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ORTH/VIM proteins that regulate DNA methylation are functional ubiquitin E3 ligases

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Summary

Appropriate methylation of genomes is essential for gene regulation. We describe here the six-member *ORTHUS* (*ORTH*) gene family of *Arabidopsis thaliana* that plays a role in DNA methylation *in vivo*. *ORTH1-5* are predicted to encode proteins each containing one PHD, two RING domains, and one SRA domain whereas *ORTHlike-1* encodes a protein with only one RING and SRA domain. cDNAs for *ORTH1*, 2, 5 and *ORTHlike-1* were isolated, and when expressed as GST fusion proteins were capable of promoting ubiquitylation *in vitro* with the E2 AtUBC11. *ORTH1* promotes ubiquitylation when paired with additional AtUBC8 family members. *ORTH1* proteins with substitutions in metal ligand binding residues in each *ORTH1* RING domain individually and *ORTH1* truncation derivatives lacking one or both RING domains were tested for ability to catalyze ubiquitylation *in vitro*. In these assays, either *ORTH1* RING domain is capable of promoting ubiquitylation. The PHD alone is not active as an E3 ligase, nor required for ligase activity. GFP-*ORTH1* and GFP-*ORTH2* are nuclear-localized in transgenic *Arabidopsis* plants. Overexpression of *ORTH1* or 2 in *Arabidopsis* leads to an altered flowering time. Inspection of DNA methylation at *FWA* and *Cen180* repeats revealed hypomethylation when *ORTH* proteins were over-expressed. Once initiated, a late-flowering phenotype persisted in the absence of the *ORTH* transgene, consistent with epigenetic effects at *FWA*. We conclude that *ORTH* proteins are E3 ligases mediating DNA methylation status *in vivo*.

Introduction

Cytosine methylation of genomic DNA is an important element in the epigenetic regulation of gene expression [for recent reviews see (Gehring and Henikoff, 2007; Henderson and Jacobsen, 2007)]. In plants, cytosine methylation can occur in CG, CHG, or CHH sequence context (where H = A, T, or C). In *Arabidopsis*, DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) is responsible for *de novo* methylation at all sites, while maintenance methylation requires multiple activities. The DNA methyltransferase MET1 is responsible primarily for maintenance of pre-existing CG methylation (Finnegan and Dennis,

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1993; Ronemus *et al.*, 1996), while maintenance of CHG methylation requires a plant-specific DNA methyltransferase, CHROMOMETHYLASE 3 (CMT3), and also a histone H3 lysine 9 methyltransferase KRYPTONITE/SUVH4 (KYP) (Henderson and Jacobsen, 2007). Small RNAs play a key role in targeting DNA methylation, especially in plants, in a process not completely understood (Wassenegger *et al.*, 1994; Henderson and Jacobsen, 2007; Huettel *et al.*, 2007; Matzke *et al.*, 2007). Asymmetric methylation of CHH is maintained in a small RNA-dependent *de novo* methylation pathway performed by DRM2 (Henderson and Jacobsen, 2007).

Plants defective in either *de novo* methylation or maintenance methylation exhibit a wide variety of phenotypic alterations. In *met1* mutants dramatic hypomethylation takes place genome-wide (Zhang *et al.*, 2006). This leads to stochastic phenotypes caused by the aberrant expression of genes silenced by methylation, including a late-flowering phenotype derived from the ectopic expression of *FWA*, altered flower development, and decreased fertility (Finnegan *et al.*, 1996; Soppe *et al.*, 2000; Kankel *et al.*, 2003).

Another family of proteins implicated in regulating DNA methylation in Arabidopsis that is much less understood, is the ORTHRUS/VARIANT IN METHYLATION (ORTH/VIM) proteins (Johnson *et al.*, 2007; Liu *et al.*, 2007; Woo *et al.*, 2007). They share domains with and are likely orthologous to the mammalian UHRF (Ubiquitin-like containing, PHD, Ring Finger) proteins (Bronner *et al.*, 2007), which are important for DNA methylation maintenance (Bostick *et al.*, 2007; Sharif *et al.*, 2007). ORTH/VIM/UHRF proteins are characterized by the presence of an SRA domain [SET (Su(var), Enhancer of Zeste, Trithorax) and RING (Really Interesting New Gene) Associated], a PHD, and a RING domain.

The SRA domain, also known as the YDG domain (for three highly conserved amino acids), as the name suggests, usually occurs in conjunction with two different types of proteins - SET domain proteins or the UHRF/ORTH/VIM proteins with one or two RING domains. The SET domain generally performs the catalytic histone methyltransferase activity of the Arabidopsis SRA-SET proteins, such as KYP (Baumbusch *et al.*, 2001). The SRA-SET proteins are unique to the plant kingdom, while the SRA-RING proteins, UHRF/ORTH/VIM, are found in many species with cytosine methylation, such as mammals, zebrafish, honeybee, and plants. The SRA domain has been reported as a methylated DNA binding domain (Unoki *et al.*, 2004; Johnson *et al.*, 2007; Woo *et al.*, 2007). Additionally, the UHRF1 SRA domain has a strong preference for hemimethylated CG (Bostick *et al.*, 2007). However, histone binding has also been ascribed to the SRA domain. The SRA domain of the murine UHRF1 binds H1, H2B and H3 and ubiquitylates histones *in vitro* (Citterio *et al.*, 2004). The human UHRF1 selectively binds to methylated H3K9 peptides, and this activity is dependent on both the PHD and SRA domains (Karagianni *et al.*, 2008). ORTH2/VIM1 has also been shown to associate with histones H2B, H3, H4, and the centromere H3 histone variant HTR12 *in vitro* (Woo *et al.*, 2007), although the domain responsible is not known.

The RING domain is an approximately 40–60 amino acid region with an octet of histidine/cysteine residues spaced to chelate two zinc atoms (Borden and Freemont, 1996). This structure binds the E2 carrying activated ubiquitin and facilitates ubiquitin transfer to substrate proteins (Lorick *et al.*, 1999). Curiously, only a few Arabidopsis RING proteins, in addition to the ORTH/VIM proteins, contain multiple RING domains out of the more than 400 predicted (Stone *et al.*, 2005). PRT1 (PROTEOLYSIS1) has two N-terminal RING domains and participates in the proteolysis of a subset of ubiquitin pathway substrates bearing specific N-terminal amino acids (Stary *et al.*, 2003). It remains to be determined whether the two RING domains can function independently in supporting ubiquitylation. Multiple members of the Arabidopsis ARIADNE family of RING proteins, like their animal counterparts, contain two RING or RING-like domains separated by a conserved region, designated IBR for in between

RING (Mladek *et al.*, 2003). Mutations in either RING domain results in lethality in *Drosophila* (Aguilera *et al.*, 2000); however, no clear evidence exists to suggest the second RING domain is independently active in ubiquitylation.

ORTH/VIM proteins also contain a PHD, which consists of an octet of histidine/cysteine residues reminiscent of the RING domain (Schindler *et al.*, 1993; Aasland *et al.*, 1995; Borden and Freemont, 1996). Searches for physiological substrates for the PHD have demonstrated binding to substrates as diverse as phosphoinositols (Gozani *et al.*, 2003) and the tri-methyl lysine 4 of histone H3 (Li *et al.*, 2006; Pena, 2006; Shi *et al.*, 2006; Wysocka *et al.*, 2006). It has also been suggested that PHD can confer E3 ligase activity to proteins, although those proteins were either determined to be RING domains or the assays were not reproducible (Lu *et al.*, 2002; Goto *et al.*, 2003; Yonashiro *et al.*, 2006).

