INTRODUCTION

Covalent modifications of core histones modulate genome function and gene expression (Strahl & Allis, 2000). Specifically, histone methylation represents one of the most complex modifications (Lachner et al., 2003; Margueron et al., 2005; Martin & Zhang, 2005). Methylation on distinct lysine residues or different degrees of methylation on the same residue may have vastly differing consequences (Barski et al., 2007; Heintzman et al., 2009). Furthermore, histones can also be methylated on arginine residues (Bedford, 2007). The dynamics of histone methylation are regulated by methyltransferases and demethylases, and there are two classes of lysine-specific histone demethylases: amine oxidases (of which LSD1 is the founding member) and JmjC domain-containing proteins (Klose & Zhang, 2007; Mosammaparast & Shi, 2010). Both classes catalyse lysine demethylation via oxidative reactions. However, LSD1 targets only mono- or dimethylated lysines and uses flavin as a cofactor, while JmjC-containing proteins can remove all three lysine methylation states and require iron [Fe(II)] and α-ketoglutarate (α-KG) as cofactors.

Recent phylogenetic analyses have demonstrated that JmjC domain-containing proteins fall into seven groups of evolutionarily conserved proteins, with six groups containing at least one additional protein domain and the seventh group harbouring only JmjC (Klose et al., 2006). The JmjN domain is found in two of the groups; in one it is separated from JmjC by other domains, while in the other, it is located adjacent to JmjC. There are indications that the JmjN and JmjC domains form one functional unit (Chen et al., 2006; Tronnersjo et al., 2007). However, the exact function of the JmjN domain remains largely unknown.

In the budding yeast, five JmjC domain-containing proteins have been identified, and four of them have been demonstrated to possess histone demethylase activity (Kim & Buratowski, 2007; Tu et al., 2007). Among these four, three (Gis1, Rph1 and Jhd2) have the JmjN domain either located closely upstream of JmjC (Gis1 and Rph1) or separated from JmjC by other domains (Jhd2), similar to the mammalian JMJD2 and JARID1 classes of proteins, respectively (Klose et al., 2006). The Gis1 transcription factor activates transcriptional reprogramming of carbon metabolism and the stress response (Cameroni et al., 2004; Wu et al., 2004; Zhang et al., 2009) when yeast cells are transitioned to nutrient starvation. The transcription activation activity of Gis1 is controlled by target of rapamycin (TOR) and protein kinase A (PKA) via the Rim15 kinase (Pedruzzi et al., 2000, 2003; Wanke et al., 2008; Zhang & Oliver, 2010), and is necessary for extension of the chronological life span of yeast (Wei et al., 2008). More recently, we have demonstrated that Gis1 is subject to selective proteolysis mediated by the proteasome to negatively regulate its transcriptional activity (Zhang & Oliver, 2010). Here, we reveal that the two Jumonji

A group of JmjC domain-containing proteins also harbour JmjN domains. Although the JmjC domain is known to possess histone demethylase activity, the function of the JmjN domain remains largely undetermined. Previously, we have demonstrated that the yeast Gis1 transcription factor, bearing both JmjN and JmjC domains at its N terminus, is subject to proteasome-mediated selective proteolysis to downregulate its transcription activation ability. Here, we reveal that the JmjN and JmjC domains interact with each other through two β-sheets, one in each domain. Removal of either or both β-strands or the entire JmjN domain leads to complete degradation of Gis1, mediated partially by the proteasome. Mutating the core residues essential for histone demethylase activity demonstrated for other JmjC-containing proteins or deleting both Jumonji domains enhances the transcription activity of Gis1, but has no impact on its selective proteolysis by the proteasome. Together, these data suggest that JmjN and JmjC interact physically to form a structural unit that ensures the stability and appropriate transcription activity of Gis1.
domains, JmjN and JmjC, in Gis1 interact with each other, and that this interaction is mediated through two β-sheets, one in each domain. Deletion of either or both β-sheets or removal of the entire JmjN domain leads to complete degradation of Gis1 and loss of its transcription activity. However, removal of both JmjN and JmjC domains from Gis1 or mutation of the core residues essential for histone demethylase activity of other JmjC-containing proteins enhances Gis1 transcription activity without any impact on its selective proteolysis. Put together, these data indicate that the JmjN and JmjC domains in full-length Gis1 form a structural unit to ensure its stability and appropriate transcriptional activity.

