

XNP/dATRX interacts with DREF in the chromatin to regulate gene expression

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ABSTRACT

The *ATRX* gene encodes a chromatin remodeling protein that has two important domains, a helicase/ATPase domain and a domain composed of two zinc fingers called the ADD domain. The ADD domain binds to histone tails and has been proposed to mediate their binding to chromatin. The putative *ATRX* homolog in *Drosophila* (XNP/dATRX) has a conserved helicase/ATPase domain but lacks the ADD domain. In this study, we propose that XNP/dATRX interacts with other proteins with chromatin-binding domains to recognize specific regions of chromatin to regulate gene expression. We report a novel functional interaction between XNP/dATRX and the cell proliferation factor DREF in the expression of *pannier* (*pnr*). DREF binds to DNA-replication elements (*DRE*) at the *pnr* promoter to modulate *pnr* expression. XNP/dATRX interacts with DREF, and the contact between the two factors occurs at the *DRE* sites, resulting in transcriptional repression of *pnr*. The occupancy of XNP/dATRX at the *DRE*, depends on DNA binding of DREF at this site. Interestingly, XNP/dATRX regulates some, but not all of the genes modulated by DREF, suggesting a promoter-specific role of XNP/dATRX in gene regulation. This work establishes that XNP/dATRX directly contacts the transcriptional activator DREF in the chromatin to regulate gene expression.

The human Alpha-Thalassemia and mental Retardation X-linked syndrome (*hATRX*) gene encodes a chromatin

remodeling protein that contains several conserved motifs. The *ATRX* protein has a helicase/ATPase domain of the SWI/SNF family at its carboxyl terminus, and this domain is characteristic of the catalytic subunits of chromatin remodeling complexes (1). At its amino terminus, the *ATRX* protein has a domain composed of a Plant Homeo Domain (PHD) and a GATA-like zinc-finger motif, known as the *ATRX-DNMT3A-DNMT3L* (ADD) domain because this configuration of Zinc fingers has been found in the DNA-methyltransferases DNMT3A and DNMT3L (2). The ADD domain of the DNMT3A and DNMT3L proteins has been shown to bind histone tails (3). This interaction has been proposed to mediate the recruitment of these proteins to chromatin (4). The ADD domain of *hATRX* is very similar to the one present in the DNMTs and recently has been reported to bind H3 when its K4, and K9 residues are methylated (5–7).

Mutations in *hATRX* are associated with several syndromes linked to the X chromosome. For this reason, most of the patients affected are males. Patients with mutations in *hATRX* have developmental defects in the urogenital system, suffer severe dysfunctions in the central nervous system, suffer from α -thalassemia, have impaired motor abilities, and display a characteristic facial dysmorphism. About 50% of the mutations detected in patients afflicted with *ATRX* syndrome are located in the ADD region, and ~30% are in the SNF2 domain (1).

Some of the phenotypes observed in patients carrying *ATRX* mutations may be due to the role of *ATRX* in the regulation of transcription. As a result, different molecular studies have focused on elucidating the role played by *ATRX* in transcription (8,9). A recent genome-wide analysis of human and mouse cells demonstrates that *ATRX* can bind G-rich tandem repeat sequences. Importantly, genes associated with these tandem repeat

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sequences are deregulated when ATRX is mutated (10). Furthermore, the same study shows that ATRX can directly bind to G-rich sequences that may form G quadruplex structures (10). In addition, mammalian ATRX is capable of interacting with other cellular factors including Heterochromatin Protein 1 (HP1), Methyl-CpG-binding protein (MeCP2), cohesin, CCCTC-binding factor (CTCF) and the Death Associated Protein-6 (DAXX) presumably acting together as a H3.3 chaperone (11–15). The interaction of ATRX with CTCF and MeCP2 results in the repression of imprinted gene expression in the postnatal mouse (15). In addition, evidence suggests that in mice, the PAR genes are positively regulated by ATRX (16). In fact, expression of the α -globin genes is affected when ATRX is mutated, and this effect is determined by the number of the tandem G-rich repeats sequences upstream of this gene, suggesting that the ATRX mechanisms that influence gene expression are complex (10). Indeed, it is not clear if all the cases involve a direct interaction with chromatin or if this activity is mediated by an interaction with other undescribed factors. Therefore, the mechanism by which ATRX regulates gene expression is currently unknown.

In *Drosophila*, XNP/ATRX is present as two isoforms (dATRX_S and dATRX_L) that are derived from the use of two alternative translational start codons (17). While some reports suggest that XNP/dATRX_L interacts with HP1 in heterochromatic regions, including the chromocenter, other studies show evidence that dATRX_L can also be located in euchromatin (18,19). The dATRX_S isoform has also been shown to be distributed in different regions across polytene chromosomes (19). In *Drosophila*, XNP/dATRX mutants act as suppressors of position effect variegation (18,19). Therefore, it is likely that XNP/dATRX is a chromatin-associated protein in the fly. However, although both XNP/dATRX isoforms conserve the ATPase/helicase domain, they lack the human ADD domain, suggesting that these proteins participate in the control of gene expression through protein–protein interactions with other cell factors that may have DNA or chromatin binding domains.

To test this hypothesis and to find putative XNP/dATRX partners, we used XNP/dATRX as a bait to screen an embryonic cDNA library through a yeast two-hybrid analysis. We identified, among several factors, that the transcription factor DREF interacts *in vitro* and *in vivo* with dATRX. DREF recognizes the consensus DNA element 5'-TATCGATA-3, which is highly prevalent in core promoters and is known as the DNA replication-related element (DRE) (20). In *Drosophila*, DREF regulates the expression of several genes that are required during the S phase of the cell cycle, such as *PCNA*, *E2F* and *DNApol- α* (22–24). Physically, DREF interacts with factors involved in chromatin structure and dynamics (25,26), and the wide spectrum of genes regulated by DREF suggests that this transcription factor acts in conjunction with a diverse array of transcriptional regulators.

The interaction between XNP/dATRX and DREF results in the regulation of *pannier* (*pnr*) gene transcription. Mechanistically, we found that DREF binds to DRE

elements present in the *pnr* promoter, where it works to maintain correct levels of *pnr* gene expression. The interaction of XNP/dATRX with DREF results in the repression of *pnr* transcription. These results suggest a dynamic interaction between XNP/dATRX and DREF to control the expression of a factor that is required for various biological processes during the development of *Drosophila*.

MATERIALS AND METHODS

Fly stocks

The wild-type flies used in this study were Oregon R(OreR), and fly stocks were maintained at 25°C with standard food. All stocks were obtained from the Bloomington, Indiana stock center. RNAi transgenic flies for DREF and XNP/dATRX on chromosome 3 were obtained from the Vienna *Drosophila* RNAi center (VDRC). *Df(3R)Exel6202* is a deficiency that does not have XNP/dATRX (deletes polytene chromosome bands 96D1-96E2). In this work, we called this deficiency *Df(ATRX)*.

Establishment of UAS-RNAi transgenic flies

The pSymp-UAST plasmid was kindly provided by Dr E. Giordano and used to make transgenic flies for expression of an inducible RNAi against XNP/dATRX. A fragment containing XNP/dATRX bases 3765–4087 was cloned into the pSymp-UAST. The resulting transgene is called *UAS-dATRXi*. Transgenic flies were made by Genetic Services Inc. Transgenic flies carrying a transgene used for the expression of an inducible RNAi for DREF (*UAS-DREFi*) were obtained from the Vienna *Drosophila* RNAi Center (VDRC).

Genetic crosses

All stocks were crossed first with *w*¹¹¹⁸ or with OreR flies in order to homogenize the genetic background. The transgene called *UAS-dATRX* is *P{Mae-UAS.6.11}XNP^{UY3132}* (27). It carries an *UAS* sequence inserted at the 5'-UTR of the XNP/dATRX gene. Transgenic RNAi lines carrying the transgenes in chromosomes 2 or 3 were established and balanced with *CyO* for chromosome 2 and with *TM2, Ubx, TM3, Ser* or *TM6B, Tb* balancers for chromosome 3. The drivers used to express the *UAS-XNP/dATRX*, *UAS-XNPi* or *UAS-DREFi* transgenes were *Act5C-Gal4*, *455.2-Gal4* (28) or *pnr-Gal4*. These drivers direct transgene expression ectopically, in the scutellum, or in the *pnr* domains, respectively.

Antibodies

Antibodies were raised in rats or rabbits against XNP/dATRX using the peptide p4:CVVRLKRVSLPKTK PAQ which recognizes XNP/dATRX_L. The polyclonal rabbit anti-DREF antibody has been previously reported (29). Polyclonal rabbit anti-Pnr antibody was kindly provided by Dr Ginés Morata.

Immunostaining of polytene chromosomes

Salivary glands from third instar larvae were fixed in solution I (PBS, 3.7% paraformaldehyde and 1% Triton X-100) and then in solution II (3.7% paraformaldehyde, 50% acetic acid). The chromosomes were spread on poly-L-Lysine coated microscope slides. Anti-DREF antibody was used at 1:200 and anti-dATRX antibody at 1:100. Secondary antibodies ALEXA fluor 488 or 568 (Invitrogen) were used at 1:500. Images were taken on a confocal laser scanning microscope (Zeiss LSM 510 META).

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSAs) were performed as previously described (30). Nuclear extracts were obtained from adult flies or embryos using the protocol described in (31). Oligonucleotide sequences are indicated in Figure 4. Competitions were performed with a 100-fold molar excess of unlabeled oligonucleotides, and 100 000 cpm of γ - 32 P ATP-labeled paired oligonucleotides were used per lane. EMSA-WB was performed as described in (32).

Pull-down assays

Pull-down assays were performed using the TNT-Quick-Coupled Transcription/Translation System (Promega) according to the manufacturer's instructions. A clone from the *Drosophila* Genomics Resource Center containing full length DREF cDNA was used for the *in vitro* translation of DREF.

