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Screening for small molecule inhibitors of embryonic pathways: Sometimes you gotta crack a few eggs

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ABSTRACT

Extract prepared from *Xenopus* eggs represents a cell-free system that has been shown to recapitulate a multitude of cellular processes, including cell cycle regulation, DNA replication/repair, and cytoskeletal dynamics. In addition, this system has been used to successfully reconstitute the Wnt pathway. *Xenopus* egg extract, which can be biochemically manipulated, offers an ideal medium in which small molecule screening can be performed in near native milieu. Thus, the use of *Xenopus* egg extract for small molecule screening represents an ideal bridge between targeted and phenotypic screening approaches. This review focuses on the use of this system for small molecules modulators of major signal transduction pathways (Notch, Hedgehog, and Wnt) that are critical for the development of the early *Xenopus* embryo. We describe the properties of *Xenopus* egg extract and our own high throughput screen for small molecules that modulate the Wnt pathway using this cell-free system. We propose that *Xenopus* egg extract could similarly be adapted for screening for modulators of the Notch and Hedgehog pathways.

1. Introduction

In this review, we will discuss the unique biological properties of the *Xenopus* egg extract system and the advantages it offers for screening small molecular modulators of complex biological pathways. We will discuss molecular pathways that have been reconstituted using *Xenopus* egg extracts and small molecule screens that have been performed using these assays. Finally, we will describe our recent studies using *Xenopus* egg extracts to identify small molecule modulators of the Wnt pathway and how this approach could be similarly adapted to other embryonic signaling pathways such as Hedgehog and Notch.

Over the past decade, there has been a great expansion in drug discovery efforts within academia as evidenced by the emergence of a new discipline known as Chemical Biology.[1] With the advent of high throughput approaches and the development of faster and cheaper technologies, industry and academia have tremendous resources and opportunities to identify novel drugs at an accelerated pace. Traditionally, pharmacological agents have been identified through enzymatic assays using purified components.[2,3] Such 'targeted screening' approaches, which have been the focus of drug discovery over the past decade or so, offer a number of advantages. First, targeted biochemical screens represent the most direct way to identify drugs based on our current understanding of a molecular target or event. Second, enzymatic assays using purified components can often be readily adapted for high throughput screening (HTS); combined with well-established colorimetric, fluorescent, and luminescent readouts, experimental set-up is often relatively straightforward. Third, with purified components, drug target identification is not a limitation. Fourth, the recent advent of increasingly sophisticated technologies has allowed investigators to acquire very precise kinetic data. Such information can facilitate the grouping of drugs into subclasses based on mechanism even at initial stages of screening and can reveal subtle drug effects as well.

Targeted screening, however, has some major limitations. The most obvious is that the investigator is restricted to the originally hypothesized target; thus, there is no potential for uncovering novel targets. Furthermore, it is exceedingly difficult to predict which compounds will work in vivo or even whether the targeted molecule will be an effective therapeutic target in the first place. An alternative approach to targeted drug screening is to screen for a specific phenotype.[1,4] Phenotypic screens can be performed in

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systems ranging from cultured cells to whole organisms. In fact, in recent years, a majority of compounds receiving FDA approval were discovered through phenotypic screens.[5] These require no a priori knowledge of the direct target of compounds, only knowledge of the desired phenotype. Representative phenotypic screens include those based on *Caenorhabditis elegans* morphology, gastrulation in zebrafish, and mitosis in cultured mammalian cells.[6–8] Screening in such complex systems pre-selects for compounds that are cell/organism permeable, reach their target, and induce a desired effect in vivo; as such, hits are proven to be active in vivo from the start. The identification of novel drug targets can lend to important insights into the biological process in question.

As with targeted screening, there are also major drawbacks to phenotypic screening. Living systems are much more difficult to manipulate in HTS format and require significantly more manual set-up. Phenotypes can often require time-consuming manual inspection or sophisticated algorithms for image analysis. Drug incubation times are longer (i.e., on the order of hours to days) such that screens require significantly more time to perform. The longer time course also increases the likelihood that the desired phenotypes may occur via non-direct mechanisms. Finding the optimal dose of drug to screen is another challenge: too low of a drug concentration can give false negative results, whereas too high of a drug concentration can produce toxicity with a valid drug candidate. Perhaps the major limitation of phenotypic screens, however, is the difficulty of drug target identification, which can represent a formidable challenge.

2. The *Xenopus* egg extract system

Cell extract-based screens, such as those performed using *Xenopus* egg extract, represents an ideal bridge between targeted and phenotypic screens. *Xenopus* egg extracts offers a powerful cell-free system to study complex biological pathways. A multitude of cellular events can be recapitulated in extracts (discussed below). In contrast to targeted screens, enzymes can be monitored in their native milieu that allows for appropriate post-translational modifications and regulation. Thus, using *Xenopus* egg extract, drugs that target enzymes directly as well as those that target unknown cofactors can be identified. In contrast to phenotypic-based screens, enzymes can be studied in a homogenous biochemical environment with minimal well-to-well, cell-to-cell, or animal-to-animal variation. The protein composition of the extract system can be readily altered via addition or depletion of individual components. Wild-type or mutant proteins can be added to extracts at precise concentrations to test their effects. Assay times range from only minutes to hours, and the option of preparing extracts that do not undergo transcription/translation decreases the number of potential mechanisms of action of drug library hits. Importantly, target identification is simplified due to the biochemical tractability of extracts.

