The Histone Deacetylase Complex 1 Protein of Arabidopsis Has the Capacity to Interact with Multiple Proteins Including Histone 3-Binding Proteins and Histone 1 Variants[1][OPEN]

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Intrinsically disordered proteins can adopt multiple conformations, thereby enabling interaction with a wide variety of partners. They often serve as hubs in protein interaction networks. We have previously shown that the Histone Deacetylase Complex 1 (HDC1) protein from Arabidopsis (*Arabidopsis thaliana*) interacts with histone deacetylases and quantitatively determines histone acetylation levels, transcriptional activity, and several phenotypes, including abscisic acid sensitivity during germination, vegetative growth rate, and flowering time. HDC1-type proteins are ubiquitous in plants, but they contain no known structural or functional domains. Here, we explored the protein interaction spectrum of HDC1 using a quantitative bimolecular fluorescence complementation assay in tobacco (*Nicotiana benthamiana*) epidermal cells. In addition to binding histone deacetylases, HDC1 directly interacted with histone H3-binding proteins and corepressor-associated proteins but not with H3 or the corepressors themselves. Surprisingly, HDC1 also was able to interact with variants of the linker histone H1. Truncation of HDC1 to the ancestral core sequence narrowed the spectrum of interactions and of phenotypic outputs but maintained binding to a H3-binding protein and to H1. Thus, HDC1 provides a potential link between H1 and histone-modifying complexes.

Regulation of gene transcription underpins plant development and dynamic responses to the environment. Transcription occurs in the context of chromatin, a highly condensed structure in which the DNA is wrapped around nucleosomes composed of histones H2A/B, H3, and H4 and further stabilized by linker histone H1(Over and Michaels, 2014; Hergeth and Schneider, 2015). Alteration of chromatin structure plays an important part in transcriptional regulation and is achieved through multiprotein complexes that recognize and instigate biochemical modifications of the DNA and/or the histones (Pfluger and Wagner, 2007; Derkacheva et al., 2013). For example, binding of repressors to so-called corepressors recruits histone deacetylases (HDAs) to the gene region (Song et al., 2005). The HDAs in turn interact with histone-binding proteins (Mehdi et al., 2016). Removal of acetyl groups from Lys residues of the core histones leads to chromatin compaction and inhibition of transcription (Kouzarides, 2007; Roudier et al., 2009). Specific recruitment at both 'ends' of the repressive protein complex generates a double lock between DNA and the nucleosome: the repressors recognize certain DNA motifs in the gene promoters, and the histone-binding proteins recognize ('read') certain histone residues and their modifications (Liu et al., 2010). A minimal HDAC complex therefore needs to combine at least three protein functions: repressor binding, histone binding, and catalytic activity. Biochemical studies in yeast (*Saccharomyces cerevisiae*) and in animal systems have provided evidence for large multiprotein complexes linking a corepressor and a HDA with several histone-binding proteins and a range of associated proteins of mostly unknown function (Yang and Seto, 2008). Plant HDAC complexes are less well characterized, but in a recent study several proteins, including corepressors and histone-binding proteins, were found to coprecipitate with a histone deacetylase, suggesting that the basic composition of plant HDAC complexes is similar to that of animal and yeast complexes (Mehdi et al., 2016).

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Histone Deacetylase Complex 1 (HDC1) protein is an important component of the plant HDAC machinery (Perrella et al., 2013). We have reported that knockout of HDC1 in Arabidopsis (*Arabidopsis thaliana*) promotes histone acetylation and gene expression, and causes a range of phenotypes, most notably hypersensitivity to abscisic acid (ABA) during germination, inhibition of leaf growth, and delayed flowering (Perrella et al., 2013). Conversely, overexpression of HDC1 desensitized the plants to ABA and increased shoot biomass even in water-limited conditions. Thus, HDC1 appeared to be a rate-limiting factor of HDAC. HDC1 is a component of native HDAC complexes in Arabidopsis (Derkacheva et al., 2013; Mehdi et al., 2016), and it directly interacts with the HDAs HDA6 and HDA19 (Perrella et al., 2013). Both HDAs have previously been reported to function in germination (Tanaka et al., 2008; Yu et al., 2011), flowering (Tanaka et al., 2008; Yu et al., 2011), and ABA-mediated responses to drought or salt (Chen and Wu, 2010; Chen et al., 2010). The phenotypes of HDC1 mutants can therefore be explained by HDC1 acting through these HDAs, but the mechanism by which HDC1 controls their apparent activity remains to be elucidated.

HDC1 is a ubiquitously expressed single-copy gene in Arabidopsis, and HDC1 homologs are present across the plant kingdom as single- or low-copy genes. The HDC1 sequence contains no known functional or structural motifs. Sequence conservation is high in a 315-amino-acid stretch within the C-terminal half of the protein, which aligns to shorter proteins in algae and fungi, including the yeast Regulator of Transcription 3 (Rxt3; see dendrogram and sequence alignment in Perrella et al., 2013). Rxt3 coelutes with the large Rpd3 HDAC complex in yeast, but its function has remained unclear (Carrozza et al., 2005a, 2005b). Sequence analysis with JPred (Drozdetskiy et al., 2015) predicts very little secondary structure for HDC1, particularly in the N-terminal part. Intrinsically disordered proteins often act as flexible adaptors for multiple protein interactions (Pazos et al., 2013). It is therefore possible that HDC1 enables multiple protein interactions in HDAC complexes.

