

Drosophila ribosomal proteins are associated with linker histone H1 and suppress gene transcription

Jian-Quan Ni,^{1,3} Lu-Ping Liu,^{1,3} Daniel Hess,¹ Jens Rietdorf,¹ and Fang-Lin Sun^{1,2,4}

¹Friedrich Miescher Institute for Biomedical Research, Basel CH-4058, Switzerland; ²Institute of Epigenetics and Cancer Research, School of Medicine, Tsinghua University, Beijing 100080, China

The dynamics and function of ribosomal proteins in the cell nucleus remain enigmatic. Here we provide evidence that specific components of *Drosophila melanogaster* ribosomes copurify with linker histone H1. Using various experimental approaches, we demonstrate that this association of nuclear ribosomal proteins with histone H1 is specific, and that colocalization occurs on condensed chromatin in vivo. Chromatin immunoprecipitation analysis confirmed that specific ribosomal proteins are associated with chromatin in a histone H1-dependent manner. Overexpression of either histone H1 or ribosomal protein L22 in *Drosophila* cells resulted in global suppression of the same set of genes, while depletion of H1 and L22 caused up-regulation of tested genes, suggesting that H1 and ribosomal proteins are essential for transcriptional gene repression. Overall, this study provides evidence for a previously undefined link between ribosomal proteins and chromatin, and suggests a role for this association in transcriptional regulation in higher eukaryotes.

[*Keywords:* Ribosomal protein; L22; histone H1; chromatin; transcription]

Supplemental material is available at <http://www.genesdev.org>.

Received September 25, 2005; revised version accepted May 8, 2006.

Transcription and translation in eukaryotes are generally believed to take place within two spatially separated cellular compartments. The processes of transcription, including initiation and elongation, are programmed by chromatin remodeling complexes in the cell nucleus (Workman and Kingston 1998; Zhang and Reinberg 2001), while the events of translation generally occur within the cytoplasm and require functional ribosome complexes (Woolford 1991; Kressler et al. 1999; Venema and Tollervey 1999; Fromont-Racine et al. 2003).

The metazoan cell nucleus is not devoid of components of the translation machinery. A number of studies have reported the presence of ribosomal proteins and other components of the translation apparatus in the nucleoplasm (Ringborg et al. 1970; Lejbkiewicz et al. 1992; Sanders et al. 1996; Lund and Dahlberg 1998; Dostie et al. 2000), and their presence has often been interpreted as indicative of their specific nuclear activity. Cook and colleagues found that newly made polypeptides in the cell nucleus colocalized with newly synthesized RNA and parts of the translation machinery, and that the level of incorporation of labeled amino acids into protein at

discrete nuclear sites was sensitive to inhibitors of both transcription and translation, arguing that the two processes are coupled (Iborra et al. 2001). This conclusion was supported by the study by Brogna et al. (2002), who further showed that many ribosomal proteins and translation factors colocalize with sites of active transcription on *Drosophila melanogaster* polytene chromosomes. In yeast, factors for translation initiation, elongation, and termination are rather strictly excluded from the nuclei (Bohnsack et al. 2002), implying that nuclear translation is unlikely. The debate about whether translation ever takes place in the nucleus has been ongoing since the 1970s (Goldstein 1970; Allen 1978; Goidl 1978; Dahlberg and Lund 2004) and is likely to continue until more concrete evidence is available.

However, the binding of ribosomal proteins to nascent/elongating Pol II RNA transcripts on chromatin has been shown by the studies both in *D. melanogaster* and budding yeast (Brogna et al. 2002; Schroder and Moore 2005). Unlike ribosomal proteins in *D. melanogaster*, the binding in *Saccharomyces cerevisiae* seems to be independent of transcript translatability, and the translation factors, such as eIF4A, eIF4E, eIF4G, eRF1, and eRF3, are all absent from the chromatin (Schroder and Moore 2005). The exact function of specific components of the translation machinery in the cell nucleus therefore remains to be defined. Notably, specific ribosomal proteins were also reported to copurify with a sub-

³Present address: Department of Genetics, Harvard Medical School, 77 Louis Pasteur Ave., Boston, MA 02115, USA.

⁴Corresponding author.

E-MAIL fang-lin.sun@fmi.ch or flsun@mail.tsinghua.edu.cn; FAX 41-0-61-697-3976 or 86-0-10-62792995.

Article published online ahead of print. Article and publication date are at <http://www.genesdev.org/cgi/doi/10.1101/gad.390106>.

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unit of chromatin assembly factor 1 (CAF1) (Schaper et al. 2001). In another case, ribosomal proteins were found to associate with complexes of the origin recognition complex (ORC)-interacting protein Yph1p (Du and Stillman 2002), implying that these proteins may play other specific roles in the cell nucleus.

Linker histone H1 is a basic component of chromatin, believed to bind to nucleosomal DNA, protecting an additional 20 base pairs (bp) of DNA, and to have a fundamental role in promoting or facilitating the condensation of nucleosome filaments into supercoiled chromatin fibers (Vignali and Workman 1998; Thomas 1999; Luger 2003; Bustin 2005). Previous studies have shown that H1 limits nucleosome mobility (Pennings et al. 1994), reduces transient exposure of DNA on the surface of nucleosomes (Polach and Widom 1995; Juan et al. 1997), and also directly occludes the binding of transcription factors, suggesting that it functions as a general repressor of transcription (Laybourn and Kadonaga 1991; Juan et al. 1997). In vivo studies suggested that H1 is also essential for the lifespan, the suppression of homologous recombination, and the transmission of apoptotic signals from the nucleus to the mitochondria following DNA double-strand breaks (Shen and Gorovsky 1996; Barra et al. 2000; Downs et al. 2003; Konishi et al. 2003). Although mutation of H1 in unicellular organisms had only limited effects on transcription (Shen and Gorovsky 1996; Hellauer et al. 2001), H1 in higher multicellular organisms appears to be essential for cell differentiation, the formation of heterochromatin (Steinbach et al. 1997; Misteli et al. 2000; Jedrusik and Schulze 2003; Vaquero et al. 2004), and the allelic repression of imprinted genes (Fan et al. 2003, 2005).

In an attempt to investigate the role of histone H1 in chromatin in vivo, we unexpectedly found that H1 copurified with a number of nuclear ribosomal proteins including L22, previously known as EAP (Epstein-Barr virus-encoded small nuclear RNA-associated protein), the translocation of which was implicated in acute myeloid leukemia (Toczyski and Steitz 1991; Nucifora et al. 1993). Further immunofluorescent staining and chromatin immunoprecipitation (ChIP) analyses demonstrated that ribosomal protein L22 and H1 are both associated with condensed chromatin. Upon depletion of H1, the association of ribosomal protein L22 and L7 with chromatin was lost. Overexpression of ribosomal protein L22 caused transcriptional repression of two-thirds of the genes suppressed by histone H1. When endogenous H1 or L22 were depleted, the transcription of several tested genes was up-regulated, supporting a role for H1 and L22 as repressors of transcription. Consistently, H1 and ribosomal proteins were lost from chromatin during transcriptional activation of endogenous genes. However, a small set of genes was up-regulated during H1 or L22 overexpression, and some lost their transcription in the absence of H1 or L22, arguing that H1 and L22 may also act as positive regulators for specific genes. This study establishes a novel relationship between nuclear ribosomal proteins and H1 on chromatin and their role in transcriptional gene regulation.

Results

Linker histone H1 copurifies with specific ribosomal proteins

Immunoprecipitation (IP) experiments aimed at copurifying the partners of *D. melanogaster* histone H1 in the cell nucleus were performed using newly derived polyclonal antibodies specifically recognizing the N terminus (amino acids 33–47, H1N) and C terminus (amino acids 242–256, H1C) of H1 (Fig. 1A,B). Since histone H1 is known to be lysine rich and to have strong DNA-binding activity (Hill et al. 1991), nuclear extracts from *D. melanogaster* Kc cells were treated with ethidium bromide (EB, see Materials and Methods) to reduce potential DNA–protein interactions (Du and Stillman 2002). IP fractions obtained using anti-H1C or anti-H1N antibodies were then separated by SDS-PAGE (Fig. 1C,D; data not shown). Mass spectrometry was used to identify the most prominent bands present between 15 and 50 kDa in the gels. In addition to core histones H2B and H3, we found that histone H1 copurified with specific 40S and 60S ribosome components (Fig. 1C,D). hnRNP48 and hnRNP36 (Matunis et al. 1993), which are known to be involved in mRNA quality control (Krecic and Swanson 1999; Lykke-Andersen 2001), were also among the pulled-down proteins (Fig. 1D). No ribosomal proteins were pulled down from cytoplasmic extracts of Kc cells by either the anti-H1N or the anti-H1C antibody (data not shown), indicating that the ribosomal proteins were unlikely to have been pulled down as a result of nonspecific interactions between the H1 antibodies and cytoplasmic ribosomal proteins.

Ribosomal proteins are present in the nucleus and interact specifically with histone H1

To further determine the specificity of the interaction between ribosomal proteins and histone H1, we transiently expressed V5-HA-tagged ribosomal proteins L7 (T-L7) and L22 (T-L22) in Kc cells. Ribosomal proteins L22 and L7 were chosen simply because they are present at high frequency in the complexes of histone H1 under differential experimental conditions (Fig. 1C,D; data not shown).