Exactly what is *Xenopus* egg extract? *Xenopus* egg extract is essentially highly active cytoplasm that can be obtained in large quantities suitable for biochemical studies.[9,10] *Xenopus* egg extract is prepared by a centrifugation step that disrupts the plasma membrane and releases the cytoplasmic fraction in an essentially undiluted form (Fig. 1). Because *Xenopus* eggs are maternally loaded with all the necessary components needed for early embryogenesis, *Xenopus* egg extract contains cytoplasmic proteins, organelles, amino acids, lipids, and nucleotides at or near physiological levels. The following biological processes have been studied in the *Xenopus* egg extract system: cytoskeletal dynamics, nuclear assembly and import, apoptosis, post-translational modifications, ubiquitin metabolism, cell cycle progression, and signal transduction pathways.[10–21] In typical preparations of *Xenopus* egg extract, biochemical pathways remain largely intact and can be readily assayed in high throughput screens. *Xenopus* egg extract can be altered using standard biochemical

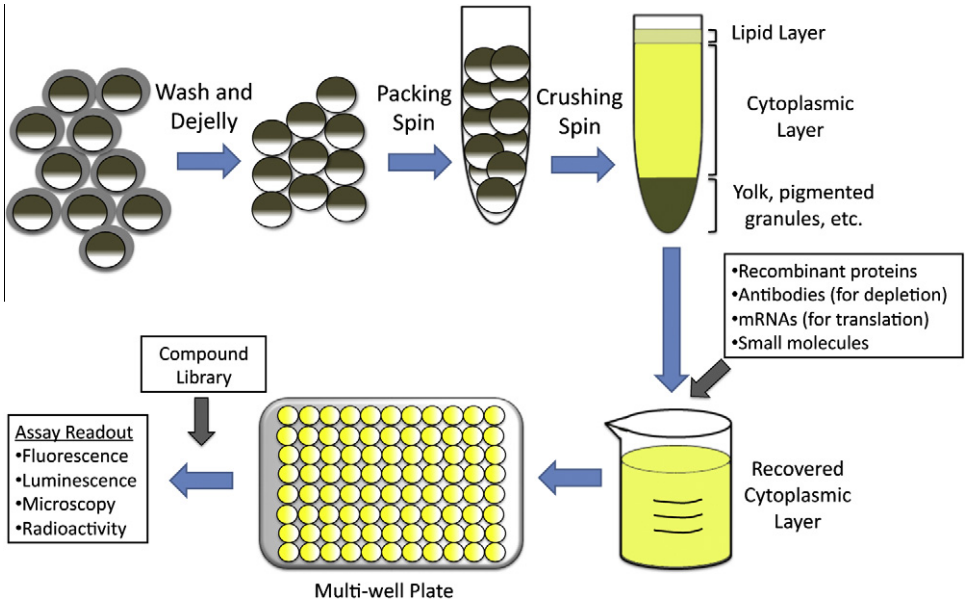


Figure 1. Preparation of *Xenopus* egg extract for HTS screening. *Xenopus* females are injected with human chorionic gonadotropin to induce egg laying into containers filled with buffer. Eggs are collected and the jelly coat removed by treatment with 2% cysteine. The dejellied eggs are washed and subjected to a packing spin (~100×*g*) to remove excess buffer. The packed eggs are then subjected to a crushing spin (>15,000×*g*) that separates the crushed egg components into three distinct layers: lipid, cytoplasmic, and pigmented granule/yolk. The cytoplasmic layer is collected and can be used for biochemical studies or for HTS. Recombinant proteins can be added directly to the egg extract; alternatively, if recombinant proteins are not readily available, mRNA encoding the desired proteins can be added instead. For the latter, the egg extract must be freshly prepared because frozen extract loses its capacity to translate mRNAs. To immunodeplete specific proteins from extract, antibodies linked to resin can be added and removed. Finally, a specific compound can be added to perturb the signaling pathway followed by screening for other compounds that either synergize with it or block its effects. Screen readouts may include fluorescence with GFP constructs, luminescence with luciferase constructs, or radioactivity using scintillation proximity assays. Microscopic analysis of cellular structures (e.g., mitotic spindle, nuclear envelope, chromosomes, etc.) can also be performed in a high throughput manner with appropriate imaging analysis software.

approaches. Recombinant or in vitro-translated proteins can be added to the extract to test the effects of increased concentrations of particular components. Alternatively, because *Xenopus* egg extract retains a high capacity for translating mRNAs, synthetic mRNAs can be added to the extract to translate the desired proteins to high levels, thereby circumventing the need for generating recombinant proteins. For loss of function studies, antibodies can be used to immunodeplete specific proteins. Alternatively, dominant-negative versions of proteins can be added either as recombinant proteins or as mRNAs. Below, we describe the reconstitution of complex biological pathways using *Xenopus* egg extract. Table 1 lists the pathways that have been successfully screened in *Xenopus* egg extract to identify small molecule modulators. Table 2 lists small molecule compounds that have been validated using *Xenopus* egg extract.

