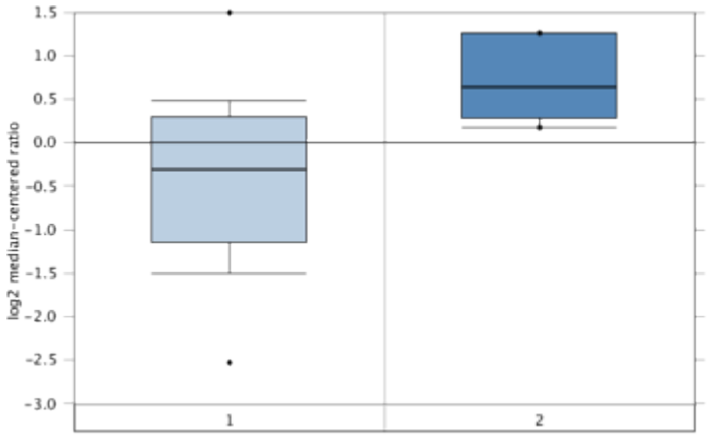


Figure 3-1. JMJD2A is overexpressed in breast cancer.

Expression of JMJD2A mRNAs in total of 593 patient samples is shown in the format of box plot. Lane 1 represents expression of JMJD2A in normal breast tissues; Lane 2 represents the expression of JMJD2A in ductal breast carcinoma. Shown are log2-transformed mRNA levels with the median. Statistical significance was calculated using Student's t-test to compare the difference of mRNA expression in normal and cancerous tissues. $p<0.01$. Microarray data was acquired from TCGA and analyzed through the ONCOMINE web portal.

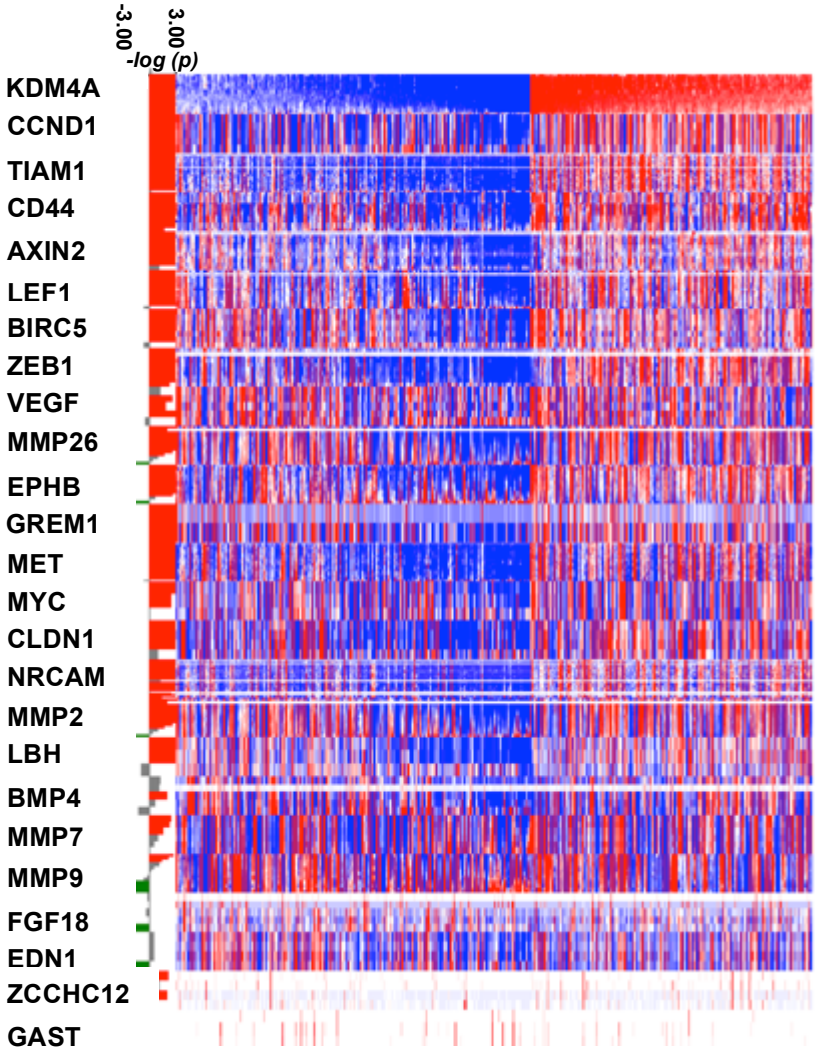


Figure 3-2. JMJD2A expression correlates with Wnt signaling pathway target genes.

TCGA Cancer Genomic Browser exon dataset for invasive breast cancer tissue was used to examine the expression of JMJD2A and major Wnt/β-catenin signaling target genes. Total patient samples=1215. The top 30% and bottom 30% patient data were used (n=729). Statistical bar is displayed on the left side of the heat map. Statistical significance ($p<0.05$) was calculated using the Wilcoxon signed-rank rest. Red means positive correlation, green means negative correlation.

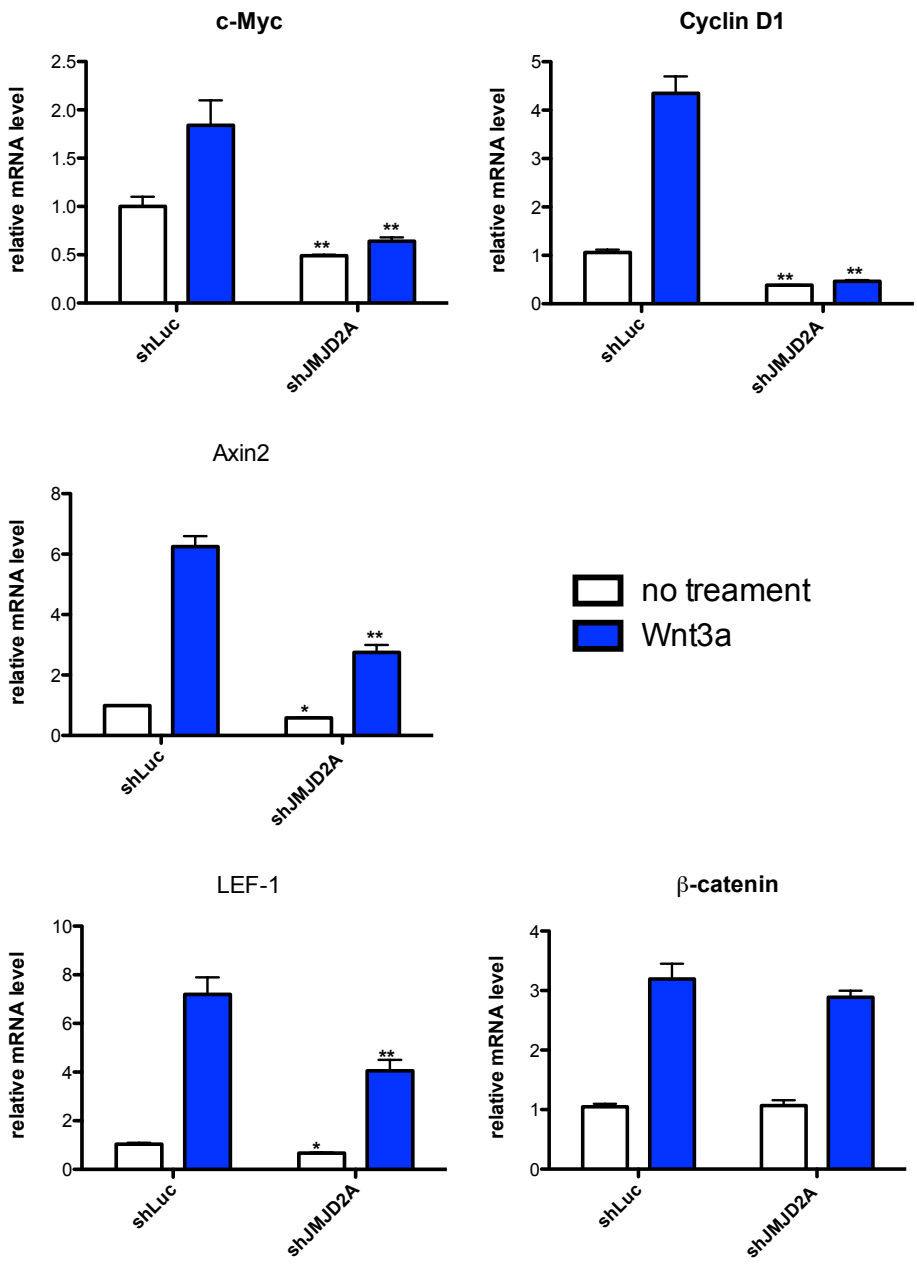


Figure 3-3. JMJD2A affects Wnt target gene expression on the mRNA level. MDA-MB-231 cells with control (shLuc) or JMJD2A knockdown were treated with Wnt3a for 6 hours. RNA was extracted for reverse transcription followed by real-time PCR to analyze the mRNA expression of Wnt target genes. The result is shown as means ± s.d. (n=3). **p<0.01, *p<0.05.

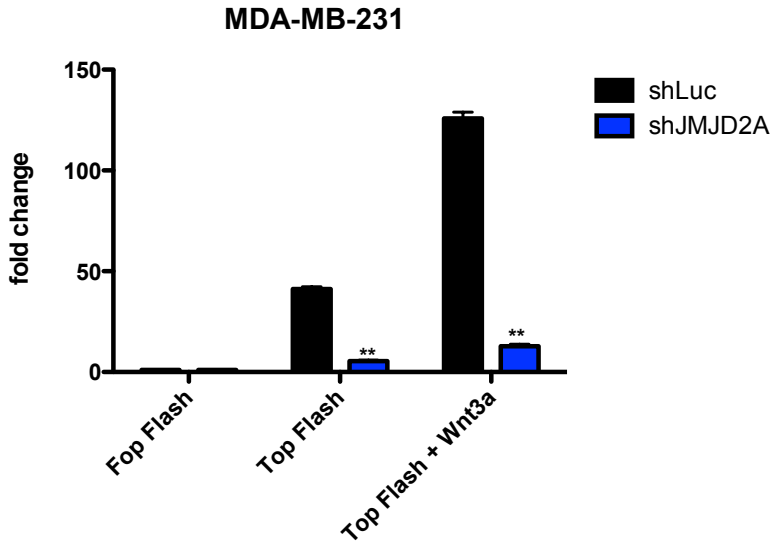


Figure 3-4. JMJD2A affects β-catenin transcriptional activity.

MDA-MB-231 control and JMJD2A knockdown cells were transfected with the TOP-Flash and FOP-Flash (negative control) luciferase reporter, and treated with Wnt3a ligand for 6 hours. The ratios between TOP-Flash and FOP-Flash were calculated 48 hours after transfection. The result is shown as means ± s.d. (n=3). **p<0.01.

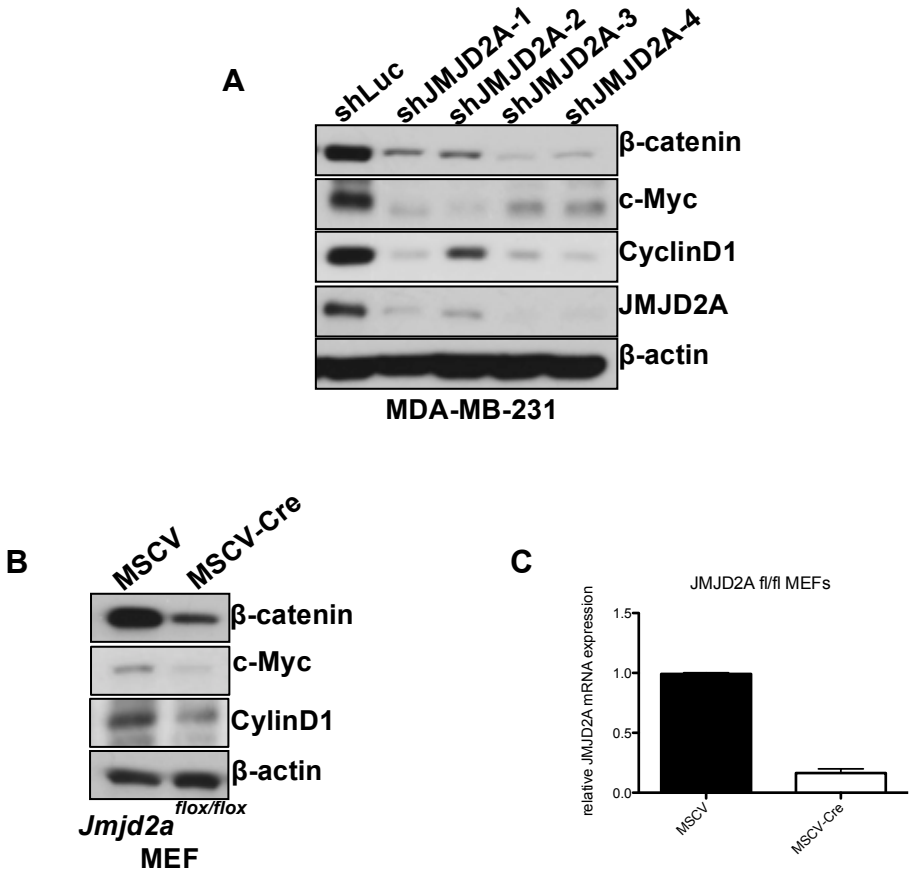


Figure 3-5. JMJD2A affects the protein level of β-catenin and its target genes.

