OsChz1 acts as a histone chaperone in modulating chromatin organization and genome function in rice

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While the yeast Chz1 acts as a specific histone-chaperone for H2A.Z, functions of CHZ-domain proteins in multicellular eukaryotes remain obscure. Here, we report on the functional characterization of OsChz1, a sole CHZ-domain protein identified in rice. OsChz1 interacts with both the canonical H2A-H2B dimer and the variant H2A.Z-H2B dimer. Within crystal structure the C-terminal region of OsChz1 binds H2A-H2B via an acidic region, pointing to a previously unknown recognition mechanism. Knockout of OsChz1 leads to multiple plant developmental defects. At genome-wide level, loss of OsChz1 causes mis-regulations of thousands of genes and broad alterations of nucleosome occupancy as well as reductions of H2A.Z-enrichment. While OsChz1 associates with chromatin regions enriched of repressive histone marks (H3K27me3 and H3K4me2), its loss does not affect the genome landscape of DNA methylation. Taken together, it is emerging that OsChz1 functions as an important H2A/H2A.Z-H2B chaperone in dynamic regulation of chromatin for higher eukaryote development.
Nucleosome is the fundamental structural unit of chromatin in eukaryotes. It is composed of almost two superhelical turns of DNA (~146 bp) wrapped around an octamer formed by two copies each of the four core histone proteins H2A, H2B, H3, and H4. Linker histone H1 binds internucleosomal DNA to stabilize adjacent nucleosomes and to mediate higher-order chromatin packaging. While chromatin appears rigid at the cytological level, nucleosomes are highly dynamic. Nucleosome assembly, disassembly, and reassembly occur not only during genome replication but also during transcription and during DNA-damage repair. Proper assembly of histones with DNA requires histone–chaperones, which function in preventing unintended interactions between the positive-electrical-charged histone and the negative-electrical-charged DNA molecules under physiological conditions. In addition to the canonical histone dynamics, histone variant incorporation constitutes a key mean in modulating nucleosome compositions.

Unlike the canonical histones that are packaged into nucleosomes primarily during DNA replication, histone variants are deposited throughout the cell cycle and are incorporated into nucleosomes largely in DNA replication-independent pathways. Among the five families of histones, the H2A family possesses the largest number of variants, including H2A.Z, H2A.X, macroH2A, H2A.W, and H2A.W9,10. From them, H2A.Z is most highly conserved across different eukaryotes during evolution. In yeast, animal, and plant, H2A.Z is found at many genes and is distributed most often around transcription start sites (TSS) with a particular preference for +1 nucleosome.

H2A.Z accumulation can affect transcription positively or negatively. Strikingly, in contrast to the extensively studied functions of H2A.Z, histone chaperones, which function in the regulation of multiple plant growth and developmental processes. Genome-wide profiling at transcriptome, nucleosome occupancy, H2A.Z enrichment, OsChz1 binding, and DNA-methylation levels demonstrate that OsChz1 plays key functions in vivo in the regulation of chromatin dynamics and genome transcription.

Results
Identification and expression analysis of OsChz1. Using the CHZ-domain sequence in Blast search, we have identified yChz1 homologs in diverse organisms from the UniProt database. While absent in some invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster*, CHZ-domain proteins are found in humans and vertebrates (Fig. 1a). Remarkably, CHZ-domain proteins are well-conserved during the green lineage evolution and are found in organisms ranging from unicellular algae to vascular higher plants (Fig. 1a). Besides the CHZ domain, which is located near to the C-terminus, the other regions of the CHZ-domain proteins share little homology, with some potential functional domains identified but failed to show a substantial association with CHZ-domain during evolution (Fig. 1a).

Most higher plants where two or more copies of CHZ-encoding genes exist in a given species, rice contains a single gene, OsChz1, which encodes the sole CHZ-domain protein in this organism. RT-PCR analysis revealed that OsChz1 is broadly expressed in various rice organs, with the highest level in flag leaf, varied intermediate levels in stem, panicle, leaf sheath, young seedling, anther, spikelet, and root, and the lowest level in mature grain (Fig. 1b). We further examined the expression pattern of OsChz1 by promoter-fusion with the encoding sequence of the visual marker β-glucuronidase (pOsChz1:GUS). The pOsChz1:GUS transgenic plants showed strong GUS signals in the root, coleoptile, and leaf blade of young seedlings (Fig. 1c), in the basal part of flag leaves (Fig. 1d), in the spikelets of panicle (Fig. 1e, f), particularly in the male and female reproductive organs such as stamens and pistil (Fig. 1g), and in grains (Fig. 1h). The GUS signal in grains dropped drastically upon grain maturation and desiccation (Fig. 1g). Taken together, the expression pattern of OsChz1 indicates that it could play important functions during rice plant growth and development, and the single copy of this gene provides a facility in its knockout for functional study in plants.

