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Reptin and Pontin function antagonistically with PcG and TrxG complexes to mediate Hox gene control

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Pontin (Pont) and Reptin (Rept) are paralogous ATPases that are evolutionarily conserved from yeast to human. They are recruited in multiprotein complexes that function in various aspects of DNA metabolism. They are essential for viability and have antagonistic roles in tissue growth, cell signalling and regulation of the tumour metastasis suppressor gene, *KAI1*, indicating that the balance of Pont and Rept regulates epigenetic programmes critical for development and cancer progression. Here, we describe Pont and Rept as antagonistic mediators of *Drosophila* Hox gene transcription, functioning with Polycomb group (PcG) and Trithorax group proteins to maintain correct patterns of expression. We show that Rept is a component of the PRC1 PcG complex, whereas Pont purifies with the Brahma complex. Furthermore, the enzymatic functions of Rept and Pont are indispensable for maintaining Hox gene expression states, highlighting the importance of these two antagonistic factors in transcriptional output.

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INTRODUCTION

Pontin (Pont) and Reptin (Rept) are paralogous ATP-dependent DNA helicases recruited as part of several chromatin-modifying multiprotein complexes thought to act in epigenetic mechanisms that control various aspects of DNA metabolism, including transcription, replication and repair. They are involved in the regulation of cell-cycle progression, in growth control by Myc, in Wnt–β-catenin signalling and in neoplastic transformations (reviewed by Gallant, 2007). Furthermore, mutation of either gene confers a lethal phenotype in all species examined so far, indicating non-redundant essential functions during development.

At the mechanistic level, the roles of Pont and Rept are poorly understood. They have essential roles in the assembly of the Ino80 chromatin remodelling complex (Jónsson *et al*, 2004), yet are found to have opposite activities in various mechanisms of transcription control: heart growth in zebrafish (Rottbauer *et al*, 2002), Wnt signalling (Bauer *et al*, 2000) and KAI1 tumour metastasis suppressor gene expression (Kim *et al*, 2005), in which Pont acts as a transcriptional co-activator and Rept as a co-repressor (reviewed by Gallant, 2007).

In most cases, Pont and Rept are found together in the same multiprotein complex. Thus, the functional antagonism of either Pont or Rept occurs within the same complex, or it is achieved through distinct antagonistically acting complexes. Although the complexes containing both Pont and Rept have been well studied, there is no evidence, so far, to show that these complexes can have both active and repressive roles in the same target genes. Contrarily, in the case of KAI1 expression, Pont collaborates with Tip60 for activation and Rept with β-catenin for repression (Kim *et al*, 2005). Thus, it is likely that whenever Pont and Rept have opposing activities on gene expression, that these activities are through the action of distinct protein complexes.

Here, we focus on a well-studied epigenetic mechanism of transcriptional control: the maintenance of Hox gene expression by opposing actions of Polycomb group (PcG) and Trithorax group (TrxG) complexes (Ringrose & Paro, 2004). In this respect, *rept* has recently been described as genetically interacting with *PcG* genes and components of the Tip60 complex (Qi *et al*, 2006), in which the authors proposed a role for the Tip60 complex in gene silencing. However, given the opposing actions of Pont and Rept in transcription, Rept having been identified in PRC1 (Saurin *et al*, 2001) and the role of Tip60 in transcriptional activation, the proposed role of Tip60 does not address the role of the opposing partner of Rept—Pont—in the complex (Kusch *et al*, 2004) and the epigenetic regulation of Hox genes by PcG and TrxG proteins. Here, we show that Rept forms a component of the embryonic PRC1 PcG complex, whereas Pont co-purifies with the Brahma complex (Brm-C) TrxG complex, and also show indispensable roles for the enzymatic activities of Pont and Rept in maintaining Hox gene expression states.

RESULTS AND DISCUSSION

Pont and Rept antagonistically contribute to Hox silencing

Rept has recently been reported to suppress variegation (Qi *et al*, 2006). We found that Pont behaves as an enhancer (supplementary Fig S1 online). The antagonistic effects of Pont and Rept on pericentric heterochromatin assembly provide a clear clue as to their roles in transcription as Su(Var) (Rept) is implicated in chromatin condensation, whereas E(Var) (Pont) acts to relax and open up chromatin (reviewed by Elgin, 1996).

