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Insulator protein Su(Hw) recruits SAGA and Brahma complexes and constitutes part of Origin Recognition Complex-binding sites in the *Drosophila* genome

Nadezhda E. Vorobyeva[1], Marina U. Mazina[1], Anton K. Golovnin[2,3], Daria V. Kopytova[1], Dmitriy Y. Gurskiy[1], Elena N. Nabirochkina[1], Sofia G. Georgieva[1,4], Pavel G. Georgiev[5] and Aleksey N. Krasnov[1,2,*]

[1]Department of Transcriptional Regulation and Chromatin Dynamics, Institute of Gene Biology, Russian Academy of Sciences, Moscow, 119334, Russia, [2]University of Oslo, Centre for Medical Studies in Russia, Moscow, 119334, Russia, [3]Department of Molecular Genetics of Drosophila, Institute of Gene Biology, Russian Academy of Sciences, Moscow, 119334, Russia, [4]Department of Transcription Factors, Engelhardt Institute of Molecular Biology, Moscow, 119334, Russia and [5]Department of the Control of Genetic Processes, Institute of Gene Biology, Russian Academy of Sciences, Moscow, 119334, Russia

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ABSTRACT

Despite increasing data on the properties of replication origins, molecular mechanisms underlying origin recognition complex (ORC) positioning in the genome are still poorly understood. The Su(Hw) protein accounts for the activity of best-studied *Drosophila* insulators. Here, we show that Su(Hw) recruits the histone acetyltransferase complex SAGA and chromatin remodeler Brahma to Su(Hw)-dependent insulators, which gives rise to regions with low nucleosome density and creates conditions for ORC binding. Depletion in Su(Hw) leads to a dramatic drop in the levels of SAGA, Brahma and ORC subunits and a significant increase in nucleosome density on Su(Hw)-dependent insulators, whereas artificial Su(Hw) recruitment itself is sufficient for subsequent SAGA, Brahma and ORC binding. In contrast to the majority of replication origins that associate with promoters of active genes, Su(Hw)-binding sites constitute a small proportion (6%) of ORC-binding sites that are localized preferentially in transcriptionally inactive chromatin regions termed BLACK and BLUE chromatin. We suggest that the key determinants of ORC positioning in the genome are DNA-binding proteins that constitute different DNA regulatory elements, including insulators, promoters and enhancers. Su(Hw) is the first example of such a protein.

INTRODUCTION

Su(Hw) is a zinc-finger protein that is responsible for the activity of the best-studied *Drosophila* insulators. Two more proteins, Mod(mdg4) and CP190, are required for the insulator function (1–3). The ENY2 protein is recruited by Su(Hw) to the insulator complex and is required for the barrier activity of Su(Hw)-dependent insulators (4). ENY2 is a small protein that plays an important role in transcription regulation, being a subunit of the DUB module of SAGA complex in *Drosophila* (5–7).

The SAGA complex is a highly conserved transcription coactivator that contains >20 protein subunits (8). In *Drosophila melanogaster*, histone acetyltransferase GCN5 is the catalytic subunit of two separate high-molecular-weight complexes, ATAC and SAGA, whereas ADA2b is the SAGA-specific subunit (9,10). SAGA is recruited to promoters of target genes through the interaction with the sequence-specific DNA-binding transcription factors (11,12). Acetylation of promoter nucleosomes by SAGA stabilizes its interactions with promoters and targets promoter nucleosomes for displacement by the SWI/SNF nucleosome remodeling complex (13,14).

SWI/SNF ATP-dependent chromatin remodeling complexes (remodelers) perform critical functions in eukaryotic gene expression control. All SWI/SNF homologs contain the bromodomain that recognizes acetylated lysines. The presence of bromodomains in SWI/SNF suggests a functional connection between histone acetylation and chromatin remodeling. BAP and PBAP are two

*To whom correspondence should be addressed. Tel: +7 499 135 9731; Fax: +7 499 135 4105; Email: krasnov@genebiology.ru

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

major subclasses of SWI/SNF remodelers in *Drosophila*. Both these complexes share several core subunits, including the Brahma ATPase, but differ in a few signature subunits: POLYBROMO, BAP170 and SAYP are specific for PBAP, whereas OSA is specific for BAP (15–18).

DNA replication begins from multiple sites distributed throughout the genome, named replication origins. The origin recognition complex (ORC) consisting of six subunits (ORC1–ORC6) binds to the replication origins and plays a critical role in the initiation of DNA replication by creating a platform for pre-RC complex assembly and replication origins firing (19). To ensure proper replication of the genome during S-phase, eukaryotic cells produce multiple replication origins, and then a certain part of them fire in the context of developmental stage or growth conditions (20). A comprehensive analysis of replication origins has shown that there is no definite sequence motif predictive of ORC binding (21). Despite the lack of apparent sequence specificity, ORCs bind to specific regions of the genome in different cell lines (22) and tissues (23), suggesting that the origins of replication are not specified by sequences alone. Sites of ORC enrichment are depleted in bulk nucleosomes (21) and undergo active nucleosome exchange (24). Consistently with this finding, replication origins were shown to be highly enriched with chromatin-remodeling complexes, including NURF and SWI/SNF (22,25). Multiple proteins were found to function as ORC chaperones in targeting ORC to certain chromatin regions (26–36).

Despite the increasing amount of data on the properties of replication origins, it is still unknown what factor(s) is responsible for the events taking place at these sites and, in particular, what is the primary determinant of ORC localization (19). In this study, we have shown that the insulator protein Su(Hw) recruits the histone acetyltransferase complex SAGA and chromatin remodeler Brahma to Su(Hw)-dependent insulators, which leads to the appearance of regions with low nucleosome density and creates conditions for ORC binding. We suggest that the key determinants of ORC positioning in the genome are DNA-binding proteins that constitute different DNA regulatory elements, including insulators, promoters and enhancers. Su(Hw) is the first example of such a protein, which determines the formation of 6% of ORC-binding sites in the genome.

MATERIALS AND METHODS

Drosophila cell culture and RNAi knockdown experiments

Drosophila S2 cells were cultured at 25°C in Schneider's insect medium (Sigma) containing 10% fetal bovine serum (HyClone). Transformation of S2 cells was performed by using Effectene Transfection Reagent (QIAGEN) according to the manufacturer's recommendations. To generate a cell line stably carrying a construct, S2 cells were placed in a selective medium with blasticidin (25 μg/ml) and cultivated for at least 1 month. RNAi experiments followed the published protocol (37). We used 15–20 μg of dsRNA per 10^6 cells; dsRNA was synthesized with an Ambion MEGA Script T7 kit, and dsRNA corresponding to a fragment of pBluescript II SK- vector was used as a control. The primers used for the synthesis of dsRNA are described in Supplementary Data.

Antibodies

Experiments were performed with antibodies against Su(Hw) (38), ADA2b (9), BAP111 and BAP170 (16), ORC2 and ORC6 (39), FLAG epitope (M2 clone, Sigma) and total histone H3 (Ab1791, Abcam). Antibodies against GCN5 (349–813 aa fragment), OSA (109–330 aa fragment), ORC3 (510–686 aa fragment) and CDC45 (138–396 aa fragment) were raised in our laboratory by immunizing rabbits with the corresponding His6-tagged protein fragments and were subsequently affinity purified. An antibody against β-tubulin, obtained by M. Klymkowsky, was from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained at the Department of Biological Sciences, University of Iowa.

Chromatin immunoprecipitation and quantitative polymerase chain reaction analysis

DNA was cross-linked (1.5% FA, 15 min) and sheared to a size of 500 bp. Approximately 3×10^6 cells or 50 mg of pupae and 10 μg of an antibody were taken for one experiment. After chromatin immunoprecipitation (ChIP), the recovered DNA was analyzed by quantitative polymerase chain reaction with Chromo4 (Bio-Rad). A detailed protocol of ChIP is given in Supplementary Data.

