Characterization of the tetrameric monocytic leukemia zinc finger protein complex

Mukta Ullah

Department of Biochemistry

McGill University, Montreal

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ABSTRACT

In human cells, histones bind DNA and form chromatin, a nucleoprotein complex important for various cellular processes. Abnormal histone modification is known to play causal roles in the development of cancer. Monocytic leukemia zinc finger protein (MOZ) is a MYST-family histone acetyltransferase whose gene is rearranged in chromosomal translocations giving rise to acute myeloid leukemia. This acetyltransferase functions as a potent transcriptional coactivator of Runx1 and Runx2, two homologous transcription factors that are important for definitive haematopoiesis and osteoblast maturation, respectively. Mouse knockouts have demonstrated that MOZ is required for the maintenance of hematopoetic stem cells, and is important for erythroid and myeloid cell differentiation.

Chapter I reviews the importance of chromatin regulation, MYST histone acetyltransferases and ties them together to explain how gene regulation is achieved. Chapter II addresses the characterization of the tetrameric MOZ complex, and suggests that its activity can be modulated by the presence of associated subunits such as BRPF1, and ING5. We show by histone acetyltransferase assays that MOZ and MORF activity is indeed enhanced by associated proteins. We have mapped the interaction domains of BRPF1 required for binding by MOZ, ING5, and Eaf6 in an effort to understand the mechanism of activation. Given that MOZ and ING proteins contribute to oncogenesis by chromosomal translocations and loss of function respectively, the characterization of the multisubunit complex provides novel mechanistic insights into its function in normal human cells and under conditions of leukemia or other cancers.

RÉSUMÉ

Dans le noyau des cellules mammifères, la liaison des histones à l'ADN forme la chromatine, un complexe nucléoprotéique d'importance cruciale pour la régulation cellulaire. Il est reconnu qu'une modification anormale des histones joue un role majeur dans le developpement de diverses pathologies, notamment des cancers. MOZ, une protéine de la famille MYST d'histones acétyltranferases, est impliquée dans de nombreuses translocations chromosomiques, qui sont responsables du développement de la leucémie myéloïde chronique. Il a été montré que MOZ est un importnat coactivateur pour les facteurs de transcription Runx1 et Runx2, qui jouent un rôle primordial dans l'hématopoëïse et la maturation des ostéoblastes, respectivements. Le knockout du gène codant pour la protéine MOZ chez les souris a démontré que cette dernière est requise pour le renouvellement des cellules souches hématopoïétique et pour la différentiation des cellules myéloïde et érythroïdes.

Le Chapitre I résume l'importance de la régulation de la chromatine et des acétyltransferases, principalement celles de la famille MYST, en les reliant afin d'expliquer les mécanismes de la régulation des gènes. Le Chapitre II traite de la caractérisation du complexe tetramérique MOZ et propose un rôle pour les sous-unités BRPF1 et ING5 dans la régulation de l'activité de ce complexe. La caractérisation du complexe de MOZ indique la présence de quatre sous-unités, telles que BRPF1, ING5 et Eaf6. De plus, ces dernières modulent l'activité enymatique de MOZ. En utilisant un essai mesurant l'activité acétyltransférase des

protéines, nous montrons que l'activité enzymatique de MOZ et MORF est effectivement augmentée en présence des autres sous-unités du complexe. De plus, on a disséqué les domaines d'interactions de BRPF1 qui sont requis pour interagir avec MOZ, ING5 et Eaf6 dans le but de mieux comprendre le méchanisme d'activation de ce complexe. Etant donné que les protéines MOZ et ING5 contribuent á l'oncogénèse via des translocations chromosomiques et une perte de fonction, repectivement, la caractérisation du complexe nous permettra d'avoir une meilleure compréhension de son méchansime de régulation dans les cellules normales et lors de la leucémogénèse ou du développement tumoral.

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Publications from the work of this thesis

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2. **Ullah, M.,** Xiao, L., Cote, J., Yang, X.J. (2006). Distinct roles of BRPF1 and ING5 in regulating activities of MOZ and MORF.
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CHAPTER I

Literature Review

1.1 Chromatin structure, function and regulation

Following the sequencing of the human genome, a plethora of proteins have been identified many of which still do not have defined functions. All the genetic information of these proteins is stored in the cell nucleus in the form of chromatin, the building block of chromosomes. With histone proteins, DNA is packaged into chromatin consisting of arrays of nucleosomes. Within each nucleosome, there is a histone octamer containing two copies each of H2A, H2B, H3 and H4 wrapped twice with 146 base pairs of DNA (Kornberg, 1974). The beads-on-a-string model represents the first level of chromosomal DNA packaging. Nucleosomes provide the first level of compaction by constraining the size of the chromosome which would otherwise span almost two meters in a human cell nucleus, and chromatin restricts the accessibility of DNA to molecular machines controlling transcription and other DNA-templated nuclear processes. As a result, chromatin structure needs to be regulated. Two of the regulating mechanisms are ATP-dependent remodeling which remodels the nucleosomal patterning along the chromatin fiber, and covalent modifications (Becker and Horz, 2002). While DNA is modified by methylation, histones are subject to a wide variety of modifications, including lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, ubiquitination, and ADP-ribosylation. Histone acetylation will be discussed in further detail.

1.2 Acetylation of histones and nonhistone proteins

In addition to the histone fold, each of the four core histones that make up the nucleosome has a short N-terminal amino acid tail that protrudes out from the DNA-histone core. These histone tails are subject to different types of covalent modifications that specify the dynamics of chromatin structure. For lysine acetylation, histone acetyltransferases (HATs) catalyze the transfer of the acetyl moiety of acetyl-co-enzyme A to the ε-$NH3^+$ groups of lysine residues on the N-terminal regions of histones. This is a dynamic and reversible process where histone deacetylases may remove the acetyl group from the acetylated lysine residue upon receiving appropriate signals. There are currently two theories as to how histone acetylation facilitates transcription. One is that acetylation of lysine residues might affect transcription by neutralizing positive histone charges and weakening histone DNA contact thereby destabilizing chromatin structure. The other hypothesis suggests that acetylation and other covalent modifications on histones are epigenetic marks for gene expression. This hypothesis is also known as the histone code (Jenuwein and Allis, 2001; Strahl and Allis, 2000; Turner, 2000).

The histone code is a combination of modifications forming a code that determines the structure of chromatin. Specific chromatin remodeling proteins or transcription factors associate with chromatin causing conformational and functional changes of DNA. First, enzymes write the code by adding or removing modifications on histone tail residues. Multisite modification needs to be considered since one modification may exclude others on a single residue (Fischle et al., 2003). For example, methylation of histone H3 at Lys9 blocks acetylation at this residue,

and acetylation of p53 at Lys382 enhances DNA binding but blocks ubiquitination; p53 is subject to many other modifications including phosphorylation that may promote acetylation or interaction with coactivators. For example, phosphorylation on Ser-15 enhances interaction with CBP while phosphorylation at Ser-33 and Ser-37 promotes acetylation; this demonstrates that phosphorylation of p53 facilitates its acetylation by promoting preliminary binding of acetyltransferases in order to stabilize the interaction with p53 and other recruited acetyltransferases (Barlev et al., 2001). Furthermore, the functional consequence of a specific modification is context dependent. For instance, acetylation of histone H4 at Lys5 and Lys12 is required for chromatin assembly and promotes chromatin compaction and gene silencing (Fischle et al., 2003). However, when Lys8 and Lys16 are also acetylated, acetylation of Lys5 and Lys12 is linked to gene activation (Fischle et al., 2003). Next, modified residues generate binding platforms for the recruitment of other protein modules (Fischle et al., 2003). For example, phosphorylated tyrosine residues recruit SH2-containing proteins including Src, PI3-kinase, GAP, and SHP2; acetylated lysine residues recruit bromodomain containing proteins found in chromatin modifying complexes as in the case of the recruitment of CBP by acetylated lysine 382 on p53; and trimethylated Lys 9 on histone H3 recruits the chromodomain of HP1 (Figure 1) (Fischle et al., 2003; Yang, 2005).

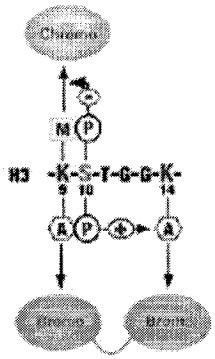

Figure 1: Effects of Neighboring Modifications. Residues 9-14 of histone H3 are modified by many enzymes. Lys 9 methylation recruits HP1 via its chromodomain while Ser 10 phosphorylation abolishes the recruitment (illustrated with an arrow marked by a red oval minus sign). When Lys 9 and Lys14 are acetylated, the TAFI bromodomain is recruited; however Ser 10 phosphorylation stimulates Lys 14 acetylation (illustrated by an arrow marked by a green plus minus sign in an oval). Adapted from Yang, 2005.

In addition to histones, HATs also acetylate non-histone proteins such as p53, non histone chromatin proteins HMG14 and HMG17, DNA binding transcription factor MyoD, GATA1, E2F, HNF-4, NF-κB and many others (Berger, 2000; Kouzarides, 2000; Yang, 2004). N^{ε}-acetylation occurs on histones, transcriptional co-regulators, in cellular proteins such as MCM3, DNA metabolic enzymes, the signaling regulator Smad7, DNA metabolic enzymes, and α-tubulin (Berger, 2000; Kouzarides, 2000). N^{ε}-acetylation has also been found in viral proteins such as the HIV TAR RNA-binding protein Tat, adenoviral oncoprotein E1A and polyomavirus large T antigen (Berger, 2000; Kouzarides, 2000). Lysine acetylation is therefore a general post-translational modification that occurs in various cellular and viral proteins.

1.3 HATs

HATs acetylate lysine residues on histones in vitro and form multiprotein complexes, such as the ADA and SAGA complexes in yeast (reviewed in (Berger, 2000; Yang, 2004)). Different complexes appear to acetylate specific histones and some can acetylate other proteins involved in gene expression. In addition to local

modifications on histone proteins in regions surrounding expressed genes, HATs may carry out more general modifications on a global scale throughout the entire genome (Berger, 2000). HATs may be divided into families (Table 1). One major group of nuclear HATs is Gcn5-related acetyltransferases (GNATs)/PCAF; these HATs have been well characterized and function as histone–acetylating transcriptional co-activators. p300/CBP HATs form a pair of homologous HATs in mammals; they constitute another major group of nuclear HATs that function as transcriptional co-activators and acetylate both histones and non-histone proteins (reviewed in (Berger, 2000; Yang, 2004)). The MYST family of HATs is more diverse than the GNAT and p300/CBP families. This thesis focuses on MYST HATs, particularly MOZ and MORF (Table 1).

1.4 The MYST family of HATs

The MYST family is named after its four founding members which include human MOZ/MORF, yeast Ybf2/Sas3, yeast Sas2, and mammalian TIP60. Additional MYST proteins include yeast Esa1, D*rosophila* MOF, D*rosophila* Enok, and mammalian HBO1 (Table 1). There is sequence similarity in the MYST domain, which is a region of homology conserved among all the family members from yeast to humans. It is comprised of an acetyl-CoA binding motif and a C_2HC zinc finger (Akhtar and Becker, 2001). The zinc finger is essential for HAT activity of Sas3, Mof, MOZ, and MORF, but Esa1 does not have this motif (Figure 2). Members of this family have roles in epigenetic control, transcriptional regulation, DNA replication, chromatin assembly, cell cycle progression, and cellular signaling.

Many HATs of the MYST family are present in multiprotein complexes and are believed to be the catalytic subunit. Sas3p is the catalytic subunit in NuA3, Esa1p in NuA4, TIP60 in the hNuA4 complex, and MOZ/MORF in the ING5/BRPF complex.

Table 1: Classification of known lysine acetyltransferases. Adapted from (Yang, 2004).

Family	HATs	Organisms	Complexes	Functions
GNAT	GCN5	*S. cerevisiae*	SAGA, SALSA, ADA	Coactivator
	PCAF	Mammals	PCAF	Coactivator
	GCN5L	Worms to mammals	STAGE, TFTC	Coactivator
	ELP3	*S. cerevisiae* to mammals	Elongator	Elongation
	HAT1	*S. cerevisiae* to mammals	HAT B	Histone deposition
	HPA2/3	*S. cerevisiae*		Unknown
	TFIIIC	*S. cerevisiae* to mammals		Transcription initiation
CBP/p300	CBP	Worms to mammals		Coactivator
	p300	Mammals		Coactivator
MYST	SAS2	*S. cerevisiae*	SAS	Gene silencing
	SAS3	*S. cerevisiae*	NuA3	Elongation
	ESA1	*S. cerevisiae*	NuA4, piccolo	Coactivator
	Mof	*Drosophila*	MSL	Dosage compensation
	ENOK	*Drosophila*		Neuroblast proliferation
	CHAMEAU	*Drosophila*		PcG-dependent silencing
	MORF	Mammals		Coactivator
	MOZ	Mammals		Coactivator
	TIP60	Mammals	TIP60	Coactivator, DNA repair
	HBO1	Mammals		Corepressor, replication
	MOF	Mammals	MAF2	Coactivator

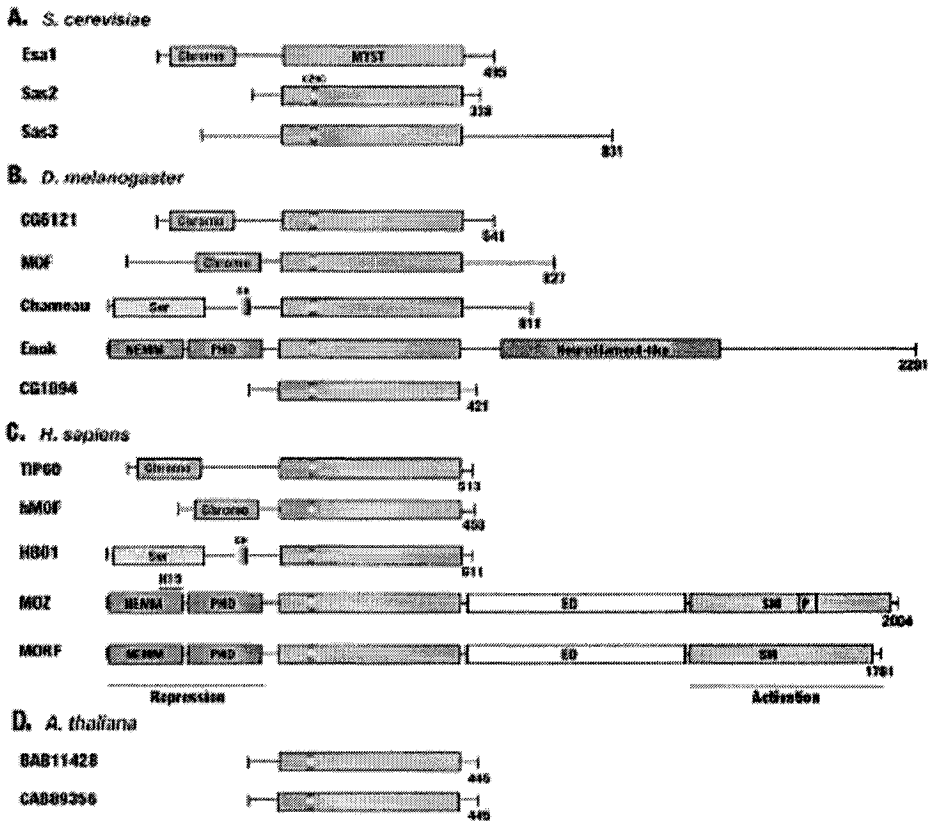

Figure 2: The functional domains of MYST family proteins from *S.cerevisiae* (A), *Drosophila* (B), human (C), *A. thaliana* (D) are depicted. Chromo, chromodomain; Ser, serine–rich domain; CH, cysteine/histidine–rich motif; H15 linker histones H1 and H5; NEMM, N-terminal part of Enok, MOZ or MORF; PHD, PHD zinc finger; ED, glutamate/aspartate rich region; SM, serine/ methionine-rich region; P, proline/glutamine stretch. Adapted from Yang, 2004.