To assess the role of Pont and Rept in the maintenance of Hox gene transcription by PcG and TrxG proteins, we first tested for genetic interactions with *PcG* genes. Males heterozygous for *pont*, *rept* or *pont* and *rept* do not show a *PcG* phenotype of extra sex combs on T2 and T3 legs, whereas heterozygosity for *pont* or *rept* significantly reduces or enhances the sex comb phenotype of Psc^{1} (Psc for Posterior sex combs) or Pc^{XT109} male heterozygotes (supplementary Table S1 online). These effects are specific as providing an extra copy of *rept* or *pont* in these backgrounds restores the *PcG* mutant phenotype. To obtain direct evidence for a role in Hox gene control, we analysed Scr (Sex combs reduced) and Ubx (Ultrabithorax) patterns in imaginal discs. *Scr* is expressed in T1 but not in the most-posterior wild-type leg discs, a pattern unaffected by heterozygosity for *pont* or *rept* (or *pont* and *rept*). Heterozygosity for *Psc* leads to *Scr* derepression in a few cells of T2 and T3 discs, and eliminating one copy of *pont* or *rept* in this context significantly reduces or, conversely, increases ectopic Scr accumulation (Fig 1A). Ubx is not detected in the epithelia of wild-type and *pont* or *rept* heterozygous wing discs, whereas it accumulates in some cells of *Psc* heterozygous discs. The additional mutation of one copy of *pont* or *rept* abolishes or exacerbates, respectively, Ubx ectopic expression (supplementary Fig S2 online). It is noteworthy that similar levels of the Hox protein are detected in *Psc* and triple *pont*, *rept*, *Psc* heterozygous discs. Thus, Pont and Rept contribute in an antagonistic manner to PcG-mediated repression of *Scr* and *Ubx*. Consistent with the opposite genetic interactions observed previously, Pont and Rept therefore behave as dominant suppressor and enhancer of PcG mutations, respectively.

As the sex comb phenotype is initially determined by the PcG mutation, these data do not distinguish between PcG-specific effects and global transcriptional effects. Thus, we studied the role of Pont and Rept in Hox silencing mediated by chromosomal integrity. Homologous pairing of regulatory elements in the *Scr* gene is crucial for silencing, and chromosomal aberrations that disrupt this pairing lead to derepression of *Scr* in the second and third thoracic segments (Southworth & Kennison, 2002). Thus, we tested for genetic interactions of *pont* and *rept* with the gain-of-function allele Scr^{Msc} (Southworth & Kennison, 2002). The number of ectopic sex comb teeth on T2 and T3 legs observed in male flies heterozygous for Scr^{Msc} significantly decreases or increases when one copy of *pont* or *rept*, respectively, is removed. These effects are countered by *pont* or *rept* genomic transgenes, and the Scr^{Msc} phenotype remains unchanged on simultaneous dosage reduction of both genes (supplementary Table S2 online). These results indicate that Pont acts as a co-activator and Rept as a co-repressor in the maintenance of Hox gene transcription, as do the TrxG and PcG proteins.

Pont and Rept antagonistically contribute to Hox expression

To investigate the possible roles of Pont and Rept in TrxG-dependent maintenance of Hox gene expression, we performed genetic interaction assays using the brm^{2} allele, which in heterozygosis results in a held-out wing phenotype at low frequency (Vazquez *et al*, 1999). Heterozygous *pont*, *rept* or *pont* and *rept* mutants do not induce such a phenotype alone, whereas the mutation of one copy of *pont* or *rept* significantly enhances or reduces the held-out wing phenotype of $brm^{2}/+$ flies, and a balanced reduction of *pont* and *rept* in triple heterozygotes results in a phenotype of penetrance similar to that in $brm^{2}/+$ flies (Fig 1Biii). Thus, *pont* and *rept* behave as dominant enhancer and suppressor, respectively, of the brm^{2} mutation.

