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Gene-Specific Targeting of the Histone Chaperone Asf1 to Mediate Silencing

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SUMMARY

The histone chaperone Asf1 assists in chromatin assembly and remodeling during replication, transcription activation, and gene silencing. However, it has been unclear to what extent Asf1 could be targeted to specific loci via interactions with sequence-specific DNA-binding proteins. Here, we show that Asf1 contributes to the repression of Notch target genes, as depletion of Asf1 in cells by RNAi causes derepression of the *E(spl)* Notch-inducible genes. Conversely, overexpression of Asf1 in vivo results in decreased expression of target genes and produces phenotypes that are strongly modified (enhanced and suppressed) by mutations affecting the Notch pathway, but not by mutations in other signaling pathways. Asf1 can be coprecipitated with the DNA-binding protein Su(H) and the corepressor Hairless and interacts directly with two components of this complex, Hairless and SKIP. Thus, in addition to playing more general roles in chromatin dynamics, Asf1 is directed via interactions with sequence-specific complexes to mediate silencing of specific target genes.

INTRODUCTION

Modulation of the chromatin structure is a key feature in transcriptional regulation. Chromatin remodeling by ATP-dependent enzymes and posttranslational histone modifications are two important mechanisms that affect transcriptional activity, by influencing the accessibility of upstream regions and promoters. A third mechanism involves the breakdown and reassembly of nucleosomes on the DNA, a process that also allows for the incorporation of histone variants, such as H3.3 (Williams and Tyler, 2007). Histone chaperones, which bind to histone heterodimers, are required both for nucleosome assembly and for their disassembly. They include the H3/H4 chaperone Anti-silencing factor 1 (Asf1), which has roles in replication-dependent and replication-independent chromatin dynamics (e.g., Robinson and Schultz, 2003; Adkins et al., 2004; Green et al., 2005; English et al., 2006; Mousson et al., 2007; Schwabish and Struhl, 2006).

In yeast, extensive Asf1-mediated exchange of histones that is independent of replication and of transcription has been detected at gene promoters and is likely to be highly significant in maintaining the balance between induction and silencing of genes (Schermer et al., 2005). Indeed, there are now several examples of Asf1 contributing to chromatin disassembly at promoters to facilitate binding of the RNA-polymerase complex (Adkins et al., 2004, 2007). Conversely, Asf1 also plays important roles in gene silencing (Sharp et al., 2001) when the reassembly of nucleosomes accompanies transcriptional repression. For example, in the absence of Asf1, there is a delay in promoter closure at the *PHO5* gene (Schermer et al., 2005). However, it remains unclear whether Asf1-mediated nucleosome reassembly occurs via a targeted mechanism, involving sequence-specific DNA-binding proteins, or whether it occurs constitutively by default.

A strong correlation between histone loss and gene activation has emerged from genome-wide studies in *Drosophila*, as it has in yeast (Bernstein et al., 2004; Lee et al., 2004; Mito et al., 2005), suggesting that transcription in higher eukaryotes is also likely to be regulated by histone loss and replacement at the promoter. However, thus far, the contribution of Asf1 to dynamic gene regulation during cell signaling in multicellular organisms has not been examined. One cell-signaling pathway with very direct effects on transcription is the highly conserved Notch pathway (Artavanis-Tsakonas et al., 1999; Schweisguth, 2004; Bray, 2006). Activation of the receptor results in the release of a nuclear-targeted intracellular fragment (Nicd), which binds directly to the CSL DNA-binding protein (Suppressor of Hairless, Su(H), in *Drosophila*) and recruits the coactivator Mastermind, resulting in the activation of target genes (Petcherski and Kimble, 2000; Wu et al., 2000; Fryer et al., 2002). CSL proteins

