Dissecting the roles of the histone chaperones reveals the evolutionary conserved mechanism of transcription-coupled deposition of H3.3

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ABSTRACT

The mammalian genome encodes multiple variants of histone H3 including H3.1/H3.2 and H3.3. In contrast to H3.1/H3.2, H3.3 is enriched in the actively transcribed euchromatin and the telomeric heterochromatins. However, the mechanism for H3.3 to incorporate into the different domains of chromatin is not known. Here, taking the advantage of well-defined transcription analysis system of yeast, we attempted to understand the molecular mechanism of selective deposition of human H3.3 into actively transcribed genes. We show that there are systemic H3 substrate-selection mechanisms operating even in yeasts, which encode a single type of H3. Yeast HIR complex mediated H3-specific recognition specificity for deposition of H3.3 in the transcribed genes. A critical component of this process was the H3 A-IG code composed of amino acids 87, 89 and 90. The preference toward H3.3 was completely lost when HIR subunits were absent and partially suppressed by human HIRA. Asf1 allows the influx of H3, regardless of H3 type. We propose that H3.3 is introduced into the active euchromatin by targeting the recycling pathway that is mediated by HIRA (or HIR), and this H3-selection mechanism is highly conserved through the evolution. These results also uncover an unexpected role of RI chaperones in evolution of variant H3s.

INTRODUCTION

Eukaryotic genomes are packed in chromatin as nucleosomes. Each nucleosome contains a histone octamer, composed of two copies of each H2A, H2B, H3 and H4 (1). In mammals, histone H3 exists as canonical or replacement types, whose incorporation into chromatin is diverged into the replication coupled (RC) and replication independent (RI) pathways (2). The canonical H3.1/H3.2, predominantly expressed during the S phase, is largely incorporated at the site of DNA replication via the RC pathway, which is mediated by the histone H3/H4 chaperone chromatin assembly factor 1 (CAF1) complex. In contrast, the replacement histone H3.3 is constitutively expressed in all phases of the cell cycle and incorporated via the RI pathway by the histone H3/H4 chaperones histone cell cycle regulation defective homolog A (HIRA) and death-domain associated protein/α-thalassaemia mental retardation syndrome X-linked (DAXX/ATRX) complexes (3,4). Asf1a and Asf1b, the two human isoforms of *Saccharomyces cerevisiae* H3/H4 chaperone Asf1, are supposed to be involved in both the RC and RI pathways (2–4).

H3.3, which differs by only five and four amino acid from H3.1 and H3.2 respectively, is known to be predominantly linked to the active euchromatic region where RNA polymerase II and transcriptionally active histone-modification marks (H3 K9/K14 acetylation and K4/K36/K79 methylation) are highly abundant (5–7). In addition, H3.3 is enriched at transcriptional regulatory elements and heterochromatic regions such as pericentric heterochromatin and telomeres. Telomere

deposition is mediated by histone chaperone DAXX and ATRX, while deposition at promoters and in the gene bodies of active genes is mediated by HIRA (2,3,8). In mammals, nucleosome assembly in the actively transcribed region is highly H3.3 specific and pathway determined. H3.3 is not deposited simply because it represents the available source of histones. However, the mechanisms explaining how H3.3 is linked to the RI pathway or how it is selectively channeled to the transcribed sites are not known.

The human HIRA forms a stable complex with UBN1 (Ubinuclein 1) and Cabin1 and interacts with ASF1a to be orthologous to yeast histone regulation (HIR) complex (composed of Hir1, Hir2, Hpc2 and Hir3 proteins) (4,9). Yeast Hir1 and Hir2 are the homologs of human HIRA, whereas Hpc2 and Hir3 are the homologs of UBN1 and Cabin1, respectively (10,11). The role of Asf1 and HIR in transcriptional regulation has been well documented in yeast. Asf1 transcriptionally activates *PHO* genes where Asf1-dependent disassembly of the nucleosomes from the *PHO* promoters is essential (12). HIR genetically interacts with the H2A/H2B chaperone facilitates chromatin transcription (FACT) complex, which functions in transcription (13). A functional link between Asf1/HIR and Set2-dependent H3 K36 methylation also implies that Asf1 and HIR are important for chromatin regulation during transcription (14–16). However, their functions linked to transcription all rely on histone-chaperoning activity that mediates eviction and deposition of histones but not histone types, as yeasts express only a single type of H3 histone. In this study, to investigate how H3.3 is specifically incorporated into the actively transcribed region, we took the advantage of yeast systems expressing either H3.1 or H3.3 as a source of new histones for transcription-coupled deposition. Our results provide evidence that H3.3 selection mechanism exists in yeast and is highly conserved through evolution. Distinct contribution of HIRA and Asf1 is essential for preferential enrichment of H3.3 during transcription.

MATERIALS AND METHODS

All yeast strains used in this study were derived from yeast strain YC73 (*MAT*a *ura3-1 leu2-3,112 trp1-1 his3-1,15 ade2-1 can1-100 TRP1::P_{GAL1}-YLR454w*) (16). Genotypes of yeast strains are listed in Supplementary Table S1. Gene deletion or replacement was performed via one-step PCR-mediated recombination using the *KanMX4* or *tc-URA3-tc* cassette. All pRS425- or YEp352GAPII-based vectors were constructed by restriction enzyme digestion or PCR. All plasmids and primers used in this study are listed in Supplementary Table S2 and S3, respectively. Experimental procedures of strain construction, plasmid construction, growth conditions, RNA isolation, reverse transcription, chromatin immunoprecipitation, real-time quantitative PCR, immunoblotting, Co-immunoprecipitation and PCR-mediated mutagenesis are described in Supplementary Materials and Methods.

RESULTS

H3.3, but not H3.1, is preferentially enriched in the actively transcribed regions in yeast

H3.3 in higher eukaryotes is the choice of selective deposition in the promoter and coding regions of transcriptionally active genes. To gain insight into the mechanism(s) of H3.3 enrichment in the transcribed region, we developed an assay that can be monitored in yeast system by modifying previous study (16). HA-tagged human H3.1 or H3.3 is expressed as a source of external histones for transcription-coupled deposition within a cell under the control of the constitutive yeast *TFA1* promoter. The yeast culture was passed sequentially through raffinose, galactose and glucose medium to transiently activate the target genes (p*GAL1-YLR454* and endogenous *GAL* genes) (Supplementary Figure S1A). The p*GAL1-YLR454* system contains *GAL1* promoter directly linked to the coding region of *YLR454* (~8 kb gene). We confirmed that the expression pattern or the level of target mRNAs was not disturbed by exogenous H3s during galactose induction (Supplementary Figure S1B). H3 deposited during transcription was detected once the genes were turned off in the glucose medium and shown as the relative value to that of raffinose medium. Deposition of new histones onto the transcribed region can be analyzed by chromatin immunoprecipitation (ChIP) using an anti-HA antibody. While the genes were activated, the α-factor was present to arrest cells in the G1 phase to exclude the possibility of incorporation of hH3.1 or hH3.3 through RC pathway. HA-tagged hH3.1 and hH3.3 were expressed at similar levels in the yeast cells (Figure 1A). However, surprisingly, the levels of each H3 incorporated into the chromatin via transcription were strikingly different in yeast, as reported previously in higher eukaryotic systems (17). Human H3.3 was efficiently incorporated into the transcribed region of the p*GAL1-YLR454* or *GAL1* genes, while hH3.1 was almost completely excluded from the nucleosome assembly, for which yH3 was preferred over hH3.1 (Figure 1B and C). The level of total H3 before and after transcriptional induction was maintained, implying that a part of preexisting yH3 was replaced by hH3.3 (Supplementary Figure S1C). Incorporation of hH3.3 was specific and coupled to transcription because the flanking regions of p*GAL1-YLR454* did not show any changes in hH3.3 occupancy (Figure 1C, left panel). Our data show that hH3.3 is selected through transcription-coupled RI pathway in yeast system despite yeast genome encoding a single type of H3 and indicate that a common and ancient mechanism exists in yeast and operates across species. Interestingly, the occupancy level of hH3.3 was always greater than that of yH3, indicating that hH3.3 was more efficient to incorporate and to replace old yH3 via transcription (Figure 1D and E).

