Abstract

**Background:** Members of TGFβ superfamily are found to play important roles in many cellular processes, such as proliferation, differentiation, development, apoptosis, and cancer. In *Drosophila*, there are seven ligands that function through combinations of three type I receptors and two type II receptors. These signals can be roughly grouped into two major TGFβ pathways, the *dpp*/BMP and activin pathways, which signal primarily through *thick veins* (*tkv*) and *baboon* (*babo*). Few downstream targets are known for either pathway, especially targets expressed in the *Drosophila* brain.

**Results:** *tkv* and *babo* both affect the growth of tissues, but have varying effects on patterning. We have identified targets for the *tkv* and *babo* pathways by employing microarray techniques using activated forms of the receptors expressed in the brain. In these experiments, we compare the similarities of target genes of these two pathways in the brain. About 500 of 13,500 examined genes changed expression at 95% confidence level (*P* < 0.05). Twenty-seven genes are co-regulated 1.5 fold by both the *tkv* and *babo* pathways. These regulated genes cluster into various functional groups such as DNA/RNA binding, signal transducers, enzymes, transcription regulators, and neuronal regulators. RNAi knockdown experiments of homologs of several of these genes show abnormal growth regulation, suggesting these genes may execute the growth properties of TGFβ.

**Conclusions:** Our genomic-wide microarray analysis has revealed common targets for the *tkv* and *babo* pathways and provided new insights into downstream effectors of two distinct TGFβ like pathways. Many of these genes are novel and several genes are implicated in growth control. Among the genes regulated by both pathways is *ultraspiracle*, which further connects TGFβ with neuronal remodeling.

**Background**

TGFβ pathways are conserved between primitive animals, such as sponges and sea anemone [1,2] and vertebrates, thus representing an ancient signal transduction pathway. In both vertebrates and invertebrates, TGFβ family members play fundamental roles in proliferation, pattern formation, apoptosis, and specification of cell fate. Mutations of various TGFβ signaling components are associated with human diseases including cancer [3].

---

Published: 08 October 2004

Received: 20 August 2004

Accepted: 08 October 2004

This article is available from: http://www.biomedcentral.com/1471-213X/4/14

© 2004 Yang et al; licensee BioMed Central Ltd.

This is an open-access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
In recent years, the core signaling components of the TGFβ pathways have been elucidated by a combination of genetics and biochemical approaches. Unique to these signaling pathways are transmembrane receptor serine-threonine kinases that are novel in animals. Signaling is initiated when dimeric ligands bind to the type I receptor or a complex of the type I and type II receptors. The type II receptor phosphorylates the type I receptor, which renders it active. R-Smads are phosphorylated by the type I receptor, the complex with a co-Smad, and translocate to the nucleus. Smads bind DNA promoter elements weakly and require co-factors for efficient regulation of target genes.

In Drosophila, seven ligands have been identified from the genomic sequence [4-6]. These ligands act through a receptor complex comprised of heterodimeric combinations of type I and type II receptors. Three type I receptors, thick veins (tkv), saxophone, baboon (babo) and two type II receptors, punt and wishful thinking (wit), interact with either of two R-Smads, mothers against dpp (mad) or dSmad2 [7-12]. Although different heteromeric combinations of receptors exist, in general, tkv transmits a dpp/BMP signal through mad, and babo transmits an activin signal through dSmad2.

The dpp and activin pathways have known functions in the brain, although our understanding of it role is rudimentary. dpp is expressed in two areas adjacent to the outer proliferation center (OPC), where it modulates wingless expression [13]. To acquire the adult pattern of projections, extensive remodeling occurs in neurons of the larval neural circuits during metamorphosis [14]. Proper neuronal remodeling is important for transformation of the larval mushroom bodies (MBs) to the adult MBs [15,16]. babo and dSmad2 (activin pathway components) are involved in neuronal remodeling, which occurs in the larval-pupal transition [17]. One target of the activin pathway identified in these studies is a subunit of the ecdysone receptor, EcR-B. Neuronal remodeling is essential for brain development in most animals and this result raises the question of possible conservation of neuronal targets in vertebrates.

In spite of intense study using classical genetic approaches and biochemical methods, very few targets of the pathway have been identified. A better understanding of the growth and patterning properties of the pathway require a more complete list of target genes. Using activated receptors (tkv and babo), we have used microarray technology to identify common targets of the BMP and activin pathways in the Drosophila brain.