Here, we used a ratiometric bimolecular fluorescence complementation (BiFC) assay in tobacco (*Nicotiana benthamiana*) epidermal cells to test the ability of HDC1 to interact with known and putative members of the HDAC machinery. We then assessed whether a truncated version of HDC1, resembling the shorter, ancestral Rxt3-like proteins, was able to maintain the identified protein interactions and to complement molecular, physiological, and developmental phenotypes of *hdc1* knockout plants. The results reveal a potential connection between linker histone H1 and histone deacetylation.

RESULTS

HDC1 Directly Interacts with Histone-Binding Protein and Associated Proteins

Based on the homology search of proteins coeluting with Rxt3 in yeast complexes and on reported phenotypes and protein interactions in plants (Supplemental Tables S1 and S2), we selected a subset of Arabidopsis proteins as candidate direct interactors with HDC1: the histone-binding proteins SHL1, ING2 and MSI1 (Müssig et al., 2000; Müssig and Altmann, 2003; Lee et al., 2009; López-González et al., 2014; Mehdi et al., 2016), the Sin3-like (SNL) corepressors SNL2 and SNL3 (Song et al., 2005; Wang et al., 2013), and the Sin3-associated protein SAP18 (Song and Galbraith, 2006). We also included the HDAs (HDA6 and HDA19; Chen and Wu, 2010), H3 variants (H3.1 and H3.3; Jacob et al., 2014), and H1 variants (H1.1, H1.2, and H1.3; Ascenzi and Gantt, 1999) in the interaction assays.

The ability of protein pairs to directly interact with each other was investigated using BiFC (Fig. 1). The proteins were fused to N- or C-terminal halves of Yellow Fluorescent Protein (YFP) and transiently coexpressed in tobacco leaves. We used a ratiometric assay (Grefen and Blatt, 2012) expressing the two fusion proteins and a full-length Red Fluorescent Protein (RFP) from the same vector (2-in-1 vector; Fig. 1A). In total, 37 pairwise interactions were assayed in almost a thousand cells. The RFP signal quantifies transgene expression in each cell, and the ratio between YFP and RFP signals allows normalization and hence direct comparison of interactions between different cells for statistical analysis. In all positive cases the complemented YFP signal was observed inside the nuclei (Fig. 1B).

To assess whether the Rxt3-like part of the protein is required and sufficient for some or all of the interactions, we generated a truncated version of HDC1 spanning amino acids 449 to 764 (Rxt3-like, RXT3L; Fig. 1C), approximately one-third of the full-length protein. Expression of GFP-fusion proteins in tobacco leaves showed that full-length HDC1 and RXT3L were exclusively located in the nuclei. Sequence analysis with PSORT (Nakai and Kanehisa, 1992) highlighted two different putative nuclear retention signals in HDC1 (KR KELKHREWGD RDKDR starting at amino acid 358 and KR RERDGDSEAE RAEKR starting at amino acid 479). Only the latter was present in RXT3L, suggesting that it is sufficient for nuclear localization. Yeast Rxt3 contains neither of the motifs and was not retained in the nuclei (Supplemental Fig. S1), suggesting that the 479 motif is necessary for nuclear retention in plant cells.

Figure 1D shows the interaction profile of HDC1 based on YFP/RFP ratios obtained from cells coexpressing HDC1 with candidate interactors. Signals were measured in at least 30 cells from three independently transformed plants. Supplemental Figure S2 shows the respective interaction profiles for SHL1, ING2, MSI1, SAP18, HDA6, and HDA19. The following observations confirmed the validity of the approach. First, for each protein a significant complementation signal was detected with at least one other protein, confirming that all fusion proteins were properly expressed. Second, the complementation signal was always observed inside the nuclei, confirming correct targeting of the fusion proteins. Third, the interaction profiles differed between the proteins tested, confirming specificity of the interactions.

