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Microarray Analysis of *Drosophila* Development During Metamorphosis

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adulthood, specific combinations of tumor suppressor genes may cooperate to control proliferation, differentiation, and survival in different cell lineages.

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17. Nf1$^{+/-}$:p53$^{+/-}$ and Nf1$^{+/-}$:p53$^{-/-}$ progeny of trans-Nf1$^{+/-}$:p53$^{+/-}$ crosses were bred with mice that were wild type at both loci. A founder male with the genotype Nf1$^{+/-}$:p53$^{-/-}$ proved to harbor a stable recombinant chromosome 11 after test crosses to three wild-type females. The recombinant (cis configuration) chromosome was maintained on a mixed C57B6/129sv background, as were mice of other genotypes used for mortality studies. For genotyping, tail DNA was subjected to two separate three-primer PCRs (one for Nf1 (*11*) and one for p53 (*8*). Samples of macroscopically recognizable tumor were fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. All immunostaining was done at room temperature on a BioTek Solutions Techmate automated immunostainer (Ventana BioTek Systems, Tucson, AZ). Buffers, blocking solutions, secondary antibodies, avidin-biotin complex reagents, chromogen, and hematoxylin counterstain were used as supplied in the ChemMate secondary detection kit (Ventana BioTek Systems). Optimum primary antibody dilutions were predetermined with known positive control tissues. A known positive control section was included in each run to ensure proper staining. Paraffin sections were cut at 3 μm on a rotary microtome and mounted on positive-ly charged glass slides (POP100 capillary gap slides; Ventana BioTek Systems) at pH 6.8. Sections were incubated in unlabeled blocking antibody solution for 5 to 10 min to block nonspecific binding of secondary antibody and then incubated for 25 min with either primary antibody (S100 protein; SMA, 1:400; myoglobin, 1:60,000; desmin, 1:100; DAKO, Carpenteria, CA) or with buffer alone as a negative reagent control. After washing in buffer, sections were incubated for 25 min with biotinylated polyvalent secondary antibody solution (containing goat antibodies to rabbit, mouse, and rat immunoglobulin). After another buffer wash, sections were incubated with three changes, 2.5 min each, of 3% H$_2$O$_2$ to inhibit endogenous tissue peroxidase activity and again washed in buffer. Sections were then incubated for 25 min with freshly prepared horseradish peroxidase–conjugated avidin-biotin complex. Sections were then washed in buffer and incubated with three changes, 5 min each, of a freshly prepared mixture of diaminobenzidine (DAB) and H$_2$O$_2$ in buffer, followed by washing in buffer and then water. Sections were then counterstained with hematoxylin, dehydrated in a graded series of ethanols and xylene, and coverslipped. Slides were reviewed by light microscopy. Positive reactions with DAB were identified as a dark brown reaction product. Sections were photographed on a Nikon Optiphot microscope (Nikon Instruments, Melville, NY).
18. Characterization of soft tissue sarcomas in cis-Nf1:p53 mice. Histopathological examination was performed on all tumors obtained from animals at the termination of the experiment. Soft tissue tumors were classified in accordance with 1994 World Health Organization criteria. Tumor masses were removed under sterile conditions and measured. Small pieces of tumor tissue were removed for histological processing, DNA isolation, and establishment of tumor cell lines. Tumor samples were fixed in either Bouin's fixative (for hematoxylin and eosin staining) or 10% formalin (for immunohistochemistry) and processed for paraffin embedding and sectioning at 5 to 7 μm. Tumor sections were immunostained with S100 antibody (anti-S100) (Novocastra), anti-desmin (Signet), anti-α-actin (Boehringer), or anti-myoglobin (Signet) and visualized by the Vectastain Elite ABC peroxidase method (Vector). Tumor DNA was genotyped by three-primer PCRs as described above.
19. Nf1/p53 tumor-derived cell lines were isolated as follows: Overlying skin and hair were removed from the tumor mass and then the tumor mass immersed briefly in Dulbecco's phosphate-buffered saline and in a solution of penicillin and streptomycin (Gibco). Small pieces of the tumor mass were minced in Dulbecco's modified Eagle's medium (DMEM) [supplemented with 10% heat-inactivated fetal calf serum (HIFCS), penicillin and streptomycin, and nonessential amino acids] (Gibco) with watchmaker's forceps and fine curved scissors. Tumor pieces were allowed to attach to 60-mm plastic tissue culture dishes, and clonal cell lines were established from tumor outgrowths after four to six passages.
20. For immunocytochemistry, tumor cells grown on coverslips in 2% HIFCS/DMEM were fixed in 4% paraformaldehyde and exposed to antibodies diluted overnight at 4°C as follows: p75 (Chemicon), 1:200; c-*neu* (Santa Cruz), 1:200; GAP-43, 1:250; S100 (Novocastra), 1:200; GFAP (Santa Cruz), 1:200; SMA (Sigma), 1:400; calponin (Sigma), 1:400; neurofilament (Chemicon), 1:200. To visualize bound antibody, we used Vectastain Elite ABC peroxidase kits (Vector Laboratories), specific for goat, rabbit, or mouse primary antibodies, according to the manufacturer's instructions. We also tested all primary antibodies with Cy3- or fluorescein isothiocyanate–conjugated fluorescent secondary antibodies (Chemicon, Sigma) to determine which method yielded little or no background staining. For immunoblots, proteins were extracted from tumor cells grown in 162-cm^2 culture flasks in Nonidet P-40 lysis buffer containing protease inhibitors (Sigma). Insoluble (SMA, GFAP) and soluble (c-*neu*, S100) fractions were subjected to SDS–polyacrylamide gel electrophoresis on 8% to 10% minigels. After protein transfer, nitrocellulose membranes were blocked with 2% bovine serum albumin in tris-buffered saline and incubated with primary antibody overnight at 4°C. Specific protein bands were visualized with Vectastain Elite ABC peroxidase kits (Vector Laboratories), or with Immun-Star kits (Bio-Rad), according to the manufacturers' instructions.
21. Supported by NIH grant NS34296 and the National Neurofibromatosis Foundation (L.F.P.) and by a grant from the Cancer Association of Greater New Orleans (K.S.V.). We thank T. Jacks and colleagues for sharing unpublished results, S. Colvin and J. Richardson for early assistance with histopathology, and members of the Parada lab for helpful discussions.