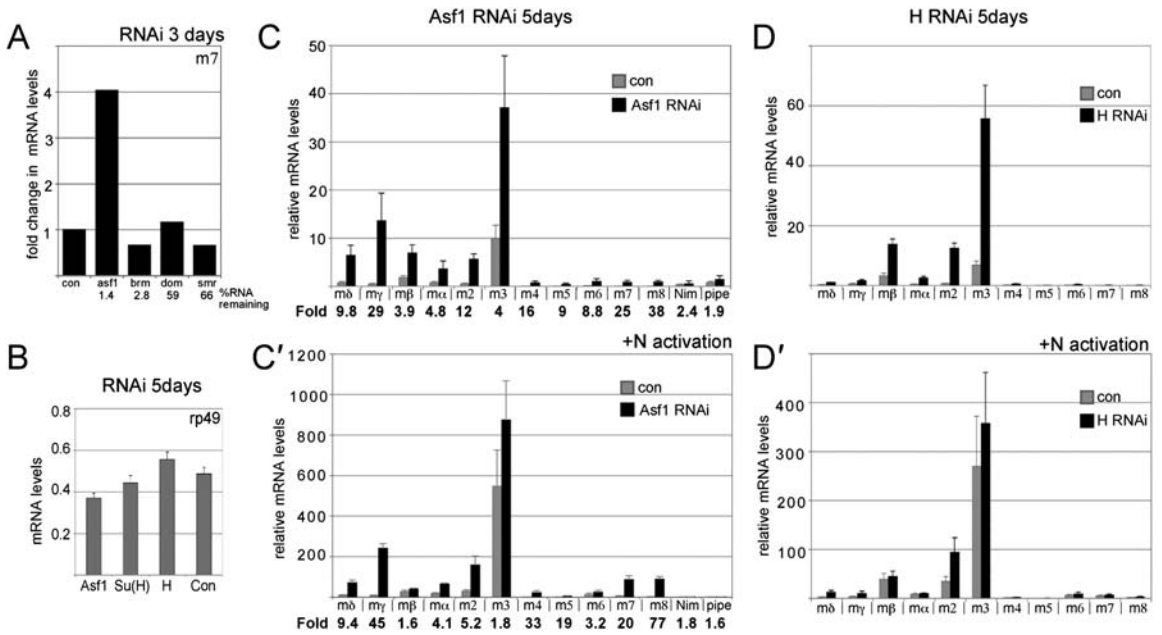

Figure 1. RNAi Depletion of Asf1 Causes Derepression of *E(spl)* Genes

(A) Fold change in *E(spl)m7* mRNA levels in S2-N cells depleted for Asf1, Brahma (brm), Domino (dom), and SMRTER (smr); the number indicates the percentage of mRNA remaining for each depleted gene. As knockdown was variable, a representative example from more than three experiments is shown. *E(spl)m7* mRNA levels in dsRNA-treated cells were quantified by real-time PCR after reverse transcription, and levels were normalized to those in control-treated cells.

(B) *rp49* mRNA levels (raw values per 50 ng RNA) are unchanged in cells depleted for Asf1, Hairless (H), or Su(H) for 5 days.

(C–D′) mRNA levels for the genes indicated in mock-treated cells (gray) or after exposure to (C and C′) Asf1 or (D and D′) Hairless dsRNA for 5 days (black). (C and D) No Notch activation; (C′ and D′) 30 min after Notch activation. *Nim* and *pipe* are repressed genes that are not inducible by Notch. Error bars in (B)–(D) indicate the standard error of the mean of more than three independent experiments.

also contribute to the silencing of target genes in the absence of Nicd, through adaptor-mediated recruitment of corepressors such as Groucho (Gro), CtBP, and SMRT (Kao et al., 1998; Zhou et al., 2000; Morel et al., 2001; Barolo et al., 2002; Nagel et al., 2005; Oswald et al., 2005). Our previous analysis indicates that the activity of Notch target genes correlates with a reduction in histone H3 density (Krejci and Bray, 2007), suggesting that nucleosome disassembly and reassembly is likely to be involved in their regulation, and prompting us to investigate whether Asf1 could play a role.

Here, we show that Asf1 contributes to the repression of Notch target genes, and that it is recruited to the DNA through interactions with the Su(H)/H complex. Thus, Asf1 is targeted to specific loci by binding to sequence-specific DNA-binding complexes, where it can promote gene silencing during development.

RESULTS

Asf1 Is Required for Repression of E(spl) Notch Target Genes

To investigate whether Asf1 contributes to the regulation of inducible genes in *Drosophila*, we used RNA interference (RNAi) to deplete S2-N cells and analyzed the levels of transcription from the 11 well-characterized Notch target genes clustered in the *E(spl)* complex (Delidakis and Artavanis-Tsakonas, 1992; Knust et al., 1992; Lai et al., 2000). We have previously established conditions for activating Notch in these cells and have shown that activation results in Su(H)-dependent stimulation of *E(spl)* gene transcription (Krejci and Bray, 2007).

