Published in final edited form as:

Genesis. 2012 March ; 50(3): 164–175. doi:10.1002/dvg.22007.

The Hitchhiker's Guide to *Xenopus* Genetics

Anita Abu-Day¹, Mustafa K. Khokha², and Lyle B. Zimmerman^{1,*}

¹Division of Developmental Biology, MRC-National Institute for Medical Research, Mill Hill, London, NW7 1AA, UK

²Department of Pediatrics and Genetics, Yale University School of Medicine, New Haven, Connecticut

Summary

A decade after the human genome sequence, most vertebrate gene functions remain poorly understood, limiting benefits to human health from rapidly advancing genomic technologies. Systematic *in vivo* functional analysis is ideally suited to the experimentally accessible *Xenopus* embryo, which combines embryological accessibility with a broad range of transgenic, biochemical and gain-of-function assays. The diploid *X. tropicalis* adds loss-of-function genetics and enhanced genomics to this repertoire. In the last decade diverse phenotypes have been recovered from genetic screens, mutations have been cloned, and reverse genetics in the form of TILLING and targeted gene editing have been established. Simple haploid genetics and gynogenesis and the very large number of embryos produced streamline screening and mapping. Improved genomic resources and the revolution in high-throughput sequencing are transforming mutation cloning and reverse genetic approaches. The combination of loss-of-function mutant backgrounds with the diverse array of conventional *Xenopus* assays offers a uniquely flexible platform for analysis of gene function in vertebrate development.

Keywords

amphibian; early development; genetics; organogenesis; *Xenopus*; *tropicalis*

Introduction

"They asked him" -- he paused and shivered -- "to tell the Truth, the Whole Truth and Nothing but the Truth.... Only, don't you see? They gave him too much of the truth drug...much too much too much..." "...Oh, I can't remember any of it now... except most of the good bits were about frogs, I remember that." Suddenly he was hooting with laughter again and stamping his feet on the ground. "You would not believe some of the things about frogs," he gasped.'

-Douglas Adams, 'Life, The Universe and Everything' (Adams, 1982)

Xenopus oocytes and embryos have been essential for elucidating the molecular basis of vertebrate axis formation, embryonic inductions, signalling pathways in organogenesis, and the cell cycle (Amaya *et al.*, 1991; Lamb *et al.*, 1993; Philpott and Yew, 2005; Whitman, 1998; Wilson and Hemmati-Brivanlou, 1995). Mass action biochemical assays, as well as sophisticated tissue manipulations such as grafts and explants, can be allied with gain-of-function overexpression via injection with synthetic mRNA. Until recently, the

*Correspondence to: Lyle B. Zimmerman, Division of Developmental Biology, MRC-National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, UK, lzimmer@nimr.mrc.ac.uk, tel. +44 208 816 2114.

conspicuously empty drawer in the frog toolbox was genetics. In addition to their classic use for analyzing gene function, mutations are invaluable for diagnosing off-target effects in other loss-of-function approaches such as morpholino antisense oligonucleotide (MO) and siRNA knockdowns (Scacheri *et al.*, 2004). Loss-of-function genetic backgrounds may also be combined with more conventional *Xenopus* approaches such as gain-of-function mRNA injection for enhancer and suppresser screens to dissect complex processes.

The last five years have seen dramatic advances in *Xenopus* genetics (Harland and Grainger, 2011), including the first screens for induced mutations in amphibians (Goda *et al.*, 2006), cloning of novel mutations (Abu-Daya *et al.*, 2010; Abu-Daya *et al.*, 2009; Geach and Zimmerman, 2010), generation of a meiotic map (Wells *et al.*, 2010) and a genomic sequence assembly (Hellsten *et al.*, 2010), reverse genetics (Goda *et al.*, 2006; Young *et al.*, 2011), and stock centers on either side of the Atlantic (see Pearl *et al.* in this issue). Recent breakthroughs in high throughput sequencing, together with improved genomic resources, are revolutionizing both forward and reverse genetic strategies. Mutation identification, formerly a rate-limiting step relying on tedious positional mapping, can now be accomplished rapidly by exome sequencing (Fairfield *et al.* 2011)(see below).

Genetic screens in invertebrates, principally *Drosophila* and *C. elegans*, underpin contemporary understanding of animal development (Nusslein-Volhard and Wieschaus, 1980; Brenner, 1974; Kemphues *et al.*, 1988). Large-scale developmental screens in vertebrates have focused primarily on zebrafish (Driever *et al.*, 1996; Haffter *et al.*, 1996; Mullins *et al.*, 1994), with more limited screens in other teleosts (Loosli *et al.*, 2000) and mouse (Garcia-Garcia *et al.*, 2005; Wansleben *et al.*, 2011). While zebrafish offer many experimental advantages, the teleost lineage includes an ancient whole-genome duplication, with subsequent sub- and neo-functionalization diversifying roles of duplicated genes (Cresko *et al.*, 2003; Force *et al.*, 1999; Postlethwait *et al.*, 2000; Ravi and Venkatesh, 2008). Systematic comparison with a tetrapod bearing a canonically organized diploid genome is crucial for a broad understanding of vertebrate gene function and applications to biomedical research. Here we discuss the features that make *X. tropicalis* ideal for combining genetic tools with *Xenopus*' broad range of functional assays, and survey recent progress.

***Xenopus* genetics and evolution**

Although the *X. laevis* mutation *anucleolate* aided pioneering animal cloning studies a half-century ago (Elsdale *et al.*, 1958; Gurdon *et al.*, 1958) and albino animals are in wide use, genetic studies in *X. laevis* have been limited to small-scale gynogenetic screens for naturally-occurring mutations (Krotoski *et al.*, 1985) and comparative mapping of immune loci (Nonaka *et al.*, 1997). Systematic genetic studies in *Xenopus* are hindered by the long generation time (1–2 years) and the peculiar speciation mechanism, where rare hybridization events can be accompanied by retention of both parental species' gene sets (allopolyploidy). Extant species form an allopolyploid series from the single known diploid *X. tropicalis* (haploid chromosome number $N=10$) to tetraploids including *X. laevis* ($N=18$) and *X. epitropicalis* ($N=20$), octoploids (e.g. *X. vestitus*, *X. wittei* etc, $N=36$) up to dodecaploids (*X. ruwenzoriensis*, *X. longipes* etc, $N=54$) (Tinsley, 1996). In many cases both parental species of higher ploidy hybrids survive and can be identified (Bewick *et al.*, 2010). While the putative hybridization event giving rise to *X. laevis* is estimated at 40 MYA (Tinsley, 1996), divergence of its dual 'alloallele' gene sets dates even earlier, from the last common ancestor of *X. laevis*' two diploid parental species, sometime after split from the *X. tropicalis* lineage 50–65 MYA (Evans *et al.*, 2004). Functional redundancy between many *X. laevis* alloalleles is retained, impeding genetic analysis and necessitating targeting both genes in

MO knockdowns, but other alloalleles show evidence of sub- and/or neo-functionalization (Chain and Evans, 2006; Hellsten *et al.*, 2007).