Yeast two-hybrid screen

Yeast two hybrid assays were performed using the BD Matchmaker library construction and screening kit (Biosciences-Clontech) according to manufacturer's instructions. The cDNA library was made from 0 to 12 h embryos. The assay was performed using the amino-terminus (amino acids 1–221) or the carboxy-terminus (amino acids 1147–1308) of XNP/dATRX as bait. Screening for positive interactions was performed by co-transformation and/or yeast mating. Positive interactions were analyzed by growth in QDO medium and X- α -galactosidase or β -galactosidase assays.

Co-immunoprecipitation assays

Embryo or adult fly nuclear extracts were prepared as described in (33). This protocol allows us to maintain the 'transcriptionally active' nuclear fraction free of membranes and DNA. Co-IP was performed as described in (34).

qRT-PCR assays

RNA was obtained using the Trizol method or the protocol reported in (35) from 0 to 12 h embryos or adult flies expressing the RNAi against XNP/dATRX or from overexpressing dATRX. Equal amounts of RNA (1 μ g) were used for first strand synthesis reactions. qRT-PCR reactions were performed using the light cycler DNA master SYBR green 1. All of the

oligonucleotide sequences used in this study are available upon request. Relative quantification was performed by the $\Delta\Delta$ Ct-method. All reactions were performed in triplicate.

ChIP, ChIP-western and Re-ChIP assays in embryos

The 0- to 14-h-old embryos were collected, dechorionated and fixed (36). The fixation reaction was stopped by adding glycine (125 mM). Embryos were washed and resuspended in lysis buffer, and sonication was performed until the size of chromatin reached between 200 and 800 bp. Pre-clearing, antibody incubations, immunoprecipitation and phenol:chloroform extractions were performed as described in (30). For the 'mock' condition, a pre-immune sera against XNP/dATRX or an anti-GFP antibody were used. The following oligonucleotides were used for *pnr- β* : FWD 5'-CGGCCTGCTAAGTGAGTG TGA-3', REV 5'-CCGGTTGGATGTGCGCTGAG-3' and for *pnr-a*: FWD 5'-CCTTCAGTACCCACAATATC GCC-3', REV 5'-GAACTAAGATTTAAACCACC-3'. qPCRs were then performed using the light cycler DNA master SYBR green 1 run in a Lyght cycler 1.5 (ROCHE), and the relative quantification was performed as follows. The percentage of INPUT was calculated using the formula:

$$\begin{aligned} \% \text{Input} &= 2^{(-\Delta C_t [\text{normalized ChIP}])} \Delta C_t [\text{normalized ChIP}] \\ &= (C_t [\text{ChIP}] - (C_t [\text{Input}] - \text{Log}_2 (\text{input dilution factor})), \end{aligned}$$

where input dilution factor = (fraction of the input chromatin saved) $^{-1}$ as described in Champion-ChIP TM qPCR Primers. Quantitative real-time PCR analysis of chromatin, SA. Biosciences 2008. All the assays were performed at least three independent times unless stated otherwise. All of the data were obtained from at least three independent experiments (37). For the ChIP-western experiments, the ChIP protocol was followed through the final wash of the agarose beads and protein complexes with TE buffer. The denatured complexes were migrated in an 8% polyacrylamide gel and transferred to a nitrocellulose membrane. WB was conducted using the anti-XNP/dATRX antibodies (described above) or the anti-DREF antibody.

For the Re-ChIP experiments, the ChIP protocol was followed through the last wash of the agarose beads and protein complexes with TE buffer, after which the agarose beads were resuspended in 100 μ l of 10 mM DTT and incubated at 37°C for 1 h to dissociate the protein complexes from the beads. The supernatant was removed and diluted in IP buffer, and immunoprecipitation was performed using the second antibody. The standard ChIP protocol was followed to obtain the DNA that was then analyzed by qPCR as described above.

RESULTS

XNP/dATRX physically interacts with DREF

To find putative XNP/dATRX interacting proteins, we performed a yeast two-hybrid screen. To achieve this, we constructed a cDNA library from 0 to 12 h embryos in

which cDNAs were linked to the sequence encoding the GAL4 activation domain. Meanwhile, the N-terminal (amino acids 1–221) and C-terminal (amino acids 1147–1308) regions of XNP/dATR_X were used as baits. The SNF2/helicase–ATPase domain was not used in this screen because it is also conserved in other factors. Several *bona fide* candidates were identified, as well as some encoded proteins involved in gene transcription and chromatin dynamics (Supplementary Table S1). Among these candidates, we decided to analyze DREF (Supplementary Figure S1) in more detail because it is a transcription factor that participates in several cellular functions and has been shown to interact with proteins involved in chromatin dynamics, such as Mi-2 (29,38).

In the yeast two-hybrid screen, the activation domain of DREF called the CR3 was found to interact with the N-terminal portion of XNP/dATR_X (Figure 1). To confirm this interaction, we performed *in vitro* GST-pull-down and co-immunoprecipitation (CoIP) experiments. In the pull-down experiments, a full length DREF or CR3 domain sequences were *in vitro* transcribed and translated to produce an S³⁵-labeled DREF protein, or the S³⁵-labeled CR3 domain, to monitor their binding with a XNP/dATR_X-N-terminal-GST fusion protein (Figure 1A). In these pull-down assays we found that

both, the S³⁵-labeled DREF protein and the S³⁵-labeled CR3 domain bound more strongly to XNP/dATR_X-N-terminal-GST coupled to a glutathione sepharose column than to GST alone (indicated in the corresponding lanes as NH3-GST in Figure 1B).

To confirm the yeast two-hybrid analysis and the results of the GST pull-downs, we performed a Co-IP experiment using protein extracts from the transcriptionally active chromatin fraction from adult flies and embryos (see ‘Material and Methods’ section) and an anti-XNP/dATR_X antibody that only recognizes the large XNP/dATR_X isoform (dATR_X_L). The presence of DREF in the proteins precipitated by the dATR_X_L antibody was determined using an anti-DREF antibody. Figure 1C shows that XNP/dATR_X could be efficiently immunoprecipitated with the antibody and that DREF co-immunoprecipitated with it. We also performed a reciprocal CoIP using the DREF antibody to immunoprecipitate proteins from the adult flies or embryo extracts and found that XNP/dATR_X also co-immunoprecipitates (Figure 1C). Intriguingly, the detection of DREF by western blot analysis after the CoIP using the XNP/dATR_X antibody suggests that most of the DREF protein interacts with XNP/dATR_X because no signal can be detected in the unbound fraction.

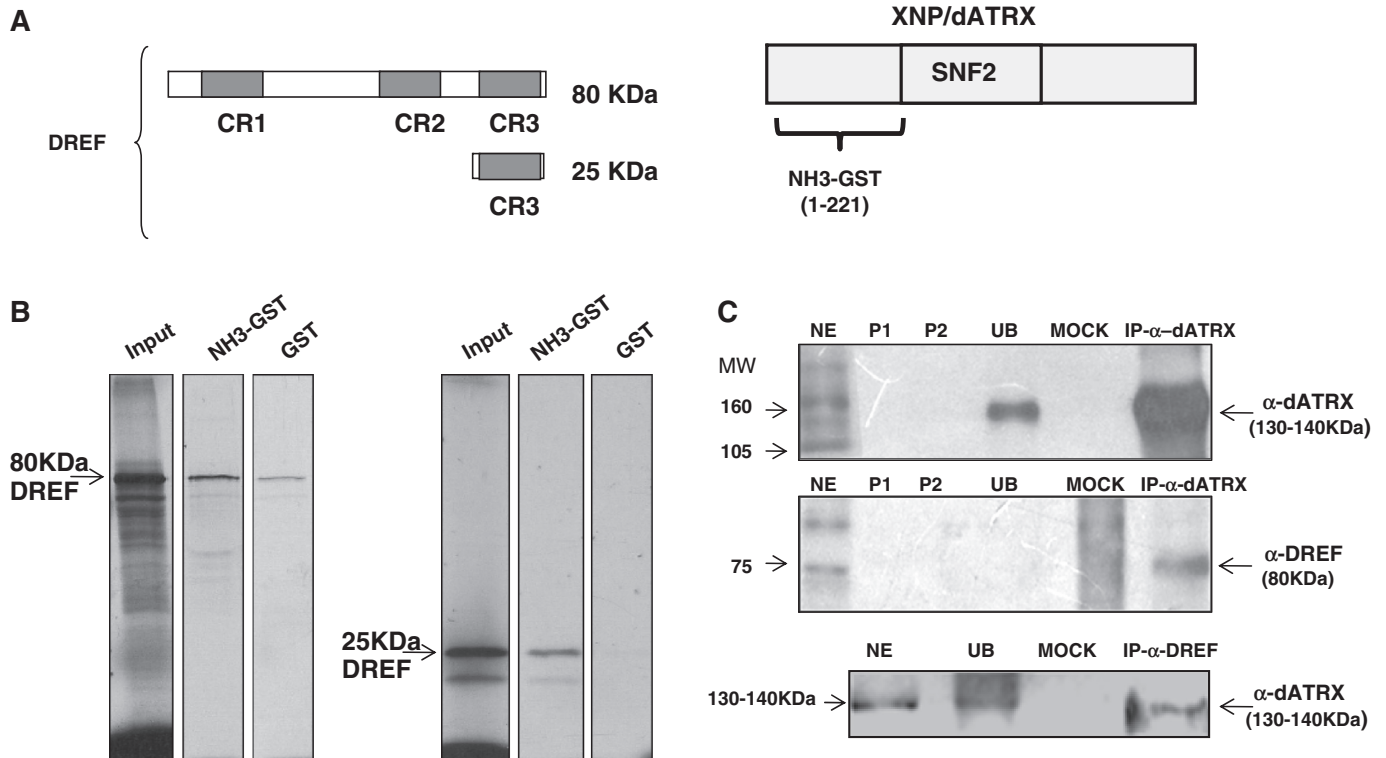


Figure 1. Physical interaction between XNP/dATR_X and DREF. (A) Representation of the fragments assayed by pull down, the three conserved regions of DREF are shown (gray boxes), CR3 corresponds to the region that interacts with XNP/dATR_X in the two-hybrid assay. On the right side, the 221 amino acids of XNP/dATR_X used for the two-hybrid and the pull-down assays is shown. (B) Pull-down assay. For both panels: the first lane shows the amount of transcription/translation labeled protein (full-length DREF or the CR3 domain) used for each experiment (input), the second lane is the experimental interaction (GST-dATR_X and dDREF) and the third lane is the control interaction (GST and dDREF) for each analyzed polypeptide. (C) CoIP of XNP/dATR_X and DREF. Transcriptional active nuclear fraction from adult flies or 0–24 h embryos (NE); P1 is the pre-clearing 1; P2 is pre-clearing 2; UB correspond to the unbound protein fraction; Mock is the incubation with an unrelated antibody, the upper and middle panels correspond to an IP performed against XNP/dATR_X. Upper panel was blotted with α-dATR_X, middle panel with α-dDREF and the lower panel again with α-dATR_X after an IP against DREF.