Nuclear extracts and immunoprecipitation

Preparation of nuclear extracts from *Drosophila* embryos and co-immunoprecipitation experiments were performed as previously (40). A control with DNase I (USB) treatment was performed for the each co-immunoprecipitation experiment, with DNase I added to the protein extract during immunoprecipitation having no effect on the observed protein interactions.

Genomic distribution analysis

Su(Hw) ChIP-Seq data (NCBI GEO GSE27679) were used to calculate the exact positions of Su(Hw) peaks in S2 cells (41). A total of 3120 peaks were determined (Supplementary Table S1). Genome-wide profiles (WIG files) of the factors of interest were downloaded from the modENCODE and NCBI website (Supplementary Table S2). To obtain the average profile of a given factor, individual profiles were calculated for each of 3120 Su(Hw)-binding sites at −5 to +5 kb relative to Su(Hw) peaks, with 1-nt resolution. The 10-kb local profiles were extracted from the WIG file. The points absent from this file were calculated by linear interpolation. The resulting 3120 individual profiles were averaged per genomic position (from −5 to +5 kb) to obtain the plot of average log2 enrichment ratio of given factors by base pair positions relative to the Su(Hw) peak. To calculate the average profile of AT content, the WIG file was generated by calculating the percentage of AT pairs in a 10-bp window at each genomic position.

RESULTS

Su(Hw) recruits the SAGA complex to Su(Hw)-dependent insulators

As shown in our previous study, the ENY2 protein binds to the zinc-finger domain of Su(Hw) and is recruited to the insulator complex (4). As ENY2 is a component of the SAGA complex (5), it was relevant to find out whether there is an association between SAGA and Su(Hw)-dependent insulators. To this end, we tested the presence of the GCN5 and ADA2b subunits of SAGA on Su(Hw)-binding sites by ChIP in *Drosophila* S2 cells. In these experiments, Su(Hw)-dependent insulators *gypsy*, 1A2, 50A, 62D, 66 E and 87 E were examined versus the 1A1 and 1A6 sites used as a negative control. The results showed that Su(Hw) and ENY2 readily interacted with these insulators, but did not bind to the 1A1 and 1A6 sites (4). As a reference site, we used the CTCF-dependent insulator Mcp, which does not bind Su(Hw) protein (Figure 1A) and therefore is appropriate as a Su(Hw)-independent control. All tested Su(Hw)-binding regions and the Mcp insulator showed significant enrichment with GCN5 and ADA2b subunits on ChIP with corresponding antibodies, providing evidence for strong binding of the SAGA complex to these regions in S2 cells (Figure 1C and E; dark bars). The binding of SAGA to the Mcp insulator is consistent with the previous finding that CTCF-dependent insulators are enriched with histone acetyltransferase complexes (42).

The role of *Drosophila* Su(Hw) in targeting the SAGA complex was evaluated in experiments on RNAi knockdown of Su(Hw) in S2 cells, verifying its efficiency by either RT–PCR or western blot analysis (Supplementary Figure S1A and B). Testing the insulators for the presence of Su(Hw) after its knockdown, we found that this protein was still present on 50A and 62D, whereas its amount on all other insulators was significantly reduced (Figure 1A). We also examined GCN5 and ADA2b recruitment to the insulators after Su(Hw) knockdown and found the SAGA complex to be depleted on all insulators except 50A and 62D (Figure 1C and E). The level of GCN5 and Ada2b on the Mcp insulator also remained unchanged.

On the whole, ~5% of Su(Hw) remained in S2 cells after its RNAi knockdown (Supplementary Figure S1A and B). This protein was still bound not only to 62D and 50A but also to some other insulators. In particular, small amounts of Su(Hw) remained on *gypsy* and 87E (Figure 1A). To completely eliminate Su(Hw) from its binding sites, we used the mutant line $Su(Hw)^{V/E8}$. Flies carrying the $su(Hw)^{v}$ alleles produce no Su(Hw) protein, whereas flies carrying the $su(Hw)^{E8}$ alleles produce a full-length protein that does not bind to DNA *in vitro* or *in vivo* owing to the point mutation of zinc-finger 7 (43,44). The pupae of this mutant line were tested in ChIP experiments with antibody against Su(Hw), using wild-type pupae as a control. In addition to known Su(Hw) insulators, we also analyzed several new Su(Hw)-binding sites (54B, 73A, 16C, 8D, 89B and 50C) selected by screening the Su(Hw) ChIP-chip data from the modENCODE project (45). The results showed that Su(Hw) bound to all test sites in wild-type pupae but was absent from these sites in the mutant line (Figure 1B). To investigate the targeting of the SAGA complex to Su(Hw)-binding sites, we performed GCN5 ChIP experiments in wild-type and mutant pupae. As in the previous case, GCN5 proved to be recruited to Su(Hw)-binding sites in wild-type pupae, while the GCN5 level on all these sites in the mutant line was drastically reduced (Figure 1D). However, the Su(Hw) mutation did not affect the level of GCN5 on the CTCF-dependent Mcp insulator (Figure 1D). Thus, we found that Su(Hw) is required for recruiting the SAGA complex to Su(Hw)-binding sites both in S2 cell and in *Drosophila* pupae. To confirm physical association of Su(Hw) and GCN5 *in vivo*, we performed co-immunoprecipitation of the nuclear extract from *Drosophila* embryos with antibodies to either protein. The anti-Su(Hw) antibody precipitated Su(Hw) and co-precipitated a portion of GCN5 and vice versa (Figure 1F). These results indicate that Su(Hw) and SAGA do interact *in vivo*.

The BAP complex is recruited to Su(Hw)-dependent insulators

It is known that SAGA and Brahma complexes play a role in histone modification and chromatin remodeling on the promoters of active genes (46,47). As we detected SAGA on Su(Hw)-binding sites, it was interesting to find out whether the Brahma complex is also recruited to these sites. Therefore, we used ChIP analysis to test Su(Hw)-binding sites for the presence of OSA, BAP111 and BAP170 subunits of the Brahma complex, which is represented in *Drosophila* by two major subclasses, BAP and PBAP. The BAP111 subunit is common to both subclasses, whereas OSA and BAP170 are specific for BAP and PBAP, respectively (15). The results of ChIP experiments confirmed the recruitment of OSA and BAP111 to Su(Hw)-dependent insulators in S2 cell (Figure 2A and B, dark bars), whereas the content of BAP170 on these sites remained near the baseline level (data not shown). OSA and BAP111 are also recruited to the Mcp insulator (Figure 2A and B, dark bars), which is in line with the previous finding that CTCF-dependent insulators are enriched with the SWI/SNF complex (25). To check the role of Su(Hw) protein in recruiting the BAP complex on Su(Hw)-binding sites, we examined the targeting of OSA and BAP111 proteins after Su(Hw) RNAi in S2 cell and found that the level of BAP subunits significantly dropped on all tested sites except 62D and 50A (Figure 2A and B). To further investigate the targeting of BAP under Su(Hw) depletion, OSA ChIP experiments were performed with wild-type and $Su(Hw)^{V/E8}$ mutant pupae. The results showed that this mutation resulted in a dramatic decrease in the level of OSA on all Su(Hw)-binding sites tested (Figure 2C). We found that Su(Hw) depletion does not affect the BAP level on the Mcp insulator (Figure 2A–C). Thus, we found that the BAP remodeling complex is recruited on Su(Hw)-binding sites in a Su(Hw)-dependent manner, as is the SAGA complex. Next, we checked whether Su(Hw) and OSA are physically associated *in vivo* by performing