METHODS

Strains and plasmids. Yeast deletion strains were generated previously (Zhang & Oliver, 2010). All plasmids used in this study are listed in Supplementary Table S1. Mutations to alanine of the conserved residues H204 and K222 were carried out by site-directed mutagenesis (Stratagene). LAKVIPPKE (amino acids 41–49 in the JmjN domain) and/or EFIITF (amino acids 282–287 in the JmjC domain) were similarly deleted. For one-hybrid assays, GIS1 or its truncated alleles, all lacking the sequence encoding the two zinc fingers, was fused with the LexA binding domain (LexA BD) in pLexPd (van der Ven et al., 2000). The DNA-binding domain of Gis1 was removed to enable all of the Gis1 protein to target the LexA operator and to maximize the sensitivity of the one-hybrid assays. For two-hybrid assays, GIS1 or JmjC (or their mutated versions) were fused with the Gal4 activation domain (Gal4 AD) in pACT2 and the LexA BD in pLexPd. Truncated GIS1 was cloned into pCM190 under the control of the tetO7 promoter for overexpression and toxicity tests. The working concentrations were 200 ng ml⁻¹ for rapamycin, 50 μM for MG132 and 20 μg ml⁻¹ for doxycycline.

Yeast one-hybrid, two-hybrid and overexpression toxicity assays. One-hybrid and two-hybrid assays were carried out in L40 (MATA hisΔ200 trp1-901 leu2-311 ade2-1 lys2::lexAop-HIS3 URA3::lexAop-lacZ GAL4) cells. The level of HIS3 expression and the β-galactosidase activity were quantified as previously described (Zhang & Oliver, 2010). Yeast transformants bearing the overexpression plasmids were grown in SMM medium under repressive conditions (plus doxycycline). Cells were resuspended in water and spotted in serial dilutions on SMM plates containing 20 μg ml⁻¹ of doxycycline. To determine the toxic effect of GIS1 overexpression on cell growth, the sequences corresponding to JmjN and JmjC domains were deleted (Fig. 1e). Put together, these data indicate that the JmjN domain is necessary for transcriptional activation by full-length Gis1, whereas the JmjN and JmjC domains together have a negative impact on this function.

The sequences corresponding to GIS1, jmjNΔ and jmjN/CΔ (lacking the two zinc fingers; see Methods) were also fused with the LexA BD and expressed in L40 cells (carrying the lexAop-HIS3 and lexAop-LacZ cassettes). On medium lacking histidine, cell growth was supported by the activity of either full-length GIS1 or jmjN/CΔ, but was significantly reduced in cells expressing jmjNΔ (H15→, Fig. 1c). Similarly, as compared with the action of full-length GIS1, the expression of β-galactosidase when activated by jmjNΔ was significantly decreased, but it was dramatically increased by the additional removal of the JmjC domain (Fig. 1d). Overexpression of GIS1 is toxic to cell growth (Pedruzzi et al., 2000; Zhang & Oliver, 2010). This toxicity is reduced when the JmjN domain is removed from GIS1 but increased when both the JmjN domain and the JmjC domain are deleted (Fig. 1e). Put together, these data confirmed that the JmjN domain has a positive, and together with the JmjC domain, a negative, effect on the function of Gis1 as a transcription activator.

