DREF Is Required for Efficient Growth and Cell Cycle Progression in Drosophila Imaginal Discs

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Based on overexpression studies and target gene analyses, the transcription factor DNA replication-related element factor (DREF) has been proposed to regulate growth and replication in Drosophila melanogaster. Here we present loss-of-function experiments to analyze the contribution of DREF to these processes. RNA interference-mediated extinction of DREF function in vivo demonstrates a requirement for the protein for normal progression through the cell cycle and consequently for growth of imaginal discs and the derived adult organs. We show that DREF regulates the expression of genes that are required for the transition of imaginal disc cells through S phase. In conditions of suppressed apoptosis, DREF activation can cause overgrowth of developing organs. These data establish DREF as a global regulator of transcriptional programs that mediate cell proliferation and organ growth during animal development.

The conversion from cellular self-renewal to differentiation, i.e., from a proliferating state to a postmitotic situation, is a fundamental step in the development of multicellular organisms. It is associated with major biochemical and metabolic transitions and coincides with global changes of gene expression. This event has to be regulated with exquisite precision, as small temporal or spatial deviations in switching from growth to differentiation phases of development can cause severe abnormalities. Furthermore, the regulation of genes that control the exit of the cell cycle and the subsequent cell fate decisions appears to be very tight, as exiting the cell cycle is a step that is rarely reversed in the adult. Failure to control growth arrest and terminal differentiation is the basis for malignant transformation and cancer. The termination of cell proliferation thus requires mechanisms that control many genes simultaneously in a coordinated, precise, and tight manner. In other situations where cells undergo major restructuring or changes of their physiology, as, for example, in the transition from a vegetatively growing bacterium to a spore or when germ line and soma separate during vertebrate development, such global changes are brought about by complex mechanisms that act at several levels of gene regulation. At the transcriptional level, events of this magnitude are mediated by enhancer binding transcription factors but also by changes of the general transcription machinery, such as sigma factors or TATA-binding protein variants (5, 12, 22).

We have previously analyzed the changes of gene expression as cells of the developing Drosophila melanogaster eye transit from a pluripotent and mitotically active precursor state to terminally differentiated cell types that comprise the adult eye (10). These studies identified a population of genes that are selectively expressed in the dividing precursor cells located anterior to the morphogenetic furrow of the eye imaginal disc.

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We and others have shown that ectopic expression of DREF can promote DNA replication in the eye imaginal disc, but overgrowth of the adult tissue was not detected, presumably because superfluous cells were eliminated from the developing tissue by apoptosis (7). DREF overexpression thus has phenotypic consequences that are similar to the effect of overexpression of E2F1 and other cell cycle regulators (2, 14, 18). In this study, we present evidence that DREF is required for the expression of cell cycle-related genes and for the control of cell proliferation during organ development in the fly.

MATERIALS AND METHODS

Fly strains. Several fly lines used in this study are gifts: UAS DREF from K. Ohno and SepGal4 and GMRGal4 from M. Mlodzik. The following fly lines were obtained from the Bloomington stock center: hid(W1); enGal4; eyeless Gal4; T80Gal4; tubGal80TS; hsFLP, Act5C::CD2::Gal4; Oregon R; and w1118.

Generation of transgenic flies. To generate the DREF RNAi construct (see schematic in Fig. 1A), a 1.28-kb genomic DNA fragment including exons 2, 3,
and 4 was amplified with primers containing EcoRI and NotI restriction sites. For amplification of a 1.15-kb cDNA fragment spanning exons 2, 3, and 4, primers containing Asp718 and NotI restriction sites were used. The two fragments were ligated in tail-to-tail orientation via their respective NotI sites, and the ligation product was cloned as an EcoRI-Asp718 fragment into the pUAST transformation vector. Two independent transgenic lines carrying upstream activation sequence (UAS) DREFRNAi were established by P-element-mediated germ line transformation (21). One line carries the RNA interference (RNAi) construct on the X chromosome and one on the third chromosome. The fly line carrying the construct on the X chromosome generates stronger DREF knockdown phenotypes than the one with the insertion on the third chromosome. Except in the experiment in which measurement of adult wing size was conducted with moderate level of DREFRNAi expression (Fig. 2), the stronger X-linked RNAi line was used throughout this study.

### In situ hybridization.
Measurement of endogenous levels of Dref mRNA in either third instar wing imaginal disc or eye disc was carried out with digoxigenin-labeled antisense mRNA probes as described previously (24).