However, we cannot discard the possibility that under our experimental conditions, the unbound DREF is unstable and degraded during the clearing procedure. Nevertheless, taken together, the results presented in this section strongly suggest that dATRX and DREF interact physically in *Drosophila*.

XNP/dATRX behaves genetically as a repressor of *pnr* function

Because *pnr* expression is likely regulated by XNP/dATRX (39), we studied the role of XNP/dATRX and DREF (see below) on gene expression. To do this, we used as a test system the modification of adult phenotypes shown by *pnr* mutant flies in conditions where the levels of ATRX or DREF are manipulated through the expression of transgenes carrying *UAS-dATRX* or sequences encoding RNA is for *XNP/dATRX (UAS-dATRXi)* or *DREF (UAS-DREFi)*. This allowed us to reduce the levels of these proteins in particular domains, according to the *Gal4*-driver used.

pnr encodes two isoforms, Pnr- α and Pnr- β , transcribed from two different promoters (40). Peña-Rangel *et al.* (39) showed that overexpression of XNP/dATRX enhances the thorax and scutellar abnormalities found in heterozygote individuals carrying the *pnr-Gal4* mutation. In *pnr-Gal4*, a transposable element carrying the *Gal4* gene is inserted just upstream of the first *pnr* exon transcribed from the *pnr*- β promoter (40). Consequently, in *pnr-Gal4* individuals, *Gal4* expression closely mimics normal *pnr*- β expression in wing imaginal discs and in adult flies (40). In homozygote *pnr-Gal4* wing discs, the levels of *pnr*- β transcripts are reduced, while those of *pnr*- α are increased (41). *pnr-Gal4* flies die as homozygotes, and only 1–3% develop into adults. *pnr-Gal4* heterozygote adult flies have a very slight dorsal thoracic cleft and disorganization of bristles, sometimes missing a few inner and post-vertical bristles (39). For this set of experiments, we also used the *pnr*¹ and *pnr*^{VX6} alleles (40). *pnr*¹ presents a stop codon at position 180 (W^{180@}), interrupting the first zinc finger found in Pnr- β , and it is considered to retain some activity (42). *pnr*^{VX6} is a small deficiency that deletes the complete *pnr* locus (40). *pnr*¹ and *pnr*^{VX6} are recessive lethal, and their adult phenotypes are normal in heterozygosis.

We found that individuals expressing the transgene *UAS-dATRX* in the *pnr* adult territory, using the *pnr-Gal4* line as a GAL4 source, showed a strong thoracic cleft and scutellar defects (Figure 2A) similar to the enhancement of the *pnr* defects found by Peña-Rangel *et al.* (39) with the overexpression of XNP/dATRX.

As *pnr-Gal4* is by itself a *pnr* allele and a *pnr* GAL4 driver, interpretation of these results could be difficult. Thus, we drove the expression of the *UAS-dATRX* transgene with two other drivers, the ectopic *Act5C-Gal4* and the *455.2-Gal4* drivers. The latter one was used to direct the *UAS-dATRX* transgene expression to the scutellum in wild-type *pnr* or in *pnr*¹ or *pnr*^{VX6} backgrounds (Figure 2). Importantly, these experiments validate *pnr-Gal4* as a suitable tool for these kinds of experiments because equivalent results can be obtained with different drivers and with different *pnr* alleles.

We found that ectopic expression of *UAS-dATRX* with the *Act5C-Gal4* driver in the presence of wild-type *pnr* or with *pnr*^{VX6} is semilethal or lethal, respectively, and surviving adult flies (when wild-type *pnr* is present) do not show any morphological defects (data not shown). With the *455.2-Gal4* driver, we found lethality that could not be attributed to the effect of *UAS-dATRX* expression (Figure 2B); however, expression of *UAS-dATRX* in the scutellum enhances the scutellar defects shown by the flies of the control genotypes (flies with the *455.2-Gal4* driver and with *pnr*¹ or *pnr*^{VX6} but without the *UAS-dATRX* transgene). These defects are not observed when the *UAS-dATRX* transgene is expressed in a wild-type *pnr* background (Figure 2B).

Based on these genetic results, we hypothesized that XNP/dATRX may act as a repressor of *pnr* expression. Therefore, we evaluated whether a reduction in XNP/dATRX levels (using RNA interference) could suppress the phenotypes found in *pnr-Gal4* flies. Flies expressing the *UAS-dATRXi* transgene with the *Act5C-Gal4* driver did not exhibit any visible defect, although there was a reduction of XNP/dATRX mRNA levels (data not shown). In contrast, the expression of *UAS-dATRXi* with the *pnr-Gal4* driver suppressed the lethal phenotype of homozygous *pnr-Gal4* flies (Supplementary Figure S2 and Figure 2C). The suppression of lethality was greater when two copies of the *UAS-dATRXi* transgene were present (Figure 2C). Similar results were obtained with a different *UAS-dATRXi* RNAi line obtained from the Vienna *Drosophila* RNAi stock collection (data not shown). In addition, *pnr-Gal4/pnr*^{VX6} heteroallelic flies do not survive well (40); however, this is alleviated with the expression of one copy of the *UAS-dATRXi* transgene (Figure 2C). Furthermore, embryonic transcript levels of *pnr* isoforms respond to XNP/dATRX levels. Through the action of the *Act5C-Gal4* driver during embryogenesis, XNP/dATRX depletion (by *UAS-dATRXi* expression) or overexpression (by *UAS-dATRX* expression) increases or decreases, respectively, the transcript levels of both *pnr* isoforms (Figure 2D). Thus in summary, overexpression of XNP/dATRX causes or enhances phenotypes that can be interpreted as a reduction of *pnr* function; meanwhile, reduction of XNP/dATRX levels rescues these phenotypes, suggesting that dATRX negatively regulates *pnr* expression.

DREF binds to DRE elements in the *pnr* control region

In silico analyses have revealed more than 250 DRE elements in the promoter regions of various genes, including genes involved in DNA metabolism, transcriptional regulation, protein synthesis and signal transduction mechanisms. Therefore, because DREF interacts with XNP/dATRX, we decided to search for DRE elements in the *pnr* regulatory region. The *pnr* locus encodes two isoforms expressed from two different promoters, named *pnr*- α and *pnr*- β (41). Two DNA elements containing the DRE consensus sequence were found in the vicinity of the *pnr* regulatory region: one –85 bp from the putative transcriptional start site of the β -isoform and the other 3.9 kb upstream of the putative *pnr*- α transcription

