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MOLECULAR MECHANISMS OF KRÜPPEL-LIKE FACTOR 4

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MOLECULAR MECHANISMS OF KRÜPPEL-LIKE FACTOR 4

by

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Dedicated to my family, who have encouraged and supported me in all my endeavors;
and to my fiancé, who has always loved me unconditionally.

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Becoming a physician/scientist has long been a dream of mine (before I even knew there was such a thing), and I hope that everyone who's helped me along the way will be proud of my future accomplishments, whatever they may be.

MOLECULAR MECHANISMS OF KRÜPPEL-LIKE FACTOR 4

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The Wnt/β-catenin pathway is a key pathway involved in the regulation of proliferation and differentiation within many tissues, including the epithelium of the intestine and of the skin. Wnt signaling is implicated in stem cell renewal. Deregulation of Wnt/β-catenin signaling is crucial early event in colorectal tumorigenesis. Earlier work in our lab demonstrated that Krüppel-like factor 4 (KLF4) interacts with β-catenin *in vivo*, repressing Wnt signaling and inhibiting tumor growth. KLF4 is an anti-proliferative transcription factor expressed in differentiated epithelial cells in the intestine. Previous studies clearly establish KLF4 as a tumor suppressor in colorectal cancer. Expression of KLF4 is downregulated in colorectal tumors, and heterozygous deletion of the Klf4 allele in a mouse model of colorectal cancer results in the formation of approximately 50% more tumors. In addition, KLF4 is important in stem cell programming. KLF4, in combination with three additional transcription factors, is sufficient to reprogram differentiated fibroblasts into embryonic stem cells. This dissertation focuses on the molecular mechanisms of KLF4-mediated transcription, both in the context of KLF4-mediated activation and KLF4-mediated repression. I demonstrate that the N-terminal transactivation domain of KLF4 recruits the co-activator p300/CBP to the promoter of the positively-regulated gene IAP, resulting in increased histone acetylation. On the negatively regulated gene, Cyclin B_1, I demonstrate that KLF4 recruits HDAC3, decreasing histone acetylation. In addition, I show that KLF4 is acetylated by p300/CBP, and that acetylation of KLF is important in the activation of target genes as well as its ability to inhibit proliferation. I demonstrate that KLF4 inhibits Wnt/β-catenin signaling by blocking β-catenin-mediated recruitment of p300/CBP to Wnt-regulated genes. Finally, I show that acetylation of β-catenin is important in its ability to interact with p300/CBP and that KLF4 inhibits β-catenin acetylation. These studies provide significant insight into the molecular mechanisms of KLF-mediated transcription, and will prove useful in the development of targeted therapies for colorectal cancer as well as providing a deeper understanding of the mechanisms behind stem cell reprogramming.

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LIST OF ABBREVIATIONS

APC	Adenomatous Polyposis Coli
APP	Amyloid Precursor Peptide
β-TrCP	β-Transducin Repeat Containing Protein
BCL9	B-Cell Lymphoma 9
BrdU	Bromodeoxyuridine
CBP	CREB Binding Protein
CH3	Cysteine/Histidine-rich domain 3
ChIP	Chromatin Immunoprecipitation
CKIα	Casein Kinase Iα
CtBP	C-terminal Binding Protein
DAPI	4',6-diamidino-2-phenylindole
Dkk1	Dickkopf-1
ES	Embryonic Stem cell
Fabp1	Fatty acid binding protein 1
FAP	Familial Adenomatous Polyposis
FHL2	Four-and-a-Half LIM domain protein
FITC	Fluorescein-5-isothiocyanate
GFP	Green Fluorescent Protein
GNU	GNU's Not UNIX
GSK-3β	Glycogen Synthase Kinase 3β
GST	Glutathione S-Transferase
hARD1	Human Arrest Defective 1
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HDC	Histidine Decarboxylase
HTML	HyperText Markup Language
IAP	Intestinal Alkaline Phosphatase
IBMX	3-Isobutyl Methylxanthine
ICAT	Inhibitor of β-Catenin And TCF
iNOS	Inducible Nitric Oxide Synthase
iPS	Inducible Pluripotent Stem cell
KAP1	KRAB-Associated Protein 1
KLF4	Krüppel-Like Factor 4
LEF	Lymphoid Erythroid Factor
LOH	Loss Of Heterozygosity
LPS	Lipopolysaccharide
LRP5/6	LDLR Related Protein 5/6
MOI	Multiplicity of Infection
MTT	Methyl Thiazol Tetrazolium
NaBT	Sodium Butyrate
N-CoR	Nuclear Co-Repressor
NLS	Nuclear Localization Sequence

OD	Optical Density
ODC	Ornithine Decarboxylase
PAF1	Polymerase II-Associated Factor complex 1
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
PHD	Plant Homeodomain
PP2A	Protein Phosphatase 2A
PSG-5	Placental Specific Glycoprotein 5
RB	Retinoblastoma protein
RK3E	Rat Kidney Epithelial Cells, E1A-immortalized
RT-PCR	Reverse Transcriptase PCR
SCF	Skp1/Cul1/F-box protein complex
SUMO	Small Ubiquitin-like Molecule
STAT	Signal Transducer and Activator of Transcription
SWI/SNF	SWItch/Sucrose Non-Fermenting
TBP	TATA Binding Protein
TCF	T-Cell factor
TGF-β	Tumor Growth Factor β
TIP	TBP Interacting Protein
TLE	Transducin-Like Enhancer Protein
TPA	12-O-tetradecanoyl phorbol-13 acetate
TSA	Trichostatin A
WT	Wild-type

CHAPTER 1: WNT/BETA-CATENIN SIGNALING, KLF4, AND COLON CANCER

The canonical Wnt/β-catenin pathway is used by organisms in a variety of contexts, including establishing the anterior/posterior axis in the developing embryo, maintaining the pluripotent state of stem cells, and controlling proliferation of epithelial cells in the intestine and the skin. KLF4 can directly interact with β-catenin and inhibit Wnt signaling (Zhang et al., 2006). Krüppel-like factor 4 (KLF4) is a transcription factor expressed in many tissues, including the intestine and the skin, where it regulates proliferation and differentiation. Deregulated Wnt signaling results in hyper-proliferation and is critical in colorectal tumorigenesis (Clevers, 2006), whereas KLF4 inhibits proliferation (Shields et al., 1996) and is frequently down-regulated in colorectal cancer (Shie et al., 2000b; Zhao et al., 2004). This chapter will present an overview of the roles of Wnt signaling and KLF4, in epithelial homeostasis of the normal intestine and in colorectal carcinoma.

OVERVIEW OF THE SMALL AND LARGE INTESTINE

In order to fully understand the role of the Wnt pathway and KLF4 in the intestine, we will first discuss the histologic structure of the small and large intestine as well as general molecular mechanisms of colorectal carcinoma.

Structure of the Small and Large Intestine

The luminal surface of the small intestine consists of an undulating epithelium that can be roughly divided into two regions: crypts and villi (**Fig. 1**). Recessed pores in the epithelium are known as crypts of Lieberkühn. Each crypt consists of a single stem cell near the bottom of crypt and transit-amplifying cells that divide three to four times as they migrate towards the epithelial surface (Schmidt et al., 1988). Projecting into the intestinal lumen are small, finger-like structures known as villi. Villi consist of mostly differentiating and fully-differentiated cells, which are ultimately sloughed off from the tip of a villus. The epithelium of the small intestine consists of only four different differentiated cell types: enterocytes, goblet cells, enteroendocrine cells, and Paneth

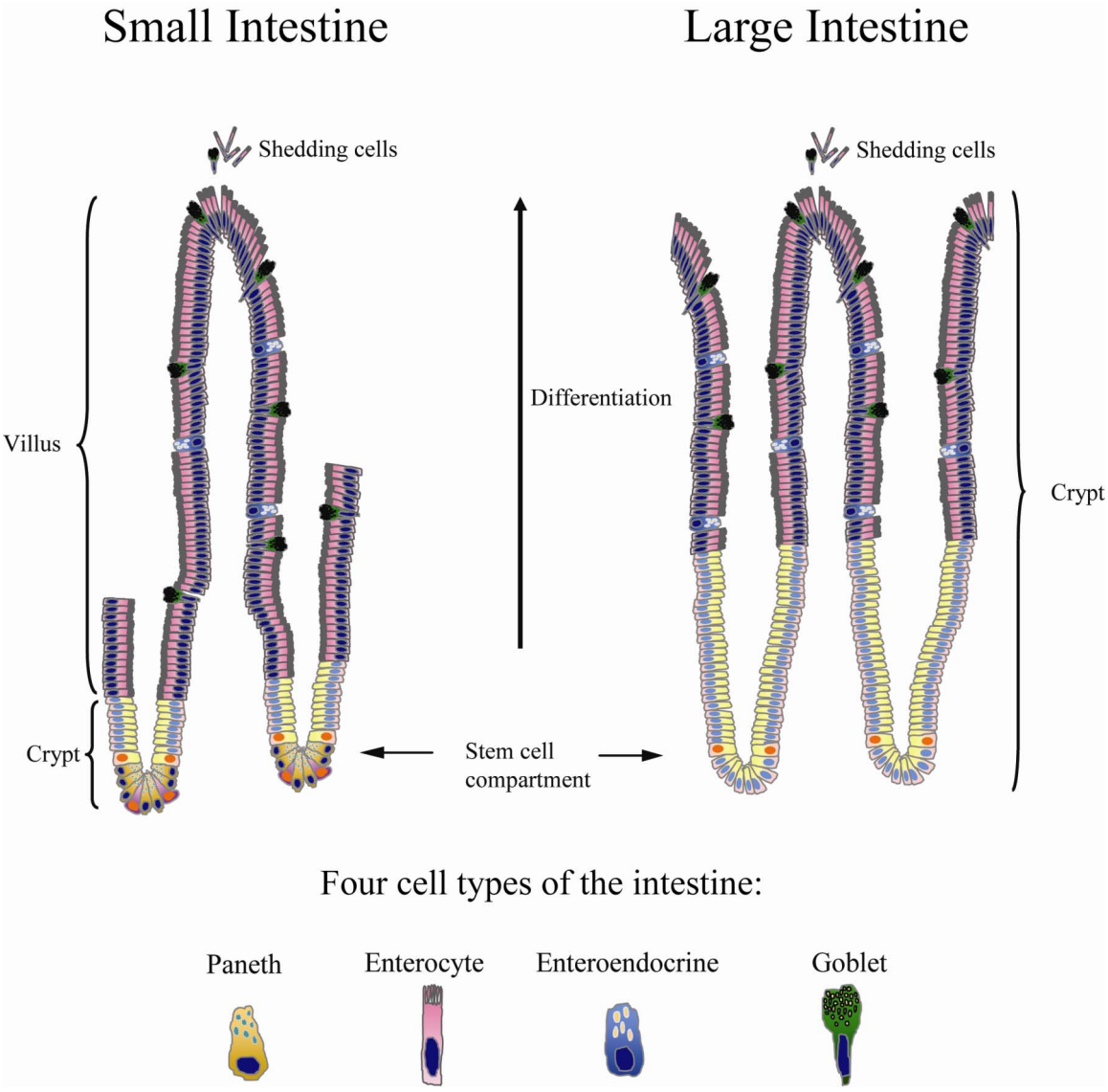

Figure 1: Structure of the small and large intestine. A, Structure of the small intestine. Cross-sectional view of the epithelium. Recessed pores in the epithelium known as crypts contain proliferative progenitor cells (yellow cytoplasm/blue nuclei), stem cells (yellow cytoplasm/orange nuclei), and differentiated Paneth cells. Finger-like structures extending into the lumen of the small intestine contain differentiated cells. Enterocytes constitute the majority of the cells found in the villus, but occasional enteroendocrine and goblet cells can be found as well. B, Structure of the large intestine. The large intestine has no villi. Instead, the large intestine is a smooth surface containing only pores into the crypts. Here, proliferative progenitors and differentiated cells are all found in the crypt. Paneth cells are absent in the large intestine.

cells. Absorptive enterocytes are by far the majority, constituting 90% of all differentiated cells; goblet cells secrete mucins and protect the epithelium; enteroendocrine cells secrete serotonin, substance P, and secretin (Hocker and Wiedenmann, 1998); and Paneth cells produce defensins and lysoyzme, which function as antimicrobial agents (Porter et al., 2002). The turnover rate of the intestinal epithelium is exceptionally high, and the lifespan of each cell is only 3 to 5 days (Potten et al., 1997). Underneath the intestinal epithelium is a basal lamina, followed by a layer of loose connective tissue and network of smooth muscle cells, neurons, lymphocytes, blood vessels, and lymphatic vessels (Gregorieff and Clevers, 2005).

The large intestine differs somewhat in that it lacks villi, and instead all proliferation and differentiation occur entirely within the crypts. The bottom two thirds of the crypt contain proliferating cells, whereas the top third contains differentiated cells. In addition, the large intestine lacks Paneth cells, and intestinal stem cells are found at the base of crypt (Booth and Potten, 2000).

Colorectal Cancer

Colorectal cancer is the third most common cancer in the United States and the second-most common cause of cancer-related death (Wolpin and Mayer, 2008). About 50% of Americans will get at least one colonic polyp by age 70 (Jemal et al., 2002). The American Society for Gastrointestinal Endoscopy recommends that all Americans over age 50 have regular colonoscopies to screen for colorectal tumors (Winawer, 2007).

Histopathologic and molecular studies of colorectal tumors at various stages led to the development of a multi-stage progression model of colorectal carcinogenesis (Fearon and Vogelstein, 1990). According to this model, all colorectal cancers originate from a single normal cell that undergoes a mutational event. This mutation gives the cell a proliferative advantage relative to neighboring cells, leading to formation of a small region hyperproliferative epithelium known as an aberrant crypt focus. Every time a cell divides, it will make several DNA copying errors. Thus hyperproliferative cells are more likely to have additional advantageous mutations occur, conferring additional proliferative advantages. These mutational events lead to the development of a pre-

cancerous lesion known as an adenoma, followed by carcinoma *in situ*, and eventually a metastatic tumor. According to this model, a minimum of five mutational events must occur in order for a metastatic tumor to develop.

Most colorectal tumors are sporadic in nature. However, approximately 10% are hereditary and inherited in an autosomal dominant manner (Lynch and de la Chapelle, 2003). The most common hereditary colorectal carcinoma syndrome is Familial Adenomatous Polyposis (FAP). In FAP, the colon of affected individuals becomes covered within hundreds of pre-cancerous adenomas by their early twenties (Haggitt and Reid, 1986). As one of these adenomas will invariably progress to cancer, removal of the entire colon is the only definitive treatment.

Early investigators quickly recognized the familial nature of FAP, and mapped the chromosomal location of the gene responsible to 5q21 (Bodmer et al., 1987; Herrera et al., 1986; Leppert et al., 1987). However, it wasn't until several years later that *APC* was definitively identified as the causative gene (Groden et al., 1991; Kinzler et al., 1991; Nishisho et al., 1991). FAP patients carry one wild-type allele and one mutant allele for the *APC* gene. However, early-stage polyps harvested from patients have lost the wild-type allele, implicating *APC* as a potential tumor suppressor gene.

Surprisingly, similar mutations in the *APC* gene occur in the majority of sporadic tumors as well (Jen et al., 1994; Miyoshi et al., 1992). *APC* is mutated in aberrant crypt foci, an early pre-cancerous lesion in the development of colorectal cancer (Powell et al., 1992), suggesting that *APC* is critical in the pathogenesis of colorectal carcinoma. Subsequent research demonstrated that the *APC* gene codes for the Adenomatous Polyposis Coli (APC) protein product, and that APC is a major regulatory component of the Wnt pathway. The Wnt pathway and its role in cancer will be discussed in greater detail in the next section.

THE WNT SIGNALING PATHWAY

A fascinating tribute to the economy of evolution is its ability to re-use the same pathway for different purposes, both in different tissues and at different times during

development. This is true for the Wnt pathway, and like many other signaling pathways, Wnt signaling has diverse functions. With few exceptions, the majority of Wnt target genes are cell type-specific, likely due to differences in the chromatin environment (Wohrle et al., 2007). Notably, this pathway and the majority of its components are preserved in many model organisms, including *Drosophila melanogaster*, *Caenorhabditis elegans*, *Xenopus laevis*, and *Mus musculus*.

The Wnt-1 gene was first identified as a preferential insertion site for the Murine Mammary Tumor Virus (MMTV). Insertion of MMTV into this gene results in overexpression of the Wnt-1 and the formation of mammary tumors (Nusse and Varmus, 1982). Later studies found that Wnt gradients are used during development in the formation of the anterior/posterior axis (Huelsken et al., 2000; Liu et al., 1999b) and deciding between an endodermal and mesodermal cell fate (Han, 1997). In addition, active Wnt signaling is crucial in stem cell renewal in the intestine (Kielman et al., 2002), bone (Reya and Clevers, 2005), blood (Reya et al., 2003), skin (Alonso and Fuchs, 2003; Lowry et al., 2005), and embryonic stem cells (Miyabayashi et al., 2007). Within the stem cell compartment of the intestine, Wnt signaling drives proliferation of transit-amplifying cells. The progressive loss of Wnt signaling as the cells migrate away from the crypts and towards the surface of the colonic epithelium is thought to induce differentiation (Clevers, 2006). *LGR5* is a Wnt target gene specifically expressed in crypt stem cells. Lineage-tracing experiments demonstrate that the Lgr5+ cell can repopulate the entire crypt and differentiate into all three cell types (Barker et al., 2007), suggesting that it is a definitive marker of intestinal stem cells.

Major Components of the Wnt Pathway

In the canonical Wnt pathway, a large number of components work together to transduce an external signal into changes in gene expression within the target cell. Wnt is a secreted ligand that binds to its receptor at the cell membrane, ultimately resulting in the stabilization of cytoplasmic β-catenin (**Fig. 2**). β-catenin then accumulates in the cytoplasm, enters the nucleus, and activates expression of Wnt target genes. β-catenin activates transcription by interacting with the TCF/LEF family of transcription factors

and recruiting co-activators such as p300/CBP, Pygopus, and BCL9/Legless. This section will explore the many components of the Wnt pathway in more detail.

Structure of β-catenin

As β-catenin is a critical molecule in mediating Wnt signaling in multiple sub-cellular compartments, it is important to briefly summarize its structure and overall function. β-catenin is a 781 amino acid protein and has several domains that are important for its function. The N-terminus (amino acids 1-127) is unstructured and is critical in regulating stability of β-catenin. The central core of the protein (amino acids 127-666) interacts with many of the known binding partners of β-catenin. The central core consists of twelve repeating sequences known as Armadillo repeats, named after the *Drosophila* homolog of β-catenin, *Armadillo*. Each repeat contains of three alpha helices, and the twelve Armadillo repeats interact with each such that the central core of β-catenin forms a superhelix. Finally, the C-terminus (667-781) contains a single alpha helix that interacts with the twelfth Armadillo repeat, followed by an unstructured region. In concert with the last few Armadillo repeats, the C-terminus is important in recruiting co-activators to activate transcription (Xing et al., 2008).

Components Involved in the Cytoplasm

Metazoan organisms have approximately 20 different Wnt proteins. Each Wnt species can have a different effect on targeted cells. Secreted Wnt binds its receptor, Frizzled (Bhanot et al., 1996), along with the co-receptor LRP5/6 (Tamai et al., 2000). Frizzled is a trans-membrane protein that activates Disheveled when bound by Wnt (Krasnow et al., 1995). β-catenin is continually synthesized, but is quickly degraded by its cytoplasmic degradation complex, keeping the level of cytoplasmic β-catenin low in unstimulated cells. In response to Wnt, Disheveled inhibits activity of the β-catenin cytoplasmic degradation complex through a mechanism that is not well understood (Wallingford and Habas, 2005). The cytoplasmic degradation complex consists of β-catenin, APC, Axin, GSK3β, CKIα, and PP2A. APC and Axin are large proteins that function as a scaffold for the assembly of other components of the complex. APC directly binds β-catenin as well as GSK3β (Rubinfeld et al., 1993; Su et al., 1993).

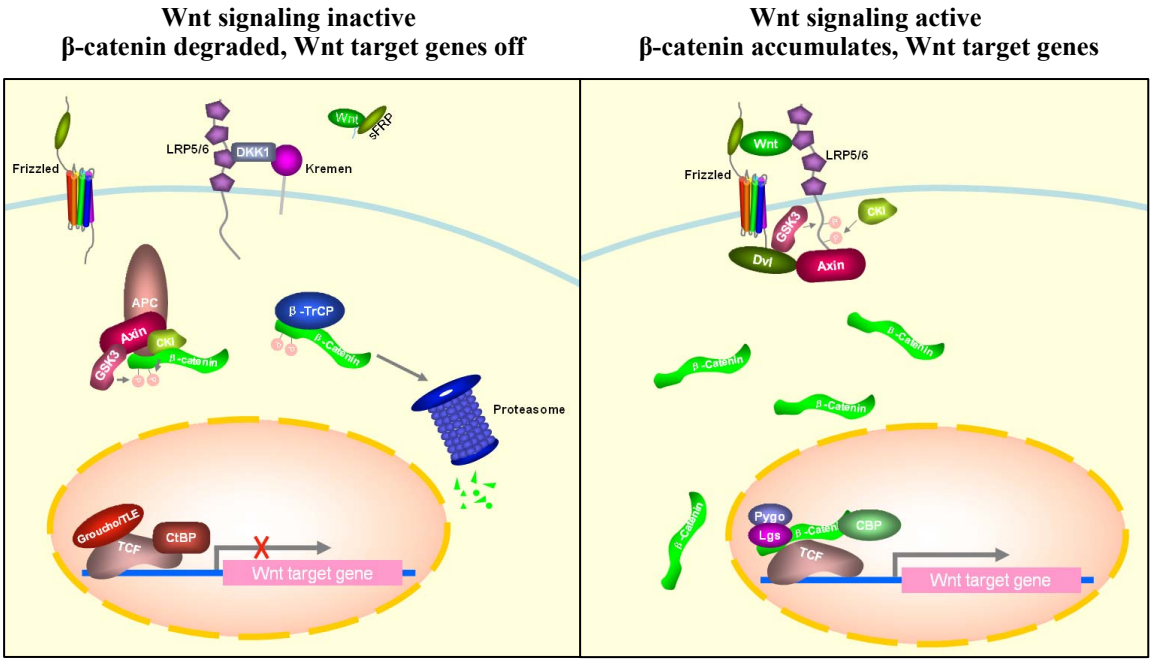

Figure 2: Canonical Wnt signaling. A, Wnt signaling inactive. In the absence of Wnt, the Frizzled receptor is inactive. β-catenin is synthesized, but rapidly degraded via a multi-step process. First, the APC/Axin complex sequentially phosphorylates β-catenin at its N-terminus. Then, β-TrCP recognizes phosphorylated β-catenin and ubiquitinates it. Finally, ubiquitinated β-catenin is targeted to the proteasome, where it is degraded. TCF/LEF family transcription factors recruit co-repressors such as Groucho/TLE and CtBP to repress transcription of Wnt target genes. B, Wnt signaling active. Wnt binds Frizzled and the co-receptor LRP5/6, inhibiting the function of the APC/Axin complex. β-catenin is no longer degraded and thus accumulates and enters the nucleus. In the nucleus, β-catenin displaces Groucho/TLE and recruits co-activators such as Pygopus, Legless, and CBP to activate transcription of Wnt target genes.

Stability of β-catenin is regulated by its N-terminal domain, and β-catenin is degraded through a multi-step process. First, CKIα phosphorylates β-catenin at serine 45. Phosphorylated S45 primes β-catenin for subsequent phosphorylation by GSK3β at serine 33, serine 37 and threonine 41 (Amit et al., 2002; Liu et al., 2002). Phosphorylation is a critical event in degrading β-catenin as mutating any of these residues stabilizes the protein (Yost et al., 1996). Then, phosphorylated β-catenin is recognized by β-TrCP. β-TrCP recruits the SCF ubiquitin ligase through its F-box domain, resulting in ubiquitination of β-catenin. Finally, ubiquitinated β-catenin enters the proteasome, where

it is rapidly degraded (Aberle et al., 1997; Liu et al., 1999a; Winston et al., 1999). These ordered phosphorylation and ubiquitination events are regulated by discrete domains within Axin (Xing et al., 2003) and APC (Yang et al., 2006). In addition, GSK3β and CKIε phosphorylate APC at a region involved in binding β-catenin, promoting the release of phosphorylated β-catenin from the degradation complex (Rubinfeld et al., 1996; Rubinfeld et al., 2001).

The cytoplasmic degradation complex maintains low levels of cytoplasmic β-catenin in the unstimulated state. However, stimulation by Wnt results in inhibition of the cytoplasmic degradation complex through multiple, cooperative mechanisms. Wnt activates the cytoplasmic protein Disheveled which then in turn inhibits GSK3β. GSK3β is then unable to phosphorylate β-catenin, preventing degradation of β-catenin. In addition, Wnt induces hyper-phosphorylation of the cytoplasmic tail of the co-receptor LRP5/6. Phosphorylated LRP5/6 recruits Axin to the cell membrane, inhibiting the formation of the degradation complex (Mao et al., 2001; Tamai et al., 2004). Finally, Wnt-mediated inhibition of GSK3β through Disheveled prevents phosphorylation of Axin, which enables Protein Phosphatase 1 to actively dephosphorylate Axin (Luo et al., 2007). Unphosphorylated Axin is less stable (Yamamoto et al., 1999) and has a significantly lower binding affinity for β-catenin (Willert et al., 1999), inhibiting function of the degradation complex. Collectively, these mechanisms function to prevent degradation of β-catenin, which then accumulates in the cytoplasm and translocates into the nucleus.

Components Involved in Nuclear Import/Export of β-catenin

Many proteins found in the nucleus contain a short peptide sequence known as a nuclear localization sequence (NLS) that facilitates entry into the nucleus. The NLS is recognized by the importin/karyopherin family of proteins, which transfer it to a nuclear pore for entry into the nucleus. β-catenin does not contain an NLS, and instead enters the nucleus unassisted by directly interacting with nuclear pore components (Fagotto et al., 1998; Yokoya et al., 1999).

