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Functional Genomic Dissection of Multimeric Protein Families in Zebrafish

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The study of multimeric protein function in the postgenomicera has become complicated by the discovery of multiple isoforms for each subunit of those proteins. A correspondingly large number of potential isoform combinations offer the multicellular organism a constellation of protein assemblies from which to generate a variety of functions across different cells, tissues, and organs. At the same time, the multiplicity of potential subunit isoform combinations presents a significant challenge when attempting to dissect the functions of particular isoform combinations. Biochemical and cell culture methods have brought us to a significant state of understanding of multimeric proteins but are unable to answer questions of function within the context of the many tissues and developmental stages of the multicellular organism. Answering those questions can be greatly facilitated in model systems in which expression can be determined over time, in the context of the whole organism, and in which hypomorphic function of each subunit can be studied individually and in combination. Fortunately, the potential for high-throughput in situ hybridization studies and antisense-based reverse genetic knockdowns in zebrafish offers exciting opportunities to meet this challenge. Some of these opportunities, along with cautions of interpretation and gaps in the existing technologies, are discussed in the context of ongoing investigations of the dimeric Na,K-ATPases and heterotrimeric G proteins. *Developmental Dynamics 228: 555–567, 2003.* © 2003 Wiley-Liss, Inc.

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INTRODUCTION

Understanding gene function in vertebrates can be achieved from an analysis of loss-of-function phenotypes and expression patterns across cell types and organs during the life span of experimental organisms. The issue of tissue-specific expression patterns can be approached by a variety of techniques, including whole-mount in situ hybridization, real-time PCR, and immunohistochemistry. Genetic analysis in placental vertebrates can be highly informative but is impeded by poor accessibility to the developing organism, especially when mutant embryos cannot successfully implant or when there is postimplantation embryonic lethality. An understanding of multi-subunit proteins such as Na,K-ATPases and heterotrimeric G proteins pose an additional problem: the evolution of multiple isoforms for each subunit necessitates a knowledge of expression patterns of individual isoforms in each tissue (Force et al., 1999). As illustrated for the 48 zebrafish hox genes (Prince, 2002), problems associated with multiple isoforms can be addressed by a combination of comparative genomics, in situ expression patterns, and hypomorphic phenotypes (Hunter and Prince, 2002; McClintock et al., 2001; reviewed by Prince and Pickett, 2002). Such experiments can provide valuable clues to which isoform combinations may be formed, where, and whether they subserve specific functions. The zebrafish has emerged as an excellent model to pursue such a functional

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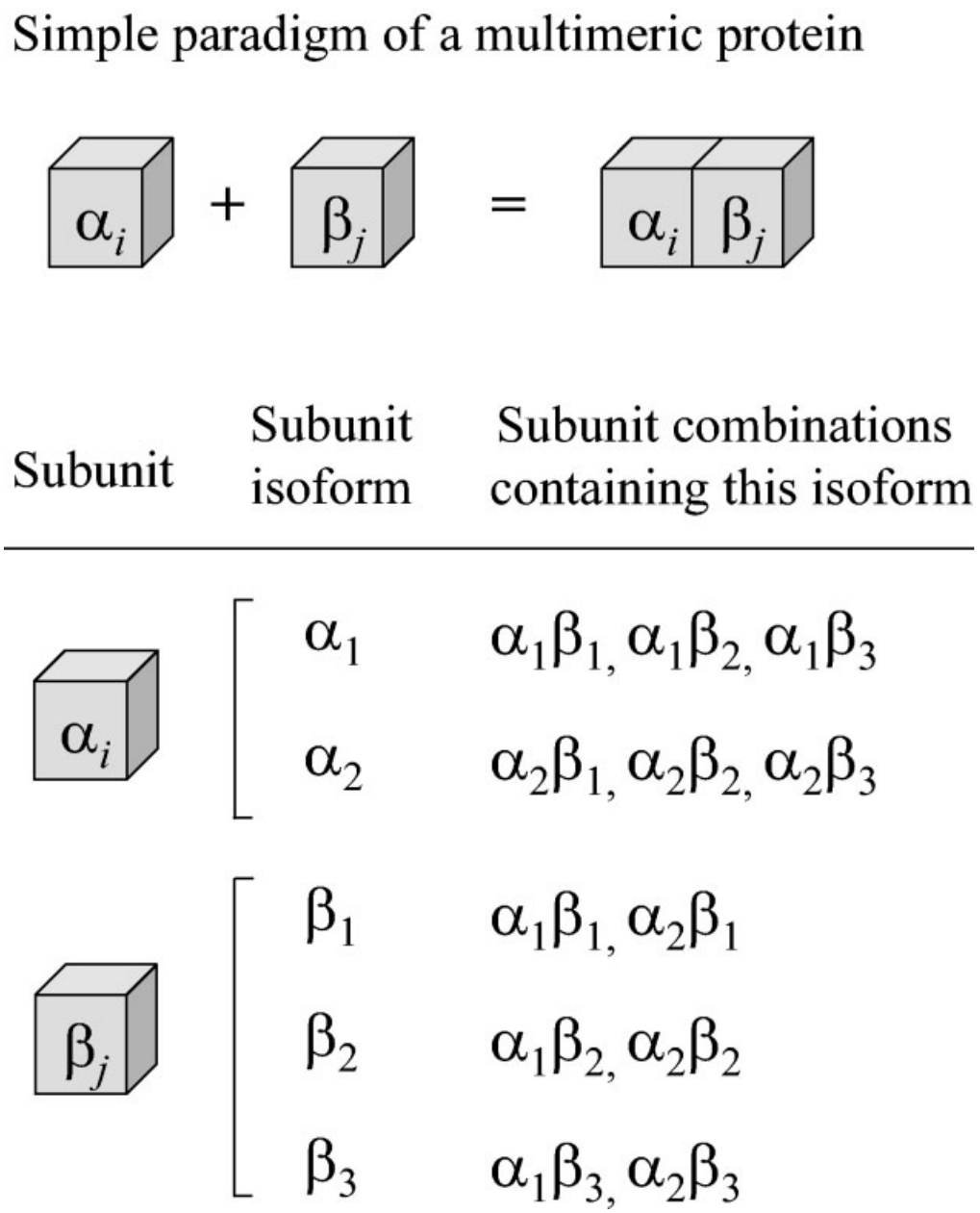

Fig. 1. Paradigm of a multimeric protein. In this simple example of a heteromultimeric protein, there are two subunits, α and β, forming a heterodimer as diagrammed in the top part of the figure. There are two α and three β subunit isoforms. The possible combinations of isoforms, which total six, are listed here by the combinations associated with each isoform.

genomic approach to a range of biological problems (Ekker and Larson, 2001). Here, we explore the potential of zebrafish functional genomics in addressing the mysteries of the function of Na,K-ATPases and G proteins. The limitations of these techniques point to a need for the development of additional technologies, particularly with respect to the analysis of gene function within individual tissues of the juvenile or adult organism.

