3 Model Organisms in the Study of Development and Disease

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The past two decades have brought major breakthroughs in our understanding of the molecular and genetic circuits that control a myriad of developmental events in vertebrates and invertebrates. These detailed studies have revealed surprisingly deep similarities in the mechanisms underlying developmental processes across a wide range of bilaterally symmetric metazoans (bilateralia). Such phylogenetic comparisons have defined a common core of genetic pathways guiding development and have made it possible to reconstruct many features of the most recent common ancestor of all bilateral animals, which most likely lived 600–800 million years ago (Shubin et al., 1997; Knoll and Carroll, 1999). As flushed out in more detail below and reiterated as a major unifying theme throughout the book, the common metazoan ancestor already had in place many of the genetic pathways that are present in modern-day vertebrates and invertebrates. This ancestor can be imagined as an advanced worm-like or primitive shrimp-like creature which had a few distinct body specializations along the nose-to-tail axis and was subdivided into three distinct germ layers (ectoderm, mesoderm, and endoderm). It also had evolved an inductive signaling system to partition the ectoderm into neural versus nonneural components and is likely to have possessed appendages or outgrowths from its body wall with defined anterior–posterior, dorsal–ventral, and proximo–distal axes, as well as light-sensitive organs, a sensory system for detecting vibrations, a rudimentary heart, a molecular guidance system for initiating axon outgrowth to the midline of the nervous system, ion channels for conducting electrical impulses, synaptic machinery required for neural transmission, trachea, germ cells, and an innate immune system.

The fact that the ancestor of vertebrate and invertebrate model organisms was a highly evolved creature which had already invented complex interacting systems controlling development, physiology, and behavior has profound implications for medical genetics. The central points that we explore in this chapter can be broadly put into two categories: *(1)* the great advantages of model organisms for identifying and understanding genes that are altered in heritable human diseases and *(2)* the functions of many of those genes and the evidence that they were present in the ancestral bilateral organisms and have remained largely intact in both vertebrate and invertebrate lineages during the ensuing course of evolution. In the course of discussing these points, we review the compelling evidence that developmentally important genes have been phylogenetically conserved and the likelihood that developmental disorders in humans will often involve genes controlling similar morphogenetic processes in vertebrates and invertebrates. A systematic analysis of human disease gene homologs in *Drosophila* supports this view since 75% of human disease genes are structurally related to genes present in *Drosophila* and more than a third of these human genes are highly related to their fruit fly counterparts (Bernards and Hariharan, 2001; Reiter et al., 2001; Chien et al., 2002).

Since its inception, the field of human genetics has focused on the identification of genes that, as single entities, can cause disease when mutated. The discovery of such new disease genes has advanced at an accelerating pace in the last decade, and the rate is now over 175 genes per year (Peltonen and McKusick, 2001). This rate is likely to accelerate even further in the near term because of the sequencing of human genome. Most of the 4000–5000 estimated human disease genes should be identified before long. In anticipation of this asymptotic discovery process, the emphasis in human genetics is shifting to understanding the function of these disease genes. An obvious avenue for functional analysis of disease genes is to study them in the closely related mouse using gene knockout techniques to assess the effects of either eliminating the gene's function or inducing specific disease-causing mutations. In some cases, this type of analysis has resulted in excellent mouse models for diseases that have phenotypes very similar to human diseases. In other cases, mouse knockout mutations have been less informative than hoped, either because the greater genetic redundancy in vertebrates masks the effect of mutations in single genes or because the mutations of interest are lethal at an early embryonic stage. Since there are limitations to the mouse system and there are deep ancestrally derived commonalities in the body plan organization and physiology of vertebrate and invertebrate model organisms, particularly flies and nematodes for which there are well-developed and powerful molecular genetic tools, these organisms are likely to play an increasingly important role in the functional analysis of human disease genes. This chapter also compares the strengths and weaknesses of several well-developed model systems, ranging from single-cell eukaryotes to primates, as tools for dissecting the function of human disease genes. We propose that multiple model systems can be employed in cross-genomic analysis of human disease genes to address different kinds of issues, such as basic eukaryotic cellular functions (e.g., yeast and slime molds), assembly of genes into various types of molecular machines and pathways (e.g., flies and nematodes), and accurate models of human disease processes (e.g., vertebrates such as zebrafish and mice).

MODEL ORGANISMS: ADVANTAGES AND LIMITATIONS OF THE VARIOUS SYSTEMS

In this section, we consider the strengths and limitations of several well-studied model organisms with regard to the analysis of human genetic disorders (see Table 3–1). In general, several model systems can be used to analyze the function of a given human disease gene. Unicellular organisms such as yeast (*Saccharomyces*) (Foury, 1997) and the facultatively colonial slime mold (*Dictyostelium*) (Firtel and Chung, 2000; Chung et al., 2001) can be used to analyze phenomena that involve important basic eukaryotic cell functions, such as metabolism, regulation of the cell cycle, membrane targeting and dynamics, protein folding, and DNA repair. Simple invertebrate systems such as *Drosophila* (Bernards and Hariharan, 2001; Reiter et al., 2001; Chien et al., 2002) or *Caenorhabditis elegans* (Aboobaker and Blaxter, 2000; Culetto and Sattelle, 2000) are excellent models for examining the coordinated actions of genes that function as components of a common molecular machine such as a signal-transduction pathway or a complex of physically interacting proteins. These proteins may or may not have highly related sequences in yeast, but if so, the value of the invertebrate system would be most pronounced if the human disease condition involved a tissue-specific requirement for the protein in question (e.g.. metabolic disorders resulting in neurological phenotypes). In contrast, mammalian systems such as the mouse (Benavides and Guenet, 2001), zebrafish (Barut and Zon, 2000; Dooley and Zon, 2000), frog, and chicken and to some extent more complex invertebrates (e.g., echinoderms and primitive chordates) are most likely to provide accurate models for the human disease state, which can be used to assess various strategies for intervening in the disease process.

Table 3–1. Strengths and Limitations of Various Model Organisms

Species	Experimental Advantages	Experimental Limitations
Yeast	Excellent genetics Very powerful second site screening Powerful molecular techniques Genes can be easily cloned Genome sequence complete Possess all basic eukaryotic cell organelles Cell cycle control similar to animals	No distinct tissues
Slime mold	Excellent genetics Very powerful second site screening Powerful molecular techniques Genes can be easily cloned Genome sequence nearing completion Simple cellular behaviors similar to animals Motility Chemotaxis	Limited cellular diversity
Nematode	Excellent genetics Hermaphrodites, self-fertilization Fast generation time Second site suppressor/enhancer screens Powerful molecular techniques Genes can be easily cloned Transposon tagging SNP mapping Rapid cosmid rescue Deletion collections span genome RNAi effective Genome sequence complete Few cells: 959 cells, 302 neurons Morphology fully characterized Serial EM reconstruction All cell lineages known Time lapse microscopy of development Laser ablation of single identified cells	Limited external morphology Less similar to human than flies (61% of *Drosophila* genes have human counterparts vs. 43% of *C. elegans* genes) Detailed direct analysis of gene expression patterns can be difficult Some embryological manipulations difficult
Fruit fly	Excellent genetics Genome sequence complete Targeted gene disruption RNAi effective Fast generation time Second site suppressor/enhancer screens Powerful molecular techniques Genes can be easily cloned Transposon tagging SNP mapping Transgenic animals easily generated Targeted misexpression of genes in space and time Mosaic analysis: determine where gene acts	Embryological manipulations difficult Targeted gene disruption still difficult, although possible
Zebrafish	Simplest vertebrate with good genetics: nearly saturated for zygotic patterning mutants Genome analysis well under way (good SNP and linkage maps) Easy examination of morphological defects (clear embryos) Embryological manipulations possible Organ systems similar to other vertebrates (e.g., eyes, heart, blood, gastrointestinal tract) Rapid vertebrate development	Not yet trivial to clone genes Cannot easily make transgenic animals No targeted gene disruption
Frog	A vertebrate Ectopic gene expression possible in early embryos, although manipulation of levels difficult Accessibility of embryo (pond no shell) Excellent experimental embryology grafting induction preparations (Keller sandwiches/animal caps, etc.) Injection of RNA into identifiable blastomeres	No genetics, although under development Difficult to create transgenic animals
Chicken	Availability, low cost Accessibility, outside of mother Well suited for embryological manipulation; transplants of limbs, notocord, neural crest Easily transfected by avian retroviruses	Limited genetics Limited genome data at present
Mouse	Mammals, brains similar to human, all homologous areas/cell types "Reverse" genetics: targeted gene knockouts by homologous recombination routine Developmental overview same as for all mammals Large mutant collection Construction of chimeric embryos possible Availability of material at all stages Source of primary cells for culture	Classic "forward" genetics difficult Early-acting mutant phenotypes difficult to study (resorbed by mother) Embryonic manipulations difficult (inside mother) Development and life cycle relatively slow (months)

(*Continued*)

Table 3–1. ***Continued***

Species	Experimental Advantages	Experimental Limitations
Monkey	Very similar to humans	Fetal experiments difficult
	Developmental connections and physiology, postnatal	No genetics
	Anatomy of learning	High cost, for both animals and facilities
	Responses to injury	
Human	Many diseases, self-reporting mutants (>5000 genetically based diseases)	Fetal material difficult
		No experimental access
	Some good family pedigrees	
	Genome sequence complete	
	Detailed behavior/ontogeny	

SNP, single nucleotide polymorphism; RNAi, RNA interference; EM, electrical microscopy.

Unicellular Organisms as Models for Eukaryotic Cell Function

All eukaryotic organisms share an organization of the cell into functionally dedicated, membrane-enclosed compartments such as the nucleus, mitochondria, endoplasmic reticulum/Golgi, and endosomes. In addition, similar mechanisms control the cell cycle, cell division, creation of cell polarity (e.g., bud site selection in yeast or polarity of chemotaxing *Dictyostelium*), and motility (*Dictyostelium*) in unicellular as well as multicellular eukaryotes. Many basic molecular biological processes are also shared by all eukaryotes, including biochemical pathways, DNA replication, DNA repair, transcriptional control, RNA processing, and protein degradation.

The best-studied unicellular eukaryotic systems are yeast (*Saccharomyces cerevisiae*) and slime molds (*Dictyostelium discoideum*). The yeast genome sequence has been completed (http://genome-www.stanford.edu/Saccharomyces/), and several additional genome-scale resources are being developed, such as collections of mutations in every gene and a comprehensive two-hybrid collection defining all two-way interactions between yeast proteins. The *Dictyostelium* genome sequence also is nearly complete (http://glamdring.ucsd.edu/others/dsmith/dictydb.html), and it is possible to knock out specific genes efficiently using the REMI method (Kuspa and Loomis, 1994). Thus, both organisms are excellent molecular systems. In addition, it is possible to carry out genetic selection schemes and screens in these organisms in which greater than a billion progeny can be generated and tested. Genetic schemes of this kind are effective at isolating potential second-site intragenic suppressor loci as well as saturating for second-site mutations which modify the phenotype of a given mutant. These unicellular systems have no equal for establishing the networks of gene action involved in basic cell biological processes.

The chief limitation of unicellular organisms as models for analyzing the function of genes involved in human disease is that pathologies that affect specific tissues, such as the nervous system or organs, or physiological functions that arise from interactions between cells cannot be assessed at the relevant organismal level. This limitation is not restricted to disease genes that do not have obvious homologs in unicellular organisms but also can apply to genes that are present in unicellular organisms but required in a more stringent fashion in certain tissues or expressed as different isoforms in different cell types. For example, defects in enzymes involved in energy metabolism can result in nervous system or muscle-specific defects (Blass et al., 2000; Darras and Friedman, 2000; Guertl et al., 2000; Palau, 2001).

Invertebrate Genetic Systems as Models for Tissue and Organ Function

The most developed invertebrate genetic organisms are fruit flies (*Drosophila melanogaster*, http://flybase.bio.indiana.edu:82/) and nematodes (*C. elegans*, http://www.expasy.ch/cgi-bin/lists?celegans.txt). These model organisms have contributed to many basic biological discoveries, including the organization of genes into independently segregating linear chromosomes, the creation of the first chromosome maps, the one gene–one protein hypothesis, the discovery that X-rays cause increased rates of mutations, the principles of pattern formation and of how genes can act hierarchically in space and time to define distinct positions and cell types, as well as the identification of many genetic pathways that subsequently have been implicated in human disease.

A major strength of these model systems is that they are well suited for second-site modifier screens. These screens can be used to isolate many components in a given genetic pathway once a single gene involved in that process has been identified. The logic of these screens is to partially cripple a process or pathway with a mutation affecting one component and then search for mutations in other genes encoding component functions in the same system. This is accomplished by screening for mutations which critically reduce the function of the pathway in a dominant fashion but only when combined with the first mutation. The cartoon of a simple crank–pulley system designed to hoist a bucket of water illustrates this principle (Fig. 3–1). If one removes any piece entirely, such as either of the gears, the machine is inoperative. If, however, one only files down the teeth on one of the

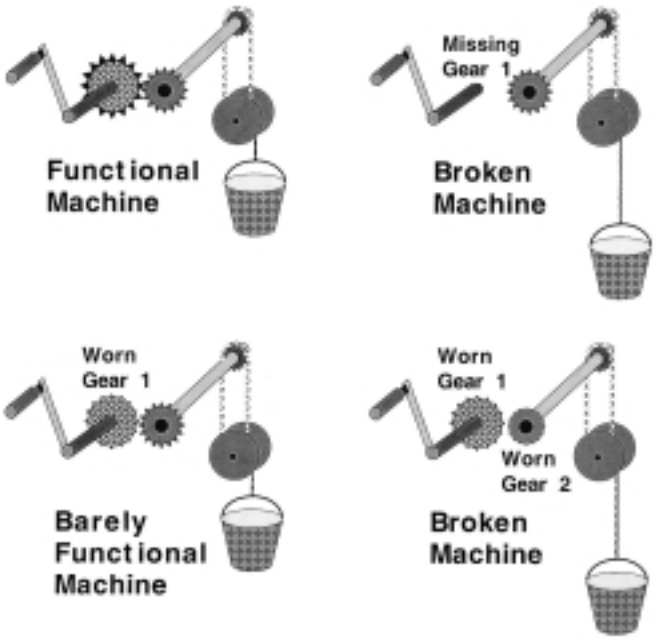

Figure 3–1. Molecular machines and the logic of genetic screens. Several genes typically function in concert as a machine to carry out a particular molecular function. In this diagram, such a "molecular machine" is depicted as a crank and gear assembly that functions to raise a bucket. In this analogy, the various components in the machine can be thought of as genes, which function together to carry out a molecular function such as passing a signal from one cell to another. If one removes either of the two gears, the machine is broken and unable to perform its task. In this complete loss-of-function situation, any further blow to the system has no further consequence. If, on the other hand, one starts out with one of the gears (gear 1) being worn such that the machine barely functions to raise the bucket, then even a small additional insult to another component (e.g., a worn gear 2) will render the machine inoperative. This latter scenario is similar to the genetic conditions one can engineer in a model genetic system wherein a partial loss-of-function mutant in one gene sensitizes the system to even a slight reduction in the function of any other component of that molecular machine. In this way, geneticists can rapidly screen for new mutants that define all the various components of the intact machine.

gears, then it is possible to get a machine that is barely working. If one then damages any other component (e.g., files down another gear), the machine fails. Thus, the barely functioning machine provides a sensitized genetic system that converts an otherwise silent recessive mutation (e.g., 50% reduction in gene dose) into a dominant read-out, which can be easily scored among large numbers of progeny (e.g., 10^5–10^6 individuals).

