Histone Chaperones ASF1 and NAP1 Differentially Modulate Removal of Active Histone Marks by LID-RPD3 Complexes during NOTCH Silencing

Yuri M. Moshkin,1 Tsung Wai Kan,1 Henry Goodfellow,3 Karel Bezstarosti,2 Robert K. Maeda,4 Maxim Pilyugin,4 Francois Karch,4 Sarah J. Bray,3 Jeroen A.A. Demmers,2 and C. Peter Verrijzer1,*

1Department of Biochemistry, Center for Biomedical Genetics
2Proteomics Center
Erasmus University Medical Center, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands
3Department of Physiology, Development and Neuroscience, University of Cambridge, Downing Street, Cambridge CB2 3DY, UK
4Department of Zoology and National Research Center Frontiers in Genetics, University of Geneva, 30 quai E. Ansermet, 1211 Geneva-4, Switzerland
*Correspondence: c.verrijzer@erasmusmc.nl
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SUMMARY

Histone chaperones are involved in a variety of chromatin transactions. By a proteomics survey, we identified the interaction networks of histone chaperones ASF1, CAF1, HIRA, and NAP1. Here, we analyzed the cooperation of H3/H4 chaperone ASF1 and H2A/H2B chaperone NAP1 with two closely related silencing complexes: LAF and RLAF. NAP1 binds RPD3 and LID-associated factors (RLAF) comprising histone deacetylase RPD3, histone H3K4 demethylase LID/KDM5, SIN3A, PF1, EMSY, and MRG15. ASF1 binds LAF, a similar complex lacking RPD3. ASF1 and NAP1 link, respectively, LAF and RLAF to the DNA-binding Su(H)/Hairless complex, which targets the E(spl) NOTCH-regulated genes. ASF1 facilitates gene-selective removal of the H3K4me3 mark by LAF but has no effect on H3 deacetylation. NAP1 directs high nucleosome density near E(spl) control elements and mediates both H3 deacetylation and H3K4me3 demethylation by RLAF. We conclude that histone chaperones ASF1 and NAP1 differentially modulate local chromatin structure during gene-selective silencing.

INTRODUCTION

Regulated modulation of the chromatin structure is essential for the transmission, maintenance, and expression of the eukaryotic genome. The combined actions of ATP-dependent chromatin-remodeling factors (remodelers), histone chaperones, and histone-modifying enzymes drive chromatin dynamics. Histones are subjected to a wide range of reversible posttranslational modifications, including acetylation, phosphorylation, methylation, and ubiquitylation (Berger, 2007). Histone modifications, in turn, can promote the recruitment of selective regulatory factors and modulate chromatin accessibility. Chromatin remodelers control DNA accessibility by mediating nucleosome mobilization either through sliding or by nucleosome (dis)assembly (Mohrmann and Verrijzer, 2005).

Whereas originally considered mainly as mere chaperones, it has become clear that histone chaperones play diverse roles during chromatin transactions (De Koning et al., 2007; Eitoku et al., 2008). Histone chaperones bind selective histones and include the highly conserved H3/H4 chaperones ASF1, CAF1, HIRA, and Spt6 and the H2A/H2B chaperones NAP1, Nucleoplasmin, and FACT (De Koning et al., 2007; Eitoku et al., 2008). Although their biochemical activity, binding and release of histones, appears rather mundane, in conjunction with other factors, histone chaperones participate in a variety of chromatin transactions and other cellular tasks. For example, yeast NAP1 participates in an extensive interaction network including a diverse set of transcription initiation/elongation factors, chromatin remodelers, RNA-processing factors, cell-cycle regulators, and other proteins (Del Rosario and Pemberton, 2008; Mosammaparast et al., 2005; Walfridsson et al., 2007; Zlatanova et al., 2007).

