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Myc interacts genetically with Tip48/Reptin and Tip49/Pontin to control growth and proliferation during *Drosophila* development

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Abstract

The transcription factor dMyc is the sole *Drosophila* ortholog of the vertebrate c-*myc* proto-oncogenes, and a central regulator of growth and cell cycle progression during normal development. We have investigated the molecular basis of dMyc function by analyzing its interaction with the putative transcriptional co-factors Tip48/Rept and Tip49/Pont. We demonstrate that Rept and Pont have conserved their ability to bind to Myc during evolution. All three proteins are required for tissue growth *in vivo*, as mitotic clones mutant for either *dmyc*, *pont* or *rept* suffer from cell competition. Most importantly, *pont* shows a strong dominant genetic interaction with *dmyc* which is manifested in the duration of development, rates of survival and size of the adult animal and in particular of the eye. The molecular basis for these effects may be found in the repression of certain target genes, such as *mfas*, by dMyc:Pont complexes. These findings indicate that dMyc:Pont complexes play an essential role in the control of cellular growth and proliferation during normal development.

Introduction

Myc proteins are essential regulators of growth, proliferation and apoptosis in metazoans (1-3). They act as transcription factors to control the expression of numerous target genes involved in growth, metabolism as well as other processes (4-7). Less is known about the molecular mechanism how Myc controls the expression of these targets. In recent years, different modes of gene activation by Myc have been proposed, notably recruitment of chromatin remodelers (8), histone acetylases (e.g. 9) or RNA pol II kinases (10), but the physiological relevance of these different factors for Myc dependent biological functions needs yet to be demonstrated. We therefore set out to study the mechanisms of Myc-controlled growth and proliferation during normal development using *Drosophila* as a model system. Initially, we focused on the interaction of Myc with two specific components of co-factor complexes, Tip48 and Tip49, because of the availability of null mutations in the corresponding genes (called *reptin* [*rept*] and *pontin* [*pont*] in flies, respectively).

Tip48 and Tip49 are closely related proteins that show a high similarity to the bacterial ATP-dependent AAA+ super family DNA helicase, RuvB. Orthologs of Tip48 and Tip49 have been identified in plants, yeast, and animals (e.g. 11, 12). Different observations strongly suggest that one major function of the Tip proteins resides in the control of transcription. Initially, vertebrate Tip49 was found as a **T**BP-**i**nteracting **p**rotein (13-16); later Tip48 and Tip49 were also shown to interact physically with the different transcription factors β-catenin (11, 14), c-Myc (12), E2F1 (only Tip49; 17), and ATF2 (only Tip48; 18), raising the possibility that the Tip proteins could bridge the basic transcription machinery and sequence-specific activators. Both proteins were also

purified as part of several multiprotein complexes involved in transcriptional regulation – the Ino80 chromatin remodeling complex in yeast (19, 20), the Polycomb repressive complex 1 (PRC1) in *Drosophila* (only Tip48; 21), the Tip60 HAT complex in vertebrates (22), and the Uri complex regulating nutrition-dependent gene expression in yeast and in vertebrates (23). Interestingly, three other proteins that were found to bind the N-terminus of c-Myc share residence with Tip48 and Tip49 in the Ino80 (BAF53, β-actin; 24) or the Tip60 complex (TRRAP, BAF53, β-actin; 24, 25). Further support for an involvement of Tip48 and Tip49 in transcription is provided by the observations that both proteins colocalize with c-Myc on the *nucleolin* promoter (26), and that elimination of Tip48 or Tip49 function in yeast rapidly affects the expression of a large number of targets (16, 27, 28). Such a transcriptional role is also consistent with the described genetic interactions between a *tip48* mutation and β-*catenin* in zebrafish, and between both *tip48* and *tip49* and a β-catenin-reporter system in *Drosophila* (11, 29); in both of these *in vivo* interactions, Tip48 behaved as a negative component and Tip49 as a positive component of the Wg signaling cascade. Similar opposing activities were also documented in a human cell line where the ability of the ß-catenin–TCF complex to activate a reporter gene was assayed (11). A potential role for Tip49 in Myc-dependent functions was addressed in a recent study that examined the consequences of co-expressing wild-type or putative dominant-negative forms of Tip49 with c-Myc. Neither form had any effect on control cells, but both enhanced the apoptosis caused by overexpressed c-Myc, and they reduced the ability of c-Myc in combination with activated Ras to transform rat embryo fibroblasts, indicating that – upon forced over-expression – Myc might require Tip49 activity (17).

In the present study we show that the physical interaction between Myc and Pont/Rept is conserved in flies, that *pont*/*rept* are essential for tissue growth *in vivo*, and that *dmyc* and *pont* show a strong genetic interaction. We further identify the gene *mfas* as a transcriptional target that is repressed by dMyc:Pont complexes. These studies provide the first evidence that Pont and Rept are essential co-factors for the normal functions of Myc *in vivo*.

Material and Methods

Fly lines and clonal analysis

Fly stocks were obtained from the Bloomington stock center, with the exception of: dm^{P0}/FM7 (30), $pont^{5.1}$/TM3, Ser (putative null allele, referred to as 'pont-' in the text; 11), $rept^{35}$/TM3 (putative null allele, referred to as 'rept-' in the text; 11), dm^{PL35}/FM7 (31); the revertant lines $pont^{rev5}$ (used as control for $pont^{5.1}$) and $rept^{rev\Delta 23}$ (used as control for $rept^{35}$) were generated in parallel with the null alleles $pont^{5.1}$ and $rept^{35}$ (with which they are isogenic) by precise excision of the P-element insertions 0229/05 and P1706 (11), respectively.

