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Mechanism of Action of ING4 as a Transcriptional Coactivator of p53

Jabez Bok

MECHANISM OF ACTION OF ING4 AS A TRANSCRIPTIONAL COACTIVATOR OF p53

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Jabez Bok

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Jabez Bok, Ph.D.
The Rockefeller University 2015

ING4 belongs to a family of proteins that have been implicated in tumor suppression and has been linked to the activation of p53 target genes in a p53-dependent manner. However the mechanism by which it interacts with p53 to activate transcription from these genes is unclear. In this study I use in vitro reconstitutions of cellular processes to biochemically dissect the activity of the ING4 complex, complementing the cell based assays that give a more physiological but less defined window into ING4's effect on p53 transcriptional activity. Purification of the ING4 complex allowed verification of previously known subunits and identification of BRPF1/2/3 as a core ING4 component. Reconstitution of the ING4 complex allowed the identification of two distinct variants of the ING4 complex which utilized either JADE or BRPF as the central scaffold subunit. Both these variants were required to recapitulate the histone acetyltransferase activity of the endogenous complex. Using these complexes, I demonstrate that the ING4 complex histone modifying activity is not directly affected by p53 but is rather indirectly modulated through p53-dependent p300 activity and enhanced by the presence of the p53-dependent trimethylated H3K4 histone mark. I also establish an ING4 complex-mediated p53-dependent

transcription system on chromatin templates and show that the ING4 complex has a direct effect on p53 dependent transcription. Additionally, the same conditions that enhance ING4 complex histone acetylation, namely p300 and trimethylated H3K4, also enhance the ING4 complex' effects on p53 dependent transcription. Taken together, these results establish ING4 as a transcriptional coactivator of p53 and suggest two mechanisms by which p53 affects ING4 complex histone modification and transcriptional activity.

To my family – David, Annabelle, Jared, Jihan and Molecule Bok.

May there always be raisins!

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List of Abbreviations

ac	Acetylation
AcCoA	Acetyl-Coenzyme A
BHIE	Reconstituted BRD1-HBO1-ING4-hEAF6 complex
BRD	Bromodomain containing
BRPF	Bromodomain and PHD finger domain containing
coIP	Coimmunoprecipitate
f:	FLAG-tag
GST	Glutathione-S-transferase
H2A	Histone H2A
H2B	Histone H2B
H3	Histone H3
H4	Histone H4
HAT	Histone acetyltransferase
HBO1	Histone acetyltransferase binding to ORC1
hEAF6	Human homologue to the yeast ESA1 associated factor 6 (EAF6)
IgG	Immunoglobulin G
ING	Inhibitor of growth protein
ING4C	ING4 complex(es) purified from HeLa cells
IP	Immunoprecipitate
JADE	Gene for apoptosis and differentiation in epithelia
JHIE	Reconstituted JADE1L-HBO1-ING4-hEAF6 complex
K#	Lysine at position indicated by following number

kB	Kilobase
kDa	Kilodalton
KMT	Lysine methyltransferase
me	Methylation
me1	Monomethylation
me2	Dimethylation
me3	trimethylation
NE	Nuclear extract
NP-40	Nonidet P-40
nuc	Nucleosome
p53 -/-	HCT 116 cells which lack p53
p53 RE	p53 response element
PCR	Polymerase chain reaction
PHD	Plant homeodomain finger
PMSF	Phenylmethylsulfonyl fluoride
qPCR	Quantitative PCR
SAM	S-adenosyl methione
TSS	Transcription start site

Chapter 1

Introduction

1.1 Histone modifications and transcription

The question of how the same genetic code can carry out such disparate activities such as differentiation, development and homeostasis is one that is of great interest. There is no difference in the genetic blueprint between a nerve cell, a liver cell, a muscle cell, or a skin cell, yet in accordance with their function, these cells demonstrate great phenotypic differences. The same phenomenon can also be observed temporally within the same cell where different reactions occur in response to different stimuli despite the same genome being present throughout. A major key to this dichotomy is through the differential expression of genes (Roeder, 2003). The mechanism by which gene expression is regulated is thus of great interest in understanding how a cell functions and in understanding and treating a variety of diseases that have their root cause in the misregulation of gene expression (Bhatia & Kleinjan, 2014).

There are many different mechanisms by which the transcriptional activation of genes may be regulated. These range from those that function at the level of DNA, such as sequence specific DNA binding transcription factors that recruit RNA polymerases and other transcriptional machinery, to those that function at the level of chromatin, such as the chromatin structure itself which affects different conformations to either repress or allow transcription (Roeder, 2005). The numerous post-translational modifications on histone proteins in chromatin such as acetylation, methylation, phosphorylation and ubiquitination

have been proposed to form a histone code that exists alongside the genetic code and allows for an additional level of transcriptional regulation (Strahl & Allis, 2000). More specifically, histone acetylation has been highly correlated with transcriptional activation (Roth, et al., 2001; Li, et al., 2007) while H3K4 trimethylation in particular is associated with the 5' regions of active genes and is strongly correlated with histone acetylation and transcription (Ruthenburg, et al., 2007a). However the precise mechanisms behind the establishment of these marks and their effect on transcription have not been fully elucidated.

1.2 The transcription factor p53

p53 is a classical transcriptional activator that binds to response elements contained within p53 target genes. As a tumor suppressor, it modulates the cellular response to a wide array of stresses (Levine, et al., 2006) and mediates the transcriptional activation of numerous target genes, amongst them those that activate the cell cycle arrest and apoptosis pathways, such as *p21* and *BAX* (Laptenko & Prives, 2006). As such, it is frequently found to be mutated or otherwise downregulated in a variety of cancers (Levine, et al., 2006).

While p53 itself has no intrinsic histone modifying capability, many p53 cofactors are capable of post-translational protein modification and have had these capabilities linked to their role as cofactors of p53-mediated transcription. The p53 cofactor p300, for example, is capable of acetylating chromatin in a p53-

dependent manner, and this acetylation is essential for p300 mediation of p53-dependent transcription (Espinosa & Emerson, 2001; An, et al., 2004). Methyltransferases such as SET1 and MLL have also been shown to affect transcription via methylation of H3K4 (Dou, et al., 2005; Jiang, et al., 2013; Tang, et al., 2013), a modification that is also affected by RAD6 ubiquitylation of H2B (Kim, et al., 2009). One pathway by which p53 regulates the transcription of its target genes is therefore through modification of histones via the activity of its cofactors.

1.3 Inhibitor of Growth (ING) proteins

1.3.1 Discovery of ING proteins

The Inhibitor of Growth (ING) family of proteins are a homologous group of proteins that when ectopically expressed in cells, as their name suggests, inhibit the growth of cells. The founding member, ING1, was first identified from a genetic suppressor element isolated in a subtractive hybridization assay that compared normal mammary epithelium and several breast cancer cell lines. A screen for genetic elements that were differentially expressed in the cancer cell lines and had an effect on cell growth resulted in the identification of ING1 (Garkavtsev, et al., 1996). Four other members of the ING family (INGs 2-5) were subsequently identified via sequence homology and characterized as having several similar structural features, foremost of which is a highly conserved PHD finger (Fig 1A & 1B) (Coles & Jones, 2009). All members were also found to have

Fig 1. The Inhibitor of Growth family of proteins.

A) Structural motifs of the ING family of proteins. PIP – PCNA interacting protein motif. PBD – Partial bromodomain. LZL – Leucine zipper-like region. NCR – Novel conserved region. NLS – Nuclear localization sequence. PHD – Plant homeodomain. PBR – Poly basic region.

B) Sequence alignment of ING family proteins showing the highly conserved PHD finger domain in yellow.

Fig 1A

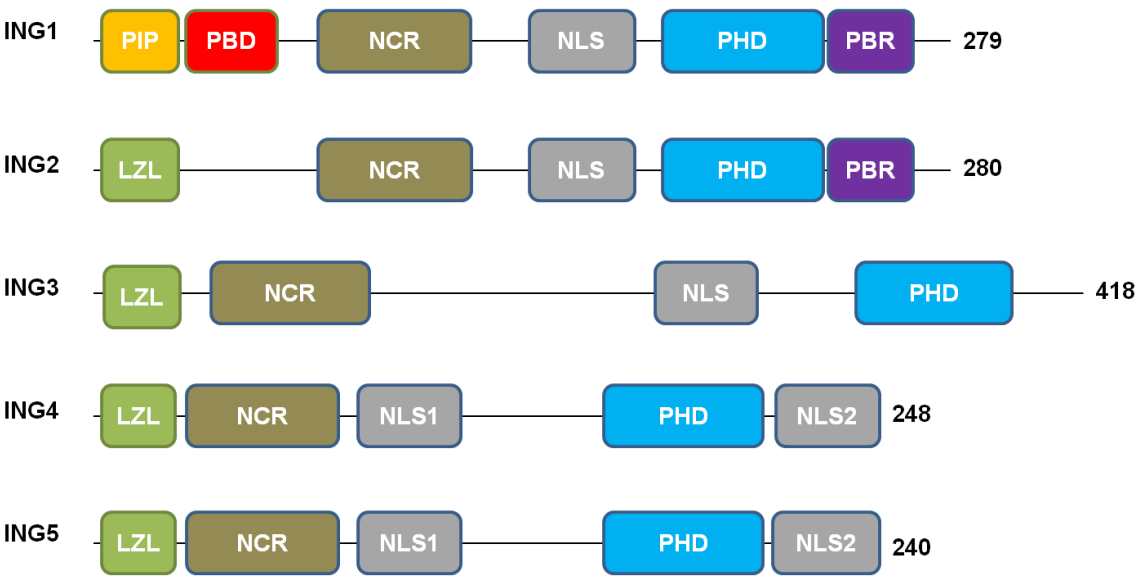

ING1
PIP
PBD
NCR
NLS
PHD
PBR
279
ING2
LZL
NCR
NLS
PHD
PBR
280
ING3
LZL
NCR
NLS
PHD
418
ING4
LZL
NCR
NLS1
PHD
NLS2
248
ING5
LZL
NCR
NLS1
PHD
NLS2
240

Fig 1B

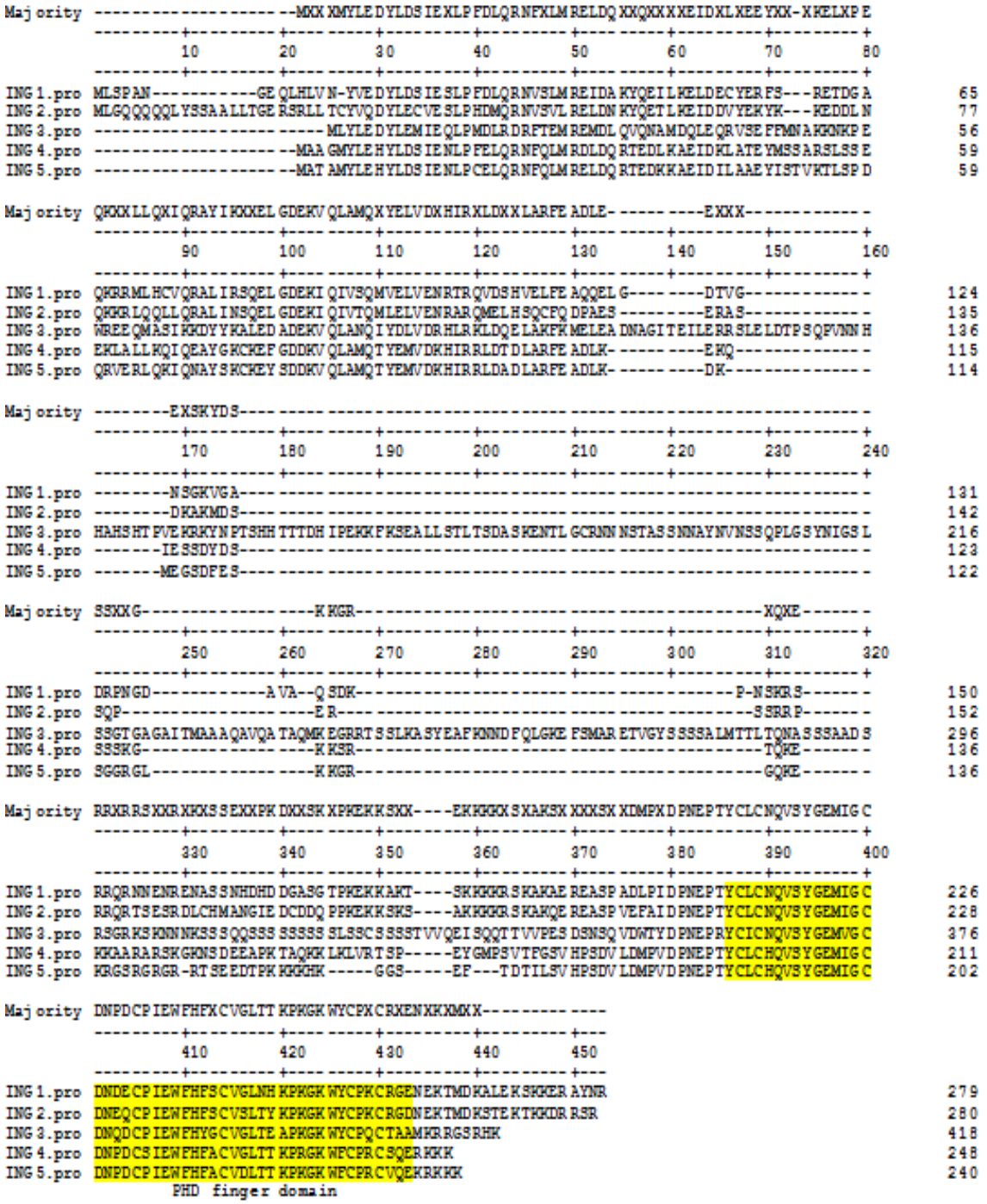


Table 1. ING complex members.

ING member	ING1	ING2	ING3	ING4	ING5
Associated HAT			TIP60	HBO1	MOZ/MORF HBO1
Associated HDAC	HDAC1/2	HDAC1/2			
Other components	RBP1 mSin3A RbAp48 RbAp46 SAP30	RBP1 RBP1-like mSin3A SAP130 RbAp46 RbAp48 SAP30 BRMS1	TRRAP p400 BRD8 EPC1/2 DMAP1 RUVBL1/2 BAF53a Actin MRG15 GAS41 hEAF6	JADE1L/2/3 hEAF6	BRPF1/2/3 JADE1L/2/3 hEAF6

the same inhibitory effect on cell growth (Shimada, et al., 1998; Nagashima, et al., 2001; Gunduz, et al., 2002; Nagashima, et al., 2003; Shiseki, et al., 2003).

1.3.2 Characteristics of ING proteins

In addition to their sequence homology and their ability to inhibit cell growth when expressed, all ING proteins have several characteristics in common. Each is a member of a multisubunit protein complex that possesses a histone modifying capability- histone deacetylase in the case of ING1 and ING2, and histone acetyltransferase in the case of INGs 3, 4 and 5 (Table 1) (Skowyra, et al., 2001; Kuzmichev, et al., 2002; Doyon, et al., 2004; Doyon, et al., 2006). Secondly, each possesses a conserved Plant Homeodomain zinc-finger (PHD finger) that has been shown in several proteins to recognize and bind preferentially to trimethylated, and to a lesser extent dimethylated, H3K4 (Shi, et al., 2006; Li, et al., 2006; Wysocka, et al., 2006). In the case of ING4, this is accomplished via an aromatic cage that interacts with the trimethylammonium group of H3K4 (Pena, et al., 2006). This ability to discriminate between trimethylated H3K4 and the other methylation states of H3K4 identifies the ING proteins as ‘readers’ of the histone code. In conjunction with the catalytic ability associated with their complexes, the ING proteins thus provide an effector pathway by which the H3K4 trimethylation mark might be translated into some downstream activity (Ruthenburg, et al., 2007a).

1.3.3 Association with p53

The ING protein family as a whole has also been implicated in the regulation of several p53-activated pathways. The ING-mediated inhibitory effects on cell growth were dependent on p53 in the majority of cases and a synergistic inhibitory effect on cell growth was observed with several of the ING proteins when they were co-expressed with p53. Additionally, all ING proteins, with the exception of ING3, have been shown to co-immunoprecipitate with p53. ING proteins thus show a physical and functional interaction with p53 (Coles & Jones, 2009). Altogether these observations suggest that the ING proteins might play a role as p53 cofactors.

1.4 The ING4 protein

1.4.1 Characteristics of the ING4 protein

The fourth member of the ING family, ING4, is of particular interest because the complex it exists in includes a histone acetyltransferase (Doyon, et al., 2006). This, together with its H3K4 trimethyl recognizing PHD finger, raises the possibility of cross-talk between trimethylated H3K4me3 and histone acetylation. Additionally, the ING4 protein complex consists of a limited number of subunits, which makes complete reconstitution of the complex a more tractable problem. Finally, unlike the ING5 complex, the ING4 complex is thought to be primarily involved in transcription regulation, rather than both transcription

and DNA replication (Doyon, et al., 2006). For these reasons the decision was made to focus this study on the fourth member of the ING family of proteins.

ING4 is frequently found to be downregulated in cancers and its expression has been inversely correlated with cancer progression and severity (Piche & Li, 2010). As with other members of the ING family, ING4 has been implicated in p53 mediated gene expression and DNA damage responses. More specifically, ING4 coimmunoprecipitates with p53 and ectopic expression of ING4 has been shown to result in reduced colony formation, cell cycle arrest and apoptosis. Activation of the *p21* and *BAX* promoters and expression of endogenous p21 and BAX were also induced by ectopic ING4 expression in a p53-dependent manner (Shiseki, et al., 2003; Zhang, et al., 2004; Doyon, et al., 2006; Avvakumov, et al., 2012). These observations suggest that ING4 can function as a transcriptional coactivator of p53 in the regulation of these pathways.

However, two other studies concluded that overexpression of ING4 also conferred inhibitory effects in the p53 mutated T47D and U-118MG cell lines (Kim, et al., 2004; Unoki, et al., 2006). Additionally, ING4 has also been associated with the transcription factors NF-κB and HIF-1α. This suggests that ING4 has additional functions independent of p53, possibly as a cofactor to other transcription factors. However the mechanism by which it functions as a cofactor

in these other cases is unlikely to be the same since in these cases it seems to function as a negative cofactor, repressing the functions of both NF-κB and HIF-1α (Garkavtsev, et al., 2004; Ozer, et al., 2005), rather than as a positive factor, as in the case of p53 and the activation of *p21* and *BAX*.

With respect to ING4's occupancy on genes, ectopically expressed ING4 was found to be enriched around the transcription start sites (TSS) of DNA damage activated promoters in response to doxorubicin treatment. This correlates with the pattern of enrichment of trimethylated H3K4 and is dependent on the trimethylated H3K4 recognizing ING4 PHD finger. This suggests that the PHD-trimethylated H3K4 interaction is important for the recruitment of ING4 to such promoters (Hung, et al., 2009) and further supports the role of ING4 as a transcriptional cofactor.

1.4.2 Structure of the ING4 protein

ING4 is a ~29kDa protein 249 amino acids in length (Fig 2A). It contains an N-terminal leucine zipper-like (LZL) region, a unique region that is conserved amongst the INGs termed the novel conserved region (NCR), a C-terminal plant homeodomain (PHD) zinc finger and two nuclear localization signals (NLS). The LZL region is thought to mediate dimerization between two ING4 molecules and potentially between ING4 and ING5 molecules (Palacios, et al., 2010; Culurgioni, et al., 2012). The ING4 PHD finger, as with all other ING PHD fingers, has been

Fig 2. The ING4 complex.

A) Domain structure of the ING4 complex subunits. PHD – Plant homeodomain finger. Bromo – bromodomain. PWWP – Pro-Trp-Trp-Pro motif. S – Serine rich region. ZF – Zinc finger. MYST – histone acetyltransferase domain. LZL – Leucine zipper like region. NCR – Novel conserved region. NLS – Nuclear localization signal. LZ – Leucine zipper.

B) Schematic representation of the JADE1S-HBO1 complex and the JADE1L-HBO1-ING4-hEAF6 complex.

