The role of the trithorax group TnaA isoforms in Hox gene expression, and in *Drosophila* late development

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Abstract

Regulation of developmental gene expression in eukaryotes involves several levels. One of them is the maintenance of gene expression along the life of the animal once it is started by different triggers early in development. One of the questions in the field is when in developmental time, the animal start to use the different maintenance mechanisms. The trithorax group (TrxG) of genes was first characterized as essential for maintaining homeotic gene expression. The TrxG gene *tonalli* interacts genetically and physically with genes and sub-units of the BRAHMA BAP chromatin remodeling complex and encodes TnaA proteins with putative E3 SUMO-ligase activity. In contrast to the phenocritic lethal phase of animals with mutations in other TrxG genes, *tna* mutant individuals die late in development. In this study we determined the requirements of TnaA for survival at pupal and adult stages, in different *tna* mutant genotypes where we corroborate the lack of TnaA proteins, and the presence of adult homeotic loss-of-function phenotypes. We also investigated whether the absence of TnaA in haltere and leg larval imaginal discs affects the presence of the homeotic proteins Ultrabithorax and Sex combs reduced respectively by using some of the characterized genotypes and more finely by generating TnaA defective clones induced at different stages of development. We found that, *tna* is not required for growth or survival of imaginal disc cells and that it is a fine modulator of homeotic gene expression.

Introduction

Homeotic (Hox) genes determine the segmental identity in *Drosophila*. In *Drosophila* Hox genes are in two complexes, the bithorax (BX-C) and the Antennapedia (ANTP-C) complexes. The initiation of Hox expression in specific segments occurs during embryogenesis and it is controlled by maternal and segmentation genes. Later on the activation or repression are
maintained in the appropriate segments by proteins encoded by genes that belong to the trithorax group (TrxG) or the Polycomb group (PcG) respectively. Several TrxG and PcG proteins are involved in chromatin dynamics (reviewed by [1]). Drosophila has two types of the SWI/SNF chromatin remodeling complex BRAHMA (BAP and PBAP), which have as a catalytic ATPase, the Brahma protein. These two types have common and specific subunits. Common subunits are Brahma and Moira, while Osa is a specific subunit of BAP. Brahma, Moira and Osa are encoded by TrxG genes [2–4].

tonnalli (tna) is a TrxG gene that was identified because it modifies brahma (brm), osa (osa) and moira (mor) [5].

tna encodes TnaA130 and TnaA123, two TnaA isoforms that presumptively have E3 SUMO ligase activity (see ahead, and [6]). These isoforms are derived either from different transcripts [7] and/or as a result of the processing of some of them [6]. TnaA130 and TnaA123 isoforms are differentially expressed during development and have specific compartmentalization within the cell [6].

SUMOylation is a post-translational modification similar to ubiquitination that adds a SUMO moiety to target proteins through the action of common activating E1 and conjugation E2 enzymes that in Drosophila are represented by single proteins. In contrast, there are several types of E3 ligases that choose or help the SUMOylation of a target protein. SUMOylation of a target protein can change its sub-compartmentalization within the cell or nucleus, can favor a change of partners and/or it can label it for degradation (revised in [8]). The PIAS (Protein Inhibitors of Activated STAT [Signal Transducers and Activators of Transcription]) family is a subgroup of E3 SUMO ligases that interact physically with E2 enzymes through a canonical 42 amino-acidic residues SP-RING (Siz/PIAS-Really Interesting) zinc finger [9]. TnaA share with the PIAS family the SP-RING [9] but this zinc finger is embedded in a unique 300 amino-acidic residues XSPRING (eXtended SP-RING) domain that is found in a few insect and vertebrate proteins and that is not present in the PIAS proteins [5]. TnaA physically interacts with Drosophila SUMO conjugating enzyme E2 in vivo and it coimmunoprecipitates with the Osa and Brm proteins from the BRM complex in embryo extracts [6].

Hox gene expression starts early at embryonic stages and prevails late in development. Therefore, it is controlled at each stage and tissue by different selected transcription factors that act on specific regulatory regions of each Hox gene (reviewed in [10]). Thus, it is probable that chromatin accessibility of these regulatory regions is under fine control involving chromatin remodelers and/or modifiers. As a TrxG gene, tna is required for the maintenance of expression of Hox genes [5], and adult animals with mutations in tna or in tna and brm, or osa mutations, show phenotypes that resemble Hox loss-of-function revealed in adult cuticular structures. One of the characteristics that make tna unique among TrxG genes is that it is required late in development [5], being the lethal phase of tna third instar larvae and pupal stages [5, 6]. As tna was identified as a brm-modifier gene and animals with tna mutant combinations reach the pharate stage and die before reaching adulthood presenting Hox loss-of-function phenotypes [5], one hypothesis is that its function is required to maintain Hox gene expression by facilitating chromatin remodeling by the BRAHMA BAP complex at these late stages of development. These facts make TnaA protein(s) interesting to study for the function they could have to ensure correct gene expression at these stages of development.

In this work we explored TnaA requirements for the expression of the Hox genes Ultrabithorax (Ubx) and Sex combs reduced (Scr), through immunostaining of the respective Hox proteins in imaginal discs of late third instar larvae with mutant tna genotypes, or in TnaA defective clones generated at different stages of development. We found that although animals derived from these experiments do present Hox loss-of-function adult cuticular phenotypes, the wild-type domains of Hox expression are not visibly altered in imaginal discs. In contrast, ectopic Hox expression is suppressed in tna mutant backgrounds, leading to the conclusion...
that TnaA finely modulates Hox gene expression in imaginal cells and that its function can only be observed when Hox gene expression is not robustly regulated.

**Material and methods**

**Ethics statement**

All animals handling was approved by the Instituto de Biotecnología, UNAM, Bioethics Committee, Permit Number 359 (2018/05/04), which follows NOM-062 animal welfare Mexican law. All efforts were made to minimize animal suffering. Animals were sacrificed by CO$_2$ euthanasia.

**Fly strains, and genetic procedures**

The lesions of *tna* alleles and the target of the interference RNAs (RNAi) used in this work are represented in Fig 1A and, unless otherwise noted, they are described in Flybase [7]. Briefly, *tna$^1*$ and *tna$^5*$ are EMS-induced mutations. In *tna$^1*$ Gln 566 changed to a stop codon [5]. *tna$^5*$ was recovered after EMS mutagenesis in a genetic screen to identify *brm*-interacting mutations. The lesion is a T for A change at base 10,857,881 (genome release version 6) [11] that correspond to the limit between exon 3 and 4 (where exon 1 is UTR) from *tna*-RD and that affects the splicing of a 451 bp intron present in all *tna* transcripts (J. A. Kennison, personal communication). *tna$^{EY22929}$* is a P[EPgy2] element insertion [12]. *tna* knockdown was achieved by expressing interference RNA (RNAi) from lines *tna$^{GD12331}$* (inserted either in chromosome 2, or in chromosome 3) from Vienna GD collection (vector pGD264, construct ID 12331 Vienna *Drosophila* Resource Center, [13]), and *tna$^{P02536}$* from Perrimon’s pVA-LIUM10-derived TRiP [14] collections using different drivers. Drivers used in this work were Act5C-GAL4 [15] for ubiquitous expression, and MS1096-GAL4 [16], and A9-GAL4 [17] were used to drive gene expression to the dorsal region of the haltere pouch. Fly cultures and crosses were performed according to standard procedures. Flies were raised on yeast-molasses media at 25˚C unless otherwise noted.

