Research

Evolutionary conservation of *otd*/*Otx2* transcription factor action: a genome-wide microarray analysis in *Drosophila*

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Abstract

Background: Homeobox genes of the *orthodenticle* (*otd*)/*Otx* family have conserved roles in the embryogenesis of head and brain. Gene replacement experiments show that the *Drosophila otd* gene and orthologous mammalian *Otx* genes are functionally equivalent, in that overexpression of either gene in null mutants of *Drosophila* or mouse can restore defects in cephalic and brain development. This suggests that *otd* and *Otx* genes control a comparable subset of downstream target genes in either organism. Here we use quantitative transcript imaging to analyze this equivalence of *otd* and *Otx* gene action at a genomic level.

Results: Oligonucleotide arrays representing 13,400 annotated *Drosophila* genes were used to study differential gene expression in flies in which either the *Drosophila otd* gene or the human *Otx2* gene was overexpressed. Two hundred and eighty-seven identified transcripts showed highly significant changes in expression levels in response to *otd* overexpression, and 682 identified transcripts showed highly significant changes in expression levels in response to *Otx2* overexpression. Among these, 93 showed differential expression changes following overexpression of either *otd* or *Otx2*, and for 90 of these, comparable changes were observed under both experimental conditions. We postulate that these transcripts are common downstream targets of the fly *otd* gene and the human *Otx2* gene in *Drosophila.*

Conclusion: Our experiments indicate that approximately one third of the *otd*-regulated transcripts also respond to overexpression of the human *Otx2* gene in *Drosophila*. These common *otd*/*Otx2* downstream genes are likely to represent the molecular basis of the functional equivalence of *otd* and *Otx2* gene action in *Drosophila*.

Background

Studies on developmental control genes involved in anterior patterning have revealed a set of homologous genes encoding transcription factors that are required for the development of the head and brain in diverse animal phyla [1-5]. A striking example for the evolutionary conservation of expression and function of such genes between invertebrates and vertebrates are the homeobox genes of the *orthodenticle* gene

family, which includes the *Drosophila orthodenticle* (*otd*) and the murine *Otx1* and *Otx2* genes [6-9]. The *Drosophila otd* gene is expressed in the anterior region of the early embryo in a domain that includes the precursors of the procephalic regions of the head, and it is also expressed in anterior brain regions and in midline CNS structures [6,10-15]. Mutational inactivation of *otd* in *Drosophila* results in defects in head structures and deletions in anterior parts of the brain as well as in ventral nerve cord defects [6,14,16]. The two *otd*-related genes in the mouse, *Otx1* and *Otx2*, are also expressed anteriorly in the embryo in nested domains that include the embryonic forebrain and midbrain [17]. Mutational inactivation of these genes results in specific defects in the head and anterior CNS; *Otx2* null mice die early in development and fail in specification of the rostral neuroectoderm and proper gastrulation [18-21]. *Otx1* null mice are viable but have spontaneous epileptic seizures and abnormalities affecting the dorsal telencephalic cortex [22].

In addition to the remarkable similarities in expression patterns and mutant phenotypes of the *otd*/*Otx* gene family, *in vivo* gene replacement experiments provide further evidence for conservation of functional properties [3,23-25]. In these cross-phylum rescue experiments, human *Otx1* or *Otx2* genes were overexpressed in *Drosophila otd* mutants and, conversely, murine *Otx1* or *Otx2* genes were replaced with the *Drosophila otd* gene in the mouse. Human *Otx1* and *Otx2* genes were able to partially rescue the brain and cephalic defects in *Drosophila*, although *Otx2* rescues at a lower frequency than *otd*, and *Otx1* rescues less efficiently still [24,25]. Similarly, the *Drosophila otd* gene coding sequence introduced into the mice *Otx1* locus was able to rescue most of the brain-patterning defects in *Otx1* mouse mutants and, when provided with the appropriate *Otx2* posttranslational control elements, also in *Otx2* mouse mutants [23,26].

Drosophila and vertebrate *otd*/*Otx* gene products share structural homology that is confined mainly to the homeodomain. The 60 amino acid residues of the fly *otd* homeodomain differ from the homeodomains of the human *Otx1* and *Otx2* protein in only three and two amino acids, respectively. It thus seems likely that most of the conserved functional action of the *otd*/*Otx* genes is mediated by the evolutionarily highly conserved homeodomain of the encoded transcription factor [25,27]. Given this highly conserved homeodomain, one might predict that the *in vivo* functional equivalence of *otd*/*Otx* genes demonstrated in the cross-phylum rescue experiments is due to the fact that both *otd* and *Otx* genes can control a comparable set of downstream target genes, irrespective of whether the *otd*/*Otx* genes are expressed in flies or in mammals [27]. However, currently little is known about the downstream targets of either *otd* or *Otx* genes in flies or in mammals, and no information on common targets of *otd* and *Otx* genes is available in any species context [27,28].

To address this issue at a genome-wide level we have combined cross-phylum overexpression experiments with expression profiling using oligonucleotide arrays. We sought to identify the common downstream target genes of fly *otd* and human *Otx2* in *Drosophila*. To this end, we used transgenic flies which carried either the fly *otd* gene or the human *Otx2* gene under the control of a heat-inducible promoter [29-33]. These experiments identified 287 annotated genes that showed highly significant ($p \leq 0.001$) changes in expression levels in response to *otd* overexpression in *Drosophila*. Among these genes, 93 also showed highly significant differential expression changes in response to *Otx2* overexpression. Moreover, the expression levels of 90 of these 93 genes were influenced in the same direction, either upregulated or downregulated, by *otd* and by *Otx2* overexpression. In summary, approximately one third of the candidate *otd* downstream target genes in *Drosophila* also respond to overexpression of the human *Otx2* gene homolog and nearly all of them display identical patterns of either up- or downregulation under both experimental conditions. From a genome-wide perspective, it is likely that the conserved genetic control of these common *otd*/*Otx2* downstream genes forms the molecular genetic basis for the striking *in vivo* functional similarity of *otd* and *Otx* gene action in *Drosophila*.

Results

In vivo overexpression and microarray analysis

In this study, transgenic fly strains carrying the *otd* coding sequence or the human *Otx2* coding sequence under the control of the heat-inducible Hsp70 promoter were used [24]. Stage 10-17 embryos were given a 25-minute heat pulse in order to overexpress the *otd* or *Otx2* genes and allowed to recover for 25 minutes (see Materials and methods). Ubiquitous overexpression of *otd* and *Otx2* was verified by whole-mount *in situ* hybridization with *otd*- or *Otx2*-specific antisense RNA probes. These experiments demonstrated that RNA was strongly overexpressed 50 minutes after the onset of heat shock in these strains (data not shown). Wild-type control flies were subjected to the identical heat-shock conditions.

Following ubiquitous overexpression of *otd* or *Otx2*, transcript profiles were analyzed using a genome-wide high-density oligonucleotide array and compared to the transcript profiles of heat-shocked wild-type control embryos. The transcripts represented on the oligonucleotide array correspond to probe sets that are complementary to approximately 13,400 annotated *Drosophila* genes according to Release 1.0 of the *Drosophila* genome [34]. For each experimental condition, several replicates were carried out (see Materials and methods). The degree of reproducibility within individual replicates is shown in scatter plots for four experimental conditions in Figure 1. A complete description of the microarray content as well as all primary data obtained in each individual microarray experiment are available as Additional data files.