β-catenin does contain a nuclear export sequence, and can freely shuttles in and out of the nucleus in response to changes in Wnt signaling. Other factors have been proposed to regulate nuclear import and export of β-catenin, although this is controversial. Some studies suggest that Axin (Cong and Varmus, 2004) and APC (Henderson, 2000; Neufeld et al., 2000; Rosin-Arbesfeld et al., 2000) actively export β-catenin from the nucleus. However, others dispute an active role for these factors, and propose that individual factors instead act as an "anchor" to retain β-catenin within their respective compartments (Krieghoff et al., 2006). According to this model, Axin and APC are cytoplasmic "anchors" (Tolwinski and Wieschaus, 2001), whereas TCF4, Pygopus, and BCL9 are nuclear "anchors" (Townsley et al., 2004)

Components Involved in Activating Transcription in the Nucleus

In the nucleus, β-catenin interacts with the TCF/LEF (T-Cell Factor / Lymphoid-Erythroid Factor) family of transcription factors (Brunner et al., 1997; van de Wetering et al., 1997), which consists of TCF-1, LEF-1, TCF-3, and TCF-4. These factors bind the consensus DNA sequence YYCAAYGG (Roose and Clevers, 1999) and are unusual in that they strongly bend DNA, at an angle greater than 90º (Giese et al., 1992; Love et al., 1995).

TCF-4 is the major TCF family member targeted by Wnt signaling in the intestine (Korinek et al., 1997), and deletion of the TCF4 gene in mice results in complete absence of proliferation (Korinek et al., 1998). TCF-1 and TCF-3 are also detectable in the adult intestine (Gregorieff and Clevers, 2005; Roose et al., 1999), but neither gives any clear intestinal phenotype when genetically deleted in mice. TCF-1 and TCF-3 function primarily as transcriptional repressors in the intestine (Merrill et al., 2004; Roose et al., 1999). Expression of LEF-1 disappears after the intestinal crypts have become fully formed, at post-natal week 2, and is undetectable in the adult (Wong et al., 2002).

In the unbound state, TCF/LEF family members recruit co-repressors such as CtBP (Brannon et al., 1999), HDAC1 (Billin et al., 2000; Kioussi et al., 2002), and Groucho/TLE (Cavallo et al., 1998; Levanon et al., 1998; Roose et al., 1998) to inhibit transcription of target genes. Groucho/TLE, in turn, interacts with hypo-acetylated

histone H3 in order to maintain a repressive chromatin environment (Palaparti et al., 1997). In response to Wnt, β-catenin enters the nucleus and binds TCF/LEF through its central armadillo repeats and displaces Groucho/TLE1 (Daniels and Weis, 2005) and HDAC1. Then, β-catenin recruits co-activators through its N- and C-terminal transactivation domains.

The N-terminal transactivation domain extends from the region just C-terminal to the regulatory region involved in its stability, to the first four Armadillo repeats (Hsu et al., 1998). This transactivation domain directly associates with BCL9/Legless, which in turn interacts with the transcriptional co-activator Pygopus (Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002). Although the precise function of Pygopus is unclear, Pygopus contains a Plant Homeodomain (PHD) near its C-terminus. Plant homeodomains interact with tri-methylated histone H3, and are thought to regulate epigenetic modifications on target genes (Soliman and Riabowol, 2007). In addition, Pygopus is clearly required in the Wnt pathway, as the *Pygopus* mutant in *Drosophila* gives a similar phenotype to the *Wingless* mutant (Thompson, 2002).

The C-terminus of β-catenin contains a strong transactivation domain that is required for Wnt signaling (Cox et al., 1999; Hecht et al., 2000; van de Wetering et al., 1997). This transactivation domain recruits p300/CBP, and recruitment of p300/CBP through this domain is required for Wnt signaling *in vivo* (Hecht et al., 2000; Takemaru and Moon, 2000). p300 and CBP are paralogous transcriptional co-activators that exert their effects through several mechanisms. They form a scaffold for the assembly of other factors; they directly interact with and recruit the basal transcriptional machinery; and they acetylate nearby histones, loosening chromatin in order to facilitate binding of other transcription factors (Goldman et al., 1997; Ogryzko et al., 1996). In addition, the C-terminal transactivation domain associates with Parafibromin, a component of PAF1 complex, and is recruited in response to binding of Pygopus. PAF1 is important in the initiation and elongation steps of transcription through its interaction with RNA Polymerase II. The association of β-catenin with the PAF1 complex is required for

transactivation, and overexpression of Parafibromin compensates for loss of Legless *in vivo* (Mosimann et al., 2006).

β-catenin also interacts with the co-activator FHL2 (Wei et al., 2003), the basal transcription factor TBP (Hecht et al., 1999), the ATP-dependent chromatin remodeling factors Brg-1/Brahma (Barker et al., 2001) and the ATP-dependent helicase TIP49a/Pontin52 (Bauer et al., 2000; Bauer et al., 1998). However, the significance of these interactions has not been fully characterized.

Other Functions of β-catenin

Prior to the discovery of its role in Wnt signaling, β-catenin was first known to be involved in cell-cell adhesion (Aberle et al., 1994). In this context, β-catenin links the membrane protein E-cadherin to the actin cytoskeleton (McCrea et al., 1991) and is an essential component of adherens junctions (Gumbiner, 1996). Although it seems plausible that cross-talk might exist between these roles of β-catenin, the consensus is that β-catenin exists in discrete pools within the cell, and that its role in cell-cell adhesion is separable from its role in Wnt signaling (Fagotto et al., 1996; Orsulic and Peifer, 1996; Sanson et al., 1996). A close paralog of β-catenin, γ-catenin (plakoglobin) performs a similar function in adherens junctions (Wahl et al., 1996). However, γ-catenin is not subject to regulation by Wnt (Shimizu et al., 2008), nor is it able to interact with TCF/LEF in the nucleus (Williams et al., 2000).

Role of Wnt Signaling in Homeostasis of the Colonic Epithelium

The organization of the epithelium of the large intestine is strongly polarized. Stem cells reside at the base, actively proliferating cells at the bottom two-thirds of the crypts, and fully differentiated cells at the top of the crypt. Mitotic pressure from actively proliferating cells is a major factor in driving differentiated cells towards the luminal surface of the large intestine, where they are shed off into the lumen. (Heath, 1996). Tight control of proliferation in the base of the crypts and the switch to differentiation is crucial in maintaining the polarity and organization of the intestinal epithelium.

Wnt signaling is strongest at the base of the crypts, with a gradual decrease towards the epithelial surface. Wnt ligands are produced by the epithelium itself as well

as by the surrounding mesenchyme, with the strongest expression nearest the base of the crypt (Gregorieff and Clevers, 2005). Basal crypts cells stain positively for nuclear β-catenin, a hallmark of active Wnt signaling (van de Wetering et al., 2002). The Wnt receptor, Frizzled, is expressed in crypt epithelial cells in a similar pattern as Wnt, whereas expression of the soluble inhibitor, Dkk-3, is highest in mesenchymal cells near the epithelial surface. Interestingly, TCF4 is expressed most highly within differentiated cells at the cell surface (Gregorieff et al., 2005). This may seem puzzling, given that TCF4 is the main transcriptional effector of Wnt/β-catenin in the intestine. However, in the absence of β-catenin, TCF4 is a strong transcriptional repressor. Thus, differentiated cells may required elevated expression of TCF4 in order to ensure continued repression of proliferative target genes.

Strong evidence implicates Wnt/β-catenin signaling as a key regulator of the organization of the intestinal epithelium. For example, TCF4-/- mice lack a proliferative compartment and die within 24 hours after birth (Korinek et al., 1998). Immunostaining of intestinal tissue from these mice demonstrates that expression of the proliferative gene c-Myc is decreased, whereas both the cell cycle inhibitor $p21^{Cip1/WAF1}$ and differentiation marker Fabp-1 are increased (van de Wetering et al., 2002). In addition, these mice lack expression of the ephrin family of receptors, EphB2 and EphB3, which are important for the proper spatial arrangement of the different cell types within the intestine (Batlle et al., 2002). Rapid, drug-inducible loss of both *APC* alleles in adult mice via the CRE/LoxP system results in deregulated proliferation and differentiation, with progenitor and differentiated cells distributed randomly throughout the crypt. These mice have also altered patterns of EphB2/3 and ephrin B expression (Sansom et al., 2004). Targeted overexpression of Dkk1, a soluble Wnt inhibitor, in the intestinal epithelium of transgenic mice results in inhibition of the proliferative compartment and an altered differentiation program (Pinto et al., 2003). Immunohistochemistry demonstrates that basal crypt cells from these mice lack nuclear β-catenin, expression of the proliferative marker Ki67, or positive BrdU staining. In addition, periodic acid/Schiff staining demonstrates an almost complete absence of goblet cells, suggesting that Wnt signaling is required for goblet cell

differentiation. Conversely, overexpression of the Wnt agonist R-spondin1 results in hyper-proliferation of crypts (Kim et al., 2005).

Wnt Signaling and Colon Cancer

As mentioned earlier, mutation of the *APC* gene is responsible for FAP syndrome (Groden et al., 1991; Kinzler et al., 1991; Nishisho et al., 1991), as well as the initiating step in the majority of sporadic colorectal tumors (Powell et al., 1992). Genetic studies using the $APC^{min/+}$ mouse model clearly demonstrate the role of mutant APC in initiating the formation of tumors in the intestine (Moser et al., 1990; Su et al., 1992). $APC^{min/+}$ mice are heterozygous for a C-terminal truncated form of APC. Loss-of-heterozygosity leaves only mutant APC, which is unable to participate in the cytoplasmic degradation complex, allowing β-catenin to accumulate and constitutively activate Wnt signaling (Korinek et al., 1997; Morin et al., 1997). Conditional deletion of the *APC* gene in the mouse adult intestine results in altered patterns of proliferation and differentiation with a "crypt progenitor-like" phenotype (Andreu et al., 2005; Sansom et al., 2004), eventually leading to the formation of tumors (Shibata et al., 1997).

In sporadic colorectal tumors with wild-type *APC*, mutations in β-catenin (Morin et al., 1997; Munemitsu et al., 1996) or Axin2 (Liu et al., 2000) are usually found. Mutations in β-catenin block degradation and are typically either a short truncation of the N-terminus or a single point mutation at one of its phosphorylated residues. Mutant β-catenin accumulates to much higher levels than wild-type β-catenin within the cell, constitutively activating Wnt signaling in a manner similar to mutant *APC*. Targeted deletion of the N-terminus of β-catenin in the intestinal epithelium of mice produces thousands of adenomatous polyps within weeks (Harada et al., 1999). Finally, in a model of colitis-associated colorectal carcinoma, where mice are given 1,2-dimethylhydrazine and dextran sulfate sodium, mice develop dysplastic lesions and invasive colorectal cancer that strongly stains for nuclear β-catenin (Wang et al., 2004b).

The most abundant isoform of TCF1 lacks a β-catenin interaction domain and is thus a constitutive repressor (Castrop, 1995; van de Wetering, 1996). Interestingly, β-catenin/TCF4 activates a TCF1 reporter construct, and restoration of wild-type APC in

APC-mutant HT29 cells results in an increase in TCF1 expression (Roose, 1999). Since TCF1 is only detectable in the base of the crypts of adult colonic epithelium, its function may be to titrate Wnt signaling, striking the appropriate balance between proliferation and differentiation. In support of this role, about 15% of $TCF1^{-/-}$ mice develop intestinal adenomas within their first year, whereas double mutant $APC^{Min/+}/TCF1^{-/-}$ mice have greatly increased numbers of tumors in both the small and large intestine, relative to $APC^{Min/+}$ mice alone (Roose, 1999).

These studies highlight the importance of aberrant Wnt/β-catenin signaling in intestinal tumorigenesis. Although much is already known about the basic mechanisms of this pathway, continued research is needed in order to identify promising targets for the development of small molecule inhibitors that can be used to treat colorectal carcinoma in humans.

Wnt Signaling and Other Diseases

In addition to colorectal cancer, aberrant Wnt signaling appears to play a role in many other cancers. For example, stabilizing point mutations of β-catenin, C-terminal truncations of APC, and mutant forms of Axin1 have been found in cancers of the liver, ovary, brain, prostate, uterus, and the skin (Roose and Clevers, 1999). Interestingly, mutations in the *APC* gene are rarely detected in prostate cancer (Watanabe et al., 1996). Instead stabilizing N-terminal mutations of β-catenin are frequently found (Voeller et al., 1998).

KRÜPPEL-LIKE FACTOR 4 (KLF4)

This section will overview what is currently known about KLF4 and its role in the regulation of homeostatic mechanisms within the intestinal epithelium, cancer, and stem cell reprogramming. It has been published as a review article (Evans and Liu, 2008).

The Krüppel-like Factor Family

Krüppel-like factors are a family of transcription factors that play an important role in many fundamental biologic processes including development, proliferation, differentiation, and apoptosis (**Fig. 3**). Krüppel-like factor family members contain three

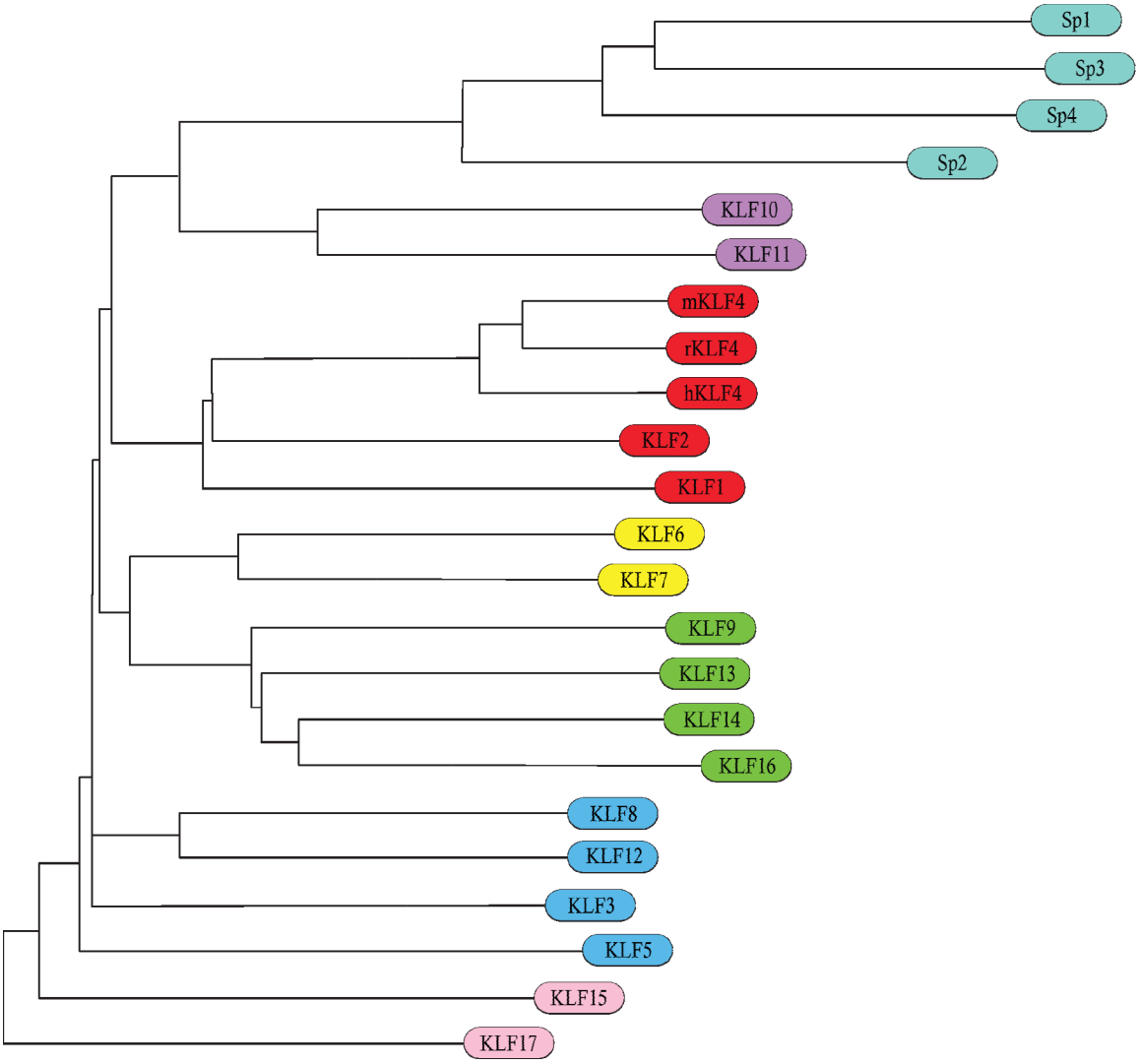

Figure 3: Phylogenetic tree of the Sp/KLF transcription factor family. Horizontal distance is proportional to the number of amino acid changes between members. KLF4 and its closest paralogs are highlighted in red. KLF4 from mouse (mKLF4), rat (rKLF4), and human (hKLF4) are indicated.

C-terminal C_2H_2-type zinc fingers that bind DNA, and are named "Krüppel-like" due to strong homology within this region to the *Drosophila* gene Krüppel. Krüppel itself is important in establishing segmentation patterns in the developing embryo. Genetic deletion of Krüppel results in the complete absence of thoracic and anterior abdominal segments (Preiss et al., 1985).

KLF4 was cloned independently by two groups, and given two different names: Gut-enriched Krüppel-like factor (GKLF) due to the fact that it was found to be highly expressed in the intestine (Shields et al., 1996), and Epithelial Zinc Finger (EZF) due to its high expression in the skin epithelium. GKLF/EZF was later renamed KLF4 to avoid confusion, as expression of KLF4 is also detectable in the lung, skin, testis, (Garrett-Sinha et al., 1996; Shie et al., 2000b; Shields et al., 1996; Ton-That et al., 1997), thymus (Panigada et al., 1999), cornea (Chiambaretta et al., 2004), cardiac myocytes (Cullingford et al., 2008) and lymphocytes (Fruman et al., 2002). In addition, KLF4 is important in development. It is detectable in the mouse embryo, with the highest expression occurring at the later stages of development (Garrett-Sinha et al., 1996; Ton-That et al., 1997).

Role of KLF4 in Homeostasis of the Colonic Epithelium

Within the adult intestine, KLF4 functions as an inhibitor of proliferation and promoter of differentiation. Consistent with this role, expression of KLF4 is greatest near the luminal surface and gradually decreases toward the base of the crypts (Shields et al., 1996; Zhang et al., 2006). $Klf4^{-/-}$ mice lack goblet cells, without affecting the number of enterocytes, suggesting that KLF4 may be specifically required for goblet cell differentiation (Katz et al., 2002). In addition, KLF4 can interact with β-catenin and antagonize Wnt signaling (Zhang et al., 2006), a key pathway in driving proliferation of the intestinal epithelium (Korinek et al., 1998). Thus, KLF4 may be important in switching transit-amplifying cells into the three differentiated cell types of the colonic epithelium (enterocytes, goblet cells, and enteroendocrine cells).

Butyrate is constantly produced in the colon by bacterial fermentation of dietary fiber (Roediger, 1980), and can induce expression of KLF4 (Chen et al., 2004; Shie et al., 2000b). In cell culture, butyrate stimulates expression of the enterocyte-specific marker Intestinal Alkaline Phosphatase (IAP) (Wang et al., 2001), and induces colon cancer cells to acquire a more differentiated, enterocyte-like phenotype (Heerdt et al., 1994). KLF4 positively regulates expression of IAP (Evans et al., 2007; Hinnebusch et al., 2004), and overexpression of KLF4 in cell culture inhibits proliferation (Shie et al., 2000b; Shields et al., 1996).

KLF4 appears to have an inhibitory effect on a wide variety of cellular processes, including protein and cholesterol synthesis, transcription, cell growth, and DNA repair (**Tables 1 and 2**) (Chen et al., 2003; Whitney et al., 2006). Moreover, KLF4 is required for cell-cycle arrest at both the G_1/S-phase (Chen et al., 2001; Yoon et al., 2003) and G_2/M-phase (Yoon and Yang, 2004) checkpoints. KLF4 simultaneously induces expression of the cyclin-dependent kinase inhibitor proteins $p21^{Cip1/WAF1}$ (Mahatan et al., 1999; Nickenig et al., 2002; Zhang et al., 2000) and $p57^{Kip2}$ (Chen et al., 2003), and represses expression of

Table 1: Transcriptional targets activated by KLF4

Factor/Condition	**References**
1200015N20Rik	Nakatake, 2006
A33 antigen	Mao et al., 2003
B2R	Saifudeen et al., 2005
Cytokeratin 4	Luo et al., 2004a
EBV ED-L2	Jenkins et al., 1998
hSMVT	Reidling and Said, 2007
IAP	Chen et al., 2003; Hinnebusch et al., 2004; Siddique et al., 2003
iNOS	Feinberg et al., 2005
Keratin 4	Jenkins et al., 1998
Keratin 19	Brembeck and Rustgi, 2000
KLF4	Dang et al., 2002; Mahatan et al., 1999
Laminin-α 3A	Miller et al., 2001
Laminin-γ 1	Higaki et al., 2002
Lefty1	Nakatake et al., 2006
Nanog	Jiang et al., 2008; Nakatake et al., 2006
Oct4	Jiang et al., 2008
$p21^{Cip1}$	Mahatan et al., 1999; Nickenig et al., 2002; Zhang et al., 2000
$p27^{Kip1}$	Nickenig et al., 2002
$p57^{Kip2}$	Chen et al., 2003
PKG-Iα	Zeng et al., 2006
Rb	Nickenig et al., 2002
Sox2	Jiang et al., 2008
SPRR1A	Luo et al., 2004a
SPRR2A	Luo et al., 2004a
Tbx3	Jiang et al., 2008
u-PAR	Wang et al., 2004a

Cyclin D_1 (Shie et al., 2000a; Shie et al., 2000b; Shie et al., 1999), Cyclin D_2 (Klaewsongkram et al., 2007), Cyclin E (Yoon et al., 2005), and Cyclin B_1 (Yoon and Yang, 2004). In addition, KLF4 represses expression of ornithine decarboxylase (ODC), an enzyme involved in the production of polyamines, a class of molecules important in proliferation (Chen et al., 2002; Chiambaretta et al., 2004). Finally, KLF4 represses expression of p53 and is important in determining whether cells undergo apoptosis or cell cycle arrest (Rowland et al., 2005).

Table 2: Transcriptional targets repressed by KLF4

Factor/Condition	**References**
Bax	Ghaleb et al., 2007
CD11d	Noti et al., 2005
Cyclin B_1	Yoon and Yang, 2004
Cyclin D_1	Shie et al., 2000a; Shie et al., 2000b; Shie et al., 1999
Cyclin E	Yoon et al., 2005
CYP1A1	Zhang et al., 1998
Fgf5	Jiang et al., 2008
HDC	Ai et al., 2004
KLF2	Jiang et al., 2008; Nakatake et al., 2006
Laminin α1	Piccinni et al., 2004
Nes	Jiang et al., 2008
ODC	Chen et al., 2002
p53	Rowland et al., 2005
PAI-1	Feinberg et al., 2005
SM22α	Adam et al., 2000
SM α-actin	Liu et al., 2003
Sp1	Kanai et al., 2006

Role of KLF4 in Homeostasis of Other Tissues

Although the importance of KLF4 in the intestine is well characterized, increasing evidence demonstrates its importance in other organs and tissues as well. $Klf4^{-/-}$ mice die soon after birth of dehydration due to defects in the epidermal barrier of the skin (Segre et al., 1999), whereas targeted overexpression of KLF4 results in early formation of the epithelial permeability barrier (Jaubert et al., 2003). Furthermore, overexpressed KLF4 synergizes with maternally-injected corticosteroids in accelerating the formation of the skin barrier, likely due to overlap between the genes targeted by KLF4 and the

glucocorticoid receptor (Patel et al., 2006). The utility of glucocorticoids in lung maturation of premature infants is well-established (1995). Thus it would be interesting to determine whether KLF4 or other Krüppel-like factors synergize with glucocorticoids in lung maturation as well.

During pregnancy, KLF4 synergizes with Sp1 in up-regulating expression of PSG-5, a protein secreted into the maternal circulation by the placenta (Blanchon et al., 2006). PSG-5 is thought to be required for maintaining a pregnancy to term and may protect the fetus from attack by the maternal immune system. In addition, expression patterns of KLF4 and PSG-5 closely overlap in the placenta, suggesting an *in vivo* role for KLF4 in the regulation of PSG-5 expression (Blanchon et al., 2001).

Human KLF4 was isolated from a umbilical vein cDNA library and is expressed in the vascular endothelium (Yet et al., 1998). Expression of KLF4 is induced by shear stress in endothelial cells (McCormick et al., 2001). In differentiated arterial smooth muscle cells, KLF4 is normally expressed at low levels (Adam et al., 2000) but is rapidly up-regulated in response to vascular injury (Liu et al., 2005).

Overexpression of KLF4 in a pro-myelocytic cell line increases expression of monocyte markers, whereas knockdown of KLF4 decreases TPA-induced overexpression of these same markers. In addition, $Klf4^{-/-}$ hematopoietic stem cells less frequently differentiate into monocytes (Feinberg et al., 2007). When fetal liver cells from $Klf4^{-/-}$ mice were transplanted into lethally-irradiated wild-type mice, they had undetectable levels of circulating inflammatory monocytes (Alder et al., 2008). Thus, KLF4 appears to be important in differentiation of both resident and inflammatory monocytes.

KLF4 is also highly expressed in the corneal epithelium. Targeted deletion of KLF4 in the eye results in corneal fragility, edema, and a lack of goblet cells in the conjunctiva (Swamynathan et al., 2007). In a cell culture model of adipocyte differentiation using 3T3-L1 cells, siRNA-mediated knockdown of KLF4 completely blocked expression of several phenotypic markers of differentiated adipocytes (Birsoy et al., 2008). Collectively, these data strongly implicate KLF4 as a factor involved in differentiation within many tissues.

Role of KLF4 in Cancer

As an anti-proliferative and anti-apoptotic factor expressed primarily in differentiated cells, it seems likely that KLF4 might be a tumor suppressor. Indeed, this has been shown to be true in the gastrointestinal tract (McConnell et al., 2007; Wei et al., 2006). However, recent evidence suggests that in certain contexts KLF4 might also act as an oncogene (Rowland and Peeper, 2006). This section will explore these two contrasting roles.

