

A TFTC/STAGA Module Mediates Histone H2A and H2B Deubiquitination, Coactivates Nuclear Receptors, and Counteracts Heterochromatin Silencing

Yue Zhao,² Guillaume Lang,¹ Saya Ito,² Jacques Bonnet,¹ Eric Metzger,³ Shun Sawatsubashi,² Eriko Suzuki,² Xavier Le Guezennec,⁴ Hendrik G. Stunnenberg,⁴ Aleksey Krasnov,⁵ Sofia G. Georgieva,⁵ Roland Schüle,³ Ken-Ichi Takeyama,² Shigeaki Kato,² László Tora,^{1,*} and Didier Devys^{1,*}

¹Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS UMR 7104, INSERM U 596, Université Louis Pasteur de Strasbourg, BP 10142-67404 ILLKIRCH Cedex, CU de Strasbourg 67404, France

²Laboratory of Nuclear Signaling, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-0032, Japan

³Universitäts-Frauenklinik und Zentrum für Klinische Forschung, Klinikum der Universität Freiburg, Breisacherstrasse 66, 79106 Freiburg, Germany

⁴Department of Molecular Biology, NCMLS, Radboud University Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands

⁵Institute of Gene Biology, Russian Academy of Sciences, Moscow 119334, Russia

*Correspondence: laszlo@igbmc.u-strasbg.fr (L.T.), devys@igbmc.u-strasbg.fr (D.D.)

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SUMMARY

Transcriptional activators, several different coactivators, and general transcription factors are necessary to access specific loci in the dense chromatin structure to allow precise initiation of RNA polymerase II (Pol II) transcription. Histone acetyltransferase (HAT) complexes were implicated in loosening the chromatin around promoters and thus in gene activation. Here we demonstrate that the 2 MDa GCN5 HAT-containing metazoan TFTC/STAGA complexes contain a histone H2A and H2B deubiquitinase activity. We have identified three additional subunits of TFTC/STAGA (ATXN7L3, USP22, and ENY2) that form the deubiquitination module. Importantly, we found that this module is an enhancer of position effect variegation in *Drosophila*. Furthermore, we demonstrate that ATXN7L3, USP22, and ENY2 are required as cofactors for the full transcriptional activity by nuclear receptors. Thus, the deubiquitinase activity of the TFTC/STAGA HAT complex is necessary to counteract heterochromatin silencing and acts as a positive cofactor for activation by nuclear receptors *in vivo*.

INTRODUCTION

Transcription initiation by RNA polymerase II (Pol II) is a highly regulated process that requires the coordinated action of numerous factors. Coactivators are multisubunit complexes that are recruited to promoters by gene-specific activators to facilitate transcription initiation either by direct interaction with general transcription factors (GTFs) or Pol II or indirectly through modification of chromatin structure (reviewed in Li et al., 2007 and Martinez, 2002).

The yeast 2 MDa SAGA coactivator complex and its *Drosophila* and human homologs, the TBP-free TAF complex (TFTC) and the SPT3/TAF9/GCN5 acetyltransferase complex (STAGA), are multisubunit complexes that facilitate access of GTFs to DNA through histone acetylation mediated by the catalytic activity of the GCN5 subunit (Martinez, 2002). Human homologs of most SAGA subunits have been identified in STAGA and TFTC, and the three-dimensional structure of TFTC can be superimposed to that of SAGA (Wu et al., 2004). In addition, biochemical and functional characterization demonstrated that the described human complexes are almost identical (hereafter called TFTC/STAGA complex; reviewed in Nagy and Tora, 2007). Thus, genetic and structural studies point to an evolutionarily conserved function of these complexes with modular organization that relates to their multiple activities in regulating gene expression.

Gcn5 and other SAGA or TFTC/STAGA subunits have been shown to play a role in ligand-dependent gene activation by nuclear receptors (Wallberg et al., 2000 and Yanagisawa et al., 2002, and references therein). These observations suggested a model in which nuclear receptors recruit, in addition to other coactivator complexes, the SAGA/TFTC/STAGA complex, and that this contributes to chromatin remodeling via a mechanism involving histone acetylation.

Biochemical studies identified new components of the yeast SAGA complex that confer additional functions to the complex (Powell et al., 2004; Rodriguez-Navarro et al., 2004; Sanders et al., 2002). A ubiquitin-specific protease, Ubp8, was shown to be incorporated into SAGA through interaction with Sgf11 and to confer a histone H2B ubiquitin protease activity to the complex (Daniel et al., 2004; Henry et al., 2003; Ingvarsdottir et al., 2005; Lee et al., 2005). H2B has been shown to be monoubiquitinated (H2Bub1) on Lys 123 in *S. cerevisiae* by the E2-conjugating enzyme Rad6 and the E3-ligase enzyme Bre1 (reviewed in Zhang, 2003). Rad6-mediated H2Bub1 was found to regulate subsequent methylation of histone H3 at Lys 4 and Lys 79 through

a “trans tail” mechanism and was shown to be required for subtelomeric gene silencing (Zhang, 2003). In addition to its role in gene silencing, H2B ubiquitination plays an important role in activation of specific genes. H2Bub1 seems to be transient at the promoter of SAGA-dependent genes, and both ubiquitination and deubiquitination of H2B are required for optimal gene activation (Daniel et al., 2004; Henry et al., 2003). Interestingly, Sus1, which was shown to be a component of both SAGA and the Sac3-Thp1 mRNA export complex (Rodriguez-Navarro et al., 2004), interacts with Sgf11 and Ubp8 and regulates the deubiquitination activity of the complex (Kohler et al., 2006). In higher eukaryotes, histone H2A is also monoubiquitinated (H2Aub1), a modification which has not been reported in yeast. H2Aub1 is more prevalent (5%–15%) when compared with that of H2Bub1 (1% to 2%). H2A ubiquitination is mediated by the Polycomb repressive complex 1 (PRC1) and is crucial for Polycomb-mediated gene silencing and X inactivation (de Napoles et al., 2004; Joo et al., 2007; Wang et al., 2004).

In this study we identified ATXN7L3, USP22, and ENY2 as the human orthologs of yeast Sgf11, Ubp8, and Sus1, respectively, and show that they are integral components of TFTC/STAGA complex. These three proteins together form a module of the TFTC/STAGA complex, which specifically removes the ubiquitin moiety from monoubiquitinated histones H2A and H2B. We demonstrate that the deubiquitination module of the *Drosophila* TFTC/STAGA complex counteracts heterochromatin silencing in vivo and both the *Drosophila* and the human deubiquitination module are required for full transcriptional activation by the androgen receptor (AR). These findings suggest that the deubiquitinase activity of the TFTC/STAGA complex, in addition to its histone acetyltransferase (HAT) activity, participates in the dynamic turnover of histone modifications during the transcription cycle and functions as a positive cofactor for activation by nuclear receptors.

