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Arabidopsis thaliana histone deacetylase 14 (HDA14) is an α-tubulin deacetylase that associates with PP2A and enriches in the microtubule fraction with the putative histone acetyltransferase ELP3

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SUMMARY

It is now emerging that many proteins are regulated by a variety of covalent modifications. Using microcystin-affinity chromatography we have purified multiple protein phosphatases and their associated proteins from *Arabidopsis thaliana*. One major protein purified was the histone deacetylase HDA14. We demonstrate that HDA14 can deacetylate α-tubulin, associates with α/β-tubulin and is retained on GTP/taxol-stabilized microtubules, at least in part, by direct association with the PP2A-A2 subunit. Like HDA14, the putative histone acetyltransferase ELP3 was purified on microcystin-Sepharose and is also enriched at microtubules, potentially functioning in opposition to HDA14 as the α-tubulin acetylating enzyme. Consistent with the likelihood of it having many substrates throughout the cell, we demonstrate that HDA14, ELP3 and the PP2A A-subunits A1, A2 and A3 all reside in both the nucleus and cytosol of the cell. The association of a histone deacetylase with PP2A suggests a direct link between protein phosphorylation and acetylation.

Keywords: histone deacetylase, histone acetyltransferase, PP2A, tubulin, ELP3, HDA14.

INTRODUCTION

The reversible addition of functional groups to amino acid side chains of proteins is regarded as the primary means of controlling cellular processes in response to signaling events. Mass spectrometry based studies have shown that protein phosphorylation is probably the most common means of modifying protein function, with upwards of 70% of all human proteins being phosphorylated (Olsen *et al.*, 2010). Given the remarkable conservation of protein kinases and phosphatases across eukaryotes, it is unlikely that this degree of proteome phosphorylation will vary much in other organisms. Similar studies examining global protein acetylation have taken this protein modification from near obscurity to prominence with the recent identification of several thousand acetylated proteins and their modified lysine residues (Choudhary *et al.*, 2009; Zhao *et al.*, 2010; Finkemeier *et al.*, 2011; Wu *et al.*, 2011). These lysine acetylation datasets revealed modified nuclear, cytosolic and mitochondrial localized proteins. Given the prevalence of covalent protein modification, it is becoming clear that many proteins contain multiple types of modifications which work in concert to control protein function (Yang and Seto, 2008).

The fundamental role of protein phosphorylation in plants is underscored by the enormous size of the protein kinase superfamily, with over 1125 annotated members in *Arabidopsis thaliana* (http://www.compbio.dundee.ac.uk/kinomer/kinomes.html). The protein phosphatases of plants have not been well characterized biochemically, but bioinformatics indicates that about 150 predicted phosphatase catalytic subunits exist in *A. thaliana* and that several phosphatase gene subclasses have either expanded, are absent, or have few representatives compared with humans (Kerk *et al.*, 2008; Moorhead *et al.*, 2009). The existing paradigm for phosphatase regulation is the utilization of

additional regulatory proteins that target to specific locations and substrates in the cell (e.g. PP1 or PP2A), or the addition of N- or C-terminal extensions to the catalytic core (e.g. PP2C). For instance, in humans there are over 200 proteins that have been found to dock type 1 protein phosphatases (PP1), and in some cases these partners have been found to alter enzyme activity, facilitate regulation by intra- or extracellularly derived signals or are substrates themselves (Moorhead *et al.*, 2007; Bollen *et al.*, 2010). PP2A functions in a similar manner, taking the form of a heterotrimer, consisting of a catalytic subunit (C) and a variable B-subunit tethered through a scaffolding A-subunit (Janssens *et al.*, 2008). Through the use of genetic screens, phosphatase inhibitors and biochemical approaches, several roles for both PP2A and PP1 have been characterized in plants, including hypocotyl and root growth, stomatal opening, cytoskeletal organization and hormonal signaling (Ayaydin *et al.*, 2000; DeLong, 2006; Takemiya *et al.*, 2006; Blakeslee *et al.*, 2008; Templeton *et al.*, 2011).

The acetylation of protein lysine residues was first noted in histones over 40 years ago (Piperno and Fuller, 1985). Investigation of the reversible acetylation of the core histone tails by histone acetyltransferases (HATs) and histone deacetylases (HDACs) ultimately established the HAT and HDAC families in eukaryotes (Pandey *et al.*, 2002) and revealed the key role that this modification plays in transcriptional regulation (Yang and Seto, 2008). The *A. thaliana* genome contains 12 HATs that form four groups based on homology to the human and yeast enzymes. Within the Gcn5-related *N*-acetyltransferase group resides ELP3, or transcriptional elongator complex protein 3. ELP3 is one of six proteins (ELP1–6) of this complex that is conserved in plants, humans and other eukaryotes and has a diverse and growing set of roles *in vivo*. There are 18 HDACs in both the human and *A. thaliana* genomes that fall into four classes based on sequence homology and requirement for Zn^{2+} or NAD^{+} as a cofactor (Pandey *et al.*, 2002; Nielsen *et al.*, 2005).

Alpha and beta tubulins form heterodimers that polymerize to produce microtubules. Alpha tubulin was the second protein found to be acetylated, with the modified residue being mapped to lysine 40 (K40). This lysine is remarkably conserved across eukaryotic α-tubulins, including *A. thaliana* α-tubulins 2, 4 and 6, but is notably absent in yeast α-tubulin and plant α-tubulins 1, 3 and 5. No acetylation was detected in a recent study on grapevine α-tubulin, but previous studies suggested K40 acetylation in maize and pine α-tubulin (Gilmer *et al.*, 1999, Wang *et al.*, 2004; Parrotta *et al.*, 2010). A series of recent works in mammalian cells has demonstrated that acetylation of this residue in α-tubulin yields a docking site for kinesins on microtubules. This in turn facilitates cargo trafficking along microtubules (Reed *et al.*, 2006). Human HDAC6 (HsHDAC6) was first characterized as the enzyme responsible for the deacetylation of this site in human α-tubulin, but additional roles for human HDAC6 continue to emerge (Hammond *et al.*, 2008).

Additional post-translational modifications of tubulin have been identified, including phosphorylation, yet a precise role for this modification is unknown (Hammond *et al.*, 2008). The phosphatase activity that controls the microtubule phosphorylation state has been attributed to PP2A, predominantly due to the observed association of this phosphatase with mammalian and algal (*Chlamydomonas reinhardtii*) microtubules (Sontag *et al.*, 1999; Yang *et al.*, 2000). In mammals PP2A associates directly with microtubules and the microtubule-binding protein tau (Sontag *et al.*, 1999). In plants, protein phosphatase inhibitors disrupt cortical microtubules suggesting that (de)phosphorylation organizes cortical arrays (Baskin and Wilson, 1997). This observation is supported by identification of Ton2 as a PP2A B″-subunit whereby mutation of this gene leads to abnormalities of the cortical microtubular cytoskeleton (Camilleri *et al.*, 2002).

Microcystin is a naturally occurring toxin that targets several PPP-type phosphatases (PP1, PP2A, PP4, PP5 and PP6) and microcystin-conjugated Sepharose has been an excellent tool for purifying phosphatases and their associated proteins from several sources (Moorhead *et al.*, 1994; Templeton *et al.*, 2011). Here we have employed microcystin-Sepharose to purify and identify several *A. thaliana* PPP enzymes and their associated regulatory subunits/binding partners. One abundant co-purifying partner was identified as a putative histone deacetylase (HDA14). Biochemical data suggest that HDA14 interacts directly with a PP2A-A subunit and functions as a tubulin deacetylase. The putative histone acetyltransferase ELP3 also co-purified on the phosphatase affinity resin and was found to be highly enriched in a GTP/taxol-stabilized microtubule fraction along with PP2A and HDA14. Of the three PP2A-A subunits in *A. thaliana*, the higher-mass A2 was the dominant form associated with GTP/taxol-stabilized microtubules. All three PP2A-A subunits, along with ELP3 and HDA14, were found to localize to both the nucleus and cytosol of the cell, consistent with these enzymes having a diverse number of roles and many substrates.

