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PRIMER

From *Drosophila* segmentation to human cancer therapy

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ABSTRACT

First described in *Drosophila*, Hedgehog signalling is a key regulator of embryonic development and tissue homeostasis and its dysfunction underlies a variety of human congenital anomalies and diseases. Although now recognised as a major target for cancer therapy as well as a mediator of directed stem cell differentiation, the unveiling of the function and mechanisms of Hedgehog signalling was driven largely by an interest in basic developmental biology rather than clinical need. Here, I describe how curiosity about embryonic patterning led to the identification of the family of Hedgehog signalling proteins and the pathway that transduces their activity, and ultimately to the development of drugs that block this pathway.

Introduction

The early part of the twentieth century saw the first concerted efforts by embryologists and biochemists to uncover the molecular basis of animal development, with a focus on the factors underlying the ability of a certain region of the developing newt embryo to induce a second body axis, a phenomenon first described by Hans Spemann and Hilde Mangold in 1924 (Spemann and Mangold, 1924). But it was the convergence of classical genetics and the emerging field of molecular biology in the latter part of the century that prompted a sea-change in developmental biology, paving the way for today's detailed understanding of the signalling pathways and gene regulatory networks orchestrating embryonic development. This knowledge has transformed our comprehension of human development and disease and helped usher in a new age of molecular medicine. Nowadays, clinicians can quickly pinpoint the genetic lesions underlying a range of congenital conditions and make use of a host of molecular markers in the diagnosis and stratification of diseases. They can deploy next-generation drugs to target the signalling pathways that drive processes such as oncogenesis, and manipulate these same pathways to generate specific cell types for use in regenerative therapies.

Remarkably, much of the knowledge upon which these advances are based originates not from clinical research but from analysis of the development of invertebrates, especially the fruit fly *Drosophila*. Gene names such as *Hippo*, *Hox*, *Klf*, *Notch*, *Pax*, *Runx*, *Wnt*, *Polycomb* and *Trithorax* abound in the medical literature, yet they have their origins in the phenotypes of mutant fruit flies, not in the manifestations of human diseases. Here, I exemplify this principle with a personal account of the characterisation of the *hedgehog* (*hh*) genes. The secreted signalling proteins encoded by these genes play key roles in the patterning of the vertebrate limb and central nervous system, as well as in a host of other processes both in the embryo and in post-embryonic life, and the aberrant activity of the Hedgehog signalling pathway underlies a number of human congenital abnormalities and cancers. Yet the analysis of the *hedgehog* gene and the pathway that transduces its activity began with studies of a seemingly unrelated and esoteric process, the segmentation of the body of the fruit fly larva.

Until the 1970s, studies of insect larval segmentation had been the preserve of a small group of experimental embryologists who used various types of manipulation (ligature, irradiation and transplantation) to decipher the principles underlying the patterning of the insect body plan and attempt to discover its molecular basis. Up to this point, there had been little focus on the genetic control of larval segmentation, with the exception of analyses of two *Drosophila* mutants, *Krüppel* (Gloor, 1950) and *fused* (Counce, 1956; Fausto-Sterling, 1971). All of this changed, however, with the publication in 1980 of the seminal paper by Nüsslein-Volhard and Wieschaus, describing the first systematic, genome-wide screen for mutations disrupting larval segmentation (Nüsslein-Volhard and Wieschaus, 1980) (see timeline in Fig. 1). There is a tendency to overuse the term 'paradigm shift' in the history of science, but here was a study more than worthy of the epithet: in one fell swoop, the authors identified most of the genes that control the subdivision and patterning of an animal along its major body axis, and in so doing they laid the foundations for the identification and characterisation of many of the key transcription factors and signalling pathways controlling vertebrate, as well as invertebrate, development. Strikingly, Nüsslein-Volhard and Wieschaus defined three distinct classes of genes, based on their mutant phenotypes: the gap genes, which when mutated cause the loss of a contiguous set of body segments; the pair-rule genes, inactivation of which results in the loss of alternating segmental units; and the segment polarity genes, mutations of which disrupt the patterning of each individual segment (Fig. 2B-D). These three classes would not have been predicted *a priori*, but their identification led at once to the notion of a hierarchy of gene regulation by which the developing embryo is progressively subdivided into successively smaller

Advocating developmental biology

This article is part of Development's advocacy collection – a series of review articles which make compelling arguments for the field's importance. The series is split into two: one set of articles, including this one, addresses the question 'What has developmental biology ever done for us?' We want to illustrate how discoveries in developmental biology have had a wider scientific and societal impact, and thus both celebrate our field's history and argue for its continuing place as a core biological discipline. In a complementary set of articles, we asked authors to explore 'What are the big open questions in the field?' Together, the articles will provide a collection of case studies looking backwards to the field's achievements and forwards to its potential, a resource for students, educators, advocates and researchers alike. To see the full collection as it grows, go to http://dev.biologists.org/content/advocating-developmental-biology.

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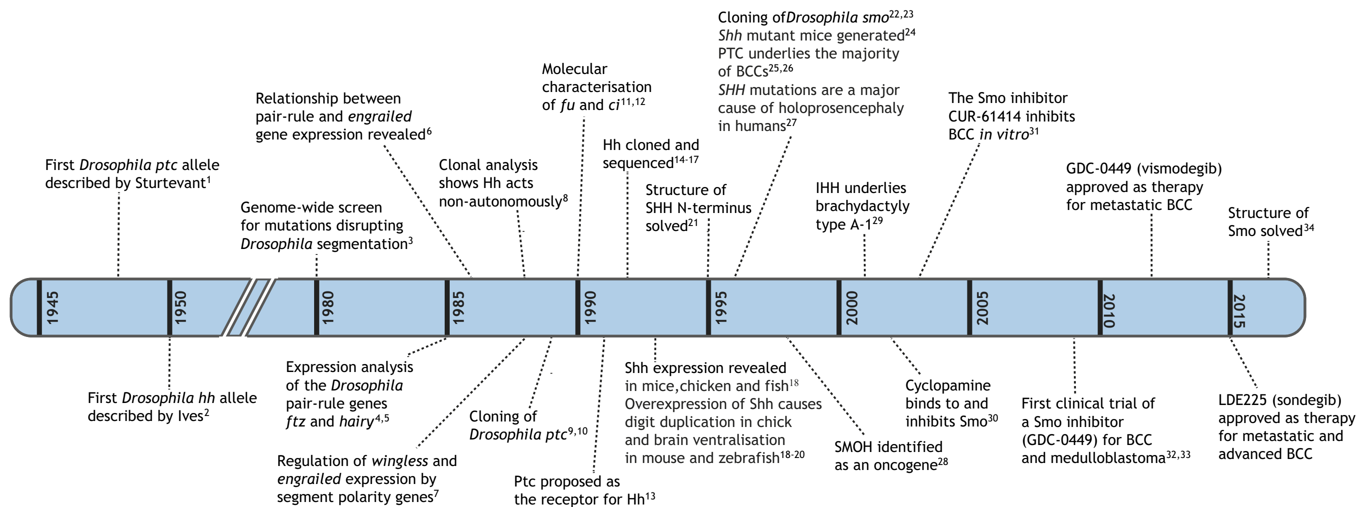

