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RESEARCH ARTICLE

The retinal determination gene *dachshund* restricts cell proliferation by limiting the activity of the Homothorax-Yorkie complex

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ABSTRACT

The *Drosophila* transcriptional co-activator protein Yorkie and its vertebrate orthologs YAP and TAZ are potent oncogenes, whose activity is normally kept in check by the upstream Hippo kinase module. Upon its translocation into the nucleus, Yorkie forms complexes with several tissue-specific DNA-binding partners, which help to define the tissue-specific target genes of Yorkie. In the progenitor cells of the eye imaginal disc, the DNA-binding transcription factor Homothorax is required for Yorkie-promoted proliferation and survival through regulation of the *bantam* microRNA (miRNA). The transit from proliferating progenitors to cell cycle quiescent precursors is associated with the progressive loss of Homothorax and gain of Dachshund, a nuclear protein related to the Sno/Ski family of co-repressors. We have identified Dachshund as an inhibitor of Homothorax-Yorkie-mediated cell proliferation. Loss of *dachshund* induces Yorkie-dependent tissue overgrowth. Conversely, overexpressing *dachshund* inhibits tissue growth, prevents Yorkie or Homothorax-mediated cell proliferation of disc epithelia and restricts the transcriptional activity of the Yorkie-Homothorax complex on the *bantam* enhancer in *Drosophila* cells. In addition, Dachshund collaborates with the Decapentaplegic receptor Thickveins to repress Homothorax and Cyclin B expression in quiescent precursors. The antagonistic roles of Homothorax and Dachshund in Yorkie activity, together with their mutual repression, ensure that progenitor and precursor cells are under distinct proliferation regimes. Based on the crucial role of the human *dachshund* homolog DACH1 in tumorigenesis, our work suggests that DACH1 might prevent cellular transformation by limiting the oncogenic activity of YAP and/or TAZ.

KEY WORDS: Dachshund, Yorkie, Homothorax, *Drosophila* eye development, Progenitor proliferation, Organ growth

INTRODUCTION

Correct organ development relies on the balance between cell proliferation, differentiation and death. The developing *Drosophila* eye is a powerful model with which to study these mechanisms and their integration because the progression through different differentiation stages and its coordination with cell proliferation and death is particularly obvious in the eye primordium (also known as the eye imaginal disc) of late instar larvae (Baker, 2007; Wolff and Ready, 1991). The eye disc derives from a group of cells set aside during embryogenesis that grows by random proliferation during the first two larval stages. It is during the third and last larval stage (L3) that retinogenesis starts at the posterior of the primordium, driven by a moving differentiation wave, called the morphogenetic furrow (MF), that progresses from posterior to anterior (Wolff and Ready, 1991). The first step in the differentiation process is the repression of the TALE-class homeodomain transcription factor *homothorax* (*hth*) in a fraction of progenitors by the Hedgehog (Hh) and the Decapentaplegic (Dpp, an ortholog of vertebrate BMP2/4) signals produced by cells at the MF (Bessa et al., 2002; Lopes and Casares, 2010). These naïve and proliferating progenitors transit through a few synchronous mitotic rounds (the first mitotic wave, FMW) into G1 quiescent cells, called eye precursors. Next, precursor cells are reached by the MF and either enter directly the differentiation pathway or are induced by those postmitotic differentiating cells to undergo a last terminal mitosis (or second mitotic wave, SMW) to finally differentiate (Wolff and Ready, 1991). Therefore, in the L3 eye primordium, three different modes of proliferation coexist along the anterior-posterior axis of the primordium: (1) asynchronous in progenitors, (2) G1 synchronized precursors anterior to the MF and (3) patterned mitoses during terminal differentiation behind the MF (see Fig. 1 for a schematic).

Among several genes and pathways that have been implicated in regulating cell proliferation of the eye disc (Kumar, 2011), the Hippo (Hpo) signaling pathway plays a central role. This pathway regulates organ growth in both *Drosophila* and mammals and has been implicated in cancer as a tumor suppressor pathway (Pan, 2010). At its core, there are the two Ser/Thr kinases – Hpo and Warts (Wts) (Udan et al., 2003; Wu et al., 2003b). When activated, this core kinase module retains the transcriptional co-activator Yorkie (Yki) in the cytoplasm, preventing Yki from executing its transcriptional program. Upon its translocation into the nucleus, Yki – which itself lacks a DNA-binding domain – forms complexes with several tissue-specific DNA-binding partners, which help to define the tissue-specific target genes of Yki. In the wing and eye imaginal discs, Yki interacts with the TEAD family transcription factor Scalloped (Sd), relieving the repressive effect of Sd on the Hpo target genes *Death-associated inhibitor of apoptosis 1* (*Diap1*), *expanded* (*ex*) and C*yclin E* (C*ycE*) (Koontz et al., 2013; Wu et al., 2008; Zhang et al., 2008). Yki also interacts with the Smad family DNA-binding transcription factor Mad to control the expression of the *bantam* (*ban*) microRNA (miRNA) in the wing imaginal disc (Oh and Irvine, 2011). Finally, Yki also forms a complex with Hth and, together, they promote proliferation and survival of eye progenitor cells by regulating directly the expression of, at least, *ban* (Nolo et al., 2006; Oh and Irvine, 2011; Peng et al., 2009; Slattery et al., 2013; Thompson and Cohen, 2006). In this tissue, although *ban* expression also requires Sd, Hth has no

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major effects on *Diap1*, *ex* and CycE expression (Peng et al., 2009; Zhang et al., 2008).

The transition from eye progenitors to precursors, in addition to the synchronization of the cell cycle, also implies an increase in the expression of the *eyes absent* (*eya*), *sine oculis* (*so*) and *dachshund* (*dac*) retinal determination genes, which are repressed by Hth in progenitors cells (Bessa et al., 2002). Their three products have been proposed to form a protein complex (Chen et al., 1997). Despite this fact, the function of *dac* is not identical to that of *eya* and *so*. Unlike *eya* or *so* (Bonini et al., 1993; Cheyette et al., 1994; Serikaku and O'Tousa, 1994), *dac* seems largely dispensable for retinal differentiation after it has been initiated, even though Dac expression is maintained at high levels throughout this process by the Hh and Dpp signals (Firth and Baker, 2009; Mardon et al., 1994). Molecularly, *dac* encodes a nuclear protein with two human homologs, DACH1 and DACH2 (Chen et al., 1997; Davis et al., 1999; Ikeda et al., 2002; Kim et al., 2002; Li et al., 2002). Dac can bind double-stranded nucleic acids (Kim et al., 2002) and is capable of activating transcription of a reporter gene in yeast (Chen et al., 1997). However, no consensus binding site or direct transcriptional target has yet been identified for Dac. Recent work indicates that DACH genes act as tumor suppressors. DACH1 expression is reduced in prostate, breast and uterine cancer, correlating with tumor progression and invasiveness (Popov et al., 2010). DACH1 inhibits oncogene-mediated breast oncogenesis in part by repressing cyclin D1 through a c-Jun DNA-binding partner (Sunde et al., 2006; Wu et al., 2006, 2007), and it prevents breast tumor stem cell expansion (Wu et al., 2011). Moreover, DACH1 expression inhibits DNA synthesis and growth in colony-forming assays in breast and prostate cancer cells and has been shown to restrict the transcriptional activity of the hormone receptors by recruiting the NCoR and HDAC1 co-repressors (Wu et al., 2009). These results point to a role of *dac*/DACH genes in cell proliferation and/or survival.

Here, we have investigated whether *dac* has a role in controlling tissue growth during *Drosophila* eye development. We have found that Dac limits tissue growth by regulating Yki-Hth activity at multiple levels. It restricts the transcriptional activity of the Yki-Hth complex and cooperates with Dpp signaling to repress *hth* expression. The antagonistic roles of *dac* and *hth* on Yki-driven proliferation together with the mutual repression between *hth* and *dac* ensure that progenitor and precursor cells are under different proliferation regimes.