RESULTS

Microcystin-sensitive phosphatase complexes

We employed microcystin affinity chromatography as a means to rapidly purify *A. thaliana* protein phosphatases that are sensitive to this cyanobacterial toxin. Figure 1 and Figure S1 and Table S1 in the supporting information show that we were able to purify, in a single step, numerous PPP-class phosphatases and associated proteins, while essentially no proteins were associated with the control matrix. One of the most abundant non-phosphatase proteins purified was a ~40-kDa band that is annotated as a histone

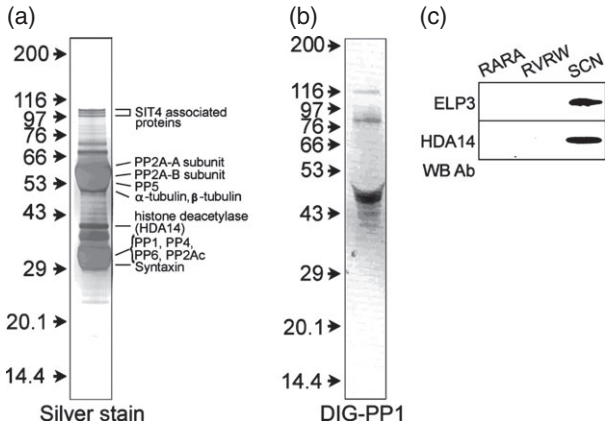

Figure 1. Purification of *Arabidopsis thaliana* microcystin-sensitive protein phosphatase complexes.
(a) Silver stain of a 4–12% gradient gel showing the microcystin-Sepharose isothiocyanate (SCN) eluted proteins. The proteins bands were excised, in-gel digested with trypsin, and analyzed by matrix-assisted laser desorption/ionisation-time of flight and tandem mass spectrometry. Identified proteins are indicated (see Table S1).
(b) The same eluted proteins were run on a 4–12% gradient gel, transferred to nitrocellulose, and probed with a 1 μg ml^{-1} digoxigenin (DIG)-PP1 probe (Tran *et al.*, 2004); PP1-binding proteins were visualized using secondary antibody to the DIG moiety. The molecular weight standards are as indicated in the figure.
(c) Proteins were sequentially eluted from a microcystin-Sepharose affinity column using a control peptide (RARA; GKKRARAADLE), followed by a PP1-docking site peptide (RVRW; (GKKRVRWADLE) and subsequently with isothiocyanate (SCN). Each sample was concentrated to an equal volume, run on SDS-PAGE, blotted and probed with ELP3 and HDA14 antibodies.

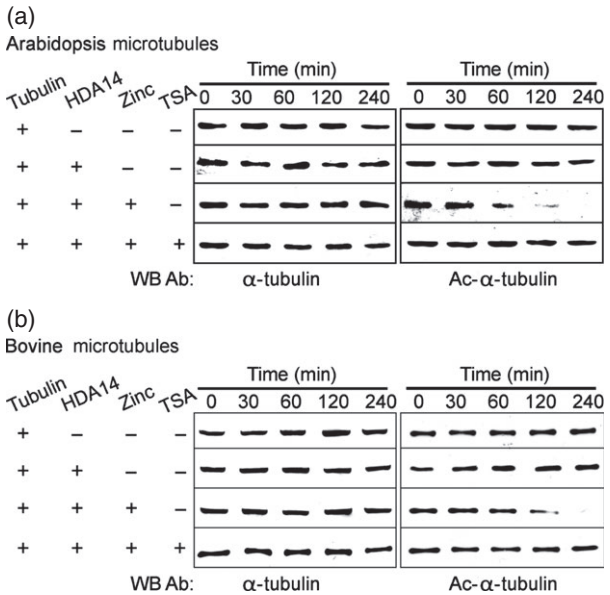

Figure 2. Histone deacetylase 14 (HDA14) is an α-tubulin deacetylase.
Histone deacetylase 14 was incubated with isolated (a) *Arabidopsis thaliana* or (b) bovine microtubules and allowed to deacetylate α-tubulin in the presence and absence of Zn^{2+} and the histone deacetylase inhibitor trichostatin A (TSA). Samples were taken at the indicated times and blotted for tubulin or K40 acetyl-α-tubulin.

deacetylase (HDA14). We cloned, expressed and purified a 6His-fusion of HDA14 and generated affinity-purified antibodies (Figure S2). As shown in Figure S1(b), these antibodies demonstrate that HDA14 is specifically retained and eluted from the microcystin matrix, indicating complex formation with a PPP-type phosphatase.

Characterization of HDA14

Annotation of the *A. thaliana* genome predicts 18 histone deacetylase (or HDA) genes (Pandey *et al.*, 2002) with the one purified here designated HDA14. Preliminary bioinformatics using the neighbor joining (NJ) tree method and the human and *A. thaliana* HDA domains indicated that HDA14 belongs to the type II HDACs (not shown). However, it was difficult to determine if HDA14 is more closely related to type IIa or IIb. For this reason, a second alignment and NJ tree were generated using the type II deacetylase domains only, and this yielded a clear clustering of *A. thaliana* HDA14, HDA15 and HDA8 with the human type IIb proteins, hsHDAC6 and hsHDAC10 (Figure S3a). Comparison of the active site signature and surrounding amino acids of the type II enzymes further supports that HDA14 is a type IIb enzyme carrying the conserved LEGGY motif, like human HDAC6 and -10. Further inspection of this region reveals that it is in fact most like the second deacetylase or B domain of hsHDAC6 (Figure S3b), the domain responsible for deacetylating α-tubulin (Haggarty *et al.*, 2003). Comparison with hsHDAC6b and hsHDAC8 (Figure S4) demonstrates conservation of amino acids that are necessary for zinc binding (Nielsen *et al.*, 2005), suggesting that HDA14 is a Zn^{2+}-dependent deacetylase, most like hsHDAC6. Human HDAC6 was originally characterized as a cytosolic α-tubulin deacetylase that through its tubulin acetylation state controls cell motility and generates the formation of a docking site for motor proteins to regulate cargo transport on microtubules (Reed *et al.*, 2006; Yang and Seto, 2008). Human HDAC6 has also been shown to directly bind β-tubulin of the α/β dimer (Zhang *et al.*, 2003). Interestingly, two proteins identified from our microcystin column elution were α- and β-tubulin (Figures 1 and S1, Table S1). Together this led us to speculate that we had purified a plant tubulin deacetylase in complex with a PPP phosphatase. To test this hypothesis we isolated both plant and bovine microtubules and performed an *in vitro* deacetylation assay. Deacetylation of α-tubulin was monitored using the classic 6-11B-1 antibody that specifically recognizes α-tubulin acetylated at K40 (Piperno and Fuller, 1985). This region is highly conserved in α-tubulins, including plant α-tubulins 2, 4 and 6, which also cluster upon phylogenetic analysis (Figure S5). Figure 2 shows that in a time- and Zn^{2+}-dependent manner, plant HDA14 deacetylates plant and bovine α-tubulin, and this can be blocked by the deacetylase inhibitor trichostatin A (TSA). In control blots, the acetylated-α-tubulin antibody could be

blocked by a peptide based on the region surrounding Ac-K40 (Figures S5a and S6), but not with a control peptide (Figure S6).