(A) MDA-MB-231 cells with control (shLuc), and JMJD2A knockdown were subjected to immunoblotting to examine the protein level of β-catenin and its target genes.

(B) *Jmjd2a*$^{flox/flox}$ MEFs infected with control (MSCV) or MSCV-Cre were subjected to immunoblotting to examine the protein level of β-catenin and its target genes.

(C) The mRNA level of JMJD2A in *Jmjd2a*$^{flox/flox}$-MSCV and *Jmjd2a*$^{flox/flox}$-MSCV-Cre MEFs as in **(B)** was validated.

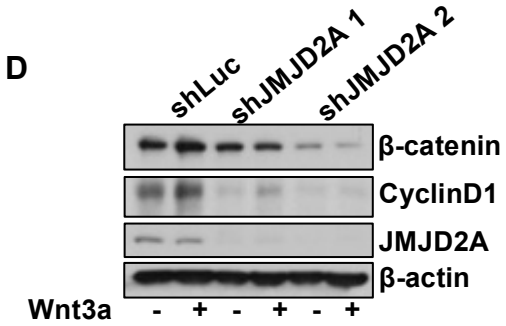


Figure 3-5. JMJD2A affects the protein level of β-catenin and its target genes (continued).

(D) MDA-MB-231 cells with control (shLuc) or JMJD2A knockdowns were treated with Wnt3a ligand like indicated for 6 hours. Cells were harvested for immunoblotting to examine the protein level of β-catenin and its transcriptional target gene cyclinD1.

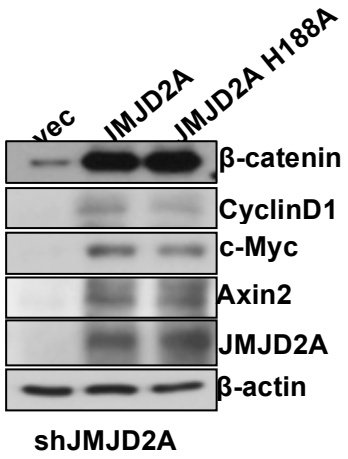


Figure 3-6. JMJD2A enzyme activity is not needed to regulate β-catenin and its target gene expressions.

Restoration of WT JMJD2A and enzyme-dead mutant H188A of JMJD2A in JMJD2A knockdown cells. Whole cell extracts of the cells were subjected to immunoblotting for β-catenin and the protein level of its downstream targets.

3.2 JMJD2A regulates the β-catenin expression through protein stability

Having shown that JMJD2A only affects the protein abundance of β-catenin but not its mRNA level, we hypothesized that JMJD2A affects β-catenin protein expression through a post-transcriptional mechanism. To assess whether JMJD2A regulates the stability of β-catenin, we measured the half-life of endogenous β-catenin by inhibiting new protein synthesis with cyclohexmide (CHX). In MDA-MB-231 control knockdown cells, the half-life of β-catenin only decreased marginally over the course of 8 hours (Figures 3-7A, B). Conversely, in the JMJD2A knockdown cells, the half-life of β-catenin dropped dramatically between 4 to 6 hours (Figures 3-7A, B). Since most of protein degradation is through proteasome, including β-catenin, therefore, we tested whether the downregulation of β-catenin under the JMJD2A deficient condition is mediated by the ubiquitin-proteasome pathway. To do this, we treated MDA-MB-231 control and JMJD2A knockdown cells with proteasome inhibitor MG132. Consistently, ablation of JMJD2A decreased the level of β-catenin and its downstream target gene cyclinD1 (Figures 3-7C,D). Nevertheless, MG132 treatment successfully blocked the degradation of β-catenin as well as its target genes, with or without Wnt3a ligand treatment (Figures 3-7C,D). These results suggest that the decreased protein level of β-catenin is mediated through the ubiquitin-proteasome pathway.

In the canonical Wnt signaling pathway, during the Wnt-off resting phase, the proteasome-mediated degradation of β-catenin occurs through several sequential steps: 1) GSK3β targets β-catenin for phosphorylation; 2)

Phosphorylated β-catenin binds and gets recognized by E3 ligase β-TrCP; 3) β-TrCP induces β-catenin ubiquitination and proteasome-induced degradation. To thoroughly study the mechanism of how JMJD2A regulates β-catenin degradation, we then examined the effect of JMJD2A on each of these critical steps in destabilizing β-catenin. In the first step, we stimulated MDA-MB-231 stable control and JMJD2A knockdown cells with Wnt3a in a time course treatment to examine the level of phosphorylation of β-catenin by GSK3β on Ser33/Ser37/Thr41. As expected, Wnt activation abrogated GSK3β-mediated phosphorylation of β-catenin in a time-dependent manner, while total level of β-catenin was induced (Figure 3-8A). Yet, in the JMJD2A knockdown cells, the levels of phospho-β-catenin were dramatically higher while the total level of β-catenin was lower, compared to its control counterparts (Figure 3-8A). To corroborate the involvement of GSK3β in facilitating the degradation of β-catenin, we restored the constitutive active β-catenin S33Y, a mutant that cannot be phosphorylated by GSK3β, into the JMJD2A knockdown cells. This restoration not only rescued the destabilized β-catenin expression seen in JMJD2A knockdowns, as well as the downstream target gene expressions (Figure 3-8B). This data indicates that JMJD2A is required to inhibit GSK3β-mediated phosphorylation of β-catenin. From these results, we hypothesized that without JMJD2A, GSK3β may have a higher binding affinity for β-catenin to induce higher phosphorylation, which eventually culminates in β-catenin degradation. To test this theory, we carried out co-IP experiment to examine the integrity of the

destruction complex and their interaction with β-catenin in control and JMJD2A ablation conditions. Compared to the control, JMJD2A deficiency significantly enhanced the interaction between β-catenin and GSK3β (Figures 3-9A,B). In addition, although Wnt stimulation abrogated the interaction between GSK3β and β-catenin in control cells, it had marginal effect on their interaction in the JMJD2A knockdown cells (Figure 3-9C). This result supports our previous observation that JMJD2A suppresses the phosphorylation of β-catenin mediated by GSK3β.

In the second step of the β-catenin degradation process, the phosphorylated β-catenin gets recognized by and binds to the F-box protein E3 ligase β-TrCP. In our co-IP experiment (Figure 3-10A), the basal interaction between β-catenin and β-TrCP was very low in the control knockdown cells (lane 1), but could be enhanced after MG132 treatment (lane 2). Expectedly, Wnt ligand treatment attenuated this interaction so that β-catenin could be stabilized for further nuclear translocation (lane 3). Contrariwise, in the JMJD2A knockdown cells, the basal interaction between β-catenin and β-TrCP (lane 5) was drastically increased, and MG132 treatment greatly enhanced this interaction (lane 6). While Wnt stimulation partially inhibited this binding, the interaction intensities between β-catenin and β-TrCP were generally higher in JMJD2A knockdown cells (lane 7).

In the third step, after β-catenin binds to β-TrCP, it acts as an E3 ligase to induce K48-linked polyubiquitination for subsequent proteasomal degradation. We performed immunoprecipitation with anti-ubiquitin antibody and immunoblotted with anti-β-catenin antibody to detected the level of ubiquitinated

β-catenin. We also treated the IP reactions with N-ethylmaleimide (NEM) deubiquitinating enzyme inhibitor to prevent protein deubiquitination. While the level of β-catenin ubiquitination was inhibited by the Wnt stimulation in the control group, it was increased by the depletion of JMJD2A, compared to its control counterpart (Figure 3-10B). Collectively, the above data suggest that JMJD2A has an indispensable role in this sequential event to induce β-catenin degradation, and that without the presence of JMJD2A, β-catenin destabilization still takes place even in the Wnt-on phase.

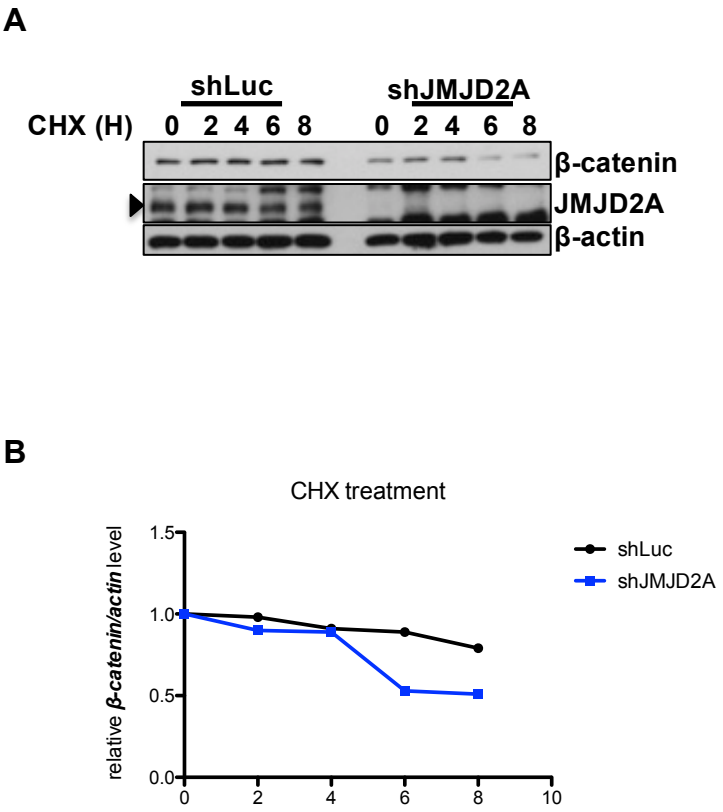


Figure 3-7. JMJD2A affects β-catenin protein level through degradation

(A) MDA-MB-231 cells with control (shLuc) and JMJD2A knockdown were treated with cyclohexmide (CHX). Cells were harvested at the indicated times for inhibition of new protein synthesis.

(B) Quantitation of the β-catenin level in **(A)** is normalized to the β-actin loading control and expressed relative to 0 h

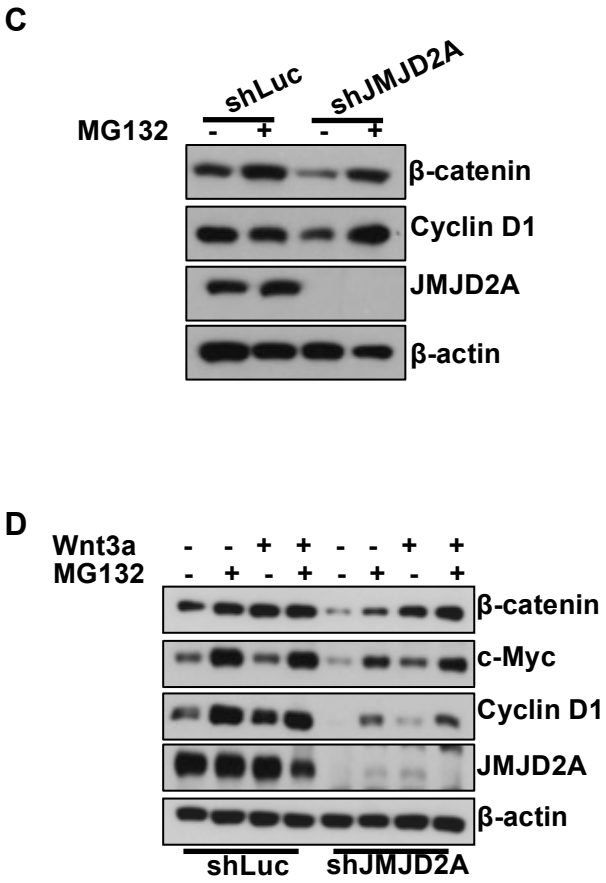


Figure 3-7. JMJD2A affects β-catenin protein level through degradation (continued)

(C) MDA-MB-231 with stable control and JMJD2A knockdown cells were treated with vehicle and MG132 for 6 h, and cells were subjected to immunoblotting.

