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Chemical ‘Jekyll and Hyde’s: Small-molecule inhibitors of developmental signaling pathways†

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Abstract

Small molecules that perturb developmental signaling pathways can have devastating effects on embryonic patterning, as evidenced by the chemically induced onset of cyclopic lambs and children with severely shortened limbs during the 1950s. Recent studies, however, have revealed critical roles for these pathways in human disorders and diseases, spurring the re-examination of these compounds as new targeted therapies. In this tutorial review, we describe four case studies of teratogenic compounds, including inhibitors of the Hedgehog (Hh), Wnt, and bone morphogenetic protein (BMP) pathways. We discuss how these teratogens were discovered, their mechanisms of action, their utility as molecular probes, and their potential as therapeutic agents. We also consider current challenges in the field and possible directions for future research.

Small molecules that can induce congenital malformations have been a source of apprehension in our society since the emergence of “thalidomide babies” during the 1950s.¹ In fact, the term “teratogen” was coined from the Greek words “teratos” and “genos” (“monster birth”) amidst this medical tragedy, in which the sedative thalidomide was used to treat morning sickness in pregnant women. Thousands of these women in at least 46 countries subsequently gave birth to children with severely shortened arms and legs, as well as other debilitating birth defects. Our view of teratogens, however, has begun to change in light of recent advances in developmental biology. Mutagenesis screens, genetic studies, and genome sequencing projects have uncovered many of the molecular processes that underlie embryogenesis, revealing several cell signaling pathways that are conserved across animal species. These signal transduction mechanisms are actuated in a highly dynamic manner during embryogenesis—often within seconds to minutes—and small molecules that can rapidly and reversibly modulate these events are now considered valuable probes for studying development. Biomedical studies have also established an intimate link between the inappropriate reactivation of developmental signaling pathways in children and adults with several human diseases, and drugs that inhibit these processes are actively being pursued as next-generation targeted chemotherapies. Thus, such compounds can have antithetical qualities in differing contexts, acting like molecular embodiments of Dr. Jekyll and Mr. Hyde.

In this tutorial review, we examine several small-molecule inhibitors of developmental signaling pathways, including both natural products and synthetic compounds. The compounds described here are meant to be representative rather than comprehensive, illustrating the various methods for discovering teratogenic chemicals, the challenges associated with determining their mechanisms of action, their utility in basic research, and

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potential applications in the clinic. In particular, we consider small molecules recently found to block the Hedgehog (Hh), Wnt, or bone morphogenetic protein (BMP) signaling pathways, three major regulators of embryonic patterning and adult physiology, as well as thalidomide and its recently identified cellular target. We discuss how these advances have fostered a reappraisal of small-molecule teratogens in biomedical research, including even thalidomide itself, and we consider future challenges in the field. Through this tutorial, we hope to encourage chemists to explore new chemical modulators of developmental pathways, thereby continuing the renaissance of small molecules that can both misshape and mend the human body.

Cyclopamine, a natural product inhibitor of the Hh pathway

Ironically, as thalidomide was being prescribed to pregnant women 60 years ago, another potent teratogen was making history in the Boise, Challis, and Sawtooth National Forests of Idaho. Sheep grazing in these areas began to give birth to lambs with cyclopic eyes, limb deformities, and other congenital abnormalities (Figure 1A), and the United States Department of Agriculture quickly dispatched scientists to investigate the local soil, water, and fauna for possible causes of this alarming outbreak.² The culprit was soon found to be a flowering plant called *Veratrum californicum*, which in its mature form resembles a cornstalk with a base of broad green leaves.³ Through extensive natural product chemistry work, Binns and his co-workers determined that a steroid alkaloid produced by *V. californicum* was the teratogenic principle, a compound they aptly named “cyclopamine” (Figure 1B)^{4, 5} Natural derivatives of cyclopamine such as jervine (11-oxo-cyclopamine) and cycloposine (3-glycosyl-cyclopamine) were also found to cause cyclopia and other birth defects when administered to pregnant ewes.^{6, 7}

How cyclopamine and its structural variants induced these profound birth defects remained a mystery for another 50 years, awaiting the genetic revolution that would transform developmental biology in the 1980s and 1990s. The first embryonic patterning genes were discovered through fruit fly mutagenesis screens conducted by Nüsslein-Volhard and Wieschaus, which identified genetic lesions associated with defects in body segment number, organization, and polarity.⁸ Many of these mutations were then mapped to specific genes, typically through linkage analysis (localization of the mutant allele relative to known genetic markers based on the distant-dependent probability that two genetic polymorphisms are co-inherited), positional cloning (the progressive isolation and characterization of partially overlapping sections of genomic DNA to identify new polymorphisms for further linkage analyses), and sequence comparisons of wildtype and mutant genomic DNA once the mutant allele was localized to genomic region less than 30 kilobases in size.⁹ Subsets of the segment polarity genes were found to constitute discrete intercellular communication mechanisms, one of which was named the “Hedgehog” pathway due to the physical resemblance of the corresponding mutant fruitfly larvae to this spiny mammal.⁸

The subsequent cloning of mammalian orthologs for each Hh pathway component and generation of mice lacking these genes provided the first clue about the cellular target of cyclopamine. In mammals, this developmental pathway is initiated by a family of secreted, Hh ligands [Sonic (Shh), Indian (Ihh), and Desert (Dhh)] (Figure 1C),¹⁰ and loss of Shh function in mouse embryos was found to cause cyclopia, limbs lacking distal structures, and other developmental abnormalities.¹¹ The phenotypic similarities between the *Shh* knockout mice and lambs exposed to cyclopamine suggested that the teratogen might act by inhibiting the Hh signaling pathway. Moreover, Hh ligands are cholesterol- and palmitoyl-modified during their biogenesis,¹⁰ presumably to regulate their movement through tissues, and it was hypothesized that cyclopamine might interfere with cholesterol ester formation. Using neural tube explants, the Beachy laboratory was able to demonstrate the ability of

cyclopamine to perturb Shh-dependent neuronal specification; however, use of recombinant Shh in these experiments ruled out the possibility that Hh ligand production was the targeted process.¹² Accordingly, Shh cholesterylization was found to be unaffected in cyclopamine-treated Shh-producing cells.¹²

