Trithorax interacts with type 1 serine/threonine protein phosphatase in *Drosophila*

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The catalytic subunit of type 1 serine/threonine protein phosphatase (PP1c) was shown to bind trithorax (TRX) in the yeast two-hybrid system. Interaction between PP1c and TRX was confirmed *in vivo* by co-immunoprecipitation from *Drosophila* extracts. An amino-terminal fragment of TRX, containing a putative PP1c-binding motif, was shown to be sufficient for binding to PP1c by *in vitro* glutathione S-transferase pull-down assays using recombinant protein and fly extracts expressing epitope tagged PP1c. Disruption of the PP1c-binding motif abolished binding, indicating that this motif is necessary for interaction with PP1. On polytene chromosomes, PP1c is found at many discrete bands, which are widely distributed along the chromosomes. Many of the sites that stain strongly for PP1c correspond to sites of TRX, consistent with a physical association of PP1c with chromatin-bound TRX. Homeotic transformations of haltere to wing in flies mutant for *trx* are dominantly suppressed by PP1c mutants, indicating that PP1c not only binds TRX, but is a physiologically relevant regulator of TRX function *in vivo*.

EMBO reports 4, 59–63 (2003)

DOI: 10.1038/embor712

INTRODUCTION

In *Drosophila*, the trithorax group (TrxG) of epigenetic regulators is required for the maintenance of normal expression of homeotic genes of the Bithorax (BX-C) and Antennapedia (ANT-C) complexes, as well as other genes, after their initial activation. By contrast, the Polycomb group (PcG) is required for the maintenance of genes in an appropriate inactive state. The consequence of these two activities is 'transcriptional memory', by which a cell 'remembers' what type of cell it is meant to be, after its initial specification (reviewed by Farkas *et al.*, 2000; Francis & Kingston, 2001; Simon & Tamkun, 2002). Disruption of this transcriptional memory can cause a remarkable respecification of segmental identity, leading to the transformation of one body segment into a different one. Mutations in *trithorax* (*trx*), the archetypal member of the TrxG family, for instance, result in phenotypes resembling those of loss-of-function mutations in multiple homeotic genes (Ingham & Whittle, 1980; Breen, 1999). The TrxG/PcG maintenance system is conserved

between flies and mammals, implying an ancient origin and an essential function. The human homologue of trithorax (TRX), ALL-1/HRX/MLL, is affected by over 30 different chromosomal fusions that lead to leukaemia (van Lohuizen, 1999), indicating that transcriptional control by TrxG genes is also crucial for normal development in humans.

Recently, much progress has been made in identifying TrxG proteins and their mode of action. TRX itself is a component of a histone acetylation complex (trithorax acetylation complex; TAC1), which acetylates core histones in nucleosomes and contains TRX, *Drosophila* CREB-binding protein (dCBP) and SET binding factor 1 (Sbf1) (Petruk *et al.*, 2001). Several other TrxG proteins have been directly linked to transcriptional activation, and several large protein complexes containing one or more TrxG proteins have been identified. For example, TRX also interacts with ASH1 (Rozovskaia *et al.*, 1999), a component of a distinct TrxG complex (Papoulas *et al.*, 1998) which might include dCBP (Bantignies *et al.*, 2000). TRX also binds SNR1, a component of the Brahma (BRM, *Drosophila* SWI/SNF) chromatin remodelling complex (Rozenblatt-Rosen *et al.*, 1998). This indicates that different TRX complexes might cooperate in changing chromatin structure by a combination of different mechanisms of nucleosome modification. However, little is known about the regulation of these complexes, which must be differentially recruited and used in different cells; this specification must then be 'remembered' in different lineages through DNA replication, cell division and differentiation. We show here that TRX both interacts genetically with and binds directly to the catalytic subunit of type 1 serine/threonine protein phosphatase (PP1c), implicating reversible phosphorylation in the control of TAC1 and transcriptional memory.

RESULTS

Isolation of TRX from a screen for PP1c-binding proteins

A yeast two-hybrid interaction screen was used to identify potential PP1c-binding proteins by using the PP1 α 87B catalytic subunit as 'bait'. Twenty-five independent complementary DNAs from 16 independent genes, which interacted with PP1 α 87B but not with control baits, were isolated from a *Drosophila* third-instar larval library (Alphey *et al.*, 1997). One of these cDNAs corresponded to *trx*. A subsequent screen for PP1 β 9C-binding proteins (Bennett *et al.*, 1999) identified 236 positive clones, of which 8 were derived from *trx*. Further two-hybrid analysis of a representative *trx* clone showed that it was capable of binding all four *Drosophila* PP1c isoforms (PP1 β 9C, PP1 α 87B, PP1 α 13C and PP1 α 96A; data not shown).

