

Evolutionarily conserved transcription factor Apontic controls the G1/S progression by inducing *cyclin E* during eye development

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During *Drosophila* eye development, differentiation initiates in the posterior region of the eye disk and progresses anteriorly as a wave marked by the morphogenetic furrow (MF), which demarcates the boundary between anterior undifferentiated cells and posterior differentiated photoreceptors. However, the mechanism underlying the regulation of gene expression immediately before the onset of differentiation remains unclear. Here, we show that Apontic (Apt), which is an evolutionarily conserved transcription factor, is expressed in the differentiating cells posterior to the MF. Moreover, it directly induces the expression of *cyclin E* and is also required for the G1-to-S phase transition, which is known to be essential for the initiation of cell differentiation at the MF. These observations identify a pathway crucial for eye development, governed by a mechanism in which Cyclin E promotes the G1-to-S phase transition when regulated by Apt.

The fly family *Drosophila* receives visual information through compound eyes. Each of the compound eyes is composed of about 800 unit eyes that are called ommatidia. An ommatidium contains eight distinct photoreceptor cells, each of which leads to projection of an axon directly to the optic lobe of the brain (1). Although there are substantial differences in the morphological appearance of *Drosophila* and vertebrate eyes, recent studies have shown that there are common underlying genetic pathways controlling retinal cell fate specification (2). Thus, *Drosophila* has proven to be an excellent model system for identifying new genes that are evolutionarily conserved in eye development.

During the eye development of *Drosophila*, a wave of morphogenesis moves from the posterior to the anterior of the eye imaginal disk (3). The crest of this wave of morphogenesis is called the morphogenetic furrow (MF), where the cell becomes coordinated with differentiation. Cells within the MF arrest in G1 phase before differentiating (4, 5). Cells located just posterior to the MF exit G1 arrest and begin to differentiate into the photoreceptor cell preclusters, and the surrounding cells enter a synchronous S phase (6). Thus, the regulation of G1-to-S phase progression is important for the eye formation in *Drosophila*. Cyclin E is expressed in a subset of cells immediately posterior to the MF, and its expression correlates with entry of these cells into S phase. The transient G1 arrest of cells in the MF of the developing eye disk requires down-regulation of *cyclin E* (7, 8).

An important developmental question is how the expression of *cyclin E* is regulated in the eye disk. Hedgehog signaling has been reported to be important for *cyclin E* expression in the posterior MF region. Cubitus interruptus (Ci) is a transcription factor that mediates Hedgehog signaling (9). Up-regulation of *cyclin E* expression by Hedgehog signaling is accomplished through binding of Ci to the *cyclin E* promoter (10). However, this conclusion has been called into question by a finding that Cyclin E accumulation occurred in *ci* mutant cells (11). Thus, the regulation of *cyclin E* in the posterior MF region remains to be clarified.

Apontic (Apt; TDF) has been identified as a bZIP protein and is required for the development of tracheae, head, heart, border cell, and central nervous system in the embryo of *Drosophila* (12–16). However, the role of Apt in the cell cycle and eye development is elusive. In this study, we show evolutionary conservation of Apt and identify *Drosophila* Apt as a key regulator of the G1-to-S transition. Apt is expressed at the MF in the eye disk and directly activates the expression of *cyclin E* at the MF to allow proper eye development. Our study elucidates the role of the evolutionarily conserved transcription factor Apt in the G1-to-S phase transition at the onset of differentiation.

Results

Evolutionary Conservation of Apt. Using evolutionary analysis, we found that Apt is conserved among species from *Drosophila melanogaster* to humans (Fig. 1A). High similarities are present in the N-terminal and the C-terminal domains among the Apt homologs (Fig. 1B and Fig. S1). Interestingly the conserved domain at the C-terminal region is a bZIP domain (14). To test DNA binding ability of this domain, we carried out electrophoretic mobility-shift assays (EMSAs) using the histidine-tagged *D. melanogaster* Apt C-terminal region containing 56 amino acids that were expressed and purified from bacteria. We found that this domain can bind to the Apt DNA binding sequence (Fig.

