EPIGENETIC REGULATION OF \textit{Hoxa1} AND \textit{Hoxa2}

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Graduate Studies and Research
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Pharmacy Graduate Program
University of Saskatchewan
Saskatoon, Saskatchewan
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ABSTRACT

*Hoxa1* and *Hoxa2* are master regulators in the development of hindbrain, ear, palate, bone and cardiovascular development. There is little information on the epigenetic regulator(s) of *Hoxa2* gene during development. In this thesis, I have determined whether regulation of *Hoxa2* is occurring via a specific epigenetic pathway, and investigated the role of DNA methylation, noncoding RNAs (microRNAs and long non-coding RNAs) and histone protein modification.

First, analysis of *Hoxa2* promoter revealed the presence of three CpG islands near the *Hoxa2* 5′ regulatory region. Using methylation specific PCR (MSP) and the bisulfite specific PCR (BSP) primers followed by DNA sequencing, I found the methylation status of CpG island 1 remains unmethylated and that the DNA methylation status of the *Hoxa2* promoter does not change with the spatio-temporal expression of *Hoxa2* during palatogenesis. These findings indicate that DNA methylation does not appear to play a key role in the epigenetic regulation of *Hoxa2* gene during palatogenesis.

My second objective was to determine whether specific miRNAs impact *Hoxa2* expression. I performed in-silico analysis and identified six miRNAs that have the potential to bind 3′UTR of the *Hoxa2* gene. The miR-669b and miR-376c were capable of down regulating *Hoxa2* expression at both transcriptional and translational level. Two direct miR-669b binding sites were identified on mouse *Hoxa2* 3′UTR. Luciferase assays showed that the two miR-669b binding sites appear to work independently of each other and that mutations within the seed sequences abrogated luciferase activity. I further analyzed the degree of sequence similarity of both miR-669b binding sites and found that binding site 1 is evolutionarily conserved between the five species (human, mouse, rat, chimpanzee and dog). In the developing mouse palate (from E13 to E15), miR-669b showed a complementary expression to that of *Hoxa2*. No direct interaction between miR-376c
and Hoxa2 3'UTR was identified. Thus my results indicated that the miR-669b likely plays a role in regulating Hoxa2 expression during palate development.

My third objective was to characterize a new lncRNA (mHotairm1) that I identified between mouse Hoxa1 and Hoxa2 intergenic region. I demonstrated that mHotairm1 is involved in recruiting MLL1/WDR5 to Hoxa1 and Hoxa2 genes and regulating their expressions. In situ hybridization histochemistry of E14 developing palate showed expression of mHotairm1 in medial edge epithelia (MEE), indicating mHotairm1 may play a role in the palatal fusion. Downregulation of mHotairm1 in NIH 3T3 cells resulted in significantly decreased expression of Hoxa1 and Hoxa2 expression, whereas treatment with ATRA resulted in increased expression of mHotairm1, Hoxa1 and Hoxa2. Using capture hybridization analysis of RNA targets (CHART) and pull down assays, I found that the TrxG protein WDR5 is associated with mHotairm1, and knockdown of mHotairm1 resulted in reduced occupancy of gene activation mark H3K4me3 and increased occupancy of gene suppression mark H3K27me3, suggesting MLL1/WDR5 complex may be playing a role in the regulation of Hoxa1 and Hoxa2 gene expression through mHotairm1.

Lastly, I found that WDR5 was sumoylated. This modification appears to be important for its interaction with mHotairm1 and MLL and for its cellular distribution, primarily to the nuclei. Following ATRA treatment, although the total WDR5 protein remained unchanged, an increase in sumoylated WDR5 was observed together with increased expression of mHotairm1, Hoxa1 and Hoxa2 gene. These findings reveal that sumoylated WDR5 with its interaction with mHotairm1 plays an important role in H3K4me3 and H3K27me3 occupancy and influencing the epigenetic regulation of Hoxa1 and Hoxa2 genes.
ACKNOWLEDGEMENTS

First I would like to acknowledge the support of my supervisor Adil J. Nazarali. Without him I would never have such great chance to study abroad. During my study at University of Saskatchewan, he gave me great support and guidance in both my academic and personal life.

I would also like to thank my external examiner Dr. Yuewen Gong for attending my defence and my committee members, Dr. Jane Alcorn, Dr. Ildiko Badea, Dr. Stanley Moore and Dr. Yuliang Wu, for their suggestions throughout my PhD study. This thesis would not have been possible without their support.

I would like to give my thanks to my lab members, especially Shaoping Ji. He generously provided me suggestions and supports on my experiments.
DEDICATION

This thesis is dedicated to all my friends and family. In particular, I would like to dedicate this thesis to my parents, Chaoyuan Bi and Liying Wei who supported me mentally and financially during my study.
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LIST OF ABBREVIATIONS

3' UTR – 3' Untranslated Region
5-Aza-CdR – 5-aza-20-Deoxycytidine
5hmC – 5-Hydroxymethylcytosine
5mC – 5-Methylcytosine
A – Adenine
Ab/Am – Antibiotic/Antimycotic
Abd-A – Abdominal-A
Abd-B – Abdominal-B
ABDS – Athabascan Brainstem Dysgenesis Syndrome
AdoMet or SAM – S-Adenosyl-l-Methionine
AGO – Argonaute
ANT-C – Antennapedia Complex
AP – Anterior-Posterior
ASD – Autism Spectrum Disorder
ASH1 – Absent Small or Homeotic Discs 1
ASH2L – Absent Small or Homeotic 2-Like
ATRA – All-trans Retinoic Acid
BCS – Bovine Calf Serum
BD – Bromo Domain
BER – Base Excision and Repair
BIG-3 – BMP-2 Induced Gene 3kb
Bmp7 – Bone Morphogenetic Protein 7
BSA – Bovine Serum Albumin
BSAS – Bosley–Salih–Alorainy Syndrome
BSP – Bisulfite Specific PCR
BX-C – Bithorax Complex
C – Cytosine
CBP – CREB-Binding Protein
CHART – Capture hybridization analysis of RNA targets
CHD8 – Chromodomain, Helicase, DNA-binding 8
ChIP – Chromatin Immunoprecipitation
CNCC – Cranial Neural Crest Cell
CNS – Central Nervous System
CREB – cAMP-Response Element Binding
CxxC – Cysteine x x Cysteine (x – any amino acid)
D – Aspartic Acid
DIG – Digoxigenin
DNMT – DNA Methyltransferase
DMEM – Dulbecco's Modified Eagle Medium
E – Embryonic Day
E1A – Early-Region 1A
EOC – Epithelial Ovarian Cancer
ES – Embryonic Stem
ESC – Embryonic Stem Cell
EZH2 – Enhancer of Zeste Homolog 2
FAM – Fluorescein Amidite

FGF1 – Fibroblast Growth Factor 1

FGF18 – Fibroblast Growth Factor 18

Fgfr3 – Fibroblast Growth Factor Receptor 3

Foxd3 – Forkhead Box D3

gDNA – Genomic DNA

GNAT – GCN5-related N-acetyltransferase

H3K4me3 – Trimethylation of Histone 3 lysine 4

H3K4me2 – Dimethylation of Histone 3 lysine 4

H3K27me3 – Trimethylation of Histone 3 lysine 27

H3T11P – Histone 3 Threonine 11 Phosphorylation

HAT – Histone Acetyltransferase

HDAC – Histone Deacetylase

HKMT – Histone Lysine Methyltransferase

hLuc – Firefly Luciferase

HMTase – Histone Methyltransferase

Hmx1 – H6 Family Homeobox 1

Hnf1b – Hepatocyte Nuclear Factor 1-Beta

HOM-C – Homeotic Complex

hRLuc – Renilla Luciferase

HRP – Horse Radish Peroxidase

HUVEC – Human Umbilical Vein Endothelial Cell

IFN – Type I Interferons
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<td>Insulin-like growth factor 1 receptor</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>ISH</td>
<td>In Situ Hybridization</td>
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<tr>
<td>ISWI</td>
<td>Imitation Switch</td>
</tr>
<tr>
<td>iPS</td>
<td>Induced Pluripotent Stem</td>
</tr>
<tr>
<td>JmjC</td>
<td>Jumonji C</td>
</tr>
<tr>
<td>KAT</td>
<td>Lysine Acetyltransferase</td>
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<tr>
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<tr>
<td>KDM</td>
<td>Lysine Demethylase</td>
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<tr>
<td>lacZ</td>
<td>β-galactosidase</td>
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<tr>
<td>Lhx5</td>
<td>LIM Homeobox 5</td>
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<tr>
<td>LncRNA</td>
<td>Long Noncoding RNA</td>
</tr>
<tr>
<td>MBD</td>
<td>Methyl-CpG Binding Domain Protein</td>
</tr>
<tr>
<td>MBP</td>
<td>Methyl-CpG Binding Protein</td>
</tr>
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<td>MEE</td>
<td>Medial Edge Epithelia</td>
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MSP – Methylation Specific PCR
Msx1 – Msh Homeobox 1
Nab1 – NGFI-A Binding Protein 1
NEM – N-Ethylmaleimide
NCOA – Nuclear Receptor Co-activator
NPC – Nuclear Pore Complex
NSL – Nonspecific Lethal
nt – Nucleotide
ORF – Open Reading Frame
PA – Pharyngeal Arch
Pax9 – Paired Box 9
PBA – 4-Phenylbutyric Acid
PcG – Polycomb Group
PHB – PHABULOSA
PHD – Plant Homeodomain
PRC – Polycomb Repressive Complex
PRE – PcG Response Element
pre-miRNA – miRNA Precursors
pri-miRNA – Primary transcript of miRNA
PRMT6 – Protein Arginine Methyltransferase 6
pRNA – Promoter-Associated RNA
PTC – Papillary Thyroid Carcinoma
PTH – Parathyroid Hormone
qRT-PCR – Quantitative Real-Time PCR
RACE – Rapid Amplification of cDNA Ends
RBBP5 – Retinoblastoma Binding Protein 5
rDNA – Ribosomal DNA
RIPA – Buffer Radioimmunoprecipitation Assay Buffer
RT – Reverse Transcription
RQ – Relative Quantity
SAE – SUMO Activation Enzyme
SAR – Ring Finger-Associated
SET – Suppressor of Variegation-Enhancer of Zeste-Trithorax
snRNA – Small Nuclear RNA
Sox6 – SRY (Sex Determining Region Y)-Box 6
SRC – Steroid Receptor Coactivator
SSC – Saline Sodium Citrate
SUMO – Small Ubiquitin-Like Modifier
SWI/SNF – Switch/Sucrose Nonfermentable
T – Thymine
TAD – Transactivation Domain
TET – Ten-eleven Translocation
TDG – Thymine DNA Glycosylase
TF – Transcription Factors
TGFβ – Transforming Growth Factor β
TRE – TrxG Response Element
TrxG – Trithorax Group
Tsg – Twisted Gastrulation
U – Uracil
Ubx – Ultrabithorax
Uch11 – Ubiquitin Carboxyterminal Hydrolase L1
VISA – Virus-Induced Signaling Adaptor
VNC – Ventral Nerve Cord
W – Tryptophan
WDR5 – WD Repeat Domain 5
WDS – WDR5 in Drosophila
Wnt5a – Wnt Family Member 5a
XCI – X-Chromosome Inactivation
Xi – Inactive X-chromosome
Xa – Active X-chromosome
Zic1 – Zic Family Member 1
CHAPTER 1

1. Summary

_Hox_ genes are evolutionarily conserved homeodomain-containing transcription factors that specify cell identity in early development (McGinnis and Krumlauf, 1992; Banerjee-Basu and Baxevanis, 2001; Mallo and Alonso, 2013). In addition to their role in embryo development, _Hox_ genes are also involved in different types of human diseases and cancer including leukemia (Alharbi et al., 2013) and breast cancer (Bhatlekar et al., 2014). In vertebrates, 39 _Hox_ genes are organized into four _Hox_ gene clusters (HoxA-D) and they encode _Hox_ proteins that regulate specific morphological diversity along the anterior-posterior (AP) axis (Akin and Nazarali, 2005; De Kumar and Krumlauf, 2016.) _Hoxa1_ and _Hoxa2_ are the first _Hox_ genes to be expressed during embryonic development (Murphy and Hill, 1991). _Hoxa1_ plays a role in hindbrain, inner ear and cardiovascular development (Makki and Capecchi, 2011; Qiao et al., 2015; Makki and Capecchi, 2012) and _Hoxa2_ is important for the development of inner ear, palate, hindbrain and bone (Minoux et al., 2013; Smith et al., 2009; Gavalas et al., 1997; Kitazawa et al., 2015). The proper expression of _Hox_ genes is important in many biological processes, making regulation of _Hox_ genes critical to living organisms. There is little information available on the mechanism(s) involved in the regulation of _Hoxa2_ gene expression. Hence, my research has involved in an investigation of the epigenetic regulation _Hoxa2_ gene expression during mouse palate development and in the NIH 3T3 cell line.

Epigenetics refers to the heritable changes in gene expression without any changes in the DNA sequence. Regulation of gene expression can occur via several mechanisms that include
noncoding RNAs (e.g. microRNAs and long non-coding RNAs), biochemical modifications of DNA such DNA methylation or via histone protein modifications (Goldberg et al., 2007).

DNA methylation which can occur on cytosine bases in CpG rich sequences and especially at the promoter regions can lead to gene silencing through the inhibition of transcription factor binding and the changing of chromatin structure into a repressive state (Domcke et al., 2015). Many studies have shown that proper DNA methylation is essential in embryonic development (Bird, 2002; Santos et al., 2005; Bartolomei and Ferguson-Smith, 2011; Lomvardas and Maniatis, 2016). Research in our laboratory has identified an important role of Hoxa2 in mouse palate development (Smith et al., 2009; Smith et al., 2013). In my study, I chose to investigate whether DNA methylation played a role in regulating the expression of Hoxa2 in early mouse palate development. Three CpG islands were found to be situated in the Hoxa2 promoter region and their methylation status did not change during the temporal expression of Hoxa2 at any stages of palatal development. Further experiments were conducted to investigate the role of miRNA and long non-coding RNA in the regulation of Hoxa2 expression.

microRNAs (miRNA) are ~22 nucleotide RNAs that can guide a RNA silencing pathway to regulate gene expression through their effect on messenger RNA (mRNA) levels (Usmani et al., 2016). The mature miRNAs interact with target mRNAs mostly within the 3' untranslated region (3' UTR) based on sequence complementarity between the miRNA and its target mRNA (Ha and Kim, 2014). Several Hox genes are regulated by miRNAs (Garaulet and Lai, 2015; Liu et al., 2013; Tang et al., 2014; Han et al., 2016); however, only one miRNA, miR-3960, has been identified to regulate Hoxa2 gene expression in primary mouse osteoblasts (Hu et al., 2011). miR-669b has previously been found to be able to bind to the 3’UTR of insulin-like growth factor1 receptor (IGF1R) and down regulate its expression (Liang et al., 2011). I performed in
silico analysis and predicted six miRNAs that can potentially bind Hoxa2 3'UTR, namely: miR-376c, miR-669b, miR-431, miR-19a, miR-298 and miR-878-3p. Experimentally, I found miR-669b and miR-376c can both down regulate Hoxa2 gene expression at the transcriptional and translational level. Moreover, two binding sites for miR-669b on Hoxa2 3' UTR were identified using a luciferase reporter assay, while no direct binding of miR-376c was found on the Hoxa2 3' UTR.

Hox genes are also regulated by epigenetic activators of the Trithorax group (TrxG) and epigenetic repressors of the Polycomb group (PcG) (Beck et al., 2010). The activation of Hox genes by TrxG can involve trimethylation of Histone 3 lysine 4 (H3K4me3) by mixed lineage leukemia 1 (MLL1)/ WD repeat domain 5 (WDR5) complex (Beck et al., 2010). Long noncoding RNAs (lncRNAs) have emerged with important regulatory roles in gene expression. LncRNAs HOTTIP and Mistral are both transcribed from Hox gene clusters and can introduce MLL1/WDR5 to nearby Hox gene promoters to induce H3K4me3 and activate gene expression (Wang et al., 2011, Bertani et al., 2011). HOTAIRM1 is an antisense intergenic transcript transcribed between human HOXA1 and HOXA2 (Zhang et al., 2009). It can activate HOXA genes that are located at the 3' end of the HOXA cluster (3' HOXA genes) and its presence is necessary during myeloid differentiation. However, HOTAIRM1 transcripts have not been reported in other species and little is known of how 3' HOXA genes are regulated by HOTAIRM1. In my study, I have identified a new transcript from the mouse HoxA cluster that shares sequence similarity with human HOTAIRM1, which we classify as the mouse Hotairm1 (mHotairm1). I further demonstrated that mHotairm1 can activate the expression of Hoxa1 and Hoxa2 by introducing MLL1/WDR5 to their promoters which in turn enhances H3K4me3 occupancy. Findings also indicate that WDR5 sumoylation could be important for its interaction with
*mHotairm1*. Taken together, my results provide additional evidence that connects lncRNAs to recruitment of epigenetic activators to promoters of target genes and for the first time provides a mechanism of the regulation of 3' *Hoxa* genes via *mHotairm1*. 
CHAPTER 2

2. Literature Review

In the following literature review, I will introduce Hox genes and the roles of Hoxa1 and Hoxa2 in development and in disease. I will also provide an overview of epigenetics and its impact on gene expression and regulation. Epigenetic mechanisms including DNA methylation, common histone post-translational modifications, miRNAs and lncRNAs, as well as the crosstalk between these will be discussed. In my PhD thesis work, sumoylation of a WDR5 protein appeared to be important in the histone methylation mediated Hox gene expression that is directed by lncRNA, hence sumoylation is also included in this literature review.

2.1 Hox genes

Homeobox genes were first discovered in the fruit fly Drosophila (Bridges and Morgan, 1923), and are evolutionarily conserved transcription factors that regulate specific morphological patterns and cellular diversity along the anterior-posterior (AP) axis (Lewis, 1978; Pearson et al., 2005; Seifert et al., 2015). Alterations in homeobox gene expression can result in the transformation of one body structure to resemble a homologous body structure in form and shape (Arlotta and Hobert, 2015; Tijchon et al., 2015). This phenomenon was first observed in Drosophila and was termed “homeotic transformation” (Lewis, 1978; Hueber and Lohmann, 2008).

In Drosophila, a single homeotic complex (HOM-C) located on chromosome 3 consists of two separate clusters, Bithorax complex (BX-C) and Antennapedia complex (ANT-C) (Fig. 2.1) (Lewis, 1978). The antennapedia class of homeobox genes are generally referred to as
“Hox” genes. Hox genes are highly conserved and clustered into multigene loci (Lewis, 1978; Ruddle et al., 1994; Santini et al., 2003; Yu et al., 2012; De Kumar and Krumlauf, 2016). In vertebrates, Hox genes have duplicated and evolved to generate multiple sets of paralogous genes which are organized in four separate chromosomal clusters (McGinnis and Krumlauf, 1992; Bailey et al., 1997; Akin and Nazarali, 2005; Duboule, 2007; De Kumar and Krumlauf, 2016). They function to govern embryonic morphogenesis as well as cell differentiation (Pineault and Wellik, 2014; Seifert et al., 2015). In the chromosomal cluster, all Hox genes are transcribed in the same 5’ to 3’ direction, and the more 3’ a Hox gene is located on the chromosome, the more anteriorly it is expressed in the developing embryo (Gaunt et al., 1988; Favier and Dollé, 1997; Lappin et al., 2006; Mallo and Alonso, 2013).

Figure 2.1. Schematic representation of Drosophila and murine Hox genes on chromosomal clusters. Hox genes are illustrated by black boxes. There are two clusters in the Drosophila HOM-C: Antennapedia (ANT-C) and Bithorax (BX-C). ANT-C is composed of: lab = labial, pb = proboscipedia, Dfd = Deformed, Scr = Sex Combs Reduced, and Antp = Antennapedia; BX-C is composed of: Ubx = Ultrabithorax, abd-A = abdominal A, Abd-B = Abdominal-B. There are 39 murine Hox genes present on four separate (Hox a, Hox b, Hox c, Hox d) chromosomal clusters. This figure is taken from Akin and Nazarali (2005) with permission.
Hox genes are very important as they regulate numerous pathways in embryonic development and other biological processes (Fig. 2.2). All homeobox genes contain a “homeodomain” which is defined as a class of protein domain that contain a conserved 60 amino acid region encoded by 180 bp homeobox sequence (Scott et al., 1989; Nazarali et al., 1992; Banerjee-Basu and Baxevanis, 2001; Anderson et al., 2012; Bürglin and Affolter, 2016). Hox proteins can enhance or suppress the expression of downstream genes through the affinity between its homeodomain and target DNA sequences (McGinnis and Krumlauf, 1992; Gehring, 1993; Kumar and Nazarali, 2001; Shah and Sukumar, 2010). The core structure of this domain consists of four alpha helices (Kissinger et al., 1990; Gehring et al., 1994; Akin and Nazarali, 2005; Bürglin and Affolter, 2016). The coordinate activity between the N-terminal region of the first helix and cofactors within a transcriptional complex is required for Hox proteins to control specific gene expression and affect cell division and cellular functions (Mann and Affolter, 1998; Akin and Nazarali, 2005; Mann et al., 2009). The second and third helix form an evolutionarily conserved helix-turn-helix motif that is responsible for the recognition and binding of target DNA sequences (Gehring et al., 1994; Akin and Nazarali, 2005; Bürglin and Affolter, 2016). The hydrophilic face of helix 3 contacts the target DNA sequence in the major groove (Kissinger et al., 1990; Banerjee-Basu and Baxevanis, 2001; Svingen and Tonissen, 2006; Bürglin and Affolter, 2016). In vitro analysis determined an element of approximately 10-12 bases with a core sequence of TAAT to be a core binding site for Hox proteins (Kissinger et al., 1990; Kumar and Nazarali, 2001; Akin and Nazarali, 2005; Breitinger et al., 2012; Beh et al., 2016).

In mice, there are four separate chromosomal clusters (Hox A, B, C, and D) composed of 39 genes in total (Fig. 2.1). These Hox genes are arranged in a 3´ to 5´ order in each cluster with synteny to the Drosophila HOM-C (Fig. 2.1), with sequence similarity between genes on
different clusters, as well as their positions on the chromosomes (Akin and Nazarali, 2005; Mallo and Alonso, 2013).

Figure 2.2. Examples of biological pathways that are regulated by Hox genes through their regulation of downstream targets. Several Hox-targeted genes that are important in development and cellular processes are shown. This figure is modified from Svingen and Tonissen (2006).

2.1.1 *Hoxa1* gene in embryonic development

In embryonic development, *Hoxa1* is one of the first *Hox* genes to be activated (Murphy and Hill, 1991). In mouse, starting from embryo day 7.5 (E7.5), *Hoxa1* expression can be detected in the primitive streak, in newly formed mesoderm, and overlying neuroectoderm (Fig. 2.3; Murphy and Hill, 1991). *Hoxa1* expression extends anteriorly, and between E7.75 and E8.25 its expression reaches the most anterior border in the presumptive hindbrain where its expression lasts for approximately 12 h (Murphy and Hill, 1991). By E8.5, *Hoxa1* has retreated from this region and remains in more posterior regions (Murphy and Hill, 1991; Makki and Capecchi, 2010). This expression pattern suggested a role of *Hoxa1* in hindbrain development. In fact,
*Hoxa1* knockout mice exhibit abnormal hindbrain development (Studer *et al.*; 1998; Helmbacher *et al.*, 1998; Tischfield *et al.*, 2005) and die shortly after birth from breathing defects (Chisaka *et al.*, 1992). *Hoxa1* null embryonic stem (ES) cells lack the ability to differentiate along a neural cell lineage following retinoic acid induction and express lower levels of neuronal differentiation markers compared to normal wild-type ES cells (Martinez-Ceballos and Gudas, 2008). *Hoxa1* acts upstream of four genes involved in hindbrain and neuron development: hepatocyte nuclear factor 1-beta (*Hnf1b*), forkhead box D3 (*Foxd3*), Zic family member 1 (*Zic1*) and LIM homeobox 5 (*Lhx5*) (Makki and Capecchi, 2011). *Hoxa1* has also been proposed as a candidate gene for autism spectrum disorder (ASD), a common neurodevelopmental condition in children and adolescents (Song *et al.*, 2011; Raznahan *et al.*, 2012). A specific *HOXA1*-A218G mutation has received particular attention in ASD since the A218G genotype has the capacity to modify the rate of cerebellar growth (Raznahan *et al.*, 2012).

![Schematic diagram of primitive streak and germ layers in mouse embryo.](https://o.quizlet.com/9elYyZW.TOVAN103uQIAqw_m.png)
In addition to the hindbrain, *Hoxa1* also affects inner ear development (Makki and Capecchi, 2010). Although researchers previously believed that *Hoxa1* expression was absent in mouse inner ear and the effects of loss of *Hoxa1* on inner ear development was indirect (Murphy and Hill, 1991), Makki and Capecchi (2010) reported significant *Hoxa1* cell lineage expression in the developing mouse otic epithelium, which raised the possibility that *Hoxa1* may have a direct role in inner ear patterning. Several genes regulating ear development have also been identified as downstream targets of *Hoxa1*. In *Hoxa1* null mice, paired box 8 (*Pax8*) and fibroblast growth factor receptor 3 (*Fgfr3*), known to be important in ear development, were identified as downstream targets of *Hoxa1* (Makki and Capecchi, 2011). A mutation in the *Hoxa1* coding sequence in pigs resulting in a truncated protein lacking the homeodomain also induced malformations of both the outer and middle ears. At the genetic level, this mutation of *Hoxa1* affected the expression of fibroblast growth factor 1 (*FGF1*) and *FGFR3* in the FGF signaling pathway, a pathway that is crucial in ear pinna development (Qiao *et al*., 2015).

Researchers have also demonstrated a previously unrecognized role of *HOXA1* in cardiovascular development in humans (Tischfield *et al*., 2005). This finding lends credence to Makki and Capecchi’s findings that show in mice all cardiac neural crest cells in the outflow tract are derived from *Hoxa1*-expressing cells (Makki and Capecchi, 2011). Further research demonstrated that *Hoxa1* knockout mice exhibit severe cardiovascular, cerebrovascular and glandular defects (Makki and Capecchi, 2012). *Hoxa1* expression in cardiac progenitor cells (Bertrand *et al*., 2011) is known to form a gene regulatory network with *Hoxb1* in the formation of the cardiac outflow tract (Roux *et al*., 2015). At the molecular level, *Hoxa1* regulates the expression of *Hnf1b, Foxd3* and *Zic1* that are necessary for neural crest specification (Makki and Capecchi, 2011; Makki and Capecchi, 2012). In humans, homozygous truncation mutations in
HOXA1 can lead to Bosley–Salih–Alorainy syndrome (BSAS) or Athabascan brainstem dysgenesis syndrome (ABDS), from which patients can exhibit facial weakness, hypoventilation, swallowing dysfunction, deafness and heart defects (Bosley et al., 2007; Holve et al., 2003; Jin and Sukumar, 2016).

2.1.2 Hoxa2 gene in embryonic development

Hoxa2 is a member of the Hox gene family that shares a 60 amino acid homeodomain and encodes a 41-kDa protein (Nazarali et al., 1992; Tan et al., 1992; Smith et al., 2009). The Hoxa2 protein binds to downstream genes to regulate their expression in a spatio-temporal manner (Akin and Nazarali, 2005). Hoxa2 gene acts as the selector gene for second branchial arch patterning (Gendron-Maguire et al., 1993; Minoux et al., 2013; Cox et al., 2014) and plays an essential role in early embryo development.

Hoxa2 is important for the development of skeletal structures derived from the second pharyngeal arch (PA) (Fig. 2.4A) and it determines the areas of skeletogenesis from the second PA mesenchyme by an inhibitory mechanism (Kanzler et al., 1998). In Hoxa2 knockout mice, the structures in the second PA are arranged as a mirror image of the first PA (Fig. 2.4A), indicating Hoxa2 may prevent the formation of the first PA (Gendron-Maguire et al., 1993). Tavella and Bobola (2009) have shown that the over expression of Hoxa2 in mice can cause failure of bones to form in the cranial base. During chondrogenesis, overexpression of Hoxa2 in cells entering chondrogenesis can impair cartilage development and lead to embryonic delay of ossification followed by a postnatal proportionate short stature with reduction in the length of the trunk and limbs (Massip et al., 2007; Deprez et al., 2012). At the molecular level, the persistent expression of Hoxa2 downregulates expression of genes controlling cell differentiation in
chondrogenesis, namely bone morphogenetic protein 7 (Bmp7), msh homeobox 1 (Msx1), paired box 9 (Pax9), sex determining region Y- box 6 (Sox6), Sox9 and Wnt family member 5a (Wnt5a) (Deprez et al., 2013). Ectopic expression of Hoxa2 in the Hox-negative cranial neural crest cells (CNCCs) in mice resulted in skeletal defects including absent or reduced bones in the skull vault and maxillary structures (Kitazawa et al., 2015).