Complex formation at microtubules

One of the major microcystin-sensitive phosphatases is PP1 and nearly all PP1-binding proteins have a short four amino acid sequence referred to as the RVXF motif that binds PP1 (Moorhead *et al.*, 2007; Bollen *et al.*, 2010). We employed far-western analysis to identify PP1-binding proteins among those eluted from, and thus highly enriched on, the microcystin matrix. The result indicated a number of potential PP1-binding proteins (Figure 1b). Consistent with the lack of an obvious RVXF motif in the HDA14 sequence, PP1 does not appear to interact with HDA14 directly in the overlay assay. Next we employed our peptide displacement method utilizing a high-affinity binding RVXF motif peptide (Moorhead *et al.*, 2008) that competitively elutes bound proteins from PP1. We found that HDA14 is retained on the matrix during RVXF peptide displacement and only elutes with isothiocyanate, which releases all other bound proteins (Figure 1c).

To further explore why HDA14 was retained on the matrix we performed an overlay using the Aα subunit of the human PP2A trimer. We have previously shown using the far-western approach that human Aα binds multiple proteins from a rat liver nuclear extract other than the classic PP2A B and C subunits (data not shown). Unfortunately, extensive efforts to produce recombinant *A. thaliana* PP2A-A subunits (A1/RCN1, A2 and A3) failed; however, given the extremely high conservation between human and plant PP2A-A subunits (~57% identical/75% similarity), we decided to use the human PR65α (or Aα) in our biochemical experiments. We probed the total protein elution from control and microcystin matrices (see Figure S1) and remarkably this showed that PR65α bound a single protein with high affinity and this protein migrated at the position of HDA14 (Figure 3).

In parallel we performed overlays on the same samples using digoxigenin (DIG)-labeled recombinant HDA14 and purified α/β-tubulin (Figure 3). Both proteins detected a single band exactly migrating at the position of HDA14, suggesting that α/β-tubulin directly binds HDA14, and supporting the idea that HDA14 docks its substrate to deacetylate it. This result also suggests that HDA14 was retained on the affinity matrix by association with PP2A-A. The overlay result also indicated that HDA14 dimerizes with itself, which was further supported by gel filtration experiments with the recombinant enzyme revealing a native mass of ~100 kDa (Figure S7), approximately double the recombinant subunit size and consistent with the known dimerization of other HDACs (Nielsen *et al.*, 2005). This result parallels blots of the endogenous plant and recombinant enzyme that show stable homodimers even during SDS-PAGE followed by western blotting (Figure S2).

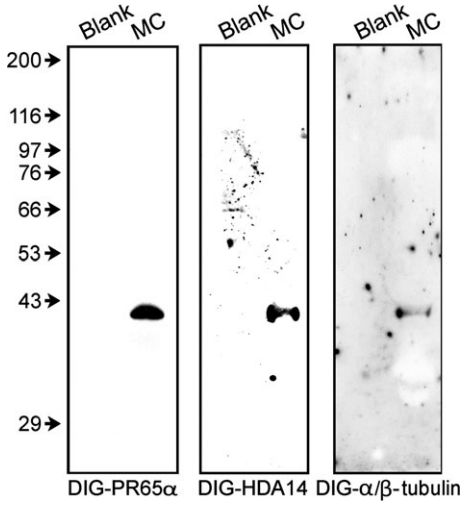

Figure 3. Histone deacetylase 14 (HDA14) binds PP2A-Aα (PR65α) and α/β-tubulin.
Equal volumes of elution from microcystin (MC)-Sepharose and control columns were run on 4–12% SDS-PAGE, blotted to nitrocellulose and probed with digoxigenin (DIG)-labeled human PP2A-Aα subunit (PR65α), HDA14 or α/β-tubulin as indicated below panels. Blank = control matrix. Molecular mass standards are indicated.

Based on Chuong *et al.*'s success in using tubulin affinity chromatography to identify plant microtubule-associated proteins, we employed this method to confirm that HDA14 and the PP2A catalytic and A-subunits could associate with tubulin *in vitro* (Chuong *et al.*, 2004). After microcystin-affinity chromatography and extensive dialysis, we incubated the microcystin-isothiocyanate eluted proteins with either α/β-tubulin or BSA coupled to Sepharose beads and determined that HDA14, PP2A catalytic (PP2Ac) and A-subunits were specifically retained on the α/β-tubulin matrix and not the control BSA matrix (Figure 4). Similarly, a pulldown with GST-tagged human PR65α using a clarified *A. thaliana* extract identified PP2Ac and HDA14 as binding proteins, but not the protein phosphatases PP4, PP1 or PP6 (Figures 5a–c and S8). This was followed by an examination of a direct interaction between recombinant HDA14 and PR65α by pulldown assay (Figure 5d). Even under stringent wash conditions GST-PR65α is specifically retained by HDA14. These results support the idea that the A-subunit of PP2A is a direct interactor of HDA14. To explore this further we performed a HDA14 immunoprecipitation from *A. thaliana* extracts (Figure 6). These results suggest that PP2A-A, PP2Ac, HDA14 and α/β-tubulin form an interaction network that excludes the phosphatase PP1. Interestingly, based on mass standards, HDA14 specifically immunoprecipitates the largest of the PP2A-A subunits, A2.

The (de)acetylation of tubulin has been well characterized in a number of eukaryotes, with one mammalian study implicating ELP3 as the enzyme responsible for acetylating α-tubulin, but biochemical verification of this is still needed

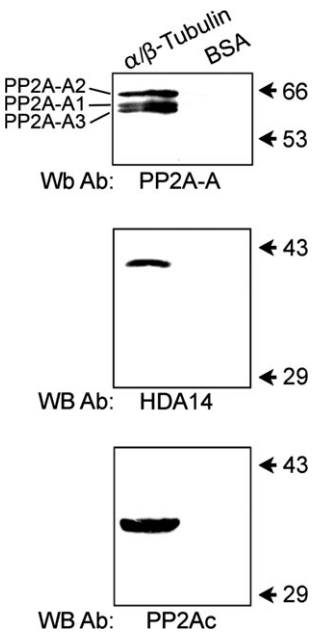

Figure 4. Tandem affinity chromatography on microcystin (MC)- and α/β-tubulin-Sepharose.
Proteins purified from a crude fraction on MC-Sepharose were subsequently chromatographed on matrix coupled with α/β-tubulin or BSA. Proteins eluted from the tubulin- or BSA-Sepharose were run on SDS-PAGE, transferred to nitrocellulose and probed with the indicated antibodies (WB Ab). A1, A2 and A3 are the characterized *Arabidopsis thaliana* PP2A-A subunits that separate based on mass as indicated (Zhou *et al.*, 2004).

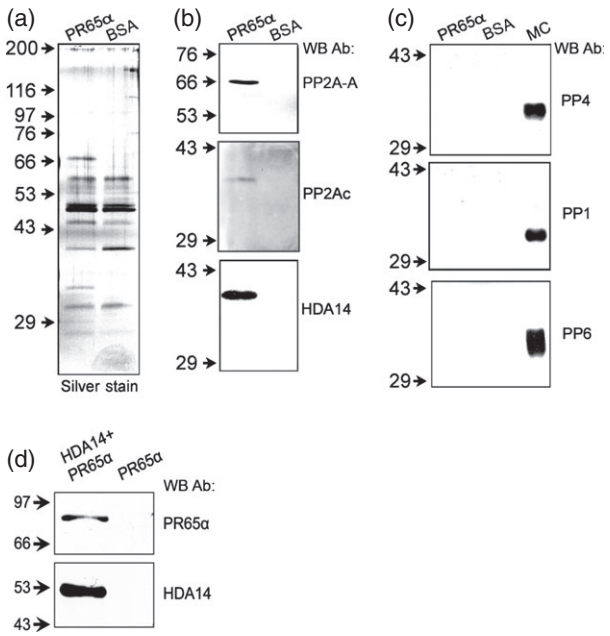

Figure 5. PP2A-Aα (PR65α) and HDA14 pulldowns.
Recombinant human PR65α and BSA (control) were coupled to Sepharose beads and mixed with a clarified *Arabidopsis thaliana* extract. The beads were pelleted, washed extensively, and bound proteins eluted with SDS-cocktail.
(a) Samples from the PR65α or BSA matrix were run on a 4–12% gradient gel and silver stained or (b) transferred to nitrocellulose and probed with the indicated antibodies.
(c) The lane marked MC contains proteins eluted from a microcystin column only and functions to show the absence of PP4, PP1 and PP6 in the PR65α pulldown.
(d) Glutathione *S*-transferase tagged PR65α was mixed with or without 6His-tagged histone deacetylase 14 (HDA14) and incubated with nickel-nitrilotriacetic acid (Ni-NTA) beads, washed stringently and eluted with imidazole and blotted as indicated.