RESULTS

USP22, ATXN7L3, and ENY2 Are Components of TFTC/STAGA Complex

Homologs of recently identified yeast SAGA subunits (i.e., Ubp8, Sgf11, and Sus1) remained to be identified in the mammalian TFTC/STAGA complex. We analyzed a highly purified TFTC fraction (Wieczorek et al., 1998) by MS-MS mass spectrometry and identified peptides corresponding to USP22, ATXN7L3, and ENY2 (see the [Experimental Procedures](#)). Human USP22 is the closest homolog of yeast Ubp8 having a similar organization to the yeast ubiquitin protease possessing an N-terminal zinc finger domain (ZnF-UBP) and a C-terminal ubiquitin carboxyl-terminal hydrolase motif (UCH-2; [Figure 1A](#)). Human ATXN7L3 is the homolog of the yeast Sgf11 protein containing a highly conserved zinc finger domain at its N-terminal end (ZnF-Sgf11; [Figure 1B](#)). We have previously described ATXN7L3, an unknown protein at that time, based on its homology to ATXN7, another subunit of the TFTC/STAGA complex (Helmlinger et al., 2004). Indeed, ATXN7 and ATXN7L3 share a second C-terminal domain, the so-called SCA7 domain (InterPro: IPR013243), which is highly conserved through all members of the ATXN7 gene family ([Figure 1B](#)). In addition, we identified a peptide corresponding to

ENY2, a 101 amino acid protein that is homologous to yeast Sus1.

To test whether the identified polypeptides are indeed TFTC/STAGA subunits, polyclonal antibodies against synthetic peptides corresponding to USP22, ATXN7L3, and ENY2 were raised. These antibodies efficiently recognized the corresponding recombinant proteins overexpressed in Sf9 cells at the expected size, 60 kDa for USP22, 42 kDa for ATXN7L3, and 13 kDa for ENY2 (see [Figure S1](#) available online). We thus analyzed whether USP22 and ATXN7L3 would be present in a highly purified TFTC preparation. Western blot analysis using anti-USP22 and anti-ATXN7L3 detected a specific signal at the expected size in the HeLa cell nuclear extract and showed that USP22 and ATXN7L3 were enriched in the purified TFTC similarly to known components of TFTC/STAGA such as TRRAP, GCN5, and TAF10 ([Figure 1C](#)). In order to confirm that USP22, ATXN7L3, and ENY2 are integral components of the TFTC/STAGA complex, we performed a series of coimmunoprecipitation experiments on HeLa cell nuclear extract using antibodies against USP22, ATXN7L3, or ENY2. Following these immunoprecipitations, we analyzed USP22 and ATXN7L3-associated proteins by western blot and identified different TFTC subunits, such as TRRAP, ATXN7, GCN5, SPT3, or TAF10 ([Figure 1D](#), lanes 2 and 3). Similarly, TRRAP, GCN5, and TAF10 were detected in an anti-ENY2 immunoprecipitation ([Figure 1E](#)). None of the TFTC subunits were detected in a control immunoprecipitation using preimmune sera ([Figure 1D](#), lane 5 and [Figure 1E](#), lane 2). TBP, a component of TFIID, but not of TFTC/STAGA, was not found associated with USP22, ATXN7L3, or ENY2. We previously showed that ATXN7 is a specific subunit of TFTC/STAGA (Helmlinger et al., 2004). Western blot analysis of complexes obtained after an ATXN7 immunoprecipitation showed that USP22 and ATXN7L3 associate with ATXN7 together with other components of TFTC/STAGA ([Figure 1D](#), lane 4). Taken together, these results indicated that USP22, ATXN7L3, and ENY2 are bona fide subunits of the TFTC/STAGA complex.

Identification of the TFTC/STAGA Deubiquitination Subcomplex

Having established that ATXN7L3, USP22, and ENY2 are components of the TFTC/STAGA complex, we next asked how these subunits interact and incorporate into TFTC/STAGA. In order to identify putative interactions between these proteins, 293T cells were transfected with expression vectors encoding Flag-tagged ATXN7L3 or USP22, or HA-tagged ENY2, and proteins were immunoprecipitated using anti-ATXN7L3 or anti-HA antibodies. Immunoblot analysis of purified complexes revealed an interaction between ATXN7L3 and USP22 ([Figure 2A](#), lane 1) and between ATXN7L3 and ENY2 ([Figure 2A](#), lanes 3 and 7), but not between ENY2 and USP22 ([Figure 2A](#), lanes 8). When the three proteins were coexpressed, they formed a stable complex that could be purified both with anti-ATXN7L3 or anti-HA antibodies ([Figure 2A](#), lanes 2 and 6). Formation of a stable TFTC module composed of ATXN7L3, USP22, and ENY2 was further confirmed by coinfection of Sf9 cells with different combinations of baculoviruses and immunoprecipitation of ATXN7L3-associated proteins ([Figure 2B](#)). Note that when USP22 and ENY2 were coexpressed in the absence of recombinant ATXN7L3, no signal corresponding

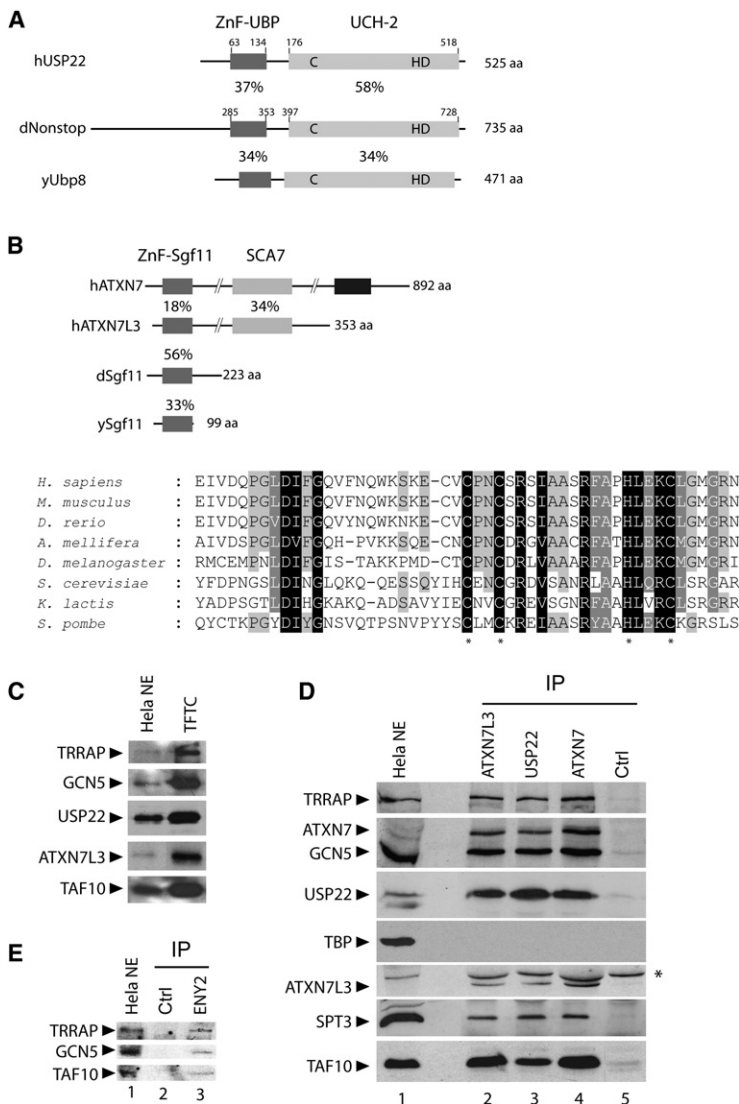


Figure 1. USP22, ATXN7L3, and ENY2 Are Integral Components of TFTC/STAGA Complexes

(A) Schematic representation of Ubp8 orthologs in human (hUSP22), *Drosophila* (dNonstop), and *S. cerevisiae* (yUbp8). Sequence identities in the N-terminal zinc finger domain (ZnF-UBP, dark gray) and in the C-terminal catalytic site (UCH-2, light gray) are indicated relative to the human USP22 sequence. Cysteine, histidine, and aspartic residues forming the catalytic triad were positioned in the proteins. SwissProt/TrEmbl/RefSeq accession numbers are as follows: *Homo sapiens* USP22 (Q9UPT9), *Drosophila melanogaster* Nonstop (NP_524140), and *Saccharomyces cerevisiae* Ubp8 (P50102).