OsChz1 can act as a histone–chaperone for both H2A-H2B and H2A.Z-H2B. To examine whether or not OsChz1 physically interacts with histone dimers H2A-H2B and H2A.Z-H2B in vitro, a glutathione S-transferase (GST) pull-down assay was performed using a recombinant N-terminal GST-tagged OsChz1 full-length protein (GST-OsChz1). The Arabidopsis histone H2A (HTA1) shares high sequence homologies with the rice H2A (HTA702), and also the Arabidopsis histone variant H2A.Z (HTA9) shares...
high sequence homologies with the rice H2A.Z (HTA713) (Supplementary Fig. 1a). Thus, we have used in our pull-down assays the Arabidopsis H2A-H2B dimer, which had been used as recombinant proteins in a previous study35, and the Arabidopsis H2A.Z-H2B dimer produced similarly. We observed that both the H2A-H2B and the H2A.Z-H2B dimers could be pulled down by GST-OsChz1, but not by GST alone (Fig. 2a). To investigate the subcellular localization of OsChz1, we designed an enhanced green fluorescent protein (GFP)-OsChz1 fusion construct driven by the maize (Zea mays) ubiquitin-1 (Ubi) promoter. Transient expression of this construct in rice protoplasts and fluorescent microscopy analyses revealed that GFP-OsChz1 was located in the cell nucleus (Fig. 2b). We further co-expressed GFP-OsChz1 together with a MYC-tagged rice histone H2A protein (HTA702-MYC) or a HA-tagged rice histone variant H2A.Z protein (HTA713-HA) in rice protoplasts, and performed co-immunoprecipitation (Co-IP) assays. Consistent with the results of our pull-down analysis, it was observed that both HTA702-MYC and HTA713-HA could be co-immunoprecipitated by GFP-OsChz1 (Fig. 2c).
To gain information about regions of OsChz1 involved in histone binding, we divided OsChz1 into four segments: an N-terminal region (OsChz1 1–78, OsChz1-N), a middle region (OsChz1 79–337, OsChz1-M) containing weak homologies with some DNA-topoisomerase2-like proteins forming the PTZ00180 superfamily (Supplementary Fig. 2), a C-terminal region (OsChz1 338–471, OsChz1-C) containing the evolutionarily conserved CHZ domain (Supplementary Fig. 3), and the CHZ-domain region (OsChz1 390–422). Our in vitro pull-down assays showed that both OsChz1-M and OsChz1-C but not OsChz1-N nor CHZ-domain exhibit binding activities to either H2A-H2B or H2A.Z-H2B (Fig. 2d). Visually, OsChz1-C seemed to pull down more efficiently H2A-H2B than H2A.Z-H2B. To verify this, we examined the binding of OsChz1-C to...
H2A-H2B and H2A.Z-H2B by isotothermal titration calorimetry (ITC) experiments. We found that OsChz1-C achieves an ~6.5-fold higher affinity for H2A-H2B (Kd of 9.8 nM) than for H2A.Z-H2B (Kd of 64.1 nM) (Fig. 2e).

Next, we examined OsChz1 function in nucleosomal deposition of H2A-H2B and H2A.Z-H2B by using in vitro assays. We assembled the (H3-H4)2-DNA tetrasome complexes as well as the H2A-containing and H2A.Z-containing nucleosomes, respectively (Supplementary Fig. 4a). In the absence of OsChz1, both the H2A-H2B and H2A.Z-H2B dimers were found readily capable to bind to the (H3-H4)2-DNA tetrasome complexes to form nucleosomes (Supplementary Fig. 4b). Thus, we examined OsChz1 activity by comparing nucleosome formation efficiency under specified conditions. For this, the (H3-H4)2-DNA tetrasome complexes were incubated, in the presence of a fixed concentration of OsChz1, with increasing amounts of H2A-H2B or H2A.Z-H2B for 30 min (Fig. 2f). A concentration-dependent efficient nucleosome assembly was observed, and this occurred significantly more efficiently in the presence of OsChz1 than in the absence of OsChz1 for both H2A-H2B and H2A.Z-H2B (Fig. 2g). This observation is in line with a histone–chaperone activity of OsChz1 that facilitates nucleosome assembly. In this assay again OsChz1 did not show any preference for H2A over H2A, and H2A-H2B displayed more efficient assembly with the (H3-H4)2-DNA tetrasome complex than did H2A.Z-H2B in either the presence or the absence of OsChz1 (Fig. 2g).

Structural basis for H2A-H2B dimer recognition by the C-terminal region of OsChz1. The absence of OsChz1 preference for H2A.Z is in contrast to the preference of yChz1 for H2A.Z reported previously. To understand the molecular basis of interaction between OsChz1 and H2A-H2B, we crystallized OsChz1-C in complex with H2A-H2B, and obtained the crystal structure of the complex at a 2.85 Å resolution by the molecular replacement (MR) method (Supplementary Table 1).

As supported by the 2Fo-Fc electron density map, residues 432–446aa of OsChz1 are well ordered in the structure (Fig. 3a). The N-terminal half of OsChz1-C forms extensive interactions with H2B, through residues located at the α3 and α4 helix regions (Fig. 3b). In detail, the main chain N atom of OsChz1 Glu432 is in contact with the side chain of H2B Asn108, while the main chain O atom of OsChz1 Glu432 directly interacts with the side chain of H2B Arg116. In addition, the carboxyl of OsChz1 Glu432 forms one hydrogen bond (H-bond) with the side chain of H2B Thr120. Next, the main chain O atom of OsChz1 Glu433 binds to the side chain of H2B Gln119. The side chain of OsChz1 Glu436 binds to H2B Thr143 via H-bond interaction, and OsChz1 Asp438 forms a salt bridge with H2B Arg123. OsChz1 Asn439 makes a contact with H2B Lys140. As supported by the 2Fo-Fc electron density map, residues 446aa of OsChz1 are well ordered in the structure (Fig. 3a).

Mutations in OsChz1 caused multiple plant developmental defects. To investigate the biological function of OsChz1, we used the CRISPR/Cas9-mediated mutagenesis to target the OsChz1 gene within the rice genome. The designed single-guide RNA (sgRNA) targets a region within the second exon of OsChz1 (Fig. 4a). The resulted mutation was identified through genotyping and sequencing analysis of transgenic rice plants. Two independent mutations, one with a 1-nt deletion (oschz1-1) and the other with a 2-nt deletion (oschz1-2) were obtained. Each of these mutations introduces a stop codon that causes premature termination of translation (Fig. 4a), implying both oschz1-1 and oschz1-2 as loss-of-function gene mutant alleles. From the transgene-free oschz1-1 and oschz1-2 homozygous mutant plants (Supplementary Fig. 8), we further sequenced and confirmed that
and OsChz1 together driven by the in tiller number per plant (Fig. 4h), a reduction of 23% in grain pattern in plants (Fig. 1b and development, which is consistent with its broad expression involved in the regulation of multiple processes of rice growth grain width (Fig. 4j). These results indicate that reduction of 22% in panicle length (Fig. 4g), a reduction of 30% number per panicle (Fig. 4i), and an increase of about 18% in

Quantitative analyses revealed that the mutants, as compared to the wild-type OsChz1-C is shown in red color and its value is the same to that in Fig. 2e, and the mutants 1M, 2M, and 3M are in blue, orange, and black, respectively. The detailed sequences of these OsChz1 mutants are listed in Supplementary Table 2. Source data underlying d are provided as a Source Data file.

