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The YPWM motif links Antennapedia to the basal transcriptional machinery

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HOX genes specify segment identity along the anteroposterior axis of the embryo. They code for transcription factors harbouring the highly conserved homeodomain and a YPWM motif, situated amino terminally to it. Despite their highly diverse functions in vivo, HOX proteins display similar biochemical properties in vitro, raising the question of how this specificity is achieved. In our study, we investigated the importance of the *Antennapedia* (*Antp*) YPWM motif for homeotic transformations in adult *Drosophila*. By ectopic overexpression, the head structures of the fly can be transformed into structures of the second thoracic segment, such as antenna into second leg, head capsule into thorax (notum) and eye into wing. We found that the YPWM motif is absolutely required for the eye-to-wing transformation. Using the yeast two-hybrid system, we were able to identify a novel ANTP-interacting protein, Bric-à-brac interacting protein 2 (BIP2), that specifically interacts with the YPWM motif of ANTP in vitro, as well as in vivo, transforming eye to wing tissue. BIP2 is a TATA-binding protein associated factor (also known as dTAFII3) that links ANTP to the basal transcriptional machinery.

KEY WORDS: ANTP, HOX, *bip2*, dTAF3, *Drosophila*, Eye-to-wing transformation, Homeotic transformation

INTRODUCTION

Homeotic genes (HOX) are selector genes that generate morphological diversity along the anteroposterior body axis during animal development (Lewis, 1978; Wakimoto and Kaufman, 1981; Lin and McGinnis, 1992; Akam, 1998; Mann and Morata, 2000). Upon ectopic expression (gain-of-function) or loss-of-function mutations in HOX genes, massive morphological changes are induced imposing a new segmental identity, transforming parts or complete segments into another one (Lewis, 1978). This finding illustrates that a single protein regulates many cellular fates in one or more segments. HOX genes encode highly conserved transcription factors that share a common sequence element of 180 bp, the homeobox, which encodes a 60 amino acid homeodomain (HD) that represents the DNA-binding domain and allows sequence-specific recognition within the regulatory region of its target genes (McGinnis et al., 1984; Scott and Weiner, 1984). However, the HD exerts a relatively low DNA-binding specificity (Ekker et al., 1994; Gehring et al., 1994). In general, the third α-helix of the HD binds a 6-bp DNA sequence containing a TAATC/GC/G recognition core (Ekker et al., 1991). This recognition sequence appears statistically once per kilobase in the genome, raising the question of how HOX proteins recognize their real target sequences among other potential target sites to achieve segmental specificity. Studies using chimeric HOX proteins indicate that the N-terminal arm of the HD that contacts the adjacent minor groove of the DNA is in some cases sufficient to provide specificity (Furukubo-Tokunaga et al., 1993; Zeng et al., 1993; Passner et al., 1999; Berry and Gehring, 2000). However, sequences outside the HD were also found to be important in conferring specificity (Lin and McGinnis, 1992; Chan and Mann, 1993; Zeng et al., 1993; Chauvet et al., 2000; Gebelein et al., 2002; Merabet et al., 2003). More recent experiments suggest that the homeodomain recognizes the DNA structure in the minor groove rather than reading a specific DNA sequence directly (Joshi et al., 2007).

Two models for HOX gene specificity, the 'widespread binding' and the 'co-selective binding' models have been proposed. The first one assumes co-operative binding on multiple monomer-binding sites, increasing the presence of one HOX protein on a cis-regulatory element, allowing the regulation of the downstream target genes (Biggin and McGinnis, 1997).

The second model proposes the regulation of target genes through protein co-factors that increase the DNA-binding selectivity and affinity (Biggin and McGinnis, 1997). One factor contributing to HOX specificity was shown to be Extradenticle (EXD). Mutations in *exd* lead to homeotic transformations without affecting the expression pattern of the HOX genes (Gonzalez-Crespo and Morata, 1995; Peifer and Wieschaus, 1990). EXD was consequently shown to act as a HOX co-factor increasing the DNA-binding specificity and target site selectivity of HOX proteins (Mann and Chan, 1996). The interaction of the HOX proteins with EXD involves a highly conserved peptide motif, the YPWM motif, which contacts the HD of EXD, as shown by structural analysis (Passner et al., 1999; Piper et al., 1999). The YPWM motif, shown to serve as a protein-protein interaction motif, is highly conserved throughout the animal kingdom and lies amino terminally to the HD. All HOX proteins share the YPWM motif except for the Abdominal B (ABD-B) class of HOX genes, which have retained only a remnant tryptophan at the corresponding position (Izpisua-Belmonte et al., 1991).

Interestingly, genetic experiments indicate that some HOX gene functions and target genes are controlled independently of *exd* (Peifer and Wieschaus, 1990; Percival-Smith and Hayden, 1998), and removal of the YPWM motif does not completely abolish EXD-HOX binding interactions (Galant et al., 2002; Merabet et al., 2003). The YPWM apparently serves other functions besides binding EXD (Chan and Mann, 1996; Merabet et al., 2003), suggesting that other

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YPWM-motif-interacting co-factors might be involved. Based on these findings, work on the HOX gene *abdominal A* (*abd-A*) revealed a function of the YPWM motif in transcriptional activation rather than DNA-binding selectivity (Merabet et al., 2003). The HOX gene *Antennapedia* (*Antp*) specifies the second thoracic segment (T2) with a pair of wings and a pair of middle legs in *D. melanogaster*. When ectopically expressed, *Antp* transforms head structures into parts of the second thoracic segment, such as the antenna into a middle leg and the dorsal head capsule into notum structures (Schneuwly et al., 1987). *Antp* further inhibits eye development by inducing cells co-expressing *eyeless* (*ey*) and *Antp* to undergo apoptosis (Plaza et al., 2001). In combination with a constitutively active form of the *Notch* receptor (N^{act}), which prevents cells from undergoing apoptosis, *Antp* is able to transform the dorsal part of the eye into the corresponding dorsal T2 appendage, the wing (Kurata et al., 2000). Using the OK-107 driver, ANTP is also capable of transforming the eye into wing structures without N^{act} (see Results).

On the basis of these results, we have analyzed the role of the YPWM motif and the DNA-binding specificity of *Antp* in inducing antenna-to-leg and eye-to-wing transformations. We found that the YPWM motif and the DNA-binding specificity of the HD are absolutely required for eye-to-wing transformations. By contrast, the transformation of the antenna into a T2 leg is largely dependent on the DNA-binding specificity, and to a much lesser extent on the YPWM motif. Based on the strict requirement of the YPWM motif to transform an eye into a wing, we screened for YPWM-motif-specific interacting co-factors. Employing the yeast two-hybrid system, we identified a novel *Antp* co-factor *bric-à-brac interacting protein 2* (*bip2*), also referred to as *dTAFII3* or *TAFII155* (Gangloff et al., 2001), which specifically interacts with the YPWM motif of *Antp* in vitro and in vivo. Using gain- and loss-of-function approaches, we show that *bip2* genetically interacts with *Antp* promoting dorsal ectopic wing development

MATERIALS AND METHODS

Fly strains and transformants

Transgenic lines were generated by standard procedures (Spradling and Rubin, 1982). Flies were reared on standard medium at 25°C. Lines used were: *ey*-Gal4 (Halder et al., 1998; Hauck et al., 1999); OK-107-Gal4 (Connolly et al., 1996); *dpp*-Gal4 (Staehling-Hampton et al., 1994); UAS-*Antp* and UAS-*AntpdHD* (Bello et al., 1998); UAS-$Antp^{Q50K}$ (Plaza et al., 2001); UAS-N^{act} (Fortini et al., 1993); UAS-*Ubx*, UAS-$Antp^{AAAA}$, UAS-*Bip2*, UAS-*Bip2-HA*, UAS-*exd*, UAS-*GFP* and *wg-lacZ* (Couso et al., 1994); *eyg-lacZ* (Jang et al., 2003); *Su(H)-lacZ* (Furriols and Bray, 2001); and *dpp-lacZ* (Halder et al., 1998).

