RETINOIC ACID AND EPIGENETIC REGULATION DURING

THE DEVELOPMENT OF THE ZEBRAFISH INTESTINE

AND IN CANCER

by

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ABSTRACT

The tumor suppressor APC is mutated in 85% of colon cancer cases. APC is necessary for normal colon homeostasis, and loss of APC function is the initiating event in colon cancer. APC is a crucial member of the β-catenin destruction complex, and it was long thought APC mutation causes disruption of this complex, removing the cell's ability to destroy excess β-catenin and activating β-catenin/Tcf/Lef-induced transcription. Recent studies, however, show that nuclear localization of β-catenin occurs only after the activation of k-ras, not during the initiation of tumorigenesis. Thus, APC must play additional roles that are disrupted when mutated to cause changes from normal colon homeostasis.

In this dissertation, we utilize the apc^{mcr} mutant zebrafish as a model to parallel the loss of APC in colon cancer to show that multiple epigenetic aberrations occur before the advent of nuclear β-catenin, showing that dysregulation of epigenetic marks correlates with the initiation of colon cancer progression. The data presented in this dissertation identify two epigenetic factors regulated by APC. We show that the histone demethylase LSD1 aberrantly represses retinoid biosynthesis, preventing terminal differentiation of the intestine. This highlights one of the epigenetic regulators that is misregulated when APC is lost and adds an epigenetic mechanism for the repression of rdh1l.

We also show that in the apc^{mcr} mutant zebrafish, retinoic acid loss causes decreased H3K27 methylation levels, adding another layer of epigenetic regulation

controlled through RA. In this case, we uncover an epistatic mechanism wherein Cox-2 regulates H3K27 methylation, independent of its role in the regulation of β-catenin, when APC is mutated.

We also used the developing zebrafish as a model to characterize the loss of the Ezh2, the histone methyltransferase responsible for H3K27 methylation. We show that loss of Ezh2 leads to several development phenotypes, including the loss of terminal differentiation of multiple cell types in the gut.

Together, the data presented in this dissertation trace the epigenetic pathways used to both regulate retinoic acid production and used by retinoic acid to promote cellular differentiation and establish APC as a regulator of histone methylation during differentiation.

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INTRODUCTION

1.1 Regulation of Intestinal Differentiation

Four types of terminally differentiated cells exist within the intestine: absorptive enterocytes (also called columnar cells), Paneth cells, goblet cells and enteroendocrine cells (*1*). (Differentiated zebrafish intestines lack Paneth cells but retain the other three cell types (*2*).) Absorptive enterocytes are the most abundant cell type in the intestine. Enterocytes functions to absorb dietary nutrients. Goblet cells are responsible for the secretion of mucus into the intestinal lumen. Enteroendocrine cells account for less than 1% of differentiated intestinal cells and, like goblet and Paneth cells, are secretory, but enteroendocrine cells secrete intestinal hormones. Paneth cells reside at the base of the crypt and secrete lysozymes and other microbicidal agents (*3*).

As the adult intestine turns over every 3 to 5 days, these cells are being constantly replaced (*4*). Stem cells reside at the bottom of a crypt and have the capability to give rise to all differentiated cell types. To retain integrity, stem cells divide and produce one new daughter stem cell and one transit-amplifying cell, a rapidly dividing intermediate. As the transit-amplifying cell travels up the crypt, it will divide in order to provide cells for repopulation. Some estimates have the transit-amplifying cells undergoing six rounds of cell division, producing up to 300 cells per crypt each day (*5*). Upon leaving the rapidly dividing zone, these cells will differentiate into one of the differentiated cell types

(*1, 4*). (Transit-amplifying cells destined to become Paneth cells will travel downward to the bottom of the crypt.) This process takes approximately 3–5 days.

The loss of retinoic acid signaling disrupts this process, causing loss of terminal differentiation in the zebrafish intestine (*6*). Although addition of exogenous retinoic acid is sufficient to restore intestinal differentiation, it is not currently known whether RA plays a role in the transition from transit-amplifying cells into differentiated cell types or whether RA functions to create and prime the transit-amplifying cell upon asymmetric stem cell division.

In addition to RA, several other pathways are involved in the repopulation of intestinal structures. Indian hedgehog (Ihh) is expressed in the intervillus region and may regulate stem cell renewal and differentiation (*7–10*). Ihh also regulates BMP production, which plays a role in the restriction of crypt numbers (*7, 12, 13*). Notch signaling has also been implicated in controlling differentiation of intestinal cells and specifically in the absorptive cell differentiation (*14–16*). Notch signaling may also be necessary for the maintenance of putative intestinal stem cells (*17*). Activation of canonical Wnt signaling has been shown to be necessary for the presence of secretory cells, and overexpression of the Wnt inhibitor Dkk-1 appears in some conditions to lead to reduced cell proliferation (*18, 19*). The transcription factor Tcf4 is thought to be the main effector of the canonical Wnt signaling pathway in the gut, and Tcf4 knockout mice die shortly after birth and lose the transit-amplifying cells of the intestine (*18*).

1.2. Colon Cancer and the Adenomatous Polyposis Coli Protein

Colorectal cancer is the third most diagnosed cancer in the U.S. annually, with an estimated 137,000 cases to be diagnosed in 2014. It kills about 50,000 men and women

annually, making it the third leading cause of cancer death (*20*). While several inherited

syndromes can give rise to colorectal cancer (most notably FAP (*21*), Lynch Syndrome

(*22*), and MUTYH-associated polyposis (*23, 24*)), these only account for 2–5% of all

colorectal cancer cases (*25*). The vast majority of colorectal cancer cases are sporadic

with no family history. Approximately 85% of colon cancers harbor a mutation in the

tumor suppressor gene adenomatous polyposis coli, or APC (*26, 27*). This gene is

mutated in the autosomal dominant FAP syndrome (*28, 29*). Carriers inherit one mutated

copy of APC, and cells within the intestine undergo either loss of heterozygosity or a

second mutation, giving rise to adenomatous polyps containing two mutated copies of

APC. These patients will develop hundreds to thousands of these polyps in the colon and

most will have prophylactic colectomies by age 40 in order to prevent colon cancer (*30*).

APC is a 312 kDa protein that contains multiple domains for interaction (*31*)

(Figure 1.1). It acts within numerous cellular processes and is most well known for its

role in the canonical Wnt signaling pathway, which is discussed below. It also

participates in cell adhesion, cell migration, and chromosomal segregation. APC contains

the Armadillo repeats, domains for binding Axin1 (SAMP repeats), the basic region, a

poorly defined region responsible for interaction with microtubules, and the EB1-binding

and DLG-binding domains (*32–36*). The c-terminal region also binds to the C-terminal

binding protein, a transcriptional corepressor (*37*). APC contains multiple 15- and 20-

amino acid repeats, responsible for binding β-catenin (*28, 36, 38*). The majority of APC

mutations occur within a 227-amino acid region known as the mutation cluster region

(MCR) (*39, 40*), and 95% of APC mutations lead to C-terminal truncations (*41*). This

eliminates multiple interaction domains, including the regions that bind to Axin and CtBP

(*42*). A C-terminal mutation in the MCR does, however, leave intact all of the 15-amino acid repeats, capable of binding β-catenin.

APC interacts with several other proteins to form the β-catenin destruction complex (Figure 1.2). The other core members of this complex include Axin1, the serine-threonine kinase GSK3β, and Casein Kinase 1 (CK1) (*43, 44*). In the absence of Wnt ligand, GSK3β and CK1 phosphorylate one threonine and three serine residues in the amino-terminal portion of β-catenin. This allows for recognition of β-catenin by an E3 ubiquitin-ligase, which leads to ubiquitination of β-catenin and subsequent degradation by the proteasome. The Wnt signaling pathway is activated when a Wnt ligand binds to a frizzled receptor and an LRP coreceptor on the cell surface. This activates a downstream signaling cascades that results in disintegration of the β-catenin destruction complex and accumulation of β-catenin (*45*). It is, however, important to note that β-catenin cannot enter the nucleus until additional phosphorylation events happen at serine 191 and serine 605, which are catalyzed by Rac1 (*46, 47*).

APC mutation is the initiating event in colon cancer (*48, 49*). It is this mutation that drives the conversion from normal epithelium to hyperplastic epithelium, which then propels the cell toward becoming adenomatous and eventually tumorigenic (Figure 1.3). It has long been held that a mutation in APC is synonymous with aberrant activation of canonical Wnt signaling since APC is an integral part of the β-catenin destruction complex (*50*). However, previous work has shown that additional events, including K-ras activation (*51*), are required for nuclear localization of β-catenin (see also *52, 53*). This is substantiated by studies showing lack of nuclear β-catenin in early adenomas (*54, 55*). Therefore, at the initiating stages of colon cancer, β-catenin may accumulate in the

cytoplasm but is not at this point nuclear, and as such the β-catenin/Tcf/Lef transcriptional activity cannot be activated. Thus, additional pathways must be in play that, upon APC mutation, are dysregulated and result in hyperplastic conditions.

One pathway that APC has been shown to regulate is the biosynthesis of the signaling molecule retinoic acid (*6, 56*). In the developing zebrafish intestine, APC mutation leads to transcriptional repression of retinol dehydrogenases, the enzymes which catalyze the oxidation of dietary retinol (Vitamin A) to retinaldehyde (or retinal) (*57*). This leads to loss of retinoic acid production in the intestine. The effect of this is loss of terminal differentiation, indicating that when APC is mutated, cells are unable to properly differentiate. This defect can be reversed upon addition of exogenous retinoic acid, indicating that retinoic acid, not β-catenin, regulates cell fating in the intestine (*6*).

The loss of retinoic acid production in the intestine causes a downstream signaling cascade that includes increases in the levels of the pro-inflammatory factor Cox-2 due to transcriptional misregulation of the transcription factor C/EBP-β (*58*). Cox-2 catalyzes the conversion of arachadonic acid into prostaglandins. One of these prostaglandins, PGE_2, antagonizes the β-catenin destruction complex (*59*), leading to increased cytoplasmic β-catenin (Figure 1.4). Loss of retinoic acid production also leads to aberrant transcription of the DNA demethylase components, which causes loss of terminal differentiation in the intestine (*60*).

1.3 Retinoid Acid Synthesis and Signaling

Retinoic acid has long been identified as a regulator of cell differentiation (*61*). It regulates or promotes the differentiation of multiple cell types, including ES cells (*62*), EC cells (*63*), regulatory T cells (*64*), mesenchymal stem cells (*65*), olfactory neurons

(*66*), and multiple types of cancer cells, including neuroblastomas (*67*) and leukemia

(*68*). That RA can differentiate cancer cells suggests that these cells are trapped in an

undifferentiated state, and it may be this aspect of their character that leads to

hyperproliferation.

Retinoic acid is derived from dietary vitamin A (retinol) (*69*). Vitamin A

deficiency on an organismal level manifests itself mainly in ocular and vision defects,

commonly night blindness, although total blindness may occur (*70, 71*). On a cellular

level, retinol is oxidized by retinol dehydrogenases to form retinal in the rate limiting step

of RA biosynthesis. Retinaldehyde dehydrogenases (also known as aldehyde

dehydrogenases or ALDHs) catalyze a second oxidation step to form retinoic acid (*69*).

(Retinoic acid can be further converted by oxidizing one or more of its carbon-carbon

double bonds, but these products are thought to be metabolically inactive and this method

used to control the amount of retinoic acid-regulated transcription (*72, 73*).)

Once retinoic acid is formed in the cell, it can be used to modulate gene

expression. A classical RA response gene would respond to retinoic acid in the following

manner (*74, 75*): RA will bind to either receptor retinoic acid receptor (binds all-trans

retinoic acid) (*76, 77*) and retinoid X receptor (binds 9-cis retinoic acid) (*78*). The

receptors may have previously heterodimerized and bound DNA in the absence of RA,

and under these conditions the heterodimer will recruit corepressors to repress RA-target

genes. The heterodimer binds DNA at retinoic acid receptor elements (RAREs). Upon

activation by retinoic acid binding, a conformational change occurs in the receptors, and

the heterodimer will either attract transcription machinery (RNA Pol II) or coactivators

(such as SWI/SNF and Nf1) to activate gene transcription (*79, 80*).

While there are genes that respond to RA in the way described, there are also genes that respond in a different fashion when RA is present. Retinoic acid can bind to other receptors, such as PPARs, promoting transcription from a unique gene set (*81, 82*). Additionally, RA may produce changes in gene expression during the differentiation process by mediating the binding of transcriptional repressors. The promoter and enhancer elements for the stem cell gene Oct4 contain RARE-motifs, which, in the presence of RA, are bound by orphan receptors that silence Oct4 (*83*). It has also been hypothesized that the RAR-RXR heterodimer can prevent transcriptional activators from binding (*84, 85*). These examples do not include the numerous secondary genes that are repressed by retinoic acid targets, many of which play a role in the regulation of differentiation and are misregulated downstream of retinoic acid loss when APC is mutated (*58, 60*). As retinoic acid is involved in the differentiation, a process involving the modification of chromatin, it is likely that RA governs additional chromatin modifiers to regulate this process.

<u>1.4 Chromatin and Chromatin Modifiers</u>

While heritable DNA sequences determine the genes present in every cell, the epigenetic makeup of each cell differs greatly dependent on cell type and developmental stage. It is these secondary marks on the DNA and histones that provide context about when gene expression is allowed (*86*). Every cell carries the same contingent of DNA, and yet that same DNA can be directed epigenetically to cause a cell to become a vast array of cell types, from differentiated neurons to intestinal stem cells. Since this process is so vital to every cell in the body and in every developmental change, it must be tightly regulated.

While we are still uncovering the "epigenetic code," it is becoming increasingly clear that there are secondary marks both to turn genes on and to turn genes off. DNA itself may undergo methylation of specific cytosine residues that serve as binding sites for repressors of gene expression (*87*), but much of the epigenetic information is encoded within posttranslation modifications of histone tails (*88, 89*). The posttranslation modification (*90*) of residues on histone tails serve as binding sites to indicate to coactivators or corepressors (depending on the modification and site) that a gene should be activated or repressed. These modifications are known as the "histone code" and being able to "read" the code allows for the proper expression of many genes, particularly those involved in development, differentiation, and patterning (*91*).

Genes involved in development are often marked with a bivalent pattern of histone methylation in a phenomenon known as poising (*92, 93*). These genes are marked in undifferentiated cells by two histone methylation marks, H3K4 methylation (H3K4me) and H3K27 methylation (H3K27me). This is of interest because these two marks have opposing functions: H3K4 methylation marks active chromatin, where H3K27 methylation is indicative of silenced chromatin (*94–96*). Work done has shown that these two marks may be found in the same nucleosome, indicating that it is not simply a wide chromatin swatch sampled that contains both marks. This bivalency allows genes to be primed to facilitate more efficient activation or repression of needed pathways as the cell differentiates (*92*). Genes that need to be activated will retain H3K4 methylation, and genes that will be silenced in the differentiated cell type will undergo removal of the H3K4 methylation, leaving behind the repressive H3K27 methyl mark. In this manner undifferentiated cells of multiple types can use chromatin markings to

prepare themselves for differentiation without committing to a specific cell fate.

The proteins responsible for laying down these marks are histone methyltranferases (HMTs) (*97*). These enzymes contain a SET domain that transfers the methyl group from the cofactor s-adenosylmethionine (SAM) to the terminal amino end of a lysine residue (*98*). Lysine residues can be methylated with one, two, or three methyl groups, although not all HMTs will attach three methyl groups to a residue. Although most lysine residues can be methylated by multiple methyltransferases, most methyltransferases are specific to one residue (*98–100*). Once methylated, the histone will remain methylated until either the histone is removed from the DNA, or, more frequently, the methyl group is removed by a histone demethylase (*101*). There are two types of histone demethylases. The first family utilizes an FAD-dependent reaction and are amine oxidases, capable only of removing mono- or dimethylation from lysine residues (*102, 103*). The more common type of histone demethylases belong to the Jumonji class of proteins. These demethylases are iron dependent and use 2-oxoglutarate in the hydroxylation of the methyl group from lysine residues (*104, 105*). Both histone methyltransferases and histone demethylases are important regulators both of development, as is shown by defects and commonly embryonic lethality in knockouts (*106–110*) and in cancer, as is evident by many of these factors being mutated or misexpressed in multiple cancer types (*111–118*). Because of their role in both of these processes, it is likely that they have an effect on cell fating.

Histone methylation plays a vital role in regulating the changing chromatin during differentiation, a process that can often be misregulated in cancer. While there appears to be a global loss of methylation at lysine 20 of histone H4 (H4K20me3) (*119*), other

histone methyl marks tend to be more cancer type- and gene-specific.   As many histone

methyltransferases and demethylases are dysregulated in cancer, these methyl marks are

frequently aberrantly placed within cancer cells. A pattern often found in tumorigenesis

is the epigenetic silencing of tumor suppressors, with histone methylation acting as a

"second hit" to the wild type copy of a tumor suppressor (*120, 121*). However, in some

cancer types histone methyltransferases are transcriptionally downregulated (or absent),

which leads to a loss of transcriptional silencing at genes that would otherwise be

repressed (*122–124*). This can cause the aberrant expression of oncogenes. The

misregulation of chromatin modifiers during cancer – and thus the aberrant placement of

chromatin marks – can lead to an increased subset of dysfunctional pathway activation

than can be accounted for solely through genetic mutations. As such, it is necessary to

determine how the mismanagement of these factors affects the progression of

tumorigenesis.

<center>1.5 Polycomb Repressive Complexes and Development</center>

Polycomb group proteins are among the chromatin modifiers that control cell fate

decisions. These genes were first discovered in fruit flies as regulators of the hox genes

and led to similar anterior-posterior patterning phenotypes when mutated (*125*). While

hox gene regulation is necessary for the developing embryo, Polycomb group proteins

also play a larger role in regulating chromatin in order for proper cell fating to occur

(*126–130*). PcG proteins comprise two repressive complexes, the Polycomb repressive

complex 2 (PRC2), which deposits the repressive H3K27 trimethyl mark, and Polycomb

repressive complex 1 (PRC1), which is responsible for recruiting further chromatin

modifiers to either repress binding of activators or further shut down the chromatin (*131*)

(Figure 1.5). PRC2 contains a histone methyltransferase, Ezh2, which adds three methyl groups to lysine 27 of histone H3 (H3K27me3) (*132*). This methyl mark is recognized by a chromodomain-containing member of PRC1 (Polycomb in flies and chromobox in vertebrates), which binds to the methylated histone (*133, 134*). It is still not fully known how PRC1 represses target genes, although several potential mechanisms exist, including directly inhibiting the binding of RNA polymerase, other members PRC1 (the Ring proteins) ubiquitylating lysine 119 on histone H2A, or the recruitment of DNA methyltransferases (*135–138*). It has also been suggested that PRC1 may also serve to mechanically compact chromatin (*139*).

