Impact of the *Xenopus* system on the mission of the NIDCR

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Craniofacial abnormalities are among the most prevalent birth defects, occurring in 1/700 live births, and present a tremendous medical and social burden. Furthermore, oral and dental health issues affect a majority of the population. Much current understanding of human craniofacial development comes from patient studies and will be immensely facilitated by studies in selected animal models, including *Xenopus*.

Overall, it appears that vertebrate craniofacial development is well conserved. Patterning of facial structures requires complex interactions between different tissue types, from the initial specification of the germ layers through morphogenesis of the facial prominences to the integration of the skeletal elements, muscles, nerves and other tissues. These processes begin very early in gestation and continue throughout life. A number of craniofacial abnormalities, including cleft palate, frontonasal dysplasia and DiGeorge syndrome, can be traced to abnormal development of a migratory, pluripotent population of cells called the neural crest. Therefore, defining the etiology of these pathologies requires an understanding of the mechanisms of neural crest formation, migration and plasticity.

Methodology useful to analysis of craniofacial development and abnormalities

Xenopus is one of the most accessible vertebrate model systems for analysis of craniofacial development. In particular, developing craniofacial structures are more readily visible in *Xenopus* than in any other vertebrate model, primarily because *Xenopus* embryos develop externally to the mother, allowing analyses of the earliest stages, and facilitating live imaging at single cell resolution. Amongst vertebrate models developing externally, *Xenopus* is more useful for craniofacial analysis than the zebrafish system, as *Xenopus* embryos are larger and easier to dissect, and the developing facial region is more accessible to imaging than the equivalent region in fish. Explants and transplants have been routine for decades; this, combined with the large clutch size (hundreds vs dozens in zebrafish) allows easy reproducibility. The ease of gain- and loss-of-function experiments in *Xenopus* has led to discoveries fundamental to biology, including Nobel Prize winning work on the cell cycle (Medicine, 2001) and water channels (Chemistry, 2003). Furthermore, experimental analyses have explored topics as varied as transcriptional control, chromatin accessibility, RNA processing, protein translation, pharmacology and synaptic plasticity. As more human mutations are uncovered, new genes, with unclear functions, will be implicated in craniofacial development.

Xenopus embryos are one of the simplest and most economical models in which to study gene function in an intact animal. Mutant alleles can be readily expressed *in vivo*; the large clutch size then allows reproducible, statistically significant phenotypic and biochemical readouts. The recent development of forward and reverse genetics in *Xenopus tropicalis* will result in new insights into craniofacial development. Several ongoing mutagenesis screens (Yale University, USA; Sanger Center, UK; National Institute of Medical Research, UK) have already produced multiple carriers of craniofacial mutations. In complementary studies, a TILLING (targeting induced local lesions in genomes) strategy is being used to identify mutations in known genes. These banks of mutations can then be used in combination with well-established embryological and molecular approaches. The availability of chemical libraries also makes *Xenopus* an extremely attractive system for studying craniofacial anomalies. *Xenopus* embryos are

aquatic and can be arrayed in multi-well dishes, allowing automation of chemical screens. Libraries of small molecules can simply be added to the media, and tadpoles can then be assayed for morphological changes visually. Finally, the increased availability of transgenic *Xenopus* lines will contribute to analysis of genes and processes associated with craniofacial abnormalities, especially when combined with chemical screening.

Recent data from *Xenopus* studies pertaining to craniofacial development

Work using *Xenopus laevis* embryos has contributed tremendously to knowledge of early steps in craniofacial development. Most notable are studies on the early induction of the neural crest. Functional studies have defined the molecules and signal transduction processes important for cell-cell and tissue-tissue interactions during neural crest development (including BMPs, FGFs and Wnts). Mechanisms underlying migration of neural crest cells can also be studied in *Xenopus*: recent work includes the molecular basis of contact inhibition and directional migration of neural crest cells. These kinds of studies are important for understanding craniofacial defects resulting from abnormal neural crest development.

Another use of *Xenopus* has been to analyze development of the primary mouth (or stomodeum) - the first opening between the pharynx and the outside of the embryo. Multiple craniofacial defects are likely to be caused by defects in this region. Recent work in *Xenopus* defined a set of steps leading to primary mouth opening, where the earliest step is local dissolution of the basement membrane. Further analysis showed that local expression of the Wnt inhibitors, Frb1 and Crescent, is necessary for basement membrane breakdown in this region. Basement membrane remodeling is essential for normal development of most organs, and pivotal in metastasis, and these unprecedented findings have proven *Xenopus* a pioneer organism, yet again.