Because flies and nematodes have closely related counterparts of many human disease genes, identification of new genes functioning as part of a common molecular process in invertebrates will help define new candidate disease genes that are likely also to be involved in the same disease process. An important point regarding the use of invertebrate systems is that it is not necessary that the phenotype resulting from reducing the activity of a pathway in the model system be similar to that of the human disease. The only critical aspect of the invertebrate model is that it faithfully identifies components acting as part of a common molecular machine. A useful example to illustrate this point is the Notch signaling pathway. The Notch pathway controls many different binary cell fate choices during development of *Drosophila* and *C. elegans* (Greenwald, 1998; Simpson, 1998). Two heavily studied phenotypes resulting from mutations in components of this pathway are notching of the wing margin in flies (Irvine, 1999; Wu and Rao, 1999) and defects in vulval development in worms (Greenwald, 1998; Wang and Sternberg, 2001). In the case of the fly, strong reduction in the activities of the ligand Delta, the Notch receptor itself, or the signal transducer Suppressor or Hairless can result in Notched wings. In the case of vertebrates, which have several paralogs of Notch pathway components, reduced function in the Delta-related ligand Delta3 (Kusumi et al., 1998; Bulman et al., 2000) or the Notch homolog Notch1 (Conlon et al., 1995) results in axial skeletal malformations (e.g., spondylocostal dysostosis) as a consequence of somite fusion defects during embryonic development. Mutations in the human *Delta3* gene were originally identified based on previous finding that mutations in mouse *Delta3* gave rise to similar spinal malformations and the fact that the human *Delta3* gene mapped within a genomic interval believed to contain the suspected disease gene. For this reasoning to hold, it was not necessary that the fly phenotype resembled that of the human disease (e.g., humans have no wings and flies do not have bony endoskeletons). The only important facts for this discovery were that mutations in different components of a common signaling pathway in humans led to similar disease phenotypes and that the components of this pathway had been defined by comprehensive saturation screening in model genetic systems.

Vertebrate Genetic Systems as Accurate Models for Human Disease

As described above, unicellular and model invertebrate systems can be of great value in defining the molecular components of pathways or processes that depend on the function of several interacting proteins. Once such components have been defined, one can ask whether similar diseases result from defects in more than one of these components in humans. In some cases, the model systems can also serve as models for the disease process itself, as in the polyglutamine repeat neurodegenerative disorders in which there are parallel correlations in *Drosophila* and humans between the length of the polyglutamine repeat and the severity and early onset of neurodegenerative phenotypes (Chan and Bonini, 2000; Fortini and Bonini, 2000). While such examples exist, model invertebrate systems cannot in general be consistently relied on to mimic the human disease state. Rather, the ability to provide an accurate model for the human disease condition is the chief strength of vertebrate systems such as the mouse (*Mus musculus domesticus*, http://www.informatics.jax.org/) and zebrafish (*Danio rerio*, http://www.ncbi.nlm.nih.gov/genome/guide/D_rerio.html).

The great advantage of the mouse system is clearly the ability to make targeted gene knockouts (mutations). The knockout phenotype of a human disease gene counterpart in mice often results in a phenotype resembling that of the human disease. There are notable exceptions to this approach, however, which may result from the significant effect of genetic background on knock-out phenotypes in mice, the genetic variation in human genetic background, or intrinsic differences between the function of mouse and human disease gene homologs. One curious trend is that a corresponding mutation in a given gene in mice and humans often results in a much stronger phenotype in humans. There are even examples in which the heterozygous loss-of-function mutation generates a dominant phenotype in humans comparable to that observed in homozygous null mice knockouts.

Although gene knock-out technology has not yet been developed for zebrafish, systematic genetic screens have been conducted for mutants disrupting various aspects of embryonic development (Driever et al., 1996; Haffter et al., 1996). Among the large number of mutants recovered in these screens, many affected embryonic patterning and formation of organ systems such as the heart (Chen et al., 1996; Stainier et al., 1996; Xu et al., 2002), digestive system (Pack et al., 1996), hematopoetic system (Ransom et al., 1996; Childs et al., 2000), bone and cartilage (Neuhauss et al., 1996; Piotrowski et al., 1996; Schilling et al., 1996), spinal chord/notochord (Odenthal et al., 1996; Stemple et al., 1996), retina (Malicki et al., 1996a; Brockerhoff et al., 1998; Daly and Sandell, 2000), auditory system (Malicki et al., 1996b; Whitfield et al., 1996), and brain (Abdelilah et al., 1996; Brand et al., 1996; Heisenberg et al., 1996; Jiang et al., 1996; Schier et al., 1996; Rodriguez and Driever, 1997). In addition, many mutations were recovered which compromised the pathfinding ability of retinal axons to be guided to their appropriate tectal targets (Baier et al., 1996; Karlstrom et al., 1996; Trowe et al., 1996). High-resolution simple sequence length polymorphisms (SSLPs) and radiation hybrid maps have also been generated for the zebrafish, which greatly aid in the genetic mapping of mutations and cloning of the affected genes (Kelly et al., 2000; Woods et al., 2000; Hukriede et al., 2001).

Nongenetic Model Systems

Although this chapter is focused on model genetic systems for studying genes involved in developmental disorders, there are some significant advantages of nongenetic systems for analyzing certain types of questions. Classic vertebrate embryological systems, for example, *Xenopus* and the chick, offer ease and access to experimental manipulations such as heterotopic transplantation and grafting, which were critical for the identification of organizing centers such as the Spemann organizer, the zone of polarizing activity (ZPA), and the apical ectodermal ridge (AER). Although classic genetic techniques are not available for these systems, some effective experimental alternatives, such as injection of normal or mutant RNAs or virus-mediated gene expression, provide important complementary systems to genetic models.

Higher vertebrate systems, such as birds, cats, ferrets, and primates, also offer advantages with regard to the postnatal development of neural connections. For example, these systems are well suited for analysis of critical periods required for experience-based formation of visual, auditory, sematosensory, and behavioral (e.g., birdsong or language) connections. As many developmental disorders in humans also result in learning or behavioral abnormalities, the more related to humans a species is, the better it can serve as a model for such complex neural functions.

RECONSTRUCTING THE COMMON ANCESTOR OF METAZOANS: OUR DISTANT REFLECTION

The detection of covert similarity in diverse body plans of bilateral animals has resulted from the great advances made in the past 20 years of developmental genetic research. For example, a series of investigations showed that all bilateralia, including humans, possess a common genetic mechanism for patterning the anterior/posterior (A/P) body axis involving the Hox cluster genes (McGinnis and Krumlauf, 1992), the dorsal/ventral (D/V) body axis (Francois and Bier, 1995; DeRobertis and Sasai, 1996), and the three derived axes of the appendages (A/P, D/V, and proximo/distal [P/D]) (Irvine and Vogt, 1997; Panganiban et al., 1997; Shubin et al., 1997). Many of the pathways involved in this discussion are covered in more detail in other sections of the book, but here we use them to illustrate the validity of studying model organisms.

Besides common axial patterning systems, other general architectural features in both vertebrates and invertebrates appear to be controlled by common genetic mechanisms. Humans and insects possess organs of very diverse appearance that serve similar functions, such as eyes for vision (Wawersik and Maas, 2000; Pichaud et al., 2001), and hearts for blood circulation (Bodmer and Venkatesh, 1998; Chen and Fishman, 2000). Traditional views have held that these structures are analogous (i.e., convergently evolved) and therefore likely to be specified by different genetic patterning systems. However, the sum of the evidence discussed below suggests that we now have good reason to call these organs homologous at the level of the genes that control their formation.

Hox Genes Determine Segment Identity along the A/P Axis: From *Drosophila* to Humans

Homeosis was defined by William Bateson (1894) as the phenomenon in which one segment of an organism is transformed in whole or in part to another. The genetic basis for these transformations of the animal body plan was partially revealed by seminal studies on homeotic selector genes (now often referred to as *Hox genes*; see Chapter 46). Mutations in Hox genes often result in homeotic transformations of the body plan in one or a few segments. A systematic collection of homeotic mutations was discovered and studied in *Drosophila* in the labortories of E.B. Lewis, Thomas Kaufman, and others. Two breakthough papers that summarize these studies are Lewis (1978) and Kaufman et al. (1980). The well-known homeotic gene *Ultrabithorax* (*Ubx*) was originally identified by mutations that transform halteres (small club-like balancing organs of flies) into an extra pair of wings. Another classical homeotic phenotype is produced by dominant mutations in the *Antennapedia* (*Antp*) gene, which transform the antenna on the head of a fly into an extra thoracic leg.

Molecular analysis of the genomes of other organisms has revealed that all bilateral animals, including humans, have multiple Hox genes (Fig. 3–2), which carry a common DNA sequence motif called the homeobox (the genesis of the *Hox* acronym). The homeobox motif encodes a similar 60–amino acid motif in Hox proteins, termed the *homeodomain*. Homeodomain proteins such as those of the Hox type are transcription factors and exert their function through activation and repres-

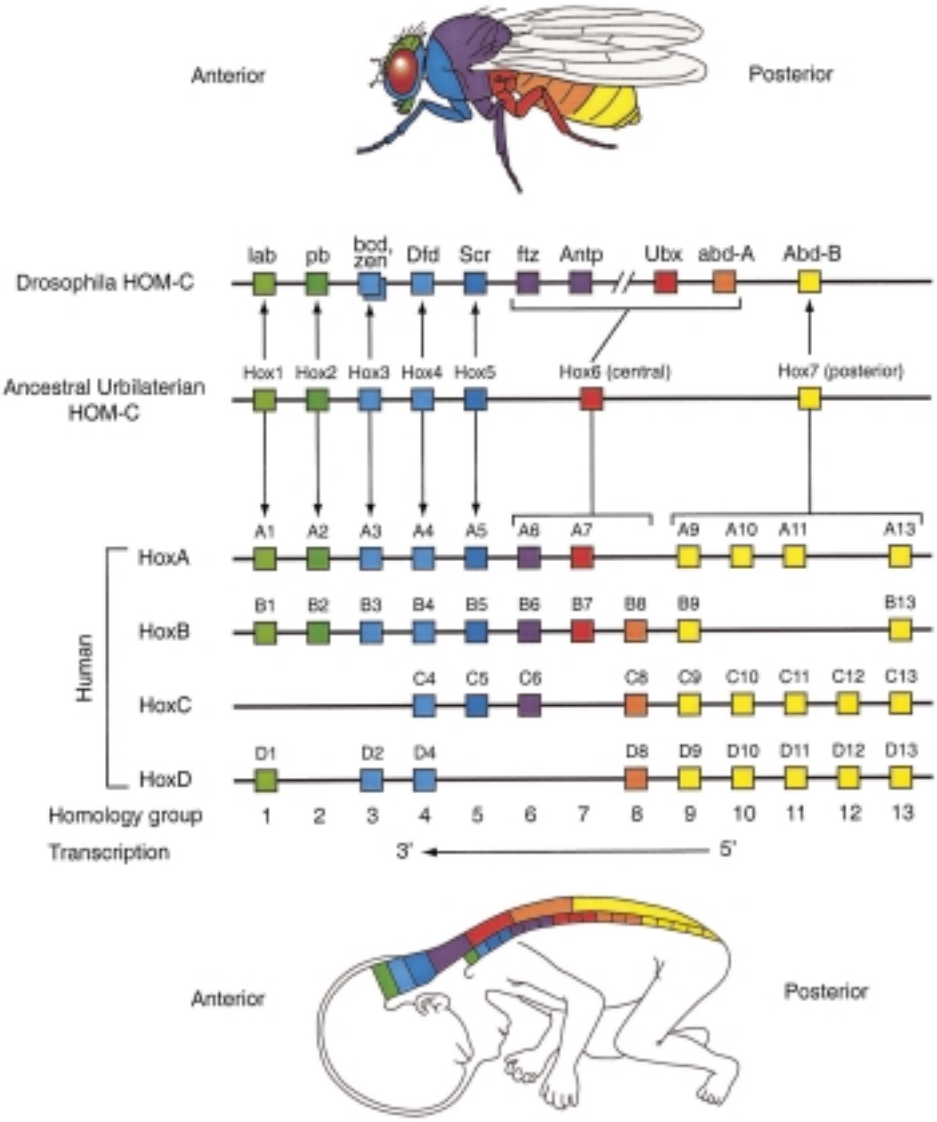

Figure 3–2. Conservation of genomic organization and expression patterns of fly and mammal Hox genes. The lower part of the figure shows the four clusters of Hox genes in mammals and the expression patterns (inferred from mouse expression studies) of the orthologous genes in a diagram of a human embryo. The colored fields in the expression diagram show the anteriormost domains of expression. The posterior extent of many Hox gene expression patterns overlap in more caudal regions. The upper half of the figure shows the *Drosophila* Hox genes aligned with their mammalian orthologs (arrows), with their corresponding expression patterns mapped onto the body plan. The composition of a hypothetical ancestral Hox cluster is shown in the middle. For some of the central and posterior Hox genes, there are no obvious orthology relationships, so groups of genes that are equally related to an ancestral gene are indicated with brackets. *Drosophila bcd*, *ftz*, and *zen* homeobox genes do not function in the Hox A/P patterning system. They represent insect homeobox genes that have recently diverged from Hox ancestors and now have novel patterning functions.

sion of multiple target genes. Interestingly, the Hox genes are arranged so that the position and order of homologous genes (e.g., *Deformed* [*Dfd*] of *Drosophila* and *HOXD4* of humans) are preserved in the Hox clusters of different animals. The functional significance of the conserved gene order in these clusters is not clearly understood at present. There is, however, evidence that the clustered arrangement has been maintained for more than 500 million years because different genes in the clusters are controlled by the same *cis*-acting DNA regulatory regions. Thus, it can be argued that the clusters function as single, complicated genetic units (Gerard et al., 1996; Gould et al., 1997; Sharpe et al., 1998). In contrast to the unique Hox cluster of *Drosophila* and most other invertebrates, humans and other vertebrates have four clusters of Hox genes (*HOXA*, *HOXB*, *HOXC*, and *HOXD*), which apparently evolved by two successive duplications of a primordial cluster.

