TnaA, an SP-RING Protein, Interacts with Osa, a Subunit of the Chromatin Remodeling Complex BRAHMA and with the SUMOylation Pathway in Drosophila melanogaster

Juan Monribot-Villanueva, R. Alejandro Juárez-Uribe, Zoraya Palomera-Sánchez, Lucía Gutiérrez-Aguilar, Mario Zurita, James A. Kennison, Martha Vázquez

1 Departamento de Fisiología Molecular y Genética del Desarrollo, Instituto de Biotecnología-Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México, 2 Program in Genomics of Differentiation, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland, United States of America

Abstract

Tonalii A (TnaA) is a Drosophila melanogaster protein with an XSPRING domain. The XSPRING domain harbors an SP-RING zinc-finger, which is characteristic of proteins with SUMO E3 ligase activity. TnaA is required for homeotic gene expression and is presumably involved in the SUMOylation pathway. Here we analyzed some aspects of the TnaA location in embryos and larval stages and its genetic and biochemical interaction with SUMOylation pathway proteins. We describe that there are at least two TnaA proteins (TnaA1,13 and TnaA1,23) differentially expressed throughout development. We show that TnaA is chromatin-associated at discrete sites on polytene salivary gland chromosomes of third instar larvae and that tna mutant individuals do not survive to adulthood, with most dying as third instar larvae or pupae. The tna mutants that ultimately die as third instar larvae have an extended life span of at least 4 to 15 days as other SUMOylation pathway mutants. We show that TnaA physically interacts with the SUMO E2 conjugating enzyme Ubc9, and with the BRM complex subunit Osa. Furthermore, we show that tna and osa interact genetically with SUMOylation pathway components and individuals carrying mutations for these genes show a phenotype that can be the consequence of misexpression of developmental-related genes.

Introduction

SUMOylation is a post-translational protein modification that can change the location, stability, activity or the interactions of the protein targets involved in many cellular processes, including cell death, cell cycle, signal transduction, and gene expression [1]. SUMOylation is the addition of SUMO (Small Ubiquitin-related Modifier) to lysine residues of the target protein in the consensus amino acid sequence YKxE (Y represents a hydrophobic amino acid) [2]. Hundreds of proteins are SUMOylated in Drosophila [3]. The SUMOylation pathway starts with processing of an immature SUMO protein by the Ulp/SENP family of proteases. Next, the SUMO protein is covalently conjugated to lysine residues in a multi-step process.[3]. The activating enzyme E1 (an Aos1/Uba2 heterodimer) generates a mature SUMO-adenylate intermediate which then forms a thioester bond between the catalytic cysteine of Uba2 and SUMO. SUMO is next transferred to the E2 conjugating enzyme (Ubc9), which transfers SUMO to the target proteins. The SUMO E3 ligases function by stimulating the activity of Ubc9 or by facilitating the formation of an Ubc9-substrate complex. Finally, proteins of the Ulp/SENP family proteases make this whole process reversible [4].

The tna gene was identified in a genetic screen designed to find brahma (brm)-interacting genes [5]. brm encodes the SNF2 type-ATPase of the BRM chromatin remodeling complexes [6,7]. The osa gene encodes an exclusive subunit of one type of BRM complexes [6,8,9]. Besides interacting with brm, tna interacts even stronger with osa. All three genes (brm, osa, and tna) are required for proper expressions of the homeotic genes [5]. Homeotic genes determine the identity of body segments in Drosophila [10,11].

The role of various components of the SUMOylation pathway have been studied in Drosophila development [12,13]. tna is involved in homeotic gene expression but little is known about the proteins encoded by this locus. tna expresses at least one putative isoform called TnaA [5]. This isoform has an XSPRING (Xtended SP-RING) domain that harbors a zinc finger of the SP-RING type [Siz/PLAS (Protein Inhibitors of Activated STAT [Signal Transducers and Activator of Transcription]-RING (Really Interesting New Gene)]. This zinc finger is present in
one of the four major groups of proteins that have SUMO E3 ligase activity [1]. The only SP-RING finger proteins with putative SUMO E3 ligase activity that have been identified in the Drosophila proteome are Suivar/2-10 [14] and TnaA [5].

Here we show that TnaA physically interacts with both Ubc9 (the SUMO E2 conjugating enzyme) and with Osa (a putative in vivo target). We determined the dynamics of different TnaA species throughout development and showed that TnaA is an embryonic nuclear protein and is also present at discrete bands on polytene salivary gland chromosomes of third instar larvae. We also found that defects in tna cause larval lethality, abnormalities in the whole protein profile and an extension of the lifespan at this stage. Finally, we found genetic interactions between tna and osa and genes encoding the SUMOylation pathway components.

Materials and Methods

Ethics Statement

All animal handling was approved by the Instituto de Biotecnología Bioethics Comitee, Permit Number 226 (2009/ 12/04), which follows NOM-062 animal welfare mexican law. All efforts were made to minimize animal suffering. Animals were sacrificed by CO2 euthanasia.

Protein Extraction and Analyses

Soluble protein extracts for the developmental Western were obtained from 1 g of Ore-R individuals from each developmental stage with Trizol (Invitrogen). For cellular localization of the TnaA proteins, soluble nuclear (SNF) and cytoplasmic fractions were obtained from Ore-R embryo collections of 3–21 hour postfertilization [15]. The SNF was also used for the TnaA coimmunoprecipitation (Co-IP) assays. For Osa Co-IP assays, a total soluble protein fraction was obtained from Ore-R embryo collections of 3–21 hour postfertilization [16]. Protein extracts from salivary glands of third instar larvae were obtained by collecting the glands in PBS buffer plus Complete protease inhibitors [EDTA-free protease inhibitor tablet (ROCHE)], and boiling them for 5 minutes in sample loading buffer. The proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose membranes for Western blot analyses. Immunoblots were done according to standard procedures and proteins of interest were detected with specific antibodies using different chemoluminiscence kits (SuperSignal West Pico Chemiluminescent Substrate from Thermo Scientific, ECL Plus Western Blotting Detection System or ECL standard procedures and proteins of interest were detected with specific antibodies using different chemoluminiscence kits (SuperSignal West Pico Chemiluminescent Substrate from Thermo Scientific, ECL Plus Western Blotting Detection System or ECL). The interaction between baits and preys was observed after the digestion of the full-length pGBK7-TnaA with NdeI and each fragment was cloned separately in NdeI-digested pGBK7T. TnaA(906–999 aa) was obtained by cloning in pGBK7T digested with NdeI and EcoRI, PCR fragments obtained from the TnaA cDNA clone [5] using the primers: a) TnaA(906–999 aa) cDNA harbouring aminoacids 1951–2600 from the Osa protein was synthesized from poly(A)-containing RNA from Ore-R embryos 3–21 hour postfertilization, according to [23]. The TnaA(906–999 aa) cDNA was subcloned into pGEM T-lok and transformed into E. coli XL1-Blue. The positive clones were sequenced before use.

