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A Genomic Switch at the Transition from Cell Proliferation to Terminal Differentiation in the *Drosophila* Eye

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Summary

Organogenesis involves cell proliferation followed by complex determination and differentiation events that are intricately controlled in time and space. The instructions for these different steps are, to a large degree, implicit in the gene expression profiles of the cells that partake in organogenesis. Combining fluorescence-activated cell sorting and SAGE, we analyzed genomic expression patterns in the developing eye of *Drosophila melanogaster*. Genomic activity changes as cells pass from an uncommitted proliferating progenitor state through determination and differentiation steps toward a specialized cell fate. Analysis of the upstream sequences of genes specifically expressed during the proliferation phase of eye development implicates the transcription factor DREF and its inhibitor dMLF in the control of cell growth in this organ.

Introduction

One of the best-studied model systems for organ development is the compound eye of *Drosophila melanogaster*. Its morphogenesis, starting from a relatively unstructured *anlage*, the eye imaginal disc, to a highly ordered sensory apparatus carrying an array of ommatidia comprised of multiple types of specialized cells, remains one of the best-understood developmental processes to date (Bonini and Fortini, 1999; Freeman, 1997; Hardie and Raghu, 2001; Kumar and Moses, 1997; Reifegerste and Moses, 1999; Salecker et al., 1998).

The eye imaginal disc initially represents an epithelium of undifferentiated asynchronously dividing cells. In the larval stages of development, cell proliferation ceases and a differentiation program initiates at the posterior margin of the disc. These events are remarkably coordinated: as imaginal disc cells lose their stem cell character and exit from the cell cycle, they start differentiation in a wave that sweeps across the eye disc from posterior to anterior (Figure 1). The border between the dividing, pluripotent cells at the anterior, and the differentiating cells at the posterior side of the eye disc, is marked by a depression in the disc epithelium, referred to as the morphogenetic furrow (MF). The developmental program that is executed after the MF has passed comprises the differentiation of several cell types: the inner photoreceptor R8 as well as four outer photoreceptors (R2 and R5 followed by R3 and R4) start differentiating immediately posterior to the MF, whereas the inner photoreceptor R7, the outer photoreceptors R1 and R6, as well as a series of accessory cells (cone cells and pigment cells) start their differentiation after one last coordinated mitosis (the second mitotic wave; Baker, 2001). After their initial specification, photoreceptor neurons start a differentiation program that includes outgrowth of axons and generation of synaptic connections in the optic lobe of the larval brain (Salecker et al., 1998). The differentiating cells form clusters, which undergo a coordinated rotation to orient the mature ommatidia properly in the plane of the eye imaginal disc. Finally, superfluous undifferentiated cells are removed by apoptotic cell death (Bonini and Fortini, 1999; Freeman, 1997).

The developing eye of *Drosophila melanogaster* has proven a supremely rich system for studies of signaling events that direct growth and patterning as well as cell determination and differentiation processes during development of a complex tissue. Mutant analysis and genetic screens have characterized the contribution of a number of different signal transduction pathways that act in conjunction to specify the patterning and development of this organ. The wealth of data that has been generated in these studies provides a detailed framework for further genetic, molecular, and genomic analyses of organ formation.

The work described here complements this framework and provides a deeper understanding of the molecular events orchestrating organ development by mapping the transcriptional landscape in the developing eye as its cells undergo transcriptome changes in a temporally and spatially defined manner.

Results

SAGE Analysis of Purified Cell Populations from Eye Imaginal Discs

To monitor the genome-wide gene transcription profiles associated with the different phases of eye development, we analyzed defined subsets of cells isolated from eye imaginal discs. These groups of cells were distinguished by the specific expression of green fluorescent protein (GFP) under the control of the Gal4-UAS system (Brand and Perrimon, 1993; Figure 1). Three distinct cell populations were purified from dissected third instar eye imaginal discs by fluorescence-activated cell sorting (FACS) of trypsin-dissociated cells (Figure 1E). The first pool (referred to as GMR^{-}; see below) contained cells from the region before the MF and represents the pluripotent, proliferative stage of eye development. The

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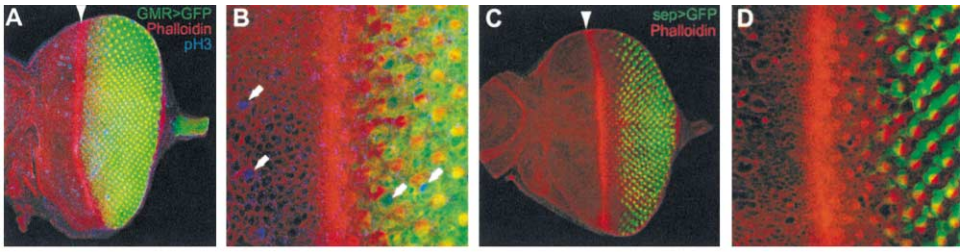

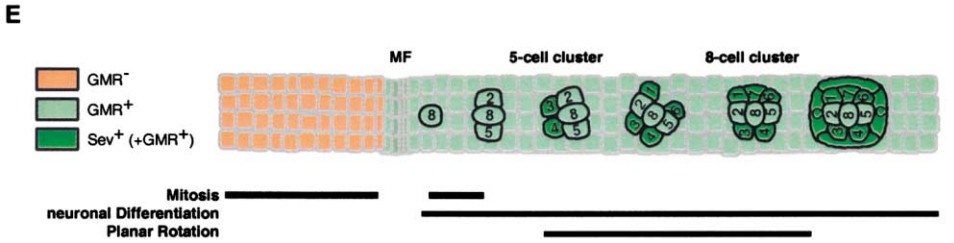

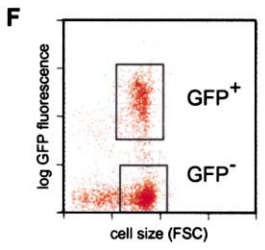

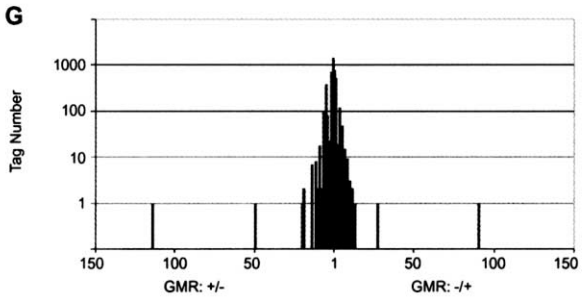

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Tag Sequence	GMR-	GMR+	Sev+	Gene Name
GCGCAGACTT	183.1	202.8	122.1	RpS9: Ribosomal protein S9
AGGCAGACCG	149.7	125.9	132.9	Ef1alpha48D: Elongation factor 1alpha48D
CAGAACCGCG	130.6	129.2	98.3	RpL32: Ribosomal protein L32
GTGGCCGTTA	85.8	69.1	37.8	RpL30: Ribosomal protein L30
CAAGAAGACC	61.0	56.8	35.6	RACK1: Receptor of activated protein kinase C 1
CGCCACTTTG	37.2	40.1	36.7	Hsp26: Heat shock protein 26
ACAAGGACAT	21.9	21.2	20.5	Act5C: Actin 5C
CCCACCTTCG	5.7	8.9	5.4	thioredoxin
CATTCCTACA	5.7	6.7	4.3	Act42A: Actin 42A
CCAACACACA	5.7	5.6	4.3	HmgZ: HMG protein Z
AAGGCCGCCG	5.7	4.5	4.3	cype: cyclope (cytochrome c oxidase subunit VIc)
GCGCTATATA	2.9	3.3	3.2	Gst2: Glutathione S transferase 2
GGCGTGAAGC	2.9	2.2	3.2	NHP2 (rRNA processing)
GTGGACAACT	2.9	2.2	3.2	RpII18: RNA polymerase II 18kD subunit