Results and discussion

Identifying targets of babo and tkv in Drosophila brains

Few targets of tkv signaling and even fewer targets of babo signaling are known in Drosophila. Though multiple ligands and type II receptors may interact with these type I receptors, the use of ligand/receptor combinations is not yet established with certainty. However, in a simplified view, tkv and babo send dpp (BMP) and activin signals. These pathways and receptors are conserved through evolution, but few downstream targets are known for these pathways in any organism.

To learn more about the growth regulatory and patterning properties of these signals in the fly brain, we used microarray technology to identify downstream targets. In these experiments, Affymetrix™ chips containing the entire protein coding capacity of the Drosophila genome (about 13,500 genes) were screened. Genomic-wide microarray analysis allows us to examine similarities and differences between two signaling pathways in a tissue where both are known to function.

Constitutively active forms of the receptors were made by single amino acid substitutions [18], rendering them active in the absence of ligand. Transformants were generated which could be transcriptionally expressed using the heat shock GAL4 driver (hs-GAL4) [19,20]. To assay for the best induction protocol, animals were heat shocked and monitored for the presence of a UAS-gfp reporter. Since additional time is required for the induction of downstream signaling targets versus the time required for appearance of the GFP reporter, we collected RNA samples from third instar larvae in a broad time period roughly 30 minutes after the peak of GFP expression. Data resulting from induced ectopic expression of tkv, and babo were compared with each other and to the control (UAS-gfp; hs-GAL4).

Three independent replicates for each treatment were generated. Hierarchical clustering (Fig 1A) and Principal Component Analysis (Fig 1B) indicated that the microarray data is highly reproducible. Only transcripts that show an expression level above 1.5 fold change at significance values of P < 0.05 (Anova) were considered to be differentially expressed. These experiments identified genes that are either regulated by both tkv and babo pathways or by one of the pathways only.

To verify the differential expression levels in response to ectopic expression of tkv and babo on microarrays, semi-quantitative real-time RT-PCR was performed on selected genes. Six among the 27 genes were picked for validation. Real time RT-PCR showed similar results similar to the microarray results for four of the six genes (Fig 2). These PCR results are consistent with those of other reported microarray experiments [21,22]. This data, with the reproducibility of the individual samples analyzed, establish the validity of our microarray data and provide a comparison of two signaling pathways in the Drosophila brain.
Figure 1
Clustering of microarray data. A. Agglomerative Hierarchical Clustering of microarray data (P < 0.05, Group T, C, B represent individual samples with ectopic expression of tkv, control, and babo respectively); B. Principal Component Analysis (PCA) of microarray data (P < 0.05, Spheres in red, blue and yellow represent individual samples with ecotopic expression of babo, tkv, and control respectively).
Overview of gene expression following ectopic expression of babo and tkv

Upon ectopic expression of tkv, 91 transcripts are detected with differential expression values in brain tissues when compared with the control (Fig 3). This corresponds to about 0.7% of the transcripts on the array. More transcripts are down regulated (n = 60) than up regulated (n = 31) in abundance levels, indicating that ectopic expression of tkv causes both repression and activation of downstream genes. Induction of activated babo results in 216 genes with differential expression values in brain tissues. Interestingly, expression levels of more transcripts are decreased (n = 126) than increased (n = 90) (Fig 3). This corresponds to about 1.6% of the transcripts on the array. Most importantly, there are 27 genes co-regulated by induction of both babo and tkv – 17 of these transcripts are down regulated and 10 of them are up regulated (Fig. 3).

Role for TGFβ signaling in neuronal remodeling

The fact that both DPP and activin signaling pathways share some common features in differentiation and growth control in various tissues suggests that both pathways might share some downstream target genes. Microarray experiments identified 27 genes (Table 1) co-
regulated by the induced expression of both tkv and babo. Among these 27 co-regulated transcripts, there are transcription factors, enzymes, transporters, signal transducers, miscellaneous proteins and four unknown genes (Fig 4). The transcription factor ultrraspiracle (usp) gene has the highest expression level increase (8.1-fold for babo, 27.3-fold for tkv), which is a subunit of a nuclear receptor [15]. USP forms a heterodimer with the nuclear ecdysone receptor (EcR) and participates in neuronal remodeling [15,23].