Previous studies have yielded some insight into the biological function of ORTH/VIM proteins. A screen for natural variation in cytosine methylation in *Arabidopsis* revealed a role for *ORTH2/VIM1* in centromeric repeat methylation (Liu *et al.*, 2007; Woo *et al.*, 2007). Additionally, the amino terminus of *ORTH2/VIM1* containing the PHD binds to NtSET1 in a yeast two hybrid experiment (Liu *et al.*, 2007). Over-expression of green fluorescent protein (GFP)-tagged *VIM1* also led to a late-flowering phenotype with increased levels of *FLC* (Liu *et al.*, 2007).

Substantial work on the role of the vertebrate ORTH/VIM homolog, UHRF1, has revealed roles in cell cycle regulation (Bonapace *et al.*, 2002; Jeanblanc *et al.*, 2005), cellular transformation (Mousli *et al.*, 2003; Jenkins *et al.*, 2005), DNA damage repair (Muto *et al.*, 2002), replication of pericentromeric heterochromatin (Papait *et al.*, 2007), liver regeneration (Sadler *et al.*, 2007), and recruiting DNMT1 to replication foci (Bostick *et al.*, 2007; Sharif *et al.*, 2007). The mechanism of many of these processes has not been thoroughly investigated, and further studies of the domains of UHRF1 and its *Arabidopsis* counterparts are essential.

Herein we report additional characterization of the *Arabidopsis* ORTH/VIM family of proteins. We demonstrate that ORTH proteins possess E3 ligase activity requiring either, but not both, RING domains for E3 activity with AtUBC8 family members *in vitro*. The PHD did not contribute to *in vitro* E3 ligase activity. As reported previously (Liu *et al.*, 2007), *ORTH1/2* over-expressing lines flower later. We further explored the molecular mechanism for this alteration and demonstrate decreased methylation at *FWA* as the likely cause for delayed flowering. *ORTH1/2* over-expression also reduces methylation at *Cen180* repeats. These data support the role of the ORTH family as E3 ligases that play a role in controlling epigenetic mechanisms at both heterochromatic and euchromatic loci in plants.

Results

ORTH Domains and Phylogenetic Analyses

Inspection of the *Arabidopsis* predicted proteome for multiple RING domain-containing proteins identified five members of the ORTHRUS/VIM family and a related protein ORTH-LIKE1 (ORL1/VIM6). ORTH proteins contain an N-terminal PHD and two RING domains flanking an SRA domain (Figure 1a, upper). ORL1/VIM6 is missing the N-terminal PHD and C-terminal RING domain, but contains the SRA and N-terminal RING domains. Proteins with both SRA and RING domains are referred to here as SRA-RING proteins. Mammalian SRA-RING proteins have an altered domain organization compared to plant ORTH proteins, with an N-terminal ubiquitin-like domain and an SRA domain that is flanked N-terminally by a PHD and C-terminally by a RING domain (Figure 1a, lower).

Additional Arabidopsis proteins contain an SRA domain without a PHD or RING domain. These include ones with a SET domain (referred to here as SRA-SET proteins) as well as those without any other described protein-protein interaction domain (SRA-only). The SRA domains of all Arabidopsis SRA-RING family members were compared with selected SRA-RING proteins from other species and to the other Arabidopsis SRA-SET and SRA-only proteins (Figure 1b). Phylogenetic analysis using the entire protein results in the same conclusions (Supplemental Figure 1 and data not shown).

Within the AtORTH family, ORTH1, 4 and 5 form a clade (Figure 1a). ORTH1 has 95% similarity to ORTH4 and 91% amino acid identity to ORTH5 across the entire protein and ORTH4 and 5 have overall 99% amino acid and 99% nucleotide identity. In contrast, ORTH1 is 70% and 62% identical to ORTH2 and ORTH3, respectively, and ORTH2 and 3 have 73% amino acid identity. Interestingly, ORTH2 and 3 (At1g57820 and At1g57800, respectively) are separated by only one predicted pseudogene, and ORTH4 and 5 (At1g66040 and At1g66050, respectively) are similarly one predicted open reading frame apart, suggesting that duplication events gave rise to the ORTH2/3 and ORTH4/5 pairs.

Comparisons of the SRA domains reveal that the SRA-RING proteins of Arabidopsis have greater similarity to SRA-RING proteins from chordates than they do to Arabidopsis SRA-SET proteins. This implies that SRA-RING proteins were present in the common ancestor of plants and animals. In contrast, the plant-specific Arabidopsis SRA-SET proteins are present in a clade with the SRA-only proteins, suggesting that they are more closely related to each other than to the SRA-RING proteins.

To determine which genes are expressed, effort was expended to isolate cDNAs for all six members. cDNAs were isolated for *ORTH1*, 2, 5 and *ORL1* using RT-PCR. Attempts to isolate cDNAs for *ORTH3* and 4 failed using TAIR (The Arabidopsis Information Resource, www.arabidopsis.org) annotations for their predicted open reading frames. Thus, the expression of *ORTH3* and *ORTH4* is uncertain. The 99% nucleotide identity between *ORTH4* and 5 coding regions makes EST/cDNA gene assignments challenging. Two *ORTH4* ESTs/cDNAs are listed on TAIR (1/08), but their sequences (GenBank EL177826 and EL265513) are 100% identical to both *ORTH4* and *ORTH5* and the clones are not available for further sequencing, so their assignment to a single locus cannot be made. The cDNA sequences that are ascribed to *ORTH5* have the few *ORTH5*-specific nucleotides, indicating that these are annotated correctly and that there is no *ORTH4* cDNA in that set. Only a single EST is listed for *ORTH3*, and this assignment appears to be correct. Thus, there is no evidence for expression of *ORTH4* and scant evidence for *ORTH3* expression. These data suggest that only a subset of ORTHs are significant contributors to ORTH activity *in vivo*.

ORTH Family Members are Active as E3 Ubiquitin Ligases

The RING domain is associated with the ability to function in ubiquitin transfer (Lorick *et al.*, 1999; Borden, 2000; Kraft *et al.*, 2005; Stone *et al.*, 2005), although not all RING proteins demonstrate *in vitro* E3 ligase activity. The murine SRA-RING domain-containing protein mUHRF1 is active in *in vitro* ubiquitylation assays with the E2 UBCH5B (Citterio *et al.*, 2004). To determine if ORTH proteins function as E3 ligases, full-length ORTH1, 2, 5 and ORL proteins were expressed in *E. coli* as glutathione-S-transferase (GST) fusions, purified on glutathione beads and tested for the ability to promote ubiquitylation *in vitro* as previously described (Kraft *et al.*, 2005; Stone *et al.*, 2005). All three ORTH proteins were active with the Arabidopsis E2 UBC11 (Figure 2a) and as expected, omission of E1, E2 or ORTH protein eliminates attachment of ubiquitin to higher molecular weight proteins. Similarly, ORL1, which lacks the C-terminal RING and N-terminal PHD, was also active (Figure 2a).