(B) Schematic representation of ATXN7 and Sgf11 orthologs in *S. cerevisiae* (ySgf11), *Drosophila* (dSgf11), and human (hATXN7L3). Sequence identities in the N-terminal zinc finger (ZnF-Sgf11, dark gray) or in the more C-terminal SCA7 domain (light gray) are shown when compared to ATXN7L3. Lower panel, sequence alignment of the ZnF-Sgf11 from different species. Conserved residues with 100%, 80%, and 60%–80% identity are indicated with black shading, dark gray shading, and light gray shading, respectively. Histidine and cysteine residues that coordinate zinc are labeled (*). SwissProt/TrEmbl/RefSeq accession numbers are as follows: *Homo sapiens* ATXN7L3 (Q14CW9), *Mus musculus* Atxn7l3 (XP_997968.1), *Brachydanio rerio* (NP_001005396.1), *Apis mellifera* (XP_393291.2), *Drosophila melanogaster* (NP_649050.1), *Saccharomyces cerevisiae* (NP_015278.1), *Kluyveromyces lactis* (XP_453582.1), and *Schizosaccharomyces pombe* (NP_001018231.1).

(C) Proteins in HeLa cell nuclear extract (HeLa NE) or in a purified TFTC fraction were detected by western blot with the indicated antibodies.

(D) HeLa cell NEs were immunoprecipitated using anti-ATXN7L3, anti-USP22, or anti-ATXN7 antibodies or preimmune serum as a control (Ctrl). Purified complexes were analyzed by immunoblotting using antibodies against the indicated TFTC/STAGA subunits. A fraction (5%) of the input nuclear extract before immunoprecipitation was loaded as a control. IgG heavy chain migrating just above ATXN7L3 is indicated (*).

(E) Complexes associated with ENY2 were analyzed by immunoprecipitation of HeLa NE using an anti-ENY2 antibody or preimmune serum as a control (Ctrl). Purified complexes and a fraction (5%) of the input nuclear extract were analyzed by immunoblotting revealed with the indicated antibodies.

to these proteins could be detected after an ATXN7L3 immunoprecipitation (Figure 2A, lane 4 and Figure 2B, lane 5).

To analyze whether ATXN7L3 is required for USP22 association with TFTC/STAGA, we have downregulated ATXN7L3 expression by an siRNA treatment. HeLa cells were transfected with siRNA against ATXN7L3, cells were grown for 48 hr, TFTC/STAGA was purified by an ATXN7 immunoprecipitation, and the levels of TFTC/STAGA subunits were compared to those of complexes purified from control cells (Figure 2C). While the amount of GCN5 did not change in the purified complexes, USP22 was significantly reduced in the complex prepared from the cells where ATXN7L3 was knocked down (Figure 2C). These results together suggest that ATXN7L3, USP22, and ENY2 form a stable subcomplex and that USP22 and ENY2 are recruited into TFTC/STAGA by ATXN7L3 (Figure 2D).

We further asked which domains of ATXN7L3 and USP22 are involved in the formation of this subcomplex. Cotransfection and coimmunoprecipitation experiments revealed that the ATXN7L3 ZnF-Sgf11 domain alone is able to interact with both ENY2 and

USP22 as efficiently as the full-length ATXN7L3 (see Figure S3B). Similar experiments using USP22 deletion mutants showed that its interaction with ATXN7L3 required the ZnF-UBP domain, but not the catalytic domain of USP22 (data not shown). Thus, the formation of this module would be driven by interactions between ATXN7L3 and USP22 through their respective ZnF-Sgf11 and ZnF-UBP domains, and between ATXN7L3 ZnF-Sgf11 and ENY2 (Figure 2D). Finally, we showed that two different TFTC/STAGA subunits, TAF5L and ATXN7, interacted with this module and may mediate its association with TFTC/STAGA (Figure 2B and Figures S2 and S3).

Deubiquitination Activity of TFTC/STAGA

It has been shown that the yeast SAGA specifically removes ubiquitin moiety from H2Bub1 (Henry et al., 2003). We thus tested whether the human TFTC/STAGA complex has a similar activity on human H2Bub1. Although it plays an important role in transcription regulation, H2Bub1 is expected to represent less than 2% of H2B in human cells. To detect H2Bub1, HEK293T cells

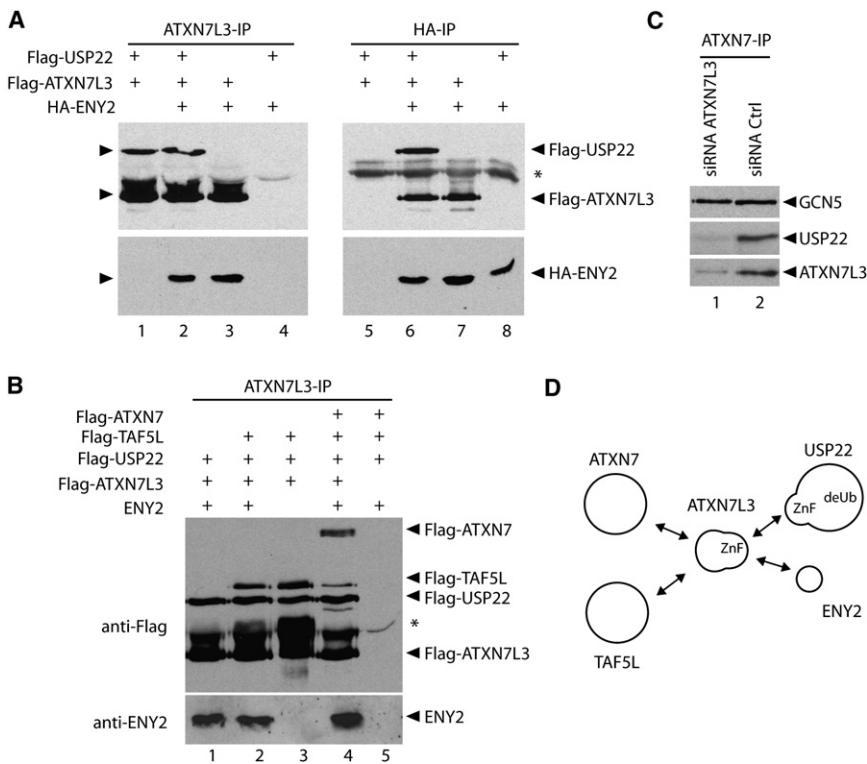


Figure 2. Interaction between Subunits of the TFTC/STAGA Deubiquitination Module

(A) 293T cells were transfected with different combinations of plasmids expressing epitope-tagged USP22, ATXN7L3, and ENY2 as indicated. Whole-cell extracts were made and proteins were immunoprecipitated using anti-ATXN7L3 or anti-HA antibodies. Purified proteins were analyzed by western blotting using anti-Flag (upper panels) or anti-HA antibodies (lower panels). IgG heavy chain is indicated (*).

(B) Sf9 cells were infected with different combinations of baculoviruses expressing ATXN7, TAF5L, USP22, ATXN7L3, and ENY2 as indicated. Immunoprecipitations using an anti-ATXN7L3 antibody were analyzed by western blotting revealed with the indicated antibodies. IgG heavy chain migrating above ATXN7L3 is indicated (*).