Lethality of individuals carrying heteroallelic combinations of *tna* alleles was determined by counting the Tb$^+$ progeny from crosses between parents with *tna* alleles balanced with *In(3LR) TM6B (tna$^+$)* carrying the larval/pupal marker Tb$^+$, and the adult markers *Dr$^{Moh}$*, or *Sb$^+$*. To evaluate pupariation and adult survival rates of heteroallelic *tna* individuals, and the survival of *tna* knocked-down flies, we performed a $\chi^2$ test (significance set at $P < 0.05$), comparing the number of *tna* heteroallelic animals (*tna/tna*) with the one of their *tna/+* siblings in each genotype. For the eclosion rate analysis, we performed a t-test ($P < 0.05$), comparing the proportion of eclosed/total heteroallelic *tna/tna* pupae (non-Tubby). At least two crosses were performed for each genotype.

Loss-of-function Hox phenotypes were scored in adult animals from *tna$^1$/tna$^{EY22929}$, with *tna* knockdown and from crosses where imaginal *tna$^1$* clones were induced (see Induction of Mitotic Clones section), and compared their appearance on control animals derived from crosses without the *tna$^1$ FRT2A* chromosome and thus *tna$^+$*. Three replicas were performed for each experiment. To evaluate the *Scr* loss-of-function phenotype we scored legs from at least 15 males of each genotype. The percentage was calculated by dividing the number of legs with less than nine teeth per sex comb over the number of total male legs scored. Statistical significance was determined with a t-test ($P < 0.001$).

To analyze the suppression effect of *tna* mutant alleles on ectopic Hox expression in imaginal discs, immunostaining with the respective Hox protein antibody was performed in at least 40 imaginal discs per genotype of interest, derived from at least three independent replicas per genotype. Statistical significance was determined using a t-test ($P < 0.05$) to compare the
number of discs from tna transheterozygous animals showing ectopic Hox suppression and the number of Pc⁺ discs showing ectopic Hox expression.

**Antibodies, production and affinity purification of TnaA antibodies**

To detect TnaA in this work, we used two polyclonal rabbit antibodies, anti-TnaAXSPRING, and anti-TnaAQTL raised against regions of the TnaAPD isoform identified and sequenced by Gutierrez et al., (2003) and reported by Flybase [11]. Rabbit anti-TnaAXSPRING was raised as the one from rat reported in Monribot-Villanueva et al., (2013) immunizing animals with a purified GST fusion protein harboring the entire XSPRING domain contained in aminoacids 433–856 of TnaA. Anti-TnaAQTL was raised against the 14-mer QTLHKRNLLPLEHS peptide.
(aminoacids 691–704) by New England Peptide. Both antibodies (Fig 1B) were affinity-purified from total sera.

For Western blot assays, affinity-purified primary rabbit anti-TnaAQT and anti-TnaAXSPRING antibodies were used at 1:3000 and 1:250 dilutions respectively. Mouse anti-β-tubulin (E7, Developmental Studies Hybridoma Bank) and anti-actin (JLA20, Developmental Studies Hybridoma Bank) were used each at 1:3000 dilution. Secondary antibodies were anti-rabbit HRP goat IgG (H+L) (65–6129) and anti-mouse HRP goat IgG/IgA/IgM (H+L) (A10668) (Invitrogen).

To detect Hox proteins Ubx and Scr, we used monoclonal antibodies FP3.38 [18] for Ubx, and 6H4.1 [19] for Scr. To detect Osa we used monoclonal Osa 15A8 [20]. These three antibodies were purchased from Developmental Studies Hybridoma Bank. Secondary antibodies anti-rabbit and anti-mouse Alexafluor 568 goat (red), and anti-rat Alexafluor 594 (Invitrogen) were used for confocal microscopy.

**Protein extraction and analyses**

Larval soluble protein extracts for Western analyses were obtained either by homogenizing whole larvae in lysis buffer (250 mM sucrose, 50 mM Tris pH 7.5, 25 mM KCl, 5 mM MgCl$_2$, Complete protease inhibitor (ROCHE), 5 mM EDTA, 1% SDS) or by inverting the anterior part of half larvae according to Cunningham et al., (2012) [21] directions, where 10–20 third instar larvae were cut in half and inverted to remove trachea, gut and adipose tissue. In this case the remaining tissue including central nervous system, imaginal discs and salivary glands was homogenized in lysis buffer (PBS, 1% Triton X-100, 1 mM MgCl$_2$, 5 mM EDTA and Complete protease inhibitor from ROCHE). Extracts obtained in either way were centrifuged at 10,000 g for 10 minutes at 4˚C to remove cell debris. The proteins were separated by SDS-PAGE and electro-transferred onto nitrocellulose membranes for Western blot analyses. Immunoblots were done according to standard procedures and proteins of interest were detected with specific antibodies with the kits Supersignal West Pico, and Femto Chemiluminescent Substrates from Thermo Scientific, according to manufacturer’s instructions.

Quantification of TnaA isoforms in mutant genotypes was done by using the densitometry measurement tool from ImageJ (Fiji). Raw values were normalized according to the respective loading control in each lane, and final values were expressed as a percentage of protein relative to the one found in wild-type animals.

**Induction of mitotic clones**

The $tna^I$ allele recombined into an FRT2A chromosome ($tna^I$ FRT2A) was a kind gift from J. A. Kennison. $tna^I$ clones were induced either with the hs-FLP [22] or the Ubx-FLP [23, 24] drivers as FLPase sources.

To induce clones in imaginal discs with heat shock, we basically used the protocol reported by [25]. Briefly, clones were induced in the progeny of the cross $tna^I$ FRT2A/TM6B, Tb, Dr X hs-FLP; +; Ubi-GFPX2 FRT2A that was set up at 25˚C. Clones were induced in the progeny at 24 h after egg laying (AEL) with a single 37.5˚C heat shock for 1 h. Some clones in haltere discs were induced in an egg collection of 4 h, applying a heat shock of 38.5˚C for 1 h to the progeny at 9 h AEL.

We also used the Ubx-FLP [24], that induces recombination through a Ubx enhancer that is active in all imaginal discs (IDE, Imaginal Disc Enhancer), identified in the PBX-41 segment [23]. Crosses were set with Ubx-FLP/Y; $tna^I$ FRT2A/TM6B/+ X +; Ubi-GFP FRT2A, or males +; FRT82B osa$^{os8}$/TM6B Dr, Tb X Ubx-FLP; +; FRT82B Ubi-mRFP at 25˚C. Animals with induced clones either with hs-FLP or with Ubx-FLP were kept at 25˚C until they reached 110
to 115 h AEL, where discs were dissected for immunostaining with the antibodies of interest and observed using confocal microscopy as stated in the next section, or animals were allowed to reach the adult stage to estimate cuticular Hox loss-of-function phenotypes.

**Immunostaining of imaginal discs and cuticle preparations**

Wing and haltere discs were obtained from male and female third instar larvae. Male first leg discs were isolated from sexed third instar larvae to study Scr expression. Immunostaining of imaginal discs were done as described by Blair (2000) [26] with some modifications. Briefly, imaginal discs were dissected in cold 1X PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Discs were washed with PBT (1X PBS with 0.2% Triton X-100), blocked for 1 h with 0.1% bovine serum albumine in PBST 1X with 250 mM NaCl at 4°C. Primary antibodies were added at appropriate dilutions overnight at 4°C, and the next day discs were washed with PBT. Secondary antibodies were added together with Hoechst (0.1 ug/ml) for 2 h at room temperature washed again with PBST and after removal of PBST, discs were mounted in 80% glycerol, 4% n-propyl gallate in 1X PBS and stored in darkness until observation in confocal microscopy.