Mitotic recombination was induced using the Flp-FRT method (32). Females *y w hs-flp*^{122}; *rept*^{06945} *FRT2A*/TM6B were crossed with males *w*; *P(ubi-GFP.nls)3L1 P(ubi-GFP.nls)3L2 FRT2A* or *w*; *M(3)i*^{55} *hs-nGFP FRT(2A)*/TM6B (33). Females *y w hs-flp*^{122}; FRT82B $pont^{5.1}$/TM6B were crossed with males *w*; *FRT82B P(Ubi-GFP(S65T)nls)3R* or *w*; FRT82B P(w^{+}, *c-myc)*87E *Sb*^{63} *M3*(96C) (34). Larvae were heat-shocked at 37°C for 1h.

ey>dm^{PL35} = "*y w dm^{PL35} tub*>FRT>(*dmyc*-cDNA)>FRT>GAL4 *ey*-Flp / Y"; ***ey>dm^{P0}*** = "*w dm^{P0} tub*>FRT>(*dmyc*-cDNA)>FRT>GAL4 *ey*-Flp / Y"; ***ey>dm^{+}*** = "*y w dm^{+} tub*>FRT>(*dmyc*-cDNA)>FRT>GAL4 *ey*-Flp / Y". These different genotypes express a rescuing *dmyc* cDNA (ref. 35) in all parts of the body except for the head capsule, where Flp-mediated recombination eliminates the cDNA and allows expression of GAL4 instead.

Molecular biology

The sequences for pGEX-dMyc (expressing GST fused to amino acids 46 to 507 of dMyc), pCasper-hsp-HA-dMyc (expressing 3 copies of the HA-epitope tag fused to full-length dMyc, H-dMyc), pUAS-HA-dMyc, pUAS-AU1-Rept (expressing one copy of the AU1-epitope tag fused to full-length Rept, A-Rept), pUAS-9E10-Pont (expressing 3 copies on the c-Myc epitope tag recognized by mAb 9E10, M-Pont) are available upon request.

Cell culture and transfection

Drosophila Schneider S2 cells were grown at 25° C using Schneider medium (GIBCO) supplemented with 10% heat-inactivated FCS and 100 U.I. of penicillin/streptomycin (GIBCO). For stable transfection $2x10^5$ cells/ml were seeded in a medium flask. The day after the medium was removed and 10 µg of pCasper-hsp-HA-dMyc and 4 µg of pcP4 plasmids (36) were mixed with the Cellfectin reagent (GIBCO) in serum free medium and added to the cells. After 16 h complete medium was added and pools of clones were selected for two weeks in α-amanitin (200 µg/ml).

For transient transfections, 10^6 S2 cells/ml were plated in a medium flask. Next day indicated amount of plasmids were transfected using the Cellfectin reagent as described above. After 48 h transfected cells were subjected to a heat shock at 37° C for 1 h and after 2 h cells were harvested and lysates were prepared for Western blot analysis.

Western blot and antibodies

Transfected S2 cells S2 cells were washed in PBS and lysed using a buffer containing 50 mM HEPES pH 7.4, 150 mM NaCl, 1mM EDTA, 0.5 % Tween-20 and protease inhibitors. Untransfected cells (5 x 10^7 cells per immunoprecipitation reaction) were lysed in 50 mM Hepes (pH 7.5) containing 0.1 % Triton X-100, 250 mM NaCl and protease inhibitors. Fifty *y w* larvae were homogenized and lysed (by shredding & sonication) in the same buffer. The different lysates were subjected to sonication for 30 seconds on ice. After measurement of the protein concentration, using the Biorad-kit, lysates were immunoprecipitated using specific antibodies bound to Protein G-Sepharose (CL-4B, Amersham Pharmacia Biotech). After immunoprecipitation proteins were separated by SDS-PAGE, electrotransferred to a nitrocellulose membrane, subjected to Western blot analysis with specific antibodies, and visualized by ECL-Plus (Amersham Pharmacia Biotech). Primary antibodies were: rat anti-HA mAb (Roche); mouse anti-AU1 mAb (Covance); mouse anti-9E10 mAb (Covance), rabbit anti-9E10 polyclonal antiserum (Santa-Cruz); rabbit anti-Pont antiserum; guinea pig anti-Rept antiserum; non-immune hybridoma supernatant for control.

In vitro binding assays

GST and GST-dMyc fusion proteins were produced in bacteria (BL21) after induction with 0.1 mM IPTG for 5 h at 37° C. Cells were washed, resuspended in STE buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) and sonicated for 2' in ice. After centrifugation the supernatants were incubated for 2 h with GST agarose beads (Amersham Pharmacia Biotech). After extensive washes with STE buffer, the GST proteins immobilized on the beads were separated by SDS-PAGE and protein concentration was estimated by staining with Coomassie blue. Equal amount of GST and GST-dMyc proteins were used for *in vitro* binding assays with the different cell lysates. Binding was performed for 3 h at 4° C. After washing, the bound material was resuspended in Laemmli sample buffer, separated in SDS-PAGE and the proteins were visualized by Western blot analysis.

Analysis of adult flies

Flies were reared under identical growth condition and were age matched (3 days old males) before weighing with a precision scale (range 0.001-10 mg; Mettler ME30). To determine ommatidial number, all ommatidia were counted from scanning electron micrographs (SEM) of the indicated number of eyes from different animals. From the same photographs, the size of the ommatidia was calculated by measuring the area of 20 ommatidia located in the center of the eye using Adobe Photoshop.

Results

Physical interaction between dMyc and Pont/Rept

The interaction with human Tip48 and Tip49 requires a short sequence element in the N-terminus of c-Myc, called "Myc Box 2" (MB2). This functionally important MB2 is conserved in dMyc (37), suggesting that dMyc also has the potential to interact with Pont and Rept. Indeed, we could observe a specific interaction between the N-terminus of dMyc and Pont or Rept *in vitro* (Sup. Fig. 1). To demonstrate these interactions in cells, we co-expressed HA-tagged dMyc (H-dMyc) with 9E10-tagged Pont (M-Pont) and/or AU1-tagged Rept (A-Rept) in *Drosophila* S2 cells and probed the lysates for the existence of different protein complexes. As shown in Figs. 1 A & B, all binary complexes could be observed. Using sequential immunoprecipitation with a 9E10 antibody, elution with 9E10 peptide, and re-precipitation with anti-AU1 antibodies, we could further demonstrate the existence of a ternary complex containing H-dMyc, M-Pont and A-Rept (Fig. 1C). While these experiments involved overexpression of the different proteins, we could also detect the interaction of endogenous dMyc with endogenous Pont or Rept (albeit more weakly), both from non-transfected S2 cells (Fig. 1D) and from 3rd instar larvae (Fig. 1E). These data demonstrate that dMyc forms complexes with Pont and Rept during normal development and that dMyc, Pont and Rept can form a ternary complex in *Drosophila* cells.

