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The Role of Cyp33 in MLL Mediated Gene Repression

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THE ROLE OF CYP33 IN MLL MEDIATED GENE REPRESSION

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ac	acetylation
AD	Activation Domain
Brd	Bromodomain
BMI1	β lymphoma Mo-MLV insertion region 1
CYPA	cyclophilin A
CsA	Cyclosporin A (CYP33 inhibitor)
CYP33	Wild type CYP33
CYP33-M	PPIase deficient mutant of *CYP33* (mutations in R191A and F196A)
ncRNA	non-coding RNA
H3	histone 3
HAT	histone acetyltransferase
HDAC1	histone deacetylase 1
HDM	histone demethylase
HMT	histone methyltransferase
me	methylation
MLL-N	Mixed lineage leukemia N terminus
MLL-C	Mixed lineage leukemia C terminus
PcG	Polycomb Group
PHD	Plant homeodomain
PPiase	cis trans prolyl isomerase activity
PRE	Polycomb Response Element
MLL-RD	MLL repression domain
RRM	RNA recognition motif
SET	SET domain contains HMT activity
TrxG	Trithorax Group

ABSTRACT

Mixed ***L***ineage ***L***eukemia (MLL) is a multidomain protein whose gene is translocated in a subset of AML leukemias. Translocation of the MLL gene is present in approximately five percent of adult acute leukemias and ten percent of pediatric leukemias (Daser, A 2004, Look, A 1997, Huret, J 2001) Patients presenting in the clinic at the time of diagnosis with an MLL fusion have been shown to respond poorly to treatment and have a worse prognosis than matched wild type MLL patients (Rubnitz, J 1994, Rubnitz, J 1999). Novel therapies therefore are needed in order to more effectively treat patients with MLL leukemias.

Cyclophilin33 (CYP33) has previously been shown to be a negative modulator of MLL gene transactivation activity (Fair, K 2001, Anderson, M 2002). It has been well established that for CYP33 to exert its repressive effects on MLL several events must occur. CYP33 binds to the 3rd PHD (PHD3) finger of MLL and increases Histone Deacacetylase1 (HDAC1) to the repression domain (RD) of MLL thereby reducing target gene expression (Xia, Z 2003, Fair, K 2001, Anderson, M 2002). It has been hypothesized that cis-trans prolyl isomerase activity is important for recruitment of HDAC1 as increased CYP33 leads to increased HDAC1 at RD domain of MLL (Fair, K 2001, Xia, Z 2003, Chen, J 2008). In cells treated with the inhibitor, Cyclosporin A

(CsA), CYP33 was not able to recruit HDAC1 to MLL (Koonce, M 2004). hPC2, BMI1 and CtBP also bind to the MLL repression domain, although the role these co-repressor proteins play in suppression of MLL target gene activation has not been elucidated (Xia, Z 2003). In this study, the co-repressor proteins (HDAC and BMI1) that bind to MLL RD were examined to determine if CYP33 leads to the recruitment of HDAC1 and BMI1 to the RD of MLL. Also CYP33 PPIase activity was examined to determine if it is essential for HDAC1 and BMI1 binding to MLL.

Once HDAC1 and BMI1 were established to bind to MLL in a PPIase dependent manner, the chromatin modifications on the H3 histone tail, which are known substrates of HDAC1, were examined to determine if the H3 Acetylation (Ac) changes in response to CYP33 levels. H3Ac (H3K9Ac, H3K14Ac) and H3K27Ac are critical marks for active gene transcription, while its counterpart H3 lysine 27 trimethylation (H3K27Me3) is a mark for gene repression. It has been proposed that one of the main roles of H3K27Ac is to block H3K27Me3 (Pasini, D 2010). This hypothesis was tested in MSA cells, a thyroid carcinoma cell line, which showed robust decrease of CYP33 protein upon knockdown by siRNA treatment and concomitant upregulation of MLL target genes. Upon modulation of CYP33 in MSA cells, it is seen that H3K27Ac inversely correlates with CYP33 expression levels. H3Ac increases upon knockdown of CYP33, but does not show a reduction upon CYP33 overexpression in MSA cell line. H3K27Me3 levels are low at MLL gene promoters in MSA cells and remain unchanged.

After studying the levels of H3Ac and H3K27Ac, HDAC1 and BMI1 recruitment to the chromatin was examined to determine if HDAC1 and BMI1 recruitment to MLL

target gene promoters occurs in a CYP33 dependent manner. Surprisingly no change was seen upon CYP33 overexpression. A decrease of HDAC1 was seen at some MLL target gene promoters upon knockdown of CYP33 by siRNA.

After knockdown of CYP33 in MSA cells, oscillations in MLL and MYC gene expression levels were observed. This could be due to regulation of CYP33 expression by a negative feedback loop. Many such regulatory networks exist in the cell. From exploring the CYP33 that is bound to the chromatin, it is clear that CYP33 is located not only at the promoters of MLL targets, but also at the promoter of housekeeping genes, *β-ACTIN* or *GAPDH*. Also CYP33 was found to bind to the promoters of *MLL* and *CYP33*, suggesting that the oscillations could be due to a negative feedback loop.

CYP33 plays a role in the repression of MLL target genes by regulating the level of HDAC1 and BMI1 recruitment to MLL, and subsequently the levels of H3Ac and H3K27Ac. Understanding the role CYP33 plays in gene transcription leads to a better knowledge of MLL mediated transactivation and may lead to new and novel therapies in MLL leukemia.

CHAPTER I

INTRODUCTION

Cancer is the leading cause of preventable death worldwide, and it is estimated that 577,190 Americans will die from cancer in 2012 (Siegel R, 2012). Although this is a discouraging number, many deaths have been prevented in the past decades due to the development of better prophylactic screening and innovative treatments. Cancer is a disease which arises from either hereditary genetic mutations or the effects of a variety of factors found in the environment, such as ultraviolet and ionizing radiation, chemical carcinogens, and viral insertions (Vogelstein B, 1993). Cancer is not caused by a single event or genetic mutation, but is the product of multiple genetic mutations and epigenetic changes often targeting several pathways (Farber E, 1984). It is a heterogeneous disease arising in otherwise normal cells that sustain altered genetic information which is stored in DNA (Marusyk A, 2010).

Cancers that arise within the blood system are termed hematopoietic malignancies. Chromosomal translocations occur in hematopoietic malignancies leading to the generation of fusion proteins with altered functions; along with point mutations chromosomal translocations lead to cancer. Translocations occur when a double strand break occurs in DNA followed by improper DNA repair in which two different

Chromosomes fragments are erroneously joined. BCR-ABL and MLL fusions are well known examples of chromosomal translocations (Rabbitts T, 1994). The MLL fusions create a chimeric protein that drives cellular processes towards a malignant phenotype (Fig. 1). Unlike other fusions that always partner with the same gene to lead to leukemic transformation such as BCR-ABL, MLL has multiple translocation partners. The N-terminal gene sequence of MLL translocates to over 60 different genetic protein partners (Rowley J, 1993; Daser A, 2004; Daser A, 2005). MLL translocations are known to occur at a higher frequency in pediatric leukemias, arising before the age of one, and also in therapy-related leukemias when compared to other leukemia subtypes (Huret J, 2001). MLL-AF4 and MLL-AF9 fusions account for the majority of childhood leukemia cases, whereas MLL-ENL accounts for the majority of MLL fusions occurring in therapy-related leukemias (Huret J, 2001; Daser A, 2004; Daser, A 2005). A subset of therapy-related leukemias arises in patients receiving topoisomerase II inhibitor treatment for a primary cancer, later developing MLL leukemia (Daser A, 2004).

Since the prognosis for patients with MLL translocations is rather poor, researchers are trying to find a cure for these leukemias, like in the case of BCR-ABL (Rubnitz J, 1994; Rubnitz J, 1999). A treatment for BCR-ABL associated leukemia was developed by studying the enzymatic function of the fusion protein and developing an inhibitor towards the kinase activity of ABL (Buchdunger E, 1998; Druker B, 1996). Treatment with imatinib, a small molecule inhibitor of BCR-ABL, leads to cancer regression and a marked increase in 5 year cancer-free survival (Deininger M, 1997; Druker B, 1996). Developing a small molecule inhibitor for the treatment of MLL fusion

leukemias requires more ingenuity as the C-terminal fusion partners are variable, and the N terminal portion of MLL-N which is retained in all MLL fusion protein does not have a domain containing intrinsic enzymatic activity. The most common partners of MLL, are AF4, AF9, and ENL, which do not have enzymatic activity, but are components of the transcriptional elongation complex. Therefore traditional enzymatic inhibitors cannot target multiple MLL fusions. New classes of inhibitors that block protein-protein interactions have been used to disrupt interactions that are important for transformation. Recent work uses inhibitors to target the Menin binding domain. Menin has been previously shown to be required for MLL leukemogenesis, and disruption of Menin-MLL interaction is believed to prevent transformation (Liedtke M, 2009; Grembecka, J 2010). Since the non translocated allele of MLL is believed to be required for leukemic transformation by the MLL fusion gene, experiments are on their way to target the SET domain in the wild type protein to inhibit leukemia (Thiel A, 2010; Schapira M, 2011).

The MLL protein was found to be necessary for stem cell immortalization *ex vivo* and for leukemogenesis in a mouse bone marrow transplantation assay (Thiel A, 2010). MLL contains a SET domain which trimethylates histone H3K4 (Milne T, 2002; Milne T, 2005a; Nakamura T, 2002). Dimethylation and trimethylation of the lysine 4 of histone H3 (H3K4me2, H3K4me3) are marks of active transcription which are thought to open chromatin and are permissive for gene transcription (Sims R, 2007; Bernstein B, 2002). Since the proposal of the histone code hypothesis (Strahl B, 2000; Jenuwein T, 2001) much work has been poured into understanding enzymatic proteins that put on

(write), bind (read), and remove (erase) marks on histone tail residues (Strahl B, 2000; Jenuwein T, 2001). A few of the ways in which histones can be modified are acetylation, methylation, phosphorylation, ubiquitination, and sumoylation (Kouzarides T, 2007). Another mechanism of chromatin modification is DNA methylation. Cytosine methylation at CpG dinucleotides directly modifies DNA and the modification is epigenetically inherited through the hemimethylase activity of the DNMT1 enzyme (Jones P, 2001).

This study focuses on changes in histone 3 acetylation (ac) and methylation (me) at MLL target genes. Acetylation of lysines on the histone tails is correlated with transcriptional activation (Kurdistani S, 2004; Wang Z, 2008). Methylation marks on the other hand have been associated with both transcriptional activation and repression of gene activity depending on the residue modified. H3K4me3 is associated with transcriptional activity, whereas trimethylation of H3K9 and/or H3K27 is correlated with transcriptional gene repression (Kurdistani S, 2004). H3 lysine residues such as H3K14, and H3K18, are acetylated, whereas H3K9 and H3K27 can be either acetylated or methylated (Roth S, 1999; Grant P, 2001; Cao R, 2002; Rea S, 2000). Histone acetyltransferases (HATs) or histone deacetylases (HDACs) function to write or erase acetyl marks respectively (Lee K, 2007; Roth S, 2001). Histone methyltransferases (HMTs) or histone demethylases (HDMs) function to write or erase methyl marks (Lee M, 2006; Cloos P, 2008).

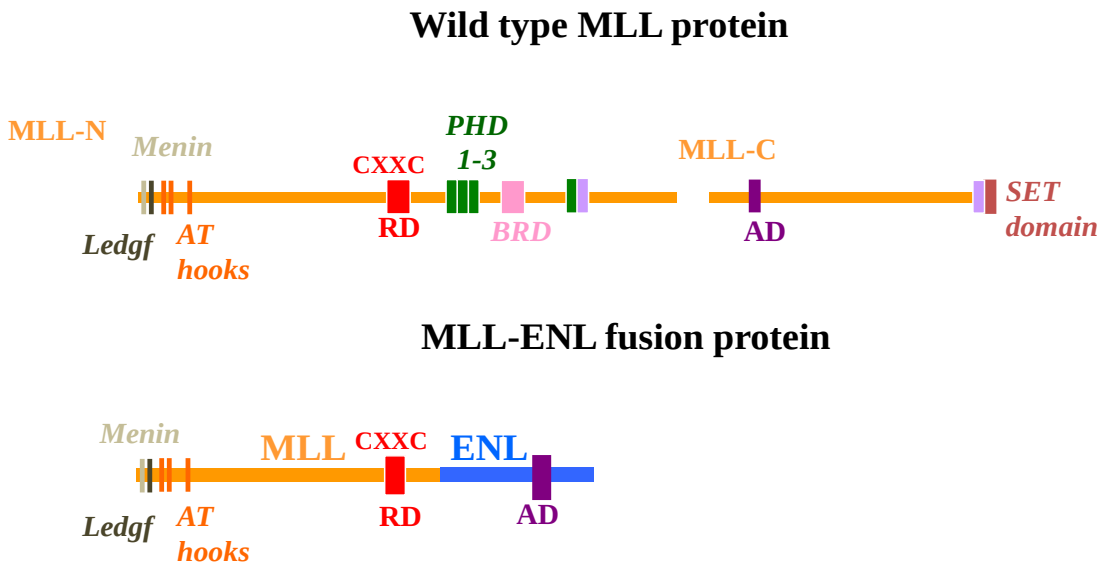


Figure 1. MLL is a large multi-domain protein that is translocated in a subset of leukemias.

A) Shown are the Menin and Ledgf binding site, the AT-hooks, the RD (repression domain), the PHD fingers, the bromodomain, and the FYRN and FYRC domains, as well as the activation domain (AD) and the SET domain. The RD binds the HDAC1, BMI1, hPC2, CtBP co-repressors, and the PAF1 component of an elongation complex. The FYRN and FYRC domains are important for MLL-N interaction with MLL-C after taspase cleavage. The AD binds CBP, a histone acetyltransferase, and MOF, another HAT with specificity for H4K16, binds nearby (Dou Y, 2005). The H3K4me3 mark is placed by the HMT activity of the SET domain of MLL. B) The MLL fusion protein is shown. The breakpoint region in the *MLL* gene occurs 5' to the PHD1 encoding sequence (Akhtar A, 2000; Akhtar A, 2001).

MLL is a multidomain protein that binds both co-activators and co-repressors. MLL is post-translationally cleaved by the protease, taspase 1 (Hsieh J, 2003). Once the full-length MLL protein is cleaved, the MLL-N and MLL-C fragments associate with one another to form a functional MLL by way of the PHD1, PHD4, and FYRN domains of MLL-N and the FYRC domain of MLL-C (Fig. 1) (Yokohama A, 2011). CYP33, a protein that binds to MLL, seems to negative modulate expression of MLL target genes, such as *CDNK1B*, *MYC*, *HOXC8*, and *HOXA9* (Anderson M, 2002; Fair K, 2001; Park S, 2010). Over-expression of CYP33 promotes recruitment of the histone deacetylase HDAC1 to the RD domain of MLL. CBP, a transcriptional co-activator that acetylates H3, has been shown to bind to the activation domain of MLL-C, whereas HDAC1, HDAC2, BMI1, hPC2, and CtBP have been shown to bind to the MLL-N repression domain (Fig. 1) (Ernst P, 2001; Xia Z, 2003; this study).

The third plant homeodomain (PHD3) of MLL-N has been shown to bind H3K4me3, a histone mark of active chromatin (Wang Z, 2010; Hom R, 2010; Park S, 2010). The enzymatic activity of the MLL SET domain on MLL-C writes the H3K4me3 mark on histone H3 (Milne T, 2002; Milne T, 2005a; Nakamura T, 2002). Therefore MLL can both place and recognize the H3K4me3 mark through two independent domains. The PHD3 is the reader and the SET domain is the writer. The PHD3 has been shown to be important for leukemogenesis, as its loss from the MLL fusion proteins is required for transformation *ex vivo* (Chen J, 2008; Muntean A, 2008). A possible explanation is that wild type MLL is a regulated activator of transcription when binding

to CYP33 switches MLL from an activator to a repressor. The fusion protein is a constitutive activator of transcription: accordingly, it is necessary to lose the PHD3 finger in the fusion protein for cellular transformation to occur.

CYP33 is a small protein that contains an RRM domain and a cyclophilin domain separated by a conserved spacer (Fig 9). The CYP33 RRM (RNA recognition motif) domain binds to the PHD3 (3rd PHD finger domain) of MLL. Repression of MLL target genes not only requires the RRM of CYP33 to bind to MLL, but also requires the cis-trans peptidyl-prolyl-isomerase (PPIase) activity of the CYP33 cyclophilin domain (Anderson M, 2002; Fair K, 2001). PPIases participate in protein folding, signal transduction, trafficking, assembly, and cell cycle regulation (Göthel S, 1999; Hunter T, 1998). Nelson *et al*. showed that Fpr4, a FKBP prolyl isomerase, regulates the cis-trans conformation of the H3K37-H3P38 prolyl bond in the Histone H3 tail. The correct conformation of this bond is necessary to allow for H3K36 methylation by SET2, and efficient transcriptional elongation (Nelson C, 2006). This provides evidence that cis-trans prolyl isomerases regulate gene transcription by targeting histone prolyl-peptide bonds.

Although it has been known that HDAC1 is recruited to MLL in a CYP33-dependent manner (Xia Z, 2003), the molecular mechanisms of this interaction at the chromatin level have not been analyzed to this point. Also the specific role of CYP33s PPIase activity is not well defined. In this study, through the use of co-immunoprecipitations (CoIPs) and chromatin immunoprecipitations (ChIPs), the question

of whether CYP33 recruits HDAC1 and BMI1 to the chromatin in a CYP33-specific PPIase-dependent manner was tested. Previously in our lab, Mark Koonce addressed the question of HDAC1 recruitment dependence on PPIase activity. General PPIase activity within the cell was inhibited by (Cyclosporin A). CsA is not specific to CYP33 but inhibits other cyclophilins that are present in the cell, including Cyclophilin A. Koonce showed that HDAC1 was not recruited to MLL by CYP33 when the PPIase activity of CYP33 was inhibited by CsA (Koonce M, 2004).

Four separate enzymatic complexes compete to either put on or take off H3K27ac or H3K27me3 histone marks: HATs, HDACs, HMTs, and HDMs (Fig. 2). The histone methyltransferase that is responsible for trimethylation of H3K27 is Ezh2, a core component of the PRC2 complex. HDAC1, the enzyme that is responsible for deacetylation of H3K27, is part of both the MLL complex and the PRC2 complex (Xia, Z 2003, Van der Vlag, J 1999).

TrxG (Trithorax Group) and PcG (Polycomb Group) proteins function antagonistically to one another (Ringrose L, 2004; Hanson R, 1999). In a simplified view, TrxG proteins maintain genes in an active state, whereas PcG proteins function to maintain genes in a repressed state. The significance of HDAC1 and its role in TrxG and PcG-mediated gene repression has not been thoroughly explored, but it has been suggested that HDAC1 is necessary to remove the H3K27ac mark to allow for PRC2 (Ezh2) to methylate H3K27 (Tie F, 2009; Pasini D, 2010). It is possible that HDACs

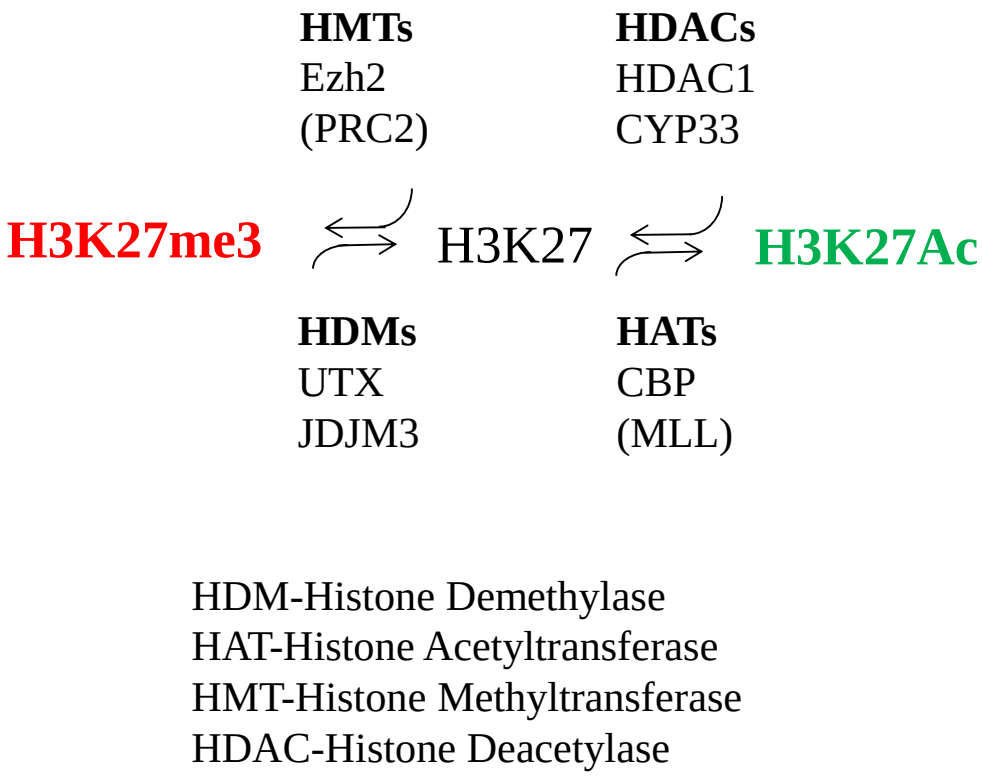


Figure 2. Various enzymes function to acetylate, methylate, deacetylate or demethylate H3K27.

Listed above are the enzymes involved in the addition or removal of acetylation or methylation from H3K27. The enzymes modifying H3K27 are often found in large complexes bound to specific promoters and their activity can be influenced by other histone modifications (see text).

have a similar function in the MLL complex to deacetylate the histone H3 tail, thereby mediating MLL gene repression.

Several possible roles for H3K27ac have been proposed. It has been proposed by groups studying both *Drosophila* and *Mouse* models that a possible role of H3K27ac is in the inhibition of H3K27 trimethylation (Tie F, 2009; Jung H, 2010; Pasini D, 2010). CBP over-expression in *Drosophila* was shown to prevent H3K27 trimethylation through a Trx-dependent mechanism (Tie F, 2009). CBP is the HAT responsible for H3K18ac and H3K27ac (Jin Q, 2011). Therefore, one of the purposes of this study was to determine if MLL-associated histone acetylation fulfills the same function in mammalian cells, and if CYP33 is part of the switch that shifts H3K27ac to H3K27me3. H3K27ac is highly correlated with transcription and has been proposed to be a mark for active enhancer elements in *Drosophila* and ES cells when paired with H3K4me1 (Wang Z, 2008; Creyghton M, 2011).

We propose that CYP33 functions to regulate MLL target gene transcription by recruiting co-repressors HDAC1 and BMI1 to the MLL RD domain in a PPIase-dependent manner. Conversely, we examined whether knockdown of *CYP33* leads to a decrease of HDAC1 and BMI1 at the promoters of MLL target genes. The recruitment of HDAC1 to MLL RD at the chromatin was previously shown to lead to decrease of H3ac levels at *HOXA9* and *HOXC8* gene promoters (Wang Z, 2010; Koonce M, 2004). It has been shown that loss of H3K27ac leads to an increase in H3K27me3 in both *Drosophila*, and mouse ES cell models (Pasini D, 2010; Tie F, 2009). The histone marks of H3 at

MLL target genes were studied, focusing especially on the acetylation and methylation of H3K27. CYP33 was over-expressed to determine if CYP33 decreased H3K27ac by recruitment of HDAC1, which led to an increase of H3K27me3 deposited by the PRC2 complex. Recruitment of co-repressors HDAC1 and BMI1 bound to MLL targets gene promoters *CDNK1B*, *MYC* and *SIX1* was monitored after over-expression of CYP33. The role of the PPIase was examined by using a PPIase mutant, called CYP33 M. Finally, H3K27 acetylation and methylation were studied at MLL target genes to determine if CYP33 affects histone acetylation or methylation levels. Through these studies it was shown that H3K27ac is decreased or increased upon CYP33 over-expression or knockdown at MLL target gene promoters. This decrease of H3K27ac is accompanied by HDAC1 and BMI1 increased recruitment to the repression domain of MLL. Therefore, CYP33 functions to repress MLL target genes in part by decreasing H3K27ac, a mark of active transcription.

CHAPTER II

REVIEW OF THE LITERATURE

CYP33 contains isomerase activity toward prolyl-peptide bonds in amino acid peptide chains. Upon binding to MLL, CYP33 is thought to negatively regulate MLL target genes in a PPIase dependent manner, thus switching MLL function from transcriptional activation to a repression. It has been shown that either Cyclosporin A (CsA), a non specific inhibitor of cis-trans prolyl isomerase activity, or Trichostatin A (TsA), which inhibits the deacetylase activity of HDACs, counteracts the ability of over-expressed CYP33 to repress transcription of MLL target genes in 293T cells (Xia Z, 2003; Fair K, 2001; Anderson M, 2002). The repressive function of CYP33 is also dependent upon the interaction between the RRM of CYP33 and the PHD3 of MLL (Anderson M, 2002; Fair K, 2001; Park S, 2010; Wang Z, 2010; Hom R, 2010). Although it has been shown that HDAC1 is recruited to MLL upon CYP33 over-expression, the molecular mechanisms by which CYP33 contributes to MLL-mediated gene repression have not been thoroughly studied (Xia Z, 2003; Fair K, 2001). The prevailing hypothesis is that CYP33 recruits HDAC1 to the chromatin which leads to a higher concentration of HDAC1 at MLL target gene promoters, which in turn leads to a decrease in H3 acetylation. Deacetylation of histone tails leads to compaction of the

nucleosomes, closing of the chromatin and subsequent repression of gene transcription (Shahbazian M, 2007).

MLL is a 430kD protein that contains multiple domains, many of which are currently under study in order to determine their function. MLL is well known for the critical role it plays in a subset of leukemias (Yu B, 1995; Hess J, 1997). Leukemia is a cancer of the blood that was diagnosed in forty four thousand Americans in 2011; two hundred seventy thousand Americans currently suffer from leukemia (Siegel R, 2012). Proteins arising from mutant genes called oncogenes can cause abnormalities in a cell leading to changes in cell proliferation, apoptosis, and/or other cellular processes that lead to cancer. Cancer cells arise from normal cells when they acquire gain-of-function mutations in proto-oncogenes and concomitantly lose the function of tumor suppressor genes. Various processes occur in cancer progression including: 1) the ability to become self-sufficient in the production of growth factors; 2) insensitivity to anti-growth signals; 3) evasion of apoptosis; 4) sustained angiogenesis; and 5) tissue invasiveness and metastasis (Hanahan D, 2000). The Mixed Lineage Leukemia gene (*MLL*) is involved in the initiation of leukemia when it becomes translocated and subsequently fused to partner genes creating an oncogene which encodes a chimeric protein; over 60 separate *MLL* partner genes in gene fusions have been documented (Daser A, 2004). MLL fusion proteins lose the ability to bind CYP33 by virtue of the loss of the PHD3 finger. CYP33 is unable to repress the promoters of MLL target genes in the presence of such fusion proteins (Chen J, 2010). Expression of MLL fusion proteins such as MLL-AF4, MLL-AF9, or MLL-ENL leads to the increased expression of MLL target genes such as

HOXC8, HOXA9, MYC, MEIS1 and *CDNK1B* (Wang Q, 2011; Xia Z, 2005; Ayton P, 2003; Ferrando A, 2003; Horton S, 2005). The over-expression of some of these genes, specifically *HOXA9* and *MEIS1*, play an important role in the leukemic phenotype (Rozovskaia T, 2001; Faber J, 2008)

MLL domains linking structure and function

MLL is a 430kD protein that is proteolytically cleaved by taspase I to form a MLL-N terminal 320kD protein (MLL-N), and an 180kD MLL-C terminal protein (MLL-C) (Hsieh, J 2003). MLL-N and MLL-C after cleavage interact through their FYRN and FYRC domains in order to maintain protein stability (Liu H, 2007; Yokohama A, 2011). MLL contains many domains currently being studied for their role in gene trans-activation. Starting from the N-terminus and working towards the C-terminus, MLL contains menin, ledgf, binding domains, along with three AT hooks. Following the AT hooks is the repression domain which contains the CXXC domain. Next are the PHD fingers, and the atypical bromodomain followed by the PHD4 and the FYRN domain. MLL-C contains the activation domain, which binds CBP, the FYRC domain and the SET domain. MLL-N contains a menin binding domain. Menin functions to promote oncogenesis in the hematopoietic lineage and to prevent oncogenesis in the endocrine lineage (Yokoyama A, 2008; Yokoyama A, 2005; Wang E, 1998). Menin functions to tether LEDGF to MLL, which is important for leukemogenesis by MLL fusions (Yokoyama A, 2008; Roudaia L, 2008). C terminal to the Menin binding domain is a series of AT hooks, a short motif consisting of an arginine-glycine-arginine-proline

tetrapeptide repeat (Aravind L, 1998; Reeves R, 1990). AT hooks are found in HMG1 proteins and help to anchor proteins to DNA (Aravind L, 1998). Multiple interactions contribute to MLL binding to its target genes. AT hooks and LEDGF binding, along with the CXXC domain (located within the repression domain) contribute to MLL binding to target genes (Cierpicki T, 2010; Erfurth F, 2008). Comprehensive, genome-wide studies have not been conducted on deletions of MLL domains and binding to target gene promoters. MLL target genes are discussed in a subsequent section.