Unlike knockdown of the other chromatin regulators tested, depletion of Asf1 led to a 4-fold increase in *E(spl)m7* mRNA levels, but it had no effect on the housekeeping genes rp49 and EF2B (Figures 1A and 1B and data not shown). More extensive analysis revealed that mRNA levels for all *E(spl)* genes were increased after Asf1 depletion in the absence of Notch activation (Figure 1C); some showed a greater than 10-fold change in expression, suggesting that these Notch targets are derepressed as they are when the corepressor Hairless is depleted (Figure 1D). In contrast, there was little effect of Asf1 depletion on several other repressed genes, including a phagocytosis receptor gene, *nimrod* (Kurucz et al., 2007). In addition to the derepression observed in resting cells, Asf1 depletion also altered the responsiveness to Notch activation. Many more of the *E(spl)* genes were susceptible to Notch activation in Asf1-depleted cells; for example, 5 of the 11 genes were expressed at greater than 20-fold higher levels after Asf1 RNAi. There was comparatively little change at the genes, such as *E(spl)m3*, which normally has the most robust response to Notch and is depleted for histones (Krejci and Bray,

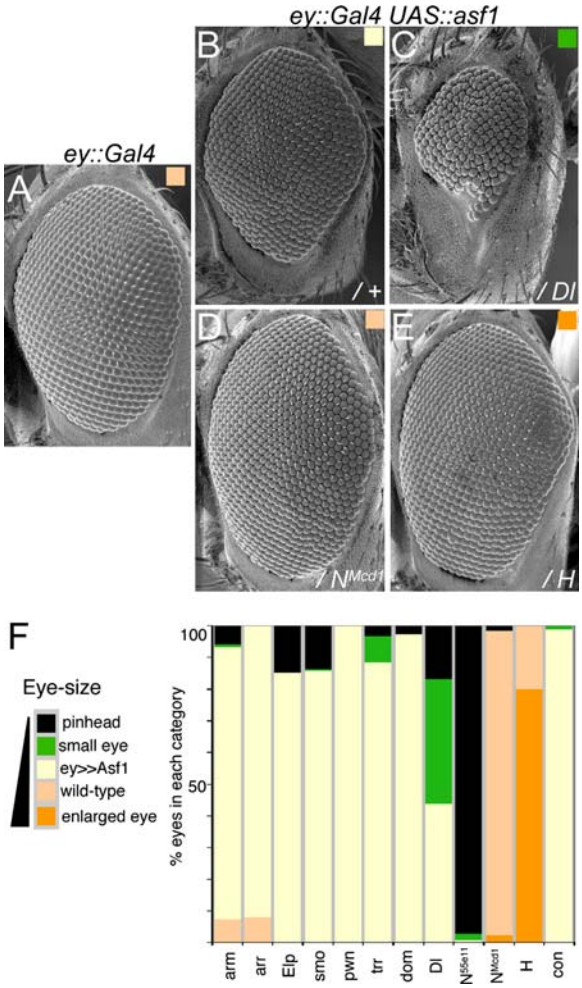

Figure 2. Asf1 Overexpression Phenotypes Are Modified by Mutations Affecting the Notch Pathway

(A–E) Scanning electron micrographs of adult eyes from the genotypes indicated; colored boxes relate to scoring shown in (F). (A) Wild-type eye size seen with *ey::Gal4* alone and with all heterozygotes tested (except *H*/+ have slightly larger eyes). (B) Expression of Asf1 (*ey::Gal4 UAS::asf1*/+) results in smaller eyes. (C) Further reduction occurs in combination with *Dl* alleles (*ey::Gal4 UAS::asf1*/+; *Dl*/+). (D and E) In combination with N^{Mcd1} (N^{Mcd1}/+; *ey::Gal4 UAS::asf1*/+) or *H* (*ey::Gal4 UAS::asf1*/+; *H*/+), eye size is restored or enlarged.

(F) Graph summarizing the range of eye sizes observed in each combination as indicated; details of alleles are given in Experimental Procedures. "con" indicates control crossed to wild-type (*yw*).

2007). Thus, it appears that Asf1 makes important contributions to the silencing of Notch target genes.

Genetic Interactions between Asf1 and Notch

Previous studies showed that overexpression of Asf1 in the *Drosophila* eye (*ey::Gal4 UAS::asf1*/+) causes a "small-eye" phenotype in which the eye is reduced in size and ommatidia are disorganized (Moshkin et al., 2002) (Figures 2A and 2B). If these small-eye phenotypes are a consequence of Asf1 altering the transcription of Notch targets, they may be modified when combined with mutations in the Notch pathway. To investigate this possibility, flies overexpressing Asf1 were crossed to alleles affecting genes central to Notch or to other signaling pathways, and the eye size was analyzed in the heterozygous progeny (Figure 2).