Significance

Cell differentiation is a critical point for eye development. Identification of genes for cell differentiation is critical for understanding the mechanism of eye formation and its evolution. Here, we show that an evolutionarily conserved transcription factor, Apt, regulates the G1-to-S phase transition by inducing *cyclin E* during eye development. We identify a pathway crucial for eye development, governed by a mechanism in which Cyclin E promotes the G1-to-S phase transition when regulated by Apt. One round of DNA replication before the onset of differentiation may be important to change epigenetic states from cell proliferation to development.

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The authors declare no conflict of interest.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE36917).

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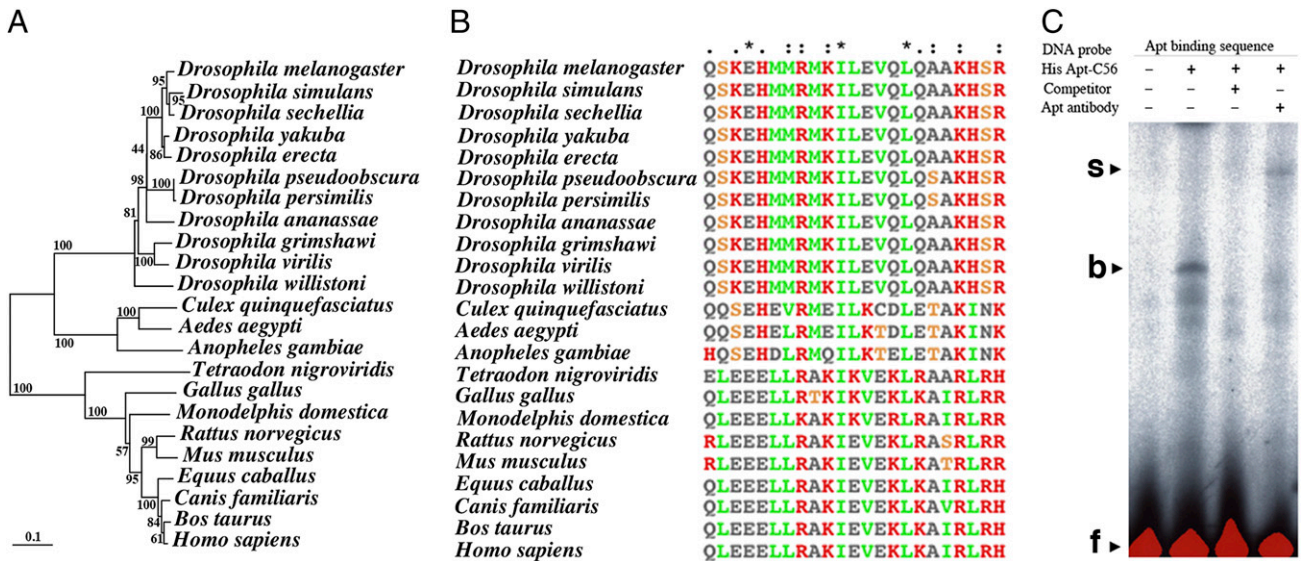


Fig. 1. Apt is an evolutionarily conserved DNA binding protein. (A) Phylogenetic tree of Apt. The tree was constructed using the neighbor-joining method performed with full-length Apt. All gaps in the amino acid sequences were excluded from the computation. Bootstrap analysis with 1,000 repetitions was used to assess the support for tree nodes. Bootstrap supporting values are indicated at the nodes. Branch lengths are proportional to sequence divergence; size bar represents substitutions per site. (B) Alignment of a DNA-binding region of *D. melanogaster* Apt between insect homologs and mammalian fibrinogen silencer binding protein (FSBP). Identical residues in all species are marked with * above the alignment; weakly conserved residues in all species are marked by a single period; strongly conserved residues in all species are marked by double period. (C) An electrophoretic mobility-shift assay was carried out using purified His-tagged Apt-C56. The DNA-Apt-C56 complex (b) and DNA-Apt-C56-antibody complex (s) were separated from the free probe (f) by electrophoresis.

1C). These data suggest that a conserved function of Apt may be achieved through the Zn finger domain.