MULTIMERIC PROTEINS

Biochemical and molecular biological approaches frequently reveal the existence of multimeric protein assemblies in which each subunit has multiple subtypes (isoforms). With increasing frequency, bioinformatic inquiry reveals the existence of additional subunit isoforms. The number of possible combinations of subunits is the product of the numbers of subunit isoforms. The simple paradigm diagrammed in Figure 1 illustrates a heterodimeric enzyme composed of α and β subunits. In this example, the first subunit has two isoforms (α_1 and α_2) and the second subunit, three (β_1, β_2, and β_3). Because the number of possible combinations of subunit isoforms is the product of the numbers of isoforms of each subunit, this arrangement is associated with $2 \times 3 = 6$ possible combinations of subunits ($\alpha_1\beta_1$, $\alpha_1\beta_2$, $\alpha_1\beta_3$, $\alpha_2\beta_1$, $\alpha_2\beta_2$, and $\alpha_2\beta_3$). The dis-

TABLE 1. Functional and Genomic Questions About Multimeric Proteins

Which subunit isoforms are expressed?
In which tissues are the individual isoforms and combinations expressed and active?
What are the functions of each combination?
When during normal development do the combinations function?
What level of functional redundancy is there among subunit isoforms?
Which combinations are important in homeostasis or in disease?

covery of additional subunit isoforms increases the number of potential functional combinations. The multiplicity of these subunit combinations raises interesting questions regarding the functional significance of multimeric protein diversity, both in embryogenesis and in adulthood. These questions are outlined in Table 1.

Limitations of Biochemical, Somatic Cell Genetic, and Transgenic Mouse Approaches

Our ability to answer the questions outlined in Table 1 has been limited in several ways. Biochemical approaches using extracts from single cell types have played a key role in the characterization of specific biochemical or cellular functions, but lack context with regard to tissue type and stage of development. Analysis of phenotypes produced in transfected mammalian cells has made it possible to study functional redundancy between subunit isoforms but is limited when evidence is lacking that specific subunit combinations form in vivo. Mouse knockouts are a valuable tool for studying gene function when the associated phenotypes can be correlated with a functional deficit. However, knockouts are of limited value when knockouts provide no visible phenotype or lead to nonspecific embryonic lethality. Two of the powerful tools of mouse transgenic technology—knock-ins of hypomorphic mutations (Marth, 1996) and the creation of organ-specific deficits during a defined time using the cre-lox system—are achievable (Sauer, 1998) but require significant investments in resources. What has been needed in the field is a vertebrate model system in which one can perform rapid whole-mount in situ hybridizations and rapid reverse genetic experiments in which a range of hypomorphic phenotypes caused by graded inhibition can be generated in a rapid, cost-effective, gene-specific manner. Such a rapid system of evaluation can be used to prioritize mouse transgenic experiments and contribute to our understanding of evolutionary conservation of mechanism.

ZEBRAFISH TO THE RESCUE

Zebrafish has proven to be eminently suited to both whole-mount in situ hybridization of specific mRNAs and to reverse genetic approaches. Whole-mount in situ expression studies in zebrafish are useful for the many genes that show distinct, high-level, tissue-specific expression patterns. This methodology can be performed in a high-throughput manner across embryonic and larval development to elegantly display patterns of expression for virtually any cloned gene (Kudoh et al., 2001; http://zfin.org/cgi-bin/webdriver?MIval=aa-xpatselect.apg). It is important to note that, although positive results in the form of distinct expression patterns are highly useful, lack of staining may be a sensitivity issue. A gene can have functionally important, low-level expression in tissues that show no apparent staining. Thus, whole-mount in situ expression patterns must be interpreted with this limitation in mind. This situation can be addressed, as needed, by more sensitive techniques based on reverse transcriptase-polymerase chain reaction (RT-PCR).

Initial efforts to use antisense technologies in zebrafish met with difficulties. For example, even though RNAi has proven effective in inhibiting specific and even heritable gene effects in *Caenorhabditis elegans* (Tavernarakis et al., 2000) and *Drosophila* (Kennerdell and Carthew, 2000), nonspecific effects render RNAi impractical as a reverse genetic tool in zebrafish (Oates et al., 2000). We know of no studies using siRNA in zebrafish. However, the creation of morpholino antisense oligonucleotides (Summerton and Weller, 1997) has made it possible to generate series of hypomorphic to null mutant phenocopies (morphants) by injection of increasing dosages of antisense morpholino-modified oligonucleotides. Morpholinos are targeted to 21–25 nucleotides regions of the 5′ untranslated region (UTR), extending just past the initiation codon, and are thought to inhibit translation by interfering with ribosome binding (Nasevicius and Ekker, 2000). This technology can also be targeted to splice donor sites (Yan et al., 2002; Mohideen and Cheng, unpublished data). Because zebrafish develop so rapidly, an entire experiment can be completed in a matter of weeks, thereby providing a particular advantage for studying large numbers of genes. Moreover, morpholinos produce suppression of maternal as well as zygotic mRNAs, thereby allowing gene function to be identified in the earliest stages of development. Finally, morpholinos are typically effective through at least the first 2 days of zebrafish development (Nasevicious and Ekker, 2000), thus producing *sustained* suppression during the critical stages of gastrulation and organogenesis. Depending upon the gene, morpholinos may inhibit gene function for 5–6 days, but some morpholinos lose functional efficacy by 50 hr (Ekker, personal communication). The specificity of phenotypes produced by antisense morpholinos can be confirmed by a variety of means, including graded phenotypes corresponding with dose, replication of the morphant phenotype using nonoverlapping morpholinos, abolition of effect using mismatched morpholinos, and mRNA rescue. In morphant analysis of multisubunit proteins, mRNA rescue can also provide a way to determine which isoforms may have overlapping vs. unique functions. A similar approach has been taken in the analysis of hox gene function (reviewed by Prince, 2002 and by Prince and Pickett, 2002), in which evolutionary origin and function of hox genes have been compared. In most instances, one or more of the zebrafish descendants of

