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Shh and Gli Signalling and Development

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PREFACE

The hedgehog signalling pathway is highly conserved and seen in organisms ranging from *Drosophila* to humans. This pathway is critical in determining cell fate decisions in a variety of different cell types. There are several vertebrate analogues of the *Drosophila* hedgehog protein of which the most widely studied is Sonic hedgehog (Shh). Shh signalling classically involves the Gli family of zinc-finger transcription factors. The Shh signalling pathway is well characterised in the development of a number of vertebrate organ systems. It could indeed be argued that the Shh and Gli signalling may well be involved at some stage in the development of all the major organ systems in vertebrates. This volume represents a concerted drive to bring together 'state of the art' reviews by leading experts in the field of Shh and Gli signalling in development from all over the world. The chapters span vertebrate organisms from zebrafish to humans and cover development of the multiple organ systems in which the Shh signalling pathway is crucial for normal development. There are chapters on the development of the central nervous system, skeletal structures, visceral organs, prostate, lung, immune system and the structures of the human face. The authors themselves span three major continents and multiple nationalities which admirably illustrates the worldwide nature of the science. The international nature of the project has been very rewarding and the quality, depth and range of the reviews included speaks for itself. It is hoped that the reader will appreciate the wide variety of scientific approaches that have contributed to our current knowledge base of the importance of Shh and Gli signalling in vertebrate development and will at the same time realise that, as with all good science, there are still more questions than answers.

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June 2006

CHAPTER 1

Introduction

Carolyn E. Fisher* and Sarah E.M. Howie

The Concept of Developmental Biology

Although no real insights into the mechanisms of development were obtained until after 1880, when experimental approaches to embryology were established, descriptive studies of embryo development have been around for millennia. Aristotle (384-322 BC) wrote a very detailed description of mammalian embryogenesis, similar to the picture we accept today, inferring that the process was driven by an *entelechy*, known as a "vital force" in later centuries. Descriptive studies continued after 1550 but there was no further serious discussion of the *mechanisms* of embryo development until the 18th and 19th centuries.

The anatomist Wilhelm Roux (1850–1924) pioneered experimental embryology, focusing on amphibian embryos, and was the first to suggest that chromosomes carry hereditary material. In 1882 he extended Darwin's theory of the struggle for existence to ontogenesis. He wrote that stronger cells leave more offspring than weaker cells, inferring that competition for space and nutrients governed development. We now know that cell reproduction is far from chaotic, and that competition for intercellular spaces is, in general, abnormal. Nevertheless, "neural Darwinism", the idea that neurites compete during growth and that only the first of the group to reach the target cell survives, is becoming established in developmental neurobiology.

Another pioneer of experimental embryology, Hans Driesch (1867–1941), discovered that cells of early sea urchin embryos "remembered" their individual locations in the cell mass—separated cells returned to their original positions—although there were no detectable physical or chemical differences among them. Lacking the understanding of the biochemistry of cell-cell interactions that we have today, Driesch concluded that a "vital force" drove embryogenesis – the idea proposed by Aristotle more than two millennia earlier. Modern-day biologists no longer believe in a "vital force"; biology is mechanistic in character.

Thanks to technological advances in the late 20th century, developmental genetics has grown in stature. The importance of these advances for understanding embryogenesis is recognised. Significantly, biologists now realise that the molecular components of many developmental pathways are present and active in adult organisms. They are not mere residues of morphogenesis; developmental pathways are important in maintaining as well as generating the adult form. In a sense, morphogenesis is never complete. As will be discussed in later chapters, developmental pathways are important in tissue repair and organ regeneration. In addition, it is now clear that these same pathways play a major role in some cancers, where mature cell types appear to "dedifferentiate", proliferating without adequate control and invading normal functioning organs. Cancer is another topic that will be covered later in the book.