Amino acids in the ID region determine the incorporation efficiency of H3

H3.1 and H3.3 differ in amino acid sequences at five residues (31, 87, 89, 90 and 96). Three residues (87, 89
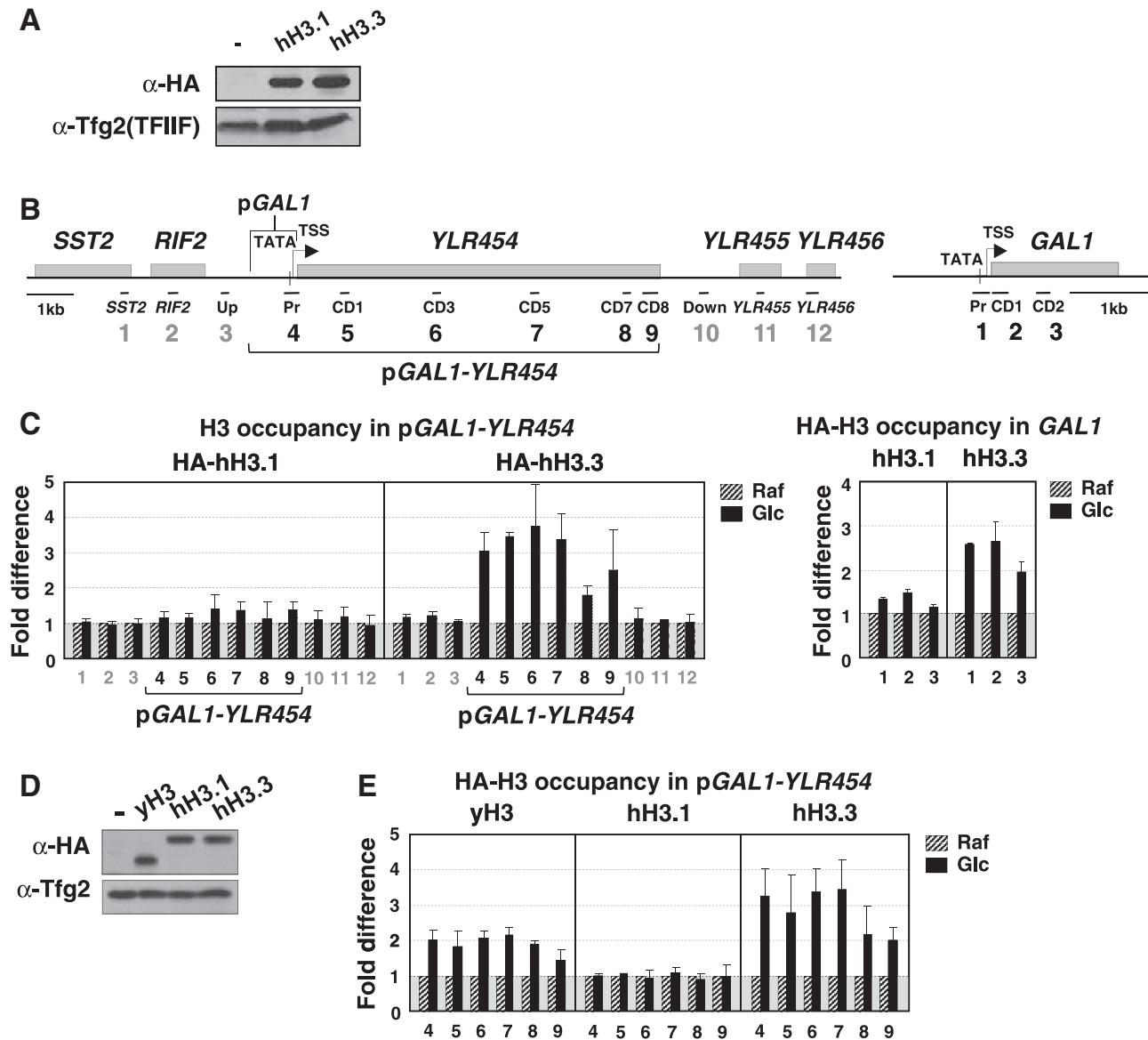
Figure 1. Histone H3.3 is enriched in the actively transcribed region. (**A**) Human H3s (hH3.1 and hH3.3) with a N-terminal 3HA tag were expressed in the wild-type yeast strain (YC73) at similar levels. Immunoblotting analysis was performed with the whole-cell extract prepared from yeasts that harbor the pRS425-p*TFA1*-HA-hH3.1 or hH3.3. Tfg2, a subunit of transcription factor IIF was used as a loading control. (**B**) The schematic diagram of the *GAL1* promoter-linked *YLR454* gene and *GAL1* with the PCR primer pairs used for ChIP. The TATA box represents the promoter and the transcription start site (TSS) is indicated by an arrow. (**C**) Human H3.3 was preferentially incorporated in the actively transcribed region. The target gene was induced by shifting the culture from raffinose- to galactose-containing medium and then subsequently repressed by addition of glucose. ChIP was performed with the 12CA5 antibody using chromatin solution prepared from the raffinose and glucose culture. Each signal was quantified with real-time quantitative PCR and normalized to the intergenic control and the input DNA. The ChIP value obtained in raffinose was arbitrarily set to 1. Raf indicates the HA-H3 level before induction of target genes, while Glc indicates HA-H3 level accumulated through transcription. The data were reported as the mean ± SD from at least three independent experiments. (**D**) Yeast H3 (yH3), human H3.1 and hH3.3 with an N-terminal 3HA tag were expressed at similar levels. (**E**) Comparison of incorporation efficiency of different HA-H3s.

and 90), clustered on the α-helix 2 of the histone core fold domain, are critical for specifying the destination of different H3 variants into the RC or RI pathways (called 'ID' region for Isoform Determinants) (Figure 2A) (5,18). Yeast H3 differs from hH3.3 at 13 residues including S(87) within ID region (Supplementary Figure S2A).