These observations indicated that cyclopamine actually abrogates cellular responses to Hh ligands, and determining the molecular basis of this inhibition became the next experimental challenge. Fortunately, several key transducers of the Hh signal had already been identified through studies in fruit flies, mice, and other model systems,¹⁰ permitting a hypothesis-based approach to target identification. These investigations established a general signaling mechanism initiated by the binding of Hh ligands to the twelve-pass transmembrane protein Patched1 (*Ptch1*), thereby inhibiting its function (Figure 1C). Since *Ptch1* normally suppresses the activity of another transmembrane component, the G protein-coupled receptor (GPCR)-like protein Smoothened (*Smo*), Hh ligand reception leads to *Smo* activation. Through mechanisms that remain unclear, activated *Smo* then regulates the activity state of the Gli transcription factors. Under basal conditions, two members of this family, Gli2 and Gli3, are sequentially phosphorylated by protein kinase A (PKA), glycogen synthase kinase 3 β (GSK3 β), and casein kinase 1 (CK1) and then proteolyzed in a proteasome-dependent manner to generate N-terminal repressors (Gli2/3R) of Hh target gene expression. The third member of the Gli family, Gli1, is a constitutively active factor that is expressed upon Hh pathway activation as part of a positive feedback loop. Active *Smo* inhibits Gli2/3 repressor formation and promotes the conversion of full-length Gli2/3 proteins into transcriptional activators (Gli2/3A), thereby inducing the expression of *Gli1* and other Hh target genes such as *Ptch1*. How this switch occurs is not well understood, but it appears to involve the *Smo*-dependent dissociation of Gli proteins from Suppressor of Fused (*Sufu*), which is required for Gli2/3 proteolysis and may also recruit histone deacetylases to Gli-binding sites to epigenetically silence those regions.^{13, 14} Recent studies have further demonstrated a critical role for the primary cilium, a microtubule-based protrusion of the plasma membrane, in Hh pathway regulation.¹⁵ This organelle is required for both Gli2/3 repressor and activator formation, and trafficking of Hh signaling proteins through the primary cilium has been observed.

To determine where cyclopamine acts within the Hh pathway, Beachy and his co-workers first assessed its epistasis with individual Hh signaling proteins. For example, they found the natural product was able to inhibit Hh target gene expression in cells derived from *Ptch1* null mice with an IC₅₀ of approximately 300 nM, demonstrating that cyclopamine acts downstream of the Hh receptor.¹⁶ In contrast, ten-fold higher concentrations of this steroid alkaloid were required to block Hh pathway activation in cells expressing a constitutively active *Smo* mutant (*Smo*M2; W539L in mice), and cells overexpressing Gli2 were completely resistant to the teratogen.¹⁶ These epistatic relationships suggested the cyclopamine acts at the level of *Smo*, by either directly inhibiting the transmembrane receptor or targeting one or more cellular proteins that regulate *Smo* activity.

To distinguish between these two possibilities, cyclopamine derivatives were synthesized to identify sites that could be modified without significantly attenuating inhibitory activity. These structure-activity relationship analyses revealed that the piperidinyl amine can tolerate long-chain alkyl substituents, allowing a variety of mechanistic probes to be generated.^{16, 17} Although the isolation of small molecule-binding proteins by affinity chromatography is perhaps the most conventional method for target identification, transmembrane proteins such as *Smo* are difficult to solubilize in their native conformations. For this reason, methods that can detect *Smo*/ligand interactions in live cells were pursued. First, a fluorescent derivative of the teratogen, BODIPY-cyclopamine (Figure 1B), was synthesized and used to probe cultured cells expressing high levels of exogenous *Smo*.¹⁷ When applied at nanomolar

concentrations, specific binding of the BODIPY-cyclopamine to these Smo-overexpressing cells could be detected, as gauged by the ability of unmodified cyclopamine to competitively inhibit this association. A cyclopamine-based photoaffinity probe, PA-cyclopamine (Figure 1B), was then prepared to assess whether the sequestration of BODIPY-cyclopamine by Smo-overexpressing cells was due to direct binding to the GPCR-like protein.¹⁷ Smo-overexpressing cells were incubated with this ¹²⁵I-labeled aryl azide reagent and briefly exposed to ultraviolet light in the absence or presence of a non-radiolabeled cyclopamine derivative. Analysis of the corresponding cell lysates by gel electrophoresis and autoradiography revealed specific labeling of Smo by PA-cyclopamine, establishing this transmembrane Hh signaling protein as the direct target of cyclopamine and its derivatives. Overexpressed SmoM2 was resistant to PA-cyclopamine crosslinking, consistent with the resistance of constitutively active Smo to this teratogen.¹⁷

The identification of Smo as the target of cyclopamine has led to the widespread application of this small molecule as a probe of Hh signaling mechanisms and Hh pathway-dependent physiology. For example, cyclopamine has been used to explore the epistatic relationships between Smo and other Hh pathway regulators,¹⁸ how Smo activity state correlates with its subcellular localization,¹⁹ and the temporal requirements of Hh signaling for anterior-posterior patterning of the vertebrate limb.²⁰ Cyclopamine has also played a critical role in pre-clinical studies establishing the potential of Hh pathway inhibitors as anti-cancer drugs. Human genetics studies in the 1990s had established a causal relationship between Hh pathway dysregulation and oncogenesis in patients with Gorlin syndrome, a genetic disorder characterized by developmental abnormalities and a propensity to develop basal cell carcinomas, medulloblastoma, and other neoplasms. Linkage analyses mapped the genetic locus for Gorlin syndrome to the human *PTCH1* gene,²¹ and several inactivating germline mutations in this Hh pathway suppressor have been found in this patient population.²² Building upon these findings, the Beachy laboratory demonstrated that cyclopamine could inhibit the growth of medulloblastoma allografts derived from *Ptch1* heterozygous/*p53* null mice, providing new hope for patients with Hh pathway-dependent tumors.²³ Since this discovery, several other cancers have been linked to uncontrolled autocrine (cancer cells responding to their own factors) or paracrine (cancer cells disseminating factors to surrounding tissues to promote a tumorigenic environment) Hh signaling, including tumors of the pancreas, prostate, lung, and blood.²⁴ Nearly all of these cancers exhibit sensitivity to cyclopamine in animal models, suggesting that Hh pathway antagonists might be efficacious against a broad spectrum of human neoplasms.

Due to the role of Hh pathway activation in oncogenesis and the success of cyclopamine in pre-clinical cancer models, the development of new Smo inhibitors is now an area of active research in both academia and industry. Drug leads include cyclopamine derivatives with improved pharmacokinetic properties, such as the semisynthetic analog IPI-926 developed by Infinity Pharmaceuticals (Figure 1D).²⁵ This derivative was prepared from cyclopamine through methylsulfonamide introduction to the A ring, reduction of the endocyclic olefin in the B ring, and cyclopropanation-mediated expansion of the D ring, resulting in a compound with improved potency, metabolic stability, and water solubility. High-throughput screens of structurally diverse chemical libraries using cell-based models of Hh signaling, have yielded other Smo inhibitors, and it appears that this GPCR-like protein is the most “druggable” target within the Hh pathway. For example, SANTs 1–4 (Smo antagonists) were discovered using cells stably transfected with a Gli-dependent firefly luciferase reporter,²⁶ and SANTs 74 and 75 were identified using transgenic zebrafish that express green fluorescent protein (GFP) upon Hh pathway activation.²⁷

At least four Smo antagonists are currently being tested in human clinical trials, and results thus far are promising. In particular, the methylsulfonylbenzamide derivative GDC-0449

