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REVIEW

Xenopus: An Ideal System for Chemical Genetics

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Summary: Chemical genetics, or chemical biology, has become an increasingly powerful method for studying biological processes. The main objective of chemical genetics is the identification and use of small molecules that act directly on proteins, allowing rapid and reversible control of activity. These compounds are extremely powerful tools for researchers, particularly in biological systems that are not amenable to genetic methods. In addition, identification of small molecule interactions is an important step in the drug discovery process. Increasingly, the African frog *Xenopus* is being used for chemical genetic approaches. Here, we highlight the advantages of *Xenopus* as a first-line in vivo model for chemical screening as well as for testing reverse engineering approaches. genesis 00:1–12 , 2012. © 2012 Wiley Periodicals, Inc.

Key words: chemical genetics; *Xenopus*; forward and reverse screens; small molecules

WHAT IS CHEMICAL GENETICS?

Chemical genetics is the identification of small molecules that can be used to study any biochemical or physiological process of interest. In this method, chemical tools are used to manipulate protein activity, providing the ability to analyze complex, multicomponent biological processes over time (Walsh and Chang, 2006). For developmental biologists, current genetic methods provide limited temporal control; thus, chemical genetics can offer a complementary approach to loss-of-function mutations.

Chemical genetics, like classical genetics, makes use of forward and reverse approaches. Forward analyses are modeled on classical genetic screens in which the search is driven by a phenotype of interest. Reverse screens are designed to target proteins of interest, either by searching for specific drug–protein interactions or by engineering chemically sensitive proteins.

Large-scale mutagenesis screens using genetic model systems have been used very successfully for many years in identifying genes involved in developmental and physiological events. However, such screens are expensive and time consuming. In addition, with respect to later development and organogenesis, these screens can be limited in scope. During embryonic development, many patterning and signaling systems are used multiple times (Davidson *et al*., 2002; Reya and Clevers, 2005; Van Raay and Vetter, 2004). Thus, if an embryo is disrupted at an early time point because of a mutation, later events using the same pathway become difficult to study. Such mutagenesis screens therefore do not allow for the assessment of the finer temporal control of protein function. The ability to have temporal control over compound addition and thus the modulation of protein function provides a more focused approach to phenotypic assays. This also means that chemical genetic screens are applicable to maternal proteins, which many traditional mutagenesis screens are not, significantly extending the opportunity to identify key endogenous players in biological processes.

Chemical genetic screens have been done on many organisms (Wheeler *et al*., 2011). In this review, we focus on the increasing use of *Xenopus laevis* for small molecule screening. We will also discuss recent devel-

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Table 1
Large Scale Chemical Screens Using Xenopus *for In Vivo* Phenotyping

Assay	Library	No. of compounds	Reference
Pigmentation	Diversity Set, NCI	1990	Tomlinson *et al*., 2009
	Gen-Plus, Microsource	960	Tomlinson *et al*., 2009
Angiogenesis/lymphangiogenesis	LOPAC, Sigma–Aldrich	1280	Kalin *et al*., 2009
TGF-β inhibitor/heterotaxia	Novel compounds	130	Dush *et al*., 2011

Table 2
Examples of Functional Validation of Chemical Screens Using Xenopus

Activity	Compound(s)	Target(s)	Reference
Inhibition of Wnt reporter lines	CCT036477	unknown	Ewan *et al*., 2010
In vitro stabilization of Axin	pyrvinium	CK1	Thorne *et al*., 2010
GSK-3 inhibition	BIO	GSK-3	Meijer *et al*., 2003
Wnt inhibition	JW67 JW74	unknown	Waaler *et al*., 2011
Wnt agonist	QS11	ARFGAP	Zhang *et al*., 2007
Wnt agonist	2-amino-4,6-disubstituted pyrimidine	unknown	Liu *et al*., 2005
Dishevelled inhibition	NSC668036	Dsh-PDZ	Shan *et al*., 2005
Dishevelled inhibition	3289–8625	Dsh-PDZ	Grandy *et al*., 2009
β-catenin-dependent transcription	PKF115–584	TCF/β-catenin interactions	Lepourcelet, 2004

opments in synthetic chemical genetics and the potential use of *Xenopus* for first-line in vivo validation.

Setting up chemical genetic screens

There are two purposes for carrying out phenotypic chemical genetic screens in model organisms. One is to promote research in a given area by obtaining small molecules that can be used to investigate fundamental questions, and the other is as a screen for pharmaceutical reagents that potentially can be used for clinical purposes.

The initial step of a chemical genetic screen is to design an assay or identify a phenotype that will be the target for the screen and to choose the relevant model organism. The screen could be to identify morphological or behavioral changes in the organism as well as alterations in gene expression. Traditional expression screens have previously demonstrated the power, ease and flexibility of *Xenopus* for the identification of gene function (Grammer *et al*., 2000). Morphological changes, such as axis formation, apoptosis, and edema are readily scored by eye. Tadpoles can then be processed for mRNA by *in situ* hybridization or protein by antibody staining. Current screening protocols can include use of tissue-specific transgenic lines such as eye-specific green fluorescent protein (GFP) reporter levels, *in situ* hybridization, microarray technology, or deep sequencing. This two-tiered approach has been nicely demonstrated in a recent study by Kalin *et al*. who identified a number of molecules involved in angiogenesis (Kalin *et al*., 2009).