Fig 2A

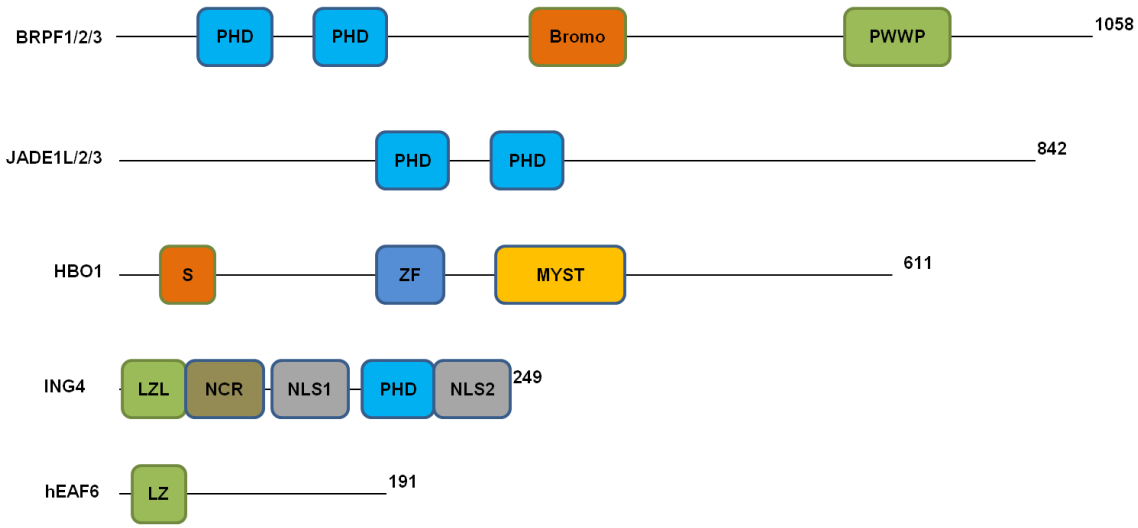
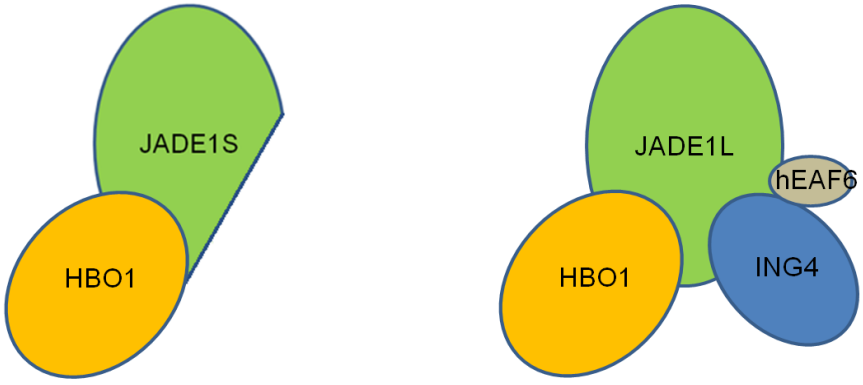

BRPF1/2/3
PHD
PHD
Bromo
PWWP
1058
JADE1L/2/3
PHD
PHD
842
HBO1
S
ZF
MYST
611
ING4
LZL
NCR
NLS1
PHD
NLS2
249
hEAF6
LZ
191

Fig 2B


JADE1S
HBO1
JADE1S Complex
JADE1L
hEAF6
HBO1
ING4
JADE1L Complex

shown to bind specifically to histone trimethylated H3K4 (Shi, et al., 2006; Palacios, et al., 2008; Hung, et al., 2009), while the first NLS (NLS1) is thought to play a role in the ING4-p53 interaction (Zhang, et al., 2005). However, another study failed to find any significant p53 interaction (Palacios, et al., 2010). The PHD domain, the NCR and the first NLS are conserved amongst all the INGs, while the LZL region is absent from ING1 and the second C-terminal NLS is present only in ING 4 and 5 (Coles & Jones, 2009).

In addition to the full length ING4, multiple splice variants of the ING4 transcript have been detected. The ING4 proteins that were expressed from three of these variants had a missing or truncated NLS1 and were shown to localize predominantly to the cytoplasm instead of the nucleus (Unoki, et al., 2006). However, other variants that also modified NLS1 did not affect the nuclear localization, but rather affected the localization of ING4 within the nucleus (Tsai, et al., 2008). Curiously, the presence of the second C-terminal NLS (NLS2) appeared to be dispensable for nuclear localization, suggesting a non-functional NLS (Unoki, et al., 2006). Three more variants that included either an intact or slightly shorter NLS1 were also shown to localize to the nucleus and to maintain their interaction to p53 and HBO1 (Raho, et al., 2007). While the existence of these naturally occurring alternatively spliced proteins has yet to be detected, these studies do suggest that the NLS1 is responsible for the nuclear localization of ING4.

1.5. The ING4 Complex

1.5.1 Components of the ING4 complex

The ING4 complex consists of ING4 itself and three additional proteins: 1) the MYST family histone acetyltransferase HBO1 (histone acetyltransferase binding to ORC1), 2) a member of the JADE (gene for apoptosis and differentiation in epithelia) protein family, JADE1L/2/3, and 3) the uncharacterized protein hEaf6 (Fig 2A), which is also present in the ING3-Tip60 complex, the ING5-HBO1 complex, and the ING5-MOZ/MORF complex (Fig 1B) (Doyon, et al., 2006). Additional interactions have also been identified with other proteins such as NF-κB and p300 (Garkavtsev, et al., 2004; Shiseki, et al., 2003), but these are lost when the ING4 complex is more stringently purified and are not thought to be integral components of the ING4 complex (Doyon, et al., 2006).

1.5.1.1 HBO1

HBO1 is a MYST family histone acetyltransferase that has been implicated in DNA replication as well as transcription (Burke, et al., 2001; Iizuka, et al., 2006; Georgiakaki, et al., 2006; Miotto & Struhl, 2006). It is a member of both ING4 and ING5 complexes (Doyon, et al., 2006) and has been shown to interact with p53 (Iizuka, et al., 2008). HBO1 is thought to be responsible for significant proportion of histone H4 acetylation in the cell at lysine residues 5, 8 and 12, but not 16, in 293 cells (Doyon, et al., 2006) and for H3K14 acetylation in

mouse primary embryonic fibroblasts and erythroblasts (Kueh, et al., 2011; Mishima, et al., 2011).

1.5.1.2 JADE

Of the JADE protein members of the complex, the JADE1 homolog has been the most extensively studied. JADE1 exists as two isoforms – a full length protein of 842 amino acids termed JADE1L, and a truncated version of 509 amino acids termed JADE1 or JADE1S. The full length JADE1L isoform is associated with the full ING4 complex while the truncated JADE1S isoform is associated only with HBO1, forming a smaller JADE1S-HBO1 complex that lacks ING4 and hEaf6 (Fig 2B). Both JADE1 isoforms possess two PHD domains, the first of which, PHD1, binds preferentially to unmethylated H3K4 and demonstrates a slight preference for trimethylated H3K36. The second PHD domain, PHD2, binds independently of H3K4 methylation status and is thought to be essential for both the JADE1S complex and the larger JADE1L/2/3-ING4 complex' association with chromatin. It is also thought to be necessary for JADE1L's inhibitory effects on cell growth (Foy, et al., 2008; Saksouk, et al., 2009).

1.5.1.3 BRPF

More recently, BRPF1/2/3, which was previously identified as a member of the ING5 complex, has been identified as an ING4 associated protein (Mishima, et al., 2011). However, the precise relationship of BRPF1/2/3 to ING4 and to the other ING4 subunits has yet to be fully elucidated. All BRPF paralogs have 2 PHD fingers, with the PHD fingers of BRPF2 (which is more commonly known as BRD1), being the most extensively studied. Similar to the JADE PHD fingers, the first BRD1 PHD finger binds preferentially to unmethylated H3K4 while the second PHD finger seems to interact with H3 independent of H3K4 methylation (Qin, et al., 2011; Lalonde, et al., 2013) and has also been shown to be capable of binding nonspecifically to DNA (Liu, et al., 2012). Based on conservation of key residues, it is possible that this is also a property of the JADE PHD2 domains, although this has not been shown. Additionally, all BRPF proteins possess a bromodomain that binds acetyl-lysines (Sanchez & Zhou, 2009), and a PWWP domain, that has an affinity for trimethylated H3K36 (Vezzoli, et al., 2010).

1.5.2 Trimethylated H3K4-dependent acetylation

Through its subunits, the ING4 complex possesses multiple chromatin readers (Table 2). These readers, combined with the histone acetylating HBO1 suggests that the ING4 complex as a whole could function as an effector,

Table 2. Chromatin binding domains of the ING4 complex.

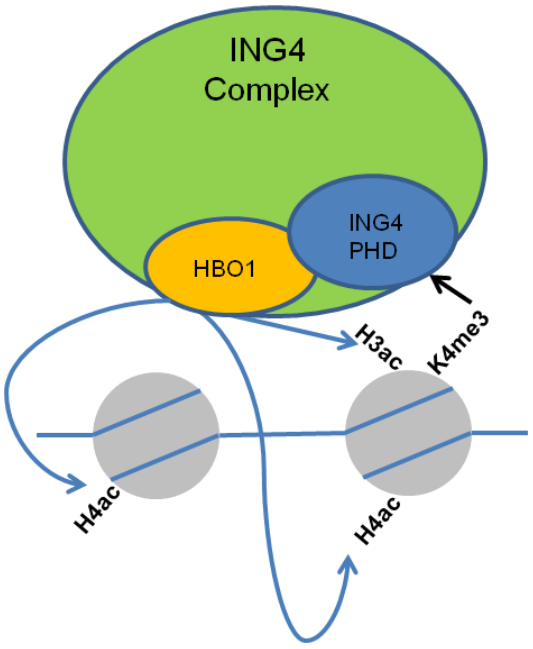
Subunit	Domain	Interacts with
BRPF2 (BRD1)	PHD1	H3K4me0
	PHD2	H3 DNA
	Bromo	Acetylated lysines
	PWWP	H3K4me36
JADE1L	PHD1	H3K4me0
	PHD2	H3
ING4	PHD	H3K4me3

mediating its histone acetylation activity in response to one or more of these marks. Indeed, similar to what has been observed with the orthologous yeast NuA3 complex (Taverna, et al., 2006), ING4 complex mediated increase in H3 acetylation in response to trimethylated H3K4 has been demonstrated on H3 peptides and reconstituted nucleosomes (Hung, et al., 2009). A similar increase was also shown with the homologous ING5 complex on oligonucleosomes purified from yeast (Saksouk, et al., 2009). There is thus an ING4 complex-mediated link between trimethylated H3K4 and H3 acetylation (Fig 3), and through this histone acetylation, between trimethylated H3K4 and transcriptional activation.

Fig 3. ING4 complex mediates H3K4me3-dependent H3 acetylation

The ING4 complex HBO1 subunit acetylates H4 independent of H3K4 methylation status. Recognition of H3K4me3 by the ING4 PHD finger mediates H3 acetylation in addition to the H3K4me3 independent H4 acetylation. Blue arrows represent histone acetylation activity by the HBO1 subunit of the ING4 complex and point to the resulting histone acetylation. The black arrow represents the interaction between the ING4 PHD finger and H3K4me3. H3ac: Acetylation on H3. H4ac: Acetylation on H4. H3K4me3: Trimethylated H3K4

Fig 3



Chapter 2

Composition and Structure of the ING4 complex

2.1 Preface

The ING4 complex was first purified by Doyon et al via TAP-tag ING4 purification and was determined to be composed of the subunits JADE1L/2/3, HBO1, ING4 itself, and hEAF6 (Fig 1B) (Doyon, et al., 2006). Other studies have looked at the interactions between the various subunits within the ING4 and homologous ING5 complexes, using co-immunoprecipitation of ectopically expressed proteins and in vitro binding of recombinant proteins (Foy, et al., 2008; Saksouk, et al., 2009; Avvakumov, et al., 2012; Lalonde, et al., 2013). These studies showed that there is a JADE1-HBO1 interaction, and that additional interactions with ING4 and hEAF6 are mediated through the C-terminal of JADE1L that is absent in the shorter JADE1S isoform (Fig 4A). This arrangement is similar to that of the ING5-BRPF complex, where the central BRPF is responsible for bridging the catalytic MOZ/MORF and ING5/hEAF6 (Fig 4B) (Ullah, et al., 2008).

A subsequent BRD1 (BRPF2) immunoprecipitation by Mishima et al showed the existence of a BRD1 containing ING4-HBO1 complex (Mishima, et al., 2011) that was undetected in the original purification (Doyon, et al., 2006). The association with ING4 and HBO1 was subsequently also shown to occur with the BRPF1 and BRPF3 paralogs (Lalonde, et al., 2013). Together these results suggest the existence of an ING4 complex that can include a BRPF subunit.

Fig 4. Interactions between ING4 and ING5 complex subunits.

A) Interactions between the subunits of the JADE1S complex and the JADE1L-ING4 complex. The shorter JADE1S protein is unable to interact with ING4 and hEAF6.

B) Interactions between the subunits of BRPF-ING5 complex**.**

Arrows represent protein interactions. Crossed out arrows represent interactions that are no longer possible for the JADE1S protein.

Fig 4A

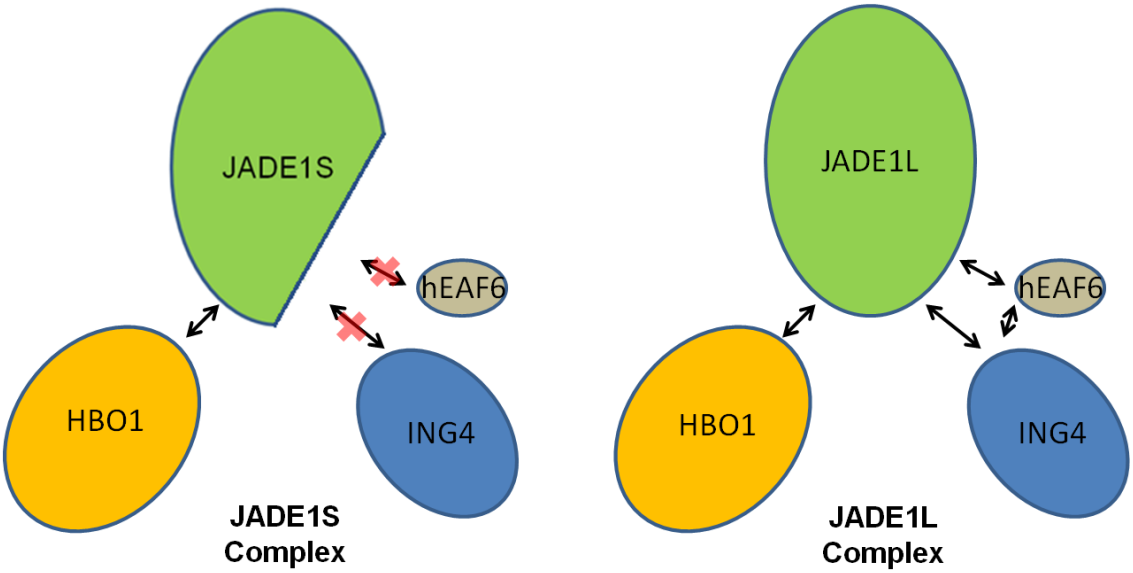
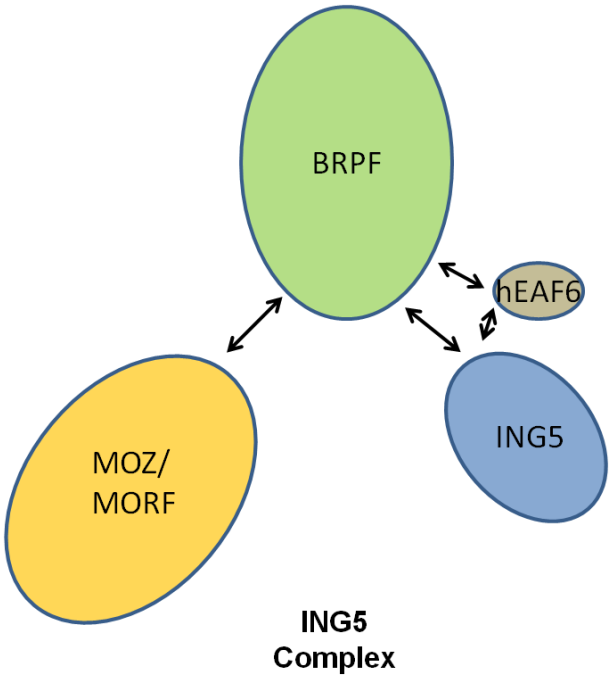

JADE1S
hEAF6
HBO1
ING4
JADE1S
Complex
JADE1L
hEAF6
HBO1
ING4
JADE1L
Complex

Fig 4B


BRPF
hEAF6
ING5
MOZ/
MORF
ING5
Complex

However many of the assays used in these studies relied upon overexpression of all subunits of interest in human 293T cells, followed by coimmunoprecipitation and analysis by Western blots (Hung, et al., 2009; Saksouk, et al., 2009; Avvakumov, et al., 2012; Lalonde, et al., 2013). While this approach allows for identification of specific proteins that are present in the immunoprecipitate, is inherently biased as the only proteins that will be identified are the proteins that are blotted for. It does not allow the conclusion that these identified proteins directly interact with each other - other yet to be identified proteins in the immunoprecipitate could be responsible for mediating the interactions of the identified proteins.

Additionally, as noted in the original ING4 complex purification, overexpression of a protein can lead to non-specific (Doyon, et al., 2006), as well as specific interactions with endogenous contaminating proteins. This problem is compounded by the large number of permutations of the ING4 complex that can occur, with possible interactions with both JADE and BRPF (Doyon, et al., 2006; Mishima, et al., 2011; Lalonde, et al., 2013). Dimerization between ING4 and ING5 proteins (Palacios, et al., 2010; Culurgioni, et al., 2012) allow for additional contamination by both ING5 as well as ING5 associated proteins such as MOZ/MORF (Doyon, et al., 2006; Ullah, et al., 2008).

The precise composition of an ING4 complex reconstituted and characterized in this manner from 293T cells is therefore unclear. In addition to the desired subunits, the final immunoprecipitate could also include endogenous JADE, BRPF, HBO1, and hEAF6 via interactions with the ING4 subunit. If ING4 can form heterodimers with ING5, this complex could also contain ING5 and ING5-associated proteins such as MOZ/MORF. A JADE-HBO1-ING4-hEAF6 complex reconstituted in this manner could therefore also include BRPF, MOZ/MORF, and ING5, leading to a heterogenous mixture of complexes rather than a homogenous purification of JADE-HBO1-ING4-hEAF6. Caution must therefore be taken in any conclusions drawn regarding the complexes purified or reconstituted in this manner.

To circumvent these risks of contamination and to obtain a better understanding of ING4 complex composition, we therefore set out to purify the ING4 complex with minimal expression of ectopic proteins. We also intended to reconstitute the ING4 complex in a non-mammalian system, allowing a cleaner analysis of protein-protein interactions without the risk of contaminating mammalian proteins.

2.2 Composition of the ING4 complex

2.2.1 Purification of ING4 and associated proteins

To purify the ING4 complex a HeLa Tet-on cell line (Tang, et al., 2013) was first established that uses doxycycline to induce expression of ectopic FLAG-tagged ING4 (f:ING4). Conditions were then established under which ectopic ING4 expressed was low – to an equivalent level as endogenous ING4 (Fig 5A). Nuclear extract (NE) from the cells were made and the FLAG-ING4 and its associated proteins were subsequently purified.

Initial attempts to purify the ING4 complex using M2-affinity purification on nuclear extract (Fig 5B) made from the f:ING4 cell line (Dignam, et al., 1983) were unsuccessful, with elution of the 'complex' at the final step containing little ING4 (Fig 5C). Analysis of ING4 levels at each step of the purification process identified the dialysis step during the nuclear extract procedure, where the salt concentration was brought down to 100mM KCl, as the point where the majority of the ING4 was lost (Fig 5D). Attempts to locate the missing ING4 were unsuccessful – treating of the pellet obtained from centrifugation extract post-dialysis with high salt buffer did not reveal the presence of any great amount of ING4 (data not shown). However, this loss could be circumvented by modifying the dialysis step to a higher salt dialysis. A dialysis to 300mM KCl resulted in a low enough salt concentration for the subsequent purification step without any observable loss of ING4 from the nuclear extract (Fig 6A & 6B).

Fig 5. ING4 complex purification from low salt nuclear extract.

A) Doxycycline induction of FLAG-tagged ING4. Doxycycline was titrated over range of concentration from 100 to 1200ng/ml to obtain expression of ectopic FLAG-ING4 (upper band) approximately equivalent to endogenous ING4 (lower band) (indicated by arrow) (Western blot with indicated antibody)

B) Outline of initial ING4 complex purification strategy from nuclear extract dialysed to 100mM KCl.

C) Silver stain of the initially purified 'ING4 complex' compared to a mock M2 affinity purification conducted on HeLa nuclear extract in which the ectopically expressed FLAG-ING4 was not expressed.

D) ING4 in nuclear extract at high and low salt showing loss of ING4 during the dialysis process. (Western blot with indicated antibody)

Fig 5A

Fig 5B

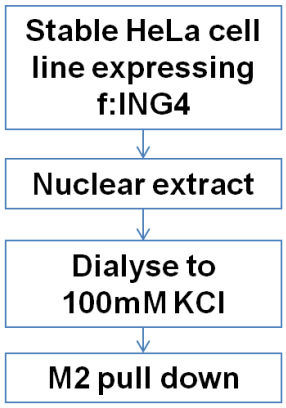


Fig 5C

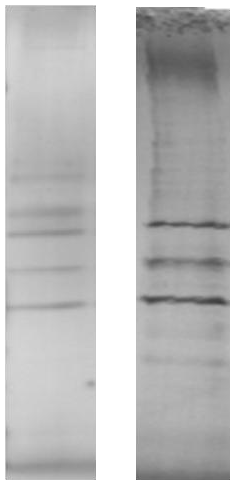
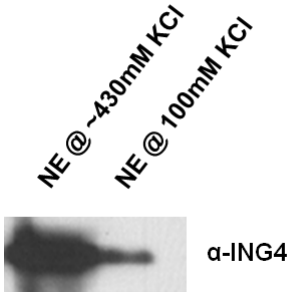


Fig 5D



ING4 complex purified from the nuclear extract contained bands that corresponded to the molecular weights of expected proteins, but also included several other contaminating bands that were also present in the mock purification (Fig 6C). A P11 ion exchange chromatography step was therefore added to the purification (Fig 6D). Both ING4 and HBO1 were found to elute from the column with the 500mM KCl fraction (Fig 6E). This fraction was dialysed again to 300mM KCl and subjected to M2 affinity purification to give a purified ING4 complex (termed ING4C, with 'ING4 complex' hereafter referring to ING4 complex(es) in general).