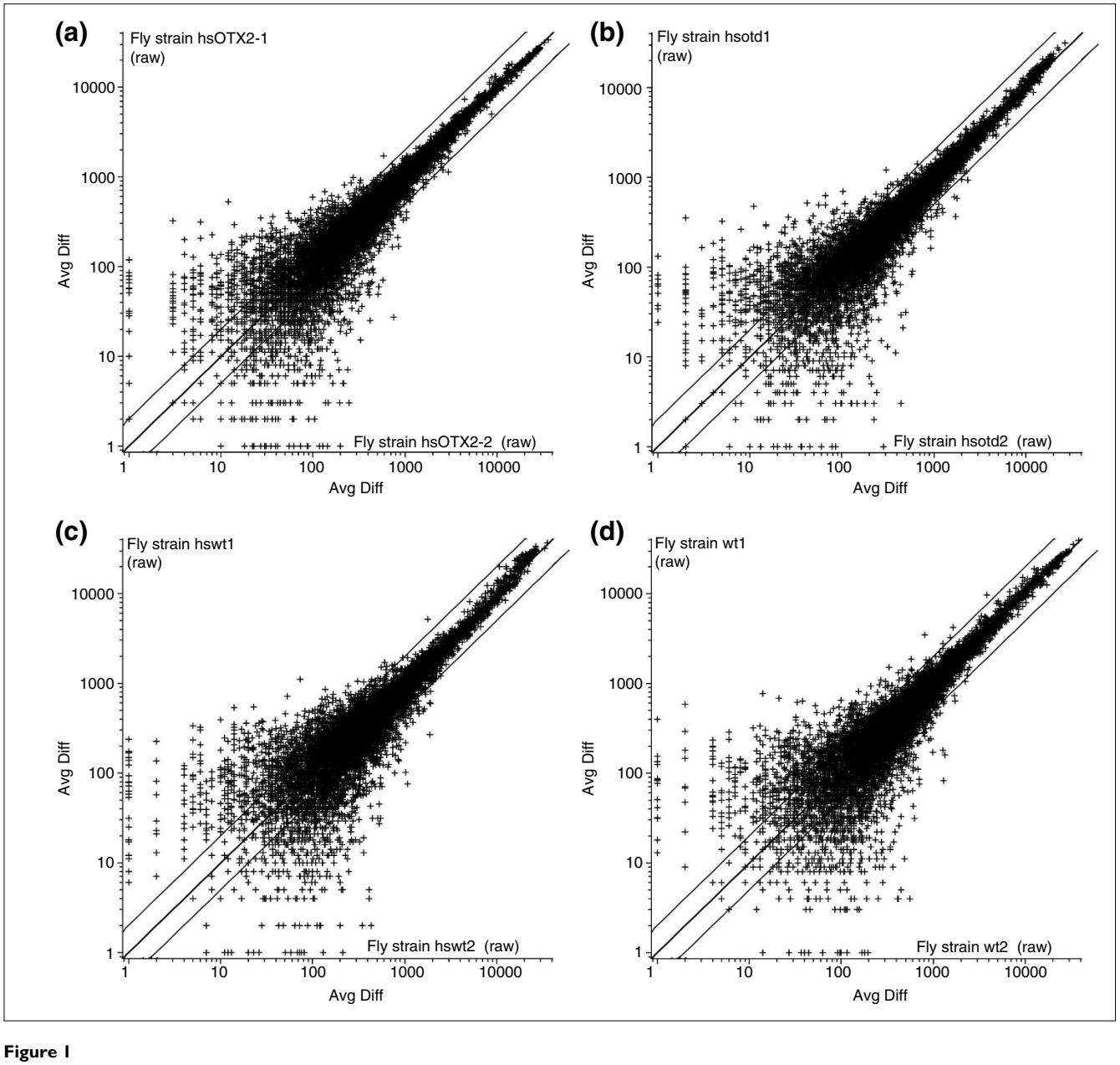

Figure 1
Normalized average difference (Avg Diff) of one pair of replicate arrays for each experimental condition in a $\log_{10}$ scale. **(a)** Heat-shocked *Otx2*; **(b)** heat-shocked *otd*; **(c)** heat-shocked wild type; **(d)** wild type. Only probe sets with positive values in both arrays are used. The central line is $y = x$, and the flanking lines indicate the difference of a factor of two.

Overview of differentially expressed transcripts

An overview of the total number of transcripts that were differentially regulated following *otd* or *Otx2* overexpression is given in Table 1. Two levels of significance for the experimental data are considered in this overview. At a significance level of $p \leq 0.001$, a total of 287 genes were found to be differentially regulated following *otd* overexpression, as compared to heat-shocked wild-type control embryos. This corresponds to 2.1% of the genes represented on the array. At a significance level of $p \leq 0.01$, a total of 762 genes were found to be differentially regulated following *otd* overexpression, as compared to heat-shocked wild-type control embryos. This corresponds to 5.7% of the genes represented on the array. In both cases, approximately a quarter of the differentially regulated transcripts corresponded to known genes, and the rest corresponded to genes that are currently characterized only by sequence information and predicted function (CG-transcripts as described by Celera Genomics [34]).

Table 1

Numbers of transcripts differentially regulated by overexpression of *otd* or *Otx2*

(a) Differential expression in response to	Total	Named transcripts	CG-transcripts*
hs*otd*	287	63	224
hs*Otx2*	682	184	498
hs*otd* and hs*Otx2*	93	21	72

(b) Differential expression in response to	Total	Named transcripts	CG-transcripts*
hs*otd*	762	165	597
hs*Otx2*	1,395	331	1,064
hs*otd* and hs*Otx2*	351	69	282

Overview of the numbers of transcripts that were differentially expressed following overexpression of *hsp-otd* (hs*otd*) or human *hsp-Otx2* (hs*Otx2*) in *Drosophila* as a result of heat shock. **(a)** Number of transcripts that were differentially expressed at a significance level of $p \leq 0.001$. **(b)** Number of transcripts that were differentially expressed at a significance level of $p \leq 0.01$. *Genes currently characterized only by sequence information and predicted function (Celera Genomics [34]).

Overexpression of the human *Otx2* gene in *Drosophila* embryos resulted in a larger number of differentially expressed transcripts than did overexpression of the *Drosophila otd* gene. At a significance level of $p \leq 0.001$, a total of 682 genes were found to be differentially expressed following *Otx2* overexpression, as compared to heat-shocked wild-type control embryos. This corresponds to 5.1% of the genes represented on the array. At a significance level of $p \leq 0.01$, 1,395 genes were found to be differentially expressed following *Otx2* overexpression as compared to heat-shocked wild-type control embryos. This corresponds to 10.4% of the genes represented on the array. Again, in both cases, approximately a quarter of the differentially regulated transcripts corresponded to known genes, and the rest were CG-transcripts.

A subset of the transcripts found to be differentially regulated following *otd* overexpression were also differentially regulated following *Otx2* overexpression. Among the transcripts that were differentially expressed at the significance level of $p \leq 0.001$, 93 transcripts were found to be differentially regulated following overexpression of either gene. This implies that 32% of the *otd*-regulated transcripts were also regulated by *Otx2*. Among the transcripts that were differentially expressed at the significance level of $p \leq 0.01$, 351 transcripts were found to be differentially regulated following overexpression of either gene. This implies that 46% of the *otd*-regulated transcripts were also regulated by *Otx2*. In the following, only genes that were differentially expressed at the significance level of $p \leq 0.001$ are considered further. We propose that these genes are potential direct or indirect downstream targets for the homeodomain transcription factors *otd* and *Otx2*.

Functional classification of differentially expressed transcripts

When ubiquitously expressed in the embryo, both *otd* and *Otx2* caused a significant transcriptional response of genes encoding a wide variety of functionally different gene products. A detailed classification of the *otd*- and *Otx2*-regulated transcripts into different functional classes was carried out according to Gene Ontology (GO) and is presented in Table 2. (In the GO classification scheme, a given gene can be grouped into more than one functional class [35]) The *otd*- and *Otx2*-regulated transcripts fall into 92 GO classes, but only about half of these classes are characterized by more than one regulated transcript.

In terms of known function, the two classes with the highest absolute and relative numbers of regulated transcripts were 'enzymes' and 'transcription factors'; this was the case for both *otd*-regulated and *Otx2*-regulated transcripts. Other functional classes with high numbers of differentially regulated genes were 'signal transduction', 'DNA binding', 'transporter', 'protein kinase', 'motor', 'ligand binding or carrier', and 'endopeptidase'; again this was the case for both *otd*- and *Otx2*-regulated transcripts. Indeed, in most cases in which a functional class was characterized by both *otd*- and *Otx2*-regulated transcripts, the relative number (n/M; see Table 2) of *otd*-regulated transcripts was similar to that of *Otx2*-regulated transcripts. For example, 2.79% of the *otd*-regulated transcripts versus 2.20% of the *Otx2*-regulated transcripts were classified under 'cell adhesion', and 3.48% of the *otd*-regulated transcripts versus 3.67% of the *Otx2*-regulated transcripts were classified under 'signal transduction'. Approximately half of both the *otd*-regulated and the *Otx2*-regulated transcripts belong to the class 'function unknown'.

Quantitative profiling of differentially expressed transcripts

Figure 2 shows the *otd*-regulated transcripts that correspond to known *Drosophila* transcripts and presents a quantitative representation of the change in expression levels for these transcripts. For clarity, these transcripts are only grouped into mother classes and not into the detailed GO classes. Most of the 63 known transcripts that were differentially expressed following *otd* overexpression showed increased expression levels; less than 20% of these transcripts were downregulated. The gene with the highest increase in expression level (78-fold) was *otd* itself, in accordance with our experimental overexpression protocol. Increases in expression levels above 10-fold were also observed for *forkhead domain 96cb* (*fd96Cb*), which encodes a nucleic-acid-binding protein, for *patched* (*ptc*), which encodes a protein

Table 2

Classification of transcripts differentially expressed in response to *Otx2* and *otd* overexpression