Fluorescent images from immunostained imaginal discs, or for detection of Hox proteins or apoptotic cells (TUNEL assay), were acquired with an Olympus Inverted FV1000, or a 2P Upright confocal FV1000 confocal microscopes with a 20X 0.75 or 60X 1.3 numerical aperture objectives. Images were processed using ImageJ (Fiji) and Adobe Photoshop CS software.

Adult cuticle preparations were processed by standard procedures by boiling flies in 10% KOH, washed in distilled water, and mounting them in glycerol 50% to observe them in a Nikon Eclipse E600 upright microscope equipped with an Amscope MU500 digital camera.

**Cell survival analyses**

To assess the role of tna on cell survival, we compared the areas of both tna<sup>−/+</sup> (GFP<sup>−/+</sup>), and tna<sup>−/−</sup> twin-spot (GFP<sup>−/−</sup>) clones induced 24 h AEL. Images were analyzed with ImageJ (Fiji) to measure the area of 13 clones and their twin spots. A paired t-test was used to assess significant differences in their respective areas (P<0.05) (S1 Fig).

To determine whether tna<sup>−/−</sup> genotype could cause cell death, we performed a TUNEL (Terminal deoxynucleotidyl-transferase-mediated dUTP Nick end Labeling) cell-death assay, with the In Situ Cell Death Detection kit TMR red, (Roche, cat. no. 12156792910), according to manufacturer instructions. Apoptotic cells in wing imaginal discs of genotype MS1096-GAL4 UAS-ras<sup>V12</sup> UAS-dlg<sup>RNAi</sup> were used as a positive control. For the cell death assay, imaginal discs were dissected, fixed and washed as for immunostaining. Confocal images were captured as stated in the previous section.

**Results**

**tna is required at late phases of development**

Pharates deficient in tna present cuticular Hox loss-of-function phenotypes [5]. To further study the effect of Hox gene expression in imaginal discs lacking TnaA at larval stages, we inspected with more detail a selected tna allelic set of combinations producing animals that die between the third instar larval stage and adulthood (Table 1). We used the tna alleles (Fig 1A) tna<sup>−</sup>[5], tna<sup>−</sup> that is a null allele [6], and tna<sup>FY29529</sup> [12] that is a P[EPgy2] element insertion at 5’ end of tna. We combined each of these alleles between them, and with two chromosomal deficiencies that uncover tna, Df(3L)378 and Df(3L)vin2. We also knocked down tna expression by inducing the expression of two different RNAi constructs, tna<sup>FPO2536</sup> from the TRiP
TnaA isoforms in late development and Hox expression in Drosophila

The genetic analyses we made involve the determination of pupal and adult survival of animals with heteroallelic tna combinations (Table 1). We evaluated the number of heteroallelic animals that reach the pupal stage, and of those we counted how many were able to eclose from their pupal cases. We found that tna1 is the strongest allele tested. This is expected because we have shown previously that tna1 is a dominant negative mutation [6]. Only between 30–40% of the expected animals with tna1 as one of the alleles in the genotype (together with tna5 or with Df(3L)378 and Df(3L)vin2), reach the pupal stage. Close to 60% of the animals with tna5 (together with any of the two deficiency chromosomes) reach the pupal stage, dying before reaching adulthood. In contrast, 80–100% of the animals with tnaEY22929 reach the pupal stage. None of the pupae with tna1 or tna5 were able to eclose from their pupal cases, with the notorious exception of combinations of these alleles with tnaEY22929 allele where some animals survive until adulthood. 45–90% of animals with tnaEY22929 eclose (with the exception of animals harboring also Df(3L)vin2 that die as pupae), and from those 30–90% do reach adulthood. Of notice, all the adult animals of tna1/tnaEY22929 genotype present the held-out wings phenotype (Fig 2A) that was the base for the identification of tna as a brm-modifier in our original screen [5]. Adult flies with tna1/tnaEY22929, or tnaEY22929/Df(3L)378 genotypes,

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Table 1. Survival of animals with tna mutant genotypes at pupal and adult stages.

| Relevant Genotypea | PUPAE | ADULTS |
|--------------------|-------|--------|
|                    | Pupariation Rateb | Eclosion Rateb | Survivalb |
|                    | (Observed/ Expected)c | (Eclosed/Total tna/tna Pupae)d | (Observed/ Expected)e |
| tna1               |                    |                   |           |
| tna5               | 147/495 (30)       | 0/147 (0)         | 0/476 (0) |
| Df(3L)378          | 115/392 (29)       | 0/115 (0)         | 0/383 (0) |
| Df(3L)vin2         | 84/215 (39)        | 0/84 (0)          | 0/174 (0) |
| tna1               |                    |                   |           |
| Df(3L)378          | 129/204 (63)       | 0/129 (0)         | 0/172 (0) |
| Df(3L)vin2         | 90/155 (58)        | 0/90 (0)          | 0/112 (0) |
| tnaEY22929         |                    |                   |           |
| tna1               | 139/132 (105) ***  | 63/139 (45)       | 37/123 (30) |
| tna5               | 132/153 (86) ***   | 117/132 (89) ***  | 110/144 (76) *** |
| Df(3L)378          | 231/253 (91) ***   | 207/231 (90) ***  | 188/207 (90) *** |
| Df(3L)vin2         | 307/380 (81) ***   | 307/307 (0)       | 307/317 (0) |

a. Relevant genotype shows the tna alleles in heteroallelic animals evaluated. The alleles carried by parental males are shown in bold at the top of each section.

b. Puparium formation, eclosion rate, and adult survival were evaluated in progeny from the same crosses in at least two independent replicas. Statistical significance in each case was determined with a χ² or t-test (P < 0.05) as stated in Material and Methods. Non-significant differences are indicated (ns).

c. The percentage (in parentheses) of tna/tna heteroallelic individuals (non-Tubby pupae or non-Sb and/or non-Dr adults) was calculated taking as 100% the tna/Balancer individuals in progeny (half of the Tubby pupae or half of the Sb and/or Dr adults).

d. Percentages of eclosed pupae (in parentheses) were calculated dividing the number of eclosed over the total number of tna/tna (non-Tubby) (non-eclosed plus eclosed) pupae.

e. The disparity between the numbers of expected pupae and adults (compare first and third columns) is caused because the balancer chromosomes from mothers and fathers have Tubby as a larval/pupal marker, and in adults one balancer chromosome carried Sb and the other one has Dr. It was not possible to distinguish which balancer chromosome carry each Tubby pupae counted, but one class carrying one of the parental balancer chromosomes is more lethal than the other. When divided by two, to calculate the expected tna/tna progeny we found a difference between the number of Tb eclosed pupae and Sb or Dr adult flies. e. g. first lane: Half of Tubby pupae = 495. Adults with Dr marker = 476.

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Table 1 and tnaGD1231 from the Vienna GD collection [13] directed towards different regions of tna mRNAs (Fig 1A).

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present Hox loss-of-function phenotypes such as loss of sex comb teeth in male first legs (Scr), and partial haltere to wing transformation (Ubx) (Fig 2A and Table 2).