RESULTS

Dac expression is complementary to that of Hth and spans the G1 precursor domain

In L3 eye discs, the expression patterns of Dac and Hth are complementary (Bessa et al., 2002; Fig. 1). Dac is expressed at low levels in proliferating progenitors, which express high levels of Hth and undergo an extended G2 phase detected by CyclinB (CycB) accumulation (Lopes and Casares, 2010). In cells approaching the MF, Dac expression increases, coinciding with the loss of Hth and CycB at the FMW. High Dac expression is detected in the G1-quiescent progenitors, straddling the MF and the SMW, but fades again coinciding with the accumulation of Hth in retinal accessory cells (Fig. 1A-A‴). Therefore, the transit from proliferating progenitors to cell cycle quiescent precursors is associated with the switch from Hth to Dac expression. However, during this transition, Hth and Dac transiently overlap at intermediate expression levels, coinciding with the FMW (Fig. 1B-C).

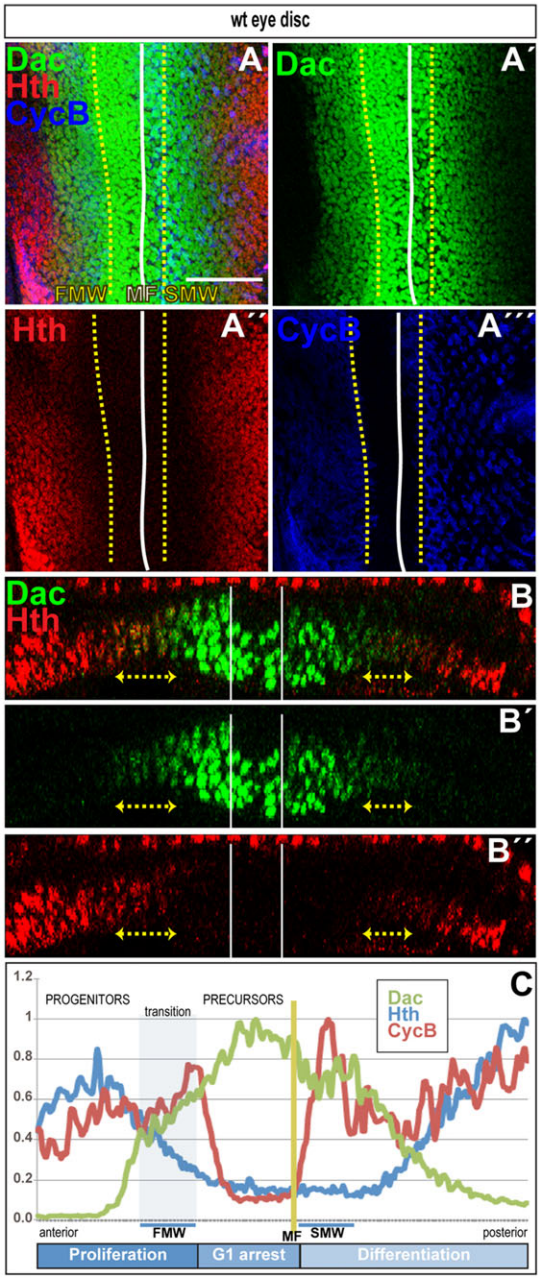

Fig. 1. Non-proliferative cells express high levels of Dac and lose Hth. (A-B″) L3 eye primordium showing the expression patterns of Dac (green in A,A′,B,B′), Hth (red in A,A″,B,B″) and CycB (blue in A,A‴). (A-A‴) Standard confocal section with anterior to the left and dorsal up on this and all subsequent eye disc panels. (B-B″) Cross section through the eye primordium with anterior to the left and apical side up. The morphogenetic furrow (MF) is indicated by the white lines. The first (FMW) and second mitotic wave (SMW) are marked by the yellow dashed lines in A-A‴. The double arrows on each side of the MF in B-B″ represent the transition domain co-expressing Dac and Hth. Scale bar: 50 µm. (C) Expression profiles of Dac (green), Hth (blue) and CycB (red) along the eye disc in A-A‴ and B-B″.

Dac prevents Hth-induced cell proliferation

The complementarity between the Hth and Dac expression patterns, and the fact that Hth has been shown to repress the premature upregulation of *dac* (Bessa et al., 2002) prompted us to test the functional significance of this repression. We first analyzed whether the repression of *dac* is necessary for Hth to promote proliferation. In agreement with this possibility, GFP-marked *hth*-expressing

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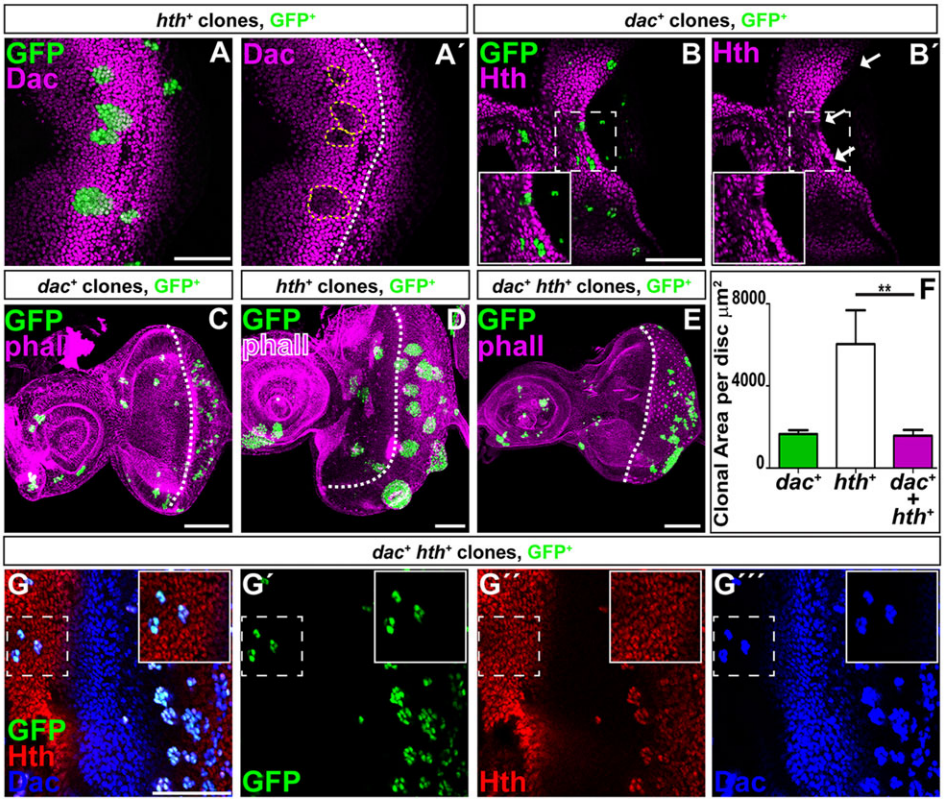

Fig. 2. Dac is sufficient to repress Hth function and expression. All panels except F show L3 eye imaginal discs, containing clones positively labeled with GFP (green) and expressing (A,A′,D) UAS-*hth*, (B-C) UAS-*dac* or (E,G-G‴) UAS-*hth* plus UAS-*dac*. Discs are stained with (A,A′) anti-Dac (magenta) or (B,B′) anti-Hth (magenta) or (G,G‴) anti-Dac (blue) and anti-Hth (red) or (C-E) phalloidin (phall), to outline tissue shape. The dashed lines in A′,C-E indicate the MF. Yellow dashed lines in A′ outline clones in A positively labeled with GFP. The crops in B,B′,G-G‴ are magnifications of the region delimited by the dashed lines. Scale bars: 50 µm. (F) Surface area in µm^2 of clones induced in the eye imaginal disc and expressing UAS-*dac*, UAS-*hth* or both. The mean for UAS-*dac* is 1658 µm^2 (n=10), for UAS-*hth* is 6035 µm^2 (n=7) and for UAS-*dac* plus UAS-*hth* is 1576 µm^2 (n=12). Error bars indicate s.e.m. **P=0.003 (Student's t-test.).