ASF1 is one of the major H3/H4 chaperones, and through association with other proteins, it contributes to diverse chromatin transactions (De Koning et al., 2007; Eitoku et al., 2008). (1) In conjunction with CAF1 and the MCM2-7 DNA helicase, ASF1 participates in replication-coupled chromatin assembly (Groth et al., 2007; Tyler et al., 1999, 2001). (2) When associated with HIRA, ASF1 participates in replication-independent chromatin assembly and histone replacement (Green et al., 2005; Zhang et al., 2007). (3) DNA-repair-associated chromatin assembly requires the cooperation between ASF1 and the H3K56 acetyltransferase Rtt109 (Chen et al., 2008). (4) ASF1 functionally cooperates with the Drosophila BRM chromatin remodeler (Moshkin et al., 2002), and (5) interaction of ASF1 with transcription activators stimulates histone eviction from promoter areas and facilitates recruitment of chromatin-specific coactivator complexes (Adkins et al., 2007). (6) ASF1 itself is one of the targets of Tousled-like kinase (TLK), which controls cell-cycle progression and chromatin dynamics (Carrera et al., 2003). (7) Finally, ASF1 is involved in developmental gene expression control by mediating transcriptional repression of NOTCH target genes (Goodfellow et al., 2007). ASF1 is recruited to E(spl) genes...
by the sequence-specific DNA-binding protein Su(H) and its associated corepressor complex, harboring Hairless (H) and SKIP. NOTCH is the central component of a highly conserved developmental signaling pathway that is present in all metazoans. NOTCH is a single-pass transmembrane protein that is activated through ligand binding, resulting in the release of the NOTCH intracellular domain (Nicd), which is targeted to the nucleus to activate gene expression (Artavanis-Tsakonas et al., 1999; Bray, 2006). The CSL (CBF1, Su(H), and Lag1) family of sequence-specific DNA-binding proteins is the key targeting factor of Nicd and coactivators and, in the absence of Nicd, corepressors. The repression of NOTCH target genes involves the sequence-specific DNA-binding proteins is the key targeting factor of Nicd and coactivators and, in the absence of Nicd, corepressors. The repression of NOTCH target genes involves multiple chromatin-modifying activities including histone deacetylases, H3K9 methyltransferases, CtBP, NcoR/SMRT, and Goucho (GRO) (Nagel et al., 2005; Perissi et al., 2008; Shi et al., 2003). In the absence of the Nicd, loss of ASF1 leads to derepression of the $E(spl)$ genes, revealing its essential role in silencing (Goodfellow et al., 2007).

The molecular mechanism by which ASF1 achieves gene-specific transcription repression and the potential roles of other histone chaperones in developmental gene regulation remains largely unknown. To address these issues, we first performed a proteomics survey of the protein interaction networks of ASF1, CAF1, HIRA, and NAP1 in Drosophila embryos. Our analysis revealed that ASF1 and NAP1 interact with two related but distinct corepressor complexes: LAF and RLAF. LAF, comprising LID/KDM5 SIN3A, PF1, EMSY, and MRG15, associates with ASF1 (forming LAF-A). RLAF, comprising LAF plus RPD3, interacts with NAP1 (forming RLAF-N). Through a combination of biochemistry and developmental genetics, we established that LAF-A and RLAF-N are tethered to NOTCH target genes by the Su(H)/H complex and mediate gene-selective silencing. Both ASF1 and NAP1 are required for the targeted removal of the positive H3K4me3 mark by facilitating LID/KDM5 recruitment to chromatin. Furthermore, NAP1 mediates nucleosome assembly at regulatory elements of NOTCH target genes and histone deacetylation by RLAF. Our results uncover extensive crosstalk between distinct histone chaperones and histone-modifying enzymes in developmental gene regulation.

RESULTS

**ASF1 Is a Hub in a Protein-Protein Interaction Network**

To determine the ASF1 regulatory network, we made an inventory of its associated proteins in developing Drosophila embryos and S2 cells. We prepared nuclear extracts (NE) from 0–12 hr embryos, encompassing up to 15 stages of embryonic development, and from S2 cells. Nuclear extracts were incubated with protein A Sepharose beads coated with either affinity-purified $\alpha$-ASF1 antibodies or $\alpha$-GST antibodies as control. Following extensive washes with a buffer containing 600 mM KCl and 0.1% NP-40, bound and unbound material were resolved by SDS polyacrylamide electrophoreses (SDS-PAGE) and visualized by Coomassie staining (Figures 1A and 1B and Tables S1 and S2 available online). Mass spectrometric analysis revealed the association of ASF1 with known as well as additional factors. Previously documented ASF1-binding proteins include the CAF1 complex (Tyler et al., 1999, 2001), HIRA and its associated factor yemalpha/UBN1 (Banumathy et al., 2009), and the core histones. TLK (Carrera et al., 2003) and MCM2 (Groth et al., 2007) were only detected in the ASF1 purification from Drosophila embryo nuclear extract (Figure 1A). The ASF1-associated factors identified in both experiments included SIN3A (Silverstein and Ekwall, 2005), histone H3K4me2/3 demethylase LID/KDM5 (Eissenberg et al., 2007; Lee et al., 2007; Secombe et al., 2007), EMSY/CG15356 (Hughes-Davies et al., 2003), PF1/CG3815 (Yochum and Ayer, 2001), and MRG15 (Yochum and Ayer, 2002). To obtain more evidence for stable association of ASF1-binding proteins, we partially purified embryo nuclear extract by conventional column chromatography prior to the ASF1 immunopurification step. ASF1 containing peak fractions on the final S300 column were pooled, immunopurified, and analyzed by mass spectrometry (Figure 1C and Table S3). SIN3A, PF1, EMSY, MRG15, and the histone H3K4 demethylase LID were confirmed as prominent ASF1-associated proteins, in addition to the well-established CAF1 and HIRA.