2.2.2 Verification of basic components

The purified ING4C was analyzed by silver stain which revealed bands that correspond to the approximate molecular weights of JADE1L/2/3, HBO1, ING4 and hEAF6 (Fig 6F, slices 5, 7, 12 & 13). Western blot further verified the presence of ING4 and HBO1 (Fig 6G). Analysis of the purified ING4C by mass spectrometry conducted by the Rockefeller University Proteomics Resource Center confirmed the identities of JADE1L/2/3, HBO1, ING4 and hEAF6, suggesting that the M2 immunoprecipitation of FLAG-ING4 was successful in pulling down the ING4 complex.

Fig 6 ING4 complex purification from high salt nuclear extract.

A) ING4 complex purification scheme from nuclear extract dialysed to 300mM KCl.

B) ING4 levels remain constant after high salt dialysis of nuclear extract. (Western blot with indicated antibodies)

C) Silver stain of the high salt purified ING4 complex compared to a mock purification.

D) High salt P11-M2 purification scheme of the ING4 complex.

E) Analysis of ING4 and HBO1 in the P11 eluted fractions. (Western blot of eluted fractions denoted by number and elution buffer used with indicated antibodies).

F) Silver stain of P11/M2 purified ING4 complex (ING4C). Proteins in each gel slice (as indicated) were identified by mass spectrometry.

G) Verification of ING4 and HBO1 in the purified ING4C compared to a mock purification. (Western blot with indicated antibodies)

H) Verification of MLL1 and associated proteins in ING4C. (Western blot with indicated antibodies)

Fig 6A

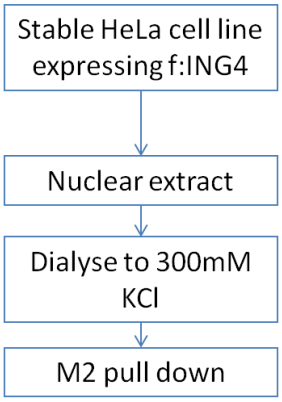


Fig 6B

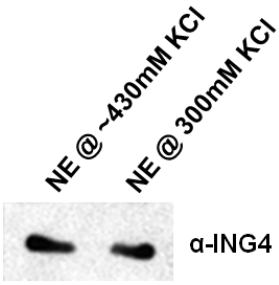


Fig 6C

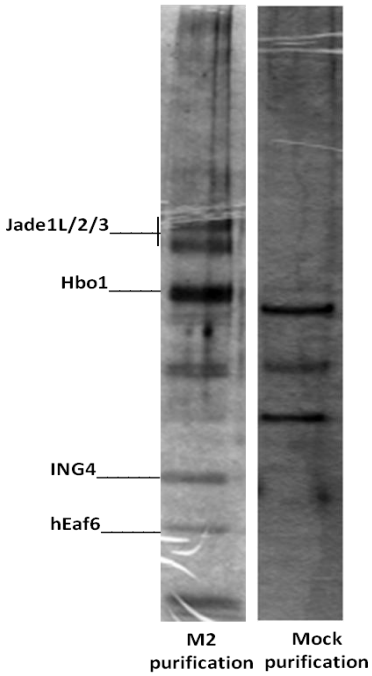


Fig 6D

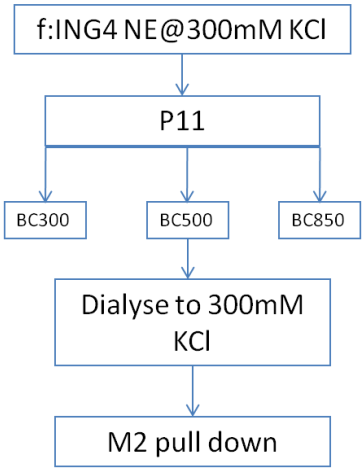


Fig 6E

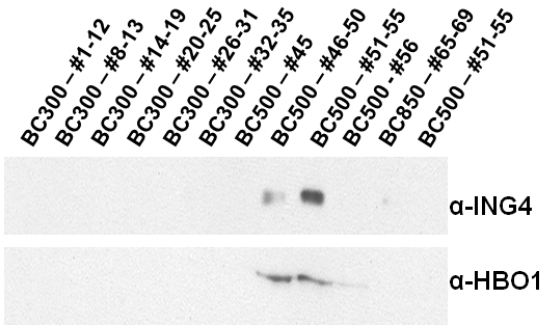


Fig 6F

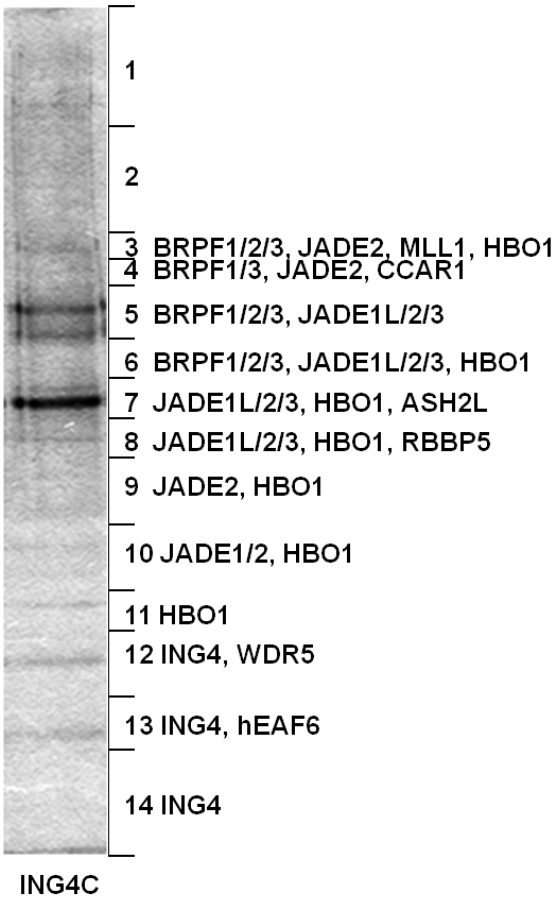


Fig 6G

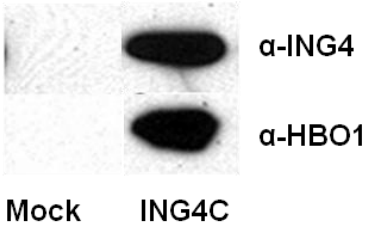
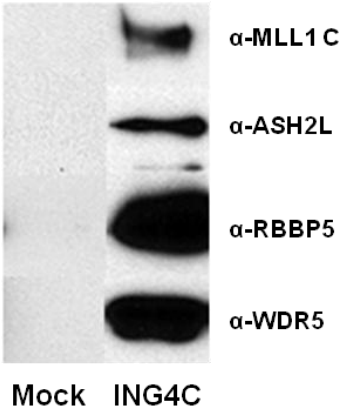


Fig 6H



2.2.3 Identification of BRPF1/2/3 as an additional subunit

In addition to bands corresponding to the proteins previously identified as being members of the ING4 complex, a larger band of ~150kDa was also observed in the silver stain (Fig 6F, slice 3). Mass spectrometry analysis identified this as BRPF1/2/3 (BRPF2 is also known as BRD1), proteins that were originally found to be members of the ING5 complex, but not the ING4 complex (Table 1). However other members of the ING5 complex such as MOZ, MORF and ING5 itself were not detected, suggesting a bona fide interaction between BRPF1/2/3 and the ING4 complex rather than contamination by the ING5 complex. BRD1 was also found to be associated with ING4 by Mishima et al (Mishima, et al., 2011), although the complex obtained there also contained many other proteins such as HSP90, TR150 and hnRNPC1. In contrast, the more stringently purified ING4 complex obtained here does not contain these other proteins. This implies that BRPF1/2/3 interact more strongly with ING4 than do these other proteins and suggests that BRPF1/2/3 are members of the core ING4 complex.

2.2.4 Association with the MLL1 complex

The analysis by mass spectrometry identified several additional proteins as MLL1, ASH2L, RBBP5, and WDR5 and CCAR1 (Fig 6F). The MLL1, ASH2L, RBBP5 and WDR5 proteins were of interest as they comprise the core MLL1 complex, which is capable of H3K4 methylation (Dou, et al., 2006). These

subunits were subsequently verified by Western blot (Fig 6H). While the aforementioned MLL1 subunits could be identified by mass spectrometry analysis and Western blot, unlike the other ING4 complex subunits they could not be easily matched to any of the visible bands on the silver stain (Fig 6F). This suggests that the MLL1 subunits are substoichiometrically associated ING4, and are not an integral part of the ING4 complex, but rather exist as a separate MLL1 complex that interacts strongly with the ING4 complex.

2.3 Structure of the ING4 complex

2.3.1 JADE1L forms the central scaffolding protein for the ING4 complex and is stabilized by HBO1

To better understand the arrangement of subunits within the ING4 complex, a reconstitution of the complex was done using baculovirus to express the desired proteins in Sf9 cells. For the purposes of this reconstitution (to determine direct interactions between individual protein subunits), this system is advantageous to using a mammalian system due to the risk of contamination with the naturally interacting endogenous mammalian proteins that would be present in a human cell line. Additionally, the reconstituted complexes were analyzed by Coomassie stain, which would reveal the presence of any contaminating Sf9 proteins that might be present in the final purification.

First, the originally identified Jade1L-HBO1-ING4-hEAF6 complex (termed JHIE) was reconstituted by infecting Sf9 cells with baculoviruses expressing JADE1L, HBO1, hEAF6 and FLAG-ING4. The resulting protein complex was purified by M2 affinity purification and Coomassie stained. This revealed bands that corresponded to the expected subunits, indicating a successful reconstitution of the JHIE complex (Fig 7A & 7B).

Different combinations of the baculoviruses were also used to provide information regarding protein-protein interactions within the complex. Omission of hEAF6 had minimal effects on the resulting complex. As expected, the hEAF6 subunit was absent but the remaining JADE1L, HBO1 and FLAG-ING4 subunits were still present in the resulting complex at comparable levels. Omission of JADE1L resulted in the complete loss of HBO1 as well as JADE1L, indicating that HBO1's association with the ING4 complex is through the JADE1L subunit. Levels of hEAF6 was also reduced but not completely absent, indicating that the hEAF6 subunit does bind directly to ING4, but that its presence in the complex is stabilized either directly or indirectly by the presence of JADE1L.

Omission of HBO1 resulted in a significant but incomplete loss of JADE1L in addition to the loss of HBO1, as well as a reduction in hEAF6 similar to what was observed in the JADE1L omission (Fig 7C). This suggests that while HBO1 is a peripheral subunit, whose association with the complex is through JADE1L, it

Fig 7. Reconstitution and purification of ING4 complex subunits.

A) JHIE and BHIE complexes reconstituted by baculovirus expression in Sf9 cells of JADE1L, HBO1, FLAG-tagged ING4, and hEAF6 or BRD1, HBO1, FLAG-tagged ING4 and hEAF6 respectively. The resulting complexes were purified through M2-affinity purification. (Coomassie stain)

B) Western blots of the reconstituted JHIE and BHIE complexes verifying the identity of ING4, HBO1 and hEAF6. (Western blot with indicated antibodies)

C) ING4 pull-downs from Sf9 cells infected with baculoviruses combinations expressing the indicated subunits. (Coomassie stain)

D) JADE1L and BRD1 are stabilized in the presence of HBO1. Purification of reconstituted JADE1L, JADE1L-HBO1, BRD1 and BRD1-HBO1 through FLAG-tagged JADE1L or FLAG-tagged BRD1. Note the increased levels of JADE1L and BRD1 obtained when they are coexpressed with HBO1. The BRD1 reconstitutions were also subjected to Western blot to verify the identity of the degraded BRD1. (Coomassie stain and Western blot with indicated antibody)

E) Purification of individual FLAG-ING4 and FLAG-HBO1 proteins via baculovirus expression in Sf9 cells. (Coomassie stain)

Fig 7A

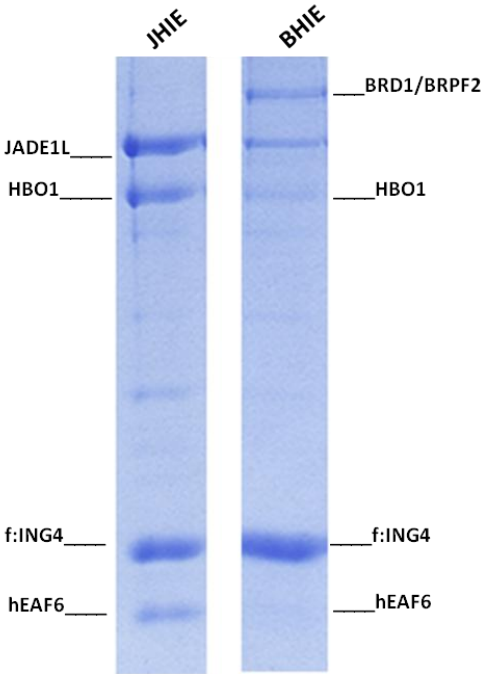


Fig 7B

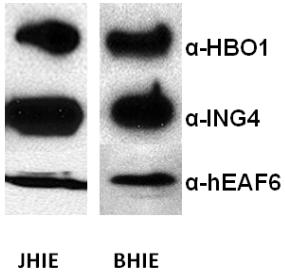


Fig 7C

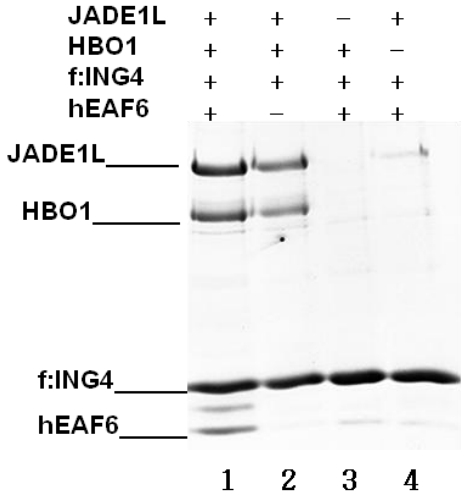


Fig 7D

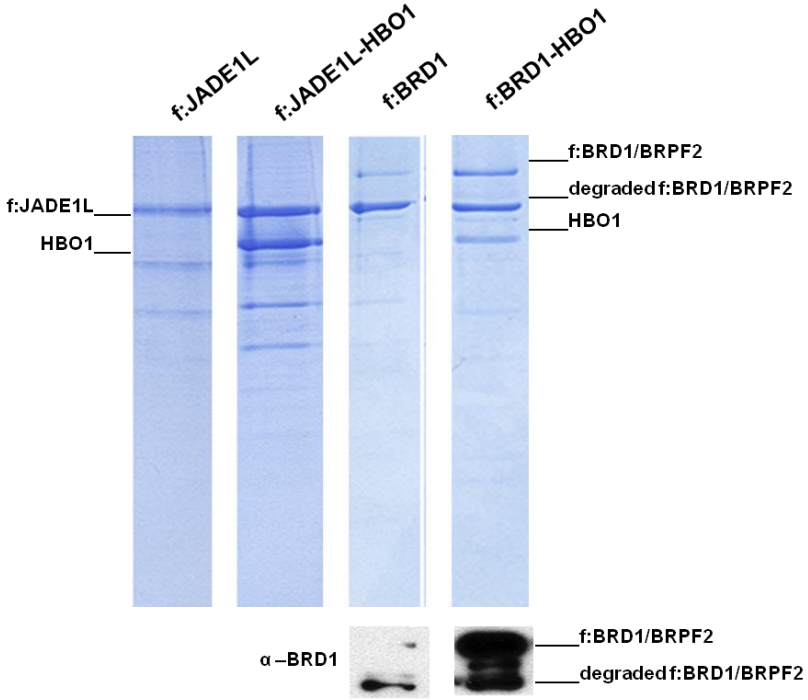
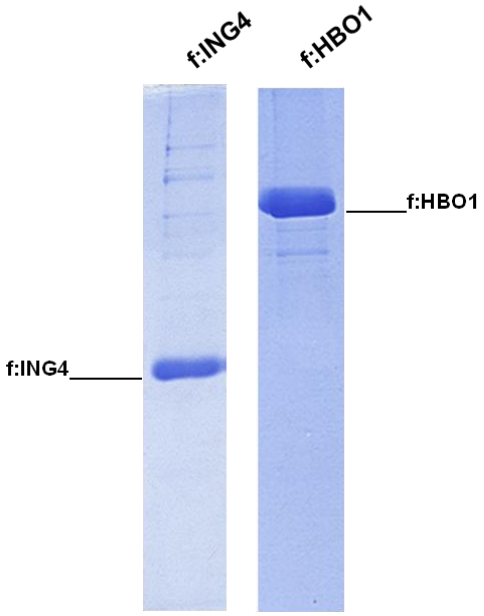

f:JADE1L
f:JADE1L-HBO1
f:BRD1
f:BRD1-HBO1
f:JADE1L
HBO1
f:BRD1/BRPF2
degraded f:BRD1/BRPF2
HBO1
α –BRD1
f:BRD1/BRPF2
degraded f:BRD1/BRPF2

Fig 7E


f:ING4
f:HBO1
f:HBO1
f:ING4

does play a role in stabilizing JADE1L. This stabilization has been previously observed when both proteins were expressed in mammalian cells (Foy, et al., 2008). The loss of hEAF6 could be due to a stabilizing effect by HBO1, but can also be interpreted as a secondary effect, since the loss of JADE1L has already been shown to adversely affect the integration of hEAF6 into the complex.

To further verify the HBO1-JADE1L interaction and stabilization, FLAG-JADE1L was expressed either alone, or together with HBO1, and M2 affinity purified (Fig 7D). As evidenced from the JADE1L-HBO1 dimer obtained, JADE1L is capable of directly interacting with HBO1. Additionally, even though identical titers of JADE1L expressing baculovirus were used in both cases, the amount of JADE1L that was obtained in the JADE1L-HBO1 purification was substantially greater than the amount of JADE1L purified alone, again supporting the stabilization of JADE1L by HBO1.

2.3.2 BRPF2 (BRD1) is an alternative scaffold to JADE1L and is stabilized by HBO1

Comparing the JADE1L-containing ING4 complex to the BRD1-containing ING5 complex, certain similarities can be observed. They both contain hEAF6, they both contain an ING protein and they both contain a MYST HAT (HBO1 in the case of ING4 and MOZ/MORF in the case of ING5). The BRPF subunit is known to function as the central scaffold for the ING5 complex, while the JADE

subunit has been shown to function as the central scaffold for the ING4 complex. Based on these similarities, it seemed possible that BRD1 might also play a similar role in an ING4 complex by replacing JADE as the central scaffold.

The ability of BRD1 to interact directly with HBO1 was first established by expressing and purifying either FLAG-BRD1 alone or together with HBO1 (Fig 7D). While expression of BRD1 on its own was minimal and heavily degraded, the addition of HBO1 resulted in increased expression and stability of BRD1 (Fig 7D, western blot of BRD1), indicating that HBO1 has a stabilizing effect on BRD1 similar to what is observed with JADE1L. For comparison, FLAG-HBO1 and FLAG-ING4 were also individually expressed and purified (Fig 7E).

A BRD1-HBO1-ING4-hEAF6 complex (termed BHIE) was then reconstituted in the absence of any JADE (Fig 7A & 7B). The success of this reconstitution indicates that a BRD1-ING4 complex could exist independently of the JADE protein or the ING5 complex, and that BRD1 could substitute for JADE as the central scaffold of the ING4 complex. This further suggests that the HeLa purified ING4 complex, which contained both JADE1L/2/3 and BRPF1/2/3, is a combination of two distinct complexes – one version that utilizes JADE as its central unit, and another that utilizes BRPF, rather than one complex that contains both JADE and BRPF (Fig 8).

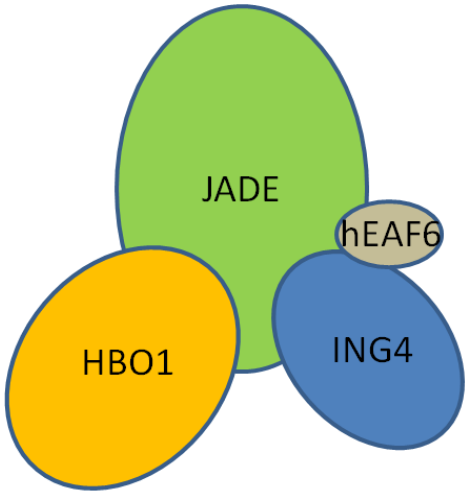
2.4 Discussion

In agreement with previous studies, the purification of an ING4 complex verifies the presence of the originally identified JADE, HBO1 and hEAF6 subunits (Doyon, et al., 2006), as well as the subsequently identified BRPF subunits (Mishima, et al., 2011, Lalonde, et al., 2013). The absence of other proteins previously identified by Mishima et al as being associated with ING4 such as HSP90 or hnRNPC1, suggests that this more stringent purification contains proteins that are more tightly associated with ING4. Additionally, the identification of the MLL1 complex as a novel interacting partner of the ING4 complex suggests a possible functional interaction between MLL1-mediated histone methylation and ING4 complex activity.

