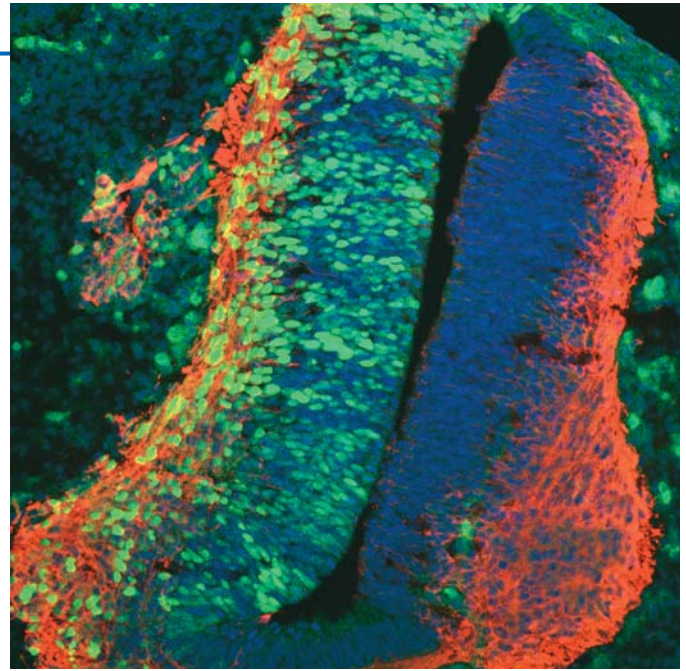


15

INTEGRATION OF SIGNALS AND GENE CONTROLS

Inherent in the full genome contained in most cells is the potential to form vastly diverse cell types, which perform an enormous variety of tasks. Each individual cell, however, employs only part of an organism's complete genetic repertoire. An array of external hormonal, metabolic, developmental, and environmental *signals* influence which genes a cell uses at any given time in its life span. Infections also can trigger many responses. A cell's response to an external signal largely depends on its properties including (1) the inventory, locations, and associations of its proteins and other molecules; (2) its shape and attachments to other cells; and (3) its chromatin structure, which facilitates or blocks access to particular genes. We can think of these properties as a cell's "memory" determined by its history and response to previous signals. Thus, for instance, a cell can respond to a signal only if it possesses a **receptor** for that signal. In addition, a cell typically receives more than one signal at a time: for example, a combination of transforming growth factor β (TGF β) and fibroblast growth factor (FGF), a hormone signal that is interpreted in light of the ambient temperature, or an electrical pulse that is modulated by local ionic conditions. The response to each signal, or condition, is often influenced by another one. This integration of signals can prevent inappropriate responses and permit more nuanced responses to multiple signals.

To understand a cell's response to one or more signals and the effect of its memory on this response, it is useful to monitor changes in the expression of all genes and changes in the locations of organelles, proteins, or other molecules. Signal-induced changes in the intracellular ionic environ-



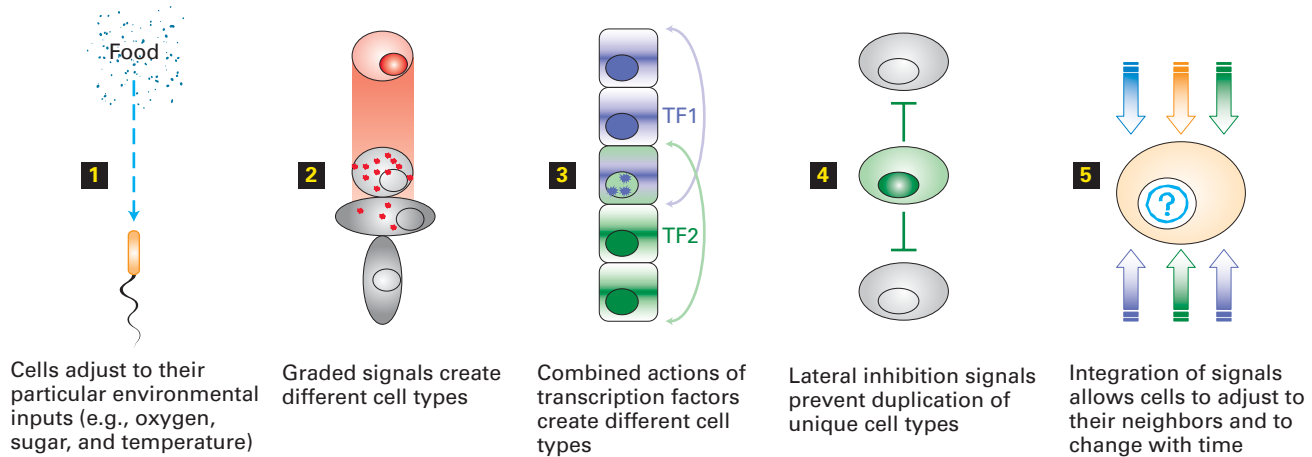
Dividing cells (blue) in the developing spinal cord will differentiate into neurons (red). Cells that were engineered to make a differentiation-inhibiting signal (green) cause persistent cell division and reduce the number of differentiated neurons at the left. [Sean G. Megason and Andrew P. McMahon. Adapted from Sean G. Megason and Andrew P. McMahon, 2002, *Development* **129**:2087–2098.]

ment, membrane potential, and cell shape also may be relevant to a cell's response. Two serious limitations have hampered efforts to obtain such a comprehensive view of the nature of cell responses to external signals. First, usually only one or a few aspects of a cell's response to a signal is easily monitored; second, determining responses in living cells in "real time" poses many technical difficulties. Technological advances are beginning to solve these problems, although neither has been completely overcome.

The major question posed in this chapter is how a cell integrates multiple signals and responds in the context of

OUTLINE

- 15.1 Experimental Approaches for Building a Comprehensive View of Signal-Induced Responses
- 15.2 Responses of Cells to Environmental Influences
- 15.3 Control of Cell Fates by Graded Amounts of Regulators
- 15.4 Boundary Creation by Different Combinations of Transcription Factors
- 15.5 Boundary Creation by Extracellular Signals
- 15.6 Reciprocal Induction and Lateral Inhibition
- 15.7 Integrating and Controlling Signals



▲ FIGURE 15-1 Signaling systems and cell responses. Cells are vibrantly alert detectors, sensing and interpreting information constantly to adjust to the environment (1) and coordinate activities with surrounding cells. A cell can respond to signals by changing the genes that it transcribes, altering the cell surface, modifying proteins and enzyme activities, moving materials between compartments, revamping its cytoskeleton, migrating, or dying. A modest number of signaling systems constitutes a core toolbox. Each system is used repeatedly in different organisms, in different tissues, and at different times. Signals are crucial in building multicellular organisms, where different cell

types are created by controlled signal transmission and reception. Cells can become different, depending on the amount of a signal (2), with a larger amount giving rise to one cell fate and a smaller amount to another. New boundaries form between cells of different types, creating tissues and demarcations within tissues. Different cell types are created by combinations of transcription factors (3). Inhibitory signals emitted by cells undergoing a differentiation step can prevent nearby cells from making the same decision (4), thus preventing duplication of structures. Cells generally integrate many signals in deciding how to proceed (5).

its memory, especially in the course of **development** and cell **differentiation** (Figure 15-1). We begin by looking at various techniques that are beginning to provide a global view of signal-induced responses. In particular, we describe how the determination of whole-genome transcription patterns is a source of new insights into responses to signals. We then consider cell responses to certain environmental perturbations in Section 15.2. The next section introduces the concept of graded regulators that cause different cell responses, depending on their concentration. This type of system allows cells at different distances from the source of a regulatory molecule to become different types of cells. We examine how such regulation creates boundaries within an epithelium in early *Drosophila* development, with cells on one side of the border taking one path of differentiation and those on the other side taking another. The creation of other boundaries by graded transcriptional activators and graded extracellular signals is discussed in Sections 15.4 and 15.5, respectively. As borders form, cells reinforce their decisions by signaling across the borders so that compatible adjacent structures form. As illustrated by the examples in Section 15.6, such signaling can either promote or inhibit particular developmental changes in adjacent cells. In the final section, we take a closer look at how signals are integrated and controlled in different cells.

Although the number of signaling pathways encountered in this chapter and others may seem overwhelming, there are actually a relatively small number of distinct pathways for transducing external signals. Primary among them are the in-

tracellular signal-transduction pathways activated by the various receptor classes listed in Table 14-1. In addition, cell–cell and cell–matrix adhesions mediated by cadherins and integrins can initiate intracellular signaling pathways (Chapter 6). The informational complexity needed to create many cell types and cell properties comes from combining signals. Elucidation of the underlying principles and mechanisms relevant to all signaling pathways forms the foundation for understanding how cells integrate signals to achieve a particular identity or other response.

15.1 Experimental Approaches for Building a Comprehensive View of Signal-Induced Responses

Several technical advances are helping investigators to discern the totality of the cellular response to signals. Perhaps the most significant advance is the sequencing of whole genomes from various organisms and subsequent analysis to identify individual genes and analyze their functions. The data amassed in these genome projects have led to the development of techniques for monitoring the effects of a signal on the expression of the entire gene set. Using gene-inactivation methods discussed in Chapter 9, researchers can mutate specific genes encoding various components of signaling pathways. The phenotypic effects of such mutations

often provide clues about the functions of pathway components and the order in which they function. In vitro studies on signaling in a variety of differentiated cell types and even complex tissues are now possible because of recent improvements in cell- and tissue-culture methods. Certain signal-induced responses can be monitored in living cells with the use of various fluorescent agents and the observation of cells in a fluorescence microscope. For instance, this technique can reveal changes in the amounts and localization of specific proteins, as well as fluctuations in H^+ or Ca^{2+} concentrations in the cytosol (see Figures 5-46 and 5-47). Development of additional fluorescent indicator dyes will allow monitoring of other molecules in living cells.

Genomic Analyses Show Evolutionary Conservation and Proliferation of Genes Encoding Signals and Regulators

In Chapter 9, we considered the difficulty and ambiguity in identifying genes within genomic sequences, especially in higher organisms. Despite the limitations, genomic analyses have been sources of exciting and sometimes surprising insights or have confirmed earlier conclusions based on the results of other types of studies.

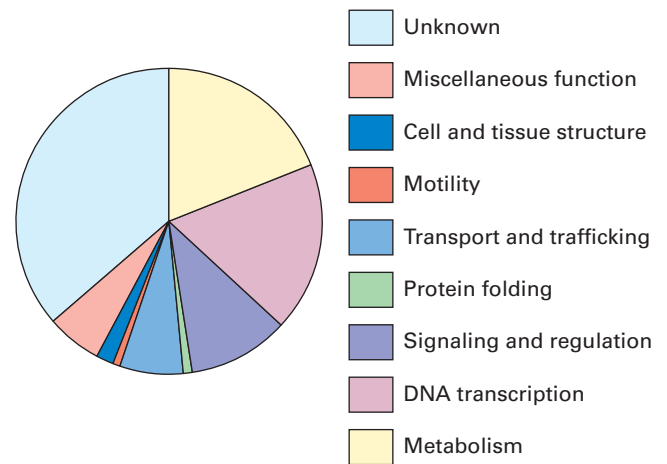
First, the total number of protein-coding genes does not correlate in any simple way with standard conceptions about animal complexity (see Figure 9-34). Humans, for instance, have only about 1.75 times as many genes as the roundworm *Caenorhabditis elegans*. Likewise, *C. elegans* has about 1.4 times as many genes as the fruit fly *Drosophila*, which exhibits a much more complex body plan and more complex behavior.

Second, genomic comparisons support the conclusion based on two decades of developmental genetics research that many regulatory genes whose encoded proteins control tissue differentiation, organogenesis, and the body plan have been conserved for hundreds of millions of years. For example, the *Pax6* gene is employed in eye development in enormously diverse organisms, such as clams, flies, and humans, and the *tinman* gene is necessary for heart development in flies and humans. As discussed in Section 15.4, the Hox gene cluster controls head-to-tail organization of the body in almost all animals examined to date. Because of the conservation of genes and proteins, the results of experiments on one organism are useful guides for research on other organisms. Indeed much of human biology and medicine has been and continues to be built on knowledge gained from a broad spectrum of experimental systems.

Third, despite the considerable commonality of genes and proteins among different animals, genomic analyses suggest that about 30 percent of the genes of each animal organism are unique to that animal. The invertebrates *Drosophila* and *C. elegans* have in common certain genes that are not recognizable in any of the other genomes analyzed to date. Flies and worms are believed to have a common ancestor that arose from an even more ancient ancestor in common with vertebrates. If this view is correct, any genes present in flies

and humans could be expected to be present in worms; likewise, any genes common to worms and humans would presumably be present in flies. Recent work has revealed that all three species have about 1500 genes in common, as expected (Figure 15-2). Contrary to expectations, however, about 1250 genes common to humans and flies are not found in worms, and about 500 genes common to humans and worms are not found in flies. Thus organism-specific gene loss occurred in the evolution of *C. elegans* and *Drosophila* subsequent to the time when the invertebrate and vertebrate lineages diverged.

Fourth, as noted in preceding chapters, duplication of certain protein-coding genes and subsequent divergence in the course of evolution have given rise to gene families. The members of a **gene family** and corresponding **protein family** have close but nonidentical sequences. Genomic analysis and findings from other studies show that the number of members in a particular protein family varies in different species. For instance, the *transforming growth factor β family* of secreted signaling proteins has 28 members in humans but only 6 in flies and 4 in worms. The semaphorins, which are signals for neural development, form a 22-member family in humans; flies have 6 members and worms have 2. Such proliferation of genes could give rise to signaling proteins that can move different distances through tissue or differ in other properties. Alternatively, the members of a gene family may be differently regulated, thus allowing rather similar proteins to be produced at different times and places. Both types of variation exist, and both allow a moderate number of types of signals to serve a multitude of purposes.



▲ FIGURE 15-2 Evolutionary conservation of core processes in human, fruit fly (*Drosophila*), and roundworm (*C. elegans*) genomes. On the basis of fairly stringent criteria for protein similarity, humans, flies, and worms have in common about 1500 genes distributed among the functional classes shown in this pie chart. About 28 percent of this common set of genes encode proteins that function in signaling or gene control. The molecular functions of about one-third of the genes and proteins common to these species are not yet known. [Adapted from J. C. Venter et al., 2001, *Science* **291**:1304.]

In Situ Hybridization Can Detect Transcription Changes in Intact Tissues and Permeabilized Embryos

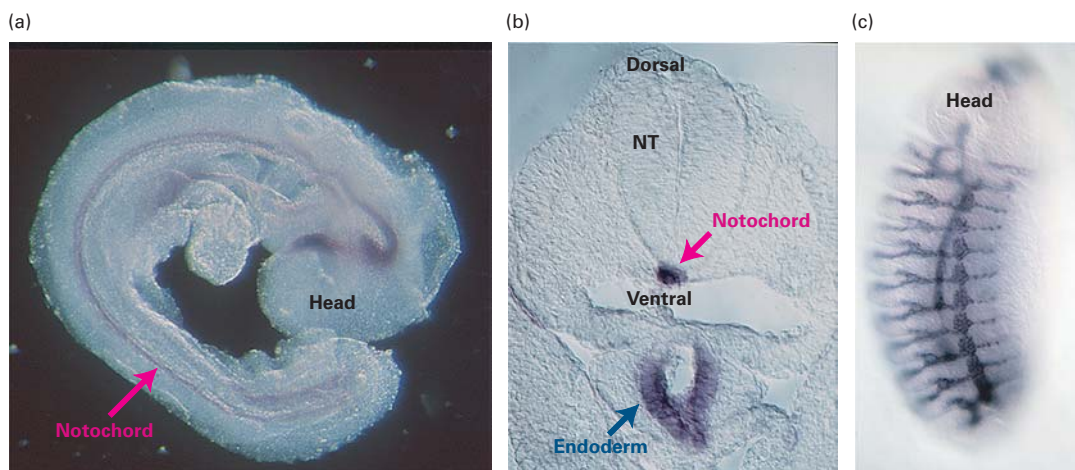
A common effect of external signals is to alter the pattern of gene expression by a cell. Signal-induced changes in the expression of particular genes is usually monitored by measuring the corresponding mRNAs or proteins in the presence and absence of a signal. The total cellular mRNA can be extracted, separated by gel electrophoresis, and subjected to **Northern blotting**, which detects individual mRNAs by hybridization to labeled complementary DNA probes (see Figure 9-26). Likewise, cellular proteins can be extracted, separated electrophoretically, and subjected to **Western blotting**, a procedure in which individual proteins separated on the blot are detected with specific antibodies (see Figure 3-35). These blotting methods are generally not sensitive enough to determine changes within a single cell. The **polymerase chain reaction (PCR)**, however, can amplify a specific mRNA from a single cell so that it is detectable (see Figure 9-24).

Both Northern blotting and PCR amplification require extracting the mRNA from a cell or mixture of cells, which means that the cells are removed from their normal location within an organism or tissue. As a result, the location of a cell and its relation to its neighbors is lost. To retain such positional information, a whole or sectioned tissue or even

a whole permeabilized embryo may be subjected to **in situ hybridization** to detect the mRNA encoded by a particular gene (Figure 15-3). This technique allows gene transcription to be monitored in both time and space. **Immunohistochemistry**, the related technique of staining tissue with fluorescence-labeled antibodies against a particular protein, provides similar information for proteins, an important advantage for obtaining ideas about protein function from its subcellular location (see Figures 5-33 and 5-45).

DNA Microarray Analysis Can Assess Expression of Multiple Genes Simultaneously

A major limitation of in situ hybridization and blotting techniques is that the mRNA or protein product of only a few genes can be examined at a time. Thus monitoring the activity of many genes by these methods requires multiple assays. In contrast, researchers can monitor the expression of thousands of genes at one time with **DNA microarrays** (see Figure 9-35). In this technique, cDNAs labeled with a fluorescent dye are made from the total mRNA extracted from the cells under study. The labeled cDNAs are then hybridized to a microscope slide dotted with spots of DNA. Each DNA spot contains a unique sequence from a particular gene, and tens of thousands of genes can be represented on a standard slide. The fluorescence of spots that



▲ EXPERIMENTAL FIGURE 15-3 In situ hybridization can detect activity of specific genes in whole and sectioned embryos.

The specimen is permeabilized by treatment with detergent and a protease to expose the mRNA to the probe. A DNA or RNA probe, specific for the mRNA of interest, is made with nucleotide analogs containing chemical groups that can be recognized by antibodies. After the permeabilized specimen has been incubated with the probe under conditions that promote hybridization, the excess probe is removed with a series of washes. The specimen is then incubated in a solution containing an antibody that binds to the probe. This antibody is covalently joined to a reporter enzyme (e.g., horseradish peroxidase or alkaline phosphatase) that produces a colored reaction product. After excess antibody has been removed, substrate for the

reporter enzyme is added. A colored precipitate forms where the probe has hybridized to the mRNA being detected. (a) A whole mouse embryo at about 10 days of development probed for *Sonic hedgehog* mRNA. The stain marks the notochord (red arrow), a rod of mesoderm running along the future spinal cord. (b) A section of a mouse embryo similar to that in part (a). The dorsal/ventral axis of the neural tube (NT) can be seen, with the *Sonic hedgehog*-expressing notochord (red arrow) below it and the endoderm (blue arrow) still farther ventral. (c) A whole *Drosophila* embryo probed for an mRNA produced during trachea development. The repeating pattern of body segments is visible. Anterior (head) is up; ventral is to the left. [Courtesy of L. Milenkovic and M. P. Scott.]

hybridize to a cDNA species is measured with an instrument that scans the slide. Fluorescing spots thus represent active genes, which have been transcribed into their mRNAs (see Figure 1-23).

Microarray experiments are commonly used to compare the mRNAs produced by two different populations of cells: for example, two distinct cell types, the same cell type before and after some treatment, or mutant and normal cells. An example of a microarray-based discovery comes from the results of studies of cultured fibroblast cells, which have long been known to initiate cell division when serum containing growth factors is added to the medium. Microarray analysis of gene expression at different times after treatment of fibroblasts with serum showed that transcription of about 500 of the 8613 genes examined changed substantially over time (see Figure 9-36). Transcriptional changes were detected within 15 minutes, with genes encoding proteins that control progression through the cell cycle becoming active first. Later, genes encoding proteins with roles in wound healing, such as clotting factors and attractants for immune-system cells, became active. The production of these proteins suggests that proliferating fibroblasts are stimulated by serum to participate in wound healing, something that had not been known. In retrospect, it makes sense, because the time during which fibroblasts are exposed to serum in an intact organism is when there is a wound. The results show the usefulness of microarrays in revealing unexpected responses by cells.

The developmental time course of gene transcription has been assessed with DNA microarrays for the nematode *C. elegans* and the fly *Drosophila*. In recent experiments, microarrays representing about 94 percent of the *C. elegans* genes were used to monitor transcription at different stages of development and in both sexes. The results showed that expression of about 58 percent of the monitored genes changes more than twofold during development, and another 12 percent are transcribed in sex-specific patterns. Findings from a similar study assessing about one-third of all *Drosophila* genes showed that transcription of more than 90 percent of them changes by twofold or more during development and that most genes are used repeatedly during development (Figure 15-5). These results clearly show that development is marked by extensive changes in transcription, with few genes exhibiting a monotonous pattern of unchanging transcription.



In the future, microarray analysis will be a powerful diagnostic tool in medicine. For instance, particular sets of mRNAs have been found to distinguish tumors with a poor prognosis from those with a good prognosis (Chapter 23). Previously indistinguishable disease variations are now detectable. Analysis of tumor biopsies for these distinguishing mRNAs will help physicians to select the most appropriate treatment. As more patterns of gene expression characteristic of various diseased tissues are recognized, the diagnostic use of DNA microarrays will be extended to other conditions. ■

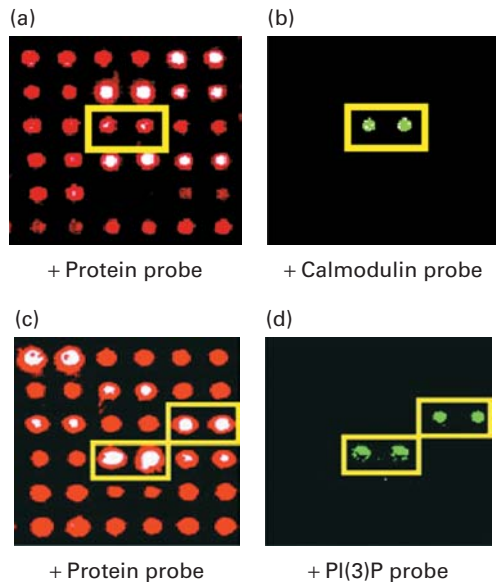
Protein Microarrays Are Promising Tools for Monitoring Cell Responses That Include Changes in Protein-Binding Patterns

A cell's response to signals can include not only changes in gene expression, but also alterations in the modifications of proteins and the associations between proteins. As discussed in other chapters, the activities of many proteins depend on their association with other proteins or with small intracellular signaling molecules (e.g., cAMP or phosphoinositides). Two common examples are the activation of adenylyl cyclase by interaction with $G_{sa} \cdot GTP$ (see Figure 13-11) and the activation of protein kinase A by binding of cAMP (see Figure 3-27). The activity of some transcriptional regulators (e.g., CREB) also depend on their associating with another protein (see Figure 13-32). The results of systematic studies are beginning to reveal protein–protein associations that are critical for cell functioning and how these associations change in response to signals. For example, scientists have produced large quantities of 5800 yeast proteins (≈ 80 percent of the total proteins) by cloning them in high-level expression vectors in yeast and purifying the individual proteins. In a technique analogous to DNA microarrays, small samples of the purified yeast proteins can be spotted on microscope slides to produce a **protein microarray**, also called a **proteome chip**.

To test the efficacy of assaying protein–protein associations on such arrays, researchers exposed the yeast protein microarray to biotin-labeled **calmodulin**, a calcium-binding protein. After excess calmodulin was removed from the microarray, binding of calmodulin to proteins in the array was detected with a fluorescent reagent specific for biotin (Figure 15-4). This experiment succeeded in detecting six proteins already known to bind calmodulin. Six other known calmodulin-binding proteins were not detected, two because they were not included in the array and four that may have been underproduced. In principle, others could be missed because proteins associate only as part of a complex of more than two proteins or because the protein fastened to the chip is in the wrong conformation for binding. Despite these possible problems, 33 other calmodulin-binding yeast proteins not previously recognized also were detected. The gene sequences corresponding to the 39 calmodulin-binding proteins detected indicate that 14 of these proteins have a common motif that may form the binding surface. The results of such experiments show that protein arrays will be a useful, if not completely comprehensive and accurate, tool for monitoring associations of proteins as indicators of cell responses.

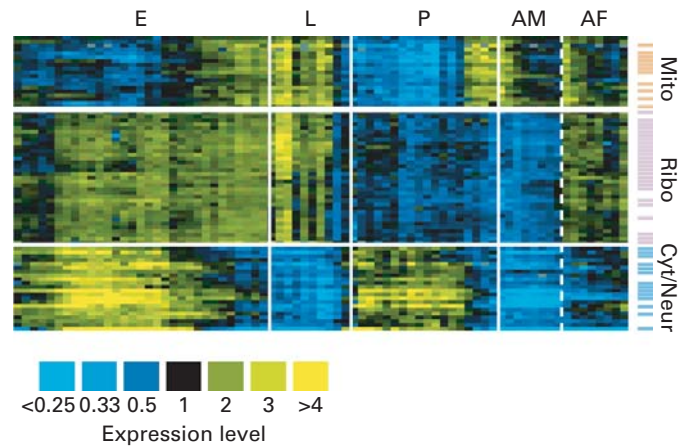
Systematic Gene Inactivation by RNA Interference

Changes in transcription at various developmental stages provide one criterion for identifying genes that play a critical role in cell regulation and differentiation. A more important



▲ EXPERIMENTAL FIGURE 15-4 Protein microarray analysis can reveal protein–protein and protein–lipid interactions. High-level expression cloning was used to produce 5800 yeast proteins, which were purified and then spotted in duplicate on microscope slides. Some 13,000 protein samples can be spotted in half the area of a standard microscope slide. A probe was prepared by covalently attaching biotin to calmodulin or to phosphatidylinositol triphosphate (IP₃). The yeast proteome chips were incubated with the biotinylated probe, and then excess probe was washed off. Calmodulin or IP₃ bound to proteins in the microarray was detected with fluorescence-labeled streptavidin, a bacterial protein that strongly and specifically binds biotin. (a) A part of a proteome chip probed to reveal the location and approximate amounts of all proteins, which are spotted in duplicate columns. (b) The same preparation probed with biotinylated calmodulin. The two green signals correspond to a calmodulin-binding protein spotted in duplicate. (c) A different field of protein spots. (d) IP₃ binds to two of the proteins in (c), each in duplicate. [Courtesy of Paul Bertone, Yale University.]

criterion, however, is the **phenotype** of cells or animals lacking the gene. Knowledge of the functions of genes and relations among them during development can be obtained by systematically eliminating the function of each gene that is normally expressed in a tissue, one at a time, and observing what goes wrong. This procedure is now theoretically possible in *C. elegans*, which is particularly susceptible to **RNA interference** (RNAi). In this technique, delivery of double-stranded RNA into worms by injection, feeding, or soaking leads to the destruction of the corresponding endogenous mRNA (see Figure 9-43). The phenotype of worms that develop from RNAi-treated embryos is often similar to the one that would result from inactivation of the corresponding gene itself. In initial studies with *C. elegans*, RNA interference with 16,700 genes (about 86 percent of the genome) yielded 1722 visibly abnormal phenotypes. The genes whose



▲ EXPERIMENTAL FIGURE 15-5 DNA microarray cluster analysis detects global changes in transcription in *Drosophila* development. The life cycle was divided into about 70 periods from early embryos through aged adults. The mRNA was extracted from animals at each period, converted into fluorescence-labeled cDNA, and hybridized to microarrays representing about 5000 genes. Computer analysis of the original microarray data grouped genes showing similar changes in expression relative to a standard reference sample. (See Figure 9-35 for general protocol of DNA microarray experiments.) The three panels shown represent only a small fraction of the genes in the microarray. Each very narrow row in a panel represents a different gene. General periods are labeled E (embryogenesis), L (larval), P (pupal), and A (adult, male or female). Each vertical line represents one of the 70 developmental periods. Yellow indicates an increase in transcription compared with the reference sample; blue, a decrease in expression; and black, no significant change in expression. Genes are grouped, or “clustered,” because they have similar rises and falls in RNA abundance during development. Clusters often reveal genes involved in similar processes. The three clusters here are mitochondrial protein genes (*top*), ribosomal protein genes (*middle*), and genes involved in the cytoskeleton and neural development (*bottom*). The clustering of a gene of unknown function with some of known function provides a hypothesis about the function of the former. [Adapted from M. Arbeitman et al., 2002, *Science* **297**:2270.]

functional inactivation cause particular abnormal phenotypes can be grouped into sets; each member of a set presumably controls the same signals or events. The regulatory relations among the genes in the set—for example, the genes that control muscle development—can then be worked out.