Apt Is Expressed at the Posterior Cells to the Morphogenetic Furrow of the Eye Disk. To study the role of Apt in eye development, we first analyzed the expression of Apt in the eye imaginal disk by immunostaining using anti-Apt antibody. In the wild-type eye disk, Apt protein was detected at the peripodial membrane (Fig. S2A), MF (Fig. S2B), and glial cells (Fig. S2C). To determine more precisely the position of expression of Apt at MF, we carried out a double-staining experiment using an MF marker, dpp-lacZ. As a result, we found that Apt was expressed at the posterior cells to MF, suggesting its role in the eye development (Fig. 2 A–C).

apt Has Cell Autonomous Effects on Eye Development. To infer the function of Apt in eye development, ideally the developing eyes of homozygous *apt*-null mutations should be examined. However, because *apt*-null homozygotes die as embryos, this simple direct experiment was not possible. Therefore, we used *apt* heterozygotes to generate homozygous mosaic cell clones by mitotic recombination, with a linked *white*⁺ (*w*⁺) transgene as a cell autonomous marker. This experiment results in patches of doubly mutant *w* and *apt* tissue in otherwise red and *apt*⁺ eyes (Fig. 2D). We cut sections of six representative homozygous retinal clones of *apt*^{PΔ3}. We found that the clones had effects on the external morphology of the eye (Fig. 2F, arrow). These clones disrupt the linear arrangement of the ommatidia rows and columns (Fig. 2E, arrow). To investigate the effect of overexpression of Apt in the eye, we used *GMR-Gal4* (Fig. 2H) or *ey-Gal4* (Fig. S2G) to drive *UAS-apt*. Overexpression of Apt in the eye imaginal disk resulted in abnormal eyes. Scanning electron microscopy revealed missing ommatidia in the *GMR-apt* individuals (Fig. 2H) and disrupted linearity of the ommatidia rows and columns in the *ey-apt* individuals (Fig. S2E). Recently, Turkel et al. (17) showed that overexpression of Apt in eye disk clones does not result in apparent tissue overgrowth and that

differentiation as revealed by Elav staining still occurs although it appears that there are missing ommatidia and pattern disruptions in the *apt*-overexpressing clone, but the surrounding wild-type tissue does not appear to be affected. Thus, both the loss of function and overexpression phenotypes indicate that *apt* has a cell autonomous function in eye development.

Identification of Apt Targets. Because Apt is a bZIP transcription factor (15), therefore, identification of its target genes would clarify its role in eye development. To identify *apt*-regulated genes, we compared expression profiles of mRNA purified from wild-type and *apt* mutant embryos. A total 323 genes were identified as significantly down-regulated in the *apt* mutant (Table S1). Among the *apt*-regulated genes, 36 were involved in neurogenesis, eye morphogenesis, tracheal development, oocyte and germ cell development, apoptosis, cell-cycle regulation, and bristle development (Fig. 3A).

We used real-time PCR to validate some of the candidate targets. Eight genes identified as putative *apt*-regulated genes in the microarray analysis exhibited lower levels of expression in *apt* mutant, compared with wild type. These genes were induced by overexpression of *apt* at the salivary gland (Fig. 3B). These results suggest that the eight genes are true candidates of Apt target genes.

Apt Controls the Expression of cyclin E at the MF. Among the eight Apt target-gene candidates, *cyclin E* is known to be expressed at the MF (7). We examined whether *apt* exhibits genetic interaction with *cyclin E*. Although animals heterozygous for *apt*-null allele were normal, transheterozygotes of *apt* and *cycE*^{JP} showed an enhanced small rough eye phenotype (Fig. S3). This genetic interaction raised the possibility that transcription of *cyclin E* is regulated by Apt. To test the regulation of *cyclin E* by Apt at the MF, we performed a double-staining experiment. In the wild-type eye disk, Apt and Cyclin E were coexpressed in cells posterior to the MF (Fig. 4 A–C). Furthermore, the expression of *cyclin E* is significantly reduced in the *apt* mutant