Fig. 1. Timeline showing some of the key discoveries in hedgehog signalling. Numbered references: (1) Sturtevant, 1948; (2) Ives, 1950; (3) Nüsslein-Volhard and Wieschaus, 1980; (4) Hafen et al., 1984; (5) Ingham et al., 1985b; (6) Howard and Ingham, 1986; (7) Martinez Arias et al., 1988; (8) Mohler, 1988; (9) Nakano et al., 1989; (10) Hooper and Scott, 1989; (11) Orenic et al., 1990; (12) Préat et al., 1990; (13) Ingham et al., 1991; (14) Lee et al., 1992; (15) Mohler and Vani, 1992; (16) Tabata et al., 1992; (17) Tashiro et al., 1993; (18) Krauss et al., 1993; (19) Riddle et al., 1993; (20) Echelard et al., 1993; (21) Hall et al., 1995; (22) Alcedo et al., 1996; (23) van den Heuvel and Ingham, 1996; (24) Chiang et al., 1996; (25) Johnson et al., 1996; (26) Hahn et al., 1996; (27) Roessler et al., 1996; (28) Xie et al., 1998; (29) Gao et al., 2001; (30) Chen et al., 2002; (31) Williams et al., 2003; (32) Von Hoff et al., 2009; (33) Rudin et al., 2009; (34) Byrne et al., 2016.

units. The big question posed by this model was: 'how is this subdivision achieved at the molecular level?' Answering this question would depend upon the development and application of new molecular biological techniques, and would lead in unexpected directions.

Cloning, transformation and *in situ* hybridisation: technical innovations drive discovery

In the early 1980s, molecular cloning of *Drosophila* genes was still in its infancy. The wealth of chromosomal rearrangements associated with the Antennapedia and Bithorax complexes made it possible to 'jump' into them from distantly located cloned sequences, before 'walking' along the chromosomal region to isolate sequences corresponding to the constituent individual genes (Garber et al., 1983; Bender et al., 1983). But this painstaking approach required great skill and resources and was beyond the capabilities of most fly labs. This all changed with the characterisation and cloning of the P-transposable element (Spradling and Rubin, 1982) bringing molecular cloning within reach of most *Drosophila* researchers. The ability to induce the transposition of P-elements allowed the recovery of 'tagged' alleles of specific genes simply by screening for the appropriate phenotype in the F1 progeny of flies in which the element had been mobilised, a so-called F1 screen. In this way, a large number of new P-element-induced alleles of the pair-rule gene *barrel* were generated, taking advantage of its allelism to the classical adult viable mutation, *hairy* (Ingham et al., 1985a,b). The genomic DNA corresponding to the *barrel*/*hairy* locus was isolated simply by screening for clones containing P-element sequences in a library of DNA fragments generated from the mutant flies.

The cloning of *hairy* was a significant achievement because it provided the means to analyse the expression of one of the enigmatic pair-rule genes. Up until this point, gene expression had traditionally been analysed using northern blotting, a method that provides good temporal, but poor spatial, resolution. It seemed likely that the pair-rule genes would be expressed during the early stages of embryogenesis, before segments become visible; but the really interesting question was where are they expressed, something northern blot analysis could not easily reveal. To address this question, Ken Howard and I optimised the technique of *in situ* hybridisation: this involved hybridisation of radioactively labelled nucleic acid probes to sections of fixed embryos and visualising the sites of hybridisation – and hence of transcript localisation – by autoradiography. We overcame two limitations of the existing protocols, first by using single-stranded RNA probes, which form more stable hybrids with their target mRNAs than DNA probes, and second by using sections of wax-embedded embryos, which are simpler to prepare than frozen sections and offer vastly superior tissue preservation.

Seeing stripes

Whilst we were perfecting our technique, Ernst Hafen and colleagues in the laboratory of Walter Gehring published the first description of the expression of another pair-rule gene, *fushi tarazu* (*ftz*): the extraordinary pattern of seven bands of transcript accumulation encircling the embryo along its antero-posterior axis neatly reflected the pair-rule mutant phenotype (Hafen et al., 1984). Within weeks we observed a similar pattern of *hairy* expression; using the *ftz* sequence kindly provided by the Gehring lab, we were able to visualise simultaneously the expression of both genes, by hybridising adjacent sections of the same embryo with one or other probe (Ingham et al., 1985b). This revealed the two genes to be expressed in almost complementary patterns, reflecting their respective 'even-skipped' (*hairy*) and 'odd-skipped' (*ftz*) mutant phenotypes (Fig. 2E,F).