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Received 28 August 2002; revised 10 October 2002; accepted 6 November 2002

Fig. 1 | TRX binds PP1α87B *in vitro* and *in vivo*. (A) Schematic representation of the domain structure of the longer TRX isoform. The shorter TRX isoform, which differs only at the N terminus, starts at residue 369. The PP1-binding domain was identified by the position of overlapping two-hybrid positives: the smallest two-hybrid clone is shown along with an overlapping clone that limits the region required for binding to PP1 in the two-hybrid system. The PHD fingers and the C-terminal SET domain are also indicated. Other conserved regions are not shown for reasons of clarity. (B) The shortest two-hybrid interactor also binds to PP1α87B in a GST pull-down assay; binding requires Phe 435 of TRX. Extracts from wild-type or *arm-GAL4 UAS HA-PP1α87B* flies were incubated with lysates from untransformed *E. coli* and *E. coli* expressing GST-TRX^[302-479], TRX^{[302-479]FA} or GST alone. TRX complexes were purified on glutathione-Sepharose and immunoblotted with anti-HA to test for co-precipitation of PP1α87B. Loading was tested by immunoblotting with anti-GST antibodies. (C) TRX co-precipitates with PP1α87B from *Drosophila* embryonic nuclear extracts. Nuclear extracts from wild-type and *arm-GAL4 UAS HA-PP1α87B* embryos were immunoprecipitated (IP) with anti-TRX antibodies (+) or with non-immune serum (-) followed by immunoblotting with anti-HA antibodies to test for co-immunoprecipitation.

trx is predicted to encode two protein isoforms (of 3,358 and 3,726 amino acids) that differ only at the amino terminus (Breen & Harte, 1991; Mazo et al., 1990). We sequenced the ends of all the *trx* two-hybrid positives; all shared the region between residues 379 and 479 of the longer isoform (between residues 11 and 111 of the smaller isoform). This suggests that residues 379–479 contain all the sequences necessary for binding to PP1c in the two-hybrid system. This region contains a consensus PP1c-binding motif (KTVTF, residues 431–435) (Egloff et al., 1997), which is found in both protein variants (Fig. 1A).

PP1 binds TRX *in vitro* and *in vivo*

The interaction of PP1α87B with TRX was confirmed *in vitro* through glutathione *S*-transferase (GST) pull-down assays. For these binding experiments the shortest interacting fragment of TRX was expressed as a GST fusion protein in *Escherichia coli*. GST-TRX^[302-479] fusion protein interacted efficiently with haemagglutinin (HA)-tagged PP1α87B from crude *Drosophila* extracts (Fig. 1B), whereas cells without the construct or cells expressing GST alone did not bind (Fig. 1B), demonstrating that residues 302–479 of TRX are sufficient for binding to PP1. To examine the importance of the putative PP1c-binding motif for interaction with PP1, a mutant form of TRX^[302-479] in which Phe 435 was replaced with alanine (TRX^{[302-479]FA}), was tested for its ability to bind PP1α87B in our pull-down assay. TRX^{[302-479]FA} failed to bind PP1, indicating that Phe 435 is crucial for interaction with PP1. To examine whether PP1 and TRX interact *in vivo* we precipitated endogenous TRX from embryonic nuclear extracts expressing HA-tagged PP1α87B and examined precipitates by blotting with anti-HA antibodies. We found that HA-PP1α87B clearly precipitated with TRX (Fig. 1C), indicating that PP1 is indeed found complexed to TRX *in vivo*.

PP1 and TRX co-localize on polytene chromosomes

To explore further the association between PP1 and TRX *in vivo*, we examined the distribution of the two proteins on polytene chromosomes of the larval salivary gland. Immunostaining experiments have shown that TRX associates with multiple sites on polytene chromosomes. The exact number of loci varies depending on the antibody used to detect TRX (Kuzin et al., 1994; Chinwalla et al., 1995). Using the antibody described by Chinwalla et al. (1995) (a gift from P. Harte), we observed approximately 60 TRX sites (Fig. 2), as reported previously. A large proportion of PP1 is found in the nucleus and is reported to associate with chromatin (Bollen & Beullens, 2002); however, the distribution of PP1 on chromosomes is not known. Anti-HA staining of chromosomes from flies expressing HA-PP1α87B showed that HA-PP1α87B is localized at many discrete sites, which are widely dispersed along the chromosomes, although the intensity of signals observed at different sites varied significantly (Fig. 2). Double staining with anti-HA and anti-TRX antibodies showed that virtually all sites staining for TRX also stain strongly for PP1, implying that PP1 is a component of the majority of TRX complexes (Fig. 2). Similar results were obtained with different TRX antibodies (a gift from A. Mazo, data not shown).