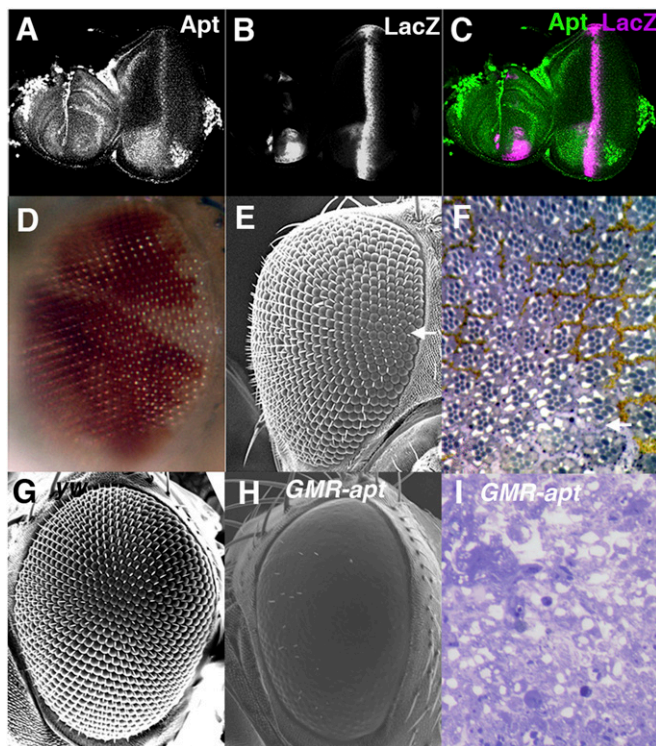


Fig. 2. Apt is expressed at the morphogenetic furrow (MF) of the eye disk and required for eye development. (A and B) The eye disk of a third instar larva of *dpp-lacZ* line was stained with anti-Apt antibody (A) and anti- β -galactosidase antibody (B). (C) Merged image of A and B. (D) External view of the mosaic eye. (E) A scanning electron micrograph of an adult with mosaic clones. In *apt^{PA3}* homozygous clones, the anterior edge of the retina was disrupted (arrow). (F) Section of adult eye with mosaic clones. Wild-type cells can be distinguished by their included pigment granules, which are brown in the pigment cells and appear blue in the photoreceptor cells. The mutant clone is indicated by an arrow. (G) Normal compound eye of wild type. (H) An abnormal eye by Apt overexpression using *GMR-GAL4* and *UAS-apt*. (I) Section of adult eye with overexpression of Apt. Development of the photoreceptor was completely disrupted.

clones (Fig. 4 D–F) whereas it is increased by overexpression of Apt at the eye disk (Fig. 4 G–I). As a control, Cyclin B is not induced by overexpression of *apt* at the eye disk (Fig. S4). These results suggest that Apt activates the expression of *cyclin E* at the MF of the eye disk.

Because the expression of *cyclin E* did not completely disappear in the *apt* mutant clones, we asked whether another transcription factor is also required for the expression of *cyclin E*. Because E2F1 has been reported for the activation of *cyclin E* (18), we examined whether *apt* exhibits genetic interaction with *e2f1*. Although animals heterozygous for *apt*-null allele were normal, transheterozygotes of *apt* and *e2f1* showed an enhanced small rough eye phenotype (Fig. S5). This genetic interaction suggests that Apt acts in combination with E2F1. The expression of *cyclin E* is induced by overexpression of *e2f1*. However, this effect is not mediated through *apt* because the expression of *apt* is not induced by overexpression of *e2f1* (Fig. S6). Taken together, these data suggest that the expression of *cyclin E* is regulated by both Apt and E2F1 in the eye disk.

Apt Directly Controls *cyclin E*. To address how Apt activates the expression of *cyclin E*, we focused on a 20-kb region of the *cyclin E* locus known to reproduce the normal *cyclin E* expression pattern in the eye disk (7). We identified one potential Apt binding sequence within the region (Fig. 5A), and this site was evolutionarily

conserved among several *Drosophila* species (Fig. 5C). Apt binding to this sequence was detected by EMSAs, using histidine-tagged Apt protein expressed and purified from bacteria. Incubation of 32 P-labeled double-stranded DNA carrying the Apt binding sequence with the full-length Apt protein yielded a slowly migrating complex. The complex formation was efficiently competed with the unlabeled oligonucleotides of the same sequence but not with the mutant oligonucleotides carrying six base substitutions (Fig. 5B).