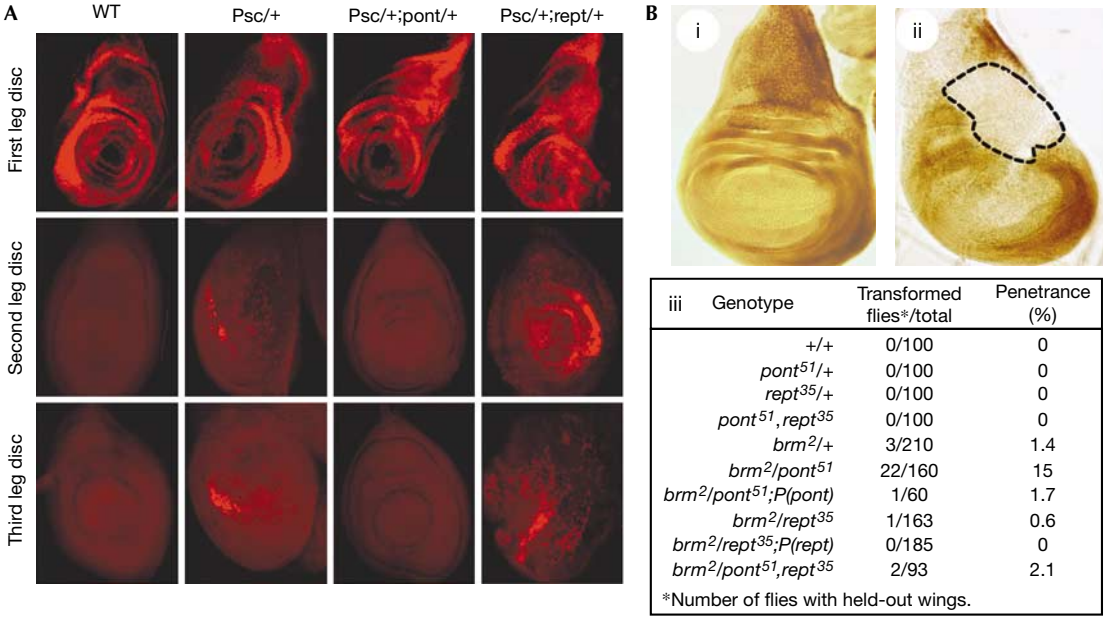
As brm^{2} reduces the activity of the *Antp* P2 promoter (Antp for Antennapedia; Vazquez *et al*, 1999), we tested whether Pont interferes with Brm at the level of *Antp* transcription in wing discs. We observed that Antp, which is usually expressed at high levels in the presumptive notum, no longer accumulates in the dorsal hinge region of brm^{2}/*pont* discs (Fig 1Bii)—the territory that gives rise to the adult structure connecting body wall and wing. A similar reduction of Antp accumulation was never observed in a sample of more than 50 $brm^{2}/+$ wing discs, which we presume is related to the low penetrance of the held-out wing phenotype in this genotype. Thus, Pont cooperates with Brm to maintain transcription at the *Antp* P2 promoter, an effect that is counteracted by Rept.

These data show that Rept, acting as a co-repressor, and Pont, acting as a co-activator, participate in epigenetic mechanisms of opposite transcriptional control of Hox genes through cooperation with PcG and TrxG proteins.

Pont and Rept ATPases are essential for Hox expression states

Members of the AAA+ family of DNA helicases, Pont and Rept contain ATP-binding and hydrolysis domains, and mutating the ATPase domain leads to dominant-negative effects over wild-type bacterial, yeast and human proteins (reviewed by Erzberger & Berger, 2006). We generated transdominant Pont and Rept point mutants in the ATPase domain ($Pont^{D302N}$ and $Rept^{D295N}$) and established Gal4-UAS-based conditional expression fly lines.

Driving $Rept^{D295N}$ by *arm*Gal4 (arm for armadillo) resulted in lethality at the pupal stage. However, in third instar wing discs



iii Genotype	Transformed flies*/total	Penetrance (%)
+/+	0/100	0
$pont^{51}$/+	0/100	0
$rept^{35}$/+	0/100	0
$pont^{51}$,$rept^{35}$	0/100	0
brm^{2}/+	3/210	1.4
brm^{2}/$pont^{51}$	22/160	15
brm^{2}/$pont^{51}$;P(pont)	1/60	1.7
brm^{2}/$rept^{35}$	1/163	0.6
brm^{2}/$rept^{35}$;P(rept)	0/185	0
brm^{2}/$pont^{51}$,$rept^{35}$	2/93	2.1

*Number of flies with held-out wings.

Fig 1 | The mutations *pont* and *rept* interfere with *PcG* and *trxG* genes in an opposite manner to control Hox gene expression. (**A**) Scr immunodetection in first, second and third instar leg discs in the indicated genetic contexts. (**B**) Antp immunodetection in (i) wild-type and (ii) brm^{2}/*pont* wing discs. The dashed area indicates Antp loss in the dorsal hinge region. (iii) Effect of *pont* and *rept* mutations on the brm^{2} dominant held-out wing phenotype. Antp, Antennapedia; Brm, Brahma; PcG, Polycomb group; Pont, Pontin; Psc, Posterior sex combs; Rept, Reptin; Scr, Sex combs reduced; TrxG, Trithorax group; WT, wild-type.