1.4.1 Esa1

Esa1 is a yeast MYST family HAT, that contributes to transcriptional activation and DNA double-stranded break repair (Bird et al., 2002). Its HAT activity is important for mediating silencing of RNA polymerase I transcribed genes at telomeres and also within the ribosomal DNA of the nucleolus (Clarke et al., 2006). Esa1 primarily acetylates histone H4 and to a limited extent, it acetylates H2A and H3. It is also involved in cell cycle progression. Temperature-sensitive mutants display an overall decrease in H4 acetylation levels, and a conditional G2/M arrest that disrupts chromosomal segregation upon the RAD9 DNA damage checkpoint (Clarke et al., 2006). Furthermore, Esa1 is an important component of the NuA4 HAT complex that is important for transcriptional activation at specific target loci, including many ribosomal protein genes (Allard et al., 1999; Doyon and Cote, 2004). Esa1 is also a component of the smaller picNuA4 complex that consists of Elp1, and Yng2 also known as $p33^{Ing1}$ (Boudreault et al., 2003; Selleck et al., 2005). The conserved EPcA domain and chromodomain of Esa1 are important for Piccolo to acetylate nucleosomes (Selleck et al., 2005).

1.4.2 Sas2/Sas3

Sas2 is involved in transcriptional silencing at all silent loci such as HML, HMR, telomeres, and ribosomal DNA in *Saccharomyces cerevisiae* (Ehrenhofer-Murray et al., 1997; Shia et al., 2005). Sas2 is the catalytic subunit of the SAS complex that also includes Sas4 and Sas5 (Xu et al., 1999). It acetylates both histones and nucleosomes and is responsible for the bulk of histone H4 lysine 16 acetylation in

vivo, but this requires the intact acetyl-CoA binding motif of Sas2 (Suka et al., 2002). The acetyl CoA binding motif was shown to be essential for both in vivo silencing function and the enzymatic activity of the SAS complex (Shia et al., 2005). Sas3 was isolated as a gene related to Sas2 and is also involved in HML silencing (Ehrenhofer-Murray et al., 1997). Sas3 was shown to be the catalytic subunit of NuA3 (John et al., 2000). The NuA3 complex acetylates histone H3 on nucleosomes, and the zinc finger in the MYST domain must be intact for this activity (John et al., 2000).

1.4.3 MOF

The D*rosophila* MYST protein Mof, is involved in dosage compensation which ensures that males with a single X chromosome have the same number of gene products as a female with two X chromosomes (Akhtar and Becker, 2000; Gu et al., 1998; Hilfiker et al., 1997). Mof acetylates lysine 16 on histone H4 to compensate male flies for the single X chromosome, and is therefore responsible for a twofold increase in transcription from the single male X chromosome by global hyperacetylation (Akhtar and Becker, 2000). The C_2HC zinc finger of Mof is identified as a nucleosome binding motif (Akhtar and Becker, 2001). In addition, Mof is found in a MSL complex containing Msl1, Msl2 Msl3, Mle and two non coding RNA molecules roX1 and roX2; this complex mediates a significant increase of H4 lysine acetylation (Smith et al., 2000). This is the only HAT complex to date that contains RNA.

1.4.4 TIP60

TIP60 (Tat-interactive protein 60kDa) interacts with the HIV1-Tat protein that typically acetylates histone H4 (Creaven et al., 1999). It is the mammalian homologue of the yeast NuA4 complex, and is involved in the cellular response to DNA damage, cell cycle control and apoptosis (Ikura et al., 2000). It participates in cell cycle arrest as a major component of the p53 pathway. Following UV irradiation, TIP60 is required for p53 dependent G1 arrest and for expression of p53 dependent genes such as p21 (Berns et al., 2004). TIP60 regulates the choice between cell cycle arrest and apoptosis following DNA damage with p300 (Tyteca et al., 2006). It is also activated by DNA-PKC upon DNA damage (Jiang et al., 2006). Overexpression of a dominant negative HAT-defective TIP60 mutant decreases DNA repair and apoptosis upon induction of DNA double strand breaks (Legube et al., 2004). TIP60 plays two roles in the p53 dependent response: first, it functions as a co-factor for p53 to activate the endogenous p21 promoter, and second, it has the capacity to inhibit Mdm2-induced ubiquitination and proteasomal degradation of p53 (Legube et al., 2004). Generally, TIP60 is associated with transcriptional activation of specific genes through local histone acetylation, however it must be noted that TIP60 was also reported to function as a co-repressor in TEL-mediated transcriptional repression (Nordentoft and Jorgensen, 2003) and as a transcriptional repressor for STAT3 (reviewed in (Yang, 2004)). TIP60 is also found upregulated in advanced prostate cancer (Culig and Bartsch, 2006). Lastly, a γ-secretase independent mechanism has been suggested for the signal transduction by amyloid precursor protein (APP) that plays a central role in Alzheimer's disease (Hass and

Yankner, 2005). Autosomal dominant mutations in APP and presenilins lead to increased plaque formation which is a well recognized pathogenic marker for Alzheimer's disease. The mechanism proposed suggests that APP recruits TIP60 to the membrane, leading to TIP60 activation by cyclin dependent kinase (CDK)–mediated phosphorylation, followed by nuclear translocation of TIP60 and Fe65, and an increase in transcriptional activity in the absence of presinilin-mediated γ-secretase cleavage (Hass and Yankner, 2005). In summary, TIP60 is involved in many diverse areas of gene regulation; some that rely on its HAT activities and others that do not.

1.4.5 HBO1

HBO1 (histone acetyltransferase binding to ORC-1) has intrinsic histone acetyltransferase activity and plays a role in DNA replication. It interacts with the Origin Recognition complex (ORC1), and minichromosome maintenance proteins (MCM2) suggesting that acetylation by HBO1 is important in the process of DNA replication (Iizuka et al., 2006). The formation of a pre-replicative complex on replication origins is important for DNA replication to occur. Since chromatin structure is believed to be influential in determining the initiation of DNA replication, HBO1 is proposed to regulate DNA initiation by its HAT activity (Iizuka et al., 2006). Indeed, acetylation status of HBO1 is modulated by cell cycle progression with highest levels of activity during synthesis of the pre-replicative complex including acetylation of histone and non-histone components of the pre-replicative complex (Iizuka et al., 2006). Next, HBO1 was shown to repress

transcription of the androgen receptor (Sharma et al., 2000). More recently, the progesterone receptor, a hormone inducible transcription factor, was reported to interact with HBO1 suggesting that it may play an important role in prostaglandin receptor signaling (Georgiakaki et al., 2006). Lastly, ING4-TAP purified complexes identified Jade1/2/3, hEaf6 and HBO1 in a complex; HBO1 is important for H4 acetylation in vivo, and is important for cell cycle progression (Doyon et al., 2006).

1.4.6 MOZ and MORF

The monocytic leukemia zinc (MOZ) finger protein is associated with leukemia. Its functional domains consist of a transcriptional repression domain at its N-terminus, PHD fingers, an acidic region, a conserved MYST domain that includes a C2HC zinc finger and an acetyl-CoA binding region, and a C-terminal Serine and Methionine rich region involved in activation (Figure 2). MOZ acetylates histones H3, H4 and H2A (Champagne et al., 2001). It was initially identified in a chromosomal translocation t(8;16)(p11;p13) that resulted in an in frame fusion of the CBP gene at 16p13 to the MOZ gene at 8p11 (Borrow et al., 1996; Panagopoulos et al., 2000); this fusion protein caused acute myeloid leukemia. MOZ is known to have other translocation partners such as TIF2, and p300 (Chaffanet et al., 1999). The p300 and CBP genes are located on chromosomes 16p13 and 22q13 respectively, and are frequently rearranged in chromosomal translocations with fusion partners MOZ, MORF and MLL (mixed lineage leukemia) (reviewed in (Yang, 2004)). CBP and its homolog p300 function in cell proliferation, differentiation, apoptosis, and hematopoetic development. These aberrant proteins

no longer retain the SM domain of MOZ/MORF, which is replaced by the C-terminal part of CBP, p300, or TIF2 (Figure 3). These fusion proteins are especially prominent in M4/M5 subtypes of AML characterized by a block in differentiation at the granulo-monocytic stage.

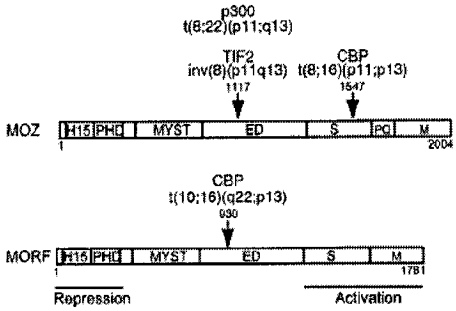

Figure 3: Schematic diagram of MOZ and MORF sequence similarity and associated chromosomal abnormalities. Arrows indicate the breakpoints. H15, linker histones H1-H5 –like module; PHD, plant homeodomain zinc fingers; MYST, MYST acetyltransferase domain; ED, Glu/Asp-rich acidic region; PQ, Pro-Gln-stretch; S, Serine rich domain; M, Methionine rich domain. Adapted from Pelletier et al., 2002.

MOZ has been shown to strongly transactivate both Runx1- and Runx2-dependent transcription (Kitabayashi et al., 2001; Pelletier et al., 2002). AML1/Runx1 mediated transcription by MOZ does not depend on its HAT activity but on its transactivation domain located at its C-terminus and the H15 linker domain (Kitabayashi et al., 2001). Similar observations were reported for MOZ interaction with Runx2 (Champagne et al., 2001; Pelletier et al., 2002). However, MOZ-CBP has been shown to inhibit Runx1-dependent transcriptional activation, and is thought to contribute to the development of leukemia by subverting its function. MOZ-AML1 levels increase during M1 myeloid differentiation but, MOZ-CBP expression inhibits differentiation of M1 murine myeloid cells into macrophages and this inhibition is mediated by the HAT domain of the CBP portion of MOZ-CBP. The mechanism underlying the inhibition of AML1 by MOZ–CBP is unclear. Several mechanisms have been proposed; studies have reported that

p300/CBP requires additional cofactors in the presence of certain transcription factors and that expression of MOZ-CBP may hinder the association of these cofactors with MOZ or p300/CBP resulting in repression (Shikama et al., 1999; Torchia et al., 1997); or repression may occur due to aberrant acetylation by MOZ-CBP or binding to acetylated proteins since mutating the bromodomain and HAT domain of CBP abolish AML1 mediated repression (Kitabayashi et al., 2001).

MOZ-TIF2 is yet another fusion protein that is a result of an inversion inv(8)(p11q13); it contains the N-terminus of MOZ (amino acids 1 to 1547) including the PHD domain, the C-terminus of TIF2, and both MYST domains (Carapeti et al., 1998). TIF2 is a member of the p160 family of coactivators of nuclear receptors known to interact with p300 and CBP. MOZ-TIF2 is a leukemia oncogene that confers properties of self-renewal to hematopoetic progenitor cells and results in acute myeloid leukemia that may be serially replanted (Huntly et al., 2004). It inhibits transcription of CBP/p300-dependent activators such as nuclear receptors including the retinoic acid receptor (RAR) and p53. The AD1 domain of MOZ-TIF2 that interacts with CBP is necessary for this transcriptional inhibitory effect (Kindle et al., 2005). The mechanism proposed for inhibition of nuclear receptors is that MOZ-TIF2 recruits CBP and depletes cellular levels of the protein such that CBP-dependent coactivators are adversely affected. The study concludes that since nuclear receptors have a role in normal haematopoiesis, MOZ-TIF2-associated AML may be caused by subverting the function of these target genes.

In mice, MOZ-TIF2 causes AML in a bone marrow transplant assay and its induction is dependent on the integrity of two functional domains, namely the CBP-

binding domain of TIF2 and the C_2HC zinc finger in the MYST domain of MOZ (Deguchi et al., 2003). Another study has reported that MOZ-TIF2 interacts with RARβ2 promoter, resulting in altered recruitment of CBP/p300 in the absence of ligand, aberrant histone modification, and inhibition of the RARβ2 gene (Collins et al., 2006). The mechanism of MOZ-TIF2 mediated repression of the RARβ2 gene proposes that in the presence of ligand (ATRA), co-repressor complexes that may occupy the RARβ2 promoter are degraded, but since MOZ-TIF2 is also present along with CBP that is aberrantly recruited, they are also degraded depleting cellular stores of CBP. Consequently, transcriptional activation by the RARβ2 does not reach its maximum potential without co-activation by CBP/p300. MOZ-TIF2 also alters histone modification at the RARβ2 promoter by induction of ligand–independent acetylation of H3K9 and H3K14 (Collins et al., 2006). This could to be due to MOZ-TIF2 acetyltransferase activity or aberrant cofactor recruitment. Also, the same study observed that MOZ-TIF2 enhances AML1 activation by recruiting CBP/p300. Similar to MOZ-TIF2, MOZ-CBP strongly inhibits RARα activation, and enhances AML-1 mediated reporter activation (Collins et al., 2006). The latter observation is in contrast to AML1 mediated repression by MOZ-CBP found by another investigator (Kitabayashi et al., 2001); possible reasons are that the latter group included the N-terminal domain of MOZ in their construct which is believed to be a repression domain (Kitabayashi et al., 2001). On a related note, MIP1 (Macrophage inflammatory promoter) has two Runx binding sites via which MOZ acts as a transcriptional co-activator important for the regulation of an inhibitor of stem cells by hematopoetic transcription factors (Bristow and Shore, 2003).