KLF4 as a Tumor Suppressor

Significant evidence implicates KLF4 as a tumor suppressor in the intestinal epithelium. In human colorectal carcinoma, expression of KLF4 is downregulated, with evidence of both hypermethylation of its promoter and loss-of-heterozygosity (Choi et al., 2006; Xu et al., 2008; Zhao et al., 2004). In patients with metastatic carcinoma, no association has been found between down-regulation of KLF4 and tumor stage or 5-year survival, suggesting that loss of KLF4 in colorectal cancer is an early event (Choi et al., 2006; Xu et al., 2008).

Examination of KLF4 expression in mouse models of colorectal cancer have yielded similar results. In adenomas from $APC^{min/+}$ mice, KLF4 is down-regulated, with expression inversely related to the size of the tumor (Dang et al., 2000; Ton-That et al., 1997). The $APC^{min/+}$ mouse develops hundreds of intestinal adenomas early in life and is a widely-used model of intestinal tumorigenesis (Moser et al., 1990; Su et al., 1992). $APC^{min/+}$ mice express a truncated form of the APC protein and have deregulated Wnt/β-catenin signaling in their intestine (Korinek et al., 1997; Morin et al., 1997). Interestingly, KLF4 interacts with β-catenin and represses Wnt signaling *in vivo*, inhibiting the growth of tumor xenografts (Zhang et al., 2006). Crossing $APC^{min/+}$ mice with $Klf4^{+/-}$ heterozygotes results in the production of significantly more intestinal adenomas than in $APC^{min/+}$ mice alone (Ghaleb et al., 2007). The most abundant isoform of TCF1 expressed in the intestine is also an antagonist of Wnt/β-catenin signaling, and $APC^{Min/+}$/$TCF1^{-/-}$ mice have a similar phenotype. These data suggest that an important

effect of decreased KLF4 expression in colorectal tumorigenesis is de-repression of Wnt signaling.

In screening human colon cancer cell lines, several point mutations have been found in the *KLF4* gene. One point mutation has significant effect on the ability to activate a $p21^{Cip1}$ reporter construct (Zhao et al., 2004). However, a comprehensive investigation to identify the location and frequency of mutations in the *KLF4* gene in human tissue samples of colorectal cancer has not yet been performed. In the HCT116 colorectal cancer cell line, KLF4 is required to prevent centrosome amplification after gamma-irradiation. Thus, loss of KLF4 may promote chromosomal instability (Yoon et al., 2005). In addition, KLF4 represses expression of ornithine decarboxylase (Chen et al., 2002), a proto-oncogene that alone is sufficient to transform NIH3T3 cells (Auvinen et al., 1992). Collectively, these data implicate KLF4 as a tumor suppressor in the colon.

Strong evidence implicates KLF4 as a tumor suppressor in the gastric epithelium as well. In gastric cancer, KLF4 is downregulated, with evidence of loss-of-heterozygosity and hypermethylation of the promoter (Cho et al., 2007; Kanai et al., 2006; Wei et al., 2005). Moreover, targeted loss of the Klf4 gene in the gastric mucosa of mice results in pre-cancerous changes in the stomach (Katz et al., 2005). Examination of both normal and cancerous gastric mucosal tissue from humans revealed an inverse relationship between expression of KLF4 and Sp1, a distantly related Krüppel-like factor family member (see **Fig. 3**). In addition, KLF4 can directly repress expression of Sp1 (Kanai et al., 2006). Since strong expression of Sp1 is correlated with poor survival in gastric cancer (Wang et al., 2003), loss of KLF4 may contribute to progression of gastric cancer.

In addition to gastric and colorectal cancer, KLF4 is down-regulated in esophageal cancer (Luo et al., 2004a; Wang et al., 2002), bladder cancer (Ohnishi et al., 2003), non-small-cell lung carcinoma (Bianchi et al., 2004), and leukemia (Kharas et al., 2007; Yasunaga et al., 2004).

KLF4 as an Oncogene

Although these data clearly demonstrate that KLF4 is a tumor suppressor in multiple tissues, there is significant circumstantial evidence that KLF4 might be an oncogene as well. KLF4 was identified as a transforming factor in a screen using E1A-immortalized rat kidney epithelial cells (RK3E), and KLF4-transformed cells produce tumors in xenografted mice (Foster et al., 1999). In addition, KLF4 is overexpressed in laryngeal squamous cell carcinoma as an early event in its progression (Foster et al., 1999). Expression of KLF4 is increased in ductal carcinoma of the breast (Foster et al., 2000) and increased nuclear staining is associated with a more aggressive phenotype and a poorer prognosis (Pandya et al., 2004). In the skin, overexpression of KLF4 results in hyperplasia and dysplasia (Foster et al., 2005), eventually leading to squamous cell carcinoma (Huang et al., 2005).

Whether KLF4 acts as a tumor suppressor or an oncogene is likely due to differences in cell context, expression patterns of other genes, and the chromatin environment of individual cells. For example, KLF4 overrides Ras^{V12}-induced senescence in primary fibroblasts and induces transformation (Rowland et al., 2005). The ability of KLF4 to induce transformation rather than cell cycle arrest depends on the status of $p21^{Cip1}$. Overexpression KLF4 alone increases expression of $p21^{Cip1}$, resulting in cell cycle arrest. However, the addition of Ras^{V12} inhibits expression $p21^{Cip1}$, allowing KLF4-mediated inhibition of p53 to predominate. Downregulation of p53 effectively blocks apoptosis and results in transformation. Thus, KLF4 can be added to a growing list of genes that can have multiple, context-dependent roles in different cancers, such as *CDKN1A* (p21), TGF-β, Ras, and *NOTCH1* (Rowland and Peeper, 2006).

Role of KLF4 in Stem Cell Renewal and Reprogramming

Recently, it was found that overexpression of KLF4 in combination with three other transcription factors could transform mouse fibroblasts into a state resembling embryonic stems cells (ES cells). These cells have been named "inducible pluripotent stem (iPS)" cells (Takahashi and Yamanaka, 2006). By replacing the open reading frame of *Fbx15*, a non-essential marker of embryonic stem cells, with a neomycin resistance

gene, authors hypothesized that overexpression of certain factors would reprogram target cells into embryonic stem cells. These cells could then be selected based on resistance to neomycin. After screening 24 potential factors, authors found that the simultaneous overexpression of Oct3/4, Sox2, c-Myc, and KLF4 can produce iPS cells. When injected into mice, these cells form teratomas containing differentiated tissues from all three germ layers, confirming their pluripotency. This approach was further refined by screening for neomycin resistance based on *Nanog* or *Oct4* expression instead of *Fbx15*. Unlike Fbx15-iPS cells, Nanog-iPS and Oct4-iPS cells can produce chimeric mice and, when injected into tetraploid blastocysts, generate live late-term embryos (Maherali et al., 2007; Okita et al., 2007; Wernig et al., 2007). Thus, Nanog and Oct4-iPS satisfy even the most stringent tests of pluripotency.

An area currently under intense investigation is understanding the molecular events that occur during stem cell reprogramming as well as the precise role of each of the four individual factors required. The importance of Oct3/4 and Sox2 in ES cell renewal is well established (Lewitzky and Yamanaka, 2007). The role of the other two factors that make up the "magic brew", c-Myc and KLF4, is less clear. Both c-Myc and KLF4 can function as oncogenes, thus one possibility is that together they confer increased proliferative capacity on potential iPS cells (Yamanaka, 2008). Since c-Myc regulates expression of a significant portion of the entire genome, its function may be to effect global changes in the chromatin environment, by recruiting histone acetyl-transferase complexes. KLF4 represses expression of c-Myc through inhibiting Wnt signaling (Zhang et al., 2006), and elevated levels of c-Myc induce apoptosis (Prendergast, 1999). Thus, one plausible mechanism is that KLF4 may maintain c-Myc expression within a narrow range in order to prevent apoptosis induced by elevated levels of c-Myc, although hypothesis this remains to be tested.

KLF4 inhibits differentiation of ES cells into erythroid progenitors, and increases their capacity to generate secondary embryoid bodies, suggesting a role for KLF4 in stem cell self-renewal (Li et al., 2005b). In concert with Oct3/4 and Sox2, KLF4 activates expression of *Lefty1*, a gene expressed in ES cells but lost during differentiation.

However, Klf4-null mice survive to term and have no detectable defects in their pluripotent stem cell population during embryogenesis (Katz et al., 2002; Segre et al., 1999), suggesting that KLF4 may be dispensable for differentiation of ES cells. In addition, human iPS cells have been produced using a slightly different "magic brew", substituting c-Myc and KLF4 with Nanog and LIN28 (Yu et al., 2007). Moreover, KLF4 positively regulates expression of Oct4, Sox2, and Nanog (Jiang et al., 2008; Nakatake et al., 2006). Thus, KLF4 and c-Myc may accelerate or increase the efficiency of the reprogramming process rather than being absolutely required (Jaenisch and Young, 2008).

A recent study found that the function of KLF4 in ES cell self-renewal is partially redundant with KLF2 and KLF5. Knockdown of all three Krüppel-like factors, but not any one individually, results in spontaneous ES cell differentiation (Jiang et al., 2008). In addition, significant overlap exists between genes regulated by Nanog and by the three Krüppel-like factors, KLF2, KLF4, and KLF5. KLF2 is the closest relative of KLF4, thus is not surprising that KLF4 and KLF2 might be redundant in some contexts, although it remains to be tested whether KLF2 can substitute for KLF4 in iPS cell reprogramming. Clearly, a complete understanding of the role of KLF4 in ES cell self-renewal and iPS cell reprogramming awaits further study.

Molecular Mechanisms of KLF4

Human and KLF4 is 470 amino acid monomeric protein and is approximately 55kD. KLF4 can be roughly divided into three separate domains: an N-terminal activation domain (Garrett-Sinha et al., 1996; Geiman et al., 2000; Yet et al., 1998), a central repressive domain (Yet et al., 1998), and a C-terminal DNA binding domain (see **Fig. 4**). The DNA binding domain consists of three successive zinc fingers. Each zinc finger contains an anti-parallel beta-sheet, followed by a short loop, and an alpha helix.

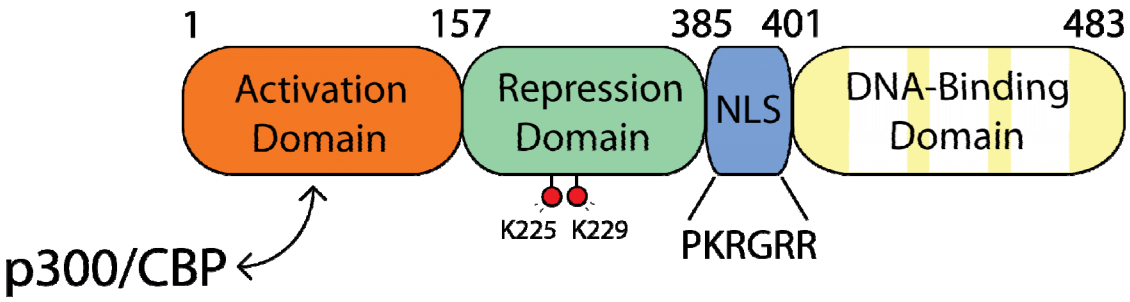

Figure 4: Functional domains of the KLF4 protein. The N-terminal transactivation domain interacts with the co-activator p300/CBP. The central repressive domain is acetylated at K225 and K229. This is followed by a basic hexapeptide nuclear localization sequence (NLS). The C-terminus contains three Krüppel-like C_2H_2 zinc fingers for binding DNA.

Two cysteine residues within the beta-sheet and two histidine residues within the alpha helix work together to coordinate a single zinc ion, which stabilizes the structure. Each zinc finger interacts with three consecutive nucleotides on a target DNA sequence, and the sequence specificity of a zinc finger protein can be increased simply by adding additional zinc fingers (Yang, 1998).

KLF4 interacts with GT-rich or CACCC elements on target genes (Philipsen and Suske, 1999; Yet et al., 1998). Although one report suggests that KLF4 prefers to bind a RRGGYGY sequence (where R = A/G and Y = C/T) (Shields and Yang, 1998), it is not clear whether this is a true consensus *in vivo*. KLF4 is exclusively nuclear, like many other transcription factors, and contains two discrete nuclear localization sequences. The first is a basic hexapeptide sequence just N-terminal to the three C-terminal zinc fingers, whereas the second is contained within the first two zinc fingers themselves (Shields and Yang, 1997).

Given the large number of genes regulated by KLF4, it is not unexpected that expression of KLF4 is highly regulated. In the HCT116 colon cancer cell line, KLF4 has a half-life of only 2 hours and is quickly degraded by the proteasome (Chen et al., 2005). However, a variety of stimuli can induce expression of KLF4 in cell culture (see **Table 3**), including serum starvation, contact inhibition (Garrett-Sinha et al., 1996), interferon-γ (Chen et al., 2000; Chen et al., 2002), sodium butyrate (Chen et al., 2004; Shie et al.,

2000b), cAMP (Birsoy et al., 2008), gastrin (Ai et al., 2007), DNA damage (Yoon et al., 2003; Zhang et al., 2000), and oxidative stress (Cullingford et al., 2008; Nickenig et al., 2002). The precise mechanism of how these stimuli increase the expression of KLF4 is unclear, although obvious possibilities include increased transcription of the *KLF4* gene, increased mRNA stability, and increased protein stability.

Although much remains to be known about how expression KLF4 is regulated, several transcription factors have been found to regulate its promoter. For example, p53 transactivates the *KLF4* gene, and p53 is required for the increase in KLF4 expression after DNA damage (Yoon et al., 2003; Zhang et al., 2000). CDX2, another protein

Table 3: Factors and conditions that modulate expression of KLF4

Factor/Condition	**References**
Increase expression	
Butyrate	Chen et al., 2004; Shie et al., 2000b
Cdx2	Dang et al., 2001; Mahatan et al., 1999
Contact inhibition	Garrett-Sinha et al., 1996; Shields et al., 1996
Endothelin-1	Cullingford et al., 2008
γ-irradiation	Yoon et al., 2003
H_2O_2	Cullingford et al., 2008; Nickenig et al., 2002
Interferon-γ	Chen et al., 2000; Feinberg et al., 2005
IBMX (cAMP activator)	Birsoy et al., 2008
KLF4	Dang et al., 2002
LPS	Feinberg et al., 2005
Methyl methanesulfonate	Zhang et al., 2000
p53	Zhang et al., 2000
Serum starvation	Garrett-Sinha et al., 1996; Shields et al., 1996
Shear stress	Hamik et al., 2007
Sp1	Mahatan et al., 1999
Sp3	Mahatan et al., 1999
TNF-α	Feinberg et al., 2005
Trichostatin A	Chen et al., 2004; Shie et al., 2000b
Decrease expression	
KLF5	Dang et al., 2002
TGF-β	Adam et al., 2000; Feinberg et al., 2005

important in intestinal differentiation, can activate a KLF4 reporter construct (Dang et al., 2001). This suggests that KLF4 may act downstream of CDX2, although more work is necessary to demonstrate this *in vivo*. KLF4 up-regulates its own expression by binding to its promoter, whereas KLF5 inhibits KLF4 expression and blocks binding of KLF4 to its promoter (Dang et al., 2002). Although both KLF4 and KLF5 are highly expressed in the intestine, their patterns of expression are markedly different. Expression of KLF5 is strongest in actively proliferating cells at the base of the crypts and expression is weakest in differentiated cells at the luminal surface (Conkright et al., 1999; Watanabe et al., 1999). KLF4 and KLF5 have several antagonizing roles in the intestinal epithelium, as reviewed elsewhere (McConnell et al., 2007).

Mechanism of Activation

A major function of KLF4 is to activate transcription of target genes. Consistently, the N-terminus of KLF4 contains a strong transactivation domain (Garrett-Sinha et al., 1996; Geiman et al., 2000; Yet et al., 1998). When directly fused to its three C-terminal zinc fingers, the N-terminal domain is sufficient to activate a synthetic reporter construct. In addition, the N-terminal domain interacts with the transcriptional co-activators p300 and CBP. This interaction is required for its function as point mutations that block interactions with CBP also completely abrogate its ability to activate transcription (Geiman et al., 2000). p300/CBP are histone acetyl-transferase (HAT) proteins, and recruitment of p300/CBP results in an increase in histone acetylation at the promoter. Acetylation of histones facilitates the recruitment of other transcription factors as well as the basal transcriptional machinery. In addition, KLF4 itself is acetylated by p300/CBP at lysines 225 and 229. Mutation of these two lysines to arginine significantly decreases the ability of KLF4 to transactivate target genes, as well as its ability to inhibit proliferation (Evans et al., 2007).

In addition to p300/CBP, KLF4 interacts with Tip60, a bi-functional cofactor that contains HAT activity, but also interacts with HDAC7 (Ai et al., 2007). Tip60 is a co-activator for several nuclear hormone receptors (Gaughan et al., 2001) as well as APP (Cao and Sudhof, 2001). However, Tip60 functions as a co-repressor for STAT3-

mediated repression by recruiting HDAC7 (Xiao et al., 2003). Krox20, another zinc finger protein, interacts with KLF4 and synergistically activates expression of the C/EBPβ gene (Birsoy et al., 2008). In addition, KLF4 interacts with the NFκB subunit p65/RelA and synergistically activates expression of iNOS (Feinberg et al., 2005). Thus, the mechanisms of transactivation by KLF4-regulated transcription may be gene-dependent.

Mechanism of Repression

In passive repression, a non-activating transcription factor competes with an activator for binding to the same sequence. On the *CYP1A1*, *HDC*, and *SP1* genes, KLF4 binds to an overlapping sequence with the activator Sp1 and displaces Sp1 from the promoter, passively repressing transcription (Ai et al., 2004; Kanai et al., 2006; Zhang et al., 1998). Since Sp1 is ubiquitously expressed and positively regulates many genes (Black et al., 2001), it is likely that KLF4 uses this mechanism to repress many of its target genes.

GAL4 fusion assays demonstrate that KLF4 contains central repressive domain in addition to its more fully characterized transactivation domain (Yet et al., 1998). This suggests that KLF4 actively represses expression of some genes, in addition to competing with activators. Indeed, KLF4 has been found to interact with and recruit HDAC1 and HDAC2 in repression of the CD11d gene promoter (Noti et al., 2005). KLF4 represses the Cyclin B_1 gene and specifically recruits HDAC3 (Evans et al., 2007). On the *TP53* gene, MUC1-C recruits KLF4, HDAC1, and HDAC3, to mediate repression (Wei et al., 2007). KLF4 inhibits Smad3-mediated activation of PAI-1 by directly competing for binding p300 (Feinberg et al., 2005). Finally, KLF4 represses transcriptional targets of Wnt signaling by directly interacting β-catenin/TCF4 (Zhang et al., 2006). These data strongly suggest that KLF4-mediated activation and repression is complex and gene-dependent.

Activation Versus Repression

A question that arises when studying transcription factors that can both activate and repress transcription is: How they can switch between these two modes? The

answer to this question is complex, but there are several possibilities. One possibility is that the function of a transcription factor depends on the absence or presence of other transcription factors at the same promoter. For example, binding of DSP1 to sequence adjacent to Dorsal or NFκB binding site can switch both Dorsal and NFκB from activators to repressors (Lehming et al., 1994). In addition, many zinc finger proteins dimerize (Mackay and Crossley, 1998), such as Sp1 with YY1 (Lee et al., 1993) and KLF1 with Sp1 or GATA-1 (Merika and Orkin, 1995). Thus, other zinc finger proteins may help enforce a state of activation or repression for KLF4 on a particular gene. On the promoter of the *HDC* gene, KLF4 interacts with Tip60. Tip60 itself can acts an activator or a repressor, so the function of KLF4 may in turn be determined by the function of Tip60 (Ai et al., 2007).

Another possibility is that binding of another factor or post-translational modifications can induce a conformational change and alter its function. For example, the binding of T3 to thyroid hormone receptor induces a conformational change that blocks its ability to interact with the co-repressor N-CoR and allows the recruitment of the co-activator p300/CBP (Horlein et al., 1995). In addition, post-translational modifications such as phosphorylation and acetylation can have an effect on the function of a transcription factor. For example, overexpression of RhoA kinase inhibits the ability of KLF4 to bind DNA *in vitro*, although it is still unclear whether RhoA directly phosphorylates KLF4 (Zeng et al., 2006). KLF4 is acetylated by p300/CBP and acetylation of KLF4 is important for its ability to transactivate the *IAP* and *CDKN1A* genes, possibly part of a feed-forward mechanism (Evans et al., 2007).

β-catenin/KLF4 Cross-talk

Our lab identified KLF4 while using affinity purification to screen for proteins that interact with β-catenin. Further investigation demonstrated that KLF4 represses Wnt signaling *in vivo*, by inhibiting double axis formation in *Xenopus* embryos and by inhibiting tumor growth in xenografts. Moreover, KLF4 represses Wnt signaling at the transcriptional level, without interfering with the interaction between β-catenin and TCF/LEF (Zhang et al., 2006). Understanding the molecular mechanisms of KLF4-

regulated transcription and its role in both homeostasis and cancer of the colon is the primary focus of this dissertation.

CHAPTER 2: SITEFIND - A SOFTWARE TOOL FOR INTRODUCING A RESTRICTION SITE AS A MARKER FOR SUCCESSFUL SITE-DIRECTED MUTAGENESIS

INTRODUCTION

During my dissertation work, I engineered many different point mutations in the primary sequence of KLF4 using site-directed mutagenesis. I wrote the computer program SiteFind to assist me in designing the primers for site-directed mutagenesis as well as screen for successful mutations. This chapter details how SiteFind works, presents several examples of its use, and has been published (Evans and Liu, 2005).

There are several methods available for mutagenesis: 1) isolate single stranded template DNA and create a mutation with one complementary primer (Hutchison et al., 1978); 2) design two sets of PCR primers that overlap the mutation site, amplify the template by two PCR reactions and then clone the two PCR fragments and the vector by three piece ligation (Stemmer and Morris, 1992); 3) site-directed mutagenesis using the QuikChange method (Hemsley et al., 1989; Kunkel, 1985; Papworth et al., 1996). All of these *in vitro* mutagenesis methods require careful design of one or more primers that cover the mutation site. Currently, QuikChange site-directed mutagenesis is the method of choice. This method requires two complementary oligonucleotide primers flanking the desired mutated nucleotide on both the sense and anti-sense strands. Furthermore, each primer must contain one to several base-pair changes within the desired region. PCR is then performed using these primers along with the gene of interest, which was previously inserted into a vector containing an antibiotic resistance gene. The extension step of the polymerase chain reaction is given sufficient time to replicate the entire circular DNA construct, with the reaction eventually ending where it started. After several rounds of PCR, the resulting mixture of newly-synthesized mutant constructs and template DNA is incubated with a methylation-specific endonuclease to remove the wild-type template DNA which contains methylated nucleotides. The mixture is then transformed into

competent bacteria, plated on antibiotic-containing agar, and grown overnight to in order to allow individual colonies to grow.

Since the bacteria was transformed with a complex mixture of undigested template DNA, successful point mutant copies of the template, and PCR side-products, it is difficult to determine which colonies contain the desired mutant construct. Restriction enzyme digestion of plasmid DNA extracted from each colony can differentiate between correct and aberrant PCR products, but it cannot distinguish between bacteria transformed with template DNA and bacteria transformed the with desired point mutant. Instead, plasmid DNA extracted from each colony must be sent to a sequencing laboratory and the sequence manually scanned for successful mutation. If the number of colonies containing template DNA is high relative to the total number of colonies, this can be an expensive and time-consuming process.

A simple method to confirm the presence of a point mutation prior to sequencing is to design the mutagenesis primer pair such that it introduces a novel restriction site, taking advantage of the redundancy of the genetic code (Arentzen and Ripka, 1984; Little and Mount, 1984; Shankarappa et al., 1992a). Thus plasmid DNA extracted from each colony can be digested with the appropriate restriction enzyme and run on an agarose gel to check for the presence of a band not found in the template DNA. However, finding the correct set of mutations in the DNA sequence that will introduce a restriction site without disturbing the corresponding amino acid sequence is not a trivial task, often requiring the investigator to manually generate hundreds of possible DNA sequences and then individually scan them for restriction sites. Even for an experienced molecular biologist, it will take time and luck to find a suitable site. SILMUT, a program written and published several years ago, can be used to discover such diagnostic restriction sites (Shankarappa et al., 1992b). The user enters a short amino acid sequence, and SILMUT determines if any of 30 of the most common, 6bp restriction sites can introduced within that sequence. To make this task much faster and less error-prone, I wrote my own, web-based computer program, called SiteFind.

In some cases, however, silent mutations in the coding sequence can have a drastic effect on the translation rate. Thus, the user must be alert to the possibility of codon bias in the organism where the DNA construct will be used.

MATERIALS AND METHODS

Reagents and Plasmids

pCS2-Flag-KLF4 was sub-cloned from pMT3-KLF4, kindly provided by Dr. Vincent Yang, and verified by sequencing (MCLab, San Francisco, CA). All restriction enzymes and ligase were obtained from New England BioLabs (Ipswich, MA). Anti-Flag monoclonal antibody (M2) was purchased from Sigma (St. Louis, MO). HeLa and 293T cells were grown in DMEM media supplemented with 10% FBS and 1% penicillin/streptomycin, and split as needed.