(Creppe *et al.*, 2009). Among the plant proteins previously identified through α/β-tubulin affinity chromatography was a single histone acetyltransferase, ELP3 (Chuong *et al.*, 2004). This enzyme is highly conserved across eukaryotes, and has been shown to reside predominantly in the cytosol of human and yeast cells (Gardiner *et al.*, 2007). It is reasonable to speculate that this microtubule-bound acetyltransferase functions as the cytosolic tubulin acetyltransferase in plants. Having started to uncover the components that regulate the acetylation state of α-tubulin, we thought it pertinent to further characterize this potential α-tubulin acetyltransferase, ELP3. Mass spectrometry had not identified ELP3 in the microcystin elution (Figure 1a and Table S1), but this protein migrates at a mass identical to the very abundant PP2A-A subunits and therefore could have been masked and not detected during analysis. Antibodies were generated against the recombinant *A. thaliana* ELP3 protein (Figure S2) and confirmed that ELP3 was specifically retained on the phosphatase affinity matrix (Figure S1b) but not through association with type 1 protein phosphatase (Figure 1c).

Efforts to obtain soluble recombinant ELP3 were unsuccessful, thus we could not attempt α-tubulin acetylation assays. In light of this we then performed assays to identify proteins that are associated with taxol-stabilized microtubule polymers (Zhang *et al.*, 2003). Consistent with our other results, PP2A-A, ELP3 and HDA14, but not another phosphatase (SEX4), were enriched in the microtubule fraction stabilized by taxol treatment (Figure 7). In a crude extract or after microcystin affinity chromatography there is an approximately equal amount of the three PP2A-A subunits (Figure S1c), while taxol stabilization of microtubules dramatically enriches the highest mass subunit, A2 (Zhou *et al.*, 2004) relative to A1 and A3. This supports the idea that PP2A-A2 is enriched at microtubules with HDA14, as already suggested by immunoprecipitation (Figure 6).

Cellular localization of ELP3, HDA14 and PP2A-A1, A2 and A3

N-terminal RFP-tagged constructs of *A. thaliana* ELP3, HDA14 and PP2A-A1 (RCN1), A2 and A3 were co-expressed with a GFP-only construct in fava bean (*Vicia faba*) epidermal leaf cells (Figure 8). The GFP expressed alone demonstrates both a cytosolic and nuclear accumulation as previously documented (Uhrig and Moorhead, 2011). The HDA14 demonstrated a predominately cytosolic localization, but also resides in the nucleus, while ELP3 is partly cytosolic with a dominant localization at the nucleus. The PP2A-A

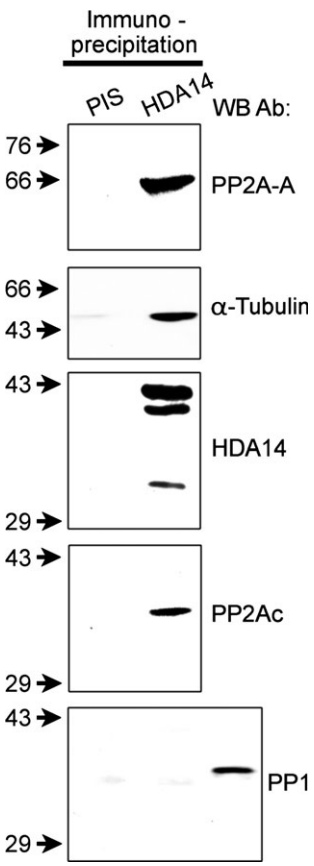

Figure 6. Immunoprecipitation of histone deacetylase 14 (HDA14) complexes.
An *Arabidopsis thaliana* crude extract was immunoprecipitated with pre-immune serum (PIS)-IgG or affinity purified HDA14-IgG and samples run on SDS-PAGE, blotted and probed with the indicated antibodies (WB Ab). For PP1 blots the positive control is an *A. thaliana* crude extract.

subunits A1 (RCN1), A2 and A3 displayed a roughly equal distribution between these two compartments. These distributions were confirmed using the nuclear marker histone 2B-YFP and were also observed in pavement cells in addition to guard cells (data not shown). Although we acknowledge that additional constructs would be required in order to conclude that the indicated distribution pattern is correct, we did follow this series of experiments with a biochemical analysis using nuclei purified from *A. thaliana* suspension cell culture. A cytosolic marker, UDP-glucose pyrophosphorylase (UGPase), confirmed that the nuclear fraction was very clean with essentially no cytosolic contaminants, whereas the histone H3 antibody revealed a dramatic enrichment of the nuclear fraction (Figure S9). Again HDA14 and ELP3 were found in both the cytosolic and nuclear fractions. These broad, diffuse distributions are consistent with proteins that maintain many cellular roles; nonetheless, some population of each resides in the cytosol where it can interact in a complex at microtubules.

DISCUSSION

Mass spectrometry based analysis of covalent modifications has provided extensive insight regarding the prevalence of protein modifications and how ultimately multiple types of protein functional groups must work in conjunction in the cell. This is especially true for protein phosphorylation and acetylation, which are now known to be prevalent in plants (Nakagami *et al.*, 2010; Finkemeier *et al.*, 2011; Wu *et al.*, 2011). With the exception of only a few studies, our biochemical understanding of the protein phosphatases in plants is limited (DeLong, 2006; Moorhead *et al.*, 2009). Here, we began our study with a biochemical approach to purify PPP-type enzymes on microcystin-Sepharose hoping to define complexes that would provide clues regarding the phosphatase function. Protein phosphatase PP2A exists as a core enzyme consisting of a catalytic subunit and scaffolding protein (A-subunit or PR65) and is thought to achieve substrate specificity through accessory B-subunits, which belong primarily to four unrelated families (DeLong, 2006; Janssens *et al.*, 2008). Although it is possible that a B-subunit exists in our complex, it may be that HDA14 functions as the 'B'-subunit, as it appears to bind the scaffolding PP2A-A protein directly. This is not unprecedented, as several proteins other than members of the 'B'-subunit families have been shown to dock A-subunits to target them to PP2A (Janssens *et al.*, 2008). Most recently large-scale screens in yeast have uncovered FIS1 and FAR11 as direct binding partners of the *Saccharomyces cerevisiae* PP2A A-subunit,

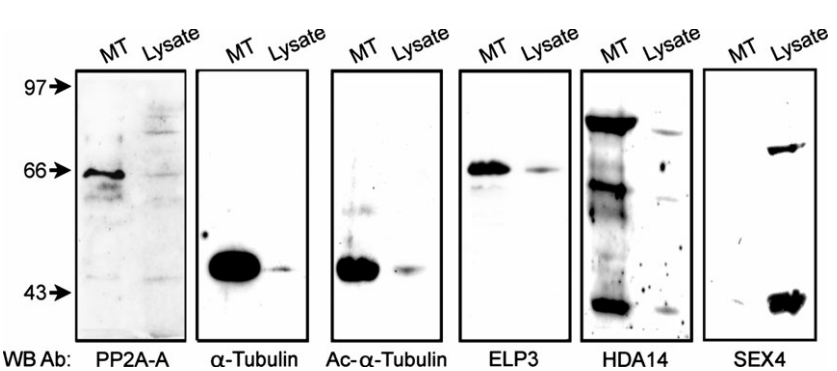

Figure 7. Histone deacetylase 14 (HDA14), PP2A-A and ELP3 bind microtubules.
Microtubules (MT) were precipitated from an *Arabidopsis thaliana* extract using a cycle of GTP/taxol precipitation and pellet solubilization. The indicated proteins were blotted (WB Ab) to examine their enrichment in the microtubule fraction compared to the crude lysate fraction.

