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Defining Synphenotype Groups in *Xenopus tropicalis* by Use of Antisense Morpholino Oligonucleotides

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To identify novel genes involved in early development, and as proof-of-principle of a large-scale reverse genetics approach in a vertebrate embryo, we have carried out an antisense morpholino oligonucleotide (MO) screen in *Xenopus tropicalis,* in the course of which we have targeted 202 genes expressed during gastrula stages. MOs were designed to complement sequence between −80 and +25 bases of the initiating AUG codons of the target mRNAs, and the specificities of many were tested by (i) designing different non-overlapping MOs directed against the same mRNA, (ii) injecting MOs differing in five bases, and (iii) performing "rescue" experiments. About 65% of the MOs caused *X. tropicalis* embryos to develop abnormally (59% of those targeted against novel genes), and we have divided the genes into "synphenotype groups," members of which cause similar loss-of-function phenotypes and that may function in the same developmental pathways. Analysis of the expression patterns of the 202 genes indicates that members of a synphenotype group are not necessarily members of the same synexpression group. This screen provides new insights into early vertebrate development and paves the way for a more comprehensive MO-based analysis of gene function in *X. tropicalis*.

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Introduction

The results of genome sequencing projects and the extensive analyses of expressed sequence tags (ESTs) have provided remarkable insights into the expression and regulation of many genes. For some species, and especially for invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster,* it has also been possible to assign functions to these genes on a genome-wide scale. Such approaches have frequently employed traditional genetic approaches [1], but in addition, RNA interference (RNAi) has been used to inhibit gene function in a systematic and high-throughput manner in *C. elegans* [2,3] and *Drosophila* [4,5], while antisense morpholino oligonucleotides (MOs) have been used in a screen for gene function in the ascidian species *Ciona intestinalis* [6].

"Reverse genetic" screens of these sorts have the advantages of speed (because one does not have to locate the mutated gene) and economy, and a similar high-throughput approach to the investigation of gene function in vertebrate embryos will be very important for a proper understanding of development and disease. Unfortunately, such an approach cannot easily be adopted in mammalian embryos, except when studying very early stages [7], because the embryos are inaccessible and the abilities of most reagents to inhibit gene function decline as the embryos grow. Zebrafish provide a useful and powerful alternative, and indeed quite extensive MO screens have been carried out in this species [8]. However, the zebrafish is not a tetrapod, and like other teleost fish it underwent a whole genome duplication event between 200 and 450 million years ago [9,10], so that some genes are likely to have retained at least partially redundant functions [11,12].

In this paper we show that the tetrapod species *Xenopus tropicalis* is a useful alternative model organism. *X. tropicalis* shares most of the advantages of *Xenopus laevis* as a model system for studying cellular, molecular, and developmental biology [13,14], and it shows a higher degree of synteny to amniotes than does the zebrafish (see http://www.metazome.net). In addition to these advantages, *X. tropicalis* is diploid (*X. laevis* is allotetraploid), its genome has been sequenced (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html), it develops more quickly than *X. laevis,* and it has a generation time of approximately 5 months compared with that of 14 months in *X. laevis*.

The technique of choice for inhibiting gene function in *Xenopus* species involves the injection of MOs [15]. MOs are frequently designed to inhibit translation of the target mRNA but can also be used to interfere with the correct splicing of a target pre-mRNA [16,17]. It is important to note that MOs, like RNAi, do not necessarily remove the gene product of interest completely, so the strategy is referred to as gene

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Abbreviations: EST, expressed sequence tag; MO, antisense morpholino oligonucleotide; RNAi, RNA interference

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Synopsis

Genome sequencing projects have provided remarkable insights into the expression and regulation of many genes. For some species, such as the invertebrates *Caenorhabditis elegans* and *Drosophila melanogaster*, it has been possible to assign functions to these genes on a genome-wide scale. For the vertebrates, similar efforts are being made in mouse and zebrafish, but work in the former species is expensive and slow, and the zebrafish experienced a whole genome duplication event, so that some genes may have retained redundant functions. Here, this study uses antisense morpholino oligonucleotides (MOs) to show that the diploid amphibian *Xenopus tropicalis* provides a powerful alternative species. The authors have designed MOs to target sequences around the initiating AUG codons of 202 genes expressed during early development and confirmed that these function in a specific manner. About 65% of the MOs caused embryos to develop abnormally, and the authors have divided the genes into "synphenotype groups," members of which cause similar loss-of-function phenotypes. Expression pattern analysis indicates that members of a synphenotype group are not necessarily members of the same synexpression group. This screen provides new insights into vertebrate development and paves the way for a comprehensive MO-based analysis of gene function in *X. tropicalis*.

"knockdown" rather than "knockout." However, MOs do offer the opportunity to study the functions of large numbers of vertebrate genes very quickly compared with the generation of genetic null mutants, and indeed a pilot screen in *X. tropicalis*, in which the functions of 26 genes were investigated, suggests that this approach might be fruitful [18].

In this paper we use MOs to inhibit the functions of 202 genes in *X. tropicalis*. We have addressed the specificity of the different MOs, assigned the observed phenotypes to different classes, and, as Niehrs and colleagues have done for gene expression patterns [19,20], subdivided the phenotypic classes into synphenotype groups [21]. Work in *Drosophila* [1] and the zebrafish [22,23] has shown that the classification of phenotypes in this way is fundamental to the analysis of development, allowing the identification of genes that are involved in similar developmental processes and which interact with each other genetically or biochemically, directly or indirectly. The expression pattern of each gene has been analyzed by in situ hybridization and by noting the representation of its associated transcripts in different cDNA libraries. All the data are accessible through a Web-based database, and our data pave the way for a more comprehensive MO-based analysis of gene function in *X. tropicalis*.

Results

Gene Selection and Experimental Conditions

Genes were selected for analysis from the Wellcome Trust/CR-UK Gurdon Institute *Xenopus tropicalis* database (http://informatics.gurdon.cam.ac.uk/online/xt-fl-db.html) [24]. BLAST searching confirmed one of our criteria that all should be conserved between *Xenopus* and mammals, and in addition, for purposes of comparison, we selected some genes that had been studied previously by mutation, knockdown, or dominant-negative technologies, and some that had not. The former category allows the comparison of phenotypes caused by injection of MOs in *X. tropicalis* with those obtained by other approaches in other species, including mouse and zebrafish, as well as *X. laevis*. The latter category was divided into one group comprising genes about which at least something is known (for example, that they are members of gene families already known to play a role in development, or have been studied in vitro, or contain a particular functional domain) and another comprising genes that are completely novel. A large proportion of genes identified through genome and EST sequencing projects are of unknown function, so analysis of this second group should provide an idea of what results might be obtained in the course of a screen designed to target the entire *X. tropicalis* transcriptome.

Unless otherwise stated, MOs were designed to complement sequence between −80 and +25 bases of the initiating AUG codon of the target mRNA (see Materials and Methods). Two different doses of MO were injected for each gene studied. First, like Kenwrick and colleagues [18], who also used *X. tropicalis*, we injected 10–15 ng of our oligonucleotides. However, we also noted that experiments in *X. laevis* can employ up to 90 ng MO [25] and that the significantly smaller embryos of *X. tropicalis* contain between a third to half as much RNA as those of *X. laevis* (unpublished data). Our results also show that 30 ng of a control MO causes no detectable effect on the development of *X. tropicalis*, beyond, in some egg batches, a slight delay in development. In an attempt to strike a balance between eliminating the gene product of interest and not causing non-specific effects, we therefore additionally used a dose of 30 ng MO in our experiments.

Overview

Embryos were examined at early to mid-gastrula (stage 10.5–12), tailbud (stage 22–28), and tadpole (stage 37–41) stages (Figure 1A–1C), and any deviations from normal development at each stage were noted (see below). MOs were modified by the addition of Carboxyfluorescein or Lissamine at their 3′ ends, allowing us to ensure that oligonucleotides were distributed evenly throughout the embryo and, to some extent, that similar amounts of MO had been injected into each embryo (Figure 1D–1F). Higher-power examination of the animal pole region of an embryo at the early gastrula stage revealed that fluorescent MOs are present in both the nucleus and the cytoplasm of cells, but are particularly highly concentrated in the nucleus (Figure 1D').

Table 1 provides an overview of the results obtained in this screen. For the purposes of this analysis we require that a particular MO should cause at least 50% of injected embryos

Table 1. Summary of Results

Gene Category[a]	% Phenotypes Observed	
	10–15 ng	30 ng
All genes ($n = 202$)	63 (48)	69
Previously studied ($n = 70$)	77 (57)	79
Partially characterized ($n = 64$)	56 (48)	61
Novel ($n = 68$)	54 (38)	59

[a]See text for definitions of these categories.
Figures in parentheses in the center column indicate the frequency of phenotypes observed when analysis is carried out at stage 30 and not tadpole stage 37–41.
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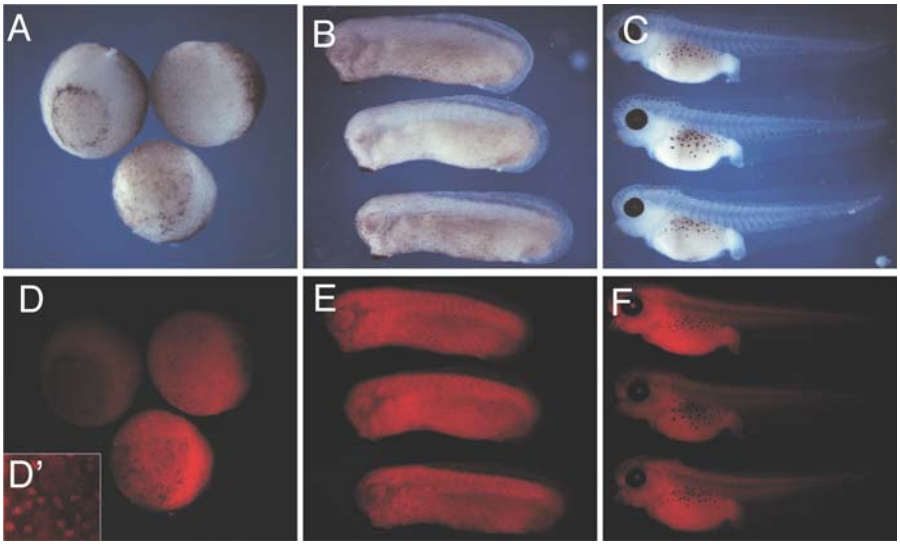

Figure 1. Embryos of *X. tropicalis* at the Stages Examined for Abnormalities Caused by Injection of MOs

Embryos had been injected at the one-cell stage with a Lissamine-labeled control MO.

(A–C) Bright field views. (D–F) Fluorescent views. D′ shows a high-power view of cells within the animal hemisphere of an embryo at the early gastrula stage.

(A and D) Early gastrula stage 10–11. (B and E) Tailbud stage 28. (C and F) Tadpole stage 41.

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($n \geq 40$) to develop in a similar abnormal fashion if the MO is to be classified as yielding a phenotype. In practice, and as described below, our results show that the results obtained at the two doses of MO used in these experiments are similar, and that the "penetrance" of the MOs is usually uniform and high. For example, of the 135 oligonucleotides that yield a phenotype following injection of 30 ng MO, 96% (127) yielded a phenotype following injection of 10 ng. At this lower concentration, 105 of the 127 (83%) showed 100% penetrance (that is, all the embryos developed in a specific abnormal fashion) and 22 (17%) displayed a phenotype in 50%–99% of cases.