Next, we analyzed the growth and developmental phenotypes of the oschz1-1 and oschz1-2 mutants. Compared to the wild-type plant (WT), both oschz1-1 and oschz1-2 mutant plants displayed similarly late-flowering and dwarf phenotypes (Fig. 4b). In addition, the mutants showed less-well developed panicles (Fig. 4c) but bigger kernels (Fig. 4d) and grains (Fig. 4e). Quantitative analyses revealed that the mutants, as compared to WT, exhibited a reduction of about 9% in plant height (Fig. 4f), a reduction of 22% in panicle length (Fig. 4g), a reduction of 30% in tiller number per plant (Fig. 4h), a reduction of 23% in grain number per panicle (Fig. 4i), and an increase of about 18% in grain width (Fig. 4j). These results indicate that OsChz1 is involved in the regulation of multiple processes of rice growth and development, which is consistent with its broad expression pattern in plants (Fig. 1b–h).

To verify that it is indeed the loss of function of OsChz1 causing the mutants phenotypes, we performed rescue experiments using a construct containing the full-length coding sequence of OsChz1 fused to the 4×MYC-tag-coding sequence, together driven by the OsChz1 native promoter (pOsChz1::OsChz1-4×MYC). This transgene was introduced into both the oschz1-1 and oschz1-2 mutants, and we obtained 20 independent transgenic lines for each complemented line. All stable transgenic lines showed similarly rescued phenotypes, and subsequently, we analyzed in detail one complemented line for each of the mutants, i.e., COM1 for oschz1-1 and COM2 for oschz1-2. Both COM1 and COM2 are phenotypically similar to WT (Supplementary Fig. 9a and b). They have expression levels of OsChz1 similar to that of WT (Supplementary Fig. 9c), and the OsChz1-MYC fusion protein was detected in COM1 and COM2 (Supplementary Fig. 9d). They also display WT-levels of heading date, plant height, tiller number, panicle length, grain number, and grain width (Supplementary Fig. 9e–j). Together, these data confirmed that the loss of function of OsChz1 is responsible for the oschz1 mutant phenotypes.

OsChz1 promotes plant flowering through the Ehd1-Hd3a/RFT1 activation pathway. Flowering represents a key developmental transition during the plant life cycle. To further investigate the flowering phenotype of the oschz1 mutants in detail, we analyzed the heading date of rice plants grown under either natural long-day (LD; at Shanghai in China) or short-day (SD; at Sanya in China) photoperiod conditions. Both the oschz1-1 and oschz1-2 mutants showed a late-flowering phenotype under either LD (Fig. 5a) or SD (Fig. 5b) photoperiods, with significantly delayed heading dates (Fig. 5c), indicating that OsChz1 promotes flowering in a photoperiod-independent manner. To gain insight into gene regulatory network underlying the mutant flowering phenotype, we analyzed the expression levels of OsChz1 and some key rice flowering-regulatory genes at 4 h time-intervals over a total of 20 h. Plants in heading period grown under natural LD

![Fig. 3 Crystal structure showing OsChz1-C binding with the H2A-H2B heterodimer.](image-url)
Interestingly, data underlying Fig. 4 were drastically reduced in oschz1-1 and oschz1-2 mutants. Similarly, the expression of *Early heading date 1* (*Ehd1*), which encodes a B-type response regulator acting as an inducer upstream of *Hd3a/RFT1* [39–41], was also reduced in the mutants. In contrast to *Ehd1*, *Hd3a*, and *RFT1*, the other examined genes showed roughly similar expression levels and patterns in the oschz1-1 and oschz1-2 mutants as compared to WT (Fig. 5d). Based on these results, we conclude that OsChz1 promotes rice flowering time through the *Ehd1-Hd3a/RFT1* pathway.
Loss of OsChz1 affects transcription and nucleosome distribution of the rice genome. To investigate the effects of loss of OsChz1 on gene transcription at genome-wide level, we performed RNA-seq analysis of the oschz1-1 mutant and WT rice plants grown under our laboratory LD photoperiods. The analysis was performed in three biological replicates. We identified the differentially expressed genes in mutant compared to WT by using DEseq2. A total of 1619 genes were detected as significantly (fold change ≥ 1.5 and padj < 0.05) misregulated, with 1619 of them upregulated and 348 of them downregulated (Fig. 6a), in the oschz1-1 mutant as compared to the control WT. Among the downregulated genes in oschz1-1, we detected Ehd1, Hda3a, RFT1, and OsChz1, confirming our RT-qPCR results (Supplementary Fig. 10a). A statistically significant degree of overlap was observed between these misregulated genes and the total protein-coding genes (PCGs) of the rice genome (Fig. 6b). In contrast, such significant overlap was not detected for transposable elements (TEs; Fig. 6b). Gene ontology (GO) analysis indicated that genes involved in response to stress, defense, and DNA replication were significantly over-represented in the upregulated genes of the oschz1-1 mutant, and that genes involved in protein phosphorylation and biotic stimulus were significantly over-represented in the downregulated genes of the oschz1-1 mutant (Supplementary Fig. 10b). The findings of a large
number of misregulated protein-coding genes involved in these different key processes are consistent with the pleiotropic growth and developmental phenotypes of the oschz1 mutants.

To gain insight into the mechanisms underlying misregulation of genome transcription, we performed an MNase digestion assay on chromatin (Supplementary Fig. 10c) and investigated nucleosome distribution in WT and oschz1-1 by MNase-seq analysis. Paired-end sequencing was performed from two independent MNase-seq libraries with two biological replicates of WT and oschz1-1, and then paired-end reads were mapped to unique positions of the rice genome. The nucleosome peaks in WT (Supplementary Fig. 10d) significantly overlapped with the previously published data. The mono-nucleosomal DNA length was roughly 146 bp in size for both WT and oschz1-1 (Fig. 6c).
Detailed mapping at the whole genome level detected in total 25,345 nucleosome-position shifts (12,540 forward shift and 12,805 reverse shift) and 37,608 nucleosome-density alterations (19,673 increased and 17,935 decreased) in oschz1-1 as compared to WT (Supplementary Table 4). The nucleosome shift was detected majorly within intergenic regions (Supplementary Table 4). In contrast, the nucleosome-density reduction occurred primarily within the gene-body region while increased nucleosome density was observed at the promoter and intergenic regions (Fig. 6d, Supplementary Table 4). These alterations of nucleosome distribution are unlikely to be associated with histone levels because histone genes are expressed at similar levels in oschz1-1 as in WT (Supplementary Fig. 10e).