C-terminal to the AT hooks, there is a conserved region called the repression domain (RD). The RD is the minimal region needed for repression of MLL target genes, as shown in a CAT reporter assay (Zeleznik-Le N, 1994). The RD domain contains the canonical CXXC motif which binds unmethylated CpGs in the DNA major groove (Birke M, 2002; Cierpicki T, 2010). Deletion of CXXC or disruption of its ability to bind unmethylated CpGs in the context of MLL fusion proteins leads to their decreased ability to transform cells (Cierpicki T, 2010; Bach C, 2009). The RD has been shown by co-immunoprecipitation (CoIP) and GST immunoprecipitation assays to bind to the proteins HDAC1, hPC2, BMI1, and CtBP (Xia Z, 2003). In the same report, it was shown that CYP33 increases the recruitment of HDAC1 to the MLL RD. Dependence on CYP33 for the binding of other repressive proteins to the MLL RD, such as HPC2, BMI1, and CtBP, was not tested (Xia Z, 2003). HDAC1 is an enzyme that functions to deacetylate both histone and non-histone substrates. Increased recruitment of HDACs to MLL target genes may have several consequences. HDAC1 can deacetylate the histone tail of H3,

leading to chromatin compaction (Shahbazian M, 2007). Also, HDACs such as SIRT2 and HDAC1 can deacetylate CBP, leading to a decrease in the ability of CBP (p300) to transactivate target genes (Stiehl D, 2007; Black J, 2008). CBP and MOF, two HAT proteins can independently bind to the activation domain of MLL and regulate histone acetylation (Dou Y, 2005; Ernst P, 2001). Although the roles of HDAC1, HPC2, BMI1, and CtBP have been studied in other complexes, the specific roles of these proteins in the MLL gene repressive mechanism are still poorly understood. Besides co-repressors, the RD binds PAF1, a component of the PAF elongation complex, and has been shown to be important for MLL recruitment and transactivation (Muntean A, 2010; Milne T, 2010). The PAF complex associates with RNA Pol II and functions to recruit histone modification enzymes (Crisucci E, 2011).

C terminal to the MLL RD lays a cluster of three PHDs. This PHD cluster is closely followed by a bromodomain and a fourth PHD (PHD4) (Fig 1). Loss of the PHD3 finger in MLL is necessary for MLL-ENL or MLL-AF9 cellular transformation (Chen J, 2008; Muntean A, 2008). The MLL fusion protein cannot transform bone marrow cells when the PHD cluster or the PHD3 alone are re-inserted into the MLL fusion protein (Chen J, 2008; Muntean A, 2008). Therefore MLL fusion proteins containing the PHD3 are not found in leukemia patients, nor cause leukemia in mouse models. Upon reintroduction of PHD3 or the PHD finger cluster into the MLL fusions MLL-ENL or MLL-AF9, CYP33 is able to bind to the fusion proteins and repress

HOXA9 expression, and the modified fusion proteins lose their ability to mediate bone marrow transformation in serial colony-plating assays (Chen J, 2008; Muntean A, 2008).

The PHD fingers have been shown to be versatile readers of different histone tail modifications (Sanchez R, 2011). MLL, like other PHD bearing proteins such as BTPF and ING2, binds specifically to H3K4me3 (Pena P, 2006; Wysocka J, 2006; Wang Z, 2010; Hom R, 2010; Park S, 2010). This is a histone modification mediated by the methyltransferase enzymatic activity of MLL which resides in its SET domain (Table 1). Hence it has been suggested that MLL is important for propagation of the H3K4me3 mark, as it can both read (bind to) and write (place) the H3K4me3 mark. Besides binding to H3K4me3, the MLL PHD3 also binds the RRM domain of CYP33. The binding of CYP33 to MLL reduces the affinity of the PHD3 for H3K4me3 (Wang Z, 2010; Park S, 2010). H3K4 trimethylation upon CYP33 over-expression is reduced at MLL target genes (Park S, 2010)

Bromodomains typically have been shown to bind acetylated (ac) lysine residues. MLL1 contains an atypical bromodomain, which is located between PHD3 and PHD4. *In vitro* experiments show that the bromodomain of MLL does not bind to ac-lysine residues (Wang Z, 2010). Instead it has been hypothesized by Wang *et al*. that the MLL bromodomain may function to inhibit the recruitment of CYP33 to MLL by steric hindrance of the CYP33 binding surface on PHD3. An extra regulatory step is isomerization of the H1628-P1629 prolyl bond which is located between the PHD3 and

Table 1: PRC1/PRC2 complex components

Polycomb Repressive Complex 2 Core Components	**General Function in the complex**
Rbp48	Binding of complex to the chromatin
EED	Binding to H3K27me3
SUZ12	Essential for histone methyltransferase activity of PRC2
EZH1/EZH2	Contains SET domain necessary for H3K27 methylation
Polycomb Repressive Complex 1 Core Components	
Pc1/Pc2 (CBX/CBX4)	Binds to H3K27me3
BMI1	Required for ubiquitination by Ring1a/1b
Ring1a/Ring1b	Ubiquiylates H2AK119
PH	

Modified from Review by Morey and Helin (*Trends in Biochemical Sciences* (2010). 323-332.)

bromodomain and is necessary to uncover the CYP33 binding surface on the PHD3 finger. This isomerization may be catalyzed by the cyclophilin domain of CYP33 (Wang Z, 2010).

Named after three proteins in which it was found, Suvar39, Enhancer of Zeste, and Trithorax, the SET domain of MLL1 is responsible for methylating H3K4 at a subset of genes within the cell (Wang P, 2009). Six HMT complexes have been found to date that can methylate H3K4. SET domain-containing proteins that methylate H3K4 include SET1A, SET1B, MLL1, MLL2, MLL3, and MLL4 (Wang P, 2009; Kouzarides T, 2007; Slany R, 2009). During development and differentiation, the enzymatic activity of the SET domain is a key function of MLL, as shown in knock-in mice expressing a truncated MLL lacking the SET domain. MLL mice expressing a truncated form of MLL do survive embryonic life, but they display a strong mutant phenotype, consisting of skeletal defects, and altered HOX gene transcription (Terranova R, 2006). As shown in Table 1, H3K4me3 is a mark of activation found on nucleosomes at the promoters of actively transcribed genes (Wang P, 2009). MLL, which is part of the Trithorax group (TrxG) of proteins, is in part responsible for maintaining a subset of genes in an active state. It does this by a variety of mechanisms. First, it can methylate H3K4me3, which is a mark associated with gene activity (Guenther M, 2007). Second, it can associate with components of the SWI/SNF chromatin remodeling complex (Rozenblatt-Rosen O, 1998). Third, it can associate with transcriptional co-activators such as CBP which lead

to acetylation of histones (Ernst P, 2001). The SET enzymatic activity has been shown to be increased in the presence of H3ac peptides (Milne T, 2002; Nakamura T, 2002).

In contrast to the TrxG family of proteins, Polycomb Group (PcG) proteins function in gene silencing. These proteins place repressive marks such as the H3K27me3 mark and H2AK119 ubiquitination, thus maintaining genes in a silent state (Fig. 2). Two well-studied PcG complexes are PRC1 and PRC2, and a further analysis of these complexes will be addressed in a subsequent section.

The role of prolyl isomerases (PPIase) in cellular function

There are three families of cis-trans prolyl isomerase proteins: cyclophilins, FK506-binding proteins, and parvulins (Handschumacher R, 1984; Harding M, 1989; Wang P, 2005). CYP33, falls into the cyclophilin protein family. Each of these families has a different structure and enzymatic active site. The structure of the cyclophilin domain-containing family of proteins consists of an eight-stranded β-barrel covered by two α-helices, with a hydrophobic pocket that can bind CsA (Cyclosporin A) (Ke H, 1993; Mikol V, 1993; Gamble T, 1996; Wang T, 2005). CsA is a natural inhibitor of the PPIase activity of cyclophilins (Takahaski N, 1989; Handschumacher R, 1984; Wang P, 2005). Cyclophilins are conserved from bacteria to humans, and the CYP33 protein is highly conserved from *Caenorhabiditis elegans* to *Homo sapiens* (Anderson M, 2002; Wang P, 2005). MLL and CYP33 interact in *Drosophila*, *mouse*, and *Homo sapiens.*

The prolyl peptide bond N terminal to the proline can adopt a cis configuration within proteins at a relatively high probability (Brandts J, 1975; Grathwohl C, 1976;

Levitt M, 1981). This is due to the intrinsic structure of the proline residue. Other amino acid residues have a much lower probability to be found in the cis conformation (10^{-3} versus 0.1-0.3 for proline) (Ramachandran G, 1976). Although a prolyl peptide bond has a higher probability to be found in either the cis or trans position within proteins, the spontaneous reaction in the absence of an enzyme is slow to reach equilibrium (Schmid F, 1993). This may lead to an additional step in the regulation of proteins with critical residues that need the peptidyl prolyl bond to be either cis or trans before a complex can form or a covalent modification can be added.

It has been shown that cyclophilins are involved in a variety of important cellular processes including: pre-mRNA splicing protein and lipid trafficking, protein complex assembly and disassembly, signal transduction, protein folding, and the regulation of gene transcription (Horowitz D, 2002; Price E, 1994; Hunter T, 1998; Anderson L, 2011; Fischer G, 1990; Nelson C, 2006; Shaw P, 2007).

Cyp33, its PPIase function, and RRM domain

CYP33 is a cis-trans prolyl isomerase which is 33 kD in size. It is a protein containing both an RRM (RNA Recognition Motif) domain and a cyclophilin domain separated by a spacer region part of which is highly conserved in amino acid sequence throughout evolution (Mi H, 1996). CYP33 belongs to the cyclophilin class of PPIases (see above). CYP33 was originally found in T-cells as a novel cyclophilin that binds RNAs (Mi H, 1996). Its cyclophilin domain is structurally similar to Cyclophilin A and binds CsA (Wang T, 2005). The RRM domain folds into five anti-parallel β-sheets and

two α-helices, as determined by x-ray crystallography (Hom R, 2010; Park S, 2010; Wang Z, 2010). It was found that CYP33 binds to poly-A and poly-U RNA sequences *in vitro* (Mi H, 1996; Solanki J, *unpublished*). CYP33 has been shown to bind to the mRNA sequence AAUAAA, which is the poly-A addition signal (Wang Y, 2008). Binding to the poly-A addition signal was shown to stimulate CYP33's PPIase activity, although no *in vivo* data was shown, and the significance of the small increase in PPIase activity by binding to mRNA was not addressed (Wang Y, 2008).

CYP33 was revealed to bind to the PHD3 finger of MLL during a yeast two-hybrid study where the PHD3 of MLL was the bait. Binding was further confirmed by CoIP experiments, GST immunoprecipitations, and imaging co-localization studies (Fair K, 2001; Anderson M, 2002). In addition to the discovery that CYP33 binds MLL, these reports also showed a functional relationship between CYP33 protein levels and MLL target gene transcript levels as the over-expression of CYP33 leads to the down-regulation of MLL target genes. This repression was dependent upon the PPIase activity of CYP33. The evidence revealed that after the addition of CsA, over-expression of CYP33 was no longer able to repress *HOXC8*, an MLL target gene (Fair, K 2001, Anderson, M 2002). Over-expression of a truncation mutant of CYP33 that lacks the PPIase domain (CYP33Δ) did not result in repression of *HOXC8* gene expression as well In a subsequent study, HDAC1 was shown to be recruited to MLL by CYP33, and both CsA and Trichostatin A (TSA), an HDAC inhibitor, could inhibit the target gene

repressive activity of CYP33 (Xia Z, 2003). In the presence of TSA or CsA, CYP33 no longer represses *HOXC8*, an MLL target gene, in 293T cells.

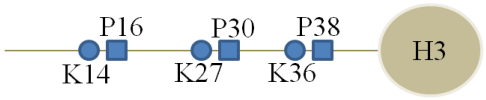
The RRM domain of CYP33 binds specifically to the MLL PHD3, but does not bind to other MLL PHD (Fair K, 2001; Hom R, 2010). Interaction of the PHD finger and the RRM domain disrupts RNA binding to RRM; CYP33 has a higher affinity for PHD3 than for RNA, according to *in vitro* data (Hom R, 2010). Results from our lab show that specific RNA sequences can disrupt the binding of PHD3 and CYP33 (Solanki J, 2011). The PHD3 binds to H3K4me3 or CYP33 in a mutually exclusive manner (Wang Z, 2010; Park S, 2010). Thus the hypothesis was proposed that when CYP33 is present, the PHD3 is unable to bind H3K4me3 and propagate the mark. Both of these studies relied heavily upon *in vitro* methods, conducting very few experiments *ex vivo* (*in tissue culture*). Most of the evidence provided in favor of the hypothesis relied on NMR and X-ray crystallography. I emphasize this to suggest that more work needs to be conducted in order to understand the complex nature and function of the RNA-CYP33 and MLL-H3K4me3 interactions.

The targets of the CYP33 PPIase activity have not been fully explored. There are two studies that look into this. A study by Wang *et al*. shows that CYP33 isomerizes the prolyl bond H1828-P1629 of MLL between the PHD3 and the bromodomain (Wang, Z 2010). They hypothesize that the z-α helix of the bromodomain blocks CYP33 binding to the PHD3 finger, and a conformation shift has to take place before CYP33 binding can occur. The identification of the prolyl bond H1628-P1629 as a substrate does not exclude

the possibility that CYP33 could have other cellular substrates, on MLL, HDAC1, histone H3 or other proteins. Histone H3 is an attractive candidate as it has been previously show to be a target of Fpr4, a FKBP PPIase in yeast (Nelson C, 2006). Since the MLL/CYP33 interaction seems to have complex functions at the chromatin level, it is reasonable to hypothesize that CYP33 could target prolyl bonds on the histone H3 tail. Using an *in vitro* chymotrypsin-coupled assay, using a histone H3 peptide substrate, this was shown to be the case. H3 is isomerized by CYP33 at H3P16 and H3P30 (Fig. 3) (Park S, 2010). The H3 A29-P30 prolyl bond is interesting for this study as H3K27 is only two amino acid residues away, and it is conceivable that the cis-trans state of this bond is important for acetylation or deacetylation of this residue. The spontaneous reaction of cis to trans in the absence of PPIase activity is slow (Nagradova N, 2010). A reasonable hypothesis is that CYP33 isomerizes H3 in order to allow deacetylation of H3K27 by HDAC1. HAT and HDAC1 have been tested for activity in the presence of CypA for the G13-P14 prolyl bond (Schultz, R. *unpublished*). In an *in vitro* assay it was shown that the activity of HDAC1 but not HAT increases towards lysine 14 in a peptide substrate in the presence of CypA.

Therefore the hypothesis that needs to be tested is whether CYP33 leads to a decrease in H3K27ac and allows for H3K27me3 (subject of this study). CYP33 could lead to a decreased H3K27ac in a couple of ways. One, CYP33 increases HDAC1 recruitment to MLL. The increased HDAC1 would lead to a reduction of H3K27ac at the

A)



B)

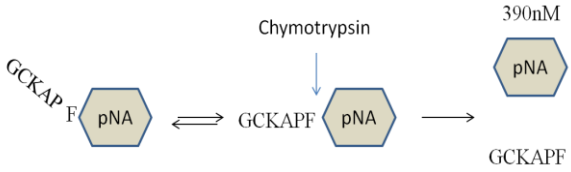


Figure 3. Histone tails such as H3 can be isomerized by the PPIase activity of cyclophilin domain containing proteins such as CYP33

A) Schematic of the histone tail showing lysine and proline residues in the N terminus of H3. B) A chymotrypsin-coupled reaction as shown above using a partial substrate of H3 shows how the CYP33 PPIase activity was assessed by Peter Breslin and Sango Park (Breslin, P *unpublished*; Park S, 2010).

promoters of MLL target genes. Two, CYP33 could isomerize H3P30 and allow for a better substrate for HDAC1, thereby decreasing H3K27ac.

MLL target genes

A well-studied group of MLL target genes are the *HOX* genes, a group of genes involved in body patterning during development. The *HOX* genes are organized in four gene clusters in mice and humans. MLL1 has been shown to regulate the more 5' genes of the *HOX* gene clusters. Well studied for their role in hematopoiesis *HOX* genes are not expressed within MSA cells (Lawrence, H 1996). MSA cells are a thyroid carcinoma cell line which will be used to study the role of repression of MLL target gene expression by CYP33. MSA cells were found to have low levels of *HOX* genes *HOXC8* and *HOXA9* and were believed to be an ideal cell line to study the role of CYP33 knockdown upon MLL mediated gene transcription.

Another theme that is emerging within the field of TrxG and PcG proteins about targeting to specific target genes within a cell is the role of non-coding RNA (ncRNA). MLL and PcG proteins have been shown to bind ncRNA. This is believed to help target TrxG and PcG proteins to specific target gene promoters, and to help maintain genes in the active (On) or repressive (Off) state (Papp B, 2006). Accordingly, there is a critical role for ncRNA in MLL-mediated maintenance of gene expression. One example is Mistral, which is a non-coding RNA that can target MLL to *HOXA6* and *HOXA7* in stem cell differentiation (Bertani S, 2011). In addition, ncRNAs have been linked to recruitment of PRC2 proteins to specific loci (Gupta R, 2010; Kotake, 2011). Therefore,

non-coding RNAs control chromatin architecture in trans and may play an important role in regulation of gene activity (Mattick J, 2001).

CYP33 also has been shown to bind to ncRNA specifically within RNA that is transcribed between coding genes *HOXC6* and *HOXC8* (Solanki J, 2011). The function of CYP33 binding to RNA is still under active investigation. Investigators Hom et al. showed that RNA binds to CYP33 with a Kd of 198μM, and PHD3 with a Kd of 1.9μM (Hom R, 2010). They hypothesize that CYP33 binds to MLL instead of RNA because the interaction of CYP33 and MLL PHD3 have a higher affinity than CYP33 has for RNA. Although Hom et al showed a high affinity bond between CYP33 and MLL, poly A or RNAs with the consensus sequence containing YAAUNY were shown to be capable of disrupting the CYP33/PHD3 finger interaction (Solanki J, 2011).

There are three studies (Wang Q, 2011; Wang P, 2009; Milne T, 2005) that explore in detail the binding of MLL and MLL fusions proteins to chromatin. These studies take a comprehensive look at MLL binding to the chromatin and try to gain a better understanding of the role played by MLL and its targets in gene expression. They vary in their conclusions about MLL target genes and their criteria for what qualifies as an MLL target gene. One paper concluded that there may be as few as 500 such target genes (Wang P, 2009), while another concluded that there may be as many as 5000 (Milne T, 2005a). Findings are based on high throughput methods such as ChIP analysis followed by sequencing (ChIP-seq).

Four MLL target genes that were examined in this study are *CDNK1B, MYC,* and *SIX1*. These have been shown to be regulated by MLL (Wang Q, 2011; Wang P, 2009; Milne T, 2005). *HOXC8* and *HOXA9* are not expressed in MSA cells and thus were not studied in this cell line, but they were examined in Mouse Embryonic Fibroblasts (MEFs). P27 which arises from the *CDNK1B* locus plays an important role in cell cycle regulation and is regulated by both MLL and MLL-fusion proteins (Xia Z, 2005). MYC, a basic helix-loop-helix transcription factor, has been shown to be a MLL target gene (Wang Q, 2011). The regulation of *c-Myc* is extremely complex, at both the levels of transcription and translation. It plays a major role in cell proliferation and also in hematopoietic cell differentiation and development. Additionally, it has been shown to be important in embryonic cell development, as it is one of the four genes that can induce pluripotent stem cells (Takahashi K, 2007). *SIX1* is a homeobox domain protein. It is a transcription factor having a role in neuronal differentiation and development; it has also been shown to be amplified in breast cancer (Reichenberger K, 2005).

MLL fusions

Much work has been conducted attempting to provide mechanistic insights into the pathogenesis of MLL leukemia, as they have particularly poorer prognoses than many other forms of this disease (Ayton P, 2001). This provides motivation in the field to develop targeted therapies for MLL leukemias. Currently, very little exists in the way of MLL directed therapy. MLL leukemias represent a small proportion of total leukemias which develop during infancy, under 1 year of age, and treatment-related leukemias.

Some patients who present with primary solid tumors, such as breast and lymphoma, are treated with topoisomerase II inhibitors. After treatment they are cured from their original cancer only to discover later that they have developed MLL leukemia (Huret J, 2001; Bhatia S, 2002).

Recent evidence supports the hypothesis from Thiel *et al.* proposing that WT MLL is necessary for the development of MLL fusion-mediated leukemia, as knocking down or knocking out of WT MLL leads to loss of transformation and leukemogenicity in mouse bone marrow cells (Thiel A, 2010). In addition, WT MLL is necessary for *HOX* gene expression regulation by MLL fusions (Thiel A, 2010). It is hypothesized that WT MLL is required to place the H3K4me3 mark and to maintain chromatin in an open conformation, since the MLL fusion lacks a SET domain (see below). More research aimed at understanding the role of WT MLL in MLL leukemia needs to be conducted. Also a report by Milne *et al.* (Milne T, 2010) showed that WT MLL1 is needed for the recruitment of the MLL fusion protein to the *HOXA9* locus, and over-expression of *HOXA9* is necessary for transformation (Ayton P, 2003). In addition to its role in leukemogenesis, MLL has an important role in both embryonic development and the development of various tissues in the adult organism (Yu H, 1995). MLL has been found to be involved in hematopoietic and neurogenic differentiation (Ernst P, 2002; Lim D, 2009).

The protein stability of WT MLL and MLL fusions has been shown to differ. WT MLL protein is degraded during G_1 and S phases of the cell cycle whereas the MLL

fusion protein remains stable throughout the cell cycle (Liu H, 2007). Forced over-expression of MLL increases H3K4me3 levels at MLL target genes during S phase, triggering recruitment of CDC45 and resulting in cell cycle arrest (Liu H, 2010). In comparison to MLL, MLL fusion proteins lose the degradation signal located on the N terminal portion of MLL; thus they are expressed at a constant level throughout the cell cycle (Liu H, 2007).

Chromatin, MLL complex, and histone tail modifications

Chromatin is comprised of nucleosomes around which 147 nucleotides of DNA are wrapped (Lugar K, 1997). A crystal structure of 2.8Å resolution revealed that nucleosome complexes are composed of DNA and octamer of four histone proteins, H3, H4, H2A, and H2B (Luger K, 1997). While histones were discovered at the turn of the 20th century, it was not until Jenuwein and Allis proposed the idea of the histone code hypothesis that the study of the histone role in gene regulation was truly brought to the forefront (Jenuwein, T 2001, Strahl B, 2000). The histone code hypothesis has had a profound effect on how transcriptional regulation is viewed. The histone code hypothesis states that the tails of histone proteins can be modified by the activities of various proteins and that the "marks" added or subtracted by these histone-modifying proteins lead to changes in the state of the chromatin. Histone tail modifications can act alone or in combination to modulate gene transcription (Strahl B, 2000; Jenuwein T, 2001).

The MLL complex has the ability to place the H3K4me3 mark at the promoter of MLL target genes. This is a mark of active transcription. H3K4me3 facilitates active

transcription through a variety of mechanisms (Deaton A, 2011). It has been shown to recruit NuRF chromatin remodeling complexes, to interact with the ING4 HAT complex, and also to directly recruit the core transcriptional machinery via interaction with TFIID (Li H, 2006; Ruthenburg A, 2007; Wysocka J, 2006; Saksouk N, 2009; van Ingen H, 2008; Vermeulen M, 2007).

Besides deposition of the H3K4me3 mark, MLL and trithorax (*Drosophila* homolog) also recruit CBP (Creb binding protein) (Ernst P, 2001; Tie F, 2009; Petruk, S 2001). Accordingly, the MLL complex can deposit H3K4me3 and H3ac in order to activate gene transcription. H3 acetylation has long been proposed to associate with active chromatin (Allfrey V, 1964; Pogo B, 1966). This idea was proposed in 1964, and in 1978 evidence showed that histone acetylation was associated with actively transcribed chromatin (Vidali G, 1978; Hebbes T, 1988; Sealy L, 1978). In 2008, genome-wide ChIP studies correlated 17 individual histone methylation and acetylation marks to active or highly active chromatin, thus underscoring the chromatin complexity within actively transcribed genes (Wang Z, 2008). H3K27ac and H3K4me3 were found in this study to be associated with highly active transcription (Wang Z, 2008). H3K27ac is located within the promoters of actively transcribed genes. Table 1 shows a brief list of the complexes that play a role in H3K27.

Histone demethylases

Earlier it had been hypothesized that the methyl mark could only be removed from the histone tail via proteolysis or dilution through histone replacement (Shi Y, 2004;

Goll M, 2002). However, it is now known that histone demethylases play a pivotal role in the regulation of histone methylation marks and that these enzymes play an important role in diseases such as cancer. UTX and JMJD3 lead to the removal of H3K27me3, whereas Jarid1A, Jarid1B, Jarid1C, and Jarid1D lead to removal of H3K4me3 (Klose J, 2006; Hong S, 2007; Swigut T, 2007). Demethylases, unlike HDACs, are generally specific for a particular histone residue. An exception is LSD1 which demethylates H3K4me2, H3K4me1, and H3K9me2,H3K8me1 (Shi Y, 2004; Shi Y, 2005). Recently, it was shown that UTX and BRM, components of the SWI/SNF chromatin remodeling complex, are necessary for demethylation and acetylation of H3K27ac in *Drosophila* (Tie F, 2012). Many proteins are still being characterized for their role in demethylation of histone tails. UTX has been shown to interact with MLL1 and MLL2 for demethylation of H3K27me3, leading to an increase in gene transcription (Lee M, 2007).

In addition to lysine, arginine residues have also been shown to be methylated. A separate class of enzymes, called PRMTs (protein arginine methyltransferases), write the arginine marks on these residues on histone tails (Di Lorenzo A, 2010). Arginine can be symmetrically or asymmetrically methylated and these lead to different transcriptional outcomes. Symmetrically methylated H3R4me2 leads to an increase in recruitment to the chromatin of WDR5, a coactivator of MLL, SET1A, SET1B, NLS1, and ATAC, whereas asymmetrically methylated H3R4me2 leads to gene repression (Migliori V, 2012). Deimination and conversion to citrulline by PADI4 inhibits methylation of arginine by CARM1. (Cuthbert G, 2004; Wang Y, 2004).

Transcriptional regulation

The accessibility of the chromatin due in part to maintaining various histone marks is an important factor related to transcriptional initiation and elongation. Of equal importance are the multimeric protein complexes, including the transcriptional cofactors that function to regulate gene transcription. The role of MLL in marking gene loci with the active mark H3K4me3 is well established (Ruthenburg A, 2007; Guenther M, 2008; Chang P, 2010). The H3K4me3 mark is recognized by CHD1. It is hypothesized that CHD1 facilitates pre-mRNA maturation by bridging spliceosomal components to H3K4me3 (Sims R, 2007). The MLL fusion partners AF4 and AF9 are components of a transcriptional elongation complex (Yokoyama A, 2010; Slany R, 2009). Therefore MLL fusions may increase transcription of MLL targets by increasing transcriptional elongation (Mueller D, 2009). CYP33 may also regulate transcription through MLL-independent mechanisms, as it has been shown to associate with XAB2 and the spliceosome (Kuraoka I, 2008; Jurica M, 2003). Transcriptional regulation is only one step in the process between transcriptional initiation and protein production.

During development transcription of MLL target genes, such as *HOXC8,* is initiated through MLL-independent mechanisms (Yu H, 1998). After initiation of *HOX* gene transcription in the early embryo MLL is critical to maintain *HOX* gene transcription in subsequent development, and prevent genes from becoming silenced in the developing embryo (Yu, B 1995). In the absence of MLL, *HOX* genes are turned off during development (Yu H, 1998).

Oscillations within cells, negative/positive feedback loops

Oscillations of gene transcription can be found in cells naturally through a variety of different mechanisms. Cell cycle genes oscillate throughout the cell cycle in rhythms that last around 24 hours in mammalian cells. By perturbing genes associated with circadian rhythms, such as *BMAL1* or *CLOCK*, these rhythms can be altered. CLOCK is a histone acetyltransferase, and it has been shown that the activity of CLOCK is important for circadian rhythm function (Doi M, 2006). MLL1 has been found in a complex with CLOCK and BMAL1 and is important for circadian gene transcription and methylation of H3K4me3 at circadian-transcribed genes (Katada S, 2010). Many cancer cells have disrupted circadian rhythms (Sahar S, 2009). The pathways of the cell cycle and circadian rhythms are not independent, but are linked through the DNA damage protein XPA, and cell cycle regulators, MYC, p21, and Wee1 (Sancar A, 2010).