### Area and cell size measurement in adult wings and eyes.
Wing size was measured by counting pixels on digital images using Adobe Photoshop. The size of cells in the adult wing was determined in the following way. The number of trichomes in areas of defined size in the posterior compartment of adult wings was counted. Cell size was estimated by calculating the wing area per trichome. Eye size was quantitated by measuring the circumference of eyes of the respective phenotypes using digital images and Adobe Photoshop.

### Clonal analysis.
Random clones were generated in larval wing discs using the FRT/FLP method (23). Flies homozygous for hsFLP (Act5C/H11022 CD2/H11022 Gal4 and UAS enhanced green fluorescent protein [EGFP]) were crossed with either flies carrying T80Gal4, UAS EGFP; tubGal80 TS. Eggs were collected for 3 h and larvae were raised at 22°C until heat shock at 37°C for 2 h for induction. After induction, larvae were kept at 25°C until dissection. To measure cell size in the wing disc using forward scatter, UAS DREFRNAi flies were crossed with flies carrying enGal4 and UAS EGFP. Cell dissociation and sorting from third instar larval wing disc were performed as described previously (14) for each experiment.

### Flow cytometry.
To express DREFRNAi in an inducible manner, we employed the Gal4/Gal80 TARGET system (13). Flies bearing UAS DREFRNAi were crossed with flies carrying T80Gal4, UAS EGFP; tubGal80 TS. Eggs were collected for 3 h and larvae were raised at 22°C until heat shock at 37°C for 2 h for induction. After induction, larvae were kept at 25°C until dissection. To measure cell size in the wing disc using forward scatter, UAS DREFRNAi flies were crossed with flies carrying enGal4 and UAS EGFP. Cell dissociation and sorting from third instar larval wing disc were performed as described previously (14) for each experiment.

### RESULTS
Loss of DREF decreases adult organ size. The lack of loss-of-function alleles for Dref has hampered the functional characterization of this gene and the analysis of its contribution to normal tissue growth. To overcome this limitation, we generated transgenic fly lines in which an inverted repeat of the Dref transcript can be expressed under the control of a yeast Gal4 upstream activating sequence (UAS DREFRNAi) (Fig. 1A). In these flies, DREF function can be ablated by RNAi in a spatially and temporally controlled manner using the Gal4/UAS system (4, 11). Two independent RNAi lines were analyzed by semiquantitative reverse transcriptase PCR (RT-PCR) (Fig. 1B). When RNAi expression was directed ubiquitously under the control of T80Gal4, both RNAi lines showed significantly decreased levels of endogenous Dref mRNA, with the construct inserted on the X chromosome causing a stronger suppression than the one residing on the third chromosome. Un-

![Image](http://mcb.asm.org/)
less stated otherwise, the stronger X-linked RNAi construct was used in the experiments described below.

We tested the efficiency of spatially restricted DREF knockdown using in situ hybridization with a Dref-specific probe that does not overlap with the double-stranded RNA construct (Fig. 1A). In the third instar larval wing imaginal disc of wild-type animals, Dref mRNA is uniformly distributed (Fig. 1C). However, when DREF RNAi was expressed in the posterior compartment using the engrailed Gal4 (enGal4) driver, Dref mRNA levels were markedly decreased in this region (Fig. 1D).

Overexpression of DREF in the posterior compartment of the wing resulted in developmental defects (Fig. 1E). Similarly, loss of DREF function, brought about by DREF RNAi expression at high levels (from the X-linked transgene), severely disrupted normal wing development (Fig. 1F). These two effects neutralized each other, and the wing developed normally when DREF RNAi was coexpressed with DREF (Fig. 1G). This result indicates that the phenotype elicited by the RNAi construct was caused by a specific decrease of Dref mRNA. Consistent results were observed in the eye, where DREF RNAi could suppress the aberrant eye phenotype elicited by DREF overexpression (Fig. 1H and I).