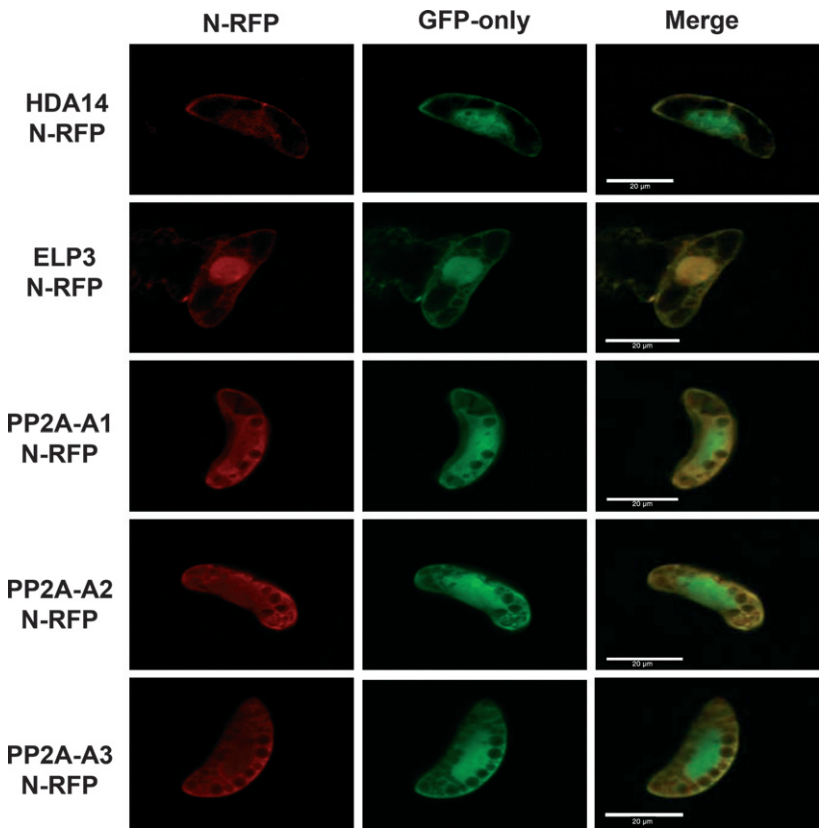

Figure 8. Cellular localization of N-terminal tagged histone deacetylase 14 (HDA14), ELP3, PP2A-A1, A2 and A3 red fluorescent protein (RFP) constructs and fluorescent GFP-only marker construct p2FGW7 in *Vicia fava* leaves.
All images are single slices of guard cells obtained using a confocal laser scanning microscope (Leica, http://www.leica.com/). The RFP images are on the left (red), GFP-only images in the center (green) and the merged image on the right (yellow). Scale bar = 20 μm.

TPD3 (Yu *et al.*, 2008). Previously PP2A has been linked to microtubule function and has been purified with the microtubule fraction of both plants and animals (Camilleri *et al.*, 2002; Chuong *et al.*, 2004). In human cells, PP2A appears to be targeted to microtubules by direct binding to tubulin, but also through the microtubule-associated protein tau (Sontag *et al.*, 1999). A similar situation may exist in plants.

Bioinformatics indicates that HDA14 is most like the well-characterized human enzyme HDAC6, which has been shown to dock β-tubulin to allow α-tubulin deacetylation (Zhang *et al.*, 2003). We noted that α/β-tubulin co-purified during microcystin chromatography, lending credence to the notion that HDA14 is the plant tubulin deacetylase. Tubulin acetylation on the highly conserved K40 was identified many years ago in human cells, and only recently has there been any elucidation of the role for this covalent modification. Although some data suggest that tubulin acetylation stabilizes microtubules (Hammond *et al.*, 2008), these data are now in question (Dompierre *et al.*, 2007). On the other hand two recent studies have provided compelling evidence that tubulin acetylation at K40 in human cells results in recruitment of the microtubule motor proteins kinesin-1 and dynein/dynactin (Reed *et al.*, 2006; Dompierre *et al.*, 2007). In human cells these motor proteins increase anterograde and retrograde cargo transport. Several over-expression studies and one knockout mouse study show that human HDAC6 is responsible for decreasing the acetylation state of α-tubulin (Hammond *et al.*, 2008; Zhang *et al.*, 2008). Although biochemical evidence is still lacking, other data strongly implicate ELP3 as the enzyme that acetylates mammalian α-tubulin, but as a component of the elongator complex (Creppe *et al.*, 2009). We purified plant ELP3 on our microcystin column and demonstrated that it is highly enriched on microtubules. Mass spectrometry did not uncover any of the elongator subunits in the microcystin elution, suggesting that ELP3 may reside at microtubules without these other partners. Support for the idea that ELP3 could operate independently of subunits 1–6 comes from the fact that an ELP3 ortholog exists in Archaea in the absence of any other elongator subunits (Versees *et al.*, 2010). Recent large scale pulldowns using a tagged ELP3 subunit in plants revealed a lack of stoichiometry among the six subunits (Nelissen *et al.*, 2010), suggesting that ELP3 may exist in different complexes with various combinations of elongator subunits, or alone. This idea is consistent with the growing number of seemingly unrelated functions for ELP3 (Creppe *et al.*, 2009).

One of our goals was to define the role of PP2A at microtubules. To this end we treated cells with the cell-permeable PPP phosphatase inhibitor calyculin A for up to 12 h, made extracts at various time points and looked for a change in the α-tubulin K40 acetylation state. We predicted

that PP2A might regulate the phosphorylation state and therefore the function of HDA14 or possibly ELP3, or even α-tubulin. At no time did we see an effect on α-tubulin (de)acetylation (data not shown). Thus, it is unclear as yet why HDA14 interacts with PP2A. It is possible that PP2A dephosphorylates HDA14 or that, as suggested above, HDA14 plays the role of PP2A 'B'-subunit to recruit PP2A to another target, such as the tubulin dimer itself. Support for a functional link between protein phosphatase activity and deacetylases comes from two other studies. The first showed that inhibitors of PPP-type protein phosphatases cause radial swelling in *A. thaliana* roots and disorganize cortical microtubules (Baskin and Wilson, 1997), while another study using the deacetylase inhibitor sodium butyrate also demonstrated radial swelling in roots (Chiatante *et al.*, 1999). Other work has indicated that multiple covalent modifications work in conjunction to alter protein function (Seet *et al.*, 2006). This is best exemplified by histones whose tails can be modified by phosphorylation, acetylation, methylation and other functional groups allowing recruitment of specific proteins in one circumstance while occluding the docking of other proteins that would bind at the same or at a nearby locale.

It is easy to imagine that acetylation, phosphorylation and possibly other known tubulin modifications work to dynamically control cargo transport in the plant cell. Given the high level of modifications of the proteome, a limited number of modifying enzymes, like PP2A, ELP3 and HDA14, undoubtedly have many substrates in the cell. Consistent with this was our observation that all three PP2A-A subunits, ELP3 and HDA14 localize to both the cytosol and nucleus and that specificity is probably conferred by targeting to substrates in specific locations, like at the microtubule.