TABLE 2. Hypothetical Determination of Functional Subunit Combinations from Expression Patterns and Morphant Phenotypes

Subunit isoform	Expression pattern		Morphant phenotype	Functional subunit combination
	Organ	Timing		
α_1	All cells	Day 1–3	No ear	$\alpha_1\beta_3$
α_2	Gut	>Day 3	None	$\alpha_2\beta_2$
β_1	Heart	>Day 2	Weak contraction	$\alpha_1\beta_1$
β_2	All cells	>Day 2	None	$\alpha_2\beta_2$
β_3	Ear	Day 2–3	No ear	$\alpha_1\beta_3$

a hox gene plays a similar role as its orthologue in mice. In one interesting exception of "function shuffling," the zebrafish Hoxb1a assumed the role of the non-orthologous murine Hoxa1 gene but not that of the orthologous murine Hoxb1 gene (McClintock et al., 2001); subsuming the appropriate expression pattern and function was based upon their evolution from a more ancient, but common, ancestor. These collective advantages seem well-suited for studying the functions of multi-subunit gene families.

Using Zebrafish Functional Genomics to Analyze Isoform Function: A Hypothetical Paradigm

A first step to using the approach outlined above is to identify all isoforms of each multimeric protein subunit. This identification can be accomplished by mining available zebrafish expressed sequence tag (EST) and genome sequence databases (http://www.genetics.wustl.edu/fish lab/frank/cgi-bin/fish/, http://www.sanger.ac.uk/Projects/D rerio/). Once potential isoforms are identified, assignment of isoform identity can be accomplished by sequence comparison and by phylogenetic and syntenic analysis (Postlethwait et al., 1998, 2000). The second step would be to search for spatial and temporal overlaps in expression of the different subunit isoforms. Anatomic and temporal overlaps in expression profiles are useful indicators of potential subunit combinations that can then be tested by morphant analysis.

This approach is illustrated by analysis of the set of hypothetical data shown in Table 2. In this example, the α_1 subunit is expressed in all cells, but only between days 1 and 3. Despite the ubiquitous expression of α_1 knockdown of this gene is found to cause an ear-specific defect. What β subunit isoforms may potentially combine with the α_1 isoform to form a functional enzyme? Each of the β isoforms is expressed in an overlapping time period, but only β_2 and β_3 are also expressed in the ear. This raises the possibility that the multimeric protein consisting of either $\alpha_1\beta_2$ or $\alpha_1\beta_3$ subunit combinations may be formed during otogenesis. Morphant analysis for β_3 shows the same phenotype as α_1, whereas knockdown of the β_2 gene does not produce an ear-specific defect. We can, therefore, conclude that the isoform combination $\alpha_1\beta_3$ is necessary for ear development. The next question is whether the α_1 isoform contributes to any other function. The only other strong morphant phenotype shown by the β subunit isoforms is a weak heart contraction phenotype caused by the β_1 morpholino. Which of the α subunits act with β_1? From these hypothetical patterns of expression, α_1 but not α_2 shares a temporal and anatomic overlap in expression with β_1; thus, $\alpha_1\beta_1$ is necessary for strong heart contraction.

What is the meaning, within the hypothetical model, of the lack of detectable morphant phenotype for isoforms α_1 and β_2? First, it is possible that target knockdown has not been achieved. If this is the case, it is possible that other morpholino or peptide nucleic acid PNA antisense oligonucleotides (Farber, personal communication) might cause an interesting morphant phenotype. It is also possible that there is truly no morphant phenotype. In that case, the isoform may (1) serve a function that is not detectable using the screening method, (2) serve no function, or (3) is active in a redundant pathway that would only be revealed when the other pathway is inactivated. The first two possibilities are less interesting than the third possibility, which has been shown for sonic hedgehog and tiggy-winkle hedgehog genes in the original study by Nasevicius and Ekker (2000), where pathway redundancy was revealed by a phenotype associated with coinjection of morpholinos for both genes, not seen with either alone.

Na,K-ATPase

The idealized example described above is directly relevant to studies designed to understand the functional significance of multiple sodium pump subunit isoforms. Na,K-ATPase is an enzyme that maintains the resting membrane potential in virtually all eukaryotic cells (Cantley, 1981). The enzyme is typically described to consist of two subunits, α and β, in equimolar amounts. Reconstitution experiments (Goldin and Tong, 1974), as well as expression studies in yeast (Horowitz et al., 1990), indicate that both subunits are essential for enzymatic activity. A putative third subunit, termed γ, has been identified that appears to be expressed predominantly in the kidney (Jones et al., 1997). A mutation in the γ subunit has been shown to cause hypomagnesemia and mislocalization of the γ subunit from the plasma membrane to the cytosol (Meij et al., 2000). Because gamma subunits have not been shown to be obligatory subunits of

TABLE 3. Expression Patterns for Na,K-ATPase in Zebrafish at 36 hpf[a]

Isoform	CNS (†)	Retina	Somite	Pronephric duct	Ear	Lens	Mucous cells	Heart
α1a.1	+	Weak		+++		++	+	++
α1α.2	Low			+++	+		+++	
α1a.3	+		++					
α1α.4	+			+++	+++		++	
α1a.5				+++	+		++	
α1b	++							
α2	Diffuse		++					+
α3a	+++							
α3b	++	+++						
β1a	+++			+++	++	++		++
β1b	+						+++	
β2a	+++	Weak						
β2b	Diffuse	Diffuse	+	+	++*			
β3a	Diffuse							
β3b	++		++					

[a]The CNS (central nervous system) column represents diverse patterns of CNS expression. hpf, hours postfertilization. The asterisk indicates highly localized.

Na,K-ATPase, however, the ensuing discussion focuses on the alpha and beta subunits. In mammals, there are four isoforms of the catalytic α subunit (Shull et al., 1986; Herrera et al., 1987; Shamraj and Lingrel, 1994) and three separate β subunit isoforms (Mercer et al., 1986; Martin-Vasallo et al., 1989; Malik et al., 1996). Expression studies have shown that each α subunit can combine in vitro with any of the β subunits to produce a functional enzyme (Jewell and Lingrel, 1991; Schmalzing et al., 1997; Crambert et al., 2000). Thus, there is the potential to form as many as 12 different α/β subunit combinations.