clones showed decreased levels of Dac in the precursor domain (Fig. 2A,A′). In the converse experiment, in which *dac* was overexpressed in GFP-marked clones, only small clones, comprising just two to three cells, were recovered (Fig. 2B-C) and in those recovered in the proliferative *hth*-expressing domain, Hth expression was cell-autonomously lost (arrows in Fig. 2B′). These clones were undergrowing compared to GFP control-marked clones (supplementary material Fig. S1), indicating that Dac is sufficient to reduce cell proliferation. Because Hth is required for the survival and proliferation of eye progenitor cells (Bessa et al., 2002; Peng et al., 2009), the growth defect of *dac*-expressing clones might result from the loss of *hth*. However, re-expressing *hth* in *dac*-overexpressing clones did not restore cell proliferation in the progenitor domain (Fig. 2E,F). On the contrary, overexpressing *dac* reduced the overgrowth of *hth*-expressing clones to levels comparable to those of *dac*-only expressing clones (Fig. 2F, P=0.003; and compare E to D). Because in these clones, *dac* overexpression did not affect the nuclear localization of ectopic Hth (Fig. 2G-G‴), Dac is therefore capable of reducing cell proliferation independently of its effect on *hth* expression.

To determine whether the repressive effect of Dac on Hth-induced proliferation is specific to the eye epithelium, we analyzed the effect of overexpressing *dac* and *hth* in the wing imaginal disc, where endogenous Dac protein is expressed only in a few restricted patches (Mardon et al., 1994). *hth*-expressing clones were recovered in the prospective hinge and notum areas of the wing imaginal disc, where *hth* is normally expressed (Azpiazu and Morata, 2000; Casares and Mann, 2000; supplementary material Fig. S2B). However, clones ectopically expressing either *dac* alone or both *dac* and *hth* grew poorly, no matter where they were induced in the wing disc (supplementary material Fig. S2A-D). Therefore, Dac reduces cell proliferation independently of its effect on *hth* expression, and it acts parallel to or downstream of Hth in imaginal epithelia.

Dac restricts Yki-mediated overgrowth

The fact that Hth promotes progenitor growth through Yki (Peng et al., 2009) raised the possibility that Dac also represses Yki-induced cell proliferation. We therefore investigated this possibility by analyzing the functional interaction between Dac and Yki. Whereas *dac*-overexpressing clones grew poorly (Fig. 3A; supplementary material Fig. S1), clones overexpressing *yki* overgrew throughout the eye disc (Fig. 3B,D). However, growth of *yki*-overexpressing clones was significantly suppressed when cells also overexpressed *dac* (Fig. 3C,D; P=0.008). Overexpressing *dac* also limited the overgrowth of *hpo* mutant clones in this tissue (Fig. 3K, P=0.0342; and compare J to I,H). To rule out the possibility that Dac blocks the ability of Yki to promote cell proliferation by repressing *hth*, we analyzed the ability of Dac to repress the growth of clones overexpressing both *yki* and *hth*. Even in the presence of a GFP-tagged form of Hth, *dac* overexpression was still able to strongly suppress the growth of *yki*+ clones (Fig. 3G, P=0.0024, and compare F and F‴ to E and E‴).

We also analyzed the effect on Yki activity of expressing *dac* ectopically in the wing disc. Overexpressing *yki* with the *nubbin*-Gal4 (*nub*-Gal4) driver, which targets the wing pouch and the inner ring (Azpiazu and Morata, 2000), doubled the size of the *nub* domain when compared with that of control *nub*-Gal4 discs expressing GFP (*nub>GFP*, supplementary material Fig. S2F,H; $P<10^{-4}$). Consistent with previous observations (Ziosi et al., 2010), Hth is required for Yki-dependent tissue growth in this tissue, as reducing Hth levels using double-stranded RNA interference (dsRNAi) constructs (*hth-IR*+) partially but significantly suppressed the growth of *nub>yki*-overexpressing wing discs (supplementary material Fig. S3, $P<10^{-4}$). This effect likely resulted from a reduction in Yki-mediated cell proliferation in the inner ring, as Hth expression is restricted to this region within the *nub*-Gal4 domain (Casares and Mann, 2000). In contrast to

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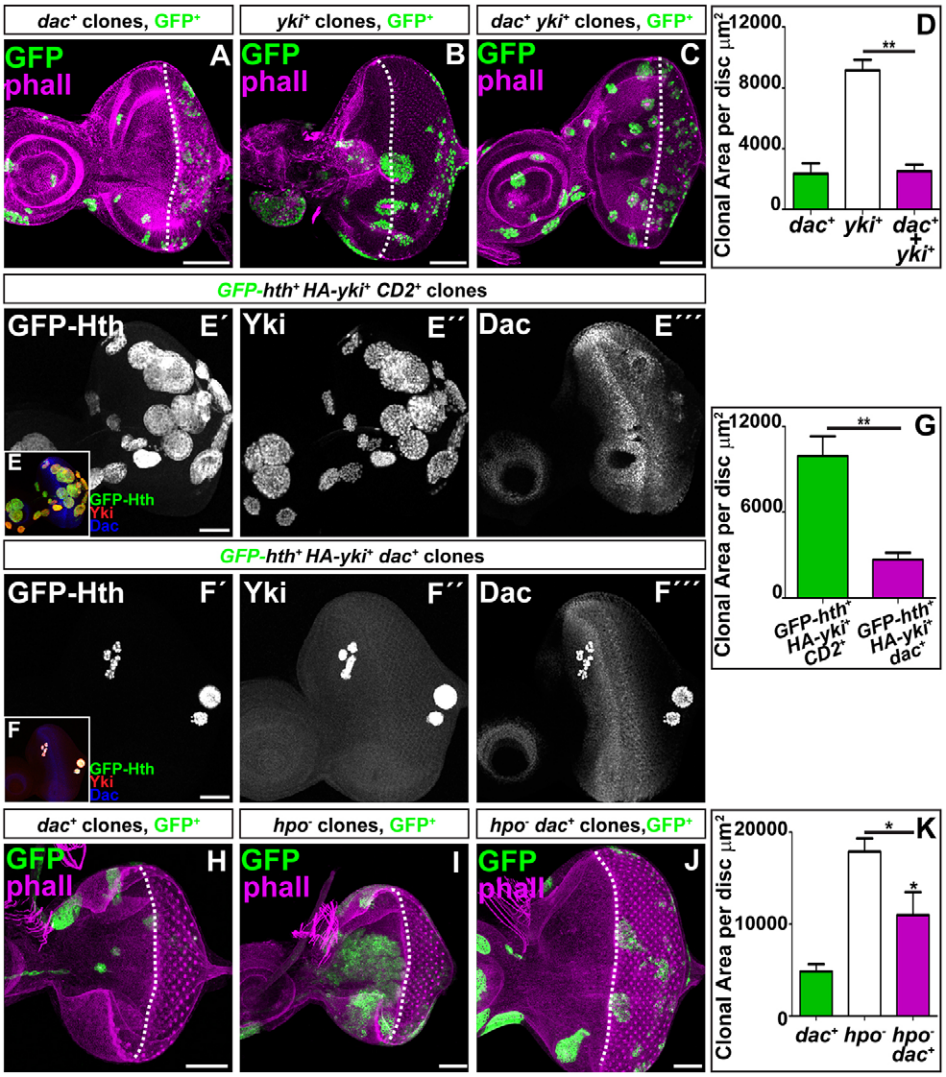