Together, these observations suggest that a dose of 10–15 ng MO will usually be sufficient to inhibit gene function, although for 4% of the genes screened a phenotype was only observed at the higher dose. In six of these eight cases the penetrance at 30 ng MO was between 80% and 100%.

Each MO was injected on three independent occasions (twice at 10–15 ng and once at 30 ng), and in every case the same phenotype was observed (except in those cases when a phenotype was only observed at 30 ng MO). These observations suggest that the phenotypes are due to gene-specific effects of the MOs and not, for example, to the injection procedure, and that the results are not influenced significantly by genetic variation between the outbred individuals used in these experiments (see Materials and Methods).

The genes studied fell into three classes (see above). For those whose functions have been previously studied, our MOs caused phenotypes in 77% (10–15 ng MO) and 79% (30 ng MO) of cases ($n = 70$; Table 1). For genes that have been partially characterized (that is, they are previously unstudied members of known gene families, or they have only been studied in vitro, or their protein products contain a known functional domain), our MOs caused phenotypes in 56% (10–15 ng) and 61% (30 ng) of cases ($n = 64$; Table 1). Finally, for "novel" genes, we observe a phenotype in 54% (10–15 ng) and 59% (30 ng) of cases ($n = 68$; Table 1).

MO Specificity: Theoretical and Experimental Considerations

How specific are the phenotypes we observe? At the end of this paper we address this point experimentally for a group of MOs that causes defects in gastrulation, but some general comments are necessary before describing the results we obtain. First, it is unlikely that our MOs exert toxic effects, because injection of 30 ng of a standard control MO has little or no effect on development (see, for example, Figure 2A, 2D, and 2G), and of the 262 MOs injected in the course of this work (some of which are "second site" MOs and excluding the additional MOs, see below), 89 have no effect on development (other than occasionally causing a slight delay) even at the higher dose of 30 ng (Table 1). We also note that MOs that are altered by five bases from their target sequences have little or no effect on development.

In addition, we have asked whether the MOs that are targeted against specific mRNAs complement sequences elsewhere in the *X. tropicalis* genome. In our experience (see below and also http://www.gene-tools.com), 25-mer MOs that differ in five nucleotides from the target sequence have no effect on the translation of the mRNA in question, so our analysis ignores sequences that differ by more than four nucleotides from a perfect match.

Theoretical calculations based on this criterion and the existing *X. tropicalis* genome assembly suggest that the probability of there being an additional MO target sequence within the vicinity of the translation start site of an mRNA is as high as 0.3 (unpublished data). This can be extended to allow for the possibility of additional interactions with intron-exon splice sites. If we assume that an "average" gene has seven exons, and that the morpholino must be centered within two or three bases of the splice site to be effective,

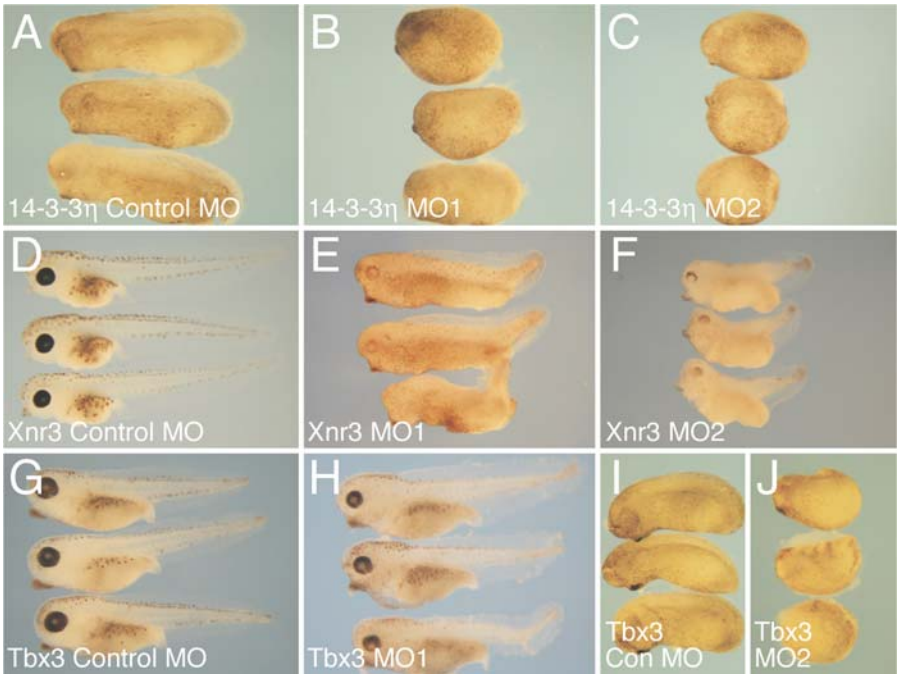

Figure 2. Examples of the Similarities between Phenotypes Caused by Second Site MOs and Those of the Primary MO Directed against Sequence around the Translation Start Site of the Target mRNA

(A–C) MOs directed against 14-3-3η. (A) Control MO; embryos develop normally.
(B) Embryos injected with MO1, directed against the translation start site of 14-3-3η, develop with a shortened antero-posterior axis.
(C) Embryos injected with MO2, directed against sequence 5′ of the translation start site of 14-3-3η, resemble those injected with MO1.
(D–F) MOs directed against Xnr3. (D) Control MO; embryos develop normally. (E) Embryos injected with MO1, directed against the translation start site of Xnr3, exhibit an upturned tail. (F) Embryos injected with MO2, directed against sequence 5′ of the translation start site of Xnr3, also have an upturned tail, but they differ slightly from those injected with MO1 because their antero-posterior axes are slightly shortened.
(G–J) MOs directed against Tbx3. (G and I) Embryos injected with control MOs develop normally. (H) Embryos injected with MO1, directed against the translation start site of Tbx3, have a normal body axis but their tails are slightly wavy. (J) Embryos injected with MO2, directed against sequence 5′ of the translation start site of Tbx3, have a more severe phenotype than those injected with MO1, in which the antero-posterior axis of the embryo is shortened. MO1, primary MOs; MO2, second site MOs.
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then this might extend the search space by (say) seven bases per exon. This would add 70 bases of search space per gene to the original 100 bases used around the translation start site, and would therefore increase the probability to ~0.5. We note, however, that the efficacies of MOs with this degree of mismatch are likely to be significantly lower than those of MOs showing a perfect match, and that these efficacies will also depend on factors such as the GC content of the target sequence and the location of the mismatches within the MO. We also note that as the criteria applied become more stringent, the probability of an MO matching an additional sequence elsewhere in the genome becomes much smaller. For example, the probability of there being an additional MO target sequence near the translation start site that differs by up to three nucleotides from a perfect match (rather than four) is approximately 0.003.

Searches for additional target sequences of our MOs by BLAST analyses of the *X. tropicalis* transcriptome are confounded by the incomplete nature of the genome, but searches of three datasets gave similar results, and the combined results from all three should set a reliable lower limit to the extent of non-specific effects of our MOs. The three datasets were (i) the complete set of Ensembl transcripts for *X. tropicalis,* (ii) the *X. tropicalis* genome sequence combined with Ensembl/JGI gene models, and (iii) EST clusters that include the complete predicted 5′ UTR. Of the 202 MOs used in this paper, we found that only 14 (6.9%) complement at least 21 out of 25 bases within the 5′ UTR of additional known or predicted open reading frames (Table 2); and of these most differ in three or four rather than one or two bases. For a further 13 MOs we could not decide whether the MO was targeting an additional gene or whether this was the intended target, perhaps obscured by sequencing errors. In the future it will be helpful to have a tool such as AMOD [26] to help in the design of *X. tropicalis* MOs.

It is possible to test the specificity of the phenotype induced by a particular MO experimentally in several ways [17]. One is to perform a "rescue" experiment, in which the MO is co-injected with a form of the target mRNA that lacks the MO-binding site. This approach is labor-intensive, and attempts to rescue the phenotype can often fail even when the phenotype is specific. This may occur, for example, if the targeted gene is expressed in a restricted manner and the presence of the gene product elsewhere in the embryo causes an over-expression phenotype [27]. Successful rescue may also depend on the concentration of rescuing RNA [28]. We have only adopted this approach to investigate the specificities of the MOs that lead to defects in gastrulation (see below).

The second approach is to target the gene in question by means of a second MO that complements a sequence different from that recognized by the first. In this paper,

Table 2. Potential Alternative Targets of the MOs Used in This Paper

Wellcome Clone Identifier	Gene Name	Alternative Targets (Number of Mismatches)		
		Ensembl Transcripts	Ensembl/JGI Gene Models	EST Clusters
TEgg054m19	*Serpin E2*	—	—	1 (1)
TEgg073l16	*Rac1*	2 (3,3)	2 (3,3)	3 (3,3,4)
TEgg078i06	Novel	—	—	1 (4)
TEgg096l10	*CD2IC*	1 (4)	—	—
TGas029e03	*Cdc42*	1 (4)	1 (4)	1 (4)
TGas050a15	*CD2LC*	1 (3)	1 (3)	1 (3)
TGas139h15	Novel	—	—	1 (0)
TGas144p20	*Wnt11*	?	—	1 (4)
TNeu074i11	*ARP2/3 1a*	—	8 (0…)	11 (0…)
TNeu103f06	*AHNAK*	—	—	1 (4)
TNeu134e01	*HoxB3*	1 (4)	?	—
TTpA004p03	*14-3-3ζ/δ*	1 (4)	?	1 (4)
TTpA007k16	*Smad5/8a*	—	1 (0)	—
TTpA010k20	*14-3-3beta*	1 (4)	—	1 (4)

Numbers indicate the number of potential additional target mRNAs found in each database; numbers in parentheses show the number of base mismatches. Question marks indicate cases where doubt exists as to a match (see text). The large numbers of perfect matches in the case of TNeu074i11/ARP2/3 1a may be due to the presence of active transposable elements.
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these second site MOs are usually designed to recognize sequence 5′ of the first MO; that is, further upstream in the 5′ UTR. If these second MOs cause a similar phenotype, one can be more confident that this is a specific effect of the loss of the target protein.

Excluding our detailed examination of the specificities of the MOs that lead to gastrulation defects (see below), second site MOs were designed for 48 of the genes which, when targeted by the first MO, resulted in abnormal development. Of these MOs, 34 yielded a phenotype following injection into embryos of *X. tropicalis*. In most cases ($n = 21$), the phenotypes resembled those caused by the first MO. In others the phenotype appeared to be a less severe version of that caused by the first MO; in only one case (Wnt5b) did the two phenotypes appear strikingly different. Second site MOs were also designed to recognize 12 genes that when targeted by the first MO did not result in a disruption of development. In seven cases no phenotype was observed with the second MO, but five of the second site MOs did cause embryos to develop abnormally (Table 3). As we discuss below, these results, together with the observation that injection of control MOs yields no phenotype, suggest that the effects of our MOs are usually specific.

The final approach is to design MOs whose complement differs from the target sequence in five nucleotides (see http://www.gene-tools.com). This approach has been adopted to investigate the specificities of the MOs that cause defects in gastrulation (see below).