Site-Directed Mutagenesis

SiteFind identified a potential *BglII* sequence overlapping with our desired R390S mutation of the KLF4 wild-type sequence [GenBank: BC010301]. Using the primer design guidelines included in the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), I chose forward primer 5'-CCAAAGAGGGGAAGATCTTCGTGGCCCCGG and reverse primer 5'-CCGGGGCCACGAAGATCTTCCCCTCTTTGG (*BglII* restriction site underlined). All primers were synthesized by Sigma-Genosys. PCR was performed using the *Pfu* Turbo DNA Polymerase (Stratagene) according to manufacturer's instructions. The PCR product was then digested with *DpnI* to remove template DNA, followed by transformation of XL-10 competent bacteria. Bacteria were then plated on ampicllin-containing Luria-Bertani (LB) agar overnight at 37°C. Individual colonies were grown in LB/Ampicillin medium for 12 hours at 37°C, and plasmid DNA was extracted using the QiaPrep MiniPrep Kit (Qiagen, Valencia, CA). Purified DNA was digested with *BglII* and *ClaI* and run on an 0.8% agarose gel for 30 min at 120V. Successful mutants, as determined by the presence of a second, 1244bp band were grown in 100mL LB/Ampicillin overnight and plasmid DNA extracted using the Qiagen MidiPrep Kit

(Qiagen). Purified DNA wasverified by sequencing. For our second mutant construct, K225/229R, SiteFind identified a potential *NheI* sequence. For this mutation, I chose forward primer 5'-CTGATGGGCAGGTTTGTGCTGAGGGCTAGCCTGACCACCCCTGGC and reverse primer 5'-GCCAGGGGTGGTCAGGCTAGCCCTCAGCACAAACCTGCCCATCAG (*NheI* restriction site underlined). I used similar methods to produce this construct, except that successful mutants were identified by restriction digest with *NheI* and *EcoRI* instead, which yields a 767bp band.

Western Blot

For Western blot, 293T cells were plated on a 12-well plate and transfected with 1ug of either pCS2 empty vector, pCS2-Flag-KLF4, pCS2-Flag-KLF4-R390S, or pCS2-Flag-KLF4-K225/229R using the calcium phosphate method. After 6 hours, the media was replaced and the cells allowed to grow for another 36 hours. Cells were lysed in standard RIPA buffer with 1% Triton X-100 and protease inhibitor cocktail. Lysate was boiled in SDS sample buffer and run on a 10% poly-acrylamide gel at 180V for 45min, and transferred to an Immobilon membrane (Millipore, Billerica, MA) at 30V overnight. After blocking in TBS-T (25mM Tris-HCl, 150mM NaCl, 0.05% Tween-20, pH 7.4) with 5% milk for 1 hr, membrane was incubated with α-Flag primary antibody (1:1000) for 1 hr, washed, and incubated with α-mouse secondary antibody (1:10,000). Membrane was then visualized using ECL buffer and exposed to film.

RESULTS

The ultimate goal of SiteFind is to search a given nucleotide sequence for any possible restriction sites that can be introduced without disturbing the amino acid sequence that it codes for. For example, the sequence CTCGAA codes for the amino acid sequence LE, or leucine-glutamate, but does not possess any common restriction site. However, by simply changing the last Adenine to a Guanine, the sequence becomes CTCGAG, which is the restriction site for *XhoI*. At the same time, the amino acid sequence is preserved, since both GAA and GAG code for glutamate. For such a short

sequence, the necessary mutations to introduce a restriction site may be obvious, but SiteFind can quickly search through much longer sequences, where potential restriction sites may be hidden in long sequence of nucleotides. I found that on the average end-user personal computer, SiteFind can handle sequences of up to approximately 400bp.

SiteFind was designed with the purpose of introducing a restriction site into a nucleotide sequence as a marker for successful point mutation via site-directed mutagenesis. Consistent with this purpose, the user can specify which amino acids should be changed in the peptide sequence and then select the potential restriction site closest to the point mutation. Ideally, these two will overlap, but this is not always possible. A novel restriction site within a few nucleotides of the point mutation is often sufficient to use as a marker.

Algorithm Optimizations

The redundancy in the genetic code means that as the length of a given amino acid sequence increases, the number of possible DNA sequences that can code for that sequence increases exponentially. Since the amino acid serine can be represented by six different codons, this means that a sequence of four serines can be represented by 6^4 (1296) different DNA sequences. To substantially reduce the number sequences that must be scanned by our program, SiteFind uses a "moving window" algorithm (See **Fig. 5A**). The "moving window" algorithm effectively breaks up a long nucleotide sequence into a series of short, non-redundant sequences that can be then searched individually. Thus, a long amino acid sequence with millions of possible nucleotide sequences can be converted into 10 or so "windows", each with only a few hundred possible sequences. The size of each "window" is determined by the length of the longest restriction site the user is searching for. In general, for a given restriction site of *n* nucleotides, the window must be at least *2n-1* nucleotides long. SiteFind then shifts the window only enough to ensure overlap between windows such that any possible restriction site is found, meaning that the window is shifted forward no more than *n* nucleotides (See **Fig. 5B**). This process is then repeated until the entire nucleotide sequence is traversed.

SiteFind was originally written in C++ as a simple command-line tool for in-house use. I subsequently rewrote the program as a Java applet embedded in a HTML web document, giving it a more intuitive, graphical interface and posted it on the Sealy Center for Cancer Cell Biology at UTMB. The source code to my Java applet is freely available and is released under the GNU Public License. SiteFind was written using TextPad v4.7.3 and compiled with the Java 1.4.2 Software Development Kit. The website was designed with Microsoft FrontPage.

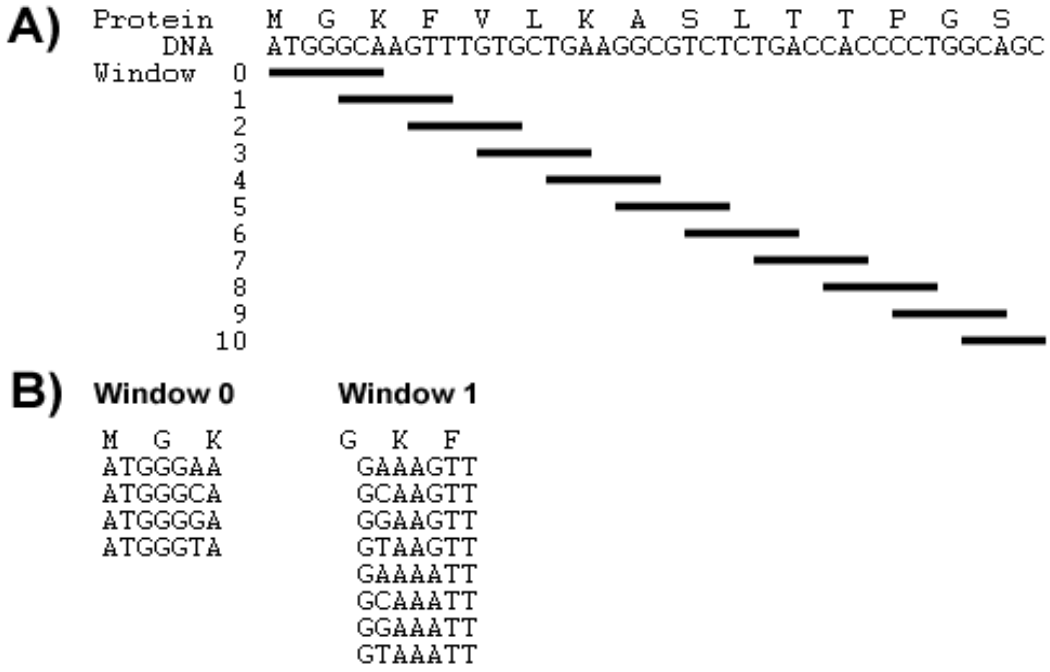

Figure 5: Example of how the algorithm is implemented with a 4 nucleotide restriction site. A, Each window is therefore 7 nucleotides, ensuring minimal overlap. B, Example of all the possible sequences generated for each of the first two search windows using the moving window algorithm.

Using SiteFind

SiteFind was designed to have an intuitive interface, with each step necessary to specify the search conditions presented in a separate window. A button labeled "Next" at the bottom right hand corner of each window allows the user to progress to the next step. The SiteFind applet loads in a browser once its webpage is visited and prints out a simple

message identifying the program name and creator. To begin, the user clicks "Next". The first window prompts the user to enter a short segment (preferably at least 15 nucleotides) of the wild-type DNA sequence, covering the region where a mutation is desired. The user is then prompted to select the correct reading frame for the sequence. After clicking "Next", the properly translated sequence is given, as shown in **Fig. 6A**.

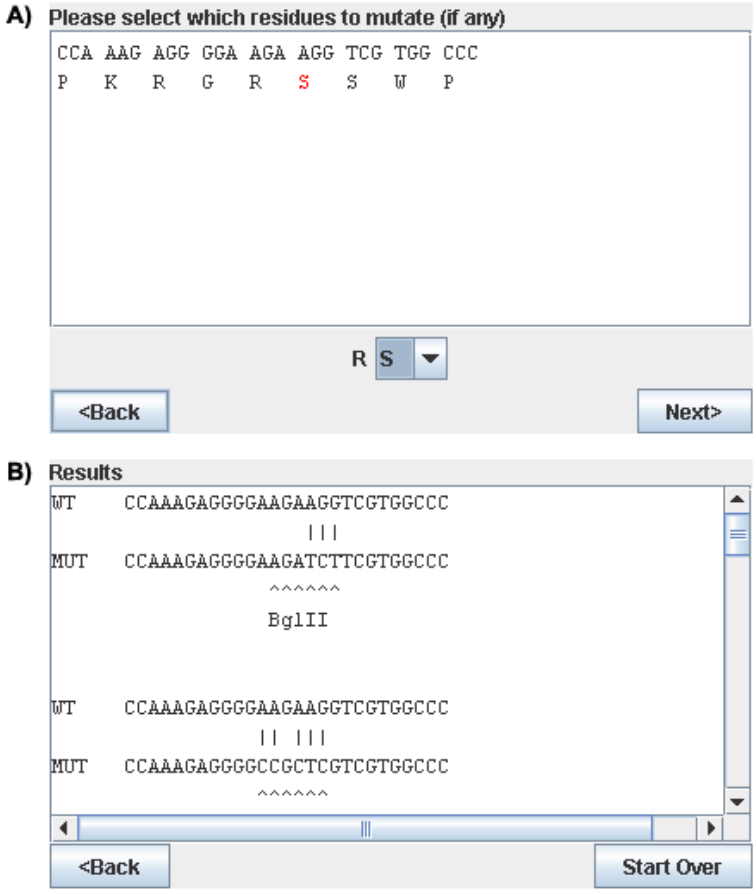

Figure 6: SiteFind screenshots. .A, Sample input, showing translated nucleotide sequence and a mutant residue highlighted in red. B, Sample output, showing a novel BglII site discovered within the sequence

The user then double-clicks the amino acid he wishes to mutate and selects from a drop-down list which residue it should be changed to. If the user wishes to mutate more than one amino acid, he can simply repeat this step. Each mutation is highlighted in red. In the next window, the user can select which restriction sites the program should search for.

Currently, SiteFind has 131 restriction sites to choose from and uses them all by default, but the user is free to remove any of these or add new ones if so desired. Any restriction sites present in the wild-type sequence are removed from the search. The next window then displays a progress bar as it searches: in most cases, the search takes no more than a few seconds. Once finished, the user can click "Next" one last time, and the results are printed in a list. A list of potential restriction sites is given, For each site, the wild-type sequence displayed, with the necessary mutant sequence displayed just under it. Any differences between the two sequences are indicated. Below the mutant sequence, the location of the introduced restriction site is clearly marked. If there are multiple locations in the sequence where a given restriction site can be introduced, only the location closest to the desired point mutation is displayed. (See **Fig. 6B**). The user can then use this information to design the appropriate primers for performing site-directed mutagenesis.

Examples of its Use

This tool is routinely used in my laboratory, such as in my work on Krüppel-like factor 4 (KLF4), a transcription factor implicated in colon cancer. Previous studies on KLF4 have shown that a single point mutation, R390S, can abolish its ability to enter the nucleus, where it is normally exclusively located (Shie and Tseng, 2001). In order to make this construct, I entered the wild-type DNA sequence corresponding to amino acids 385-393 into SiteFind and then specified the desired mutation R390S. Using the default settings, SiteFind found 10 restriction sites that we could use as a marker. I chose *BglII* since no *BglII* site was present in our original construct, and it required the mutation of only three nucleotides. Using this information, I was then able to design the proper primers for site-directed mutagenesis.

After transformation of competent bacteria with the PCR product, I plated the bacterial on LB/Amp+ agar overnight. The following day, I picked several colonies and isolated their plasmid DNA. The plasmid DNA was then digested with *ClaI*, which is present in the vector backbone, and *BglII*. Since *BglII* is neither present within the vector backbone nor the wild-type KLF4 sequence, *BglII* should only cut successfully mutated plasmid DNA, yielding a 1244bp fragment (See **Fig. 7A**). As shown in **Fig. 7B**, wild-

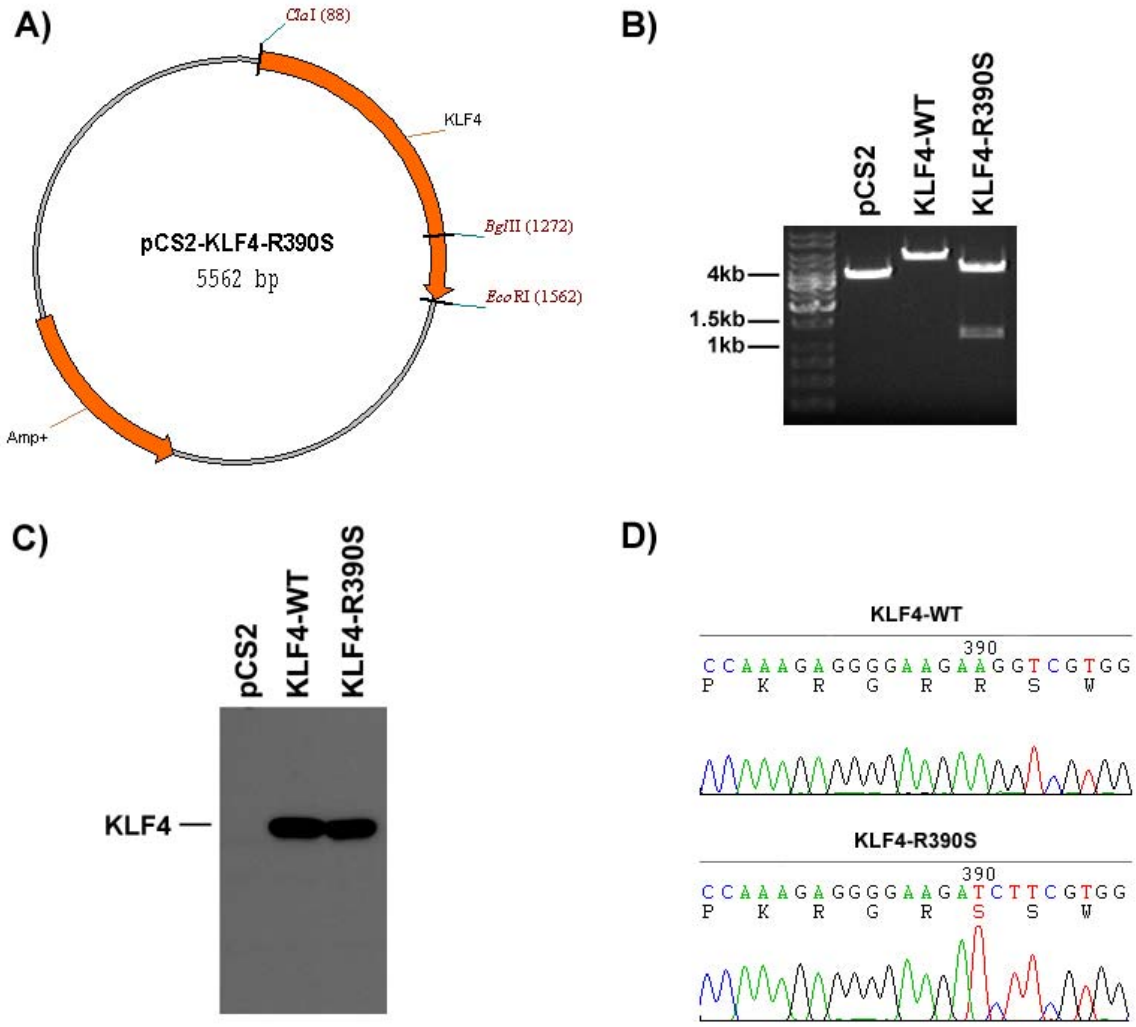

Figure 7: KLF4 R390S mutant has a novel *BglII* restriction site. A, pCS2-KLF4-R390S construct diagram. B, *ClaI* / *BglII* restriction digest of both wild-type and successfully mutated plasmid DNA. C, α-Flag Western blot showing expression of mutant construct in 293T cells. D, Sequencing result of the mutation, mutated residues are highlighted in red.

type plasmid DNA yields only one fragment, whereas successfully mutated DNA yields a second, 1.2kb fragment.

To confirm that the mutant construct is expressed, I transfected 293T cells, lysed the cells 48-hours post-transfection, and performed an α-Flag Western blot with the lysate. **Fig. 7C** demonstrates that both the wild-type and mutant constructs express a protein of identical size, whereas transfection with an empty vector yields no Flag-tagged protein whatsoever. This is expected since a point mutation should have no detectable effect on the molecular weight. Finally, I verified the mutant construct by sequencing (See. **Fig. 7D**).

To demonstrate that SiteFind can be used to design multiple point mutation, I produced a double point mutation of KLF4, mutating two successive lysines (K225/K229) to arginine. Using SiteFind, I decided to introduce an *NheI* site just 3' to the second point mutation. After PCR and plasmid purification, I digested the mutant construct with *NheI* and *EcoRI*. *NheI* should only cut the mutant construct, producing a 767bp fragment (See **Fig. 8A**). As expected *NheI* cuts the mutant construct to produce a second fragment of approximately 750bp, whereas the wild-type plasmid yields only one fragment (See **Fig. 8B**). I confirmed expression of this construct in 293T cells, and as expected, wild-type and K225/229R mutant KLF4 produce bands of an identical size (See **Fig. 8C**). Finally, I verified our construct by sequencing (See **Fig. 8D**).

DISCUSSION

There are several programs available for designing primers for site-directed mutagenesis. Most of these programs are used to calculate the annealing temperature and to predict secondary structures. They cannot be used to design a restriction site. SiteFind is designed specifically for this.

In an easy-to-use, graphical interface, the user is prompted to enter the desired template nucleotide sequence. Then, the translated amino acid sequence is given and the user is able to select which amino acids to mutate. After that, the user can specify which restriction sites to search for, and even add additional sites if so desired. Finally, after a

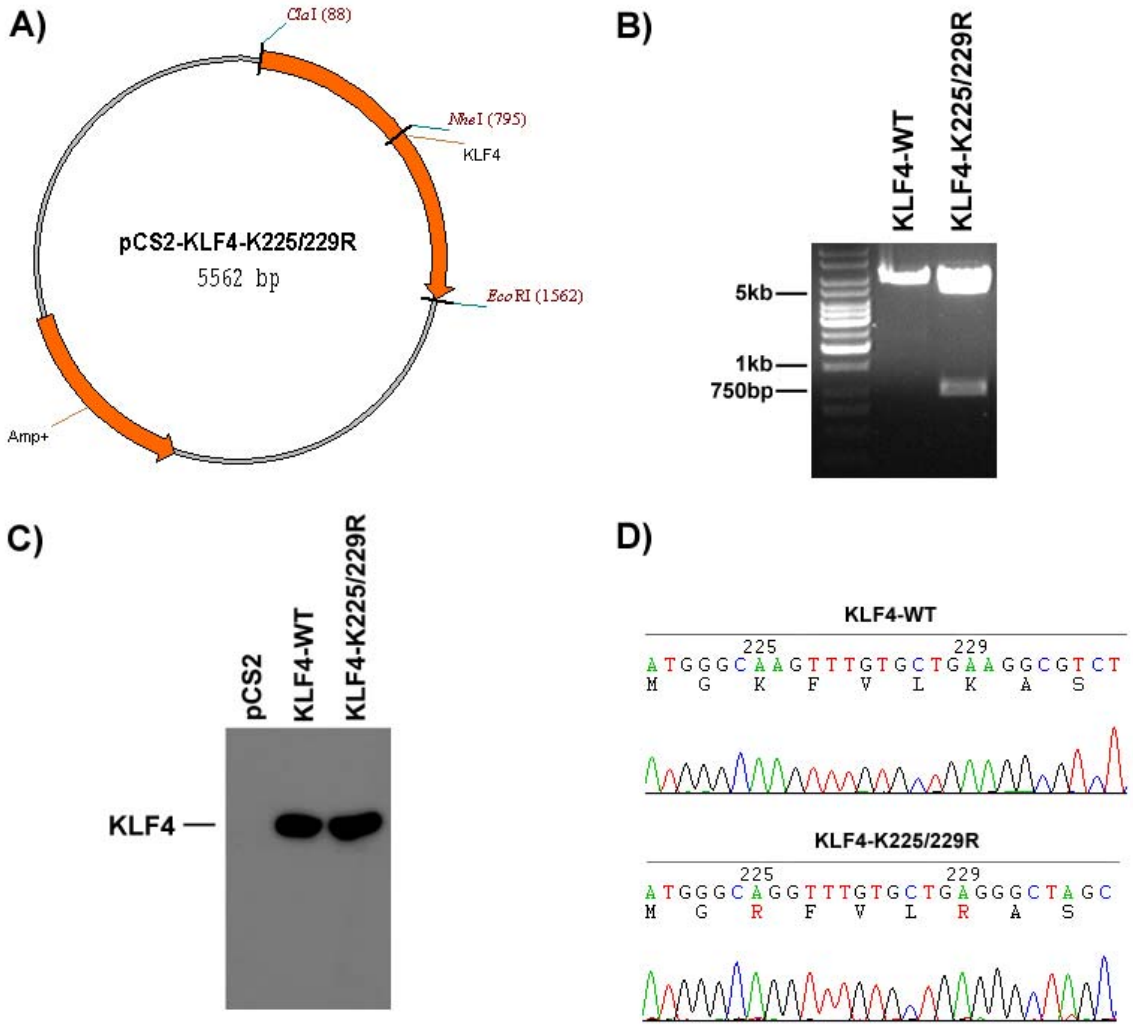

Figure 8: KLF4-K225/229R mutant has a novel NheI restriction site. A, pCS2-KLF4-K225/229R construct diagram. B, NheI / EcoRI restriction digest of both wild-type and successfully-mutated plasmid DNA. C, α-Flag Western blot showing expression of mutant construct in 293T cells. D, Sequencing results of the mutation, mutated residues are highlighted in red.

few seconds, a list of potential restriction sites is given. For each site, only the location closest to the desired point mutation and involving the fewest number of mutations is given. This substantially reduces the amount of information the user has to process prior to selecting the optimal sequence for site-directed mutagenesis, saving both time and money. Furthermore, SiteFind can be used for any type of mutagenesis and places no limits on the number of point mutations in the mutant sequence.

As the sequence length increases, the time required to search through every possible DNA sequence increases exponentially. Thus, searching sequences longer than15 base-pairs quickly becomes infeasible. Our "moving window" algorithm is a novel way to drastically reduce the time required for a search, and yet does so without missing any potential sites. Because SiteFind implements this algorithm, it can process much longer sequences.

Shankarappa et. al. have published a computer program called SILMUT (Shankarappa et al., 1992b). SILMUT is a simple command-line program that can search a short amino acid sequence for the 30 most common, 6 base-pair restriction sites. It does this by translating each restriction site in all three frames and compares every possible translation with the user-specified amino acid sequence. Primer Generator is a web-based program that performs a function similar to SiteFind (Turchin and Lawler, 1999). However, the Primer Generator requires the user to manually type in both the wild-type sequence and desired mutant amino acid sequence and to manually pick from hundreds of output sequences. Furthermore, it is not suitable for nucleotide sequences longer than 15bp.

In contrast, SiteFind, automatically translates the input nucleotide sequence and allows the user to graphically select which residues to mutate. Furthermore, our window algorithm enables SiteFind to quickly and efficiently work with sequences of any length. For each restriction site, if multiple locations are found, SiteFind only gives the location closest to the desired point mutation: this means much less information for the user to parse in order to choose the best restriction site and sequence. Although not specifically designed for it, SiteFind could be used to make translational fusions between two

different coding sequences. The user can specify that SiteFind give every location found for each restriction enzyme, and then run a search on a portion of both sequences. Then, through manual comparison, the user could select a restriction site found within both sequences and design the appropriate primers for introducing the necessary mutations.

SiteFind is a useful tool for performing site-directed mutagenesis, enabling the user to introduce a novel restriction site into the mutated nucleotide sequence for use as a marker of successful mutation. The "moving window" is a novel algorithm that enables SiteFind to work efficiently with sequences up to 400bp. In order to demonstrate its utility, I introduced a point mutation, R390S, into the wild-type sequence of KLF4 while simultaneously introducing a novel *BglII* restriction site. This mutant DNA could be cut by *BglII*, as expected, and expressed a full-length protein in 293T cells. For a double point-mutation, K225/229R, we introduced a novel *NheI* restriction site. This mutant DNA could be cut by *NheI*, as expected, and expresses a full-length protein in 293T cells.

CHAPTER 3: MOLECULAR MECHANISMS OF KLF4-MEDIATED TRANSCRIPTION

INTRODUCTION

Some of the data shown in this chapter has been published (Figures 9, 12 and 13) (Evans et al., 2007). KLF4 is a transcription factor that modulates the expression of many genes. For example, KLF4 activates the expression of Intestinal Alkaline Phosphatase (IAP) and $p21^{Cip1}$, and represses the expression of Cyclin B_1 (Hinnebusch et al., 2004; Yoon and Yang, 2004; Zhang et al., 2000). Although it is not fully understood how KLF4 regulates gene expression, it was previously demonstrated that KLF4 and CBP (CREB Binding Protein) interact (Geiman et al., 2000). CBP and its close homologue, p300, are transcriptional co-activators (Eckner et al., 1994) that contain a catalytic histone acetyltransferase (HAT) domain (Ogryzko et al., 1996). Although p300 and CBP interact with many other proteins, they do not bind DNA directly. Instead, they are recruited to specific regions of DNA by sequence-specific transcription factors. p300/CBP are then able to facilitate the recruitment of the basal transcriptional machinery as well as increase localized histone acetylation through their HAT domain. Acetylated histones have long been associated with the activation of transcription (Allfrey et al., 1964) and are thought to loosen DNA-histone interactions in order to facilitate binding of additional transcription factors.