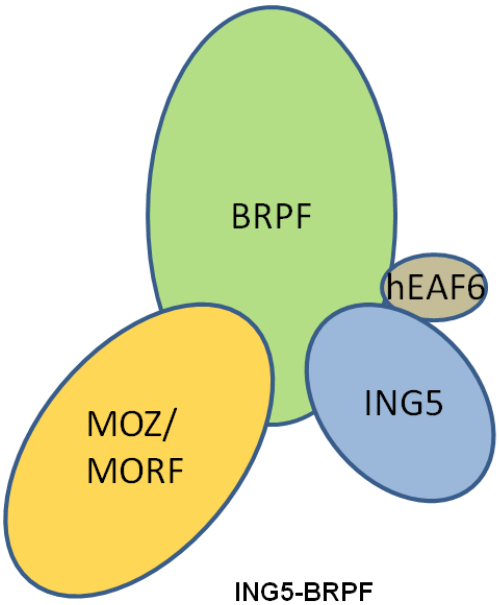
From the reconstitutions of JHIE and BHIE complexes, it can be concluded that two possible versions of the ING4 complex are possible-the previously identified JHIE complex and the new BHIE complex. In contrast to previous studies that used coimmunoprecipitation of overexpressed subunits from human 293T cells with detection by Western blot (Saksouk, et al., 2009; Lalonde, et al., 2013), the successful reconstitution of the complex in Sf9 cells in the absence of other mammalian subunits strongly suggests that all the interactions between member subunits are direct, that BRD1 is capable of substituting for JADE as the central scaffolding subunit, and that the BHIE complex is a version of the ING4 complex that can exist independently of JADE, MOZ/MORF or any other protein.

Fig 8. Schematic representations of the original JADE-ING4 and BRPF-ING5 complexes and the proposed BRPF-ING4 complex.

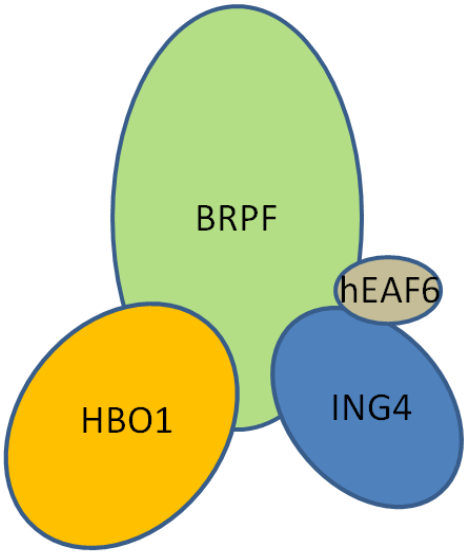
The originally identified ING4 complex consists of JADE, HBO1, ING4 and hEAF6. The originally identified ING5-BRPF complex consists of BRPF, MOZ/MORF, ING5 and hEAF6. Based on sequence homology, the presence of BRPF (but not MOZ/MORF or ING5) in a purified ING4 complex suggests the existence of a BRPF-HBO1-ING4-hEAF6 complex.



ING4 –JADE Complex



ING5-BRPF Complex



Proposed ING4-BRPF Complex

While much progress has been made in identifying the key members of the ING4 complex, as well as the various regions on the ING4 complex subunits that are responsible for their interactions with one another, more work remains to be done. The possibility of an ING4-ING4 or ING4-ING5 dimer raises the possibility of an ING4 complex-ING4 complex or ING4 complex-ING5 complex dimer (Palacios, et al., 2010), allowing for a whole array of permutations of scaffolding and catalytic subunits, as well as an expansion of the number of histone marks that can be read by such a dimer compared to a single ING4 or ING5 complex. However the existence of such a dimeric complex and its effect on chromatin has yet to be established.

Chapter 3

Histone modifying activities of the ING4 complexes

3.1 Preface

In conjunction with ING4, HBO1 has been shown to acetylate H4 independent of trimethylated H3K4, while recognition of trimethylated H3K4 by the ING4 PHD finger mediates acetylation of H3. (Saksouk, et al., 2009; Hung, et al., 2009). The specific acetylated histone lysine residues that the ING4 complexes are responsible for seems dependent on the cell lines used. Its H4 HAT activity on nucleosomes is the same in 293T and MCF cells, where a knockdown of HBO1 results in a global decrease of acetylation at H4K5, K8 and K12 but not K16 (Doyon, et al., 2006). ChIP assays have linked overexpression of various members of the ING4 complex to an increase in acetylation at H4K5, K8, and K12 residues, as well as H3K9, K14 and K23 (Saksouk, et al., 2009; Hung, et al., 2009; Avvakumov, et al., 2012; Mishima, et al., 2011; Lalonde, et al., 2013).

In contrast, a knockout of HBO1 in a mouse primary embryonic fibroblast cell line correlated only to a decrease in acetylation at H3K14, with acetylation at H3K9 and H4K5 actually being increased while acetylation at H4K8 and H4K12 were unchanged (Kueh, et al., 2011). The actual sites which HBO1 is responsible for acetylating may therefore vary between cell lines. Additionally, the limitation of cell based assays are such that while the overall effects of protein overexpression, knockdown or knockouts can be determined, there is no way to tell if this is due to a direct effect of the ING4 complex itself, or is instead

mediated indirectly through some other protein or protein complex. This uncertainty is something that must be taken into account when trying to elucidate the actual acetylation targets of the ING4 complex.

On an individual level HBO1 and ING4 have also been shown to interact physically and directly with p53, although the direct ING4-p53 interaction appears to be weak (Shiseki, et al., 2003; Zhang, et al., 2005; Palacios, et al., 2010). Thus far however, these interactions have only been studied in isolation, looking at each protein individually, rather than in the context of the ING4 complex. Similarly, the effects of p53 have only been examined on immunoprecipitated HBO1 HAT activity rather than in the context of a defined ING4 complex, and on free histones rather than on the more physiologically relevant chromatin substrate (Iizuka, et al., 2008).

To better identify the direct histone modifying activities of the ING4 complexes identified, we therefore aimed to set-up histone modifying assays in a biochemically defined system using the previously purified and reconstituted ING4 complexes. These assays would ultimately be performed on chromatin which would also allow a direct examination of ING4 complex histone modifying activity on a more physiological substrate. This would also allow any effects that the JADE and BRPF subunits might have on the activity of the ING4 complex to be elucidated. We also aimed to study ING4 complex activity in conjunction with

p53 and trimethylated H3K4 to directly determine if these conditions, as previous studies have suggested, would affect ING4 complex histone modifying activity.

3.2 The central scaffold subunit confers H3 or H4 HAT preference to the ING4 complex HAT activity.

Using the purified endogenous ING4 ‘complex’ which contains both JADE as well as BRPF (designated ING4C) as well as the reconstituted JHIE and BHIE versions, we conducted HAT assays to analyze the catalytic capabilities. HAT assays utilizing ING4C on recombinant histones and purified HeLa nucleosomes show that ING4C acetylates free histones but that in the more restrictive and physiologically relevant context of a nucleosomal substrate, acetylation is restricted to histones H3 and H4, as has been previously reported (Doyon, et al., 2006) (Fig 9A).

Next we examined the acetylation by the ING4 complex in the context of chromatin. Chromatin was assembled using a p208ML p53 DNA template (Fig 9B), recombinant histones and the ACF/NAP1 system (An & Roeder, 2004) (Fig 9C). While ING4C was found to acetylate H3 and H4 equally well, the reconstituted BHIE and JHIE complexes demonstrated a strong preference for H3 and H4 acetylation respectively (Fig 9D). These observations further suggest that the purified ING4C is actually a combination of JADE-based and BRPF based-ING4-HBO1-hEAF6 complexes and that the net activity of ING4C is a

Fig 9. HAT activity of the ING4 complex.

A) HAT activity of ING4C on 200ng of nucleosomes purified from HeLa cells or 200ng of individual recombinant histones using ^{3}H radiolabeled acetyl-CoA. ING4C acetylates free histones, but is restricted to H3 and H4 in the context of nucleosomes. (Autoradiograph shows incorporation of ^{3}H into nucleosomes/histones. Coomassie stain of the corresponding substrates used is included as a loading control).

B) p208ML p53 DNA template containing five p53 response elements used to assemble chromatin.

C) Chromatin assembly and HAT assay outline. The DNA template, purified chromatin assembly factors and recombinant histone octamers were incubated for 3 hours at 27'C to assemble the chromatin. p53 was added and incubated for a further 20 minutes at 27'C to allow binding to the p53 binding sites present on the DNA. Finally the HAT of interest and acetyl-CoA was added and incubated at 30'C to allow the histone acetylation reaction to occur.

Fig 9A

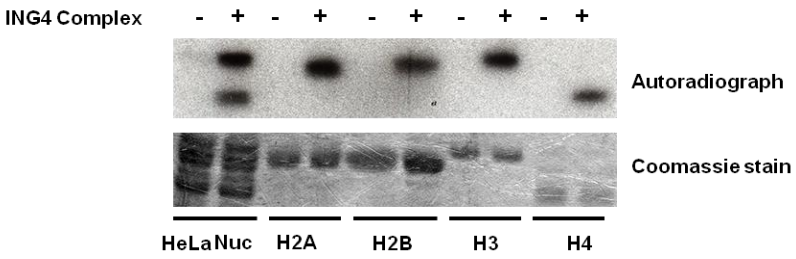


Fig 9B

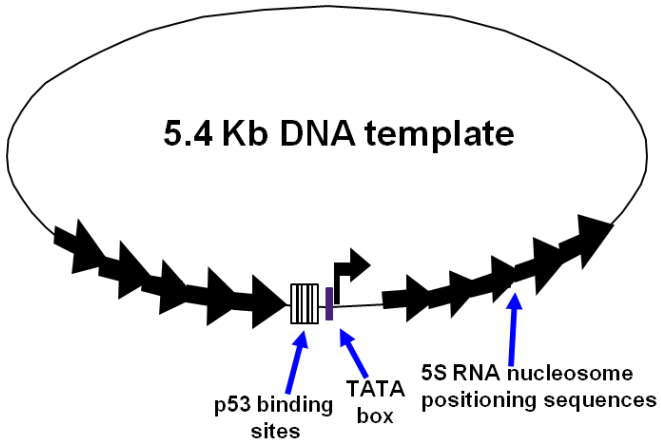


Fig 9C

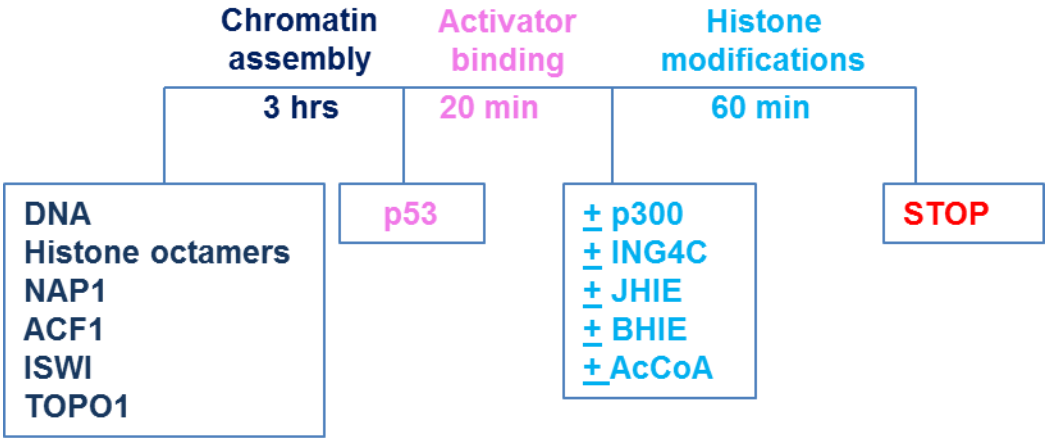


Fig 9. (continued)

D) ING4 complex HAT assay on chromatin. ING4C acetylates H3 and H4 in the context of chromatin, while JHIE preferentially acetylates H4 and BHIE preferentially acetylates H3. p53 has no detectable effect on HAT activity. (Autoradiograph)

E) Time course of ING4C acetylation of chromatin using low levels of ING4C . No detectable p53 effect on ING4C acetylation. (Autoradiograph)

F) JHIE/BHIE HAT assay on chromatin. A very slight p53 dependent signal can be observed with a long exposure. (Western blot with indicated antibody. The top row is a short exposure of the blot for two minutes while the bottom row is a longer exposure of the blot for thirty minutes)

Fig 9D

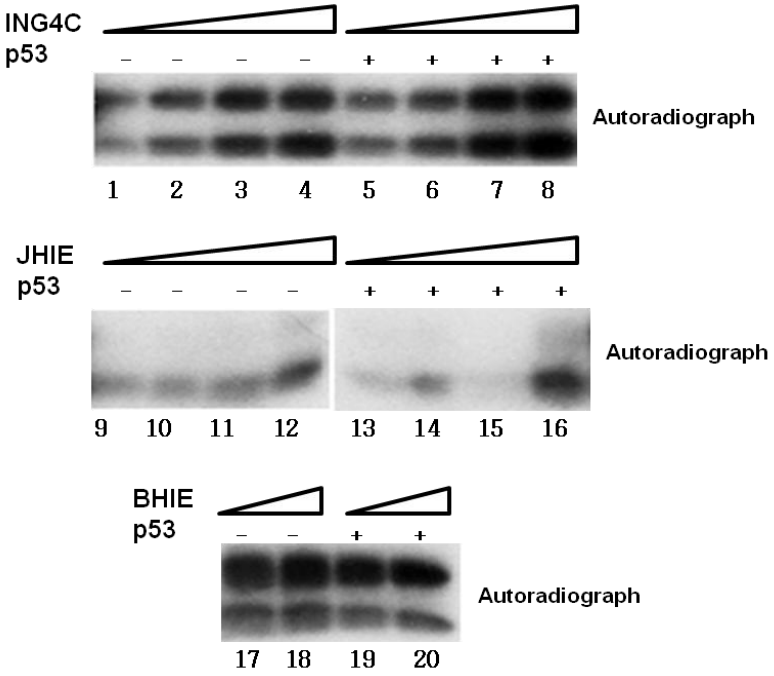


Fig 9E

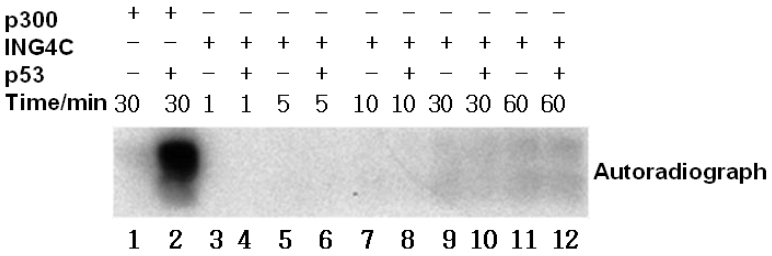
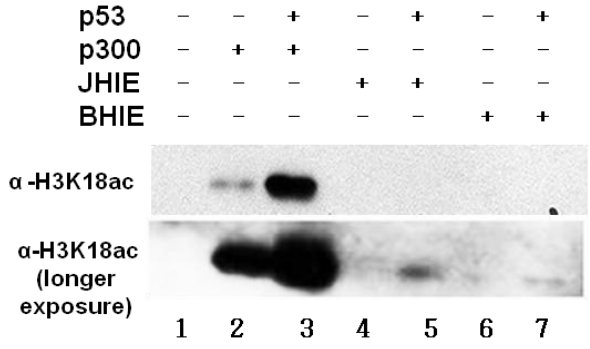


Fig 9F



combination of JHIE (acetylating H4) and BHIE (acetylating H3) HAT activity. These observations also reveal a control over ING4 complex HAT specificity that is mediated by the central subunit (either JADE1L or BRD1) similar to what has been shown with the ING5 complex (Lalonde, et al., 2013).

3.3 H3 acetylation by the ING4 complex is enhanced by trimethylated H3K4

As a link between trimethylated H3K4 and ING4 mediated HAT activity has been observed, we next investigated the effect of H3K4me3 on the HAT activity of our ING4 complexes. In agreement with previously reported results, ING4C HAT activity on H3 peptides bearing the various K4 methylation states was increased when H3K4 was di- or trimethylated (Fig 10A) (Saksouk, et al., 2009; Hung, et al., 2009).

We further showed that this H3K4me3-dependent increase in H3 acetylation also occurred in the context of chromatin via HAT assays on reconstituted chromatin. Chromatin was assembled with recombinant histones using either H3 that was unmodified at K4 or that bore the methyl-lysine analogue of trimethylated K4 (Lauberth, et al., 2013). Using equivalent amounts of chromatin (Fig 10B), some increase in H3 acetylation in the presence of trimethylated H3 could be detected with ING4C (Fig 10C). This increase can be seen more clearly in the case of Western blots done against individual acetyl-

Fig 10. H3K4me3-dependent ING4 complex HAT activity.

A) ^{3}H-labeled acetyl-CoA HAT assay on H3 peptides containing the N-terminal 1-21 residues of H3 with different K4 methylation states provided by the Rockefeller University Proteomics Center. Counts per minute of each sample was assayed with scintillation. Results are from two independent experiments for me0 and me1 and three independent experiments for me2 and me3. Error bars indicate the standard deviation.

B) H3K4me0 and H3K4me3 analog chromatin assemblies (chrom) used in subsequent assays. (Coomassie stain)

C) HAT assay on chromatin assembled with recombinant unmodified histones or with histones with the H3K4me3 analog. A slight increase in activity is observed in the presence of H3K4me3. (Autoradiograph)

D) Acetylation on H3K18 and H3K9 after chromatin acetylation by ING4C, JHIE and BHIE. ING4C. ING4C acetylation of H3K9 and BHIE acetylation of H3K9 and H3K18 is enhanced by H3K4me3. (Western blot. The top four rows are short, two minute exposures of the blots while the bottom four rows are longer, thirty minute exposures of the same blots)

Fig 10A

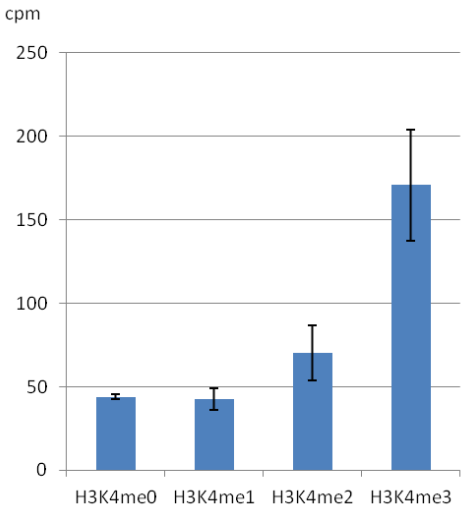


Fig 10B

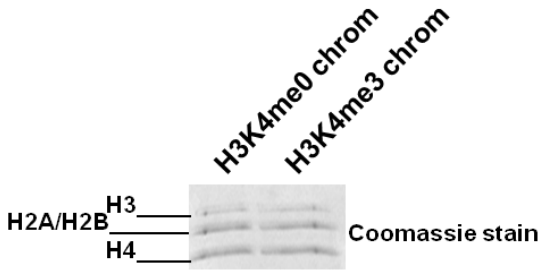


Fig 10C

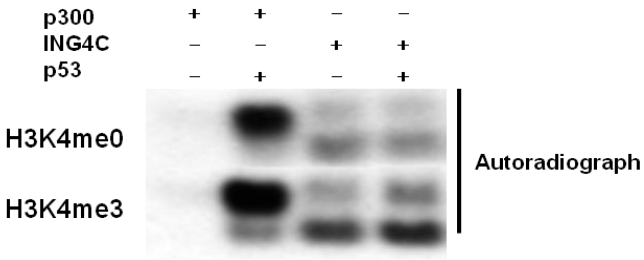
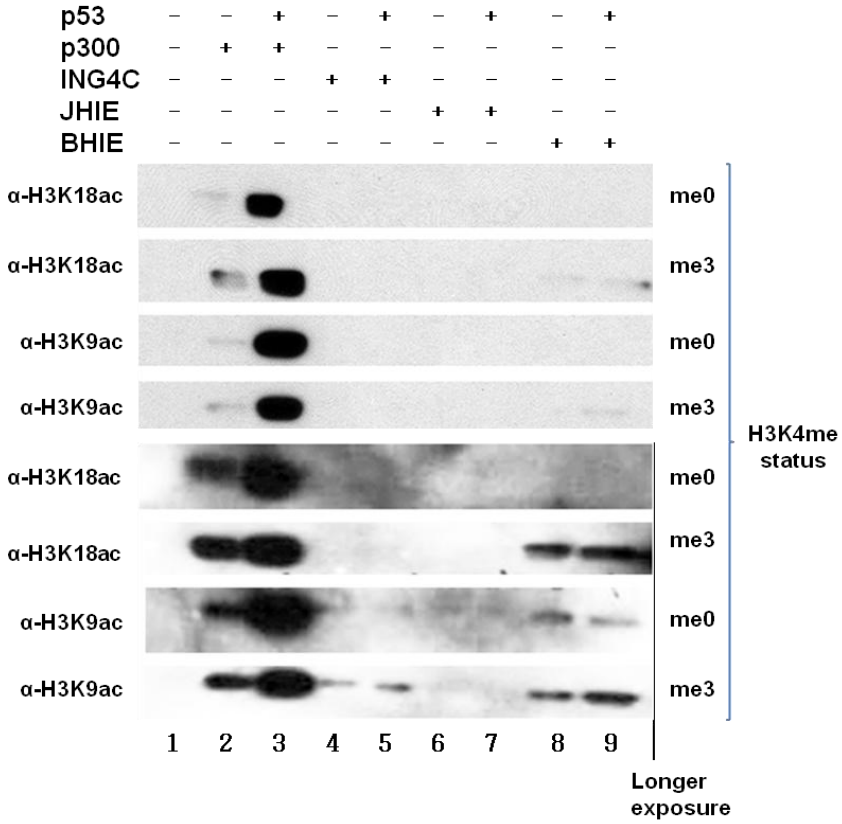


Fig 10D


p53 – – + – + – + – +
p300 – + + – – – – – –
ING4C – – – + + – – – –
JHIE – – – – – + + – –
BHIE – – – – – – – + +
α-H3K18ac
me0
α-H3K18ac
me3
α-H3K9ac
me0
α-H3K9ac
me3
α-H3K18ac
me0
α-H3K18ac
me3
α-H3K9ac
me0
α-H3K9ac
me3
H3K4me status
1 2 3 4 5 6 7 8 9
Longer exposure

lysines. These blots revealed that acetylation of H3K9 by ING4C and H3K9 and H3K18 by BHIE, but not by JHIE, was increased in the presence of trimethylated H3K4 (Fig 10D). This is consistent with the observation that BRD1 mediates HBO1 H3 HAT specificity, while JADE1L mediates HBO1 H4 HAT specificity. Western blots were also conducted for H3K14 acetylation, but no signal was detected (data not shown).