PP1 antagonizes *trx* function in *Drosophila*

Flies mutant for *trx* show transformations of segmental identity, in which posterior identity is transformed to a more anterior

Fig. 2 | Endogenous TRX and epitope-tagged PP1 α 87B proteins co-localize on salivary-gland polytene chromosomes. Merging of (A) the green signal representing TRX with (B) the red signal representing PP1 α 87B identifies sites where these proteins co-localize (C). The relative signals of TRX and PP1 α 87B vary between the sites, but most TRX loci co-localize with strong PP1 α 87B staining. The inset is an enlarged view of one chromosomal region (indicated by arrows) where this is clearly visible.

identity, similar to the transformations caused by loss-of-function mutations in homeotic genes (Breen, 1999). We investigated the functional interaction between TRX and PP1 by examining the effect of PP1 levels on homeotic transformations exhibited by *trx* mutants. Heteroallelic combinations of amorphic *trx* mutations *trx*^{E2} or *trx*^{B11} with *trx*¹, a viable hypomorphic mutation that produces reduced levels of TRX (Chinwalla et al., 1995, a gift from P. Harte), result in the partial transformation of wing to haltere structures and of pigmented to non-pigmented abdominal segments. Although the penetrance of abdominal transformations in these transallelic combinations is variable, the penetrance of wing to haltere transformations is reproducible and can be classified into three categories: no transformation, weak transformation and strong transformation (see Fig. 3 and Table 1). About 50–55% of *trx*¹/*trx*^{E2} and *trx*¹/*trx*^{B11} flies show strong transformation, 35% show weak transformation and 10–15% show no transformation (Fig. 3, Table 1).

To investigate the role of PP1c we examined the frequency of transformations in *trx*¹/*trx*^{E2} and *trx*¹/*trx*^{B11} flies that were also heterozygous for mutations in PP1 α 87B. PP1 α 87B is the major PP1c isoform in *Drosophila*, contributing about 80% of the total PP1 activity (Dombrádi et al., 1990). For these experiments we used

Fig. 3 | PP1 antagonizes *trithorax* function. (A) The range of homeotic transformation phenotypes in *trx*¹/*trx*^{B11} and *trx*¹/*trx*^{E2} flies. Part of the third thoracic segments and abdominal segments are shown; anterior is to the right, dorsal is at the top. The haltere, a balancing organ on the third thoracic segment, is indicated by arrowheads. a, b, Wild-type halteres with no visible transformation. c–e, Weak transformations of haltere to wing, with minor changes in haltere size and shape, and the presence of extra bristles. Abdominal transformations are also visible (arrowed) in these flies. f–h, Strong transformations of haltere to wing, consisting of more severe defects, namely size and shape changes resulting in wing-like transformations and the presence of necrotic tissue. (B, C) Transformations in *trx*¹ flies are dominantly suppressed by loss of one copy of PP1 α 87B. Overexpression of PP1 α 87B has no effect on *trx*-dependent transformations. Black columns, strong transformation; blue columns, weak transformation; yellow columns, no transformation. See Table 1 for data.

two different mutant alleles of PP1 α 87B: PP1 α 87B^{87Bg-6}, a protein-null mutation, and PP1 α 87B¹, a point mutation with reduced PP1 activity. Both PP1 α 87B^{87Bg-6} and PP1 α 87B¹ dominantly suppressed *trx*-dependent homeotic transformations: there were more than twice the number of *trx* flies with no visible transformations when one copy of PP1 α 87B was mutated (Fig. 3, Table 1). We also examined the effect of PP1 α 87B overexpression on the frequency of homeotic transformations by using the Gal4–UAS system (Parker et al., 2001). Modest overexpression of UAS PP1 α 87B by using *arm-GAL4* in either a wild-type or *trx* mutant background had no phenotypic effect, indicating that PP1 levels are not limiting for TRX:PP1 function.