In addition to conservation of primary sequence and chromosomal organization, Hox gene expression patterns are conserved in diverse animals. Persistent expression of Hox genes in discrete zones on the A/P axis is required to remind embryonic cells of their axial position long after the initial genetic cues are gone. Hox expression zones typically have sharp anterior boundaries, with less well-defined posterior boundaries. The order of anterior boundaries of Hox expression along the A/P axis of the embryo and the timing of activation during development are generally colinear with the order of the genes on the chromosome (Zákány and Duboule, 1999). It is interesting to note that the same Hox gene can have a slightly offset boundary of expression in different tissues, which is especially true for vertebrate embryos (Fig. 3–2). Within the same tissue, however, the relative expression boundaries of different Hox cluster members are almost always preserved.

Conservation of Hox protein sequence and expression patterns suggested that vertebrate Hox genes controlled axial patterning in a manner similar to that in flies. This was confirmed when mouse Hox mutants were obtained and homeotic transformations found in the mutant embryos. For example, in *Hoxc-8* homozygous mutant mice, the most obvious transformations were attachment of the eighth pair of ribs to the sternum and the appearance of a fourteenth pair of ribs on the first lumbar vertebra (Le Mouellic et al., 1992).

Studies in both *Drosophila* and mouse show that Hox loss-of-function mutants generally result in transformations in which more posterior body structures resemble more anterior ones (McGinnis and Krumlauf, 1992). Conversely, many gain-of-function mutations in which a posterior gene is inappropriately expressed in a more anterior region result in the replacement of anterior stuctures with stuctures characteristic of more posterior regions. For example, when *Drosophila* Ubx protein, which is normally confined to the posterior most abdominal region of the fly embryo, is provided ubiquitously under the control of a heat shock promoter, all head and thoracic segments attain a more posterior (abdominal-like) identity. The ability of a more posterior Hox gene to impose its function on more anterior genes is called *posterior prevalence*, or *phenotypic suppression.*

D/V Patterning in *Drosophila*

Establishment of the D/V axis in *Drosophila* is initiated by a cascade of maternally acting genes functioning in both the oocyte and surrounding follicle cells. These genes ultimately create a nuclear gradient of the *rel*-related transcription factor encoded by the *dorsal* gene (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). The Dorsal nuclear gradient is directly responsible for subdividing the embryo into three primary territories of zygotic gene expression: a ventral zone giving rise to mesoderm, a lateral zone giving rise to neuroectoderm, and a dorsal zone giving rise to dorsal ectoderm and amnioserosa (Fig. 3–3). Dorsal activates expression of genes in ventral and lateral regions of the embryo in a threshold-dependent fashion (reviewed in Rusch and Levine, 1996). High levels of Dorsal are required for activating expression of mesoderm-determining genes such as *snail* (Kosman et al., 1991; Leptin, 1991; Rao et al., 1991; Ray et al., 1991; Thisse et al., 1991; Ip et al., 1992b) and *twist* (Jiang et al., 1991; Kosman et al., 1991; Leptin, 1991; Rao et al., 1991; Ray et al., 1991), whereas lower levels are required to activate genes such as *rhomboid* (*rho*) (Kosman et al., 1991; Leptin, 1991; Rao et al., 1991; Ray et al., 1991; Ip et al., 1992a), *ventral nervous system defective* (*vnd*) (Mellerick and Nirenberg, 1995), *intermediate nervous system defective* (*ind*) (McDonald et al., 1998; Weiss et al., 1998), *short gastrulation* (*sog*) (Francois et al., 1994), and *brinker* (*brk*) (Jazwinska et al., 1999a, 1999b) in the neuroectoderm. The absence of Dorsal defines the dorsal domain since Dorsal represses expression of key genes required for the establishment of dorsal cell fates, such as *decapentaplegic* (*dpp*) (Ray et al., 1991; Jiang et al., 1993; Huang et al., 1993, 1995), *zerknüllt* (*zen*) (Rushlow et al., 1987; Doyle et al., 1989; Ray et al., 1991; Jiang et al., 1992), *tolloid* (*tld*) (Kirov et al., 1994), and *twisted gastrulation* (*tsg*) (Mason et al., 1994).

Mesoderm Specification in *Drosophila*

High levels of Dorsal activate expression of the mesoderm-determining genes *snail* and *twist* (Jiang et al., 1991; Kosman et al., 1991; Leptin, 1991; Rao et al., 1991; Ray et al., 1991; Ip et al., 1992b; see Chapter 34). The *twist* gene encodes a basic helix-loop-helix (bHLH) transcription factor (Thisse et al., 1988), which activates expression of mesoderm-specific target effector genes such as the homeodomain genes *tinman* (Bodmer, 1993; Lee et al., 1997; Yin et al., 1997), *bagpipe* (Azpiazu and Frasch, 1993), and the fibroblast growth factor (FGF) receptor tyrosine kinase *heartless* (Beiman et al., 1996; Gisselbrecht et al., 1996). *snail*, however, encodes Zn^{2+} finger transcription factor (Boulay et al., 1987), which represses expression of neural genes such as *rho* (Kosman et al., 1991; Leptin, 1991; Rao et al., 1991; Ip et al., 1992a), *vnd* (Mellerick and Nirenberg, 1995), and *sog* in ventral cells (Francois et al., 1994). The dual requirement for activation of mesoderm genes and repression of genes specifying alternative fates (e.g., neural genes) is typical of cell fate specification in many settings. This theme of combined activation and repression is echoed in both the neural and non-neural regions of the ectoderm.

Specification of the Lateral Neural Ectoderm in *Drosophila*

Genes required for neural development are expressed in the lateral region of the *Drosophila* embryo. Some of these "neural" genes encode transcription factors that promote neural fates, such as genes of

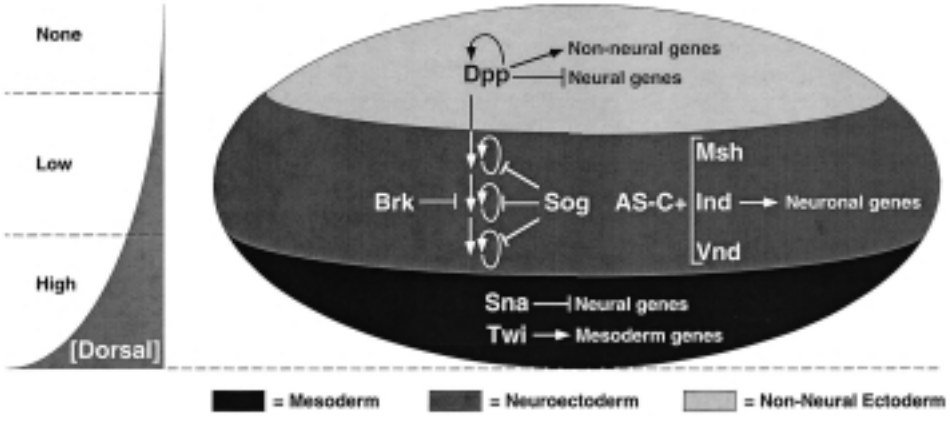

Figure 3–3. Subdivision of the *Drosophila* embryonic dorsal–ventral axis into three primary subdomains. High levels of the maternal morphogen Dorsal specify mesoderm (black ventral domain), intermediate of Dorsal define the neuroectoderm (dark gray lateral domain), and the absence of Dorsal specifies the epidermis (light gray dorsal domain).

the achaete-scute complex (ASC) (Cabrera et al., 1987; Jimenez and Campos-Ortega, 1990; Campuzano and Modolell, 1992; Skeath and Carroll, 1992) and homeodomain protein genes *vnd* (Skeath et al., 1994), *ind* (McDonald et al., 1998; Weiss et al., 1998), and *msh* (D'Alessio and Frasch, 1996). These latter three genes are expressed in three nonoverlapping stripes within the neuroectoderm and are required for the formation of the three primary rows of neuroblasts which derive from those regions. As in the case of the mesoderm, repression also plays an important role in establishing the neural ectoderm since mutations in the repressor *brk* result in ectopic expression of dorsal ectodermal genes, such as *dpp* laterally (Jazwinska et al., 1999b; Rushlow et al., 2001; Zhang et al., 2001).

Sog encodes a secreted antagonist of bone morphogenetic protein (BMP; see Chapter 24) signaling (Francois et al., 1994) and acts in parallel with *brk* to prevent BMP signaling from spreading into the neuroectoderm (Biehs et al., 1996). Sog blocks the activity of the BMP Screw (Scw) (Neul and Ferguson, 1998; Nguyen et al., 1998), which is expressed ubiquitously in the early embryo and acts in concert with Dpp to define peak levels of BMP signaling (Arora et al., 1994). By blocking Scw, Sog interferes with an invasive positive feedback loop of BMP signaling created by Dpp diffusing laterally and activating its own expression in the neuroectoderm (Biehs et al., 1996; Bier, 1997). As discussed further below, this interplay between Sog and Dpp is important for the primary subdivision of the ectoderm into neural versus nonneural domains and has been highly conserved during the course of evolution (Bier, 1997). Thus, as in the case of mesoderm specification, neural genes act by both promoting appropriate neural fates and suppressing the alternative epidermal fate.

Specification of the Dorsal Nonneural Ectoderm

The absence of Dorsal defines the nonneural ectoderm by virtue of Dorsal acting as a repressor of dorsally expressed genes such as *dpp* and *zen* in ventral and lateral cells (Rushlow et al., 1987; Doyle et al., 1989; Ray et al., 1991; Jiang et al., 1992, 1993; Huang et al., 1993, 1995). The key gene involved in development of dorsal cells is *dpp*, the homolog of vertebrate BMP2/4 (Padgett et al., 1987). To achieve maximal levels of BMP signaling, another BMP family member, Screw (Scw), is also required (Arora et al., 1994). Dpp is essential for BMP signaling in dorsal cells in that the lack of Dpp cannot be compensated for by increasing the levels of Scw. Scw appears to function in more of a helper capacity, however, since elevating Dpp levels can rescue *scw* mutants (Arora et al., 1994). BMP signaling plays two roles in specifying the nonneural ectoderm: it activates expression of genes required for dorsal cell fates, such as *zen* (Ray et al., 1991), and it suppresses expression of neural genes (Skeath et al., 1992; Biehs et al., 1996; von Ohlen and Doe, 2000). One of the genes activated by BMP signaling is *dpp* itself, which results in a positive feedback autoactivation loop (Biehs et al., 1996).

As described in more detail below, a variety of evidence suggests that Dpp acts in a dose-dependent fashion to specify at least two different dorsal cell fates (Ferguson and Anderson, 1992a, b; Wharton et al., 1993; Biehs et al., 1996; Jazwinska et al., 1999b). In this model, peak Dpp activity specifies the dorsalmost cell type (amnioserosa), while lower levels of Dpp signaling specify dorsal nonneural ectoderm.