(D) Cells in **(C)** were treated with MG132 proteasome inhibitor and Wnt3a ligand simultaneously for 6 h. Cells then were subjected to immunoblotting to examine the protein level of β-catenin and its downstream target genes.

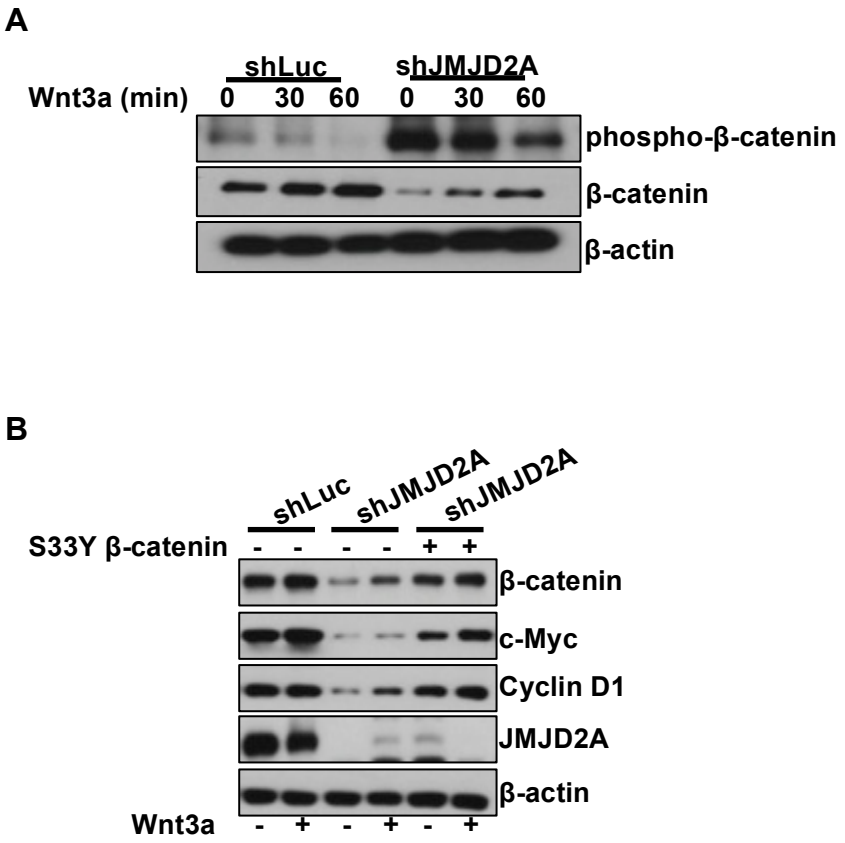


Figure 3-8. JMJD2A affects GSK3β-mediated phosphorylation of β-catenin.

(A) MDA-MB-231 cells with stable control (shLuc) and JMJD2A knockdown were treated with Wnt3a for the indicated time points. Phosphorylated β-catenin (Ser33/Ser37/Thr41) level was examined by using Western blot analysis

(B) Cells in **(A)** were transiently transfected with vector and S33Y β-catenin, and stimulated with Wnt3a ligand like indicated. The cells were subjected to immunoblotting to examine the levels of β-catenin and its downstream target genes.

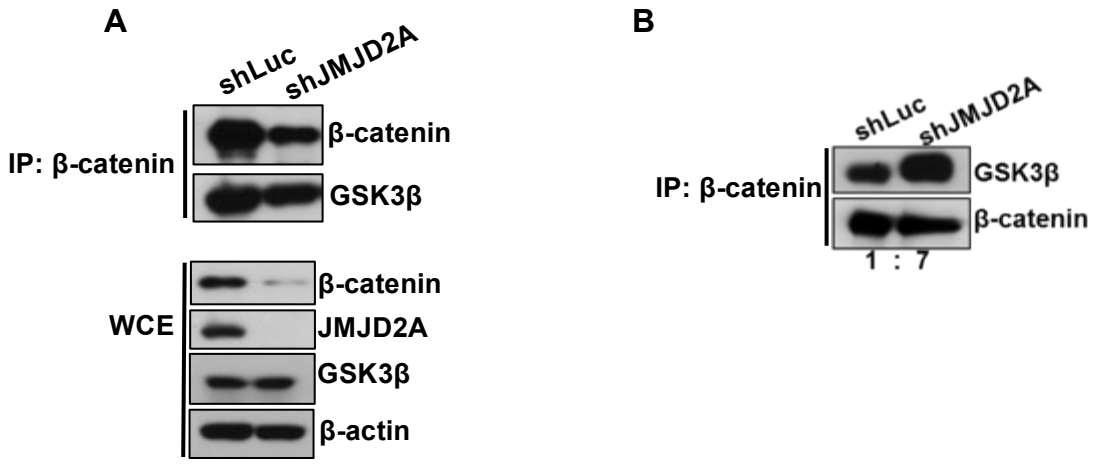


Figure 3-9. JMJD2A affects the integrity of the destruction complex.

(A) MDA-MB-231 with control and JMJD2A knockdowns were immunoprecipitated with anti-β-catenin antibody to examine level of interaction with GSK3β.

(B) Samples in **(A)** were reloaded to normalize the levels of immunoprecipitated β-catenin in the control and JMJD2A knockdown. The sample-loading ratio is indicated on the bottom of the IP blots. The interaction intensity between β-catenin and GSK3β was re-immunoblotted after the levels of immunoprecipitated β-catenin were normalized.

C

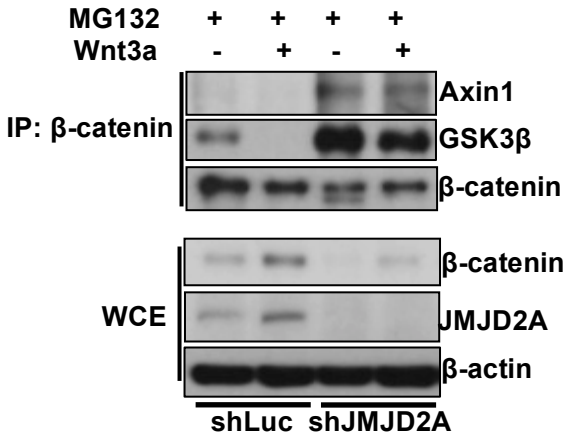


Figure 3-9 JMJD2A affects the integrity of the destruction complex (continued)

(C) MDA-MB-231 with control and JMJD2A knockdowns were immunoprecipitated with anti-β-catenin antibody to examine their intensities of interaction with GSK3β. MG132 and Wnt3a were treated simultaneously as indicated.

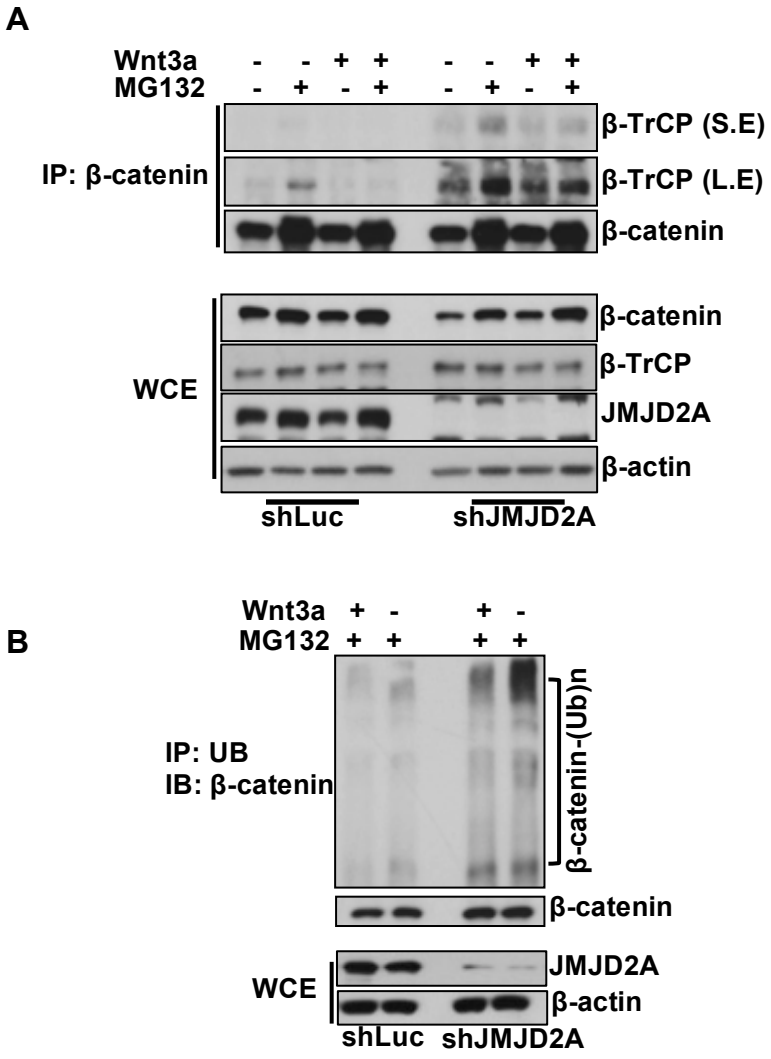


Figure 3-10. Ablation of JMJD2A induces the binding between β-catenin and β-TrCP.

(A) MDA-MB-231 cells with stable control (shLuc) and JMJD2A knockdown were treated with Wnt3a and MG132 simultaneously for 6 h, as indicated. The cells were subjected to β-catenin IP to examine its interaction with E3 ligase β-TrCP. S.E indicates short exposure time, and L.E indicates long exposure time.

(B) Cells from **(A)** were treated with Wnt3a ligand and MG132 as indicated. The cells were immunoprecipitated with anti-Ubiquitin to examine the endogenous β-catenin ubiquitination.

3.3 JMJD2A and β-catenin interact in the cytosol and nucleus

Since we have already shown that JMJD2A regulates the β-catenin degradation through regulating its interaction with the destruction complex, then we asked the question whether JMJD2A binds to β-catenin. We performed reciprocal co-immunoprecipitation experiments, and we were able to observe that exogenously expressed of JMJD2A interacted with endogenous β-catenin in HEK293T cells (Figures 3-11A,B). We further used the split-luciferase assay to confirm their interaction. In the control group (no Wnt ligand treatment), the luminescence signal was low, as quantified by bioluminescence (Figure 3-13B). After stimulating the cells with Wnt3a, JMJD2A and β-catenin showed significant higher interaction and full reconstitution of the split-luciferase enzyme with enhanced luminescence signal (Figure 3-11C). Since previous data showed that the enzyme-dead mutant H188A of JMJD2A has the same effect on β-catenin protein stability as the WT, we then aimed to study whether the enzyme activity of JMJD2A is needed for its interaction with β-catenin. To avoid the possibility that point mutation is not sufficient enough to affect protein-protein interaction, we generated JMJD2A truncation that is missing its catalytic Jumonji C (delC) domain, instead of using point mutation H188A enzyme-dead mutant. Consistent with our observation, the catalytic truncation of JMJD2A did not affect its binding with β-catenin (Figure 3-12), further corroborating that the enzyme activity of JMJD2A is not needed to exert its function in the Wnt signaling pathway. We then strived to confirm whether this interaction still occurs under the physiological condition by performing endogenous immunoprecipitation for JMJD2A and β-

catenin. Since Wnt ligand is capable of stabilizing β-catenin, we treated the IP reactions with Wnt3a ligand. The amount of β-catenin that was bound to JMJD2A increased substantially with the treatment of Wnt3a ligand (Figure 3-13). This data confirmed our IP result that the interaction of JMJD2A and β-catenin can be heightened by the activation of the Wnt-signaling pathway.