(C) HeLa cells were transfected with siRNA against ATXN7L3 or a control (Ctrl) siRNA. TFTC/STAGA complexes were purified using an anti-ATXN7 antibody and analyzed by western blotting.

(D) Schematic representation of interactions between different subunits of the TFTC deubiquitination module.

were cotransfected with expression vectors encoding a Flag-tagged histone H2B (Flag-H2B) and/or an HA-tagged ubiquitin (HA-Ub). Cells were grown for 48 hr, and recombinant histone H2B was immunoprecipitated using an anti-Flag antibody. When testing the purified proteins by immunoblotting, the anti-HA antibody specifically detected H2Bub1 (Figure 3A, lanes 4 and 5), while the anti-Flag antibody revealed the unmodified histone H2B and a faint signal corresponding to H2Bub1. The specific detection of H2Bub1 was also confirmed by the absence of signal when Flag-H2B or HA-Ub was omitted from the transfection reactions (Figure 3A, lanes 2 and 3).

To characterize the deubiquitination activity of TFTC/STAGA, we used the immunopurified fraction of H2B, which contains both unmodified H2B and H2Bub1 as a substrate in the *in vitro* deubiquitination assay. Two independent TFTC preparations decreased the level of H2Bub1 significantly, when analyzed with an anti-HA antibody, while the level of unmodified H2B was equal in each reaction (Figure 3B, compare lane 2 to lanes 3 and 4). The levels of H2Bub1 were further decreased when the amount of purified TFTC was increased in the deubiquitination assay, suggesting a dose-dependent effect (Figure 3B, lanes 4 and 5). Because in mammalian cells an important fraction of histone H2A is monoubiquitinated on Lys 119, we investigated the deubiquitination activity of TFTC/STAGA on the H2Aub1. Recombinant Flag-H2A-HA-Ub was prepared as described above for H2Bub1 and used as a substrate in an *in vitro* deubiquitination assay. Two different TFTC preparations could deubiquitinate H2Aub1 with a high efficiency (Figure 3C). From these results we conclude that the human TFTC/STAGA complex deubiquitinates H2Aub1 and H2Bub1.

Both *nonstop*, the *Drosophila* Homolog of USP22, and *dSgf11*, the *Drosophila* Homolog of ATXN7L3, Counteract Genomic Silencing Mediated by Heterochromatin

To characterize the *in vivo* function of the TFTC/STAGA deubiquitination activity, we followed a genetic approach in *Drosophila*. The *Drosophila* homolog of USP22 is called *nonstop* (Figure 1A) and was shown to be required for glial cell and neuronal development (Poeck et al., 2001). To determine a possible role of *nonstop* in the modulation of chromatin structure *in vivo*, we have used the well-defined *In(1)W^{m4h}* system that allows the identification of modifiers of position effect variegation (PEV) (Tartof et al., 1984). The *In(1)W^{m4h}* inversion brings the *white* locus close to a heterochromatic breakpoint leading to variegated expression of the *white* gene and a mosaic pattern of eye pigmentation. We found that mutation of one *nonstop* copy (*not⁰²⁰⁶⁹*, 75D4) or a deficient line, lacking the chromosomal region containing *nonstop* (*Df[3L]ED225*, 75C1-75D4), gave significant decrease in pigment area in this PEV model, resulting in a 2-fold decrease of pigment levels in *In(1)W^{m4h}* (Figure 4A). In good agreement, we also showed that overexpression of full-length (FL) Nonstop increased eye pigmentation to wild-type levels in the *In(1)W^{m4h}* PEV model (Figure 4B). To further investigate which domain of Nonstop is required for the effect on PEV modification, we constructed Nonstop N- or C-terminal truncation mutations. Transgenic fly lines expressing only the deubiquitinase catalytic domain of Nonstop (UCH-2) had significant increase in pigment area in the eye when tested in the *In(1)W^{m4h}* PEV model (although weaker than FL), whereas there was no modification of PEV in transgenic fly lines expressing only the ZnF-UBP domain of Nonstop

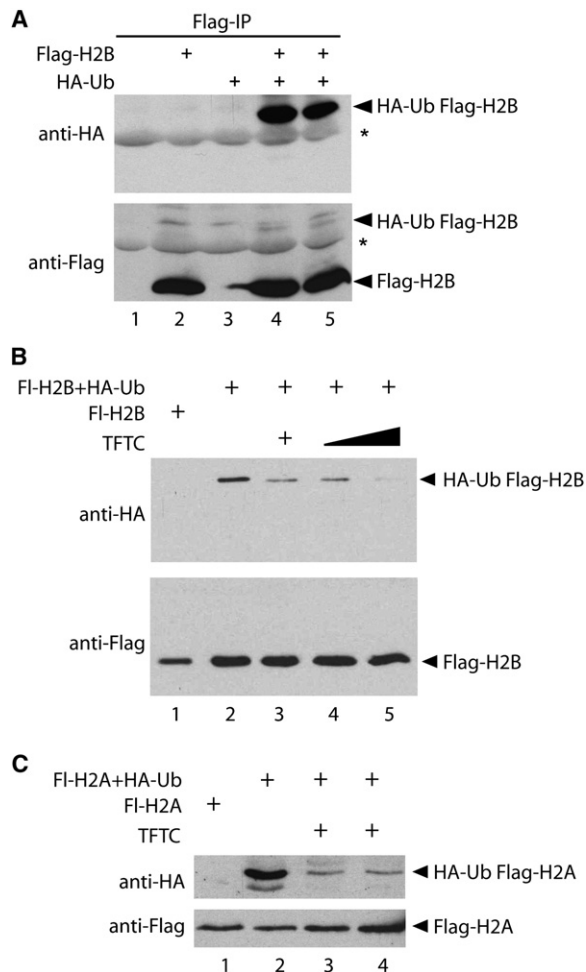


Figure 3. Deubiquitination Activity of the TFTC/SAGA Complex

(A) Purification of recombinant H2B and monoubiquitinated H2B (H2Bub1). HEK293T cells were cotransfected with plasmids encoding Flag-H2B and HA-Ub. Proteins from whole-cell extracts were immunopurified with an anti-Flag antibody and analyzed by immunoblotting with the indicated antibodies. H2Bub1 is specifically detected with the anti-HA antibody just above the IgG light chain (*).

(B) The purified TFTC complex removes ubiquitin from H2Bub1. Recombinant H2B, unmodified and monoubiquitinated, was eluted by peptide competition and used as a substrate in an in vitro deubiquitination assay using two independent TFTC preparations (in lane 3 a different preparation was used than in lanes 4 and 5). Recombinant H2B and H2Bub1 were analyzed by immunoblotting revealed with anti-Flag and anti-HA antibodies as indicated.

(C) Recombinant ubiquitinated histone H2A was purified as H2Bub1 in (A) and used in an in vitro deubiquitination assay with two independent TFTC preparations (lanes 3 and 4) as in (B).

(Figure 4B). These data strongly suggest that the deubiquitination activity of Nonstop is required for the modification of PEV in the *In(1)W^{m4h}* system.