Conveniently, one species in the genus, *Xenopus (Silurana) tropicalis*, is a canonical diploid vertebrate. Its genome, at 1.5×10^9 bp in ten chromosomes, is among the smallest among tetrapods, and is highly syntenic to those of amniotes, including humans (Hellsten *et al.*, 2010). The shorter generation time (4–6 months versus 1–2 years for *X. laevis*) also makes it more amenable to genetic analysis. In 1990, Marc Kirschner, then at UCSF, first brought *X. tropicalis* into the US for developmental biology research. Over the next decade several groups, particularly those of Rob Grainger at Virginia and Enrique Amaya at Cambridge, began piloting genetic approaches.

Mutagenesis strategies

Since screening, phenotyping and mapping can all be time consuming, it is important to balance inducing abundant mutations with obtaining interpretable single gene phenotypes and simplifying mutation identification. A variety of chemicals, radiation, transgene insertions, and most recently targeted gene editing can induce sequence lesions. The stage of germline targeted (mature sperm or mitotic spermatogonia) also has practical implications. As described below, the labour of positionally cloning mutations has been greatly reduced by improvements in the *X. tropicalis* genome assembly, gynogenetic shortcuts, and whole exome sequencing.

Since development of transgenesis in *Xenopus* (Kroll and Amaya, 1996), insertional mutagenesis has been attractive for directly cloning disrupted loci. Indeed, this approach has already yielded a dramatic forelimbless phenotype (Abu-Daya *et al.*, 2010) (see below). Remobilization of transposon-based transgenes (Yergeau *et al.*, 2010) shows promise for efficiently generating new insertions, and is compatible with attractive gene-trap and enhancer-trap schemes (Bronchain *et al.*, 1999; Yergeau and Mead, 2007). However, recovery of phenotypes from dedicated insertional screens in *X. tropicalis* has yet to be reported; whether the rate of gene disruption approaches that of chemical mutagenesis remains a key question.

To date, published screens for induced mutations in *X. tropicalis* have used chemical mutagenesis, specifically *in vitro* alkylation of post-mitotic sperm using *N*-nitroso-*N*-ethylurea (ENU) (Goda *et al.*, 2006). ENU creates point mutations forming both nulls (Abu-Daya *et al.*, 2009) and milder hypomorphic alleles (Geach and Zimmerman, 2010) more typical of human disease variants. Since dosage of the toxic and labile ENU is difficult to control, *in vitro* mutagenesis of sperm offers a practical opportunity to select a dose producing both high rates of dominant lethal abnormalities visible in the days after *in vitro* fertilization as well as viable F1 carriers to raise. However, this founder generation will be mosaic for induced mutations, which affects choice of screen schemes (see below). Since a given *in vitro* ENU adduct forms on only one strand of the mature sperm double helix, it is not ‘fixed’ on the complementary strand until DNA replication and repair post-fertilization. 25% or less of the F1 germline carries any given mutation induced *in vitro*, and different germ cells in an F1 individual may carry different constellations of mutations. Alternatively, mutagenizing mitotic spermatogonia by injecting adult males with ENU produces non-mosaic F1 offspring, but animals need several months or longer to recover after treatment before breeding.

Forward Genetic Screens: Inbreeding and Gynogenesis

Gynogenesis, or generation of viable offspring without contribution from a paternal genome (Tompkins, 1978), has been used extensively in zebrafish screens and mapping (Johnson *et*

al., 1995; Johnson *et al.*, 1996; Streisinger *et al.*, 1986) and is an enormous advantage for *Xenopus* genetics. Simple and efficient, it reduces time and colony space to breed mutations or transgenes to homozygosity, and also provides a useful rapid mapping shortcut (Khokha *et al.*, 2009) (see below). The first screens in both *X. laevis* and *X. tropicalis* used gynogenesis to uncover naturally-occurring mutations in outbred animals (Krotoski *et al.*, 1985; Noramly *et al.*, 2005); several mutations were also uncovered via orthodox inbreeding (Grammer *et al.*, 2005).

In a conventional forward screen scheme, phenotypes are uncovered in the third generation following mutagenesis (Figure 1A)(Driever *et al.*, 1996; Haffter *et al.*, 1996). After *in vivo* mutagenesis of G0 males and two outcross generations, F2 siblings are randomly paired and their offspring inspected for recessive phenotypes in simple Mendelian ratios (25% mutant offspring from 1/4 of the F2 random sibling pairs). In addition to the colony space and time required to raise two generations to sexual maturity, this design is not practical with the mosaic F1 produced by *in vitro* mutagenesis: since <1/4 of F2 offspring of mosaic F1 are carriers of a given recessive allele, <1/16 random F2 sibling pairs will produce phenotypic clutches.

In contrast, gynogenesis allows recessive phenotypes to be uncovered in the offspring of heterozygous carrier females (Goda *et al.*, 2006; Khokha *et al.*, 2009; Krotoski *et al.*, 1985; Noramly *et al.*, 2005). As with other amphibians and fish, *Xenopus* oocytes do not complete second meiosis by ejecting the second polar body until shortly after fertilization. UV-inactivation of the sperm genome followed by *in vitro* fertilization yields haploid embryos, which typically develop for several days with posterior defects and truncations, but relatively complete anterior structures. Haploids can be efficiently rescued to viable diploids with a simple icewater cold shock to suppress polar body formation and retain both maternal sister chromatids (Goda *et al.*, 2006; Khokha *et al.*, 2009). Alternatively, haploids can be rescued by allowing the first round of zygotic DNA replication, then blocking cytokinesis with cold shock or pressure (Tompkins and Reinschmidt, 1991), but with significantly lower efficiency. Androgenetic haploids, with no maternal genetic contribution, can also be made by UV-irradiation of eggs and fertilization with untreated sperm (Elsdale *et al.*, 1960).

Gynogenesis minimizes colony space and generation time required for screens. With *X. tropicalis*' large number of eggs (up to 9000 from one ovulation), screening and much preliminary mapping, or even cloning by direct sequencing, are possible using individual adult female carriers rather than families. Gynogenetically-derived embryos (gynogenotes) also provide extremely useful rapid low-resolution map information (see below). In the first *X. tropicalis* gynogenetic screen for naturally-occurring mutations, Grainger and colleagues isolated 10 heritable defects affecting diverse developmental processes from wild-caught females (presumably outbred with high allele diversity)(Noramly *et al.*, 2005).