The crucial function of p300/CBP in transactivation is well established. For example, p53 interacts with p300 on both the *GADD45* and *CDKN1A* ($p21^{Cip1}$) promoters, increasing localized histone acetylation (An et al., 2004; Barlev et al., 2001). In a similar fashion, KLF6 interacts with p300 and promotes histone acetylation on the *CDKN1A* ($p21^{Cip1}$) gene (Li et al., 2005a). Thus it is likely that p300/CBP are similarly important for KLF4-mediated transactivation. As p300 and CBP share 63% homology at the amino acid sequence level, we postulated that KLF4 could directly interact with p300 as well as CBP.

MATERIALS AND METHODS

Reagents and Plasmids

pMT3-KLF4 was a kind gift from Vincent Yang. KLF4 was sub-cloned from the pMT3 vector into the pCS2 vector with the addition of an N-terminal FLAG sequence (DYKDDDDK). Flag-p300 and pRC-CMV-mCBP-HA have been previously described (Bhakat et al., 2004; Luo et al., 2004b). pGEX-GST-p300-CH3 (residues 1780-1891) is a gift from William Weis (Daniels and Weis, 2002). HDACs 1, 2, and 3 have been described previously (Bhakat et al., 2004; Luo et al., 2004b; Sui et al., 2004; Zhang et al., 2006). HDACs 4, 5, and 6 were kind gifts from Stuart L. Schreiber. IAP-Luciferase has been previously described (Wang et al., 2001). The TOPFlash reporter has been previously described (Zhang et al., 2006).

Deletion mutants KLF4(1-366), KLF4(350-483), KLF4ΔN(155-483), KLF4ΔC(1-392), and KLF4-N(1-157) were generated by PCR and cloned into the pCS2 vector. Point mutant KLF4-R390S was described in the previous chapter. Point mutant KLF4-E93/95/96V was introduced by site-directed mutagenesis using primers designed with the assistance of the software program SiteFind (see **Chapter 2**) (Evans and Liu, 2005), using the following primers: 5'-GGCCCTCCTAGCCCGACGCGTGACCGTGGTGTTCAACGACCTCC-3' (Forward) and 5'- GGAGGTCGTTGAACACCACGGTCACGCGTCGGGCTAGGAGGGCC-3' (Reverse). Cyclin B_1-Luciferase was produced by amplifying the proximal 2.4kb of the Cyclin B_1 gene from genomic DNA using PCR. The resultant 2.4kb fragment was then inserted into the pGL3-basic (Promega, Madison, WI) reporter. All constructs were verified by sequencing.

Cell Culture

HEK293T, HT29, and HeLa cells were grown in DMEM medium (Mediatech) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. LS174T cells were grown in RPMI medium (Mediatech, Manassas, VA) supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. Stable cell lines LS174T-tet/on,

LS174T-tet/on-KLF4, and LS174T-tet/on-KLF4-K225/229R were grown normal RPMI medium, supplemented with 1ug/mL Zeocin and 0.25ug/mL Blasticidin.

Transient Transfection

293T cells were plated in a 12 well plate and transiently transfected with DNA using the calcium phosphate method. Briefly, plasmid DNA was diluted in 100uL of CaCl2, 100ul of 2xHEPES-buffered saline was slowly added drop-wise, and the resultant mixture was added to the cells. After 3-18 hours, fresh media was added and cells were grown for an additional 36 hours. Lipofectamine (Invitrogen, Carlsbad, CA) transfections were performed following the manufacturer's directions.

Stable Cell Line Selection

Stable cell line LS174T-tet/on-KLF4 was produced by transient transfection of LS174T-tet/on cells with the pcDNA4-tet/on-KLF4 construct using Lipofectamine. Stable clones were selected for with Zeocin (1ug/mL) added to the culture medium. After approximately 2 weeks, individual colonies were isolated and selected for based on their level of KLF4 expression after the addition of doxycycline (1ug/mL) for 48 hours.

RT-PCR

LS174T-tet/on-KLF4 cells were plated approximately $1x10^6$ cells per plate in a $10cm^2$ dish. The following day, doxycycline (1ug/mL) was added to the culture medium. After 48 hours incubation, RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA). RT-PCR was performed as described previously (Zhang et al., 2006). The following primers were used: β-Actin: 5'-CAACCGCGAGAAGATGAC-3', 5'-AGGAAGGCTGGAAGAGTG-3'; $p21^{Cip1}$: 5'-CGACTGTGATGCGCTAATGG-3', 5'-AGAAGATCAGCCGGCGTTTG-3'; Cyclin B_1: 5'-GCAGCACCTGGCTAAGAATG-3', 5'-GCCACAGCCTTGGCTAAATC-3'; IAP: 5'-CCATTGCCGTACAGGATGGAC-3', 5'-CGCGGCTTCTACCTCTTTGTG-3'.

Western Blot

Cells were washed in PBS, and lysed in the appropriate volume of lysis buffer (50mM HEPES, 100mM NaCl, 2mM EDTA, 1% glycerol, 50mM NaF, 1mM Na3VO4,

1% Triton X-100, protease inhibitor cocktail, pH 7.6) for 30min at 4ºC, followed by centrifugation at 13,200rpm for 10min in a table-top centrifuge. Samples were boiled in 6X SDS sample buffer for 5min, then loaded onto a 10-12% polyacrylamide gel. Gels were run for 45min at 180V, and then transferred to a Immobilon PVDF membrane (Millipore, Billerica, MA) for 3 hours at 30V. Membranes were blocked in TBS-T (25mM Tris, 150mM NaCl, 0.05% Tween-20, pH 7.4) with 5% milk for 1 hour and then incubated with the appropriate antibody overnight at 4ºC. Membranes were washed three times with TBS-T, and the appropriate secondary antibody was then added for 1 hour. After three washes in TBS-T, membranes were visualized in electrochemiluminescent (ECL) buffer (Luminol diluted in 0.1M Tris, 0.05% H_2O_2, pH 8.6) and exposed to film (Phenix, Candler, NC).

Time Course Assays

For butyrate treatment, LS174T cells were plated on a 12-well plate, $1x10^5$ cells/well. Starting the following day, cells were treated with 5mM sodium butyrate for various time periods such that all cells were ready for harvest at the same time. Cells were then lysed, and Western blot was performed on the lysate as described above.

IAP Enzymatic Assay

HT29 cells were harvested after treatment with 5mM sodium butyrate for various time points, and 40μL of cell lysate was incubated with 200μL of p-nitrophenyl phosphate (Sigma, A3469) in a 96-well plate for 1-3 hours. The reaction was stopped by the addition of 50μL 3M NaOH and the yellow enzymatic product was quantitated by measuring absorbance in a Vmax 96-well plate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 405nm.

Immunoprecipitation Assays

293T cells were transiently transfected in a 12-well plate with 0.5ug of empty vector (pCS2) or the appropriate KLF4 construct with or without 0.5ug of p300 plasmid DNA. Cells were washed with PBS and lysed in Triton lysis buffer (50mM HEPES, 100mM NaCl, 2mM EDTA, 1% Glycerol, 1% Triton, 50mM NaF, 1mM Na_3VO_4,

protease inhibitor cocktail, pH 7.4) for 30min at 4ºC. Lysate was collected and centrifuged at 13,200rpm for 10min. Supernatant was diluted 1:1 in PBS and added to 20uL of M2-agarose beads (F7425, Sigma, St. Louis, MO). After gentle rocking overnight at 4ºC, beads were washed three times in PBS-T, and the remaining bound proteins were eluted either with 20uL 0.2mg/mL Flag peptide for 20 min, or by boiling in 40ul 2X SDS sample buffer for 5 min. Western blot was then performed as described earlier.

GST Pull-Down Assays

E. coli were transformed with GST and GST-CH3-p300 plasmids via heat shock and plated on Luria-Bertani agar supplemented with Ampcillin (LB/Amp+). The next day, a single colony was inoculated into 200mL of LB/Amp+ media and grown overnight with shaking. The next day, 20mL of media was diluted into 180mL of fresh LB/Amp+ media and grown for 1 hour. 20uL of 1M IPTG was added to final a concentration of 100uM, and grown for an additional 5 hours. Cells were then spun at 6000g for 15min at 4ºC, and resuspended in 15mL of PBS. Cells were then sonicated five times for 30 seconds each in a Branson digital sonifier with a 102C probe at 40% amplitude. Triton X-100 was added to a final concentration of 1% and kept at 4ºC with gentle rocking for 30min. Cellular debris was then pelleted at 20,000g for 30min. 2mL of supernatant was incubated with 500uL of glutathione sepharose (506405, BioWorld, Dublin, OH) overnight at 4ºC with gentle rocking. Sepharose beads were then pelleted with gentle centrifugation and washed three times in PBS-T (Phosphate-buffered saline with 1% Triton X-100, pH 7.4). 20uL of washed Sepharose beads containing purified GST or GST-CH3-p300 protein were incubated with 200uL of cell lysate diluted in 200uL of PBS-T. After gentle rocking overnight at 4ºC, beads were washed three times in PBS-T. Bound proteins were eluted by boiling in 40uL 2x SDS sample buffer for 5min. Western blot was then performed with the eluted protein as described above.

Time Course Assays

LS174T-tet/on-KLF4 cells were plated at $1x10^5$ cells/well in a 12-well plate. Starting the following day, doxycycline (1μg/mL) was added to the appropriate wells

such that all wells were harvested at the same time. Then, Western blot was performed using a $p21^{Cip1}$ (2947, Cell Signaling, Danvers, MA), Cyclin B_1 (4135, Cell Signaling), β-Actin (A1978, Sigma), or KLF4 antibody (Zhang et al., 2006).

Luciferase Assays

IAP-Luciferase reporter was used as described in (Wang et al., 2001). HEK293T cells were transiently transfected in a 12-well plate with 0.2μg of the appropriate reporter and 0.5μg of empty vector (pCS2+) or the appropriate KLF4 construct with or without 0.5μg of p300 plasmid DNA. Total DNA transfected in each well was 1.2μg and was normalized using pCS2 DNA as needed. Two days later, cells were harvested and Luciferase activity measured. Conditions were done in triplicate and each experiment was carried out at least two times.

Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed according to the protocol developed by (Nowak et al., 2005) with some modifications. $1x10^6$ LS174T-tet/on-KLF4 cells were plated on $10cm^2$ dishes. The following day doxycycline was added to a final concentration of 1μg/mL and cells were grown for an additional 36 hours. The cells were crosslinked with disuccimidyl glutarate (20593, Pierce, Rockford, IL) and formaldehyde at room temperature. Cells were pelleted at 3000rpm for 1 min and resuspended in 900μL L1 buffer (50mM Tris, 2mM EDTA, 0.1% IGEPAL, 10% glycerol, 1mM DTT, 1mM PMSF, protease inhibitor cocktail, pH 8.0) and allowed to sit on ice for 15 min. After centrifugation at 4000rpm for 5 min, supernatant was removed and the nuclear pellet was resuspended in 500μL of ChIP lysis buffer (1% SDS, 10mM EDTA, 50mM Tris, protease inhibitor cocktail, pH 8.0). Cell lysate was sonicated 4 times for 10 seconds at setting 5 on a Branson Sonifier 150 on ice, with a 30 second break between sonciations. After centrifugation at 13200rpm for 10min at 4ºC, supernatant was transferred to a fresh tube. Absorbance at 260nm (A_{260}) of a 1:50 dilution of each sample was measured using a spectrophotometer to estimate DNA content. For each assay, 100μL of the most dilute sample was used and the more concentrated samples were diluted in lysis buffer so that

each condition received the same amount of total DNA. Lysate was diluted and incubated with 4μg of the appropriate antibody overnight, followed by incubation with 100μL of Protein A agarose/salmon sperm DNA 50% slurry (16-157, Millipore) for 3 hours at 4ºC. Beads were then washed with a series of washes and bound DNA-protein complexes were eluted and decrosslinked. DNA was then purified by phenol/chloroform extraction and ethanol precipitation. Pelleted DNA was resuspended in 20μL TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and 1μL was used for PCR. Optimal cycling parameters to ensure operation in the linear range were primer-specific, but typically 32-40 cycles were done. The following primers were used: Cyclin B_1: 5'-TCTTGCCCGGCTAACCTTTCCAGG-3', 5'-TTCCGCCGCAGCACGCCGAGAAGA-3'; IAP: 5'-CCACAAGACACTGTGAGCCACACC-3', 5'-AAGTGGGGACACCAGGAACCGGCT-3'; GAPDH: 5'-ATGCCAGGAGCCAGGAGATG-3', 5'-TGAGAGGCGGGAAAGTTGGG-3'. Antibodies used include p300 (sc-584, Santa Cruz, Santa Cruz, CA), HDAC3 (611124, BD Biosciences, San Jose, CA), and KLF4 (Zhang et al., 2006).

RESULTS

Previous reports implicated KLF4 as a negative regulator of the cell cycle. To test this function in colon cancer cells, we established a doxycycline-inducible KLF4 stable LS174T cell line and monitored the expression of $p21^{Cip1}$ and Cyclin B_1 after treatment with doxycycline over a time course. As shown in **Fig. 9A**, expression of KLF4 increased after doxycycline treatment. Expression of $p21^{Cip1}$ increased in a similar manner, whereas expression of Cyclin B_1 was strongly downregulated at 48 hours, suggesting that KLF4 represses the cell cycle by differentially regulating expression of these genes. To demonstrate treatment with doxycycline does not have a direct effect on the expression of these proteins, we performed the same experiment using the parental cell line, LS174T-tet/on, and found no effect on either $p21^{Cip1}$ or Cyclin B_1 expression. We also examined the effect of KLF4 overexpression on the level of mRNA for these

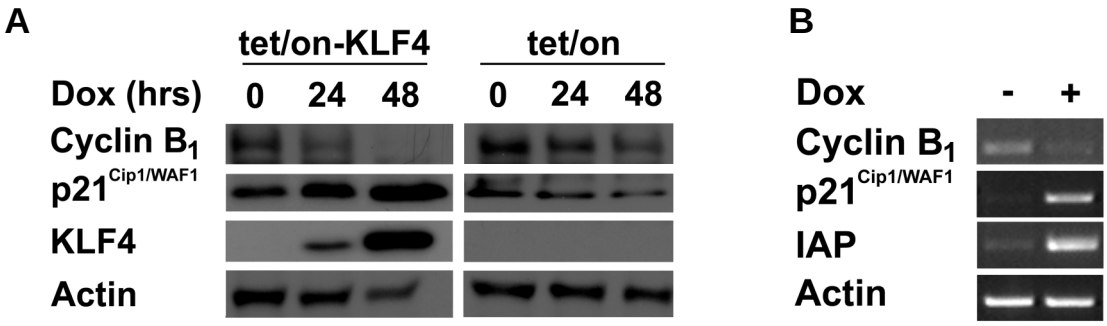

Figure 9: A, KLF4 represses Cyclin B_1 and activates $p21^{Cip1}$ expression in a LS174T doxycycline-inducible cell line. LS174T-tet/on-KLF4 cells were treated with 1ug/mL doxycycline for various time periods prior to harvest. Western blot was then performed on the cell lysate with antibodies shown. B, RT-PCR for several KLF4 target genes in LS174T-tet/on-KLF4 cells after treatment with doxycycline for 48 hours.

target genes using semi-quantitative RT-PCR (**Fig. 9B**). After 48 hours treatment with doxycycline, $p21^{Cip1}$ mRNA was significantly increased, whereas the level of Cyclin B_1 mRNA decreased, suggesting that KLF4 regulates the expression of these genes by modulating their transcription. Furthermore, expression of KLF4 also resulted in strong induction of IAP, a known target of KLF4 and a marker of enterocyte differentiation (Hinnebusch et al., 2004; Wang et al., 2001).

KLF4 Interacts with the CH3 domain of p300/CBP Through its N-terminus

The CH3 domain of p300/CBP is a cysteine/histidine-rich domain known to interact with many different transcription factors (Kalkhoven, 2004). A previous report demonstrated that KLF4 interacts with CBP through its N-terminal transactivation domain (Geiman et al., 2000). To confirm these results, Flag-tagged, full-length KLF4 and various mutants were co-expressed with full-length HA-tagged CBP in 293T cells. KLF4 was immunoprecipitated and an anti-HA Western blot was performed to detect CBP. As shown in **Fig.10A**, Full-length CBP could interact with KLF4, a C-terminal deletion (1-366), and the N-terminus alone (1-157), but not with ΔN (155-483) or the C-terminal DNA-binding domain (350-483). To test whether KLF4 interacts with p300 as well, Flag-tagged KLF4 was overexpressed in 293T cells, and cell lysate was incubated with GST-p300(CH3 domain). Wild-type KLF4, interacts with the CH3 domain of p300

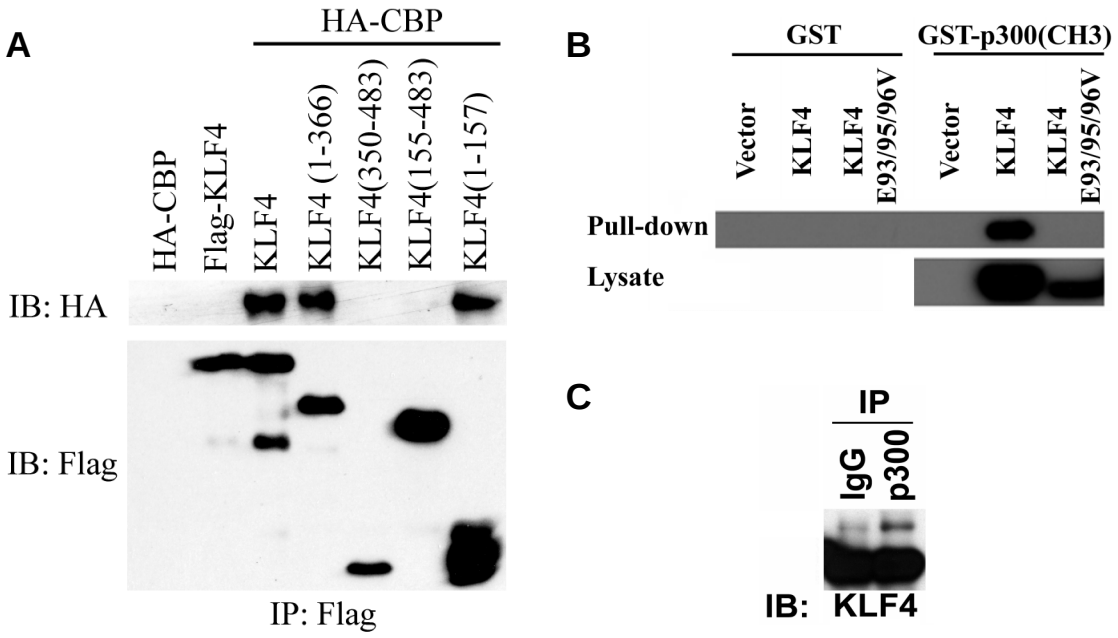

Figure 10: N-terminus of KLF4 is required for interaction with p300/CBP. A, wild-type or N-terminal mutant KLF4 (E93/95/96V) was overexpressed in 293T cells. Lysate was harvested and incubated with GST-p300(CH3 domain) protein or GST alone. Anti-Flag Western blot was then performed on eluate the detect KLF4 protein. B, Flag-tagged, full-length KLF4 (1-483) and various mutants were co-expressed with full-length HA-tagged CBP in 293T cells. KLF4 was immunoprecipitated using M2-agarose (anti-Flag) beads. Bound CBP protein was detected using an HA antibody. C, Endogenous KLF4 and p300 interact *in vivo*. p300 was immunoprecipitated from HCT116 cells, which express detectable levels of KLF4 protein, and eluate was probed for KLF4. IgG was used as a negative control.

(**Fig. 10B**). However, a triple point mutation (E93/95/96V) within the N-terminus of full-length KLF4 abolished interactions with GST-CH3-p300. GST was included as negative control. To demonstrate that p300 and KLF4 interact *in vivo*, p300 was immunoprecipitated from HCT116 cells, which express detectable levels of KLF4. After IP, KLF4 was detectable in the eluate (**Fig. 10C**). Collectively, these data suggesting that KLF4 interacts with p300/CBP through its N-terminal domain and that a triple point mutation in the N-terminus abolishes this interaction.

The N-terminal Domain of KLF4 is Required for Both Activation and Repression

KLF4 interacts with p300/CBP through its N-terminal transactivation domain. To test whether this interaction is important for its function, I used Luciferase reporters for several known target genes. As shown in **Fig. 11A**, KLF4 activates the IAP-Luciferase reporter and synergizes with p300. However, KLF4-E93/95/96V, which is unable to interact with p300/CBP, cannot activate the reporter and does not synergize with p300. In addition, co-expression of E1A, which binds and sequesters p300/CBP (Lundblad et al., 1995), completely blocks KLF4-mediated activation of the reporter. These data suggest that the interaction between KLF4 and p300/CBP is required for KLF4-mediated activation of the IAP reporter.

To test whether this interaction is required for KLF4-mediated repression as well, I used a Cyclin B_1 reporter and a TOPFlash reporter. KLF4 represses the Cyclin B_1 reporter, whereas KLF4-E93/95/96V cannot (**Fig. 11B**). TOPFlash is a widely used reporter for Wnt/β-catenin signaling and contains three consensus TCF binding sites. β-catenin activates this reporter approximately 25-fold. Wild-type KLF4 dose-dependently inhibits β-catenin mediated activation of the TOPFlash reporter, whereas KLF4-E93/95/96V is unable to repress the reporter (**Fig. 11C**). These data suggest that the N-terminal domain of KLF4 is necessary for repression as well as activation.

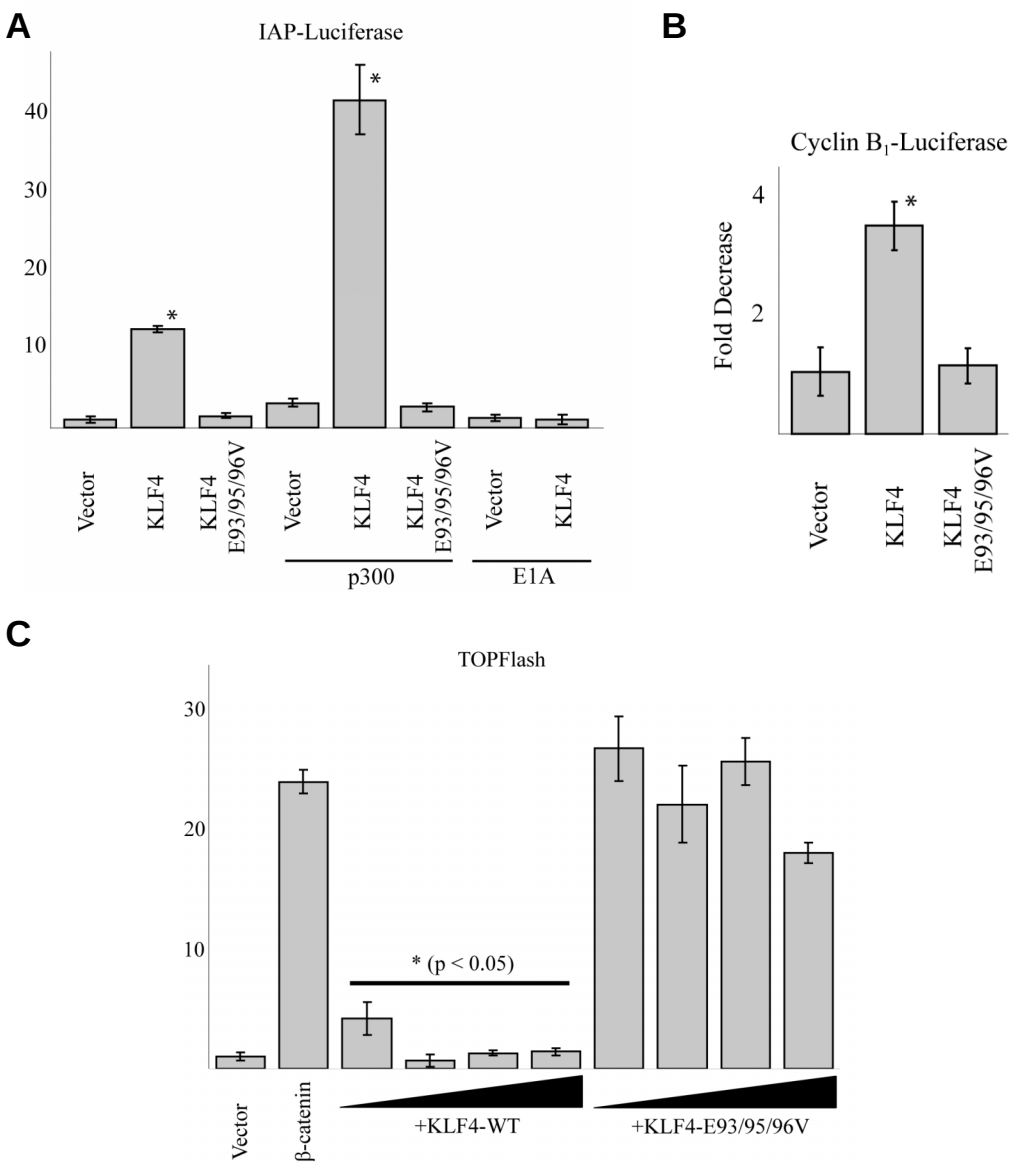

Figure 11: N-terminus of KLF4 is required for both activation and repression of several reporters. A, IAP-Luciferase assay. KLF4 or CBP-binding mutant KLF4 (E93/95/96V) were co-transfected with IAP reporter in 293T cells. Mutant KLF4 was unable to activate reporter and did not synergize with p300. The p300/CBP antagonist E1A blocked KLF4-mediated activation of the IAP reporter. B, Cyclin B_1 Luciferase assay. Wild-type KLF4 represses Cyclin B_1 reporter, whereas mutant KLF4 cannot. C, TOPFlash assay. β-catenin activates reporter, whereas increasing amounts of KLF4 (0.1 to 0.5ug) inhibit activation. Mutant KLF4 cannot repress reporter. *, different from control ($p < 0.05$ by Student's t-test).

KLF4 Differentially Modulates Histone Acetylation at the Promoter of Target Genes

Since a major function of p300 is to acetylate histones (Ogryzko et al., 1996) and histone acetylation can have a potent effect on gene expression (Allfrey et al., 1964), I hypothesized that KLF4 might activate gene expression via recruitment of p300 to the target promoter. To test this, I performed ChIP assays in the doxycycline-inducible LS174T-tet/on-KLF4 cell line. Overexpression of KLF4 results in increased occupancy of p300 at the IAP promoter, whereas KLF4 does not affect p300 occupancy at the Cyclin B_1 promoter (**Fig. 12A**). KLF4 itself occupies the promoter of the both these genes (**Fig. 12B**), suggesting that KLF4 specifically recruits p300 to the promoter of activated target genes.