The first dramatic result was that the heterozygous combination of a *Notch* loss-of-function allele (N^{55e11}) and Asf1 overexpression caused a severe reduction in the eye/head capsule ("pin-head") and resulted in lethality. Thus, the effects of Asf1 overexpression were strongly enhanced by a decrease in *Notch* function (Figure 2F). Significant enhancement of the Asf1 phenotype also occurred with *Delta* loss-of-function alleles (Figure 2C), but not with alleles affecting Hedgehog (*smo*), EGF-R (*Egfr*), or Wingless (*arm, arrow*) pathways or with alleles affecting the SET domain protein Trithorax-related (*trr*), the histone exchange factor Domino (*dom*), or the cell adhesion protein Pawn (*pwn*) (Figure 2F). Complementary results were obtained by using mutant alleles that increase Notch signaling: both a loss-of-function *Hairless* (*H*) allele and a gain-of-function *Notch* allele (N^{Mcd1}) suppressed the small-eye defect caused by Asf1 overexpression (Figures 2D–2F). These findings are fully consistent with the results of RNAi-mediated Asf1 depletion, and they suggest that Asf1 is involved in repression of Notch target genes. As *asf1* mutant cells failed to proliferate, we were unable to obtain clones of homozygous mutant cells to test the effects of eliminating Asf1 on Notch target genes in the eye.

Asf1-Mediated Repression of Notch Target Genes in the Wing

To investigate whether interactions between Notch and Asf1 occur in other tissues, we asked whether Asf1 overexpression also perturbed Notch function in the *Drosophila* wing (Figure 3). Expression of Asf1 in the developing wing pouch (*sd::Gal4*/+; *UAS::asf1*/+) resulted in margin loss/wing nicks and mild vein thickening, characteristics of reduced *Notch* function (Figure 3B) (*Notch*/+ heterozygous flies have mild wing nicks due to reduced signaling at the dorsal/ventral (d/v) organizer of the wing). The Asf1 overexpression phenotypes were strongly enhanced when the levels of Notch were reduced; thus, wings had extensive scalloping/margin loss and more extensive vein thickening (Figure 3D) (*sd::Gal4*/N^{55e11}; *UAS::asf1*/+). Wing phenotypes, similar to the eye phenotypes, produced by Asf1 expression were thus enhanced by reduced Notch.

To further assess whether Asf1 affects expression of target genes regulated by Notch (e.g., *cut*) (Neumann and Cohen, 1996) or by other pathways (e.g., *spalt*) (de Celis et al., 1996a), we analyzed the effects of overexpressing Asf1 in wing discs. In wild-type discs, Notch-dependent expression of Cut is detected in a stripe along the d/v boundary (Figure 3E). This was interrupted and reduced in discs in which Asf1 was overexpressed (Figure 3G). In contrast, there was no visible effect on Spalt under these conditions (Figures 3F and 3H). Similar results were obtained when Asf1 was expressed in a more limited domain (by using *ptc::Gal4*) (Figures 3I and 3J),

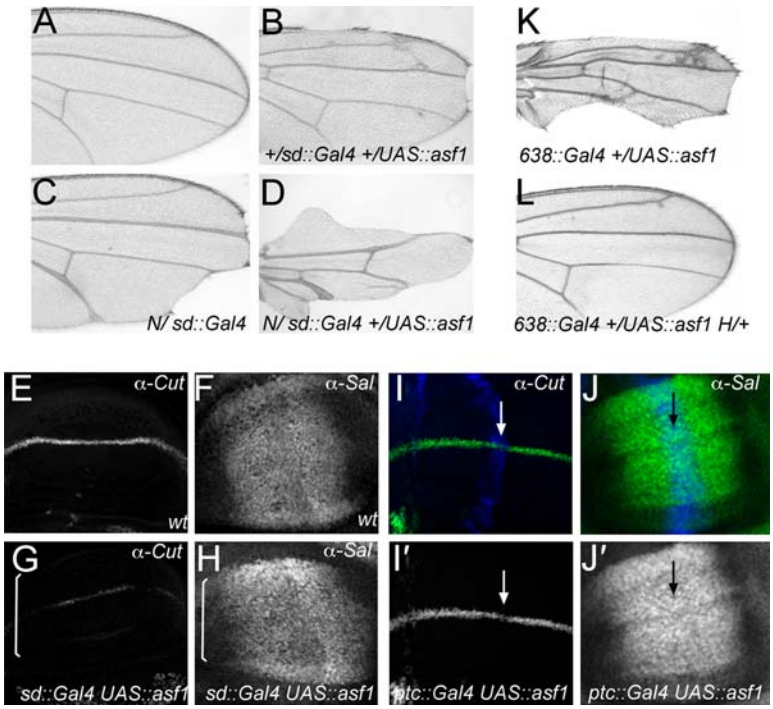

Figure 3. Asf1 Overexpression Affects Notch Activity in the Wing

(A–D) Adult wings from the genotypes indicated. (B) Asf1 overexpression causes loss of wing margin tissue (*sd::Gal4/+; UAS::asf1/+*). (C) Mild wing nicks are present in Notch heterozygotes (*sd::Gal4/N^{55e11}*). (D) Phenotypes of Asf1 overexpression are strongly enhanced in combination with *N/+* (*N^{55e11}/sd::Gal4; UAS::asf1/+*).