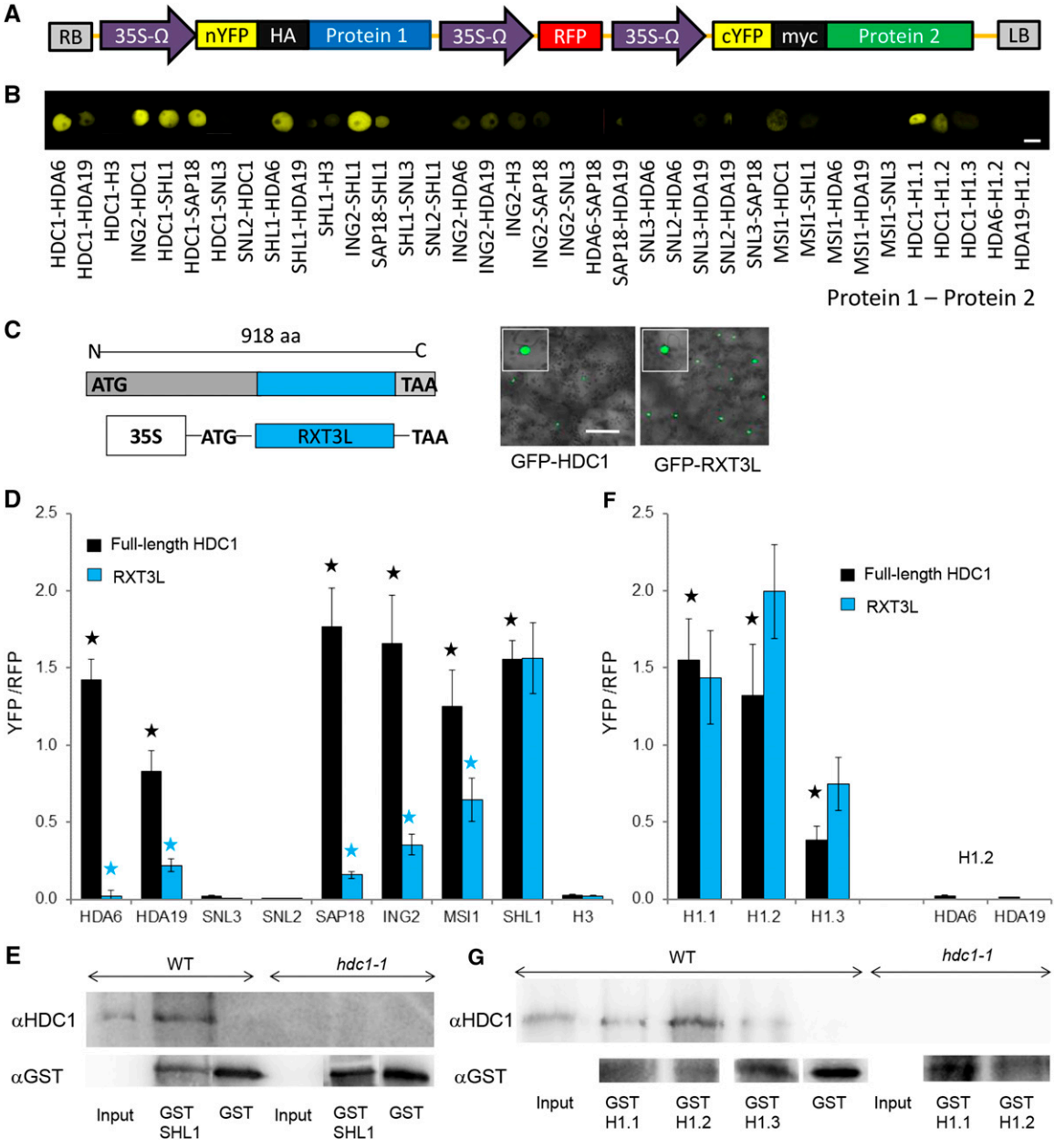

Figure 1. HDC1 directly interacts with several different proteins, and the truncated RXT3L fully maintains the capacity to interact with H3-binding protein SHL1 and with H1 linker histone variants. A, The 2-in-1 vector for ratiometric BiFC contains N- and C-terminal halves of YFP (nYFP, cYFP) and full-length RFP. B, Representative YFP signals in nuclei of tobacco epidermis cells transformed with the indicated protein pairs. Bar = 10 μm. C, Schematic representation of the truncation construct RXT3L representing a conserved (blue) C-terminal part of full-length HDC1. As for full-length HDC1, GFP-fusion protein of RXT3L shows nuclear localization. Bar = 50 μm. D and F, YFP/RFP signal ratio determined in tobacco leaf cells after transient transformation with 2-in-1 BiFC vector containing full-length HDC1 (black bars) or RXT3L (blue bars) together with other proteins. Tested interactors include HDAs HDA6 and HDA19, Sin3-like corepressors SNL2 and SNL3, Sin3-associated protein SAP18, H3-binding proteins SHL1, ING2, and MSI1 (D), as well as H3 and H1 variants H1.1, H1.2, and H1.3 (F). Bars are means ± SE ($n \geq 30$ cells from three independently transformed plants). Black stars (for full-length HDC1) indicate a significant ($P < 0.05$) difference from the signal obtained with SNL3 or H3 (negative controls). Blue stars (for RXT3L) indicate significant ($P < 0.05$) difference from the signal obtained with full-length HDC1. The two bars on the right in F are signals obtained for cells transformed with H1.2 and HDA6 or HDA19. E and G, Western blots showing in vivo pulldown of HDC1 in nuclei-enriched protein samples from wild-type (WT) or HDC1 knockout plants (*hdc1-1*) using GST-SHL1 (E) or GST-H1 variants (G) as bait. The top panels show the membrane probed with HDC1 antibody (αHDC1). The bottom panels show the membranes reprobed with GST antibody (αGST). As labeled, lanes contain total nuclear protein (Input, positive control), pull-down with GST-SHL1 or GST-H1, and pull-down with GST alone (negative control).