A central unresolved issue concerning Na,K-ATPase is whether the Na,K-ATPase subunit isoforms have unique or overlapping functions. Chromosomal dispersion of Na,K-ATPase genes, coupled with differing expression patterns, suggests specialized functional roles for each of the α and β subunit isoforms (Blanco and Mercer, 1998). However, several lines of evidence suggest that different Na,K-ATPase isoforms may share at least partially overlapping functions. First, each of the four Na,K-ATPase α subunit isoforms is capable of replacing the endogenous α_1 subunit of ouabain-poisoned HEK 293 cells and rescuing the cells from ouabain cytotoxicity (Dahl et al., 2000). Second, mice lacking a functional Na,K-ATPase β_2 gene die at ~2 weeks of age (Magyar et al., 1994). Introduction of the β_1 subunit gene into β_2 knockout mice rescues the mice from juvenile lethality (Weber et al., 1998). Although the mice still exhibit many physiological deficits, these results suggest that the β_1 gene can partially compensate for loss of the β_2 gene in certain cell types. Finally, many different combinations of α and β subunits have been expressed in heterologous cell types (Levenson, 1994). Analysis of the different α/β subunit combinations indicates only subtle biochemical differences (Jewell and Lingrel, 1991; Schmalzing et al., 1997; Crambert et al., 2000), suggesting that the different isoenzymes may not markedly differ in terms of their substrate binding or ion transport properties.

The ability to determine whether different Na,K-ATPase genes have unique or redundant functions has been limited by several factors. While most animal tissues express multiple Na,K-ATPase α and β subunit genes, it is not yet clear whether all possible α and β subunit combinations actually form in vivo. Biochemical purification of individual isoenzymes has not succeeded to date. This difficulty has made it inherently difficult to compare functional properties of native Na,K-ATPase enzymes. In addition, the biochemical assay normally used to characterize Na,K-ATPase activity is performed under nonphysiological conditions. The failure to distinguish differences in substrate binding or ion transport properties among specific subunit combinations produced in transfected cells may reflect the fact that the enzymatic assays are not sufficiently sensitive to measure the biochemical differences between the various enzymes or that specific regulatory factors are not present in transfected cells or are lost during enzyme purification. The use of knockout mice to study Na,K-ATPase gene function in vivo is appealing, because by creating mouse mutants, it should be feasible to link a particular Na,K-ATPase gene with a specific physiological function. However, knockouts of either the Na,K-ATPase α_1 or α_2 genes in mice result in embryonic lethality (James et al., 1999), making it difficult to study the functional properties of these genes.

Zebrafish represent a model system that holds great potential for elucidating the details of Na,K-ATPase gene function. One advantage of the zebrafish system is that evidence for the pairing of specific α and β subunit isoforms can be obtained by examining tissues that express only a single α or β subunit. For example, three Na,K-ATPase genes are expressed in zebrafish heart: α_1a.1, α_2, and β_1a (Table 3). Assuming that both of the α subunits in

heart must pair with the β_1a subunit, it seems likely that enzymes composed of α_1a.1/β_1a and α_2/β_1a subunit combinations are active in heart. A distinctive set of α and β subunit genes are expressed in mucous cells, including α_1a.1, α_1a.2, α_1a.4, and β_1b. Mucous cells, therefore, are likely to express three different Na,K-ATPase isoenzymes that are composed of each of the three α_1-like subunits in combination with the β_1b subunit. This analysis points to a minimum of 14 distinct Na,K-ATPase isozymes in zebrafish. Similar types of analyses have not been systematically carried out in mammalian systems.

Another advantage of the morpholino gene knockdown approach is that it is possible to generate hypomorphs of specific subunit isoforms. By creating Na,K-ATPase hypomorphs, it should be possible to produce recognizable phenotypes without the lethality associated with the homozygous null mutations cited above. The morpholino-based approach allows us to correlate a gene with a visible physiological, developmental, or behavioral phenotype. To perform such an analysis, we have identified, cloned, and sequenced full-length zebrafish Na,K-ATPase cDNAs and determined when and where during embryogenesis the genes are expressed.

Zebrafish Na,K-ATPase Genes

From zebrafish EST and genomic databases, we have identified a cohort of genes encoding zebrafish α and β subunits. The sequence of full-length clones indicates that zebrafish contain nine distinct Na,K-ATPase α, and six β subunit genes (Rajarao et al., 2001, 2002; Blasiole et al., 2002). Sequence comparisons, gene mapping, and phylogenetic analysis have shown that, of the nine α subunit genes, six are orthologous to the mammalian α_1 subunit gene, one to the mammalian α_2 subunit, and two to the mammalian α_3 subunit. No zebrafish counterpart of the mammalian α_4 gene has been identified to date. Of the six β subunit genes, two are mammalian β_1 orthologues, two are β_2 orthologues, and two are β_3 orthologues.

Analysis of the zebrafish genome indicates that zebrafish have undergone an additional round of genome duplication compared with mammals (Amores et al., 1998; Postlethwait et al., 1998). In the absence of gene loss, we would predict that each mammalian α and β subunit gene should have two zebrafish orthologs. Consistent with this view, we have identified two zebrafish orthologs of the rat α_3, β_1, β_2, and β_3 subunit genes. In contrast, we have found only a single ortholog of the rat α_2 subunit, suggesting that the second α_2 ortholog was lost during zebrafish evolution. Recent work suggests that many duplicate genes have been lost during zebrafish evolution (Postlethwait et al., 2000). Six of the zebrafish α subunit genes appear to be orthologous to the mammalian α_1 subunit gene. Thus, for the α_1 gene subfamily, there must have been additional gene duplication events in addition to the genome-wide duplication. Because our mapping data places five of the six α_1-like genes within a tight cluster on LG1, we surmise that these loci have arisen by local, tandem duplications that have arisen after the hypothesized genome duplication event. Retention of a high proportion of Na,K-ATPase α and β subunit gene duplicates suggests that these genes may possess specialized, rather than redundant, functional properties. It should be noted that it is possible, and even likely, that some of the duplicate genes may have evolved functions not present in mammals.