Fig. 3. Dac blocks Yki-induced tissue growth. All panels, except D,G and K, show L3 eye imaginal discs containing clones positively labeled with (A-C,H-J) GFP (green) or (E-F‴) GFP-Hth (green in E,F; gray in E′,F′) and expressing (A,H) UAS-*dac*, (B) UAS-*yki*, (C) UAS-*dac* plus UAS-*yki*, (E-E‴) UAS-*yki*, UAS-*GFP-hth* and UAS-*CD2* or (F-F″) UAS-*yki*, UAS-*GFP-hth* and UAS-*dac*, or (I) mutant for the $hpo^{42\text{-}47}$ allele or (J) mutant for the $hpo^{42\text{-}47}$ allele and expressing UAS-*dac*. Discs are stained with (A-C,H-J) phalloidin (phall, magenta) to outline tissue shape or (E-F‴) anti-Yki (red in E,F; gray in E″,F″) and anti-Dac (blue in E,F; gray in E‴,F‴). The dashed lines in A-C,H-J indicate the MF. Scale bars: 50 µm. (D) Surface area of clones expressing UAS-*dac*, UAS-*yki* or both. The mean for UAS-*dac* is 2359 µm^2 (n=17), for UAS-*yki* is 9159 µm^2 (n=2) and for UAS-*dac* plus UAS-*yki* is 2517 µm^2 (n=59). Error bars indicate s.e.m. **P<0.008 (Student's t-test). (G) Surface area of clones expressing UAS-*yki*, UAS-*GFP-hth* and UAS-*CD2* or UAS-*dac*. The mean for UAS-*yki*, UAS-*GFP-hth* and UAS-*CD2* is 9924 µm^2 (n=27), and for UAS-*yki*, UAS-*GFP-hth* and UAS-*dac* is 2681 µm^2 (n=11). Error bars indicate s.e.m. **P<0.0024 (Student's t-test). (K) Surface area of clones expressing UAS-*dac* or mutant for $hpo^{42\text{-}47}$ or mutant for $hpo^{42\text{-}47}$ and expressing UAS-*dac*. The mean for UAS-*dac* is 4854 µm^2 (n=10), for $hpo^{42\text{-}47}$ is 17,870 µm^2 (n=22) and for UAS-*dac* and $hpo^{42\text{-}47}$ is 11,000 µm^2 (n=6). Error bars indicate s.e.m. *P=0.0342 between clones mutant for $hpo^{42\text{-}47}$ with or without UAS-*dac* or P=0.0128 between clones with UAS-*dac* and mutant or wild type for *hpo* (Student's t-test).

overexpressing *yki*, expressing *dac* ectopically with *nub*-Gal4 reduced the size of this domain by half compared with that of *nub>GFP* controls (supplementary material Fig. S2E,H; $P<10^{-4}$) and significantly suppressed the overgrowth resulting from *yki* overexpression (supplementary material Fig. S2G,H; $P<10^{-4}$). The presence of *dac* was also able to prevent the growth of clones co-expressing *yki* and a *GFP*-tagged form of *hth* (supplementary material Fig. S4; P=0.0019). Dac is unlikely to inhibit the overgrowth by promoting apoptosis of *yki*-overexpressing cells, because we detected similar low levels of apoptosis in *nub>dac, nub>yki* and *nub>dac+yki* wing discs (supplementary material Fig. S2I-L′). Taken together, we conclude that Dac blocks the ability of Hth and Yki to promote cell proliferation. Dac might act downstream of or in parallel to Yki-Hth. Alternatively, Dac might restrict the activity of the Yki-Hth transcriptional complex.

Dac restricts tissue growth by limiting Yki function

To investigate whether Dac is required to limit tissue growth in the eye disc, we analyzed the effects of loss of Dac function in clones of a null allele. *dac* mutant clones located in internal regions of the primordium showed a delay in the onset of photoreceptor differentiation and abnormal ommatidial arrangements, as reported previously (Mardon et al., 1994). When we compared the size of *dac* mutant clones, marked by the absence of GFP, with that of their wild type 'twin' clones, marked by two copies of GFP, we found that *dac* mutant clones were, on average, 1.7 times larger than the twin clones (P=0.0069; Fig. 4A,B). Thus, *dac* restricts tissue growth in the eye disc.

If one of the functions of Dac is to restrict the ability of the Hth-Yki complex to promote cell proliferation, decreasing Yki levels should suppress the growth of *dac* mutant cells. To test this possibility, we downregulated Yki function in two ways. First, we knocked Yki expression down by the use of a dsRNAi construct (*yki-IR+*). *yki-IR+* clones were seldom recovered in the anterior region of the eye primordium and, when recovered, were very small (Fig. 4D,I). When *dac* mutant clones were simultaneously *yki-IR+*, the recovery rate and size of the clones was reduced to that of *yki-IR+*- only clones (Fig. 4I, $P<10^{-4}$; and compare E to C). Second, we overexpressed the upstream Hpo pathway regulator *ex*, as increased Ex levels are expected to block Yki activity by promoting its phosphorylation (Hamaratoglu et al., 2006; Hariharan and Bilder, 2006; McCartney et al., 2000) and through direct binding (Badouel et al., 2009; Oh et al., 2009). As expected, clones overexpressing *ex* grew poorly (Fig. 4G,I), and suppressed the growth of *dac* mutant clones (Fig. 4I, $P<10^{-4}$; and compare H and F). We conclude that Yki function is required for the growth of *dac* mutant tissues.

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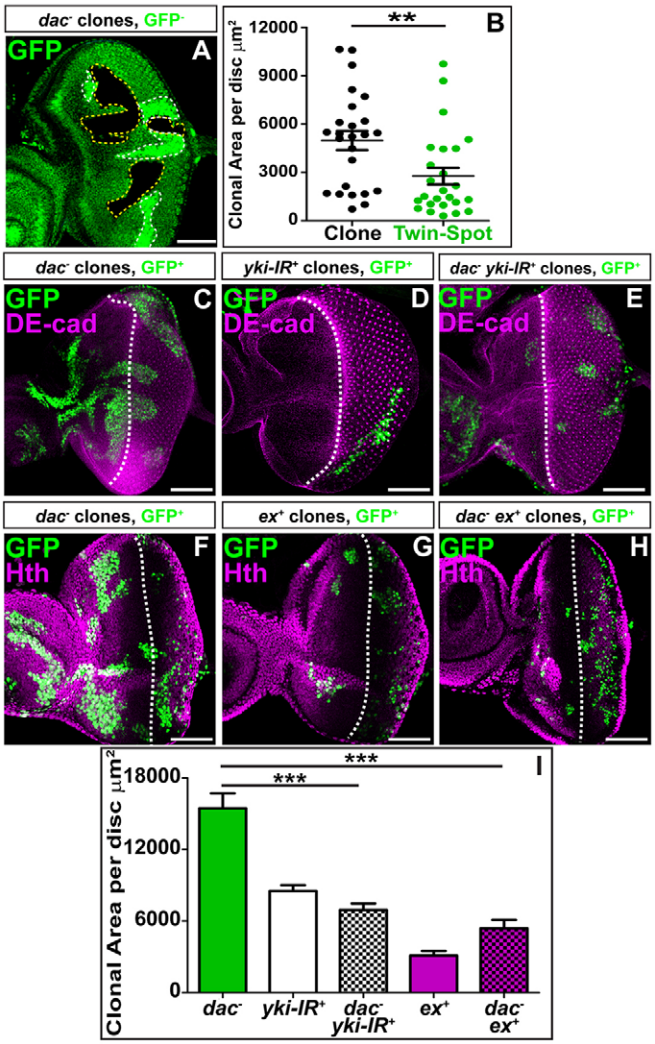

Fig. 4. Overgrowth of *dac* mutant tissues requires Yki. All panels, except B and I, show L3 eye discs. (A) *dac*^{3} mutant clones marked by the absence of GFP (green) and outlined by yellow dashed lines. Wild-type twin spots are outlined by white dashed lines. (B) Surface area of *dac*^{3} mutant clones (no GFP) and their wild-type twin spots (two copies of GFP) per disc in µm^{2}. The mean for *dac*^{3} is 4987 µm^{2} and for wild-type twin spot is 2775 µm^{2} (*n*=25). Error bars indicate s.e.m. ***P=0.0069 (Student's *t*-test). (C-H) Clones labeled with GFP (green) and (C,F) mutant for *dac*^{3} or (D) expressing UAS-*yki-IR*$^{4005R-2}$, (E) mutant for *dac*^{3} and expressing UAS-*yki-IR*$^{4005R-2}$, (G) expressing UAS-*ex* or (H) mutant for *dac*^{3} and expressing UAS-*ex*. Discs are stained with (C-E) anti-DE-Cad to outline tissue shape (magenta) or (F-H) anti-Hth (magenta). The dashed lines indicate the MF. Scale bars: 50 µm. (I) Surface area in µm^{2} of clones for the five genotypes in C-H. The mean for *dac*^{3} is 15,470 µm^{2} (*n*=17), for UAS-*yki-IR* is 8519 µm^{2} (*n*=23), for *dac*^{3}, UAS-*yki-IR* is 6939 µm^{2} (*n*=29), for UAS-*ex* is 3103 µm^{2} (*n*=35) and for *dac*^{3}, UAS-*ex* is 5389 µm^{2} (*n*=8). Error bars indicate s.e.m. ***P<10^{-4} (Student's *t*-test).