We next assessed the function of the Apt-binding site in *cyclin E* by transgenic reporter assays. To do this assay, reporter genes were constructed that carried the endogenous promoter and the *cyclin E* regulatory element, containing a wild-type Apt-binding site (*cycEPlacZ*) or a mutated site (*cycEMPlacZ*) (Fig. 5A). Although *cycEPlacZ* with the wild-type binding site recapitulated the *cyclin E* expression in the MF (Fig. 5 D–F), base substitutions in the Apt-binding site in *cycEMPlacZ* abolished the lacZ expression (Fig. 5 G–I). Taken together, these results demonstrate a direct role for Apt in the activation of *cyclin E* and suggest that Apt controls eye development through directly binding to the Apt site in its regulatory region.

Apt Is Required for the G1-to-S Phase Transition. To determine whether up-regulation of *cyclin E* by Apt in the eye imaginal disk is important for the G1-to-S phase transition, we ectopically expressed Apt by using *GMR-Gal4* and *UAS-apt* and monitored S phase by BrdU labeling. Ectopic expression of Apt resulted in an increase in the number of BrdU-labeled cells in the eye disk (Fig. 5K), compared with the control (Fig. 5J). The M-phase cells

Neurogenesis Genes	Eye morphogenesis Genes	Oocyte and germ cell Genes
<i>Neurotactin (Nrt)</i>	<i>eyes absent (eya)</i>	<i>par-6</i>
<i>Fasciclin3 (Fas3)</i>	<i>dacapo (dap)</i>	<i>pumilio (pum)</i>
<i>Lim3</i>	<i>three rows (thr)</i>	<i>zero population growth (zpg)</i>
<i>torso (tor)</i>	<i>neuralized (neur)</i>	Cell cycle Genes
<i>longitudinals lacking (lola)</i>	<i>anterior open (aop)</i>	<i>cyclinE (cycE)</i>
<i>Frazzled (fra)</i>	<i>Star (S)</i>	<i>expanded (ex)</i>
<i>Protein tyrosine phosphatase</i>	<i>Suppressor of variegation 2-10</i>	<i>Hairless (H)</i>
<i>69D (Ptp69D)</i>	<i>(Su(var)2-10)</i>	<i>singed (sn)</i>
<i>gliotactin (glec)</i>	<i>hedgehog (hh)</i>	<i>Dic2</i>
<i>nervous fingers 1 (nerfin-1)</i>	<i>cyclinE (cycE)</i>	<i>RNA-binding protein 9 (Rbp9)</i>
<i>scribbler (sbb)</i>	Tracheal development Genes	<i>Cip4</i>
<i>Sox21b</i>	<i>Lamin (Lam)</i>	<i>Cyclin dependent kinase</i>
<i>smell impaired 35A (smi35A)</i>	<i>three rows (thr)</i>	<i>subunit 30A (Cks30A)</i>
<i>Sema-2a</i>	<i>dacapo (dap)</i>	Apoptosis Genes
<i>PAK-kinase (Pak)</i>	Bristle development Gene	<i>Rox8</i>
	<i>singed (sn)</i>	<i>grim</i>

Gene Symbol	Fold changes <i>yw:apt^{PA3}</i>	Fold changes <i>UAS-apt/sg-GAL4:yw</i>
<i>apt</i>	2.18±0.067	19.09±0.051
<i>cycE</i>	11.4±0.038	2.06±0.028
<i>glec</i>	1.94±0.086	1.69±0.009
<i>dap</i>	4.41±0.059	1.20±0.031
<i>sn</i>	4.18±0.029	2.21±0.029
<i>Ptp69D</i>	2.57±0.089	2.21±0.069
<i>neur</i>	3.04±0.033	1.73±0.066
<i>Su(var)2-10</i>	2.37±0.094	1.46±0.121
<i>Cks30A</i>	4.58±0.026	1.73±0.053