D/V Patterning in Frogs and Fish

The unfertilized *Xenopus* embryo is visibly subdivided into two hemispheres, a pigmented half known as the vegetal hemisphere and a nonpigmented half known as the animal hemisphere. The A/P and D/V axes are established by a coupled mechanism, which is initiated by the point of sperm entry in *Xenopus* embryos. Fertilization takes place in the animal hemisphere of the egg near the boundary with the vegetal hemisphere and triggers a rotation of the egg cortex away from the point of sperm entry (Fig. 3–4; reviewed in Moon and Kimelman, 1998). The ensuing cortical rotation is believed to result in the activation and displacement of latent dorsalizing factors that previously resided at the vegetal pole of the embryo. A primary response to the cortical activation event is a graded nuclear localization of the Wingless/Wnt pathway (see Chapter 22) signal transducer β-catenin

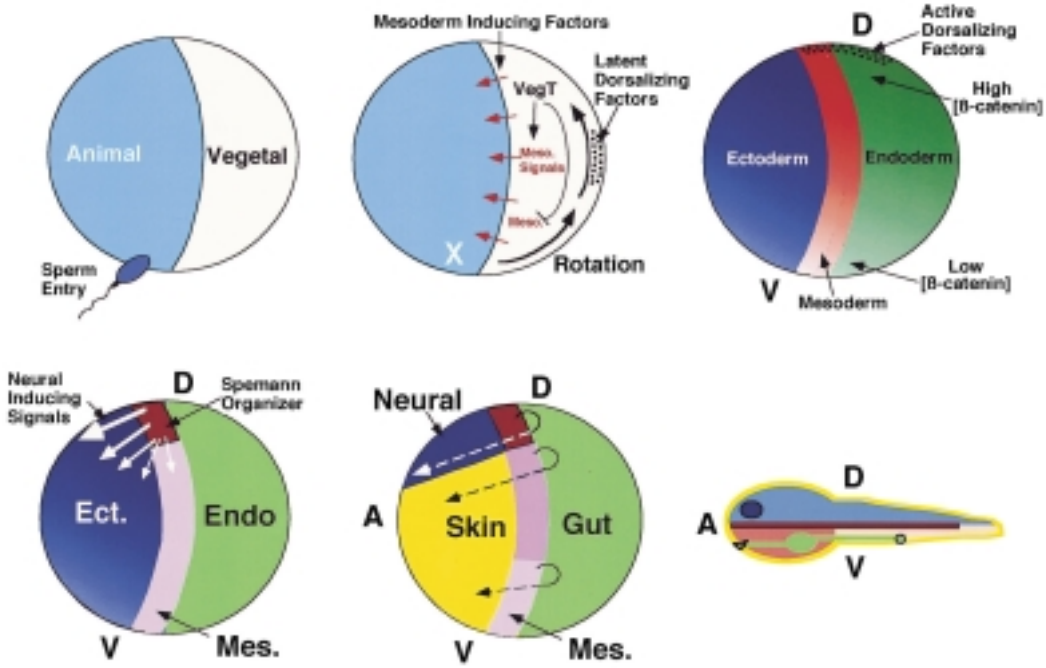

Figure 3–4. Dorsal–ventral patterning of the early *Xenopus* embryo. The point of sperm entry (lower left) defines the future dorsal pole on the opposite side of the embryo by triggering rotation of the cortex and redistribution/activation of putative latent dorsalizing factors. High levels of β-catenin that accumulate in the nuclei of dorsal cells are required for activating expression of genes in dorsal regions. These dorsalizing factors act in concert with mesoderm-inducing factors produced by the vegetal (white domain) hemisphere to induce a band of patterned mesoderm (red domain) within the animal hemisphere (blue domain). The remaining cells of the animal hemisphere will form the ectoderm (purple domain). The transcription factor VegT, which is expressed in vegetal cells, activates expression of the mesoderm inducing factors but prevents these cells from responding to those factors and directs them instead to become endoderm (green domain). A combination of dorsalizing and mesoderm inducing factors defines a dorsal domain of mesoderm known as the Spemann Organizer, which becomes the source of neural inducing substances such as Chordin and Noggin. The lateral spread of neural inducing substance coupled with their subsequent delivery to overlying cells following involution of the mesoderm (arrows) during gastrulation permits cells to follow their default preference to become neural ectoderm (dorsal purple domain) rather than to give rise to epidermal ectoderm (yellow domain).

(Larabell et al., 1997; Medina et al., 1997), which may occur in a signal (e.g., Wnt) independent fashion (Miller et al., 1999). The maximum point of β-catenin activation defines the dorsal pole of the embryo in much the same fashion that the structurally unrelated Dorsal (and nuclear factor κB [NFκB] family member) initiates patterning along the D/V axis of *Drosophila* embryos (see above). β-Catenin then activates dorsal expression of target genes such as *siamois* (Brannon and Kimelman, 1996; Carnac et al., 1996; Brannon et al., 1997; Fan et al., 1998; Nelson and Gumbiner, 1998), *twin* and *Xnr-3* (Moon and Kimelman, 1998). In addition, the levels of gene expression driven by β-catenin/T-cell transcription factor *siamois* response element are greatest in the dorsalmost cells and diminsh ventrally, suggesting that this enhancer element can sense a β-catenin activity gradient (Brannon et al., 1997). β-Catenin also appears to play a similar role in intiating D/V patterning in early zebrafish embryos (Sumoy et al., 1999).

Establishment of the Marginal Zone and Mesoderm

Following fertilization, a band of equatorial cells, which lie within the animal hemisphere immediately adjacent to the vegetal hemisphere (referred to as marginal cells), are induced to become mesoderm. This inductive event requires the concerted action of FGF (see Chapter 32) and most likely a transforming growth factor-β (TGF-β)/Activin-like signal (see Chapter 24) emanating from the vegetal cells (Fig. 3–4; reviewed in Kimelman and Griffin, 1998, 2000). Vegetal cells cannot themselves respond to these signals by virtue of the fact that they express the transcription factor VegT, which promotes endodermal cell fates, suppresses mesodermal cell fates, and activates expression/activity of secreted TGF-β/Activin/Nodal-related mesodermal inducing factors (Zhang and King, 1996; Zhang et al., 1998; Stennard, 1998; Clements et al., 1999; Xanthos et al., 2001). In response to the nonautonomous induction by vegetal hemisphere–derived signals, marginal cells activate expression of various mesoderm-determining genes such as *brachyury* (Wilkinson et al., 1990; Smith et al., 1991; Conlon et al., 1996; Smith, 2001) and the vertebrate homologs of the *Drosophila twist* (Hopwood et al., 1989; Chen and Behringer, 1995) and *snail* (Nieto et al., 1992; Smith et al., 1992; Essex et al., 1993; Hammerschmidt and Nusslein-Volhard, 1993; Carver et al., 2001; Ciruna and Rossant, 2001) genes. The vertebrate *snail* and *twist* genes may function similarly to the invertebrate counterparts as expression of mesodermal markers is lost in *twist*$^-$ mice (Chen and Behringer, 1995), while ectopic expression of ectodermal markers but normal mesdermal gene expression is observed in *snail*$^-$ mice (Carver et al., 2001). Depending on their D/V position, marginal cells give rise to different derivatives, including blood (ventral), muscle (lateral), and notochord (dorsal). The function of *twist* in specifying mesodermal derivatives may be very ancient as a *C. elegans twist* (Harfe et al., 1998) gene is required for the formation of nonstriated muscle (Corsi et al., 2000) and a *twist*-related gene is expressed in mesodermal cells in the jellyfish (Spring et al., 2000). Twist also plays an important developmental role in humans as mutations in this gene lead to dominant inheritance of Saethre-Chotzen syndrome (el Ghouzzi et al., 1997; Howard et al., 1997) and possible recessive inheritance of Baller-Gerold syndrome (Seto et al., 2001). Twist may activate FGF receptor (GFGR) expression in humans as it does in *Drosophila* since mutations in the *FGFR-2* and *FGFR-3* genes also can lead to Saethre-Chotzen syndrome (Lajeunie, 1997; Paznekas et al., 1998).

Establishment of a Dorsal Neural Inducing Center: The Spemann Organizer

As a result of the combined action of mesoderm-inducing factors and transcription factors such as Siamois (Brannon and Kimelman, 1996; Carnac et al., 1996; Brannon et al., 1997; Fan et al., 1998; Nelson and Gumbiner, 1998) and its target gene *goosecoid* (Blum et al., 1992; De Robertis et al., 1992; Steinbeisser and De Robertis, 1993), expressed only in dorsal regions of the embryo, dorsal marginal cells begin to express several secreted neuralizing factors, such as Chordin (Sasai et al., 1994) and Noggin (Smith and Harland, 1992; Lamb et al., 1993; Smith et al., 1993). The first evidence for the existence of such neural inducing substances was provided by the classical embryological transplantation experiments of Spemann and Mangold (1924), who showed that the dorsal mesoderm of amphibian embryos could induce surrounding ventral ectodermal cells to assume neural fates. These neural inducing factors are secreted from the marginal zone and may diffuse in a planar fashion into the neighboring ectoderm and/or may be delivered to overlying dorsal ectodermal cells following invagination of the mesoderm during gastrulation.

Following the landmark work of Spemann and Mangold (1924), a great deal of effort was expended in trying to determine the molecular identity of the neural inducing factor(s). A variety of substances and factors were tested for neural inducing activity, and while many substances could induce second neural axis formation, none of these studies led to isolation of an endogenous neural inducing factor. The first endogenous neural inducer was Noggin, which was identified in a screen for *Xenopus* proteins capable of inducing second neural axes (Smith and Harland, 1992). A subsequent study, based on cloning of genes expressed differentially in the Spemann organizer region of the embryo, identified several other factors with neural inducing activities, including Chordin (Sasai et al., 1994), which is the vertebrate counterpart of *Drosophila sog* (Francois and Bier, 1995).

BMP Signaling Suppresses the Default Ectodermal Fate in Vertebrates and Invertebrates

A variety of evidence indicates that the vertebrate neural inducers Noggin and Chordin and the *Drosophila* counterpart of Chordin (Sog) function by blocking BMP signaling in the neuroectoderm. First, *Drosophila* Dpp and its vertebrate homolog BMP4 are expressed at high levels only in the nonneural ectodermal regions of the embryo (Arendt and Nubler-Jung, 1994), while the neural inducers are expressed in, or adjacent to, neuroectodermal regions of the embryo (Francois and Bier, 1995). Second, Sog and Chd bind to BMPs and prevent these ligands from activating their receptors (Piccolo et al., 1996; Chang et al., 2001; Ross et al., 2001; Scott et al., 2001). Finally, Sog and Chordin function equivalently in cross-species experiments in which Sog can induce a secondary neural axis in *Xenopus* embryos and Chordin can oppose Dpp signaling in *Drosophila* (Holley et al., 1995; Schmidt et al., 1995; Yu et al., 2000).

Although the historical term *neural inducers* connotes a positive action of these factors, they actually function by a double negative mechanism to promote neural fates. Cell dissociation and reaggregation experiments using *Xenopus* ectoderm revealed that BMP4 signaling actively suppresses a default preference of vertebrate ectodermal cells to become neural (Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995) and that neural inducers such as Chordin and Noggin function by inhibiting this negative action of BMP4 signaling (reviewed in Hemmati-Brivanlou and Melton, 1997). Likewise, in *Drosophila* embryos, several neural genes, including the critical neural promoting genes of the ASC, are ectopically expressed in *dpp*$^-$ mutant embryos (Biehs et al., 1996), while ectopic Dpp expression suppresses expression of neural genes in the neuroectoderm. In genetically sensitized *sog*$^-$ mutant embryos, the autoactivating function of BMP signaling can lead to the spread of *dpp* expression into the neuroectoderm, which then activates expression of Dpp targets and represses expression of neural genes (Biehs et al., 1996). Furthermore, patterning defects in *chordino* mutant zebrafish embryos, which lack function of the *chordin* gene (Schulte-Merker et al., 1997), are strikingly similar to those observed in sensitized *sog*$^-$ mutant embryos. BMP4 expression autoactivates and expands into the dorsal ectoderm (Hammerschmidt et al., 1996) in *chordino*$^-$ embryos. The high degree of evolutionary conservation in Dpp/BMP4 and Sog/Chordin function suggests that this patterning system was active in the most recent common ancestor of vertebrates and invertebrates and that the ancestral form of Sog/Chordin protected the neuroectoderm from invasion by Dpp/BMP signaling, permitting cells to follow the default preference of neural development.

Sog and Chordin Also Act as Long-Range Morphogens in the Nonneural Ectoderm

As mentioned above, there is strong evidence that BMP signaling is graded in the dorsal region of the embryo and that different levels of BMP activity define distinct dorsal tissues in a threshold-dependent fashion. Since the level of *dpp* mRNA appears uniform throughout

the dorsal zone and *scw* is expressed evenly throughout the embryo (Arora et al., 1994), it has been speculated that a posttranscriptional mechanism is responsible for establishing graded Dpp activity. One mechanism by which this BMP activity gradient might form is long-range diffusion of the antagonist Sog into the dorsal region from the adjacent neuroectodermal domain where it is produced (Francois et al., 1994). Consistent with Sog functioning as a morphogen to define distinct thresholds of BMP signaling in dorsal cells, the gene dose of *sog* determines the width of cells experiencing peak levels of BMP activity (Biehs et al., 1996). This model received additional support when it was found that a metalloprotease known as Tolloid (Shimell et al., 1991), which specifically cleaves and inactivates Sog in vitro (Marques et al., 1997), is expressed in the dorsal region. The combination of Sog expression in the lateral neuroectoderm and Tld degradation of Sog dorsally provides a source and sink configuration, which could create a ventral-to-dorsal concentration gradient of Sog protein in the dorsal region, which in turn generates a reciprocal BMP activity gradient (e.g., highest dorsally and lowest ventrally).

Direct support for the hypothetical Sog gradient in dorsal cells has recently been obtained by histochemical methods (Srinivasan et al., 2002). As predicted, Tolloid proteolysis limits the accumulation of Sog dorsally, which is required to form a stable concentration gradient of Sog. In addition, these studies revealed that Dynamin-mediated endocytosis acts in parallel with Tld-dependent proteolysis to remove active Sog from dorsal cells. Cumulatively, these observations lend strong support to the model that a Sog concentration gradient in dorsal cells creates a reciprocal BMP activity gradient, which partitions the dorsal region into high versus low BMP activity zones. These two domains then give rise, respectively, to an extraembryonic tissue similar to the amnion (amnioserosa) and epidermis proper.

It seems likely that Chordin also acts as a long-range morphogen in vertebrate embryos. First, there are vertebrate homologs of the various *Drosophila* genes involved in sculpting the BMP activity gradient in the nonneural ectoderm, such as the vertebrate counterpart of Tolloid, Xolloid (Piccolo et al., 1997). There is also evidence that BMP signaling plays a role in long-range patterning of the mesoderm and ectoderm in vertebrates. For example, in zebrafish BMP2/4 mutants (e.g., *swirl*$^{-}$), patterning along the entire D/V axis of the embryo is disrupted (Hammerschmidt et al., 1996). Furthermore, as in *Drosophila*, there is no evidence for an asymmetric distribution of BMP2/4 protein or mRNA in the vertebrate nonneural ectoderm and adjacent mesoderm, suggesting that a posttranslational mechanism may also be necessary in vertebrates to establish a gradient of BMP activity, which may be generated by inhibitors such as Chd and Noggin (Jones and Smith, 1998; Blitz et al., 2000). For example, Chordin can block a BMP response far from the site of RNA injection, whereas in control experiments where a truncated dominant negative BMP receptor was injected, a response was elicited only within the progeny of injected cells (Blitz et al., 2000). In addition, cell transplantation experiments indicate that the zebrafish *chordino* gene acts nonautonomously since transplanted wild-type cells restricted to dorsal anterior structures of *chordino* mutants can restore normal patterning along the entire length of the axis (Hammerschmidt et al., 1996).