In the course of these experiments, we also noticed that (upon strong overexpression) M-Pont can compete with A-Rept for binding to dMyc *in vitro*, and that the M-Pont:dMyc complex is more resistant to increasing NaCl concentrations than the A-

Rept:dMyc interaction (Sup. Fig. 1). These observations suggest that Pont can also interact with dMyc in the absence of any bound Rept, although the physiological significance of such a complex is currently unclear.

Function of pont *and* rept in vivo

If the physical interaction with Pont and Rept is important for dMyc function, we would expect mutations in *pont* and *rept* to affect some of the processes that are controlled by dMyc. However, currently there is only limited information available concerning the function of Tip48 and Tip49 in metazoans: flies carrying null mutations in *pont* or *rept* were reported to die during early larval stages (11), whereas overexpression of putative dominant negative versions of Tip49 in vertebrate cells showed no defects (12, 17). A closer inspection of larvae mutant for *pont* or *rept* further indicated that both genes might also control growth and proliferation. Homozygous $pont^{-/-}$ or $rept^{-/-}$ larvae hatch at normal rates, showing that the zygotic product of neither gene is essential for embryogenesis. The mutant larvae live for up to seven days, but grow only minimally in size (Fig. 2A); this phenotype is similar to that of mutants in growth-controlling genes (e.g. 38) and to *dmyc* null mutants (39; data not shown). The defects of $pont^{-/-}$ $rept^{-/-}$ double mutants are very similar to the single mutants, although these larvae don't survive for longer than five days after egg-deposition (Fig. 2A). Clones of $pont^{-/-}$ or $rept^{-/-}$ cells located within phenotypically wild-type imaginal discs grow poorly, indicating that the observed defects are cell-autonomous. Furthermore, they suffer from a phenomenon termed “cell competition”: when surrounded by phenotypically wild-type cells, only few small $pont^{-/-}$ or $rept^{-/-}$ clones survive at 72 hours after the induction of the clones (Fig.

2B), and none can be observed at 120 hours (data not shown). In contrast, when the cells surrounding the *pont* or *rept* clones are growth-impaired (because they carry a dominantly acting mutation in a gene coding for a ribosomal protein, a so-called *Minute* mutation), the mutant clones thrive even at 120 hours after induction (Fig. 2C). Only few growth-controlling genes have been demonstrated to suffer from cell competition, notably the *Minute* loci and *dmyc* (30, 35, 40). This similarity of mutant phenotypes is consistent with the possibility that dMyc, Pont and Rept control similar biological processes, as is the observation that all three genes are expressed in very similar patterns during embryogenesis (dynamically regulated in the mesoderm & midgut) and larval development (ubiquitously) (11, 30, 37). It should be noted that *pont* and *dmyc* also show some differences in their mutant phenotypes. Unlike *dmyc*-mutant cells, $pont^{-/-}$ cells are not smaller than wt control cells – which is consistent with an involvement of Pont in additional processes that are less affected by *dmyc* mutations (e.g. in the control of cell cycle progression).

Genetic interaction of dmyc *with* pont/rept in vivo

The following sections provide direct evidence for a genetic interaction between *dmyc* and *pont* (and to a lesser extent *rept*) *in vivo*. Since the only available *pont* and *rept* alleles are recessive lethal, we tested for dominant interactions between *pont* and/or *rept* and hypomorphic *dmyc* alleles (called *dm*). The dm^{P0} allele is caused by a P-element insertion in the *dmyc* promoter which results in a reduced *dmyc* expression; dm^{P0}/Y mutant males are characterized by a slightly delayed development, thin bristles, and a weak reduction in body size and viability (30; Table 1). The severity of this phenotype is

dramatically enhanced when such dm^{P0}/Y mutant flies also carry one mutant allele of *pont*: the viability drops from 54% to 12%, the surviving flies take 2.4 days longer for their development and, despite the extended period, ultimately eclose with a significantly lower weight (Table 1); none of these defects are seen in control crosses with dm^{+} flies. *rept* does not show any dominant interaction with dm^{P0} and the *pont rept* double mutant chromosome behaves like a *pont* single mutant. In addition, dm^{P0}/Y; $pont^{-/+}$ flies, but none of the other genotypes, occasionally show notches in the posterior wing margin (not shown). A closer inspection reveals a significant decrease in the total wing area of dm^{P0}/Y; $pont^{-/+}$ flies as compared to control, consistent with the functions of dMyc and Pont in growth control. A large part of this size difference can be attributed to a decrease in cell size (Sup. Table 1).

The interaction of dmyc *with* pont *&* rept *is essential for eye development*

The most striking manifestation of the genetic interaction between *dmyc* and *pont* is seen in the eye. Around 90% of dm^{P0}/Y; $pont^{-/+}$ flies have small, irregularly shaped, and slightly rough eyes. Penetrance and expressivity of the phenotype are variable, but the anterior and ventral portions of the eye are always most defective (Fig. 3C). Interestingly, the same defect can also be observed in dm^{P0}/Y flies, albeit at a very low frequency (30; unpublished data), but not in dm^{+}/Y flies that are heterozygous for *pont*. A quantitative analysis of adult eyes revealed a significant reduction in ommatidial size in all dm^{P0}/Y

mutant flies, which is significantly exacerbated by heterozygosity for *pont* (Table 2). In addition, dm^{P0}/Y; $pont^{-/+}$ eyes contain a reduced number of ommatidia, indicating an essential role of the *dmyc*/*pont* interaction for proliferation and growth during development.

Similar genetic interactions were also observed with a second independent *dmyc* allele. ey>dm^{PL35} flies express the allele dm^{PL35} specifically in the eye (Materials & Methods); these eyes are only moderately affected in their appearance, but heterozygosity for *pont* induces similar defects as described above (Sup. Fig. 2; Table 2). In addition, heterozygosity for *rept* also results in a weak reduction of ommatidial number in ey>dm^{PL35}eyes, providing the first evidence that Rept might also act as a positive co-factor for dMyc.