Fig. 3. Apt target genes identified by microarray. (A) Manual annotation of the list of putative Apt targets reveals numerous genes involved in neurogenesis, eye morphogenesis, tracheal development, oocyte and germ-cell development, apoptosis, cell-cycle regulation, and bristle development. (B) Quantitative PCR analysis of selected regulated genes identified by microarray analysis. Fold change of mRNA level between *yw* and *apt* mutant embryos or *yw* and Apt overexpression (*sg[Gal4/+; UAS-apt/+]*) salivary gland. Each PCR was carried out in duplicate, and the average of the two was normalized to tubulin.

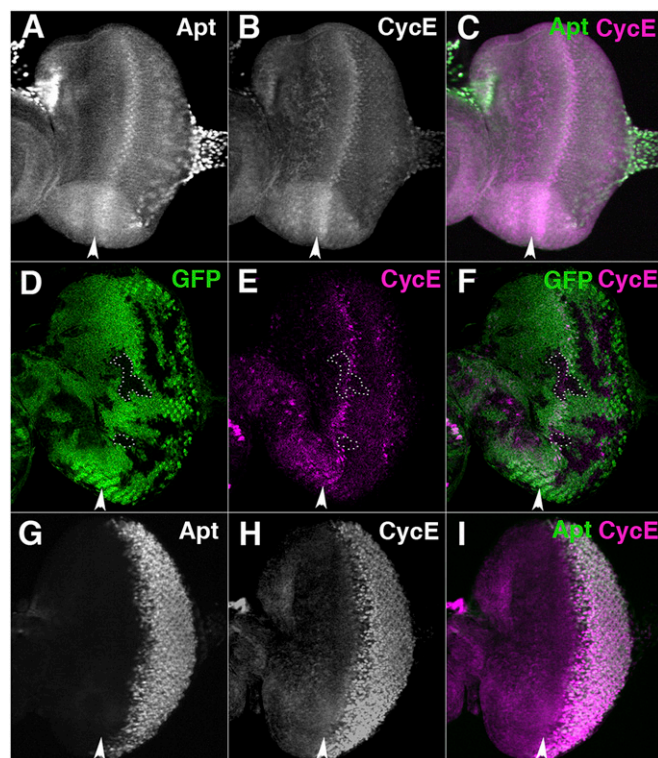


Fig. 4. Apt controls the expression of Cyclin E. (A–C) The expression of Apt (A) and Cyclin E (B) at the wild-type eye disk of a third instar larva. (D–F) Decreased Cyclin E expression (E) in *apt* mutant clones in the eye disk (D). *apt*^{Δ3} homozygous clones visualized by the lack of GFP marker staining is outlined with dotted lines. (G–I) The expression of Cyclin E (H) was induced by overexpression of Apt (G). Eye disk of a third instar larva from wild-type (A–C), *ey-FLP; FRT42D,Ubi-GFP/FRT42D,apt*^{Δ3} (D–F), and *GMR-apt* (G–I) fly was stained with anti-Apt antibody (A and G), anti-GFP antibody (D) and anti-Cyclin E antibody (B, E, and H). (C, F, and I) Merged images of A and B, D and E, and G and H, respectively. Arrowheads indicate the morphogenetic furrow (MF).

were not affected by ectopic expression of Apt as revealed by phosphohistone H3 staining (Fig. S7). From these data, we propose a model in which Apt directly activates the expression of *cyclin E* and is required for the G1-to-S phase transition at the onset of differentiation (Fig. 5L).

Discussion

Microarray analyses suggested that the bZIP transcription factor, Apontic, regulates the genes required for the neuron, trachea, oocyte, germ cell, bristle and eye development, apoptosis, and cell-cycle regulation. Among these candidates for Apt target genes, we show here that Apt directly controls the expression of *cyclin E* and is required for the G1-to-S phase transition during eye development.