Once the assay has been designed, a library of compounds is chosen. There are many sources for such libraries as discussed in Wheeler and Brandli, 2009. Of course, the choice of the library depends on the researchers' initial goals and will greatly affect the nature of the positive hits. For example, the screen may be designed to test analogs of known compounds with identified target proteins [e.g., screening a library of pyrimidine-based Wnt agonists, (Liu *et al*., 2005)]. Alternatively, the screen could be designed with a functional goal [e.g., disruption of thyroid hormone function, (Fini *et al*., 2007)]. Finally, the screen could be designed with a biological process in mind [e.g., molecules that affect melanocyte development, (Tomlinson *et al*., 2009a)]. Some recent examples of chemical screens using Xenopus are shown in Table 1.

Once the screen has been carried out and hits obtained, these hits need to be validated. This can be done in a number of ways including determining the mechanism of action, identifying potential targets, and testing for interactions. Compound effects can be assessed in secondary assays or by showing similar effects in other organisms. Validation of drug–protein interactions is likely the most challenging part of this procedure. Specificity and off-target effects can also be difficult to assess. Use of *Xenopus* can improve the efficiency of validation: first, perturbations of conserved signaling pathways leads to stereotypical phenotypes which are well documented, and, second, overexpression of a putative protein target in *Xenopus* can allow the researcher to do simple "competition" assays. Table 2 gives some examples of functional validation carried out using Xenopus.

The *Xenopus* model and why it is a useful addition to chemical genetics

Xenopus has a long history in the fields of developmental biology, teratology and toxicology but is relatively new to chemical genetics. Frog Embryo Teratogenesis Assay–*Xenopus* (FETAX) is an assay used for many years to screen for effects of known chemicals on early developmental stages (Longo *et al*., 2008; Richards and Cole, 2006) and protocols exist to study the effects of small molecules on metamorphosis. However, until recently, chemical genetic screens had not been carried out in *Xenopus* embryos, and screens using zebrafish have led the way in vertebrates. In recent years, the use of genetics in *Xenopus tropicalis* (Goda *et al*., 2006) and work by the authors and others in chemical genetic phenotypic screens have shown *Xenopus* to be an excellent model system for such screens (Adams and Levin, 2006; Longo *et al*., 2008; Tomlinson *et al*., 2005, 2009a; Wheeler and Brandli, 2009). In fact, *Xenopus* is the only tetrapod vertebrate to have free-living embryos in which embryonic development is neither *in utero* nor *in ovo* and thus is the highest order in which high-throughput screens can be carried out (Wheeler and Brandli, 2009).

Xenopus has many of the advantages that are necessary to carry out phenotypic screens. Its eggs can be easily obtained in large numbers at any time during the year by simple hormone injection, and they can then be synchronously fertilized. This facilitates biochemical, pharmacologic, and statistical analyses. The embryos develop in the petridish in simple salt solutions at room temperature. *X. laevis* embryos are bigger than zebrafish but can still be screened in 96-well plates (Tomlinson *et al*., 2005, 2009a; Wheeler and Brandli, 2009). Compounds can be added to the media, and the vitelline membrane around the embryo is highly porous and accessibility of compounds to the embryo is usually good. Moreover, *Xenopus* embryos are easy to microinject so cell-impermeable compounds can also be simply tested in vivo. In addition, the detailed fate map for *Xenopus* facilitates injection of compounds, as they can be easily targeted to specific areas of the embryo (Dale and Slack, 1987). This is not the case with zebrafish, in which cell migration and mixing during gastrulation prevent targeted injections.

Also unique to *Xenopus* among model organisms is the use of *Xenopus* oocytes as “laboratories” for the study of ion translocators, neurotransmitter receptors, second messenger cascades, calcium-dependent events, and cytoskeletal rearrangements (examples of screens include Peterson *et al*., 2001; Verma *et al*., 2004; Wignall *et al*., 2004). In addition, *Xenopus* oocyte and egg extracts are used extensively to study DNA damage and cell cycle progression (Peterson *et al*., 2006; Dupre *et al*., 2008). As a result, chemical screens making use of these biochemical properties of *Xenopus* have been particularly successful. However, using *Xenopus* for in vivo chemical screening is a comparatively recent development (Tables 1 and 2).

Forward screens using *Xenopus*

The Wheeler and Brandli labs have shown *Xenopus* to be a useful model for developmental chemical genomic screens (Brändli, 2004; Tomlinson *et al*., 2005).

Figure 1a outlines how we do a large-scale screen with *Xenopus* embryos. A full description of the methodology can be found in Tomlinson *et al*. (2012). Unless interested in specific early developmental effects or gastrulation phenotypes, we apply compounds postgastrulation. Our previous studies have shown that the degree of nonspecific toxicity of compounds is much lower if applied at this time (Tomlinson *et al*., 2005). Embryos are viewed under a low-magnification dissecting microscope. Phenotypes easily scorable include pigmentation, effects on the eye (i.e., small eye) and effects on shape of the head, body, and fin (Fig. 1b).