Immunofluorescent staining experiments using formaldehyde-fixed Kc cells showed the expected distribution pattern of tagged ribosomal proteins within cells; i.e., cytoplasmic and nucleolar localization (Fig. 2A,B; Supplementary Fig. S1). However, ribosomal proteins L22 and L7 in most cells are distributed within both the nucleus and cytoplasm. In ~10% of cells, ribosomal proteins showed a predominant nuclear localization, as indicated by their overlap with DAPI signals (Fig. 2A,B, middle panels). Although ribosomal proteins fused with green fluorescent protein (GFP) or LacZ tags have been shown to be functional (Tsay et al. 1994; Stage-Zimmermann et al. 2000; Milkereit et al. 2001; Gadal et al. 2002), we performed cosedimentation experi-

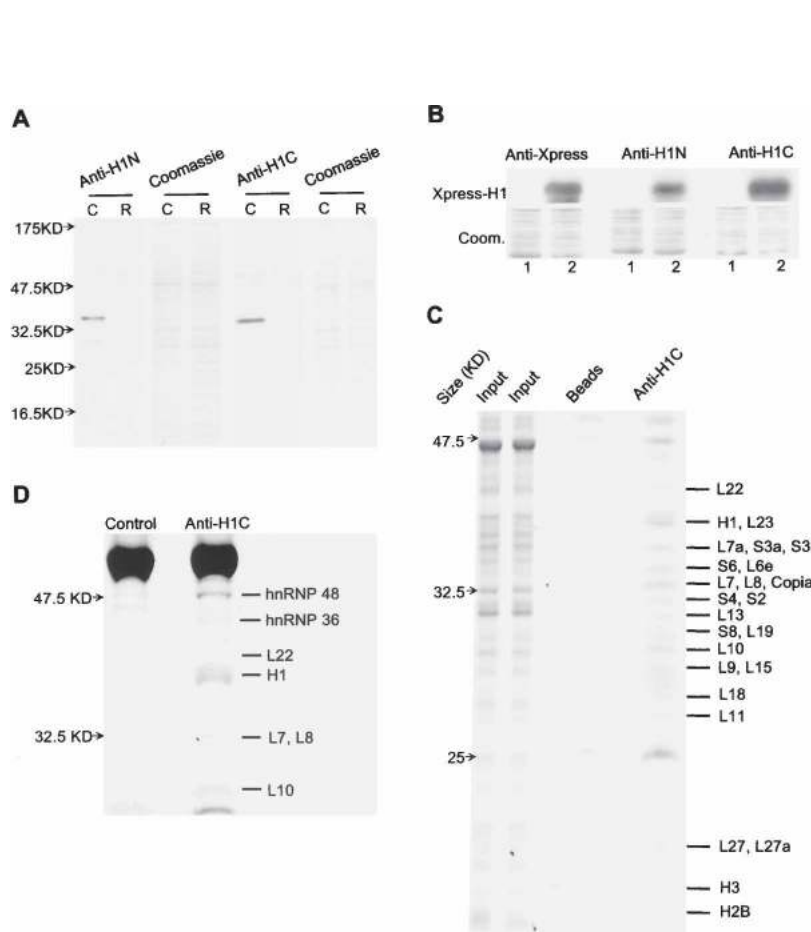


Figure 1. *Drosophila* histone H1 copurifies with 40S and 60S ribosomal subunit proteins. (A) Anti-H1N and anti-H1C antibodies specifically recognize H1. Extracts from control Kc cells (C) and Kc cells depleted of H1 using an RNAi approach (R) were separated using 15% SDS-PAGE and subjected to Western analysis with anti-H1N or anti-H1C antibodies. The same membranes stained with Coomassie blue are also shown. The positions of molecular weight markers (in kilodaltons) are indicated to the left of the gels. (B) Anti-H1N and anti-H1C antibodies specifically recognize bacterially expressed *Drosophila* histone H1. (Top panel) Western blot analysis of bacterial cell extracts from control bacteria not expressing *Drosophila* H1 (lanes 1) and bacteria expressing His-Xpress-tagged histone H1 (lanes 2) with anti-Xpress, anti-H1N, and anti-H1C antibodies. (Bottom panel) The same membranes stained with Coomassie blue. (C) *Drosophila* Kc cell nuclear extract not treated with EB was used to perform IP reactions with anti-H1C antibodies. As controls, anti-H1C antibodies preblocked with protein-A-Sepharose beads (Beads) were used. (D) *Drosophila* Kc cell nuclear extract treated with 300 μ g/mL of EB was used to perform IP reactions with anti-H1C antibodies. As controls, anti-H1C antibodies preblocked with the peptide used to raise the antibody were used. IP fractions were resolved by 15% SDS-PAGE, stained with Gelcode Blue Staining Reagent (Pierce), and photographed. Prominent bands were excised and identified by mass spectrometry (as indicated to the right of the gels). Size markers are shown to the left. C also shows diluted inputs.

ments in sucrose density gradients to confirm that the tagged ribosomal proteins were functional in our case. Indeed, T-L22 was present in both cytoplasmic 60S ribosomes and polysomes (Fig. 2C). Using the same approach, we then performed an experiment with nuclear extract from Kc cells. Notably, nuclear fractions containing histone H1 all contained T-L22, regardless of the salt conditions used [e.g., 360 mM $(\text{NH}_4)_2\text{SO}_4$ or 300 mM NaCl] (Fig. 2D; data not shown), supporting the notion of a specific interaction between nuclear ribosomal proteins and histone H1. This was also supported by the results of the IP experiments, in which both the tagged and endogenous nuclear ribosomal proteins could reverse pull-down histone H1 (Fig. 2E,F).

To exclude that the ribosomal proteins interacting with H1 were cytoplasmic contaminants, we isolated native histone H1 from the nuclei of Kc cells (see Materials and Methods) and mixed it with the cytoplasm collected from T-L22 cells. Immunoblotting of fractions collected following sucrose gradient sedimentation showed that the native H1 failed to interact with cytoplasmic 40S, 60S, or 80S ribosomes (Fig. 2G), implying that the interaction between H1 and ribosomal proteins was indeed specific to the nucleus.

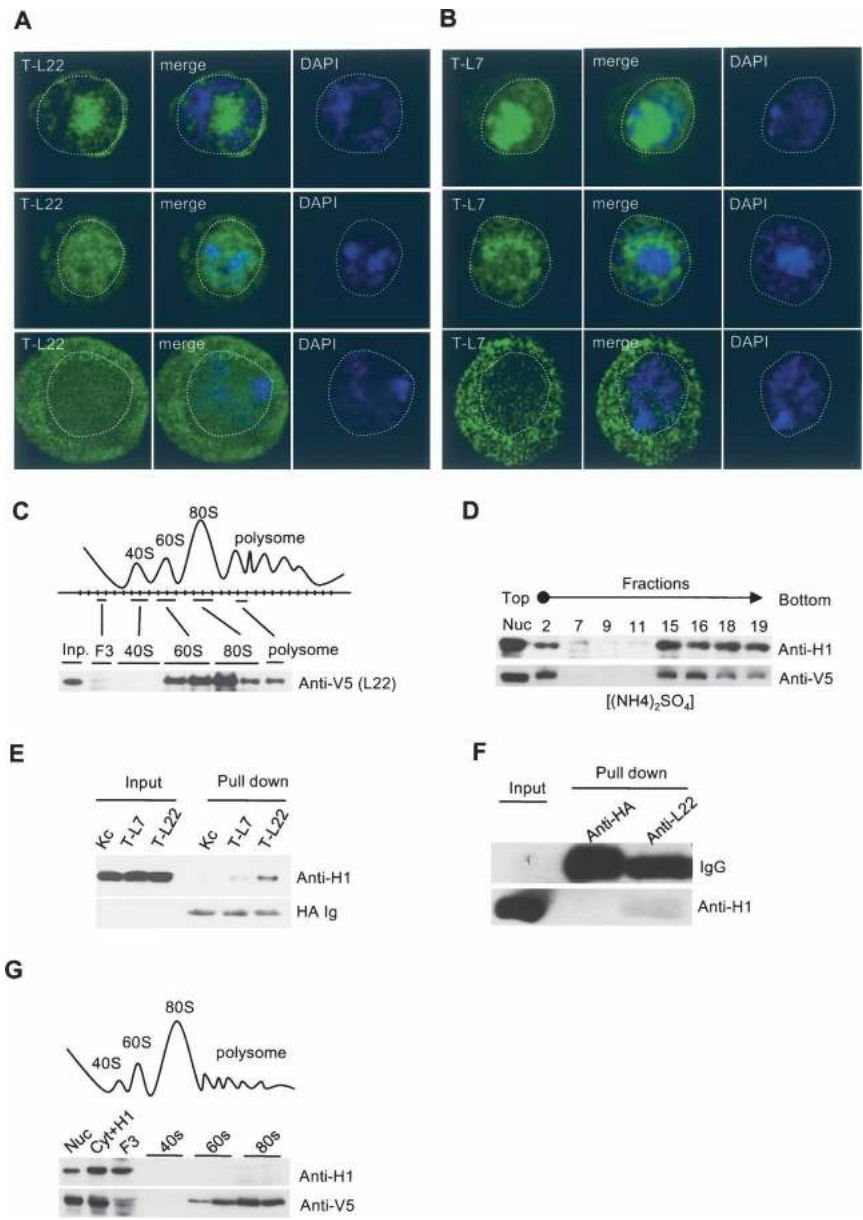
Endogenous ribosomal proteins are associated with condensed chromatin, and colocalize with H1 in the cell nucleus

To examine the association of nuclear ribosomal proteins and histone H1 in vivo, we derived specific antibodies against the N termini (1–15; L22N) and C termini (285–299; L22C) of *D. melanogaster* ribosomal protein L22 (see Supplementary Fig. S2 for antibody specificity verification).

Using these specific antibodies, we performed immunofluorescent staining experiments to study the cytoplasmic distribution of endogenous ribosomal protein L22 in Kc cells. While most cells showed both a nuclear and cytoplasmic localization, L22 in ~10% of cells appeared to be predominantly distributed in the nucleus (Fig. 3A,B; Supplementary Fig. S3A). Nucleolar localization of the endogenous L22 was also evident (Fig. 3B; Supplementary Fig. S3A). This differential distribution pattern of ribosomal protein L22 in the cells seems to be associated with the cell cycle. Interestingly, the nuclear ribosomal protein L22 often colocalized with intensive DAPI signals, which represent the most condensed chromatin in the cell nucleus. In some cells, the localization of L22 almost fully overlapped with DAPI staining (Fig. 3A,B; Supplementary Fig. S3).