In addition, I performed ChIP using an acetylated-histone H4-specific antibody. As shown in **Fig. 12B**, induction of KLF4 expression results in an increased level of acetylated H4 on the IAP promoter, suggesting that KLF4 increases histone acetylation via recruiting p300 to the IAP promoter. In contrast, binding of KLF4 to the Cyclin B_1 promoter results in an overall decrease of histone H4 acetylation at its promoter,

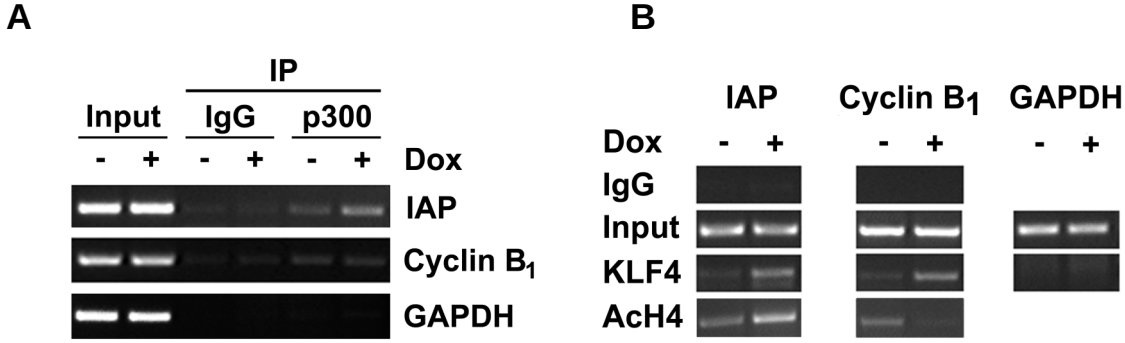

Figure 12: KLF4 differentially modulates histone acetylation at the promoter of target genes. A, ChIP assay in LS174T-tet/on-KLF4 cells. KLF4 binds the proximal promoter of IAP and Cyclin B_1 and modulates local histone H4 acetylation. KLF4 did not bind to the promoter of an unrelated gene, GAPDH. B, ChIP as in previous panel, using a p300-specific antibody. KLF4 recruits p300 to the IAP promoter but not the Cyclin B_1 promoter.

consistent with its inability to recruit p300 to this promoter. As a control, I performed PCR after ChIP for the housekeeping gene GADPH. Binding of KLF4 binding to this gene was undetectable, although its DNA was present in the input fraction. These results suggest that KLF4 differentially recruits p300 and regulates histone acetylation at the promoter of its target genes.

KLF4 recruits HDAC3 to repress Cyclin B_1 expression

My discovery that overexpression of KLF4 differentially modulates histone acetylation was compelling, so E decided to investigate the role of histone deacetylases (HDACs) using the HDAC inhibitor sodium butyrate. Previous reports have shown that butyrate can inhibit proliferation and induce differentiation, thus it seemed logical that inhibition of deacetylation should have a similar effect as KLF4 on the expression of IAP, Cyclin B_1, and $p21^{Cip1}$. I treated HT29 cells with butyrate and measured the enzymatic activity of IAP. Treatment with butyrate for 48 hours resulted in a dramatic increase in IAP enzymatic activity (**Fig. 13A**). In addition, I treated LS174T cells with butyrate for various times and measured the changes in $p21^{Cip1}$ and Cyclin B_1 expression via Western blot. Butyrate treatment resulted in a gradual increase in $p21^{Cip1}$ expression, peaking at 24 hours (**Fig. 13B**). On the other hand, butyrate had a minimal effect on Cyclin B_1 expression for the first 12 hours, but at 24 hours post-treatment, expression of Cyclin B_1 was dramatically reduced. This suggests that within 24 hours, butyrate had induced cell cycle arrest. Furthermore, inhibition of histone deacetylases by butyrate resulted in a rapid increase in histone H4 acetylation, peaking at 6-12 hours.

To probe the role of histone deacetylases more specifically, I investigated the ability of various HDACs to synergize with KLF4 in repressing a Cyclin B_1-Luciferase reporter (**Fig. 13C**). Co-transfection of the reporter and KLF4 alone resulted in ~17-fold repression of the reporter. Co-transfection of the reporter and HDACs 1 through 6 individually resulted in modest repression (1.3 to 3.3-fold repression), and co-transfection of KLF4 and most HDAC plasmids resulted in no further increase in repression relative to KLF4 alone. However, co-transfection of HDAC3 and KLF4 resulted in 66-fold repression of the reporter, suggesting that KLF4 synergizes with HDAC3 in repressing

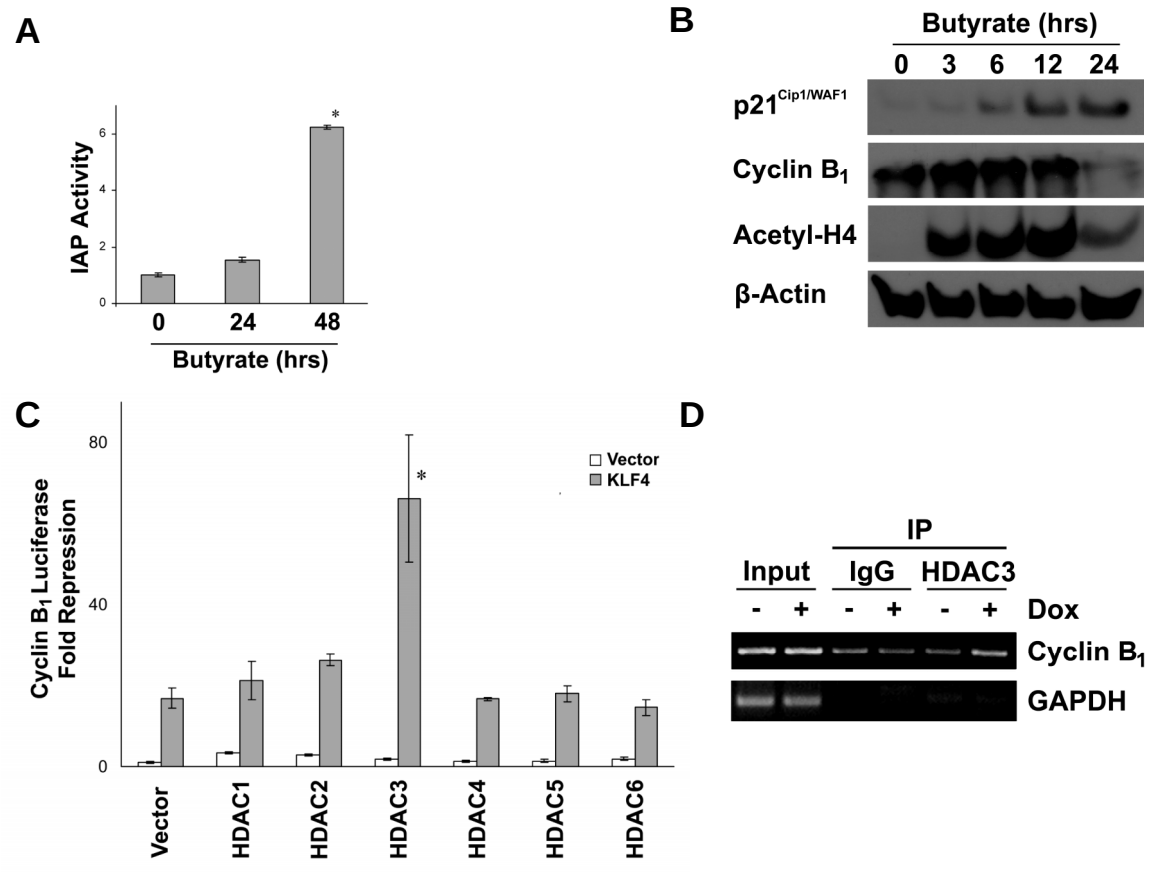

Figure 13: KLF4 recruits HDAC3 to repress Cyclin B_1 expression. A, IAP enzymatic assay. HT29 cells were treated with sodium butyrate for various time periods and then cell lysate was incubated with p-nitrophenyl phosphate, an artificial substrate of IAP that produces a yellow color when cleaved. Absorbance was then measured at 405nm. B, Butyrate time course assay. LS174T cells were treated with butyrate for various time periods and Western blot was performed using labeled antibodies. Actin was included as a loading control. C, Cyclin B_1 Luciferase assay. Empty vector (clear bars) or KLF4 (shaded bars) was co-transfected with the Cyclin B_1-luciferase reporter, along with various HDAC plasmids. D, ChIP, LS174T-tet/on-KLF4 cells. ChIP was performed as in Fig. 12B, but using an HDAC3-specific antibody. GADPH was included as a negative control. *, different from control ($p < 0.05$ by Student's t-test).

the Cyclin B_1 reporter. To confirm this interaction on native chromatin, I performed ChIP using an HDAC3-specific antibody. As seen in **Fig. 13D**, overexpression of KLF4 in a doxycycline-inducible cell line resulted in increased occupancy of HDAC3 on the Cyclin B_1 promoter, suggesting that KLF4 can recruit HDAC3 to native chromatin.

R390S mutation does not affect nuclear localization of function of KLF4

KLF4 contains two nuclear localization sequences (NLSs). One is contained within the C-terminal zinc fingers, whereas another is a basic hexapeptide sequence, PKRGRR, just N-terminal to the three zinc fingers (see **Fig. 4**) (Shields and Yang, 1997).One report suggests that a single point mutation of the hexapeptide NLS (PKRGRR to PKRGRS, or R390S) is sufficient to abolish nuclear localization of KLF4 and thus unable to regulate transcription. As this mutation might be a useful negative control, I decided to test the sub-cellular localization of this mutant in HeLa cells. After

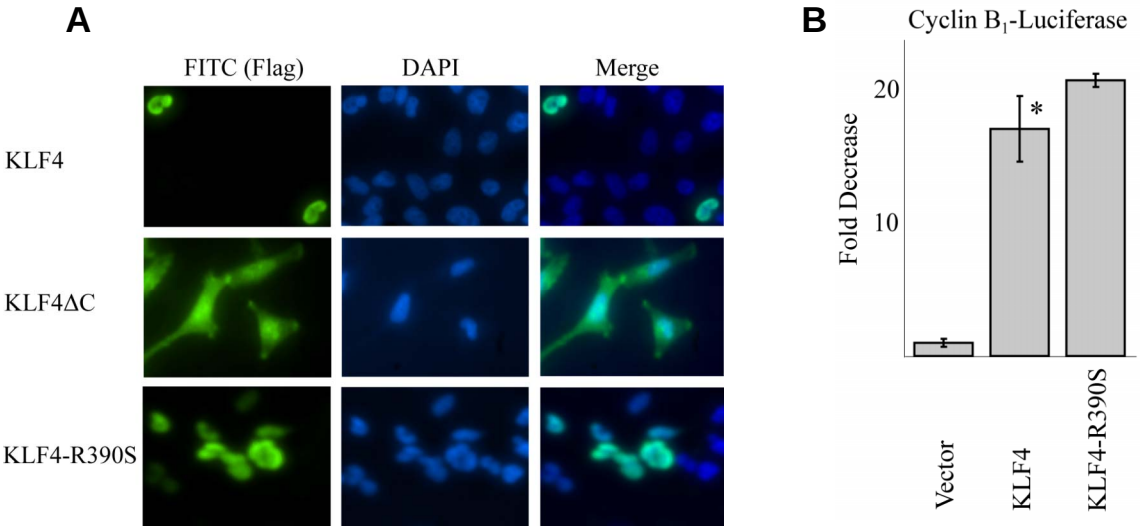

Figure 14: A single point mutation in the hexapeptide nuclear localization sequence, R390S, does not affect nuclear localization nor function of KLF4. A, Immunofluorescence. HeLa cells were transfected with Flag-tagged KLF4, KLF4-R390S, or KLF4ΔC(1-392). Cells were then fixed and stained with Flag antibody. B, Cyclin B_1 Luciferase assay. Both wild-type and R390S KLF4 can repress a Cyclin B_1 reporter. *, different from control ($p < 0.05$ by Student's t-test).

transfection, I fixed and immunostained the cells with a Flag antibody. Wild-type KLF4 is found exclusively in the nucleus (**Fig. 14A**), as expected. KLF4ΔC, which lacks all three zinc fingers, is found throughout the cell, suggesting that the zinc fingers are important for nuclear localization. The R390S point mutation, however, appeared to have no effect as it was still localized to the nucleus.. Moreover, both wild-type and R390S mutant KLF4 were able to repress the Cyclin B_1 reporter, suggesting that the R390S does not affect its ability to repress transcription either (**Fig. 14B**). These results suggest that the R390S mutation is insufficient to abolish nuclear localization of KLF4.

DISCUSSION

In this study, I found that KLF4 interacts with the HAT proteins p300 and CBP *in vivo*. KLF4 interacts with p300/CBP through its N-terminus, and mutation of three acidic residues within the N-terminal transactivation domain to valine abolishes this interaction. A functional N-terminal domain appears to required for KLF4-mediated activation and repression, as the E93/95/96V triple point mutation abolishes the function of KLF4 on the IAP, Cyclin B_1, and TOPFlash reporters. Moreover, my ChIP experiments are in agreement with previous reports that KLF4 directly regulates expression of both IAP and Cyclin B_1. In addition, I report the novel finding that binding of KLF4 to these gene targets has a differential effect on localized histone H4 acetylation. KLF4 recruits p300 to the IAP promoter and results in an increase in histone H4 acetylation. Conversely, overexpression of KLF4 results in a decrease of histone H4 acetylation at the Cyclin B_1 promoter. Occupancy of p300 is unaffected, and instead, occupancy of the histone deacetylase HDAC3 is increased. Given that histone acetylation is typically associated with gene activation and deacetylation with repression, this presents a possible mechanism for how KLF4 can function as both an activator and repressor of gene transcription.

A recent report found a direct interaction between KLF4 and MUC1 on the p53 promoter and demonstrated that MUC1-C can recruit HDAC3 to the same promoter, resulting in localized histone deacetylation (Wei et al., 2007). This suggests that

recruitment of HDAC3 might be a general mechanism for KLF4-mediated repression. Given the recent finding that KLF4 can repress the Wnt pathway (Zhang et al., 2006), a key pathway in the early stages in colon cancer development (Morin et al., 1997), clearly a greater understanding of the molecular mechanisms of the tumor suppressor protein KLF4 will give further insight into its role in colon carcinogenesis, as well as gastrointestinal and stem cell biology.

CHAPTER 4: KLF4 IS ACETYLATED BY P300/CBP AND ACETYLATION IS IMPORTANT FOR KLF4-MEDIATED TRANSACTIVATION AND INHIBITION OF PROLIFERATION

INTRODUCTION

All of the data shown in this chapter has been published (Evans et al., 2007). In the previous chapter, I demonstrated that KLF4 interacts with p300 and CBP (CREB Binding Protein). p300 and CBP are close homologues and transcriptional co-activators (Eckner et al., 1994) that contain a catalytic histone acetyltransferase (HAT) domain (Ogryzko et al., 1996). Although p300/CBP were originally found to acetylate histones, subsequent reports have shown that p300/CBP can acetylate transcription factors as well (Chan and La Thangue, 2001). Acetylation of transcription factors can have diverse effects on their activity, such as increasing the DNA binding affinity of p53 (Gu and Roeder, 1997), increasing binding between β-catenin and TCF4 (Levy et al., 2004), and delaying nuclear export of STAT3 (Wang et al., 2005). p300 acetylates KLF1, another Krüppel-like factor family member (Zhang and Bieker, 1998), increasing its interaction with the SWI/SNF chromatin remodeling complex (Lee et al., 1999; Zhang et al., 2001). Thus I hypothesized that KLF4 might be a target for acetylation as well, and that acetylation of KLF4 may have an effect on its ability to regulate transcription.

MATERIALS AND METHODS

Transient transfections, Western blotting, and luciferase assays were performed as described in the previous chapter.

Reagents and Plasmids

pCS2-Flag-KLF4, Cyclin B_1-Luciferase, Flag-p300, HA-CBP, GST-p300-CH3 were described in the previous chapter. Deletion mutants KLF4Δ1-154(ΔN), KLF4Δ155-399(ΔM), KLF4Δ402-483(ΔC), KLF4Δ367-483, KLF4Δ158-350, KLF4Δ393-483, KLF4 Δ158-242, and KLF4Δ158-268 were generated by PCR and cloned into the pCS2 vector. Point mutant KLF4-K225R/K229R was described in

Chapter 2. Point mutant KLF4-K225R and KLF4-K229R were introduced by site-directed mutagenesis using primers designed with the assistance of the software program SiteFind (see **Chapter 2**) (Evans and Liu, 2005), using the following primers: KLF4-K225R: 5'-CTGATGGGCAGGTTTGTGCTGAAGGCTAGCCTGACCACCCCTGGC-3' (Forward) and 5'-GCCAGGGGTGGTCAGGCTAGCCTTCAGCACAAACCTGCCCATCAG-3' (Reverse); KLF4-K229R: 5'-GGGCAAGTTTGTGCTGAGGGCTAGCCTGACCACCCCTGGC-3' (Forward) and 5'-GCCAGGGGTGGTCAGGCTAGCCCTCAGCACAAACTTGCCC-3' (Reverse). All constructs were verified by DNA sequencing.

Cell Culture

HEK293T, LS174T-tet/on, and LS174T-tet/on-KLF4 were cultured as described in the previous chapter. Stable cell line LS174T-tet/on-KLF4-K225/229R was produced exactly as described for LS174T-tet/on-KLF4 in the previous chapter.

RT-PCR

RT-PCR was performed as described in the previous chapter. Densitometry was performed using the *Quantity One* software (BioRad, Hercules, CA). The following additional primers were used: KLF4: 5'-CCGGTGACCGCATGTGCCCCAAGATTAAG -3' (Forward), 5'-TTGAATTCTTAAGGTTTCTCGCCTGTGTGAGT-3' (Reverse)

Immunoprecipitation/Acetylation Assays

293T cells were transiently transfected in a 12-well plate with 0.5ug of empty vector (pCS2) or the appropriate KLF4 construct with or without 0.5ug of p300 plasmid DNA. Two days later, 5mM sodium butyrate and 5mM Nicotinamide (Acros Organics, Morris Plains, NJ) were added to cells 6 hrs. prior to harvest to inhibit deacetylases. Cells were washed with PBS and lysed in lysis buffer supplemented with 5mM sodium butyrate and 5mM nicotinamide for 30min at 4ºC. Lysate was collected and centrifuged at 13,200rpm for 10min. Supernatant was diluted 1:1 in PBS and added to 20uL of M2-

agarose beads (Sigma, F7425). After gentle rocking overnight at 4ºC, beads were washed three times in PBS-T, and the remaining bound proteins were eluted either with 20uL 0.2mg/mL Flag peptide for 20 min, or by boiling in 40ul 1X SDS sample buffer for 5 min. Western blot was then performed as described earlier using either an acetylated lysine (9941S, Cell Signaling) or Flag (F3165, Sigma) antibody.

RESULTS

KLF4 is Acetylated by p300/CBP at Lysines 225 and 229

Given that KLF4 can interact with both p300 and CBP, I postulated that KLF4 could be acetylated by p300/CBP as well. To test this, I overexpressed Flag-tagged KLF4 and p300 or CBP in HEK293T cells, immunoprecipitated KLF4 using a Flag-specific antibody conjugated to agarose beads, followed by elution and Western blot with an acetyl-lysine-specific antibody. I found that KLF4 was indeed acetylated by both

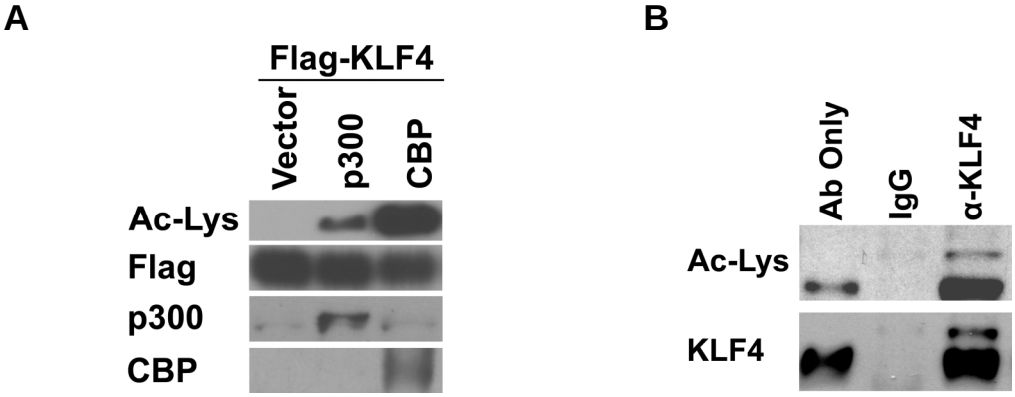

Figure 15: KLF4 is acetylated *in vivo* by p300/CBP. A, IP/Western blot. Flag-tagged KLF4 and full-length p300 or CBP were overexpressed in 293T cells. After IP with Flag antibody, a 55kD protein was detected via Western blot using an acetyl-lysine-specific antibody. Western blots confirmed similar levels of expression of KLF4 in each condition, and the expression of p300 and CBP where indicated. B, Endogenous IP, HCT116 cells. Endogenous KLF4 was immunoprecipitated from HCT116 cells using KLF4 antibody and probed via Western blot with anti-KLF4 and anti-acetylated lysine antibody. Upper band is KLF4. Anti-KLF4 antibody alone (without cell lysate) and mouse IgG (with cell lysate) were included as negative controls.

p300 and CBP (**Fig. 15A**). Additional Western blots confirm the expression of p300 and CBP when co-transfected with KLF4.

To assess the physiologic relevance of my results, I immunoprecipitated endogenous KLF4 from HCT116 cells (**Fig. 15B**). After IP with an anti-KLF4 antibody, KLF4 was detected as a band slightly heavier than the IgG band, which is present due to the immunoprecipitation step. Using control pre-immune mouse IgG or anti-KLF4 (rabbit) diluted in PBS (no lysate) for immunoprecipitation, I was unable to detect KLF4, confirming the specificity of the KLF4 band. Via Western blot with an acetylated-lysine specific antibody, I detected acetylated KLF4, confirming that KLF4 is acetylated *in vivo*.

Next, I attempted to identify which lysines are targeted for acetylation by p300/CBP. As the KLF4 amino acid sequence contains 18 lysines, I devised a strategy using three deletion mutants, either lacking the N-terminal transactivation domain, the middle region, or the C-terminal DNA binding domain of KLF4 (see **Fig. 16A**). I could then determine which mutants could still be acetylated. I performed the same Flag-IP/Acetyl-lysine Western Blot with each KLF4 mutant as was done with wild-type KLF4 in the previous figure. Acetylation is detectable in the ΔN and ΔC mutants, whereas acetylation in the ΔM mutant is absent, suggesting that the middle region contains the acetylation target (**Fig. 16B**). KLF4ΔM lacks a total of 8 lysines, so I produced several more deletion mutants encompassing this domain. Acetylation is not detectable in the KLF4Δ158-350 construct, which lacks 5 lysines within in the middle region, whereas mutants KLF4Δ367-483 and KLF4Δ393-483 is still acetylated (**Fig. 16C**). KLF4Δ158-242 and KLF4Δ158-268, lacking the first 2 and 3 lysines of the M region, respectively, cannot be acetylated. Thus, I conclude that the acetylation target is K225/K229, as these are the only two lysines absent in KLF4Δ158-242. Using full-length KLF4, I mutated each lysine (K225 and K229) to arginine, individually as well as simultaneously and found that all three point mutants are not acetylated by p300 (**Fig. 16D**), suggesting that the presence of both lysine residues is required for acetylation by p300. To exclude the possibility that these point mutations inhibited the ability of KLF4 to interact with p300, I incubated GST-CH3-p300 protein with cell lysate containing the KLF4 double point

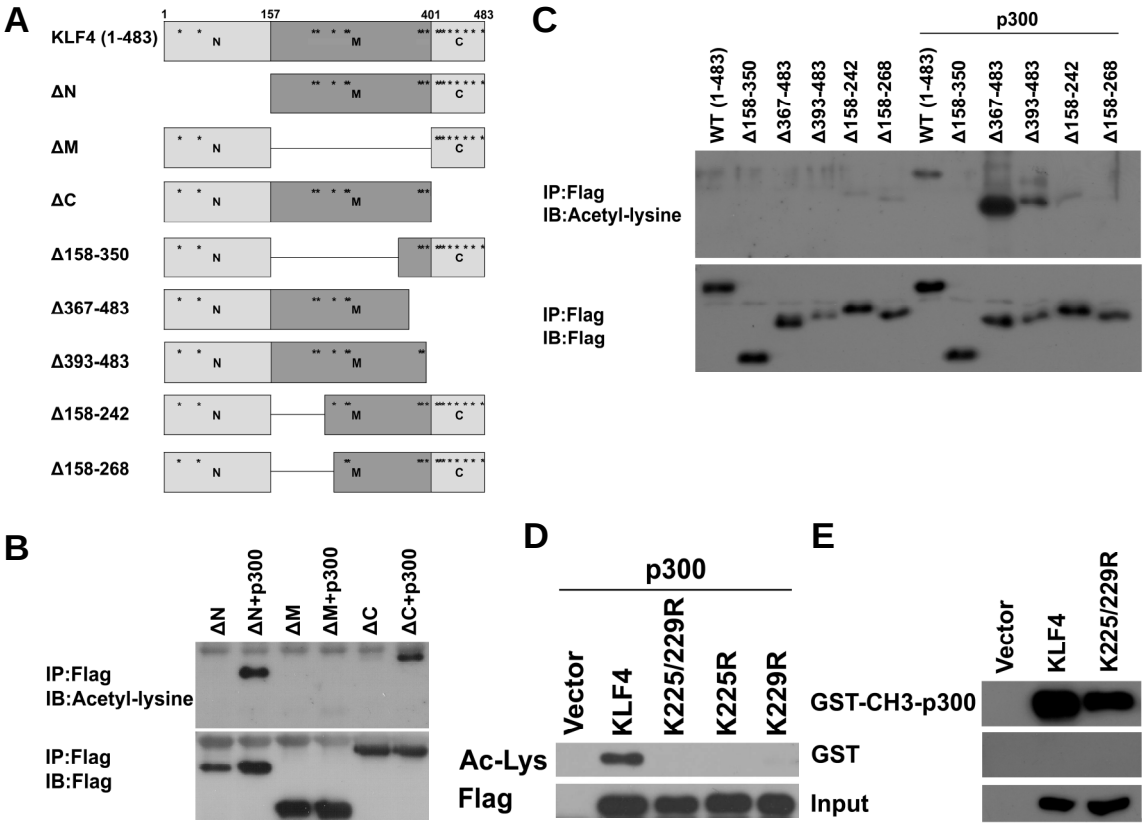

Figure 16: KLF4 is acetylated by p300/CBP at K225/229. A, Diagram of constructs used for mapping acetylated lysine residues in KLF4. Each of the 18 lysines found within the wild-type sequence are marked with a '*'. B, KLF4 is acetylated within in the middle region (residues 155-400). KLF4 deletion mutants ΔN, ΔM, and ΔC were overexpressed with full-length p300 in 293T cells. After IP with Flag antibody, a Western blot with an acetyl-lysine specific antibody was performed. C, KLF4 is acetylated within the proximal 193 amino acids of the middle region. The same experiment was performed as in *B*, this time using constructs Δ158-350, Δ367-483, Δ393-483, Δ158-242, and Δ158-268. D, KLF4 is acetylated at K225/229. Mutation of either lysine 225 or 229 abolished recognition of KLF4 with an acetyl-lysine-specific antibody. E, Point mutation does not abrogate interaction with p300 *in vitro*. GST-p300 (CH3 domain) was incubated with Flag-tagged KLF4 or KLF4-K225R/K229R. After pull-down with GST beads, KLF4 was detected via Western blot using a Flag antibody.

mutant and found that KLF4-K225R/K229R still interacts with the CH3 domain of p300 *in vitro* (**Fig. 16E**).