(Figure 1D), developed by Genentech and Curis through high-throughput screening and medicinal chemistry, was able to regress metastatic basal cell carcinomas in the majority of patients enrolled in a Phase I trial.²⁸ A patient with metastatic medulloblastoma was also treated with GDC-0449, resulting in a rapid, dramatic but transient reduction in tumor burden (Figure 1E).²⁹ While these outcomes validate the Hh pathway as a therapeutic target, the relapse observed in the medulloblastoma case may reflect a general propensity of tumors with ligand-independent Hh pathway activation to acquire resistance to Smo inhibitors. The GDC-0449-insensitive medulloblastoma cells were found to express a mutant Smo allele (D473H) that was not detected in the original tumors, and the expression of this Smo variant in Hh-responsive cells was sufficient to convey resistance to both GDC-0449 and a cyclopamine derivative.³⁰

Subsequent studies have revealed additional Smo mutations that can convey drug resistance,^{31, 32} as well as evidence for the circumvention of Smo antagonists by genetic lesions in downstream or parallel signaling processes.^{30, 32} While these findings delineate the limitations of current Hh pathway-targeting therapies, such challenges should be surmountable through the coordinated efforts of chemists, cell biologists, and oncologists. For example, new structural classes of Smo antagonists can be used to regain pharmacological control of GDC-0449-insensitive forms of Smo,³¹ and inhibitors that act downstream of this GPCR-like signaling protein may also be effective countermeasures. The development of Hh pathway-targeting small molecules will therefore be an area of active research for some time to come.

IWRs and XAV939, synthetic antagonists of the Wnt pathway

The Wnt pathway is a second intercellular signaling process that was discovered through fruit fly mutagenesis screens and plays a major role in embryogenesis and oncogenesis.^{33, 34} In fact, the name “Wnt” was coined as a combination of Wingless, the secreted factor that initiates the signaling pathway in fruit flies and promotes wing development, and Int-1, a vertebrate homolog of Wingless that has been linked to mammary tumors in mice. A myriad of tissues and organs require the Wnt pathway for their development, including the brain, spinal cord, kidneys, lungs, limbs, and eyes.³⁴ Wnt signaling has also been implicated in stem cell self-renewal, and genetic lesions that constitutively activate the Wnt pathway can cause gastric and colorectal tumors, melanomas, hepatocellular carcinomas, and other human cancers.³⁵

Given the critical roles of Wnt pathway activation in tissue formation and cancer, small molecules that specifically inhibit this signaling process could be valuable probes of embryonic patterning and structural leads for next-generation chemotherapies. Mechanistic studies in fruit flies and other model organisms have revealed a number of possible drug targets, uncovering interesting parallels between the Hh and Wnt pathways at the molecular level (Figure 1C and 2A).^{33, 34} For example, both Hh and Wnt ligands are lipid-modified, the latter containing two palmitoyl groups, and the Frizzled (Fzd) family of Wnt receptors are the closest structural relatives of Smo. Wnt pathway activity is similarly dictated by a phosphorylation- and proteasome-dependent balance of transcriptional repressors and activators, in this case the TCF/LEF (T cell-specific transcription factor/lymphoid enhancer binding factor) class of repressors and their transcriptionally active complexes with the co-activator β -catenin.

The signal transduction model that has emerged from these studies centers around a “ β -catenin destruction complex” composed of the scaffolding protein Axin1, the large, multi-functional protein adenomatous polyposis coli (APC), GSK3 β , and CK1 (Figure 2A).^{33, 34} In the absence of Wnt ligands, β -catenin is constitutively phosphorylated by the destruction

complex, targeting it for degradation by the proteasome. The binding of Wnts to Fzd family members and LDL receptor-like proteins (LRPs) on the surface of responding cells leads to phosphorylation of the LRP cytoplasmic tail, recruitment of Axin1 to the plasma membrane, and the phosphorylation-dependent activation of the Dishevelled (Dvl) family of multimodular polypeptides. Activated Dvl then inhibits the β -catenin destruction complex through mechanisms that are not yet clear, allowing the co-activator to associate with TCF/LEF proteins in the nucleus and promote Wnt target gene expression. As with Hh signaling, Wnt target genes include pathway regulators as part of feedback mechanisms. For example, *Axin2* is expressed in response to Wnt pathway activation to create a negative feedback loop.

Genetic mutations that disrupt any of these signaling proteins can produce developmental defects. For example, loss of *Wnt4* causes kidney defects, *Fzd4* mutations are associated with impaired vascular growth, and mice lacking *Dvl1* and *Dvl2* exhibit spinal cord abnormalities.³⁴ Pathway-activating mutations also are potentially oncogenic, a risk underscored by presence of loss-of-function mutations in APC or stabilizing mutations in β -catenin in nearly all hereditary and sporadic colorectal carcinomas.³⁵ Yet the identification of small molecules that either phenocopy or counteract these genetic perturbations has proven to be a challenging endeavor. For example, while Smo is the most “druggable” signaling protein in the Hh pathway, no chemical inhibitors of Fzd function have been reported to date. This difference in pharmacological sensitivity may reflect the greater evolutionary diversification of Wnt pathway components in comparison to Hh signaling regulators. There are at least 19 Wnt ligands, 10 Fzd and 2 LRP co-receptors, 4 Dvl proteins, 2 Axin homologs, and 4 TCF/LEF family members in humans, many of which are co-expressed in specific tissues and likely have redundant functions. The pharmacological modulation of Wnt target gene expression might therefore require small molecules that can perturb multiple isoforms of a given Wnt pathway component.

The first unbiased screen for Wnt pathway inhibitors was reported by the Lum group, which surveyed approximately 200,000 compounds using a cell line stably transfected with a *Wnt3a* expression construct and a TCF/LEF-dependent firefly luciferase reporter.³⁶ In principle, the constitutive Wnt signaling associated with this line could be blocked by compounds that inhibit either Wnt ligand production or responsiveness, and both types of antagonists were discovered in this screen. Lum and his co-workers determined that the former set of compounds, benzothiazole-based inhibitors of Wnt production (IWPs), target the acyltransferase Porcupine to prevent the palmitoylation of Wnt ligands. The inhibitors of Wnt response (IWRs) could be further subdivided into two structurally distinct pharmacophores, a group of bicyclo[2.2.1]heptene-containing quinoline derivatives and a collection of quinazolininediones with IC₅₀s in the 200–300 nM range (Figure 2B). These latter antagonists have provided unexpected insights into the biochemical processes that regulate Wnt target gene expression and new strategies for targeting Wnt pathway-dependent cancers, and we therefore focus on our discussion on the IWRs.