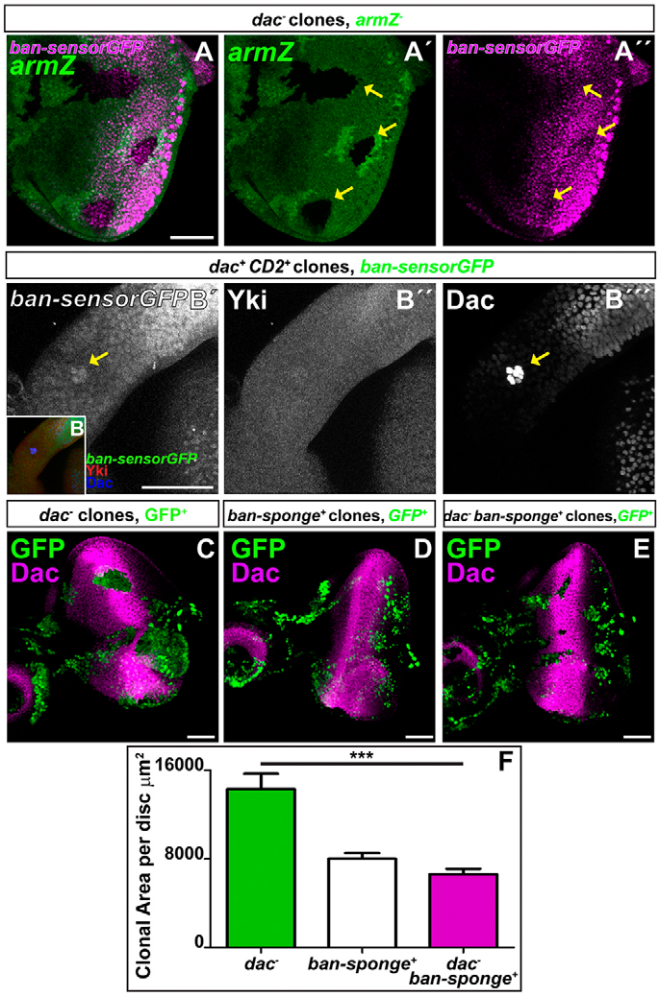

Fig. 5. *ban* expression is affected by Dac levels and is required for the growth of *dac* mutant clones. All panels, except F, show L3 eye discs. (A-A″,B-B‴) Discs expressing the GFP-*ban* sensor (magenta in A,A″, green in B and gray in B′) and containing (A-A″) *dac*^{3} mutant clones marked by the absence of *arm*-LacZ (green in A,A′) or (B-B‴) clones expressing UAS-*dac*, stained with anti-Yki (red in B and gray in B″) and anti-Dac (blue in B and gray in B‴). Yellow arrows in A′, A″, B and B‴ indicate the (A′,A″) reduction or (B,B‴) accumulation of the GFP *ban* sensor in clones mutant for *dac* or overexpressing *dac*, respectively. (C-E) clones labeled with GFP (green) and (C) mutant for *dac*^{3} or (D) expressing UAS-*ban-sponge* or (E) mutant for *dac*^{3} and expressing UAS-*ban-sponge*. Discs are stained with anti-Dac (magenta). Scale bars: 50 µm. (F) Surface area in µm^{2} of clones for the three genotypes in C-E. The mean for *dac*^{3} is 14,300 µm^{2} (*n*=22), for UAS-*ban-sponge* is 8026 µm^{2} (*n*=45) and for *dac*^{3}, UAS-*ban-sponge* is 6618 µm^{2} (*n*=29). Error bars indicate s.e.m. ***P<0.0001 (Student's *t*-test).

Dac restricts tissue growth by inhibiting *ban* expression

Hth and Yki together ensure the maintenance and proliferation of the eye progenitor population by up-regulating *ban* (Peng et al., 2009). We therefore tested whether Dac restricts Yki-Hth-dependent cell proliferation by preventing *ban* expression. To do so, we used a GFP *ban* sensor that is repressed by the *ban* miRNA. Consequently, the GFP levels are inversely proportional to the levels of the *ban* miRNA (Brennecke et al., 2003). Thus, in wild-type eye discs, the expression of the GFP ban sensor was complementary to the expression of a *lacZ* enhancer trap insertion in the *ban* locus (*ban*-LacZ) (supplementary material Fig. S5A-A″), with low expression levels in proliferating progenitors and higher levels in differentiating photoreceptors (Peng et al., 2009). *dac* mutant clones recovered posterior to the MF showed reduced expression of the GFP ban sensor (Fig. 5A-A″), associated with an increase in *ban*-LacZ levels (supplementary material Fig. S5B-B″). By contrast, loss of *dac* had only weak effects on the expression of Yki target genes that require the Yki DNA-binding partner Sd but not Hth (Peng et al., 2009; Zhang et al., 2008). Thus, a *lacZ* enhancer trap insertion in the *Diap1* gene (*Diap1*-LacZ) was only weakly upregulated at the border of some *dac* mutant clones located in internal regions of the primordium (supplementary material Fig. S5C-C″). CycE levels were not significantly affected by

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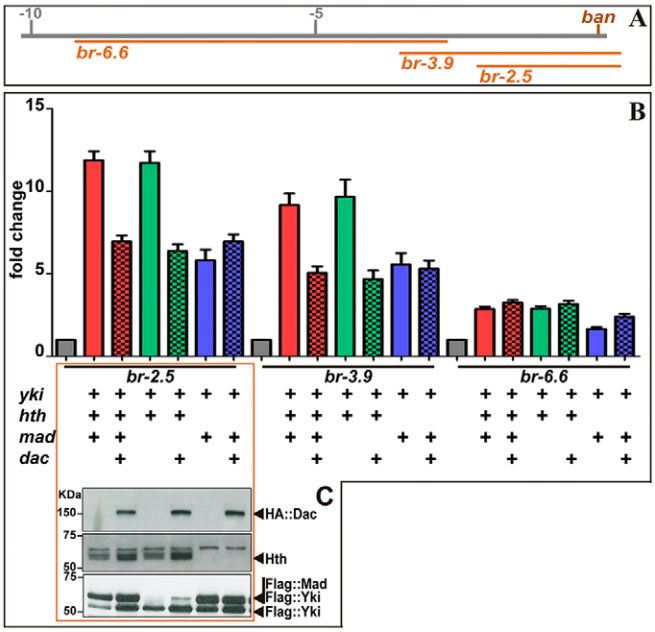

Fig. 6. Dac limits Yki-Hth-mediated transcriptional stimulation on restricted *ban* enhancers. (A) Schematic of the *ban* locus. Orange lines indicate the *ban* reporter constructs (Oh and Irvine, 2011) used to perform the transcriptional assays in B. (B) Graph of the fold changes of firefly luciferase over *Renilla* luciferase control in Dmel cell extracts transfected with the indicated *ban* luciferase reporters and the nuclear factors Yki, Hth, Mad and Dac. Error bars indicate s.e.m.; *n*=4. (C) Western blots from Dmel cell extracts transfected with the indicated nuclear factors Yki, Hth, Mad and Dac and blotted with (upper panel) anti-HA, which marks HA::Dac, (middle panel) anti-Hth or (lower panel) anti-Flag, which marks Flag::Yki (upper and lower bands) and Flag::Mad (upper band).

removing *dac* function (supplementary material Fig. S5E-E‴), whereas Ex levels were slightly reduced (supplementary material Fig. S5D-D″). Conversely, clones expressing *dac* ectopically in the progenitor domain upregulated the GFP ban sensor (Fig. 5B-B‴). Furthermore, expressing *dac* ectopically along the anterior-posterior boundary of the wing disc, using the *patched*-Gal4 (*ptc*-Gal4) driver, resulted in increased levels of *ban* sensor activity along this domain (supplementary material Fig. S6, compare B and B′ to A). Thus, Dac inhibits *ban* expression.