Next, we investigated the nucleosome occupancy at the misregulated genes in oschz1-1 (Fig. 6e). The nucleosome occupancy within the genic regions of upregulated genes was found decreased significantly (Fig. 6f), whereas that of downregulated genes barely changed (Fig. 6g). Similar conclusions were obtained when nucleosome occupancy just adjacent to TSS was analyzed (Supplementary Fig. 11). We further confirmed the correlation of nucleosome-occupancy decrease with upregulated genes by quantitative PCR analysis on several random-selected genes (Supplementary Fig. 12). This association between the decrease of nucleosome occupancy and the upregulation of gene expression is in agreement with the general inhibitory role of nucleosome on transcription, as reported in the previous studies in Arabidopsis, rice, and maize.

In contrast, for downregulated genes, a statistically significant correlation with nucleosome-occupancy changes could not be established, albeit some slight increase of nucleosome occupancy distantly upstream of TSS (Fig. 6g, Supplementary Fig. 11). Examination of the downregulated flowering genes Ehd1, Hd3a, and RFT1 also did not show a significant change of nucleosome occupancy in oschz1-1 (Supplementary Fig. 13).

OsChz1 affects H2A.Z levels within the rice genome. While the alteration of nucleosome distribution observed in oschz1-1 is consistent with the histone-chaperone function of OsChz1, they cannot specify whether or not OsChz1 plays a role in the regulation of chromatin content of histone variant H2A.Z within the rice genome. Therefore, we analyzed genome-wide H2A.Z enrichment in WT and oschz1-1 by ChIP-seq experiments. As compared to WT, in oschz1-1 we detected a total of 5310 decreased H2A.Z peaks (corresponding to 5270 genes), with a consecutive decrease of H2A.Z over the entire gene-body region (Fig. 7a). Examination of all the misregulated genes revealed that both upregulated and downregulated genes exhibit lower levels of H2A.Z in oschz1-1 as compared to WT (Supplementary Fig. 14).

To characterize in more details about the H2A.Z decrease, we categorized genes into nine classes based on H2A.Z levels (low, medium, or high) at TSS and at gene body in all of their different combinations, as described in Coleman-Derr and Zilberman. A decrease of H2A.Z in oschz1-1 compared to WT was found ubiquitous for all of these nine classes of genes (Fig. 7b), indicating that OsChz1 promotes H2A.Z deposition without any specific class-preference of genes marked by different H2A.Z-enrichment patterns.

Next, we analyzed gene length in relation with H2A.Z enrichment. It was found that shorter genes (<2 kb) contain significantly higher levels of H2A.Z than longer genes (>3 kb) within the gene body (Fig. 7c). We further compared gene expression in relation to H2A.Z enrichment. In WT, gene expression levels were found negatively correlated with H2A.Z levels at either TSS or gene body or both (Fig. 7d). This observation is consistent with previous studies together supporting a static repressive effect of H2A.Z on gene transcription. We intersected these classes with genes that lost H2A.Z in oschz1-1 and misregulated, and then plotted their distribution. This revealed that loss of H2A.Z in oschz1-1 tends to occur over genes containing high level of H2A.Z, whereas the upregulated genes tend to belong to gene classes containing medium and high H2A.Z levels over gene bodies (Fig. 7e).

A significant loss of H2A.Z was observed in a subset of genes exhibiting reduced nucleosome occupancy in oschz1-1 (Group 2, Fig. 7f). Meanwhile, a higher number of genes showed separately either a loss of H2A.Z (Group 3, Fig. 7f) or a reduced nucleosome occupancy (Group 1, Fig. 7f). To further investigate H2A.Z enrichment independently from nucleosome occupation changes, we analyzed H2A.Z levels after normalization with the nucleosome density (Group 4, Fig. 7f). The nucleosome occupancy and the upregulation of gene expression is in agreement with the general inhibitory role of nucleosome on transcription, as reported in the previous studies in Arabidopsis, rice, and maize. In contrast, for downregulated genes, a statistically significant correlation with nucleosome-occupancy changes could not be established, albeit some slight increase of nucleosome occupancy distantly upstream of TSS (Fig. 6g, Supplementary Fig. 11). Examination of the downregulated flowering genes Ehd1, Hd3a, and RFT1 also did not show a significant change of nucleosome occupancy in oschz1-1 (Supplementary Fig. 13).

OsChz1 binds chromatin regions containing repressive histone marks. To investigate the localization of OsChz1 in chromatin, we performed ChIP-seq analysis by using the pOsChz1:OsChz1-4×MYC transgenic line (COM1) to detect OsChz1-MYC and using oschz1-1 to eliminate background signal (Fig. 8a). Data analysis with MACS2 allowed us to define 4865 OsChz1-occupied peaks, corresponding to 3099 genes. The OsChz1-occupied peaks showed a preference for promoter and first exon compared to a control set of peaks randomly selected from the genome (Fig. 8b). Consistently, OsChz1 peaks contained higher levels of histone H2A.Z when compared to a random set of
control genes or all protein-coding genes (Fig. 8c). A significant overlap was observed for the OsChz1 binding with genes showing loss of H2A.Z (alone or together with reduced nucleosome occupancy) but not with those genes exhibiting reduced nucleosome occupancy alone (Fig. 8d). It is likely that some nucleosome-occupancy changes may not require a steady binding of OsChz1 on chromatin. We further investigated the preferences of OsChz1-occupied genes for histone modifications recently published data from similar stage of rice plant development. Obviously, OsChz1-occupied genes containing higher levels of H3K27me3 over the whole gene region when compared to a random control set of genes or genes averages, especially in gene body (Fig. 8e). Similarly, OsChz1-occupied genes tend to show higher enrichment of H3K4me3 over the whole gene region when compared to a random control set of genes or gene averages. By contrast, the lower levels of histone modifications H3K36me3 and H3K4me3 were detected in those genes when compared to a random control set.
of genes (Fig. 8e). Altogether, these results indicate that OsChz1 preferentially localizes to the regions that contained high levels of repressive histone marks.