KEY CONCEPTS OF SECTION 15.1

Experimental Approaches for Building a Comprehensive View of Signal-Induced Responses

- About one-third of the genes in humans, flies (*Drosophila*), and roundworms (*C. elegans*) are unique to each animal.

- The presence and distribution of specific mRNAs and proteins can be detected in living cells by in situ hybridization and immunohistochemical staining.
- DNA microarray analysis allows the full complement of genes to be examined for transcriptional changes that occur in response to environmental changes or extracellular signals and in development (see Figure 15-5).
- Protein microarrays are proving useful in detecting and monitoring changes in protein–protein associations (see Figure 15-4).
- The function of a gene found to be activated under certain conditions can be tested by inactivating it and observing the resulting phenotype.
- Signals, altered environmental conditions, or infection generally evoke not a single response by cells but multiple changes in the pattern of gene transcription, protein modifications, and associations between proteins. By monitoring the totality of these individual responses, researchers are developing comprehensive views of how and why cells respond.

15.2 Responses of Cells to Environmental Influences

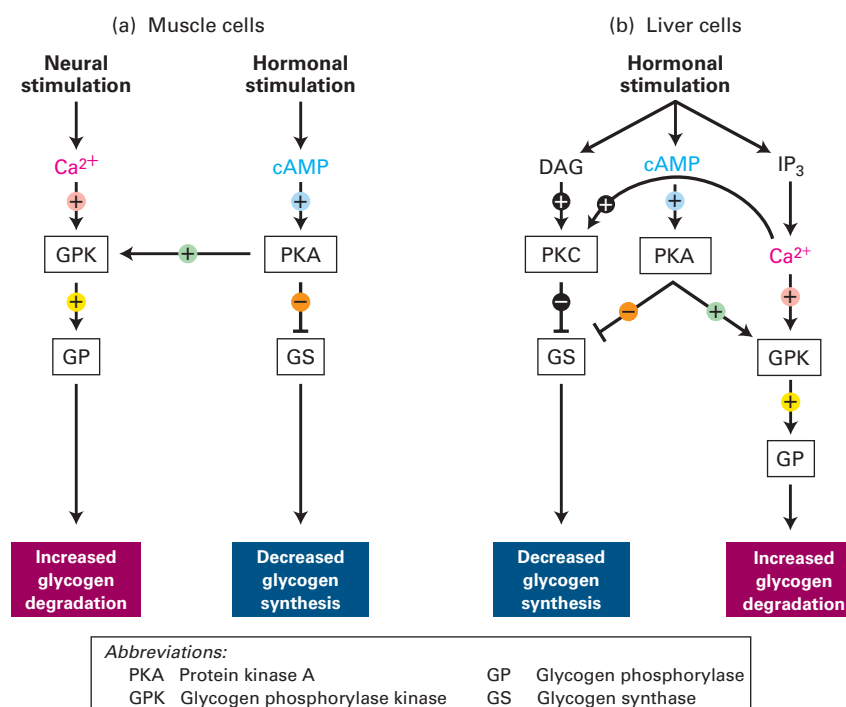
Much of this chapter deals with signaling pathways that control how cells change in the course of development. The mature cells of some tissues (e.g., blood and skin) have a relatively short life span compared with that of other types of cells and are constantly being replaced by the differentiation and proliferation of stem cells (Chapter 22). In a sense, such

tissues never stop developing. The mature cells of other tissues, such as the brain, are long lived; after such tissues reach maturation, there is little additional differentiation. Mature cells in all tissues, however, are changing constantly in response to metabolic or behavioral demands as well as to injury or infection. In this section, we consider several ways that cells respond to variations in the demand for two environmental inputs—glucose and oxygen—or in their levels.

Integration of Multiple Second Messengers Regulates Glycogenolysis

One way for cells to respond appropriately to current physiological conditions is to sense and integrate more than one signal. A good example comes from glycogenolysis, the hydrolysis of glycogen to yield glucose 1-phosphate. In Chapter 13, we saw that a rise in cAMP induced by epinephrine stimulation of β -adrenergic receptors promotes glycogen breakdown in muscle and liver cells (see Figure 13-17). In both muscle and liver cells, other second messengers also produce the same cellular response.

In muscle cells, stimulation by nerve impulses causes the release of Ca^{2+} ions from the sarcoplasmic reticulum and an increase in the cytosolic Ca^{2+} concentration, which triggers muscle contraction. The rise in cytosolic Ca^{2+} also activates *glycogen phosphorylase kinase* (GPK), thereby stimulating the degradation of glycogen to glucose 1-phosphate, which fuels prolonged contraction. Recall that phosphorylation by cAMP-dependent **protein kinase A** also activates glycogen phosphorylase kinase. Thus this key regulatory enzyme in glycogenolysis is subject to both neural and hormonal regulation in muscle (Figure 15-6a).



◀ **FIGURE 15-6 Integrated regulation of glycogenolysis mediated by several second messengers.** (a) Neuronal stimulation of striated muscle cells or epinephrine binding to β -adrenergic receptors on their surfaces leads to increased cytosolic concentrations of the second messengers Ca^{2+} or cAMP, respectively. The key regulatory enzyme, glycogen phosphorylase kinase (GPK), is activated by Ca^{2+} ions and by a cAMP-dependent protein kinase A (PKA). (b) In liver cells, β -adrenergic stimulation leads to increased cytosolic concentrations of cAMP and two other second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Enzymes are marked by white boxes. (+) = activation of enzyme activity, (–) = inhibition.

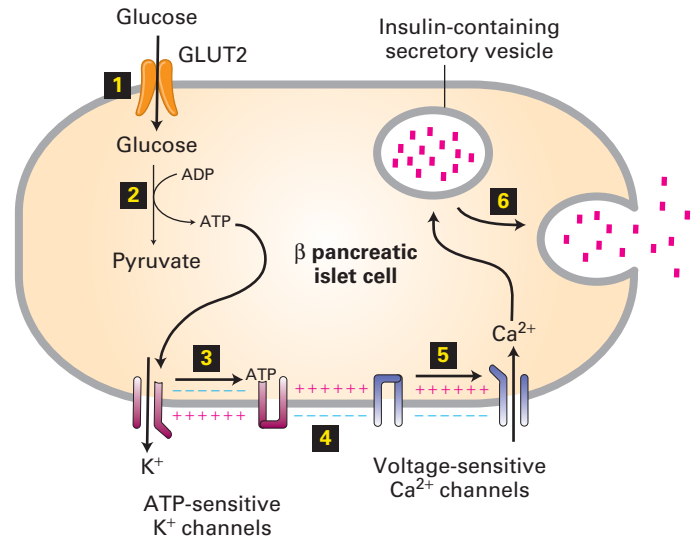
In liver cells, hormone-induced activation of phospholipase C also regulates glycogen breakdown and synthesis by the two branches of the inositol-lipid signaling pathway. Phospholipase C generates two second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (see Figure 13-28). DAG activates **protein kinase C**, which phosphorylates glycogen synthase, yielding the phosphorylated inactive form and thus inhibiting glycogen synthesis. IP_3 induces an increase in cytosolic Ca^{2+} , which activates glycogen phosphorylase kinase as in muscle cells, leading to glycogen degradation. In this case, multiple intracellular signal-transduction pathways are activated by the same signal (Figure 15-6b).

The dual regulation of glycogen phosphorylase kinase results from its multimeric subunit structure $(\alpha\beta\gamma\delta)_4$. The γ subunit is the catalytic protein; the regulatory α and β subunits, which are similar in structure, are phosphorylated by protein kinase A; and the δ subunit is the Ca^{2+} -binding switch protein calmodulin. Glycogen phosphorylase kinase is maximally active when Ca^{2+} ions are bound to the calmodulin subunit and at least the α subunit is phosphorylated. In fact, the binding of Ca^{2+} to the calmodulin subunit may be essential to the enzymatic activity of glycogen phosphorylase kinase. Phosphorylation of the α and β subunits increases the affinity of the calmodulin subunit for Ca^{2+} , enabling Ca^{2+} ions to bind to the enzyme at the submicromolar Ca^{2+} concentrations found in cells not stimulated by nerves. Thus increases in the cytosolic concentration of Ca^{2+} or of cAMP or of both induce incremental increases in the activity of glycogen phosphorylase kinase. As a result of the elevated level of cytosolic Ca^{2+} after neuron stimulation of muscle cells, glycogen phosphorylase kinase will be active even if it is unphosphorylated; thus glycogen can be hydrolyzed to fuel continued muscle contraction in the absence of hormone stimulation.

Insulin and Glucagon Work Together to Maintain a Stable Blood Glucose Level

In the regulation of glycogenolysis, neural and hormonal signals regulate the same key multimeric enzyme. In contrast, the maintenance of normal blood glucose concentrations depends on the balance between two hormones that elicit different cell responses. During periods of stress, the epinephrine-induced increase in glycogenolysis in liver cells leads to a rise in blood glucose. During normal daily living, however, the blood glucose level is under the dynamic control of **insulin** and **glucagon**.

Insulin and glucagon are peptide hormones produced by cells within the islets of Langerhans, cell clusters scattered throughout the pancreas. Insulin, which contains two polypeptide chains linked by disulfide bonds, is synthesized by the β cells in the islets; glucagon, a monomeric peptide, is produced by the α cells in the islets. Insulin *reduces* the level of blood glucose, whereas glucagon *increases* blood glucose. The availability of glucose for cellular metabolism is regulated during periods of abundance (following a meal) or

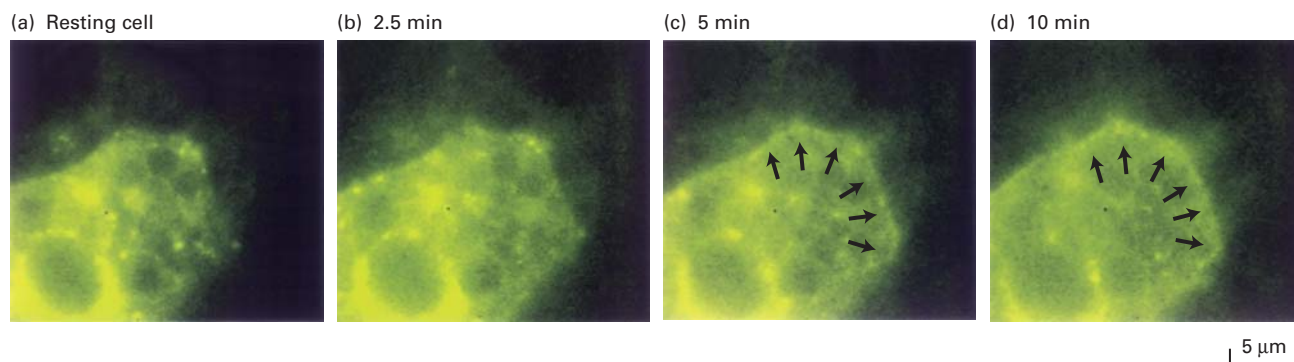


▲ FIGURE 15-7 Secretion of insulin from pancreatic β cells in response to a rise in blood glucose.

The entry of glucose into β cells is mediated by the GLUT2 glucose transporter (**1**). Because the K_m for glucose of GLUT2 is ≈ 20 mM, a rise in extracellular glucose from 5 mM, characteristic of the fasting state, causes a proportionate increase in the rate of glucose entry (see Figure 7-3). The conversion of glucose into pyruvate is thus accelerated, resulting in an increase in the concentration of ATP in the cytosol (**2**). The binding of ATP to ATP-sensitive K^+ channels closes these channels (**3**), thus reducing the efflux of K^+ ions from the cell. The resulting small depolarization of the plasma membrane (**4**) triggers the opening of voltage-sensitive Ca^{2+} channels (**5**). The influx of Ca^{2+} ions raises the cytosolic Ca^{2+} concentration, triggering the fusion of insulin-containing secretory vesicles with the plasma membrane and the secretion of insulin (**6**). Steps **5** and **6** are similar to those that take place at nerve terminals, where a membrane depolarization induced by the arrival of an action potential causes the opening of voltage-sensitive Ca^{2+} channels and exocytosis of vesicles containing neurotransmitters (see Figure 7-43). [Adapted from J.-Q. Henquin, 2000, *Diabetes* **49**:1751.]

scarcity (following fasting) by the adjustment of insulin and glucagon concentrations in the blood.

After a meal, when blood glucose rises above its normal level of 5 mM, the pancreatic β cells respond to the rise in glucose or amino acids by releasing insulin into the blood (Figure 15-7). The released insulin circulates in the blood and binds to insulin receptors on muscle cells and adipocytes (fat-storing cells). The insulin receptor, a **receptor tyrosine kinase (RTK)**, can transduce signals through a phosphoinositide pathway leading to the activation of **protein kinase B** (see Figure 14-27). By an unknown mechanism, protein kinase B triggers the fusion of intracellular vesicles containing GLUT4 glucose transporters with the plasma membrane (Figure 15-8). The resulting increased number of GLUT4 on the cell surface increases glucose influx, thus lowering blood glucose. When insulin is removed, cell-surface GLUT4 is internalized by endocytosis, lower-



▲ EXPERIMENTAL FIGURE 15-8 Insulin stimulation of fat cells induces translocation of GLUT4 from intracellular vesicles to the plasma membrane. In this experiment, fat cells were engineered to express a chimeric protein whose N-terminal end corresponded to the GLUT4 sequence, followed by the entirety of the GFP sequence. When a cell is exposed to light of the exciting wavelength, GFP fluoresces yellow-green, indicating the position of GLUT4 within the cell. In resting cells (a), most

GLUT4 is in internal membranes that are not connected to the plasma membrane. Successive images of the same cell after treatment with insulin for 2.5, 5, and 10 minutes show that, with time, increasing numbers of these GLUT4-containing membranes fuse with the plasma membrane, thereby moving GLUT4 to the cell surface (arrows) and enabling it to transport glucose from the blood into the cell. Muscle cells also contain insulin-responsive GLUT4 transporters. [Courtesy of J. Bogan.]

ing the level of cell-surface GLUT4 and thus glucose import. Insulin stimulation of muscle cells also promotes the uptake of glucose and its conversion into glycogen, and it reduces the degradation of glucose to pyruvate. Insulin also acts on hepatocytes to inhibit glucose synthesis from smaller molecules, such as lactate and acetate, and to enhance glycogen synthesis from glucose. The net effect of all these actions is to lower blood glucose back to the fasting concentration of about 5 mM.

If the blood glucose level falls below about 5 mM, pancreatic α cells start secreting glucagon. The glucagon receptor, found primarily on liver cells, is coupled to G_s protein, like the epinephrine receptor (Chapter 13). Glucagon stimulation of liver cells activates adenylyl cyclase, leading to the cAMP-mediated cascade that inhibits glycogen synthesis and promotes glycogenolysis, yielding glucose 1-phosphate (see Figure 15-6b). Liver cells can convert glucose 1-phosphate into glucose, which is released into the blood, thus raising blood glucose back toward its normal fasting level.



Diabetes mellitus results from a deficiency in the amount of insulin released from the pancreas in response to glucose (type I) or from a decrease in the ability of muscle and fat cells to respond to insulin (type II). In both types, the regulation of blood glucose is impaired, leading to persistent hyperglycemia and numerous other possible complications in untreated patients. Type I diabetes is caused by an autoimmune process that destroys the insulin-producing β cells in the pancreas. Also called insulin-dependent diabetes, this form of the disease is generally responsive to insulin therapy. Most Americans with diabetes mellitus have type II, but the underlying cause of this form of the disease is not well understood. ■

Oxygen Deprivation Induces a Program of Cellular Responses

In glycogenolysis, the activity of preexisting proteins was regulated by the integration of multiple signals. Organisms that require oxygen respond to oxygen deprivation, a single stimulus, in multiple ways, some occurring rapidly and others taking longer to develop. In addition, over evolutionary time, animals that live at high altitude (e.g., llamas, guanacos, alpacas) became adapted to low oxygen. This adaptation entailed single amino acid changes in the β -globin chain that increased the oxygen affinity of hemoglobin in these animals compared with that of hemoglobin in other animals.

Among the rapid responses to low oxygen (*hypoxia*) is dilation of blood vessels, permitting increased blood flow. This response is regulated by nitric oxide, cyclic GMP, and protein kinase G (see Figure 13-30). A rapid shift in metabolism, called the Pasteur effect, also occurs when cells are deprived of adequate oxygen. First observed in yeast cells, this response accelerates the anaerobic metabolism of glucose when aerobic metabolism and oxidative phosphorylation slows owing to low oxygen. Burning more carbohydrates compensates for the reduced ATP yield from anaerobic metabolism. Phosphofructokinase 1, the third enzyme in glycolysis, is inhibited by ATP and stimulated by AMP; so, when the cell is short on energy, glycolysis increases (see Figure 8-12). The adjustment is rapid, inasmuch as it does not require the synthesis of new molecules.

Slow adaptive responses to low oxygen at the level of the whole organism include increasing the production of erythrocytes, which is stimulated by **erythropoietin** produced in the kidney (see Figure 14-7). Transcription of the erythropoietin gene is regulated primarily by *hypoxia-induced factor 1 (HIF-1)*, a transcriptional activator. The amount of HIF-1 increases drastically as the partial pressure of oxygen

decreases from 35 mm Hg to zero, a range typical of normal fluctuations. The nature of the oxygen sensor that causes the increased expression of HIF-1 is not yet known, but it probably requires a protein that has a heme-containing oxygen-binding site somewhat like that in hemoglobin. In addition to regulating the erythropoietin gene, HIF-1 coordinately activates the transcription of several other genes whose encoded proteins help cells respond to hypoxia (Figure 15-9). One of these proteins, vascular endothelial growth factor (VEGF), is secreted by cells lacking oxygen and promotes local *angiogenesis*, the branching growth of blood vessels. Expression of VEGF requires not only HIF-1 but also a Smad transcription factor, which is activated by a TGF β signal. The ability of HIF-1 to control different genes in different cell types presumably results from this type of combinatorial action.

The results of recent studies revealed that the degradation of HIF-1 is controlled by an oxygen-responsive prolyl hydroxylase. HIF-1 is a dimer composed of two subunits, α and β . The β subunit is abundant in the cytosol under high or low oxygen conditions but, when oxygen is plentiful, the α subunit (HIF α) is ubiquitinated and degraded in proteasomes (Chapter 3). Ubiquitination is promoted by the von Hippel-Lindau protein (pVHL), which binds to a conserved “degradation domain” of HIF-1 α . The binding of pVHL in turn is facilitated by hydroxylation of a proline in the pVHL-binding site on HIF-1 α . The prolyl hydroxylase catalyzing this reaction requires iron and is most active at high oxygen, leading to degradation of the α subunit and no transcriptional activation by HIF-1. At low oxygen, when hydroxylation does not occur, active dimeric HIF-1 forms and is translocated to the nucleus. The *hypoxia-response pathway* mediated by HIF-1 and its regulation by pVHL have been

conserved for more than half a billion years, given that it is the same in mammals, worms, and insects.



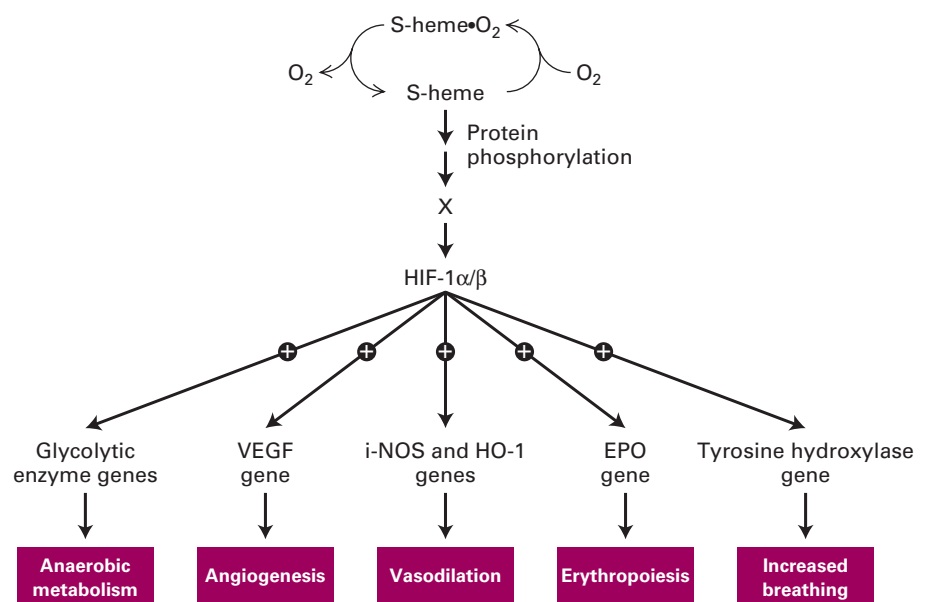
Hypoxia affects the growth of blood vessels, particularly the small capillaries, whose exact pattern, unlike that of major blood vessels like the aorta, is not genetically determined. Angiogenesis, the branching growth of the vasculature, is stimulated by hypoxia, thus ensuring that all cells are in adequate proximity to the oxygenated blood supply. Growing tumors stimulate angiogenesis to ensure their own blood supply (Chapter 23). Understanding the signals that control angiogenesis could potentially lead to the development of therapeutic agents that stimulate angiogenesis in a transplanted or diseased organ that is receiving insufficient blood or that inhibit angiogenesis in developing tumors, thereby suffocating them. ■

KEY CONCEPTS OF SECTION 15.2

Responses of Cells to Environmental Influences

- Glycogen breakdown and synthesis is regulated by multiple second messengers induced by neural or hormonal stimulation (see Figure 15-6).
- A rise in blood glucose stimulates the release of insulin from pancreatic β cells (see Figure 15-7). Subsequent binding of insulin to its receptor on muscle cells and adipocytes leads to the activation of protein kinase B, which promotes glucose uptake and glycogen synthesis, resulting in a decrease in blood glucose.
- The binding of glucagon to its G protein-coupled receptor on liver cells promotes glycogenolysis and an increase

► **FIGURE 15-9 Model of hypoxia response pathway mediated by hypoxia-induced factor 1 (HIF-1).** The local oxygen concentration is sensed by an unknown mechanism probably requiring a heme-associated protein (S-heme). The oxygen-deprived form of the sensor activates intermediates (X), which in turn stimulate increased production of both subunits of hypoxia-induced factor 1 (HIF-1). HIF-1 activates transcription of genes whose encoded proteins mediate short-term and long-term responses to an oxygen deficit. HIF-1 activates different target genes in different cells, indicating that it probably acts in combination with other gene-regulating proteins. VEGF = vascular endothelial growth factor; i-NOS = inducible nitric oxide synthase; HO-1 = heme oxygenase-1; EPO = erythropoietin. [Adapted from H. Zhu and H. F. Bunn, 2001, *Science* **292**:449; see also W. G. Kaelin, Jr., 2002, *Genes & Dev.* **16**:1441.]



in blood glucose by the cAMP-triggered kinase cascade (similar to epinephrine stimulation under stress conditions).

- Two oxygen-sensing mechanisms help cells respond to oxygen deprivation: one mechanism activates transcription of hypoxia-induced factor 1 (HIF-1), and the other promotes stabilization of HIF-1 by inhibiting its proteasomal degradation.

- When cellular oxygen is low, HIF-1 acts with tissue-specific transcription factors to activate the expression of various target genes in different tissues (see Figure 15-9). One protein induced by this pathway is vascular endothelial growth factor, which stimulates local angiogenesis to increase the blood supply and thereby oxygen to oxygen-deprived cells.

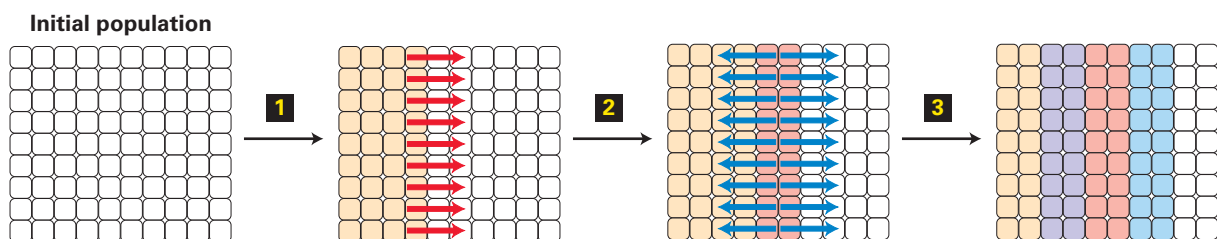
15.3 Control of Cell Fates by Graded Amounts of Regulators

In a developing tissue, each cell must learn how to contribute to the overall organization of the tissue. Frequently, cells in a particular position within the developing embryo must divide, move, change shape, or make specialized products, whereas other nearby cells do not. In modern developmental biology, the term **induction** refers to events where one cell population influences the fate of neighboring cells. Figure 15-10 schematically depicts how a series of inductive signals can create several cell types, starting from a population of initially equivalent cells. Induction may create tissue types at specific sites (e.g., formation of a lens near the site at which the retina will grow) or cause changes in the shape of cells at a specific location. For example, changes in the shape of cells in the center but not at the periphery of the neural plate give rise to the neural tube from which our central nervous system develops. Cell orientation is critical, too. If

an epithelium produces appendages, such as feathers or bristles, all of them may need to point in the same direction. All these properties of cells are coordinated by integrating signals in the developing organism, and each cell interprets the signals that it receives in light of its previous experience and state of differentiation.

Some extracellular inductive signals move through tissue and hence can act at a distance from the signaling cell; some signals are tethered to the surface of the signaling cell and thus can influence only the immediate neighboring cells. Still other signals are highly localized by their tight binding to components of the extracellular matrix. The transmission rate of a signal depends on the chemical properties of the signal, the properties of the tissue through which it passes, and the ability of cells along the way to take up or inactivate the signal. The distance that an inductive signal can move influences the size and shape of an organ. For instance, the farther a signal that induces neuron formation can move, the more neurons will form.

In this section and those that follow, we will see how quantitative differences in external signals and transcription factors can determine cell fates and properties. We begin by distinguishing two basic mechanisms of inductive signaling and then, by way of example, examine in some detail early stages of *Drosophila* development. To learn how signals work during cell interactions in development, transgenic animals are used to observe the effects of increasing or decreasing gene function in specific cells. For example, if a cell can send a signal even if a certain gene function is removed, the gene is not required for sending the signal. Removing the same gene function from a cell that normally receives the signal may reveal a requirement of the gene for signal reception or transduction. In this way, even when a novel protein is being studied, it is possible to deduce its place in a signaling pathway. These gene manipulation methods are especially advanced in *Drosophila* (Figure 15-11) but are increasingly being adapted for other experimental organisms.



▲ **FIGURE 15-10** Simplified model of sequential induction of cell types in an epithelium. Step 1: Starting from a population of equivalent cells (white), an initial event (e.g., cell movements or a polarized signal) creates a second population of cells (tan) that secretes a signal (red arrows). This signal reaches only some of the cells in the adjacent field of cells. Step 2: The

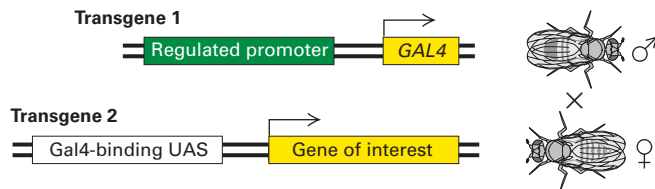
cells capable of receiving and interpreting the red signal now form a new cell type (pink) that secretes a different signal (blue arrows) that moves away from the red cells in both directions. Step 3: The blue signal induces still more cell types (purple and blue). Note that the effect of the blue signal differs, depending on whether it acts on white cells or on tan cells.

(a) Ectopic gene expression with spatial/temporal control

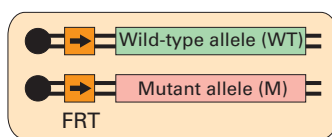
A fly gene of interest is linked to a regulated promoter in order to express the gene in a time or place that is not normal. If the overexpression is lethal, no transgenic flies can be obtained (see part [b] for a solution to this problem). A variation is to use a heat-inducible promoter from a “heat shock” gene; a pulse of heat (37° C, 30 min) will cause expression in all cells.

**(b) Spatially and temporally regulated ectopic gene expression using GAL4**

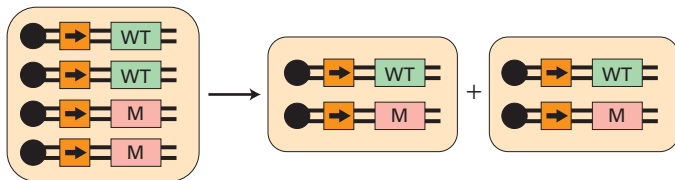
One transgenic fly codes for the yeast transcription factor Gal4 under the control of a tissue-specific promoter. Another carries a transgene that can respond to Gal4 because it contains a UAS sequence to which Gal4 binds. After crossing the two flies, any gene that had been attached to the UAS sequence will fall under the control of Gal4 and be expressed in that specific tissue. This allows each transgenic fly line to survive, even if the activation of the gene will prove lethal in the progeny of the cross.