With regard to chemical screening, a recent study identified multiple compounds affecting cell migration. By combining chemical structure predictions and enzymatic assays using *Xenopus* lysates, the authors identified an activity that inhibited matrix metalloproteinases (MMPs). They then performed loss-of-function analyses; by knocking down several MMPs, confirming the drug target. Finally, they were able to extrapolate their findings to a human melanoma cell line, illustrating the ease of using *Xenopus* as a whole animal assay system for drug discovery.

Due to the unusual demands of metamorphosis, *Xenopus* also provides a fascinating example of developmental plasticity. Craniofacial alterations during metamorphosis are similar to changes that occur in regeneration, remodeling and wound healing. Thus, studying these transitions may be extremely informative. Recent studies have begun applying molecular tools to these questions.

Selected references

Neural crest induction:

Monsoro-Burq AH, Wang E, Harland R. Msx1 and Pax3 cooperate to mediate FGF8 and WNT signals during *Xenopus* neural crest induction. *Dev Cell.* 2005. Feb;8(2):167-78.

Neural crest migration:

Carmona-Fontaine C, Matthews HK, Kuriyama S, Moreno M, Dunn GA, Parsons M, Stern CD, Mayor R. Contact inhibition of locomotion in vivo controls neural crest directional migration. *Nature*. 2008 Dec 18;456(7224):957-61. Epub 2008 Dec 10.

Primary mouth formation:

Dickinson A. and Sive H. The Wnt antagonists, Frzb-1 and Crescent regulate primary mouth formation by modulating the basement membrane. *Development* 2009 Apr;136(7):1071-81. Epub 2009 Feb 18.

Genetic screens:

Grammer TC, Khokha MK, Lane MA, Lam K, Harland RM. Identification of mutants in inbred *Xenopus tropicalis*. *Mech Dev.* 2005 Mar;122(3):263-72.

Goda T, Abu-Daya A, Carruthers S, Clark MD, Stemple DL, Zimmerman LB. Genetic screens for mutations affecting development of *Xenopus tropicalis*. *PLoS Genet.* 2006 Jun;2(6):e91.

Chemical Screens:

Tomlinson ML, Guan P, Morris RJ, Fidock MD, Rejzek M, Garcia-Morales C, Field RA, Wheeler GN. A chemical genomic approach identifies matrix metalloproteinases as playing an essential and specific role in *Xenopus* melanophore migration. *Chem Biol.* 2009 Jan 30;16(1):93-104.

Skull development:

Gross JB, Hanken J. Cranial neural crest contributes to the bony skull vault in adult *Xenopus laevis*: insights from cell labeling studies. *J Exp Zoolog B Mol Dev Evol.* 2005 Mar 15;304(2):169-76.

Slater BJ, Liu KJ, Kwan MD, Quarto N, Longaker MT. Cranial osteogenesis and suture morphology in *Xenopus laevis*: a unique model system for studying craniofacial development. *PLoS One.* 2009;4(1):e3914.

Xenopus **grants funded by the Institute:**

According to NIH RePORTER Search Tool, in the fiscal year of 2011, the National Institute of Dental and Craniofacial Research (NIDCR) funded two grants for projects involving Xenopus. These grants total to $1,000,854.

2011 *Xenopus* White Paper - Community Needs:

Executive Summary

Xenopus**: An essential vertebrate model system for biomedical research:**

Model animals are crucial to advancing biomedical research. Basic studies in vertebrate animals rapidly accelerate our understanding of human health and disease. Among the commonly used model animals, the frog, *Xenopus,* has great impact because of its close evolutionary relationship with mammals. Moreover, the remarkable experimental repertoire of the *Xenopus* system has made it a cornerstone of neurobiology, physiology, molecular biology, cell biology, and developmental biology.

Current NIH investment in research using *Xenopus*:

Consistent with its broad utility, the NIH has made a large and continuing investment in *Xenopus* research. Indeed, a search of the NIH rePORT database for R01 or equivalent grants using the search term "*Xenopus*" returned **678 grants for a total of**

over $217,000,000 for FY09-10. The NIH has also recently demonstrated its commitment to *Xenopus* community resources by approving $2.5 million to establish the National *Xenopus* Resource in Woods Hole, MA and a similar amount to maintain and expand Xenbase, the *Xenopus* Community's online database.

Xenopus as a model system for human disease gene function

Given the tremendous power of the *Xenopus* system, the pace of new biological discovery by the *Xenopus* Community is vigorous. Using *Xenopus*, we have significantly improved our understanding of human disease genes and their mechanisms of action, justifying the NIH's investment. For example:

Xenopus embryos are used for *in vivo* analysis of gene expression and function:

Congenital Heart Disease – *PNAS* 2011. 108, 2915-2920
CHARGE Syndrome – *Nature* 2010. 463, 958-962.
Bardet-Biedl and Meckel-Gruber Syndromes – *Science* 2010. 329, 1337-1340.
Hereditary hypotrichosis simplex – *Nature* 2010. 464, 1043-1047.
Hutchison-Gilford Progeria – *Dev. Cell* 2010. 19, 413-25.
Cutis laxa – *Nat Genet.* 2009. 41, 1016-21.
Colorectal cancer – *Genome Res.* 2009. 19, 987-93.
Nephronophthisis – *Hum Mol Genet.* 2008. 17, 3655-62; *Nat Genet.* 2005. 37, 537-43.