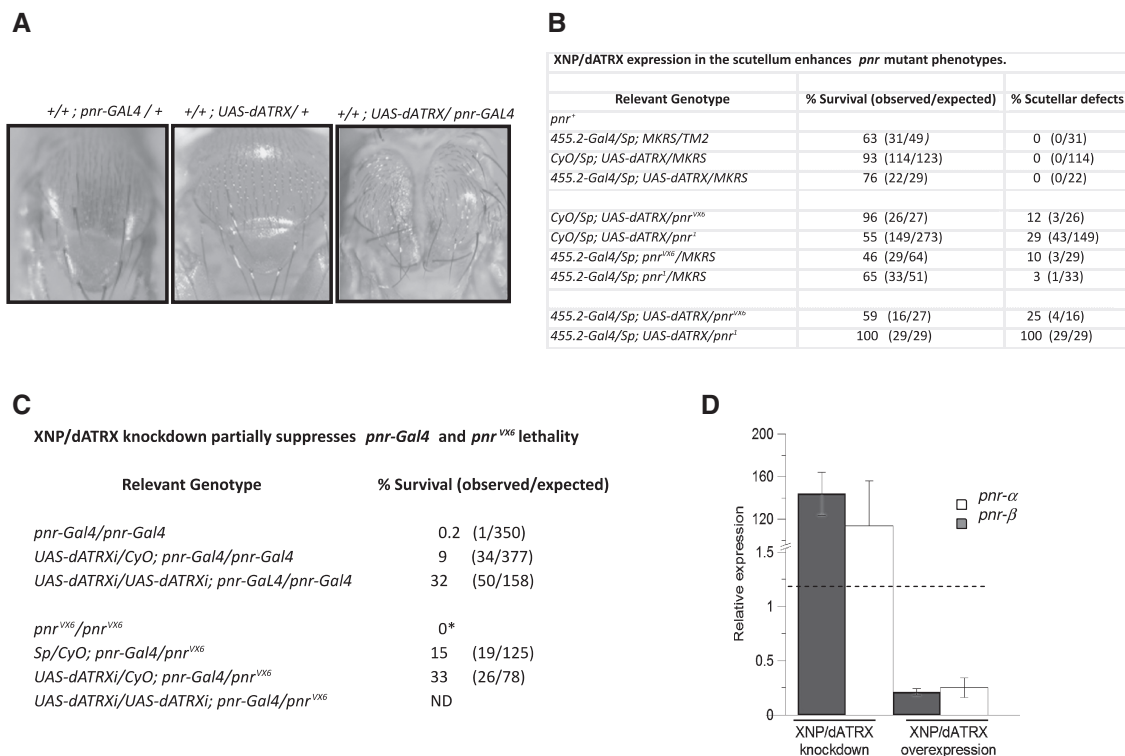


Figure 2. Modification of *pnr* mutant phenotypes by XNP/dATRX dosage. (A) Enhancement of the thoracic cleft phenotype by the expression of the *UAS-dATRX* transgene in the *pnr*-expression domain. The genotypes of each organism are indicated at the top. Note the enhancement of the thorax cleft defect in the *UAS-dATRX/pnr-Gal4* fly. This result is in agreement with Peña-Rangel *et al.* (39) where they used a different *P-UAS* construct that overexpresses XNP/dATRX in the *pnr* expression domain (27). (B) Enhancement of *pnr*^{VX6} and *pnr*¹ scutellar defects by the expression of the *UAS-dATRX* transgene in the scutellum. Relevant genotypes are indicated. The scutellar defects consists of a reduction in the scutellum size and deformations in the scutellar border (data not shown). No defects in the macrochaetes were observed in these flies. (C) XNP/dATRX depletion rescues the lethality of *pnr* mutant flies. *Homozygous *pnr*^{VX6} flies die as embryos (40). Homozygous *pnr-Gal4* or *pnr-Gal4/pnr*^{VX6} adult flies survive in the presence of one or two copies of the *UAS-dATRXi* transgene. (D) Modification of *pnr* transcript levels by XNP/dATRX. Expression of transgenes was driven by the *Act5C-Gal4* driver. Depletion of XNP/dATRX by ectopic expression of the *UAS-dATRXi* transgene increases the transcript levels of the two *pnr* isoforms in *Drosophila* embryos. On the contrary, ectopic expression of XNP/dATRX by the *UAS-dATRX* transgene, reduced both *pnr* mRNA levels. The quantification of the mRNA of mutant embryos was performed by RT-qPCR compared with the relative mRNA levels of wild-type embryos of the same age. The relative expression levels are adjusted to one, which is the level of each *pnr* isoform transcript in wild-type flies. Data are from three different experiments.

start site (Figure 3A). We named these two elements *Dre-pnrα* and *Dre-pnrβ*. Next, to determine whether oligonucleotides containing the putative DRE elements were shifted by proteins in adult fly nuclear extracts, we performed an EMSA analysis. As a positive control, we used the DNA polymerase-α DRE element (*Dre-polα*), a known DREF binding site. As a non-competing element, we used the same *Dre-polα* sequence with a three-base mutation in the consensus element, as reported by Ida *et al.* (21; Figure 3A). Figure 4B shows that proteins in the nuclear extracts formed a complex with either *Dre-pnrα*, *Dre-pnrβ* or *Dre-polα*: in all three cases the radioactive binding signal was inhibited by a 100-fold molar excess of the *Dre-polα* non-radioactive oligonucleotide but not by the mutated *Dre-pm* oligonucleotide at the same molar excess (Figure 3B). These results suggested that the two DRE elements present in the *pnr* control region could be recognized by a nuclear factor and that this interaction could be inhibited with the *DRE-polα* oligonucleotide but not by the mutated *Dre-pm* oligonucleotide. Next, we assessed whether DREF was part of the shifted complexes. Initially, we

used the DREF antibody to identify super-shifted complexes; however, this antibody was not able to produce any super-shift, even with the positive control *Dre-polα* oligonucleotide. After DREF antibody incubation with the nuclear extracts and with each oligonucleotide, the formation of the complex was reduced, suggesting that the DREF antibody sequestration inhibits the putative DREF–DNA interaction (data not shown). Therefore, to assess the presence of DREF in these complexes, we performed an EMSA–western experiment. Indeed, we found that the DREF antibody recognizes the complexes bound at the *Dre-pnrα* and *Dre-pnrβ* oligonucleotides (Figure 3C). No signal was detected in a similar western blot analysis performed with an antibody which does not recognize DREF. To confirm that DREF binds to the *Dre-pnr* elements, we incubated nuclear extracts with or without the different oligonucleotides to allow the formation of the complexes. The presence of DREF in the formed complexes was determined by western blot analyses with the anti-DREF antibody in the nuclear protein extracts separated electrophoretically under native conditions (Figure 3D). These results show

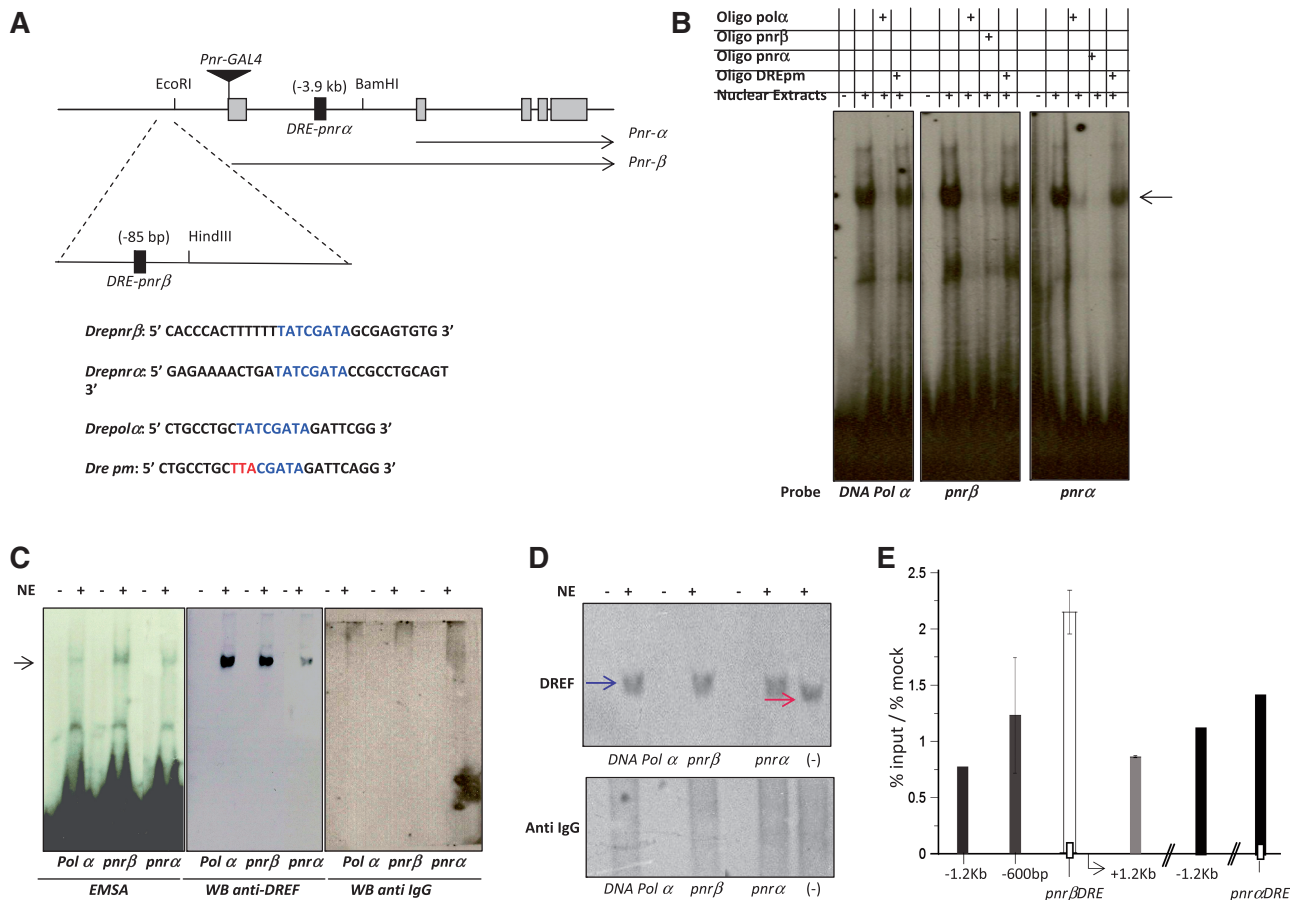


Figure 3. DREF binds to two DRE elements in the *pnr* control region. (A) Localization of the two DRE (*Dre-pnr α* and *Dre-pnr β*) consensus sequences near the transcription initiation site of the *pnr- β* (–85 bp) and *pnr- α* (–3.9 kb) transcripts. The sequence of the two *Dre pnr* elements, the *Dre-Pol α* , *Dre-pnr α* and *Dre-pnr β* oligonucleotides. The central panel shows a western blot using an anti-DREF antibody after the transfer of the EMSA to a nitrocellulose membrane. The right panel shows a western blot after the transfer of the DRE-protein complexes, but using a not related antibody as a control. (D) Native DREF is shifted in the presence of the DRE oligonucleotides. Western blots are shown after the transfer of total nuclear proteins previously incubated with the DRE oligonucleotides and separated in a native polyacrilamide gel. Note that in the presence of the different DRE oligonucleotides the band recognized by the DREF antibody is shifted when compared to the one without DNA indicated as (–). A control western blot using an unrelated antibody is also shown. (E) ChIP assays using a specific antibody against DREF to determine the distribution DREF factor around and on the putative *pnr- α* and *pnr- β* DRE in chromatin derived from embryos. Graphs show results from three independent immunoprecipitation reactions ($n = 3$). ChIP signals quantified by means of quantitative polymerase chain reaction, are presented as percentage of the input/percentage of the mock antibody. As it can be observed in the figure, the *pnr- β* chromatin region shows the maximum enrichment when the precipitation was carried out with the DREF antibody.