We then tested whether the effects of *dac* on growth depend on *ban* levels. *dac* mutant clones expressing a *bantam-sponge* (*ban-sponge*) tagged with *dsRed*, which prevents direct *ban* mRNA cleavage (Becam et al., 2011), did not grow better than wild-type clones expressing the *ban-sponge* (Fig. 5F, and compare D to E). Moreover, the presence of the *ban-sponge* suppressed the growth of *dac* mutant clones (Fig. 5F, $P<0.0001$; and compare E to C). Conversely, expressing *ban* ectopically restored the growth of *nub>dac*-overexpressing tissues (supplementary material Fig. S6C-F). We conclude that Dac restricts tissue growth in the eye disc mainly by inhibiting *ban* expression.

Dac inhibits Yki-Hth-mediated transcriptional stimulation of *ban*

We next investigated the mechanism by which Dac restricts the expression of *ban* by testing the effect of Dac on the *ban* enhancers *br-2.5*, *br-3.9* and *br-6.6* (Fig. 6A; Oh and Irvine, 2011). Their transcriptional activity was measured by luciferase assays in Dmel cells transfected with Yki and Hth or with Mad or with both Hth and Mad in the absence or presence of Dac, as Dmel cells do not express *dac* (data not shown). As reported previously (Oh and Irvine, 2011), cotransfection of Yki and Hth or Mad stimulated the transcription of luciferase driven by all three *ban* enhancers (Fig. 6B). Interestingly, cotransfecting Mad did not enhance the Yki-Hth-dependent expression of luciferase on either of the enhancers, indicating that the Yki-Mad and Yki-Hth complexes do not cooperate in *ban* expression. Strikingly, the presence of cotransfected Dac significantly reduced the Yki-Hth-dependent transcriptional stimulation of the *br-2.5* and *br-3.9 luciferase* reporters from 11.7- to 6.4-fold ($P=0.0006$) and from 9.7- to 4.7-fold ($P=0.0052$), respectively (Fig. 6B). However, although the *br-6.6 luciferase* reporter also responded to Yki-Hth, luciferase levels were not affected by cotransfected Dac, suggesting that Dac restricts Yki-Hth-dependent transcriptional stimulation in an enhancer-specific manner. The inhibitory effect of Dac on the expression of the *br-2.5* and *br-3.9* reporters was not due to Dac-mediated loss of Hth, as cotransfecting Dac did not downregulate Hth (Fig. 6C). Moreover, this effect depended on Hth, as expressing *dac* did not affect the Yki-Mad-mediated transcriptional stimulation on any of the three *ban* enhancers (Fig. 6B). Co-transfected Dac also significantly reduced the Yki-Hth-dependent transcriptional stimulation of the *br-2.5 luciferase* reporter in DL2 cells, from 6.9- to 3.9-fold ($P<0.0001$), but not the basal expression level of this reporter. This effect requires both Yki and Hth, as Dac did not affect the Yki- or Hth-dependent transcriptional stimulation of this reporter (supplementary material Fig. S7). Taken together, these results suggest that Dac represses the transcriptional activity of Hth and Yki on restricted *ban* enhancers.

Dac and Tkv cooperate to restrict proliferation and Hth expression in the precursor domain

Because ectopic Dac represses Hth expression, we asked whether Dac ensures the G1 synchronization in precursor cells prior to photoreceptor differentiation by restricting Hth expression. However, unlike loss of *eya*, which caused Hth accumulation (supplementary material Fig. S8A-A″), loss of *dac* in the internal region of the eye primordium was not sufficient to derepress Hth (supplementary material Fig. S8B-B″; Fig. 7A,A′), nor to cause CycB accumulation in the G1 domain (Fig. 7D,D′). Thus, the overgrowth of *dac* mutant clones does not result from ectopic Hth expression but likely from de-repression of Yki-Hth transcriptional activity in the transition domain where Hth and Dac overlap ahead of the MF.

Because Dpp has been shown to contribute to Hth repression (Bessa et al., 2002; Lopes and Casares, 2010), we investigated whether removing the function of the Dpp receptor Thickveins (Tkv) in *dac* mutant clones was sufficient to fully de-repress Hth and allow proliferation in the precursor domain. As reported previously (Lopes and Casares, 2010), cells mutant for *tkv* could still downregulate Hth (Fig. 7B,B′) and CycB (Fig. 7E,E′), indicating that they become arrested in G1. By contrast, double mutant clones for *tkv* and *dac* accumulated Hth (Fig. 7C,C′) and CycB (Fig. 7F,F′) even in cells close to the MF. We conclude that Dac and Tkv synergize to repress Hth and ensure the transition between progenitor and precursor cells, promoting the entrance into G1 prior to photoreceptor differentiation.

DISCUSSION

In this report, we show that Dac controls tissue growth in the eye disc by regulating Yki-Hth activity at multiple levels. It counteracts Yki-Hth-dependent *ban* expression and cooperates with Dpp signaling to repress *hth* expression (Fig. 8).

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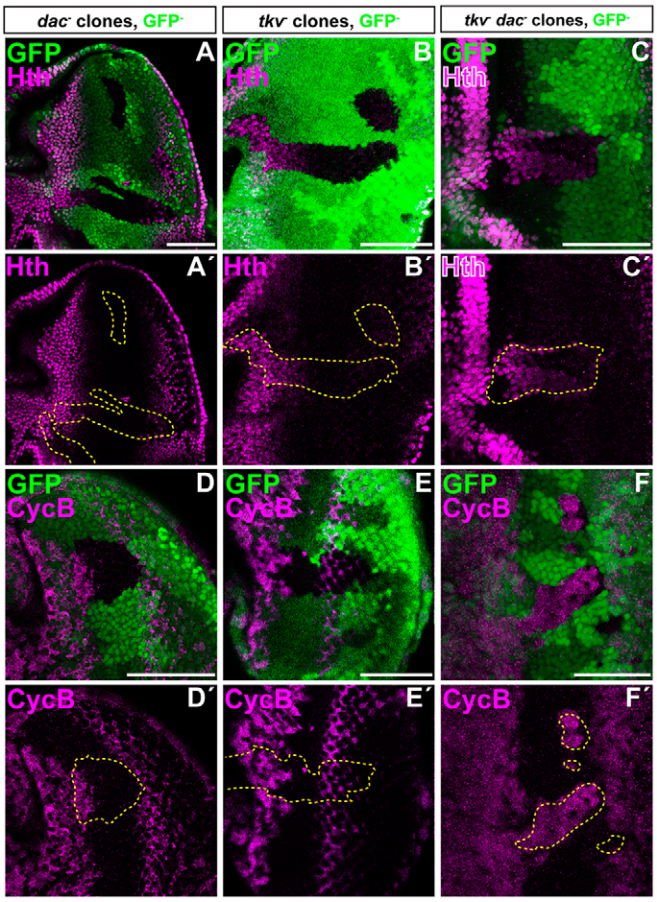

Fig. 7. Loss of *dac* and *tkv* derepresses Hth and CycB. All panels show L3 eye imaginal discs. Mutant clones for (A,A′,D,D′) dac^3, (B,B′,E,E′) tkv^4 or (C,C′,F-F′) both tkv^4 and dac^3, marked by the absence of GFP (green in A,B,C, D,E,F) or outlined by yellow dashed lines in A′,B′,C′,D′,E′,F′. Discs are stained with (A-C′) anti-Hth (magenta) or (D-F′) anti-CycB (magenta). Scale bars: 50 µm.