To determine how amino acid differences in the ID region influence H3 selectivity, we tested a set of H3 ID mutants for their abilities to incorporate into chromatin

via transcription. Interestingly, we found that a single amino acid substitution of A(87) to S [hH3.3(S)] or substitution of both I(89) and G(90) to V and M [hH3.3(VM)] in the hH3.3 backbone decreased their deposition to a level similar to that of yH3. In fact, hH3.3(S) corresponds to yH3 in terms of the ID code. Importantly, the incorporation efficiency was predominantly determined by three residues regardless of 12 different residues outside of ID region. A triple substitution [hH3.3(SVM)], which renders
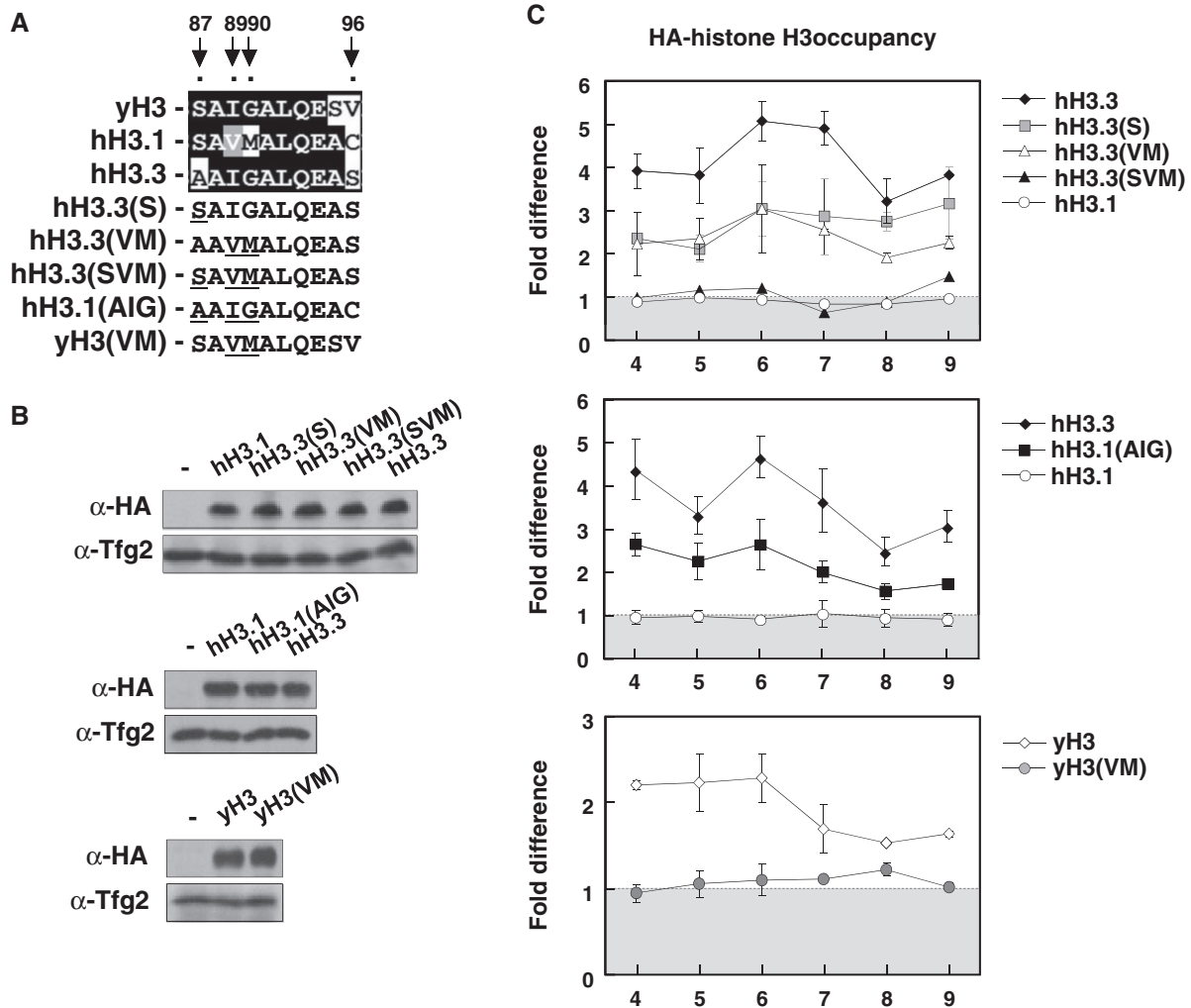
Figure 2. Amino acid residues in the ID region are important for variant-specific H3 deposition. (**A**) The amino acid alignment showing sequence difference in ID region among yH3, hH3.1 and hH3.3. H3 point mutants used in figure (**C**) and S2*B-D* are shown in parallel. (**B**) The expression levels of the H3s under the *TFA1* promoter were similar. (**C**) Incorporation of HA-tagged H3s was analyzed by ChIP. Data points of the HA-H3 occupancy obtained from the glucose sample were plotted.

it to hH3.1, completely abolished incorporating ability even though all three mutants were expressed at similar levels (Figure 2B and C, top panel and Supplementary Figure S2B). This suggests that the three residues in the ID region cooperatively create a synergetic effect on selection of H3 for nucleosome assembly during transcription. The histone deposition system in yeasts was more compatible with A-IG explaining why hH3.3 was more efficient in nucleosome incorporation than yH3 (Figure 1E). Reversely, when corresponding residues of hH3.1 were changed into H3.3-like residues [hH3.1(AIG)], the incorporation efficiency was increased (Figure 2C, middle panel and Supplementary Figure S2C), but not to the level of hH3.3, implying that C(96) may have an adverse effect on RI pathway-mediated deposition. Furthermore, the substitution of two H3.3-like residues of yeast H3 (89 and 90) with those of hH3.1, yH3(VM), resulted in lack of incorporation (Figure 2C, bottom panel and Supplementary Figure S2D). Taken together, our data show that deposition efficiency of H3 variants was solely determined by positive selection through decoding

the amino acid sequences in the ID region. In this regard, H3.3 with A-IG was a more preferred substrate than yH3 (S-IG) for transcription-coupled nucleosome assembly. Yeast H3 could be a reconciled form with both RI and RC traits.

The whole complex of yeast HIR proteins is required for selective deposition of hH3.3