A Conserved Mechanism for Partitioning the Neuroectoderm into Three Primary Rows?

After being specified by neural inducers, the neuroectoderm is partitioned into three non-overlapping rows of homeobox gene expression, which give rise to the three primary rows of neuroblasts. As in the case of the Hox genes, homologs of these three neuroblast determination genes exist in vertebrates (*Nkx-2*, *Gsh*, *Msx*) and invertebrates (*vnd*, *ind*, *msh*) and are expressed in the same order relative to the midline of the nervous system (reviewed in Bier, 1997; Arendt and Nubler-Jung, 1999). Although the nervous system forms dorsally in vertebrates and ventrally in invertebrates, the fact that the D/V polarity of the neural plate is inverted during invagination of the neural tube results in the final orientation of the nervous system being similar in both organisms (Fig. 3–5). For example, in both classes of organisms, the outermost row of neuroectodermal cells, which express *msh* or *Msx*, form nearest epidermal cells expressing *dpp* or *BMP4*.

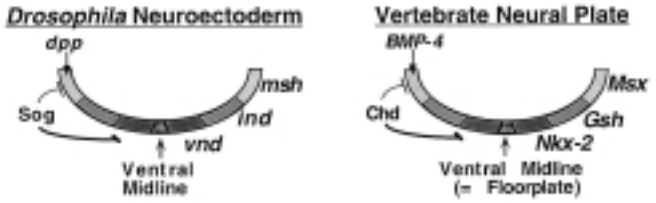

Figure 3–5. Conservation of dorsal–ventral patterning within the neuroectoderm. The *Drosophila* homeobox containing neuroblast determining genes *vnd, ind,* and *msh* are expressed in three adjacent stripes along the dorsal ventral axis of the CNS (left panel). *vnd* (dark gray) is expressed nearest the future ventral midline (hatched) of the CNS and *msh* (light gray) is expressed adjacent to epidermal cells producing Dpp. Vertebrate orthologues of the *Drosophila* neuroblast determining genes (Nkx-2 ⇔ vnd; Gsh ⇔ Ind; Msx ⇔ Msh) are expressed in the same relative position with respect to the future ventral midline of the CNS (= floorplate) and the epidermis (which expresses BPM-4, the vertebrate orthologue of Dpp). In both organisms, neuroectodermal cells contain BMP antagonists (e.g., Sog in *Drosophila* and Chordin in vertebrates).

In *Drosophila*, where the functional interrelationships of these three genes have been well studied, mutants lacking function of any of these genes fail to form neuroblasts derived from the corresponding region. In addition to these genes promoting neuroblast fates appropriate to the three rows of neuroblasts, they engage in cross-regulatory interactions reminiscent of the posterior dominance exhibited by the Hox genes. In this current case, the ventral genes are dominant in the sense that *vnd* represses expression of *ind*, which represses expression of *msh*. Whether a similar cross-regulatory relationship contributes to defining the mutually exclusive patterns of the corresponding vertebrate neuroblast identity genes remains to be determined.

Appendage Outgrowth and Axis Patterning

Appendages typically develop within the context of an already well-organized embryo or larva. The A/P and D/V axes of the appendage therefore derive from the preexisting body axes. Because appendages emerge as outgrowths from the body wall, they have a third direction of polarity, the P/D axis. Although there are significant differences in the structure of appendages forming in vertebrates and invertebrates as well as in the molecular mechanisms underlying their formation, a core set of genetic pathways appears to have defined the primary axes of all appendages (Fig. 3–6).

A/P Axis

Early in appendage development of both vertebrates and invertebrates, A/P axis formation involves creation of a posterior source of the secreted short-range signal Hedgehog (Hh; see Chapter 16). The mechanisms for generating the posterior source of Hh appear to be different in vertebrates and invertebrates, but the effect of Hh is similar, which is to activate expression of a longer-range secondary BMP signal. This posterior source of Hh in vertebrate limbs was identified by classical transplantation experiments (Saunders and Gasseling, 1968) similar to those that defined the Spemann organizer and named the ZPA. The subsequent graded spread of BMPs across the appendage defines positions along the A/P axis, which ultimately leads to the formation of specific structures such as bones in a human hand or veins in a fly wing (reviewed in Pearse and Tabin, 1998; Capdevila and Izpisua Belmonte, 2001).

D/V Axis

Narrow stripes of cells separating the dorsal (e.g., back of the hand) and ventral (e.g., palm) surfaces of limb primordia play critical roles in orchestrating the outgrowth and patterning of vertebrate and invertebrate appendages. These cells arise in response to localized activation of the Notch signaling pathway (see Chapter 39) at the interface between the dorsal and ventral surfaces of the appendage, the AER (reviewed in Capdevila and Izpisua Belmonte, 2001). In both *Drosophila* and vertebrate systems, glycosyl transferases in the Fringe family are required to activate Notch ligands along the margin of the appendage (Irvine, 1999; Wu and Rao, 1999).

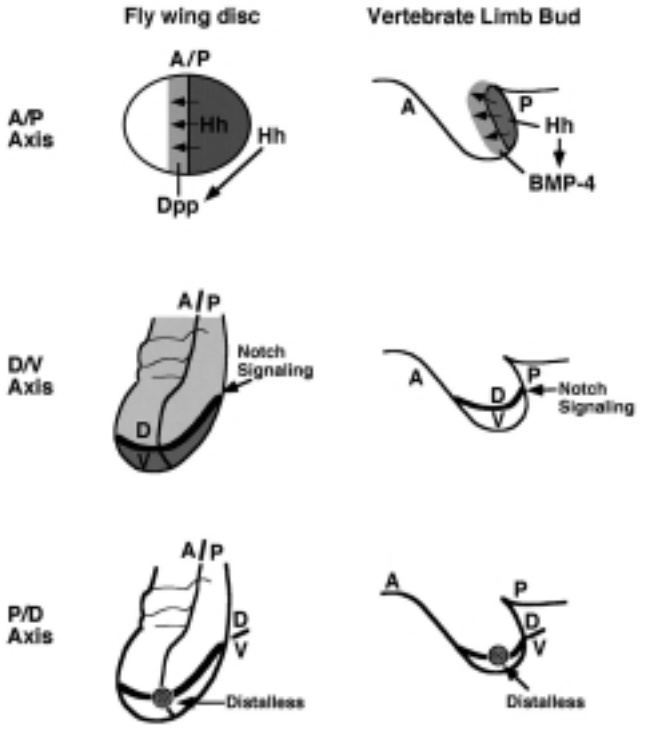

Figure 3–6. Similarities in patterning the primary axes of vertebrate and invertebrate appendages. Anterior–posterior (A/P) axis (top panels): in the primordia of *Drosophila* and vertebrate limbs, posterior cells express the short range signal Hh (dark gray domains), which induces expression of the longer range BMP morphogens (light gray domains). Dorsal–ventral (D/V) axis (middle panels): cells at the interface between dorsal and ventral domains of *Drosophila* and vertebrate limbs are defined by activation of the Notch signaling pathway (black stippled lines). Proximodistal (P/D) axis (bottom panels): Expression of *Distalless* gene (concentric circles), which is activated at the distal tip of *Drosophila* and vertebrate appendages, is required for distal outgrowth of the appendages.

P/D Axis

As appendages grow out from the body wall, they express the homeodomain protein Distalless (Dll) at their distal tips. Dll is also expressed in other tissues of developing animals. In animal systems where function of Dll genes has been determined, it has been found that Dll function is required for appendage outgrowth in many, but not all, cases. The fact that Dll is expressed at the distal tip of all body wall outgrowths, including the tube feet of starfish (Panganiban et al., 1997), suggests that this gene performed a function required to initiate such outgrowth in the bilateral ancestor of vertebrates and invertebrates (Panganiban, 2000; Zerucha and Ekker, 2000).

Early Heart Development

Although the issue of early heart development remains unresolved (see Chapter 9), there are a few examples of genes that are apparently conserved to primarily specify the development of one organ. The term *master control gene* has been coined to denote this class of embryonic patterning genes (Halder et al., 1995). Interestingly, some of these master control proteins also contain homeodomain motifs that are distantly related to the original homeodomain signature found in Hox transcription factors, while others are transcription factors of other types. As seen below, it has been argued that these genes control the development of specific organs, but it is also possible that these genes control regional identities in certain germ layers which just happen to develop functionally similar organs in vertebrates and invertebrates.

One of the so-called master control genes is required for the development of a blood-pumping organ in many animals whose hearts are of diverse shapes and sizes. This work began with the study of a *Drosophila* homeobox gene that was expressed in both dorsal mesoderm and the dorsal vessel (the insect equivalent of the heart). The dorsal vessel is a tubular muscle that circulates hemolymph within the open body cavity (Frasch, 1999). The *Drosophila* heart gene was named *tinman*, after the character in *The Wonderful Wizard of Oz* (Baum, 1997), who believes he lacks a heart. Mutations in *tinman* resulted in dead larvae that were missing the dorsal vessel, along with other dorsal mesoderm derivatives (Azpiazu and Frasch, 1993; Bodmer, 1993).

Homology cloning revealed that mice have *tinman*-like genes, one of which is called *Nkx2.5* or *Csx*. The *Nkx2.5/Csx* gene is expressed in the fetal heart primordia (Komuro and Izumo, 1993; Lints et al., 1993), a pattern that is similar to *tinman* gene expression in *Drosophila*. Targeted mutation of *Csx/Nkx2.5* results in embryonic lethality, and embryonic heart development is arrested at the initial stage of heart looping (Lyons et al., 1995). There is also evidence from human genetics that the human *NKX2-5* gene (localized to chromosome 5q35) is required for normal heart morphogenesis. Several cases of familial congenital heart disease with defects in the morphology of the atrial septum and in atrioventricular conduction have been associated with both haploinsufficiency and gain-of-function mutations in the *NKX2-5* gene (Schott et al., 1998). All of this information has led to the proposal that the Csx/NKX2-5/Tinman-like proteins are ancestral determinants of heart and surrounding visceral mesoderm. Ranganayakulu et al. (1998) indicated that the common function of genes in this class may be to specify a positional identity in visceral mesoderm, which in both flies and mice happens to develop into a blood-pumping organ, and that the common ancestor of mammals and insects did not have a blood-pumping organ truly homologous to that in present-day animals.

In addition to heart primordia, the mesodermal layer of the embryo gives rise to muscle, bone, and connective tissues. While the earliest events in specification of the mesoderm vary in different animal groups, one common denominator has been found in the development of skeletal muscle cells: a MADS box gene, *MEF2* (*D-MEF2* in the fly), is an early marker of skeletal muscle lineage in both insects and vertebrates (Lilly et al., 1994, 1995). In vertebrates, MEF2 activates and stabilizes the expression of such well-known muscle-specific genes as the bHLH homologs *Myf5*, *MyoD*, *MRF4*, and *Myogenin* (Brand-Saberi and Christ, 1999). In *Drosophila*, mesoderm fates are initially controlled by Twist and Snail proteins, and Twist directly activates *D-MEF2* (Lilly et al., 1994, 1995; Taylor et al., 1995). D-MEF2 and its vertebrate homologs are required for the completion of myogenesis in all muscles (Baylies et al., 1998; Brand-Saberi and Christ, 1999). Key features of this system have been preserved through millions of years of evolution. Such features include conservation of the MEF2 MADS domain, which mediates sequence-specific DNA binding, and conservation of DNA target sites in regulatory regions of the muscle-specific genes (Lilly et al., 1994, 1995).

Specification of Eye Organ Primordia

Another example of conservation of developmental patterning pathways was shown in a series of experiments that revealed a striking similarity in the mechanisms underlying the formation of eyes and photoreceptor cells in different animals (or the regions of the head that develop those organs, as seen below). As is often the case in genetics, relevant mutations proved crucial for unraveling the molecular pathways underlying eye development. Two such mutations have been known for quite some time: the *Aniridia* defect in humans (Hanson and Van Heyningen, 1995) and the *Small eye* (*Sey*) mutation in mice and rats (Hill et al., 1991; Walther and Gruss, 1991). The human *Aniridia* syndrome is characterized by a reduction in eye size and absence of the iris in heterozygotes. A similar defect is seen in mice that are heterozygous for the *Small eye* mutation. Mice homozygous for *Small eye* completely lack eyes and die in utero.

Molecular analysis revealed that the same gene, *Pax6*, was affected in both the *Aniridia* and the *Small eye* syndromes. Pax6 belongs to a paired box/homeodomain family of transcriptional regulators (see Chapter 59). As expected, the Pax6 protein is abundantly expressed in the eye from the earliest stages until the end of eye morphogenesis: initially in the optic sulcus and subsequently in the eye vesicle, lens, retina, and finally cornea (Hill et al., 1991; Walther and Gruss, 1991). In *Drosophila*, the genes *eyeless* (*ey*) and *twin of eyeless* (*toy*) encode proteins that are homologs of Pax6 (Quiring et al., 1994; Czerny et al.,

1999). Both *ey* and *toy* are expressed at high levels in the cells that will form a photoreceptor field of the *Drosophila* eye, as well as in other regions of the developing nervous system. Weak mutations in *eyeless* lead to the reduction or complete loss of compound eyes, whereas strong mutations are lethal when homozygous (Quiring et al., 1994). Even more striking was the observation that targeted expression of the mouse *Pax6* genes in various fly tissues led to the formation of small ectopic *Drosophila* eyes on the wings, legs, and antennae (Halder et al., 1995). These results demonstrate that the *Pax6* and *eyeless* genes are not only required but sufficient to promote eye development, and they have been called master control genes for eye morphogenesis.