Acetylation by p300 Regulates KLF4-Mediated Transactivation

Given that acetylation of transcription factors often has an effect on its transcriptional activity (Chan and La Thangue, 2001), I decided to investigate the ability of acetylation-deficient KLF4 to activate the target gene IAP (Hinnebusch et al., 2004). Co-transfection of an IAP-luciferase construct with wild-type KLF4 in 293T cells strongly activates (~12-fold) the reporter (**Fig. 17A**). The addition of p300 results in a synergistic activation of the IAP-luciferase construct (**Fig. 17B**), with an approximately ~40-fold increase of luciferase activity relative to baseline. However, acetylation-deficient KLF4-K225R/K229R is only able to activate the reporter approximately 50% as strongly as wild-type KLF4, a result maintained after co-transfection with p300. I found a similar decrease in activation for both single point mutants as well.

These results suggest that acetylation of KLF4 by p300 is important for transactivation. Thus, I decided to investigate whether acetylation is important for KLF4-mediated repression as well. To test this, I used a Cyclin B_1-luciferase construct since Cyclin B_1 is negatively regulated by KLF4. As seen in **Fig. 17C**, wild-type KLF4 represses the Cyclin B_1-luciferase reporter and KLF4-K225R/K229R is similarly effective at repressing this reporter. Furthermore, overexpression of p300 (**Fig. 17D**) appears to have a minimal effect on the ability of wild-type or lysine mutant KLF4 to repress the Cyclin B_1 reporter, suggesting that p300 is important for KLF4-mediated activation but not for repression.

Lysine Residues 225 and 229 of KLF4 are Important for Induction of Endogenous $p21^{Cip1}$ and IAP

To further test the functional significance of these lysine residues in transcriptional activation by KLF4, I established a doxycycline-inducible stable cell line that expresses KLF4-K225R/K229R. After treating these cells with doxycycline for various amounts of time, I harvested the RNA and performed RT-PCR. I found that overexpression of wild-type KLF4 results in an ~10-fold induction of IAP mRNA at 48

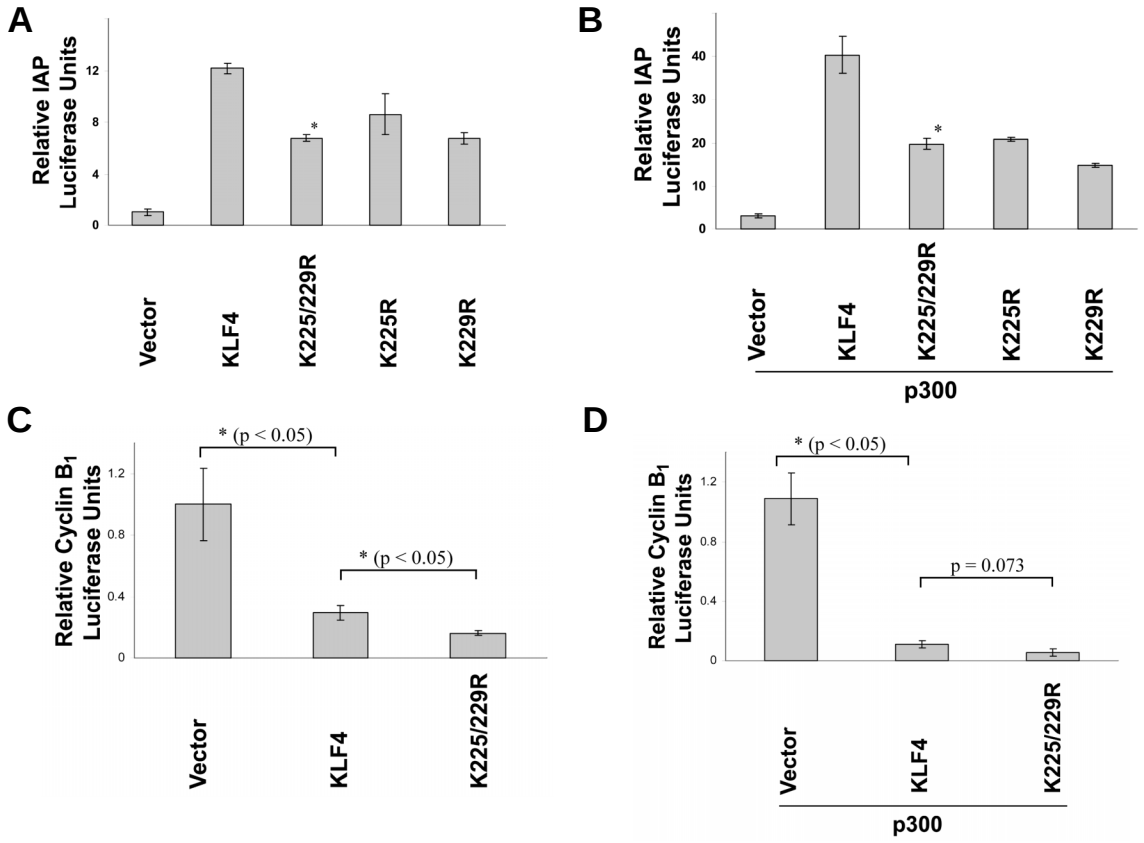

Figure 17: Acetylation by p300 regulates KLF4-mediated transactivation. A, Lysine mutant KLF4 is less effective at activating the IAP-Luciferase reporter. Empty vector, wild-type or lysine mutant KLF4 was co-transfected into 293T cells with IAP luciferase plasmid. After 48 hours, luciferase activity was measured. B, Addition of p300 results in super-activation of the IAP reporter. Full-length p300 plasmid was co-transected into 293T cells, and luciferase activity measured as in panel A. C, Lysine mutant KLF4 is still a strong repressor of the Cyclin B_1 luciferase reporter. Wild-type or lysine mutant KLF4 was co-transfected into 293T cells. After 48 hours, luciferase activity was measured. D, Addition of p300 had no effect on KLF4-mediated repression of the Cyclin B_1 gene. Same experiment as in panel C, but with the addition of full-length p300. For A and B: *, different from wild-type KLF4 ($p < 0.05$ by Student's t-test). For C and D: *, different from control or different from wild-type KLF4 ($p < 0.05$ by Student's t-test).

hours post-treatment, whereas overexpression of KLF4-K225R/K229R resulted in only a ~2-fold induction of IAP (**Figs. 18A and 18B**). Similarly, wild-type KLF4 resulted in an ~8-fold induction of $p21^{Cip1}$, whereas KLF4-K225R/K229R produced almost no change. I performed RT-PCR for KLF4 mRNA as well. KLF4 mRNA is undetectable prior to treatment, but quickly increases after treatment with doxcycline. β-Actin was used as a loading control. Western blots demonstrate similar levels of expression of KLF4 for both the wild-type and mutant KLF4 stable cell lines.

Lysine Residues 225 and 229 of KLF4 are Important for KLF4-Mediated Inhibition of Cellular Proliferation

My lab previously demonstrated that overexpression of KLF4 represses cellular proliferation using an MTT (Methyl thiazol tetrazolium) assay (Zhang et al., 2006). Thus, I decided to test the ability of double lysine mutant KLF4 to repress cellular proliferation as well. Without treatment with doxycycline, the lysine mutant stable cell line proliferated at a rate similar to our wild-type KLF4 stable cell line (**Fig. 18C**). After 3 days treatment with doxycycline, expression of wild-type KLF4 approximately halves the rate of proliferation, as was seen previously. However, double lysine mutant KLF4 is significantly less effective at repressing cellular proliferation, suggesting these residues are important for KLF4-mediated inhibition of proliferation.

DISCUSSION

In this study, I make the novel observation KLF4 is acetylated by the HAT proteins p300 and CBP. Although several other intensely-studied transcription factors have been shown to be acetylated by HAT proteins, acetylation of KLF4 has not been reported previously. Moreover, I find that KLF4 is acetylated *in vivo*, as after immunoprecipitation of endogenous KLF4 from HCT116 colon cancer cells, I could detect KLF4 with an acetyl-lysine specific antibody. Through deletion mutation and site-directed mutagenesis of full-length KLF4, I identify the acetylated lysines as K225 and K229. However, both single point mutants, K225R and K229R, are unable to be acetylated by p300. This was not due to mutations preventing an interaction between KLF4 and p300, as a double lysine mutant (K225R/K229R) KLF4 still interacts with

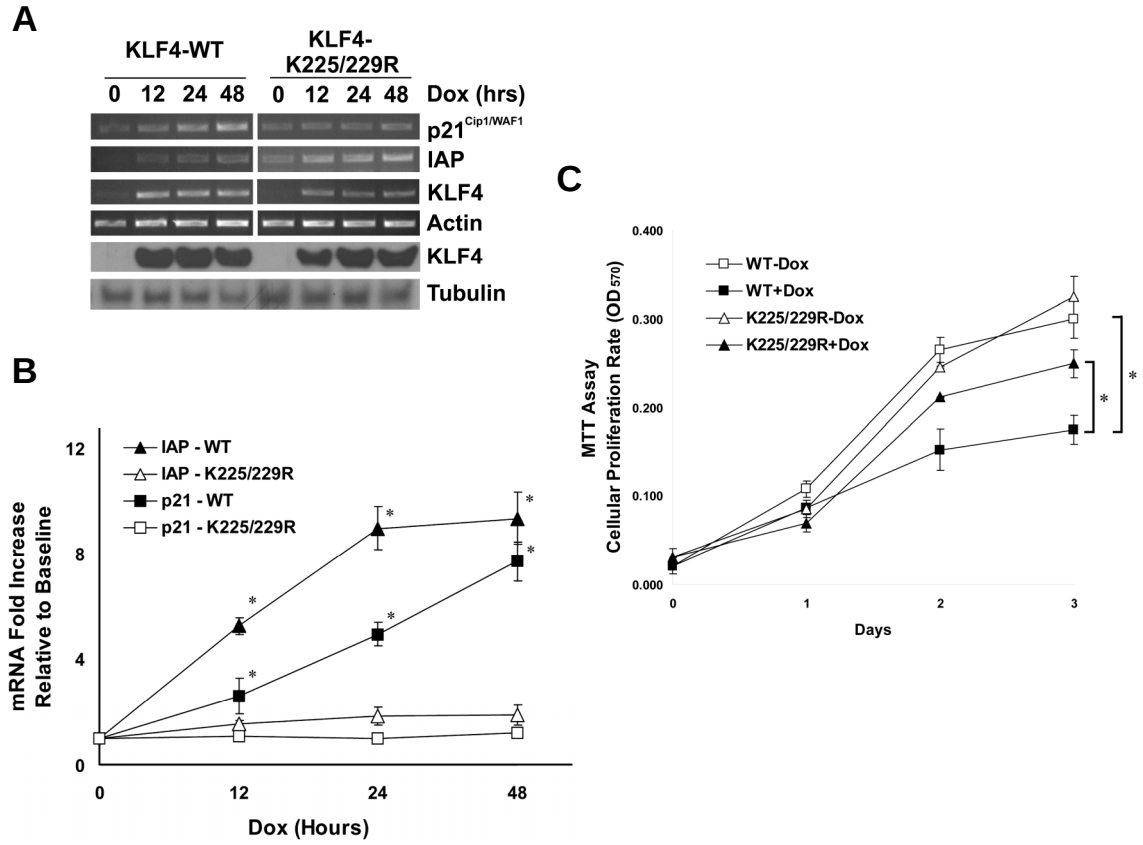

Figure 18: Lysine mutant KLF4 is defective in activating expression of endogenous genes and repressing proliferation. A, RT-PCR for p21Cip1/WAF1, IAP, KLF4, and Actin in wild-type KLF4 and lysine mutant KLF4 stable cell lines after treatment with doxycycline for specified time periods. Overexpression of wild-type KLF4 strongly increased the endogenous levels of p21Cip1/WAF1 and IAP mRNA, whereas lysine mutant KLF4 only weakly activated these genes. Western blots were performed using anti-Flag antibody to confirm similar levels of expression of wild-type and mutant KLF4, anti-tubulin was used as a loading control. B, Graphical representation of data from panel A, using densitometric analysis of images taken of ethidium bromide stained agarose gels from at least three separate PCR runs. *, different from control ($p < 0.05$ by Student's t-test). C, MTT Assay. Wild-type KLF4 and lysine mutant KLF4 stable cell lines were plated in a 96-well plate and treated with doxycycline for the specified time periods. At each time point, cells were incubated with MTT reagent and resultant blue product was measured using an absorbance plate reader at OD570. Wild-type KLF4 effectively repressed cellular proliferation after treatment for three days, whereas lysine mutant KLF4 was significantly less effective at repressing cellular proliferation. *, different from control or different from wild-type KLF4 ($p < 0.05$ by Student's t-test).

GST-tagged p300. I speculate that both lysines constitute part of a recognition sequence for p300/CBP, and that mutation of either residue abolishes recognition. This possibility has some precedence. For example, Tat is acetylated by p300, and mutation of specific residues surrounding the acetylated lysine prevented acetylation by p300 (Dormeyer et al., 2005). Moreover, p300 acetylates only certain lysine residues within the C-termini of the histone H2A, H2B, H3, and H4 proteins, despite the presence of many other nearby lysines, implying a high degree of substrate specificity (Schiltz et al., 1999).

Acetylation of KLF4 appears to be important in KLF4-mediated transactivation as mutation of either K225 or K229 within the KLF4 amino acid sequence results in decreased activation of an IAP reporter construct, and significantly reduces KLF4-mediated induction of IAP and $p21^{Cip1}$ mRNA, as measured by RT-PCR. However, acetylation appears to be dispensable for KLF4-mediated repression as double lysine mutant KLF4 is also effective at repressing a Cyclin B_1 reporter construct. It is unclear specifically how acetylation of KLF4 results in increased transactivation. One possibility is that KLF4 mediates protein-protein interactions with other transcriptional co-activators and that the acetyl group on these key lysines is required for such interactions. For example, p300/CBP contain a bromodomain that interacts specifically with acetylated lysines. Thus, acetylation of KLF4 may be part of a feed-forward mechanism, ensuring the continued occupancy of p300/CBP on target activated genes. Another possibility is that acetylated KLF4 inhibits interactions with co-repressor proteins, similarly ensuring that KLF4 continues to function in a context favoring transactivation.

CHAPTER 5: KLF4 INHIBITS BETA-CATENIN ACETYLATION AND RECRUITMENT OF P300/CBP

INTRODUCTION

In the canonical Wnt pathway, Wnt binds its receptor at the cell membrane, resulting in the stabilization of β-catenin. Then, cytoplasmic β-catenin accumulates, enters the nucleus, and activates Wnt-regulated genes through its interaction with the TCF/LEF family of transcription factors (Brunner et al., 1997; van de Wetering et al., 1997). In the absence of nuclear β-catenin, TCF/LEF family members recruit co-repressors, such as CtBP (Brannon et al., 1999), HDAC1 (Billin et al., 2000; Kioussi et al., 2002), and Groucho/TLE (Cavallo et al., 1998; Levanon et al., 1998; Roose et al., 1998), in order to inhibit transcription and maintain a repressive chromatin environment (Palaparti et al., 1997). In response to Wnt signaling, β-catenin enters the nucleus and binds TCF/LEF through its central Armadillo repeats. β-catenin displaces Groucho/TLE1 from TCF/LEF (Daniels and Weis, 2005) and recruits co-activators through its N-terminal and C-terminal transactivation domains.

At the N-terminus of β-catenin a regulatory region critically involved in regulating its stability. This is immediately followed by an N-terminal transactivation domain, which extends through the first four Armadillo repeats (Hsu et al., 1998). The N-terminal transactivation domain associates with BCL9/Legless, which in turn recruits the transcriptional co-activator Pygopus (Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002). Moreover, the interaction between β-catenin and BCL9 is required for Wnt signaling *in vivo* (Thompson et al., 2002).

The C-terminus of β-catenin contains a strong transactivation domain that is also required for Wnt signaling *in vivo* (Cox et al., 1999; Hecht et al., 2000; Takemaru and Moon, 2000; van de Wetering et al., 1997). The C-terminus of β-catenin interacts with the co-activator p300/CBP (Hecht et al., 2000; Miyagishi et al., 2000; Sun et al., 2000; Takemaru and Moon, 2000), Parafibromin (Mosimann et al., 2006), the ATP-dependent chromatin remodeling factor Brg-1/Brahma (Barker et al., 2001), the histone acetyl-

transferase TRRAP, the DNA helicase p400, and several members of the SET1-type histone methyl-transferase complex (Sierra et al., 2006).

Strong evidence suggests that β-catenin is acetylated by p300/CBP. *In vivo*, β-catenin is acetylated in the distal colon of mice in response to hyper-proliferation induced by infection with *Cibrobacter rodentium* (Umar et al., 2007). p300/CBP acetylates β-catenin at lysine 49 (Wolf et al., 2002), a residue frequently mutated in thyroid anaplastic cancer. Acetylated β-catenin more strongly activates the TOPFlash reporter (Winer et al., 2006), an index of β-catenin/TCF transcriptional activity. In addition, the co-activator FHL2 increases the level of acetylated β-catenin and synergizes with β-catenin in activating TOPFlash (Labalette et al., 2004).

Our lab recently identified KLF4 as a novel β-catenin binding protein using affinity purification. We found that KLF4 decreases growth of tumor xenografts, and subsequent investigation demonstrated that KLF4 inhibits double axis formation in the *Xenopus* embryo, suggesting that KLF4 represses Wnt signaling *in vivo*. In addition, KLF4 directly represses Wnt signaling within the nucleus, independent of interactions between β-catenin and TCF/LEF (Zhang et al., 2006). However, the precise mechanisms of KLF4-mediated repression of Wnt signaling are unclear. Given that KLF4 can directly interact with p300/CBP (**Chapter 3**) (Evans et al., 2007; Geiman et al., 2000), I hypothesized that that KLF4 represses Wnt signaling by competing with β-catenin for binding p300/CBP. Since β-catenin is acetylated by p300/CBP, I hypothesized that KLF4 inhibits acetylation of β-catenin as well.

MATERIALS AND METHODS

Transient transfections, GST pull-downs, ChIP, immunoprecipitation, and immunofluorescence were performed as described in the previous chapters.

Reagents and Plasmids

pMT3-KLF4, pCS2-Flag-KLF4, KLF4ΔN, KLF4ΔM, KLF4ΔC, SuperTOPFlash, pCIN4-Flag-p300, pRC/RSV-HA-CBP, HDAC1-6, pCS2-Flag-β-catenin and pGEX-GST-p300-CH3 were described in the previous chapters. GST-TCF4(1-65) was a gift

from Dr. Jun Yang. pcDNA3-Myc-β-catenin has been previously described (Zhang et al., 2006). ICAT was cloned from a SW480 cDNA library using PCR and inserted into the pcDNA4-TO-2xFlag vector using the following primers 5'-CCGAATTCTATGAACCGCGAGGGAGCT-3' (Forward) and 5'-CCCTCGAGCTACTGCCTCCGGTCTTC-3' (Reverse).

Cell Culture

293T, HCT116, and LS174T-tet/on-KLF4 were cultured as described in the previous chapters.

Western blot

Western blotting was performed as described in previous chapters. The following antibodies were used: acetyl-K49-β-catenin (9534S, Cell Signaling), acetyl-lysine (9441S, Cell Signaling), mouse anti-Flag (F1804, Sigma,), rabbit anti-Flag (F7425, Sigma), HA (11867423001, Roche, Indianapolis, IN), and β-catenin (C2206, Sigma).

Adenovirus Construction

Ad-GFP and Ad-KLF4 Adenoviruses were prepared according to the protocol described in (He et al., 1998). Briefly, pCS2-Flag-KLF4 was cut by *HindIII* and *XbaI*, and inserted into the pAdTrack vector. The GFP-KLF4 cassette was then transferred into the pAdEasy vector via homologous recombination in the *E. Coli* BJ5183 cell line after electroporation and selection on kanamycin agar plates. Resistant colonies were then grown in 200mL LB/Kan+ and pAdEasy-GFP-KLF4 DNA was harvested using a MidiPrep (Qiagen). DNA was then transfected into 293-Ad cells using the calcium phosphate method. Several days later, Adenovirus-containing media was harvested and used to re-infect fresh 293-Ad cells. After several rounds of enrichment, high-titer Adenovirus was collected and stored at -80ºC. Viral titers were determined based on the percentage of infected cells expressing GFP, and estimated to be $1x10^8$ PFU/mL. HCT116 cells were infected at a MOI of 10.

Chromatin Immunoprecipitation

ChIP was performed as described the previous chapters. For the SuperTOPFlash promoter, the following primers were used: 5'-CAACGCGTGTACGGGAGGTACTTGGAG-3' (Forward) and 5'-CAGGATCCGTGGCTTTACCAACAGTAC-3' (Reverse). The following antibodies were used: Flag (F7425, Sigma), β-catenin (C2206, Sigma), p300 (sc-584, Santa Cruz), Acetylated Histone H3 (06-599, Millipore)

RESULTS

KLF4 Inhibits Interaction Between β-catenin and p300/CBP

Both KLF4 and β-catenin interact with the CH3 domain of p300/CBP (Daniels and Weis, 2002; Evans et al., 2007). To test whether KLF4 could compete with β-catenin for binding p300 *in vitro*, I co-transfected β-catenin and KLF4 in 293T cells and incubated the cell lysate with a GST fusion protein containing the CH3 domain of p300 (GST-CH3-p300). Individually, KLF4 and β-catenin interact with p300, as expected. However, overexpression of KLF4 effectively blocks binding of β-catenin (**Fig. 19A**), suggesting that KLF4 might inhibit binding between β-catenin and p300. To confirm the specificity of this interaction, I tested the ability of KLF4 to inhibit β-catenin binding to the N-terminus of TCF4. KLF4 has no effect on this interaction (**Fig. 19B**). To test whether KLF4 could inhibit the binding of endogenous β-catenin to p300, I used the cell lines HCT116 and LS174T, which have elevated levels of free β-catenin. Expression of KLF4 using an adenovirus inhibited binding of endogenous β-catenin to p300 (**Fig. 19C**). Similarly, expression of KLF4 in the doxycycline-inducible stable cell line LS174T-tet/on-KLF4 similarly inhibited interactions between β-catenin and GST-CH3-p300 (**Fig. 19D**). Collectively, these data suggest that KLF4 specifically inhibits binding between β-catenin and p300 *in vitro*.

To more fully explore these findings, I decided to investigate whether KLF4 could inhibit interactions between β-catenin and full-length CBP. To test this, I

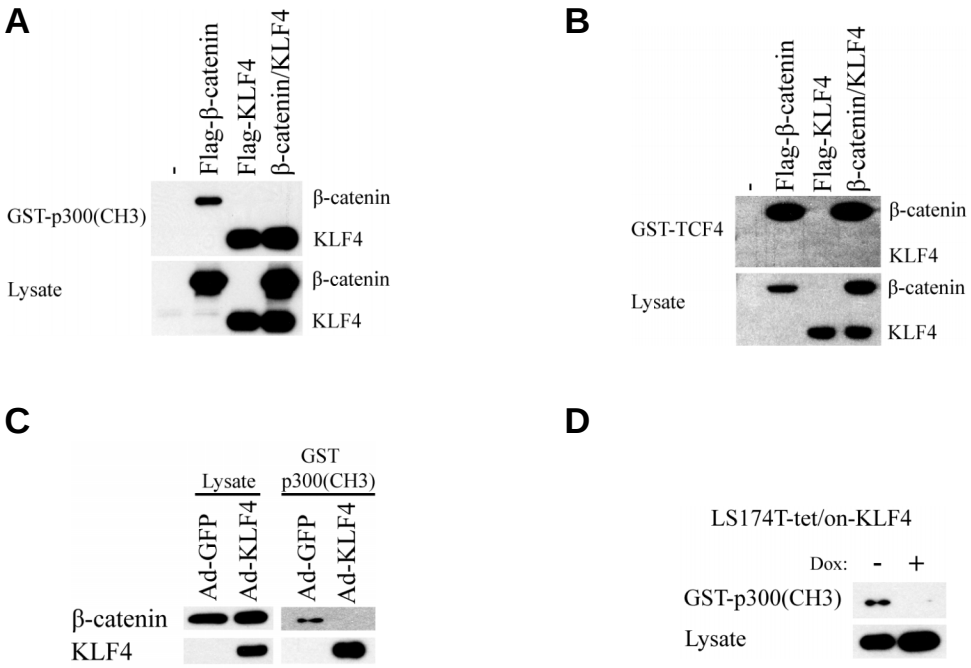

Figure 19: KLF4 competes with β-catenin for binding p300 in vitro, but not TCF4. A, GST pull-down assay. GST-CH3-p300(1710-1890) was incubated with 293T lysate overexpressing Flag-β-catenin and Flag-KLF4. GST beads were washed, and eluate probed with Flag antibody. B, GST pull-down assay. Similar conditions as in A, but using GST-TCF4(1-65). C, GST pull-down assay. HCT116 cells were infected with an Adenovirus expressing GFP (control) or KLF4. 48-hours post-infection, lysate was incubated with GST-CH3-p300 beads, washed, and eluate probed with β-catenin antibody, to detect endogenous β-catenin, or Flag antibody, to detect KLF4. D, GST pull-down assay. LS174T-tet/on-KLF4 cells were treated with doxycycline for 48 hours to induce expression of KLF4. Binding of endogenous β-catenin to GST-CH3-p300 was detected as in C.

immunoprecipitated HA-tagged CBP from 293T cells and performed an anti-Flag Western blot to detect β-catenin. Full-length CBP interacts with β-catenin, as expected. However, KLF4 significantly decreases the amount of β-catenin detected after immunoprecipitation (**Fig. 20A**). As a positive control, I co-transfected ICAT, a protein known to interfere with binding between β-catenin and p300 (Daniels and Weis, 2002).