In keeping with their role in cell fating, full organism knockouts of Suz12, Eed, and Ezh2, the core members of PRC2, cause embryonic lethality in mice (*106, 140, 141*). The knockouts of the core components of PRC1 do not seem to share the same embryonic lethality, although they do cause physical malformations (*142–144*). It has been theorized that PRC2 knockouts are embryonic lethal but PRC1 knockouts are not because redundancy of PRC1 components in the mouse genome.

Many of the Polycomb group proteins have been implicated in cancer (*145–149*). The majority of these cases have an increase of Polycomb activity. An increase in activity leading to a hyperproliferative phenotype and a loss of activity giving rise to lethality indicates that an optimal window of Polycomb activity is necessary for homeostasis (*150–153*). However, it is not solely the levels of these factors that must be tightly regulated, but also the activity and the localization. Proper localization of Polycomb group proteins (and thus the H3K27me3 mark) will turn off cell fating genes not required for differentiated cell types during development and differentiation (*154,*

155). Should this mark not be found at genes that should be turned off, genes may

differentiate into the improper cell type, or they will fail to undergo differentiation at all.

This mark may also be improperly found at tumor suppressor genes such as cell cycle

regulators, which should be turned on in order to maintain proper cell functions upon

differentiation (*156*). The H3K27me3 mark is found at genes in multiple different

pathways that regulation differentiation, among them Wnt signaling, Hedgehog, Notch,

retinoic acid signaling, FGF signaling, and the TGF pathway (*157–160*). With this vast

array of pathways under its control, it is obvious that regulation of the H3K27me3 mark –

and thus both the expression and localization of the factors that place it – is of vital

importance to the proper development of an organ and an organism.

1.6 Preview

The work presented in this dissertation focuses on the use of epigenetics to

control differentiation with the intestine, on a general scale and within the context of

mutated APC. It also establishes further mechanisms for the regulation of retinoic acid

biosynthesis and how RA regulates downstream effectors of retinoic acid signaling.

Chapter 2 focuses on the histone demethylase LSD1 and the role that it plays in the

epigenetic regulation of the retinoic acid biosynthetic machinery. This adds an additional

misregulated player that contributes to tumorigenesis when APC is mutated in cancers.

Chapter 3 will introduce another epigenetic mark, H3K27 methylation, that is regulated

by APC and misregulated downstream of loss of retinoic acid production. We present an

epistatic mechanism through which retinoic acid regulates this mark, which presents an

additional paradigm for the regulation of chromatin modifiers by retinoic acid. Chapter 4

describes the loss of ezh2 in the developing zebrafish embryo. This is of interest because

loss of ezh2 has not been previously described in vertebrate development. This chapter

also highlights ezh2's role in the developing intestine.

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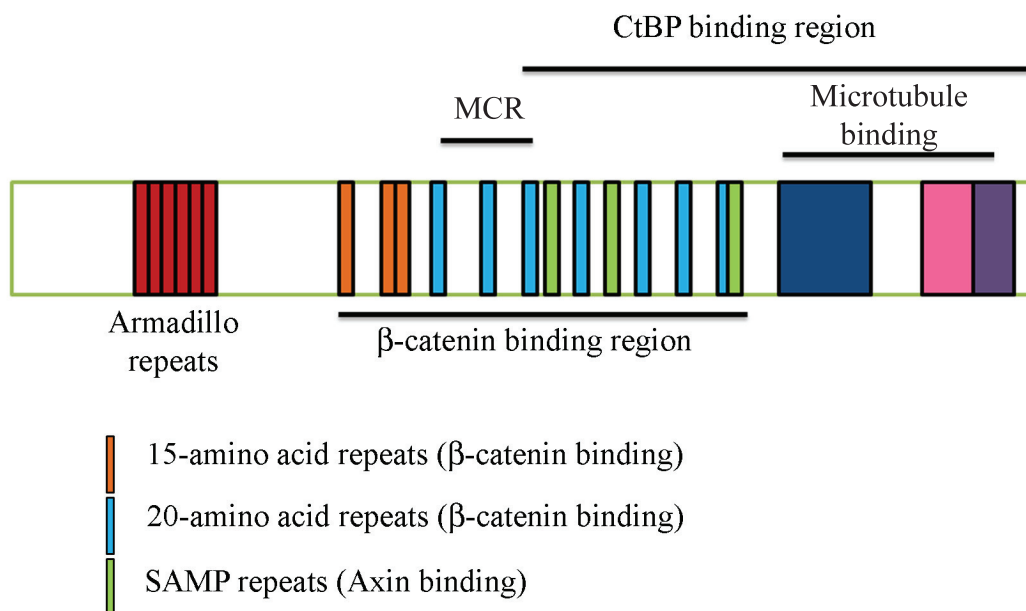
Figure 1.1 Insights from the structure of APC. APC is a 2483 residue protein containing domains for interaction with multiple factors, including Axin (SAMP repeats) and β-catenin (15- and 20-amino acid repeats). The majority of mutations in colorectal cancer are c-terminal truncations that occur in an area within exon 15 known as the mutation cluster region (MCR). These truncations eliminate the SAMP repeats and the CtBP-binding region of APC, but leave intact several β-catenin-binding domains.

Figure 1.2 The role of APC in canonical Wnt signaling. A, in the absence of Wnt ligand, the β-catenin destruction complex, consisting of APC, Axin, GSK3β, and CK1, phosphorylates β-catenin at serine and threonine residues at the N-terminal end of the protein, signaling for degradation of β-catenin by the proteasome. B, in the presence of Wnt ligand, Frizzled receptors and LRPs at the cell membrane are activated and disrupt the β-catenin destruction complex, allowing for accumulation of β-catenin in the cytoplasm.
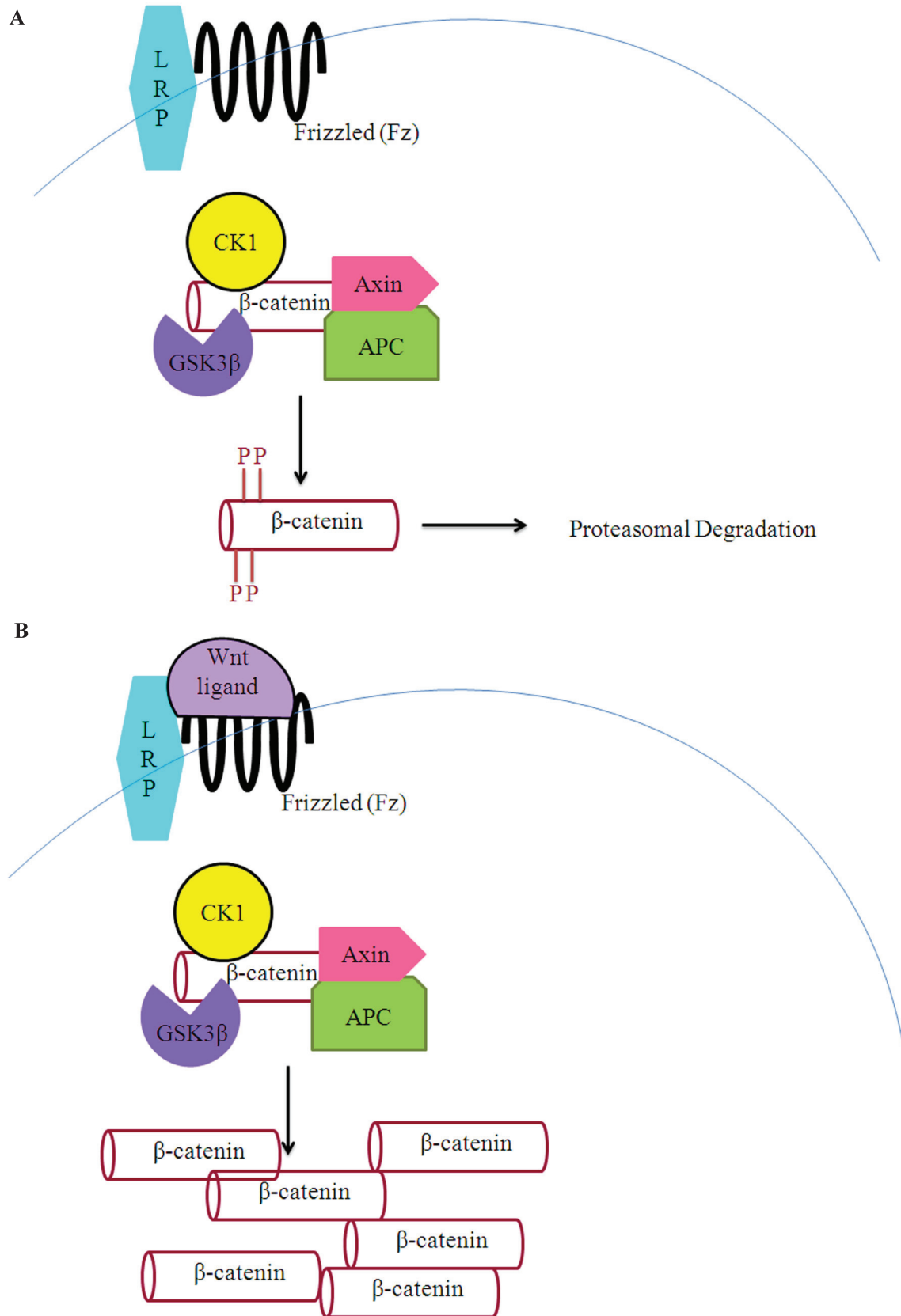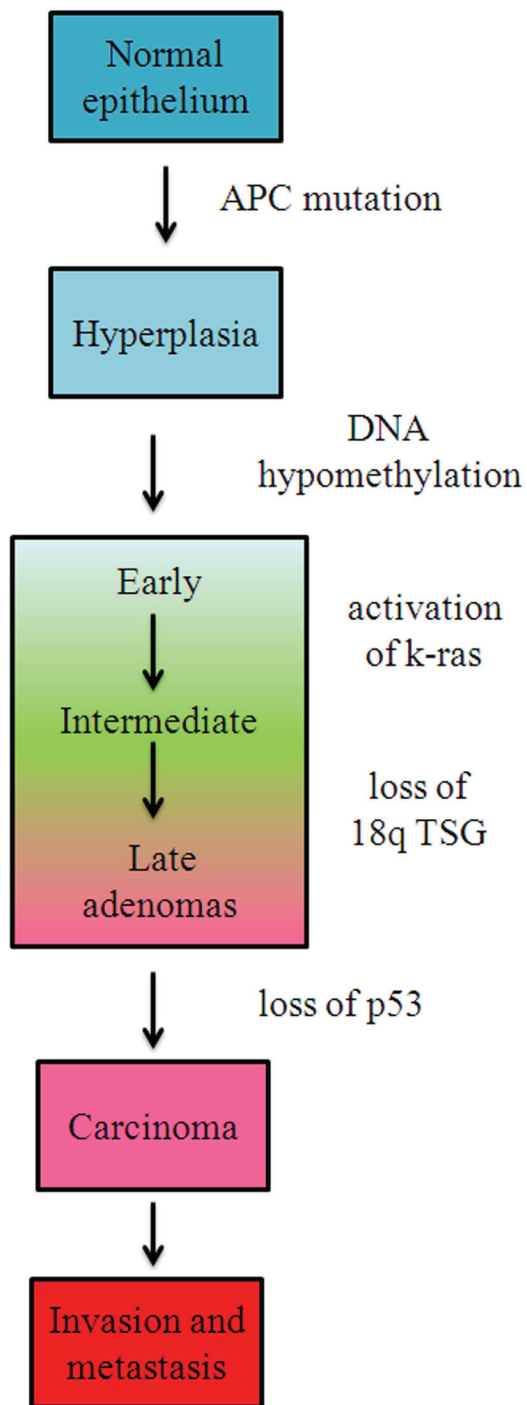
Figure 1.3 Colon cancer progression. Schematic displaying the progression of colon cancer, from normal epithelium to metastasis, with corresponding genetic, epigenetic, and cell signaling pathway aberrations.

$$APC^{mut}$$

Retinoic Acid

β–catenin

C/EBP-β
transcription factor

Tcf/Lef

HIGH Cox-2

Activation of
Wnt target genes

HIGH PGE$_2$
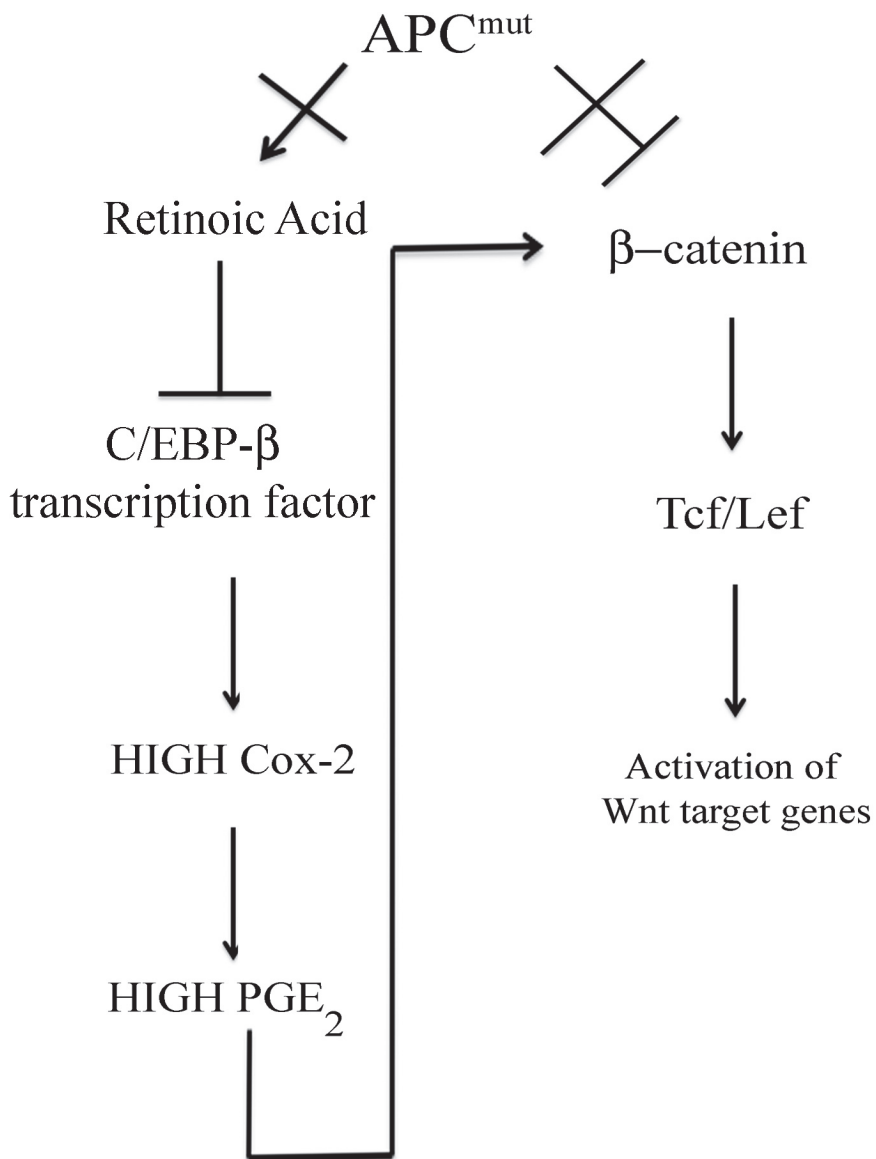
Figure 1.4 Mutation of APC gives rise to loss of retinoic acid production, causing downstream accumulation of β-catenin. APC acts as a member of the β-catenin destruction complex and as a regulator of retinoic acid production. When APC is mutated, loss of retinoic acid leads to increased levels of PGE2, an inflammatory molecule that interferes with the destruction complex and leads to increased cytoplasmic β-catenin.
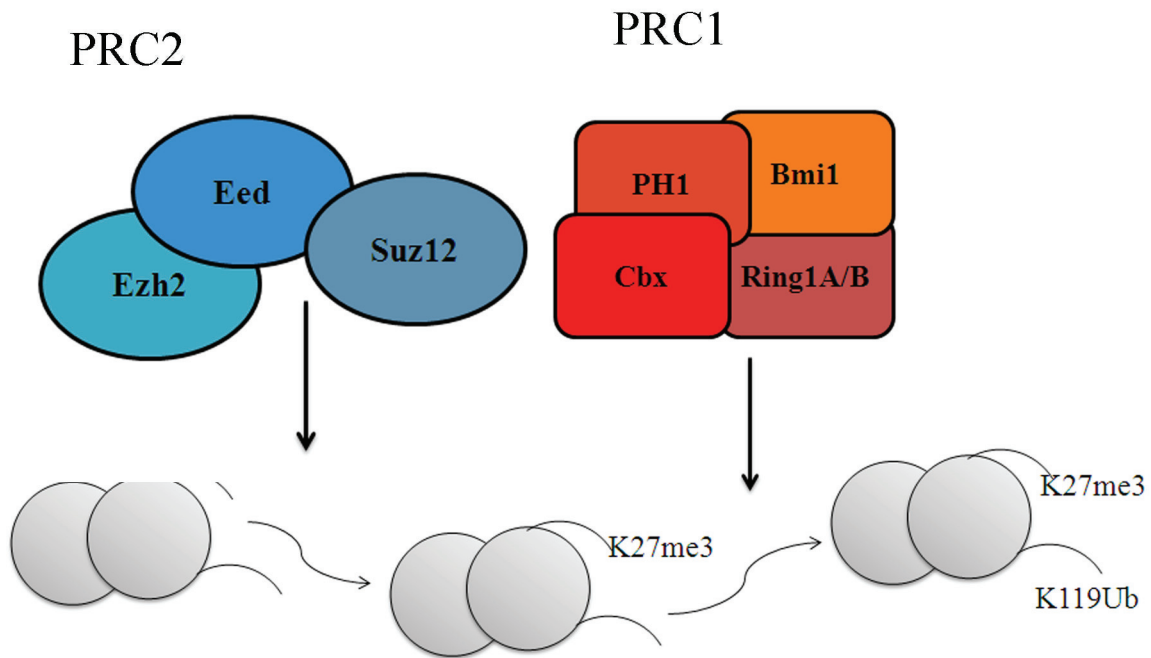
Figure 1.5 Polycomb repressive complexes act to repress transcription. The Polycomb Repressive Complex 2 (PRC2; shown in blue) is responsible for the deposition of the H3K27me3 mark by the histone methyltransferase Ezh2. This mark is recognized by Chromobox, a chromodomain-containing member of the Polycomb Repressive Complex 1 (PRC1; shown in red). PRC1 members Ring1A and Ring1B are ubiquitin-ligases that may ubiquitylate H2AK119 to further inhibit transcription.