Antibody staining

Staged larvae were dissected in cold PBS and fixed in PEM [100 mM Pipes (pH 6.9), 2 mM $MgSO_4$, 1 mM EGTA, 4% formaldehyde] for 25 minutes on ice. After washing with PBT (PBS containing 0.3% Triton X-100), blocking was performed in PBTB (PBT with 2% NGA, normal goat serum) for 2 hours at 4°C. Antibody staining was performed by using a primary rabbit anti-VG at 1/500 (Williams et al., 1991), primary anti-β-Gal at 1/500, primary anti-ANTP (MAB 8c11) at 1/1000 or primary anti-EYA at 1/200 (Bonini et al., 1993) overnight at 4°C. For immunofluorescence detection, a dichlorotriazinyl amino fluorescein (DTAF)-conjugated donkey anti-IgG (Jackson ImmunoResearch) antibody was used. The preparations were mounted in Vectashield (Vector Laboratories) and examined by confocal microscopy using a Leica (TCS NT) microscope.

Yeast two-hybrid system

A *Drosophila* third larval instar cDNA library in the pACT vector (a generous gift from Dr Elledge, Dana-Faber Cancer Institute, Boston, MA) was screened with an *Antp* bait that corresponds to the region of aa 280-304 of the ANTP protein, including the YPWM motif and the N-terminal arm of the HD. The screen was performed as described previously (Bartel and Fields, 1995) in L40 yeast cells (Matα, trp1, leu2, his3 LYS2::lexA-lacZ). Around 2×10^6 clones were screened for β-galactosidase activity. Quantification of the protein-protein interaction was performed as described by Bartel and Fields (Bartel and Fields, 1995) after co-transformation in L40 cells. Oligonucleotides coding for the following peptides were cloned with *Eco*RI and *Bam*HI into the pBTM116 vector (Bartel and Fields, 1995):

LexA-YPWM-N-term, PSPLYPWMRSQFGKCQERKRGRQT;
LexA-AAAA-N-term, PSPLAAAARSQFGKCQERKRGRQT;
LexA-YPWM-, PSPLYPWMRSQFGKCQE; and
LexA-AAAA-, PSPLAAAARSQFGKCQE.

LexA-YPWM-HD was cloned with *Sma*I and *Sal*I into pBTM116. Amino acids 279-348 of the ANTP protein were used. Bip2-235 was cloned as an *Xho*I fragment from aa 853-1088 into pACT (from screen).

In all constructs, the splice variant generating the longer linker arm (eight amino acids) was used.

Pull-down experiments and co-immunoprecipitation

A fusion construct ANTP-YPWM-HD-GST was amplified by PCR. This fragment was subcloned into pGEX-KG (Pharmacia). The resulting GST fusion was expressed in *E. coli* and extracted according to Pharmacia's recommendations. For analyzing protein-protein interactions, 10 μg of GST fusion protein were incubated with 50 μg of a 50% slurry of glutathione Sepharose 4B beads in incubation buffer [12 mM HEPES (pH 7.9), 4 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM KCl, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonylfluoride] for 20 minutes at room temperature, washed, and resuspended in a total volume of 30 μl containing 10 μl of [^{35}S]-methionine-labeled rabbit reticulocyte lysates in incubation buffer for 40 minutes on ice. Beads were then washed four times with 1 ml of washing buffer [0.5% NP 40, 1 mM EDTA, 20 mM Tris-HCl (pH 8.0), 0.1 M NaCl] at room temperature. Beads were then recovered in SDS-PAGE loading buffer, and proteins were analyzed by SDS-PAGE followed by fluorography.

Reticulocyte lysate proteins were produced using the TNT reticulocyte lysate synthesis kit (Promega). Co-immunoprecipitation was performed using third instar larval nuclear extracts. The hemagglutinin (HA)-tagged BIP2 protein was bound to the Anti-HA (3F10) Affinity Matrix (Roche Applied Science). After preabsorbing the Anti-HA Affinity Matrix, 200 μg of nuclear extract was added to the matrix and incubated for 2 hours at 4°C. The matrix was washed twice with buffer I [50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM PMSF, and protease inhibitors mix]. The precipitate was eluted with SDS sample buffer and analyzed by western blotting, using a monoclonal mouse anti-ANTP antibody (4C3).

Cloning procedure and plasmids

Standard molecular biology methods (Sambrook et al., 1989) and yeast protocols (Bartel and Fields, 1995) were used as previously described. The *bip2* cDNA was subcloned into the pBSKII vector with *Not*I and sequenced using the Big Dye Terminator Cycle Sequencing Kit and an ABI 373A automated sequencer (Perkin Elmer/Applied Biosystems). To generate UAS-*bip2*, the full-length *bip2* cDNA in pBS-SKII was subcloned into the *Not*I site of pUAST (Brand and Perrimon, 1993). To generate UAS-*bip2-HA*, the C-terminal 200 bp of the *bip2* cDNA were replaced by the same *bip2* that was sequenced, fused to the HA coding sequence by PCR. The *bip2*-HA cDNA was then subcloned via *Not*I and *Asp*718 into pUAST. To generate pTV2-bip2-SceI-6kb, 6.2 kb of the genomic *bip2* region, with an additional *Sce*I restriction site, was amplified by PCR and subcloned into the *Not*I site of the pTV2 vector (Rong and Golic, 2000).

Generation of *bip2* mutants by homologous recombination

bip2 has been mapped to position 102B on the fourth chromosome. We took w^{1118}; CyO/Sp; hs-Flp, hs-Cre/TM6B,Hu flies and crossed them to the y^1, w^{1118}; pTV2-*bip2-Sce*I-6kb/pTV2-*bip2-Sce*I-6kb stock. The offspring were heat shocked (HS) for 1 hour at 37°C at 3 hours of development. From the heat-shocked offspring, CyO non-TM6b, Hu females that had lost the w^+ marker in the eye were selected. The females were crossed back to y^1, w^{1118} males. The progeny was screened for CyO and w^+, indicating a transposition

of the mini-white gene from the second chromosome to another location within the genome. The two flies recovered from 60,000 flies screened were balanced over ci^D, spa^{pol} on the fourth chromosome. The second line with homologous recombination on the fourth chromosome was recombined with an HS-Flp transgene on the first chromosome, and crossed to the line Rb_e00710 with a razor Bac vector insertion 1.1 kb 5′ to the initiation ATG of the *bip2* gene (kindly provided by Exelixis). Both lines (Rb_e00710 and the line from the homologous recombination) harbor an FRT sequence oriented in the same direction. The offspring were heat shocked after 3 hours of development for 1 hour at 37°C. The heat-shocked flies (F1) were crossed back to a fourth chromosome balancer *yw*; ci^D, spa^{pol}/ey^D. F2 flies with dark red eyes and ci^D, spa^{pol} were selected and balanced over ci^D, spa^{pol}. The recombined chromosomes harbor the mini-white from of the pTV2 vector, whereas the mini-white gene from the piggy bac vector was deleted. The two mini-white mutants can be distinguished by their eye colour (pTV2, dark red; piggy bac, orange), therefore the flies with the dark eye colour were selected and later screened by PCR for recombination of the markers.

RESULTS

The YPWM motif of ANTP is essential for the eye-to-wing transformation

It was previously shown that the combined ectopic expression of *Notch* (*N*) and *Antp* in the eye imaginal disc by the *ey* enhancer driving Gal4 (*ey*-Gal4) induces the ectopic outgrowth of T2 specific appendages (wings and legs) on the adult head (Kurata et al., 2000) (Fig. 1A-C). The transformation of the antenna into leg is not complete, as can be seen in Fig. 1C. This is owing to the fact that the driver used (*ey*-Gal4) is not expressed in the entire antennal disc. Misexpression of *Antp* alone by *ey*-Gal4 promotes antenna-to-leg transformation but does not induce wing development. Instead it interferes with endogenous eye morphogenesis by inducing apoptosis (Kurata et al., 2000; Plaza et al., 2001). By contrast, activated *N* signalling alone leads to hyperplasia of the eye, but no ectopic wings or legs are observed, suggesting that *N* activation prevents apoptosis during normal eye development (Kurata et al., 2000). Kurata et al. proposed that *N* signalling modulates the ability of the precursor cells to respond to different developmental signals, allowing *Antp* to promote wing and leg development.