Because both yUbp8/ySgf11 and their human homologs USP22/ATXN7L3 seem to exert their activity in a common sub-complex, next we set out to identify the *Drosophila* homolog of ySgf11 or ATXN7L3. The *CG13379* gene product (hereafter called dSgf11) was identified as a homolog of yeast Sgf11 and human ATXN7L3 (Figure 1B). We then investigated the effect

of mutations of the *dSgf11* gene on the *In(1)W^{m4h}* PEV system. *dSgf11*, similarly to *nonstop*, acted as an enhancer of PEV as its mutations led to decreased *white* expression assessed by eye pigmentation (Figure 4C). In contrast, mutation of *dRpd3*, a histone deacetylase, had no visible effect on PEV in the *In(1)W^{m4h}* system (Figure 4C). These results together suggest that both Nonstop and its partner dSgf11 are required to counteract heterochromatic gene silencing in vivo.

The Subunits of the *Drosophila* TFTC/STAGA Deubiquitination Module Are Required for Androgen Receptor-Mediated Transactivation

The fact that TFTC/STAGA has been implicated as a chromatin-modifying cofactor for activated transcription and that both Nonstop and dSgf11 counteract heterochromatic gene silencing suggested that the deubiquitination module of dTFTC/STAGA may play a coactivator role for transcriptional activators by modifying chromatin. To test this possibility, we developed an androgen receptor (AR)-associated position effect variegation (AR-PEV) model in *Drosophila*. In this system, the AR or a truncated AR harboring the ligand-independent AF-1 domain (AR[AF-1]) is expressed in the *Drosophila* eye using the *glass multimer reporter (GMR)* gene promoter (Figure 5A). A reporter construct that contains the *white* gene and a gene encoding the green fluorescent protein (GFP) controlled by eight AR-responsive elements (AREs) was inserted into a heterochromatic region leading to a mosaic red eye phenotype (Figures 5A and 5B). In this system, the AR counteracted silencing by heterochromatin spreading in a ligand-dependent manner (data not shown). The ligand-independent AR(AF-1) led to higher transactivation of the reporter genes and was hereafter used to analyze the AR-dependent alteration of chromatin structure. Two different mutations of *nonstop* (*not⁰²⁰⁶⁹* and *Df[3L]ED225*) gave significant decrease (2.2-fold) in the pigment area in this AR-dependent PEV model (Figure 5B). In good agreement, we also show that overexpression of Nonstop increased eye pigmentation to wild-type levels in the AR(AF-1)-PEV model (data not shown).

We further analyzed the effects of Nonstop on AR-dependent gene activation in vivo by testing AR(AF-1)-mediated transactivation on the GFP reporter gene expression system. In order to be able to detect GFP expression, we used additional transgenic lines expressing AR(AF-1) in the *Drosophila* eye using a GMR GAL4 driver in a GAL4-UAS system. The ARE-GFP reporter was inserted in euchromatic region and further mobilized into pericentric heterochromatin leading to variegated expression of GFP reporter (Y.Z., K.-I.T., and S.K., unpublished data). In agreement with our results obtained with the above described AR-dependent PEV model, this reporter system confirmed again that *nonstop* loss-of-function mutations, *not⁰²⁰⁶⁹* and *Df[3L]ED225*, dominantly reduce AR(AF-1) transactivation of the reporter transgene inserted in pericentric heterochromatin (Figure 5C). In contrast, but consistent with the loss-of-function effects, *nonstop* overexpression (*UAS-nonstop*) significantly increased GFP transactivation by AR(AF-1) (Figure 5C).

Having established that overexpression of Nonstop-mediated AR-dependent gene activation in pericentric region, we investigated whether this activity requires the association of Nonstop with TFTC/STAGA complex. To this end, we examined how

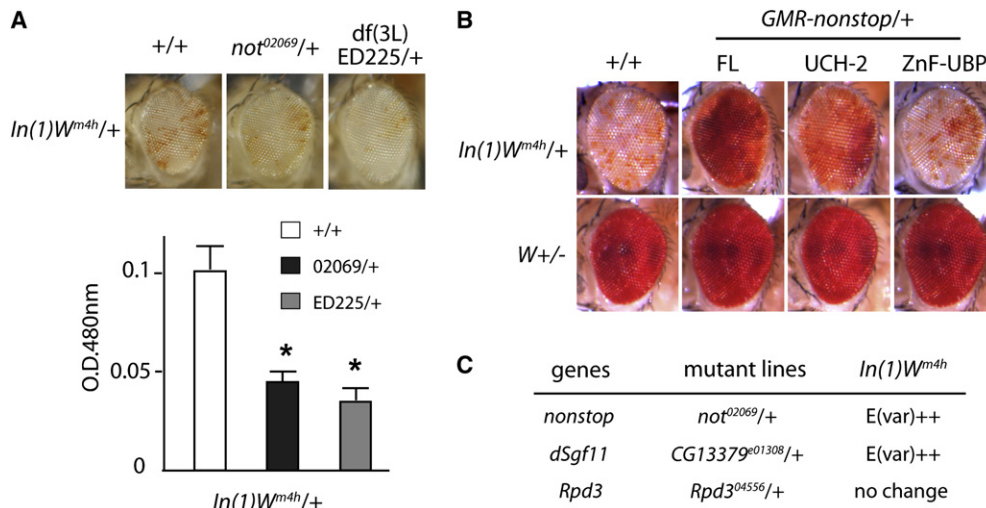


Figure 4. *Drosophila* Nonstop Counteracts Heterochromatic Gene Silencing

(A) Two different fly lines carrying *nonstop* loss-of-function mutation (*not⁰²⁰⁶⁹*) or deficiency line including *nonstop* gene location (*Df[3L]ED225*) or wild-type flies (*+/+*) were crossed with *In(1)W^{m4h}* flies. Modification of PEV was analyzed by areas of eye pigmentation in the progeny (upper panels) and by optical density (OD) measurement at 480 nm (lower panel). Average values of more than three independent measurements are shown with SD. **p* < 0.01 when compared to *+/+* control (Student's *t* test).

(B) The catalytic domain of Nonstop is required for its modification effect on PEV. *In(1)W^{m4h}* flies were crossed with wild-type flies (*+/+*) or with flies overexpressing full-length Nonstop (FL), Nonstop catalytic domain (UCH-2), or Nonstop ZnF-UBP (ZnF-UBP) (upper panels). Effect of Nonstop overexpression was assessed by modification of eye pigmentation. Flies carrying the *white* gene in its normal locus were used as a control (lower panels).

(C) Both Nonstop and Sgf11 are enhancers of PEV. Heterozygous males from the lines bearing mutations of *dSgf11* and *Rpd3* were mated to (*In[1]W^{m4h}*) females. Eye pigmentation was analyzed in 2- to 3-day-old male progeny.

mutations of different TFTC/STAGA components affect Nonstop-mediated AR(AF-1) transcriptional activation *in vivo* using the second GFP transactivation system. As shown in Figure 5D, Nonstop overexpression enhanced AR(AF-1) transactivation of the ARE-GFP reporter gene expression. In good agreement with our biochemical interaction studies, mutations of *dSgf11* (*CG13379^{e01308}*) and *e(y)2* (*Df(1)m259-4*) significantly reduced GFP expression (Figure 5D). Importantly, the coactivation activity of Nonstop was not modified by mutation of *dTAF5* (*Taf5^{EY01764b}*), which is a specific component of TFIID. Taken together, these results suggest that the deubiquitination activity of *Drosophila* TFTC/STAGA, mediated by Nonstop, requires the components of the TFTC/STAGA deubiquitination module to play an important role in AR-dependent transactivation *in vivo*.