Previous studies have shown that the Drosophila activin signaling pathway partially mediates neuronal remodeling through regulating EcR-B1 expression [17]. Two independent mutations that block neuronal remodeling in the mushroom bodies (MBs) during pupation were found to reside in babo and dSma2 [17], both of which have been shown to participate in the activin signaling pathway [7,9]. Further, mutations in these signaling components reduce the expression of EcR-B1, and restoration of EcR-B1 expression rescues neuronal remodeling defects. These observations led to the model that the Drosophila activin signaling results in induction of the EcR-B1 isoform. Upon binding of ecdyson to the EcR-B1/USP heterodimeric receptors, neuronal remodeling is initiated via transcriptional activation of downstream target genes [17]. Our microarray analysis shows that high level expression of usp is also induced by ectopic expression of tkv and babo. In addition, we find that EcR-A expression is repressed by the induction of babo. Using real-time PCR, we confirmed that EcR-B1 is induced by ectopic expression of babo (1.5 fold), a more modest change than the increases on usp by tkv and babo. These finding suggest that Drosophila activin signaling mediates neuronal remodeling by regulation of both EcR-B1 and usp expression, while inhibiting EcR-A induction.

BMP-like pathways, as well as activin pathways, have been implicated in neuronal remodeling [13,17]. PUNT and WTI have been shown to have a redundant function in inducing EcR-B1 expression during brain development. In mutant clones, levels of EcR-B1 were unaffected, unless both receptors were mutant. These results are consistent with our findings that activated tkv and babo both induce EcR-B1, although it is not known which receptor combinations or ligands are responsible for these effects.

dpp (and presumably tkv) has other known roles in organizing the visual center of the brain [13]. It has been shown that wingless, acting through dpp, is an important participant in organizing the optical centers of the brain [13]. wingless is expressed at the tips of the crescent shaped OPC. Fourteen hours later, wingless induces dpp expression in adjacent cells, in two spots in each brain hemisphere. These dpp expressing cells also express fasciclin II. BrdU staining shows that wingless, dpp, and fasciclin II expressing cells proliferate throughout larval development. However, a reduction of wingless or dpp results in a reduction in the rate of proliferation in the OPC, resulting in smaller optic lobes of the brain. Loss of wingless also results in a severe reduction of the medulla, where the photoreceptor axons R7 and R8 migrate. Another defect noted in wingless mutant animals is that the OPC derived precursor cells had failed to assume their proper neuronal fate.

**Transcription factors regulated by both DPP and activin pathways**

Besides usp, two other transcription factor genes, CG7839 and TIIIFβ, are up regulated by tkv and babo. Both are implicated in growth processes. CG7839 has 30% homology over 1016 residues to C. elegans F23B12.7, which shows a slow growing phenotype in RNAi experiments [24]. TIIIFβ is part of the RNA transcriptional machinery, and 28% of glioblastomas and 80% of astrocytomas show amplification of this gene. Perhaps part of the growth potential of the tkv and babo TGFβ pathways operate through these transcription factors.

Two transcription factors, CG14422 and Antennapedia (antp), are down regulated by both pathways during brain...
development. *antp* is a well-studied Hox gene in *Drosophila*, which controls many developmental decisions, most notably, the differentiation of the antennae and legs from homologous structures [25]. The enormous diversity of body plans in animals is partially due to the variations that Hox transcription factors regulate gene expression. Most animals have one or more clusters of Hox genes, and each Hox gene controls the development of a specific region of the body plan [26]. In *Drosophila*, differences between segments, such as the presence or absence of appendages, are often controlled by Hox transcription factors. The role of *antp* in brain development is not known, but it is tempting to speculate that both *dpp* and activin might regulate brain development, at least partially, through interaction with the Hox gene *antp*. Determining the mechanisms by which Hox proteins regulate gene expression will be important for understanding animal development and pattern formation.

### Table 1: Changes in transcript levels of the coregulated genes by both tkv and babo pathways after ectopic expression of tkv and babo. (*FC* represents the fold changes in gene expression levels between tkv/babo ectopic expression and control. Positive values indicate that the relative expression level of a gene is increased (upregulation) and negative values indicate a decrease (downregulation)).