Table 3. Comparison of Results Obtained with First and Second Site MOs

Phenotype Observed with First MO	Phenotype Observed with Second MO	*n*
+	+	34[a]
+	—	14
—	+	5
—	—	7

[a]In 21 of these cases the observed phenotype was the same or similar to that obtained with the first MO.
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Expression Patterns, Phenotypic Classes, and Synphenotype Groups

Niehrs and Pollet [20] have introduced the concept of a "synexpression group," a set of genes that are all expressed in a very similar pattern and are all believed to function in the same developmental process. In this paper, in an analogous manner, we have grouped the various phenotypes we observe, in the hope that this too might help identify genes involved in the same process. A similar approach has been adopted by Chen and colleagues in describing the effects of over-expressing genes in *Xenopus* embryos [21], and as described above, the classification of phenotypes in this way has long been known to be an essential component of forward genetic analyses of development [1,22,23].

Our initial classification divided embryos into seven phenotypic classes based on the time of embryonic lethality, the length and shape of the main body axis, and, for the seventh class, the ability to swim. The members of these classes were then divided into 19 synphenotype groups (Figure 3), each of which comprises genes whose loss-of-function phenotypes resemble each other particularly closely. This exercise may help in coming to understand how the genes function, individually and collectively, in the generation of the early embryo.

We describe the seven phenotypic classes and their subdivision into different synphenotype groups, below. These results are summarized in Table 4 and illustrated in Figures 4–13. In some cases a gene that has been targeted by two different MOs may appear in two different groups, because the phenotype caused by the first MO may be more or less extreme than that caused by the second. In other cases it is possible that a gene might be classified as belonging to more than one synphenotype group, and this is indicated in the third column of Table 4.

Table 4. Phenotypic Classes and Synphenotype Groups Identified in This Study

Phenotypic Class	Synphenotype Group	Alternative Group	Wellcome Clone Identifier	Gene Name	Accession Number	Representation in Egg/Gas/Neu cDNA Libraries
1. Gastrula	1. Gastrula defects		TGas061b15	*cD1LIC*	CT030328.1	0.4/0.5/0.7
			TNeu143c03	*Dp71.2*	CR848277.2	1.1/0.5/0.9
			TNeu073g23	*E-Cad*	AL779506.2	4.3/7.2/5.4
			TGas142e24	*HMG20b*	CR761881.2	0.2/0.9/0.9
			TEgg130i17	*HP1beta*	CR760924.2	2.2/0.6/0.9
			TGas055a16	Novel	CR761559.2	0.0/0.6/0.0
			TEgg040a13	Novel Zn finger	CR762039.2	5.0/6.6/9.5
			TEgg137c14	*Suv39h1*	AL877771.2	1.5/0.4/0.0
			TGas012b13	*Wnt5b*	CR760740.2	0.0/0.7/0.2
2. Short axis	2.1 Involution defects		TNeu036f01	*cD2HC.2*	AL661523.2	—
			TNeu087g20	*Frizzled8.2*	AL805138.2	0.0/0.4/1.1
			TEgg003f20	Novel.2	CR848500.2	3.0/1.0/0.6
			TGas124h10	*Xnr1.2*	CR761456.2	0.0/0.1/0.0
	2.2 Gastrula or neurula defects		TEgg065g11	*Anillin*	AL860870.2	0.9/0.1/0.4
			TEgg140p11	Novel	CR761019.2	0.9/0.1/0.6
			TGas080g08	Novel	CR848087.2	0.0/0.4/0.0
			TNeu097d01	Novel	AL801792.2	0.0/0.1/0.2
			TNeu127e06	*PAR6B*	CR760394.2	0.0/0.6/0.2
			TEgg126n13	*Rad51*	CR761167.2	0.7/0.6/0.2
			TEgg104h10	*Rb1*	AL863992.2	0.7/1.3/0.4
			TNeu052c18	*Smad4a*	AL673301.2	0.0/0.5/0.6
			TTpA012g23	*Tbx3.2*	BX703861.1	0.0/0.1/0.6
	2.3 Short axis surviving to tailbud		TTpA010k20	*14-3-3beta*	CR760847.2	3.3/4.8/10.2
			TTpA018g17	*14-3-3epsilon*	CR848241.2	2.2/4.0/7.6
			TGas138o08	*14-3-3eta*	CR848634.2	0.0/0.5/2.0
			TGas138o08	*14-3-3eta.2*	CR848634.2	0.0/0.5/2.0
			TTpA008p09	*14-3-3theta*	CT025504.2	3.5/6.3/10.4
			TTpA008p09	*14-3-3theta.2*	CT025504.2	3.5/6.3/10.4
			TNeu074i11	*ARP2/3 1a*	CR926215.2	11.5/26.5/17.1
			TEgg076c16	*Delangin.2*	AL865073.2	0.9/0.8/0.4
			TGas086e13	*Dp427p1*	AL960406.2	—
			TNeu143c03	*Dp71*	CR848277.2	1.1/0.5/0.9
			TEgg139n15	*DTC50; Dynactin 2*	CR760618.2	0.4/0.3/0.2
			TEgg033o10	*Exostosin1*	AL873459.2	3.0/0.9/0.4
			TNeu054b08	*Fbxl11*	AL678939.2	0.0/0.0/0.4
			TNeu087g20	*Frizzled8*	AL805138.2	0.0/0.4/1.1
			TEgg017g13	Novel.2	CR848520.1	0.7/0.1/0.2
			TGas066o14	Novel	CR926381.2	—
			TGas128o11	Novel	CR762195.2	0.0/0.4/0.0
			TNeu095k24	*Smad7*	CX822076.1	0.0/0.4/0.4
	2.4 Short axis surviving to tadpole		TNeu142f12	*ActivinbetaB.2*	AL803838.2	0.0/0.0/0.2
			TNeu103f06	*AHNAK*	CR760312.2	0.0/0.9/0.4
		2.5	TEgg020c18	*Chimerin 1*	CT030309.1	1.5/0.0/0.0
			TEgg018e06	*Dlgh1*	CR942446.2	1.1/0.1/0.0
			TGas086e13	*Dp427p1.2*	AL960406.2	—
			TGas087l14	*Frizzled6*	AL959523.2	0.2/0.1/0.0
			TNeu065e03	*Frizzled7*	CR759995.1	1.1/0.5/0.6
			TGas034n12	*Fullback; NRH1*	AL654321.1	0.0/2.0/3.3
			TGas087m13	*ING5*	CT030479.1	1.3/1.9/0.7
		2.5	TEgg135f07	*Integrinbeta1*	CT485679.1	5.9/3.7/4.1
			TGas028k15	*Lim1*	CX494294.2	—
			TEgg019e13	Novel	CR761216.2	1.7/0.5/0.7
			TEgg140p11	Novel.2	CR761019.2	0.9/0.1/0.6
			TGas076l13	Novel	AL959005.2	0.0/0.4/0.2
			TGas083e14	Novel	AL681066.2	2.8/2.0/0.6
			TGas096p02	Novel	CR761683.2	0.0/0.6/0.4
			TNeu018d13	Novel	AL638307.2	0.2/1.2/1.9
		6.4	TNeu122d11	Novel	CT030602.1	0.0/2.1/2.6
			TEgg056i10	Novel; 67-11-3	CR761068.2	0.7/1.4/0.9
			TGas010f07	Novel; Tsp101	CR848169.2	0.2/0.9/0.4
			TGas098h07	Novel Zn finger	CR762202.2	5.0/9.4/10.8
			TGas106k21	Novel Zn finger	AL629390.2	0.0/0.3/0.0
			TGas136i03	*p32INGL*	CR761858.2	4.3/1.3/1.3

Table 4. continued

Phenotypic Class	Synphenotype Group	Alternative Group	Wellcome Clone Identifier	Gene Name	Accession Number	Representation inEgg/Gas/Neu cDNA Libraries
			TNeu034a23	*p53*	AL656057.1	0.9/2.6/2.4
		2.5	TEgg133h21	*Padi2*	CX845163.1	0.2/0.2/0.0
			TGas097o15	*PKClambda; 14-3-3zeta*	CR848145.2	0.7/1.5/0.2
			TGas008e09	*Rb2; p130*	AL630010.2	0.0/0.2/0.0
		6.1	TNeu050m05	*Smad3*	CX891579.1	0.0/0.0/0.2
			TEgg055l20	*Suv39h2*	AL958755.2	2.2/0.5/0.2
			TNeu117j03	*Tbx2*	AL791963.2	0.0/0.2/1.1
			TNeu117j03	*Tbx2.2*	AL791963.2	0.0/0.2/1.1
			TGas124n10	*Tbx6*	CR942588.2	0.2/1.3/3.3
			TNeu139i18	*TCTEX1*	AL781811.2	0.9/0.6/1.3
			TNeu131c18	*TGIF2*	AL792964.2	0.2/0.7/0.4
			TEgg012b23	*TIEG2*	CT030382.1	2.0/0.5/1.3
			TGas116l23	*Xbra*	CR761440.2	0.0/4.1/1.9
			TGas116l23	*Xbra.2*	CR761440.2	0.0/4.1/1.9
			TNeu061i04	*Xbra3*	CR760217.2	0.0/1.0/0.6
			TGas097d22	*Xnr3.2*	AL971028.2	0.0/0.3/0.0
	2.5 Normal body, short tail		TGas049g17	*Blimp1*	AL649398.2	0.0/0.1/0.2
			TNeu087f10	*Cdx2; CAD2*	CR760338.2	0.0/0.2/0.6
			TNeu123l16	*Mu2*	AL802310.2	0.2/4.4/5.0
			TGas144p20	*Wnt11*	CT025383.1	0.2/0.5/0.4
	2.6 Proportionately small		TGas029e03	*Cdc42*	BX727142.1	6.1/7.7/7.2
		6.4	TGas076c07	Novel	CT030100.2	0.0/0.2/0.0
			TNeu123g11	Novel	CR761938.2	0.0/0.6/0.9
			TNeu072l04	*RBBP1.2*	CR848405.2	0.4/0.2/0.6
3. Late degradation	3. Degradation after tailbud		TGas053l04	*CPSF4*	CR761660.2	2.0/1.2/0.9
4. Curved body axis	4. Curved body axis		TTpA018g17	*14-3-3epsilon.2*	CR848241.2	2.2/4.0/7.6
			TEgg077g12	*ARP6*	CR760917.1	2.2/0.3/0.4
			TGas050a15	*cD2LC*	CR761866.2	1.1/1.3/3.0
5. Ventral defect	5.1 normal length, ventral oedema		TNeu142f12	*ActivinbetaB*	AL803838.2	0.0/0.0/0.2
			TNeu069m07	*Anf-1*	CR760108.2	0.0/0.1/0.2
			TNeu045c17	*BMP4*	CR761955.1	0.0/1.7/1.1
			TEgg021o06	*Smad1*	AL855227.2	4.8/3.9/1.3
	5.2 Normal length, ventral reduction		TNeu118d19	*Wnt8*	CR760475.2	0.0/2.6/0.7
6. Bent axis	6.1 Short body, up-turned tail, and dorsalized	2.4	TEgg076c16	*Delangin*	AL865073.2	0.9/0.8/0.4
		2.4	TGas128m18	*Flamingo1.2*	EF012769.1	—
			TNeu059i07	*Frizzled2*	AL677424.2	1.7/1.2/1.1
		2.4	TGas077n04	Novel	CR760737.2	0.0/1.0/0.0
		2.4	TNeu089n21	Novel	CR761967.2	0.4/0.0/0.4
		2.4	TNeu072l04	*RBBP1*	CR848405.2	0.4/0.2/0.6
		2.4, 5.1, 7.1	TEgg068f10	*REEP4.2*	CR926301.2	2.2/0.3/0.0
		2.4	TGas073h08	*Smad6*	AL784104.2	—
		2.4	TTpA011k01	*Suv420h1; SET8*	CR760868.2	1.3/0.4/0.4
		2.4	TGas124n10	*Tbx6.2*	CR942588.2	0.2/1.3/3.3
	6.2 Short body, up-turned tail, and ventralized	6.3	CX843311	*BS69.2*	CX843311.2	—
		2.4	TGas124j21	*MARK2; Par1*	BQ389144.1	0.2/0.8/1.3
		2.4	TEgg003f20	Novel	CR848500.2	3.0/1.0/0.6
		2.4	TEgg048b09	*VegT.2*	CR760687.2	5.7/8.5/1.3
	6.3 Normal body length, wavy tail	2.4	TNeu036f01	*cD2HC*	AL661523.2	—
			TEgg096l10	*cD2lC.2*	CT030381.1	1.1/1.6/0.6
			TGas128i12	*Dach2l*	AL959426.2	0.0/0.2/0.0
			TEgg061a19	*FGFR4*	AL630886.2	1.7/1.8/1.1
			TEgg061a19	*FGFR4.2*	AL630886.2	1.7/1.8/1.1
			TNeu065e03	*Frizzled7.2*	CR759995.1	1.1/0.5/0.6
			TGas131h15	*Hox-7.1; Msx1*	CR855795.2	0.0/2.4/2.8
			TTbA033a09	*Mu1b*	CF224028.1	0.0/0.0/0.0
			TEgg017g13	Novel	CR848520.1	0.7/0.1/0.2
			TGas055a16	Novel.2	CR761559.2	0.0/0.6/0.0
		2.4	TGas128o11	Novel.2	CR762195.2	0.0/0.4/0.0
			TNeu109g23	Novel	AL875318.2	2.2/0.1/0.4
			TEgg118f13	*Smad2*	AL867605.2	2.6/0.5/0.9
			TTpA007k16	*Smad5/8a*	CX909167.1	0.4/0.0/0.0
			TTpA012g23	*Tbx3*	BX703861.1	0.0/0.1/0.6
			TNeu139i18	*TCTEX1.2*	AL781811.2	0.9/0.6/1.3
			TNeu055p01	*TGIF1*	CR848339.2	0.2/0.5/1.9
	6.4 Bent-up tail or arched back	2.5	TGas030a23	*aD1LC*	AL646908.2	0.2/0.9/0.6
		2.4	TGas061b15	*cD1LIC.2*	CT030328.1	0.4/0.5/0.7