Figure 1. SAGE Analysis of Sorted Cell Populations from Eye Imaginal Discs

(A–D) GFP fluorescence marks differentiating cells in eye discs dissected from *w; GMRGal4/UAS-eGFP* larvae (A and B). Anterior is left in all panels. The confocal images in (A)–(D) show eye imaginal discs stained with TRITC-phalloidin (red) to visualize the actin cytoskeleton and anti-phospho histone 3 (pH3, blue) to mark mitotic cells (arrows in [B]). The undifferentiated cells in the anterior part of the disc lack GFP fluorescence (green) and are proliferating, as indicated by the high number of pH3-positive cells. Expression of the GFP transgene starts in the MF (arrowhead) and marks cells of the second mitotic wave (pH3-positive cells posterior to the furrow) as well as postmitotic differentiating cells. (B) shows a magnified view of the same specimen as in (A). Note that GFP fluorescence becomes detectable a few hours after translation of the GFP protein due to slow maturation of the fluorophore. It is therefore detected in situ only after ommatidial preclusters are formed. Since the dissociation of the tissue by trypsinization takes 3–4 hr, it can be expected that all cells that expressed the GFP transgene within the intact tissue exhibited fluorescence when sorting was performed. Dissociation and sorting was done on discs from which the antennal disc (most anterior structure) was removed. In eye discs from *w; UAS-eGFP/+; sepGal4/+* larvae, GFP expression is confined to subsets of postmitotic differentiating photoreceptors and cone cells (C and D).

(E) Schematic showing domains of organogenesis in the eye imaginal disc. Cells anterior to the MF (GMR^- pool shown in red) proliferate and grow in an unsynchronized manner. Within the MF, cells enter an extended G1 phase. The prospective R8 cell is specified and starts differentiation within the MF. It then recruits R2, R3, R4, and R5 into ommatidial preclusters while the other cells undergo one round of coordinated mitosis. After this second mitotic wave, photoreceptor precursor cells R1, R6, R7, and four cone cells (labeled C) are recruited into the ommatidial clusters and start differentiation. Concurrent with their assembly, the ommatidial clusters rotate in the plane of the disc. The cells in which the GMRGal4 driver is active (GMR^+ pool) are shown in green and the Sev^+ cells are in dark green.

(F) Sorting profile of cells derived from eye imaginal discs of GMRGal4, UAS-eGFP larvae. 800,000 cells each were used for the GMR^+ and GMR^- libraries; 80,000 cells were used for the Sev^+ library. Cells were gated for morphology (forward scatter/side scatter) to exclude dead cells and cell aggregates.

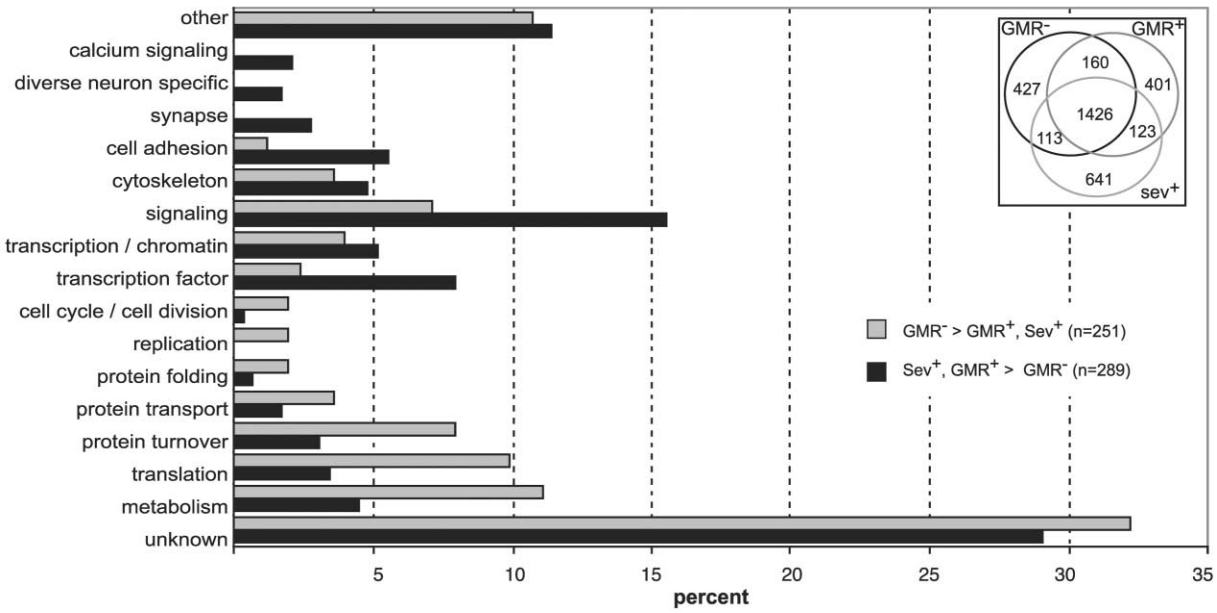


Figure 2. Functional Classification of Region-Specifically Expressed Genes

Functional classification of transcripts enriched in each library as compared to the other two. Genes coding for transcripts enriched in any one library were classified according to their known or predicted function. The fraction of genes belonging to the indicated functional class is represented as the percentage of all analyzed genes coding for enriched transcripts. Transcripts were considered "enriched" when their expression levels were 3-fold or higher in one library as compared to the other two. Tags with no or unclear annotation (about 20% of the cases in each library) were disregarded in the analysis. Note the high representation of genes involved in protein turnover (translation, modification, and degradation) as well as genes involved in cell proliferation (replication, mitosis, and cytokinesis) within the genes encoding enriched transcripts in GMR^-. See Figure 3 for examples. The inset shows the distribution of 3291 tags present at least twice in any one library. The largest fraction (1426) corresponds to tags that are present in all three libraries in similar amounts. For a complete overview of the described libraries, tag annotations, and functional classification, see Supplemental Tables S1 and S2. Note that several genes appear to be exclusively expressed in the Sev^+ library, even though the Sev^+ cell pool is part of the larger GMR^+ cell pool. The Sev^+ population constitutes a relatively small fraction of cells within the GMR^+ pool. As a consequence, genes that are represented in Sev^+ might be present in GMR^+ at levels that are below the detection limit of our analysis.