3. Reconstitution of complex biological pathways in *Xenopus* egg extracts

3.1. Cell cycle

The *Xenopus* egg extract system has been particularly valuable in elucidating mechanistic details of the cell cycle. A fertilized *Xenopus* egg undergoes many rounds of synchronous cell divisions in the absence of cell growth, thereby subdividing a single-celled egg into a similarly sized embryo of ~4000 cells within the first 8 h of development. Proteins and mRNAs required for cell division are maternally loaded into the egg so as to allow cell divisions to take place without the need for transcription. In groundbreaking experiments performed over the past few decades, *Xenopus* egg extract has been used to reconstitute major cell cycle events: entry into mitosis, cyclin degradation, mitotic spindle assembly, and chromosome segregation.

Work by Murray and Kirschner demonstrated that addition of Cyclin B is sufficient to drive *Xenopus* egg extract into mitosis and that fluctuations in Cyclin B levels largely mediate progression through the embryonic cell cycle.[19,20,22,23] Using this same system, the detailed molecular basis for Cyclin B degradation was uncovered.[19,20,22,23] Studies in *Xenopus* egg extract identified Cyclin B and Cdk1 as the molecular components of Maturation Promoting Complex.[24] Two decades later, studies in this system were key to formulating a biochemical and mathematical description of the mechanism by which Cyclin B and Cdk1 impart an oscillatory nature to the cell cycle.[25]

Using this system, ubistatin was identified by King and co-workers as a cell cycle inhibitor in a small molecule screen of >100,000 compounds.[10] Cyclin B is degraded upon exit from mitosis in a process mediated by the Anaphase-Promoting Complex, an E3 ubiquitin ligase. For a HTS assay, Cyclin B was fused to luciferase and added to extract. Its proteolysis was then monitored to identify compounds that blocked its degradation. Subsequent experiments in a purified biochemical system showed that ubistatin inhibited

Table 1
Cell biological processes in which *Xenopus* egg extract have been used to screen for small molecule modulators

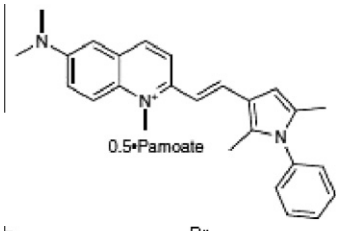
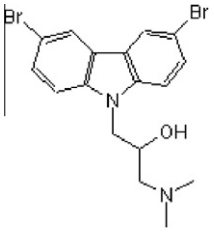
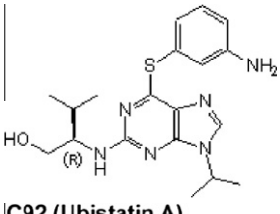
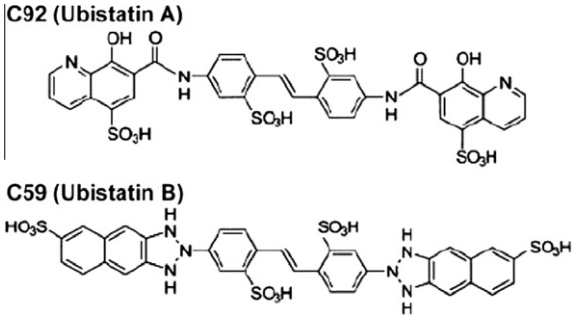
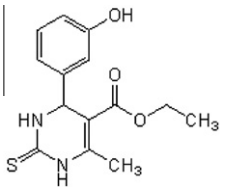
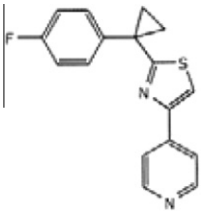
Pathway	Assay	Compound	Structure	Molecular target	Refs.
Wnt	Altered degradation of β-catenin-firefly luciferase and Axin–Renilla luciferase fusion proteins as assayed by luminescence	Pyrvinium		Casein kinase 1α	80–82
Actin polymerization	Altered fluorescence intensity of pyrene-actin monomers that is normally enhanced upon polymerization	Wiskostatin		WASP	53,108,109
Cell cycle/microtubule dynamics	Microscopic analysis of altered spindle formation (visualized using rhodamine-labeled microtubules)	Diminutol		NQO1	47,110
Cell cycle	Altered degradation of cyclin-B1-luciferase fusion protein assayed by luminescence	Ubistatins		Ubiquitin	10

Table 2
Cell biological processes in which *Xenopus* egg extract have been used to validate small molecule inhibitors

Pathway	Assay	Compound	Structure	Molecular target	Refs.
Cell cycle/microtubule dynamics	Microscopic analysis of altered spindle formation (visualized using rhodamine-labeled microtubules)	Monastral		Kinesin eg-5	111–113
Cell cycle/microtubule dynamics	Microscopic analysis of altered spindle attachment by rhodamine-labeled kinesin (eg-5)	FCPT		Kinesin, eg-5	114

cell cycle progression by blocking the binding of ubiquitylated substrates to the proteasome.[10]

3.2. Nuclear assembly and disassembly

Elucidation of the mechanism underlying nuclear assembly and disassembly has been greatly facilitated by the development of an in vitro system using *Xenopus* egg extract. The nuclear membrane serves to physically separate the genomic DNA from the cytoplasm. The nuclear pore complex mediates the trafficking of macromolecules between the nucleus and cytoplasm. *Xenopus* egg extract contains large amounts of disassembled nuclear components including an abundance of nuclear pores. Pioneering work by Lohka and Masui demonstrated that incubation of chromatin with *Xenopus* egg extract spontaneously induced the formation of a nuclear structure around demembranated sperm nuclei.[26,27] Nuclei formed in vitro in this manner are indistinguishable from eukaryotic nuclei observed in cultured cells and organisms (as visualized by phase contrast and immunofluorescence microscopy). Using this in vitro system, nuclear import activity can be readily measured by assaying for accumulation of substrates within the reconstituted nuclei.[28,29] *Xenopus* egg extract was also shown to reconstitute nuclei using purified lamda DNA as template.[30] This breakthrough allowed for identification of discrete intermediates in chomatin assembly.