As a further demonstration of specificity, the ectopic expression of M-Pont in the eyes of *ey*> dm^{P0}; $pont^{-/+}$ flies (Materials & Methods) fully rescued their defects (Table 2, Sup. Fig. 2). Ectopic expression of H-dMyc also rescued the morphology defect of *ey*>dm^{P0}; $pont^{-/+}$ eyes, but in addition produced gain-of-function phenotypes such as an increase in ommatidial size. These gain-of-function effects are more pronounced in an *ey*>dm^{P0} or *ey*>dm^{+} background (Table 2, Sup. Fig. 2), and they illustrate the documented abilities of dMyc to promote both growth and apoptosis (30; L. Montero & PG, submitted).

In contrast, ectopic expression of A-Rept in *ey*>dm^{P0}; $pont^{-/+}$ flies enhanced penetrance and expressivity of their eye defects (Table 2, Sup. Fig. 2). Even when overexpressed in an *ey*>dm^{P0} or an *ey*>dm^{+} background, A-Rept induced a slight

reduction in ommatidial number and a rough eye appearance (Table 2 and data not shown); qualitatively similar results were obtained with two independent transgenes in *ey>dm*$^{+}$ flies. These results indicate that A-Rept cannot substitute for Pont; instead, overexpression of A-Rept seems to have a dominant-negative effect on ommatidial number in an *ey>dm*$^{+}$; *pont*$^{+/+}$ background, and on ommatidial size and number in an ey>*dm*^{P0}; *pont*$^{-/+}$ background. The basis for this effect is not known, but it is possible that the imbalance of Rept to Pont levels alters the composition of the multiprotein complexes that mediate the functions of these two proteins, and thereby interferes with their function. It is further conceivable that endogenous Pont levels are relatively lower than Rept levels, which would explain why mild Pont overexpression does not cause any dominant-negative effects, and why heterozygosity for *pont* results in a stronger genetic interaction with *dmyc* than heterozygosity for *rept*.

Cellular basis of the eye defect in dmyc/pont *mutant flies*

A comparison of control, *dm*^{P0}/Y and *dm*^{P0}/Y; *pont*$^{-/+}$ third instar larval eye imaginal discs revealed no obvious defects in any of the three genotypes with respect to overall shape and the pattern of differentiating ommatidia (Sup. Fig. 3). However, all *dm*^{P0}/Y mutant discs are significantly smaller than the control discs, and the *pont*, but not the *rept* mutation, further reduces their size; these data are in excellent agreement with the effects of the different genotypes on adult eye size (Table 2 and data not shown). This size reduction presumably reflects a reduction in cell size in the *dm*^{P0}/Y eye discs as

compared to control discs. Heterozygosity for *pont* further reduces this cell size and additionally reduces the number of cells.

This effect on cell number could be caused by a decreased rate of cell division and/or an increased rate of apoptosis. The different dm^{P0} genotypes contain similar numbers of mitotic cells (Sup. Fig. 3 and data not shown), suggesting at most minor differences in cell cycle progression rates. However, a difference of only 5% in cell doubling time would be sufficient to explain the differences in cell number between the genotype with the fewest cells (dm^{P0}/Y; $pont^{-/+}$) and the genotype with the most cells (*y w*), and such a difference would have escaped our detection. On the other hand, we did not find any indications for an involvement of apoptosis in this phenotype, as dm^{P0}/Y; $pont^{-/+}$ and dm^{P0}/Y; $pont^{+/+}$ discs do not show any difference in the number of apoptotic cells; in addition, expression of the viral caspase inhibitor p35 did not suppress the morphological defects in *ey*>dm^{P0}/Y; $pont^{-/+}$ eyes, nor did it affect the number of ommatidia in these eyes (Sup. Fig. 4). Thus, we consider a proliferation defect in dm^{P0}/Y; $pont^{-/+}$ flies the most likely explanation for the observed reduction of ommatidia in the adult eyes.

Molecular basis of the dMyc:Pont interaction

An explanation for the observed genetic interaction might be found in the genes whose expression is controlled by dMyc:Pont complexes. To identify such genes we combined microarrays with an RNAi approach in S2 cells. As shown in Sup. Fig. 5, Pont and Rept were found to control largely similar genes, but there is surprisingly little overlap with the dMyc targets; in particular, the genes activated by dMyc through canonical Myc

binding sites called “E-boxes” (7, 41) were not significantly affected by RNAi against *pont* or *rept*. However, 11 genes were significantly and in the same direction affected by RNAi against all three proteins (8 up- and 3 down-regulated; Sup. Table 2). While we deem it unlikely that the misregulation of these genes explains all of the observed defects in the *dm*/Y; *pont*$^{-/+}$ mutants, they may serve as examples for a larger class of genes that are controlled by dMyc:Pont[:Rept] complexes. To confirm these microarray results, we focused on one model target, *midline fasciclin* (*mfas*). Mfas is involved in cell-cell adhesion; its levels are up-regulated upon RNAi against *dmyc*, *pont* or *rept*, and rapidly down-regulated upon dMyc-overexpression *in vivo* (42). Quantitative real-time PCR experiments showed that *mfas* levels are increased in *dm*^{P0}/Y; *pont*$^{-/+}$ double mutant eye discs, but not in single-mutant or wild-type control discs, confirming that *mfas* is also a target of dMyc:Pont complexes *in vivo* (Sup. Fig. 6). To determine whether this effect is direct, we overexpressed H-dMyc in S2 cells and carried out chromatin immunoprecipitation experiments. As shown in Sup. Fig. 7, under these conditions both H-dMyc and endogenous Pont were found to be associated with a broad region of the *mfas* promoter (which does not contain any recognizable dMyc binding sites). These data suggest that dMyc:Pont complexes bind to certain target promoters such as *mfas* through as yet unidentified sequence motifs and mediate the transcriptional repression of these genes. Impairment of this process (through a combination of mutations in *dmyc* and *pont*) may then contribute to a reduction in cellular growth and proliferation.