Unsurprisingly, chemical mutagenesis greatly increased the yield of phenotypes. A pilot gynogenetic screen of 192 mosaic F1 females derived from *in vitro* mutagenesis yielded 77 candidate post-neurulation phenotypes (Goda *et al.*, 2006). More than 35 of these selected for further analysis have proven to be heritable, with only a handful not recovered despite mosaicism of the females used for gynogenesis. Nonspecific gastrulation defects were filtered out by focusing on post-neurulation organogenesis and differentiation phenotypes up to Nieuwkoop & Faber stage 45 (4 days post-fertilization). Phenotypes affecting a variety of processes including neural crest migration and differentiation, cardiogenesis and heart function, primitive haematopoiesis, eye and ear development, axis elongation, left-right asymmetry and others were described. Nearly all similar phenotypes sorted into different complementation groups, with the exception of two independently-isolated non-

complementing alleles (*mrs lot^{mh187}* and *mrs lot^{mh19}*). Interestingly, these map to an interval containing the giant protein *titin*, a large enough target to hit twice even in a small screen.

Mapping Frog Mutations

In addition to positionally cloning forward mutations, linkage analysis helps prove that loci known by specific sequence or transgene insertion, rather than secondary mutations, are responsible for specific phenotypes. Meiotic recombination events are the currency of genetic mapping, and relative to other vertebrate genetic models, *X. tropicalis*' abundant eggs translate to unparalleled wealth: a single mating can produce enough meioses to clone a mutation by conventional positional methods. In the past few years, addition of an *X. tropicalis* genome assembly (Hellsten *et al.*, 2010) and meiotic map (Wells *et al.*, 2010), accompanied by a series of breakthroughs in genomics technologies, have greatly simplified mutation identification.

Gynogenetic centromere linkage for low-resolution linkage assignment

Rather than scan the entire genome for linked polymorphisms, a simple shortcut has been developed to take advantage of the small number of chromosomes and ease of gynogenesis in *X. tropicalis*. In a small number of PCR reactions, this method can rapidly locate most *X. tropicalis* mutations to a genetic region (Khokha *et al.*, 2002), which could then be inspected for candidate genes.

As seen in Figure 2, gynogenotes contain the sister chromatid products of meiosis I, held together during recombination with non-sister chromatids at their centromeres, meaning that nearby regions are always homozygous, while progressively more distal regions are more likely to be heterozygous due to intervening recombination. Likewise, phenotypically mutant embryos are homozygous at the mutant locus. Two useful pieces of information fall out of this observation. First, the phenotypic ratio in gynogenetically-derived embryos reflects the gene-centromere distance (Johnson *et al.*, 1995; Johnson *et al.*, 1996; Reinschmidt *et al.*, 1985; Streisinger *et al.*, 1986; Thiebaud *et al.*, 1984), with centromeric loci giving up to 50% mutant gynogenotes, decreasing to a plateau of ~10% (~40 cM) beyond which multiple intervening crossovers predominate (Khokha *et al.*, 2009). Second, even distal mutations usually appear linked to one of the 10 *X. tropicalis* centromeres when analyzed in pools of gynogenetic embryos. With the exception of very telomeric loci, most homozygous mutant gynogenotes will have no crossovers between the mutant locus and their chromosome's centromere, and even when multiple intervening crossovers are present, half of these restore parental linkage. Linkage to a specific centromere is only disrupted when a locus is so distal that multiple intervening recombination events are present in the majority of mutant gynogenotes. In practice, assigning a mutation to a specific chromosome usually requires only interrogating two pools of mutant and wild type gynogenote DNA with polymorphic markers corresponding to the 10 *X. tropicalis* centromeres (Figure 3E). The genetic locations of the ten *X. tropicalis* chromosomes have been identified and confirmed cytogenetically (Khokha *et al.*, 2009) and used to map mutations to specific chromosomes in a small number of PCR reactions (Geach and Zimmerman, 2010).

Higher resolution mapping to specific genes can be accomplished using conventional positional cloning methods as well as candidate gene approaches (Abu-Daya *et al.*, 2009; Geach and Zimmerman, 2010). Efficiency of these strategies has greatly increased with improved genome contiguity and annotation and meiotic map marker density, and they remain useful for evaluating candidate genes. However, since these approaches are shared by other systems, and because they are being replaced by sequence-based direct identification/mapping, conventional high-resolution mapping will not be discussed further here.

Reverse genetics and rapid mapping by high throughput sequencing

In contrast to conventional ‘forward’ genetics (isolate a phenotype, then identify the responsible gene), reverse genetics refers to engineering or identifying a specific mutated sequence for subsequent functional analysis. While antisense morpholino oligonucleotides can effect knockdown of specific gene functions in early development, later embryonic events are less accessible, off-target effects can be problematic, and it can be challenging to obtain reproducible and complete depletion of gene products.

Next generation sequencing technologies have revolutionized mutation identification for reverse genetic strategies. In one strategy, Targeting Induced Local Lesions In Genomes (TILLING), animals carrying mutations in genes of interest are identified from mutagenesis-derived populations. TILLING was initially developed using non-sequence based methods (McCallum *et al.*, 2000; Till *et al.*, 2003), and adapted for *X. tropicalis* using capillary sequencing of specific amplicons (Goda *et al.*, 2006). However PCR amplification introduces allele bias and other errors, hindering high-throughput screens (Showell *et al.*, 2011).

‘Exome sequencing’ has provided a critical breakthrough. Focus on the genome’s protein-coding space is obtained using solution hybridization of sheared genomic DNA to a set of synthetic baits representing the entire exon set (‘exome’, Figure 3I) or a subset of specific genes of interest (Fairfield *et al.*, 2011; Gnirke *et al.*, 2009). Coding regions of individual mutagenesis-derived carrier animals can then be subjected to high throughput sequencing and bioinformatically inspected to identify the mutations they carry for either forward or reverse genetic applications. With collaborators, our groups have used this method to identify multiple chemically-induced nonsense and essential splice mutations per F2 frog, and to confirm heritability of these lesions in the next generation. In addition to reverse genetic applications, mutations found by forward genetics can often be directly identified by exome sequencing. One variation involves simultaneous direct identification and mapping, by exome sequencing pools of phenotypically mutant embryos (Figure 3H–K). Even if assignment of a responsible mutation is ambiguous, linkage information is obtained from the increasing SNP homozygosity in the mutation-containing interval.