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Introduction to Morphogens: Shh

The term *morphogens* was coined by the mathematician Allan Turing in 1952 to denote graded signals released by 'organisers' such as the notochord and Zone of Polarising Activity (ZPA) in the developing limb bud. To qualify as a morphogen, a signal must fulfil two criteria: to form a concentration gradient, and to elicit distinct responses at different concentrations. Cells encounter different concentrations of a morphogen according to their distance from the organiser that secretes it. Different transcription factors are therefore induced, committing the cells to different fates.[1] At least four models of morphogen transport have been proposed.[2]

Chemoattractants and chemorepellents also form graded signals, guiding cell migration and various cellular processes, but they are "guidance cues" not morphogens. Cells respond to chemoattractant and chemorepellent *gradients* rather than absolute concentrations. Also, these signals act by regulating cytoskeletal and membrane dynamics, not by signalling to nuclei.[3]

The first morphogens identified were the transcription factors encoded by the *Drosophila* genes *bicoid* and *hunchback*, which operate in the embryo before cellularization, forming concentration gradients along the anterior-posterior axis.[1] Morphogenesis genes are highly conserved across species. They include members of the Wnt family (wingless in *Drosophila*) and *decapentaplegic* (Dpp) in *Drosophila* appendage development;[4,5] bone morphogenic proteins (BMPs); fibroblast growth factors (FGFs); members of the TGFβ family, such as Squint in early zebrafish embryogenesis;[6] and Hh genes. Sonic Hedgehog (Shh), one of three mammalian homologues of Hh, has been shown to act as both a morphogen and a guidance cue.[7]

In *Drosophila*, Hh functions as a short-range morphogen during wing development whereas Dpp acts over a long range. Imaginal discs (wings) comprise anterior (A) and posterior (P) compartments. Cells in the latter express *engrailed* (en), which induces Hh synthesis. Hh is secreted into the A compartment, inducing transcription of several genes including Patched (Ptc), Dpp and en.[8] In anterior cells bordering the A-P boundary (the disc lumen), Dpp organises the wing's A-P axis and is required for disc development and patterning.[5] After A-P subdivision the imaginal disc is divided into Dorsal-Ventral (DV) compartments, the border between which develops into the wing margin. DV patterning involves the Notch and wingless signal transduction pathways. Wg acts as a morphogen inducing target gene expression and patterning activities of the dorsal/ventral boundary.[9]

Morphogens also play a role during vertebrate development. For example, squint promotes the formation of mesoderm and endoderm in zebrafish embryos;[6] and Shh acts directly at long range to pattern the ventral neural tube in chicks. Shh is also involved in limb bud formation but whether it acts as a morphogen in this context is unclear.

The Hh Pathway in *Drosophila*

The Hh pathway was first recognised as important during segmentation in *Drosophila*.[10] An elegant study by Ingham and colleagues led to a now widely-accepted model of Hh signalling in *Drosophila*;[11] a simplified version is shown in Figure 1.

Hh signalling is absolutely dependent on smo. Smo is inhibited by the protein Ptc, which acts indirectly and substoichiometrically. The mechanism might involve the transport of an endogenous modulator of smo, but this has not been identified, nor has Ptc transport activity been characterised.[12] However, it is generally held that Hh removes the inhibition of smo by binding to Ptc. Hh stimulation of cells stabilises smo, which accumulates at least 10-fold and becomes more highly phosphorylated.[13]

Evidence suggests that intracellular localisation of smo-containing organelles depends partly on costal-2 protein (cos 2). Cos-2 tethers a group of segment polarity proteins to cytoskeletal microtubules, and full-length Ci is bound to these. Smo and cos-2 may interact directly.[14] Recruitment of cos-2 to smo causes Ci to dissociate from the cytoskeleton, preventing its cleavage to the transcriptional repressor form Ci^{75} (CiR). When smo is activated, however, the Ci/protein complex dissociates and full-length Ci is translocated to the nucleus, where it activates target genes containing Ci-binding sites. A detailed analysis of smo has been published.[15]