CHAPTER 2

THE ROLE OF LSD1 IN THE REGULATION
OF RETINOIC ACID BIOSYNTHESIS

2.1 Abstract

Loss of APC in the intestine leads to loss of intestinal terminal differentiation, a state precipitated by lack of retinoic acid production. The retinoic acid biosynthetic machinery is aberrantly repressed, leading to loss of retinoic acid, which does not allow the cells to undergo proper cell fate transitions. LSD1 is a histone demethylase that can act to repress genes by removing the activating histone methyl mark H3K4me2. Genes lacking the H3K4me2 mark are transcriptionally repressed, a phenomenon often seen in cancer to silence tumor suppressors and genes required for differentiation. In this study, we show that LSD1 has abnormal expression patterns when APC is mutated and that it directly represses rdh1l, a member of the retinoic acid biosynthetic machinery. We see that inhibition of LSD1 activity can restore intestinal differentiation, identifying another key player in the regulation of differentiation in the intestine. We also demonstrate that LSD1 acts to repress rd1hl in a Lef1-dependent manner, indicating additional players in the repression of retinoid biosynthesis.

2.2 Introduction

The methylation of lysine residues on histones not only indicates active or inactive chromatin states (*1, 2*), but also signals to proteins involved in transcription that binding is accessible or that binding sites are blocked (*3*). This system is tightly regulated so as to allow for specific changes during development and differentiation (*4*). Because of this important role, transcriptional regulation by histone methylation is often hijacked within cancer cells to regulate gene programs to increase tumorigenesis (*5*).

Methylation at lysine 4 of histone H3 (H3K4me) is an activating methyl mark often associated with euchromatin (*6*). Di- and trimethylated H3K4 (H3K4me2 and H3K4me3, respectively) are found enriched at actively transcribed promoters, whereas monomethylated H3K4 (H3K4me1) more often marks enhancers of genes (*7*). These marks can be left by multiple methyltransferases, including multiple MLL proteins (members of the Trithorax group family) (*8, 9*) and the SET1 histone methyltransferase (*10*). H3K4 methylation has the ability to recruit transcriptional activators, such as the ATP-dependent nucleosome remodelers CHD1 and NURF (*11, 12*). The highest levels of H3K4 methylation are found at the transcriptional start sites of highly expressed genes, further linking H3K4 methylation and transcriptional activation (*13, 14*).

Until the discovery of LSD1, it was presumed that lysine methylation on histones was an irreversible mark. However, the discovery of LSD1 showed the dynamic nature of this mark and allowed for exploration of histone demethylation (*15*). LSD1 is a flavin-dependent amine oxidase that can demethylate mono- or dimethylated histone H3 lysine 4 or lysine 9, dependent on context (*16*). When acting as a repressor, LSD1 complexes with the transcriptional corepressors CoREST and HDAC1/2 (*17, 18*) and removes the

activating H3K4 dimethyl mark. LSD1 has been shown to be misregulated in multiple

cancer types (*19, 20*), suggesting a role for this chromatin modifier in tumorigenesis.

APC mutation is the initiating step in colon cancer (*21, 22*). Previous work has

established that this is due to its regulation of retinoic acid biosynthesis, and when RA is

not produced in the cell, the cell cannot properly differentiate (*23*). Retinol

dehydrogenases, the enzymes responsible for the catalysis of retinol to retinal, the first

step in retinoic acid biosynthesis, are transcriptionally repressed when APC is mutated

(*24*). The misregulation of these genes is a key step in the initiation of colon

tumorigenesis. APC regulates the expression of these genes by relieving their repression

by CtBP (*25*). APC interacts with CtBP through the c-terminal region frequently lost in

colon cancers, and thus, when mutated, it is unable to target CtBP1 for proteasomal

degradation (*26*). CtBP is a transcriptional corepressor that recruits additional repressors

in order to silence genes (*27, 28*). It is known to interact with several histone

deacetylases and histone methyltransferases to methylate lysine 9 of histone H3, a mark

of silenced chromatin (*29*). CtBP has also been linked to LSD1, although this

relationship is less clear (*28*).

The TCF/LEF proteins are a family of transcription factors that activate Wnt

signaling and are often bound to β-catenin to activate the transcription of Wnt target

genes (*30*). Certain TCF/LEF complexes have also been shown to bind to CtBP in order

to repress genes (*31–33*). One family member, LEF1, has been reported to interact with

multiple other partners. LEF1 acts independently of Wnt signaling to bind ETS family

members (*34*), cooperates with SMADs to coordinate BMP gene regulation (*35, 36*), and

acts in concert with CDX1 to autoregulate the Cdx1 promoter (*37*). Previous work done

in the lab has shown that lef1 levels are elevated in the apc mutant zebrafish and that lef1

binds to the *rdh1l* promoter (*38*).

While we have shown that the RA biosynthetic machinery is dysregulated through

a CtBP-dependent mechanism when APC is absent, we have not been able to show a

comprehensive mechanism for this phenomenon. This study highlights the role of one

player, LSD1, in the regulation of the RA biosynthetic machinery and explains a

mechanistic basis for the role of defective cell fating when APC is mutated.

2.3 Materials and Methods

2.3.1 Wild type and *apc* heterozygous *Danio rerio* (zebrafish)

Wild type (Tü strain) and *apc* heterozygous *Danio rerio* (zebrafish) were

maintained on a standard 14 hour/10 hour light/dark cycle. (*apc* heterozygous zebrafish

were a kind gift from Anna Pavlina-Haramis and Hans Clevers.) Fertilized embryos were

collected following natural spawning and allowed to develop at 28 ^{o}C. All embryos were

raised in 0.003% phenylthiourea to inhibit pigment formation starting at 24 hpf.

2.3.2 Morpholino injections

Antisense and control morpholino oligonucleotides were obtained from Gene

Tools, LLC. LSD1 morpholino (GGTCTGACTTCTTATTGGACAACAT) was

solubilized in 1x Danieau buffer and injected at the 1-cell stage using 1 nl of 0.25 mM

concentration. Control injection (CCTCTTACCTCAGTTACAATTTATA) was done

using control morpholino at similar final concentration.

2.3.3 Drug treatments

Embryos were placed in 3 mM LSD1 inhibitor pargyline (Sigma) or water control starting at 75% epiboly and drug was replaced every day.

2.3.4 RNA extraction and quantitative RT-PCR

RNA from whole embryos or adenoma tissue was isolated using Trizol (Invitrogen). cDNA was reverse-transcribed using Superscript III (Invitrogen). A template-free negative control was included in each experiment. RT-PCR was performed on a Roche lightcycler. Primers were *rdh1l*: 5'- GCTGCATCTGGATGTGACTG-3', 5'-ACTCGACCCTTGGCTTTCTT-3', zebrafish *lsd1*, 5'-CCCTTAAGCACTGGGATCAG-3', 5'- ACACGAGTAGCCATTCCTTACTG-3', 28s rRNA 5;-CCTCACGATCCT TCTGGCTT-3, 5'-AATTCTGCTTCACAATGATA-3', human *LSD1* 5'- ATGTTATCTGGGAAGAAGGC-3', 5'-GACCCAGGCACGACAGTA-3', 18s rRNA, 5'- CGATTGGATGGTTTAGTGAGG-3', 5'- AGTTCGACCGTCTTCTCAGC-3', *DHRS9*: 5'-TGGAAACTTGGCAGCCAGAA-3', 5'-CCAGAGACCTTTCTCCCCAA-3'.

2.3.5 Whole mount *in situ* hybridization

Zebrafish embryos were fixed in sucrose-buffered 4% paraformaldehyde, rinsed in PBST and dehydrated through an ethanol series, and stored in 100% methanol at -20 ^{o}C. Whole mount *in situ* hybridizations were carried out by standard protocol as previously described (*21*). cDNAs were cloned into the pCR-TOPO-II vector (Invitrogen) and riboprobes for the indicated genes were made with digoxigenin-labeled UTP (Roche).

2.3.6 Tissue samples and western blotting

Matched adenoma and uninvolved tissue samples were taken from FAP patients.

Samples were dounce-homogenized in IPH buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5

mM EDTA, 0.5% NP-40, Protease inhibitors, phosphatase inhibitors), further

homogenized in the FastPrep 120 (Thermo Scientific), boiled, and spun to pellet the

debris. For blotting, protein extracts were boiled in Nupage sample buffer (Invitrogen)

and then separated on a 4–12% denaturing polyacrylamide gel. Proteins were then

transferred onto a PVDF membrane, which was then immunoblotted in the antibodies α-

LSD1 (Abcam; ab-17721) and α-β-actin (Novus Biologicals).

Zebrafish embryos were collected at 72 hpf and placed in an SDS lysis buffer (10

mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton-X, 0.1% SDS) and 1x protease inhibitor

mixture (Sigma). The embryos were homogenized using the Tissuelyzer II (Qiagen).

Lysates were analyzed as above.

2.3.7 siRNA knockdown

SW480 cells were cultured as recommended by the ATCC. For silencing of

LEF1, the sequences of the small interfering RNAs were: sense, 5'-

CACCUCAGGUCAAACAGGAdTdT-3', antisense, 5'-

UCCUGUUUGACCUGAGGUGdTdT-3'. Transfection experiments were performed

using Lipofectamine 2000 (Invitrogen) according to the manufacturer's

recommendations. Following transfection with 100 nM or 200 nM siRNA, cells were

incubated for 72 hours and then harvested for chromatin immunoprecipitation.

2.3.8 Chromatin immunoprecipitation

ChIP was performed as described earlier (*39*). ChIP in SW-480 cells were

performed with antibody α-LSD1 (Abcam; ab-17721) and rabbit IgG (control). Primers

for *RDH1L* promoter were 5'-CATAATATGTGCGTGTGCAGG -3' and 5'-

GCTTTAGCCAATCACAACTGTC-3'.

2.4 Results

Previous work done in the lab has shown that CtBP is a key player in the

transcriptional repression of *RDH1L* when APC is mutated (*23*). Though CtBP

associates with multiple transcriptional co-repressors, we had not yet determined an

epigenetic mechanism for the repression. As CtBP had been shown to interact with the

lysine demethylase LSD1, we wanted to assess whether LSD1 may play a role in the

repression of *rdh1l*. We began by determining the effect of APC mutation on LSD1

levels. In order to do this, we obtained colon polyp samples and grossly uninvolved

tissue from patients harboring an APC mutation and probed for expression of LSD1 by

Western blotting (Figure 2.1). In patient 1, grossly uninvolved tissue (U) had relatively

low expression of LSD1 and high expression of LSD1 in adenoma tissue (A). Patients 2

and 3 displayed little to no expression of LSD1 in uninvolved tissue and showed

increased expression in adenomas. Patient 4 displayed no change in LSD1 expression,

indicating that while LSD1 is often misexpressed, it is not solely responsible for the

effect observed when APC is mutated. Similarly, lsd1 protein was upregulated in apc[mcr]

zebrafish embryos compared to very low levels of expression in apc wild type siblings

(Figure 2.1).

We also wanted to observe the effect of APC mutation on LSD1 transcript levels.

Quantitative RT-PCR was performed on RNA extracted from adenomas and grossly uninvolved tissue (Figure 2.1). Several of the patients displayed significantly increased levels of *LSD1* mRNA in the adenoma samples, indicating that *LSD1* is misregulated transcriptionally when APC is mutated. As with protein levels, not all samples show effects in *LSD1* RNA levels when APC is mutated, testifying to the need of addition players in the progression of colon carcinogenesis. As in adenoma samples, we found that *lsd1* is transcriptionally upregulated when apc is mutated in zebrafish when assayed by whole mount *in situ* hybridization (Figure 2.1).

Previous studies have shown that the retinol dehydrogenases are misregulated at a transcriptional level when APC is mutated. This loss of RDH gene product in cells leaves them incapable of differentiating, indicating that RA is necessary for proper cell fate decisions (*22*). Because LSD1 can play a role in epigenetic silencing through its role in regulating histone methylation, we wanted to determine whether it plays a role in the regulation of retinol dehydrogenases when APC is mutated. To determine whether lsd1 plays a role in the regulation of *rdh1l*, we injected lsd1 morpholino into apc^{mcr} zebrafish embryos and tested *rdh1l* transcript levels by RT-PCR (Figure 2.2). Loss of lsd1 increased levels of *rdh1l*, indicating that lsd1 plays a role in its transcriptional regulation. We also used the lsd1 inhibitor pargyline in order to abrogate the enzymatic demethylase activity of lsd1 and tested *rdh1l* transcription by RT-PCR. Inhibition of lsd1 increased levels of *rdh1l*, although not to the same extent as morpholino knockdown (Figure 2.2).

We have previously demonstrated that addition of exogenous retinoic acid is capable of rescuing terminal differentiation defects in apc^{mcr} zebrafish (*21*). Because lsd1

plays a role in the regulation of retinol dehydrogenases, we wanted to determine whether inhibition of lsd1 was also capable of rescuing terminal differentiation. We used the apc^{mcr} zebrafish to study this role of lsd1. To ascertain the role of lsd1 in intestinal differentiation, we used pargyline to inhibit the activity of lsd1 and performed *in situ* hybridization for the terminal differentiation marker *fabp2* (*i-fabp*) (Figure 2.2). Zebrafish harboring the apc mutation do not display expression of this marker, but when these embryos are treated with pargyline, they regain expression of *fabp2*, indicating that histone lysine demethylation by lsd1 plays a role in the differentiation of the zebrafish intestine.

Previous work in the lab has shown that during normal intestinal differentiation, lef1 and lsd1 interact to form a transcriptional activation complex that activates the *rdh1l* promoter in the presence of RA (*40*). Like lsd1, when apc is mutated lef1 is aberrantly expressed (*38*) and as the *rdh1l* promoter contains lef1 binding sites, we posited that lsd1 acts directly to repress transcription of *rdh1l*. To test this, we performed chromatin immunoprecipitation on SW480 cells and examined LSD1 occupancy (Figure 2.3). We found that LSD1 does bind the *RDH1L* promoter, suggesting a direct repression of the gene. This is consistent with the data that depletion of lsd1 by morpholino increases expression of *rdh1l*. When LEF1 siRNA was transfected into the cells, LSD1 binding at the *RDH1L* promoter was abolished, indicating that LEF1 presence is necessary for LSD1 binding and suggesting that LEF1 and LSD1 are interacting to suppress *RDH1L* transcription. (All IP work done by Kunal Rai.)

2.5 Discussion

Retinoic acid is required for proper cell fating. We have previously shown that

the retinol dehydrogenases are misregulated when APC is mutated, leading to loss of

retinoic acid production and loss of terminal differentiation in the intestine (*22*).

Restoration of retinoic acid signaling by the addition of exogenous retinoic acid is

capable of restoring differentiation to the intestine, indicating that RA is responsible for

this differentiation. What remains unclear, however, is how the machinery of retinoic

acid production is regulated. In this study, we identified a factor, LSD1, that regulates

the expression of some of this machinery at an epigenetic level.

In order to determine the role of LSD1 in the regulation of retinoic acid

production when APC is mutated, we utilized the apc^{mcr} zebrafish line as well as

adenoma samples from FAP patients that harbor a germline APC mutation. We found

that LSD1 is increased at the mRNA and protein level when APC is absent, both in the

context of apc^{mcr} zebrafish and in patient adenomas. This novel finding created a

platform whereby we could elucidate the epigenetic regulation of retinoic acid production

machinery. As this was evaluated in adenomas, where β-catenin is not yet nuclear, and in

apc^{mcr} zebrafish, which also lack nuclear β-catenin, this demonstrates that misregulation

of LSD1 occurs outside of an activated Wnt context. This also adds another layer of

chromatin modification that is misregulated when APC is mutated. As we observe more

of these factors being misregulated in the context of APC mutation, it brings into light a

larger role for APC in the regulation of chromatin. As it is becoming obvious that

changes in chromatin are necessary for the changes that occur during differentiation, this

highlights a vital function for APC – independent of β-catenin and Wnt signaling – in this

process.

LSD1 is a lysine demethylase that can demethylate H3K4me2 (*11, 12*). We wanted to determine whether LSD1 was acting within this role to repress the retinoic acid biosynthetic machinery when APC was mutated. To do this, we manipulated lsd1 levels and activity in apc^{mcr} zebrafish to test the levels of *rdh1l* transcript. *Rdh1l* levels are high in wild type siblings, but very low in zebrafish embryos containing mutated apc. When these embryos are injected with lsd1 morpholino, the *rdh1l* level rises, indicating that the high levels of lsd1 in apc^{mcr} zebrafish play a role in this misregulation. Similarly, treatment of apc^{mcr} embryos with pargyline, an inhibitor of lsd1 demethylase activity, also leads to increased levels of *rdh1l* transcript. This finding indicates that the demethylase activity of lsd1 is necessary for the misregulation of *rdh1l*. Importantly, neither of these actions completely restores *rdh1l* levels, indicating that lsd1 is one of multiple factors regulating expression of *rdh1l*. Because levels of other chromatin modifiers are misregulated when apc is absent, it is likely that lsd1 acts in concert with such factors to aberrantly repress the *rdh1l* promoter.

In order to determine whether this was a direct effect, we performed chromatin immunoprecipitation on SW480 cells to determine if LSD1 occupies the *RDH1L* promoter. SW480 cells are colon cancer cells harboring an APC mutation that closely mimics the mutation found most frequently in human colon cancers. We found that LSD1 does occupy the *RDH1L* promoter in these cells. This result, taken in concert with the finding that LSD1 activity is necessary, suggests that LSD1 actively demethylates histones at the *RDH1L* promoter to repress transcription. This provides the basis of an epigenetic mechanism for the transcriptional repression of *RDH1L* when APC is mutated.