Table 1. Percentage eye-to-wing and antenna-to-leg transformation in the various genotypes

Genotype*	Eye-Wing	Antenna-Leg
UAS-N^{act}; UAS-*Antp*	40% (68/172)	28% (48/172)
UAS-N^{act}; UAS-$Antp^{AAAA}$	0% (0/517)	19% (98/517)
UAS-*exd*; UAS-N^{act}; UAS-*Antp*	2% (3/191)	3% (6/191)
UAS-*exd*; UAS-N^{act}; UAS-$Antp^{AAAA}$	0% (0/157)	4.5% (7/157)
UAS-*bip2*; UAS-N^{act}; UAS-*Antp*	82% (94/114)	13% (15/114)
UAS-*bip2*; UAS-N^{act}; UAS-$Antp^{AAAA}$	0% (0/108)	24% (26/108)
UAS-*Nact*; UAS-$Antp^{Q50K}$	0% (0/127)	20% (25/127)

*All transgenes were activated by *ey*-Gal4.

In order to determine whether the YPWM motif of *Antp* is essential for the homeotic transformations induced by *Antp*, we generated transgenic lines carrying a constitutively active form of the Notch receptor (N^{act}) (Fortini et al., 1993) and a mutated version of the ANTP protein in which the YPWM motif was substituted by four alanines (ANTP^{AAAA}). None of the flies expressing UAS-N^{act}; UAS-$Antp^{AAAA}$ under the control of the *ey*-Gal4 enhancer showed any eye-to-wing transformation, whereas about 20% of the flies showed antenna-to-leg transformation (Fig. 1D,E, Table 1). These data are in line with the finding that, in the *fushi tarazu* gene of the beetle Tribolium (which has a YPWM motif), the AAAA mutation does not abolish the ability to induce antenna-to-leg transformations in *Drosophila* (Lohr and Pick, 2005). Interestingly, legs induced by $Antp^{AAAA}$ showed second leg identity (Fig. 1E), indicating a strict requirement of the YPWM motif for eye-to-wing but not antenna-

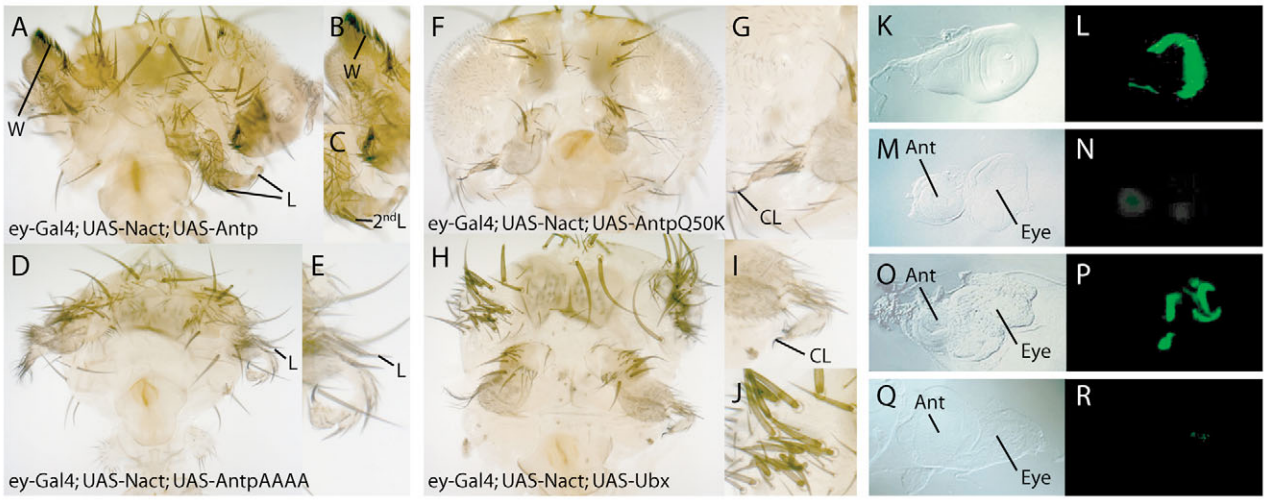

Fig. 1. The eye-to-wing transformation is specific for *Antp* and dependent on the YPWM motif. (**A**) Ectopic wings (W) and legs (L) induced on the head of *ey*-Gal4; UAS-N^{act}; UAS-*Antp* flies. (**B**) Higher magnification of the ectopic wing (W) in A, showing the marginal bristles of the triple row. (**C**) Higher magnification of the antenna-to-leg transformation in A. The apical bristles on the tibia indicate second leg identity (2nd L). (**D**) Head of an *ey*-Gal4; UAS-N^{act}; UAS-$Antp^{AAAA}$ fly showing no wing structures. (**E**) Higher magnification of the antenna-to-leg transformation, showing the tibial apical bristles (L). (**F**) Head of an *ey*-Gal4; UAS-N^{act}; UAS-$Antp^{Q50K}$ fly. No ectopic wings are formed. (**G**) Higher magnification of the transformed arista with a claw (CL). (**H**) The head of an *ey*-Gal4; UAS-N^{act}; UAS-*Ubx* fly shows no ectopic wing. (**I**) Higher magnification of the ectopic leg with a claw (CL). (**J**) Higher magnification of ectopic bristles induced in the eye part of the head. (**K**,**M**,**O**,**Q**) Bright-field micrograph of third instar discs and (**L**,**N**,**P**,**R**) the VG protein distribution visualized by immunostaining of the corresponding disc to its left. Ant, antennal discs; Eye, eye discs. (K-N) VG is expressed in the wing pouch in wild-type wing (K,L) discs but not in the eye-antennal disc (M,N). (O,P) VG is ectopically induced in the eye disc of *ey*-Gal4; UAS-N^{act}; UAS-*Antp* larvae. (Q,R) There is no detectable VG protein induced in eye-antennal disc of *ey*-Gal4; UAS-N^{act}; UAS-$Antp^{AAAA}$ larvae.

to-second leg transformation. Experiments using a *Distal-less*-Gal4 driver indicate the AAAA mutation is capable of inducing some antenna-to-leg transformation, but to a much lesser extent than the YPWM construct (R. Fünfschilling, M. Seimiya and W.J.G., unpublished).

vestigial (*vg*) the selector gene for wing development is expressed in the wing and haltere imaginal discs but not in the eye or leg imaginal discs (Kim et al., 1996). Kurata et al. previously showed that *Antp* driven in combination with N^{act} in the eye-antennal disc is able to induce ectopic VG expression. We therefore investigated whether wing development is induced but not maintained in *ey*-Gal4; UAS-N^{act}; UAS-$Antp^{AAAA}$ larvae. Immunofluorecence analysis of third instar imaginal discs revealed that animals carrying both transgenes under the control of *ey*-Gal4 did not induce VG (Fig. 1Q,R), in contrast to the control animals expressing wild-type *Antp* in combination with N^{act} (Fig. 1O,P). These data indicate that the YPWM motif is required for the ectopic induction of the wing selector gene *vg* in the eye imaginal disc.

The eye-to-wing transformation is specific for the *Antp* HOX gene

It was shown that homeotic genes in *Drosophila* have similar effects when ectopically expressed in the eye-antennal disc (Casares and Mann, 1998; Yao et al., 1999). Most HOX genes are able to transform the distal part of the antenna into leg structures (Casares et al., 1996; Kuhn et al., 1993; Kuziora, 1993; Mann and Hogness, 1990) by repressing *homothorax* (*hth*) (Yao et al., 1999). In order to test the specificity of *Antp* toward wing and leg development, we performed gain-of-function experiments with several other homeotic genes, such as *Sex combs reduced* (*Scr*), *Ultrabithorax* (*Ubx*) and *Abdominal-B* (*Abd-B*). Misexpression of UAS-N^{act}; UAS-*Ubx* by *ey*-Gal4 did not induce any ectopic wing outgrowth in the eye, but it showed antenna-to-leg transformation, which, however, could not be attributed to a particular thoracic segment (Fig. 1H-J). The same results were obtained with *Scr* and *Abd-B* (data not shown). In addition, we found that changing the DNA-binding specificity of the *Antp* HD ($Antp^{Q50K}$) abolishes the capacity of *Antp* to induce ectopic wing tissue in the eye and also reduces the capacity to transform the antenna into a leg. Only an arista-to-tarsus transformation is observed (Fig. 1F,G). When an *Antp* gene lacking the HD is co-expressed with N^{act}, these flies show the same phenotype as *ey*-Gal4; UAS-N^{act} flies, i.e. enlarged eyes (data not shown). Thus, among the different HOX genes tested, only *Antp* is able to induce ectopic wing development, a transformation dependent on the YPWM motif and the DNA-binding specificity of the protein.