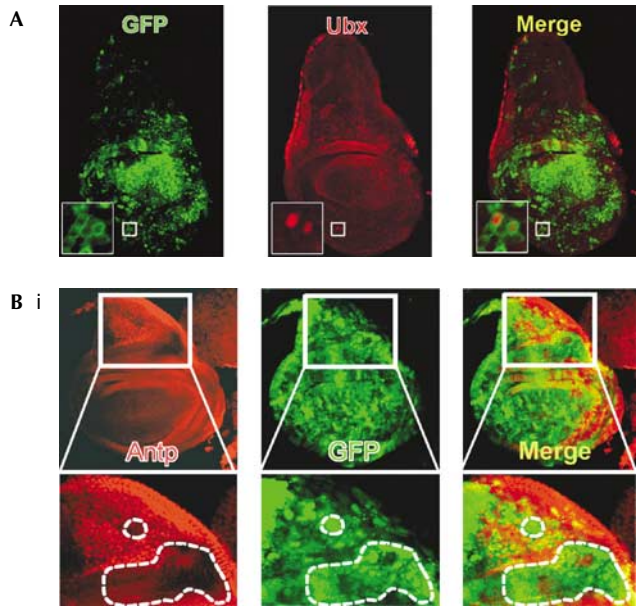
expressing $Rept^{D295N}$ using *arm*Gal4, several transdominant Rept-expressing cells also ectopically expressed Ubx (Fig 2A, insets), whereas driving either wild-type Rept or $Pont^{D302N}$ using *arm*Gal4 had no effect on Ubx (not shown). Thus, expression of ATPase-deficient Rept leads to *Ubx* derepression. Conversely, although expressing $Pont^{D302N}$ by *arm*Gal4 is viable, the resulting adults showed the *brm* held-out wing phenotype, whereas expression of wild-type Pont did not (Fig 2Bii). The penetrance of this phenotype in *arm*Gal4/UAS$pont^{D302N}$ adults is significantly greater than that observed in brm^{2} heterozygotes (compare Fig 2Bii and iii). Furthermore, expression of $Pont^{D302N}$ by *arm*Gal4 in brm^{2} heterozygotes shows an even stronger penetrance (35.5%) of the wing phenotype, indicating functional cooperation between Brm and enzymatically active Pont, and a direct role for Pont in maintaining *Antp* transcription. Indeed, immunostained wing discs showed a marked absence of Antp in dorsal hinge cells expressing $Pont^{D302N}$ (Fig 2Bi). Thus, eliminating Rept enzymatic activity leads to Ubx derepression and that of Pont to Antp repression. This loss of maintenance of Hox expression occurs in an otherwise wild-type background and, hence, the two paralogous proteins have clear opposing direct ATPase-dependent actions on Hox gene transcription.

To better understand at what level Pont and Rept act on Hox gene regulation, we studied their effect on an *mw* (miniwhite) reporter gene under the control of the *iab-7* PcG response element (PRE; Mishra *et al*, 2003). Silencing of *mw* by PRE was partly suppressed by mutation of either *rept* or the PcG gene *Asx* (Additional sex combs), an effect that was compounded in *rept/Asx* transheterozygotes (supplementary Fig S3 online). Conversely, mutation of *pont* resulted in increased silencing of *mw* by the iab-7 PRE (supplementary Fig S3 online). These data indicate that Rept and Pont have direct opposing activities on PRE-regulated gene transcription, as do the *PcG* and *TrxG* genes.

Rept forms an integral component of PcG PRC1

Rept has previously been identified by mass spectrometry in PRC1 (Saurin *et al*, 2001), although this was not investigated in further detail. Furthermore, it remains unknown whether Pont is also present in PRC1. To address whether PRC1 represents the first example of Rept in a purified protein complex without its paralogue, we purified PRC1 from *Drosophila* embryos (Fig 3A,B). Through numerous independent purifications, Rept, but not Pont, co-purified with PRC1 (Fig 3B). PRC1 comprises several proteins, of which only four are known PcG (Saurin *et al*, 2001). To determine whether Rept associates in PRC1 through direct interaction with PcG or with non-PcG members of PRC1, we performed complex reconstitution assays. Infection of Sf9 cells with baculoviruses encoding Flag-Polyhomeotic (F-Ph), Psc, Pc and dRing allows reconstitution of the PRC1 core complex (PCC; Francis *et al*, 2001). In addition, by co-infecting baculovirus encoding either Pont or Rept, we investigated whether either of these could incorporate into the reconstituted PCC.