Knowing the epigenetics behind the repression of *RDH1L* unlocks LSD1 as a potential drug target in the treatment of syndromes with mutated APC. While further examination in other retinoic acid responsive tissues, such as the eye, would be required to determine whether this phenomenon is extant in tissues other than the intestine and how inhibition of LSD1 activity would affect the homeostasis of these tissues, it is likely that this effect would be concentrated in tissues negatively affected by APC loss.

These results also have implications for the formation of an LSD1/LEF1 complex in the absence of wild type APC. These factors appear to form a complex that activates *RDH1L* when APC is present (*40*), but switches the gene to a repressed state when APC is mutated. Although it has been previously shown that LSD1 is capable of switching between demethylating methylated H3K9 (and thus activating) and methylated H3K4 (and thus repressing), loss of APC adds a new context wherein this switch can take place. Though it is unclear what is causing this switch, the implications of this change directly affect the synthesis of retinoic acid and terminal differentiation of the intestine.

Rdh1l loss disrupts retinoid biosynthesis, a process required for the terminal differentiation of the intestine. Because LSD1 directly represses transcription of *rdh1l*, we wanted to determine whether inhibition of LSD1 activity would be capable of restoring terminal intestinal differentiation. Apc^mcr zebrafish lack staining for *fabp2*, a marker for terminal intestinal differentiation, but apc^mcr embryos treated with pargyline show restored staining of *fabp2*. This suggests that LSD1, through its direct transcriptional repression of *rdh1l*, is one of several factors responsible for loss of terminal differentiation when APC is mutated. This further cements LSD1 as a potential drug target when APC is mutated. As inhibition of LSD1 demethylase activity is

sufficient to restore intestinal differentiation in the absence of APC, abrogation of LSD1

demethylase activity may restore colon cancer cells with mutated APC to a more

differentiated and less proliferative state.

We propose a model whereby aberrant expression of LSD1 in the presence of

mutated APC forms a complex containing Lef1, which leads to direct repression of rdh1l,

leading to loss of retinoic acid production and loss of terminal differentiation in the

intestine (Figure 2.4).

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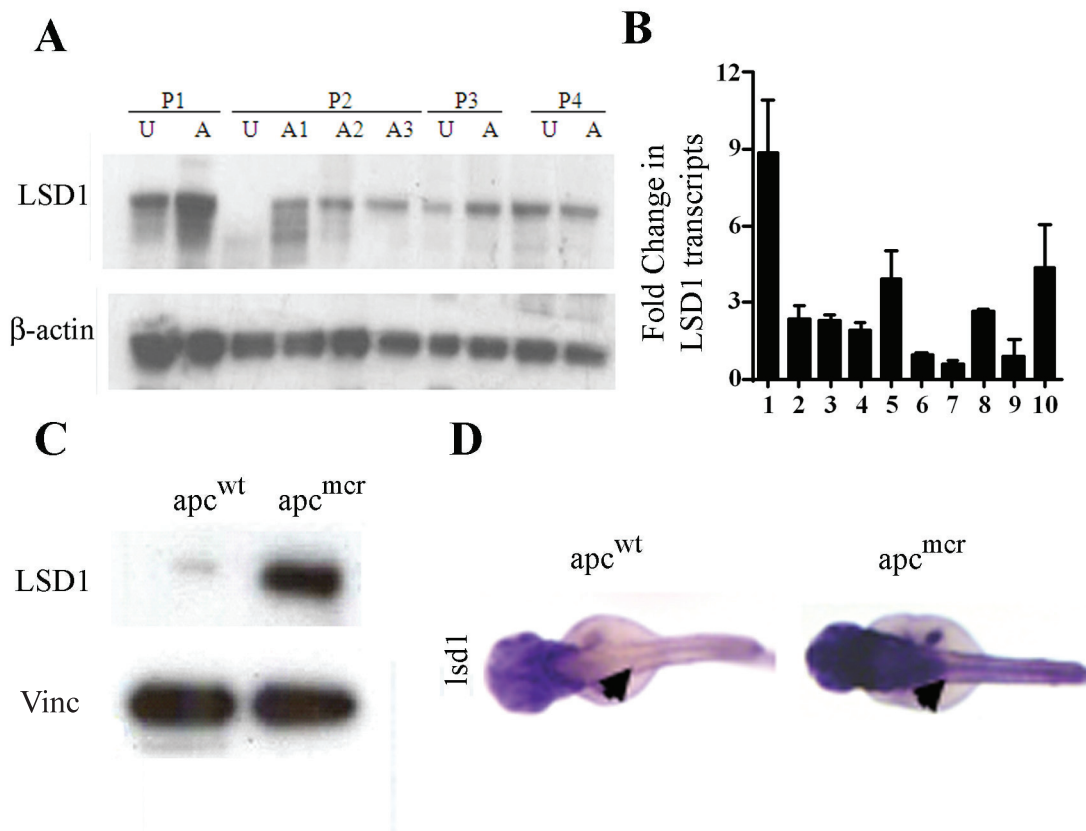
Figure 2.1 APC mutation causes increase in LSD1 transcript and protein levels. A, western blot of grossly uninvolved tissue (U) and adenomas (A). In three of four samples, LSD1 levels are increased in adenoma compared to uninvolved tissue. B, quantitative RT-PCR of RNA extracted from adenomas. Numbers indicate individual adenoma samples. Samples normalized to grossly uninvolved tissue; error bars indicate standard deviation between technical replicates. Many samples have increased levels of LSD1 transcript. C, western blot of protein extracted from apc mutant zebrafish. Lsd1 is upregulated in apc mutants compared to wild type siblings. D, whole mount *in situ* hybridization of LSD1 in apc mutants and wild type siblings. Lsd1 is increased in apc mutants at the RNA level. Arrow indicates zebrafish intestine. (Adapted from *38*)

A

B
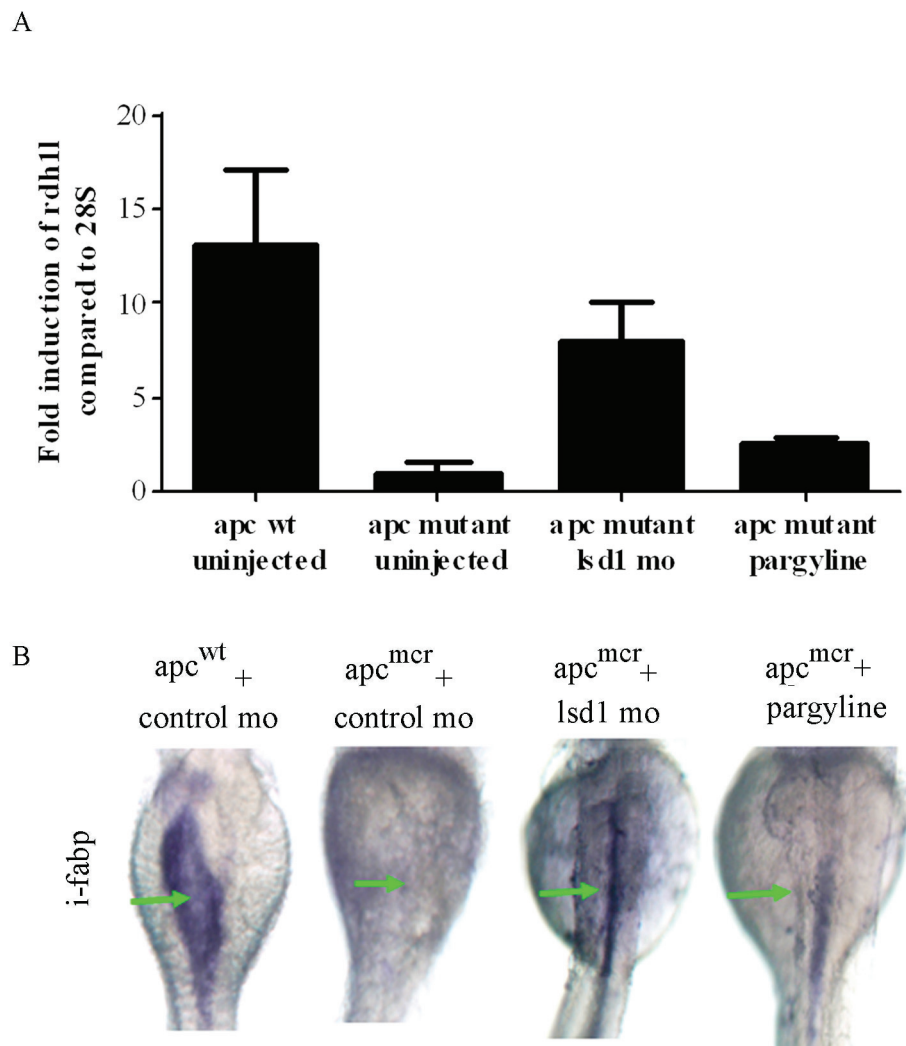
Figure 2.2 Inhibition or loss of LSD1 in APC mutant background restores intestinal differentiation and increases *rdh1l* levels. A, quantitative RT-PCR of apc mutant embryos treated with pargyline or injected with lsd1 morpholino. Error bars indicate standard deviation between biological replicates. *Rdh1l* is increased in mutants where lsd1 activity is lacking, although it is not restored to the level of wild type embryos. B, whole mount *in situ* hybridization of apc mutant embryos treated with lsd1 inhibitor pargyline or injected with lsd1 morpholino. Arrows indicated zebrafish intestine. *I-fabp* is lost in apc mutant embryos control injected, but it is restored in mutants where lsd1 activity is lacking. (Adapted from *38*)
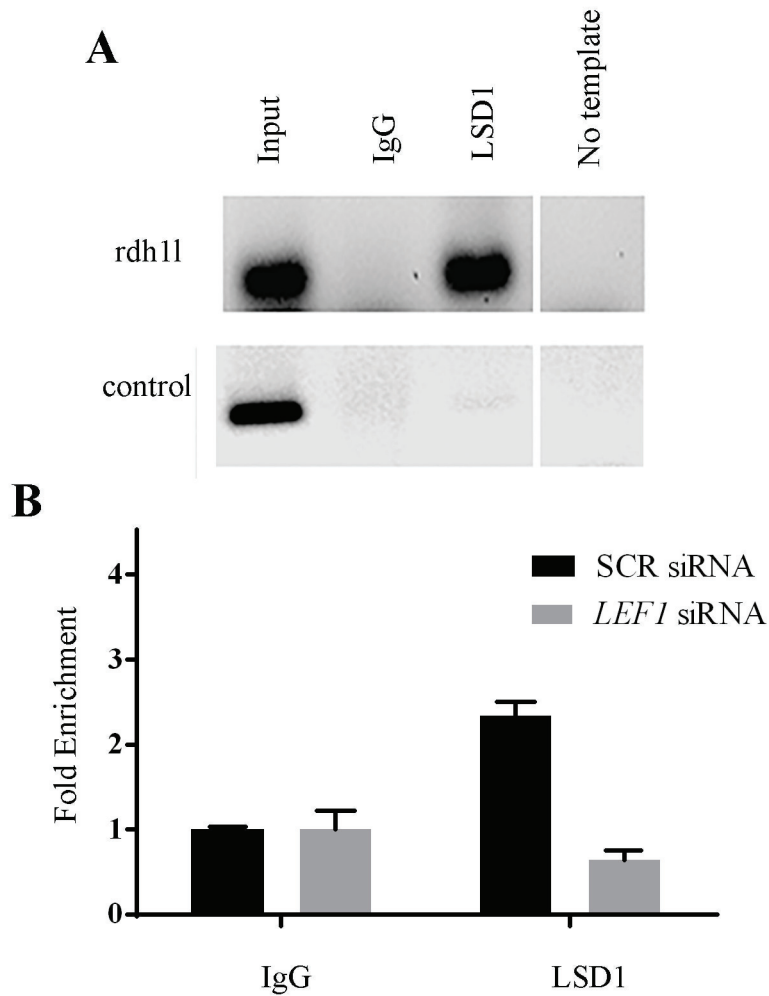
Figure 2.3 LSD1 binds to the *RDH1L* promoter in a manner dependent on interaction with Lef1. A, RT-PCR of chromatin IP. The LSD1 pulldown amplifies at the RDH1L promoter. B, quantization of ChIP pulldown under conditions of LEF1 depletion. LSD1 binds to the *RDH1L* promoter only when LEF1 is present. Error bars indicate standard deviation between biological replicates. (Adapted from *38*)
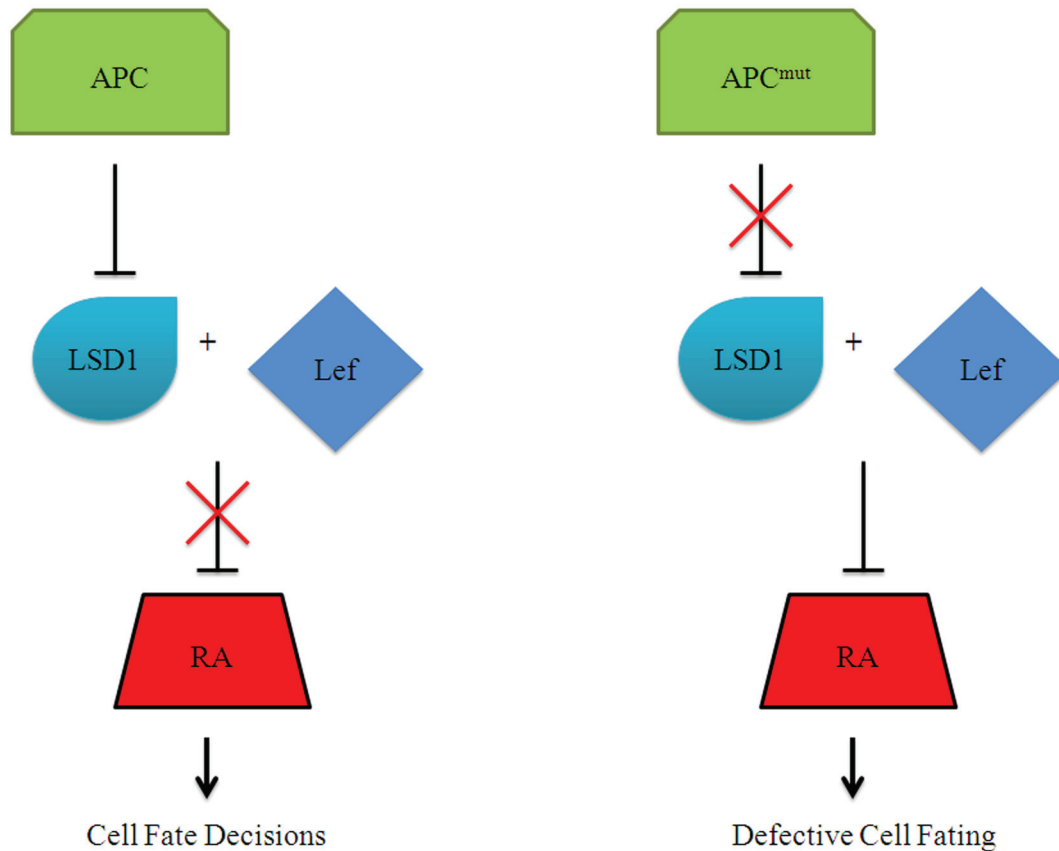
Figure 2.4 Model. Schematic displaying the effect of APC mutation in cell fating. When APC is wild type, LSD1 is repressed. This allows for the rdh1l promoter to remain in the activated state, leading to increased levels of retinoic acid and proper cell fating. When APC is mutated, LSD1 is expressed at high levels. It interacts with Lef1 and represses the rdh1l promoter, which shuts down retinoic acid production and leads to defective cell fating.

CHAPTER 3

APC AND RETINOIC ACID IN THE REGULATION

OF POLYCOMB-MEDIATED HISTONE

METHYLATION IN THE INTESTINE

3.1 Abstract

The c-terminal truncation of APC is the initiating event in 85% of colorectal cancer cases. Although it was long thought that this event was synonymous with the nuclear localization of β-catenin and subsequent transcription of TCF/Lef targets, more recent work has shown that additional events are needed for β-catenin to be found in the nucleus and that this happens further in the progression of tumorigenesis. Recent work has also clarified the ability of APC, through its regulation of retinoic acid biosynthesis, to control the chromatin modifying DNA demethylase components. The work presented herein expands upon the role of APC and retinoic acid in the control of chromatin by introducing an additional epigenetic mark controlled by APC: H3K27 trimethylation. We show that APC, through its regulation of retinoic acid biosynthesis, regulates the levels of H3K27me3. However, unlike the DNA demethylase components, the factors responsible for the deposit of H3K27me3 are not regulated transcriptionally by retinoic acid, but are regulated through Cox-2, a downstream target of RA signaling. Although we have previously shown that, in the absence of APC, increased levels of Cox-2 leads to an increase in β-catenin levels, the Cox-2-mediated regulation of H3K27me3 occurs

independent of β-catenin.

3.2 Introduction

APC is necessary for the proper differentiation of intestinal cells (*1*). This is due to the fact that APC is a major regulator in the biosynthesis of retinoic acid, and when RA is lost, cells are no longer able to properly differentiate (*2*). Upon addition of exogenous RA, gut cells once again undergo terminal differentiation. However, the mechanisms by which RA influences cell fating remain unclear.

APC, through its control of RA biosynthesis, can control cell fating through the transcriptional regulation of chromatin modifiers, as indicated by the finding that APC mutation results in increased levels of the DNA demethylase components (*3*). This misregulation caused defective cell fating and was rescued by the addition of RA. Because downstream effects of RA can be felt amongst chromatin modifiers, it is possible that it is through this mechanism that RA prepares cells to promote proper cell fate decisions.

The Polycomb group proteins are chromatin modifiers that are necessary for proper embryonic development (*4–6*). Polycomb group proteins were identified in flies as regulators of Hox genes (*7*) and, when mutated in flies, give rise to phenotypes wherein body segments are aberrantly placed. The Hox genes regulate anterior-poster body patterning (*8*) and are found in vertebrates in seven or eight clusters, determined by genome duplication (*9, 10*). Hox gene clusters are found and regulated linearly in the genome (*11*), with the genes with the smallest numerator regulating patterning most anterior in the body and the genes with the largest numerator most posterior (*12, 13*). Several of these genes are transcriptionally regulated by retinoic acid and dysregulated

upon mutation of APC (*14*).