Functional class	n^{otd}	n^{otd}/N (%)	n^{otd}/M (%)	n^{Otx2}	n^{Otx2}/N (%)	n^{Otx2}/M (%)
Function unknown (7,108)	143	2.01	49.83	311	4.38	45.60
Enzyme (1,872)	34	1.82	11.85	88	4.70	12.90
Transcription factor (940)	23	2.45	8.01	69	7.34	10.12
Signal transduction (462)	17	3.68	5.92	24	5.19	3.52
DNA binding (306)	14	4.58	4.88	27	8.82	3.96
Transporter (498)	12	2.41	4.18	19	3.82	2.79
Motor (406)	11	2.71	3.83	22	5.42	3.23
Protein kinase (365)	10	2.74	3.48	25	6.85	3.67
Ligand binding or carrier (581)	9	1.55	3.14	28	4.82	4.11
Endopeptidase (413)	8	1.94	2.79	25	6.05	3.67
Nucleic acid binding (369)	8	2.17	2.79	21	5.69	3.08
Cell adhesion (328)	8	2.44	2.79	15	4.57	2.20
Structural protein (335)	7	2.09	2.44	18	5.37	2.64
Actin binding (157)	6	3.82	2.09	10	6.37	1.47
RNA binding (292)	4	1.37	1.39	13	4.45	1.91
Transmembrane receptor (251)	4	1.59	1.39	9	3.59	1.32
Chaperone (195)	3	1.54	1.05	14	7.18	2.05
Cell cycle regulator (190)	3	1.58	1.05	12	6.32	1.76
Ion channel (214)	3	1.40	1.05	7	3.27	1.03
Protein phosphatase (91)	3	3.30	1.05	6	6.59	0.88
DNA repair protein (65)	3	4.62	1.05	4	6.15	0.59
Transcription factor binding (64)	2	3.13	0.70	11	17.19	1.61
Cytoskeletal structural protein (121)	2	1.65	0.70	6	4.96	0.88
DNA replication factor (42)	2	4.76	0.70	5	11.90	0.73
Defense/immunity protein (64)	2	3.13	0.70	4	6.25	0.59
G-protein linked receptor (103)	2	1.94	0.70	3	2.91	0.44
Receptor (97)	2	2.06	0.70	2	2.06	0.29
Cytochrome P450	2	14.29	0.70	0	0.00	0.00
Storage protein (25)	1	4.00	0.35	3	12.00	0.44
Peptidase (97)	1	1.03	0.35	3	3.09	0.44
Lysozyme (8)	1	12.50	0.35	2	25.00	0.29
Cyclin-dependent protein kinase (11)	1	9.09	0.35	2	18.18	0.29
GABA-B receptor (1)	1	100.00	0.35	1	100.00	0.15
Enzyme inhibitor (121)	1	0.83	0.35	1	0.83	0.15
Ecdysteroid hormone receptor (2)	1	50.00	0.35	0	0.00	0.00
3′,5′-cyclic-nucleotide phosphodiesterase (1)	1	100.00	0.35	0	0.00	0.00
FK506 binding (2)	1	50.00	0.35	0	0.00	0.00
Peptidylprolyl isomerase (3)	1	33.33	0.35	0	0.00	0.00
Neurotransmitter transporter (29)	1	3.45	0.35	0	0.00	0.00
Steroid hormone receptor (16)	1	6.25	0.35	0	0.00	0.00
Acid phosphatase (5)	1	20.00	0.35	0	0.00	0.00
Arginine-tRNA ligase (2)	1	50.00	0.35	0	0.00	0.00
Carboxypeptidase (1)	1	100.00	0.35	0	0.00	0.00
Caspase activator(1)	1	100.00	0.35	0	0.00	0.00
Protein tyrosine phosphatase (9)	0	0.00	0.00	4	44.44	0.59
Protein serine/threonine kinase (43)	0	0.00	0.00	4	9.30	0.59
Chromatin binding (16)	0	0.00	0.00	4	25.00	0.59
Ubiquitin conjugating enzyme (12)	0	0.00	0.00	3	25.00	0.44
Structural protein of ribosome (136)	0	0.00	0.00	3	2.21	0.44
Casein kinase I (6)	0	0.00	0.00	3	50.00	0.44
Calcium binding (18)	0	0.00	0.00	3	16.67	0.44
Ubiquitin (14)	0	0.00	0.00	2	14.29	0.29
Translation factor (70)	0	0.00	0.00	2	2.86	0.29
Transcription co-repressor (3)	0	0.00	0.00	2	66.67	0.29
GTP binding (14)	0	0.00	0.00	2	14.29	0.29
Glutathione transferase (7)	0	0.00	0.00	2	28.57	0.29
Furin (2)	0	0.00	0.00	2	100.00	0.29
Electron transfer (35)	0	0.00	0.00	2	5.71	0.29
Ubiquitinyl hydrolase 1 (2)	0	0.00	0.00	1	50.00	0.15
Ubiquitin-specific protease (5)	0	0.00	0.00	1	20.00	0.15
Ubiquitin-like conjugating enzyme (1)	0	0.00	0.00	1	100.00	0.15
Tubulin-tyrosine ligase (7)	0	0.00	0.00	1	14.29	0.15

Table 2 *(continued)*

Classification of transcripts differentially expressed in response to *Otx2* and *otd* overexpression

Functional class	n^{otd}	n^{otd}/N (%)	n^{otd}/M (%)	n^{Otx2}	n^{Otx2}/N (%)	n^{Otx2}/M (%)
Transmembrane receptor protein tyrosine phosphatase (4)	0	0.00	0.00	1	25.00	0.15
Transmembrane receptor protein tyrosine kinase (7)	0	0.00	0.00	1	14.29	0.15
Transcription factor, cytoplasmic sequestering (1)	0	0.00	0.00	1	100.00	0.15
Transcription co-activator (2)	0	0.00	0.00	1	50.00	0.15
Thioredoxin (4)	0	0.00	0.00	1	25.00	0.15
Spermidine synthase (1)	0	0.00	0.00	1	100.00	0.15
SNF1A/AMP-activated protein kinase (1)	0	0.00	0.00	1	100.00	0.15
SH3/SH2 adaptor protein (2)	0	0.00	0.00	1	50.00	0.15
Sarcosine oxidase (2)	0	0.00	0.00	1	50.00	0.15
Ribulose-phosphate 3-epimerase (1)	0	0.00	0.00	1	100.00	0.15
Receptor signaling protein tyrosine phosphatase (1)	0	0.00	0.00	1	100.00	0.15
Protein tagging (2)	0	0.00	0.00	1	50.00	0.15
Prenylated protein tyrosine phosphatase (1)	0	0.00	0.00	1	100.00	0.15
Phosphoserine phosphatase (1)	0	0.00	0.00	1	100.00	0.15
Multicatalytic endopeptidase (4)	0	0.00	0.00	1	25.00	0.15
mRNA (guanine-N7)-methyltransferase (1)	0	0.00	0.00	1	100.00	0.15
Mitochondrial processing peptidase(1)	0	0.00	0.00	1	100.00	0.15
MAP kinase kinase (3)	0	0.00	0.00	1	33.33	0.15
Inositol-1,4,5-triphosphate receptor (1)	0	0.00	0.00	1	100.00	0.15
Electron transfer flavoprotein (1)	0	0.00	0.00	1	100.00	0.15
Effector caspase (3)	0	0.00	0.00	1	33.33	0.15
DNA-directed RNA polymerase III (7)	0	0.00	0.00	1	14.29	0.15
Cyclin (5)	0	0.00	0.00	1	20.00	0.15
CDP-diacylglycerol-serine O-phosphatidyltransferase (1)	0	0.00	0.00	1	100.00	0.15
Caspase (5)	0	0.00	0.00	1	20.00	0.15
cAMP-dependent protein kinase regulator (1)	0	0.00	0.00	1	100.00	0.15
cAMP-dependent protein kinase catalyst (3)	0	0.00	0.00	1	33.33	0.15
cAMP-dependent protein kinase (1)	0	0.00	0.00	1	100.00	0.15
Amine oxidase (flavin-containing) (7)	0	0.00	0.00	1	14.29	0.15
3-oxo-5-alpha-steroid 4-dehydrogenase (1)	0	0.00	0.00	1	100.00	0.15

Genes that were differentially expressed following ubiquitous overexpression of *otd* or human *Otx2*, grouped according to Gene Ontology (GO) functional classes. *n*, Number of transcripts detected that belong to an individual class. *N*, Number of transcripts represented on the chip for each functional class; the value of *N* for each functional class is given in parentheses following the class name. *n*/*N* x 100, Percentage of transcripts that were differentially regulated for each functional class relative to the total number of transcripts in that class represented on the chip. *M*, Total number of differentially expressed transcripts (of all classes) following overexpression of *otd* or human *Otx2* ($p \leq 0.001$); for *otd* and *Otx2*, *M* is 287 and 682 respectively. *n*/*M* x 100, Percentage of transcripts that were differentially regulated in each functional class relative to the the total number of differentially regulated transcripts for *otd* and *Otx2*.

involved in signal transduction, for *picot*, which encodes a transporter, and for *cortactin* and *Regulator of cyclin A1* (*Rca1*), which encode gene products of currently unknown molecular function. Only two transcripts showed increases in the 5-10-fold range, namely *sugar transporter1* (*sut1*) encoding a protein involved in sugar transportation, and *scraps* (*scra*) encoding an actin-binding protein. The majority of the upregulated transcripts had increases in the 2-5-fold range. The transcript with the most marked decrease in expression was *eyegone* (*eyg*), encoding a transcription factor known to be involved in eye development.

Figure 3 shows the *Otx2*-regulated transcripts that correspond to known *Drosophila* genes and presents a quantitative representation of their expression level changes. Again, these transcripts are grouped into mother classes and not into detailed GO classes. As was the case for *otd* overexpression, most of the known transcripts that were differentially expressed following *Otx2* overexpression showed increased expression levels. For example, in the functional class of 'enzyme', 45 out of 49 transcripts were upregulated. In total, less than 13% of the 184 *Otx2*-regulated known transcripts were downregulated. Increases in expression levels above 10-fold were observed for 23 genes and for 6 of these genes, *retained* (*retn*), *SMC2*, *licorne* (*lic*), *Rtc1*, *Hairless* (*H*) and *deadhead* (*dhd*), the increases were greater than 50-fold. Twenty-two transcripts showed increases in the 5-10-fold range, and, similarly to the *otd* overexpression situation, increases of 2-5-fold dominated in most of the functional classes. The transcript with the most marked decrease in expression was once again *eyg*.

Common candidate downstream genes of *otd* and *Otx2*

Ninety-three transcripts were differentially expressed in response to both *otd* overexpression and *Otx2* overexpression.