Table 4. continued

Phenotypic Class	Synphenotype Group	Alternative Group	Wellcome Clone Identifier	Gene Name	Accession Number	Representation inEgg/Gas/Neu cDNA Libraries
		2.4	TGas020f13	*DNALC4*	CR762240.2	1.3/1.2/1.1
		2.4	TGas028g07	*DOC-1*	AL652680.2	0.4/0.2/0.2
		2.4	TGas047c23	*EED*	CR848605.2	4.8/1.2/1.3
		2.4	TGas092f08	*FGFR3; CEK2*	AL964086.2	0.0/0.1/0.2
		2.4	TGas092f08	*FGFR3.2; CEK2.2*	AL964086.2	0.0/0.1/0.2
		2.4	TEgg061a13	Novel	CR761145.2	0.7/0.0/0.0
		2.4	TEgg078i21	Novel.2	AL878749.2	0.2/0.0/0.0
		2.4	TEgg098o12	Novel	AL865292.2	0.4/0.0/0.0
		2.4	TNeu062k05	Novel	CR848314.2	1.1/0.1/0.4
		2.4	TNeu104f22	Novel	AL660492.2	0.0/0.6/0.9
		2.4	TGas076f10	*Novel; NOP 7 associated 1*	CR761697.2	0.0/0.4/0.4
		2.4	TGas143j10	Novel Zn finger	CR848612.2	2.4/1.7/0.7
		2.4	TEgg004p11	*Par3B*	AL849138.2	0.7/0.2/0.0
		2.4	TGas080l23	*RxRbeta*	CR926364.2	0.4/1.7/1.3
		2.4	TTpA011h07	*Serpina1*	BX704113.1	0.0/0.0/0.0
		2.4	TEgg110h11	*ST13; novel*	CR942468.2	0.2/0.8/0.0
		2.4	TGas097d22	*Xnr3*	AL971028.2	0.0/0.3/0.0
	6.5 Bent-down tail		TTpA010k20	*14-3-3beta.2*	CR760847.2	3.3/4.8/10.2
			TGas058k09	*CPSF2*	AL681922.2	0.0/1.2/0.7
			TNeu132f09	*EDIL3*	AL785940.2	0.9/0.4/0.9
		2.4	TGas031a08	*Lefty-b*	AL649862.2	0.0/0.4/0.0
			TEgg078i21	Novel	AL878749.2	0.2/0.0/0.0
			TGas083e14	Novel.2	AL681066.2	2.8/2.0/0.6
			TGas141c24	Novel	CR761833.2	0.0/1.2/0.2
			TNeu053k08	Novel.2	CR760048.2	0.0/0.0/0.2
			TNeu062k05	Novel.2	CR848314.2	1.1/0.1/0.4
			TGas106k21	Novel Zn finger.2	AL629390.2	0.0/0.3/0.0
		6.2	TNeu123j18	*PAR6A*	CR855473.2	2.8/0.4/0.6
		6.2	TEgg032k01	*Smad10*	CT025227.1	1.1/0.3/0.2
		2.4	TGas012b13	*Wnt5b.2*	CR760740.2	0.0/0.7/0.2
7. Motility defects	7.1 Mildly kinked tail and motility defects		TEgg066c16	*CC1*	CR761074.2	0.9/0.1/0.0
			TNeu136h04	*HMG17*	CR761935.2	8.3/10.2/20.3
			TNeu108m10	*HoxC8*	CR926189.2	0.0/0.0/0.6
			TEgg068f10	*REEP4*	CR926301.2	2.2/0.3/0.0
			TGas064l01	*Tinp1*	CR848589.2	0.7/2.0/2.4
			TEgg131f10	*VHLH*	CR761285.2	0.2/0.2/0.0
	7.2 Swimming in circles		TEgg021k02	*Aurora A*	CR760668.2	16.7/1.2/1.5
			TNeu102i09	*FrzA*	CR926172.2	0.0/0.0/0.6
			TNeu104l10	*Mu1a*	CR760351.2	0.0/1.2/0.7
	7.3 Normal appearance but paralyzed		TNeu102i09	*FrzA.2*	CR926172.2	0.0/0.0/0.6
			TNeu053k08	Novel	CR760048.2	0.0/0.0/0.2
			TNeu098a04	Novel	CR760171.2	0.0/0.4/0.6
			TEgg043a17	Novel Zn finger	CR926298.2	3.9/1.8/2.6
			TEgg058h11	Novel Zn finger	CR761015.2	3.0/1.3/0.9

The third column in the Table indicates cases where a phenotype might be assigned to an alternative synphenotype group. In the fifth column the suffix ".2" indicates that the data pertain to the use of a second site antisense MO. The final column in the Table indicates the frequency with which each transcript is represented in the Egg, Gastrula, and Neurula cDNA libraries described by Gilchrist and colleagues [24], represented as a percentage multiplied by 100. The numbers of sequenced clones in each library are: Egg: 45,948; Gastrula: 112,307; Neurula: 53,822.

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Table 4 also provides an insight into the temporal expression pattern of each gene, by indicating how frequently its transcripts are represented in cDNA libraries derived from egg, gastrula, and neurula cDNA libraries [24]. This is of interest because it is possible that the products of maternally expressed genes are more difficult to deplete than are newly expressed zygotic transcripts [29]. Our analysis, however, reveals no clear relationship between the maternal levels of expression of a gene and the likelihood of an MO directed against that gene producing a phenotype (unpublished data).

The Phenotypic Classes

Gastrula defects. The members of this class, targeted by nine MOs, exhibit defects during gastrula stages and all die before the mid-neurula stage (Figure 4). The phenotypes falling into this class are very similar, allowing the genes to be classed as a single synphenotype group. The specificities of the MOs that cause these defects are examined in the next section of this paper, and we are currently investigating whether the observed phenotype derives, for example, from defects in germ layer specification or in the cell cycle.

Shortened axes. This class comprises 71 genes (targeted using a total of 78 MOs) whose loss-of-function phenotypes are characterized by a shortening of the anterior-posterior body axis (Figures 5–7). These genes have been divided into six synphenotype groups, including: (i) involution defects (four MOs); (ii) gastrula/neurula defects (nine MOs); (iii) short

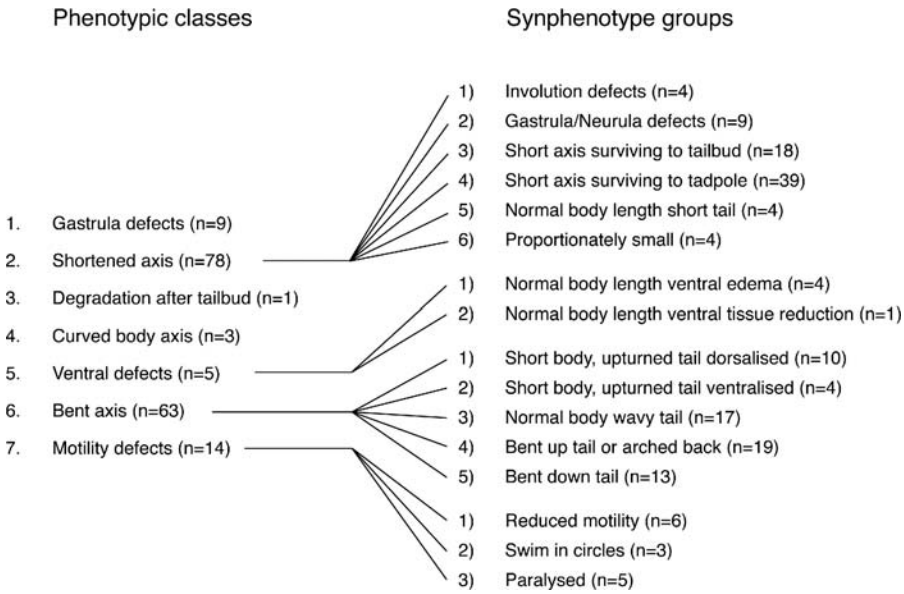

Figure 3. The Phenotypic Classes and Synphenotype Groups Defined by This Work
doi:10.1371/journal.pgen.0020193.g003

axis with death occurring after tailbud stages (18 MOs); (iv) short axis surviving to tadpole stages (39 MOs); (v) normal body length with short tail (four MOs); and (vi) proportionately small (four MOs). The observed phenotypes may derive from (among other things) defects in germ layer specification, organizer function, DNA damage, or cell death.