The Polycomb repressive complex 2 lays down the H3K27 trimethyl mark (H3K27me3) (*15*). This mark represses transcription and is often found at key developmental regulator genes (*4–6, 16*). However, there is not yet a clear method of genome-wide recruitment for PRC2. While several processes have been discovered for specific loci (*17–19*), these methods do not seem to bear out on a global level. Although Polycomb responsive elements (PREs) have been discovered in flies (*20, 21*), the method of recruitment to these sites is in question, and no PRE has been discovered in mammals. While the method of recruitment is unclear, there is no doubt that it would need to be kept under tight regulation during development and differentiation to ensure proper placement of H3K27me3. The need for proper regulation of these factors is corroborated by the loss of PRC2 members being embryonic lethal in mice (*22–24*).

Knowing the role that RA plays in the regulation of chromatin modifiers to promote proper cell fating and the role that retinoic acid plays in the transcriptional regulation of Hox genes, we wanted to determine whether it plays a role in the regulation of H3K27me3, the mark laid down by PRC2. This study will describe the role of RA in the regulation of H3K27 trimethylation and how this mark is affected when APC is lost.

3.3 Materials and Methods

3.3.1 Wild type and apc heterozygous *Danio rerio* (zebrafish)

Wild type (Tü strain) and *apc* heterozygous *Danio rerio* (zebrafish) were maintained on a standard 14 hour/10 hour light/dark cycle. (*apc* heterozygous zebrafish were a kind gift from Anna Pavlina-Haramis and Hans Clevers.) Fertilized embryos were collected following natural spawning and allowed to develop at 28 $^{\circ}$C. All embryos were

raised in 0.003% phenylthiourea to inhibit pigment formation starting at 24 hpf.

3.3.2 Morpholino microinjections

Antisense and control morpholino oligonucleotides were obtained from Gene Tools, LLC. APC splice blocking morpholino (AGATGTATCTTACCTTCTGCACCTC) was solubilized in 1x Danieau buffer and injected at the 1-cell stage using 3 nl of 0.25 mM concentration. Ezh2 splice blocking morpholino (ATTGATCTAACCTCTCTGGTTCCAC) and control morpholino (ATATACATTGTCTCACCTCCATCTC) were solubilized in 1x Danieau buffer and injected at the 1-cell stage using 2 nl of 0.75 mM concentration.

3.3.3 Drug treatments

Embryos were treated with 1 µm all-trans retinoic acid (Sigma) or DMSO for 1 hour at 6, 30, and 56 hpf. NS-398 treatment was similar except at 10 µM (Cayman Chemical). Embryos were chemically dechorionated and treated with 10 µM diethylaminobenzaldehyde (Sigma) at 30 hpf consistently until protein harvesting at 96 hpf.

3.3.4 RNA extraction/RT-PCR

RNA was isolated from embryos using an RNeasy kit (Qiagen) and then synthesized with the Superscript III kit (Invitrogen). RT-PCR was performed on a Roche light cycler. Primers were *ezh2* 5'-GGTATTGAACGTGAAATGGAAAT-3', 5'-TGTAAGGCATTTGTTCAGAGAGG-3'; *utx* 5'-CTGGATGCACCATACAGTGG-3', 5'-ATGGCGTAGCTGTCCTTGTT-3'; *suz12l* 5'- CGGTGAAGTCCTACTCCCTTC-3', 5'-CTGGTGGTCATGCCATTGT-3'; *ezh1* 5'-AGGATGGAGGAAGTGTCAGG-3', 5'-

CTCTCTTCTTCCACTCCAGCA-3'; *eed* 5'-AGACACTCCCACCAACACG-3', 5'-CACTTCTTGGACTTCCACTTCC-3'; *ef1α* 5'-CCTTCGTCCCAATTTCAGG-3', 5'-CCTTGAACCAGCCCATGT-3', *i-fabp* 5'- CAACGTGAAGGAAGTCAGCA-3', 5'-GGTGACGCCCAGAGTAAAGT-3'; 28s rRNA 5'-AAACCAACCCGGAGAAGC-3', 5'-CGCGAGATTTACACCCTCTCT-3'.

3.3.5 Whole mount *in situ* hybridization

Zebrafish embryos were fixed in sucrose-buffered 4% paraformaldehyde, rinsed in PBST and dehydrated through an ethanol series, and stored in 100% methanol at -20 ^{o}C. Whole mount *in situ* hybridizations were carried out by standard protocol as previously described (*2*). cDNAs were cloned into the pCR-TOPO-II vector (Invitrogen), and riboprobes for the indicated genes were made with digoxigenin-labeled UTP (Roche).

3.3.6 Cell culture and siRNA knockdown

SW480 cells were purchased from ATCC and grown according to the guidelines of the manufacturer. Transfection experiments were performed with Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's recommendation. Transfection was performed at the time of seeding and after transfection, cells were incubated for 120 hours and then harvested for western blot analysis.

β-catenin and control siRNA were obtained from Cell Signaling.

3.3.7 Western blotting

Zebrafish embryos were collected at 72 or 96 hpf and placed in an SDS lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton-X, 0.1% SDS) and 1x

protease inhibitor mixture (Sigma). The embryos were homogenized using the

Tissuelyzer II (Qiagen). Lysates were quantified using the DC protein assay (Bio-Rad).

Samples were run through Tris-glycine 4-12% gradient NuPage gels using the MES

buffer (Invitrogen) and transferred to PVDF membrane. Blots were probed using the

following primary antibodies: rabbit α-H3K27me3 (Millipore), mouse α-H3K27me3

(Abcam), rabbit α-H3 (Abcam), and rabbit α-beta-catenin (Abcam).

Protein samples from cells were harvested at 120 hours posttransfection in RIPA

buffer (50 mM HEPES, pH 7.6, 1 mM EDTA, 0.7% deoxycholate, 1% NP-40, 0.5 M

LiCl and 1.5x protease inhibitors) and spun down to pellet debris. Lysates were

quantified and analyzed as with zebrafish embryos above.

Blots were quantified by calculating intensity in Adobe Photoshop for H3K27me3

and control (histone H3). Means and standard deviations were calculated using

Microsoft Excel.

3.4 Results

APC mutation has been shown to dysregulate multiple chromatin modifying

pathways (*3, 25*). This dysregulation has caused loss of terminal differentiation in the

intestine, indicating a role for chromatin in the process of differentiation. It is likely that

APC regulates other chromatin marks and modifiers in order to promote proper cell

fating. We wanted to examine the effects of apc loss on the regulation of histone 3 lysine

27 methylation. To evaluate the effect of apc mutation on H3K27 methylation levels,

apc^{mcr} zebrafish and apc wild type siblings were grown to 72 hpf, at which point protein

was extracted from whole embryos. Extracts blotted for levels of H3K27me3 show

decreased levels of this histone methylation in apc^{mcr} embryos, indicating a role for apc in

the regulation of histone methylation (Figure 3.1). Because APC is a member of the β-catenin destruction complex (*26*), we wanted to determine whether the activation of the β-catenin/TCF/Lef signaling pathway was playing a role in this change in histone methylation. To test this, β-catenin was knocked down in SW480 cells. While β-catenin protein levels are greatly diminished, H3K27me3 levels are unchanged (Figure 3.1), suggesting that apc regulates this histone methylation through a β-catenin-independent pathway.

One of the hallmarks of apc loss in zebrafish is loss of terminal differentiation in the intestine (*2*). While these embryos retain expression of the primordial gut marker gata6, they lose expression of i-fabp, the marker for terminally differentiated gut. We wanted to determine whether the decreased levels of H3K27me3 under conditions of APC mutation contribute to this loss of cell fating. To assay this, we used a splice-blocking morpholino for ezh2, the H3K27 methyltransferase. Wild type embryos injected with ezh2 morpholino phenocopy the apc mutant phenotype in loss of terminal differentiation of the intestine. Whole mount *in situ* hybridization for gata6 is unchanged in ezh2 morphants, but i-fabp is deficient in these embryos (Figure 3.2). We have previously shown that in conditions of apc mutation, it is loss of retinoic acid production that leads to loss of terminal gut differentiation (*27*). This defect can be rescued by the addition of all trans retinoic acid to the developing zebrafish. Because ezh2 morphants also experience a loss of terminal gut differentiation, this raised the question of whether ezh2 morphant zebrafish are also retinoic acid deficient. In order to test the relationship between retinoic and ezh2, ezh2 morphants were treated with retinoic acid to determine i-fabp levels. Quantitative RT-PCR of i-fabp in ezh2 morphants treated with retinoic acid

shows no significant increase as compared to ezh2 morphants that are control treated (Figure 3.2), indicating that both retinoic acid and ezh2 are necessary for terminal differentiation of the intestine or that retinoic acid is upstream of ezh2 activity.

Many of the defects observed in apc^{mcr} zebrafish are caused by loss of retinoic acid and can therefore be restored by the addition of exogenous RA. The ezh2 morphant appears to phenocopy some of these defects, but these defects cannot be rescued by RA. This led us to question whether ezh2 and RA are in convergent pathways or whether they reside in the same pathway, with RA acting upstream of ezh2's regulation of H3K27 methylation. To examine this, wild type zebrafish were injected with apc morpholino and treated with all-trans retinoic acid. Protein blots show that apc morphants treated with ATRA have increased levels of H3K27me3, whereas apc morphants control treated with DMSO have very low levels of H3K27me3 (Figure 3.3). To determine whether RA was sufficient for the regulation of H3K27me3, wild type embryos were treated with DEAB, an inhibitor of retinoic acid biosynthesis, and protein was extracted from whole embryos at 96 hpf. Protein blots show that inhibition of retinoic acid biosynthesis is sufficient to decrease in the levels of H3K27me3 (Figure 3.3).

Previously examined key regulators of differentiation-inducing programs have been determined to be transcriptionally misregulated when APC is mutated (*3*). In these cases, loss of retinoic acid is responsible for changes in the transcript levels of the components. To determine whether RA plays a role in the transcriptional regulation of PRC2 components (Ezh2/Ezh1, Suz12, and Eed) or the H3K27 demethylase, Utx, RNA was extracted from apc^{mcr} zebrafish and wild type siblings, and we performed quantitative RT-PCR for the above mentioned transcripts. None of the transcripts

showed significant changes when APC is mutated (Figure 3.4). PRC2 interacting

partners Rbbp4, Jarid2a, and Jarid2b also show no significant change in transcript levels

when apc is mutated (data not shown).

As retinoic acid does not directly affect transcription of PRC2 components, we

explored other pathways it affects. Previous work done in the lab has elucidated a

pathway whereby the loss of retinoic acid signaling caused by mutation of APC affects

Cox-2 (*28*). We wanted to determine if it was this role of RA that was affecting levels of

H3K27me3. To do so, we treated apc^mcr zebrafish embryos with the Cox-2 inhibitor NS-

398. Similar to the results we saw with treatment with exogenous RA, blocking Cox-2

increased levels of H3K27me3 in the apc mutants (Figure 3.4), indicating that Cox-2 is

an intermediary in the regulation of histone methylation by RA.

3.5 Discussion

We have previously shown that APC plays a role in the regulation of certain

chromatin marks and by so doing, promotes proper cell fating decisions (*3*). As such,

when APC is mutated, defective cell fating occurs. In this study we investigate the role

of APC in the regulation of an additional chromatin mark, the trimethylation of histone

H3 at lysine 27, and how it affects cell fating in the intestine.

This work demonstrates that loss of APC results in decreased levels of

H3K27me3. The H3K27me3 mark occupies numerous pathways involved in the

differentiation of the intestine, and misregulation of these pathways has the potential to

cause defective cell fating. The fact that there are decreased levels of this chromatin

mark is of interest because it increases the collection of chromatin marks and modifiers

that are misregulated in the context APC mutation. While misregulation of chromatin

marks is something often seen in cancer, loss of methylation at histone 3 lysine 27 has the profound potential to cause complications in the transition from progenitor to terminally differentiated cells. The occupancy of this mark at most of the pathways that regulate the formation of multiple types of differentiated intestinal cells (*4*) highlights the necessity of proper regulation of this mark during this transition. Loss of H3K27me3 suggests that these pathways cannot be turned off, leading to aberrant transcription of signaling pathways for multiple cell types.

Hypomorphism of ezh2 in the zebrafish causes loss of intestinal differentiation, a retinoic acid deficient phenotype and one hallmark of APC mutation. However, in apc^mcr zebrafish and other retinoic acid deficient models, treatment with exogenous retinoic acid rescues this loss of differentiation, which does not occur in ezh2 morphants. This is the first model we have examined in which RA lacks the ability to restore intestinal differentiation. This implies that retinoic acid requires other cellular factors to promote terminal differentiation, among them being ezh2. It also signifies that H3K27me3 is necessary for terminal differentiation of the intestine.

We have determined from previous work that defective cell fating due to mutated APC is mediated through a loss of retinoic acid signaling. We have also previously shown that chromatin modifiers responsible for other chromatin marks are regulated by RA, so we wanted to determine whether RA also plays a role in the regulation of the H3K27me3 mark. We show that treatment of APC deficient fish with RA is sufficient to increase levels of H3K27me3, indicating that RA does play a role in the regulation of this mark. Chemical inhibition of RA biosynthesis is also sufficient to decrease levels of H3K27me3, although not to the extent that APC mutation does, indicating that there may

be other players involved when APC is mutated. Although we have previously identified chromatin modifiers through which RA controls cell fating, these findings give additional insights into how RA controls differentiation. As the knowledge of RA regulating chromatin modifiers grows, we will be able to better understand the pathways through which it regulates cellular differentiation events.

The previous findings are all consistent with the method by which APC mutation affects the DNA demethylase components. As is common with retinoic acid regulation, the DNA demethylase components are transcriptionally regulated by RA, and thus are misregulated at the transcriptional level when APC is mutated. However, the regulators of H3K27 trimethylation differed in this case. None of the PRC2 components (Ezh2, Ezh1, Eed, or Suz12) nor the H3K27 demethylase (Utx) are transcriptionally affected by loss of APC. We also saw no change in the PRC2 interacting partners Rbbp4 or Jarid2 (two isoforms, Jarid2a and Jarid2b, exist in zebrafish) (*29, 30*). We conclude that there must be additional players in the APC-mediated regulation of the H3K27me3 and that RA does not regulate the PRC2 components directly. This diverges from the way RA regulates many components, adding new layers through which retinoic acid regulates chromatin modifiers during differentiation. This may also explain why addition of exogenous retinoic acid failed to rescue gut differentiation in ezh2 morphants.

Previous work done in the lab has shown that RA regulates C/EBP-β and through this regulation affects the expression of the proinflammatory protein Cox-2 (*28*). Because we see that RA regulates PRC2 indirectly, we wanted to determine whether it took advantage of this pathway to regulate H3K27 methylation. We saw that this mark changed when treated with Cox-2 inhibitor, indicating that RA uses its control of this

pathway to govern levels of H3K27me3. This also led us to ask whether β-catenin plays a role in this regulation. We have shown previously that the misregulation of Cox-2 when APC is absent can lead to an upregulation of β-catenin, and we wanted to determine whether this played a role in the regulation of H3K27me3 (*28*). We found that knockdown of β-catenin when APC is mutated does not affect H3K27me3 levels, which denotes that Cox-2 plays additional roles not related to β-catenin stabilization in the context of mutated APC. This may reveal an additional role for Cox-2 in the regulation of cell fating and epigenetics. This is additional evidence that the misregulation of H3K27 methylation takes places in the absence of activated Wnt and thus clarifies further pathways aberrantly regulated upon APC mutation that are not dependent on β-catenin. This also raises the potential for H3K27 trimethylation to be regulated in response to increased inflammatory signals. Colon cancer is frequently associated with inflammation in the intestine, and patients who have a family history are twice as likely to develop colon cancer if they already suffer from inflammatory bowel diseases (*31*). Although multiple proinflammatory pathways affect colon cancer development, Cox-2 is an important factor in colon tumorigenesis (*32*). Other work has shown that, in macrophages, levels of H3K27me3 can be affected through proinflammatory pathways (*33*), although in this study levels of histone demethylases were changed, an effect we do not see when APC is mutated. However, the implications of Cox-2 involvement in the regulation of H3K27 trimethylation add potential new insights for how inflammation promotes colon tumorigenesis.

Our data present a continuation of the story we began with Rai et al. in demonstrating that APC controls cell fate decisions through controlling chromatin

modifiers. However, these data present a unique aspect in that, unlike the DNA

demethylase described in Rai et al., retinoic acid affects H3K27 methylation indirectly

through the proinflammatory protein Cox-2 (Figure 3.5).