As tna locus harbors different transcripts that produce different isoforms, mainly TnaA$^{130}$ and TnaA$^{123}$, we were interested in correlate which of them was present in larvae of the genotypes studied. We performed Western analyses with anti-TnaA antibodies (Fig 1B) that detect both TnaA$^{130}$ and TnaA$^{123}$ isoforms (TnaA$^{xspring}$) or TnaA$^{123}$ preferentially (TnaA$^{qtl}$) in protein soluble extracts from larvae of some of the genotypes tested and from its siblings that carried a wild type tna allele for comparison (Fig 1C–1F). Animals with combinations that include either one of these deficiencies, survive up to the third instar larva stage in significant percentages (from 30–90% of the expected individuals, Table 1) and then die as pupae. This fact allows us to analyze protein extracts from these
mutant third instar larvae, making it easier to determine which TnaA isoforms were affected specifically with the \textit{tna} mutant alleles we were testing, given that combined with the deficiency chromosomes, these alleles would be the only source of TnaA. We also analyzed soluble extracts from larvae with \textit{tna} knockdown by the expression of \textit{tna}\textsubscript{JF02536} at 28˚C (Fig 1G).

We found that TnaA\textsubscript{130} disappeared in all the mutant genotypes tested (Fig 1), particularly when one of the alleles is \textit{tna}\textsubscript{EY22929}, while TnaA\textsubscript{123} disappears (\textit{tna}\textsubscript{1}/\textit{tna}\textsubscript{5}, Fig 1C and 1H), or it is still detected at a much lower concentration than in a wild-type condition (see for example, \textit{tna}\textsubscript{1}/\textit{tna}\textsubscript{EY22029}, or \textit{tna}\textsubscript{EY22929}/Df(3L)\textsubscript{Df(3L)378}, Fig 1E, 1F and 1H) with antibodies (TnaA\textsubscript{QTL} and TnaA\textsubscript{XSPRING}). We also found that RNAi expression from \textit{tna}\textsubscript{JF02536} at 28˚C, knocked down \textit{tna} expression almost 90% (see ahead, Fig 1G). In particular, we compared \textit{tna}\textsubscript{EY22929}/Df(3L)\textsubscript{vin2} and \textit{tna}\textsubscript{EY22929}/Df(3L)\textsubscript{378} (Fig 1D and 1F) because they give totally different results regarding survival to adulthood (Table 1). While 90% of \textit{tna}\textsubscript{EY22929}/Df(3L)\textsubscript{378} animals survive to adult stages, none of the \textit{tna}\textsubscript{EY22929}/Df(3L)\textsubscript{vin2} animals survive to this stage. In contrast, animals from both genotypes reach the pupal stage with the difference that 100% of \textit{tna}\textsubscript{EY22929}/Df(3L)\textsubscript{vin2} die before they eclose (Table 1). Western analyses are in agreement with these findings given that \textit{tna}\textsubscript{EY22929}/Df(3L)\textsubscript{378} larvae still present some detectable levels of TnaA\textsubscript{123} protein (Fig 1F and 1H), that may account for their 90% survival to adulthood, meanwhile in

| Relevant Genotype | Antp P2 Held-out wings | Ubx Haltere to wing | Scr < 9 teeth/sex comb |
|-------------------|-------------------------|---------------------|------------------------|
| OreR              | 0/72 (0)                | 0/72 (0)            | 0/48 (0)               |
| \textit{tna}\textsubscript{EY22929} | 0/62 (0)                | 0/62 (0)            | 0/44 (0)               |
| \textit{tna}\textsubscript{1}/\textit{tna}\textsubscript{EY22929} | 54/54 (100)            | 15/54 (28)          | 30/39 (77)             |
| \textit{tna}\textsubscript{EY22929}/Df(3L)\textsubscript{378} | 0/63 (0)                | 7/63 (11)           | 32/37 (87)             |

\textit{tna} knockdown\textsuperscript{c}

- \textit{Act5-GAL4} 0/85 (0) 0/85 (0) 0/43 (0)
- \textit{tna}\textsubscript{JF25036} 0/96 (0) 0/96 (0) 0/32 (0)
- \textit{Act5-GAL4}\textsubscript{>}\textit{tna}\textsubscript{JF25036} 0/136 (0) 22/136 (16) 4/29 (14)
- \textit{A9-GAL4} NA 0/110 (0) NA
- \textit{A9-GAL4}\textsubscript{>}\textit{tna}\textsubscript{JF25036} NA 53/345 (15) NA
- \textit{MS1096-GAL4} NA 0/95 (0) NA
- \textit{MS1096-GAL4}; \textit{Ubx}\textsuperscript{d} NA 19/87 (22) NA
- \textit{MS1096-GAL4}\textsubscript{>}\textit{tna}\textsubscript{JF25036} NA 12/152 (8) NA
- \textit{MS1096-GAL4}\textsubscript{>}\textit{tna}\textsubscript{JF25036}/\textit{Ubx}\textsuperscript{d} NA 122/142 (86) NA

\textit{Adults from mitotic imaginal clones}\textsuperscript{NA}

- \textit{hs-FLP}; \textit{tna}\textsubscript{1} FRT2A/\textit{Ubi-GFP} FRT2A
  - No heat shock 0/47 (0) 0/47 (0) 0/49 (0)
  - Heat shock\textsuperscript{d} 23/159 (15) 1/55 (2) 2/49 (4)
- \textit{Ubx-FLP}; \textit{tna}\textsubscript{1} FRT2A
  - 0/29 (0) 0/29 (0) 0/37 (0)
- \textit{Ubx-FLP}; \textit{tna}\textsubscript{1} FRT2A/\textit{Ubi-GFP} FRT2A
  - 34/50 (68) 7/50 (14) 0/33 (0)

Percentages are in parentheses. NA is non applicable. The number of individuals showing the indicated Hox loss-of-function phenotypes is statistically significant (t-test, P<0.01, see Material and methods). Statistical test was not applicable (NA) for the evaluation of phenotypes of adults from mitotic imaginal clones induction because in principle it was not known how many clones were induced in each case.

\textsuperscript{a}. Adult individuals with at least one partially transformed haltere.
\textsuperscript{b}. Adult (\textit{tna}\textsubscript{1}/\textit{tna}\textsubscript{EY22929}) or pharate (\textit{Act5-GAL4}\textsubscript{>}\textit{tna}\textsubscript{JF25036}) males with less than 9 sex comb teeth per leg.
\textsuperscript{c}. Flies expressing \textit{tna} RNAi from \textit{tna}\textsubscript{JF25036} were raised at 28˚C.
\textsuperscript{d}. Heat shock was applied as established in Material and Methods at 24 AEL.

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tna^{EY22929}/Df(3L)vin2 larvae, TnaA_{123} isoform is barely detectable (Fig 1D and 1H) and they present 100% of lethality in adult stages.

These results indicate two findings. First, the presence of TnaA_{130} is not required for survival to adulthood, because animals lacking it, reach this stage (tna^1/tna^{EY22929}, and tna^{EY22929}/Df(3L)378). We noticed that although tna^1/tna^{EY22929} animals present a slightly higher amount of TnaA_{123} than the one found in tna^{EY22929}/Df(3L)378 animals, the latter ones have a better adult survival (90% compared to 30% of tna^1/tna^{EY22929}). This difference may be due to the fact that tna^1 is a dominant negative [6]. The second finding is that the P[EPgy2] element insertion in the tna^{EY22929} allele, is affecting the expression of tna-RA transcript that would be encoding TnaA_{130}. Moreover, one of our hypotheses was that TnaA_{123} could originate from TnaA_{130} processing [6], but with these data, we support the hypothesis that TnaA_{123} and TnaA_{130}, are translated from different transcripts, being those tna-RD and tna-RA respectively.