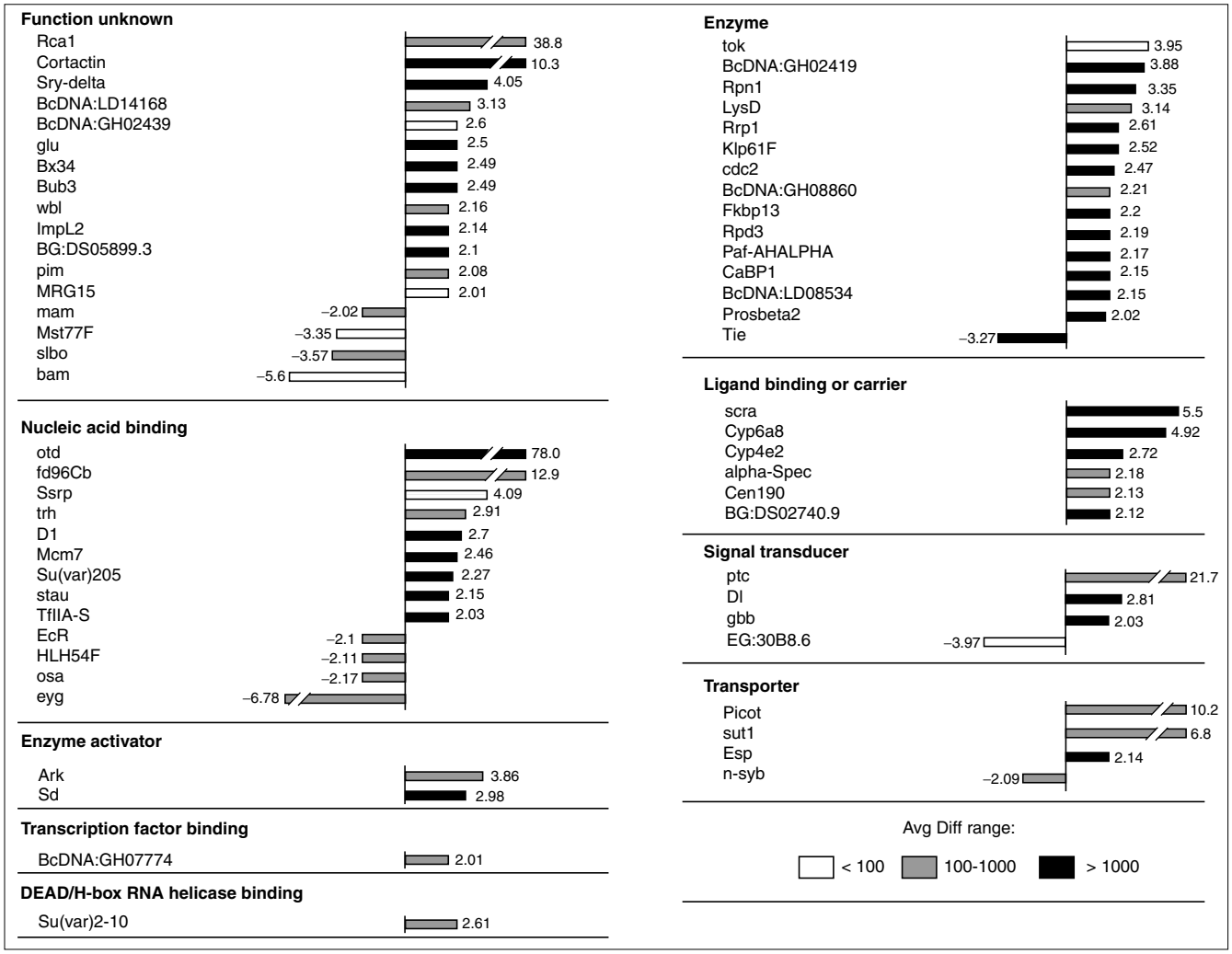

Figure 2

Known transcripts differentially expressed in response to overexpression of *otd*, grouped according to functional classes. Bars represent the fold change between differentially expressed transcripts in heat-shocked wild-type embryos and heat-shocked *otd* embryos. Positive values indicate that the relative expression level of a gene is increased (upregulated) following *otd* overexpression and negative values indicate a decrease (downregulated). Absolute average difference (Avg Diff) values are given for the *otd* overexpression condition as follows: white bars, Avg Diff < 100; gray bars, Avg Diff from 100-1,000; black bars, Avg Diff > 1,000.

This indicates that approximately one third of the *otd*-regulated genes in *Drosophila* also respond to overexpression of the human *Otx2* gene homolog. Figure 4 shows the expression levels for these transcripts, which are likely to represent the common downstream target genes for *otd* and *Otx2*. Twenty-one of these transcripts correspond to known *Drosophila* genes and 72 correspond to annotated CG-transcripts. The expression levels of all of the known transcripts were influenced in the same manner by overexpression of *otd* and *Otx2*, in that a given downstream target gene was either upregulated in both cases or downregulated in both cases. Moreover, for most of these transcripts the absolute expression levels were similar in response to *otd* and to *Otx2*. Two marked exceptions were *pimple* (*pim*), which was upregulated 12.4-fold following *Otx2* overexpression and 2.1-fold following *otd* overexpression, and *eyg*, which was downregulated 77.6-fold following *Otx2* overexpression (but see PCR data below) and downregulated 6.8-fold following *otd* overexpression. Similarly, the expression levels of 68 of the CG transcripts were influenced in the same manner by overexpression of *otd* and *Otx2*. Only in the three remaining cases were transcripts upregulated by overexpression of one of the *otd*/*Otx* transgenes and downregulated by overexpression of the other. Thus, approximately one third of the candidate *otd* downstream target genes in *Drosophila* are controlled in a comparable manner by the human *Otx2* gene homolog.

There are a number of interesting genes among these common candidate genes. The four known transcripts in class 'ligand binding or carrier', *scra*, *Kinesin-like protein at*

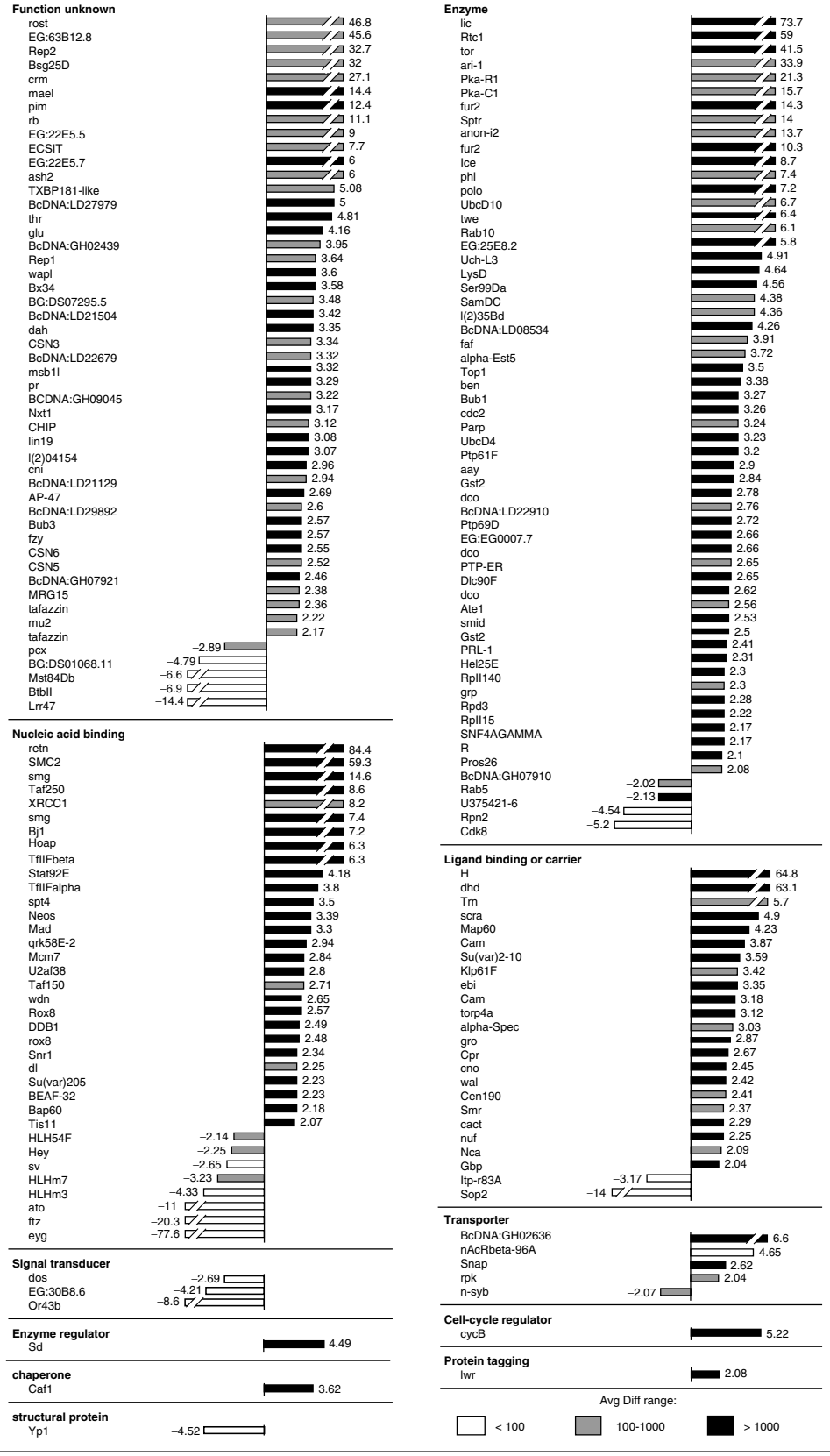

Figure 3 *(see legend on the next page)*

61F (*Klp61F*), *alpha-Spectrin* (*alpha-spec*) and *Centrosomal protein 190kD* (*Cen190*), are all involved in actin or microtubule binding or movement [36-39]. This finding is intriguing as one of the *Otx2* downstream genes identified in the mouse is a tropomyosin gene, which also encodes an actin-binding protein [40]. Among the four known transcripts in the class 'nucleic acid binding' are the genes *Minichromosome maintenance 7* (*Mcm7*) and *Suppressor of variegation 205* (*Su(var)205*) [41,42] which encode chromatin-binding proteins, and the genes *eyg* and *HLH54F*, which encode transcription factors [43,44]. The four known transcripts in the functional class 'enzymes' are *Lysozyme D* (*LysD*), *cdc2*, *Rpd3*, and *BcDNA:LD08534* [45-48]. Although the *cdc2* gene product is classified as 'enzyme', it also acts at the G2/M transition of the mitotic cell cycle [47]. Moreover, *Rpd3* encodes a histone deacetylase which is involved in chromatin structure [46]. In the class 'transporter' the *n-synaptobrevin* (*n-syb*) gene, encoding a SNAP receptor, is involved in synaptic-vesicle docking and fusion and is expressed in the embryonic CNS [49]. In the class 'signal transducer', the gene EG:30B8.6 encodes a putative GABA-B receptor [50]. Finally, the gene *Segregation distorter* (*Sd*), classified as 'enzyme regulator', encodes a Ran GTPase activator [51]. Among the transcripts of known genes are several genes whose precise functional role is not well defined at the molecular level. These are the *Bx34* and *MRG15* genes [52,53] which encode components of the nucleus and the *gluon*, *Bub3* and *pim* genes which are all involved in mitosis. *gluon* encodes a putative component of the condensin complex, and *gluon* mutants show peripheral nervous system defects during embryogenesis [54]. The gene product of *Bub3* is localized to the kinetochore and may function in the mitotic checkpoint [55]. *pim* is expressed in the embryonic CNS and encodes a protein implicated in mitotic sister-chromatid separation [56].