RESULTS

The JmjN domain is necessary for transcription activation by full-length Gis1

Our previous analysis of the Gis1 transcription factor (Zhang & Oliver, 2010) has revealed two transcription activation domains (TAD1 and TAD2) interspersed by an unstructured region, with the JmjN and JmjC domains located at the N terminus and the DNA-binding domain (two zinc fingers) at the C terminus (Fig. 1a). Selective proteolysis of Gis1 by the proteasome removes both the JmjN domain and the JmjC domain and disrupts TAD2. To elucidate the function of the two Jumonji domains, the sequences corresponding to JmjN (1–100) or both JmjN and JmjC (1–330) were removed from GIS1. Truncated versions of GIS1, named jmjN and jmjN/CA (Fig. 1a), were expressed under the control of the endogenous GIS1 promoter in a centromeric plasmid. Exponentially growing gis1Δ deletion cells bearing these plasmids were treated with rapamycin, and the transcript levels of Gis1-target genes were assayed by Northern analysis. Transcription activation of SSA3 and GRE1, seen in cells expressing the full-length GIS1, was completely abolished in cells expressing jmjN (Fig. 1b). In contrast, when both the JmjN domain and the JmjC domain were removed, transcription activation of SSA3 and GRE1 was increased by two- to fourfold (Fig. 1b). These data indicate that the JmjN domain is necessary for transcriptional activation by full-length Gis1, whereas the JmjN and JmjC domains together have a negative impact on this function.

To find out why the JmjN domain is necessary for transcription activation by Gis1, we initially assayed the transcript level of GIS1 in cells expressing different truncated GIS1 alleles. As shown in Fig. 2(a), the transcript...
The JmjN domain is required for the transcription activity of Gis1. (a) Diagrammatic view of full-length GIS1 and its truncated alleles (jmjNΔ and jmjN/CΔ). C/C, coiled-coil domain; ZinF, zinc fingers; TAD1, -2, transcription activation domains 1 and 2. (b) Northern analysis of SSA3/GRE1 transcripts. Exponentially growing gis1Δ cells bearing wild-type or truncated GIS1 (jmjNΔ and jmjN/CΔ) were treated with rapamycin (Rapa) for 0, 1 and 3 h. Transcript levels of SSA3 and GRE1 were quantified, and normalized against the level of ACT1. Fold regulation at time 0 was set at 1. (c) Growth assay of L40 cells expressing wild-type or truncated GIS1 (jmjNΔ and jmjN/CΔ) in pLexPd. Exponential cells bearing the constructs were spotted in serial dilutions on plates which were incubated at 30 °C for 3–5 days. HIS+, SMM medium supplemented with histidine; HIS−, SMM medium without histidine. (d) Normalized β-galactosidase activities activated by the above GIS1 constructs in L40 cells. Data were obtained from quadruplicates (two biological and two technical replicates); error bars, sd. (e) Growth assay of cells overexpressing GIS1 and its truncated alleles in pCM190. Dox+, 20 μg doxycycline ml⁻¹; Dox−, no doxycycline.

level of GIS1 increased marginally (less than twofold) in cells treated with rapamycin. Removal of either JmjN or both JmjN and JmjC had little effect on the transcript level of GIS1. These truncated alleles were then tagged with myc13 and expressed under the control of the GIS1 promoter in exponentially growing cells treated with rapamycin. We have previously demonstrated that full-length Gis1 is subject to selective proteolysis mediated by the proteasome, producing multiple variants. The extent of the proteolysis increases with nutrient starvation or rapamycin treatment (Zhang & Oliver, 2010). Surprisingly, very little of either the full-length protein or its shorter variants was detected when the JmjN domain was deleted from Gis1 (Fig. 2b). With both the JmjN and JmjC domains removed, the levels of the Gis1 protein and those of its variants were similar to those seen for the full-length (wild-type) protein. This indicates that the JmjN domain is required to maintain Gis1 integrity. To find out whether the instability caused by removal of the JmjN domain was related to the proteasome, the myc13-tagged GIS1 alleles were also expressed in drug-sensitive pdr5Δ cells treated with the proteasome inhibitor MG132. The jmjNΔ truncated protein accumulated with MG132 treatment, in a manner similar to the full-length Gis1 (Fig. 2c) and to the jmjN/CΔ-deleted Gis1 (Zhang & Oliver, 2010). This confirms that the JmjN domain is required for the presence of intact Gis1; without it, the stability of Gis1 is much reduced (Fig. 2b). Recently, Yu et al. (2010) have demonstrated that removal of the JmjC domain alone from GIS1 results in a much lower level of Gis1 protein. Together, these data suggest that the JmjN and JmjC domains are both required for the presence of intact Gis1. In the absence of either domain, the stability of full-length Gis1 is much decreased. Furthermore, in addition to accumulation of full-length Gis1 after MG132 treatment, substantial amounts of v3 and v5, and a new variant above v2 (Fig. 2c),
were detected in cells expressing wild-type GIS1. Similarly, increasing levels of v3 were seen in cells expressing the jmjNΔ truncated GIS1. These data indicate that Gis1 is not exclusively processed by the proteasome.