Hoxa2 also plays a role in ear development where a loss of Hoxa2 function in mice can cause an abnormal appearance of the external ear, a condition that is known as microtia (Kanzler et al., 1998; Santagati et al., 2005). Several bones in the middle ear are affected in Hoxa2−/− mice, including stapes, the styloid bone and the lesser horn of the hyoid bone (Fig. 2.4B; Kanzler et al., 1998; Minoux and Rijli, 2010). In the mouse the whole auricle is derived from the Hoxa2-expressing second PA neural crest-derived mesenchyme and Hoxa2 spatially organises cell proliferation during external ear development (Minoux et al., 2013). Further genetic analyses showed that Hoxa2 regulates the expression of bone morphogenetic protein 4 (Bmp4), bone morphogenetic protein 5 (Bmp5), twisted gastrulation (Tsg) (Minoux et al., 2013; Cox et al., 2014) and H6 family homeobox 1 (Hmx1) (Rosin et al., 2016) in the developing pinna. In human studies, several mutations of HOXA2 coding region have been related to microtia (Alasti et al., 2008; Monks et al., 2010). In some cases, HOXA2 haploinsufficiency (with only one functional copy of HOXA2 gene and the other copy inactivated by mutation) is also known to cause microtia and hearing loss (Brown et al., 2013; Jin and Sukumar, 2016).
Figure 2.4. Schematic diagram of mouse pharyngeal arches (PA) and developing ear. A. Schematic of E10.5 mouse embryo was shown with specific Hox code in each PA (a). Inactivation of Hoxa genes in NCCs resulted in a Hox-free ground pattern in all four PAs (b). B. Schematic diagram of the effect of Hoxa2 on PA2 derived lower jaw and middle ear structures. D – dentary bone; G – gonial bone; G* – modified gonial bone; I and I2 – incus and its duplicated counterpart; LH – lesser horns of the hyoid bone; M and M2 – malleus and its duplicated counterpart; Mc and Mc2 – Meckel's cartilage and its duplicated counterpart; SP – styloid process; S – stapes; T and T2 – tympanic bone and its duplicated counterpart. Figure taken from Minoux and Rijli (2010) with permission.
A previous study from our laboratory has shown that *Hoxa2* plays a direct role in murine palate development (Smith *et al.*, 2009). In mice at embryonic day 11.5 (E11.5), the CNCC-derived cells bud out of the maxillary prominences to form two downward projecting structures of the palatal shelves. These shelves then undergo significant growth and grow vertically down the sides of the tongue until E13.5. At this point the two shelves begin to ascend and grow horizontally above the tongue. At E14.5, the palatal shelves contact one another and fuse completely at E15.5 (Ferguson, 1988) (Fig. 2.5). *In vivo* *Hoxa2* protein is expressed in both epithelial and mesenchymal cells of the developing murine secondary palate (Nazarali *et al.*, 2000). Our laboratory has further demonstrated a spatial and temporal expression of *Hoxa2* mRNA and protein within the mouse palate from E12.5 to E15.5 (Smith *et al.*, 2009). During development, palatal shelves emerge, elevate, and then fuse. The *Hoxa2* expression pattern changes during this period as well, being highly expressed at the beginning of palatal development, peaking at E13.5 and declining significantly after E14.5 (Smith *et al.*, 2009) (Fig. 2.5). Increased cell proliferation and decreased fusion rates were also observed in *Hoxa2* null mice. Several genes involved in palate development, including *Msx1*, *Bmp4*, *Barx1* and *Ptx1*, have been identified as downstream targets of *Hoxa2* within the palate (Smith *et al.*, 2009).

During nervous system development, *Hoxa2* expression affects development of hindbrain and cerebellum through its impact on rhombomeres (Gavalas *et al.*, 1997). In the vertebrate central nervous system (CNS), oligodendrocytes form myelin sheath. Our laboratory has shown that *Hoxa2* is expressed throughout oligodendrogenesis (Nicolay *et al.*, 2004). In a mouse model of *Hoxa2* loss-of-function, early stages of oligodendrogenesis does not appear to be altered in the spinal cord (Nicolay *et al.*, 2004), although over expression of *Hoxa2* inhibits oligodendrogenesis throughout the brain (Miguez *et al.*, 2012). In addition, we have shown that
overexpression of *Hoxa2* in CG4 oligodendroglial cells increased cell proliferation but delayed CG4 oligodendroglial cell differentiation (Wang *et al.*, 2011).

As summarized above, *Hoxa1* and *Hoxa2* genes control many important embryonic developmental processes, hence it would be of great value to investigate how expression of *Hoxa1* and *Hoxa2* are regulated, especially at the epigenetic level.

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**Figure 2.5. Mouse palate development and *Hoxa2* expression.** The developing secondary palate first extends vertically down the sides of the tongue from E11.5-E12.5 (A and B). This is followed by the two palatal shelves ascending horizontally above the tongue at E13.5 (C) and extending towards each other at E14.5 (D). The red dots in the figure indicate the expression of *Hoxa2* at different stages of mouse palate development. The density of the dots represents the relative expression of *Hoxa2*. Figure taken from W. Zhang’s M.Sc., Thesis 2003.
2.1.3 Hox gene activation by all-trans retinoic acid (ATRA)

All-trans retinoic acid (ATRA) is derived from vitamin A. It can activate the early expression of 3' Hox paralogs and is important in embryo development (Daftary and Taylor, 2006; Cunningham and Duester, 2015). ATRA can activate gene expression via its cognate receptors, including retinoic acid receptors (RARs) and retinoid X receptors (RXRs). RAR and RXR can form a heterodimer complex and bind to target gene at a retinoic acid response element (RARE) located in enhancer regions (Cunningham and Duester, 2015). Without activation of RA binding, unliganded RAR-RAX heterodimer bind to RARE sequences to maintain repressed transcription. The unliganded RAR-RAX heterodimer can also recruit co-repressors to further help maintain repressed transcription. Upon the binding of ATRA to RAR-RXR heterodimer, a conformational change is induced in the heterodimer which leads to the recruitment of gene co-activators and further induces gene expression (Daftary and Taylor, 2006; Cunningham and Duester, 2015). RARE sequences are found in many Hox genes and ATRA induces sequential activation of Hox genes that correlates with their positions on the chromosomal cluster (Kashyap et al., 2011; Cunningham and Duester, 2015). Following ATRA exposure, epigenomic reorganization of the Hox cluster occurs quickly (Kashyap et al., 2011). The 3'-Hoxa1 RARE is essential for ATRA mediated activation of Hoxa and Hoxb clusters (Kashyap et al., 2011). Important roles of ATRA have been reported in development including central nervous system development (Daftary and Taylor, 2006), limb development and organ development (Cunningham and Duester, 2015).
2.2 Epigenetics

Epigenetics refers to the heritable changes in gene expression without any changes in DNA sequences and is a major mechanism that regulates gene expression changes in response to gene-environment interactions (Holliday, 2006). At the molecular level, epigenetic pathways include regulation by noncoding RNAs and biochemical modifications of the DNA and histone proteins, such as methylation, acetylation and phosphorylation (Skinner, 2011; Yao et al., 2016). The importance of epigenetic modifications has long been recognized in the areas such as stem cell research, cancer (Pogribny, 2010) and developmental biology (Skinner, 2011; Yao et al., 2016).

2.2.1 DNA methylation

DNA methylation is one of the most widely studied aspects in epigenetic mechanism. Proper DNA methylation is essential for mammalian embryonic development and is involved in gene repression, regulation of parental imprinting and X-chromosome inactivation (Bird, 2002; Santos et al., 2005; Bartolomei and Ferguson-Smith, 2011; Lomvardas and Maniatis, 2016). It is also closely related to diseases like cancer, neurodegenerative diseases, psychiatric disorders and cardiovascular diseases, and is a potential therapeutic target in many diseases (Abdelfatah et al., 2016; Tang et al., 2016; Li et al., 2016; Fries et al., 2016; Napoli et al., 2016; Zhong et al., 2016). DNA methylation occurs on cytosine bases throughout the genome but is most relevant when present in sequences rich in CpG dinucleotides, which are called CpG islands, often found in promoter regions. Methylated DNA contains the covalent addition of a methyl group to cytosine residues at CpG dinucleotides. The enzymes that catalyze this reaction are the DNA methyltransferases (DNMTs). Mammalian DNMTs are comprised of three regions: (1) an N-
terminal regulatory region which guides the localization of the enzymes to the nucleus and mediates their interactions with other proteins, DNA and chromatin; (2) a central linker region; and (3) a C terminal catalytic region which catalyzes the transfer of the methyl group from a cofactor molecule S-adenosyl-l-methionine (AdoMet or SAM) to the C5 position of the cytosine residue (Fig. 2.6; Jurkowska et al., 2011; Uysal et al., 2015).

Figure 2.6. Schematic diagram of cytosine’s methylation and demethylation processes. DNA methylation on 5-cytosine (5mC) is carried out by DNMTs. 5mC can be demethylated by the ten-eleven translocation (TET) family of DNA hydroxylases and converted back to cytosine through thymine DNA glycosylase (TDG)/ base excision and repair (BER) mechanism. 5mC can also be deaminated and converted into thymine (T) and further be replaced by unmethylated C through BER mechanism. Figure modified from Xu et al., 2014.
### Table 2.1. List of structures and functions of DNMTs

<table>
<thead>
<tr>
<th>DNMTs</th>
<th>Structure</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT1</td>
<td>√</td>
<td>Copy pre-existing methylation patterns from parental strand onto newly synthesized daughter strand.</td>
<td>Bestor, 1992; Uysal et al., 2015</td>
</tr>
<tr>
<td>DNMT2</td>
<td>√</td>
<td>Transfer RNA methylation.</td>
<td>Goll et al., 2006; Jeltsch et al., 2016</td>
</tr>
<tr>
<td>DNMT3a</td>
<td>√</td>
<td>Introduce new DNA methylation patterns in mammalian development and in germ cells.</td>
<td>Okano et al., 1999; Uysal et al., 2015</td>
</tr>
<tr>
<td>DNMT3b</td>
<td>√</td>
<td>Facilitate DNMT3a and DNMT3b in DNA methylation.</td>
<td>Uysal et al., 2015; Basu et al., 2016</td>
</tr>
<tr>
<td>DNMT3L</td>
<td>√</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2.2 List of proteins that bind to methylated DNA

<table>
<thead>
<tr>
<th>Methyl-DNA binding Domain</th>
<th>Major group members</th>
<th>Binding preference</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBDs</td>
<td>MeCP2, MBD1, MBD2</td>
<td>Single 5mC</td>
<td>Transcriptional repressors; interact with gene co-repressors</td>
<td>Du et al., 2015; Wood and Zhou, 2016</td>
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<tr>
<td></td>
<td>MBD3</td>
<td>5mC, 5hmC, C</td>
<td>Transcriptional repressors</td>
<td>Shimbo et al., 2013; Du et al., 2015</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>MBD4</td>
<td>Single 5mC</td>
<td>DNA damage repair</td>
<td>Laget et al., 2014</td>
</tr>
<tr>
<td>The methyl-CpG binding zinc-finger proteins</td>
<td>Kaiso</td>
<td>Two methyl-CpG</td>
<td>Transcriptional repressors</td>
<td>Sasai et al., 2010; Bogdanović and Veenstra, 2009</td>
</tr>
<tr>
<td></td>
<td>ZBTB4, ZBTB38</td>
<td>Single 5mC</td>
<td>Transcriptional repressors</td>
<td></td>
</tr>
<tr>
<td>SET and SAR family proteins</td>
<td>UHRF1</td>
<td>Hemi-methylated DNA</td>
<td>Associate with DNMT1; DNA damage repair; recruitment of KDAC</td>
<td>Unoki et al., 2004; Berkyurek et al., 2014; Yang et al., 2013</td>
</tr>
<tr>
<td></td>
<td>UHFR2</td>
<td>5hmC</td>
<td>DNA damage repair</td>
<td>Liu et al., 2016b; Yang et al., 2013</td>
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</tbody>
</table>
In mammals, five different DNMTs, namely DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L have been identified and amongst all five DNMTs, the three active DNMTs are DNMT1, DNMT3a and DNMT3b (Table 2.1; Uysal et al., 2015). During DNA replication, hemimethylated DNA is created with only the parental strand having methylation marks while the newly synthesized strand remains unmethylated. DNMT1 preferentially recognizes hemimethylated DNA over unmethylated DNA and can copy pre-existing methylation patterns onto the new DNA strand to remethylate the daughter strand (Bestor, 1992; Uysal et al., 2015). In this way DNA methylation can be preserved during DNA replication. The de novo methyltransferases DNMT3a and DNMT3b are mainly responsible for establishing DNA methylation patterns in mammalian development and in germ cells by introducing cytosine methylation to previously unmethylated CpG sites (Okano et al., 1999). Unlike DNMT1, they do not show any significant preference between hemimethylated and unmethylated DNA (Okano et al., 1998; Uysal et al., 2015). Besides methylation of CpG sites, DNMT3a can also methylate cytosine residues at non-CpG sites but the biological function of these methylation sites remain unknown (Arand et al., 2012; Shirane et al., 2013). DNMT3s are also able to read other epigenetic marks, like trimethylation of histone 3 lysine 4 (H3K36me3), to guide DNA methylation (Rondelet et al., 2016). In recent years, a function in the DNA demethylation pathway has also been identified for DNMT3a and DNMT3b. It has been reported that under low SAM concentrations, DNMT3a and DNMT3b can convert 5-methylcytosine (5mC) into T, which could further be replaced by an unmodified C by the base excision and repair (BER) mechanism (Chen et al., 2013; van der Wijst et al., 2015). DNMT3L does not contain methyltransferase active site motifs and thus lack DNA methyltransferase activity but it is also present in mammals and is functionally related to DNMT3a and DNMT3b and modulates their
catalytic activity (Suetake et al., 2004; Uysal et al., 2015). It can bind to the N terminus of Histone 3 to bring about DNA methylation and the recognition of unmethylated H3K4 by DNMT3L is important for the methylation function of DNMT3a (Basu et al., 2016; Hu et al., 2009; Ooi et al., 2007). Due to a lack of an N-terminal regulatory domain, DNMT2 cannot catalyze DNA methylation. However, a functional study of DNMT2 showed that it is involved in methylation of transfer RNA through its C-terminal catalytic domain (Goll et al., 2006; Jeltsch et al., 2016).

![Diagram](image)

**Figure 2.7. Mechanisms of DNA-methylation-mediated gene repression.** (a) DNA methylation directly inhibit the binding of some transcription factors (TF). (b) Methyl-CpG-binding proteins (MBPs) can recognize methylated DNA and recruit co-repressor molecules to silence transcription. They can also modify surrounding chromatin structure to achieve gene silencing.
DNA methylation usually occurs at the transcription start site and generally prevents transcription in two ways (Fig 2.7). First it can directly inhibit the binding of transcription factors or regulators to the DNA sequence (Fig 2.7a). Secondly, it can also indirectly recruit methyl-CpG binding proteins (MBPs) which bind to and recognize 5-methylcytosines, to change chromatin into a repressive state (Fig 2.7b) (Bogdanovic and Veenstra, 2009; Deaton and Bird, 2011; Moore et al., 2013; Domcke et al., 2015). DNA methylation can also occur within the coding region of the gene and was initially believed to inhibit transcriptional elongation (Klose and Bird, 2006) although later evidences showed that this methylation was not associated with transcriptional repression (Jones, 2012). However, it has been demonstrated that gene body methylation is positively correlated with active transcription, and further research has suggested that it may play a role in gene splicing (Shukla et al., 2011; Jones, 2012). The function of methylation within the coding region however, needs further investigation (Hellman and Chess, 2007; Jones, 2012; Yang et al., 2014). Based on their functional domains, there are three families of proteins that bind to methylated DNA (Table 2.2), namely; (1) Methyl-CpG binding domain proteins (MBDs) including MeCP2, MBDs 1-6, SETDB1, SETDB2, BAZ2A and BAZ2B (reviewed by Fan and Hutnick, 2005; Moore et al., 2013; Du et al., 2015); (2) the methyl-CpG binding zinc-finger proteins of the Kaiso family where so far only three members have been described; Kaiso, ZBTB4 and ZBTB38 (Prokhortchouk et al., 2001; Sansom et al., 2007; Moore et al., 2013; Du et al., 2015); and finally (3) SET (Suppressor of variegation-enhancer of zeste-trithorax) and the ring finger-associated (SAR) family composed of UHRF1 and UHFR2 (Pichler et al., 2011; Moore et al., 2013; Du et al., 2015). Due to amino acid changes at critical positions in the methyl-CpG binding domain, MBD3 is the only MBD protein that binds to methyl-CpG, 5-hydroxymethylated DNA (5hmC) as well as unmethylated DNA (Saito and Ishikawa, 2002;
Yildirim et al., 2011; Shimbo et al., 2013). MBD4 can also act as a DNA repair enzyme (Walsh and Xu, 2006; Laget et al., 2014). MeCP2, MBD1 and MBD2 are transcriptional repressors and these MBD proteins were found to interact with co-repressors such as histone deacetylases, methyltransferases, and chromatin remodeling factors that regulate gene expression by acting on histone proteins (Sasai and Defossez, 2009; Du et al., 2015; Wood and Zhou, 2016). Similar to MBDs, ZBTB4 and ZBTB38 require only one methylated CpG for binding, while Kaiso requires two methyl-CpGs for binding (Sasai et al., 2010; Bogdanović and Veenstra, 2009). UHRF1 acts as gene repressor via recruitment of the KDAC complex to the promoters (Unoki et al., 2004). UHRF1 is associated with DNMT1 as it also has a preferential binding affinity for hemi-methylated DNA and specifically recruits DNMT1 to hemi-methylated sites during DNA replication (Bostick et al., 2007; Berkyurek et al., 2014). UHRF1 may also be important in histone modification (Sasai and Defossez, 2009; Qin et al., 2015). UHRF2 is a paralog of UHRF1 but have structural differences at the SRA domains. Due to this structural difference, UHRF2 has higher affinity to 5-hydroxymethylated cytosine (Liu et al., 2016b). UHRF1 and UHRF2 are also involved in DNA damage repair (Luo et al., 2013; Yang et al., 2013). Researchers also find DNA methylation in actively transcribed genes, thus it may also play a positive role in transcription regulation (Weber et al., 2007).

DNA methylation is especially important in mammalian embryo development. Mutation in mouse Dnmt1 gene causes extensive demethylation of the genome and leads to embryonic lethality shortly after gastrulation (Li et al., 1992; Kurihara et al., 2008; Arand et al., 2012). Dnmt3b−/− mice have multiple developmental defects and embryos die at embryonic day E9.5 while Dnmt3a−/− mice die shortly after birth (Okano et al., 1999). Loss of Dnmt3a in mouse embryonic cardiomyocytes alters multiple signaling pathway in cardiomyocytes and inhibits
function of embryonic cardiomyocytes (Fang et al., 2016). DNMTs are necessary for bovine parthenogenetic preimplantation embryo development as DNMT inhibitors can inhibit this process (Zhang et al., 2015). Unlike knockout of DNMTs, single knockout of MBDs in mice display only mild changes. This could be because MBDs can compensate each other or the silencing of genes by DNA methylation can also occur through different pathways (Sasai and Defossez, 2009; Du et al., 2015). MeCP2 is related to neural development as mutation of MeCP2 can lead to severe neurodevelopmental disorders in females (Amir et al., 1999; Yang et al., 2016). Similar to DNMT1 mutants, UHRF1 knockout mice die during early embryogenesis (Sharif et al., 2007). Hence all this evidence suggest that proper DNA methylation status is critical for embryonic development. As reviewed above, Hox genes are also essential in embryonic development. Thus it would be interesting to investigate how expression of Hox genes is controlled by DNA methylation during embryonic development.

2.2.2 Histone modification

Histone modifications can lead to either the activation or the silencing of gene transcription. Posttranslational histone modifications normally occur on the N-terminal tails of Histone H3 and H4. There are many types of modifications of histone proteins, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation and ADP-ribosylation (Jenuwein and Allis, 2001; Spivakov and Fisher, 2007; Bannister and Kouzarides, 2011; Canovas and Ross, 2016). Histone modifications can regulate gene expression in three ways. First, they can regulate chromatin structure to alter DNA accessibility (Abel and Zukin, 2008; Canovas and Ross, 2016). Second, they serve as a signal by integrating responses to multiple biochemical signaling cascades and recruit the transcriptional machinery and chromatin
remodeling complexes (Abel and Zukin, 2008; Badeaux and Shi, 2013). Third, histone modifications can also mediate epigenetic changes in gene expression (Abel and Zukin, 2008; Moore et al., 2013). Below, I review three common histone modifications: histone acetylation, histone phosphorylation and histone methylation.

### 2.2.2.1 Histone acetylation

Histone acetylation occurs on a lysine residue in histones H2A, H2B, H3 and H4 and is associated with transcriptional activation by opening the chromatin structure (Graves et al., 2016). Histone acetylation not only takes place on the N-terminal tail of histone proteins, but also to a lesser extent in the globular histone core (Bannister and Kouzarides, 2011; Graves et al., 2016). Histone acetylation of lysine is regulated by lysine acetyltransferases (KATs, also known as histone acetyltransferases, HATs) and lysine deacetylases (KDACs, also known as histone deacetylases, HDACs). The KATs can transfer an acetyl group to the lysine side chain, which neutralizes the lysine’s positive charge to weaken the electrostatic interactions between histones and the DNA phosphodiester backbone (Bannister and Kouzarides, 2011; Canovas and Ross, 2016) (Fig 2.8). Based on the structural and functional similarity of their catalytic domains, KATs are grouped into five families: p300/CBP family, the MYST-family, the GCN5-related N-acetyltransferase (GNAT) superfamily, the nuclear receptor co-activator (NCOA)/steroid receptor coactivator (SRC) family and transcription-initiation-related factor KATs (Sheikh, 2014; Canovas and Ross, 2016). CREB-binding protein (CBP) and its paralog p300 were originally identified to bind the cAMP-response element binding (CREB) protein (Chrivia et al., 1993) and the adenovirus early-region 1A (E1A) protein (Eckner et al., 1994), respectively. Researchers further discovered that CBP/p300 also possess histone acetyltransferase activity
(Bannister and Kouzarides, 1996) and H3K14, H3K18, H3K27, H4K5 and H4K8 have all been identified as their targets (reviewed by Tie et al., 2009; Valor et al., 2013). CBP/p300 play important roles in cell growth and embryo development. Loss of function of CBP and p300 are both lethal in mice (reviewed by Goodman and Smolik, 2000; Philip et al., 2015). KATs from all five families have been found to be important in neural development (Sheikh, 2014).

Figure 2.8. Mechanisms of gene regulation by histone acetylation and histone phosphorylation. DNA molecules are negatively charged. Histone acetylation and phosphorylation can neutralize the positive charge of histone. The interactions between histones and DNA are then weakened, making DNA accessible to transcription factors. Without acetylation/phosphorylation, chromatin is compacted and DNA is inaccessible to transcription factors.
KDACs are transcriptional repressors that reverse lysine acetylation, restore the positive charge of the lysine residue and thus stabilize the local chromatin architecture (Bannister and Kouzarides, 2011). KDACs are categorized into classical KDACs (KDAC1-11) and non-classical KDACs based on their enzymatic mechanisms. Classical KDACs all share a conserved deacetylase domain whereas the catalytic function of non-classical KDACs is NAD$^+$-dependent (Das Gupta et al., 2016). KDACs are important in embryonic development. Deletions of KDAC1, 2, or 3 are all lethal in mice (Montgomery et al., 2008; Guan et al., 2009; Dovey et al., 2010; Lv et al., 2014). KDACs are also important in cell differentiation. Inhibition of KDACs activities can lead to early differentiation of embryonic stem cells (ESCs) (Lv et al., 2014).

2.2.2.2 Histone phosphorylation

Similar to histone acetylation, histone phosphorylation also activates gene expression. It occurs on serines, threonines and tyrosines of the N-terminal histone tails (Rossetto et al., 2012; Sawicka and Seiser, 2014). Protein kinases and phosphatases are the two groups of enzymes that add and remove the modification, respectively. Histone kinases transfer a phosphate group from ATP to the hydroxyl group of the target amino-acid, add negative charge to the histone and change the chromatin structure to increase DNA accessibility and activate the gene (Fig. 2.8) (Bannister and Kouzarides, 2011; Brehove et al., 2015). Histone phosphorylation is important in DNA damage repair, transcription regulation and chromatin remodeling (Rossetto et al., 2012; Sawicha and Seiser, 2014).
2.2.2.3 Histone methylation

Histone methylation is associated with both transcriptional activation and gene silencing. Histone methylation occurs on lysines and arginines without changing the charge of the histone protein. There are three levels of histone lysine methylation: mono-, di- and tri-methylation (Ng et al., 2009; Bannister and Kouzarides, 2011). Histone lysine methyltransferase (HKMT) contain a so-called SET domain that catalyses the transfer of a methyl group from SAM to a lysine’s ε-amino group (Bannister and Kouzarides, 2011; Fan et al., 2015). HKMTs are relatively specific enzymes with different enzymes within a family catalyzing different sites and levels of histone methylation. There are generally two classes of lysine demethylases (KDMs): the Jumonji C (JmjC) class and PHF8 (KDM7) families (Bannister and Kouzarides, 2011; Krishnan et al., 2011). Methylation of histone on different lysine residues can result in different regulation functions. It is reported that methylation on histone H3K4, H3K36, and H3K79 are associated with transcriptional activation while the di- and tri-methylation on histone H3K9, H3K27 induce transcriptional inhibition (Fig 2.9) (Feng et al., 2007; Nguyen and Zhang, 2011). Different degrees of residue methylation may result in different biological functions as well. H4K20 methylation has three states (mono-, di- and trimethylation). Mono- and di-methylation of H4K20 are involved in DNA replication in cell cycle and DNA damage repair, while trimethylation of H4K20 is related to heterochromatin maintenance (Jørgensen et al., 2013).
2.2.2.4 Histone modification complexes

Histone modification enzymes often form complexes with other proteins to carry their activity. These complexes are divided into two groups: the polycomb group (PcG) proteins and trithorax group (TrxG) proteins. The PcG and the TrxG proteins are epigenetic regulators responsible for the repression and activation, respectively of a group of genes important in development and cell fate specification. They are important for these genes to maintain previously established states of gene expression over multiple cell generations (Schuettengruber et al., 2011). The PcG and TrxG were first identified in Drosophila where they were shown to be required for the long-term repression and activation of the Antennapedia class of homeobox genes (Jurgens, 1985; Mallo and Alonso, 2013). The DNA regulatory elements that recruit PcG and TrxG factors to chromatin are referred to as PcG and TrxG response elements (PREs and TREs), respectively. Some TrxG and PcG components possess direct histone modification
activity while other TrxG and PcG proteins affect histone marks (Schuettengruber et al., 2007). PcG proteins are comprised of two types of epigenetic regulators: Polycomb Repressive Complex 1 (PRC1) and PRC2. PRC1 contains Bmi1, Ring1A, Ring1B, Cbx and Phc, and is responsible for ubiquitination of histone H2A at lysine 119, which is essential for Hox gene silencing (Cao et al., 2005; Mallo and Alonso, 2013; Piunti and Shilatifard, 2016). PRC2 is composed of Suz12, EED (embryonic ectoderm development) and Ezh1/2. Ezh2 catalyzes the di- and tri-methylation of histone H3 at lysine 27 which also acts to silence gene expression (Cao et al., 2002; Piunti and Shilatifard, 2016) and this mark is specifically recognized by PRC1 (Schuettengruber et al., 2011; Piunti and Shilatifard, 2016). There are generally two classes of TrxG: one is composed of SET domain factors and the other includes components of ATP-dependent chromatin remodeling complexes (Schuettengruber et al., 2011; Geisler and Paro, 2015). The SET domain histone modification complexes include COMPASS, COMPASS-like, TAC1 and absent small or homeotic discs 1 (ASH1) complexes. COMPASS and COMPASS-like complexes have H3K4 trimethylase activity and are composed of histone methyltransferase (SET1A, SET1B, MLL1-4) and subunits including WD repeat domain 5 (WDR5), retinoblastoma binding protein 5 (RBBP5), absent small or homeotic 2-like (ASH2L) and DPY30. TAC1 and ASH1 complexes both coupled to CBP and counteract PcG silencing. ATP-dependent chromatin remodeling complexes includes switch/sucrose nonfermentable (SWI/SNF), imitation switch (ISWI) and various chromodomain helicase (CHD)-containing complexes that can recognize the histone tail modifications to facilitate active transcription (Schuettengruber et al., 2011; Geisler and Paro, 2015).

PcG and TrxG proteins play important roles in embryonic development and Hox gene regulation. PRC2 deficiency is fatal because single knockout of Suz12, EED or Ezh2 results in
early embryonic lethality in mice (Pasini et al., 2004; Faust et al., 1995; O’Carroll et al., 2001; Cheedipudi et al., 2014; Kadoch et al., 2016). Mutation of Ring1B (member of PRC1) also leads to embryonic lethality (Voncken et al., 2003; Morey et al., 2015) while other PRC1 deficiencies are less severe, only resulting in developmental abnormalities (reviewed by Jones and Wang, 2010; Morey et al., 2015). In ES cells, Hox gene promoters often have ‘bivalent domains’, that is, they display both H3K4me3 and H3K27me3 marks. These bivalent domains appear to keep proper expression status of Hox genes (Bernstein et al., 2005; Montavon and Duboule, 2013). PcG and TrxG protein have a “ying-yang” effect on the bivalent domains. Mutation of PcG genes results in abnormal Hox gene expression and leads to irregular ES cell differentiation in both human and mouse (Lee et al., 2006; Boyer et al., 2006; Morey et al., 2015).

### 2.2.2.5 Histone methyltransferase complex MLL1/WDR5

Among all the PcG and TrxG protein complexes, MLL1/WDR5 complex is of special interest in my study (discussed later in the thesis). MLL1/WDR5 complex belongs to the TrxG protein complexes and is specifically responsible for the methylation of H3K4. Mutations in MLL1 are associated with several acute lymphoblastic and myelogenous leukemias from where it derives its name. MLL1 is an H3K4 methyltransferase that belongs to the evolutionarily conserved SET1 family. In humans, this gene is located on chromosome 11 and its cDNA is ~12 kb in length. *MLL1* gene encodes a protein of ~4000 amino acids in length which can be digested in cells by taspase into two fragments: a 320 kDa N-terminal fragment and a 180 kDa C-terminal fragment (Zhang et al., 2013). The two cleaved peptides remain associated to form a heterodimer.
MLL1 protein contains several conserved domains which are related to its functions in chromatin-mediated transcriptional regulation. The N-terminal fragment contains three DNA-binding AT-hooks, followed by a cysteine-rich region with homology to DNA methyltransferases, referred to as the CxxC domain, followed by three plant homeodomain (PHD) zinc-finger-like motifs, and finally a bromo domain (BD) region. In the C-terminal fragment there is a transactivation domain (TAD) which can interact with CBP, a Win motif known as WDR5 binding site, and a SET domain which is responsible for the histone methyltransferase activity (Rasio et al., 1996; Nakamura et al., 2002; Zhang et al., 2013; Cosgrove and Patel, 2010) (Fig. 2.10A). MLL1 is a H3K4 specific methyltransferase and the CxxC domain may play a role in targeting MLL1 to active genes because this domain selectively binds to non-methylated CpG islands (Birke et al., 2002; Long et al., 2013; Bina et al., 2013). The PHD domains aid to recognize lysine residues on chromatins (Taverna et al., 2007; Lalonde et al., 2014). Further research has showed that the TAD domain may also contribute to the specificity of MLL1. The TAD domain of MLL1 can interact with lysine acetyltransferases CBP/p300 which contains a number of protein-binding domains that mediate transcription factor recruitment (Cosgrove and Patel, 2010; Wang et al., 2013). In this way, MLL1 can increase the binding of other transcriptional activators to help activate gene expression. In the TrxG, MLL1 forms complex with other components, including WDR5, RBBP5, ASH2L and DPY30 (Zhang et al., 2013; Shinsky et al., 2015). These proteins can stimulate catalytic activity and product specificity of SET1-familiar methyltransferase, including MLL1. Deletion of any of these genes in yeast leads to a similar phenotype as observed in SET1 mutants (Nagy et al., 2002; Ernst and Vakoc, 2012).
Figure 2.10. Regulation of MLL1 by the core complex subunits. (A) MLL1 domain architecture. MLL1 contains the following functional domains: AT-hooks, CxxC, PHD, BD, TAD, Win motif and SET. Taspase can cleave MLL1 between BD and TAD into two peptide fragments. (B). Interaction of important subunits in MLL1 complex (Figure modified from Ernst and Vakoc, 2012).