The Human Deubiquitination Module and Its Activity Are Positive Modulators of AR-Mediated Transactivation in Human Cells

The fact that *Drosophila* Nonstop, Sgf11, and E(y)2 increased *in vivo* transactivation activity of the AR on silenced reporter transgenes suggested that these proteins play a coactivator role in AR activation. To further investigate this hypothesis, we analyzed putative interactions of USP22 or ATXN7L3 with AR in mammalian cells. Cotransfection in HEK293T cells and coimmunoprecipitation experiments revealed that AR interacted with ATXN7L3 in a ligand-dependent manner, similarly to GCN5, while interaction between AR and USP22 was detected also in the absence of ligand (see Figure S6A). As AR and USP22 interacted *in vivo*, we next tested whether AR could be a substrate of the TFTC/STAGA deubiquitination activity. Coexpression of

increasing amounts of USP22 with AR in 293T cells revealed that the AR steady-state levels were not modified, indicating that AR is not a substrate of USP22 (see Figure S6B).

In order to determine whether USP22, ATXN7L3, and ENY2 are required for AR-dependent transcriptional activity in human cells, we performed AR-dependent reporter assays. 293T cells were transfected with an AR-dependent reporter gene together with expression vectors encoding AR alone, or together with USP22, ATXN7L3, and ENY2 in the presence or absence of ligand (Figures 6A and 6B). USP22, ATXN7L3, and ENY2 significantly enhanced AR-mediated transactivation in the presence of ligand (2.5- to 3.5-fold compared to AR alone), similarly to the effect of the GCN5 HAT (Figures 6A and 6B). Furthermore, we observed that USP22 dominantly increased also ER- or GR-mediated transactivation in similar reporter assay analyses (data not shown). These results suggested that USP22, ATXN7L3, and ENY2 can function as coactivators for nuclear receptor-mediated transactivation. We next examined whether the deubiquitination activity of USP22 is required for coactivation of AR transcriptional activity. To this end we constructed an expression vector encoding a USP22 mutant in which the two conserved histidine residues in the catalytic site (His box) were mutated to alanines. Such mutations in the active site of ubiquitin-specific proteases were shown to result in undetectable levels of catalytic activity (Hu et al., 2002). Expression of this inactive USP22 catalytic mutant did not change the ligand-induced activation of the AR-dependent luciferase reporter (Figure 6A), further demonstrating that USP22 deubiquitination activity is required for its nuclear receptor coactivation function. In good agreement with our observations in *Drosophila*, ATXN7L3, ENY2, and USP22,

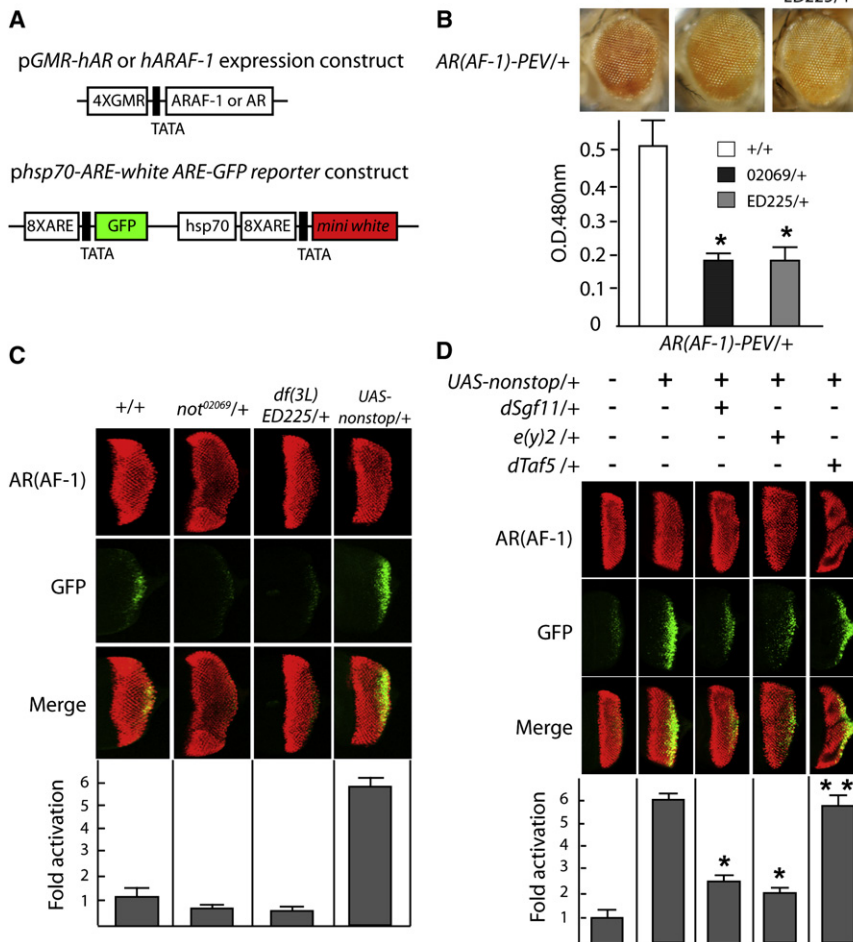


Figure 5. The Androgen Receptor Coactivation Function of Nonstop Requires dSgf11 and dE(y)2

(A) Schematic representation of the expression and reporter constructs. The expression constructs include the full-length human androgen receptor (hAR) or hAR(AF-1) driven by four GMR binding sites. The reporter construct harbors GFP and *white* reporter genes driven by the hsp70 promoter in which eight AREs were introduced.

(B) AR(AF-1)-PEV flies were crossed with *nonstop* mutant lines, and progeny were analyzed as in Figure 4A.

(C) Flies expressing AR(AF-1) in the eye using a GMR GAL4 driver and carrying an ARE-GFP reporter in pericentric heterochromatin were crossed with lines harboring *nonstop* loss-of-function (*not*⁰²⁰⁶⁹, *Df*[3L]ED225) or gain-of-function (*UAS-nonstop*) mutations as indicated. Expression of AR(AF-1) was assessed by immunostaining using an anti-AR antibody (upper panels). The effect of *nonstop* mutations or overexpression on AR(AF-1)-mediated transactivation was assessed by examination of GFP expression (middle panels). Merge images are shown in lower panels. (D) Flies carrying ARE-GFP reporter and AR(AF-1) expression transgenes were crossed with flies carrying *UAS-nonstop* and loss-of-function mutations of *dSgf11* (*CG13379*^{e01308}), *e(y)2* (*Df*[1]m259-4), and *dTaf5* (*Taf5*^{EY01764b}). Analysis of AR(AF-1) transactivation was performed on eye disc of the third instar larvae from the progeny. Average values of four independent measurements are shown with SD. *p < 0.001 and **p > 0.10 when compared to *UAS-nonstop*/+ control (Student's t test).

but not the USP22 catalytic mutant, increased transactivation also by the ligand-independent AR(AF-1) in similar reporter assays (Figure 6C).