**(c) Creating mosaic tissues with clones of cells that lack a gene function**

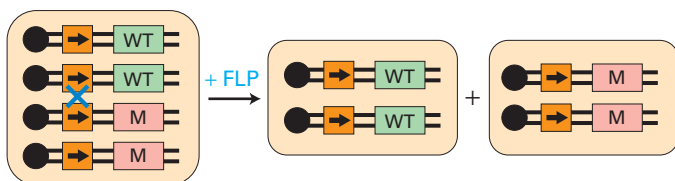
Yeast FLP recombinase, acting upon FRT sequences inserted near the centromere, can be used to create clones of homozygous mutant cells in flies that are heterozygous for a recessive mutation. Recombinase is produced at a specified time using a heat-inducible promoter. Just enough recombinase is induced to cause an occasional recombination event. The effects of lost gene function are then assessed.



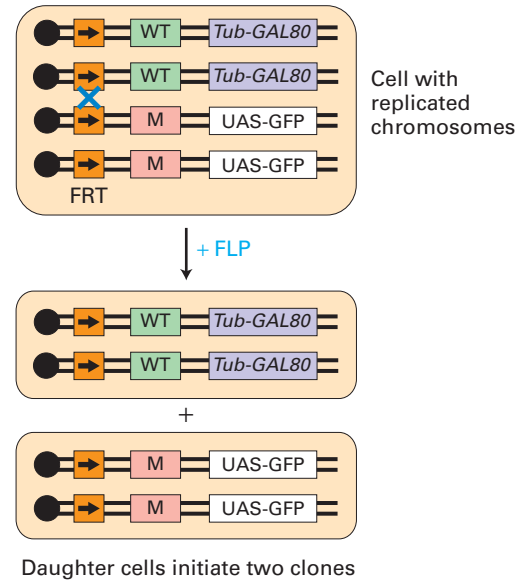
Normal cell division creates more heterozygous cells:



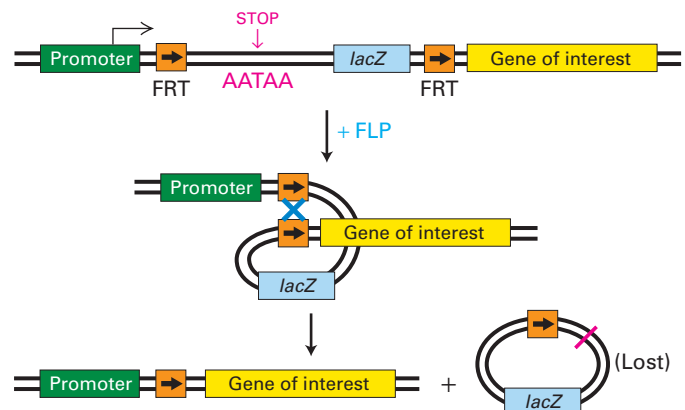
Division after recombination creates a clone of mutant cells and a clone of wild-type cells:

**(d) Creating marked mutants to assess the effects of lost gene function in single cells**

The goal is to mark mutant cells created by FLP recombination as in (c). Flies are made that carry a gene encoding the yeast protein Gal80 under the control of a *tubulin* promoter that is active in all cells. This transgene is located on the chromosome that carries the wild-type allele of the gene of interest. All cells are engineered to make the yeast transcription factor Gal4 using a constitutive promoter (not shown). After recombination, Gal80 is present in the wild-type cell, where it blocks the activity of Gal4. In the mutant cell, no Gal80 is made and Gal4 acts on a UAS sequence to activate production of a fluorescent protein (GFP). In this way the mutant cells are marked with fluorescence at the same time that they lose their wild-type allele.

**(e) Inducibly activating a gene function in clones of cells**

The goal is to activate the gene of interest in a clone of cells during development. All cells initially express *lacZ*, which is under the control of a constitutive promoter, and therefore stain blue. When a modest pulse of FLP recombinase is produced using a heat-inducible promoter active in all cells, enough recombinase is made in a few cells to act on FRT sites and cause a deletion of the DNA between the FRTs. This removes the *lacZ* gene at the same time that it joins a promoter to the gene of interest. The resulting clone of cells that expresses the gene of interest can be identified because the cells do not express *lacZ*. The effect of the gene of interest is then assessed in the clone.



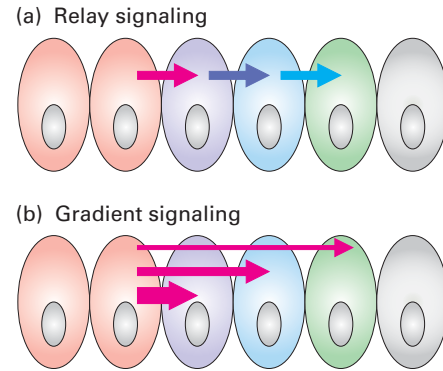
◀ **FIGURE 15-11 Gene manipulation in analysis of signaling systems.**

(a) In the simplest case, a gene is activated with the use of a promoter that is specific to certain cells or, as with “heat shock” promoters, that is inducible in all cells. This procedure has the limitation that the transgene may be detrimental and transgenic animals cannot be isolated. (b) An improvement is to make two lines of flies transgenic, one carrying the gene of interest under the control of an upstream activating sequence (UAS, X-ref) from yeast. The UAS is activated when the yeast transcription factor GAL4 is present, as it is when the UAS-bearing flies are crossed with flies having GAL4 expressed in certain cells. (c) The opposite goal is to remove a gene’s function selectively from certain cells. The yeast recombinase FLP acts on FRT sequences that have been inserted near the base of a chromosome. Starting from a fly that is heterozygous for a mutation of interest, mutant/wild-type, one can obtain clones of cells that are homozygous for either the mutant or the wild-type allele. (d) It is difficult to recognize and analyze small clones of cells obtained as in part (c) if the cells are not marked. In this refinement, the recombination removes GAL80, a protein that inhibits GAL4 function, at the same time as the mutant allele is made homozygous. The unleashed GAL4 then activates a UAS that drives the production of a fluorescent protein. A mutant cell, like the neuron shown, can be analyzed to see the effect of the mutation on, for example, wiring the brain. (e) To activate a gene in small, randomly generated clones of cells, FLP is again used but this time to remove an intervening transcriptional termination sequence that prevents the gene of interest from being active. At the same time, a *lacZ* or other marker gene is removed; so the clone of cells with the gene turned on is identifiable by the lack of marker gene expression.

Inductive Signaling Operates by Gradient and Relay Mechanisms

In some cases, the induction of cell fates includes a binary choice: in the presence of a signal, the cell is directed down one developmental pathway; in the absence of the signal, the cell assumes a different developmental fate or fails to develop at all. Such signals often work in a *relay mode*. That is, an initial signal induces a cascade of induction in which cells close to the signal source are induced to assume specific fates; they, in turn, produce other signals to organize their neighbors (Figure 15-12a). Alternatively, a signal may induce different cell fates, depending on its concentration. In this *gradient mode*, the fate of a receiving cell is determined by the amount of the signal that reaches it, which is related to its distance from the signal source (Figure 15-12b). Any substance that can induce different responses depending on its concentration is often referred to as a **morphogen**.

The concentration at which a signal induces a specific cellular response is called a *threshold*. A graded signal, or morphogen, exhibits several thresholds, each one corresponding to a specific response in receiving cells. For instance, a low concentration of an inductive signal causes a cell to assume fate A, but a higher signal concentration causes the cell to assume fate B. In the gradient mode of



▲ **FIGURE 15-12 Two modes of inductive signaling.** In the relay mode (a), a short-range signal (red arrow) stimulates the receiving cell to send another signal (purple), and so on for one or more rounds. In the gradient mode (b), a signal produced in localized source cells (red arrows) reaches nearby cells in larger amounts than the amounts reaching distant cells. If the receiving cells respond differently to different concentrations of the signal (indicated by width of the arrows), then a single signal may create multiple cell types.

signaling, the signal is newly created, and so it has not built up to equal levels everywhere. Alternatively, the signal could be produced at one end of a field of cells and destroyed or inactivated at the other (the “source and sink” idea), so a graded distribution is maintained.

Mesoderm Cell Fates in *Xenopus* Blastula Studies with activin, a TGFβ-type signaling protein that determines cell fate in early *Xenopus* embryos, have been sources of insight into how cells determine the concentration of a graded inductive signal. Activin helps organize the **mesoderm** along the dorsal/ventral (back/front) axis of an animal. The **endoderm** and **ectoderm** form first after fertilization of a *Xenopus* oocyte; the mesoderm forms slightly later. These three distinct cell populations (germ layers) make up the *blastula*, a hollow ball of cells.

Specific genes are used as indicators of the tissue-creating effects of signals such as activin. For instance, a low concentration of activin induces expression of the *Xenopus brachyury* (*Xbra*) gene throughout the early mesoderm. *Xbra* is a transcription factor necessary for mesoderm development. Higher concentrations of activin induce expression of the *Xenopus goosecoid* (*Xgsc*) gene. *Xgsc* protein is able to transform ventral into dorsal mesoderm; so the local induction of *Xgsc* by activin causes the formation of dorsal, rather than ventral, mesodermal cells near the activin source. Using ³⁵S-labeled activin, scientists demonstrated that *Xenopus* blastula cells each express some 5000 type II TGFβ-like receptors that bind activin. Findings from additional experiments showed that maximal *Xbra* expression was achieved when about 100 receptors were occupied. At a concentration of activin at which 300 receptors were occupied, cells began expressing higher levels of *Xgsc*. Similar results were

obtained with blastula cells experimentally manipulated to express sevenfold higher levels of the activin type II receptor. These findings indicate that blastula cells measure the absolute number of ligand-bound receptors rather than the ratio of bound to unbound receptors, and confirm the importance of signal concentration.

Vulva Development in *C. elegans* An example of cell-fate determination by a combination of graded and relayed signals is the development of the vulva of the nematode worm *C. elegans*. This structure develops from a group of epidermal vulval precursor cells (VPCs) whose fates are controlled by an inductive signal from a nearby cell called the anchor cell. All the VPCs have the potential to become any of three different cell types: 1° and 2°, which refer to different vulval cell types, and 3°, which is a nonvulval type. A set of cells, such as the VPCs, is called an *equivalence group* if each cell in the set has equal capacity to form more than one cell type. The inductive signal secreted by the anchor cell is LIN-3, which is similar to vertebrate epidermal growth factor (EGF). Like the EGF receptor, the receptor for LIN-3 is a receptor tyrosine kinase, called LET-23, that acts through a Ras–MAP kinase pathway (see Figure 14-21).

The results of early studies suggested that LIN-3 was a graded signal inducing the 1° fate in the nearest VPC (nor-

mally P6.p) and the 2° fate in P5.p and P7.p, which are located slightly farther away from the anchor cell (Figure 15-13a). If determination of the 2° fate depended solely on a graded LIN-3 signal, then mutant P5.p and P7.p cells lacking the receptor for LIN-3 would be expected to assume the nonvulval 3° fate. Surprisingly, when this experiment was done, the mutant cells took on their normal 2° fate (Figure 15-13b). The most likely explanation of these results is that, when the P6.p cell takes on the 1° fate, it sends out a different signal that normally works with moderate levels of LIN-3 to ensure the production of 2° cells. This second, relayed signal appears to be a ligand for LIN-12, a Notch-type receptor. Stimulation of LIN-12 on the P5.p and P7.p cells induces expression of a phosphatase that inactivates MAP kinase and affects other regulators as well, thus preventing the 1° fate choice.

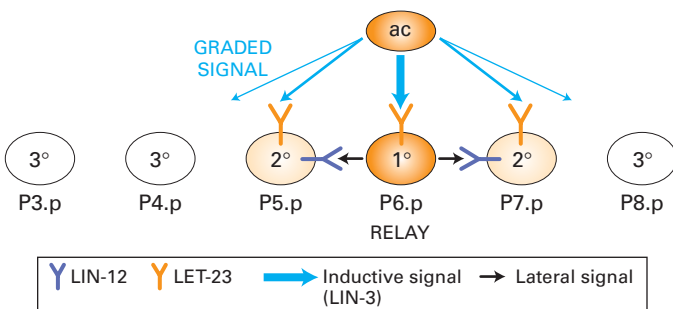
In glycogenolysis, signal integration is at the level of a two-subunit protein, with each signal acting on one of the subunits. In vulval cell-fate determination, the activity of a single kinase, MAP kinase, is controlled by two pathways: signaling from an EGF-type receptor activates MAP kinase; signaling from a Notch-type receptor deactivates it. The convergence of these two pathways on MAP kinase elegantly allows the formation of multiple adjacent cell types.

Morphogens Control Cell Fates in Early *Drosophila* Development

Drosophila has been particularly useful in studying morphogens for three reasons. First, graded regulatory molecules are used extensively in the development of the early *Drosophila* embryo and in growth of the legs and wings. Second, a fertilized egg develops into an adult fly in only about 10 days. Third, powerful genetic screens have identified many developmental mutants with dramatic abnormal phenotypes. Some of these defects have been found to arise from mutations in genes encoding morphogens; others arise in genes encoding signal-transduction proteins.

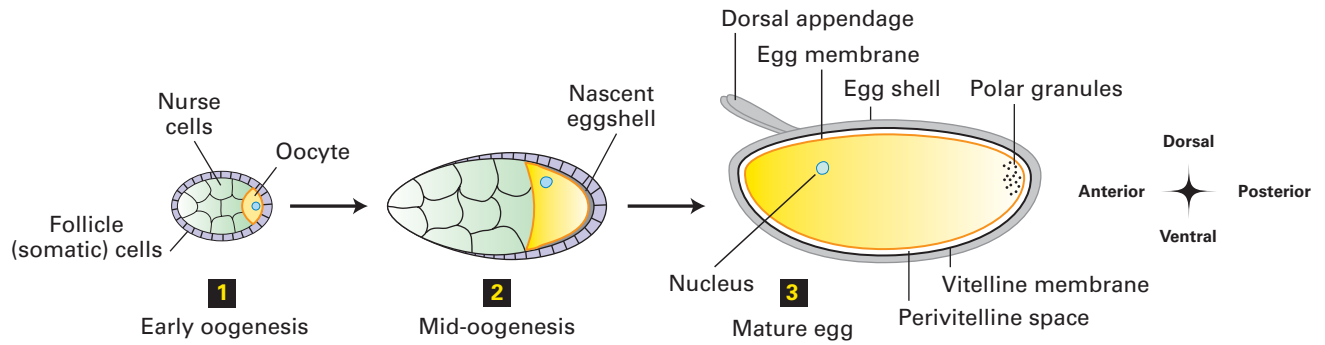
To understand how morphogens determine cell fates in the early fly embryo, we first need to set the scene. Oogenesis begins with a stem cell that divides asymmetrically to generate a single **germ cell**, which divides four times to generate 16 cells. One of these cells will complete meiosis (see Figure 9-3), becoming an **oocyte**; the other 15 cells become *nurse cells*, which synthesize proteins and mRNAs that are transported through cytoplasmic bridges to the oocyte (Figure 15-14). These molecules are necessary for maturation of the oocyte and for the early stages of **embryogenesis**. At least one-third of the genome is represented in the mRNA contributed by the mother to the oocyte, a substantial dowry. Each group of 16 cells is surrounded by a single layer of somatic cells called the *follicle*, which deposits the eggshell. The mature oocyte, or egg, is released into the oviduct, where it is fertilized; the fertilized egg, or **zygote**, is then laid.

The first 13 nuclear divisions of the *Drosophila* zygote are synchronous and rapid, each division occurring about every 10 minutes. This DNA replication is the most rapid



▲ FIGURE 15-13 Gradient and relay signaling in *C. elegans* vulva development.

The anchor cell (ac) sends a signal to the vulval precursor cells (P3.p–P8.p), all of which have equivalent potential to form any of three cell types—1°, 2°, 3°. The EGF-related signal, LIN-3, is received by the LET-23 receptor. Cells receiving the highest amount of signal form 1°, cells receiving a moderate amount form 2°, and cells receiving little or none form 3°. The three cell fates are distinguished by following their subsequent patterns of cell division. The effects of the graded LIN-3 signal are further controlled by a signal relay. After the anchor cell sends LIN-3 to the nearest cell, normally P6.p, the P6.p cell then sends a different signal to its neighbors, which turns out to be a ligand for LIN-12. The demonstration of the relay effect came from genetically removing LET-23 receptor from just the P5.p and P7.p cells, which prevents them from responding to the LIN-3 signal. If direct LIN-3 graded signal accounted for all the cell types, P5.p and P7.p should take on the 3° fate. However, they still manage to form 2° cells; so another system must operate. This other system is the signal for LIN-12 from the P6.p cell. [Adapted from J. S. Simske and S. K. Kim, 1995, *Nature* **375**:142.]



▲ FIGURE 15-14 Development of a *Drosophila* oocyte into a mature egg. A single germ cell gives rise to fifteen nurse cells (green) and a single oocyte (yellow) (1). The early oocyte is about the same size as the neighboring nurse cells; the follicle, a layer of somatic cells, surrounds the oocyte and nurse cells. The nurse cells begin to synthesize mRNAs and proteins necessary for oocyte maturation, and the follicle cells begin to form the egg shell. Midway through oogenesis (2), the oocyte has increased in size considerably. The mature egg (3) is surrounded by the completed eggshell (gray). The nurse cells have been discarded,

but mRNAs synthesized and translocated to the oocyte by the nurse cells function in the early embryo. Polar granules located in the posterior region of the egg cytoplasm mark the region in which germ-line cells will arise. The asymmetry of the mature egg (e.g., the off-center position of the nucleus) sets the stage for the initial cell-fate determination in the embryo. After its release into the oviduct, fertilization of the egg triggers embryogenesis. [Adapted from A. J. F. Griffiths et al., 1993, *An Introduction to Genetic Analysis*, 5th ed., W. H. Freeman and Company, p. 643.]

known for a eukaryote, with the entire 160 Mb of chromosomal DNA copied in a cell-cycle S phase that lasts only 3 minutes. Because these nuclear divisions are not accompanied by cell divisions, they generate a multinucleated egg cell, a **syncytium**, with a common cytoplasm and plasma membrane (Figure 15-15a). As the nuclei divide, they begin to migrate outward toward the plasma membrane. From about 2 to 3 hours after fertilization, the nuclei reach the surface, forming the syncytial blastoderm; during the next hour or so, cell membranes form around the nuclei, generating the cellular blastoderm, or blastula (Figure 15-15b). All future tissues are derived from the 6000 or so epithelial cells on the surface of the blastula. Soon some of these cells move inside, a process termed gastrulation, and eventually develop into the internal tissues.

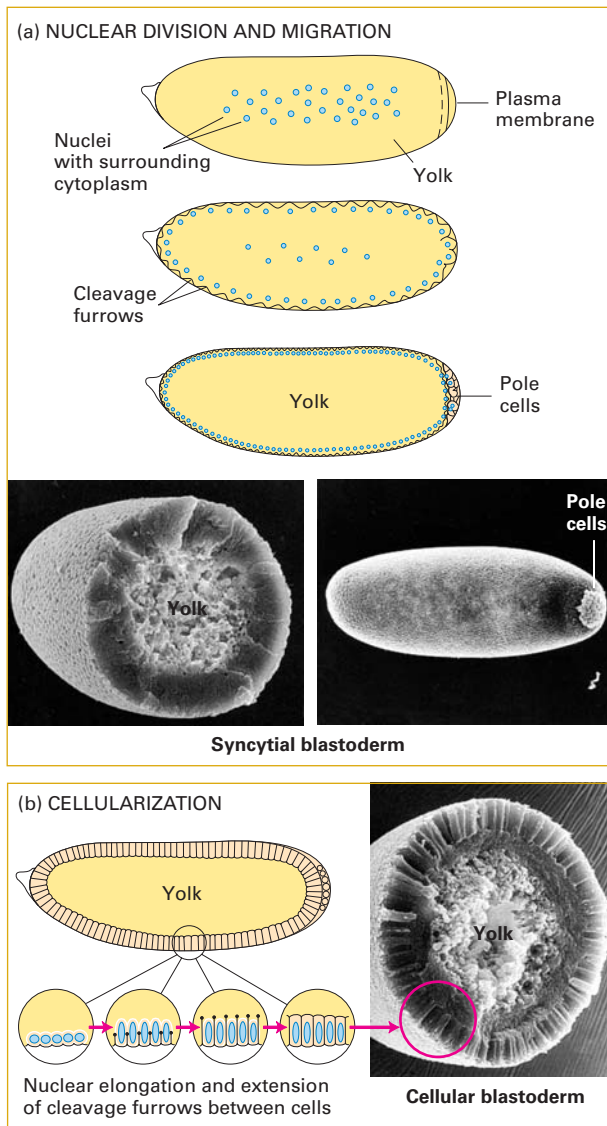
The syncytial fly embryo is about 100 cells long, head to tail, and about 60 cells around. Within 1 day of fertilization, the zygote develops into a larva, a segmented form that lacks wings and legs. Development continues through three larval stages (~4 days) and the ~5-day pupal stage during which metamorphosis takes place and adult structures are created (Figure 15-16). At the end of pupation, about 10 days after fertilization, the pupal case splits and an adult fly emerges.

The initially equivalent cells of the syncytial embryo rapidly begin to assume different fates, leading to a well-ordered pattern of distinct cell identities. These early patterning events set the stage for the later development and proper placement of different tissues (e.g., muscle, nerve, epidermis) and body parts, as well as the shapes of the appendages and the organization of cell types within them. Because the early embryo is initially symmetric side to side, the creation of differences among cells is a two-axis prob-

lem: *dorsal/ventral* (back/front) and *anterior/posterior* (head/tail). Different sets of genes act on each axis; so every cell learns its initial fate by responding to input from both dorsoventral-acting and anterioposterior-acting regulators in a kind of two-dimensional grid. As we will see, both regulatory systems begin with information and molecules contributed to the oocyte as a dowry from the mother. When the mature egg is laid, it is already asymmetric along both axes (see Figure 15-14).

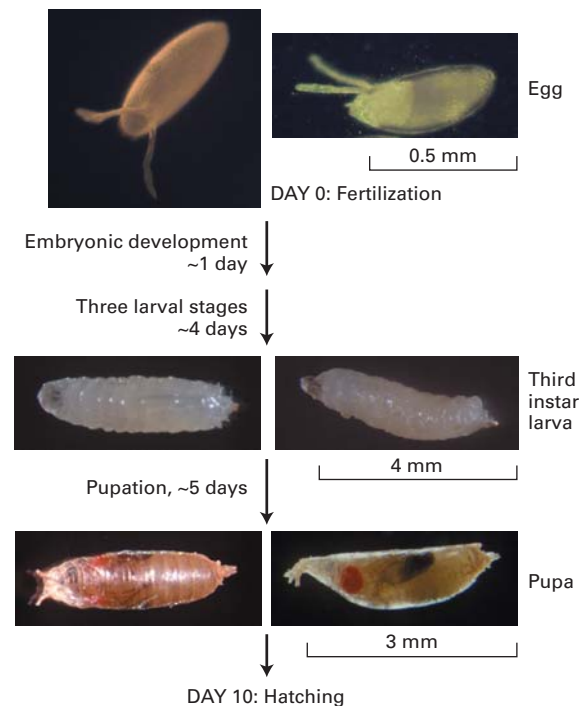
Because the early fly embryo is a syncytium, regulatory molecules can move in the common cytoplasm without having to cross plasma membranes. Some molecules form gradients, which are used in the earliest stages of cell-fate determination in *Drosophila* before subdivision of the syncytium into individual cells. Thus transcription factors, as well as secreted molecules, can function as morphogens in the syncytial fly embryo. Syncytia are less common in the early development of other animals and in later stages of fly development; in these stages, patterning events are controlled largely by interactions between cells mediated by extracellular signals, which may act in a graded or relay mode.

To decipher the molecular basis of cell-fate determination and global patterning, investigators have (1) carried out massive genetic screens to identify all the genes having roles in the organizing process, (2) cloned mutation-defined genes; (3) determined the spatial and temporal patterns of mRNA production for each gene and the distribution of the encoded proteins in the embryo; and (4) assessed the effects of mutations on cell differentiation, tissue patterning, and the expression of other regulatory genes. The principles of cell-fate determination and tissue patterning learned from *Drosophila* have proved to have broad applicability to animal development.

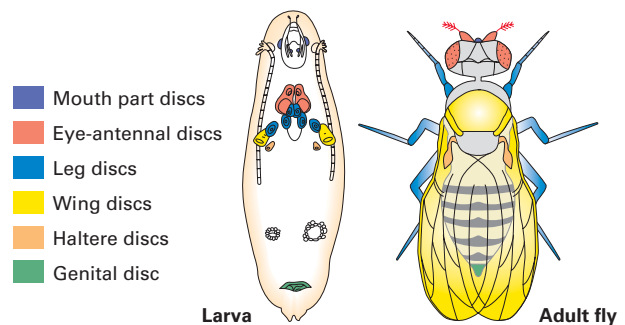


▲ **FIGURE 15-15 Formation of the cellular blastoderm during early *Drosophila* embryogenesis.** Stages from syncytium (a) to cellular blastoderm (b) are illustrated in diagrams and electron micrographs. Nuclear division is not accompanied by cell division until about 6000 nuclei have formed and migrated outward to the plasma membrane. Before cellularization, the embryo displays surface bulges overlying individual nuclei, which remain within a common cytoplasm. No membranes other than that surrounding the entire embryo are present. After cellularization, cell membranes are evident around individual nuclei. Note the segregation of the nuclei of so-called pole cells, which give rise to germ-line cells, at the posterior end of the syncytial blastoderm. [See R. R. Turner and A. P. Mahowald, 1976, *Devel. Biol.* 50:95; photographs courtesy of A. P. Mahowald; diagrams after P. A. Lawrence, *The Making of a Fly*, 1992, Blackwell Scientific, Oxford.]

(a) *Drosophila* developmental stages



(b) Imaginal discs, precursors to the adult



▲ **FIGURE 15-16 Major stages in the development of *Drosophila*.** (a) The fertilized egg develops into a blastoderm and undergoes cellularization in a few hours. The larva, a segmented form, appears in about 1 day and passes through three stages (instars) over a 4-day period, developing into a prepupa. Pupation takes ~4–5 days, ending with the emergence of the adult fly from the pupal case. (b) Groups of ectodermal cells called imaginal discs are set aside at specific sites in the larval body cavity. During pupation, these give rise to the various body parts indicated. Other precursor cells give rise to adult muscle, the nervous system, and other internal structures. [Part (a) from M. W. Strickberger, 1985, *Genetics*, 3d ed., Macmillan, p. 38; reprinted with permission of Macmillan Publishing Company. Part (b) Adapted from same source and J. W. Fristrom et al., 1969, in E. W. Hanly, ed., *Park City Symposium on Problems in Biology*, University of Utah Press, p. 381.]

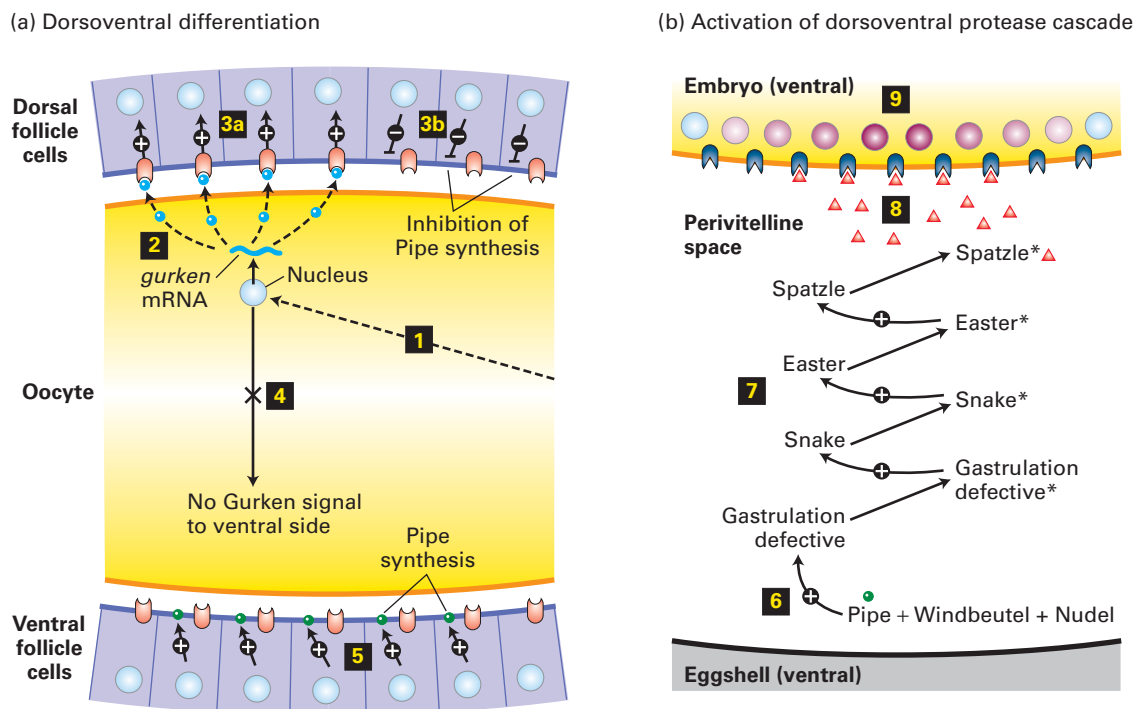
Reciprocal Signaling Between the Oocyte and Follicle Cells Establishes Initial Dorsoventral Patterning in *Drosophila*

Initial dorsal/ventral patterning in *Drosophila* is controlled by the events of oogenesis. Indeed, the shape of the mature oocyte is an accurate predictor of the dorsal/ventral orientation of the embryo. The process begins when the nucleus of the early oocyte moves slightly, perhaps randomly, toward what will become the anterior and dorsal side of the mature egg (Figure 15-17a). That loss of symmetry triggers the polarization of signals that coordinate the dorsal/ventral axes of the oocyte, embryo, and surrounding eggshell. Such coordination is necessary so that the structures on the eggshell become aligned properly with structures of the growing embryo. For example, breathing tubes in the eggshell must connect with appropriate regions of the embryo.