To test whether DREF is required for normal organ growth, we analyzed the consequences of DREF knockdown in the developing wing and eye. In the fly, the effect of transgenes on tissue growth can be conveniently assessed by overexpressing them under the control of enGal4 in the posterior compartment of the wing. In such a setting, the size of the anterior compartment serves as an internal wild-type control. We analyzed a Drosophila line carrying the moderately expressing DREF RNAi transgene on the third chromosome to assess wing disc growth in a DREF loss-of-function situation. Using this allele, we avoided the massive growth and developmental defects observed in wings in which DREF function was ablated more dramatically (Fig. 1F). Such conditions of limited knockdown of DREF expression did not affect patterning, but growth of the posterior compartment was significantly reduced, indicating that wild-type levels of DREF are critically required for normal tissue growth (Fig. 2A to C). The observed reduction in wing size correlates with a smaller cell size in the posterior compartment, as revealed by a higher density of trichomes in the area of DREF RNAi expression (Fig. 2D). At higher levels of DREF RNAi expression, more severe phenotypes manifested themselves, possibly including patterning defects. This is consistent with recent reports that implicate DREF in mitogen-associated protein kinase-dependent vein differentiation (27). Whether such aberrant patterning phenotypes are a primary consequence of DREF deficiency or an indirect effect of growth defects cannot be judged based on the evidence presently available.

The expression pattern of Dref in the developing Drosophila eye imaginal disc is consistent with its proposed predominant function in cell proliferation. Dref mRNA is expressed at high levels in the dividing and growing cells of the eye imaginal disc, which are located anterior to the morphogenetic furrow (MF) (Fig. 3A). The MF consists of cells that have arrested in G1 phase of the cell cycle in a coordinated fashion. Posterior to the MF, some cells become determined and differentiate into photoreceptors, while others undergo one more cell division

FIG. 3. DREF is required in proliferating cells during eye development. (A) In situ hybridization in the third instar larval eye imaginal disc visualizes the Dref expression pattern during eye development. Dref mRNA was detected in regions where cells undergo cell division. (B) The arrowhead demarcates the position of the second mitotic wave. Anterior is to the left. When UAS DREF RNAi was driven by GMR-Gal4 (B) or SepGal4 (C) in postmitotic cells, no discernible developmental defects were observed. Panel C serves as a control for the eye shown in panel D. (E and F) DREF RNAi expression was induced in the entire larval eye disc under the control of eyegless Gal4, resulting in severe developmental defects. Images were taken at the same magnification.
and are thus part of the “second mitotic wave” (3). DREF expression is low posterior to the second mitotic wave, suggesting that it is not required for normal photoreceptor differentiation. Consistent with this notion, expression of DREFRNAi in differentiating cells of the eye did not interfere with normal eye development (Fig. 3B and D). However, when expressed in the whole-eye imaginal disc, including areas of active cell proliferation, DREFRNAi induced drastically aberrant phenotypes. These ranged from small, rough eyes to the complete loss of the organ (Fig. 3F). We conclude that DREF function is required for normal growth and cell proliferation in the eye but does not contribute significantly to the patterning and differentiation processes that shape the eye after cell proliferation has ceased.

DREF is required for clonal growth. To observe the effect of DREF in cell proliferation directly, we generated random EGFP-marked clones of cells expressing DREFRNAi in third instar wing imaginal discs. Such clones were significantly smaller and less abundant than control clones expressing only EGFP (Fig. 4). Furthermore, clones that were generated at earlier stages of larval development almost never survived through the third instar larval stage, while control clones were found abundantly (data not shown). These results suggest that cell clones expressing DREFRNAi had a growth disadvantage and were eliminated in the course of wing disc growth.

Loss of DREF impairs cell cycle progression during imaginal disc development. To investigate whether the requirement of DREF for organ growth might reflect a function in cell cycle regulation, we assessed the cell cycle profile of wing imaginal disc cells in which DREF was knocked down. To this end, we induced DREFRNAi expression ubiquitously in third instar larvae using the TS-Gal80 TARGET system (13). At various time points after DREFRNAi induction, we analyzed the cell cycle distribution of dissociated wing disc cells using fluorescence-activated cell sorter (FACS) analysis. While the cell cycle profile did not change in control cells, among cells expressing DREFRNAi, the cell population residing in the G2 phase of the cell cycle was progressively lost over a time course of 16 h. This result suggests that DREF function is required for cells to progress through late G1 phase or S phase efficiently (Fig. 5A and B).