Table 1 | Data used to generate the graph in Fig. 3

Genotypes of the flies scored	Strong haltere transformations (%)	Weak haltere transformations (%)	No haltere transformations (%)	Total flies scored
1. <i>trx</i> ¹ / <i>trx</i> ^{B11}	49	34	16	100
2. <i>trx</i> ¹ PP1 α 87B ^{87Bg-6} / <i>trx</i> ^{B11}	28	29	43	148
3. <i>trx</i> ¹ PP1 α 87B ¹ / <i>trx</i> ^{B11}	27	24	49	130
4. <i>trx</i> ¹ / <i>arm-Gal4</i> UAS-HA–PP1 α 87B <i>trx</i> ^{B11}	37	50	13	100
5. <i>trx</i> ¹ / <i>trx</i> ^{E2}	57	34	9	105
6. <i>trx</i> ¹ PP1 α 87B ^{87Bg-6} / <i>trx</i> ^{E2}	36	42	22	92
7. <i>trx</i> ¹ PP1 α 87B ¹ / <i>trx</i> ^{E2}	31	39	30	94
8. <i>trx</i> ¹ / <i>arm-Gal4</i> UAS-HA–PP1 α 87B <i>trx</i> ^{E2}	55	37	7	110

DISCUSSION

Our studies showing genetic interactions between PP1 and TRX implicate reversible phosphorylation, regulated by PP1 and one or more unknown kinases, in the control of TRX function and transcriptional memory. We have shown that PP1 binds TRX *in vitro* and *in vivo* and that the two proteins co-localize on polytene chromosomes. We therefore propose that PP1 is part of one or more TRX chromatin-associated complexes, in which PP1 exerts an effect on TRX-dependent transcriptional control. One such complex might be TAC1, which is reported to contain about 90% of total TRX protein (Petruk et al., 2001). However, PP1 might also be part of other complexes, containing dCBP or SNF1 for instance. PP1 is found at other discrete sites that do not contain TRX, indicating that there are other PP1c-binding proteins that can recruit PP1 to different chromosomal regions.

The exact role of TRX-bound PP1 is not yet known. TRX, which contains four serine-rich domains, could itself be a direct target of PP1. However, it is not known whether TRX is a phosphoprotein, and because the exact role of TRX is unclear it is difficult to predict what the effect of phosphorylation might be. It is possible that TRX is an anchor for other chromatin-associated proteins and that it targets PP1 to some other member of this complex, such as dCBP. Mammalian CBP has been found to be phosphorylated in both quiescent and dividing cells (Yaciuk & Moran, 1991), but surprisingly few studies have addressed the role of phosphorylation in controlling CBP activity. Phosphorylation of the carboxy terminus of CBP has been reported to repress its histone acetylation activity (Ait-Si-Ali et al., 1998; Impey et al., 2002), whereas phosphorylation at the N terminus by protein kinase C (at Ser 89) has the opposite effect (Yuan & Gambée, 2000). Further studies will need to examine the phosphorylation state of dCBP in TRX complexes, but one possibility is that PP1 dephosphorylates CBP to allow patterns of transcription to be reprogrammed.

A role for phosphorylation in the control of TAC1 activity is implied by the presence of Sbf1 in the TAC1 complex. Sbf1 belongs to a subset of myotubularin-related dual-specificity phosphatases that are catalytically inactive and have been proposed to act as 'anti-phosphatases' by binding to and protecting substrates from dephosphorylation (Laporte et al., 2001). Myotubularin phosphatases seem primarily to dephosphorylate phosphatidylinositol 3-OH mono-phosphatase but can also dephosphorylate phosphoserine and phosphothreonine residues (Laporte et al., 2001), indicating that Sbf1 might protect against the effects of PP1. Identification of targets of Sbf1 might therefore help to identify relevant PP1 substrates.

METHODS

Yeast two-hybrid screen. The two-hybrid screens for PP1 α 87B and PP1 β 9C-binding proteins were performed as described (Alphey et al., 1997; Bennett et al., 1999; Bennett & Alphey, 2002).