To better understand how the IWRs block cellular responses to Wnt ligands, the Lum laboratory investigated the effects of these compounds on known Wnt signaling proteins.³⁶ Both IWR classes reduced β -catenin levels in *Wnt3a*-stimulated fibroblasts, suggesting that they promote the degradation of this TCF/LEF co-activator. Subsequent studies of upstream signaling proteins revealed that the IWRs had no effect on APC, Dvl, or GSK3 β levels. Nor did they alter Dvl phosphorylation state in DLD-1 colorectal cancer cells, an immortalized line that has a truncating mutation in APC and therefore constitutive Wnt pathway activation. However, both quinoline and quinazolininedione IWRs significantly increased *Axin2* levels in these cells without inducing *de novo* synthesis of this scaffolding protein. Based on these observations, Lum and his co-workers proposed that the IWRs inhibit Wnt

pathway activation by stabilizing Axin proteins and promoting β -catenin degradation, perhaps by targeting the Axins directly.

Independent studies by scientists at Novartis revealed a second small-molecule Axin stabilizer and clarified the mechanism by which this occurs.³⁷ Using a similar high-throughput Wnt3a signaling assay, they identified a trifluoromethylphenylpyrimidine derivative called XAV939 that could inhibit Wnt target gene expression in human embryonic kidney cells with an IC₅₀ of approximately 50 nM, as well as block Wnt pathway activity in colorectal cancer cell lines harboring truncating mutations in APC (DLD-1 and SW480 cells) (Figure 2B). As with the IWRs, XAV939 decreased β -catenin levels and caused accumulation of Axin1 and Axin2. XAV939 also significantly promoted β -catenin phosphorylation, consistent with a model in which the compound potentiates β -catenin destruction complex activity by increasing Axin protein levels. To elucidate the mechanism by which XAV939 stabilizes the Axin proteins, the Novartis team then immobilized an XAV939 derivative onto crosslinked agarose beads and used this affinity chromatography matrix to isolate XAV939-binding proteins from cell lysates. Mass spectrometry-based characterization of the proteins that specifically bound to the XAV939-functionalized matrix revealed several poly(ADP-ribose) polymerases (PARPs) such as PARP1, PARP2, tankyrase1 (TNKS1), and tankyrase2 (TNKS2), suggesting that PARsylation of one or more signaling proteins might negatively regulate the Wnt pathway. Compound titration studies further established that XAV939 bound to TNKS1 and TNKS2 with approximately ten-fold greater affinity than to PARP1 and PARP2, suggesting that the former PARP family members might be the key XAV939 targets with respect to Wnt pathway inhibition. Accordingly, subsequent crystallographic studies of the PARP domain of TNKS2 bound to XAV939 show that the trifluoromethyl group in the inhibitor makes productive, non-polar contacts with TNKS2 side chains near the nicotinamide adenine dinucleotide cofactor-binding site, whereas structural alignments indicate that this XAV939 moiety would clash with the regulatory domain in PARP1.³⁸

Validating a small molecule-binding protein as its physiologically relevant target is a critical but non-trivial task in chemical genetics, frequently requiring multiple lines of experimental evidence. In the case of XAV939, the Novartis scientists found that co-depletion of TNKS1/2 through short interfering RNAs (siRNAs) recapitulated the effects of XAV939 on Axin levels, but the combined knockdown of PARP1/2 did not.³⁷ Interestingly, siRNA-mediated loss of either TNKS isoform alone also failed to stabilize the Axin proteins, suggesting that TNKS1/2 regulate the Wnt pathway in a redundant manner. The Novartis team further showed that: (1) XAV939 inhibited the auto-PARsylation of TNKS1/2 *in vitro* with nanomolar potency; (2) XAV939 blocked the TNKS-dependent PARsylation and subsequent ubiquitination of Axin proteins in live cells; (3) a 12-amino acid, N-terminal region of the Axin proteins was required for TNKS binding; and (4) Axin proteins lacking this TNKS-binding domain were expressed in an XAV939-insensitive manner. They also found that the IWRs could potentially inhibit TNKS1/2 but not PARP1/2, revealing that these structurally distinct Axin stabilizers share a common mechanism of action. Taken together, these observations indicate that XAV939 and the IWRs block Wnt target gene expression by preventing the TNKS1/2-dependent PARsylation of Axin proteins, stabilizing these scaffolding macromolecules and allowing them to promote β -catenin phosphorylation and degradation.

It is important to note that the role of TNKS1/2 in Axin homeostasis and Wnt pathway regulation was not known until the discoveries of XAV939 and the IWRs. These investigations therefore illustrate how small-molecule screens can not only identify new chemical modulators of cell signaling pathways but also uncover regulatory mechanisms that have eluded conventional biological approaches. In this case, the functional redundancy

of TNKS1 and TNKS2 masked the consequences of losing either isoform alone through genetic perturbations, whereas the ability to pharmacologically silence both enzymes allowed their actions on the Axin proteins to come to light. The studies by the Lum and Novartis groups also led to the unexpected finding that increased Axin levels can counteract β -catenin function even in the absence of wildtype APC, since the compounds were able to block Wnt pathway activation in cell lines expressing truncated forms of APC. Whether this holds true for the full range of APC mutations remains to be determined, but these results suggest that the IWRs and XAV939 could lead to a new class of targeted chemotherapies that are effective against at least a subset of the APC-deficient tumors that are so prevalent in colorectal cancer, as well as other Wnt pathway-dependent neoplasms. TNKS1/2 antagonists might therefore complement other chemotherapies that appear to work at least in part by attenuating Wnt target gene expression, such as cyclooxygenase inhibitors. These non-steroidal anti-inflammatory drugs have demonstrated chemopreventive activities against cancers of the colon, breast, prostate, and lung,³⁹ and they are believed to block prostaglandin E2-mediated stabilization of β -catenin.⁴⁰

Dorsomorphin, a small-molecule inhibitor of BMP signaling

As illustrated by the preceding examples, inhibitors of developmental signaling pathways have been identified through the serendipitous exposure of embryos to these teratogens outside of the laboratory or the high-throughput screening of compound libraries using cell-based assays. These differing modes of discovery highlight the dual challenges of achieving target specificity and screening efficiency in chemical genetic studies. The ability of cyclopamine to faithfully recapitulate Hh loss-of-function phenotypes in fetal lambs provides a measure of its pathway selectivity, yet identifying *V. californicum* as the source of the cyclopia-inducing teratogen and isolating cyclopamine as the active principle were time-consuming, labor-intensive processes. In contrast, the use of cell-based Wnt signaling reporters facilitated the rapid screening of large compound libraries for pathway antagonists, but the efficacy of the IWRs and XAV939 as Wnt pathway-specific inhibitors in whole organisms remains to be established.

Phenotypic screens that employ whole animals can realize both research goals if the organisms can be cultured in microplate formats and compound delivery is facile. While chemical screens involving fruit flies and worms have been described, zebrafish embryos have emerged as a leading animal model for such studies.⁴¹ Zebrafish zygotes are amenable to growth in 96-well microplates, and they readily take up small molecules dissolved in their culture medium. They also are readily obtained in large numbers, achieve an essentially complete body plan within a few days, and are optically transparent throughout this process, enabling one to visualize vertebrate embryogenesis in real time with single-cell resolution. These attributes have prompted numerous zebrafish screens for compounds that perturb various aspects of embryonic development, including haematopoiesis, vascularization, and cardiac morphogenesis. When such screens yield small molecules with clear modes of action, these compounds can often be applied rapidly to investigate biochemical mechanisms *in vivo* and to establish pre-clinical support for new drug therapies.