| Gene/synonym | Signal | FC* | Molecular function | P      |
|--------------|--------|-----|--------------------|--------|
| **Transcription factors** |        |     |                    |        |
| usp          | 137 8 | 27.3| transcription factor, DNA binding, ligand-dependent nuclear receptor, ecdysteroid hormone receptor | 0.0001 |
| CG7839       | 34 1.7| 1.6 | transcription factor | 0.0007 |
| TllIfβ       | 249 1.2| 1.5 | RNA polymerase II transcription factor | 0.0000 |
| CG14422      | 12 -3.8| -2.9| RNA binding/nucleic acid binding/transcription regulator | 0.0115 |
| Antp         | 25 -1.5| -1.4| specific RNA polymerase II transcription factor | 0.0228 |
| **Enzymes and enzyme regulators** |        |     |                    |        |
| ia2          | 276 7.3| 5.8 | protein tyrosine phosphatase | 0.0000 |
| CG1827       | 26 2.3| 1.8 | N4-(beta-N-acetylglucosaminy1)-L-asparaginase | 0.0010 |
| ninaC        | 22 1.8| 1.9 | myosin ATPase, protein serine/threonine kinase | 0.0127 |
| G-ix65A      | 259 1.8| 1.4 | heterotrimeric G-protein GTPase | 0.0028 |
| Sucb         | 142 1.6| 1.5 | succinate-CoA ligase | 0.0096 |
| CG7288       | 125 1.5| 1.6 | ubiquitin-specific protease | 0.0040 |
| CG9813       | 69 1.1| 1.5 | peroxidase | 0.0000 |
| CG9236       | 5 2.6| 3.4 | calcium-dependent protein serine/threonine phosphatase | 0.0199 |
| **Transporters** |        |     |                    |        |
| CG8533       | 12 -2.6| -3.5| glutamate-gated ion channel | 0.0000 |
| CG6293       | 49 -2.5| -1.4 | L-ascorbate:sodium symporter | 0.0004 |
| Acpa         | 126 -1.5| -1.3 | sodium/potassium-exchanging ATPase | 0.0003 |
| Fatp         | 219 -1.4| -1.5 | long-chain fatty acid transporter | 0.0003 |
| **Signal transducers** |        |     |                    |        |
| usp          | 137 8 | 27.3| transcription factor, DNA binding, ligand-dependent nuclear receptor, ecdysteroid hormone receptor | 0.0001 |
| ninaC        | 22 1.8| 1.9 | myosin ATPase, serine/threonine kinase, calmodulin binding | 0.0127 |
| CG8533       | 12 -2.6| -3.5| glutamate-gated ion channel | 0.0000 |
| **Structural protein** |        |     |                    |        |
| CG144289     | 50 50 | -2.8| extracellular matrix/structural molecule | 0.0023 |
| **Miscellaneous proteins** |        |     |                    |        |
| CG2807       | 105 -2.3| -1.7 | pre-mRNA splicing factor | 0.0034 |
| CG32423      | 428 -2.1| -2.1 | RNA binding | 0.0000 |
| XRCC1        | 20 -1.4| -3.8 | DNA repair protein | 0.0000 |
| Cyp9f2       | 104 -1.5| -1.8 | cytochrome P450 | 0.0012 |
| Cyp9f3       | 70 -1.5| -1.7 | pseudogene | 0.0038 |
| **Unknown**  |        |     |                    |        |
| CG3857       | 186 -2.4| -3.0 | NA | 0.0000 |
| CG3855       | 117 -1.5| -1.7 | NA | 0.0009 |
| CG31150      | 69 -1.5| -1.4 | NA | 0.0160 |
| CG33187      | 125 -1.3| -1.6 | NA | 0.0035 |
Other genes regulated by tkv and babo pathways

Many of the other genes that are significantly regulated by tkv and babo are evolutionarily conserved throughout animal phyla. Quantitative analysis of transcript levels indicates that TGFβ controls some genes that encode kinases and phosphatases that might be involved in signaling pathways. For example, iα2, a transmembrane receptor protein phosphatase [27], has the highest level of transcriptional change among these kinases and phosphatases. Antibodies to the human version of the gene are often indicative of diabetes [28-30]. NinaC is a protein serine/threonine kinase [31] with calmodulin binding activity [32]. CG9236 is a calcium-dependent protein serine/threonine phosphatase, which is down regulated. It is strongly related to C. elegans F30A10.1, which is involved in negative regulation of body size. If the function of the protein has also been conserved, then down-regulation by the TGFβ-like pathways would allow growth in the developing brain. Other kinases and phosphatases co-regulated by both TGFβ pathways are G-ialpha65A (G-ialpha65A), a G-protein coupled receptor protein involved in neuroblast cell division and cell size control [33,34], and CG 9236, a calcium-dependent protein serine/threonine phosphatase [27].