We then asked whether the TFTC/STAGA deubiquitination activity is required for endogenous AR-dependent gene activation in vivo. To this end, we turned to LNCaP prostate tumor cells in which the AR is constitutively expressed and analyzed the androgen-induced expression of the endogenous AR target gene *KLK2* after downregulation of USP22. Transfection of LNCaP cells with siRNA against USP22 led to a 55% reduction of USP22 mRNA and protein levels (Figure 6D and data not shown). Induction of *KLK2* expression by the synthetic AR agonist R1881 in LNCaP cells transfected with control siRNA was 3-fold and significantly reduced in cells transfected with siRNA against USP22 (1.6-fold) (Figure 6D). This result together with the observed interaction between the AR and both ATXN7L3 and USP22 suggested that these proteins are recruited together to the promoters of AR-dependent genes upon ligand induction. To test this hypothesis, chromatin was prepared from LNCaP cells treated with or without ligand and subjected to chromatin immunoprecipitation (ChIP) using anti-AR and anti-USP22 antibodies. Recruitment of the AR was significantly increased at the promoter region of *KLK2* gene after ligand addition

(Figure 6E). Similarly, USP22 associated with the chromatin of *KLK2* promoter in a ligand-dependent manner (Figure 6E). No significant association of AR and USP22 with chromatin at an intergenic region could be detected, and the amount of histone H3 at *KLK2* promoter remained constant, further demonstrating specificity (Figure 6E). Taken together, these results indicate that upon ligand induction TFTC/STAGA is recruited to the promoter of AR-dependent genes where its deubiquitination activity facilitates gene expression.

DISCUSSION

In this study, we identified three subunits of the 2 MDa GCN5-containing TFTC/STAGA complex. We show that the ubiquitin protease USP22 forms a subcomplex with ATXN7L3 (*ySgf11* homolog) and ENY2 (*ySus1* homolog). Importantly, we demonstrate that the catalytic domain of the ubiquitin protease together with *dSgf11* is necessary to modify PEV in the *Drosophila* eye, pointing to the involvement of the deubiquitination module in chromatin remodeling. Furthermore, we show that the subunits of the deubiquitination subcomplex are required for the full activity of the ligand-induced AR and other nuclear receptors in vivo. Thus, to our knowledge we established for the first time the

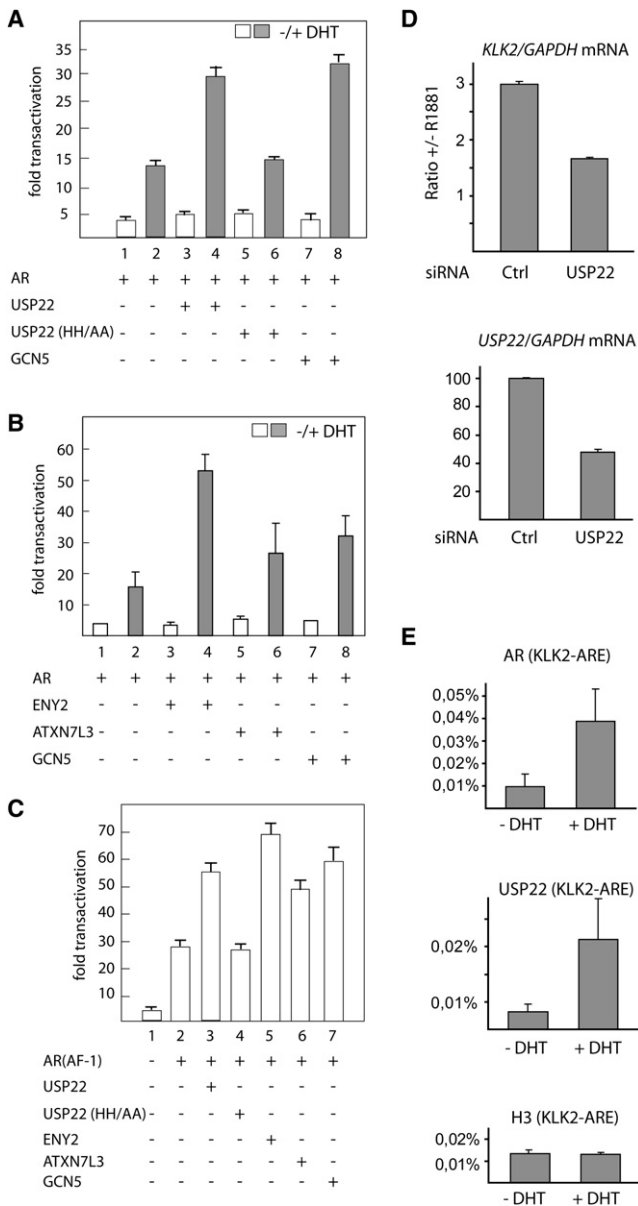


Figure 6. Androgen Receptor-Dependent Transactivation Requires the Deubiquitination Activity of USP22, ATXN7L3, and ENY2 in Human Cells

(A and B) 293T cells were cotransfected with plasmids expressing the full-length androgen receptor (AR) together with USP22, USP22(HH/AA), GCN5 (A), ATXN7L3, or ENY2 (B) expression plasmids with AR-dependent reporter in the absence (white bars) or presence (gray bars) of ligand (DHT). GCN5, ATXN7L3, ENY2, and USP22, but not a USP22 catalytic mutant, mediate AR-induced transcriptional activity.

(C) Similar results were obtained using the ligand-independent AF-1 domain (AR[AF-1]). In (A)–(C), the error bars represent mean \pm SD ($n \geq 4$).

(D) LNCaP cells were transfected with either control (Ctrl) or USP22 siRNA and treated with or without ligand (R1881). Induction of *KLK2* expression is expressed as the ratio of *KLK2* mRNA levels normalized to *GAPDH* levels between cells treated and untreated with R1881 (upper panel). *USP22* downregulation was assessed by quantitative RT-PCR. *USP22* levels are presented as a percentage of the mean of control cells after normalization to *GAPDH* mRNA levels (lower panel).

deubiquitination activity of the metazoan TFTC/STAGA HAT complexes as a second positive chromatin-modifying function.

TFTC/STAGA Complex Has a Modular Organization that Links the Deubiquitination Function to HAT, Activator Binding, and Transcription Initiation

An overlay of the yeast SAGA and the human TFTC structures indicated, in good agreement with their similar subunit composition (Nagy and Tora, 2007), a high degree of structural conservation in size and shape (Wu et al., 2004). Several SAGA subunits have been localized in one of the five domains of the complex (Wu et al., 2004). Gcn5 was detected in the central region of SAGA and colocalized with Ada2 and Ada3, indicating that this central domain harbors the HAT activity. We have now identified a module of TFTC/STAGA, which is responsible for the deubiquitination activity of this complex on H2Aub1 and H2Bub1. This module is composed of three subunits, among which ATXN7L3 (hSGF11) interacts with TAF5L and ATXN7 and allows the recruitment of both USP22 and ENY2 to TFTC/STAGA. Because yTAF5 localizes close to the Gcn5-containing HAT module, it is tempting to speculate that a functional connection may exist between the HAT and the deubiquitination modules. Along these lines it is interesting to note that neither free USP22 nor a stable recombinant subcomplex, composed of TAF5L, ATXN7L3, ENY2, and USP22, can deubiquitinate H2Aub1 or H2Bub1 in vitro (see Figure S4), suggesting that additional TFTC subunits are required for the regulation of this activity.

Interestingly, the two different TFTC/STAGA subunits, ATXN7 (ySgf73 homolog) and ATXN7L3 (ySgf11 homolog) share a conserved domain and may have a related function within the complex. A polyglutamine expansion in ATXN7 has been identified as the mutation responsible for an inherited neurodegenerative disorder, Spinocerebellar ataxia type 7 (SCA7). It remains to be investigated whether a polyglutamine expansion in ATXN7 deregulates the TFTC/STAGA deubiquitination activity and whether this would account for chromatin modifications and transcriptional alterations previously identified in SCA7 models (Helmlinger et al., 2006).