Therefore, chromosomal rearrangements in Runx1 and MOZ genes may contribute to the development of leukemia by alteration of MIP1 expression (Bristow and Shore, 2003).

Recently, mouse MOZ knockout models were designed to investigate whether MOZ plays a role in normal haematopoiesis since MOZ fusion proteins result in leukemia. Truncation of the mouse MOZ gene in mice results in their death at birth (Katsumoto et al., 2006; Thomas et al., 2006). MOZ is widely expressed during embryonic development and in all adult organs with stronger expression in the thymus and lung. In addition, the total number of lymphoid progenitors and total T cell production is reduced in the MOZ mutant thymus; however these cells are still able to undergo normal T cell maturation. Mutating the MOZ gene results in the absence of long-term repopulating stem cells and a reduction in the number of multipotent cells able to form spleen colonies (Thomas et al., 2006). Lastly, pharyngeal segmental identity defects and Hox expression defects are observed in zebra fish carrying mutations in the MOZ gene (Miller et al., 2004).

Our lab identified and characterized MOZ-related factor (MORF) (Champagne et al., 1999). MORF shares significant sequence similarity with MOZ including an N-terminal region containing two C4HC3 PHD fingers, a putative HAT domain, an acidic region and a C-terminal SM-rich domain (Figure 3) (Champagne et al., 1999). A transcriptional activation domain is located at the SM rich region of MORF while the repression domain is located at the N-terminal region suggesting involvement in both positive and negative regulation of gene expression. MORF however does not include a proline-glutamine stretch in the serine methionine rich

domain as does MOZ. Tissue profiling revealed that MORF was ubiquitously expressed similar to MOZ (Champagne et al., 1999). A MORF mutation in mice called Querkopf, demonstrated the function of MORF *in vivo* via a gene trapping vector; homozygotes display defects in calvarial bone and cerebral cortex development, suggesting that MORF is important for osteogenesis and neurogenesis (Thomas and Voss, 2004; Thomas et al., 2000). Querkopf also shares similarity to MOZ in its PHD finger, MYST HAT domain, and C-terminal SM rich domains; it is a histone acetyltransferase that demonstrates substrate specificity, because it acetylates histone H3 and H4. After that, MORF coactivation potential was tested upon osteoblast specific transcription factors. Indeed, MORF coactivated Runx1 as well as Runx2 via its C-terminal SM domain *in vitro* and *in vivo* validating its function as a transcriptional co-regulator (Pelletier et al., 2002). Also, MORF is a component of the transcriptional coactivator nuclear receptor PPARα-interacting complex (PRIC) (Surapureddi et al., 2002). Since PPARα is involved in fatty acid metabolism and is also thought to be involved in inhibition of osteoblast differentiation by Runx2 (Jeon et al., 2003), MORF may act as a co-regulator to determine differentiation of mesenchymal cells to specific cell lineages.

Similar to MOZ, MORF has been reported to form fusion proteins as a result of chromosomal translocation in humans. For example, the CBP gene at 16p13 fuses to 10q22 forming a MORF chimera that leads to childhood AML (Panagopoulos et al., 2001). The MORF-CBP protein retains the zinc fingers, two nuclear localization signals, the HAT domain, and a portion of the acidic domain of MORF; the HAT domain and bromodomain of CBP are also present (Panagopoulos

et al., 2001). Recently, MORF was found disrupted in a case of uterine leiomyomata (Moore et al., 2004). Rearrangement of 10q22 involved breakpoints at 17q. The translocation in uterine leiomyomata disrupts MORF at the NH_2-terminal between the H15 domain (that is homologous to the linker histones H1 and H5) and the PHD fingers of MORF, the C2HC zinc finger, or the acetyl-CoA binding site of the histone acetyltransferase domain (Moore et al., 2004). Mapping of the 17q21 breakpoint by fluorescent *in situ* hybridization revealed possible candidates including the gene of GCN5L2, a HAT that is able to bind p300 and CBP.

The MOZ and MORF fusion proteins are believed to contribute to leukemic and other forms of oncogenesis by causing misdirected histone acetyltransferase activity which alters transcriptional regulation. Our lab has shown that both these HATs possess transcriptional repression and activation domains, making it likely that a chromosomal rearrangement may separate these domains or join them to another gene which would in turn subvert the function of effector genes. MOZ and MORF fusion proteins may contribute to leukemogenesis either by aberrant acetylation of histones or transcription factors, or by interacting with transcription complexes via the activation or repression domains. Still, the mechanism underlying the oncogenic nature of these fusion proteins is not clear.

1.4.7 Components of the MOZ/MORF complex

The MOZ complex was purified by ING5-TAP purification and MOZ, BRPF1/2/3 , and Eaf6 were identified as associated proteins by mass spectrometry (Doyon et al., 2006). The latter protein is found in the NuA3 and NuA4 complex, but very little is

known about its function. More is known about BRPF1/2/3 and ING5, so they will be discussed in more detail here.

The ING family of tumor suppressor

There are five ING proteins in the ING family of tumor suppressors. ING1 was the first to be identified followed by ING2, ING3, ING4, and ING5 proteins (He et al., 2005). They all share a conserved PHD domain at their C-terminus which is implicated in chromatin remodeling (He et al., 2005). One of their biological functions is as a histone acetyltransferase co-factor. In *Saccharomyces cerevesiaie*, there are three ING orthologs, Yng1, Yng2, and Yng3. Yng1 is a stable component of the NuA3 complex comprised of the MYST Sas3 HAT that acetylates H3 and H4 (Howe et al., 2002). Yng2 is part of the NuA4 complex, a multi-subunit complex composed of the MYST Esa1 protein, which is involved in the acetylation of H2A and H4 and is linked to cell cycle progression (Choy et al., 2001; Sterner and Berger, 2000). Deletion of Yng2 results in slow growth and increased sensitivity to UV irradiation (Loewith et al., 2000). Both Yng1 and Yng2 are required for maintaining HAT activity of NuA3 and NuA4, respectively. In addition, the depletion of Yng1 severely compromised HAT activity on free histones and resulted in the inability of NuA3 to acetylate nucleosomes (Howe et al., 2002). These findings suggest that Yng proteins facilitate interactions between HAT complexes and chromatin in yeast (Howe et al., 2002).

Mammalian ING3, ING4 and ING5 are integral components of MYST HAT complexes. ING3 is a stable component of the TIP60/NuA4 complex and is

required for acetylation of chromatin substrates (Doyon et al., 2006). Next, the loss of ING4 has been associated with brain tumor growth and angiogenesis (Garkavtsev et al., 2004). However, ING4-TAP purified complexes revealed HBO1 association with Jade1/2/3 paralogs and Eaf6 (Doyon et al., 2006); HBO1 was found to be involved in the progression of cells in S phase and for the majority of histone H4 acetylation (Doyon et al., 2006). The ING3 versus ING4 complexes have different acetylation specificities. TIP60/ING3 targets nucleosomal histones H4 and H2A while the HBO1/ING4 complex prefers histones H4 and H3. Depletion of TIP60 and HBO1 arrests cells at the G2/M boundary. Next, ING5 is present in two distinct complexes: HBO1-JADE complex and MOZ/MORF-BRPF complexes. Both complexes were purified by ING5 tandem affinity purification, and associated proteins were identified by mass spectrometry. The HBO1-ING5 complex is responsible for histone H4 specific HAT activity, whereas the MOZ/MORF-ING5 complex targets histone H3 (Doyon et al., 2006). MCM proteins are also specific to the ING5-HBO1 complex. This implicates ING5–HAT complexes as important regulators of DNA replication, initiation, and movement along the replication fork.

Another mechanism by which ING proteins are involved in cell cycle regulation is via the p53 tumor suppressor. ING1, ING2 and ING3 suppress growth by p53 dependent transcriptional activation of the p21/Waf1 gene, a cyclin dependent kinase inhibitor involved in cell cycle arrest at the G1/S phase (Garkavstev, 1998). The role of ING1 in UV-induced apoptosis is also p53 dependent; $p33^{ING1b,}$ an ING1 isoform, enhances transactivation of the proapoptotic Bcl-2 family protein Bax upon ultraviolet irradiation (Cheung and Li, 2002). ING1

is also a stable component of the mSin3/HDAC complex and interacts with PCAF, CBP, and p300 (Kuzmichev et al., 2002). In addition, both $p29^{ING4}$ and $p28^{ING5}$ also mediate cell cycle arrest in a p53-dependent manner by promoting acetylation of lysine 382 on p53 (Shiseki et al., 2003). In summary, ING proteins are cofactors of histone acetyltransferases and histone deacetylases that mediate acetylation of cellular components, core histones, and the p53 tumor suppressor thereby linking ING proteins to transcription regulation, DNA repair, and apoptosis. Thus, ING proteins are important components of chromatin modifying complexes.

The BRPF family of co-regulators

There are three members in the BRPF (bromodomain- and PHD finger containing) 1/2/3 family of regulatory proteins. BRPF1 and BRPF2 are known as BR140 and BRD1 respectively. The BRPF proteins may have a role in regulating general transcription machinery. BFPF1 was initially copurified with an integrin (Thompson et al., 1994), however no functional relationship between the two was established. It has features characteristics of a gene regulatory protein, including zinc fingers, a bromodomain, a leucine zipper, a PWWP domain, and is localized in the nucleus (Figure 4) (Thompson et al., 1994). Domain I is suggested to interact with ING proteins since it is found in other proteins that share binding with ING proteins such as Jade1/2/3, yNto1, yEpl1/EPC1/EPC2 and yDep1/BRMS1-like/SDS3 (Figure 4) (Doyon et al., 2006). Domain M is suggested to bind MYST family proteins since it is shared by proteins that associate with MYST HATs, and is absent in proteins that associate with HDAC complexes such as yDep1/BRMS1-like/SDS3. Two regions

in BRPF1 correspond to DNA binding domains of transcription factors, including a zinc finger of TFIIIA and five additional zinc finger motifs (Thompson et al., 1994). There is homology in the cysteine rich region of BRPF1 with AF10, and AF17 which are MLL partner genes (McCullagh et al., 1999). BRPF1 also has a bromodomain which has been identified in other transcriptional activators such as the human brahma protein (BRM) and the yeast protein SNF2/SWI2 (Figure 4) (McCullagh et al., 1999). LIN49 and LIN59 genes in *C.elegans* are related to *Drosophila* trithorax group proteins which are implicated in chromatin remodeling. LIN49 is structurally similar to human BRPF1 (Chamberlin and Thomas, 2000); it is required for normal development of mating structures of the adult male tail, for normal morphology and function of the hindgut cells in both males and hermaphrodites, and for maintenance of structural integrity in the hindgut and egg-laying system in adults (Chamberlin and Thomas, 2000). LIN-49 has a bromodomain which recognizes acetyl-lysine residues, and a cysteine rich region, similar to BRPF1. Functional analysis of BR140 has not yet been reported, so the characterization of LIN-49 is the first for this class of bromodomain containing proteins.

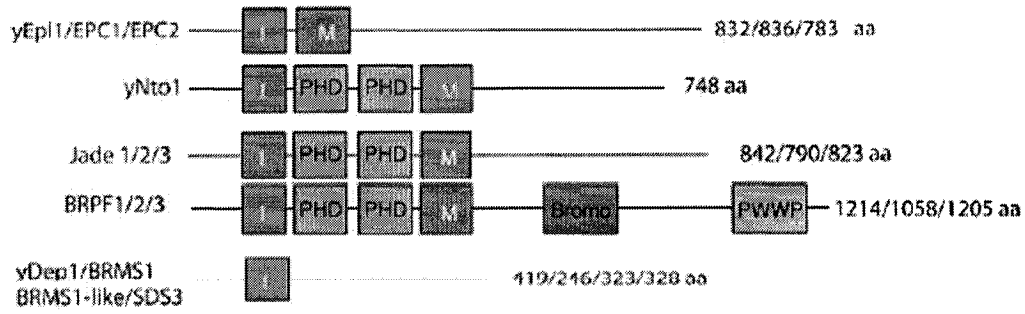

Figure 4: Schematic representation of different proteins that share similar domains as BRPF1/2/3. Adapted from Doyon et al., 2006.

1.5 Runx transcription factors

The Runx genes are reported to function as tumor suppressors and as dominant oncogenes in a context-dependent manner. Runt domain genes are found in phylogenetically diverse organisms (Zhang et al., 1997). The most highly conserved feature of the Runx protein is the 128 amino acid Runt domain; it is a DNA-binding domain that specifically recognizes the consensus-binding site TGT/cGGT. The DNA binding ability of Runx is enhanced by CBFß, the non-DNA binding component of the CBF (core binding factor) complex, when it forms a heterodimer with Runx genes (Bushweller, 2000). The mammalian family of Runt-domain transcription factors is comprised of three members, Runx1, Runx2 and Runx3. They interact with a range of co-activators or co-repressors to regulate haematopoiesis, osteogenesis, and cell cycle progression.

Runx1/AML1/Cbfa2 is a regulator of haematopoiesis and is essential for the development of myeloid and lymphoid lineages. Knockout of Runx1 or CBFβ in mice is lethal at midgestation; the central nervous system undergoes hemorrhaging

and necrosis (Tracey and Speck, 2000). Human germline mutations of Runx1 display autosomal dominant familial platelet disorder with predisposition to acute myeloid leukemia (Michaud et al., 2002). The most frequently observed Runx1 translocation is the t(8;21) which results in the fusion of the N-terminal portion of Runx1, including the Runt domain to a hetererologous partner ETO (Lutterbach et al., 2000; Nucifora et al., 1994). Many studies have reported that Runx1 fusion proteins act as dominant–negative inhibitors of the normal Runx1 gene. A C-terminal truncation leads to loss of transactivation potential but does not affect DNA binding, therefore creating a potential competitive inhibitor of Runx1. The consequence of the fusion of Runx1 to ETO is repression of Runx1-dependent transcription by recruitment of corepressors (Okuda et al., 1998; Yergeau et al., 1997). Similarly, TEL-Runx1 has also been shown to function as a constitutive repressor of Runx target genes (Guidez et al., 2000). The main oncogenic feature of Runx1 translocations is immature development of hematopoetic cells, resulting in self-renewal instead of differentiation. Runx transcription factors are also required for gene silencing in mice. More specifically, Runx1 and Runx3 are required for CD4 silencing in vivo (Taniuchi and Littman, 2004).