To establish the in vivo significance of the interactions between ASF1 and some of the ASF1-associated factors, we used a genetic assay. Overexpression of ASF1 in the fly eye leads to mildly disrupted eye development characterized by reduced size and disorganized facets, providing us with a convenient tool for genetic interaction analysis (Goodfellow et al., 2007; Moshkin et al., 2002). To drive ASF1 overexpression in the developing eye, we used an eyeless-Gal4, UAS-ASF1 fly strain (ey-ASF1). The ey-ASF1 line was crossed with available mutants, namely $lid$ and Sin3A. In heterozygous combinations, two different $lid$ mutant alleles ($lid^{D424}$ and $lid^{D690/7}$) and the Sin3A$^{D269}$ allele showed significant suppression of the ey-ASF1 rough-eye phenotype (Figure 1D). These genetic interactions indicate that ASF1 cooperates with SIN3A and LID in vivo. These results suggest that the network of ASF1-interacting factors is substantially more extensive than previously anticipated.

**Proteomic Characterization of ASF1-Interacting Factors**

Sephacryl S-300 size-exclusion chromatography revealed that the ASF1-interacting proteins SIN3A, PF1, EMSY, and LID eluted in fractions corresponding to apparent molecular masses ranging from ~440–900 kDa (Figure 2A). Thus, their migration is consistent with their incorporation into large multisubunit assemblages. The chromatin remodelers BRM and ISWI served as a reference. Because ASF1 forms two mutually exclusive complexes with CAF1 and HIRA, we wondered whether the ASF1 interactors might associate with either of these two complexes. To test this hypothesis, we immunopurified CAF1 and HIRA followed by mass spectrometric analysis (Figures 2B and 2C and Tables S4 and S5). ASF1 and a number of previously uncharacterized CAF1- or HIRA-interacting proteins were identified. Importantly, none of the additional ASF1-associated factors were detected, suggesting that they bound neither CAF1 nor HIRA. We note that the CAF1 and HIRA purifications shared a few proteins, suggesting potential crosstalk between these two histone chaperones.

Next, we purified biotin-tagged LID (bioLID) from S2 cells using NeutrAvidin-coated beads and immunopurified LID, PF1, and EMSY from embryo nuclear extracts using the appropriate
that binding of ASF1 or RPD3 to LID and its associated factors (LAF) is mutually exclusive. (R)LAF components engage in additional interactions, linking individual subunits to gene silencing, replication, chromatin remodeling, and other pathways (see Tables S7–S9).

In summary, we made a proteomic survey of factors that associate with the histone H3/H4 chaperones ASF1, CAF1, or HIRA. In addition to known associations, our analysis uncovered several additional binding partners. Our observations emphasize that, rather than acting as generic factors, distinct histone chaperones are involved in highly specific chromatin processes. Here, we focus on an ASF1-interacting complex harboring the histone H3K4me2/3 demethylase LID/KDM5, which we named LAF. In addition, we identified RLAF, comprising LAF plus the histone H3 deacetylase RPD3. However, association of RPD3 or ASF1 appears to be mutually exclusive: ASF1 binds LAF, but not RLAF. Finally, neither HIRA nor CAF1 interacts with LAF, suggesting that its association with ASF1 is selective.

The Histone H3/H4 Chaperone ASF1 Binds LAF, whereas the H2A/H2B Chaperone NAP1 Binds RLAF

To further characterize the interaction between LAF and ASF1, we performed a series of stringent coimmunoprecipitations (coIPs) from embryo NE. Western immunoblotting showed that SIN3A, PF1, EMSY, MRG15, and LID are stably associated with ASF1 (Figure 3A). CAF1 and HIRA were also readily

affinity-purified antibodies, followed by mass spectrometric analysis (Figures 2D–2G and Tables S6–S9). Our results revealed that RPD3, LID, SIN3A, PF1, EMSY, and MRG15 form a stoichiometric complex, which we refer to as RLAF. The following RLAF subunits have been implicated in gene repression: (1) SIN3A, a transcriptional corepressor that is targeted to a variety of promoters (Silverstein and Ekwall, 2005), (2) PF1, a PHD-finger protein, (3) MRG15, which has been implicated in gene repression together with PF1 and SIN3A (Yochum and Ayer, 2001, 2002), (4) EMSY, originally identified as a human BRCA2-interacting protein that can mediate transcriptional repression (Hughes-Davies et al., 2003), and (5) LID/KDM5, a JARID1-type demethylase that can remove the active H3K4me2/3 mark (Eissenberg et al., 2007; Lee et al., 2007; Secombe et al., 2007). (6) Finally, RPD3 is a histone deacetylase (Pile et al., 2002) with a significant mascot score in our RLAF purifications but that is absent from our ASF1 purifications. Thus, it appears