Whole mount in situ hybridization analysis has shown that all 15 zebrafish Na,K-ATPase genes are expressed during embryogenesis (Canfield et al., 2002; Blasiole et al., 2002). The most striking aspect of this analysis was that no two α subunit genes were found to exhibit the same spatial distribution pattern, and likewise, each β subunit exhibited a unique pattern of expression. Expression at 36 hours postfertilization (hpf) is summarized in Table 3. Based on these distinct expression profiles, we were able to deduce with a high degree of certainty that at least 14 of the 54 possible different α/β subunit combinations are likely to be produced in zebrafish embryos. For example, the sole β subunit strongly expressed in pronephric duct, β_1a, is likely to associate with each of the four α subunits expressed in this tissue. Knowledge gained from these types of experiments comprise a framework for morpholino-based gene knockdown experiments designed to analyze Na,K-ATPase gene function.

Analysis of Na,K-ATPase Gene Function

We are exploring a strategy that combines targeted gene knockdowns with mRNA rescue to learn whether individual Na,K-ATPase genes perform unique or redundant functions. In this approach, mRNAs produced from different Na,K-ATPase genes can be analyzed for their ability to rescue a morphant phenotype produced by targeted knockdown of one specific Na,K-ATPase gene. The ability of mRNA from one gene to rescue a morphant derived by knockdown of another gene would provide evidence that the rescuing mRNA and knocked-down gene possess overlapping functions. In settings where the rescuing gene is not actually expressed in the cell type being rescued, experimental rescue may not reflect function of that subunit in vivo. Functional divergence in this case can be achieved by an evolutionary change in the tissue-specificity of expression. The inability of an mRNA to rescue a morphant, on the other hand, would provide evidence that the knocked-down gene and rescuing mRNA encode Na,K-ATPase subunits with distinct biological functions. This type of complementation analysis should provide a much clearer picture of which Na,K-ATPase genes are functionally distinct and which are redundant. It is important to note in this context that the Na,K-ATPase α and β subunit genes show distinct expression patterns. Differences in gene expression patterns result in spatial segregation of the various enzyme subunits. It is, therefore, possible that the different genes can compensate for each other if they were in the same tissue, but because the genes are spatially separated, they appear to be functionally divergent due to expression

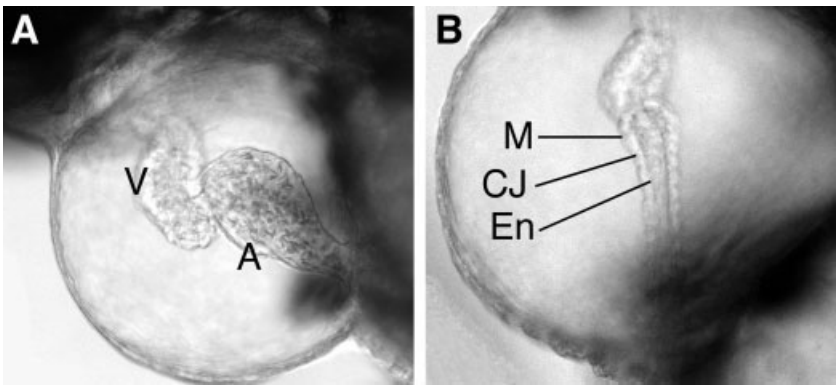

Fig. 2. Cardiac phenotype of Na,K-ATPase β_1a morphants. **A:** At 0.7 ng. **B:** At 1.0 ng. Embryos are shown at 72 hours postfertilization. View is from left side of embryo, with anterior oriented toward upper left of each panel. The morpholino used was complementary to nt −1 to +24 of the mRNA, numbered relative to the initiation codon. The no morpholino control was difficult to compare due to lack of pericardial edema. Note the decreased complexity of the heart in both chambers at the higher dose. Similar results were obtained using a nonoverlapping morpholino complementary to nt −28 to −4. A, atrium; CJ, cardiac jelly; EN, endocardium; M, myocardium; V, ventricle.

pattern differences. Knockout experiments in mice do not support this view. In mice, the α_1 and α_2 subunits are both expressed in the heart; however, null mutations at either of the two alleles produces embryonic lethality (James et al., 1999). In the mRNA cross-rescue experiments, microinjected mRNA will be heterogeneously distributed throughout the developing embryo, ensuring that the knocked-down gene and the rescuing mRNA are expressed in the same cell and tissue types. This type of approach should, therefore, allow for genetic complementation to occur in morphant embryos, regardless of whether the two genes are spatially separated in wild-type embryos. When complementation occurs between subunits that by whole-mount in situ hybridization do not appear to be present in the tissue in question, it will be important to determine by RT-PCR and real-time PCR of microdissected tissues whether there actually is expression in those tissues that was below the sensitivity of the whole-mount technique.

Morpholino-based gene knockdowns provide a potentially powerful new approach to understanding the function of the Na,K-ATPase β subunit. The Na,K-ATPase β subunit is a class II-type membrane protein containing a single transmembrane segment and an extracellular amino-terminal domain. Several lines of evidence suggest that the β subunit is an essential component of Na,K-ATPase. The β subunit has been shown to be absolutely required for Na,K-ATPase activity when purified subunits are reconstituted in lipid-containing vesicles (Goldin and Tong, 1974) or when enzyme activity is produced from cDNAs transfected into yeast (Horowitz et al., 1990). Understanding the functional role of the β subunit has proven elusive, however, because β subunits seem to contribute to neither the Na,K-ATPase catalytic cycle nor the ouabain sensitivity of the enzyme. Biosynthetic studies performed in *Xenopus* oocytes and transfected mammalian cells suggest a potential role for the β subunit in the folding and membrane-targeting of the enzyme (McDonough et al., 1990; Geering, 2001). The possibility that the β subunit may modulate enzyme activity has also received considerable attention (Geering, 2001). A major limitation in the analysis of the β subunit is the lack of a biological assay that measures β subunit function. The rodent Na,K-ATPase α subunit has been shown to confer a selectable phenotype, ouabain resistance, upon ouabain-sensitive primate cells (Kent et al., 1987; Emanuel et al., 1988). In this assay, the naturally ouabain-resistant rodent α_1 subunit can substitute for the endogenous α subunit of primate cells to form a functional Na,K-ATPase (Emanuel et al., 1988). In contrast, the β subunit does not confer a selectable phenotype so that when β subunits are introduced into mammalian cells (all of which contain native Na,K-ATPase), the activity of the transfected β subunit cannot be distinguished from that of the resident β subunit present in the recipient cell. In an attempt to understand β subunit function, mice carrying a null mutation at the β_2 subunit locus have been generated by homologous recombination (Magyar et al., 1994). However, homozygous β_2 knockout mice die shortly after birth. Whereas the β_2 gene knockout affected the morphologic integrity of several brain compartments, the physiological basis for these alterations has not been elucidated.