CG3857 and CG7986 are two novel proteins that have homologs in C. elegans and in vertebrates. While their molecular functions are not currently known, the C. elegans CG7986 homolog F41E6.13 is involved in positive regulation of growth. RNAi experiments with the C. elegans homolog of CG3857, Y54E2A.2, revealed no mutant phenotype. Four transporters (Atpa, Fatp, CG8533, CG6293) are transporters regulated by the tkv and babo pathways. Fatp is a long-chain fatty acid transporter. Atpa is a sodium/potassium-exchanging ATPase, while CG8533 is a glutamate-gated ion channel and CG6293 is a L-ascorbate:sodium symporter.

Conclusions

Microarray experiments revealed that 27 genes are co-regulated in both tkv and babo signaling pathways in the developing Drosophila brain. One of the most striking developmental events in the fly brain is neuronal remodeling. These results indicate usp is positively regulated by
tkv and babo, and thus adds another important link to their roles in brain remodeling. Many of the 27 genes are strongly conserved in other species. If their biological functions are also conserved, then the RNAi experiments in their C. elegans counterparts show that several of them are involved in growth regulation. This is particularly useful since few downstream targets of BMP or activin signaling pathways are known, particularly the targets that execute their growth regulatory properties. Not surprisingly, mutational analysis of several of these genes has not been done, but the genetic tools in Drosophila make this relatively straightforward. Further characterization of these downstream genes may provide insights into the integration of tkv and babo signaling pathways in Drosophila brain development, and provide hints into their functions in other organisms.

**Methods**

**Fly stocks**

For over-expression of constitutively activated tkv, virgin females from UAS-CA-tkv were crossed to hs-Gal4 males. For over-expression of constitutively activated babo and the control, UAS-CA-babo and UAS-gfp were crossed to hs-Gal4 flies. The larvae were raised in standard medium at 25°C.

**Heat shock treatment and RNA purification**

Wandering third-instar larvae were heat-shocked to induce ectopic expression of tkv, babo, and the gfp control (UAS-gfp; hs-GAL4). Animals were heat shocked at 37°C for 1 hour, followed by cooling to room temperature for 30 minutes, and then kept at 25°C for one hour to allow expression before dissection. Approximately 150-200 larvae were dissected and the brains were collected in a drop of PBT (PBS, 0.01% Tween-20, pH 7.4) on Sylgard (Dow Corning). Total RNA was extracted from the tissue using the Trizol™ reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol.

**Preparation of labeled cRNA**

Total RNA from each of nine independent samples (three tkv, three babo and three gfp) was prepared for hybridization according to the Affymetrix GeneChip® Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). The Superscript Choice System kit (Invitrogen, Gaithersburg, MD) was used to make complementary DNA (cDNA) from 5 µg. First strand synthesis was primed with a 17-mer oligonucleotide primer containing a T7 RNA polymerase promoter sequence on the 5’ end (Genset Oligos, La Jolla, CA). Second strand products were cleaned with the GeneChip® Sample Cleanup Module (Affymetrix, Santa Clara, CA) and used as a template for in vitro transcription (IVT) with biotin-labeled nucleotides (Bioarray High Yield RNA Transcript Labeling Kit, Enzo Diagnostics, Farmindale, NY). The copy RNA (cRNA) product was cleaned with the GeneChip® Sample Cleanup Module (Affymetrix, Santa Clara, CA) and a 20 µg aliquot was heated at 94°C for 35 min in fragmentation buffer provided with the Cleanup Module (Affymetrix, Santa Clara, CA).

**Microarray hybridization**

Fifteen µg of adjusted cRNA from each sample was hybridized for 16 hr at 45°C to an Affymetrix (Santa Clara, CA) Drosophila GeneChip 1 array. After hybridization, each array was stained with a streptavidin-phycocerythrin conjugate (Molecular Probes, Eugene, Oregon), washed and visualized with a Genearray™ Scanner (Agilent Technologies, Palo Alto, CA). Images were inspected visually for hybridization artifacts. In addition, quality assessment metrics were generated for each scanned image and evaluated based on empirical data from previous hybridizations and on the signal intensity of internal standards that were present in the hybridization cocktail. Samples that did not pass quality assessment were eliminated from further analyses.