A traditional view, based on the drastic differences observed in eye development and structure in mammals, insects, and mollusks, holds that eye organs evolved independently in different phyla (von Salvini-Plawen and Mayr, 1977). Indeed, this is partly true as the organization of the organ has diverged extensively in different animal lineages. However, the current evidence suggests that a variety of modern animals specify fields of photoreceptor cells using the same *Pax6* controls that triggered the development of the ancestral eye. Recently, *Pax6* homologs have also been identified in other triploblastic animals (e.g., flatworms, nematodes) and even in cnidarians (Callaerts et al., 1999 and references therein). Deep conservation in the visual system is further supported by the fact that all animals use opsins as photoreceptor proteins (Goldsmith, 1990). However, it is also possible that the *Pax6* and *eyeless* genes specify a head regional identity that includes an eye organ in both vertebrate and invertebrate lineages that just happens to include the eye as a specialization of that region. Evidence for this is found in the fact that nematodes, which have no eyes, also conserve a *Pax6*-like gene that is expressed in the head region (Chisholm and Horvitz, 1995); in addition, ablation of *Pax6/eyeless* gene function in *Drosophila* results in headless flies (Jiao et al., 2001).

As described above and in other chapters of this volume, the existence of so many common genetic pathways between distantly related organisms suggests that the ancestor of all bilaterally symmetric animals was a sophisticated creature, with many architectural and organ-specifying genetic systems already in place (De Robertis and Sasai, 1996; Knoll and Carroll, 1999). Figure 3–7 shows a proposed diagram of that ancestral worm-like creature.

Nervous System Wiring

Genes controlling other developmental and physiological functions (see Chapter 71) have also been highly conserved during the evolution of the bilateralia. For example, attractive and repulsive guidance factors directing early outgrowth of axons in the CNS toward or away from the CNS midline have been highly conserved (Kaprielian et al., 2001). A class of factors that act as attractants for most commissural axons, guiding them to the midline, are the netrins (Serafini et al., 1994). In addition, netrins repel a subset of axons from the midline. Analysis of mutants lacking the function of genes encoding the netrins and netrin receptors have revealed a similar requirement for these factors in midline guidance in *C. elegans* (Hedgecock et al., 1990; Ishii et al., 1992; Leung-Hagesteijn et al., 1992), *Drosophila* (Harris et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996; Keleman and Dickson, 2001), and mice (Serafini et al., 1996; Fazeli et al., 1997; Leonardo et al., 1997). In all three organisms, loss-of-function *netrin* mutants result in failure of commissural axons to be attracted to the midline as well as failure of a subset of projections to avoid the midline (Hedgecock et al., 1990; Harris et al., 1996; Mitchell et al., 1996; Serafini et al., 1996). Similarly, the attractive and repulsive effects of the Netrins are mediated by two distinct types of Netrin receptor in all three species. Netrin receptors most closely similar in amino acid sequence to the *C. elegans* Unc-40 receptor are required to mediate the attractive component of the Netrins (Hedgecock et al., 1990; Kolodziej et al., 1996; Fazeli et al., 1997) whereas receptors most similar to the *C. elegans* Unc-5 receptor are necessary in the subsets of axons that are repelled by the midline (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992; Leonardo et al., 1997; Keleman and Dickson, 2001).

Another clear example of a phylogenetically conserved system for midline guidance is axon repulsion mediated by the Slit/Robo signaling system (reviewed in Rusch and Van Vactor, 2000; Guthrie, 2001). Slit is secreted from midline cells (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999) and in a dose-dependent fashion repels Robo-expressing axons from the midline (Kidd et al., 1998a, b). Axons that are most sensitive to the Slit repellent express multiple isoforms of the Robo receptor, while those less sensitive express fewer isoforms (Simpson et al., 2000). Since commissural axons that do cross the midline express Robo, they would be prevented from crossing if it were not for the action of the *commisureless* gene (Tear et al., 1996), which is responsible for down-regulating Robo protein levels in appropriate axons near the midline (Kidd et al., 1998b). This transient down-regulation of Robo allows the attraction mediated by Netrin signaling to overcome the repulsion by low Robo signaling. Once the commissural axons cross the midline, they reexpress Robo on the cell surface and are prevented from recrossing the midline. Since axon fibers expressing differing numbers of Robo isoforms are differentially sensitive to Slit repulsion, they are chased to different distances from the midline and end up following one of three major radially organized axon bundles. In addition to midline repulsion mediated by Slit/Robo activity, a group of Ig domain–containing repellents known as the Semaphorins (Kolodkin et al., 1993) also act in vertebrates and invertebrates to divert axons from the midline (reviewed in Giger and Kolodkin, 2001).

Nervous System Function

Given that the common ancestor of the bilateralia had in place genetic systems for specifying and wiring the nervous system, it is not surprising that it also appears to have evolved the basic molecular processes required for the proper physiological properties of neurons, such as ion channels required for action potential generation and conduction as well as the complex secretory machinery required for release of neurotransmitters.

Ion channels are one of the best studied classes of proteins known. Ever since the mathematical formations of Hodgkin and Huxeley

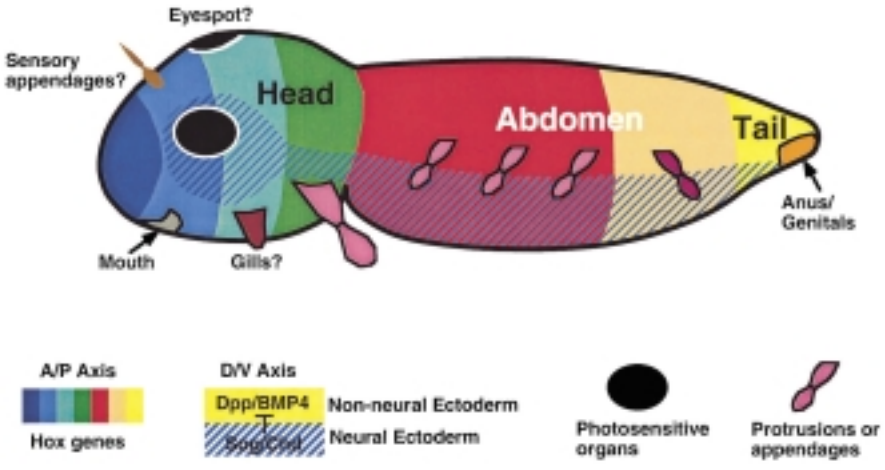

Figure 3–7. Conserved developmental patterning systems. Examples of patterning mechanisms that have been conserved since the divergence of invertebrate and vertebrate lineages include the following: determination of segmental identity along the A/P axis by a series of related Hox genes, subdivision of the ectoderm into neural versus non-neural domains via suppression of BMP signaling in neural domains, speciation of light sensitive primordia by *Eyeless/Pax6*, and patterning the primary axes of protrusions from the body wall (e.g., patterning A/P axis by Hh->BMP signaling, defining border between D/V territories by Notch signaling, and promoting appendage outgrowth by Distalless).

(1952), modeling axons as leaky cables containing voltage gated ion channels, electrophysiological studies have defined detailed in vivo kinetic parameters of ion channels that underlie various electrical phenomena such as the voltage-dependent propagation of action potentials and release of neurotransmitters in presynaptic nerve terminals, the rapid and slow chemical responses of postsynaptic cells to neurotransmitters, and the conduction of electrical impulses in muscle and heart (reviewed in Pallotta and Wagoner, 1992). The similarities in the voltage-dependent properties of action potential propagation in vertebrate in invertebrate axons suggested that similar types of ion channel were involved in defining the electrical behavior of neurons in diverse species. The identification of genes encoding a broad variety of ion channels has confirmed this prediction as there are clear counterparts to vertebrate voltage-gated Na^+, K^+, Ca^+, and Cl^- channels as well as homologs of chemically activated channels such as the acetylcholine, glutamate, GABA, and many peptide transmitters in invertebrates such as *Drosophila* and *C. elegans*. Sequence comparison of these various ion channel proteins reveals that the most recent common ancestor of bilateralia had already evolved specialized prototypes for each of these channel families. Not surpisingly, a variety of neurological disorders in humans have been associated with alterations in ion channel function (reviewed in Cooper and Jan, 1999).

The mechanism by which neurotransmitter-containing vesicles are released following depolarization of axon terminals and Ca^{2+} entry has also been very well studied in both vertebrate and invertebrate systems (Wu and Bellen, 1997; Fernandez-Chacon and Sudhof, 1999; Li and Schwarz, 1999; Lin and Scheller, 2000). Specialized protein complexes have been identified which are required for the vesicle docking (Sec1), fusion (Ca^{2+} activation of the soluble *N*-ethylmaleimide-sensitize factor [NSF] attachment protein [SNAP] receptor [SNARE] complex: Ca^{2+}-bound Synaptotagmin, Synaptobrevin, and SNAP25) of synaptic vesicles at defined release sites in the plasma membrane, followed by ATP-dependent dissociation of the core complex (αSNAP, βSNAP, NSF) and Dynamin-mediated endocytosis of vesicular components. As in the case of ion channels, counterparts of nearly all components identified in vertebrate systems are also present in *Drosophila* and *C. elegans* (Wu and Bellen, 1997; Fernandez-Chacon and Sudhof, 1999; Li and Schwarz, 1999; Lloyd et al., 2000). In a genomewide survey, it was found that *Drosophila* vesicle release proteins on average share approximately 70% amino acid identity with their vertebrate counterparts (Lloyd et al., 2000). The diversity and functional equivalence of homologous ion channel genes and components required for Ca^{2+}-dependent synaptic release strongly suggest that the ancestor of all bilateralia possessed a sophisticated interconnected nervous system and that the basic properties of the nervous system function are shared by all its descendents.

Immune Function

Another striking example of a highly conserved physiological process is the innate immune response, which is mediated by the Toll signaling pathway (reviewed in Wasserman, 2000). The core pathway in both vertebrates and *Drosophila* is initiated by ligand binding to the Toll receptor and assembly of a membrane complex including a conserved kinase, which phosphorylates a cytoplasmic protein in the IκB family (Cactus in *Drosophila*), leading to release and nuclear translocation of a bound transcription factor in the NFκB family (Dif or Dorsal in *Drosophila*). The liberated NFκB-related protein then activates genes that mediate innate immunity (Karin, 1999; Wasserman, 2000). The targets of innate immunity are quite different in vertebrates and flies (e.g., genes mediating cell proliferation, cell–cell signaling, environmental stress, and inflamatory responses in vertebrates [Li et al., 2001] and bactericidal Cecropins in flies [Ip et al., 1993; Meng et al., 1999; Rutschmann et al., 2000]), but this simple immune system is absolutely required for survival in mammals, whereas loss of the antigen-specific component of the highly specialized vertebrate immune system (e.g., B cell– and T cell–mediated) leads to a less severe and conditionally viable form of immune suppression.

Organism-Specific Thematic Variations

Although we have stressed the similarities of the patterning processes acting in vertebrate and invertebrates in this section, it is also important to note that there are organism-specific variations, which in some cases are quite surprising given the overall conservation of patterning mechanisms. For example, while molecules in the BMP family are expressed in the dorsal region of the developing vertebrate neural tube (where they play a key role in patterning cell fates and suppressing alternative ventral fates) and other regions of the nervous system (e.g., Mowbray et al., 2001), the expression patterns of clear counterparts of these genes can vary significantly between mouse, *Xenopus*, zebrafish, and chicken. Similarly, although the *chordin* and *noggin* genes are expressed in the Spemann organizer equivalent of a chick embryo (Henson's node), these factors do not appear to play as primary a role in establishing neural cell fates by inhibiting BMP signaling in the chick (Connolly et al., 1997; Streit et al., 1998). Other factors/pathways derived from Henson's node may have taken over this primary neural inducing activity (Alvarez et al., 1998; Streit and Stern, 1999; Sasai, 2001). Thus, it is important to bear in mind that even mammalian systems may not always provide accurate models for the role of developmentally important genes in humans.

A BROAD SPECTRUM OF HUMAN DISEASE GENES HAVE INVERTEBRATE COUNTERPARTS

Given the high degree of evolutionary conservation in the genetic circuitry controlling developmental processes in vertebrates and invertebrates, as well as basic physiological processes, a natural question is whether other genes might also be members of conserved molecular machines. Genome-scale gene sequence comparisons indicate that there are many related protein-coding sequences across genomes as diverse as yeast, nematodes, flies, and vertebrates (Table 3–2) (Lander et al., 2001). More focused analyses of genes implicated in genetic forms of human disease indicate that they also have high levels of sequence conservation in model organisms such as fruit flies and nematodes. For example, a systematic analysis of *Drosophila* counterparts of human diseases gene listed in the OMIM database revealed that approximately 74% of all human disease gene entries had matches in flies with expectation values (e values) of $\leq 10^{-10}$. As would be predicted from the greater similarity of the fly versus yeast genome to humans, only 50% of the human disease genes with matches in *Drosophila* ($e \leq 10^{-10}$) also had hits with yeast proteins at comparable stringency. Statistical matches in this probability range typically indicate that matching sequences are nearly certainly related by descent from a common ancestral gene but do not suggest that the genes necessarily carry out equivalent functions. For example, members of large gene families, such as the G protein–coupled receptors, receptor tyrosine kinases, or transcription factor subclasses (e.g., homeobox, helix-loop-helix, and zinc finger), often match other functionally distinct members, with e values in this range. Although e values cannot be used alone to deduce the functional equivalence of two related gene sequences, in general, genes which have been shown to function in cross-phylum experiments have counterparts with e values in the range

Table 3–2. Genome Comparisons of Model Organisms

Organism	Transcriptome Size	Percentage of Genes Similar to a Human Gene	Cellular Complexity	Genetic Screening	Generation Time
Yeast	6,200 genes	46%	1 cell	$>10^9$ progeny	2 hours
Nematode	18,300 genes	43%	959 cells	10^6–10^7 progeny	3 days
Fly	14,400 genes	61%	$>10^6$ cells	10^5–10^6 progeny	10 days
Mouse	30,000–80,000 genes	95%–97%	$>10^9$ cells	10^2–10^3 progeny	6 weeks

Source: Lander et al. (2001). *Nature* 409: 860–921.