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Figure 2. Colocalization and interaction of ribosomal proteins with histone H1 in the cell nucleus. (A,B) Distribution of V5-HA-tagged ribosomal proteins L22 (T-L22) (A) and L7 (T-L7) (B) in individual Kc cells. The localization of tagged proteins is illustrated in green (FITC) and DAPI staining is in blue. The cell nucleus is defined by the dotted line. (C) Ribosomal protein T-L22 is incorporated into 60S and 80S ribosomes and polysomes in the cytoplasm. The input from cytoplasmic extracts (Inp.) and fractions from sucrose gradient sedimentation were assayed by Western blot with anti-V5 antibodies to reveal the tagged T-L22 protein. The lane labeled F3 represents fraction 3 of the gradient. The A_{254} absorbance profile shows the distribution of 40S, 60S, 80S, and polysomes in the gradient. (D) Ribosomal protein T-L22 is present in all nuclear fractions containing histone H1. Selected fractions (as indicated) were resolved on a 15% SDS-PAGE gel and subjected to Western blot analysis using antibodies against H1N (anti-H1) and V5 (anti-V5; to reveal T-L22). Diluted nuclear extract (Nuc) was used as a control. (E) T-L7 and T-L22 reverse pull-down histone H1 in the cell nucleus. Nuclear extracts from Kc cells (or Kc cells expressing T-L7 or T-L22) were subjected to IP using anti-HA antibodies. IP products were resolved by 15% SDS-PAGE and subjected to Western blotting using anti-H1N antibodies to detect H1. The inputs (0.5% of total nuclear extract) from different cell lines are shown, and the amount of anti-HA antibodies used in each reaction is indicated by HA Ig. (F) Antibody against the C termini of endogenous L22 (anti-L22) pulls down endogenous histone H1. Anti-HA polyclonal antibody was used as a negative control. (G) No interaction occurs between nuclear histone H1 and 40S, 60S, or 80S ribosomes from the cytoplasm of T-L22 Kc cells. Cytoplasmic extract from cell line T-L22 premixed with purified H1 (Cyt + H1) was separated by sucrose gradient sedimentation. The top and bottom panels show Western blot analysis of gradient fractions with H1N (Anti-H1) and anti-V5 antibodies, respectively. Nuclear extract (Nuc), input extract (Cyt + H1), and fraction 3 (F3) from the gradient were also loaded onto the 15% SDS-PAGE as controls.



The distribution of L22 was also determined in wild-type c1-8 cells, derived from a *D. melanogaster* third-instar larval wing imaginal disc (Peel et al. 1990). In contrast to embryonic Kc cells, L22 was found to overlap with DAPI only in <5% of wing disc cells. The majority of cells showed both a nuclear and cytoplasmic distribution (Fig. 3C; Supplementary Fig. S3B) with only partial overlap with DAPI, implying that the nuclear localization of L22 may be developmentally regulated.

Using the same antibodies, we next compared the distribution of L22 with that of histone H1 in the cell nucleus. In Kc cells expressing GFP-tagged H1 (H1-GFP), the nuclear fraction of L22 and H1 was partially colocal-

ized when L22 was distributed in both cytoplasm and nucleus (Fig. 4A; Supplementary Fig. S3C). However, in cells in which L22 was mostly nuclear, it almost entirely overlapped with H1 in regions of highly condensed chromatin, as indicated by DAPI staining (Fig. 4A). The results support the view that histone H1 and ribosomal protein L22 are associated with each other on chromatin in vivo.

ChIP assay confirms that ribosomal proteins are associated with chromatin

Histone H1 is a known component of chromatin (Zlatanova and Van Holde 1992; Wolffe 1997; Vignali and

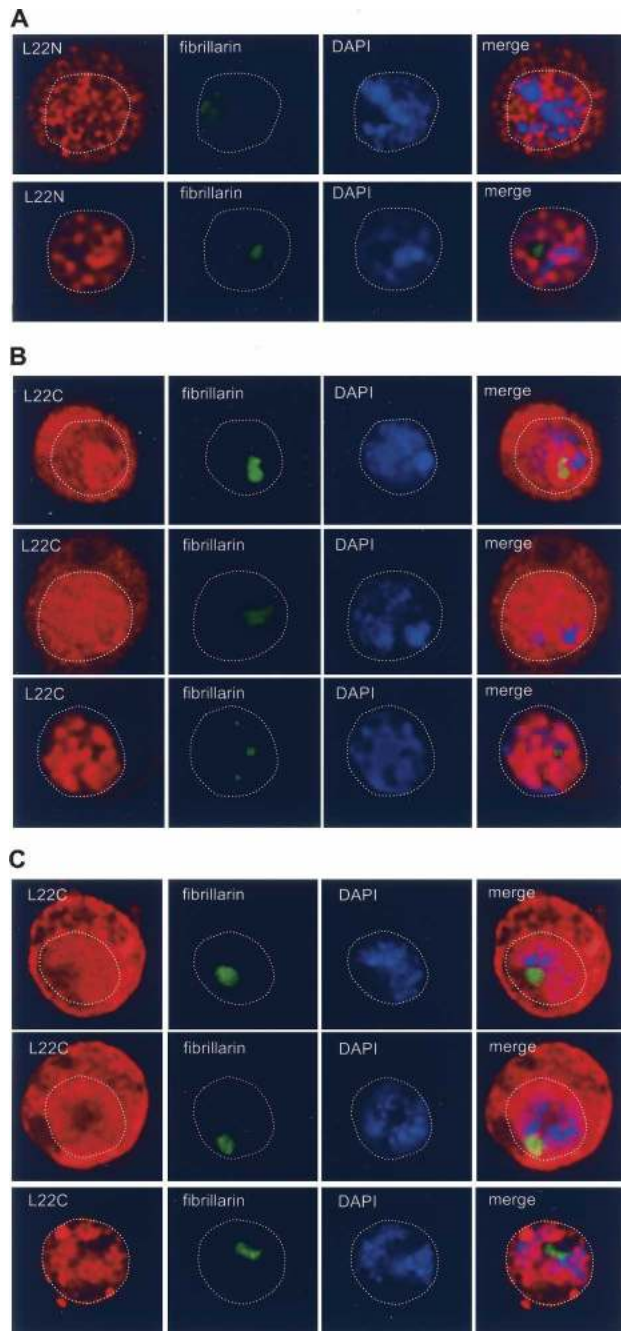


Figure 3. Localization of endogenous ribosomal protein L22 in the cell nucleus. (A,B) The dynamic localization of L22 in embryonic Kc cells, as determined by anti-L22N (A) and anti-L22C (B) antibody (red), is compared with anti-fibrillarin (green), a nucleolar marker. DAPI is indicated in blue. (C) The distribution of L22 in larval wing imaginal disc cells cl-8. The anti-L22C antibody (red), anti-fibrillarin (green), and DAPI are all indicated. The cell nucleus is defined by the dotted line.

Workman 1998; Thomas 1999). To confirm that the interaction between histone H1 and ribosomal proteins is associated with chromatin in the cell nucleus, we performed ChIP analysis using both wild-type Kc cells and the stable Kc cell line T-L22 to detect any physical as-

sociation of L22 with chromatin. ChIP analysis was performed using anti-L22C, anti-HA (to detect T-L22), and anti-H1 antibodies, and fragmented chromatin extracts from formaldehyde-fixed cells. The resulting isolated ChIP DNA was subjected to PCR analysis with primers specific for genes known to be enriched for H1 binding (J.-Q. Ni and F.-L. Sun, unpubl.). The results show that most tested genes were enriched with both H1 and L22 (Fig. 4B; Supplementary Fig. S4A), thus confirming that ribosomal protein L22 and H1 are indeed associated with chromatin *in vivo*.

The association of ribosomal proteins on chromatin is H1-dependent

We next determined whether the presence of histone H1 is essential for the association of ribosomal proteins on chromatin. Using wild-type Kc cells and an RNA interference (RNAi) procedure (see Materials and Methods), we depleted histone H1 by ~80%, and these cells were then used in further ChIP analysis. As controls for the ChIP assay, we used polyclonal anti-H3 (positive control) and anti-HA antibodies (negative controls). We chose four genes, *CG8066*, *Act57B*, *Klp38B*, and *CG4914*, known to bind histone H1 and ribosomal protein L22 on chromatin in Kc cells (Fig. 4B; Supplementary Fig. S4A), to monitor changes in binding of H1 and ribosomal proteins in H1-depleted cells. Depletion of histone H1 resulted in increased transcription of *Act57B*, *CG8066*, and *CG4914* (~10-fold, fourfold, and 2.5-fold, respectively), and a fourfold decrease in transcription of *Klp38B* (J.-Q. Ni and F.-L. Sun, unpubl.). PCR analysis of the ChIP DNA showed that the association of H1 was dramatically reduced following H1 depletion, and association of ribosomal proteins L22 and L7 with chromatin was reduced by several-fold in *CG8066*, *Act57B*, and *Klp38B* (Fig. 4C), suggesting that H1 is required for the association of ribosomal proteins with chromatin. For *CG4914*, only a minor change in L22 and L7 association was observed, possibly because some H1 remained associated with this gene following RNAi treatment (Fig. 4C). The same results were also observed in Kc cells expressing the tagged L22 (Supplementary Fig. S4B).