One notable example of this research paradigm is the discovery of dorsomorphin as an inhibitor of BMP signaling by the Peterson laboratory.⁴² The BMPs constitute the largest subset of the transforming growth factor- β (TGF- β) superfamily of secreted signaling proteins, which also includes the Activins, Nodals, growth and differentiation factors (GDFs), anti-Müllerian hormone (AMH), and isoforms of TGF- β itself.⁴³ Originally identified as extracellular factors that promote bone and cartilage formation,⁴⁴ the BMPs are now known to regulate a wide range of embryological processes, such as dorsoventral patterning and limb development.^{45, 46} Inappropriate activation of BMP signaling is also

associated with fibrodysplasia ossificans progressiva (FOP), a human connective tissue disorder.^{47, 48} Like all other TGF- β superfamily members, BMP ligands are initially expressed in as disulfide-stabilized, homo- or heterodimeric precursor proteins, which are then proteolytically processed to release the biologically active factor. The mechanisms by which cells respond to TGF- β -type ligands are also conserved, with the dimeric signaling proteins promoting the assembly of heterotetrameric receptor complexes that are composed of two types of serine/threonine receptor kinases (Figure 3A). Within each ligand-bound complex, the two constitutively active type II receptors phosphorylate and trigger the two type I receptors. The latter then phosphorylate downstream effectors called R-Smads, which associate with the cofactor Smad4 and translocate to the nucleus to drive target gene expression.

There are seven type I (activin receptor-like kinases ALK-1 through 7) and five type II (ACVR2A, ACVR2B, TGFBR2, BMPR2, and AMHR2) receptors in mammals, and individual members of the TGF- β superfamily bind preferentially with specific receptor combinations.⁴³ In turn, the activated heterotetrameric receptors selectively interact with subsets of the five mammalian R-Smad proteins to elicit distinct cellular responses. The BMPs tend to actuate transmembrane complexes composed of ALK-2, 3, or 6 type I receptors and BMPR2 or ACVR2A type II receptors, subsequently recruiting R-Smads 1, 5, and 8 as transcriptional co-activators. Other TGF- β superfamily members such as Activins, Nodals, and TGF- β isoforms prefer alternative ensembles of receptor kinases and R-Smad proteins. Selectively inhibiting cellular responses to BMP ligands therefore requires an ability to target the appropriate subset of TGF- β superfamily pathway components.

To achieve this goal, Peterson and his co-workers tested over 7,500 compounds for their ability to dorsalize zebrafish embryos, a phenotype associated with defective BMP signaling.⁴² Their screen identified one pyrazolopyrimidine derivative that induced significant embryo dorsalization (Figure 3B), as gauged by morphological defects, the expanded expression of genes associated with dorsal tissues [*paired box gene 2a* (*pax2a*) and *krox20*], and reduced transcription of a ventral marker [*even-skipped-like 1* (*eve1*)]. The compound was consequently named dorsomorphin to reflect its effects on embryogenesis. The Peterson laboratory then sought to confirm that dorsomorphin acts through the selective inhibition of BMP signaling by assessing functional interactions between the small molecule and known BMP pathway regulators. For example, they were able to demonstrate that dorsomorphin could rescue embryo ventralization caused by silencing of the expression of chordin, an endogenous BMP antagonist. Dorsomorphin also blocked the BMP-dependent phosphorylation of R-Smad1/5/8 in cultured cells with an IC₅₀ of 470 nM but not TGF- β 1-induced activation of Smad2. Similarly, the pyrazolopyrimidine analog could antagonize the activity of constitutively active mutants of ALK2/3/6, whereas it had no effect on analogous forms of ALK4/5/7. These observations strongly support a model in which dorsomorphin inhibits BMP type I receptor function while leaving other TGF- β superfamily signaling pathways intact. This molecular mechanism has since been confirmed by crystallographic studies of the kinase domain of ALK2 complexed with dorsomorphin, which reveal interactions between the teratogen's pyrazolopyrimidine ring and residues within the ATP-binding pocket that normally recognize the adenine base.⁴⁹

While the embryonic phenotypes induced by dorsomorphin treatment provide experimental evidence for selectivity of this compound for BMP signaling, its targeting of type I BMP receptor kinases raises the concern that other kinases might be perturbed as well. Indeed, dorsomorphin is structurally identical to a compound previously reported to inhibit AMP-activated protein kinase (AMPK);⁵⁰ however, loss of AMPK function in zebrafish embryos does not cause dorsalization and is therefore unlikely that the primary effects of this teratogen can be attributed to AMPK inhibition.⁴² To further minimize possible off-target

effects, Peterson and his co-workers conducted a structure-activity-relationship (SAR) analysis of the dorsomorphin pharmacophore, identifying substituents that influence compound potency and BMP pathway selectivity.⁵¹ These studies led to the optimized compound LDN-193189, which replaces with 4-pyridinyl and ether functionalities in dorsomorphin with 4-quinoline and piperazine groups, respectively (Figure 3B). LDN-193189 is approximately 100 times more potent than dorsomorphin against BMP signaling, and it has increased selectivity for this developmental pathway relative to AMPK-dependent signaling. This dorsomorphin analog also exhibits superior pharmacokinetic properties, such as a nearly 10-fold increase in plasma half-life. Subsequent investigations further revealed that dorsomorphin and LDN-193189 have “off-target” effects on vascular endothelial growth factor receptor (VEGFR) signaling and therefore angiogenesis, prompting additional SAR studies using zebrafish embryos.⁵² These efforts have resulted in dorsomorphin analogs with even greater BMP pathway specificity (*e.g.* DMH1; Figure 3B), as well as selective VEGFR antagonists.

The optimization of dorsomorphin through medicinal chemistry has facilitated the use of this compound and its derivatives to modulate BMP function *in vivo*. For example, genetic studies had previously suggested a role for BMP signaling in systemic iron metabolism, as mice lacking hepatic Smad4 function fail to express hepcidin, a liver-secreted peptide hormone that systemically regulates iron homeostasis by promoting degradation of the iron exporter ferroportin.⁵³ Consistent with this model, dorsomorphin can inhibit hepcidin expression in cultured hepatocytes challenged with BMPs, iron, or the inflammatory cytokine interleukin-6 (IL-6).⁴² The teratogen can also reduce basal hepcidin transcription in adult mice, thereby elevating ferroportin expression in macrophages and the intestinal epithelial cells, decreasing intracellular iron levels within these cell types, and increasing serum iron concentrations. These results suggest that dorsomorphin might be an effective treatment of diseases caused by dysregulated iron homeostasis, such as hypothermia and anemia. In addition, since the production of proinflammatory cytokines by macrophages is impaired by low intracellular iron, dorsomorphin and its structural analogs could constitute a new therapeutic strategy for controlling inflammation. Accordingly, the Cherayil group has shown that dorsomorphin can block inflammatory responses to *Salmonella typhimurium* infection in mouse models and that LDN-193189 can minimize the intestinal inflammation that arises in IL-10 knockout mice treated with piroxicam.⁵⁴