that the migration of DREF is shifted in the presence of the *Dre-pnr α* , *Dre-pnr- β* and *Dre-pol α* oligonucleotides suggesting, as in the EMSA analysis, that DREF found in the nuclear extracts interacts with *Dre-pnr α* , *Dre-pnr- β* and *Dre-pol α* elements. To complement these data, we performed ChIP experiments using chromatin obtained from 0 to 14 h developed embryos. DREF was detected at the *Dre-pnr β* elements and at the DRE-PCNA element, which was used as a positive control, indicating that DREF binds the *Dre-pnr β* element *in vivo* (Supplementary Figure S3). In addition, we performed ChIP experiments with several PCR primer sets over the *pnr α* and *pnr β* promoter region and found that the DREF

signals peaks at the two putative DRE elements but it is enriched at the *DRE-pnr β* element (Figure 3E). The detection of DREF at the *DRE-pnr α* element was marginal (Figure 3E and Supplementary Figure S3). However, we cannot exclude the possibility that this element could be recognized by DREF at other developmental stages. We also performed a bioinformatics search for DRE elements in the putative regulatory regions of the *pnr* homologs in other *Drosophila* species. Assuming that *pnr* also encodes two isoforms in these other species, we found that a putative *Dre-pnr β* element is conserved in other *Drosophila* species (Supplementary Figure S4). A putative *Dre-pnr- α* element was found to be conserved

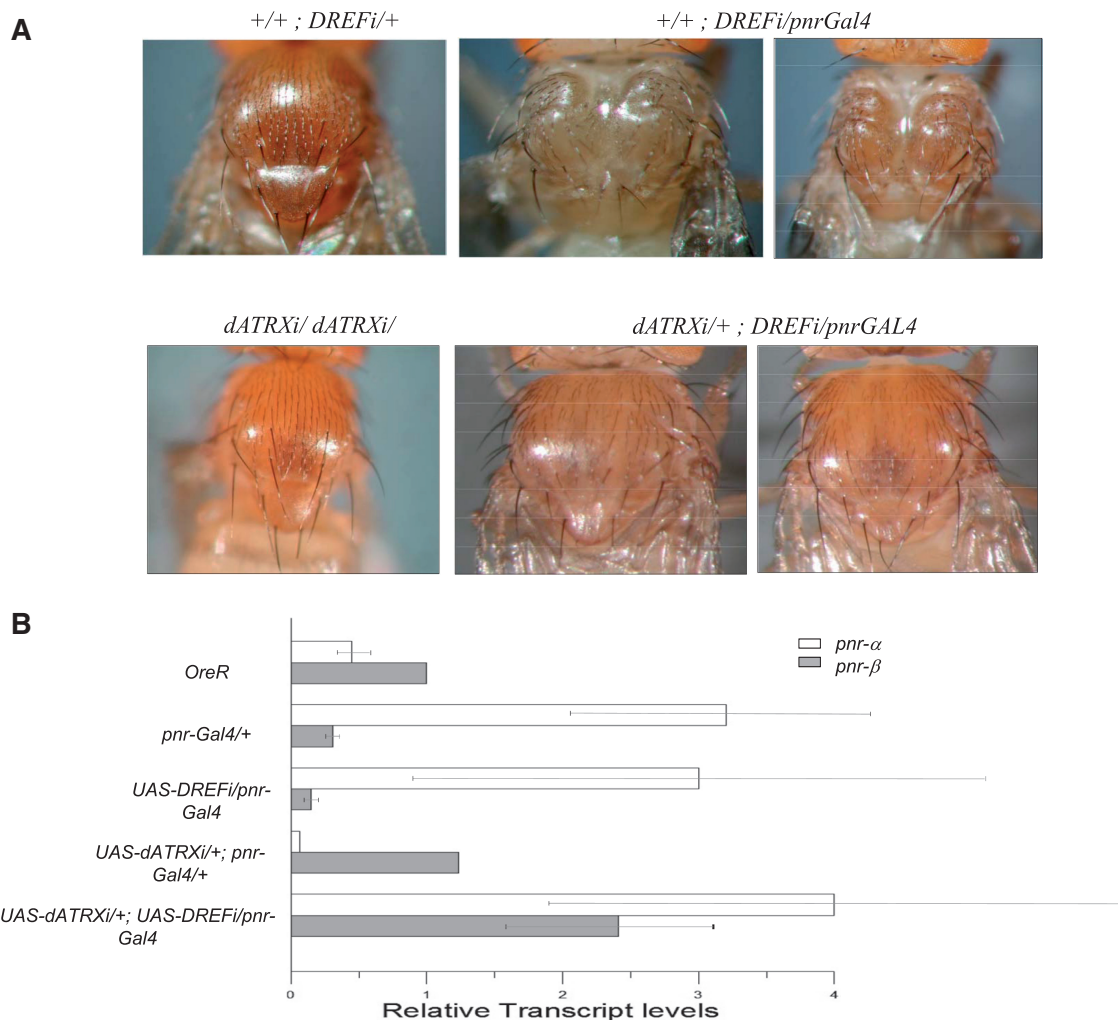


Figure 4. Suppression of the thoracic cleft induced by DREF knockdown in *pnr-Gal4* heterozygous flies, by depletion of XNP/dATRX. (A) *UAS-DREFi* (top panel, left) or *UAS-dATRXi* (bottom panel, left) have a normal thorax, meanwhile flies with *UAS-DREFi* expression in the *pnr* domain driven by the *pnr-Gal4* driver (middle and right top panels) show strong thoracic cleft defects. Depletion of XNP/dATRX in these flies, accomplished by the expression of the *UAS-dATRXi* transgene in the *pnr* domain, alleviates this defect (middle and right bottom panels). (B) *pnr* transcript levels are influenced by DREF and XNP/dATRX dosages. *pnr-α* and *pnr-β* transcript levels in the thorax of flies depleted of DREF and DREF and XNP/dATRX in the *pnr* expression domain (genotypes as in Figure 4A). Note that in the thorax of DREF depleted flies there is a significant reduction of the *pnr-β* transcript levels. These levels are recovered when DREF and XNP/dATRX are simultaneously depleted in the *pnr* expression domain and partially recovered the ratio between *pnr-α* and *pnr-β* mRNAs. The quantification was obtained from three independent experiments and each cDNA was amplified three times by qRT-PCR. The *Dmp8* transcript levels were used as reference control, which is not affected by the *XNP/dATRX* levels.

only to a minor extent. Taken together, these results suggest that DREF recognizes at least one *DRE* element located upstream of the *pnr-β* promoter.

Genetic interaction between DREF and XNP/dATRX in *pnr* gene regulation

In the previous sections, we showed that DREF binds directly to XNP/dATRX and to the *DRE* sites at the *pnr* promoter, suggesting a possible role of DREF in the control of *pnr* gene expression. Therefore, we investigated the effect of knocking down DREF using the *pnr-Gal4* driver. We used the transgenic *UAS-DREFi* line from the Vienna *Drosophila* RNAi stock collection (43). A reduction in the DREF protein level was detected in the

thorax of adult flies expressing the *UAS-DREFi* RNAi transgene (Supplementary Figure S5). Interestingly, individuals expressing the *UAS-DREFi* transgene with the *pnr-Gal4* driver showed a dramatic enhancement of thoracic cleft defects (Figure 4A and Table 1). We also noticed a reduction in macrochaete number in the *pnr* expression domain, as well as the presence of thinner bristles when compared to the ones in *pnr-Gal4/+* flies without the transgene (Figure 4A and Table 1).

Depletion of DREF in flies expressing the *UAS-DREFi* transgene with the *455.2-Gal4* driver, causes the disappearance of macrochaetes in the scutellum (Supplementary Figure S6, middle panel). Interestingly, when a mutant *pnr* allele such as *pnr^I* (Supplementary Figure S6, right panel) or *pnr^{VX6}* (data not shown) is

Table 1. DREF and XNP/dATTRX knockdowns modify *pnr* thorax cleft and macrochaete defects

Relevant genotype	Thoracic cleft defects (%)	Macrochaete (%)
+/+; <i>UAS-DREFi/TM3</i>	0 (0/177) ^a	0 (0/177)
+/+; <i>pnr-Gal4/MKRS</i>	0 (0/160)	0 (0/160)
+/+; <i>pnr-Gal4/UAS-DREFi</i>	100 (86/86)	100 (86/86)
<i>UAS-dATTRXi/+; pnr-Gal4/UAS-DREFi</i>	0 (0/73) ^b	65 (48/73)

^aFlies with thoracic cleft and absence of macrochaetes from the total number of flies counted for each genotype.

^bThe reduction of the thoracic cleft phenotype and the absence of macrochaetes are shown in Figure 4A.

added, the scutellum is smaller, with irregular borders. These results, together with the binding of DREF in the promoter region of *pnr-β*, suggest that DREF is a positive regulator of *pnr* expression.

Because our data show that XNP/dATTRX and DREF may be antagonistic *pnr* transcriptional regulators, we tested the effect of *XNP/dATTRX* knockdown in the *UAS-DREFi/pnr-Gal4* flies, expecting that the enhancement of the thoracic cleft presented by DREF depleted *pnr-Gal4* flies (Figure 4A, upper panel) would be suppressed by the depletion of XNP/dATTRX. Indeed, we found that this was the case. Figure 4A (lower panel) shows a reduction of the thoracic cleft defect of *UAS-DREFi/pnr-Gal4* flies when the levels of XNP/dATTRX were diminished (Table 1).