In contrast to interphase extract, which promotes nuclear assembly, mitotic *Xenopus* egg extract promotes nuclear disassembly.[26,31] Mitotic *Xenopus* egg extract can be prepared from unactivated eggs in the presence of a calcium chelator or addition of recombinant Cyclin B to drive interphase extract into mitosis.[23,30] Addition of intact nuclei to mitotic extract results in nuclear envelope breakdown and vesicularization, lamin solubilization, and chromosome condensation. Thus, *Xenopus* egg extract represents a powerful biochemical system to study the number membrane.[13]

3.3. DNA replication and repair

Although studies in yeast have provided critical insights into proteins involved in the initiation of DNA replication, our understanding of the biochemical mechanisms and temporal events of eukaryotic DNA replication has been driven in large part by work using *Xenopus* egg extract. A large stockpile of material that is capable of supporting a rapid and complete round of chromosomal DNA replication is present within *Xenopus* egg extract, thus making it a powerful system to study this process. This in vitro system has been used in pioneering studies on the initiation of DNA replication.[32] Demembranated sperm chromatin added to *Xenopus* egg extract undergoes a single round of semiconservative DNA replication. DNA is replicated efficiently but is only observed upon efficient nuclear assembly.[33] Evidence that structural components of the nucleus are essential for replication initiation comes from studies in which lamins are immunodepleted; nuclear assembly is not inhibited, but the nuclei formed are not capable of initiating DNA replication.[34,35] Subsequent advances have led to the development of modified *Xenopus* egg extract capable of replicating chromosomal DNA in the absence of nuclei.[36] Such extract supports the replication of small DNA (e.g., plasmids). This feature has made it possible to study the effects of DNA topology and sequence on DNA replication. Studies using such modified *Xenopus* egg extract along with studies using traditional 'nuclear assembly' egg extract have provided detailed insights into the formation of the pre-replication complex and its mechanism of activation.[37]

It is imperative for the survival of an organism that genomic integrity is preserved. To accomplish this goal, eukaryotic cells utilize numerous types of DNA damage and replication checkpoints. Biochemical studies using *Xenopus* egg extract have helped to identify the components and mechanisms involved in these checkpoints. For example, the functions of key regulators of the DNA checkpoint pathways in the mammalian DNA damage response, such as ATM and ATR, have been illuminated using *Xenopus* egg extract.[38] Using the *Xenopus* egg extract system, Walter and Newport demonstrated that uncoupling of MCM helicase (a helicase essential for DNA replication) and DNA polymerase in response to UV irradiation is necessary for checkpoint activation.[39]

3.4. Microtubule polymerization

Xenopus eggs, which are abundant in proteins involved in regulation of microtubules, have been instrumental in our understanding of microtubule dynamics.[40] Microtubule ends undergo phases of polymerization ('rescue') and depolymerization ('catastrophe') in a stochastic fashion in a process known as 'dynamic instability' that was first coined by Mitchison and Kirschner based on their studies of *Xenopus* egg extract.[11] The biochemical tractability of the *Xenopus* egg extract system has led to the identification of numerous microtubule-associated proteins that modulate microtubule polymerization.[41]

The ability to reconstruct a complete, functional mitotic spindle in a test tube using *Xenopus* egg extract has been a watershed for the biochemical interrogation of spindle formation and function.[42]

During mitosis, microtubules undergo dramatic reorganization to mediate sister-chromatid separation during cell division. Because the cell cycle state can be synchronized in *Xenopus* egg extract, this system has been invaluable in our understanding of the role of microtubules in the formation of the mitotic spindle and chromosome segregation.[43,44] The addition of fluorescent tubulin to *Xenopus* egg extract made it feasible to perform time-lapse image analysis of the mitotic spindle to study its dynamic properties.[44,45] Using *Xenopus* egg extract, Rebecca Heald in the lab of Eric Karsenti showed that a bipolar spindle can spontaneously assemble around DNA-coated beads, suggesting that bipolarity is an intrinsic property of microtubules that form around chromatin during mitosis.[46] Finally, taking advantage of the capacity for *Xenopus* egg extract to support mitotic spindle formation, a high throughput screen for inhibitors of mitotic spindle assembly led to the identification of the compound diminutol.[47]

3.5. Actin dynamics

The actin cytoskeleton has been successfully reconstituted using *Xenopus* egg extract. As with microtubules, actin nucleation and the rate of polymerization are regulated by numerous actin-binding proteins.[48] Our understanding of actin dynamics and its interacting partners has been advanced by the successful reconstitution of actin in egg extract.[12,49,50] *Listeria monocytogenes* is an intracellular bacterium that utilizes an actin-based mechanism in which an elongated structure containing actin filaments ('comet tail') is assembled to propel the bacterium within the host cell. Using *Xenopus* egg extract, Theriot and co-workers were able to observe the actin-based motility of *Listeria monocytogenes*.[12] Depleting *Xenopus* egg extract of actin depolymerizing factor (ADF)/cofilin (XAC) resulted in increased length of the comet tail, demonstrating that XAC is involved in actin filament turnover during comet tail formation.[51]