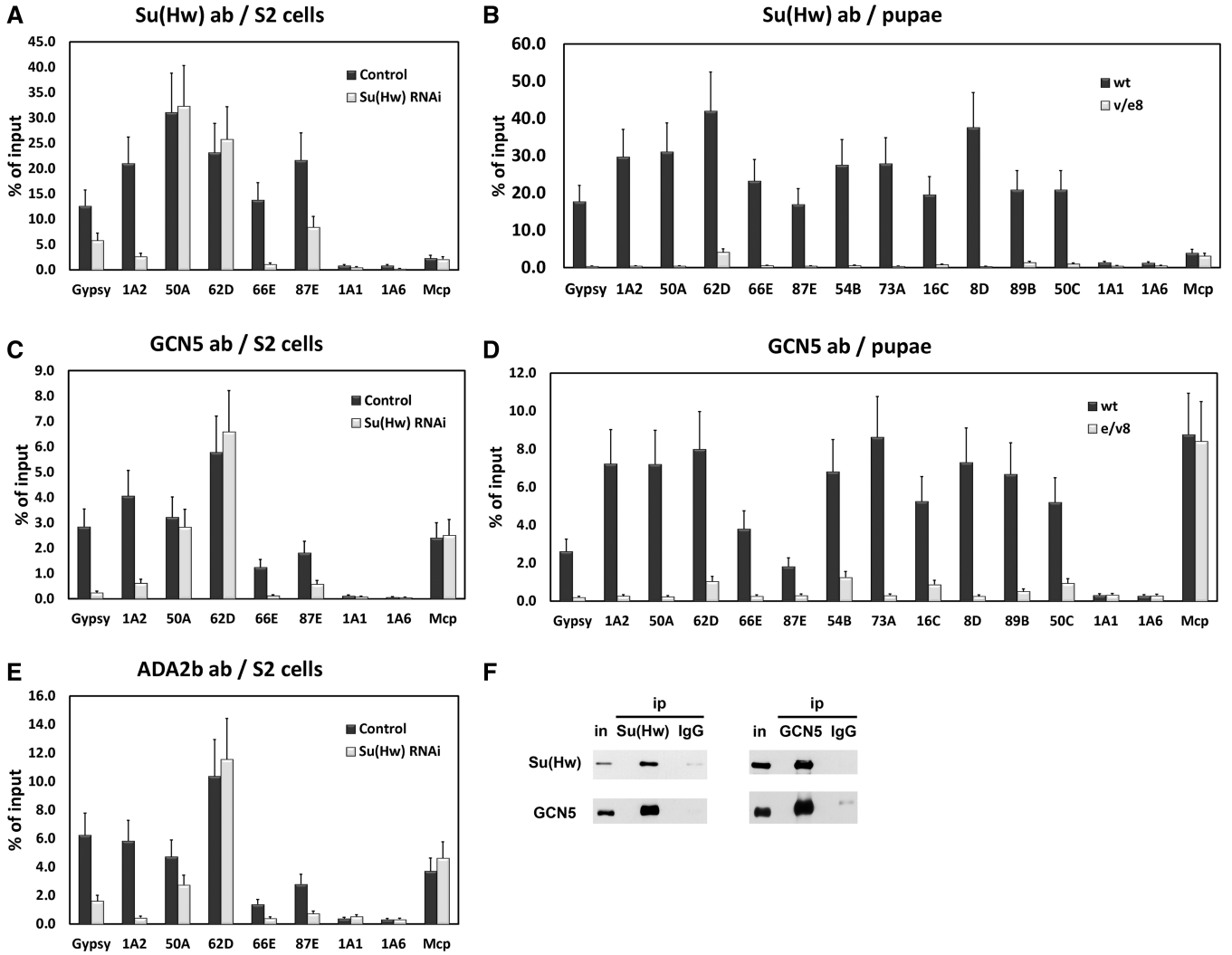

Figure 1. Su(Hw) recruits histone acetyltransferase complex SAGA. (**A**, **C**, **E**) The levels of (A) Su(Hw), (C) GCN5 and (E) ADA2b on Su(Hw)-binding sites in control S2 cells (dark bars) and after Su(Hw) knockdown (light bars) as evaluated by ChIP analysis. The results are expressed as the percentage of DNA input. Error bars show standard errors of the means from three replicate experiments. Sites 1A1 and 1A6 were used as a negative control. The Mcp insulator was used as a reference site. (**B**, **D**) The levels of (B) Su(Hw) and (D) GCN5 on Su(Hw)-binding sites in wild-type (dark bars) and $Su(Hw)^{V/E8}$ mutant pupae (light bars) as evaluated by ChIP analysis. (**F**) Co-immunoprecipitation of Su(Hw) and GCN5 from *Drosophila* embryo nuclear extract by rabbit antibodies against each of these proteins; IgG from rabbit pre-immune serum was used as a negative control. Ten percent portions of the input nuclear fraction (in) and immunoprecipitated fraction (ip) were resolved by SDS–PAGE and western blotted with antibodies against Su(Hw) and GCN5.

co-immunoprecipitation of nuclear extract from *Drosophila* embryos. The anti-Su(Hw) antibody proved to precipitate not only Su(Hw) but also a portion of OSA and vice versa (Figure 2D), indicating that Su(Hw) and OSA associate *in vivo*. However, the interaction between them is weak, and it may well be that Su(Hw) and BAP complex co-associate on insulators indirectly, through an as yet unknown mediator protein or protein complex.

Su(Hw) is required for creating regions with low nucleosome density

As we detected SAGA and Brahma complexes on Su(Hw)-binding sites, it was relevant to determine whether chromatin remodeling takes place on these sites. To assess chromatin structure, we performed a ChIP experiment using an antibody against histone H3 and found that the level of this protein was lower on Su(Hw)-binding sites than on control sites 1A1 and 1A6 (Figure 2E and F, dark bars). This result is consistent with the previous data that binding sites for insulator-associated proteins are regions of reduced nucleosome density (48). To find out whether Su(Hw) binding is a prerequisite for histone eviction, we knocked down Su(Hw) by RNAi in S2 cell and then analyzed histone H3 binding by ChIP assay, which revealed a significant increase in its level on Su(Hw)-dependent insulators (Figure 2E), while the level of histone H3 on the CTCF-dependent insulator Mcp remained unchanged. An analysis of histone H3 binding in $Su(Hw)^{V/E8}$ mutant pupae confirmed the fact that Su(Hw) is required for chromatin remodeling on Su(Hw)-binding sites (Figure 2F).