Runx2/Cbfa1 is required for development of the skeleton and haploinsufficiency causes cleiodocranial dysplasia while Runx2 overexpression is common in bone metastatic cancers (reviewed in (Schroeder et al., 2005)). Runx2 is important for mesenchymal condensation, osteoblast differentiation from mesenchymal stem cells, chrondrocyte hypertrophy, and vascular invasion of developing skeletons (Komori, 2005). It is regulated by many transcriptional co-

factors (Figure 5). The primary activation domain of Runx proteins is a proline-serine-threonine (PST) rich region that is distal to the NLS (Figure 5). This domain induces transcriptional activation of a heterologous promoter when fused to an appropriate DNA binding domain (reviewed in (Schroeder et al., 2005)). The amino terminus of Runx proteins may be necessary to reach maximal transcriptional activation but is not sufficient to activate a heterologous promoter. This region contains a QA domain – a polyglutamine and polyalanine rich domain that is not present in Runx1 or Runx3.

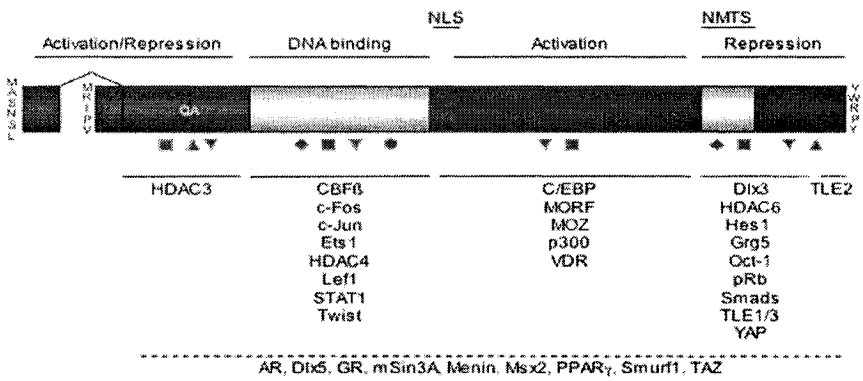

Figure 5: Runx2 functional domains. Adapted from Schroeder et al., 2005.

Runx2 on human chromosome 6p21 is subject to amplification in osteosarcomas (Man et al., 2004). However, the consequences of gene expression of the amplicon remain to be examined. On the other hand, Runx2 overexpression interferes with murine T-cell differentiation at an immature CD8-positive stage, although the cells that accumulate in the preleukemic phase are non-proliferative (Vaillant et al., 2002). Further evidence in favor of Runx2 oncogenic potential are

exogenous factors that signal through Runx2 such as regulators of tumor cell growth, including members of the fibroblast-growth factor family (Lee et al., 2003; Xiao et al., 2002), and insulin like growth factor-1 that upregulate Runx2 transcription (Zhao et al., 2004). These factors regulate Runx2 through signaling pathways that are dependent on phosphatidylinositol 3-kinase (PI3K), AKT and protein kinase C, which are frequently activated in cancer. Runx2 is also involved in the regulation of genes associated with tumor progression, invasion, and metastasis, such as osteopontin, bone sialoprotein, and collagensases (matrix metalloproteinase 13), all of which have been implicated in metastasis and invasion (reviewed in (Shore, 2005)).

Recent reports indicate that Runx2 may be implicated in breast cancer. Runx2 is expressed in breast cancer cells and is required for the development of osteolytic lesions. Some studies indicate that Runx2 is deregulated in breast cancer cells or in prostate cancer cells affecting bone metastasis, while others report that Runx2 is also expressed in normal mammary epithelial and prostate tissues (reviewed in (Shore, 2005)). Accumulating evidence suggests that Runx2 has a role in normal mammary gland. Since breast cancers preferentially metastasize to bone and express many genes important for bone remodeling such as RANK, RANKL, Vitamin D, bone sialoprotein, osteopontin and calcitonin, it is postulated that Runx2 may have a role in breast cancer associated osteolytic lesions (Shore, 2005). In addition, Runx2 stimulates the mouse β-casein gene promoter which contains a Runx2 binding site suggesting that Runx2 contributes to mammary gland specific casein genes and validates further that its deregulation may be involved in breast

cancer (Inman et al., 2005). Interestingly, a mouse implantation model demonstrated that disruption of Runx2 does indeed abolish the formation of osteolytic lesions in MDA-MB-231 cells (Javed et al., 2005). Additionally, bone morphogenetic proteins (BMPs) regulate the expression and activation of Runx2 (Jeon et al., 2006). The Smad family of proteins function downstream of BMP and are thought to trigger osteoblast differentiation by inducing Runx2 gene expression. Recently, Smurf1, an E3 ubiquitin ligase, was shown to be responsible for ubiquitin-dependent Runx2 degradation (Jeon et al., 2006). Smads mediate Runx2 acetylation in response to BMP-2, and acetylation protects Runx2 from Smurf-1 mediated degradation. Runx2 acetylation is required for stabilization and for transactivation activity. The amino terminus of Runx2 also has a repressive domain, suggesting involvement as a potent transcriptional repressor. Repression and activation by Runx family members is cell-type specific and depends on associated cofactors.

Runx3/Cbfa3 has a role in gastric and neuronal development. In Runx3 -/- mice, the gastric mucosa undergoes hyperplasia due to loss of TGF-β growth inhibitory activity and TGF-β mediated apoptosis (Torquati et al., 2004). Runx3 inactivation in gastric cancers occurs by hemizygous deletion and gene silencing by promoter hypermethylation (Ku et al., 2004). The gastric mucosa of Runx3 knockout mice undergoes hyperplasia because of stimulation of proliferation and suppression of apoptosis, processes that are accompanied by a decreased sensitivity to TGF-β (Li et al., 2002). Runx3 is therefore defined as a gastric tumor suppressor. Runx3 is also a target of the acetyltransferase activity of p300 which protects it from Smurf-mediated degradation (Jin et al., 2004). p300 is reported to acetylate lysine

residues on Runx3 with the help of TGF-β superfamily signaling enhancing the level of acetylation and the stability of Runx3. Since the same lysine residues are targeted by ubiquitin ligase Smurfs, TGF-β suppresses Runx3 degradation through competitive acetylation.

1.6 Rationale for the thesis project

There is substantial evidence supporting that MOZ/MORF fusion proteins are involved in leukemia. These two acetyltransferases are members of the MYST family, which have not been as well studied as GCN5, PCAF, p300, and CBP. The GCN5/PCAF family has been well characterized and components of their complexes have also been identified. Also well studied is the p300/CBP family of transcriptional coactivators, which is known to acetylate a wide range of transcription factors. However, the MOZ/MORF tetrameric complex has only recently been identified. Moreover, the MOZ-associated proteins BRPF1, ING5, and Eaf6 have not been so well characterized. Thus far, it is accepted that ING5 is a tumor suppressor and members of this family are implicated in modulating HAT activity. It is also known that MOZ is a potent transcriptional coactivator of Runx1 and Runx2; however, how they all fit into an overall scheme of gene regulation is yet to be determined. In order to comprehend the mechanistic function of MOZ in gene expression and leukemogenesis, further characterization of its tetrameric complex needs to be conducted. This is the subject of my thesis project, described in the next chapter.

CHAPTER II

DISTINCT ROLES OF BRPF1 AND ING5 IN REGULATING ACTIVITIES OF MOZ AND MORF

Mukta Ullah, Lin Xiao, Jacques Côté, and Xiang Jiao Yang

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ABSTRACT

Monocytic leukemia zinc finger protein (MOZ) is a MYST-family histone acetyltransferase whose gene is rearranged in chromosomal translocations giving rise to acute myeloid leukemia. This acetyltransferase functions as a potent transcriptional coactivator of Runx1 and Runx2, two homologous transcription factors that are important for definitive haematopoiesis and osteoblast maturation, respectively. Mouse knockouts have demonstrated that MOZ is required for the maintenance of hematopoetic stem cells, and is important for erythroid and myeloid cell differentiation. The characterization of the tetrameric MOZ complex suggests that its activity can be modulated by the presence of associated subunits such as BRPF1, and ING5. We show here by histone acetyltransferase assays that MOZ and MORF activity is indeed enhanced by associated proteins. We have mapped the interaction domains of BRPF1 that are required for binding by MOZ, ING5, and Eaf6 in an effort to understand the mechanism of activation. Given that MOZ and ING proteins contribute to oncogenesis by chromosomal translocations and loss of function respectively, the characterization of the multisubunit complex provides novel mechanistic insights into its function in normal cells and under conditions of leukemia or other cancers.

INTRODUCTION

MOZ and MORF, MYST family HATs, are both involved in chromosomal translocations associated with acute myeloid leukemia. They have intrinsic HAT activity and are ubiquitously expressed (Champagne et al., 1999; Champagne et al., 2001). MOZ was initially isolated as a fusion protein with CBP t(8;16)(p11;q13) that caused acute myeloid leukemia (Borrow et al., 1996). MOZ-CBP inhibits the differentiation of M1 myeloid precursor cells into macrophages (Kitabayashi et al., 2001). Other MOZ fusion proteins also include MOZ-p300 and MOZ-TIF2 (Carapeti et al., 1998; Chaffanet et al., 2000). The latter confers properties of self-renewal to hematopoetic progenitor cells and results in acute myeloid leukemia that can be serially replanted (Huntly et al., 2004). It inhibits transcription of CBP/p300-dependent activators such as nuclear receptors including the retinoic acid receptor (RAR) and p53 (Deguchi et al., 2003; Huntly et al., 2004).

MOZ/MORF physically interacts with and regulates Runx1 and Runx2-dependent transcription via the transactivation domain (Pelletier et al., 2002). Human germline mutations of Runx1 display autosomal dominant familial platelet disorder with predisposition to acute myeloid leukemia (Michaud et al., 2002). Runx2 is required for development of the skeleton and haploinsufficiency causes cleiodocranial dysplasia while Runx2 overexpression is common in bone metastatic cancers (Schroeder et al., 2005). Although the interaction between MOZ/MORF and Runx transcription factors confirm that MOZ is a transcriptional coactivator, little is known regarding mechanistic regulation. Recently, the MOZ complex was purified by ING5-TAP purification and MOZ, BRPF1/2/3 , and Eaf6 were identified as the

associated proteins by mass spectrometry (Doyon et al., 2006). This was a much anticipated finding since there was speculation that MOZ/MORF probably did exist in a protein complex, as in the case of yeast NuA3 and NuA4 complexes, and the GCN5 complex. The involvement of ING proteins in histone acetylation is evident. Yng1 is a stable component of the NuA3 complex which is comprised of the MYST Sas3 HAT that acetylates H3 and H4 (Howe et al., 2002). In addition, the depletion of Yng1 severely compromised HAT activity on free histones and resulted in the inability of NuA3 to acetylate nucleosomes. Yng2 is part of the NuA4 complex, a yeast multi-subunit complex composed of the MYST Esa1 protein, which is involved in the acetylation of H2A and H4 and is linked to cell cycle progression (Choy et al., 2001; Sterner and Berger, 2000). This suggests that Yng proteins facilitate interactions between HAT complexes and chromatin (Howe et al., 2002). Similarly, we hypothesize that MOZ/MORF complex subunits will influence HAT activity of the complex. There are three members in the BRPF (bromodomain- and PHD finger containing) 1/2/3 family of regulatory proteins (Thompson et al., 1994). BRPF1 and BRPF2 are known as BR140 and BRD1 respectively. They have features characteristic of a gene regulatory protein, including zinc fingers, a bromodomain, a leucine zipper, a PWWP domain, and are localized in the nucleus.

In this study, we evaluated the molecular mechanisms underlying MOZ/MORF HAT activity in the presence of BRPF1, ING5 and Eaf6. We show that MOZ/MORF is significantly influenced by the presence of BRPF1. We characterize the interaction domains of BRPF1 in an effort to comprehend the molecular mechanism of BRPF1-dependent coactivation of the tetrameric complex.

Finally, we show that transcription factors dependent on MOZ/MORF coactivation are also influenced by BRPF1.

MATERIALS and METHODS

Plasmid construction. Plasmid constructs containing BRD1, ING5, and Eaf6 were subcloned by PCR into the EcoRI/HindIII sites of pcDNA3.1-HA based vectors, and into pAcSG2-HA based baculovirus vectors. The full length Flag-MOZ and Flag-MORF-HAT expression plasmids were generated as fusion proteins using the Bacmid system (Champagne et al., 2001). Mutants were generated by PCR with Expand thermostable DNA polymerase (Roche). Expression plasmids for MBP-fusion proteins were cloned by inserting the DNA fragment into pMAL-C_2, a vector that contains the maltose binding (MBP) tag (New England Biolabs). Other baculovirus and MBP fusion expression plasmids used were constructed by previous members in the lab (Pelletier et al., 2002). The 6OSEII-luciferase reporter is under the control of six tandem copies of the osteocalcin–specific repeat element. The GM10-luciferase reporter contains a GM-CSF promoter fragment upstream from the luciferase coding sequence. This promoter has binding sites for AML1.

Protein expression and purification. The expressed fusion protein, Flag-MORF-HAT was affinity purified on M2 agarose and eluted with Flag peptide (Sigma, 0.4 mg/ml). HA-tagged Eaf6, ING5, and BR140 were co-purified in Sf9 cells. For all protein affinity purifications in Sf9 cells, buffer B (20 mM Tris-HCl pH 8.0, 10% glycerol, 5 mM $MgCl_2$, 0.1% NP-40 and protease inhibitors) containing 0.25M KCl was used as lysis and washing buffers. To verify complex formation, purified F-MORF-HAT and HA-tagged proteins were resolved by reducing SDS-PAGE and stained with Coomassie Blue R-250 (Bio-Rad).

To examine the interaction of MOZ with BR140, Eaf6 and ING5 in vivo, an expression plasmid for Flag-tagged MOZ was transfected into 293 cells along with expression plasmids for HA-BR140, HA-ING5 as well as HA-EAF6. A total of 10μg of plasmid was used to transfect 5-10 x 10^5 cells (10 cm dish) with 20μl of SuperFect (Qiagen) transfection reagent. Cells were washed twice with PBS 48 hours post-transfection, and collected in 0.9 ml buffer K (20 mM sodium phosphate pH 7.0, 30 mM sodium pyrophosphate, 0.1% NP-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na_3VO_4, and protease inhibitors) containing 0.15 M KCl. This buffer was used as lysis and washing buffers to remove unbound proteins. Purifications were performed by immunoprecipitating on M2 Agarose beads (Sigma). Beads were washed four times with 0.3 ml buffer K (150 mM), and bound proteins were eluted with the same buffer containing Flag peptide (Sigma, 0.4 mg/ml). Eluted proteins were subsequently resolved by SDS-PAGE and transferred to nitrocellulose membrane for Western blotting analysis with anti-Flag or anti-HA antibody. Blots were developed with Supersignal chemiluminescent substrates (Pierce).

