HDC1 interacts with SHL1 and H1

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The Histone Deacetylase Complex (HDC) 1 protein of Arabidopsis thaliana has the capacity to interact with multiple proteins including histone 3-binding proteins and histone 1 variants.

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1-sentence summary:
A conserved ancestral domain of the intrinsically disordered protein HDC1 can directly interact with H3-binding proteins and with H1 variants, thus providing a means for mediating between histones at the core-nucleosome and at the linker DNA.

Author contributions:
G. P. performed and analysed most of the experiments. C. C. and K. P. assisted with the BiFC assays, M.A.A. quantified leaf phenotypes and N. A. M. assisted with cloning and transformation. G.P., M.A.H. and A.A. planned the experiments. A.A. wrote the paper with input from all authors.

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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 (HDC) 1 protein from Arabidopsis thaliana interacts with histone deacetylases and quantitatively determines histone acetylation levels, transcriptional activity and several phenotypes, including ABA-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 BiFC assay in tobacco epidermal cells. In addition to binding histone deacetylases, HDC1 directly interacted with histone H3-binding proteins and co-repressor associated proteins, but not with H3 or the co-repressors themselves. Surprisingly, HDC1 was also 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.
INTRODUCTION

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 comprised of histone octamers comprised of histones H2A/B, H3 and H4, and further stabilised 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 multi-protein 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 co-repressors recruits histone deacetylases (HDAs) to the gene region (Song et al., 2005). The HDAs in turn interact with histone binding proteins (Mehdi et al., 2015). Removal of acetyl groups from lysine 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 and in animal systems have provided evidence for large multi-protein complexes linking a co-repressor and a histone deacetylase with several histone-binding proteins and a range of associated proteins of mostly unknown functions (Yang and Seto, 2008). Plant HDAC complexes are less well characterised but in a recent study several proteins, including co-repressors and histone-binding proteins, were found to co-precipitate 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., 2015).

Histone Deacetylation 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 *A. 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, over-expression 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 *A. thaliana* (Derkacheva et al., 2013; Mehdi et al., 2015) and it directly interacts with the histone deacetylases 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 et al., 2010; Chen and Wu, 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 co-elutes with the large Rpd3 HDAC-complex in yeast but its function has remained unclear (Carrozza et al., 2005a; Carrozza et al., 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 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 co-eluting with Rxt3 in yeast complexes and on reported phenotypes and protein interactions in plants (Supplemental Tables 1 and 2), we selected a subset of *A. thaliana* proteins as candidate direct interactors with HDC1: the histone-binding proteins SHL1, ING2 and MSI1 (Mussig et al., 2000; Mussig and Altmann, 2003; Lee et al., 2009; Lopez-Gonzalez et al., 2014; Mehdi et al., 2015), the Sin3-like (SNL) co-repressors 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 histone deacetylases (HDA6, HDA19; (Chen and Wu, 2010)), H3 variants (H3.1., 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 Bimolecular Fluorescence Complementation (BiFC, Figure 1). The proteins were fused to N- or C-terminal halves of Yellow Fluorescent Protein (YFP) and transiently co-expressed 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, Figure 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 normalisation 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 (Figure 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, Figure 1C), approximately a 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 aa 358, and KR RERDGDSEAE RAEKR starting at aa 479). Only the latter was present in RXT3L suggesting that it is sufficient for nuclear localisation. Yeast ScRXT3 contains neither of the motifs and GFP-ScRXT3 was not retained in the nuclei (Supplemental Figure 1), suggesting that the 479 motif is necessary for nuclear retention in plant cells.

Figure 1 D shows the interaction profile of HDC1 based on YFP/RFP ratios obtained from cells co-expressing HDC1 with candidate interactors. Signals were measured in at least 10 cells from three independently transformed plants. Supplemental Figure 2 shows the respective interaction profiles for SHL1, ING2, MSI1, SAP18, HDA6 and HDA19. The following
observations confirmed the validity of the approach. Firstly, for each protein a significant complementation signal was detected with at least one other protein confirming that all fusion proteins were properly expressed. Secondly, the complementation signal was always observed.
inside the nuclei, confirming correct targeting of the fusion proteins. Thirdly, the interaction profiles differed between the proteins tested, confirming specificity of the interactions.