Gene editing *in vivo* with sequence-specific nucleases

While TILLING by exome sequencing is now recovering large numbers of *X. tropicalis* mutations, there has also been a breakthrough in targeted mutation of specific genes (‘genome editing’) using synthetic sequence-specific enzymes such as Zinc Finger Nucleases (ZFNs). The conventional ZFN design is a fusion of the non-specific nuclease domain of FokI and zinc-finger motifs engineered to bind and digest a specific genomic target (Miller *et al.*, 2007; Urnov *et al.*, 2010). The resulting double strand breaks are usually imperfectly repaired, with insertions and deletions causing frameshift mutations (Bibikova *et al.*, 2002).

Young et al used this method to heritably mutate the *noggin* locus as well as a GFP transgene in *X. tropicalis* (Young *et al.*, 2011). After careful design and precharacterization of ZFNs in yeast reporter assays, they injected ZFN mRNA into fertilized eggs, resulting in the visible mosaic deletion of the GFP reporter and recovery of mutated *noggin* alleles in the next generation. While building and validating these ZFN constructs is laborious, several variations in sequence-specific nuclease design are under active development (Christian *et al.*, 2010; Kim *et al.*, 2009; Maeder *et al.*, 2009; Meng *et al.*, 2008; Miller *et al.*, 2011) and show promise for targeted mutagenesis in *Xenopus*.

Insights from mapped mutations

A number of groups have now mapped genes underlying mutations from *X. tropicalis* forward screens, and it is possible to make some general observations, illustrated with vignettes. First, forward screens efficiently recover disease models: >70% of mapped mutations (10/14) correspond to orthologs of human disease genes. Second, efficient gynogenetic screens recover mutations throughout the genome, including very telomeric regions (e.g. *komimi*, ~40cM or 70Mb from the centromere). Third, *X. tropicalis* screens are not redundant with zebrafish screens: fewer than half of the identified phenotypes have fish correlates (5/14).

Limb Development

One process where fish screens offer few insights is limb development. Limb formation in metamorphosing frogs is unique among vertebrate organogenesis processes, which otherwise occur during a brief coordinated period in early development. In contrast, *X. tropicalis* tadpoles grow for several weeks as feeding, swimming larvae with all other organ systems functioning before the first limb buds appear, eventually giving rise to appendicular skeletal structures homologous to those of mammals and other tetrapods. The *xenopus de milo* (*xdm*) mutation is caused by a random reporter transgene insertion disrupting the *nephronectin* (*npnt*) locus (Abu-Daya *et al.*, 2010), resulting in a specific and complete abrogation of forelimb bud formation in homozygous tadpoles (Figure 4A–D). This effect was upstream of the earliest known marker of the forelimb field, *tbx5*, but did not perturb hindlimb formation. In mouse, *npnt* is a secreted $\alpha 8 \beta 1$ integrin ligand required for development of the metanephros, a renal organ not found in frog, but mouse limbs were not affected by the knockout (Brandenberger *et al.*, 2001; Linton *et al.*, 2007), and *xdm* pronephros and mesonephros appear normal. This striking frog phenotype identifies a novel step upstream of the earliest previously-known gene function in limb subtype initiation.

Muscle and Cardiac formation

Screens have identified a number of *X. tropicalis* mutations disrupting skeletal and/or cardiac muscle function, both because paralysis phenotypes are easily scored, and because sarcomere assembly requires many unusually large proteins, providing a good genetic target. *Xenopus* embryos are particularly well suited to cardiogenesis studies, since in the absence of heart function they survive to swimming tadpole stages on diffused oxygen, unlike mammals where contractility defects cause early lethality. The three-chambered amphibian heart also more closely resembles that of mammals than the two-chambered fish heart.

The *muzak* (*muz*) mutation completely lacks heartbeat, and is caused by a truncation of the cardiac specific myosin heavy chain gene *myh6* (Figure 4E–H); skeletal muscle is unaffected (Abu-Daya *et al.*, 2009). *Muzak* permits study of the roles of heartbeat, bloodflow, and myofibrils in cardiac morphogenesis. While cardiac *myh* mutants have been identified in zebrafish, teleost subfunctionalization has produced distinct atrial and ventricular myosin heavy chains, and awkward double mutants are required for analysis of a comparable phenotype. Despite loss of a major structural component and complete ablation of beating, early steps in cardiac development such as looping and chamber formation occur surprisingly normally, but later features such as valves and ventricular trabeculation were not detected. By reconstructing serial plastic sections of cardiac regions at different stages, it was possible to create 3-D projections of heart development in normal and non-contractile mutant hearts. In the absence of *myh6*, the mutant heart showed dilated cardiac chambers and malformed endocardium, which is compressed with drastically reduced lumen (Figure 4G, H).

Likewise, *Xenopus* tadpoles excel in studies of skeletal muscle formation. As somites are added sequentially during development, multiple stages in sarcomerogenesis can be observed in a single embryo, conveniently imaged in the transparent tadpole tail. Paralysis phenotypes specifically affecting muscle structure are easily diagnosed by reduced birefringence under polarized light (Figure 4I,J). The *dicky ticker* (*dit*) mutation, characterized by complete paralysis and lack of heartbeat (Goda *et al.*, 2006) maps to the *unc45b* muscle specific myosin chaperone (Geach and Zimmerman, 2010). At early stages of myofibrillogenesis in *dit*, myh protein can be detected in poorly ordered fibres, and a few thick filaments are present (Figure 4K,L), but appearance of Z-discs and associated α -actinin staining was substantially delayed. This suggests modifications to current models of myofibrillogenesis, which assume that Z-discs form early and independent of sarcomeric myosin, are needed.

Inner ear development

At tailbud stages, the embryo's head becomes transparent as yolk is metabolized, facilitating observation of sensory structures such as the inner ear. Screens have identified many mutations affecting formation of the otoconia, calcitic crystals attached to inner ear stereocilia which provide mass for detection of balance and acceleration (Figure 4M-P); otoconial phenotypes usually also result in behavioural abnormalities in swimming and orientation. Correct formation of otoconia reflects proper otic vesicle patterning as well as extensive intracellular trafficking to secrete proteins required for crystal formation. The *seasick* (*ssk*) mutation (Figure 4N) is characterized by overgrowth of individual crystals, and is caused by a mutation in the vesicle transport adaptor protein subunit *ap3 δ 1*, part of the human disease complex Hermansky-Pudlack Syndrome; no zebrafish models for the HPS complex are currently known. Consistent with a vesicle transport disorder, *ssk* embryos also show punctate distribution of pigment-bearing melanosomes in melanocytes, although the stellate melanocyte morphology is not affected. In another mutant, *komimi* (*kom*), a single large otoconium forms over the two otic sensory epithelia (Figure 4O). *Komimi* results from a splicing mutation in *otoconin 90*, previously thought not to be a major protein in frog otoconia (Pote *et al.*, 1993), but here shown to be critical for proper formation. Another phenotype, *bunny* (*bun*, Figure 4P), displays a smaller otic vesicle with a single sensory epithelium, probably reflecting an earlier defect in patterning the otic vesicle.