Next, we investigated potential factors involved in H3 choice. Previously, we demonstrated that Asf1 and HIR complex are responsible for disassembly and reassembly of nucleosomes by taking histones from a histone pool (new histones) or recycling chromatin histones (old histones) (16). In higher eukaryotes, HIRA is known to support the H3.3 incorporation (3,19,20). We thus tested the potential contribution of the HIR complex (composed of Hir1, Hir2, Hir3 and Hpc2) to histone variant selection. To do this, the occupancy of hH3.1 or hH3.3 was analyzed by ChIP in the absence of components of the HIR complex. hH3.1 and hH3.3 were expressed at similar
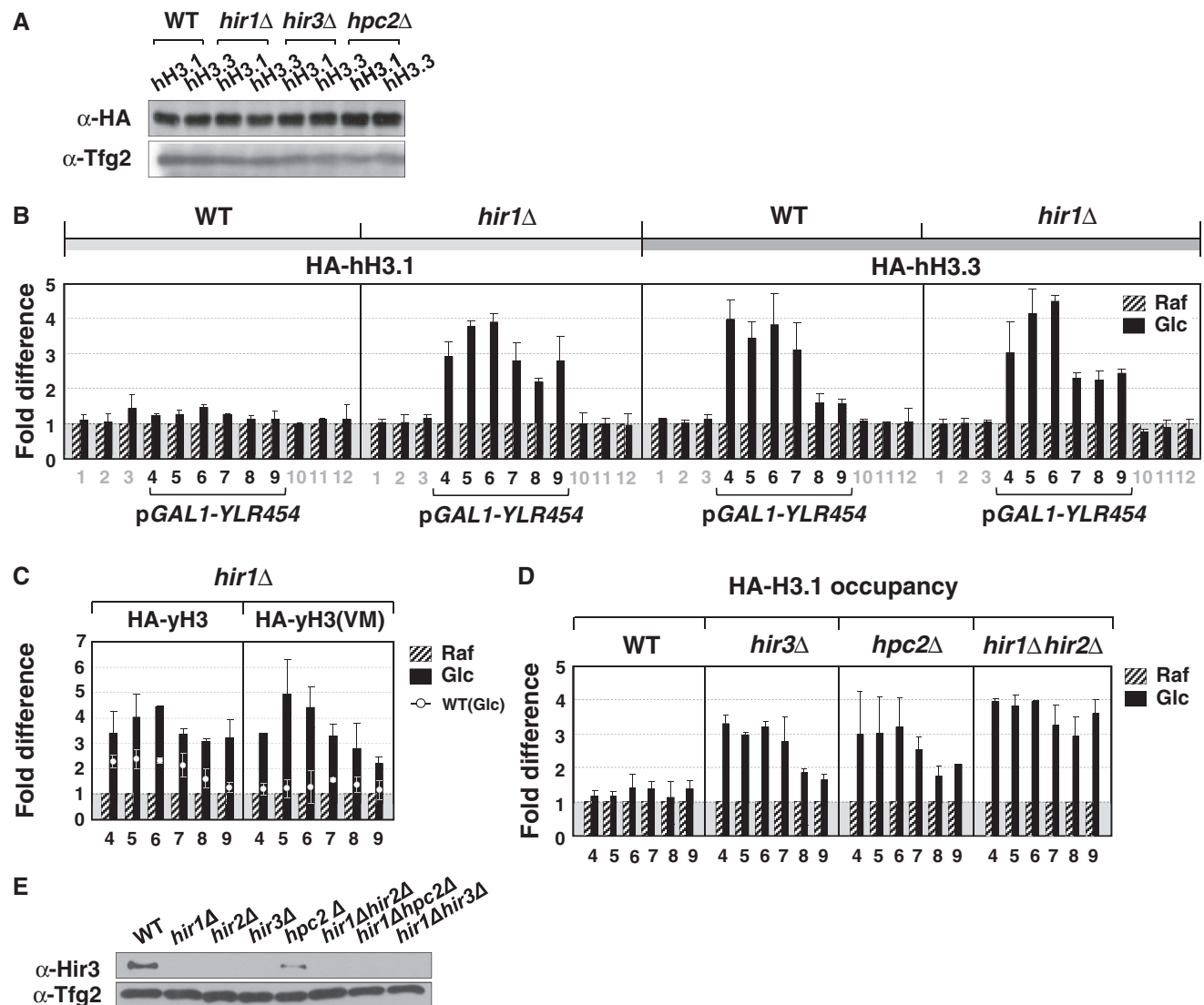
Figure 3. The yeast HIR complex is involved in the selective incorporation of hH3.3 during transcription. (**A**) The expression levels of hH3.1 and hH3.3 in the wild-type (YC73), *hir1Δ* (YC199), *hir3Δ* (YC264) and *hpc2Δ* (YC265) detected by immunoblotting analysis. (**B** and **C**) The selectivity of H3 toward H3.3 is lost in the absence of *HIR1*. The occupancy of HA-tagged H3s [hH3.1 and hH3.3 in (B) or yH3 and yH3(VM) in (C)] was analyzed by ChIP. The occupancy levels of HA-yH3s in wild-type yeasts are plotted as open circles (C). (**D**) Human H3.1 incorporation is abnormally increased during transcription in the absence of HIR subunits (*hir3Δ*, YC264; *hpc2Δ*, YC265; *hir1Δhir2Δ*, YC293). HA-hH3.1 incorporation was analyzed within p*GAL1-YLR454* by ChIP. Yeast strains were arrested appropriately by α-factor. (**E**) Deletion of HIR complex subunits results in an overall loss of Hir3 protein level. The Hir3 protein level in the wild-type (YC73), single (*hir1Δ*, YC199; *hir2Δ*, YC208; *hir3Δ*, YC264; *hpc2Δ*, YC265) or double deletion mutants (*hir1Δhir2Δ*, YC293; *hir1Δhpc2Δ*, YC297; *hir1Δhir3Δ*, YC296) was detected by immunoblotting analysis with an anti-Hir3 antibody.

levels in all mutant backgrounds (Figure 3A). As shown in Figure 3B, to our surprise, the occupancy of hH3.1 in the transcribed region was drastically increased in *hir1Δ*, as high as that of hH3.3, showing H3.1 itself was not inherently defective in deposition into chromatin through transcription. On the other hand, the level of hH3.3 was barely altered by lack of Hir1 subunit and was maintained similarly to that of wild-type (Figure 3B and Supplementary Figure S3A). In the case when HA-yH3 is expressed as a source of a new histone pool, deposition of new yH3 increases further by *hir1Δ* in a compensatory manner as the deposition of old yH3 mediated by HIR (recycling) is absent (16). We confirmed this observation by performing ChIP with expression of HA-yH3 in *hir1Δ* (Figure 3C). In

addition, similar to hH3.1, yH3(VM), which lacked incorporation in wild-type background, gained deposition on loss of Hir1 protein (Figure 3C).

When Hir1 protein was ectopically expressed in *hir1Δ*, aberrant incorporation of hH3.1 was suppressed and H3.3 selectivity was restored (Supplementary Figure S3B and C). The H3 selectivity for nucleosome assembly was completely lost in *hir1Δ* without detrimental effect on normal assembly of histones. In this background, H3s were able to incorporate regardless of their H3 type as long as they were supplied from new histone pool. Our data indicate that *hir1Δ* is defective in H3 selection in addition to recycling of chromatin. We also analyzed the level of hH3.1 in the backgrounds of individual or double

deletion and found that hH3.1 increased in these backgrounds (Figure 3D and Supplementary Figure S3D), indicating that whole complex of HIR is required for H3 selection. Deletion of any subunit of the HIR complex significantly reduced Hir3 protein levels, showing that each subunit is required for the integrity or the stability of the HIR complex (Figure 3E). Taken all, our data suggest that old yH3 is normally recycled, but this recycling pathway becomes negligible when hH3.3 is present, as HIR complex prefers histones in the order of hH3.3 > yH3 > hH3.1. New H3 from a histone pool normally competes for yH3 being recycled from chromatin by HIR, where hH3.3 was most efficient to outcompete yH3 on HIR, while hH3.1 was not.