Because both endogenous JMJD2A and β-catenin exist in two cellular pools—cytosol and nucleus, we next examined their interaction in each of these two pools upon Wnt ligand treatment. The fractionated portions of the cells were immunoprecipitated with anti-JMJD2A antibody. In the cytosolic fraction, JMJD2A bound to β-catenin under normal resting conditions, and its interaction was abrogated upon Wnt3a treatment (Figure 3-14A). On the contrary, in the nuclear fraction under resting condition, JMJD2A and β-catenin did not interact since most of the protein resided in the cytosol prior to Wnt stimulation. After Wnt3a ligand treatment, their interaction was enhanced in the nucleus due to the translocation of these two proteins into the nucleus (Figure 3-14A). To ascertain that JMJD2A and β-catenin interact and translocate to the nucleus upon the activation of Wnt signaling pathway, we used FRET assay to further confirm this observation. After stimulating the cells with Wnt3a in a time-course manner, we observed FRET activity, indicated by the yellow fluorescence signal, in the cytosol at time 0 for the Wnt3a treatment (Figure 3-14B,C), confirming our IP results that cytosolic JMJD2A and β-catenin interact in resting cells. As the stimulation of Wnt3a progressed, both JMJD2A and β-catenin translocate from the cytosol to the nucleus, and their interaction and FRET activity was

significantly heightened, indicated by the red-orange fluorescence signal (Figures 3-14 B,C). Thus, we concluded that β-catenin interacts with JMJD2A in both the cytoplasm and the nucleus, and Wnt activation promotes their interaction in the nucleus by translocating both proteins to the nucleus.

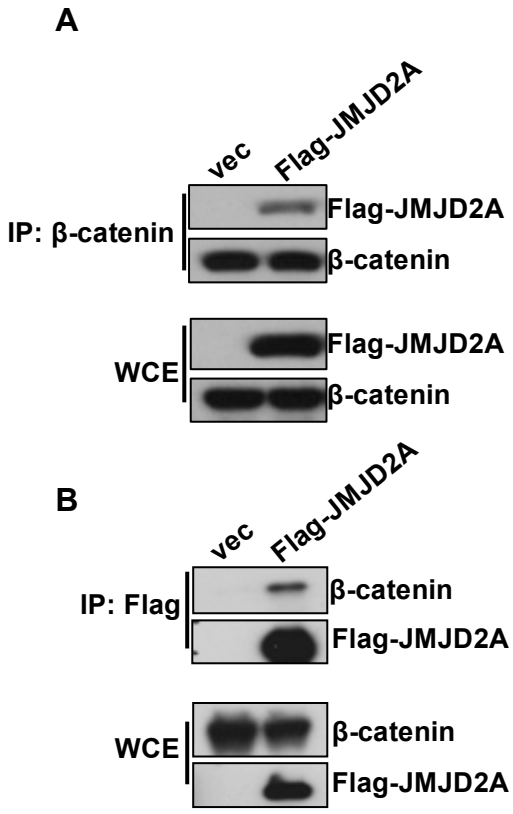


Figure 3-11. JMJD2A and β-catenin interact with each other.

(A) Transient transfection of Flag-JMJD2A was performed in HEK293T cells. Anti-Flag antibody was used for immunoprecipitation.

(B) HEK293T cells were transiently transfected with Flag-JMJD2A, and anti-β-catenin antibody was used for immunoprecipitation.

C

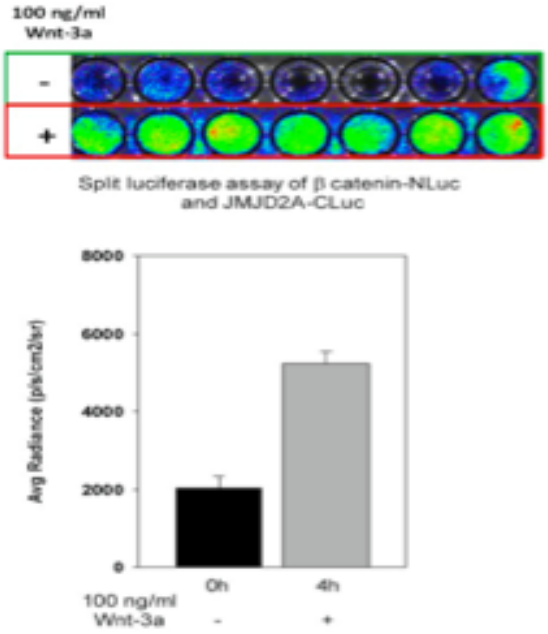


Figure 3-11. JMJD2A and β-catenin interact with each other.

(C) β-catenin was constructed to the N-terminal of luciferase plasmid while JMJD2A was constructed to the C-terminal of luciferase plasmid, and these plasmids were transfected into the MDA-MB-231 cells, and treated with Wnt3a like indicated. Luciferase activities would be fully reconstituted if the two proteins interact. The upper panel shows representative images of the luminescence signal and the lower panel presents the quantitative results. (Data done by Dr. Yuan-Soon Ho's group)

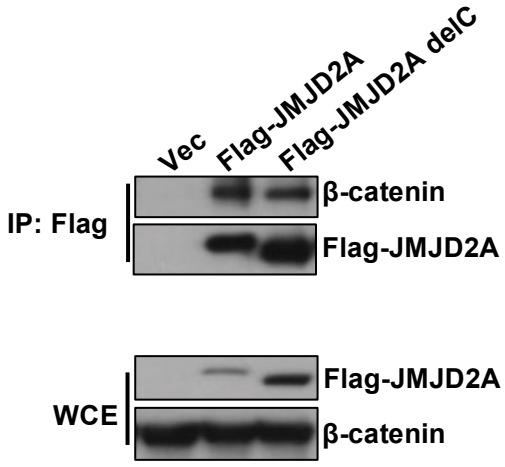


Figure 3-12. Enzyme-dead JMJD2A mutant has no effect on JMJD2A and β-catenin interaction.

WT Flag-JMJD2A and Flag-JMJD2A delC were transiently transfected in HEK293T cells. Immunoprecipitation was carried out with anti-Flag antibody to examine the interaction between Flag-JMJD2A and β-catenin

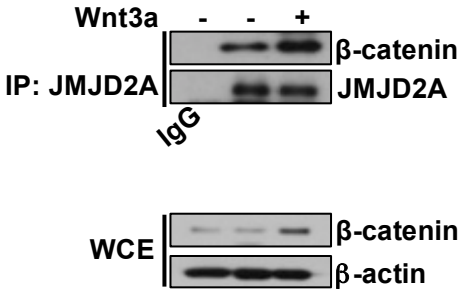


Figure 3-13. JMJD2A and β-catenin interact endogenously

293T cells were treated with vehicle and Wnt3a ligand for 6 h. Cells were immunoprecipitated with anti-JMJD2A antibody, and immunoblotted with anti-β-catenin antibody.

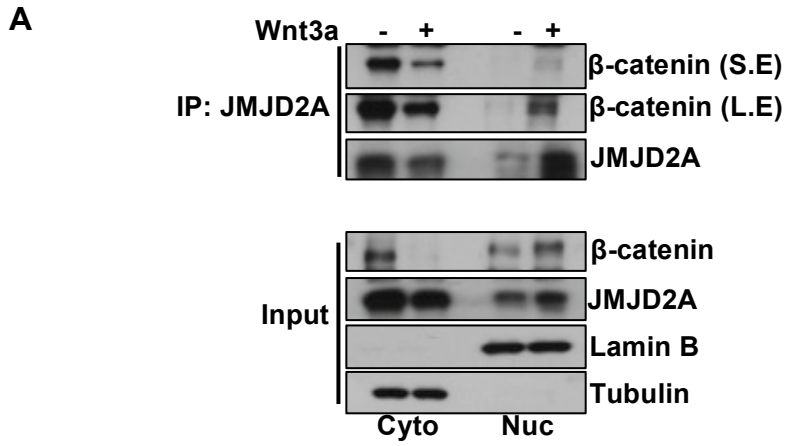


Figure 3-14. JMJD2A and β-catenin interact in the nucleus upon Wnt activation.

(A) Nuclear fractionation was harvested from 293T cells treated with vehicle and Wnt3a ligand. The cytosolic and nuclear fractions were immunoprecipitated with anti-JMJD2A antibody. Input lysates were subjected to immunoblotting to for detecting nuclear (LaminB) and cytosol (Tubulin) markers.

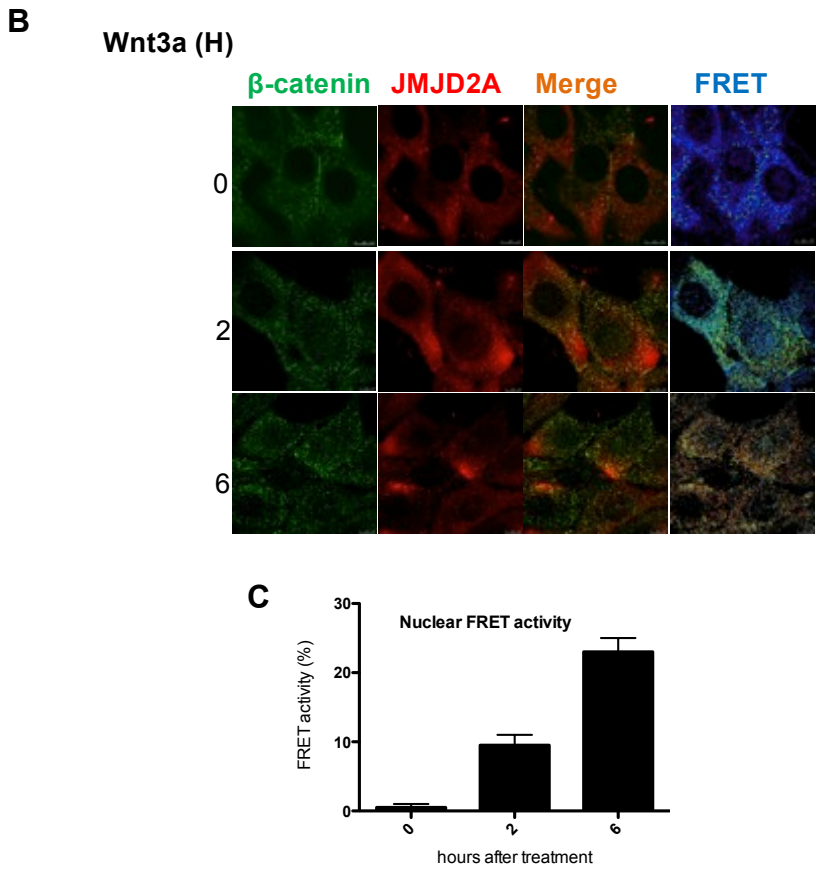


Figure 3-14. JMJD2A and β-catenin interact in the nucleus upon Wnt activation (continued)

(B) MDA-MB-231 cells were stimulated with Wnt3a ligand at the indicated time points, and stained JMJD2A (red), β-catenin (green) for immunofluorescence to examine their expression. FRET activity was detected at different time point of Wnt3a treatment for JMJD2A-β-catenin protein interaction. Yellow fluorescence indicates low FRET activity while red-orange fluorescence indicates high FRET activity. Representative imagines are shown. (Data done by Dr. Yuan-Soon Ho's group)

(C) The percentage of FRET activity was quantitated based on cell counting for positive FRET activity cells among all the cells in the 3 randomly chosen field.

3.4 JMJD2A affects the nuclear translocation of β-catenin

While the event of nuclear translocation of β-catenin has been well established to be the major outcome of the activated Wnt signaling pathway, the nuclear transport route of β-catenin has not been clearly defined. Even though many other nuclear imported proteins rely on the classical Ran/importin import machinery, β-catenin has no NLS sequence motif to bind to the import receptor complex to shuttle its nuclear movement (Fagotto et al., 1998). Many studies suggest that specific binding partners of β-catenin can influence its nuclear-cytoplasmic partitioning (Jamieson et al., 2014). Since our data indicate that JMJD2A translocates from the cytosol to the nucleus with β-catenin upon Wnt stimulation, we then asked the question whether JMJD2A plays a role in β-catenin nuclear localization. To do this, we examined β-catenin nuclear localization in the MDA-MB-231 JMJD2A knockdown cells. We conducted immunofluorescence assay to examine the localization of β-catenin. Wnt3a treatment promoted the nuclear translocation of both JMJD2A and β-catenin in the control cells (Figure 3-15A). Deficiency of JMJD2A caused significant degradation of β-catenin, even after Wnt3a stimulation. Combination treatment of MG132 and Wnt3a inhibited the degradation of β-catenin, but did not promote the nuclear translocation of β-catenin (Figure 3-14A). To validate this result, we performed nuclear fractionation to examine the two pools of β-catenin expressions. Consistently, while ablation of JMJD2A abrogated the expression of β-catenin in both pools (Figure 3-15B), MG132 restored the β-catenin expression in JMJD2A knockdown (Figure 3-15C). However, Wnt3a could not successfully

induce the nuclear translocation of MG132-stabilized β-catenin in JMJD2A knockdown (Figure 3-15C). These data collectively demonstrated that β-catenin nuclear localization depends on the expression of JMJD2A.