EXPERIMENTAL PROCEDURES

Materials

Unless specifically indicated in brackets, all reagents were purchased from Sigma (http://www.sigmaaldrich.com) or GE Healthcare (http://www.gehealthcare.com/).

Sequence and phylogenetic analysis

Sequence alignments and phylogenetic analysis used annotated GenBank entries for sequences and were performed using ClustalX. Neighbor joining trees were generated using the tree building function of ClustalX as in (Kerk *et al.*, 2008). The GI numbers are listed in figure legends.

Affinity chromatography

A microcystin-Sepharose matrix was prepared and used as described (Moorhead *et al.*, 1994). *Arabidopsis thaliana* suspension cells were cultured and a soluble protein extract prepared as described (Chen *et al.*, 2006). Typically, 300 g of cells yielding 3000 mg of protein was pre-cleared by incubating, end-over-end, for 30 min at 4°C with 20 ml of Sepharose CL-4B matrix. The clarified crude extract was then divided in half and incubated for 2 h at 4°C end-over-end with 2 ml microcystin-Sepharose or control matrix [coupled with 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) instead of aminoethanethiol microcystin] equilibrated in Buffer A (25 mM TRIS-HCl pH 7.5, 0.1 mM EGTA, 0.1% (v/v) β-mercaptoethanol). Columns were washed identically and in parallel with Buffer A plus 350 mM NaCl until essentially no protein eluted from the columns and both matrices were eluted identically with 3 M sodium isothiocyanate (NaSCN) (Moorhead *et al.*, 1994). Eluted protein was dialyzed against Buffer A and then concentrated using a Centriprep-20 to 25 μl. Samples were run on 4–12% SDS-PAGE gradient gels, stained with colloidal blue and individual bands excised and proteins were identified by mass spectrometry (see Table S1 and Tran *et al.* (2004) for details). For RVXF motif peptide elution, the matrix was incubated with the peptides as described in Moorhead *et al.* (2008) and samples concentrated to equal volumes for SDS-PAGE and blotting.

For tandem microcystin-Sepharose followed by α/β-tubulin-Sepharose chromatography, proteins eluted from microcystin-Sepharose with isothiocyanate (SCN), were dialyzed extensively, divided in half and incubated end-over-end for 2 h at 4°C with equal volumes (50 μl) of bovine tubulin-Sepharose or BSA-Sepharose (control) matrices, each coupled with 50 μg protein. Proteins were coupled to CH-Sepharose following the manufacturer's instructions. Matrices were washed with 100 column volumes of Buffer B (50 mM HEPES pH 7.5, 1 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 0.5 mM DTT, 2.5 μg ml^{-1} leupeptin, 2.5 μg ml^{-1} pepstatin, 350 mM KCl) plus 0.05% (v/v) NP-40. The columns were then washed with 50 column volumes of Buffer B to remove detergent. Proteins bound to the matrix were eluted by boiling the matrix in 100 μl SDS-cocktail and equal volumes of each analyzed for binding proteins by western blot.

For PR65α (human PP2A-Aα, GI:143811355) subunit pulldowns from a crude fraction, an *A. thaliana* extract was prepared as above, divided in half, and 100 μg of purified 6His-PR65α recombinant protein was added to one half with no protein added to the other half (the control sample). The two samples were incubated end-over-end for 2 h at 4°C, after which nickel-nitrilotriacetic acid (Ni-NTA) matrix (25 μl) was directly added to each and incubated for 1 h. The 25 μl of matrix was recovered and washed with 200 column volumes of Buffer B plus 0.05% (v/v) NP-40. The matrices were then washed with 40 column volumes of wash Buffer B, and eluted with three column volumes of elution buffer (25 mM TRIS-HCl pH 7.5, 300 mM imidazole pH 7.5). Samples were boiled in SDS-PAGE cocktail for analysis by western blotting.

For pulldowns using purified proteins, human *PR65α* was cloned into pGEX-6p for expression in bacteria as a GST-tagged protein. This version of PR65α (~91 kDa) was purified to homogeneity and 5 μg mixed with 5 μg of 6His-HDA14 (see Figure S2), diluted to 0.2 ml in 25 mM TRIS pH 7.5, 150 mM NaCl, 10 mM imidazole and incubated end-over-end for 1 h at 4°C in the presence of 20 μl of Ni-NTA matrix. The 20 μl of matrix was recovered and washed with 100 matrix volumes of 25 mM TRIS pH 7.5, 1 M NaCl, 30 mM imidazole, 0.5% (v/v) Tween-20 and then eluted with two matrix volumes of SDS-PAGE cocktail for analysis by western blotting.

Immunoprecipitations, western and far-western blotting

Antibodies to HDA14 and ELP3 were generated in rabbits against the purified recombinant proteins using the method described in Chen *et al.* (2006). Antibodies to *A. thaliana* PP4, PP6, PP1 and PP2Ac were generated with peptides coupled to KLH as described in Ferrar *et al.* (2012). Peptides used to generate antibodies are shown in Figure S8. Antibodies to HDA14 and ELP3 were affinity purified by nitrocellulose membrane, while antibodies to PP1, PP4, PP6 and PP2Ac were affinity purified after coupling peptide to CH-Sepharose

as described in Ferrar *et al.* (2012) and used at 1, 3, 0.25 and 3 μg ml^{-1} respectively (see Figure S8). Antibodies to the PP2A-A subunit were generated in a rabbit with the peptide DTPMVRRAAASKLGEFAK coupled to KLH as in Ferrar *et al.* (2012) and used as a crude serum at 2000-fold dilution. Tubulin and acetyl-α-tubulin (6-11B-1) antibodies were purchased from Sigma (T9026) and Abcam (ab24610; http://www.abcam.com/), respectively and used as recommended. Acetylation of α-tubulin was confirmed by blocking the 6-11B-1 antibody with an acetylated α-tubulin-K40 peptide (DGQMPSD-AcK-TVGGGD) and control peptide (RKRKNSRVTASEDDEI). Antibodies to UGPase and histone H3 were from Agrisera (http://www.agrisera.com/) and Chemicon International (http://www.millipore.com/), respectively, and SEX4 antibody was used as in Kerk *et al.* (2006). For immunoprecipitations, equal amounts of either affinity-purified HDA14, ELP3 or pre-immune serum-purified IgG (10 μg) were covalently coupled to Protein-A Sepharose (Ulke-Lemee *et al.*, 2007). An *A. thaliana* extract was prepared and pre-cleared with Sepharose CL-4B before being incubated with antibody-coupled Protein-A Sepharose for 2.5 h at 4°C. Beads were then washed with PBS containing 300 mM NaCl and 0.5% (v/v) NP-40, followed by PBS alone, and samples were eluted by boiling in SDS-PAGE cocktail. Far-westerns were performed by DIG-labeling purified proteins for 10 min as described in the manufacturer's instructions (Roche, http://www.roche.com/) and used as described for PP1 (Tran *et al.*, 2004) at the following concentrations (HDA14, 1 μg ml^{-1}; PR65α, 1 μg ml^{-1}; PP1γ, 1 μg ml^{-1}; α/β-tubulin, 1 μg ml^{-1}).

Tubulin deacetylation assays

Microtubules were purified from bovine brain or *A. thaliana* suspension cells as in Chuong *et al.* (2004) and deacetylated using 1 μg HDA14 with or without 40 μM $ZnCl_2$ and 10 μM TSA as indicated. After incubation at 37°C for the times shown, samples were withdrawn and the reaction stopped by boiling in SDS-PAGE cocktail.

Microscopy

All red fluorescent protein (RFP) fusion constructs were created as previously described (Uhrig and Moorhead, 2011) using the plant expression vector pB7WGR2 (http://gateway.psb.ugent.be/). Transient co-expression of N-terminal RFP-tagged HDA14, ELP3, PP2A-A1 (RCN1), PP2A-A2 and PP2A-A3 with marker constructs was also performed as previously described (Uhrig and Moorhead, 2011) using *V. faba* leaves. Marker constructs employed included cytosol/nuclear targeted GFP-only (vector p2FGW7; GFP with no targeting motif) and C-terminal YFP-tagged histone 2B (H2B-YFP).