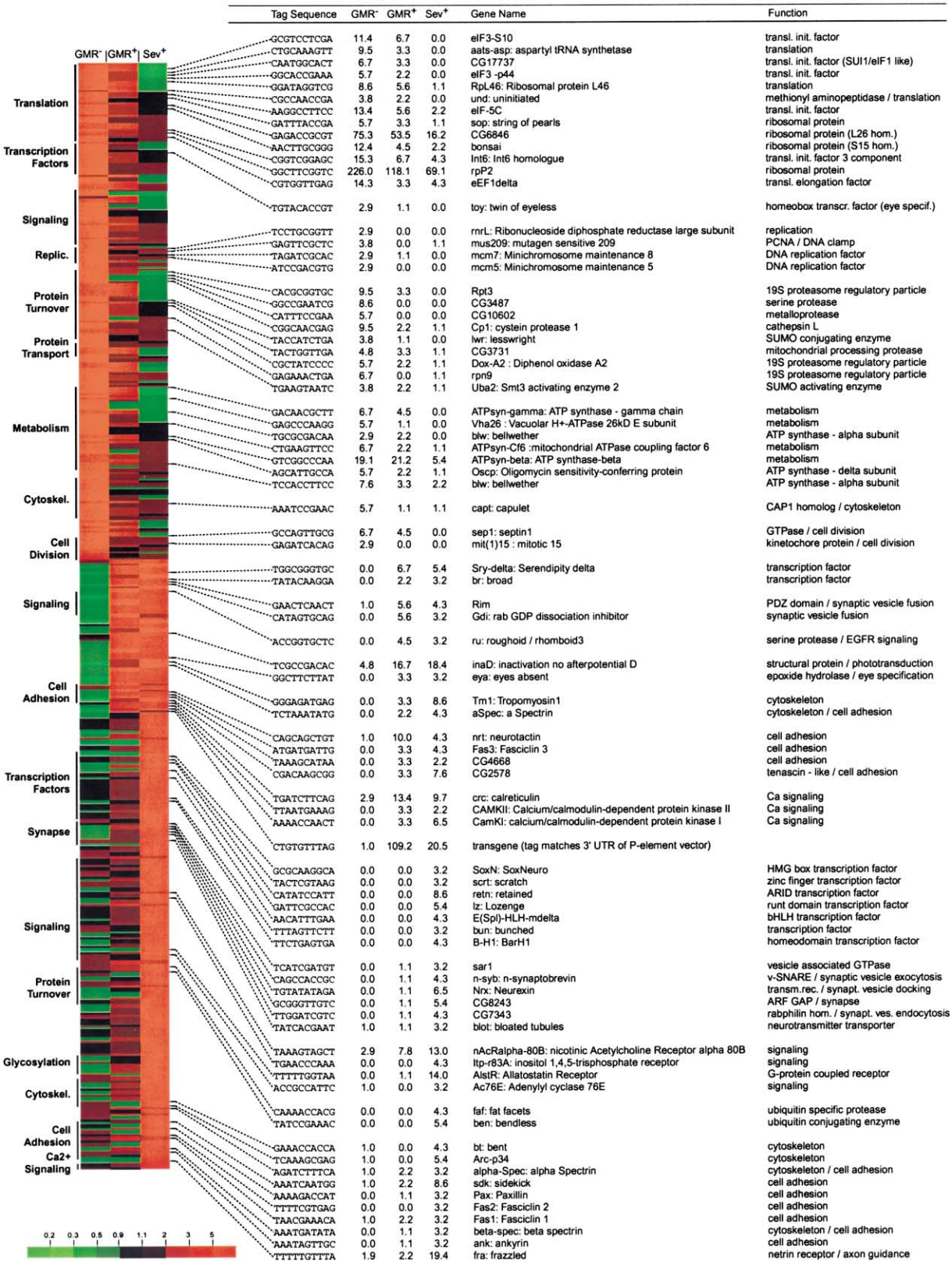
second pool of cells (GMR^+) includes cells in the morphogenetic furrow, the second mitotic wave, as well as cells engaged in differentiation and patterning programs. Expression of GFP under the control of the GMR-Gal4 driver (Hay et al., 1997; Moses and Rubin, 1991) is restricted to the second pool of cells and can be used to distinguish the two cell populations (Figures 1A and 1B). The third cell pool that was isolated represents a late stage of organogenesis, a group of already determined cells that are undergoing differentiation into specialized photoreceptor and cone cells (Figures 1C and 1D). These cells were sorted based on GFP expression under the control of the *sevenless* enhancer/promoter (using sepGal4; Therrien et al., 1999), which is transiently active in R3/R4 photoreceptor precursors and whose expression during ommatidial development becomes confined to R1, R6, R7, and the cone cells (Tomlinson et al., 1987).

The transcriptome of the three cell pools was quantitatively analyzed by serial analysis of gene expression (SAGE; Velculescu et al., 1995). SAGE was chosen as a method, since it allows accurate genome-wide quantification of mRNA levels in minute amounts of cellular material (St Croix et al., 2000; Virlon et al., 1999), without the need for amplification of the RNA pool by strategies that are prone to distortion of relative RNA representation. SAGE libraries were constructed from the sorted GMR^-, GMR^+, and Sev^+ cell pools. Close to 20,000 tags were sequenced from each library, generating expression data for 4,279 different genes (tags present twice or more times in the 57,441 tags of the combined libraries).

SAGE tags were annotated using recently described databases (Jasper et al., 2001; available at http://ccb-research20.urmc.rochester.edu) and by BLAST searches against the *Drosophila* genome. Similar to our results in

(G) Distribution of up- or downregulated SAGE tags when comparing GMR^+ to GMR^- libraries. To calculate ratios, a value of 0.5 was assigned to tags not detected in one of the libraries. Most transcripts are present in equal amounts in both libraries (less than three times up- or downregulated).

(H) Examples for tags that are represented at unchanged frequencies in the three compared libraries. Relative tag numbers (number of specific tags per 20,000 tags sequenced) and the corresponding genes are shown. Note that tag numbers of specific genes are comparable across the three libraries for a wide range of expression levels.