Protein protein interaction assays. The interaction between MBP-MORF-HAT, MBP-MOZ-HAT and their deletion mutants with *in vitro* synthesized BRD1 and its deletion mutants was examined in the presence of [^{35}S]Methionine obtained using the TNT-T7 coupled reticulocyte lysate kit (Promega). MBP-expression plasmids were incubated in 20 μl of amylose resin for 30 minutes at 4°C. Next, they were washed twice in Buffer B (250 mM) before incubation with *in vitro* synthesized transcribed and translated product. Following rotation at 4°C for 1 hour, the beads

were washed with Buffer B (250 mM) four times, eluted with 10% maltose, and then loaded on a 10% SDS-PAGE gel to be processed by autoradiography.

HAT assays. Purified combinations of HA tagged BR140, -Eaf6, -ING5 and Flag-MORF- HAT from Sf9 insect cells were mixed in a 20 µl reaction containing 50 mM Tris-HCl pH 8.0, 10% glycerol, 1 mM DTT, 0.1 mM EDTA, 1 mM PMSF,10 mM sodium butyrate and 2.5 nCi [^{3}H]acetyl-CoA. The reactions were incubated at 30°C for 10 minutes, and were then immediately spotted onto p81 Whatmann paper. They were air-dried for 30 minutes, and then washed in buffer (50 mM $NaHCO_3$-Na_2CO_3, pH 9.2) at 30°C with agitation (70 rpm) for 1 hour. The filter was air-dried for 1 hour before quantification of HAT activity was performed by scintillation counting. Further details may be found in (Pelletier et al., 2003).

Reporter gene assays. Plasmids were prepared using double CsCl gradient ultra-centrifugation, butanol extraction, and ethanol precipitation. Human embryonic kidney 293 cells were transiently transfected with a luciferase reporter (200 ng) and/or mammalian expression plasmids of varying concentrations with Superfect (Qiagen) transfection reagent. pBluescript (KSII)(+) was used to normalize the total amount of plasmids used in each transfection and CMV-βgal (50 ng) was cotransfected for normalization of transfection efficiency. Cells were harvested 48 hours post-transfection, and luciferase activity was quantified using Galacto-Light Plus (Tropix, Perkin-Elmer) as the substrate. The chemiluminescence from

activated Luciferin or Galacto-Light Plus was measured on a luminometer plate reader (Dynex).

Antibodies. Anti-HA (Sigma) and anti-Flag (Sigma) antibodies were used in 1:2000 dilution. Secondary anti-mouse and anti-rabbit horse radish peroxidase (Amersham Biosciences) were used in 1:5000 dilution.

Cell culture. Human embryonic kidney 293 cells were cultured in Dulbecco's modified Eagle's medium, containing 10% FBS (Invitrogen), and 1% streptomycin/penicillin at 37°C. Sf9 cells were cultured in suspension in Graces' insect medium supplemented with 1% Pluronic acid F-68, 10% FBS, and 1% streptomycin/penicillin at 27°C.

RESULTS

Components of the MOZ tetrameric complex

It is well accepted that HATs exist in vivo as components of protein complexes. The well characterized GCN5 and PCAF complexes have been identified and characterized. For example, Yng1 protein is a stable component of the NuA3 complex which is comprised of the MYST Sas3 HAT (Howe et al., 2002), and Yng2 is part of the NuA4 complex (Choy et al., 2001; Sterner and Berger, 2000). Yng proteins facilitate interactions between HAT complexes and chromatin (Howe et al., 2002). Recently, ING5-TAP purified complexes in Hela S3 cells revealed three new HAT complexes (Doyon et al., 2006). ING4 and ING5 associated with HBO1, and ING5 associated with MOZ/MORF, both of which are MYST family HATs (Doyon et al., 2006). BRPF1/2/3 (bromodomain and PHD finger containing protein) and Eaf6 were also revealed in the ING5-MOZ/MORF purified complex. To establish this complex in mammalian cells, we performed coimmunoprecipitation analysis. Flag-tagged MOZ was transiently cotransfected with HA-BRPF1, HA-ING5, and HA-Eaf6. Cell lysates were prepared, and were immunoprecipitated with M2 agarose beads for Flag tagged proteins. Western blot analysis with HA antibodies indicated that MOZ coimmunoprecipitated with BRPF1 (Figure 1, lane11). ING5 does not associate with MOZ directly, but indirectly through BRPF1 (Figure 1, lane 14). Eaf6 (Esa1-associated factor) does not associate with this complex unless MOZ, BRPF1, and ING5 are present (Figure 1, lane 17). This confirms that the complex is stable in mammalian systems. Furthermore, antibodies raised in rabbits against BRPF1, BRPF3, and ING5 were used to verify

endogenous interaction of the complex. We successfully immunoprecipitated BRPF1, BRPF3, and MOZ with the respective antibodies in 293 nuclear cell extracts; western analysis revealed that this complex does exist under physiological conditions (data not shown).

Identification of the binding site of BRPF1 on MOZ and MORF

To map the domain(s) of MOZ and MORF that mediate binding to BRPF1, we expressed MORF fragments as MBP-tagged fusion proteins in E.Coli. Further details on construct design may be found in (Pelletier et al., 2002). MBP-pull down assays with *in vitro* transcribed and translated BRPF1 in the presence of [^{35}S]methionine demonstrates whether the interaction is direct. Figure 2A (left panel), Figure 2B (left panel), and Figure 2C (left panel) are schematic diagrams of the constructs used for each binding assay (Champagne et al., 1999; Pelletier et al., 2002). The Coomassie stained gels demonstrate that the levels of purified protein are comparable (Figure 2A, right panel), (Figure 2B, right panel), and (Figure 2C, right panel). As shown in Figure 2A (right panel), lane 1 indicates that the *in vitro* transcribed product is specific since it migrates to approximately 120kDa. Lane 2 is the negative control indicating that non-specific interaction does not occur between MBP and BRPF1. BRPF1 directly interacts with the HAT domain of MORF between residues 361 and 716 (Figure 2A, lane 4), and not with the N-terminus, serine rich or methionine rich domains of MORF.

Next, to verify which region of MORF-HAT is important for binding by BRPF1, we used MORF-HAT deletion constructs designed by a previous member in

the lab (Champagne et al., 1999). MBP-A is intact MORF-HAT from residues 361-716; MBP-B is truncated MORF-HAT from residues 460-716; and MBP-C is MORF-HAT with a deletion from residues 553 to 588 so that the acetyl CoA binding site is no longer intact (Figure 2B, left panel). The *in vitro* transcribed and translated product is specific to BRPF1 (Figure 2B, lane 1). Lane 3 indicates that the intact MORF-HAT domain binds BRPF1 as expected. The MBP-B and MBP-C also bind BRPF1 indicating that the acetyl-CoA binding motif is not required for the interaction. Also, to verify whether BRD1 and BRPF3, share similar binding specificity as BRPF1 , *in vitro* transcription and translation of BRD1 and BRPF3 in the presence of [^{35}S]methionine was included to conduct binding assays as above. Figure 2C (right panel) indicates the three *in vitro* transcribed and translated products corresponding to the three BRPF homologs (lane 1-3). We show that they share similar binding to MOZ and MORF HAT domains (Figure 2C, lane 5-6, lane 8-9 and lane 11-12). This shows that all BRPF family members interact with MOZ and MORF, indicating that perhaps they share a conserved function.

Effect of BRPFs and ING5 on acetyltransferase activity of MOZ and MORF

Since it is established that subunits in a HAT complex influence enzymatic activity of the complex; we speculated that MOZ/MORF HAT activity might also be modulated by other components in the complex. In order to determine the contribution of each subunit in the MOZ/MORF complex, we co-expressed recombinant proteins in Sf9 insect cells. In Sf9 insect cells, proteins have a greater chance to be properly folded and processed while in mammalian cells, there is also

the possibility that other proteins associating with the complex may be pulled down during co-immunoprecipitiation. Given that the MORF-HAT domain binds BRPF1, we used Flag tagged MORF-HAT to assemble the complex in Sf9 cells; also the full length protein has been reported to be difficult to express in Sf9 cells (Pelletier et al., 2002). Combinations of HA-BFPF1, HA-ING5, and HA-Eaf6 were used to infect Sf9 insect cells (Figure 3A). Flag-MORF-HAT was immunoprecipitated on M2 Agarose beads, and purified complexes were visually observed by Coomassie staining. MORF-HAT associates with BRPF1 (Figure 3A, lane 2) confirming that the association is specific in Sf9 cells. ING5 does not associate with MOZ unless BRPF1 is present (lane 5) again showing that the association is indirect. Eaf6 associates with the MORF complex only when ING5 and BRPF1 are also present (lane 8); viruses expressing Eaf6 and ING5 did not yield high protein levels and as a result the band intensity is very faint. This demonstrates that perhaps ING5 stabilizes the association of Eaf6 in the complex. At this point, the function of Eaf6 is not clear.

It has been reported that MOZ and MORF possess HAT activity (Pelletier et al., 2002), and HAT activity is known to be modulated by subunits present in the complex. This is true in the case of NuA3 (Howe et al., 2002), and also for the hNuA4/TIP60 HAT complex in which ING3 is required for acetylation of chromatin substrates (Doyon et al., 2004). Thus, an interesting possibility is that ING5 and/or BRPF1 modulates HAT activity of the complex and contributes to histone acetylation specificity. In order to evaluate the contribution of each subunit to HAT activity of the tetrameric complex, we performed histone acetyltransferase assays.

In these assays, [^{3}H] acetyl-CoA is used as the coenzyme. Labeled histones are separated from non-labeled histones by retention on p81 Whatman phosphocellulose filter, followed by liquid scintillation counting (Pelletier et al., 2003). The purified eluates from SF9 insect cells as listed in Figure 3A were used in HAT assays. The assay clearly indicates that BRPF1 influences HAT activity of the complex by approximately 500 fold (Figure 3B, lane 2), and addition of ING5 further increases HAT activity (lane 5). Addition of all four subunits including Eaf6 increases HAT activity more than with just BRPF1 alone (lane 8). This assay confirms that HAT activity is enhanced when MOZ/MORF are in a tetrameric complex. It is in agreement with previous studies done with yeast NuA3 and NuA4. One important experiment will be to monitor the HAT activity of the complex with nucleosomes since they are physiologically representative. Additionally, since BRPF1 demonstrates this potential to increase HAT activity of the complex, we examined whether BRPF1 could regulate transcription when tethered to a promoter. For this, a series of constructs were engineered to express BRPF1 and its deletion mutants fused to the Gal4 DNA-binding domain. However, there was no evident increase in transcription (data not shown). Similarly, we fused ING5 and Eaf6 to Gal4 DNA-binding domains, but they too did not possess intrinsic activity (data not shown).

Mapping the MOZ, ING5, and Eaf6 binding sites on BRPF1

To ascertain how BRPF1 coactivates MOZ such that there is an increase in MOZ HAT activity, functional domains of BRPF1 were analyzed in an effort to shed some light on this mechanism. To locate MOZ, ING5, and Eaf6 binding sites on BRPF1,

deletion mutants were constructed in HA-tagged expression vectors (Figure 4A). BR1 is the full length protein BRPF1 (1-1214 aa); BR2 (1-660 aa) spans two zinc finger domains, the I domain which is shared by proteins that bind ING family members, and includes the M domain which is believed to mediate binding to MYST family of proteins; BR3 (505-845 aa) includes only the M and bromodomain; BR4 (845-1214 aa) includes the C-terminal region of BRPF1; BR5 (1-552 aa) includes the I domain to the beginning of the M domain; BR6 (1-301 aa) includes only the I domain; BR7 (260-552 aa) and BR8 (260-471 aa) include the zinc finger region. MOZ interacts with deletion mutants BR1 (lane 1), BR2 (lane 2), and BR5 (lane 5); weak binding was observed with BR6 but this was evident only with very long exposure times (lane 6) suggesting that the I domain is required for binding, but the M domain may potentiate stronger interaction (Figure 4B, right panel). BR6 and BR7 were difficult to express (lane 6-7) as shown in the extract. To confirm these results, we performed *in vitro* transcription and translation of BR1, BR2, BR3, BR4, and BR6 in the presence of [^{35}S]methionine (Figure 4B, right panel, lanes 1-5). The Coomassie stained gel confirms that protein levels are comparable (Figure 4B, lanes 6-15). For the binding assay, the MOZ-HAT (510-810 aa) domain fused to MBP was used to assess interaction. As shown in Figure 4B (right panel) BR1, BR2, and BR6 directly associate with the HAT domain of MOZ confirming that the I domain is necessary for this interaction (lanes 7, 9, and 11). The intensity of bands in lanes 9 and 11 are poor, but longer exposures do confirm that BR1, BR2, and BR6 interact with MOZ-HAT (data not shown). Next, ING5 interacts strongly with BR1 (Figure 4C, lane 1), BR2 (lane 2), BR3 (lane 3) which includes only the M and

bromodomain, but weakly associates with BR5 (lane 5), and BR6 (lane 6); the latter two associations are only visible upon longer exposure times (data not shown). This may be due to the fact that BR5 and BR6 both expressed poorly as shown in the extract (lane 5-6); optimization of conditions is still required. This suggests that ING5 may have two binding sites; the binding site located at the I domain potentiates a weak interaction but is sufficient, while binding with the M domain is important for establishing a stronger association. Next, Eaf6 requires only the M domain for association (Figure 4D, lanes 9-11). Mutants that do not include the M domain do not show binding to Eaf6. Although BRPF1 does have binding sites for EAF6, it is not stable enough to interact with MOZ in a tetrameric complex unless ING5 is present; this suggests that ING5 may act to stabilize Eaf6. This may be one reason why ING5 association with the M domain is stronger; because it is required to stabilize interaction with Eaf6. To further explore the association between subunits in the complex, Flag-MOZ and HA-BRPF1, HA-ING5, and HA-Eaf6 were transfected into NIH 3T3 cells to conduct immunofluorescence studies. The localization of these proteins in the presence of a subset of the subunits in the complex or all the subunits in the complex is an indicator of the role that each has to play in regulating MOZ activity. We found that MOZ is localized in the nucleus, and that ING5 can recruit BRPF1 to the nucleus to associate with MOZ (data not shown).