There followed a frenzy of activity as we analysed the expression of both *hairy* and *ftz* in embryos mutant for the various gap and pair-rule genes. The results of these experiments provided molecular evidence of the segmentation hierarchy that Nüsslein-Volhard and Wieschaus had proposed. A key finding was the relationship between pair-rule gene expression and function and the spatial regulation of the *engrailed* (*en*) gene (Howard and Ingham, 1986). Although originally classified as a pair-rule gene on the basis of its mutant phenotype (Nüsslein-Volhard and Wieschaus, 1980), the expression of *en* resembled much more that expected of segment

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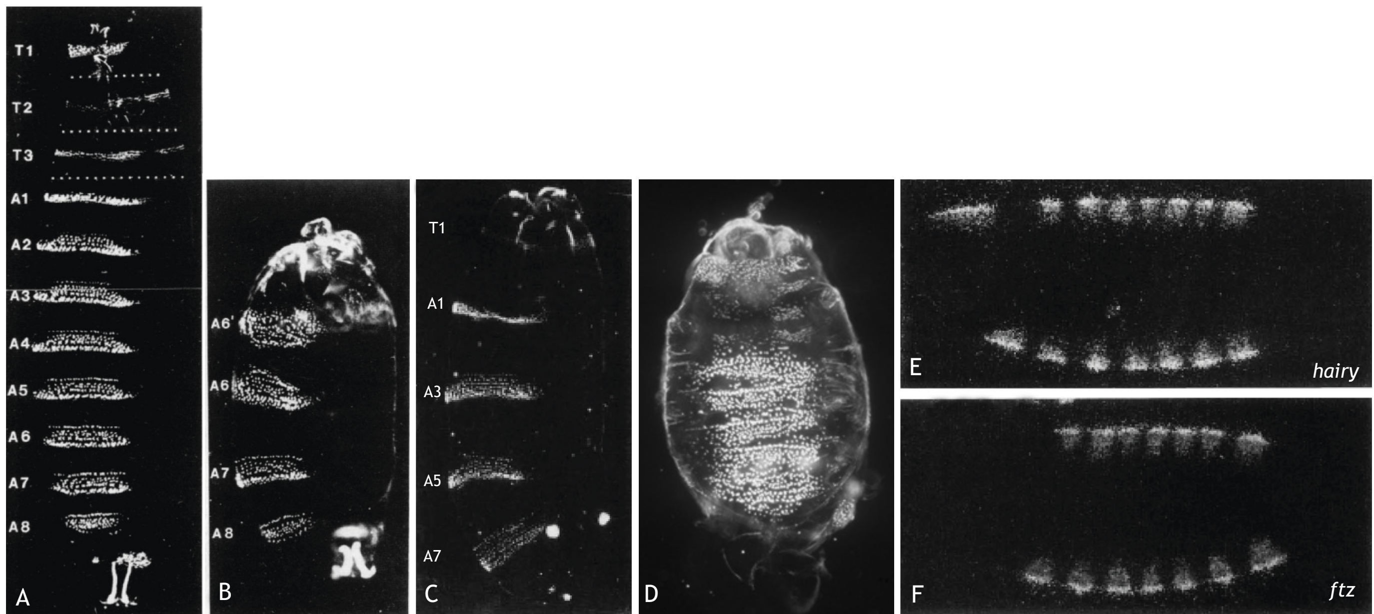

Fig. 2. Mutant phenotypes and expression patterns of *Drosophila* segmentation genes. (A-D) Dark-field photomicrographs of larval cuticles. The wild-type (A) ventral cuticle is decorated with 11 belts of denticles that mark the three thoracic (T1-3) and eight abdominal (A1-8) segments. In gap mutant embryos, contiguous segments are deleted, as exemplified by the *Krüppel* mutant (B), which lacks segments T1-A5, replaced by a mirror image duplicated A6 segment (reproduced from Ingham et al., 1986). In pair-rule mutants, alternate segments are deleted as exemplified by the *hairy* mutant (C), which lacks abdominal segments A2, A4, A6 and A8 (the so called 'even-skipped' phenotype) as well as T1 and T3 (reproduced from Howard et al., 1988). In segment polarity mutants, all segmental units are present, but parts of each segment are deleted, as exemplified by the *hedgehog* mutant (D), which lacks the posterior 'naked' cuticle in each segment, giving rise to a continuous lawn of denticles in the abdominal region. (E,F) Dark-field photomicrographs of autoradiographs of longitudinal sections of wild-type blastoderm embryo revealing the localisation of *hairy* (E) and *fushi tarazu* (F) transcripts (white grains) (reproduced from Howard et al., 1988). Anterior to the left, dorsal top.

polarity genes, as subsequently epitomised by the pattern of expression of *wingless* (*wg*), a canonical member of this class (Baker, 1987). Together with Peter Lawrence and Alfonso Martinez-Arias, we showed that each narrow stripe of cells that expresses *en* (Kornberg et al., 1985) corresponds to the anterior boundary of each morphologically distinguishable metameric unit, termed a parasegment (Ingham et al., 1985c). These domains of *en* are first established and coincide with the anterior boundaries of expression of the *ftz* and *even skipped* (*eve*) genes around 3 h after fertilisation, at the cellular blastoderm stage of embryogenesis (Ingham et al., 1988). Moreover, their activation depends upon the activity of these and other pair-rule genes; conversely, we showed that the narrow segmental stripes of *wg* expression are established at the posterior of each *ftz* and *eve* stripe at the blastoderm stage, reflecting the repression of *wg* transcription by the products of these two pair-rule genes (Ingham et al., 1988). Visualising gene expression thus allowed us to reveal the crucial phase of pair-rule gene activity, before their mutant phenotypes became morphologically apparent.

Maintaining boundaries

Unlike the pair-rule genes, the accumulation of transcripts and protein products of which is extremely transient and peaks around the cellular blastoderm stage, the expression of *en* and *wg* persists at parasegment boundaries for much of the subsequent stages of embryonic development. To address how these patterns of expression are maintained, we used *in situ* hybridisation to analyse the expression of *wg* and *en* expression in embryos mutant for either gene (Martinez Arias et al., 1988). The results revealed a mutual interdependence of *wg* and *en* transcription, suggesting that signals are exchanged between the two cell populations. The contemporaneous discovery that *wg* is the *Drosophila* orthologue of the mouse proto-oncogene *Int-1* (Rijsewijk et al., 1987), which was accordingly renamed *Wnt1*, provided a potential candidate for one of these signals. Biochemical analysis had revealed that the murine *Int-1* gene product enters the secretory pathway in tissue culture cells (Papkoff et al., 1987), so a plausible hypothesis was that Wg protein is secreted and signals across the parasegment boundary to maintain *en* expression. The mechanistic basis for the maintenance of *wg* transcription by *en* function in neighbouring cells, on the other hand, was less clear: *en* encodes a homeodomain protein and all the evidence pointed to it acting as a classic transcription factor (Desplan et al., 1985). This suggested that *en* might regulate the expression of a gene encoding a secreted 'factor X' that would signal in the opposite direction to the Wg protein to maintain *wg* transcription, but the identity of the gene encoding 'factor X' was not obvious. In any event, we were at the time more intrigued by the effects of another segment polarity mutation, *patched* (*ptc*).