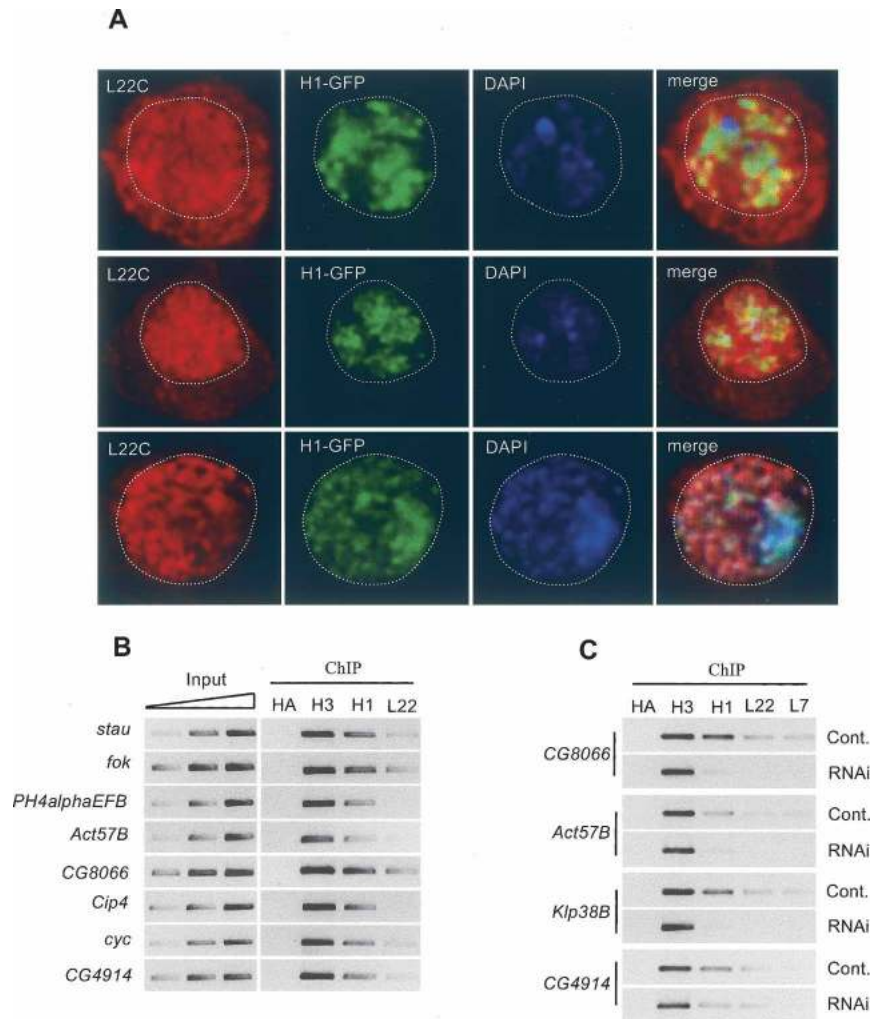
Histone H1 and nuclear ribosomal protein L22 in histone modifications

To understand the biological function of the association of ribosomal proteins with H1 on chromatin, we investigated the relationship between histone H1, ribosomal protein L22, and core histone modifications, including hallmarks of active chromatin, such as histone H3K4 methylation and H4 acetylation, and of inactive chromatin, such as H3K9 methylation.

Histone H1 has been suggested to be a repressor of specific histone modifications in mammals (Herrera et al. 2000; Gunjan et al. 2001; Vaquero et al. 2004). Using Kc cells expressing H1-GFP, we compared the H1 localization pattern with that of histone H3K4 methylation

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Figure 4. Ribosomal protein L22 is associated with chromatin enriched in histone H1 binding. (A) Colocalization of nuclear L22 with H1. L22 (L22C) is shown in red, H1-GFP is in green, and DAPI is indicated in blue. The cell nucleus is defined by a dotted line. (B) Seven H1-enriched genes are bound by L22 (two showed weak binding). Fragmented chromatin extracts from formaldehyde-fixed wild-type Kc cells were immunoprecipitated with polyclonal anti-HA antibodies (HA, used as mock control), anti-H3, anti-H1N, and anti-L22C antibodies. The resulting isolated ChIP DNA was subjected to PCR analysis with primers specific for genes known to be enriched in H1 binding (names/CG numbers indicated to the left of the panel). PCR products from the input DNA (0.1%, 0.2%, 0.4% of total chromatin DNA) were loaded into the first three lanes (on the left). (C) The association of ribosomal protein L22 and L7 with chromatin depends on histone H1. Chromatin extracts from control Kc cells (Cont.) and dsH1RNA-treated cells (RNAi) were used to perform ChIP assays. Anti-HA (HA) polyclonal antibodies were used as a negative control, and polyclonal antibodies against histone H3 (H3) were used as a positive control. Anti-L22C (L22) and anti-L7 polyclonal (L7) antibodies were used to immunoprecipitate L22- or L7-associated chromatin. ChIP DNA was subjected to PCR analysis with primers specific for genes *CG8066*, *Act57B*, *Klp38B*, and *CG4914*. The PCR products in B and C were run in a 1.5% agarose gel, stained with EB, and photographed.



and H4K8 acetylation, both hallmarks of active chromatin. The results showed that H1 is largely excluded from domains where histone H3K4 is hypermethylated, or H4K8 is hyperacetylated (Fig. 5A), supporting the notion that histone H1 may be a repressor of histone modifications in *D. melanogaster*.

Using wild-type Kc cells, we also compared the localization of L22 with H3K4 methylation. The results showed that in cells where L22 was distributed in both the nucleus and cytoplasm, the nuclear fraction of L22 seemed to partially overlap with H3K4 methylation (Supplementary Fig. S3D), consistent with a role for ribosomal proteins in active transcription (Brognia et al. 2002; Schroder and Moore 2005). However, in cells where L22 was mainly localized in the nucleus, L22 was largely excluded from chromatin where H3K4 was hypermethylated (Fig. 5A).

The above result implies that ribosomal protein L22, like histone H1, may also be associated with inactive chromatin or transcriptional repression. To test this hypothesis, we next attempted to determine whether the dosage of histone H1 and ribosomal proteins affects global histone modifications. Extracts from stably trans-

duced Kc cells overexpressing T-L22 and cells overexpressing H1-GFP were used to perform the assays. The results showed that ectopic expression of histone H1 caused a global reduction in H3K4 methylation, acetylation of H4 at Lys 8 and Lys 12, and pan-acetylated histone H4, all hallmarks of active chromatin (Fig. 5B). However, the global level of H3K9 methylation, which is believed to be associated with pericentric heterochromatin, seemed not to be affected (Fig. 5B).

Using the same experimental strategy, we then analyzed the impact of overexpression of T-L22 on histone modifications. In contrast to histone H1, overexpression of T-L22 caused only a minor reduction in histone H4K8, H4K12, and pan-acetylated H4, and no change in global H3K4 methylation (Fig. 5C). This difference from histone H1 may be due to the differential expression level of H1 and L22 in Kc cells or the suppression effect of L22 on chromatin predominantly occurring in cells where L22 is mainly present in nucleus, the percentage of such cells being rather low within the entire cell population. Alternatively, the result may imply that ribosomal proteins only play a minor role in the regulation of histone modifications.

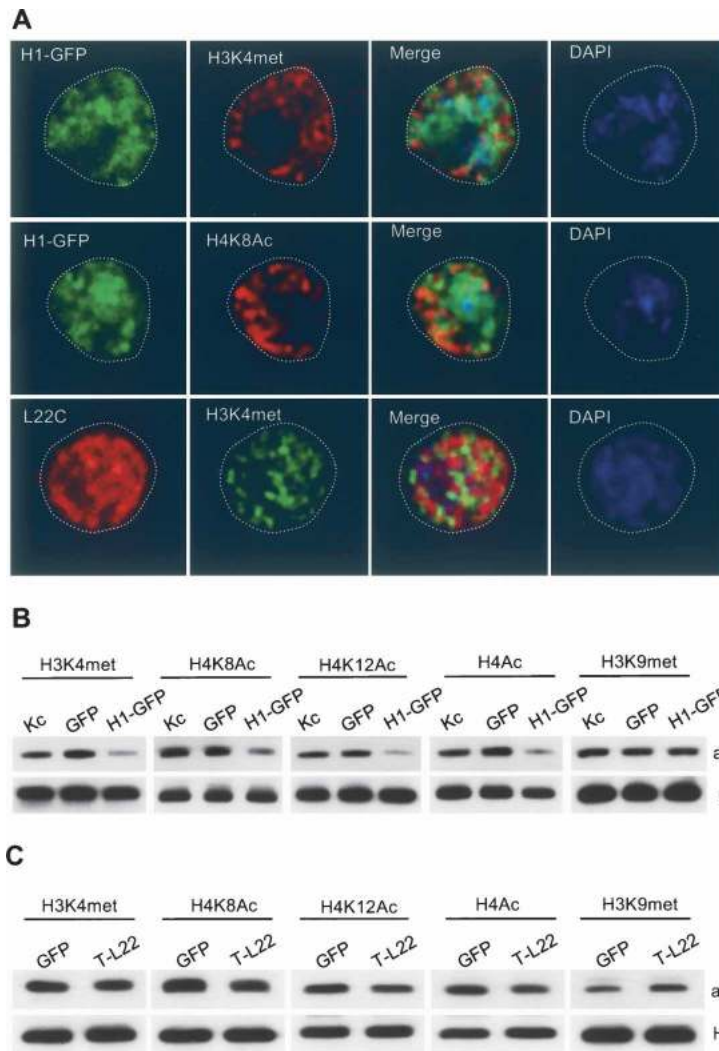


Figure 5. H1 and L22 in the suppression of histone modifications. (A) The *top* two panels show the distribution of H1 (H1-GFP; green) compared with that of methylated H3K4 (H3K4met; red) and acetylated H4K8 (H4K8Ac; red). The *bottom* panel compares the localization of L22 (L22C) with that of methylated H3K4 (H3K4met; green). DAPI is in blue. The cell nucleus is marked with cycles. (B) Overexpression of H1 causes global suppression of histone modifications. Equal amounts of cell extracts from control Kc cells (Kc), or cells expressing GFP (GFP) or GFP-tagged H1 (H1-GFP) were used in Western blot assays. Antibodies against the methylated (met) or acetylated (Ac) histones H3 and H4 are indicated at the *top* of the panel. Anti-H3 (H3) antibody was used as a loading control. (C) Overexpression of L22 slightly affects histone acetylation. Extracts from Kc cells expressing GFP or T-L22 were subjected to Western analysis with the same antibodies as in B.