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Microarray Analysis of *Drosophila* Development During Metamorphosis

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Metamorphosis is an integrated set of developmental processes controlled by a transcriptional hierarchy that coordinates the action of hundreds of genes. In order to identify and analyze the expression of these genes, high-density DNA microarrays containing several thousand *Drosophila melanogaster* gene sequences were constructed. Many differentially expressed genes can be assigned to developmental pathways known to be active during metamorphosis, whereas others can be assigned to pathways not previously associated with metamorphosis. Additionally, many genes of unknown function were identified that may be involved in the control and execution of metamorphosis. The utility of this genome-based approach is demonstrated for studying a set of complex biological processes in a multicellular organism.

The generation of vast amounts of DNA sequence information, coupled with advances in technologies developed for the experimental use of such information, allows the description of biological processes from a global genetic perspective. One such technology, DNA microarrays, permits the simultaneous monitoring of thousands of genes (*1*). DNA

microarrays have been particularly useful for analyzing gene expression profiles in single-celled organisms and in tissue culture (2). Here we describe the use of this method to study gene expression patterns in the multicellular organism *Drosophila* as it undergoes a dramatic developmental transition.

Drosophila metamorphosis is characterized by diverse developmental phenomena, including cellular proliferation, tissue remodeling, cell migration, and programmed cell death. Cells undergo one or more of these processes in response to the hormone 20-hydroxyecdysone (ecdysone), which initiates metamorphosis at the end of the third larval instar and before puparium formation (PF) via a transcriptional hierarchy (3, 4). Additional pulses of ecdysone further coordinate these processes during the prepupal and pupal phases of metamorphosis. Larval tissues such as the gut, salivary glands, and larval-specific muscles undergo programmed cell death and subsequent histolysis. The imaginal discs undergo physical restructuring and differentiation to form rudimentary adult appendages such as wings, legs, eyes, and antennae. Ecdysone also triggers neuronal remodeling in the central nervous system (CNS) (3).

In this study, we examined wild-type patterns of gene expression in *D. melanogaster* during early metamorphosis by assaying whole animals at stages that span two pulses of ecdysone (Fig. 1). We constructed microarrays containing 6240 elements that included more than 4500 unique cDNA expressed sequence tag (EST) clones along with a number of ecdysone-regulated control genes having predictable expression patterns (1, 5). These ESTs represent approximately 30 to 40% of the total estimated number of genes in the *Drosophila* genome (6). In order to gauge expression levels, we hybridized microarrays with fluorescent probes derived from polyA$^+$ RNA isolated from developmentally staged animals (Fig. 1). We examined time points relative to PF, which lasts approximately 15 to 30 min during which the larvae cease to move and evert their anterior spiracles. We analyzed 19 arrays representing six time points relative to PF: one time point before the late larval ecdysone pulse [≥18 hours before PF (BPF)], one time point just after the initiation of this pulse (4 hours BPF), and

time points at 3, 6, 9, and 12 hours after PF (APF). The prepupal pulse of ecdysone occurs 9 to 12 hours APF.

In order to manage, analyze, and disseminate the large amount of data, we generated a searchable database (7) that includes the average expression differential at each time point. Our analysis set consisted of all elements that reproducibly fluctuated in expression threefold or more at any time point relative to PF, leaving 534 elements containing sequences represented by 465 ESTs and control genes (8). More than 10% of the genes represented by the ESTs displayed threefold or more differential expression during early metamorphosis. This may be a conservative estimate of the percentage of *Drosophila* genes that change in expression level during early metamorphosis, because of the stringent criteria used for their selection (8).