Late degradation. This class contains only one member: *CPSF4*. Loss-of-function individuals develop normally until the tailbud stage, after which time they begin to disintegrate (Figure 8). This is a more severe phenotype than that observed for zebrafish *CPSF4,* in which null mutants are characterized by a lack of brachial arches [30]. There may be different requirements for the gene in the two species, or perhaps there is some functional redundancy in zebrafish that derives from its partially duplicated genome [9].

Curved body axis. Loss-of-function of the three genes in this class causes the body axes of embryos to curve either to the left or the right (Figure 9). This phenotype may derive from defects in notochord or somites, or in laterality.

Ventral tissue defects. This class contains five genes whose loss-of-function phenotypes are characterized by defects in ventral tissues (Figure 10). Its members can be divided into two synphenotype groups, those with ventral oedema at the tadpole stage ($n = 4$) and those with a reduction in ventral tissue ($n = 1$). The former group may exhibit defects in heart or kidney development, or osmoregulation; the latter may show defects in ventral patterning.

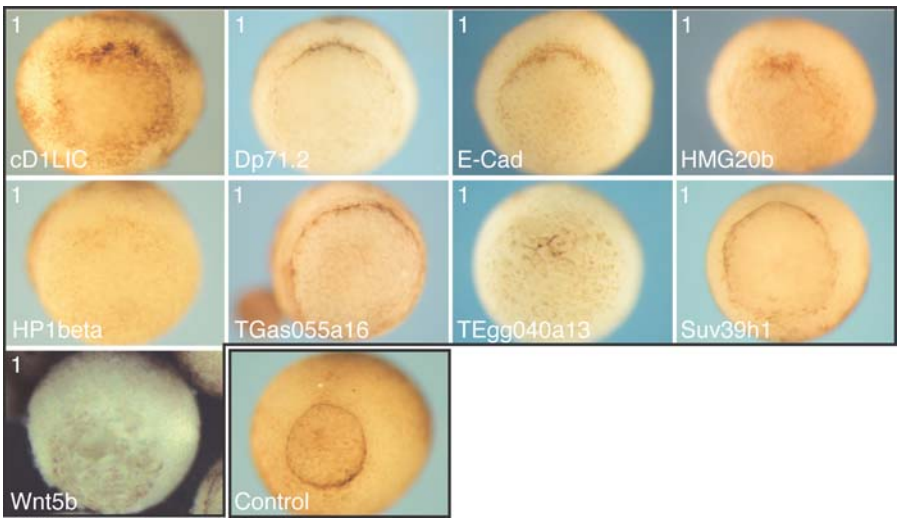

Figure 4. The Nine Members of the Gastrula Defect Phenotypic Class
Note that the blastopore in control embryos is closing normally, but is either absent or severely delayed in embryos in which the functions of the indicated genes are inhibited. All embryos shown are at gastrula stage 10.5 to 11.5. In this figure and in Figures 5–13, the number in the top left hand side of each panel represents the synphenotype group to which the embryos belong, and the name of the gene in question is shown bottom left.
doi:10.1371/journal.pgen.0020193.g004

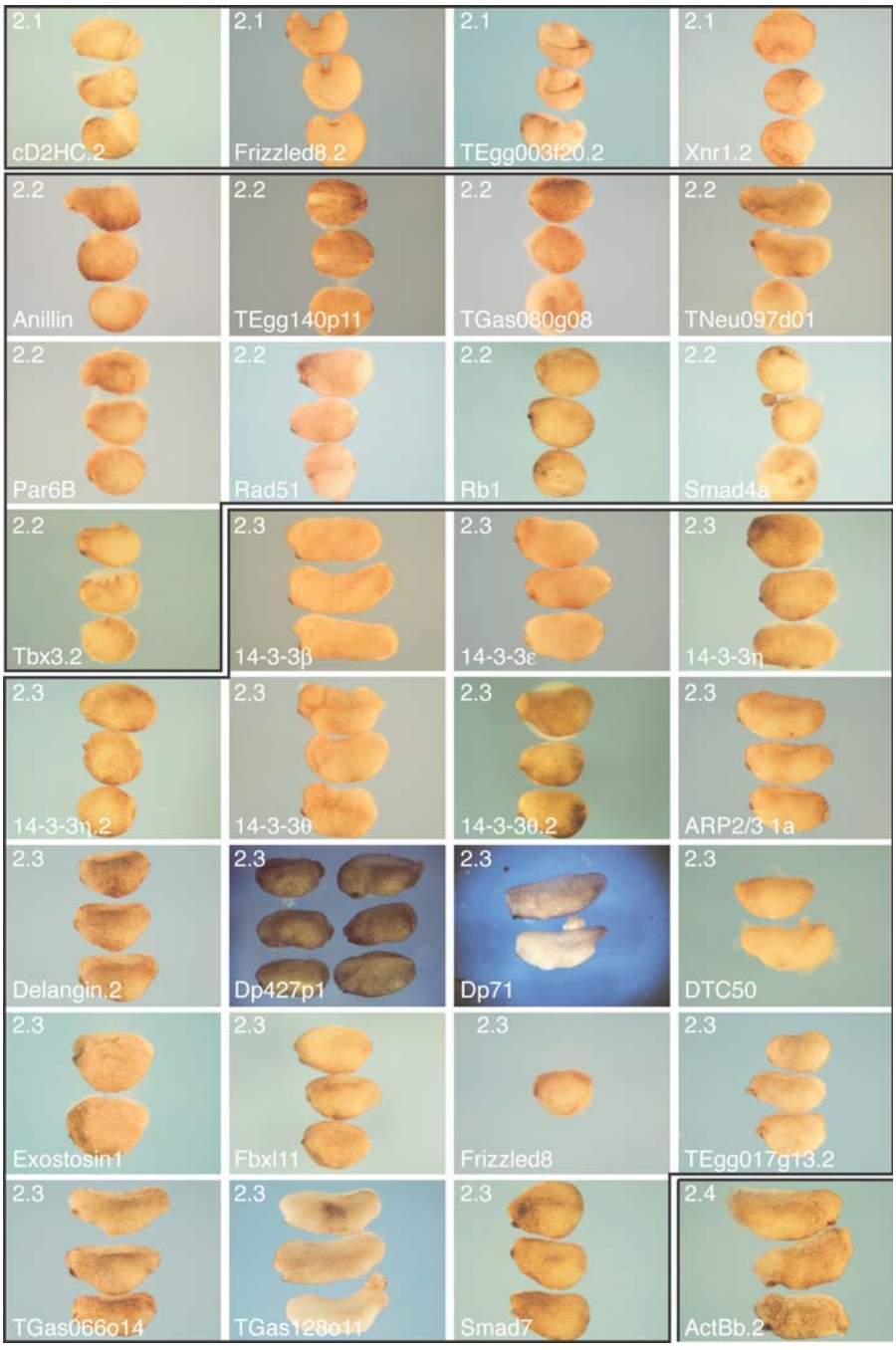

Figure 5. The First 32 Members of the Shortened Axis Phenotypic Class

This class can be subdivided into six synphenotype groups, as indicated in Figure 3 and Table 4. Members of the first three synphenotype groups (involution defects, gastrula or neurula defects, and short axis surviving to tailbud) are shown at tailbud stage (stage 24–28), while the member of the second synphenotype group shown here (short axis surviving to tadpole) is shown at tadpole stage 35–41. Lines in this and subsequent figures demarcate the different synexpression groups.

doi:10.1371/journal.pgen.0020193.g005

Bent axis. The 61 genes in this class (targeted by 63 MOs) can be divided into five synphenotype groups, all of which are characterized by a bent antero-posterior body axis (Figures 11 and 12). The groups are (i) short dorsalized body with upturned tail (ten MOs); (ii) short ventralized body with upturned tail (four MOs); (iii) normal body and wavy tail (17 MOs); (iv) arched back with bent-up tail (19 MOs); and (v) bent-down tail (13 MOs). All these phenotypes are likely to derive from defects in neural tube, somites, or notochord.

Motility defects. This class comprises genes whose loss-of-function causes embryos to develop apparently normally (or perhaps with slight defects in tail development) but whose motility is abnormal, greatly reduced, or absent (Figure 13). The 14 genes in this class can be divided into three synphenotype groups: (i) mildly kinked tail with greatly reduced motility (six MOs); (ii) embryos swim in circles (three MOs); and (iii) embryos are paralyzed and cannot swim even when prodded with a pair of forceps (five MOs). In all these

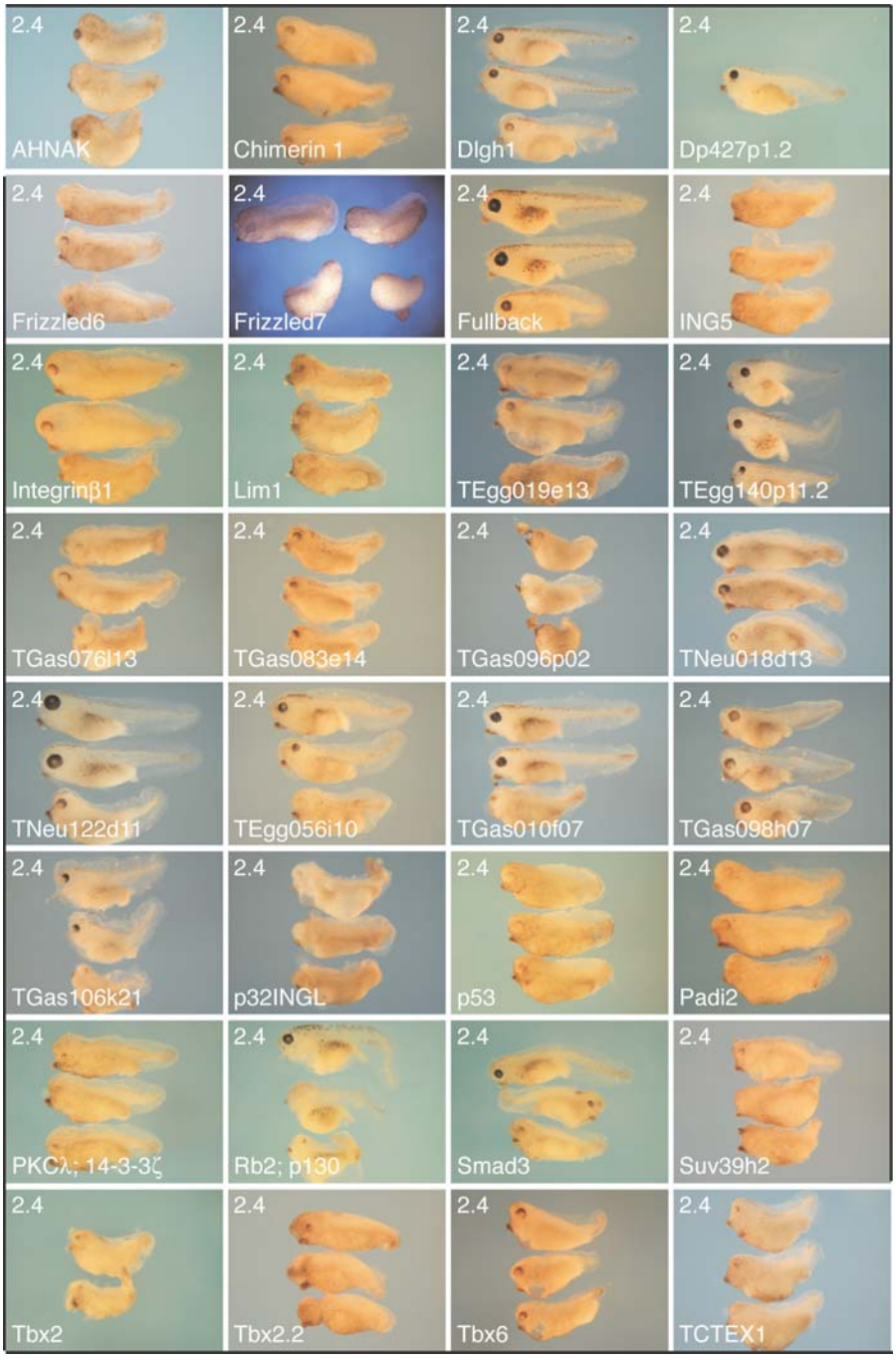

Figure 6. The Second 32 Members of the Shortened Axis Phenotypic Class

This class can be subdivided into six synphenotype groups, as indicated in Figure 3 and Table 4. The figure shows examples of the fourth synphenotype group (short axis surviving to tadpole) at tadpole stages 35–41.

doi:10.1371/journal.pgen.0020193.g006

cases, embryonic heartbeat was normal. Effects are likely to derive from defects in muscle or the nervous system, although those that swim in circles may exhibit defects in balance or laterality. This is under investigation.