Circadian rhythms are controlled by a series of negative feedback loops (Ko C, 2006). These feedback loops are regulated by light/dark stimuli. Circadian rhythms oscillate in synchronous cells in yeast (Klevecz R, 2004). Normal circadian feedback loops can be simple: Gene A is expressed and its protein product negatively regulates its own expression with delays in several steps of transcription, translation and protein transport to the nucleus. Feedback loops also can be complex and have multiple gene

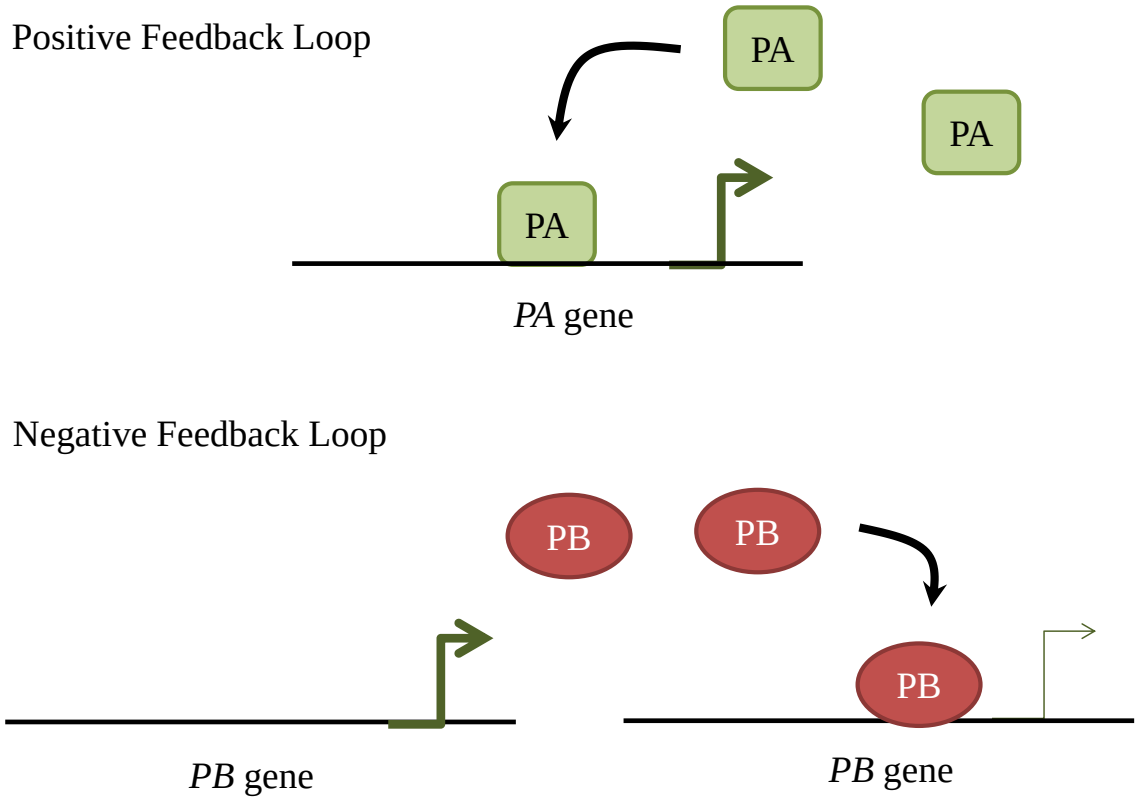


Figure 4. Representation of simple positive and negative feedback loops.

An example of a positive feedback loop is *PA* gene encoding the PA protein which binds its promoter and stimulates its transcription. An example of a negative feedback loop is the *PA* gene encoding the PA protein, a repressor which binds to its promoter and decreases its transcription. A negative or positive feedback loop can operate through a single gene, or through multiple genes and proteins and pathways.

regulatory networks. Gene A regulates Gene B and Gene C, which then negatively regulates Gene A. Figure 3 shows examples of positive and negative feedback loops.

MLL and the cell cycle, senescence, and MLL protein stability

Lastly, when studying MLL it is worth noting that it is cleaved into N- and C-terminal fragments by a protease, taspase1 (Hsieh J, 2003). This taspase-mediated cleavage is conserved in evolution as the *Drosophila* Trx (MLL homolog), is also cleaved by a taspase homolog (Capotosti F, 2007). The FYRN and FYRC domains along with PHD fingers 1 and 4 are responsible for proper interaction of MLL-N and MLL-C (Hsieh J, 2003; Yokohama A, 2011). MLL-N when overexpressed in cells by itself, apart from MLL-C, is unstable (Liu H, 2007; Yokohama A, 2011). The stability of MLL-N and MLL C-proteins is regulated through separate degradation mechanisms (Yokohama A, 2011).

In the cell cycle, MLL is regulated by protein degradation. During the S and G1 phases of the cell cycle, MLL is degraded by SCF and APC respectively, causing a reduction in MLL target gene transcription (Liu H, 2007). MLL has also been shown to be important for gene transcription after mitosis. In the absence of MLL, a large number of genes that are silenced during mitosis suffer a delay in their reactivation during G_1 (Blobel G, 2009). Consequently, MLL plays a broader role in the regulation of the cell cycle than previously thought.

Senescence is a process by which a cell irreversibly exits the cell cycle (Kuilman T, 2010). A couple of studies show that inhibition of cyclophilins, through CsA, lead to

senescence. CsA treatment induces senescence in renal tubule epithelial cells through induction of p21 and p16, and differentiation in K562 cells (Sawafuji K, 2003; Jennings P, 2007). P27 has also been linked to Rb mediated senescence (Alexander K, 2001). It was also found that knocking down MLL leads to a p53-dependent cellular senescence response through upregulation of p21 in human derived fibroblasts (Caslini C, 2009).

PRC2 complex

The PRC2 complex consists of three core proteins along with many accessory proteins: EED, Suz12, and Ezh2 (Cao R, 2004; Kuzmichev A, 2002; Montgomery N, 2005). A fourth protein, PHF1, has been shown to be necessary for efficient H3K27 trimethylation (Cao R, 2008; Sarma K, 2008). HDAC1 is also part of the PRC2 complex, yet its role is not well understood (Table 2). The homolog of HDAC1, RPD3 has been found to be a part of the drosophila PRC2 complex (Tie F, 2001; Tie F, 2003). HDAC1 has also been shown to be necessary for EED repression (van der Vlag J, 1999). Several reports have shown that Ezh2 is over-expressed or deregulated in aggressive breast and complex are over-expressed or lead to tumorgenesis in a variety of cancers including breast, prostate, bladder, colon, lung, pancreatic cancer, sarcoma and lymphomas (Chang C, 2012; Kleer C, 2003; Wilson B, 2010). Also beside Ezh2 being an oncogene, it has also been shown to have a role as a tumor suppressor. Ezh2 and Suz12 were shown to be decreased in a variety of T-ALLs (Ntziachristos P, 2012; Zhang J, 2012). Ezh1 is a closely related homolog of Ezh2 in mammals. Ezh1 has methyltransferase activity for

Table 2. A list of selected histone marks that are written or removed by TrxG and PcG proteins and the complex that places or removes the marks.

Histone Mark	Known Function	Complex/Enzyme that places the mark	Complex/Enzyme that removes mark
H3K4me3	Transcriptional initiation at the gene promoter	MLL complex/SET domain of MLL	Jarid1A-D Complex
H3K9ac/ H3K14ac	Gene transcription, allows for open chromatin	GCN5	HDACs
H3K27ac	High rate of transcription	CBP	HDACs
H3K9me3	Heterochromatin	SUV39H1	JMJD1A, JMJD2C
H3K27me3	Gene repression	PRC2 complex/Ezh2	UTX, JMJD3
H2AK119ub	Gene repression may be through chromatin compaction	PRC1 complex /RING1A/B	DUBs

Red-Marks that lead to the repression of gene transcription

Green-Marks that lead to the activation of gene transcription

H3K27me3, and upon knockout or knockdown in ES cells, Ezh1 can partially compensate for Ezh2 (Shen X, 2008). Ezh1 was also shown to be able to compensate for Ezh2 in adult hematopoesis but it is unable to compensate for it in the fetal liver where it is expressed at a lower level. Therefore Ezh2 is indispensable for the fetal liver hematopoesis but it can be knocked down with little effect in adult hematopoesis in mice (Mochizuki-Kashio M, 2011).

The prevailing hypothesis in the literature is that H3K27me3 is deposited upon the chromatin by Ezh2 within the context of the PRC2 complex. Once the H3K27 mark is deposited, hPC2, a component of the PRC1 complex, can then bind to the H3K27me3 with its chromodomain (Fischle W, 2003). This leads to repression of gene transcription by multiple mechanisms (see PRC1 section below) that are not completely understood. PRC2 recruitment to the chromatin has been studied extensively and yet many questions remain unanswered.

PREs (Polycomb Response Elements) are regions at which both PcG and TrxG proteins bind to chromatin (Ringrose L, 2003). PREs in *Drosophila* are better defined than mammalian PREs as far as which proteins recruit PcG and TrxG proteins. In *Drosophila* DNA sequence motifs allow for recruitment of the proteins PHO, Zeste, and GAGA, which in turn recruit PRC2 (Mendenhall E, 2010; Wang L, 2004; Tolhuis B, 2006; Simon J, 1993). Recently, certain GC rich sequences have been shown to have PRE properties in mammalian systems. (Mendenhall E, 2010). A region between

HOXD11 and *HOXD12* is a possible example of a PRE element to be defined in humans (Woo C, 2011).

Activating chromatin marks prevent PRC2 from methylating H3K27me3. Different studies have shown that the H3K36me3, H3K4me3, and H3K27ac marks antagonize PcG protein functions (Tie F, 2009; Pasini, D 2010; Yuan W, 2011). One report demonstrates that Suz12 can no longer bind H3K27me3 when an H3K4me3 or H3K36me3 mark is present on histone H3 (Yuan W, 2011). Thus histone marks can antagonize and prevent efficient PRC2 complex binding and/or activity.

Multiple chromatin marks can have a different outcome that individual marks alone. A common example is when H3K4me3 and H3K27me3 are found together. H3K4me3 and H3K27me3 are present together in the so-called bivalent domains which are associated with regions poised for transcription. Pluripotent ES cells contain many genome regions with bivalent domains that are resolved into H3K4me3 or H3K27me3 upon differentiation, depending on the cell type (Cui K, 2009). Bivalent domains are associated with poised differentiation genes in ES cells and are found at the transcriptional start sites of *Sox, Fox, Pax, Irx,* and *Pou* gene family members (Bernstein B, 2005).

PRC2 activity is important in regulating the decision between stem cell self-renewal and differentiation (Surface L, 2010; Kamminga L, 2006). A hypothesis that has arisen in the field of cancer study is the idea of the cancer stem cell. This concept was

proposed 150 years ago by Cohnheim and Durante (Wicha S, 2006). It is an attractive hypothesis for two main reasons. Firstly, it goes a long way toward explaining why cancers go into remission and then relapse. Secondly, it helps to explain why cancers that are treated often relapse in a more aggressive form. Conventional cancer treatments, such as chemotherapy or radiation, selectively kill rapidly proliferating cells. Cells that are quiescent or slowly cycling have a survival advantage over proliferating cells. These quiescent or slowly-cycling cells are subject to additional mutations. These escapees may become resistant to additional rounds of treatment due to mutations that provide the cells with resistance to apoptosis, leading to an aggressive relapsed disease.

MLL and its homologues have not only been implicated in leukemia but also in many other cancers, such as prostate, diffuse large B-cell lymphoma, breast, and pancreatic cancer (Grasso C, 2012; Lohr J, 2012; Ellis M, 2012; Mann K, 2012).

PRC1 complex

PRC1 is a complex comprised of four core proteins: hPC2 (CBX4), BMI1, RING1A/B, and PH. Other orthologs of CBX4 include CBX2 (hPC1), CBX7, CBX8 (hPC3). The mechanism of PRC1 repression remains under investigation. PRC1 has been shown to inhibit transcription, and it mediates ubiquitination of lysine 119 of the histone H2A and its variants (H2AK119ub) (King I, 2002; Sparmann A, 2006), which may increase the binding of histone H1 to nucleosomal spacer DNA (Jason L, 2005). Also RING1B has been shown to compact chromatin independently of histone ubiquitination (Eskeland R, 2010). Finally CBX7, a component of PRC1, has been

shown to recruit DNMTs to promoters of CBX7 target genes, promoting DNA hypermethylation in cases of cancer (Mohammad H, 2009).

BMI1, which is part of the PRC1 complex, has been extensively studied for its role in maintaining the stem cell phenotype. The stem cell phenotype includes the ability to self renew, and to differentiate into many cell lineages (multipotency) upon appropriate stimulation. In hematopoietic stem cells, BMI1 has been shown to be required for maintaining the stem cells phenotypes (Park I, 2003). BMI1 and hPC2, two of the components of PRC1, are found associated with MLL (Xia Z, 2003). BMI1 also has a role in cancer and was shown to cooperate with c-MYC to promote the generation of B and T cell lymphomas (Jacobs J, 1999). BMI1 has also been shown to be a downstream target of c-myc (Sparmann A, 2006). Ring1a and Ring1b contain a RING finger domain that functions as an E3 ubiquitin ligase. Ring1a/1b and BMI1 have ubiquitin ligase activity specific to H2AK119ub. Bmi1 and Ring1A through H2A ubiquitination plays an important role in HOX gene silencing (Cao R, 2005).

CBX8, a PRC1 component, has been shown to play a role in MLL-AF9 leukemogenesis (Tan J, 2011). Briefly, it was shown that CBX8 (also known as hPC3) is necessary for transformation of MLL-AF9 cells. By knockdown or knockout of *CBX8*, MLL-AF9-mediated transformation can be prevented; the expression of the *HOX* genes is reduced in such knockout cells upon transduction of *MLL-AF9*. The investigators believe that this phenomenon is independent of the PRC1 complex because if they knockdown RING1B expression by shRNA, MLL-AF9 can still transform cells (Tan J, 2011). Also

BMI1 has been shown not to be required for MLL-AF9 transformation, but loss of BMI1 in PLZF-RARα, and AML1-ETO trigger senescence and prevents transformation (Smith L, 2011).

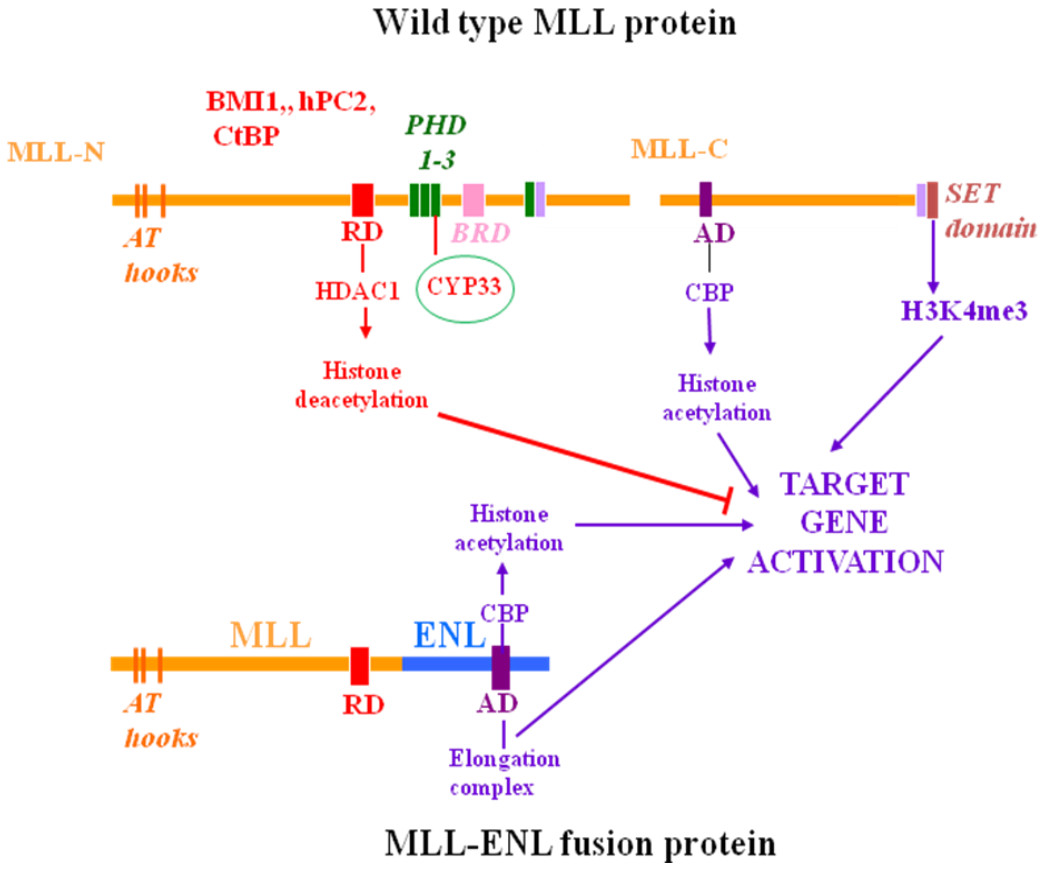

Wild type MLL protein
BMI1,, hPC2,
CtBP
PHD
1-3
MLL-N
MLL-C
SET
domain
AT
hooks
RD
BRD
AD
HDAC1
CYP33
CBP
H3K4me3
Histone
deacetylation
Histone
acetylation
TARGET
GENE
ACTIVATION
Histone
acetylation
CBP
MLL
ENL
AT
hooks
RD
AD
Elongation
complex
MLL-ENL fusion protein

Figure 5. Multiple domains of MLL function together to regulate MLL target gene transcription.

The domains that are pertinent to this study are shown above. In brief, what has been shown prior to this study is that HDAC1 binding to MLL is increased in the presence of CYP33, which subsequently leads to deacetylation of MLL target genes. CYP33 also has been shown to decrease H3K4me3 at MLL target genes. This mark is placed by the SET domain of MLL. The mechanism by which CYP33 functions to lead to decreased H3K4me3 is currently under investigation.

Also indicated in the above diagram are the proteins that compete for binding to the PHD3 finger. CYP33, when bound, reduces the affinity of the MLL PHD3 for H3K4me3. CYP33, when bound to MLL, promoters the recruitment of HDAC1, which can then deacetylate appropriate residues on the histone tails or on other proteins. CBP, a histone acetyltransferase, binds to the AD of MLL and can acetylate H3K18 and H3K27. Also shown is the fusion protein, MLL-ENL, which is able to activate MLL target gene expression, but is unable to bind CYP33 or to repress MLL target genes transcription, as it lacks the PHD3.

CHAPTER III

MATERIALS AND METHODS

Cloning

pCEP4, pCEP4-CYP33, and pCEP4-CYP33 M were cloned by Dr. Peter Breslin, Wei Wei, and Dr. Ute Osmers (Park S, 2010). Using these plasmids as a template, HA-CYP33 and HA-CYP33 M were cloned into the HA CMV vector using the following strategy. HA- CYP33 and HA-CYP33 M were pcr amplified from pCEP4-CYP33 and pCEP4-CYP33 M using primers containing Sal1 and Kpn1 restriction sites flanking the coding sequence of CYP33 (Table 8). High fidelity Pfu DNA polymerase from *Stratagene* was used to avoid mutations. After addition of buffer and dNTPs, the solution was run on a BioRad Thermocycler (iCycler) using a program of 95^{o}C. for 5 minutes for a single cycle, then 35 cycles of 95^{o}C., 60^{o}C., and 72^{o}C. for 45 seconds each, followed by a 72^{o}C. extension step for 7 minutes. TA cloning using 0.5µL of Taq Polymerase from *Promega* and 1µL dATP was then performed at 72^{o}C. for 30 minutes. The product was then separated on a 1.8% agarose gel to check for the correct product size of 1400bp. The 1400bp band was excised from the gel and DNA purification was performed using the *Promega* Gel Extraction Kit. The excised band was subcloned into pGEMT. Subcloning was achieved using 3µl of the PCR cloned KpnI/SalI CYP33 or CYP33 M fragment a

1µL of pGEMT, along with 1µl of T4 DNA ligase and 5µl of 2X ligase buffer from *Fermentas*. Prior to cloning the insert from pGEMT into HA-CMV, the pGEMT plasmid containing CYP33 or CYP33 M was cut with Sal1 to determine orientation and then sent to an outside source for sequencing. Clones containing the correct sequence were then cut with Kpn1 and Sal1. The Kpn1/Sal1 CYP33 or CYP33 M fragment was then ligated into HA-CMV vector.

siRNA construction

siRNA's were purchased using IDT's predesigned DsiRNA. siRNA's were diluted using siRNA dilution buffer which is comprised of 30mM HEPES, 100mM potassium acetate, in DEPC water pH adjusted to 7.5. The siRNA dilution buffer is then filter sterilized and the siRNA were diluted to 100µM. siCYP33 (2) was used for MSA cell knockdown and timecourse experiments. Both siCYP33 (1) and siCYP33 (2) target the RRM domain of CYP33. The sequences for the double stranded RNAs are shown below.

siCYP33(1)	5'-rArGrCrUrArUrCrGrArCrArArCrArUrGrArArUrGrArArUCT-3'
	5'- rArGrArUrUrCrArUrUrCrArUrGrUrUrCrUrCrGrArUrArGrCrUrGrC-3"
siCYP33 (2)	5'-rGrArGrArCrArUrCrArCrArGrArUrArUrUrCrArGrArUrUCC-3
	5'-rGrGrArArUrCrUrGrArArUrArUrCrUrGrUrGrArUrGrUrCrUrCrCrA-3'

Table 3: Expression plasmids used in this study

Plasmid	Epitope	Insert	Origin
pGEMT		N/A	Promega
HA CMV	HA	N/A	Clontech
HA-CYP33	HA	AA 1-301	Subcloned from pCEP4
HA-CYP M	HA	AA 1-301 (R191A,F196A)	Subcloned from pCEP4
pCEP4	FLAG	N/A	Invitrogen
pCEP4-CYP33	FLAG	AA 1-301	Dr. Ute Osmers
pCEP4-CYP33 M	FLAG	AA 1-301 (R191A,F196A)	Dr. Ute Osmers
pIC-MLL-N	FLAG	AA 1-2665	Dr. James Hiesh
MLL RD-PHD	FLAG, GAL4	AA 1088-1371, 1394-1630	Dr. Keri Fair

Co-immunoprecipitation

In order to study the interactions of proteins, co-immunoprecipitation (CoIP) was performed. Cells expressing the protein of interest were scraped off the culture dishes after rising them with 1mL of PBS. After spinning down the cells at 1500 r.p.m. the supernatant was removed. To lyse the cells 1mL of IPH buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 5mM EDTA, 0.5% NP40). Cells were incubated on ice for 1 hr., vortexing every 15 mins to break up the cells. The lysate could then be frozen at -20^{o}C. overnight and the protocol continued the next day. To clear the cellular debris, the lysates were then spun down at 12,000 × g. at 4^{o}C. for 30 minutes. The supernatant was collected and placed in a clean microcentrifuge tube; the cellular debris was discarded. The protein concentration was then quantitated using BCA or Lowry high methods. Between 1mg to 2.5mg of protein was used for protein pulldown and 150µg to 200µg was used as input.

1mg to 2.5mg of the cell lysates were diluted to a final volume of 1mL using IPH buffer. The lysates were precleared by adding 100µl of washed Protein A agarose beads (*Santa Cruz* sc2001) for 10 minutes at 4^{o}C. The lysates were then centrifuged at 12,000 r.p.m. for 30 seconds and the supernatant was placed into a 1.5mL microcentrifuge tube. Sixty µL of washed Flag-M2 beads (*Sigma*) were added to the lysate. For Gal 4 pulldowns, 1µg of Gal4 Ab (see Ab table) was added along with 60µL of Protein A beads. The mixture was then incubated for 2 hours to overnight at 4^{o}C with gentle rocking.

The Protein A/antibody complex was then collected by centrifuged at 12,000 × g. for 30 seconds and the supernatant removed. Eight hundred µl of IPH was added to wash the complex followed by rotation for 3 minutes and centrifugation. Three washes allowed for non-specific interactions to be removed. The beads were then resuspended in SDS-loading dye, followed by Western Blotting as described.

RNA extraction

Cells were detached from the culture dishes using 1 to 3 mL of Trypsin-EDTA (*Gibco*). Cells were spun down at 2000r.p.ms and trypsin was removed. RNA was extracted using *Trizol*® Reagent (*Gibco*). One mL of *Trizol* Reagent was added to the cells. During timecourse experiments in which CYP33 was knocked down using siRNA, or inhibited using CsA, once cell lysates were suspended in the *Trizol* Reagent they immediately were placed in the -80^{o}C. freezer, and the protocol was continued after all

timepoints were collected. Freezing down the *Trizol*/cell mixture resulted in no reduction of RNA quality.

One hundred µL of chloroform was added to the *Trizol* RNA mixture. After 2 to 3 minutes, *Trizol*/chloroform was centrifuged at 12,000 rpms in the cold room at 4 ^{o}C. The upper liquid phase containing the RNA, was carefully removed as to not disrupt the interphase layer. The RNA was then precipitated by using 250µL of 2-isopropanol. Samples were incubated at room temperature for at least 10 minutes, and then they were centrifuged at 12,000 r.p.m. for 10 minutes at 4^{o}C. The supernatant was removed and 75% Ethanol-DEPC H_20 was added to wash the pellet. The pellet was detached by briefly vortexing. The pellet was then centrifuged at 6500 r.p.m. at room temperature for 5 minutes. The supernatant was removed completely and the pellet was air dried for 5 minutes, after which 25µL of DEPC H_20 was added to the pellet and the solution was placed at 55^{o}C. for 10 minutes. RNA concentration was then determined using the *Nanodrop 2000c*.

cDNA synthesis

Six µg of RNA was converted to cDNA using *Applied Biosystem's* High Capacity cDNA Reverse Transcription Kit (Part No: 4368814). RNA was treated with 3µL of DNAseI (*Fermentas* Cat No) and 3µL of DNaseI Buffer in a total volume of 30µL. Samples were incubated for 30 minutes at 37^{o}C. Afterwards, 3µl of 50mM EDTA was added, and the samples were mixed thoroughly and then incubated for 10 minutes at 65^{o}C. to inactivate DNaseI.

High capacity cDNA kit from *AB* was used for the reverse transcriptase reaction. Briefly, 10× RT buffer, 25× dNTPs, 10× Random Primers, *MultiScribe*™ Reverse Transcriptase, and of RNase Inhibitor, were mixed together to make a 2× master mix. The 2× master mix was then added to the above DNAase1 treated reaction. For the negative control, the same master mix was generated, minus the *MultiScribe*™ Reverse Transcriptase.

Real Time PCR

cDNA or ChIP lysates were diluted 1 to 3 or 1 to 5 with ddH_2O, and 7µL was placed into a 96 well Dot #650-PCR plate already containing 18µL of a master mix containing 12µL *GoTaq*® qPCR Master Mix (Cat # A6001), 1µL F/R primers, 0.25µL CRX Reference Dye, and 4.75µL Nuclease-Free Water. RT-PCR sealing film cengrifuging at 1000 r.p.m. in a *Beckman* GPR Centrifuge for 5 minutes, the plate was inserted into an ABI7300 Real Time PCR machine and run 40 to 50 cycles. A temperature of 60^{o}C. was used for primer annealing unless otherwise indicated. The dissociation or melting curve was obtained in order to show that the product was unique and not a primer dimer; primer dimers would melt at a much lower temperature due to its small size and GC content. Cycle threshold (Ct) values were obtained using either manual or automatic setting using *ABI7300 SDS* system software. Relative values were obtained using a ΔΔCt method. Standard deviations were calculated, taking into account variation in both the standards and samples. To determine if changes in mRNA levels reached significance, a student t test using *GraphPad's* student t test online was used.

Western blot assays

IPH buffer (recipe shown in co-immunoprecipitation) was used to lyse cells in order to isolate proteins for both Western Blotting and Co-IP. Proteins were quantified by BCA assay (see below), and equal amounts of protein were loaded onto an SDS-PAGE gel. Proteins were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Tris-HCl-glycine running buffer. After electrophoretic separation, proteins were transferred onto a PVDF membrane. CAPS buffer (10mM CAPS Sigma C2632 pH to 11, 15% MeOH) or transfer buffer (25mM Tris, 200mM gylcine, 20% MeOH) were used to transfer proteins depending on the protein of interest; CAPS was used for the transfer of MLL or Jarid1b and transfer buffer was used for all other proteins. Using CAPS buffer, transfer was accomplished at 25V. overnight followed by 70V. for 1 hour using cold solutions at 4^{o}C. Transfer for proteins under 200kD was conducted at room temperature for 1 hour at 100V. After transfer, the membrane was placed in PBST (PBS Gibco 0.05% Tween20) and 5% nonfat dry milk for one hour at RT to block non-specific bands. After removing the blocking solution, PBST/2.5% milk was added along with the appropriate primary antibody, followed by incubation overnight at 4^{o}C. or 3 hours at room temperature. The membrane was rinsed three times for 2 minutes each in TBST followed by addition of the appropriate secondary antibody. The membrane was incubated for 45 minutes in secondary antibody before being removed and then washed in TBST for 5 minutes three times and then rinsed in TBS once for one minute before detection with *Supersignal ECL* from *Pierce*. Signals were visualized using a *FugiFilm* Intelligent Dark Box LAS 3000.