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Figure 2. Protein-Protein Interaction Networks of ASF1-Associated Factors

(A) Copurifying ASF1-associated factors display overlapping elution patterns during Sephacryl S-300 size-exclusion chromatography. The indicated fractions were resolved by SDS-PAGE, followed by immunoblotting. Voided volume (void) and elution of the markers ferritin (440 kDa) and aldolase (158 kDa) are indicated. Detection of the BRM subunit of the Drosophila SWI/SNF complexes and ISWI serve as additional references.

(B and C) Immunopurification of CAF1 and HIRA from embryo NE using affinity-purified antibodies as described in Figure 1A. See Tables S4 and S5 for mass spectrometric details. Background proteins are indicated with an asterisk. ASF1-associated factors are labeled in red, whereas proteins shared by CAF1 and HIRA complexes are labeled in blue.

(D) Identification of LID-associated factors from NEs prepared from S2 cells expressing bio-tagged LID using Neutravidin beads. For details, see Table S6. Proteins present in ASF1 purifications are labeled in red.

(E–G) Immunopurification of LID, PF1, and EMSY from NEs using affinity-purified antibodies as described in Figure 1A. Mass spectrometry details are in Tables S7–S9. Proteins present in ASF1 purifications are labeled in red.
detected, whereas RPD3, NAP1, or the unrelated USP7 did not coimmunoprecipitate with ASF1. These results confirm our proteomics analysis and substantiate the notion that ASF1 forms a separate complex with LAF, lacking RPD3. We refer to this complex as LAF-A. In conclusion, ASF1 engages in three mutually exclusive associations involving HIRA, CAF1, and LAF. As predicted by our proteomics analysis, LAF does not associate with CAF1 or HIRA but binds ASF1 or RPD3 (Figures 3B and 3C).

Mass spectrometric analysis of LID- and EMSY-associated factors revealed NAP1, albeit with low mascot score (Tables S6, S7, and S9). Indeed, coIPs using antibodies against PF1, EMSY, and LID, followed by western immunoblotting, readily revealed the association of NAP1 (Figure 3C). Next, we immunopurified NAP1 from NE prepared from Drosophila embryos and S2 cells, followed by mass spectrometry detection (Figures 4A and 4B and Tables S10 and S11). NAP1 associates with multiple factors, including RLAf, Cohesin, RFC, and THO complexes. CoIPs confirmed that NAP1 interacts with RLAf, but not with ASF1 (Figure 4C). NAP1 binding to RLAf is substoichiometric (Figures 2D–2G), suggesting that a portion of RLAf associates with NAP1, forming RLAf-N. In conclusion, LID/KDM5 is part of at least three mutually exclusive complexes: LAF-A, RLAf, or RLAf-N (Figure 4D).