About midway through *Drosophila* oogenesis, the production of Gurken, a signal similar to epidermal growth factor, begins. Because of the off-center location of the nucleus,

Gurken is produced on the dorsal side of the oocyte (Figure 15-17b). The receptor for Gurken, a receptor tyrosine kinase like the EGF receptor, is present on the surfaces of all the follicle cells that abut the oocyte. The dorsal Gurken signal activates its receptors only in dorsal follicle cells, leading to changes in their appearance and to repression of the *pipe* gene within them. Because of this dorsal repression, Pipe protein is produced only in ventral follicle cells. Pipe is an enzyme that catalyzes sulfation of glycosaminoglycans (GAGs), the polysaccharide chains that are added to proteins to form proteoglycans (Chapter 6).

Pipe protein promotes ventral cell fates, probably by activating a still unknown signal that triggers a series of proteolytic cleavages in the perivitelline space on the ventral side of the by now mature egg. The ensuing chain of events has some similarity to the blood-clotting cascade, each protein cleaving and thereby activating the next one in the series. The outcome of the cleavages is the production of a ligand called Spätzle only on the ventral side (Figure 15-17c). By this time, the egg has been fertilized and early nuclear division is



▲ FIGURE 15-17 Dorsoventral axis determination in *Drosophila*. This process relies on two signal systems, one in follicle cells and the other in the oocyte or embryo or both, plus a proteolytic cascade within the perivitelline space. (a, b) Movement of the oocyte nucleus creates an initial asymmetry (1). The dorsal location of the oocyte nucleus ultimately results in the production of Pipe protein only in ventral follicle cells (2–5). (c) Subsequent events in the perivitelline space along the ventral surface generate a gradient of active Spätzle (6–9). By this time, the egg has been fertilized and the embryo is a syncytium with many nuclei; the region around only one nucleus is shown. Activation of Toll on the embryo's surface

by Spätzle causes Dorsal protein to enter the nucleus (10 and 11) where it activates transcription of specific target genes, depending on its concentration. (d) The concentration of Spätzle—hence Toll activation and nuclear localization of Dorsal—is greatest along the ventral midline, conferring ventral fates (e.g., muscle) on cells in this region. More laterally, less Dorsal enters the nuclei, and in consequence the cell fates are different (e.g., neural). Dorsal cell fates arise where no Dorsal enters the nucleus. Mutants lacking Toll receptor, or Dorsal, form only dorsal cell types. [Adapted from Gilbert and Hashimoto, 1999, *Trends Cell Biol.* 9:102.]

underway. Spätzle binds to its transmembrane receptor, called Toll, on the ventral embryo surface. Thus the signaling has come full circle: Gurken ligand produced dorsally in oocyte → activation of its EGF-like receptor on dorsal follicle cells, and then back through the protease cascade on the ventral side → Spätzle ligand → activation of its receptor, Toll, on the ventral side of the embryo. The net effect is to coordinate the eggshell structures produced by the follicle cells with the embryo structures produced inside.

Within the embryo, association of the cytosolic domain of activated Toll with two proteins (Tube and Pelle) leads to phosphorylation of Cactus protein. In the absence of Toll signaling, Cactus binds to a transcription factor called Dorsal and traps it, but phosphorylated Cactus is rapidly degraded by the proteasome. The newly freed Dorsal is able to enter the nuclei of the embryo's cells and activate the transcription of different target genes, depending on its concentration (see Figure 15-17c). Spätzle and Dorsal thus function as graded regulators, inducing ventral fates where their concentration is highest and other fates laterally as their concentration diminishes (Figure 15-17d). Dorsal function reaches its peak after cellularization has taken place.

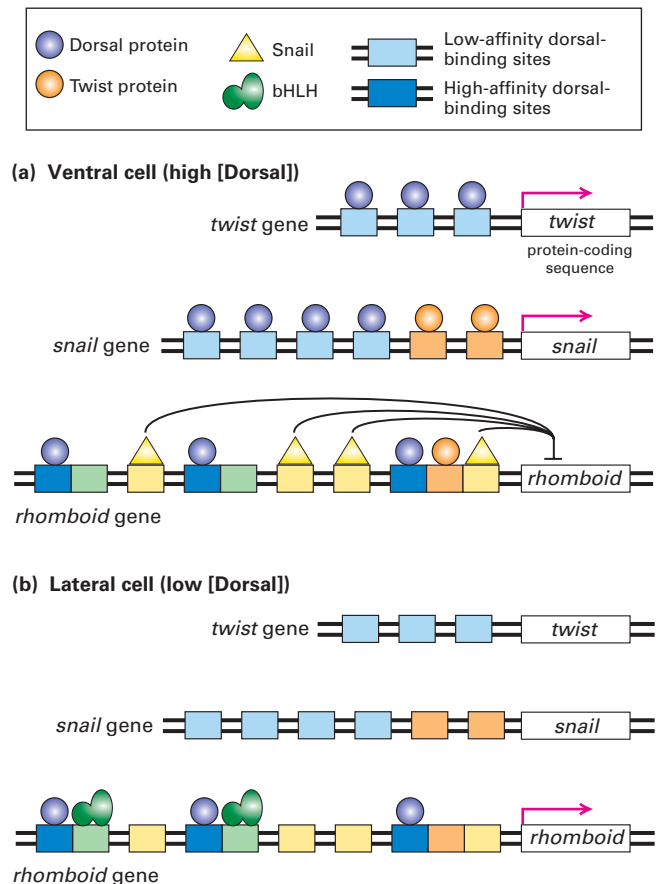
The central features of the *Toll-Dorsal pathway* in flies, which are analogous to those of the mammalian *NF-κB pathway* discussed in Chapter 14, exist in mammals and probably in all animals. Dorsal is similar to the NF-κB transcription factor; Cactus, to its inhibitor, I-κB; and the Toll receptor, to the receptor for interleukin 1, which acts through Tube and Pelle equivalents to cause the phosphorylation of I-κB and the release of NF-κB (see Figure 14-28). NF-κB is a critical regulator of genes required for immune responses in mammals and insects and appears to function in mammalian development as well. It nicely exemplifies the utilization of one signal-transduction pathway to accomplish multiple tasks, such as patterning in development plus the immune response to infection or injury. This phenomenon appears to be fairly common and partly explains the small number of signaling pathways that have evolved over biological time despite the increasing complexity of organisms.

Nuclear Dorsal and Decapentaplegic, a Secreted Signal, Specify Ventral and Dorsal Cell Fates

The remarkable series of steps depicted in Figure 15-17 results in a gradient in the nuclear localization of the transcription factor Dorsal. The concentration of nuclear Dorsal decreases gradually from highest in cells at the ventral midline to lower values in lateral cells and eventually to none in dorsal cells. Mutants lacking *dorsal* function cannot make cells with ventral character; so the entire embryo develops dorsal structures. (Note that fly genes are named according to their mutant phenotypes; thus the *dorsal* gene controls ventral fates.) Once inside the nucleus, Dorsal controls the transcription of specific target genes by binding to distinct high- and low-affinity regulatory sites and by interacting in a combinatorial fashion with other transcription factors. Dorsal represses the transcription

of *decapentaplegic* (*dpp*), *tolloid*, *short gastrulation*, and *zerknüllt* and activates the transcription of *twist*, *snail*, *single-minded*, and *rhomboid*. Each of these genes contains a unique combination of cis-acting regulatory sequences to which Dorsal and other transcription factors bind.

Figure 15-18 illustrates how nuclear Dorsal specifies different target-gene expression patterns, depending on its con-



▲ FIGURE 15-18 Activation of different target genes by Dorsal subsequent to Toll signaling. Activation of Toll by Spätzle leads to a graded nuclear localization of Dorsal protein (see Figure 15-17d). The resulting Dorsal concentration gradient (ventrally high and dorsally low) can lead to different patterns of gene expression. Shown here are three target genes that have either high-affinity (dark blue) or low-affinity (light blue) Dorsal-binding sites. (a) In ventral regions where the concentration of Dorsal (purple) is high, it can bind to low-affinity sites in *twist* and *snail*, activating the transcription of these genes. Twist protein (orange) also activates the transcription of *snail*, which encodes a repressor (yellow) that prevents the transcription of *rhomboid* in this region. (b) In lateral regions, the Dorsal concentration is not high enough for the binding of Dorsal to the low-affinity sites regulating *twist* and *snail*. The binding of Dorsal to *rhomboid* is facilitated by the presence of high-affinity sites and the synergistic binding of bHLH heterodimeric activators (green) to neighboring sites. The sharp boundary in the expression of Rhomboid causes formation of distinct cell types in ventral vs. lateral regions. [See A. M. Huang et al., 1997, *Genes & Dev.* **11**:1963.]

centration. For instance, the *twist* gene, which contains three low-affinity Dorsal-binding sites, is expressed most ventrally where the Dorsal concentration is highest. As the Dorsal concentration decreases, it falls beneath the threshold necessary to activate the transcription of *twist*. The *rhomboid* gene, which is expressed only in lateral regions, is controlled through a complex cis-acting regulatory region that contains three high-affinity Dorsal-binding sites. Two of these sites are adjacent to regulatory sequences that bind proteins containing the basic helix-loop-helix (bHLH) motif, which is present in numerous transcription factors. As Twist contains a bHLH motif, it appears that it acts cooperatively with Dorsal to induce transcription of the *rhomboid* gene in lateral cells. The *rhomboid* control region also contains four binding sites for Snail, a transcriptional repressor. The production of Snail is induced only at high concentrations of Dorsal because the *snail* gene contains only low-affinity Dorsal-binding sites within its control region. Because Snail is localized ventrally, its repressor activity defines a sharp ventral-lateral boundary in the transcription of *rhomboid*. This example nicely illustrates how two transcriptional regulators can collaborate to create a sharp boundary between cell types, something to be discussed further in Sections 15.4 and 15.5.

The dorsal/ventral patterning produced by Dorsal is extended by Decapentaplegic (Dpp). This secreted signaling protein belongs to the TGF β family, which is found in all animals (Chapter 14). Because transcription of the *dpp* gene is repressed by Dorsal, Dpp is produced only in the dorsal-most cells of the early fly embryo, which lack Dorsal in their nuclei. A combination of genetic and molecular genetic evidence suggests that Dpp acts as a morphogen to induce the establishment of different ectoderm cell types in the dorsal region of the embryo. For instance, complete removal of Dpp function leads to a loss of all dorsal structures and their conversion into more-ventral ones. Embryos carrying only one wild-type *dpp* allele show an increase in the number of cells assuming a ventral fate, whereas embryos with three copies of *dpp* form more dorsal cells.

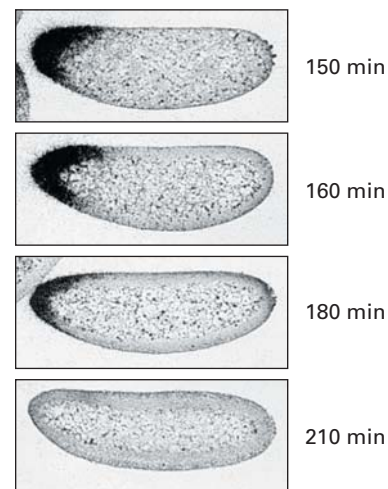
Thus two graded secreted signals, Spätzle and Dpp, play critical roles in determining the dorsal/ventral axis in *Drosophila* and in inducing further patterning within the dorsal and ventral regions. Spätzle, acting through nuclear-localized Dorsal, a transcription factor, induces ventral fates and controls the production of Dpp, which induces dorsal fates. Unlike Dorsal, which functions only in early development, the Dpp signal is used repeatedly in later development, participating in many processes such as appendage development, gut formation, and eye development. The Spätzle signal also has other functions, which are discussed later.

The frog TGF β family members called BMP2 and BMP4 have inductive effects similar to those of Dpp protein and indeed are the vertebrate proteins most closely related in sequence to Dpp. Most or all components of the TGF β signaling pathway, including Smad transcription factors, appear to be present and participating in development in all animals

(see Figure 14-2). As discussed in Section 15.5, the vertebrate proteins also control patterning along the dorsal/ventral axis, although the axis is flipped in vertebrates compared with invertebrates. Loss of TGF β signaling, owing to mutations in TGF β receptors or Smad proteins, contributes to the onset of cancer (Chapter 23).

Transcriptional Control by Maternally Derived Bicoid Protein Specifies the Embryo's Anterior

We turn now to determination of the anterior/posterior axis in the early fly embryo while it is still a syncytium. As in determination of the dorsal/ventral axis, specification of anterior/posterior cell fate begins during oogenesis. The initial asymmetry also involves so-called *maternal mRNAs*, which are produced by nurse cells and transported into the oocyte. In this case they become localized in discrete spatial domains (see Figure 15-14). For example, *bicoid* mRNA is trapped at the most anterior region, or anterior pole, of the early fly embryo (Figure 15-19). The anterior localization of



▲ EXPERIMENTAL FIGURE 15-19 Maternally derived *bicoid* mRNA is localized to the anterior region of early *Drosophila* embryos.

All embryos shown are positioned with anterior to the left and dorsal at the top. In this experiment, in situ hybridization with a radioactively labeled RNA probe specific for *bicoid* mRNA was performed on whole-embryo sections 2.5–3.5 hours after fertilization. This time period covers the transition from the syncytial blastoderm to the beginning of gastrulation. After excess probe was removed, probe hybridized to maternal *bicoid* mRNA (dark silver grains) was detected by autoradiography. Bicoid protein is a transcription factor that acts alone and with other regulators to control the expression of certain genes in the embryo's anterior region. [From P. W. Ingham, 1988, *Nature* 335:25; photographs courtesy of P. W. Ingham.]

bicoid mRNA depends on its 3'-untranslated end and three maternally derived proteins. Embryos produced by female flies that are homozygous for *bicoid* mutations lack anterior body parts, attesting to the importance of Bicoid protein in specifying anterior cell fates.

Bicoid protein, a homeodomain-type transcription factor, activates expression of certain anterior-specific genes discussed later. In the syncytial fly embryo, Bicoid protein spreads through the common cytoplasm away from the an-

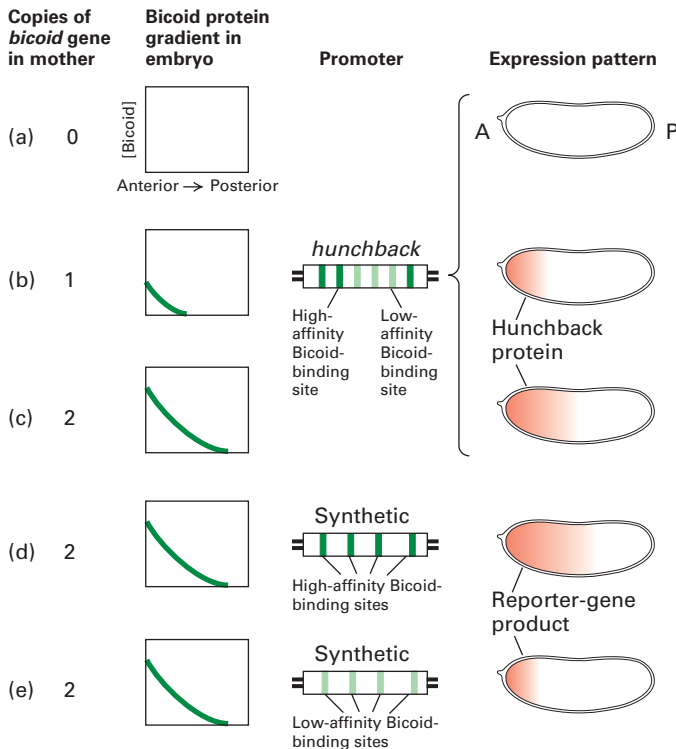
terior end where it is produced from the localized mRNA. As a result, a Bicoid protein gradient is established along the anteroposterior axis of the syncytial embryo. Evidence that the Bicoid protein gradient determines anterior structures was obtained through injection of synthetic *bicoid* mRNA at different locations in the embryo. This treatment led to the formation of anterior structures at the site of injection, with progressively more posterior structures forming at increasing distances from the injection site. Another test was to make flies that produced extra anterior Bicoid protein; in these flies, the anterior structures expanded to occupy a greater proportion of the embryo.

Bicoid protein promotes transcription of the *hunchback* (*hb*) gene from the embryo's genome. Transcription of *hunchback* is greatest in the anterior of the embryo where the Bicoid concentration is highest. Mutations in *hunchback* and several other genes in the embryo's genome lead to large gaps in the anteroposterior pattern of the early embryo; hence these genes are collectively called **gap genes**. Several types of evidence indicate that Bicoid protein directly regulates transcription of *hunchback*. For example, increasing the number of copies of the *bicoid* gene expands the Bicoid and Hunchback (Hb) protein gradients posteriorly in parallel (Figure 15-20a–c). Analysis of the *hunchback* gene revealed that it contains three low-affinity and three high-affinity binding sites for Bicoid protein. The results of studies with synthetic genes containing either all high-affinity or all low-affinity Bicoid-binding sites demonstrated that the affinity of the site determines the threshold concentration of Bicoid at which gene transcription is activated (Figure 15-20d, e). In addition, the number of Bicoid-binding sites occupied at a given concentration has been shown to determine the amplitude, or level, of the transcription response.

Findings from studies of Bicoid's ability to regulate transcription of the *hunchback* gene show that variations in the levels of transcription factors, as well as in the number or affinity of specific regulatory sequences controlling different target genes, or both, contribute to generating diverse patterns of gene expression in development. These findings thus parallel those on the Dorsal transcription factor discussed previously. Similar mechanisms are employed in other developing organisms.

Maternally Derived Translation Inhibitors Reinforce Bicoid-Mediated Anteroposterior Patterning

Cell types at the posterior end of the fly embryo are controlled by a different mechanism—one in which control is at the translational level rather than the transcriptional level. As just discussed, transcription of the embryo's *hunchback* gene, which promotes anterior cell fates, produces an anteriorly located band of *hunchback* mRNA and Hunchback protein because of the anterior → posterior gradient of maternally derived Bicoid protein. In addition, however, *hunch-*



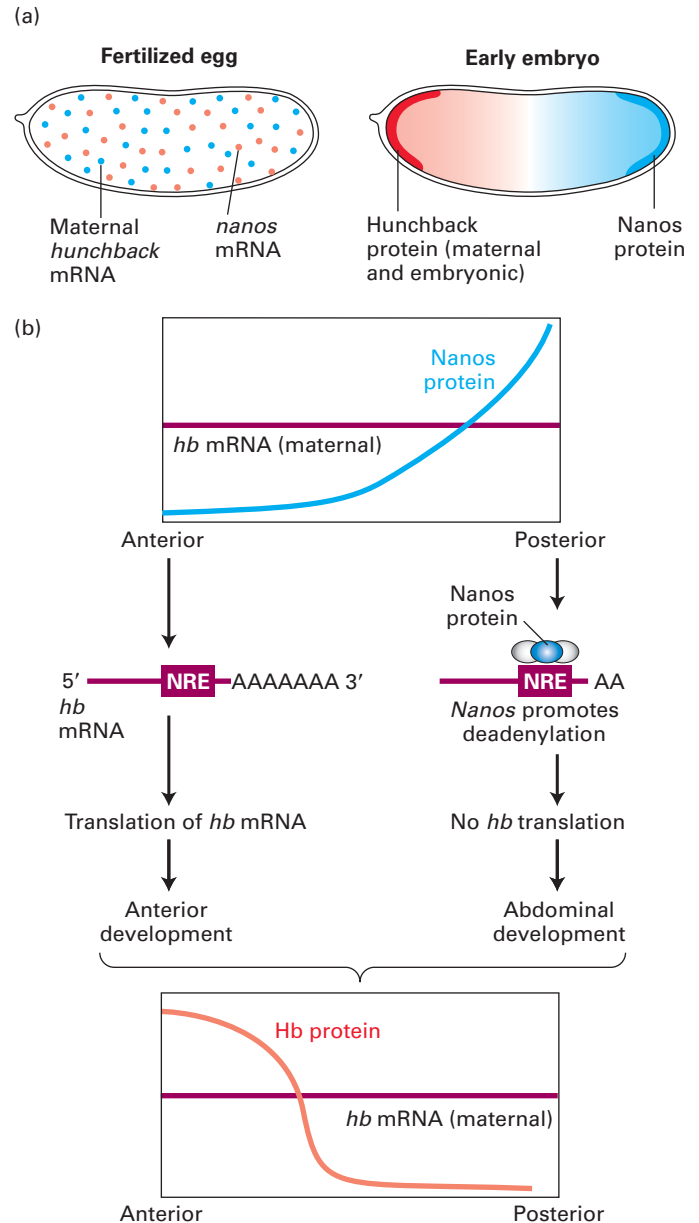
▲ EXPERIMENTAL FIGURE 15-20 Maternally derived Bicoid controls anterior/posterior expression of the embryonic *hunchback* (*hb*) gene. (a–c) Increasing the number of *bicoid* genes in mother flies changed the Bicoid gradient in the early embryo, leading to a corresponding change in the gradient of Hunchback protein produced from the *hunchback* gene in the embryo's genome. The *hunchback* promoter contains three high-affinity and three low-affinity Bicoid-binding sites. Transgenic flies carrying a reporter gene linked to a synthetic promoter containing either four high-affinity sites (d) or four low-affinity sites (e) were prepared. In response to the same Bicoid protein gradient in the embryo, expression of the reporter gene controlled by a promoter carrying high-affinity Bicoid-binding sites extended more posteriorly than did transcription of a reporter gene carrying low-affinity sites. This result indicates that the threshold concentration of Bicoid that activates *hunchback* transcription depends on the affinity of the Bicoid-binding site. Bicoid regulates other target genes in a similar fashion. [Adapted from D. St. Johnston and C. Nüsslein-Volhard, 1992, *Cell* 68:201.]

back mRNA synthesized by nurse cells also is present in the early embryo. Even though this maternal *hunchback* mRNA is uniformly distributed throughout the embryo, its translation is prevented in the posterior region by another maternally derived protein called Nanos, which is localized to the posterior end of the embryo. Nanos protein not only blocks translation of maternal *hunchback* mRNA in the posterior region. The set of genes required for Nanos protein localization is also required for germ-line cells to form at the posterior end of the embryo. The evolutionary conservation of this Nanos function in flies and in worms may indicate an ancient system for forming germ-line cells. Related proteins exist in vertebrates, but their functions are not yet known.

Figure 15-21 illustrates how translational regulation by Nanos helps to establish the anterior → posterior Hunchback gradient needed for normal development. Translational repression of *hunchback* mRNA by Nanos depends on specific sequences in the 3'-untranslated region of the mRNA, called Nanos-response elements (NREs). Along with two other RNA-binding proteins, Nanos binds to the NRE in *hunchback* mRNA. Although the precise mechanism by which repression is achieved is not known, repression inversely correlates with the length of the poly(A) tail in *hunchback* mRNA, which is determined by the balance between the opposite processes of polyadenylation and deadenylation. In wild-type embryos, the length of the poly(A) tail increases immediately before translation of *hunchback* mRNA. The results of genetic and molecular studies suggest that Nanos promotes deadenylation of *hunchback* mRNA and thereby decreases its translation. In the absence of Nanos, an accumulation of maternal Hb protein in the posterior region leads to the failure of the posterior structures to form normally, and the embryo dies. Conversely, if Nanos is produced in the anterior, thereby inhibiting the production of Hb from both maternal and embryonic *hunchback* mRNA, anterior body parts fail to form, again a lethal consequence.

Nanos protein localization in the posterior embryo is intimately coupled to the regulation of translation of *nanos* mRNA. The *nanos* mRNA that is not located at the posterior is not translated due to a protein called Smaug that binds the 3' UTR of *nanos* mRNA. Localization of *nanos* mRNA at the posterior depends on other proteins as well. One of these is Oskar, whose maternally provided mRNA is transported to the posterior by kinesin, a motor protein that moves along microtubules (Chapter 20). Therefore the kinesin controls, after several intervening steps, the localized activity of a transcription factor (Hunchback).

Translational control due to the action of an inhibitor, mRNA localization, or both, may be widely used strategies for regulating development. For instance, specific mRNAs are localized during the development of muscle cells (see Figure 12-31) and during cell division in the budding yeast *Saccharomyces cerevisiae* (see Figure 22-22). Similar mechanisms operate during the development of *C. elegans*. Even more intriguing is the discovery that Bicoid protein binds



▲ **FIGURE 15-21 Role of Nanos protein in excluding maternally derived Hunchback (Hb) protein from the posterior region of *Drosophila* embryos.** (a) Both *nanos* (red) and *hunchback* (*hb*) (blue) mRNAs derived from the mother are distributed uniformly in the fertilized egg and early embryo. Nanos protein, which is produced only in the posterior region, subsequently inhibits translation of maternal *hb* (blue) mRNA posteriorly. (b) Diffusion of Nanos protein from its site of synthesis in the posterior region establishes a posterior → anterior Nanos gradient. A complex of Nanos and two other proteins inhibits translation of maternal *hb* mRNA. As a consequence, maternally derived Hb protein is expressed in a graded fashion that parallels and reinforces the Hb protein gradient resulting from Bicoid-controlled transcription of the embryo's *hb* gene (see Figure 15-20). [See C. Wreden et al., 1997, *Development* **124**:3015.]

not only to DNA to promote transcription of the embryonic *hunchback* gene but also to *caudal* mRNA (which encodes another *Drosophila* protein having a role in early-patterning events) and regulates its translation.

Toll-like Signaling Activates an Ancient Defense System in Plants and Animals

Before continuing our account of signaling and gene control in development, we digress briefly to consider the connection between innate immunity and the Toll-Dorsal signaling pathway discussed previously (see Figure 15-17c). Recall that this pathway has many parallels with the NF- κ B pathway in mammals, which is intimately involved in immune responses. Discovery of these parallels was the first hint that Toll signaling might function in nondevelopmental contexts. More recently, researchers have found that the Toll receptor, its ligand Spätzle, and other pathway components are required for the expression of an antifungal peptide (drosomycin) in fly larvae and adults. Stimulation of a different Toll-like receptor triggers the production of an antibacterial peptide (diptericin) in flies. Discovery of a mammalian Toll-like receptor that controls the production of anti-inflammatory cytokines stimulated exploration of the whole set of similarities.

Toll signaling now appears to be one of the most evolutionarily conserved processes known. All the components between Toll and the activation of Dorsal have been largely conserved. There are 8 *Drosophila* proteins related to Toll plus Toll itself and 10 Toll-related human proteins that control the production of a wide variety of antimicrobial peptides in flies and cytokines in mammals. These molecules provide a rapid, nonspecific defense against infection by a wide array of pathogens. The adaptive immune response mounted by vertebrates, involving antibodies and T cells, is directed against specific pathogens but is slower to develop than the innate, nonspecific response.

Most remarkably, parts of the Toll pathway and its function in immunity are readily recognizable in plants. For instance, *Arabidopsis* has about 100 proteins containing domains similar to the cytosolic domains of Toll that transduce the intracellular signal. At least some of these proteins are required for resistance to tobacco mosaic virus, and an *Arabidopsis* protein similar in sequence to I- κ B is required for resistance to downy mildew fungus. However, the Toll-like signaling in *Arabidopsis* appears to act through transcription factors that are unrelated to Dorsal or NF- κ B.

Because the Toll-based innate immunity system appears to be present in both plants and animals, it may be more than a billion years old. In the course of their long evolution, at least some of the genes having roles in the basic survival function of immunity have been adapted to serve as developmental regulators—a nice example of biological parsimony in the use of genetic resources.