As we have previously reported, HDC1 can directly interact with the deacetylases HDA6 and HDA19. No direct interaction was found for HDC1 with the corepressor SNL3 or SNL2, but a strong YFP signal was recorded when HDC1 was coexpressed with SAP18. SAP18 also failed to directly interact with SNL3 or SNL2 (Supplemental Fig. S2). However, SNL2, SNL3, and SAP18 all produced a signal with HDA19, confirming correct expression/folding of the fusion proteins.

HDC1 showed interaction with the H3-binding proteins SHL1 and ING2 but not with H3 itself. As expected, SHL1 and ING2 both produced YFP signals with H3 (Supplemental Fig. S2). They also showed very strong interaction with each other. In addition, SHL1 produced YFP signals when coexpressed with the HDAs or with SAP18. BiFC also showed direct interaction between HDC1 and the H3-binding protein MSI1.

HDA19 displayed the broadest interaction profile (Supplemental Fig. S2). The strongest signal was obtained with HDC1. Complementation signals with SNL3, SNL2, and SAP18 were weaker than with HDC1 and SHL1 but significantly higher than the signals produced by SNL3 with HDC1 or other proteins. Despite previous reports showing pull-down of MSI1 with HDA19, we did not record a BiFC signal for these two proteins, suggesting that their interaction is indirect potentially via HDC1. HDA6 had a more selective interaction profile. It strongly interacted with HDC1 and SHL1 but failed to produce BiFC signals with the other proteins tested (Supplemental Fig. S2).

In summary, the BiFC study identified HDC1 and SHL1 as potential hubs for multiple protein scaffolding in HDAC complexes. To confirm native HDC1-SHL1 assembly, we carried out in vivo pull-down assays with protein extracts from Arabidopsis leaves using SHL1 as bait. As shown in Figure 1E, SHL1-GST (but not GST alone, first negative control) pulled down native HDC1 (detected with HDC1 antibody) in protein extracts from wild-type plants but not from *hdc1-1* knockout plants (second negative control). Statistically significant SHL1-HDC1 interaction was confirmed in three independent pull-down experiments (Supplemental Fig. S3). HDC1 was not recovered in pull-down assays using a truncated version of SHL1 (amino acids 21–137) spanning the histone-binding bromo-adjacent homology domain (Supplemental Fig. S4). Thus, the bromo-adjacent homology domain is not involved or not sufficient for the interaction of SHL1 with HDC1. Motivated by our previous finding that HDC1-mediated growth enhancement was maintained under salt stress (Perrella et al., 2013), we also tested interaction between SHL1 and HDC1 in leaf tissue collected from plants subjected to salt (150 mM NaCl for 24 h). Using full-length SHL1 as a bait, HDC1 was successfully pulled down from salt-treated wild-type plants but not from salt-treated *hdc1-1* plants (Supplemental Fig. S5).

HDC1 Interacts with H1

Originally intended as a negative control, we included the linker histone H1 (variant H1.2) in the BiFC assays. To our surprise we found a strong YFP complementation signal for HDC1 with H1.2 (Fig. 1F). The interaction was specific because HDC1 did not interact with H3 (see above) and H1.2 did not interact with HDA6 or HDA19 (Fig. 1F, right bars). Upon further testing we found that HDC1 also produced a strong complementation signal with the histone variant H1.1, which is very similar to H1.2, and a weaker signal with the more distinct H1.3 (Fig. 1F). In vivo interaction between HDC1 and H1 was confirmed by pull-down assays with protein extracts from Arabidopsis leaves using the H1 variants as bait. As shown in Figure 1G, GST-tagged H1.2 (but not GST alone, first negative control) pulled down native HDC1 (detected with HDC1 antibody) in protein extracts from wild-type plants but not from *hdc1-1* knockout plants (second negative control). HDC1 bands were fainter when GST-H.1.1 or GST-H1.3 was used as bait. Pull-downs were repeated four times, and statistical analysis of relative band intensities confirmed consistent binding of HDC1 by H1.2 ($P = 0.001$), more variable binding by H1.1 ($P = 0.06$), and no binding by H1.3 (Supplemental Fig. S4). Pull-down of HDC1 with H1.2 also was achieved using leaf material from plants that had been subjected to salt (Supplemental Fig. S5). HDC1 was not recovered in pull-down assays with truncated versions of H1.2 representing the N-terminal (amino acids 1–60), globular (amino acids 61–129), or C-terminal (amino acids 130–273) parts of H1.2 (Supplemental Fig. S4), indicating that none of these parts alone is sufficient for interaction.