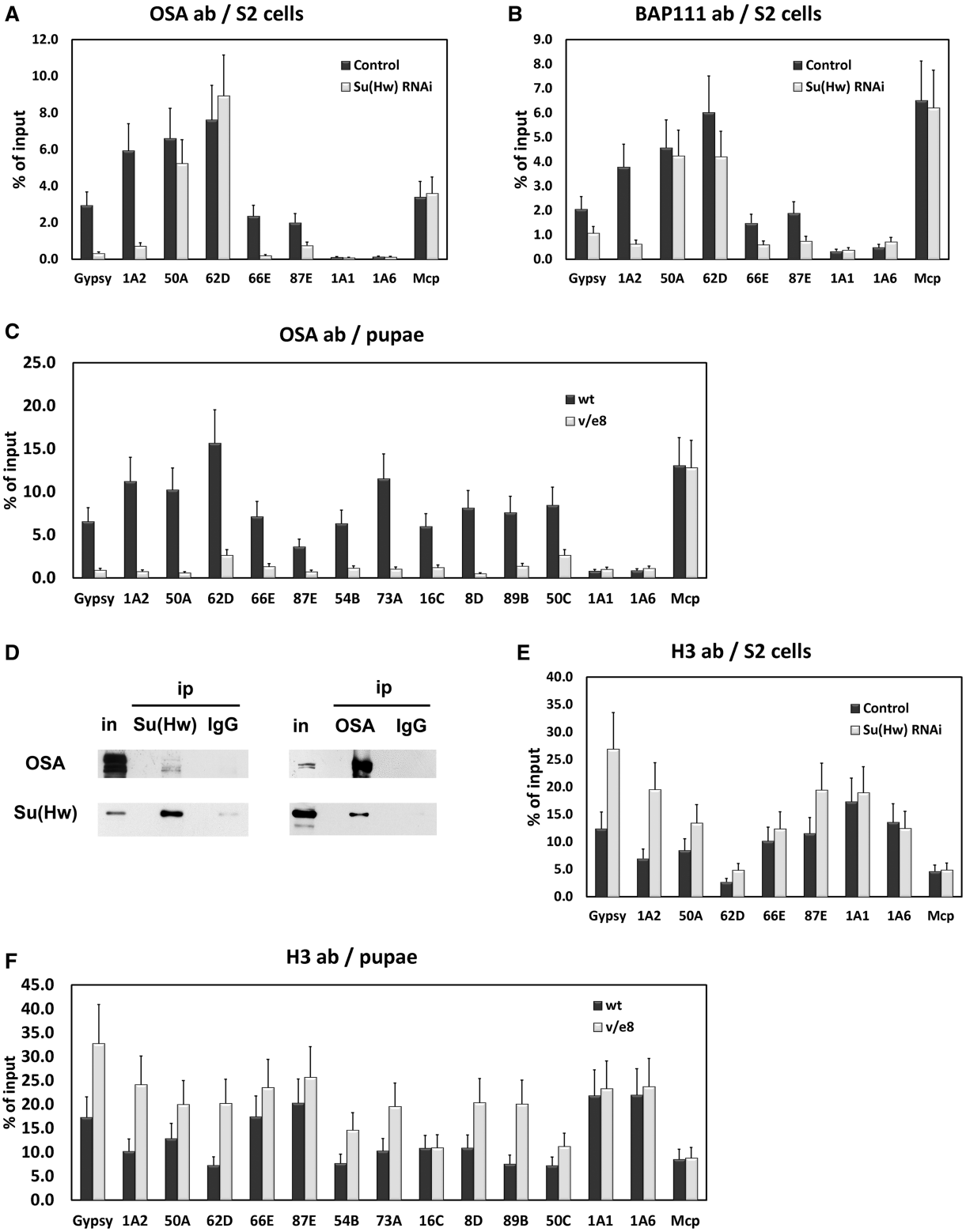

Figure 2. Su(Hw) recruits chromatin remodeler Brahma. (**A**, **B**) The levels of (A) OSA and (B) BAP111 on Su(Hw)-binding sites in control S2 cell (dark bars) and after Su(Hw) knockdown (light bars) as evaluated by ChIP analysis. Results are expressed as percentage of DNA input. Error bars show standard errors of the means from three replicate experiments. Sites 1A1 and 1A6 were used as a negative control. The Mcp insulator was used as a reference site. (C) The levels of OSA on Su(Hw)-binding sites in wild-type (dark bars) and $Su(Hw)^{V/E8}$ mutant pupae (light bars) as evaluated by ChIP analysis. (**D**) Co-immunoprecipitation of Su(Hw) and OSA from *Drosophila* embryo nuclear extract by rabbit antibodies against each of these proteins; IgG from rabbit pre-immune serum was used as a negative control. Ten percent portions of the input nuclear fraction (in) and immunoprecipitated fraction (ip) were resolved by SDS–PAGE and western blotted with antibodies against Su(Hw) and OSA. (**E**, **F**) Histone H3 levels on Su(Hw)-binding sites in (E) *Drosophila* S2 cells and (F) pupae as evaluated by ChIP analysis. The dark bars refer to control cells or wild-type pupae; light bars, to Su(Hw)-knockdown cells or $Su(Hw)^{V/E8}$ mutant pupae.

Whole-genome analysis of Su(Hw)-binding sites for association with other factors

To gain an insight into Su(Hw)-associated chromatin organization in general, we developed a software allowing analysis of ChIP-Seq and ChIP-chip data on any set of genomic positions. We identified the exact positions of 3120 Su(Hw) peaks in the genome and then calculated the average genomic distributions of different factors in the regions between −5 and +5 kb relative to a Su(Hw) peak (see 'Materials and Methods' section). This approach allowed us to visualize ChIP-Seq data and calculate the average level of the factor of interest on Su(Hw) peaks relative to surrounding sequences (the background level). To substantiate this approach, we calculated average profiles of CP190 and Mod(mdg4) proteins, which are known to strongly associate with Su(Hw) (3,49). Distinct peaks of CP190 and Mod(mdg4) proved to coincide with Su(Hw) genomic positions, confirming the strong association between these proteins (Figure 3A). As a negative control, we calculated the profile of Pol II around Su(Hw)-binding sites and revealed no colocalization of Pol II with Su(Hw) genomic positions (Figure 3C). This result is consistent with the fact that promoter association has been reported for Class I insulator proteins, but not for Su(Hw) (48,50). In addition, we performed ChIP with an antibody against Pol II in S2 cell and observed no enrichment in Pol II on Su(Hw)-binding sites (data not shown). At the next step, we considered genome-wide data on chromatin structure at Su(Hw) peaks. As shown previously, binding sites for insulator-associated proteins are regions of reduced nucleosome density, although nucleosome depletion on Su(Hw)-binding sites is not as significant as on CTCF sites (48). Analyzing new high-quality ChIP-Seq data on histones H1, H3 and H4 in S2 cells, we found that Su(Hw)-binding sites are regions with a low nucleosome density (Figure 3B).

ORC is recruited to Su(Hw)-binding sites

Although Su(Hw)-binding sites do not contain Pol II, they share certain properties with the promoters of active genes, such as the ability to recruit the histone acetyltransferase complex SAGA and chromatin remodeler Brahma; in addition, both promoters and Su(Hw)-binding sites are regions with a low nucleosome density. Therefore, it could be assumed that these sites may have some other properties in common with the promoters of active genes. To check this assumption, we used our bioinformatics approach to analyze the genomic distribution of different factors included in the modENCODE database and revealed a strong association of ORC2 protein and MCM2-7 helicase complex with Su(Hw)-binding sites (Figure 3C). ORC and MCM2-7 complexes frequently localize near the transcription start sites of actively transcribed genes (51) and play a critical role in the formation and firing of replication origins (20). The sites of ORC enrichment in *Drosophila* are depleted in bulk nucleosomes (21) and undergo active nucleosome turnover as shown by the CATCH-IT technique (24). We analyzed the CATCH-IT profiles for Su(Hw)-binding sites and revealed a peak of nucleosome turnover at all test time points (20, 40 and 60 min), which confirmed the active state of chromatin at these sites (Figure 3D).

ORCs frequently localize near the transcription start sites of actively transcribed genes and bind preferentially to AT-rich regions (51,52). Meanwhile, Cayrou *et al.* (53,54) have found that replication origins are generally associated with higher GC content, as follows from the results of nascent strand analysis (55). We decided to find out whether Su(Hw)-binding sites are similar in this respect. First, we analyzed the AT content of all ORC-binding sites in the *Drosophila* genome and found that ORC-binding sites contain both GC-rich and AT-rich regions (Figure 3E), with a GC-rich region occupying the central position and AT-rich regions being located on both sides of it. Thus, our finding consolidates all previous data about the AT/GC content of replication sites. Next, we calculated the AT content of all Su(Hw)-binding sites and found them to be structurally similar to ORC-binding sites in this respect (Figure 3E).

To verify the results of the bioinformatics assay, we used ChIP analysis in *Drosophila* S2 cells to test for the presence of ORC2, ORC3 and ORC6 proteins on Su(Hw)-binding sites. The results confirmed the recruitment of all these subunits of the ORC complex to Su(Hw)-binding sites (Figure 4A, dark bars; Supplementary Figure S2A and B). To check the role of Su(Hw) protein in this process, we examined the targeting of ORC3 after RNAi knockdown of Su(Hw) in S2 cell and found that the level of this subunit dropped on all tested Su(Hw)-dependent insulators except 62D and 50A (Figure 4A). Analysis of ORC3 binding in $Su(Hw)^{V/E8}$ mutant pupae, in which this protein was absent from its binding sites, showed that Su(Hw) is strongly required for ORC binding (Figure 4C). To test for the protein–protein interaction between Su(Hw) and the ORC complex, we performed a co-immunoprecipitation experiment with corresponding antibodies and found that the anti-Su(Hw) antibody precipitated not only Su(Hw) but also a portion of ORC3 and vice versa (Figure 4B). The anti-Su(Hw) antibody co-precipitated ~20% of GCN5 (Figure 1F), 2% of ORC3 (Figure 4B) and 0.5% of OSA (Figure 2D). Thus, co-IP experiments confirmed that Su(Hw) associates with SAGA, BAP and ORC complexes. The strong interaction of Su(Hw) with GCN5 may be regarded as evidence for its direct association with the SAGA complex, whereas the weak interaction of Su(Hw) with BAP and ORC complexes could be explained by some indirect protein interactions. In addition, the fairly weak interaction of Su(Hw) and ORC may also be explained by the fact that Su(Hw) constitutes only a small proportion of ORC-binding sites in the genome (see later in the text).