Verification of microarray expression data with RT-PCR

To confirm the differences in gene expression levels after heat-shock induced overexpression of *otd* and human *Otx2* as compared to heat-shocked wild-type embryos, quantitative reverse transcription polymerase chain reaction (RT-PCR) was carried out on selected candidate target genes. Changes in expression levels were determined for eight genes that were differentially regulated by *otd* or human *Otx2*, namely *scra*, *LysD*, *glu*, *Rpd3*, *pim*, *n-syb*, *eyg* and *otd*. The genes *wunen* (*wun*) and *Scc1*, whose expression levels remained unchanged in response to *otd* or *Otx2* overexpression, were used as controls. As indicated in Table 3, these experiments showed that the changes in relative expression level, as measured by RT-PCR, are generally consistent with the data obtained with the oligonucleotide arrays. An exception is the data on the response of the *eyg* gene to *Otx2* overexpression; RT-PCR data indicate a weak downregulation (-1.62) whereas oligonucleotide array data indicate a strong downregulation (-77.6).

Discussion

Common downstream target genes for *otd* and *Otx*

Cross-phylum gene replacement experiments have shown that the fly *otd* gene and the homologous human *Otx* genes are functionally equivalent *in vivo*, in that overexpression of either gene in *Drosophila otd* null mutants can lead to the restoration of defects in cephalic and brain development [23-26]. We have used a combination of transgenic overexpression genetics and functional genomics to gain insight into the equivalence of *otd* and *Otx* gene expression in *Drosophila* at a comprehensive, genome-wide level. Using inducible overexpression and quantitative transcript imaging through oligonucleotide arrays representing the total number of 13,400 currently annotated *Drosophila* genes, we have identified hundreds of candidate downstream genes for both the fly *otd* gene and the human *Otx2* gene. A comparison of these candidate downstream genes reveals that both *otd* and *Otx* genes appear to control an overlapping set of genes; we refer to these genes as common downstream genes. The number of identified common downstream genes for *otd* and *Otx2* depends on the statistical level of significance used to determine whether a given gene showed differential expression in response to transgene overexpression. If the analysis is restricted to highly significant ($p \leq 0.001$) datasets, we find 93 common downstream genes, equivalent to 32% of the candidate *otd* downstream genes or approximately 1% of transcripts in the annotated fly genome. If, in contrast, the analysis is based on significant ($p \leq 0.01$) datasets, we find 351 common downstream genes, equivalent to 46% of the candidate *otd* downstream genes or approximately 3% of transcripts in the annotated fly genome. In either case, a substantial, but far from complete, set of the *otd* regulated genes are common downstream targets of both fly and human transgenes.

It is interesting that, at the genome-wide transcript level, the *Otx2* gene does not appear to be able to replace *otd* action in full; over half of the transcripts that are influenced by *otd* overexpression are not influenced by *Otx2* overexpression. Given the pronounced differences in amino acid sequence

Figure 3 *(see figure on the previous page)*
Known transcripts differentially expressed in response to overexpression of *Otx2*, grouped according to functional classes. Bars represent the fold change between differentially expressed transcripts in heat-shocked wild-type embryos and heat-shocked *Otx2* embryos. Positive values indicate that the relative expression level of a gene is increased (upregulated) following *Otx2* overexpression and negative values indicate a decrease (downregulated). Avg Diff values are given for the *Otx2* overexpression condition as follows: white bars, Avg Diff < 100; gray bars, Avg Diff from 100-1,000; black bars, Avg Diff > 1,000.

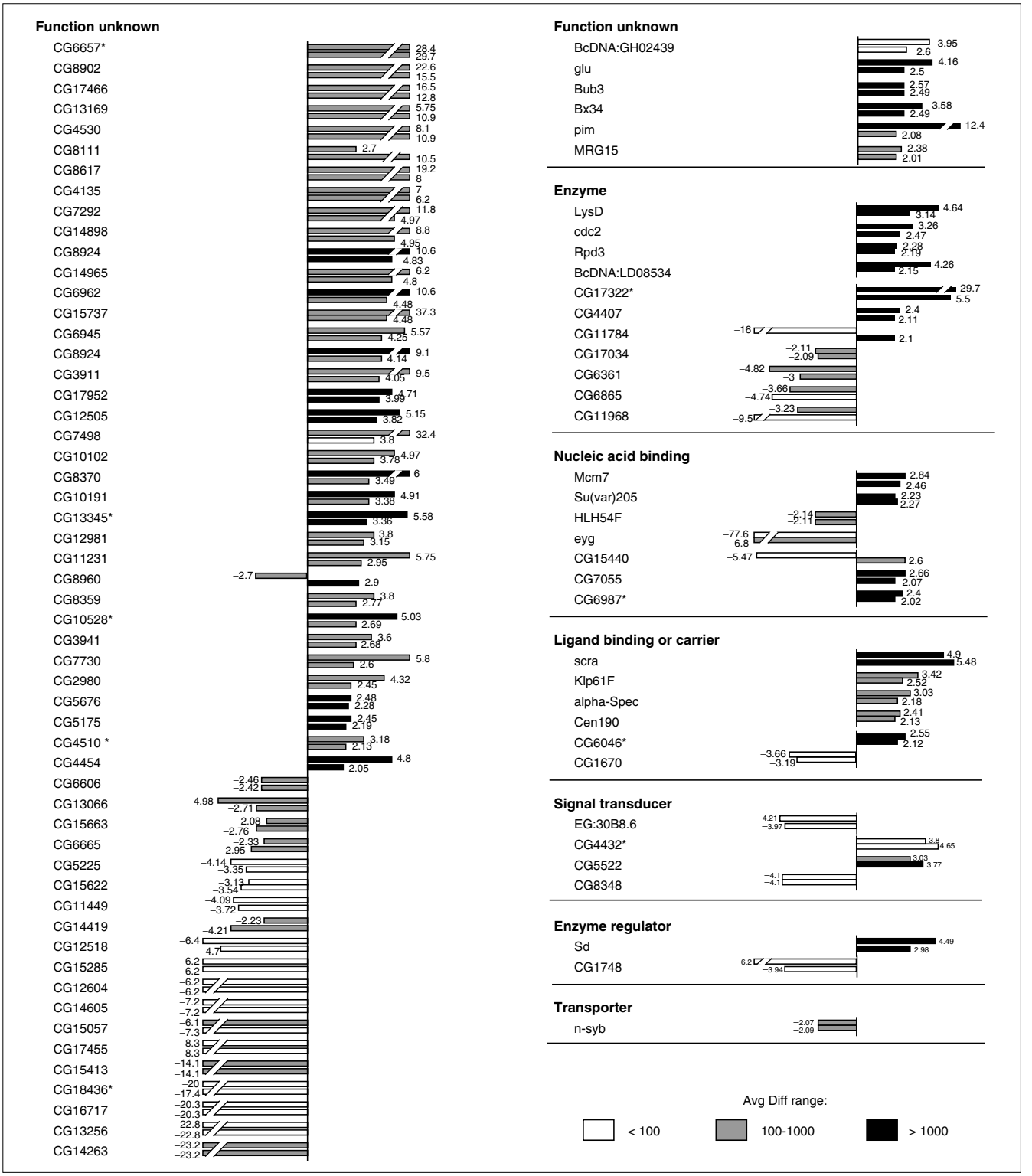

Figure 4

Transcripts differentially expressed in response to overexpression of *otd* and in response to overexpression of human *Otx2*, grouped according to functional classes. Bars represent the fold change between differentially expressed transcripts in heat-shocked wild type embryos and heat-shocked *otd* or heat-shocked *Otx2* embryos. The upper bars represent the fold change of differentially expressed transcripts following overexpression of *Otx2* and the lower bars represent the fold change of differentially expressed transcripts following overexpression of *otd*. Positive values indicate that the relative expression level of a gene is increased (upregulated) following *otd* overexpression and negative values indicate a decrease (downregulated). Avg Diff values are given for the *otd* overexpression condition as follows: white bars, Avg Diff < 100; gray bars, Avg Diff from 100-1,000; black bars, Avg Diff > 1,000.