Wing induction is inhibited by the overexpression of *exd*

So far, EXD is the only HOX co-factor known to bind the YPWM motif. To test whether *exd* is required to promote wing versus leg development, we co-expressed *exd* in combination with the wild-type *Antp* or the $Antp^{AAAA}$ transgene in the presence of N^{act}, as described above. We found that only 2% of flies carrying the *ey*-Gal4; UAS-N^{act}; UAS-*Antp;* UAS-*exd* transgenes showed wing structures in the eye region, as compared with 40% of flies without *exd* (Table 1). Similarly, *exd* inhibited the antenna-to-leg transformation, as only 3% of the flies expressing *exd* showed antenna-to-leg transformation compared with 28% without *exd* (Table 1). Furthermore, *exd* also inhibits the antenna-to-leg transformation induced by $Antp^{AAAA}$ from 19% to 4.5% (Table 1). This inhibition is caused by EXD, which is localized in the nuclei as shown by antibody staining. Both proteins, EXD and ANTP, are found to be co-expressed in the nucleus of the eye-antennal disc cells (see Fig. S1 in the supplementary material). The suppression of the antenna-to-leg transformation by *exd* is in line with previous studies that show that nuclear EXD is incompatible with distal leg development (Casares and Mann, 1998). We further do not attribute the observed effect to a dilution of the GAL4 protein by the addition of a third UAS transgene into the system, as the addition of an UAS-*bip2* transgene instead of UAS-*exd* enhances the eye-to-wing transformation (see below). As the co-expression of *exd* in combination with *Antp* and N^{act} reduces the frequency of eye-to-wing transformations, these results indicate that *exd* is not acting in combination with *Antp* to induce ectopic wing development.

Identification of a new ANTP-interacting protein

Because the YPWM motif of ANTP plays an essential role in ectopic wing development, we screened for proteins that specifically interact with the ANTP YPWM motif using the yeast two-hybrid system (Bartel and Fields, 1995). We screened a third instar *Drosophila* cDNA library fused to the Gal4 activation domain with a bait composed of the LexA DNA-binding domain fused to the ANTP YPWM motif and the N-terminal arm of the HD (LexA-YPWM-N-term) (Fig. 2A). We identified two identical cDNA clones (BIP2-235; amino acid 853-1088) of the gene *bip2* (Gangloff et al., 2001). The clone fused to the Gal4 activation domain interacted specifically with the ANTP bait and not with the empty vector (Fig. 2B).

BIP2 specifically interacts with the YPWM motif

To identify the protein domain of ANTP that is essential for the interaction with BIP2, several deletion constructs of ANTP were assayed for interaction (Fig. 2A). The interaction between the YPWM full-length ANTP-HD (LexA-YPWM-HD, aa 279-348) and BIP2-235 is similar to the interaction observed using the ANTP YPWM-N bait used in the screen (Fig. 2B,C, compare lanes 1 and 2). Then, we compared the relative interactions of various ANTP baits carrying deletions and/or substitutions of the N-terminal arm of the HD and/or the YPWM motif to BIP2-235. Substituting the YPWM motif of the LexA-YPWM-N-term bait by four alanines (LexA-AAAA-N-term) reduced the interaction (Fig. 2B,C, compare lanes 2 and 3), whereas deleting the N-terminal arm (LexA-YPWM-) had only a mild effect on the interaction (Fig. 2B,C, compare lanes 2 and 4). By contrast, substituting the YPWM motif and deleting the N-terminal arm abolished the interaction (Fig. 2C, lane 5). Although the yeast two-hybrid analysis shows that the major interaction occurs with the YPWM motif and that the N-terminal arm of the HD exerts a minor effect only, BIP2-235 is able to interact with the complete ANTP HD lacking the YPWM motif, indicating additional interaction surfaces (data not shown).

BIP2 forms a complex with ANTP depending on the YPWM motif in vivo

In order to confirm the interaction found in vitro between ANTP and BIP2, and its YPWM-motif dependence, we used a co-immunoprecipitation assay. As shown in Fig. 2E, we were able to co-immunoprecipitate ANTP protein (detected with a anti-ANTP antibody) with BIP2 by using an anti-HA-affinity-matrix aimed at isolating a hemagglutinin (HA)-tagged BIP2 protein (BIP2-HA) from larval nuclear extracts (overexpressing wild-type *Antp* and *bip2-HA* upon heat shock treatment; HS-Gal4>UAS-*Antp*; UAS-*bip2-HA*; Fig. 2E, lane YHA). When the YPWM motif mutated version of ANTP ($ANTP^{AAAA}$) is co-expressed, no complex