Tag Sequence	GMR⁻	GMR⁺	Sev⁺	Gene Name	Function
GCGTCCTCGA	11.4	6.7	0.0	eIF3-S10	transl. init. factor
CTGCAAAGTT	9.5	3.3	0.0	aats-asp: aspartyl tRNA synthetase	translation
CAATGGCACT	6.7	3.3	0.0	CG17737	transl. init. factor (SUI1/eIF1 like)
GGCACCGAAA	5.7	2.2	0.0	eIF3 -p44	transl. init. factor
GGATAGGTCG	8.6	5.6	1.1	RpL46: Ribosomal protein L46	translation
CGCCAACCGA	3.8	2.2	0.0	und: uninitiated	methionyl aminopeptidase / translation
AAGGCCTTCC	13.4	5.6	2.2	eIF-5C	transl. init. factor
GATTTACCGA	5.7	3.3	1.1	sop: string of pearls	ribosomal protein
GAGACCGCGT	75.3	53.5	16.2	CG6846	ribosomal protein (L26 hom.)
AACTTGCGGG	12.4	4.5	2.2	bonsai	ribosomal protein (S15 hom.)
CGGTCGGAGC	15.3	6.7	4.3	Int6: Int6 homologue	transl. init. factor 3 component
GGCTTCGGTC	226.0	118.1	69.1	rpP2	ribosomal protein
CGTGGTTGAG	14.3	3.3	4.3	eEF1delta	transl. elongation factor
TGTACACCGT	2.9	1.1	0.0	toy: twin of eyeless	homeobox transcr. factor (eye specif.)
TCCTGCGGTT	2.9	0.0	0.0	rnrL: Ribonucleoside diphosphate reductase large subunit	replication
GAGTTCGCTC	3.8	0.0	1.1	mus209: mutagen sensitive 209	PCNA / DNA clamp
TAGATCGCAC	2.9	1.1	0.0	mcm7: Minichromosome maintenance 8	DNA replication factor
ATCCGACGTG	2.9	0.0	0.0	mcm5: Minichromosome maintenance 5	DNA replication factor
CACGCGGTGC	9.5	3.3	0.0	Rpt3	19S proteasome regulatory particle
GGCCGAATCG	8.6	0.0	0.0	CG3487	serine protease
CATTTCCGAA	5.7	0.0	0.0	CG10602	metalloprotease
CGGCAACGAG	9.5	2.2	1.1	Cp1: cystein protease 1	cathepsin L
TACCATCTGA	3.8	1.1	0.0	lwr: lesswright	SUMO conjugating enzyme
TACTGGTTGA	4.8	3.3	1.1	CG3731	mitochondrial processing protease
CGCTATCCCC	5.7	2.2	1.1	Dox-A2 : Diphenol oxidase A2	19S proteasome regulatory particle
GAGAAACTGA	6.7	0.0	1.1	rpn9	19S proteasome regulatory particle
TGAAGTAATC	3.8	2.2	1.1	Uba2: Smt3 activating enzyme 2	SUMO activating enzyme
GACAACGCTT	6.7	4.5	0.0	ATPsyn-gamma: ATP synthase - gamma chain	metabolism
GAGCCCAAGG	5.7	1.1	0.0	Vha26 : Vacuolar H+-ATPase 26kD E subunit	metabolism
TGCGCGACAA	2.9	2.2	0.0	blw: bellwether	ATP synthase - alpha subunit
CTGAAGTTCC	6.7	2.2	1.1	ATPsyn-Cf6 :mitochondrial ATPase coupling factor 6	metabolism
GTCGGCCCAA	19.1	21.2	5.4	ATPsyn-beta: ATP synthase-beta	metabolism
AGCATTGCCA	5.7	2.2	1.1	Oscp: Oligomycin sensitivity-conferring protein	ATP synthase - delta subunit
TCCACCTTCC	7.6	3.3	2.2	blw: bellwether	ATP synthase - alpha subunit
AAATCCGAAC	5.7	1.1	1.1	capt: capulet	CAP1 homolog / cytoskeleton
GCCAGTTGCG	6.7	4.5	0.0	sep1: septin1	GTPase / cell division
GAGATCACAG	2.9	0.0	0.0	mit(1)15 : mitotic 15	kinetochore protein / cell division
TGGCGGGTGC	0.0	6.7	5.4	Sry-delta: Serendipity delta	transcription factor
TATACAAGGA	0.0	2.2	3.2	br: broad	transcription factor
GAACTCAACT	1.0	5.6	4.3	Rim	PDZ domain / synaptic vesicle fusion
CATAGTGCAG	0.0	5.6	3.2	Gdi: rab GDP dissociation inhibitor	synaptic vesicle fusion
ACCGGTGCTC	0.0	4.5	3.2	ru: roughoid / rhomboid3	serine protease / EGFR signaling
TCGCCGACAC	4.8	16.7	18.4	inaD: inactivation no afterpotential D	structural protein / phototransduction
GGCTTCTTAT	0.0	3.3	3.2	eya: eyes absent	epoxide hydrolase / eye specification
GGGAGATGAG	0.0	3.3	8.6	Tm1: Tropomyosin1	cytoskeleton
TCTAAATATG	0.0	2.2	4.3	aSpec: a Spectrin	cytoskeleton / cell adhesion
CAGCAGCTGT	1.0	10.0	4.3	nrt: neurotactin	cell adhesion
ATGATGATTG	0.0	3.3	4.3	Fas3: Fasciclin 3	cell adhesion
TAAAGCATAA	0.0	3.3	2.2	CG4668	cell adhesion
CGACAAGCGG	0.0	3.3	7.6	CG2578	tenascin - like / cell adhesion
TGATCTTCAG	2.9	13.4	9.7	crc: calreticulin	Ca signaling
TTAATGAAAG	0.0	3.3	2.2	CAMKII: Calcium/calmodulin-dependent protein kinase II	Ca signaling
AAAACCAACT	0.0	3.3	6.5	CamKI: calcium/calmodulin-dependent protein kinase I	Ca signaling
CTGTGTTTAG	1.0	109.2	20.5	transgene (tag matches 3' UTR of P-element vector)	
GCGCAAGGCA	0.0	0.0	3.2	SoxN: SoxNeuro	HMG box transcription factor
TACTCGTAAG	0.0	0.0	3.2	scrt: scratch	zinc finger transcription factor
CATATCCATT	0.0	0.0	8.6	retn: retained	ARID transcription factor
GATTCGCCAC	0.0	0.0	5.4	lz: Lozenge	runt domain transcription factor
AACATTTGAA	0.0	0.0	4.3	E(Spl)-HLH-mdelta	bHLH transcription factor
TTTAGTTCTT	0.0	0.0	3.2	bun: bunched	transcription factor
TTCTGAGTGA	0.0	0.0	4.3	B-H1: BarH1	homeodomain transcription factor
TCATCGATGT	0.0	1.1	3.2	sar1	vesicle associated GTPase
CAGCCACCGC	0.0	1.1	4.3	n-syb: n-synaptobrevin	v-SNARE / synaptic vesicle exocytosis
TGTATATAGA	0.0	1.1	6.5	Nrx: Neurexin	transm.rec. / synapt. vesicle docking
GCGGGTTGTC	0.0	1.1	5.4	CG8243	ARF GAP / synapse
TTGGATCGTC	0.0	1.1	4.3	CG7343	rabphilin hom. / synapt. ves. endocytosis
TATCACGAAT	1.0	1.1	3.2	blot: bloated tubules	neurotransmitter transporter
TAAAGTAGCT	2.9	7.8	13.0	nAcRalpha-80B: nicotinic Acetylcholine Receptor alpha 80B	signaling
TGAACCCAAA	0.0	0.0	4.3	Itp-r83A: inositol 1,4,5-trisphosphate receptor	signaling
TTTTTGGTAA	0.0	1.1	14.0	AlstR: Allatostatin Receptor	G-protein coupled receptor
ACCGCCATTC	1.0	0.0	3.2	Ac76E: Adenylyl cyclase 76E	signaling
CAAAACCACG	0.0	0.0	4.3	faf: fat facets	ubiquitin specific protease
TATCCGAAAC	0.0	0.0	5.4	ben: bendless	ubiquitin conjugating enzyme
GAAACCACCA	1.0	0.0	4.3	bt: bent	cytoskeleton
TCAAAGCGAG	1.0	0.0	5.4	Arc-p34	cytoskeleton
AGATCTTTCA	1.0	2.2	3.2	alpha-Spec: alpha Spectrin	cytoskeleton / cell adhesion
AAATCAATGG	1.0	2.2	8.6	sdk: sidekick	cell adhesion
AAAAGACCAT	0.0	1.1	3.2	Pax: Paxillin	cell adhesion
TTTTCGTGAG	0.0	0.0	3.2	Fas2: Fasciclin 2	cell adhesion
TAACGAAACA	1.0	2.2	3.2	Fas1: Fasciclin 1	cell adhesion
AAATGATATA	0.0	1.1	3.2	beta-spec: beta spectrin	cytoskeleton / cell adhesion
AAATAGTTGC	0.0	1.1	3.2	ank: ankyrin	cell adhesion
TTTTTGTTTA	1.9	2.2	19.4	fra: frazzled	netrin receptor / axon guidance