Serendipity, sequence and surprise

We had singled out the *ptc* mutant for analysis based on its atypical phenotype: whereas most segment polarity mutant embryos are, like *wg*, associated with a loss of naked cuticle, giving rise to a 'lawn' of denticles – small darkly pigmented spikey processes – on their ventral surface (Fig. 2), embryos mutant for *ptc* are characterised by a loss of one or two denticle rows from each segment, and an expansion of the smooth cuticle characteristic of the posterior portion of each segment. Intriguingly, we found that in *ptc* mutants, the domain of *wg* expression is expanded in each segment with an additional domain of *en* expression appearing anterior to the expanded *wg* domain (Martinez Arias et al., 1988) (Fig. 3C,D).

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It was on the basis of these effects that we decided to clone *ptc*, and were greatly helped by the discovery that *ptc* is allelic to a classical mutation called *tufted*, which causes outgrowths in the wing. The implications of this finding were profound both biologically and logistically: biologically, because it established a role for *ptc* in the patterning of the imaginal disc that formed the wing, as well as in the embryo; and logistically, because it provided a facile way of isolating transposon-tagged alleles of *ptc*, namely through an F1 screen of P-element-mutagenised chromosomes *in trans* to the viable *tuf* allele. Using these new alleles, Isabel Guerrero, Alicia Hidalgo and I proceeded to clone the locus (Nakano et al., 1989). Sequencing of the *ptc* cDNA by Yoshiro Nakano led to the unexpected prediction that *ptc* encodes a multi-pass transmembrane protein, resembling a transporter or ion channel (Nakano et al., 1989), a conclusion supported by Joan Hooper and Matthew Scott, who had independently cloned and characterised *ptc* using a different strategy (Hooper and Scott, 1989).

Hedgehog – the elusive signal

Whilst we puzzled over the significance of the Ptc structure, we set about studying the spatial and temporal regulation of *ptc* expression, using the now greatly simplified method of non-radioactive *in situ* hybridisation (Hidalgo and Ingham, 1990). We found that *ptc* has a complex and dynamic pattern of expression that depends upon the function of both *ptc* itself as well as another member of the segment polarity class, namely *hedgehog* (*hh*). In the absence of Ptc function, the pattern fails to resolve from an initially broad distribution into two narrow stripes, whereas in the absence of Hh function, the broad domain of expression dissipates and the two narrow stripes are never established (Fig. 4D). At the same time, we showed that *wg* transcription, which coincides with one of the two stripes of *ptc*, is not maintained in *hh* mutants (Fig. 4B).

Although a mutant *hh* allele had first been described in 1950 (Ingham, 2016), little was known about its function. In a paper published in late 1988, however, Jym Mohler described a genetic mosaic analysis of the requirement for Hh activity in developing imaginal discs (Mohler, 1988); this revealed what Mohler referred to as a 'domineering' non-autonomous effect of loss of Hh function from the posterior compartment of the wing, an effect consistent with Hh functioning as a secreted signal, an inference subsequently confirmed when the *hh* gene was cloned and sequenced by Mohler and three other groups (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Tashiro et al., 1993). Based on this finding and our own observations of the dependence of both *ptc* and *wg* transcription on Hh function, we postulated that *hh* encodes the mystery 'factor X', secreted by *en*-expressing cells to maintain expression of *wg* and, as we showed, *ptc*, in neighbouring cells (Hidalgo and Ingham, 1990). Notably, we also found that *ptc* and *wg* transcription become independent of Hh function in the absence of Ptc. Accordingly, we proposed that Ptc acts to repress the transcription of both *wg* and *ptc* and that their expression is promoted by Hh binding to and thereby repressing Ptc (Ingham et al., 1991) (Fig. 4E). This model of Ptc as the receptor for Hh was *avant garde* for two reasons: first, the structure of Ptc was unlike that of any known receptor for a secreted signalling molecule; and second, it ran counter to the prevailing view of receptors being activated by their ligands. Nevertheless, it provided a parsimonious explanation both for the pattern of expression of *ptc* and for the effects of loss of Ptc and Hh functions, exemplifying the power of genetic analysis to generate hypotheses unconstrained by conventional wisdom based on biochemical knowledge.

From Patched to the nucleus – the Hh signal transduction pathway revealed

With this model in mind, we set out to identify the downstream components of the putative Hh signalling pathway. Our analysis of *ptc* and *wg* expression had already identified another segment polarity gene *cubitus interruptus* (*ci*) as a positive regulator of both these putative Hh targets (Hidalgo and Ingham, 1990) and analysis of embryos mutant for the segment polarity gene *fused* (*fu*) suggested that it plays a similar role (Forbes et al., 1993; Ingham, 1993). The molecular characterisation of these genes had shown them to encode, respectively, a protein with zinc fingers characteristic of a transcription factor (Orenic et al., 1990), and a serine-threonine kinase (Préat et al., 1990), both typical components of signal transduction pathways. A study by Pat Simpson and Yves Grau had previously revealed that removal of both maternal and zygotic (MZ) function of the *costal-2* (*cos-2*; also known as *costa*,