Discussion

This study provides the first evidence that Tip49/Pont (and possibly Tip48/Rept) are essential partners for Myc during normal development, and that they play an important role in the control of Myc-dependent transcription, growth and proliferation. These conclusions are supported by four lines of evidence. First, we show that dMyc physically interacts with Rept and Pont *in vitro*, in cells, and in larvae. While ternary complexes containing dMyc, Rept and Pont can exist, we also provide evidence that dMyc can associate with Pont in the absence of Rept, although it is unclear whether such complexes lacking Rept have any physiological role *in vivo*. The stronger genetic interaction with *pont* raises the possibility that some of dMyc's functions might be mediated by such complexes, but the large degree of overlap between the targets of Pont and Rept and the fact that in most biochemically purified complexes Tip48 is accompanied by Tip49 suggest that most often these two proteins function together.

Second, flies lacking zygotic *pont* or *rept* gene products arrest their growth early during larval development, and mitotic clones homozygous mutant for *pont* or *rept* suffer from the same type of cell competition as do *dmyc* clones. These characteristics indicate a requirement for Pont/Rept for cellular proliferation and growth, which is consistent with their functioning as co-factors for dMyc.

Third, *pont* shows a strong dominant genetic interaction with *dmyc*. The causes for this interaction are likely to be defects in cellular growth and proliferation. The control of growth is most sensitive to variations in dMyc levels, since the moderate reduction of dMyc activity achieved in hypomorphic *dmyc* alleles already results in a decrease in cell size but not cell numbers. Removal of one copy of the *pont* gene exacerbates the growth

defect and results in a reduction of cell numbers. We did not find any indication that apoptosis contributes to this reduction in cell number, and therefore conclude that the cell number defects primarily reflect a proliferation defect. It is important to stress that none of these defects are seen in flies that are heterozygous for *pont* but wild type for *dmyc*, arguing strongly against purely additive effect of the *pont* and *dmyc* mutations. Although we cannot strictly rule out the possibility that Pont and dMyc act in parallel growth-controlling pathways, such a dominant genetic interaction is indicative of close functional connections. We have not observed a dominant effect of the *pont* mutation on dMyc-overexpression phenotypes (data not shown), suggesting that Pont is not limiting in situations of mildly increased dMyc levels. However, using a vertebrate tissue culture system (Rat1 cells) Wood and colleagues have previously demonstrated that dominant-negative Pont/Tip49 inhibits the ability of human c-Myc to transform Rat1 fibroblasts in conjunction with activated Ras (12). Overexpression of a dominant-negative protein mutant potentially allows a stronger reduction of Pont/Tip49 activity than can be obtained in a heterozygous $pont^{+/-}$ situation, and thus these experiments further reinforce our observation of a genetic interaction between *myc* and *pont*.

Fourth, we have shown that the expression of several genes, including *mfas*, is increased upon down-regulation of either *dmyc*, *pont* or *rept* in S2 cells, and in *dmyc*/*pont* double mutant eye imaginal discs *in vivo*. ChIP experiments further suggest that *mfas* is a direct transcriptional target of both Pont and dMyc.

Taken together, these data strongly argue that dMyc:Pont complexes are essential regulators of proliferation and growth *in vivo*, and that they act at least partly by repressing the expression of target genes such as *mfas*. A similar repressive function has

recently also been found for *Xenopus* Pont and Rept (43); it was proposed that the well characterized repression of the transactivator Miz1 by c-Myc is mediated by Pont and Rept. While it is tempting to speculate that *Drosophila* Pont functions analogously, no fly homolog of Miz1 has been identified. We currently also don't know which of the reported Pont-containing complexes (see introduction) is responsible for the observed effect.

The function of Rept is less clear, as a *rept* mutant shows only a weak interaction, and only with one *dmyc* allele. On the other hand, overexpression of Rept strongly enhances the *dmyc*/*pont* mutant phenotypes. This could indicate that Rept also acts as antagonist of Pont and of dMyc:Pont complexes, analogously to what has been proposed for the interaction between Rept/Pont and β-Catenin (11). Alternatively, overexpression of Rept functions in a dominant-negative fashion, possibly by titrating Pont and/or other factors away from the multiprotein complexes in which they normally reside; in addition, Rept might be relatively more abundant than Pont such that heterozygosity for *rept* does not show any effects in most situations. While we currently cannot rule out either explanation, our identification of *mfas* as a common target for dMyc, Pont and Rept is more consistent with the latter possibility.

In conclusion, we have shown here that Pont, and possibly Rept, assist dMyc in the control of cellular proliferation and growth, presumably in part by repressing the expression of certain target genes. This constitutes the first demonstration of the physiological significance of any transcriptional co-factor for the function of Myc during normal development.

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Figure legends

Table 1. Phenotypes of male *y w* and dm^{P0} mutant flies carrying a *pont* and/or *rept* mutation. [a] Viability is calculated as % of the total of the expected genotype; [b] from egg deposition until eclosion; ***** values differing significantly ($p < 0.01$) from their control ("dm^{P0} / Y; + / +"). Standard deviations are calculated based on the total number of the animals reported (indicated in parenthesis). None of the "*y w*/Y;..." genotypes differed significantly from "*y w*/Y; + / +". Note that relevant comparisons can only be made within the "dm^{+}" (*y w*) and the dm^{P0} flies, since the two lines are not isogenic.

Table 2. Effect of the *pont*:*dmyc* interaction on eye and ommatidial size. *nd*: not determined. Standard deviations (+/-) are calculated based on the total number of the animals (reported in parenthesis). Values differing significantly from their control (lines 1, 5, 9, 13, 17, 21 for samples 2-4, 6-8, 10-12, 14-16, 18-20, 22-24, respectively) are marked with ***** ($p < 0.01$) and ****** ($p<0.05$), respectively. The genotypes are explained in the text.