For tna knockdown, two UAS-RNAi constructs, tna^{JF02536} and tna^{GD12331}, were expressed at 18, 25 and 28˚C with the ubiquitous driver Act5C-GAL4. A higher percentage of lethality is observed by increasing tna RNAi expression at higher temperatures (Table 3). RNAi expression from either tna^{JF02536} or tna^{GD12331} alleles caused lethality of pupae and pharates, being males more sensitive than females. tna^{JF02536} induction caused more lethality than tna^{GD12331} induction and then it was further characterized. The effectiveness of the interference, caused by the expression of the RNAi from tna^{JF02536}, was confirmed by the low protein levels found in these larvae (Fig 1G). Females from these experiments reached adulthood in higher percentages than males, but they die within 10 days after eclosion (Table 3). As for tna^1/tna^{EY22929} animals, knocking down tna through RNAi, result in Ubx and Scr loss-of-function phenotypes at, albeit low, measurable penetrance (Fig 2B and 2C and Table 2). Act5C-Gal4 is a strong ubiquitous driver. To study the effect of knocking down tna in a restricted spatial domain within the haltere, we induced the expression of tna RNAi to the dorsal region of the haltere pouch with the GAL4 drivers MS1096 and A9 (see Material and methods). The animals from these experiments showed ectopic bristles located mainly in the dorsal region of the haltere capitellum. To test whether this partial transformation was caused by a reduction in Ubx expression, we tested whether tna knockdown (by expressing RNAi from tna^{JF02536}), enhances the loss-of-function Ubx phenotypes observed in animals with the null Ubx^1 allele [27].

### Table 3. Survival of flies with tna knockdown.

| Relevant Genotype | Survival to adulthood of flies bred at: |
|-------------------|----------------------------------------|
|                   | 18˚C | 25˚C | 28˚C |
| Act5C-GAL4/+a     |      |      |      |
| tna^{GD12331} F^b | 95/105 (91)% | 81/127 (64)% | 4/98 (4)% |
| M^b               | 107/114 (94)% | 46/97 (47)% | 0/112 (0)% |
| tna^{JF02536} F   | 81/115 (70)% | 107/222 (48)% | 5/125 (4)% |
| M                 | 1/109 (1)% | 1/183 (1)% | 3/169 (2)% |

a. Act5C-GAL4 driver directs ubiquitous expression of tna^{GD12331} or tna^{JF02536} (Fig 1A).

b. Female (F) or male (M) adult progeny expressing the indicated RNAi. The proportion indicates the survival of adult flies expressing the indicated RNAI with respect to the survival of the healthier class in the cross (which is not expressing the RNAI) at the indicated temperature. Percentages of each proportion are in parentheses. Note that survival is more affected in males than in females and it diminishes by increasing the breeding temperature, where RNAI expression is increased. Data are from three independent crosses for each genotype, n = 3, and are statistically significant (χ^2 test, P < 0.01), except the ones labeled ns (non-significant).

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result of this genetic combination (Table 2) was the appearance of a single ectopic bristle in the dorsal distal pouch region of the transformed halteres (Fig 2B). tna knockdown enhanced the penetrance and the expressivity of the Ubx\(^1\) phenotype (from 22 to 86\%, observed as a higher percentage of halteres with a single larger bristle than the one present in halteres of Ubx\(^1/\) halteres genotype) (Table 2, Fig 2B).

In conclusion, reduction of TnaA dosages caused by different tna null or hypomorphic alleles or by knocking down its expression, causes different grades of lethality through development and, when animals are able to form cuticles, homeotic defects caused by loss-of-function of several Hox genes are evident at different extents (penetrances and expressivities).

### tna\(^1\) mitotic clones induced either at embryonic or larval stages survive normally

Previously we showed that loss of maternal tna function is completely rescued paternally and loss of both maternal and zygotic functions caused lethality primarily at the third larval instar [5]. To further investigate the role of tna in imaginal disc cells, we generated tna deficient mitotic clones. We recombinated the lesion in tna\(^1\) from a tna\(^1\) FRT2A chromosome (gift from J. A. Kennison, see Methods), with the FLP/FRT system, expressing the FLPase either by heat shock at different times of development (Fig 3A), or under the control of an enhancer active in all discs (Imaginal Disc Enhancer, IDE) [24], identified in the Ubx PBX-41 segment [23]. With both methods, we were able to get tna \(^{-}\) GFP \(^{-}\) clones that we checked by immunostaining with the anti-TnaA \((XSPRING)\) antibody (Fig 3B). We were able to induce tna \(^{-}\) mutant clones in all the discs and we found in general that these clones survive well in all types of imaginal discs. We

![Fig 3. Induction of TnaA defective mitotic clones in third instar imaginal discs at different times of development does not cause cell death. (A) Timeline of heat shock pulses (black triangles After Egg Laying, AEL) applied to induce mitotic recombination in animals bearing hs-FLP (hs-FLP; +; tna\(^1\) FRT2A/Ubi-GFPX2 FRT2A). (B) Immunostaining of TnaA with the TnaA \((XSPRING)\) antibody in a wing disc where mitotic clones were induced. DNA was stained with Hoechst (blue) to show nuclear presence. GFP (green) marks the tna \(^{-}/\) tna \(^{-}\) and that did not recombine (medium green intensity), and the tna \(^{-}/\) tna \(^{+}\) (strong green intensity) cells result of the recombination event. GFP marks the tna \(^{+}/\) tna \(^{-}\) clone, as corroborated by the absence of TnaA immunostaining (red). (C) TUNEL death assay (red) performed in imaginal discs with tna \(^{-}\) clones (GFP \(^{-}\)). Hoechst (blue), (tna\(^{-}\) cells are GFP \(^{-}\), green) (lower panels). As a positive cell death control (red), apoptotic cells were detected in larval discs of the genotype MS1096-GAL4 UAS-ras\(^V12\) UAS-dlg\(^{RNAi}\) (see text) (upper panels). Note also that in general here and in the next figures, the number and size of the tna \(^{-}/\) tna \(^{-}\) (GFP \(^{-}\)) cells compared to the ones in the tna \(^{-}/\) tna \(^{-}\) (GFP \(^{+}\)) clone seems to be similar, showing that tna \(^{-}/\) tna \(^{-}\) cells do not present an obvious defect.](https://doi.org/10.1371/journal.pone.0206587.g003)
did not notice any change in the size or in the frequency of appearance of the GFP\textsuperscript{+} tna\textsuperscript{-} clones compared to GFP\textsuperscript{+} tna\textsuperscript{+} twin spots in discs (GFP panels in Fig 3B, 3C and S1 Fig), with the exception of clones in haltere discs, where we noticed often that the GFP\textsuperscript{+} twin spot was slightly larger than the tna\textsuperscript{-} GFP\textsuperscript{+} cells (e.g. GFP panel in Fig 4A). Neither, we observed any effect in cells in the vicinity within the border of tna\textsuperscript{-} clones. We also evaluated cell death by TUNEL in clones made in the wing disc (Fig 3C), finding that there was no difference in survival between tna\textsuperscript{+}/tna\textsuperscript{-}, tna\textsuperscript{-}/tna\textsuperscript{+} cells, and tna\textsuperscript{+}/tna\textsuperscript{+} mutant clones, while we detect cell death in the dorsal pouch of wing discs where apoptosis and overgrowth are induced by disrupting apical-basal cell polarity (Fig 3C) as reported by [28].

Thus, according to the results of these genetic and immunostaining assays, in general tna does not seem to influence cell survival, or cell number or size in imaginal discs.