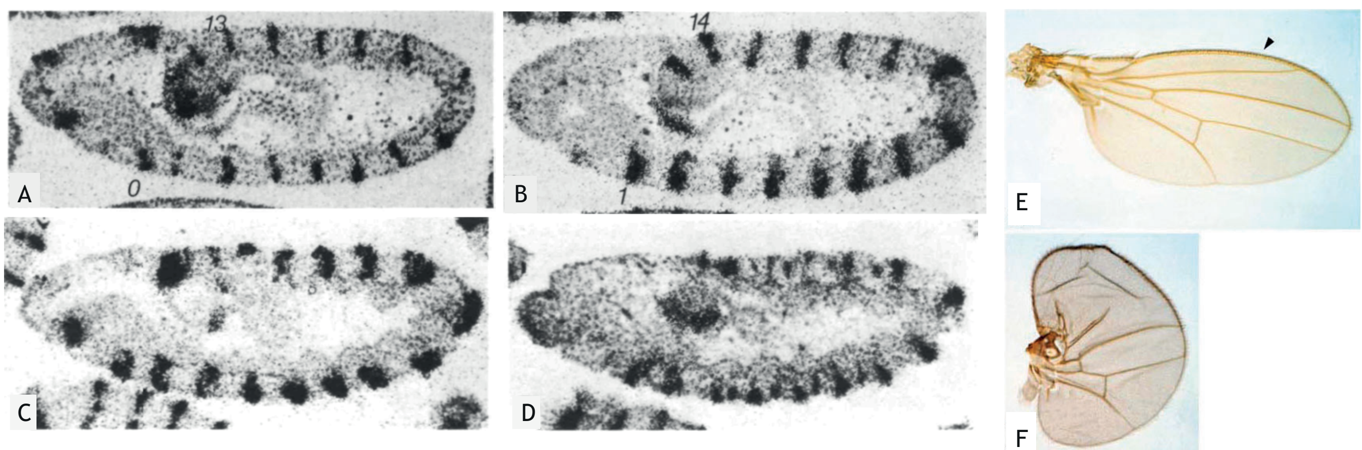

Fig. 3. Effects on gene expression and adult phenotypes of *patched* mutations. (A,B) The distribution of *wg* (A) and *en* (B) transcripts in adjacent longitudinal sections of a stage 11 wild-type embryo revealed by *in situ* hybridisation with ^{35}S-labelled radioactive probes (reproduced from Martinez Arias et al., 1988). (C,D) Sections of a similar stage *ptc* null mutant embryo, hybridised with the same probes: the domain of *wg* expression in each segment is significantly expanded (C) and additional domains of *en* expression are present in the middle of each segment (D) (reproduced from Martinez Arias et al., 1988). (E,F) Wings from wild-type (E) and ptc^{tuf}/ptc^{S2} mutant (F) adult flies showing the duplication of the anterior compartment region and disruption of the anterior venation pattern caused by *ptc* dysfunction.

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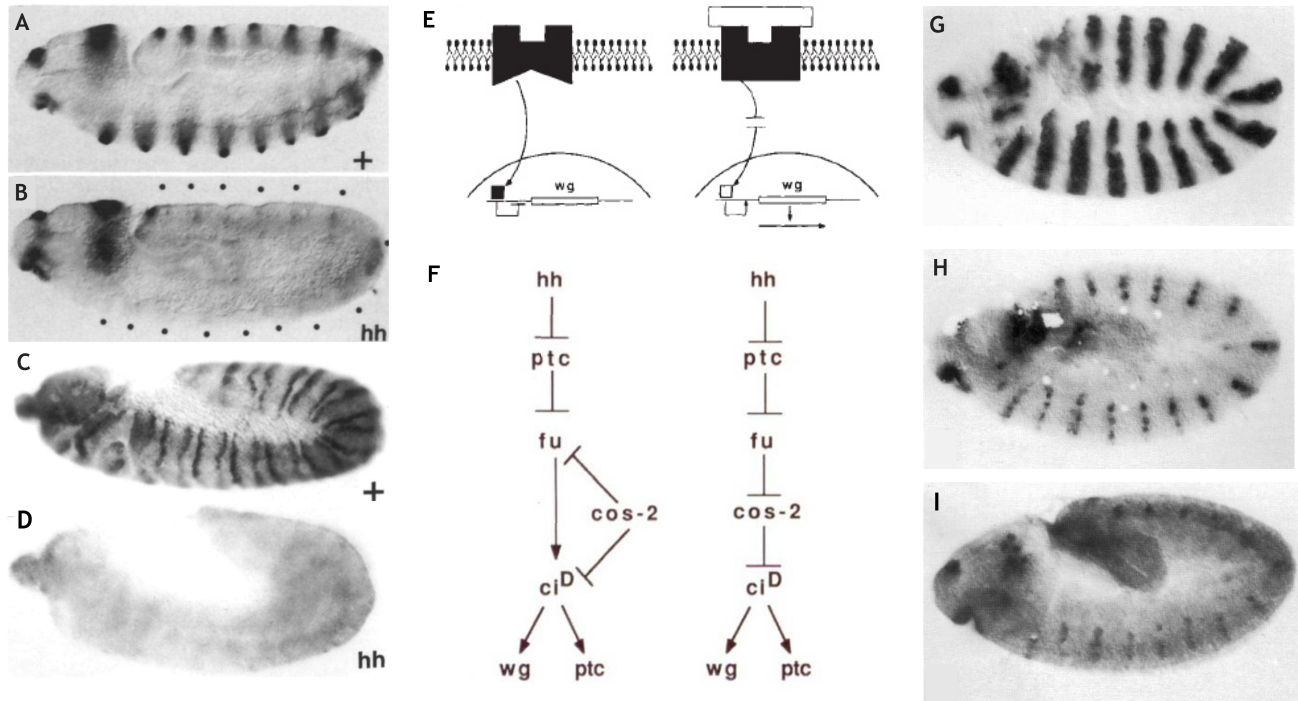

Fig. 4. The role of Hh in signalling at the parasegment boundary and the Hh signal transduction pathway revealed by genetic epistasis analysis. (A-D) The patterns of *wg* (A,B) and *ptc* (C,D) transcript accumulation are revealed by non-radioactive whole-mount *in situ* hybridisation of wild-type (A,C) and *hh* null mutant (B,D) stage 11 embryos. The stripes of *wg* expression at each parasegmental boundary (marked by dots in B) are completely absent, as are the stripes of *ptc* transcript that normally flank the *en* expression domains. Data reproduced from Hidalgo and Ingham (1990). (E) Model of the interaction between Hh (T-shape) and Ptc (black shape in plasma membrane) proposed by Ingham et al. (1991). According to this model, Ptc actively represses *wg* transcription (by an unknown mechanism). Activation of *wg* transcription is achieved by relieving this repression through Hh binding to and inactivating Ptc. (F-I) Subsequent epistasis analysis of *ptc* and *fused* (*fu*) and *cubitus interruptus* (ci^D) mutants by Forbes et al. (1993) led to the elaboration of the putative signal transduction pathway shown in F. *fu;ptc* (H) and $ptc;ci^D$ (I) double mutants both show a loss of the typical broad domains of *wg* transcript accumulation seen in *ptc* single mutants (G). It follows from this that Fu and Ci proteins both act downstream of Ptc (F). The order in which they appear in the pathway reflects the evidence that Ci acts as a transcription factor. The analysis could not discriminate between Cos-2 acting in series or in parallel, hence the two alternative pathways shown in F (reproduced from Forbes et al., 1993).

cos) gene, originally identified by Robert Whittle through its adult phenotype (Whittle, 1976), results in an embryonic phenotype similar to that of *ptc* loss of function (Grau and Simpson, 1987). Prompted by this finding, we also analysed the expression of *wg* and *ptc* in MZ *cos-2* mutant embryos and found a corresponding effect on their expression. Based on these data and on the epistasis relationships between *ptc*, *fu* and *ci*, we proposed the first putative versions of the Hh signal transduction pathway, whereby the products of the latter two genes act downstream of Ptc to promote target gene expression, whilst Cos-2 was proposed to antagonise Fu and Ci function (Fig. 4F; Forbes et al., 1993).