The ability to perform gene knockdown/mRNA rescue in zebrafish now provides a strategy for development of an in vivo biological assay for Na,K-ATPase β subunit function. Our cloning experiments identified six zebrafish β subunit genes (Rajarao et al., 2001, 2002). For our initial gene knockdown experiments, we focused on the β_1a gene. During embryogenesis, β_1a transcripts are robustly expressed in pronephric duct, otic vesicle, lens, and heart (Canfield et al., 2002), suggesting that the β_1a gene is a reasonable candidate for a gene knockdown approach. It should be noted that some morphant phenotypes may be refractory to mRNA rescue because of the relatively short half-life of injected mRNAs, and poor distribution of large mRNAs among the blastomeres of the injected embryo. This problem may potentially be addressed by identifying earlier surrogate phenotypes, by transgenic rescue, or by developing longer-lived, biologically active derivatives of mRNA.

To knockdown β_1a expression, two independent, nonoverlapping β_1a morpholinos targeted to the 5′ UTR were synthesized and injected into single cell embryos. As shown in Figure 2, both morpholinos produced essentially the same morphant phenotype, and each acted in a dose-dependent manner. The most notable effect of the β_1a morpholino was on cardiac function, which occurs in 100% of embryos injected with 0.7 ng or more of β_1a morpholino. The ma-

jor difference between the lower and higher morpholino doses is that the defective cardiac phenotype occurs earlier and with greater severity at the higher doses. In 48 hpf, embryos injected with 0.7 ng of morpholino, the heart chambers appear enlarged, whereas at 1.0 ng of morpholino, the walls of the ventricle appear to lack the normal muscular trabeculae that normally criss-cross the heart chambers, causing the lumen to be diminished (Fig. 2). At later time points, pooling of blood occurs in the sinus venosus and heart chambers, associated with a complete loss of circulation (not shown). These defects appear to be similar to the *pipe heart* and *tango* mutants described in the Tübingen mutant screen (Chen et al., 1996). As expected, β_1a sense morpholino and a β_1a antisense morpholino containing a four base mismatch did not produce noticeable defects in cardiac morphology or function. These experiments support the specificity of the β_1a morpholinos in causing cardiac dysfunction. Rescue experiments are in progress.

A major challenge we now face is to understand how a decrease in Na,K-ATPase β_1a expression levels causes a defect in cardiac function. One approach will be to learn whether the β_1a gene knockdown causes a defect in the electrical activity of the heart. By analyzing sections of zebrafish heart, we should also be able to determine whether there are structural abnormalities in morphant vs. normal hearts such as in the morphology of the myocardial cell layer, and whether there are gross abnormalities in cell size, shape, or concentric cell growth within the developing myocardium. In this context, it is interesting to note that the Na,K-ATPase α_1a.1 and α_2 genes are also expressed in the developing myocardium (Canfield et al., 2002). Because the β_1a gene is the only β subunit gene expressed in heart, it seems likely that enzymes composed either of α_1a.1/β_1a or α_2/β_1a combinations are formed in the zebrafish myocardium. These experiments were performed by Dr. Manzoor Mohideen in the Cheng lab. It will clearly be of interest to determine whether knockdown of the α_1a.1 or α_2 genes phenocopy the β_1a morphant, and whether the two α subunit genes play functionally distinct roles in either heart development or function.

Preliminary data from studies that use the same approach presented elsewhere in this issue (Blasiole et al., 2002) indicate that sodium pump genes play distinct and unexpected roles in ear development. Together, these experiments are beginning to offer tantalizing clues regarding the roles of sodium pump subunit isoforms in various aspects of zebrafish embryogenesis.

HETEROTRIMERIC G PROTEIN SIGNALING

Heterotrimeric G proteins are membrane-associated proteins required for receptor signaling in all eukaryotic cells. Members of this G protein family function as heterotrimers composed of α β, and γ subunits. Over the past decade, several well-done reviews document the remarkable progress made in identifying families of subunit isoforms, surveying their potential interactions, and elucidating their mechanistic roles in the signaling process (Yan et al., 1996; Hamm, 1998; Ford et al., 1998; Wess, 1997; Horowitz et al., 2000; Pierce et al., 2002; Albert and Robillard, 2002; Landry and Gies, 2002; Robishaw et al., 2003). Nevertheless, significant gaps in our understanding of these pathways remain. For instance, the large number of α β, and γ subunits has raised fundamental questions regarding how the G proteins are assembled to control the specificity of the several hundred receptor signaling pathways that are now known to exist. The currently identified 16 α , 5 β, and 12 γ subtypes (Horowitz et al., 2000) have the potential to form 960 different heterotrimeric isoform combinations. How many G protein α, β, and γ subunit combinations actually exist in vivo? Because the individual subunits are structurally diverse and differentially expressed, one can reasonably predict that specific G protein α, β, and γ isoform combinations play specialized roles in particular receptor-signaling pathways. Despite this exciting possibility, that prediction has been difficult to address using traditional approaches and models.

Attempts to purify individual G protein α, β, and γ subunit isoform combinations have been unrewarding. This difficulty reflects the deleterious effects of the detergent required to solubilize the G proteins. Detergent speeds up the innate tendency of the G protein subunits to dissociate (Wilcox et al., 1995) and disrupts any physical organization of the signaling systems that is likely to be present in the intact cell (Neubig, 1994; Ray et al., 1996; Huang et al., 1997). As a result, the biochemical approach produces artifactual mixing and exchange of G protein α, β, and γ subunits between signaling pathways that may not normally have access to each other. Transfection and reconstitution approaches, however, have provided important structure-function information, including identification of regions of the α, β, and γ subunits important for interaction with certain receptors and effectors (Yan et al., 1996; Wess, 1997; Hamm, 1998; Ford et al., 1998). Unfortunately, comparison of the biochemical properties of various G protein α, β, and γ subunit combinations have not revealed striking functional differences (Iniguez-Lluhi et al., 1992; Ueda et al., 1994; Richardson and Robishaw, 1999; Hou et al., 2000; McIntire et al, 2001). This finding may reflect the high homology among various members of the α, β, and γ subunit families that allows them to readily substitute for one another when they are brought together or the lack of unidentified factors that operate in a cell-specific context. Regardless, biochemical approaches to date have fallen critically short of establishing roles of specific G protein α, β, and γ subunit combinations in particular signaling pathways in the context of the organism.