To interpret these data, genes were grouped according to similarity of expression patterns by two methods (Fig. 2). The first relied on pairwise correlation statistics (9), and the second relied on the use of self-organizing maps (SOMs) (10). Several ecdysone-regulated control genes are shown from different areas of the "clustergram" in Fig. 2A and from the clusters in Fig. 2B. The two clustering methods gave essentially the same results and agree with published RNA blot analyses of these transcripts. Furthermore, each clustering method grouped genes with similar expression profiles. In addition to the genes shown in Fig. 2, all other known ecdysone-controlled genes that were detected with these arrays behaved as expected, and duplicate control genes clustered together, demonstrating the consistency of the results.

Differentially expressed genes fall into two main categories. The first category contains genes that are expressed at ≥18 hours BFP (before the late larval ecdysone pulse) but then fall to low or undetectable levels during this pulse. This category is colored red in the first column of Fig. 2A and has an initial negative slope for the curves given in Fig. 2B. These genes are potentially repressed by ecdysone and make up 44% of the 465 ESTs identified in this set. The second category consists of genes expressed at low or undetectable levels before the late larval

ecdysone pulse but then are induced during this pulse (indicated by green in the first column of Fig. 2A and by an initial positive slope in the Fig. 2B curves). These genes are potentially induced by ecdysone and make up 31% of the 465 ESTs. Consequently, 75% of genes that changed in expression by threefold or more did so during the late larval ecdysone pulse that marks the initial transition from larva to prepupa. This result is consistent with the extreme morphological changes that are about to occur in these animals. Although only two general categories are marked in Fig. 2A, there are clearly discrete subdivisions of gene expression within these categories. Figure 2 has been annotated with individual gene names or BLAST hits that are available at (7).

Relatively little is known about basic metabolic processes during metamorphosis. Most work has focused on the alcohol dehydrogenase gene, which is known to be repressed by ecdysone (3, 10), but a few studies have included the ecdysone-inducible glucose dehydrogenase gene and the ecdysone-repressible urate oxidase locus (3, 11, 12). All three of these genes were present in our controls and behaved as expected. Nine genes encoding enzymes in the glycolytic pathway are present on the array and are down-regulated during the late larval ecdysone pulse (Fig. 3A). Also, the expression levels of genes encoding enzymatic constituents of the citric acid cycle, oxidative phosphorylation, amino acid metabolism, fatty acid oxidation and synthesis, glycogen synthesis and breakdown, and the pentose phosphate pathway are reduced [see (7)]. Thus, some tissues must be responding to the initiation of metamorphosis by tempering their metabolic activity. This reduction may represent an early response in certain tissues that are destined to undergo programmed cell death, or it may reflect a global response to the transition from an active larva to a sessile prepupa.

Gene expression changes during metamorphosis also foreshadow both larval muscle breakdown and adult myogenesis. At approximately 2 hours APF, the anterior larval musculature begins to break down (13). This breakdown lasts until approximately 6 hours APF. Genes encoding both structural and reg-

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Fig. 1. Developmental stages analyzed in this study. The late larval and prepupal ecdysone pulses are indicated by the black bars at the top. Mid–third instar larvae (≥18 hours BPF) that had not yet been exposed to the late larval ecdysone pulse and late third instar larvae (4 hours BPF) exposed to this ecdysone pulse were selected with the "blue gut" method (29). Puparia (at the PF stage) were isolated by picking immobile white larvae with everted anterior spiracles. They were then incubated at 25°C on grape agar plates for 3, 6, 9, or 12 hours. Hybridizations to microarrays were done as described (30).
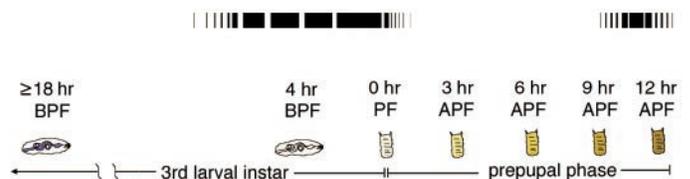
ulatory components of muscle formation are down-regulated as early as 4 hours BPF (Fig. 3B). In addition to the repression of genes encoding components of thin and thick filaments, genes encoding other muscle-specific molecules are also repressed, including factors that compose the mesh in which these filaments lie and regulatory factors involved in the specification of muscle tissue (Fig. 3B). The mRNAs of all these repressed genes decrease substantially many hours before histolysis of the anterior larval muscles and therefore predict the occurrence of this morphological event well before it begins.

Twenty-four hours APF, adult myogenesis is well underway (*14*). Figure 3B shows several genes that are involved in visceral mesoderm differentiation and muscle development. The genes *DMef-2*, *bagpipe*, and *tinman* (*15*) are all up-regulated at 12 hours APF from the baseline at PF, coincident with the prepupal pulse of ecdysone. We suggest that induction of these regulatory factors initiates the development of the adult musculature that will establish itself several hours later.