One key player missing from this scheme was the product of the *smoothened* (*smo*) gene. Also isolated in the Nüsslein-Volhard and Wieschaus screen, *smo* homozygous mutants display a weak and variable *hh*-like phenotype (Nüsslein-Volhard et al., 1984) that had led to them being overlooked in our earlier studies. By making germline clones, we subsequently found that MZ *smo* mutants have a phenotype indistinguishable from that of *hh* nulls (van den Heuvel and Ingham, 1996), and, whereas *ptc* mutations are epistatic to *hh* loss of function, *smo* mutations are epistatic to loss of *ptc* (Hooper, 1994; Quirk et al., 1997), placing *smo* downstream of the Hh receptor in the pathway. Paradoxically, however, cloning of *smo* revealed that it encodes a member of the G protein-coupled receptor superfamily (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). As such, Smo is the most proximal pathway component to Ptc, and acts as the obligate transducer of all Hh activity.

From flies to vertebrates: collaborating to hedge(hog) our bets!

Our attention next turned to the evolutionary conservation of the pathway: although taken for granted now, the existence of Wnt signalling in vertebrates and the conservation of Engrailed across multiple phyla (Patel et al., 1989) was seen as remarkable at the time, and emboldened us to bet that Hh signalling might also operate in other species. This proposition was encouraged by two observations: first, the intriguing spatial relationship between the expression domains of engrailed 2 (*En2*), a murine orthologue of *en*, and *Wnt1* at the mid-hindbrain boundary; and, second, the capacity of *Wnt1*-expressing cells to induce ectopic *En2* expression when transplanted into the forebrain (Bally-Cuif et al., 1992). These findings encouraged the thought that regulatory interactions similar to those at the *Drosophila* parasegment boundary might also be at play in the vertebrate brain and could potentially be meditated by Hh orthologues. With this in mind, Andrew McMahon, Cliff Tabin and I decided to maximise our chances of success by joining forces in a simultaneous effort to isolate vertebrate *hh* orthologues from our favourite vertebrate model systems, namely mouse, chick and fish, respectively. This international collaboration proved to be the secret

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of our success: the synergistic interaction between our groups accelerated progress towards our shared goal.

The McMahon lab was the first to isolate a cDNA clone with significant sequence identity to *Drosophila hh* that corresponded to what would subsequently be designated the mouse desert hedgehog (*Dhh*) gene. Analysis of its expression by *in situ* hybridisation led to initial disappointment, as little, if any, transcript could be detected in the mouse embryo, except in the testes (Bitgood and McMahon, 1995), not an organ in which we had anticipated an *Hh* homologue to be expressed. Undeterred, Stefan Krauss, a post-doctoral fellow in my laboratory, used this *Dhh* clone to screen a zebrafish cDNA library. This yielded a single clone that showed significant sequence identity to both *Dhh* and *Drosophila hh*, but did not appear to be a *Dhh* orthologue. At the same time, Cliff Tabin's lab had identified three distinct *Hh*-related sequences from chicken by degenerate PCR, reinforcing the notion that our zebrafish clone was probably not a *Dhh* orthologue. This was soon confirmed when Stefan analysed its spatiotemporal expression pattern by *in situ* hybridisation: the results, although not at all what we had predicted, were nonetheless spectacular. The gene subsequently designated sonic hedgehog (*Shh*) was found to be expressed throughout the notochord and floorplate of the neural tube (Krauss et al., 1993) (Fig. 5C), two tissues known to have embryonic organising activities (Placzek et al., 1993). The McMahon and Tabin labs quickly isolated cDNAs of the orthologous gene from mouse and chicken, respectively, and confirmed that this pattern of expression is conserved across the vertebrates (Echelard et al., 1993; Riddle et al., 1993). In addition, the Tabin lab noted strong expression of *Shh* in the posterior of the developing limb buds, in a region that corresponds precisely to that of another well-characterised embryonic organiser, the zone of polarising activity (ZPA). Within a matter of weeks, overexpression studies had shown that *Shh* can mimic the effects of the ZPA, causing digit duplications in the developing chick limb (Riddle et al., 1993), as well as of the notochord and floorplate, causing the ventralisation of the dorsal brain (Echelard et al., 1993; Krauss et al., 1993). Almost overnight, Hh signalling was thus elevated from an esoteric process in the *Drosophila* embryo to one of the major regulators of animal embryogenesis, and there followed an explosion of interest in this fascinating signalling pathway.

The human dimension

Between 1987 and the cloning of the vertebrate Hedgehogs in 1993, there were just 18 publications referring to the *Drosophila hh* gene. In the following 7 years, over 540 papers featuring *Shh* were published. This 30-fold increase in output reflects the pervasive reach of Hh signalling in the development of all vertebrate species, including humans. From the patterning of the limb bud to the branching of the lung buds (Pepicelli et al., 1998), Hh proteins play a multitude of essential roles in building the embryo. In several contexts, Hh proteins pattern structures in a dosage-dependent manner, a defining property of the elusive morphogen, long sought after by experimental embryologists (Lawrence, 2001). The classic example of this is in the ventral neural tube, where a gradient of Shh activity emanating from the axial midline structure, the notochord, specifies a series of progenitor domains from which distinct neuronal subtypes differentiate (Dessaud et al., 2008). This role in patterning the ventral CNS extends into the brain, as graphically illustrated by the generation of *Shh* mutant mice, the most striking phenotypic manifestation of which is cyclopia (Chiang et al., 1996). Cyclopia is the most severe form of holoprosencaphaly, a relatively common human birth anomaly; in line with this, mutations in the