Xenopus egg extract is an ideal system to dissect the components of signal transduction pathways that regulate the dynamic properties of actin. The actin cytoskeleton is regulated by a diverse set of membrane-proximal cues that recruit and activate Wiskott Aldrich Syndrome Protein (WASP) family members, which associate with the Arp2/3 complex to initiate the polymerization of new actin filaments. Using *Xenopus* egg extract, Cdc42, a member of the Rho family of GTPases, and phosphatidylinositol 4, 5-bisphosphate have been shown to activate the Arp2/3 complex through WASP to induce actin assembly.[49,50] Taking advantage of this system, the Kirschner group purified Toca-1, a member of the evolutionarily conserved PCH protein family, from *Xenopus* egg extract and demonstrated that it is required for Cdc42-mediated activation of WASP.[52] The fluorescence of pyrene-labeled actin monomers increases 20- to 30-fold upon polymerization, and provides a fast and quantitative assay for actin polymerization. Taking advantage of this property, an HTS was performed in *Xenopus* egg extract that identified a cyclic peptide (Wiskostatin) that maintains WASP in an autoinhibitory state via an allosteric mechanism.[53]

3.6. Apoptosis

Programmed cell death (apoptosis) has been successfully reconstituted using *Xenopus* egg extract.[54] Apoptosis is mediated by the activation of a cascade of serine proteases of the caspase family.[55] These activated caspases ultimately impinge on a large number of cellular targets, thereby leading to cell death. In *Xenopus* egg extract, apoptosis occurs spontaneously when the extract is allowed to incubate at room temperature for an extended period of time.[56] Alternatively, apoptosis can be induced in *Xenopus* egg extract upon addition of purified cytochrome c to extract depleted of the mitochondrial fraction. In such a system, markers of apoptosis (e.g., caspase activation, chromatin condensation, and nuclear fragmentation) can be readily monitored. Using this system, the Kornbluth group demonstrated a link between decreased nutrient status, caspase activation, and apoptotic cell death.[57] *Xenopus* egg extract thus represents a powerful tool to study apoptosis.

4. The Wnt pathway

4.1. Overview of the Wnt pathway

The Wnt pathway controls many aspects of embryonic development and tissue maintenance.[58] Wnt dysregulation leads to various developmental defects and has been linked to many types of cancer in humans.[58,59] In the absence of the Wnt ligand, cytoplasmic β-catenin levels are kept low by the β-catenin destruction complex, which is composed of Axin, adenomatous polyposis coli (APC), glycogen synthase kinase 3 (GSK3), and casein kinase 1α (CK1α).[60] Axin is a scaffolding protein that brings together the destruction complex components and is the limiting factor in β-catenin degradation. CK1α primes β-catenin for subsequent phosphorylation by GSK3, targeting β-catenin for ubiquitin-mediated proteolysis. Wnt signaling is initiated when Wnt ligands bind Frizzled receptors (Fz) and the co-receptor, low-density lipoprotein-related receptor 5/6 (LRP5/6). As a consequence, the β-catenin destruction complex is recruited to the plasma membrane, and β-catenin degradation is inhibited. Cytoplasmic accumulation of β-catenin leads to its translocation into the nucleus, where it turns on Wnt target gene transcription by activating T-cell factor/lymphoid enhancer factor (Tcf/Lef) family proteins. In the absence of β-catenin, Tcf/Lef acts as a transcriptional repressor when bound to Groucho. In the presence of β-catenin, Groucho is displaced and Tcf/Lef interacts with other nuclear proteins to initiate efficient transcription of Wnt target genes.[61]

The Wnt pathway is constitutively active in many human cancers, particularly colorectal cancer.[62] In the intestinal epithelial cells of familial adenomatous polyposis patients, the APC gene is inactive or defective.[63,64] Consistent with a role for Wnt signaling in transformation of epithelial cells, loss of APC in mice also leads to the formation of intestinal polyps.[65] Remarkably, over 80% of sporadic colorectal cancers have mutations in APC and 10% in β-catenin.[66] To date, all of the mutations in the Wnt pathway characterized in colorectal cancer lead to abnormal accumulation of β-catenin and chronic activation of the Wnt pathway. In addition to being the initiating event in colon cancer formation, continual Wnt pathway activation is thought to be required for maintenance of late-stage colon cancers.[67] Inhibition of Wnt signaling by RNAi or expression of a dominant-negative Tcf/Lef in colon cancer cell lines reduces their growth and reverses the epithelial–mesenchymal transition.

Aberrant Wnt signaling is also involved in cancers such as hepatocellular carcinoma, ovarian cancer, prostate cancer, and Wilms tumor.[60] Thus, inhibition of Wnt signaling may represent an effective therapeutic modality for the treatment of many common human cancers. Currently, there are no small compounds in late clinical trials or in clinical use that inhibit the Wnt pathway.