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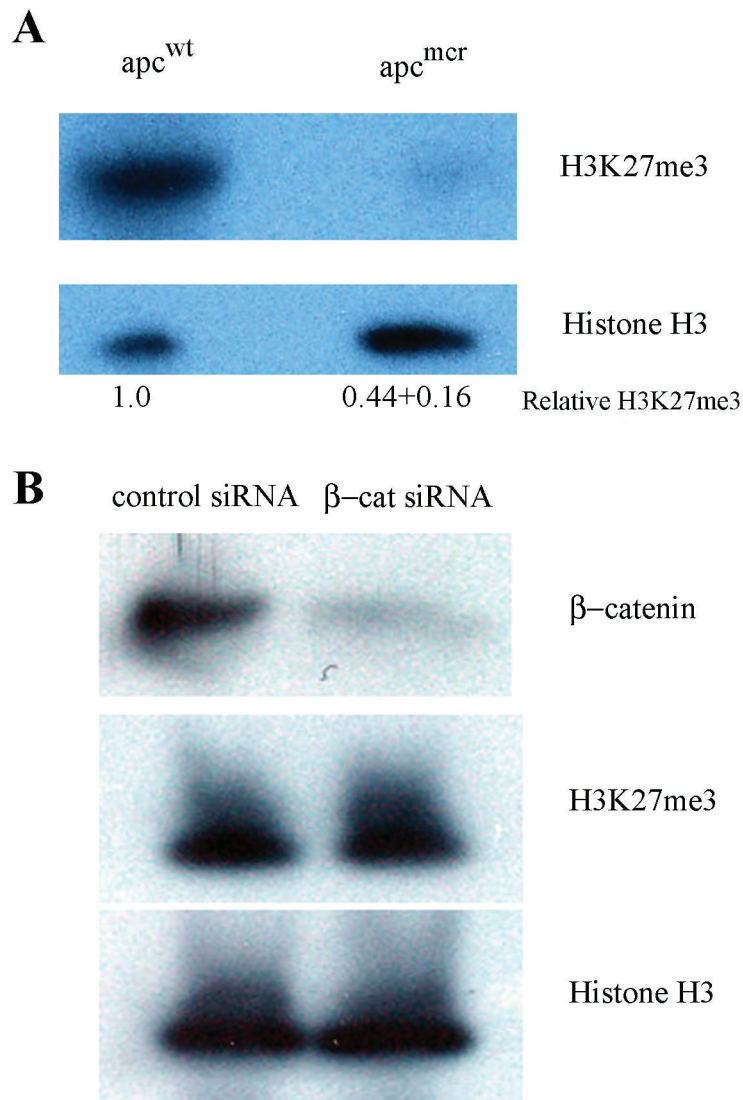
Figure 3.1 APC mutation causes decreased H3K27 methylation in a β-catenin-independent manner. A, western blot of apc mutant zebrafish shows decreased levels of H3K27 methylation compared to wild type siblings. Levels of H3K27me3 are quantified and normalized to apc wild type. Relative levels and standard deviation are shown (n = 5). Protein extracted at 72 hpf. B, western blot of SW480 cells transfected with β-catenin siRNA. Although knockdown of β-catenin is achieved, H3K27 trimethylation is unaffected.
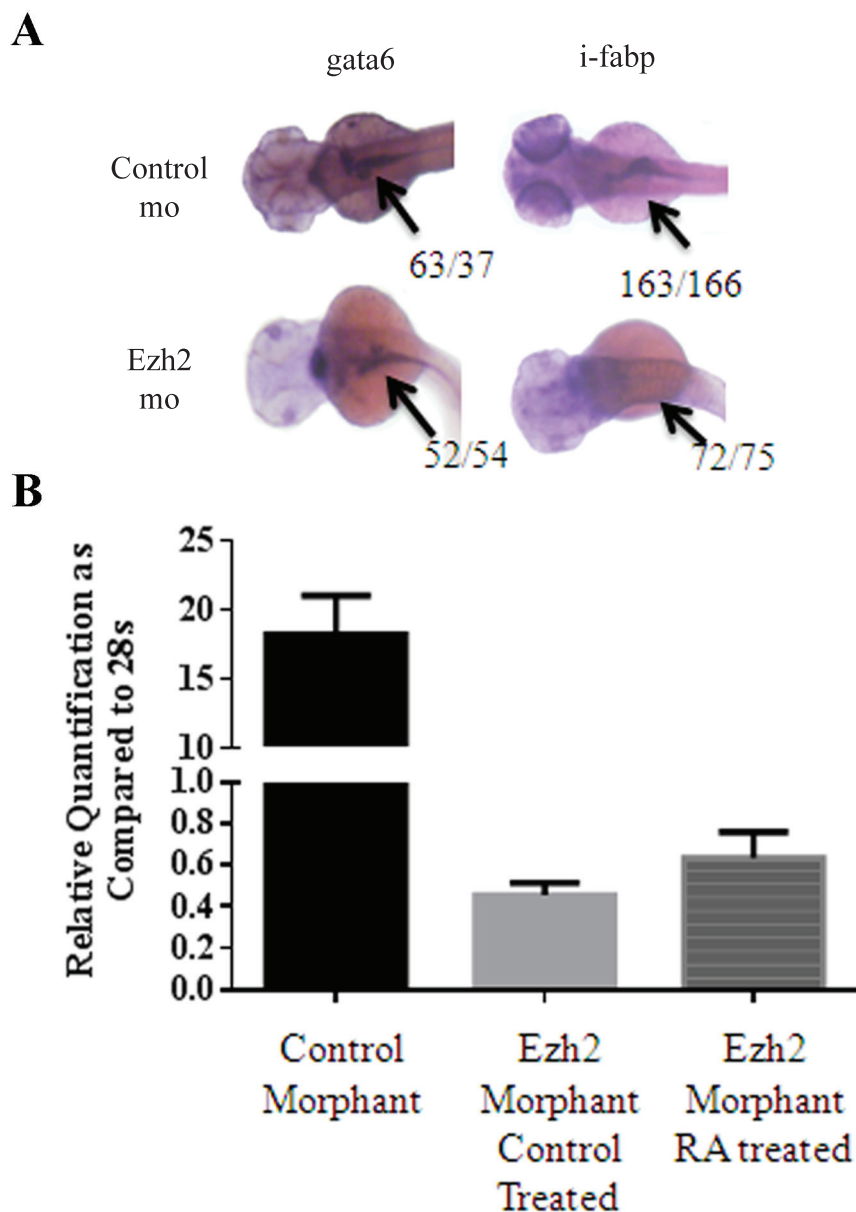
Figure 3.2 Hypomorphism of ezh2 causes loss of terminal differentiation in the zebrafish intestine, a defect not capable of rescue by retinoic acid. A, whole mount *in situ* hybridization of zebrafish injected with ezh2 morpholino have lost expression of ifabp, the marker for terminal differentiation of the intestine. Ezh2 morphants retain expression of gata6, the marker for primordial gut. Embryos fixed at 72 hpf. Arrow indicated zebrafish intestine. B, quantitative RT-PCR of ezh2 morphant embryos treated with retinoic acid. Control morphants have high levels of i-fabp, but ezh2 embryos, whether treated with RA or DMSO, display low levels of i-fabp. RNA extracted at 72 hpf.

A

	WT DMSO	WT RA	apc mo RA	apc mo DMSO
H3K27me3				
H3				
Relative H3K27me3	1.0	0.84±0.56	0.76±0.29	0.27±0.36

B

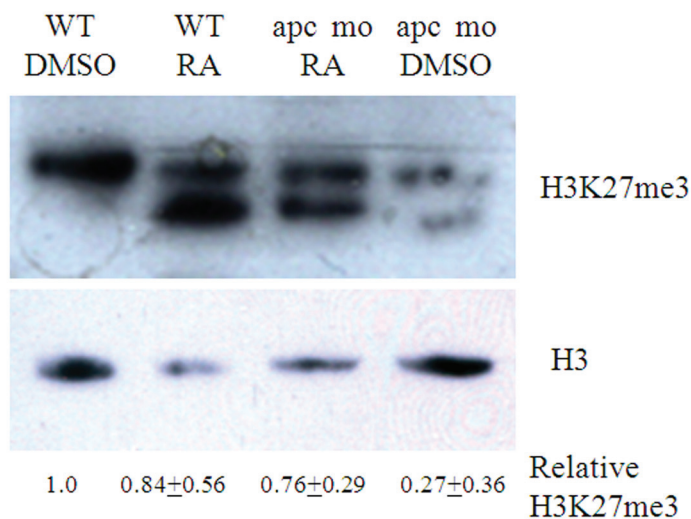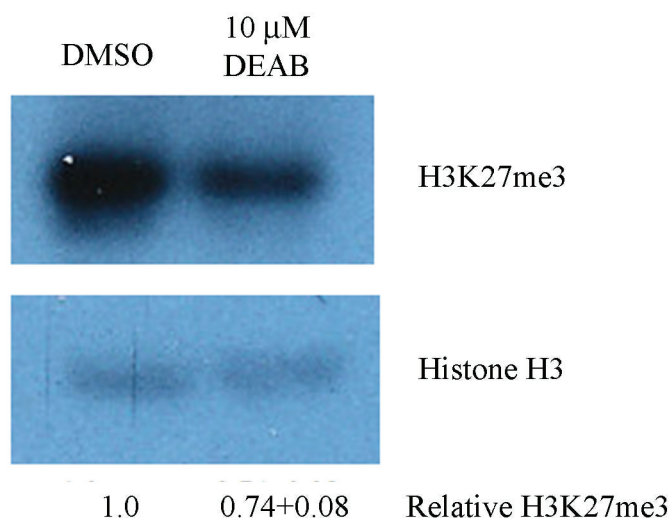|	DMSO	10 μM DEAB
H3K27me3		
Histone H3		
Relative H3K27me3	1.0	0.74+0.08

Figure 3.3 Retinoic acid increases levels of H3K27me3 in apc-deficient embryos, whereas inhibition of retinoic acid biosynthesis recapitulates apc-deficient defects. A, western blot of wild type embryos were injected with apc morpholino and treated with retinoic acid. Embryos treated with retinoic acid have H3K27 methylation levels similar to wild type embryos, whereas control treated embryos have very low levels of H3K27me3. Levels of H3K27me3 are quantified and normalized to wild type control (DMSO) treated. Relative levels and standard deviation are shown (n = 3). Protein extracted at 72 hpf. B, western blot of wild type embryos treated with DEAB. DEAB treated embryos have decreased levels of H3K27me3 compared to control treated embryos. Levels of H3K27me3 are quantified and normalized to wild type control (DMSO) treated. Relative levels and standard deviation are shown (n = 4). Protein extracted at 96 hpf.

A

B

	apc^wt	apc^mcr	apc^wt	apc^mcr
	DMSO	DMSO	NS-398	NS-398

H3K27me3

Histone H3

1.0   0.48±0.12   1.37±0.60   1.86±1.0

Figure 3.4 RA does not directly transcriptionally regulate PRC2, but employs an intermediate pathway including cox-2. A, quantitative RT-PCR of apc mutant embryos and wild type siblings. PRC2 subunits and the H3K27 demethylase, utx, show no significant change in expression between the two states; error bars indicate standard deviation between biological replicates. RNA extracted at 72 hpf. B, western blot of apc mutant embryos treated with the cox-2 inhibitor NS-398. Apc mutants treated with NS-398 have increased levels of H3K27me3 compared to control treated mutants. Levels of H3K27me3 are quantified and normalized to wild type control (DMSO) treated. Relative levels and standard deviation are shown (n = 3).
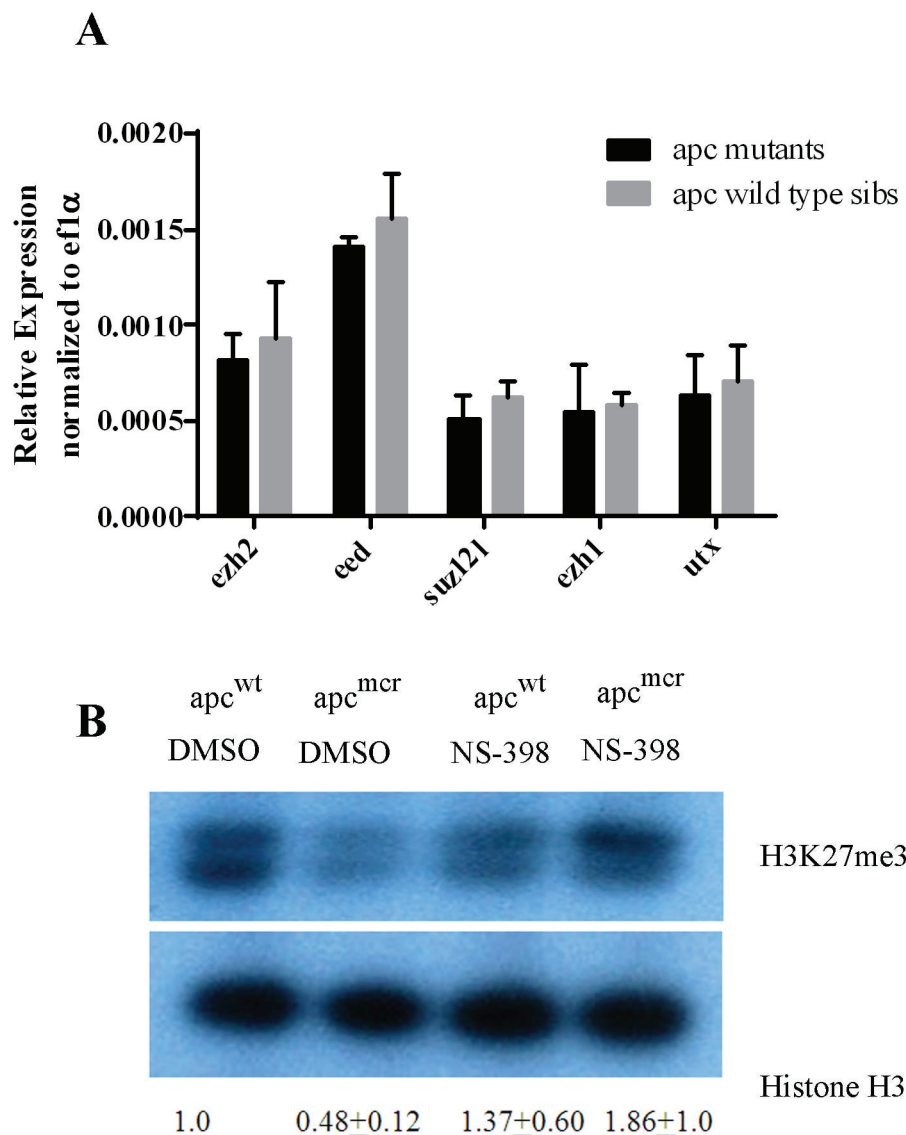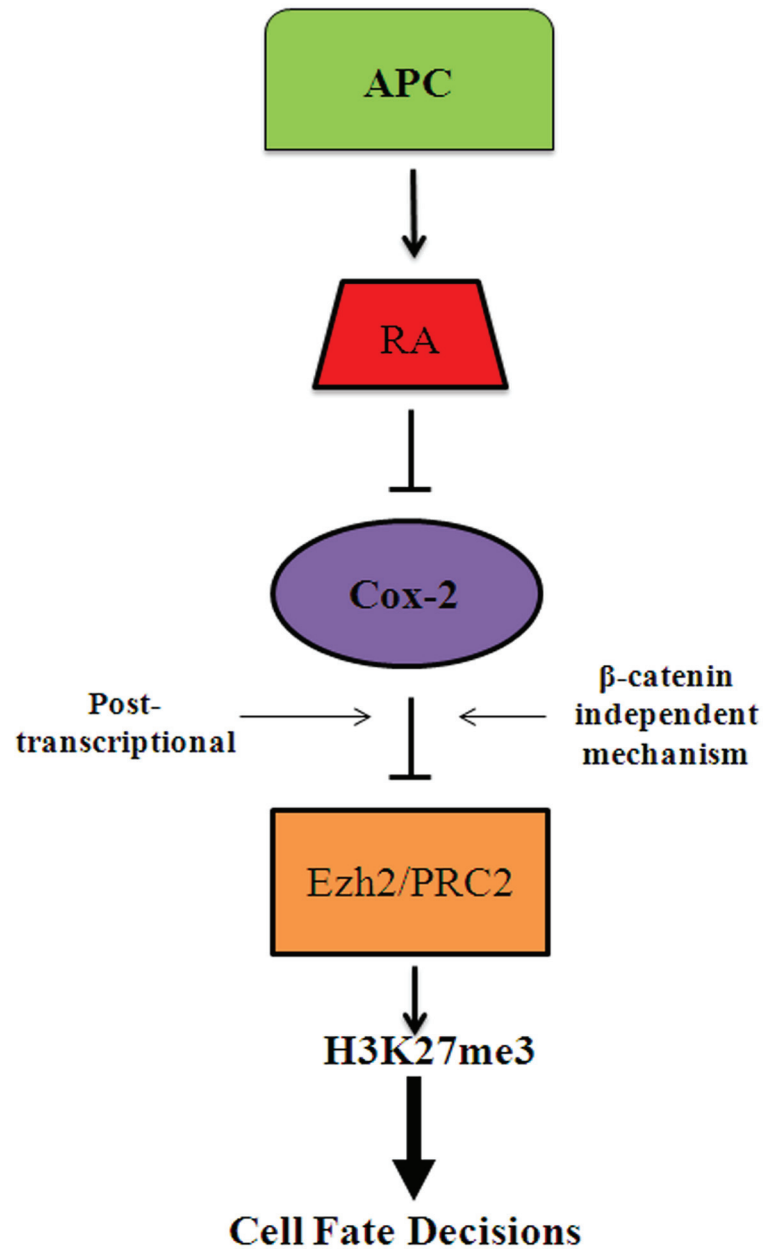
Figure 3.5 Model. APC controls levels of retinoic acid through its regulation of the transcription of retinoid biosynthetic machinery. RA controls levels of Cox-2, which regulates the levels of H3K27me3 through a posttranscriptional method that is independent of β-catenin.

CHAPTER 4

THE ROLE OF EZH2 AND HISTONE METHYLATION
IN THE DEVELOPING EMBRYO

4.1 Abstract

The Polycomb group proteins are necessary for the development of the embryo and play a role in cellular differentiation. This family is divided into two complexes, Polycomb repressive complex 1 and Polycomb repressive complex 2, and acting in concert these complexes regulate gene expression to allow for proper embryogenesis. While knockouts in *Drosophila* have been characterized, PRC2 knockout mice are embryonic lethal. While this supports their necessity in the regulation of development, it leaves gaps in our understanding of the specific roles that they play in the regulation of development. Here, we use zebrafish to present a model of vertebrate development with decreased levels of ezh2, the catalytically active subunit of PRC2. This study characterizes loss of ezh2 in the developing vertebrate embryo. We show that loss of ezh2 leads to morphological defects in the spine and loss of cartilage formation in the jaw and fins. This model also shows that loss of ezh2 allows for normal development of some organ systems but negatively affects the development of others, highlighting tissue specificity for ezh2. This study also brings into light the role of ezh2 in the development of the zebrafish intestine.

4.2 Introduction

Proper regulation of chromatin during development and cellular programming is necessary to enact programs for cell differentiation and stem cell maintenance (*1*). The transcriptional timing of differentiation programs must be carefully regulated in order to ensure proper development in both organogenesis and for development of the organism as a whole (*2*). When these programs are dysregulated, the animal can fail to properly form organ systems and may become nonviable (*3–5*). The Polycomb group proteins are among the factors that regulate this system to provide proper development.

The Polycomb group proteins were first discovered in flies to regulate the Hox genes (*6–8*). Hox genes are developmental transcription factors that regulate the anterior-posterior axis and segmental identity (*9*). While the Polycomb group proteins are necessary regulators during development, multiple phenotypes are seen when they are lost. Some of the defects observed include defects in neural tube formation, seen in Jarid2-null mice (*10*); left-right asymmetry, in chicken embryos devoid of Pcl2 (*11*); and a susceptibility to lung and liver tumorigenesis, in adult mice lacking Cbx7 (*12*). Knockout of some of the Polycomb group proteins display no defects at all (*13*), whereas others, including all of the core members of members of PRC2, are indispensible for development (*3, 14–16*).