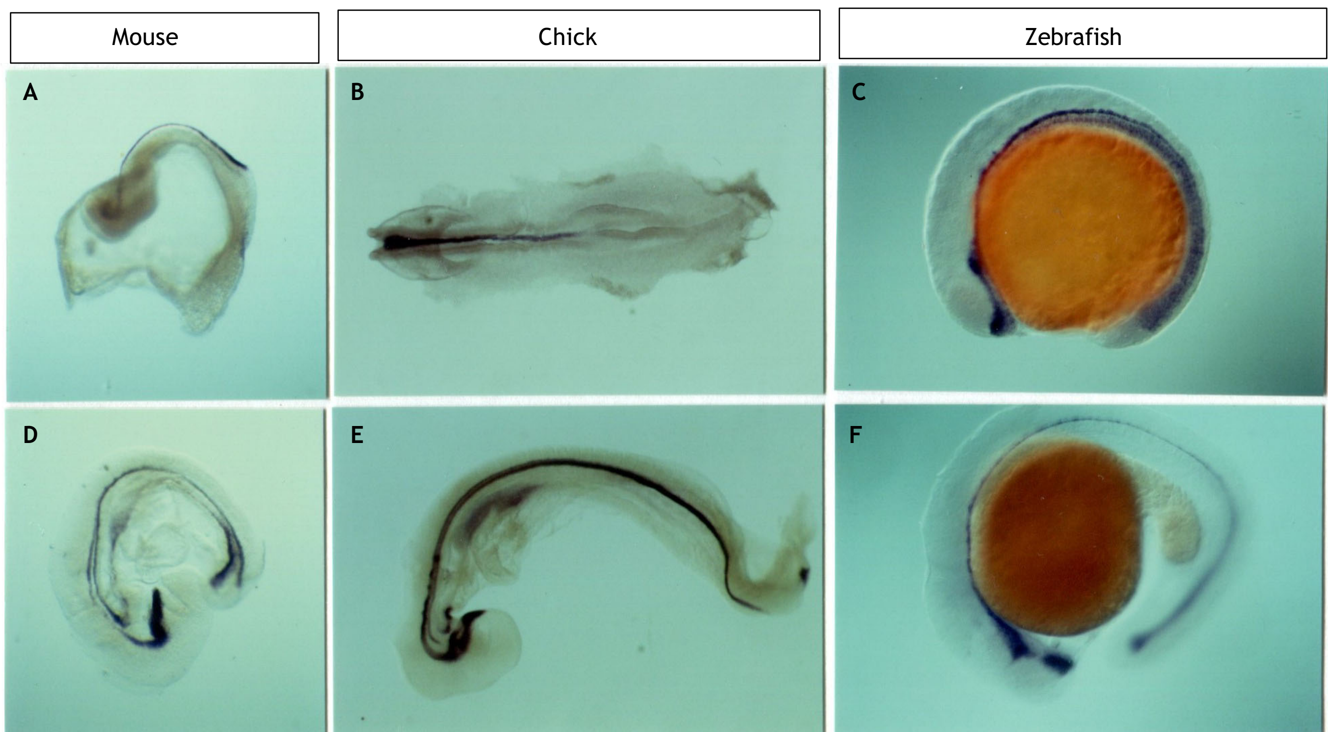

Fig. 5. Conserved patterns of Shh expression from mammals to teleosts. (A-C) At early somitogenesis (~8-10 somites) stages, *Shh* transcript accumulates throughout the axial mesoderm (presumptive notochord) in mouse, chicken and zebrafish embryos and is already detectable in the forming floorplate of the zebrafish embryo. (D-F) By later somitogenesis stages, transcript is detectable throughout the ventral floor of the brain and neural tube but is beginning to dissipate from the notochord. Images reproduced from figure 3 of Fietz et al. (1994).

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human *SHH* gene have been found to be the most prevalent genetic defect underlying this condition (Dubourg et al., 2007). Subsequent studies have uncovered connections between Hh signalling and a variety of other human pathologies: mutations in Indian hedgehog (*IHH*), for instance, underlie brachydactyly type A-1, a shortening or loss of the middle phalanges (Gao et al., 2001), reflecting the role of IHH in controlling chondrocyte proliferation and differentiation (Gao et al., 2009)

The capacity of Shh to programme specific cell types was anticipated in a number of patents filed following its cloning (Ingham et al., 1993). A biotech start-up company, Ontogeny, licensed this intellectual property, initially with the aim of identifying small molecule pathway agonists that could be used to induce the directed differentiation of stem cells into specific cell types. But in 1996 one of two human orthologues of *Drosophila ptc* was identified as a tumour suppressor gene, switching the focus of Hh-based therapeutics discovery away from regenerative medicine and towards oncology. Coming from different directions, Matthew Scott and colleagues (Johnson et al., 1996) and a human genetics consortium led by Brandon Wainwright and Allen Bale (Hahn et al., 1996), had converged on the discovery that loss-of-function mutations in human patched 1 (*PTCH1*) account for the majority of basal cell carcinomas (BCCs), a type of skin tumour that is the most prevalent form of cancer among Caucasians (Fig. 6A,B). Moreover, gain-of-function mutations in the human orthologue of *smo* (*SMO*; previously known as *SMOH*) were discovered in the majority of those BCCs not associated with loss-of-function *PTCH1* mutations (Xie et al., 1998) implicating *SMOH* as an oncogene. These findings now put Hh signalling in the frame as a potential anti-cancer drug target. In particular, SMOH seemed an attractive target given that it encodes a member of the G protein-coupled receptor superfamily, long favoured as drug targets by pharmaceutical companies. A proof of principle was provided by a key insight of Philip Beachy and colleagues into the effects of a naturally occurring alkaloid known as cyclopamine (CycA). This had been discovered many years earlier through its teratogenic effects on lambs born to sheep that grazed on the desert lily *Veratrum californicum* (Chen, 2016). Beachy and colleagues realised that the cyclopic phenotype of the *Shh* mutant mouse closely resembles that caused by exposure to CycA (Cooper et al., 1998); consistent with such a view, CycA was subsequently shown to bind to and specifically inhibit Smo (Chen et al., 2002) (see Fig. 6E). The effective concentration of CycA, however, precludes its therapeutic use, stimulating the quest for more potent Smo inhibitors, initially by Curis, a company formed by the merger of Ontogeny with two other biotechs. Key to this endeavour was the development of cell-based assays of Hh activity that employed a reporter gene that responds to Hh activity. The reporter gene incorporated binding sites for Gli proteins (Sasaki et al., 1997), the vertebrate orthologues of the *Drosophila* Ci proteins that we had shown function as transcriptional activators of *hh*-target genes such as *ptc*, through interaction with specific binding sites upstream of their promoters (Alexandre et al., 1996). Using this assay, Jeff Porter and his colleagues at Curis screened a library of 140,000 compounds, identifying both agonists and antagonists of the pathway (Frank-Kamenetsky et al., 2002), including CUR-61414, a Smo inhibitor that was subsequently shown to elicit complete regression of BCC-like lesions cultured *in vitro* (Williams et al., 2003).

