Preface

Developmental biology is one of the most exciting and fast-growing fields today. In part, this is so because the subject matter deals with the innately fascinating biological events—changes in form, structure, and function of the organism. The other reason for much of the excitement in developmental biology is that the field has truly become the unifying melting pot of biology, and provides a framework that integrates anatomy, physiology, genetics, biochemistry, and cellular and molecular biology, as well as evolutionary biology. No longer is the study of embryonic development merely "embryology." In fact, development biology has produced important paradigms for both basic and clinical biomedical sciences alike.

Although modern developmental biology has its roots in "experimental embryology" and the even more classical "chemical embryology," the recent explosive and remarkable advances in developmental biology are critically linked to the advent of the "cellular and molecular biology revolution." The impressive arsenal of experimental and analytical tools derived from cell and molecular biology, which promise to continue to expand, together with the exponentially developing sophistication in functional imaging and information technologies, guarantee that the study of the developing embryo will contribute one of the most captivating areas of biological research in the next millennium.

There is a demonstrated need for students of developmental biology to be knowledgeable of the breadth and depth of the available experimental methodologies, by necessity derived from multiple disciplines, which are applicable to the study of the developing embryo. In particular, because developmental biology deals with multiple model systems, from organismal to tissue and cell levels, as well as a wide range of "change"-related biological activities, the investigator is often frustrated as to how his/her findings relate to those obtained in another model system and/or by using different reagents or functional markers. Compared to other more strictly defined fields of biological research, the number of "reference" publications that deal specifically with the practical aspects of experimental developmental biology are, however, relatively scarce.

Developmental Biology Protocols grows out of the need for a comprehensive laboratory manual that provides the readers the principles, background, rationale, as well as the practical protocols, for studying and analyzing the events of embryonic development. This three-volume set, consisting of 142 chapters, is intentionally broadin scope, because of the nature of modern developmental biology. Information is grouped into the following topics: (1) systems—production, culture, and storage; (2) developmental pattern and morphogenesis; (3) embryo structure and function; (4) cell lineage analysis; (5) chimeras; (6) experimental manipulation of embryos; (7) application of viral vectors; (8) organogenesis; (9) abnormal development and teratology; (10) screening and mapping of novel genes and mutations; (11) transgenesis production and gene knockout; (12) manipulation of developmental gene expression and function; (13)

analysis of gene expression; (14) models of morphogenesis and development; and (15) in vitro models and analysis of differentiation and development.

Throughout *Developmental Biology Protocols*, the authors have consistently striven for a balanced presentation of both background information and actual laboratory details. It is believed that this highly practical format will permit readers to bring the concepts and principles we present into their personal research practices in a most efficient manner. Specifically, the wide range of model systems and multidisciplinary experimental techniques presented here should lower the "activation energy" for the student of developmental biology to become a contributing member of this exciting scientific discipline. In addition, teachers of developmental biology at all levels should also readily find relevant and useful information to enrich the experience of their students.

The practice of developmental biology is currently in a state of constant change, reflecting the close relationship of the field to other rapidly developing fields of biological research, particularly cell and molecular biology, and imaging and information technology. The materials presented in this three-volume set are therefore the beginning of a project that will involve continuous update and upgrade to reach and enhance the scientific endeavors of developmental biologists at large.

The production of *Developmental Biology Protocols* would not have been possible without the outstanding work of the contributing authors who share here with the readers the hands-on wisdom they have earned in the laboratory. We are grateful for their intellectual contributions as well as their remarkable tolerance to our constant reminders. Tom Lanigan and his staff at Humana Press worked diligently on the project to ensure a final product of the highest quality. Chuck, our young son, persevered throughout the gestation period of the project, and constantly demonstrated to us the meaning of "developmental biology."

Our final, heartfelt thanks go to Lynn Stierle, who expertly and singlehandedly maintained the massive organization of the manuscripts and the correspondence (snail-mail and e-mail), as well as the sanity of the editors! Michelle Levinski also provided valuable assistance in proofreading the final production.