The JmjN and JmjC domains interact with each other

Selective proteolysis by the proteasome requires the substrate protein to have an unstructured region flanked by structured domains, which protect the protein from complete degradation (Piwko & Jentsch, 2006). We initially analysed the amino acid sequences of the Gis1 protein using GlobPlot (Linding et al., 2003) and found that the N-terminal Jumonji domain contained the only globular region in the entire protein (Fig. 3a), with disordered structures and sequences of low complexity distributed between the coiled-coil domain and the C-terminal DNA-binding domain. Both the JmjN domain and the JmjC domain are required for the stability of the full-length Gis1.
protein, suggesting that the two domains form a structural unit. Structural analysis of a histone demethylase of the mammalian JMJD2 class has also suggested that the JmjN and JmjC domains come into contact with each other (Chen et al., 2006). We employed a yeast two-hybrid assay to find out whether the two domains interact in vivo. The sequences corresponding to JmjN and JmjC were fused with the Gal4 AD in pACT2 and the LexA BD in pLexPd, respectively. These plasmids were co-transformed into L40 cells (see Methods). As seen in Fig. 3(b), when both JmjN and JmjC were expressed, the expression of HIS3 was sufficient to allow cell growth on medium lacking histidine (HIS−). Slow growth was observed when JmjN was expressed simultaneously, and no cell growth was seen when JmjC was expressed in both plasmids, suggesting that JmjN may interact weakly with itself and that JmjC does not interact with itself at all. Similarly, strong β-galactosidase activity was detected only when JmjN and JmjC were expressed together (Fig. 3c), confirming the physical interaction between JmjN and JmjC.

The JmjN and JmjC domains interact through two β-sheets, one in each domain

An earlier structural analysis of a histone demethylase of the mammalian JMJD2 class has suggested that the JmjN and JmjC domains come into contact through two β-sheets, one in each domain (Chen et al., 2006). Sequence alignments and secondary structure analysis have demonstrated that Gis1 has two similar β-strands in its JmjN and JmjC domains (Fig. 4a). To find out whether these two β-strands are essential for the interaction between JmjN and JmjC, the 27 nt corresponding to IVKVIPPKE was deleted from the JmjN domain (named jmjN1) and the 18 nt encoding EFITTF was similarly removed from the JmjC domain (named jmjC1). jmjN1 and jmjC1 were fused with the GAL4 AD and the LexA BD, respectively, and expressed in the above two-hybrid system. As shown in Fig. 4(b), deletion of either or both β-strands abolished the expression of HIS3, which supports cell growth on medium lacking histidine. Expression of the β-galactosidase activity seen with the wild-type JmjN and JmjC domains was also abolished when either or both β-sheets were removed (Fig. 4c), indicating that the two β-sheets are crucial to the interaction between the two domains.