Chromatin immunoprecipitation

Adherent cells, 293T or MSA, were trypsinized in 3mL Trypsin-EDTA 0.05%. One mL was used for RNA analysis and the rest diluted to 10mL and fixed with formaldehyde. Formaldehyde was added to a final concentration of 1% and the cells were fixed for 10 minutes by shaking. To stop the reaction, 1mL of 1.25M glycine was added and the solution was mixed by inversion. After 5 minutes, cells were spun down at 1750 r.p.m. for 5 minutes, then washed twice with ice-cold PBS and centrifuged at 4 ^{o}C. at 1750 r.p.m. Supernatant was removed and the pellet was resuspended in 0.5mL Cell Lysis Buffer (solutions for the ChIP protocol are described at the end of this section) plus 5µL of 100× protease inhibitor cocktail (*Roche* Complete Cat. No. 04 693 116 001) and the solution was placed on ice for 15 minutes, with vortexing every 5 minutes. The nuclear pellet was centrifuged at 1750 r.p.m. for 5 minutes and then lysed in 0.5mL of Nuclear Lysis Buffer plus 5µL of 100× protease inhibitor.

The nuclear lysis was aided by sonication using a *Branson Sonifier250* using one of the protocols below. Sonication was empirically tested by taking individual cell lines and sonicating them using various parameters in order to determine the optimal protocol necessary to fractionate the DNA to between 200 and 1000 bp. Six second pulses were performed for the indicated duration. Samples were placed on ice between replications to prevent the heating of samples.

Table 4. Sonication Condtions

Cell Line	Sonication protocol
HEK293	(Output 4) four rounds of 6 second pulses
MSA	(Output 4) eight rounds of 6 second pulses
MEF	(Output 5) three rounds of 6 second pulses

After sonication, solution was centrifuged at 10,000 r.p.m. at 4^{o}C. for 10 minutes. Twenty-five µL of undiluted sample was used for Input. Input was removed and frozen at -20^{o}C. to be used at a later time. Fifty µL was then diluted using 450µL of ChIP Dilution Buffer plus 4.5µL of 100× protease inhibitors to a 2mL flat bottom microcentrifuge tube. To the diluted sample, 5µL of Antibody and 20µL of Protein A magnetic beads (*Millipore* Cat. 16-661) were added. The samples were then placed into a 50mL falcon tube and rotated at 4^{o}C. overnight. The magnetic beads were pelleted using a magnetic rack. The Protein A/Ab/chromatin complex was then washed with low salt, high salt, LiCl, and TE buffer for 5 minutes, pelleting the complexes in between.

After the washes, all the supernatant was removed, and to both Input and samples 100µl of ChIP Elution Buffer plus 1µl of 10µM proteinase K was added. Samples and Input were then incubated at 62^{o}C. for 2 hours to reverse the crosslinking and to degrade the protein. Samples were heated for 10 minutes at 95^{o}C. to inactivate the Proteinase K, and then cooled down to room temperature. Beads were removed by pelleting in the magnetic rack, and the samples removed and placed into a microcentrifuge tube. DNA

purification kit from *Molebio* was used to extract the DNA. Fifty µL of TE was used to elute samples, which were diluted either 1:3 or 1:7. Seven µl were used to evaluate a number of target genes using real time PCR.

Table 5. ChIP solutions

low salt	0.1%	SDS (Sodium Dodecyl Sulfate)
	1.0%	Triton X-100
	2mM	EDTA
	20mM	Tris HCl pH 8.0
	150mM	NaCl
high salt	0.1%	SDS (Sodium Dodecyl Sulfate)
	1.0%	Triton X-100
	2mM	EDTA
	20mM	Tris HCl pH 8.0
	500mM	NaCl
LiCl	1.0%	NP40
	1.0%	Deoxoycholic acid (Sodium Salt)
	1mM	EDTA
	10mM	Tris HCl pH 8.0
TE	10mM	Tris HCl pH 8.0
	1mM	EDTA
Nuclear Lysis Buffer	1.0%	SDS
	10mM	EDTA
	50mM	Tris HCl pH 8.0
Cell Lysis Buffer	3mM	MgCl2
	10mM	NaCl
	10mM	Tris pH 7.4
	0.1%	NP40
ChIP Dilution Buffer	0.01%	SDS
	1.1%	Triton X-100
	1.2mM	EDTA
	16.7mM	Tris HCl pH 8.0
	150mM	NaCl

ChIP solutions were made and then filtered through a 0.22µm vacuum sterile filter in order to remove all contaminants.

ATP cell viability and proliferation assay

Cell Titre Glo (*Promega*) assay was used to determine ATP levels in the cell after CYP33 knockdown or over-expression. Eight hundred thousand MSA cells were transfected overnight with siGFP or siCYP33 was using *Lipofectamine 2000* at 4µl per 60mm dish. For over-expression studies, cells were transfected with pCEP4 vector, CYP33, or CYP33 Mut using 10µl of *Lipofectamine* in a 60mm dish. Cells were transfected for 12 hours, and afterwards MSA medium (DMEM/F12 Gibco) containing transfection was removed and replaced by fresh medium. After 24 hours, cells were trypsinized, and 5,000 cells were plated in a Falcon white opaque 96 well plate together with 70µl of DMEM/F12 complete medium. Twenty-four hours later, the *Cell Titre Glo* assay (*Promega*) was performed. Cells were incubated for 30 minutes at room temperature with slow shaking to allow cells to reach RT. Cell *Cell Titer Glo* reagent was prepared and stored at -20^{o}C. until use; the reagent thawed and kept on ice during use. The buffer and substrate were combined according to manufacturer's protocol. After thawing the reagent (buffer and substrate), 70µl of *Cell Titer Glo* reagent was added to 70µl of medium for 10 minutes before the luminescence was detected using a *PolarSTAR Omega* microplate reader.

Cell culture

The HEK 293T cell line was purchased from American Tissue Culture Collection (ATCC) and was grown in DMEM (*Gibco*), 10% Fetal Bovine Serum (*Gibco*) and 1% Penicillin/Streptomycin (*Hyclone*). Cells were maintained at around 50-60% confluency.

The MSA cell line, a human thyroid carcinoma cell line, kindly provided by Dr. Shoji Nakamori (Osaka National Hospital, Japan), was cultured in DMEM F/12 (1:1) high glucose (*GIBCO*), supplemented with 10% FBS and 1% penicillin/streptomycin at 60-70% cell density for routine maintenance. Mouse embryonic fibroblasts, that are wild type for MLL, $MLL^{+/+}$ or fibroblasts that lack a functional MLL, in which LacZ has been inserted into the third exon of MLL (Yu, B 1995). MEFs were grown in DMEM (*GIBCO*), supplemented with 10% FBS, 1% penicillin/streptomycin and 0.1mM β-mercaptoethanol. Cell lines used in this study are shown below.

Table 6. Cell lines used in this study

Cell Lines (Obtained from)	**Reason Used/Methods**
293T	Cells are easy to transfect/CoIP
MEF $MLL^{+/+}$ (S. Korsmeyer)	Can use cell line to determine MLL-dependent effects/ChIP
MEF $MLL^{-/-}$ (S. Korsmeyer)	See above/ChIP
MSA	Repressed *HOX* gene cluster/RT-PCR and ChIP

Table 7. Antibodies used in this study

Antibody	**ChIP/CoIP/Western**	**Company/Cat No**
H3K27 acetylation	ChIP	Millipore/07-360
H3K27 trimethylation	ChIP	Millipore/07-449
H3K4 trimethylation	ChIP	Millipore/07-473
H3 Pan aceytylation	ChIP	Millipore/06-599
H3	ChIP	Abcam/ab1791
Mouse IgG	ChIP	Millipore/12-371b
Rabbit IgG	ChIP	Millipore/ChIP Kit
CYP33	ChIP/CoIP	Abnova/H00010450-A01
BMI1	ChIP	Millipore/17-664
BMI1	Western	Millipore/05-637
HDAC1 clone E210	ChIP/CoIP	Millipore/05-614

FLAG	Western/CoIP	GenScript/A00013
HA-tag	Western/CoIP	Abcam/ab9110
ACTIN	Western	MP/69100
HOXC9	Western	Abcam/ab50839
MLL N	Western	Millipore/05-764
GAL4	Western/CoIP	Millipore/06-262

Table 8. Primers used in this study

Human Primer Table

Primer Name	**RT-PCR/ChIP**	**Sequence 5'-3'**
CDNK1B(P27)F	RT PCR	CCGGCTAACTCTGAGGACAC
CDNK1B(P27)R	RT PCR	CTTCTGAGGCCAGGCTTCTT
MYC F	RT PCR	CCTACCCTCTCAACGACAGC
MYC R	RT PCR	CTCTGACCTTTTGCCAGGAG
SIX1 F	RT PCR	AGCTCCTGGCGTGGCCCATA
SIX1 R	RT PCR	CGCGGAGGCCAAGGAAAGGG
CCNA2 F (CyclinA2)	RT PCR	ACTGTTGTGCATGCTGTGGTGCT
CCNA2 R (CyclinA2)	RT PCR	CACGGGACAAAGCTGGCCTGA
β-ACTIN F	RT PCR	CGTACCACTGGCATCGTGAT
β-ACTIN R	RT PCR	GTGTTGGCGTACAGGTCTTTG
GAPDH F	RT PCR	ATGGCAAATTCCATGGCACCGT
GAPDH R	RT PCR	ATGGTGGTGAAGACGCCAGT
B2M F (beta 2 microglobulin)	RT PCR	TGCTGTCTCCATGTTTGATGTATCT
B2M R	RT PCR	TCTCTGCTCCCCACCTCTAAGT
MEIS1 F	RT PCR	CAGAAAAAGCAGTTGGCACA
MEIS1 R	RT PCR	TCATGCCCATTCCACTCATA
HOXA9 F	RT PCR	TCCCACGCTTGACACTCACACTTT
HOXA9 R	RT PCR	AGTTGGCTGCTGGGTTATTGGGAT
HOXC8 F	RT PCR	TGGAAACCTGAAGGAGATGTGGGT
HOXC8 R	RT PCR	AAACAGGGAAGGAGAGGAAGGCAT
CYP33 F	RT PCR	TCCAGGCCAGTTTGGTCAGATGAT
CYP33 R	RT PCR	CCGGCTTGTTCCCAATCTTGATGT
MLL F	RT PCR	ATGTGGAAAGTGTGATCGCTGGGT

MLL R	RT PCR	TGCCGCTCAGTACAGTTCACACAA
CDNK1A(P21) F	RT PCR	GCAGACCAGCATGACAGATTT
CDNK1A(P21) R	RT PCR	GGATTAGGGCTTCCTGGA
BMI1 F	RT PCR	CTGGTTGCCCATTGACAGC
BMI1 R	RT PCR	CAGAAAATGAATGCGAGCCA
CDNK1B(P27)F	ChIP	CCGGCTAACTCTGAGGACAC
CDNK1B(P27)R	ChIP	ATACGCCGAAAAGCAAGCTA
MYC F	ChIP	TCCGCCCACCGGCCCTTTAT
MYC R	ChIP	TCAGCGCGATCCCTCCCTCC
SIX1 F	ChIP	ACCAGGTTGCCAGATTCGTTGGC
SIX1 R	ChIP	TGCCATTACTCATGCCCTCAAAG
CCNA2 F (CyclinA2)	ChIP	CCTGCTCAGTTTCCTTTGGT
CCNA2 R (CyclinA2)	ChIP	ATCCCGCACTATTGAAATG
ACTIN F	ChIP	TGCACTGTGCGGCGAAGC
ACTIN R	ChIP	TCGAGCCATAAAAGGCAA
GAPDH F	ChIP	TACTAGCGGTTTTACGGGCGCACGT
GAPDH R	ChIP	TCGAACAGGAGGAGCAGAGAGCGA
B2M F (beta 2 microglobulin)	ChIP	AGACTTCCCAAATTTTGCCATCCTA
B2M R	ChIP	AAAGGCCTGAAATGTTAGTGTTGAGT
CYP33	ChIP	TGAACCAGGAGGACGGCGAG
CYP33	ChIP	TGAACCTTGGGGGCAAGTGG
MLL	ChIP	GCGTCCCGGGAACGTGTGTAA
MLL	ChIP	GATGGAGGCGTAGGGAGCCG
CYP33 Sal	Cloning	**GTCGAC**CGCCACCACCAAGCGCGTCTTGTA
CYP33 Kpn1	Cloning	**GGTACC**CACCCTCGGTGCTGCCTCCT

Mouse Primer table

Primer Name	**RT-PCR/ChIP**	**Sequence 5'-3'**
Actin F	RT-PCR	GGCTGTATTCCCCTCCATCG
Actin R	RT-PCR	CCAGTTGGTAACAATGCCATGT
Cyp33 F	RT-PCR	CATCAAGATTGGGAACAAGCCAGC
Cyp33 R	RT-PCR	AAGCTGCTTCCCTTGAAGCCAAAG
Mll F	RT-PCR	GCATCGATGACAACCGACAGTG
Mll R	RT-PCR	CACAGCCATATGCACATTCTTC
Cdnk1b(p27) F	RT-PCR	GAACTAACCCGGGACTTGGAGAAGC

Cdnk1b(p27) R	RT-PCR	TAACCCAGCCTGATTGTCTGACGAG
HoxA9 F	RT-PCR	TACTATGTGGACTCCTTCCTGC
HoxA9 R	RT-PCR	TTCCACGACGCACCAAACAC
Cyp33 F	ChIP	CCCGACCTAAGGGGAGGGGC
Cyp33 R	ChIP	AGGCCCAGCAAGCCCTCCAT
Mll F	ChIP	TTCCCTCCAGGCCCTGTCCG
Mll R	ChIP	TCGGCCTCTGCCACCTCCTC

Table Legend: RT PCR primers were designed spanning introns when possible to aviod DNA contamination. ChIP primers were designed to known promoters. For less established promoters the region upstream of the transcription start site was amplified.

CsA or 5-azacytadine treatment

MSA cells were treated with concentrations of 5-Azacytadine (5-Aza) for 72 hours, replacing the medium and 5-Aza every 24 hours. A concentration of 500 nM, 250nM, or 5μM 5-Aza was used. These concentrations were determined empirically by using serial dilutions starting from 500μM. Cells were treated at 90% confluency with 5-Aza. After 48 hours, cells were replated at 40% confluency. After 96 hours, cells were harvested and RT PCR was performed to determine mRNA levels of MLL target genes.

MSA cells and MEFs were treated with Cyclosporin A (CsA) (*Sigma*) to a final concentration of 5uM or 10μM for the indicated timepoints before cells were trypsinized and collected in *Trizol* Reagent for RNA isolation. Treatment with CsA to determine mRNA levels for MLL target genes independent of oscillation proceeded for 96 hours, with replacement of CsA every 24 hours.

Dectection of senescence associated β -galactosidase (SA β-gal)

X-Gal at pH 6 detects endogenous β-galactosidase and is used as a marker for cellular senescence.Cells were washed twice with PBS before being fixed in 2% formaldehyde and 0.2% glutaraldehyde diluted in PBS to cover the cells for 3-5 minutes. Afterwards the cells were washed with PBS to remove the fixing solution. Staining solution consisted of 1mg/ml X-Gal dissolved in dimethylformamide, 40mM of citric acid/sodium phosphate buffer at pH 6.0, 5mM potassium ferrocyanide, 5mM potassium ferricyanide, 150mM sodium cloride, and 2mM magnesium chloride. The pH of the citric acid/Na phosphate buffer is crucial for the dection of SA β-gal. Cells were incubated at 37^{o}C. in a non-CO_2 incubator. Blue color is detected after 12 to 16 hours after incubation. X-Gal at pH 6 detects endogenous β-galactosidase and is used as a marker for cellular senescence.

Proliferation Assay

MSA cells were transfected using *Lipofectamine 2000* with vector alone (pCEP4), pCEP4-CYP33, or pCEP4-CYP33 M for over-expression and siGFP (control) or with two siRNAs specific for CYP33-labeled siCYP33 (1) siCYP33 (2) that target the RRM domain. Twelve μg of DNA for over-expression or 100nM for siRNA were used. Cells were then counted and replated daily with fresh medium for 9 days (DMEM/F12).

BCA assay for protein

The *Pierce* BCA Protein Assay Kit was used to determine protein concentration for Western Blots and Co-IPs. A standard curve using BSA was created using varying concentrations ranging from 0μg to 80μg of protein and diluted to 100μL with H_20 into a

5mL Falcon polystyrene round bottom tube. Five µl of unknown protein sample was also added to a clean 5mL tube and diluted to 100µL with ddH_20. A master mix of the working reagent was made by the addition of 50:1 Reagent A to Reagent B and vortexed until well mixed. Two mL were then added to each standard and sample and the tubes were placed at 37^oC. for 30 minutes. After 30 minutes, samples were placed in a 1 cm cuvette and standards were read on a *Beckman* DU530 Life Science UV/Vis spectrophotometer at 562nm wavelength in order to create a standard curve. After the generation of a standard curve, samples were read.

Transfection of plasmids and siRNA

Transfection of plasmids and siRNA was conducted using *Lipofectamine 2000* reagent. Twelve µg of DNA or 100nM of siRNA were used for transfection. For transfection, 0.8 million cells were plated in a 60mm dish for siRNA, or 2 million cells for a 10cm dish in DMEM/12 complete (see cell culture section). When the cells were attached, 4µl *Lipofectamine 2000* for siRNA or 20µl for plasmids, along with the corresponding DNA, was added. For example, for pCEP4, CYP33, and CYP33 M, 12µg of DNA was added to 1.5mL DMEM/F12 in a 15ml Falcon tube. Twenty µl of *Lipofectamine 2000* was added to 1.5ml of DMEM/F12 in a 15ml Falcon tube. Both DNA sample and solution containing *Lipofecatmine 2000* were vortexed for 5 seconds. After 5 minutes, DNA and *Lipofectamine* were combined. The solution was vortexed for 5 seconds and samples were incubated at room temperature for 30 minutes before being added drop wise onto cells. After 12 hours, medium was replaced with fresh media.

Cell cycle analysis

MSA cells were removed from 10cm plates using Trypsin EDTA 0.05%. After centrifuging the cells at 2500 r.p.m. for 5 minutes, the trypsin solution was removed and cells were washed once with PBS. The cell pellet was loosened and 1mL of 70 % ice cold EtOH was added drop wise while vortexing. The cells were fixed for 30 minutes on ice. The cell pellet was then centrifuged at 2000 r.p.m. for 5 minutes. The pellet was again washed with 2mL of PBS and centrifuged at 2000 r.p.m. The supernatant was removed and 0.25mL of RNAse A in PBS was added (final concentration of 20μg/mL). Cells were incubated at 37^{o}C. for 15 minutes. The pellet was then cooled down for 5 minutes. After this cool down period, 0.25mL of PI in PBS (final concentration of 50μg/mL) was added. The tube was wrapped in foil and kept dark for 30 minutes. Flow cytometry of the PI-stained cells was performed and DNA content was determined.

CHAPTER IV

RESULTS

Modulation of cellular CYP33 concentration leads to a change in expression of MLL target genes, and a subsequent change in H3 acetylation

The cyclophilin domain of CYP33 contains an enzymatic activity that promotes isomerization of prolyl-peptide bonds between their cis and trans conformation: cis-trans prolyl isomerase (PPIase) activity. Both deletion of the cyclophilin domain or inhibition of the PPIase activity by Cyclosporin A (CsA) have been previously shown to render CYP33 incapable of reducing *HOXC8* transcription (Fair K, 2001; Anderson M, 2002; Park S, 2010). CsA treatment inhibits the recruitment of HDAC1 to the RD of MLL in the presence of over-expressed CYP33 (Koonce M, 2004). Due to the indiscriminate nature of PPIase inhibition of all cyclophilins by CsA, a more specific means of testing the function of the CYP33 PPIase activity was needed. Therefore a CYP33 PPIase mutant (CYP33 M) was created to test this hypothesis. CYP33 M was a mutation generated by Dr. Peter Breslin, S.J., in a plasmid encoding CYP33. The mutant CYP33 protein was shown to be enzymatically dead in a cis-trans prolyl isomerase activity assay (Breslin, P and Schultz, R *unpublished* results). CYP33 M contains mutations in two

residues lying in the active site of the cyclophilin domain that are critical for its enzymatic activity (R191A and F196A) (Fig 9). These amino acid residues were chosen for modification based on homology of the amino acid sequence of the CYP33 cyclophilin domain to that of cyclophilin A (CYPA). Mutations of the corresponding residues in CYPA render the protein enzymatically inactive, retaining less than 1% of wild-type activity (Zydowsky, L 1992).

In order to confirm that CYP33 was overexpressed or knocked down, the protein and mRNA levels of CYP33 in MSA cells were tested by Western Blot and quantitative PCR respectively. A six-fold increase or reduction of CYP33 protein levels was observed based on densitometric analysis, compared to ACTIN protein levels (Figs. 6A, 6B). The Western Blot showed a similar trend to that of the CYP33 mRNA levels (Figs. 6C, 6D). Knockdown of CYP33 in MSA cells leads to increased expression of *MLL* target genes *CDNK1B*, *MYC*, and *SIX1*, whereas over-expression of CYP33 leads to a decrease in *MLL* target gene expression (Figs. 6E, 6F). Surprisingly, the expression of the housekeeping genes ACTIN and GAPDH, which are not known targets of MLL, also increased upon CYP33 knockdown, and were repressed upon CYP33 over-expression (Figs 6E, 6F).

ChIP was conducted in order to determine if, upon knockdown of CYP33, changes occurred in histone modifications, H3ac (K9ac and K14ac), H3K27ac and H3K27me3 (Figs. 7, 8) at the chromatin of the affected genes. In order to obtain a more accurate picture of how the histone modification marks change upon over-expression or

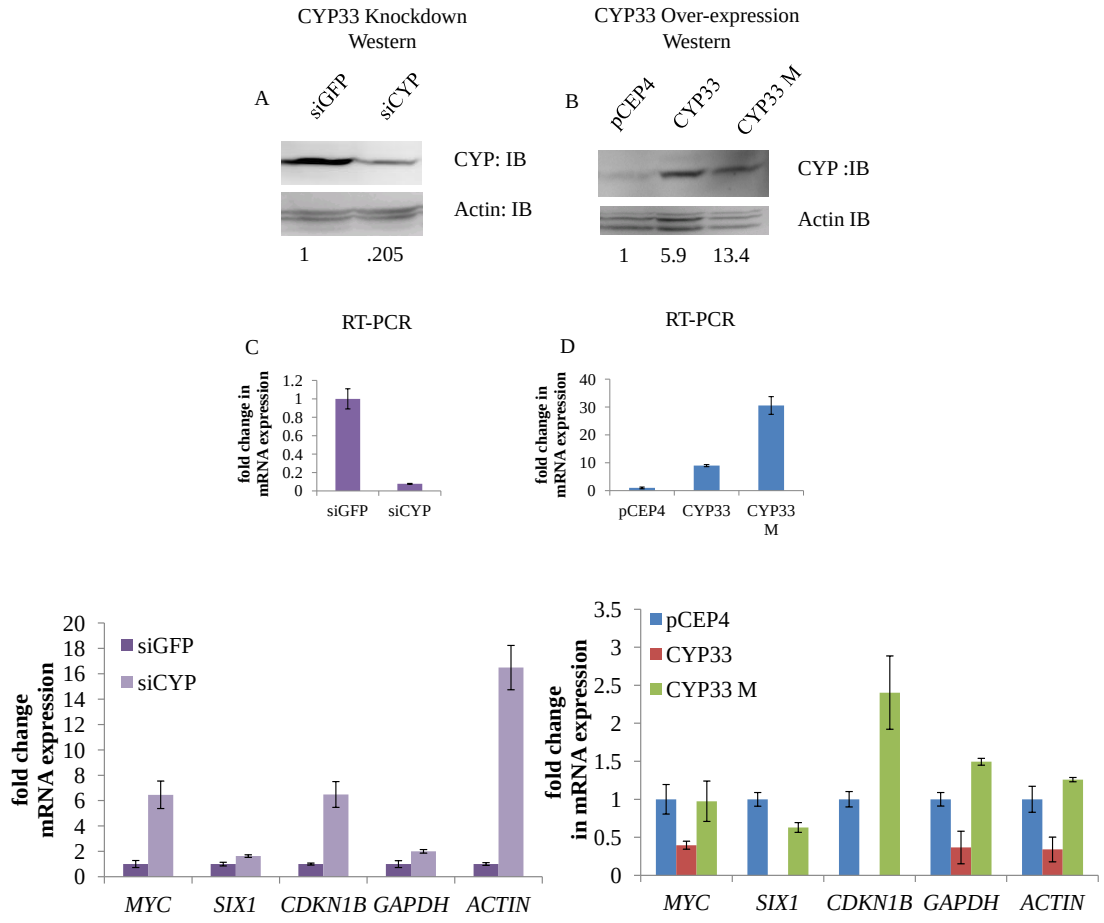


Figure 6. Modulation of CYP33 levels leads to changes in MLL target gene expression.

Either siGFP, siCYP33(2) (A,C,E) or pCEP4, pCEP4-CYP33, or pCEP4- CYP33 M (Table 3) (B,D,F) were transfected into MSA cells for 36 hours (siRNA), or 48 hours (CYP33 over-expression). RT-PCR and Western Blots were used to confirm CYP33 knockdown or CYP33 over-expression. Relative band intensity estimated by average densitometry is shown below the Western blots and represents three independent experiments. Real time RT-PCR was conducted to determine mRNA levels in over-expressed or knocked down cells. Standard deviations are from triplicate real-time PCRs.

knockdown of CYP33, the marks were normalized first to total H3 levels before the modifications are compared between the controls and treatments. Two acetylation antibodies were used: a pan acetylation antibody (H3ac) which recognizes histone tails with the dual marks of H3K9ac and H3K14ac, and an antibody against H3K27 acetylated histone tails.

Upon over-expression of CYP33, H3K27ac decreases at the *CDNK1B, MYC, SIX1* and *ACTIN* promoters (Fig. 7C). At the *MYC*, *CDNK1B*, and *GAPDH* promoters, over-expression of CYP33 M does not significantly decrease H3K27ac compared with the vector control indicating that the PPIase activity is important for reduction of H3K27ac (Fig. 7C). Upon over-expression of CYP33 M in MSA cells, there is a decrease in H3K27ac at the *SIX1* and *ACTIN* promoters compared to the vector control, but not as strong a decrease as upon CYP33 wild-type over-expression (Fig. 7C). Upon knockdown of CYP33, *MYC*, *SIX1*, *P27*, and *ACTIN* promoters show a significant increase of H3K27ac. Only H3K27ac at the *GAPDH* promoter doesn't increase. This lack of increase may be due to an already high level of H3K27ac at the *GAPDH* promoter. Therefore, further inhibition of CYP33, by knockdown does not increase H3K27ac at the *GAPDH* promoter. For all promoters tested, H3K27me3 does not significantly change upon CYP33 over-expression or knockdown (Figs. 7B, 7D). In MSA cells at the promoters tested, the levels of H3K27me3 is very low comparable to the IgG control.