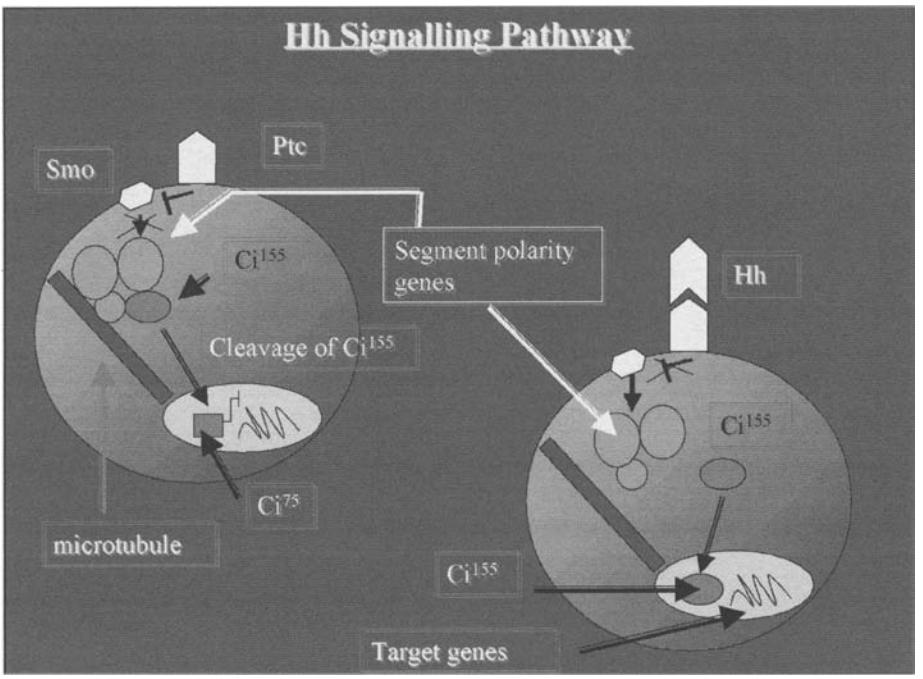

Figure 1. In the absence of ligand binding, Ptc-1 inhibits the activity of smo, allowing Ci to be cleaved to form a transcriptional repressor. When Hh binds to Ptc-1 this inhibition of smo is repressed. This allows full-length Ci to be translocated to the nucleus, where it acts as a transcription factor for various genes.

The smo-Cos-2 complex also contains Fu (Fused), and Fu kinase activity is needed for Hh signalling. Fu phosphorylates Cos-2 at the two positions induced by Hh stimulation.[16] A primary function of activated smo appears to be the inhibition of suppressor of fused (Su(fu)), activating Fu; this may happen indirectly via Cos-2.[15] The stability of Fu kinase is an absolute requirement for positive regulation by Cos-2. Therefore, the Hh-induced stabilisation of smo results in recruitment of both Fu and Cos-2.[13] Fu is dispensable if Su(fu) is lost. Su(fu) negatively regulates Ci by localising it in the cytoplasm, either through cytoplasmic anchoring or nuclear export; it might also inhibit Ci function in the nucleus.[17]

CiR (the N-terminal proteolytic fragment of Ci that suppresses transcription) retains the zinc finger-mediated DNA binding specificity but lacks nuclear export signals, a cytoplasmic anchoring sequence and a transcriptional activation domain.[17,18] *Drosophila* protein kinase A (dPKA) is required, along with Cos-2 and Fu, to process Ci^{155} to Ci^{75} in vivo. Intact Ci (Ci^{155}) is found in cells carrying mutations in these genes. It can activate the transcription of Hh target genes if normal Fu is present. Loss of Fu also causes accumulation of Ci, but in this situation Ci cannot activate Hh target genes.[19]