(E–J′) Expression of (E, G, and I) Cut and (F, H, and J) Spalt (Sal) in (E and F) wild-type and Asf1-overexpressing discs of genotypes (G and H) *sd::Gal4/+; UAS::asf1/+* (the bracket indicates the region of Asf1 expression) and (I and J) *ptc::Gal4 UAS::GFP/UAS::asf* (the blue domain and the arrow indicate the region of Asf1 expression).

(K and L) (K) Higher levels of Asf1 overexpression with 638::Gal4 produce severe wing defects (59% wings [n = 57]), which are suppressed in combination with (L) H/+ (*638::Gal4/+; UAS::asf1/+; H^{P1}/+*) (89% wild-type, 11% nicks [n = 85]).

where a local loss of Cut, but not Spalt, expression was seen (Figures 3I′ and 3J′). Stronger expression of Asf1 resulted in more pronounced Notch-like phenotypes and loss of Cut expression (Figure 3K and data not shown), which could be rescued by a reduction in *Hairless* function (Figure 3L). Under these conditions, where Asf1 was expressed more strongly, some more generalized effects of Asf1 were sometimes detected, compatible with its proposed role as a histone chaperone during replication. The replication defects became more severe at even higher levels of expression (29°C). Similarly, clones of cells mutant for *asf1* failed to proliferate. Thus, as in yeast, Asf1 appears to have roles in replication-dependent as well as replication-independent chromatin dynamics in *Drosophila*. By moderating the levels of Asf1 expression, we have been able to uncouple these requirements, revealing a contribution to repression of Notch target genes.

Asf1 Interacts with Su(H)/H Complexes

Complexes implicated in repression at Notch targets are formed by the CSL/Su(H) DNA-binding protein in conjunction with adaptor proteins, such as SKIP and Hairless, which recruit general corepressors, including SMTR or Gro and CtBP (Zhou et al., 2000; Morel et al., 2001; Barolo et al., 2002; Nagel et al., 2005). On polytene chromosomes from *Drosophila* salivary glands, Asf1 is detected at most Su(H)-enriched sites, suggesting that these proteins are present at the same loci (Figure 4A). Asf1 is also bound at many other loci, and it is strongly enriched at centromeres and telomeres, reflecting its multiple roles in chromatin dynamics.

The colocalization of Su(H) and Asf1 on polytene chromosomes prompted us to test whether Su(H) and/or associated factors could copurify with Asf1 in immunoprecipitation (IP) experiments. For these experiments, we used extracts prepared from *Drosophila* embryos and immunoprecipitated Su(H) or Asf1 by using moderate salt conditions. Under these conditions, Asf1 was detected in Su(H) IP experiments (Figure 4B), and, conversely, Su(H) was precipitated with Asf1 (Figure 4C), as was the corepressor Gro, but not CtBP (Figures 4D and 4E). To exclude the possibility that the interaction between Asf1 and the Su(H) complex was mediated by the independent binding of both protein complexes to DNA, IP experiments were performed in the presence of ethidium bromide (EtBr), a DNA-intercalating drug that dissociates proteins from DNA. This treatment did not affect the interaction of Asf1 with Su(H) (Figure 4F). Thus, these data suggest that Asf1 is present in protein complexes containing the sequence-specific DNA-binding protein Su(H) and the Gro corepressor. We also observed a significant suppression of the Asf1-induced small-eye phenotype in flies that were also heterozygous for a strong *gro* allele (*gro^{E48}*) (Figure S1A; see the Supplemental Data available with this article online) and an enhancement by Hairless proteins that retained a Gro-binding domain (Figure S1B), which agrees with a model linking Gro to Asf1-mediated repression. We therefore examined whether any of the proteins in the Su(H) repression complex are able to bind to bacterially produced Asf1 (fused to glutathione S-transferase, GST). Of those tested, both Hairless and the adaptor protein SKIP were bound to GST-Asf1, but not to GST alone or to GST-CAF1p55 (a component of chromatin assembly factor 1). Neither Gro nor Su(H) itself showed direct interactions with Asf1 in this assay.