Other methods

Microtubule co-assembly was performed as a modification of the method described in Korolev *et al.* (2005). Briefly, *A. thaliana* suspension cells were lysed by French press in 50 mM PIPES, pH 6.9, 5 mM EGTA, 5 mM $MgSO_4$ and centrifuged at 55 000 ***g*** for 90 min to remove cellular debris. The supernatant was made to 25% (v/v) glycerol, 20 mM taxol and 1 mM GTP and incubated at 26°C for 40 min. The sample was then centrifuged at 72 000 ***g*** for 90 min to pellet the microtubules and associated proteins, the pellet was washed once, and microtubules pelleted again and boiled in SDS-PAGE cocktail for analysis by western blotting. Nuclei were prepared from *A. thaliana* suspension cells by lysing in sucrose buffer and centrifuging through a sucrose cushion as in Ulke-Lemee *et al.* (2007). Cloning, protein expression and purification of HDA14, ELP3 and human PR65α are provided in Appendix S1 (Supporting Methods).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Affinity purification of protein phosphatase complexes on microcystin and control matrices.

Figure S2. Characterization of HDA14 and ELP3 (HAT) antibodies.

Figure S3. *Arabidopsis thaliana* HDA14 is most similar to a type IIb enzyme with an active site sequence motif most like human HDAC6.

Figure S4. Informatics indicates *Arabidopsis thaliana* HDA14 is a Zn^{2+}-dependent enzyme most like human HDAC6.

Figure S5. Alpha tubulin N-terminal sequences surrounding lysine 40 (K40).

Figure S6. Control blot for acetyl-α-tubulin (6-11B-1) antibody.

Figure S7. HDA14 is a homodimer during Superdex 200 gel filtration chromatography.

Figure S8. Characterization of antibodies for *Arabidopsis thaliana* PP1, PP4, PP6 and PP2Ac.

Figure S9. Biochemical localization of HDA14 and ELP3 to the cytosol and nuclei.

Table S1. Microcystin-Sepharose purified proteins identified by mass spectrometry.

Appendix S1. Supporting methods.

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REFERENCES

Ayaydin, F., Vissi, E., Meszaros, T., Miskolczi, P., Kovacs, I., Feher, A., Dombradi, V., Erdodi, F., Gergely, P. and Dudits, D. (2000) Inhibition of serine/threonine-specific protein phosphatases causes premature activation of cdc2MsF kinase at G2/M transition and early mitotic microtubule organisation in alfalfa. *Plant J.* **23**, 85–96.

Baskin, T.I. and Wilson, J.E. (1997) Inhibitors of protein kinases and phosphatases alter root morphology and disorganize cortical microtubules. *Plant Physiol.* **113**, 493–502.

Blakeslee, J.J., Zhou, H.W., Heath, J.T., Skottke, K.R., Barrios, J.A., Liu, S.Y. and DeLong, A. (2008) Specificity of RCN1-mediated protein phosphatase 2A regulation in meristem organization and stress response in roots. *Plant Physiol.* **146**, 539–553.

Bollen, M., Peti, W., Ragusa, M.J. and Beullens, M. (2010) The extended PP1 toolkit: designed to create specificity. *Trends Biochem. Sci.* **35**, 450–458.

Camilleri, C., Azimzadeh, J., Pastuglia, M., Bellini, C., Grandjean, O. and Bouchez, D. (2002) The Arabidopsis TONNEAU2 gene encodes a putative novel protein phosphatase 2A regulatory subunit essential for the control of the cortical cytoskeleton. *Plant Cell*, **14**, 833–845.

Chen, Y.M., Ferrar, T.S., Lohmeier-Vogel, E.M., Morrice, N., Mizuno, Y., Berenger, B., Ng, K.K., Muench, D.G. and Moorhead, G.B. (2006) The PII signal transduction protein of Arabidopsis thaliana forms an arginine-regulated complex with plastid N-acetyl glutamate kinase. *J. Biol. Chem.* **281**, 5726–5733.

Chiatante, D., Di Iorio, A., Maiuro, L. and Scippa, S.G. (1999) Effect of water stress on root meristems in woody and herbaceous plants during the first stage of development. *Plant Soil*, **217**, 159–172.

Choudhary, C., Kumar, C., Gnad, F., Nielsen, M.L., Rehman, M., Walther, T.C., Olsen, J.V. and Mann, M. (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science*, **325**, 834–840.

Chuong, S.D., Good, A.G., Taylor, G.J., Freeman, M.C., Moorhead, G.B. and Muench, D.G. (2004) Large-scale identification of tubulin-binding proteins provides insight on subcellular trafficking, metabolic channeling, and signaling in plant cells. *Mol. Cell. Proteomics*, **3**, 970–983.

Creppe, C., Malinouskaya, L., Volvert, M.L. *et al.* (2009) Elongator controls the migration and differentiation of cortical neurons through acetylation of alpha-tubulin. *Cell*, **136**, 551–564.

DeLong, A. (2006) Switching the flip: protein phosphatase roles in signaling pathways. *Curr. Opin. Plant Biol.* **9**, 470–477.

Dompierre, J.P., Godin, J.D., Charrin, B.C., Cordelieres, F.P., King, S.J., Humbert, S. and Saudou, F. (2007) Histone deacetylase 6 inhibition compensates for the transport deficit in Huntington's disease by increasing tubulin acetylation. *J. Neurosci.* **27**, 3571–3583.

Ferrar, T., Chamousset, D., Nimick, M., DeWever, V., Andersen, J., Trinkle-Mulcahy, L. and Moorhead, G.B.G. (2012) Taperin (c9orf75), a mutated gene in nonsyndromic deafness, encodes a vertebrate specific, nuclear localized protein phosphatase one alpha (PP1α) docking protein. *Biology Open*, **1**, 128–139.

Finkemeier, I., Laxa, M., Miguet, L., Howden, A.J. and Sweetlove, L.J. (2011) Proteins of diverse function and subcellular location are lysine acetylated in Arabidopsis. *Plant Physiol.* **155**, 1779–1790.

Gardiner, J., Barton, D., Marc, J. and Overall, R. (2007) Potential role of tubulin acetylation and microtubule-based protein trafficking in familial dysautonomia. *Traffic*, **8**, 1145–1149.

Gilmer, S., Clay, P., MacRae, T.H. and Fowke, L.C. (1999) Acetylated tubulin is found in all microtubule arrays of two species of pine. *Protoplasma*, **207**, 174–185.

Haggarty, S.J., Koeller, K.M., Wong, J.C., Grozinger, C.M. and Schreiber, S.L. (2003) Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. *Proc. Natl Acad. Sci. USA*, **100**, 4389–4394.

Hammond, J.W., Cai, D. and Verhey, K.J. (2008) Tubulin modifications and their cellular functions. *Curr. Opin. Cell Biol.* **20**, 71–76.

Janssens, V., Longin, S. and Goris, J. (2008) PP2A holoenzyme assembly: in cauda venenum (the sting is in the tail). *Trends Biochem. Sci.* **33**, 113–121.

Kerk, D., Conley, T.R., Rodriguez, F.A., Tran, H.T., Nimick, M., Muench, D.G. and Moorhead, G.B. (2006) A chloroplast-localized dual-specificity protein phosphatase in Arabidopsis contains a phylogenetically dispersed and ancient carbohydrate-binding domain, which binds the polysaccharide starch. *Plant J.* **46**, 400–413.