Figure 1. Rept and Pont associate with dMyc *in vivo*. A, B, M-Pont and/or A-Rept were transiently transfected into S2 cells stably expressing H-dMyc (as indicated). *Upper*

panels, whole cell lysates were immunoprecipitated with anti-AU1 antibodies (for A-Rept, panel **A**) or anti-HA antibodies (recognizing H-dMyc, panel **B)**, followed by immunoblotting with anti-tag antibodies to detect the proteins indicated on the right. *Lower panels*, immunoblots of whole cell lysates to reveal the relative expression levels of the indicated proteins. Positions of H-dMyc, M-Pont and A-Rept, respectively, are indicated. The first lane in the upper panel of **B** contains lysate of non-transfected S2 cells. **C**, M-Pont and A-Rept (lanes 1, 3, 5) or M-Pont alone (lanes 2, 4, 6) were transiently transfected into S2 cells stably expressing H-dMyc. Cell lysates were incubated with 9E10 antibodies and the immunoprecipitate eluted with the 9E10 peptide (lanes 3, 4). The eluate was then re-immunoprecipitated using anti-AU1 antibodies (lane 5 and 6) and the immunoprecipitate was analyzed by immunoblotting. **D**, **E**, S2 cell lysates (**D)** or third instar larval extracts (**E)** were incubated with anti dMyc antibodies or control hybridoma supernatant. Immunoprecipitates were blotted with anti-dMyc antibodies, anti-Pont or anti-Rept antisera as indicated. The rightmost lanes show immunoblots of whole cell lysates. Asterisks indicate the migration of the endogenous proteins in panel **E**.

Figure 2: Consequences of *pont* and/or *rept* inactivation *in vivo*. **A**, the sizes of $pont^{-/-}$, $rept^{-/-}$ or $rept^{-/-}$ $pont^{-/-}$ homozygous mutant larvae are shown in comparison with those of wild type larvae. Larvae of the indicated genotypes were reared at 25°C and photographed at the indicated times after egg-deposition (in days). No *rept pont* double mutant larvae are alive after day 5. **B, C**, analysis of mitotic *pont* or *rept* clones in 3rd instar imaginal wing discs. Seventy-two hours after their induction, clones of cells homozygous mutant for *pont* (**B**) or *rept* (not shown) are consistently smaller than their

corresponding twin clones, or even completely absent. If the clones are induced in animals heterozygous for a *Minute* mutation, their growth is less disadvantaged and their size is still important 120 h after induction in wing imaginal discs, as shown for clones homozygous mutant for *rept* (**C**). A *pont* mutant clone is shown in higher magnification in the inset in panel **B** (outlined with white dots, the corresponding twin spot is marked by bright colour and outlined with a solid line). In panel **C**, the $rept^{-/-}$ clones are marked by the absence of colour; the corresponding twin spots do not survive.

Figure 3. Heterozygosity for *pont* or *rept* results in eye defects in hypomorphic *dmyc* mutants. Representative scanning electron micrographs of adult eyes. Genotypes are: dm^{P0}/Y; +/+ (**A**), dm^{P0}/Y; $rept^{-/+}$ (**B**), dm^{P0}/Y; $pont^{-/+}$ (**C**), dm^{P0}/Y; $rept^{-/+}$ $pont^{-/+}$ (**D**), *ey>*dm^{PL35}/Y; +/+ (**E**), *ey>*dm^{PL35}/Y; $rept^{-/+}$ (**F**), *ey>*dm^{PL35}/Y; $pont^{-/+}$ (**G**), *ey>*dm^{PL35}/Y; $rept^{-/+}$ $pont^{-/+}$ (**H**). All pictures are shown at the same magnification; anterior is to the left.

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Table 1

Genotype	Viability (%) [a]	Development [b] (days)	Weight (mg)
y w / Y; + / +	98 (339)	10.3 ± 0.6 (339)	863 ± 56 (47)
y w / Y; *pont* $^{-}$ / +	108 (281)	10.1 ± 0.6 (281)	854 ± 37 (27)
y w / Y; *rept* $^{-}$ / +	92 (487)	10.0 ± 0.5 (487)	828 ± 44 (44)
y w / Y; *rept* $^{-/+}$ *pont* $^{-/+}$	108 (363)	10.1 ± 0.4 (363)	852 ± 46 (33)
dm^{P0} / Y + / +	54 (287)	10.6 ± 0.4 (287)	772 ± 53 (46)
dm^{P0} / Y; *pont* $^{-}$ / +	**12 (55)***	**13.0 ± 0.8 (55)***	**693 ± 65 (21)***
dm^{P0} / Y; *rept* $^{-}$ / +	60 (301)	10.8 ± 0.4 (301)	774 ± 33 (29)
dm^{P0} / Y; *rept* $^{-/+}$ *pont* $^{-/+}$	**20 (81)***	**12.4 ± 0.7 (81)***	**702 ± 70 (25)***

Table 2

	Genotype	Number of ommatidia	Ommatidial size (μm^2)	Area eye discs ($\mu m^2 x10^3$)
1	*y w*/Y; +/+	735 ± 18 (5)	246 ± 5 (5)	1.86 ± 0.26 (33)
2	*y w*/Y; *pont*$^{-/+}$	748 ± 12 (5)	241 ± 6 (5)	1.74 ± 0.31 (51)
3	*y w*/Y; *rept*$^{-/+}$	727 ± 12 (5)	244 ± 5 (5)	1.73 ± 0.19 (20)
4	*y w*/Y; *rept*$^{-/+}$ *pont*$^{-/+}$	726 ± 12 (5)	240 ± 9 (5)	nd
5	*dm*^{P0}/Y; +/+	722 ± 18 (5)	217 ± 8 (5)	1.44 ± 0.15 (33)
6	*dm*^{P0}/Y; *pont*$^{-/+}$	561 ± 89 (7)*	157 ± 16 (7)*	0.90 ± 0.17 (51)
7	*dm*^{P0}/Y; *rept*$^{-/+}$	695 ± 33 (5)	200 ± 5 (5)**	1.51 ± 0.24 (17)
8	*dm*^{P0}/Y; *rept*$^{-/+}$ *pont*$^{-/+}$	532 ± 82 (7)*	162 ± 7 (7)*	nd
9	*ey>dm*^{PL35}/Y; +/+	685 ± 35 (5)	194 ± 10 (5)	nd
10	*ey>dm*^{PL35}/Y; *pont*$^{-/+}$	591 ± 39 (5)*	174 ± 7 (5)**	nd
11	*ey>dm*^{PL35}/Y; *rept*$^{-/+}$	637 ± 10 (5)**	192 ± 12 (5)	nd
12	*ey>dm*^{PL35}/Y; *rept*$^{-/+}$ *pont*$^{-/+}$	583 ± 72 (5)**	156 ± 9 (5)*	nd
13	*ey>dm*$^{+}$; +/+	729 ± 10 (5)	244 ± 8 (4)	nd
14	*ey>dm*$^{+}$; UAS-H-dMyc/+	615 ± 17 (5)*	329 ± 27 (5)*	nd
15	*ey>dm*$^{+}$; UAS-M-Pont/+	737 ± 14 (5)	234 ± 7 (5)	nd
16	*ey>dm*$^{+}$; UAS-A-Rept/+	562 ± 88 (5)*	226 ± 13 (5)	nd
17	*ey>dm*^{P0}/Y; UAS-lacZ/+	738 ± 40 (10)	208 ± 16 (10)	nd
18	*ey>dm*^{P0}/Y; UAS-dMyc*/+	615 ± 90 (10)*	295 ± 11 (10)*	nd
19	*ey>dm*^{P0}/Y; UAS-M-Pont/+	754 ± 37 (10)	216 ± 7 (10)	nd
20	*ey>dm*^{P0}/Y; UAS-A-Rept/+	535 ± 108 (10)*	201 ± 26 (10)	nd
21	*ey>dm*^{P0}/Y; *pont*$^{-/+}$	514 ± 34 (4)	169 ± 19 (4)	nd
22	*ey>dm*^{P0}/Y; *pont*$^{-/+}$ UAS-H-dMyc	661 ± 31 (3)*	312 ± 4 (3)*	nd
23	*ey>dm*^{P0}/Y; *pont*$^{-/+}$ UAS-M-Pont	729 ± 46 (3)*	227 ± 5 (3)*	nd
24	*ey>dm*^{P0}/Y; *pont*$^{-/+}$ UAS-A-Rept	198 ± 95 (3)*	167 ± 20 (3)	nd