In contrast to the histolyzing larval muscles, the CNS undergoes dramatic differentiation and restructuring during early metamor-

phosis. The majority of the CNS is composed of adult-specific neurons that reorganize at this time by extending processes and establishing new connections (*3*). Several genes known to be involved in neuronal-specific processes are differentially regulated during the late larval ecdysone pulse. For example, the *Drosophila neurotactin* and *plexin A* genes are induced (Fig. 3C). These genes are involved in axonal pathfinding and in establishing synaptic connections. The *neurotactin* (*nrt*) gene product is involved in growth cone guidance and is localized to the cell surface at points of interneuronal cell contact in the presumptive imaginal neurons within the larval CNS (*16*). Nerve cord condensation does not occur normally in the late third instar CNS of *nrt* mutant animals. In prepupae, *nrt* is expressed in a tissue- and cell type–specific manner: it is restricted to a small set of ocellar pioneer neurons in the brain, photoreceptors of the eye, and some sensory neurons in the developing wing (*16*). We suggest that *nrt*, like the control genes induced from ≥18 hours BPF to PF, is regulated by the late larval ecdysone pulse. The *plexin A* gene belongs to a family of genes that encode Ca^{2+}-dependent homophilic cell adhesion

molecules first identified in the vertebrate CNS and PNS (*17*). *Drosophila* PLEXIN A also acts as a receptor for class I semaphorins, and both loss of function and overexpression experiments demonstrate that PLEXIN A is involved in axon guidance and repulsion of adjacent neurons (defasciculation) (*17*). Many neurons defasciculate in response to ecdysone during nervous system remodeling (*3*), and we suggest that an increase in *plexin A* expression may be partly responsible for this response. Several more differentially expressed neuronal-specific molecules are shown at (*7*). These genes provide several new candidates for factors that are involved in the neuronal outgrowth and morphological remodeling responses to ecdysone.

Larval-specific tissues such as the aforementioned larval muscles, the midgut, and the salivary glands undergo programmed cell death during metamorphosis. Genes involved in programmed cell death were identified in these experiments. The apoptosis-activating *reaper* gene has previously been shown to be ecdysone-inducible (*18*), and this is reflected in our data. We also observed expression of the *Drosophila caspase-1* gene during the prepupal ecdysone pulse but not during the
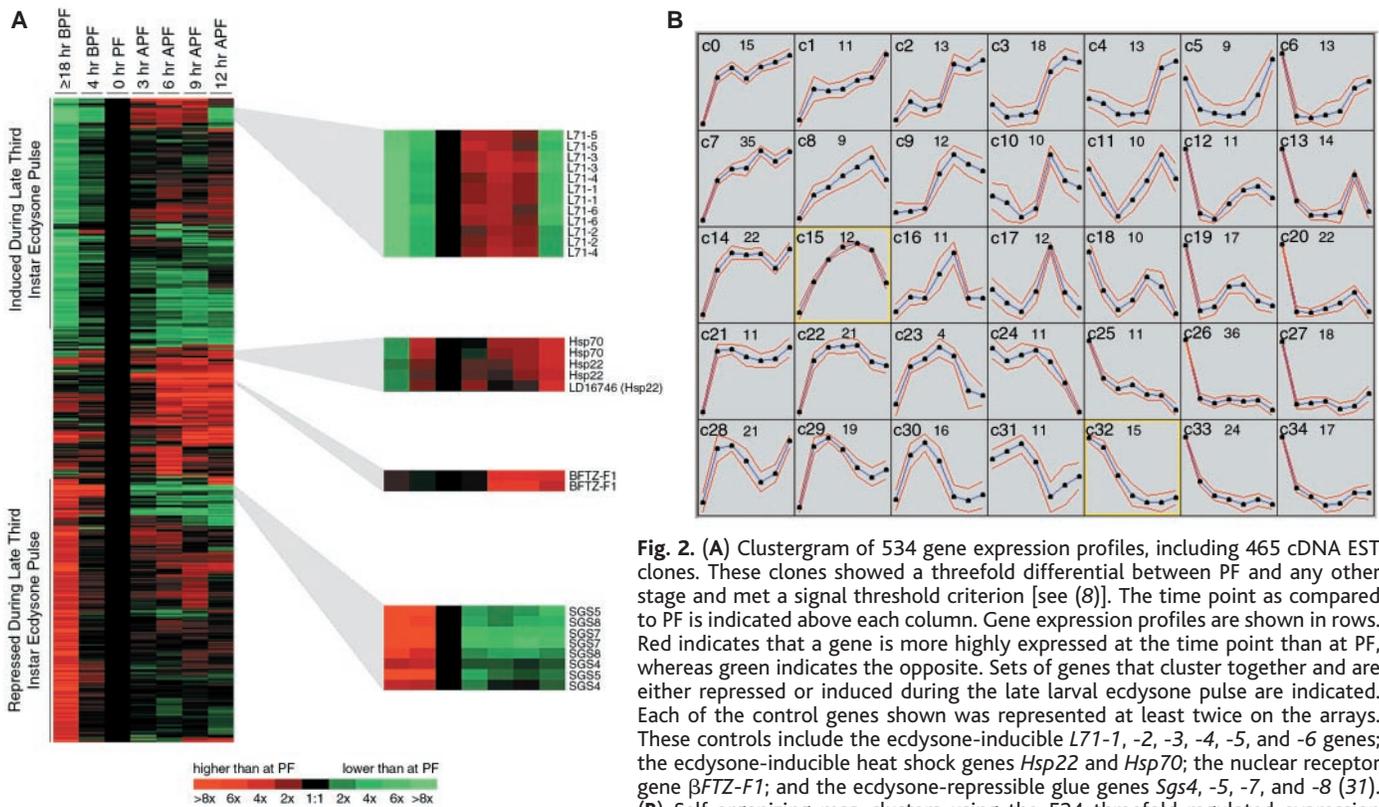
Fig. 2. (**A**) Clustergram of 534 gene expression profiles, including 465 cDNA EST clones. These clones showed a threefold differential between PF and any other stage and met a signal threshold criterion [see (*8*)]. The time point as compared to PF is indicated above each column. Gene expression profiles are shown in rows. Red indicates that a gene is more highly expressed at the time point than at PF, whereas green indicates the opposite. Sets of genes that cluster together and are either repressed or induced during the late larval ecdysone pulse are indicated. Each of the control genes shown was represented at least twice on the arrays. These controls include the ecdysone-inducible *L71-1*, *-2*, *-3*, *-4*, *-5*, and *-6* genes; the ecdysone-inducible heat shock genes *Hsp22* and *Hsp70*; the nuclear receptor gene β*FTZ-F1*; and the ecdysone-repressible glue genes *Sgs4*, *-5*, *-7*, and *-8* (*31*). (**B**) Self-organizing map clusters using the 534 threefold regulated expression profiles. In this method, the user specifies the number of clusters desired and an SOM algorithm groups them into discrete clusters (*10*). The label in the upper left corner of each cluster indicates the cluster number (c0 to c34), whereas the number in the top center of each box indicates how many elements are in each cluster. Two of the 35 clusters containing control genes are indicated by yellow boxes. Cluster 15 contains the *L71-1* to *L71-6* genes, and cluster 32 contains the *SGS4* to *SGS8* genes. Time points are represented in these graphs from left to right as diagrammed in Fig. 1A (≥18 hours and 4 hours BPF, PF, and 3, 6, 9, and 12 hours APF). Blue lines indicate the mean expression profiles; red lines indicate SD. A complete list of the genes in each cluster can be obtained at (*7*).