We asked whether ORTH proteins require a specific E2 or E2s for activity. UBC11 is a member of the UBC8 family of Arabidopsis E2s (Kraft *et al.*, 2005). ORTH1 was tested with additional E2s of the UBC8 family (UBC8, 10, 28, 29, 30), as well as with members of three other E2 subgroups (UBC27, 36, 32 and 34) (Kraft *et al.*, 2005). ORTH1 shows robust activity with UBC8 family members UBC8, 10 and 11 (Figure 2b). ORTH1 also shows activity, albeit diminished, with other more divergent UBC8 family members UBC28 and 29 (Figure 2b). ORTH1 was not active with the other E2s tested; UBC34 (Figure 2b) and UBC27, 30, 32 and 36 (data not shown). Thus, recombinant GST-ORTH1 is most active *in vitro* with E2s from the UBC8 subfamily.

A single RING domain is sufficient for robust *in vitro* ubiquitylation activity

To determine the domain associated with the E3 ligase activity of ORTH1, codons for conserved metal ligand residues in the PHD and each RING domain were individually targeted for mutagenesis. These residues have been shown for many RING proteins to be essential for ligase activity (Lorick *et al.*, 1999; Stone *et al.*, 2005). Codons for the PHD metal-ligand binding positions 3–6 or for the N-terminal RING metal ligand binding position 4 and 5 of the N-terminal or C-terminal RINGs were mutagenized to encode alanine, creating proteins called mPD for mutant PHD, mNR for mutant N-terminal RING and mCR for mutant C-terminal RING (Figure 3a). When these GST-ORTH1 fusion proteins were tested for ubiquitylation activity with UBC8, all three retained robust activity in end-point assays (Figure 3b). Time course experiments to detect differences in ubiquitylation rates showed that activity of each mutant protein was largely unaffected relative to wild-type GST-ORTH1 (Figure 3c), indicating that alteration of one RING domain or the PHD does not dramatically interfere with the *in vitro* ubiquitylation activity of ORTH1.

To further characterize the RING domain responsible for E3 ligase activity of ORTH1, the open reading frame was truncated to express GST-ORTH1 proteins lacking one or more domains; lacking either the C-terminal RING only (C-Trunc, Figure 4b), the C-terminal RING and SRA domains (P/NR, Figure 4c), or the PHD and N-terminal RING domains (SRA/CR, Figure 4d). These truncations were assayed with UBC11 in *in vitro* ubiquitylation assays and compared to the activity of the full-length protein (Figure 4a). All truncated proteins exhibited E3 ligase activity with UBC11, demonstrating that *in vitro* either RING domain can provide E3 ligase activity. The autoubiquitylation of these proteins can be seen in the anti-GST blots, visualized as slowed migration in the complete reactions only, resulting from covalent addition of ubiquitin to the GST-ORTH fusion protein (Figure 4, below each activity blot).

The contribution of the PHD to ubiquitylation activity was additionally tested by comparing the reactions of a truncated ORTH1 protein with only the PHD to that of the identical protein except with substitutions in four of the eight metal ligand binding residues in the PHD (mPHD, Figure 4e). In these reactions, there were faint higher molecular weight anti-ubiquitin immunoreactive bands, but the profile was identical between reactions with or without the ORTH-PHD (-E3 lanes) and reactions containing either ORTH-PHD or ORTH-mPHD (T lanes). There does not appear to be any ubiquitylation activity associated with the closely related PHD, at least in these *in vitro* assays.

GFP-ORTH1 and GFP-ORTH2 are nuclear-localized

To determine the intracellular localization of ORTH proteins, constructs for expression of ORTH1 and ORTH2 in fusion with GFP were made and stably transformed into Arabidopsis. GFP was visualized by fluorescence microscopy and compared to DAPI nuclear staining. Both GFP-ORTH2 and GFP-ORTH1 co-localized with DAPI-staining nuclei (Figure 5a and b, respectively). Thus ORTH1 and ORTH2 are located primarily in nuclei.

Overexpression of ORTH1 or ORTH2 affects DNA methylation-mediated gene silencing

While producing plants for intracellular localization studies, we observed that GFP-ORTH1 and GFP-ORTH2 expressing transgenic plants exhibited delayed flowering, while GFP-ORL plants did not (data not shown and Supplemental Figure 2). Similarly, Liu *et al* (2007) recently reported that expression of GFP-ORTH2/VIM1 under the control of a constitutive promoter leads to a mild to moderate late flowering phenotype, while expression of GFP-ORL/VIM6 does not. To further explore the molecular basis of this effect, multiple T1 plants were analyzed for *FWA* expression. *FWA* is a homeodomain transcription factor completely silenced by DNA methylation in all cells of wild-type plants, except in the developing endosperm. Hypomethylation of the *FWA* promoter leads to ectopic expression causing a late flowering phenotype (Soppe *et al.*, 2000). Using RNA extracted from leaf tissue, no expression of *FWA* is observed in wild-type plants, while varying levels of expression are seen in almost all independent T1s transformed with either *GFP-ORTH1* or *GFP-ORTH2* by real time PCR (polymerase chain reaction) of cDNA relative to *ACTIN* (Figure 6a). Further analysis by Southern blotting confirmed hypomethylation at the *FWA* promoter. Increased digestion of genomic DNA by the methylation-sensitive enzyme *CfoI* was present in all GFP-ORTH1- and GFP-ORTH2-expressing lines, compared to the negative control, untransformed Col ecotype (Figure 6b, top panel).

A correlation is observed between *FWA* expression levels and *FWA* methylation levels throughout the independent T1s for both GFP-ORTH1 and GFP-ORTH2. Higher *FWA* expression is generally coincident with decreased methylation (GFP-ORTH2 #16), and lower *FWA* expression coincident with a more wild-type level of methylation (GFP-ORTH2 #10). Noticeable late-flowering phenotypes are only observed in lines with the highest expression of *FWA* (GFP-ORTH2, #12, 15, and 16, data not shown).

To test if the late-flowering phenotype was the function of a dominant negative form of ORTH protein caused by the GFP tag, an expression construct for untagged-ORTH1 was also introduced into Col under control of the 35S promoter. Flowering time, expressed as the leaves present prior to transition to flowering, was measured in segregating T2 seedlings derived from independent T1s, and five of the ten lines had an average leaf number higher than wild type (Supp. Figure 3a). This late-flowering phenotype was also associated with decreased methylation at the *FWA* locus (Supp. Figure 3b, top panel). Therefore, plants over-expressing either ORTH1/VIM3 or ORTH2/VIM1 exhibit a late-flowering phenotype likely caused by the ectopic expression of a hypomethylated *FWA* locus.

Loss of *ORTH2/VIM1* leads to decreased methylation at the heterochromatic *Cen180* repeats (Woo *et al.*, 2007). To test if overexpression of ORTH family members affects methylation at the *Cen180* repeats in addition to the *FWA* locus, *Cen180* Southern blots were performed on similarly digested genomic DNA from wild-type Columbia and transgenic lines. Decreased *Cen180* DNA methylation was observed in GFP-ORTH1, GFP-ORTH2, and untagged-ORTH1 lines (Figure 6b, Supplemental Figure 3b; bottom panels).