In forward screens, it is important to target a specific phenotype or organ to identify compounds with defined, specific effects or modes of action. Mutagenesis screens often examine a phenotype of the whole embryo. For instance, in *Drosophila*, phenotypic changes in the outer cuticle pattern led to insight into important patterning and morphogenesis pathways (Nusslein-Volhard and Wieschaus, 1980). In some of the initial *Xenopus* screens, pigmentation was looked at, as changes in pigment pattern of the embryo are easily scorable. Pigment cells are derived from pluripotent neural crest cells and can give rise to melanoma. So effects on pigment can relate to issues of neural crest induction, cell morphology, cell migration, cell biochemistry, as well as cancer biology (Blackiston *et al*., 2011; Tomlinson *et al*., 2009a,b; White *et al*., 2011).

In our screen we identified a number of compounds that affect pigment cell migration (Tomlinson *et al*., 2009a). Further analysis of one of these showed it to be a novel matrix metalloproteinase (MMP) inhibitor (Tomlinson *et al*., 2009b). MMPs are a family of proteins known to have important roles in cell migration, inflammation, angiogenesis, and cancer (Page-McCaw *et al*., 2007). Another compound (NSC210627) was characterized as an inhibitor of dihydroorotate dehydrogenase (DHODH). This led to the identification of leflunomide, a structurally distinct DHODH inhibitor, which phenocopied NSC210627 in *Xenopus* and zebrafish. Other compounds were also identified that affect pigment cell morphology, and the production of pigment and their cellular targets have begun to be determined (Tomlinson *et al*., 2009a). In a powerful validation of this approach, subsequent tests on melanoma cell lines and

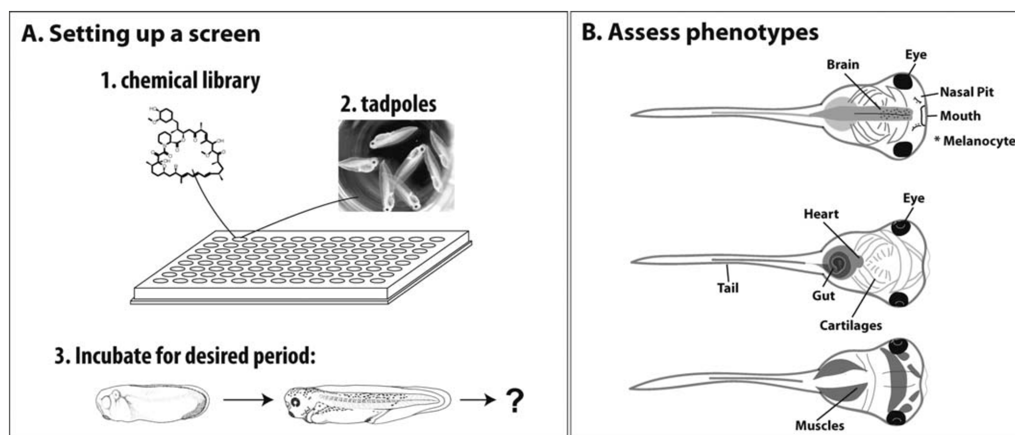

FIG. 1. Screening chemical libraries using *Xenopus.* (**A**) Chemical libraries are arrayed in 96-well plates. Multiple tadpoles can be cultured in each well and are added at a stage of interest. Tadpoles are then cultured until desired maturity. (**B**) The transparency of the free-swimming tadpoles allows easy assessment of a variety of organs some examples of which are shown.

in vivo mouse models showed leflunomide to be a potent inhibitor of melanoma growth (White *et al.*, 2011). Human clinical trials are now underway.

In another screen small molecule regulators of lymphatic and blood vascular system development were identified (Kalin *et al.*, 2009). Interestingly, when the same library was used on zebrafish the screen identified fewer of the antiangiogenic compounds known to be in the library than the *Xenopus* screen thus suggesting *Xenopus* could be more sensitive in recovering compounds with vascular activity.

A recent study identifying the TGF-β inhibitor heterotaxin nicely illustrates the power of *Xenopus* for in vivo screening (Dush *et al.*, 2011). The establishment of the left-right axis in vertebrates is dependent on TGF-β signaling. In *Xenopus,* phenotypic readouts of TGF-β inhibition are well established; heterotaxia (reversal of organ laterality) was easily scored by examining looping of the heart and guts. In addition, molecular and biochemical consequences of TGF-β perturbation were also easily assessed by expression analysis and western blotting. Although the authors do not identify the direct drug–protein interaction in this study, they place the action upstream of the Smad2 phosphorylation. We expect future studies could include testing TGF-β receptors and ligands in competition with heterotaxin, which may be able to distinguish selectivity of the compound.

Screening with random libraries has its advantages and disadvantages. Random libraries are unbiased and may reveal novel compounds or novel pathways. However, screening with random libraries can rapidly become unwieldy. How does one limit the number of compounds screened? It is possible to screen pools of compounds as shown by Dush *et al.* (2011). But, this may limit the efficacy of each individual molecule and obscure its function. Furthermore, identification and validation of protein interactions can be particularly difficult, as target proteins are usually unknown. Additional means are often necessary, as in a recent cell culture study designed to identify compounds that antagonize Wnt signaling. Huang *et al.* used affinity capture experiments in which the compound (XAV939) was immobilized and used as molecular "bait," thus identifying tankyrase as a target protein (Huang *et al.*, 2009). When using *Xenopus,* one could imagine subsequent analyses would then include experiments examining *tankyrase* itself (Chang *et al.*, 2005; Liu *et al.*, 2005).