CDC45 is loaded onto the pre-RC complex on Su(Hw)-binding sites

To ensure proper replication of the genome during S-phase, eukaryotic cells produce multiple replication origins and then a certain part of them fire in the context of developmental stage or growth conditions

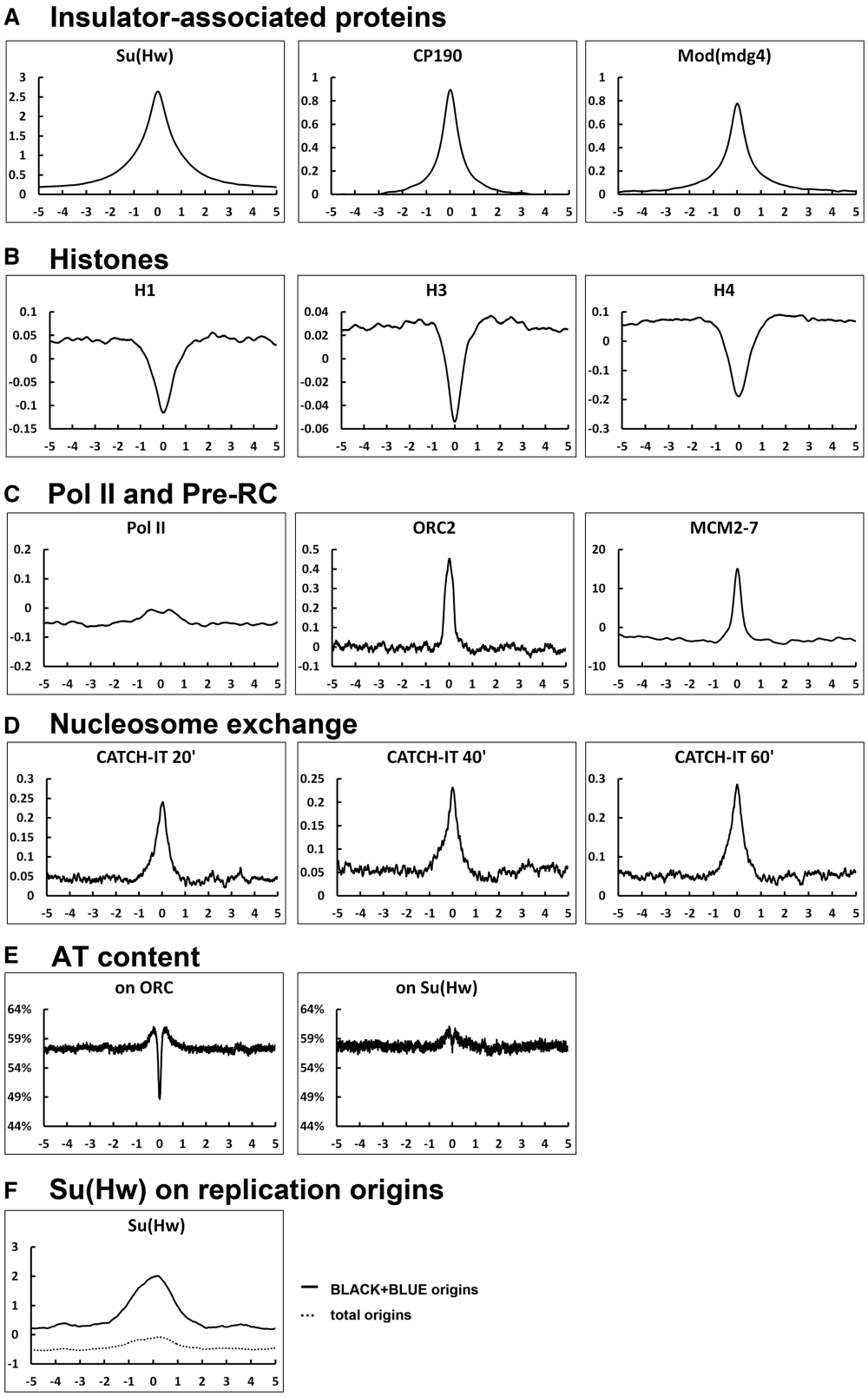

Figure 3. Whole-genome analysis of Su(Hw)-binding sites for association with other factors. (**A–D**) Plots of average log2 enrichment ratios for indicated factors at positions −5 to +5 kb relative to Su(Hw) peaks: (A) insulator-associated proteins Su(Hw), CP190 and Mod(mdg4) in S2 cells; (B) histones H1, H3 and H4 in S2 cells; (C) Pol II, ORC2 and MCM2-7 in S2 cells; (D) nucleosome exchange profiles: CATCH-IT at 20-, 40- and 60-min time points. (**E**) The distribution of average AT contents of total ORC and Su(Hw)-binding sites. (**F**) Plots of average log2 enrichment ratios for Su(Hw) at positions −5 to +5 kb relative to replication origins. Profiles were calculated taking into account either all replication origins (dotted line) or only the origins localized in BLACK and BLUE chromatin (solid line).