To investigate whether transcription-mediated H3 selection ability is a specific nature attributed to HIR complex, we examined other chromatin factors as well. Chd1 is a switch/sucrose nonfermentable (SWI/SNF) family chromatin remodeler and involved in transcriptional elongation by targeting chromatin (21). In particular, it was known to function in the H3.3 deposition in the male pronucleus of *Drosophila* (22). However, unlike the HIR complex, *chd1Δ* did not affect incorporation of either hH3.1 or hH3.3 (Supplementary Figure S4A). We also tested Rtt106, a histone chaperone functionally related to the HIR complex and involved in chromatin function (23). Although Rtt106 is involved in chromatin dynamics during active transcription, it had marginal effects on H3 selectivity (Supplementary Figure S4B). These data indicate that H3.3 introduction during transcription is specifically mediated by the HIR complex.

The human HIRA and yeast HIR complex are functionally conserved

The human counterpart of yeast Hir1 and Hir2 is HIRA, with its N- and C-termini corresponding to Hir1 and Hir2, respectively. To determine whether HIRA complex functions with similar mechanism for positive selection of H3.3, we tried functional complementation of yeast mutants by expression of human HIRA. We generated four HIRA truncation mutants containing the N-terminal N1(1–420), N2(1–729), C-terminal C1(421–1017) or C2(730–1017) domain, as well as the full length HIRA (Figure 4A). Each of them was introduced into the *hir1Δ* background, and their expressions were confirmed by immunoblotting with an anti-FLAG antibody (Figure 4B). Next, we analyzed the occupancy of hH3.1 in *hir1Δ* cells expressing each HIRA construct. As shown in Figure 4C, and Supplementary Figure S5, hH3.1 incorporation in *hir1Δ* was significantly suppressed by the full length HIRA in comparison with *hir1Δ* cells harboring empty vectors. Specifically, HIRA-N2(1–729) containing both WD40 repeats and the B domain, important for Asf1 interaction, was sufficient to inhibit H3.1 deposition in *hir1Δ*, indicating that HIRA-N2(1–729) can function for variant selection in the yeast system in place of Hir1. However, HIRA-N1(1–420), HIRA-C1(421–1017) and HIRA-C2(730–1017) were defective in suppression of *hir1Δ* phenotype. Furthermore, hH3.1 incorporation in *hir1Δhir2Δ* was substantially suppressed by the expression

of HIRA (Figure 4D). These data provide evidence for functional conservation between yeast HIR and human HIRA, and more importantly, implicate a distinct role of HIRA complex in the selection of H3 variants during transcription.

The conserved C-terminal HUN domain of yeast Hpc2 subunit plays an important role in H3.3 selection by affecting the stability of the HIR complex

Yeast Hpc2 contains CDI and CDII domains that are conserved among yeast species. The CDI and CDII domains are required for the repression of histone genes and the stability of Hpc2 protein, respectively (24). Hpc2 carries additional HUN (Hpc2-UBN1) domain within residues 569–601, displaying 46% identity to the N-terminal corresponding domain (residues 132–167) of human UBN1 (Figure 5A). The function of this HUN domain remains elusive despite of being highly conserved from yeast to human (25). Intrigued by the strong conservation through evolution, we examined the HUN domain of Hpc2 by generating yeast strains expressing Hpc2-ΔC mutant (missing C-terminal 43 amino acids) from its own genomic locus (Supplementary Figure S6). Interestingly, deletion of HUN domain abrogated H3 selection (Figure 5B). We next constructed plasmids for ectopic expression of wild-type and two Hpc2 truncation mutants, Hpc2-ΔN (residues 80–625) and Hpc2-ΔC (residues 1–558) (Figure 5A). All constructs were introduced into the *Hpc2Δ* background, and their expression was confirmed by immunoblotting with an anti-FLAG antibody (Figure 5C). We analyzed the occupancy of hH3.1 in *hpc2Δ* cells expressing each Hpc2 construct. Hpc2-ΔC lacking the C-terminal HUN domain was defective in suppression of *hpc2Δ* phenotype, whereas Hpc2-FL and Hpc2-ΔN completely suppressed hH3.1 incorporation in *hpc2Δ* (Figure 5D). Hir3 is the yeast ortholog of human Cabin1, which is the largest subunit containing the tetratricopeptide repeats (TPRs) and serves as a scaffold platform in HIRA/UBN1/Cabin1 complex (11). The loss of H3 selectivity in *hpc2Δ*-expressing Hpc2-ΔC mutants coincided with the loss of the functional HIR complex attributed by significant reduction of Hir3 proteins (Figure 5E and F). Our data suggest that the evolutionarily conserved HUN domain of Hpc2 subunit is indispensible for the HIR complex stability and for the preferential deposition of hH3.3.

HIR and Asf1 play distinctive roles in the selective accumulation of hH3.3 during transcription