Last, since some previous studies documented anti-correlation between H2A.Z and DNA methylation\(^{16,20,51,52}\), we performed bisulfite sequencing (BS-seq) and generate single-nucleotide resolution maps of cytosine methylation. It was found that the DNA-methylation levels in all of the three nucleotide sequence contexts (CG, CHG, and CHH; with H being A, C, or T) are lower at OsChz1-binding peaks as compared to the control set of peaks over the genome (Fig. 8f), suggesting that OsChz1 binds with a preference for low DNA-methylation regions. The CG, CHG, and CHH methylation in WT was found at 43.8%, 20.3%, and 2.4%, respectively; and that in oschz1-1 was found at 42.6%, 20.1%, and 2.5%, respectively. These comparable levels between WT and oschz1 indicate that the loss of OsChz1 barely
effects the methylation landscape of the rice genome (Supplementary Fig. 15a). To further investigate the relationship between histone H2A.Z and DNA methylation, we plotted the levels of DNA methylation over the 5,270 genes with reduced H2A.Z deposition in oschz1-1. At these regions, the level of methylation in all cytosine contexts was unaffected in OsChz1-occupied genes (not significant). Whether or not OsChz1 is capable of binding to H2A.Z dimers, with a similar or higher affinity for the canonical H2A than for the variant H2A.Z. This differs from yChz1, which was shown to bind preferentially the H2A.Z-H2B dimer over the H2A-H2B dimer28. Two structural domains were reported as responsible for the binding preference of yChz1 to H2A.Z over H2A: a middle region, embracing the CHZ-motif, binds the highly conserved H2A.Z-specific residues Gly98 and Ala57 and dictates a modest preference for H2A.Z-H2B; and the C-terminal region harboring the DEF/Y motif engages an arginine finger and a hydrophobic pocket in H2A.Z-H2B and enhances majorly the binding preference for H2A.Z-H2B35. Both the sequence involved in H2A.Z Gly98-binding and the DEF/Y motif of yChz1 are poorly conserved in CHZ-domain proteins from higher eukaryotes (Supplementary Fig. 5).

Our crystal structure analysis of the OsChz1-H2A-H2B complex showed that OsChz1 binds H2A-H2B via an acidic binding surface. Mutagenesis unraveled that the OsChz1 residues Glu432 + Glu433, Glu436 + Glu 438 + Asn439 + Asp440, or Asn444 + Val445 + Glu446 all are functionally involved in the OsChz1 binding with H2A.Z-H2B as well as with H2A.Z-H2B. In vitro nucleosome assembly assay also indicated that OsChz1 could enhance the assembly of both H2A.Z-H2B and H2A.Z-H2B with a (H3–H4)2-DNA tetrasome. The H2A residues Tyr59, and Leu67 involved in binding with OsChz1 in the OsChz1-H2A.Z-H2B complex are conserved in H2A.Z, likely providing an explanation for OsChz1 in chaperoning both H2A.Z-H2B and H2A.Z-H2B. These residues are also conserved in the variant H2A.W and H2A.X (Supplementary Fig. 1b), which marks constitutive heterochromatin and can be phosphorylated upon DNA damage, respectively. Whether or not OsChz1 binds to H2A.W and H2A.X remains unknown. Moreover, the N-terminus-longer OsChz1-C showed higher binding-affinity to H2A.Z-H2B over H2A.Z-H2B, but both the molecular basis and the functional significance of this preference remains uncertain. Last, the OsChz1-M fragment also displayed binding activity to H2A.Z-H2B and H2A.Z-H2B, raising the question of whether one OsChz1 molecule binds two molecules of H2A.Z-H2B/H2A.Z-H2B. Future investigation about these different issues will extend our knowledge on the function and molecular mechanisms of OsChz1 in histone chaperoning.

At nucleosome level, the OsChz1-H2A.Z-H2B complex structure predicts that the binding is located at the disk face of the nucleosome where the C-terminus of OsChz1 prolongs into the acidic pocket of nucleosome and the N-terminus counteracts with the C-terminus α2 helix of histone H4. This differs from the NAP1 histone–chaperone that shields the DNA-binding surface of H2A.Z. Analyses of the yNAP1–H2A.Z-H2B complex structure showed that a yNAP1 homodimer forms an acidic binding surface and engages two copies of H2A.Z34 or a single H2A.Z-H2B heterodimer. The yNAP1–H2A.Z-H2B inhibitory conformation was proposed to serve in avoiding inappropriate binding of H2A.Z-H2B with DNA and/or in facilitating H2A.H2B transport and storage in the cell. OsChz1 is localized exclusively to the nucleus, which is in agreement with the idea that Chz1 acts primarily in the nucleus in nucleosome assembly and/or in the replacement of H2A.Z-H2B by H2A.Z-H2B from the nucleosome. The fact that deprivation of Chz1 caused some specific mutant phenotypes in both yeast28 and rice (this study) further underlined some distinct biological functions of Chz1 versus NAP1. Distinct from the sole OsChz1, NAP1 is represented by a family of 5–6 members in rice or Arabidopsis. Interestingly, a recent study showed that the Arabidopsis NRPL1 and NRPL2 negatively regulate H2A.Z abundance in chromatin and their loss causes over-accumulation of H2A.Z genome-wide, especially at heterochromatin regions normally depleted for H2A.Z in wild-type plants. In parallel to specific functions, it is worth to note that redundant functions also exist between Chz1 and NAP1. Analyses of the yeast mutants revealed that yChz1 and yNAP1 can reciprocally substitute for the binding to H2A.Z-H2B and simultaneously lose of both yChz1 and yNAP1 aggravates defects in H2A.Z deposition onto nucleosomes.