Specificity Revisited: Apoptosis

As discussed above, interpretation of the experiments described in this paper, and indeed of all experiments making use of MOs, requires that the effects of the MOs are specific. Several criteria for specificity are described above, and these lead us to conclude that the effects of the MOs are usually specific. To investigate this important issue in more detail, we first asked if the possible non-specific effects of MOs might include the induction of apoptotic cell death. Apoptosis might, in particular, underlie later phenotypes such as those exhibited in the "short axis surviving to tadpole" synphenotype group.

To address this possibility we examined embryos from several synphenotype groups using TUNEL staining [31]. Our results (Figure 14) indicate that the Gene Tools standard

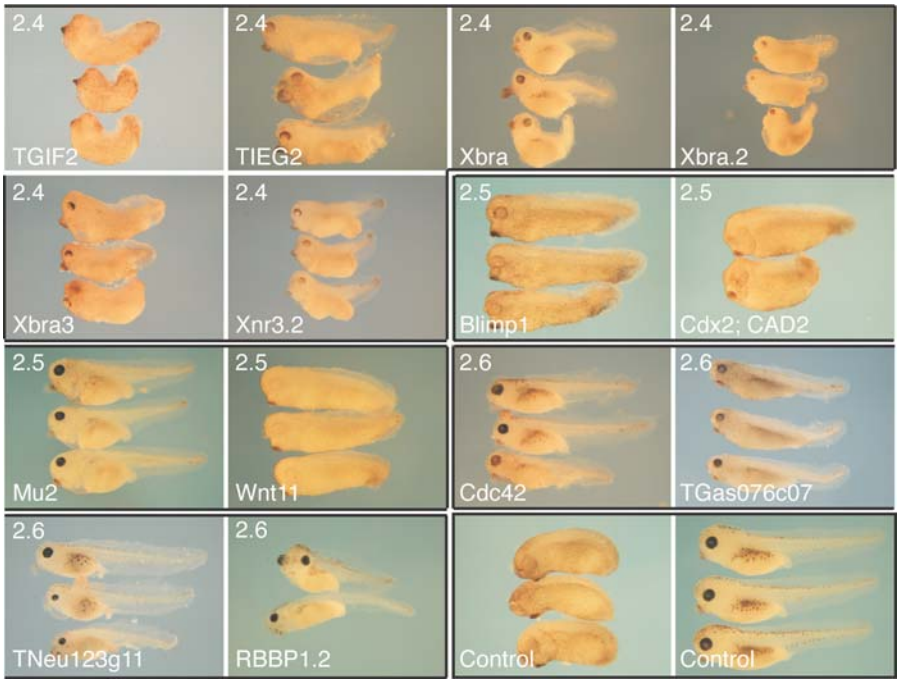

Figure 7. The Final 14 Members of the Shortened Axis Phenotypic Class

This class can be subdivided into six synphenotype groups, as indicated in Figure 3 and Table 4. The figure shows examples of the second three synphenotype groups (short axis surviving to tadpole, normal body short tail, and proportionately small) at tadpole stages 35–41.

doi:10.1371/journal.pgen.0020193.g007

control MO does not cause an increase in apoptosis at any stage. MOs directed against *BMP4, Flamingo1, Frizzled2, Hox7.1, ING5, p32INGL, Smad1,* and *Tbx6* do not cause significant changes in levels of apoptosis at tailbud stages, even though the MOs in question have usually induced a phenotype by this time. Although MOs directed against *Xbra, Xbra3, Xnr3,* and *Tbx3* do cause a significant increase in apoptosis by tailbud stages, we note that both *Xbra* and *Tbx3* have previously been implicated in programmed cell death [32–34], although the role of Xnr3 in apoptosis remains to be resolved.

Together, these results suggest that the phenotypes we observe do not derive from non-specific apoptotic cell death.

Specificity Revisited: The Gastrula Defects

As a final attempt to investigate the question of the specificity of MOs, we decided to concentrate specifically on the class of gastrula defects, and to ask, for each member of the class, whether a second site MO yields the same phenotype, whether an MO with five altered bases causes a phenotype, and whether the phenotype can be rescued by expression of a form of the target mRNA that lacks sequence complementary to the MO in question. The results of these experiments are shown in Figure 15 and Table 5. They indicate that for all nine MOs that cause gastrula defects, alteration of five bases causes no phenotype to occur in injected embryos, while five of the nine MOs prove to exert specific effects in the sense that the phenotype can be rescued by injection of the cognate mRNA. As discussed above, the latter is a particularly strict test and will underestimate the degree of specificity quite significantly.

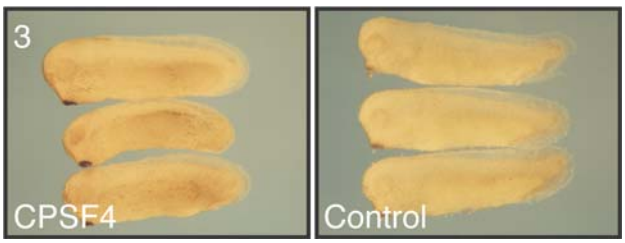

Figure 8. *CPSF4,* the Sole Member of the Late Degradation Phenotypic Class

Embryos injected with MOs targeting this gene appear perfectly normal to early tailbud stage 30 but then rapidly disintegrate. Embryos are shown at the tailbud stage (stage 24–28).

doi:10.1371/journal.pgen.0020193.g008

It was possible to rescue the effects of six out of eight of the second site MOs, indicating that their effects too are specific. However, the second site MOs almost invariably yielded a less severe phenotype than did the corresponding first site MOs. This observation is consistent with the results described above (Figure 2 and Table 3), and with the fact that it is the first site MOs that are designed to be the optimum antisense oligonucleotide for the mRNA in question (see Discussion).

Significantly, a less severe phenotype was also observed using second site MOs in a series of experiments investigating the expression of genes such as *Chordin, Xbra, Wnt11, Wnt8, Sox17,* and *E-Cadherin* (unpublished data). In these experiments, injection of an MO with five altered bases caused little alteration in gene expression, while first site MOs directed against, for example, Dp71, HP1beta, and TEgg040a13 caused significant down-regulation of mesodermally expressed genes such as *Chordin, Xbra,* and *Wnt11.* Second site MOs caused much less dramatic down-regulation in the cases of HP1beta and TEgg040a13, consistent with their lack of effect on development (Figure 15), but the second site MO directed

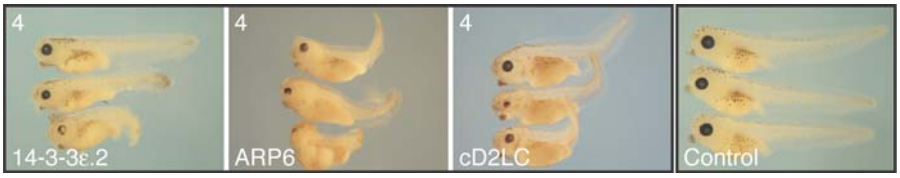

Figure 9. The Three Members of the Curved Body Axis Phenotypic Class
Embryos are shown at the tadpole stage (stage 35–41).
doi:10.1371/journal.pgen.0020193.g009

against Dp71, which disrupted gastrulation, also interfered with mesodermal gene expression.

Synphenotype and Synexpression Groups

Genes within a synphenotype group do not necessarily belong to the same synexpression group [20]. This may be illustrated by referring to two synphenotype groups within the class of genes required for normal motility. Thus, of the three genes whose loss-of-activity causes embryos to swim in circles, one *(AuroraA)* is expressed at highest levels in the head (Figure 16A), another *(FrzA)* is expressed most strongly in muscle, heart, pronephros, and otic vesicle (Figure 16B), and the third *(Mu1a)* is expressed in the brain and neural tube but absent from muscle (Figure 16C). Similarly, of the genes whose loss-of-function causes paralysis, one *(TEgg043a17)* is expressed almost ubiquitously (Figure 16D), another (*TNeu053K08)* is highly expressed in muscle (Figure 16E), and yet another *(TNeu098a04)* is most strongly expressed in the posterior neural tube (Figure 16F). These observations are discussed below.

Web Access

The results described in this paper can be accessed through a database at http://rd.plos.org/pgen__0057__0001__Xenopus__morpholino__screen. For each gene we have provided (i) the Sanger Institute clone identifier; (ii) the accepted gene name, where available; (iii) MO sequence(s); (iv) phenotypic class and synphenotype group; (v) a description of the phenotype; (vi) images of the phenotype; (vii) images of the expression pattern of the targeted gene; and (viii) the temporal expression profiles of the genes derived from their representation in *X. tropicalis* cDNA libraries.

Discussion

In this paper we use MOs to investigate by reverse genetics the functions of 202 genes in *X. tropicalis*. We have assessed the specificities of 60 of the MOs by injecting a second antisense oligonucleotide, compared the functions of the *X. tropicalis* genes with those of their orthologs in other species (including the mouse), and divided the genes, based on their loss-of-function phenotypes, into seven phenotypic classes and 19 synphenotype groups. As we discuss below, it is possible that members of the same synphenotype group are involved in the same developmental pathway. We have also tested in some detail the specificities of the MOs that cause defects to occur during gastrulation. Together, our results suggest that a large-scale attempt to inhibit the functions of all genes expressed during gastrula stages of *X. tropicalis* is feasible and that it should shed light on gene function in other organisms.

Specificity of MOs

Meaningful interpretation of the results presented in this paper requires that our MOs are non-toxic and act in a specific manner. First, as described above, our experiments indicate that injection of antisense MOs does not cause non-specific phenotypes to form, and there is no evidence for non-specific apoptotic programmed cell death in the embryo (Figure 14).