Finally, to test if *FWA* hypomethylation was stable in subsequent generations, T2 plants from two GFP-ORTH1 (#2, #3) and GFP-ORTH2 (#8, #16) lines were planted. Flowering time was measured, and plants were genotyped for the presence of the ORTH transgene. As expected, the average flowering time of all T2 lines delayed relative to wild type Columbia (Figure 6c). In addition, the presence or absence of the GFP-ORTH expressing transgene did not affect flowering time (Figure 6c). This shows that ORTH over-expression in the T1 generation leads to stable and heritable late-flowering phenotype.

Discussion

The Arabidopsis ORTH/VIM family contains six family members; however, ORL1/VIM6 lacks the N-terminal PHD and C-terminal RING domain present in the other five family members. In addition, ORL1 over-expression does not have the same effect as over-expression of ORTH1 or ORTH2, suggesting that ORL1 may have a unique function [this work and (Liu *et al.*, 2007)]. Of the ORTH1-5 proteins, only three may contribute significant activity *in vivo* because *ORTH3/VIM5* and *ORTH4/VIM4* appear to have very low or limited expression. Information on their expression cannot be determined from available microarray studies. *ORTH3/VIM5* sequences are not on the most commonly used expression array (ATH1), and *ORTH4* and *ORTH5* are 99% identical in the coding region and cannot be distinguished on the current arrays. Among the expressed ORTH/VIM proteins, there are differences in expression patterns. Using *in situ* hybridization, Liu *et al.* (2007) detected RNA in maturing or mature embryos from *ORTH2/VIM1* and *ORL1/VIM6*, but not *ORTH5/VIM2*. Thus, only a subset of the ORTH proteins is likely to be present at significant levels *in vivo* at any given time.

Previous work by many laboratories has demonstrated that the presence of a RING domain confers E3 ligase activity (Lorick *et al.*, 1999; Kraft *et al.*, 2005; Stone *et al.*, 2005). Consistent with these reports, we show here that ORTH1, 2, 5, and ORL1 show a clear ability to function as E3 ligases *in vitro*. The related mammalian SRA-RING proteins, mouse UHRF1/Np95 and human UHRF2/NIRF, were active *in vitro* with the human E2s, Ubch5b (Citterio *et al.*, 2004) and Ubch5a, respectively, and UHRF2 was not active with Ubch7 (Mori *et al.*, 2004). The UBC8 family of Arabidopsis is more similar to the human Ubch5 E2s than any other Arabidopsis E2 family (Kraft *et al.*, 2005), and thus the E2-E3 pairing of UHRFs and AtORTH family members appears to be shared.

ORTH1-5 are distinguished from most other Arabidopsis RING proteins and from the chordate SRA-RING proteins in containing two RING domains. Activity assays with truncated ORTH1 proteins lacking one or more domains, or with full-length ORTH1 proteins with amino acid substitutions in one RING domain showed that either RING domain alone is functional in the transfer of ubiquitin. However, it still is possible that both RING domains function synergistically in the transfer of ubiquitin to the endogenous target(s) or that one RING domain has a novel function *in vivo*.

mUHRF1/Np95 interacts with and ubiquitylates core histones *in vitro* and *ex vivo* with a preference for H3 (Citterio *et al.*, 2004). UHRF2/NIRF interacts with and ubiquitylates PCNP (PEST-containing nuclear protein), a protein of unknown function, *in vitro*, and enhances its ubiquitylation *in vivo* (Mori *et al.*, 2004). Arabidopsis ORTH2/VIM1 expressed in plants interacted with recombinant histones H2B, H3, H4 and a centromere-localized H3, HTR12 (Woo *et al.*, 2007), although a direct interaction was not demonstrated, as another protein in complex with ORTH2/VIM1 could be binding to the histones. In our hands, GST-ORTH1 was able to ubiquitylate histone H3 *in vitro*; however, another Arabidopsis non-ORTH RING E3 also shared this ability (unpublished data). Thus, we could not determine whether ORTH proteins specifically ubiquitylate histone H3 in our assays. ORTH2/VIM1 interacts with tobacco SET1, but whether SET1 is a ORTH substrate was not tested (Liu *et al.*, 2007). Clearly, further analysis to determine additional interacting proteins or characterize ORTH-containing complexes will allow testing for ORTH-mediated ubiquitylation.

The SRA domain of SRA-RING and SRA-SET proteins has been shown to be a methylated DNA binding domain (Unoki *et al.*, 2004; Johnson *et al.*, 2007; Woo *et al.*, 2007). The presence of this domain in a proteome correlates with the presence of methylated DNA in a genome. For example, SRA domain-containing proteins are not predicted from the annotated genomes of *Drosophila melanogaster*, *Caenorhabditis elegans*, *Schizosaccharomyces pombe* and

Saccharomyces cerevisiae by BLAST and SMART searches, and CpG methylation has not been reliably detected in their DNA. The SET domain associated with the SRA domain is recognized to be required for protein methyltransferase activity, most notably but not exclusively onto histone lysyl ϵ -amino groups (Trievel *et al.*, 2002; Marmorstein, 2003; Ng *et al.*, 2007). Those organisms lacking SRA domains contain SET domain proteins as well as methylated histones. *C. elegans* protein Maternal-effect sterile protein 4 (MES-4) and *D. melanogaster* protein Mes4, have either/both a PHD and/or RING domain(s), and the N-terminal MES-4 PHD is required for chromosome association (Bender *et al.*, 2006). A SET domain in association with a RING and/or PHD without an SRA domain is also observed in human and mouse proteins, but not in plants. Thus, it appears the SRA domain is specific to organisms with cytosine methylation, while SET domains are more widespread.

The ORTH PHD fits the PHD consensus (SMART, $e=1.77e^{-05}$), including the spacing limitations, nature of the metal ligands and the characteristic tryptophan residue one residue N-terminal to the seventh metal ligand. Here we present *in vitro* data to support the hypothesis that the PHD does not function as an E3 ligase. However, it remains to be determined if the PHD can function as an E3 *in vivo*, or possesses E3 ligase activity with an untested E2. Recently, Liu *et al* (2007) reported that ORTH2/VIM1 PHD was required and sufficient for interaction with NtSET in yeast two-hybrid assays. Thus, the ORTH/VIM PHD may be a protein interaction domain.

It is interesting that both loss of *ORTH2/VIM1* (Woo *et al.*, 2007) and expression of *35S::ORTH1* or *35S::GFP-ORTH2* lead to loss of DNA methylation. A similar phenomenon was seen with *GL2 (GLABRA2)* expression, where *35S::GL2* had the same phenotype as a loss-of-function line (Ohashi *et al.*, 2002). The mechanism responsible is not understood. One possibility is that there are decreased *ORTH* mRNA levels rather than increased, due to co-suppression in the 35S expression lines. However, endogenous *ORTH1* mRNA levels were not substantially changed in GFP-ORTH1 lines tested (data not shown), eliminating this as a mechanism. Another possibility is that over-expression leads to a dominant negative effect, for example, by sequestering other proteins. The large GFP tag is not responsible for the dominant negative effect, because expression of untagged ORTH1 also results in a late flowering phenotype, but over-expression of untagged and tagged ORTH proteins could reduce the free levels of other proteins. Finally, expression of hUHRF1 is regulated through the cell cycle, with highest expression just before S phase (Miura *et al.*, 2001). It is possible that the phenotype is derived from 35S driven expression throughout the cell cycle.