Figure 3. Major Changes of the Transcriptome during Organogenesis in the *Drosophila* Eye

Expression profile of 372 genes that show significant changes across the analyzed libraries. Analysis was limited to tags with expression levels higher than 0.01% and with 3-fold or higher expression differences between GMR$^-$ and GMR$^+$/Sev$^+$ libraries. Representation is limited to tags with unambiguous annotation and known function of the corresponding genes. Tags are clustered by function as well as expression ratios (see Experimental Procedures). Expression ratios over mean are color coded. Tags highly expressed in GMR$^+$ but not in GMR$^-$ and Sev$^+$ are not included. See Supplemental Table S2 for a complete list of analyzed tags. Supplemental Data also contains a comparison of

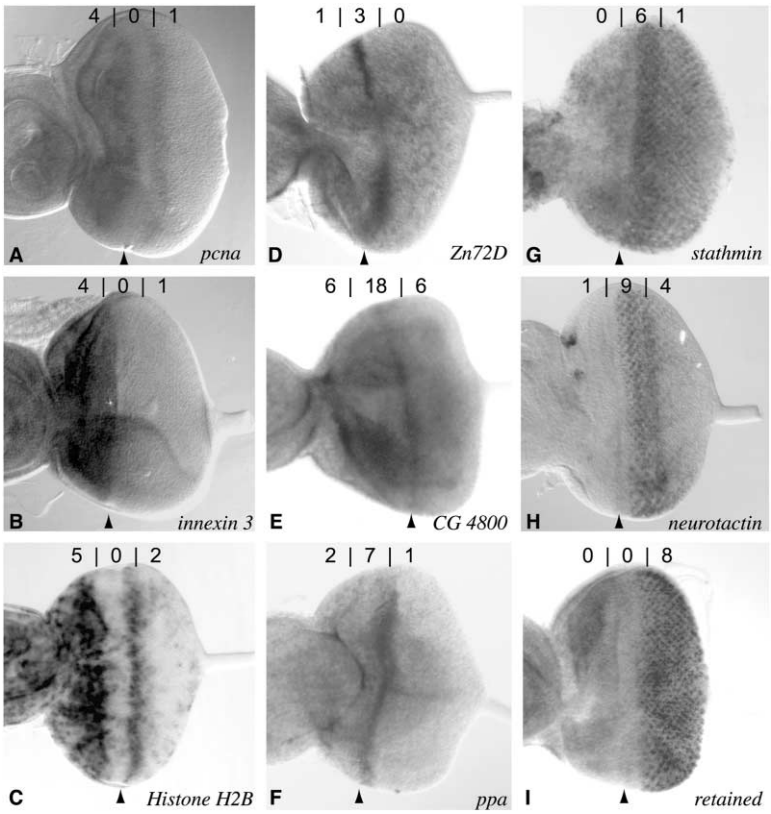


Figure 4. Validation of SAGE Data by In Situ Hybridization

Examples of RNA in situ hybridizations on transcripts found by SAGE to be enriched in specific cell populations of the eye imaginal disc (anterior is left in all cases). The mRNAs analyzed are indicated at the bottom of each panel. The numbers at the top indicate the frequency of tags corresponding to the analyzed gene in the GMR^-, GMR^+, and Sev^+ libraries, respectively.

(A–C) Genes represented by GMR^--specific tags are expressed at higher levels anterior to the MF. PCNA is the *Drosophila* homolog of proliferating cell nuclear antigen and is involved in DNA replication. Innexin 3 is a component of gap junctions in *Drosophila*. Histone H2B is expressed in S phase of the cell cycle. Its expression pattern recapitulates the domains of cell proliferation in the eye disc.

(D–F) Tags found exclusively in the GMR^+ population are representative of genes expressed within the MF. *Zn72D* encodes an RNA binding zinc finger protein of unknown function. CG4800 is a homolog of TCTP (translationally controlled tumor protein) with unknown function. *ppa* (*partner of paired*) encodes a ubiquitin ligase.

(G–I) Tags present in the GMR^+ and Sev^+ libraries, or exclusively in the Sev^+ library, were representative of genes expressed in developing photoreceptors. Stathmin, a microtubule binding protein, is required for nervous system development in the embryo. The cell adhesion protein neurotactin is involved in axon guidance. *retained* encodes an ARID transcription factor. For further in situ hybridizations and references on specific genes, see Flybase links in Supplemental Table S2.

the analysis of embryonic expression patterns (Jasper et al., 2001), about 20% of the identified tags had no match to the *Drosophila* genome. Six percent had multiple matches and 4% matched the genome in regions without predicted genes. A large fraction (34% of all tags) matched the genome 3′ to a predicted gene, indicating alternative 3′ end processing and incomplete annotation of the genome sequence (based on release 2).

The majority of tags appeared at comparable frequency in the three libraries, indicating constant expression levels of the corresponding genes (see examples in Figures 1H and 2). A tag derived from the transgene RNAs encoding GFP and Gal4 was abundant in the GMR^+ and Sev^+ libraries, while found only once in the GMR^- library (Figure 3), illustrating the validity of the data and the purity of the sorted cell preparations. We confirmed the SAGE data by performing RNA in situ hybridization on eye imaginal discs for selected genes that were differentially represented in the different libraries (Figure 4; see Supplemental Figure S2 at http://www.developmentalcell.com/cgi/content/full/3/4/511/DC1). These experiments corroborated the differential expression of virtually all genes for which an informative signal could be obtained (28 out of 29). For many other genes, our data matched earlier reports of specific expression in the analyzed cell populations (e.g., *toy*, Czerny et al., 1999; *capt*, Benlali et al., 2000; *sdk*, Nguyen et al., 1997; *lz*, Flores et al., 1998; *mdelta*, Cooper and Bray, 2000; *B-H1*, Higashijima et al., 1992; *ru*, Wasserman et al., 2000).

transcripts expressed in the eye with embryo-specific transcripts as analyzed previously (Jasper et al. 2001; Supplemental Table S3).

Examples of analyzed genes are shown on the right. The first column of the table shows the tag sequences. Tag numbers in the three libraries (normalized to 20,000 tags) are shown in the next columns. The right-hand columns show the name and function of representative genes. Probably due to alternative splicing and/or 3′ end processing, some genes are represented by more than one tag. A significant fraction of the transcripts enriched in GMR^- cells code for proteins involved either in replication, cytokinesis, and cell cycle regulation or in protein synthesis and degradation. Several genes known to be expressed at higher levels in cells anterior to the furrow were identified in our analysis (e.g., *capt* and *toy*).

Postmitotic differentiating photoreceptors express cell adhesion molecules and proteins involved in synaptic differentiation, Ca^{2+} signaling, and axon guidance to a higher degree. Consistent with the literature, transcripts for cell-specific transcription factors such as *lz*, *E(Spl)-HLH-mdelta*, and *B-H1* were identified exclusively in the Sev^+ population.