Although this Hh pathway has become widely accepted and has been mapped out in detail, some observations challenge it. In *Drosophila*, whilst smo protein is distributed throughout the imaginal disc, it accumulates in wing compartments and clones of cells lacking Ptc, but is reduced in cells overexpressing Ptc, even in the absence of Hh signalling. Also, cell-surface levels of smo increase in response to Hh stimulation whereas Ptc levels decrease. This suggests that most smo does not colocalise with Ptc, making it unlikely that Ptc-smo binding, if it occurs in vivo, is important in Hh signalling.[20] Some workers have gone so far as to suggest that the first step in the Hh pathway (modification of smo activity by Hh and Ptc) should be

reconsidered. Currently it is hypothesised to involve changes in smo concentration, localisation, phosphorylation, conformation or binding to small molecules related to cyclopamine, i.e., changes in isolated smo molecules. Now it seems possible that Ptc and Hh might act primarily, or partly, through smo partners such as cos-2 instead of smo itself.[21]

In *Drosophila*, Hh regulates cell proliferation and differentiation in essential patterning events such as embryonic segmentation, appendage formation, and development of the eye and regions of the brain; either directly, or indirectly via recruitment of Dpp and wingless. Before they can execute such roles, Hh molecules are matured by autocatalytic cleavage. The products are Hh-Np (the N-terminal polypeptide), the functional signal, and a C-terminal polypeptide that appears to have no function other than catalysing the autoproteolysis. The signalling peptide (Hh-Np) is modified at its N- and C-termini by palmitoyl and cholesteryl adducts, respectively.[22] Although many proteins are lipid-modified, Hh and its vertebrate homologues are unique in being modified by cholesterol addition.[23]

The action of Hh on distant cells in developing tissues involves: (a) the transmembrane transporter-like protein Dispatched (Disp), which is required for releasing Hh from cells; (b) the heparan sulphate proteoglycans (HSPs) Dally-like (Dlp) and Dally, which are required for extracellular Hh transport; and (c) HSP biosynthesis enzymes such as Sulfateless and *tout velu*.[24-26] *Tout velu* is required for moving cholesterol-modified Hh.[27] The ability of Hh to attach to membranes via the C-terminal cholesterol may be critical for increasing the distance over which the morphogen acts.[23] *Dispatched*, a distant relative of Ptc, is predicted to encode a 12-pass transmembrane protein with a sterol-sensing domain. Its role in trafficking cholesterol-modified Hh might be executed through a secretory pathway, so that the active form arrives at the cell surface, or through the displacement of cholesterol-modified Hh from the lipid bilayer.[23] If *dispatched* is absent during the development of imaginal discs, normal levels of Hh are produced but it is not released from posterior cells and accumulates instead. Moreover, *Drosophila dispatched* mutants lacking both maternal and zygotic activity have a segment polarity phenotype identical to Hh mutants, demonstrating that this molecule is critical for proper Hh pathway signalling.[25]

The Shh Pathway in Vertebrates

The Hedgehog pathway in vertebrates parallels that in *Drosophila* but there are two or more homologues of some components, consistent with divergence of function. Mammals have two Ptc receptors (Ptc-1 and Ptc-2), though only the former is definitely involved in Hh signalling. It is confined to target cells and is upregulated in response to Hh. Ptc-2 is coexpressed with Hh but its transcription is independent of pathway activation.[28] Mammals also have three Hh proteins, *Sonic* (Shh), *Indian* (Ihh), *and Desert* (Dhh) *Hedgehog*, which differ in their tissue-specific expression patterns and in their roles during development. The mammalian homologues of *Drosophila* Ci are the three Gli molecules (Gli 1-3), which regulate the transcription of Hh-responsive genes both positively (Gli 2) and negatively (Gli 3).