In this study, over-expression of either *ORTH1* or *ORTH2* phenocopy the *vim1* mutant at the *Cen180* repeats, implicating both proteins in the same pathway. However, the *35S::ORTH* lines have a more severe phenotype than *vim1*, since these lines lose CpG methylation additionally at *FWA*, as well as at the heterochromatic *Cen180* repeats. This result implicates the ORTH family members in activities outside of centromeric DNA methylation maintenance. In addition to affecting flowering time, over-expression of ORTH2/VIM1 affects root growth (Liu, *et al.*, 2007).

While the target for ubiquitylation by the ORTH/VIM family of proteins remains uncertain, it is clear that this family has an important role in DNA methylation and subsequent gene regulation. While single insertion lines have no obvious macroscopic phenotype [unpublished data and (Liu *et al.*, 2007; Woo *et al.*, 2007)], T-DNA insertions in *ORTH2/VIM1* have decreased methylation at centromeric repeats and the *vim1-2* allele exhibited increased centromeric decondensation by FISH hybridization (Woo *et al.*, 2007). Generation of multiple knockout lines in ORTH family members will be required to further elucidate ORTH *in vivo* function. Additional work to understand the intricacies of how ORTHs regulate DNA

modifications will not only prove valuable in plants but aid in the understanding of the orthologous chordate proteins as well.

Experimental Procedures

Identification of Arabidopsis ORTH proteins

The RING domain of Arabidopsis CIP8 was used in BLAST searches against the complete non-redundant Arabidopsis genome (TAIR, The Arabidopsis Information Resource, April 16, 2003, <http://www.arabidopsis.org>; see also (Stone *et al.*, 2005)). BLAST searches identified five proteins each with two RING domains, a PHD, and an SRA domain as well as one protein with only a single RING and SRA domain. The SMART database was used to analyze retrieved sequences (Simple Modular Architecture Research Tool, version 4.0 05/28/2004, <http://smart.embl-heidelberg.de/>) followed by manual inspection to confirm the presence of the complete PHD/RING/SRA domains.

Phylogenetic Analysis

The ClustalX program was used to generate an alignment of the ORTH protein sequence. The alignment was generated using a PAM350 protein matrix, with gap opening and gap extension penalty parameters of 35.0 and 0.75, respectively, in pairwise alignment and 15.0 and 0.3, respectively, in the multiple alignments (Thompson *et al.*, 1997). MacClade sequence editor (Sinauer Associates, Inc., Sunderland MA) was used to manually edit the alignment. The phylogenetic trees were created by PAUP* (Phylogenetic Analysis Using Parsimony version 4.0, Sinauer Associates, Inc.) using the neighbor-joining method with 1000 bootstrap replicates.

Cloning and Mutagenesis

Arabidopsis ORTH cDNAs were cloned by reverse transcription (RT) reactions followed by polymerase chain reactions (PCR) to amplify the predicted open reading frame (ORF) and recombined into Gateway pDONR vector (Invitrogen, Carlsbad, CA). RNA was isolated from either *Arabidopsis thaliana* ecotype Col-0 10 day-old seedlings or floral tissue from 6 to 7-week-old plants using Qiagen RNeasy plant RNA extraction kit (Qiagen, Valencia, CA) per manufacturer instructions. DNA sequence was identical to the predicted ORF (<http://www.arabidopsis.org>). ORTHs were expressed in bacteria as glutathione-S-transferase fusions from pDEST15-based plasmids (Invitrogen, Carlsbad, CA). Quik-Change site-directed mutagenesis (Stratagene, La Jolla, CA) was used to make a series of point mutations in RING domain metal ligand codons. Truncations were made with gene-specific primers with in-frame stop codons using cDNA clones as template and the sequences were verified. Positions of truncations (nucleotide relative to ATG) are as follows: PHD only, nt 198; PHD and N-terminal RING nt 675; no C-terminal RING nt 1476. The protein with the SRA and C-terminal RING domains contains nucleotides 676 to stop. *ORTH1* and *ORTH2* coding regions were recombined into the pGWB6 binary transformation vector for localization studies (Nakagawa *et al.*, 2007). Over-expression of tagless ORTH1 was achieved by recombination into pYL TAP C-t but with a stop codon present (Rubio *et al.*, 2005).

Protein Expression and Purification

GST-ORTH fusions were expressed in *E. coli* strain BL21 AI or BL21-pLysS. Transformed cells were grown at 37 °C for 2 to 3 hours or to an OD₆₀₀ of 0.4 – 0.6 before induction with 0.2% arabinose or 0.5mM IPTG, respectively, for 2 to 3 hours at 25°C. Cells were harvested by centrifugation and lysed in a buffer containing 25 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% Triton X-100. For purification, glutathione agarose (Sigma, St. Louis, MO) was added to cleared lysates and incubated for one hour at 4 °C. Beads were then washed four times with

wash buffer containing 25 mM Tris-HCl pH 7.5, 300 mM NaCl, 0.1% Triton X-100. GST fusion proteins were eluted with elution buffer containing 25 mM Tris-HCl pH 8.1, 50 mM Glutathione, and 0.1% Triton X-100. Glycerol was added to the eluted protein to a final concentration of 40%. Proteins were stored at -80°C until needed.

***In Vitro* Ubiquitylation Assay**

Ubiquitylation reactions (30 μL) containing 50 mM Tris-HCl, pH 7.5; 10 mM MgCl_2 ; 0.05 mM ZnCl_2 ; 1 mM ATP; 0.2 mM dithiothreitol; 10 mM phosphocreatine; 0.1 unit creatine kinase (Sigma, St. Louis, MO); 50 ng yeast E1 (Boston Biochem, Cambridge, MA); 250 ng of recombinant Arabidopsis E2 (Kraft *et al.*, 2005); 500 ng of GST-ORTH; and 2 μg ubiquitin (Sigma, St. Louis, MO) were incubated at 30°C for 2 h. For histone ubiquitylation assays, 2 μg of crude bovine nucleosomes (Sigma, St. Louis, MO) were added to the ubiquitylation reaction. Reactions were stopped by adding 6 μL of 5x SDS-PAGE sample buffer (125mM Tris-HCl, pH 6.8, 20% [v/v] glycerol, 4% [w/v] SDS, and 10% [v/v] β -mercaptoethanol) and analyzed by SDS-PAGE followed by western blotting using ubiquitin and/or GST antibodies as described previously (Stone *et al.*, 2005).

Plant Growth Conditions and GFP visualization

All transgenes were expressed in ecotype Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col). Seed was surface sterilized with 30% (v/v) bleach and 0.1% (v/v) Triton X-100 and grown on GM containing 1% (w/v) agar with 1X Murashige and Skoog salts (Sigma Chemical Co) under continuous light. Seedling roots were examined for GFP expression using a Nikon Optiphot 2 fluorescent microscope with a Nikon Super High Pressure Mercury Lamp power supply model HB-10101AF (Nikon, Melville, NY). Images were captured by a QImaging Retiga 1300 camera and QCapture 2.68.2 software (Quantitative Imaging Corporation, Burnaby, British Columbia).