Ciliogenesis

Cilia have emerged as a key cellular structure for receiving and integrating intercellular signals, in addition to their other medically important roles as motile structures lining the mammalian airway, kidney and elsewhere. Ciliogenesis is an ideal genetic target in that it requires hundreds of genes, many of which have poorly-characterized functions, and is uniquely accessible in the tadpole's external mucociliary epidermis (Hayes *et al.*, 2007). Functional motile epidermal cilia generate an easily-detected fluid flow, causing even anaesthetized embryos to 'glide' across surfaces, or can be assayed using microscopic beads. The *grinch* mutation (Grammer *et al.*, 2005) abrogates epidermal ciliary flow, and produces stubby cilia which are reduced in number in epidermal multiciliate cells (Figure 4Q-T).

Future Prospects

While *X. tropicalis* genetics effectively recovers novel gene functions and disease models, it is clear that few *Xenopus* groups have the resources to initiate large-scale screens. However, NIH support for TILLING projects and stock centers for strain distribution means that the frog community will soon have access to a large number of identified null mutations as well as lines made by targeted gene editing with synthetic sequence-specific nucleases. Attractive focused applications of these loss-of-function genetic backgrounds include rescue with

engineered BAC transgenes, for example to create conditional floxed alleles for analysis of specific later steps in development, tagged proteins for biochemical analysis, or gain-of-function suppressor/enhancer for interacting factors in complex processes (Bai *et al.* 2010). Stock center-based TILLING programs may also be able to give other genetic projects a one- or two-generation head start, by making excess mutagenized stock available for other types of screens. The simplicity of haploid genetics and gynogenesis, together with direct sequencing to identify mutations, means that even small-scale screens can recover useful phenotypes for studying vertebrate gene function.

Acknowledgments

For animated discussions and seminal contributions, we would especially like to thank Richard Harland, Amy Sater, Enrique Amaya, Rob Grainger and Takuya Nakayama, and Derek Stemple and Samantha Carruthers, as well as past and present members of our own laboratories. MKK is supported by NIH NICHD 1R01DE018824, 1R01DE018825-01A1; AA and LBZ are supported by UK Medical Research Council U117560482, NIH 1R01 HD054354-01 and 1R01 DC011901-01.

References

- Abu-Daya A, Nishimoto S, Fairclough L, Mohun TJ, Logan MP, Zimmerman LB. The secreted integrin ligand nephronectin is necessary for forelimb formation in *Xenopus tropicalis*. *Dev Biol.* 2010; 349:204–212. [PubMed: 20977901]
- Abu-Daya A, Sater AK, Wells DE, Mohun TJ, Zimmerman LB. Absence of heartbeat in the *Xenopus tropicalis* mutation muzak is caused by a nonsense mutation in cardiac myosin myh6. *Developmental biology.* 2009; 336:20–29. [PubMed: 19769958]
- Adams D. Life, The Universe and Everything. 1982
- Amaya E, Musci TJ, Kirschner MW. Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. *Cell.* 1991; 66:257–270. [PubMed: 1649700]
- Bai X, Kim J, Yang Z, Jurynek MJ, Akie TE, Lee J, LeBlanc J, Sessa A, Jiang H, DiBiase A, Zhou Y, Grunwald DJ, Lin S, Cantor AB, Orkin SH, Zon LI. TIF1gamma controls erythroid cell fate by regulating transcription elongation. *Cell.* 2010; 142:133–143. [PubMed: 20603019]
- Bewick AJ, Anderson DW, Evans BJ. Evolution of the closely related, sex-related genes DM-W and DMRT1 in African clawed frogs (*Xenopus*). *Evolution.* 2010; 65:698–712. [PubMed: 21044062]
- Bibikova M, Golic M, Golic KG, Carroll D. Targeted chromosomal cleavage and mutagenesis in *Drosophila* using zinc-finger nucleases. *Genetics.* 2002; 161:1169–1175. [PubMed: 12136019]
- Brandenberger R, Schmidt A, Linton J, Wang D, Backus C, Denda S, Muller U, Reichardt LF. Identification and characterization of a novel extracellular matrix protein nephronectin that is associated with integrin alpha8beta1 in the embryonic kidney. *J Cell Biol.* 2001; 154:447–458. [PubMed: 11470831]
- Brenner S. The genetics of *Caenorhabditis elegans*. *Genetics.* 1974; 77:71–94. [PubMed: 4366476]
- Bronchain OJ, Hartley KO, Amaya E. A gene trap approach in *Xenopus*. *Curr Biol.* 1999; 9:1195–1198. [PubMed: 10531033]
- Chain FJ, Evans BJ. Multiple mechanisms promote the retained expression of gene duplicates in the tetraploid frog *Xenopus laevis*. *PLoS Genet.* 2006; 2:e56. [PubMed: 16683033]
- Christian M, Cermak T, Doyle EL, Schmidt C, Zhang F, Hummel A, Bogdanove AJ, Voytas DF. Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics.* 2010; 186:757–761. [PubMed: 20660643]
- Cresko WA, Yan YL, Baltrus DA, Amores A, Singer A, Rodriguez-Mari A, Postlethwait JH. Genome duplication, subfunction partitioning, and lineage divergence: Sox9 in stickleback and zebrafish. *Dev Dyn.* 2003; 228:480–489. [PubMed: 14579386]
- Driever W, Solnica-Krezel L, Schier AF, Neuhauss SC, Malicki J, Stemple DL, Stainier DY, Zwartkruis F, Abdelilah S, Rangini Z, Belak J, Boggs C. A genetic screen for mutations affecting embryogenesis in zebrafish. *Development.* 1996; 123:37–46. [PubMed: 9007227]