The high abundance of a tag corresponding to transgenic transcripts exclusively in GMR^+ and Sev^+ cells demonstrates the specificity of the analysis and the purity of the sorted cell populations.

Gene	Function	SAGE Data	Distribution of motifs in Promoter region
		GMR⁻|GMR⁺|Sev⁺	-1kb
mus209	PCNA, replication	4 | 0 | 1	
eEF1d	translation elongation	15 | 3 | 4	
aats-asp	Asp-tRNA synthetase	10 | 3 | 0	
bonsai	ribosomal protein S15	13 | 4 | 2	
eIF-3S10	translation initiation	12 | 6 | 0	
rpn9	proteasome regulation	7 | 0 | 1	
rpt3	proteasome regulation	10 | 3 | 0	
prosMA5	proteasome subunit	6 | 4 | 1	
dox-A2	proteasome regulation	6 | 2 | 1	
lesswright	SUMO conjugating enzyme	4 | 1 | 0	
mRpL5	mitochondrial ribosomal protein	3 | 2 | 1	
REG	**proteasome activator**	**3 | 1 | 0**	
sdk	cell adhesion	1 | 2 | 8	
fasciclin 3	cell adhesion	3 | 4 | 9	
CG2578	tenascin - like / cell adhesion	0 | 3 | 7	
alpha actinin	cell adhesion / cortical cytoskeleton	0 | 3 | 1	
roughoid	EGF signaling activator	0 | 4 | 3	
paxillin	cytoskeleton / cell adhesion	0 | 1 | 3	
Rim	synaptic vesicle exocytosis	1 | 5 | 4	
n-synaptobrevin	synaptic vesicle docking / fusion	0 | 1 | 4	
rabGDP diss.inh.	neurotransmitter release	0 | 5 | 3	
CG1861	mint1 hom. / synaptic vesicle exocytosis	0 | 5 | 6	
Appl	synapse	0 | 9 | 9	
lozenge	runt domain transcription factor	0 | 0 | 5	
rough	homeodomain transcription factor	0 | 0 | 2	
retained	ARID transcription factor	0 | 0 | 8	
BarH1	homeodomain transcription factor	0 | 0 | 4	
mdelta	bHLH transcription factor	0 | 0 | 4	
Calcineurin B2	Ca^{2+} signaling	1 | 0 | 3	

DRE	DRE-like	potential Glass binding site	novel element
: TATCGATA	: TATCGATN : NATCGATN	: GCRRSNGNNNNGNNNRNRNNG	: WGWGWGNGYG

Figure 5. Coregulated Genes Share Common *cis*-Acting Elements

Schematic representation of the promoter of region-specifically expressed genes. For clarity, only a subset of promoters containing the identified motifs is shown. Tables with all genes analyzed is included in Supplemental Figures S1A and S1B. For each gene, tag frequencies in the three libraries are shown. The position of three different DNA motifs identified by the AlignACE algorithm is depicted. Complete AlignACE results are included in Supplemental Tables S4 and S5. The motif defined as a potential Glass binding site has significant similarity to the Glass binding element (5′-GTGGAAACCCTTGAAATGCCTTT-3′) as described (Moses and Rubin, 1991). Note the high frequency of DREs and DRE-like elements in GMR⁻-specific genes. The novel element WGWGWGNGYG has similarity to GAGA-like elements.

Transcriptome Changes in Cells at the Transition from Proliferation to Differentiation

Classification of the differentially expressed genes into functional categories based on published data or on sequence similarities provides an overview of the general changes in cellular functions as cells transit from proliferation to the patterning and differentiation stages of organ development (Figure 2). Not surprisingly, many of the genes that are downregulated upon cessation of cell proliferation and at the onset of differentiation encode proteins involved in DNA replication and cell proliferation (Figure 3). These include genes specifically induced at the transition from G1 to S phase of the cell cycle, such as *pcna* (*mus209*) and *ribonucleoside-diphosphate reductase* (*rnrL*; Duronio and O'Farrell, 1994), as well as the replication licensing factors *mcm2* and *mcm5* (Treisman et al., 1995).

Other genes that are expressed at elevated levels in

the proliferating cells of the GMR$^-$ pool encode products with functions in metabolism and the regulation of protein synthesis (Figures 2 and 3). This is consistent with the reported deleterious effect of mutations in some of these genes on cell proliferation and growth, such as for *und* (Cutforth and Gaul, 1999), *eif4A*, *Asp-tRNA synthetase*, *bellwether*, and *bonsai* (Galloni and Edgar, 1999). The similar expression patterns of a group of proteasome subunits (Figures 2 and 3) can be rationalized by the high degree of regulated protein turnover in proliferating tissues. Altogether, we identified 93 genes that are upregulated significantly in the GMR$^-$ pool and that have tentatively assigned functions in cell growth and proliferation (Figure 3; Supplemental Table S2).

When eye imaginal disc cells enter the MF, they transit from the growth phase to the patterning phase of organogenesis and initiate specific differentiation programs. Consistent with this change of function, the cells posterior to the furrow upregulate specific cell adhesion and signal transduction molecules (Figures 2 and 3). These include proteins involved in the regulation of cellular adhesiveness and the cortical cytoskeleton such as Paxillin, Spectrin, Ankyrin, and α-Actinin, which show elevated expression levels in the GMR$^+$ and Sev$^+$ libraries (Figure 3). It is conceivable that such proteins mediate dynamically changing cell contacts as ommatidial clusters undergo rotation movements within the plane of the epithelium. Furthermore, differentiation markers such as genes involved in synaptic organization and axonal pathfinding begin to be upregulated in the GMR$^+$ library and are yet more highly represented in the Sev$^+$ library. Many of the mRNAs that are most prevalent in the latter library are involved in neuronal differentiation and signaling (Figure 3). Genes that are selectively transcribed in differentiating photoreceptors, as identified by their exclusive expression in the Sev$^+$ cell population, include the cell type-specific transcription factors *rough*, *lozenge*, *BarH1*, and *E(spl)mdelta* (Figure 3; Supplemental Table S2). *rough* encodes a homeodomain transcription factor expressed in photoreceptors R2, R3, R4, and R5 (Kimmel et al., 1990), whereas *lozenge* encodes a Runt domain transcription factor known to be expressed in cone cells and in all photoreceptors that arise from the second mitotic wave (R1, R6, and R7; Flores et al., 1998). The homeodomain transcription factor BarH1 is specifically expressed in R1 and R6 cells (Higashijima et al., 1992). E(spl)mdelta is a bHLH transcription factor expressed in R4 and R7 (Cooper and Bray, 2000). These transcription factors act in combination with specific signaling events to direct cell fate decisions within ommatidial clusters (Simon, 2000). The expression of the AT-rich interaction domain (ARID) transcription factor Retained, in a subset of photoreceptors as identified here (Figure 4I), might contribute to this combinatorial genetic control of cell specification.

In summary, the group of genes that was identified by SAGE to be specifically expressed in the differentiating cells of the eye imaginal disc overlaps to a significant degree with the regulators of photoreceptor differentiation previously identified by genetic means. This underscores the reliability of the method and supports the notion that genes that were designated as differentiation specific by SAGE, but have not yet been characterized genetically, may make important contributions to eye development. A further analysis of these genes thus holds the promise of providing significant new insights into the molecular biology of retinal development.