Targeted screens with small libraries of compounds of a known functionality are also powerful. An example of this is the recent use of histone deacetylase (HDAC) inhibitors to show that HDAC activity is required for left-right patterning (Carneiro *et al.*, 2011). Because *Xenopus* is quite amenable to whole animal analyses, this approach can uncover previously unknown phenotypes for specific protein activities.

Levin *et al.* have also developed an interesting chemical genetic strategy using *Xenopus* (Levin *et al.*, 2006). They use known pharmacologic compounds to correlate specific families of proteins with a chosen biological phenotype. They have taken advantage of a hierarchical structure that can be shown for drug reagents in a number of fields, such as ion transport, neurotransmitter function, metabolism, and the cytoskeleton. They call this an "inverse drug screen" (Levin *et al.*, 2006). The advantage is that it is more efficient than carrying out an exhaustive screen with large numbers of drugs and instead quickly reveals a manageable number of specific molecular candidates that can then be validated and targeted. They have used this method to determine whether the ion flow was important for embryonic left/right patterning (Adams and Levin, 2006; Adams *et al.*, 2006) and to uncover novel prenervous system roles for

the neurotransmitter serotonin (Fukumoto *et al.*, 2005; Levin *et al.*, 2006). They first test for a specific phenotype using known global inhibitors of a cellular function, that is, ion transport. If this causes a phenotype, they then test more specific drugs that target subset pathways of this global inhibitor. Thus, they use a general ion channel inhibitor to show a phenotype and then narrow this down to a specific ion channel or pump (Adams and Levin, 2006). Recently they have also used ion channel drugs to identify new cell types (Blackiston *et al.*, 2011).

Future screens that can be or are being done will make use of *Xenopus*' evolutionary proximity to mammals. Therefore, screens looking at aspects of limb development and regeneration (in the eye, tail, or limb) can be envisaged. Assay systems are also being developed to screen for compounds that affect learning and behavior (Blackiston *et al.*, 2010).

Reverse engineering of chemically dependent systems

As described above, broad chemical screening methods are very useful. However, one drawback is that target specificity can be difficult to validate, particularly if the initial chemical library contains a broad range of compounds, rather than compounds chosen in a pharmacophore or similarity screen. In addition, drug discovery approaches are most readily applied to enzymes, which have clear active sites for targeting inhibition. Many other proteins, such as transcription factors or structural proteins are hard to regulate in this fashion. As a result, chemical screens can be difficult to generalize. Recently, rationally designed methods have become more widespread and developmental biologists are adapting these synthetic strategies to regulate specific protein activities (Nielsen and Schreiber, 2008).

Ideally, drug–protein interactions should be highly specific, with no off-target effects, and function should be regulated rapidly and reversibly. Demonstrating specificity, or eliminating the possibility of off-target effects, is difficult. However, this is also a problem that arises with open-ended screening described above. The best test for specificity is comparison with a genetic mutant (Liu *et al.*, 2007). However, this is often impossible. Although not ideal, the ease of knockdown and overexpression in *Xenopus* often provides a good correlation with specificity. As a result, *Xenopus* assays are frequently used to validate compounds that affect Wnt and other pathways (Liu *et al.*, 2005; Meijer *et al.*, 2003). The drug–protein interaction will also ideally be rapid and reversible; in some cases, competitive inhibitors can be used to test both speed and reversibility, as with small molecule effectors of Hedgehog signaling (Frank-Kamenetsky *et al.*, 2002).

Finally, for generality, the approaches should be applicable to a wide range of targets. For example, the Shokat lab recently designed a system in which a single amino acid substitution in the kinase domain of the Trk receptor results in inhibition of the receptor by a small molecule inhibitor PP1 (Chen *et al.*, 2005). The Ginty and Shokat labs then produced Trk-A knock-in mice that could be combined with a PP1 analogue for rapid, specific and reversible inhibition of TrkA in vivo (Chen *et al.*, 2005). In principle, this approach could be easily generalized; however, to our knowledge, this strategy has not yet been applied to *Xenopus* studies.

Controlling transcription with small molecules

As noted above, proteins such as transcription factors are notoriously difficult to target chemically (compare to kinases, above, which have clear active sites). Researchers frequently bypass this problem by fusing transcription factors with small-molecule-sensitive moieties. In a method that is now routine in *Xenopus*, the ligand-binding domains of hormone receptors such as the glucocorticoid (GR), estrogen and ecdysone receptors can be fused to transcription factors. In the absence of ligand, these chimeric proteins are sequestered. Ligand binding induces a conformational change in the protein, uncovering nuclear localization signals (Fig. 2a). This approach, initially developed for in vivo use by Kolm and Sive in 1995, has been very successful for ligand-dependent transcriptional activation (Kolm and Sive, 1995, summarized in Table 3). In *Xenopus,* fusion constructs can be easily expressed as mRNA and injected into specific tissues, allowing tissue and temporal analysis of transcription factor targets. When combined with activator (VP16) or repressor (engrailed) fusions, this system can be used to turn transcription factor targets on or off in response to the drug dexamethasone (Horb and Thomsen, 1999; Rones *et al.*, 2000). Further refinement of this system makes use of GR fusions with a yeast transcriptional activator, Gal4, which recognizes an upstream activating sequence (UAS) that is absent in vertebrates. Hartley *et al.* had previously demonstrated the utility of Gal4-UAS systems in stable *Xenopus* transgenics (Hartley *et al.*, 2002). Transgenic animals are then produced carrying two plasmids, one encoding the Gal4-GR fusion and the second carrying the UAS driving expression of the target gene. In principle, a tissue-specific promoter could be used to drive expression of the Gal4-GR fusion protein [Fig. 2b, (Hartley *et al.*, 2002)]. Tissue-specific transgenic lines have also been developed for *Xenopus tropicalis* using Gal4-progesterone receptor fusions (GAL4PR) which can be induced using low doses of the synthetic progesterone analogue RU486 (Chae *et al.*, 2002).