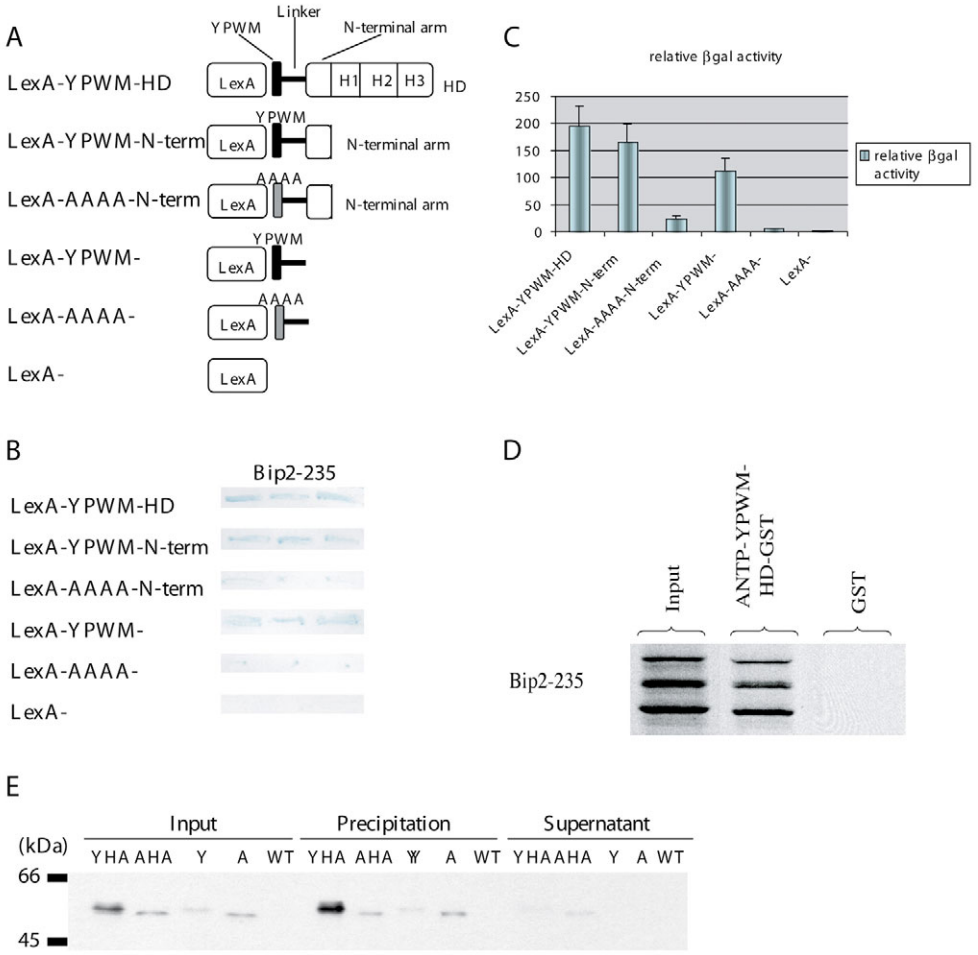

Fig. 2. *Drosophila* BIP2 interacts directly with ANTP via the YPWM motif. (**A**) Schematic of the *Antp* constructs used fused to the LexA DNA-binding domain. LexA-YPWM-HD consists of the YPWM motif, the linker region and the HD with α-helices 1-3 (H1-H3). LexA-YPWM-N-term consists of the YPWM motif, the linker and the N-terminal arm of the HD. LexA-AAAA-N-term is the same construct as LexA-YPWM-N-term with the YPWM motif substituted by four alanines. LexA-YPWM- consists of the YPWM motif and the linker region. LexA-AAAA- is the same construct as LexA-YPWM- with the YPWM motif substituted by four alanines. LexA- is an empty vector. (**B**) X-gal filter-lift experiment. (**C**) Relative β-gal activity. The β-gal experiment was repeated four times independently using three samples of each interaction tested. (**D**) Glutathione-S-transferase (GST) pull-down experiments. The ANTP-YPWM-HD-GST fusion protein (amino acid 279-356) was produced in *E. coli* and purified with glutathione sepharose beads. The BIP2-235 protein (amino acids 853-1088) was produced in a rabbit reticulocyte lysate and labelled with [^{35}S]-methionine. The BIP2 protein domain found in the yeast two-hybrid screen (BIP2-235) is also able to interact with ANTP-HD fused to GST in vitro. The synthesis of the BIP2-235 protein gives three bands, likely to be due to different methionine start codons used for protein synthesis by the reticulocyte lysate. (**E**) Co-immunoprecipitation of ANTP and BIP2. The BIP2 protein was tagged with the hemaglutinin (HA) epitope and immunoprecipitated with an anti-HA antibody. Co-immunoprecipitated ANTP protein was detected by using a mouse monoclonal anti-ANTP antibody. Upon mutating the YPWM motif of ANTP, ANTP is not co-immunoprecipitated with BIP2-HA, unlike the wild-type ANTP protein. The larvae used were hs>*bip2-HA*, *Antp* (YHA), hs>*bip2-HA*, *Antp*^{AAAA} (AHA), hs>*bip2*, *Antp* (Y), hs>*bip2*, *Antp*^{AAAA} (A), and wild type (WT), as a control.

between this modified ANTP protein and BIP2 is formed (Fig. 2E, lane AHA), which shows an in vivo requirement of the YPWM motif for the ANTP-BIP2 complex formation. As expected the ANTP^{AAAA} protein can be detected in the supernatant lane (Fig. 2E, lane AHA of the supernatant). To demonstrate the specificity of the interaction in the cell extracts, we co-expressed the wild-type and mutated ANTP protein in combination with BIP2 not fused to HA (HS-Gal4>UAS-*Antp*; UAS-*Bip2* and HS-Gal4>UAS-*Antp*^{AAAA}; UAS-*Bip2*). As seen in Fig. 2E lanes Y and A, the weak protein detection represents non-specific binding to the anti-HA-affinity matrix.

To further confirm the direct interaction between ANTP and BIP2, we performed glutathione S-transferase (GST) pull-down experiments. The isolated clone of the BIP2 protein, BIP2-235, was expressed in vitro in reticulocyte lysate and labelled with [^{35}S]-methionine. The protein was then tested for interaction with an ANTP protein consisting of the YPWM motif and the HD fused to GST (GST-ANTP-YPWM-HD, amino acids 279-356). The BIP2-235 protein interacted with the GST-ANTP-YPWM-HD fusion protein but not with GST alone (Fig. 2D). We also performed the above-mentioned experiment using a YPWM motif-mutated, GST-fusion protein. Owing to technical difficulties, we were not able to directly compare the interactions, but we could observe a weak interaction of the BIP2 protein and the YPWM motif-deleted ANTP HD (data not shown). Taken together, these results demonstrate that BIP2 is able to interact directly with ANTP, implicating the YPWM motif. Although the interaction is mainly due to the YPWM motif, other parts of the ANTP HD are also involved in the interaction.

bip2 acts as a co-factor of *Antp* by enhancing eye-to-wing transformation

BIP2 was first identified as a Bric-à-brac (BAB)-interacting protein encoding a TATA-box-binding protein associated factor (TAF) (Albright and Tjian, 2000), also referred as dTAFII3 or dTAFII155 (Gangloff et al., 2001). BIP2 is a member of the TFIID complex for transcription initiation and is the *Drosophila* homolog of TAF3. The BIP2 transcript and protein are ubiquitously expressed during embryogenesis and are widely expressed in all third instar imaginal discs (Gangloff et al., 2001).

We next addressed the question of a functional relevance of the BIP2/ANTP complex. Our results and findings from others (Gangloff et al., 2001) indicate a broad expression of BIP2 throughout development, including a maternal contribution, suggesting several functions played by this gene in various cells at different stages, rendering the analysis difficult. As no *bip2* mutants were available, we decided to generate mutants by the homologous recombination technique (Rong and Golic, 2000). The resulting *bip2* mutants have a 1.2 kb (–1.1 kb 5′ to +152 bp 3′ of the start ATG) deletion around the start ATG, deleting the first exon harbouring the Histone Fold Domain (HFD) (data not shown). The two alleles, $bip2^{3}$ and $bip2^{4}$, obtained are late embryonic or first instar larval recessive lethals without any obvious morphological defects (data not shown). The analysis of cuticles did not reveal any visible phenotype, as observed in HOX or *exd* mutations, probably because of the strong maternal contribution (data not shown). As *bip2* lies at position 102B on the fourth chromosome, we were not able to perform a germline or somatic clonal analysis, instead we used a gain-of-function approach to address the question of a functional relevance of *bip2* in *Antp*-induced transformations. Upon ectopic co-expression of *bip2* in combination with *Antp* and N^{act}, we found that co-expression of *bip2* strongly enhanced the penetrance of eye-to-wing transformation, whereas *exd* had the opposite effect (Table 1). About 80% of the flies carrying *ey*-Gal4; UAS-N^{act}; UAS-*Antp*; UAS-*bip2* transgenes showed eye-to-wing transformation compared with 40% without co-expression of *bip2* (Table 1). The opposite effect was observed when comparing the antenna-to-leg transformation. The co-expression of *bip2* in combination with *Antp* and N^{act} reduced the fraction of flies showing antenna-to-leg transformation from 28% to 13% (Table 1). No obvious effect of *bip2* could be observed in combination with the $ANTP^{AAAA}$ protein, neither on eye-to-wing nor on antenna-to-leg transformation (Table 1), indicating a requirement of the YPWM motif for the ANTP/BIP2 interaction and for the specificity observed by adding BIP2 protein. Owing to the fact that opposite effects are seen in combination with wild-type ANTP, and no effect with $ANTP^{AAAA}$, rules out an unspecific effect of BIP2 by either influencing the transcriptional machinery or sequestering the Gal4 protein. We conclude from these results that the phenotypes observed are due to the interaction of *Antp* with *bip2*, as transgenic flies expressing UAS-N^{act}; UAS-*bip2* under the control of *ey*-Gal4 showed the same phenotype as flies expressing UAS-N^{act} alone (data not shown). Interestingly, the ectopic wing formation is not entirely dependent on an activated form of the *N* receptor. Using the eye-specific OK-107-Gal4 driver to express *Antp* ectopically in the eye disc, we found 8% (10/132) of flies having an eye-to-wing transformation (Fig. 3G), in addition to the previously described eye-reduction phenotype (Fig. 3E,F). The penetrance of the eye-to-wing transformation is increased upon ectopic co-expression of *bip2* to 24% (27/111). No ectopic wings could be observed when the YPWM motif-mutated (AAAA) ANTP protein was expressed by the OK-107 enhancer, instead the flies showed a strong eye-reduction phenotype with a similar penetrance to the wild-type *Antp* transgene (data not shown). We can exclude the possibility that the YPWM motif dependence to induce eye-to-wing transformation is due to low protein levels, because the line shows a similar penetrance of the eye-reduction phenotypes as the wild-type *Antp* transgenic lines. Although we have never seen any ectopic wing formation using an $ANTP^{AAAA}$ mutant protein, we are not able to entirely exclude a quantitative rather than a qualitative effect of the YPWM motif. In order to address this question, we repeated the above described experiment using an additional UAS-*Gal4* insert to enhance the ectopic protein expression (OK-107-Gal4; UAS-*Gal4*; UAS-$Antp^{AAAA}$), but could never observe any eye-to-wing transformation. Instead a high degree of lethality was observed making the analysis difficult. These data strongly suggest that *bip2* acts as a co-factor of *Antp* promoting ectopic wing development.