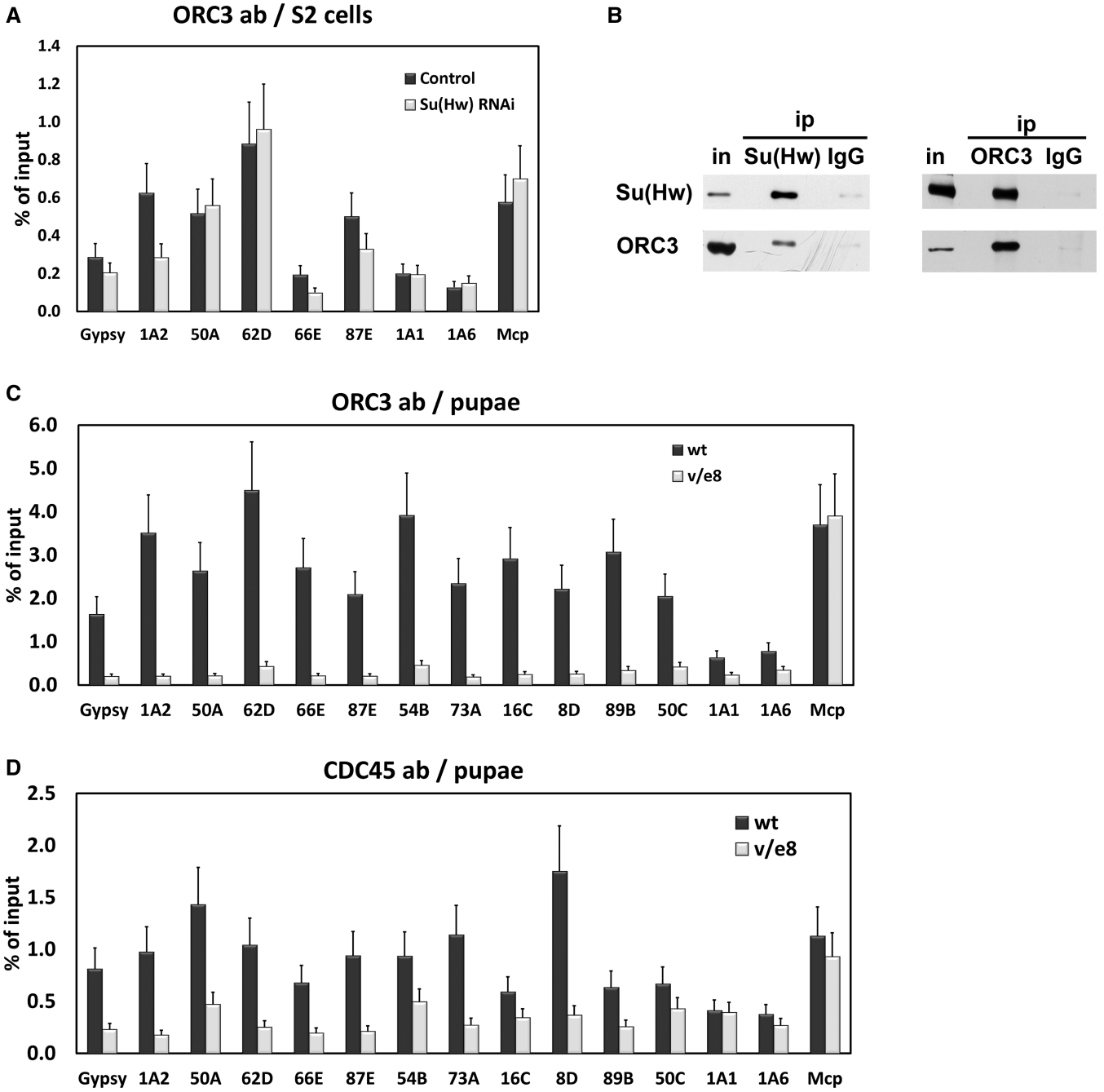

Figure 4. Su(Hw) recruits ORC. (**A**) The levels of ORC3 on Su(Hw)-binding sites in control S2 cell (dark bars) and after Su(Hw) knockdown (light bars) as evaluated by ChIP analysis. The results are expressed as the percentage of DNA input. Error bars show standard errors of the means from three replicate experiments. Sites 1A1 and 1A6 were used as a negative control. The Mcp insulator was used as a reference site. (**B**) Co-immunoprecipitation of Su(Hw) and ORC3 from *Drosophila* embryo nuclear extract by rabbit antibodies against each of these proteins; IgG from rabbit pre-immune serum was used as a negative control. Ten percent portions of the input nuclear fraction (in) and immunoprecipitated fraction (ip) were resolved by SDS–PAGE and western blotted with antibodies against Su(Hw) and ORC3. (**C**, **D**) ORC3 and CDC45 levels on Su(Hw)-binding sites in wild-type pupae (dark bars) and $Su(Hw)^{V/E8}$ mutant pupae (light bars) as evaluated by ChIP analysis.

(20). Therefore, it was relevant to find out whether Su(Hw)-binding sites may be used as replication origins. We used ChIP analysis to test Su(Hw)-binding sites for the presence of the CDC45 protein, which is known to be recruited to pre-RC before loading of DNA polymerase and to mark replication origins ready to fire (56–58). The results of ChIP experiments in pupae confirmed the recruitment of CDC45 to Su(Hw)-binding sites, although the content of CDC45 on different sites varied considerably (Figure 4D): in particular, it was close to the background level on three sites (16C, 89B, 50C), whereas site 8D showed considerable enrichment with this protein. On the other hand, the content of ORC3 level on these sites was similar and significantly exceeded the background

level (Figure 4C). An analysis of CDC45 level in $Su(Hw)^{V/E8}$ mutant pupae revealed that Su(Hw) is required for CDC45 loading onto Su(Hw)-binding sites, whereas Su(Hw) depletion does not affect the CDC45 level on the Mcp insulator. Our bioinformatics approach showed that the MCM2-7 helicase complex is associated with Su(Hw)-binding sites (Figure 3C). Thus, we found that Su(Hw)-binding sites recruit the ORC and MCM2-7 complexes, and then CDC45 is loaded onto pre-RC in part of these sites.

Su(Hw)-binding sites constitute considerable part of ORC-binding sites in BLACK and BLUE chromatin

We found that all tested Su(Hw)-binding sites recruited SAGA, Brahma and ORC complexes. In addition, analysis of ChIP-Seq data confirmed whole-genome association of these complexes with nucleosome-free regions at Su(Hw)-binding sites. Therefore, it may be concluded that considerable part of Su(Hw)-binding sites recruit the ORC complex.

Meanwhile, recent studies have demonstrated that majority of replication origins associate with the promoters of active genes, whereas Su(Hw) tends to be localized in transcriptionally inactive chromatin regions termed BLACK and BLUE chromatin according to the color-coded classification (59). We calculated the distribution of replication origins in different chromatin types and found that 80% of replication origins are localized in RED or YELLOW chromatin, whereas 12 and 5% are in BLUE and BLACK chromatin, respectively (Supplementary Figure S2C). In contrast, 86% of Su(Hw)-binding sites are localized in BLACK and BLUE chromatin (Supplementary Figure S2C).

We calculated the average profile of Su(Hw) protein on all ORC-binding sites in the *Drosophila* genome and found no considerable enrichment of Su(Hw) on these sites (Figure 3F, dotted line). Thus, the majority of replication origins localized in RED and YELLOW chromatin do not contain Su(Hw), on average. As the Su(Hw) protein preferentially localizes in BLACK and BLUE chromatin, we calculated the average Su(Hw) profile on replication origins within these chromatin regions and revealed a distinct peak of Su(Hw) (Figure 3F, solid line). Thus, a considerable part of replication origins localized in BLACK and BLUE chromatin contain Su(Hw). Analysis of the whole-genome Su(Hw) profile showed that Su(Hw) is present on 6% of all ORC-binding sites in the *Drosophila* genome.

Artificial Su(Hw) recruitment is sufficient for subsequent recruitment of SAGA, Brahma and ORC

To further investigate the role of Su(Hw) in the positioning of replication origins, it was important to determine whether the presence of Su(Hw) is sufficient for recruiting the SAGA, Brahma and ORC complexes to Su(Hw)-binding sites. To this end, we constructed an S2 cell line stably expressing $3 \times$ FLAG-tagged GAL4-binding domain fused to the N-terminus of Su(Hw) and assessed its ability to recruit GCN5, OSA and ORC3 subunits to GAL4 DNA-binding sites. An S2 cell line stably expressing $3 \times$ FLAG_GAL4BD alone was used as a negative control. Both plasmid constructs used for generating these cell lines contained $10 \times$ GAL4-binding sites ($10 \times$ UAS) and blasticidin-resistance gene (Supplementary Figure S2D). ChIP analysis with an anti-FLAG antibody showed that $3 \times$ FLAG_GAL4BD bound to $10 \times$ UAS more effectively than did $3 \times$ FLAG_GAL4BD_Su(Hw) (Figure 5A). Nevertheless, the GCN5, OSA and ORC3 subunits were much more actively recruited to $10 \times$ UAS in the $3 \times$ FLAG_GAL4BD-Su(Hw) line than in the $3 \times$ FLAG_GAL4BD line (Figure 5B). This is evidence that Su(Hw) is necessary and sufficient for the recruitment of SAGA, Brahma and ORC complexes to Su(Hw)-binding sites.