A

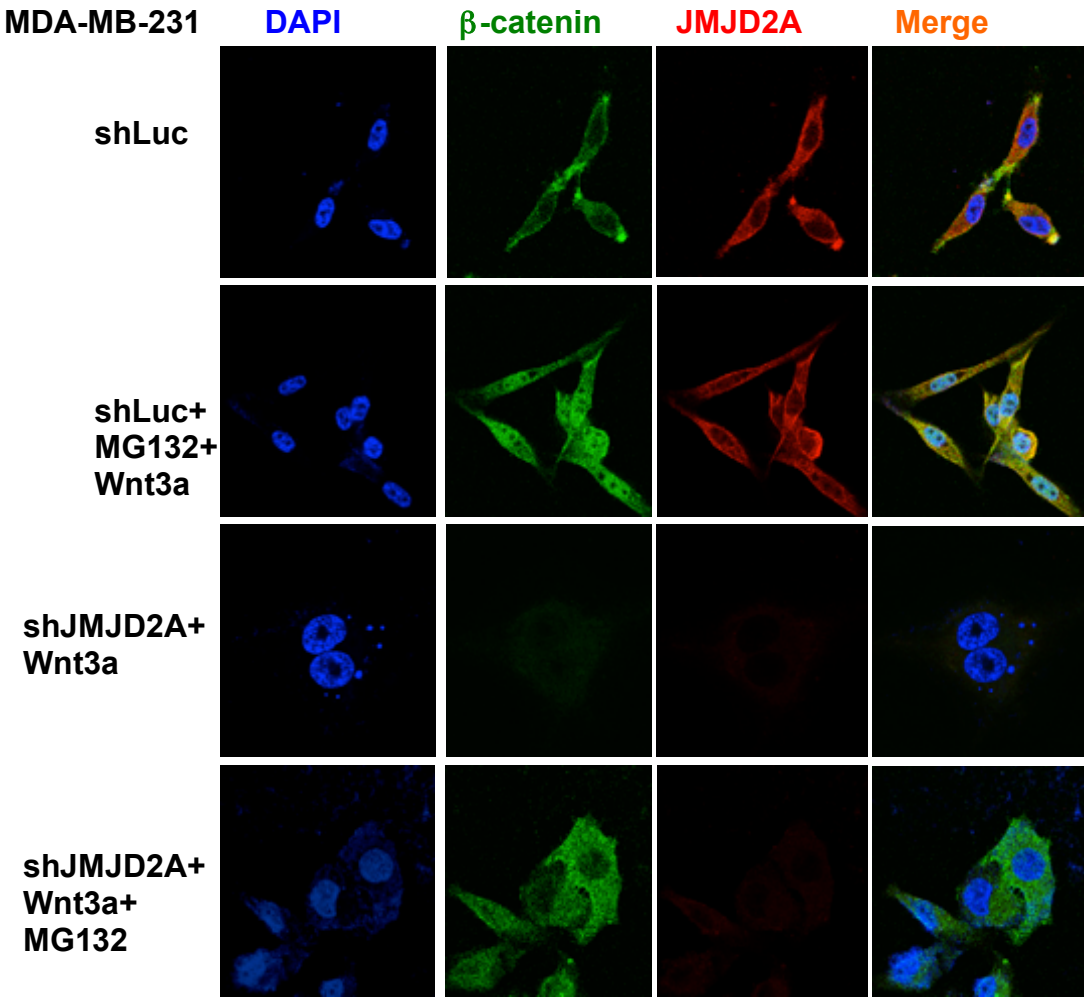


Figure 3-15. JMJD2A affects the nuclear translocation of β-catenin.

(A) Immunofluorescence staining for JMJD2A (red), β-catenin (green), and nuclei (DAPI, blue) was performed in shLuc (control) and shJMJD2A cells that were treated with Wnt3a and MG132, like indicated.

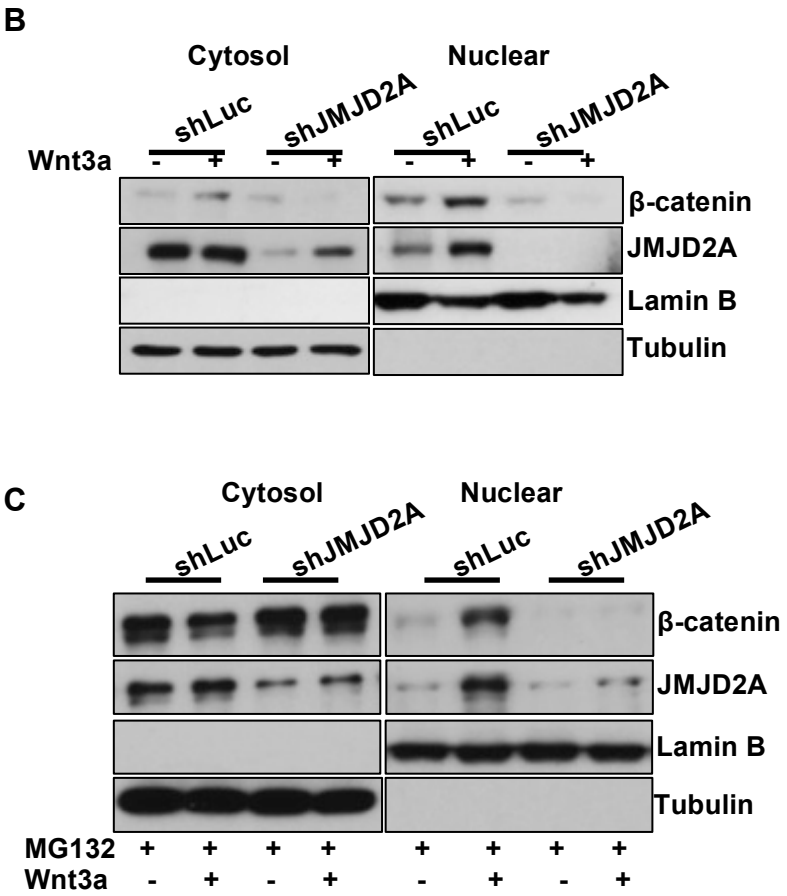


Figure 3-14. JMJD2A affects the nuclear translocation of β-catenin (continued).

(B) Nuclear fractionation assay separated the cytosolic pool and the nuclear pool of JMJD2A and β-catenin. Immunoblotting was performed to examine the expression of β-catenin and JMJD2A in both of the pools. Lamin B (nuclear) and Tubulin (cytosol) were blotted to serve as fractionation markers.

(C) Cells in **(B)** were treated with MG132 and Wnt3a simultaneously for 6 h, like indicated. Cells were then subjected to nuclear fractionation assay and immunoblotting to examine the cytosolic and nuclear pool of JMJD2A and β-catenin.

3.5 JMJD2A-mediated Wnt/β-catenin signaling in cancer progression

JMJD2A is known to be overexpressed in a variety of cancer (Guerra-Calderas et al., 2014), we aimed to confirm the biological significance of JMJD2A *in vitro*. We performed cell proliferation assay by using multiple types of cancer cell lines (PC-3 prostate, Hep3B liver, BT474 Her2+ breast). Consistently, in all four cell lines, the deficiency of JMJD2A greatly diminished cell proliferation (Figure 3-16A). Its important role in cancer progression is further validated in migration and invasion assay, using the *in vitro* cell line models (Figure 3-16B-E, PC-3, and Hep3B results showed similar trend, but data is not shown). Among all the studies regarding the oncogenic function of JMJD2A, most of the experiments were executed *in vitro*. Therefore, we examined the role of JMJD2A in tumor growth and metastasis *in vivo.* We first subcutaneously injected MDA-MB-231 cells into the nude mice. The tumors isolated from the JMJD2A knockdown groups were significantly smaller compared to the control group (Figure 3-17). Similarly, in the tail vein injection experiment, which mimics *in vivo* metastasis, we also observed much less number of lung lesions in the JMJD2A deficient group compared to the control group (Figure 3-18). Since our data demonstrate that JMJD2A is heavily involved in the Wnt signaling pathway, and greatly affects the stability of β-catenin, the keystone in this pathway, we then sought to determine whether the biological significance of JMJD2A is through regulating Wnt signaling pathway. To do this, we infected the control knockdown cells with vector, JMJD2A knockdown cells with vector and S33Y β-catenin to stably restore the expression of degraded β-catenin (Figure 3-19A). Even though

deficiency of JMJD2A attenuated cell proliferation, migration and invasion ability of the cancer cells, restoration of constitutive active β-catenin S33Y rescued these defects, to a level similar to that of the control knockdowns (Figure 3-19B-F). These results reveal that JMJD2A affects cancer progression through regulating the stability of β-catenin.

Since Wnt signaling pathway is known to control stem cell maintenance and self-renewal, we hypothesized that JMJD2A could also be implicated in stem cell regulation. We used sphere-forming assay and the percentage of ALDH+ cells, which a classical cancer stem cell marker (Visvader and Lindeman, 2008), to study the effect of JMJD2A in cancer stem cell maintenance *in vitro*. We used Hep3B, a hepatocyte carcinoma and classical *in vitro* cell line model for the sphere-forming assay (Figure 3-20A). While JMJD2A deficiency included a very small population of ALDH+ cells and failed to grow cancer stem cell spheres, restoration of S33Y β-catenin clearly rescued the sphere formation ability and amplified the ALDH+ population, to a level similar to the control cells (Figure 3-20B-D). These results, therefore, support that JMJD2A has a role in stem cell maintenance through regulating β-catenin stability, and further highlighted the importance of JMJD2A in the Wnt signaling pathway.

A

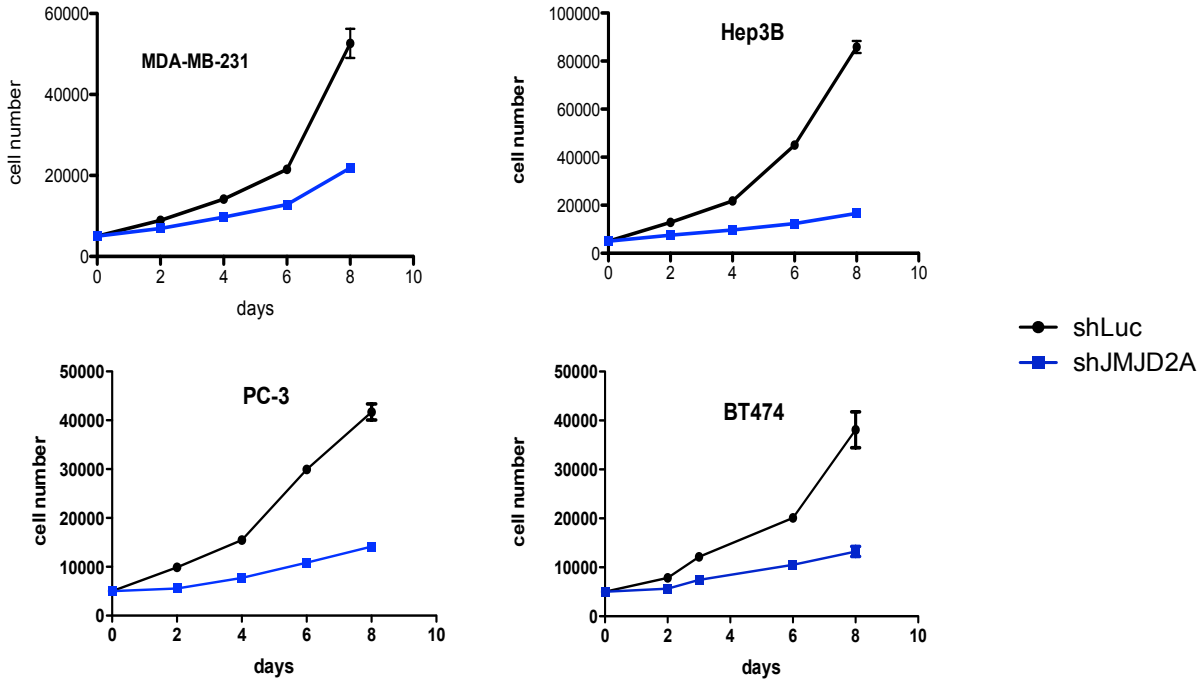


Figure 3-16. JMJD2A has an oncogenic role *in vitro*

(A) MDA-MB-231, Hep3B, PC-3 and BT474 control and JMJD2A knockdown cell lines were seeded in triplicates. Cells were counted every 2 days for 8 days total. The result is shown as means ± s.d. (n=3).