Asf1 functions by delivering the H3/H4 heterodimer to HIRA for assembly of nucleosomes (4,9). To address the role of Asf1 in H3 selectivity, we next analyzed H3 occupancy in the *hir1Δ* background with an additional deletion of *ASF1* (*asf1Δhir1Δ*). As shown in Figure 6A and B and Supplementary Figure S7A, incorporation of hH3.3 and hH3.1, which had been induced by *hir1Δ*, was completely abrogated in *asf1Δhir1Δ*. Incorporation of new H3 histone was significantly reduced regardless of H3 type, indicating that the initial introduction of new H3 subunit to chromatin was affected by *asf1Δ*. To further confirm this, we
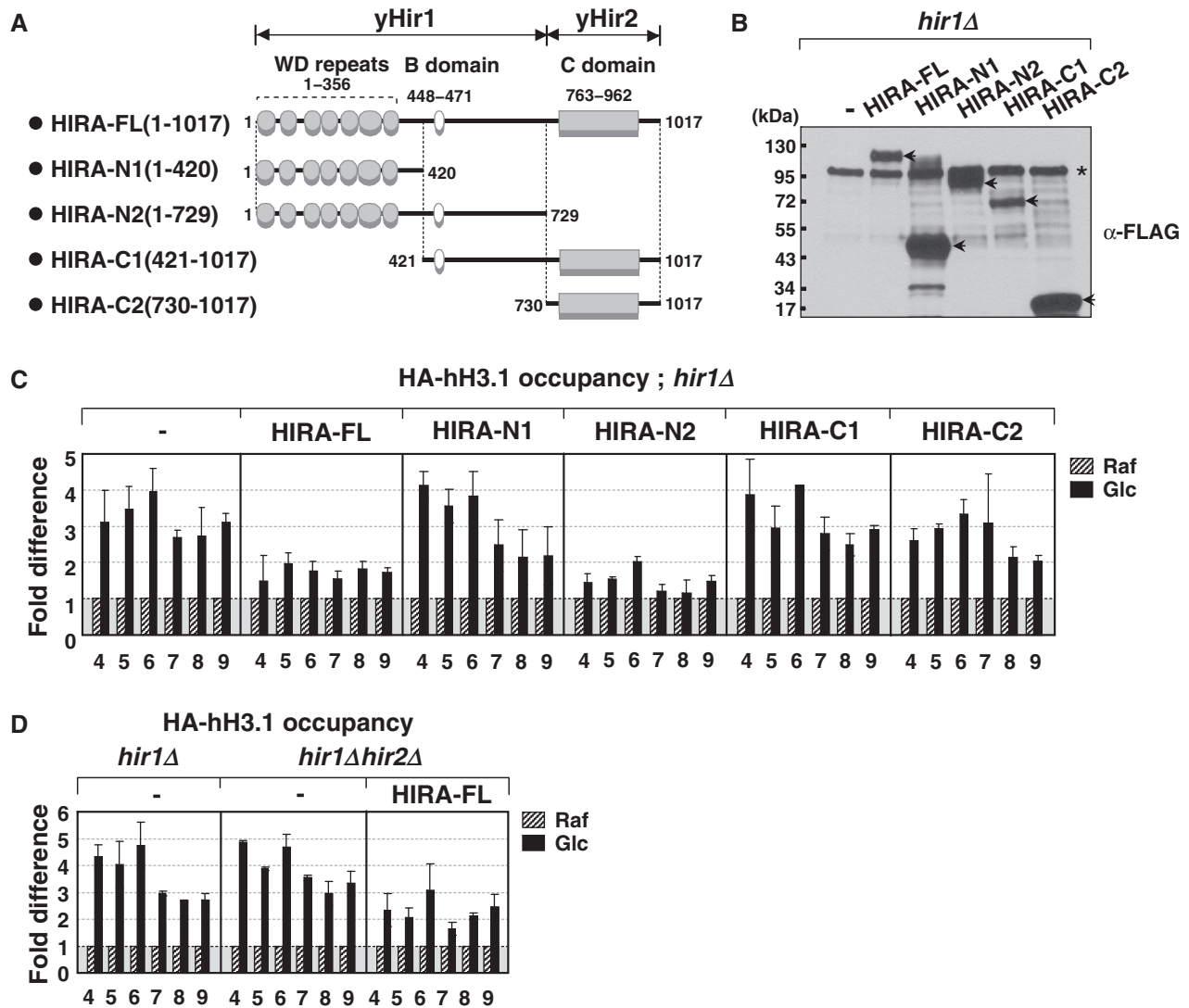
Figure 4. Human HIRA functionally complements the yeast HIR phenotype. (**A**) The schematic diagram of the domain structure of human HIRA. Filled circle, 7 × WD40 repeat (residues 1–356); open oval, B domain (residues 448–471); filled box, Hir2-like domain (residues 763–962). Protein domains were analyzed using InterProScan software (http://www.ebi.ac.uk/InterProScan/). (**B**) All human HIRA constructs were expressed in *hir1Δ*. The immunoblotting analysis was performed with the whole cell extract prepared from *hir1Δ* (YC199) expressing indicated HIRA constructs. The arrows indicate the position of full-length and truncated HIRA proteins. The asterisk indicates a nonspecific band. Protein molecular mass markers are shown. (**C**) Expression of human HIRA partially suppressed hH3.1 incorporation in *hir1Δ*. HIRA-FL and the HIRA-N2(1–729) partially suppressed hH3.1 incorporation in *hir1Δ*. The incorporation of HA-hH3.1 in yeast strains indicated in Figure 4B was analyzed by ChIP. (**D**) Expression of human HIRA partially suppresses hH3.1 incorporation in *hir1Δhir2Δ* (YC293). The incorporation of HA-hH3.1 in *hir1Δ* and *hir1Δhir2Δ* carrying YEp352GAPII (empty vector) or YEp352GAPII-HIRA-FL(1-1017)-FLAG was analyzed by ChIP.

supplemented the mutant with Myc-tagged wild-type Asf1 or Asf1 V94R that lacks H3/H4 binding. The V94R mutant mimics the *asf1Δ* phenotype, as it exhibits sensitivity to genotoxic agents and to high temperature and a defect in transcriptional silencing, demonstrating that proper contact between Asf1 and H3/H4 is critical (26). Experssion of WT Asf1 recovered the occupancy levels of both hH3.1 and hH3.3, thus resulting in a phenotype resembling *hir1Δ*. However, *asf1Δhir1Δ* supplemented with Asf1 V94R remained defective for the incorporation of any type of H3 (Figure 6C and Supplementary Figure S7B). Yeast Asf1 contains a highly conserved N-terminal core domain required for its interaction with both H3/H4 and HIR/CAF1 chaperones. Its C-terminal region is