Yeast Two-hybrid Assays

Drosophila TnaA and Ubc9 cDNAs were cloned in the EcoRI and SalI, and EcoRI and XhoI sites of the pGBK7 and pGADT7 vectors, respectively. pGBK7-TnaA was digested with BamHI and religated to obtain the TnaA(1–432 aa) fragment (1–432 aa), TnaA(379–927 aa) and TnaA(929–1073 aa) were obtained after the digestion of the full-length pGBK7-TnaA with NdeI and each fragment was cloned separately in NdeI-digested pGBK7T, TnaA(906–999 aa) and TnaA(1–432 aa) were obtained by cloning in pGBK7T digested with NdeI and EcoRI, PCR fragments obtained from the TnaA cDNA clone [5] using the primers: a) TnaA(1–906) Forward 5′-GGACAGGCTCATATGGCCAAGATCT-3′ and Reverse 5′-GGACAGGCTCATATGGCCAAGATCT-3′, b) TnaA(1–432 aa) Forward 5′-GGACAGGCTCATATGGCCAAGATCT-3′ and Reverse 5′-GGACAGGCTCATATGGCCAAGATCT-3′, c) TnaA(906–999 aa) cDNA harbouring aminoacids 1951–2600 from the Osa protein was synthesized from poly(A)-containing RNA from Ore-R embryos 3–21 hour postfertilization, according to [23]. The TnaA(906–999 aa) cDNA was subcloned into pGEM T-lok and transformed into E. coli XL1-Blue. The positive clones were sequenced before use.

Fly Strains, Genetic Procedures, and Larval Staging

Unless otherwise noted, all mutations are described in Flybase [17]. Briefly, tna1, tna2, osa1 and osa2 are EMS-induced mutations. In tna1 Gin 566 changed to a stop codon [5]. tna2 was recovered after EMS mutagenesis in a genetic screen to identify bm-1 interacting mutations (J. A. K., unpublished results). The lesion in the bm-1 allele (Arg 104 to His) is located in a region that has been involved in the interaction between ubiquitin-conjugating enzymes with the HECT or RING ubiquitin E3 ligases [24]. The bm-1 and bm-2 were both derived from imprecise excision of P-elements inserted in the 5′ regulatory zone [23,26]. smt304493 is a P-element insertion 10 bp upstream of the first exon of smt3 [27]. Fly cultures and crosses were performed according to standard procedures. Flies were raised on cornmeal-molasses media at 25°C unless otherwise noted. Media were supplemented with 0.05% of glycerol and 0.2 mM PMSE.
bromophenol blue to stage third instar larvae according to the gut dye clearance [28].

Immunostaining of Ring Glands, Salivary Glands, and Polytene Chromosomes of Third Instar Larvae

Immunostaining of ring and salivary glands were done as described by [29], and the immunostaining of polytene salivary gland chromosomes was done as reported by [30]. For immunostaining of polytene salivary gland chromosomes, the TnaA_XSPRING antibodies were preabsorbed with fixed 0–3 hour embryos [31]. Polytene chromosomes and salivary and ring glands images were captured on a Leitz DMIRB inverted phase microscope equipped with a Leica TCS NT laser confocal imaging system, a Zeiss Inverted Axiovert fluorescent microscope, a Leica Aristoplan fluorescent microscope or an Olympus Inverted confocal FV1000 microscope. Images were processed using Image J.

Results

TnaA130 and TnaA123 in Space and Time throughout Development

The tna gene produces several large transcripts that are differentially expressed from embryo through adult stages [5,17]. The main large transcript is 6.1 kb and it peaks at the pupal stage [5]. Translation of this transcript predicts a protein product of 127 kDa that we named TnaA [5]. To study TnaA, we prepared two affinity-purified antibodies: TnaA_XSPRING that was raised against the amino-terminal region and TnaA_XSPRING that was raised against the XSPRING domain (Fig. 1 and Material and Methods). Both antibodies recognize the same proteins on adult male soluble extracts and they were used indistinctly along this work (Fig. 2A).

Two main TnaA protein products, one of 130 kDa (TnaA130) and another one of 123 kDa (TnaA123) are present in varying abundance throughout development (Fig. 2B). The abundance does not correspond to the tna mRNA expression pattern [5] suggesting posttranscriptional regulation. We sometimes observe another protein heavier than TnaA130 in embryos of 3–21 h (Fig. 2B). These three Tna species we found, are consistent with the three Tna polypeptides described in Flybase [17]. Nevertheless we cannot discard the possibility that TnaA could be posttranslationally modified. For example, we determined using the SUMOsp 2.0 program [32] that TnaA has two putative SUMOylation sites and one putative SUMO Interacting Motif (SIM) [33] (data not shown). In extracts isolated from 0–3 hour embryos, we detected very low levels of TnaA130 while TnaA123 was not detected. In extracts isolated from 3–21 hour embryos, we detected a TnaA form larger than TnaA130 and the levels of both TnaA130 and TnaA123 increased, reaching maximums in the first larval instar. Decreases in the abundances of both proteins were observed in second and third instar larvae, with the levels of TnaA123 higher than those of TnaA130. Both forms abundance decreased substantially in pupae and TnaA130 was observed again at the pharate stage meanwhile TnaA123 is not detected. In adult flies of both sexes, TnaA130 and TnaA123 were both highly abundant at about equal levels. The appearance of TnaA123 was always preceded by the presence of TnaA130.