In mammalian cells, WDR5 seems essential for all forms of H3K4 methylation whereas knockdown of RBBP5 affects primarily H3K4me3 and H3K4me2 (Dou et al., 2006; Ernst and Vakoc, 2012). ASHL2 and DPY-30 have more specific role on H3K4me3 modification (Jiang et al., 2011; Dou et al., 2006; Tremblay et al., 2014). Research in yeast has also found that WDR5 and RBBP5 proteins are essential for the stability of SET1 as depletion of either subunit leads to SET1 degradation (Steward et al., 2006). WDR5 binds directly to MLL1 through the Win motif in MLL1 in a 1:1 ratio (Patel et al., 2008; Shinsky et al., 2014) and bridges MLL1 with RBBP5 and ASH2L. The hinge region in RBBP5 then binds directly to the SPRY domain of ASH2L,
and ASH2L in turn binds to DPY30 (reviewed by Ernst and Vakoc, 2012) (Fig. 2.10B). MLL1 enzyme activity is relatively weakened in the absence of any of the associated subunits mentioned above. Research has revealed that the MLL1-WDR5-RBBP5 complex has only a mild enzyme activity and is only capable of mono-methylation (Patel et al., 2009; Shinsky et al., 2014; Shinsky et al., 2015). Adding ASH2L to the complex leads to a significant increase in H3K4 methyltransferase activity and shifted the product specificity to di- and tri-methylation. The addition of DPY30 further increased the activity of the complex (Patel et al., 2009; Ernst and Vakoc, 2012).

2.2.3 MicroRNA

MicroRNAs are ~22 nucleotide (nt) RNAs first discovered in *C. elegans* that can guide a RNA silencing pathway to regulate gene expression through their effect on mRNA (Lee et al., 1993; Felekkis et al., 2010; Usmani et al., 2016). Currently, many studies focus on miRNA regulated gene expression. Researchers have made several advancements on the roles of miRNAs in the areas of embryo development (Yan and Jiao, 2016; Green et al., 2015) as well as diseases including cancer (He et al., 2015; Usmani et al., 2016), cardiovascular disease (Elia and Condorelli, 2015), neurodevelopmental disease (Ardekani and Naeini, 2010) and metabolic disease (Deiuliis 2015).

2.2.3.1 microRNA biogenesis

*In vivo*, miRNA is synthesized as follows (Fig. 2.11): Initially a long primary transcript of the miRNA gene (pri-miRNA) composed of at least one hairpin-like miRNA precursor is cleaved by the nuclear microprocessor complex and the endonuclease Drosha complex in the
nucleus to form 50–120 nucleotide hair-pin secondary structures, named miRNA precursors (pre-miRNA) (Ke et al., 2003; Kocerha et al., 2009; Ha and Kim, 2014). The 60- to 90-nt pre-miRNAs form the stem-loop structures which are then transported by Exportin-5 from the nucleus to the cytoplasm where the pre-miRNA hairpin stem regions are excised by the endonuclease Dicer into 18 to 22 nt double strand RNAs (Bartel, 2004; Macfarlane and Murphy, 2010; Ha and Kim, 2014). One of the two strands is the mature miRNA and the other counterpart is called miRNA*. The mature miRNAs bind to the miRNA-containing RNA-induced silencing complex (miRISC) and interact with target mRNAs mostly within the 3' untranslated region (3' UTR) based on sequence complementarity between the miRNA and its target mRNA (Maes et al., 2009; Ha and Kim, 2014). Argonaute (AGO), a large protein family, is a key component of RISCs (Carmell et al., 2002; Tang, 2005; Ha and Kim, 2014). AGO is associated with Dicer as well as the target sites of RISCs to cleave the target mRNAs (Tang, 2005; Ha and Kim, 2014). The most important determinant of miRNA function is the degree of complementarity between the proximal (5') region (also known as “seed” region or the “nucleus”) of the miRNA and the mRNA (Brennecke et al., 2005; Felekkis et al., 2010; Valinezhad Orang et al., 2014). MiRNAs can silence gene expression by triggering the degradation of the target mRNA or blocking translation of the target mRNA (Ipsaro and Joshua-Tor, 2015; Jonas and Izaurralde, 2015). Evidences show that 66-90% of miRNA-mediated gene repressions in mammalian cells are through the degradation of target mRNAs (Eichhorn et al., 2014; Jonas and Izaurralde, 2015). GW182 is a key protein in the miRNA-mediated mRNA degradation. After the recognition of mRNA target by RISCs, AGO protein interacts with GW182 protein, which then binds to the cytoplasmic deadenylase complexes PAN2-PAN3 and CCR4-NOT and triggers the cellular 5'-3' mRNA decay pathway. The deadenylated mRNAs are then decapped and finally degraded.
(Valinezhad Orang et al., 2014; Jonas and Izaurralde, 2015). Originally researchers had proposed that miRNAs can inhibit translation at both initiation and post-initiation steps (Olena and Patton, 2010) but the use of ribosome profiling method ruled out the mechanisms occurring post-initiation (Huntzinger and Izaurralde, 2011; Jonas and Izaurralde, 2015). The molecular mechanism for the miRNA inhibited translation initiation remains to be resolved (Jonas and Izaurralde, 2015). A mRNA transcript can have multiple miRNA binding sites and be repressed simultaneously by different miRNA’s. The same or different miRNAs bind within the same 3’UTR and can act cooperatively to enhance repression (Doench and Sharp, 2004; Felekkis et al., 2010).

Figure 2.11. miRNA biogenesis and function. Transcribed by RNA polymerase II, long primary transcripts (pri-miRNA) are then processed in the nucleus into hairpins (pre-miRNA) by Drosha and DGCR8. Exportin 5 exports the pre-miRNA to cytoplasm where it is cleaved into a mature dsRNA duplex. One strand that is stably associated with Argonaute (AGO) is packaged into the RISC to target and regulate mRNAs. The other strand is unwound and degraded. The target mRNA is either degraded or translation is repressed. This figure is taken with permission from from Jung and Suh (2015).
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<td>Darda et al., 2015</td>
</tr>
<tr>
<td></td>
<td>miR-196b</td>
<td>HOXA9</td>
<td>Lung cancer</td>
<td>Yu et al., 2015</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Chronic myeloid leukemia</td>
<td>Liu et al., 2013</td>
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<td></td>
<td></td>
<td></td>
<td>MLL-rearranged leukemia</td>
<td>Li et al., 2012</td>
</tr>
<tr>
<td></td>
<td>miR-126,</td>
<td>HOXA9</td>
<td>Bone marrow</td>
<td>Shen et al., 2008</td>
</tr>
<tr>
<td></td>
<td>miR-145, let-7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>miR-193a-3p</td>
<td>HOXC9</td>
<td>Bladder cancer</td>
<td>Lv et al., 2015</td>
</tr>
<tr>
<td></td>
<td>miR-135a</td>
<td>HOXA10</td>
<td>Epithelial ovarian cancer</td>
<td>Tang et al., 2014</td>
</tr>
<tr>
<td></td>
<td>miR-100</td>
<td>HOXA1</td>
<td>Small cell lung cancer</td>
<td>Xiao et al., 2014</td>
</tr>
<tr>
<td></td>
<td>miR-99</td>
<td>HOXA1</td>
<td>Epithelial cell proliferation and migration</td>
<td>Chen et al., 2013</td>
</tr>
<tr>
<td></td>
<td>miR-10</td>
<td>HOXD10</td>
<td>Non-metastatic breast cancer</td>
<td>Ma et al., 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ovarian cancer</td>
<td>Nakayama et al., 2013</td>
</tr>
<tr>
<td></td>
<td>miR-130a</td>
<td>HOXA5</td>
<td>Human umbilical vein endothelial cells</td>
<td>Chen and Gorski, 2008</td>
</tr>
<tr>
<td></td>
<td>miR-221</td>
<td>HOXB5</td>
<td>Papillary thyroid carcinoma</td>
<td>Kim et al., 2008</td>
</tr>
<tr>
<td></td>
<td>miR-7, miR-218</td>
<td>HOXB3</td>
<td>Breast cancer</td>
<td>Li et al., 2012</td>
</tr>
</tbody>
</table>
2.2.3.2 Regulation of Hox genes by miRNAs

Studies show that miRNAs can regulate the expression of Hox genes and play important roles in embryonic development. Some miRNA genes are transcribed from Hox gene clusters like mir-10, mir-196 and iab-4 (Lagos-Quintana et al., 2011; Aravin et al., 2003; Woltering and Durston, 2008; Yekta et al., 2004; Hornstein et al., 2005). These miRNAs are expressed in patterns that approximate the characteristic expression of Hox genes. Other miRNAs have also been found to regulate Hox genes. Below I have summarized research on miRNAs and Hox gene regulation that has so far been investigated in several species including insects, mouse, chicken, fish and human cell lines (see Table 2.3).

In Drosophila, three homeotic genes within BX-C encode homeobox proteins. They are Ultrabithorax (Ubx), Abdominal-A (Abd-A) and Abdominal- B (Abd-B) (Martin et al., 1995; Garaulet and Lai, 2015). Two groups of Drosophila miRNAs are transcribed from BX-C and can regulate Hox gene expression (Garaulet and Lai, 2015). The iab-4–5p and iab-4–3p are located at the end of the iab-4 locus between abd-A and Abd-B genes (Aravin et al., 2003; Garaulet and Lai, 2015). Ronshaugen et al., (2005), demonstrated direct targeting of miR–iab-4–5p in Ubx 3' UTR and showed that iab-4 miRNA down regulates Ubx activity in vivo. Over expression of mir-iab-4-5p attenuates Ubx protein levels and induces the transformation of halteres into wings (Ronshaugen et al., 2005). iab-4 miRNA was also predicted to repress the downstream targets of the homeobox genes (Grun et al., 2005). Thus iab-4 miRNA may fine-tune the development of Drosophila via both homeobox genes and their downstream targets (Chopra and Mishra, 2006). The miRNA iab-8 is a transcript produced on the sense strand of the coding region of BX-C but antisense to iab-4 (Tyler et al., 2008; Gummalla et al., 2012; Garaulet and Lai, 2015). Ubx appears to be a much stronger target of miR-iab-8-5p than of miR-iab-4-5p (Tyler et al., 2008;
Ectopic expression of iab-8 leads to haltere to wing transformation which is similar to what occurs after over expression of mir-iab-4-5p (Tyler et al., 2008; Bender, 2008). Abd-A is also regulated by iab-8 where deletion of mir-iab-8 influenced the expression of both Ubx and Abd-A in the posterior larval ventral nerve cord (VNC) and disrupted CNS patterning and reproductive behavior in Drosophila (Garaulet et al., 2014).

miRNAs are also very important in Zebrafish development. It has been shown that Dicer1, the microRNA-producing enzyme, is essential for Zebrafish development. Zebrafish embryos with blocked Dicer activity display abnormal morphogenesis during gastrulation, brain formation, somitogenesis, and heart development (Giraldez et al., 2005; He et al., 2011a). miR-10 gene is located 5′ of the Hox-4 genes and binds to Zebrafish Hox genes. In Zebrafish, miR-10c is located on the same primary transcript as Hoxb4a and is expressed in a Hox-4 like pattern in the spinal cord (Woltering and Durston, 2008; Mallo and Alonso, 2013). Binding sites of miR-10 have been experimentally confirmed to occur in both Zebrafish Hoxb1a and Hoxb3a 3′ UTR and in the open reading frame (Woltering and Durston, 2008). It has been reported that miR-10 represses Hoxb1a and Hoxb3a gene expression and its overexpression leads to loss of function phenotypes for both genes (Woltering and Durston, 2008). miR-196 is another hox-cluster miRNA that down regulates hoxb8a expression in zebrafish by targeting its 3′ UTR. An over expression or knockdown of miR-196 leads to abnormal embryo development in zebrafish (He et al., 2011b; Mallo and Alonso, 2013).

In cultured human Hela cells, miR-196 down regulates the expression of HOXB8, HOXC8, HOXD8, and HOXA7 by binding to 3′ UTR regions (Yekta et al., 2004). In patients with chronic myeloid leukemia, lower levels of miR-196b expression were detected and further experiments showed that miR-196b targets HOXA9 and reduces cell proliferation rate (Liu et al.,
miR-196b mediated regulation of HOXA9 expression has also been reported in mesenchymal-like-state non-small cell lung cancer cells (Yu et al., 2015). HOXB9 is also regulated by miR-196a in head and neck squamous cell carcinoma (Darda et al., 2015). Other experiments showed targeting of HOXD10 mRNA by miR-10 in cultured human non-metastatic breast cancer (SUM149) cells (Ma et al., 2007). Luciferase assays in HCT 116 cells demonstrated that miR-10a strongly repressed luciferase activities mediated by 3’ UTR vectors derived from HOXA3 and HOXD10 (Han et al., 2007). In ovarian cancer cells, over expression of miR-10b down regulated the expression of HOXD10 and accelerated the migration and invasion activities of these cancer cells (Nakayama et al., 2013). HOXA10 is another gene important in epithelial ovarian cancer (EOC). A down regulation of HOXA10 by miR-135a can lead to enhanced cell apoptosis and inhibition of cell growth and adhesion in EOC-derived cell lines (Tang et al., 2014). Down regulation of both antiangiogenic homeobox genes GAX and HOXA5 by miR-130a was observed in human umbilical vein endothelial cells (HUVECs), though the antagonism is weaker for HOXA5 than what was observed for GAX (Chen and Gorski, 2008). miR-221 is highly expressed in human papillary thyroid carcinoma (PTC) cell lines and altered the expressions of many target genes (Kim et al., 2008). HOXB5 is one of the most significantly affected gene and was identified as a direct target of miR-221 (Kim et al., 2008). miR-126, miR-145, and let-7 are all found to target the full-length HOXA9 cDNA and can block HOXA9 biological functions. More interestingly, miR-126 regulates HOXA9 by binding to the homeobox region rather than the 3’ UTR (Shen et al., 2008). Two tumor suppressor microRNAs, miR-7 and miR-218, were found to target HOXB3 in human breast cancer cell lines and further regulate the expression of tumor suppressor genes RASSF1A and Claudin-6 (Li et al., 2012). Three members of miR-99 family (miR-99a, miR-99b and miR-100) can bind directly
to HOXA1 mRNA. An over expression of miR-99 family led to reduced HOXA1 expression and caused reduced proliferation and cell migration (Chen et al., 2013; Xiao et al., 2014). miR-193a-3p can directly regulate the expression of HOXC9 and plays an important role in bladder cancer chemoresistance. In bladder cancer cell lines, ectopic expression of miR-193a-3p reduced HOXC9 expression and led to chemoresistance to several drugs (Lv et al., 2015).

In mouse embryos, Hoxb8 is directly cleaved by miR-196 (Yekta et al., 2004). miR-196 was also shown to regulate Hoxb8 in both mouse and chick hindlimb development (Brock et al., 2009). Observations by McGlinn and colleagues showed that loss of function of miR-196 in chick embryos led to extensive skeletal defects including homeotic transformations (McGlinn et al., 2009). Hox genes, Hoxa7 and Hoxc8, have also been identified as targets of miR-196 in mouse embryo development (Wong et al., 2015). miR-130a regulates Hoxa3 expression at the protein level in the chick and is involved in the regulation of cell proliferation in tumorigenesis associated with the chicken Marek’s disease (Han et al., 2016). miR-181 is strongly upregulated during differentiation of skeletal muscle cells. miR-181 can downregulate Hoxa11 protein expression and is suggested to play a role in skeletal-muscle differentiation (Naguibneva et al., 2006). In mouse embryo development, miR-130a and miR-221 were shown to regulate both airway branching and lung microvascular development by targeting Hoxa5 and Hoxb5, respectively (Mujahid et al., 2013). While a significant number of miRNAs have been reported to regulate Hox gene expression, only one miRNA has been reported to directly target Hoxa2. Hu and colleagues identified a novel miR-3960 from primary mouse osteoblasts and instead of targeting the 3’ UTR region, it targets Hoxa2 coding region. Together with miR-2861 which targets the Hoxa2 downstream gene, Runx2, miR-3960 can form a regulatory feedback loop and
plays an important role in osteoblast differentiation (Hu et al., 2011). Whether there are any specific miRNAs that target Hoxa2 3’ UTR still remain to be discovered.

2.2.4 Long noncoding RNA (lncRNA)

Although 70-90% of the mammalian genome is believed to be transcribed, only 1% of the genome encodes protein (Lee, 2012). Researchers previously considered these noncoding transcripts as transcriptional noise, but are beginning to realize that many of these noncoding transcripts have functions, and are now classified as long noncoding RNAs (lncRNAs). LncRNAs are a group of RNAs that do not encode proteins, and are comparably longer than other short noncoding RNAs, such as microRNAs and tRNAs. A majority of lncRNAs share the same transcriptional machinery with mRNAs, namely; (1) their transcription is catalyzed by RNA polymerase II; (2) lncRNAs also have 5' methylguanosine cap and 3' polyadenylation and often undergo splicing (Mercer and Mattick, 2013). However, lncRNAs usually lack an extended open reading frame (ORF). It is difficult to determine the exact number of human lncRNAs. Researchers estimate there are at least 5,000 to 15,000 long noncoding transcripts with many lncRNAs yet to be annotated (reviewed by Mercer and Mattick, 2013). Although there are a large number of lncRNAs, individual lncRNA tend to express in significantly lower quantities compared to their protein-coding counterparts, making these difficult to detect (Cabili et al., 2011). LncRNAs can be transcribed between or within coding genes. Many lncRNAs are transcribed from the antisense of coding genes (Mercer and Mattick, 2013).

LncRNAs have emerged with important regulatory roles in gene expression. An important question that needs addressing is, how do lncRNAs regulate gene expression? At the structural level, lncRNAs carry their regulatory function by interacting with other molecules,
including DNA, RNA and protein. Proteins are the most common partner of lncRNAs, with at least 15% of expressed proteins having RNA binding capacity (Baltz et al., 2012). Proteins usually do not recognize a specific lncRNA sequence but tend to interact with their secondary structures (Mercer and Mattick, 2013). Significant evidence indicates that lncRNAs can recruit chromatin-modifying proteins to specific promoter sites. The indication that lncRNAs may be crucial accessory factors for Polycomb function first arose when researchers found the interacting factors of Xist: PRC2 (Zhao et al., 2008). In fact, many histone methyltransferases (HMTases) lack DNA binding domain but usually have RNA binding capacity, indicating lncRNAs may play an important role in guiding HMTases to certain chromatin loci (Bernstein and Allis, 2005). Some lncRNAs can also interact with mRNAs. For example, AS Uchl1 is a lncRNA anti-sense to mouse ubiquitin carboxyterminal hydrolase L1 (Uchl1). It can interact with Uchl1 mRNA to regulate its stability and translation (Carrieri et al., 2012). Although there is currently little evidence for direct binding of lncRNA to DNA, researchers have reported a lncRNA, the pRNA (promoter-associated RNA) which forms a DNA:RNA triplex with ribosomal DNA (rDNA) promoter to be specifically recognized by the DNA methyltransferase DNMT3b (Schmitz et al., 2010). LncRNAs are biologically important in many processes. Below I will review several examples of lncRNAs that have important biological functions.

2.2.4.1 LncRNAs and X-Inactivation

In mammals to balance the dosage of X-linked genes between the two genders, a majority of genes on one X-chromosome are inactivated in females, known as X-chromosome inactivation (XCI) (Lyon, 1961; Yue et al., 2015). This inactivation process is driven by a series of lncRNAs, including Xist, Tsix, RepA and Jpx (Froberg et al., 2013; Yue et al., 2015). Xist is
one of the first lncRNAs identified to induce chromosome-wide silencing on inactive X (Xi) (Penny et al., 1996). In the active X (Xa), Tsix is expressed which is antisense to Xist and can block the expression of Xist (Lee, 2000). As mentioned earlier, Xist can silence X-chromosome by recruiting PRC2 to specific gene sites. At the 5' end of Xist, there is a repeat motif identified as “Repeat A” (Zhao et al., 2008). This motif directly interacts with EZH2, the histone methyltransferase in PRC2, which trimethylates histone H3 at lysine 27. With the help of Xist, PRC2 is initially localized to “PRC2 strong sites” (e.g. the bivalent domains and CpG islands) along the Xi. It then migrates to other parts of the chromosome and eventually silences the entire chromosome (Pinter et al., 2012; Valencia and Wutz 2015; Goodrich et al., 2016). A recent study also showed that Xist can silence transcription through KDAC3. Moreover, KDAC3 is critical for Xist-mediated recruitment of PRC2 across Xi (McHugh et al., 2015). Expression of Xist is regulated by other noncoding RNAs. Jpx and Ftx are two noncoding RNAs encoded upstream of Xist promoter that act as activators of Xist (Nora et al., 2012; Goodrich et al., 2016). In Xa, Tsix controls Xist expression by methylating its promoter and modulating its chromatin structure (Sado et al., 2005). Evidence showed that Tsix can interact with PRC2 and establishes H3K27me3 on Xist as well as recruit DNMT3a to enhance hypermethylation on Xist promoter (Sado et al., 2005; Ohhata et al., 2008; Furlan and Rougeulle, 2016).

### 2.2.4.2 LncRNAs from Hox loci

There are several lncRNAs that have been identified to be transcribed from Hox clusters (Fig. 2.12). Investigation on the transcriptional activity of the human HOX loci showed that many of the intergenic regions are actively transcribed and most of the transcripts from the intergenic regions are lncRNAs (Rinn et al., 2007; De Kumar and Krumlauf, 2016). Up to 74%
of these lncRNAs are transcribed from the opposite-strand of *HOX* genes. Rinn and colleagues concluded that *Hox* lncRNAs play important biological roles based on the following key facts: 1. Some lncRNAs are conserved in evolution; 2. Like *HOX* genes, these lncRNA have different expression patterns along the A-P axes depending on their physical location on the chromosome; 3. These lncRNAs possess specific sequence motifs based on their site-specific expression patterns (Rinn *et al.*, 2007; De Kumar and Krumlauf, 2016).

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**Figure 2.12. Noncoding transcripts originating from the four Hox clusters.** Relative positions of non-coding transcripts (green) are shown on the basis of human Hox coding genes (red) as landmark. Arrows indicate the direction of transcription.
Table 2.4. Summary of IncRNAs transcribed from the HOX gene cluster.

<table>
<thead>
<tr>
<th>LncRNA</th>
<th>Binding partner</th>
<th>Epigenetic marks induced</th>
<th>Target gene regulation</th>
<th>Functions and Systems</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HOTAIR</strong></td>
<td>PRC2 (Suz12 and EZH2)</td>
<td>H3K27me3</td>
<td>HOXD8, HOXD9, HOXD10, HOXD11</td>
<td>Human primary foreskin fibroblast cells</td>
<td>Rinn et al., 2007</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>DNA methylation</td>
<td>PTEN</td>
<td>Human laryngeal squamous cell cancer</td>
<td>Li et al., 2013</td>
</tr>
<tr>
<td></td>
<td>PRC2 (EZH2)</td>
<td>H3K27me3</td>
<td>P21</td>
<td>Cell cycle disorder</td>
<td>Liu et al., 2016a</td>
</tr>
<tr>
<td></td>
<td>PRC2</td>
<td>H3K27me3</td>
<td>WIF-1</td>
<td>Activate Wnt pathway in cancer cells</td>
<td>Ge et al., 2013; Li et al., 2016</td>
</tr>
<tr>
<td><strong>HOTTIP</strong></td>
<td>TrxG (WDR5)</td>
<td>H3K4me3</td>
<td>Hoxa10, Hoxa11, Hoxa13</td>
<td>Mouse and chick embryo development</td>
<td>Wang et al., 2011</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>—</td>
<td>P21</td>
<td>Regulate cell growth and apoptosis in colorectal cancer</td>
<td>Lian et al., 2016</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>—</td>
<td>cyclin D1, CDK4 and β-catenin</td>
<td>Activate the Wnt/β-catenin pathway in osteosarcoma</td>
<td>Li et al., 2016</td>
</tr>
<tr>
<td><strong>Mistral</strong></td>
<td>TrxG (MLL1)</td>
<td>H3K4me3</td>
<td>Hoxa6, Hoxa7</td>
<td>Stem cell differentiation</td>
<td>Bertani et al., 2011</td>
</tr>
<tr>
<td><strong>HOTAIR M1</strong></td>
<td>—</td>
<td>—</td>
<td>HOXA1, HOXA4, HOXA5</td>
<td>Myeloid differentiation and maturation</td>
<td>Zhang et al., 2009; Zhang et al., 2014</td>
</tr>
<tr>
<td><strong>HOXA-AS2</strong></td>
<td>PRC2</td>
<td>H3K27me3</td>
<td>P21, PLK3, DDIT3</td>
<td>Gastric cancer</td>
<td>Xie et al., 2015</td>
</tr>
</tbody>
</table>
HOTAIR was the first lncRNA to be studied in human HOX clusters. HOTAIR is a HOX-C loci antisense transcript expressed in posterior and distal sites (Rinn et al., 2007). It has been reported to bind to Suz12 and EZH2, components of PRC2 and induces H3K27me3 in HOXD locus in human primary foreskin fibroblast cells (Rinn et al., 2007). HOTAIR expression is related to several types of cancers. HOTAIR promoter contains estrogen response elements and its expression can be induced by estradiol in the human breast cancer cell line MCF7 (Bhan et al., 2013). HOTAIR is essential for the growth of many cancer cells and knockdown of HOTAIR induces apoptosis in these cancer cells (Bhan et al., 2013; Kong et al., 2015; Tang et al., 2016). In human laryngeal squamous cell carcinoma cell line Hep-2, HOTAIR suppresses the expression of PTEN, a tumor suppressor gene, via DNA methylation (Li et al., 2013). Knockdown of HOTAIR in Hep-2 cells also leads to apoptosis and can inhibit the invasion of Hep-2 cells (Li et al., 2013). Other genes and pathways regulated by HOTAIR in cancer cells include p21, NF-κB and Wnt/β-catenin pathway (Liu et al., 2016a; Özeş et al., 2016; Li et al., 2016). Expression of HOTAIR also increased proliferation and invasion of several tumor cells (Fang et al., 2016, Lee et al., 2016). HOTAIR is also involved during heart failure (Greco et al., 2016).

HOTTIP is an antisense transcript transcribed from 5' to HOXA13 and is required for the activation of 5' HOXA genes (Wang et al., 2011). HOTTIP is expressed in distal human fibroblasts and in posterior sites of mouse and chick embryos. Knockdown of HOTTIP in chick embryo causes shortened forelimbs. ChIP-seq results showed that the down regulation of HOTTIP decreased H3K4me3 occupancy in 5' HOXA genes, from where HOTTIP is transcribed. Further results showed that HOTTIP can bind directly to WDR5, a protein interacting with histone methyltransferase MLL in TrxG. HOTTIP is essential for the recruitment
of MLL1 to 5' HOXA genes through WDR5 (Wang et al., 2011). Like HOTAIR, HOTTIP also plays a very important role in cancer but through regulation of different HOX genes. HOTTIP regulated HOXAI3 expression contributes to the progression of several cancers (Ren et al., 2015; Li et al., 2015b; Zhang et al., 2016). HOTTIP can also regulate the expression of p21 and Wnt/β-catenin pathway and enhance tumor cell proliferation and migration (Li et al., 2016; Cheng et al., 2015; Lian et al., 2016).

Mistral is another IncRNA found in HoxA cluster. It is an antisense transcript located between Hoxa6 and Hoxa7 in mice (Bertani et al., 2011). Similar to HOTTIP, it can activate nearby gene expression (Hoxa6 and Hoxa7) by interacting with MLL1. In this way it induces H3K4me3 modification in Hoxa6 and Hoxa7 and this process is important in stem cell differentiation (Bertani et al., 2011).

A model has been suggested by Rinn et al. (2007) that transcription of IncRNA in cis (e.g. HOTTIP) may recruit TrxG proteins such as MLL1 and WDR5 to chromatin, leading to H3K4me3 and gene activation (Fig. 2.13A). While IncRNAs transcribed but acting in trans may interact with PRC2 and result in H3K27me3 modification and gene silencing (Fig. 2.13B). The later findings on HOTTIP and Mistral support this model.

Other IncRNAs identified from the Hox cluster include HOTAIRM1 and HOXA-AS2. HOTAIRM1 is an antisense intergenic transcript transcribed between human HOXA1 and HOXA2 (Zhang et al., 2009). It can activate 3' HOXA genes and its presence is necessary during myeloid differentiation and myeloid maturation (Zhang et al., 2009; Zhang et al., 2014). HOXA-AS2 is a lncRNA located between the HOXA3 and HOXA4 genes in the HOXA cluster (Zhao et al., 2013). Its expression was found in NB4 promyelocytic leukemia cells and human peripheral blood neutrophils. HOXA-AS2 expression is responsive to all-trans retinoic acid (ATRA)
induction in NB4 cells and can negatively regulate ATRA-induced NB4 cell apoptosis by regulating caspase 8 and 9 pathway (Zhao et al., 2013). HOXA-AS2 can also recruit PRC2 to P21, PLK3 and DDIT3 genes to induce H3K27me3 and silence their expression in gastric cancer cells (Xie et al., 2015).

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**Figure 2.13.** Model of long ncRNA regulation of chromatin domains via histone-modification enzymes. (A) Transcription of IncRNAs in cis may recruit TrxG proteins to chromatin, leading to H3K4 tri-methylation, open the chromatin structure and transcriptionally activate neighboring HOX genes. (B) IncRNAs acting in trans, in contrast, can recruit PRC2 to chromatin, causing a compacted chromatin structure and lead to H3K27 methylation and gene repression.
2.2.5 Cross talk between epigenetic regulators

Thus far I have reviewed how each individual epigenetic regulator can influence gene expression. The reality is usually that the regulation of gene expression is not controlled simply by one epigenetic regulator but is interrelated with many different epigenetic regulators having a coordinated affect on gene expression. Hence, more often multiple epigenetic regulators are involved in regulating the expression of a single gene. One good example is the regulation of gene expression via lncRNA mediated histone methylation that was reviewed above. Histone methylation and histone acetylation are also closely related. It has been reported that TrxG proteins can interact directly with KAT CBP and monomethylation of H3K4 can enhance H3K27 acetylation through CBP (Tie et al., 2014).