diverged and dispensable for Asf1's known functions, such as DNA replication, DNA damage response and chromatin silencing. We also found that the conserved core domain (Asf1ΔC) was sufficient to support hH3.3 incorporation (Figure 6D and Supplementary Figure S7C and D). These data show that Asf1 normally allows influx of H3 in the wake of polymerase to assemble nucleosomes, but it does not influence the choice of H3 type, indicating that Asf1 is functioning epistatically over HIR (HIRA)-mediated H3.3 incorporation. Taken all, using yeast system, we show yeast HIR and human HIRA complexes are likely to take yH3 (S-IG) through the recycling pathway avoiding an influx of H3.1 (S-VM), but prefer H3.3 (A-IG) over yH3, leading
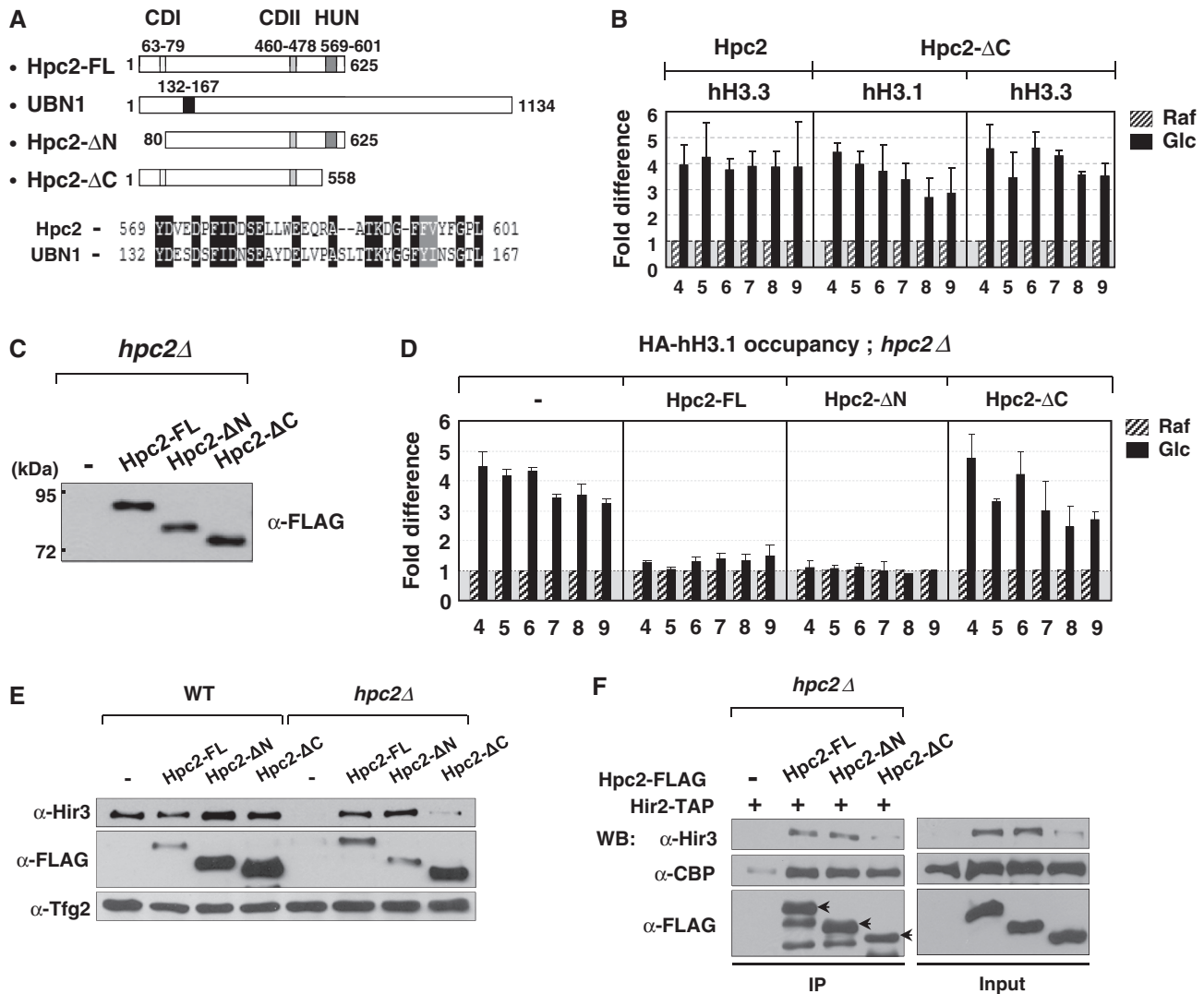
Figure 5. The C-terminal HUN domain of Hpc2 is essential for the selective incorporation of hH3.3 during transcription by maintaining the stability of the HIR complex. (**A**) The schematic diagram of the domain structure of yeast Hpc2 and human UBN1. Boxes indicate three domains (N-terminal CDI, C-terminal CDII and HUN) conserved among Hpc2 homologs of yeast species and higher eukaryotes. The amino acid alignment (bottom) shows sequence similarity of HUN domains between yeast Hpc2 and human UBN1. (**B**) Human H3.1 and H3.3 incorporations in yeast strains expressing wild-type Hpc2-13Myc (YC298) or Hpc2ΔC-13Myc (YC299) were analyzed by ChIP. (**C**) Expression of Hpc2-FL-FLAG, Hpc2-ΔN-FLAG and Hpc2-ΔC-FLAG in *hpc2Δ* (YC265) was confirmed by immunoblotting by using an anti-FLAG antibody. The molecular mass markers are shown. (**D**) The Hpc2-ΔC lacking C-terminal HUN domain fails to suppress hH3.1 incorporation in *hpc2Δ*. The incorporation of HA-hH3.1 was analyzed by ChIP. (**E**) Lack of C-terminal HUN domain of Hpc2 causes a significant decrease in the Hir3 protein level. The Hir3 protein level in wild-type (YFC193) and *hpc2Δ* (YC302)-expressing Hpc2-FL-FLAG, Hpc2-ΔN-FLAG and Hpc2-ΔC-FLAG was detected by immunoblotting analysis with an anti-Hir3 antibody. (**F**) Co-immunoprecipitation assay of Hpc2, Hir2 and Hir3. The whole-cell extract from *hpc2Δ* cells (YC302) expressing indicated proteins was prepared. Co-IP was performed with anti-Flag antibody followed by immunoblotting with anti-Hir3 or anti-CBP (to detect TAP) antibodies.

to selective accumulation of H3.3 in the transcribed region.

DISCUSSION

Our data provide insights into the molecular mechanisms for selective incorporation of the histone variant H3.3 in the actively transcribed regions according to the H3 ID (5). H3 assembled into nucleosomes behind elongating polymerase are either from a free histone pool or from preexisting chromatin. If a gene is packed with H3.1 nucleosomes, during the first few rounds of transcription,

H3.3 can rapidly incorporate into the chromatin by replacing old histones because it efficiently outcompetes H3.1 for HIRA/UBN1/Cabin1 complex, which is responsible for recycling of chromatin histones (Figure 6E). Once a gene is covered with sufficient H3.3 nucleosomes, equilibrium between new H3.3 and old H3.3 might be established for formation of nucleosomes, thus maintaining the consistent histone modification and transcription status by preventing saturation. Loss of Asf1 or Asf1 V94R led to a complete loss of deposition of both types of H3 via the RI pathway, suggesting that Asf1 is required for assembling nucleosomes by providing H3 regardless of histone type,
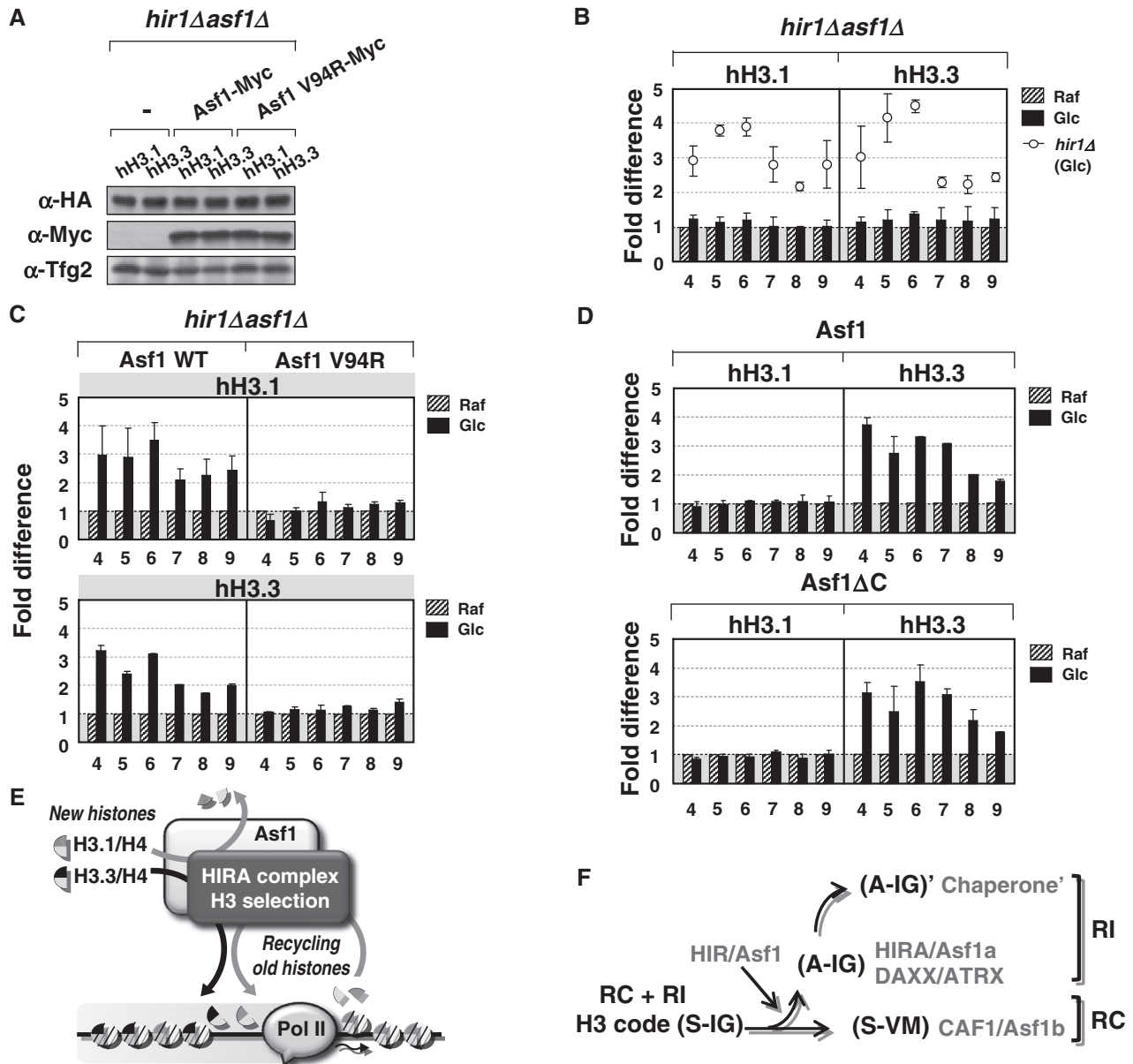
Figure 6. The histone chaperone HIR complex and Asf1 cooperate together for selective accumulation of hH3.3 during transcription. (**A**) Wild-type Asf1 and the Asf1 V94R mutant with a C-terminal 13Myc tag were expressed in *asf1Δhir1Δ* (YC252). All yeasts expressed HA-H3 at similar levels. (**B**) ChIP analysis of HA-hH3.1 and hH3.3 incorporation in *asf1Δhir1Δ* (YC252). The data points of the HA-hH3.1 and hH3.3 occupancy obtained in *hir1Δ* (glucose sample) from Figure 3B were depicted as open circles and shown for comparison. (**C**) ChIP analysis of HA-hH3.1 and -hH3.3 incorporation in *asf1Δhir1Δ* expressing either wild-type or Asf1 (V94R) mutant. (**D**) ChIP analysis of HA-hH3.3 and HA-hH3.1 incorporation in cells expressing wild-type Asf1 or yAsf1ΔC. (**E**) A model suggesting that Asf1 and HIRA complex cooperate for selective accumulation of hH3.3 in the active genes during transcription. Asf1 and HIRA complex are required for disassembly and reassembly of nucleosomes on the path of polymerase II, allowing for the incorporation of new histones. HIRA complex normally mediates recycling of histones and deposits old H3 to assemble nucleosomes. In this model, hH3.3, but not hH3.1, efficiently binds to HIRA complex and competes with recycling histones to incorporate into nucleosomes. (**F**) A potential role of RI histone chaperones in the evolution of histone H3 variants.