**Influence of TnaA in Hox expression in larval imaginal discs**

Our next goal was to study Hox expression in tna\textsuperscript{-} cells in the region of imaginal discs that will become the adult cuticle where loss-of-function homeotic transformations have been characterized. In particular, we focused on Ubx and Scr expression that is affected in pharates with tna mutant genotypes [5], in adults with the genotype tna\textsuperscript{+}/tna\textsuperscript{EY22929} (Fig 2A, Table 2), or where tna expression has been knocked down through the expression of the RNAi from tna\textsuperscript{JF02536} (Fig 2B and 2C, Table 2).

![Fig 4. Ubx protein is present in TnaA or Osa defective mitotic clones in haltere discs. TnaA (GFP\textsuperscript{-} in A and C and Fig 3B) and Osa (RFP\textsuperscript{-} in E and E' and S3 Fig) defective mitotic clones induced by expressing FLPase either from hs-FLP (A and B) or Ubx-FLP (C, D, E', and F') in haltere imaginal discs. Almost the whole disc is shown in C and D to note that the absence of TnaA (GFP\textsuperscript{-}) in any region of the disc does not affect the presence of Ubx, which is observed in a wild-type pattern. Ubx protein was immunostained with monoclonal antibody FP3.38 [18] (red signal in B, and D, and green in F, and F'). Note that no decrease or absence of the Hox protein Ubx is observed in any of the TnaA\textsuperscript{-} or Osa\textsuperscript{-} clones (labeled with pointed white shapes).](https://doi.org/10.1371/journal.pone.0206587.g004)
In a wild-type haltere disc, Ubx is expressed strongly in the pouch (the region that will become the haltere of the adult fly), being the most prominent the posterior compartment [29] (see for example, Fig 4D). In these regions, Ubx represses several genes that direct wing development [30]. The halteres from the animals with the tna mutant aforementioned genotypes, present a mild haltere-to-wing transformation indicated by the presence of ectopic bristles in the haltere capitellum (Fig 2A and 2B). This resembles a loss-of-function phenotype of Ubx [30]. Taking into account these observations, we made tna' (GFP) clones in haltere discs by inducing recombination either with an hs-FLP (Fig 4A), or with the Ubx-FLP (Fig 4C). We dissected haltere discs from animals where clones were induced, and they were immunostained for Ubx (Fig 4B and 4D, red signal). Other animals from the same experiments were allowed to reach the adult stage to evaluate the presence of the ectopic bristles in the haltere (Ubx phenotype) (Table 2). We found that the Ubx' phenotype was present in the halteres of adult flies where clones were induced (2% to 14% when recombination was induced either with heat shock or with the Ubx-FLPase, respectively, Table 2). The presence of this transformation corroborate that cells producing the mutant phenotype in the haltere disc were hit by the recombination event. We tried several protocols to induce tna' clones at different moments of development (with heat shock at 9 or 24 h AEL as stated in Fig 3A) (Fig 4A) or by getting tna' clones in several regions of the disc (by driving recombination with the Ubx-FLP that is expressed widely in imaginal discs, Fig 4C). We specially looked for clones in the region of the haltere disc that produces the ectopic bristle in the adult halteres in tna mutant animals from the same experiments (Table 2), and we could not detect a reduction Ubx (Fig 4B and 4D) in any of the tna' (GFP) clones induced with any of the treatments tested.

Scr is normally expressed in the prothoracic leg imaginal disc mostly in a crescent-shaped region in the anterior half of the disc, and in marginal regions near the disc stalk, specially on the posterior side [19] (for example, Fig 5A). tna mutant adults (e. g. tna1/tnaEY22029) with decreased TnaA levels (Fig 1E), present Scr loss-of-function phenotype [5] (Fig 2A, and Table 2 this work). This phenotype is observed in adult males as a reduction in the number of sex comb teeth in the prothoracic T1 first leg. We immunostained for Scr, leg discs of tna1/tnaEY22029 animals finding that decreased TnaA level does not affect immunostaining of Scr (S2 Fig).

Therefore, we generated tna' (GFP) clones in leg discs, inducing recombination by expressing the FLPase under the control of a heat shock promoter (Fig 5B and 5B') (see Material and methods). Some animals were allowed to reach the adult stage, finding that 4% of the first leg of males analyzed have less than nine sex comb teeth (compared to a mean of 10.5 teeth per comb in wild type), phenotype caused by Scr loss-of-function (Table 2). tna' (GFP) clones from leg discs of animals of the same experiment were immunostained for Scr (Fig 5C and 5C', red signal) and, as for the tna1/tnaEY22029 discs, we could not find any tna' clone where the signal of Scr immunostaining was reduced.

Osa is a subunit of the BRAHMA chromatin remodeling complex BAP. The osa gene interacts strongly with tna in a genetic assay of Hox gene expression in pharates and adults [5]. Thus, we also made osa' (RFP') clones in haltere (Fig 4E and 4E', and S3 Fig) discs by inducing recombination of the strong loss-of-function osa308 allele [31]. In these Osa' clones, as in the ones for tna, Ubx levels, estimated by immunostaining are not affected (Fig 4F and 4F' green signal), indicating that the requirement of Osa, in the majority of these cells, is not essential for keeping the levels of these Hox proteins.

The penetrance of the Ubx and Scr loss-of-function phenotypes in animals derived from the experiments where the tna' clones were induced, was very low (in the best of cases, 14% of halteres with an ectopic bristles, when tna' clones were induced with Ubx-FLPase, Table 2), and we wondered whether we were not being able to detect by immunostaining the specific cells...
affected in the haltere or leg discs that would give origin to the transformed tissue in each case (the ectopic bristle found in the transformed halteres or the reduction of the number of sex comb teeth in the first leg of males, Table 2). Thus, we studied the effect of tna mutations in a derepressed homeotic background caused by loss of function of Polycomb.

_Ubx_ is normally expressed in the haltere disc while in the wing disc (Fig 6A, shows wing disc organization), _Ubx_ expression is observed only in discrete areas such as the peripodial membrane [18] and it is not expressed in the epithelia. _Scr_ is normally expressed in the first prothoracic leg imaginal disc (T1), but not in the second and third thoracic leg discs (T2 and T3) (Fig 7A). _Ubx_ and _Scr_ expression is derepressed in imaginal discs of animals harboring PcG loss-of-function mutations such as _Pc<sup>3</sup>_ (Figs 6C, 6C' and 7B) [32, 33]. Derepression of both, _Ubx_ (compare Fig 6B, 6C and 6C') and _Scr_ (compare Fig 7A and 7B) can be observed in wing, T2 and T3 leg discs of _Pc<sup>3</sup>_ heterozygote animals respectively. _tna_ mutations suppress derepression of both Hox genes evaluated in cuticles from _Pc<sup>3</sup>_ pharate animals [5]. To investigate how _tna_ influences _Ubx_ and _Scr_ expression in this context, we immunostained _Ubx_ and _Scr_ proteins in imaginal discs derived from _Pc<sup>3</sup>_ (Figs 6C, 6C' and 6H and 7B and 7E respectively) and _Pc<sup>3</sup>/tna<sup>EY22929</sup> (Figs 6C, 6D, 6D' and 6G and Fig 7C and 7E respectively) animals. As expected, we found ectopic production of both _Ubx_ [94% (44/47) of _Pc<sup>3</sup>_ wing discs with

Fig 5. _Scr_ proteins are present in TnaA defective mitotic clones in prothoracic T1 leg imaginal discs. _Scr_ immunostaining pattern in wild type _OreR_ prothoracic leg discs (A). The disc region from where the sex combs in the first leg are originated in the male is labeled by a pointed rectangle (A) and amplified in A'. TnaA (GFP in B-B' and Fig 4B) defective mitotic clones induced by expressing FLPase from the _hs-FLP_ chromosome in T1 leg imaginal discs. _Scr_ protein was immunostained with the monoclonal antibody 6H4.1 [19] (red signal in A-A', and C-C'). Note that no decrease or absence of _Scr_ is observed in the TnaA' clones (labeled with pointed white shapes C, and amplified in C).