In addition, epigenetic regulators have the ability to affect each other. First, the ability of DNA methylation and histone modification to regulate gene expression suggests that the expression of lncRNA and miRNA may be regulated by these modifications (Han et al., 2007). It is predicted that over 90% of the human miRNA promoters are located 1,000 bp upstream of the mature miRNA (Zhou et al., 2007). In human colorectal cancer cell line CRC, hsa-miR-9 gene is included within 1,000 bp of a CpG island which is hypermethylated (Bandres et al., 2009). The hsa-miR-9 expression is downregulated in primary CRC compared to matched normal colorectal epithelial tissues (Bandres et al., 2006). Both DNA demethylation and H3 deacetylation upregulate the expression of the mature hsa-miR-9 (Bandres et al., 2009). Han et.al. (2007) have reported 13 miRNAs in colon cancer cells HCT116 that are regulated by DNA methylation. Moreover, a high level of CpG site demethylation is essential for the re-expression of these miRNAs (Han et al., 2007). When comparing patients with leukemia to healthy individuals, a higher degree of DNA methylation in the CpG islands of miR-196b promoter and a lower
expression of miR-196b was observed in patients with chronic myeloid leukemia (Liu et al., 2013). Research has shown that miR-127 is induced from its own promoter after treatment with demethylating drug 5-aza-20-deoxycytidine (5-Aza-CdR) and 4-phenylbutyric acid (PBA) in both LD419 and T24 cells. This induction is observed together with a decrease in DNA methylation levels and an increase in histone H3 acetylation as well as histone H3K4 methylation (Saito et al., 2006). Scott and colleagues observed that when they inhibited histone deacetylase, 40% of tested miRNAs showed significant changes in expression level within 5 h of treatment (Scott et al., 2006). LncRNA HOTAIR mediates changes in histone methylation status on miR-205 promoter to regulate miR-205 expression in bladder cancer cells (Sun et al., 2015).

While DNA methylation and histone modification can regulate the expression of miRNAs and lncRNAs, several researchers have found that miRNAs can also influence DNA methylation and histone modification. First, miRNAs themselves can target genes that control epigenetic pathways. Clues have been found for miR-140 to target histone deacetylase 4 in mice (Tuddenham et al., 2006). Ezh2, described earlier as a component of PRC2, has its expression regulated by miR-26a, miR-101, miR-205 and miR-214 (reviewed by Sato et al., 2011). Bmi1 belongs to PRC1 and is regulated by miR-128 (Godlewski et al., 2008) and mir-203 (Wellner et al., 2009). In prostate cancer, down regulation of miR-449a causes overexpression of KDAC-1 (Noonan et al., 2009). miR-29 family of miRNAs are shown to directly target DNMT 3a and 3b (Fabbri et al., 2007). miR-142-3p downregulates the expression of TrxG proteins ASH1L and MLL1 in thyroid follicular tumorigenesis (Colamaio et al., 2015). An intriguing experiment presented that miR-165 and miR-166 can interact with the newly processed PHABULOSA (PHB) mRNA and induce DNA methylation (Bao et al., 2004). Another research study showed that the accumulation of miRNA: target-RNA duplexes hypermethylate the genes encoding
target RNAs, leading to gene silencing (Khraiwesh et al., 2010). miRNAs can also regulate lncRNA expression. For example, iab-4 non-coding RNA is a substrate for miRNA (Bender, 2008). miR-192 and miR-204 can directly suppress expression of HOTTIP in hepatocellular carcinoma (Ge et al., 2015).

2.3 WDR5

The WD40 repeat (also known as the WD or beta-transducin repeat) proteins are a group of proteins containing a highly conserved core/repeating units of approximately 40 amino acids that usually terminate with tryptophan (Trp, W) - aspartic acid (Asp, D) dipeptide (Neer et al., 1994). WD repeat proteins normally contain 4-16 WD repeats (Li and Roberts, 2001). The WD repeat proteins are a large family of proteins present in all eukaryotes and involved in a variety of cellular activities including cell division (Li et al., 2015a), transmembrane signalling (Neer et al., 1994), autophagy (Grimmel et al., 2015), gene regulation (Sun et al., 2015) and apoptosis (Li et al., 2015a). These proteins play key roles in the formation of protein-protein complexes in nearly all the major pathways and organelles unique to eukaryotic cells, associating them with many genetic diseases (Smith, 2008).

WDR5 is a protein belonging to the WD repeat protein family. It was first identified in 2001 and named BIG-3 (BMP-2 induced gene 3kb, Gori et al., 2001). Later it was renamed WDR5 (WD repeat protein 5) as it contains seven WD-40 repeats (Gori et al., 2005). As a member of the WD repeat protein family, WDR5 is hypothesized to have the three common features shared by WD repeat proteins (Li and Roberts, 2001) and these are: 1) the WD repeat domains are folded into beta propellers; 2) the WD repeat domains can reversibly assemble to multiple protein complexes and serve as a platform without any catalytic activities; 3) WDR5
may be involved in regulating cellular functions, such as cell division, cell-fate determination, gene transcription, and mRNA modification. In fact, WDR5 is linked to various diseases including leukemia (Ali et al., 2014), breast carcinogenesis (Dai et al., 2015), prostate cancer (Kim et al., 2014) and pulmonary hypertension (Chen et al., 2015).

2.3.1 Structure of WDR5

Since the first structural determination of WD repeat protein in 1995 (Wall et al., 1995), hundreds of different WD repeat proteins have been identified (Neer and Smith, 2000). These WD repeat proteins share a common beta-propeller structure composed of several four-stranded anti-parallel beta sheets/blades (Li and Roberts, 2001; Smith, 2008). Each WD repeat sequence encodes a structure of four beta anti-parallel strands but the repeat structure is not equivalent to a single blade. Rather, each repeat consists the first three strands of one blade and the last strand of the previous blade (Fig. 2.14e; Li and Roberts, 2001; Smith, 2008). In this way, the molecule can be more stabilized (Neer et al., 1994). These repeats also have a high percentage of hydrophobic residues to form the contact surface between the blades (Neer and Smith, 2000). The WD domain structure has two ends: the narrower end is often called the top, and wider end is called the bottom. In addition, there is a central tunnel that varies in shape and diameter based on the number of blades included (Neer and Smith, 2000).

The crystal structure of WDR5 is usually studied together with its associated complexes. Like other WD40 repeat proteins, WDR5 exhibits a typical propeller-like structure with seven blades and a narrow central tunnel (Han et al., 2006). Below I summarize important structures of WDR5 that interact with different proteins and complexes.
**Figure 2.14. Structure of WDR5.** (a). Top and side views of crystal structure of WDR5 bound to N terminus of H3. H3 is bound to top face (the narrower end) of the β propeller of WDR5 (Taken from Couture et al., 2006 with permission). (b). Top and side views of crystal structure of WDR5 bound to MLL1. Similar to H3, MLL1 is also bound to top face of the β propeller of WDR5. (Taken from Song and Kingston, 2008 with permission) (c). Top and side views of crystal structure of WDR5 bound to RBBP5. RBBP5 is bound to the bottom face (the wider end) of the β propeller, (opposite site of H3 binding site). (d). Side view of crystal structure of WDR5 bound to KANSL1 and KANSL2. KANSL1 is bound to top face the β propeller of WDR5 and KANSL2 is bound to the bottom face (Taken from Dias et al., 2014 with permission). (e). WDR repeat proteins share a common beta-propeller structure composed of several anti-parallel beta sheets/ blades. The four-stranded anti-parallel beta sheets encoded by the same WD repeat sequence is marked in the same color. Each blade is composed of the first three parallel sheets of one WD repeat and the last parallel sheet of the previous WD repeat (Adapted from Valeyev et al., 2008).

**Recognition of Histone 3** - The top face (the narrower end) of the β propeller of WDR5 can recognize H3 protein, specifically the first three amino acids, Ala-Arg-Thr (ART, immediately preceding Lys4) of H3 (Fig. 2.14a, Han et al., 2006). There are several key amino acids in WDR5 involved in the binding to H3. The carboxylate side chain of Asp107 in WDR5 hydrogen bonds to the free amino group of Ala1 in H3. Ser91 and Cys261 in WDR5 also form a hydrogen bond to the backbone amide and carbonyl oxygen of Arg2 in H3 peptide. Tyr131 in WDR5 interacts with Ala1 in H3 via van der Waals contact (Han et al., 2006 and Couture et al., 2006). WDR5 have varied binding capacities to H3K4 with different degrees of methylation (Wysocka et al., 2005). Although the methyl groups of H3K4me2 are located on the surface of WDR5 with no obvious contact with WDR5, Han and colleagues believed that H3K4 dimethylation (H3K4me2) has the strongest binding affinity to WDR5 because the two methyl groups of Lys4 can form an extra pair of nonconventional hydrogen bonds with the carboxylate oxygen of Glu322 in WDR5. Compared with H3K4me2, H3K4 monomethylation (H3K4me1) has only one nonconventional hydrogen bond with Glu322, causing reduced interaction with
WDR5. The extra methyl group in H3K4me3 causes loss of the hydrogen bond, resulting in weaker affinity with WDR5 (Han et al., 2006 and Schuetz et al., 2006).

**MLL1-WDR5-RBBP5 complex** - As mentioned earlier, WDR5 together with RBBP5 and MLL can form an H3K4 methyltransferase complex. MLL proteins can bind WDR5 through a Win motif. More detailed structure analysis indicated that Win motif of MLL1 binds WDR5 at the top face of the β propeller, which is the same binding pocket for H3 (Fig. 2.14b, Patel et al., 2008; Song and Kingston, 2008). There are several key amino acids in MLL1 Win motif that interact with WDR5. Gly3762 and Ser3763 of the MLL1 form multiple hydrogen bond and van der Waals interactions with Ala47, Ala65, Gly89, Ile90, and Asp107 in WDR5. The side chain of Arg3765 of MLL1 inserts into the central tunnel of WDR5 and form hydrogen bond and hydrophobic interactions with Ser91, Phe133, Ser175, Ser218, Cys261, Phe263, and Ile305 in WDR5. In addition, the Arg3765 is positioned between Phe133 and Phe263 of WDR5. These facts make Arg3765 of MLL1 the key amino acid for WDR5 binding. Ala47 and Tyr260 in WDR5 interacts with Ala3766 of MLL1 and Tyr-260 in WDR5 makes van der Waals contacts with Ala3766, Glu3767, and Val3768 in MLL1. His3769 of MLL1 interacts with WDR5 residues: Lys259, Phe149, Asp172, Pro173, and Tyr191 (Patel et al., 2008; Song and Kingston, 2008).

RBBP5 consists of an N-terminal β-propeller domain and a C-terminal hinge region. RBBP5 binds to WDR5 through its hinge region and more precisely, the segment located between residues 371 and 380 (Odho et al., 2010; Avdic et al., 2011). RBBP5 binds to a V-shaped cleft formed by the junction of blades 5 and 6 of WDR5 at its bottom face (the wider end) of the β propeller, which is on the opposite site of H3 and MLL1 binding sites (Fig. 2.14c). Similar to the binding of H3 and MLL1, WDR5 binds RBBP5 by maintaining several hydrogen
bonds and van der Waals interactions. The Gln289 side chain in WDR5 makes van der Waals contacts with Glu374 in RBBP5. WDR5 can form two hydrophobic pockets for RBBP5 binding. Pocket 1 is created by amino acid Leu249, Tyr228, Leu240 and Lys250 in WDR5 to make hydrophobic interactions with Val375 in RBBP5. And pocket 2 consists of Phe-266, Val-268, and Leu-288 in WDR5 to bind Val377 in RBBP5. Val 375 in RBBP5 also forms two hydrogen bonds with Gln289 side chain in WDR5. Asp376 and Ser379 in RBBP5 all form hydrogen bonds with Asn225 in WDR5 and WDR5′s Pro224 carbonyl group hydrogen bonds to Thr378 in RBBP5. There is also interaction between the RBBP5 Val380 and the WDR5 Arg181 (Odho et al., 2010; Avdic et al., 2011).

**KANSL1/WDR5/KANSL2 complex** - The nonspecific lethal (NSL) complex is a distinct multi-protein complex. Together with the KAT MOF (male absent on the first), they form an evolutionarily conserved KAT complex (Dias et al., 2014). WDR5, KANSL1 and KANSL2 are all subunits of NSL complex and both KANSL1 and KANSL2 can interact with WDR5 (Zhao et al., 2013; Dias et al., 2014). Interestingly, KANSL1 and KANSL2 binds to opposite sides of WDR5 β propeller like MLL1 and RBBP5 (Fig. 2.14d, Dias et al., 2014). KANSL1 interacts with WDR5 by a region centered on Arg592 in a similar manner to MLL proteins and H3. Arg592 in KANSL1 inserts into the central tunnel of the β propeller of WDR5 and form hydrogen bonds with Phe133, Phe263, Ser91, and Cys261 in WDR5. Thr587 and Val589 in KANSL1 form additional hydrogen bonds with Lys67, Ala65, and Gly89 in WDR5. Arg594 in KANSL1 makes a salt bridge with Asp107 in WDR5 and Val596 in KANSL1 makes a hydrophobic interaction with Tyr191, Pro216, and Leu234 in WDR5. Mutation on Arg592 in KANSL1 lead to unstable interaction with WDR5 and has resulted in protein degradation (Dias et al., 2014). KANSL2, like RBBP5, binds to the bottom face of the β propeller of WDR5.
Leu411, Val413, and Val414 of KANSL2 insert into a hydrophobic cleft between blades 5 and 6 of the WDR5 domain. The mutation of either Leu411 or Val413 in KANSL2 is sufficient to disrupt its interaction with WDR5 (Dias et al., 2014).

**LncRNA binding interface on WDR5** - LncRNA HOTTIP and RBBP5 have shared binding cleft on WDR5 - the cleft between blades 5 and 6. Mutations of WDR5 on Tyr228, Leu240, Lys250, and Phe266 can significantly reduce its ability to bind HOTTIP. And among these mutations, F266A only affected its binding to HOTTIP without any defects in binding of MLL1 and RBBP5 (Yang et al., 2014).

### 2.3.2 Biological functions of WDR5

#### 2.3.2.1 WDR5 is an important subunit in several protein complexes

WDR5 is required as a scaffold for multi-protein complex interactions. It plays a role in facilitating the assembly of many chromatin-modifying complexes. The function of WDR5 as a subunit of the Histone methyltransferase complex MLL/WDR5/RBBP5 was first discovered in 2005 (Wysocka et al., 2005) and is now becoming the most well studied function of WDR5. WDR5 is important for the recognition of H3 and for linking the methytransferase MLL to the rest of the WRAD complex. WDR5 is specifically and directly associated with methylated H3K4 and knockdown of WDR5 in human cells affects global H3K4 methylation levels (Wysocka et al., 2005). Together with the SET1-family complex, they achieve transcriptional activation via methylation of H3K4.

WDR5 is also a subunit of the histone acetyltransferase complex MOF/NSL. MOF is a member of the MYST family of histone acetyltransferases and is important in DNA damage repair and the maintenance of cell cycle and genomic stability (Zhao et al., 2013). MOF/NSL
complex can acetylate H4 on K16, K5, and K8 (Zhao et al., 2013). MOF directly interacts with KANSL1. WDR5 interacts with both KANSL1 and KANSL2 and brings the MOF/NSL complex together (Dias et al., 2014). In Drosophila, a point mutation of NSL1 that disrupts its interaction with WDS (WDR5 in Drosophila) is entirely lethal to females and partially lethal to males (Dias et al., 2014).

Since histone methylation on H3K4 and histone acetylation are all gene activation marks and both MLL/SET complex and MOF/NSL complex share a common WDR5 subunit, a question that needs addressing: is there any crosstalk between these two modifications? Indeed, there is a crosstalk between MOF-mediated H4K16 acetylation and MLL/SET-mediated H3K4 methylation. Mutation or knockdown of MOF led to a reduced global histone H3K4 di-methylation level in human cells while overexpression of MOF promoted H3K4 mono-, di- and tri-methylation. In contrast, the knockdown of RBBP5 only affected global H3K4 methylation level but had less effect on H4K16 acetylation. These findings indicate that MOF/NSL mediated H4 acetylation contributes to H3K4me2 by MLL/SET complexes (Zhao et al., 2013). More interestingly, WDR5 interacts with PKN1-mediated histone H3 threonine 11 phosphorylation (H3T11P), which in turn recruits MLL/SET complex leading to H3K4me3 occupancy (Kim et al., 2014). Since WDR5 is also a subunit of MOF/NSL complex, it is highly possible that H3T11P can also recruit MOF/NSL complex leading to H4 acetylation.

WDR5 is also reported to be a component of a CHD8 (chromodomain, helicase, DNA-binding) complex which is a group of ATP-dependent chromatin remodeling enzymes critical for the regulation of chromatin structure by modulating the contacts between histones and DNA (Thompson et al., 2008).
2.3.2.2 Role of WDR5 in regulation of Hox gene expression

As a component of multiple histone modification complexes, WDR5 plays an important role in gene regulation. Hox genes are well known targets of MLL/SET complex. Knockdown of WDR5 led to decreased H3K4me3 occupancy over the promoter regions of HOXA9 and HOXC8 and resulted in decreased expression of HOXA9 and HOXC8 (Wysocka et al., 2005). Hoxa2 and Hoxa10 are also targets of MLL/SET complex. The arginine methyltransferase PRMT6 (protein arginine methyltransferase 6) mediated H3R2 methylation can inhibit recruitment of WDR5 and H3K4 trimethylation and overexpression of PRMT6 resulted in decreased Hoxa2 and Hoxa10 expression (Hyllus et al., 2007). The lncRNA HOTTIP interacts with WDR5 and induces H3K4me3 on 5' HOXA genes. Knocking down of WDR5 greatly abolished transcription of HOTTIP and 5' HOXA genes in primary foreskin fibroblast cells (Wang et al., 2011).

2.3.2.3 WDR5 is important in cell differentiation and development

WDR5 is also important for bone growth and skeletal development. WDR5 is expressed in osteoblasts, osteocytes as well as growth plate chondrocytes (Gori et al., 2001). The overexpression of Wdr5 in a murine prechondroblastic cell line led to increased cAMP production and parathyroid hormone (PTH) binding as well as accelerated formation of mineralized nodules, which are all signs of increased osteoblastic differentiation (Gori et al., 2001). The persistent acceleration of osteoblast differentiation caused by overexpression of WDR5 was confirmed by postnatal analyses of transgenic mice and suggested that this effect is associated with the activation of the canonical Wnt pathway (Gori et al., 2006). Wdr5 suppression resulted in significantly decreased alkaline phosphatase activity, Runx2 and osteocalcin expression, and absence of mineralized matrix formation, which are all indicators of
inhibited osteoblast differentiation (Zhu et al., 2008). WDR5 can also affect chondrocyte differentiation by modulating the expression of Twist-1 and fibroblast growth factor 18 (FGF18) (Gori et al., 2009). Down regulation of Wdr5 in developing chick limb confirmed that Wdr5 is required in vivo for endochondral bone development (Zhu et al., 2010). The down regulation of Wdr5 resulted in impaired chondrocyte maturation, delayed endochondral bone development and shortened skeletal elements (Zhu et al., 2010). In ameloblastoma, abnormal bone resorption and bone formation are observed. Patients with ameloblastoma showed down regulated WNT-related bone-forming genes including WDR5 and Runx2, further supporting that WDR5 is important in bone formation (Sathi et al., 2012).

The transcription and regulatory networks in pluripotent ES cells are complicated and critical for the maintenance of ES cell differentiation and self-renewal and WDR5 is critical in maintaining epigenetic status in these networks. The expression of WDR5 has been found to be upregulated during the formation of induced pluripotent stem (iPS) cells and downregulated upon ES cell differentiation (Ang et al., 2011). Also higher levels of Wdr5 expression and H3K4me3 occupancy were observed in ES cells compared to somatic cells and tissues, suggesting a role for Wdr5 in ES and iPS cell maintenance (Ang et al., 2011). The knockdown of Wdr5 induced changes in ES cell morphology and resulted in loss of self-renewal. Furthermore, the depletion of pluripotency transcription factor Oct4 led to down regulated Wdr5 expression and decreased global H3K4me3 occupancy (Ang et al., 2011). In addition, the binding of lncRNA and WDR5 are important for ES cell pluripotency and differentiation. The binding of lncRNAs appears to be able to stabilize WDR5 protein and several ES cell lncRNAs that can bind WDR5 have been shown to be essential for maintaining ES cell fate (Yang et al., 2014). In mouse ES cells, expression of LincRNA1230 can block the localization of Wdr5 at the promoters
of early neural genes and inhibit the cells from adopting a neural cell fate (Wang et al., 2016). As a component of KAT complex MOF/NSL, WDR5 is also important in facilitating the maintenance of H4K16ac in ES cells (Li et al., 2012; Mu et al., 2015).

2.3.2.4 Essential role of WDR5 in antiviral signaling pathway

Viral infection can cause the assembling of a virus-induced complex on the outer membrane of mitochondria and activate NF-KB and IRF3 to induce type I interferons (IFNs) (Wang et al., 2010). WDR5 is associated with virus-induced signaling adaptor (VISA), the key protein for assembling the virus-induced complex, and is translocated from nucleus to the mitochondria outer membrane. Knockdown of WDR5 disrupted the formation of VISA associated virus-induced complex and inhibited the trigger of NF-KB and IRF3 expression, suggesting that WDR5 plays an important role in cellular antiviral response (Wang et al., 2010).

2.4 Sumoylation and its role in nuclear protein translocation

Sumoylation is a posttranslational modification by an approximately 10 kDa Small Ubiquitin-like Modifier (SUMO) proteins. This family of small proteins can modify the function of their target protein by covalently attaching to and detaching from them. Three enzymes are involved in sumo conjugation process: an E1 activating enzyme, an E2 conjugating enzyme and an E3 sumo ligase. Sumoylation is involved in several important cellular processes, including cell cycle, subcellular transportation, regulation of transcription and protein stability (reviewed by Hay, 2005; Kumar and Zhang, 2015).

Among all the important functions of sumoylation, I have focused on its role in protein translocation. SUMOylation has emerged as a major mediator of nucleo-cytoplasmic
translocations of proteins. RanGAP1, the first SUMO substrate being identified, is a cytoplasmic protein (Matunis et al., 1996). The sumoylated RanGAP1 can activate the small GTPase protein Ran, a key player in the nuclear pore complex (NPC) that controls the nucleocytoplasmic trafficking of proteins (Mahajan et al., 1997). In HEK293 cells, five sumoylation sites have been identified on CDC73 protein yet only the sumoylation on K136 is located within the nuclear localization sequence and is responsible for the nuclear localization of CDC73 (Lamoliatte et al., 2014). The serine/threonine kinase GSK3β is a protein important in neuronal development. GSK3β can be sumoylated and is present in both cytoplasm and nucleus under normal conditions. A mutation of GSK3β that inhibit sumoylation resulted in exclusion of GSK3β in the nucleus (Eun Jeoung et al., 2008; Berndt et al., 2012). Three lysine residues of the IGF1R can be SUMO-1 sumoylated and the mutations of these SUMO-1 sites prohibited the translocation of IGF1R into the nucleus (Sehat et al., 2010). Sumoylation can also translocate proteins from nucleus to cytoplasm, examples include MEK1 and TEL. The cytoplasmic localization of both proteins are dependent on their sumoylation and disruption of sumoylation prevents the nuclear export of both proteins (Sobko et al., 2002; Wood et al., 2003). SUMO activation enzyme (SAE) can rapidly shuttle in and out of nucleus when it is not sumoylated at the C-terminus of SAE2 subunit. Sumoylation at the C-terminus of SAE2 subunit prevents the protein from being exported out from the nucleus (Truong et al., 2012).
CHAPTER 3

3. Goal of the project

3.1 Hypotheses to be tested:

The Hoxa2 gene has been found to play a very important role in palate development in mice (Smith et al., 2009). DNA methylation is an epigenetic pathway that can regulate gene expression through the methylation of CpG islands on gene promoter. I first hypothesized that CpG islands exist on Hoxa2 promoter and DNA methylation of the Hoxa2 gene promoter will regulate Hoxa2 expression in the developing mouse palate.

MicroRNAs are small noncoding RNAs that can lead to gene silencing and have been found to regulate hox genes in embryo development. Yet only one miRNA has been reported to directly target Hoxa2 gene. My second hypothesis is that specific miRNAs will directly bind Hoxa2 3' UTR and down regulate Hoxa2 expression at both transcriptional and translational level.

Hotairm1 is a lncRNA identified in human that is transcribed between Hoxa1 and Hoxa2 and can activate the expression of Hoxa1 and Hoxa2. The regulatory mechanisms of Hotairm1 remain unstudied. My second hypothesis is that a noncoding transcript exists in the mouse genome between Hoxa1 and Hoxa2 genes and that can regulate their expression. This lncRNA will regulate Hoxa1 and Hoxa2 genes expression via histone modification. WDR5 is sumolylated and this modification is important in lncRNA induced histone methylation of Hoxa1 and Hoxa2 genes.
3.2 Objectives:

To test my first hypothesis, my objectives are to investigate the DNA methylation status of *Hoxa2* promoter in developing mouse palate and to determine whether *Hoxa2* expression will be regulated by DNA methylation in this process.

To test my second hypothesis, my objectives are to determine if specific miRNA binding sites are present on 3' UTR of *Hoxa2* mRNA and to determine if the miRNA's identified from above, are expressed in the developing mouse palate and whether this correlates with *Hoxa2* expression.

To test my third hypothesis, my objectives are to characterize a lncRNA transcribed between *Hoxa1* and *Hoxa2* and determine whether this lncRNA plays a role in regulating *Hoxa1* and *Hoxa2* expression. To identify the histone modification complex that interacts with the lncRNA. To investigate whether WDR5 is sumoylated and whether this modification plays a role in the function of WDR5.
CHAPTER 4

4. Materials and methods

4.1 Materials

4.1.1 Chemicals and reagents

Chemicals, reagents, kits, antibodies, miRNA mimic and siRNA, primers, vectors, enzymes and equipments used in the materials and methods section are listed below.

Table 4.1. List of chemicals, reagents and equipments.

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**Commercially Available Kits**

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**Antibodies**

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**Commercially Available miRNA mimic and siRNA**

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**Commercially Available Primers**

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### Table 4.2. Names and addresses of distributors

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### 4.1.2 Cell lines

In this study, NIH 3T3 (ATCC CRL-1658) and EG7 (ATCC CRL-2113) cell lines were used. NIH 3T3 is a mouse embryonic fibroblast cell line that exhibits expression of both *Hoxa1*
and Hoxa2. In addition, Hoxa2 promoter was demonstrated to be unmethylated in this cell line (X. Wang, PhD Thesis, 2013). EG7 cell line is derived from the murine T-cell lymphoma EL-4 transfected with cDNA for ovalbumin (OVA). Previous studies from our laboratory has shown Hoxa2 promoter to be highly methylated with no Hoxa2 expression in the EG7 cell line (X. Wang, PhD Thesis, 2013).

4.1.3 Animals

Female timed-pregnant CD-1 mice were obtained from Animal Resources Centre, University of Saskatchewan. All procedures were approved by the University Committee on Animal Care and Supply at the University of Saskatchewan.

4.2 Methods

4.2.1. Cell culture conditions

The mouse embryonic fibroblast NIH 3T3 cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% bovine calf serum (BCS) and 1% antibiotic/antimycotic (Ab/Am). The mouse lymphocyte EG7 cell line was cultured in DMEM supplemented with 10% FBS, 110 mg/L sodium pyruvate and 1% Ab/Am. Both cell cultures were incubated with 5% CO₂ and 100% relative humidity at 37° C.

4.2.2. DNA methylation analysis

4.2.2.1 Sodium bisulfite modification

Genomic DNA samples were extracted from NIH 3T3 cell line, EG7 cell line and E12 to E15 mouse palate tissue using PureLink® Genomic DNA Mini Kit (Invitrogen). Genomic DNA
was modified with bisulfite reagent for DNA methylation analysis using the EZ DNA methylation kit (Zymo Research, USA). According to the manufacturer’s instructions, 2 µg of genomic DNA was modified per sample. NanoVue UV/visible Spectrophotometer (GE Healthcare) was used to determine the concentration and purity of bisulfite treated DNA samples. Bisulfite treated DNA samples were stored at -20°C.

4.2.2.2 Methylation specific PCR

After sodium bisulfite modification of genomic DNA, DNA methylation within promoter associated CpG islands was determined with methylation specific PCR (MSP) primers listed in Table 4.3. The principle used to design MSP primers are shown in Figure 4.1. Bisulfite treatment of DNA can convert unmethylated cytosine (C) into uracil (U) while the methylated C will remain as C. In this way the methylation status of a CpG island can be identified by its sequence after bisulfite conversion. MSP primers were designed to include at least one CpG site in the primer sequence and based on the sequence differences between methylated and unmethylated CpG island following bisulfite treatment. Two sets of primers were designed for each CpG island, the methylated primer set and the unmethylated primer set. In total, six pairs of MSP primers were designed to detect methylation status of the three CpG rich regions of the Hoxa2 promoter (Table 4.3).

For the PCR amplification (a total of 50µl), 200 ng of bisulfite-modified DNA, 2 × EpiTect MSP master mix (Qiagen), 200 µM of each dNTP and 500 nM of each forward and reverse primer was used. The PCR cycle for region 1 and 3 (MPS-CpG1 and MPS-CpG3) started with a Taq activation step at 95°C for 10 min, followed by 35 cycles of 94°C for 15sec, 53°C for 30sec, 72°C for 30sec and a final extension step at 72°C for 10 min. The PCR cycle for region 2
(MPS-CpG2) had 1 cycle of 95°C for 10 min, followed by 35 cycles of 94°C for 15 sec, 55°C for 30 sec, 72°C for 30 sec and a final extension step at 72°C for 10 min.

**Figure 4.1. Bisulfite determination of DNA methylation.** Under the treatment of bisulfite conversion, the methylated cytosine (C) in the genomic DNA will remain as C, while unmethylated C will be converted into uracil (U). Based on this, methylation specific primers (MSP) can be designed for methylated and unmethylated sequences. During PCR, U will be replaced with thymine (T). BSP primers are designed in the sequences that lack CG sites.