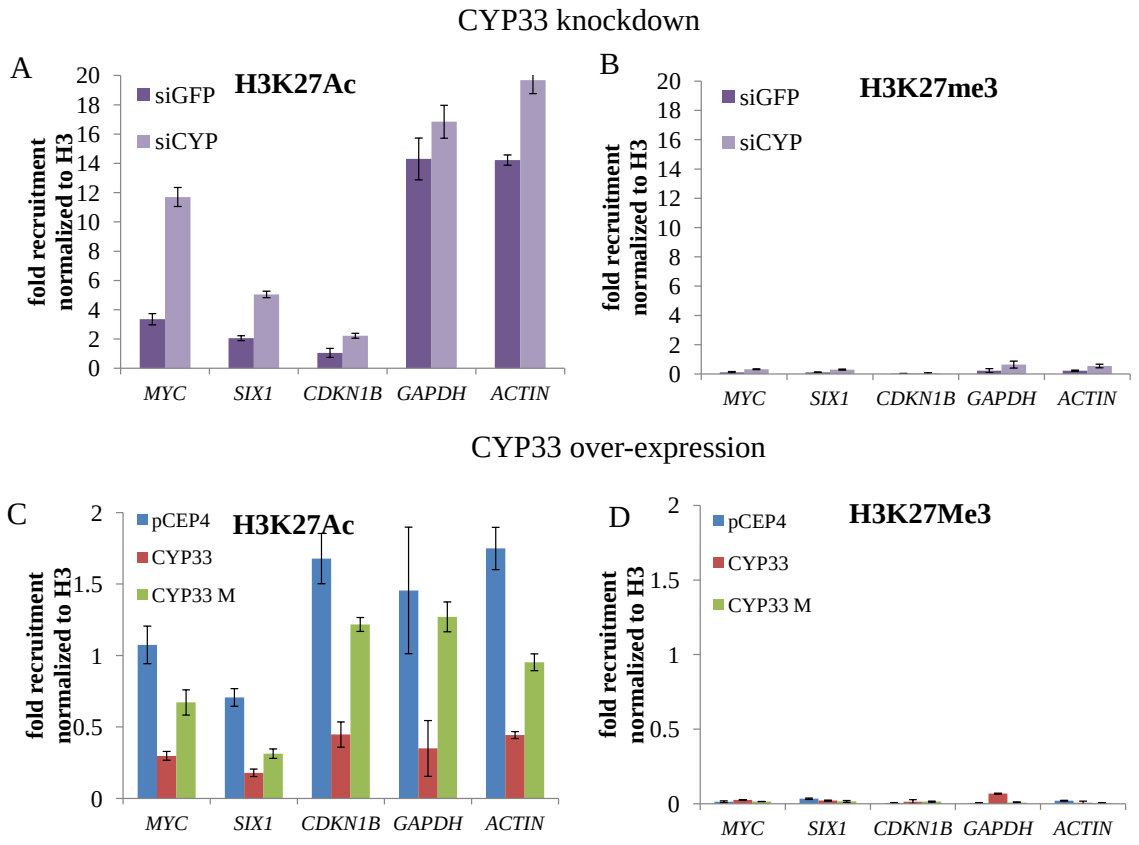


Figure 7. Modulation of CYP33 levels change H3K27ac at several gene promoters while H3K27me3 remains low.

(A,B) siGFP, siCYP33(2), or (C,D) pCEP4, pCEP4-CYP33, or pCEP4-CYP33 M were transfected into MSA cells. ChIP was conducted in MSA cells 36 hours after transfection for siRNA or 48 hours after transfection for CYP33 over-expression, as this was when it was observed that knockdown or over-expression led to a increase/decrease of MLL target genes. ChIP was conducted on MSA samples using H3K27ac or H3K27me3 specific antibodies. H3K27ac level change at the *GAPDH* promoter does not reach significance upon CYP33 knockdown. Shown is one of three representative experiments, and error bars shown are standard deviations +/- of triplicate PCRs.

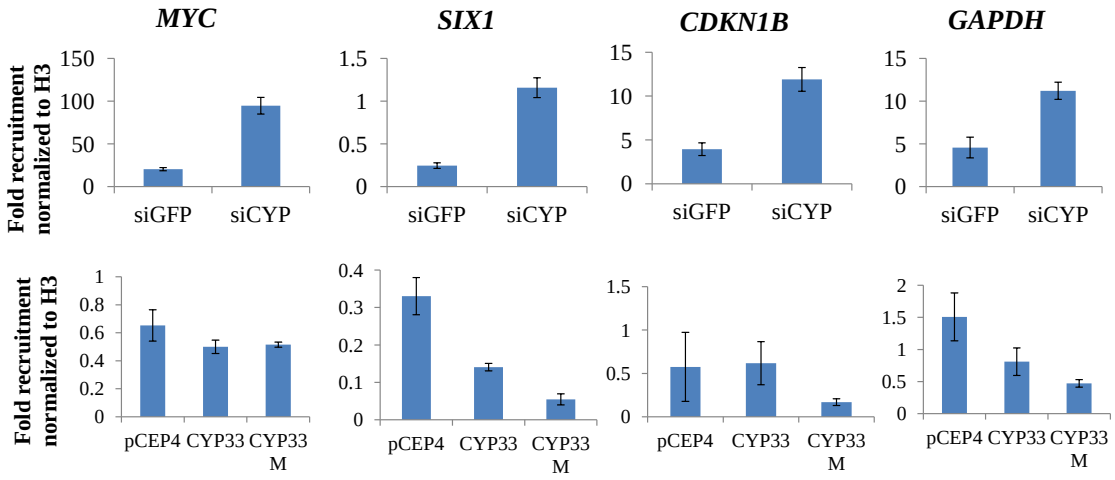


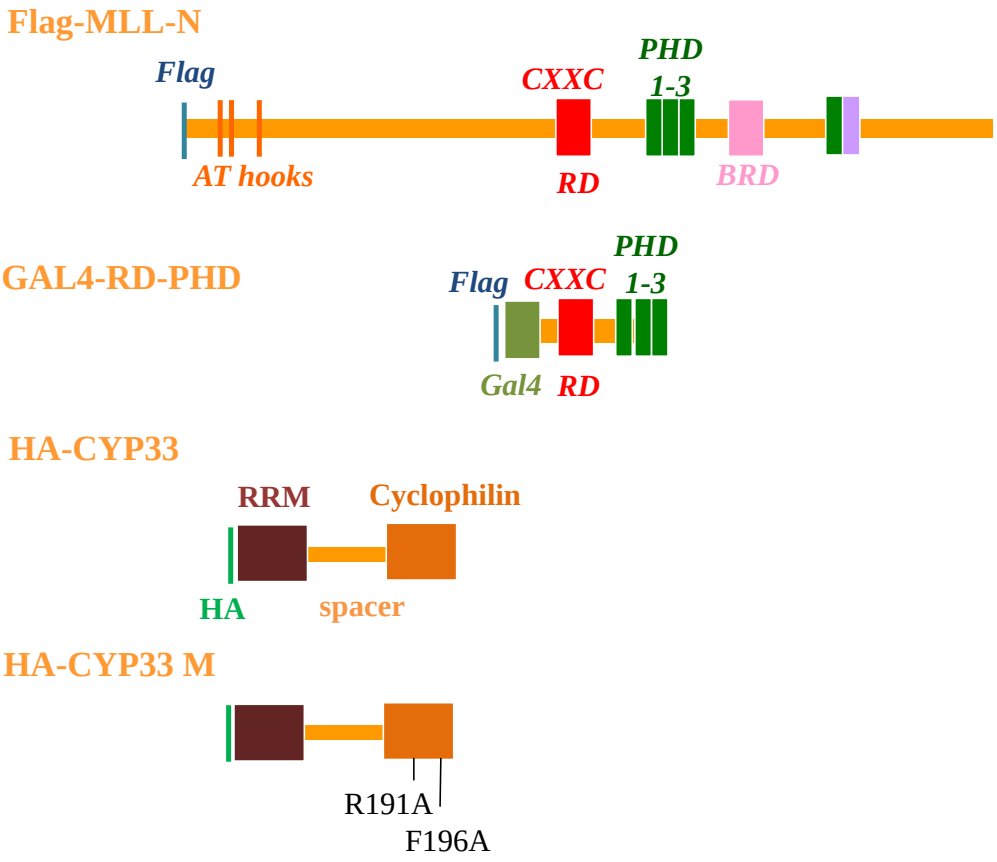
Figure 8. Modulation of CYP33 levels also leads to changes in H3ac levels. siGFP, siCYP33(2), pCEP4, pCEP4-CYP33, or pCEP4-CYP33 M were transfected into MSA cells similar to Figs. 11 and 12. ChIP was conducted in a similar manner to Figure 7 but using α-H3ac (H3K9 and H3K14) antibody. Shown is a representative of two independent experiments.

Global H3ac (H3K9, H3K14) was also tested to determine if it decreases with CYP33 over-expression as was shown in previous reports (Wang, Z 2010, Park, S 2010). H3ac, like H3K27ac, increased upon the knockdown of CYP33 (Figs. 7A, 8A). Upon CYP33 over-expression, H3ac decreases slightly or remains the same. CYP33 and CYP33 M overexpression reduces H3ac at the *SIX1* and *GAPDH* promoters, which is not in accordance with the RT-PCR data obtained. H3ac is not as changed upon CYP33 over-expression as H3K27ac (Figs. 8F, 10B). Upon knockdown of CYP33, however, H3ac shows a greater increase than H3K27ac at *CDKN1B*, *MYC*, *SIX1*, and *GAPDH* promoters (Figs. 7, 8).

Over-expression of CYP33 increases recruitment of HDAC1 and BMI1 to the RD domain of MLL in a PPIase-dependent manner

In order to test the interaction of MLL with CYP33 and its dependence on the CYP33 PPIase activity, a Co-IP experiment using Flag tagged MLL-N, and HA tagged CYP33 and CYP33 M was performed. Domain maps of the CYP33, CYP33 M, and MLL constructs that were used in the CoIP experiments are shown (Fig. 9A).

Before conducting the CoIPs, the abilities of CYP33 and CYP33 M to repress the MLL target genes *HOXC8* and *MYC* were again confirmed in the HEK293T cell line by over-expressing CYP33 or CYP33 M and conducting real time RT-PCR to measure *HOXC8* and *MYC* mRNA levels (Fig. 9B). Over-expression of CYP33 can down-regulate *MYC* and *HOXC8* transcription (Fig 9B). Upon over-expression of CYP33 M, *HOXC8* mRNA is expressed at a level close to that of the vector alone, whereas *MYC*


Flag-MLL-N
Flag
AT hooks
CXXC
RD
PHD
1-3
BRD
GAL4-RD-PHD
Flag
CXXC
PHD
1-3
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RD
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Cyclophilin
HA
spacer
HA-CYP33 M
R191A
F196A

B

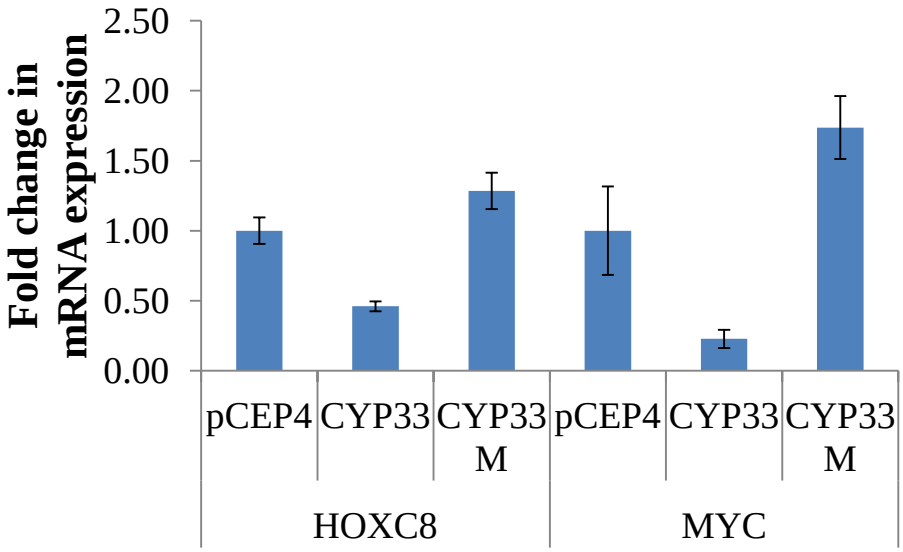

Fold change in mRNA expression
2.50
2.00
1.50
1.00
0.50
0.00
pCEP4
CYP33
CYP33 M
pCEP4
CYP33
CYP33 M
HOXC8
MYC

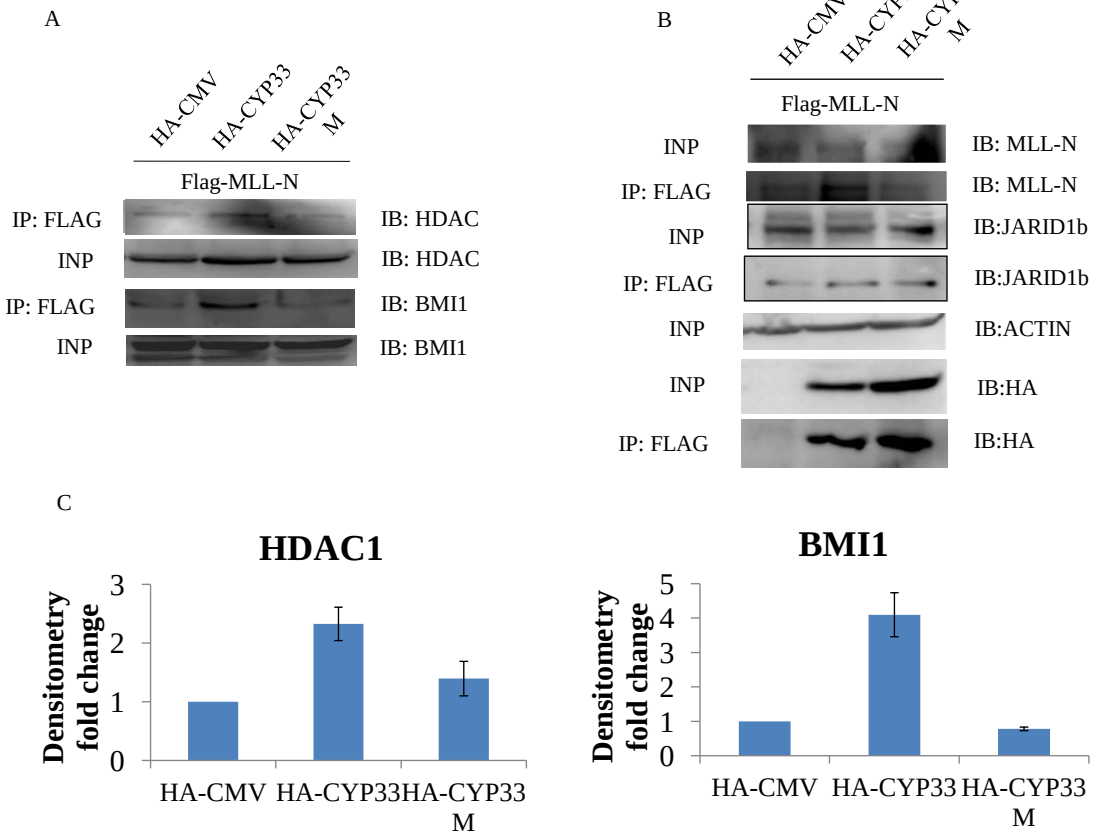
Figure 9. The PPIase activity of CYP33 is necessary to decrease the expression of MLL target genes *HOXC8* and *MYC* upon CYP33 over-expression.

A) Schematic of the over-expression constructs used in Figures 10 and 11. CYP33 contains two domains, an RRM domain and a cyclophilin domain, separated by a spacer that is evolutionarily conserved. The MLL protein contains many domains including but not limited to the repression domain colored red and the PHD fingers shaded in green. The PHD fingers are separated by an atypical bromodomain (pink). The GAL4 construct contains only the repression domain (RD) and the first three plant homeodomain fingers (PHD fingers) amino acid residues 1088-1371 and 1394-1600. B) pCEP4 vector, pCEP4-CYP33 or pCEP4-CYP33 M was transfected into 293T cells. RNA was extracted 48 hours after transfection and real-time PCR was performed using the ΔΔCt method using B2M as a control. *HOXC8* and *c-MYC* are two representative MLL target genes that show down-regulation upon CYP33 over-expression, but they are not down-regulated by CYP33 M over-expression. Error bars show +/- standard deviation for triplicate PCRs.

mRNA appears to have increased above that of the vector alone (Fig. 9B) suggesting that CYP33 M may act in a dominant negative manner by replacing the endogenous CYP33 in its complex with MLL. These results confirm previous published data, which conclude that the PPIase activity of CYP33 is necessary for MLL target gene repression (Anderson, M 2001, Fair, K 2002, Xia, Z 2003, Park, S 2010, Wang, Z 2010).

Co-IP experiments were also performed to determine whether the recruitment of BMI1 and HDAC1 proteins to MLL is dependent on the PPIase activity of CYP33. A plasmid expressing Flag-tagged MLL-N (amino acid residues 1-2665) was transfected together with an empty vector (HA-CMV), or expression plasmid constructs for HA-CYP33 or HA-CYP33 M in HEK293T cells. Flag-tagged MLL was immunoprecipitated, and the precipitate probed using α-HDAC1 or α-BMI1 antibodies in immunoblots. As is shown in Fig. 10A, CYP33 over-expression enhances the recruitment of HDAC1 and BMI1 to MLL-N (Fig. 10A). These results confirm previous findings that CYP33 enhances HDAC1 recruitment to MLL (Xia, Z 2003) and extends these observations to BMI1. Jarid1b, an H3K4me3 demethylase, and MLL were also blotted after immunoprecipitation (Fig. 10B). Interestingly, Jarid1b was also shown to bind to MLL (Fig. 10B). However, Jarid1b recruitment to MLL-N, did not increase upon CYP33 over-expression.

By over-expressing either CYP33 or CYP33 M, it is possible to determine if the recruitment of CYP33 is dependent upon the cis-trans prolyl isomerase activity of


A
HA-CMV
HA-CYP33
HA-CYP33 M
Flag-MLL-N
IP: FLAG
IB: HDAC
INP
IB: HDAC
IP: FLAG
IB: BMI1
INP
IB: BMI1
B
HA-CMV
HA-CYP33
HA-CYP33 M
Flag-MLL-N
INP
IB: MLL-N
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IB: MLL-N
INP
IB:JARID1b
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HDAC1
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0
1
2
3
HA-CMV
HA-CYP33
HA-CYP33 M
BMI1
Densitometry fold change
0
1
2
3
4
5
HA-CMV
HA-CYP33
HA-CYP33 M

Figure 10. Recruitment of HDAC1 and BMI1 to the MLL N is increased by CYP33 over-expression in a PPIase dependent manner.

A) pIC-MLL N-terminal construct, along with either HA-CMV (vector), HA CYP33 or HA CYP33 M was transfected into 293T cells. MLL-N is flag-tagged at its N-terminal end (Figure 9). Forty-eight hours after transfection, cells were harvested and lysed. Input (INP) blots are indicated to distinguish them from immunoprecipitated blots (IP:FLAG). (IB) Immunoblot denotes the antibody used in western detection The HDAC1 and BMI1 blots are representative of three independent experiments. B) Flag pulldowns followed by blotting for HA was conducted to shown equal recruitment of HA-CYP33 and HA-CYP33 M to the MLL PHD3. C) Quantification of HDAC1 in input versus HDAC immunoprecipitated was conducted using *Multigauge* Software that is a companion to the *FujiFilm* detection device. Error bars are standard deviations and the p value between CYP33 and CYP33 M is highly significant for both HDAC1 ($p=.0169$) and BMI1 ($p=.0009$) using the Student t test.

CYP33. Binding of CYP33 M to MLL-N is similar to the binding of CYP33 as shown by HA antibody (Fig. 10B). The recruitment of either HDAC1 or BMI1 to MLL-N is not increased when CYP33 M is over-expressed, compared with expression of the vector control. The relative amounts of HDAC1 and BMI1 precipitated with MLL-N were quantified from three independent experiments (Fig. 10C). CYP33 increases HDAC1 and BMI1 recruitment to MLL-N in a PPIase dependent manner.

In a recent paper (Wang, Z 2010), it was proposed that isomerization of a prolyl bond residue (H1628-P1629) that lies between the PHD fingers and the bromodomain of MLL, is necessary for the bromodomain to move away from the CYP33 biding site on the MLL PHD3. If this hypothesis is correct, the CYP33 PPIase activity may only be necessary for binding of CYP33 to MLL-N, but may not be necessary for recruiting HDAC1 and BMI1 to MLL. In order to determine if the CYP33 PPIase activity is necessary to recruit HDAC1, or BMI1 to the RD of MLL independently of the bromodomain, a construct that contains only the RD and the PHD fingers, but not the bromodomain, was used for Co-IP. This construct N- terminal tagged with a Gal4 domain was used to test the recruitment of HDAC1 and BMI1. In the absence of the bromodomain, HDAC1 recruitment still requires the PPIase activity of CYP33, whereas BMI1 binding to MLL no longer requires the CYP33's PPIase activity (Fig. 11A).

Even when using the smaller construct MLL RD-PHD, HDAC1 bind MLL in a PPIase-dependent manner, suggesting that the PPIase target is either within the RD, the PHD finger domain, or HDAC1 itself. This confirms previous data which showed that

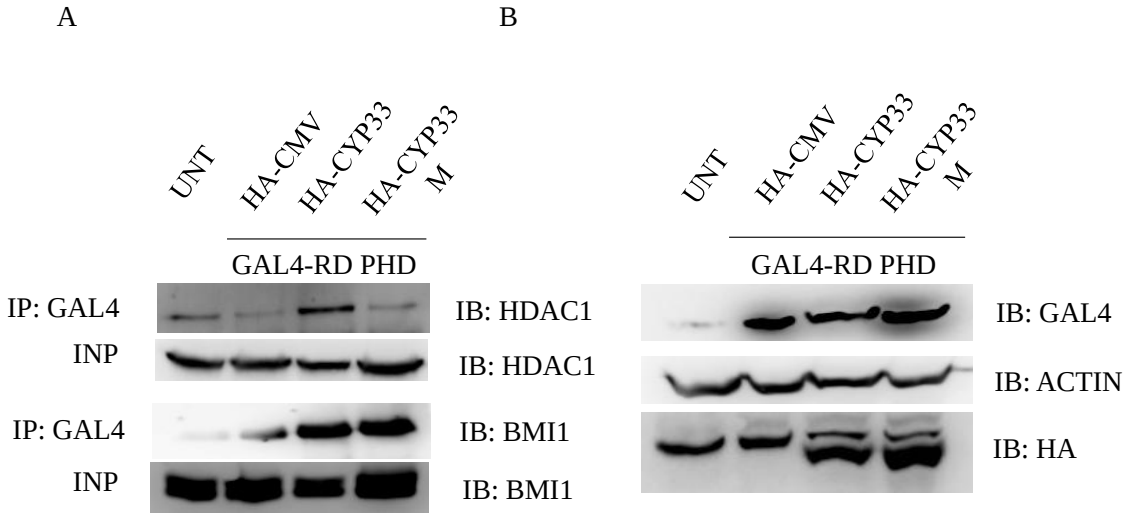


Figure 11. CYP33 over-expression results in recruitment of HDAC1 and BMI1 to the RD domain of MLL.

A, B) The GAL4-FLAG RD PHD , along with either HA-CMV (vector), HA-CYP33 or HA-CYP33 M was transfected into 293T cells. (UNT) denotes untransfected 293T cells. The GAL4-FLAG-RD PHD is tagged at its N-terminus (Figure 9). Forty-eight hours after transfection, cells were harvested and lysed. Input (INP) blots are indicated to distinguish them from immunoprecipitated blots (IP:GAL4). (IB) Immunoblot denotes the antibody used in western detection Anti-GAL4 was used for immunoprecipitation and either HDAC1 or BMI1 were immunoblotted. Representative of two independent experiments.

CYP33 overexpression was able to recruit HDAC1 to the RD-PHD finger domain (Xia, Z 2003). In contrast, BMI1 binding to the RD-PHD MLL no longer shows a dependence on the PPIase activity of CYP33, but is still increased upon CYP33 or CYP33 M overexpression (Fig. 11). This was surprising, as this suggests that for BMI1, another function of CYP33, separate from its PPIase activity is involved in regulating binding of BMI1 to the RD of MLL.

The recruitment of BMI1 and HDAC1 to chromatin at MLL target genes does not change upon CYP33 or CYP33 M over-expression

ChIP was used to test whether CYP33 promotes the recruitment of HDAC1 and BMI1 to the chromatin of MLL target genes (Fig. 12). Surprisingly, BMI1 and HDAC1 binding to the *MYC, CDNK1B,* or *SIX1* promoters does not change upon CYP33 or CYP33 M overexpression. The recruitment of HDAC1 and BMI1 is also not dependent upon the PPIase activity of CYP33, since at the *MYC, CDNK1B,* and *SIX1* promoters, the recruitment of these proteins after CYP33 M over-expression is very close to vector control. BMI1 binding does not change either upon CYP33 knockdown. HDAC1 recruitment on the other hand, is decreased upon knockdown of CYP33 at *MYC* and *CDNK1B* promoters, but not at *SIX1* promoters. This inconsistency between the Co-IP data and the ChIP data will be explained in more detail in the discussion section.

Knockdown of CYP33 by siRNA or PPIase activity inhibition by CsA, leads to oscillations in MLL target gene expression

During knockdown of CYP33 it was observed that, depending upon the timepoint at which the cells were collected after siRNA transfection, the expression of MLL target

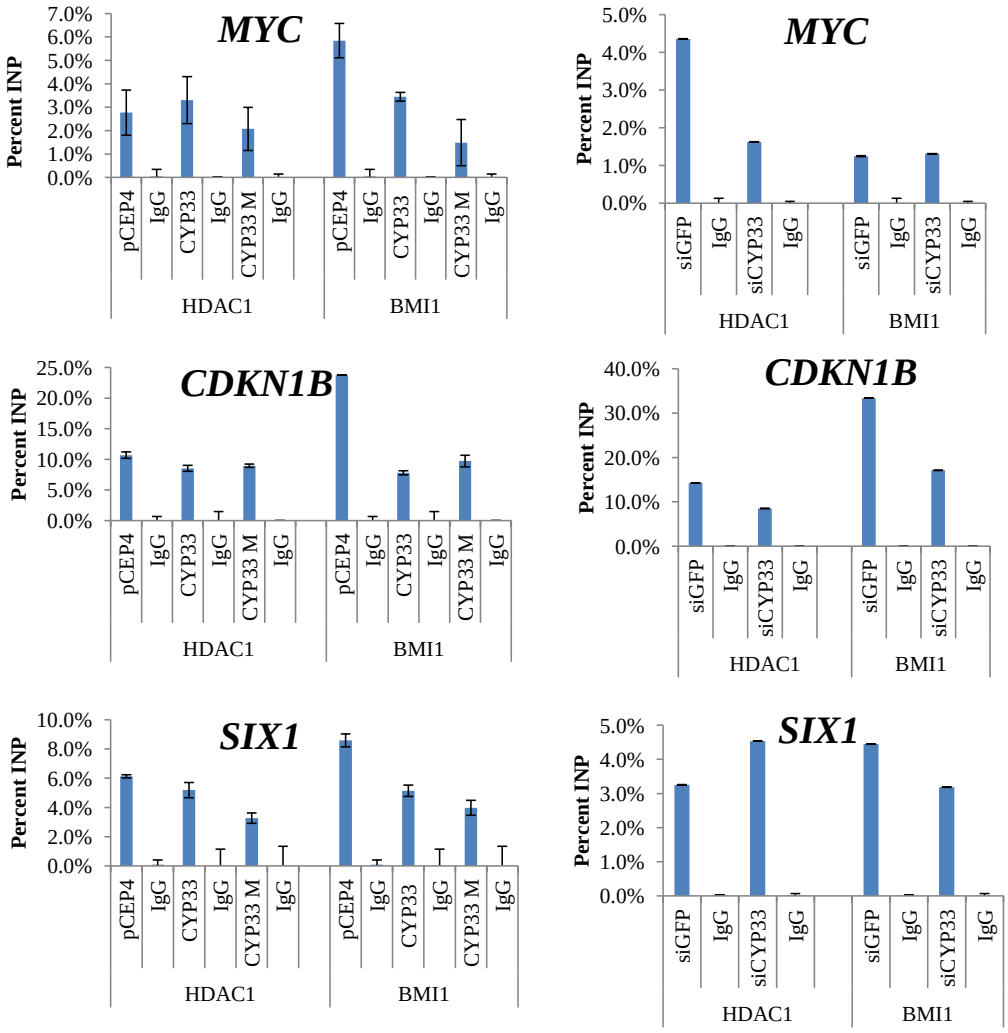


Figure 12. Recruitment of BMI1 and HDAC1 to the chromatin does not increase upon CYP33 over-expression.

CYP33 was either over-expressed or knocked down in MSA cells using the indicated plasmids as previously described in Fig 6. 48 hours after overexpression or 36 hours after knockdown ChIP was conducted to test for BMI1 or HDAC1 recruitment to the chromatin using α-HDAC, α-BMI1 or an antibody against mouse IgG as a negative control.