We determined the levels of *pnr-α* and *pnr-β* transcripts in the thorax of these flies (Figure 4B). We found that in heterozygous *pnr-Gal4* flies, the *pnr-β* transcript level is reduced and the *pnr-α* transcript level is increased, in accordance with previous studies in which the transcript levels of the different isoforms were studied (41). When DREF is depleted in the *pnr* domain, *pnr-β* transcript levels are further diminished, supporting the positive role of DREF in *pnr-β* expression. On the contrary, when XNP/dATTRX is depleted, the *pnr-β* transcript level is increased in comparison to the level found in wild-type flies, supporting the negative role of XNP/dATTRX in *pnr* expression. In this latter condition, we found a decrease in the *pnr-α* transcript level compared to the wild-type level probably due to indirect effects of *Pnr-β* on *pnr-α* transcription (41). The differences between the transcript levels of *pnrα* in embryos versus the wing disc when XNP/dATTRX is depleted could be attributed to differences in *pnr* regulation during development. Interestingly, depletion of both DREF and XNP/dATTRX in the *pnr-Gal4* domain recovers the ratio between *pnr-α* and *pnr-β* transcript levels (Figure 4B). These results support the idea that besides their physical interaction, DREF and XNP/dATTRX also interact genetically and have antagonistic roles in *pnr* transcription. In addition, the depletion of XNP/dATTRX does not affect DREF protein levels, suggesting that dATTRX does not regulate DREF gene expression (Supplementary Figure S7).

On the other hand, the simultaneous overexpression of XNP/dATTRX and knockdown of DREF cause lethality and the organisms die during the embryo development. We also analyzed the effect in *Drosophila* of the simultaneous overexpression of both XNP/dATTRX and DREF using the *pnr-GAL4* driver. Intriguingly, we also found that this combination is lethal. Different possibilities may explain these two results, since both XNP/dATTRX and DREF independently may regulate different set of genes (as it is suggested polytene chromosomes immunostainings, see ahead). Therefore it is possible that in either of these two conditions creates a dramatic de-regulation in gene expression during the fly development leading to organism lethality.

A recent publication (44) has shown that DREF depletion in the notum and in other subdomains of the wing disc reduces or eliminates bristles. This phenotype is due in part to the influence of DREF on *PCNA* gene expression and affects the endoreplication process of bristle development, which results in a lack of bristles. The reduction in the number and size of macrochaetes in *UAS-DREFi/pnr-Gal4* flies found in our study (Supplementary Figure S6) may be related to a cell proliferation defect independent of *pnr*. Nevertheless, the disruption of the thorax is only observed in *UAS-DREFi/pnr-Gal4* flies (Figure 4, upper panel). Our genetic analyses indicate that we can separate these two phenotypes. We think that DREF depletion on the *PCNA* gene regulatory sequences would lead to the bristle defects, meanwhile depletion of this molecule on the *pnr* regulatory sequences would lead to the notum defects.

XNP/dATTRX represses transcription of some, but not all, of the genes regulated by DREF

The genetic results suggest that XNP/dATTRX negatively regulates the expression of *pnr*, which requires DREF to be properly expressed. Therefore, we studied the transcript levels of other genes that are positively regulated by DREF in conditions where XNP/dATTRX levels were altered. To perform this analysis, we used the *Act5C-Gal4* driver to overexpress or reduce XNP/dATTRX levels ectopically. We measured the mRNA levels of *E2F*, *osa* and *PCNA* from embryonic total RNA through qRT-PCR. These three genes are positively regulated by DREF. As a control, we used the *p8/TTDA* transcript (31), which we found to be unaffected by XNP/dATTRX. We also analyzed the transcript of the ribosomal protein *rp49*, which is generally used as a reference control to measure mRNA levels in *Drosophila*. However, it should be noted that genes encoding ribosomal proteins in mammals have been reported to be regulated by DREF (45).

In flies that ectopically express XNP/dATTRX, we detected a reduction on the *E2F* and *osa* transcript levels (Figure 5C and D). However, the transcript levels of *PCNA* and *rp49*, which are also positively regulated by DREF, were enhanced (Figure 5B and E) This result is intriguing because E2F acts as an activator of *PCNA* transcription. At the same time, it has also been reported that E2F may act as a repressor of *PCNA* expression in some

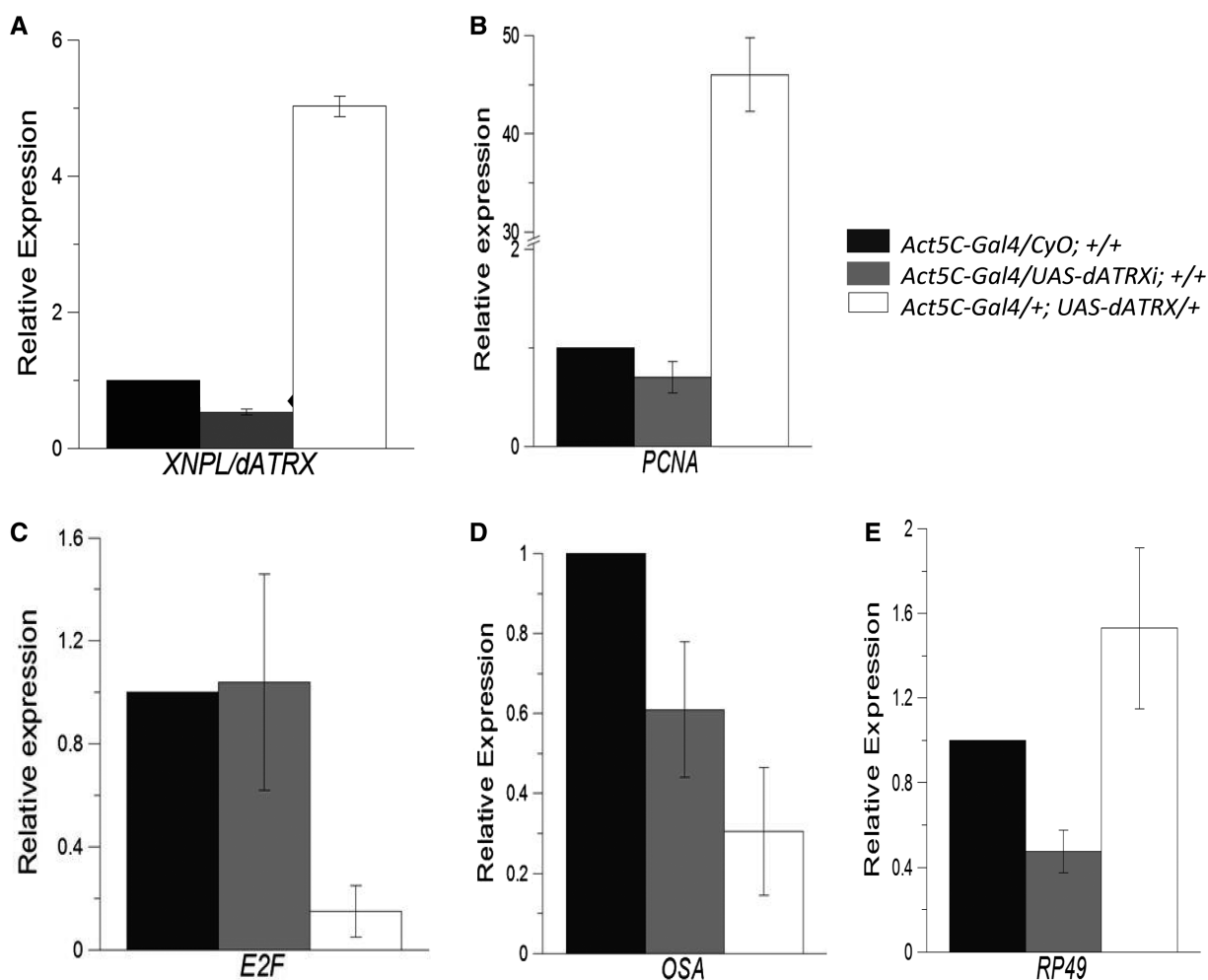


Figure 5. Effect on the mRNA levels of different genes regulated by DREF by the overexpression and depletion of *XNP/dATRX*. Total RNA from flies either overexpressing *XNP/dATRX* or an *XNP/dATRX* RNAi construct under the control of the *Act5C-Gal4* driver (ectopic expression) or without it, was purified from embryos and used in quantitative RT-PCR analysis for different gene transcripts. The genotypes are indicated for each bar color. Black bars indicate the quantification of the specific mRNA in flies with only the *Act5C-Gal4* driver; gray bars indicate flies expressing the *XNP/dATRX* RNAi and white bars correspond to flies overexpressing *XNP/dATRX*. As an internal control we used the mRNA levels of the *Dmp8* transcript, which is not affected by the *XNP/dATRX* levels. The relative expression values are adjusted to 1, which correspond to *Act5C-Gal4/CyO; +/+* organisms. (A) Quantification of mRNA levels of *XNP/dATRX*. Note that the levels of the specific mRNA is reduced when the RNAi against *XNP/dATRX* is expressed (*RNAi-dATRX/Act5C-Gal4*) and increased by the overexpression of *XNP/dATRX* in the *UAS-XNP/dATRX* (*UY3132* line). (B-E) Quantification of the mRNA levels of *E2F*; *osa*; *PCNA*; *rp49* in flies overexpressing *XNP/dATRX* or a RNAi against *XNP/dATRX*. In all cases the analyzed RNA was prepared from three independent experiments and each cDNA preparation amplified three times for posterior qRT-PCR.

fly tissues (46). Thus, it is possible that the effect on *PCNA* mRNA levels may be indirect and not a direct consequence of *XNP/dATRX* ectopic expression. To determine whether dATRX is found at the *PCNA-DRE* element in embryonic chromatin, we performed a ChIP experiment using specific dATRX antibodies. We found that dATRX is at the *PCNA-DRE* element (data not shown), suggesting that dATRX may have a direct impact on *PCNA* transcript levels; however, its interaction with DREF in this region is not clear because Re-ChIP experiments using DREF and dATRX antibodies pulled down the *PCNA-DRE* only marginally (Figure 6).

Interestingly, in *XNP/dATRX*-depleted flies, the *E2F*, *osa* and *PCNA* transcript levels do not respond significantly to the reduction of *XNP/dATRX* dosage (Figure 5). This result contrasts with the *pnr*

transcriptional deregulation that we have found as a consequence of *XNP/dATRX* depletion (Figure 2C), suggesting that *pnr* gene expression is particularly sensitive to *XNP/dATRX* levels. We also found that *rp49* transcript levels are reduced under *XNP/dATRX* depletion, suggesting that *XNP/dATRX* may be a positive factor for *rp49* expression. Taken together, these data show that *XNP/dATRX* can act as a repressor for some, but not all, DREF regulated genes, such as *pnr*, and suggest that *XNP/dATRX* exerts a promoter-specific effect on gene expression.