### 4.2.2.3 Bisulfite specific PCR and sequencing

After sodium bisulfite modification of genomic DNA, bisulfite specific PCR (BSP) amplification was carried out. BSP primers were designed in the sequences that lack CG sites so the CpG island, whether methylated or not, can be amplified by the same BPS primer and the sequence difference can be tested by DNA sequencing (Fig. 4.1). For the PCR amplification (a
total of 50µl), 200 ng of bisulfite-modified DNA, 10×EconoTaq buffer, 2.5 units of EconoTaq DNA polymerase (Invitrogen), 200 µM of each dNTP and 500 nM of each forward and reverse primer (Table 4.3) were used. The PCR cycle started with one step at 95°C for 3min, followed by 35 cycles of 95°C for 30sec, 55°C for 30sec, 72°C for 40sec and a final extension step at 72°C for 10 min. DNA samples were run on a 1% agarose gel and target bands were excised from the gel and purified using QIAquick Gel Extraction kit (Qiagen). The purified DNA samples were then cloned into pGEM® T-easy vector (Fig.4.2, Promega). The ligation system (10 µl) was set up with 5µl 2x Rapid Ligation Buffer, 1 µl pGEM® T-easy vector, 1 µl T4 DNA ligase and 3 µl gel purified PCR product (pGEM®-T Easy Vector System I, Promega). Ligation was carried at 10°C over night, followed by 5 min incubation at 70°C to inactivate T4 DNA ligase. Ligation product was then transformed into DH5α™ Competent Cells (Subcloning Efficiency™, Invitrogen). For each transformation, 5 µl ligation product was incubated with 50 µl DH5α™ competent cells for 40 min on ice and heat shocked at 42°C for 90 s. Cells were then incubated on ice for 2 min and recovered in 200 µl LB medium (10g Bacto-Tryptone, 5g Bacto-yeast extract and 10g NaCl in 1L medium, PH 7.4) for 30 min at 37°C. The cells were then spread in LB-ampicillin agar plates (10 ml LB per plate with 1% ampicillin and 1.5% agar) with 20mM IPTG and 80µg/ml X-gal for blue-white screening. The plates were incubated at 37°C overnight and each single white clone was picked and cultured in 4ml LB with 1% ampicillin overnight at 37°C. Cells were then collected and plasmids were purified with GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) as per manufacture’s protocol. The plasmids containing BSP amplification fragments were sequence analyzed with an ABI PRISM™ system using M13 forward primer at the National Research Council, Plant Biotechnology Institute, Saskatoon, SK.
**Figure 4.2. pGEM® T-easy vector map (Promega).** pGEM®-T Easy Vector is a linearized vector with a single 3’-terminal thymidine (T) at both ends. PCR products generated by certain polymerases are adenine (A) tailed and can be ligated to pGEM®-T Easy Vector based on A-T pairing. pGEM®-T Easy Vector has the α-peptide coding region of the enzyme β-galactosidase (lacZ) which allows blue/white screening on indicator plates. The pGEM®-T Easy Vector also provides three single-enzyme digestions (EcoRI, BstZI and NotI) for release of the insert. Figure taken from Promega http://embed.widencdn.net/img/promega/ws62vit9cf/640px/1473VA05_6B.jpeg?u=7fvzhm.

### 4.2.3. Prediction and analysis of miRNA binding sites on mouse Hoxa2 3' UTR

The website http://www.microrna.org was used to predict miRNAs that may bind to mouse 3' UTR of Hoxa2 gene, and comparisons made with the binding site present in mouse 3' UTR Hoxa2 gene with other species including human, rat, chimpanzee and dog using Genbank (NCBI).
4.2.4. RNA Isolation and Reverse Transcription (RT)

For the mRNA study, total RNA was isolated from NIH 3T3 cells, mouse embryo palate shelves (E12 to E15) and mouse tissue samples (head, forelimb, hindlimb and tail) from E13 embryos using RNeasy Protect Mini Kit (Qiagen). Each sample was reverse transcribed to cDNA using QuantiTect® Reverse Transcription Kit (Qiagen). Genomic DNA (gDNA) elimination reaction was set up to a total volume of 14 µl with 2 µl 7x gDNA Wipeout Buffer, 500ng template RNA and incubated for 2 min at 42°C and placed on ice immediately. Reverse-transcription master mix was prepared with 1 µl Quantiscript Reverse Transcriptase, 4 µl 5x QuantiScript RT Buffer and 1 µl RT Primer Mix and added to gDNA eliminated RNA template from the previous step. The reverse transcription was then carried out by incubating the system at 42°C for 1 h and 3 min at 95°C to inactivate Quantiscript Reverse Transcriptase. cDNA samples were then stored at -20°C for further experiments. For miRNA study, total RNA including small RNA was isolated from NIH 3T3 cell culture and mouse embryo palate shelves (E12 to E15) using miRNeasy Mini Kit (Qiagen) and was reverse transcribed into cDNAs using miScript II RT Kit (Qiagen). A total volume of 20 µl reverse-transcription reaction containing 4 µl 5x miScript HiSpec Buffer, 2 µl 10x miScript Nucleics Mix, 2 µl miScript Reverse Transcriptase Mix and 500 ng template RNA was incubated for 60 min at 37°C followed by 5 min incubation at 95°C to inactivate miScript Reverse Transcriptase Mix. cDNA samples were stored at - 20°C for further experiments.

4.2.5. Quantitative real-time PCR (qRT-PCR)

In miRNA study, *Hoxa2* gene expression was detected using FAM (fluorescein amidite)-labeled Taqman gene expression primers (Applied Biosystems). VIC-labeled β-actin Taqman
primers were used as endogenous control. PCR reaction started with a 2 min initiation at 50°C and 10 min denaturation at 95°C, followed by 40 cycles with each cycle consisting of 15 s at 95°C and 70 s at 60°C (Smith et al., 2009). Mature miRNAs were detected with individual miRNA SYBR® green primers (Qiagen). Small nuclear RNA (snRNA) RNU6B was used as control gene of miRNA expression and was detected with RNU6B SYBR® green primers (Qiagen). Amplification system of 25µl was prepared with miScript SYBR® Green PCR Kit (Qiagen) with 25ng cDNA input and the reaction cycles were as follows: 15 min initial activation at 95°C, followed by 40 cycles of 15s at 94°C, 30s at 55°C and 30s at 70°C in 7300 ABI detection system. All reactions were run in replicates of 2, n=4.

For the IncRNA study, Hoxa1, Hoxa2 and mHotairm1 expressions were detected using primers as listed in Table 4.1 and Table 4.3. β-actin was detected using primers listed in Table 4.3 as control house keeping gene. SYBR® Green PCR Master Mix (Applied Biosystems) was used and the reaction cycles were as follows: 10 min initiation at 95°C, followed by 40 cycles of 15s at 95°C and 60s at 60°C in 7300 ABI detection system. All reactions were run in replicates of 2, n=3.

Relative quantity (RQ) value was determined by the $2^{(\Delta\Delta C_T)}$ method described in the (Smith et al., 2009) and from Applied Biosystems User Bulletin (ABI PRISM 7700 Sequence Detection System, Applied Biosystems, 2001).

4.2.6. Western blot analysis

Protein samples were collected from NIH 3T3 cells and E13 Hoxa2−/− mice embryos with radioimmunoprecipitation assay buffer (RIPA buffer). Samples were then quantified using a Bio-Rad DC Protein Assay kit and denatured for 10 min with 2X loading buffer. Each sample was
electrophoresed in a 10% polyacrylamide-SDS gel and transferred to a PVDF transfer membrane (Thermo Fisher Scientific) using Xcell II Blot Module (Invitrogen) for wet transfer in 1X transfer buffer containing 12 mM Tris, 96 mM glycine and 20% methanol, for 1.5 h at 20V/150 mAmp.

In miRNA study, the blotting and detection of Hoxa2 protein was performed as described in Smith et al. (2009). Briefly, PVDF membranes were blocked in 3% skim milk (in PBS) at 4 °C for 1 h and then incubated with the rabbit anti-Hoxa2 antiserum (Hao et al., 1999) at a dilution of 1:1000 in 3% skim milk overnight at 4 °C. Membrane were then washed 3 x 10 min with 0.1% Tween-20 in PBS (PBST) and incubated for 1 h at room temperature with horse radish peroxidase (HRP) conjugated secondary goat anti-rabbit IgG (1:3000 in 5% skim milk, Bio-Rad). Visualization of proteins was performed by a Clarity™ Western ECL Blotting Substrate Kit (Bio-Rad) as per manufacture’s instructions, followed by exposure of the membranes using Syngene imaging system (Syngene, Cambridge, UK). The same membranes were probed with GAPDH antibody as an internal control (sc-365062, 1:3000, Santa Cruz®) for 1h at room temperature and secondary anti-mouse IgG antibody (1:3000, Bio-Rad®) for 1h at room temperature.

In lncRNA study, to detect WDR5 protein, transfer membranes were blocked in 3% bovine serum albumin (BSA) for 1 h (in PBST) followed by overnight incubation with anti-WDR5 antibody (ab22512, 1:1000, Abcam®) at 4°C, and subsequent incubation with secondary anti-rabbit IgG antibody (1:3000, in 5% skimmed milk in PBST, Bio-Rad®) for 1 h at room temperature. Sumoylated protein was detected by overnight incubation with SUMO1 21C7 antibody (1:500, in 3% BSA, Developmental Studies Hybridoma Bank) at 4°C and secondary anti-mouse IgG antibody (1:3000, in 5% skimmed milk, Bio-Rad®) for 1h at room temperature.
4.2.7. MiR-669b and miR-376c over expression

NIH 3T3 cells (6 × 10⁴/well) were seeded in a 24-well plate in 0.5 ml culture medium. HiperFect Transfection Reagent, (6 µl, Qiagen®) and 300 ng of either miRNA-669b mimic, miR-376c mimic (Qiagen®) or control siRNA (Qiagen®) were applied to each well based on HiperFect transfection protocol (Qiagen®). Cells were then incubated under normal growth conditions and RNA samples were isolated after 24 h and 48 h incubation. Protein samples were collected after 24 h incubation.

4.2.8. Hoxa2-3'UTR mutation vector construction

The vector pEZX-MT01-Hoxa2 (GeneCopoeiaTM) contained full length mouse Hoxa2 3'UTR. QuikChange Lightening Site-Directed Mutagenesis Kit (Agilent Technologies) was used to carry out site directed mutations on Hoxa2 3'UTR. pEZX-MT01-Hoxa2mut1 was generated using forward primer 669b1F and reverse primer 669b1R (Table 4.3) with an AA-CG mutation (Fig. 5.5A). pEZX-MT01-Hoxa2mut2 was generated using forward primer 669b2F and reverse primer 669b2R (Table 4.3) with an AA-CG mutation (Fig.5.5A). Mutation reaction was set up (50 µl) with 5 µl of 10x reaction buffer, 100ng pEZX-MT01-Hoxa2 vector template, 125 ng of forward and reverse primers, 1 µl of dNTP mix, 1.5 µl of QuikSolution reagent and 1 µl of QuikChange Lightning Enzyme. The mutation PCR started with 2 min incubation at 95°C, followed by 18 cycles of 20s at 95°C, 10s at 60°C and 3 min at 68°C, and a 5 min extension at 68°C. The PCR products were then digested with 2 µl of Dpn I restriction enzyme at 37°C for 5 min to digest the parental vector DNA. Mutated vectors were then transformed into XL10-Gold ultracompetent cells as described in manufacturer’s protocol (QuikChange Lightening Site-Directed Mutagenesis Kit, Agilent Technologies). Each single clone was picked and incubated in
4 ml LB medium with 1% Kanamycin over night at 37°C. Plasmid was purified from bacterial culture using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) as per manufacture’s protocol and target mutation was confirmed via sequencing with an ABI PRISM™ system at the National Research Council, Plant Biotechnology Institute, Saskatoon, SK. A double mutation vector pEZX-MT01-\textit{Hoxa2mut1+2} containing both mutation1 and mutation2 was also generated.

4.2.9. Luciferase assay

The vector pEZX-MT01 (Genecopoeia) encoding \textit{firefly} luciferase (hLuc) and \textit{renilla} luciferase (hRLuc) was used as negative control in luciferase assay (Fig. 4.3A). NIH 3T3 cells (6 \times 10^4) were seeded in each well of a 24-well plate in 0.5 ml culture medium and cultured for 24 h. After 24 h incubation, either miR-669b mimic (Qiagen), miR-376c mimic (Qiagen) or control siRNA (Qiagen) was co-transfected with one of the following luciferase vectors: pEZX-MT01-\textit{Hoxa2}, pEZX-MT01-\textit{Hoxa2mut1}, pEZX-MT01-\textit{Hoxa2mut2}, pEZX-MT01-\textit{Hoxa2mut1+2} or blank pEZX-MT01 vector using lipofectamine 2000 (Invitrogen). For each transfection, 3 \mu l lipofectamine 2000 (Invitrogen) was incubated together with 300 ng miRNA mimic/siRNA control and 1 \mu g luciferase vector in 100 \mu l DMEM for 20 min and then added into cell cultures. The vector pEZX-MT01 (Genecopoeia) encoding hLuc and hRLuc was used as negative control in luciferase assay. After 24h post-transfection, cells were washed with PBS and lysed using LucPair miR Duo-Luciferase Assay Kit (GeneCopoeia). \textit{Firefly} and \textit{Renilla} luciferase activities were determined for each transfection using a luminometer. The \textit{firefly} luciferase signals were normalized to the \textit{Renilla} luciferase signal.
Figure 4.3. Mechanism of Duo-Luciferase assay. (A) pEZX-MT01 vector map. Hoxa2 3'UTR is inserted as miR target. pEZX-MT01 vector encodes firefly luciferase (hLuc) and renilla luciferase (hRLuc). hLuc mRNA is merged with Hoxa2 3'UTR and is used as an indicator of miRNA effects. hRLuc is expressed independently and is used as an internal control. Vector map taken from Genecopoeia (B) Mechanism of luciferase assay. Hoxa2 3'UTR is attached to firefly luciferase mRNA. If Hoxa2 3'UTR is targeted by miRNAs, the entire mRNA may be cleaved and reduced luminescence will be detected.
4.2.10. Mouse Hotairm1 (mHotairm1) sequence analysis

Three pairs of overlapping primers (Mush 147F, Mush 147R, Mush 138F, Mush 138R, Mush 245F, Mush 245R) were designed (Fig. 4.4) and PCR were carried out using cDNA samples from NIH 3T3 cells. For the PCR amplification (a total of 50µl), 50 ng of cDNA, 10×EconoTaq buffer, 2.5 units of EconoTaq DNA polymerase (Invitrogen), 50 µM of each dNTP and 500 nM of each forward and reverse primer (Table 3.1) were used. The PCR cycle started with one cycle at 95°C for 3 min, followed by 30 cycles of 95°C for 30 sec, 52°C for 30 sec, 72°C for 40 sec and a final extension step at 72°C for 10 min. The PCR fragments were run on a 1.2% agarose gel and purified from the gel using QIAquick Gel Extraction kit (Qiagen). The DNA samples were then cloned into pGEM T-easy vector as described in 4.2.2.3 for subsequent sequence analysis with an ABI PRISM™ system at the National Research Council, Plant Biotechnology Institute, Saskatoon, SK.

4.2.11. MHotairm1 silencing in NIH 3T3 cells

NIH 3T3 cells (6 ×10⁴) were seeded in each well of a 24-well plate in 0.5 ml culture medium. siRNA (150 ng of hotairm1 siRNA or control siRNA) and 6 µl HiperFect Transfection Reagent (Qiagen) were incubated in 100 µl DMEM medium for 20 min and then added to each well. Cells were then incubated under normal growth conditions and RNA samples were isolated after 72 h incubation. SYBR green real-time PCR was carried out with mHotairm1 primers mush 137F and mush138R (Table 4.3). Hoxa1 and Hoxa2 expression were detected using Hoxa1 and Hoxa2 SYBR primers (Table 4.1, Integrated DNA Technologies).
4.2.12. Effect of all-trans Retinoic acid (ATRA) on \textit{mHotairm1} expression

NIH 3T3 cells were seeded in 100 mm cell culture dish and grown to 70% confluency. NIH 3T3 cells were treated with ATRA (10^{-6} M) for 24 h. RNA was isolated, reverse transcribed to cDNA, followed by real-time PCR to detect \textit{mHotairm1}, \textit{Hoxa1} and \textit{Hoxa2} expression as indicated in 4.2.4 and 4.2.5. Protein samples were collected and WDR5 protein was detected using western blot analysis as indicated in 4.2.6.

\begin{center}
\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{primer的设计.png}
\caption{ Primer, siRNA and probe design for \textit{mHotairm1}. Three pairs of primers were designed to cover the predicted mouse \textit{hotairm1} sequence. Violet: primer Mush 147F/R; Blue: primer Mush 138F/R; Green: primer Mush 245F/R. Primer names were marked above sequence. Three probes (Probe 1, 2 and 3, underlined sequences in red) were designed against \textit{mHotairm1} for CHART experiment and Probe C is complimentary to Probe 2. CHART Probe names were marked below sequence. \textit{mHotairm1} siRNA is marked in grey and \textit{mHotairm1} \textit{in situ} probe is marked in yellow.}
\end{figure}
\end{center}
4.2.13. *In situ* hybridization histochemistry

Mouse head samples were dissected and immediately frozen in isopentane on dry ice. *In situ* hybridization histochemistry was carried out using IsHyb In Situ Hybridization (ISH) Kit (BioChain) as per manufacture’s protocol. Frozen palatal sections (20 µm) were collected on slides and fixed immediately in 4% paraformaldehyde in PBS for 10 min. The samples were then rinsed with RNase free PBS and treated with proteinase K digestion (10 µg/ml in PBS) for 5 sec. When the slides were dry, pre-hybridization solution was added and slides were incubated for 3h at 50°C. Slides were then washed with 2x Saline Sodium Citrate (SSC) for 10 min at 45°C, 1.5x SSC for 10 min at 45°C and 0.2x SSC twice for 20 min at 37°C. Slides were blocked with 1x blocking solution for 1 h at room temperature. Labeled 100 ng of digoxigenin (DIG) tagged *mhotairm1* probe (Fig. 4.4, Table 4.3, IDT) or random control probe was added to the slides, respectively and incubated over night at 37°C. Slides were then rinsed with SSC buffer and PBS and blocked for 30 min at room temperature with 0.1% Triton 100, 1% sheep serum in PBS. The anti-DIG-fluorescein, Fab fragments (1:50, Roche) was added and incubated for 3h at room temperature and washed 3 times with PBS.

4.2.14. Capture hybridization analysis of RNA targets (CHART)

The genomic binding sites of *mHotairm* Inc RNA were identified by using the CHART technique developed by Simon *et al.*, (2011) and was modified as described here. NIH 3T3 cells (10^7) were collected and cross-linked in 10 ml PBS with 1% formaldehyde for 10 min at room temperature. Cells were washed 3 times with PBS and dounce homogenized in 4 ml sucrose buffer (0.3M sucrose, 1% Triton X-100, 10mM HEPES pH 7.5, 100 mM KOAc, 0.1 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 1× protease inhibitor, 1 mM DTT, 10 unit/ml RNase
inhibitor). The homogenized sample was added to the top of 4 ml glycerol buffer (25% glycerol, 10 mM HEPES pH 7.5, 100 mM KOAc, 1 mM EDTA, 0.1 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 1× Roche protease inhibitor tablet, 1 mM DTT, 10 unit/ml RNase inhibitor) and centrifuged at 1000 g for 15 min at 4°C. The pellet (nuclei) was collected and cross-linked in 10 ml PBS with 3% formaldehyde for 30 min at room temperature. Nuclei pellet was then washed with PBS for 3 times and resuspended in 1 ml WD100 solution (100 mM NaCl, 10 Mm Heps pH 7.5, 2 Mm EDTA, 1 Mm EGTA, 0.2% SDS and 0.1% N-Lauroylsarcosine). The resuspended nuclei pellet was sonicated using Branson digital sonifier 250 (Branson) with 10% input pulse, 3 cycles with 2 s pulse “on” and 30 s pulse “off” to shear DNA to 2-3 kb fragments. Sheared sample was centrifuged at 16000 g for 10 min at room temperature and 50 µl supernatant was collected as 5% input. The rest of supernatant was adjusted to hybridization conditions (20 mM HEPES, pH 7.5, 817 mM NaCl, 1.9 M urea, 0.4% SDS, 5.7 mM EDTA, 0.3 mM EGTA, 0.03% sodium deoxycholate, 5× Denhardt’s solution) and pre-cleared with ultralink-streptavidin resin (Pierce). Three biotin labeled oligo probes against mHotairm1 were designed (Fig. 4.4, Table 4.3) and were added (16 nM each) and hybridized with 55 °C for 20 min, 37 °C for 10 min, 45 °C for 60 min and followed by 37 °C for 30 min. The bound material was captured using streptavidin beads overnight at room temperature. The product was rinsed five times with WB250 (250 mM NaCl, 10 mM Heps, pH 7.5, 2 mM EDTA, 1 mM EGTA, 0.2% SDS, 0.1% N-lauroylsarcosine). For protein sample detection, beads were suspended in RIPA buffer and boiled with 4x loading buffer for SDS-PAGE and western blot analysis as described in 4.2.6. For DNA sample detection, 100 µl reverse buffer (1 mg/ml proteinase K, 0.5% SDS and 100 Mm Tris pH 7.4) was added to the beads and incubated at 55°C for 1 h followed by 65 °C for 30 min. DNA was then isolated using GeneJET PCR Purification kit (ThermoFisher Scientific) and Hoxa1, Hoxa2,
Hoxa3, Hoxa5 and Hoxa13 promoters were identified by PCR using primers listed in Table 4.3 (Simon et al., 2011).
Figure 4.5. Capture hybridization analysis of RNA targets (CHART). Chromatin was crosslinked and sheared. Biotin labelled complementary oligonucleotides were used to purify the targeted RNA together with its DNA and protein targets using streptavidin beads. Reversibly cross-linked CHART-enriched material can then go through different analysis, e.g. DNA analysis (left) and protein analysis (right) (taken from Simon et al., 2011 with permission).

4.2.15. GST fusion protein pull down experiments

4.2.15.1 GST fusion protein expression vector construction

WDR5 and MLL1 (amino acid 3810-3963) were amplified from NIH 3T3 cDNA samples using PCR primers containing EcoR I (GAATTC) and BamH1 (GGATCC) restriction enzyme digestion sites (Table 4.3, Mouse MLL13810 start, Mouse MLL1 3963 stop, Mouse WDR5 start, Mouse WDR5 stop). PCR amplification system (a total of 50 µl) were prepared with 50 ng cDNA, 10×EconoTaq buffer, 2.5 units of EconoTaq DNA polymerase (Invitrogen), 50 µM of each dNTP and 500 nM of each forward and reverse primer (Table 4.3). The PCR cycle started with one cycle at 95°C for 3 min, followed by 30 cycles of 95°C for 30 sec, 53°C for 40 sec, 72°C for 1 min and a final extension step at 72°C for 10 min. The PCR products were run on a 1.0% agarose gel and purified from the gel using QIAquick Gel Extraction kit (Qiagen). The DNA samples were then cloned into pGEM T-easy vector (Promega) and sequenced with an ABI PRISM™ system at the National Research Council, Plant Biotechnology Institute, Saskatoon, SK. The correct clone sequences were then digested from T-easy vector using FastDigest™ EcoRI and FastDigest™ BamHI restriction enzymes (Thermo Scientific™) at 37°C for 20 min. The digested DNA fragments were run on a 1% agarose gel and target bands were excised from the gel and purified using QIAquick Gel Extraction kit (Qiagen). The purified DNA samples were then cloned into pGEX-6p-1 vector (Fig. 4.6. GE Healthcare). This started with the incubation of 100 ng pGEX-6p-1 vector with 50 ng of purified DNA fragment (WDR5 or MLL1) at 65°C for 5 min. Vector/DNA fragment mix were then placed on ice and a ligation (10 µl) was
set up by adding 5 µl of 2x Ligation buffer and 0.5 µl of T4 DNA ligase (Promega) to the mix. Ligation was carried at 16°C overnight followed by 5 min incubation at 70°C to inactivate T4 DNA ligase. Ligation product was then transformed into DH5α™ Competent Cells (Subcloning Efficiency™, Invitrogen) as described in 4.2.2.3. Single clone was picked and incubated in 4ml LB medium with 1% ampicillin overnight at 37°C. Plasmid was then extracted from bacteria culture and target fragment insertion was tested by digesting the plasmid DNA with FastDigest™ EcoRI and FastDigest™ BamHI restriction enzymes (Thermo Scientific™) at 37°C for 20 min. The digested DNA fragments were run on a 1% agarose gel. DH5α™ cells with correct WDR5 and MLL1 expression vectors were stored at -80°C in LB medium with 25% glycerol.

Figure 4.6. pGEX-6p-1 vector map (GE Healthcare). WDR5 and MLL1 (amino acid 3810-3963) were cloned into pGEX-6p-1 vector using EcoRI and BamHI restriction enzymes to link with GST (Figure taken from www.snapgene.com/resources)
4.2.15.2 GST fusion protein expression and purification

The bacterial DH5α™ cells with WDR5 and MLL1 expression vectors were cultured in 4 ml LB medium with 1% ampicillin overnight at 37°C. The cells were then subcultured (1:100 v/v) into LB medium with 1% ampicillin for 2 h at 37°C. Protein expression was induced following treatment with IPTG (1mM) for 2h at 26°C.

DH5α™ cells were collected by centrifuging at 3000rpm for 3 min and vortexed with 1ml lysis buffer (1mM DTT, 0.5 mg/ml lysozyme, 1% triton X-100 and 1x proteinase inhibitor in RIPA buffer) and incubated on ice for 20 min. The samples were then sonicated using Branson digital sonifier 250 (Branson) (10% input pulse, 5 cycles with 2 s pulse “on” and 30 s pulse “off”). Following sonication, samples were centrifuged at 12000 rpm for 10 min at 4°C and supernatant was collected. Glutathione agarose beads were washed with RIPA buffer. A 100 µl of glutathione agarose beads (Pierce™, ThermoFisher Scientific) were added into each supernatant collected from the previous step and incubated for 2 h at room temperature to purify GST fused protein. The beads were collected by centrifuging at 3000rpm for 1 min and washed for three times with RIPA buffer. The purification of GST fused protein was tested by running the purified beads on an SDS-PAGE gel, followed by coomassie blue staining.

4.2.15.3 GST fusion protein pull down

For protein sample pull down, the purified glutathione agarose beads with protein were added to NIH 3T3 cell lysates (in RIPA buffer) and incubated at 4°C overnight. Pull down samples were collected by centrifuging at 3000rpm for 2 min and washed three times with RIPA buffer. Protein samples pulled down with GST fused MLL1 (amino acid 3810-3963) were tested
with WDR5 antibody following western blot analysis as described in 4.2.6. GST was used as a negative control.

For RNA sample pull down, NIH 3T3 cells were lysed in cell lysis buffer with 100 mM KCl, 5 mM MgCl₂, 10 mM Hepes (pH 7.0), 0.5% Nonidet P-40, 1 mM DTT, 2 mM Vanadyl ribonucleoside complexes solution, 25 µg/ml protease inhibitor and 100 unit/ml RNase inhibitor. Lysates were vortexed and centrifuged at 12000 rpm at 4°C for 10 min. Purified glutathione agarose beads with WDR5/MLL1 were added into the supernatant and incubated at 4°C for 3 h. Beads were then collected by centrifuging at 3000 rpm for 2 min and washed 3 times with cell lysis buffer. RNA samples were recovered using RNeasy Protect Mini Kit (Qiagen) and converted to cDNA as described in 4.2.4. The existence of *mHotairm1* was tested by PCR using mush 138 forward and reverse primers as listed in Table 4.3.

### 4.2.16 Chromatin Immunoprecipitation (ChIP)

NIH 3T3 cells transfected with either *hotairm1* siRNA or control siRNA were cross linked with 1% formaldehyde in cell culture media for 10 min at room temperature. Glycine was added to a final concentration of 125 mM and incubated with shaking for 5 min at room temperature. Cells were washed two times with 10 ml cold PBS. Cells were collected and lysed with Lysis Buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA pH 8, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail). Cell lysates were sonicated using Branson digital sonifier 250 (Branson) with 20% input pulse, 3 cycles of 5s pulse “on” and 30s pulse “off” to shear DNA to lengths between 200 and 1000 bp. Sheared DNA samples were centrifuged for 30 s at 8,000 g at 4°C and supernatant was collected. Input represents 5% of supernatant.
Samples were then incubated with 10 µg anti-Histone H3 (tri methyl K4) antibody (Abcam), 10 µg H3K27trime antibody (Abcam), 10 µg Histone3 antibody (Abcam) or 10µg normal rabbit IgG (Santa cruz) respectively, at 4°C overnight. Antibody/histone complex were collected with 20 µl protein A agarose slurry (Santa Cruz). Beads were washed three times with 1 ml wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA pH8, 150mM NaCl and 20mM Tris-HCl pH8). Histone complex from the antibody was eluted with 100 µl elution buffer (1% SDS and 100 mM NaHCO3) and histone-DNA crosslinks were reversed by heating at 65°C in 5M NaCl for 4 h. Resulting DNA was then recovered by incubating with proteinase K and extracted by phenol/chloroform. Samples were resuspended in 20 µl of MiliQ distil water, and 1:50 was used for qPCR with Hoxa1, Hoxa2 promoter primers listed in Table 4.3. qPCR was performed in a final volume of 25 µl containing 50 ng of ChIP DNA or 50 ng of input DNA as template, 400 ng of each primer, and 12.5 µl of SYBR green PCR master mix (Applied Biosystems). The amplification consisted of 10 min at 95°C followed by 35 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s in 7300 ABI detection system. The fold enrichment of each protein at the Hoxa1 and Hoxa2 gene promoter was determined by the 2(-ΔΔCt) method described in the Applied Biosystems User Bulletin (ABI PRISM 7700 Sequence Detection System, Applied Biosystems, 2001) and are shown as the fold increase relative to input, N=3.

4.2.17 Separation of nucleus and cytoplasm

NIH 3T3 cells were collected and washed in cold PBS. The cells were homogenized in 0.5 ml sucrose buffer (0.3M sucrose, 1% Triton X-100, 10mM Hapes pH 7.5, 100 mM KOAc, 0.1 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 1× Roche protease inhibitor tablet, 1 mM DTT, 10 unit/ml RNase inhibitor). The homogenized sample was added to the top of 0.5 ml glycerol
buffer (25% glycerol, 10 mM Hepes pH 7.5, 100mM KOAc, 1 mM EDTA, 0.1 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 1× protease inhibitor, 1 mM DTT, 10 unit/ml RNase inhibitor) and centrifuged at 1000g for 15 min at 4°C. The supernatant was collected as cytoplasm and the pellet was collected as nucleus (Simon et al., 2011).