**LAF-A and RLAf-N Repress Transcription of NOTCH Target Genes**

Recently, we described that ASF1 functions in the NOTCH-signalling pathway by contributing to the repression of NOTCH target genes (Goodfellow et al., 2007). Therefore, we set out to test whether the LAF complexes might also be involved in this process. First, we used a well-established genetic approach by analyzing the effect of combining the heterozygous Notch allele N55e11 in trans with either lidk06801 or Sin3A08269. We found that mutations in lid or Sin3A suppressed the dominant thickening of the wing vein, caused by N55e11 (Figure 5A, left panels). In contrast, heterozygous lidk06801 or Sin3A08269 enhanced the truncations of the L5 wing vein in flies heterozygous for the HP141 mutation in transcriptional corepressor Hairless (Figure 5A, right panels). These observations suggest that, like ASF1, LID and SIN3A contribute to repression of NOTCH target genes in vivo. ASF1 is recruited to the E(spl) NOTCH target genes by the sequence-specific DNA-binding Su(H)/H corepressor complex (Goodfellow et al., 2007). Purification of NAP1-interacting factors revealed subunits of the Su(H)/H corepressor complex SKIP and CtBP (Figures 4A and 4B). Moreover, CtBP was also detected in LID and PF1 purifications (Figures 2E and 2F). Therefore, we decided to test whether LAF complexes interact with Su(H)/H corepressor. To this end, we performed a series of coIPs with antibodies against Su(H). Like ASF1, LAF was efficiently coimmunoprecipitated with Su(H) as well as RPD3 and NAP1 (Figure 5B). In contrast, HIRA or USP7 were not bound by Su(H). Conversely, Su(H) was also present in PF1, EMSY, and LID immunopurifications (Figure 5B). We conclude that LAF-A, RLAf, and RLAf-N bind the DNA-binding recruiter Su(H).
To address the role of the LAF complexes in gene expression control, we treated S2 cells with dsRNA directed against individual LAF subunits ASF1, NAP1, and RPD3. This leads to a strong reduction of the targeted protein levels, without appreciable destabilization of the others (Figure S1). Next, we extracted RNA and, to monitor gene expression, used reverse transcription followed by real-time PCR (RT-qPCR) using gene-selective primers. Strikingly, depletion of ASF1, NAP1, individual LAF subunits, or RPD3 resulted in a clear derepression of the selective primers. Strikingly, depletion of ASF1, NAP1, and RPD3. This leads to impaired LID recruitment to the chromatin structure at the E(spl)m4 enhancers and promoters. Rather, they might play major roles in bulk histone H3K4 deacetylation. Because LID is an H3K4me2/3 demethylase, we tested whether LAF complexes might be involved in the control of global H3K4 methylation levels. We performed RNAi-mediated depletion of individual LAF subunits, as well as ASF1, NAP1, and RPD3. In addition, HIRA knockdown involved in the control of global H3K4 methylation levels. We performed RNAi-mediated depletion of individual LAF subunits, as well as ASF1, NAP1, and RPD3. In contrast to loss of ASF1 or NAP1, HIRA depletion had no effect on NOTCH target gene expression. Collectively, these results established a physical and functional connection between LAF complexes and Su(H)/H corepressor complex in NOTCH target silencing.

Therefore, we tested whether the Su(H)/H complex might facilitate LID recruitment to E(spl) genes by chromatin immunoprecipitations (ChIPs) quantified by qPCR. Chromatin was extracted from S2 cells expressing bioLID that were depleted for H, ASF1, NAP1, and RPD3. We found that loss of H, ASF1, NAP1, or LAF subunits, except RPD3, significantly impaired LID recruitment to the E(spl) enhancers and promoters (Figures 6A and S3). Previously, we reported that ASF1 is recruited to the E(spl) genes by the Su(H)/H corepressor complex (Goodfellow et al., 2007). Therefore, we tested whether NAP1 might also be targeted by the Su(H)/H complex in S2 cells. Indeed, loss of H, but not of ASF1 or LID, caused a clear reduction in NAP1 binding to E(spl) regulatory elements (Figures 6B and S4). Thus, the Su(H)/H complex plays a key role in tethering the histone chaperones ASF1 and NAP1, as well as the LAF complexes.

To examine the impact of LAF-A and RLAF-N on local chromatin structure at the E(spl)m4, m7, and m8 enhancers and...
promoters, we performed a series of ChIP-qPCR experiments combined with RNAi-mediated protein depletion. Changes in H3K4me3 and H3 acetylation levels were normalized against those of histone H3, as determined by using modification-independent antibodies. All ChIP data presented here are the results of at least three fully independent biological replicates. Loss of ASF1, NAP1, individual LAF subunits, or H caused increased H3K4me3 levels at enhancers and promoters of the E(spl)m4, m7, and m8 genes (Figures 6C and S5). In contrast, depletion of RPD3 did not affect H3K4me3. However, when we examined

Figure 5. LAF-A and RLAF-N Are Required for Repression of NOTCH Target Genes
(A) Lid and Sin3A interact genetically with the Notch pathway. Mutations in lid (lid^{k06801/+}) and Sin3A (Sin3A^{08269/+}) suppress wing vein thickening in flies heterozygous for Notch mutant allele (N^{55e11/+}). Arrows mark the distal part of L4 and L5 where the phenotypes are most evident. Mutations in lid (lid^{k06801/+}) and Sin3A (Sin3A^{08269/+}) enhance truncations of the L5 wing vein in flies heterozygous for the Hairless mutant allele (H^{141/+}). (Arrow) The end of L5 in H^{141/+}. (Arrowheads) Additional truncations in transheterozygous mutants.
(B) LAF, ASF1, NAP1, and RPD3 bind Su(H). Immunoprecipitations with either control (α-GST) or α-Su(H) antibodies, followed by washes with a buffer containing 200 mM KCl and 0.1% NP-40, were resolved by SDS-PAGE and analyzed by western blotting. Ten percent of the input material was loaded.
(C) Su(H) binds PF1, EMSY, and LID, as detected by colIPs performed as described above.
(D) RLAF-N and LAF-A are selectively required for silencing of NOTCH target genes. To knock down the indicated (R)LAF subunits, ASF1, NAP1, and HIRA as a control, S2 cells were incubated with dsRNAs directed against the appropriate mRNAs. RNA was extracted and quantified by RT-qPCR, using primers selective for E(spl)m4, E(spl)m7, E(spl)m8, and the cdc2 and elf23 controls. For normalization, we performed qPCR for 18S rRNA on progressively diluted samples. Normalized mRNA levels were expressed relative to those in mock-treated cells. All results are based on three independent biological replicate experiments. Error bars represent SE of mean.
Histone Chaperones in Histone Mark Removal