Truncation of HDC1 Protein to the Yeast Rxt3-Like Core Weakens Most Interactions But Does Not Impact on Binding of SHL1 or H1

A 315-amino-acid stretch in the C-terminal half of the 918-amino-acid-long HDC1 protein aligns to the shorter Rxt3-like proteins in algae and fungi (Perrella et al., 2013). This part of the protein also is more conserved within higher plants than the rest of the protein, and it contains a highly conserved motif of unknown function (PF08642, 602–650 amino acids in HDC1). To assess whether the Rxt3-like part of the protein is required and sufficient for some or all of the interactions within the plant protein complex, we carried out ratiometric BiFC assays and compared the YFP/RFP ratios obtained with RXT3L (Fig. 1, D and F, blue bars) with those obtained for full-length HDC1 (black bars). The complementation signals obtained for RXT3L with HDA6, HDA19, ING2, MSI1, or SAP18 were significantly lower than those obtained for full-length HDC1, although still significantly larger than the ones obtained for each protein with SNL3 (Fig. 1D). Thus, the truncated protein maintains some affinity for these partners, but the interaction is considerably weakened. Strikingly, the truncated RXT3L protein fully retained the ability to directly interact with SHL1, generating a similarly high YFP/RFP signal as full-length HDC1. RXT3L also fully retained the ability to interact with the H1 variants (Fig. 1F). The strong signals obtained with