LDN-193189 has even demonstrated an ability to counteract the widespread ossification of postnatal tissues in a mouse model of FOP.⁵⁵ Patients with this congenital disorder are prone to progressive calcification of their muscles and connective tissues in response to injury or viral infection, resulting in joint fusions and decreased life expectancy.⁴⁷ Linkage analysis studies of families affected by FOP has revealed heterozygous mutations in the BMP type I receptor ALK-2,^{48, 56} and the expression of these mutants in cultured cells leads to Smad1/5/8 phosphorylation and BMP target gene expression.⁵⁵ These findings indicate that FOP is caused at least in part by the constitutive activation of ALK-2, suggesting that inhibitors of this receptor kinase might help remediate this debilitating disease. To explore this possibility, Bloch and his co-workers established a FOP model using postnatal day 7 (P7) transgenic mice engineered to have Cre recombinase-inducible expression of a constitutively active form of ALK-2 (Q207D; caALK-2).⁵⁵ When the hindlimbs of these mice were injected with adenovirus carrying Cre, they developed bony calluses that encased the tibia and fibula and frequently fused with the femur and pelvis to severely restrict limb mobility (Figure 3C). The penetrance of this heterotopic ossification phenotype by P30 was 100%. In comparison, caALK-2 transgenic mice injected with the Cre adenovirus and treated with LDN-193189 exhibited significantly slower disease progression. At P30, two-thirds of the mice remained free of ectopic bone, and joint fusion had not occurred in the remaining mice. Equally important, wildtype and transgenic mice treated with this

dorsomorphin analog did not present any skeletal, morphological, or physiological abnormalities, consistent with the pathway selectivity of this compound.

Thalidomide, a sedative with teratogenic, anti-inflammatory, and anti-cancer activities

As discussed in the introduction of this tutorial review, thalidomide is perhaps the most infamous small molecule known to disrupt embryonic patterning (Figure 4A). Since the discovery of thalidomide's teratogenic activity in the 1950s,^{1, 57} numerous laboratories have pursued the mechanism by which this sedative causes shortened or absent limbs (phocomelia and amelia, respectively; Figure 4B), ear and eye defects, and other developmental abnormalities, resulting in a myriad of opposing hypotheses and models. In fact, the thalidomide story is a cogent reminder of how difficult it can be to determine the cellular target (or targets) of a bioactive compound. This decades-long effort is not simply an academic exercise, for even though thalidomide is no longer prescribed as a sedative, this teratogen and its synthetic analogs have re-entered the clinic as an effective treatment for inflammation associated with leprosy, arthritis, and Crohn's disease and for cancers such as AIDS-related Kaposi's sarcoma and multiple myeloma.^{58, 59} For example, approximately 90% of multiple myeloma patients treated with the thalidomide derivative lenalidomide and dexamethasone experience significant reductions in the number of cancer cells in their blood.⁶⁰ Understanding how thalidomide perturbs embryonic development could shed light on how this drug remediates these human disorders or guide medicinal chemistry efforts to selectively eliminate its teratogenicity, depending on whether these biological activities share a common molecular mechanism.

There are several reasons why thalidomide has been challenging to study. First, thalidomide is typically prepared as a racemic mixture, and although it is generally believed that the (*S*)-stereoisomer is the teratogenic agent, the two enantiomers interconvert under physiological conditions.⁶¹ Second, thalidomide rapidly hydrolyzes *in vivo*, and it is a substrate for the cytochrome P450 group of enzymes, giving rise to over 20 metabolic products.⁵⁹ Third, thalidomide exhibit species-selective activity; while it induces embryonic limb defects in humans, other primates, rabbits, and chickens, it does not cause similar abnormalities in rats or mice.^{62–64} This latter characteristic is why thalidomide was approved as a clinical sedative in Europe and Canada sixty years ago, as only rodent models were required at the time to assess deleterious effects.

As a result of these complicating factors, nearly all hypotheses about the mechanism of thalidomide have been descriptive rather than molecular. It has been proposed by various laboratories that thalidomide generates reactive oxygen species that prevent limb outgrowth,⁶⁵ antagonizes cell adhesion molecule-mediated signaling,⁶⁶ causes neurotoxicity,⁶⁷ intercalates DNA to induce genomic damage,⁶⁸ inhibits cytokine production,⁶⁹ and blocks angiogenesis.⁷⁰ Of these models, the latter two have gained the most acceptance, and thalidomide analogs are frequently characterized in terms of their immunomodulatory and anti-angiogenic properties. The two activities, however, appear to be independent of each other,⁷¹ and their relative contributions to current thalidomide-based therapies undoubtedly varies between medical contexts and how the immunomodulatory and/or anti-angiogenic activities of thalidomide might be related to its effects on limb development remains unclear.

This mechanistic impasse was finally overcome last year by Handa and his co-workers, who used affinity chromatography to identify the first direct protein target of thalidomide.⁷² These studies employed the carboxylic thalidomide derivative FR259625 conjugated to ferrite-glycidyl methacrylate beads, a magnetic support that allows greater target protein

recovery and exhibits less non-specific binding than standard agarose-based matrices (Figure 4A).⁷³ When cell lysates were incubated with these beads and thalidomide-binding proteins were eluted with soluble teratogen, two polypeptides were identified by proteolytic digestion and tandem mass spectrometry-based sequencing: cereblon (Crbn), a protein previously associated with mild mental retardation, and damaged DNA-binding protein 1 (Ddb1). Using purified recombinant proteins, the Handa laboratory subsequently determined that thalidomide interacts directly with Crbn with a dissociation constant of 8.5 nM, while Ddb1 associates with Crbn. Since Ddb1 is a subunit of E3 ubiquitin ligase complexes that contain the scaffolding protein Cullin 4 (Cul4A or Cul4B), the E2 ubiquitin-conjugating enzyme-interacting protein Regulator of cullins 1 (Roc1), and various substrate receptors,⁷⁴ they also examined whether Crbn associates with E3 ligase components. Indeed, Cul4A and Roc1 co-immunoprecipitated with epitope-tagged Crbn, and interactions between Crbn and Ddb1 could be blocked by co-expression of the E3 ubiquitin ligase substrate receptor Ddb2.