4.2. Use of *Xenopus* egg extract system for studying the Wnt pathway

Xenopus embryos have played an important role in our understanding of the Wnt pathway and its role in early vertebrate development.[68] The pathway is activated in the dorsal side of the embryo, an important step in organizing the tissue axis of the embryo. Ectopic expression of Wnt induces a secondary dorsal axis, and inhibition of the pathway reduces dorsalization of the embryo.[68] This phenotypic readout is simple and clear, and it has been used with great success to study the roles of Wnt components.

Xenopus egg extract is a biochemically tractable, in vitro system that has been used to reconstitute cytoplasmic aspects of the Wnt pathway.[21] The regulated degradation of β-catenin, which is central to Wnt signaling, has been reconstituted using cytoplasmic egg extract. Extract prepared from *Xenopus* eggs is transcriptionally inactive, which is likely due to inaccessibility of the assembled chromatin to RNA polymerase II.[69–72] In contrast, *Xenopus* egg extract has a high capacity for translation and can readily translate exogenously added mRNA.[19,20,22,23,73,74] The translational capacity of extract can be inhibited by addition of cycloheximide or by simply freeze-thawing the extract.[19,20,22,23] Thus, β-catenin stability in extract can be directly assessed without complications from changes in its steady-state levels.[21]

In addition, known components of the Wnt pathway, such as Dsh and LRP6, can be added as purified proteins directly into the system to effect changes in the kinetics of β-catenin degradation, thereby allowing for quantitative analysis of the Wnt pathway.[21,75] Using a biochemical approach that involved depleting and supplementing various Wnt components in *Xenopus* egg extract, the regulation of β-catenin turnover and the role of APC-axin-β-catenin interactions were examined; these data were used to develop a mathematical model of the Wnt pathway.[21,76]

4.3. HTS screening of the Wnt pathway using *Xenopus* extract

Previous studies have shown that activation of the Wnt pathway promotes degradation of Axin and stabilization of β-catenin; conversely, inhibition of the Wnt pathway promotes stabilization of Axin and turnover of β-catenin.[75–79] Because Axin and β-catenin turnover represent independent readouts for Wnt signal transduction, and their stabilities are regulated in opposite directions, measuring changes in both of their levels represents a powerful approach to monitor Wnt pathway activity. Addition of a recombinant form of the intracellular domain of LRP6, which had been previously shown to promote degradation of Axin and stabilization of β-catenin, resulted in activation of the Wnt pathway.[75,80]

A high throughput screen using *Xenopus* egg extract to identify small molecules that inhibit the Wnt pathway was recently undertaken.[80,81] To facilitate detection of β-catenin and Axin levels in a high throughput format, β-catenin and Axin proteins were fused to firefly and Renilla luciferase, respectively. The high throughput screen identified pyrvinium, an FDA-approved drug, as an inhibitor of the Wnt pathway. Inhibition of the Wnt pathway by pyrvinium was validated by in vivo studies.[80] Injection of pyrvinium into developing *Xenopus* embryos induced ventralization and blocked Xwnt8-induced secondary axis formation, confirming that the compound was active in an organism. Further studies showed that pyrvinium was active in *Drosophila* and *C. elegans*, indicating that the molecular target of pyrvinium was conserved across phyla[80] Reconstitution studies using purified components ultimately identified casein kinase 1α (CK1α) as the cellular target of pyrvinium and suggested that pyrvinium may allosterically activate CK1α. Further studies have subsequently shown that pyrvinium is biologically active in mouse models.[82]

5. Future studies of other embryonic signaling pathways using *Xenopus* egg extract

The Wnt, Hedgehog, and Notch pathways play critical roles in regulating cell fate during the embryonic development of metazoans.[58,83,84] Not surprisingly, these signaling pathways are required to maintain stem renewal and homeostasis in adult organs. Together, these pathways are hypothesized to be inappropriately regulated in the vast majority of solid human tumors.[85,86] Recent evidence strongly suggests the existence of a population of stem cells (cancer stem cells) capable of generating all of the cell types found in a given tumor.[87,88] Given their roles in stem cell renewal and differentiation, it has been predicted that targeting these three pathways would have a major impact on regenerative medicine and cancer therapy. Currently, there are no drugs for general use in the clinic that target Wnt, Hedgehog, or Notch signaling pathways.

There are strong structural and regulatory parallels between Wnt, Hedgehog, and Notch signaling pathways (Fig. 2). All three pathways ultimately impinge on a transcription factor so as to regulate a program of gene expression that alters cellular behavior and fate. Many of the genes regulated by these three pathways are involved in cellular growth and proliferation.[86,89,90] For all three pathways, proteolysis of a critical transcription factor occurs via the ubiquitin-proteosome system. In the case of Wnt and Hedgehog, proteolysis of the transcription factor occurs in the absence of ligand and is inhibited in a ligand-dependent manner.[58,60,83,91] For Notch signaling, proteolysis occurs in a ligand-dependent manner to generate Notch intracellular domain (NICD), the active transcription factor. Growing evidence, however, suggests that ubiquitin-mediated turnover may limit the activity of the liberated NICD.[84,92–94] Thus, as with the Wnt and Hedgehog pathways, proteolysis may represent a mechanism for regulating Notch signaling. As described above for the Wnt pathway, small molecules that modulate the turnover of transcription factors that mediated Hedgehog and Notch signaling could similarly represent attractive strategies for targeting these two pathways. A brief description of the Hedgehog and Notch pathways is given.