As we have previously reported, HDC1 can directly interact with the deacetylases HDA6 and HDA19. No direct interaction was found for HDC1 with the co-repressors SNL3 or SNL2 but a strong YFP-complementation signal was recorded when HDC1 was co-expressed with SAP18. SAP18 also failed to directly interact with SNL3 or SNL2 (Supplemental Figure 2). However, SNL2, SNL3 and SAP18 all produced a signal with HDA19 confirming correct expression/folding of the fusion proteins.

HDC1 showed interaction with the histone-binding proteins SHL1 and ING2, but not with H3 itself. As expected, SHL1 and ING2 both produced YFP signals with H3 (Supplemental Figure 2). They also showed very strong interaction with each other. In addition, SHL1 produced YFP signals when co-expressed 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 Figure 2). 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 Figure 2).

In summary, the BiFC study identified HDC1 and SHL1 as a potentially important interaction hub in HDAC complexes. To confirm native HDC1-SHL1 assembly we carried out in vivo pulldown assays with protein extracts from A. thaliana leaves using SHL1 as bait. As shown in Figure 1E, SHL1-GST (but not GST alone, 1st negative control) pulled down native HDC1 (detected with HDC1-antibody) in protein extracts from wildtype plants, but not from hdc1-1 knockout plants (2nd negative control). Statistically significant SHL1-HDC1 interaction was confirmed in three independent pulldown experiments (Supplemental Figure 3). HDC1 was not recovered in a pulldown assays using a truncated version of SHL1 (amino acids (aa) 21-137) spanning the histone-binding bromo-adjacent homology (BAH) domain (Supplemental Figure 4). Thus the BAH 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 wildtype plants but not from salt-treated
hdc1-1 plants (Supplemental Figure 5).

**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
(Figure 1F). The interaction was specific because HDC1 did not interact with H3 (see above) and
H1.2 did not interact with HDA6 or HDA19 (see right bars in Figure 1F). 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 (Figure 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, 1st negative control) pulled down native HDC1 (detected with HDC1-antibody) in
protein extracts from wildtype plants, but not from hdc1-1 knockout plants (2nd negative control).
Fainter HDC1 bands were seen when GST-H.1.1 or GST-H1.3 were used as baits. Pulldowns 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 Figure 4). Pull-down of HDC1 with H1.2 was also achieved using leaf material from
plants that had been subjected to salt (Supplemental Figure 5). HDC1 was not recovered in
pulldown assays with truncated versions of H1.2 representing the N-terminal (aa 1-60), globular
(aa 61-129) or C-terminal (aa 130-273) parts of H1.2 (Supplemental Figure 4), indicating that
neither of these parts is alone 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- aa stretch in the C-terminal half of the 918-aa long HDC1 protein aligns to the shorter Rxt3-
like proteins in algae and fungi (Perrella et al., 2013). This part of the protein is also more
conserved within higher plants than the rest of the protein, and it contains a highly conserved motif
of unknown function (PF08642, 602-650 aa 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
(blue bars in Fig. 1D and Fig. 1F) 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 SHL1 and H1 also proved that lower signals with the other proteins were not due to weak expression of the RXT3L-YFP 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 figure 6).

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 wildtype plants under the control of the 35S promoter. Two homozygous lines from each background were used for the experiments. qPCR analysis with primers in the RXT3L domain (Supplemental Figure 7) 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 wildtype 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; over-expression of RXT3L caused enhanced shoot fresh weight both in wildtype 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 wildtype (Fig. 2C). Another phenotype of hdc1-1 is compact rosette appearance due to shortened petioles (see inserts in Fig. 2D). 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 wildtype plants due to short petioles.
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 multi-protein 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 histone deacetylases, histone-binding proteins and associated proteins of unknown function. Particular 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 co-repressor SNL3, which only made close contact with HDA19. The interaction profile suggests that HDC1 associates with the ‘histone-binding end’ of the complex (Supplemental Figure 8). 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 8, 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 thaliana 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 it is induced by low light and drought (Ascenzi and Gantt, 1999; Rutowicz et al., 2015). At the phenotypic level, triple knock-out/down 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 (DDM) 1, 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 Figure 8). 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 the RXT3L part. It is tempting to consider that the latter phenotypes are evolutionary 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 thaliana Col-0 background. hdc1-1 and HDC1-overexpressing lines have been characterised 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 PAR. 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 Image J.
Entry clones with full-length HDC1, HDA6, HDA19, SAP18, SHL1, ING2, H3, SNL2 and 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 3). 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 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), 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 (Thermofisher), pGEX-4T1 (GE) and a modified pGEX vector containing also a C-terminal histidine 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 *Nicotiana benthamiana* was achieved by leaf infiltration (Geelen et al., 2002). For ratiometric BiFC assays and co-localization studies, each construct was co-expressed 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. Co-localization plane and line scans were evaluated using Zeiss LSM510AIM software (v3.2).