which is consistent with the hypothesis that Asf1 buffers and transports histones to RC and RI chaperones, facilitating chromatin assembly through chaperone–chaperone interactions (2,9).

It is noteworthy that our observation that deletion of HIR subunits resulted in concomitant induction of hH3.1 deposition in hH3.1-expressing yeasts without changes in hH3.3 level in hH3.3-expressing yeasts is seemingly contrast to the reports that show genomic or functional depletion of HIRA results in loss of H3.3 deposition in the transcriptionally active chromatin in higher eukaryotes (3,20,27,28). In yeast, deletion of both HIR and Asf1 (*hir1Δ/asf1Δ*) gave a pattern that is more related to HIRA depletion in higher eukaryotes than deletion of HIR alone (*hir1Δ*). Continous deposition of hH3.3 in *hir1Δ* implies that a supplementary histone chaperoning activities exist to allow influx of hH3.3 (or hH3.1) in yeasts. A potential candidate is yeast Asf1, which

functions more independently than human Asf1a that has an intimate connection with HIRA (29). Another chaperoning candidate is yeast CAF1, which is recruited to genes in a transcription-dependent manner although its role in transcription is not known (30). Besides, other chromatin factors such as ATP-dependent chromatin remodeling factors, Chd1, Isw1, Spt6, Rtt106, Set2, etc. may contribute to supplementary pathways for nucleosome assembly in yeasts (22,31–35).

According to the structure of the Asf1/H3/H4 complex (26,36), Asf1 interacts with the C-terminal α3 helix of H3 and the C-terminal tail of H4 through the conserved globular core domain, and these interactions could occur independently of its interaction with other chaperone partners. In this structure, the ID region of H3 is exposed on the surface and not occupied by Asf1, indicating that there is other factor(s) that allow the pathway-specific charge of different H3 variants. Recently, the structure of the histone-binding domain of DAXX that mediates H3.3-specific recognition was solved (37,38). DAXX covers the solution-accessible region of H3.3/H4 dimer and makes direct contact with the H3.3 ID region. More precisely, DAXX uses a hydrophobic pocket and a more polar surface to recognize A87 and G90 respectively, indicating that theses two residues are the most critical determinants of H3 specificity that allow DAXX to deposit H3.3 in the pericentric and telomeric heterochromatin. Unfortunately, neither the structure of HIRA and H3.3/H4 complex nor the biochemical evidence of direct binding between HIRA and H3.3 ID region has been provided. As the A-IG was the most efficient combination for HIRA-dependent deposition in the transcribed region, similar contact might be expected between HIRA and H3.3. Nonetheless, we could not exclude the possibility that subunits other than HIRA within complex contribute to the H3.3 recognition specificity. Obviously, posttranslational modification of histone subunits is another possibility that affects the specificity of chaperone–histone interaction.

Curiously, our assay system provides a quantitative analysis of the variant histones for chromatin incorporation via the RI pathway, which can be precisely decoded by amino acids in the ID region. A-IG was most efficient for the RI deposition, followed by S-IG and S-VM, a note that is consistent with the phylogenetic relationships of H3s (39). In this aspect, a fungal (Ascomycetes such as *S.cerevisiae*) type of H3 (S-IG) is the ancestral histone that can perform both the RC and RI nucleosome assembly. During evolution, it diverged to canonical H3 (S-VM) and replacement H3 (A-IG), each taking a more specialized role, with the requirement of the division of labor in bulk packaging and transcription. The role of canonical H3 might be further ensured by taking C at 96 in case of H3.1. In this study, unexpectedly, we show that yeast RI histone chaperones are more compatible with A-IG code. This observation suggests that RI machineries might contribute more actively than was previously thought to structural and functional diversification of their histone substrates in accordance with the expansion of chromatin domain variation during evolution (Figure 6F). They might play a role in selection and evolution of replacement histones that provide the best partnerships. With the discovery of more H3 variants with A-IG code, more RI-type chaperones wait to be assigned for the roles in structure and function of chromatin domains based on their cognate partners (18,40). Further studies will provide the structural and functional details of histone–chaperons and their recruitment across a wide range of genome.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–3, Supplementary Figures 1–7, Supplementary Materials and Methods and Supplementary References [41–47].

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