Genetic approaches to the problem of subunit combinations have great potential. If one were able to diminish the expression of individual subunits in an organism, an associated phenotype would provide strong evidence of function. Gene targeting approaches in mice have begun to demonstrate important

functional differences among G protein α (Offermanns, 2001) and γ (Schwindinger et al., 2003) subunit genes. However, the large sizes of these gene families and the even larger number of combinatorial possibilities have limited the scope of this approach. Thus, more than 10 years after their discovery, more than half of the members have yet to be targeted for knockout in mice. For this purpose, the use of morpholino antisense inhibition of specific G protein subunit genes in zebrafish offers a unique opportunity to rapidly screen for interesting loss-of-function phenotypes that can then be targeted for knockout in mice.

Diversity of G Protein α, β, and γ Subtypes

A primary advantage of zebrafish for the study of heterotrimeric G proteins is that it has a molecular complexity close to that of humans and mice. Both the human and mouse genomes contain 16 α, 5 β, and 12 γ subunit genes (Horowitz et al., 2000). Although the zebrafish genome is not yet complete, analysis of the available zebrafish databases has identified at least 12 α, 5 β, and 10 γ subunit genes, which in most cases show striking homology to their mammalian counterparts (> 90% amino acid homology; J.D. Robishaw and T.C. Leung, unpublished observations). By contrast, other genetically tractable models, such as yeast, fungi, and nematode, do not show this degree of multiplicity. For instance, the *S. cerevisiae* and *Dictyostelium* genomes contain only one β and one γ subunit gene (Zhang et al., 2001; Dohlman, 2002). Similarly, the *C. elegans* genome includes only two β and two γ subunit genes (Jansen et al., 2002). These observations suggest that vertebrates have evolved an additional level of complexity at the level of assembly of the G protein heterotrimer that make them unique. The similar diversity of G protein subunits between human, mouse, and zebrafish allows the use of zebrafish as a functional genomics tool to dissect the ontogeny of this important class of multi-subunit proteins.

Differential Expression of G Protein Subunit Isoforms

An important step in deciphering which G protein α, β, and γ subunit combinations actually exist in the intact organism is to determine what factors control their assembly. One way to regulate their assembly is to restrict the expression of certain subunits to a given time and place. Therefore, study of their expression patterns is a prerequisite for understanding which G protein α, β, and γ subunit combinations are operative in the context of the organism.

Zebrafish offer several specific advantages for this purpose. Whole-mount in situ hybridization can be used as a powerful screening tool to identify temporal and spatial patterns of gene expression (Jowett, 1999, 2001). Moreover, because embryos are optically transparent, any changes in gene expression can be readily correlated to specific developmental events. Recently, our laboratory has established proof-of-principle by showing that G protein α, β, and γ subunits exhibit distinctive patterns of gene expression during embryogenesis that can be used to infer potential associations and functions. As an example, we have identified a novel G protein γ subunit, designated γ_{15}, in zebrafish. At 24 hpf, the γ_{15} subunit is expressed in a very localized region of the diencephalon that gives rise to the pineal gland (epiphysis). Intriguingly, we have identified a specific β subtype whose expression pattern overlaps with the γ_{15} subunit in this tissue. Taken together, these findings suggest a mechanism in which regulated expression can be used to produce a novel G protein α, β, and γ trimer that may have a specialized role in the pineal gland. Through subsequent analysis of promoter regions and a search for potential enhancers, it should be possible to identify and test those regulatory features responsible for coordinate expression in transgenic fish (Long et al., 1997). Through subsequent analysis of their loss-of-function phenotypes, it should be possible to directly link this novel G protein trimer to a particular function and signaling pathway, as described in more detail below. Thus, zebrafish provides an unique opportunity to study the factors controlling the assembly of this important class of heterotrimeric proteins. This type of information not only suggests the makeup of the G protein α, β, and γ complex but also provides an entry point for functional studies in the context of the whole organism.

Functional Heterogeneity of G Protein Isoforms

The functional significance of the rich diversity of G protein α, β, and γ subtypes remains enigmatic. Although combinatorial association predicts the existence of several hundred G protein α, β, and γ trimers, their specific roles in particular receptor signaling pathways have been difficult to show using transfection and reconstitution approaches. Recently, a gene targeting approach was used to provide the first conclusive proof that receptors recognize G protein trimers on the basis of their α, β, and γ subunit combinations in the organism. Notably, despite the expression of a wide variety of γ subtypes in the brain, we showed that genetic disruption of the γ_7 subtype compromised the assembly of a specific G protein trimer that is required for signaling by the D_1 dopamine receptor (Schwindinger et al., 2003). This result, however, points to a large void in the field. In the vast majority of cases, it is not known which receptors activate which particular G protein α, β, and γ trimers in the context of the organism; nor is it understood how the specificity is encoded in this interaction. Unfortunately, the large number of combinatorial possibilities precludes the timely application of a gene targeting approach in mice. However, the recent emergence of a morpholino antisense strategy provides the opportunity to address these questions in a timely manner in zebrafish.

There is little information available on the roles of G protein α, β, and γ subtypes during the development of any vertebrate organism (Malbon, 1997; Schwindinger and Robishaw, 2001). Nonetheless, consistent with their multiplicity and diversity, mor-

pholino "knockdown" of the α or γ subtype would be expected to give a more discrete phenotype, whereas "knockdown" of the β subtype would be likely to show a more generalized phenotype. Moreover, a certain subset of α, β, and γ subtypes would be expected to yield overlapping phenotypes, thereby providing much-needed information on their functional associations and roles in particular receptor-signaling pathways.