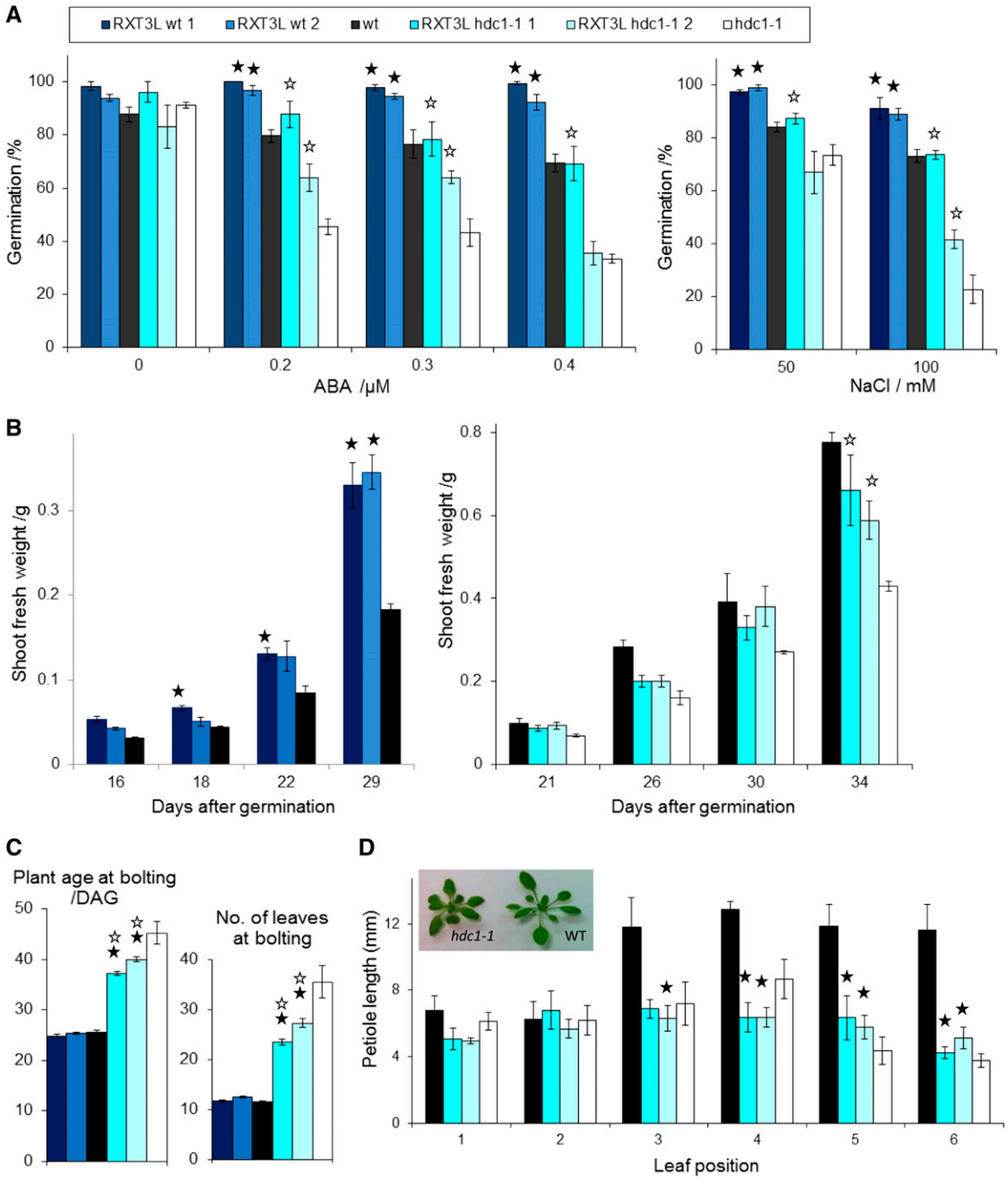

Figure 2. RXT3L complements germination and growth phenotypes of *hdc1* but only partially recovers flowering and is unable to restore petiole extension. Phenotypes for Arabidopsis wild type (wt; black), HDC1 knockout line (*hdc1-1*; white), two independent lines expressing RXT3L in wild-type background (RXT3Lwt 1 and 2; dark and light blue), and two independent lines expressing RXT3L in *hdc1-1* background (RXT3Lhdc1-1 1 and 2; dark and light turquoise) Significant differences ($P < 0.05$) for RXT3L-expressing lines against their respective background are indicated with black stars for the wild type and with white stars for *hdc1-1*. A, Germination rates on agar containing different concentrations of ABA and NaCl. Bars are means ± SE of at least three plates containing 50 seeds each. *hdc1-1* was significantly different from the wild type in all conditions other than control ($P < 0.05$). B, Shoot fresh weight of plants grown in short days at the indicated days after germination. Bars are means ± SE of three plants harvested each day. *hdc1-1* was significantly different from the wild type from day 26 onward ($P < 0.05$). C, Plant age and number of rosette leaves at bolting (1 cm stem length). Plants were grown in long days. Bars are means ± SE of 15 plants. *hdc1-1* was significantly different from the wild type for both parameters ($P < 0.05$). D, Petiole length of true rosette leaves 1 to 6. Plants were grown in short days. Bars show average petiole length of leaves from three plants ± SE. *hdc1-1* was significantly different from the wild type for leaves 3 to 6 ($P < 0.05$). Inset, Picture of *hdc1-1* and wild-type plants (3 weeks old).

SHL1 and H1 also proved that lower signals with the other proteins were not due to weak expression of the YFP-RXT3L fusion protein. The ability of RXT3L to bind SHL1 and H1 was further confirmed in reciprocal in vitro pull-down experiments using each of the proteins as bait (Supplemental Fig. S6).

RXT3L Partially Restores HDC1 Functions in Plant Growth and Development

We have previously reported that knockout or overexpression of HDC1 causes a range of phenotypes during plant germination, vegetative growth, and flowering (Perrella et al., 2013). To assess the ability of the RXT3L part of the protein to mediate downstream effects of HDC1-dependent histone deacetylation, we expressed RXT3L in the HDC1 knockout line *hdc1-1* and in wild-type plants under the control of the 35S promoter. Two homozygous lines from each background were used for the experiments. Quantitative PCR analysis with primers in the RXT3L domain (Supplemental Fig. S7) confirmed the presence of RXT3L transcript in the overexpressing and complemented lines.

Figure 2 shows that the truncated protein was able to carry out functions of full-length HDC1 in germination and growth but was less effective in replacing HDC1 in other functions such as flowering and petiole length. Figure 2A shows that overexpression of RXT3L decreased the ABA and NaCl sensitivity of germinating seeds both in wild-type background and in *hdc1-1* background, thus mimicking full-length HDC1 (Perrella et al., 2013). RXT3L also reproduced the growth enhancement reported for full-length HDC1; overexpression of RXT3L caused enhanced shoot fresh weight both in wild-type background and in *hdc1-1* background (Fig. 2B). We have shown before that enhanced biomass is due to larger leaf size, not to changes in the plastochron (Perrella et al., 2013).

RXT3L only partially complemented the delayed flowering phenotype of *hdc1-1*; plant age and number of leaves at bolting were significantly lower than in *hdc1-1* but still significantly higher than in the wild type (Fig. 2C). Another phenotype of *hdc1-1* is compact rosette appearance due to shortened petioles (Fig. 2D, inset). Petiole length can be rescued by expression of full-length HDC1 (Perrella et al., 2013) but was not restored by expression of RXT3L in *hdc1-1* (Fig. 2D). Thus, plants expressing RXT3L in *hdc1-1* background were larger than the knockout plants (growth effect) but bulkier than HDC1-complemented or wild-type plants due to short petioles.

DISCUSSION

We are only just beginning to appreciate the complexity and regulatory functions of protein interactions in the nucleus. How DNA and histones recruit the enzymes that modify and regulate them in a dynamic manner is an active area of research, and understanding how these interactions affect chromatin structure, DNA accessibility, and gene transcription remains a challenge. To fully understand the mechanism of histone deacetylation within the context of multiprotein complexes, it is essential to investigate those members for which the molecular function is unknown. HDC1 is particularly important because both knockout and overexpression produce measurable effects on histone acetylation levels, gene expression, and downstream phenotypes (Perrella et al., 2013). The lack of obvious structural features suggests that HDC1 is intrinsically disordered and could act as a flexible link between multiple proteins.

The results of our BiFC study strengthen this hypothesis. We found that HDC1 has the ability to directly interact with several different types of proteins, including HDAs, histone-binding proteins, and associated proteins of unknown function. Particularly strong interaction was found with the H3-binding protein SHL1, which itself showed a capacity to interact with multiple other proteins. Neither HDC1 nor SHL1 directly interacted with the corepressor SNL3, which only made close contact with HDA19. The interaction profile suggests that HDC1 associates with the 'histone-binding end' of the complex (Supplemental Fig. S8). It is likely that depending on cell type, developmental stage, and environmental conditions, native complexes dynamically assemble into different subsets of the prototype shown in Supplemental Figure S8 and incorporate additional partners not tested here.