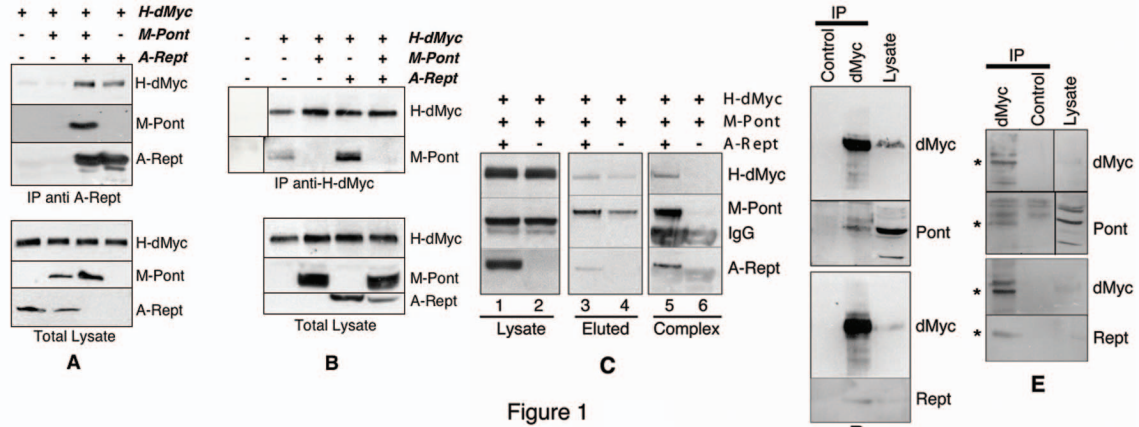
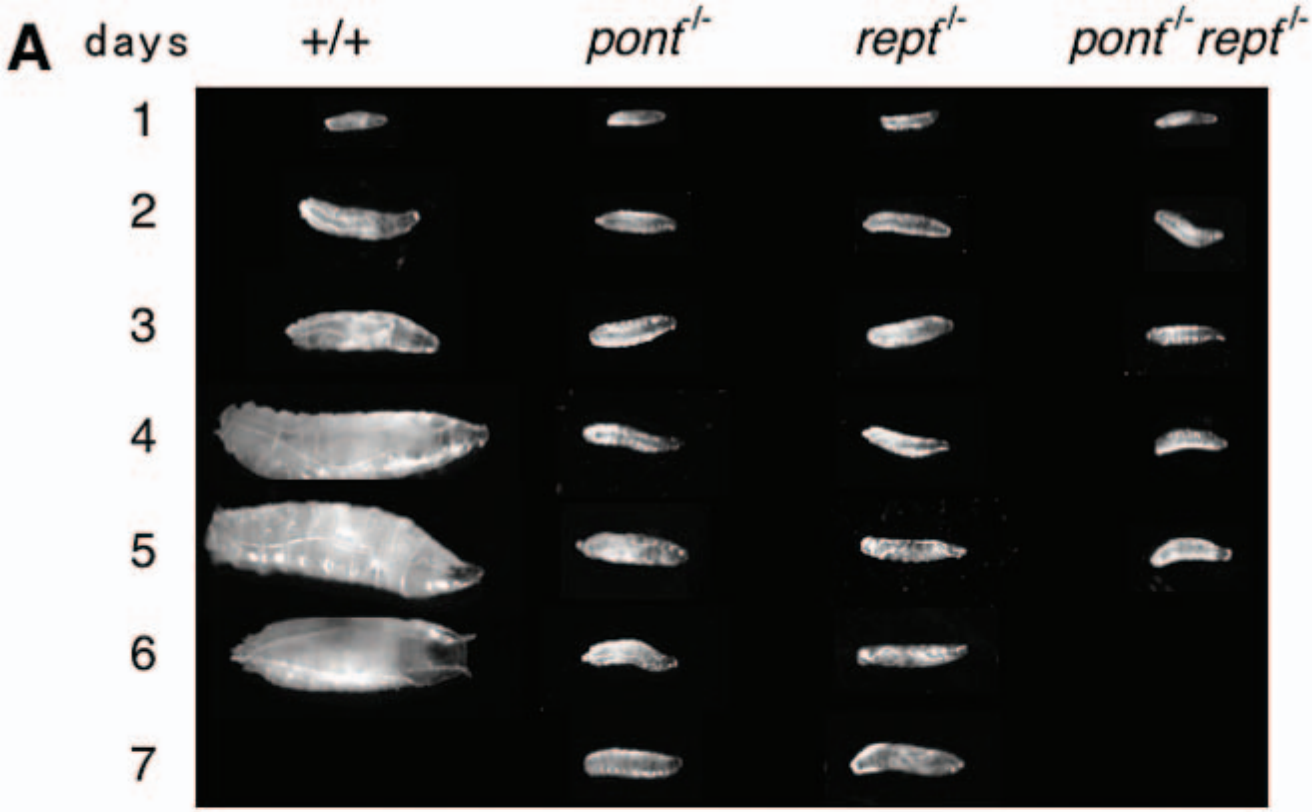
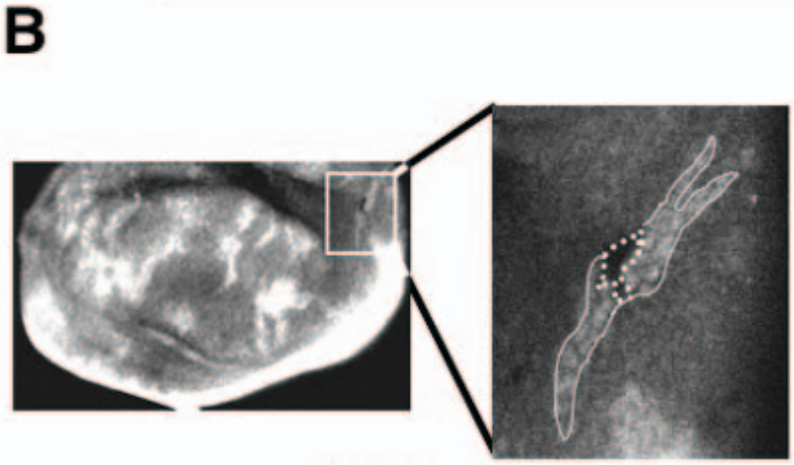