Polycomb group proteins reside in two complexes: the Polycomb repressive complex 1 (PRC1) and the Polycomb repressive complex 2 (PRC2) (*17*). PRC2 lays down the repressive H3K27me3 mark (*18*), which is recognized by a member of PRC1 containing a chromodomain (*19*). PRC1 then lays down further repressive marks (*13, 20*). In this manner the Polycomb group proteins reprogram chromatin to set in motion

pathways that promote proper cell differentiation.

PRC2 is composed of 4 proteins: the catalytically active histone methyltransferase Ezh2, Eed, zinc finger and regulatory factor Suz12, and histone binding protein RbAp46/48 (*17*), although RbAp46/48 may not be consistently found with the other complex members (*21*). Although there are individual cases of how PRC2 is recruited to silence certain genes (mostly notably ncRNA) (*22–24*), there is no clear mechanism of action for the recruitment of this complex on a genome-wide scale.   During development, this complex occupies many genes involved in pathways necessary for development, including Hedgehog, Fox, and Sox pathways; FGF; and Notch signaling (*25*).   Thus, proper regulation and recruitment of PRC2 during development is vital for development.

Ezh2 contains a SET domain and is responsible for methyltransferase activity observed in PRC2. Unlike many histone methyltransferases, Ezh2 is capable of placing 3 methyl groups on a lysine residue (*26, 27*). Ezh2 also has the ability to bind RNA at high affinity (*28*), although it does so at low specificity, human Ezh2 being able to bind RNA from multiple species (*29*). It has been hypothesized that this binding ability is required for recruitment to specific gene loci.

A tight regulation must be kept on these factors as is shown by their frequent mutation in multiple cancer types (*30–35*). Ezh2 has been found mutated in several cancer types, including lymphoma, bladder, prostate, and breast cancer (*36–46*). Ezh2 knockout mice are embryonic lethal, indicating the necessity of this factor in early development (*3*). In order to study the role of H3K27me3 regulation, we utilized developing zebrafish to study the effect of loss of Ezh2 (the H3K27 methyltransferase) to

better understand its role.

4.3 Materials and Methods

4.3.1 Wild type *Danio rerio* (zebrafish)

Wild type (Tü strain) *Danio rerio* (zebrafish) were maintained on a standard 14

hour/10 hour light/dark cycle. Fertilized embryos were collected following natural

spawning and allowed to develop at 28 $^{\circ}$C. All embryos were raised in 0.003%

phenylthiourea to inhibit pigment formation starting at 24 hpf unless otherwise indicated.

4.3.2 Morpholino and mRNA microinjections

Antisense and control morpholino oligonucleotides were obtained from Gene

Tools, LLC. Ezh2 splice blocking morpholino

(ATTGATCTAACCTCTCTGGTTCCAC) and control morpholino

(ATATACATTGTCTCACCTCCATCTC) were solubilized in 1x Danieau buffer and

injected at the 1-cell stage using 2 nl of 0.75 mM concentration. The zebrafish *ezh2*

construct was cloned into the pCR-TOPO-II vector (Invitrogen). The construct with

linearized with EcoRV (Fermentas) and transcribed using the T7 mMessage mMachine

(Ambion) according to the manufacturer's protocol. mRNA was injected at 25 ng/µl

with morpholino as above.

4.3.3. RNA extraction/PCR

RNA was isolated from embryos using an RNeasy kit (Qiagen) and then

synthesized with the Superscript III kit (Invitrogen). Primers for verification of *ezh2*

splicing were 5'- GGAGCGTACTGACATCTT -3' and 5'-

TGGAGAACTCAGAAAAGCCA-3'.

4.3.4 Whole mount *in situ* hybridization

Zebrafish embryos were fixed in sucrose-buffered 4% paraformaldehyde, rinsed in PBST, dehydrated through an ethanol series, and stored in 100% methanol at -20 ^{o}C. Whole mount *in situ* hybridizations were carried out by standard protocol as previously described (*47*). cDNAs were cloned into the pCR-TOPO-II vector (Invitrogen) and riboprobes for the indicated genes were made with digoxigenin-labeled UTP (Roche).

4.3.5 Alcian blue staining

72 hpf embryos were fixed and dehydrated as above. Dehydrated embryos were bleached in 30% hydrogen peroxide and stained in an Alcian blue solution (1% concentrated hydrochloric acid, 70% ethanol, and 0.1% Alcian blue). Embryos were cleared in acidic ethanol.

4.3.6 Western blotting

Zebrafish embryos were collected at 72 hpf and placed in an SDS lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton-X, 0.1% SDS) and 1x protease inhibitor mixture (Sigma). The embryos were homogenized using the Tissuelyzer II (Qiagen). Lysates were quantified using the DC protein assay (Bio-Rad). Samples were run through Tris-glycine 4–12% gradient NuPage gels using the MES buffer (Invitrogen) and transferred to PVDF membrane. Blots were probed using the following primary antibodies: rabbit α-H3K27me3 (Millipore), mouse α-H3K27me3 (Abcam), and rabbit α-H3 (Abcam).

Blots were quantified by calculating intensity using Adobe Photoshop for H3K27me3 and control (histone H3). Means and standard deviations were calculated

using Microsoft Excel.

4.4 Results

The histone methyltransferase Ezh2 is responsible for the deposition of the H3K27me3 mark (*18*). It is also a member of the Polycomb group proteins, a group of developmental regulators best known for their regulation of hox gene regulation during development (*6*). As such, it would be compelling to know how Ezh2 affects vertebrate development. However, murine knockouts of Ezh2 are embryonic lethal, and thus we have previously not known how loss of Ezh2, and thus the H3K27 trimethyl mark, affects development (*3*). To answer this question, we opted to use the developing zebrafish as a model to study the role of Ezh2 in development and particularly in the differentiation of the gut. We performed whole mount *in situ* hybridization on uninjected wild type embryos to determine the expression pattern of ezh2 in the developing embryo. Ezh2 is ubiquitously expressed as early as 1 hour postfertilization (hpf). It continues to be so through approximately 24 hpf, at which point in time the marker becomes increasingly anterior. By 5 days post fertilization (dpf), ezh2 is found diffusely in the brain of the embryo (Figure 4.1).

In order to better characterize the loss of H3K27 methylation in developing zebrafish, morpholino knockdown of ezh2, the H3K27 methyltransferase, was performed. RT-PCR of the affected region of ezh2 in ezh2 morpholino injected embryos shows decreased splicing of ezh2 pre-mRNA (Figure 4.2). Embryos injected with control morpholino do not show loss of ezh2 pre-mRNA splicing. Protein extracted from ezh2 morphants and blotted for H3K27me3 shows decreased levels of H3K27me3 compared to control morphant embryos (Figure 4.2).

We wanted to further characterize the loss of ezh2 in the developing zebrafish embryo. Brightfield microscopy of embryos grown without PTU injected with ezh2 morpholino shows that loss of ezh2 delays development at the 24 hpf stage. At 48 hpf, these embryos lack the pigmentation now present in the control morpholino injected embryos. They also retain a curvature of the spine, whereas control morphant embryos have completed the straightening of the spine. By 72 hpf, the ezh2 morphant embryos have developed pigmentation, but they display a spectrum of morphological phenotypes (Figure 4.2). While all embryos display a loss of cartilage formation in the jaw and fins, a subset of embryos displays an indentation in the head, suggesting abnormalities in brain development. The more severe phenotypes have curvature of the spine and often have a shortened body, indicative of spinal malformations, similar to what is observed in PRC1 knockouts in mice. At its most severe, ezh2 hypomorphism manifests as extreme curvature of the spine, a collapsed jaw, heart edema, and body lengths approximately half the size of control-injected embryos.

The expression pattern of ezh2 guided our search to determine how partial ezh2 loss affects development. Because ezh2 is predominantly expressed in the brain, we wanted to determine whether the loss of ezh2 caused brain formation defects. Whole mount *in situ* hybridization of five early brain markers, stained at 18 and 30 hpf, showed no change in ezh2 morphants in four of the markers, with a slight downregulation of zash1a when ezh2 is decreased (Figure 4.3). No signal was lost completely in the ezh2 morphant.

Due to the gross morphological defects caused by deficiency in ezh2 by 72 hpf, we wanted to determine whether this deficiency manifested in developmental defects on

later developing organ systems. Whole mount *in situ* hybridization shows that ezh2 morphants have normal staining for fabp10, a marker for development of liver, and insulin, the marker for endocrine pancreas (Figure 4.4). Normal staining in these markings indicates that while developmental defects exist in the ezh2 morphant, total organism development is not delayed. However, while some markers develop normally, whole mount *in situ* hybridization of myoD, the marker for somite development, is affected by ezh2 loss, and alcian blue staining of 72 hpf ezh2 morphants indicates a total loss of cartilage formation (Figure 4.4). These defects can be partially or completely rescued by coinjection with *ezh2* mRNA (Figure 4.5).

We show in Chapter 3 that APC mediates regulation of H3K27me3. As APC is responsible for the differentiation of the zebrafish intestine, we were interested in determining whether ezh2 was also involved in this process and whether this affected multiple cell types or it was unique to the enterocyte lineage. While whole mount *in situ* hybridization of gata6, the marker for primordial gut development, was unaffected in ezh2 morphant embryos, three markers for differentiated cells in the intestine were affected by ezh2 hypomorphism. Two markers for enterocytes, i-fabp (fixed at 72 hpf) and pept1 (fixed at 102 hpf) and a marker for goblet cells, agr2 (fixed at 98 hpf) were absent in the ezh2 morphant (Figure 4.4), indicating that ezh2 plays a role in the proper differentiation of multiple cell types in the developing intestine. (The expression of i-fabp could be partially restored by coinjection with ezh2 mRNA, as shown in Figure 4.5.)

4.5 Discussion

Mouse knockouts of the histone methyltransferase Ezh2 are embryonic lethal, corroborating its necessity in early development (*3*). Studies have shown that Ezh2

coordinates the gene expression during differentiation of stem cells (*48*), indicating a role

for Ezh2 in cell fate decisions, and this may explain its necessity during early

development. Because of the inability to study Ezh2 loss in the developing mouse, we

used the developing zebrafish embryo to study ezh2 and how its loss affects early

development.

Whole mount *in situ* hybridization of ezh2 mRNA shows that ezh2 is expressed

ubiquitously in the embryo from 1 hpf through 24 hpf. This ubiquitous expression is

consistent with the need for ezh2 in early embryonic development. After 24 hpf, ezh2

mRNA expression becomes consistently anterior up to 4 dpf, with expression becoming

diffuse through the brain from 5 dpf and older. This may suggest that as the embryo ages

and has more developed organ systems, the need for ezh2 decreases. This would support

a role for ezh2 in the development and proper cell fate decisions in organogenesis. Other

studies have shown that ezh2 expression is associated with highly proliferative tissue and

is less needed in more differentiated tissues (*49*). This is consistent with ezh2 being

expressed ubiquitously in the early development of the zebrafish embryo, as cells are

rapidly dividing to form organ systems and anatomical structures.

Morpholino knockdown of ezh2 provided an approximately 50% of knockdown

of H3K27me3, which was used as a readout for ezh2 activity. These hypomorphic

embryos displayed a range of phenotypes, including the lack of cartilage formation in the

jaw and fins and a curvature of the spine resulting in a "curly tail" phenotype. The lack

of cartilage formation is reminiscent of retinoic acid deficient zebrafish (particularly the

apc[mcr] zebrafish line) (*47*). The tail phenotype seen was reminiscent of the spinal

malformations that occur in PRC1 knockout mice (*31, 50, 51*). Vertebral abnormalities

are also common defects seen in hox mutant mouse models (*52–54*). Several hox genes were transcriptionally misregulated in the ezh2 morphant (data not shown), so these types of patterning malformations in the spine provide additional evidence that ezh2 is playing a role in the regulation of hox genes in the developing zebrafish.

Ezh2 hypomorphism displayed a variety of different phenotypes with regards to development and organogenesis. Although ezh2 is largely expressed in the brain at later stages, early brain markers did not show defects, indicating that an incomplete knockdown is insufficient to disrupt brain development or that lack of ezh2 can be compensated for in early brain development. At the 72 hpf stage, endocrine pancreas and liver were also not affected by lack of ezh2. However, underdeveloped somite formation and loss of cartilage formation in the jaw and fins were observed in the ezh2 morphant. These results show that hypomorphism of ezh2 does not delay total embryonic development. This is at odds with what is seen in the mouse knockout, but not inconsistent with hypomorphism of other chromatin modifiers in developing zebrafish (*55, 56*). While gradients of these modifiers display tissue specific developmental defects, not experiencing a total loss of these factors allows for study in a manner that is unavailable in mammalian systems. Underdeveloped somite formation and loss of cartilage development are two phenotypes that are seen in retinoic acid deficient models (*51, 57, 58*). These results are striking because the ezh2 morphant appears to be retinoic deficient, but the data presented in Chapter 3 preclude rescue by treatment with exogenous RA. The results presented in this chapter give additional implications that RA is responsible for the regulation of H3K27me3, the mark laid down by ezh2 enzymatic activity, but also adds evidence that ezh2 is a downstream effector of retinoic acid

signaling. Both retinoic acid and ezh2 are required for proper development and differentiation in certain organ systems.

While we had previously seen a phenotype in the developing gut similar to apc[mcr] zebrafish, we wanted to further characterize what effect the hypomorphism of ezh2 had on the developing intestine. Primordial gut develops normally in ezh2 morphants, but markers for two types of differentiated cells (enterocytes and goblet cells) are absent in ezh2 morphants, indicating that loss of ezh2 affects multiple types of differentiated cells. This suggests that ezh2 is playing a role at either the stem cell level, affecting the transition into transit-amplifying cells, or at the transit-amplifying cell level, causing lineage specification, and when ezh2 is lost, these cells are not able to successfully complete differentiation. While these results state that ezh2 is necessary for the rudimentary regulation of cell differentiation, this has far reaching implications for the misregulation of ezh2 in cancer. Several cancer types have decreased expression or activity of ezh2, and we present a new model wherein the readout of ezh2 activity, H3K27me3, is decreased when APC is lost. The loss of H3K27me3 leads to inability of cells to differentiate, which is a hallmark of carcinomas (*59, 60*). Treatments that reverse this inability to differentiate have the potential to be more effective treatments and would be more specific for cancer cells than current therapies.

These findings allow for new insights into the role of ezh2 in development. Of particular interest is the tissue specific defects seen, as they open up novel areas of research for how ezh2 plays a role in the differentiation, both in development and with the potential for adult tissues with high levels of turnover.

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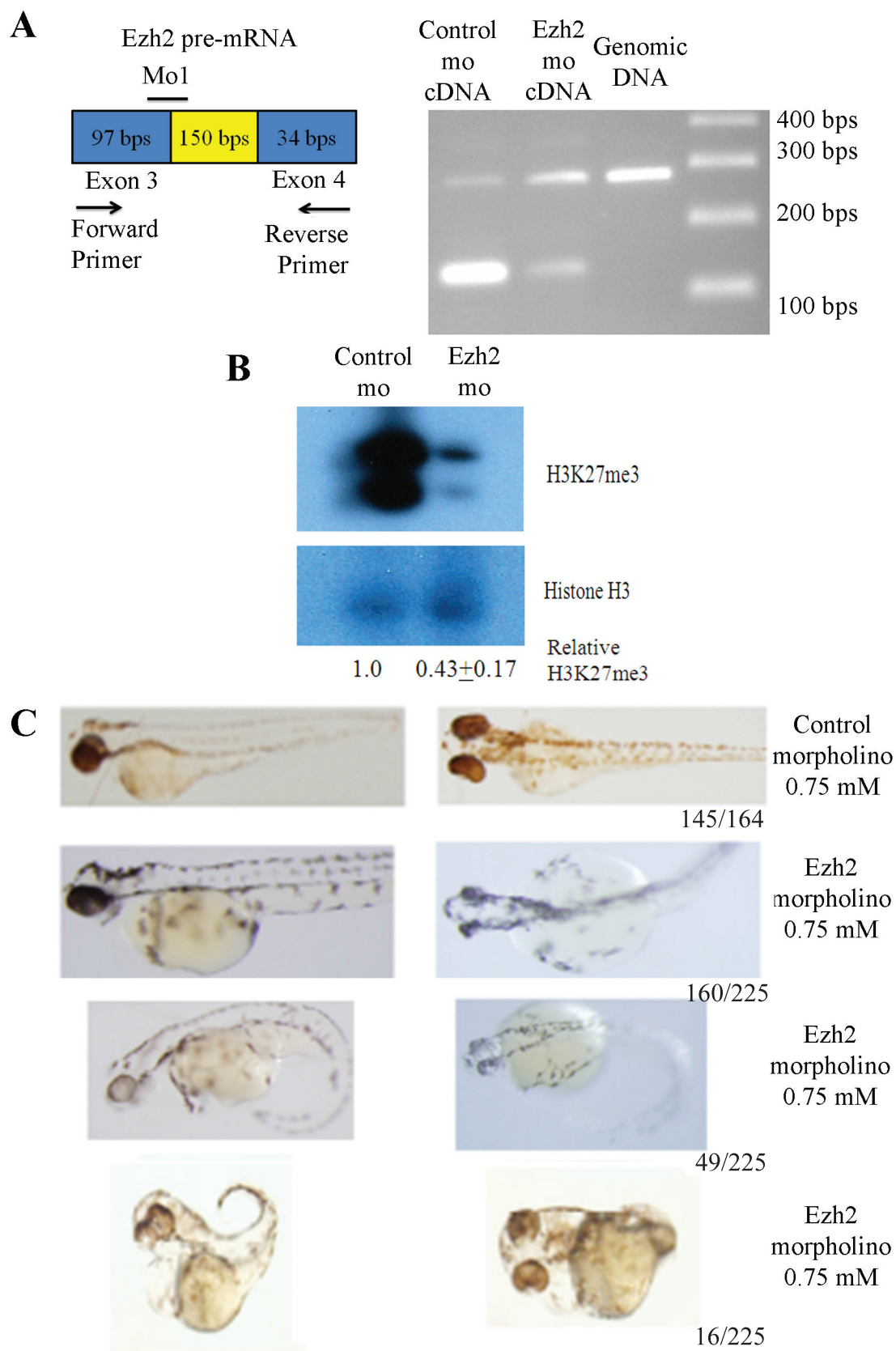
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Figure 4.1 Ezh2 is expressed ubiquitously in the early embryo and becomes increasingly anterior as development progresses. Whole mount *in situ* hybridization of ezh2 at the given developmental timepoints. Ezh2 is expressed as early as 1 hour postfertilization and continues to be expressed ubiquitously until 24 hpf. It becomes increasingly anterior and is diffusely expressed in the head at 5 dpf.