The Regulation of *cyclin E* Expression at the MF. In the wild type, Cyclin E protein accumulates in a stripe of cells posterior to the MF (7). Cyclin E was not induced by N, Hh, or Dpp signal in the eye disk because Cyclin E accumulation occurred in *Su(H)* mutant cells or *Mad*¹⁻² *Su(H)* *ci* mutant cells (11). Ci, Mad, and *Su(H)* are the transcriptional targets of Hh, Dpp, and N signaling, respectively (19). A prior work suggested that the expression of *cyclin E* requires activation by both E2F/DP and tissue-specific activators (18). In this study, we have shown that Apt and Cyclin E are coexpressed at the posterior cells to MF. The expression of Cyclin E was reduced in the *apt* mutant clones and induced by overexpression of Apt at the eye disk. Apt

directly activated the expression of *cyclin E* at the posterior cells to MF. These data suggest that Apt and E2F1 function together in the activation of *cyclin E* in the eye disk.

How Apt Controls the G1-to-S Phase Transition. Our experiments involving ectopic expression of Apt in a *GMR-Gal4/UAS-apt* fly demonstrate that *apt* expression can induce S phase. Ectopic expression of Apt at the eye disk, therefore, has a similar effect as that of ectopic expression of *cyclin E* from a heat-inducible transgene (*hs-cyclin E*) (7), suggesting that Apt and Cyclin E work on the same pathway. Cyclin E is essential and rate limiting for the G1-to-S phase transition (7, 20–22).

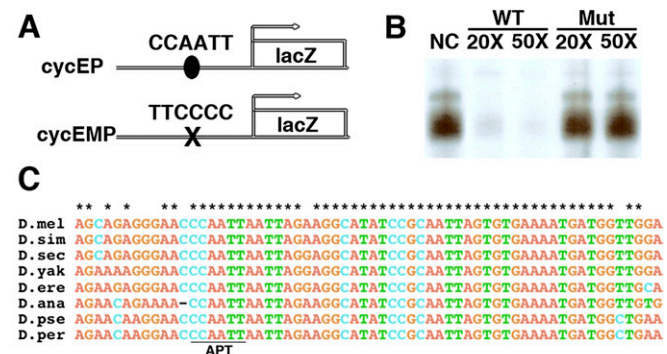


Fig. 5. *cyclin E* is a direct downstream target of *apt*. (A) Schematic illustration of the *lacZ* reporter constructs driven by either the *cyclin E* promoter carrying the wild type or the mutant Apt binding site. (B) Unlabeled DNA containing the consensus (WT) competed with labeled consensus oligonucleotides for binding to Apt protein. Mutated (Mut) oligonucleotides served as negative controls. NC, no competition. (C) Apt binding sites are conserved at the *cyclin E* promoter among several *Drosophila* species. Asterisk indicates nucleotides conserved in all listed species. Apt binding sites are indicated by underline. (D–F) The reporter *cycEPlacZ* (E) was coexpressed with the endogenous *apt* (D) in the eye disk. (G–I) The expression of reporter *cycEPlacZ* (H). Eye disk of a third instar larva was stained with anti-Apt antibody (D and G) and anti-β-galactosidase antibody (E and H). (F and I) Merged images of D and E and of G and H, respectively. (J and K) Increased population of S-phase cells in the *GMR-apt* eye imaginal disk (arrow). BrdU incorporation was examined using eye discs prepared from wild-type (J) and *GMR-apt* (K) flies. (L) Model for Apt-mediated regulation of G1-to-S phase at the MF.

directly activated the expression of *cyclin E* at the posterior cells to MF. These data suggest that Apt and E2F1 function together in the activation of *cyclin E* in the eye disk.

Our finding indicates that Apt controls G1-to-S phase progression by inducing *cyclin E* expression in the eye imaginal disk.

Apt Function During Development. What is the function of Apt during development? Apt was expressed at trachea, head, heart, and CNS and was required for the development of these tissues (12–15). However, how Apt controls the development of these tissues was unknown. In this study, we identified the candidates for Apt target genes using microarray analysis. Many of these genes can be assigned to specific aspects of the development of these tissues.