Developmental Functions of G Proteins

Elucidation of the network of signaling pathways involved in vertebrate development remains a challenge. Although G proteins are used extensively to regulate development in lower eukaryotes (Zhang et al., 2001; Dohlman, 2002; Jansen et al., 2002), their importance in higher eucaryotes has been much more difficult to elucidate. In large part, this finding reflects the difficulty of studying early embryogenesis in most vertebrate models. Traditionally, the mouse has been a choice vertebrate species for studying the genetics of development. Gene "knockouts" of many of the G protein α subtypes have pointed to their importance in embryogenesis (Offermanns, 2001); and similar studies are now under way for the β and γ subtypes (Schwindinger et al., 2003). Nevertheless, the mouse model has many disadvantages for this type of analysis. Predominant among these are the large sizes and combinatorial possibilities of the G protein subunit genes; the intrauterine development of embryos that prevents direct observation of developmental events; and the difficulty of studying mutants with embryonic lethality. For this reason, investigators are turning to other models. As first described by Streisinger et al. (1981), zebrafish have many advantages for embryologic studies. Zebrafish have a short generation time, which permits the whole analysis to be completed in weeks rather than years; and at a fraction of the cost. Moreover, they produce large clutches of synchronized embryos, which facilitate genetic manipulation, and the transparent nature of embryos allows visual screening for developmental abnormalities that may occur as the result of genetic manipulation. Recent studies provide proof-of-principle that zebrafish are a useful system to dissect out the functional roles of the *edg 5* and *cxcr4* receptors in development (Kupperman et al., 2000; David et al., 2002). Because these classic, seven transmembrane-domain receptors have been shown to act through G proteins in other systems, it is only a matter of time before this system is used to decipher the roles of specific G protein α, β, and γ subtypes in these signaling pathways in zebrafish.

There is a growing awareness that G proteins serve as points of convergence for multiple signals originating from diverse types of signaling pathways (Radhika and Dhanasekaran, 2001). Intriguingly, there is some evidence to suggest that nonclassic, seven transmembrane-domain receptors such as smoothened (Hammerschmidt and McMahon, 1998) and frizzled (Liu et al., 1999) may also require G proteins for some of their actions during development. For example, in zebrafish, a possible role for the G protein α_i subtype in the ventral specification of forebrain and somitogenesis has been suggested in *hedgehog* signaling (Hammerschmidt and McMahon, 1998); and in *Xenopus*, the α_i subtype has been implicated in *smoothened*-mediated pigment aggregation in melanophores (DeCamp et al., 2000). In another instance, recent studies show that frizzled receptor chimeras containing the ligand binding domain of the β-adrenergic receptor are able to induce primitive endoderm formation in mouse cell cultures in response to isoproterenol; this effect is blocked by pertussis toxin, suggesting the involvement of the G proteins (Liu et al., 1999). Collectively, these data suggest that at least some FRZ receptors are bona fide G protein coupled receptors that interact directly with G proteins to mediate their effects. Therefore, the study of G protein subtype morphants will also test whether G proteins are involved in these nonclassic signaling pathways. Those components that are part of the same signaling pathway would be expected to produce similar phenotypes.

The breadth of biological processes regulated by the G proteins is remarkable. The emerging picture suggests that the α, β, and γ subunit composition of the G proteins may provide a mechanism to propagate this range of biological responses. Zebrafish offers several specific advantages for deciphering how the G proteins receive, integrate, and process the multitude of signals required for the development of an organism. Because many diseases (e.g., cancer, heart disease) are associated with re-induction of embryonic genes, this information is expected to eventually lead to the design of more selective diagnosis and therapeutic treatments.

WHENCE FUNCTIONAL GENOMIC ZEBRAFISHING?

Reverse genetic approaches in zebrafish represent a powerful system for dissecting the unique functions of multimeric protein assemblies in the context of organism biology. Despite that power, limitations to this approach need to be kept in mind. First, morpholinos are generally effective for only 2 to 4 days in zebrafish. New methods are needed to extend the time of sequence-specific knockdown effects, especially for late-acting genes. Ideally, graded hypomorphic series will become possible in zebrafish that not only last through adulthood but also can be induced at any time in specific tissues during the lifespan of the individual. In the mouse, these advances have been achieved using a variety of transgenic technologies (reviewed by Tuveson and Jacks, 2002). Zebrafish embryonic stem (ES) -like cells have now been grown in culture (Sun et al., 1995) and germline chimeras have been made (Ma et al., 2001). More recently, ES-like cells have been shown to remain pluripotent and germ-line competent after selection of homologous insertion of a targeting vector. The frequency of homologous recombination achieved in zebrafish is comparable to that of mouse ES cells

(Collodi, personal communication; Fan et al., 2003). This progress suggests that the goal of an array of knockout technologies, including knock-in, conditional, and tissue-specific transgenesis in zebrafish may be reached within the next few years. The combination of these tools with the ability to perform large-scale forward genetic screens in zebrafish will greatly facilitate the genetic dissection of many biological functions, including those associated with multiple subunit isoforms. A promising technology, though without well-documented reproducibility, is the use of photo-mediated gene activation using caged RNA/DNA in zebrafish embryos (Ando et al., 2001). Uncaging of morpholinos in specific tissues would also be a highly desirable development. Technologies targeting specific tissues in the adult are particularly important to develop for modeling human diseases, particularly cancer, in which a significant somatic genetic component applies.

The proposed use of zebrafish to dissect the function of multimeric proteins is not an end-all. Rather, as promising results are derived from zebrafish, complementary work with other organisms, including other vertebrates, as well as other (including biochemical) approaches, will be indicated. Nonetheless, the proposed functional genomics work in zebrafish, in conjunction with a consideration of evolutionary issues (elegantly discussed by Prince, 2002) is bound to lead to a deeper understanding of the combinatorial power of multisubunit proteins. In the face of a tidal wave of gene expression and gene network analyses, we expect the growing functional genomic toolbox of the zebrafish to contribute strongly to our understanding of how complex protein assemblies work in the whole vertebrate organism. In the past, we have encouraged the scientific community to apply zebrafish forward genetics (mutagenesis screens) in the dissection of biological functions outside of development (Cheng and Moore, 1997). Similarly, the reverse genetics approaches in zebrafish described here can be applied to any biological problems for which the investigator can come up with a readily detectable hypomorphic phenotype.

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