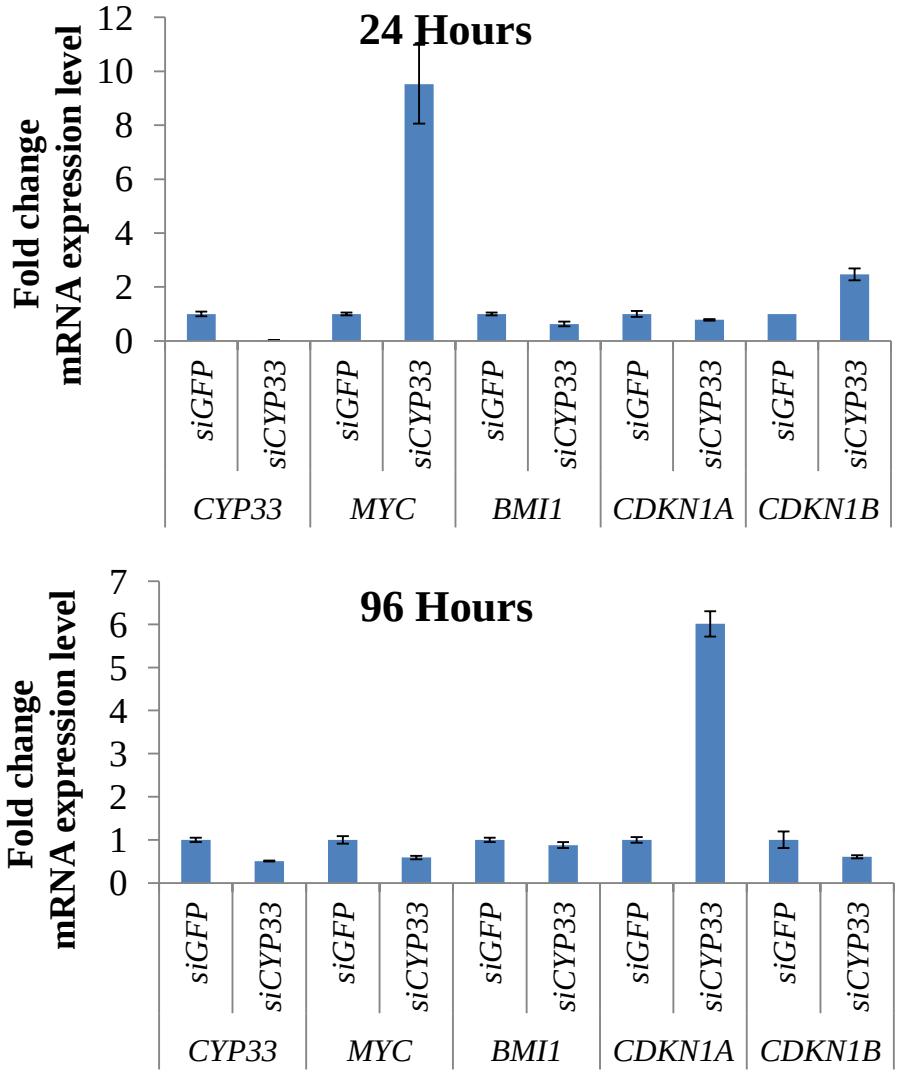


Figure 13. Knockdown of CYP33 leads to increased MLL target gene mRNA expression.

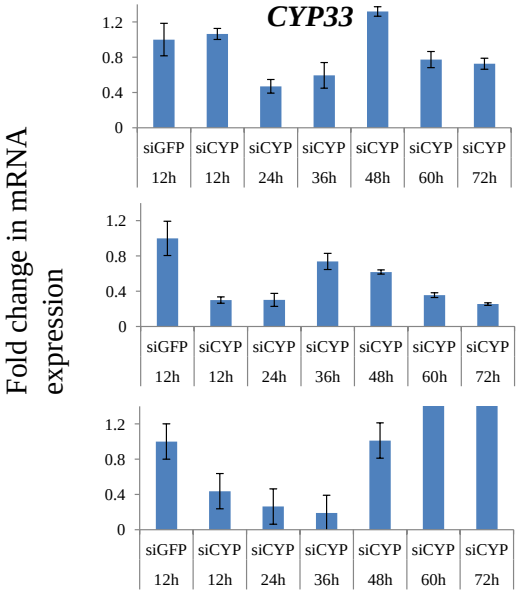
Following 24 hours or 96 hours after transfection of siGFP or siCYP33 were transfected as described in Fig 6, RNA was collected and real-time RT PCR was conducted to explore whether knockdown of *CYP33* by siRNA increases MLL target gene expression.

genes, including *CYP33*, was either increased as expected, or decreased, when compared to the control (Fig. 13). After 96 hours, *CYP33* is repressed two-fold, as are *MYC* and *CDNK1B*. Expression of *CDKN1A*, a gene repressed by MYC, is increased after 96 hours. This, along with inconsistencies in *CYP33* knockdown, led us to conduct a more detailed timecourse experiment to explore whether knockdown of CYP33 by siRNA led to oscillations in MLL target gene expression.

In three independent experiments, oscillations showed a regular period were observed in the expression of MLL target genes, but the periodicity of these oscillations differed in each experiment (Fig. 14). The more intense the knockdown of *CYP33*, as shown by RT-PCR, the longer the period and the higher the peaks and valleys of CYP33 and MLL target genes. After finding that there were oscillations in *CYP33*, *MLL* and *MYC* gene expression, their target genes expression was studied to determine whether it oscillated as well. As indicated in Fig. 14, both the expression of MLL target genes and c-MYC target genes, such as *BMI1* and *CDKN1A*, oscillate in response to changes in CYP33.

In order to test whether the inhibition of the PPIase activity of CYP33 also led to oscillations in gene expression, MSA cells were treated with CsA. A timecourse experiment was set up to determine the optimal time for CsA treatment of MSA cells (Fig. 15A). Based on data from the MEF cells, a concentration of 10 μM CsA was selected for the experiment as this gave the most robust increase of MLL target genes. The shortest time possible that yielded an increase in MLL target gene expression was

A


CYP33
Fold change in mRNA expression
1.2
0.8
0.4
0
siGFP 12h
siCYP 12h
siCYP 24h
siCYP 36h
siCYP 48h
siCYP 60h
siCYP 72h

B

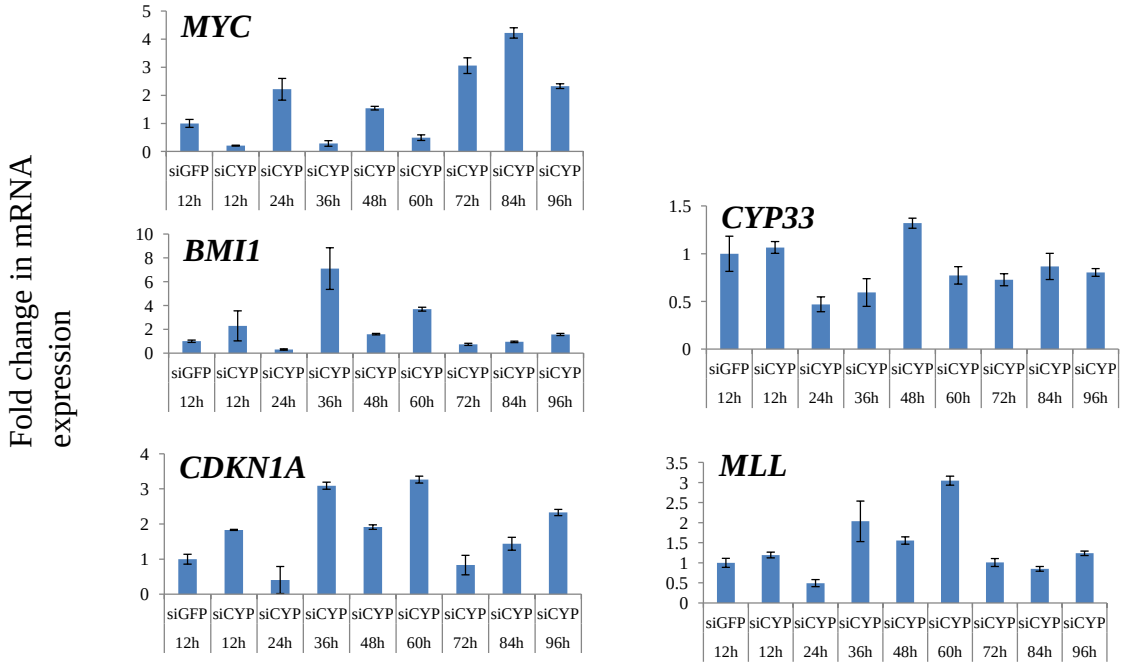

MYC
BMI1
CDKN1A
CYP33
MLL
Fold change in mRNA expression
siGFP 12h
siCYP 12h
siCYP 24h
siCYP 36h
siCYP 48h
siCYP 60h
siCYP 72h
siCYP 84h
siCYP 96h

Figure 14. Knockdown of CYP33 by siRNA leads to oscillations in expression of MLL target genes.

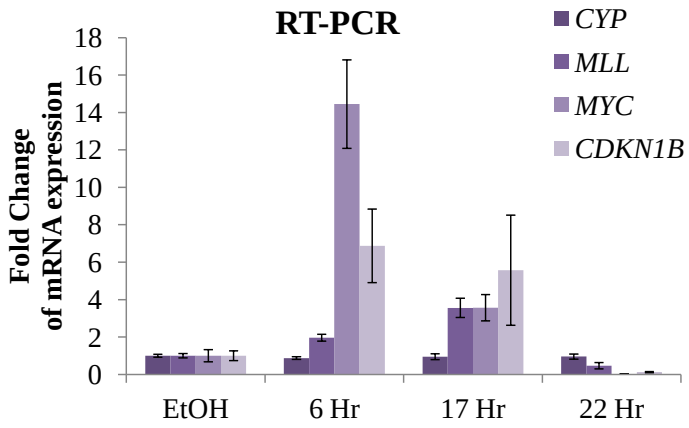
A) siGFP (control) or siCYP33(2) were transfected into MSA cells. The x-axis shows time in hours after transfection. At each timepoint, siGFP was set as 1. Results of three independent experiments are shown. B) The expression of target genes of MLL or c-MYC is shown for experiment 1 from part A. Time is indicated as hours after the start of transfection. Each time-point was normalized relative to siGFP at the corresponding time, although only siGFP at 12 hours is shown for simplicity. Shown is a representative of three experiments.

used. A short time was used because CsA is a general inhibitor of cyclophilins and has off-target effects that may influence the results. MLL target gene expression increased after only 6 hours of treatment, and this was the treatment time used in subsequent MSA treatments. After 6 hours, a 100-fold increase over the EtOH (control) was observed for *CDNK1B* gene transcription, and a 20-fold increase over control was seen for *MYC* gene transcription (Fig. 15B). The levels quickly diminished and returned to near control levels by 18 hours. After 42 hours, an additional increase of 10-fold was seen for *MYC* showing an oscillation of its mRNA transcription levels, with diminishing amplitude. The oscillations of *CDNK1B* differed in that after 30 hours, a robust decrease in *CDNK1B* was seen when compared to control. With time, *CDNK1B* levels recovered, and after 72 hours an increase is again seen compared to control.

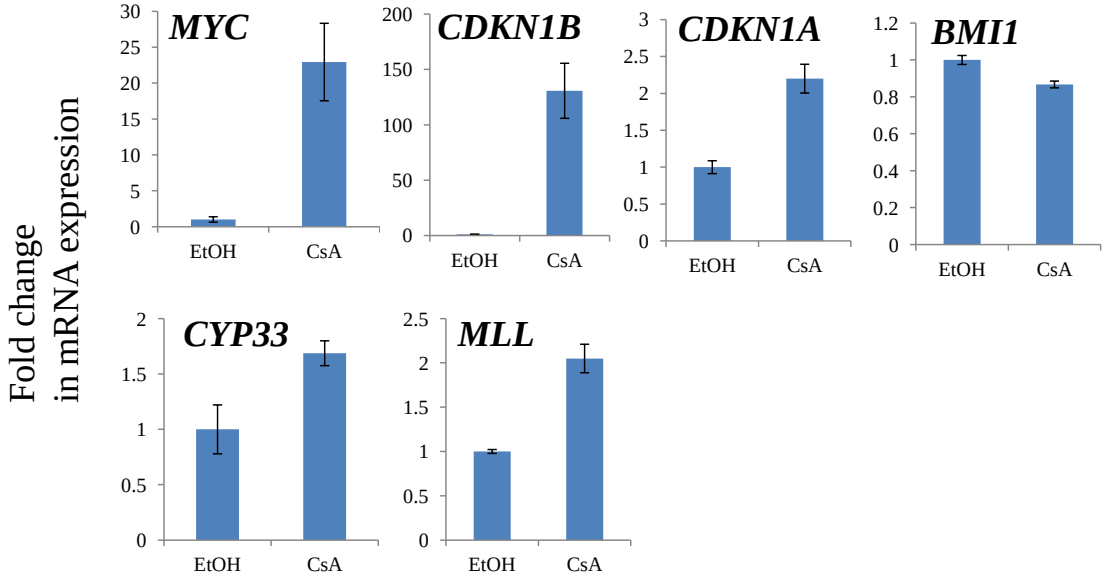
Both increase and decrease of CYP33 protein levels causes decreased cell growth in MSA cells.

It was observed that upon *CYP33* over-expression or knockdown, MSA cell growth was decreased. To quantify the decreased cell proliferation, viable cells were counted and *Cell Titre-Glo*, an assay to measure cellular ATP levels, was conducted. One hundred thousand cells were transfected with either CYP33, CYP33 M or 2×10^4 cells were transfected with siRNA to *GFP* or *CYP33*. Two separate siRNAs, targeting different nucleotide sequences of *CYP33* were used to discount off-target effects. Every other day cells were counted using Trypan Blue exclusion in order to determine live cell numbers, which were subsequently re-plated. As is shown, a decrease in cell growth was observed up to 5 days after transfection (Fig. 16A). After 5 days cells regained the ability

A


RT-PCR
CYP
MLL
MYC
CDKN1B
Fold Change of mRNA expression
18
16
14
12
10
8
6
4
2
0
EtOH
6 Hr
17 Hr
22 Hr

B


Fold change in mRNA expression
MYC
CDKN1B
CDKN1A
BMI1
CYP33
MLL
EtOH
CsA

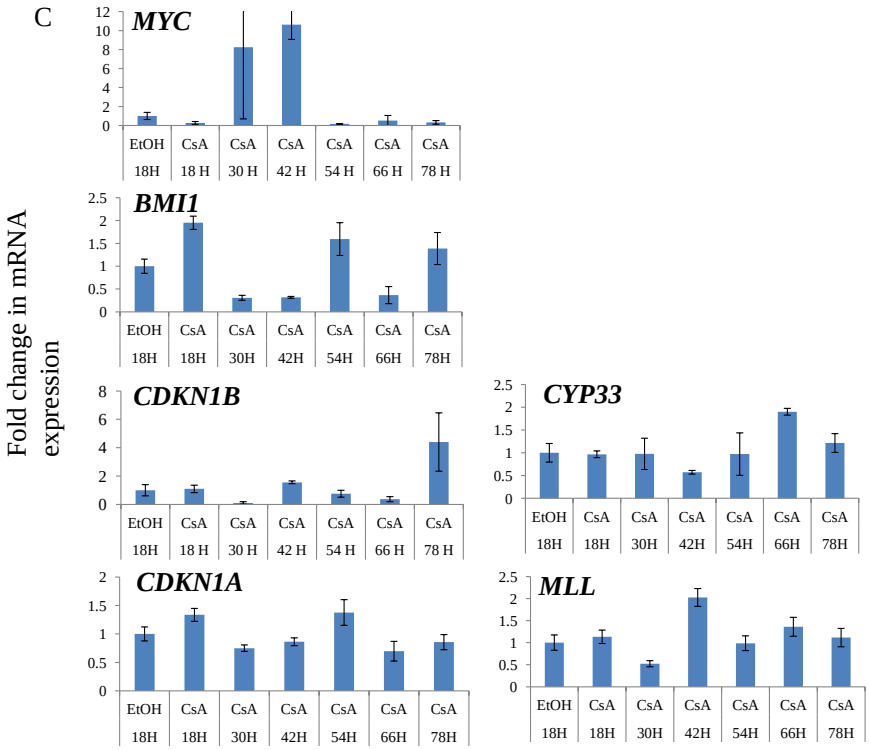


Figure 15. Treatment with CsA (a cyclophilin prolyl isomerase inhibitor) leads to oscillations in the expression of MLL target genes.

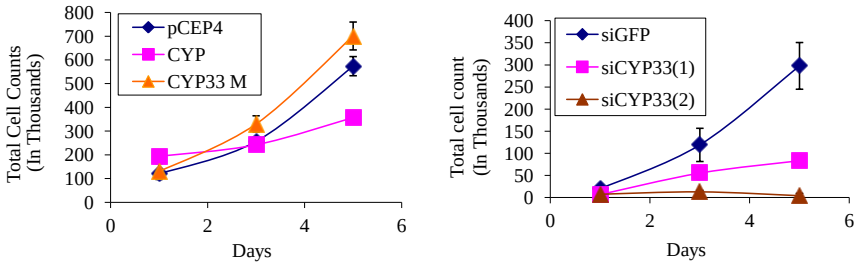
A) MSA cells were treated for the indicated periods of time with CsA, followed by RNA collection and RT-PCR. B) RNA was collected and RT-PCR was performed after 6 hours of CsA treatment. C) Cell were washed to remove CsA, and then collected every 12 hours, 18h, 30h, 42h, 54h, 66h, 78h. After collection, RNA was prepared and RT-PCR was conducted. CsA treatment was normalized to EtOH control at each timepoint, but only EtOH control at 18H is shown for simplicity. *c-MYC* and *CDNK1B* are *MLL* target genes. *BMI1* and *CDKN1A* are c-MYC target genes. Expression MLL and CYP33 were also shown to oscillate upon inhibition of CYP33 by CsA. This is a representative of two independent experiments.

to grow at a rate similar to control cells. Therefore, either over-expression of WT CYP33 or knockdown by siRNA causes decreased cell growth. In addition to cell growth, cellular morphology changed after CYP33 knockdown, with cells becoming flatter and having a greater number of projections (Fig. 16B).

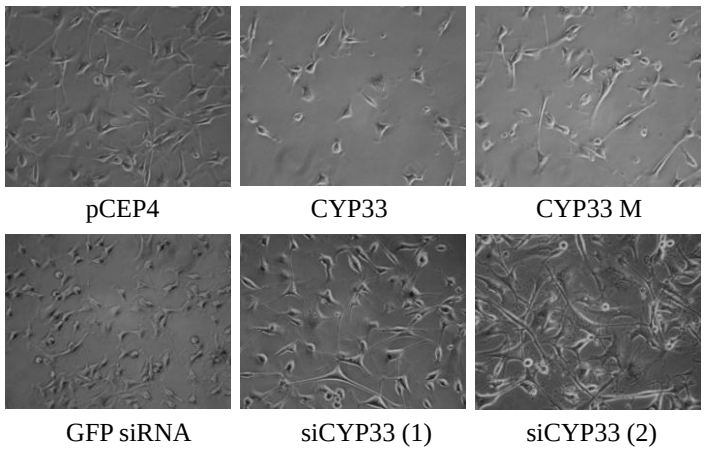
In order to further quantify this phenomenon which is also seen in 293T cells, MSA cells were transfected with either vector (pCEP4), CYP33, or CYP33 M expressing plasmids. Also, siGFP or siCYP33 were transfected into MSA cells. ATP detection was conducted using a *Cell Titre-Glo* assay according to the manufacturer's specifications. The *Cell Titre-Glo* assay is useful for estimation of ATP levels within the cell and thus can be used as a surrogate marker of cell viability and/or cell proliferation. A decrease in ATP levels was observed in cells over-expressing WT CYP33, but not in cells over-expressing CYP33 M (Fig. 16C). Increasing CYP33 protein levels by over-expression decreased ATP levels more than two-fold, whereas ATP levels remained the same upon CYP33 M over-expression. Upon CYP33 knockdown by siRNA in MSA cells, a slowdown in cell growth was also observed, but no change in ATP levels between siGFP and siCYP were observed (Fig. 16A).

Consequently CYP33 levels inversely correlate with ATP levels within a cell upon CYP33 over-expression. The decreased ATP levels correlated with changes in cell proliferation, both being dependent upon CYP33 PPIase activity. Upon knockdown of

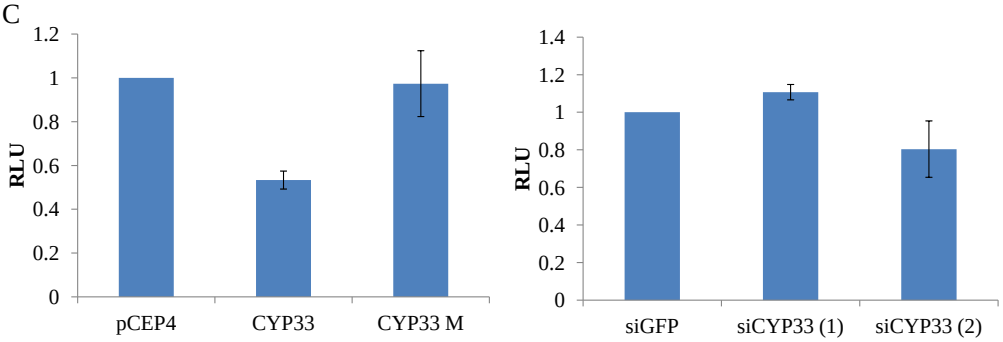
A


Total Cell Counts (In Thousands)
pCEP4
CYP
CYP33 M
Days
Total cell count (In Thousands)
siGFP
siCYP33(1)
siCYP33(2)
Days

B


pCEP4
CYP33
CYP33 M
GFP siRNA
siCYP33 (1)
siCYP33 (2)

C


RLU
pCEP4
CYP33
CYP33 M
RLU
siGFP
siCYP33 (1)
siCYP33 (2)

D

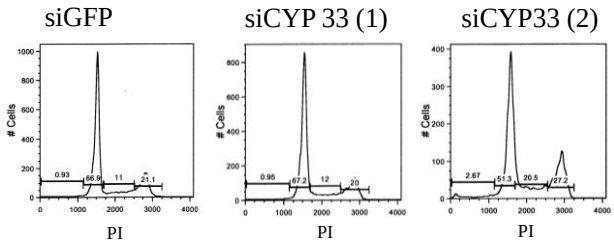

siGFP
siCYP 33 (1)
siCYP33 (2)
Cells
PI

Figure 16. Growth of MSA cells is sensitive to over-expression or down-regulation of CYP33.

A,B) Measurement of cell growth by counting cells upon CYP33 over-expression or knockdown, by two different siRNAs (siCYP33 (1), siCYP33 (2)). Also pCEP4, CYP33 or CYP33 M were transfected into MSA cells. Cells were plated in 3 separate plates and counted. Standard deviations are shown. C) *Cell Titre-Glo* assay (*Promega*). This assay assesses the level of ATP within the cell which is a surrogate marker of cell growth/death. As shown, knockdown does not change the levels of ATP within the cell, whereas over-expression of CYP33 decreases ATP levels. The standard deviation of three independent experiments is shown. D) PI staining followed by flow cytometric analysis.

CYP33 by siRNA, there is also a decrease in cell proliferation but no corresponding decrease or increase in ATP levels. A decrease in ATP levels is not likely due to apoptosis, as PI staining followed by FACS analysis showed no significant sub-G_1 cell population (Fig. 16D).

It was also observed that upon siRNA knockdown of CYP33, cells became flatter and larger, with longer projections, although this was not quantified. These are common characteristics of senescent cells. In order to test whether knockdown of CYP33 causes an increase in senescence, senescence associated β-galactosidase (SA β-gal) assay was performed. An increase from 1-2% of cells staining positive for β-galactosidase among siGFP control cells to 8-10% positive cells in siRNA to CYP33 was observed (Fig. 17). Shown below are representative fields (Fig. 17).

Density of nucleosomes depends on CYP33 levels in MSA cells

CYP33 over-expression leads to no change or a slight increase in the H3 ChIP signal at the promoters of the MLL target genes *CDNK1B* and *MYC*, as well as at the *GAPDH* promoter, whereas knockdown of CYP33 or over-expression of CYP33 M leads to decreased histone H3 occupancy at *CDNK1B*, *MYC*, *SIX1*, *GAPDH*, and *ACTIN*. This is possibly due to a dominant negative effect of the CYP33 mutant in which CYP33 M competes with CYP33 for binding to MLL target gene promoters. The H3 ChIP signal is a surrogate for nucleosome occupancy. Nucleosomes can either be displaced from promoters, by ATP dependent nucleosome remodeling complexes, or upon rounds of cell division can be replaced due to increased activity of gene transcription. CYP33 over-

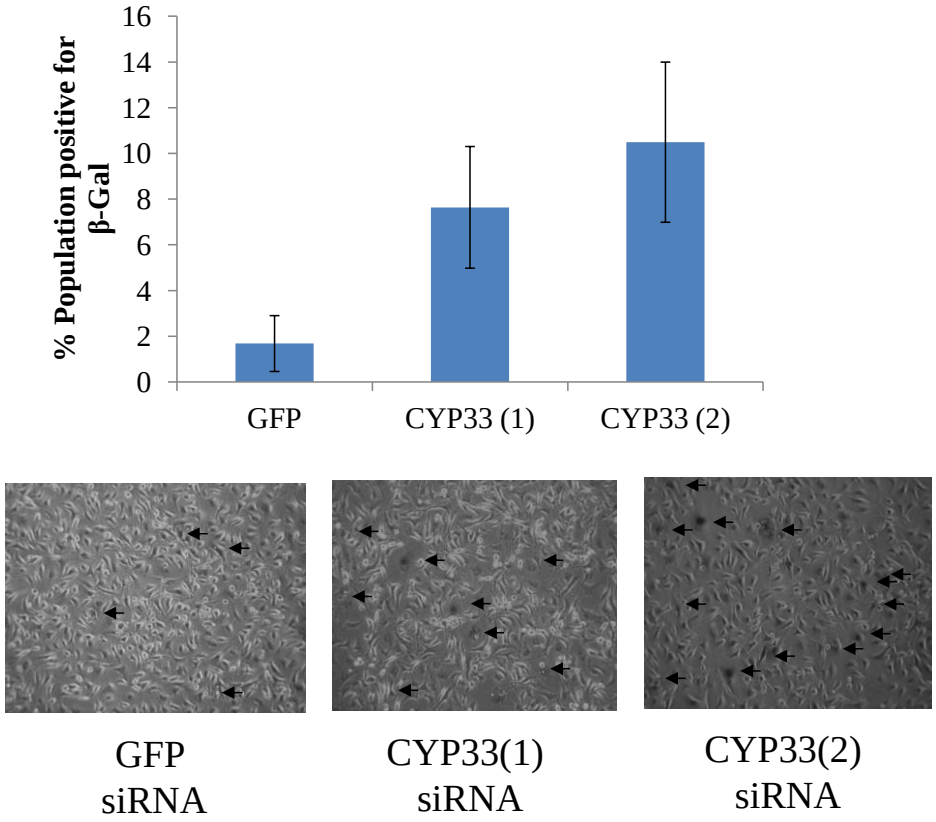


Figure 17. Knockdown of CYP33 leads to an increase in the senescence marker SA β-galactosidase.

Twenty-four hours after knocking down CYP33 using two separate siRNAs (CYP33(1) or CYP33(2) and using siGFP as a control, cells were incubated and treated with X-Gal as a substrate to examine senescence-associated β-galactosidase (SA β-galactosidase). Ten fields were counted for cells that were positive or negative for staining with X-Gal. The percent population staining positive is shown in the graph. The error bars are standard deviation from the ten fields tested. Arrows point to cells that stained positive.

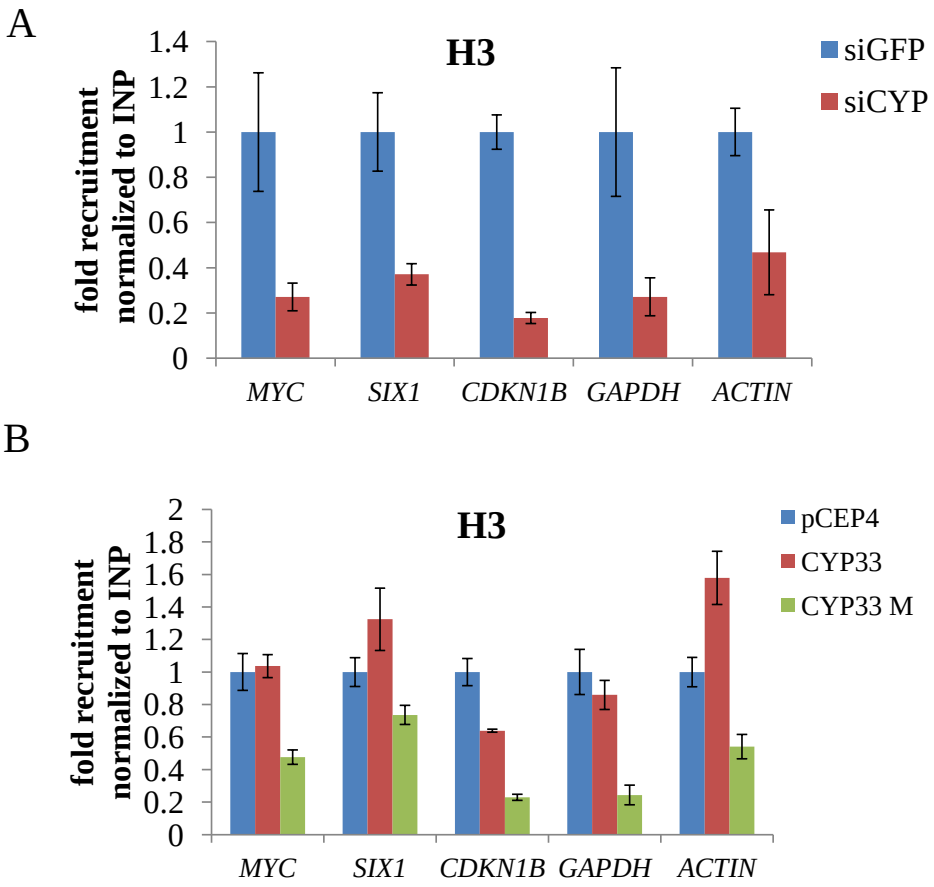


Figure 18. H3 levels at MLL target gene promoters change upon CYP33 knockdown, or CYP33 M over-expression.