**Generation of expression values**

Microarray Suite version 5 (Affymetrix, Santa Clara, CA) was used to generate *.cel files. Probe Profiler™ version 1.3.11 software (Corimbia Inc, Berkeley, CA) was used to convert cel file intensity data into quantitative estimates of gene expression for each probe set. For each probe set, a probability statistic is generated. Genes not significantly expressed above background in any of the samples (P > 0.05) were considered absent. Absent genes were removed from the data set and not included in further analyses.

**Data analysis**

**Tests of Significance**

Gene expression levels were subjected to a 1-way analysis of variance (Anova) for 3 treatments (B, C, T) and 3 replicates using AnalyzeIt Tools, a custom software program developed by the Interdisciplinary Center for Biotechnology Research (ICBR, University of Florida), for the analysis of microarray data. In this software, the statistical package, R, serves as the backend for Anova. Genes were considered to have a significant treatment effect if P-level was less than 0.05.

The expression values of those genes that were considered to have a significant treatment effect were normalized by performing a Z-transformation [35], thereby generating a distribution with mean 0 and standard deviation of 1 for each gene. Hierarchical clustering, K-Means clustering and Principal Component Analysis were performed on normalized values using GeneLinker™ Gold 3.1 (Predictive Patterns, Kingston, Ontario).
To eliminate noise from low-level expression, spots quantified less than 5 were replaced by value 5. The following criteria were used to filter the data. Only transcripts with the fold change difference over 1.5 (tvl or babo) average/Control average or Control average/tvl or babo average) and statistically significant (P <= 0.05, analysis of variance (Anova)) were considered as differentially expressed. Analyzed Tools and notations in Flybase were used for classification of genes by gene ontology in molecular function and biological process categories.

**Real time RT-PCR**

Two independent total RNA samples were generated for each of the three experimental conditions (two tvl, two babo and two gfp). Each of the samples was analyzed three independent times, resulting in six repeats. These six repeats were averaged and the tvl and babo samples were compared with the gfp controls. Approximately 1 μg of the each total RNA was used for first strand cDNA reaction using Superscript First Strand Synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For real-time PCR, the reaction consisted of cDNA first strand template, primer mix, Rox (Invitrogen, Carlsbad, CA) and SYBR Green PCR Master Mix (Invitrogen, Carlsbad, CA) in a total volume of 25 μl. Three reactions per template were performed in parallel. Actin 42F was used as an internal standard to generate a standard curve and to normalize the amount of cDNA samples. The fold change (as presented in Fig 2) was calculated from the average real time PCR data: (tvl or babo) average/Control average or Control average/(tvl or babo) average. The experiments were performed using a Rotor Gene 3000 (Corbett Research, Sydney, Australia). To validate the specificity of PCR reaction, a melting curve was produced by denaturation of PCR end products from 60 to 99°C at 0.5°C/min steep and the end products were also assayed with 1.5% agarose gel electrophoresis after cycling.

**Authors' contributions**

MY and DN carried out experiments in the project and YF assisted in the experimental design. RWP and MY prepared the manuscript.

**Acknowledgements**

We thank Mick Popp and Li Liu of UFRCS (University of Florida) for microarray and data analysis. Fly stocks were received from the Bloomington Stock Center. We thank members of the Padgett lab for comments on the manuscript. MY was a Busch postdoctoral fellow and YF was a postdoctoral fellow for research abroad from Japan Society for Promotion of Science. This work was supported by grants from the National Institutes of Health to RWP.

**References**

1. Finnerty JR, Pang K, Burton P, Paulson D, Martindale MQ: Origins of bilateral symmetry: *Hox* and *Dpp* expression in a sea anemone. Science 2004, 304:1335-1337.

2. Suga H, Ono K, Miyata T: *Multiple TGF*-β receptor related genes in sponge and ancient gene duplications before the parazoan-eumetazoan split. FEBS Lett 1999, 453:129-133.

3. Derynck R, Akhurst R, Balmain A: TGFβ signaling in tumor suppression and cancer progression. Nat Genet 2001, 29:117-129.