Su(Hw)-binding sites account for direct positioning of SAGA, Brahma and ORC

To test the role of Su(Hw) binding sites in positioning of replication origins, we used the 'yellow' regulatory system as a model. The 'yellow' gene is responsible for dark pigmentation of adult cuticle and bristles. Two upstream enhancers provide for its activation in the body cuticle and wing blades (60). We used the Wen-Ben-$4 \times$ SBS-Y construct, in which the third Su(Hw)-binding site from *gypsy* retrotransposon was $4 \times$ multiplexed ($4 \times$ SBS) and inserted 900 bp upstream of the 'yellow' transcription start site, separating the wing (Wen) and body (Ben) enhancers from the promoter (Supplementary Figure S2E). In all seven Wen-Ben-$4 \times$ SBS-Y transgenic lines, the $4 \times$ SBS insertion completely blocked the wing and body enhancers (data not shown), which was evidence for the functional activity of this insulator. Next, we used ChIP analysis to test for the presence of OSA, GCN5 and ORC3 on $4 \times$ SBS in transgenic pupae. The results showed that $4 \times$ SBS readily recruited SAGA, Brahma and ORC (Figure 5C), suggesting that Su(Hw)-binding sites govern the positioning of these complexes. Thus, Su(Hw)-binding sites are capable of directly recruiting the ORC complex, which is in line with our results obtained in experiments with an artificial Su(Hw) recruitment.

Su(Hw), CTCF, GAF and BEAF32 possess common chromatin remodeling and ORC-recruiting properties

Having found that Su(Hw)-binding sites comprise 6% of ORC-binding sites in the *Drosophila* genome, we assumed that the remaining 94% of these sites are constituted by other DNA-binding proteins that bind to different regulatory elements, including promoters and insulators. To test this assumption, we analyzed Class I insulator proteins such as CTCF, GAF and BEAF32 for chromatin remodeling and ORC-recruiting properties. We also analyzed random sites as a negative control. To additionally validate our software, we analyzed the distribution of RNA Polymerase II on binding sites for the test proteins and confirmed the known fact that Su(Hw) belongs to Class II insulators, which do not associate with Pol II, whereas CTCF, GAF, and BEAF32 belong to Class I insulators, which frequently colocalize with Pol II in the genome (Figure 6A). Whole-genome analysis of ORC2 and MCM2-7 for Su(Hw) and other insulator proteins showed that ORC2 and MCM2-7 colocalized with

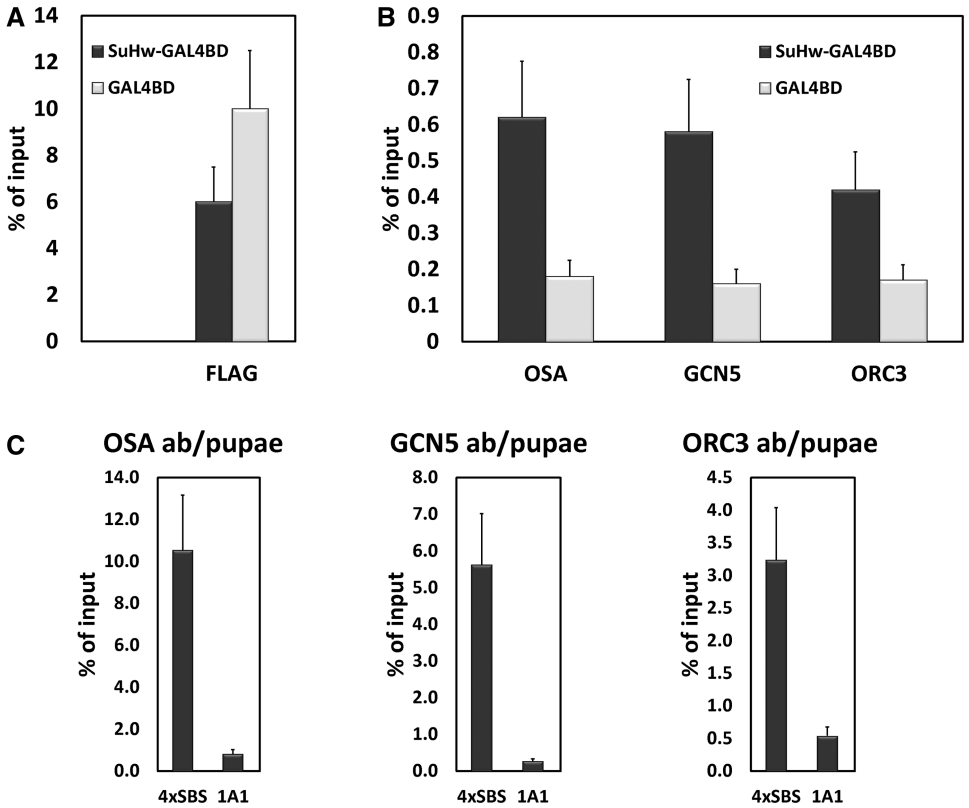

Figure 5. Su(Hw) is sufficient for recruiting SAGA, BAP and ORC. (**A**) Recruitment of 3 × FLAG-GAL4BD–SuHw (dark bar) or 3 × FLAG-GAL4BD alone (light bar) to 10 × UAS as evaluated by ChIP with an antibody against the FLAG epitope in two S2 cell lines stably expressing those proteins. (**B**) Recruitment of OSA, GCN5 and ORC3 to 10 × UAS in S2 cell lines stably expressing 3 × FLAG-GAL4BD–SuHw (dark bars) or 3 × FLAG-GAL4BD alone (light bars). (**C**) Recruitment of OSA, GCN5 and ORC3 to the 4 × Su(Hw)-binding site in transgenic pupae.

binding sites for all insulator proteins tested, whereas analysis of random sites revealed no enrichment on these sites (Figure 6A). Su(Hw)-binding sites showed weaker activity in ORC recruitment, compared with other insulators, which is consistent with chromatin structure on Su(Hw) sites (see later in the text). We analyzed histone density (histones H1, H3, H4) (Figure 6B) and dynamic exchange (CATCH-IT) (Figure 6C) on Su(Hw), dCTCF, BEAF-32, GAF and random sites and found that all tested insulator proteins have common chromatin remodeling properties. Class I insulator proteins are more active than Su(Hw) in this respect, which correlates with pre-RC loading ability of these regions. Thus, CTCF, GAF and BEAF32 have common chromatin remodeling and ORC-recruiting properties and, therefore, are good candidates for proteins that play a role in the positioning of ORC, as does Su(Hw). We consider that the high activity of Class I binding sites may be explained by the fact that these sites coincide with promoters of active genes, and therefore the calculated profiles represent the result of coordinated action of both Class I insulator proteins and transcriptional activators. In this respect, Su(Hw)-binding sites provide a unique model for investigating molecular mechanisms of ORC positioning, which is not complicated by transcription-specific events and factors.

DISCUSSION

The results of this study show that Su(Hw) recruits the histone acetyltransferase complex SAGA and chromatin remodeler Brahma to Su(Hw)-binding sites, which results in the appearance of regions with low nucleosome density and creates conditions for ORC binding. The depletion of Su(Hw) leads to a dramatic drop in the levels of SAGA, Brahma and ORC subunits and a significant increase in nucleosome density on Su(Hw)-binding sites. Su(Hw) is necessary and sufficient for chromatin remodeling and ORC recruitment. Our bioinformatics approach shows that the MCM2-7 helicase complex is associated with Su(Hw)-binding sites (Figure 3C). The results of ChIP experiments provide evidence for the recruitment of CDC45 to Su(Hw)-binding sites and the necessity of Su(Hw) for this (Figure 4D). Thus, Su(Hw) creates conditions for ORC binding, the MCM2-7 helicase complex binds to the ORC platform and then CDC45 loads onto pre-RC. Different Su(Hw)-binding sites recruit different levels of CDC45, whereas ORC3 levels on all these sites are more or less similar (Figure 4C). Thus, Su(Hw) creates the platform for Pre-RC assembly on all tested Su(Hw)-binding sites, and then some of these sites are further activated.