After transfection of various plasmids as described in Figure 6, ChIP was conducted in order to determine Histone H3 levels as a surrogate marker for nucleosome density. Each transfection was normalized first to Input, and then vector control (siGFP, or pCEP4) was set as 1. An antibody directed against mouse IgG was used as a negative control, but is not shown. A) CYP33 knockdown using siRNA. B) CYP33 or CYP33 M over-expression in MSA cells. Shown is a representative of three experiments.

expression does increase H3 levels at the *SIX1* and *ACTIN* gene promoters. When comparing the effects of CYP33 to CYP33 M over-expression, there is a significant change in H3 levels at all gene promoters tested (Fig. 18).

Expression of housekeeping genes changes upon CYP33 knockdown or over-expression

In order to accurately measure the mRNA levels traditionally reference genes like *GAPDH* and *ACTIN* are used. However, the standard reference genes *GAPDH* and *ACTIN* both changed more than four fold over many different experiments (Fig. 19) after over-expression or knockdown of CYP33. Other standard reference genes described in the literature were tested (Chen, D 2011). Transcription of a panel of reference genes, *TCBP*, *RNU47*, *RNU48*, *GAPDH*, and *ACTIN*, also showed variations upon either CYP33 over-expression or knockdown (Fig. 19) (Chen, D 2011). Beta 2 microglobulin (B2M), a commonly used reference gene, was also tested. B2M, an MHC class I molecule, upon CYP33 over-expression showed the smallest variation and was consequently used as the reference gene in this study (Fig. 19).

In addition to changes in *CDNK1B* and *MYC* gene expression, the mRNA levels of *MLL* target genes, *ACTIN* and *GAPDH* relative to *B2M* also increases upon knockdown of CYP33 by siRNA (Fig. 6). H3K27ac is increased at *ACTIN* and *GAPDH* promoters, but not as much as at *CDNK1B*, *MYC*, or *SIX1* promoters, and the increase in H3K27ac at *GAPDH* is not significant (Fig. 7). This may be due to an already high level of H3K27ac at the *ACTIN* and *GAPDH* promoters.

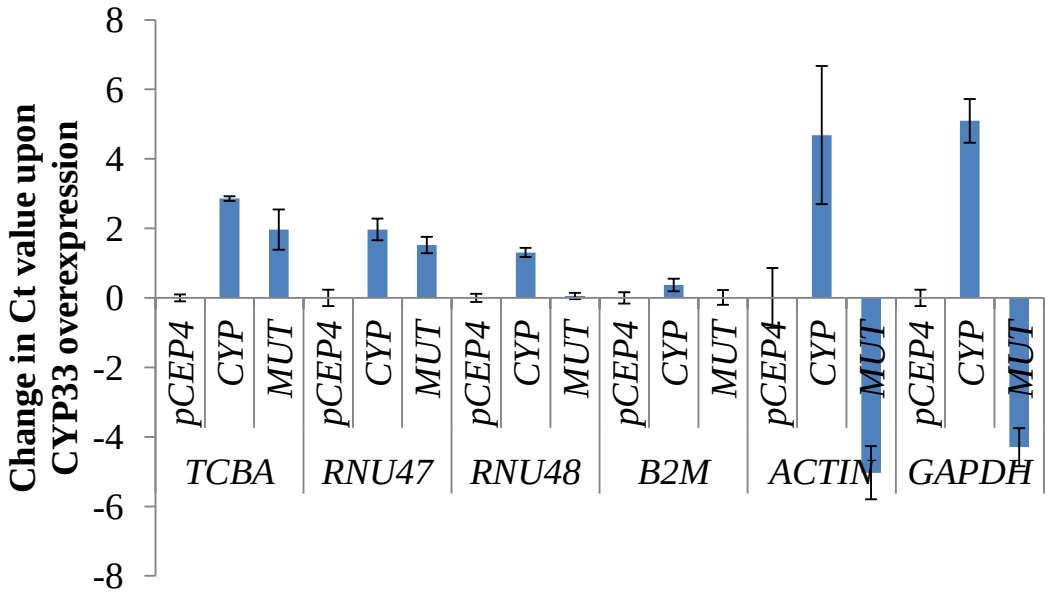
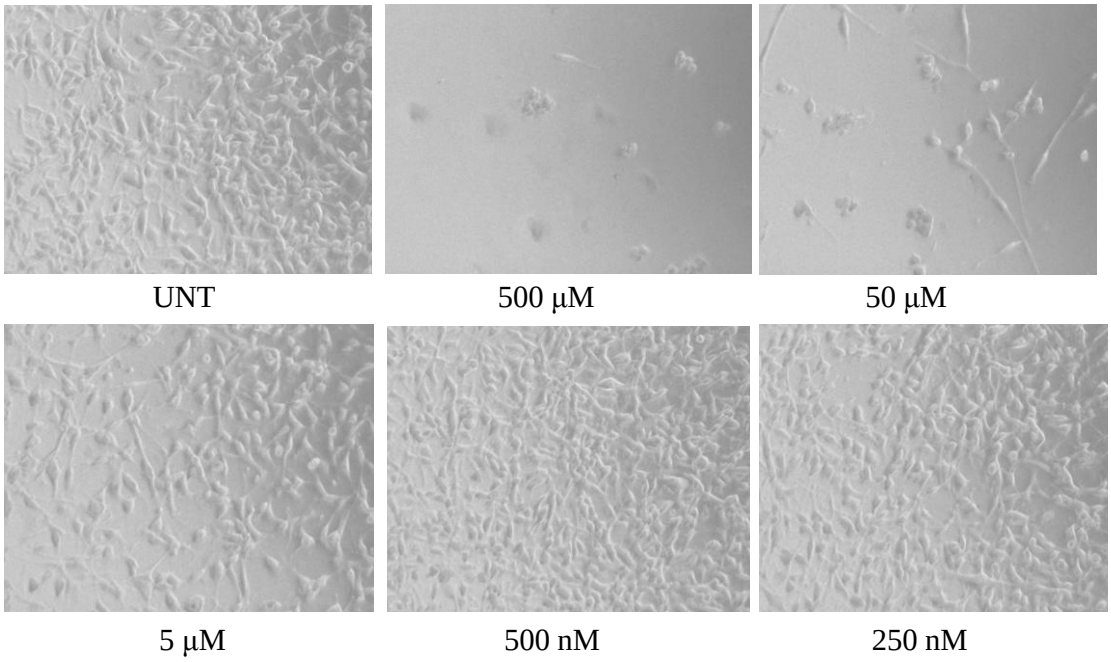


Figure 19. Various reference genes change expression upon CYP33 over-expression.

pCEP4, pCEP4-CYP33 (CYP) or pCEP4-CYP33 M (MUT) were transfected into MSA cells. After 48 hours, cells were trypsinized and RT-PCR was conducted to determine a reference gene that does not change upon CYP33 overexpression in MSA cells. Shown is the Ct (cycle threshold value) of the vector control, and how CYP33 leads to an increase or decrease of the cycle threshold. The error bar represents plus/minus standard deviations from triplicate PCRs.

A


UNT
500 µM
50 µM
5 µM
500 nM
250 nM

B

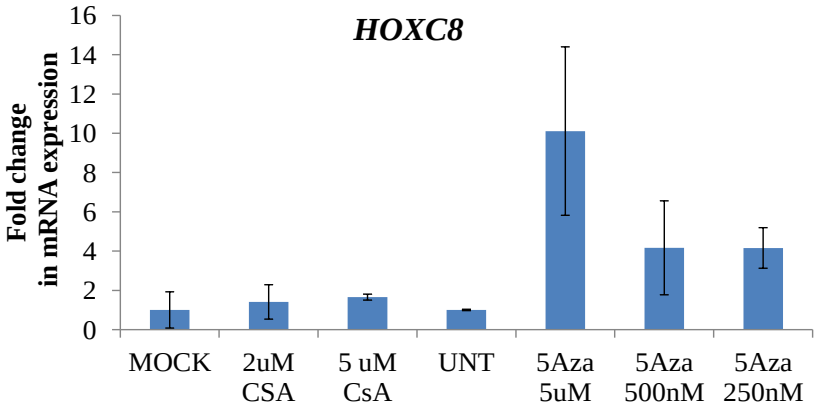

HOXC8
Fold change in mRNA expression
16
14
12
10
8
6
4
2
0
MOCK
2uM CSA
5 uM CsA
UNT
5Aza 5uM
5Aza 500nM
5Aza 250nM

Figure 20. *HOXC8* expression is increased upon 5-Azacytadine treatment in MSA cells.

A) A kill curve for MSA cells using various concentrations of 5-Azacytadine (5-Aza). Various conentrations of 5-Aza were used. For concentrations above 5uM of treatment with 5-Aza cells detached from the plate and stopped cellular growth/proliferation. B) Mock (EtOH), CsA (Cyclosporin A), UNT (untreated), or 5-Aza, a DNMT methylatransferase inhibitor, was used to treat MSA cells every 24 hrs. for 72 hrs. After 72 hrs., cells were harvested for RNA, and real-time RT PCR was performed.

HOX genes such as *HOXA9* could not be used as genes to study the effect of knocking down CYP33 as in the MSA cell line since *HOX* genes remained silent even after CYP33 knockdown in these cells. Consequently, since no gene expression was detected upon knockdown of CYP33, it was hypothesized that *HOX* genes could be silenced by other mechanisms independent of histone modifications, such as DNA methylation. In order to test if the *HOX* gene cluster was silenced by DNA methylation, the cells were treated with 5-azacytadine (5-Aza), a DNMT (DNA methyltransferase) inhibitor. A kill curve showed that treating MSA cells with concentrations of 5-Aza above 5μM caused cells to detach from the plate, round up and stop proliferating (Fig. 20). Concentrations of 5μM, 500nM, and 250nM were used. Cells were treated for 72 hours in medium plus EtOH (vehicle) or 5-Aza. Due to the short half-life of the inhibitor every 24 hours the media along with EtOH or 5-Aza was replaced. After 72 hours, cells were harvested, RNA was collected, and real time RT PCR was performed. Compared to controls, *HOXC8* mRNA levels increased in MSA cells treated with 5-Aza suggesting that DNA methylation inhibits *HOXC8* gene transcription in MSA cells(Fig. 20).

CYP33 is recruited to MLL target gene promoters when MLL is present in the cells

Mouse Embryonic Fibroblasts (MEFs) that either contain WT MLL protein (MEF $MLL^{+/+}$), or possessing a truncated MLL in which LacZ is fused to MLL in-frame within Exon 3 of MLL (MEF $MLL^{-/-}$), were used to test whether CYP33 is recruited to the promoter of *MLL* target genes in an MLL-dependent manner (Yu, B 1995). $MLL^{-/-}$ is

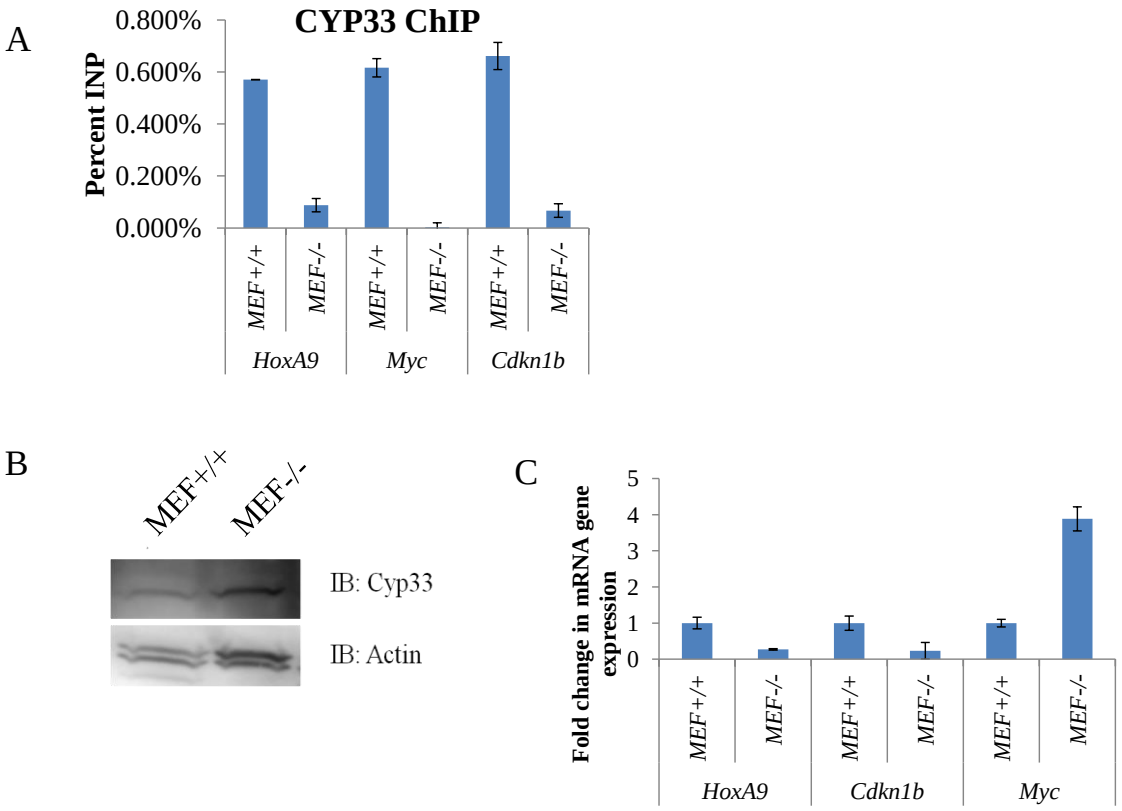


Figure 21. CYP33 is recruited to chromatin in MLL$^{+/+}$ MEFs but not in MLL$^{-/-}$ MEFs.

A) Either mouse IgG or α-CYP33 antibody was used for ChIP in cells that have wild-type (WT) MLL (MLL$^{+/+}$) or MLL in which a LacZ construct is inserted into Exon 3 (Yu, B 1995) and produces a protein lacking both a PHD finger and a SET domain. Such cells are MLL-null (MEF$^{-/-}$). ChIP for CYP33 binding at *Myc*, *HoxA9* and *Cdkn1b* gene promoters. Percent input is shown. B) A Western Blot for CYP33 is shown for the ChIP to demonstrate that CYP33 is expressed at equal levels in the two cell lines. C) mRNA expression of MLL target genes are shown in MLL$^{+/+}$ or MEF$^{-/-}$. Shown is a representative of two experiments.

devoid of the ability to bind CYP33 or activate MLL target genes. As mentioned above, CYP33 binds to MLL through the former's RRM domain. The RRM domain also has the ability to bind to RNA (Mi, H 1996, Hom, R 2010). It is therefore possible that CYP33 can be recruited to chromatin through RNA, independently of MLL.

In order to test the hypothesis that CYP33 recruitment to chromatin is mediated by MLL, ChIP was conducted in $MLL^{+/+}$ MEFs and $MLL^{-/-}$ MEFs to determine whether CYP33 can still be recruited to the chromatin in cells lacking MLL. There is no recruitment of CYP33 to chromatin in $MLL^{-/-}$ MEFs, supporting the hypothesis that CYP33 binds to chromatin through MLL as opposed to recruitment to chromatin through RNA (Fig. 21). Shown by Western Blot, CYP33 was still expressed at similar levels in $MEF^{-/-}$ MEF showing that loss of recruitment of CYP33 at MLL target gene promoters is not due to a lower concentration of CYP33 protein within $MLL^{-/-}$ MEFs (Fig. 21B). This is especially necessary as a control because in this study we found evidence that CYP33 may auto-regulate its own gene expression (Figs. 21, 22). *HoxA9*, *Cdnk1b*, and *Myc* are transcribed in $MLL^{-/-}$ MEFs, but CYP33 does not bind to their promoters. The lack of CYP33 binding to these promoters in $MLL^{-/-}$ MEFs is not due to silencing of gene transcription. In $MLL^{-/-}$ MEFs, *Myc* mRNA is higher when compared to $MLL^{+/+}$ MEFs (Fig. 21C). In $MLL^{-/-}$ MEFs, both *HoxA9* and *Cdnk1b* were shown to produce mRNA gene transcripts, albeit at a reduced level in comparison to $MLL^{+/+}$ MEFs (Fig. 21C). Hence, these data support the hypothesis that CYP33 is recruited to MLL target genes by MLL or via an MLL-dependent mechanism.

A. MSA Cells ChIP

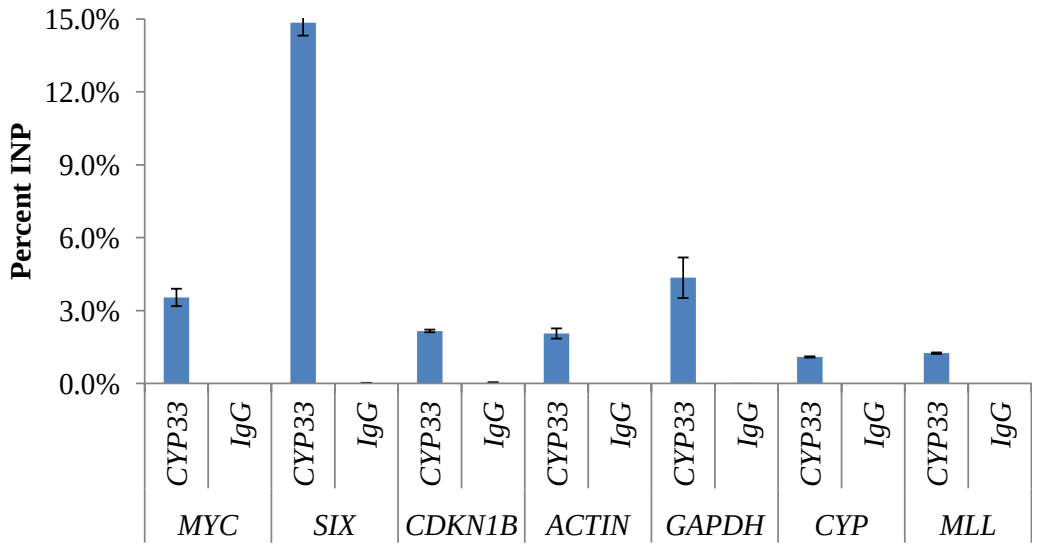


Figure 22. CYP33 is recruited to the promoter of various genes in MSA cells.

ChIP for CYP33 recruitment to MLL target gene promoters, *CDNK1B*, *MYC*, *CYP33*, and to housekeeping genes such as *ACTIN* and *GAPDH* in MSA cells. IgG is used as a control for non-specific interactions. Percent input is shown for CYP33 or IgG ChIP. Representative of two independent experiments is shown. Error bars are standard deviations from triplicate real time PCR reactions.

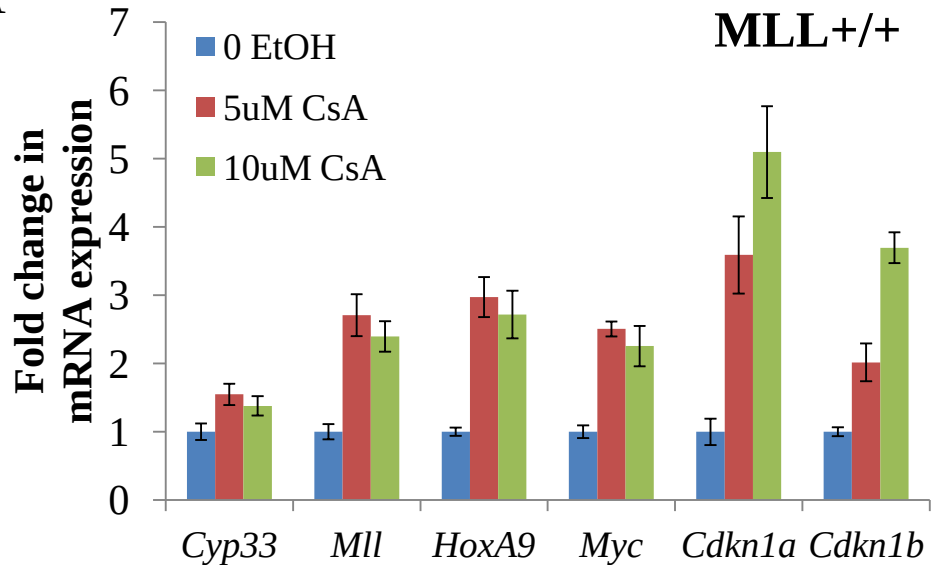
To study *CYP33* and *MLL* target gene promoters in the human system, the MSA cell line was used. The MSA cell line is a thyroid carcinoma-derived cell line in which the 5'-*HOX* genes are silenced. *CDNK1B*, *SIX1*, and *MYC* genes whose promoters are established MLL targets were studied along with *CYP33* and *MLL* promoters for CYP33 recruitment. CYP33 binds to *CDNK1B*, *SIX1*, *MYC*, *CYP33* and *MLL* promoters in the MSA cells as shown by ChIP (Fig. 22). CYP33 is recruited to MLL target genes in both murine and human cell lines, and in MEFs it does so in a MLL-dependent manner. It is important for understanding the knockdown and over-expression studies that were conducted in this study to show that CYP33 is recruited at MLL target genes in MSA and MEF cell lines.

In order to test whether the PPIase activity of CYP33 was responsible for the increase in MLL target genes observed after the knockdown of CYP33, Cyclosporin A (CsA) was used to inhibit the PPIase activity of cyclophilins. MLL target gene expression was determined by real time PCR. MEF cells were treated for 96 hours with EtOH or either 5μM or 10μM CsA. When inhibiting CYP33 with CsA, only in WT MEF cells is expression of Mll target genes *Cdnk1b*, *HoxA9*, *Myc*, as well as the Myc target gene *Cdkn1a* increased (Fig. 23). For *Cdkn1a* and *Cdnk1b*, an additional increase is seen for 10μM versus 5μM. Therefore, the response observed was dose-dependent.

In the MLL null MEFs, inhibition of CYP33 using up to 10uM CsA did not lead to an increase of *MLL* target gene expression (Fig. 23). These results support the previously tested hypothesis that CYP33 effects on *Mll* target genes are dependent on

Mll. Several previously reported MLL target genes, *HoxA9*, *Myc*, *Cdnk1b*, were tested. *Cyp33*, and *Mll*, which are not known to be MLL target genes, were also tested as these were shown to have recruitment of CYP33. *Cdnk1a* is regulated by *Myc* and its mRNA levels increase upon CsA treatment. *Cdnk1a* and *Cdnk1b*, the expression of which may lead to cellular senescence (Alexander, K 2001), will be discussed in greater detail below. As mentioned above, CYP33 is not recruited to the chromatin in the absence of MLL (Fig. 21). Thus it can be inferred that CYP33 must bind to MLL in order to exert its repressive effects and the absence of MLL negates any of the repressive effects attributable to CYP33.

A


MLL+/+
0 EtOH
5uM CsA
10uM CsA
Fold change in mRNA expression
0
1
2
3
4
5
6
7
Cyp33
Mll
HoxA9
Myc
Cdkn1a
Cdkn1b

B

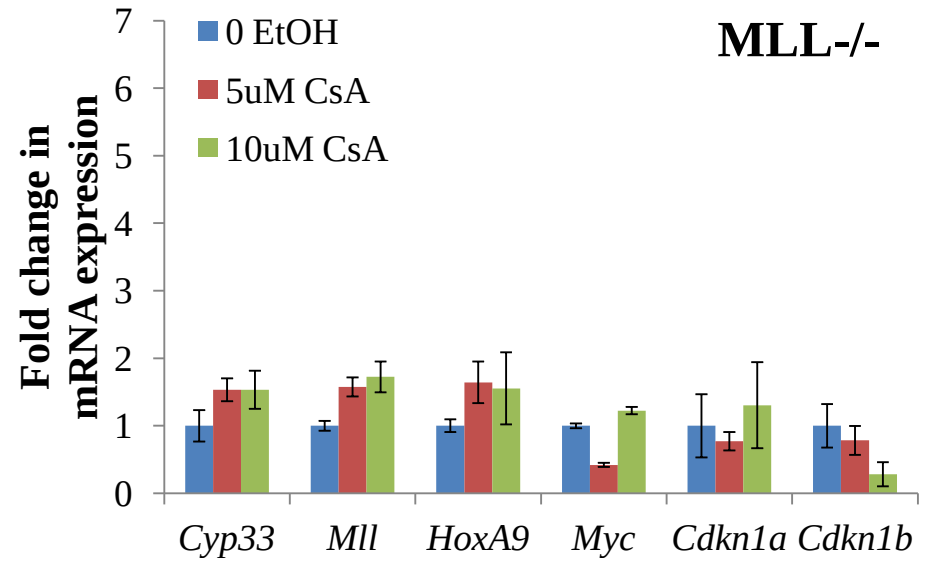

MLL-/-
0 EtOH
5uM CsA
10uM CsA
Fold change in mRNA expression
0
1
2
3
4
5
6
7
Cyp33
Mll
HoxA9
Myc
Cdkn1a
Cdkn1b

Figure 23. Upon treatment with CsA, expression of MLL, CYP33 and MLL target genes is increased in MEF$^{+/+}$ but not MEF$^{-/-}$.

MEF$^{+/+}$ or MEF$^{-/-}$ cells were treated for 96 hours with either EtOH (control) or 10μM CsA. Twenty four hours after treatment cells were typsinized and plated at a lower density in order to avoid confluency. After 96 hours, RNA was collected and real-time PCR was performed. A) *Mll*, *Cyp33*, *c-Myc*, *Cdnk1a*, *Cdnk1b*, and *HoxA9* before and after treatment with CsA in MEF$^{+/+}$. B) *Mll*, *Cyp33*, *c-M*yc, *Cdnk1b*, and *HoxA9* before and after treatment with CsA in MEF$^{-/-}$. Error Bars derived from standard deviations from triplicate real-time PCRs. A representative of two independent experiments is shown.

.

CHAPTER V

DISCUSSION

It has been proposed that CYP33 functions to recruit HDAC1 to chromatin in order to repress MLL target genes (Wang Q, 2011; Xia Z, 2003). CYP33 or CYP33 M was over-expressed to determine if HDAC1 or BMI1 were recruited to MLL in a CYP33 PPIase dependent manner. Prior to this study, it was hypothesized that CYP33 would lead to an increase in HDAC1, a reduction in H3K27ac, and an increase in PRC2 binding to MLL target genes, such as *CDNK1B* and *MYC* (Fig. 24). This would allow PRC2 to trimethylate H3K27, resulting in gene silencing. Upon knockdown of CYP33 by siRNA the opposite would be hypothesized to occur. PRC2 binding to target genes decreases and an increase in H3K27ac and H3K4me3 would be observed. This suggests a reduction in HDAC1 recruitment and the ability of the SET domain of MLL to methylate H3K4 and of the MLL-associated CBP to acetylate the histones at MLL promoters; acetylation of H3K27 would prevent its methylation by the PRC2 complex (Tie F, 2009; Pasini D, 2010; Jung H, 2010) (Fig. 24).