Cell size as measured by forward light scatter supports the notion that the predominant consequence of DREF abrogation in mitotic cells is a defect in cell cycle regulation rather than growth. We used two genotypes to assess the effect of

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**FIG. 4.** DREF is required for clonal growth in the wing imaginal disc. (A and B) GFP-marked clones were generated using the heat shock Flip-out method (see Materials and Methods), and clones expressing DREFRNAi were compared with control GFP-expressing clones for growth. Clones with decreased DREF function frequently did not survive or grew to a smaller size. (C and D) Graphs show the clone sizes and numbers in wing discs.
FIG. 5. Loss of DREF interferes with normal cell cycle progression in vivo. EGFP (A) or DREFRNAi and EGFP (B) expression was induced ubiquitously in third instar larvae using the Gal4/Gal80 TARGET system. The DNA content of GFP-positive cells as measured by FACS analysis is shown in the histograms. Suppression of DREF function caused a progressive loss of the cell population in G2/M phase of cell cycle throughout the time course of the experiment. The level of EGFP expression is depicted in the graphs in the far right and left panels and serves as a measure for the expression of the UAS transgenes. (C) Forward scatter of cells expressing DREFRNAi in a spatially restricted area under the control of engrailed promoter. DREFRNAi was expressed together with EGFP in the posterior compartment of the larval wing disc. Cells in the posterior compartment of the wing disc expressing DREFRNAi were substantially larger than those in the control wing disc. (D) Cell size measured by forward scatter after expression of DREFRNAi using inducible system (see panels A and B). Cell sizes were compared between the two different genotypes indicated under the same condition. Compared to the nonexpressing fraction of wing disc cells (left panel), cells expressing DREFRNAi became bigger than cells in the control wing discs (right panel).
DREF suppression on cell size. First, we expressed DREFRNAi along with a GFP marker under the control of enGal4 in the posterior part of wing imaginal discs (Fig. 5C). GFP-positive G1/S cells of this genotype were larger than control cells in which GFP was expressed alone. The size of the anterior cells, in which enGal4 is not active, was not affected. To rule out possible differentiation or developmental effects of long-term DREF suppression as a cause for the change in the size of the G1/S cell population, we also analyzed wing imaginal disc cells in which DREFRNAi was expressed for just 16 h using the inducible TARGET system (Fig. 5D). Again, cells in which DREF activity was thus suppressed shifted to a larger size compared to control cells. These findings suggest that the inefficient progression into or through S phase is not a result of cells not reaching a critical size threshold. It rather seems that cells lacking DREF activity accumulate in G1 and continue to grow to a bigger size than cells in a wild-type disc. A requirement for DREF for cell cycle regulation is also supported by the identity of its target genes (see below).

**DREF induces cell cycle-related target genes in vivo.** Next, we examined whether DREF-regulated gene expression might account for the cell cycle effects described above. This possibility is supported by the prevalence of DRE sites in the 5′ region of genes involved in cell growth and proliferation (10). To analyze potential DREF-inducible changes in expression of such potential DREF targets, we ubiquitously expressed either wild-type DREF or DREFRNAi in larvae and performed semi-quantitative RT-PCR analysis (Fig. 6). Consistent with previous observations (8, 16, 19, 20), overexpression of wild-type DREF increased mRNA levels of genes that are known to promote G1-S transition and that are required for S phase, including cyclin E, cyclin A, dE2F1, myb, the DNA polymerase α gene, and PCNA. The same genes were down-regulated in loss-of-function conditions for DREF. Our studies identified the orc2 gene (origin recognition complex subunit 2) as a novel DREF target. Its 5′ promoter region was found to bear three putative DREF binding sites (48 bp before the start codon), and our RT-PCR results showed that overexpression of DREF increased orc2 transcript levels and loss of DREF reduced them in vivo. These results demonstrate that DREF is sufficient and required to induce the expression of genes involved in S-phase progression in vivo and suggest that the absence of the DREF-induced gene expression program is the cause for the reduced size of DREFRNAi-expressing tissues. To test whether DREF would specifically be required for S phase or might also affect other stages of the cell cycle, we measured the RNA levels of cyclin B and string (the Drosophila homolog of cdc25) as representative regulators of the G2-M transition in DREF gain- and loss-of-function conditions. As opposed to the effect on S-phase genes, gain of DREF function did not result in up-regulation of these mitotic genes, indicating that their expression is not controlled by the transcription factor. This is consistent with the absence of recognizable DREF DNA in the RNA preparations. RT-PCR for the ribosomal rp49 mRNA served as an internal reference measurement. The gene hlc, which encodes a helicase, was not activated by DREF expression, confirming that increasing levels of DREF did not cause a general up-regulation of transcription.
binding motifs in the respective promoter regions. cyclin B and string expression levels were modestly reduced in the DREF knockdown background. This effect is most likely indirect and explained by the smaller fraction of cells that reach the G2-M phase in conditions of reduced DREF activity (Fig. 5B).