The homologues of Hh, Ptch, smo and Ci are well conserved but those of Cos2 and Fu are less so. They have not been functionally linked to pathway regulation, suggesting that certain *Drosophila* routing mechanisms may be less important in mammals. SuFu, however, is conserved, and does have pathway regulatory functions. This is demonstrated by loss of function in zebrafish;[29] also, Cheng and Bishop (2002) showed that SuFu can enhance the binding of Gli proteins to DNA.[30]

As in *Drosophila*, Hh proteins undergo autocatalytic cleavage to an active 19kDa ligand with cholesterol covalently linked to the C-terminus. Caveolin-1 may be a Ptc-binding partner in *Drosophila*:[31] caveolins are the major constituents of caveolae, nonclathrin-coated membrane invaginations important in endocytosis and intracellular trafficking. This might imply that the cholesterol moiety is involved in directing intracellular transport, and cell culture experiments have shown that cholesterol-modified Hh remains bound to the cell surface, suggesting limited movement in vivo.[23] Nevertheless, cholesterol-modified Shh in vertebrates is

thought to spread Shh activity rather than anchor it in one place; Lewis et al (2001) demonstrated that Shh-Nu (sonic that could not be cholesterol-modified) in mice had a restricted range of signalling in comparison to wild type Shh.[32]

This conflict of evidence might have been resolved by the discovery in vertebrates of inhibitors of Hh signalling, such as Hip1 (hedgehog interacting protein 1) and GAS-1 (growth arrest specific-1). These proteins have no *Drosophila* homologues. The former encodes a membrane-bound glycoprotein that binds Shh, and the latter is a Wnt-inducible mouse gene expressed in areas that respond to but do not express Shh.[33,34]

Hh proteins are involved in neural tube formation in vertebrates. In mammals, Shh activity at the midline patterns the ventral neural tube and somites, and is involved in the development of left-right asymmetry. It has polarising activity in the limb, acting at both short (posterior limb identities) and long (anterior limb identities) distances. It is involved in maintaining stem cells in postembryonic tissues and acts as a pathogenic mitogen in some endodermally-derived human cancers, which account for 25% of all cancer deaths.[35,36] Shh also regulates morphogenesis of many other organs (see below).

Gli Transcription Factors

Gli molecules are evolutionarily conserved, with homologues identified in invertebrates and in all vertebrate species analysed so far.[37] Humans and mice have three Gli genes that are candidates for mediating downstream activities of Shh but their precise roles are not fully determined.

Generally, expression of Gli1 is highly restricted compared to Gli2 and Gli3, and it is transcriptionally regulated by Hh signalling, whereas the others are less reliant on Hh for transcription. Gli1 only activates Shh transcription, whereas Gli2 and 3 are bi-functional and Hh signalling regulates their activities post-transcriptionally. Data from the many studies in mice with defective Gli genes show that Gli1 expression is tightly controlled by the activities of Gli2 and 3.[38] Gli genes are never expressed in Shh-expressing organiser cells during embryogenesis. Normally Gli1 is expressed in cells adjacent to the organiser, consistent with its role as a transcriptional activator of the Shh signal. Gli3 is usually situated opposite the organiser, possibly limiting its range.

First indications that transcription factors play a role in establishing cell fates in response to a morphogen came from studies on the spinal cord. Here, Gli 1-3 are expressed in partially overlapping patterns and establish the initial stripes of homeodomain transcription factor expression in the ventral neural tube in response to Shh produced by the notochord and floorplate, promoting the specification of several ventral cell types.[39] In the frog neural plate, widespread expression of Gli2/3R (repressors) abolishes neuronal differentiation.[40] In mice, inactivation of Gli2 results in absence of the floor plate, probably partly due to inefficient activation of the transcription factor HNF3β, which regulates floor plate identity.[41] Also, high expression of Gli3R in chick neural tube abolishes ventral cell differentiation.[42]

The importance of Gli factors during embryogenesis has been assessed in single and double knockout mice. Gli1-/- mice have no obvious defects, indicating that Gli1 is dispensable for embryogenesis.[43] Since Gli2-/- mice have phenotypes similar to but milder than Shh-/- mutants, it appears that Gli2 is the major transducer of Shh signalling.[38] These mice have severe skeletal abnormalities including no vertebral bodies or intervertebral discs, and shortened limbs.[44] Gli3-/- mutants have defects, such as polydactyly, distinct from those of Gli2-/- and Shh-/-. Xt mutant mice have alterations within the Gli3 locus, and Xt/Xt embryos display enhanced polydactyly in the fore and hind limbs. Heterozygotes show preaxial polydactyly of the hindlimbs.