4. Adams MD, Celniker SE, Holt RA, Lewis SE, Richardson S, Asbsburg M, Keegan SM, Sutton GG, Worman JH, Yandell MD, Zhang Q, Chen LX, Brandon RC, Rogers YH, Blazey RG, Champa M, Pfeffer BD, Wan KH, Doyle C, Baxter E, Gelt H, Nelson CR, Gabor GL, Abril JF, Agabayani A, Hj J, Andrews-Pfannkoch C, Baldwin D, Ballew RM, Basu A, Baxendale J, Bayraktaroglu L, Beasley EM, Beeson KY, Benos PV, Berman BP, Bhandari D, Bolskakov S, Borkova D, Botchan MR, Bouck J, Brooke PM, Broecker P, Burris KC, Busam DB, Butler H, Cadieu E, Center A, Chandra I, Cherry JM, Cawley S, Dahlke C, Davenport LB, Davies P, de Pablois B, Delcher A, Deng Z, Devices AD, Dew I, Dizot SM, Dodson K, Doup LE, Downes M, Doughty R, Dopp BC, Dure P, Durbin RJ, Evangelista CG, Ferraz C, Ferreira S, Fleischmann W, Fosler C, Gabrielle AE, Garn NS, Gelbhart WM, Glasser K, Gilead A, Gong F, Gorrell JH, Gu Z, Guan P, Harris M, Harris NL, Harvey D, Heiman TJ, Hernandez J, Houck J, Hostin D, Houston KA, Howland T, Wei MH, Ibegwam C, Jialai M, Kadom F, Karpen GH, Kjellmann JA, Kimmel BE, Kodira CD, Kraft K, Krazivitz S, Kulp D, Lai Z, Lasko P, Lei Y, Levitsky AA, Li J, Li Z, Liang Y, Lin X, Liu X, Matei B, McIntosh TC, McLeod MP, McPherson D, Mekulov G, Milishina VN, Moberry C, Morris J, Mostreffli A, Mount SM, Muy M, Murphy B, Murphy L, Musny DM, Nelson DL, Nelson DR, Nelson KA, Nixon K, Nusskern DR, Pacley JM, Palazzolo M, Pittman GS, Pan S, Pollard J, Puri V, Reese MG, Reineart K, Remington K, Saunders RD, Scheeler F, Shen H, Shue BC, Sidenkam P, Simpson M, Skupsik MP, Smith T, Spier E, Spradling AG, Stapleton M, Strong R, Sun E, Svirskas R, Tector C, Turner R, Venter E, Whew AH, Wang X, Wang ZY, Wassarman DA, Weinstock GM, Weissenbach J, Williams SM, Woodgate T, Worley KC, Wu D, Yang S, Yao QA, Ye J, Yeh RF, Zaveri JS, Zhan M, Zhang G, Zhao Q, Zheng L, Zheng XH, Zhong FN, Zhong W, Zhou X, Zhu S, Zhu X, Smith HO, Gibbs RA, Myers EW, Rubin GM, Venter JC: The genome sequence of *Drosophila melanogaster*. Science 2000, 287:2185-2195.

5. Parker L, Stathakis DG, Arora K: Regulation of BMP and activin signaling in *Drosophila*. Proc Natl Acad Sci USA 2004, 101:37-101.

6. Raftery LA, Sutherland DJ: *TGF*-β family signal transduction in *Drosophila* development: from Mad to Smads. Dev Biol 2001, 235:251-268.

7. Das P, Inoue H, Baker JC, Beppu H, Kawabata M, Harland RM, Miyazono K, Padgett RW: *Drosophila* dsmad2 and Atv-I transmit activin/TGFβ signals. Genes Dev 1999, 4:123-134.

8. Brummel TJ, Twombly V, Marques G, Wranala J, Newfeld SJ, Artisano L, Massague J, O’Connor MB, Gelhart WM: Characterization and relationship of Dpp receptors encoded by the saxophone and thick veins genes in *Drosophila*. Cell 1994, 78:251-261.

9. Brummel T, Abdullah S, Haery TE, Shimell MJ, Merriman J, Raferty L, Wranala JL, O’Connor MB: The *Drosophila* Activin receptor Baboon signals through dsmad2 and controls cell proliferation but not patterning during larval development. Genes Dev 1999, 13:98-111.

10. Xie T, Finelli AL, Padgett RW: The *Drosophila* saxophone gene: a serine-threonine kinase receptor of the TGF-β superfamily. Science 1994, 263:1756-1759.

11. Ruberte E, Marcy T, Nellen D, Affolter M, Basler K: An absolute requirement for both the type II and type I receptors, pum and thick veins, for dpp signaling in *Drosophila*. Cell 1992, 69:889-897.

12. Marques G, Bao H, Haery TE, Shimell MJ, Duchek P, Zhang B, O’Connor MB: The *Drosophila* BMP type II receptor Wishful Thinking regulates neuromuscular synapse morphology and function. Neuron 2003, 38:529-543.