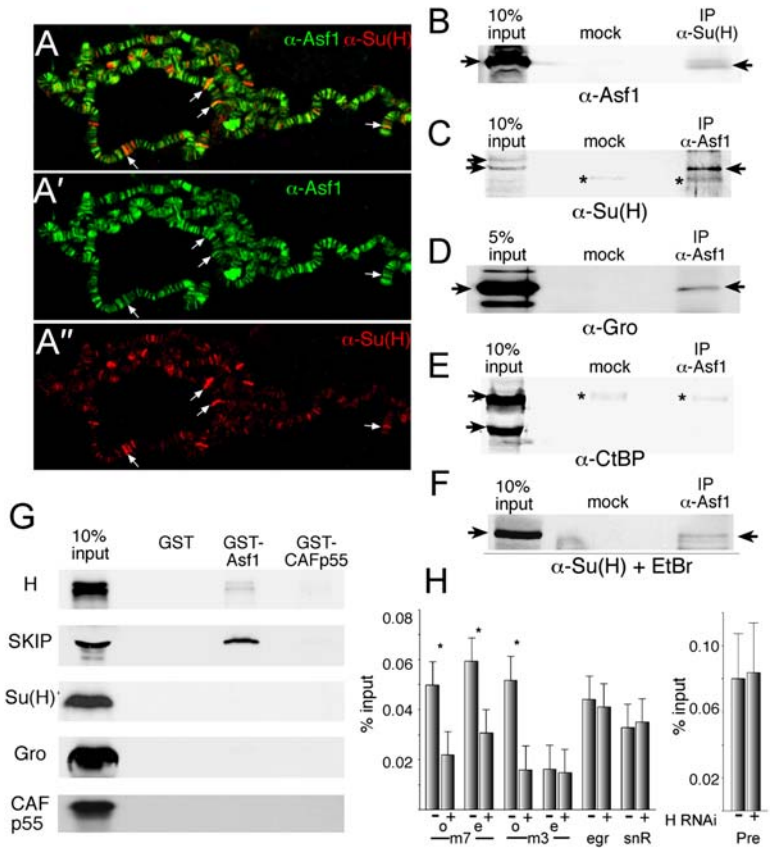

Figure 4. Asf Is Recruited to Su(H) Complexes

(A–A″) Polytene chromosomes stained to detect (A and A′) Asf1 (green) and (A and A″) Su(H) (red); arrows indicate some sites at which the two proteins colocalize.

(B–F) Western blots to detect proteins present in immunoprecipitates from nuclear extracts with (B) Su(H) or (C–F) Asf1 antibodies. In (F), immunoprecipitation (IP) was carried out in the presence of EtBr. Western blots were probed with antibodies against (B) Asf1, (C and F) Su(H), (D) Gro, or (E) CtBP. For lanes labeled "mock," IPs were performed with control antisera. The asterisk in (C) and (E) indicates the nonspecific band present in mock and experimental IPs. Input lanes contain the indicated amount of total extract used for IP.

(G) Protein interactions detected by GST pull-down experiments. The input lane contains 10% of the indicated ^{35}S-labeled protein included in each pull-down experiment; GST lanes show the bound fraction with the indicated GST fusion protein.

(H) ChIP with anti-Asf1 antibodies in control cells (−) and cells treated with Hairless dsRNA (+). Enrichment of fragments in the ChIP corresponding to enhancer (e) and ORF (o) sequences from *E(spl)m7* and *E(spl)m3* and to promoter regions of *eiger* (egr) and *snRNP69D* (snR) were quantified by using real-time PCR and were expressed relative to the total input. One-way ANOVA shows significant changes in the binding of Asf1 to the target genes after Hairless depletion (the asterisk indicates significant reduction in Asf1 ChIP levels; $p < 0.01$ revealed by Fisher LSD post hoc analysis). The residual binding (0.02%) signal for Asf1 is lost under conditions in which Asf1 is ablated by RNAi, suggesting that it is due to Hairless-independent recruitment, possibly through binding to nucleosomes (data not shown). Binding of Polycomb to the *bxd*-PRE was not affected by Hairless knockdown, as determined by ChIP with anti-Polycomb antibodies. Error bars represent 95% confidence intervals from three independent experiments.