Next, we investigated the subcellular location of the TnaA proteins in nuclear and cytoplasmic fractions from 3–21 hour embryos (Fig. 2C, upper panel). The largest subunit of RNA polymerase II and β-tubulin were used to test the purity of the fractions (Fig. 2C, middle and lower panels). We found that TnaA130 was enriched in the nuclear fraction whereas TnaA123 was enriched in the cytoplasmic fraction (Fig. 2C). It has been shown that SUMO is present in prothoracic gland nuclei [29] in third instar larvae. tna mutant individuals arrest development at the larval-pupal transition which is where less TnaA protein is expressed (see ahead). This suggests that TnaA may be expressed in specific tissues relevant for metamorphosis. We immunostained salivary (Fig. 2D, upper panel) and ring glands (Fig. 2D, lower panel) from third instar larvae with the TnaA_XSPRING antibody and we found that TnaA was present most highly within the nucleus of the secretory cells of salivary glands and in prothoracic gland cells.

TnaA is Critical for Larval Development

While we can detect TnaA130 and TnaA123 in Ore-R and in tna+/+ or tna+/+ individuals, TnaA130 is barely detectable, and TnaA123 decreases dramatically in tna+/+ larvae (Fig. 3A, left panel). The tna1 mutation changes Gln 566 to a stop codon, is recessive lethal [5] and behaves as a dominant negative. tna1 is a much stronger dominant enhancer of nsa1 than is a deficiency of the tna region (Table 1). tna1 would produce a truncated protein of 62 kDa that we have been able to observe in heterozygous tna+/+ salivary glands soluble extracts (Fig. 3A, right panel). The molecular lesion of tna1 has not been determined, but it behaves genetically as a hypomorphic allele and its product can be detected in tna1/tna1 third instar larval extracts (Fig. 3A, left panel).

To better understand tna function we studied the lethality of tna1/tna1 animals. The tna1/tna1 larvae (Fig. 3B) did not have melanotic tumors as observed in ior or aos1 mutant individuals [34,35,36], nor are they a larger size as observed for smt3 knockdowned larvae [29]. We found that 65% of tna1/tna1 individuals reach the third instar larval stage (Fig. 3C), but only 41% pupated and only 8% of the expected individuals reached the pharate stage. No tna1/tna1 individuals eclosed as adults (Fig. 3C). We also noticed that the tna1/tna1 third instar larvae that did not pupate often survived long after their heterozygous tna1+/+ or tna1/ tna1/ tna1/ tna1/+ siblings larvae pupated. Some of these tna1 tna1 third instar larvae have an extended lifespan of at least two weeks (Fig. 3C). A similar extension of larval lifespan was previously observed in animals with reduced levels of SUMO [29], Aos1 (one of the E1 subunits) [34] or Ubch9 (E2) [35,36].

Given the abnormal behavior of tna1/tna1 larvae and knowing that the TnaA profile is altered (Fig. 3A), we characterized the protein profile of their salivary glands (Fig. 3D). We staged the larvae by feeding them with bromophenol blue [28] and divided them in early (blue) and late (white) larvae. All tna1/tna1 larvae remained as early larvae (blue). They were collected 24 hours after they crawled from the food to obtain their salivary glands and we determined their protein profile (Fig. 3D). Although tna1/tna1 larvae remained blue, the protein profile differed from both the early and late wild-type Ore-R salivary glands obtained under the same conditions. Differences in the quantity and quality of proteins present in tna1/tna1 salivary glands fall mostly in the range over 72 kDa (Fig. 3D).

TnaA is Chromatin-associated at Discrete Sites on Polytene Salivary Gland Chromosomes

We have shown that TnaA123 is nuclear in Drosophila embryos (Fig. 2C) and that TnaA (probably TnaA123) is mainly nuclear in salivary and ring glands from third instar larvae (Fig. 2D). We immunostained polytene salivary gland chromosomes of third instar larvae and found that TnaA is associated with discrete sites (Fig. 4A). The number of TnaA sites suggests that TnaA might be required for the transcription of more than just the homeotic genes. Interestingly, most of the TnaA signals detected on polytene salivary gland chromosomes are located in interbands which are thought to have decondensed chromatin where transcription can
Because of the strong genetic interactions between tna and osa [5], we coimmunostained for TnaA and Osa on polytene salivary gland chromosomes. TnaA colocalizes with Osa at some sites, but not at others (Fig. 4C, upper and bottom panels). We do not know whether this is because TnaA is not required at all genes regulated by Osa, or whether it is due to an interaction between TnaA and Osa that is more transient than Osa localization.

**TnaA Physically Interacts with Ubc9 and with Osa**

SUMO E3 ligases function for selection of SUMOylation targets and/or for enhancement of the SUMO conjugation process. TnaA has an SP-RING zinc finger that is also present in a subclass of SUMO E3 ligases that includes the PIAS proteins in mammals [37] and Su(var)2–10 in Drosophila [14]. Since the SP-RING in the PIAS proteins physically interacts with Ubc9 [38,39], we explored whether TnaA physically interacts with Drosophila Ubc9, using yeast two-hybrid assays and pull-down assays.

For the yeast two-hybrid assays we first used the full-length TnaA protein (Fig. 1) fused to the yeast GAL4-DNA binding domain as “bait”, and the full-length Drosophila Ubc9 protein (Fig. 5A) fused to the GAL4-activation domain as “prey”. We found that the full-length TnaA protein was able to activate the transcription of at least two reporter genes in the absence of a “prey” (Fig. 5B), and as a consequence the full-length TnaA protein could not be used to test for the Ubc9 interaction in this assay. We then split the TnaA protein into five fragments that cover the whole TnaA protein (Fig. 1). Two out of the five fragments contain the SP-RING zinc finger (TnaAXSPRING2 and TnaA SP-COO Qless). The other fragments have different TnaA regions that include the two glutamine-rich domains (TnaANH2–2), the bipartite nuclear location signal (TnaA QLess) and the carboxy-ending (TnaA COO). We found that the TnaAXSPRING2 fragment interacted with Ubc9 in the yeast two-hybrid assay while the other fragments, including TnaA SP-COO Qless, did not interact (Fig. 5B). These results show that the TnaA SP-RING zinc finger is necessary but not sufficient for the TnaA interaction with Ubc9 in this assay.