Xenopus egg extracts are used for *in vitro* biochemical studies:

Fanconi Anemia – *Mol. Cell.* 2009. 35, 704-15; *Science.* 2009, 326, 1698-701.
C-myc oncogene – *Nature*. 2007. 448, 445-51.
BRCA1 – *Cell*. 2006. 127, 539-552

Xenopus oocytes are used to study gene expression and channel activity:

Rapid-onset dystonia-parkinsonsim – *Nature* 2010. 467, 99-102.
Trypanosome transmission – *Nature* 2009. 459, 213-217.
Epilepsy, ataxia, sensorineural deafness – *N Engl J Med.* 2009. 360, 1960-70.
Catastrophic cardiac arrhythmia (Long-QT syndrome) – *PNAS* 2009. 106,13082-7.
Megalencephalic leukoencephalopathy – *Hum Mol Genet.* 2008. 17, 3728-39.

Xenopus as a model system for understanding basic biological processes:

Xenopus also plays a crucial role in elucidating the basic cellular and biochemical mechanisms underlying the entire spectrum of human pathologies. Just a small fraction of the many recent discoveries are highlighted here:

Xenopus contributes to our understanding of vertebrate genome organization.
(*Science.* 2010. *328, 633-636*).

Xenopus egg extracts reveal fundamental aspects of cell division.
(*Cell.* 2010. 140, 349-359; *Nature*. 2008. 453, 1132-6; *Science*. 2008. 319, 469-72).

Xenopus reveals new aspects of eukaryotic nuclear structure and function.
(*Cell*. 2010. 143, 288-98; *Science. 2010.* 318, 640-643).

Xenopus embryos are used for studies of Wnt and TGF-β signal transduction.
(*Science*. 2010. 327, 459-463; *Cell*. 2009. 136,123-35).

Xenopus embryos are used for studying mucociliary epithelia.
(*Nat Cell. Biol.* 2009 11 1225-32; *Nature*. 2007. 447, 97-101).

Xenopus embryos are used for studying development of the vasculature.
(*Cell*. 2008.135, 1053-64).

Xenopus egg extracts provide key insights into DNA damage responses.
(*Mol Cell.* 2009. 35,704-15; *Cell.* 2008.134, 969-80).

Xenopus embryos link telomerase to Wnt signaling.
(*Nature*. 2009. 460, 66-72).

Xenopus are used for small molecule screens to develop therapeutics.

(*Nat Chem Biol.* 2010. 6, 829-836; *Blood*. 2009. 114, 1110-22; *Nat Chem Biol.* 2008. 4, 119-25).

Despite its demonstrated utility and despite the recent investments by the NIH, *Xenopus* still lacks many resources that are considered entirely essential for other model systems. It is the consensus of the *Xenopus* community that their biomedical research could be greatly accelerated by the development of key resources of use to the entire *Xenopus* research community.

At the 2010 International *Xenopus* Conference, developmental, cell, and molecular biologists gathered to discuss the resources needed and the priority that should be assigned to each. There was broad community-wide consensus that eleven resources are currently needed, and these were prioritized into two categories: Immediate Needs and Essential Resources:

The Immediate Needs of the *Xenopus* research community:

1. **Generation of the *Xenopus* ORFeome:**
   - -Will enable genome-wide *in vivo* analyses of gene function.
   - -Will enable genome-wide *in vivo* analyses of protein localization.
   - -Will enable, when combined with transgenesis, the first large-scale biochemical determination of protein-protein interactions in specific tissues and at specific embryonic stages.
   - -Will facilitate more-rapid functional characterization of specific proteins.

2. **Improvement of the *Xenopus* genome sequence:**
   - -Will accelerate molecular studies by providing a complete catalogue of *Xenopus* genes.
   - -Will enable completion of the *Xenopus* ORFeomes.
   - -Will enable genomic analyses & systems biology approaches for novel gene discovery.
   - -Will facilitate proteomics approaches and peptide analysis.