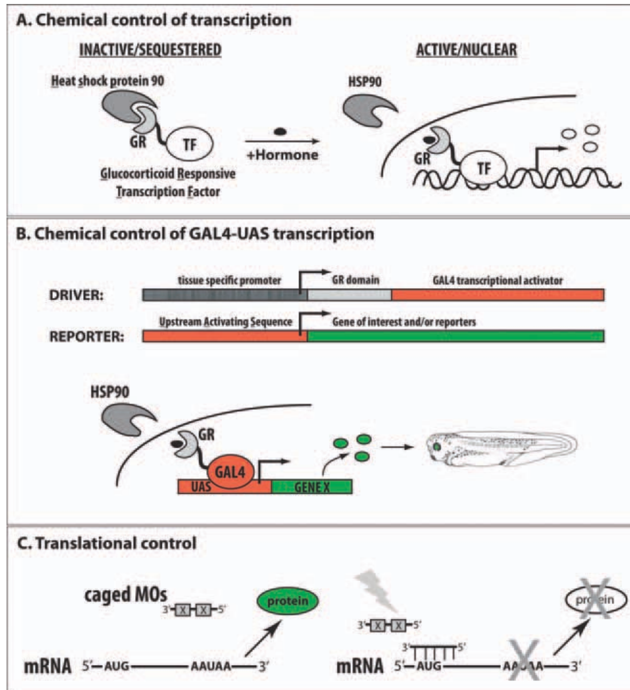

FIG. 2. Chemical control of transcription and translation. (**A**) The ligand-binding domains of hormone receptors such as the glucocorticoid receptor (GR) can be fused to transcription factors. In the absence of ligand, these chimeric proteins are sequestered by heat shock protein-90 (HSP90). Ligand binding induces a conformational change, leading to release of the protein and subsequent transcriptional activation. (**B**) Transgenic animals carrying two plasmids, one encoding the "driver," a GR-GAL4 fusion, and the second carrying the upstream activating sequence (UAS) "reporter," driving expression of the target gene. A tissue-specific promoter could be used to drive expression of the Gal4-GR fusion protein. This process will allow for temporal and spatial control of expression. (**C**) Several antisense methods use synthetic oligonucleotides to block translation. To improve the specificity of antisense morpholino oligonucleotides (MOs), several groups have designed caged MOs, which are unable to bind to their complementary target. Irradiation leads to uncaging and release of the MO, which is then free to inhibit translation.

Although GR-fusions are very useful for inducing gene expression, there is always the potential complication of interaction between endogenous GR signaling and the exogenous fusion proteins. A second commonly used system makes use of the tetracycline repressor protein from *E. coli.* The tetracycline repressor (TetR) protein recognizes the tetracycline operator (tetO/TRE) sequence that can be used to drive gene expression. In the presence of tetracycline, or its analogue doxycycline, the repressor protein loses its affinity for the target sequence resulting in loss of expression, or a "drug off" system. This system has also been modified to a "drug on" system, where a mutant variant of the repressor only binds to the target system in the presence of drug (rTtA). This approach is widely used in mouse and has been adapted to *Xenopus* (Das and Brown, 2004). However, this method has not been exploited in *Xenopus,* in part due to the complexities of maintaining transgenics. In addition, transgenic approaches in other organisms, such as mouse, has demonstrated that introduced DNA is often subject to local effects, such as gene silencing or "leaky" expression due to nearby promoter elements (Clark *et al*., 1994).

Table 3
Controlling Transcription With Small Molecules

Method	Compound	Reference
Hormone responsive transcription		
Glucocorticoid receptor (GR) fusion	Dexamethasone	Kolm and Sive, 1995
Estrogen receptor (ER) fusion	β-estradiol	
combined with engrailed repressor	Dexamethasone	Horb and Thomsen 1999
combined with VP16 activation	Dexamethasone	Rones et al., 2000
Bipartite GAL4/UAS systems		
GR-GAL4 fusion	Dexamethasone	Hartley et al., 2002
Progesterone receptor (PR) fusion	Progesterone	Chae et al., 2002
Bipartite tetracycline repressor/operator	Tetracycline/	Das and Brown 2004
rTtA activator/TRE promoter	Doxycycline	Rankin et al., 2011
Light sensitive transcriptional activation	Photosensitive doxycycline	Cambridge et al., 2009

Most recently, Rankin *et al*. developed two versatile *Xenopus* transgenic lines, one ubiquitously expressing the doxycycline responsive rTtA and a second expressing the responsive TRE-promoter driving a construct expressing an activated thyroid hormone receptor (Rankin *et al*., 2011). These two lines are being made available through the *Xenopus* Stock Centre (University of Portsmouth, UK; see also Pearl *et al*., this issue). In the future, they can be combined with additional lines such as tissue-specific rTtA or TRE-promoters driving other genes. A new library of efficient transgenesis tools are also now available in *Xenopus;* these incorporate GAL4-UAS, recombination capabilities and tissue specificity (Love *et al*., 2011). Furthermore, they could, in principle, be used in combination with injection of mRNA or plasmids expressing the drivers or targets. Combining these approaches will improve specificity of the transgenes while reducing ectopic or "leaky" effects.