Table 3

Comparison of change folds between oligonucleotide arrays and RT-PCR

	Avg Diff			Change fold			
				hs*otd*		hs*Otx2*	
Transcript	hs*wt*	hs*otd*	hs*Otx2*	Array	RT-PCR	Array	RT-PCR
scra	251	1375	1229	5.5	1.3	4.9	1.6
LysD	525	1646	2436	3.1	1.6	4.6	4.0
glu	479	1196	1991	2.5	1.8	4.2	10.9
Rpd3	1170	2562	2673	2.2	2.0	2.3	2.5
pim	118	246	1467	2.1	1.4	12.4	8.0
n-syb	612	293	296	-2.1	-1.5	-2.1	-1.5
eyg	1552	229	10	-6.7	-1.4	-77.6	-1.6
wun	885	/	884	/	/	1.0	1.0
Scc1	724	723	/	1.0	1.0	/	/
otd	84	6555	108	78.0	119.4	1.3	1.5

RT-PCR was carried out on cDNA derived from heat-shocked wild type (hs*wt*), heat-shocked *otd* (hs*otd*) or heat-shocked *Otx2* (hs*Otx2*) embryos. Change folds determined by RT-PCR are represented as the mean value of eight independent replicates, derived from two different cDNA preparations. *wun* is used as a control for the comparison of the RT-PCR data between heat-shocked wild type and heat-shocked *Otx2*. *Scc1* is used as a control for the comparison of the RT-PCR data between heat-shocked wild type and heat-shocked *otd*.

between the OTD and OTX2 proteins, this may not be altogether surprising. The OTD and OTX2 proteins consist of 548 and 289 amino acids, respectively. Shared homology between them is restricted to the homeodomain and to a short domain immediately upstream of the homeodomain as well as a tripeptide at the amino terminus [25]. Moreover, as *Otx* genes cannot completely replace the *otd* gene in cross-phylum rescue experiments *in vivo*, a complete correspondence of *otd* downstream genes and common *otd*/*Otx* downstream genes might not be expected [3,24,25]. However, approximately one third of the *otd*-regulated genes do also respond to *Otx2* overexpression. We suggest that these common downstream genes are likely to explain the overlapping roles of the *otd*/*Otx* genes in cross-phylum rescue experiments *in vivo*. These target genes reflect the evolutionarily conserved roles of the members of the *otd*/*Otx* gene family in *Drosophila*. To investigate this further, it will now be important and interesting to carry out similar functional genomic analyses of *otd* and *Otx* gene action in a mammalian system such as the mouse [27].

otd overexpression: a genomic perspective on candidate downstream genes

The experiments reported here identify approximately 300 genes that showed highly significant ($p \leq 0.001$) changes in expression levels in response to *otd* overexpression in *Drosophila*. The genomic perspective of these identified *otd* downstream target genes reveals several features of *otd* action at a higher level of insight. First, this finding indicates that the *otd* gene product, a homeodomain transcription factor, regulates a limited and distinct set of candidate downstream genes. At a significance level of $p \leq 0.001$, 287 genes were found to be differentially regulated, corresponding to approximately 2.1% of the transcripts in the annotated fly genome. At a significance level of $p \leq 0.01$, 762 genes were found to be differentially regulated, corresponding to approximately 5.7% of the transcripts in the annotated fly genome. This is further evidence for the notion that homeoproteins in *Drosophila* control only a subset and not the majority of the genes in the genome [30]. Indeed, in similar experiments in which the homeobox gene *labial* (*lab*) was overexpressed using the same heat-shock protocol as described here, 6.4% of the genes represented on the array used were shown to be differentially regulated at a significance level of $p \leq 0.01$ [30]. (It should however, be noted that the array used in these *lab* overexpression experiments represents only 10% of the genes in the fly genome.) Thus the relative number of putative *otd* targets appears to be in the same range as the number of putative *lab* targets.

Second, these experiments show that the OTD homeodomain transcription factor acts on numerous candidate target genes that also encode transcription factors, consistent with the idea that homeodomain proteins act through a cascade of transcription factors which regulate the expression of their own subset of downstream genes [57]. Currently, we do not know which of the downstream target genes are direct OTD targets and are, thus regulated directly by OTD protein binding to DNA regulatory sequences, and which are indirect targets. At present, little is known about temporal response of putative target genes following pulsed expression of a transcription factor. Some studies have been carried out on the basis of the assumption that direct targets respond immediately whereas indirect targets respond with

a delay due to the time required for intermediary gene expression. Nasiadka and Krause used a kinetic approach to identify direct and indirect targets of the ectopically expressed homeodomain transcription factor *fushi tarazu* (*ftz*) [58]. Their results show that target genes respond to pulses of *ftz* expression within two distinct temporal windows. Direct responses (no intermediary gene transcription is required) are 50% complete within about 18 minutes after heat shock. Indirect responses do not reach the same level of response until 26 minutes after heat shock. Assuming that *otd* expression follows a similar kinetic profile to *ftz*, it is likely that we have identified primary targets as well as genes whose response was caused by indirect effects requiring intermediate transcription.

Third, these results show that the primary consequence of *otd* overexpression in *Drosophila* is the upregulation of its downstream target genes. Indeed more than 80% of the genes that were differentially expressed following *otd* overexpression showed increased expression levels. This contrasts with the action of the homeotic gene *lab*; overexpression of *lab* under comparable conditions resulted in an approximately equal number of upregulated and downregulated target genes [30].

The majority of potential downstream target genes of *otd* are annotated CG-transcripts and, hence, correspond to predicted genes which have not yet been studied in detail in an *in vivo* context. This is surprising given the fact that numerous classical genetic screens for genes involved in cephalic and CNS embryogenesis have been carried out [59]. This may indicate that many of the genes involved in those aspects of cephalic and CNS embryogenesis that are under the control of *otd* in *Drosophila* have not yet been identified. Alternatively, this finding may reflect specific constraints of the overexpression experiment. For example, the overexpression protocol used makes it difficult to control OTD protein concentration and stability. As different levels of a homeoprotein may have different developmental consequences, the relatively high level of OTD protein attained may influence target genes that are not affected by the endogenously attained protein level [60,61]. Moreover, the fact that *otd* overexpression is not accompanied by simultaneous overexpression of cofactors, which can act together with homeodomain transcription factors to determine their *in vivo* target specificity, may also lead to nonspecific activation of target genes [62].

Functional genomics of a human transgene overexpressed in *Drosophila*

In several cases, human transgenes have been overexpressed in *Drosophila* in order to gain insight into the evolutionary conservation of developmental control gene action [24,25,63-66]. This has also been the primary goal of the overexpression of human *Otx2* in *Drosophila* reported here. In addition to the identification of common *otd*/*Otx* downstream genes, the genomic level of analysis reported here has uncovered remarkable similarities in the activity of the human transgene in the fly as compared to that of its fly homolog. Thus, *otd* and *Otx2* both upregulate most of their target genes upon overexpression. Moreover, the target genes of both transcription factors fall into the same functional categories. For example, the classes 'enzymes' and 'transcription factors' had the highest absolute and relative number of transcripts.

The striking difference in the action of the two transgenes is that overexpression of human *Otx2* causes expression changes in many more downstream genes than does overexpression of the fly *otd* gene. The experiments reported here identify approximately 700 genes that showed highly significant ($p \leq 0.001$) changes in expression levels in response to *Otx2*; this is more than double the number observed in response to *otd*. It is unlikely that this difference is due to corresponding differences in the expression levels attained for *Otx2* versus *otd* transcripts. Indeed, the transcript abundance of *otd* was higher than that of *Otx2* in these experiments (see Materials and methods). Nevertheless, these data should be interpreted with caution, as several explanations, not mutually exclusive, are possible for the observation that more genes respond to overexpression of *Otx2*. First, only one single-transgenic strain of *otd* and only one single-transgenic strain of *Otx2* were used. Thus, strain differences or insertion effects might account for the fact that more genes show differential expression following overexpression of *Otx2* compared to overexpression of *otd*. Second, it is conceivable that overexpression of *Otx2* affects more downstream genes in *Drosophila* than *otd* because the OTX2 transcription factor binds to many more DNA regulatory regions than does OTD. The smaller OTX2 protein might, therefore, have a lower specificity for target gene regulatory regions. Similarly, the OTX2 protein might be more promiscuous than OTD in its interactions with the numerous cofactors that determine target specificity. Third, it has been shown that the DNA-binding specificity of homeoproteins is low *in vitro*. But given that the homeodomain is conserved and *Otx2* rescues the *otd* phenotype, this suggests that they should recruit a similar subset of cofactors and regulate a common subset of downstream genes, at least in those tissues where *otd* is endogenously expressed. Furthermore, the *Otx2* product, which is not a fly protein, could influence the expression of a small number of transcription factors that are not affected by OTD and which then regulate the expression of their own subset of downstream genes. Whatever the molecular basis for this unexpected difference in the result of *Otx2* and *otd* overexpression may be, its discovery is a further demonstration of the new level of insight that can be attained from a genome-wide functional perspective.

Materials and methods

Embryos

The wild type was *Drosophila melanogaster* Oregon-R. For overexpression of *otd*, we used the *hsp-otd* line 5A generated

by Royet and Finkelstein [67]. For overexpression of human *Otx2*, we used the *hsp-Otx2* line generated by Leuzinger *et al*. [24]. All fly stocks were kept on standard cornmeal/yeast/agar medium at 25°C. Embryos were collected overnight for 12 h on grape juice plates, kept for a further 4 h at 25°C and then subjected to a 37°C heat shock for 25 min, followed by a recovery period of 25 min at 25°C before RNA isolation. Therefore, at the time of RNA isolation these embryos were at embryonic stages 10-17 [29]. Embryos younger than embryonic stage 10 were not used, as heat shock in these earlier stages results in lethality [68]. Embryos used for *in situ* hybridization studies were collected and heat-shock treated in the same way.

Whole-mount *in situ* hybridization

For *in situ* hybridization, digoxigenin-labeled sense and antisense *otd/Otx2* RNA probes were generated *in vitro*, with a DIG labeling kit (Roche Diagnostics) and hybridized to whole-mount embryos following standard procedure [69]. Hybridized transcripts were detected with an alkaline-phosphatase conjugated anti-digoxigenin Fab fragment (Roche Diagnostics) using Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma) as chromogenic substrates.

High-density oligonucleotide arrays and hybridization

In this study, a custom-designed *Drosophila* oligonucleotide array (roDROMEGAa, Affymetrix, Santa Clara, CA) was used. It contains 14,090 sequences representing *Drosophila*-specific transcripts, prokaryotic control sequences and custom-chosen sequences for transgenes such as *gal4*, *gfp*, and *lacZ*. Of the sequences included, 13,998 correspond to *Drosophila*-specific transcripts annotated by Celera Genome Release 1 [34] and deposited in SWISS-PROT/TrEMBL databases. These 13,998 sequences represent approximately 13,400 genes in the *Drosophila* genome and therefore some genes are represented by more than one probe set. Each sequence is represented on the array by a set of 14 oligonucleotide probes (25mers) matching the sequence. To control the specificity of hybridization, the same probes are represented on the array with a single nucleotide mismatch in a central position. As such, each sequence is represented by 14 perfect match and 14 mismatch probes. The average difference (Avg Diff) between the perfect-match hybridization signal and the mismatch signal is proportional to the abundance of a given transcript [32]. RNA was isolated, labeled, and hybridized to the arrays as described [29,30] with minor modifications.