XNP/dATRX regulates *pnr* gene expression by binding DREF in chromatin

We have shown that both, DREF and *XNP/dATRX* are *pnr* transcriptional regulators. Thus, we investigated

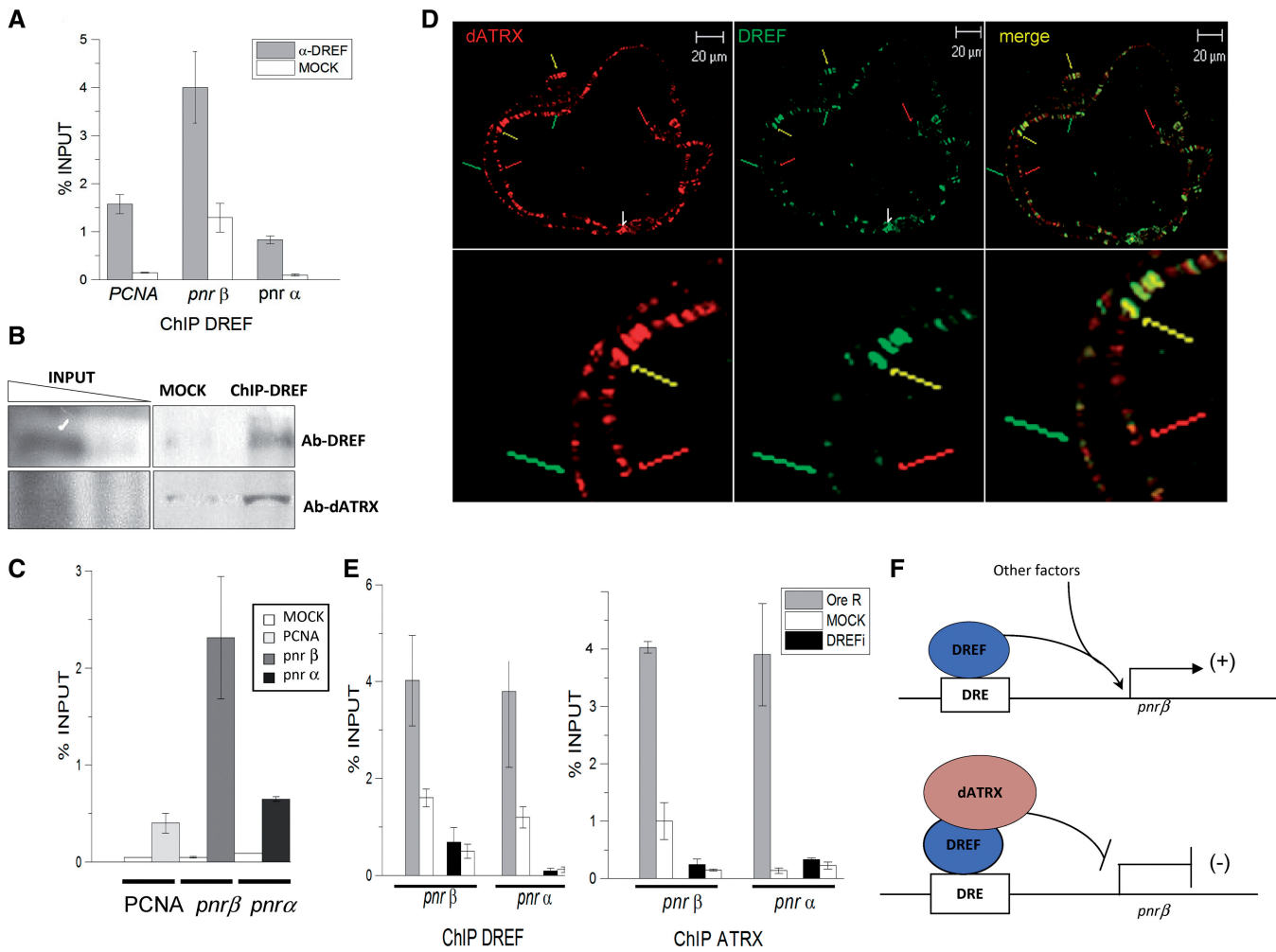


Figure 6. A XNP/dATRAX–DREF complex binds the *DRE-pnrβ* element. (A) DREF–ChIP assay from embryos overexpressing XNP/dATRAX using an *Act5C-Gal4* driver. Note that even when XNP/dATRAX overexpression reduces *pnr* mRNA levels (Figure 2), DREF is still located at the *pnr-β* and *pnr-α-DRE* elements. The ChIP experiment was performed by triplicate ($P = 0.001$). (B) ChIP–western assay. ChIP was performed with the anti-DREF antibody and the precipitated chromatin analyzed in a typical western blot to search for the presence of DREF and dATRAX. Note that both proteins can be detected. (C) Re-ChIP experiment using the DREF antibody for the first ChIP and then the XNP/dATRAX antibody for the Re-ChIP. An unrelated antibody was used as negative control for the first ChIP and then immunoprecipitated with the dATRAX antibody (white bars, MOCK). The Re-ChIP shows the average of three independent experiments ($P = 0.01$). (D) Polytene chromosomes prepared from wild-type larvae were simultaneously stained with the anti-DREF (green signal) and anti-XNP/dATRAX antibodies (red signal). Several merge bands containing both factors can be visualized (yellow arrows), regions where only XNP/dATRAX is present (red arrows) and regions where only DREF is present (green arrows). (E) ChIP experiments using the DREF and XNP/dATRAX antibodies in wild-type and DREFi knocked-down embryos. The genotype of the organisms tested is *Act5C-Gal4/+; UAS-DREFi/+*. OreR corresponds to immunoprecipitated chromatin from wild-type organism (gray bars). DREFi corresponds to immunoprecipitated chromatin from embryos expressing the anti DREF RNAi (black bars). Mock is the incubation with unrelated antibody (white bars). Antibodies used for each IP are indicated at the bottom of the figure. (F) Model on the role of DREF and XNP/dATRAX in the transcriptional regulation of *pnr-β*. Among other factors that regulate *pnr* gene expression during development, the interaction between DREF and dATRAX may participate in the establishment of an active or negative effect in gene regulation.

whether the negative effect of XNP/dATRAX on *pnr* occurred via a direct interaction with DREF or through an independent mechanism. The fact that XNP/dATRAX genetically and physically interacts with DREF supports the hypothesis that the repression of *pnr* by XNP/dATRAX occurs via a physical contact with DREF. Overexpression of XNP/dATRAX in the *pnr* expression domain enhanced the thoracic cleft phenotype, as was observed during DREF knockdown (Figures 2 and 4). In this situation, XNP/dATRAX may be sequestering DREF and preventing it from interacting with *DRE* elements, thereby inhibiting transcription.

ChIP assays of *pnr* chromatin from XNP/dATRAX overexpressing flies, show that in spite of XNP/dATRAX increased levels, DREF still occupies the *DRE-pnrβ* element, suggesting that XNP/dATRAX did not prevent the association of DREF with this *DRE* site (Figure 6A). To determine whether XNP/dATRAX interacts with DREF at the *DRE-pnrβ* element, we performed ChIP–western and Re-ChIP experiments. The DREF antibody was used in a typical ChIP assay with wild-type-embryo chromatin, and the precipitated proteins were analyzed by western blot analysis to identify DREF and XNP/dATRAX. We found that both

DREF and XNP/dATR_X can be identified in the precipitated chromatin by the specific antibodies (Figure 6B), indicating that XNP/dATR_X can be co-localized together with DREF in the chromatin. In Re-Chip assays, in which we first precipitated the chromatin with the DREF antibody and then with the XNP/dATR_X antibody and then quantified the precipitated *DRE-pnr* element region by qPCR, we found that both XNP/dATR_X and DREF are bound to this element *in vivo* (Figure 6C). Interestingly, in this Re-Chip assay, there was no XNP/dATR_X enrichment at the *DRE-PCNA* element, contrary to the XNP/dATR_X enrichment found at the *DRE-pnr-β* element, suggesting that the XNP/dATR_X transcriptional effect on *PCNA* (Figure 5), may be an indirect effect. It is important to note that Re-ChIP does not necessarily mean that XNP/dATR_X is binding through DREF. The chromatin in these experiments is fragmented to 200- to 800-bp range, and therefore it is equally possible that XNP/dATR_X is binding at neighboring DREF sites and is brought down by intervening DNA. However, the depletion of DREF reduces the occupancy of XNP/dATR_X at the *pnr DRE* sites (see ahead).

To support the idea that DREF and XNP/dATR_X can be located at the same chromosomal positions, we performed co-immunostaining experiments in polytene chromosomes to identify DREF and XNP/dATR_X location sites. XNP/dATR_X and DREF co-localize in around half of the sites (Figure 6D). In fact, we even observed co-localization of XNP/dATR_X and DREF in the so-called pericentric focus (Figure 6D, white arrow, 18). However, some regions showed no co-localization of the two factors (Figure 6D), suggesting a highly dynamic behavior of both factors in the chromatin and that XNP/dATR_X may interact with other factors besides DREF to bind specific chromatin regions (see below).

As previously mentioned, XNP/dATR_X lacks the ADD motif. Therefore, we hypothesized that XNP/dATR_X requires an interaction with other factors like DREF to bind to specific regions of the chromatin. Thus, we depleted DREF using RNAi and the *Act5C-Gal4* driver in embryos and performed ChIP assays to determine the occupancy of DREF and ATRX in the *DRE-pnr* elements. We found that when DREF was depleted, the occupancy of both dATR_X and DREF in the *DRE-pnr* elements was reduced (Figure 6E). This result indicates that dATR_X requires the presence of DREF to bind to the chromatin at the *DRE* element in the *pnr-β* promoter.