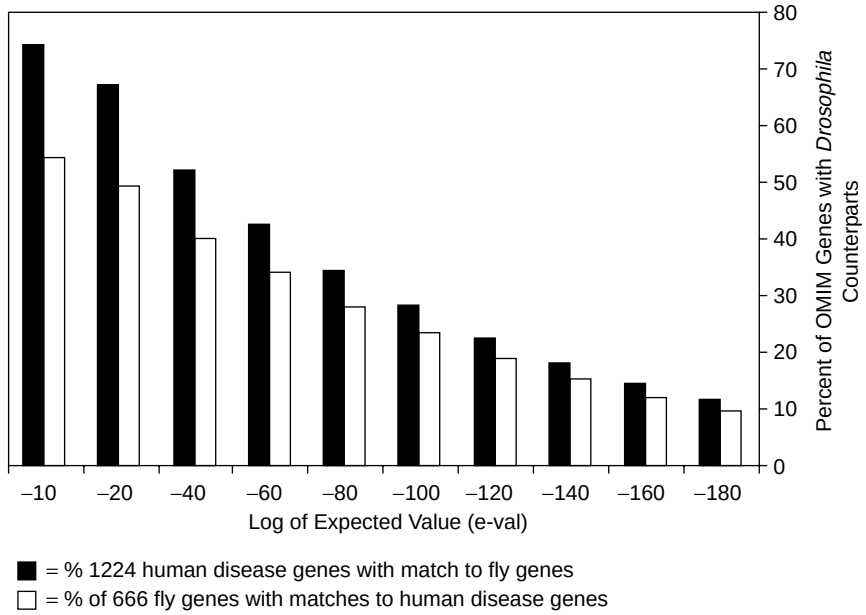

Figure 3–8. Percentage of human disease genes with fly counterparts. Black bars show percentage of 1224 human disease genes with match to fly genes; white bars show percentage of 666 fly genes with matches to human disease genes.

of $\leq 10^{-100}$. Nearly 30% of human disease genes have matches to genes at this stringent level of sequence similarity (Fig. 3–8). This high degree of cross-species sequence similarity suggests that the *Drosophila* homologs of human disease genes will frequently share important functional characteristics with their human counterparts.

Another important indication that model organisms will be of widespread utility in analyzing the function of conserved molecular machines is that a very broad spectrum of human disease genes have invertebrate counterparts. In the case of *Drosophila*, there are matches to diseases in categories as diverse as cancer, cardiac diseases, neurological diseases, immune dysfunction, metabolic disorders, and, as highlighted in this review, developmental disorders. Furthermore, these human disease genes encode proteins acting in virtually every known biochemical capacity ranging from transcription factors to signaling components to cytoskeletal elements to metabolic enzymes. Thus, it would appear that genes involved in development are likely typical rather than special in being highly conserved functionally during evolution of the bilateralia.

Examples of Human Diseases Caused by Mutations in Developmental Patterning Genes

Disease Phenotypes Associated with Mutations in Hox Genes

Despite the scarcity of available mutations in human and mouse Hox genes, it is possible to make a few generalizations about the observed effects of such genetic lesions. In many cases, mutations involving one or several mammalian Hox genes do result in homeotic transformations, but they are also associated with loss of axial structures and organs and other nonhomeotic malformations (Mark et al., 1997). Part of the reason for the highly complex mutant phenotypes is that Hox genes are involved in an elaborate system of intra-cluster interactions and intercluster redundant functions.

Hox genes are not required solely for the proper development of the rostrocaudal main body axis. In mammals, the posteriormost members of the *HOXC*, *HOXD*, and *HOXA* clusters (*HOXC9-13*, *HOXD9-13*, and *HOXA11-13*, respectively) are expressed in developing limb buds (Zákány and Duboule, 1999). Many of the same genes from the *HOXD* and *HOXA* clusters are also expressed in external genitourinary structures (Peterson et al., 1994; Kondo et al., 1997). The limb and genital defects observed in mice and humans that possess mutations in the posterior Hox genes indicate that these expression patterns are crucial for the proper development of the mentioned body parts.

Several groups have reported heterozygous and homozygous synpolydactyly phenotypes that co-segregated with an expansion in a 15-residue polyalanine stretch in exon 1 of the *HOXD13* gene (Akarsu et al., 1996; Muragaki et al., 1996) (Table 3–3). A significant increase of the penetrance and severity of the phenotype correlated with increasing expansion size. Interestingly, the family with the largest expansion included affected males with hypospadias, which is not a fea-

Table 3–3. Mutations in Human HOX Genes and Associated Phenotypes

Disease	Human Gene	Fly Gene	e Value	Component
Heterozygous synpolydactyly: Fingers 3/4 and toes 4/5, with polydactyly in the cutaneous web between digits.	*HOXD13*	*Abd-B*	6×10^{-13}	Transcription factor
Homozygous synpolydactyly: Short hands/feet. Complete soft tissue syndactyly of all four limbs. Preaxial, mesoaxial, and postaxial polydactyly of hands. Loss of tubular shape of carpal, metacarpal, and phalangeal bones.				
Single bone in zeugopod: Radial appearance. Monodactyly with biphalangeal digit and absence of carpal ossification in four limbs. Hypoplastic male external genitalia and cryptorchidism.	*HOXD9-13* deletion	*Abd-B*		
Hand-foot-genital syndrome: Small hands and feet, short great toes, abnormal thumbs. Short first metacarpal and metatarsal, short fifth fingers, carpal and tarsal fusions, small pointed distal phalanx of first toe. Müllerian duct fusion (bicornuate or didelphic uterus). Displaced urethral opening and displaced urethral orifices in bladder wall. Hypospadias.	*HOXA13*	*Abd-B*	4×10^{-12}	Transcription factor
Hand-foot-genital syndrome: Velopharyngeal insufficiency. Persistent ductus botalli.	*HOXA11-13*	*Abd-B*	3×10^{-17}	Transcription factor

ture of classic synpolydactyly but conforms to the genital expression of the gene in mammals.

Two different intragenic *HOXD13* deletions that resulted in premature stop codons have been associated with a phenotype with some features of synpolydactyly and a novel foot malformation (Goodman et al., 1998). Such truncations would eliminate the function of the HOXD13 protein, which suggested that this synpolydactyly phenotypic variant was due to haploinsufficiency for the *HOXD13* gene. Finally, monodactylous limbs and abnormal genitalia were observed in two unrelated patients that were heterozygous for deletions spanning the whole *HOXD* cluster and nearby loci (Del Campo et al., 1999).

Mutations in the posterior genes of the *HOXA* cluster also result in abnormal limb and genital development. The classic hand-foot-genital syndrome is associated with heterozygosity for a nonsense mutation in the homeodomain of HOXA13 (Mortlock et al., 1996) (Table 3–3). This nonsense mutation may generate a truncated protein that would be unable to bind DNA; thus, it is possible that haploinsufficiency for HOXA13 is the mechanism leading to the phenotype. The importance of a diploid dose of the *HOXA* genes is further suggested by the phenotype of a patient with a large deletion spanning the *HOXA* cluster. This patient possessed features of the hand-foot-genital syndrome and other anomalies, possibly caused by deficiency of other members of the cluster (Devriendt et al., 1999).

Conserved Signaling Pathways in Vertebrates and Invertebrates Are Targets for Disease

Systematic genetic analyses of pattern formation in *Drosophila*, *C. elegans*, and *dictyostelium* have uncovered a surprisingly limited number of distinct signaling systems involved in cell fate development. These pathways include the TGF-β-related/BMP, receptor tyrosine kinase (RTK), Notch, Toll, G protein–coupled receptor, Hedgehog (Hh), Wingless (Wg) and Janus kinase/signal transducer and activator of transcription (JAK/STAT) signal-transduction networks. In addition, several signaling systems have been implicated in axonal pathfinding and synapse formation, including by the Netrin, Round About (Robo), Semaphorin, Neuroglian, and BMP-mediated pathways. Disease-causing mutations have been identified in components of nearly all of these major signaling pathway categories (Reiter et al., 2001). Consistent with the high degree of evolutionary conservation between vertebrate and invertebrate genetic systems, many human diseases associated with mutations in signal-transduction pathways lead to developmental disorders, as illustrated by the diseases covered in this volume. Since signaling pathways are also intimately tied to regulation of the cell cycle, another common consequence of disrupting signaling systems is failure of growth control and cancer.

One notable trend among diseases associated with mutations in components of signaling pathways is that defects in extracellular components such as ligands or in ligand-specific receptor subunits often result in limited developmental defects while mutations in more downstream intracellular components, which mediate the action of many ligands, often result in cancer (Reiter et al., 2001). For example, in the BMP pathway, mutations affecting the human BMP2/4 ligand and BMP5/7-specific type I receptor lead to morphological defects such as brachydactyly and hereditary hemoragic telangiectasia, whereas mutations in the shared BMP2 type II receptor or the signal transducer SMAD4 cause cancer (Table 3–4). Similarly, in the case of the RTK pathway, mutations in genes encoding FGFR chain isoforms lead to restricted conditions such as achondroplasia, while mutations in RAS, the cytoplasmic transducer of all RTKs, lead to cancer (Table 3–5).

CROSS-GENOMIC ANALYSIS OF HUMAN DISEASE GENE FUNCTION USING MODEL SYSTEMS

Model genetic systems have long been appreciated for their value in delineating basic biological mechanisms and uncovering fundamental principles of molecular organization. When work was initiated on such model systems as *Drosophila* and *C. elegans*, the deep genetic homologies between these organisms and humans were not yet evident. The expectations of these studies were largely to provide detailed examples of how various biological processes might be carried out with the hope that these concepts would be helpful in dissecting similar but mechanistically distinct processes in humans. One of the reasons we have gone into such detail in describing the similarities between vertebrate and invertebrate development is that the idea that the common ancestor of bilateral animals was such a highly evolved creature, which had already invented most of the morphogenetic systems in existence today, was initially a great surprise to us all. Prior to these revelations, the images that the field had conjured up of this ancestor were more along the lines of a facultatively colonial organism such as a slime mold.

As the image of our common ancestor has come into clearer focus, it has become increasingly apparent that model systems initially chosen for their experimental advantages might actually be good models for genes involved in human disease. Since the molecular devices which suffer insults causing disease states in humans were likely to have been present in the ancestor of the bilateralia and a high proportion of known human disease genes (e.g., $\approx$30%) have extremely good matches ($e \leq 10^{-100}$) to genes present in flies, it seems likely that flies, worms, and humans share many genetic systems involved in the formation and function of these systems. An important challenge now is to find the most effective ways to exploit the deep functional homologies between model genetic systems and humans to help solve defined problems in medical genetics.

"Closing the Loop"

Given that completed genome sequences now exist for nematodes, flies, mice, and humans and given all of the functional homologies described above, the time is now ripe to use cross-genomic approaches to help answer specific questions in medical genetics. Many types of question could in principle benefit from cross-genomics. For example, there are situations in which *(1)* the function or mechanism of action of the disease genes is unknown, *(2)* the effector targets of a gene (e.g., a transcription factor or an E-3 ubiquitin ligase) are unknown, and *(3)* the identity of a human second-site modifier locus is unknown. In addition, only about 20% of the estimated 4000–5000 disease genes have yet been identified.

In this section, we will discuss three examples that illustrate the potential utility of model systems in addressing explicit questions regarding human diseases. In general, the goal is to create mutants in the human disease gene counterpart in the model organism and conduct genetic screens to identify new candidate genes in humans that may play an important role in disease etiology. The final goal in each case is to "close the loop" between the model system and humans by having an explicit test in mind to validate the relevance of candidate

Table 3–4. BMP Pathway Diseases

Disease	Human Gene	Fly Gene	e Value	Component
Fibrodysplasia ossificans progressiva	*BMP2*	*dpp*	6×10^{-76}	Ligand
Fibrodysplasia ossificans progressiva	*BMP4*	*dpp*	2×10^{-76}	Ligand
Brachydactyly type C	*BDC*	*dpp*	3×10^{-36}	Ligand
Acromesomelic dysplasia Hunter-Thompson type	*CDMP1*	*dpp*	3×10^{-36}	Ligand
Hereditary hemorrhagic telangiectasia-2	*ALK1*	*sax*	1×10^{-132}	Specific type I receptor
Persistent müllerian duct syndrome type II	*AMHR*	*wit*	2×10^{-52}	Specific type II receptor
Colorectal cancer, familial nonpolyposis, type 6	*TGFBR2*	*put*	8×10^{-70}	General type II receptor
Polyposis, juvenile intestinal	*JIP*	*med*	1×10^{-10}	Cytoplasmic transducer
Pancreatic cancer	*SMAD4*	*med*	1×10^{-108}	Cytoplasmic transducer

Table 3–5. RTK Pathway Diseases

Disease	Human Gene	Fly Gene	e Value	Signaling Component
Obesity with impaired prohormone processing	*PC1*	*Fur1*	1×10^{-165}	Protease: ligand activation?
Crouzon's syndrome: achondroplasia, craniosynostosis	*FGFR3*	*htl*	1×10^{-129}	Receptor
Pfeiffer's syndrome	*FGFR1*	*htl*	1×10^{-124}	Receptor
Venous malformations, multiple cutaneous and mucosal	*TIE2*	*htl*	6×10^{-63}	Receptor
Apert's syndrome: Beare-Stevenson cutis gyrata	*FGFR2*	*htl*	1×10^{-131}	Receptor
Mast cell leukemia: mastocytosis, piebaldism	*KIT*	*htl*	6×10^{-65}	Receptor
Diabetes mellitus: insulin-resistant, leprechaunism, Rabson-Mendenhall syndrome	*INSR*	*InR*	1×10^{-300}	Receptor
Renal cell carcinoma	*MET*	*Alk*	6×10^{-53}	Receptor?
Predisposition to myeloid malignancy	*CSF1R*	*CG8222*	7×10^{-70}	Receptor?
Elliptocytosis-1	*EPB41*	*cora*	1×10^{-130}	Cyoskeletal scaffolding?
Ehlers-Danlos syndrome type X	*FN1*	*Ptp10D*	5×10^{-39}	Tyrosine phosphatase
Colon cancer	*PTPG1*	*Ptp99A*	4×10^{-46}	Tyrosine phosphatase
Bladder cancer	*HRAS*	*Ras85D*	2×10^{-74}	Cytoplasmic transducer
Colorectal adenoma	*RASK2*	*Ras85D*	1×10^{-78}	Cytoplasmic transducer
Colorectal cancer	*NRAS*	*Ras85D*	6×10^{-73}	Cytoplasmic transducer

genes or allelic variations identified in model systems with respect to a specific question(s) in human medical genetics.