3.4 Interaction with p53

3.4.1 ING4 and the ING4 complex are capable of direct physical interactions with p53

ING4 has been shown to physically associate with p53 in the cell, although the assays in these cases have consistently used ectopically overexpressed ING4 (Shiseki, et al., 2003; Zhang, et al., 2005). To verify that the physical association is something that occurs normally with natural levels of ING4, endogenous ING4 was immunoprecipitated from doxorubicin treated HCT116 cells that contain functional p53. p53 was found to coIP with ING4 (Fig 11A). These results are consistent with, but do not prove, a direct interaction since the contribution of other proteins to this association have not been ruled out.

In vitro, a direct interaction has also been mapped out between purified p53 and ING4, although not in the context of the ING4 complex (Zhang, et al.,

2005). To verify that this interaction can also take place in the context of the more physiologically relevant ING4 complex, binding assays were conducted with bacterially purified GST-p53 and the ING4 complex as well as with purified ING4 on its own (Fig 11B). These combined results showed that p53 can directly bind both to ING4 as isolated subunit and also to the ING4 complex as a whole.

Interactions between HBO1 and p53 have also been noted and were verified in vitro using purified components. In fact, every subunit of the ING4 complex that was tested in an in vitro binding assay was observed to interact directly with p53 despite stringent wash conditions (Fig 11B). This begs the question of whether any of these p53 interactions are actually valid associations that occur in vivo, rather than non-specific interactions to some especially tractable region of p53. Regardless, these assays show that both ING4 as well as the ING4 complex is capable of binding directly to p53, at least under the conditions used in the in vitro assays both here and in the literature.

3.4.2 p53 does not directly affect ING4 complex HAT activity on chromatin

It has been shown that p300, another p53 cofactor that is a histone acetyltransferase and interacts with p53, is capable of increased acetylation of H3 and H4 in the context of chromatin in the presence of p53. In fact, while p300

Fig 11. ING4 complex interacts with p53.

A) p53 coIPs with ING4. Endogeneous ING4 IP from HCT 116 cells treated with doxorubicin. p53 association analyzed by Western blot.

B) In vitro binding using purified ING4 complex subunits and GST-p53. Binding to p53 was analyzed by Western blots against the FLAG-tagged subunit.

Fig 11A

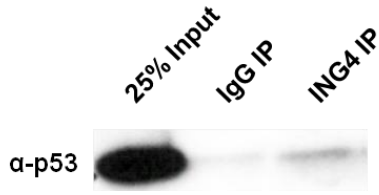
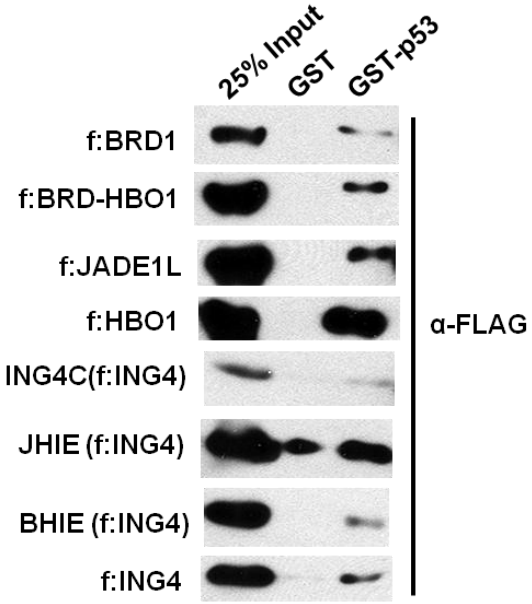


Fig 11B



is able to acetylate free histones or nucleosomal substrates on its own, its ability to acetylate chromatin is almost completely dependent on the presence of an activator such as p53 (Espinosa & Emerson, 2001; An, et al., 2004). In light of this p53-dependent activity by a p53-associated histone acetyltransferase, it seemed possible that the ING4 complex, interacting with p53 and containing a histone acetyltransferase, might also have a p53 affected/dependent chromatin acetylation.

To test this hypothesis, an ING4 complex HAT assay was carried out using chromatin assembled from recombinant histone octamers and purified factors. While, as previously observed in the literature, p300 acetylation of chromatin was strongly dependent on the presence of p53, histone acetylation by the ING4 complex seemed to be completely independent of p53, being equally strong in the presence of or absence of p53 (Fig 9D). Moreover, no p53 effect was observed even when lower amounts of the complex were used in a kinetic acetylation assay (Fig 9E compare lanes 9 with 10, and 11 with 12), which has been previously used to elucidate an activator-dependent histone acetylation on a similarly promiscuous HAT (Ikeda, et al., 1999). This p53 independence contrasts sharply with p300 mediated chromatin acetylation, which is strongly dependent on p53 (Fig 9E lanes 1 and 2). An extremely small effect can possibly be observed at H3K18 if the Western blot is exposed for an extended period of time, but this effect is so slight as to be negligible (Fig 9F compare lanes 4 with 5 for JHIE and lanes 6 with 7 for BHIE). For comparison, the strong p53-dependent

p300 acetylation of these residues is also shown here (Fig 9F compare lanes 2 and 3).

Therefore, despite the potential direct physical interaction capable of forming between the ING4 complex and p53, the observed acetylation of chromatin by the ING4 complex under these assay conditions does not require and is not measurably influenced by the presence of p53. This is in contrast with a previous study that reported an inhibition of HBO1 HAT activity by p53 in vitro. However, as previously noted, that study used a crude HBO1 immunoprecipitate rather than purified ING4 complex and studied its effects on free histones rather than the more physiologically relevant chromatin substrate (Iizuka, et al., 2008).

3.4.3 A synergistic p300-BHIE HAT activity that is dependent on p53

Cooperative effects have been observed between various p53 cofactors, such as p300 with PRMT1 and CARM1 (An, et al., 2004), MLL1 with MOF (Dou, et al., 2005), and p300 with SET1 (Tang, et al., 2013). Since both p300 (Espinosa & Emerson, 2001; An, et al., 2004) and ING4 (Shiseki, et al., 2003; Zhang, et al., 2004; Doyon, et al., 2006) have been implicated in p53-dependent transcription from *p21* or a *p21* promoter it seemed possible that both proteins might work together. An attempt was thus made to see if there a cooperative HAT effect might be observed by combining the two activities together.

To this end a HAT assay on chromatin was conducted using p300 and the JHIE and BHIE versions of the ING4 complex. Levels of p300 had to be substantially decreased from what is usually used in p300 HAT assays as the robust p300 acetylation of chromatin could be expected to mask any additional effect from the ING4 complexes that might be seen. Levels of JHIE and BHIE were also titrated to determine optimal conditions (data not shown). Under these conditions an increased histone acetylation was observed, with greater H3K9 and H3K18 acetylation levels being observed when p300, JHIE and BHIE were used together (Fig 12A lane 15), compared to when either was used individually (Fig 12A lanes 3, 5 and 10). This effect is not simply a sum of the independent HAT activities since acetylation by JHIE and BHIE alone under these conditions is negligible, but rather a synergistic effect of their respective HAT activities.

To further investigate this synergistic effect, the assay was also done using JHIE or BHIE individually in combination with p300. The greatest HAT synergy was observed with when both p300 and BHIE were used together (Fig 12A compare lane 14 with 13). This is consistent with earlier observations regarding the HAT specificity of BHIE and suggested that BHIE HAT activity might be enhanced by p300. Moreover, this HAT synergy by p300 and BHIE is strongly dependent on p53 (Fig 12A lanes 12 & 15), as is p300 HAT activity (Fig 12A lanes 2 & 3), whereas, as shown earlier, BHIE HAT activity on its own is not

Fig 12. Cooperative p300 and ING4 complex HAT activity on chromatin.

A) A p53-dependent p300-ING4 complex HAT synergy on both H3K4me0 and H3K4me3 containing chromatin. (Western blot with the indicated antibodies)

B) Scheme for ordering the p300 and BHIE acetylation on chromatin.

C) Ordered reactions of p300 and BHIE. p300 or BHIE or both were added to the reaction as indicated to obtain sequential, simultaneous or individual acetylation by p300 and/or BHIE. Maximal synergy is obtained with p300 followed by BHIE acetylation. (Western blot with indicated antibodies)

Fig 12A

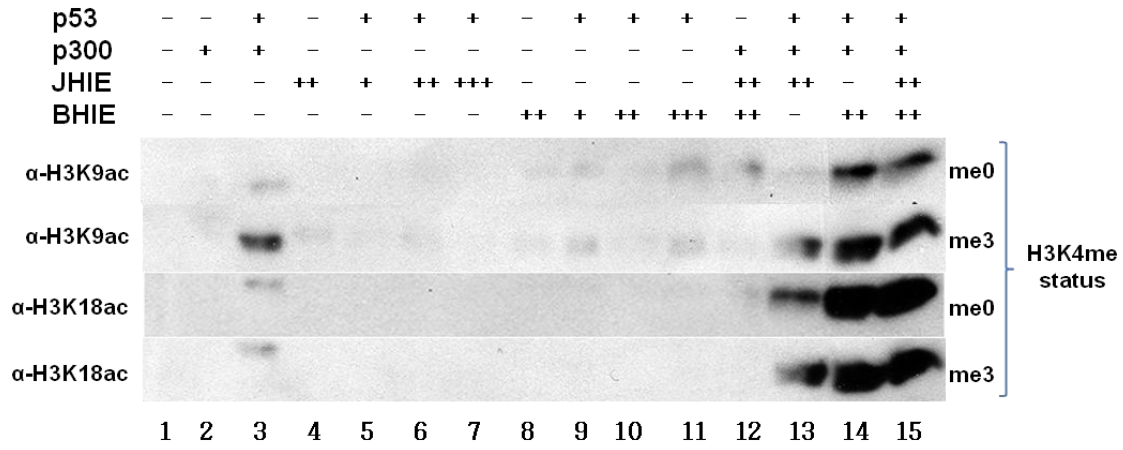


Fig 12B

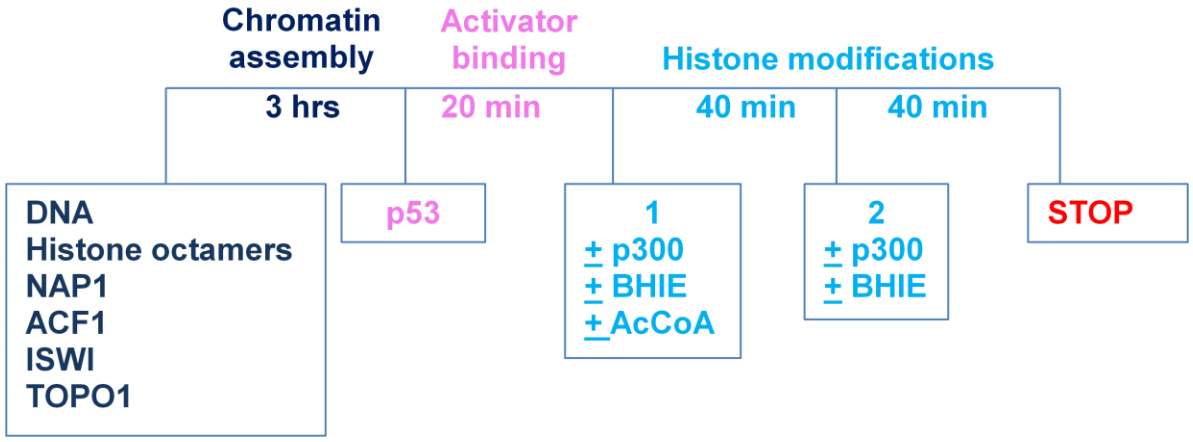
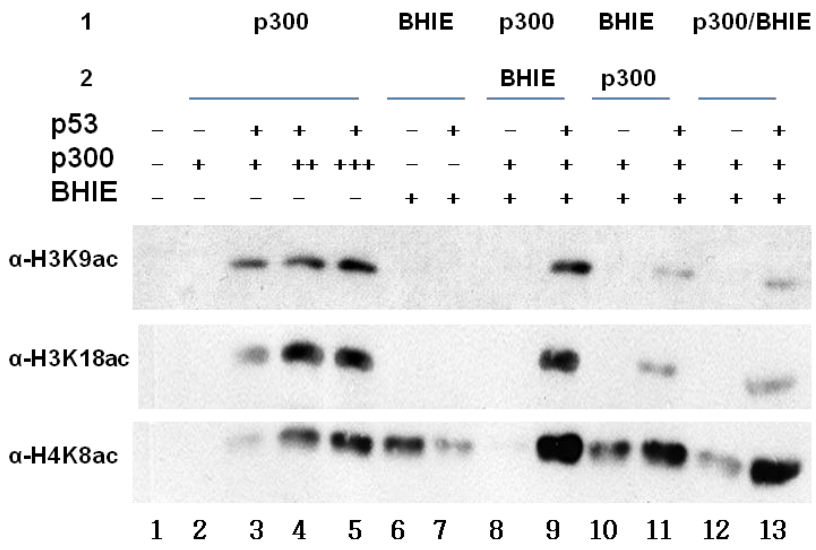


Fig 12C


1
p300
BHIE
p300
BHIE
p300/BHIE
2
BHIE
p300
p53 – – + + + – + – + – + – +
p300 – + + ++ +++ – – + + + + + +
BHIE – – – – – + + + + + + + +
α-H3K9ac
α-H3K18ac
α-H4K8ac
1 2 3 4 5 6 7 8 9 10 11 12 13

p53-dependent (Fig 9D, compare lanes 17 & 18 with 19 & 20). A comparative analysis with templates assembled with H3K4me3 revealed no additional effect of trimethylated H3K4 (Fig 12A, compare H3K4me0 with H3K4me3 rows), suggesting that this synergy is not enhanced by the ING4 PHD finger-trimethylated H3K4 interaction.

One possible explanation for the p300-BHIE HAT synergy is that some level of histone acetylation by p300 might be a prerequisite for maximal BHIE HAT activity. If this were the case, the synergistic effect would be most noticeable if the HAT reaction were ordered, with p300 histone acetylation taking place first, followed by BHIE histone acetylation. To test this, the HAT reaction by p300 and BHIE was staggered, with one HAT added before the other (Fig 12B). As predicted, a p300 addition followed by BHIE addition showed the greatest synergy (Fig 12C lane 9), compared to BHIE followed by p300 (Fig 12B lane 11), BHIE and p300 added simultaneously (Fig 12C lane 13), or either p300 or BHIE added individually (Fig 12C lanes 3 & 7). As before, this activity was strongly dependent on p53 (Fig 12C lanes 8 & 9). Note that use of higher concentrations of p300 does mask this synergistic effect (Fig 12C compare lanes 4 & 5 with lane 9), again emphasizing the need to avoid p300 saturation of the system.

3.5 Synergistic MLL1 complex-ING4 complex activity

Having established that the core MLL1 complex was also present in the purified ING4 complex, the next question was if the MLL1 complex was catalytically active. A lysine methylation assay utilizing the ING4 complex-MLL1 complex purified from HeLa cells were therefore conducted on chromatin assembled with recombinant histones. This assay determined that the MLL1 complex was indeed catalytically active, being capable of monomethylating, but not trimethylating H3K4, as has been previously reported for this complex (Fig 13B lane 6) (Tang, et al., 2013).

Acetylation and methylation reactions require acetyl-CoA and SAM respectively as cofactors. By controlling when/if these cofactors are added to the reaction, the ability of the ING4 complex-MLL1 complex to acetylate or methylate histones can be ordered or restricted and the effects of the histone acetylation on H3K4 methylation and vice versa can be examined (Fig 13A). So, for example, incubating the complexes with SAM for a time period, and then adding acetyl-CoA for a subsequent time period would result in methylation occuring first, followed by acetylation. From this assay, as would be expected, addition of acetyl-CoA resulted in H3 and H4 acetylation (Fig 13B lanes 2-5). However, addition of acetyl-CoA also led to an increase in monomethylation of H3K4 when added together with SAM, compared with SAM alone (Fig 13B compare lanes 2-4 with 6). This increase was greatest when acetyl-CoA was added

Fig 13. H3K4 methylation of the MLL1 complex associated with ING4.

A) Scheme for ordering the methyltransferase and acetyltransferase of the ING4 complex and associated MLL1 complex present in ING4C. The order of the methylation and acetylation is determined by controlling when the appropriate substrate is added in – ^{3}H labelled acetyl-coA for acetylation and S-adenosyl methione (SAM) for methylation.

B) ING4C-MLL1C HAT/KMT assay on chromatin. (Western blots with indicated antibodies to determine methylation and autoradiography to determine acetylation)

Fig 13A

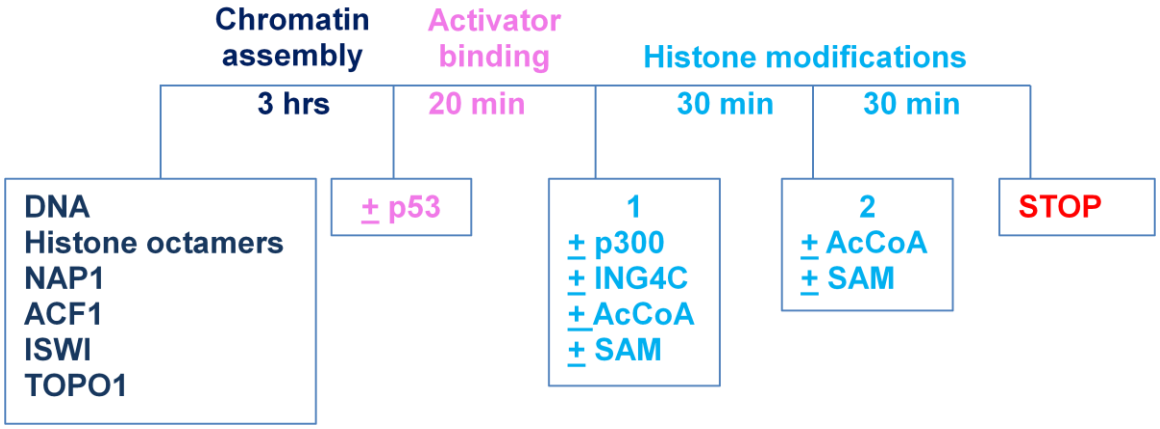
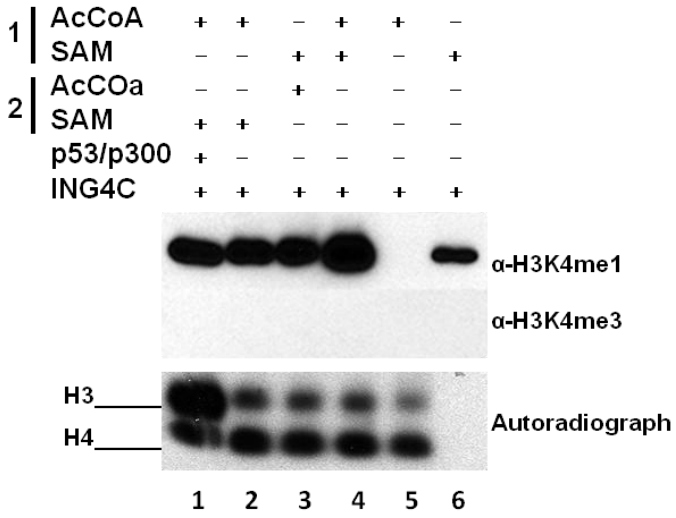


Fig 13B



simultaneously with SAM (Fig 13B lane 4). Conversely, addition of SAM had no significant effect on histone acetylation, regardless of the order of the reactions (Fig 13B compare lanes 2-4 with 5). To see if this enhancement could be enhanced by further histone acetylation, p300 and the p53 required for p300 chromatin acetylation were added, but no further increase in K4 methylation was observed (Fig 13B lane 1). Therefore the ING4 complex HAT activity enhances MLL1 complex mediated H3K4 methylation, similar to what has been seen between p300 and SET1 complex H3K4 methylation (Tang, et al., 2013).

3.6 Discussion

From this study we can conclude that the primary determinant of histone acetylation specificity in the ING4 complex is the choice of the scaffold subunit that holds the complex together. A BRD1 complex preferentially acetylates H3 while a JHIE complex preferentially acetylates H4, similar to what has been shown for the ING5 complex (Lalonde, et al., 2013). In contrast, purified ING4C, which contains both JADE and BRPF proteins, acetylates both H3 and H4 equally. This is consistent with the hypothesis that the activity of the purified ING4C is in fact a combination of the activities of two distinct ING4 complexes.

Using chromatin assembled with recombinant histones and purified factors, the HAT assays with both ING4C and the recombinant JHIE and BHIE have verified that H3 acetylation is increased by trimethylated H3K4. These

assays further identify H3K9 and H3K18 as two of the residues where this increase in acetylation takes place, and further establish H3K18 as an ING4C and BHIE acetylation target. More specifically, these assays show that this trimethylated H3K4-dependent activity occurs with BHIE and with ING4C (which we hypothesize contains the BRPF version as well as the JHIE version of the ING4 complex) but not with JHIE.