- Elsdale T, Fischberg M, Smith S. A mutation that reduces nucleolar number in *Xenopus laevis*. *Exo Cell Res.* 1958; 13:642–643.
- Elsdale TR, Gurdon JB, Fischberg M. A description of the technique for nuclear transplantation in *Xenopus laevis*. *J Embryol Exp Morphol.* 1960; 8:437–444. [PubMed: 13726553]
- Evans BJ, Kelley DB, Tinsley RC, Melnick DJ, Cannatella DC. A mitochondrial DNA phylogeny of African clawed frogs: phylogeography and implications for polyploid evolution. *Mol Phylogenet Evol.* 2004; 33:197–213. [PubMed: 15324848]
- Fairfield H, Gilbert GJ, Barter M, Corrigan RR, Curtain M, Ding Y, D'Ascenzo M, Gerhardt DJ, He C, Huang W, Richmond T, Rowe L, Probst FJ, Bergstrom DE, Murray SA, Bult C, Richardson J, Kile BT, Gut I, Hager J, Sigurdsson S, Mauceli E, Di Palma F, Lindblad-Toh K, Cunningham ML, Cox TC, Justice MJ, Spector MS, Lowe SW, Albert T, Donahue LR, Jeddelloh J, Shendure J, Reinholdt LG. Mutation discovery in mice by whole exome sequencing. *Genome Biol.* 2011; 12:R86. [PubMed: 21917142]
- Force A, Lynch M, Pickett FB, Amores A, Yan YL, Postlethwait J. Preservation of duplicate genes by complementary, degenerative mutations. *Genetics.* 1999; 151:1531–1545. [PubMed: 10101175]
- Garcia-Garcia MJ, Eggenschwiler JT, Caspary T, Alcorn HL, Wyler MR, Huangfu D, Rakeman AS, Lee JD, Feinberg EH, Timmer JR, Anderson KV. Analysis of mouse embryonic patterning and morphogenesis by forward genetics. *Proc Natl Acad Sci U S A.* 2005; 102:5913–5919. [PubMed: 15755804]
- Geach TJ, Zimmerman LB. Paralysis and delayed Z-disc formation in the *Xenopus tropicalis* unc45b mutant dicky ticker. *BMC Dev Biol.* 2010; 10:75. [PubMed: 20637071]
- Gnirke A, Melnikov A, Maguire J, Rogov P, LeProust EM, Brockman W, Fennell T, Giannoukos G, Fisher S, Russ C, Gabriel S, Jaffe DB, Lander ES, Nusbaum C. Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. *Nat Biotechnol.* 2009; 27:182–189. [PubMed: 19182786]
- Goda T, Abu-Daya A, Carruthers S, Clark MD, Stemple DL, Zimmerman LB. Genetic screens for mutations affecting development of *Xenopus tropicalis*. *PLoS genetics.* 2006; 2:e91. [PubMed: 16789825]
- Grammer TC, Khokha MK, Lane MA, Lam K, Harland RM. Identification of mutants in inbred *Xenopus tropicalis*. *Mechanisms of development.* 2005; 122:263–272. [PubMed: 15763207]
- Gurdon JB, Elsdale TR, Fischberg M. Sexually mature individuals of *Xenopus laevis* from the transplantation of single somatic nuclei. *Nature.* 1958; 182:64–65. [PubMed: 13566187]
- Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, Kane DA, Odenthal J, van Eeden FJ, Jiang YJ, Heisenberg CP, Kelsh RN, Furutani-Seiki M, Vogelsang E, Beuchle D, Schach U, Fabian C, Nusslein-Volhard C. The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development.* 1996; 123:1–36. [PubMed: 9007226]
- Harland RM, Grainger RM. *Xenopus* research: metamorphosed by genetics and genomics. *Trends Genet.* 2011
- Hayes JM, Kim SK, Abitua PB, Park TJ, Herrington ER, Kitayama A, Grow MW, Ueno N, Wallingford JB. Identification of novel ciliogenesis factors using a new in vivo model for mucociliary epithelial development. *Dev Biol.* 2007; 312:115–130. [PubMed: 17961536]
- Hellsten U, Harland RM, Gilchrist MJ, Hendrix D, Jurka J, Kapitonov V, Ovcharenko I, Putnam NH, Shu S, Taher L, Blitz IL, Blumberg B, Dichmann DS, Dubchak I, Amaya E, Detter JC, Fletcher R, Gerhard DS, Goodstein D, Graves T, Grigoriev IV, Grimwood J, Kawashima T, Lindquist E, Lucas SM, Mead PE, Mitros T, Ogino H, Ohta Y, Poliakov AV, Pollet N, Robert J, Salamov A, Sater AK, Schmutz J, Terry A, Vize PD, Warren WC, Wells D, Wills A, Wilson RK, Zimmerman LB, Zorn AM, Grainger R, Grammer T, Khokha MK, Richardson PM, Rokhsar DS. The genome of the Western clawed frog *Xenopus tropicalis*. *Science.* 2010; 328:633–636. [PubMed: 20431018]
- Hellsten U, Khokha MK, Grammer TC, Harland RM, Richardson P, Rokhsar DS. Accelerated gene evolution and subfunctionalization in the pseudotetraploid frog *Xenopus laevis*. *BMC Biol.* 2007; 5:31. [PubMed: 17651506]
- Johnson SL, Africa D, Horne S, Postlethwait JH. Half-tetrad analysis in zebrafish: mapping the *ros* mutation and the centromere of linkage group I. *Genetics.* 1995; 139:1727–1735. [PubMed: 7789773]