histone H3ac levels, we obtained strikingly different results. Depletion of NAP1, RPD3, or H caused a strong increase in H3ac levels at enhancers and promoters of the E(spl) genes (Figures 6D and S6). SIN3A depletion had only a minor effect on H3ac at the enhancers of E(spl) genes. However, neither ASF1 nor other LAF subunits affected histone H3ac. In agreement with its importance as part of the Su(H)/H recruiting complex, loss of H caused both increased H3K4me3 and H3ac levels.

We also examined the effect of LAF-A and RLAF-N on histone density at the E(spl) regulatory elements. Strikingly, depletion of NAP1, but not ASF1, caused a strong loss of histone H2B and H3 at the E(spl) promoters and enhancers (Figures 6E, 6F, S7, and S8). This result suggests that NAP1 mediates a higher nucleosome density at the silenced E(spl) genes. Loss of NAP1 did not affect general histone density (data not shown). Loss of H also resulted in a drop of histone levels at these loci, again emphasizing its importance for recruitment of the silencing machinery. Other (R)LAF subunits did not appreciably affect local histone density.

Collectively, these results revealed an intricate interplay between sequence-specific DNA-binding factors, selective histone chaperones, and histone-modifying enzymes in targeted control of histone density and the removal of positive histone marks during developmental gene silencing (Figure 7). We established that the Su(H)/Hairless coressor complex tethers LAF-A and RLAF-N chromatin-modifying activities to NOTCH target genes. ASF1, NAP1, and all LAF subunits assisted in LID recruitment to the E(spl) genes and removal of the positive H3K4me3 mark, whereas RPD3 played no role. Conversely, only NAP1 and SIN3A were critical for H3 deacetylation by RPD3. Thus, NAP1 cooperates with RLAF, stimulating both H3K4 demethylation by LID and H3 deacetylation by RPD3. Moreover, NAP1, but not ASF1, directs a higher histone density at the repressed E(spl) regulatory elements. Thus, ASF1 and NAP1 differentially modulate chromatin to mediate gene-selective silencing.

**DISCUSSION**

Our results emphasize that, rather than generic, redundant factors, histone chaperones play highly specialized roles in gene-specific regulation. We dissected the molecular mechanism underpinning coordinate silencing of NOTCH target genes by the histone H3/H4 chaperone ASF1 and the H2A/H2B chaperone NAP1 (Figure 7). ASF1 interacts with LAF, comprising SIN3A, PF1, EMSY, MRG15, and the histone H3K4me2/3 demethylase LID/KDM5, forming LAF-A. A closely related complex, RLAF that includes the deacetylase RPD3, does not bind ASF1. Instead, RLAF associates with NAP1, forming RLAF-N. The chaperones ASF1 and NAP1 link, respectively, LAF and RLAF to the Su(H)/H DNA-binding complex, tethering them to the E(spl) genes. Both ASF1 and NAP1 bind the SKIP subunit of the Su(H)/H complex (Figures 4A and 4B; Goodfellow et al., 2007). Thus, at least in part, ASF1 and NAP1 facilitate H3K4me3 demethylation activity at the E(spl) genes through LID recruitment. Other LAFs might provide additional links to the Su(H)/H complex by contacting GRO and CIBP, which themselves associate with the Su(H)/H complex (Nagel et al., 2005).

For example, mammalian PF1, MRG15, and SIN3A have been reported to bind GRO (Yochum and Ayer, 2001). Here, we identified CIBP in LID, PF1, and NAP1 immunopurifications, providing an additional contact between the Su(H)/H complex and (R)LAF.

ASF1 does not bind RLAF and has no effect on histone H3 deacetylation by RPD3. In contrast, NAP1 does associate with RLAF and stimulates both H3K4 demethylation by LID and H3 deacetylation by RPD3. SIN3A had a mild effect, but the other LAF subunits played no apparent role in deacetylation. Finally, NAP1 depletion caused a dramatic loss of histones at the E(spl) regulatory elements, whereas ASF1 depletion had no effect on local histone density.