FWA Expression

Six micrograms of RNA extracted from adult leaf tissue (Trizol, Invitrogen, Carlsbad, CA) was converted to cDNA (First Strand, Invitrogen, Carlsbad, CA). *FWA* transcript levels were analyzed by semi-quantitative real-time PCR, using primers JP3130 (CCCACCAAGATCTGAAGTCC) and JP3133 (CAGGTGCAATGGTGGTGTAT) and internal primer M12 (CCTTCGGGATTTTCGATAGTGCCA). *ACTIN* was used to standardize cDNA levels and was quantified with primers JP2452 (TCGTGGTGGTGAGTTTGTAC) and JP2453 (CAGCATCATCACAAGCATCC) and visualized with Brilliant SYBR Green QPCR Master Mix (Statagene, La Jolla, CA).

Southern Blot Analysis

DNA was extracted from leaves or flowers of recently bolted plants using a slightly modified CTAB/sarkosyl method (Bernatzky and Tanksley, 1986). For *Cen180* Southern blots, one half to one microgram of DNA was digested with *HpaII* overnight and run for 4 hr on a 1 % agarose gel, and transferred to membrane. The membrane was incubated overnight with radiolabeled probe for *Cen180* bp repeats (Vongs *et al.*, 1993). After washing, the membrane was exposed to film for four hours. *FWA* Southern blots were performed as previously described (Chan *et al.*, 2004).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Aasland R, Gibson TJ, Stewart AF. The PHD finger: implications for chromatin-mediated transcriptional regulation. *Trends Biochem Sci* 1995;20:56–59. [PubMed: 7701562]
- Aguilera M, Oliveros M, Martinez-Padron M, Barbas JA, Ferrus A. Ariadne-1: a vital *Drosophila* gene is required in development and defines a new conserved family of ring-finger proteins. *Genetics* 2000;155:1231–1244. [PubMed: 10880484]
- Baumbusch LO, Thorstensen T, Krauss V, Fischer A, Naumann K, Assalkhou R, Schulz I, Reuter G, Aalen RB. The *Arabidopsis thaliana* genome contains at least 29 active genes encoding SET domain proteins that can be assigned to four evolutionarily conserved classes. *Nucleic Acids Res* 2001;29:4319–4433. [PubMed: 11691919]
- Bender LB, Suh J, Carroll CR, Fong Y, Fingerman IM, Briggs SD, Cao R, Zhang Y, Reinke V, Strome S. MES-4: an autosome-associated histone methyltransferase that participates in silencing the X chromosomes in the *C. elegans* germ line. *Development* 2006;133:3907–3917. [PubMed: 16968818]
- Bernatzky R, Tanksley SD. Toward a saturated linkage map in tomato based on isozymes and random cDNA sequences. *Genetics* 1986;112:887–898. [PubMed: 17246322]
- Bonapace IM, Latella L, Papait R. Np95 is regulated by E1A during mitotic reactivation of terminally differentiated cells and is essential for S phase entry. *J Cell Biol* 2002;157:909–914. [PubMed: 12058012]
- Borden KL, Freemont PS. The RING finger domain: a recent example of a sequence-structure family. *Curr Opin Struct Biol* 1996;6:395–401. [PubMed: 8804826]
- Borden KL. RING domains: master builders of molecular scaffolds? *J Mol Biol* 2000;295:1103–1112. [PubMed: 10653689]
- Bostick M, Kim JK, Esteve PO, Clark A, Pradhan S, Jacobsen SE. UHRF1 plays a role in maintaining DNA methylation in mammalian cells. *Science* 2007;317:1760–1764. [PubMed: 17673620]
- Bronner C, Achour M, Arima Y, Chataigneau T, Saya H, Schini-Kerth VB. The UHRF family: oncogenes that are drugable targets for cancer therapy in the near future? *Pharmacol Ther* 2007;115:419–434. [PubMed: 17658611]
- Cao X, Jacobsen SE. Role of the *Arabidopsis* DRM methyltransferases in de novo DNA methylation and gene silencing. *Curr Biol* 2002;12:1138–1144. [PubMed: 12121623]
- Chan SWL, Zilberman D, Xie Z, Johansen LK, Carrington JC, SEJ. RNA silencing genes control de novo DNA methylation. *Science* 2004;303:1336. [PubMed: 14988555]
- Citterio E, Papait R, Nicassio F, Vecchi M, Gomiero P, Mantovani R, Di Fiore PP, Bonapace IM. Np95 is a histone-binding protein endowed with ubiquitin ligase activity. *Mol Cell Biol* 2004;24:2526–2535. [PubMed: 14993289]
- Finnegan EJ, Dennis ES. Isolation and identification by sequence homology of a putative cytosine methyltransferase from *Arabidopsis thaliana*. *Nucleic Acids Res* 1993;21:2383–2388. [PubMed: 8389441]
- Finnegan EJ, Peacock WJ, Dennis ES. Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development. *Proc Natl Acad Sci USA* 1996;93:8449–8454. [PubMed: 8710891]
- Gehring M, Henikoff S. DNA methylation dynamics in plant genomes. *Biochim Biophys Acta* 2007;1769:276–286. [PubMed: 17341434]
- Goto E, Ishido S, Sato Y, Ohgimoto S, Ohgimoto K, Nagano-Fujii M, Hotta H. c-MIR, a human E3 ubiquitin ligase, is a functional homolog of herpesvirus proteins MIR1 and MIR2 and has similar activity. *J Biol Chem* 2003;278:14657–14668. [PubMed: 12582153]