In PCC, a Coomassie blue-stained protein migrating at the size of Rept slightly immunoreacted with antibodies to *Drosophila* Rept (Fig 3C,D) and might correspond to endogenous Sf9 Rept. Co-infection with PCC and Rept viruses resulted in depletion of recombinant Rept from the nuclear extract on incubation with anti-Flag matrix and strong enrichment of Rept in the purified complex (Fig 3D). Conversely, co-infection with PCC and Pont viruses resulted in neither the depletion of Pont from the nuclear



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Genotype	Transformed flies*/total	Penetrance (%)
UAS*pont*^{D302N}/+	0/62	0
UAS*pont*/*arm*Gal4	0/68	0
UAS*pont*^{D302N}/*arm*Gal4	6/112	5.3
UAS*pont*^{D302N}/+;*brm*^{2}/+	0/88	0
UAS*pont*^{D302N}/*arm*Gal4;*brm*^{2}/+	16/45	35.5

*Number of flies with held-out wings.

Fig 2 | Pont and Rept enzymatic activities are essential for the maintenance of Hox gene expression. (**A**) Ubx derepression by Rept^{D295N}. Confocal section showing GFP (left) and Ubx staining (centre) in an *arm*Gal4/UAS*rept*^{D295N};UAS*GFP*/+ wing disc. The insets focus on cells expressing Rept^{D295N} (green) resulting in Ubx derepression (red). Note that high Ubx staining around the periphery of the disc is due to Ubx expression in the peripodial membrane. (**B**) (i) Antp repression by Pont^{D302N}. Stack of confocal sections showing Antp (left) and GFP (centre) expression in an *arm*Gal4/UAS*pont*^{D302};UAS*GFP*/+ wing disc. The lower panels are magnified views of the boxed areas and highlight sites of Antp repression (dashed areas). (ii) Held-out wing phenotype induced by Pont^{D302N} and genetic interactions with *brm*^{2}. Antp, Antennapedia; Arm, Armadillo; Brm, Brahma; GFP, green fluorescent protein; Pont, Pontin; Rept, Reptin; Ubx, Ultrabithorax.