late larval pulse (Fig. 3C). This gene is also an activator of apoptosis, and mutants display melanotic tumors and larval lethality (*19*). Induction of a cell death inhibitor gene, *thread* (also known as *Diap1*), was observed during the late larval pulse but not the prepupal pulse (Fig. 3C). The DIAP1 protein includes inhibitor-of-apoptosis (IAP) domains and was identified as a factor that can block *reaper* activity (*20*). Because different tissues begin apoptosis at different stages of development, we expect changes in the expression of inhibitors and activators of apoptosis to be tissue-specific. For example, the expression profiles we observed for the *caspase-1* activator and the *Diap1* inhibitor are those expected in tissues such as the larval salivary glands. Tissue-specific information on the induction of these genes will be important to understanding the coordination of apoptosis during metamorphosis.

The expression levels of genes involved in cellular differentiation also dynamically change during metamorphosis. The gene *headcase* is expressed in all proliferating imaginal cell lineages (*21*). According to our results, this gene is induced during the prepupal ecdysone pulse but does not substantially change ex-
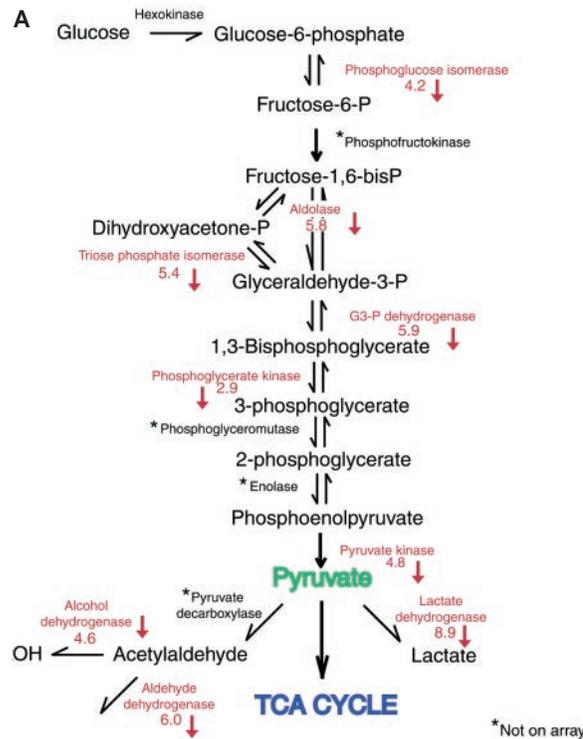
pression levels during the late larval ecdysone pulse. Imaginal tissues in *headcase* null mutants appear normal in size and shape but fail to differentiate normally (*21*). These mutants are invariably pupal lethal and show pleiotropic effects in adult tissues. The predominant *headcase* loss of function phenotype is defective head development. Mutants can display deletion of the head capsule, leaving only a protruding proboscis. Another gene expressed in this manner with a known role in ecdysone-mediated differentiation of imaginal discs is *IMP-L2* (*22*). These data demonstrate that factors required for cellular differentiation during metamorphosis are present in our data set. There are 29 other EST sequences encoding novel genes that display a greater than threefold induction from PF to 12 hours APF but do not display a threefold or greater change in expression level during the late larval ecdysone pulse. Perhaps some of these genes, such as *headcase* and *IMP-L2*, are involved in differentiation of adult-specific tissues.