Both H1 and L22 are repressors of gene transcription

To test whether, like histone H1, ribosomal proteins are involved in transcriptional repression, we performed microarray analysis using total RNA extracted from Kc cells stably overexpressing GFP (control), T-L22, or H1-GFP. The results showed that >1344 genes were significantly affected when overexpressing H1-GFP. Upon overexpression of T-L22, 1161 genes were affected. Notably, among the genes affected by H1-GFP and T-L22, >1000 genes were commonly affected by both proteins (Fig. 6A; Supplementary Tables 1, 2), and nearly 70% of these commonly affected genes (690/1007 genes) were down-regulated (Fig. 6A,B), supporting an overlapping role of ribosomal proteins and histone H1 in transcriptional repression.

To ensure that the suppression caused by histone H1 and ribosomal proteins is due to their binding to the affected genes, we next performed ChIP experiments to detect the presence of these proteins on chromatin using formaldehyde prefixed wild-type Kc cells and specific antibodies against histone H1 and ribosomal protein L22. Among nine randomly selected genes whose transcription was affected at least ninefold in cells overexpressing

H1-GFP and T-L22, seven genes showed the presence of both histone H1 and L22 on their chromatin (Fig. 6C), supporting a direct role for H1 and L22 in their transcriptional regulation. The other two showed the presence of H1, but with less, or no, binding of L22, implying that the altered transcription of these genes may be due to secondary effects.

To demonstrate that the endogenous H1 and ribosomal proteins are essential for transcriptional repression, we depleted histone H1 and L22 separately in wild-type Kc cells using an RNAi approach (see Materials and Methods). Among the six tested genes, four (*LysX*, *CG5973*, *CG8066*, and *CG8936*) showed up-regulation when depleted of H1 and L22, while two others were down-regulated (Fig. 6D). The results confirm that ribosomal protein L22 and H1 are indeed associated with transcriptional repression. However, the loss of transcription of specific genes in the absence of H1 or L22 and the up-regulation of a small set of genes upon H1 and L22 overexpression argue for a positive role by H1 and L22 in the transcription of specific genes, although this positive effect may be secondary.

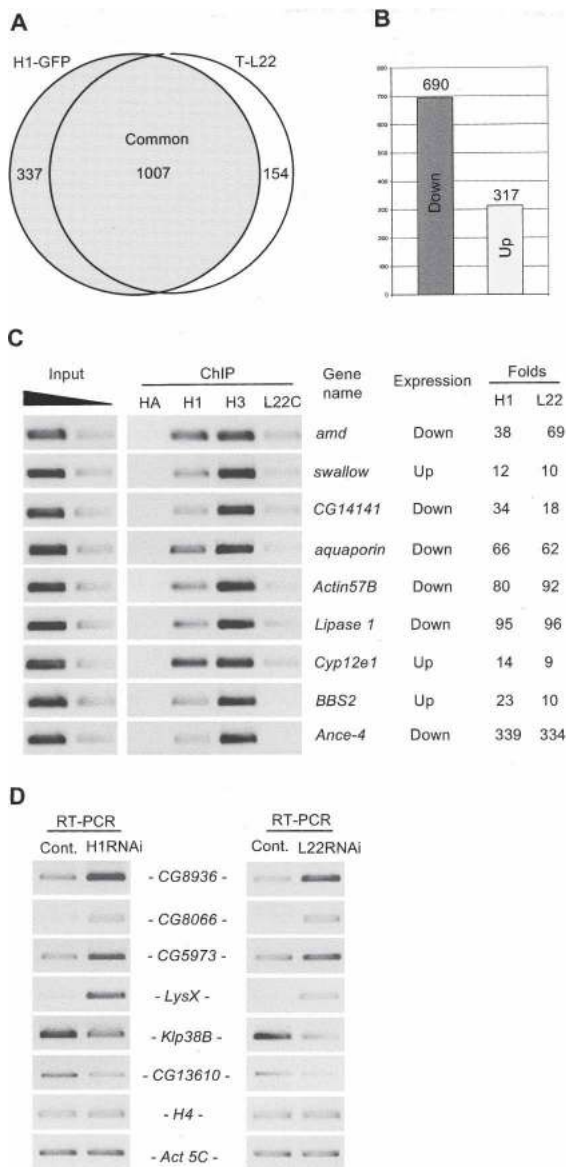


Figure 6. Overexpression of H1 and L22 causes transcriptional repression of the same set of genes. (A) The number of genes specifically affected in cells overexpressing H1 (H1-GFP), in cells overexpressing T-L22, and those commonly affected (common) are all indicated in the Venn diagram. (B) The number of genes down-regulated (Down; gray bar) and up-regulated (Up; blank bar) by H1 and L22 of the 1007 commonly affected genes. (C) ChIP analysis shows that the affected genes are directly associated with H1 and L22. The antibodies used to perform the assays, the names/CG numbers of the genes tested, the fold change in their transcription, and the PCR products from the diluted inputs (0.4, 0.05%) are all indicated. The PCR products were run in a 1.5% agarose gel, stained with EB, and photographed. (D) Deletion of L22/H1 resulted in up-regulation and down-regulation of endogenous genes. Total RNA isolated from control (Cont.), L22-depleted (L22RNAi), and H1-depleted (H1RNAi) Kc cells was used for RT-PCR analysis. The names of the tested genes are indicated. The RT-PCR products were run in a 1.5% agarose gel, stained with EB, and photographed.

Overall, the results support the hypothesis that H1 and L22 are involved in transcriptional repression.

The loss of histone H1 and nuclear ribosomal proteins on chromatin is coupled to the transcriptional hyperactivation

We next examined the role of H1 and ribosomal proteins in the transcription of heat-shock-inducible genes, whose transcription increases at least 100-fold upon heat shock (Lis and Wu 1993), and which thus serve as another system to study the relationship between H1 and ribosomal protein L22 and the process of transcription. Using cell extracts from heat-shocked and non-heat-shocked wild-type Kc cells, and antibodies against histone H1 (H1N) and pan-acetylated histone H4 (H4Ac), anti-HA antibodies, and anti-L22C, we performed ChIP analysis to detect any change in H1 and ribosomal protein L22 binding on local chromatin before and after transcription was initiated (Fig. 7A). The efficiency of the heat-shock treatment was monitored by RT-PCR, which confirmed the dramatic increase in the transcription of the heat-shock genes (data not shown). To detect changes in H1 and L22 binding on chromatin, we performed PCR analysis using ChIP DNA and primers covering the transcribed regions of three heat-shock genes, *Hsp70Aa* (CG31366), *Hsp70Ab* (CG18743), and *Hsp23*, and one non-heat-shock gene, *CG8066*. Both H1 and ribosomal protein L22 were associated with chromatin of the *Hsp70* and *Hsp23* genes before heat-shock treatment; however, binding decreased at least 10-fold following heat-shock treatment (Fig. 7A). Histone H4 acetylation in the heat-shock genes increased more than fivefold after heat shock, presumably because of the loss of histone H1 and/or L22, which would result in a relatively “open” chromatin structure. The non-heat-shock gene *CG8066* showed no changes in L22 or histone H1 enrichment, or in histone H4 acetylation, after heat-shock treatment (Fig. 7A). Using the stable cell line expressing tagged L22 (T-L22), we performed similar heat-shock experiments and obtained the same result (Supplementary Fig. S5).

The relationship between ribosomal proteins and transcriptional activation of the heat-shock genes was also verified using specific antibodies against ribosomal protein L7, another ribosomal protein associated with the H1 complex. The results show that, similar to L22, L7 was associated with the chromatin of heat-shock genes before their activation, but the enrichment was reduced after transcription was initiated (Fig. 7B). Overall, the above results support a role for histone H1 and ribosomal proteins in transcriptional repression of endogenous genes.

Ribosomal protein L22, but not H1, is associated with nuclear RNA transcripts

A previous study showed an association of functional ribosomes with newly synthesized RNA on *Drosophila*