To find the functional consequences of the interaction between the two β-sheets, either or both β-strands were removed from full-length Gis1 and expressed from the Gis1 promoter in gis1Δ cells. Exponentially growing cells were treated with rapamycin. The transcription activation of Gis1 targets seen in cells expressing the full-length Gis1 was dramatically reduced in cells expressing any of the deletion alleles (Fig. 4d). These alleles were also tagged with myc13, and the protein levels of Gis1 and its variants were assayed. As shown in Fig. 4(e), no significant amount of full-length Gis1 or any of its variants was detected in cells expressing the truncated alleles, indicating that removing either β-strand from the Jumonji domains leads to accelerated degradation of the Gis1 protein. To find whether this instability is related to the proteasome, these myc13-tagged alleles were also expressed in pdr5Δ cells treated with MG132 to inhibit the proteasome function. Significant amounts of the full-length Gis1 protein were seen in cells expressing the truncated alleles, although at lower levels than those observed in cells bearing the wild-type Gis1 (Fig. 4f). Cells treated with MG132 were then washed and resuspended in medium containing 100 μg Cyh ml−1. As shown in Fig. 4(g), Gis1 bearing both deletions had a much shorter half-life than the full-length protein (note the different timescales). These data confirmed that JmjN and JmjC interact to form a structural unit to ensure the stability of the full-length Gis1 as well as its selective proteolysis mediated partially by the proteasome.

Mutating the core residues of JmjC enhances transcription activation by Gis1

Removal of both the JmjN domain and the JmjC domain does not change the proteolytic processing of Gis1 by the proteasome (Fig. 2b) but does increase its ability to activate transcription (Fig. 1b), indicating that the Jumonji domains negatively regulate transcription activation by Gis1. Two residues (H204 and K222) in JmjC of Gis1 were each mutated to Ala. The two residues are conserved in the family of the JHDM3/JMJD2 proteins (Klose et al., 2006) and are essential to the histone demethylase activity of other JmjC-containing proteins (Tsukada et al., 2006). The mutated alleles of JmjC were expressed in the yeast two-hybrid system together with wild-type JmjN. As shown in Fig. 5(a), the mutant alleles of JmjC activated the expression of LacZ to an extent similar to their wild-type counterpart, suggesting that the interaction between the JmjN and JmjC domains is not affected by these mutations. These mutations were also introduced into full-length Gis1, and their effects on protein fate and the ability of Gis1 to activate transcription were determined. Both mutant alleles gave rise to the same levels of full-length and shorter variants of Gis1 protein as their wild-type counterpart (Fig. 5b), indicating that these mutations have no effect on the proteolytic processing of Gis1. However, transcription activation of GRE1 by the mutant alleles was increased by 1.5- to twofold as compared with the wild-type Gis1 protein (Fig. 5c). In contrast, transcription activation of SSA3 by the mutants remained the same as that observed in wild-type Gis1 (Fig. 5c). The mutant alleles of Gis1 lacking the two zinc fingers were also fused with the LexA BD, and transformed into L40 cells. As compared with wild-type Gis1, the β-galactosidase activity in cells expressing the mutant alleles increased by 50–100 % (Fig. 5d). Of the two mutant alleles, K222A exhibited higher transcription activation (Fig. 5c, d). These data suggest that the JmjC domain of Gis1 could possess a histone demethylase activity that has a negative impact on transcriptional activation by Gis1, depending on the promoter context.
DISCUSSION