Other genes known to be involved in cellular differentiation exhibited changes in level of expression during metamorphosis. For example, *corkscrew* is induced during the late

larval ecdysone pulse (Fig. 3C). This gene encodes a protein tyrosine phosphatase that is involved in receptor tyrosine kinase signaling during photoreceptor differentiation (*23*). *short-sighted* encodes a bZIP transcription factor homologous to a mouse transforming growth factor–β (TGF-β)–responsive gene and acts in the *decapentaplegic* pathway (*24*). This gene is induced during the late larval ecdysone pulse and then further induced during the prepupal pulse. *tolkin* encodes a TGF-β homolog and is induced during the late larval ecdysone pulse but not during the prepupal pulse (Fig. 3C). *tolkin* is expressed in imaginal discs during metamorphosis and causes pupal lethality when mutated (*24*). These results establish potential connections between known signal transduction pathways and ecdysone-initiated metamorphosis.

Description of wild-type development is a first step in understanding metamorphosis from a global perspective. However, we also wish to understand the composition of the genetic hierarchy that leads to metamorphosis. To test whether we are able to identify new targets of transcription factors identified in the ecdysone genetic hierarchy, we prematurely expressed the ecdysone-induced nucle-

Fig. 3. (**A**) Glycolysis. Enzymes corresponding to genes represented on the arrays are listed in red next to the reactions they catalyze. The fold repression of these genes during the initiation of metamorphosis (≥18 hours BPF versus PF) is also shown by numbers in red. (**B**) Structural and regulatory genes involved in myogenesis. (**C**) Genes encoding molecules that are involved in controlling CNS restructuring, apoptosis, and cellular differentiation during metamorphosis.

ar receptor DHR3 at ≥18 hours BPF. DHR3 is responsible for the coordination of part of the transcriptional program controlling metamorphosis and can act as either a repressor or an activator of transcription, depending on the target gene (*25*). DHR3 can induce βFTZ-F1, a nuclear receptor that is active during midprepupal development and is responsible for the difference in the genetic response to ecdysone between the late larval and prepupal ecdysone pulses (*25*, *26*). β*FTZ-F1* induction is confirmed by our microarray results (Fig. 4). Several other genes were induced by DHR3 when it was expressed at ≥18 hours BPF. One of these is represented by a novel EST (LD24139) that was induced from 3 to 9 hours APF during wild-type development (Fig. 4). ESTs representing 12 other DHR3-induced genes that have less than threefold induction at 3 to 9 hours APF are listed at (*7*). Some of these additional genes may not normally be DHR3 targets or may be induced by DHR3 at other stages during development.

DHR3 has been shown to inhibit the induction of ecdysone-inducible genes, and with E75B it can act as a repressor of the β*FTZ-F1* gene (*25*). DHR3 was expressed before the ecdysone-inducible genes were up-regulated but was still capable of repressing genes (Fig. 4). Four out of seven such genes belong to the cytochrome P450 (*CYP*) class of genes (Fig. 4) (*27*). Three of these CYP genes are normally repressed during the late larval pulse, and this repression begins before DHR3 induction occurs (approximately 4 hours BPF). Thus, DHR3 cannot be solely responsible for their repression, although it may contribute to it. One function of cytochrome P450 molecules is hydroxylation of steroids (*27*), and the depletion of transcripts of the *CYP* genes may provide a mechanism by which production of the biologically active form of ecdysone (20-hydroxyecdysone) is stifled at PF. Regulation of these *CYP* genes

within the ecdysone hierarchy further suggests that they may have a role in controlling the ecdysone genetic cascade.