Controlling translation with small molecules

For many years, developmental studies in *Xenopus* were primarily limited to overexpression of DNAs, mRNAs or proteins. As a result, determining the activity of a protein was comparatively straightforward. It was more difficult to assess the specific in vivo requirements. RNA-based strategies, such as short interfering

Table 4
Controlling Translation With Antisense Approaches

Method	Oligos	Reference
Antisense morpholino oligonucleotides (MO)	Synthetic	Heasman *et al*., 2000
Antisense phosphorothioate oligos	Modified	Woolf *et al*., 1990
Various modified oligonucleotides locked nucleic acids phosphorothioates phosphoramidates	Modified	Lennox *et al*., 2006
Peptide nucleic acids (platinum conjugates)	Synthetic	Dodd *et al*., 2011
2′-O-methyl oligonucleotides	Modified	Schneider *et al*., 2011
UV-activated morpholino oligonucleotides	Synthetic	Deiters *et al*., 2010

RNAs, have been used to good effect in other systems (Paddison *et al*., 2002). These methods have not worked well in *Xenopus.* A recent study suggests that this is due to a lack of the Argonaute-2 (Ago2) protein in embryos. Ago2 is necessary for dicer dependent nuclease activity and Lund *et al*. have shown that expression of exogenous Ago2 is sufficient for RNAi activity in *Xenopus* (Lund *et al*., 2011). However, for many years RNAi seemed impossible in *Xenopus;* therefore, studies in *Xenopus* have made use of synthetic nonnatural oligonucleotides to target RNA activity, summarized in Table 4.

In 2000, Heasman *et al*. showed that use of 25-base antisense morpholino oligonucleotides (MOs) efficiently blocked translation of target proteins, resulting in loss-of-function phenotypes (Heasman *et al*., 2000). These MOs contain a morpholino-phosphorodiamidate backbone instead of a deoxyribose-phosphate backbone and are therefore resistant to RNase H-degradation, resulting in very stable interactions. Over the past decade, this use of synthetic antisense oligonucleotides to induce loss-of-function phenotypes has become routine in *Xenopus* (Tomlinson *et al*., 2008; Garcia-Morales *et al*., 2009).

Several additional antisense approaches, which have not been as broadly utilized, use the same strategy of eluding RNase H-sensitivity. These include locked nucleic acids (LNA) in which an extra link between the 2′-O and the 4′-C "locks" the ribose group, phosphorothioate substituted nucleic acids (PS), peptide nucleic acids (PNAs), in which the bases are linked to the backbone by methylene carbonyl bonds (Hanvey *et al*., 1992; Obika *et al*., 2001; Wahlestedt *et al*., 2000; Woolf *et al*., 1990) and fully modified 2′-O-methyl oligonucleotides (Schneider *et al*., 2011). Each of these methods has been validated in *Xenopus*. A recent paper compares the efficacy of knockdowns, suggesting that LNA compounds are the most active and specific (Dodd *et al*., 2011; Lennox *et al*., 2006). Thus, together with MOs, *Xenopus* biologists have a range of methods to regulate translation of target proteins. New refinements such as photoactivation, described further below, are improving the spatial and temporal resolution of these methods (see below, and Fig. 2c) (Deiters *et al*., 2010).

Table 5
Controlling Protein Function With Engineered Domains

Method	Compound	Reference
Inducible dimerization	FK1012	Yang *et al*., 1998 Pownall *et al*., 2003
Inducible stabilization	Rapamycin	Liu *et al*., 2006

Controlling protein function with small molecules

Methods that act on DNA and RNA are limited by the cellular machinery and are dependent on RNA production, translation and protein degradation rates. As outlined above, the most rapid way to control gene function is to use small molecules that act directly on target proteins, either by regulating protein availability, activity or stability, uses in Xenopus summarized in Table 5. Thus, it would be ideal to have generalizable approaches such as the chemical targeting of kinases by the Shokat and Ginty labs (Chen *et al*., 2005). One approach that could be easily applied to many proteins is to use ligand-dependent stabilization or degradation domains.

We and others have exploited several alternative ways to control protein activity by fusing a destabilizing peptide tag to the protein of interest. In the absence of drug, the fusion protein is unstable and rapidly degraded. The addition of drug restores protein stability and function. We have previously used this system to regulate GSK-3b in vivo (Liu *et al*., 2007). In this approach, the peptide moiety, derived from the FK506/rapamycin binding (FRB) domain, has been engineered to be unstable in the absence of drug (Banaszynski *et al*., 2006; Stankunas *et al*., 2003). The relevant drug then acts as a "chemical chaperone" which restores stability and function of the fusion protein (Fig. 3a). Two additional systems have been developed using directed evolution (error-prone PCR) to generate unstable variants of FKBP-12 (FKBP*) and dihydrofolate reductase (DHFR*), (Banaszynski *et al*., 2006). These systems have not yet been tested, and pharmacokinetics and pharmacodynamics will need to be assessed.