Osa is a subunit of some BRM complexes, and the osa gene strongly interacts with tna [5]. Since it was found that Osa is modified by SUMO in Drosophila embryos [3], we thought that TnaA might be involved in Osa SUMOylation. We searched for SUMOylation consensus sites (ψKxE) in the Osa protein sequence [2713 aa] using the SUMOsp 2.0 program [32] and found eight putative SUMOylation sites (Fig. 5A), six of them located within a segment located from amino acids 1951 to 2600 surrounding the C2 domain [40]. We will refer to the fragment with the six putative SUMOylation sites as OsaC2 in this work. We synthesized the OsaC2 cDNA from polyA+ RNA of 3–21 hour embryos and fused it to the GAL4-activation domain to use as “prey” in the yeast two-hybrid assay. We tested the six TnaA baits already described (including full-length TnaA), and found that baits harbouring the SP-RING (TnaAXSPRING2 or TnaA SP-COO Qless) did not interact with the OsaC2 prey. Although TnaANH2–2 (and to a lesser extent, full-length TnaA) interacted with OsaC2, these baits also interacted with pGADT7 or pGADT7-SV40 negative control samples, preventing us from concluding whether the interactions with OsaC2 are bona fide. In contrast, we found that the TnaAQless bait cleanly interacts physically with OsaC2 (Fig. 5B).

Although the TnaAXSPRING2 region interacted physically with Ubc9 in the yeast two-hybrid assays, we wanted to test for TnaA/Ubc9 physical interactions in Drosophila embryos. We performed pull-down assays using as bait a purified GST-Ubc9 fusion protein incubated with a nuclear protein extract from 3–21 hour embryos where we know TnaA is present (Fig. 2B). After extensive stringent washing, the presence of TnaA amongst the GST-Ubc9-interacting proteins was assessed by Western analyses with the TnaAXSPRING antibody (Fig. 5C). As expected, we found that full-length TnaA does not interact with Ubc9.
TnaA from nuclei of *Drosophila* embryos interacts with full length GST-Ubc9, confirming the results that we obtained with the yeast two-hybrid assays using TnaA fragments and further suggesting that these proteins interact *in vivo*.

In all reported cases it is known that only a fraction of the whole pool of a SUMOylatable protein in a cell is SUMOylated, either because of spatial restrictions (the target protein should be located where the SUMO and the SUMOylation enzymes are) or because fine regulation constricts the amount of the SUMOylated protein [4]. We showed that OsaC2 interacts with a fragment of TnaA (TnaA<sub>QLess</sub>) in a yeast two-hybrid assay (Fig. 5B). To test whether this interaction can be observed with the full-length proteins in *Drosophila* embryos, we performed TnaA or Osa coimmunoprecipitation assays from total or nuclear protein extracts from 3–21 hour embryos. For this purpose, we first showed that the TnaA<sub>XSpring</sub> and Osa antibodies are able to immunoprecipitate TnaA and Osa, respectively (Fig. S1), and that the control proteins Hsp70 and Cdk7 do not coimmunoprecipitate with TnaA or with Osa, respectively (Fig. S2). Interestingly, we found that TnaA coimmunoprecipitates with a fraction of Osa found in nuclear protein extracts from 3–21 hour embryos (Fig. 5D), and that reciprocally, Osa coimmunoprecipitates with TnaA from a total protein extract of 3–21 hour embryos (Fig. 5E). Since we found...
that TnaA interacts physically with Osa and with Ubc9 (Fig. 5) we tried to test whether TnaA has SUMO E3 ligase activity on the OsaC2 fragment using a mammalian in vitro assay (Active Motif kit). Although OsaC2 is SUMOylated in this assay, we were not able to get convincing evidence that TnaA has SUMO E3 ligase activity under these conditions (data not shown).

tna and osa Genetically Interact with Components of the SUMOylation Pathway

**tna** geneticaly interacts with *hsm* and *osa* [5]. Transheterozygous adult flies carrying mutations in combinations of any of these three genes have a strong held-Out wing phenotype [5,8] (Fig. 6). This phenotype appears to result from reduced expression from the P2 promoter of the homeotic gene *Antp* [8]. The interactions with *tna* might be a consequence of reduced SUMOylation of Osa (and/or
Table 1. Genetic interactions of tna and osa with SUMOylation pathway genes.

| Genotype | Number of flies with HWO* | Penetration (%) |
|----------|---------------------------|-----------------|
| +/osa1   | 9/265                     | 3               |
| +/osa2   | 0/303                     | 0               |
| +/smt3^{plesio} | 0/389                | 0               |
| +/lwr4^{ts} | 1/133                  | 1               |
| +/lwr5   | 0/297                     | 0               |
| +/tna1   | 0/341                     | 0               |
| tna1/osa1 | 116/624                 | 19              |
| tna1/osa2 | 327/334                 | 98              |
| Df(3L)lxd6/osa1 | 16/115              | 14              |
| tna1/osa2 | 140/334                 | 42              |
| tna1/osa2 | 44/249                   | 18              |
| tna1/lwr4^{ts} | 66/118               | 56              |
| lwr4^{ts}/+; tna1/+ | 69/150             | 46              |
| lwr1^{ts}/+; tna1/+ | 71/282             | 25              |
| lwr1^{ts}/+; tna1/+ | 177/343            | 52              |
| smt3^{plesio}/+; tna1/+ | 57/264             | 22              |
| smt3^{plesio}/+; tna1/+ | 0/216              | 0               |
| lwr1^{ts}/+; osa1/+ | 1/147              | 1               |
| lwr1^{ts}/+; osa2/+ | 0/124              | 0               |
| lwr1^{ts}/+; osa2/+ | 0/212              | 0               |
| lwr1^{ts}/+; osa2/+ | 0/256              | 0               |

*For expressivity of held-out wing phenotype (HWO) see Fig. 6.