Finally, we hope that these volumes will find their place on the laboratory shelves, with their pages well soiled and their contents tried and tested, and prove their utility as an everyday resource for the students of developmental biology, the most exciting discipline of biology for many decades to come!

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2

Drosophila as a Genetic Tool to Define Vertebrate Pathway Players

Nancy M. Bonini

I. Introduction

In many instances, the strength of *Drosophila melanogaster* genetics can be used to enhance our understanding of complex vertebrate signaling systems. The general success of this approach is underscored by the large number of vertebrate signaling components whose very names derive in part from the names of *Drosophila* mutants. Examples include the vertebrate pathway components *Sonic Hedgehog*, *Son of Sevenless*, *Lunatic Fringe*, *Notch*, the SMAD family of transforming growth factor-β (TGF-β) signaling, and many others. Given the powerful genetics of *Drosophila melanogaster* (*see* **ref.** ***1***), it can be of interest to test functional equivalence of vertebrate homologs with fly genetic pathway components, or to re-create in *Drosophila* transgenic models for vertebrate or human gene function. If such complementation can be established, then the strength of *Drosophila* genetics can be brought to bear on defining additional components of the particular pathway of interest; for example, through enhancer and suppressor screens. Subsequently, one can then clone such modifier genes from *Drosophila*, as a springboard from which to identify their vertebrate counterparts. To establish a genetic model for a vertebrate gene function in *Drosophila*, there are a number of considerations with respect to expressing foreign genes in the fly, establishing whether and how the foreign proteins function, and using the transgenic lines in genetic screens.

Examples of functional complementation in flies with vertebrate genes include the ability of domains of human bone morphogenetic protein to substitute in the related fly protein Dpp ***(2)***, effects of vertebrate *fringe* homologs to establish boundaries like the fly gene ***(3)***, functional complementation of *orthodenticle* homeobox gene homologs ***(4–6)***, and functional complementation of mammalian counterparts of eye determination genes *eyeless* and *eyes absent* ***(7,8)***. In addition, dominant effects can be generated in flies with vertebrate genes, such as phenocopying fly homeotic mutants with the appropriate vertebrate *Hox* homologs ***(9,10)***, and generating genetically tractable human disease models by expressing mutant human disease proteins in flies ***(11)***.

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2. Experimental Approaches

2.1. Design of Constructs for Transformation

As noted, there are a number of examples of expressing vertebrate cDNA counterparts of a particular gene of interest in flies. In general, it appears that simply taking a human, mouse, or other vertebrate cDNA and expressing it in flies will usually generate a functional protein. Thus, it has not proven necessary to be overly concerned about possible differential codon usage from flies to vertebrates. However, because the insect body plan is quite different from the typical mammalian body plan, gene regulatory sequences cannot be transferred between species so easily (but *see* **refs. *10*** and ***12***). Consequently, care must be taking to select an expression system compatible with *Drosophila* to achieve adequate protein levels in the relevant tissues, as discussed below.

An important consideration is the ability to detect the foreign protein when expressed in flies, especially when one considers that the *Drosophila* genome displays position effects such that some transgenic insertions will express at higher levels than others as a result of the location in which the transgene has inserted in the genome ***(13)***. Transformation vectors can include insulators, such that the transgene will be much less sensitive to genomic position effects ***(14)***. Otherwise, it is typically necessary to generate a number of different transgenic lines in order to obtain a sufficient number with strong expression; a minimum estimate is about four lines. Having different lines that express at weak, moderate, or strong levels to give a weak, moderate, or strong phenotype, respectively, can be of benefit, however, especially when performing genetic screens for modification of the phenotype (e.g., *see* **refs. *15–17***). If the experiments require the construction of a large number of genetic stocks, having the ability to detect expression of the foreign protein can be extremely valuable to allow selection of strongly expressing transgenic lines. In addition, if a phenotype is not observed, unless transgene expression can be monitored directly, it might be difficult to distinguish whether the foreign protein does not function in flies or if there is simply a technical problem with expression.