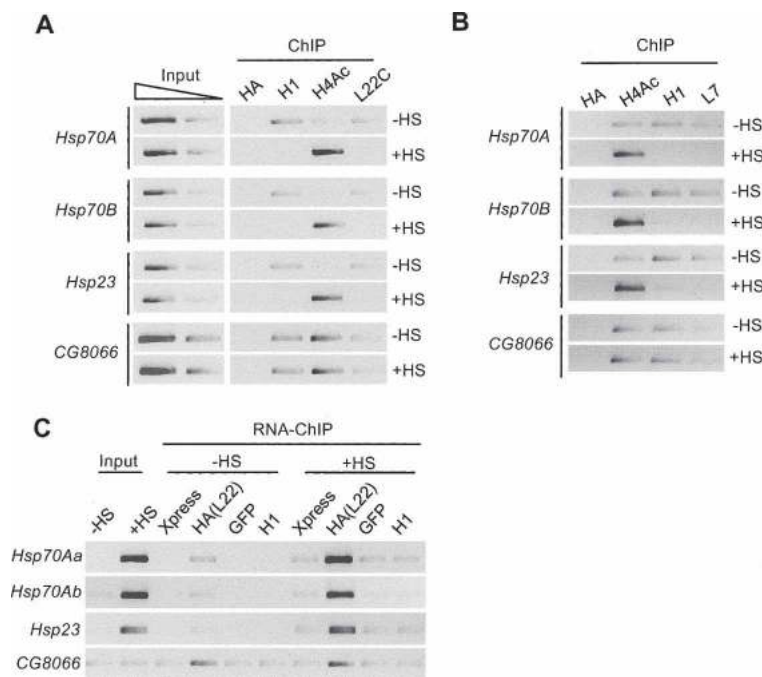


Figure 7. (A) Loss of association of L22 with heat-shock genes correlates with the absence of H1 on chromatin. ChIP assays were performed using chromatin extract from fixed non-heat-shocked (-HS) and heat-shocked (+HS) wild-type Kc cells. Antibodies against H1N (H1), pan-acetylated histone H4 (H4Ac), and anti-L22C were used in the assay as indicated. Anti-HA antibodies (HA) were used as a negative control. The PCR products from the diluted input (0.4% and 0.1%) are shown to the left. The names of the tested genes are indicated. (B) ChIP analysis performed using wild-type Kc cells and antibodies against endogenous L7, H1N, H4Ac, and HA. Chromatin extract from non-heat-shocked (-HS) and heat-shocked (+HS) Kc cells was treated with the antibodies shown. The genes tested are indicated on the left. (C) Ribosomal protein L22 becomes associated with newly synthesized *Hsp70Aa*, *Hsp70Ab*, and *Hsp23* transcripts after heat shock. Nuclei from formaldehyde-fixed T-L22 cells without (-HS) or with heat-shock (+HS) treatment were used to perform RNA-ChIP analysis using anti-Xpress (negative control), anti-HA (to reveal L22), anti-GFP (negative control), and anti-H1N (H1) antibodies. Anti-Xpress (monoclonal) was used as control for anti-HA (monoclonal). Anti-GFP (polyclonal) was used as control for anti-H1 (polyclonal). RT-PCR products from the input RNA are shown in the two lanes on the left of the gel.

polytene chromosomes isolated from salivary gland cells (Brognia et al. 2002). We wondered whether such an association also occurs in Kc cells, which are derived from *D. melanogaster* embryos. Using cells stably expressing T-L22, we analyzed the presence of ribosomal protein T-L22 and H1 on transcripts of the heat-shock genes *Hsp70Aa*, *Hsp70Ab*, and *Hsp23*. The non-heat-shock gene *CG8066* was used as a control. RNA-ChIP analysis shows that T-L22 became associated with *Hsp70* and *Hsp23* after heat shock (Fig. 7C), confirming association of this ribosomal protein with newly synthesized RNA transcripts. However, histone H1 was not found with the RNA transcripts tested, either before or after heat shock (Fig. 7C). This result confirms the presence of ribosomal proteins on newly synthesized nuclear RNA. On the other hand, the above result also implies that the interaction between histone H1 and ribosomal proteins is chromatin based, but does not occur through RNA transcripts.

Discussion

This study provides evidence that specific nuclear ribosomal proteins interact with histone H1 and are associated with chromatin. Several experimental approaches were used to corroborate this finding. Pretreatment of nuclear extract with EB or DNase I prior to IP experiments did not affect the interaction between H1 and ribosomal proteins, arguing against a DNA-mediated interaction. The immunolocalization and ChIP experiments all support their interaction and presence on chromatin in vivo.

The results from this work further suggest that *Drosophila* nuclear ribosomal proteins and H1 are involved in transcriptional gene repression. This conclusion is based on the following evidence: (1) The localization of ribosomal protein L22 overlaps with the known repressor of transcription, H1, at highly condensed chromatin regions; (2) overexpression of either H1 or L22 resulted in transcriptional repression of nearly 70% of the commonly affected genes; and (3) depletion of either H1 or L22 leads to up-regulation of the tested genes. In addition, the presence and absence of ribosomal proteins and H1 on chromatin in heat-shock genes also support this hypothesis. However, we also noticed that one-third of the affected genes in cells overexpressing H1 or L22 were up-regulated, and two out of six endogenous genes showed down-regulation in the absence of H1 or L22. This result may indicate a positive role for H1 and L22 in the transcription of specific genes; one cannot exclude the possibility that these positive effects may be secondary, for example, as a consequence of gain or loss of H1 and L22 suppression on specific transcription repressors.

The suppressive effects of H1 and L22 on transcription appeared to overlap in vivo, consistent with our biochemical assays showing that H1 and nuclear L22 are in the same complexes. Given that nearly 90% of the affected genes overlapped when H1 and L22 were overexpressed, one might have expected to see a strong colocalization pattern of the two proteins in the cell nucleus. However, the immunolocalization experiments demonstrated that rather a small percentage of cells fully overlapped; most cells showed a partial or weak colocaliza-

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tion, implying that the association could be a transient event during the cell cycle.

The suppressive effects of L22 on transcription seems to be mediated by the transcriptional repressor, H1. It has been shown that the recruiting of H1 to promoters by DNA-binding proteins or other chromatin remodeling complexes directly causes transcriptional repression in vivo (Lee et al. 2004; Vaquero et al. 2004). The fact that overexpression of H1 resulted in a several-fold reduction in global histone modifications associated with active chromatin, while overexpression of L22 only had minor effect on these modifications, further implies that H1 likely plays an important role in modulating chromatin structure. L22 and other proteins associated with H1 may be utilized to facilitate the H1-associated higher-order packaging of chromatin and transcriptional repression. The fact that depletion of L22 alone resulted in derepression of H1-repressed genes, at least argues that the presence of L22 on chromatin is involved in the H1-mediated repression in vivo. Other possibilities, such as an H1 or histone modification-independent repression mechanism, should not be fully excluded.

Our pull-down assay showed that a number of other ribosomal proteins, including specific components of 40S, also associate with H1. It is not clear whether these H1-associated ribosomal proteins are also involved in transcriptional repression. Overexpression or depletion of some of these proteins seem to affect cell viability dramatically (data not shown). This study confirmed that at least two H1-associated ribosomal proteins, L22 and L7, are associated with chromatin. It remains to be seen whether these ribosomal proteins interact with H1 alone or as a component of fully assembled ribosomes. The fact that mutated histone H1 is shifted into the nucleolus when it fails to bind chromatin (J.-Q. Ni and F.-L. Sun, unpubl.) supports the possibility that the association of ribosomal proteins on chromatin may be a part of the ribosome assembly/maturation process either before the partially assembled particles are imported into the nucleolus or before they are exported to the cytoplasm for protein synthesis.

Our finding that L22 is associated with newly synthesized nuclear Pol II RNA transcripts after gene activation is consistent with recent studies performed in *Drosophila* and budding yeast, in which ribosomal proteins were found to bind to nascent RNA transcripts on active chromatin (Brogna et al. 2002; Schroder and Moore 2005). What might be the function of ribosomal proteins association with Pol II RNA transcripts? Since functional ribosomes are the only known means to detect termination codons in mRNA, ribosomes have been proposed to be linked to nonsense-mediated mRNA decay (NMD)/mRNA surveillance, a phenomenon in which mRNA degradation is triggered by premature codons in mRNAs (Maquat 1995; Hilleren and Parker 1999; Muhlemann et al. 2001; Wilusz et al. 2001; Schell et al. 2002; Wagner and Lykke Andersen 2002; Wilkinson and Shyu 2002; Baker and Parker 2004). However, several studies in mammals and yeast indicate that NMD does not occur in the nucleus (Kuperwasser et al.

2004), and the most recent study also does not favor the interpretation that the association of yeast ribosomal proteins with sites of active transcription reflects the presence of active ribosomes (Schroder and Moore 2005). This binding may reflect other specific functions of the nuclear ribosomal proteins, for example, modulating nuclear pre-mRNA splicing as suggested by Wool (1996). Tasheva and Roufa (1995) showed previously that human ribosomal protein S14 can bind to its own pre-mRNA transcripts and regulate its transcription. Studies by a number of other groups have demonstrated that ribosomal proteins inhibit pre-mRNA splicing when they are present in excessive amounts (Bozzoni et al. 1984; Presutti et al. 1991; Fewell and Woolford 1999; Vilardeell et al. 2000; Ivanov et al. 2005). Further studies are required to verify all these possibilities.

Overall, the findings in this work, in particular that specific ribosomal proteins interact with H1, associate with chromatin, and are involved in transcriptional gene repression, provide strong support for the hypothesis that the machineries of gene expression, encompassing chromatin organization, transcription, mRNA transport, and protein synthesis, are likely to be highly coordinated (Maniatis and Reed 2002; Jensen et al. 2003). We further propose that histone H1 is one of the key regulators linking these cellular machineries.

Materials and methods

Constructs

Vectors for expression of L7 and L22 in Kc cells were constructed by subcloning the RT-PCR-amplified coding regions of L7 and L22, fused with an HA tag, into pIB/V5-His-TOPO (Invitrogen). pIB/V5-His-TOPO constructs expressing GFP were constructed by subcloning the GFP coding sequence from pcDNA3.1/NT-GFP-TOPO (Invitrogen). The pIB/V5-His-TOPO construct expressing H1-GFP was constructed by fusing the GFP-tag with the *D. melanogaster* H1 coding sequence, and then subcloning into the vector. Primer sequences are provided.

Preparation of stable Kc cell lines

All Kc cells were grown in a 25°C incubator. Transfection of constructs into Kc cells was performed according to a standard protocol (Invitrogen) with some modifications. Briefly, 1×10^6 Kc cells (in 2 mL of Schneider's *Drosophila* Medium [Gibco], with 10% fetal calf serum and 200 mM glutamine), were first seeded into a six-well plate for 1 h at 25°C. Purified plasmid DNA (5 µg) was then diluted into 100 µL of serum-free medium (Gibco) and mixed with 100 µL of serum-free medium containing 8 µL of cellfectin (Invitrogen). The mixture was incubated for 40 min at room temperature. After removing the medium from the six-well plate, the cells were washed once with 2 mL of serum-free medium, and then with 0.8 mL of serum-free medium plus the 200 µL of medium containing plasmid DNA and cellfectin. The remaining procedures followed the standard Invitrogen protocol (available online at <http://www.invitrogen.com/transfection/celltypes>).