4.2.18 Immunoprecipitation (IP)

NIH 3T3 cells were lysed in RIPA buffer with protease inhibitor cocktail (Thermo Fisher Scientific) and sumoylation/ubquitilation protector N-ethylmaleimide (NEM). Cell samples were collected and vortexed and kept on ice for 5 min. Agarose protein A beads were pre-cleaned with RIPA buffer. Cell lysate was centrifuged for 15 min at 12000 g at 4°C and supernatant collected. For WDR5 antibody IP, 10µg WDR5 antibody (ab22512, rabbit IgG, Abcam) was added to the cell lysate to precipitate WDR5 at 4°C over night. Normal rabbit IgG antibody (10µg, Santa Cruz) was used as negative control. Pre-cleaned agarose protein A beads were then added and samples were incubated at 4°C on shaker for 1h. Samples were centrifuged at 8000 g for 30 s and the precipitates were washed three times with RIPA buffer. The collected samples were denatured at 100 °C for 10 min for subsequent SDS-PAGE (10%) separation. Protein samples were transferred and immobilized onto a PVDF transfer membrane. Membrane was blocked in 3% BSA (in PBS) at 4 °C overnight. SUMO-1 antibody (1:500, Developmental Studies Hybridoma Bank) was used to detect sumoylated WDR5. For SUMO1 antibody IP, 10µg SUMO1 antibody (mouse IgG, Developmental Studies Hybridoma Bank) was used in IP and normal mouse IgG (Santa Cruz) was used as negative control. Western blot analysis was carried out using WDR5 antibody (WDR5 A-6, mouse IgM, Santa Cruz).
4.2.19 Statistical data analysis

All statistical analyses were performed using the GraphPad 5.0 software (GraphPad Prism). Statistical analysis on qRT-PCR of miRNAs and gene expressions were evaluated using one way analysis of variance (ANOVA) followed by Bonferroni post-tests. Student’s T-Tests was used in the analysis of ChIP experiment and luciferase assay for the comparison between experimental groups and control groups. A significant p-value of less than or equal to 0.05 was used to denote significant difference.
Table 4.3. Sequences of primers and probes used in this study.

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<tr>
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<td>Probe C: Biotin-5’ AGAGTTGGAACGTAGATGTTG (Antisense to Probe 2)</td>
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<tr>
<td><strong>mHotairm1 siRNA</strong></td>
<td>5’ GGAAGAGAUUGGAAACGUAGAUUGU</td>
</tr>
<tr>
<td><strong>In situ Probes</strong></td>
<td>5’ DigN/ACTCCGTTATTGACCTAGAAACTAGCAGCTTGGTAAGGGAAC</td>
</tr>
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CHAPTER 5

5. Results and analysis of data

5.1 Methylation status of Hoxa2 promoter region in developing mouse palate, NIH 3T3 cells and EG7 cells.

DNA methylation was the first epigenetic mechanism that I investigated in the regulation of Hoxa2 gene expression in mouse palate development. Analysis of the Hoxa2 promoter revealed the presence of three CpG islands (Fig. 5.1). The three sets of methylation specific primers used for the analysis of the CpG islands are listed in the materials and methods section (Table 4.3). Methylation specific PCR (MSP) carried out for all three CpG rich regions showed that all three CpG regions in the Hoxa2 promoter were unmethylated in developing palate as well as in NIH 3T3 cells, while these regions remained methylated in EG7 cells, (Fig. 5.2). A further bisulfite specific PCR (BSP) amplification and sequencing was carried out to examine the methylation status of each CpG site. A region close to the transcription start site (Region 1, Fig. 5.1) with 14 CpG sites was selected for further analysis. I found that all 14 CpG sites were unmethylated in both NIH 3T3 cells and in developing palate samples (Fig. 5.3A, Appendix 1-5); however, in the same CpG rich region 1 of the Hoxa2 promoter in EG7 cells, 10 of the 14 sites were methylated (Fig. 5.3B, Appendix 6).

Since Hoxa2 is expressed in both the developing mouse palate (Smith et al., 2009) and NIH 3T3 cells but not in EG7 cells (X. Wang, PhD Thesis, 2013), my results indicate that an unmethylated promoter of Hoxa2 may be required for its expression. However, since Hoxa2 expression changes temporally at different stages of mouse palate development (Smith et al., 2009), and that the Hoxa2 promoter remains unmethylated throughout mouse palate
development, DNA methylation does not appear to be a regulator for *Hoxa2* expression during mouse palatogenesis, therefore, I focused on other epigenetic regulators, such as miRNAs and lncRNAs.

**Figure 5.1. Analysis of CpG rich regions in *Hoxa2* gene promoter.** All the predicted CpG sites in the region 2000 bp upstream of the transcriptional start site were separated into three methylation specific regions: region 1 from -489 to -624, region 2 from -1019 to -1169, and region 3 from -1244 to -1360 (transcriptional start site begins at 0). CpG sites are highlighted with light grey. Predicted methylated cytosines of CpG dinucleotides are shown as red letters (predicted with Methylator, Bhasin *et al.*, 2005, [http://bio.dfci.harvard.edu/Methylator/](http://bio.dfci.harvard.edu/Methylator/)).
Figure 5.2. MSP amplification for three CpG islands in *Hoxa2* promoter. MSP was done for DNA samples collected from NIH 3T3 cells, EG7 cells and developing mouse palatal tissue (E12-E15). M = PCR fragment amplified with designed methylated primers. U = PCR fragment amplified with designed unmethylated primers. The presence of a PCR band in M indicates the region is methylated and PCR band in U indicates the region is unmethylated.

Figure 5.3. BSP and sequencing results for the CpG rich region 1. (A). Schematic of CpG methylation status in NIH 3T3 cells and in E12 to E15 palatal samples. All 14 CpG sites remained unmethylated. (B). Schematic of CpG methylation status in EG7 cells. 10 out of 14 CpG sites were methylated in EG7 cells. Each circle represents a CpG site. The number under each circle represents the position of each CpG site on mouse *Hoxa2* promoter (transcriptional start site begins at 0). Blank circles: unmethylated CpG site; Black circles: methylated CpG site.
5.2 Regulation of *Hoxa2* gene expression by miRNAs

5.2.1 miRNA binding sites prediction and sequence analysis.

MicroRNAs play an important role in the regulation of *Hox* gene expression in several biological and pathological processes, mainly via binding to the 3' UTR of *Hox* genes (reviewed in section 1.2.3). To study the regulation of *Hoxa2* gene expression by miRNAs, I first used an online software (http://www.microrna.org) to predict miRNAs that bind to mouse *Hoxa2* 3' UTR. Six miRNAs were predicted to bind to mouse *Hoxa2* 3' UTR as shown in Figure 5.4. The stem-loop sequence and mature sequence of all six miRNAs are shown in Table 5.1. Detailed predicted binding sequences and structures between miRNAs and mouse *Hoxa2* 3' UTR are shown in Figures 5.5-5.10 (A, B) for each individual miRNA. I also analyzed the sequence similarity for each miRNA binding site among several species (Figs. 5.5–5.10C). Most predicted miRNA binding sites are evolutionally conserved except the binding site 2 of miR-669b (Fig. 5.5C)

GAACAUUAAAGCATAACAAAGCUUCACAAACAAAGCCUUUGACCGGGG

miR-669b binding site 1

UUUGCCUCUUUUAAUCUGGAGUUGAUUUCGUUUUUGUUUGCUUCUGAUUCA

miR-376c

CCCCUCUCUCACAAAUUGUGAGGACUUCCGUUUAAUGUCUCCCGCUACAC

miR-669b binding site 2

GUUUUUAAGGAAGCCACUCUCUGGAAAUUGUGGCUGUCUCAGUGUUUUUACACA

miR-431

GAACCCACCAAGCUUCUGUAGUAAUUUCUGAAAAACAAAAACAGGGGGCUC

miR-873-3p

AGAAAGUGACCAUUAUUGUCUUGUCACUUUCUGUUUAAUUUUGUACCAUAUA

miR-19a

GAAUGCAUUGCAUGCGUAUUUUGGUAGAAUAUAUCUCUUUGCUUAUAA

miR-298
Figure 5.4. Predicted binding sites of miRNA on mouse Hoxa2 3' UTR (prediction from http://www.microrna.org). Full length mouse Hoxa2 3' UTR sequence is shown (5' to 3'). Colour lines beneath the nucleotide sequence represents binding sites of respective miRNAs.

Table 5.1. Mouse miRNA stem-loop sequence and mature sequence. The sequence data is from miRBase (http://www.mirbase.org/).

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<th>Mature sequence 5'-3'</th>
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<td>UGUCUUGCAGGGCGUCAUGCA</td>
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<td>GGCGAGGAGGGCUUGUUCUU CCC</td>
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<td>UGUGCAAAUCUAUGCAAAAAC UGA</td>
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<td>UGCAAGCUUUAAUCUAUGUUGG AUGUCAAGACAGUGAAACUU AAGUGCAUGACACCACACUGG GUAGAGAGGGCUCA</td>
<td>GCAUGACACCACACACUGGGUAG A</td>
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A

3’ UGUACGUGUACGUGUGUUUGA 5’  miR-669b

5’ CAAAAACAAAGCUUCACAACAACA 3’  Hoxa2 3’ UTR binding site 1

3’ UGUACGUGUAC-UGUGUUUGA 5’  miR-669b

5’ UGUUUCCUGAAACAAACAACA 3’  Hoxa2 3’ UTR binding site 2

B

mfe: -19.5 kcal/mol

miR-669b binding site 1

mfe: -14.2 kcal/mol

miR-669b binding site 2

C

miR-669b

Human 5’ AUUAAAACAAAAACAAAGCUUCACAACAAAAACUUCCUU 3’
Mouse 5’ AUUAAAACAAAAACAAAGCUUCACAACAAAAACUUCCUU 3’
Rat 5’ AUUAAAACAAAAACAAAGCUUCACAACAAAAACUUCCUU 3’
Chimpanzee 5’ AUUAAAACAAAAACAAAGCUUCACAACAAAAACUUCCUU 3’
Dog 5’ AUUAAAACAAAAACAAAGCUUCACAACAAAAACUUCCUU 3’

Hoxa2 3’ UTR miR-669b binding site 1

miR-669b

Human 5’ CCUUUAUGUGAUAU--CCUGAGA--GCA--GUUGAGGCCUU 3’
Mouse 5’ CCUUUAUGUGAUAU--CCUGAGA--GCA--GUUGAGGCCUU 3’
Rat 5’ CCUUUAUGUGAUAU--CCUGAGA--GCA--GUUGAGGCCUU 3’
Chimpanzee 5’ CCUUUAUGUGAUAU--CCUGAGA--GCA--GUUGAGGCCUU 3’
Dog 5’ CCUUUAUGUGAUAU--CCUGAGA--GCA--GUUGAGGCCUU 3’

Hoxa2 3’ UTR miR-669b binding site 2
Figure 5.5. Prediction and analysis of miR-669b binding sites on *Hoxa2* 3' UTR. (A) The predicted target seed sequences for miR-669b in mouse *Hoxa2* 3' UTR is marked in red (http://www.microrna.org). The underlined “AA” in blue in both binding sites were mutated to “CG” for luciferase assays. (B) Predicted binding structures of miR-669b to mouse *Hoxa2* 3' UTR (RNAhybrid, Rehmsmeier *et al.*, 2004). mfe: minimal free energy. *Hoxa2* 3' UTR sequence is labelled in red and miR-669b is labelled in green. (C) The degree of sequence similarity within the seed targets for miR-669b were shown for *Hoxa2* genes from five different species. Complementary regions between miR-669b and *Hoxa2* 3' UTR were highlighted in red.
Figure 5.6. Prediction and analysis of miR-376c binding site on Hoxa2 3' UTR. (A) The predicted target seed sequences for miR-376c in mouse Hoxa2 3' UTR is marked in red (http://www.microrna.org). (B) Predicted binding structures of miR-376c to mouse Hoxa2 3' UTR (RNAhybrid, Rehmsmeier et al., 2004). mfe: minimal free energy. Hoxa2 3' UTR sequence is labelled in red and miR-376c is labelled in green. (C) The degree of sequence similarity within the seed targets for miR-376c were shown for Hoxa2 genes from five different species. Complementary regions between miR-376c and Hoxa2 3' UTR were highlighted in red.
Figure 5.7. Prediction and analysis of miR-431 binding site on Hoxa2 3' UTR. (A) The predicted target seed sequences for miR-431 in mouse Hoxa2 3' UTR is marked in red (http://www.microrna.org). (B) Predicted binding structures of miR-431 to mouse Hoxa2 3' UTR (RNAhybrid, Rehmsmeier et al., 2004). mfe: minimal free energy. Hoxa2 3' UTR sequence is labelled in red and miR-431 is labelled in green. (C) The degree of sequence similarity within the seed targets for miR-431 were shown for Hoxa2 genes from five different species. Complementary regions between miR-431 and Hoxa2 3' UTR were highlighted in red.
Figure 5.8. Prediction and analysis of miR-19a binding site on Hoxa2 3' UTR. (A) The predicted target seed sequences for miR-19a in mouse Hoxa2 3' UTR is marked in red (http://www.microrna.org). (B) Predicted binding structures of miR-19a to mouse Hoxa2 3' UTR (RNAhybrid, Rehmsmeier et al., 2004). mfe: minimal free energy. Hoxa2 3' UTR sequence is labelled in red and miR-19a is labelled in green. (C) The degree of sequence similarity within the seed targets for miR-19a were shown for Hoxa2 genes from five different species. Complementary regions between miR-19a and Hoxa2 3' UTR were highlighted in red.
Figure 5.9. Prediction and analysis of miR-878-3p binding site on Hoxa2 3' UTR. (A) The predicted target seed sequences for miR-878-3p in mouse Hoxa2 3' UTR is marked in red (http://www.microrna.org). (B) Predicted binding structures of miR-878-3p to mouse Hoxa2 3' UTR (RNAhybrid, Rehmsmeier et al., 2004). mfe: minimal free energy. Hoxa2 3' UTR sequence is labelled in red and miR-878-3p is labelled in green. (C) The degree of sequence similarity within the seed targets for miR-878-3p were shown for Hoxa2 genes from five different species. Complementary regions between miR-878-3p and Hoxa2 3' UTR were highlighted in red.
Figure 5.10. Prediction and analysis of miR-298 binding site on Hoxa2 3' UTR. (A) The predicted target seed sequences for miR-298 in mouse Hoxa2 3' UTR is marked in red (http://www.microrna.org). (B) Predicted binding structures of miR-298 to mouse Hoxa2 3' UTR (RNAhybrid, Rehmsmeier et al., 2004). mfe: minimal free energy. Hoxa2 3' UTR sequence is labelled in red and miR-298 is labelled in green. (C) The degree of sequence similarity within the seed targets for miR-298 were shown for Hoxa2 genes from five different species. Complementary regions between miR-298 and Hoxa2 3' UTR were highlighted in red.
5.2.2 The predicted miRNAs are expressed in NIH 3T3 and EG7 cells.

To study the role of these miRNAs in the regulation of *Hoxa2* expression, I first needed to determine whether these miRNAs were expressed in the NIH 3T3 cells, EG7 cells and in the developing mouse palate. Expressions of all six miRNAs predicted to bind *Hoxa2* 3’ UTR were examined in both NIH 3T3 and EG7 cells using specific miRNA primers (Qiagen®) (Table 4.1). miR-298 has the highest expression in NIH 3T3 cells followed by miR-431. Both miR-298 and miR-431 have low expression in EG7 cells. miR-669b, miR-376c and miR-19a all have higher expression in NIH 3T3 cells than in EG7 cells. In contrast, miR-878-3p is expressed higher in EG7 cells than in NIH 3T3 cells (Fig. 5.11). Generally, the miRNA expression levels in EG7 cells are much lower than in NIH 3T3 cells. One explanation would be because EG7 is a cell line with a high degree of DNA methylation (X. Wang, PhD Thesis, 2013), thus transcription activities may be relatively low compared to that in NIH 3T3 cells. This result fits with the hypothesis that the DNA methylation status can impact the expression of miRNAs (Han *et al*., 2007; Bandres *et al*., 2009; Liu *et al*., 2013). miR-431 and miR-298 are strongly expressed in NIH 3T3 cells, indicating that these miRNAs have a less possibility to regulate *Hoxa2* expression based on the fact that *Hoxa2* is also highly expressed in NIH 3T3 cells.
miRNA expression in NIH 3T3 and EG7 cell lines. miRNA expression levels in NIH 3T3 and EG7 cells were measured by qRT-PCR as described in section 4.2.5, p76. miRNA expressions were relative to snRNA RNU6B. Relative quantitative expression of miR-431 in EG7 cells were normalized to 1. miR-431 and miR-298 had relatively high expressions in NIH 3T3 cells. Overall, expressions of miRNAs in EG7 cells were relatively low. E - EG7 cells, N - NIH 3T3 cells.
5.2.3 The predicted miRNAs are expressed in the developing mouse palate

All six miRNAs were expressed in the developing mouse palate (Fig. 5.12). Interestingly, three of them, miR-669b, miR-376c and miR-431 exhibited gradual increase in expression from stages E12 to E15 during palatal development. These miRNAs had a lower expression at E12, when Hoxa2 begins its expression in mouse palate (Smith et al., 2009). Their expression continued to increase in the developing palate from E13 to E15 (Fig. 5.12) when Hoxa2 expression gradually declines (Smith et al., 2009). The counter and opposite expression levels of these miRNAs to Hoxa2 gene during mouse palatogenesis indicates that these miRNAs may have a high potential to regulate Hoxa2 expression during palatal development. For other three miRNAs, miR-19a expression also showed significant differences during some of the stages and may play a role in palate development; however, miR-298 and miR-878-3p did not show significant expression changes during palate development (Fig. 5.12). Taken together with the miRNA expression levels during palate development and miRNA expression in NIH 3T3 and EG7 cell lines, I speculated that miR-669b and miR-376c had a higher possibility to regulate Hoxa2 expression. Hence, I chose these two miRNAs for further investigation.
Figure 5.12. miRNA expression in the developing mouse palate. miRNA expression levels in wild-type mouse palate shelves from E12 to E15 were measured by qRT-PCR as described in section 4.2.5, p76. miRNA expressions were relative to snRNA RNU6B. Relative quantitative expression of miRNAs at E12 were normalized to 1. Relative quantitative expression during mouse palate development of miRNA: (A) miR-669b; (B) miR-376c; (C), miR-431; (D) miR-19a; (E) miR-878-3p and (F) miR-298. (G) Expression level of Hoxa2 gene in developing mouse palate. Hoxa2 gene expressions were relative to β-actin. Figure taken from Smith et al., 2009 with permission. RQ: relative quantity of miRNA expression. Bars represent mean ±SEM, n =3. *p ≤ 0.05 between bars indicated by brackets. **p ≤ 0.01 between bars indicated by brackets.

5.2.4 miRNA-669b and miR-376c down regulate Hoxa2 expression in NIH 3T3 cells.

To investigate whether miR-669b and miR-376c impact Hoxa2 gene expression, I transfected NIH 3T3 cell line with the respective miRNA mimic. NIH 3T3 cells transfected with miR-669b mimic reduced Hoxa2 mRNA expression by ~30% after 24h of transfection (Fig. 5.13A). Although miR-376c mimic significantly reduced expression of Hoxa2 after 24h of transfection compared to mock treated cells (transfection reagent only), it did not reach significance when compared to control miRNA treated cells. These differences were not observed after 48h of transfection with either miR-669b or miR-376c mimic, possibly due to the degradation of the miRNA mimics. Western blot analysis showed both miR-669b and miR-376c mimics down regulated Hoxa2 protein expression after 24h of transfection (Fig. 5.13B).
Figure 5.13. Effect of miR-669b and miR-376c mimics on Hoxa2 expression in NIH 3T3 cells. NIH 3T3 cells were transfected with miR-376c mimic, miR-669b mimic, control miRNA and mock treatment, respectively. (A) After 24h and 48h of transfection, total RNAs were isolated and qRT-PCR was carried out using Hoxa2 Taqman primers as described in 4.2.5. Bars represent mean ±SEM, n =3. *p ≤ 0.05 between bars indicated by brackets. (B) NIH 3T3 cells were transfected with miR-376c mimic, miR-669b mimic, control miRNA or mock treatment, and whole cell lysates were collected for western blot analysis after 24h. GAPDH was used as a loading control.
5.2.5 miR-669b binds directly to mouse Hoxa2 3'UTR

Since both miR-669b and miR-376c repress Hoxa2 expression in NIH 3T3 cells (Fig. 5.13), I further investigated whether this effect on Hoxa2 expression is due to direct binding of miRNAs to Hoxa2 3' UTR. To answer this question, I carried out dual-luciferase assays in NIH 3T3 cells. The vectors pEZX-MT01 (Genecopoeia®) encoding firefly luciferase (hLuc) and renilla luciferase (hRLuc) pEZX-MT01-Hoxa2 (Genecopoeia®) containing full length mouse Hoxa2 3' UTR were used in these experiments. If Hoxa2 3' UTR was being targeted by the miRNAs, the translation of firefly luciferase will be affected and any change in enzyme activities will be detected (Fig. 4.3). Renilla luciferase was used as an internal control to normalize firefly luminescence. In the experimental group, pEZX-MT01-Hoxa2 was co-transfected with miR-669b mimic, miR-376c mimic or control miRNA, respectively. A significantly reduced luminescence signal was observed in cells transfected with miR-669b mimic, indicating miR-669b can bind directly to mouse Hoxa2 3' UTR (Fig. 5.14). No significant down regulation of firefly luciferase activity was observed in samples transfected with miR-376c (Fig. 5.14). Hence, the down regulation of Hoxa2 expression in NIH 3T3 cells following transfection of miR-376c mimic does not appear to be through direct binding of miR-376c to Hoxa2 3'UTR. For the control group, I used pEZX-MT01 blank vector without Hoxa2 3'UTR and found no significant difference in NIH 3T3 cells transfected with miR-669b mimic, miR-376c mimic or control miRNA (Fig. 5.14).

miR-669b has two predicted binding sites on Hoxa2 3'UTR and a AA-CG mutation was engineered in the predicted seed sequences (Fig. 5.5A) on pEZX-MT01-Hoxa2 vector (pEZX-MT01- Hoxa2 mut1 and pEZX-MT01- Hoxa2 mut2, Fig. 5.5A). A construct with both predicted binding sites that were mutated was also generated (pEZX-MT01-Hoxa2 mut1+2). Luciferase
assay showed that mutation at single binding site alone was not sufficient to block the effect of miR-669b on Hoxa2 3'UTR, only after both binding sites were mutated, miR-669b mimic could no longer reduce the luciferase activity, suggesting that both predicted binding sites are required for the effective miR-669b function (Fig. 5.15).

Figure 5.14. Luciferase assay revealed direct interactions between miR-669b and mouse Hoxa2 3'UTR. NIH 3T3 cells co-transfected with luciferase vector and miRNA mimics. Luciferase reporter vectors with (pEZX-MT01-Hoxa2) or without Hoxa2 3'UTR (pEZX-MT01) were co-transfected with miR-669b mimic, miR-376c mimic or control miRNA, respectively. Firefly luminescence signal was normalized to renilla luminescence signal. Y-axis shows ratio of firefly and renilla luminescence signal. Bars represent mean ±SEM, n = 4. ***p ≤ 0.001 compared to control miRNA.
Figure 5.15. Luciferase assay revealed that seed sequence mutations affect miR-669b binding capacity to Hoxa2 3' UTR. Firefly luminescence signal was normalized to renilla luminescence signal. Y-axis shows ratio of firefly and renilla luminescence signal. Three different vectors with mutations in seed binding sites of Hoxa2 3' UTR (pEZX-MT01- Hoxa2 mut1, pEZX-MT01- Hoxa2 mut2 and pEZX-MT01-Hoxa2 mut1+mut2) were tested for the luciferase assay. Bars represent mean ±SEM, n =3. ***p ≤ 0.001 compared to control miRNA.

The findings above show that the overexpression of both miR-669b and miR-376c decreases the expression of Hoxa2 at transcriptional and translational level. Using luciferase reporter assay, two direct miR-669b binding sites were revealed in mouse Hoxa2 3' UTR and both sites proved to be functional. miR-376c does not have direct binding sites on Hoxa2 3' UTR. The effect of miR-376c on Hoxa2 expression may be due to the binding of miR-376c to another part of the Hoxa2 gene, or due to an indirect effect.
5.3 *mHotairm1* IncRNA regulates *Hoxa1* and *Hoxa2* gene expression via an epigenetic mechanism

*HOTAIRM1* is known to activate 3' HOXA genes (HOXA genes located at the 3' end of HOXA loci) in human cells (Zhang *et al.*, 2009), yet no similar transcript has been reported in other species and little is known about how 3' HOXA genes are regulated by *HOTAIRM1*. In the following study, I identified the existence of a noncoding transcript in mouse that shares sequence similarity with human *HOTAIRM1*, and referred it as the mouse *Hotairm1* (*mHotairm1*). I further investigated the roles and mechanisms of *mHotairm1* in regulating the expression of two 3' Hoxa genes in mice, namely *Hoxa1* and *Hoxa2*.

5.3.1 Predicted mouse *Hotairm1* sequence analysis

To investigate the existence of *mHotairm1* noncoding transcript in mouse, first I analyzed the human *HOTAIRM1* sequence using BLAST with mouse RefSeq RNA, and found a predicted transcript in mouse that shared some sequence similarity with human *HOTAIRM1* (Fig. 5.16C). This transcript in mouse is transcribed between *Hoxa1* and *Hoxa2* (Fig. 5.16A), and it is 522 nt long (Fig. 5.16B) with two exons and one intron that undergoes splicing during transcription (Fig. 5.16D). Next, three pairs of overlapping primers were designed (Fig. 4.4, Table 4.3) and PCR was carried out for a cDNA library that was generated from NIH 3T3 cells. The amplified DNA fragments were sequenced and a 462 nt sequence was identified, hereafter I refered it as *mHotairm1* [Fig. 5.16B (sequence in orange), Appendix 7-9].
**A**

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**B**

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GGCCCCCTCCCGGCGCCCTCCCAACAGCCCCAGCTGGAAGTTGGAACGTAGATGTGGTG
GGGGGTGGGGGAGGGAAAGGAAGAGTTGGAACGTAGATGTGGTG
GTAAGGGGAACCTCCAGTTCAAGCTGGGAGATTATCTGAGATGGAAGTGGCA
CTTTGCTTGCTCCACACTCAAAAGCTTGGGCTAGGGCGTGGGAGGAGGAGGACGTGGCG
CTTTTCCTCTCCCACTCCACTTCATTCATGCTGATCGACTAGCAGCGGG
CATTCCCTTAGGGGGGAAAATCCA
```

**C. Sequence similarity between human and mouse** *Hotairm1*.