Although deletion of the Gli1 zinc finger domain leads to no obvious abnormalities in the embryo, Gli1-/-Gli2+/- mice have reduced viability and exhibit lung and neural tube defects that are not found in either Gli1-/- or Gli2+/- mice.[43] This indicates that Gli1 has a physiological role in Shh signalling. Perhaps Gli2 and/or Gli3 can compensate for the lack of Gli1 function during embryogenesis.

Roles for Shh in Vertebrates

The importance of Shh signalling during development, in adult organisms, and in pathological processes, should not be underestimated. Although Shh signalling has been analysed in detail in relatively few organs/systems such as the CNS, limbs, lungs, eyes and the reproductive system, the pathway appears to have important roles in nearly every organ. Many of these are covered in detail in subsequent chapters.

CNS

Shh acts as a morphogen during development of the early vertebrate ventral neural tube. Later, in the dorsal brain, it acts as a mitogen on progenitors of the cerebellum, tectum, neocortex and hippocampus.[45] General consensus attributes dorsoventral specification of the neural tube to Shh secreted by the notochord inducing differentiation of the floor plate; the latter starts to express Shh in response to the notochordal signal.[46] An alternative proposal is that because the floor plate, notochord and dorsal endoderm share a common origin in Henson's node, all are sources of Shh.[47] Details notwithstanding, it is clear that Shh influences the development of, and many cell fates within, the CNS and associated structures.

A study on chick embryos by Ahlgren and Bronner-Fraser demonstrated the importance of Shh in craniofacial development, dealt with in a later chapter: branchial arch structures are lost and there are subsequent brain anomalies.[48] Somite development in Shh null mice has been investigated by Borycki et al, who demonstrated that Shh is critical in activating myogenic determination genes and that it is required for survival of sclerotome cells as well as ventral and dorsal neural tube cells.[49] Weschler-Reya and Scott implied a role for Shh during development of granule cells. They demonstrated that Shh, which is made by Purkinje cells, regulates the division of granule cell precursors.[50] A mitogenic action of Shh was also found by Rowitch et al, who suggested temporal restrictions on Shh-mediated cell proliferation.[51]

The three Gli genes are expressed in partially overlapping domains in the neural tube; Gli2 and 3 are proposed to mediate initial Hh signalling and to regulate Gli1. All have activator function but only Gli2 and 3 have potent repressor functions, and each appears to be regulated differently. Details of the role(s) of the Gli proteins during CNS development are dealt with in various subsequent chapters.

Limbs

Shh and Gli gene functions during limb bud formation have been studied extensively. Briefly, the ZPA (zone of polarising activity) signalling centre in the posterior limb bud is necessary for A-P patterning, and defects resulting from ZPA transplants can be mimicked by misexpression of Shh.[52]

Gli genes are expressed only in the mesenchyme during limb formation. However, only Gli3 appears to have a role in limb development, its major function being establishment of A-P asymmetry. It also represses Shh expression in the anterior margin of the limb bud; loss of Gli3 function results in ectopic Shh expression, induction of Gli1 in adjacent cells, and preaxial polydactyly. Despite the lack of limb defects in Gli1 mutant mice, Gli1 is always upregulated in the anterior region of limb buds adjacent to Shh-expressing cells in polydactylous animals, implying a mediating role in Shh signalling.[53]

All Gli genes are expressed in developing bones; Gli2 and 3 are essential for normal development. In Gli2-/- mice, bone ossification is delayed and long bones are shortened;[44] in Gli3-/-, the length and shape of most bones are altered and sometimes the radius and tibia are missing.[54]