Finally, to test whether Hairless contributes to the recruitment of Asf1 in vivo, we performed chromatin immunoprecipitation (ChIP) with anti-Asf1 antibodies in cells with and without RNAi-mediated depletion of Hairless and assayed for association with two *E(spl)* genes, *m3* and *m7*. The *E(spl)m7* gene is silenced in the S2 cells and is strongly affected by Asf1 depletion, whereas *E(spl)m3* is expressed in S2 cells, is highly induced by Notch activation, and is more mildly affected by Asf1 depletion. Of the two genes, the greatest effects were seen for *E(spl)m7*; binding of Asf1 to both enhancer and ORF fragments strongly decreased in ChIP after Hairless depletion (Figure 4H). A decrease was also seen at the *E(spl)m3* ORF region, but not at the *E(spl)m3* enhancer. This enhancer is found to have very low histone coverage in these cells, and we found that it shows only small Asf1 occupancy levels. The decrease in Asf1 from ORFs of both *E(spl)m3* and *E(spl)m7* after Hairless depletion may indicate that Asf1 spreads from the site of recruitment. Binding of Asf1 to *E(spl)m7* and *E(spl)m3* regions was confirmed by using affinity-purified anti-Asf1 antibodies raised in a different species (data not shown). Loss of Hairless does not affect the binding of Asf1 to other loci that do not require Su(H)/H for their regulation, such as *eiger* or *snRNP69D*. Similarly, there was no change in the levels of Polycomb protein associated with *bxd*-PRE after Hairless knockdown. Together, these data support the model that recruitment of Asf1 to Notch targets requires Hairless.

DISCUSSION

The density and precise positioning of nucleosomes are important factors in determining the transcriptional activity of a locus. It is now evident that most nonnucleosomal histones in cells are likely to be complexed with chaperones (Loyola and Almouzni, 2004). It is therefore not surprising that the histone chaperone Asf1 is important for chromatin dynamics and has been shown to have multiple roles in transcription as well as in the disassembly and reassembly of chromatin during replication (Mousson et al., 2007). These include gene-specific roles in repression, activation, and transcription elongation (Sutton et al., 2001; Adkins et al., 2004; Zabaronick and Tyler, 2005; Schwabish and Struhl, 2006). For example, Asf1 is required for nucleosome disassembly and transcription activation at the yeast *PHO5*, *PHO8*, *ADY2*, and *ADH2* promoters (Adkins et al., 2004, 2007). However, the

mechanisms responsible for targeting Asf1 to these loci remain unclear. Here, we have demonstrated that Asf1 can be specifically recruited to target loci by interactions with sequence-specific DNA-binding transcription factors. We have shown that Asf1 is present in a complex with Su(H), the central DNA-binding protein in the Notch pathway, and that it interacts directly with two proteins found in CSL complexes, Hairless and SKIP. Importantly, we found that Asf1 plays a significant role in the repression of Notch target genes. Thus, contrary to effects at many of the inducible loci examined in yeast, our data demonstrate a requirement for Asf1 in silencing rather than in activation of these inducible genes.

As the global corepressor Gro is also coprecipitated with Asf1 and is implicated in Asf1-mediated repression through genetic interactions, Gro and Asf1 may cooperate in the repression of Notch target genes. Gro has been postulated to exert long-range repressive effects (Courey and Jia, 2001) by nucleating a transcriptionally silent chromatin state, in a similar manner to its yeast relative Tup1. For example, at the STE6 locus, Tup1 recruitment results in increased nucleosomal density and local nucleosome positioning (Cooper et al., 1994). The recruitment of the histone chaperone Asf1 with Gro to Su(H)/H DNA-binding complexes could facilitate a similar localized increase in histone deposition and participate in the spreading of repressed chromatin (Courey and Jia, 2001; Song et al., 2004). Furthermore, as we have previously shown that Su(H)/H complexes engage in comparatively low-stability interactions with target loci (Krejci and Bray, 2007), we suggest that Asf1 could be critical for translating these transient interactions into stable silencing. However, thus far, our analysis has focused on relatively few targets and tissues; thus, it remains to be determined whether Asf1 is recruited to all targets regulated by Su(H)/H, or whether there are additional factors that influence its recruitment at specific loci. Similarly, it will be important to determine whether other sequence-specific complexes are able to bind directly to Asf1.

In conclusion, our results show that the histone H3/H4 chaperone Asf1 contributes to selective silencing of genes in *Drosophila*, through interactions with the Su(H)/H DNA-binding protein complexes. In this way, chaperones can act as gene-selective regulators that contribute to the control of gene expression by developmental signaling pathways.