Not all tested genes preceded by putative DREF binding sites were induced in response to DREF expression in vivo. For example, the helicase gene hlc carries three DREs in its upstream region (37 bp before its start site), but RT-PCR data showed no changes in its mRNA levels with either DREF or DREFRNAi overexpression. This finding suggests that for some genes DREF may not be sufficient, and additional inputs might be required for their transcriptional activation. Alternatively, it is possible that not all computationally identified DREF binding sites in the promoter regions are functional in the cells tested. It seems clear that DREF overexpression does not cause an indiscriminate and global activation of transcription.

Growth promotion by DREF gain of function. Our results indicate that DREF directs a gene expression program that should promote cell proliferation in developing imaginal discs and increase organ size. Accordingly, gain-of-function situations for DREF may be expected to result in tissue overgrowth. It has been difficult to directly test this hypothesis, as DREF overexpression resulted in an increase in cell cycle markers in the developing disc that was accompanied by widespread apoptosis (7). The resulting adult organ thus typically did not show overgrowth. We reasoned that higher than wild-type levels of DREF activity might cause problems during replication and result in cell death that is initiated by common cell cycle checkpoints and developmental safeguards.

We wanted to examine whether a DREF-driven growth program would become apparent in conditions of suppressed apoptosis. Thus, we conducted genetic interaction experiments by crossing flies in which DREF was overexpressed in cells of the developing eye imaginal disc with flies carrying a homozygous viable loss-of-function allele of the proapoptotic gene head involution defective, hid(W3) (1, 6). In agreement with our hypothesis, eyes expressing DREF in hid mutant backgrounds

FIG. 7. DREF-induced overgrowth in adult eyes. (A) Ectopic expression of DREF in a hid homozygous mutant background promotes overgrowth in the adult eye. The eye of hid mutants is phenotypically wild type (B). Panel C shows an eye of the wild-type Oregon R strain. (D) Eyes of flies that overexpress DREF under the control of GMRGal4 in a wild-type background show decreased size. (E) The outlines of the eyes shown in panels A to D were traced and overlaid to illustrate size differences. (F) The bar graph displays sizes of nine adult eyes per genotype as measured by their circumference. DREF overexpression in a hid mutant background caused bigger adult eyes (*, P < 10⁻⁶). All images were taken at the same magnification. Anterior is left in all pictures.
grew larger than eyes in control animals (Fig. 7A and B). In addition to increased eye circumference, as quantified in figure Fig. 7F, DREF-overexpressing eyes frequently displayed bulged-out areas of overgrowth (Fig. 7A, arrowhead) when both copies of wild-type hid were eliminated. These data demonstrate that, consistent with its molecular targets and its effects on cells, DREF overexpression is sufficient to promote tissue growth during larval development.

**DISCUSSION**

Here we present a loss-of-function analysis that supports a function of DREF as a regulator of a proliferative cell state. Based on these studies, we propose the following: (i) DREF function is required in proliferating but not postmitotic cells; (ii) loss of DREF prevents cells from effectively completing S phase; (iii) the inefficient progression into or through S phase is not caused by a cell growth defect in G1; and (iv) the inefficient progression of DREF-deficient cells through S phase is due to suboptimal expression of replication factors and G1-S regulators, including PCNA, Orc2, and E2F.

The size of DREF-deficient cells in proliferating imaginal disc tissues is slightly larger than that of wild-type cells, which we interpret as a consequence of delayed S-phase entry. In contrast, the size of cells in the adult wing that has developed in conditions of a partial loss of DREF function brought on by moderate RNAi expression is slightly decreased. This phenotype of postmitotic wing cells may be mediated by effects of DREF on functions other than cell cycle progression. In addition to cell cycle regulators, DREF has been proposed to regulate the expression of proteins involved in anabolic functions (such as ribosomal proteins and tRNA synthetases) (10).

In postmitotic cells, defects in the expression of such genes might cause a growth defect that is not apparent in cycling DREF-deficient cells where regulators of G1-S progression appear to be limiting.

Ectopic activation of DREF can drive even differentiating cells into S phase (7). Such a forced entry into the cell cycle may cause the induction of apoptosis. Indeed, when a known mediator of cell death, Hid, is inactivated, DREF-induced apoptosis is suppressed and tissue overgrowth can be observed. Whether the mammalian relative of DREF that has recently been identified has similar functions and might, as such, contribute to malignant overgrowth, for example, in a p53 mutant background, is an intriguing possibility that remains to be examined.

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