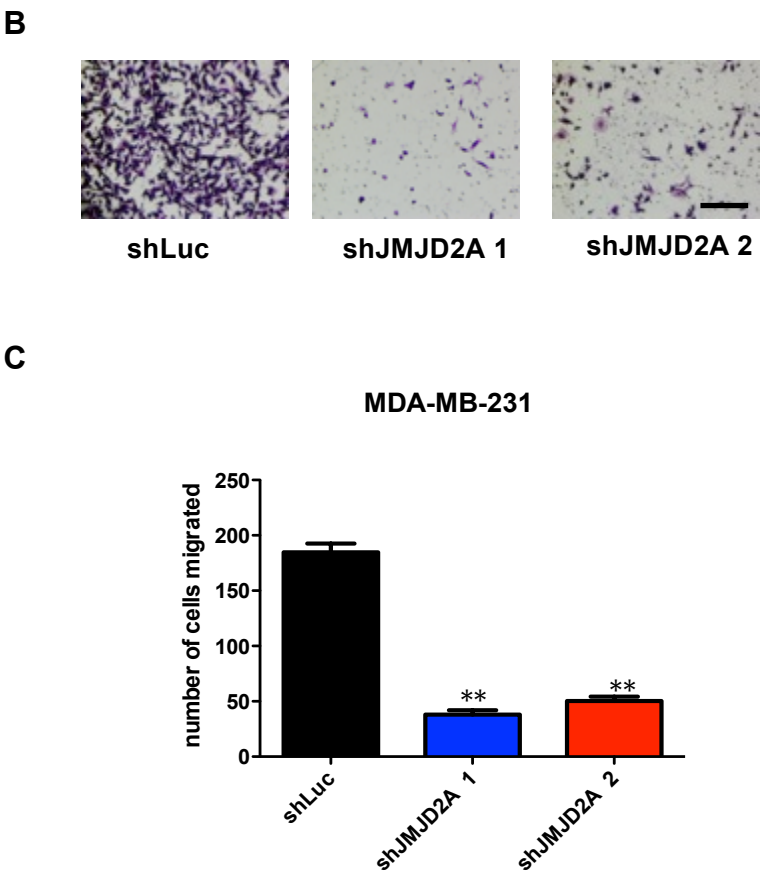


Figure 3-16. JMJD2A has an oncogenic role *in vitro* (continued)

(B) Representative images of migration assay of MDA-MB-231 cells with stable control (shLuc) and JMJD2A knockdown.

(C) Bar graph to quantitate the number of cells migrated in **(A).** The result is shown as means ± s.d. (n=3). **p<0.01. Scale bar, 100 μm.

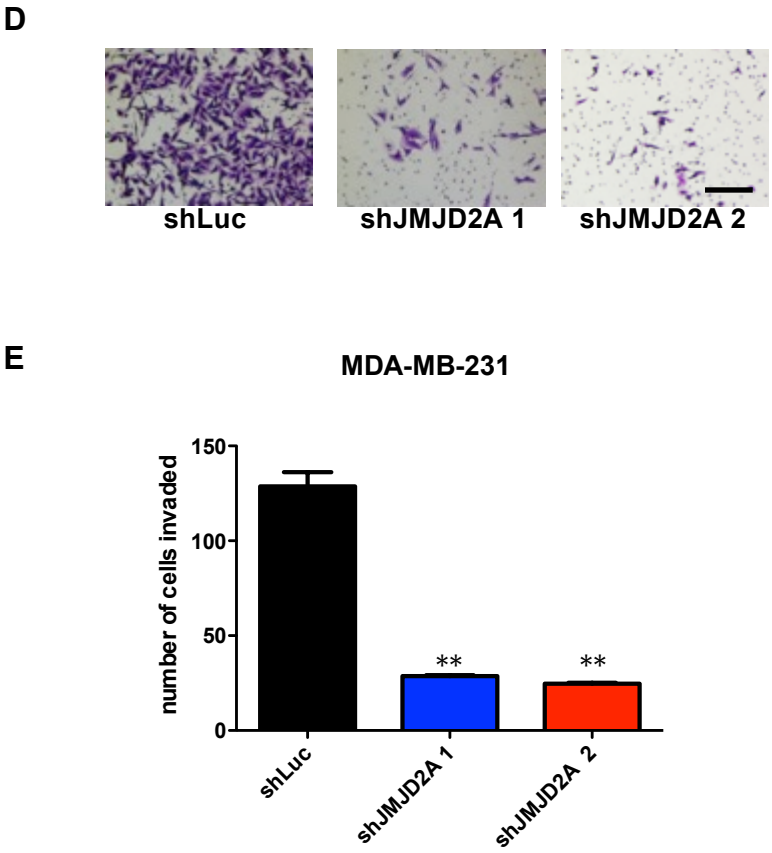


Figure 3-16. JMJD2A has an oncogenic role *in vitro* (continued)

(D) Representative images of invasion assay of MDA-MB-231 cells with stable control (shLuc) and JMJD2A knockdown.

(E) Bar graph to quantitate the number of cells invasion in **(D).** The result is shown as means ± s.d. (n=3). **p<0.01. Scale bar, 100 μm.

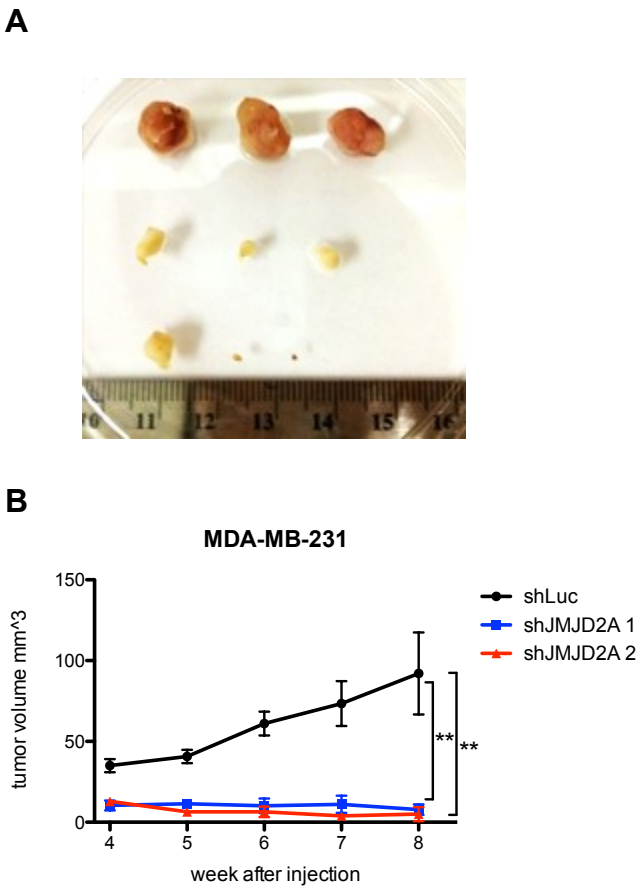
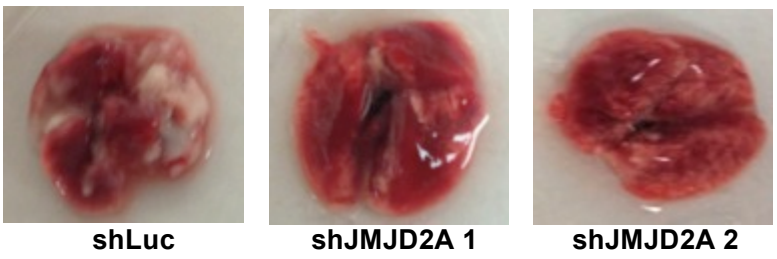


Figure 3-17. JMJD2A affects tumor growth *in vivo*

(A) MDA-MB-231 cells with stable knockdown of control (shLuc) and JMJD2A were subcutaneously injected into nude mice. Tumors were isolated after 8 weeks.

(B) Tumors were measured weekly by the caliper, and the result shown as means ± s.d. (n=3). **p<0.01

A



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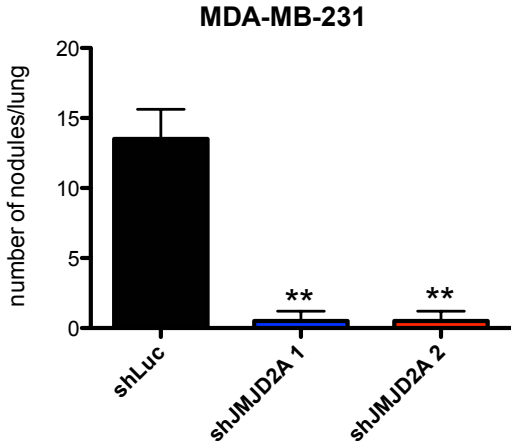


Figure 3-18 JMJD2A affects lung metastasis *in vivo*

(A) MDA-MB-231 with stable control (shLuc) and JMJD2A knockdowns were injected into the nude mice through tail vein. Representative images are shown for metastatic lung nodules.

(B) Metastatic lung lesions were counted. The result is shown as means ± s.d. (n=3). **p<0.01

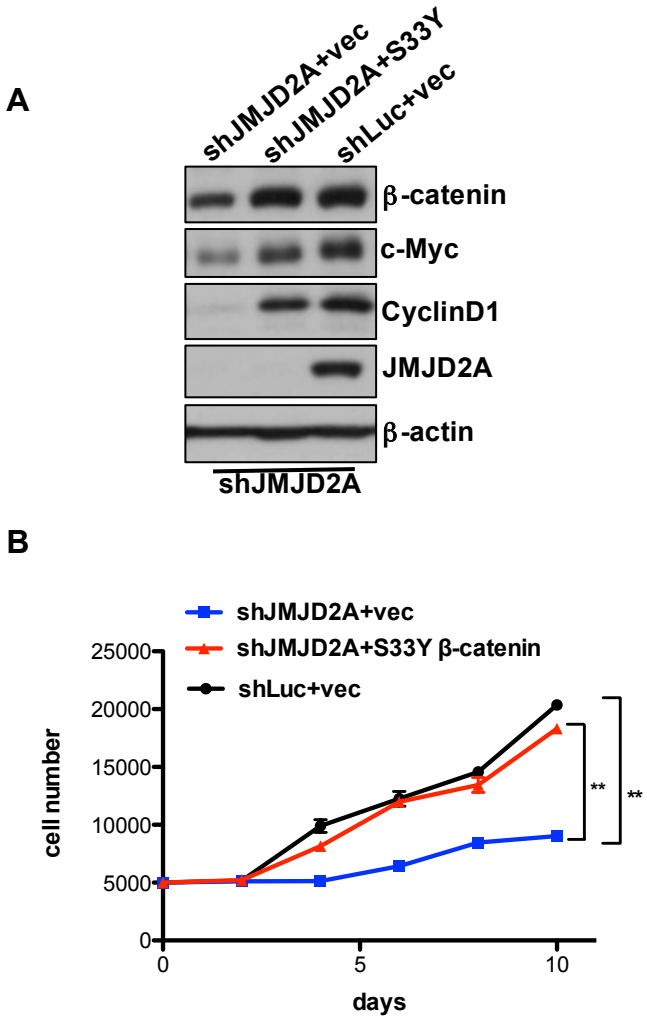
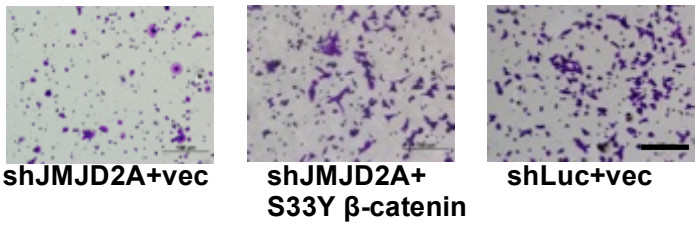


Figure 3-19. JMJD2A is essential for oncogenic phenotypes through stabilizing β-catenin.

(A) MDA-MB-231 shJMJD2A cells were stability infected with empty vector or S33Y non-degradable β-catenin. Cells were then subjected to immunoblotting to examine the efficiency of β-catenin restoration and the expression of its downstream target genes.