These results support a model in which Crbn targets certain cellular proteins for ubiquitination and proteasome-dependent degradation, with thalidomide inhibiting the action of Crbn-dependent E3 ligase complexes on factors that regulate limb development (Figure 4C). Consistent with this idea, the Crbn-containing complex can undergo autoubiquitination *in vitro* and this activity is blocked by the teratogen.⁷² To demonstrate that Crbn is the relevant target of thalidomide *in vivo*, Handa and his colleagues investigated the function of this protein in animal models. Although teleosts have not been traditionally used to study thalidomide, they were first able to establish that zebrafish embryos grown in the presence of this compound exhibit pectoral fin and otic vesicle defects that are reminiscent of the limb and ear malformations observed in thalidomide-treated tetrapods (Figure 4D). Zebrafish Crbn could be specifically purified from embryo lysates using their FR259625 affinity matrix, and loss of Crbn expression using antisense morpholino oligonucleotides induced similar developmental phenotypes. Most importantly, the Handa group was able to identify point mutations in Crbn (Y384A and W386A) that render its autoubiquitination activity insensitive to thalidomide, and the overexpression of this protein in zebrafish embryos prevented fin and otic vesicle defects upon teratogen exposure. Limb development in thalidomide-exposed chick embryos was similarly rescued upon overexpression of the teratogen-resistant Crbn mutant within the forelimb field. Thus, the teratogenic effects of thalidomide are due at least in part to its direct abrogation of Crbn-dependent protein ubiquitination and degradation.

The precise mechanisms by which Crbn inhibition disrupts limb development await further study, but the Handa laboratory has uncovered a few clues.⁷² In particular, limb growth along the proximodistal axis is promoted by mutually reinforcing signals between the ectoderm and mesoderm at the distal-most tip of the developing limb bud (Figure 4E). *Fibroblast growth factor 8* (*Fgf8*) is expressed in a region called the apical ectodermal ridge (AER) and *Fgf10* is produced by the underlying mesenchyme, and the two FGF signaling processes compose a feedback loop that is required for cell proliferation and therefore limb outgrowth.⁷⁵ Thalidomide significantly reduces the expression of these FGF family members in zebrafish fin and chick limb buds, and these effects are reversed by overexpression of the *Crbn* Y384A/W386A mutant.⁷² Which substrates of the Crbn-containing E3 ligase regulate *Fgf8* and/or *Fgf10* expression remain to be determined; however, it is likely that they are several steps removed from the actual transcription of these genes. Previous studies have shown that thalidomide upregulates the expression of BMP ligands in the developing chick limb,⁷⁶ which in turn can inhibit *Fgf8* expression and induce apoptosis within the AER.⁷⁷ Thus, the inhibition of Crbn by thalidomide might ultimately perturb multiple developmental signaling pathways in a tissue-specific manner.

How the effects of thalidomide on Crbn-dependent ubiquitination might translate into its therapeutic activities is also an important question that remains unanswered. Does the ability of thalidomide analogs to inhibit Crbn function correlate with their immunomodulatory or anti-angiogenic activities? Or do the compounds target distinct subsets of Crbn substrates? While the pharmaceutical industry hopes to develop thalidomide-based anti-inflammatory and anti-cancer therapies that are non-teratogenic, it is not clear how mechanistically separable these activities will be. For instance, the ability of thalidomide to block Crbn-dependent protein ubiquitination and degradation may be related to its action against multiple myeloma, which is responsive to proteasome inhibitors such as bortezomib (Velcade®).⁷⁸ The discovery of Crbn as a primary target for thalidomide teratogenicity therefore sets the stage for future investigations of this valuable yet potentially debilitating drug.

Concluding remarks

As evidenced by the case studies described in this tutorial review, chemical modulators of embryonic patterning mechanisms are no longer just molecular curios for developmental biology textbooks. The emerging roles of Hh, Wnt, BMP, and other developmental pathways in human disease have spurred efforts to elucidate and pharmacologically regulate the molecular events that constitute these processes, and inhibitors of these pathways are now recognized as valuable tools for signal transduction research and leads for targeted therapies. The ability of compounds to perturb protein function rapidly and reversibly is a particularly useful asset, since the time scales at which developmental processes transpire are difficult to achieve with conventional genetic approaches. Moreover, pharmacological tools can overcome the functional redundancy that is manifest in Hh, Wnt, BMP, and other signaling pathways by simultaneously targeting homologous proteins.

The examples discussed above exemplify the promise of small-molecule teratogens as next-generation chemotherapies, yet the clinical use of these agents is associated with certain risks and challenges. In addition to the clear need to prevent fetal exposure to these drugs, it is possible that chemical modulators of these developmental pathways will have unintended effects on post-natal tissue homeostasis and regeneration since these processes frequently recapitulate developmental mechanisms. For example, Hh signaling regulates bone growth⁷⁹ and hair follicle cycling,⁸⁰ and Wnt signaling is required for renewal of the intestinal epithelium.⁸¹ The effects of teratogens on normal adult physiology are likely to be reversible, and it is possible that dysregulated cells associated with human disease will be more sensitive to these compounds than healthy adult tissues. Technologies that help target these therapies to diseased cells could also help mitigate any adverse responses. However, extra caution should be exercised with pediatric treatments. As illustrated by the permanent bone defects observed in juvenile mice treated with a Hh pathway inhibitor,⁸² teratogenic therapies in children can lead to life-long developmental deficits. Yet despite these risks, small-molecule inhibitors of developmental signaling pathways provide new, long-awaited hope for many patients afflicted with diseases that currently lack effective treatments.

Our tutorial review also highlights several of the technical challenges associated with identifying and characterizing compounds that target developmental pathways. While thalidomide and cyclopamine are two of the best known teratogens due to their storied origins, the discovery of new developmental pathway modulators cannot rely on such pharmacological accidents. Deliberate, well-designed screening campaigns will be integral to this effort, using either *in vitro*, cell-based, or *in vivo* models. As was undertaken to discover synthetic Hh pathway inhibitors, high-throughput screens that utilize cell lines containing pathway-specific reporters will likely be the primary means of identifying such compounds. Miniaturizable into 384- or even 1536-well microplate formats, such assays are

readily automated and can be used to evaluate hundreds of thousands of molecules in days. On the other hand, triaging the numerous hits from these cell-based screens to identify compounds that are pathway-selective and effective *in vivo* can be a laborious, time-consuming process. For every GDC-0449-like compound, there are dozens of molecules that can block Hh target gene expression in non-specific ways or that fail to recapitulate these effects in animal models. Screens that utilize purified components *in vitro* can help direct these chemical genetic efforts toward pathway-specific proteins, but this approach also eliminates the possibility of discovering novel biological regulators of these signaling mechanisms. For example, identification of the IWRs and XAV939 as Wnt pathway antagonists was the key to revealing the role of TNKS1/2-dependent PARsylation in Axin homeostasis. Organismal screens such as that used to discover dorsomorphin can provide an alternative means for incorporating pathway-specificity into the primary assay, as well as appropriate pharmacokinetic properties, although the throughput rate for animal-based studies are currently orders of magnitude lower than *in vitro* or cell-based investigations. Technologies that allow cell-based assays to comprehensively survey multiple signaling pathways in tandem or that increase the throughput of whole-organism screens would help overcome some of these limitations.