Kerk, D., Templeton, G. and Moorhead, G.B. (2008) Evolutionary radiation pattern of novel protein phosphatases revealed by analysis of protein data from the completely sequenced genomes of humans, green algae, and higher plants. *Plant Physiol.* **146**, 351–367.

Korolev, A.V., Chan, J., Naldrett, M.J., Doonan, J.H. and Lloyd, C.W. (2005) Identification of a novel family of 70 kDa microtubule-associated proteins in Arabidopsis cells. *Plant J.* **42**, 547–555.

Moorhead, G., MacKintosh, R.W., Morrice, N., Gallagher, T. and MacKintosh, C. (1994) Purification of type 1 protein (serine/threonine) phosphatases by microcystin-Sepharose affinity chromatography. *FEBS Lett.* **356**, 46–50.

Moorhead, G.B., Trinkle-Mulcahy, L. and Ulke-Lemée, A. (2007) Emerging roles of nuclear protein phosphatases. *Nat. Rev. Mol. Cell Biol.* **8**, 234–244.

Moorhead, G.B., Trinkle-Mulcahy, L., Nimick, M., De Wever, V., Campbell, D.G., Gourlay, R., Lam, Y.W. and Lamond, A.I. (2008) Displacement affinity chromatography of protein phosphatase one (PP1) complexes. *BMC Biochem.* **9**, 28.

Moorhead, G.B., De Wever, V., Templeton, G. and Kerk, D. (2009) Evolution of protein phosphatases in plants and animals. *Biochem. J.* **417**, 401–409.

Nakagami, H., Sugiyama, N., Mochida, K., Daudi, A., Yoshida, Y., Toyoda, T., Tomita, M., Ishihama, Y. and Shirasu, K. (2010) Large-scale comparative phosphoproteomics identifies conserved phosphorylation sites in plants. *Plant Physiol.* **153**, 1161–1174.

Nelissen, H., De Groeve, S., Fleury, D. *et al.* (2010) Plant Elongator regulates auxin-related genes during RNA polymerase II transcription elongation. *Proc. Natl Acad. Sci. USA*, **107**, 1678–1683.

Nielsen, T.K., Hildmann, C., Dickmanns, A., Schwienhorst, A. and Ficner, R. (2005) Crystal structure of a bacterial class 2 histone deacetylase homologue. *J. Mol. Biol.* **354**, 107–120.

Olsen, J.V., Vermeulen, M., Santamaria, A. *et al.* (2010) Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. *Sci. Signal.* **3**, ra3.

Pandey, R., Muller, A., Napoli, C.A., Selinger, D.A., Pikaard, C.S., Richards, E.J., Bender, J., Mount, D.W. and Jorgensen, R.A. (2002) Analysis of histone acetyltransferase and histone deacetylase families of Arabidopsis thaliana suggests functional diversification of chromatin modification among multicellular eukaryotes. *Nucleic Acids Res.* **30**, 5036–5055.

Parrotta, L., Cai, G. and Cresti, M. (2010) Changes in the accumulation of alpha- and beta-tubulin during bud development in Vitis vinifera L. *Planta*, **231**, 277–291.

Piperno, G. and Fuller, M.T. (1985) Monoclonal antibodies specific for an acetylated form of alpha-tubulin recognize the antigen in cilia and flagella from a variety of organisms. *J. Cell Biol.* **101**, 2085–2094.

Reed, N.A., Cai, D., Blasius, T.L., Jih, G.T., Meyhofer, E., Gaertig, J. and Verhey, K.J. (2006) Microtubule acetylation promotes kinesin-1 binding and transport. *Curr. Biol.* **16**, 2166–2172.

Seet, B.T., Dikic, I., Zhou, M.M. and Pawson, T. (2006) Reading protein modifications with interaction domains. *Nat. Rev. Mol. Cell Biol.* **7**, 473–483.

Sontag, E., Nunbhakdi-Craig, V., Lee, G., Brandt, R., Kamibayashi, C., Kuret, J., White, C.L. III, Mumby, M.C. and Bloom, G.S. (1999) Molecular interactions among protein phosphatase 2A, tau, and microtubules. Implications for the regulation of tau phosphorylation and the development of tauopathies. *J. Biol. Chem.* **274**, 25490–25498.

Takemiya, A., Kinoshita, T., Asanuma, M. and Shimazaki, K. (2006) Protein phosphatase 1 positively regulates stomatal opening in response to blue light in Vicia faba. *Proc. Natl Acad. Sci. USA*, **103**, 13549–13554.

Templeton, G.W., Nimick, M., Morrice, N., Campbell, D., Goudreault, M., Gingras, A.C., Takemiya, A., Shimazaki, K. and Moorhead, G.B. (2011) Identification and characterization of Atl-2, an Arabidopsis homologue of an ancient protein phosphatase 1 (PP1) regulatory subunit. *Biochem. J.* **435**, 73–83.

Tran, H.T., Ulke, A., Morrice, N., Johannes, C.J. and Moorhead, G.B. (2004) Proteomic characterization of protein phosphatase complexes of the mammalian nucleus. *Mol. Cell. Proteomics*, **3**, 257–265.

Uhrig, R.G. and Moorhead, G.B. (2011) Two ancient bacterial-like PPP family phosphatases from Arabidopsis are highly conserved plant proteins that possess unique properties. *Plant Physiol.* **157**, 1778–1792.

Ulke-Lemee, A., Trinkle-Mulcahy, L., Chaulk, S., Bernstein, N.K., Morrice, N., Glover, M., Lamond, A.I. and Moorhead, G.B. (2007) The nuclear PP1 interacting protein ZAP3 (ZAP) is a putative nucleoside kinase that complexes with SAM68, CIA, NF110/45, and HNRNP-G. *Biochim. Biophys. Acta*, **1774**, 1339–1350.

Versees, W., De Groeve, S. and Van Lijsebettens, M. (2010) Elongator, a conserved multitasking complex? *Mol. Microbiol.* **76**, 1065–1069.

Wang, W., Vignani, R., Scali, M., Sensi, E. and Cresti, M. (2004) Post-translational modifications of alpha-tubulin in Zea mays L are highly tissue specific. *Planta*, **218**, 460–465.

Wu, X., Oh, M.H., Schwarz, E.M., Larue, C.T., Sivaguru, M., Imai, B.S., Yau, P.M., Ort, D.R. and Huber, S.C. (2011) Lysine acetylation is a widespread protein modification for diverse proteins in Arabidopsis. *Plant Physiol.* **155**, 1769–1778.

Yang, X.J. and Seto, E. (2008) Lysine acetylation: codified crosstalk with other posttranslational modifications. *Mol. Cell*, **31**, 449–461.

Yang, P., Fox, L., Colbran, R.J. and Sale, W.S. (2000) Protein phosphatases PP1 and PP2A are located in distinct positions in the Chlamydomonas flagellar axoneme. *J. Cell Sci.* **113**(Pt 1), 91–102.

Yu, H., Braun, P., Yildirim, M.A. *et al.* (2008) High-quality binary protein interaction map of the yeast interactome network. *Science*, **322**, 104–110.

Zhang, Y., Li, N., Caron, C., Matthias, G., Hess, D., Khochbin, S. and Matthias, P. (2003) HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo. *EMBO J.* **22**, 1168–1179.

Zhang, Y., Kwon, S., Yamaguchi, T. *et al.* (2008) Mice lacking histone deacetylase 6 have hyperacetylated tubulin but are viable and develop normally. *Mol. Cell. Biol.* **28**, 1688–1701.

Zhao, S., Xu, W., Jiang, W. *et al.* (2010) Regulation of cellular metabolism by protein lysine acetylation. *Science*, **327**, 1000–1004.

Zhou, H.W., Nussbaumer, C., Chao, Y. and DeLong, A. (2004) Disparate roles for the regulatory A subunit isoforms in Arabidopsis protein phosphatase 2A. *Plant Cell*, **16**, 709–722.