ASF1 has been proposed to function in chromatin assembly by acting as a donor that hands off the H3/H4 tetramer to either CAF1 or HIRA (De Koning et al., 2007). Because LAF-A does not associate with either CAF1 or HIRA, this might explain that ASF1 does not modulate nucleosome density at the E(spl) genes. In conclusion, the H3/H4 chaperone ASF1 mediates silencing of NOTCH target genes by (1) providing a connection between LAF and the Su(H)/H tether and (2) facilitating H3K4 demethylation by LID. The H2A/H2B chaperone NAP1 participates in E(spl) silencing by (1) linking RLAF to Su(H)/H, (2) facilitating H3K4 demethylation by LID, (3) facilitating H3 deacetylation by RPD3, and (4) directing high nucleosome density at repressed loci. The functioning of the H2A/H2B chaperone NAP1 in demethylation and deacetylation of histone H3 provides an example of trans-histone regulation.

LID and its interacting factors appear to work in a context-dependent manner. For example, LID facilitates activation of dMYC target genes in a manner independent of its demethylase activity (Secombe et al., 2007). Suggestively, we observed a genetic interaction between ASF1 and dMYC, indicating a potential role for LAF-A (data not shown). Recently, it has been suggested that selective RLAF subunits could interact with a homolog of GATA zinc-finger domain-containing protein 1 to facilitate expression of targets by inhibition of RPD3 activity (Lee et al., 2009). In mammalian cells, LID homolog RBP2 and MRG15 have been implicated in transcription elongation by restricting H3K4me3 levels within transcribed regions (Hayakawa et al., 2007). Our identification of SIN3A as a LAF and RLAF subunit provides a molecular explanation for the recent observation that SIN3A is involved in genome-wide removal of both H3K4 methyl and acetyl marks (van Oevelen et al., 2008). Collectively, these findings suggest that LID and RPD3 enzymatic activities can be modulated through association with specific partners. Our proteomics analysis of the LID, PF1, and EMSY interaction networks further emphasizes the diverse involvement of LAFs in regulation of chromatin dynamics.

In conclusion, our results emphasize the close interconnectivity between distinct chromatin transactions and reveal cooperation between histone chaperones and targeted histone modifications during developmental gene control. Our proteomic survey of ASF1, CAF1, HIRA, and NAP1 provides a starting point for the functional analysis of the regulatory networks in which these chaperones participate. As illustrated by our analysis of LAF-A and RLAF-N, specific protein-protein associations and gene
Figure 6. LAF-A and RLAF-N Differentially Modulate Removal of Active Histone Marks at NOTCH Target Genes

(A) Su(H)/H complex, ASF1, NAP1, and LAF subunits, but not RPD3, mediate LID recruitment to E(spl) genes. LID binding to E(spl) enhancers and promoters was analyzed by ChIP-qPCR. Crosslinked chromatin was prepared from bio-LID-expressing S2 cells, which were either mock treated or incubated with dsRNA directed against the indicated mRNAs. bio-LID was precipitated using NeutrAvidin beads. The abundance of E(spl)m4, E(spl)m7, and E(spl)m8 enhancer and promoter DNA in the precipitates of treated cells was expressed relative to mock. Here, we depict the results for the E(spl)m4 gene. Similar results were obtained for E(spl)m7 and E(spl)m8 (see Figure S3). All ChIP data in this manuscript are the result of at least three independent experiments. Error bars represent SE of mean.

(B) The Su(H)/H complex mediates NAP1 recruitment to E(spl) genes. ChIPs were performed with α-NAP1 antibodies on either mock-treated S2 cells or cells depleted for NAP1, ASF1, LID, or H. Results for the E(spl)m4 gene are shown, and similar results were obtained for E(spl)m7 and E(spl)m8 (see Figure S4). Error bars represent SE of mean.

(C) H, ASF1, NAP1, and LAF subunits, but not RPD3, are required for H3K4me3 demethylation at NOTCH target gene enhancers and promoters. Crosslinked chromatin was isolated from S2 cells that were either mock treated or incubated with dsRNA directed against the indicated mRNAs. H3K4me3 enrichment levels at the enhancers and promoters of E(spl) genes were determined by ChIP-qPCR and normalized to histone H3. H3K4me3/H3 ratio in mock-treated cells was set at 1. Results for the E(spl)m4 gene are shown. For E(spl)m7 and E(spl)m8, see Figure S5. Error bars represent SE of mean.