While other groups have linked the JADE-containing ING4 complex to a trimethylated H3K4-dependent increase in H3 acetylation (Hung, et al., 2009; Saksouk, et al., 2009; Avvakumov, et al., 2012), those links were made based on ChIP assays or ING4 complexes whose precise composition remains unclear. In the former case, while ChIP assays can show an increase in a histone mark in response to an increase in JADE, the limitations of cell-based assays mean that there is no way to tell if the effect is a direct one. In the latter case, possible contamination with the BRPF-based ING4 complex means that the H3 acetylation may not be due to the JADE-containing ING4 complex at all, but rather by a BRPF-containing ING4 complex.

The acetylation of H3K14 has also been linked to the ING4 complex through overexpression, knockdowns and knockouts of various ING4 complex members (Saksouk, et al., 2009; Kueh, et al., 2011; Mishima, et al., 2011). However, in contrast to this in vivo correlation, our in vitro HAT assays using

purified ING4 complexes and chromatin assembled with recombinant naïve (unmodified) histones failed to show any H3K14 acetylation under any of the conditions examined (p53, p300, trimethylated H3K4). H3K14 acetylation may therefore not be a direct target of the ING4 complex, but could instead be a subsequent downstream effect of ING4 complex activity. For example, a subsequent recruitment of MOZ/MORF, which has been shown to acetylate H3K14 (Doyon, et al., 2006), could account for such a link between the ING4 complex and H3K14 acetylation. Alternatively, given the number of chromatin recognizing domains present in the ING4 complex, it is possible that there is a histone mark missing from the naïve chromatin used here that is a prerequisite for H3K14 acetylation by the ING4 complex.

ING4 effects on cell growth and activation of p53 target genes is dependent on p53 (Shiseki, et al., 2003; Zhang, et al., 2004) and a direct physical interaction between p53 and components of the ING4 complex has been shown in this study and elsewhere (Zhang, et al., 2005, Iizuka, et al., 2008). However no direct p53-dependent effect on ING4 complex histone acetylation of any significance was observed in any of the in vitro HAT assays. What then accounts for the ING4 dependency on p53 that is observed in cells? One pathway by which this might be accomplished is through the establishment of trimethylated H3K4 via the SET1 complex, a pathway that has been shown elsewhere to be p53-dependent (Tang, et al., 2013). Another pathway is the one that has been established here, through ING4 complex synergy with p300 (Fig

14). So while there is no direct effect of p53 on ING4 complex HAT activity, there are at least two indirect pathways by which a p53 effect on ING4 complex activity is apparent. Future studies involving the integration of all of these pathways might further uncover additional mechanisms by which p53 affects ING4 complex activity.

Lastly we have described here a novel interaction between the ING4 complex and the MLL1 complex and shown that ING4 complex activity is capable of enhancing H3K4 monomethylation by the MLL1 complex. However the functional role of this H3K4 monomethylation in the context of ING4 complex activity is unclear. It could be a precursor to H3K4 di- or trimethylation, leading in turn to additional ING4 complex H3 acetylation in a feed-forward loop, or it could act as a mark that serves to recruit some other protein or protein complex. These are two possible avenues of further investigation, although both would require the identification and incorporation of additional interacting factors.

Chapter 4

Mediation of transcription by the ING4 complex

4.1 Preface

Members of the ING4 complex have been linked to transcriptional activity in various ways. As mentioned previously, overexpression of ING4 leads to activation of *p21* and *BAX* genes in a p53-dependent manner (Shiseki, et al., 2003; Zhang, et al., 2004) and overexpression of HBO1 was found to have a similar effect (Avvakumov, et al., 2012). Knockdowns of HBO1 in 293T cells decrease overall levels of H4K5, K8 and K12 acetylation (Doyon, et al., 2006) while knockouts of HBO1 and BRD1 in mice lead to a depression of transcriptional activity of various genes and an overall decrease in H3K14 acetylation (Kueh, et al., 2011; Mishima, et al., 2011).

ING4 and its complex members have been further linked to transcription activity by establishing their presence on genes via ChIP assays. Ectopically expressed ING4, JADE1L, BRD1 and HBO1 have been shown to localize around the TSS of genes, to colocalize with various histone modifications, and to correlate with actively transcribed genes. ING4 colocalizes with acetylated H3K9 and trimethylated H3K4 on *SMC4* (Hung, et al., 2009) while JADE1L was shown to colocalize with trimethylated H3K4 and acetylated H3K14 and H4K8 at the TSS of *EXT1* and *FOXP4* as well as further down the body of the genes where it colocalized instead with trimethylated H3K36 (Saksouk, et al., 2009). The catalytic subunit HBO1 tends to colocalize with BRD1 on genes that are transcriptionally active (Mishima, et al., 2011) and correlates with acetylated

H3K14 on the coding region of genes (Kueh, et al., 2011). Altogether these results suggest that ING4 and its complex members correlate with transcription and transcriptionally associated histone acetylation.

With regard to p53-associated transcription, endogenous HBO1, BRD1 and its BRPF1 paralog, as well as the ING5 protein, have been shown to bind to the region around the TSS of the p53 target *p21* gene (Avvakumov, et al., 2012; Lalonde, et al., 2013). Taken together with the more general links made between ING4 complex subunits and transcriptional activation as well as the observed link between ING4 and *p21* expression, these results suggest that ING4 and ING4 complex subunits could potentially act in conjunction with p53 to activate a p53 target gene.

Caution, however, must be taken in the extrapolation of these results to the ING4 complex. While JADE1L, HBO1 and BRD1 are members of the ING4 complex, they are not found exclusively in the ING4 complex. So while results involving these proteins can tentatively suggest ING4 complex enrichment and activity, the only protein that can be used to conclusively extrapolate ING4 complex enrichment and activity is ING4 itself. Additionally, the use of ectopically overexpressed proteins in the ChIP assays may not reflect the enrichment that occurs normally with the endogenous protein. Finally, previous ING4 and ING4

complex subunit ChIPs that have looked at *p21* occupancy have only looked at occupancy on *p21* in the absence of p53 activation.

Therefore the first thing we aimed to do was to study the enrichment of endogenous ING4 (and thus the ING4 complex) on a p53 target gene and to correlate this with p53-dependent gene activation. We then planned to complement these cell-based correlations by directly investigating the contribution of the ING4 complex to transcription in the context of a p53-dependent in vitro transcription system from chromatin templates. Guided by our histone modification studies, we would then use this system to look at conditions that affect the ING4 complex histone modifying activity to see if these might be translated into an effect on transcription.

4.2 ING4 enrichment on *p21*

4.2.1 ING4 recruitment to *p21* correlates with gene activation

To better determine the activity of the ING4 complex in association with transcription under endogenous protein conditions, ING4 ChIP assays were conducted against endogenous ING4 in HCT116 cells (which contain wild type p53) that had been with treated with the DNA damaging reagent doxorubicin in order to activate transcription of p53 target genes. The enrichment of ING4 around the TSS of *p21* (where HBO1, BRPF1/2 and ING5 have previously been

shown to localize (Avvakumov, et al., 2012; Lalonde, et al., 2013) was subsequently examined by quantitative PCR.

Under conditions of doxorubicin treatment, ING4 was enriched at the *p21* TSS and the region immediately downstream compared with untreated cells (Fig 14B). This enrichment was further accompanied by an increase in acetylated H3K9 (Fig 14C). As this region is also enriched with trimethylated H3K4 (Tang, et al., 2013), ING4 occupancy on *p21* correlates with trimethylated H3K4 (which is recognized by the ING4 PHD finger), acetylated H3K9 (which is acetylated by HBO1 in the ING4 complex), and p53 gene activation. This is consistent with the results of the in vitro HAT assays, which show that the ING4 complex binds to trimethylated H3K4 and acetylates H3K9.

4.2.2 ING4 recruitment to *p21* is dependent on p53

While p53 has no direct effect on ING4 complex chromatin acetylation in vitro, this study has shown than an indirect p53-dependent effect does exist. It is thus possible that p53 would also have an indirect effect on recruitment and histone acetylation of ING4 on *p21* in the cell. To investigate this possibility, an ING4 ChIP was conducted on HCT116 p53 knockout (p53 -/-) cells. As can be seen, the absence of p53 caused a substantial reduction in occupancy of ING4 on *p21* compared to the p53-containing HCT116 cells (Fig 14D). The increase in levels of acetylated H3K9 from the untreated to the treated cells were also

Fig 14. ING4 enrichment around the *p21* transcription start site (TSS) correlates with gene activation by p53 and correlates with increased H3K9ac enrichment.

A) Diagram of *p21* gene with location of sites targeted by ChIP primers indicated by arrows. Sites were selected based on observations that members of the ING4 complex tend to localize to the area immediately around the TSS. (Red line: TSS. p53 RE: p53 response element. The ladder shows the distance in Kb from the TSS)

B-E) ChIP assays on HCT 116 cells treated and untreated with doxorubicin. Results are from two independent assays. Error bars show the standard error. (con: control (untreated) cells. dox: cells treated with 0.5mM doxorubicin for 4 hours. p53 -/-: p53 knockout cell line.)

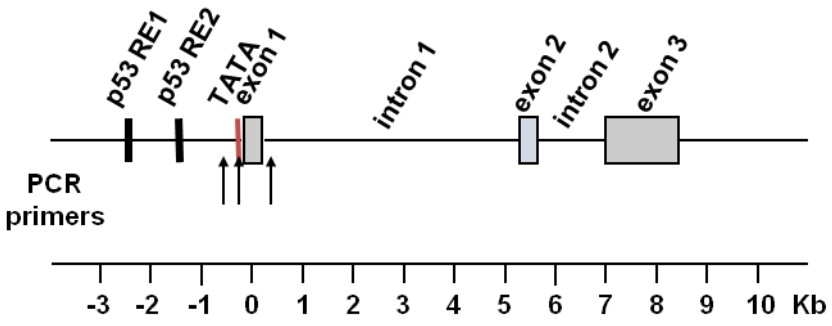
B) Doxorubicin treatment causes an increase in ING4 enrichment at the TSS and immediately downstream of the TSS of *p21* compared to control cells.

C) Doxorubicin treatment causes an increase in H3K9ac enrichment that correlates with ING4 enrichment compared to control cells.

D) In the absence of p53, ING4 enrichment upon doxorubicin treatment is decreased compared to control cells.

E) In the absence of p53, H3K9ac enrichment upon doxorubicin treatment is decreased compared to control cells.

Fig 14A


p53 RE1
p53 RE2
TATA
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intron 1
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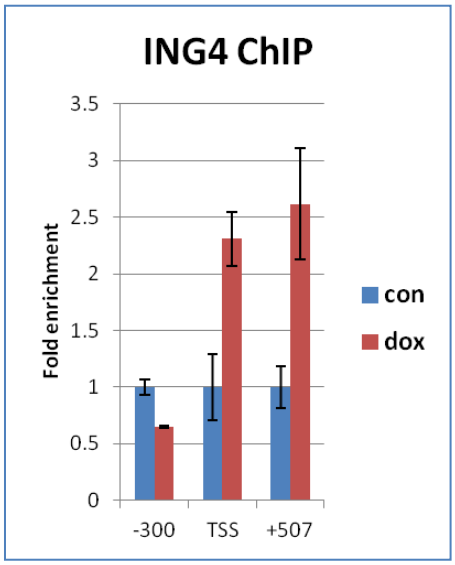
primers
-3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 Kb

B


ING4 ChIP
Fold enrichment
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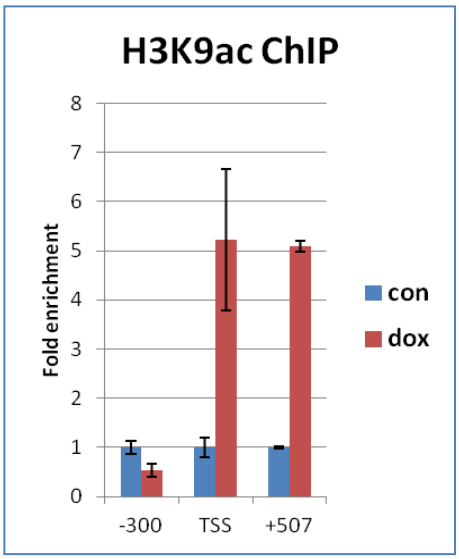
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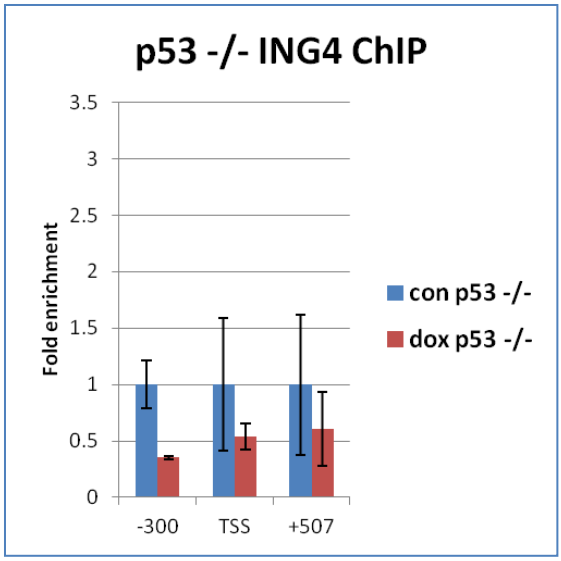
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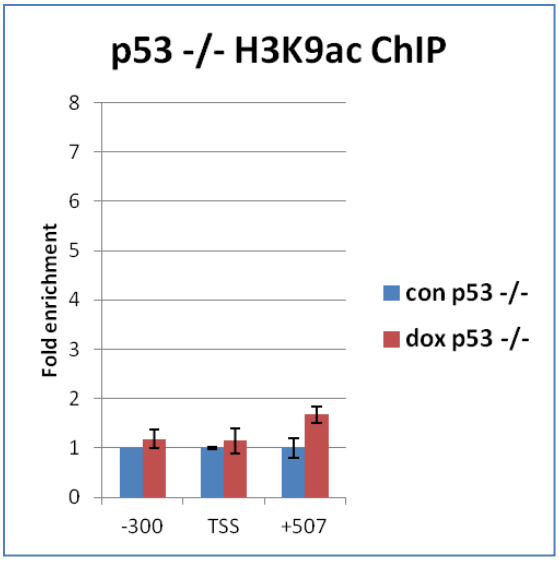
p53 -/- H3K9ac ChIP
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reduced (Fig 14E), which is what would be predicted if such acetylation were due to the ING4 complex. Thus, in the cell the recruitment of ING4 to *p21* is dependent on p53, as is the enrichment of H3K9 acetylation upon doxorubicin treatment.

4.3 p53-dependent, ING4 complex-mediated in vitro transcription

The described cell-based assays have provided clues as to the activity of the ING4 complex with respect to transcription in general and p53-mediated transcription in particular. However, the recurring limitation with such cell-based assays is that they are unable to show a direct causative effect of the ING4 complex on the transcriptional activation of target genes (p53 or otherwise). This difficulty is compounded by the multiple roles that ING4 and its complex are suspected to play in the cell. In addition to its role in p53-dependent gene activation, ING4 has also been implicated in the repression of NF-κB and HIF-1α pathways (Coles & Jones, 2009), while HBO1 plays a vital role in DNA replication (Burke, et al., 2001) and regulation of genes involved in erythropoiesis and embryonic patterning (Mishima, et al., 2011; Kueh, et al., 2011). The multiplicity of possible ING4 complex effects in the cell thus makes it difficult to elucidate ING4's precise role in p53 mediated transcription in such as system.

The strategy used here to resolve this dilemma is to study the effects of the ING4 complex in conjunction with p53 via an in vitro chromatin transcription system (Fig 15A). The use of such an in vitro system allows for the elimination of extraneous variables and indirect effects of the ING4 complex on p53-mediated transcription by changes in the expression of other genes or effects on DNA replication and the cell cycle. This allows for more focused conclusions on the ING4 complex' direct effects on p53-mediated transcription to be drawn.

4.3.1 The ING4 complex has a direct effect on p53-mediated transcription from chromatin templates

In vitro transcription assays on chromatin showed that while p53 alone had a minimal effect (Fig 15B lane 2), addition of the ING4C with p53 substantially enhanced transcription to a level comparable to what is seen with p300 (Fig 15B lanes 3 & 4). This demonstrates that ING4C does indeed function directly to enhance p53-mediated transcription and that ING4, in the context of its complex, does function as a transcriptional coactivator for p53. This effect was also observed using both of the reconstituted JHIE and BHIE. While JHIE or BHIE alone resulted in some p53-dependent transcription, transcription levels increased when both were used together (Fig 15C). Note, however, that this required titration of proteins since higher concentrations of JHIE and BHIE inhibit transcriptional activity (Fig 15D). This suggests that both the JADE and BRD

Fig 15. ING4 complex effects on p53-mediated transcription from chromatin templates.

A) Outline of in vitro transcription assay from chromatin templates.

B) ING4C enhances p53-dependent transcription on chromatin templates. 30ng of p300 and 20ng of ING4C were used.

C) JHIE and BHIE cooperate to enhance p53-dependent transcription. 7.5 ng of JHIE and 6ng of BHIE were used.

D) Excess JHIE and BHIE inhibit transcription. JHIE titration is from 15-60ng. BHIE titration is from 15-120ng.

Fig 15A

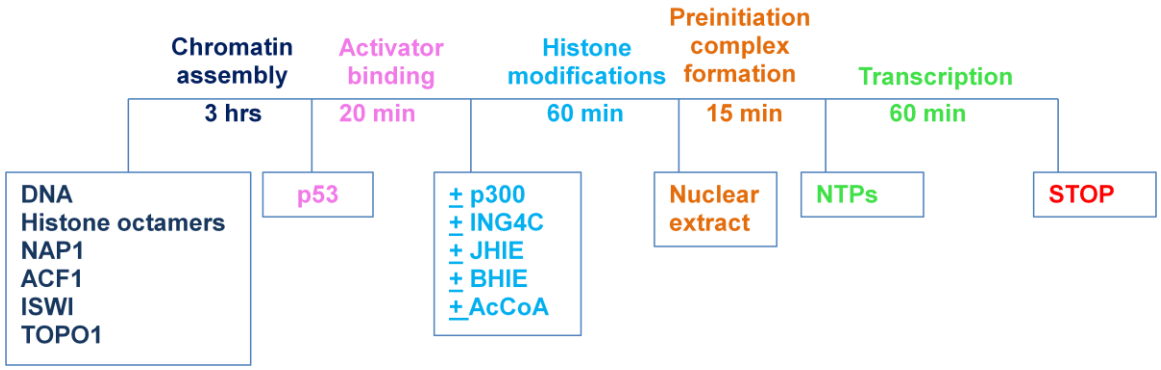


Fig 15B

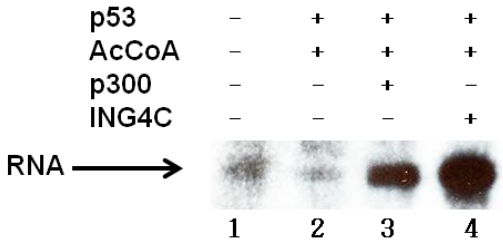


Fig 15C

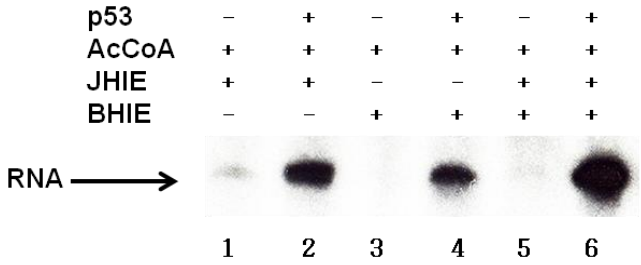
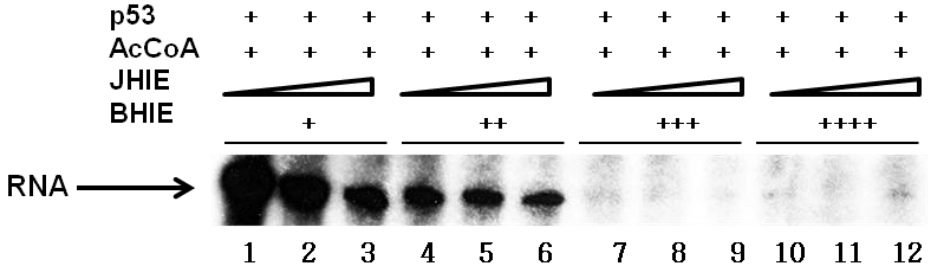


Fig 15D



versions of the ING4 complex are required for optimal HBO1/ING4-based p53 coactivator functions, and that JHIE and BHIE are both required to properly reconstitute purified ING4C activity.

Previous work has established the importance of joint H3 and H4 acetylation by p300 for p53-dependent transcription from chromatin (An, et al., 2004). In the in vitro transcription assays done here however, the addition of ING4C or JHIE and BHIE in the absence of p300 is sufficient to allow p53-dependent transcription. This suggests that it is the HAT activity of the ING4 complex that is important for its effect on chromatin transcription, and that the H3 and H4 acetylation supplied by ING4C or jointly by JHIE and BHIE is sufficient to allow this transcription. The fact that JHIE, which gives predominantly H4 acetylation, and BHIE, which gives mostly H3 acetylation, resulted in less transcription on their own than together supports the importance of both H3 and H4 acetylation for transcription from chromatin (Fig 15C).