H-dMyc
M-Pont
A-Rept
IP anti A-Rept
Total Lysate
A
IP anti-H-dMyc
B
IgG
1 2 3 4 5 6
Lysate
Eluted
Complex
C
IP
Control
dMyc
Lysate
Pont
Rept
D
E

Figure 1

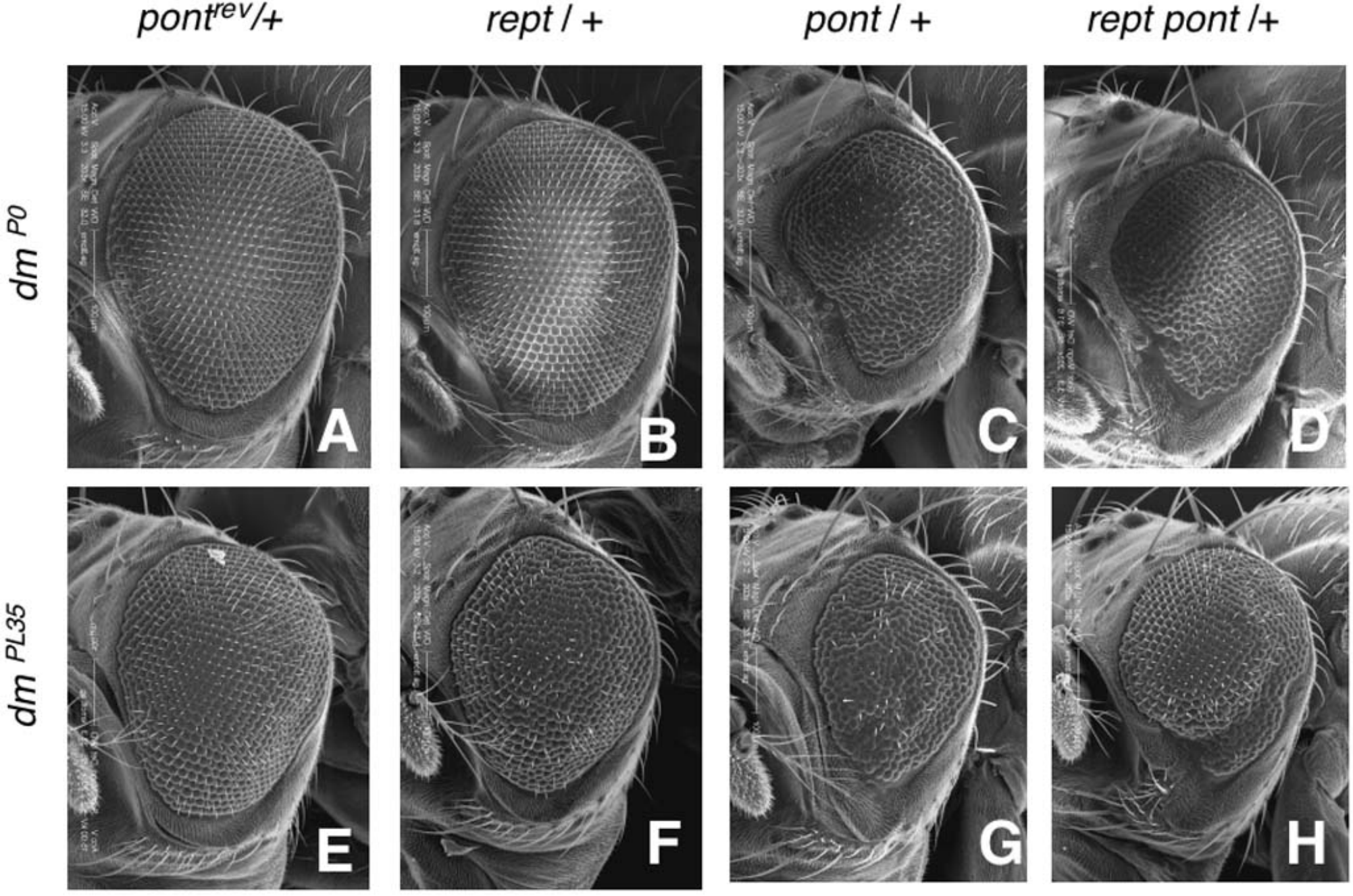




pont-/- clones

rept-/- clones
in M+/- background

Figure 2


pont^rev/+
rept / +
pont / +
rept pont /+
dm P0
dm PL35
A
B
C
D
E
F
G
H