In rice, loss of OsChz1 caused genome-wide modifications of nucleosome occupancy, including nucleosome-position shifts and local nucleosome-density alterations. It was found that genes with decreased nucleosome occupancy are upregulated and vice versa genes with increased nucleosome occupancy are downregulated in expression in the oschz1 mutant. This result is consistent with the previous knowledge that nucleosome compaction is inhibitory to DNA access by transcription factors. In spite of a diminished binding preference of OsChz1-C for H2A.Z-H2B in vitro, decreased levels of H2A.Z were majorly detected within gene-body chromatin in oschz1-1. Thus, similar to yChz1, OsChz1 also plays a role in H2A.Z deposition into chromatin. H2A.Z incorporation into chromatin is catalyzed by SWR1-c, and coordinatively yChz1 acts in delivering H2A.Z to SWR1-c. Components of SWR1-c are conserved in eukaryotes and studies of some SWR1-c components in Arabidopsis mutants have also demonstrated their roles in H2A.Z incorporation into chromatin. Future studies will be necessary to investigate precise roles of Chz1 and
SWR1-c during H2A.Z deposition in plants as well as in other higher eukaryotes. A genome-wide anti-correlation between DNA methylation and H2A.Z was reported previously in Arabidopsis16 and rice20 as well as animals52,57. In Arabidopsis, against this general anti-correlation rule, some specific chromatin regions are characterized by co-existence of DNA methylation and H2A.Z. Nonetheless, in some of these specific regions, H2A.Z interacts with the DNA-demethylase ROS1 to prevent hypermethylation of DNA34. In contrast to the prediction by the anti-correlation rule, the decreased level of H2A.Z was observed without any detectable increase of DNA methylation in the rice oschz1 mutant (this study). Also, loss of H2A.Z in Arabidopsis has only a minor effect on DNA methylation20, and the wrp-1–wrp-2 mutant displays H2A.Z accumulation but largely unaltered DNA methylation or even slight increases of CG/CHG methylation at some TEs34. H2A.Z accumulation but largely unaltered DNA methylation or even slight increases of CG/CHG methylation at some TEs34. H2A.Z accumulation but largely unaltered DNA methylation or even slight increases of CG/CHG methylation at some TEs34. H2A.Z accumulation but largely unaltered DNA methylation or even slight increases of CG/CHG methylation at some TEs34. H2A.Z accumulation but largely unaltered DNA methylation or even slight increases of CG/CHG methylation at some TEs34. H2A.Z accumulation but largely unaltered DNA methylation or even slight increases of CG/CHG methylation at some TEs34.

The anti-correlation between DNA methylation and H2A.Z is primarily caused by the exclusion of H2A.Z from methylated DNA20. In our study, we detected OsChz1 binding with a preference to chromatin regions enriched by the repressive histone marks H3K27me3 and H3K4me2. H2A.Z and H3K27me3 are functionally linked and both are involved in transcription repression58. While H3K4me3 marks active transcription, H3K4me2 is also associated with transcription repression in rice and in Arabidopsis49. Consistent with the association of OsChz1 with these repressive histone marks, loss of OsChz1 caused massive upregulation of genes in the oschz1 mutant. Many of the misregulated genes in oschz1 are predicted as functionally involved in response to diverse stimuli. This is in line with H2A.Z as a major player in plant response to phytohormones and environmental cues22,29. Plant flowering time is tightly controlled by complex gene networks that integrate endogenous and environmental cues39,40. Loss of OsChz1 caused downregulation of the rice floregins Hd3a and RFT1 as well as their upstream activator Ehd1. Nucleosome density and distribution were found not significantly changed at chromatin of these flowering genes. Slight increase of H2A.Z was noticed at 5′-end of Ehd1 and 3′-end of RFT1 (Supplementary Fig. 13), which is in line with the repressive role of H2A.Z on transcription but is in contrast to the global function of OsChz1 in H2A.Z deposition. So far, it is unclear whether OsChz1 has regulated the expression of these flowering genes directly or indirectly. Transcription of Hd3a/RFT1/Ehd1 is regulated, positively or negatively, by several histone modifications39,61,62. As a histone–chaperone, OsChz1 may affect these modifications. While its precise function remains to be determined, the human and vertebrate CHZ-domain protein HIRIP3 had been identified as an interacting protein of the H3.3-chaperone HIRA and had been shown to also bind H3 and H2B in vitro63. In the same line of assumptions, sequences in addition to CHZ-domain (e.g., OsChz1-M) may also contribute to OsChz1 binding with histones and/or with other regulatory proteins. Last, in addition to flowering time, several other agronomic traits (e.g., plant architecture, reproductive capacity, and grain size) are affected by loss of OsChz1. Molecular mechanisms underlying these effects are still obscure. Future studies will be necessary to address these different issues, which will undoubtedly shed further light on molecular mechanisms of CHZ-domain proteins in multilayer regulations of chromatin remodeling, gene transcription, and organism growth and development.

Methods
Phylogenetic analysis. The full-length amino acid sequences of CHZ-domain proteins were used for the phylogenetic tree analysis. Homology sequence searches were carried out in the database of UniProt (https://www.uniprot.org/). The phylogenetic analysis was conducted by using MEGA version 7.0.26 with bootstrap setting at 1000 replicates and illustrated by using FigTree version 1.4.3. The conserved protein domains were identified by conducting searches in NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

RT-PCR. Total RNA was extracted from different tissues of rice (Oryza sativa ssp. japonica cv. Nipponbare) using an RNAprep pure Plant Kit (Tiangen Biotech, Beijing, China) and RNA was reverse-transcribed using PrimeScriptTM1. RT reagent kit (TaKaRa, RR047A). qRT-PCR reactions were carried out on CFX Connect™ Real-Time System (Bio-Rad). Primers specific to the genes examined are listed in Supplementary Table 5. Each group data comprised three biological replicates and Ubiquitin5 was used as a reference gene to normalize the gene expression data.

Pulldown assay. Full-length and truncated versions of OsChz1 were PCR amplified and cloned into the pGEX-6P-1 (GE Healthcare, Milwaukee, WI, USA). The PCR primers are listed in Supplementary Table 5. Recombinant GST-fusion proteins were produced in Escherichia coli BL21 (DE3) and purified54. The Arabidopsis histone H2A (HTA1; AT5G54640) and H2B (HTB1; AT1G07790) dimer was obtained from a previous study27, and the co-expressed H2A.Z (HTA9; AT1G74400) and H2B (HTB1) dimers produced similarly. Briefly, bacteria cells expressing the histone dimers were disrupted by high-pressure cell disruptor in the lysis buffer containing 20 mM Tris-HCl pH 8.0 and 250 mM NaCl. After centrifugation at 34,000 × g for 60 min, the supernatant was loaded onto the SP column (GE Healthcare), and eluted by running a linear gradient of 10 column volumes of the elution buffer containing 20 mM Tris-HCl pH 8.0 and 1 M NaCl. Frctions were collected, concentrated, and then loaded into the pre-equilibrated column (Superose64). Increase 200 10/300, GE Healthcare) with the buffer containing 20 mM Tris-HCl pH 8.0 and 1 M NaCl. For the GST pulldown assay, beads (GE Healthcare) were used as a reference. The full-length DNA was added to 100 μl protoplast suspension and then mixed with 40% PEG buffer (40% (W/V) PEG4000, 0.4 M mannitol, 100 mM CaCl2, pH 5.7) for 20 min. After adding W5 buffer to dilute the mixture and removing the supernatant, the protoplasts were incubated with DAPI staining. The protoplasts were observed under a confocal microscope.