**Pull-Down Assays**
Protein pulldown were performed as previously described (Perrella et al, 2013). In short, histidine (His)-fused proteins, GST-fused proteins and GST were expressed in Escherichia coli BL21 cells. After induction with 0.5mM isopropyl b-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 pulldowns purified proteins were bound to Glutathione-Sepharose resin and applied to a microcolumn. Nuclei enriched plant lysates were incubated overnight at 4C. For in vitro pulldowns purified proteins bound to Glutathione-Sepharose resin were incubated with His-fused proteins for 4 hours at 4C. 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 life sciences). Incubation with αHDC, αGST (GE Healthcare) or αHis (Cell Signalling 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 hour at room temperature. Finally the membrane was covered with ECL Dura HRP reagent (Thermo Fisher Scientific) and the proteins were detected using a chemiluminescence imaging platform (Fusion FX, Peqlab). Band intensities were quantified using Image J software.

Data analysis
Data were collated and analysed in Excel spreadsheets. Means were calculated across replicates and relevant comparisons were tested using Student 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 in 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); YDL076C (ScRXT3).

Supplemental Material
Supplemental File 1 contains Supplemental Figures:
Supplemental Figure 1: Subcellular localisation of GFP-fusion protein expressed in tobacco.
Supplemental Figure 2: Interaction profiles of different HDAC complex proteins.
Supplemental Figure 3. Quantification of HDC1 interaction with H1.2 and SHL1 in *A. thaliana*.

Supplemental Figure 4. Truncated versions of H1.2 and SHL1 are not binding HDC1.

Supplemental Figure 5. HDC1 interaction with H1.2 and SHL1 in salt-treated *A. thaliana* plants.

Supplemental Figure 6. Reciprocal pulldown of Rxt3L/SHL1 and Rxt3L/H1.2.

Supplemental Figure 7: Transcript levels of the RXT3-like part of HDC.

Supplemental Figure 8: Visual summary of protein interactions assayed in this study.

Supplemental File 2 contains Supplemental Tables:

Supplemental Table 1: Proteins co-eluting in the *S. cerevisiae* Rpd3L complex

Supplemental Table 2: Information on selected candidates for interaction with HDC1

Supplemental Table 3: Primers used for genotyping and cloning

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FIGURE LEGENDS

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 is 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 is 50 µm.

D, F: YFP/RFP signal ratio determined in tobacco leaf cells after transient transformation with 2-in-1 BIFC vector containing full length HDC1 (grey bars) or RXT3L (blue bars) together with other proteins. Tested interactors include histone deacetylases HDA6 and 19, Sin3-like co-repressors SNL2 and 3, 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 ≥ 30 cells from three independently transformed plants). Black asterisks (for full-length HDC1) indicate a significant (p < 0.05) difference to the signal obtained with SNL3 or H3 (negative controls). Blue asterisks (for RXT3L) indicate significant (p < 0.05) difference to 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, 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 (B) or GST-H1 variants (D) as bait. The upper panels show the membrane probed with HDC1 antibody (αHDC1). The bottom panels show the membranes re-probed with GST antibody (αGST). As labelled, lanes contain HDC1 only (Input, positive control), pull-down with GST-SHL1 or GST-H1, and pull-down with GST alone (negative control).

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 thaliana wildtype (wt; black), HDC1-knockout line (hdc1-1, white), two independent lines expressing RXT3L in wt background (RXT3Lwt1,2, dark and light blue) and two independent lines expressing RXT3L in hdc1-1 background (RXT3Lwt1,2dark and light turquoise). Significant differences (p < 0.05) for Rxt3L-expressing lines against their respective background are indicated with black asterisks for wildtype, and with white asterisks 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 wildtype 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 wildtype from day 26 onwards (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 wildtype 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 wildtype for leaves 3-6 (p < 0.05). Insert: Picture of hdc1-1 and wild type plants (3-weeks old).
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