Next, I tested the ability of KLF4 to inhibit the recruitment of p300 by β-catenin on the SuperTOPFlash promoter using chromatin immunoprecipitation (ChIP). As shown in **Fig. 20B**, TCF4 binds the SuperTOPFlash promoter, but not SuperFOPFlash, which contains mutant TCF binding sites, confirming the specificity of our assay. Overexpression of KLF4 has no effect on occupancy of TCF4 on the SuperTOPFlash promoter, nor does KLF4 inhibit β-catenin occupancy (**Fig. 20C**). However, KLF4 strongly inhibits occupancy of p300, with a concomitant decrease in histone H3 acetylation. These findings are consistent with my GST pull-down and immunoprecipitation assays, suggesting that KLF4 inhibits β-catenin/TCF4-mediated transactivation through blocking the recruitment of p300/CBP.

KLF4 Inhibits β-catenin Acetylation

Since KLF4 inhibits binding between β-catenin and p300/CBP, and β-catenin is acetylated by p300/CBP, I hypothesized that KLF4 might inhibit β-catenin acetylation as well. I transfected flag-tagged β-catenin and CBP in 293T cells. β-catenin was then immunoprecipitated and acetylated β-catenin detected using a general acetyl-lysine antibody. Acetylated β-catenin is clearly detectable, whereas co-transfection with KLF4 significantly blocks β-catenin acetylation (**Fig. 21A**). Similar results were found in endogenous β-catenin using an antibody recognizing specifically K49-acetylated β-catenin (**Fig. 21B**). Since KLF4 interacts with p300/CBP through its N-terminal transactivation domain, I hypothesized that this domain might be required for blocking β-catenin acetylation. I deleted various domains of KLF4 and tested their ability to inhibit acetylation of β-catenin. As shown in **Fig. 21C**, deletion of N-terminus (KLF4ΔN) abrogates the ability of KLF4 to block β-catenin acetylation, whereas deletion of the

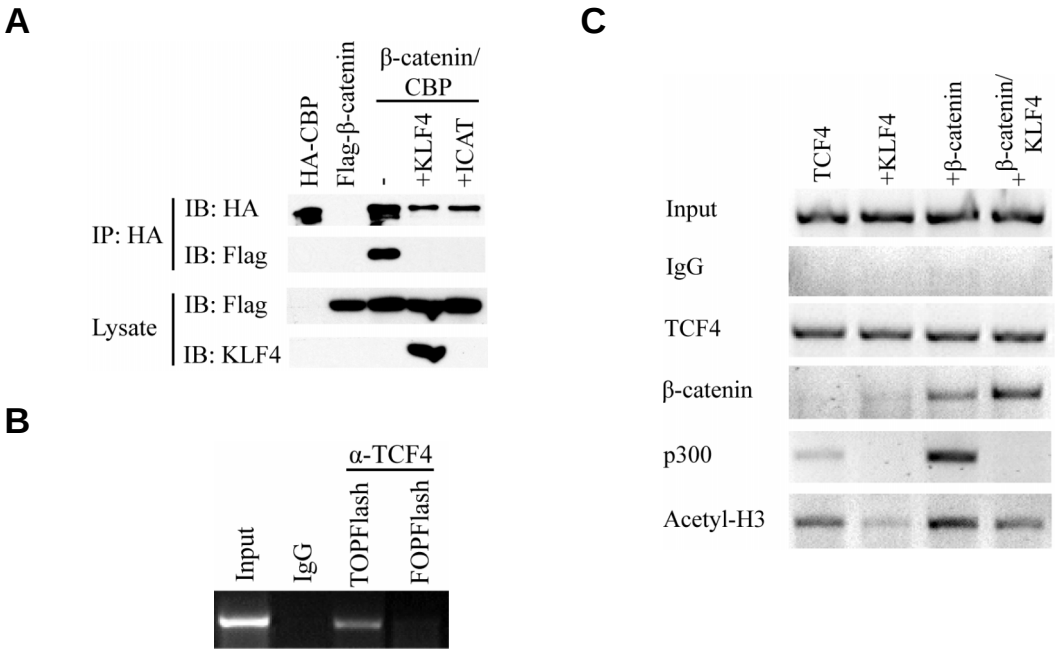

Figure 20: KLF4 inhibits interaction between β-catenin and p300/CBP *in vivo*. A, HA-IP. HA-tagged, full-length CBP and Flag-tagged β-catenin were transfected in 293T cells with or without ICAT or KLF4. CBP was immunoprecipitated using HA antibody, and bound β-catenin was detected via Flag Western blot. Additional HA and Flag Western blots were performed to demonstrate similar levels of expression in cell lysate and amount of CBP immunoprecipitated in each condition. B, ChIP. 293T cells were transfected with Flag-TCF4 and SuperTOPFlash or SuperFOPFlash reporter. 24 hours later, cells were analyzed using anti-Flag ChIP. SuperFOPFlash was used as a negative control, as it contains mutant TCF binding sites in its promoter. C, ChIP. 293T cells were transfected with Flag-TCF4, with or without untagged KLF4 and Myc-tagged β-catenin. TCF4 and β-catenin were immunoprecipitated with a Flag and β-catenin antibody, respectively.

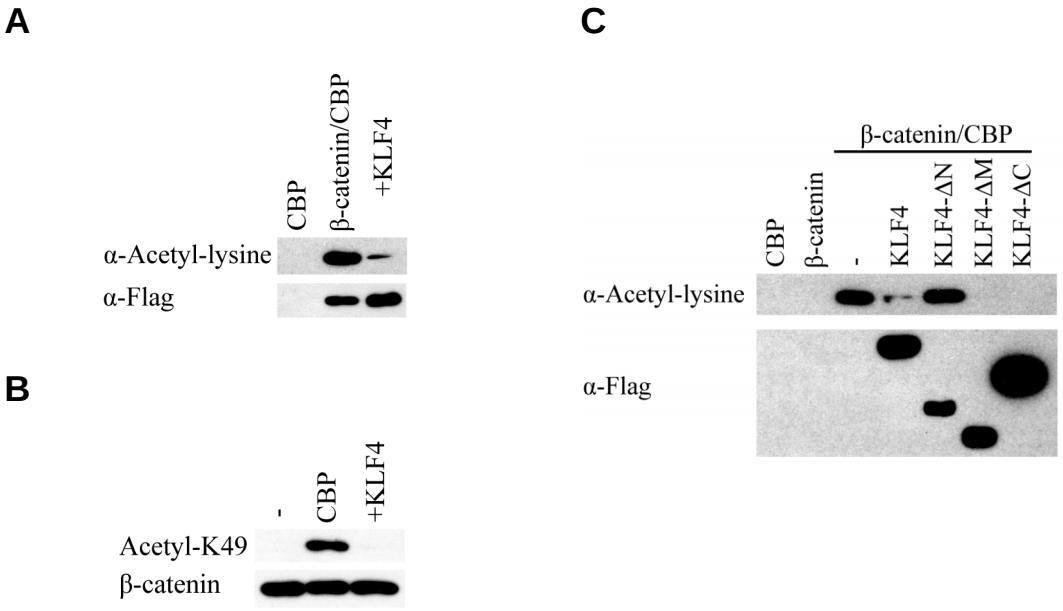

Figure 21: KLF4 inhibits CBP-mediated acetylation of β-catenin. A, β-catenin acetylation assay. Flag-tagged β-catenin and CBP were transfected in 293T cells with or without KLF4. β-catenin was immunoprecipitated using Flag antibody, and acetylated β-catenin detected using a general acetylated lysine antibody. Flag Western blot was performed to detect total β-catenin level after IP. B, β-catenin acetylation assay. Similar experiment as in A, but various domain deletions of KLF4 were co-transfected with β-catenin and CBP. Flag Western blot was performed on cell lysate to detect expression of each KLF4 construct. C, Endogenous K49-acetylated β-catenin Western blot. 293T lysate was transfected with CBP with or without KLF4, and lysate was probed with anti-K49-acetyl-β-catenin antibody. Membrane was then stripped and reprobed with β-catenin antibody to demonstrate total level of β-catenin in each condition.

middle or C-terminal domains does not. These data suggest that KLF4 inhibits β-catenin acetylation through its N-terminal domain.

DISCUSSION

In this study, I investigated the molecular mechanisms of KLF4-mediated inhibition of β-catenin/TCF4-mediated transcription. Using GST pull-down assays and IP, I consistently found that KLF4 inhibits the interaction between β-catenin and p300/CBP. Using ChIP, I demonstrate that KLF4 blocks the recruitment of p300 to the SuperTOPFlash promoter, resulting in decreased histone acetylation. These data suggest a clear model of KLF4-mediated inhibition of Wnt signaling, where KLF4 interferes with recruitment of p300/CBP to β-catenin/TCF target genes.

ICAT (Inhibitor of β-catenin and TCF) is a small protein that inhibits Wnt signaling by interfering with interactions between β-catenin and p300/CBP through binding the C-terminal transactivation domain of β-catenin (Daniels and Weis, 2002). However, ICAT interferes with β-catenin/TCF4 binding, whereas KLF4 does not (**Fig. 20C**), suggesting that KLF4 uses a different mechanism than ICAT to inhibit Wnt signaling.

β-catenin is acetylated by p300/CBP, and I further demonstrate that KLF4 interferes with interactions between β-catenin and p300/CBP by demonstrating that KLF4 inhibits acetylation of β-catenin as well. As lysine 49 is the primary acetylation site of β-catenin, and is frequently mutated in at least some cancers (Wolf et al., 2002), these results give additional insight into the role of β-catenin in cancer.

Wnt signaling is deregulated in many cancers. Overexpression of KLF4 inhibits the tumor growth *in vivo* (Zhang et al., 2006), and heterozygous deletion of the Klf4 gene predisposes $APC^{min/+}$ mice to additional intestinal tumors (Ghaleb et al., 2007). Recruitment of p300/CBP by β-catenin is critical to the transactivation of Wnt/β-catenin target genes and KLF4 inhibits the recruitment of p300/CBP and blocks β-catenin acetylation. These studies give additional insight into how KLF4 functions as a tumor suppressor, and I anticipate that small molecular inhibitors based on the crystal structure

of the β-catenin/KLF4 complex will prove useful in the treatment of many cancers, including colorectal cancer.

CHAPTER 6: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

GENERAL CONCLUSIONS

The tumor suppressor KLF4 represses activity of the Wnt pathway (Zhang et al., 2006), a key pathway involved in the early stages in the development of colorectal cancer (Morin et al., 1997). In addition, KLF4 is a crucial factor involved in reprogramming differentiated fibroblasts into embryonic stem cells (Takahashi and Yamanaka, 2006). Thus, a greater understanding of the molecular mechanisms of KLF4 will provide insight into its role in colon carcinogenesis, as well as gastrointestinal and stem cell biology.

This dissertation investigates the molecular mechanisms of KLF4-mediated transcription in several different contexts. I demonstrate that KLF4 interacts with the co-activator p300/CBP *in vivo* through its N-terminus, and this interaction is critical for activation of target genes. ChIP experiments show that KLF4 recruits p300 to the IAP promoter, resulting in localized histone acetylation (see **Fig. 22A**). A functional N-terminal domain is required for both KLF4-mediated activation and repression, as the point mutations within the N-terminus abrogate the function of KLF4 on the IAP, Cyclin B_1, and TOPFlash reporters. Moreover, HDAC3 is important for KLF4-mediated repression, as HDAC3 synergizes with KLF4 in repressing a Cyclin B_1 reporter and HDAC3 occupancy is increased on the Cyclin B_1 promoter when KLF4 is overexpressed, with a concomitant decrease in histone acetylation (see **Fig. 22B**).

I make the novel observation that KLF4 is acetylated by p300/CBP *in vivo*. Using KLF4 deletion mutants and site-directed mutagenesis of full-length KLF4, I identified K225 and K229 as the target acetylated lysines. Acetylation of KLF4 is important in KLF4-mediated transactivation as mutation of either K225 or K229 resulted in decreased activation of an IAP reporter construct and significantly decreased the ability of KLF4 to increase expression of IAP and $p21^{Cip1}$. In addition, I show that acetylation of KLF4 is dispensable for KLF4-mediated repression as double lysine mutant KLF4 is still effective at repressing a Cyclin B_1 reporter construct.

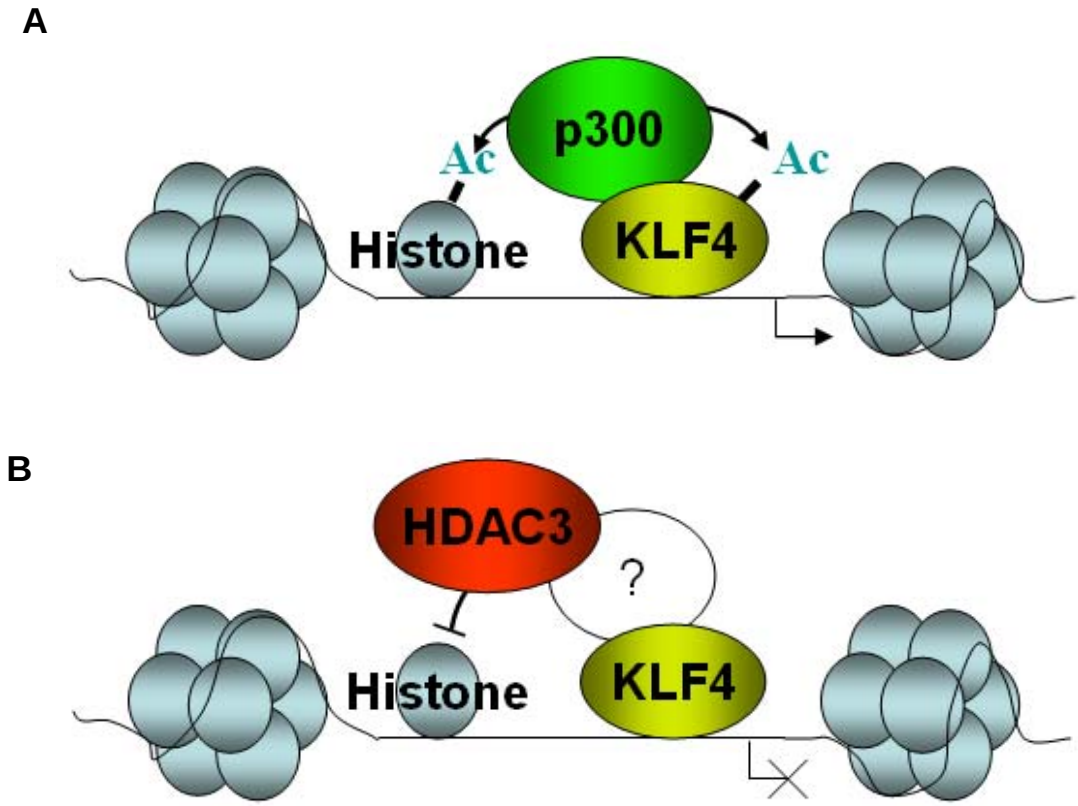

Figure 22: Model of KLF4-mediated transcription. A, Model of KLF4-mediated activation. KLF4 binds its target sequence and then recruits the co-activator p300. p300 acetylates KLF4 in addition to acetylating neighboring histones such has histone H4. Histone acetylation promotes localized unwinding of DNA and allows other transcription factors and the basal transcriptional machinery to bind. B, Model of KLF4-mediated repression. KLF4 binds its target sequence and then recruits co-repressor proteins, including HDAC3, to the promoter, resulting in an overall decrease in transcription of the target gene.

I also investigated the molecular mechanisms of KLF4-mediated inhibition of Wnt/β-catenin signaling. Using several different approaches, I demonstrate that KLF4 inhibits the interaction between β-catenin and p300/CBP and blocks the recruitment of p300/CBP to target genes. In addition, I show that KLF4 inhibits acetylation of β-catenin at lysine 49. The role of β-catenin acetylation at lysine 49 is not entirely clear, although several possibilities will be discussed in the following section.

These studies give additional insight into how KLF4 functions as a tumor suppressor, and the information provided in this dissertation will prove useful in the treatment of many cancers, particularly colorectal cancer.

FUTURE DIRECTIONS

How Does Acetylation Increase the Transactivation Potential of KLF4?

It is unclear specifically how acetylation of KLF4 results in increased transactivation. One possibility is that KLF4 mediates protein-protein interactions with other transcriptional co-activators and that the presence of an acetyl group on these key lysines is required for such interactions. p300/CBP contains a bromodomain that interacts with acetylated lysines. Thus, acetylation of KLF4 may be part of a feed-forward mechanism that ensures the continued occupancy of p300/CBP on target promoters, after its initial recruitment by KLF4. Several approaches could test this hypothesis. One approach is to perform *in vitro* binding studies between the p300/CBP bromodomain and acetylated / non-acetylated KLF4. Using full-length p300/CBP is another option. Another approach is to perform time-course ChIP analysis of wild-type and mutant KLF4 and measure the ability of each to recruit and retain p300/CBP at the promoter of target genes.

Another possible explanation is that acetylated KLF4 interacts with additional co-activators or that acetylation inhibits interactions with co-repressor proteins, ensuring that KLF4 functions in a context favoring transactivation. During my dissertation work, I performed affinity purification analysis to identify novel KLF4 binding proteins (**Fig. 23A**). One method to test this hypothesis would be to investigate the ability of acetylated

and non-acetylated KLF4 to interact with these newly-identified binding proteins, such as N-CoR, KAP-1, Rb, or hSNF2H (**Fig. 23B**). Another, more comprehensive method would be to perform two separate affinity purification experiments using acetylated and non-acetylated KLF4. Or alternatively, one could use a two-step, subtractive affinity purification approach, where non-acetylated KLF4 would be used to pre-clear cell lysate, followed by affinity purification using acetylated KLF4.

There is currently no published crystal structure for KLF4. A crystal structure of KLF4, either unbound or bound to DNA, would be highly informative. A crystal structure of acetylated KLF4 would also be useful, and provide a strong complement to my data.

How and in What Context Do KLF4 and HDAC3 interact?

KLF4 and HDAC3 synergize in repressing a Cyclin B_1 reporter, and overexpression of KLF4 results in increased HDAC3 occupancy on the Cyclin B_1 promoter. One explanation for these results is that KLF4 and HDAC3 directly interact. However, it is well known that N-CoR interacts with HDAC3 and strongly potentiates its enzymatic activity (Karagianni and Wong, 2007). Thus, another possibility is that KLF4 and HDAC3 interact indirectly, though N-CoR. Future studies should confirm the ability of KLF4 to interact with HDAC3 through N-CoR, followed by experiments using N-CoR siRNA or N-CoR$^{-/-}$ cell lines to test the requirement of N-CoR and HDAC3 for KLF4-mediated repression.

As acetylated proteins are often be de-acetylated by HDACs, it is possible that HDAC3 deacetylates KLF4. Future studies should also test whether HDAC3, or other HDACs can deacetylate KLF4 and whether this is important in switching KLF4 from a transcriptional activator to a repressor.

What is the Role of β-catenin Acetylation in Wnt Signaling?

I demonstrate that KLF4 blocks acetylation of β-catenin, using a general acetyl-lysine antibody and an antibody that recognized K49-acetylated β-catenin. However these findings could be more fully explored. For example, if KLF4 represses β-catenin

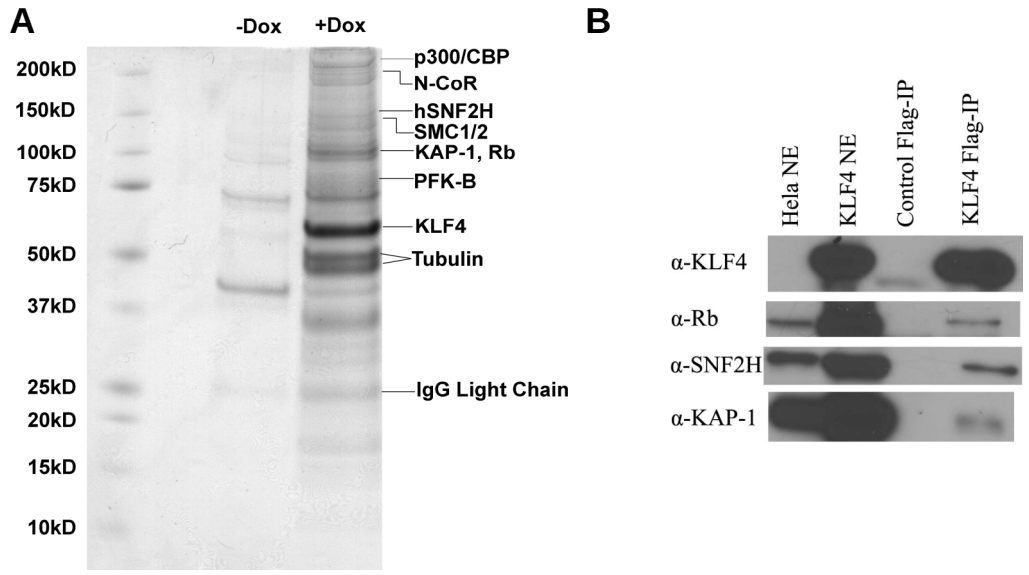

Figure 23: Identification of novel KLF4-binding proteins. A, Affinity purification assay. Doxycycline-inducible cell line LS174T-tet/on-KLF4 was treated with or without doxycycline for 48 hours. For each condition, 40 15cm^2 plates were harvested, and nuclear extract was immunoprecipitated with Flag-conjugated agarose beads (M2-agarose, Sigma). Eluate was run on 4-15% NuPAGE gel, stained with Coomassie, and individual bands identified using MS/MS. Putative binding proteins are labeled to the right of the gel. B, Western blot. Eluate from affinity purification assay was probed with indicated antibodies to confirm putative binding proteins identified in A.

acetylation, knockdown of KLF4 expression using siRNA or shRNA should increase the level of acetylated β-catenin.

Although K49 appears to be the primary acetylation target, several reports indicate that β-catenin can be acetylated at K345 (Levy et al., 2004) and K670/K671 (Shah et al., 2006) as well. Future studies should definitively identify which lysines are acetylated by p300/CBP and investigate what is the role of acetylation in β-catenin-mediated transactivation. In addition, the role of acetylation should be more fully characterized *in vivo* using a mutant β-catenin that cannot be acetylated in well established models of Wnt signaling, such as suppression of the *Wingless* phenotype in *Drosophila* or double axis formation in *Xenopus*.

Interestingly, lysine 49 is frequently mutated in at least some cancers (Wolf et al., 2002). Previous reports found that K49R mutant β-catenin is not phosphorylated at S45 (Li et al., 2008) and is less susceptible to ubiquitination (Aberle et al., 1997; Winer et al., 2006), suggesting that acetylation at K49 may function to stabilize β-catenin by preventing degradation by the APC degradation complex. Mutation of the phosphorylation sites involved in β-catenin degradation (S33/S37/T41/S45) do not affect acetylation at K49 (Wolf et al., 2002). In addition, S45-phosphorylated β-catenin is still acetylated (Umar et al., 2007), whereas K49R mutant β-catenin is not phosphorylated at S45 (Li et al., 2008). These data suggest that acetylation of may act upstream of phosphorylation in the regulation of β-catenin stability. Future studies should investigate if acetylation of β-catenin regulates its stability.

In summary, I show that KLF4 is acetylated by p300/CBP and recruits p300/CBP to positively regulated genes to modulate histone acetylation, whereas on negatively regulated genes, KLF4 recruits HDAC3. KLF4 inhibits binding between β-catenin and p300/CBP on β-catenin target genes and additionally blocks β-catenin acetylation. These studies provide further understanding of the molecular function of the tumor suppressor protein KLF4 and its role in cancer and stem cell reprogramming.

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VITA

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While at UTMB, Paul received several honors. In 2002, Paul was recipient of the Truman Graves Blocker, Jr. fellowship. In 2006, Paul received the Barbara Bowman Scholarship, the GSBS Associates Christian Fleischmann Travel Award, and the Sealy Center for Cancer Cell Biology Pre-Doctoral Training Fellowship. In 2007, the SCCCB fellowship was renewed for another year. In addition, Paul received the Marianne Blum, PhD. Endowed Scholarship and the James E. Beall II Memorial Scholarship. In 2008, Paul was nominated Who's Who Among American Colleges and Universities.

Paul has presented his research at Digestive Disease Week, the National Science Research Forum, the National M.D./PhD Student Conference, and SCMS/SCCCB Science Forum. In addition, Paul gained significant teaching experience while at the UTMB. During his graduate school years, he tutored 1st and 2nd year medical students and mentored several graduate students rotating through his lab.

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Publications

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