(D) RPD3, H, and NAP1, but not LAF-A, are required for histone H3 deacetylation at enhancers and promoters of E(spl) genes. ChIP-qPCR experiments were performed as described above. The H3Ac/H3 ratio is strongly increased upon RNAi knockdown of H, NAP1, and RPD3 at both enhancers and promoters of E(spl) genes. SINSA only modestly affected H3ac at enhancers, but not at promoters. Neither other LAF subunits nor ASF1 affected H3ac at these sites. Results for the E(spl)m4 gene are shown. For E(spl)m7 and E(spl)m8, see Figure S6. Error bars represent SE of mean.
targeting provide an intricate network of combinatorial gene expression control.

**EXPERIMENTAL PROCEDURES**

**Protein Purification, Mass Spectrometry, and In Vitro Interaction Assays**

Embryo NEs were prepared from 0–12 hr old *Drosophila* embryos or S2 cells, and immunopurifications were performed as described (Chalkley and Verrijzer, 2004). In brief, extracts were incubated with affinity-purified antibodies coupled by dimethylpimelimidate crosslinking to protein A Sepharose beads (GE Healthcare 17-0963-03). We also used S2 cells transfected with BirA biotin ligase and bio-tagged LID. Biotinylated LID was purified on NeutAvidin coated beads (Pierce 29202). Immuno- and biotin-purified complexes were washed extensively with HEMG buffer (25 mM HEPES-KOH [pH 7.6], 0.1 mM EDTA, 12.5 mM MgCl2, 10% glycerol, and cocktail of protease inhibitors) containing 600 mM KCl and 0.1% NP-40 (HEMG/600). Anti-GST antibodies were used for mock purifications. Proteins were eluted from the beads with 100 mM NaCitrate buffer (pH 2.5), resolved by SDS-PAGE, and visualized by Coomasie staining. Mass spectrometry was performed as described using an LTQ-Orbitrap hybrid mass spectrometer (ThermoFischer) (Wilm et al., 1996). Data analysis was performed using the Mascot search algorithm (version 2.1; MatrixScience) searching against the FlyBase database (http://flybase.bio.indiana.edu; version FB2007_03, released November 1, 2007). NE fractionation by (NH4)2SO4 precipitation, POROS Heparin, and Sephacryl S-300 size-exclusion chromatography were performed as described (Mohrmann et al., 2004).

**Antibodies and Immunological Procedures**

For antigen production, we cloned PF1 (encoding aa 437–600), EMSY (aa 173–385), LID (aa 426–678 and 1226–1414), CAF1-105 (aa 431–583), and full-length NAP1 and CAF1-55 cDNAs into pGEX-4T-1. GST fusion protein expression, purification, and subsequent immunization were performed as described (Chalkley and Verrijzer, 2004). The remaining antibodies have been described: a-ASF1 (Moshkin et al., 2002); a-SIN3A and a-RPD3 (Pile et al., 2002); a-HIRA (Loppin et al., 2005); a-BRM, a-USP7, and a-ISWI (Mohrmann et al., 2004); and a-Su(H) (sc-15813, Santa Cruz Biotechnology). CoIPs and immunoblotting were performed using standard procedures (Mohrmann et al., 2004).

**Drosophila Genetics**

Fly strains for *li10424*, *lid^D08B07*, *Sin3^A8269*, *rdp3^A556*, and *N55e11* were obtained from the Bloomington stock center (http://flystocks.bio.indiana.edu/). ey::Gal4, USA::asf1/CyO and *H^R^-Tet* loss-of-function allele were described previously (Goodfellow et al., 2007; Moshkin et al., 2002). Further information on gene structure and chromosomal location of genes used is present at FlyBase (http://www.flybase.org/). All crosses were performed at 25°C and repeated at least three times.

**RNAi, RT-qPCR Expression Analysis, and ChIP-qPCR**

RNAi and RT-qPCR experiments were performed as described (Mohrmann et al., 2007). In brief, *Drosophila* S2 cells were cultured in Schneider’s media (Invitrogen 21720-024) and treated with double-stranded RNA (dsRNA) for 4 days. Double-stranded RNA for LAF-A subunits, RPD3, and HIRA were synthesized using an Ambion Megascript T7 kit according to the manufacturer’s protocol. RNA samples from three fully independent experiments were prepared and analyzed by RT-qPCR as described (Mohrmann et al., 2007). ChIP-qPCR assays were performed as described (Mohrmann et al., 2007; van der Knaap et al., 2005). In brief, crosslinked chromatin was prepared from S2 cells and sheared by sonication to an average length of 0.5 kb. Chromatin was then incubated with x-H3K4me3(ab1791, Abcam), x-H3ac (17-615, E(Spl)m7, and E(Spl)m4 gene). Similar results were obtained for E(spl)m7 and E(spl)m8 (see Figures S7 and S8). Error bars represent SE of mean.

**SUPPLEMENTAL DATA**

Supplemental Data include seven figures and 11 tables and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00516-4.

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