It is of course possible to use *in situ* hybridization to detect expression of the transcript for the transgene. A disadvantage of this approach is that it is, in general, more laborious than detecting protein expression and, moreover, does not indicate whether the protein is being translated appropriately in the fly. In some cases, an antibody to the foreign protein may already be available; one can then test for crossreactivity to potential fly counterparts to determine the utility and limitations of the antibody. When testing for antibody crossreactivity, it is frequently necessary to preadsorb an antibody against fixed fly tissue (e.g., a 1:10 antibody dilution preadsorbed with 50 µL of 4% paraformaldehyde-fixed, dechorionated, devitellinized embryos) to lower potential background crossreactivity. This is particularly necessary for rabbit antisera, which are notorious for giving a high background on fly tissue. It is also necessary to determine whether the antibody to the vertebrate counterpart crossreacts to the fly counterpart; if so, one must be able to distinguish expression of the vertebrate counterpart from the fly gene by some other means, such as expression in a novel tissue where the fly gene is not normally expressed or by tagging the vertebrate protein with a peptide domain to which antibodies are available.

An alternative approach is to tag the foreign protein with a small peptide sequence for which antibodies are available. Examples include FLAG, c-Myc, and hemaglutinin

(HA), for which antibodies can be purchased commercially; alternatively, fusion to a protein with endogenous fluorescence, such as green fluorescent protein (GFP) or one of its derivatives, can be used. If electron microscopy is ultimately of interest, then a glutaraldehyde-resistant epitope tag is particularly useful, as many antibodies lose reactivity to tissue treated with glutaraldehyde [although we have successfully performed immunoelectron microscopy with HA-tagged protein ***(11)***]. Such epitope tags can be added by polymerase chain reaction (PCR) or by subcloning into various commercially available vectors which have these epitopes upstream of a number of convenient restriction enzyme sites. We have added HA and GFP to the N- or C-terminus of a number of proteins successfully ***(18)***. Frequently, we add HA to the C-terminus using PCR. To do this, we design a C-terminal primer which deletes the stop codon and adds a linker of a few small amino acids (glycine and alanine, plus a convenient restriction site), followed by the HA sequence and a stop codon. One can also multimerize the exogenous tag (3–5×) to boost sensitivity of detection ***(19)***. If the tag is at the C-terminus, then one can be assured that the entire protein is being produced if the introduced protein can be detected with the relevant antibody. Alternatively, Western immunoblotting can be used to confirm the synthesis of a protein of the appropriate size.

2.2. Expression Systems

There are a number of different expression systems available, the simplest of which couples a standard transformation vector with an appropriate promoter. Such a promoter may be conditional, such as a heat-shock promoter which is inducible by heat pulsing the animal at 37°C for a short time ***(20)***. Alternatively, it may be a constitutive promoter expressed in a tissue of interest, such as actin or ubiquitin which will be expressed in most cells of the animal ***(21)***, or a promoter that targets gene expression to a particular tissue, such as the *gmr (glass multiple reporter)* or *sevenless* promoter elements which target gene expression to developing eye cells ***(22–24)***. Such constructs have the advantages of simplicity and, depending on the promoter used, yield a transgenic line with a constant and consistent phenotype. Conditional promoters allow one to express the protein at any desired time; however, in general, expression will vary over time (although the heat-shock promoter can give a constant basal level of expression at normal growth temperatures, depending on insertion site, which can be sufficient for a phenotype at normal growth temperatures [e.g., **ref. *25***]). If one suspects that ubiquitous or early expression of the protein may be lethal to the animal, then conditional or tissue-restricted expression is essential.

Another approach is a two-component system, the GAL4-UAS system ***(26)***. In this system, the gene of interest is cloned downstream of the yeast UAS–GAL4 DNA-binding regulatory sequences in a fly transformation vector pUAST, and transgenic lines are generated. Then, upon crossing the transgenic line to any of a large collection of fly lines that express GAL4 in tissue-specific patterns, one can express the gene of interest in different tissues at different times of development. One advantage of this system is versatility, as there are many GAL4 lines with different expression patterns available from *Drosophila* stock centers or research laboratories. In this system, a *UAS–lacZ* tester strain can be used to monitor promoter strength and tissue-specific expression of the GAL4 lines being used. In addition, an advantage is the ease of determining the viability or other features of the phenotype—even if expressing the protein widely is