4.3.2 ING4 complex-mediated transcription activity is enhanced by trimethylated H3K4

Having established that trimethylated H3K4 has a positive effect on ING4C and BHIE HAT activity, it seemed possible that this could also translate to a trimethylated H3K4-dependent effect on ING4C transcriptional activity. To

investigate this possibility, in vitro transcription assays were conducted with chromatin templates assembled with the trimethylated H3K4 analogue (Simon, et al., 2007) and carried out in conjunction with p300. As before, levels of p300 had to be titrated down to prevent masking of any ING4 complex contributions. ING4C used was similarly titrated to determine optimal concentrations (data not shown).

Under these conditions with reduced levels of p300, the presence of trimethylated H3K4 was crucial to ING4C-dependent as well as p300-dependent p53-mediated transcription (Fig 16A lane 4). This activity was dependent on p53 and acetyl-CoA, with no significant transcriptional activity observed in the absence of p53 or acetyl-CoA (Fig 16A compare lanes 4 with 7 & 9). The p53-dependency is consistent with ING4's role as a p53 coactivator rather than as an independent transcription activator of its own. The acetyl-CoA dependency further suggests that it is the ING4C HAT activity that is responsible for its contribution to transcription. As with the reconstituted JHIE and BHIE, the amount of ING4C used here is critical, as too much of it seems to inhibit transcriptional activity (Fig 16A compare lanes 4 & 5).

4.3.3 ING4 complex-mediated transcription activity is enhanced by p300

The synergy previously noted between p300 and the ING4 complex HAT activity, and the established link between histone acetylation and transcription, suggested the possibility of transcriptional synergy between p300 and the ING4 complexes. In fact addition of both p300 and ING4C resulted in a slight increase in transcriptional activity (Fig 16A compare lanes 3 & 4 with 6). As the HAT synergy between p300 and the ING4 complex was most apparent when they were added sequentially to the reaction, the in vitro transcription assay was similarly modified so that p300 was allowed to acetylate chromatin prior to the addition of JHIE and BHIE (Fig 16B). This ordered reaction, as with the HAT activity, gave greater levels of transcription than either p300 or JHIE and BHIE alone (Fig 16C lanes 4, 5, 7 & 8) and is dependent on p53 (Fig 16C lanes 8 & 9). This correlation between HAT synergy and transcription synergy further supports the link between histone acetylation and transcription.

This cooperative effect between p300 and the ING4 complex also allows lower levels of p300 to be used than is normally required to see the p53-dependent transcription in the p300 only system. So while it is possible to overcome the lack of other HATs by using an excess of p300, the inclusion of the JHIE and BHIE, which reflects the more physiological condition found in the cell

Fig 16. Factors that enhance ING4 complex histone acetylation enhance ING4 complex-mediated p53-dependent transcription.

A) In vitro transcription assay on chromatin comparing chromatin incorporating naïve (unmodified) histone octamers and chromatin incorporating the H3K4me3 analogue, with reduced levels of p300 (12.5ng) and ING4C (6 or 12ng).
B) Outline for of in vitro transcription assay with sequential p300 and JHIE/BHIE histone modification.
C) In vitro transcription assay on chromatin modified by p300 or JHIE/BHIE individually, or sequentially by p300 then by JHIE/BHIE. 12.5ng of p300 was used with 7.5 or 15ng of JHIE and 6 or 12ng of BHIE.

Fig 16A

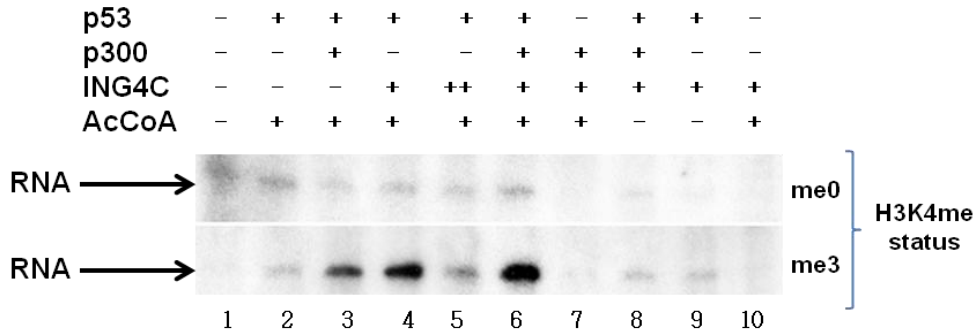


Fig 16B

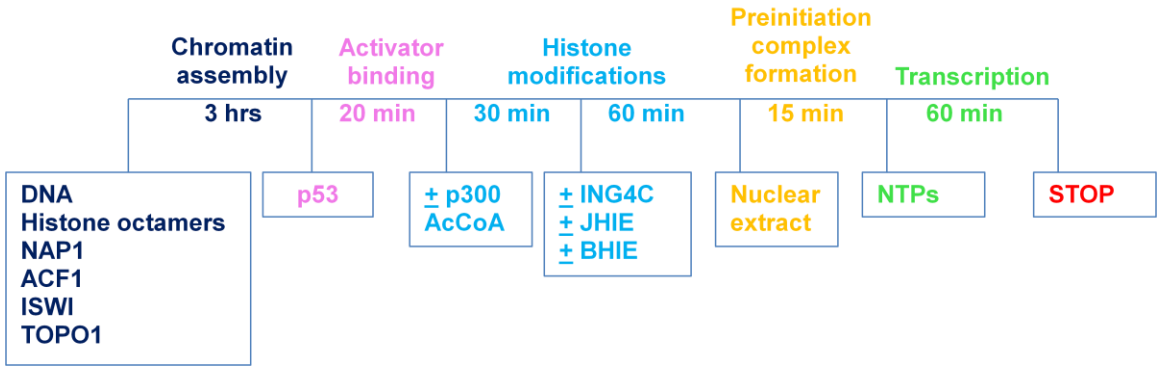
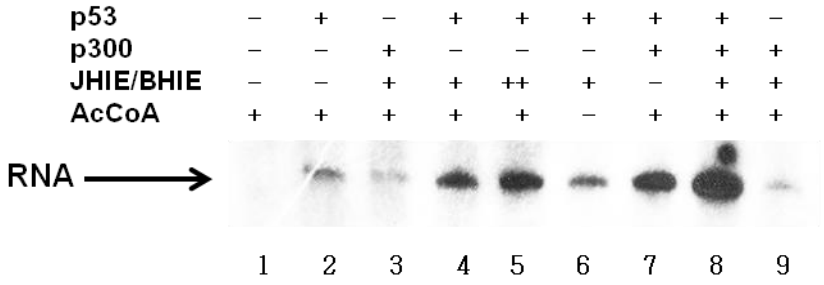


Fig 16C



where all these HATs are present, allows transcriptional activity to be obtained with ~3 fold lower concentration of p300.

4.4 Discussion

The ChIP assays conducted here show that endogenous ING4 enrichment on the *p21* promoter is enhanced upon activation of the *p21* gene. This is consistent with the hypothesized role of ING4 and ING4C in the activation of genes. In keeping with what is known about ING4C histone acetylation activity, this enrichment is correlated with trimethylated H3K4 and H3K9 acetylation. This is what would be expected if ING4C were mediating transcription by recognizing trimethylated H3K4 and acetylating histones. Furthermore, by using a p53 knockout cell line, this study establishes that the association of ING4 and acetylated H3K9 on the *p21* TSS in response to doxorubicin treatment is dependent on p53.

Note that while these assays show the p53 dependency of ING4 recruitment to *p21*, it does not mean that this dependency is direct. In fact the data available suggest that ING4 is recruited primarily to the TSS of *p21* rather than the p53 response elements (Avvakumov, et al., 2012; Lalonde, et al., 2013). This is a different enrichment pattern from p300, which is recruited to the p53 response elements (Dr Wei Yi Chen, Rockefeller University, unpublished data). The recruitment of p300 and the ING4 complex to different regions of *p21* may

be indicative of the different pathways by which they are recruited by p53. p300 is directly recruited by p53 and colocalizes with p53 at the p53 response elements upon gene activation. In contrast, the HAT activity of the ING4 complexes is not directly dependent on p53, and ING4 is not recruited to the p53 response elements. Rather, it is recruited to the TSS where it colocalizes instead with trimethylated H3K4.

Having established that ING4 is indeed associated with a p53 target gene under conditions of gene activation and that this association is dependent on p53, we then turned to an in vitro approach in order to overcome the limitations of cell-based assays. In contrast to all previously conducted assays associating the ING4 complexes with transcription, the in vitro transcription system established here demonstrates conclusively the direct effect of the ING4 complexes on p53-mediated transcription. This directly demonstrates its role as a transcriptional coactivator of p53. Furthermore it shows that in the context of this transcription system, in the absence of p300, ING4 complex activity is necessary for p53-mediated transcription and that this activity only occurs when acetyl-CoA, a necessary cofactor for acetylation is present. Lastly it shows that ING4 complex activity is capable of supporting p53-mediated transcription without any other ectopically added HATs, with the caveat that contributions from endogenous proteins in the nuclear extract used for the transcription cannot be ruled out.

In conjunction with what is known about the requirements for p300 H3 and H4 acetylation from the transcription assays done elsewhere using p300 and p53 (An, et al., 2004), and the known H3 and H4 acetylation specificity of the ING4 complexes, the logical conclusion would be that in this ING4 complex-dependent p300 independent system, the necessary H3 and H4 acetylation is being supplied by the ING4 complex. This is further corroborated by the fact that transcription attempted with the reconstituted JHIE or BHIE alone, which give predominantly H4 alone or H3 acetylation alone respectively, is substantially reduced, while using both JHIE and BHIE to supply the both H3 and H4 acetylation maximizes the transcription.

In contrast to the basal ING4 complex HAT activity on chromatin, which is p53 independent, the ING4 complex-mediated transcription of chromatin is highly dependent on p53. Histone acetylation by the ING4 complex is therefore insufficient to potentiate transcription on its own, but in conjunction with p53 allows transcription from chromatin, potentially as a function of ING4 complex histone acetylation. In fact, the ING4 complex-mediated effect on transcription correlates exactly with the HAT activity of the ING4 complex. Where greater histone acetylation is obtained by using both JHIE and BHIE complexes together, greater transcription is also observed. Where trimethylated H3K4 enhances the HAT activity of the ING4 complex, ING4 complex-mediated transcription in the presence of trimethylated H3K4 is also enhanced. Finally when HAT synergy is obtained by allowing sequential acetylation by p300 followed by the ING4

complex, transcriptional synergy is also obtained. Altogether, these observations provide strong, if circumstantial, evidence that the ING4 complex HAT activity, which is enhanced by p53-dependent p300 histone acetylation and p53-dependent trimethylated H3K4, is what is responsible for its effect on p53-mediated transcription.

However, this does not rule out other mechanisms by which the ING4 complex might also affect p53-dependent transcription. Acetylation of non-histone proteins could also play a role, or the ING4 complex might recruit other proteins/protein complexes and activities, such as the MLL1 complex and its H3K4 methylation activity. In fact, given the complexity of the transcriptional processes and the wide array of interactions that the ING4 complex is responsible for, it is very likely that there are other mechanisms yet to be discovered by which the ING4 complex affects transcription.

The establishment of an in vitro transcription system from chromatin that is dependent on both p53 and the ING4 complex is one that will be of great use in the further study of the ING4 complex and its contributions to p53-mediated transcription, allowing for biochemical in addition to cell based analyses of ING4 complex activity. The use of naïve (unmodified) chromatin templates in conjunction with other histone modifiers such as p300, as analyzed here, or other cofactors (such as SET1) known to function with p53 would allow a more

comprehensive approach to studying p53-mediated transcription. This, in turn would more accurately reconstitute and reflect the in vivo activities of p53 gene activation.

Chapter 5

Models and perspectives

5.1 Mechanism of ING4 complex p53-dependency

The premise behind the hypothesis that ING4 acts as a p53 cofactor was that its inhibitory effects on cell growth through cell cycle arrest and apoptosis were dependent on p53. This premise was further supported by current studies showing p53-dependent enrichment of ING4 and ING4 associated H3K9 acetylation at the TSS of *p21* upon conditions that activate *p21*. However, biochemical dissection of ING4 complex activity on chromatin revealed no significant direct effect of p53 on the HAT activity of the ING4 complex. So while ING4 is functionally dependent on p53 in terms of its effects in the cell, its ability to acetylate histones in the context of chromatin is not directly dependent on p53. How then does this functional p53 dependency come about? We propose here two non-mutually exclusive pathways by which p53 can indirectly effect ING4 complex HAT activity and thereby, to ING4 complex-mediated activation of p53 target genes.

5.1.1 Synergy of the ING4 complex with p53-dependent p300 activity

While p53, on its own, does not affect ING4 complex HAT activity on chromatin in any observable way, the ING4 complex and p300 together exhibit synergistic HAT activity that is dependent on p53. The fact that optimal HAT activity was achieved when the HATs were added sequentially, with p300 first and BHIE second, suggests that the p53-dependent p300 HAT activity primes the chromatin for subsequent ING4 complex-mediated histone acetylation (Fig

17), which affects subsequent transcription. One possible explanation for this priming can be drawn from the observation that this synergistic effect is seen primarily with the bromodomain containing BHIE version of the ING4 complex rather than the JHIE version of the ING4 complex which does not contain a bromodomain. This suggests that the bromodomain recognition of a p300 acetylated lysine residue on a histone tail may be responsible for the enhanced BHIE HAT activity observed. This might function to stabilize the protein complex on chromatin in the same way that bromodomain recognition of acetyl-lysines stabilizes p300 on chromatin (Tang, et al., 2013).

Another possibility for this synergistic HAT activity is based on the ING4 association with p300 (Shiseki, et al., 2003). A physical interaction between the ING4 complex and p300 might allow an alternative pathway by which p53 could indirectly affect ING4 complex activity. In this case, the p53 would recruit p300 which would in turn interact with the ING4 complex and enhance its activity on chromatin.

Further investigation will be required to determine if either or both of these possibilities are indeed responsible for the p53-dependent p300-ING4 complex effects on histone acetylation and transcription. A bromodomain mutation would allow analysis of its contribution to the p300-ING4 complex synergy while an HBO1 mutation that results in inactivation of the catalytic MYST domain would

Fig 17. p53-dependent ING4 complex HAT activity through p300.

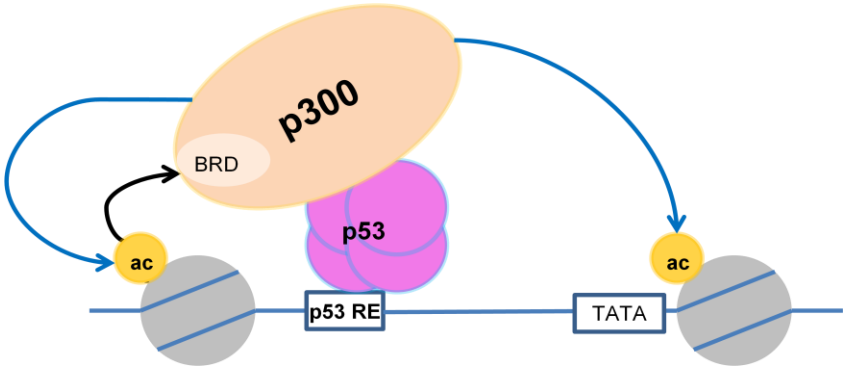
i) p53 binds to p53 response elements (p53 RE) and recruits p300. p300 acetylates histones and is stabilized on chromatin by p300 bromodomain-acetyl lysine interactions.

ii) The BRPF bromodomain of the ING4 complex interacts with the acetyl-lysines and allows additional histone acetylation by the ING4 complex. The ING4 complex may be further stabilized on chromatin by possible interactions with p300.

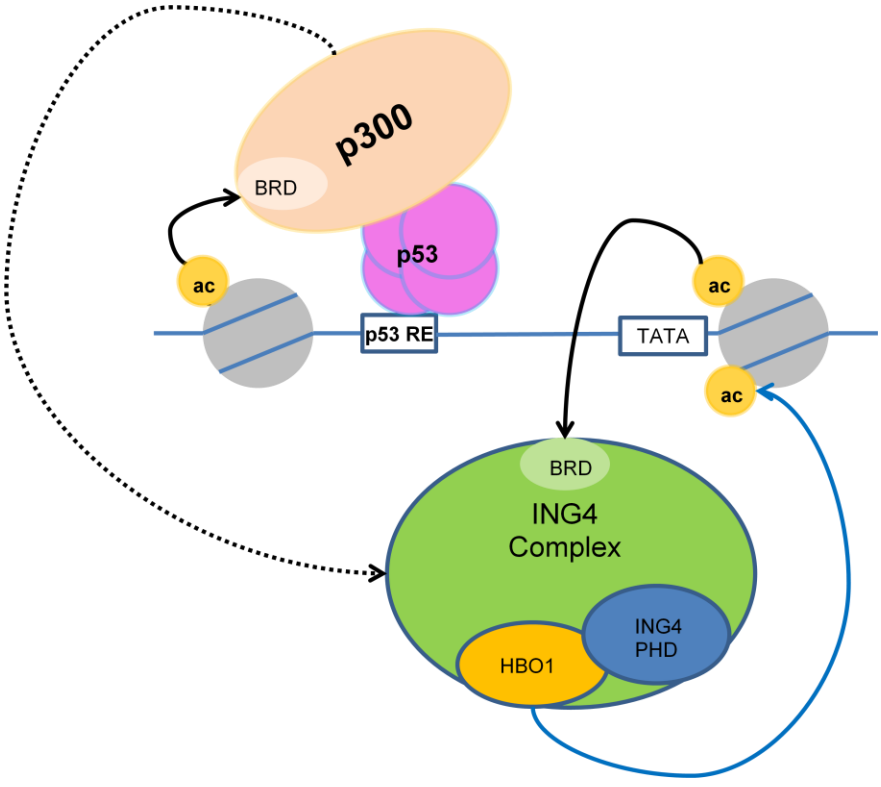
Blue arrows represent histone acetylation activity. Black arrows represent physical interactions. Dashed black arrow represents a possible (but unconfirmed) physical interaction. ac: Histone acetylation.

Fig 17

i.


p300
BRD
p53
ac
ac
p53 RE
TATA

ii.


p300
BRD
p53
ac
ac
p53 RE
TATA
ac
BRD
ING4
Complex
ING4
PHD
HBO1

allow a more conclusive link to be made between the ING4 complex HAT activity and its effects on both the observed HAT synergy as well its contributions to transcription. The determination of the acetyl-lysines that are bound by the BRPF bromodomain and whether it binds acetylated lysines in general or is restricted to specific acetylated lysines on H3 and H4 would also be interesting.

5.1.2 Cooperativity with p53-dependent H3K4 trimethylation

While not shown here, the link between p53 and H3K4 trimethylation on p53 target genes have been made elsewhere. What has been shown here is that H3K4 trimethylation does positively affect ING4 complex HAT activity. This allows for another pathway by which p53 could indirectly affect ING4 complex HAT activity on chromatin. It has been shown that p53 recruits p300, which in turn stabilizes the SET1 complex on chromatin and leads to trimethylation of H3K4 (Tang, et al., 2013). As an extension to that model, the ING4 complex could then be recruited to the chromatin through ING4 PHD finger recognition of the trimethylated H3K4, which allows subsequent histone H3 acetylation and transcription activation by the ING4 complex in conjunction with p53 and p300 (Fig 18).

Fig 18. p53-dependent ING4 complex HAT activity through H3K4me3.

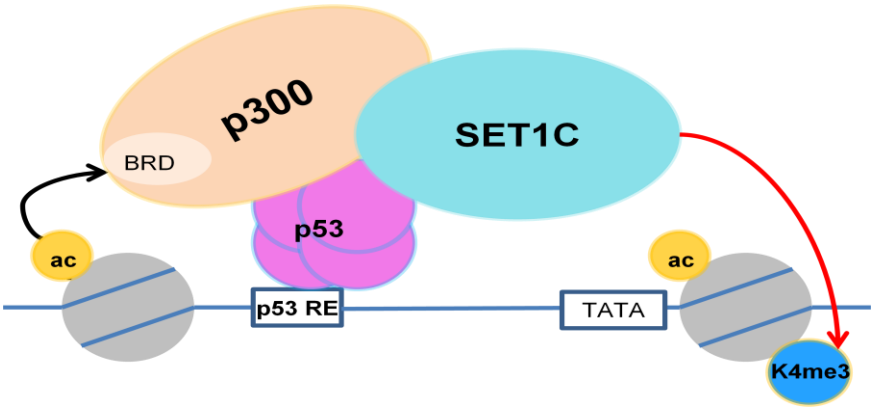
i) p300 is recruited to chromatin by p53 and is stabilized on the chromatin via bromodomain-acetyl lysine interactions. The SET1 complex (SET1C) is recruited by p53 and p300 and trimethylates H3K4.

ii) The ING4 complex binds to trimethylated H3K4 via its ING4 PHD finger, allowing additional histone acetylation by the ING4 complex.