(B) Cells in **(A)** were used for cell proliferation assay. 5000 cells for each sample were seeded in triplicates. Cell numbers were counted every 2 days for 10 days in total. The result is shown as means ± s.d. (n=3). **p<0.01

C



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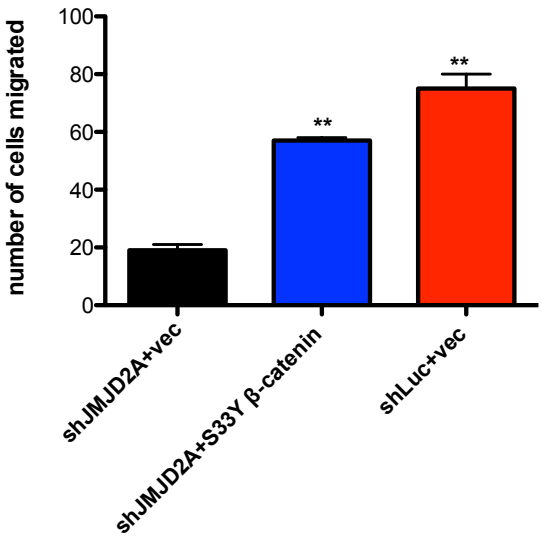


Figure 3-19. JMJD2A is essential for oncogenic phenotypes through stabilizing β-catenin (continued).

(C) Representative images of migration assay of MDA-MB-231 shLuc stably expressing empty vector, shJMJD2A expressing empty vector and shJMJD2A expressing the degradation resistant mutant S33Y β-catenin as in **(A)**.

(D) Bar graph to quantitate the number of cells migrated in **(C).** The result is shown as means ± s.d. (n=3). **p<0.01. Scale bar, 100 μm.

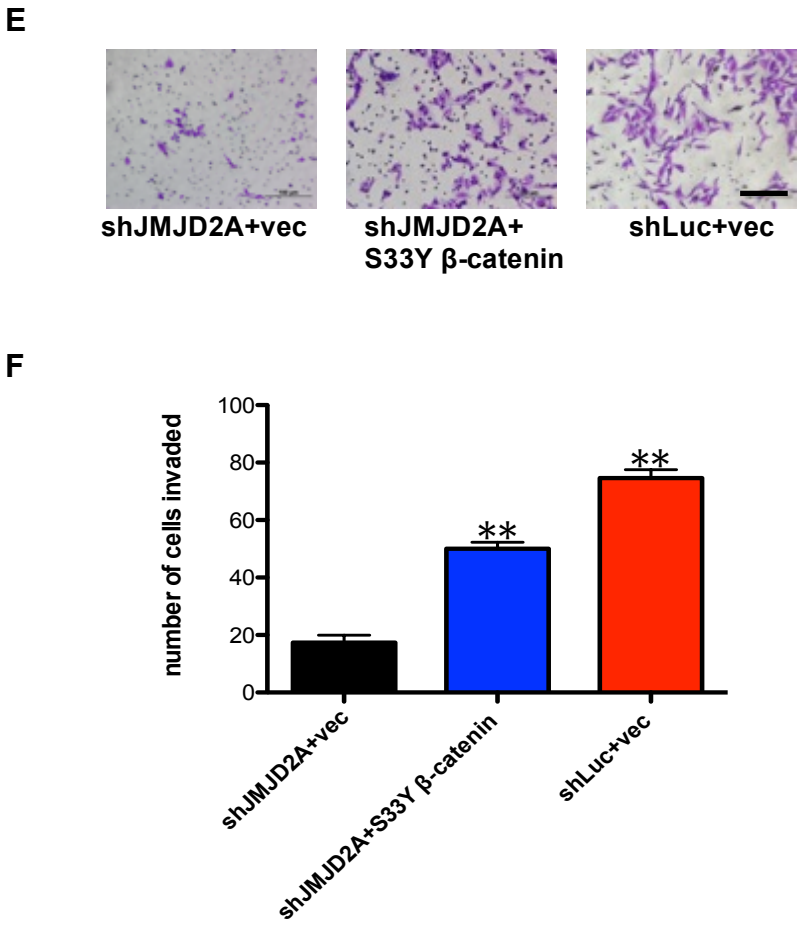


Figure 3-19. JMJD2A is essential for oncogenic phenotypes through stabilizing β-catenin (continued).

(E) Representative images of invasion assay of MDA-MB-231 shLuc stably expressing empty vector, shJMJD2A expressing empty vector and shJMJD2A expressing the degradation resistant mutant S33Y β-catenin as in **(A)**. Scale bar, 100 μm.

(F) Bar graph to quantitate the number of cells invaded in **(C).** The result is shown as means ± s.d. (n=3). **p<0.01.

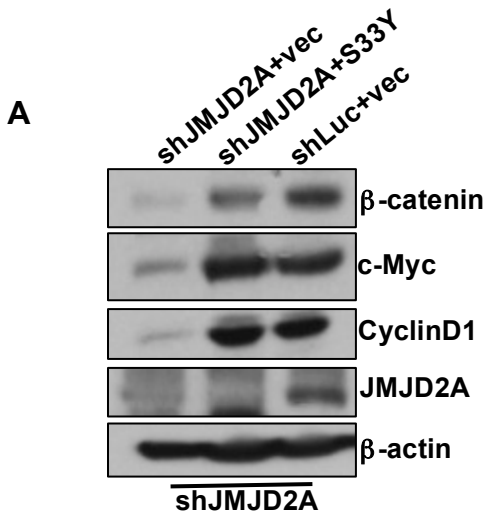
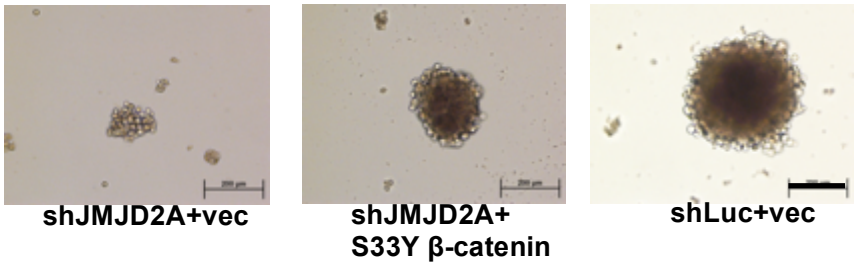


Figure 3-20. JMJD2A is essential for cancer stem cell maintenance through stabilizing β-catenin

(A) Hep3B shJMJD2A cells were stability infected with empty vector or S33Y non-degradable β-catenin. Cells were then subjected to immunoblotting to examine the efficiency of β-catenin restoration and the expression of its downstream target genes.

B



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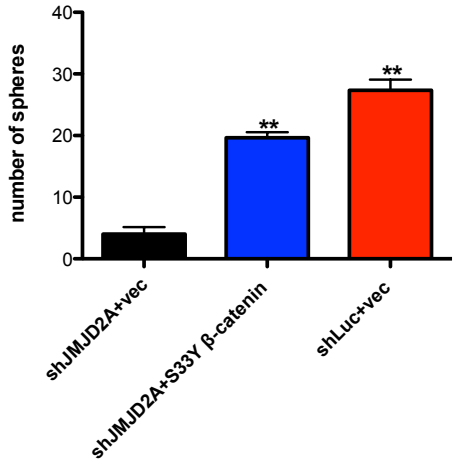

Figure 3-20. JMJD2A is essential for cancer stem cell maintenance through stabilizing β-catenin (continued)

(B) Representative images of sphere formation of Hep3B shLuc stably expressing empty vector, shJMJD2A expressing empty vector and shJMJD2A expressing the degradation resistant mutant S33Y β-catenin as in **(A)**. Scale bar, 200 μm.

(C) Bar graph to quantitate the number of sphere formation in **(B).** The result is shown as means ± s.d. (n=3). **p<0.01.

D

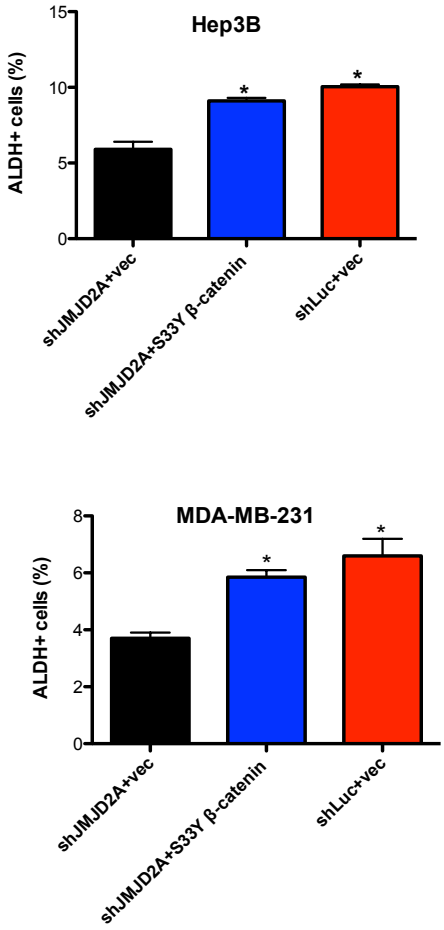

Figure 3-20. JMJD2A is essential for cancer stem cell maintenance through stabilizing β-catenin (continued).

(D) Quantitative results of ALDH+ cell populations in Hep3B and MDA-MB-231 shLuc stably expressing empty vector, shJMJD2A expressing empty vector and shJMJD2A expressing the degradation resistant mutant S33Y β-catenin. The result is shown as means ± s.d. (n=3). *p<0.05.

Chapter 4

Discussion

JMJD2A regulates chromatin organization by demethylating histone lysine residues. This alteration in the compaction of chromatin leads to changes in gene transcription. Recently, the function of JMJD2A in cancer has started to emerge. Most of these studies have focused on the role of JMJD2A in regulating gene transcription, which eventually leads to aberrant expression of oncogenes or tumor suppressors. In these studies, the oncogenic function of JMJD2A relies on its demethylase enzyme activity to change the histone methylation status of its target genes. However, whether the enzyme activity of JMJD2A is absolutely required to exert its oncogenic activity remains unclear. Recently, there have been few studies that unexpectedly found that JMJD2A may act through protein-protein interaction to elicit cancer progression, which is independent of its catalytic activity (Li et al., 2014; Mallette et al., 2012a). Searching for non-catalytic functions of JMJD2A and its binding partners may increase our knowledge in designing inhibitors that target not only its enzymatic activity, but also protein-protein interactive domain to fully thwart tumorigenesis caused by JMJD2A.

In this study, we unveil that other than functioning as a histone-modifying enzyme, JMJD2A, acting independently of its enzyme activity, participates in the canonical Wnt/β-catenin signaling transduction pathway. The Wnt/β-catenin pathway is known to promote cancer progression through the nuclear accumulation of β-catenin for it to serve as a coactivator in the nucleus for TCF1 to turn on gene transcription. Our results show that the presence of JMJD2A is essential for regulating the protein stability of β-catenin, thereby controlling the

expression of β-catenin target genes. In the nucleus, β-catenin is recruited to the promoters of Wnt target genes, and through cooperating with the TCF transcription factor and recruiting other coactivators such as BCL9, MLL1/2, and p300/CBP, they serve as essential factors to turn on the Wnt target gene transcriptions. Even though JMJD2A is known as a histone-modifying protein that regulates gene transcription, however, supported by our data, JMJD2A itself does not regulate the transcription of Wnt target genes through modulating histone methylation since its enzyme activity is not needed (Figure 3-6). The restoration of constitutive active, non-degradable β-catenin in JMJD2A knockdown cells rescued the expression of downstream target genes (Figure 3-8B), suggesting that JMJD2A affects Wnt target genes through regulating the protein stability of β-catenin.