Of the three systems, the original, derived from an unstable variant of the FKBP-rapamycin binding motif (FRB*), has been validated using a "knock in" strategy in mouse, in which glycogen synthase kinase-3β (GSK-3β) is replaced by a fusion protein, $GSK\text{-}3\beta^{FRB^*}$ (see schematic Fig. 3a, (Stankunas *et al*., 2003). In the absence of rapamycin or rapamycin analogues, these mutant ani-

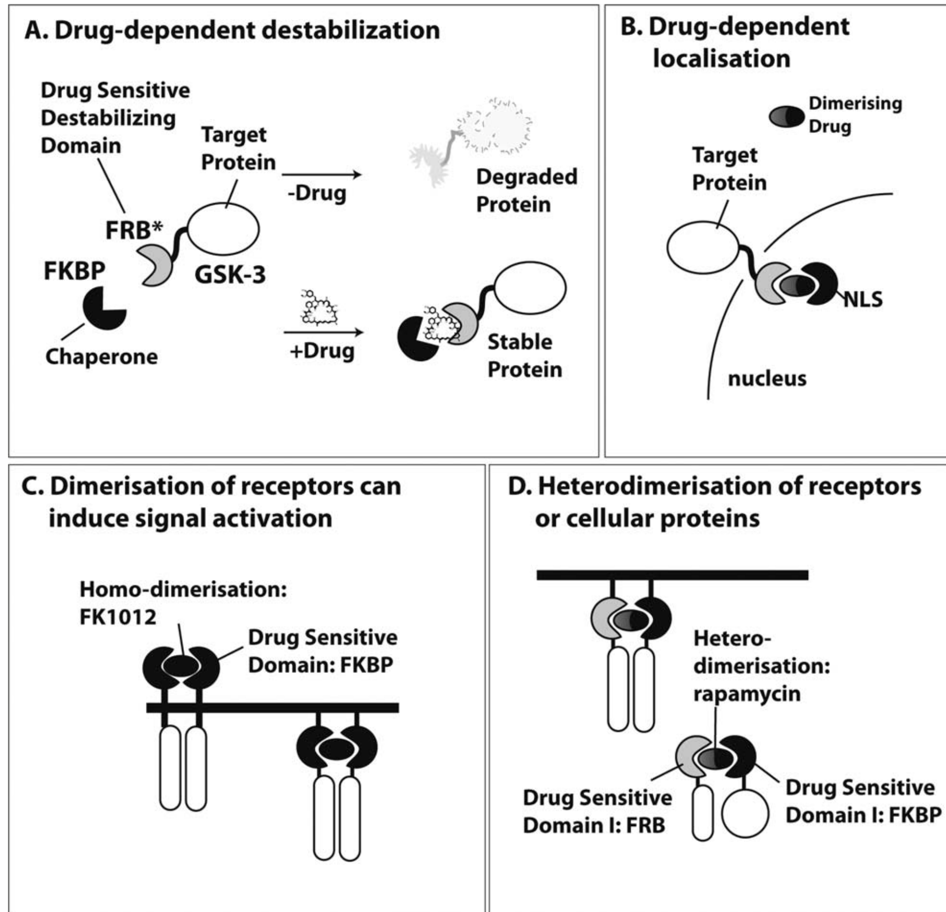

FIG. 3. Chemically engineered control of protein interactions. (**A**) Target proteins are fused to a drug-sensitive destabilizing domain. In this case, the target protein, GSK-3 is fused to the rapamycin binding domain FRB*. In the absence of drug, the fusion protein is unstable and rapidly degraded. The addition of drug promotes dimerization with the chaperone protein FK506-binding protein (FKBP). This interaction restores protein stability and function. (**B**) Similar drug–protein interactions can be used to regulate dimerization. In this schematic, the rapamycin or rapamycin analogues induce dimerization of the FRB domain to FKBP. FKBP can be tagged with a subcellular tag, such as a nuclear localization signal (NLS). In this scenario, the FRB*-tagged protein can be simultaneously stabilized and relocalized. (**C**) This approach has been used successfully to induce dimerization of receptors such as insulin, platelet-derived growth factor (PDGF) and fibroblast growth factor receptors (FGF). Receptors are fused to the FKBP protein. Dimerization is then induced using a synthetic compound, FK1012, which binds two molecules of FKBP with high affinity. Dimerization of these receptors leads to activation of signaling pathways in the absence of endogenous ligands. (**D**) Heterodimerization of receptors, or cellular proteins, can also be induced using rapamycin, as in (B). In this case, two different proteins are tagged with FKBP and FRB, respectively. Addition of rapamycin induces heterodimerization of stoichiometric levels of protein (as opposed to tagging both proteins with FKBP, after (C).

mals are identical to conventional loss-of-function alleles; treatment with drug restores protein stability and function, thus rescuing embryonic phenotypes (Liu *et al*., 2007; Stankunas *et al*., 2003). Washout of the drug or use of a competitive FK506-derived inhibitor allows reversible on/off cycles of protein activity. To avoid off-target effects, analogs of rapamycin and FK506 have been developed (MaRap and Shld-1, respectively). Neither MaRap nor Shld-1 can bind to the natural targets of the parental drugs (Banaszynski *et al*., 2006; Stankunas *et al*., 2003).