KEY CONCEPTS OF SECTION 15.3

Control of Cell Fates by Graded Amounts of Regulators

- The influence of one cell population on the developmental fate of another one nearby is called induction. Both diffusible signaling molecules and direct cell–cell contacts mediate induction.
- Morphogens are signals that act in a graded fashion: a cell that receives more of a signal takes on one fate, a cell that receives less takes on a different fate, and so forth. Other signals act in a relay fashion: a signal induces one cell to produce a different signal that instructs cells farther away from the original signal source (see Figure 15-11).
- In *Drosophila* syncytial embryos, morphogens are used at several stages to induce different cell types along both axes. Asymmetries created in the fly egg during oogenesis trigger events that determine both the dorsoventral and the anterioposterior axes in fly embryos.
- Two signal systems—one acting during oogenesis and the other acting in the early fly embryo—plus a proteolytic cascade within the perivitelline space lead to the graded nuclear localization of Dorsal, a transcription factor: ventral nuclei receive the most Dorsal; lateral nuclei, less; and dorsal nuclei, none (see Figure 15-17). These early events control development along the dorsoventral axis.
- In ventral cells, Dorsal turns off genes (e.g., *decapentaplegic*, or *dpp*) needed to make dorsal structures. Dpp, a TGF β -type signal produced in dorsal cells, functions in graded fashion to specify dorsal cell types. Dpp homologs in vertebrates (BMP proteins) also act in dorsoventral patterning.
- Early anterioposterior patterning in *Drosophila* produces an anterior $\rightarrow$ posterior gradient of Hunchback (Hb), a transcription factor that promotes anterior cell fates. Transcription of the embryonic *hb* gene is activated by maternally derived Bicoid protein, which is localized to the anterior (see Figure 15-20).
- Nanos protein inhibits the translation of maternal *hb* mRNA in the posterior of the fly embryo (see Figure 15-21). Synthesis of Nanos from maternal *nanos* mRNA is restricted to the posterior by translational control linked to motor protein-mediated transport of other regulators to the posterior pole.

15.4 Boundary Creation by Different Combinations of Transcription Factors

In Section 15.3, we saw that maternally derived Bicoid plays a key role in initiating the transcription of gap genes (e.g., *hunchback*) in the anterior region of the early fly embryo; other maternal factors prevent the translation of *hunchback* mRNA in the posterior. Further specification of cell fates in *Drosophila* is controlled by *transcription cascades* in which

one transcription factor activates a gene encoding another transcription factor, which in turn acts to promote the expression of a third transcription factor. Such a transcription cascade can generate a population of cells that may all look alike but differ at the transcriptional level.

Transcription cascades have both a temporal and a spatial dimension. At each step in a cascade, for instance, RNA polymerase and ribosomes can take more than an hour to produce a protein, depending on the length of the corresponding gene. Spatial factors come into play when cells at different positions within an embryo synthesize different transcription factors. In this section, we continue the story of early patterning in *Drosophila*, which illustrates both the spatial and the temporal aspects of transcription cascades. The principles learned from the study of *Drosophila* development broadly apply to the creation of form and pattern in all organisms including plants, as discussed at the end of this section. Errors in the genes that control organization, boundary formation, and cell type are associated with many human diseases.

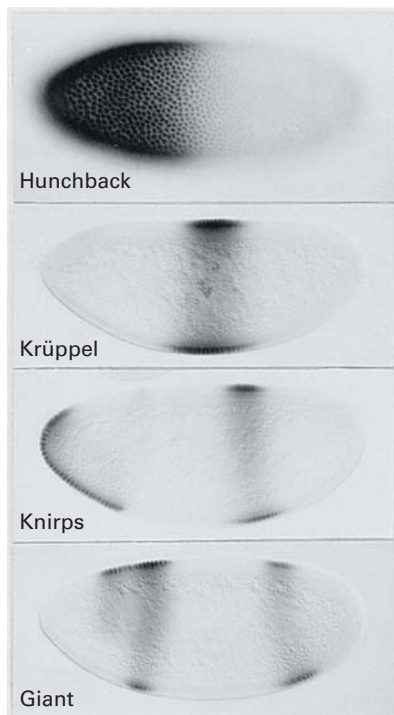
Drosophila Gap Genes Are Transcribed in Broad Bands of Cells and Regulate One Another

The rough outline of cell fates that is laid down in the syncytial fly embryo is refined into a system for precisely controlling the fates of individual cells. The discovery of the relevant regulators came from a genetic screen for mu-

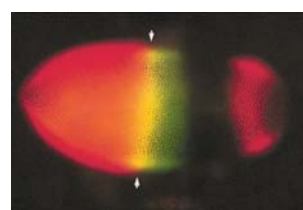
tants with altered embryo body segments. The embryonic body segments go on to grow into the familiar striped pattern seen on any passing hornet. In addition to *hunchback*, four other gap genes—*Krüppel*, *knirps*, *giant*, and *tailless*—are transcribed in specific spatial domains, beginning about 2 hours after fertilization and just before cellularization of the embryo is complete (Figure 15-22a).

All the gap-gene proteins are transcription factors. Because these proteins are distributed in broad overlapping peaks (Figure 15-22b), each cell along the anterior/posterior axis contains a particular combination of gap-gene proteins that activates or represses specific genes within that cell. Indeed, something like a battle ensues, because some gap proteins repress the transcription of genes encoding other gap proteins. Although they have no known extracellular ligands, some gap proteins resemble **nuclear receptors**, which are intracellular proteins that bind lipophilic ligands (e.g., steroid hormones) capable of crossing the plasma membrane. Most ligand–nuclear receptor complexes function as transcription factors (see Figure 11-44). The sequence similarity between gap proteins and nuclear receptors suggests that gap genes may have evolved from genes whose transcription was controlled by signals that could cross membranes, such as the steroid hormones. The use of such signal-controlled genes, rather than transcription cascades, could explain how early cell-fate specification operates in animals that do not have a syncytial stage.

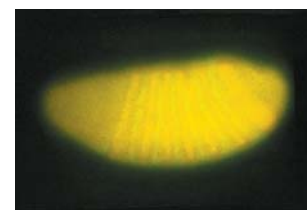
(a) Gap-gene proteins



(b) Hunchback and Krüppel



(c) Even-skipped and fushi tarazu



▲ EXPERIMENTAL FIGURE 15-22 Gap genes and pair-rule genes are expressed in characteristic spatial patterns in early *Drosophila* embryos.

Fixed, permeabilized embryos were stained with fluorescence-labeled antibodies specific for a particular protein. All embryos shown are positioned with anterior to the left and dorsal at the top. (a) These syncytial embryos were stained individually for the indicated gap-gene-encoded proteins. Transcription of the *Krüppel*, *knirps*, and *giant* gap genes is regulated by Hunchback, Bicoid, and Caudal. (b) This syncytial embryo was doubly stained to visualize Hunchback protein (red) and Krüppel protein (green). The yellow band identifies the region in which expression of these two gap proteins overlaps. (c) Double staining of an embryo at the beginning of gastrulation reveals even-skipped protein (yellow) and Fushi tarazu protein (orange). These two pair-rule gene products are expressed in alternating stripes. See text for discussion. [Part (a) adapted from G. Struhl et al., 1992, *Cell* 69:237. Parts (b) and (c) courtesy of M. Levine.]

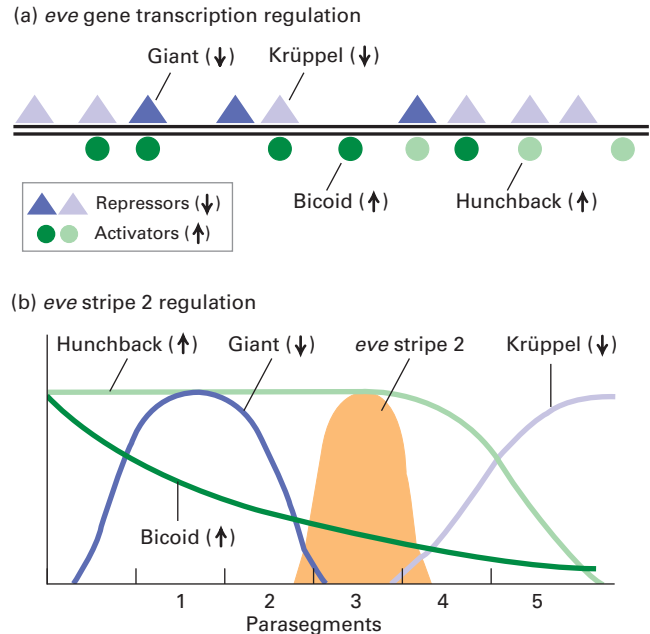
The fates of cells distributed along the anterior/posterior axis are specified early in fly development. At the same time, cells are responding to the dorsal/ventral control system. Each cell is thus uniquely specified along both axes, in a two-dimensional grid. If each of the five gap genes were expressed in its own section of the embryo, at just one concentration, only five cell types could be formed. The actual situation permits far greater diversity among the cells. As noted earlier, the amount of each gap protein varies from low to high to low along the anterior/posterior axis, and the expression domains of different gap genes overlap. This complexity creates combinations of transcription factors that lead to the creation of many more than five cell types. Remarkably, the next step in the *Drosophila* development process generates a repeating pattern of cell types from the rather chaotic non-repeating pattern of gap-gene expression domains.

Combinations of Gap Proteins Direct Transcription of Pair-Rule Genes in Stripes

Our vertebrae and the body segments of an insect are both examples of a commonly employed tactic in animal structure and music: repeats with variations. The first sign of such repeats in fly embryos is a pattern of repeating stripes of transcription of eight genes collectively called **pair-rule genes**. The body of a larval fly consists of 14 segments, and each pair-rule gene is transcribed in half of its primordia, or seven stripes, separated by “interstripes” where that pair-rule gene is not transcribed (Figure 15-22c). Mutant embryos that lack the function of a pair-rule gene have their body segments fused together in pair-wise fashion—hence the name of this class of genes. The expression stripes for each pair-rule gene partly overlap with those of other pair-rule genes; so each gene must be responding in a unique way to gap-gene and other earlier regulators.

The transcription of three “primary” pair-rule genes is controlled by transcription factors encoded by gap and maternal genes. Because gap and maternal genes are expressed in broad, nonrepeating bands, the question arises: How can such a nonrepeating pattern of gene activities confer a repeating pattern such as the striped expression of pair-rule genes? To answer this question, we consider the transcription of the *even-skipped (eve)* gene in stripe 2, which is controlled by the maternally derived Bicoid protein and the gap proteins Hunchback, Krüppel, and Giant. All four of these transcription factors bind to a clustered set of regulatory sites, or **enhancer**, located upstream of the *eve* promoter (Figure 15-23a). Hunchback and Bicoid activate the transcription of *eve* in a broad spatial domain, whereas Krüppel and Giant repress *eve* transcription, thus creating sharp posterior and anterior boundaries. The combined effects of these proteins, each of which has a unique concentration gradient along the anteroposterior axis, initially demarcates the boundaries of stripe 2 expression (Figure 15-23b).

The initial pattern of pair-rule stripes, which is not very sharp or precise, is sharpened by autoregulation. The Eve



▲ **FIGURE 15-23 Expression of the Even-skipped (*Eve*) stripe 2 in the *Drosophila* embryo.** (a) Diagram of an 815-bp enhancer controlling transcription of the pair-rule gene *eve*. This regulatory region contains binding sites for Bicoid and Hunchback proteins, which activate the transcription of *eve*, and for Giant and Krüppel proteins, which repress its transcription. Enhancer is shown with all binding sites occupied, but in an embryo occupation of sites will vary with position along the anterior/posterior axis. (b) Concentration gradients and of the four proteins that regulate *eve* stripe 2. The coordinated effect of the two repressors (↓) and two activators (↑) determine the precise boundaries of the second anterior *eve* stripe. Only in the orange region is the combination of regulators correct for the *eve* gene to be transcribed in response to the stripe 2 control element. Further anterior Giant turns it off; further posterior Krüppel turns it off and the level of Bicoid activator is too low. Expression of other stripes is regulated independently by other combinations of transcription factors that bind to enhancers not depicted in part (a). [See S. Small et al., 1991, *Genes & Devel.* 8:827.]

protein, for instance, binds to its own gene and increases transcription in the stripes, a positive autoregulatory loop. This enhancement does not occur at the edges of stripes where the Eve protein concentration is low; so the boundary between stripe and interstripe is fine-tuned.

Each primary pair-rule gene is regulated by multiple enhancers that are organized in modules. Each stripe is formed in response to a different combination of transcriptional regulators acting on a specific module, so the nonrepeating distributions of regulators can create repeating patterns of pair-rule gene repression and activation. If even one enhancer is bound by an activating combination of transcriptional regulators, the presence of other enhancers in an inactive, “off” state (not bound to a regulator) will not prevent transcription. For instance, in Eve stripe 2, the right combination and amounts of Hunchback and Bicoid create an “on” state that activates

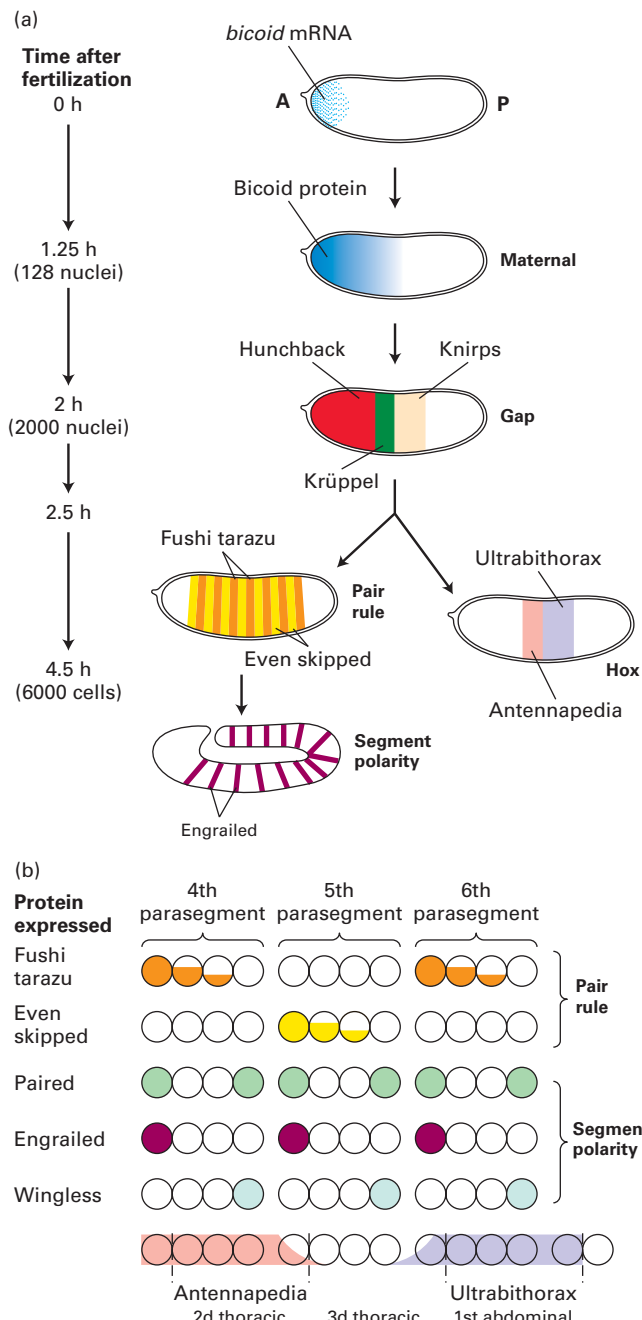
transcription even though other enhancers are present in the inactive state. In each stripe, at least one enhancer is bound by an activating combination of regulators. Note that this system of gene control is flexible and could be used to produce nonrepeating patterns of transcription if that were useful to an animal.

Similar responses to gap and maternal proteins govern the striped patterns of transcription of the two other primary pair-rule genes, *runt* and *hairy*. Because the enhancers of *runt* and *hairy* respond to different combinations of regulators, the *eve*, *runt*, and *hairy* expression stripes partly overlap one another, with each stripe for any one gene offset from a stripe

for another gene. Subsequently, other (secondary) pair-rule genes, including *fushi tarazu* (*ftz*) and *paired*, become active in response to the Eve, Runt, and Hairy proteins, which are transcription factors, as well as to maternal and gap proteins. The outcome is a complex pattern of overlapping stripes.

In early embryos each segment primordium is about four cells wide along the anteroposterior axis, which corresponds to the approximate width of pair-rule expression stripes. With pair-rule genes active in alternating four-on four-off patterns, the repeat unit is about eight cells. Each cell expresses a combination of transcription factors that can potentially distinguish it from any of the other seven cells in the repeat unit. Complete segmentation of the embryo into repeat units in which each cell exhibits a unique transcriptional pattern depends on activation of a third set of genes, the **segment-polarity genes**. These genes, which include *engrailed* (*en*) and *wingless* (*wg*), also are expressed in stripes, but the stripes are narrower and appear once in each segment primordium. Because some segment-polarity genes encode components of cell–cell signaling systems, they are discussed in Section 15.5.

Figure 15-24a schematically depicts the distribution of some key regulators in the *Drosophila* embryo during the first few hours after fertilization. When all the segmentation genes have been turned on, the resulting single-cell accuracy of cell-fate specification is impressive, as the example in Figure 15-24b illustrates.



◀ **FIGURE 15-24 Summary of sequential, spatially localized expression of selected genes in early development of the *Drosophila* embryo.** (a) Maternal *bicoid* mRNA is

localized at the anterior pole of the egg, but Bicoid protein, which is synthesized soon after fertilization, diffuses to form a gradient in the fly syncytium. In most cases, an mRNA and its corresponding protein are present in the same regions of the embryo. Specific combinations of Bicoid and various gap-gene products, including Hunchback, Krüppel, and Knirps, control transcription of the pair-rule genes such as *fushi tarazu* (*ftz*) and *even-skipped* (*eve*). Gap gene products are shown in discrete bands but actually they overlap. The pair-rule proteins demarcate 14 stripes corresponding to the parasegments (an offset form of the segment primordia). The segment-polarity gene *engrailed* (*en*) is expressed at the anterior end of each parasegment; it and other segment-polarity genes participate in patterning of each parasegment. Cellularization occurs after 2.5 hours, and gastrulation occurs at about 4.5 hours. By this time, each parasegment consists of four belts of cells. (b) Within a parasegment, each belt of cells (represented by a circle) is characterized by expression of a unique set of proteins encoded by pair-rule and segment-polarity genes. Shown here are the locations of three pair-rule proteins and two segment-polarity proteins in three parasegments (4–6). These expression patterns act as positional values that uniquely characterize each cell belt in a parasegment and determine where Hox genes such as *Antennapedia* (*Antp*) and *Ultrabithorax* (*Ubx*) are transcribed. Hox genes give the repeating body segments their distinct shapes and appendages (indicated at the bottom). Hox gene expression is regulated by gap, pair-rule, and segment polarity genes.

Maternal and Zygotic Segmentation Proteins Regulate Expression of Homeotic (Hox) Genes

As we have just seen, the spatially controlled transcription of pair-rule genes sets up repeating units within the initial sheet of cells composing the early *Drosophila* embryo. Later, these repeating units must diversify: only some of them will produce appendages, and they will specialize internally as well. This early diversification of body segments depends on the **Hox genes**, which are important switches that control cell identities and indeed the identities of whole parts of an animal.

Mutations in Hox genes often cause **homeosis**—that is, the formation of a body part having the characteristics normally found in another part at a different site. For example, flies develop legs on their heads instead of antennae. Loss of function of a particular Hox gene in a location where it is normally active leads to homeosis if a different Hox gene becomes derepressed there; the result is the formation of cells and structures characteristic of the derepressed gene. A Hox gene that is abnormally expressed where it is normally inactive can take over and impose its own favorite developmental pathway on its new location (Figure 15-25).

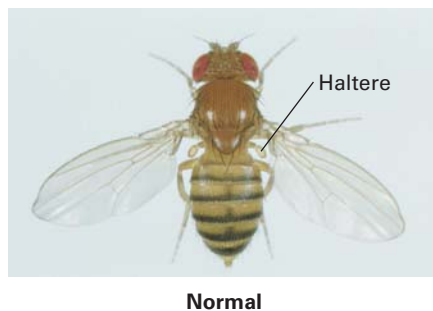
Hox genes encode highly related transcription factors containing the **homeodomain** motif. Classical genetic studies in *Drosophila* led to the discovery of the first Hox genes (e.g., *Antennapedia* and *Ultrabithorax*). Corresponding genes with similar functions (orthologs) have since been identified in most animal species. Each Hox gene is transcribed in a particular region along the anterioposterior axis in a remarkable arrangement where the order of genes along the chromosomes is colinear with the order in which they are expressed along the anterior/posterior axis. At one end of the complex are head genes, and genes expressed with progressively more posterior boundaries are in order, with “tail” genes last. Hox-gene expression domains can overlap (see Figure 15-24). In *Drosophila*, the spatial pattern of Hox-gene transcription is regulated by maternal, gap, and pair-rule transcription factors. The protein encoded by a particular Hox gene controls the organization of cells within

the region in which that Hox gene is expressed. For example, a Hox protein can direct or prevent the local production of a secreted signaling protein, cell-surface receptor, or transcription factor that is needed to build an appendage on a particular body segment.

Drosophila Hox proteins control the transcription of target genes whose encoded proteins determine the diverse morphologies of body segments. Vertebrate Hox proteins similarly control the different morphologies of vertebrae, of repeated segments of the hindbrain, and of the digits of the limbs. The association of Hox proteins with their binding sites on DNA is assisted by cofactors that bind to both Hox proteins and DNA, adding specificity and affinity to these interactions.

When Hox genes are turned on, their transcription must continue to maintain cell properties in specific locations. As in the *even-skipped* gene, the regulatory regions of some Hox genes contain binding sites for their encoded proteins. Thus Hox proteins can help to maintain their own expression through an autoregulatory loop.

Another mechanism for maintaining normal patterns of Hox-gene expression requires proteins that modulate chromatin structure. These proteins are encoded by two classes of genes referred to as the Trithorax group and Polycomb group. The pattern of Hox-gene expression is initially normal in Polycomb-group mutants, but eventually Hox-gene transcription is derepressed in places where the genes should be inactive. The result is multiple homeotic transformations. This observation indicates that the normal function of Polycomb proteins is to keep Hox genes in a transcriptionally inactive state. The results of immunohistological and biochemical studies have shown that Polycomb proteins bind to multiple chromosomal locations and form large complexes containing different proteins of the Polycomb group. The current view is that the transient repression of genes set up by patterning proteins earlier in development is “locked in” by Polycomb proteins. This stable Polycomb-dependent repression may result from the ability of these proteins to assemble inactive chromatin structures (Chapter 11). Polycomb complexes contain many proteins, including histone



▲ EXPERIMENTAL FIGURE 15-25 Misexpression of the *Ultrabithorax* (*Ubx*) gene leads to development of a second pair of wings in *Drosophila*. Like other Hox genes, *Ubx* controls the organization of cells within the region in which it is expressed (see Figure 15-24b). Mutations in Hox genes often lead to the formation of a body part where it does not normally

exist. In this case the loss of *Ubx* function from the third thoracic segment allows wings to form where normally there are only balancer organs called halteres. [From E. B. Lewis, 1978, *Nature* **276**:565; photographs courtesy of E. B. Lewis. Reprinted by permission from *Nature*, copyright 1978, Macmillan Journals Limited.]

deacetylases, and appear to inactivate transcription by modifying histones to promote gene silencing.

Whereas Polycomb proteins repress the expression of certain Hox genes, proteins encoded by the Trithorax group of genes are necessary for maintaining the expression of Hox genes. Like Polycomb proteins, Trithorax proteins bind to multiple chromosomal sites and form large multiprotein complexes, some with a mass of $\sim 2 \times 10^6$ Da, about half the size of a ribosome. Some Trithorax-group proteins are homologous to the yeast Swi/Snf proteins, which are crucial for transcriptional activation of many yeast genes. Trithorax proteins stimulate gene expression by selectively remodeling the chromatin structure of certain loci to a transcriptionally active form (see Figure 11-37). The core of each complex is an ATPase, often of the Brm class of proteins. There is evidence that many or most genes require such complexes for transcription to take place.

Flower Development Also Requires Spatially Regulated Production of Transcription Factors



The basic mechanisms controlling development in plants are much like those in *Drosophila*: differential production of transcription factors, controlled in space and time, specifies cell identities. Our understanding of cell-identity control in plants benefited greatly from the choice of *Arabidopsis thaliana* as a model organism. This plant has many of the same advantages as flies and worms for use as a model system: it is easy to grow, mutants can be obtained, and transgenic plants can be made. We will focus on certain transcription-control mechanisms regulating the formation of cell identity in flowers. These mechanisms are strikingly similar to those controlling cell-type and antero-posterior regional specification in yeast and animals.

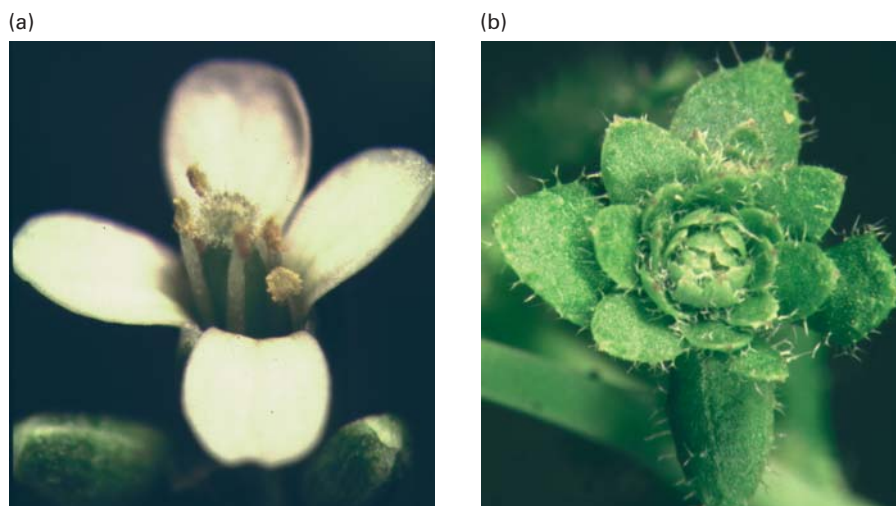
Floral Organs A flower comprises four different organs called sepals, petals, stamens, and carpels, which are arranged in con-

centric circles called whorls. Whorl 1 is the outermost; whorl 4, the innermost. *Arabidopsis* has a complete set of floral organs, including four sepals in whorl 1, four petals in whorl 2, six stamens in whorl 3, and two carpels containing ovaries in whorl 4 (Figure 15-26a). These organs grow from a collection of undifferentiated, morphologically indistinguishable cells called the floral **meristem**. As cells within the center of the floral meristem divide, four concentric rings of primordia form sequentially. The outer-ring primordium, which gives rise to the sepals, forms first, followed by the primordium giving rise to the petals, then the stamen and carpel primordia.

Floral Organ–Identity Genes Genetic studies have shown that normal flower development requires three classes of *floral organ–identity genes*, designated A, B, and C genes. Mutations in these genes produce phenotypes equivalent to those associated with homeotic mutations in flies and mammals; that is, one part of the body is replaced by another. In plants lacking all A, B, and C function, the floral organs develop as leaves (Figure 15-26b).

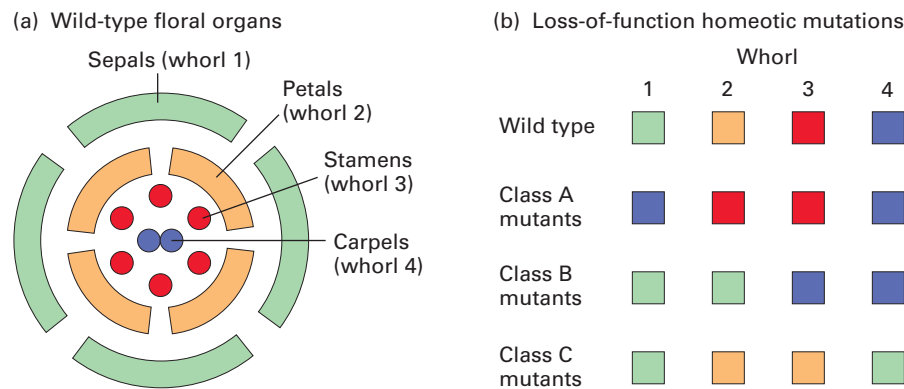
Figure 15-27 summarizes the loss-of-function mutations that led to the identification of the A, B, and C gene classes. On the basis of these homeotic phenotypes, scientists proposed a model to explain how three classes of genes control floral-organ identity. According to this ABC model for specifying floral organs, class A genes specify sepal identity in whorl 1 and do not require either class B or class C genes to do so. Similarly, class C genes specify carpel identity in whorl 4 and, again, do so independently of class A and B genes. In contrast with these structures, which are specified by only a single class of genes, the petals in whorl 2 are specified by class A and B genes, and the stamens in whorl 3 are specified by class B and C genes. To account for the observed effects of removing A genes or C genes, the model also postulates that A genes repress C genes in whorls 1 and 2 and, conversely, C genes repress A genes in whorls 3 and 4.

To determine if the actual expression patterns of class A, B, and C genes are consistent with this model, researchers



◀ EXPERIMENTAL FIGURE 15-26 Mutations in floral organ–identity genes produce homeotic phenotypes.