We have addressed the question of specificity by designing additional, non-overlapping MOs against 60 of the mRNAs targeted in this screen. For the 48 genes where the first MO yielded a phenotype (at a dose of 10–15 ng), the second site MO perturbed development in 34 of the cases, and in 21 of these the phenotype was the same or similar (Figure 2 and Table 3). In the remaining 13 cases, our analysis of the

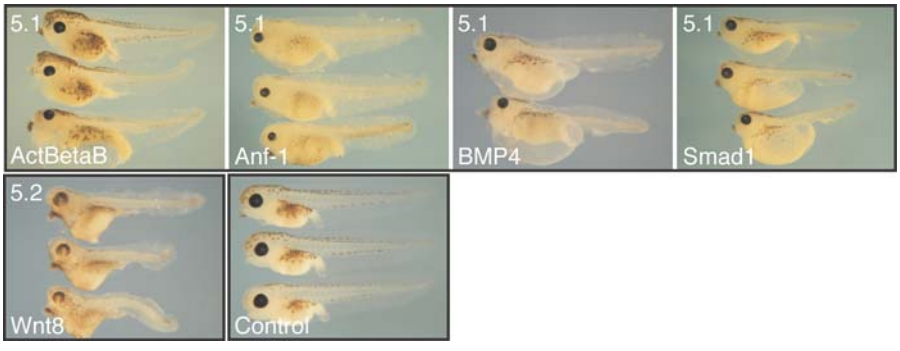

Figure 10. The Five Members of the Ventral Defects Phenotypic Class
This class can be subdivided into two synphenotype groups, as indicated in Figure 3 and Table 4. All embryos are shown at the tadpole stage (stage 35–41).
doi:10.1371/journal.pgen.0020193.g010

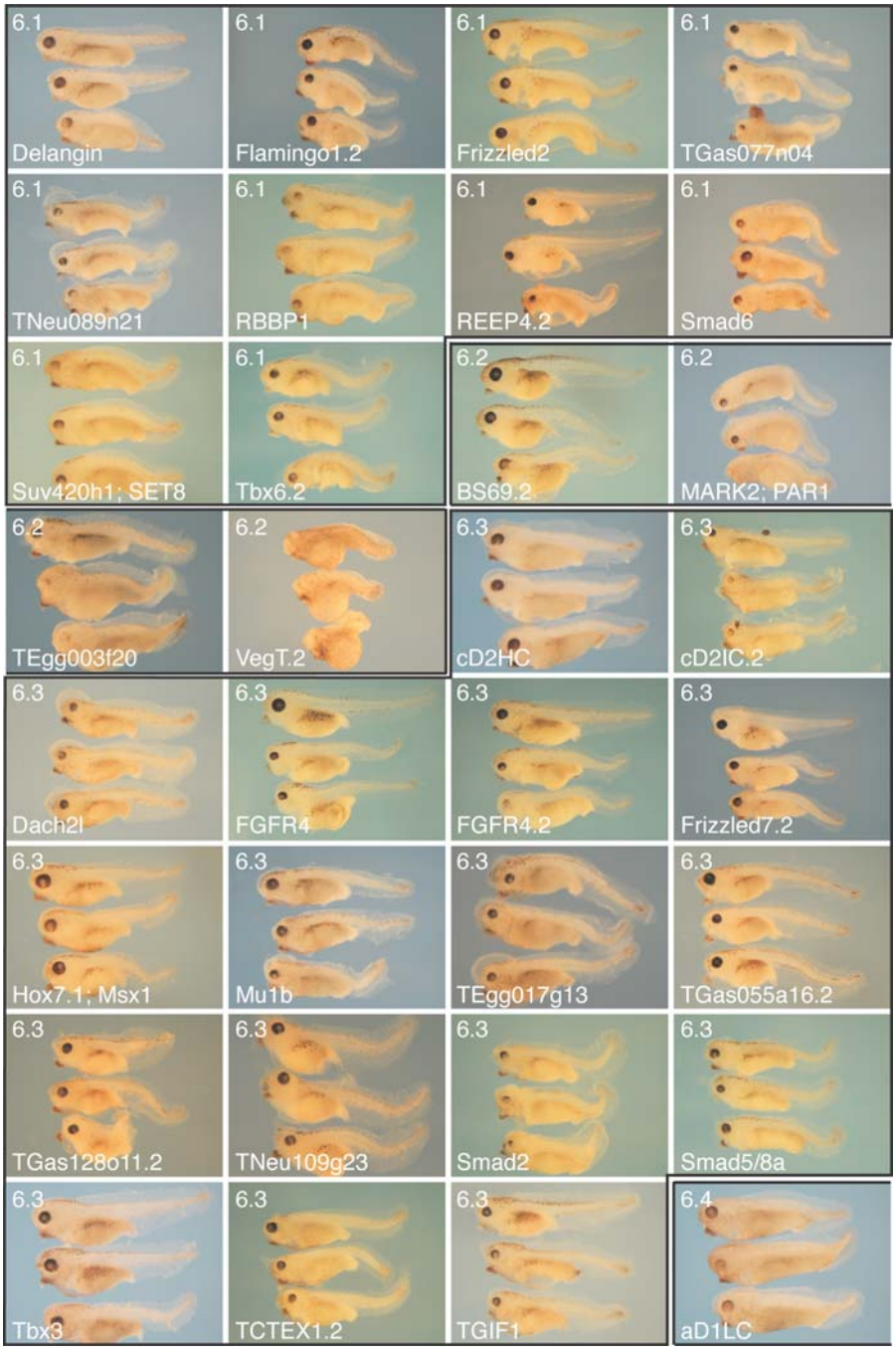

Figure 11. The First 32 Members of the Bent Axis Phenotypic Class

This class can be subdivided into five synphenotype groups, as indicated in Figure 3 and Table 4. All embryos are shown at the tadpole stage (stage 35–41).

doi:10.1371/journal.pgen.0020193.g011

gastrula class of defects (Figure 15 and Table 5) suggests that the disruption of development observed using the second site MO might represent a weaker manifestation of that caused by the first MO.

There were 14 cases in which the first MOs yielded a phenotype but the second MOs did not. In these examples, we suspect that the results obtained with the first site MOs are the more reliable. These were designed as the optimal MOs for the mRNAs in question (see Materials and Methods), and as discussed above, it is likely that the second MOs, designed to be non-overlapping with the first while being targeted to a similar region of the mRNA, are less effective, perhaps because of RNA secondary structure (see also Figure 15 and Table 5). In support of this idea, we note that in three of the five cases where the gene had been studied previously, the first MO phenotypes resembled those obtained by other means in *Xenopus* or in other vertebrates. These include *Frz2, Wnt8,* and *Wnt11* (Table 3).

Our experiments investigating the specificities of the nine MOs that cause defects in gastrulation (Table 5 and Figure 15)

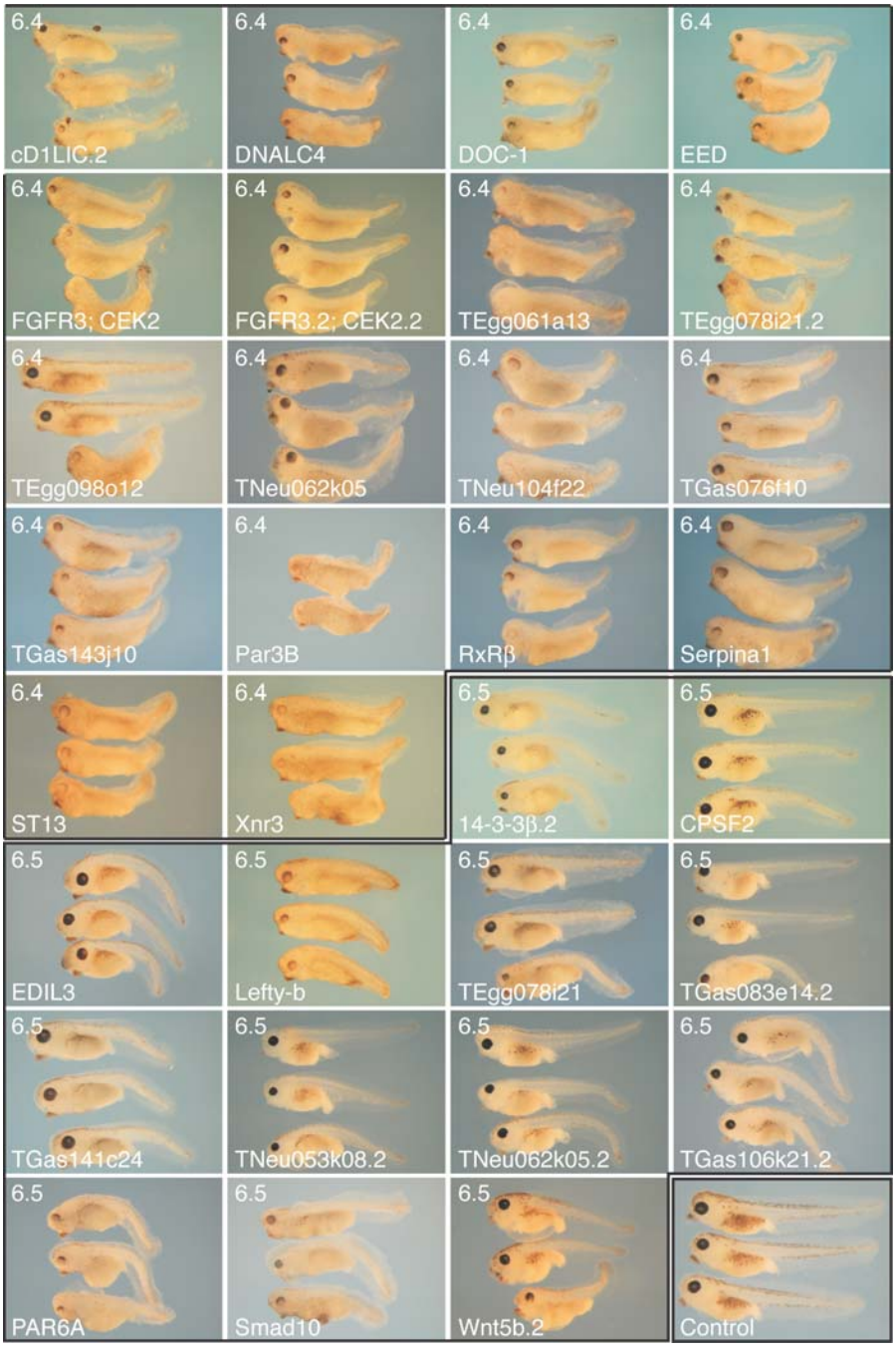

Figure 12. The Final 31 Members of the Bent Axis Phenotypic Class

This class can be subdivided into five synphenotype groups, as indicated in Figure 3 and Table 4. All embryos are shown at the tadpole stage (stage 35–41).

doi:10.1371/journal.pgen.0020193.g012

are also consistent with the idea that MOs are specific in their effects, because oligonucleotides that differ in five nucleotides from their target sequences have no effect, and the effects of the specific MOs can frequently be rescued by injection of the cognate mRNA. We also observe that the effects of our first site MOs are usually stronger than those of the second site MOs, as noted above.