A Genetic Program of Cell Growth and Proliferation Regulated by DREF

We reasoned that the coordinated regulation of groups of genes at specific stages of organogenesis might correlate with the presence of similar regulatory sequence motifs in their promoter regions. To identify such putative *cis*-acting elements, we employed an unbiased computational approach that would identify nonrandom sequence patterns in sequences proximal to the transcription start site of coregulated genes. Such algorithms have been employed successfully to identify genetic regulatory networks in the yeast genome (Bussemaker et al., 2001; Roth et al., 1998; Tavazoie et al., 1999). We used the AlignACE server (http://atlas.med.harvard.edu/cgi-bin/alignace.pl) to screen for nonrandom patterns within 1,000 bp upstream of the transcription start site of a set of 23 coregulated growth-related genes as well as a set of 23 differentiation-specific genes (see Supplemental Figures S1A and S1B for a complete list of analyzed genes). In this way, we identified one DNA element (TATCGATA) that occurs in the upstream regions of genes implicated in cell growth and proliferation ahead of the MF. This motif is identical to the previously described DNA replication-related element (DRE; Hirose et al., 1993). DREs, in combination with E2F-responsive elements, control expression of genes involved in DNA replication including *pcna* (Hirose et al., 1993; Yamaguchi et al., 1995). DREF, the transcription factor that binds to DREs, acts as a regulator of DNA synthesis in the *Drosophila* eye imaginal disc (Hirose et al., 1996, 2001; Yamaguchi et al., 1995) and is expressed predominantly in proliferating cells of the eye disc (Hirose et al., 2001). We confirmed the AlignACE results by searching for DREs in the upstream region of a larger group of GMR$^-$-specific genes as well as in the 23 differentiation-specific genes used for the second AlignACE search. Strikingly, 14 of 41 tested GMR$^-$-specific genes contain a perfect match and 10 more contain a sequence closely resembling the 8 bp consensus DRE sequence within 1,000 bp of their transcription start site (Figure 5; Supplemental Figures S1A and S1B). In many cases, DREs or DRE-related sequences are found clustered with other DREs or with consensus binding sequences for E2F, another cell cycle-promoting transcription factor (Figure 5 and data not shown). In contrast, only 1 out of 23 tested differentiation-specific genes contained a DRE in the examined promoter regions (Figure 5; Supplemental Figure S1B). However, in the upstream sequences of this group of genes, a different motif resembling the binding site for the transcription factor Glass was found frequently (Figure 5). Glass is required for photoreceptor differentiation and is expressed in all cells posterior to the MF (Moses and Rubin, 1991; Ellis et al., 1993). Additionally, we identified a novel motif present recurrently in upstream regions of GMR$^+$/Sev$^+$-enriched genes but only rarely in promoters of GMR$^-$-enriched genes (Figure 5). Lists of nonrandom motifs identified by the AlignACE algorithm in promoters of the GMR$^-$ as well as of the GMR$^+$/Sev$^+$-enriched genes are included in Supplemental Tables S4 and S5.

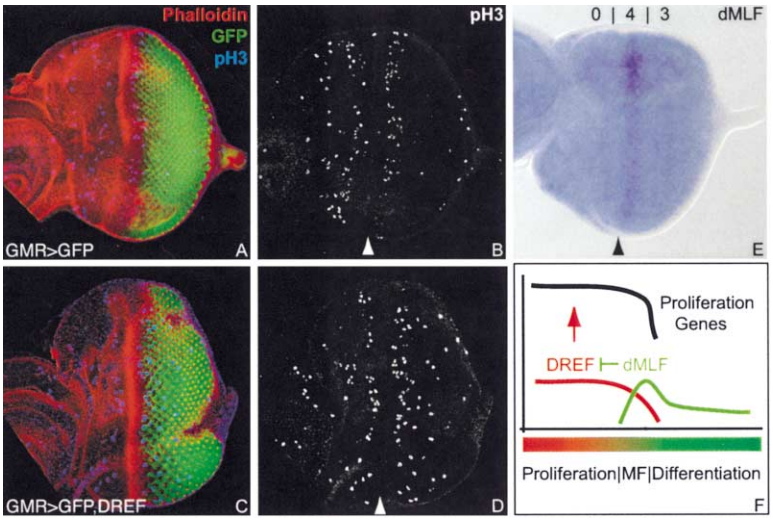


Figure 6. Regulation of Organ Growth by the DREF/dMLF System

DREF induces ectopic cell proliferation in postmitotic cells (A–D). Staining for phospho histone 3 (pH3) reveals an increased frequency of mitoses posterior to the second mitotic wave in GMR-GAL4, UAS eGFP/UAS DREF eye imaginal discs (C and D) as compared to GMR-GAL4, UAS eGFP/+ discs (A and B). Images are projections of confocal stacks including only GFP-positive cells and excluding the peripodial membrane. When the number of mitotic figures posterior to the MF is normalized to rows of ommatidial clusters, this increase amounts to nearly 40% (7.5 ± 0.8 to 10.4 ± 0.5). The negative regulator of DREF, dMLF, is expressed at high levels in cells of the morphogenetic furrow (MF; arrowhead in [E]). This suggests specific downregulation of DREF function at the MF by dMLF, thus reducing the expression of DREF-regulated proliferation-specific genes at the onset of cellular differentiation (F).

The prevalence of DREs in genes that are associated with the proliferative state of the GMR^- cell population suggests that the transcription factor DREF, possibly in concert with E2F, regulates a genetic program of cellular proliferation and growth during the early stages of eye development. In such a scenario, the downregulation of genes containing DRE sequences in their promoter region in the cells in and behind the MF (represented by the GMR^+ and Sev^+ pools) is likely to be a consequence of a suppression of DREF activity. One mechanism to explain the downregulation of DREF activity in the MF involves a known inhibitor of DREF, myelodysplasia/myeloid leukemia factor (*dmlf*; Ohno et al., 2000). As indicated by the increased presence of dMLF-derived SAGE tags in the GMR^+ and Sev^+ libraries, and confirmed by in situ hybridization (Figure 6E), dMLF expression is specifically upregulated in the MF and to a lesser degree posterior to the MF, thus coincident with the proposed suppression of DREF activity. Induction of *dmlf* in the MF might thus limit DREF function when cells prepare for differentiation (Figure 6F). To test this model, we ectopically expressed DREF in the cells behind the MF. Earlier reports suggested that DREF overexpression leads to increased DNA synthesis behind the MF (Hirose et al., 2001). Additionally, we found a significant increase of mitotic cells in this area, as visualized by immunostaining for phosphorylated histone 3, a specific marker for mitotic cells (Figures 6A–6D). These data thus suggest a function of the DREF/dMLF system in the control of a cell growth and proliferation program during organogenesis.

Discussion

SAGE has been established as a valuable genomic transcription profiling approach in a variety of studies ranging from the investigation of tumorigenesis and metastasis to the mapping of transcriptome changes during retinal differentiation in mice (Blackshaw et al., 2001; Saha et al., 2001). In combination with in vivo labeling and cell sorting methods in *Drosophila*, as described here, SAGE provides a powerful tool that will facilitate the analysis of the genetics and molecular biology of development in this important model organism. The plethora of tissue- and cell-specific driver lines available in *Drosophila* makes this approach to genome-wide expression profiling very versatile. Thus, the generation of a cell- and stage-specific transcriptome map of *Drosophila* development both in wild-type and specific mutant situations becomes possible. As shown here, the combination of these experimental approaches with bioinformatic analyses will provide fresh insight into the genetic regulatory networks governing development of multicellular organisms.