Shh signalling is also involved in chondrogenesis and smooth muscle differentiation, with Shh and Ihh participating in the differentiation of chondrogenic precursor cells into chondrocytes.[55] The Hh family also plays a role in joint formation.[56]

Reproductive Tract

Hh signalling is critical in the development and differentiation of the gonads and accessory sex glands.[57] In females, Ihh, rather than Shh, is the important molecule. In murine mammary gland development there appears to be a complete absence of Shh; Ihh is localised exclusively to the epithelium. During puberty it is found in undifferentiated epithelial 'body cells' at the tips of terminal end buds of elongating ducts.[58] The role of Hh in somatic and germline stem cell proliferation in adult Drosophila ovary is well-characterised,[59] but it is unclear whether Hh-signalling is involved in vertebrate ovaries.

In the adult male, Desert hedgehog (Dhh) signalling is essential for spermatogenesis and for development of Leydig cells, peritubular cells and seminiferous tubules; Shh appears to have no role. Male Dhh-/- mice lack mature sperm but no expression is observed in the female ovary during early or late stages of development.[60]

Shh is necessary for normal prostate development but not initial organogenesis. Specifically, it provides the signal for prostate ductal budding, a testosterone-dependent process, and is involved with ductal patterning.[61] All three Gli genes are expressed during ductal budding; their levels decline postnatally, becoming low in the adult.[62] Prostate development is covered in detail later in the book.

Lung and Visceral Organs

Lung bud morphogenesis begins in mice at E9.5 as an endodermal outbudding of the developing gut tube, the A-P patterning of which is governed by Shh. Normal lung development depends on Shh signalling and Gli transcription factors; Shh -/- murine embryos fail to form lungs, Gli3 is essential for proper pulmonary development, and Gli1 is known to act downstream of Shh signalling in lung.[63] Shh is essential during early stages of pulmonary branching morphogenesis but it does not appear to be important in the subsequent differentiation of specialised lung cells such as Clara cells. Shh signalling is also required for proper separation of the trachea and esophagus. It is also pivotal in digestive tract morphogenesis and differentiation; epithelial Shh regulates the formation of stomach glands, connective tissue and smooth muscle, and stratification of mesenchyme.[64] Lung development and the role of Shh in visceral organs are subjects of later chapters.

Eye

Much work has been done on eye development in *Drosophila*, *Xenopus*, chick, zebrafish and mouse, and in all cases Hh signalling regulates morphogenesis to some extent. The retinal determination gene in *Drosophila*, *eyes absent* (Eya), represents a crucial link between Hh signalling and photoreceptor differentiation: Hh acts as a binary switch, initiating retinal morphogenesis by inducing Eya expression.[65] In *Xenopus*, misexpression of Tbx2 and Tbx3 results in defective eye morphogenesis. Tbx2/3 expression is thought to be regulated by Gli-dependent Hh signal-transduction.[66] In zebrafish eye development (covered later in the book), the eye phenotype of the sonic-you (syu) mutant is consistent with multiple roles for Hh during retinal development.[67] Generally, Hh signalling regulates eye morphogenesis and photoreceptor differentiation and plays a role in defining the proximal-distal and dorsal-ventral axes in the eye.

Other Roles

Other roles of Shh in vertebrate morphogenesis include those in tooth development, covered in a later chapter. Attenuation of Shh signalling by means of a function-blocking Ab markedly delays tooth germ development and demonstrates that Shh is required for ameloblast and odontoblast maturation.[68] Shh is also vital for tongue formation; if signalling is disrupted early in rat embryogenesis (E12) then no tongue forms.[69] It is also important in renewing and maintaining tastebuds.[70] Liu et al[69] propose that high concentrations of Shh result in formation and maintenance of papillae, while low concentrations activate between-papillae genes that maintain a papilla-free epithelium. Shh signalling is essential for forming the olfactory