The *Antp* allele $Antp^{Cephalothorax}$ allele shows eye-to-wing transformation

To find out whether the ANTP/BIP2 complex plays a role in vivo, we searched through the literature for *Antp* alleles inducing ectopic wing transformations. Scott et al. found some transformations

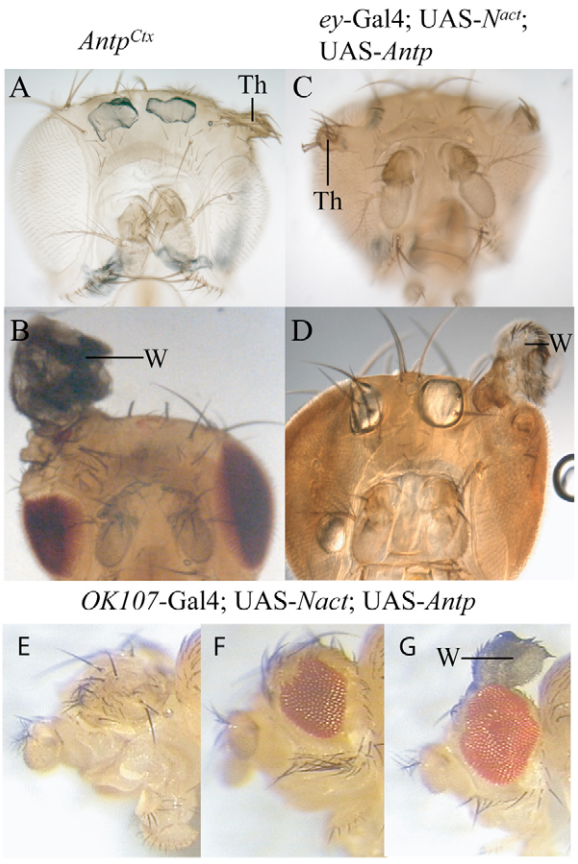

Fig. 3. $Antp^{Ctx}$ show similar adult phenotypes to ectopic *Antp* in combination with N^{act} in eye imaginal discs. (**A**,**B**) Head of $Antp^{Ctx}$ an adult showing an ectopic dorsal thoracic structure (Th in A) and wing tissue (W in B) on the dorsal side. (**C**,**D**) *ey*-Gal4>UAS-N^{act}; UAS-*Antp* head showing some ectopic thoracic tissue (Th in C) and a wing (W in D) growing out of the dorsal side. (**E**,**F**) Heads of OK-107-Gal4>UAS-*Antp* flies showing the eye reduction phenotype. (**G**) The addition of *bip2* increases the frequency of formation of ectopic wings (W) formed on the head of OK-107>UAS-*bip2*; UAS-*Antp* flies. *Antp* is able to induce ectopic wings without N^{act}.

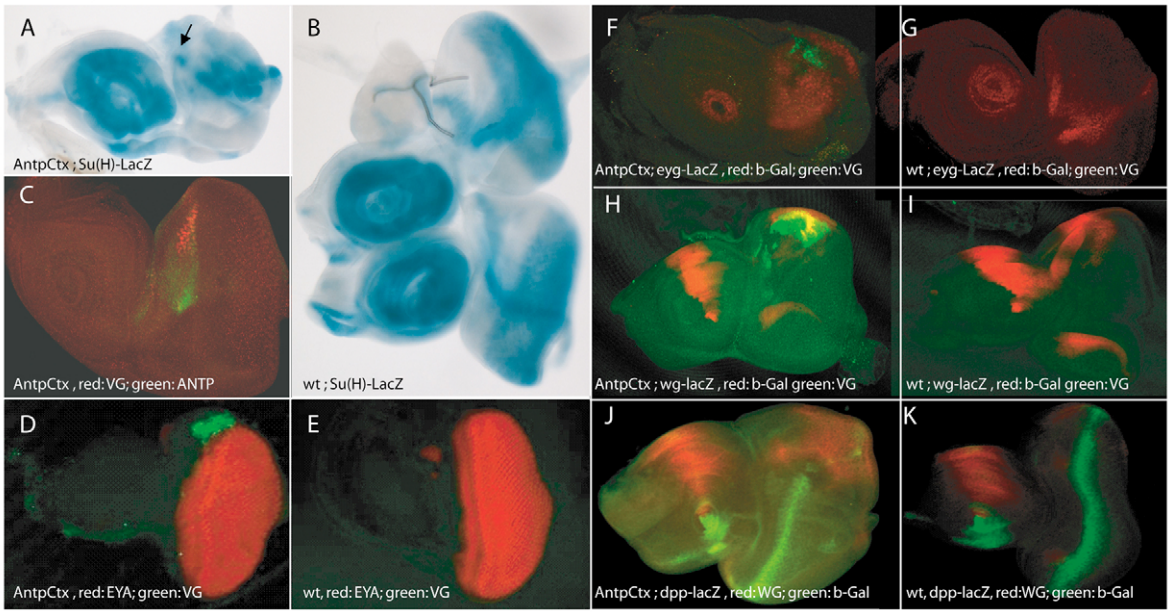

Fig. 4. The *Antp^{Ctx}* eye-antennal disc is transformed into various parts of the wing disc. (**A**) *Su(H)-lacZ* staining on an *Antp^{Ctx}* eye-antennal disc shows ectopic *N* signaling (arrow). (**B**) *Su(H)-lacZ* staining on wild-type eye-antennal discs showing active *Notch* signaling. (**C**) *Antp^{Ctx}* eye-antennal disc showing ectopic VG protein (red) and ectopic ANTP protein (green). (**D**,**F**,**H**,**J**) *Antp^{Ctx}* eye-antennal disc. (**E**,**G**,**I**,**K**) Wild-type eye-antennal disc. (D,E) Ectopic VG (green) represses EYA (red) cell non-autonomously in the *Antp^{Ctx}* eye-antennal disc (D). (F,G) Ectopic VG (green) and ectopic EYG (*eyg-lacZ*; β-Gal in red) in the *Antp^{Ctx}* eye-antennal disc (F) indicate a transformation toward notum identity. (H,I) Ectopic VG (green) is co-expressed with ectopic WG (*wg-lacZ*; β-Gal in red) in the *Antp^{Ctx}* eye-antennal disc (H). The localization of the ectopic VG protein corresponds to the dorsal eye region where the ectopic wings are formed. (J,K) Ectopic WG (red) is co-expressed with ectopic DPP (*dpp-lacZ*; β-Gal in red) in the *Antp^{Ctx}* eye-antennal disc, indicating transformation to wing disc cells (J).

caused by the *Antp^{Cephalothorax}* (*Antp^{Ctx}*) allele corresponding to the phenotypes obtained by co-expression of *Antp* in combination with *N^{act}* (Fig. 3A-D) (Scott et al., 1983), namely ectopic wings and ectopic thoracic outgrowths on the dorsal rim of the eye (Fig. 3A-D), head capsule-to-thorax (Fig. 5B) and antenna-to-leg transformations, and eye-reduction (Fig. 5B, Fig. 3B, data not shown). *Antp^{Ctx}* is an *Antp* gain-of-function allele with a chromosomal translocation between the *Antp* locus 84B1,2 and 35B, where Su(H) maps to (Scott et al., 1983). In order to characterize the molecular events involved in these transformations, we tested whether the *Notch* signalling pathway is activated in these transformed discs. Using a Su(H)-reporter construct [Su(H)-lacZ] indicating the activation of the Notch pathway (Furriols and Bray, 2001), we found the *N* signalling pathway ectopically activated in transformed *Antp^{Ctx}* eye imaginal discs (Fig. 4A,B). Additionally, ectopic ANTP and VG protein can be observed in the dorsal part of *Antp^{Ctx}* eye imaginal discs (Fig. 4C), where the ectopic wings will form (Fig. 3B, Fig. 5A). Consistent with the adult phenotype, the neuronal identity marked by the Eyes absent (EYA) protein is repressed in the transformed part of the discs, marked by the presence of VG protein (Fig. 4D,E). Interestingly, the repression of neural fate is non-cell autonomous to *vg* expressing cells, indicating that cells adjacent to the VG expressing clones are also transformed.

The *Antp^{Ctx}* allele transforms the eye imaginal disc into wing disc identity

The adult *Antp^{Ctx}* phenotypes suggest that the eye disc is transformed into wing disc tissue. In order to analyze this observation further, we used several different markers for wing disc identity, namely *wingless-lacZ* (*wg-lacZ*) and *eyegone-lacZ* (*eyg-lacZ*) markers for wing pouch and notum identity, respectively (Aldaz et al., 2003; Couso et al., 1994; Jang et al., 2003; Phillips and Whittle, 1993). As seen in Fig. 4F, reduced eye discs show broad ectopic *eyg* expression that is not co-expressed with ectopic VG, marking thoracic/notum identity. The co-expression of VG and *wg-lacZ* seen in Fig. 4H illustrates a transformation of the eye disc toward wing pouch identity. We find co-expression of SRF-β-Gal, a wing pouch marker, in the ectopic VG domain (data not shown). In agreement with the morphology of the eye imaginal disc, head capsule toward thorax transformation, marked by strong *eyg* expression, is strongly linked to eye-loss phenotype, as indicated by a reduced disc size (Fig. 5B). By contrast, ectopic wings, marked by VG and WG co-expression, are associated with slightly reduced eyes only and eye imaginal discs (Fig. 3B,D,G, Fig. 5A).