In our genomic analysis of eye development, we obtained data for 4,279 different transcripts, representing more than 30% of the predicted genes in the genome. These include genes for transcription factors and other regulatory proteins that are generally expressed at comparatively low levels. Nevertheless, at the current depth of analysis, very rare messages are not represented. 9,234 tags appear only once, and more sequencing or further purified cell populations would be required to gain statistically relevant information on weakly expressed messages or messages that are present only in subgroups of eye cells. How the number of genes covered by our analysis compares to the complete transcriptome of eye imaginal disc cells is difficult to estimate at this time, as the fraction of transcriptionally active genes in the analyzed cells is unknown.

While photoreceptor differentiation in the developing eye has been studied extensively and a host of genes has been implicated in this process, we are still largely ignorant about the molecular mechanisms that execute other aspects of *Drosophila* eye development, such as the generation of epithelial planar polarity. Our SAGE analysis suggests numerous candidate genes whose role in this aspect of eye development can now be studied.

The transcriptional changes described here reflect the dramatic transition of cell function at the onset of differentiation in vivo when cell proliferation ceases. In the area of the eye disc that is populated by uncommitted stem cells, the predominant gene expression programs are tailored for growth and cell division, with prominent expression of genes involved in metabolism, protein synthesis, DNA replication, cytokinesis, and protein degradation. Our data suggest that many of these genes are coregulated by the transcription factor DREF, which would thus function as a major regulator of tissue growth in organogenesis. The upregulation of the DREF inhibitor dMLF in the MF and the observation that forced overexpression of DREF in cells posterior to the furrow leads to ectopic cell mitoses provides a compelling model for a switch that turns off the proliferation-specific gene expression program.

The relationship of the DREF/dMLF system with the Ras signaling pathway remains unclear. Similar to its homologs in higher organisms, *Drosophila* Ras is a key regulator of cell growth and proliferation (Karim and Rubin, 1998; Prober and Edgar, 2000). In the postmitotic cells of the developing *Drosophila* eye, however, Ras has a different function and specifies photoreceptor cell fate. One might thus speculate that it is the absence of DREF activity in the cells behind the MF that causes Ras activation to be interpreted as a differentiation rather than a proliferation signal. For a detailed analysis of the relationship between Ras and DREF, mutations in the *dref* gene will have to be isolated and characterized.

Experimental Procedures

Fly Strains and Genetics

The fly strains *w; GMRGal4* and *w; sepGal4* were gifts from Marek Mlodzik. To induce expression of GFP in subsets of cells as described in the text, these strains were crossed to *w; UASeGFP* flies generated in our laboratory. Progeny was grown at 18°C and collected at the third instar larval stage. The strain carrying *UAS DREF* was a gift from Katsuhito Ohno.

Cell Sorting and SAGE

Eye discs were dissected from third instar larvae in ice-cold PBS. Dissociation and sorting were performed as described (Neufeld et al., 1998). Briefly, cells were dissociated by incubating discs for 3–4 hr at 25°C in 10× trypsin-EDTA (Sigma), 1× PBS and fluorescent cells were separated from GFP^- cells using a fluorescence-activated cell sorter (MoFlo; Cytomation). Cells were sorted into ice-cold PBS, sedimented by short centrifugation (2 min, 5,000 rpm in a tabletop centrifuge), and lysed in Dynal lysis/binding buffer (Dynal mRNA direct kit). pA^+ RNA was then purified by incubating the lysate with magnetic poly-dT beads (Dynal), and double-stranded cDNA was generated directly on the beads. We generated SAGE libraries from 800,000 GMR^+, 800,000 GMR^-, and 80,000 Sev^+ cells. The SAGE protocol used is described (Jasper et al., 2001) and can be downloaded at http://ccb-research20.urmc.rochester.edu. Sequencing of SAGE concatemers was performed at the Genomics Core Facility of EMBL and at Agencourt Bioscience Corporation.

In Situ Hybridization and Immunostaining

In situ hybridization was performed with digoxigenin-labeled RNA probes following standard protocols (Tautz and Pfeifle, 1989).

Mitotic cells in eye imaginal discs were detected by fixing discs in 4% paraformaldehyde/0.1% Triton X-100/PBS for 15 min at room temperature (RT), then blocking them in several changes of BBT (0.1% BSA/0.1% Triton X-100/PBS) for a total time of 1 hr at RT and incubating them overnight at 4°C in BBT with rabbit anti-phospho histone 3 antibody (Upstate Biotech; 1:1000 diluted). After washing in BBT, the samples were incubated with fluorescent secondary antibodies for 2 hr at RT, washed in BBT, and mounted on glass slides. Imaging was performed on a Leica SP2 confocal microscope.

Clustering and Graphical Representation of Expression Levels

Tags with unambiguous annotations were assigned to functional groups by virtue of the known or implied function of their corresponding genes (as derived from Flybase). By using the "sort" function in Microsoft Excel, tags were also clustered for differential expression (using logical tests to define tags with 3-fold or higher expression in one library as compared to others). Monte Carlo simulations using the SAGE2000 program indicate that, in a pair-wise comparison of two libraries with 20,000 tags each, a gene that is represented at three tags in one and zero tags in the second library can be regarded as differentially expressed with a p value of 0.1. Fifty-eight percent of the genes that were assigned as differentially expressed between GMR^- and Sev^+ have p values of 0.1 and lower. The monitoring of expression levels across three libraries leads to a further enhancement of significance (since the third library constitutes an independent sampling). Nevertheless, it should be noted that expression differences for specific genes with tags present at frequencies of less than five in 20,000 tags should be confirmed by in situ hybridization. As shown in Figure 4, differential expression of almost all of the tested genes could be validated in this way (see also Supplemental Figure S2).

To represent the distribution of tag abundances across libraries graphically, tag numbers (normalized to 20,000) were converted to relative expression levels over mean (calculating the ratio of normalized tag number to the average tag number across the three libraries; this value was multiplied by three to correct for distorted representation that occurs when tag numbers are very high in one library, but not present in others). A tag number of 0.5 was assigned to tags that were not present in a library. Colors were assigned to distinct ranges of relative expression over mean (see Figure 3).

Promoter Analysis

AlignACE searches were performed on 5′ sequences (1 kb upstream of transcription start site) of selected coregulated genes. Genes were selected to be representative of the different functional groups shown in Figure 3. The sequences were obtained from GadFly (http://hedgehog.lbl.gov:8002/cgi-bin/annot/query/). Motifs defined by the AlignACE algorithm were selected for high MAP score, the presence of the motif in all the submitted fragments, and similarity to known motifs, and subsequently tested for presence in the upstream region of a larger group of selected genes. All tested genes are listed in Supplemental Figures S1A and S1B. Motifs found to be present in considerably higher frequency in one group of coregulated genes as compared to the other group were considered "specific" for this group. Sequence analysis was performed using the DNAMAN sequence analysis package.

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