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Ubx signal, Fig 6B, 6B' and 6G] and Scr proteins [93 (40/43) and 85% (33/39) of T2 and T3 Pc3 leg discs respectively with Scr signal, Fig 7B and 7E], while in discs from Pc3/tna EY22929 animals, tna haploinsufficiency suppresses close to 95% (2/42) Ubx ectopic expression (Fig 6D and 6H) and 83 to 87% Scr ectopic expression (9/52, and 6/47, positive Scr T2 and T3 immuno stained leg discs, respectively) (Fig 7C and 7E). In a few cases [6% (2/42), Fig 6E and 6E'], Ubx suppression was not total. A few cells remain that still have detectable Ubx immunostaining signal, (Fig 6E and 6E'). In the case of Scr, the suppression was clearly observed in both Pc3/tna EY22929 T2 and T3 leg discs (Fig 7C, in comparison to Fig 7B and 7E), while Scr expression in T1 leg disc looks normal (Fig 7 compare Fig 7A or 7B and 7C, see also Fig 7E).

In the same way, we tested whether osa haploinsufficiency was able to suppress Hox protein immunostaining in Pc3 discs. Indeed, we found that ectopic expression of Ubx (Fig 6B) and Scr (Fig 7B) in Pc3 wing discs was almost totally suppressed when a copy of osa308 was introduced (Pc3/osa308 discs) [83% (6/35) of suppression for Ubx, Fig 6F–6H, and 75% (12/49), and 78% (11/51) Scr suppression in T2 and T3 leg discs respectively, Fig 7D–7E].

In summary tna and osa are required to finely tune Hox expression and these subtle differences are not observed in the endogenous regions of Hox expression in the imaginal discs. A different situation is observed when Ubx and Scr expression is forced out of these regions and then, the requirements of tna and osa genes are revealed by the strong suppression of the ectopic Hox expression when these TrxG genes are inactivated.

Discussion

In this work we studied the role of the TrxG gene tna on Hox gene expression in larval imaginal discs. First, we characterized the production of TnaA isoforms in different tna mutant genetic backgrounds. We also analyzed the Hox loss-of-function phenotypes present in adults with some of these tna mutant backgrounds. We found that the TnaA123 isoform is essential for larval, pupal, and adult survival. In contrast, we found, through mitotic clonal analyses,
that tna is not required for individual cell survival in imaginal discs. Neither, we found decreased Hox expression in these tna-defective imaginal cells, although adult animals derived from these experiments do present the already characterized Hox loss-of-function phenotypes. We found that tna-defective function suppresses ectopic Hox expression in imaginal discs in a Pc-defective background, indicating that tna is a fine modulator of Hox gene expression. Below we discuss some possible mechanisms to explain how tna might be implicated in the expression of Hox and other genes.

**TnaA isoforms have dedicated functions related to the survival of post-embryonic stages**

TrxG genes comprehend a functional diverse group that include among others, regulators of transcriptional initiation and elongation to maintain developmental gene expression (recently reviewed in [1]). tna encodes a group of proteins present in multicellular organisms, with a zinc SP-RING finger, characteristic of a type of SUMO E3-ligases. Besides the inherent complexity of the TrxG genes, biochemical studies are revealing that as tna, some genes of the
group, encode several protein isoforms that may have dedicated functions. Up to date, we have detected at least three different protein products (two of them being TnaA$_{130}$ and TnaA$_{123}$) that may be the result of the expression from different promoters, alternative splicing, or post-translational modifications ([6] and this work). We have characterized that in embryos, TnaA$_{130}$ is cytoplasmic, while TnaA$_{123}$ is mainly nuclear [6]. One of the questions derived from this evidence is to determine whether or not these different isoforms are equivalent in function. In this work, we characterized the effect of reducing the main TnaA proteins (TnaA$_{130}$ and TnaA$_{123}$) on the expression of the Hox genes Ubx and Scr in imaginal discs. Through analyses of, first, the lethality shown by tna mutant animals that die since larval and pupal stages, second, the presence of homeotic transformations in the survivors with some of these genotypes, and third, the protein characterization produced by animals with different tna mutant alleles, we were able to make the following observations discarding previous hypotheses. TnaA$_{123}$ is not a processing product of TnaA$_{130}$. We can eliminate TnaA$_{130}$ and still be able to observe TnaA$_{123}$. TnaA$_{130}$ does not affect the organism survival significantly, while TnaA$_{123}$ must be at least detectable, to allow animals to reach the adult stages revealing for the first time a dedicated function for this isoform.

What proved to be more difficult was to determine whether the larval or pupal lethality in these tna mutant animals, was caused by a problem in Hox gene expression in imaginal discs at these developmental stages. Nevertheless, adult cuticles of tna mutant survivors do show Hox loss-of-function phenotypes. Third instar larvae with stronger tna alleles show no detectable TnaA$_{130}$ and TnaA$_{123}$ isoforms and die as late larvae or early pupal stages. In that sense, from these experiments we cannot discard the possibility that both, TnaA$_{130}$ and TnaA$_{123}$, could be contributing for proper Hox expression.

It is probable that TnaA may be required in two phases during development. In the first phase, the maternal deposition of TnaA might be important to establish the early chromatin landscape for Hox gene expression, in a similar way as the TrxG gene Utx. The Utx protein (Ubiquitously transcribed tetratricopeptide repeat protein X chromosome) is a demethylase of the lysine 27 of histone H3 deposited by the PcG [34, 35]. Animals without both, maternal and zygotic Utx, die as larvae and do not maintain Hox expression, attributed to the fact that some cells cannot initiate the maintenance of Hox gene expression at early stages during the onset of zygotic gene transcription [34, 35]. Animals carrying maternal but no zygotic Utx reach adulthood, have weak loss-of-function phenotypes of diverse Hox, and die just after eclosing, revealing the Utx requirement for viability [34, 35].

Previously, we showed that loss of maternal tna function is completely rescued paternally and loss of both maternal and zygotic functions caused lethality primarily at the third larval instar [5]. It will be important to determine whether depleting TnaA at early stages could result in a reduction in Hox expression. In this work we show that tna is required for viability at larval, pupal and adult stages, and for ectopic Hox expression in imaginal discs. Is tna a gene necessary to initiate the maintenance of Hox gene expression as Utx? It is probable that tna could participate in this mechanism and later on for viability. This would explain the weak Hox phenotypes and the lethality at late developmental stages of tna mutant animals, resembling the ones observed in individuals lacking Utx zygotic expression.

Robust regulatory networks allow proper Hox gene expression that masks fine regulation mediated by TnaA

A central contribution of the present work is that wild-type domains of Hox gene expression are not visibly altered in tna mutant larval imaginal discs, in spite of the adult mutant Hox phenotypes presented by these animals. These results make us consider that first, robust regulatory
networks protect proper Hox gene expression and that the role of fine modulators such as TnaA is difficult to analyze in this scenario. Second, tna might be required in particular stages of development that we did not explore here, and third, that the tna mutant cells in imaginal discs that will produce the cuticular adult mutant phenotypes, might be reading very subtle differences in the Hox protein levels that we could not detect by immunostaining.