These early findings encouraged further screens for more potent inhibitors: one of these, GDC-0449, developed in collaboration with Genentech, was evaluated in a Phase I clinical trial in patients with metastatic or locally advanced BCC, both of which are refractory to surgical or radiation therapy. Over 50% of this patient cohort was reported to respond favourably to oral dosing with GDC-0449 (Von Hoff et al., 2009). In addition, similar trials showed efficacy in the treatment of patients with medulloblastoma (see Fig. 6C,D), a paediatric brain tumour also associated with Hh pathway activity (Rudin et al., 2009). In 2012, following further Phase II and III clinical trials (Sekulic et al., 2012), GDC-0449, now

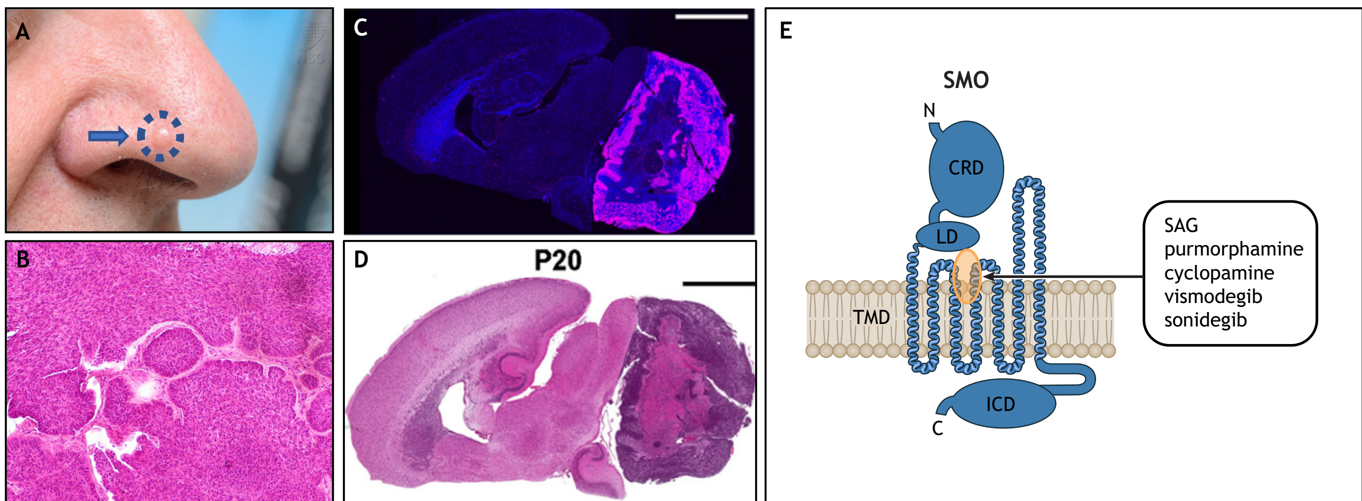

Fig. 6. Pathological effects of Hh pathway dysregulation and therapeutic targeting of Smo. (A) A basal cell carcinoma on the author's nose (arrow). These are invariably associated with ectopic activity of the HH signalling pathway in basal keratinocytes, due to loss of PTCH function or ectopic activation of SMOH. The cause of this particular tumour was not determined. (B) Histological section of the tumour shown in A following surgical removal, showing the massive over-proliferation of the darkly stained basal keratinocytes. (C,D) A medulloblastoma in the brain of a postnatal day 20 mouse induced by targeted cerebellar expression of the oncogenic *Smo*^{M2} mutant allele using the *hGFAP* promoter; the images show Haematoxylin & Eosin-stained (D) and PCNA-stained (C) sagittal sections and are reproduced from Lang et al. (2016). (E) Schematic of the Smo protein within the lipid bilayer, showing the cysteine rich (CRD) and linker (LD) domains of its large extracellular region, its seven alpha-helical transmembrane domains (TMD) and its intracellular domain (ICD). The orange oval indicates the approximate site occupied by small molecule agonists such as smoothened agonist (SAG) and purmorphamine as well as antagonists such as cyclopamine, vismodegib and sonidegib. Based on Byrne et al. (2018).

known as vismodegib, received US Food and Drug Administration (FDA) approval as a therapy for metastatic BCC. A second Smo inhibitor, LDE225, isolated by a team at Novartis (Pan et al., 2010) also showed promising results in a Phase I trial, exhibiting anti-tumour activity against both BCCs and medulloblastomas (Rodon et al., 2014). Following successful Phase II and III trials, LDE225, now known as sonidegib, received FDA approval for use in the treatment of metastatic and advanced BCC in 2015. The implication of aberrant Hh signalling in a number of other cancers, including prostate and small cell lung cancer, has since encouraged exploration of the therapeutic potential of Smo inhibitors in their treatment (Pietanza et al., 2016; Ross et al., 2017).

Conclusion

The road from the study of fruit-fly segmentation to the development of anti-cancer drugs has been a long, exciting and somewhat tortuous one. Starting from a fundamental question in biology – 'how many genes are required to pattern an embryo and how do they function?' – the journey took us from a heroic genetic screen, through the harnessing of transposable elements and the visualisation of gene transcripts in developing embryos to the discovery of the deep evolutionary conservation and diverse functions of Hh signalling, as well as its central role in a variety of human diseases. Few in 1980 could have imagined where the discovery of the segment polarity genes would ultimately lead (see also Wiese et al., 2018), but the power of curiosity-driven research to generate unpredictable insights is amply illustrated by the story I have recounted here. This is not the first example of the importance of basic developmental biology research for medical science and it will surely not be the last.

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Competing interests

P.W.I. has a minor shareholding in Curis Inc., the company that developed vismodegib in collaboration with Genentech.

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