Role of the MOZ tetrameric complex in regulating Runx2-dependent transcription

Since MOZ and MORF are known to coactivate Runx2-dependent transcription (Pelletier et al., 2002), we verified whether this could be functionally linked to their association with ING5/BRPF1. Reporter gene assays with 6OSEII-luciferase promoter, that has binding sites for Runx2, show coexpression of MOZ, BRPF1, and ING5 leads to greater Runx2-dependent transcription than with just MOZ and BRPF1, or MOZ and ING5 (Figure 5A). This suggests that MOZ/MORF coactivation potential can be further enhanced by ING5 and BFPF1 proteins supporting that the tetramer complex indeed enhances activity of the associated HAT. To make certain that this effect is specific to ING5 and not other ING family members; we tested ING3 and ING4 in reporter assays (Figure 5B). ING4 does not function as a coactivator of Runx2 while ING3 is intermediate, confirming that the increase in coactivation is partly dependent on ING5.

BRPF1 interacts with MOZ to increase Runx2-dependent transcription. However, mapping analysis of BRPF1 interaction domains suggest that it mediates binding with MOZ via the I domain although the M domain may potentiate stronger interaction. To corroborate this further, we verified whether Runx1/2 dependent transcription potential could still be increased by BRPF1 mutants. Runx1 dependent transcription increases with BR1 as expected; BR2 (includes M domain) potentiates greater Runx1-dependent transcription compared to BR6, however not to levels that are obtained with full length BRPF1, (Figure 6A). This indicates that the M domain is required to exert a significant coactivation potential upon MOZ. Since the levels

of transcription did not reach the levels of that with full length protein, the full length protein must be more stable. Coactivation potential of the BRPF1 mutants upon MOZ was similar for Runx2-dependent transcription (Figure 6B). These assays need to be repeated to verify that they are consistent.

Figure 1: Tetrameric MOZ complex

293 human embryonic kidney cells were transiently transfected with Flag-MOZ and HA-BRPF1, HA-ING5, and HA-Eaf6. Immunoprecipitation by M2 agarose was followed by western blot analysis with anti-HA antibodies and anti-flag antibodies (1:5000). Western blots show that MOZ associates with HA-BRPF1, and indirectly with HA-ING5 via HA-BRPF1, and HA-Eaf6 when only BRPF1 and ING5 are both expressed. The arrows indicate migration of the proteins.

Figure 1

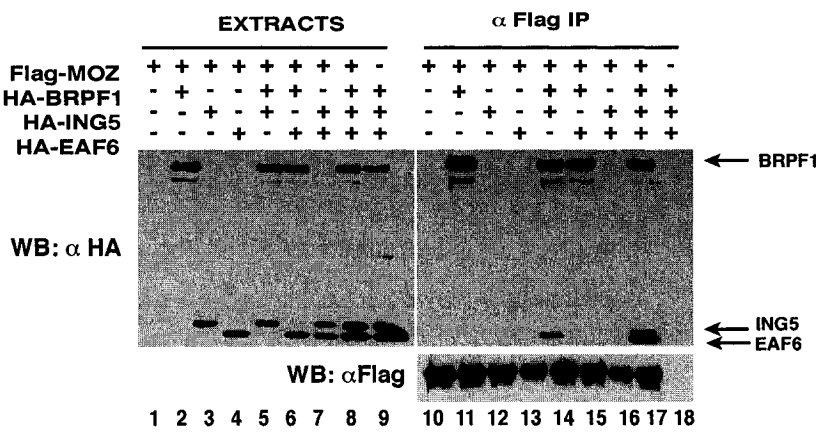

EXTRACTS
α Flag IP
Flag-MOZ
HA-BRPF1
HA-ING5
HA-EAF6
WB: α HA
BRPF1
ING5
EAF6
WB: αFlag
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 2: Interaction of BRPF1 with MOZ and MORF

(A) Schematic representation of MORF and its fragments (left panel). Bold lines depict MORF-fragments, and numbers indicate position of the amino acids. BRPF1 binding is summarized at the right (left panel). Bacterial extracts expressing MBP, MBP-N, MBP-HAT, MBP-Serine, and MBP-Methionine fragments were incubated with amylose agarose in the presence of [^{35}S]methionine labeled BRPF1, and specifically bound proteins were analyzed by reducing SDS-PAGE, and stained with Coomassie blue (right panel) prior to autoradiography (right panel).

(B) Schematic representation of MORF HAT mutants (left panel). Numbers indicate the position of amino acids. Bacterial extracts expressing MBP fusion MORF-HAT mutants were incubated with amylose agarose in the presence of [^{35}S]methionine labeled BRPF1. BRPF1 specifically bound proteins were analyzed by reducing SDS-PAGE, and stained with Coomassie blue prior to autoradiography (right panel).

(C) Schematic representation of MOZ and MORF-HAT mutants (left panel). BRPF1, BRPF2, and BRPF3 were labeled with [^{35}S]methionine and incubated with MOZ-HAT or MORF-HAT MBP-fusion proteins (right panel). They show a similar pattern of binding as that of BRPF1. Specifically bound proteins were analyzed by reducing SDS-PAGE, and stained with Coomassie blue prior to autoradiography (right panel)

Figure 2

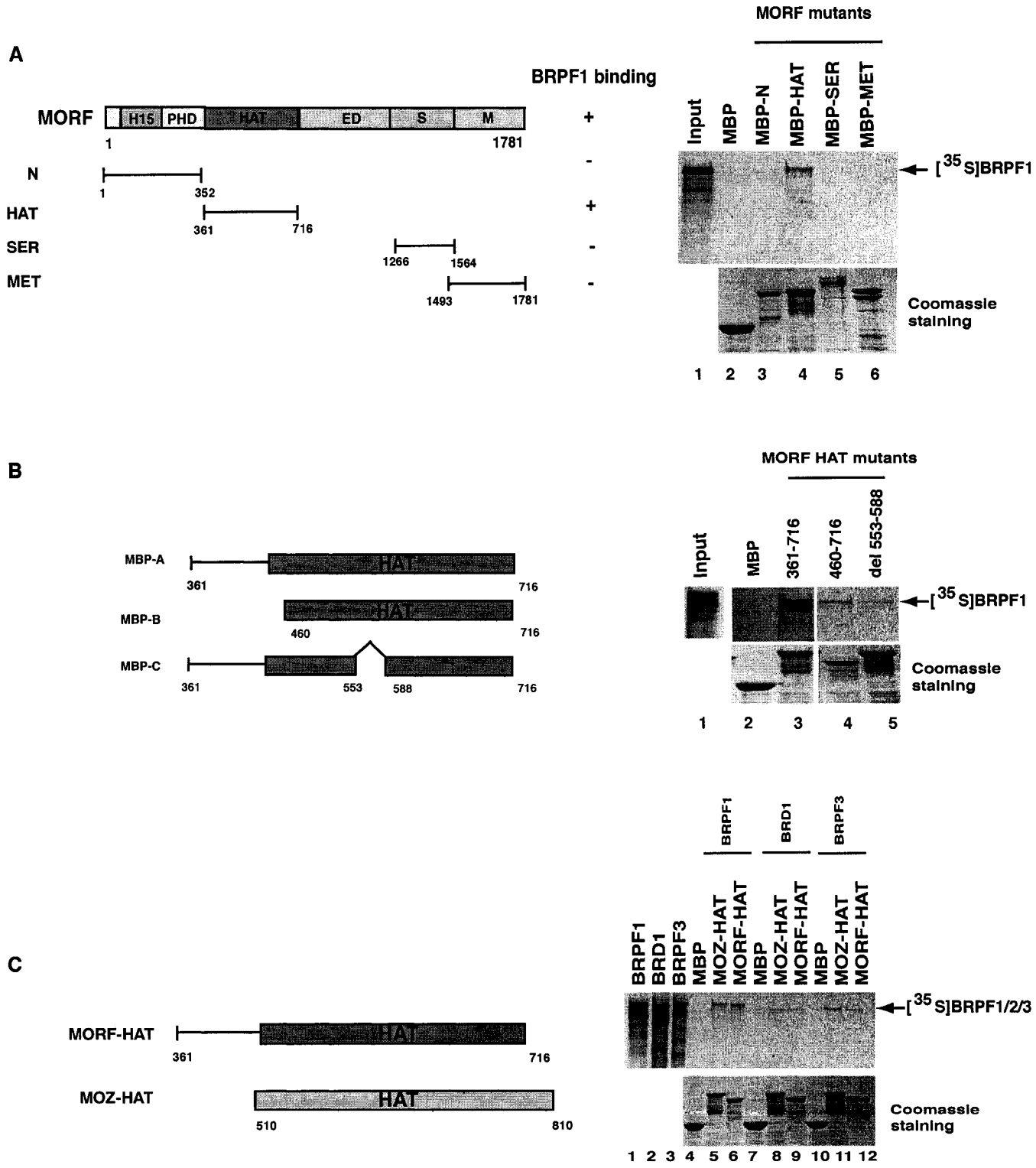
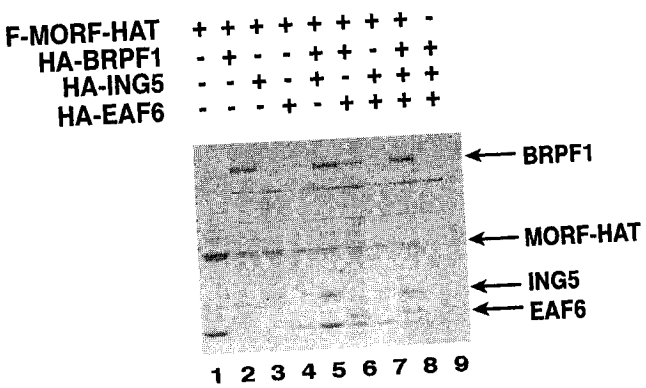

A
BRPF1 binding
MORF
H15
PHD
HAT
ED
S
M
1
1781
N
1
352
-
HAT
361
716
+
SER
1266
1564
-
MET
1493
1781
-
MORF mutants
Input
MBP
MBP-N
MBP-HAT
MBP-SER
MBP-MET
[35S]BRPF1
Coomassie staining
1 2 3 4 5 6
B
MBP-A
HAT
361
716
MBP-B
HAT
460
716
MBP-C
361
553
588
716
MORF HAT mutants
Input
MBP
361-716
460-716
del 553-588
[35S]BRPF1
Coomassie staining
1 2 3 4 5
C
MORF-HAT
HAT
361
716
MOZ-HAT
HAT
510
810
BRPF1
BRD1
BRPF3
BRPF1
BRD1
BRPF3
MBP
MOZ-HAT
MORF-HAT
MBP
MOZ-HAT
MORF-HAT
MBP
MOZ-HAT
MORF-HAT
[35S]BRPF1/2/3
Coomassie staining
1 2 3 4 5 6 7 8 9 10 11 12

Figure 3: Components of the MOZ tetrameric complex influence histone acetyltransferase activity.

(A) SF9 insect cells were infected with recombinant baculovirus vectors expressing the coding sequences for F-MORF-HAT, HA-BRPF1, HA-ING5, and HA-Eaf6. Lane 1-9 represent different combinations of subunits in the complex. Immunoprecipitation by M2 agarose and elution with Flag peptide were performed to obtain purified complexes that were then subjected to SDS-PAGE followed by Coomassie staining. ING5 and Eaf6 do not express well.

(B) HAT assay of MORF-HAT complexes by p81-filter binding assays. Immunoprecipitated eluates from SF9 insect cells (1-9 from A) were incubated with free histones in the presence of [^{3}H] acetyl-CoA. HAT activity is measured in disintegrations per minute (dpm).

A


F-MORF-HAT + + + + + + + + -
HA-BRPF1 - + - - + + - + +
HA-ING5 - - + - + - + + +
HA-EAF6 - - - + - + + + +
BRPF1
MORF-HAT
ING5
EAF6
1 2 3 4 5 6 7 8 9

B

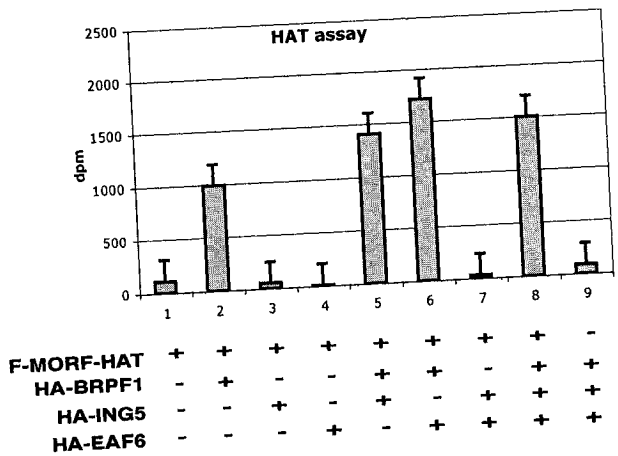

HAT assay
dpm
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8 9
F-MORF-HAT + + + + + + + + -
HA-BRPF1 - + - - + + - + +
HA-ING5 - - + - + - + + +
HA-EAF6 - - - + - + + + +

Figure 4: BRPF1 Mapping Analysis

(A) Schematic representation of BRPF1 and its deletion mutants. Numbers indicate the position of amino acid residues. BRPF1 domains are labeled as follows: Zn finger, Zinc finger; I, I domain; M, M domain, Bromo, bromodomain; and PWWP domain, conserved proline-tryptophan-tryptophan-proline motif required for binding methylated residues. The binding ability of MOZ, ING5, and Eaf6 is summarized at the right.

(B) Mammalian 293 cells transfected with Flag-MOZ and BRPF1 deletion mutants were immunoprecipitated with M2 agarose specific for the Flag tag (left panel). Bound proteins were eluted with Flag peptide, and were blotted with anti-HA antibody (Sigma). BR6 and BR7 were difficult to express (lane 6-7) as shown in the extract. *In vitro* transcription and translation of BRPF1 deletion constructs BR1, BR2, BR3, BR4, and BR6 were used in binding assays with MOZ-HAT fused to MBP (right panel). Input lanes 1-5 indicate that the *in vitro* transcription and translation products were expressed. The intensity of bands in lanes 9 and 11 is poor, but longer exposures do confirm that BR1, BR2, and BR6 interact with MOZ-HAT (data not shown).

(C) Interaction of BRPF1 mutants with ING5. Similar manipulations as in (B) were executed for immunoprecipitation and immunoblotting. BR5, BR6, BR7, and BR8 express poorly (lane 4-8) as shown in the extract. Conditions need to be improved to optimize protein levels.

(D) Interaction of BRPF1 mutants with Flag-EAF6. Similar manipulations as in (B) were executed for immunoprecipitation and immunoblotting. BR6 is very poorly expressed (lane 6); the protein has been consistently difficult to express in all experiments. In the extract (lane 1-8), nonspecific bands appeared along the bottom of the blot.