Without Wnt ligand stimulation, the molecular mechanism of β-catenin degradation occurs through several steps of sequential event (*Introduction 1.8).* Nevertheless, our data suggest that JMJD2A deficiency drives this degradation event, even under Wnt stimulation. Without JMJD2A, the interaction between β-catenin and the other components of the destruction complex, such as Axin1 and GSK3β, increases (Figures 3-9A,B). This increased binding causes higher GSK3β-mediated phosphorylation on β-catenin (Figure 3-8A). This phosphorylation motif is considered as the “degron” that can be recognized by the F-box E3 ligase β-TrCP, which targets its substrate protein for K48-linked proteasomal ubiquitination and degradation. Consistently, we observed that the increased phosphorylation on β-catenin, caused by JMJD2A ablation, enhanced

its binding with β-TrCP, which eventually leads to higher level of β-catenin ubiquitination (Figures 3-10A,B). The presence of JMJD2A, thus, becomes relevant in maintaining the stability of β-catenin, and may serve as a negative regulator for the binding between the destruction complex and β-catenin for subsequent β-catenin nuclear accumulation. Some unaddressed questions still remain. What is the role of JMJD2A in the destruction complex? What is the mechanism that JMJD2A utilizes to release β-catenin from being targeted by the destruction complex? Besides residing in the cytosol, the destruction complex components can also be anchored at the cellular membrane once cells are stimulated with Wnt ligands, resulting in the release of β-catenin from the destruction complex (Gao et al., 2014). By using immunofluorescence and membrane fractionation assays, we have data to indicate that JMJD2A can also be localized in the cellular membrane, and that JMJD2A interacts with GSK3β endogenously as well (data not shown). One possible mechanism could be that upon Wnt ligand treatment, JMJD2A could be localized to the cellular membrane, while also bringing GSK3β to the membrane to phosphorylate LRP5/6 Wnt signaling receptor. Without JMJD2A, Axin1 itself may not be sufficient enough to relocate GSK3β to the membrane to phosphorylate LRP5/6 receptor, thereby releasing β-catenin from the destruction complex. Another possibility is that GSK3β and JMJD2A may compete each other for β-catenin binding. GSK3β binds to the “degron” motif of β-catenin to mediate its phosphorylation. And since JMJD2A also binds to β-catenin, it is likely that JMJD2A binds to the “degron” motif to prevent GSK3β-mediated

phosphorylation. Future experiments will be needed to address these questions to get a clearer mechanism on how JMJD2A keeps the destruction complex from interacting with β-catenin.

Since JMJD2A is known as a histone-modifying protein, previous study has demonstrated that JMJD2A should mainly be localized in the nucleus while having a marginal fraction in the cytosol (Zhang et al., 2005). Nevertheless, our findings have surprisingly revealed that in the resting cells, without Wnt ligand stimulation, both JMJD2A and β-catenin are mainly localized in the cytosol. In unstimulated cells, JMJD2A and β-catenin are found to interact with each other in the cytoplasmic pool, and this interaction can be heightened when the Wnt signaling pathway is activated. Interestingly, a closer analysis shows that upon Wnt ligand stimulation, both β-catenin and JMJD2A translocate to the nucleus and the intensity of their interaction increases in the nucleus. Consistent with our previous observation that the enzyme activity of JMJD2A is not needed, deletion of the JMJD2A catalytic domain still interacted with β-catenin, similar to that of the WT. Together, our findings suggest that Wnt activation not only drives the nuclear accumulation of β-catenin, but as well as JMJD2A nuclear translocation.

Although the nuclear localization of β-catenin has been established for nearly twenty years, the exact nuclear import route that β-catenin undertakes has not been clear. Some studies indicate that β-catenin binds with the nuclear pore complex, whereas others propose that the other coactivators (Bcl9/Pygopus) of the TCF complex are important to recruitment β-catenin to the (Behrens et al., 1996; Townsley et al., 2004). In our study, we demonstrate that while the

presence of JMJD2A governs the nuclear translocation of β-catenin, the exact mechanism still remains to be found. Since JMJD2A is known to possess two NLS sequences, we postulated that JMJD2A may bring β-catenin into the nucleus through the traditional nuclear import machinery. To test this hypothesis, we deleted the two bipartite NLS sequences that reside within the C-terminal of JMJD2A. However, deletion of the NLS sequences in JMJD2A did not inhibit its nuclear translocation or the nuclear movement of β-catenin (data not shown). This means that the translocation of JMJD2A and β-catenin do not rely on the traditional nuclear import machinery. This raises the possibility that protein-protein interaction may be involved in mediating their nuclear accumulation. Future studies should focus on deciphering the mechanism of how JMJD2A induces the translocation of β-catenin through protein-protein interaction. One approach to accomplish this would be to generate truncations of JMJD2A to map for its binding region with β-catenin. We found out that the individual deletion of each of the four domains in JMJD2A did not affect its binding with β-catenin (data not shown). Therefore, different fragments that exclude larger regions of JMJD2A should be used to study their interaction (Figure 4-1). Once we identify the binding region, this truncation mutant of JMJD2A can be used to gauge the nuclear translocation of β-catenin to study whether this protein-protein interaction is responsible in promoting the nuclear localization of β-catenin. Furthermore, this binding region motif can potentially provide a consensus sequence of JMJD2A to guide future studies in searching for other non-histone protein binding partners to exert its oncogenic role.

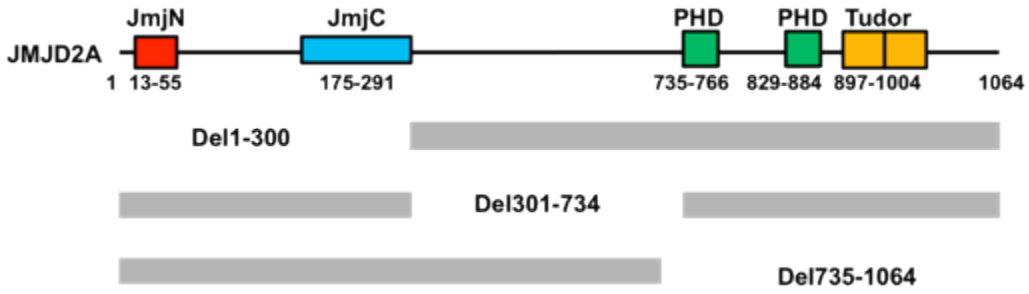

Figure 4-1: JMJD2A truncation fragment scheme.

Diagram represents the truncated fragments that we will use to study the JMJD2A-β-catenin interaction. The first fragment excludes the JmjN and JmjC domains. The second fragment excludes that linker region between catalytic core (JmjN and JmjC) and the site-recognition domains (PHD and Tudor). The third fragment excludes both the PHD and Tudor domains.

Another question in the JMJD2A field is how it shuttles between cytosol and nucleus. Although JMJD2A is endowed with two NLS sequences, there has not been any clear indication that they are responsible for the nuclear translocation of JMJD2A (Tan et al., 2008). Some studies suggest that, just like β-catenin, the nuclear movement of JMDD2A may be mediated by protein-protein interaction (Caceres et al., 1997; Furuyama and Bruzik, 2002). Since our data strongly suggest that the nuclear translocation of β-catenin relies on the expression of JMJD2A, one conundrum to be answered is whether the JMJD2A-β-catenin interaction is also important for the nuclear translocation of JMJD2A. To do this, we can generate stable β-catenin knockdown cells to examine the nuclear-cytoplasmic movement of JMJD2A.

In our study, we were able to demonstrate, by using *in vitro* cell lines, that JMJD2A has a role in maintaining cancer stem cell traits and population through stabilizing β-catenin. To extend this observation, we should further conduct *in vivo* experiment. We can use our previously generated *in vitro* cell lines to isolate ALDH+ cells for nude mice subcutaneous injection to observe tumor engraftment incidence and growth.

Although by utilizing *in vitro* cell lines, we were able to show that JMJD2A is a critical mediator in the Wnt signaling pathway. Genetically engineered mouse models are still lacking to ultimately attest this notion. Like mentioned in the previous sections, *Jmjd2a* conventional knockout mice are embryonic lethal since it has a stellar function in development. This has hindered additional analyses to characterize the oncogenic function of JMJD2A *in vivo*. Up to date, only one

group has generated the JMJD2A heart conditional knockout mice by breeding *Jmjd2a*$^{fl/fl}$ with α-*MHC-Cre* (Zhang et al., 2011). By exploiting the technology of conditional knockout *Cre-lox* system, we can knockout the expression of JMJD2A in different mouse tissues and systems that are known to be tightly regulated by the Wnt signals, such as hair follicles, neural and hematopoietic systems (van Amerongen and Berns, 2006). The generation of these conditional knockout mice could potentially provide the final verdict on the importance of JMJD2A in mediating the activity of the Wnt/β-catenin signaling transduction *in vivo*. The generation of these mice could also be used to test JMJD2A inhibitors in the future.

Since previous studies have already generated solid data to demonstrate that JMJD2A exerts its oncogenic capability through its catalytic activity, many efforts have focused on designing inhibitors to target its enzymatic core (Guerra-Calderas et al., 2014). However, some previous studies, and our current study all have proven that the function JMJD2A in cancer is beyond its catalytic function, and its protein-protein interaction is also central for its oncogenic role. Therefore, these catalytic-targeting inhibitors would not completely shut down JMJD2A activities. The therapeutic effort should also be focused on designing small molecule inhibitors that can disrupt the binding region of JMJD2A with other proteins to completely block its oncogenic functions. By using the crystal structure, we can identify the protein-protein interactive “hot spots”, which are residues that contribute more to protein-protein binding than others, for JMJD2A. Computational and high-throughout screening methods can then be used to

identify compounds that are highly selective and specific. There are many effective and promising inhibitors that have been identified by using this approach (Arkin et al., 2014; Chan et al., 2013).

In summary, our study presents a new non-enzymatic and non-histone role for histone demethylase JMJD2A in mediating the activity of Wnt/β-catenin signaling pathway. In our model, JMJD2A is important in maintaining the protein stability of β-catenin and its nuclear translocation. JMJD2A also has crucial roles in tumorigenesis and cancer stem cells maintenance through stabilizing β-catenin. Our study highlights a new oncogenic role of JMJD2A in signaling transduction pathway and provides therapeutic strategy to target protein-protein interaction interface of JMJD2A.

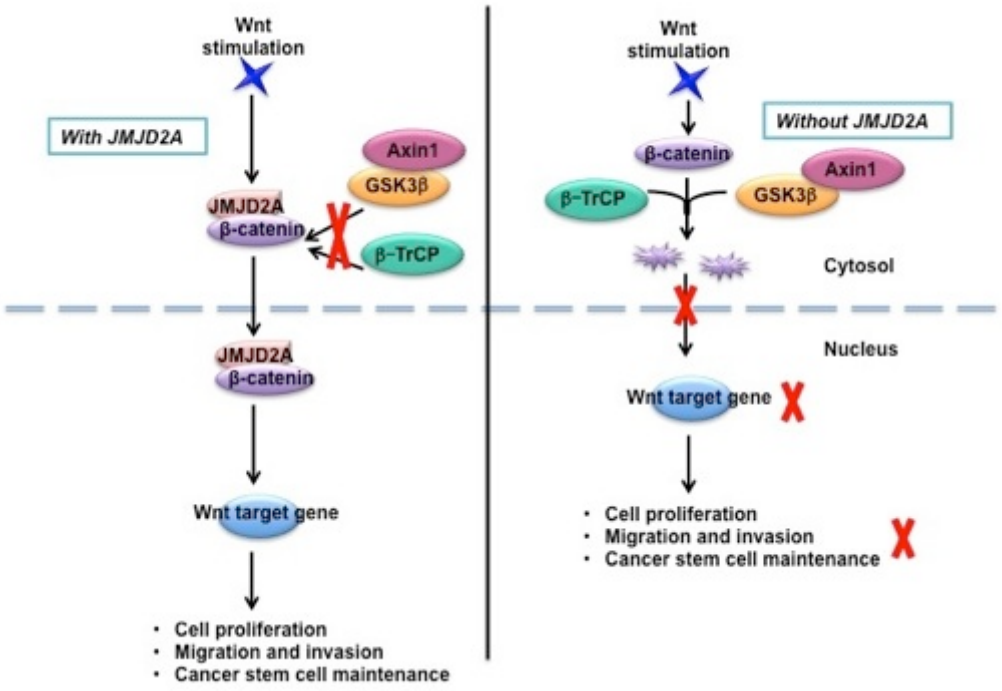

Figure 4-2. Schematic diagram of the working model.

In cells with JMJD2A protein expression (left panel), Wnt treatment induces the canonical Wnt/β-catenin signaling activation. JMJD2A binds to β-catenin, and stabilizes its expression by preventing it from binding to the destruction complex. The two then translocate to the nucleus, where β-catenin acts as an coactivator to turn on Wnt target gene expressions, which eventually leads to increased cell proliferation, migration, invasion and cancer stem cell maintenance. On the other hand, when cells are depleted from JMJD2A expression (right panel), under Wnt stimulation, β-catenin still binds to the destruction complex, which eventually leads to its degradation, hence shuts down the Wnt signaling pathway.

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Vita

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