**Reciprocal crosses were done in all cases with no observed differences except in the crosses with tna1 males and smt3^{plesio} females, or with smt3^{plesio} males and tna1 females. At least 100 flies were examined for each genotype. Flies that do not present the held-out wing phenotype include tna1, tna2, or tna deficiencies Df(3L)lxd6 and Df(3L)lxd6, osa1, lwr4^{ts}, and lwr5^{ts}, or other combinations of interacting genes listed above have more than one SIM and could be either SUMOylation targets, readers of the SUMO mark, or proteins that help TnaA exert its function(s).**

Discussion

The presence of the SP-RING, the physical interaction of SUMO E2 conjugating enzyme Ubc9 with TnaA, and the genetic interaction of tna with genes encoding SUMOylation pathway proteins suggest that TnaA may be involved in the SUMOylation pathway to activate transcription. TnaA may also have other functions not directly related to SUMOylation. These other functions may or may not act together with SUMOylation to positively regulate gene expression.

TnaA Function in Gene Expression Involving the SUMOylation Pathway

Gene expression involves the integration of many regulatory mechanisms. Recently, many examples of SUMOylation and/or ubiquitylation during transcriptional regulation have been described [4]. These examples include the clearance of activators to favor transcription cycles in inducible genes [41] and the assembly of different proteins into a complex [42,43]. Most of the tna interacting genes (osa, brahma, moza, kohd, ashd, and kismed) [5] encode subunits of complexes involved in chromatin remodeling and transcription by RNA polymerase II, suggesting that SUMOylation may be important at multiple aspects of gene regulation in Drosophila. Typically, SUMO-tagged proteins are recognized by a binding partner that contains a SIM (SUMO Interaction Motif) [33]. All of the proteins encoded by the tna interacting genes listed above have more than one SIM and SUMOylation sites (data not shown) and could be either SUMOylation targets, readers of the SUMO mark, or proteins that help TnaA exert its function(s).

SUMO E3 ligases are required for the enhancement and/or for the specificity of the SUMOylation tagging on targets. In this work we utilized different approaches to show that TnaA is involved in the SUMOylation pathway possibly as a SUMO E3 ligase. We showed a TnaA physical interaction with Ubc9 and genetic interactions between tna and osa with SUMOylation pathway genes. SUMOylated Osa is found in early embryos (0–3 hour) [3] and embryonic TnaA and Osa commmunoprecipitate reciprocally (this work). We also showed that a GST-Ubc9 fusion physically interacts with native near TnaA from Drosophila embryos. Hence, we suggest that Osa is a good candidate to be a TnaA SUMOylation target in vivo. Our data suggest that TnaA-dependent SUMOylation of Osa and/or of other target(s), particularly proteins associated with Osa (e.g. other BRM complex subunits, histones, or others, see ahead), may be required for correct gene expression including homoeotic genes. Osa is a large protein of around 280 kDa with an ARID domain which binds AT-rich sequences, LXXLL domains [8] that could help it to interact with nuclear receptors and has eight putative SUMOylation target sequences, six of them in the Osa-A3 fragment (Fig. 5A). In humans there are three proteins related to Osa, BAF250a, BAF250b and BAF200/ARID2 [44] and it was reported that...
BAF250b could be in a complex that has E3 ubiquitin ligase activity on histone H2B [45].

Originally \( tna \) was identified in a screen to find Brm-interacting proteins [5]. Although we did not study here whether Brm can be SUMOylated, it has been reported that mammalian SUMO-2 can be acetylated at K33 to inhibit some SUMO-SIM interactions [46]. Interestingly, these authors also show that the bromodomain of p300, besides recognizing acetylated histones [47], can bind the SUMO acetylated form, opening the question of whether other bromodomains, such as the one present in the Brahma protein, would be able to recognize a putative \( \text{Drosophila} \) acetylated SUMO when present in any of its interactor proteins.

\( Tna \) may also be promoting homeotic gene expression by inactivation through SUMOylation of a PcG protein. Indeed, SUMOylation of the PcG protein Scm (encoded by the \( \text{Sex comb on midleg} \) gene) decreases its levels at the PRE (Polycomb Response Element) located upstream the \( Ubx \) homeotic gene. SUMO compromised animals show a reduction of \( Ubx \) expression and it has been suggested that \( Tna \) may be involved in Scm SUMOylation to promote homeotic gene expression [48].

Other \( Tna \) Interactors and SUMO-independent Functions of \( Tna \)

We found that \( TnaA_{130} \) is mainly cytoplasmic and \( TnaA_{123} \) is mainly nuclear. Although most studied SUMO enzymes and targets are in the nucleus, there are some examples of SUMOylation of proteins in the cytoplasm [49]. As \( TnaA_{130} \) always precedes

**Figure 4.** \( Tna \) is located on polytene salivary gland chromosomes of third instar larvae and sometimes colocalizes with \( Osa \). (A) Immunostaining of \( Tna \) in \( \text{Ore-R} \) (wild type) polytene salivary gland chromosomes of third instar larvae. \( Tna^{\text{AXSPRING}} \) antibody (1:50, red) and DNA (Sytox, green). Amplification in B is indicated (pointed white rectangle). (B) \( Tna \) is located in chromatin interbands. (C) \( Tna \) and \( Osa \) colocalize in some sites on polytene salivary gland chromosomes of third instar larvae (blue arrows in the top panels) but in others do not (purple arrows in the bottom panels). \( Tna^{\text{AXSPRING}} \) antibody (1:50, red) and \( Osa \) (1:50, green). No signal was detected when no primary antibody was added (data not shown).