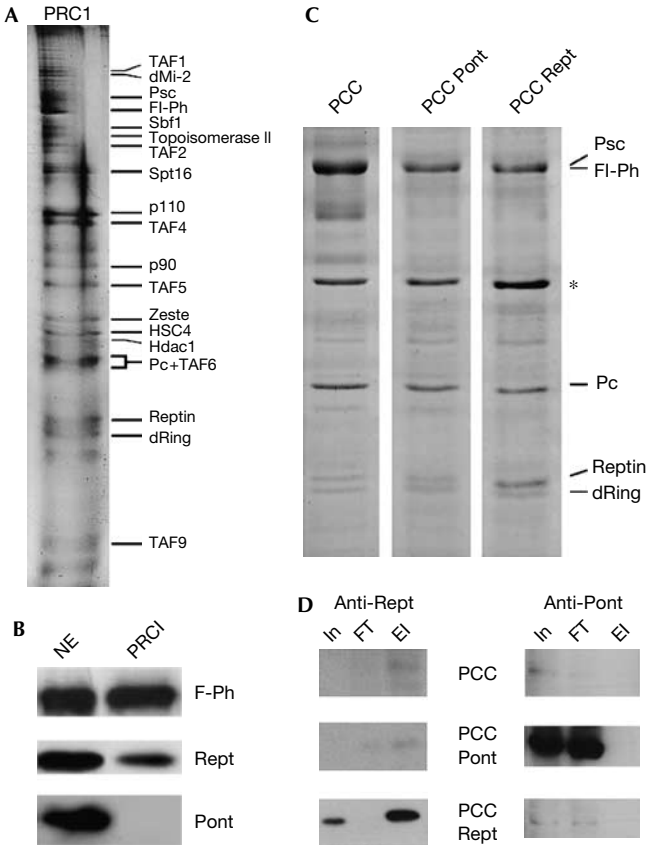

Fig 3 | Rept is an integral component of PRC1. (**A**) SDS–PAGE (silver stain) of PRC1. Protein bands are labelled according to Saurin *et al* (2001). (**B**) Western analyses of PRC1. NE: 20 μg nuclear extract; PRC1: 15 μl PRC1. (**C**) SDS–PAGE (Coomassie blue) of reconstituted PCCs from Sf9 cells expressing Flag-Polyhomeotic (F-Ph), Psc, Pc, dRing (PCC), and Pont (PCC-Pont) or Rept (PCC-Rept). *Nonspecific Sf9 protein. (**D**) Western analyses of Rept (left) and Pont (right) profiles during purification of PCCs shown in (**C**). In: 1.5 μl (15 μg) Sf9 nuclear extract; FT: 1.5 μl anti-Flag affinity column flow through; El: 3 μl purified complex. PCC, PRC1 core complex; Pont, Pontin; Psc, Posterior sex combs; Rept, Reptin; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

extract by anti-Flag matrix nor its enrichment in the purified PCC (Fig 3D). Thus, Rept physically interacts with PRC1 PcG proteins, whereas Pont does not, placing Rept as an authentic PRC1 component.

Pont associates with TrxG Brm-C

So far, Pont has not been described in any known TrxG complex. Given the genetic interactions with *brm*, the antagonism with Rept in Hox control and its absence in PRC1, we used column chromatography purifications to determine whether Pont associates with Brm in a TrxG complex (Fig 4A). In nuclear extracts from the embryo, two independent Pont antibodies—Pont53 (Bauer *et al*, 2000) and Pont67 (this study)—showed, besides the predominant 50 kDa monomeric form, a series of slowly migrating species, of which one, of 170 kDa, became highly enriched following BioRex-70 chromatography (data not shown). This 170 kDa protein is Pont and not an unrelated protein crossreacting with our antibodies as it is present in Flag-Pont purifications from Sf9 extracts, is recognized by both Pont and Flag antibodies and was positively identified by nano-ESI-IT ion trap mass spectrometry (supplementary Fig S4 online). By following the purification profiles of Brm and Pont during extract fractionation, we found that 170 kDa Pont always co-purifies with Brm (data not shown) and, at the Mono Q step, 170 kDa Pont and Brm purifies away from 50 kDa Pont and Rept (Fig 4B). Furthermore, by fractionating through a glycerol gradient, we found that Pont purifies in the same complex as Brm (Fig 4C). The Pont–Brm glycerol gradient peak, analysed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), western blot and multidimensional protein identification technology (Washburn *et al*, 2001),