We also discovered that HDC1 has the capacity to bind H1. H1 is positioned at the edge of nucleosomes, binds to both the nucleosome core and the linker DNA, and correlates with more condensed, less accessible, and transcriptionally silent DNA (Ascenzi and Gantt, 1999). In Arabidopsis H1 is encoded by three genes (Ascenzi and Gantt, 1999; Wierzbicki and Jerzmanowski, 2005). H1.1 and H1.2 share 85% identity at the DNA level in the nuclear domain, indicating they might be result of gene duplication. H1.3 is more divergent and is induced by low light and drought (Ascenzi and Gantt, 1999; Rutowicz et al., 2015). At the phenotypic level, triple knockout/knockdown of the H1 genes leads to developmental abnormalities with a reduction of plant size, delayed flowering, and embryo lethality (Jerzmanowski et al., 2000). Arabidopsis H1s have been found to directly interact with the DNA glycosylase DEMETER, which regulates genomic imprinting by demethylating MEDEA promoter in the endosperm (Rea et al., 2012). Furthermore, loss of H1 alters DNA methylation patterns with different effects on euchromatin and heterochromatin (Wierzbicki and Jerzmanowski, 2005; Zemach et al., 2013). The exact role of H1 in DNA modification remains to be elucidated, but it has been proposed that it restricts the access of the DNA methyltransferase to the nucleosome (Zemach et al., 2013). The block imposed by H1 proteins, mainly within long transposable elements, was overcome by the Swi/Snf chromatin remodeler Decrease of DNA Methylation 1 (DDM1), and it was suggested that DDM1 facilitates access of DNA methylases by removing H1 from the DNA.

Based on the above, an interaction between HDC1 and H1 could be functionally interpreted in two ways. In the first hypothesis, HDC1 establishes a physical link between HDAC complexes and H1, thereby enhancing chromatin condensation and repression of the target genes. In the second hypothesis, HDC1 removes H1, similar to DDM, thereby facilitating access of HDAs to the core histone tails. Both functions would benefit from a flexible structure of HDC1. These hypotheses now need to be tested in a genetics approach.

Due to the lack of predicted structural motifs or homology to known functional domains in HDC1, it is impossible to pinpoint specific binding sites. In a first gene truncation approach, we found that the capacity to interact with SHL1 and with H1 was fully maintained by the conserved RXT3L part of HDC1 while other interactions were weakened. This could indicate that HDC1 is positioned with the Rxt3-like part at the edge of the nucleosome and the N-terminal part reaching deeper into the complex (Supplemental Fig. S8). The phenotypic spectrum of RXT3L indicates that flowering and petiole extension require the full interaction capacity of HDC1, while regulation of germination and growth can be achieved with the partial interaction spectrum maintained by RXT3L. It is tempting to consider that the latter phenotypes are evolutionarily older and may therefore already been enabled by shorter Rxt3-like proteins in algae, whereas the former, linked to the complex morphology and development of higher plants, required considerable sequence extension of HDC1 to enable a broader protein interaction profile. The results presented here provide a basis for further dissecting the structure-function relationship of HDC1 in different species and for identifying specific target genes that underpin its diverse physiological and developmental functions.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Treatments

All transgenic lines were generated in Arabidopsis (*Arabidopsis thaliana*) Columbia-0 background. *hdc1-1* and HDC1-overexpressing lines have been characterized before (Perrella et al., 2013). Homozygous RXT3L-expressing lines were generated from the progeny of wild-type and *hdc1-1* plants transformed with RXT3L part under the control of 35S promoters (see "Cloning Procedures"). Plants were grown and treated in controlled growth rooms at a temperature of 22°C and a light intensity of 150 μmol photosynthetically active radiation. Plants were grown either in long days (16 h light) or in short days (10 h light) as indicated in text and figure legends. Germination, growth, and flowering assays were carried out as described before (Perrella et al., 2013). Petiole and leaf blade length were measured by ImageJ (http://imagej.nih.gov/ij/).

Cloning Procedures

Entry clones with full-length HDC1, HDA6, HDA19, SAP18, SHL1, ING2, H3, SNL2, SNL3, H1.1, H1.2, H1.3, H3.3, MSI1, RXT3L, and ScRXT3 with or without stop codon were generated by PCR amplification using primers that contained attB1 and attB2 sites or attB3 and attB4 (Supplemental Table S3). For cloning of the RXT3L part, the HDC1 gene sequence from bp 1345 to 2292 was amplified. Gel-purified PCR products were introduced into pDONR207/221 (Life Technologies) using BP-clonase II according to the manufacturer's instructions and transferred to destination vectors by recombination using LR-clonase II (Life Technologies). The reaction product was used to transform TOP10 (Thermo Fisher Scientific) bacterial cells. Antibiotic marker-resistant colonies were isolated and verified by restriction digest analysis and sequencing. The following plasmids were generated and used in this study: 2x35S::RXT3L in pMDC032 (Curtis and Grossniklaus, 2003); 35S::GFP-HDC1, 35S::GFP-RXT3L, and 35S::GFP-ScRXT3 in pH7WGF2 (Karimi et al., 2002); and 35S:nYFP-protein1/cYFP-protein2 in pBiFCt-2in1-NN (Grefen and Blatt, 2012). For protein expression, the following plasmids were used: pET-Dest42 and pET300/NT-Dest (Thermo Fisher Scientific), pGEX-4T1 (GE), and a modified pGEX vector also containing a C-terminal His tag (Strugnell et al., 1997).