5.1. The Hedgehog pathway

Hedgehog ligands are synthesized as precursor proteins that undergo autocleavage to generate the active ligands. In the absence of Hedgehog, the twelve-transmembrane protein Patched (Ptch) represses the activity of the seven-transmembrane protein Smoothened (Smo). Binding of Hedgehog to its receptor, Ptch, relieves inhibition of Smo activity by Ptch via a largely uncharacterized mechanism. Smo, which is the rate-limiting step in Hedgehog signaling, regulates the activity of all known downstream effectors upon binding of Hedgehog to Ptch.[95] Hedgehog signaling ultimately alters the activity and stability of members of the GLI family of transcription factors in a manner that is tightly regulated by a large molecular weight protein complex (Fig. 2)[83,91] The GLI proteins subsequently regulate a large number of Hedgehog target genes including *Cyclin D1*, *Myc*, and *Bcl-2*.[90]

Dysregulation of the Hedgehog pathway has been implicated in multiple types of human solid tumors. GLI1 was initially identified as a gene that is amplified in malignant glioma and was subsequently shown to be upregulated in other malignant cancers such as basal cell carcinoma.[96–98] Ptch mutations have been implicated in basal cell carcimona, in particular Gorlin syndrome, a rare inherited disease.[99,100] The most clinically advanced drug that targets the Hedgehog pathway, the Smo inhibitor GDC-0449, is currently in clinical trials and has been shown to be effective for the treatment of basal cell carcinoma.[101] Resistance to GDC-0449, however, due to mutations in Smo that block its binding to the drug, has been reported.[102] Patients with mutations in Smo (or elevated GLI) would likely benefit from compounds that target the Hedgehog pathway further downstream.

5.2. The Notch pathway

Notch receptors are single-pass type I transmembrane glycoproteins.[84,103] Notch ligands (Delta/Delta-like, Serrate/Jagged, Lag-2) are themselves single-pass transmembrane receptors. Thus, activation of the Notch pathway requires close juxtaposition of cells. An unusual aspect of Notch signaling is that binding of Notch receptor to its ligand induces successive proteolytic events. The first cleav-