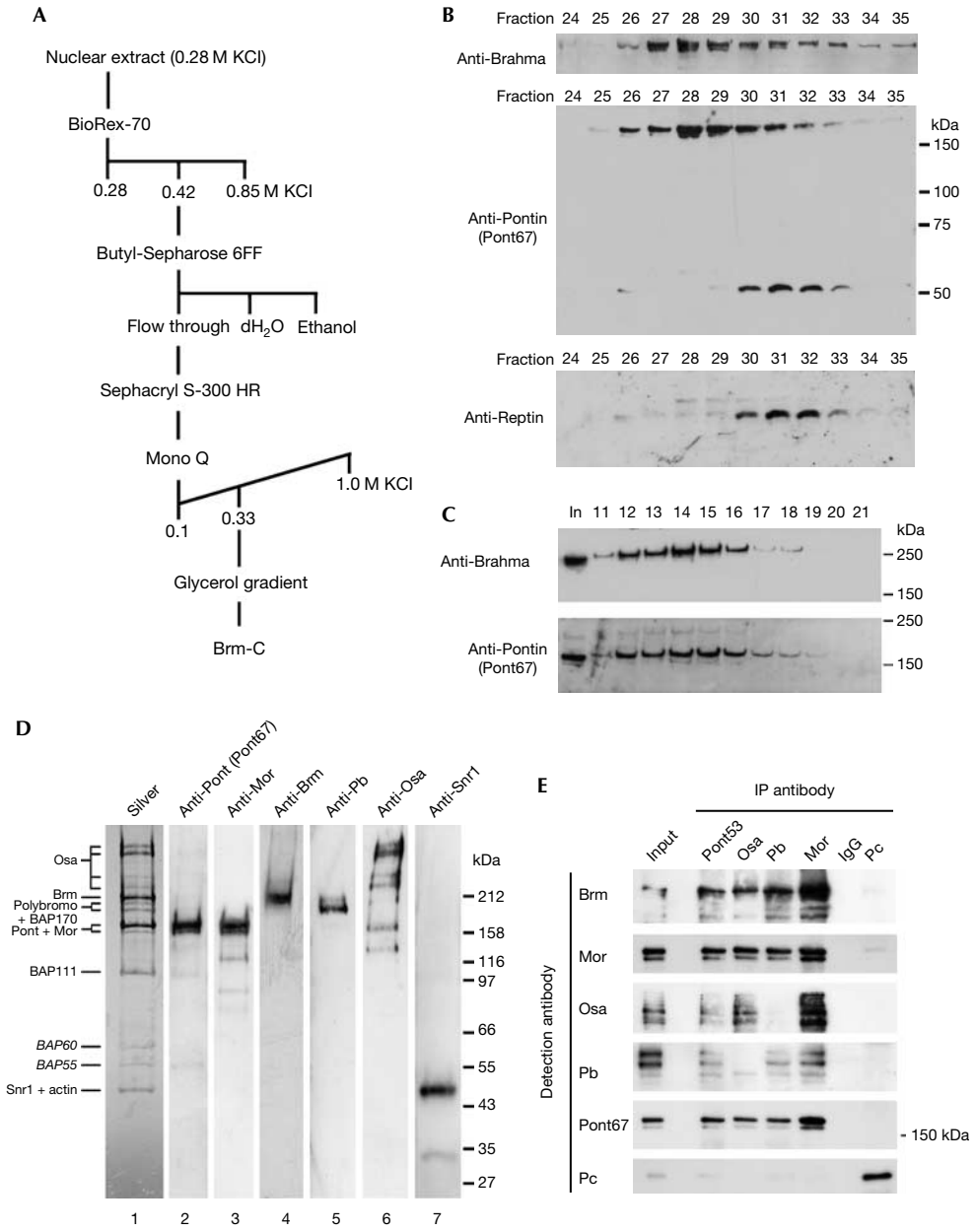

Fig 4 | Pont co-purifies with the Brahma complex. (**A**) Purification scheme of Brm-C. (**B**) Western analyses of Mono Q fractions in which 50 kDa Pont and Rept purify away from the 170 kDa Pont and Brm peak. (**C**) Western analyses of peak fractions from glycerol gradient sedimentation of Mono Q fraction 28. (**D**) 4–15% SDS–polyacrylamide gel electrophoresis (silver staining, lane 1) and western analyses (lanes 2–7) of the Brm–Pont peak (glycerol gradient fraction 14). Note that the antibody used in lane 2 (Pont67) does not crossreact with Mor (J. Antao and R. Kingston, personal communication). (**E**) Western analyses of proteins precipitated from 0.42 M BioRex-70 fraction by antisera to Pont (IP, Pont53; western, Pont67), Osa, Pb, Mor, Pc or rabbit IgG. Input, 2.5 μl (10 μg); IP, 10 μl (1/10). Brm-C, Brahma complex; IP, immunoprecipitation; Pont, Pontin; Rept, Reptin.

corresponds to a highly purified complex containing 13 prominent protein species, identified as Pont and all Brm-C components (Fig 4D; supplementary Table S4 online). At least two distinct Brm-C components exist in *Drosophila*, BAP and PBAP, which contain shared subunits and either Osa (BAP) or Pb and BAP170 (Mohrmann *et al*, 2004). Both Pb and Osa antibodies co-precipitated 170 kDa Pont, and Pont53 equally co-precipitated 170 kDa Pont (revealed by Pont67), Osa and Pb, as well as all members of Brm-C tested (Fig 4E), indicating that 170 kDa Pont interacts with both BAP and PBAP complexes.

Pont has not previously been found in Brm-C by using classical mass spectrometry of bands excised from gels (Papoulas *et al*, 1998; Kal *et al*, 2000). However, the unbiased and sensitive multidimensional protein identification technology identified all