doi:10.1371/journal.pone.0062251.g004
Figure 5. TnaA interacts with Drosophila Ubc9 and with Osa. (A) Schemes of Drosophila Ubc9 and OsaC2 used in biochemical assays. In the Osa protein, the ARID, the C1 and C2 domains (grey boxes), the SUMO interacting motif (SIM) and the OsaC2 fragment (dark line) are indicated. Forward (black circles) and inverted (grey circles) putative SUMOylation consensus sites in these proteins are indicated. For TnaA baits see Fig. 1. (B) TnaA interaction with Ubc9 and OsaC2 in yeast two-hybrid assays. Yeast colony complementation of growth controls in SD-Trp/2-Leu media due to the presence of pGBKT7 (Trp+) and pGADT7, (Leu+) plasmids (left) in the same yeast cells. Interaction assay in QDO +3-AT (SD-Trp/2-Leu/2-Ade/2-His+3-AT) media (right). Growth is observed when baits and preys interact, allowing GAL4 reconstitution with the consequent ADE2 and HIS3 reporter genes transcription. Baits were TnaA fragments (Fig. 1) fused to the DNA-binding domain of GAL4 in pGBKT7. Ubc9 and OsaC2 were preys fused to the GAL4 activation domain in pGADT7. Human p53 (p53) and Lamin C (Lam) interactions with SV40 are positive and negative controls, respectively. (C) TnaA interaction with Ubc9 by pull-down. The assays were done with 10 μg of each GST or GST-Ubc9 as baits and with 500 μg of soluble nuclear fraction from 3–21 hour embryos. 10 and 20% of the extract are shown as Input. TnaA was detected by Western analysis with TnaAXSPRING antibody (1:100) when GST-Ubc9 was used as bait. The 130 kDa weight marker is indicated (left) and increasing exposures of the same membrane are shown. Cdk7 was detected only in the Input lanes (antibody dilution, 1:1000). (D) Coimmunoprecipitation of Osa with TnaA antibodies from nuclear extracts obtained from 3–21 hour embryos. 10 and 20% of the extract are shown as Input. TnaA was detected by Western analysis with TnaAXSPRING antibody (1:100) when GST-Ubc9 was used as bait. The 130 kDa weight marker is indicated (left) and increasing exposures of the same membrane are shown. Cdk7 was detected only in the Input lanes (antibody dilution, 1:1000). (E) Coimmunoprecipitation of TnaA with Osa antibodies from total extracts obtained from 3–21 hour embryos. Osa antibodies
the appearance of TnaA through development (developmental Western, Fig. 2B), we think that TnaA may be processed to enter the nucleus to SUMOylate its targets. Notably, SUMOylation pathway proteins with well known nuclear activities also SUMOylate targets in the cytoplasm [50]. Thus, with what we know at present, we cannot discard the possibility that TnaA can also function in the cytoplasm. We also found that tna interacts with the γTub23C gene that encodes an isoform of γ-tubulin [51] and with taranis (tara) [5]. The significance of the interaction of tna with tara and γTub23C is currently unknown.

It is possible that TnaA could be necessary for BRM complex(es) function(s) regardless of SUMOylation, and that independently, SUMOylation could be required for function of other BRM complex(es) components. We cannot neither rule out the possibility that TnaA may have other functions independent of its possible role in the SUMOylation pathway, as has been
reported for the PIAS proteins, known SP-RING SUMO E3 ligases [52,53,54]. The SP-RING plays a key role in this PIAS activity. The TnaA SP-RING is immersed in a 300-aminoacid region that we called the XSPRING domain that is shared with the vertebrate proteins Zimp7 and Zimp10 [KIAA1086 and KIAA1224 respectively, 5]. Although TnaA is related to the PIAS proteins because it has an SP-RING, it does not have the SAP (Scaffold attachment factor-A/B, Actinus and PIAS domain) nor the PINIT motif that are PIAS signature domains.

The SAP and PINIT motifs in the PIAS proteins confer functions related to structural anchoring and transcriptional regulation. In mammals it has been shown that PIAS1 promotes the transcriptional repressive activity of Mxt1 through regulating its location in a SUMO-independent way [53], it controls the stability of Mxt1 by preventing its ubiquitinization [55] and it regulates the transcriptional activity of GATA4 [56]. Similarly, in Xenopus, XPIASy down-regulate XSmad2 transcriptional activity independently from XPIASy SUMO E3 ligase activity [57].

Although human Zimp7, human Zimp10, and Drosophila TnaA do not have these other PIAS signature motifs they have transcriptional activation domains [58,59] (Fig. 5B). The presence of a transcriptional activation domain could explain why we could not use the TnaA-Gal4 DNA-binding domain fusion in the yeast two-hybrid system (Fig. 5B). This suggests that TnaA, besides its possible role in the SUMOylation pathway, has other functions in Drosophila transcriptional activation.

TnaA in Drosophila Development

We described a genetic interaction between tna, osa, and SUMOylation pathway genes. TnaA interacts physically with Ub9 through the SP-RING supporting the genetic interaction data. Animals derived from osa and tna mutant germline clones die at different stages of development. While the osa ones do not survive embryogenesis [8] the tna ones die mostly as third instar larvaes [5]. A pool of Osa is found SUMOylated in embryos of 0–3 hour of development when zygotic expression has not started [3] and TnaA is barely detectable (overexposure of Fig. 2B, data not shown). Moreover, when we studied the tna and smt3 interaction, we found a tna maternal effect. The held-out wings phenotype in smt3/+; tna/+ adults is observed when the mother is tna defective, but we do not observe this when the mothers have low dosages of SUMO (Table 1). We think it is probable that SUMOylated Osa plays a role at early stages of development. SUMOylation of embryonic Osa can happen in the maternal germline or in the embryo with the help of the maternally-inherited SUMOylation pathway machinery. This machinery may include TnaA if TnaA is involved in SUMOylation or another protein with a SUMO-related function. It is also possible that smt3/+; tna/+ embryos derived from smt3 mothers do not present the held-out wings phenotype because the SUMOylation pathway can compensate even with low dosages of SUMO. On the other hand, if TnaA is related to SUMOylation, embryos derived from tna mothers would lack correct SUMOylation of specific targets (such as Osa) causing later the appearance of the held-out wings phenotype.

Why do tna mutant animals die at later stages of development? One possibility is that proteins other than TnaA can exert its function on particular targets, such as Osa, or that they could only impact the TnaA targets in earlier stages of development, but not in later stages. SUMO is required for metamorphosis [29]. As the majority of tna mutant animals die as larvae or pupae and cannot proceed to metamorphosis (Fig. 3C) [5], and as TnaA is in prothoracic gland nuclei of third instar larvaes (Fig. 2D) obvious candidates for regulation by tna would be the ecdysone-pathway, ecdysone-regulated or patterning genes.

The relevance of SUMOylation (and of genes like tna) in different developmental processes is just starting to emerge. The requirement of SUMOylation and of tna to maintain gene expression makes that the next challenges will be to find the SUMOylation and tna targets in vivo and to understand the consequences of this modification in proteins involved in chromatin dynamics and in gene expression.