An argument to explain why the effect of tna mutations can only be observed in ectopic but not on wild-type regions of Hox expression derives from the robustness of regulatory networks. For example, Ubx has several enhancers (and at the end, all Hox genes) that ensure proper Ubx expression in time and space. Some of them are active in haltere discs in redundant spatial patterns which allows to buffer changes in Ubx expression levels due to natural variation [36]. Then it is possible that TnaA modulates the expression of only some components of these regulatory networks in imaginal discs, and when those components fail to function, the other ones “compensate” for Hox gene expression. This compensation mechanism has been observed in experiments studying the effect of loss-of-function alleles of TrxG genes. In these experiments, although the TrxG function is totally removed in mitotic clones, Hox expression (particularly Ubx) is partially restored in a “patchy” way, probably by these compensation mechanisms [34, 37]. Taking in account this situation it makes sense that it was in a Pc-defective background where we were able to observe the suppression by tna mutations of Ubx and Scr ectopic expression in imaginal discs.

That tna suppresses the extra-sex-combs adult cuticular phenotype in Pc-defective individuals caused by derepression of Scr is known [5], but this effect in imaginal discs was analyzed until this work. The suppression effect was observed in imaginal discs harboring the tnaEY22029 allele that affects primarily the production of the TnaA130 isoform (this work), or in adult animals harboring the tna1 allele [5], that lack both TnaA130 and TnaA123 isoforms (this work). This is also similar to the effect of a null brm mutation in the suppression ectopic Scr expression caused by Pc mutants in imaginal discs [2].

To study the developmental window of tna requirement for Hox gene expression, we made clones at different stages of development, finding that tna may be required at early stages (3–4 h AEL). Animals with clones generated at this time did not survive. In contrast, clones generated later (24–48 h AEL) do survive, and adults present a reduced number of sex comb teeth similarly to the Scr loss-of-function phenotype presented by tna hypomorphic and knocked down mutants. This suggests that tna requirements may be biphasic as has been shown for other TrxG genes such as Utx (discussed in the previous section).

Individuals with mutations in RNA polymerase II and transcriptional factors that facilitate initiation [38], or elongation [39] present, as tna mutant individuals, Hox loss-of-function phenotypes. Of these, kismet (kis) is a TrxG gene involved in elongation that was identified because it suppresses ectopic expression of Scr in Pc heterozygotes [40]. kis clones induced during larval development do not show homeotic transformations, meanwhile clones induced earlier at the cellular blastoderm stage, show a reduction on sex comb teeth [41]. Many evidences points towards the possibility that TnaA could be required as a co-factor of the BRAHMA BAP chromatin remodeling complex, but it will also be possible that it targets other components of the general transcriptional machinery.

### TnaA on gene expression mediated by the BAP complex, and other general transcription factor targets

The BRAHMA BAP complex is required for the expression of multiple genes at different times of development [42]. TnaA physically interacts with the E2 Ubc9 SUMO-conjugating enzyme and with the subunits of the BRAHMA BAP complex, Osa and Brm [6]. TnaA could be
modifying the assembly, the recruitment, or the remodeling function of the complex by stimulating the SUMOylation of one or more BAP subunits to facilitate Hox gene expression at a specific time, or cell-type. In fact, we have shown that TnaA co-localizes sometimes with the BAP subunit Osa in polytene chromosome bands of third instar larvae, but not in others [6], meaning that TnaA is required for function of the BAP complex at some gene targets but not in others, or that the co-localization of TnaA with Osa is transient. On the other side, TnaA may act on targets other than the BRAHMA BAP complex, meaning that epistatic relationships at different levels can contribute to the phenotypes derived from TnaA function(s) (S4 Fig).

Although TnaA itself is not a subunit of the BAP complex, tna defective clones in imaginal discs, resemble the behavior of defective clones in some subunits of the BAP complex. We compared our results particularly with the ones obtained with brm and osa clones, because TnaA physically and genetically interacts with the BAP subunits Brahma and Osa [5, 6]. Kassis et al. (2017) have recently published an excellent summary of results involving the clonal analyses of TrxG genes, and we only note here that germ line and/or somatic clones for mutant brm, osa, Snf5-related 1 (Snr1), and moira (mor) [3, 4, 31, 43, 44], all of them encoding BAP subunits, do not present the same phenotypes, and show requirements at different times of development. For example, analyses of brm clones suggest defects in cell division and in adult peripheral nervous system [43]. osa clones in the germ line produce embryos with segmentation defects [4], and somatic clones in the wing imaginal discs, have defects in venation, and in cell growth and viability [31]. These clones do not present homeotic transformations although adult individuals with hypomorphic brm or osa mutations do have homeotic phenotypes, e.g. [40]. All these evidences, indicates that the BAP complex acts on different gene targets influenced by other factors, including TnaA.

In conclusion, these and other differences and mechanisms, may account for the diverse developmental requirements observed in the clonal analyses of different TrxG genes. As other TrxG genes which have functions in the regulation of genes other than the Hox, tna could have targets not related to Hox expression that are essential for larval or pupal survival. tna epistatic relationships, may involve different TnaA isoforms that could be required for the expression of different gene targets or at diverse times of development (S4 Fig).

If TnaA is influencing BRAHMA BAP complex function, it may act close to the promoter or on regulatory elements such as enhancers where BAP complexes are remodeling chromatin. TnaA may also have other targets than the BAP complex and have a wider target specificity as has been shown for other E3 SUMO ligases (reviewed in [8]). These are questions still unanswered and for example, chromatin immunoprecipitation experiments with TnaA antibodies that recognize specific isoforms at different times of development, will be helpful to start to determine the range of action of these TrxG proteins.

Supporting information

S1 Fig. Cell survival is not affected in TnaA defective mitotic clones. (A) tna1/tna1 (GFP^-), and tna+/tna^+ (GFP^+) clones in a wing disc showing an example of the areas affected by clone-induction. (B) Comparison of the area of 13 tna1/tna1 (GFP^-), and tna+/tna^+ (GFP^+) adjacent clones from independent events of clone-induction in wing discs. There were no significant (NS) differences between correspondant areas (t-test, P>0.05). (TIF)

S2 Fig. TnaA level does not affect Scr immunostaining in leg imaginal discs. TnaA (red) and Scr (green) immunostaining (red) of tna1/+ or tna22029/+ (upper panel), or tna1/tna22029 (lower panel) leg discs. DNA is stained with Hoescht (left) and images with merged
TnA and Scr signals is shown (extreme right). Note that TnA level diminishes in tna/tnaEY22029 leg discs, although the Scr signal looks normal, and 77% of adult tna/tnaEY22029 animals present a loss-of-function Scr phenotype (Table 2).

(TIF)

S3 Fig. Osa defective mitotic clones in haltere discs. osa308 mitotic clones were induced with the Ubx-FLPase. Immunostaining of Osa with the anti-Osa15A8 (dil. 1:200) in a haltere disc where mitotic clones were induced. DNA was stained with Hoechst (blue) to show nuclear presence. RFP (red) marks the osa+/osa− cells that did not recombine (middle red intensity), and the osa+/osa+ cells result of the recombination event (strong red intensity). RFP marks the osa308+/osa308 clone, as corroborated by the absence of Osa immunostaining (green).

(TIF)

S4 Fig. Possible TnaA targets that can influence gene expression involved in organism survival and Hox loss-of-function phenotypic outcomes. Representation of TnaA target proteins that can influence the transcription of different genes. Epistatic relationships, can contribute to the Hox loss-of-function and organism survival phenotypes studied in this work.

(TIF)

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