Protein expression in protoplasts and Co-IP assay. Protein isolation and transformation were performed using 10-day-old rice leaf sheaths and stems grown at ~28 °C in the dark, based on established procedure65. The full-length cDNA of rice H2A (HTA702), H2A.Z (HTA713), and OsChz1 were amplified and cloned into the pRTVeHA, pRTVeMYC, and pRTVgFP vectors95, respectively. The leaf sheaths and stems were cut into 0.5-mm strips and incubated in enzyme solution (1.5% cellulose RS, 0.3% macerozyme, 0.4 M mannitol, 2 mM MES, pH 5.7) for 4 h in the dark. After removing the tissues, the mesophyll protoplasts were collected and suspended in the suspension medium (0.4 M mannitol, 20 mM CaCl2, 5 mM MES, pH 5.7) for 1 h to release the protoplasts. After flow through filter, the protoplasts were collected and resuspended in the suspension medium (0.4 M mannitol, 20 mM CaCl2, 5 mM MES, pH 5.7). For transformation, 10 μl plasmid DNA was added to 100 μl protoplast suspension and then mixed with 40% (W/V) PEG buffer (40% (W/V) PEG4000, 0.4 M mannitol, 100 mM CaCl2, pH 5.7) for 20 min. After adding W5 buffer to dilute the mixture and removing the supernatant, the protoplasts were incubated 12–16 h in W5 buffer for protein expression. Expression of GFP-OsChz1 in the protoplasts was detected by fluorescence and imaged under a confocal microscope. For Co-IP, GFP-OsChz1 was co-expressed together with HTA702-HA or HTA713-MYC in rice protoplasts. The protoplasts were harvested in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 2 mM DTT, protease inhibitor cocktail) and sonicated at 4 °C for five times, 20 s each. Total protoplast extract was immunoprecipitated by anti-GFP antibody (M20004, Abmart, 1:100 dilution) in combination with pre-cleared magnetic protein A beads (10002D, Invitrogen) at 4 °C for 2 h. The immunoprecipitates were washed two or
three times with the buffer containing 2 mM KH2PO4, 10 mM Na2HPO4, 140 mM NaCl, 2.7 mM KCl, 0.05% (w/v) Tween 20, and analyzed by western immunoblot using a monoclonal antibody (Abcam) at a 1:1000 dilution and incubation (1:5000 dilution). Signals were detected using Chemiluminescence Imaging System (ChemiScope 3600 Mini, CIINX).

**ITC and protein crystal structure determination.** DNA fragments encoding 6xHis-SUMO-tagged wild-type or mutated OsChz1-C were cloned into the pET28a vector (Novagen). Primers used in cloning are listed in Supplementary Table S3. For octamer formation, one molar of the H3-H4 tetramer was truncated to a tetramer using the Qiagen High-fidelity PCR kit, and the supernatant was dialyzed against 0.2 M sodium citrate, 0.1 M HEPES pH 7.0, 1 mM EDTA, the immunoprecipitation complex was eluted from the beads twice with 10 mM EDTA pH 8.0, 1 M NaCl, 1 M EDTA, 1 mM β-mercaptoethanol, and proteinase K (Roche) under shaking for 30 min at 4°C. Theocabaculum tumefaciens EHA105. The Agrobacterium strain was used to transform WT and oschz1-1 through callus inoculation, plant regeneration, and selection.29 Next-generation plants were obtained through self-pollination of parents, and plant genotypes were verified through genomic PCR analysis.

Rice plants were grown in the paddies under natural conditions at two locations characterized by different latitudes: Shanghai under LD conditions and Sanya under SD conditions. Plant flowering time was recorded only in the first panicle growth stage, after the flag leaf had emerged, and the panicle traits were measured during maturation stage. Each measurement of agronomic characteristics was performed based on an average of 20 individual plants per sample.

**Genome-wide profiling analyses.** Thirty-day-old rice seedlings were grown in artificial growth chambers under an LD photoperiod (14 h light at 30 °C and 10 h dark at 20 °C) for 2 weeks before ChIP-seq, MNase-seq, and RNA-seq analyses. For RNA-seq, total RNA was extracted from over 5 seedlings using an RNeasy plant kit (QIAGEN), followed by de-proteinization (0.02 M EDTA, 0.1 M Tris-HCl pH 6.5, 1 mM iodoacetamide) and Illumina fragmentation (http://illumina.com). The suspension was then filtered through a Miracloth (Millipore), and nuclei were collected through centrifugation at 3000 × g for 20 min at 4°C. The pellets were washed twice and resuspended in the MNase buffer (50 mM Tris-HCl pH 7.5, 25 mM MgCl2, 1 mM CaCl2). Chromatin was digested with 2 units of micrococcal nuclease (Sigma) for 10 min at 37°C. The reaction was stopped with 10 mM formaldehyde, under vacuum for 15 min. Cross-linking was stopped by incubation in 0.125 M glycine under vacuum treatment for 10 min. About 1 g powdered leaf material was resuspended in Nuclear Extraction Buffer (50 mM HEPES pH 7.5, 1 mM EDTA pH 8.0, 150 mM NaCl, 1 mM β-mercaptoethanol, and proteinase K (Roche)) under shaking for 30 min at 4°C. The suspension was then filtered through a Miracloth (Millipore), and nuclei were collected through centrifugation at 3000 × g for 20 min at 4°C. The pellets were washed twice and resuspended in the MNase buffer (50 mM Tris-HCl pH 7.5, 25 mM MgCl2, 1 mM CaCl2). Chromatin was digested with 2 units of micrococcal nuclease (Sigma) for 10 min at 37°C. The reaction was stopped with 10 mM formaldehyde, under vacuum for 15 min. Chromatin was washed twice with 1 M NaCl, 1 M EDTA, and sequencing adaptors were added, and then the adapter- 150 bp genomic DNA was amplified using PCR to enrich the segments to obtain the cDNA library. There were three replicates for each genotype. The library was sequenced on an Illumina HiSeq3000 instrument via the custom service of GEN-ERGY BIO (Shanghai, China).