We have also validated this approach in *Xenopus* using expression of a chemically dependent luciferase protein and found that stabilization of the target protein was both dose-dependent, temperature-dependent and reversible (Liu *et al*., 2006). Because the drugs directly regulate stability, and hence availability, of the target protein, this system affords much more rapid switching of protein function than transcription-based methods (e.g., Tet-on systems). Furthermore, conditional protein stabilization via the small molecules Shld-1 or rapamycin could be combined with tet-inducible systems to decrease "leakiness" of the transcriptional activator (Almogy and Nolan, 2009).

Finally, because the rapamycin or rapamycin analogues induce dimerization of the FRB domain to the

FK506-binding protein (FKBP), this system can also be used to induce ligand-dependent dimerization (Fig. 3b). In this case, the FRB*-tagged protein is simultaneously stabilized and relocalized when coexpressed with FKBP carrying a subcellular localization tag. This interaction can be reversed using a competitive inhibitor, FK506M, which binds to FKBP, dissociating the rapalogue. Variations on these systems have been used very successfully to induce homodimerization or heterodimerization of receptors (Fig. 3c,d, respectively), including insulin, platelet-derived growth factor (PDGF) and fibroblast growth factor receptors (FGFRs) in vivo (Pownall *et al*., 2003; Yang *et al*., 1998).

We feel the frog would be ideal to rapidly test these systems in parallel. Ideally, we would determine which domain works the best for each target protein. It is also conceivable that the three different destabilizing domains could be used in parallel. For example, if we had three different destabilized fusion proteins (X-FRB*, Y-FKBP*, and Z-DHFR*), we could then use the three different drugs to control timing of three different protein activities, within the same biological system. A long-term application would then be the ability to do "order-of-addition" experiments in any biological process.

Photoactivation reduces signal-to-noise

Precise spatial and temporal control of protein activity is notoriously difficult to achieve in vivo. However, recently, a number of synthetic approaches make use of photoswitchable compounds to refine chemical control. In these approaches, the chemically dependent molecules could be introduced throughout the organism. These would remain quiescent until light-activation "uncages" the activating compounds. Because light can be focused on single cells or specific tissues, these approaches would allow fine control over spatiotemporal protein activity. *Xenopus* is an ideal system for testing and application of these systems, because they are easy to microinject, develop *ex utero,* and are reasonably transparent as swimming tadpoles.

To provide a light-sensitive transcriptional control system, Cambridge *et al*. recently designed two caged analogues of tetracycline: caged doxycyline and caged cyanodoxycline (Cambridge *et al*., 2009). The second compound has the additional advantage of reduced membrane permeability, to increase perdurance within the cell after uncaging. Both compounds are soluble in aqueous solutions and are activated at a wavelength of 330–350 nM, thus GFP excitation could be used as an orthogonal system. Cambridge *et al*. went on to validate these compounds in *Xenopus* using the existing tet-on transgenics (Das and Brown, 2004). Therefore, these compounds should be very useful for in vivo activation of next generation Tet-dependent transgenes (Das and Brown, 2004).

To improve the specificity of antisense morpholino oligonucleotides, Shestapolov *et al*. designed MOs with a short complementary blocking MO that is attached with a photosensitive linker. Irradiation with 360-nm light releases the short fragment, thus freeing the antisense MO (Shestopalov *et al*., 2007). Although this approach seems to work well in zebrafish, it does have the disadvantage that one must synthesize two MOs, in addition to coupling them together. Furthermore, releasing the MOs results in two distinct MO molecules, which could lead to toxicity or off target effects (Deiters *et al*., 2010; Shestopalov *et al*., 2007). More recently, Deiters *et al*. have designed a new caging approach for MOs (Fig. 2c), (Deiters *et al*., 2010). They hypothesized that incorporation of a photosensitive group, 6-nitropiperonyloxymethyl (NPOM), would first disrupt DNA:RNA duplexes. NPOM could then be simply removed by UV irradiation (at 365 nM). As a proof of principle, they showed that they could readily target expression of green fluorescent protein (expressed as an mRNA) in a light-sensitive manner (Deiters *et al*., 2010).

Finally, photoregulation of small molecules can allow precise spatial and temporal control of signaling. Morckel *et al*. reasoned that the addition of the NPOM group to Rockout, a known Rho kinase inhibitor, would render it photoactivatable (Morckel *et al*., 2012). They then treated tadpoles, uncaging the compound on the left versus the right side during gut morphogenesis. These experiments revealed a specific right-sided requirement for Rho kinase in gut looping. These exciting new tools, which should be broadly generalizable, demonstrate the power of combining chemical approaches with developmental studies.

CONCLUSION

In summary, *Xenopus* is an excellent system for a variety of chemical screens. Tadpoles can be visually assayed for morphological changes ranging from heterotaxia, body size and shape, melanocyte migration, skeletal development and organogenesis. By combining chemical structure predictions and enzymatic assays using *Xenopus* lysates, researchers can correlate phenotypes with biochemical activities. Even more powerful, chemical screens can be designed to utilize transgenic animals or overexpression of target proteins. New technologies can also be readily assessed and refined in *Xenopus*, since overexpression and transgenic approaches are routine and economical. All of these approaches allow validation of drug–protein interactions, which can be an extremely challenging part of drug discovery. Finally, *Xenopus* as an in vivo system can provide great insights into the pharmacology of identified compounds as well as anticipating off-target effects (Vandenberg *et al*., 2011).

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