(a) Flowers of wild-type *Arabidopsis thaliana* have four sepals in whorl 1, four petals in whorl 2, six stamens in whorl 3, and two carpels in whorl 4. (b) In *Arabidopsis* with mutations in all three classes of floral organ–identity genes, the four floral organs are transformed into leaf-like structures. [From D. Weigel and E. M. Meyerowitz, 1994, *Cell* **78**:203; courtesy of E. M. Meyerowitz.]



▲ EXPERIMENTAL FIGURE 15-27 Phenotypic analysis identified three classes of genes that control specification of floral organs in *Arabidopsis*. (a) Diagram of the arrangement of wild-type floral organs, which are found in concentric whorls. (b) Effect of loss-of-function mutations leading to transformations of one organ into another. Class A mutations affect organ identity

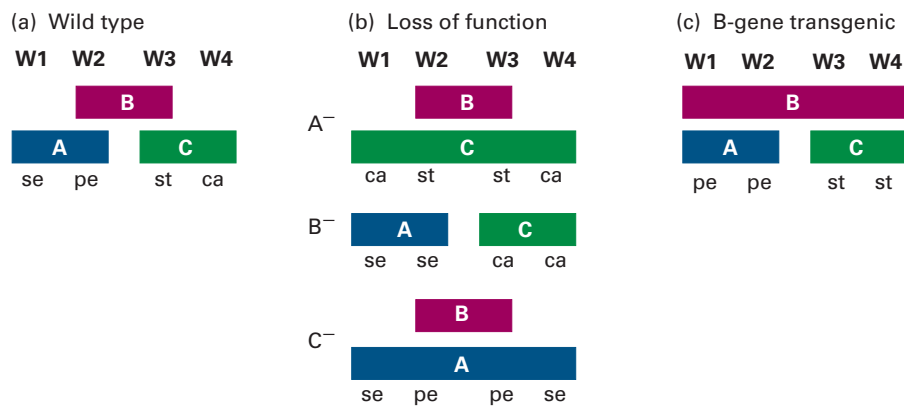
in whorls 1 and 2: sepals (green) become carpels (blue) and petals (orange) become stamens (red). Class B mutations cause transformation of whorls 2 and 3: petals become sepals and stamens become carpels. In class C mutations, whorls 3 and 4 are transformed: stamens become petals and carpels become sepals. [See D. Wiegand and E. M. Meyerowitz, 1994, *Cell* **78**:203.]

cloned these genes and assessed the expression patterns of their mRNAs in the four whorls in wild-type *Arabidopsis* plants and in loss-of-function mutants (Figure 15-28a, b). Consistent with the ABC model, A genes are expressed in whorls 1 and 2, B genes in whorls 2 and 3, and C genes in whorls 3 and 4. Furthermore, in class A mutants, class C genes are also expressed in organ primordia of whorls 1 and 2; similarly, in class C mutants, class A genes are also expressed in whorls 3 and 4. These findings are consistent with the homeotic transformations observed in these mutants.

To test whether these patterns of expression are functionally important, scientists produced transgenic *Arabidopsis* plants in which floral organ-identity genes were expressed in inappropriate whorls. For instance, the introduction of a transgene carrying class B genes linked to an A-

class promoter leads to the ubiquitous expression of class B genes in all whorls (Figure 15-28c). In such transgenics, whorl 1, now under the control of class A and B genes, develops into petals instead of sepals; likewise, whorl 4, under the control of both class B and class C genes, gives rise to stamens instead of carpels. These results support the functional importance of the ABC model for specifying floral identity.

Sequencing of floral organ-identity genes has revealed that many encode proteins belonging to the MADS family of transcription factors, which form homo- and heterodimers. Thus floral-organ identity may be specified by a combinatorial mechanism in which differences in the activities of different homo- and heterodimeric forms of various A, B, and C proteins regulate the expression of subordinate downstream genes necessary for the formation of the differ-



▲ EXPERIMENTAL FIGURE 15-28 Expression patterns of class A, B, and C genes support the ABC model of floral organ specification. Depicted here are the observed expression patterns of the floral organ-identity genes in wild-type, mutant, and transgenic *Arabidopsis*. Colored bars represent the A, B, and

C mRNAs in each whorl (W1, W2, W3, W4). The observed floral organ in each whorl is indicated as follows: sepal = se; petals = pe; stamens = st; and carpels = ca. See text for discussion. [See D. Wiegand and E. M. Meyerowitz, 1994, *Cell* **78**:203, and B. A. Krizek and E. M. Meyerowitz, 1996, *Development* **122**:11.]

ent cell types in each organ. Other MADS transcription factors function in cell-type specification in yeast and muscle (Chapter 22). ■

KEY CONCEPTS OF SECTION 15.4

Boundary Creation by Different Combinations of Transcription Factors

- Gradients of transcription factors, produced from maternal mRNAs in the early *Drosophila* embryo, control the patterned expression of embryonic genes, leading to segmentation of the embryo along the anteroposterior axis.
- Target genes whose regulatory regions contain multiple enhancers are expressed preferentially in specific regions of the embryo, depending on the amounts and combinations of the transcription factors that control them (see Figure 15-23).
- Early patterning events, utilizing maternal, gap, pair-rule, and segment-polarity genes, generate a unique pattern of transcription factors in different regions along the anteroposterior axis of *Drosophila* embryos (see Figure 15-24). These transcription factors are expressed transiently and play an essential role in establishing the domains in which different Hox genes are expressed.
- Hox genes, which encode transcription factors, control the unique morphologic characteristics of different regions along the anteroposterior axis in most or all animals.
- Misexpression of Hox genes causes homeotic transformations—the development of body parts in abnormal positions.
- Hox expression patterns are sometimes maintained through positive autoregulatory loops, and through modulation of chromatin by proteins encoded by Polycomb-group and Trithorax-group genes.
- Three classes of genes (A, B, and C) participate in specifying the identity of the four organs constituting a flower (see Figure 15-27). The patterns of expression of these genes, many of which encode transcription factors, are consistent with the ABC genetic model.

15.5 Boundary Creation by Extracellular Signals

As the syncytial fly embryo becomes cellular and undergoes gastrulation, the movement of proteins and mRNAs through the common cytoplasm of a syncytium is over. Further cell-fate specification is controlled primarily by cells communicating with one another through secreted extracellular signals. In this section, we examine how three signaling path-

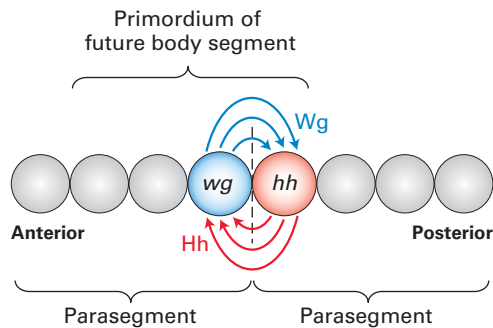
ways, activated by Hedgehog (Hh), Wingless (Wg, a member of the Wnt family), and TGF β , create boundaries between cell types during *Drosophila* development. The Wingless and Hedgehog proteins are encoded by segment-polarity genes, so named because they affect the orientation of surface features of the cuticle, such as bristles. The events discussed here are representative of what happens in virtually all tissues and all animals to specify cell types and create boundaries between different types.

Two Secreted Signals, Wingless and Hedgehog, Create Additional Boundaries Within Segments of Cellular Fly Embryos

As we saw in Section 15.4, the 14 segment primordia in the early *Drosophila* embryo are defined by various pair-rule proteins, with each protein located in seven stripes that alternate with stripes of cells that do not make the protein. The segment-polarity gene *engrailed*, which encodes a transcription factor, is expressed in the most anterior cell in each primordium, forming 14 Engrailed stripes. Transcription of *engrailed* is activated and repressed by various pair-rule proteins. In each eight-cell repeat unit established by the pair-rule proteins, *engrailed* is transcribed in cells 1 and 5. Recall that the pair-rule proteins produced in cells 1 through 4 differ from those produced in cells 5 through 8 (see Figure 15-24b). Although the transcriptional regulation of *engrailed* is the same in cell 1 in all the eight-cell repeats, it cannot be the same in cells 1 and 5 of a repeat. Thus two different combinations of pair-rule proteins must activate transcription of *engrailed*; so a seemingly simple repeating pattern masks a striking difference in regulation.

Another segment-polarity gene called *wingless* becomes active at about the same time as *engrailed*. It also is expressed in single-cell-wide stripes, adjacent to the Engrailed stripes and just one cell farther anterior (see Figure 15-24b). Wingless is a secreted signaling protein, a member of the Wnt protein family found in most or all animals. With the production of Wingless, the cells of the fly embryo stop ignoring one another and begin communicating through signals. In adjacent Engrailed-producing cells, the Wingless signal maintains the expression of another segment-polarity gene called *hedgehog* (*hh*), which also encodes an external signal. Expression of *hedgehog* is initially activated by Engrailed, a transcription factor that has both activating and repressing abilities. Engrailed activates *hedgehog* directly and represses a gene encoding a repressor of *hedgehog*, thereby indirectly promoting *hedgehog* expression. In the fly embryo, the Wingless and Hedgehog signals, produced in adjacent stripes of cells, form a positive feedback loop, with each maintaining expression of the other across the boundary (Figure 15-29).

The Wingless and Hedgehog signals control which cell types form in which positions, creating additional boundaries beyond those established by pair-rule proteins. Even



▲ **FIGURE 15-29 Role of Hedgehog (Hh) and Wingless (Wg) in boundary creation between parasegments in *Drosophila* embryo.** Hedgehog is necessary to maintain *wingless* transcription, and, conversely, Wingless is required to maintain *hedgehog*. These two secreted signals play a key role in patterning the epidermis. Both signaling proteins act on cells in addition to those indicated by the arrows. They act through the signal-transduction pathways shown in Figures 15-31 and 15-32. [See M. Hammerschmidt et al., 1997, *Trends Genet.* 13:14.]

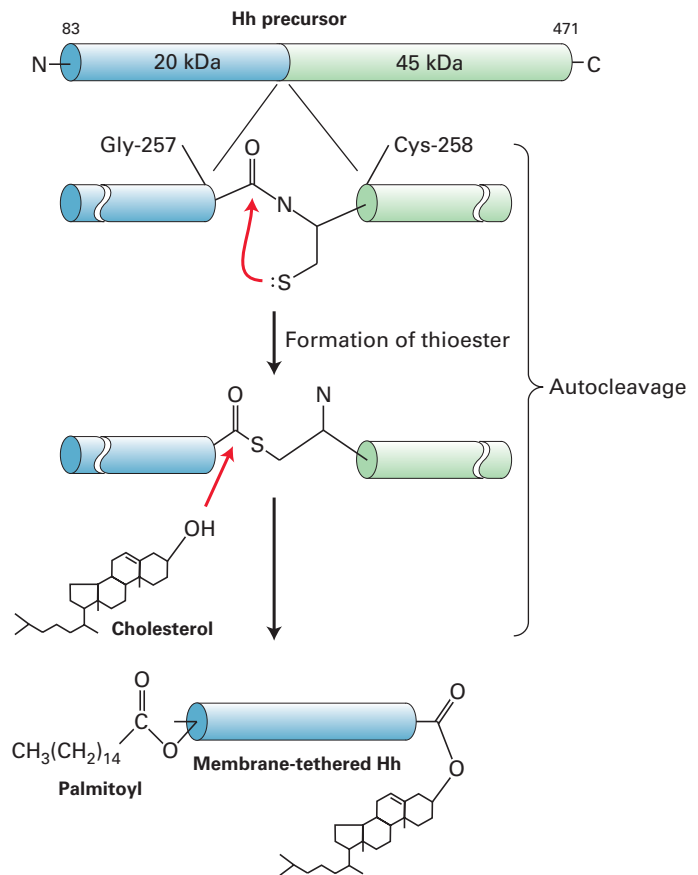
before Wingless- or Hedgehog-induced morphological features are evident, the prospective cell fates can be detected by the production of specific transcription factors. Both Hedgehog and Wingless can act as morphogens, with different concentrations inducing different fates in receiving cells (see Figure 15-11b). Cells that receive a large amount of Wingless turn on certain genes and form certain structures; cells that receive a smaller amount turn on different genes and thus form different structures. The same idea applies to the effects of different amounts of Hedgehog on receiving cells. As Wingless and Hedgehog are secreted from their source cells, they theoretically could move and signal in both directions. Recent work, however, shows that a signal can act mostly in one direction, anterior in the case of Wingless. This directional preference results from active destruction of much of the Wingless protein that moves posteriorly.

Having seen when the *Drosophila* Hedgehog and Wnt-type signals first begin to act in fly development, we take a closer look at the operation of these pathways. Both pathways participate in the development of many different tissues in *Drosophila* and most other animals.

Hedgehog Signaling, Which Requires Two Transmembrane Proteins, Relieves Repression of Target Genes

The Hedgehog signal is secreted from cells as a 45-kDa precursor protein. Cleavage of this secreted precursor produces a 20-kDa N-terminal fragment, which is associated with the plasma membrane and contains the inductive activity, and a 25-kDa C-terminal fragment. A series of elegant experiments demonstrated how the N-terminal Hedgehog fragment, which does not contain any hydrophobic sequences, acquires an affinity for the membrane. As depicted in Figure 15-30,

this process includes adding cholesterol to a glycine residue, splitting the molecule into two fragments, and leaving the N-terminal signaling fragment with an attached hydrophobic cholesterol moiety. The C-terminal domain of the precursor, which catalyzes this reaction, is found in other proteins and may promote the linkage of these proteins to membranes by the same autoproteolytic mechanism. A second modification to Hedgehog, the addition of a palmitoyl group to the N-terminus, makes the protein even more hydrophobic. Together, the two modifications may tether Hedgehog to cells, thereby affecting its range of action in tissue. Spatial restriction plays a crucial role in constraining the effects of powerful inductive signals.



▲ **FIGURE 15-30 Processing of Hedgehog (Hh) precursor protein.** Removal of the N-terminal signal peptide from the initial translation product yields the 45-kDa Hh precursor consisting of residues 83–471 in the original protein. Nucleophilic attack by the thiol side chain of cysteine 258 (Cys-258) on the carbonyl carbon of glycine 257 (Gly-257) forms a thioester intermediate. The C-terminal domain then catalyzes the formation of an ester bond between the β -3 hydroxyl group of cholesterol and glycine 257, cleaving the precursor into two fragments. The N-terminal signaling fragment (tan) retains the cholesterol moiety and is modified by the addition of a palmitoyl group to the N-terminus. These two hydrophobic anchors tether the signaling fragment to the membrane. [Adapted from J. A. Porter et al., 1996, *Science* 274:255.]

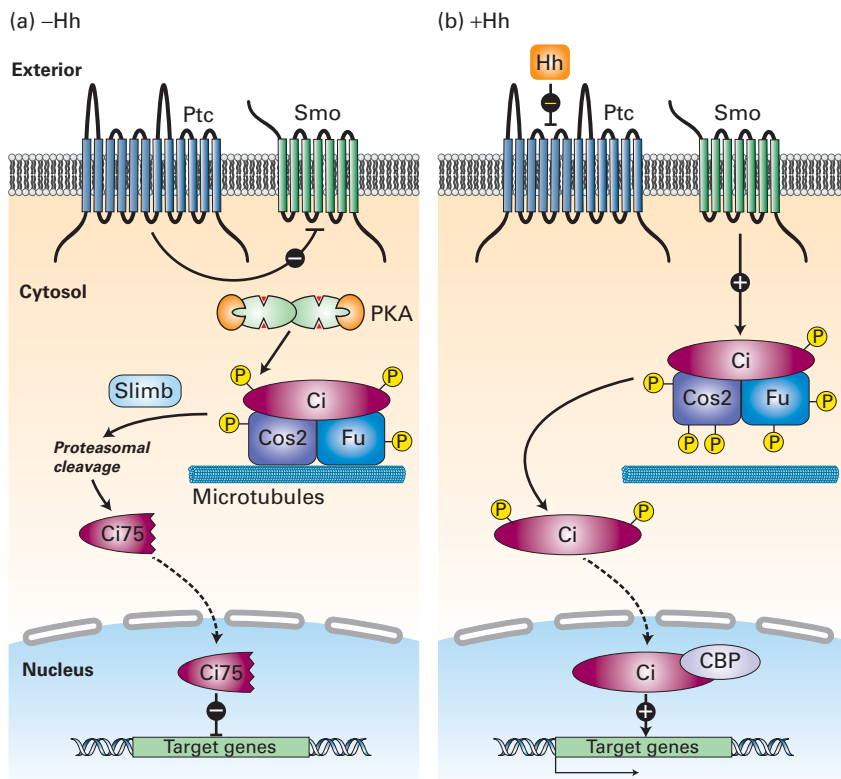
Findings from genetic studies in *Drosophila* indicate that two membrane proteins, Smoothed (Smo) and Patched (Ptc), are required to receive and transduce a Hedgehog signal to the cell interior. Smoothed has 7 membrane-spanning α helices, similarly to G protein-coupled receptors (Chapter 13). Patched is predicted to contain 12 transmembrane α helices and is most similar to Niemann-Pick C1 protein (NPC1). These proteins may act as pumps or transporters. As discussed in Chapter 18, NPC1 protein is necessary for normal intracellular movement of sterols through vesicle-trafficking pathways. In humans, mutations in the NPC1 gene cause a rare, autosomal recessive disorder marked by defects in the lysosomal handling of cholesterol.

Drosophila embryos with loss-of-function mutations in the *smoothed* or *hedgehog* genes have very similar phenotypes. Moreover, both genes are required to activate transcription of the same target genes (e.g., *wingless*) during embryonic development. Loss-of-function mutations in *patched* produce a quite different phenotype, one similar to the effect of flooding the embryo with Hedgehog. Thus Patched appears to antagonize the actions of Hedgehog and vice versa. These findings and analyses of double mutants suggest that, in the absence of Hedgehog, Patched represses target genes by inhibiting a signaling pathway needed for gene activation. The additional observation that Smoothed is required for the transcription of target genes in mutants lacking *patched* function places Smoothed downstream in the pathway. The binding of Hedgehog evidently prevents

Patched from blocking Smoothed action, thus activating the transcription of target genes.

The results of recent studies have shown that, in the absence of Hedgehog, Patched is enriched in the plasma membrane, but Smoothed is in internal vesicle membranes. When cells receive a Hedgehog signal, both Patched and Hedgehog move from the cell surface into internal vesicles, whereas Smoothed moves from internal vesicles to the surface. The similarity of Patched to Niemann-Pick C1 protein, the covalent joining of cholesterol to Hedgehog, and the ability of cholesterol analogs such as cyclopamine to block reception of a Hedgehog signal all suggest a possible link between sterol metabolism and Hedgehog signaling. Indeed, one interesting idea is that developmental regulation by the Hedgehog system evolved from earlier cell components needed to control vesicle composition and movement.

Figure 15-31 depicts a current model of the *Hedgehog* pathway. Although the signal-transduction mechanisms are only partly understood, the pathway includes a cytoplasmic complex of proteins consisting of Fused (Fu), a serine-threonine kinase; Costal-2 (Cos-2), a microtubule-associated kinesin-like protein; and Cubitus interruptus (Ci), a transcription factor. In the absence of Hedgehog, when Patched inhibits Smoothed, these three proteins form a complex that binds to microtubules in the cytoplasm. Proteolytic cleavage of Ci in this complex generates a Ci fragment that translocates to the nucleus and represses target-gene expression. In the presence of Hedgehog, which relieves the



◀ **FIGURE 15-31 Operational model of the Hedgehog (Hh) signaling pathway.**

(a) In the absence of Hh, Patched (Ptc) protein inhibits Smoothed (Smo) protein by an unknown mechanism. In the absence of Smo signaling, a complex containing the Fused (Fu), Costal-2 (Cos2), and Cubitus interruptus (Ci) proteins binds to microtubules. Ci is cleaved in a process requiring the ubiquitin/proteasome-related F-box protein Slimb, generating the fragment Ci75, which functions as a transcriptional repressor. (b) In the presence of Hh, inhibition of Smo by Ptc is relieved. Signaling from Smo causes hyperphosphorylation of Fu and Cos2, and disassembly of the Fu/Cos2/Ci complex from microtubules. This leads to the stabilization of a full-length, alternately modified Ci, which functions as a transcriptional activator in conjunction with CREB binding protein (CBP). The exact membrane compartments in which Ptc and Smo respond to Hh and function are unknown; Hh signal causes Ptc to move from the surface to internal compartments while Smo does the opposite. [After K. Nybakken and N. Perrimon, 2002, *Curr. Opin. Genet. Devel.* **12**:503.]

inhibition of Smoothed, the complex of Fu, Cos-2, and Ci is not associated with microtubules, cleavage of Ci is blocked, and an alternatively modified form of Ci is generated. After translocating to the nucleus, this Ci form binds to the transcriptional coactivator CREB-binding protein (CBP), promoting the expression of target genes. In addition to these components, protein kinase A participates in controlling Hedgehog-responsive target genes, which become inappropriately active when protein kinase A is inactivated. Phosphorylation of Ci by protein kinase A appears to stimulate the proteolytic cleavage of Ci.



Hedgehog signaling, which is conserved throughout the animal kingdom, functions in the formation of many tissues and organs. Mutations in components of the Hedgehog signaling pathway have been implicated in birth defects such as cyclopia, a single eye resulting from union of the right and left brain primordia, and in multiple forms of human cancer. ■

Wnt Signals Trigger Disassembly of an Intracellular Complex, Releasing a Transcription Factor

As noted previously, the *Drosophila* segment-polarity gene *wingless* encodes a protein that belongs to the Wnt family of secreted signals. Inactivation of *wingless* causes segment-polarity defects very similar to those caused by the loss of *hedgehog* function. This observation is logical because Hedgehog and Wingless form a positive feedback loop, with each protein maintaining production of the other (see Fig-

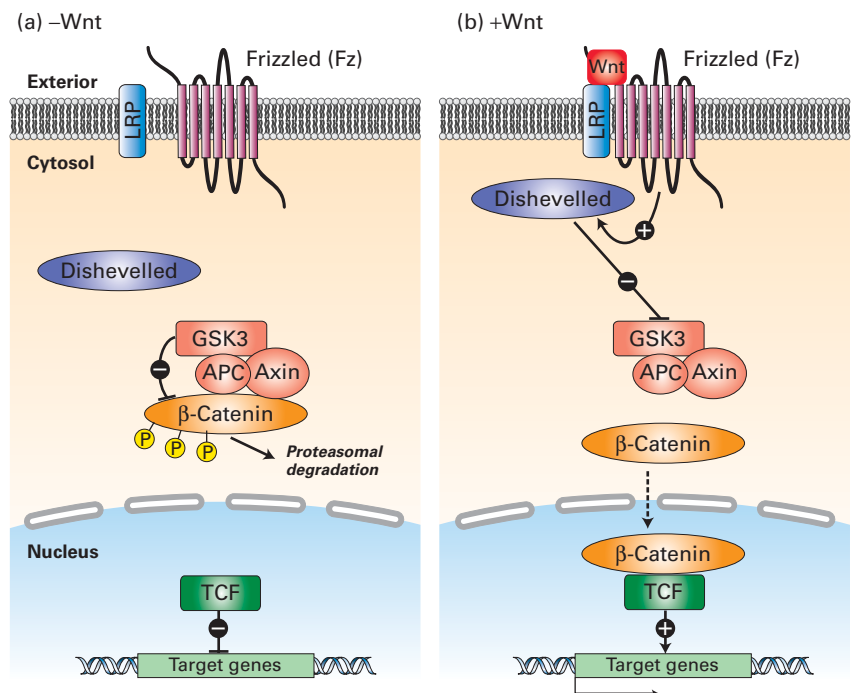
ure 15-29). The first vertebrate Wnt gene to be discovered was a mouse gene called *Wnt-1* (formerly *int-1*). Activation of *int-1* by insertion of a mouse mammary tumor virus (MMTV) provirus leads to mammary cancer. Hence *Wnt-1* is a **proto-oncogene**, a normal cellular gene whose inappropriate expression promotes the onset of cancer (Chapter 23). The word Wnt is an amalgamation of *wingless*, the corresponding fly gene, with *int* for MMTV integration.

Genetic studies in *Drosophila* and *C. elegans*, studies of mouse proto-oncogenes and tumor-suppressor genes, and studies of cell junction components have all contributed to identifying many components of the Wnt signal-transduction pathway. Like Hedgehog proteins, Wnt proteins are modified by the addition of a hydrophobic palmitate group near their N termini, which may tether them to the plasma membrane of secreting cells and limit their range of action. Wnt proteins act through two cell-surface receptor proteins: Frizzled (Fz), which contains seven transmembrane α helices and directly binds Wnt; and Lrp, which appears to associate with Frizzled in a Wnt signal-dependent manner, at least in frog embryos. Mutations in the genes encoding Wingless, Frizzled, or Lrp (called Arrow in *Drosophila*) all have similar effects on the development of embryos. Frizzled protein and the Smoothed protein in Hedgehog signaling have sequence similarities, and both bear some resemblance to the G protein-coupled receptors discussed in Chapter 13. To date, however, evidence for G protein involvement downstream of Smoothed or Frizzled remains indirect and not compelling.

A current model of the *Wnt pathway* is shown in Figure 15-32. The central player in intracellular Wnt signal transduction is called β -catenin in vertebrates and Armadillo in

► FIGURE 15-32 Operational model of the Wnt signaling pathway.

(a) In the absence of Wnt, the kinase GSK3 constitutively phosphorylates β -catenin. Phosphorylated β -catenin is degraded and hence does not accumulate in cells. Axin is a scaffolding protein that forms a complex with GSK3, β -catenin, and APC, which facilitates phosphorylation of β -catenin by GSK3 by an estimated factor of >20,000. The TCF transcription factor in the nucleus acts as a repressor of target genes unless altered by Wnt signal transduction. (b) Binding of Wnt to its receptor Frizzled (Fz) recruits Dishevelled (Dsh) to the membrane. Activation of Dsh by Fz inhibits GSK3, permitting unphosphorylated β -catenin to accumulate in the cytosol. After translocation to the nucleus, β -catenin may act with TCF to activate target genes or, alternatively cause the export of TCF from the nucleus and perhaps its activation in cytosol. [After R. T. Moon et al., 2002, *Science* 296:644; see also The Wnt Gene Homepage, www.stanford.edu/~rnusse/wntwindow.html.]



Drosophila. This remarkable protein functions both as a transcriptional activator and as a membrane–cytoskeleton linker protein (see Figure 6-7). In the absence of a Wnt signal, β -catenin is phosphorylated by a complex containing GSK3, a protein kinase; the adenomatosis polyposis coli (APC) protein, an important human tumor suppressor; and Axin, a scaffolding protein. Phosphorylated β -catenin is ubiquitinated and then degraded in proteasomes. In the presence of Wnt, β -catenin is stabilized and translocates to the nucleus. There, it is believed to associate with the TCF transcription factor to activate expression of particular target genes (e.g., *wg*, *cyclin D1*, *myc*, and metalloprotease genes), depending on cell type. Recent evidence suggests that β -catenin acts by a different mechanism in which it controls the export of TCF from the nucleus and perhaps its activation in the cytosol.

Findings from genetic studies have shown that Wnt-induced stabilization of β -catenin depends on Dishevelled (Dsh) protein. In the presence of Wnt, Dsh and the Lrp membrane protein appear to interact with components of the phosphorylation complex, thereby inhibiting the phosphorylation and subsequent degradation of β -catenin (see Figure 15-32b). The importance of β -catenin stability and location means that Wnt signals affect a critical balance between the three pools of β -catenin in the cytoskeleton, cytosol, and nucleus.

Wnt signals help control numerous critical developmental events, such as gastrulation, brain development, limb patterning, and organogenesis. The regulated movement of Wnts through tissue is critical to establishing properly placed boundaries between different cell types. As is discussed in Chapter 23, disturbances in signal transduction through the Wnt pathway and many other developmentally important signaling pathways are associated with various human cancers.

Gradients of Hedgehog and Transforming Growth Factor β Specify Cell Types in the Neural Tube

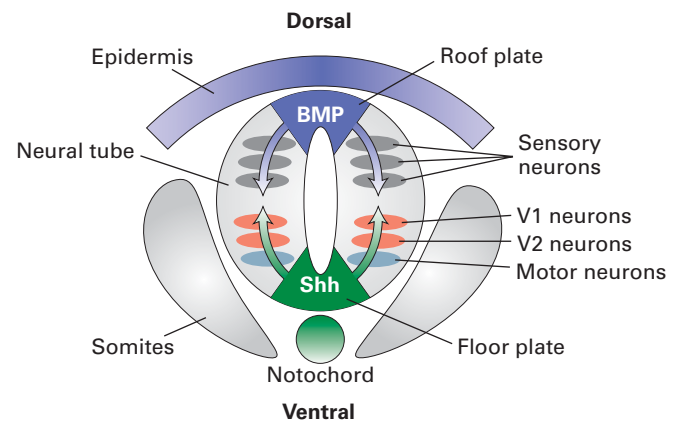
As we have seen in *Drosophila*, many developmental signals act in a graded fashion, inducing different cell fates depending on their concentration. The same phenomenon exists in vertebrates, for example, in the development of the mammalian central nervous system from the neural tube, which forms early in embryogenesis. The neural tube is a simple rolled-up sheet of cells, initially one cell thick. Cells in the ventral part will form motor neurons; lateral cells will form a variety of interneurons. The different cell types can be distinguished prior to morphological differentiation by the proteins that they produce.