Apt and Cyclin E were evolutionarily conserved from *Drosophila* to humans. However, the function of Apt in human was unknown. In humans, the expression of *cyclin E* is related to many cancers (23). Furthermore, Apt overexpression suppresses cancer metastasis in *Drosophila* (24). The role of Apt in the regulation of *cyclin E* might be a conserved function because its human homolog fibrinogen silencer binding protein (FSBP) is also a cancer-related factor and is also expressed in many tissues, including heart, brain, lung, liver, skeletal muscle, kidney, and pancreas (25, 26). The discovery of a direct connection between cell differentiation and Cyclin E provides insights into the mechanism by which Apt promotes tumor formation.

Materials and Methods

Microarray Analysis. The *apt* heterozygote or mutant embryos were identified using *CyOact-GFP* balancer chromosome. Total RNA was extracted separately for each of the heterozygous and homozygous *apt* mutant embryos using the QIAGEN RNeasy isolation kit. Biotinylated cRNA probes were prepared from total RNA of each sample following the Affymetrix IVT labeling protocol. The labeled cRNA preparations were then fragmented and hybridized to Affymetrix *Drosophila* GeneChip 1.0 microarrays. The hybridized arrays were washed, stained, and scanned following the Affymetrix GeneChip Expression Analysis 2000 manual. All microarrays were scaled to the same target signal using the “All Probe Sets” scaling option. Probes with fold-change >2.0 and *P* values <0.05 were considered to be differentially expressed (Table S1). Raw and normalized microarray expression data are available on the NCBI Gene Expression Omnibus (GEO) Web site (www.ncbi.nlm.nih.gov/geo) under accession code GSE36917.

Real-Time PCR. Real-time PCR was conducted with the primers listed in Table S2 and Bio-Rad iQ SYBR Green Supermix (cat. no. 170–8882) on a DNA Engine real-time PCR machine (Bio-Rad).

Clonal Analysis. The *apt^{Pa3}* and *apt^{Pa4}* clones were generated using ey:FLP (27) or hs:FLP (28). For heat-shock experiments, clones were induced 24 h before dissection by a single incubation at 37 °C for 1 h. Discs were dissected from wandering third instar larvae.

Microscopy and Immunohistochemistry. Scanning electron microscopy was performed as previously described (29). Adult eye sections were prepared as previously described (30). Eye disk immunohistochemistry was performed as previously described (31). BrdU was performed as described (32). The primary antisera used were rabbit anti-Apt (1:1,000), rabbit anti-GFP (1:100), mouse anti-BrdU (1:100; 33281A; BD Biosciences), mouse anti-Cyclin E (4:50; gift of H. Richardson, Peter MacCallum Cancer Center, Melbourne), rat anti-Cyclin E (1:500; gift of H. Richardson), rabbit anti-β-galactosidase (1:2,000; Cappel), mouse anti-Cyclin B (1:50 F2F4; DSHB), and rabbit anti-phosphohistone H3 (1:1,000; 9701; Cell Signaling Technologies). The secondary antibodies used were as follows: Cy3-conjugated anti-mouse IgG (The Jackson Laboratory), Cy5-conjugated anti-rat IgG (The Jackson Laboratory), and Alexa 488 goat anti-rabbit IgG conjugate (Molecular Probes).

Generation of Transformants. The *cycE*PlacZ was constructed by inserting a 3,957-bp genomic fragment containing the upstream region and the transcription start site of *cyclin E* with an Apt-binding site (CCAATT) into a CaSpeR-AUG-β-gal vector. The *cycE*MPlacZ was made from *cycE*PlacZ and contained a mutated Apt-binding site (TTCCCC). The *sgP[Gal4]* driver strain was used for misexpression in the salivary glands (33).

Electrophoretic Mobility-Shift Assay. A gel mobility-shift assay was performed as described previously (15), except that electrophoresis was carried out on a 4% polyacrylamide gel in 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 380 mM glycine, and 5% (vol/vol) glycerol. The probe or functional competitor was 5'-GAGGGAAC-CCAATTAAATTAGAAGGCA-3'. The mutant competitor was 5'-GAGGGAACCTTC-CCAATTAGAAGGCA-3'. Underlined regions indicate the recognition sequence of Apt and its mutant derivative, respectively.

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