- Gozani O, Karuman P, Jones DR, Ivanov D, Cha J, Lugovskoy AA, Baird CL, Zhu H, Field SJ, Lessnick SL, Villasenor J, Mehrotra B, Chen J, Rao VR, Brugge JS, Ferguson CG, Payraastre B, Myszka DG, Cantley LC, Wagner G, Divecha N, Prestwich GD, Yuan J. The PHD finger of the chromatin-associated protein ING2 functions as a nuclear phosphoinositide receptor. *Cell* 2003;114:99–111. [PubMed: 12859901]
- Henderson IR, Jacobsen SE. Epigenetic inheritance in plants. *Nature* 2007;447:418–424. [PubMed: 17522675]
- Huettel B, Kanno T, Daxinger L, Bucher E, van der Winden J, Matzke AJ, Matzke M. RNA-directed DNA methylation mediated by DRD1 and Pol IVb: a versatile pathway for transcriptional gene silencing in plants. *Biochim Biophys Acta* 2007;1769:358–374. [PubMed: 17449119]
- Jeanblanc M, Mousli M, Hopfner R. The retinoblastoma gene and its product are targeted by ICBP90: a key mechanism in the G1/S transition during the cell cycle. *Oncogene* 2005;24:7337–7345. [PubMed: 16007129]
- Jenkins Y, Markovtsov V, Lang W. Critical role of the ubiquitin ligase activity of UHRF1, a nuclear RING finger protein, in tumor cell growth. *Mol Biol Cell* 2005;16:5621–5629. [PubMed: 16195352]
- Johnson L, Cao X, Jacobsen S. Interplay between two epigenetic marks. DNA methylation and histone H3 lysine 9 methylation. *Curr Biol* 2002;12:1360–1367. [PubMed: 12194816]
- Johnson LM, Bostick M, Zhang X, Kraft E, Henderson I, Callis J, Jacobsen SE. The SRA methyl-cytosine-binding domain links DNA and histone methylation. *Curr Biol* 2007;17:379–384. [PubMed: 17239600]
- Kankel MW, Ramsey DE, Stokes TL, Flowers SK, Haag JR, Jeddeloh JA, Riddle NC, Verbsky ML, Richards EJ. Arabidopsis MET1 cytosine methyltransferase mutants. *Genetics* 2003;163:1109–1122. [PubMed: 12663548]
- Karagianni P, Amazit L, Qin J, Wong J. ICBP90, a novel methyl K9 H3 binding protein linking protein ubiquitination with heterochromatin formation. *Mol Cell Biol* 2008;28:705–717. [PubMed: 17967883]
- Kraft E, Stone SL, Ma L, Su N, Gao Y, Lau OS, Deng XW, Callis J. Genome Analysis and Functional Characterization of the E2 and RING domain E3 ligase Ubiquitination Enzymes of *Arabidopsis thaliana*. *Plant Physiol* 2005;139:1597–1611. [PubMed: 16339806]
- Li H, Ilin S, Wang W, Duncan EM, Wysocka J, Allis CD, Patel DJ. Molecular basis for site-specific read-out of histone H3K4me3 by the BPTF PHD finger of NURF. *Nature* 2006;442:91–95. [PubMed: 16728978]
- Liu X, Yu Y, Ruan Y, Meyer D, Wolff M, Xu L, Wang N, Steinmetz A, Shen WH. The SET- and RING-associated domain proteins in heterochromatinization. *Plant J* 2007;5:914–926. [PubMed: 17892444]
- Lorick KL, Jensen JP, Fang S, Ong AM, Hatakeyama S, Weissman AM. RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc Natl Acad Sci USA* 1999;96:11364–11369. [PubMed: 10500182]
- Lu Z, Xu S, Joazeiro C, Cobb MH, Hunter T. The PHD domain of MEKK1 acts as an E3 ubiquitin ligase and mediates ubiquitination and degradation of ERK1/2. *Mol Cell* 2002;9:945–956. [PubMed: 12049732]
- Marmorstein R. Structure of SET domain proteins: a new twist on histone methylation. *Trends Biochem Sci* 2003;28:59–62. [PubMed: 12575990]
- Matzke M, Kanno T, Huettel B, Daxinger L, Matzke AJ. Targets of RNA-directed DNA methylation. *Curr Opin Plant Biol* 2007;10:512–519. [PubMed: 17702644]
- Miura M, Watanabe H, Sasaki T, Tatsumi K, Muto M. Dynamic changes in subnuclear NP95 location during the cell cycle and its spatial relationship with DNA replication foci. *Exp Cell Res* 2001;263:202–208. [PubMed: 11161719]
- Mladek C, Guger K, Hauser MT. Identification and characterization of the ARIADNE gene family in Arabidopsis. A group of putative E3 ligases. *Plant Physiol* 2003;131:27–40. [PubMed: 12529512]
- Mori T, Li Y, Hata H, Kochi H. NIRF is a ubiquitin ligase that is capable of ubiquitinating PCNP, a PEST-containing nuclear protein. *FEBS Lett* 2004;557:209–214. [PubMed: 14741369]
- Mousli M, Hopfner R, Abbady AQ. ICBP90 belongs to a new family of proteins with an expression that is deregulated in cancer cells. *Brit J Cancer* 2003;89:120–127. [PubMed: 12838312]

- Muto M, Kanari Y, Kubo E, Takabe T, Kurihara T, Fujimori A, Tatsumi K. Targeted disruption of Np95 gene renders murine embryonic stem cells hypersensitive to DNA damaging agents and DNA replication blocks. *J Biol Chem* 2002;277:34549–34555. [PubMed: 12084726]
- Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka K, Jinbo T, Kimura T. Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *J Biosci Bioeng* 2007;104:34–41. [PubMed: 17697981]
- Ng DW, Wang T, Chandrasekharan MB, Aramayo R, Kertbundit S, Hall TC. Plant SET domain-containing proteins: structure, function and regulation. *Biochim Biophys Acta* 2007;1769:316–329. [PubMed: 17512990]
- Ohashi Y, Oka A, Ruberti I, Morelli G, Aoyama T. Entopically additive expression of GLABRA2 alters the frequency and spacing of trichome initiation. *Plant J* 2002;29:359–369. [PubMed: 11844112]
- Papait R, Pistore C, Negri D, Pecoraro D, Cantarini L, Bonapace IM. Np95 is implicated in pericentromeric heterochromatin replication and in major satellite silencing. *Mol Biol Cell* 2007;18:1098–1106. [PubMed: 17182844]
- Pena PV, Davrazou F, Shi X, Walter KL, Verkhusha VV, Gozani O, Zhao R, Kutateladze TG. Molecular mechanism of histone H3K4me3 recognition by plant homeodomain of ING2. *Nature* 2006;442:100–103. [PubMed: 16728977]
- Ronemus MJ, Galbiati M, Ticknor C, Chen J, Dellaporta SL. Demethylation-induced developmental pleiotropy in *Arabidopsis*. *Science* 1996;273:654–657. [PubMed: 8662558]
- Rubio V, Shen Y, Saijo Y, Liu Y, Gusmaroli G, Dinesh-Kumar SP, Deng XW. An alternative tandem affinity purification strategy applied to *Arabidopsis* protein complex isolation. *Plant J* 2005;41:767–778. [PubMed: 15703063]
- Sadler KC, Krahn KN, Gaur NA, Ukomadu C. Liver growth in the embryo and during liver regeneration in zebrafish requires the cell cycle regulator, *uhrf1*. *Proc Natl Acad Sci USA* 2007;104:1570–1575. [PubMed: 17242348]
- Schindler U, Beckmann H, Cashmore AR. HAT3.1, a novel *Arabidopsis* homeodomain protein containing a conserved cysteine-rich region. *Plant J* 1993;4:137–150. [PubMed: 8106082]
- Sharif J, Muto M, Takebayashi S, Suetake I, Iwamatsu A, Endo TA, Shinga J, Mizutani-Koseki Y, Toyoda T, Okamura K, Tajima S, Mitsuya K, Okano M, Koseki H. The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA. *Nature* 2007;450:908–912. [PubMed: 17994007]
- Shi X, Hong T, Walter KL, Ewalt M, Michishita E, Hung T, Carney D, Pena P, Lan F, Kaadige MR, Lacoste N, Cayrou C, Davrazou F, Saha A, Cairns BR, Ayer DE, Kutateladze TG, Shi Y, Cote J, Chua KF, Gozani O. ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. *Nature* 2006;442:96–99. [PubMed: 16728974]
- Soppe WJ, Jacobsen SE, Alonso-Blanco C, Jackson JP, Kakutani T, Koornneef M, Peeters AJ. The late flowering phenotype of *fwa* mutants is caused by gain-of-function epigenetic alleles of a homeodomain gene. *Mol Cell* 2000;6:791–802. [PubMed: 11090618]
- Stary S, Yin XJ, Potuschak T, Schlogelhofer P, Nizhynska V, Bachmair A. PRT1 of *Arabidopsis* is a ubiquitin protein ligase of the plant N-end rule pathway with specificity for aromatic amino-terminal residues. *Plant Physiol* 2003;133:1360–1366. [PubMed: 14551326]
- Stone SL, Hauksdottir H, Troy A, Herschleb J, Kraft E, Callis J. Functional analysis of the RING-type ubiquitin ligase family of *Arabidopsis*. *Plant Physiol* 2005;137:13–30. [PubMed: 15644464]
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997;25:4876–4882. [PubMed: 9396791]
- Triebel RC, Beach BM, Dirk LM, Houtz RL, Hurley JH. Structure and catalytic mechanism of a SET domain protein methyltransferase. *Cell* 2002;111:91–103. [PubMed: 12372303]
- Unoki M, Nishidate T, Nakamura Y. CBP90, an E2F-1 target, recruits HDAC1 and binds to methyl-CpG through its SRA domain. *Oncogene* 2004;7601–7610. [PubMed: 15361834]
- Vongs A, Kakutani T, Martienssen RA, Richards EJ. *Arabidopsis thaliana* DNA Methylation Mutants. *Science* 1993;260:1926–1928. [PubMed: 8316832]