DISCUSSION

XNP/dATR_X interacts physically with DREF to regulate *pnr* gene expression

In *Drosophila*, little is known about the genes regulated by XNP/dATR_X and how it is recruited to chromatin sites. In this work, we demonstrated that XNP/dATR_X interacts with the trans-activation domain of the transcription factor DREF and that this interaction mediates XNP/dATR_X recruitment to chromatin at the *pnr* promoter. Furthermore, depletion of DREF significantly reduces

the occupancy of dATR_X in these chromatin regions and suggests that XNP/dATR_X occupancy at the *DRE* sites depends on the presence of DREF. This observation also suggests that the interaction between the two proteins at the *pnr-β* promoter results in the repression of *pnr* transcription.

Mammalian ATRX has two important domains: the helicase/ATPase SNF2 domain and the ADD domain. As mentioned before, only three proteins share the ADD domain: ATRX, DNMT3a and DNMT3L. The ADD domain of the DNMTs and hATR_X are able to bind to histone tails, thus providing a model for how these proteins bind to chromatin (5–7). XNP/dATR_X, as well as its counterpart in *Caenorhabditis elegans*, lacks the ADD domain present in the mammalian protein. Several studies have shown that XNP/dATR_X is a chromatin-associated protein (17–19), but there is no evidence of direct binding to DNA sequences or to histones. For this reason, the identification of target sequences or promoters of XNP/dATR_X has been elusive. We hypothesized that XNP/dATR_X requires an interaction with other proteins to reach its target sites/promoters and to achieve its functions in chromatin. We found that one of these factors is DREF. DREF is a transcriptional activator that was initially found to regulate the expression of several genes required for DNA replication and cell proliferation (47,48). Recently, it has been demonstrated that DREF also activates the expression of many other genes in *Drosophila* and in human cells and that putative *DRE* motifs are present at many promoter regions in the fly genome (20).

The region of DREF that interacted with the amino terminus of XNP/dATR_X corresponded to the transactivation domain of the protein (CR3 region). XNP/dATR_X_L bound to HP1 α through a motif that covers amino acids 245–249 (17). Therefore, the DREF interaction with XNP/dATR_X did not overlap with the HP1 α interaction site. Moreover, it is important to mention that this interaction only occurs between DREF and the long isoform of XNP/dATR_X because the short isoform does not contain this region. Further experiments with both isoforms of XNP/dATR_X will be necessary to address the biological importance of both isoforms. CoIP experiments also confirmed the association between the two proteins, suggesting that this interaction took place *in vivo* in the cell nucleus (Figure 1C). Additionally, immunofluorescence analysis of polytene chromosomes indicated that both proteins co-localized at several, but not all, chromatin regions, suggesting that there may be other factors in addition to DREF that mediate XNP/dATR_X recruitment to chromatin (Figure 6D). In fact, we identified several putative candidates using our two-hybrid screen (Supplementary Table S1).

It was initially reported that the longer isoform of XNP/dATR_X is preferentially located in the chromocenter and less abundantly in other chromosomal regions (17). The short isoform apparently has a different distribution and is located in many regions on polytene chromosomes. In addition, it has recently been reported that XNP/dATR_X is required for HP1 α deposition in pericentric β -heterochromatin of the X chromosome (19). However,

a second recent report challenged those results (18) because the authors found that XNP/dATR_X is preferentially found in euchromatic regions. Furthermore, XNP/dATR_X seems to co-localize with the H3.3 histone variant (which is a histone enriched in euchromatic regions) in polytene chromosomes (18). Moreover, it has recently been shown that hATR_X interacts with the H3.3 histone in embryonic stem cell telomeres, and together with DAXX, it is important for the incorporation of H3.3 at the telomeres (49,50). Our experiments provide further support for the euchromatic localization of XNP/dATR_X. Thus, it could be possible that XNP/dATR_X represses the expression of genes located in euchromatin. However, a positive impact on gene expression cannot be discarded, and it would be interesting if XNP/dATR_X also interacts with the H3.3 histone at the *DRE* sites. Nevertheless, our results in *Drosophila* suggest that DREF is one of the factors that interacts with XNP/dATR_X to regulate gene expression and that it localizes to euchromatic regions.

The DREF-XNP/dATR_X interaction has a functional relevance in the regulation of *pnr*

A previous report from another group (39) is in accordance with our own data which demonstrate that the ectopic expression of dATR_X in the *pnr* expression domain enhances *pnr* loss-of-function phenotypes, suggesting that XNP/dATR_X may act as a *pnr* negative transcriptional regulator. Specifically, reduction of the two XNP/dATR_X isoforms with RNAi suppressed mutant phenotypes caused by different *pnr* alleles. This repressive role of XNP/dATR_X in *pnr* expression is also supported by the fact that ectopic expression of XNP/dATR_X in the embryo causes a reduction in the mRNA levels of the *pnr* isoforms and that the XNP/dATR_X depletion by RNAi increases the *pnr* transcripts level. However, the DREF knockdown in the *pnr-Gal4* expression domain enhances thoracic cleft defects caused by a reduction in *pnr-β* levels, and these defects are suppressed by the simultaneous XNP/dATR_X knockdown. Thus, these factors interact genetically, and they have antagonistic roles in *pnr* regulation. Moreover, we also found that DREF can bind to the *DRE* element that is located in the *pnr-β* control region. Based on these results, we suggest that DREF activates *pnr-β* gene expression because its depletion enhances the loss of function phenotypes found in *pnr-Gal4* flies. XNP/dATR_X is targeted to the *DRE* element at the *pnr-β* promoter through its direct interaction with DREF and this interaction negatively regulates *pnr* (Figure 6F). However, this model opens the question of why XNP/dATR_X depletion restores the *pnr-β* transcript levels in DREF-depleted thoraxes (Figure 4B) given the assumption that lowering DREF dosages would reduce consequently the amount of XNP/dATR_X at the *DRE* regulatory sequence. A possible explanation is that the *DRE* element controlling *pnr-β* is very sensitive to changes in the DREF-XNP/dATR_X ratio (Figure 4B). We also think that other factors binding to the regulatory regions of *pnr*, may influence DREF and XNP/dATR_X location at the *DRE* and moreover DREF

and/or XNP/dATR_X, could be regulating the expression of upstream genes, therefore indirectly influencing *pnr* gene expression.

Interestingly, it has been reported that DREF associates with TRF2 and can recognize the *PCNA* core promoter (24). Therefore, it is possible that DREF may act as a co-activator that is necessary for the expression of genes required for cell proliferation, which are also regulated by TRF2. The *DRE* element, we identified at the *pnr-β* promoter overlaps with an identified sequence that is occupied by TRF2 (51). These data and our molecular and genetic assays suggest that DREF likely activates *pnr-β* expression through its interaction with TRF2. Therefore, it is possible that the mechanism of *pnr* gene inactivation by XNP/dATR_X occurs via the interference of the interaction between DREF and TRF2.

XNP/dATR_X has a possible dual role in transcriptional regulation

In this work, we present data indicating that in addition to *pnr*, the ectopic expression of XNP/dATR_X also represses *E2F* and *osa* transcription. However, this was not true for other genes, such as *PCNA*, which requires DREF for its expression. In fact, in some examples, we found an increase in the level of specific mRNAs when XNP/dATR_X was overexpressed. Contrary to the results observed with *pnr*, XNP/dATR_X depletion did not affect the mRNA levels of *PCNA*, *E2F* and *osa*; however, *rp49* mRNA levels were decreased in this condition (Figure 5). These results suggest that there is a selective effect of dATR_X in gene regulation and that XNP/dATR_X may be required for both gene activation and gene repression. A recent microarray analysis of a mouse model that lacks ATR_X in the forebrain identified several overexpressed genes, as well as some genes that were downregulated. The functions of both sets of genes were diverse, but these results suggest that in mice, ATR_X is required for gene activation and repression (15,16). In addition, the forebrain transcript analysis in the ATR_X mutant mice indicated that ATR_X is not a global regulator of gene expression, as it seems that ATR_X acts through other factors like MeCP2, cohesin and CTCF in the regulation of imprinted genes in the postnatal mouse brain (15,16). A global analysis of changes in transcript levels caused by the XNP/dATR_X overexpression and depletion in *Drosophila* will be required to achieve a comprehensive view of the influence of XNP/dATR_X on gene regulation.

Pnr belongs to the GATA family of transcription factors, which has been conserved over the course of evolution. For example, there are six GATA family members in mammals. Therefore, it is important to assess whether ATR_X regulates some of these factors in vertebrates. This is of particular interest because some patients afflicted with ATR_X syndrome suffer from α -thalassemia, and it is known that the GATA 1 and GATA 2 factors are involved in the regulation of the globin genes (52). Moreover, it has recently been reported that human ATR_X is recruited to G-rich tandem repeat sequences that have the potential to form G-quadruples, and these

may have a negative impact on genes in the vicinity, such as the α -globin genes (10). It will be interesting in future studies to determine if XNP/dATRAX is also recruited to G-rich sequences.

In conclusion, our results show that DREF is required for the proper expression of *pnr* and that XNP/dATRAX binds to DREF at the *DRE* sites, resulting in the repression of *pnr* gene expression. This establishes a connection between a chromatin remodeling factor (XNP/dATRAX) and a transcriptional activator (DREF) in the regulation of an important gene (*pnr*). In addition, these studies in *Drosophila* open new avenues for the study of human pathologies generated by mutations in hATRAX. For example, human cells code for a DREF homolog, and as hATRAX interacts with multiple factors to achieve its functions, it will be important to determine if hATRAX functionally interacts with DREF in humans. These types of studies will allow us to assess whether genes regulated by DREF are affected in cells derived from patients afflicted with the ATRX syndrome.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table S1, Supplementary Figures S1–S7.

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