lethal, one may be able to obtain transgenic lines because expression is only induced when the transgenic line is crossed to a GAL4 expression line. Conversely, the fact that crosses must be made in order to express the transgene represents a disadvantage of the GAL4–UAS system. Furthermore, the double-insert line of interest is not of itself stable unless one takes the trouble to generate an appropriate stable recombinant fly line. This requirement can become particularly unwieldy when testing the phenotype of a foreign gene in a fly mutant background—performing a single experiment can require many crosses to assemble a complex combination of mutant alleles and transgenic constructs. Again, one must consider the different potential uses of the transgenic line in the long run to determine which approach or approaches will be best suited for the experiments.

2.3. Testing for Function

There are a number of ways to test for function of a foreign protein in transgenic *Drosophila*. If testing homologs of a known fly gene for which mutants exist, then one test for function is ability of the foreign gene to rescue the fly mutant phenotype. If the fly counterpart has dominant effects or if one might expect dominant effects as a result of the function of the protein in vertebrates (such as for a dominant oncogene or disease gene), then another test is to determine whether the vertebrate homolog can induce similar dominant phenotypes in flies. There are examples of dominant oncogenic mutations leading to a form of the protein that also functions dominantly in the fly ***(27–33)***.

In some cases, expression of vertebrate genes in flies has demonstrated that a conserved function of the vertebrate and fly genes is autoregulation; thus, the vertebrate protein (frequently a transcription factor) turns on expression of the endogenous fly counterpart ***(9,34)***. If one has mutants in the fly gene involved, then it is possible to test for functional conservation in the genetic background of a protein null of the fly gene and, hence, address broader aspects of functional conservation (e.g., **ref. *5***).

When expressing a foreign gene in the fly in a tissue that normally does not express any such gene, one must consider if screens to identify interacting proteins will be useful for understanding the function of the gene in its normal cellular context. It is important to assess whether any phenotypic effects observed in the fly accurately reflect conserved functions of the vertebrate protein under scrutiny. For example, will vertebrate anti-apoptotic genes block *Drosophila* programmed cell death? Will the vertebrate homolog, like its fly counterpart, direct ectopic tissue formation in the fly? If the vertebrate cDNA induces a dominant effect, is that effect the result of elevated levels of a normal activity of the protein (a hypermorphic effect) or of a new activity of the protein that may have little to do with its normal function (a neomorphic effect). Neomorphic effects, for example, might be the result of subcellular mislocalization of the vertebrate protein in the fly. To what degree does the pathology of a human disease gene reflect biological effects known to occur in humans or vertebrate models, and can these effects be faithfully replicated in the fly model? These are, of course, specific issues that vary for any one gene of interest, and they are critical to consider.

2.4. Genetic Screens for Modifier Mutations

A major goal of expressing a foreign protein in flies is to be able to apply *Drosophila* genetics to further understand the biological problem. The basic idea is to find mutations in fly genes that enhance or suppress the phenotype, and use these mutations

to identify vertebrate genes that function in the same pathways or biological process. By this means, one can, therefore, define additional genes that elucidate or indirectly influence the biological pathway of interest.

There are two general approaches for identifying modifier mutations: (1) to screen collections of existing mutations or deficiencies to define interacting genes and (2) do a *de novo* mutagenesis in flies to define interacting genes. Usually, both approaches are performed as screens for dominantly modifying mutations on the autosomes and recessive or dominant mutations on the X chromosome. These screens allow direct analysis of modifying effects in the progeny of mass fly matings, enabling a large number of potential mutants to be rapidly screened relative to other methods.