Taken together, our results demonstrate the utility of DNA microarrays in determining the genetic foundations of metamorphosis. The identities of the differentially expressed genes discovered in this study suggest several points of coordination between the ecdysone-regulated pathways that control the temporal aspects of metamorphosis and the developmental pathways that control the specification and differentiation of particular cell types and tissues. Despite the experimental restrictions imposed by the use of whole animals, we observed changes in the abundance of transcripts that correlate with the late larval or prepupal ecdysone pulses (or both) for genes whose activities were not known to be influenced by this hormone. Further studies are now needed to delineate the relationship between the ecdysone-regulated genetic hierarchies and the functions of both the known and the novel genes that are differentially expressed during metamorphosis. For example, a next step is to distinguish genes that are directly regulated by ecdysone from those that are secondary targets of ecdysone-regulated factors.
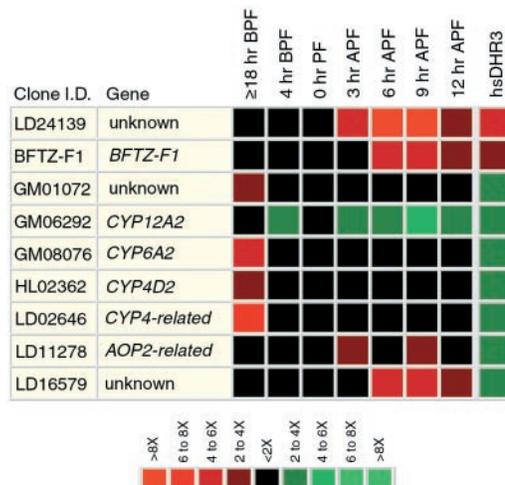
Data produced on a genomic scale can be used to similarly assist in deciphering the complex genetic networks that control other stages of *Drosophila* development. Great strides have of course been made in defining these networks by use of mutations and expression constructs. The resulting stick diagrams describing these networks must, however, be incomplete, in part because inactivation of the majority of genes does not result in obvious mutant phenotypes (*6*). Genomic approaches have the potential to expand these stick diagrams to include all functional genes. Integrating and visualizing data derived from genomic studies present a substantial challenge. Nonetheless, combining the powerful molecular and genetic approaches that *Dro-*

sophila offers with genomic information will inevitably produce a reasonably complete picture of gene regulation and its implications for metazoan development.

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5. Specific DNA primers were used to amplify ecdysone-regulated control genes by polymerase chain reaction (PCR). Vector-specific primers were used for 5853 cDNA EST clones. More than 30,000 cDNA EST clones were selected for low levels of redundancy by the bioinformatics group at the Berkeley *Drosophila* Genome Project (BDGP) to obtain this set of 5853. This set was arrayed into 96-well plates by Research Genetics. These clones were obtained from embryonic (LD), female germ line (GM), or head (HL) cDNA libraries provided by the Berkeley/Howard Hughes Medical Institute *Drosophila* EST Project. Forward and reverse vector-specific primers were made for either the pBS vector (ESTf, GAACAGCTATGACCATGAT-TACGCC; ESTr, CGGCCAGTGAAT TGTAATACGACTC) or the pOT2 vector (OTf, AATGCAGGT TAACCTG-GCT TATCG; OTr, AACGCGGCTACAAT TAATACATA-ACC). All PCR primers were picked with Primer3 software developed at the Whitehead Institute and are available at www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi. PCR reactions were done in 96-well plates with reaction volumes of 100 μl per well under the following conditions: 0.2 μg each of forward and reverse primers, 2 mM MgCl$_2$, 10 μl of 10× PCR buffer, 0.25 mM each dNTP, 1.5 U of Taq DNA polymerase, and 0.025 U of Pfu DNA polymerase, for 35 cycles at 94°C for 30 s, at 65°C for 30 s, and at 72°C for 5 min. Several microliters of bacterial liquid culture containing the cDNA EST clone were transferred from a master plate to each well in the PCR reaction plate by means of a 96-pin transfer device. All PCR reactions were analyzed by agarose gel electrophoresis. The following categories of amplified product were obtained: no product, 731; light single band, 604; double band, 221; double band with one much fainter than the other, 412; streak, 5; other anomaly, 19; and single intense band, 3861. These PCR products were then mechanically spotted on glass microscope slides. More detailed information is available at cmgm.stanford.edu/~kpwhite and in K. P. White and K. C. Burtis, Drosophila *Methods*, B. Sullivan, M. Ashburner, R. S. Hawley, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, in press).
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8. In a control experiment to determine biological variability, animals were taken from different vials at the 0 hour PF stage and polyA$^+$ RNA was prepared

Fig. 4. Genes regulated by DHR3 at ≥18 hours BPF. This experiment was repeated four times. LD24139 was up-regulated in all four experiments, as was β*FTZ-F1*. These two genes are also highly induced during midprepupal development. Also shown are genes repressed twofold or more by DHR3 at ≥18 hours BPF. Four of these genes (represented by ESTs GM06292, LD02646, GM08076, and HL02362) belong to the *cytochrome P450* gene family. The gene represented by LD11278 encodes a factor related to a mammalian antioxidant protein (AOP2). Three other *CYP* genes are repressed during the onset of metamorphosis but are not repressed by DHR3 (HL05525, HL02362, and HL01094) (*32*).