the previously known components of Brm-C together with 170 kDa Pont. This study is the first, to our knowledge, to describe a 170 kDa Pont form in *Drosophila* and might explain why Pont has not previously been found in Brm-C, especially because the predicted molecular weight of Pont is 50 kDa. Alternatively, it is feasible that conditions used by other groups to purify Brm-C destroyed the interaction with Pont, although our immunoprecipitation conditions show that this interaction is stable up to 600 mM salt. However, 170 kDa Pont is only barely detectable by western blotting in our nuclear extracts when compared with 50 kDa Pont, becoming enriched following initial fractionations. Thus, if Pont is associated with Brm-C (and not an integral component of it), then small changes in extract preparation could reduce or eliminate the quantity of 170 kDa Pont in the extract and thus in the complex. Nonetheless, our data show that 170 kDa Pont robustly associates with Brm-C throughout the purification scheme and also through specific immunoprecipitations with Pont or Brm-C antibodies. The nature of 170 kDa Pont is puzzling; this species could not have been generated from a longer transcript, as it is identified by Flag and Pont antisera, as well as mass spectrometry when the open reading frame complementary DNA fused to a Flag tag is expressed and purified from Sf9 cells (supplementary Fig S4 online). Given the identified molecular weights of Pont (50, 110 and 170 kDa), we believe that these higher molecular weight species are reducing/denaturing-resistant multimers of the monomeric 50 kDa protein, although the nature of this multimerization requires experimental confirmation.

The co-purification of Pont with Brm in Brm-C indicates that the genetic interaction of *pont* with *brm* in Hox control translates the functional cooperation of the two factors within Brm-C. PRC1 is believed to stabilize chromatin structure by counteracting remodelling by Brm-C (reviewed by Francis & Kingston, 2001). The finding that Rept and Pont co-purify separately in these functionally antagonistic complexes affords an explanation for their opposite effects on Hox gene transcription. Although ATP hydrolysis by Pont and Rept is essential for maintaining Hox gene transcription (Fig 2), we did not detect any activity in *in vitro* chromatin remodelling assays (data not shown). Thus, it seems likely that these proteins use ATP hydrolysis for some purpose other than ATP-dependent chromatin remodelling.

PcG and TrxG proteins not only regulate the expression patterns of Hox, but also those of many other genes (Ringrose & Paro, 2007 and references therein). Of particular interest is the involvement of PcG PRC1 and PRC2 in prostate cancer progression (Varambally *et al*, 2002; Berezovska *et al*, 2006). The unambiguous role of Rept in promoting prostate cancer metastasis through KAI1 repression (Kim *et al*, 2006) and its role in PcG repression of Hox genes (this study) converge two crucial transcriptional repression pathways. Our findings, that the enzymatic activities of Pont and Rept are indispensable for maintaining transcriptional states, highlight the importance of these two transcriptionally antagonistic factors and open up new avenues of research towards a better understanding of the mechanisms that dictate the balance of transcriptional fate during development and the progression of cancer.

METHODS

Flies and manipulations. All stocks except F-Ph$^{71\text{-}51A}$ (R. Kingston, Boston, MA, USA), *pont* and *rept* lines (Bauer *et al*, 2000) were obtained from Bloomington Stock Center (Bloomington, IN, USA). Primer sequences used to generate cDNA encoding Pont^{D302N} and Rept^{D295N} are available on request. Immunodetections were performed according to standard procedures. Antibodies to Antp (4C3) and Scr (6H4.1) were from Developmental Studies Hybridoma Bank (University of Iowa, IA, USA) and those to Ubx (FP3.38) were from R. White (Cambridge, UK). Images were obtained using a Zeiss Confocal 510 Meta microscope.

Protein biochemistry. PRC1 was purified from embryos according to Shao *et al* (1999). PCCs, including 10 μM $ZnCl_2$ in buffers, were purified according to Francis *et al* (2001).

Brm-C was purified by using a multistep fast protein liquid chromatography purification strategy and is described in detail in the supplementary information online.

Immunoprecipitations were carried out from 800 μg of 0.42 M BioRex-70 fraction used for Brm-C purification, dialysed against PBS. Following preclearing with protein A Sepharose (50 μl per reaction), 25 μl anti-Pont (Pont53), 25 μl anti-Pb, 13 μl anti-Mor, 100 μl anti-Osa (Developmental Studies Hybridoma Bank), 25 μl anti-Pc (dN-19; Santa Cruz, Heidelberg, Germany) or 50 μg rabbit IgG (Sigma-Aldrich, Chimie, Lyon, France) were added and incubated overnight at 4 °C. Immunocomplexes, isolated by binding to protein A Sepharose (50 μl) for 30 min, were centrifuged and extensively washed: ten times in PBS, eight times in PBS-650 and twice in PBS-100. Immunocomplexes were eluted with 90 μl of 0.1 M glycine pH 2.0, neutralized by 10 μl of 1 M Tris.Cl pH 8.0 and analysed by 8% SDS–PAGE.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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