Figure 4

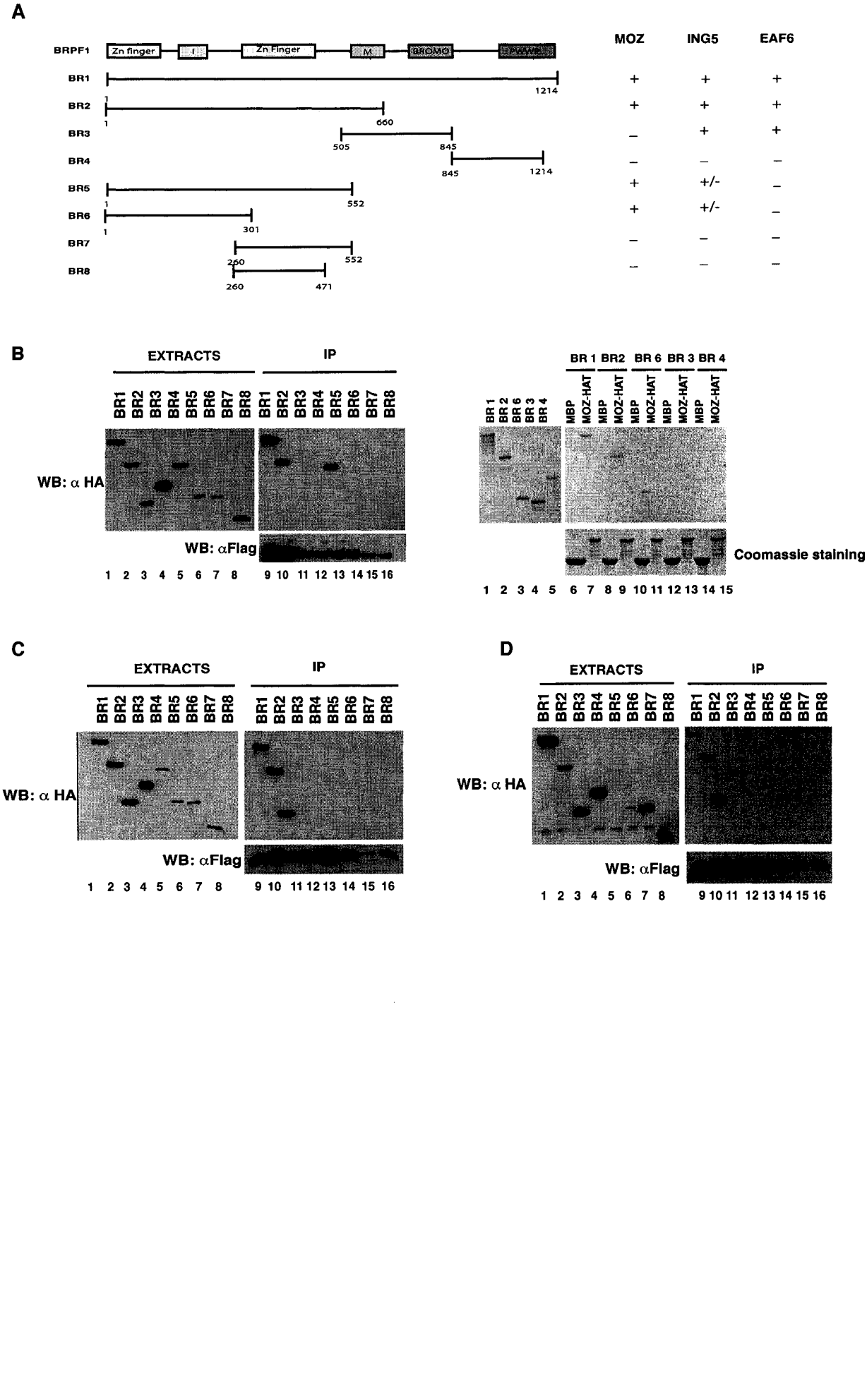

A
BRPF1
Zn finger
Zn Finger
BROMO
BR1
BR2
BR3
BR4
BR5
BR6
BR7
BR8
1214
660
505
845
552
301
260
471
MOZ
ING5
EAF6
B
EXTRACTS
IP
WB: α HA
WB: αFlag
Coomassie staining
MBP
MOZ-HAT
C
D

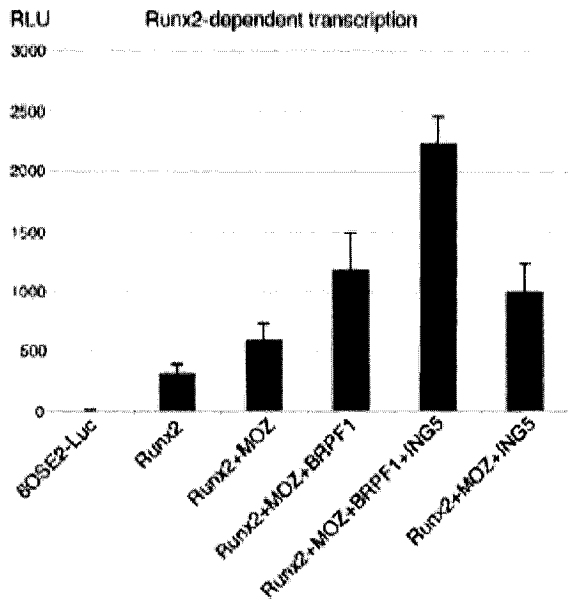
Figure 5: MOZ, BRPF1, and ING5 promote Runx2-dependent transcription.

(A) A luciferase reporter construct containing six Runx2 binding sites was cotransfected with the indicated expression vectors, and cell extracts were analyzed for luciferase activity (mean of three different experiments). BRPF1 bridges MOZ and ING5 in vivo and the complex stimulates Runx2-dependent transcription.

(B) A reporter construct containing six Runx2 binding sites was co-transfected with the indicated expression vectors and analyzed for luciferase activity.

Figure 5

A


RLU
Runx2-dependent transcription
3000
2500
2000
1500
1000
500
0
6OSE2-Luc
Runx2
Runx2+MOZ
Runx2+MOZ+BRPF1
Runx2+MOZ+BRPF1+ING5
Runx2+MOZ+ING5

B

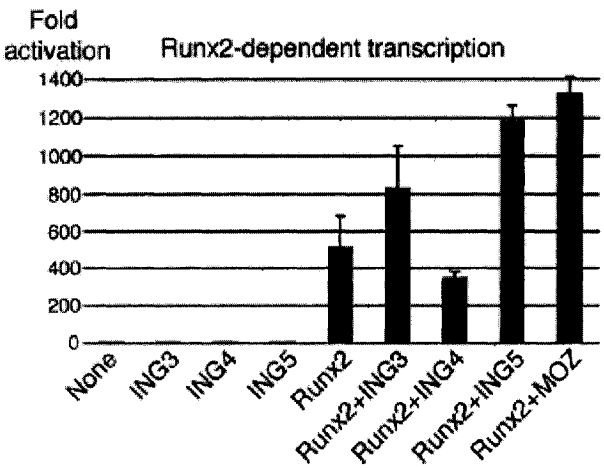

Fold activation
Runx2-dependent transcription
1400
1200
1000
800
600
400
200
0
None
ING3
ING4
ING5
Runx2
Runx2+ING3
Runx2+ING4
Runx2+ING5
Runx2+MOZ

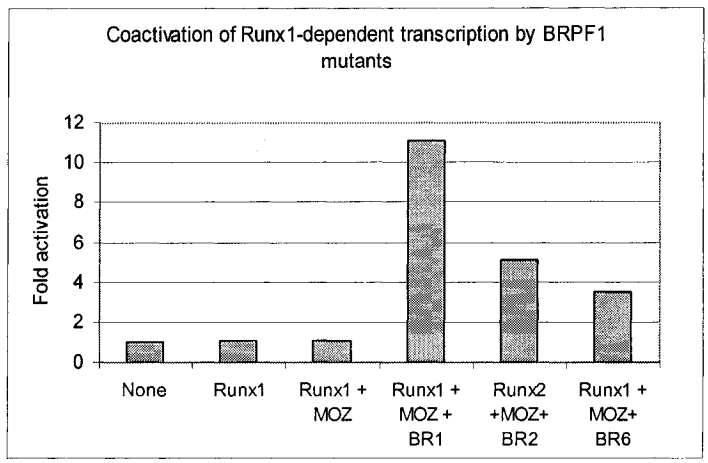
Figure 6: Effects of BRPF1 mutants on Runx1 and Runx2 dependent transcription

(A) The GM10-luciferase reporter contains a GM-CSF promoter fragment upstream from the luciferase coding sequence. This promoter has binding sites for AML1; it was cotransfected with the indicated expression vectors, and cell extracts were analyzed for luciferase activity.

(B) A luciferase reporter, 6OSEII-luciferase, containing six Runx2 binding sites was cotransfected with the indicated expression vectors, and cell extracts were analyzed for luciferase activity.

Figure 6

A


Coactivation of Runx1-dependent transcription by BRPF1 mutants
Fold activation
12
10
8
6
4
2
0
None
Runx1
Runx1 + MOZ
Runx1 + MOZ + BR1
Runx2 +MOZ+ BR2
Runx1 + MOZ+ BR6

B

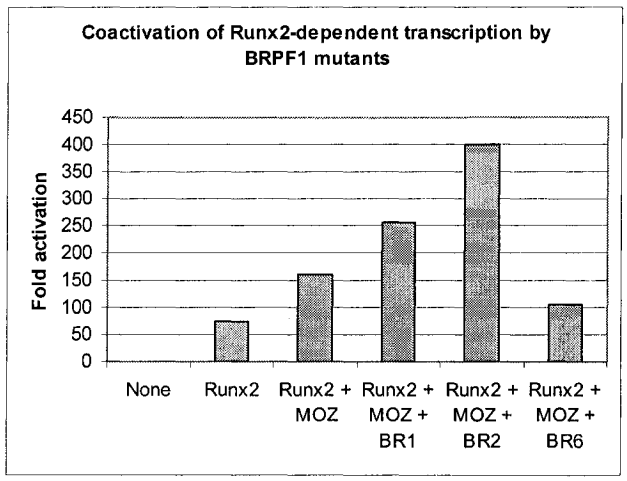
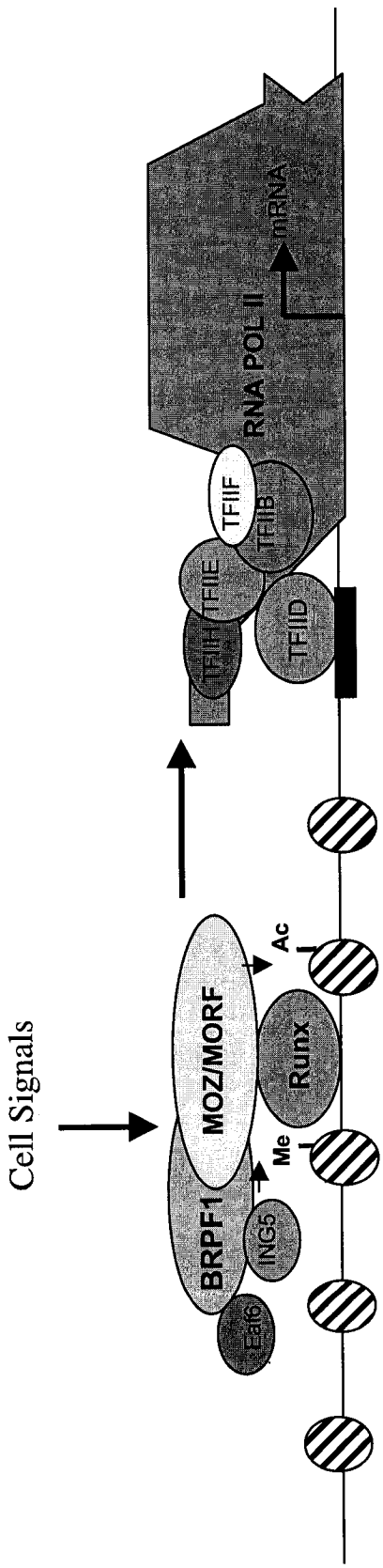

Coactivation of Runx2-dependent transcription by BRPF1 mutants
Fold activation
450
400
350
300
250
200
150
100
50
0
None
Runx2
Runx2 + MOZ
Runx2 + MOZ + BR1
Runx2 + MOZ + BR2
Runx2 + MOZ + BR6

Figure 7: Schematic representation of the MOZ/MORF tetrameric complex

The MOZ/MORF tetrameric complex consists of BRPF1, ING5, and Eaf6. Transcription factors, such as Runx, bind their consensus sequence on DNA. Transcriptional coactivators such as MOZ/MORF are recruited, and they increase Runx-dependent transcription. When tetrameric MOZ is present, Runx-dependent transcription increases. Suggested mechanisms include the recruitment of the complex to methylated H3K4 on promoter regions via the PHD domain of ING5. This enforces the complex to remain near promoter regions so that MOZ can further coactivate Runx-dependent transcription. Another possibility is the recruitment of BRPF1 via its bromodomain to acetylated lysine residues on the promoter. BRPF1 may also target the complex to nucleosomal regions via its PWWP domain.

Figure 7


Cell Signals
BRPF1
MOZ/MORF
ING5
Eaf6
Runx
Ac
Me
TFIIH
TFIIE
TFIIF
TFIIB
TFIID
RNA POL II
mRNA

DISCUSSION

One way to decipher the function of multisubunit complexes is to associate the loss of the subunit with the phenotype. Following the discovery that MOZ and MORF associate with BRPF1/2/3, ING5, and Eaf6 (Doyon et al., 2006), we became interested in characterizing their molecular association. Many reports have identified that human ING proteins associate with specific HAT or HDAC complexes. Human ING1 and ING2 are purified as stable components of the mSin3 HDAC complexes, also HBO1 is found in an ING4 complex (Doyon et al., 2006). Most HATs are capable of acetylating free histones in solution but cannot acetylate chromatin substrates such as nucleosomes. For example, yeast Pho23, Yng1, Yng2 and human ING3 are important for enabling associated HATs in their respective complexes to modify chromatin substrates (Boudreault et al., 2003; Doyon et al., 2004; Selleck et al., 2005). Similarly, we found that components of the MOZ/MORF tetrameric complex modulate histone acetyltransferase activity. These observations suggest that ING proteins are partly responsible for modulating histone acetylation specificity.