Graded concentrations of Sonic hedgehog (Shh), a vertebrate equivalent of *Drosophila* Hedgehog, determine the fates of at least four cell types in the chick ventral neural tube. These cells are found at different positions along the dorsoventral axis in the following order from ventral to dorsal: floor-plate cells, motor neurons, V2 interneurons, and V1 interneurons. During development, Shh is initially expressed at high levels in the notochord, a mesoderm structure in direct contact with the ventralmost region of the neural

tube (Figure 15-33). On induction, floor-plate cells also produce Shh, forming a Shh-signaling center in the ventral-most region of the neural tube. Antibodies to Shh protein block the formation of the different ventral neural-tube cells in the chick, and these cell types fail to form in mice homozygous for mutations in the *Sonic hedgehog* (*Shh*) gene.

To determine whether Shh-triggered induction of ventral neural-tube cells is through a graded or a relay mechanism, scientists added different concentrations of Shh to chick neural-tube explants. In the absence of Shh, no ventral cells formed. In the presence of very high concentrations of Shh, floor-plate cells formed; whereas, at a slightly lower concentration, motor neurons formed. When the level of Shh was decreased another twofold, only V2 neurons formed. And, finally, only V1 neurons developed when the Shh concentration was decreased another twofold. These data strongly suggest that in the developing neural tube different cell types are formed in response to a ventral $\rightarrow$ dorsal gradient of Shh. The accumulating evidence for gradients does not rule out additional relay signals that may yet be discovered.

Cell fates in the dorsal region of the neural tube are determined by BMP proteins (e.g., BMP4 and BMP7), which belong to the TGF β family. Recall that Dpp protein, a *Drosophila* TGF β signal, is critical in determining dorsal cell fates in early fly embryos. Indeed, TGF β signaling appears to be an evolutionarily ancient regulator of dorsoventral patterning. In vertebrate embryos, BMP proteins secreted from ectoderm cells overlying the dorsal side of the neural tube promote the formation of dorsal cells such as sensory neurons (see Figure 15-33). Thus cells in the neural tube sense multiple signals that originate at opposite positions on the dorsoventral axis, and measure the signals from both origins to decide on a course of differentiation.



▲ FIGURE 15-33 Graded induction of different cell types in the neural tube by Sonic hedgehog (Shh) and BMP signaling.

Shh produced in the notochord induces floor-plate development. The floor plate, in turn, produces Shh, which forms a ventral $\rightarrow$ dorsal gradient that induces additional cell fates. In the dorsal region, BMP proteins secreted from the overlying ectoderm cells act in a similar fashion to create dorsal cell fates. [See T. M. Jessell, 2000, *Nature Rev. Genet.* 1:20.]

Cell-Surface Proteoglycans Influence Signaling by Some Pathways

How do signals move through or around cells embedded in tissues? The full answer is not known, but the distance that a signal can move has important implications for the size and shape of organs. A signal that causes neurons to form, for example, will create more neurons if its range of movement increases. The binding of signaling proteins to cell-surface **proteoglycans** not only affects the range of signal action but also facilitates signaling in some cases. A proteoglycan consists of a core protein to which is bound **glycosaminoglycan** chains such as heparin sulfate and chondroitin sulfate (see Figure 6-22). Proteoglycans are important components of the extracellular matrix. Some are embedded in the plasma membrane by a hydrophobic transmembrane domain or tethered to the membrane by a lipid anchor.

Evidence for the participation of proteoglycans in signaling comes from *Drosophila sugarless (sgl)* mutants, which lack a key enzyme needed to synthesize heparin (and chondroitin) sulfate. These mutants exhibit the phenotypes associated with defects in Wingless signaling and have greatly depressed levels of extracellular Wingless protein, a Wnt signal. Mutations in *dally* and *dally-like*, both of which encode core proteins of cell-surface proteoglycans, also are associated with defective Wingless signaling.

The Wnt pathway is not the only signaling pathway affected in *sugarless* and other *Drosophila* mutants with defective proteoglycan synthesis. For instance, such mutants have phenotypes (e.g., absence of a heart or trachea) that are associated with loss-of-function of Heartless and Breathless, which are receptor tyrosine kinases that bind FGF-like signaling proteins. These mutants also appear to have defective TGF β signaling in metamorphosis, though not in embryos, suggesting specific actions of the proteoglycans. In Chapter 14, we saw that the type III TGF β receptor is a cell-surface proteoglycan. Although not absolutely required for TGF β signaling, the type III receptor binds and concentrates TGF β near the surface of a cell in which it is produced, thereby facilitating signaling from the type I and type II receptors (see Figure 14-2).

KEY CONCEPTS OF SECTION 15.5

Boundary Creation by Extracellular Signals

- Three *Drosophila* segment-polarity genes, *engrailed*, *wingless*, and *hedgehog*, are expressed in one-cell-wide stripes in each of the 14 body-segment primordia.
- Engrailed, a transcription factor, activates transcription of *Hedgehog*, which encodes a secreted signaling protein.
- Wingless, a secreted signaling protein in the Wnt family, is produced in stripes adjacent to the Engrailed/Hedgehog stripes. Wingless and Hedgehog maintain expression of each other's genes in a positive feedback loop (see Figure 15-29).

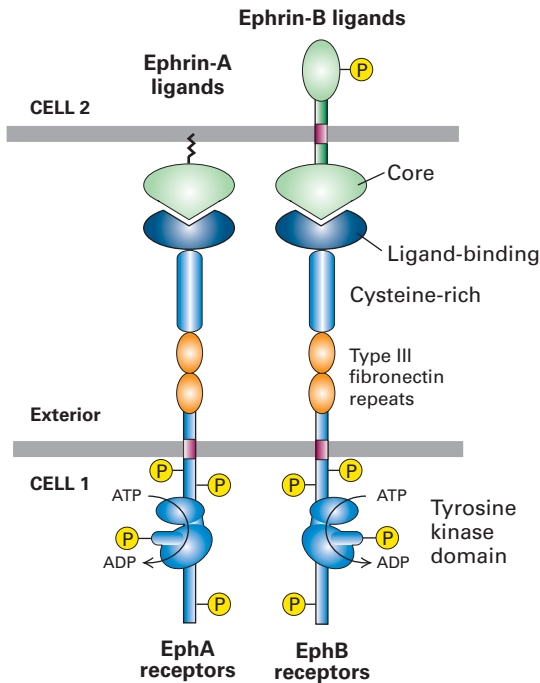
- Both Hedgehog and Wingless/Wnt contain lipid anchors that can tether them to cell membranes, thereby reducing their signaling range.
- The Hedgehog signal acts through two cell-surface proteins, Smoothened and Patched, and an intracellular complex containing the Cubitus interruptus (Ci) transcription factor (see Figure 15-31). An activating form of Ci is generated in the presence of Hedgehog; a repressing form is generated in the absence of Hedgehog. Both Patched and Smoothened change their subcellular location in response to Hedgehog binding to Patched.
- Wingless and other Wnt signals act through two cell-surface proteins, the receptor Frizzled and coreceptor Lrp, and an intracellular complex containing β -catenin (see Figure 15-32). Wnt signaling promotes the stability and nuclear localization of β -catenin, which either directly or indirectly promotes activation of the TCF transcription factor.
- Gradients of two external signals, a ventralizing Sonic hedgehog signal and a dorsalizing TGF β signal, induce different cell types in the vertebrate neural tube (see Figure 15-33).
- Cell-surface proteoglycans bind some extracellular signaling proteins, restricting their range of action and presenting them to nearby receptor proteins.
- Cancer and birth defects occur when Hh, TGF β , or Wnt signaling systems do not work properly.

15.6 Reciprocal Induction and Lateral Inhibition

In the development of an organism, cells must “talk” with one another to ensure a proper division of labor. The outcome of these cell–cell conversations can be an agreement about which cell should follow what differentiation pathway. For instance, negotiation between two initially equivalent cells can send each down distinct developmental paths. One cell preventing the other from following a particular path is called *lateral inhibition*, a process that prevents the duplication of structures at the expense of something not forming. Alternatively, two cells with distinct fates can send and receive signals between themselves, inducing further differentiation. Such *reciprocal induction* is common in the formation of internal organs. In this section, we consider two signaling systems that mediate such dialogues between cells.

Cell-Surface Ephrin Ligands and Receptors Mediate Reciprocal Induction During Angiogenesis

Perhaps the simplest type of reciprocal induction is between cells that interact through two cell-surface proteins, each of



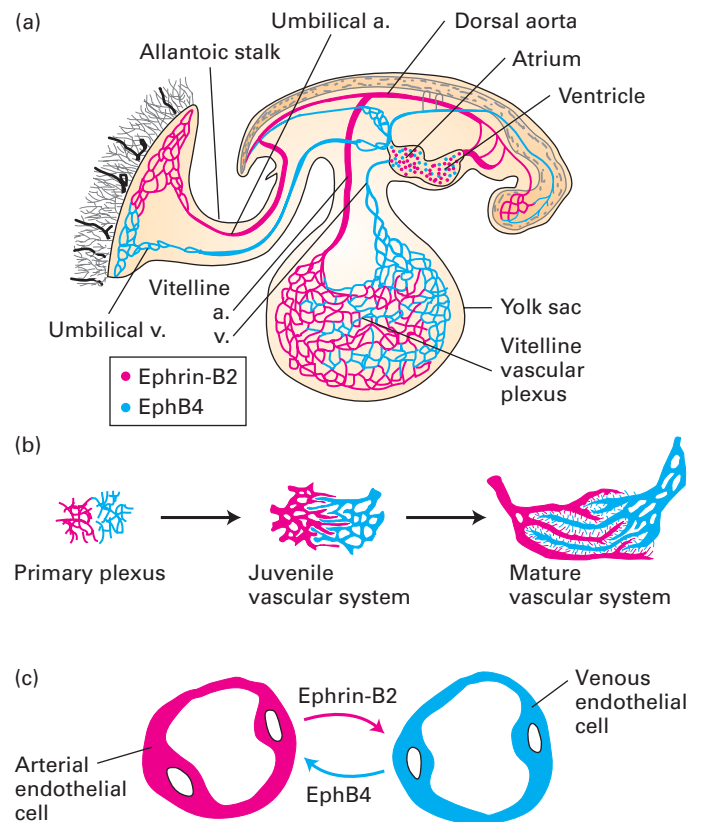
▲ **FIGURE 15-34 General structure of Eph receptors and their ligands.** The cytosolic domain of Eph receptors has tyrosine kinase activity. Within the Eph receptor family, the receptors exhibit some 30–70 percent homology in their extracellular domains and 65–90 percent homology in their kinase domains. Their ligands, the ephrins, either are linked to the membrane through a hydrophobic GPI anchor (class A) or are single-pass transmembrane proteins (class B). The core domains of various ephrin ligands show 30–70 percent homology. Ephrin-B ligands and their receptors can mediate reciprocal signaling. [Adapted from V. Dodelet and E. Pasquale, 2000, *Oncogene* **19**:5614; see J. G. Flanagan and P. Vanderhaegen, 1998, *Ann. Rev. Neurosci.* **21**:309.]

which can act as a receptor and ligand. To illustrate this phenomenon, we consider the role of the ephrins, a family of cell-surface ligands, and the Eph receptors in the development of mammalian blood vessels.

The Eph receptors, a novel type of receptor tyrosine kinase, have two classes of ligands (Figure 15-34). Ephrin-A ligands are tethered to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. These ephrin ligands play a crucial role in forming connections between neurons in the developing nervous system. Ephrin-B ligands are single-pass transmembrane proteins. The results of biochemical experiments showed that ephrin-B ligands stimulate tyrosine phosphorylation of EphB receptors and of their own cytosolic domain. These observations led to the intriguing notion that ephrin-B ligand/EphB receptor complexes promote bidirectional reciprocal interactions. Strong support for this hypothesis has come from the study of blood vessel formation.

Blood vessels, arteries and veins, form a complex network of branched structures in the adult. An early network

of vessels is remodeled during angiogenesis as larger branches assemble from smaller ones and vessels become surrounded by support cells. Knockout mice lacking ephrin-B2 exhibit striking defects in angiogenesis. This finding led scientists to explore the pattern of expression of ephrin-B2 and its receptor, EphB4, in the developing embryo (Figure 15-35). In normal embryos, ephrin-B2 is expressed only in arteries; EphB4, only on veins. Although ephrin-B2 is expressed only on arterial capillaries, venous capillaries also fail to undergo angiogenesis in *ephrin-b2* knockouts. These data suggest that interaction between an arterial cell producing ephrin-B2 and a venous cell producing EphB4 causes the induction of *both* cells (see Figure 15-35c). In other words, ephrin-B2 and EphB4 each functions as both a ligand and a receptor to control the development of both veins and arteries.



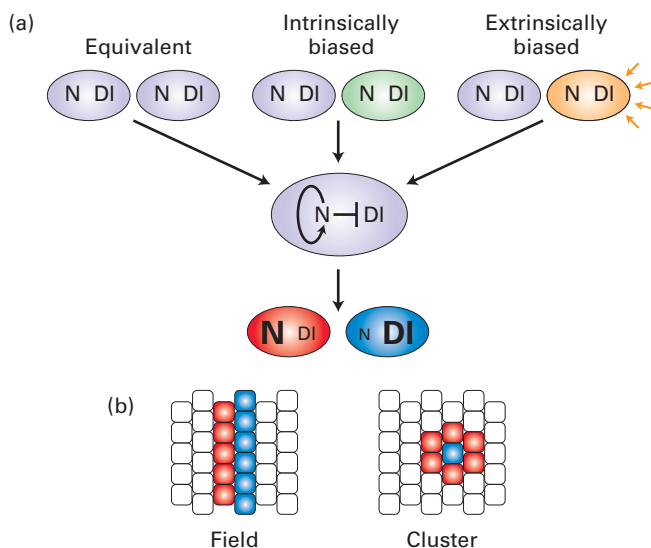
▲ **FIGURE 15-35 Reciprocal induction mediated by ephrin-B2 and its receptor, EphB4, in angiogenesis in the yolk sac.**

(a) Ephrin-B2 (red) is expressed on arteries and EphB4 (blue) on veins in the early mouse embryo. (b) The early vascular network is remodeled during angiogenesis. In *ephrin-B2* knockout mice, angiogenesis is blocked at the primary plexus stage. The absence of ephrin-B2 thus interrupts the development of both arteries and veins. (c) Formation of intercalating arteries and veins results from interactions between developing arterial and venous endothelial cells mediated by ephrin-B2 (arterial) and EphB4 (venous). These reciprocal interactions induce the development of both cell types. [Adapted from H. U. Wang et al., 1998, *Cell* **93**:741.]

The Conserved Notch Signaling Pathway Mediates Lateral Inhibition

Now we shift our attention to lateral inhibition, which causes adjacent developmentally equivalent or near-equivalent cells to assume different fates. Genetic analyses in *Drosophila* and *C. elegans* revealed the role of the highly conserved *Notch/Delta pathway* in lateral inhibition. The *Drosophila* proteins Notch and Delta are the prototype receptor and ligand, respectively, in this signaling pathway. Both proteins are large transmembrane proteins whose extracellular domains contain multiple EGF-like repeats and binding sites for the other protein. Although Delta is cleaved to make an apparently soluble version of its extracellular domain, findings from studies with genetically mosaic *Drosophila* have shown that the Delta signal reaches only adjacent cells.

Interaction between Delta and Notch triggers the proteolytic cleavage of Notch, releasing its cytosolic segment,



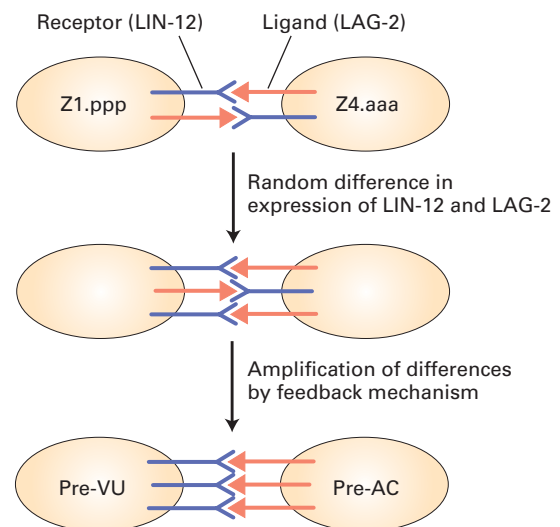
▲ FIGURE 15-36 Amplification of an initial bias to create different cell types by Notch-mediated lateral inhibition.

(a) A difference between two initially equivalent cells may arise randomly (*left*). Alternatively, interacting cells may have an intrinsic bias (*center*) or an extrinsic bias (*right*). For instance, cells that have received different proteins in an asymmetric cell division will be intrinsically biased; those that have received different signals (orange) will be extrinsically biased. Regardless of how the small initial bias arises, Notch becomes predominant in one of the two cells, promoting its own expression and repressing production of its ligand Delta in that cell. In the other cell, Delta predominates. The outcome is reinforcement of the small initial difference. (b) Notch-mediated lateral inhibition may create a sharp boundary in an initial field of cells, such as along the edge of the developing *Drosophila* wing, or distinguish a central cell from a surrounding cluster of cells, as in neural precursor establishment. [Adapted from S. Artavanis-Tsakonas et al., 1999, *Science* **284**:770.]

which translocates to the nucleus and regulates the transcription of specific target genes (see Figure 14-29). In particular, Notch signaling activates the transcription of *Notch* itself and represses the transcription of *Delta*, thereby intensifying the difference between the interacting cells (Figure 15-36a). Notch-mediated signaling can give rise to a sharp boundary between two cell populations or can single out one cell from a cluster of cells (Figure 15-36b). Notch signaling controls cell fates in most tissues and has consequences for differentiation, proliferation, the creation of cell asymmetry, and apoptosis. In the immune system, for instance, Notch signaling helps prevent the formation of T cells that attack an individual's own proteins. Here, we describe two examples of Notch signaling in cell-fate determination.

Determination of AC and VU Cell Fates in *C. elegans* Two equivalent cells, designated Z1.ppp and Z4.aaa, in roundworms can give rise to an anchor (AC) cell or a ventral uterine (VU) precursor cell. The results of laser ablation studies showed that, if either the Z1.ppp or Z4.aaa cell is removed, the remaining cell always becomes AC. In worms lacking functional LIN-12, the *C. elegans* homolog of Notch, both cells become AC. Conversely, constitutive activation of LIN-12 in both Z1.ppp and Z4.aaa results in both cells becoming VU. Thus LIN-12 activity levels specify AC and VU cell fates.

Both Z1.ppp and Z4.aaa produce the receptor, LIN-12, and its ligand, the Delta homolog LAG-2, at similar levels (Figure 15-37). As development proceeds, one cell begins to express more receptor through random fluctuations in pro-



▲ FIGURE 15-37 Determination of different cell fates by lateral inhibition in *C. elegans* development. LIN-12, a Notch homolog, and LAG-2, a Delta homolog, regulate interactions between two equivalent cells, designated Z1.ppp and Z4.aaa. Either cell can assume a ventral uterine (VU) or anchor (AC) fate. See text for discussion. [Adapted from I. Greenwald, 1998, *Genes & Dev.* **12**:1751.]

tein levels or differences in the ambient level of signaling through the pathway. The cell receiving a slightly higher signal begins to increase its expression of the receptor and decrease its expression of the ligand. In the neighboring cell, now exposed to a reduced level of ligand, expression of the receptor falls and that of the ligand increases. In this way, the initial asymmetry resulting from a random event is amplified, finally leading to the commitment of one cell as a pre-VU cell and its partner as a pre-AC cell. When formed, the AC cell begins sending out a LIN-3 signal that functions in vulva development. Notch-mediated lateral inhibition also operates in that process when the P6.p cell inhibits the neighboring P5.p and P7.p cells (see Figure 15-12b).

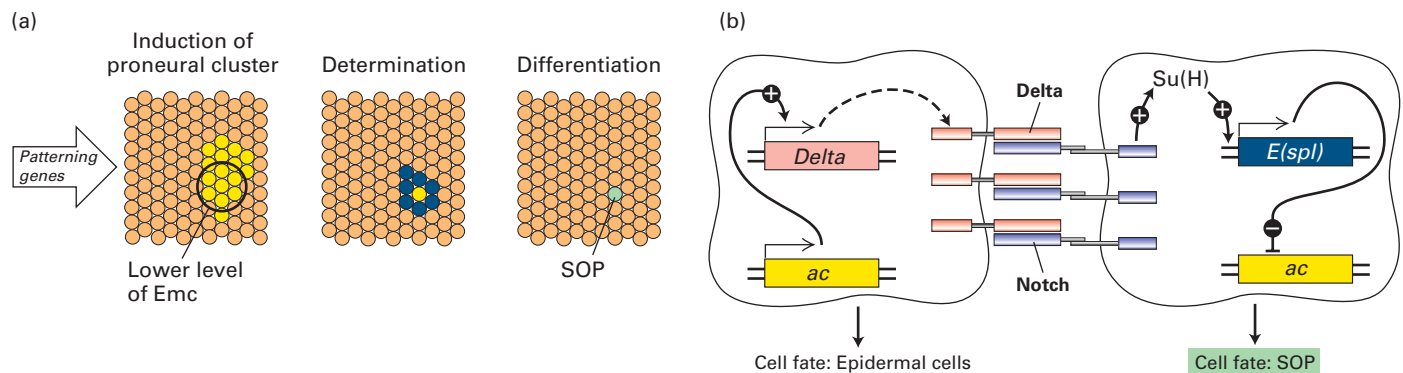
Neuronal Development in *Drosophila* and Vertebrates

Loss-of-function mutations in the *Notch* or *Delta* genes produce a wide spectrum of phenotypes in *Drosophila*. One consequence of such mutations in either gene is an increase in the number of neuroblasts in the central nervous system. In *Drosophila* embryogenesis, a sheet of ectoderm cells becomes divided into two populations of cells: those that move inside the embryo eventually develop into neuroblasts; those that remain external form the epidermis and cuticle. As some of the cells enlarge and then loosen from the ectodermal sheet to become neuroblasts, they signal to surrounding cells to prevent their neighbors from becoming neuroblasts—a case of lateral inhibition. Notch signaling is used for this in-

hibition; in embryos lacking the Notch receptor or its ligand, all the ectoderm precursor cells become neural.

The role of Notch signaling in specifying neural cell fates has been studied extensively in the developing *Drosophila* peripheral nervous system. In flies, various sensory organs arise from proneural cell clusters, which produce bHLH transcription factors, such as Achaete and Scute, that promote neural cell fates. In normal development, one cell within a proneural cluster is somehow anointed to become a *sensory organ precursor* (SOP). In the other cells of a cluster, Notch signaling leads to the repression of proneural genes, and so the neural fate is inhibited; these nonselected cells give rise to epidermis (Figure 15-38). Temperature-sensitive mutations that cause functional loss of either Notch or Delta lead to the development of additional SOPs from a proneural cluster. In contrast, in developing flies that produce a constitutively active form of Notch (i.e., active in the absence of a ligand), all the cells in a proneural cluster develop into epidermal cells.

To assess the role of the Notch pathway during primary neurogenesis in *Xenopus*, scientists injected mRNA encoding different forms of Notch and Delta into embryos. Injection of mRNA encoding the constitutively active cytosolic segment of Notch inhibited the formation of neurons. In contrast, injection of mRNA encoding an altered form of Delta that prevents Notch activation led to the formation of too many neurons. These findings indicate that in vertebrates, as in *Drosophila*, Notch signaling controls neural precursor cell fates.



▲ FIGURE 15-38 Role of Notch-mediated lateral inhibition in formation of sensory organ precursors (SOPs) in *Drosophila*.

(a) Extracellular signaling molecules and transcription factors, encoded by early-patterning genes, control the precise spatiotemporal pattern of proneural bHLH proteins such as Achaete and Scute (yellow). Most cells within the field express Emc (orange), a related protein that antagonizes Achaete and Scute. A small group of cells, a proneural cluster, produce proneural bHLH proteins. The region of a proneural cluster from which an SOP will form expresses lower levels of Emc, giving these cells a bias toward SOP formation. Interactions between these cells, leading to accumulation of E(spl) repressor proteins in neighboring cells (blue), then restrict SOP formation to a single cell (green). (b) Initially, *achaete* (*ac*) and other proneural genes are transcribed in all the cells within a proneural cluster, as are

Notch and *Delta*. Achaete and other proneural bHLH proteins promote expression of *Delta*. When one cell at random begins to produce slightly more Achaete (left), its production of *Delta* increases, leading to stronger Notch signaling in all its neighboring cells (right). In the receiving cells, the Notch signaling pathway activates a transcription factor designated Su(H), which in turn stimulates expression of *E(spl)* genes. The E(spl) proteins specifically repress transcription of *ac* and other proneural genes. The resulting decrease in Achaete leads to a decrease in *Delta*, thus amplifying the initial random difference among the cells. As a consequence of these interactions and others, one cell of a proneural cluster is selected as a SOP; all the others lose their neural potential and develop into epidermal cells.

KEY CONCEPTS OF SECTION 15.6

Reciprocal Induction and Lateral Inhibition

- Ephrins are cell-surface signaling ligands whose receptors (Ephs) are receptor tyrosine kinases.
- B-class ephrins and their receptors (EphB), each acting as both a ligand and a receptor, can mediate reciprocal induction in adjacent cells (see Figure 15-35).
- Interactions between the Notch receptor and its transmembrane ligand Delta are used in lateral inhibition, which can control the choice between two alternative cell fates.
- An initial random or biased fluctuation in signaling between two adjacent equivalent cells can trigger a regulatory circuit that amplifies the initial difference, leading eventually to one cell expressing only Delta ligand and the other expressing only Notch receptor (see Figure 15-36).
- In *C. elegans*, Notch-mediated lateral inhibition causes two initially equal cells to follow different developmental paths.
- In *Drosophila*, newly formed neural precursor cells (neuroblasts and sensory organ precursors) express high levels of Delta and use Notch signaling to prevent their neighbors from becoming neural precursors (see Figure 15-38). Similar Notch-mediated lateral inhibition controls the balance between neural and non-neural cells in the development of *Xenopus laevis*.

trol the availability of active signals. The examples of signal integration and modulation described in this section illustrate some general mechanisms used in a wide variety of contexts.

Competence Depends on Properties of Cells That Enable Them to Respond to Inductive Signals

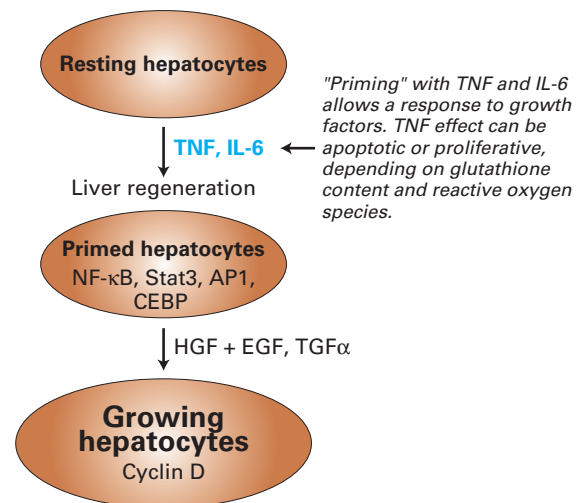
Early embryologists noted that cells differ in their ability to respond to various inductive signals. The ability to respond to a particular signaling molecule, referred to as *competence*, depends on several properties of the receiving cell: the presence of receptors specific for the signal, the ability of these receptors to activate specific intracellular pathways, the presence of transcription factors that stimulate the expression of the genes required to implement the appropriate response, and a chromatin structure that makes these genes accessible for transcription.

In some cases, the reception of one signal may make cells competent to receive another. After a part of the liver is damaged or removed surgically, increased amounts of two signals, tumor necrosis factor (TNF) and interleukin-6 (IL-6), are produced as part of the response to liver damage. These signals cause hepatocytes to enter a “primed” state in which the cells increase their production of certain transcription factors (e.g., NF- κ B, Stat3, AP1, and CEBP) but do not divide. Primed cells are competent to respond to a combination

15.7 Integrating and Controlling Signals

Cells change their properties rapidly in response to signals, both during and after development. The segregation and progressive restriction of cell potential during development are changes that take place as an organism grows and generates vast numbers of new cells that must be organized into new tissues and shapes. The cells in some adult tissues (e.g., blood, gut epithelia, and skin) also continue to proliferate and differentiate. These cells build on a substantial preexisting framework and have less “original” construction of tissues to do. Both dividing and nondividing cells in adult tissues remain highly responsive to hormones and other signaling molecules and to environmental changes.