Supporting Information

Figure S1 The TnaAXSPRING and Osa antibodies immuno-precipitate TnaA and Osa proteins, respectively. (A) TnaA was immunoprecipitated from 3–21 hour embryo-soluble nuclear fraction (500 μg) using TnaAXSPRING antibody (1 μg). The Western was revealed with TnaAXSPRING (1:100). The three panels correspond to films with increasing exposure times. Input (In), Preclearing (Pcl1), Unbound (Ub), and Bound (B). Mock (M) where the immunoprecipitation was done with the equivalent amount of a preimmune serum instead of TnaAXSPRING. (B) Osa protein was immunoprecipitated from 3–21 hour embryos soluble extract (3.7 μg) with the Osa antibody (1 μg). For Osa detection, the Western was revealed with Osa antibody (1:1000). Lanes are labeled as above. The equivalent amount of an irrelevant antibody was used as mock. Molecular weight markers are indicated (left).

Figure S2 Negative controls of TnaA and Osa immuno-precipitations. (A) TnaA antibodies do not coimmunoprecipitate Hsp70 (Bound, lane B) from a 3–21 hour embryos soluble nuclear fraction (immunoprecipitation shown in Fig. S1A), meanwhile Hsp70 is present in the input (In) and unbound (Ub) samples. The other lanes are preclearing 1 (Pcl1), and mock (M) samples. (B) The Osa antibody do not coimmunoprecipitate Cdk7 (immunoprecipitation shown in Fig S1B). The assays were done as in (A). Lanes are labeled as above.

Acknowledgments

We thank the Bloomington Drosophila Stock Center for providing stocks. We also thank Luis Gutiérrez for TnaA cDNA clones in pGEK vectors, J. Riesgo-Escovar for providing the Gold collection Ub9 clone, V. Valadez-Graham for assistance in this work, Carmen Muñoz for technical assistance and R. Vera, and J. A. Kassis for critical reading of the manuscript. We thank the two anonymous reviewers for valuable suggestions.

Author Contributions

Conceived and designed the experiments: JMV LGA JAK MV. Performed the experiments: JMV RAJU ZPS. Analyzed the data: JMV RAJU ZPS LGA JAK MV. Contributed reagents/materials/analysis tools: MZ JAK MV. Wrote the paper: JMV JAK MV.

References

1. Geiss-Friedlander R, Melchior F (2007) Concepts in SUMOylation: a decade on. Nat Rev Mol Cell Biol 10: 947–956.
2. Rodríguez MS, Dargemont C, Hay RT (2001) SUMO-1 conjugation in Drosophila transcriptional activation. J Biol Chem 276: 12654–12659.
3. Nie M, Xie Y, Loo JA, Courey AJ (2009) Genetic and proteomic evidence for roles of Drosophila SUMO in cell cycle control, Ras signaling, and early pattern formation. PLoS One 4: e5905.
4. Gareau JR, Lima CD (2010) The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. Nat Rev Mol Cell Biol 11: 861–871.
5. Gutiérrez L, Zurita M, Kennison JA, Vázquez M (2003) The Drosophila trithorax group gene small (tau) interacts genetically with the Brahma remodeling complex and encodes an SR-RING finger protein. Development 130: 343–354.

6. Kennison JA, Tamkun JW (1980) Dosage-dependent modifiers of Polycomb and Antennapedia mutations in Drosophila. Proc Natl Acad Sci USA 85: 0156–0159.

7. Tamkun JW, Deuring R, Scott MP, Kissingier M, Pattatucci AM, et al. (1992) brat/bra: a regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator Snf2/Swi2. Cell 68: 561–572.

8. Vázquez M, Moore L, Kennison JA (1999) The trithorax group gene soa encodes an ARID-domain protein that genetically interacts with the Brahma chromatin-remodeling factor to regulate transcription. Development 126: 733–742.

9. Mohrman L, Langenberg K, Kriegveld J, Kal Aj, Heck Aj, et al. (2004) Differential targeting of two distinct SWI/SNF-related Drosophila chromatin-remodeling complexes. Mol Cell Biol 24: 3077–3089.

10. Lawrence PA, Morata G (1994) Homeobox genes: their function in Drosophila segmentation and pattern formation. Cell 78: 181–189.

11. Maeda R, Karch F (2006) The ABC of the BX-C: the bithorax complex explained. Development 133: 1413–1444.

12. Talamillo A, Sanchez J, Barrio R (2008) Functional analysis of the Drosophila Smt3 SUMOylation pathway. Drosophila Biochem Soc Trans 36: 868–873.

13. Cooper H, Paterson Y (2004) Preparation of polyclonal antisera. In: Bonifacio J, Sullivan W, Ashburner M, Hawley R, editors. Drosophila: a laboratory manual. New York: Cold Spring Harbor Laboratory Press. 389–411.

14. Hari KL, Cook KR, Karpen GH (2001) The Drosophila Snf2/a-tel2 locus regulates chromosome structure and function and encodes a member of the PIAS protein family. Genes Dev 15: 1334–1348.

15. Kamakaka RT, Tyree CM, Kadonaga JT (1991) Accurate and efficient RNA polymerase II transcription with a soluble nuclear fraction derived from Drosophila embryos. Proc Natl Acad Sci USA 88: 1024–1028.

16. Elling LK, Daniel C, Papoulas O, Deuring R, Sarto M, et al. (1998) Genetic analysis of brahma: the Drosophila homolog of the yeast chromatin remodeling factor SWI2/SNF2. Genetics 148: 251–265.

17. McQuilton P, St Pierre SE, Thurmood J, Consortium F (2012) FlyBase 10.1: the basics of navigating FlyBase. Nucleic Acids Res 40: D706–D714.

18. Smith D, Corcoran L (2001) Expression and purification of glutathione-S-transferase fusion proteins. In: Ausubel F, Brent R, Kingston R, Moore D, Seidman J, Smith J, Struhl K, Editors. Current Protocols in Molecular Biology. New York: John Wiley and Sons. 16.7.1–16.7.

19. Cooper H, Paterson Y (2004) Preparation of polyclonal antiserum. In: Bonifacio J, Sullivan W, Ashburner M, Hawley R, editors. Drosophila: a laboratory manual. New York: Cold Spring Harbor Laboratory Press. 389–411.