The yeast and the *Drosophila* homolog of hENY2 (Sus1 and E[y]2, respectively) were identified as subunits of SAGA and an anchoring and mRNA export (AMEX) complex, homolog of the yeast Sac3-Thp1-Sus1 complex (Kurshakova et al., 2007; Rodriguez-Navarro et al., 2004). Sus1 or dE(y)2 as subunits of AMEX function together with SAGA/TFTC in the anchoring of a subset of transcription sites to the nuclear pore complexes (NPCs) to achieve efficient transcription and mRNA export (Cabal et al., 2006; Kurshakova et al., 2007). The association of USP22 and ENY2 in the same TFTC/STAGA module would therefore

(E) LNCaP cells were incubated with or without ligand (DHT) and subjected to ChIP using antibodies against AR (upper panel), USP22 (middle panel), or unmodified histone H3 (lower panel). Precipitated DNA was quantified by real-time qPCR using primers flanking the promoter region of *KLK2* gene (–343 to –90). The values are expressed as percentage of the amount of immunoprecipitated DNA normalized to the respective input DNA signal. Background signals obtained from an intergenic region were subtracted for the quantification of AR and USP22 association with the specific region. All results are representative of at least two independent experiments. In (D) and (E), the error bars represent mean \pm SD ($n = 2$).

suggest that two activities, histone deubiquitination and mRNA export, may also be coregulated within this complex. Although we showed that TFTC/STAGA deubiquitinates H2Aub1 and H2Bub1, USP22 could act on other nonhistone targets as suggested by the accumulation of several ubiquitinated proteins after inactivation of *Drosophila nonstop* (Poeck et al., 2001).

Different histone deubiquitinases involved in transcriptional regulation have been identified in metazoan. *Drosophila* USP7 specifically deubiquitinates H2Bub1 and regulates Polycomb-mediated silencing (van der Knaap et al., 2005). Recently, two ubiquitin proteases, Ubp-M/USP16 and 2A-DUB/KIAA1915/MYSM1, were identified as specific H2A deubiquitinases regulating *Hox* gene expression and AR-dependent gene activation (Joo et al., 2007; Zhu et al., 2007). In contrast to the specific recognition of either H2Aub1 or H2Bub1 by these enzymes, we showed that TFTC/STAGA is active on both H2Aub1 and H2Bub1 in vitro. Thus, it seems that, in addition to these enzymes that seem to be active only at specific target genes, TFTC/STAGA has a broader specificity to counteract heterochromatin silencing and to regulate gene activation by nuclear receptors. Alternatively, the action of TFTC/STAGA on either H2Aub1 or H2Bub1 could be restricted to specific genes in vivo.

The Functional Link between the HAT and the Deubiquitination Activities of TFTC/STAGA in Gene Activation

Genetic and biochemical analysis in yeast and *Drosophila* suggested that the 2 MDa GCN5-containing complexes have multiple roles in the cells: global deposition of histone H3 acetylation marks along the entire chromosomes and targeted acetylation of histones at specific loci. Our results obtained with the ubiquitin protease mutants in both the *In(1)W^{m4h}* PEV system or the AR-dependent PEV model suggest that, similarly to its HAT activity, the deubiquitination module of SAGA/TFTC is influencing both the global chromatin architecture, as a modifier of PEV, and the gene-specific function of the complex, as a nuclear receptor coactivator. This intriguing parallel raises the following questions: is the TFTC/STAGA HAT activity in vivo functionally connected with deubiquitination activity within the complex, and which activity is needed first? Interestingly, all the subunits of the deubiquitination module of TFTC/STAGA are able to function as coactivators during the activation process of AR, similarly to what has been demonstrated for individual subunits of the HAT module (Meng et al., 2004; vom Baur et al., 1998) or for the entire 2 MDa HAT complexes (Yanagisawa et al., 2002). In this respect the HAT and the deubiquitination modules seem to fulfill similar functions suggesting that the two activities together are necessary to achieve full activation of nuclear receptors on chromatin templates. Histone acetylation is thought to open up the chromatin, which may suggest that the deubiquitination activity of the complex would work on templates, which have been previously acetylated by the complex.

Our study pinpoints the monoubiquitination of histone H2A and H2B as a mark that is timely regulated for appropriate transcription in metazoan organisms and further emphasizes that the precise turnover of chromatin modifications is crucial for the control of gene expression. The association of both HAT and deubiquitination activities in the TFTC/STAGA complex provides

an attractive mechanism by which the so called “cross-talk” between given histone marks is coordinated within the same regulatory complex. Further mechanistic studies will be required to address the exact link between these activities and the other chromatin-modifying complexes to understand how these sequential events participate in chromatin remodeling and gene activation.

EXPERIMENTAL PROCEDURES

Plasmids, Fly Stocks, and Genetics

Plasmid construction, generation of transgenic flies, fly stocks, and crossing schemes are presented in the Supplemental Experimental Procedures.

Mass Spectrometry

Mass spectrometry was performed using LTQ-FT mass spectrometer (Thermo Electron, Bremen, Germany) essentially as described previously (Olsen et al., 2004). Peptides identified were as following: SPHIPYK, DFFLSDR, and ITSNC TIGLR corresponding to USP22; SHLSLVGTASGLGSNKK and SLLTTQCGVI SEHTK corresponding to ATXN7L3; and TFLAQHASL corresponding to ENY2.

Antibodies

The anti-USP22 (2391 and 2392) and anti-ENY2 (2584 and 2585) polyclonal antibodies were obtained by immunization of rabbits with peptides corresponding to residues 2–15 (VSRPEPEGEAMDAE) of human USP22 and to residues 50–66 (CKEVIKEKGLEHVTVD) of human ENY2. Rabbits and mice immunization with ATXN7L3 peptides corresponding to residues 278–296 (LQWDGSSDLSPSDSGSSKT) and to residues 335–353 (KKKPKPPAPPTPS IYDDIN) produced the 2326 polyclonal and the 2ATX-2B1 monoclonal antibodies and the 2325 polyclonal and 1ATX-2D7 monoclonal antibodies, respectively.

In Vitro Deubiquitination Assay

293T cells transfected with Flag-H2A or Flag-H2B (gift from R.G. Roeder) and/or HA-Ub (gift from I. Dikic) plasmids were collected in phosphate buffer saline 1X (PBS1X) containing 20 mM N-ethylmaleimide (NEM) and 1 mM PMSF. Whole-cell extracts prepared in modified RIPA buffer (50 mM Tris-HCl [pH 7.9], 150 mM NaCl, 5 mM EDTA, 15 mM MgCl₂, 1% NP40, 0.5% sodium deoxycholate, 1 mM DTT, 1X protease inhibitor, 1 mM PMSF, and 20 mM NEM) were immunoprecipitated using M2 beads (Sigma). After extensive washing, bound proteins were eluted with an excess of free Flag peptide. Eluted proteins were used as a substrate in a deubiquitination reaction with different amounts of highly purified TFTC complex prepared as described (Wieczorek et al., 1998). The reaction was performed in 100 mM Tris-HCl (pH 7.9), 5% glycerol, 1 mM DTT, and 1 mM EDTA for 2 hr at 37°C. Unmodified and monoubiquitinated recombinant H2A or H2B were analyzed by immunoblotting using anti-Flag and anti-HA antibodies.

Supplemental Data

Supplemental Data include six figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at <http://www.molecule.org/cgi/content/full/29/1/92/DC1/>.

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