Data analysis

Probe arrays were scanned with a commercial confocal laser scanner (Hewlett-Packard). Pixel intensities were measured, and expression signals were analyzed with commercial software (GENECHIP 3.1, Affymetrix). Data processing was carried out using RACE-A (F. Hoffmann-La Roche), Access 97 and Excel 97 (Microsoft) software. Scatter plots were prepared using GeneSpring™ software version 4.1 (Silicon Genetics, Redwood City, CA). For quantification of relative transcript abundance, Avg Diff value was used [32]. Four replicates were carried out for *hsp-otd* and *hsp-Otx2*. Three and five replicates were done for heat-shocked wild type and wild type respectively. All arrays were normalized against the mean of the total sums of Avg Diff values across all 16 arrays. In order to avoid huge fold changes, genes with a normalized Avg Diff below 20 were automatically assigned an Avg Diff of 20 (RACE-A protocol). An unpaired *t*-test for each individual gene was carried out for the following pairwise comparisons: heat-shocked wild type versus wild type, heat-shocked wild type versus heat-shocked *otd*, and heat-shocked wild type versus heat-shocked *Otx2*. For differential transcript imaging, only transcripts that had highly significant or significant changes in Avg Diff ($p \leq 0.001$ and $p \leq 0.01$, respectively) and whose changes were in the two-fold and above range are presented. Additionally, the higher mean Avg Diff of a pairwise comparison for a given transcript had to be above or equal to 50. To obtain a comprehensive analysis of the number and identity of genes differentially regulated by *otd/Otx2*, candidates that were already differentially expressed in heat-shocked wild-type embryos compared to non-heat-shocked wild-type controls were excluded from further analysis (data not shown [30]). For a comprehensive list of all genes with their fold changes and significance levels, see Additional data.

RT-PCR

Poly(A)$^+$ RNA (300 ng) was isolated from embryos of wild type, heat-shocked wild type, heat-shocked *otd* and heat-shocked *Otx2* (mRNA isolation kit; Roche Diagnostics) and reverse transcribed with AMV-RT and random hexamers (RT-PCR kit; Roche Diagnostics). PCR was performed with 100 pg template DNA and gene-specific primers (Seq Web, Winsconsin Package Version 10.0, GCG) on a light cycler (LightCycler, Roche Diagnostics). Continuous fluorescence observation of amplifying DNA was possible using SYBR Green I (Roche Diagnostics) After cycling, a melting curve was produced by slow denaturation of the PCR end products, to validate the specificity of amplification. To compare the relative amounts of PCR products we monitored the amplification profile on a graph, displaying the log of the fluorescence against the number of cycles. Relative fold changes for a given gene under both conditions (heat-shocked *otd* versus heat-shocked wild type or heat-shocked *Otx2* versus heat-shocked wild type) were calculated using the fit point method (Light Cycler Manufacturer, Roche).

Quantification of *otd* and human *Otx2* transcripts by RT-PCR

Plasmids containing fly *otd* or human *Otx2* cDNA were linearized with appropriate restriction enzymes and purified. The concentrations of the linearized plasmids were spectrophotometrically quantified using a GeneQuant RNA/DNA calculator (Pharmacia Biotech) and serial dilutions were made. To quantify the concentration of the *otd* and *Otx2*

transcripts from heat-shocked *otd* and heat-shocked *Otx2* embryos, a standard curve was established using the serial dilution of the corresponding linearized plasmid on a light cycler (Roche). RT-PCR was carried out when the standard curve was established. Thereafter, the steady-state concentrations of the *otd* and human *Otx2* were calculated in relation to their standard curves, using the second derivative maximum method (Roche). This showed that the concentrations of *otd* and *Otx2* transcripts were 1.5 x 10^{-6} μg/μl and 3.6 x 10^{-7} μg/μl, respectively.

Additional data

The following additional data files are available: a list of the genes on the microarray; Primary data (Avg Diff values, both raw and normalized) for each microarray experiment: heat-shocked *otd* embryos (replicates 1, 2, 3, 4); heat-shocked *Otx2* embryos (replicates 1, 2, 3, 4); heat-shocked wild-type embryos (replicates 1, 2, 3); wild-type embryos (replicates 1, 2, 3, 4, 5); normalization factors for each replicate; comparisons between pairs of experiments, including the fold change for each gene and the results of a *t*-test: heat-shocked wild-type embryos compared with heat-shocked *otd* embryos; heat-shocked wild-type embryos compared with heat-shocked *Otx2* embryos; heat-shocked wild-type embryos compared with wild-type embryos.

These data have been submitted to the Gene Expression Omnibus at the National Center for Biotechnology Information [70], accession numbers GSM1351-GSM1366 (platform accession GPL70, series accession GSE32).

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References

1. Finkelstein R, Boncinelli E: **From fly head to mammalian forebrain: the story of *otd* and *Otx*.** *Trends Genet* 1994, **10:**310-315.
2. Thor S: **The genetics of brain development: conserved programs in flies and mice.** *Neuron* 1995, **15:**975-977.
3. Sharman AC, Brand M: **Evolution and homology of the nervous system: cross-phylum rescues of *otd*/*Otx* genes.** *Trends Genet* 1998, **14:**211-214.
4. Holland LZ, Holland ND: **Chordate origins of the vertebrate central nervous system.** *Curr Opin Neurobiol* 1999, **9:**596-602.
5. Galliot B, Miller D: **Origin of anterior patterning. How old is our head?** *Trends Genet* 2000, **16:**1-5.
6. Finkelstein R, Smouse D, Capaci TM, Spradling AC, Perrimon N: **The orthodenticle gene encodes a novel homeo domain protein involved in the development of the *Drosophila* nervous system and ocellar visual structures.** *Genes Dev* 1990, **4:**1516-1527.
7. Simeone A, Gulisano M, Acampora D, Stornaiuolo A, Rambaldi M, Boncinelli E: **Two vertebrate homeobox genes related to the Drosophila empty spiracles gene are expressed in the embryonic cerebral cortex.** *EMBO J* 1992, **11:**2541-2550.
8. Simeone A, Acampora D, Mallamaci A, Stornaiuolo A, D'Apice MR, Nigro V, Boncinelli E: **A vertebrate gene related to orthodenticle contains a homeodomain of the bicoid class and demarcates anterior neuroectoderm in the gastrulating mouse embryo.** *EMBO J* 1993, **12:**2735-2747.
9. Acampora D, Simeone A: **The TINS Lecture. Understanding the roles of Otx1 and Otx2 in the control of brain morphogenesis.** *Trends Neurosci* 1999, **22:**116-122.
10. Gao Q, Wang Y, Finkelstein R: **Orthodenticle regulation during embryonic head development in *Drosophila*.** *Mech Dev* 1996, **56:**3-15.
11. Cohen SM, Jurgens G: **Mediation of *Drosophila* head development by gap-like segmentation genes.** *Nature* 1990, **346:**482-485.
12. Grossniklaus U, Cadigan KM, Gehring WJ: **Three maternal coordinate systems cooperate in the patterning of the *Drosophila* head.** *Development* 1994, **120:**3155-3171.
13. Schmidt-Ott U, Gonzalez-Gaitan M, Jackle H, Technau GM: **Number, identity, and sequence of the *Drosophila* head segments as revealed by neural elements and their deletion patterns in mutants.** *Proc Natl Acad Sci USA* 1994, **91:**8363-8367.
14. Wieschaus E, Perrimon N, Finkelstein R: ***orthodenticle* activity is required for the development of medial structures in the larval and adult epidermis of *Drosophila*.** *Development* 1992, **115:**801-811.
15. Finkelstein R, Perrimon N: **The *orthodenticle* gene is regulated by *bicoid* and *torso* and specifies *Drosophila* head development.** *Nature* 1990, **346:**485-488.
16. Klambt C, Jacobs JR, Goodman CS: **The midline of the *Drosophila* central nervous system: a model for the genetic analysis of cell fate, cell migration, and growth cone guidance.** *Cell* 1991, **64:**801-815.
17. Simeone A, Acampora D, Gulisano M, Stornaiuolo A, Boncinelli E: **Nested expression domains of four homeobox genes in developing rostral brain.** *Nature* 1992, **358:**687-690.
18. Suda Y, Matsuo I, Kuratani S, Aizawa S: **Otx1 function overlaps with Otx2 in development of mouse forebrain and midbrain.** *Genes Cells* 1996, **1:**1031-1044.
19. Matsuo I, Kuratani S, Kimura C, Takeda N, Aizawa S: **Mouse Otx2 functions in the formation and patterning of rostral head.** *Genes Dev* 1995, **9:**2646-2658.
20. Ang SL, Jin O, Rhinn M, Daigle N, Stevenson L, Rossant J: **A targeted mouse Otx2 mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain.** *Development* 1996, **122:**243-252.
21. Acampora D, Mazan S, Lallemand Y, Avantaggiato V, Maury M, Simeone A, Brulet P: **Forebrain and midbrain regions are deleted in Otx2-/- mutants due to a defective anterior neuroectoderm specification during gastrulation.** *Development* 1995, **121:**3279-3290.
22. Acampora D, Mazan S, Avantaggiato V, Barone P, Tuorto F, Lallemand Y, Brulet P, Simeone A: **Epilepsy and brain abnormalities in mice lacking the *Otx1* gene.** *Nat Genet* 1996, **14:**218-222.
23. Acampora D, Avantaggiato V, Tuorto F, Barone P, Reichert H, Finkelstein R, Simeone A: **Murine *Otx1* and *Drosophila otd* genes share conserved genetic functions required in invertebrate and vertebrate brain development.** *Development* 1998, **125:**1691-1702.
24. Leuzinger S, Hirth F, Gerlich D, Acampora D, Simeone A, Gehring WJ, Finkelstein R, Furukubo-Tokunaga K, Reichert H: **Equivalence of the fly *orthodenticle* gene and the human *OTX* genes in embryonic brain development of *Drosophila*.** *Development* 1998, **125:**1703-1710.
25. Nagao T, Leuzinger S, Acampora D, Simeone A, Finkelstein R, Reichert H, Furukubo-Tokunaga K: **Developmental rescue of *Drosophila* cephalic defects by the human *Otx* genes.** *Proc Natl Acad Sci USA* 1998, **95:**3737-3742.
26. Boyl PP, Signore M, Acampora D, Martinez-Barbera JP, Ilengo C, Annino A, Corte G, Simeone A: **Forebrain and midbrain development requires epiblast-restricted Otx2 translational control mediated by its 3´ UTR.** *Development* 2001, **128:**2989-3000.
27. Reichert H, Simeone A: **Developmental genetic evidence for a monophyletic origin of the bilaterian brain.** *Phil Trans R Soc Lond B Biol Sci* 2001, **356:**1533-1544.