Figure 4.2 Ezh2 hypomorphism leads to decreased levels of H3K27me3 and morphological defects. A, schematic displaying the location of ezh2 splice block morpholino and RT-PCR assaying that splicing. Embryos injected with ezh2 morpholino show more unspliced RNA than do embryos injected with control morpholino. B, western blot of ezh2 morphant embryos. Ezh2 morphants show decreased levels of H3K27me3. Levels of H3K27me3 are quantified and normalized to control injected embyro sample. Relative levels and standard deviation are shown (n = 4). C, brightfield microscopy of ezh2 morphant phenotypes. Embryos grown in the absence of phenylthiourea.
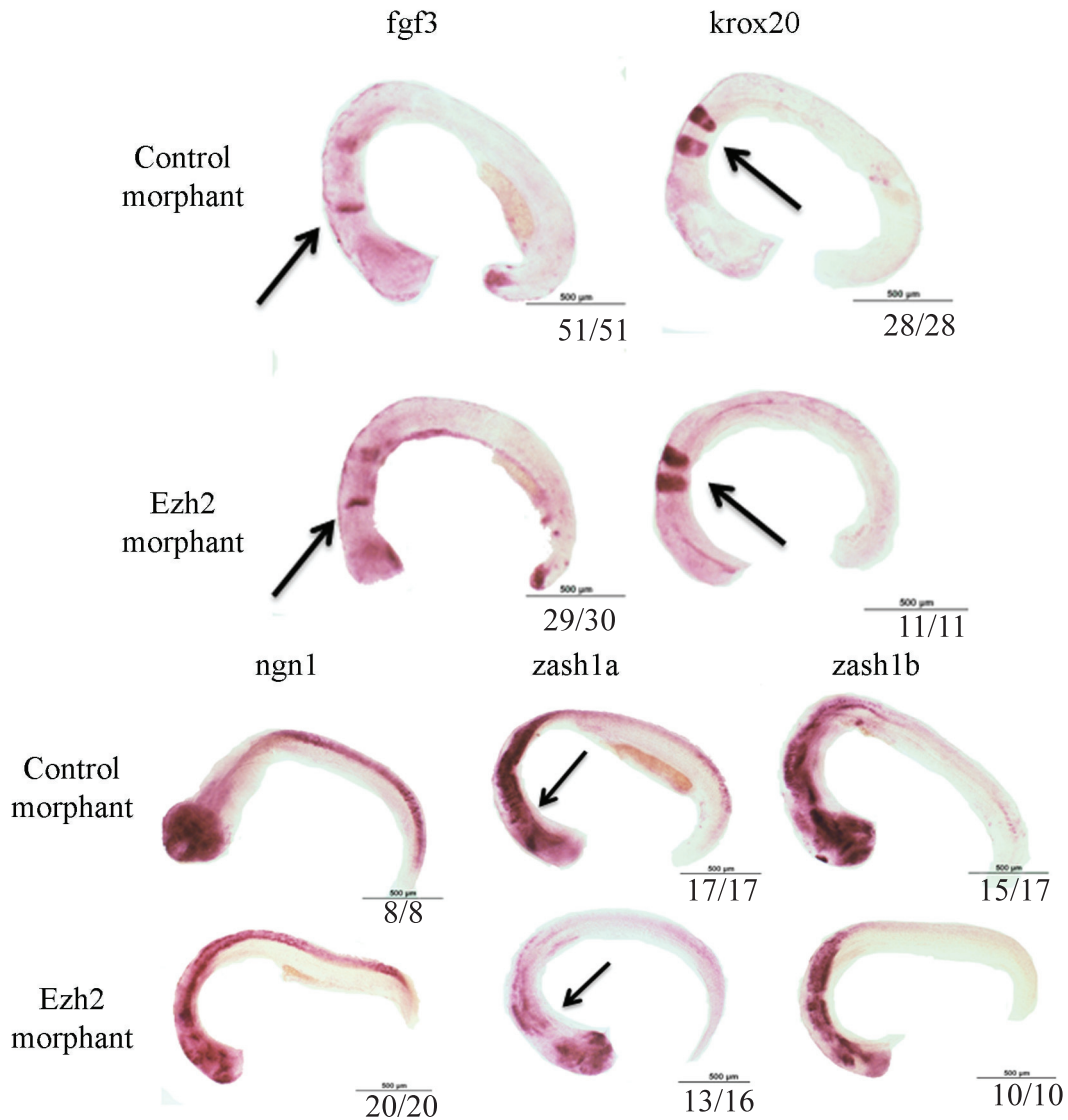
A

Ezh2 pre-mRNA

Mo1

97 bps	150 bps	34 bps

Exon 3   Exon 4

→   ←
Forward   Reverse
Primer   Primer

Control mo cDNA   Ezh2 mo cDNA   Genomic DNA

400 bps
300 bps
200 bps
100 bps

B

Control mo   Ezh2 mo

H3K27me3

Histone H3

Relative H3K27me3

1.0   0.43±0.17

C

Control morpholino 0.75 mM

145/164

Ezh2 morpholino 0.75 mM

160/225

Ezh2 morpholino 0.75 mM

49/225

Ezh2 morpholino 0.75 mM

16/225

Figure 4.3 Hypomorphism of ezh2 does not affect early brain development.
Whole mount *in situ* hybridization of early brain markers at 18 hpf (fgf3, krox20)
and 30 hpf (ngn1, zash1a, zash1b). Arrows indicate expression (fgf3, krox20) and
changes in expression (zash1a). Hypomorphism of ezh2 does not affect expression
of fgf3, krox20, ngn1, or zash1b. There is slight but not total loss of zash1a
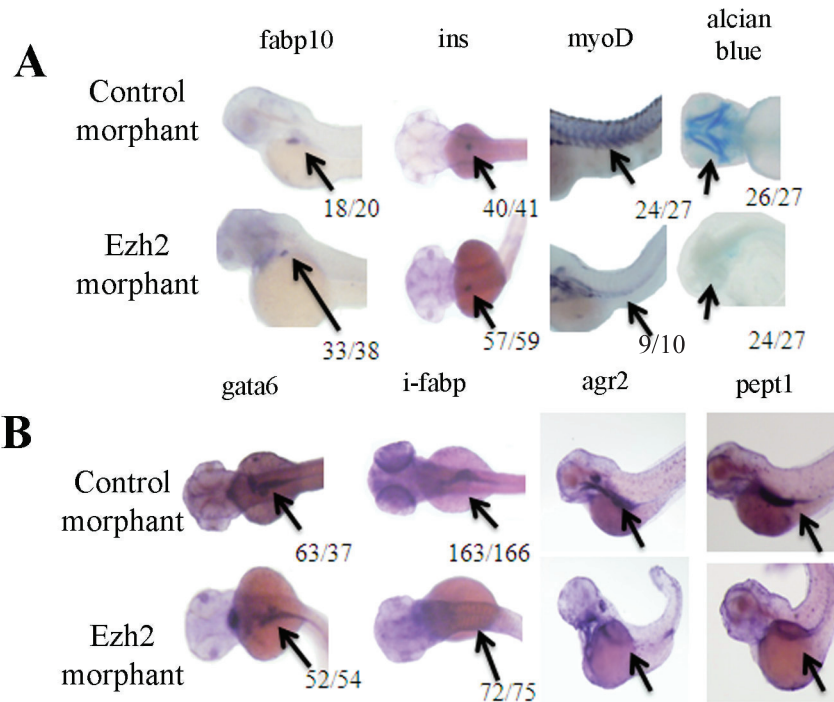expression.

Figure 4.4 Hypomorphism of ezh2 allows for normal development of liver and endocrine pancreas, but causes loss of cartilage formation, decrease in skeletal muscle, and loss of multiple cell lineages in the intestine. A, whole mount *in situ* hybridization and alcian blue staining of ezh2 morphants at 72 hpf. Liver (fabp10) and endocrine pancreas (ins) develop normally in the ezh2 morphant, but skeletal muscle (myoD) and cartilage (shown by Alcian Blue staining) formation are impaired. Arrows indicate zebrafish liver (fabp10), pancreas (ins), somites (myoD), and jaw (alcian blue). B, whole mount *in situ* hybridization of ezh2 morphants at 72 hpf (gata6, ifabp), 98 hpf (agr2), and 102 hpf (pept1). While primordial gut develops in the ezh2 morphant, multiple terminally differentiated intestinal cell lineages are lost. Arrows indicate zebrafish intestine.
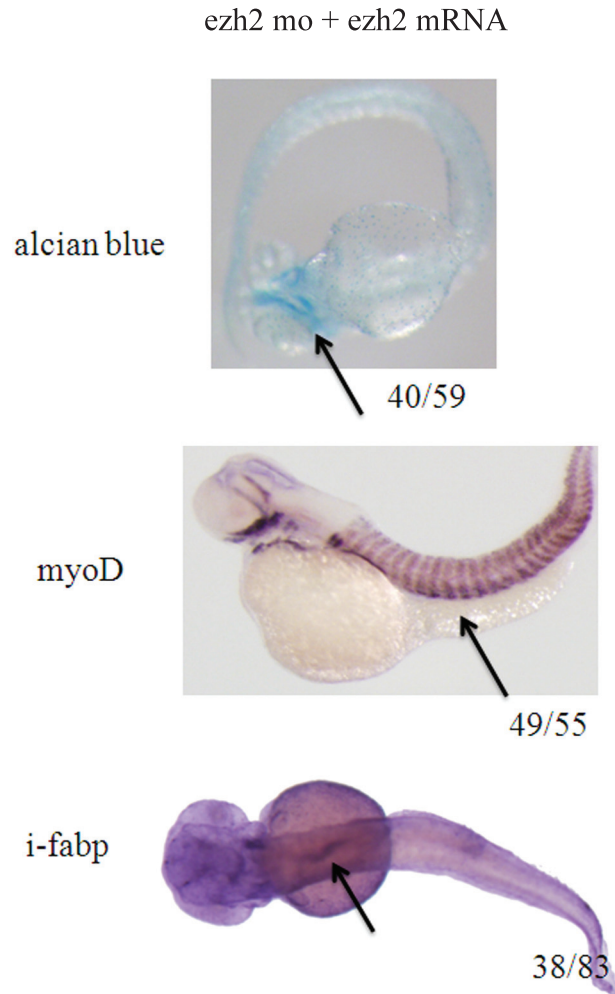
ezh2 mo + ezh2 mRNA

alcian blue

40/59

myoD

49/55

i-fabp

38/83

Figure 4.5 Ezh2 morphant phenotypes can be rescued by injection of ezh2 mRNA. Whole mount *in situ* hybridization and alcian blue staining of embryos coinjected with ezh2 morpholino and ezh2 mRNA. Top panel, alcian blue staining. Arrow indicates staining in jaw. Ezh2 morphants coinjected with ezh2 mRNA have cartilage formation in the jaw. Second and third panels, *in situ* hybridizations of myoD (arrow indicates zebrafish somites) and i-fabp (arrow indicates zebrafish intestine), 2 marker lost in ezh2 morphants. Coinjection with ezh2 mRNA restores these marks partially (i-fabp) or completely (myoD).

CHAPTER 5

CONCLUSION

5.1 Summary and Perspectives

This dissertation produces new insights into how epigenetics plays a role in the differentiation process of the intestine. In doing so, it further characterizes how retinoic acid is able to carry out the daunting task of regulating intestinal differentiation. The data paint a clear picture of two epigenetic marks that deviate when APC is mutated, displaying a broader role for APC in the control of epigenetics during the differentiation process. They also introduce a role for the histone methyltransferase Ezh2 in the regulation of differentiation in the intestine and describe how partial loss of Ezh2 affects the developing animal.

This study traces the path of retinoic acid in its role in the developing intestine. We show that retinoic acid biosynthesis is controlled through APC by the histone demethylase LSD1. LSD1 is misregulated when APC is absent and aberrantly demethylates the promoter of rdh1l, effectively turning off transcription. This is one of many factors that is responsible for the repression of retinoic acid biosynthesis when APC is mutated (*1, 2*), but this adds a direct mechanism by epigenetic repression. It also adds a potential drug target for the treatment of mutated APC syndromes. We also show that retinoic acid controls the levels of H3K27 trimethylation in the developing embryo, adding another level of epigenetic regulation under the control of retinoic acid as well as

suggesting potential mechanisms for RA-mediated differentiation. We establish an epistatic mechanism whereby retinoic acid, through its regulation of the expression of cyclooxygenase-2, regulates H3K27me3. In this mechanism, unlike what we have previously seen with the DNA demethylase components, RA does not control the levels of PRC2 transcripts, introducing a novel paradigm whereby RA may control chromatin modifiers (Figure 5.1).

The roles of APC in the regulation of retinoic acid and intestinal differentiation and in the regulation of chromatin modifiers that are involved in this process instruct proper cellular differentiation and, when absent, the progression of tumorigenesis. Progenitor-like cells contain higher levels of LSD1, lower levels of the RA biosynthetic machinery, and lower levels of H3K27me3. APC drives a transition, through its regulation of retinoic acid production and chromatin modifiers, that gives rise to a differentiated cell, with higher levels of RA, more H3K27me3 to shut down improper signaling pathways, and less LSD1 (Figure 5.1). When APC is absent, in the case of cancer, this transition cannot happen, leaving the cancer cell in a more progenitor-like state, which leads to defective cell fating and hyperproliferation. The chromatin state of these cells is of particularly importance. This work shows that APC mutation leads to a more permissive chromatin state, containing less H3K27me3, a hallmark of undifferentiated cells. The inability to regulate H3K27 methylation – and thus the multiple pathways required for differentiation that this mark has been shown to occupy – highlights the undifferentiated state of a cell harboring an APC mutation.

This study also shows that both retinoic acid and Ezh2 are necessary for the terminal differentiation of the intestine in developing zebrafish. Ezh2 plays a role in the

regulation of intestinal differentiation, either at the stem cell or transit-amplifying cell level, as is shown by the absence of multiple differentiated cell types when Ezh2 is knocked down.

In addition to its role in the intestine, we show morphological and developmental defects of Ezh2 absence in a vertebrate. A loss of ezh2 leads to a curvature of the spine, a phenotype similar to the vertebral defects seen in the loss of PRC1 components in mice (*3–5*), as well as decrease in skeletal muscle formation and loss of cartilage in fin and jaw, additional retinoic acid deficient phenotypes (*6–8*). Although RA is incapable of rescuing the loss of terminal differentiation in the intestine in the ezh2 morphant, it is obvious from these phenotypes that retinoic acid plays a role in the regulation of PRC2 in the developing intestine, albeit upstream of PRC2.

Taken together, these findings not only increase our understanding of how histone modifiers plays a role in the regulation of intestinal differentiation and the effects of their aberration in cancer, they also highlight the importance of understanding the regulation of epigenetic marks and the mechanism by which they are deposited. While we provide an epistatic mechanism for the regulation of H3K27 methylation, it is still unclear how these marks are misregulated when APC is mutated. Such an understanding would open new areas of study and provide for potential drug targets in FAP and other mutated APC syndromes.

5.2 Future Directions

The data presented in this dissertation allow for several interesting new areas of study. While we continue to increase our knowledge of which factors are misregulated when APC is mutated, it is still unclear how loss of APC affects each of these factors.

We show that LSD1 is aberrantly expressed in conditions of APC mutation, but it is unclear why it is misexpressed. As it acts upstream of retinoic acid loss, it is not controlled, as many factors misregulated in APC mutation, by retinoic acid, so APC must have additional ways of controlling epigenetic regulators. We also do not have a clear idea of which factors can cause LSD1 to switch substrate preference from methylated H3K9 to methylated H3K4. Future work to examine additional roles of APC in the regulation of upstream regulators of RA biosynthesis will shed light on how the epigenetic regulators of cellular differentiation are controlled as well as elucidate further mechanisms and pathways that cells use to undergo the transition from stem to terminally differentiated cell.

Of great interest would be the underlying mechanism of how Cox-2 regulates H3K27 trimethylation, regarding both the pathways Cox-2 employs to regulate this mark and the process through which the effectors of the mark are modified. We have explored theories on both counts, but all have proved inconclusive. We were unable to determine whether localization of H3K27me3 changes in apc^{mcr} zebrafish, a method of regulation that has the potential to affect the expression of multiple differentiation pathways. We also explored the effect of multiple prostaglandins, the final products of Cox-2 enzymatic activity (*9, 10*), on H3K27me3, but saw either no effect or an effect inconsistent with Cox-2 overexpression. More exploration with the prostaglandins may prove fruitful in determining how H3K27 methylation is misregulated in the absence of APC. Another area of study could include the regulation of Ezh2 by the Akt pathway. Akt is overexpressed in early adenomas when APC is mutated (*11*). Ezh2 has been shown to be phosphorylated by Akt. This phosphorylation affects its activity, causing decreases in

H3K27 trimethylation (*12*). This pathway may be aberrantly activated in cells with APC mutation.

Further exploration into the roles of ezh2 in development could also prove fruitful. Determining where ezh2 affects differentiation in the intestine (at the stem cell- or transit amplifying cell-level) could provide insights into how the pathways governing these processes are regulated. This also has the potential to reveal stem cell markers in the intestine, a field currently poorly understood. Other studies have shown that ezh2 contains an RNA-binding domain (*13*). This is consistent with previous findings that PRC2 can be recruited by long noncoding RNAs, but it raises questions as to whether, through this domain, Ezh2 can play regulatory roles unrelated to its histone methyltransferase activity. Determining whether catalytic mutants of Ezh2 or RNA-binding mutants of Ezh2 would be able to rescue the defects seen in the guts of ezh2 morphants would provide unique perspectives into what Ezh2 is doing in the developing intestine. It could also provide insight into any regulation of intestinal differentiation by long noncoding RNAs, a method not currently seen in intestinal development.

5.3 References

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Figure 5.1 APC controls cell fating through chromatin modifiers. A, schematic representation of data presented within this dissertation. APC uses multiple chromatin modifiers, both to control RA biosynthesis, and through RA to regulate cell fating. Black lines indicate functions that promote proper cell fating; red lines indicate functions that lead to defective cell fating. B, implications of APC control over regulation of differentiation. APC, through its control of retinoic acid production and chromatin modifiers, is responsible for the transition from a progenitor cell to a differentiated cell.

A

B