Culture of wing imaginal disc cells

Drosophila larval wing imaginal disc cells *cl-8* were grown in M3 medium (Sigma #S-8398) supplemented with 2.5% fetal calf

serum, 2.5% fly extract, 10 $\mu\text{g}/\text{mL}$ insulin, and antibiotics (100 U/mL penicillin G, 100 $\mu\text{g}/\text{mL}$ streptomycin).

RNAi in *Kc* cells

The coding sequence of *Drosophila* histone H1/L22 was first amplified with primers containing the gene sequence plus the sequence of a T7 promoter (see Supplemental Material). Single-stranded RNA (ssRNA) was then produced using a MEGAscript T7 kit (Ambion). To prepare double-stranded RNA (dsRNA), the ssRNAs were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) at a concentration of 10 $\mu\text{g}/\mu\text{L}$ for 30 min at 65°C and for 5 min at 95°C, and the tube was then immediately placed into a glass beaker filled with water at 75°C and allowed to cool slowly to room temperature. The products were then aliquoted at 10 $\mu\text{L}/\text{tube}$ and stored in a -80°C freezer.

RNAi was performed according to the protocol of Dixon (<http://dixonlab.biochem.med.umich.edu>). For H1 RNAi, 1×10^6 *Kc* cells cultured at 25°C were suspended in 1 mL of prewarmed (25°C) serum-free medium and seeded into one well of a six-well plate. H1 dsRNA (45 μg) was then added to each well and gently mixed. After 1 h incubation at 25°C, a further 2 mL of complete *Kc* cell culture medium was added to each well. The medium was removed after 2 d of incubation in a 25°C incubator, and the cells were washed once with serum-free medium before adding another 1 mL of fresh serum-free medium containing 45 μg of H1 dsRNA. Subsequent procedures were as described above, and the RNAi treatment was performed for the third time on day 4. Cells were harvested on day 8.5 for further analysis.

For L22, 45 μg of L22 dsRNA was added into *Kc* cells. Cells were collected on day 8.5 for further analysis.

Antibodies

Drosophila histone H1 and L22 peptide sequences were as follows: H1 N terminus (CAGTKAKKSATPSHP; H1N), H1 C terminus (CATAKKPKAKTTAAKK; H1C), L22 N terminus (MAPTAKTNGDGTKTA; L22N), L22 C terminus (YFRISND DEDDDAE; L22C), and L7 N terminus (DFGNREDQINR). Injection of rabbits with these peptides and antibody purification were performed by Eurogentec (<http://www.eurogentec.com>). Anti-V5 and anti-Xpress monoclonal antibodies were purchased from Invitrogen. Anti-H4Ac, anti-H3K4met (polyclonal antibodies), anti-H3K9met, anti-H4K8Ac, and anti-H4K12Ac were purchased from Upstate Biotechnology. Anti-H3, anti-GFP, anti-fibrillarin, anti-H3K4met, and anti-HA monoclonal antibodies (HA-m) were all purchased from Abcam. Anti-HA polyclonal antibodies (HA-p) were purchased from Sigma.

Western blots

Kc cells were lysed in NP-40/300 mM NaCl buffer (1% NP-40, 300 mM NaCl, 50 mM Tris at pH 7.8). Bacteria were lysed in denaturing buffer (8 M urea, 100 mM NaH_2PO_4 , 10 mM Tris at pH 8.0). The protein concentration of the supernatant was measured using Coomassie Plus Protein Assay Reagent (Pierce). For SDS-PAGE, 20 $\mu\text{g}/\text{lane}$ were loaded for *Kc* cell extracts, and 30 $\mu\text{g}/\text{lane}$ were loaded for bacterial extracts. For modification checking, cells were lysed in HEMGN buffer (25 mM Hepes at pH 7.6, 0.1 mM EDTA, 12.5 mM MgCl_2 , 10% glycerol, 0.1% NP-40, 1 mM DTT, 0.3 M KCl), mixed with Laemmli buffer (Bio-Rad), and boiled for 5 min; 2 μg was used for loading. After electrophoresis, proteins were transferred from the gel onto Hybond-P PVDF membrane (Amersham), then hybridized with pri-

mary antibodies at the dilutions indicated: H1N (1:10,000), H1C (1:10,000), anti-Xpress (1:5000), anti-V5 (1:10,000), L22N (1:100), L22C (1:100), H3K4met polyclonal antibodies (1:3000), H4K8Ac (1:3000), H4K12Ac (1:2000), H3K9met (1:2000), and anti-H4Ac (1:5000). The secondary antibodies used were peroxidase-conjugated affininure goat anti-rabbit IgG (H + L) (1:10,000) and peroxidase-conjugated affininure goat anti-mouse IgG (H + L) (1:10,000). The ECL detection system (Amersham) was used to detect signals on the blots. Loading on the gel was monitored by staining the same membrane with Coomassie blue (Coomassie Plus Protein Assay Reagent, Pierce).

Immunofluorescence staining

Immunofluorescence staining was performed according to a standard procedure (Harlow and Lane 1999). *Kc* cells (100 μL ; $6 \times 10^6/\text{mL}$) were seeded on a polylysine slide for 10 min at room temperature, and fixed with 4% formaldehyde for 12 min. The primary antibodies used were anti-H1C (1:500), anti-H1N (1:500), anti-L22N (1:10), anti-L22C (1:10), anti-V5 (1:500), anti-H3K4met polyclonal (1:500), anti-H4K8Ac (1:300), anti-H3K4met monoclonal (1:200), and anti-fibrillarin (1:400). The DNA-staining marker, DAPI (Sigma), was used at a concentration of 1×10^{-4} $\mu\text{g}/\mu\text{L}$. Secondary antibodies coupled to FITC (green, 1:100 dilution) and anti-rabbit Texas red (red, 1:400 dilution) were purchased from Milan. Images were taken under a deconvolution microscope (Olympus, $\times 71$ model) and processed using Adobe Photoshop software.

Purification of H1 complex

Nuclear extracts from *Kc* cells used for IP were prepared following a standard protocol (for details, see Abmayr et al. 2003). *Kc* cells (4×10^8) were collected and washed once with $1 \times$ PBS, then with 10 mL of hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM DTT, protease inhibitors); the pellet was then suspended in 5 mL of hypotonic buffer, placed for 10 min on ice, homogenized, and pelleted by centrifugation (3600g, 15 min at 4°C). The supernatant (cytoplasmic extract) was collected. The pellet (nuclei) was then further purified by resuspension in 5 mL of buffer A1 (60 mM KCl, 15 mM NaCl, 15 mM Tris at pH 7.8, 1 mM EDTA, 0.1 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 0.5 mM DTT, 0.5% Triton X-100, 0.2 mM PMSF), and then gently layered onto 5 mL of buffer A2 (buffer A1 + 0.3 M sucrose). Cell debris was removed by centrifugation (9000g, 15 min at 4°C). The nuclear pellet was washed with 5 mL of buffer A2 (60 mM KCl, 15 mM NaCl, 15 mM Tris at pH 7.8, 0.5 mM spermidine, 0.15 mM spermine, 0.5 mM DTT, 0.5% Triton X-100, 0.2 mM PMSF) to remove EDTA and EGTA. The nuclei were then resuspended in 3 mL of NP-40/300 mM NaCl buffer with 300 $\mu\text{g}/\text{mL}$ EB, vortexed three times, and frozen/thawed on dry ice; this procedure was repeated an additional three times. The lysate was centrifuged at 19,000g for 15 min at 4°C. The supernatant was then precleared using protein-A-sepharose beads (Amersham) at 4°C (50 μL beads/mL lysis buffer). IP experiments were performed with anti-H1C (10 μg), 35 μL of protein A beads, and 600 μL of nuclear extract (1 $\mu\text{g}/\mu\text{L}$). As a control, 10 μg of anti-H1C antibodies were preblocked with 1 μg of the peptide used to derive the H1C antibodies. IP was performed in NP-40/300 mM NaCl buffer (see above) with overnight incubation at 4°C, followed by washing with NP-40 buffer for 6×8 min at 4°C. The pellets were boiled in Laemmli buffer (Bio-Rad) and loaded onto a 15% SDS PAGE gel. The gel was stained with Gelcode Blue Stain Reagent (Pierce) and photographed.

For digestion of the immunoprecipitate with RNase A and

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DNase I, the pellet was washed with NP-40/300 mM buffer five times, then resuspended in RNase A buffer and digested with 100 $\mu\text{g}/\text{mL}$ RNase A for 30 min at 25°C. After removing the RNase A buffer by centrifugation, DNase I buffer (10 mM Tris at pH 7.5, 2.5 mM MgCl_2 , 0.5 mM CaCl_2) and DNase I (100 U/mL) were added and incubated for 30 min at 25°C. DNase I buffer was removed and the pellet was washed once with wash buffer before being suspended in Laemmli buffer, boiled, and loaded onto a 15% PAGE gel. The gel was stained and photographed.

Sucrose gradient and polysome analysis

Nuclear extract was prepared using 2×10^8 T-L22 cells. Cells were washed once with 1 \times PBS and then with 10 mL of hypotonic buffer. The pellet was resuspended in 2 mL of hypotonic buffer (HB), incubated for 10 min on ice, homogenized, and then pelleted by centrifugation (3600g, 15 min at 4°C). The supernatant (cytoplasmic extraction) was collected. The pellet was then resuspended and homogenized in buffer HB and gently loaded onto buffer HB + 0.3 M sucrose. The nuclei were purified by centrifugation (9000g, 15 min at 4°C), washed once with buffer HB, and resuspended in 1 mL of buffer B (15 mM HEPES at pH 7.6, 110 mM KCl, 3 mM MgCl_2 , 0.1 mM EDTA, 1 mM dithiothreitol). Ammonium sulfate (4 M at pH 7.6) was then added to a final concentration of 0.36 M. The lysis of nuclei was performed by gently vortexing the tube for 1 h at 4°C (Topol et al. 1985). The extract was centrifuged at 22,000g for 30 min at 4°C. The supernatant was loaded into Spectra/PorCE (Cellulose Ester) Float A Lyzer in dialysis buffer (20 mM Tris-HCl at pH 7.4, 80 mM NaCl, 5 mM MgCl_2) for 10 h at 4°C. Dialyzed nuclear extract (450 μL , 2 $\mu\text{g}/\mu\text{L}$) was loaded onto a 17%–51% linear sucrose density gradient with 20 mM Tris-HCl (pH 7.4), 80 mM NaCl, and 5 mM MgCl_2 . The lysates were centrifuged at 36,000 rpm (SW41 rotor, Beckman) for 6 h at 4°C. Thirty fractions (400 $\mu\text{L}/\text{tube}$) were then collected from the top to the bottom (numbered from 1 to 30) using a gradient collector (FRAC-100, Pharmacia) with continuous monitoring by a UV/Vis detector (UA-6, IG instrument) measuring absorbance at A_{254} .