We further analyzed the expression of another signaling pathway, the *decapentaplegic* (*dpp*) pathway. Transdetermination experiments have revealed 'weak points' representing cells that are plastic and capable of altering their normal selector gene expression (Maves and Schubiger, 2003). These weak points can be defined by strong *dpp* expression. When *wg* is ubiquitously expressed in leg discs, cells with strong *dpp* expression (dorsal cells) are able to change fate and express VG protein, representing a leg-to-wing transdetermination (Maves and Schubiger, 2003). By analyzing the *Antp^{Ctx}* eye-antennal imaginal disc for ectopic *wg* and *dpp* expression, we could observe domains co-expressing both signalling molecules (Fig. 4J,K), indicating a general eye-to-wing transformation (transdetermination) induced by *Antp*.

These data suggest that the eye imaginal disc in *Antp^{Ctx}* flies is transformed into different parts of the wing disc, indicating a more general homeotic transformation toward T2.

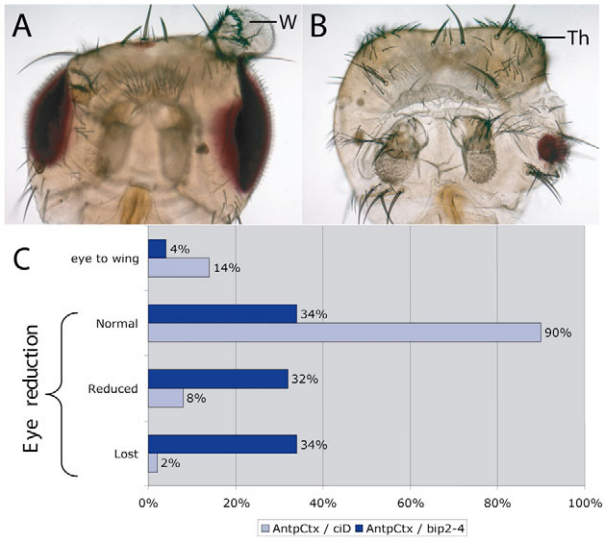

Fig. 5. The *bip2*^{4} mutant genetically interacts with *Antp*^{Ctx}. (**A**) Head of an *Antp*^{Ctx}/+; *ci*^{D}/+ fly with an ectopic wing (W) on the dorsal head. The wing shows the characteristic wing margin bristles. (**B**) Head of an *Antp*^{Ctx}/+; *bip2*^{4}/+ fly with the head capsule transformed into dorsal thorax (Th). One eye is missing and one eye is strongly reduced. Flies showing an ectopic wing on the dorsal head regularly show a normal or mildly reduced eye and no head capsule-to-thorax transformation. Flies with a head capsule-to-thorax transformation mostly show severely reduced or lost eyes. The eye reduction was therefore used as a measure of the two phenotypes shown above. (**C**) Analysis of eye-to-wing transformation and the strength of eye reduction in the progeny of *Antp*^{Ctx} females crossed to *yw; bip2*^{4}/*ci*^{D} males. The number of F1 flies counted were: *Antp*^{Ctx};+; *ci*^{D}/+, 233; *Antp*^{Ctx}/+; *bip2*^{4}/+, 234. Only 4% of the flies with a single *bip2* gene copy (*Antp*^{Ctx};+; *ci*^{D}/+) show ectopic wings on their dorsal head, compared with 14% with two wild-type *bip2* gene copies (*Antp*^{Ctx}/+; *bip2*$^{+}$/*bip2*$^{+}$; see Table 2). Normal (slightly reduced) sized eyes, reduced (strongly reduced) and missing eyes were counted. Flies with a single *bip2* gene copy show a stronger eye reduction phenotype than do flies harbouring two gene copies.

bip2 genetically interacts with *Antp*^{Ctx}

As the *Antp*^{Ctx} allele shows ectopic wing tissue on the head, we tested whether the newly created loss-of-function *bip2* mutant genetically interacts with the *Antp*^{Ctx} allele, by reducing the frequency of eye-to-wing transformations. We compared *Antp*^{Ctx}/+;*ci*^{D}/+ to *Antp*^{Ctx}/+;*bip2*^{4}/+ flies. After crossing *Antp*^{Ctx}/CyO females to *yw; bip2*^{4}/*ci*^{D} males, 14% of the *Antp*^{Ctx}/+; *ci*^{D}/+ flies show ectopic wings on the head as compared with 4% in *Antp*^{Ctx}/+; *bip2*^{4}/+ transheterozygous flies (Table 2, Fig. 5C). *bip2* also influences the eye-reduction phenotype. Comparing the different transheterozygous flies, we could not only observe a change in the penetrance of ectopic wing formation, but also a change in head phenotype (represented in Fig. 5A-C). Flies showing an ectopic wing on the head almost always show a normal or slightly reduced eye, whereas flies with a head capsule-to-thorax transformation mostly have reduced or lost eyes (Fig. 5A,B). In order to quantify these phenotypes, we monitored the eye phenotypes of the above mentioned transheterozygous flies. Reducing the gene dosage of *bip2* increases the eye reduction phenotype. *Antp*^{Ctx}/+; *bip2*^{4}/+ flies show stronger eye-reduction/eye-loss phenotypes than do *Antp*^{Ctx}/+; *ci*^{D}/+ flies. Ninety percent of the eyes of *Antp*^{Ctx}/+; *ci*^{D}/+ flies show a normal to weakly reduced eye phenotype, whereas only 34% of the eyes of *Antp*^{Ctx}/+; *bip2*^{4}/+ flies show a normal eye phenotype. By contrast, 34% of the eyes of *Antp*^{Ctx}/+; *bip2*^{4}/+ flies show an eye-loss phenotype, compared with 2% without the *bip2*^{4} mutation (Table 2, Fig. 5C). Similar results were obtained in the reciprocal cross: *yw; bip2*^{4}/*ci*^{D} females × *Antp*^{Ctx}/CyO males (data not shown). In conclusion, the *bip2* loss-of-function mutation reduces the frequency of *Antp*^{Ctx} flies showing ectopic wing outgrowth on the head and enhances the *Antp*-induced eye reduction phenotype – which we used as a measure for strong head capsule-to-thorax transformation. Consistent with the reduction of ectopic wings in a *bip2*^{4} mutant background, overexpression experiments of *bip2* show an increase of ectopic wing outgrowth in combination with *Antp* and *N*^{act} (see above).

We also tested whether *bip2*^{4} would have a similar effect on another *Antp* gain-of-function allele, *Antp*^{73b}. *Antp*^{73b} shows an almost complete antenna-to-leg transformation with dorsal head capsule-to-thorax transformation, but no ectopic wing structures (Garber et al., 1983). We could not observe any effect of *bip2*^{4} on the *Antp*^{73b} allele, in agreement with the gain-of-function experiment with *bip2* counteracting the antenna-to-leg transformation by *Antp*.

DISCUSSION

The YPWM motif is required for eye-to-wing transformation

In this study, we used a gain-of-function approach to express the homeotic selector gene *Antp* in combination with a constitutively active form of the *Notch* receptor. In this context, *N* prevents *Antp*-induced apoptosis in the eye and allows the cells to adopt a new developmental fate of the dorsal second thoracic segment, the wing. This peculiar situation allows the study of two *Antp*-dependent functions at the same time: the ventral antenna-to-leg and the dorsal eye-to-wing transformation. Using this approach, we found a differential requirement for the YPWM motif of ANTP: the YPWM motif of *Antp* is strictly required for the eye-to-wing, but less stringently required for the antenna-to-second leg transformation. A similar differential requirement of peptide motifs was also found for the YPWM motif of ABD-A (Merabet et al., 2003) and for the QA motif of UBX (Hittinger et al., 2005).