- Wassenegger M, Heimes S, Riedel L, Sanger HL. RNA-directed de novo methylation of genomic sequences in plants. *Cell* 1994;76:567–576. [PubMed: 8313476]
- Woo HR, Pontes O, Pikaard CS, Richards EJ. VIM1, a methylcytosine-binding protein required for centromeric heterochromatinization. *Genes Dev* 2007;21:267–277. [PubMed: 17242155]
- Wysocka J, Swigut T, Xiao H, Milne TA, Kwon SY, Landry J, Kauer M, Tackett AJ, Chait BT, Badenhorst P, Wu C, Allis CD. PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. *Nature* 2006;442:86–90. [PubMed: 16728976]
- Yonashiro R, Ishido S, Kyo S, Fukuda T, Goto E, Matsuki Y, Ohmura- Hoshino M, Sada K, Hotta H, Yamamura H, Inatome R, Yanagi S. A novel mitochondrial ubiquitin ligase plays a critical role in mitochondrial dynamics. A novel mitochondrial ubiquitin ligase plays a critical role in mitochondrial dynamics. *EMBO J* 2006;25:3618–3626. [PubMed: 16874301]
- Zhang X, Yazaki J, Sundaresan A, Cokus S, Chan SW, Chen H, Henderson IR, Shinn P, Pellegrini M, Jacobsen SE, Ecker JR. Genome-wide high-resolution mapping and functional analysis of DNA methylation in arabidopsis. *Cell* 2006;126:1189–1201. [PubMed: 16949657]

Figure 1. ORTH domains and phylogenetic analysis

(a). Domain organization of plant SRA-RING (top) and chordate SRA-RING (bottom) proteins.

(b). Phylogenetic tree of the SRA domain-containing proteins. The tree is based on the SRA domain only. Included in the analysis are all SRA domain-containing proteins of Arabidopsis and select SRA-RING proteins of other organisms. ORTH1/VIM3 (At5g39550), ORTH2/VIM1 (At1g57820), ORTH3/VIM5 (At1g57800), ORTH4/VIM4 (At1g66040), ORTH5/VIM2 (At1g66050), and ORL/VIM6 (At4g08590). Arabidopsis SRA-domain only proteins are listed by their AGI code (At5g47150, At5g47160). Arabidopsis SRA-SET domain-containing proteins are listed as previously designated with the abbreviation SUVH (Baumbusch et al., 2001). Rice SRA-RINGs are listed by NCBI locus code. NCBI locus codes for other organisms are as follows: HsICBP90 (AAF28469), MmNP95 (AAK55743), XINP95like (AAH72079), MmNIRF (AAH60241), HsNIRF(NP_690856), DrNP95like (AAT68031). At (*Arabidopsis thaliana*), Os (*Oryza sativa*), Hs (*Homo sapiens*), Mm (*Mus musculus*), Xl (*Xenopus laevis*), Dr (*Danio rerio*).

Figure 2. ORTH Family Members Possess E3 Ligase Activity

(a-b) *In vitro* ubiquitylation assays. The complete reaction (T) contains E1, 6HIS-E2 and GST-tagged E3, ubiquitin and ATP. The reaction was fractionated by SDS-PAGE, the proteins transferred to a membrane, and the products visualized with anti-ubiquitin antibodies. Free ubiquitin migrates at 5.5 kDa and has migrated off of the bottom of the gel. Anti-ubiquitin bands visualized in the -E3 lane correspond to E2-Ub adducts. Activity was determined by comparing -E3 reactions to complete reactions for the presence of high molecular weight proteins immunoreactive with anti-ubiquitin antibody (Stone et al., 2005, Kraft et al., 2005). The experiments were repeated at least two times with similar results.

(a) GST-ORTH proteins were tested with the E2 UBC11.

(b) GST-ORTH1 was tested with different E2s to determine E2-E3 specificity. The E2 used is listed below its corresponding western blot.

Figure 3. Substitutions in a single zinc-chelating domain do not affect *in vitro* ubiquitylation activity of full length ORTH1

(a). Schematic of the ORTH1 protein and amino acid changes introduced. Codons for the internal CHCC of the ORTH1 proteins mPD or mNR or the internal HC of mCR were altered to encode for alanine.

(b). Single domain mutations have no effect on ubiquitylation in endpoint assays. GST-tagged ORTH1 and mutant proteins were tested for ubiquitylation activity as described in Figure 1 to determine the contribution of each domain. Ubiquitylated proteins were visualized by anti-ubiquitin western. Anti-GST western (below) illustrate equal input of the E3 ORTH1 at time zero.

(c). Time course of ubiquitylation activity of wild-type ORTH1 (WT, bottom left) and mutant ORTH1 proteins (designated as in (a)). Aliquots of assays were removed at times indicated (in minutes) and stopped with SDS.

Figure 4. ORTH1 proteins with either RING domain, but not the PHD, have E3 ligase activity
 Truncations of ORTH1 (name and schematic form under their respective western blots) were expressed as GST fusions and tested for *in vitro* ubiquitylation activity.
 (a-e) Top, anti-ubiquitin immunoblot to detect ubiquitylated products; bottom, anti-GST to visualize E3 ligase. Reactions lacking either E1, E2 or E3 (-E1, -E2, -E3, respectively) are compared to comparable reaction with all components (T).
 (e) mPHD has the CCHC of the core metal ligands, positions 3-6, all changed to alanine. Full length ORTH1 assays (a) were included for comparison.

Figure 5. ORTH1 and 2 are Nuclear-localized

Confocal analysis shows GFP-ORTH1 (a) and GFP-ORTH2 (b) are localized to nuclei (top panels). Corresponding DAPI (middle panels) and DIC images (bottom panels) are included to show position of nuclei and cellular organization/outline.

Figure 6. The late-flowering phenotype of *GFP-ORTH1* and *GFP-ORTH2* plants is caused by decreased DNA methylation

(a) Relative *FWA* levels from seven *GFP-ORTH1* and sixteen *GFP-ORTH2* independent T1 plants, compared to Col.

(b) Southern blots for *FWA* (upper) and *Cen180* bp repeats (lower) with DNA from eight *GFP-ORTH1* and fifteen *GFP-ORTH2* independent T1 plants. Col (Col) DNA is fully methylated (m), while *met1-3* (*met*) DNA is severely unmethylated (u).

(c) Flowering time analysis for the T2 generation of two independent *GFP-ORTH1* (#2, #3) and *GFP-ORTH2* (#8, #16) lines. The number of leaves produced before the transition from vegetative tissue was counted in at least 26 plants from each line. The plants were then genotyped for the presence (+ ORTH) or absence (-ORTH) of the transgene.