Su(Hw) belongs to Class II insulator proteins and, unlike Class I insulator proteins, is not associated with

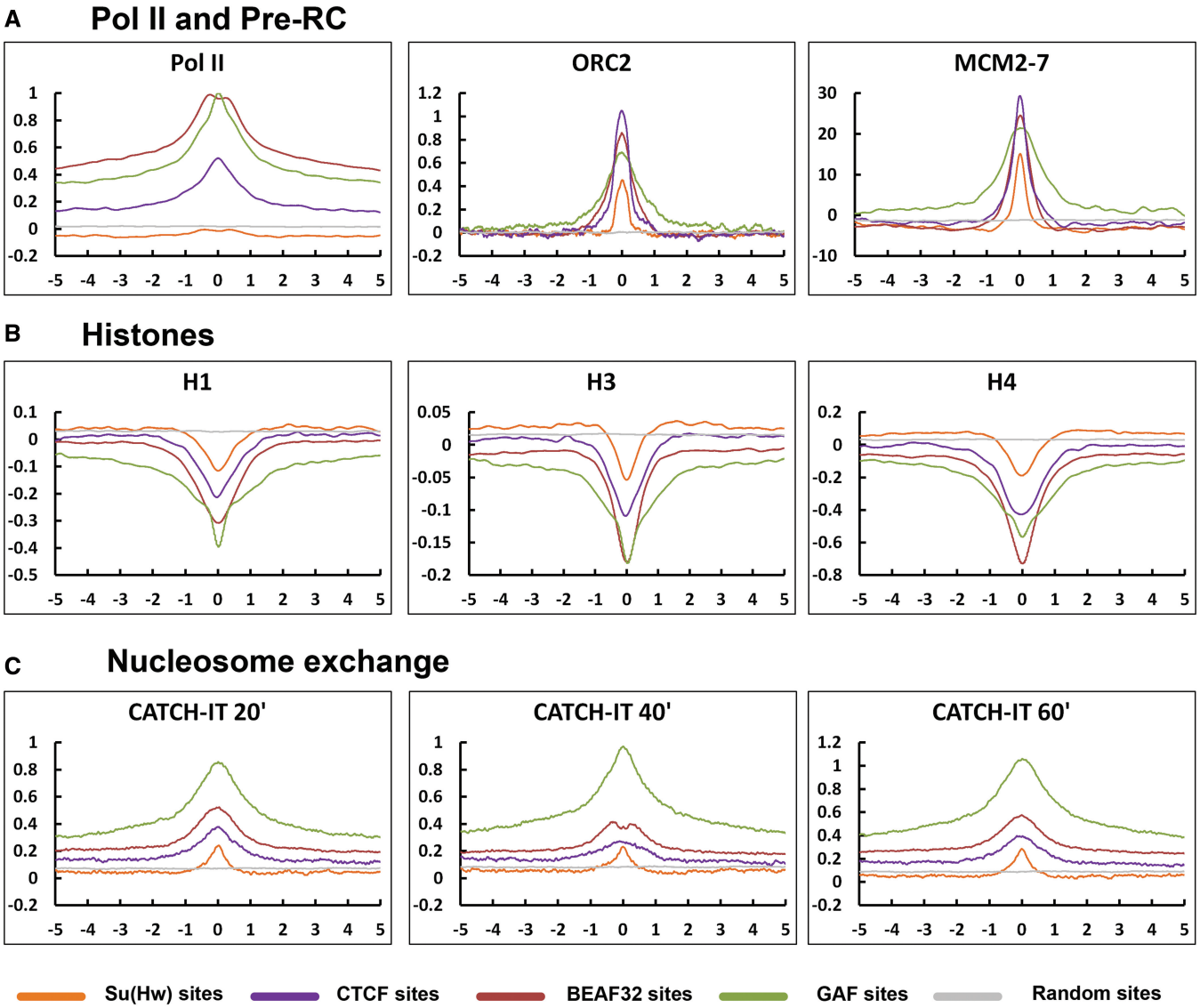

Figure 6. Whole-genome analysis of Su(Hw), CTCF, GAF and BEAF32-binding sites. (**A**–**C**) Plots of average log2 enrichment ratios for indicated factors at positions −5 to +5 kb relative to Su(Hw), CTCF, GAF and BEAF32-binding sites. Random sites were used as a negative control. (A) Pol II, ORC2 and MCM2-7 in S2 cells. (B) Histones H1, H3 and H4 in S2 cells. (C) Nucleosome exchange profiles. CATCH-IT profiles at 20-, 40- and 60-min time points.

Pol II or promoters of active genes (48,50). Moreover, Su(Hw) tends to localize in transcriptionally inactive chromatin regions termed BLACK and BLUE chromatin according to color-coded classification (59). Nevertheless, we have found that Su(Hw) interacting with Su(Hw)-dependent insulators uses the same mechanism of chromatin opening as in the case of transcriptional activators binding to promoter sequences, namely, the recruitment of SAGA and Brahma complexes to a given genomic site. Our data also show that the ORC complex is recruited to Su(Hw)-dependent insulators and to the promoters of active genes. Moreover, analysis of chromatin structure provides evidence for active histone turnover at Su(Hw)-binding sites, which is a characteristic feature of replication origins (24). As we have revealed common chromatin remodeling and ORC-recruiting properties in regulatory elements so different as promoters and Su(Hw)-dependent insulators, it can be assumed that the cell has a universal mechanism for positioning replication origins to any open chromatin region. This allows the cell to assemble multiple replication origins at definite positions determined by specific DNA-binding proteins that create proper chromatin structure for ORC binding. As shown in several studies, open chromatin is associated with all known classes of active DNA regulatory elements, including promoters, enhancers, silencers, insulators and locus control regions (61,62). Thus, this universal mechanism may allow the cell to connect transcription and replication events at various regulatory elements of the genome, thereby coupling different levels of transcriptional regulation with the replication program. We suggest that the key determinants of ORC positioning in the genome are

DNA-binding proteins constituting different DNA regulatory elements, including insulators, promoters and enhancers. Su(Hw) is the first example of such a protein, which determines the formation of 6% of ORC-binding sites in the genome. As for the majority of replication origins that are localized to RED and YELLOW chromatin, the DNA-binding transcription activators are probable candidates for such proteins because many transcription factors have been shown to be involved in interactions with pre-RC components and in ORC targeting (27,29,30,63). Our bioinformatics approach provides evidence that Su(Hw) and Class I insulator proteins have common chromatin remodeling and ORC-recruiting properties, indicating that CTCF, GAF and BEAF32 are also probable candidates for such proteins.

It has been shown that replication origins are highly enriched with chromatin-remodeling complexes, including NURF and SWI/SNF (22,25), which suggests a role for chromatin remodeling in ORC recruitment. We have found that Su(Hw) recruits the histone acetyltransferase complex SAGA and chromatin remodeler Brahma to Su(Hw)-binding sites, which results in the appearance of regions with low nucleosome density. On this basis, it appears that the same protein that determines ORC positioning is also responsible for the loading of chromatin remodeling complex and formation of low-nucleosome regions. These data further support the idea that chromatin remodeling has a role in ORC positioning.

Analyzing the AT content of total ORC-binding sites in the *Drosophila* genome, we have found that ORC-binding sites contain both GC-rich and AT-rich regions. This is in agreement with previous findings that ORC binds preferentially to AT-rich regions (51) and that the *Drosophila* Orc6 subunit is required for ORC binding and prefers polyA sequences (52). We suggest that the GC-rich region in central position is used as a specific binding site for proteins constituting replication origins. Cayrou *et al.* (53,54) also suggest that GC-rich regions may be used as specific binding sites for factors controlling nascent strand synthesis. We found that the nucleotide structure of Su(Hw)-binding sites is similar to that of total ORC-binding sites, which is additional evidence that Su(Hw) is one of the proteins responsible for ORC positioning.

We consider that ORC lacks apparent sequence specificity (19,21) because the positions of replication origins are determined by a variety of proteins that have different binding sites on the DNA. In support of this idea, we have shown that a Su(Hw)-binding site itself can govern the positioning of the ORC-binding site *in vivo*. Thus, Su(Hw) appears to be the first example of a protein, which determines ORC positioning in genome. Su(Hw) constitutes 6% of all ORC-binding sites, and these 6% are preferentially localized in BLACK and BLUE chromatin.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2, Supplementary Figures 1 and 2 and Supplementary Methods.

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Conflict of interest statement. None declared.

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