28. Boncinelli E, Morgan R: **Downstream of Otx2, or how to get a head.** *Trends Genet* 2001, **17:**633-636.
29. Leemans R, Egger B, Loop T, Kammermeier L, He H, Hartmann B, Certa U, Hirth F, Reichert H: **Quantitative transcript imaging in normal and heat-shocked *Drosophila* embryos by using high-density oligonucleotide arrays.** *Proc Natl Acad Sci USA* 2000, **97:**12138-12143.
30. Leemans R, Loop T, Egger B, He H, Kammermeier L, Hartmann B, Certa U, Reichert H, Hirth F: **Identification of candidate downstream genes for the homeodomain transcription factor Labial in *Drosophila* through oligonucleotide-array transcript imaging.** *Genome Biol* 2001, **2:**research0015.
31. Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H, Brown EL: **Expression monitoring by hybridization to high-density oligonucleotide arrays.** *Nat Biotechnol* 1996, **14:**1675-1680.
32. Lipshutz RJ, Fodor SP, Gingeras TR, Lockhart DJ: **High density synthetic oligonucleotide arrays.** *Nat Genet* 1999, **21:**20-24.
33. Rubin GM, Yandell MD, Wortman JR, Gabor Miklos GL, Nelson CR, Hariharan IK, Fortini ME, Li PW, Apweiler R, Fleischmann WEA: **Comparative genomics of the eukaryotes.** *Science* 2000, **287:**2204-2215.
34. Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, Scherer SE, Li PW, Hoskins RA, Galle RF, *et al.*: **The genome sequence of *Drosophila melanogaster*.** *Science* 2000, **287:**2185-2195.
35. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, *et al*: **Gene ontology: tool for the unification of biology. The Gene Ontology Consortium.** *Nat Genet* 2000, **25:**25-29.
36. Barbosa V, Yamamoto RR, Henderson DS, Glover DM: **Mutation of a Drosophila gamma tubulin ring complex subunit encoded by discs degenerate-4 differentially disrupts centrosomal protein localization.** *Genes Dev* 2000, **14:**3126-3139.
37. Lee JK, Coyne RS, Dubreuil RR, Goldstein LS, Branton D: **Cell shape and interaction defects in alpha-spectrin mutants of *Drosophila melanogaster*.** *J Cell Biol* 1993, **123:**1797-1809.
38. Heck MM, Pereira A, Pesavento P, Yannoni Y, Spradling AC, Goldstein LS: **The kinesin-like protein KLP61F is essential for mitosis in *Drosophila*.** *J Cell Biol* 1993, **123:**665-679.
39. Field CM, Alberts BM: **Anillin, a contractile ring protein that cycles from the nucleus to the cell cortex.** *J Cell Biol* 1995, **131:**165-178.
40. Zakin L, Reversade B, Virlon B, Rusniok C, Glaser P, Elalouf JM, Brulet P: **Gene expression profiles in normal and *Otx2-/-* early gastrulating mouse embryos.** *Proc Natl Acad Sci USA* 2000, **97:**14388-14393.
41. Wakimoto BT: **Beyond the nucleosome: epigenetic aspects of position-effect variegation in *Drosophila*.** *Cell* 1998, **93:**321-324.
42. Feger G: **Identification and complete cDNA sequence of the missing *Drosophila* MCMs: DmMCM3, DmMCM6 and DmMCM7.** *Gene* 1999, **227:**149-155.
43. Treisman JE: **A conserved blueprint for the eye?** *BioEssays* 1999, **21:**843-850.
44. Ledent V, Vervoort M: **The basic helix-loop-helix protein family: comparative genomics and phylogenetic analysis.** *Genome Res* 2001, **11:**754-770.
45. Regel R, Matioli SR, Terra WR: **Molecular adaptation of *Drosophila melanogaster* lysozymes to a digestive function.** *Insect Biochem Mol Biol* 1998, **28:**309-319.
46. Wallrath LL: **Unfolding the mysteries of heterochromatin.** *Curr Opin Genet Dev* 1998, **8:**147-153.
47. Su TT, O'Farrell PH: **Size control: cell proliferation does not equal growth.** *Curr Biol* 1998, **8:**R687-R689.
48. The FlyBase Consortium. **The FlyBase database of the *Drosophila* Genome Projects and community literature.** *Nucleic Acids Res* 1999, **27:**85-88.
49. Saitoe M, Schwarz TL, Umbach JA, Gundersen CB, Kidokoro Y: **Absence of junctional glutamate receptor clusters in *Drosophila* mutants lacking spontaneous transmitter release.** *Science* 2001, **293:**514-517.
50. Benos PV, Gatt MK, Murphy L, Harris D, Barrell B, Ferraz C, Vidal S, Brun C, Demaille J, Cadieu E, *et al*: **From first base: the sequence of the tip of the X chromosome of *Drosophila melanogaster*, a comparison of two sequencing strategies.** *Genome Res* 2001, **11:**710-730.
51. Crow JF: **Unmasking a cheating gene.** *Science* 1999, **283:**1651-1652.
52. Zimowska G, Aris JP, Paddy MR: **A *Drosophila* Tpr protein homolog is localized both in the extrachromosomal channel network and to nuclear pore complexes.** *J Cell Sci* 1997, **110:**927-944.
53. Bertram MJ, Pereira-Smith OM: **Conservation of the MORF4 related gene family: identification of a new chromo domain subfamily and novel protein motif.** *Gene* 2001, **266:**111-121.
54. Prokopenko SN, He Y, Lu Y, Bellen HJ: **Mutations affecting the development of the peripheral nervous system in *Drosophila*: a molecular screen for novel proteins.** *Genetics* 2000, **156:**1691-1715.
55. Dobie KW, Hari KL, Maggert KA, Karpen GH: **Centromere proteins and chromosome inheritance: a complex affair.** *Curr Opin Genet Dev* 1999, **9:**206-217.
56. Philip AV: **Mitotic sister-chromatid separation: what *Drosophila* mutants can tell us.** *Trends Cell Biol* 1998, **8:**150.
57. Kablar B, Vignali R, Menotti L, Pannese M, Andreazzoli M, Polo C, Giribaldi MG, Boncinelli E, Barsacchi G: ***Xotx* genes in the developing brain of *Xenopus laevis*.** *Mech Dev* 1996, **55:**145-158.
58. Nasiadka A, Krause HM: **Kinetic analysis of segmentation gene interactions in *Drosophila* embryos.** *Development* 1999, **126:**1515-1526.
59. Bate M, Martinez-Arias A: *The Development of* Drosophila Melanogaster. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1993.
60. Hoppler S, Bienz M: **Specification of a single cell type by a *Drosophila* homeotic gene.** *Cell* 1994, **76:**689-702.
61. Cribbs DL, Benassayag C, Randazzo FM, Kaufman TC: **Levels of homeotic protein function can determine developmental identity: evidence from low-level expression of the *Drosophila* homeotic gene *proboscipedia* under Hsp70 control.** *EMBO J* 1995, **14:**767-778.
62. Mann RS, Affolter M: **Hox proteins meet more partners.** *Curr Opin Genet Dev* 1998, **8:**423-429.
63. Hartmann B, Hirth F, Walldorf U, Reichert H: **Expression, regulation and function of the homeobox gene empty spiracles in brain and ventral nerve cord development of *Drosophila*.** *Mech Dev* 2000, **90:**143-153.
64. Yang X, Li DM, Chen W, Xu T: **Human homologue of *Drosophila* lats, LATS1, negatively regulate growth by inducing G(2)/M arrest or apoptosis.** *Oncogene* 2001, **20:**6516-6523.
65. Gunawardena S, Goldstein LS: **Disruption of axonal transport and neuronal viability by amyloid precursor protein mutations in *Drosophila*.** *Neuron* 2001, **32:**389-401.
66. Parkes TL, Elia AJ, Dickinson D, Hilliker AJ, Phillips JP, Boulianne GL: **Extension of *Drosophila* lifespan by overexpression of human SOD1 in motorneurons.** *Nat Genet* 1998, **19:**171-174.
67. Royet J, Finkelstein R: **Pattern formation in *Drosophila* head development: the role of the orthodenticle homeobox gene.** *Development* 1995, **121:**3561-3572.
68. Walter MF, Petersen NS, Biessmann H: **Heat shock causes the collapse of the intermediate filament cytoskeleton in *Drosophila* embryos.** *Dev Genet* 1990, **11:**270-279.
69. Tautz D, Pfeifle C: **A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene hunchback.** *Chromosoma* 1989**, 98:**81-85.
70. **NCBI Gene Expression Omnibus** [http://www.ncbi.nlm.nih.gov/geo/]