- Johnson SL, Gates MA, Johnson M, Talbot WS, Horne S, Baik K, Rude S, Wong JR, Postlethwait JH. Centromere-linkage analysis and consolidation of the zebrafish genetic map. *Genetics*. 1996; 142:1277–1288. [PubMed: 8846904]
- Kemphues KJ, Priess JR, Morton DG, Cheng NS. Identification of genes required for cytoplasmic localization in early *C. elegans* embryos. *Cell*. 1988; 52:311–320. [PubMed: 3345562]
- Khokha MK, Chung C, Bustamante EL, Gaw LW, Trott KA, Yeh J, Lim N, Lin JC, Taverner N, Amaya E, Papalopulu N, Smith JC, Zorn AM, Harland RM, Grammer TC. Techniques and probes for the study of *Xenopus tropicalis* development. *Dev Dyn*. 2002; 225:499–510. [PubMed: 12454926]
- Khokha MK, Krylov V, Reilly MJ, Gall JG, Bhattacharya D, Cheung CY, Kaufman S, Lam DK, Macha J, Ngo C, Prakash N, Schmidt P, Tlapakova T, Trivedi T, Tumova L, Abu-Daya A, Geach T, Vendrell E, Ironfield H, Sinzelle L, Sater AK, Wells DE, Harland RM, Zimmerman LB. Rapid gynogenetic mapping of *Xenopus tropicalis* mutations to chromosomes. *Dev Dyn*. 2009; 238:1398–1346. [PubMed: 19441086]
- Kim HJ, Lee HJ, Kim H, Cho SW, Kim JS. Targeted genome editing in human cells with zinc finger nucleases constructed via modular assembly. *Genome Res*. 2009; 19:1279–1288. [PubMed: 19470664]
- Kroll KL, Amaya E. Transgenic *Xenopus* embryos from sperm nuclear transplantations reveal FGF signaling requirements during gastrulation. *Development* (Cambridge, England). 1996; 122:3173–3183.
- Krotoski DM, Reinschmidt DC, Tompkins R. Developmental mutants isolated from wild-caught *Xenopus laevis* by gynogenesis and inbreeding. *J Exp Zool*. 1985; 233:443–449. [PubMed: 3973558]
- Lamb TM, Knecht AK, Smith WC, Stachel SE, Economides AN, Stahl N, Yancopolous GD, Harland RM. Neural induction by the secreted polypeptide noggin. *Science*. 1993; 262:713–718. [PubMed: 8235591]
- Linton JM, Martin GR, Reichardt LF. The ECM protein nephronectin promotes kidney development via integrin $\alpha 8 \beta 1$ -mediated stimulation of Gdnf expression. *Development*. 2007; 134:2501–2509. [PubMed: 17537792]
- Loosli F, Koster RW, Carl M, Kuhnlein R, Henrich T, Mucke M, Krone A, Wittbrodt J. A genetic screen for mutations affecting embryonic development in medaka fish (*Oryzias latipes*). *Mech Dev*. 2000; 97:133–139. [PubMed: 11025214]
- Maeder ML, Thibodeau-Beganny S, Sander JD, Voytas DF, Joung JK. Oligomerized pool engineering (OPEN): an 'open-source' protocol for making customized zinc-finger arrays. *Nat Protoc*. 2009; 4:1471–1501. [PubMed: 19798082]
- McCallum CM, Comai L, Greene EA, Henikoff S. Targeting induced local lesions IN genomes (TILLING) for plant functional genomics. *Plant Physiol*. 2000; 123:439–442. [PubMed: 10859174]
- Meng X, Noyes MB, Zhu LJ, Lawson ND, Wolfe SA. Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. *Nat Biotechnol*. 2008; 26:695–701. [PubMed: 18500337]
- Miller JC, Holmes MC, Wang J, Guschin DY, Lee YL, Rupniewski I, Beausejour CM, Waite AJ, Wang NS, Kim KA, Gregory PD, Pabo CO, Rebar EJ. An improved zinc-finger nuclease architecture for highly specific genome editing. *Nat Biotechnol*. 2007; 25:778–785. [PubMed: 17603475]
- Miller JC, Tan S, Qiao G, Barlow KA, Wang J, Xia DF, Meng X, Paschon DE, Leung E, Hinkley SJ, Dulay GP, Hua KL, Ankoudinova I, Cost GJ, Urnov FD, Zhang HS, Holmes MC, Zhang L, Gregory PD, Rebar EJ. A TALE nuclease architecture for efficient genome editing. *Nat Biotechnol*. 2011; 29:143–148. [PubMed: 21179091]
- Mullins MC, Hammerschmidt M, Haffter P, Nusslein-Volhard C. Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. *Curr Biol*. 1994; 4:189–202. [PubMed: 7922324]
- Nonaka M, Namikawa C, Kato Y, Sasaki M, Salter-Cid L, Flajnik MF. Major histocompatibility complex gene mapping in the amphibian *Xenopus* implies a primordial organization. *Proc Natl Acad Sci U S A*. 1997; 94:5789–5791. [PubMed: 9159152]

- Noramly S, Zimmerman L, Cox A, Aloise R, Fisher M, Grainger RM. A gynogenetic screen to isolate naturally occurring recessive mutations in *Xenopus tropicalis*. *Mechanisms of development*. 2005; 122:273–287. [PubMed: 15763208]
- Nusslein-Volhard C, Wieschaus E. Mutations affecting segment number and polarity in *Drosophila*. *Nature*. 1980; 287:795–801. [PubMed: 6776413]
- Philpott A, Yew PR. The *Xenopus* cell cycle: an overview. *Methods Mol Biol*. 2005; 296:95–112. [PubMed: 15576928]
- Postlethwait JH, Woods IG, Ngo-Hazelett P, Yan YL, Kelly PD, Chu F, Huang H, Hill-Force A, Talbot WS. Zebrafish comparative genomics and the origins of vertebrate chromosomes. *Genome Res*. 2000; 10:1890–1902. [PubMed: 11116085]
- Pote KG, Hauer CR 3rd, Michel H, Shabanowitz J, Hunt DF, Kretsinger RH. Otoconin-22, the major protein of aragonitic frog otoconia, is a homolog of phospholipase A2. *Biochemistry*. 1993; 32:5017–5024. [PubMed: 8494877]
- Ravi V, Venkatesh B. Rapidly evolving fish genomes and teleost diversity. *Curr Opin Genet Dev*. 2008; 18:544–550. [PubMed: 19095434]
- Reinschmidt D, Friedman J, Hauth J, Ratner E, Cohen M, Miller M, Krotoski D, Tompkins R. Gene-centromere mapping in *Xenopus laevis*. *The Journal of heredity*. 1985; 76:345–347. [PubMed: 4056365]
- Scacheri PC, Rozenblatt-Rosen O, Caplen NJ, Wolfsberg TG, Umayam L, Lee JC, Hughes CM, Shanmugam KS, Bhattacharjee A, Meyerson M, Collins FS. Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proc Natl Acad Sci U S A*. 2004; 101:1892–1897. [PubMed: 14769924]
- Showell C, Carruthers S, Hall A, Pardo-Manuel de Villena F, Stemple D, Conlon FL. A Comparative Survey of the Frequency and Distribution of Polymorphism in the Genome of *Xenopus tropicalis*. *PLoS One*. 2011; 6:e22392. [PubMed: 21829622]
- Streisinger G, Singer F, Walker C, Knauber D, Dower N. Segregation analyses and gene-centromere distances in zebrafish. *Genetics*. 1986; 112:311–319. [PubMed: 3455686]
- Thiebaud CH, Colombelli B, Muller WP. Diploid gynogenesis in *Xenopus laevis* and the localization with respect to the centromere of the gene for periodic albinism ap. *J Embryol Exp Morphol*. 1984; 83:33–42. [PubMed: 6594425]
- Till BJ, Reynolds SH, Greene EA, Codomo CA, Enns LC, Johnson JE, Burtner C, Odden AR, Young K, Taylor NE, Henikoff JG, Comai L, Henikoff S. Large-scale discovery of induced point mutations with high-throughput TILLING. *Genome Res*. 2003; 13:524–530. [PubMed: 12618384]
- Tinsley RCK, H R. *The Biology Of Xenopus*. 1996
- Tompkins R. Triploid and gynogenetic diploid *Xenopus laevis*. *J. exp. Zool*. 1978; 203:251–256.
- Tompkins R, Reinschmidt D. Experimentally induced homozygosity in *Xenopus laevis*. *Methods Cell Biol*. 1991; 36:35–44. [PubMed: 1811144]
- Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet*. 2010; 11:636–646. [PubMed: 20717154]
- Wansleeben C, van Gurp L, Feitsma H, Kroon C, Rieter E, Verberne M, Guryev V, Cuppen E, Meijlink F. An ENU-mutagenesis screen in the mouse: identification of novel developmental gene functions. *PLoS One*. 2011; 6:e19357. [PubMed: 21559415]
- Wells D, Gutierrez L, Xu Z, Krylov V, Macha J, Blankenburg K, Hitchens M, Bellot L, Spivey M, Kowis A, Ye Y, Pasternak S, Owen J, Tran T, Slavikova R, Tumova L, Tlapakova T, Seifertova E, Scherer S, Sater A. A Genetic Map of *Xenopus tropicalis*. *Developmental Biology*. 2010
- Whitman M. Smads and early developmental signaling by the TGFbeta superfamily. *Genes Dev*. 1998; 12:2445–2462. [PubMed: 9716398]
- Wilson PA, Hemmati-Brivanlou A. Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature*. 1995; 376:331–333. [PubMed: 7630398]
- Yergeau DA, Kelley CM, Kuliyev E, Zhu H, Sater AK, Wells DE, Mead PE. Remobilization of Tol2 transposons in *Xenopus tropicalis*. *BMC Dev Biol*. 2010; 10:11. [PubMed: 20096115]
- Yergeau DA, Mead PE. Manipulating the *Xenopus* genome with transposable elements. *Genome biology*. 2007; 8 Suppl 1:S11. [PubMed: 18047688]