Effects of BRPF1 and ING5 on activities of MOZ and MORF

Our data is in agreement with the hypothesis that components of a complex influence activity of the associated HAT/HDAC. We show by western analysis (Figure 1) and Coomassie staining (Figure 3A) that BRPF1 binds MOZ and MORF. BRPF1 mediates the indirect interaction between MOZ and ING5 (Figure 1). Binding assays indicate that BRPF1 interacts with the HAT domain of MOZ and

MORF (Figure 2A). *In vitro* binding of BRPF1 to MORF-HAT mutants confirm that it does not require the acetyl-CoA binding domain for this interaction (Figure 2B). All BRPF family members demonstrate binding to MOZ- and MORF-HAT domains suggesting that they may have redundant functions (Figure 2C). HAT assays demonstrate that BRPF1, ING5, and Eaf6 do indeed influence HAT activity of the complex. In fact, HAT activity is enhanced in the presence of BRPF1 and ING5 suggesting that they potently coactivate MOZ (Figure 3B). It is not clear how either BRPF1 or ING5 cause this increase in activity, other than the fact that ING proteins facilitate HAT enzymes to function on chromatin. Hence, the molecular mechanism of the observed coactivation remains to be elucidated. Initially, upon sequence similarity, it was speculated that ING5 associated with the I domain, and MOZ associated with the M domain. However, out results suggest otherwise. Deletion analysis of BRPF1 demonstrates that MOZ requires the I domain for binding, but the M domain may potentiate stronger association (Figure 4B). ING5 binds weakly to the I domain and strongly to the M domain suggesting the possibility of two ING5 binding sites on BRPF1 (Figure 4C), and Eaf6 only requires the M domain (Figure 4D). These results differ from the model proposed when the complex was identified (Doyon et al., 2006). Also, BRPF1 and ING5 coactivate MOZ such that Runx2-dependent transcription increases even further (Figure 5A). The mechanism of transcriptional coactivation of Runx2 is most likely different than the mechanism that potentiates an increase in HAT activity of the complex.

Role of BRPF1 in assembling the tetrameric complex of MOZ

The subunit structure of the MOZ/MORF tetrameric complex is analogous to NuA3 in yeast. Both complexes contain Eaf6, and NuA3 contains Nto1, a double PHD finger protein related to human BRPF protein (Howe et al., 2002). Both share similar specificity for histone H3 (Howe et al., 2001). NuA3 and MOZ both acetylate lysine 14 on H3. Yeast Epl1 was shown to bridge Yng2 and Esa1 together facilitating acetylation of chromatin substrates in NuA4 (Boudreault et al., 2003). Similarly, EPC1 links Tip60 and ING3 in human cells (Doyon et al., 2004) and JADE links ING4 to HBO1 (Doyon et al., 2006) bridging the ING protein and the HAT enzyme together. Amino acid sequence similarity supports that the function of BRPF1/2/3 in the MOZ complex is analogous to that of yeast Epl1 and EPC1 (Doyon et al., 2006). They all share an I domain to bind ING proteins and an M domain that mediates interaction with MYST family proteins.

Mechanisms of BRPF1-mediated stimulation of MOZ and MORF activities

Upon speculating on the functional domains of BRPF1, there are a few ways by which it may modulate the coactivation potential of MOZ and hence the activity of the complex. Figure 6 schematically illustrates a proposed mechanism by which BRPF1 may either improve HAT activity of the complex or coactivation potential of MOZ upon transcription factors in general. More specifically, the PWWP domain has been involved in chromatin targeting of proteins and perhaps, the PWWP domain of BRPF1 targets MOZ more efficiently to promoter regions (Chen et al., 2004). It is characterized by the presence of a highly conserved proline-tryptophan-

trypotophan-proline motif and is a module of 100 to 150 amino acids found in many chromatin associated proteins. Deletion of PWWP domains in DNA methyltransferases Dnmt3a and Dnmt3b1 compromises their ability to methylate major satellite repeats (Chen et al., 2004). There is evidence that the PHD domain of NURF is important for H3K4me3-binding since it couples NURF-mediated ATP-dependent chromatin remodeling to H3K4 methylation; this maintains Hox gene expression patterns during development (Wysocka et al., 2006). Additionally, ING2, a subunit of mSin3a-HDAC1, binds trimethylated H3K4 via the PHD domain on ING2 and stimulates an increase in the associated HDAC activity (Pena et al., 2006; Shi et al., 2006). The ING5 PHD domain was also shown to bind trimethylated H3K4 (Shi et al., 2006). The mechanisms of PHD-mediated protein regulation are not clear. However, the PHD finger is demonstrating its importance as a chromatin-binding module such as in the case of the BPTF PHD finger of NURF (Li et al., 2006). One possible mechanism by which subunits in the MOZ/MORF complex enhance transcriptional coactivation is perhaps by its recruitment to promoter regions via the H3K4me3 association with the ING5 PHD domain (Figure 6); this interaction may increase retention of the MOZ/MORF complex at promoter regions. It may also facilitate the ability of MOZ to acetylate lysine residues on histone H3 when it is recruited to nucleosomes by the ING5 PHD domain, thereby providing another mechanism of increasing activation of Runx2-dependent transcription. .

Still another possibility is that MOZ acetylates lysine residues on promoter regions creating docking sites for bromodomain containing proteins such as other HATs like CBP, whose recruitment may further enhance coactivation by MOZ; in

this way MOZ may behave as a preliminary HAT that sets off a cascade of acetyltransferase activity. Acetylated lysine residues on histones located on promoter regions may recruit BRPF1 via its bromodomain. The bromodomain is highly conserved throughout evolution and is associated with many transcription co-factors that have roles in histone acetylation such as GCN5, p300/CBP, and TAFII250. Hence, the recruitment of BRPF1 via its bromodomain may stabilize the association with MOZ/MORF; in this way, BRPF1 may regulate HAT activity. Although BRPF1 does not require its bromodomain to interact with MOZ/MORF, it may stabilize the complex by simply retaining the complex near promoter regions. Interestingly, many proteins with bromodomains have been implicated in chromosomal rearrangements giving rise to AML. The putative zinc finger domain and leucine zipper region of BRPF1 are very similar to those in AF10 and AF17 (Iizuka et al., 2006), and may be targets of chromosomal translocations as well, thus making BRPF1 a potential target of chromosomal-associated translocations.

Possible roles of MOZ and MORF complexes in DNA repair and replication

In addition, ING5 has been shown to activate apoptosis in a p53 dependent manner by enhancing acetylation of lysine 382 and recruiting HATs such as CBP to induce p21/Waf1 expression (Shiseki et al., 2003). This is reminiscent of the significant role that ING family tumor suppressors have in modulating p53 function. It is then possible to surmise that the MOZ/MORF complex may be recruited to p53 responsive promoters via ING5 leading to chromatin remodeling and induction of p53 responsive genes making it indirectly associated with apoptosis and cell cycle

regulation. In agreement with this idea, other MYST HATs such as TIP60 function as coactivators by promoting p53 dependent activation of the p21 endogenous promoter (Legube et al., 2004). On a related note, CBP-dependent activators such as nuclear receptors and p53 are inhibited by MOZ-TIF2; this oncogenic fusion protein depletes cellular levels of CBP (Kindle et al., 2005). Though it was shown that wild type MOZ does not stimulate the transcriptional activation of nuclear receptors, MORF and CBP have both been shown as components in the PPARα complex (Surapureddi et al., 2002). This makes it possible for MOZ to regulate certain nuclear receptor target genes by indirect recruitment via other coactivators. Also, nuclear receptors have been reported to be important for normal haematopoiesis and deregulation by MOZ fusion proteins is associated with AML thus linking nuclear receptor deregulation to MOZ (Tenen et al., 1997).

Lastly, we cannot dismiss the idea that the MOZ/MORF complex may have a role in DNA replication. The yeast NuA3 complex has been physically and functionally linked to the yeast FACT complex, which plays a role in transcription elongation and DNA replication (Utley and Cote, 2003). The catalytic subunit of NuA3, Sas3, was also shown to be part of a distinct protein complex linked to DNA polymerase ϵ that includes a bromodomain and a PWWP domain both of which are also found in BRPF1/2/3 (Tackett et al., 2005). Also, Sas3 in NuA3, contributes to cell cycle progression (Howe et al., 2001). As alluded to earlier, NuA3 may be a homolog of the MOZ/MORF complex, so perhaps the MOZ/MORF complex has a role in DNA replication.

Clinical relevance to acute myeloid leukemia

Chromosomal translocations in MOZ, MORF and particularly Runx1 are among the most frequent in human leukemia. MOZ is a potent transcriptional coactivator of both Runx1 and Runx2 genes which regulate haematopoiesis and osteoblast maturation suggesting that MOZ or MORF deregulation contribute to cancer. Since DNA binding transcription factors are not useful targets for the development of small molecule inhibitors for therapeutic intervention in cancer, Runx associated factors such as MOZ or HDAC's may be potential secondary therapeutic targets. In fact, HDAC inhibitors have already been discovered such as TSA, valproxin, and trapoxin (Rodriquez et al., 2006). Recently, it was reported that MOZ mutant mice display defects in long term reconstituting hematopoetic stem cells (Thomas et al., 2006). The ETS family transcription factor PU.1 is essential for maintenance of hematopoetic stem cells and for the development of myeloid and lymphoid lineages (Iwasaki et al., 2005), and mice with hypomorphic PU.1 alleles are predisposed to AML due to a decrease in PU.1 expression (Rosenbauer et al., 2004). MOZ-null mice exhibit defects in haematopoiesis similar to that observed in PU.1 deficient mice suggesting a functional link between the two (Iwasaki et al., 2005). Interestingly, MOZ was discovered to physically interact with PU.1 and strongly stimulate PU.1-dependent transcriptional activation (Katsumoto et al., 2006). This suggests that MOZ is a coactivator of PU.1. So far, only Runx2 has been identified to be transcriptionally coactivated by MOZ and MORF, and PU.1 has only recently been identified as a transcription factor coactivated by MOZ. Since MOZ fusion proteins are involved in leukemogenesis, it is quite possible for MOZ fusion proteins

to deregulate PU.1 and trigger leukemia. This will hopefully direct investigators to consider the possibility of therapies directed towards stem cells to target AML. Moreover, MOZ and MORF are expressed ubiquitously, but exhibit more elevated expression in the thymus, lung, testis and ovaries (Borrow et al., 1996; Champagne et al., 1999). Thus, it is our belief that many more transcription factors responsive to MOZ coactivation will be identified in the near future. MOZ has only recently become important in cancer, and unraveling its associated proteins leads us one step closer to deciphering its mechanistic functions. Its fusion proteins cause acute myeloid leukemia and now MOZ is demonstrating its importance in hematopoetic stem cells. Understanding the mechanisms by which MOZ and MORF activate transcription will unravel how MOZ and MORF fusion proteins deregulate haematopoiesis in leukemia.

Future directions

We have shown that subunits in the MOZ/MORF tetrameric complex participate in its coactivation. However, mechanisms behind HAT activation of the complex remain to be deciphered. Since we now have antibodies for BRPF1 and BRPF3, we can perform immunoprecipitation to examine the endogenous HAT complex and its activity under physiological conditions. We also need to assess whether components of the complex influence specificity between histones and nucleosomes and between which lysine residue(s) will be acetylated and on which histone(s). We have constructed expression plasmids for BRPF1 mutants in baculovirus vectors that may be used to verify which domain(s) of BRPF1 are necessary for complex formation

with MOZ, and for regulating HAT activity. Following that, HAT assays with nucleosomes and free histones will determine which domain(s) of BRPF1 and/or ING5 are responsible for specificity of acetylation. Reporter gene assays that include all the BRPF1 mutants will need to be repeated to verify which part of BRPF1 is required for MOZ coactivation potential, and whether there is more than one functional domain involved. Also, the function and mechanism of Eaf6 remains obscure. It is a subunit of yeast NuA3 H3-specific HAT complex and the NuA4 complex; however its role has not been determined. Lastly, the similarities and differences among the BRPF1/2/3 family members need to be examined using similar experimental approaches outlined in this thesis.

The characterization of the MOZ/MORF tetrameric complex contributes significantly to the knowledge of MOZ/MORF regulation; however the possibility remains that MOZ/MORF may exist as subunits in other complexes. So far, many HATs have been demonstrated specificity for H3 acetylation, including Gcn5p, Sas3p, Hpa2p and Nut1p (Sterner and Berger, 2000). Gcn5 is the catalytic subunit of many HAT complexes; therefore it is possible that MOZ/MORF may be discovered in different complexes in the future. This is likely, since it may contribute to the maintenance of the histone code, through which diverse histone modifications determine protein function. Another possibility is that these different MOZ/MORF HAT complexes may regulate different levels of transcription, or other DNA-templated nuclear processes. Different complexes may mediate different levels of histone acetylation throughout the genome. For example, Gcn5 mediates high levels of acetylation in promoter regions, but lower levels in surrounding

regions (Vogelauer et al., 2000). This may explain why Gcn5 functions in different complexes. These complexes may function interdependently. Correspondingly, we anticipate the identification of additional MOZ/MORF complexes by either tandem affinity purification, or microarray analysis.

Appendix A: List of Abbreviations

aa	amino acid
AML	acute myeloid leukemia
APP	amyloid precursor protein
ATRA	all-*trans*-retinoic acid
BRPF	bromodomain and PHD containing finger protein
BMP	bone morphogenetic proteins
CBP	CREB binding protein
CBF	core binding factor
CDK	cyclin dependent kinase
DMEM	dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic Acid
DTT	dithiothreital
Eaf6	Esa1-associated factor 6
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene(oxyethylenenitrilo)tetaacetic acid
FBS	fetal bovine serum
G1	Gap1 phase
G2	Gap2 phase
GNAT	Gcn5-related acetyltransferases
HA	hemaglutinnin
HAT	histone acetyltransferases
HBO1	histone acetyltranferase binding to ORC1

HDAC	histone deacetylase
HMG	high mobility group box
HP1	heterochromatin protein 1
HRP	horseradish peroxidase
ING	inhibitor of tumor growth
IP	immunoprecipitation
M	mitosis
MgCl2	magnesium chloride
MBP	maltose binding protein
MCM2	minichromosome maintenance proteins
MLL	mixed lineage leukemia
MOF	male absent on the first
MOZ	monocytic leukemia zinc finger protein
MORF	MOZ related factor
MSL	male-specific lethal complex
NaCl	sodium chloride
NaF	sodium fluoride
Na3VO4	sodium orthovanadate
NP-40	nonidet P40
NuA3	nucleosomal acetyltranferase of histone H3
NuA4	nucleosomal acetyltranferase of histone H4
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PI3K	phosphatidylinositol 3-kinase
PHD	plant homeodomain finger
PKC	protein kinase C
PST	Pro-Ser-Thr
PWWP	Pro-Trp-Trp-Pro motif
RAR	retinoic acid receptor
Sas2	something about silencing 2
Sas3	something about silencing 3
SDS	sodium dodecyl sulfate
SM	serine methionine rich domains
TAP	tandem affinity purification
TIF2	transcriptional intermediary factor
TIP60	tat-interactive protein 60kDa
TGF	transforming growth factor
Tris	tris[hydroxymethyl]aminomethane
UV	ultraviolet

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