GST pull-down assays. The shortest fragment of *trx* (nucleotides 1745–2275) that bound PP1 in the yeast two-hybrid system was subcloned from pACT as a *Bgl*II fragment into the *Bam*HI site of pGEX-5X-1 in frame with an N-terminal GST tag to generate TRX^[302–479]. TRX^[302–479]FA was made by site-directed mutagenesis based on the polymerase chain reaction. Bacterial cell lysates expressing GST-tagged TRX^[302–479] or TRX^[302–479]FA were incubated with either *arm-GAL4 UAS-HA-PP1 α 87B* flies or wild-type (*Oregon R*) flies and GST-labelled protein was precipitated with glutathione-Sepharose 4B resin (Amersham Biosciences). Precipitates were examined by SDS-PAGE followed by immunoblotting with anti-HA 12CA5 (Roche Diagnostics) and anti-GST (a gift from I. Farkas) antibodies.

Immunoprecipitation from *Drosophila* extracts. Immunoprecipitation from 2–18-h-old *arm-GAL4 UAS-HA-PP1 α 87B* and *Oregon R* *Drosophila* embryonic nuclear extracts was performed as described by Rozenblatt-Rosen et al. (1998), with minor modifications. Nuclear extracts were prepared from ~200 μ l of dechorionated embryos of each genotype and incubated for 2 h with anti-TRX antibodies (Chinwalla et al., 1995) diluted 1:40, followed by protein G bound to GammaBind Plus Sepharose (Amersham Biosciences) for 2 h. The presence of HA-PP1 α 87B in precipitates was tested by immunoblotting with anti-HA 12CA5 antibodies (Roche Diagnostics).

Immunocytochemistry. The salivary glands of *arm-GAL4 UAS-HA-PP1 α 87B* third-instar larvae raised at 25 °C were dissected as described by Kennison (2000). Immunostaining of polytene chromosomes was performed as described by Zink & Paro (1989), with minor modifications. Double labelling of polytene chromosomes was performed with rabbit anti-TRX antibody (Chinwalla et al., 1995) at 1:50 dilution and anti-HA 12CA5 antibody (Roche Diagnostics) diluted 1:100. Identical results were obtained with anti-TRX antibody (a gift from A. Mazo) at 1:20 dilution. Primary antibodies were detected with preadsorbed Cy3-conjugated anti-rabbit and fluorescein-isothiocyanate-conjugated anti-mouse antibodies (Jackson ImmunoResearch) at 1:500 and 1:200 dilution, respectively.

Fly strains and genetic crosses. Flies were raised at 25 °C on standard agar–cornmeal–molasses medium. Mutant alleles of *PP1 α 87B* and *trithorax* are described in FlyBase (<http://www.flybase.org>). The line *arm-GAL4 UAS HA-PP1 α 87B* is essentially identical to *arm-GAL4 UAS HA-PP1 β 9C* (Parker et al., 2001) but with the *PP1 α 87B* coding region in place of that of *PP1 β 9C*. *trx*¹ *PP1 α 87B^{87Bg-6}/TM6B* and *trx*¹ *PP1 α 87B¹/TM6B* lines were generated by recombination and crossed to *trx*^{B11}/TM6B and *trx*^{E2}/TM6B flies. Penetrance and expressivity of haltere transformation phenotypes were quantified in the progeny: *trx*¹ *PP1 α 87B^{87Bg-6}/trx^{B11}*, *trx*¹ *PP1 α 87B¹/trx^{B11}*, *trx*¹ *PP1 α 87B^{87Bg-6}/trx^{E2}* and *trx*¹ *PP1 α 87B¹/trx^{E2}* lines compared with *trx*¹/*trx*^{B11} and *trx*¹/*trx*^{E2} controls. *trx*^{B11} *UAS HA-PP1 α 87B/TM6B* and *trx*^{E2} *UAS HA-PP1 α 87B* lines were generated by recombination and crossed to *arm-GAL4/arm-GAL4*; *trx*¹/TM6B flies. The penetrance and expressivity of haltere transformation phenotypes were quantified for *arm-GAL4/+*; *trx*^{B11} *UAS HA-PP1 α 87B/trx¹* and *arm-GAL4/+*; *trx*^{E2} *UAS HA-PP1 α 87B/trx¹* flies compared with *trx*^{B11}/*trx*¹ and *trx*^{E2}/*trx*¹ controls.

ACKNOWLEDGEMENTS

We thank T. Breen, A. Shearn and the Bloomington *Drosophila* Stock Center for fly strains, and T. Breen and H. White-Cooper for advice and technical help. This work was supported by grant G117/255 from the UK MRC and grant 43/G11827 from the UK BBSRC, with additional support from the Royal Society. L.A. is an MRC Senior Research Fellow; A.R. is an Oxford University Satcherd Scholar.

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