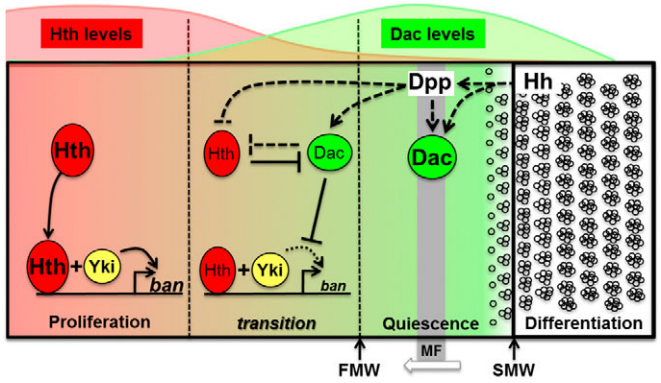

Fig. 8. Model of the mechanism by which Dac controls cell proliferation in the eye disc by regulating Hpo pathway activity at multiple levels. Prior to photoreceptor differentiation, Hh expressed by photoreceptor cells triggers Dpp expression in the MF (in gray). Together, they induce high *dac* expression (Firth and Baker, 2009) and in concert with Dac, prevent *hth* expression to promote cell cycle quiescence in this domain. In the transition domain, where Dac and Hth are expressed at intermediate levels, Dac restricts the transcriptional activity of the Yki-Hth complex on Yki targets such as *ban* to limit cell proliferation. By contrast, in the proliferation domain, Hth expressed at high levels prevents *dac* expression and, together with Yki, promotes *ban* expression to allow for cell proliferation.

Dac is an inhibitor of Yki-Hth transcriptional activity

Our work demonstrates that Dac does not act as a general Yki inhibitor, but rather limits the ability of the co-transcription factor Yki and the DNA-binding partner Hth to activate transcription on the enhancers of growth-promoting genes, such as *ban*. First, Dac acts in parallel to or downstream of Yki and Hth, as Dac is capable of preventing Yki- and/or Hth-dependent cell proliferation (Figs 2, 3; supplementary material Figs S2, S4). Second, Dac limits the expression of *ban* (Fig. 5; supplementary material Figs S5, S6), a direct target of Yki-Hth (Peng et al., 2009) (Fig. 6). Third, Yki and its downstream target *ban* promote growth in *dac* mutant tissue (Figs 4, 5). Fourth, in Dmel and DL2 cells, Dac restricts Yki-Hth- but not Yki-Mad-mediated transcriptional stimulation (Fig. 6). Fifth, like Hth, Dac has no major effect on Ex, *Diap1* and CycE expression (supplementary material Fig. S5).

To exert this function, Dac could be directly recruited to Yki targets through binding to Hth. Alternatively, Dac might be brought to other DNA-binding sequences to counteract Yki-Hth-dependent gene expression. The finding that the *br-6.6* enhancer responds to Yki-Hth but not to Dac (Fig. 6) gives support to this second possibility. Moreover, DACH1 has been shown to interact with a DNA-binding sequence that resembles the FOX (Forkhead box-containing protein) binding site (Zhou et al., 2010), but also counteracts the effect of Ras, ErbB2 and Myc on the cyclin D1 promoter through binding to c-Jun or CREB (Sunde et al., 2006; Wu et al., 2006, 2007). Because DACH1 interacts with the nuclear receptor co-repressor (NCoR), mSin3A and histone deacetylases (HDACs) (Popov et al., 2009; Song et al., 2003; Tskvitaria-Fuller et al., 2003; Wu et al., 2003a,b), the interaction of Dac with Hth might bring general co-repressors of the transcriptional machinery to the *ban* enhancers.

Surprisingly, Dac not only limits *ban* expression in the presumptive thorax where Yki and Hth promote tissue growth (supplementary material Fig. S3; Ziosi et al., 2010), but also affects the expression of the *ban* sensor in the wing blade (supplementary material Fig. S6). In this tissue, Yki has been shown to control *ban* expression through its interaction with Sd and Mad (Oh and Irvine, 2011; Slattery et al., 2013). Although Dac has no effect on Yki-Mad-mediated transcriptional stimulation of the *ban* enhancers *br-2.5* and *br-3.9* (Fig. 6), in the wing blade, ectopic Dac might limit *ban* expression by restricting the activity of the Yki-Mad or Yki-Sd complexes on additional enhancers. In addition, Dac appears to regulate the expression of distinct sets of patterning genes depending of the cellular context, as *dac* is also required for establishing the segmental pattern of Notch ligand and fringe expression in the leg imaginal disc, for sensory organ, genitalia and sex comb development and for proper neuronal differentiation (Atallah et al., 2014; Keisman and Baker, 2001; Martini et al., 2000; Miguel-Aliaga et al., 2004; Okamoto et al., 2012; Rauskolb, 2001).

Dac and Hth control the pattern of cell proliferation in the eye disc

In the progenitor domain, Dac levels are kept low as a result of high levels of Hth expression (Bessa et al., 2002). This regulation sustains the Yki-Hth-induced proliferation of progenitors (Peng et al., 2009), which would otherwise be restricted by the inhibitory effect of Dac. In cells approaching the MF, while Hth is repressed, Dac is upregulated (Fig. 1). During this transition, both Dac and Hth are transiently co-expressed, and Dac might alter the transcriptional properties of Yki and Hth on specific target genes, such as *ban*. Interestingly, although Hth has been reported to regulate the

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proliferation of progenitor cells in conjunction with Yki (Peng et al., 2009), our data argue that Hth additionally contributes to this proliferation by repressing Dac, because Dac could potentially block Yki function even in the presence of Hth (Fig. 3). In addition, we have found that Dac is sufficient to repress Hth (Fig. 2). However, in the transition from proliferating progenitors to cell cycle quiescent precursors, Dac, expressed at high levels by Dpp and Hedgehog (Hh) (Firth and Baker, 2009), requires the Dpp signaling pathway to synergistically repress Hth expression (Fig. 7). Thus, inhibitory interactions involving Dac and Hth coexist prior to photoreceptor differentiation at two levels, transcriptional and functional: Hth represses *dac*; this repression is alleviated by Dpp produced at the MF and reinforced by increasing levels of Dac. In addition, in the transition domain, where both Dac and Hth are co-expressed (Fig. 1), Dac inhibits the transcriptional activity of Hth and Yki. These multiple inhibitory interactions might constitute a double safe mechanism to guarantee that the transition from progenitors to precursors is perfectly coupled to the change in the proliferative status (high to low *yki* activity) prior to photoreceptor differentiation. Consistently, altering Dac or Hth levels affects tissue growth (Figs 2, 3). Moreover, Dac and Dpp signaling are together necessary to ensure the synchronous exit of progenitors from the FMW, guaranteeing that precursors are all in the G1 phase (Fig. 7). In this way, the Dac-dependent change in the proliferation status would allow proper neuronal differentiation. Dac might also regulate the ordered progression of neurogenesis in the nervous system. In the optic lobe, as in the eye disc (Fig. 1), Dac expression levels inversely correlate with Yki-dependent cell proliferation during the transition from proliferating neuroepithelial cells into differentiating lamina neurons (Kawamori et al., 2011).