Compounds that arise from either cell- or organism-based screening strategies also require target identification to realize their maximum utility in basic and clinical science. Unfortunately there is still no 'silver bullet' for this process, which can take weeks or years, depending on potency, biological stability, and promiscuity of the small molecule and the nature of its physiological target. In most cases, unbiased methods for the biochemical or genetic identification of teratogen-binding proteins will not be sufficient, and an intimate knowledge of the pathway of interest can determine the success or failure of the study. For instance, the discovery of Smo as the cyclopamine target would have been very difficult to achieve through affinity chromatography since Smo is a transmembrane protein expressed at relatively low levels and cyclopamine has a modest, high-nanomolar potency (although greater than most hits from high-throughput chemical screens). Demonstrating this interaction required a combination of epistatic mapping with known Hh signaling proteins and hypothesis-driven experiments that directly probed for binding between the steroid alkaloid and GPCR-like receptor. Similarly, although XAV939-functionalized affinity matrices led to the isolation of several PARP enzymes, recognizing the significance of this biochemical association and deciphering the role of TNKS1/2 in Axin protein regulation was enabled by concurrent, in-depth analyses of XAV939-dependent effects on Wnt pathway components. Even in the rare circumstances where a single teratogen-binding protein is revealed by unbiased approaches, mutually reinforcing, well controlled biological experiments such as those used to investigate the thalidomide target Crbn are essential to conclusively establish its physiological relevance. Thus, teratogen target discovery is as much an exercise in cell and developmental biology as it is in chemistry and biochemistry.

Yet despite the multidisciplinary nature of the field, chemists are perhaps best positioned to advance its frontiers. Chemical screening efforts are of course limited by the availability of high-quality compound collections, and the ongoing development of small-molecule libraries with improved structural diversity is an important initiative. Moreover, identifying the cellular target of a screening hit, developing it into a useful cell biological probe, or generating a suitable reagent for *in vivo* studies almost always requires the synthesis of tens if not hundreds of chemical analogs. Synthetic derivatives of cyclopamine, the IWRs, XAV939, dorsomorphin, and thalidomide were essential for determining their binding proteins and/or achieving compound selectivities and potencies necessary for biological studies. The thalidomide studies even involved the preparation of novel polymeric beads for the affinity purification of its target. An ability to create non-natural molecules is therefore

integral to the discovery and exploration of novel teratogens, a skill that is still largely limited to scientists formally trained in organic chemistry.

Given the continually emerging roles of developmental signaling pathways in cancer, inflammation, fibrosis, and other human disorders, an investment by the chemistry community in small-molecule inhibitors of these processes is likely to reap substantial rewards. The dramatic effects of thalidomide and GDC-0449 on multiple myeloma and metastatic basal cell carcinoma, respectively, illustrates the potential of teratogen-based therapies. In addition, the emergence of GDC-0449-resistant tumors underscores the urgent need for a variety of inhibitors that collectively target signaling proteins throughout each developmental pathway. This current state of affairs must have been unimaginable during the 1950s, as few could have conceived that any medical benefit might arise from the one-eyed lambs birthed by *V. californicum*-ingesting ewes or the devastating limb defects in children exposed *in utero* to thalidomide. While there is still much work to be done, happy endings to these stories now appear to be within reach, and chemists have an opportunity to make key contributions to these next chapters.

Biographies

Tomoyo Sakata received her B.S. (1992) and M.S. (1994) degrees in Organic Chemistry from Nagoya University, and a Ph.D. degree in Chemical Ecology from Kyoto University (1997). She pursued postdoctoral studies in Kyoto University, the State University of New York, University of Wisconsin, and Genomics Institute of the Novartis Research Foundation, gaining broad research experience in molecular probe development and drug discovery. She joined the Chen Lab as a Research Associate in 2010 and is currently working on discovery and characterization of novel Hedgehog pathway inhibitors.

James K. Chen received his A.B. (1991) and Ph.D. (1999) degrees in Chemistry and Chemical Biology from Harvard University, working with George Whitesides and Stuart Schreiber, respectively. After postdoctoral studies with Philip Beachy at the Johns Hopkins School of Medicine, he joined the faculty at the Stanford University School of Medicine in 2003, where he is currently an Associate Professor of Chemical and Systems Biology and Executive Director of the Stanford High-Throughput Bioscience Center. His research interests include the molecular mechanisms of embryonic patterning and the development of chemical probes to interrogate these processes.

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Figure 1. Pharmacological inhibition of Hh signaling

(A) Cyclopic lamb resulting from *in utero* exposure to the natural product cyclopamine. Photo courtesy of the USDA-Agricultural Research Service, Poisonous Plant Research Lab, Logan, Utah. (B) Chemical structures of cyclopamine and synthetic derivatives used to identify its cellular target. Individual ring systems within the cyclopamine skeleton are labeled A–F. (C) Schematic representation of the Hh pathway, showing the trafficking of signaling proteins through the microtubule-containing primary cilium and nucleus (dashed box). Key phosphorylation events are indicated by the black circles and the putative Gli activation step is depicted by the red diamond. (D) Chemical structures of selected Smo antagonists currently being evaluated in human clinical trials. (E) Response and relapse of

metastatic medulloblastoma (dark signals) to GDC-0449 therapy. Reprinted with permission from the Massachusetts Medical Society (Ref. 29, copyright 2009).

Figure 2. Pharmacological inhibition of Wnt signaling

(A) Schematic representation of the Wnt pathway, with key phosphorylation events indicated by the black circles and the nucleus depicted by the dashed box. (B) Chemical structures of the Wnt pathway inhibitors IWR-1, IWR-3, and XAV939.

Figure 3. Pharmacological inhibition of BMP signaling

(A) Schematic representation of the BMP pathway, with key phosphorylation events indicated by the black circles and the nucleus depicted by the dashed box. (B) Chemical structures of dorsomorphin and its chemical analogs LDN-193189 and DMH1. (C) Reduction of heterotopic ossification by LDN-193189 in a mouse model of fibrodysplasia ossificans progressiva. X-ray images of mice with ectopic expression of ALK2-Q207D in their left hindlegs are shown, with soft tissue calcification resulting in animals treated with vehicle alone (arrowheads). Adapted by permission from Macmillan Publishers Ltd: Nature Medicine (Ref. 42, copyright 2008).

Figure 4. Thalidomide and its mechanism of action

(A) Chemical structures of thalidomide and an affinity matrix conjugated to its carboxyl derivative FR259625. (B) Limb deformities caused by *in utero* exposure to thalidomide. Reprinted with permission from the British Medical Journal Publishing Group, Ltd. (Ref. 57, copyright 1992). (C) Inhibition of Crbn-dependent ubiquitination of a putative protein that is required limb development and possibly angiogenesis and immune responses. (D) Pectoral defects (arrowheads) in thalidomide-treated zebrafish embryos. Adapted with permission from the American Association for the Advancement of Science (Ref. 72, copyright 2010). (E) Schematic representation of the Fgf8/Fgf10 feedback loop that promotes limb outgrowth, with the Fgf8-expressing apical epidermal ridge depicted in orange and the underlying Fgf10-expressing mesenchyme depicted in blue.