The addition of the well-known HOX co-factor *exd*, that has been shown to bind via the YPWM motif, antagonizes the eye-to-wing transformation, indicating a YPWM-motif-dependent *Antp* function, independent of *exd*. We cannot exclude the possibility that *exd* has an *Antp*-independent effect by repressing wing development, as is the case for leg development (Casares and Mann, 1998), but the overexpression of *exd* fused to a nuclear localization signal does not interfere with endogenous wing development (Jaw et al., 2000). We cannot distinguish whether deleting the YPWM motif of *Antp* changes its DNA-binding selectivity or whether *Antp* loses its transactivation potential, as the direct targets of *Antp* genes in the eye-to-wing transformation remain to be identified. Nevertheless,

Table 2. Percentage of ectopic wings and eye-reduction phenotype

Genotype	Eye-Wing	Normal eyes	Reduced eyes	Lost eyes
Antp^{Ctx}/+; *bip2*$^{+}$/*bip2*$^{+}$	14% (32/233)	90% (419/466)	8% (37/466)	2% (10/466)
Antp^{Ctx}/+; *bip2*^{4}/*bip2*$^{+}$	4% (10/234)	34% (159/468)	32% (150/468)	34% (159/468)

we favour the later possibility, although it was shown that mutating the YPWM motif of HOXA5 does not interfere with the transcriptional activity of the protein (Zhao et al., 1996).

We found *bip2* acting as an *Antp* co-factor for ectopic wing formation, linking *Antp* to an activating TFIID complex and to the basal transcriptional machinery. Previously it has been shown that HOX gene activity regulation might play in important role in HOX-dependent gene regulation (Li and McGinnis, 1999; Li et al., 1999; Merabet et al., 2003). *bip2* might also provide target gene specificity by linking *Antp* to a specific TFIID complex, which might confer specificity through promoter selectivity, as was shown for other TAF-complexes (Verrijzer and Tjian, 1996).

In summary, our data indicate that the YPWM motif is a more generally used protein-protein interaction interface interacting with at least two, but probably more protein co-factors, judging from the numerous *exd*-independent HOX functions that have been found.

bip2 acts as a co-factor of *Antp* promoting eye-to-wing transformation

The YPWM-motif dependence of the *Antp*-specific eye-to-wing transformation implies the existence of a novel YPWM-motif-interacting protein, as the YPWM motif is considered to be a protein-interaction domain. Using the yeast two-hybrid system, we found a new ANTP-interacting protein, encoded by the *bip2* gene (Gangloff et al., 2001), *Drosophila* TBP-associated factor 3 (dTafII3/dTAFII$_1$55; BIP2 – FlyBase). Several lines of evidence indicate that *bip2* might be a novel *Antp* co-factor interacting with the YPWM motif. (1) In our gain-of-function experiments, *bip2* behaves as an *Antp* co-factor promoting ectopic wing development, and the *bip2* loss-of-function mutation genetically interacts with the *Antp* allele $Antp^{Ctx}$, reducing the frequency of eye-to-wing transformations. (2) *bip2* acts as a co-factor for an *Antp* function requiring the YPWM motif. (3) BIP2 interacts in vitro with the YPWM motif in a yeast two-hybrid assay and shows an in vivo requirement of the YPWM motif for the ANTP-BIP2 interaction in a co-immunoprecipitation assay.

bip2 (*dTAFII3*) is a member of the TBP-associated TFIID complex in the basal transcriptional machinery, and belongs to the class of histone-like TATA-binding protein (TBP)-associated factors (TAF) with two homologues in yeast, humans and mice (Gangloff et al., 2001). The *bip2* gene codes for a protein with two distinct domains, a Histone Fold Domain (HFD) at the N terminus and a Plant Homeodomain (PHD) finger at the C terminus. The HFD is a domain initially found in histones involved in the formation of histone dimers (Aasland et al., 1995), whereas the PHD has been recently shown to specifically interact with three-methylated histone H3 at lysine 4 (Li et al., 2006; Pena et al., 2006; Shi et al., 2006; Wysocka et al., 2006). BIP2 forms a histone-like dimer with TAF10 (dTAFII24) (Gangloff et al., 2001). This dimer formation is conserved from yeast to humans (Gangloff et al., 2001). *bip2* and its homologues have been identified as members of the TBP-containing TFIID complex (Gangloff et al., 2001), linking ANTP to the basal transcriptional machinery. But, BIP2 might also be a part of a TBP-free TAF-containing complex (TFTC), a histone acetyl transferase complex (HAT). The human homologue of BIP2 and TAF10, and TAF10 itself are found to co-immunoprecipitate with GCN5 (PCAF – FlyBase), the acetyl transferase of the TFTC HAT complex (Georgieva et al., 2000; Grant et al., 1998; Martinez et al., 1998; Ogryzko et al., 1998; Wieczorek et al., 1998), and BIP2 harbours a PHD domain implicated in reading specific histone codes (Li et al., 2006; Pena et al., 2006; Shi et al., 2006; Wysocka et al., 2006). Furthermore, *Drosophila* has two paralogous genes encoding TAF10 homologues, *Taf10* and *Taf10b*, which are differentially expressed during development (Georgieva et al., 2000). BIP2 specifically forms a dimer with TAF10 and not with TAF10b (Gangloff et al., 2001); TAF10 was found to be present in both TFIID and TFTC-like complexes, whereas TAF10b was only identified in TFIID complexes (Georgieva et al., 2000). These results raise the possibility of ANTP being linked to a histone acetylase complex. The link unravelled between *Antp* and *bip2* raises numerous questions, including which complex incorporating *Antp* is present to perform its wing promoting function? Interestingly, Katsuyama and co-workers found a novel gene *winged eye* (*wge*) implicated in the eye-to-wing transformation (Katsuyama et al., 2005). *wge* seems to be downstream of *Antp* in the developmental process of eye-to-wing transformation (Katsuyama et al., 2005). *wge* codes for a bromo-adjacent homology domain (BAH)-containing protein (Katsuyama et al., 2005). The BAH domain has frequently been associated with other domains, such as bromodomains, PHD fingers, and Suppressor of variegation 3-9, Enhancer of zeste and Trithorax (SET) domains, in proteins that are suggested to be involved in the epigenetic regulation of gene expression (Callebaut et al., 1999). This indicates that epigenetic regulation of so far unknown genes is involved in eye-to-wing transformation.

Antp is able to induce ectopic wings

It has previously been shown that *Antp* in combination with N^{act} is able to induce ectopic wings by transforming eye-to-wing tissue (Kurata et al., 2000). Although endogenous wing development is considered to be independent of *Antp* (Carroll et al., 1995), we found that *Antp* is the only HOX gene tested so far that is able to transform the eye into wing, which is in line with the fact that *Antp* specifies the entire second thoracic segment. Furthermore, $Antp^{Ctx}$ is the only homeotic gain-of-function allele found that induces ectopic wings on the head (Scott et al., 1983).

Several lines of evidence indicate that *N* supports *Antp* in inducing ectopic wings, by preventing eye cells from undergoing apoptosis and in allowing them to adopt a new developmental fate. First, wings formed by ectopic expression of *Antp* in combination with N^{act}, or wings found on $Antp^{Ctx}$ heads, show the same characteristic triple row of bristles at the wing margin (Fig. 4B,C). These bristles are found only when *vg* is ectopically co-expressed in combination with *wingless* (*wg*), not when in combination with N^{act} (Baena-Lopez and Garcia-Bellido, 2003). Second, we found that *N* alone does not induce ectopic wings, and that eye-to-wing transformation can also be achieved without the action of *N*, by using another eye-specific driver, OK-107-Gal4 (see Hauck et al., 1999), indicating that *N* is not absolutely required for ectopic wing induction. Using different markers for different parts of the wing disc, we found parts of the eye disc to be transformed into most wing disc identities from wing pouch to notum, indicating an eye-to-dorsal T2 transformation, rather than the eye-to-wing pouch transformation seen in adult flies.

The known HOX co-factors *exd* and *hth* code for DNA-binding proteins that have been shown to increase DNA-binding specificity. *bip2*, however, encodes a member of the basal transcriptional machinery without any DNA-binding capacity, indicating a different mechanism of action, i.e. by linking *Antp* directly to the transcriptional machinery. In summary, we propose that ANTP interacts directly with BIP2, activating, in turn, a subset of genes that are implicated in wing development.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/9/1669/DC1

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