Young JJ, Cherone JM, Doyon Y, Ankoudinova I, Faraji FM, Lee AH, Ngo C, Guschin DY, Paschon DE, Miller JC, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Harland RM, Zeitler B. Efficient targeted gene disruption in the soma and germ line of the frog *Xenopus tropicalis* using engineered zinc-finger nucleases. *Proc Natl Acad Sci U S A*. 2011; 108:7052–7057. [PubMed: 21471457]

Figure 1. Screen strategies

(A) (left column) Conventional 3-generation mutagenesis and screen scheme. *In vivo* mutagenesis of adult G0 males followed by successive outcrosses gives groups of F2 siblings, half carrying a given recessive allele (grey frogs). ¼ of random F2 sibling matings uncover the phenotype (black tadpoles) in 25% of their offspring. (B) (right column) Gynogenetic screen scheme. Dissociated testes are mutagenized *in vitro* and used to fertilize eggs, producing F1 founders mosaic for induced mutations (half-grey frog), which are then outcrossed to produce non-mosaic F2 carriers (grey). Gynogenesis from individual F2 carrier females uncovers recessive phenotypes (black tadpoles) in non-mendelian ratios of ~10–50% depending on gene-centromere distance.

Figure 2. Haploid genetics and gynogenesis

(A) As in normal oogenesis, recombination in meiosis I between non-sister chromatids from blue and red parental strains produces (B) oocytes arrested in meiosis II containing both sister chromatids from one parental strain, plus the 1st polar body. (C) To create gynogenetic haploids, sperm are UV-irradiated to block paternal genetic contribution, with the resulting zygote (D) completing second meiosis by ejecting the second set of sister chromatids in the 2nd polar body to give a haploid (E, Hoechst-stained karyotype below). Haploids can be rescued by allowing DNA replication to take place, then blocking cytokinesis with cold shock to form completely homozygous (isogenic) double haploids. Alternatively, (G) cold shock immediately after fertilization with UV-irradiated sperm suppresses polar body

formation to create 'early cold shock' gynogenetic diploids retaining both sets of sister chromatids. Due to recombination (A), gynogenotes are not homozygous at all loci, but rescue to viable diploidy is very efficient.

Figure 3. High throughput mutation mapping strategies

(A) 'red' strain frog showing two chromosomes, one of which carries a mutation (*), crossed to a polymorphic 'blue' strain animal differing at many sequence loci. (B) Both parental chromosomes are inherited by hybrid 'map cross' offspring, and recombine in meiosis. (C) Gynogenetic diploids with recombined sister chromatid pairs are obtained from mapcross females and sorted into phenotypically mutant and wild type pools. In the homozygous mutant pool, red strain alleles dominate near the mutant locus, with increasing frequency of blue alleles further from the mutation; wild type usually show both alleles. (D) Recessive phenotypes mapping close to centromeres appear in up to 50% of gynogenotes, with frequency of wild type heterozygotes increasing with crossovers for more distal loci,

providing gene-centromere distance. For all but very distal mutations, linkage to one of the 10 *X. tropicalis* centromeres can be detected. (E) A genomic region can then be inspected for candidate genes to evaluate (F). (G–K) Alternatively, mutations can be mapped or directly identified by whole-exome sequencing of carrier DNA from a toe clip. Pools of mutant and wild type embryo DNA are enriched for coding sequences by hybridization with synthetic biotinylated RNA baits and sequenced. (J) Sequences are then bioinformatically inspected for homozygous SNPs in the mutant pool to identify candidate genes or define a linked interval.

Figure 4. Selected *X. tropicalis* phenotypes

(A–D) The *xenopus de milo* (*xdm*) mutation reveals an unexpected requirement for *nephronectin* in limb subtype initiation upstream of the earliest known forelimb marker, *tbx5*; (C) WISH showing *tbx5* forelimb field expression (arrow) and loss in mutant (D, arrow). (E–H) Heartbeat is absent in the cardiac myosin *myh6* mutation *muzak* (*muz*) stained for sarcomeric myosin (green) and phalloidin (red). Despite absence of beating/contractility, heart looping and chamber formation are relatively normal, but (G,H) reconstruction from serial sections shows the endocardium has collapsed. (I,J) mutations affecting muscle structure (*mrs lot* (*mlo*)) disrupt birefringence to polarized light. (K,L) myosin chaperone *unc45b* mutant *dicky ticker* (*dit*) shows disordered sarcomere ultrastructure. (M–P)

Mutations affecting inner ear development and the formation of otoconial crystals include *seasick* (*ssk*), in the vesicle transport adaptor protein *ap3 δ 1*, and *komimi* (*kom*), in the *oc90* gene, as well as *bunny* (*bun*) where the otic vesicle is reduced in size. (Q–T) Multiciliate epidermal cells are affected in *grinch* (*gri*), with stubby cilia reduced in length and number in the mutant as seen stained with α -acetylated tubulin (Q,R) and by SEM (S,T).