13. Kaplingth K, Kunze S: Pattern formation in the visual centers of the *Drosophila* brain: wingless acts via decapentaplegic to specify the dorsalventral axis. Cell 1994, 78:437-448.

14. Truman JW: Metamorphosis of the central nervous system of *Drosophila*. J Neurobiol 1990, 21:1072-1084.

15. Lee T, Marticke S, Sung C, Robinow S, Luo L: Cell-autonomous requirement of the USP/Ecr-R edysone receptor for muscle body neuronal remodeling in *Drosophila*. Neuron 2000, 28:807-818.
16. Lee T, Lee A, Luo L: Development of the Drosophila mushroom bodies: sequential generation of three distinct types of neuron from a neuroblast. Development 1999, 126:4065-4076.
17. Zheng X, Wang J, Haery TE, Wu AY, Martin J, O'Connor MB, Lee CH, Lee T: TGFβ signaling activates steroid hormone receptor expression during neuronal remodeling in the Drosophila brain. Cell 2003, 112:303-315.

Wieser R, Wrana JL, Massague J: GS domain mutations that constitutively activate TβRI, the downstream signaling component in the TGF-β receptor complex. Embo J 1995, 14:2199-2208.
19. Brand AH, Perrimon N: Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 1993, 118:401-415.
20. D’Avino PP, Thummel CS: Ectopic expression systems in Drosophila. Methods Enzymol 1999, 306:129-142.
21. Leemans R, Egger B, Loop T, Kammermeier L, He H, Hurtmann B, Cerra 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 U S A 2000, 97:12138-12143.
22. Zinke I, Schutz CS, Katzenberger JD, Bauer M, Pankratz MJ: Nutrient control of gene expression in Drosophila: microarray analysis of starvation and sugar-dependent response. Embo J 2002, 21:6162-6173.
23. Yao TP, Segraves WA, Oro AE, McKeown M, Evans RM: Drosophila ultraspireacle modulates ecdysone receptor function via heterodimer formation. Cell 1992, 71:63-72.
24. Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, Kanapi N, Le Bot N, Moreno S, Sohrmann M, Welchman DP, Zipperlen P, Ahringer J: Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature 2003, 421:231-237.
25. Casares F, Mann RS: Control of antenvers versus leg development in Drosophila. Nature 1998, 392:723-726.
26. McGinnis W, Krumlauf R: Homeobox genes and axial patterning. Cell 1992, 68:283-302.
27. The FlyBase Consortium: The FlyBase database of the Drosophila genome projects and community literature. Nucleic Acids Res 2003, 31:172-175.
28. Bonifacio E, Lampasona V, Genovese S, Ferrari M, Bosi E: Identification of protein tyrosine phosphatase-like I2A (islet cell antigen 512) as the insulin-dependent diabetes-related 37/40K autoantigen and a target of islet-cell antibodies. J Immunol 1995, 155:5419-5426.
29. Borg H, Fernlund P, Sundkvist G: Protein tyrosine phosphatase-like protein I2A-antibodies plus glutamic acid decarboxylase 65 antibodies (GADA) indicates autoimmunity as frequently as islet cell antibodies assay in children with recently diagnosed diabetes mellitus. Clin Chem 1997, 43:2358-2363.
30. Morahan G, Huang D, Yu WP, Cui L, DeAizpurua H, Pallen CJ: Localization of the genes encoding the type I diabetes autoantigens, protein-tyrosine phosphatases I2A and IAR. Mamm Genome 1998, 9:593-594.
31. Ng KP, Kambara T, Matsumura M, Burke M, Ikebe M: Identification of myosin III as a protein kinase. Biochemistry 1996, 35:9392-9399.
32. Porter JA, Minke B, Montell C: Calmodulin binding to Drosophila NinaC required for termination of phototransduction. Embo J 1995, 14:4450-4459.
33. Cai Y, Yu F, Lin S, Chia W, Yang X: Apical complex genes control mitotic spindle geometry and relative size of daughter cells in Drosophila neuroblast and pl asymmetric divisions. Cell 2003, 112:51-62.
34. Wolfgang WJ, Quan F, Goldsmith P, Unson C, Spiegel A, Forte M: Immunolocalization of G protein alpha-subunits in the Drosophila CNS. J Neurosci 1990, 10:1014-1024.
35. Draghičić S, Khazri P, Martins RP, Ostermeier GC, Krawetz SA: Global functional profiling of gene expression. Genomics 2003, 81:98-104.