CYP33 decreases H3K27ac at MLL target genes upon CYP33 over-expression

MLL regulates the expression of many transcription factors and cell cycle regulators which are determinants of cell fate. This study focused on the molecular

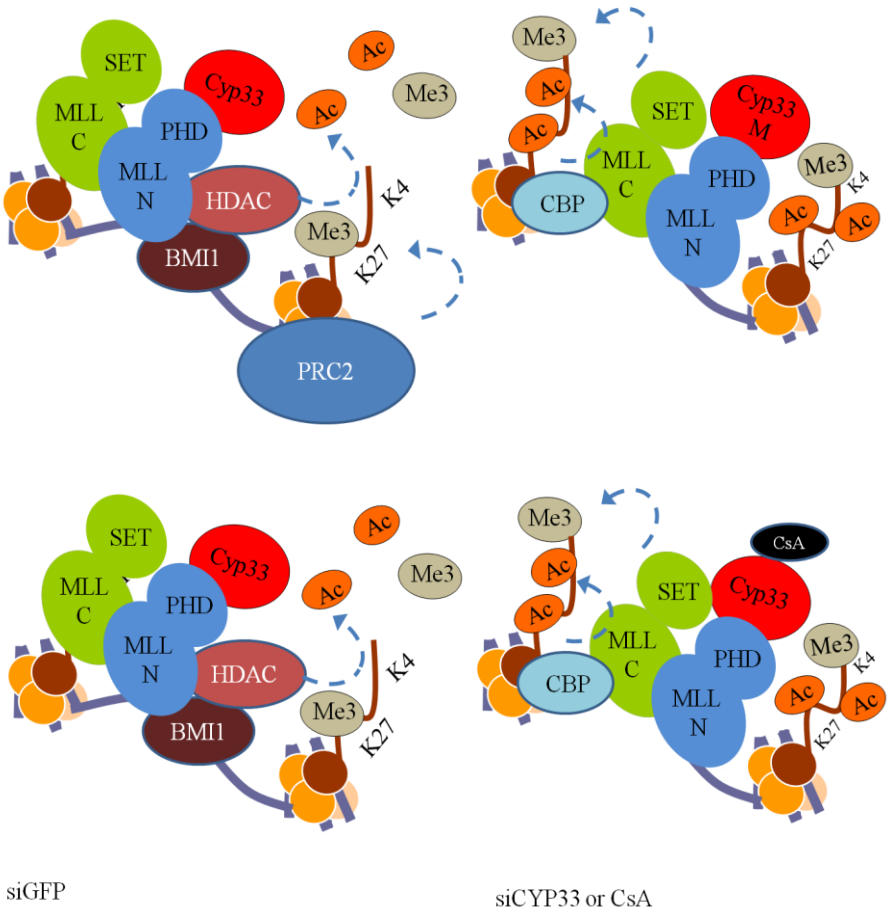


Figure 24. Working model of CYP33-mediated gene repression mechanism.

It was hypothesized that following over-expression of CYP33, HDAC1, BM11, hPC2, and CtBP would be recruited to the RD of MLL in a PPIase-dependent manner (upper left). This would lead to repression of gene transcription by reducing H3ac levels, including H3K27ac. PRC2 then could methylate H3K7me3 (upper left). Upon over-expression of CYP33-M (PPiase mutant), HDAC1 is no longer recruited to the chromatin (upper right). Upon knockdown of CYP33 or inhibition by CsA, HDAC would be removed; the HAT activity of CBP would be restored, leading to histone acetylation followed by increased gene expression (lower right).

mechanism of down-regulation of select MLL target genes, *CDNK1B*, *MYC*, and *SIX1*, by CYP33 and MLL (Wang Q, 2011; Wang P, 2009; Milne T, 2005). Although previous studies determined the critical residues that are important for the interaction between CYP33 and MLL *in vitro*, the mechanism behind the down-regulation of MLL target genes has yet to be elucidated (Wang Z, 2010; Park S, 2010). CYP33 has been shown to be an important negative regulator of MLL activity and is dependent upon HDAC activity for its repressive function, as tricostatin A prevents repression of MLL target genes in cells over-expressing CYP33 (Anderson M, 2001; Fair K, 2002; Wang Z, 2010; Park S, 2010).

CYP33 has been previously shown to regulate H3ac (K9ac and K14ac) and H3K4me3 (Park S, 2010; Wang Z, 2010). Upon over-expression of CYP33, but not of a CYP33 PPIase mutant, H3ac decreases at *HOXC8* and *HOXA9* promoters and H3K4me3 is reduced at *CDKN1B* and *HOXC8* (Park S, 2010; Wang Z, 2010). The H3K27 status was not examined in these studies. H3K27ac along with H3K9 and H3K14 acetylation is found at the promoters of active genes (Kurdistani S, 2004). H3K27ac is preferentially located at genes that are actively transcribed and, along with H3K4me1, at active enhancers (Rada-Iglesias A, 2011; Pekowska A, 2011; Melgar F, 2011; Creyghton M, 2010). In this study, H3K27ac was analyzed and found to be decreased at the *CDNK1B*, *MYC*, and *SIX1* promoters upon CYP33 over-expression. CYP33-M overexpression did not result in decreased H3K27ac at the promoters of MLL target genes, which implies that the decrease in acetylation is dependent on the PPIase activity of CYP33. H3ac

(H3K9 and H3K14) levels, did not change in a CYP33 PPIase dependent manner, but tended to decrease upon CYP33 or CYP33-M overexpression. Conversely, upon knockdown of CYP33, H3ac levels (including H3K27ac) are increased at a number of known MLL-regulated genes such as *CDNK1B*, *MYC*, and *SIX1*. *ACTIN* and *GAPDH*, two housekeeping genes which were also tested, showed similar upregulation to the known MLL target genes. Upon over-expression of CYP33 in MSA cells, only H3K27ac correlated with transcriptional mRNA levels; changes in H3K9 and H3K14 acetylation levels did not. Upon knockdown of CYP33, both H3ac and H3K27ac increased at the MLL target genes *CDNK1B, MYC* and *SIX1*. This is in contrast to what was seen previously upon CYP33 over-expression in which H3ac was decreased to a significant degree. One possible explanation for the differences observed is that H3ac may be lower than H3K27ac and H3K9 or H3K14 may not respond to HDAC1 mediated CYP33 decrease to as great a degree as H3K27. Another possible explanation is that HOX genes, which were tested and found to decrease H3ac may respond differently to CYP33, than MYC, SIX1 and P27. This is feasible as in Park *et al*, upon CYP33 over-expression HOXC8 decreased H3ac, whereas at the P27 promoter H3ac did not decrease (Park S, 2010; Wang Z, 2010)

Since H3K27ac decreases at *CDNK1B*, *MYC*, and *SIX1* promoters after CYP33 overexpression, these studies also examined whether the H3K27me3 repressive histone mark increased concordantly. H3K27me3 was detected at very low levels in the control MSA cells at the *CDNK1B*, *MYC*, and *SIX1* gene promoters as well as *ACTIN* and

GAPDH. There also failed to be any significant differences before and after knockdown or over-expression of CYP33. It can therefore be concluded that repression of MLL target genes by CYP33 in the MSA cell line is not mediated by a typical PRC2-dependent mechanism as was first hypothesized (Fig. 24). Although this does not exclude the possibility that H3K27me3 were already at their minimal levels and upon CYP33 knockdown could not be reduced further. Also it may take longer to deposit H3K27me3, after the HDAC1 has removed the acetyl mark from H3K27. Therefore upon a more sustained level of CYP33 over-expression for a longer time, H3K27me3 may be regulated by CYP33. Alternatively, this study does not exclude that CYP33 may play a role in mediating PRC2 deposition of H3K27me3 in other cells lines or in other target genes within MSA cells. It only shows that in the MSA cell line, at the MLL gene promoters tested, H3K27me3 is not regulated by CYP33.

The methylation of H3K27me3 by Ezh2 in the PRC2 complex may be mis-regulated or blocked in the MSA cell line, as Ezh2 was bound at the MLL target gene promoters in these cells, although there is little H3K27me3 present at MLL target genes (this study). Mis-regulation of Ezh2 has been previously shown, as in many cancer cell lines Ezh2 is over-expressed or mutated causing a change in H3K27me3 (Hock H, 2012; Simon C, 2012; Ernst T, 2010). Commonly believed to be an oncogene, Ezh2, has also been suggested to function as a tumor suppressor in myeloid malignancies, such as MDS and T-ALL (Nikoloski G, 2010; Ntziachristos P; 2012).

BMI1 and HDAC1 are recruited to the MLL-RD in a PPIase dependent manner

Previously it was shown that over-expression of CYP33 leads to increased recruitment of HDAC1 to the RD of MLL (Xia, Z 2003). In the current study, CYP33 over-expression was shown to lead to increased recruitment of both HDAC1 and the polycomb group protein BMI1 to MLL-N. Over-expression of CYP33-M, which lacks PPIase activity, failed to increase recruitment of HDAC1 or BMI1 to the RD of MLL-N. Two separate constructs encoding MLL-N (amino acids 1-2665) and a MLL RD-PHD peptide (amino acids 1088-1371,1394-1630) which contains the RD and PHD finger 1-3 cluster, but lacks the bromodomain were used in order to determine if the binding of HDAC1 and BMI1 to the MLL-RD depended on other domains besides the MLL-RD and the MLL PHD3, upon CYP33 or CYP33 M overexpression.

An immunoblot for HA shows that HA-CYP33 and HA-CYP33 M are similarly recruited to MLL-N (Fig 10). This is in contrast to what was shown *in vitro* by Wang et al. (2010). These investigators reported that a PPIase deficient mutant of CYP33 cannot bind a cassette containing the MLL PHD3 and bromodomain, and that the CYP33-RRM domain alone does not bind to the PHD-Bromodomain cassette due to the lack of PPIase activity. They hypothesize that H1628-P1629 is isomerized by CYP33 PPIase domain in order to enact a conformational change between the PHD3 and bromodomains. This then will allow for CYP33 to bind. There are a couple of reasons why CYP33 M overexpressed in living cells may be able to bind to the MLL-N PHD3 whereas *in vitro* it is unable to do so. 1) The complex composition between an in-vitro experiment and an

experiment conducted within cell culture may open the bromodomain rendering the PPIase activity of CYP33 unnecessary for the RRM of CYP33 to bind to the PHD3 of MLL-N. 2) Another cyclophilin, such as CYPA may be able to provide the necessary PPIase activity. Our results imply that the PPIase dependence of HDAC or BMI1 binding to MLL-N, is not explained by differences in CYP33 recruitment to the MLL PHD3.

HDAC1 recruitment to the MLL RD showed dependence on the PPIase activity in both MLL-N and MLL RD-PHD constructs. This suggests that the PPIase target regulating recruitment of HDAC1 is within either the MLL RD-PHD finger construct, HDAC1 itself, or histone H3. BMI1 recruitment on the other hand, unlike HDAC1, shows a difference in its recruitment between the MLL-N and the MLL RD-PHD construct. Increased binding of BMI1 to the MLL RD-PHD construct no longer depends upon the PPIase activity of CYP33. This suggests that another property of CYP33 aside from its PPIase activity is necessary to promote BMI1 recruitment. CYP33 contains other domains besides the cyclophilin domain: the RRM and the conserved spacer, which may function to promote recruitment of BMI1 to the MLL RD-PHD finger construct. In addition, the cyclophilin domain may have a function separate from its enzymatic activity that helps bring BMI1 to MLL. Nevertheless, binding of BMI1 to the full length MLL-N requires the CYP33 PPIase activity and this requirement must involve a PPIase target, outside the MLL amino acid sequences encompassing the RD and PHD cluster, which can inhibit PPIase independent BMI1 recruitment. The recruitment of HDAC1 and

BMI1 to the RD of MLL and the subsequent decrease of H3K27ac are consistent with a model in which CYP33 regulates H3 acetylation levels by promoting recruitment of HDAC1 to the MLL RD. The role of BMI1 recruitment is not entirely clear, and will need further studies for its clarification.

HDAC1 and BMI1 recruitment to the chromatin does not change upon CYP33 over-expression

Due to the dependence of BMI1 and HDAC1 recruitment to MLL on CYP33 and its PPIase activity, it was expected that HDAC1 and BMI1 recruitment to the chromatin at MLL target gene promoters would change upon CYP33 over-expression or knockdown. Nevertheless, the BMI1 and HDAC1 recruitment showed no increase at the chromatin level after over-expression of CYP33 contrary to the hypothesis derived from the Co-IP data. One possible explanation for these results is that HDAC1 and BMI1 associate with the chromatin separate from but close to MLL. Upon CYP33 overexpression HDAC1 and BMI1 bound close to MLL, through a change in conformation in their protein complexes, through chromatin bending, or through partial dissociation from their binding sites, are able to interact with the RD domain of MLL and mediate gene repression at the MLL target genes. Therefore the role of CYP33 is not in recruitment of HDAC1 and BMI1 to the chromatin but in recruitment of HDAC1 and BMI1 to the RD of adjacent MLL.

Surprisingly, HDAC1 and BMI1 are recruited to MLL by CYP33 as shown by CoIP, but neither HDAC1 nor BMI1 are increased upon CYP33 over-expression to the

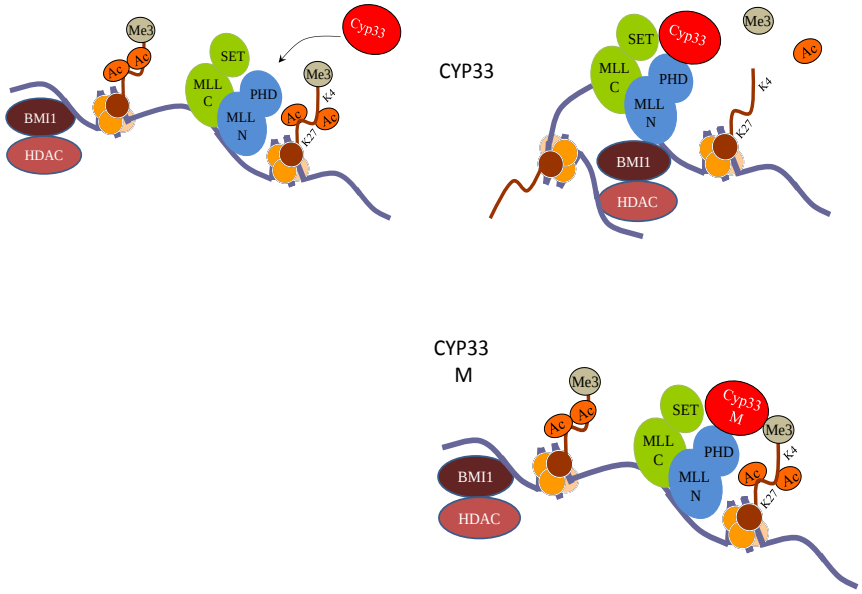


Figure 25. New working model for the mechanism of CYP33-mediated gene repression.

Upon over-expression of CYP33 or CYP33 M, CYP33 binds to MLL via the RRM domain by interacting with the PHD3 domain, as has been previously described (Fair, K 2001, Anderson, M 2002). HDAC1 and BMI1 were shown hereinto be recruited to MLL upon CYP33 overexpression. HDAC1 and BMI1 are bound to chromatin in untransfected MSA cells and CYP33 or CYP33 M over-expression does not increase binding above vector control levels. Therefore, CYP33 instead brings the already bound HDAC1 and BMI1 into a complex with MLL through an as yet undefined mechanism. CYP33 M, which is unable to increase HDAC1 and BMI1 recruitment because it lacks PPIase activity, allows H3K27ac and H3ac to remain at steady state levels; thus it does not decrease gene transcription.HDAC1 binding to chromatin is decreased. This reconciles the observed differences between the CoIP and ChIP data.

chromatin at *MLL* target genes *CDNK1B*, *MYC*, and *SIX1*. This suggests another model and hypothesis (Fig. 25) in which BMI1 and HDAC1 are not soluble before being recruited to chromatin in MSA cells but are bound to the chromatin at MLL target genes in MSA cells at endogenous levels of CYP33. Recruitment of HDAC1 and BMI1 to the RD of MLL occurs upon CYP33 over-expression, and only when it is in a complex with MLL is HDAC1 able to reduce H3K27ac levels, perhaps through the inhibition of MLL-associated CBP HAT activity. Upon knockdown at specific genes, *CDNK1B* and *MYC*, HDAC1 binding to chromatin is decreased. This reconciles the observed differences between the CoIP and ChIP data, but leads to additional hypothesis that need to be tested.

Knockdown of CYP33 by siRNA or inhibition of CYP33 by CsA leads to oscillations in gene transcription

Due to the oscillations seen in MSA cells for *CYP33* mRNA gene transcription as measured using mRNA steady-state levels, it was hypothesized that CYP33 and MLL might be part of a negative feedback loop. In conducting ChIP at the *CYP33* promoter in MSA cells or MEFs, it was found that CYP33 binds at the chromatin of the *CYP33* promoter, supporting the hypothesis of regulation of the *CYP33* gene by CYP33 and MLL. Therefore CYP33 through MLL may participate in a negative feedback loop that would be the cause of the oscillations observed upon CYP33 knockdown. The simplest feedback loop within this context would be the MLL protein transactivating the *CYP33* gene, leading to an increased level of CYP33 protein, which inhibits MLL transactivating

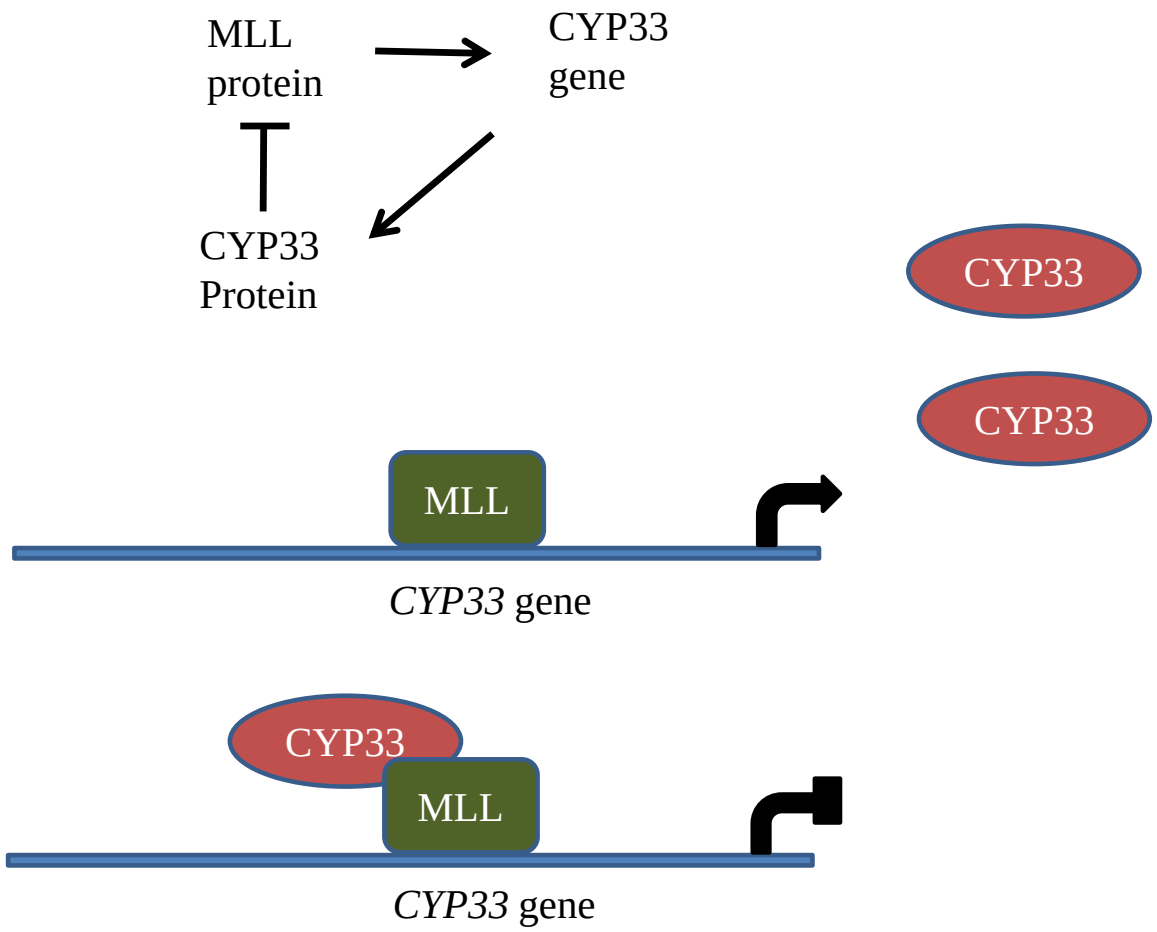


Figure 26. CYP33 recruitment to the CYP33 promoter and oscillations of *CYP33* mRNA transcripts upon CYP33 knockdown suggests the existence of a negative feedback loop regulating the expression of CYP33.

As diagramed above, the negative feedback loop involving CYP33 may involve the MLL protein regulating the transcription of the CYP33 gene. Therefore upon CYP33 knockdown with siRNA, MLL can then lead to increased levels of CYP33 mRNA. This leads to increased CYP33 protein levels, which then inhibit CYP33 transcription.

activity on the *CYP33* promoter (Fig. 26). The oscillations in CYP33 levels in turn produce oscillations in the expression of MLL-regulated genes, as well as in the expression of several housekeeping genes which are not known MLL targets (see section on housekeeping genes below).

Circadian rhythms are natural oscillations that also may contribute to CYP33 and MLL-mediated oscillations. It was recently discovered that MLL regulates circadian rhythm function by regulating the transcription of the *CLOCK* and *BMAL1* genes (Katada S, 2010). CLOCK and BMAL1 proteins function in a series of feedback loops to regulate genes involved in synchronizing the light/dark daily cycle with the mammalian cell functions. Circadian rhythm disruption is increasingly being found to be linked to cancer (Sahar S, 2009). This may contribute to natural oscillations within cancer cells that may go undetected in many studies. The period of oscillation caused by CYP33 is much longer than the 24 hour circadian period, and they are more likely caused by a separate negative feedback loop.

Modulation of CYP33 leads to changes in cell proliferation and senescence

MSA cells were found to be reliant on CYP33 for proliferation. Since both overexpression and knockdown of MLL led to a slowdown of cell growth by inhibition of the cell cycle (Liu H, 2007; Mishra B, 2009), it was not surprising that CYP33 also led to a decrease in cell proliferation/growth. CYP33 over-expression or knockdown led to a decrease in cell doubling time in MSA cells. Although no obvious cell cycle inhibition

was seen using FACS analysis of propidium iodide stained cells, a more comprehensive study needs to be conducted to dissect the role of CYP33 in cell cycle regulation.

CYP33 over-expression led to decreased cellular ATP levels while over-expression of CYP33-M resulted in ATP levels similar to ones of the vector control. Knocking down CYP33 did not lead to increased PI staining of living cells, an indicator of apoptosis. ATP levels were also unchanged. An increase in SA β-galactosidase activity was observed after CYP33 knockdown in MSA cells, compared to siGFP controls. This suggests that CYP33 at control levels may function to prevent senescence in MSA cells. The increase in senescence could be due to the increase in *CDKN1A* and *CDNK1B* expression that is seen upon CYP33 knockdown (Fig 14,15,23).

CYP33 affects nucleosome density within MSA cells

CYP33 does not only affect H3K27ac levels but also affects the H3 levels within target gene promoters, potentially reflecting nucleosome density at these sites. Reduction in H3 levels could also represent a reduced ability of the H3 antibody to bind to the histone tail; this is known as epitope masking. Upon knockdown of CYP33, H3 levels decreased, and when comparing the effect of over-expression of CYP33 or CYP33 M, the density of nucleosomes after CYP33 overexpression was always higher than that seen with CYP33-M overexpression (Fig. 14). MLL interacts with the SWI/SNF chromatin remodeling complex, yet the functional consequences of this interaction have not been elucidated (Rozenblatt–Rosen O, 1998). It is conceivable that the CYP33-MLL complex functions to regulate chromatin remodeling at MLL target genes, leading to decreased

nucleosome occupancy at MLL promoters. This hypothesis could be tested by performing nucleosome remodeling assays in the presence and absence of CYP33 (Jaskelioff M, 2009).

All chromatin marks in this study were normalized to histone H3. Normalization allows for one to look at changes in histone modifications versus changes in the nucleosome density or epitope masking. This allows for a better comparison between two groups that have been differentially treated. In this study when looking at CYP33 or CYP33 M, and siGFP or siCYP33, H3 was used to normalize the marks between the two groups.

Housekeeping genes *ACTIN* and *GAPDH* are also regulated by CYP33

Surprisingly, *ACTIN* and *GAPDH* transcription were up-regulated after CYP33 knockdown, and decreased after CYP33 overexpression (Fig 7). Concordant with increased transcription H3K27ac and H3ac increased at the promoter of *ACTIN* upon CYP33 knockdown in MSA cells. H3K27ac at the *ACTIN* and *GAPDH* promoters were also found to be decreased after CYP33 over-expression (Fig. 8).

CYP33 binds to known MLL target genes such as *CDNK1B* and *MYC*, and it was found through ChIP that CYP33 binds to *β-ACTIN*, *GAPDH*, and *B2M* promoters. In addition to an increase in mRNA upon CYP33 knockdown, *ACTIN* and *GAPDH* followed a similar pattern to the MLL target genes *p27*, *MYC*, and *SIX1* in that H3K27ac and H3ac changed according to the mRNA levels within cells. It has been shown that MLL binds to housekeeping gene promoters during mitosis and that MLL is necessary to

allow for SET1-dependent transcription activation in the early G_1 phase of the cell cycle (Blobel G, 2009). Therefore, it is possible that CYP33 regulates *ACTIN* and *GAPDH* through MLL binding during mitosis, changing the ability of these genes to be turned on in the G_1 phase of the cell cycle.

It is possible that CYP33 could have functions that are independent of its binding to PHD3 of MLL to regulate housekeeping genes. CYP33 has been found to bind to RNA as well as to both a XAB2 protein complex with function in transcription coupled excision repair and in alternative splicing, and in the large spliceosome protein complex (Kuraoka I, 2008; Mesa A, 2008). XAB2 has been shown to bind to the RRM domain of CYP33 through tetratricopeptide repeats (TTPR) (Kuraoka I, 2008). Because of the involvement of CYP33 in these complexes it has been hypothesized that it may play a role in alternative splicing, but this has not been tested as yet. It is possible that CYP33 bind other proteins with TTPRs mediating its MLL-independent functions (Kuraoka I, 2008).

The molecular mechanism by which MLL regulates the reactivation of mitotic genes remains unclear. In mitotic chromatin at MLL target genes, H3K4me3 levels are similar in both control and MLL-knockdown cells. This suggests that the mechanism of MLL regulation of mitotic chromatin is independent of the MLL methyltransferase activity. The proposed function of MLL is to tether key co-regulatory proteins, WDR5, RbBP5, or ASH2L to active gene promoters while they are transiently silenced in mitotic

chromatin (Blobel G, 2009). Blobel et al. propose that MLL acts as a placeholder for the HMT SET1 complex that is displaced during mitosis (Blobel G, 2009).

General Conclusions

CYP33 has been previously thought to be recruited to MLL target gene promoters by interaction with the PHD3 of MLL. This study shows that CYP33 is recruited to the promoters of MLL target genes like *HOXA9*, *MYC*, and *CDKN1B*, through an MLL dependent mechanism, since in the absence of MLL, CYP33 is not recruited to these target gene promoters.

CYP33 overexpression leads to recruitment of HDAC1 and BMI1 to the RD of MLL in a CYP33 PPIase dependent manner. HDAC1 was previously shown to bind MLL in a PPIase dependent manner using CsA (Koonce, M 2004). As CsA is a general inhibitor of all cyclophilin family PPIase's, in this study we used a CYP33 PPIase mutant lacking cis-trans prolyl isomerase activity (CYP33-M). Using a CYP33mutant deficient in PPIase activity, we showed that it is the PPIase of CYP33 that is important for HDAC1 recruitment and extended the previous studies to include another important co-repressor, BMI1, showing that CYP33 regulates BMI1 recruitment to MLL as well.

However, the binding of HDAC1 and BMI1 to MLL does not lead to a similar increase in recruitment of these co-repressors to chromatin at the MLL target gene promoters, since upon CYP33, or CYP33 M overexpression HDAC1 and BMI1 recruitment to the chromatin is unchanged.

It has been shown that H3Ac (H3K9ac and H3K14ac) is decreased upon CYP33 over-expression (Wang Z, 2010). In this study H3K27ac, as a mark of activation, was also studied in addition to H3ac. CYP33 over-expression or knockdown leads to changes in H3K27ac, and knockdown of CYP33 leads to a marked increase of H3ac and H3K27ac marks at MLL gene promoters. Decreased H3K27ac at the promoters of MLL target genes, *CDNK1B*, *MYC*, and *SIX1* leads to a decrease in mRNA gene expression (Park S, 2010; this study).

The initial hypothesis of this study was that H3K27ac prevents H3K27me3 and upon over-expression of CYP33, H3K27ac would decrease, and H3K27me3 would increase. In the MSA cell line at the MLL promoters studied this turned out not to be the case as H3K27me3 was very low and remained unchanged after CYP33 over-expression or knockdown. So, gene repression after CYP33 overexpression does not depend on H3K27 methylation.

It was observed in this study that CYP33 inhibition by CsA, or its knockdown by siRNA, leads to oscillations of MLL target gene expression. The levels of *MYC*, *BMI1*, *CDKN1A*, and *CDKN1B* mRNAs were all shown to oscillate. Expression of *CYP33* and *MLL* was also shown to oscillate upon knockdown or CsA inhibition by CYP33.

CYP33 was also shown to bind to the *CYP33* gene promoter by ChIP in MSA and MEFs cells (Fig. 21, 22). These two observations suggest that a regulatory negative

feedback loop exists, involving the *CYP33* gene promoter, and the MLL protein, through which CYP33 regulates its own gene transcription.

Understanding the functions of the co-regulators of MLL such as HDAC1, BMI1, and CYP33 is important for gaining further insight into the regulation of MLL and MLL-fusion leukemias. By studying CYP33, which is a critical regulator of MLL, a better understanding of the molecular functions of MLL and MLL-fusions is possible.

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VITA

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