20. Koelle MR, Horvitz HR (1996) EGL-10 regulates G protein signaling in the nervous system and shares a conserved domain with many mammalian proteins. Cell 84: 115–125.

21. Vikis HG, Guan KL (2004) Glutathione-S-conjugate transferase assay for studying protein interactions. Methods Mol Biol 261: 175–186.

22. Sambrook J, Fritsch E, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. 770.

23. Kerscher O (2007) SUMO junction-what's your function? New insights through genetically with Brachia chromatin-remodeling complexes in Drosophila melanogaster. Dev Biol 280: 407–420.

24. Tang Z, Hecker CM, Scheschonka A, Betz H (2008) Protein interactions in the PIAS protein family. Genes Dev 15: 1334–1348.

25. Koelle MR, Horvitz HR (1996) EGL-10 regulates G protein signaling in the nervous system and shares a conserved domain with many mammalian proteins. Cell 84: 115–125.

26. Iyengar S, Farnham PJ (2011) KAP1 protein: an enigmatic master regulator of transcription and during activation of inducible genes in yeast. Genes Dev 25: 1765–1769.

27. Takahashi Y, Iwase M, Strunnikov AV, Kikuchi Y (2008) Cytoplasmic SUMOylation of tumor suppressor p53. Mol Cell 31: 713–718.

28. Takahashi Y, Yasuda H (2001) Involvement of PIAS1 in the SUMOylation of tumor suppressor p53. Mol Cell 6: 713–718.

29. Huang L, Ohno S, Tanda S (2005) The lesion mutation activates Rel-related proteins, leading to overproduction of larval hemocytes in Drosophila melanogaster. Dev Biol 280: 407–420.

30. Chinn H, Ring BC, Sorrentino RP, Kalamanz M, Garza D, et al. (2005) dush-9 negatively regulates the Toll/NEPH pathways in larval hematopoiesis and drosomycin activation in Drosophila. Dev Biol 288: 60–72.

31. Kahyo T, Nishida T, Yasuda H (2001) Involvement of PIAS1 in the SUMOylation of tumor suppressor p53. Mol Cell 6: 713–718.

32. Rytinki MM, Kaikkonen S, Pehkonen P, Jaaskelainen T, Palvimo JJ (2009) PIAS function as a ubiquitin ligase that regulates androgen receptor-dependent transcription. J Biol Chem 284: 41311–41317.

33. Kerscher O (2007) SUMO junction-what's your function? New insights through genetically with Brachia chromatin-remodeling complexes in Drosophila melanogaster. Dev Biol 280: 407–420.

34. Kanakousaki K, Gibson MC (2012) A differential requirement for SUMOylation of tumor suppressor p53. Mol Cell 6: 713–718.

35. Va´zquez M, Cooper MT, Zurita M, Kennison JA (2008) Yeast PIAS-type Ull1/Siz1 is composed of SUMO ligase and regulatory domains. J Biol Chem 280: 35822–35828.

36. Lu X, Treiser P, Matsamuru T, Treisman JE, Tanese N (2010) Mammalian SWI/SNF/SFN-related BAF250/ARID1 is an E3 ubiquitin ligase that targets histone H2B. Mol Cell 36: 1673–1683.

37. Ullmann R, Chien CD, Advantaggiati ML, Muller S (2012) An acetylation switch regulates SUMO-dependent protein interaction networks. Mol Cell 46: 759–770.

38. Dhalluin C, Carlson JE, Zeng L, He C, Aggarwal AK, et al. (1999) Structure and ligand of a histone acetyltransferase bromodomain. Nature 399: 491–496.

39. Takahashi Y, Kikuchi Y (2005) Yeast PIAS-type Ull1/Siz1 is composed of core SUMOylation by PIAS-type Ull1/Siz1 homologue Tub23C. Cell Cycle 7: 1738–1744.

40. Castillo-Llueva S, Tatham MH, Jones RC, Jallay EG, Edmondson RD, et al. (2010) SUMOylation of the G1/Pase Rac1 is required for optimal cell migration. Nat Cell Biol 12: 1078–1085.

41. Vázquez M, Cooper MT, Zurita M, Kennison JA (2008) yTa23C interacts genetically with Brahma chromatin-remodeling complexes in Drosophila melanogaster. Genetics 180: 835–843.

42. Sharrocks AD (2006) PIAS proteins and transcriptional regulation-more than just SUMO E3 ligases? Genes Dev 20: 734–738.

43. Lee H, Quima JC, Prasanth KV, Swan VA, Economides KD, et al. (2006) PIAS1 confers DNA-binding specificity on the Mx1 homoeoprotein. Genes Dev 20: 784–794.

44. Ryrinski MM, Kaikononen S, Pekkonen P, Jaksela T, Palvimo J (2009) PIAS proteins: pleiotropic interactors associated with SUMO. Cell Mol Life Sci 66: 3029–3041.

45. Song YJ, Lee H (2011) PIAS1 negatively regulates ubiquitination of Mx1 homoeoprotein independent of its SUMO ligase activity. Mol Cells 32: 221–226.

46. Belagali NS, Zhang M, Garcia AH, Berger DH (2012) PIAS1 is a GATA4 SUMO ligase that regulates GATA4-dependent intestinal promoters independent of SUMO ligase activity and GATA4 SUMOylation. PLoS One 7: e43717.

47. Daniels M, Shimizu K, Zorn AM, Ohnuma S (2004) Negative regulation of Smad2 by PIASy is required for proper Xenopus mesoderm formation. Development 131: 5613–5626.

48. Huang CNC, Bolado J, Liu X, Le J, Sharma M, et al. (2005) hZimp7, a novel PIAS-like protein, enhances androgen receptor-mediated transcription and interacts with SWI/SNF-like BAF complexes. Mol Endocrinol 19: 2915–2929.

49. Huang CNC, Bolado J, Liu X, Le J, Sharma M, et al. (2005) hZimp7, a novel PIAS-like protein, enhances androgen receptor-mediated transcription and interacts with SWI/SNF-like BAF complexes. Mol Endocrinol 19: 2915–2929.