One approach is to look for enhancers or suppressors by crossing the flies bearing the foreign gene of interest to a collection of *Drosophila* deficiency chromosomes. This collection, available from the Bloomington *Drosophila* Stock Center, consists of about 190 fly lines, which uncover, in total, approximately 70–80% of the *Drosophila* euchromatin. By this approach, one searches for regions of the chromosomes that harbor genes that, when reduced in dosage by 50%, will modify the phenotype of interest. Thus, to test all regions of the genome uncovered by available deficiencies, one simply performs fly crosses and examines the resulting progeny flies. Once a deficiency region of interest is found, then the genetic interaction can be confirmed and the cytological region of the chromosome narrowed down as much as possible using smaller available deficiencies. Eventually, one can test for interactions with all available known mutations in the region and/or perform a mutagenesis to define genes in the region. Hay et al. ***(15)*** have successfully used this strategy to identify a conserved gene that is involved in programmed cell death pathways.

A disadvantage of this technique is that the deficiency lines tend to show variable genetic background effects; that is, it is difficult to determine whether any observed effect on the phenotype of interest is the result of the deficiency itself or to the fact that the cross is made between nonisogenic fly lines. Thus, the success of the approach can depend on the strength and variability of the phenotype being modified. If the modifier effect is very strong, then this approach can be quite successful; however, if the modifier effect is subtle, then it can be difficult to distinguish modification of the phenotype in the widely variable backgrounds of the deficiency lines. Another disadvantage is that eventually after narrowing down a region to the smallest possible extent, it may still be necessary to perform many molecular biological manipulations before having a defined gene in hand.

A variation on this approach is to look for modifier mutations among the large collection of P-element-induced mutation lines ***(36,37)***. Should an interaction be found by using P-element-induced mutations to look for dosage-sensitive modifier interactions, then the gene can easily be cloned if the P element has inserted into it or nearby. In addition, some of the P lethals have been generated using reporter gene constructs, such that one can stain the line for the reporter gene expression (β-galactosidase), which may reveal interesting aspects of the expression pattern of the potentially interacting gene. However, the P-element-induced mutations have a similar background problem as the deficiency lines, which, again, can often be too variable in practice to make such a screen successful (e.g., *see* **ref. *17***).

In general, both deficiencies and the P lethals test for the same type of interaction: an interaction resulting from reduction of a gene dosage by 50%. An alternative approach

is to use a point mutagen (ethyl methanesulfonate [EMS] is commonly used in *Drosophila*) to identify dominantly interacting mutations. With a point mutagen, one can obtain both loss-of-function mutations that reduce gene function and gain-of-function mutations resulting from single amino acid substitution in a critical region of the interacting protein. Thus, using EMS as a mutagen may select for different types of interacting mutations than a deficiency or P-element lethal screen. X-ray mutagenesis can also be of interest, because X-rays will, in general, produce chromosomal rearrangements that can affect very large genes or gene complexes as well as result in gain-of-function mutations, depending on the particular rearrangement (*see* **ref. *17***). Most crucially, by doing such a mutagenesis, one has greater control over the genetic background: one can select an isogenic background that, when crossed to the line of interest, gives a uniform phenotype such that the effect of any modifier interaction will be readily seen. Such an approach has proven successful for a number of different types of modifier screens ***(16,17)***.

By any of these approaches, the real challenge comes in the analysis of the modifiers obtained to identify those that are most interesting with respect to the question of interest. In all of these approaches, it is essential to have good controls to eliminate modifiers that interact with the expression system or the promoter expressing the gene rather than with the protein being expressed, and so forth. Thus, secondary screens are critical to classify mutants to distinguish those modifiers more directly involved in the question of interest, from those that are only peripherally involved. An excellent example of this is **ref. *38***, where 30,000 mutagenized chromosomes were screened for modification of a *sevenless* receptor tyrosine kinase mutant phenotype. Of seven complementation groups identified, four of seven also modified the mutant phenotype of a second tyrosine kinase receptor (the EGF receptor), thus defining those genes that were common signaling components of receptor tyrosine kinase pathways. Some argue that the best approach is to do different types of modifier screens and then focus on those subsets of new genes that are repeatedly identified in multiple screens, indicating that they are likely to be centrally important in the biological pathway of interest.

By these means, one can apply the ease and rapidity of genetics in a simple model system like *Drosophila* to questions of fundamental interest and importance in vertebrates. With the advent of genomic sequencing, the importance of model systems like *Drosophila* to reveal protein function and define biological pathways becomes ever more important.

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