Essential Resources for *Xenopus* research community:

In addition to these most-pressing needs, the community has identified nine other Essential Resources that should be developed as soon as possible, so that *Xenopus* biologists can more effectively fulfill the missions of the NIH. The *Xenopus* community considers all of these additional resources to be essential, but understands that priorities must be set, and therefore ranks these as indicated below:

3. **Improvement of long-range contiguity in the *Xenopus laevis* genome**
4. **Improvement of *Xenopus* antibody resources**
5. **Loss of function: Zinc Finger Nucleases/TILLING**
6. **Loss of function: Small inhibitory hairpin RNAs**
7. **Novel loss of function/knockdown/knockout technologies**
8. **Intergenic annotation of the *Xenopus* genome**
9. **Improvements of the *X. tropicalis* genome – long range contiguity**
10. **Additions and improvements to Xenbase: the *Xenopus* Model Organism Database**
11. **Frogbook: A comprehensive resource for methods in *Xenopus* biology**

Community Recommendations for Attaining Resources:

The *Xenopus* Community feels that in order to attain these much needed resources it will be imperative to renew the PAR-09-240/1: "Genetic and Genomic Analyses of *Xenopus*". This mechanism can help to direct funding to the establishment of resources that will accelerate research by the entire community. Development of research resources is essential to the NIH mission, but because such work is not hypothesis-driven, these proposals fare poorly in standard CSR study sections. Moreover, the standard study sections typically lack the depth of expertise that is needed to properly evaluate these proposals. The "Genetics and Genomic Analyses of *Xenopus*" PAR allows for a focused and expert review of resource development proposals, and its renewal will help to ensure a continuing return on the current NIH investment in biomedical research using *Xenopus*.

The *Xenopus* Community also feels that, given the ease with which massive amounts of biological samples can be obtained using this organism, a new PAR to support systems biology using *Xenopus* is warranted. A new PAR in this area would allow all biomedical researchers to exploit the emerging genomic resources for *Xenopus* to perform systems-level analyses *in vivo*, in a vertebrate, and in a cost-effective manner. Such research would generate significant advances into the "New Biology" described below.

Anticipated Gains for Biomedical Research:

Xenopus as an animal model continues to have a broad impact for biomedical research. Given its already long history of large-scale screens of gene function and its broad use in molecular, cell, and developmental biology, the establishment of additional community-wide resources will greatly facilitate the impact of *Xenopus* as a premier vertebrate model for systems-level analyses.

The National Research Council and the National Academy of Sciences have recently called on the United States "to launch a new multiagency, multiyear, and multidisciplinary initiative to capitalize on the extraordinary advances recently made in biology". This report (http://www.nap.edu/catalog.php?record_id=12764) recommends the term "New Biology" to describe an approach to research where "physicists, chemists, computer scientists, engineers, mathematicians, and other scientists are integrated into the field of biology." The promise of systems-level analysis in *Xenopus*, combined with its already proven strengths, make *Xenopus* the ideal model organism for pursuing "New Biology."

Specifically, genome improvements will provide *Xenopus* researchers with the ability to perform genome-wide screens for biological activities that will in turn allow the rapid assembly and analysis of gene regulatory networks and their relationship to phenotypes. The ORFeome will greatly facilitate such genome-wide screening by allowing all ORFs to be rapidly analyzed or large numbers of proteins to be tagged for analysis of protein-protein interaction or for *in vivo* visualization. Using extracts and biochemical purification coupled with mass-spectrometry and genomic sequence, protein interactomes can be rapidly identified and validated. *Xenopus* offers a unique resource because it is the only *in vivo* vertebrate animal model that couples vast amounts of biological material and a sequenced genome, thus cell-type specific interactomes can also be identified. Large-scale genetic screens will identify important novel genes in developmental pathways, especially given the relatively simple genome of *X. tropicalis* compared to zebrafish. Finally, the flexibility of both *Xenopus* extracts and embryos make this system ideal for chemical biology screens.

Identifying gene-regulatory networks, interactomes, and novel genes will be only the first steps. The well-established power of *Xenopus* for rapid analysis of gene function will then allow deeply mechanistic analyses to complement the systems-level

approaches described above. It is the combination of these characteristics that distinguishes *Xenopus* from other vertebrate model systems such as mouse and zebrafish and allows for a systems-level approach to understanding biological mechanisms. The tremendous impact of the *Xenopus* model cannot be realized, however, without the immediate development of community-wide research resources. This White Paper presents the needed resources, and we look to the NIH for guidance in how to best achieve these goals.

For complete details of the 2011 *Xenopus* White Paper, please visit
http://www.xenbase.org/community/xenopuswhitepaper.do

Appendix

Project Number	Project Title	Activity	Principal Investigator	Organization Name	Total Cost
3R01DE018824-02S1	CHARACTERIZATION AND CLONING OF X. TROPICALIS CRANIOFACIAL MUTANTS	R01	KHOKHA, MUSTAFA K	YALE UNIVERSITY	$200,003
5U01DE016754-06	PRACTIONERS ENGAGED IN APPLIED RESEARCH AND LEARNING	U01	VENA, DON	EMMES CORPORATION	$800,851
				Total	$1,000,854