```
Mouse 51 CTGCCTTCTGCGCGGCTTCTGCGC---TGCGCTGCTCCTGCA-----CCAGATGTGGGGG 102
Human 173 CTGCCTTCTGCGCGGCTTCTGCGC---TGCGCTGCTCCTGCA-----CCGCCACCAGCTGGGAGGTGGGG 231
Mouse 103 GATGGGAGGCCCCTCGCGGCA---GCCCTTCTCCACCCAGCGAGAAAGCTGGAACGTGGCA 159
Human 232 GCTGGGAGGCTCCTCCCCCTCCTCCCCTCCCCCACCCTCCCTCCCTCCCTCCTGAAAAAGATGGAACGTGGCG 291
Mouse 160 AGAGGTCTGTTTTCTGAAACCATCCACAGCTGGGAGATTATACTCCACACTGAAAAAT 219
Human 292 AGAGGTCTGTTTTCTGAAACCATCCACAGCTGGGAGATTATACTCCACACTGAAAAAT 351
Mouse 220 GGGG------GTGTGGGAAAGGAAAGAGATGAGTGGAAACGTTAGATGTTGAAACAAATG 273
Human 352 GGGGAGGATTTATGGGGGAAAACGTTGGAACGTAGATGTGGTG 403
```
D. Comparation of mHotairm1 RNA sequence to mouse genome.

(a)
Figure 5.16. **Sequence analysis of mouse Hotairm1.** A. Mouse Hotairm1 is transcribed between Hoxa1 and Hoxa2. It has two exons, and one intron that is spliced during transcription. B. The 522 nt predicted transcript in mouse that shared sequence similarity with human HOTAIRM1 was listed. Sequence identified by cloning and sequencing of mouse Hotairm1 transcript (462 nt) is shown in orange. Splicing happen at nucleotide 163, with AG (marked in blue) the 5’ splicing signal and G (marked in green) the 3’ splicing signal. C. Analysis of sequence similarity between human and mouse Hotairm1. mHotairm1 RNA sequence was blasted with human HOTAIRM1 RNA sequence. D. mHotairm1 RNA sequence was blasted with mouse genomic DNA. Two ranges were found (a) and (b), indicating that mHotairm1 is spliced.
5.3.2 Expression of mHotairm1 in mouse tissues and cell lines

To further confirm the existence of mHotairm1 and to study its function, I investigated the expression of mHotairm1 in mouse embryonic tissues. Also, I investigated whether the expression of mHotairm1 in NIH 3T3 cells is induced by an activator of Hox gene expression, namely, the all-trans retinoic acid (ATRA) (Bertani et al., 2011).

5.3.2.1 mHotairm1 is expressed in several different mouse tissues

The expression of mHotairm1 was examined in the mouse developing palate using real-time PCR. In the mouse, palatogenesis occurs between E12 to E15 (Smith et al., 2009; 2013) and my findings showed that mHotairm1 expression exhibited the highest expression at E12, and gradually decreased with the lowest expression at E15 (Fig. 5.17A). mHotairm1 is also expressed in the head, forelimbs, hindlimbs and tail region of E13 embryos with the highest expression in the tail (Fig. 5.17B).

5.3.2.2 In situ hybridization histochemistry of mHotairm1 in mouse palate

To determine precisely where the mHotairm1 transcript is being expressed in the developing mouse palate, I utilized in situ hybridization histochemistry (ISH). The mHotairm1 transcript appeared to be primarily expressed in the medial edge epithelial cells of the developing palate at E14 and E15 (Fig. 5.18). The location of its expression at the seam of the medial edge epithelia prior to palatal fusion may indicate its role in facilitating palatal fusion. Further experiments will need to be carried out to identify the role of mHotairm1 in palatal development.
Figure 5.17. Mouse *Hotairm1* expression in the developing palate (E12 to E15) and mouse tissues from E13 embryos. A. *mHotairm1* expression levels in wild-type mouse palatal shelves from E12 to E15 were quantitated with qRT-PCR. B. *mHotairm1* expression detected in head, forelimbs, hindlimbs and tail region from E13 embryos by qRT-PCR. Bars represent mean ±SEM, n =3.
Figure 5.18. *In situ* hybridization histochemistry of *mHotairm1* in mouse palate. A-B: Schematic diagram of E14 and E15 mouse palatal shelves. PS: palatal shelves. MES: middle edge seam. C: Distribution of *mHotairm1* in E14 and E15 mouse palate detected by *in situ* hybridization histochemistry. Green staining represents the expression of *mHotairm1* transcript at E14 (a,b) and E15 (d,e). (c,f): control in situ probes were used and no staining was observed. Scale bar indicate 5 microns.
5.3.2.3 ATRA induces *mHotairm1* expression

ATRA is a known inducer for the expression of *Hox* genes and has also been reported to induce IncRNA transcription (Bertani *et al.*, 2011). To determine how *mHotairm1* is regulated, NIH 3T3 cells were treated with ATRA. Results showed that the expression of *mHotairm1* as well as that of *Hoxa1* and *Hoxa2* were significantly increased after 24 h treatment with ATRA (Fig. 5.19). These results confirmed that *mHotairm1* is a mouse noncoding transcript that is present in mouse embryonic tissues and that its expression can be induced by ATRA in the NIH 3T3 cell line.

Figure 5.19. All-trans retinoic acid induces *mHotairm1, Hoxa1* and *Hoxa2* expression. NIH 3T3 cells were treated with 10^{-6}M ATRA. After 24h, RNA was isolated and *mHotairm1* (A), *Hoxa1* (B) and *Hoxa2* (C) expression were quantified using qRT-PCR. Bars represent mean ±SEM, n =3. *p ≤ 0.05, **p ≤ 0.01 compared to DMSO treated control group.
These findings show that \textit{mHotairm1} is expressed differently in different mouse embryonic tissues. It is also expressed in NIH 3T3 cells and its expression can be induced by ATRA, which agrees with the findings of Zhang 	extit{et al} (2009) that human HOTAIRM1 can be induced by ATRA in myeloid differentiation.

\subsection*{5.3.3 \textit{mHotairm1} regulates \textit{Hoxa1} and \textit{Hoxa2} expression via histone methylation}

The confirmation of the existence of \textit{mHotairm1} led to my next research question: what is the function of this lncRNA? The human \textit{HOTAIRM1} is known to regulate 3’ HOXA genes, and several lncRNAs transcribed within Hox cluster genes are also known to regulate expression of nearby \textit{Hox} genes (Wang \textit{et al}., 2011, Bertani \textit{et al}., 2011). Hence, I further investigated whether \textit{mHotairm1} regulates the expression of nearby \textit{Hoxa1} and \textit{Hoxa2} genes and what if any, were regulatory mechanisms involved.

\subsubsection*{5.3.3.1 Knockdown expression of \textit{mHotairm1} leads to decreased expression of \textit{Hoxa1} and \textit{Hoxa2}}

To determine what impact \textit{mHotairm1} had on mouse \textit{Hoxa1} and \textit{Hoxa2} expression, NIH 3T3 cells were transfected with \textit{mHotairm1} siRNA. Exposure to \textit{mHotairm1} siRNA decreased \textit{mHotairm1} expression in NIH 3T3 cells after 72h, and resulted in significantly decreased expression of both \textit{Hoxa1} and \textit{Hoxa2} genes (Fig. 5.20).
Knockdown of mHotairm1 leads to decreased expression of Hoxa1 and Hoxa2. NIH 3T3 cells were transfected with 150 ng of mHotairm1 siRNA and control siRNA, respectively. After 72 h of transfection, total RNAs were isolated, reverse transcribed and quantified using qRT-PCR with Hoxa1, Hoxa2 and mHotairm1 primers. Bars represent mean ±SEM, n =3. **p ≤ 0.01 compared to respective control.

5.3.3.2 mHotairm1 interacts with promoters of 3' HoxA genes and WDR5.

To determine the potential mechanism of how blocking the expression of mHotairm1 causes the down expression of Hoxa1 and Hoxa2 expression, I carried out a “CHART” experiment (Simon et al., 2011) to investigate any interactions between mHotairm1, Hoxa1 and Hoxa2 promoter and the H3K4 methyltransferase complex MLL1/WDR5. DNA samples bound to mHotairm1 probes were collected from the “CHART” experiment and tested using PCR. Probe 1, 2, 3 were designed to detect mHotairm1 and Probe C (anti-sense to Probe 2) was used as the control probe (Fig. 4.4, Table 4.3). Probe 2 showed much stronger interaction with mHotairm1 (Fig. 5.21 and Fig. 5.22) compared to the control probe, indicating a specific recognition of
mHotairm1 by Probe 2. PCR results from the DNA fragments pulled down by Probe 2 showed a strong interaction between mHotairm1 and the promoters of 3' HoxA genes (Hoxa1, Hoxa2, Hoxa3 and Hoxa5) but not with 5' HoxA gene (Hoxa13) when compared to Probe C (Fig. 5.21). Protein samples pulled down by Probe 2 also showed strong interaction between mHotairm1 and WDR5, a subunit in TrxG complex (Fig. 5.22A). No interaction was observed between mHotairm1 and CBP (CREB-binding protein), a histone acetyltransferase. These results indicate that there is strong interaction between mHotairm1 and promoters of 3' HoxA genes including Hoxa1 and Hoxa2, and the TrxG complex MLL1/WDR5.

![PCR results from HoxA promoters](image)

**Figure 5.21.** PCR amplification of CHART enriched DNA fragments. DNA fragments that can interact with mHotairm1 were pulled down together with mHotairm1 using gene specific Probe 1, 2, 3. Probe C was used as control. Each pull down sample was PCR tested with Hoxa1, Hoxa2, Hoxa3, Hoxa5 and Hoxa13 promoter primers (see Table 4.3 for probe and primer sequences).
Figure 5.22. Western blot of CHART enriched protein samples. A. Protein samples interacting with mHotairm1 was pulled down together with it using gene specific Probe 1, 2, 3. Probe C was used as control. Each pull down sample was tested with WDR5 antibody in western blot. NIH 3T3 cell lysate was used as a positive control. Two WDR5 bands were observed in NIH 3T3 cell lysate, a WDR5 band at ~37KDa and a modified WDR5 band at ~ 50KDa. Only ~50KDa bands were seen in CHART enriched protein samples. B. No CBP band was detected in western blot in samples pulled down by mHotairm1 specific probe 2.
5.3.3.3 *mHotairm1* binds directly to MLL1/WDR5 complex.

### 5.3.3.3.1 Construction of GST fusion protein vectors.

To further confirm the interaction between *mHotairm1* and MLL1/WDR5 complex, GST pull down assay was used. WDR5 and MLL1 (amino acid 3810-3963) coding cDNAs were successfully amplified from NIH 3T3 cDNA samples, and cloned into T-easy vectors and further confirmed with sequencing (Fig. 5.23, Appendix 10-11). From T-easy vectors, the two fragments were then cloned into pGEX-6p-1 vector that contains a GST tag. MLL1 (3810-3963), WDR5 and GST protein expression were successfully induced with IPTG in DH5α *E. coli* (Fig. 5.24A-C). All GST fused proteins were purified with Glutathione agarose beads. Single bands were observed in purified samples (Fig. 5.24D).

### 5.3.3.3.2 Binding of *mHotairm1* to MLL1/WDR5 complex

To confirm the interaction between mHotairm1 and MLL1/WDR5 complex, I carried out GST pull down experiments. Using GST fused MLL1 (3810-3963) and WDR5 proteins, the RNA pulled down were converted into cDNA and amplified with *mHotairm1* primers mush138 F and mush138 R (Table 4.3). PCR results indicated that *mHotairm1* can be pulled down with both GST-MLL1 (3810-3963) and GST-WDR5, but not with control GST (Fig. 5.24E).
A. DNA and protein sequence of full length mouse WDR5