Plant Transformation

Plasmids were inserted by heat shock into *Agrobacterium tumefaciens* strain GV3101 pMP90 (Koncz and Schell, 1986). Agrobacterium-mediated transformation of Arabidopsis was performed by the floral dip method (Clough and Bent, 1998). Transient transformation of tobacco (*Nicotiana benthamiana*) was achieved by leaf infiltration (Geelen et al., 2002). For ratiometric BiFC assays and colocalization studies, each construct was coexpressed with p19 protein of *Tomato bushy stunt virus*, encoding for a suppressor of gene silencing (Voinnet et al., 2003).

Confocal Microscopy

Fluorescence in tobacco epidermal cells was assessed 2 d after infiltration using a CLSM-510-META-UV confocal microscope (Zeiss). For single protein localization, GFP fluorescence was excited at 488 nm with light from an argon laser and collected after passage through an NFT545 dichroic mirror with a 505-nm long-pass filter. RFP fluorescence was excited at 543 nm with light from a helium neon laser and was collected after passage through an NFT545 dichroic mirror and a 560- to 615-nm band-pass filter. YFP fluorescence was excited at 514 nm with light from an argon laser and collected using lambda mode between 520 and 550 nm. Colocalization plane and line scans were evaluated using Zeiss LSM510AIM software (v3.2).

Pull-Down Assays

Protein pull-downs were performed as described previously (Perrella et al., 2013). In short, His-fused proteins, GST-fused proteins, and GST were expressed in *Escherichia coli* BL21 cells. After induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside, cells were harvested and sonicated in lysis buffer. GST proteins were affinity purified using Glutathione-Sepharose resin (GE Healthcare) according to the manufacturer's instructions. His-fused proteins were purified using Nickel-NTA resin (Sigma). For pull-downs purified proteins were bound to Glutathione-Sepharose resin and applied to a microcolumn. Nuclei-enriched plant lysates were incubated overnight at 4°C. For in vitro pull-downs purified proteins bound to Glutathione-Sepharose resin were incubated with His-fused proteins for 4 h at 4°C. After several washes, pulled-down proteins were eluted in Laemmli buffer. For western blots, the protein samples were boiled, loaded onto SDS-PAGE gel, and transferred to nitrocellulose membrane (GE Healthcare Life Sciences). Incubation with αHDC1, αGST (GE Healthcare), or αHis (Cell Signaling Technology) was overnight at dilutions of 1:4000, 1:5000, or 1:2000, respectively. Secondary antibody conjugated with horseradish peroxidase was applied for at least 1 h at room temperature. Finally, the membrane was covered with ECL Dura HRP reagent (Thermo Fisher Scientific) and the proteins detected using a chemiluminescence imaging platform (Fusion FX; Peqlab). Band intensities were quantified using ImageJ software.

Data Analysis

Data were collated and analyzed in Microsoft Excel spreadsheets. Means were calculated across replicates, and relevant comparisons were tested using Student's *t* test. Numbers of replicates and the *P* values are indicated in the figure legends.

Accession Numbers

Sequence data for genes used in this study can be found in the GenBank/EMBL libraries and in The Arabidopsis Information Resource or

the Saccharomyces Genome database under the following accession numbers: AT5G08450 (HDC1), AT5G63110 (HDA6), AT4G38130 (HDA19), AT2G45640 (SAP18), AT5G15020 (SNL2), AT1G24190 (SNL3), AT4G39100 (SHL1), AT1G54390 (ING2), AT1G09200 (H3.1), AT4G40030 (H3.3), AT1G06760 (H1.1), AT2G30620 (H1.2), AT2G18050 (H1.3), AT5G58230 (MSI1), and YDL076C (ScRXT3).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Subcellular localization of GFP-fusion protein expressed in tobacco.

Supplemental Figure S2. Interaction profiles of different HDAC complex proteins.

Supplemental Figure S3. Quantification of HDC1 interaction with H1.2 and SHL1 in Arabidopsis.

Supplemental Figure S4. Truncated versions of H1.2 and SHL1 are not binding HDC1.

Supplemental Figure S5. HDC1 interaction with H1.2 and SHL1 in salt-treated Arabidopsis plants.

Supplemental Figure S6. Reciprocal pull-down of Rxt3L/SHL1 and Rxt3L/H1.2.

Supplemental Figure S7. Transcript levels of the RXT3-like part of HDC.

Supplemental Figure S8. Visual summary of protein interactions assayed in this study.

Supplemental Table S1. Proteins coeluting in the *S. cerevisiae* Rpd3L complex.

Supplemental Table S2. Information on selected candidates for interaction with HDC1.

Supplemental Table S3. Primers used for genotyping and cloning.

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