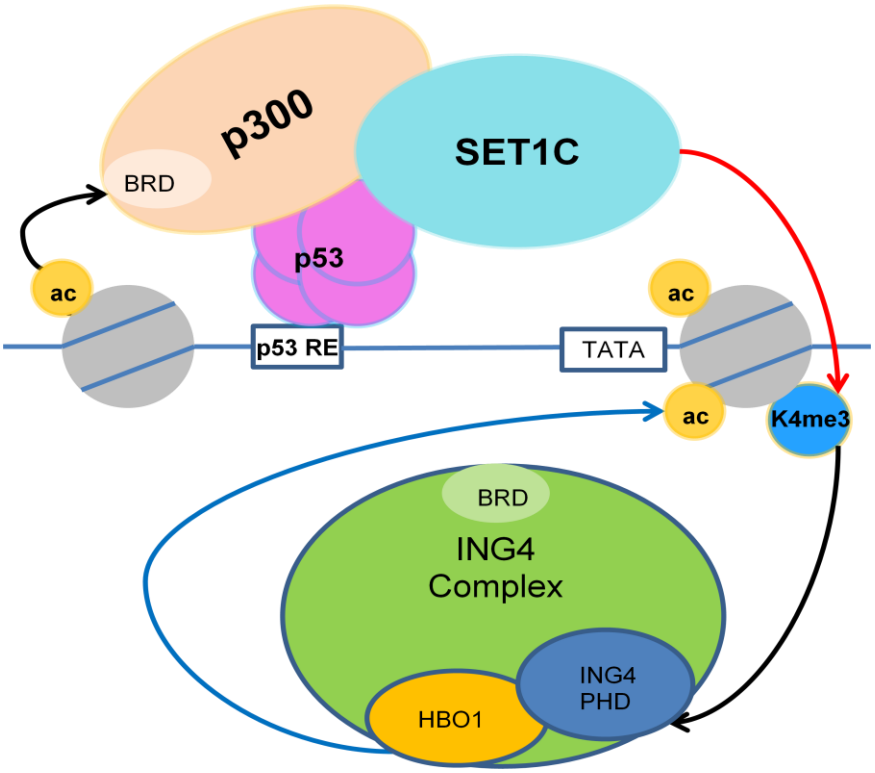
Blue arrow represents histone acetylation activity. Black arrows represent physical interactions. Red arrow represents H3K4 trimethylation activity. ac: Histone acetylation. K4me3: Trimethylated H3K4

Fig 18

i.


p300
SET1C
BRD
p53
ac
p53 RE
TATA
ac
K4me3

ii.


p300
SET1C
BRD
p53
ac
p53 RE
TATA
ac
ac
K4me3
BRD
ING4
Complex
ING4
PHD
HBO1

An interesting future experiment to consider would be to employ SET1 complex-mediated H3K4 trimethylation in conjunction with the p53-dependent, p300-ING4 complex dependent chromatin acetylation and transcription system used here. Such an experiment would integrate all histone modifications into one contiguous set of reactions and allow a more cohesive conclusion to be drawn rather than via extrapolating from individual experiments.

5.2 Expanding the ING4 complex epigenetic vocabulary

5.2.1 Simultaneous reading from differently marked histones

The multiplicity of histone readers present in the ING4 complex, some of which read mutually exclusive histone marks (ING4 PHD recognizing trimethylated H3K4 and the JADE and BRPF PHD1 recognizing unmethylated H3K4) begs the question of how these multiple readers integrate their input on chromatin. The size of the histone reading modules and the crystal structures available for modules binding to histone tails preclude the possibility of multiple modules reading the same histone tail at the same time due to steric hindrance. The possibility exists, however, of simultaneous binding of multiple histone reading modules to different histone tails, either within the same nucleosomes, or on multiple nucleosomes.

One intriguing possibility that this suggests is this use of the JADE/BRPF PHD1 and the ING4 PHD fingers to simultaneously bind to nucleosomes at a trimethylated H3K4-unmethylated H3K4 boundary. In conjunction with HBO1 HAT activity, this could be one possible mechanism by which acetylation of histones on unmethylated H3K4 could be influenced by the trimethylation of H3K4 on an adjacent nucleosome. This acetylation could in turn stimulate methylation of the unmethylated H3K4 by the MLL1 or SET1 complex, providing a pathway by which H3K4 methylation could be spread outwards from an existing trimethylated H3K4 bearing nucleosome (Fig 19).

5.2.2 ING4-ING4/ING4-ING5 complex dimerization

The ability of the ING4 LZL region to form homodimers with the LZL regions of other ING4 molecules and the predicted ability to form heterodimers with the LZL regions of ING5 molecules raises the possibility of ING4-ING4 and ING4-ING5 (as well as ING5-ING5) complex dimmers. This would dramatically expand the number of histone binding domains and catalytic histone modifying domains that could be assembled into a single entity. For example, a dimer that contains both JADE as well as BRPF versions of the ING4 complex would be possible, combining the H3 acetylation preference of BRPF complex with the H4 aceylation preference of JADE complex. A dimer that contains both HBO1 and

Fig 19. Exploiting the H3K4me3-H3K4me0 nucleosomal boundary through binding of the ING4 complex to adjacent H3K4me3 and H3K4me0 containing nucleosomes.

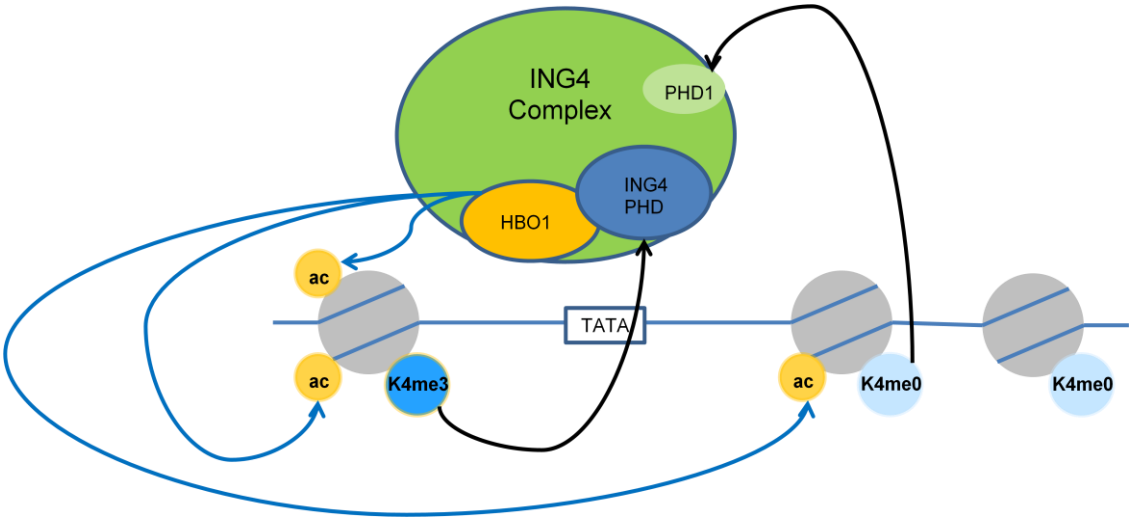
i) The ING4 complex binds to adjacent nucleosomes via ING4 PHD interactions with H3K4me3 and JADE/BRPF PHD interactions with H3K4me0 at the H3K4me3-H3K4me0 nucleosome boundary. The ING4 complex acetylates H3 & H4 on the H3K4me3 nucleosome but only H4 on the H3K4me0 nucleosome.

ii) Acetylation on the H3K4me0 nucleosome allows a p300 stabilized SET1 complex to trimethylated H3K4, creating a new H3K4me3-H3K4me0 boundary.

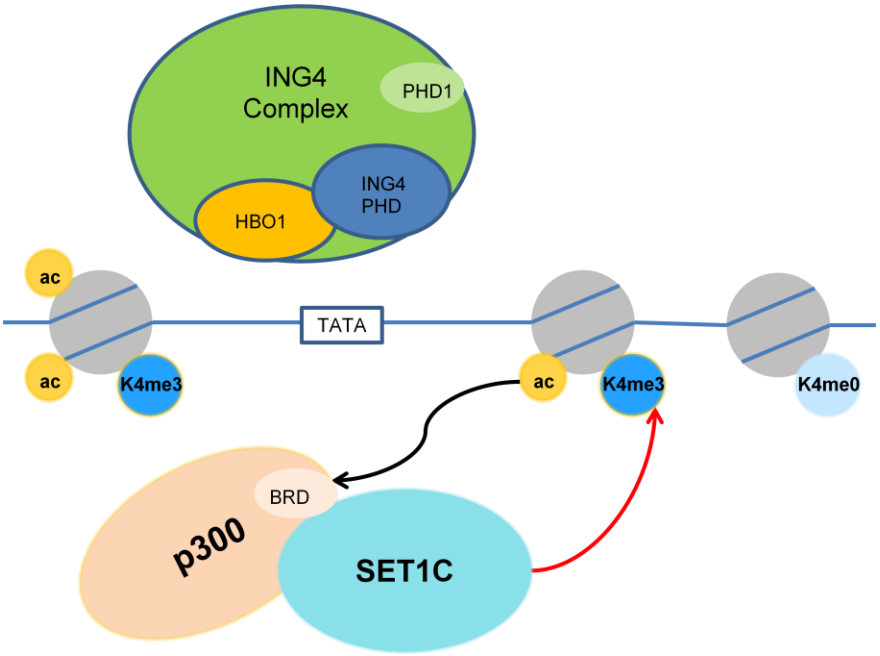
iii) The ING4 complex binds to the newly trimethylated H3K4 nucleosome and adjacent H3K4me0 nucleosome.

Blue arrows represents histone acetylation activity. Black arrows represent physical interactions. Red arrow represents H3K4 trimethylation activity. ac: Histone acetylation. K4me3: Trimethylated H3K4. K4me0: Umethylated H3K4

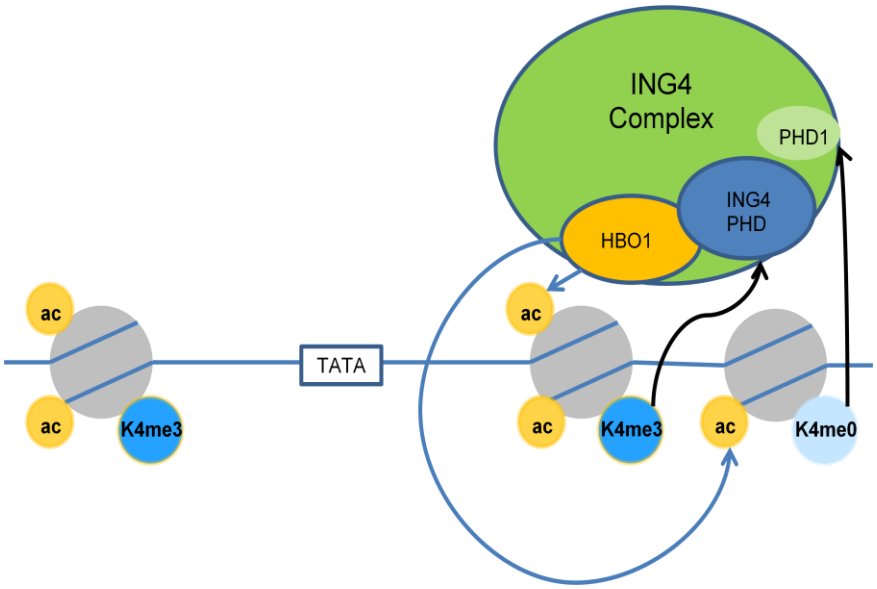
i.


ING4
Complex
PHD1
ING4
PHD
HBO1
ac
ac
TATA
K4me3
ac
K4me0
K4me0

ii.


ING4
Complex
PHD1
ING4
PHD
HBO1
ac
ac
TATA
K4me3
ac
K4me3
K4me0
BRD
p300
SET1C

iii.


ING4
Complex
PHD1
ING4
PHD
HBO1
ac
ac
K4me3
TATA
ac
ac
K4me3
ac
K4me0

MOZ/MORF would also be possible, leading to coordination between the H3 and H4 acetylation by HBO1, and MOZ/MORF-mediated H3K14 acetylation (Ullah, et al., 2008).

The increased availability of multiple histone binding domains would also increase the affinity of the overall dimer for chromatin (Ruthenburg, et al., 2007b), as well as allowing the specific recognition of a more extensive combination of histone marks. Dimerization would likewise increase the physical distance over which nucleosomes could be bound and modified by the dimerized complex, compared to a single undimerized complex. Further studies looking beyond the dimerization of ING4 to dimerization of the ING4 complex would therefore be useful in studying the full scope of what the ING4 complex can do in terms of coordinated recognition and modification of histones.

5.2.3 Effects of recognition of other histone marks

While this study has focused primarily on the recognition of trimethylated H3K4 and its effects on the HAT activity of the ING4 complex, the effects of many other histone marks that are recognized by the ING4 complex on ING4 complex activity have yet to be fully elucidated. The BRD1 bromodomain recognition of acetylated lysines has been proposed as a possible mechanism for the p300-ING4 complex HAT synergy, but has yet to be investigated. The

specificity of such recognition to a particular lysine or lysines on histones has also yet to be discovered.

Similarly, while the effect of trimethylated H3K4 recognition compared to unmethylated recognition of H3K4 by the ING4 complex on H3 acetylation has been examined on several specific H3 residues, the same has yet to be done for H4, with studies to date looking at overall H4 acetylation via radiography rather than at specific residues. An ING4 complex modulated H4 acetylation specificity in response to unmethylated vs trimethylated recognition of H3K4 remains a possibility to be elucidated.

Lastly, while the recognition of the JADE PHD1 and BRPF PWWP domain to trimethylated H3K36 has been noted, no further study of the effects of such recognition on the HAT activity of the ING4 complex has yet been conducted. Investigating the effects of this mark on ING4 complex activity would be useful to better understand the mechanism by which the ING4 complex mediates transcription.

Chapter 6

Methods and Materials

6.1 cDNAs

ING4 and JADE1L cDNAs were amplified from HeLa cell RNA by RT-PCR. HBO1 (Gene accession number: BC032640) and hEAF6 (BG702589) cDNAs were purchased from Open Biosystems, while BRD1 (BC030007) cDNA was purchased from ATCC.

6.2 Antibodies

The following antibodies were used for ChIP, co-IP and immunoblots: ING4 (Dr Zhanyun Tang, Rockefeller Univeristy), JADE1L/PHF17 (Abcam), BRD1/BRPF2 (Abcam), HBO1 (Santa Cruz Biotechnology), hEAF6/C1orf149 (Santa Cruz Biotechnology), H3K4me3 (Abcam), H3K4me1 (Abcam), H4K8ac (Abcam), H3K9ac (Abcam), H3K14ac (Millipore), H3K18ac (Abcam), MLL1C (Dou, et al., 2005), RBBP5 (Bethyl Laboratories), WDR5 (Bethyl Laboratories), ASH2L (Bethyl Laboratories) and p53 (Santa Cruz Biotechnology).

6.3 Purification and reconstitution

A stable HeLa/Tet-on cell line (Tang, et al., 2013) expressing FLAG-ING4 was obtained from Dr Zhanyun Tang. Doxoycline (Sigma) was used to induce expressing of FLAG-ING4 for 36 hours and the treated cells lysed in SDS sample buffer and blotted for ING4 to determine conditions where ectopic ING4 expression was approximately equal to endogenous ING4 expression. Under

these conditions nuclear extracts were prepared from the cells as previously described (Dignam, et al., 1983) except that extract was dialyzed to 300mM rather than 100mM KCl. The extract was fractionated through a phosphocellulose (Whatman P11) column equilibrated with BC300 (BC0 (20mM Tris-HCl (ph 7.9), 0.2mM EDTA, 20% glycerol, 0.2mM phenylmethylsulfonyl fluoride (PMSF) and 10mM β-mercaptoethanol) with 300mM KCl) with 0.1% NP-40, and the BC500 fraction (BC0 with 500mM KCl) containing ING4 and HBO1 was dialyzed in BC0 to a final salt concentration of 0.3M KCl. This fraction was cleared by centrifugation and the supernatant incubated for eight hours with anti-FLAG M2 agarose (Sigma), washed three times with BC300 with 0.1% NP-40 (Sigma) and eluted with BC150 (BC0 with 150mM KCl) with 0.1% NP-40 containing 0.3 mg/ml 3xFLAG peptide (Sigma). The complex was then analyzed by silver staining or concentrated with an Amicon Ultra 10K centrifugal filter and sent for mass spectrometry analysis.

cDNAs were cloned into pFASTBAC1 vectors either directly, or after modification to include an N-terminal FLAG epitope. Baculoviruses were generated according to the manufacturer's instruction (Invitrogen). Sf9 cells were infected with the desired baculoviruses and incubated for 72 hours. The infected cells were collected and resuspended in BC300 with 0.1% NP-40 and sonicated for 2.5 minutes in a Diagenode bioruptor. Cell debris was removed by centrifugation and the supernatant incubated with M2 agarose beads (Sigma) for

four hours. The complex was then washed and eluted as described for the ING4 complex purification.

The p208ML DNA template along with p300, NAP1 and the chromatin assembly factors ACF1, ISWI and TOPO1, as well as FLAG-tagged p53 and GST tagged p53 were purified as described (An, et al., 2004). Unmodified recombinant histones together with histones containing the semi-synthetic H3Kc4me3 (Simon, et al., 2007) were assembled into histone octamers and combined with the DNA template and incubated with chromatin assembly factors to assemble chromatin as described (An & Roeder, 2004). HeLa nucleosomes were purified from HeLa cell nuclear pellets as described (Owen-Hughes, et al., 1999).

6.4 Histone modification assays

HAT assays were carried out in 10mM HEPES (pH 7.9), 30mM KCl, 2.5mM DTT, 0.25mM EDTA, 5mM sodium butyrate and 5mM $MgCl_2$ with 2ul of 0.5mM ^3H acetyl-CoA or unlabeled acetyl-CoA added together with the HATs in a total volume of 31.5ul. For the assays on HeLa nucleosomes and free histones, 4ul ING4C, 8ul JHIE or 8ul BHIE was added to 200ng of substrate and incubated with at 30'C for one hour (Fig 9A). For assays on chromatin, 50ng of p53 was incubated with 95ng of DNA assembled into chromatin for 20 minutes at 27'C, followed by addition of p300, ING4C, JHIE or BHIE and incubated for one hour at 30'C. For the titration assay 10, 20, 40 or 80ng of ING4C, 5, 10, 20 or 40ng of

JHIE or 40 or 60ng of BHIE, as measured by the amount of HBO1 present in the complex were used (Fig 9D). For the time course, 10ng ING4C and 200ng p300 were used (Fig 9E), while for the other chromatin HAT assays 200ng of p300, 80ng of ING4C, 60ng JHIE and 60ng BHIE was used (Fig 9F, Fig 10C & 10D). For the assays examining p300 and ING4 complex HAT activity, 25, 50 or 100ng of p300, and 7.5, 15 or 30ng of JHIE or BHIE complex was used (Fig 12A & 12C).. For the KMT/HAT assay 120ng ING4C, 70ng p300 and 50ng p53 was used and where indicated, SAM was added to a final concentration of 50µM SAM (Fig 13B). Samples were run on a 15% acrylamide gel and analyzed by immunoblot or autoradiography as indicated.

For the H3 peptide HAT assay, biotinylated H3 peptides with different K4 methylation states were synthesized by the Rockefeller core facility and conjugated to streptavidin agarose beads (Thermo Scientific) by incubation of 25 µg of peptide with 25 ul of beads for 3 hours at room temperature, followed by extensive washing with PBS (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 and 1.8mM KH_2PO_4, pH 7.4) with 0.1% Triton-X (Sigma). 250ng of H3 peptide was incubated with 2ul of 0.5mM ^3H acetyl-CoA and 8ul ING4C for one hour at 30'C, washed extensively with TBS (150mM NaCl, 2.5mM KCl, 25mM Tris-HCl, pH 8), and the cpm measured by scintillation (Fig 10A).

6.5 p53 coIP and in vitro binding assays

~25x10^6 HCT 116 cells were treated with 0.5µM doxorubicin for 24 hours to activate p53, harvested, pelleted, then lysed by suspension in BC150 with 0.1% NP-40 and sonication on ice. The suspension was cleared by centrifugation and precleared with Protein A sepharose (Amersham). 5ul of ING4 antibody or IgG control antibody was then added to the supernatant and incubated overnight at 4'C. Protein A sepharose was incubated with the supernatant for 4 hours at 4'C, then extensively three times with BC300 with 0.1% NP-40. Samples were eluted from the beads with SDS sample buffer and analyzed by immunoblot.

For the in vitro binding assays, 1µg GST-p53 or control GST protein was incubated with 400ng of indicated ING4 complex subunit/s in BC150 with 0.1% NP-40 for 2 hours at 4'C. Gluthathione 4B sepharose was subsequently incubated with the sample for 1 hour at 4'C, then washed five times with BC150 with 0.1% NP-40. Samples were eluted with SDS sample buffer and analyzed by immunoblot.

6.6 ChIPs

ChIP assays were performed as described (Lauberth, et al., 2013) with 5ul of antibodies and enrichment at three sites around the TSS of p21 measured by qPCR. ING4 and H3K9ac values were normalized to the values in untreated

Table 3. Primers used in ChIP qPCR assays.

Region (bps from TSS)	Forward Primer	Reverse Primer
-300	TCTAACAGTGCTGTGTCCTCCT	CCCACGAAGTGAGCCACAAATCT
TSS	TATATCAGGGCCGCGCTG	GGCTCCACAAGGAACTGACTTC
+507	CCAGGAAGGGCGAGGAAA	GGGACCGATCCTAGACGAACTT

cells. Primers used for the qPCR are listed in Table 3. The TSS and +507 primers were adapted from Donner et al (Donner, et al., 2007)

6.7 Transcription assays

In vitro transcription assays from chromatin templates were performed essentially as described (An & Roeder, 2004). Briefly, 40ng of DNA template assembled into chromatin was incubated with 50ng of p53 for 20 minutes at 27'C followed by addition of indicated HATs and acetyl-CoA and a further incubation at 30'C for one hour. 5ul of HeLa nuclear extract was then incubated with the reaction for 15 minutes to allow PIC formation, followed by the addition of nucleotides and a further 60 minute incubation to allow transcription. For sequential modifications, p300 was incubated for 30 minutes, followed by incubation with the indicated complexes for one hour at 30'C.

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