```
ATG GCC ACA GAG GAG AAG AAG CCA GAG ACA GAG GCT GCA AGA GCA CAG
MAT E K K P E T A A R A Q
CCC ACT CCT TCC TCA TCA GCC ACA AGC AAG CCC ACA CCA GGT T AA G
PT P S S S A T Q S K P T P V K
CCA AAC TAT GCC CTG AAG T T C ACC CTG GCT GGC AAC ACC AAA GCT GTG
P N Y A L K F T L A G H T K A V
TCC TCT GTG AAG TTC AGC CCC AAT GGG GAA TGG T T G GCA AGT TCA TCT
SS V K F S P N G E W L A S S
GCT GAT AAA CTC ATT AAA ATT TGG GAA GCA TAT GAT GGA AAG TTT GAG
AD KL IK I W G A Y D G K F E
AAA ACT ATA TCT GGT CAC AAA CTG GGA ATA TCT GAT GTA GCG TGG TCA
KT ISA G H K L G I S D V A W S
TCA GAT TCT AAC CTC CTT GTG TCT GCC TCT GAT GAT AAA ACT TTG AAG
SD S N L L V S A S D D K T L K
ATT TGG GAC GTG AGT TCC GGC AAG TGT CTG AAG ACC CTG AAG GCC CAC
IW D V S S G K C L K T L K G H
AGT AAC TAC GTC TTD TGC TGC AAC TTC AAC CCC CAG TCC AAC CTC ATC
SN Y V F C C N F N Q S N L I
GTC TCA GGG TCT TTT GAT GAA AGT GTG AGG ATA TGG GAC GTG AAG ACA
V S G S F D E S V R I W D V K T
GGG AAG TGC CTC AAG ACT TTG CCT GCC CAT TCG GAC CCA GTC TCA GCC
G K C L K T L P A H S D P V S A
GTT CAT TTC AAC CGT GAT GGA TCA T T G A T T G T T C A G T A G TAT GAT
V H F N R D G S L I V S S S Y D
GCC CTC TGC CGA ATC TGG GAC ACC GCC TCT GCC CAG TGT CTG AAG ACA
GL C R I W D T A S G Q C L K T
CTC ATT GAT GAT GAC AAT CCT CCA GTG TCC TTT GAT AAG TCC TCT CCA
L I D D D N P P V S F V K F S P
AAT GGC AAA TAC ATC CTC GCT GCA ACT TTG GAC AAC ACA CTG AAG CTC
NGK Y I L A A T L D N T L K L
TGG GAC TAC AGC AAG GGC AAG TGC CTG AAG ACA TAC ACT GCC CAC AAG
W D Y S K K C L K K Y T G H K
AAT GAG AAG TAC TGC ATA TTT GCC AAC TTC TCC GTG ACA GGC GGG AAG
NE K Y C I F A N F S V T G K
TGG ATT GTG TCT GGT TCT GAA GAT AAC CTG GTG TAT ATC TGG AAT CTG
W I V S G S E D N L V Y I W N L
CAG ACC AAC GAG ATT CTG CAG AAG TGG CAG GGT CAC ACA GAT GTT GTG
Q T K E I V O K L Q G H T D V V
ATT TCC ACG GCT TGC CAC CCG ACA GAG AAC ATC ATT GCC TCA GCA GCG
I S T A C H P T E N I I A S A A
TTA GAG AAC GAC AAA ACA ATC AAA CTG TGG AAG AGT GAC TGC TAA
LEN DK T I K L W K S D C
```
B. DNA and protein sequence of mouse MLL1 (amino acid 3810-3963)

ATG CCC ATG AGA TTC CGG CAC TTG AAG AAG ACT TCT AAG GAG GCG GTT
M P M R F R H L K K T S K E A V
GGT GTC TAC AGG TCT CCC ATC CAT GGT CGG GTT CTT TCC TGT AAG AGA
G V Y R S P I H G R G L F C K R
AAC ATC GAT GCA GGA GAG ATG GTG ATT GAA TAC GCC GGC AAC GTC ATC
N I D A G E M V I E Y A G N V I
CGC TCC ATC CAG ACA GAC AAG CGT GAG AAG TAC TAT GAC AGC AAG GGC
R S I Q T D K R E K Y Y D S K G
ATT GGT TGC TAC ATG TCC CGA ATT GAT GAC TGG GAG GTA GTG GAT GCC
I G C Y M F R I D D S E V V D A
ACC ATG CAT GGA AAT GCT GCA CGC TTC ATC AAT CAC TCT TGT GAG CCT
T M H G N A A R F I N H S C E P
AAC TGC TAC TCC CGG GTC ATC AAT ATT GAT GGG CAG AAG CAC ATT GTC
N C Y S R V I N I D G Q K H I V
ATC TCC GCC ATG CGT AAG ATC TAC CGG GGG GAG GAG CTC ACC TAT GAC
I F A M R K I Y R G E E L T Y D
TAT AAG TTC CCC ATT GAG GAC GCC AGC AAC AAG CTA CCC TGC AAC TGT
Y K F P I E D A S N K L P C N C
GGC GCC AAA AAA TGC CGC AAG TTC CTG AAC TAA
G A K K C R K F L N

Figure 5.23. Coding sequence of WDR5 and MLL1 (amino acid 3810-3963). DNA sequences were grouped in triplets with correlating amino acids marked under DNA sequences in bold letters. DNA sequencing confirmed successful cloning of WDR5 (A) and MLL1 (amino acid 3810-3963) (B).
Figure 5.24. GST fusion protein pull down of mHotairm1. (A-C) Coomassie blue staining show expressions of GST and GST fused MLL1 (3810-3963) and WDR5 induced with IPTG in DH5a (Pointed with red arrow). Lane numbers indicate different clones. (D) GST fusion proteins were purified with Glutathione agarose beads. P- protein samples from the pellet of E coli cell lysate, S- protein samples from the supernatant of E coli cell lysate, BP- beads purified protein sample from the supernatant of E coli cell lysate. Target bands are pointed with red arrow. (E) PCR amplicant of mHotairm1 (pointed with red arrow) from GST fusion protein pull down. Bands in MLL1 (3810-3963) and WDR5 pull down indicate direct interaction between mHotairm1 and MLL1/WDR5 complex. NIH 3T3 cDNA sample was used as a positive control.
5.3.3.4 *mHotairm1* can affect H3K4me3 and H3K27me3 marks on *Hoxa1* and *Hoxa2* chromatin.

Results above confirmed an interaction between *mHotairm1* and TrxG MLL1/WDR5 complex, next, I wanted to determine whether this interaction also impacts the bivalent domain marks H3K4me3 and H3K27me3. H3K4me3 is an activation mark and its occupancy on the promoters of some *Hox* genes is enhanced when expression of the *Hox* gene is increased, while at the same time, gene repressive mark H3K27me3 has decreased occupancy (Wang *et al.*, 2011; Bertani *et al.*, 2011). The reverse occurs when *Hox* gene expression is inhibited resulting in a lower occupancy of H3K4me3 and higher occupancy of H3K27me3. Chromatin immunoprecipitation (ChIP) were carried out as described in 4.2.16 (p. 90) and experiments showed that siRNA induced suppression of *mHotairm1* expression in NIH 3T3 cells (Fig. 5.20), resulted in a decreased occupancy of the activating mark H3K4me3 and an increased occupancy of repressive mark H3K27me3 in both *Hoxa1* and *Hoxa2* chromatin samples (Fig. 5.25 A-D). The occupancy of Histone 3 (H3) on both *Hoxa1* and *Hoxa2* gene chromatin samples was not affected by the down regulation of *mHotairm1* (Fig. 5.25 E, F). These results confirmed that *mHotairm1* does indeed regulate the expression of *Hoxa1* and *Hoxa2* via histone methylation impacting bivalent domain marks H3K4me3 and H3K27me3.
Figure 5.25. *mHotairm1* affects the bivalent histone methylation marks, H3K4me3 and H3K27me3, at *Hoxa1* and *Hoxa2* chromatin sites. qChIP was carried out as described in 4.2.16. Relative occupancy represents the fold enrichment of H3K4me3 (A,B), H3K27me3 (C,D) and H3 (E,F) on both *Hoxa1* and *Hoxa2* chromatin relative to input. Histone 3 was used as a positive control (E,F). Bars represent mean ±SEM, n = 3. *p ≤ 0.05 compared to respective control.
The above findings demonstrate that \textit{mHotairm1} can recruit histone methyltransferase complex MLL1/WDR5 to \textit{Hoxa1} and \textit{Hoxa2} gene and induce H3K4me3 to activate their expression. These findings are similar to the findings of \textit{HOTTIP} (Wang \textit{et al.}, 2011) and \textit{Mistral} (Bertani \textit{et al.}, 2011), which can positively regulate neighbouring \textit{Hox} genes through the recruitment of MLL1/WDR5 complex.

5.3.4 \textbf{WDR5 is sumoylated and this modification maybe important for the function of WDR5.}

5.3.4.1 \textbf{MLL1 specifically interacts with 50 kDa WDR5.}

Using a WDR5 antibody in western blot, two distinct WDR5 bands at ~37KDa and at ~50KDa were observed in NIH 3T3 cell lysates (Fig. 5.22). In addition, GST-MLL1 (3810-3963) protein pull down of WDR5 confirmed a direct interaction between MLL1 and WDR5 but interestingly, only predominantly with the ~50 kDa isoform (Fig. 5.26). This ~50 kDa band was suspected to represent a modification of WDR5 and since only the ~50 kDa band co-precipitated with MLL1, this modification may be biologically important. The size difference between the ~50 kDa band and the unmodified WDR5 (~37 kDa) suggested the modification might be sumoylation, which is ~ 12 kDa in size (Kumar and Zhang, 2015). Indeed, in the presence of the ubiquitin/SUMO protector NEM, protein samples showed a stronger band intensity further supporting this hypothesis (Fig. 5.26A). Thus I hypothesized this post-translation modification to be sumoylation and additional experiments were carried out to test this hypothesis.
Figure 5.26. GST fused MLL1 (3810-3963) pull down of modified WDR5. A. Western blot showing modified WDR5 bands at ~ 50kDa in samples pulled down with GST fused MLL1 (3810-3963). The unmodified WDR5 band (~37kDa) was not observed. NEM: ubiquitin/SUMO protector N-ethylmaleimide. GST was used as a negative control. B. Loading control of GST and MLL1 (3810-3963). Coomassie blue staining showing equal loading of GST and GST fused MLL1 used in the pull down experiment above.
5.3.4.2 Sumoylated WDR5.

I carried out immunoprecipitation assays and established that the protein samples precipitated with WDR5 antibody could be detected with a SUMO1 antibody in Western blot assays (Fig. 5.27A). Similarly, protein samples precipitated with SUMO1 antibody could also be identified with the WDR5 antibody (Fig. 5.27B). Hence, the modification of WDR5 as it exists in NIH 3T3 cells is most likely sumoylation. Experiments using mass spectrometry to confirm sumoylation of WDR5 will be needed to characterize this further.

![Figure 5.27](image)

**Figure 5.27. Reciprocal co-immunoprecipitation of WDR5 and SUMO1.** A. SUMO1 antibody detected a band in protein samples immunoprecipitated with WDR5 antibody at the exact size as modified WDR5 seen in NIH 3T3 nucleus. B. WDR5 antibody also detected a band in protein samples immunoprecipitated with SUMO1 antibody at the size of modified WDR5. IgG antibody was used as the negative control.
5.3.4.3 Cellular distribution of WDR5 in NIH 3T3 cells.

Although SUMO modification of proteins has multiple functions, one important function is their role in nuclear-cytosolic transport (Eun Jeoung et al., 2008; Berndt et al., 2012; Lamoliatte et al., 2014). I investigated this further by first performing immunocytochemistry to determine the distribution profile of WDR5 in NIH 3T3 cells. Immunocytochemistry revealed that WDR5 is distributed throughout the cell in both cytoplasm and nuclei (Fig. 5.28). Subsequently, nuclei and cytoplasm were isolated from the NIH 3T3 cells using the method described in section 4.2.17. Western blot analysis showed that the modified WDR5 protein is present in the nuclei whereas both forms of WDR5 exist in the cytoplasm (Fig 5.29). The proteins β-actin (only expressed in cytoplasm) and Histone 3 (H3, only expressed in nucleus) were used to verify the separation of nuclei and cytoplasm.

We can speculate that since only the modified WDR5 is present in the nuclei, this SUMO modified WDR5 may play a role in translocation of the protein from the cytoplasm to the nuclei.
Figure 5.28. WDR5 is present in both cytoplasm and nuclei in NIH 3T3 cells. NIH 3T3 cells were fixed and immunostained with a WDR5 antibody (shown in red, a and c). Nuclei were labelled with DAPI (shown in blue, b-c, e-f). A blank group without primary antibody (WDR5 antibody) is used as negative control (d). Arrows point to nuclei and arrowheads point to cytoplasm. Scale bars = 3 microns.

Figure 5.29. WDR5 proteins in NIH 3T3 cell cytoplasm and nuclei. Western blot analysis shows modified WDR5 protein band (~50kD) in nuclei, cytoplasm and NIH 3T3 whole cell lysates. Unmodified WDR5 protein band (~37kD) was only observed in the cytoplasm and NIH 3T3 cell lysates. β-Actin protein was observed only in cytoplasm and NIH 3T3 whole cell lysates. H3 protein was observed in nuclei and NIH 3T3 whole cell lysates.
5.3.4.4 Sumoylated WDR5 is responsive to ATRA induction.

Since ATRA induces the expression of \( m\text{Hotairm1} \) as well as \( \text{Hoxa1} \) and \( \text{Hoxa2} \) (Fig. 5.19), I further investigated whether ATRA would have any effect on post-translation modification of WDR5. Interestingly, ATRA treatment appeared to show a stronger band intensity of the modified WDR5 (~50 kDa) protein in the nuclei of NIH 3T3 cells (Fig 5.30). As \( m\text{Hotairm1} \) can positively regulate \( \text{Hoxa1} \) and \( \text{Hoxa2} \) expression through the regulation of H3K4me3 occupancy on their promoters, it is highly possible that exposure to ATRA which induces \( \text{Hoxa1} \) and \( \text{Hoxa2} \) expression is through the increase of both modified WDR5 and \( m\text{Hotairm1} \) expression. In summary, my results reveal a new a gene regulatory pathway in which \( m\text{Hotairm1} \) working closely with H3K4 methylation complex and sumoylated WDR5, regulates \( \text{Hoxa1} \) and \( \text{Hoxa2} \) expression.

**Figure 5.30. Effect of ATRA on the expression sumoylated WDR5.** After ATRA treatment, an increased sumoylated WDR5 in NIH 3T3 nuclei was observed. \( \beta \)-actin bands were observed in cytoplasm and NIH 3T3 cell lysates. Histone 3 bands were observed in the nuclei and NIH 3T3 cell lysates.
These findings suggest that WDR5 is sumoylated and this modification plays an important role in its interaction with *mHotairm1* and MLL1. This modification may help WDR5 to translocate from cytoplasm to the nucleus. Further experiments are needed to study the function of sumoylated WDR5.
CHAPTER 6

6. Discussion

6.1 DNA methylation

DNA methylation is an important epigenetic mechanism in mammalian embryo development (Bird, 2002; Santos et al., 2005; Bartolomei and Ferguson-Smith, 2011; Lomvardas and Maniatis, 2016). DNA methylation which primarily occurs on cytosine bases in GpG rich sequences of the promoter regions leads to gene silencing through the inhibition of transcription factor binding and the changing of chromatin structure into a repressive state (Domcke et al., 2015). Our lab has been interested in Hoxa2 regulation in mouse palatal development and has revealed a spatio-temporal expression pattern of Hoxa2 during palatogenesis (Nazarali et al., 2000; Smith et al., 2009, 2013). It is not known whether this differential expression of Hoxa2 is regulated by a specific epigenetic mechanism(s). The genome is comprised of many CpG rich regions and many are located near 5′ regulatory regulatory regions of genes. My analysis of Hoxa2 promoter revealed three CpG islands close to Hoxa2 5′ regulatory regulatory region. I have used several approaches to study these CpG islands. First I used MSP to study the three CpG islands within the Hoxa2 promoter region. The advantage of this method is that by designing methylated and unmethylated specific primers, the methylation status can be tested by a simple PCR. However, since PCR primers are usually only 18-24nt long, the CpG sites that can be covered by forward and reverse primers are limited. This also resulted in higher degree of primer sequence similarity between methylated and unmethylated primers. A critical PCR condition is needed for MSP primers to selectively bind and amplify specific methylated/unmethylated target sequences. To develop optimal PCR conditions for all sets of
MSP primers, DNA samples from NIH 3T3 cells and EG7 cells were used as unmethylated and methylated control, respectively. It has previously been shown in our lab that the Hoxa2 promoter is unmethylated in NIH 3T3 cells and that it remains highly methylated in the EG7 cells (Wang and Nazarali, unpublished). After testing several different annealing temperatures, I was able to develop optimal PCR cycle conditions for each pair of MSP primer set. In the developing mouse palate, DNA samples collected at all four stages of palatogenesis were recognized by unmethylated primers but not with methylated primers, suggesting Hoxa2 promoter is most likely unmethylated during palate development.

The limitation of using MSP is that only CpG sites recognized by the primers can be tested. To examine the methylation status of each single CpG site, a BSP method was used. BSP is a method that uses PCR to amplify a certain CpG island, whether methylated or unmethylated, and the methylation status of each individual CpG site can then be analyzed by DNA sequencing. This method requires a longer experimental process but the advantage is that each GpG site within the CpG island can be analyzed. Since the CpG region-1 is closest to Hoxa2 transcription start site and likely most relevant to Hoxa2 expression, this region was selected for further BSP analysis. The results of BSP showed that all 14 CpG sites tested in the CpG region-region 1 were unmethylated in NIH 3T3 cells and in developing mouse palate from E12 to E15, whereas 10 of the 14 CpG sites were found to be methylated in EG7 cells. These results supported my findings from the MSP experiments. From these observations we can conclude that during mouse palate development the Hoxa2 promoter within the CpG-region 1 primarily remains unmethylated and that the DNA methylation status of the Hoxa2 promoter does not change with the spatio-temporal expression of Hoxa2 during palatogenesis.
6.2 Regulation of *Hoxa2* gene expression by miRNAs

Currently, there are several studies that focus on miRNA regulation of gene expression (Yan and Jiao, 2016; Green *et al.*, 2015; He *et al.*, 2015; Usmani *et al.*, 2016). Regulation of gene expression by miRNAs is complex because a specific gene can be regulated by many different miRNAs and whereas a specific miRNA can also regulate expression of several different genes (Doench and Sharp, 2004; Felekkis *et al.*, 2010). Generally, miRNAs bind to the 3'UTR of a gene, hence I performed in-silico analysis and identified six miRNAs that had the potential to bind 3'UTR of the *Hoxa2* gene. Following this, I investigated expression profiles of these miRNAs in mouse palatal tissues, and in cell lines in which *Hoxa2* expression is already studied (Smith *et al.*, 2009; Wang and Nazarali unpublished). Since these predicted miRNAs were considered to downregulate *Hoxa2* expression, I primarily focused on miRNA expression patterns that were complementary to that of the *Hoxa2* expression. The two miRNAs: miR-431 and miR-298 showed high expressions in NIH 3T3 cells at the same time as *Hoxa2* expression. Thus these two miRNAs are unlikely to down regulate *Hoxa2* in NIH 3T3 cells. All six miRNAs exhibited low expression levels in EG7 cells, possibly due to globally repressed transcription activity and high DNA methylation status of gene promoters in EG7 cells (Wang and Nazarali unpublished). I then investigated the expression of of all six miRNAs in developing mouse palate at stages E12 to E15. Three miRNAs (miR-669b, miR-376c and miR-431) exhibited increased expression from E13 to E15 in mouse palate, during the same period when *Hoxa2* expression is declining (Smith *et al.*, 2009). Since miR-431 showed high expression in NIH 3T3 cells, I continued further investigations with only miR-669b and miR-376c.

To investigate whether miR-669b and miR-376c are able to down regulate the expression of *Hoxa2*, I transfected synthesized mature miRNAs: miR-669b mimic and miR-376c mimic into
NIH 3T3 cell cultures. Overexpression of both miRNAs by miRNA mimics resulted in the downregulation of Hoxa2 expression at both transcriptional and translational level. At this stage, it was not known whether miR-669b and miR-376c miRNAs bound directly to the 3’UTR of Hoxa2 or had an indirect effect on Hoxa2 expression via regulation of upstream genes that may control Hoxa2 expression. A luciferase expressing vector carrying the 3’UTR of Hoxa2 was used in luciferase assays to determine whether miR-669b and miR-376c miRNAs bound to the Hoxa2 3’UTR. Luciferase assays showed that miR-669b appears to directly bind to Hoxa2 3’UTR since mutations within the seed sequences abrogated luciferase activity, however miR-376c had no effect on luciferase assay and so its effect on Hoxa2 expression may likely be via an indirect effect. Although most miRNAs bind to the 3’UTR of their target genes, some miRNAs are also known to bind to coding regions of mRNA (Hu et al., 2011). Hence, it is possible that miR-376c may affect Hoxa2 expression by interacting with other regions of the Hoxa2 mRNA. An additional possibility is that miR-376c may be regulating genes upstream of Hoxa2 which in turn is regulating Hoxa2 expression. One such possibility is the Krox20 gene that is upstream of Hoxa2 (Nonchev et al., 1996). In human pluripotent stem cells, miR-376c has been reported to inhibit the expression of SMAD4 gene in the TGF-β (transforming growth factor β) pathway (Liu et al., 2014). SMAD4 is known to bind directly to PAX6 (paired box 6) gene promoter and suppress its expression (Liu et al., 2014). Thus, overexpression of miR-376c would lead to the downregulation of SMAD4 expression which in turn would lead to an upregulation of PAX6 expression. Pax6 has been reported to induce the expression of Nab1 (NGFI-A Binding Protein 1), a Krox20 repressor in the chick embryo (Kayam et al., 2013). Hence, overexpression of miR-376c could lead to an upregulation Nab1 expression via Pax6 and subsequent downregulation of Krox20 expression. Krox20 is a known upstream gene of Hoxa2 and can directly activate its
expression (Nonchev et al., 1996). Thus, downregulation of Krox20 would induce downregulation of Hoxa2 expression. Hence, an indirect link between miR-376c and Hoxa2 gene expression may exist via genes upstream of Hoxa2 (Fig 6.1).

![Figure 6.1. A putative indirect link between miR-376c and Hoxa2 expression.](image)

Researchers in the past have generally put a higher emphasis on comparing differences within coding regions of genes between various species. However, many researchers are beginning to realize that the non-coding regions have important regulatory roles and sequence differences in these regions could lead to differentially regulated gene expression between various species (Barrett et al., 2012). There are two predicted miR-669b binding sites on mouse Hoxa2 3’UTR. My mutation experiments showed that the two binding sites appear to work independently from each other. I analyzed the degree of sequence similarity for both miR-669b binding sites and found that the binding site 1 is evolutionally conserved among the five species.
examined, human, mouse, rat, chimpanzee and dog. However, the binding site 2 in mouse only exhibited sequence similarity with that in rat and not with the other three species examined (Fig. 5.5). Since the binding site 1 is present in all five species examined, it is possible that miR-669b has more conserved role in regulating Hoxa2 gene expression via this binding site. While it is known that one miRNA may have hundreds of gene targets, having a single miRNA binding site may not be sufficient to strongly inhibit gene expression (Stark et al., 2005). Thus, it is also possible that the regulation of Hoxa2 by miR-669b would not be as strong in other species compared to mouse or rat which has two miR-669b binding sites on Hoxa2 3'UTR.

Interestingly, evidences support genomic evolution of 3'UTR region to mitigate and augment the effects of miRNA on gene expression regulation (Stark et al., 2005; Thomsen et al., 2010). Some mRNAs have evolved to have different lengths of 3'UTRs so that they have different ‘visibilities’ to miRNAs in different tissues (Stark et al., 2005; Thomsen et al., 2010). Thomsen and colleagues found different 3' UTR splicing exist in several Hox genes in Drosophila (Thomsen et al., 2010). For example, the longer form of Ubx mRNA has eight miR-iab4/8 binding sites and is expressed mainly in the developing CNS. Expression levels of the longer Ubx mRNA with multiple miR-iab4/8 binding sites is very sensitive to miR-iab4/8 regulations. The shorter form of Ubx mRNA with only three miR-iab4/8 binding sites in the 3'UTR is mainly expressed in germ band elongation and removal of miR-iab4/8 binding sequence did not have a significant effect on the expression of the short form of the Ubx transcript (Thomsen et al., 2010). Differential splicing of Hoxa2 3'UTR has not been reported; however, bioinformatic analysis has revealed high frequency of alternative polyadenylation in mammals (Brett et al., 2000; Di Giammartino et al., 2011). Hence, it is still possible that Hoxa2 may also have differentially spliced 3'UTR. Having a single miR-669b binding site in human
HOXA2 3'UTR may make it less ‘visible’ to miR-669b, although my experiments showed that each single miR-669b binding site on Hoxa2 3'UTR is sufficient to confer the repression of Hoxa2 expression when miR-669b was over expressed. Possessing two binding sites may enhance the regulatory role of miR-669b on Hoxa2 expression under normal biological conditions.

In my study, I have also found that in the developing mouse palate (from E13 to E15), miR-669b has a complementary expression to that of Hoxa2. At the E12 stage, both Hoxa2 and miR-669b have relatively low expression in palate indicating that miR-669b may not be responsible for regulating Hoxa2 expression at this stage in mouse palate. However, it is highly probable that miR-669b may play a role in down regulating Hoxa2 expression towards the end of mouse palate development when Hoxa2 palatal expression declines and miR-669b expression is the highest. Since Hoxa2 can also regulate ear and nervous system development (Minoux et al., 2013; Cox et al., 2014; Gavalas et al., 1997; Wang et al., 2011), it may be of interest to investigate whether miR-669b plays a role in these developmental processes. Many pathological processes, including cancer, neurological diseases and cardiovascular diseases impact specific miRNA expression changes, making them a promising biomarker and potential diagnostic tool for a variety of diseases (Chi and Zhou, 2016; Stoicea et al., 2016; Wallace et al., 2016). The regulatory roles of miRNAs also render them promising therapeutic targets in many diseases (Broderick and Zamore, 2011). The mechanisms by which miRNAs regulate gene expression are still not fully understood and further studies on miRNAs will lead to a better understanding of biological processes.

In summary, my investigations identified two miRNAs, miR-669b and miR-376c, that regulate Hoxa2 expression in mouse NIH 3T3 cells. MiR-669b directly binds to mouse Hoxa2
3'UTR to down regulate its expression, also two functional miR-669b binding sites were identified on Hoxa2 3'UTR. No direct interactions between miR-376c and Hoxa2 3'UTR was identified. miR-376c possibly binds to other regions of Hoxa2 mRNA or other regulatory genes upstream to Hoxa2 which in turn may regulate Hoxa2 expression (Fig. 6.1). miR-669b is expressed in developing mouse palate with a complementary expression profile to Hoxa2 and may have regulatory role in mouse palate development.

6.3 lncRNA mHotairm1 regulation of Hoxa1 and Hoxa2 expression

Following the discovery of the lncRNA:Polycomb repressive complex 2 (PRC2) interaction (Zhao et al., 2008), significant interest has been generated to characterize the role of lncRNAs in epigenetic activation/inactivation of gene expression (Cao, 2014). Additionally, investigation on the transcriptional activity of the human HOX loci showed that many of the intergenic regions are actively transcribed and most of transcripts from the intergenic regions are lncRNAs (Rinn et al., 2007). Hox lncRNAs likely play important biological roles based on the fact that: 1) Some lncRNAs are conserved during evolution; 2) Like HOX genes, lncRNAs in HOX cluster have different expression patterns along the A-P axes depending on their physical location on the chromosome (Rinn et al., 2007). However, very few lncRNAs from Hox clusters have been characterized. In my study, I have found a new lncRNA located between mouse Hoxa1 and Hoxa2 and further demonstrated its ability to recruit MLL1/WDR5 to nearby target genes and to regulate these gene expressions. My results provide additional evidence to support the hypothesis raised by Rinn et al. that transcription of lncRNA in cis may recruit TrxG proteins such as MLL1 and WDR5 to chromatin, leading to H3K4me3 induction and gene activation (Rinn et al., 2007).
6.3.1 Identification of mHotairm1

The existence of HOTAIRM1 in human was reported by Zhang and colleagues in 2009 (Zhang et al., 2009). In GenBank database that I searched, a similar long non-coding transcript was found to potentially exist in the mouse. I made several attempts to identify the full length of mHotairm1. First, based on the predicted mHotairm1 sequence provided by GenBank, I designed three pairs of PCR primers that covered 462bp of the predicted mHotairm1 sequence in total. Each pair of primer set were designed to have overlapping region with the next pair. All three pairs of primers successfully amplified DNA fragments from cDNA samples generated from total RNA isolated from NIH 3T3 cells. Each amplified fragment was sequenced and matched exactly with the predicted sequence (Fig. 5.16B, Appendix 7-9). The analysis of the predicted mHotairm1 sequence showed that this transcript has two exons and splicing occurs between nucleotide 162 and 165. Primer set Mush-138 covered a length from nucleotide 131 to 268 (Table 4.3, Appendix 8) which include the splicing site. The fact that primer set Mush-138 is only able to amplify DNA fragment from cDNA sample but not from genomic DNA (Appendix 12) further supported the existence of mHotairm1 and ruled out a possible contamination from genomic DNA. This primer set was also used to identify mHotairm1 in further experiments.

I then tried to identify the 3' and 5' end of mHotairm1 using Rapid Amplification of cDNA Ends (RACE) approach. The strategy of RACE experiment is to add special adaptor sequences to both 3' and 5' end of mRNAs and construct a cDNA library. Then by using gene specific primers and adaptor primers, target gene fragments can be amplified with their 3' and 5' ends. Unfortunately, I was not able to successfully amplify either 3' or 5' end of mHotairm1. I have tried different mHotairm1 specific primers but none of amplification products turned out to be from mHotairm1. The possible reasons are: in RACE experiments, only one gene specific
primer is used in target fragment amplification, which reduced the specificity of amplification compared to normal PCR, and secondly, IncRNAs usually have significantly lower levels of expression compared to protein-coding genes (Cabili et al., 2011; Derrien et al., 2012), which makes them even more difficult to amplify in a RACE experiment. However, I was able to identify the majority of the mHotairm1 sequence (462nt) and confirm the existence of a new long noncoding transcript located between Hoxa1 and Hoxa2 in mouse.

6.3.2 mHotairm1 expression in cell lines and mouse tissues

Most IncRNAs have tissue specific expression (Derrien et al., 2012). mHotairm1 expression was identified in different mouse embryonic tissues (Fig 5.17). In the developing palate (E14) mHotairm1 expression is localized to the medial edge epithelia (MEE) (Fig 5.18A). In the developing mouse palate at E14 the two vertical palate shelves begin elevating above the tongue and grow towards each other. The two palate shelves eventually contact each other at the MEE. The MEE layers from the two palate shelves then merge together to form the medial edge seam (MES) and finally fuse together (Ferguson, 1988). This process is regulated by a series of cellular and biochemical reactions and is a very important step in palate development (Ferguson, 1988; Jin and Ding, 2006, Smith et al., 2013, Lan et al., 2015). In situ hybridization histochemistry of E14 developing palate showed the expression of mHotairm1 in MEE (Fig. 5.18), indicating mHotairm1 may play a role in the palatal fusion. mHotairm1 expression in E15 palatal shelves was only faintly detected by in situ hybridization histochemistry, which agrees with the real-time PCR data where mHorairm1 expression has significantly declined. The expression of mHotairm1 in E12 and E13 mouse palatal shelves was not detectable with in situ probes, possibly due to a low abundance of IncRNAs at these stages.
6.3.3 Regulation of Hoxa1 and Hoxa2 expression by mHotairm1

Although the presence of non-coding transcripts was initially considered to be “noise” and of little consequence, they have gained importance as more information is gathered on their important regulatory roles (Rinn and Chang, 2012; Bonasio and Shiekhattar, 2014). As Rinn and colleagues (2007) have suggested, the lncRNAs transcribed from Hox loci can either up or down regulate target gene expression. Thus to study the function of mHotairm1, I first tested whether a change in the expression of mHotairm1 could influence the expression of nearby genes such as Hoxa1 and Hoxa2. I chose to down regulate expression of mHotairm1 with siRNA in mouse fibroblast cell line NIH 3T3 where Hoxa1, Hoxa2, as well as mHotairm1 are all expressed. My results showed that downregulation of mHotairm1 resulted in significant downregulation of Hoxa1 and Hoxa2 expression.

A well characterized activator of Hox gene expression, all-trans retinoic acid (ATRA) has also been shown to induce the expression of Hox loci derived lncRNAs (Zhang et al., 2009; Bertani et al., 2011). Moreover, the induction of 3’ HOX gene expression by ATRA is attenuated if the regulatory lncRNA HOTAIRM1 is silenced (Zhang et al., 2009). As well Bertani and colleagues (2011) have reported that the transcription of lncRNA Mistral precedes its target gene Hoxa6 and Hoxa7 expression following ATRA treatment. These evidences support induction of Hox genes via the effect of ATRA on lncRNA expression. My results showed that ATRA induces the expression of mHotairm1, Hoxa1 and Hoxa2 in NIH 3T3 cells. Based on the findings of Bertani et al (2011) and Zhang et al (2009), it is highly probable that ATRA induces the expression of mHotairm1 further leads to an increase in the expression of Hoxa1 and Hoxa2. Thus my findings indicate that Hoxa1 and Hoxa2 have the same expression trend as that of mHotairm1 and that mHotairm1 can positively regulate the expression of Hoxa1 and Hoxa2.
6.3.4 *mHotairm1* activates *Hoxa1* and *Hoxa2* expression through histone methylation

LncRNAs are involved in numerous cellular processes, including ES cell pluripotency, cellular pathway regulation and cell-cycle regulation (Rinn and Chang, 2012). They are also important during development (Schmitz *et al*., 2016) and in certain diseases, such as cardiovascular disease (Archer *et al*., 2015), nervous system diseases (Briggs *et al*., 2015) and cancer (Schmitt and Chang, 2016). Despite all the different cellular processes and diseases that involve lncRNAs, one fundamental rule has emerged: lncRNAs drive the formation of ribonucleic-protein complexes to regulate gene expression (Rinn and Chang, 2012). One large group of protein partners that bind to lncRNAs are the chromatin and DNA modification complexes. These complexes include DNA methylation complex, PRC1 and PRC2, TrxG complex and HDACs (reviewed by Rinn and Chang, 2012), which closely link lncRNA with other epigenetic marks. The lncRNAs transcribed from *Hox* loci have thus far been reported to bind to two histone methylation complexes: PRC2 which tri-methylates H3K27 and suppresses gene expression (Rinn *et al*. 2007), and MLL1/WDR5 complex, which belongs to TrxG and tri-methylates H3K4 to activate gene expression (Wang *et al*., 2011; Bertani *et al*., 2011). Since my results show that *mHotairm1* plays a role in activating the expression of *Hoxa1* and *Hoxa2*, I speculated that *mHotairm1* may achieve its gene activation role by recruiting MLL1/WDR5 complex to the chromatin of *Hoxa1* and *Hoxa2*.

Capture hybridization analysis of RNA targets (CHART) developed by Simon *et al* (2011) is method used to enrich endogenous RNAs along with their associated proteins and their DNA targets. Similar to ChIP assays, samples are cross-linked and chromatin is sheared into small fragments. Then instead of using antibodies targeting chromatin proteins to enrich target fragments as in ChIP, affinity-tagged oligonucleotides are used to enrich target lncRNA together.
with its protein partners and chromatin targets. In this way, CHART can achieve two goals at the same time: to study the genomic binding sites of a lncRNA and to study which protein complex is associated with this lncRNA. The advantages of CHART over ChIP in the study of lncRNA function are: (1) CHART can allow for the study of protein, DNA and RNA from the same coprecipitated sample; (2) CHART is able to identify the genomic target of lncRNA. The key step in CHART experiment is to design a lncRNA specific oligo probe to specifically bind to and enrich the target RNA. Since lncRNAs have secondary structures and are usually bound to proteins, the regions on the lncRNA that can be recognized by a complimentary oligo probe are limited. Additionally, the oligo probe still should be long enough to specifically recognize target lncRNA. Thus, I designed three 25-mer mHotairm1 specific probes targeting 3’ (Probe 1), middle (Probe 2) and 5’ (Probe 3) part of mhotairm1 (Fig. 4.4). The samples enriched with three mHotairm1 specific probes were compared with samples pulled down with a control probe (Probe C) to rule out any background interference and nonspecific binding. Probe 2 showed the strongest binding to mHotairm1. The DNA samples retrieved from Probe 2 pull down showed that Hoxa1 and Hoxa2 as well as additional 3’ HoxA genes are all targets of mHotairm1 while the 5’ HoxA gene Hoxa13 did not specifically interact with mHotairm1. This result is supported by Wang et al’s (2011) finding that in HoxA cluster, 3’ lncRNAs primarily interact with 3’ HoxA genes while 5’ lncRNAs primarily interact with 5’ HoxA genes. The protein samples retrieved from the same pull down sample showed that the TrxG protein WDR5 is associated with mHotairm1, suggesting MLL1/WDR5 complex may play a role in the regulation of Hoxa1 and Hoxa2 gene expression by mHotairm1. Since histone acetylation is also a gene activation marker, I examined the presence of histone acetyltransferase CBP and found no interaction between mHotairm1 and CBP. This, however, does not rule out the possibility that histone acetylation is
involved in mHotairm1 induced expression of Hoxa1 and Hoxa2, since there are other histone acetyltransferases that may be able to interact with WDR5 (Zhao et al., 2013; Dias et al., 2014). As CHART probes were DNA probes designed complementary to the sequence of mHotairm1, they may also have the abilities to bind to the genomic DNA, especially in region where mHotairm1 is transcribed. To reduce the background due to the direct binding of genomic DNA to CHART probes, DNA samples can be eluted with RNase-H instead of biotin. In this way, only the DNA bound through RNA should be eluted from the resin, but not the DNA that is directly bound to the oligo probe (Simon et al., 2011).

WDR5 is a subunit of MLL1/WDR5 complex and is also involved in other functional complexes (Zhao et al., 2013; Dias et al., 2014). Thus, to confirm the involvement of histone methylation in the regulation of Hoxa1 and Hoxa2 expression by mHotairm1, I wanted to determine whether there is an interaction between mHotairm1 and MLL1. Unfortunately, I was not able to find a good antibody that could recognize MLL1 in western blots, partially because MLL1 is a very large and complex protein. Thus, I was not able to determine the interaction between MLL1 and mHotairm1 in CHART experiment. However, I designed a pull down experiment that would confirm the interaction between mHotairm1 and MLL1/WDR5 complex. A GST tagged WDR5 (full length) and MLL1 (amino acid 3810-3963) expression vectors were constructed and a prokaryotic system (E.coli) was used to produce WDR5 and MLL1 proteins. As the mammalian MLL1 cDNA is ~12 kb in length and MLL1 protein contains ~4000 amino acids (Zhang et al., 2013), it is almost impossible to produce a full length MLL1 protein in E.coli. Bertani and colleagues reported that amino acids 3810 to 3963 is the critical region of MLL1 that binds lncRNA Mistral (Bertani et al. 2011), thus I selected this region of MLL1 in the pull down experiment. GST tag was used for the purification of proteins produced in E.coli and purified
WDR5 and MLL1 (3810-3963) proteins were applied to NIH3T3 whole cell lysate. Both WDR5 and MLL1(3810-3963) could pull down mHotairm1, which indicates that mHotairm1 is closely associated with histone methytransferase complex MLL1/WDR5. However, it is not clear which of the two proteins bind directly to mHotairm1, since the fragment of MLL1 used in pull down experiments contains the Win motif, which is the region on MLL1 that also binds to WDR5 (Zhang et al., 2013). Hence possibility exists that the MLL1 pulled down mHotairm1 is due to its interaction with WDR5.

There is also some debate in the literature as to which specific protein in the TrxG complex is key for the interaction with lncRNA. Wang and colleagues reported that lncRNA HOTTIP can only bind to WDR5 but not MLL1 (Wang et al., 2011). This is supported by Yang and colleagues who have identified several critical amino acids of WDR5 that are key in the binding of lncRNA HOTTIP (Yang et al., 2014). However, Bertani et al. (2011) demonstrated that Mistral binds directly to MLL1 only and not to WDR5. Nevertheless, my findings confirmed that mHotairm1 interacts with MLL1/WDR5 complex to target Hoxa1 and Hoxa2 genes.

To further support the above findings that mHotairm1 can recruit MLL1/WDR5 complex to Hoxa1 and Hoxa2 genes and regulate their expression, I downregulated the expression of mHotairm1 in NIH 3T3 cells and investigated its impact on H3K4me3 and H3K27me3’s occupancy on Hoxa1 and Hoxa2 chromatin using ChIP. The knockdown of mHotairm1 resulted in reduced occupancy of gene activation mark H3K4me3 and increased occupancy of gene suppression mark H3K27me3, which correlates with my previous finding that downregulation of mHotairm1 leads to decreased Hoxa1 and Hoxa2 expression.

These findings together suggested that mHotairm1 can regulate Hoxa1 and Hoxa2 expression through the recruitment of histone methytransferase complex MLL1/WDR5. Hence,
the regulatory mechanism of mHotairm1 appears to be very similar to Mistral (Bertani et al. 2011) and HOTTIP (Wang et al., 2011), other two lncRNAs identified within the HoxA cluster.

6.3.5 Modification of WDR5

Another significant aspect of my work is the finding that WDR5 is sumoylated and this modification is important for its function. Several pieces of information led me to suggest that WDR5 is being sumoylated. Firstly, the molecular mass difference between the modified and unmodified WDR5 is ~13KDa, which is close to the molecular mass of sumoylation (~11KDa) (Hay, 2005; Kumar and Zhang, 2015). Secondly, the 50KDa protein identified in my experiments is protected by NEM, a ubiquitin/SUMO protector (Fig. 5.26A). To confirm that WDR5 is sumoylated, I first used immunoprecipitation (IP) to test whether proteins immunoprecipitated by WDR5 antibody can be recognized by SUMO1 antibody, and proteins IP by SUMO1 antibody can be recognized by WDR5 antibody. In WDR5 antibody precipitated sample, a 50KDa band was detected by SUMO1 antibody, which is at the same molecular weight as that of the modified WDR5 in NIH 3T3 cells. To avoid the influence by IgG heavy chain, which is also at 50KDa, I used a rabbit WDR5 antibody (IgG) for IP and mouse SUMO1 antibody (IgG) for western blot. The sample precipitated with SUMO1 antibody can also be detected by WDR5 antibody at 50KDa. Again to avoid the influence of IgG heavy chain, a mouse SUMO1 antibody (IgG) was used for IP and a mouse WDR5 antibody (IgM) was used in western blot. The IP experiment supported the hypothesis that WDR5 is being sumoylated. This finding is supported by Nayak et al.’s work demonstrating that WDR5 is sumoylated in an in vitro transcription/translation system (Nayak et al., 2014). I further tried to use mass spectrometry to confirm my finding and to determine which amino acid is sumoylated to better
study the function of sumoylated WDR5. I enriched the modified WDR5 using immunoprecipitation and ran the protein sample on a SDS-PAGE gel that was subsequently stained with coomassie blue. Unfortunately, the IgG heavy chain from the WDR5 antibody also showed up at 50KDa in the coomassie blue stained gel, thus I was not able to distinguish the modified WDR5 from IgG.

Even though I could not conclusively determine which amino acid in WDR5 is sumoylated, I was able to show the importance of this modification from my studies. From the CHART experiment, only modified WDR5 (~50KDa) was observed in samples pulled down with mHotiarm1 specific probes, even when two WDR5 protein (modified and unmodified) bands were detected in NIH 3T3 cell lysates. In addition, when I used GST-fused MLL1 (amino acid 3810-3963) to pull down protein samples from NIH 3T3 cell lysates, again only the modified WDR5 band was observed in western blot assays. Immunocytochemistry showed that WDR5 proteins were present in both cytoplasm and nucleus of NIH 3T3 cells. I then analyzed the distribution of modified and unmodified WDR5 in NIH 3T3 cells. Results indicated that there is only modified WDR5 in the nucleus while both forms exist in the cytoplasm. This result further supported the importance of the modification of WDR5 and indicated that this modification may facilitate WDR5 translocation to the nucleus. Sumoylation have multiple functions for proteins and translocation of proteins to nucleus is one of its key functions (Kumar and Zhang, 2015), which further supports my findings. During the separation of cytoplasm and nucleus, only weak unmodified protein band of WDR5 could be observed in cytoplasm (Fig. 5.28, Fig. 5.29), which is possibly due to the dilution of sample during the separation. Because for the separation method I used, protein samples in the cytoplasm are much more diluted compared to nucleus samples and
may be the reason why I observed a lower concentration of the unmodified WDR5 in the cytoplasm.

Treatment with ATRA (all-trans retinoic acid) also impacted sumoylated WDR5, appearing to increase the quantity of sumoylated WDR5 in the cell nucleus but not influence the total quantity of WDR5 protein in NIH 3T3 cells. These results indicate that ATRA is able to influence the distribution of modified WDR5 in NIH 3T3 cells and most likely facilitates its translocation to the nucleus. Together, all these findings suggested that sumoylated WDR5 with its interaction with \textit{mHotairm1} plays an important role in impacting changes in H3K4me3 and H3K27me3 occupancy in epigenetic regulation of \textit{Hoxa1} and \textit{Hoxa2} genes.

In conclusion, my research has found that \textit{mHotairm1} is a new noncoding transcript in mouse that regulates the expression of \textit{Hoxa1} and \textit{Hoxa2} through the recruitment of MLL1/WDR5 to their chromatin. In NIH 3T3 cells, only sumoylated WDR5 is present in the nuclei and interacts with MLL1 and \textit{mHotairm1}. Following treatment with ATRA, there is an increased sumoylated WDR5 in the nucleus, indicating this modified WDR5 plays a key role in ATRA induced expression of \textit{Hoxa1} and \textit{Hoxa2} (Fig. 6.2)
Figure 6.2. Schematic model of mHotairm1 regulation of Hoxal and Hoxa2 expression via MLL1/sumoylated WDR5 mediated histone-modification. Sumoylated WDR5 translocates to cell nuclei and binds to the MLL1 histone methyltransferase complex and lncRNA Hotairm1 to induce H3K4me3 occupancy on both Hoxal and Hoxa2 gene promoters and enhance their expression.

6.4 Future directions

In my experiments, a direct regulation of Hoxa2 gene expression by the microRNA miR-669b was demonstrated in NIH 3T3 cells. I have also demonstrated expression of miR-669b in the developing mouse palate using real-time PCR which has a complementary expression profile to Hoxa2 in the developing palate. However, it is not known whether miR-669b has direct role in regulating Hoxa2 expression in the developing palate. To study the function of miR-669b in mouse palate development, first an overexpression of miR-669b in mouse embryonic palate mesenchyme (MEPM) cell cultures generated from developing mouse palate would be needed. It
would be necessary to show whether \textit{Hoxa2} expression, at both transcriptional and translational level, is downregulated in the MEPM cell cultures after overexpression of miR-669b.

To gain an overview of the spatial distribution profile of miR-669b and \textit{Hoxa2} in all four stages of developing mouse palate, an \textit{in situ} hybridization histochemistry system would need to be undertaken. Locked nucleic acids miRNA probes would be used to achieve higher specificity for the detection of miRNA (Nielsen, 2012; McEwen \textit{et al}., 2016). Mouse palatal organ culture has previously been developed in our lab (Smith \textit{et al}., 2009) and I would use these to determine the role of miR-669b in mouse palatal development by overexpressing miR-669b in mouse palate organ cultures. It is already known that loss of \textit{Hoxa2} function in mice decreases palatal fusion rates and increases in cell proliferation rates (Smith \textit{et al}., 2009). Whether overexpression of miR-669b influencing palatal growth and fusion would also need to be tested.

Further investigation into the function of \textit{mHotairm1} is also required. To study the function of \textit{mHotairm1} in mouse palate development, an improved \textit{in situ} hybridization histochemistry system with higher sensitivity is required so that the distribution of \textit{mHotairm1} in all four stages of developing mouse palate can be examined. It has been suggested that the sensitivity of traditional RNA \textit{in situ} hybridization is too low to visualize lncRNAs in many cases (Raj \textit{et al}., 2008). Recent years, approaches have been made to achieve single molecule RNA \textit{in situ} hybridization, methods including the usage of multiple probes against one target RNA (Raj \textit{et al}., 2008; Bayer \textit{et al}., 2015), usage of chemically modified nucleic acids in probes to strengthen binding specificity (Li \textit{et al}., 2013) and the amplification of fluorescent signals (Larsson \textit{et al}., 2010; Shah \textit{et al}., 2016). These technologies may help better visualize \textit{mHotairm1} expression in developing palate.
Downregulation of \textit{mHotairm1} would be achieved in mouse palatal fusion cultures using siRNAs and the impact of \textit{mHotairm1} on palate fusion and proliferation rates can then be examined. Also \textit{mHotairm1} knockout mice can be generated and the effect of \textit{mHotairm1} on mouse embryo development can be studied in detail. The consequence of loss-of-function of \textit{mHotairm1} on \textit{Hoxa1} and \textit{Hoxa2} gene expression as well as their downstream targets can be examined. The function of \textit{mHotairm1} can also be studied at the cellular level. LncRNAs transcribed from the Hox loci have been reported to be able to regulate cell cycle, cell differentiation and apoptosis (Liu \textit{et al.}, 2016; Lian \textit{et al.}, 2016; Bertani \textit{et al.}, 2011; Zhang \textit{et al.}, 2009). Downregulation of \textit{mHotairm1} can be achieved in different cell lines using siRNAs and the influence on cellular activities can be examined.

In my study, the sumoylation of WDR5 was demonstrated by immunoprecipitation but the amino acid where sumoylation may occur has not been identified. Mass spectrometry would be used to determine the amino acid residue that is sumoylated and the key step in this method would be to enrich modified WDR5 protein samples. I previously used immunoprecipitation to accumulate modified WDR5 but unfortunately it was not of sufficient purity with contamination from the IgG heavy chain which also showed up at 50KDa position. In a future experimental design, GST fused MLL1 (amino acid 3810-3963) could be used to accumulate the modified WDR5 protein sample for mass spectrometry as my finding showed there is an interaction between MLL1 and modified WDR5. After identifying the site of sumoylation on WDR5, mutation can be introduced to the amino acid and the role of sumoylation can be examined more thoroughly. The mutated WDR5 can be tagged with GFP and transfected into NIH 3T3 cell lines or MEPM cells to examine the consequence of loss of sumoylation on WDR5, which may affect
the cellular distribution of WDR5, its interaction with lncRNA and MLL1, and the regulation of gene expression.
CHAPTER 7

7. References


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Appendix 1. DNA sequence chromatogram of *Hoxa2* promoter CpG island 1 from E12 mouse palate genomic DNA
Appendix 2. DNA sequence chromatogram of *Hoxa2* promoter CpG island 1 from E13 mouse palate genomic DNA
Appendix 3. DNA sequence chromatogram of Hoxa2 promoter CpG island 1 from E14 mouse palate genomic DNA
Appendix 4. DNA sequence chromatogram of Hoxa2 promoter CpG island 1 from E15 mouse palate genomic DNA
Appendix 5. DNA sequence chromatogram of Hoxa2 promoter CpG island 1 from NIH 3T3 cell line genomic DNA
Appendix 6. DNA sequence chromatogram of *Hoxa2* promoter CpG island 1 from EG7 cell line genomic DNA
Mush 147-F: TGACTTGGAGCACTGGGA
Mush147-R: CTCTTGCCAGTTTCTGCTTTCT

Appendix 7. DNA sequence chromatogram of cDNA fragments amplified with mHotairm1 primer Mush 147 F+R
Mush138-F: CCCACCCAGCCCAGAAAAG
Mush138-R: GTTTCAAACATCTACGTTC

Mush 138 (131-268 bp)
CCCCACCCAGCCCAGAAGCTGAACTGGCAAGAGGTCTGTCTTTTCCTGAACCCAT
CCACAGCTGGGAGATTAATCAACCACACTGAAAATGGGGGTGTGGGGGAGGGAA
AGGAAGAGTTTGGAACGTAGATGTGGTTGAAAC

Appendix 8. DNA sequence chromatogram of cDNA fragments amplified with mHotairm1 primer Mush 138 F+R
Mush245-F: GAAAGGAAGAGTTGGAACGTAGA
Mush245-R: TGAGACTCAGGCCATAGGTTA

Mush 245 (236-478 bp)
GAAAGGAAGAGTTGGAACGTAGATGTTGAAACAAATGTGTATAAATAAATGAATT
TTTGATAACTCCGTTATTGACCTAGAAACTAGCAGCTTGGTAAGGGAACTCCATTCC
ACTCCACTCGTCCTAGAACTGGAAGTTTTTGTAGGCACTTTTCCTCTCCACACTCAAA
AGCTTGGGCTAGGGCCAACCTCGAGTGGCCTGCAAGCCCATTTCATTACTAATGTAACT
CTATGGCCTGAGTCT
Appendix 9. DNA sequence chromatogram of cDNA fragments amplified with mHotair m1 primer Mush 245 F+R
Appendix 10. DNA sequence chromatogram of MLL1 (amino acid 3810-3963) cloning.
WDR5 (1-640 nt)
WDR5 (495-1005 nt)
Appendix 11. DNA sequence chromatogram of full length WDR5 cloning.
Appendix 12. *mHotairm1* can only be amplified from cDNA samples using much138 F+R primers. cDNA samples were reverse transcribed from total RNA samples collected from E13 mouse palate. Genomic DNA (gDNA) samples were also collected from E13 mouse palate. *mHotairm1* specific primer much138 F+R were used in PCR. A 138bp DNA fragment can only be amplified from cDNA samples but not from genomic DNA.
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