Two out of seven groups of JmjC domain-containing proteins also bear JmjN domains, either located close to JmjC or separated from JmjC by other domains (Klose et al., 2006). Structural analysis of mammalian JMJD2-class proteins has suggested that the JmjN and JmjC domains come into contact through two β-strands (Chen et al., 2006). However, it remains unknown whether the interaction takes place in vivo and what the functional implication of the interaction may be. Here, using the yeast Gis1 transcription factor as a model, we demonstrate that the JmjN domain physically interacts with JmjC, and that the two β-strands (one in each domain) are essential for their interaction. The function of this interaction is to ensure the stability of the full-length Gis1 protein and its selective
proteolysis. Failure of this interaction leads to faster degradation of Gis1 (Fig. 4g) and abolition of Gis1-dependent transcription (Fig. 4d). Recently, the analysis of the crystal structure of Rph1 has revealed that its JmjN and JmjC domains come into contact through the two conserved β-strands, one in each domain (Chang et al., 2011). Jhd2, another JmjC-containing protein in budding yeast, has also been shown to be degraded by the proteasome through polyubiquitination (Mersman et al., 2009). The JmjN domain of Jhd2, separated from JmjC by a plant homeodomain (PHD), is required for its protein stability (Huang et al., 2010). Put together, these data indicate that the JmjN and JmjC domains, located close together or separated from each other, form a structural unit through physical interaction to maintain the stability of JmjC-containing proteins. Contrary to our predictions, Yu et al. (2010) have reported that deletion of the entire JmjC domain has no effect on transcription activation by Gis1. This is may be due to the different starvation conditions and techniques used in the two studies. Yu et al. (2010) assayed the levels of SSA3 and GRE1 transcripts in gis1Δ deletion cells expressing GIS1 or its mutated alleles treated with rapamycin (Rapa) for 0, 0.5 and 2 h. Quantification and normalization are the same as described in Fig. 1(b). Moreover, transcriptional changes were not quantified in their study, making it difficult to compare the two studies. For example, in their study, upon glucose limitation, the SSA3 transcript level is noticeably lower in cells expressing GIS1DjmjC as compared with that seen in cells bearing the wild-type GIS1 (Yu et al., 2010). However, those authors state that the JmjC domain is dispensable for SSA3 induction under glucose limitation.

There is controversy on whether the JmjC domain of Gis1 possesses histone demethylase activity. It has been revealed that the position-three Fe(II)-binding residue in the JmjC domain of Gis1 is mutated (H292Y; Klose et al., 2006). A similar mutation in the fission yeast protein Epa1 renders it inactive as a histone demethylase (H305A; Tsukada et al., 2006). Experimental assays of H3 methylation in the budding yeast indicate that Gis1 has histone demethylase activity (Tu et al., 2007), whereas Yu et al. (2010) have reported that mutation of H204A or K222A in JmjC has no effect on SSA3.

Fig. 5. Mutation of the core residues essential for histone demethylase activity enhances the transcription activity of Gis1. (a) Normalized β-galactosidase activities activated by expressing JmjN and mutated JmjC. Data were obtained from quadruplicates (two biological and two technical replicates); error bars, sd. (b) Western analysis of Gis1 protein products encoded by the GIS1 ORF, GIS1H204A and GIS1K222A. These constructs were tagged with myc13 and expressed from the native GIS1 promoter. α-myc, anti-myc13 antibody; α-tub, anti-tubulin antibody. gis1Δ cells bearing either construct were grown to mid-exponential phase. (c) Northern analysis of SSA3 and GRE1 transcripts in gis1Δ deletion cells expressing GIS1 or its mutated alleles treated with rapamycin (Rapa) for 0, 0.5 and 2 h. Quantification and normalization are the same as described in Fig. 1(b). (d) Normalized β-galactosidase activities activated by GIS1 or its mutated alleles in L40 cells. Data were obtained from quadruplicates (two biological and two technical replicates); error bars, sd.
transcription activation by Gis1. Although mutation of H204A or K222A had little effect on transcription activation of SSA3 in our analysis, these mutations, especially K222A, increased the transcription activation of GRE1 (Fig. 5c) and exhibited higher transcription activation by Gis1 in the one-hybrid assay (Fig. 5d), suggesting that the JmjC domain of Gis1 could have histone demethylase activity. However, further evidence, such as the methylation level at the GRE1 locus, is necessary to demonstrate that the Junomii domain of Gis1 does possess histone-specific demethylase activity.

ACKNOWLEDGEMENTS

The experimental cost of this study was supported by a Biotechnology and Biological Sciences Research Council grant (BB/C505140/2) to S. G. O. Z. Q. is grateful to her family, the Cambridge Overseas Trust and Lucy Cavendish College for financial support.

REFERENCES


Edited by: S. D. Harris