Sedimentation of cytoplasmic extracts from T-L22 was performed as described by Pelczar and Filipowicz (1998). We used 2×10^8 cells, which were washed once with 1 \times PBS, then with 10 mL of hypotonic buffer; the pellet was resuspended with 2 mL of hypotonic buffer, incubated on ice for 10 min, then homogenized and pelleted by centrifugation (3600g, 15 min for 4°C). The supernatant was collected, and 450 μL (4 $\mu\text{g}/\mu\text{L}$) was loaded onto a 17%–51% linear sucrose density gradient prepared in 20 mM Tris-HCl (pH 7.4), 80 mM NaCl, 5 mM MgCl_2 . The lysates were centrifuged for 6 h at 4°C at 36,000 rpm in an SW41 rotor (Beckman). Thirty fractions (400 $\mu\text{L}/\text{tube}$) were collected as described above. A 40- μL sample from each of the selected fractions was boiled in loading buffer and run on a 15% SDS PAGE gel.

Interaction between histone H1 and cytoplasmic ribosomes

For purification of nuclear histone H1, 1×10^9 Kc cells were washed once with 1 \times PBS, then suspended in 20 mL of low-salt buffer (10 mM HEPES at pH 7.9, 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM DTT, 0.5% Triton X-100, protease inhibitors), placed for 10 min on ice, homogenized, and pelleted by centrifugation (3600g, 15 min for 4°C). The pellet was washed twice with wash buffer (10 mM Tris, 1 mM EDTA, 0.5 mM EGTA, 0.2 M NaCl, protease inhibitors) and then resuspended in 2 mL of extract buffer (50 mM Tris, 50 mM EDTA at pH 7.4). Thirty microliters of 98% H_2SO_4 was added, the mixture was incubated for 1 h on

ice, and then spun at 19,000g for 15 min at 4°C. The supernatant was precipitated with acetone and dissolved in NP-40 buffer, mixed with Laemmli buffer, and then loaded onto a 15% SDS-PAGE gel, electrophoresed at 150 V for 100 min, and stained with CuCl_2 (0.3 M); protein bands were isolated and eluted from the gel using an Electro-Eluter (model 422, Bio-Rad). Purified proteins were precipitated using methanol-chloroform (<http://wolfson.huji.ac.il/purification/Protocols/ProteinPrecipitation.html>) and then dissolved in standard NP-40 buffer (1% NP-40, 150 mM NaCl, 50 mM Tris at pH 7.8), and stored at -80°C .

The interaction between H1 and ribosomes in cytoplasm extracted from cells expressing T-L22 was determined as follows: Cytoplasmic extract obtained as described above was adjusted to a final concentration of 150 mM NaCl with 3 M NaCl stock solution, and NP-40 was then added to a final concentration of 1%. NP-40 buffer (80 μL) containing 30 μg of purified histone H1 or 80 μL of NP-40 buffer control was then added separately to 400 μL of cytoplasmic extract. The mixes were rotated for 2 h at 4°C, after which 450 μL (4 $\mu\text{g}/\mu\text{L}$ protein) of extract was used for sucrose density gradient centrifugation as described above. A 30- μL sample from each selected fraction was used for Western analysis.

ChIP

ChIP analysis followed an Upstate Biotechnology protocol (<http://www.upstate.com>) with some modifications. Approximately 2×10^8 Kc cells were fixed in 1% formaldehyde, and the reaction was terminated by adding 2.5 M glycine to a final concentration of 0.125 M. The Kc cells were washed once with 5 mL of hypotonic buffer, and then resuspended with 5 mL of hypotonic buffer, incubated in ice for 10 min, homogenized, and pelleted by centrifugation. The nuclei were purified as described above and were then resuspended in 3 mL of sonication buffer (50 mM Tris at pH 8.0, 10 mM EDTA, 1% SDS, protease inhibitors). Subsequent steps were as described in the Upstate ChIP protocol. The sizes of the chromatin fragments after sonication (Branson, sonifier 250, setting at 0–1) were checked in an agarose gel using DNA purified from the chromatin fractions and digested by proteinase K (100 $\mu\text{g}/\text{mL}$) for 2 h at 45°C, followed by a phenol/chloroform extraction. In our experiments, we used fractions with a chromatin size range between 0.3 and 0.8 kb. Chromatin fractions were diluted 10 times, then 100- μL aliquots were used in each ChIP reaction. Five micrograms of each of the following antibodies were used in IP reactions: polyclonal anti-GFP (mock control), polyclonal anti-H1N, polyclonal anti-L22C, polyclonal anti-L7, polyclonal anti-H3, polyclonal anti-HA, monoclonal anti-HA, and monoclonal anti-Xpress (mock control). ChIP DNA was precipitated using 2 μL of color precipitant and ethanol. The pellet was dissolved in 80 μL of 1 \times TE; 2 μL was used in each 50- μL PCR reaction. The number of cycles used for amplification was between 30 and 35, which was within the linear range of the amplification judged by different number of cycles and the amount of input DNA used (data not shown). From a total of 50 μL of PCR products, 6 μL was loaded onto a 2% agarose gel, stained with EB, and photographed. Signals were quantified using a Molecular Dynamics PhosphorImager and data were analyzed using ImageQuant version 5.2 software. The primer sequences used to amplify ChIP DNA are given in Supplemental Material.

For the heat-shock experiments in ChIP and RNA-ChIP assays (see below), 2×10^8 Kc cells in two T_{75} flasks were incubated for 1 h in a 37°C water bath and then immediately fixed with 1% formaldehyde. Subsequent processing was as described above.

RNA-ChIP

RNA-ChIP was performed as described for the ChIP procedures, but with the addition of 0.5 U/ μ L RNASIN in all the buffers used. Nuclei from T-L22 Kc cells were first isolated from 1% formaldehyde-fixed cells and used for chromatin fragmentation. Fragment size was between 0.3 and 0.8 kb. The amount of chromatin extract and antibodies used in each reaction was the same as in ChIP assays. After IP, washing, and elution, the precipitated RNA/DNA pellets were resuspended in 70 μ L of H₂O (nuclease-free) with 1 μ L of 40 U/ μ L RNASIN, 5 μ L of 1 M Tris-HCl (RNase-free) (pH 7.5), 20 μ L of 50 mM (RNase-free) MgCl₂, and 4 μ L of 10 U/ μ L DNase I (RNase-free). The mixture was incubated for 30 min at 37°C and extracted once with phenol/chloroform (5:1). RNA was precipitated with ethanol and dissolved in 30 μ L of nuclease-free water. Twenty-seven microliters of the RNA was used for a 60- μ L cDNA synthesis reaction; 2 μ L from a total of 60 μ L of cDNA reaction was used in each RT-PCR reaction. The PCR reactions were performed between 30 and 32 cycles, which was within the linear range of the amplification judged by different number of cycles and the amount of template used (data not shown). Of 50 μ L of product, 6 μ L was loaded onto a 2% agarose gel, stained with EB, and photographed. The primer sequences used are given in the Supplemental Material.

RT-PCR

Total RNA from 4×10^6 non-heat-shocked and heat-shocked Kc cells was isolated using Trizol reagent (Invitrogen). Total RNA was then digested with DNase I, phenol/chloroform extracted, and precipitated with ethanol. Total RNA (5 μ g) was used to synthesize cDNA in a volume of 20 μ L (SuperScript II Reverse Transcriptase, Invitrogen). For each 50- μ L PCR reaction, 2 μ L of cDNA was used for 20–25 cycles, which is within the linear range of the PCR amplification judged by different amplification cycles (data not shown). PCR products (6 μ L) were loaded onto a 2% agarose gel, stained with EB, and photographed. The primer sequences used for RT-PCR are provided in Supplemental Material.

Microarray analysis

Extraction of total RNA was performed following a standard protocol. Total RNA was isolated from two or three independent populations of Kc cells that express GFP, H1-GFP, and T-L22. In brief, cells were resuspended in Trizol reagent by pipetting and were extracted with phenol-chloroform. The precipitated RNA was washed and then dissolved in RNase-free water. Five micrograms of total RNA from each experimental sample was reverse-transcribed using the SuperScript Choice cDNA synthesis kit from Stratagene. One microgram of double-stranded cDNA was in vitro transcribed using the Affymetrix IVT kit and labeled by the incorporation of biotinylated-UTP. Fifteen micrograms of cRNA was then fragmented and hybridized to Affymetrix DG GeneChips as per the manufacturer's instructions (Affymetrix).

Acknowledgments

We thank in particular Witold Filipowicz and Barbara Hohn, who provided many useful suggestions throughout this study. We thank Dr. Ramesh Pillai for his technical help, and Dr. Edward Oakeley for his assistance with the microarray analysis. We thank Michaele Schwaiger for providing us with the *cl-8* cells, our colleague Tian-Ke Wang for his help at the early stage

of the work, and Dirk Schübeler and Sarah Elgin for their critical reading of the manuscript. This work was supported by the Novartis Research Foundation.

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Genes Dev. 2006, **20**:

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