**Nucleosome reconstitution and histone deposition assay.** The 187 bp DNA template containing a single Wdnon 601 sequence was prepared by PCR from plasmid using an unlabeled primer (Supplementary Table S5). The histone octamers, H3-H4 tetramers, H2A-H2B, and H2AZ-H2B dimers were reconstituted based on established procedure.62 Briefly, DNA fragments encoding H3 and H4 were cloned into the pETDuet-1 vector (Novagen) for co-expression. The co-expressed H3-H4 tetramer was purified successively by S column (GE Healthcare), butyl column (GE Healthcare), and size-exclusion chromatography (Superdex 200 16/600, GE Healthcare). For octamer formation, one molar of the H3-H4 tetramer was mixed with two molars of the H2A-H2B dimer was incubated in the refolding buffer containing 2 M NaCl, 10 mM Tris-HCl at pH 7.5, 1 mM EDTA, and 5 mM 2-mercaptoethanol. The reaction octamer was purified through Superdex 200 16/600 (GE Healthcare). The reconstitution reaction mixture with the histone octamers/ tetramers was used for the immunoprecipitation assay using anti-MYC (M20002L, Abcam) at a 1:300 dilution. After gradual washes using the low-salt buffer (50 mM HEPES pH 7.5, 5 mM EDTA pH 8.0, 150 mM NaCl, 1 mM β-mercaptoethanol, and proteinase K (Roche)) under shaking for 30 min at 4°C. The nucleosome reconstitution and histone deposition assay were performed following the manufacturer’s instructions of VAHTSTM Universal DNA Library Prep Kit (Vazyme). Two replicates for each genotype were obtained and sequenced.

For ChiP-seq, about 5 g rice seedlings were harvested and fixed in the buffer containing 4 M sucrose, 2 mM formaldehyde, under vacuum for 15 min. Cross-linking was stopped by incubation in 0.125 M glycine under vacuum treatment for 10 min. About 1 g powdered leaf material was resuspended in Nuclear Extraction Buffer (50 mM HEPES pH 7.5, 1 mM EDTA pH 8.0, 150 mM NaCl, 1 mM β-mercaptoethanol, and proteinase K (Roche)) under shaking for 30 min at 4°C. The suspension was then filtered through a Miracloth (Millipore), and nuclei were collected through centrifugation at 3000 × g for 20 min at 4°C. The pellets were washed twice and resuspended in the MNase buffer (50 mM Tris-HCl pH 7.5, 25 mM MgCl2, 1 mM CaCl2). Chromatin was digested with 2 units of micrococcal nuclease (Sigma) for 10 min at 37°C. The reaction was stopped with 10 mM formaldehyde, under vacuum for 15 min. Chromatin was washed twice with 1 M NaCl, 1 M EDTA, and sequencing adaptors were added, and then the adapter- 150 bp genomic DNA was amplified using PCR to enrich the segments to obtain the cDNA library. There were three replicates for each genotype. The library was sequenced on an Illumina HiSeq3000 instrument via the custom service of GEN-ERGY BIO (Shanghai, China).

For BS-seq, total genomic DNA was prepared using the DNeasy plant maxi kit (QIAGEN). Sodium bisulfite treatment, library construction, and sequencing were performed via the custom service of GENERGY BIO (Shanghai, China). Two replicates for each genotype were obtained and sequenced.

**Seq-data analyses.** FastQC v.0.11.7 was used to check read quality and size. Raw reads were trimmed to remove bases with low quality score and from adapters with following filtering short reads by OUTADAPT v.1.10.31. Then, the cleaned reads were aligned to the reference genome of ‘Nipponbare’ (japonica) rice (MSU7; https://rice.plantbiology.msu.edu). A total of 10 million reads for each sample were then extracted for genome library construction following the manufacturer’s instructions of VAHTSTM Universal DNA Library Prep Kit (Vazyme). Two replicates for each genotype were obtained and sequenced.

For BS-seq, total genomic DNA was prepared using the DNeasy plant maxi kit (QIAGEN). Sodium bisulfite treatment, library construction, and sequencing were performed via the custom service of GENERGY BIO (Shanghai, China). Two replicates for each genotype were obtained and sequenced.
to obtain unique reads. FeatureCounts v1.6.2 was used to assign aligned reads to genes. Differentially expressed genes (DEGs) were identified by the DESeq2 v1.22.24 based on the combined thresholds set as fold change ≥2.5 and p-value (adjusted p-value) ≤0.05. Genome ontology analysis of DEGs was conducted in CARMO online (http://bioinfo.sibs.ac.cn/carmo/Gene_Annotation.php) and significantly enriched biological functions were identified as a p-value ≤0.05.

For RNA-seq and ChIP-seq, cleaned paired reads were mapped to the reference genome by using Bowtie2 v2.3.4.16. Samtools v1.9 was used to remove reads with low mapping quality (Q < 20) and potential PCR duplicates. The normalized reads (RPKM) for profiles were calculated with DeepTools v3.0.27. Enrichments along the gene body and around TSS and TTS were examined using the Heatmap tool in DeepTools. Average density profile was plotted in R and a heatmap was generated using the plotHeatmap tool in DeepTools. Bed format files were generated using BED Tools v2.25.0. DANPOS v2.1.3 was used to position methylation regions (DMRs). The methylation levels for each sample were generated using BED Tools v2.25.0. The peaks were annotated to features by using ChIPseeker v1.18.0. For BS-seq, Bismark v0.22.1 was used to map trimmed paired-end reads to the rice reference genome and to extract methylated regions (DMRs). The methylation levels for profiles and distribution were conducted by using ViewBS v0.19.5.

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