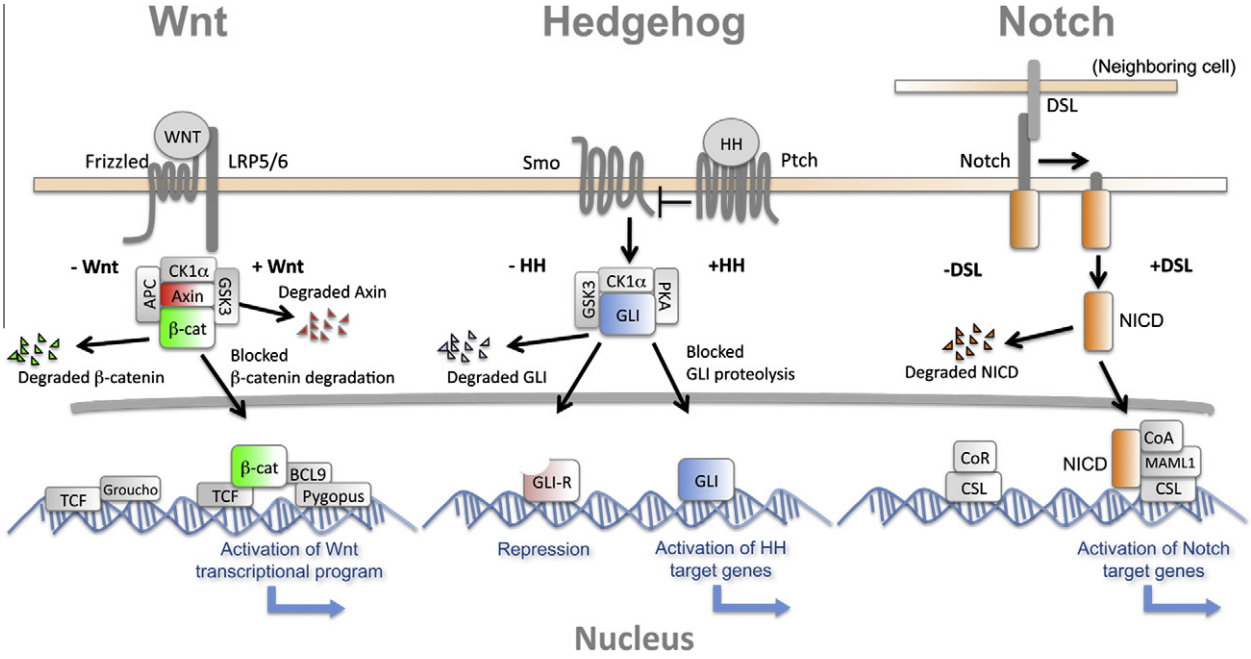


Figure 2. Schematic of the Wnt, Hedgehog, and Notch signaling pathways highlighting cytoplasmic degradation of key transcriptional mediators. For Wnt signaling, the transcriptional coactivator, β-catenin, is degraded in the absence of a Wnt signal, whereas the scaffold protein, Axin, is stable. Binding of Wnt ligands to the Frizzled and LRP5/6 co-receptors results in inhibition of β-catenin degradation whereupon it enters the nucleus. Transcription factors such as TCF, BCL9, and Pygopus form a complex with nuclear β-catenin that leads to activation of a Wnt transcriptional program. In contrast, Axin degradation is stimulated upon Wnt pathway activation. Members of the GLI family of transcriptional factors are the mediators of canonical Hedgehog (HH) signaling. In the absence of HH, GLI proteins are degraded or converted to a lower molecular weight form (GLI-R), which acts as a transcriptional repressor. Proteolysis of GLI occurs in a cytoplasmic complex containing several kinases including glycogen synthase kinase 3 (GSK3), casein kinase 1α (CK1α), and protein kinase A (PKA). The binding of HH to its receptor, Patched (Ptch), relieves inhibition of the seven membrane-spanning protein, Smoothened (Smo), by Ptch via an unknown mechanism. The uninhibited Smo protein subsequently inhibits GLI proteolysis, promoting accumulation of the full-length form and subsequent activation of HH target genes. Notch signaling is initiated upon binding of the transmembrane Notch protein to the Delta/Serrate/LAG-2 (DSL) family of plasma transmembrane ligands present in the membrane of adjacent cells. Binding results in a series of proteolytic cleavage events that ultimately release the Notch intracellular domain (NICD) into the cytoplasm followed by its translocation into the nucleus. In the absence of NICD, the CBF1/Suppressor of Hairless/LAG1 (CSL) family of DNA binding proteins associates with corepressors (CoR) to inhibit Notch target gene transcription. Nuclear NICD interacts with CSL and the transcriptional coactivator Mastermind (MAML1) to recruit transcriptional coactivators (CoA) to initiate transcription of Notch target genes.

age, dependent on ligand activation, is mediated by an ADAM metalloprotease and occurs in the extracellular domain to generate a membrane-anchored Notch extracellular truncation (NEXT) fragment (Bozkulak and Weinmaster, 2009; van Tetering et al., 2009). This NEXT fragment is a substrate for a gamma-secretase, which cleaves the protein within the transmembrane domain. The freed Notch ICD (NICD) subsequently translocates into the nucleus to initiate transcription of Notch target genes.

Blocking gamma secretase activity has been the primary focus for development of therapeutics against the Notch pathway, and several gamma secretase inhibitors (GSIs) are in early clinical trials.[104] Side effects of GSIs, however, including debilitating diarrhea resulting from treatment-induced differentiation of mucus-producing Goblet cells, represent a major hurdle for their further development. The carboxy terminus of NICD contains a PEST domain, which regulates its intracellular stability. The importance of this sequence is suggested by the identification of mutations within this domain in certain Notch-driven tumors.[105,106] These mutations are predicted to increase NICD steady-state levels, thereby resulting in enhanced pathway activation. Interestingly, mutations that alter NICD stability have been shown to be resistant to the effects of GSIs.[107] This observation suggests that promoting the degradation of NICD may be an alternative strategy for inhibiting Notch-driven tumors with stabilizing mutations of NICD.

6. Summary

Xenopus egg extract represents an attractive system that combines the advantages of a phenotypic screen (not limited by preconceived hypothesis as to the appropriate drug target) with the tractability of a biochemical approach (more quantitative and reproducible) for high throughput screening. As discussed herein, the *Xenopus* egg extract system has been exploited to reconstitute a wide range of complex biological processes. *Xenopus* egg extract has been successfully used in high throughput screens (or further validation) for small molecule modulators of the cell cycle, microtubule polymerization, and actin polymerization (Tables 1 and 2).

We have previously shown that the Wnt pathway can be reconstituted in *Xenopus* egg extract and successfully screened for small molecular modulators of β-catenin degradation. Similar to the Wnt pathway, other 'embryonic' pathways (e.g., Hedgehog and Notch) play critical roles in early metazoan development. A common feature of all three is that proteolysis of key transcriptional mediators controls the extent of signaling through the pathway. Thus, as for the Wnt pathway, a screening strategy using *Xenopus* egg extract represents an attractive approach to identify small molecule modulators of the Hedgehog and Notch pathways. Evidence for the feasibility of such an approach comes from our recent studies showing that the transcriptional mediators of the Hedgehog (Gli1) and Notch (NICD) pathways readily undergo robust degradation in *Xenopus* egg extract.[81]

As with any biochemical approach, an obvious drawback to using the *Xenopus* extract system to screen for inhibitors of Wnt, Hedgehog, and Notch pathways is that one cannot screen for modulators of cellular morphology. In addition, because one is limited to screening for cytoplasmic events of these pathways, it is not feasible to screen for compounds that selectively modulate activation of particular receptor subtypes or other events at the level of the receptor (e.g., cell-to-cell communication). Specific transcriptional programs

are ultimately initiated upon activation of the Wnt, Hedgehog, and Notch signaling pathways. To date, however, a system in which transcriptional responses can be readily measured in a high throughput fashion has not been developed. Regardless, the roles of Hedgehog and Notch pathways, like the Wnt pathway, have been well characterized during *Xenopus* embryonic development; thus, in vivo validation of compounds can also be readily performed using *Xenopus* embryos. Although we have focused on screening for small molecule modulators of pathways in which proteolysis plays a central role, we expect that other signaling pathways could similarly be interrogated using *Xenopus* egg extract.

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