Discussion in this chapter and in Chapters 13 and 14 calls attention to the enormous scientific effort that has been made in identifying the components of signaling pathways, how they transduce signals, and the resulting cellular responses. Many current projects are aimed at learning how multiple signaling pathways are mustered to control normal tissue growth and function in embryonic and adult tissues. In many circumstances, the appropriate response depends on the ability of receiving cells to integrate multiple signals and to con-



▲ **FIGURE 15-39 Priming of resting hepatocytes for later responses to signaling molecules that induce growth.** Injury to the liver or removal of part of the liver leads to priming of cells in response to interleukin-6 (IL-6) and tumor necrosis factor (TNF) signals. Primed cells increase their production of the indicated transcription factors but do not divide. Subsequent increase in the blood level of hepatocyte growth factor (HGF) induces primed cells to produce cyclin D, which is required for cell division (Chapter 21). HGF acts in conjunction with epidermal growth factor (EGF) and transforming growth factor α (TGF α).

of three signals that together induce the synthesis of cyclin D and mitosis (Figure 15-39).

Liver regeneration is an important survival tactic after a part of the liver is damaged or poisoned, but unregulated liver growth would lead to an unduly large organ or possibly cancer. In the primed state, hepatocytes can measure their own physiological state, their location within a tissue, their proximity to other cells, the need for healing, and the spatial organization of cells within a structure. On the basis of this assessment, the cells can respond most appropriately to subsequent signals.

Some Signals Can Induce Diverse Cellular Responses

Several classes of cell-surface receptors discussed in Chapters 13 and 14 are linked to more than one intracellular signal-transduction pathway (see Table 14-1). Multiple intracellular signaling possibilities are most evident with G protein-coupled receptors, cytokine receptors, and receptor tyrosine kinases. This phenomenon raises a general question: What governs how a cell responds to a signal that can be transduced by multiple pathways? Conversely, if the signaling pathway is the same in many cell types, why does one cell respond by dividing, another by differentiating, and still another by dying? For instance, signaling through the RTK-Ras-MAP kinase pathway (see Figure 14-16) is used repeatedly in the course of development, yet the outcome in regard to cell-fate specification varies in different tissues. If there is no specificity beyond the ligand and receptor, an activated Ras might substitute for any signal. In fact, activated Ras can do so in many cell types. In one DNA microarray study of fibroblasts, for instance, the same set of genes was transcriptionally induced by platelet-derived growth factor (PDGF) and by fibroblast growth factor (FGF), suggesting that exposure to either signaling molecule had similar effects. The PDGF receptor and the FGF receptor are both receptor tyrosine kinases, and the binding of ligand to either receptor can activate Ras.

Several mechanisms for producing diverse cellular responses to a particular signaling molecule seem possible in principle: (1) the strength or duration of the signal governs the nature of the response; (2) the pathway downstream of the receptor is not really the same in different cell types, for example because different complements of transcription factors are present in the receiving cells; and (3) converging inputs from other pathways modify the response to the signal.

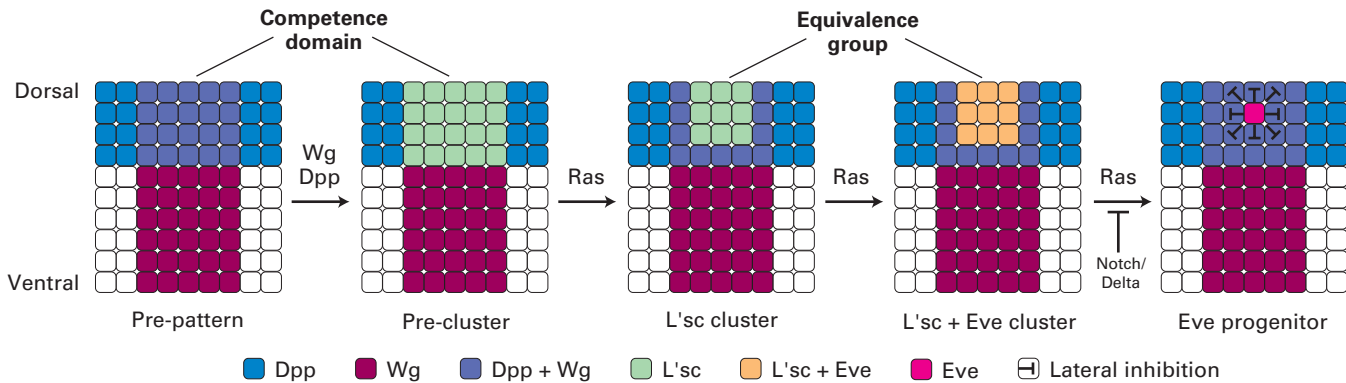
Differences in Signal Strength or Duration Evidence supporting the use of the first mechanism comes from studies with PC12 cells, a cultured cell line capable of differentiating into adipocytes or neurons. Nerve growth factor (NGF) promotes the formation of neurons, whereas epidermal growth factor (EGF) promotes the formation of adipocytes.

Strengthening the EGF signal by prolonging exposure to it causes neuronal differentiation. Although both NGF and EGF are RTK ligands, NGF is a much stronger activator of the Ras-MAP kinase transduction pathway than is EGF. The EGF receptor can apparently activate this pathway only after prolonged stimulation.

Differences in Downstream Pathways Signaling through cell type-specific pathways downstream of an RTK has been demonstrated in *C. elegans*. In worms, EGF signals induce at least five distinct responses, each one in a different type of cell. Four of the five responses are mediated by the common Ras-MAP kinase pathway; the fifth, hermaphrodite ovulation, employs a different downstream pathway in which the second messenger inositol trisphosphate is generated. Binding of IP_3 to its receptor (IP_3R) in the endoplasmic reticulum membrane leads to the release of stored Ca^{2+} from the ER (see Figure 13-29). The rise in cytosolic Ca^{2+} then triggers ovulation. This alternative pathway was discovered with a genetic screen that implicated IP_3R , a Ca^{2+} channel, in EGF signaling—a good example of how a mutation in an unexpected gene can lead to a discovery.

Integration of Signals The third way that the same signaling ligand/receptor pair can produce diverse effects on cells is to integrate more than one signal, as occurs in *Drosophila* muscle development. Figure 15-40 depicts the convergence of signal inputs that leads to the formation of a single muscle precursor cell, which is defined by its ability to transcribe the *even-skipped* gene. Early in muscle development, the *Drosophila* Wnt signal Wingless (Wg) and the TGF β signal Decapentaplegic (Dpp) prime a cell to make it competent to receive a subsequent signal that is transduced through the MAP kinase pathway downstream of Ras. The Wingless signal is produced in circumferential belts, and the Dpp signal is produced in two longitudinal bands at right angles to the Wnt belts.

One group of cells on each side of each body segment receives both the Wingless and the Dpp signals and thus become competent to respond to an unidentified RTK signal that activates Ras. In these cells, signal integration takes place during transcription of the *eve* gene. The transcription of *eve* is activated when a short 312-bp *eve* enhancer (not the same one described in Figure 15-32) is bound by two muscle-specific transcription factors and by three signal-induced transcription factors: TCF factor by Wingless, Mad by Dpp, and Pnt by an RTK acting through Ras. Thus tissue-specific and signal-responsive information is integrated through the action of five regulators on one short piece of DNA in specifying a cell type. As the result of lateral inhibition, eventually only one *eve*-expressing cell in each initial group of competent cells is left (see Figure 15-40). That single cell will develop into a particular muscle fiber by recruiting other cells and fusing with them.



▲ **FIGURE 15-40 Sequential action of critical signals in *Drosophila* muscle development.** Signal transduction through the RTK pathway is governed by Wnt and TGF β signals. Wingless (Wg) is produced in a stripe of cells running in a belt around part of each body segment of the embryo (purple). Decapentaplegic (Dpp) is produced in a band of dorsal cells running from head to tail on each side of the embryo (blue), a band that is created by the dorsal/ventral signaling system described in Section 15.3. A patch of cells in each body segment will receive both signals; only these cells (green) are competent to respond to the (unidentified) RTK signal that activates intracellular signaling from Ras. All the cells in the patch activate a gene called *L'sc*, though

further signaling restricts *L'sc* and then *eve* transcription to a more restricted set of cells called the pre-cluster (orange). Within the pre-cluster, a central cell begins to use Notch signaling to surrounding cells to repress *L'sc* and *eve* transcription there until only one cell is left making *eve* products (red). That single cell will develop into a particular muscle by recruiting other cells and fusing with them; two such cells are created in each body segment by this elaborate process. Both require RTK-mediated signaling; one cell uses the *Drosophila* EGF receptor (DER) and the EGF-type receptor called Heartless (Htl), and the second cell uses only Htl. [See Halton et al., 2000, *Cell* **103**:63–74.]

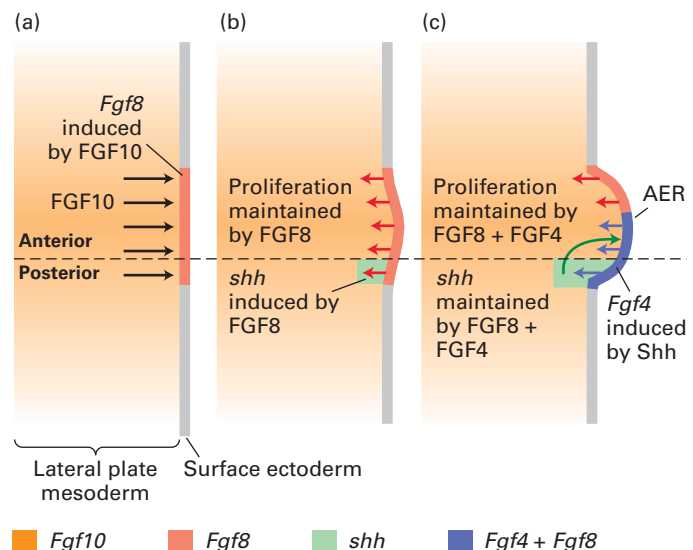
Limb Development Depends on Integration of Multiple Extracellular Signal Gradients

Vertebrate limbs grow from small “buds” composed of an inner mass of mesoderm cells surrounded by a sheath of ectoderm. Secreted signals from both cell layers coordinate limb development and instruct cells about their proper fates within limbs. The first signal, fibroblast growth factor 10 (FGF10) is secreted from the lateral trunk mesoderm and initiates outgrowth of a limb from specific regions of the em-

bryo's flank. Implantation of a bead soaked in FGF10 into places in the flank where a limb does not normally form causes an extra limb to grow; so FGF has remarkable inductive capabilities.

There are three dimensions to a limb: anterior/posterior (thumb to little finger), dorsal/ventral (palm versus back of hand) and proximal/distal (shoulder to fingers). An embryonic cell that knows its position along each of these dimensions is well along toward knowing what to do. A different signaling system operates in each of the three dimensions; so,

► **FIGURE 15-41 Integration of three signals in vertebrate limb development along proximal/distal and anterior/posterior axes.** Each limb bud grows out of the flank of the embryo. (a) A fibroblast growth factor (FGF) signal, probably FGF10, comes from the mesoderm in specific regions of the embryo's flank, one region for each limb. FGF10 acts on a local region of surface ectoderm called the apical ectodermal ridge (AER) because it will form a prominent ridge. (b) The ectoderm that receives a FGF10 signal is induced to produce FGF8, another secreted signal. At the posterior end of the limb bud, FGF8 induces transcription of the *Sonic hedgehog* (*Shh*) gene. (c) Shh signaling induces transcription of the gene encoding FGF4 in the AER. FGF8 and FGF4 promote continued proliferation of the mesoderm cells, causing outgrowth of the limb bud. Shh also stimulates this outgrowth and confers posterior characteristics on the posterior part of the limb. Development along the dorsal/ventral axis depends on a Wnt signal that is not shown here.



by reading the three signals, a cell appears to learn where it is within the limb bud and to act accordingly.

In response to FGF10, a local region of surface ectoderm becomes a signaling center, the *apical ectodermal ridge* (AER), at the tip of emerging limb bud (Figure 15-41). This region secretes FGF8 and later FGF4, both of which drive persistent division of mesoderm cells and therefore continued limb outgrowth. The FGF8 signal also induces the production of Sonic hedgehog in the posterior limb bud. The FGF signals tell cells their distance from the distal limb bud, and Shh tells cells that they are posterior. If Shh is added to the anterior part of the bud, the limb that eventually forms will have two posterior patterns of bones and no anterior. Along the dorsal/ventral axis, a Wnt signal instructs cells to form ventral cell types. The Wnt, FGF4, and FGF8 signals promote the transcription of *Shh*, and Shh signaling promotes the transcription of the *Fgf4* and *Fgf8* genes. Thus the signals are mutually reinforcing in cells that are close enough; cells too far from one of the reinforcing signals will cease making their own signal. In this way, the strength and movement of signals is tied to the eventual size and shape of the limb.

A cell in the midst of the limb bud, wondering what to do, is assailed by this brew of signals. By integrating the information from all of them, each cell begins to learn how to proceed. The main developmental task in the formation of limbs and organs is to organize a few cell types (e.g., mesenchyme, vascular, epithelial) into complex multicellular structures. The signals discussed herein provide the initial guidelines for this building process, instructing cells about their location relative to the coordinates of the limb and causing specific transcription factors to become active in proper parts of the limb.

Signals Are Buffered by Intracellular and Extracellular Antagonists

In real life, organisms experience wide variations in their environments and must adapt or die. Most multicellular organisms have highly variable numbers of cells, experience a wide range of temperatures, and must endure periods of nutritional deprivation, environmental toxicity, injury, and competition with their own and other species. Signal systems with rigid requirements for temperature and physiological conditions would be ill adapted to the real world. Scientists are beginning to recognize ways in which signals are regulated so that the outcome in regard to cell fates is correct.

Inducible Antagonists One way that cells can modulate signal activity is by producing *inducible antagonists*. The idea is that a signal induces the transcription of genes in the receiving cells. Among the induced genes is one encoding an antagonist that reduces the effect of the signal. If, by chance, too low an amount of signal gets through, the amount of antagonist also is diminished and the net target-gene induction is preserved. Similarly, too much signal, or signal transduction, will be corrected by greater production of antagonist.

Examples of both intracellular and extracellular (secreted) inducible antagonists are known. Such inducible antagonists have been found for the Hedgehog, Wnt, TGF β , RTK, and cytokine receptor signaling pathways.

In Chapter 14, we describe how inducible intracellular antagonists modulate signaling from TGF β receptors (see Figure 14-3) and from cytokine receptors (see Figure 14-14). The Hedgehog pathway, discussed in Section 15.5, also is regulated by an intracellular antagonist. Recall that, in the absence of a Hedgehog signal, Patched inhibits Smoothened and prevents intracellular signaling (see Figure 15-31). One of the target genes transcriptionally activated by Hedgehog signaling is *patched*; the resulting increased level of Patched in the plasma membrane reduces downstream signaling and turns off the transcription of Hedgehog target genes.

In other cases, a signal is controlled by an inducible secreted antagonist that binds the signal's receptor without activating it. For instance, the secreted protein encoded by the *Drosophila argos* gene competes with signals that activate the EGF receptor (EGFR), a receptor tyrosine kinase. Transcription of *argos* is stimulated by EGFR ligands, making Argos a type of inducible antagonist. When bound, Argos blocks dimerization of the EGF receptor, which is necessary for subsequent signal transduction.

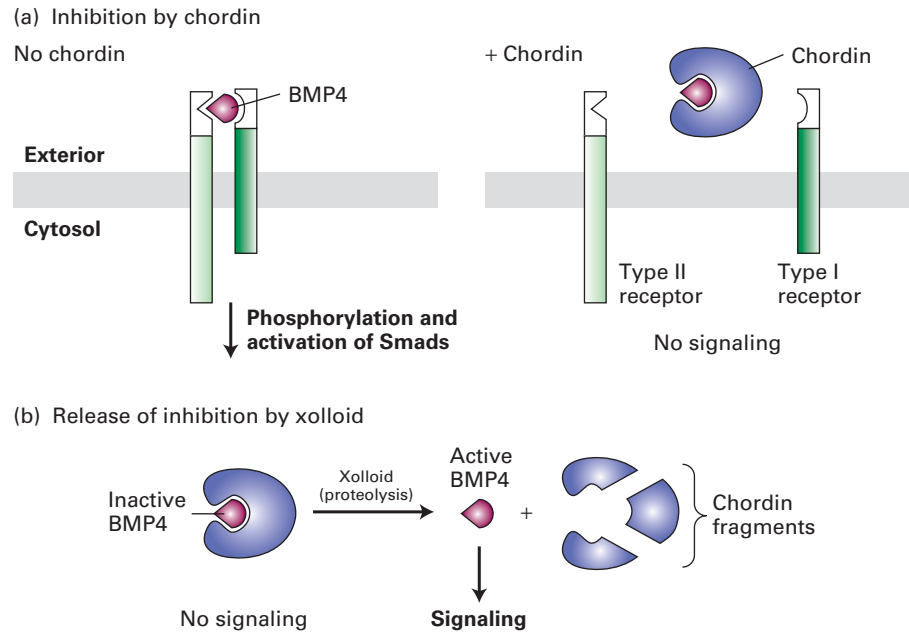
The Argos buffering system is used to create different cell types during dorsal/ventral patterning in early fly embryos. Recall that Gurken is a ligand for the EGF receptor on dorsal follicle cells (see Figure 15-17). A consequence of Gurken binding to the EGF receptor is transcriptional activation of the *spitz* and *vein* genes, both of which encode ligands that also bind to the EGF receptor and activate it. In this way, the effect of Gurken is amplified. In dorsal midline cells where the Gurken concentration is highest and EGFR signaling is strongest, the transcription of *argos* is also induced. Argos protein *reduces* signal transduction from EGF receptors in the midline; only in the two flanking regions does EGFR activity continue at high levels. This system creates two distinct populations of cells: those in which the EGF receptor is active flanking a central population of cells in which the EGF receptor is turned off. The highest level of the Gurken signal leads to the lowest level of signal transduction from the EGF receptor.

Antagonists Not Induced by the Signal Signaling pathways are also controlled by secreted antagonists that are not induced by the signal itself. One effect of such an antagonist can be to sharpen or move a boundary between cell types. A signal coming from source cells is progressively less potent with distance; at some point, it falls below a threshold amount and is without effect. If a secreted antagonist comes from the opposite direction, it will block the action of the signal even in cells receiving above-threshold amounts.

We see an example of this effect in the formation of neural cells in vertebrate development. Normally, secreted TGF β proteins prevent the formation of neural cells in a part of early frog embryos called the animal cap. In *Xenopus* embryos, the

► **FIGURE 15-42 Modulation of BMP4 signaling in *Xenopus* by chordin and Xolloid.**

(a) Chordin binds BMP4, a TGF β -class secreted protein signal, and prevents it from binding to its receptor. (b) Xolloid specifically cleaves chordin in the chordin–BMP4 complex, releasing BMP4 in a form that can bind to its receptor and trigger signaling. Similar regulation of Dpp (related to BMP) signaling in *Drosophila* is by Sog (related to chordin) and Tolloid (related to Xolloid). [See S. Piccolo et al., 1997, *Cell* 91:407.]



animal cap produces BMP4, a member of the TGF β family of signals, and will therefore not produce neural tissue. The effect of signals and other regulators on neural induction can be tested by exposing parts of the animal cap in *Xenopus* embryos to individual proteins and seeing whether neural cells form. This type of experiment revealed the ability of *chordin protein* to antagonize BMP4 and induce neural cell identity, an indication of the presence of dorsal mesoderm. The addition of chordin to *Xenopus* animal caps induces the formation of neural cells; this neural induction by chordin is reversed by the addition of an excess of BMP4 protein. On this basis, neural cell fate is looked on as the default state. Only when BMP signaling is successful can other cell types form. Together, these data led to a simple model in which chordin prevents BMP from binding to its receptor. In principle, inhibition could occur by the direct binding of chordin to BMP receptors or to BMP molecules themselves. The results of a series of biochemical studies demonstrated that chordin binds BMP2 and BMP4 homodimers or BMP4/BMP7 heterodimers with high affinity ($K_D = 3 \times 10^{-10}$ M) and prevents them from binding to their receptors (Figure 15-42). Chordin-mediated inhibition of BMP signaling is relieved by Xolloid protein, a protease that specifically cleaves chordin in chordin–BMP complexes, releasing active BMP.

Wnt signaling also is modulated by secreted antagonists, including a special group of secreted proteins, called Frizbees, that are related to the Frizzled receptor in this pathway. Wnt signals bind to a cysteine-rich domain in the extracellular domain of Frizzled receptors, thus activating downstream signaling (see Figure 15-32). Frizbee proteins have the extracellular domain of Frizzled but lack its transmembrane and cytosolic domains. By tightly binding Wnt signals, Frizbees soak up the signal, so less is available to activate Frizzled receptors.

We conclude by mentioning Cerberus, a champion secreted antagonist discovered in frog embryos. This protein is called Cerberus, after the mythological guardian dog with three heads, because it has binding sites for three different types of powerful signals—Wnt, Nodal, and BMP. The binding of these signals by Cerberus prevents activation of their respective receptors. By inactivating Wnt, Nodal, and BMP signals having roles in the development of the trunk and tail of the body, Cerberus promotes head development.

KEY CONCEPTS OF SECTION 15.7

Integrating and Controlling Signals

- In liver regeneration, early signals change cells into a primed state in which they are competent to respond to subsequent signals that cause growth and mitosis.
- Most components of the RTK–Ras–MAP kinase signaling pathway are used repeatedly in the course of development and are evolutionarily conserved in a broad spectrum of animals.
- In embryonic muscle development in *Drosophila*, three types of signals converge to promote differentiation (see Figure 15-40). These signals induce the production of three transcription factors that combine with two tissue-specific transcription factors to activate transcription of the *even-skipped* gene, which marks a muscle precursor cell.
- At least one signaling protein acts along each axis of the developing limb bud. Cells respond to the combination of signals, and the signals reinforce the production of other signals to coordinate growth and patterning in three dimensions (see Figure 15-41).

- Inducible antagonists include intracellular and secreted proteins whose production is induced by a particular signal. The antagonist protein then feeds back, opposing the ongoing action of the signal. Inducible antagonists provide buffering in most signaling pathways to compensate for excess or inadequate signals.
- Signals can also be controlled by antagonists that are not induced by the signal itself. By binding to signals, these antagonists prevent the signals from binding to their specific receptors and activating them (see Figure 15-42).

PERSPECTIVES FOR THE FUTURE

The remarkable interplay of signals and gene controls during development is a process of pattern formation. Simple masses of cells become startlingly beautiful, functional structures such as eyes, lungs, hearts, and wings. For this transformation to happen, cells must respond to a rich mix of short-range and long-range signals and integrate the information to make the right decisions. Only a few signaling systems are used repeatedly to organize cells into tissues. For the most part, the signaling systems are common to all animals, thus vastly simplifying the problem of learning the underlying molecular mechanisms. This manifestation of our common evolutionary origins reduces the problem of understanding biological regulation—but the remaining challenges are still enormous.

To more fully understand how cells acquire their fates and participate in morphogenesis will require a comprehensive view of their molecular responses *in vivo*. The advent of DNA microarray technology for simultaneously monitoring the transcription of thousands of genes—indeed all of the transcription units in a genome—is a major step forward in “reading the minds” of cells. A signal can now be assessed for its ability to redirect a cell’s activities by measuring all the signal-induced changes in gene expression. Exciting as these new insights are, their limitations also are apparent. For instance, transcripts are currently measured in extracts that represent an average of many cells isolated without great temporal precision—from embryos that change in seconds or minutes. Therefore we can look forward to continuing advances as methods improve for observing responses in living cells. Moreover, DNA microarray analysis measures only transcription, not RNA splicing or translation into protein; nor are protein localization and modification and protein–protein associations assessed by this technology. These other levels of gene control are still measured mostly on a gene-by-gene basis. Advances in protein microarray technology that enable monitoring of all proteins at once, as well as protein–protein interactions, will contribute tremendously to our understanding.

The rapid fluxes in cell responses that occur during signaling events, the integration of information from multiple signals, and the dynamic feedback control loops are all dif-

ficult to comprehend without precise measurements of signal concentrations, reaction rates, and equilibrium states. More precise quantitation will be necessary to understand signaling circuitry properly. Crude statements of genes being on or off or of proteins being phosphorylated or not will have to be replaced by more precise information, because in fact the changes entail gradations over orders of magnitude, not simply all-or-none phenomena.

The elaborate switching systems in cells, usually many of them working at the same time, have a degree of complexity that requires computer modeling. This field, sometimes referred to as systems biology, aims to produce models that successfully describe the normal responses of cells to stimuli and that predict how cells will respond to additional or different perturbations. An engineering approach of this sort holds the exciting promise that cells could be directed to perform tasks, such as improved immunity or healing, that are useful. The field of molecular cell biology increasingly is becoming a computational science, but with the special fascination that the signals are building life.

KEY TERMS

angiogenesis 620	maternal mRNA 629
chordin protein 652	morphogen 623
development 612	pair-rule gene 634
equivalence group 624	protein microarray
floral organ–identity gene 637	(proteome chip) 615
gap gene 630	reciprocal induction 644
Hedgehog pathway 641	segment-polarity gene 635
homeosis 636	sensory organ
Hox gene 636	precursor (SOP) 647
inducible antagonist 651	syncytium 625
induction 621	Toll-Dorsal pathway 628
<i>in situ</i> hybridization 614	transcription cascade 632
lateral inhibition 644	Wnt pathway 642

REVIEW THE CONCEPTS

1. Describe three different pathways in which EGF-type receptors participate.
2. What enzyme catalyzes the degradation of glycogen to glucose 1-phosphate? Describe how the subunit structure of this enzyme allows for integration of multiple signals.
3. People with Type 1 diabetes do not produce insulin. Describe what happens to GLUT4 glucose transporters in Type 1 diabetics just after a meal. Most patients with Type 2 diabetes produce normal amounts of insulin and have insulin receptors present on insulin-responsive cells. Yet these patients are defective in glucose uptake, and hence have abnormally

high blood glucose levels. Speculate on the nature of the defect in these patients.

4. Compare and contrast the action of morphogens involved in dorsal-ventral specification in *Xenopus laevis* and *Drosophila melanogaster*.

5. Using in situ hybridization with a dorsal-specific probe, where in the syncytial *Drosophila* embryo would one expect to find *dorsal* expressed? Using immunohistochemistry with an anti-dorsal antibody, where would you expect to find Dorsal protein expressed?

6. A microarray analysis of wildtype vs. *dorsal* mutant embryos could be expected to yield information on all genes regulated by the Dorsal protein. Why? Other than new genes regulated by Dorsal, one would expect to see changes in regulation of previously identified genes. Which genes would be increased or decreased in expression in *dorsal* mutants?

7. Deleting the 3' UTR of the *bicoid* gene would yield what phenotype in a mutant fly? Why?

8. How does the motor protein kinesin ensure that proper post-translational controls take place in the anterior end of the *Drosophila* embryo? If kinesin functioned only during embryo development, what would the phenotype of a *kinesin* mutant embryo be?

9. What is the evidence that the Toll-based innate immunity system may be more than a billion years old?

10. How can the group of five gap genes specify more than five types of cells in *Drosophila* embryos?

11. What is homeosis? Give an example of a floral homeotic mutation and describe the phenotype of the mutant and the normal function of the wild-type gene product.

12. Compare and contrast the receptor systems responsible for recognition of the Hedgehog and Wnt secreted ligands.

13. What is the evidence that a gradient of Sonic hedgehog leads to development of different cell types within the avian neural tube?

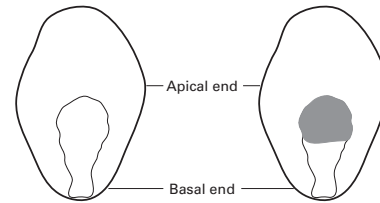
14. The finding that *ephrin b2* knockouts contain striking defects in both arterial and venous capillaries supports the idea that ephrin B2 and its receptor, EphB4, are involved in a reciprocal induction. How?

15. Give a specific example of a signal-buffering inducible antagonist, and describe how its actions lead to signal buffering.

ANALYZE THE DATA

The entire genome sequence of rice (*Oryza sativa*) has been determined, allowing investigators to search the rice genome for gene sequences homologous to *Drosophila* genes involved in developmental processes. A putative *bicoid*

homolog was found in the rice genome that encodes a homeodomain-type transcription factor. This rice gene was used as a probe against rice embryos in an in situ hybridization experiment, shown below. Plant embryos are contained within seeds. The rice embryos used in these experiments were in the globular stage of development. At this stage, the apical and basal ends of the future plant are being specified.



a. What is the expression pattern of the putative rice *bicoid* gene?

b. Speculate as to the function of the putative rice *bicoid* gene.

c. From the above results, what phenotype do you think might develop if one microinjected mRNA molecules transcribed from the putative rice *bicoid* gene into the basal end of the embryo?

d. From the above results, what would the expected expression pattern be in rice if one could identify a putative *nanos* homolog from rice?

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