Dac or DACHs and cancer

Altered expression of the human Dac ortholog DACH1 has been reported in a variety of human tumors (Popov et al., 2010). DACH1 has an instructive role in preventing proliferation, because its expression inhibits oncogenic transformation of breast epithelial cell lines (Wu et al., 2006) and breast tumor stem cell expansion (Wu et al., 2011). Based on the high levels of conservation between *Drosophila* Yki and Dac and their human counterparts, our work suggests that mammalian DACH proteins might also inactivate the transcriptional activity of YAP and/or TAZ (also known as WWTR1) on a subset of target genes by interacting with specific YAP or TAZ DNA-binding partners. In agreement with this possibility, DACH1 and YAP or TAZ regulate cyclin D1 expression (Wu et al., 2006, 2007; Zhou et al., 2011). Therefore, we propose that oncogenic transformation associated with increased YAP or TAZ transcriptional activity could result not only from perturbation of YAP or TAZ localization or levels (Pan, 2010), but also from inactivation of the tissue-specific DACH co-repressors.

MATERIALS AND METHODS

Fly strains and genetics

The fly stocks used were dac^3, UAS-dac^F (Mardon et al., 1994), UAS-*HA:dac*^{F} (Tavsanli et al., 2004), eya^{E8} (Bonini et al., 1998), tkv^4, $hpo^{42–47}$ (Wu et al., 2003b), UAS-*hth-GFP* (Casares and Mann, 2000), UAS-*yki* (Huang et al., 2005), UAS-*yki:HA* (Sidor et al., 2013), UAS-*yki-IR*$^{4005R-2}$ (NIG), UAS-*hth-IR* (VDRC #12763), UAS-*ex* (Udan et al., 2003), *GFP-ban* sensor (Brennecke et al., 2003), *ban*-lacZ (Spradling et al., 1999), UAS-*ban*$^{EP(3)3622}$ (Rorth et al., 1998), UAS-*ban-sponge* (Becam et al., 2011), *Diap1::lacZ* (Hay et al., 1995), *ptc*-Gal4 (Tang and Sun, 2002), *nub*-Gal4 (Calleja et al., 1996) and *da*-Gal4 (Wodarz et al., 1995). Mutant clones for dac^3 or tkv^4 or tkv^4, dac^3 or eya^{E8} marked by the absence of GFP or *arm-lacZ* were generated through mitotic recombination (Xu and Rubin, 1993). The MARCM technique (Lee and Luo, 1999) was used to induce clones expressing UAS-*yki-IR*$^{4005R-2}$ or UAS-*ex* or mutant for hpo^{42-47} and expressing UAS-*dac*. Larvae were heat-shocked for 1 h at 37°C between 48 and 72 h after egg laying. Gain-of-function experiments using UAS-*dac*, UAS-*hth-GFP* or UAS-*yki* were performed either using the flip-out method for clonal analysis (Struhl and Basler, 1993) or using the *nub*-Gal4 driver for expression in the prospective blade and distal hinge regions of the wing disc. Crosses carrying UAS-*dac* and the corresponding controls were raised at 18°C, whereas others were raised at 25°C. To analyze the effect of *dac* on *ban* activity, *ptc*-Gal4 females were crossed to w^-; *GFP-ban* sensor/CyO; UAS-*dac*/TM6B males.

Immunohistochemistry

Imaginal discs were dissected and fixed according to standard protocols. The primary antibodies used were mouse anti-Dac (1:100, mAbdac2.3, DSHB), rabbit anti-CycB (1:10, Jacobs et al., 1998), guinea pig anti-CycE (1:1000, a gift from T. Orr-Weaver, Whitehead Institute, Cambridge, MA, USA), guinea pig anti-Hth (1:5000, Casares and Mann, 1998), rabbit anti-Hth (1:500, Kurant et al., 1998), mouse anti-GFP (1:1000, A11120, Invitrogen), rabbit anti-GFP (1:1000, A11122, Invitrogen), mouse anti-β-galactosidase (1:200, Z378B, Promega), rabbit anti-β-galactosidase (1:1000, 55976, Cappel), rabbit anti-activated caspase 3 (1:150, 9661L, Cell Signaling), rat anti-DE-Cad (1:50, CAD2, DSHB), rabbit anti-Ex (1:200, a gift from A. Laughon, University of Wisconsin, Madison, WI, USA) and rat anti-Yki (1:200, Genevet et al., 2010). Rhodamine-conjugated phalloidin (Sigma) was used at a concentration of 0.3 µM. Fluorescently labeled secondary antibodies were from Jackson Immunoresearch (1:200). Imaging was carried out on Leica SP2 or SP5 confocal microscopes.

Quantification

The NIH ImageJ program was used to perform measurements. The surface area of *dac* mutant clones and their sibling twin spots was calculated in µm^2 for each clone outlined separately. The total area of clones positively marked by GFP was calculated as the sum of GFP signals per disc area. The surface area of wing disc blades was calculated as the ratio of the surface area of the *nub>GFP* or *nub>dac*-expressing area over the total surface area in µm^2 for each disc. Statistical analysis was performed with GraphPad Prism5 software, using Student's *t*-test.

Molecular biology

Genomic DNA extracted from UAS-*HA-dac*^{F} flies using the NucleoSpin® Tissue kit (Macherey-Nagel) was used to amplify *HA-dac* using the oligonucleotides 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTC-GAAGGAGATAGAACCATGGATTCTGTGACAAGTGAACAG-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTATTAGTTGGCGCT-GCCGAAG-3′ and the Phusion DNA Polymerase (Thermo Scientific). PCR products were then inserted in pDONR™221 using the Gateway BP method (Invitrogen). After sequencing the pEntry-*dac*, Gateway LR reaction was performed to transfer *HA-dac* into pAHW.

Drosophila cell assays and western blotting

Schneider *Drosophila* line 2 (DL2) cells obtained from Dr Paul Scotti (Horticulture Research, Auckland, NZ) and kindly provided by L. Teixeira (Instituto Gulbenkian de Ciência, Portugal) were cultured in Schneider's medium supplemented with 10% fetal bovine serum and 1× penicillin-streptomycin (Gibco). Dmel-2 cells (Invitrogen) were kindly provided by M. Bettencourt-Dias (Instituto Gulbenkian de Ciência, Portugal) and cultured in Express Five® SFM medium with 2 mM L-glutamine and 1× penicillin-streptomycin (Gibco). Transient transfections in six-well plates and luciferase reporter assays were performed using Effectene (Qiagen) and the Dual Luciferase Assay System (Promega), respectively, according to the manufacturer's instructions. Two independent experiments were performed in duplicate. 125 ng of *pAc5.1-Hth* (Culi and Mann, 2003), *pAc5.1-3xFlag::Mad*, *pGL3-br-6.6-luciferase*, *pGL3-br-3.9-luciferase*, *pGL3-br-2.5-luciferase* (Oh and Irvine, 2011), *pAct5C-3xFlag::Yki* (Zhang et al., 2008), *pAFW*, *pAHW* and *pAct5C-HA::Dac* and 25 ng of *pAct5C-3xFlag::Renilla-luciferase* (Invitrogen) were transfected and cells were incubated for 72 h (Dmel cells) or 48 h (DL2 cells) before extraction.

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To detect protein expression, cells were lysed (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA pH 7.4, 1% NP-40) in the presence of protease (Roche #04693159001) and phosphatase inhibitors (Sigma #S6508 and S7920). Extracts were boiled and run on 8% SDS-PAGE gels, and transferred to PVDF membranes (Amersham Hybond™-P, GE Healthcare). Blots were blocked in TBST (10 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween-20), 5% non-fat milk for 1 h at room temperature and incubated with rabbit anti-Hth (1:5000, Kurant et al., 1998) or mouse anti-HA (1:1000, Covance, 11 MMS101P) or mouse anti-Flag (1:500, F1804, Sigma) in TBST, 1% non-fat milk for 1 h at room temperature. After three washes, blots were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch) and revealed by ECL using Amersham Hyperfilm ECL (GE Healthcare).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

All authors contributed to the design and interpretation of the data and critically revised the manuscript and approved the version to be published. C.B.-P. and F.J. contributed to the acquisition of the data. F.C. and F.J. drafted the manuscript.

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Supplementary material

Supplementary material available online at
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