Primary Congenital Glaucoma

Mutations in the human *CYP1B1* gene, which encodes a P-450 protein, cause primary congenital glaucoma (PCG) with high penetrance (Stoilov et al., 1997) as a result of a developmental defect in the formation of the trabecular meshwork, which drains fluid from the eye to maintain intraocular pressure. Curiously, several Saudi Arabian pedigrees have been identified in which some individuals with homozygous or compound heterozygous *CYP1B1* mutant alleles do not develop the glaucoma phenotype (Bejjani et al., 2000). Genetic mapping analysis indicated that unaffected individuals share a modifier locus on the short arm of chromosome 8, which compensates for the loss of *CYP1B1* function. The identity of this second-site suppressor(s) locus remains elusive, however, since the existing inbred pedigrees provide only an approximate map position for this gene(s).

The closing-the-loop goal for PCG is to use a model genetic system to help identify the human PCG suppressor locus. One approach is to make mutations in the single highly related *Drosophila* homolog of CYP1B1 (*cyp18a*) and then conduct genetic screens to identify suppressor loci of *cyp18a* loss-of-function mutants in *Drosophila* that have human counterparts on chromosome 8p. We have generated such loss-of-function mutations (L. Reiter and E. Bier, unpublished) and are collaborating with Dr. Bassem Bejjani (Baylor College of Medicine, Houston, TX) to determine whether this locus or other candidate suppressor loci we identify in *Drosophila* might be homologs of human genes that protect unaffected individuals with mutant copies of the *CYP1B1* gene from developing PCG.

Angelman Syndrome

Angelman syndrome (AS) may hold the best promise for closing the loop between flies and humans. AS causes severe mental retardation and other abnormalities, resulting from inactivation of the human *UBE3A* gene (Matsuura et al., 1997), which encodes an E3 ligase that conjugates ubiquitin to specific protein targets that are to be degraded. We have made mutants in the apparent *Drosophila* structural homolog (*d-as*) of *UBE3A* (L. Reiter, M. Bowers, and E. Bier, unpublished data) and are now screening for second-site modifiers of these loss-of-function *d-as* mutants with the goal of identifying candidate proteins that might be substrates of *UBE3A*-targeted degradation and cause AS phenotypes when over produced. Such candidate *d-as* degradation targets will be analyzed by our collaborator, Dr. A. Beaudet (Baylor College of Medicine), who will test whether levels of the human counterparts of these potential targets are altered in AS patients or mouse models.

Alzheimer Disease

Dr. Jane Wu and colleagues (Washington University, St. Louis, MO) identified two antioxidant proteins (thiol-specific antioxidant and proliferation associated gene) that physically interact with the Presenilin (Psn) protein (J. Wu, personal communication). We have coexpressed these proteins with Psn in *Drosophila* using the GAL4/UAS system and found that this results in a strong synergistic reduction in Notch signaling (L. Reiter, M. Wangler, M. McElroy, and E. Bier, unpublished data). We are currently trying to see whether TSA and PAG also interact with mutations in the *Drosophila* homolog of the β-amyloid gene. As a closing-the-loop goal, we will collaborate with various members in the Alzheimer field to determine whether human TSA/PAG-related genes are mutated in any of the five familial forms of Alzheimer disease. We have identified five of the 20 TSA/PAG-related genes in humans which map to intervals harboring new suspected Alzheimer loci (L. Reiter, M. McElroy, and E. Bier, unpublished data).

Multi-tier Cross-genomic Analysis of Human Disease Gene Function

As discussed above, unicellular organisms such as yeast and slime mold can be used to analyze important basic eukaryotic cellular functions such as metabolism, regulation of the cell cycle, membrane targeting and dynamics, protein folding, or DNA repair, while simple invertebrate systems such as flies or nematodes are excellent models for examining the coordinated actions of genes that function as components of a common molecular machine. The primary strength of mammalian systems such as the mouse, zebrafish, frog, and chicken is that they can provide the most accurate models for the human disease state. Given that the different model genetic systems have different strengths and limitations, more than one such system will typically offer advantages for analyzing the function of a given human disease gene. For example, all three levels of genetic systems could make important contributions to the analysis of PCG. As the mutant gene (*CYP1B1*) in PCG is a *P-450* gene, which is a member of a protein class present in yeast, one could attempt to establish assays in yeast that distinguish the function of wild-type versus mutant forms of the gene or to identify endogenous yeast genes that are required for the effect of the human *CYP1B1* gene. Because PCG in humans results from a failure to form the trabecular meshwork, which normally drains fluid from the eye, one would obviously need to turn to a multicellular organism to establish a system in which to analyze the developmental function of *CYP1B1*. Invertebrate models such as the fly (see above) are useful for identifying other genes acting together with the *P-450* gene to carry out its developmental function and to help identify human modifier loci but will not necessarily provide an accurate model for glaucoma (e.g., fly eyes do not have a morphological equivalent of the trabecular meshwork). Finally, the mouse knock-out (which exists for the homolog of *CYP1B1*) is best suited for analyzing the primary event responsible for the failure of the trabecular meshwork to develop.

We anticipate that cross-genomic studies will become an integral part of the analysis of human disease gene function. As this field

grows, an important goal should be to coordinate studies across the various genetic tiers. For example, in analyzing developmental disorders, one could use model unicellular and invertebrate organisms to identify candidate proteins interacting with the human protein of interest as part of the molecular machines that carry out cellular or developmental functions. The developmental role of these new genes could then be evaluated in a vertebrate model (e.g., by knocking them out alone or in combination in mice) and by asking whether mutations in the human counterparts of these genes result in developmental disorders. One interesting question in this regard would be whether compound heterozygosity for several of these genes in mice could lead to disorders similar to known multigenic disorders in humans. Once new medically relevant target genes have been identified through such a closing-the-loop process, these new genes would become themselves substrates for a second round of cross-genomic analysis. This need not be a purely cyclical process since as the mechanism of disease gene action becomes better defined, it should become increasingly possible to ask more hypothesis-driven questions, which again should in principle be addressed in one or another model system. Such an integrated use of multiple genetic systems should prove far more powerful than reliance on any single system.

Genetic Semantics: Cross-species Translation of Developmental Defects

Bioinformatics is another very important field that will undoubtedly help shape the future of medical genetics. As data sets derived from cross-genomic analyses accumulate, one interesting challenge will be to use bioinformatics tools to make new links between mutant phenotypes in model organisms and human disease phenotypes. This new area of interface between computational and experimental fields could be referred to as "genetic semantics" in that the problem is essentially to translate between the languages of two very different phenotype categories. In the case of model systems, systematic screens typically identify loss-of-function mutations affecting a particular process. One great advantage of such systematic screens is that they can saturate for all genes involved in that process. The famous screen carried out by Nusslein-Volhard and Wieschaus (1980) for developmental patterning mutants in *Drosophila* is a classic example of such a saturating screen. The phenotypes, or lexicon in the linguistic analogy, used to categorize gene function in such screens are often lethal and involve major defects, such as loss of entire sections of the body plan or organs. The equivalent of such homozygous mutations in human counterparts of these genes would typically not be identified as diseases in humans as they would lead to early prenatal lethality.

Because mutations in human genes that completely ablate early crucial developmental functions will not be identified by this phenotype, they tend to be found due to mutations that result in subtle recessive defects or dominant phenotypes resulting from loss of only one gene copy (e.g., haploinsufficiency). Many human disease phenotypes are indeed so subtle that they are known only as a result of the self-reporting tendency of afflicted humans and the remarkable finely honed diagnostic skills of experienced clinicians. The lexicon of this exquisitely subtle language of human disease phenotypes bears little similarity to that of the coarse tongue of loss-of-function genetics in model systems. Because the number of self-reporting mutant humans is significant (e.g., $>10^9$), human genetics is often quasi-saturating in that mutations in many components of various systems have been identified. For example, if one considers inherited cardiac diseases, mutations in nearly all of the known components involved in heart muscle contraction (e.g., actin, myosin, myosin kinase, tropomyosin, and troponin) and electrical conduction (e.g., Na^+, K^+, and Ca^{2+} channels) have been identified. Similarly, if one considers peripheral neuropathies, mutations in several protein components of myelin and peripheral nerve have been assigned to similar but distinct disease subtypes. Signaling pathways provide another example of quasi-saturation in human genetics, as exemplified by the RTK/mitogen-activated protein kinase and BMP pathways (Tables 3–4, 3–5) in which mutations in multiple components have been recovered.

The significant linguistic differences between the genetics of model systems and human disease notwithstanding, any genes which are altered in both humans and model systems will most likely perform the same or very similar molecular functions. How then can one translate between these disparate languages? One way to address this question is to cluster genes into groups using phenotypic similarities in one system and then ask whether the phenotypes associated with mutations in counterparts of these genes in the other system share anything. Text comparing algorithms such as internet search engines could be modified in principle for such purposes, and several commercially available software packages have similar capabilities. As discussed earlier, the Notch pathway illustrates a simple form of this idea. In *Drosophila*, loss-of-function mutations in the Notch pathway lead to a multitude of phenotypes, including hyperplasia of the nervous system at the expense of epidermal cell fates, disruption of D/V patterning of appendages and loss of marginal structures, as well as thickened wing veins. In *C. elegans*, mutants in the Notch (lin12) pathway lead to transformations of cell fates within the vulval cell lineage in which two cells that ordinarily would communicate via Notch signaling to generate two different cell types both develop with the default fate. There are several Notch-related receptors in mice and humans, and mutations in one of these receptors (Notch1) or in one of the Delta-related Notch ligands (Delta3) cause defects in somite segregation, which result in fusion of adjacent somites and subsequent spinal malformations. Given the conservation of signaling pathway organization during evolution, it would be reasonable to ask whether mutations in other components of the Notch pathway might lead to spinal malformations in humans. This seems likely since the mouse knock-out of a gene encoding a glycosyltransferase related to the *Drosophila fringe* gene exhibits spinal malformation phenotypes similar to those observed in Notch1 or Delta3 knockouts. Thus, in this case, one translation of the genetic lexical item Notch is excess neural development in flies, vulval defects in worms, and spinal malformation in humans.

It will not necessarily always be the case that one can identify mutations in all components of a pathway based on there being a shared disease phenotype. For example, as discussed above, a variety of ligands can funnel through a more limited number of receptors whose function may be mediated by only one or a few cytoplasmic transducing molecules. In addition, since humans often have several highly related copies of a gene, which can be expressed in very different patterns, the phenotypes resulting from loss-of-function mutations in various components of a pathway can range from specific developmental conditions (e.g., brachydactyly) to more general loss of cellular growth control (e.g., cancer). Although these factors will complicate phenotypic translation attempts, one can imagine factoring relevant data into clustering programs, such as known gene expression data gathered from the mouse. It is also possible to conduct the analysis in the reverse direction by clustering human diseases based on shared phenotype and then asking whether the counterpart genes in the various model organisms share previously unappreciated similarities. Finally, one can search for patterns of similarity between the phenotypes in mutants of homologous components in more than two organisms (e.g., compare clusters of fly to worm phenotypes and then search carefully for similarities between the disease phenotypes in the human counterparts of this set of genes). Phenotypic homology searches of this kind are likely to uncover hidden genetic relationships that would otherwise remained buried in the vast data fields of the postgenomic era in much the same way that sequence alignment programs such as MIME and Beauty have extracted critical functional information from raw amino acid sequence data (e.g., shared protein motifs).

SUMMARY AND PERSPECTIVES

An important practical consequence of the fact that vertebrates and invertebrates derived from a shared, highly structured, bilateral ancestor is that many types of complex molecular machine which were present in this creature have remained virtually unchanged in both lineages. Given that three-quarters of all known genes which cause disease when mutated in humans have counterparts in model systems such as *Drosophila*, it seems very likely that these genes will often perform similar functions in the context of similar molecular pathways or protein complexes in model organisms and humans. These deep

homologies between genetic networks can be exploited to understand the function of genes which can cause disease in humans when altered and should be very useful for identifying new genes in humans involved in disease states.

With the completion of the human genome project and the discovery of many of the most important genes involved in heritable disorders, the primary emphasis in human genetics is shifting to understanding the function of these disease genes. Model organisms ranging from yeast to mice offer distinct advantages for cross-genomic analysis of different aspects of human disease gene function. If unicellular organisms such as yeast and slime molds have closely related sequences to a given human disease gene of interest, these powerful model systems are ideal for conducting systematic screens for new genes that interact with the disease gene as part of a common eukaryotic pathway or cellular process. Since developmental disorders by definition involve interactions between cells in multicellular organisms, there is also a need for model genetic systems such as *Drosophila* and *C. elegans* that can define genes acting at the organismal level. The great advantage offered by these model genetic systems is the ability to design second-site modifier screens to identify new genes involved in a given developmental process or pathway. It is not necessary that these model organism mimic the human disease state as long as the genetic screens are successful in identifying proteins which function as part of a conserved molecular device. Finally, vertebrate model systems such as the mouse or zebrafish are essential for providing accurate models for the human disease state.

As cross-genomic approaches become a routine component in the analysis of human disease function, an interesting and important challenge will be to integrate studies in the various systems into complementary comparative programs. A critical element of this integration will be the use of computational methods to search through large phenotypic and gene expression data sets to extract hidden relationships between individual genes and genetic networks. The next decade should prove to be a very fertile period for forging this new field of comparative functional genomics.

Model Organism Genome Websites

Yeast: http://genome-www.stanford.edu/Saccharomyces/
Slime mold: http://glamdring.ucsd.edu/others/dsmith/dictydb.html
Fly: http://flybase.bio.indiana.edu:82/
Worm: http://www.expasy.ch/cgi-bin/lists?celegans.txt
Zebrafish: http://www.ncbi.nlm.nih.gov/genome/guide/D_rerio.html
Mouse: http://www.informatics.jax.org/
Human disease genes (OMIM): http://www.ncbi.nlm.nih.gov/Omim/

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