EXPERIMENTAL PROCEDURES

RNA Interference

Drosophila S2-N cells are a stable Notch-expressing S2 cell line containing a Cu^{2+}-inducible pMT-Notch construct (Fehon et al., 1990). dsRNA was transcribed with the MEGA script T7 kit (Ambion) by using 750 bp PCR fragments flanked by T7 promoter sequences as a template, followed by precipitation and a 10 min annealing step at 65°C. In a 6-well plate, the medium was replaced with 30 μg dsRNA diluted in 250 μl Optimem (Invitrogen) for 30 min, followed by the addition of 2 ml culture medium. A total of 64 hr after transfection, cells were harvested (3 days of RNAi) (Figure 1A) or were split, and they were allowed to grow for an additional 48 hr before being induced with Cu^{2+}. A total of 16 hr later, they were harvested before, or 30 min after, Notch activation (5 days of RNAi) (Figures 1B–1D). Conditions for Notch activation with EDTA have been described previously (Krejci and Bray, 2007).

RNA Isolation and Quantification

RNA was isolated by Trizol (Ambion). Reverse transcription was performed with M-MLV reverse transcriptase (Promega) and oligo-dT primers or random hexamers. Levels of cDNA were quantified by real-time PCR with QuantiTec Sybr Green PCR mix (QIAGEN) and an AbiPrism machine. The calibration curve was constructed from serial dilutions of genomic DNA, and values for all genes were normalized to the levels of the housekeeping gene rp49. To allow for comparison among primer sets, a constant amount of genomic DNA (standard DNA) was used in each real-time PCR run for additional normalization.

Genetic Interactions

ey::Gal4 UAS::asf1/CyO stock was described previously (Moshkin et al., 2002). Interactions were tested with the following loss-of-function alleles: N^{55e11}, N^{mcd1}, Dl^{rev10}, smo^{3}, arm^{1}, arr^{2}, $Egfr^{f2}$, H^{P141}, trr^{3}, dom^{9}, pwn^{14}, and gro^{E48}. The alleles listed above, *sd::Gal4*, and *ptc::Gal4* are described in Flybase (http://flybase.bio.indiana.edu/). *638::Gal4* was a gift of Isabel Guerrero. All crosses were performed at 25°C unless indicated otherwise, and eye phenotypes were scored for more than 40 progeny of at least 2 independent experiments. For scanning electron micrographs, flies were mounted on stubs by using double-sided tape, dessicated, and coated with gold/palladium (15 nm thick) by using a Polaron E5000 Sputter Coater. For analysis of wing phenotypes, female flies were collected in 100% ethanol, and the wings removed and mounted in Euparal. Immunofluorescence of imaginal discs was performed as described previously (de Celis et al., 1996b). Primary antibodies were mouse anti-Cut (1/20) (Blochlinger et al., 1990, Developmental Biology Hybridoma Bank), rat anti-Spalt (1/300) (de Celis et al., 1996a), and rabbit anti-GFP (1/500; Molecular Probes). Fluorescent (FITC, Cy3, and Cy5)-conjugated secondary antibodies were purchased from Jackson Immunological.

Biochemical Interactions and Colocalization on Polytene Chromosomes

For coimmunoprecipitations, *Drosophila* embryo nuclear extract was incubated with Protein A Sepharose for 2 hr at 4°C in binding buffer (20 mM HEPES-KOH [pH 7.6], 150 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol, 0.05% NP-40, and 1 mM DTT) in the presence of affinity-purified anti-Asf1 (Moshkin et al., 2002), anti-Su(H) (Santa-Cruz), or control antibodies. Protein A beads were washed five times with ten bed volumes of binding buffer, and precipitated proteins were detected on western blots with antibodies raised against Asf1 (Moshkin et al., 2002), Su(H) (Santa Cruz), Gro (Delidakis et al., 1991), and CtBP. For some experiments, *Drosophila* embryo nuclear extracts were preincubated with 50 μg/ml ethidium bromide (EtBr) on ice for 30 min. Precipitates were removed by centrifugation at 4°C for 10 min, and the supernatant was used in immunoprecipitation experiments. GST pull-downs were performed under similar conditions as coimmunoprecipitation experiments. Bacterially expressed GST-Asf1, GST-CAF1p55, and GST proteins were incubated with ^{35}S-methionine-labeled H, SKIP, Su(H), Gro, and CAF1p55 proteins (synthesized with the Promega transcription/translation system), and complexes were isolated via glutathione agarose. Colocalization on polytene chromosomes was performed according to the methods described by Corona et al. (2004). Chromatin immunoprecipitation experiments were performed by using affinity-purified anti-Asf1 and anti-Pc antibodies according to the Upstate protocol.

Supplemental Data

Supplemental Data include additional experimental details and results of genetic interactions with Groucho and are available at http://www.developmentalcell.com/cgi/content/full/13/4/593/DC1/.

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