Clone I.D.	Gene	≥18 hr BPF	4 hr BPF	0 hr PF	3 hr APF	6 hr APF	9 hr APF	12 hr APF	hsDHR3
LD24139	unknown								
BFTZ-F1	*BFTZ-F1*								
GM01072	unknown								
GM06292	*CYP12A2*								
GM08076	*CYP6A2*								
HL02362	*CYP4D2*								
LD02646	*CYP4-related*								
LD11278	*AOP2-related*								
LD16579	unknown								

>8X 6 to 8X 4 to 6X 2 to 4X <2X 2 to 4X 4 to 6X 6 to 8X >8X

independently. We labeled one sample with Cy3 and the other with Cy5, then hybridized both samples to the same array. The mean normalized ratio of Cy3: Cy5 signal intensity for more than 3500 genes was 0.996 with a SD of 17.6% of the mean and a SE of 0.003. Few genes fluctuated greater than 2.0-fold and no genes on the array appeared to be differentially expressed more than 2.5-fold in this single experiment. Each value in our data set represents the average of replicate experiments. Therefore, we consider a threefold differential to be a conservative criterion for gene selection. Five hundred and sixty genes exhibited a threefold or greater differential relative to PF. Twenty-six of these genes were then culled from this set because of a lack of reproducibility, usually due to signal that was below threshold (leaving 534 genes). The threshold was set at a signal intensity of 1000. The value of 1000 is typically between 2 and 3 SDs above the mean background value in these microarray experiments.

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30. Poly A$^+$ RNA was isolated from whole animals at PF, ≥18 hours, and 4 hours BPF and at 3, 6, 9, or 12 hours APF. Fluorescent cDNA was produced with random hexamer primers and Superscript II reverse transcriptase in the presence of Cy3 or Cy5 fluorescent-tagged dUTP. This cDNA was hybridized to the arrays for 8 to 12 hours at 65°C in humidified incubation chambers. Arrays were then washed for 1 min in 1× saline sodium citrate (SSC) and 0.03% SDS, then rinsed for 15 to 30 s in 0.06× SSC, spun dry, and scanned with a confocal laser array scanner. All EST sequences discussed in the text were confirmed by resequencing. Approximately 150 clones were resequenced by spot-checking and confirmation. This resequencing revealed an error rate of approximately 15% in this version of the uniEST collection.
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Impact of El Niño and Logging on Canopy Tree Recruitment in Borneo

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Dipterocarpaceae, the dominant family of Bornean canopy trees, display the unusual reproductive strategy of strict interspecific mast-fruiting. During 1986–99, more than 50 dipterocarp species dispersed seed only within a 1- to 2-month period every 3 to 4 years during El Niño–Southern Oscillation events. Synchronous seed production occurred across extensive areas and was essential for satiating seed predators. Logging of dipterocarps reduced the extent and intensity of these reproductive episodes and exacerbated local El Niño conditions. Viable seed and seedling establishment have declined as a result of climate, logging, and predators. Since 1991, dipterocarps have experienced recruitment failure within a national park, now surrounded by logged forest.

Global climatic cycles, such as El Niño–Southern Oscillations (ENSO), affect diverse ecological processes including community dynamics and landscape disturbances in tropical regions (*1*). Dipterocarpaceae, a monophyletic group of trees (*2*), dominate low- to mid-elevation tropical forests in Southeast Asia and can contribute ≥70% of canopy biomass (*3*, *4*). Of the 257 species on the island of Borneo, most are insect-pollinated, obligate outcrossers that flower asynchronously on supra-annual cycles (*5*). Dipterocarps produce single-seeded fruits; are dispersed by wind, water, or gravity; and germinate within days of dispersal. Primary or secondary dispersal agents have not been documented (*4*, *6*).

Mast-fruiting, the supra-annual production of large seed crops interspersed by irregular periods of low seed production, has been challenged as a distinct biological phenomenon (*7*). Therefore, the geographic, taxonomic, and temporal extent of synchronous reproduction in plants must be quantified to address these claims (*7*, *8*). Our 14-year investigation tests assertions that Bornean dipterocarps display mast-fruiting over large spatial scales (*9*). Five dipterocarp genera and 54 sympatric dipterocarp species were monitored for fruit production, seed damage, and seedling establishment (*10*) across two watersheds (15 km²) within Gunung Palung National Park (GPNP; 90,000 ha), West Kalimantan (146,760 km²; Indonesian Borneo).

To assess the logging impact on dipterocarp recruitment across West Kalimantan, we also conducted 3 years of field research within 12 logging concessions, compiled a decade of logging records across all 72 timber concessions, collected dipterocarp seed export data covering a 30-year period, digitized land use maps, and assessed Landsat TM images of the GPNP region from 1988 to 1998.

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