Of the 12 genes for which the first MO yielded no phenotype, the second MO did cause abnormal development in five cases. Of all the phenotypes observed in this study, these, representing just 2% of the total, are perhaps the most likely to be caused by non-specific effects, because the second site MOs may be less effective than the first site MOs in targeting the desired gene product. However, we again note that three of these five genes *(Flamingo1, nodal-related 1,* and *VegT)* have been investigated previously in *Xenopus* or in other vertebrates and that the loss-of-function phenotypes in all three cases resemble those obtained in this study. We suspect, therefore, that the specificity of MOs is, in general, high, and the absence of a phenotype may reflect the complexity of

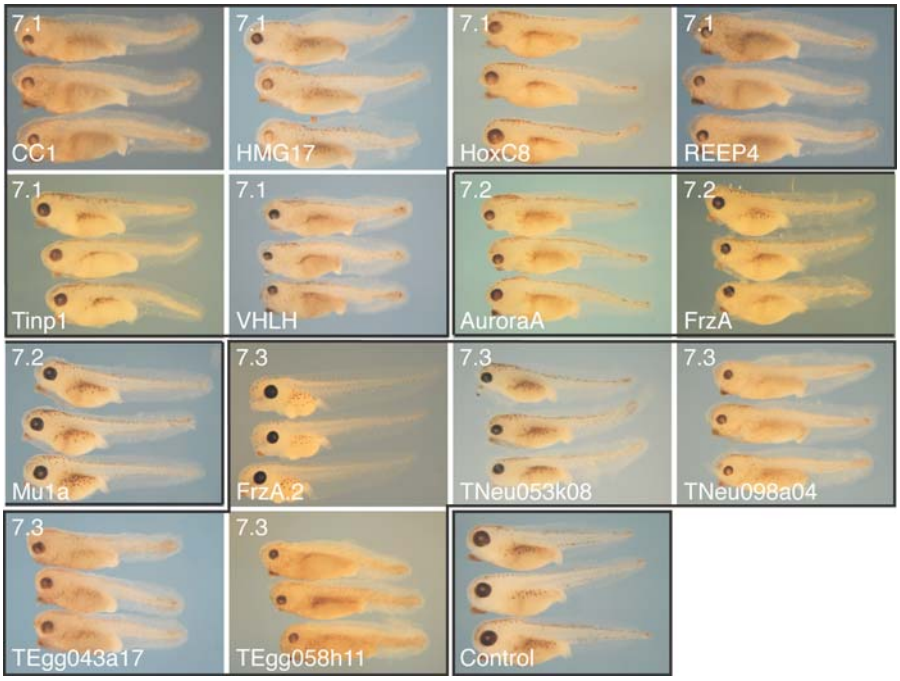

Figure 13. The 14 Members of the Motility Defects Phenotypic Class
This class can be subdivided into three synphenotype groups, as indicated in Figure 3 and Table 4. All embryos are shown at the tadpole stage (stage 35–41).
doi:10.1371/journal.pgen.0020193.g013

mRNA secondary structure. We conclude that MOs will provide valuable information about gene function in the early embryo of *X. tropicalis* and will be able to inform future analyses of gene function in other vertebrate embryos.

Comparison with Other Functional Screens

How does the screen described in this paper compare with others carried out in *Xenopus* and in other species? The most obvious comparison is with a smaller scale *X. tropicalis* MO screen carried out by Kenwrick and colleagues [18]. These authors targeted 26 genes expressed in the neural plate and neural tube of *X. tropicalis,* and like us required that greater than 50% of injected embryos should develop abnormally for a particular MO to be classified as causing a phenotype. This more limited study reported a lower frequency of loss-of-function phenotypes (23%) than that reported here (62%–67%). However, we note that Kenwrick and colleagues [18] allowed their embryos to develop only to stage 30 and used a dose of MO corresponding to our lower concentration. If we apply the same restrictions to our own larger dataset, using only the first site MOs, we observe loss-of-function phenotypes in 48% of cases (97 out of 202), a figure that is closer to that of Kenwrick et al. [18] and which indicates that many of the phenotypes we observe are detectable only after the tailbud stage. We also note that if we include in this analysis only the 68 novel genes, our frequency of loss-of-function phenotypes falls to just 38% (see Table 1).

MOs have also been used to study gene function in the ascidian *Ciona intestinalis* [6]. Loss-of-function of 40 of the 200 genes tested in these experiments caused embryos to develop abnormally in $\geq$50% of cases, and of these genes many had counterparts in mouse and human embryos. At 20%, the frequency of loss-of-function phenotypes in *Ciona* is again lower than that observed in this paper with *X. tropicalis,* although a higher frequency might have been observed if embryos had been allowed to develop for longer or if a higher dose of MO had been used.

Other large-scale screens have been performed in *C. elegans* and *D. melanogaster*. In *C. elegans,* genome-wide RNAi screens have revealed early embryonic mutant phenotypes in 9% of the genes studied [2,3]; as in our own experiments, it is likely that additional phenotypes would become apparent at later stages of development. In *Drosophila,* RNAi screens have been used to search for novel components of signaling pathways such as the JAK/STAT and Wnt pathways [4,5,35]. These targeted screens inevitably yield fewer phenotypes, but they are very effective in identifying regulators of the signaling pathways in question. A similar targeted screen has also been carried out in the zebrafish, where MOs have been used to study the functions of the homologs of genes expressed in human haematopoietic stem cells. In these experiments, 23% (14/61) of the MOs caused haematopoietic defects in the developing embryos [8].

It is difficult to make comparisons with the frequencies of embryonic phenotypes obtained in mouse embryos, because the mouse data frequently refer to embryonic lethal phenotypes (and many of our milder *X. tropicalis* phenotypes may not be lethal), and because mutants in which a phenotype is not observed, or which have only a mild postnatal phenotype, are less likely to be published. We note, however, that one study has used a modified gene-trap approach to analyze the functions of 60 genes encoding secreted and membrane proteins and that loss-of-function of one third of these causes embryonic and postnatal death [36]. Another gene-trap study, which more resembles ours in the sense that gene selection was more random, observed

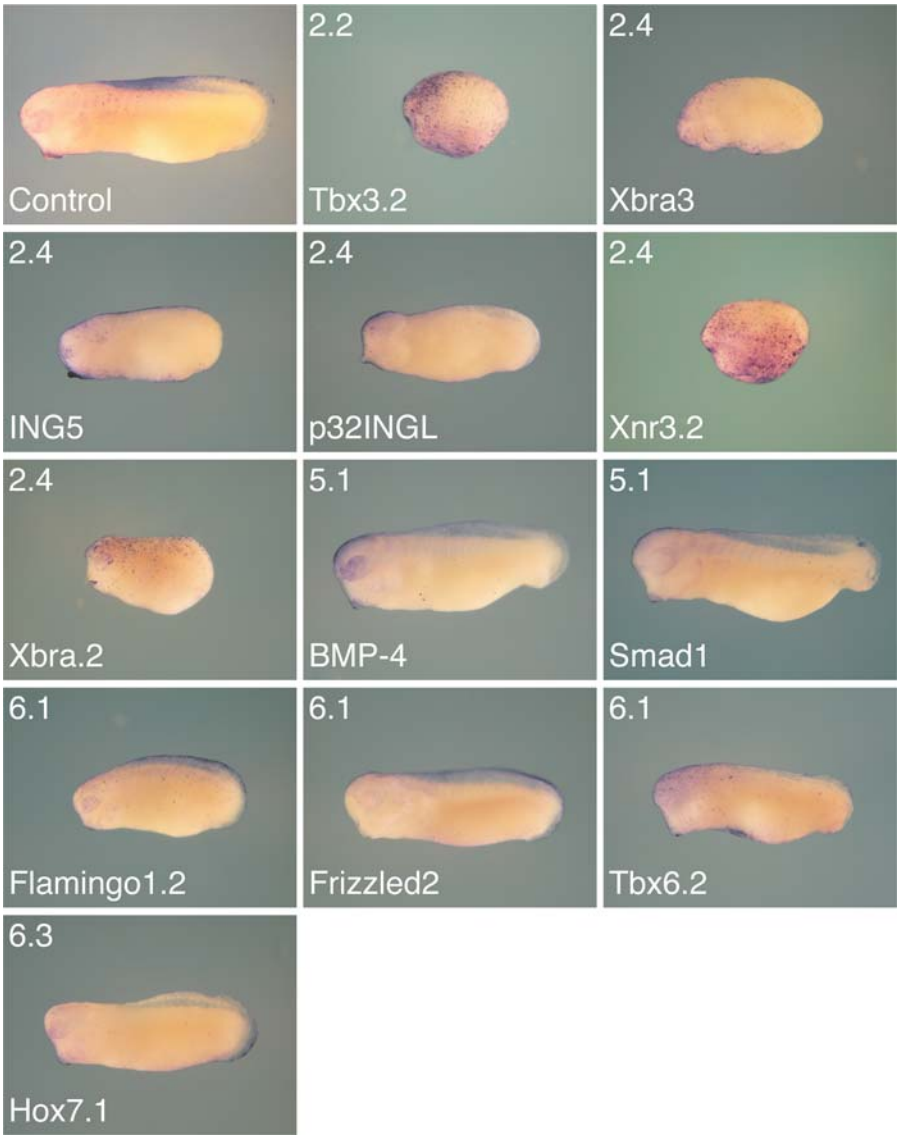

Figure 14. Apoptosis Is Not a Non-Specific Response to Injection of MOs

Embryos were injected at the one-cell stage with 15 ng of the indicated MO and allowed to develop to the equivalent of the tailbud stage, when they were examined by TUNEL staining. Note that only the Tbx3.2, Xnr3.2, and Xbra.2 MOs caused a level of apoptosis that exceeded the level observed in control embryos (injected with 30 ng of the Gene Tools control MO).

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phenotypes in 59% of integrations [37], a figure that resembles the 66% observed in the present work.

Comparisons with Loss-of-Function Phenotypes in Different Species

To ask whether the loss-of-function phenotypes we observe in *X. tropicalis* might allow us to predict gene function in other species (including mammals), we compared our results with those obtained by the removal of the orthologous gene in other vertebrate species, including the zebrafish and the mouse.

Some of the genes studied here have also been investigated in *X. laevis,* usually by dominant-negative approaches. These include Xbra [38], the FGF receptor [39,40], Frizzled8 [41], Xwnt8 [42], and the BMP receptor [43–45]. In each case the phenotype caused by our MOs in *X. tropicalis* resembles that observed in *X. laevis,* although the *X. tropicalis* phenotype is sometimes weaker. For example, the *Xtbra* MO does not produce as severe a shortening of the antero-posterior axis as does the dominant-interfering construct Xbra-En^{R} [38], and the individual MOs targeted against different FGF receptors do not yield phenotypes as dramatic as that caused by the dominant-negative FGF receptor XFD [39,40]. In each case it is likely that the dominant-negative construct is capable of inhibiting the function of more than one gene product. For example, Xbra-En^{R} is likely to inhibit the activities of all three known *Xbra* genes in the *X. laevis* genome [46–48] while the MOs directed against the *X. tropicalis* genes will target only one of the two homologs we have identified in that species (Table 4). That said, there are cases where the *X. laevis* and *X. tropicalis* phenotypes are very similar. These include, for example, loss-of-function of Frizzled 8, which in both species causes shortening of the antero-posterior axis and defects in neural tube closure [41].

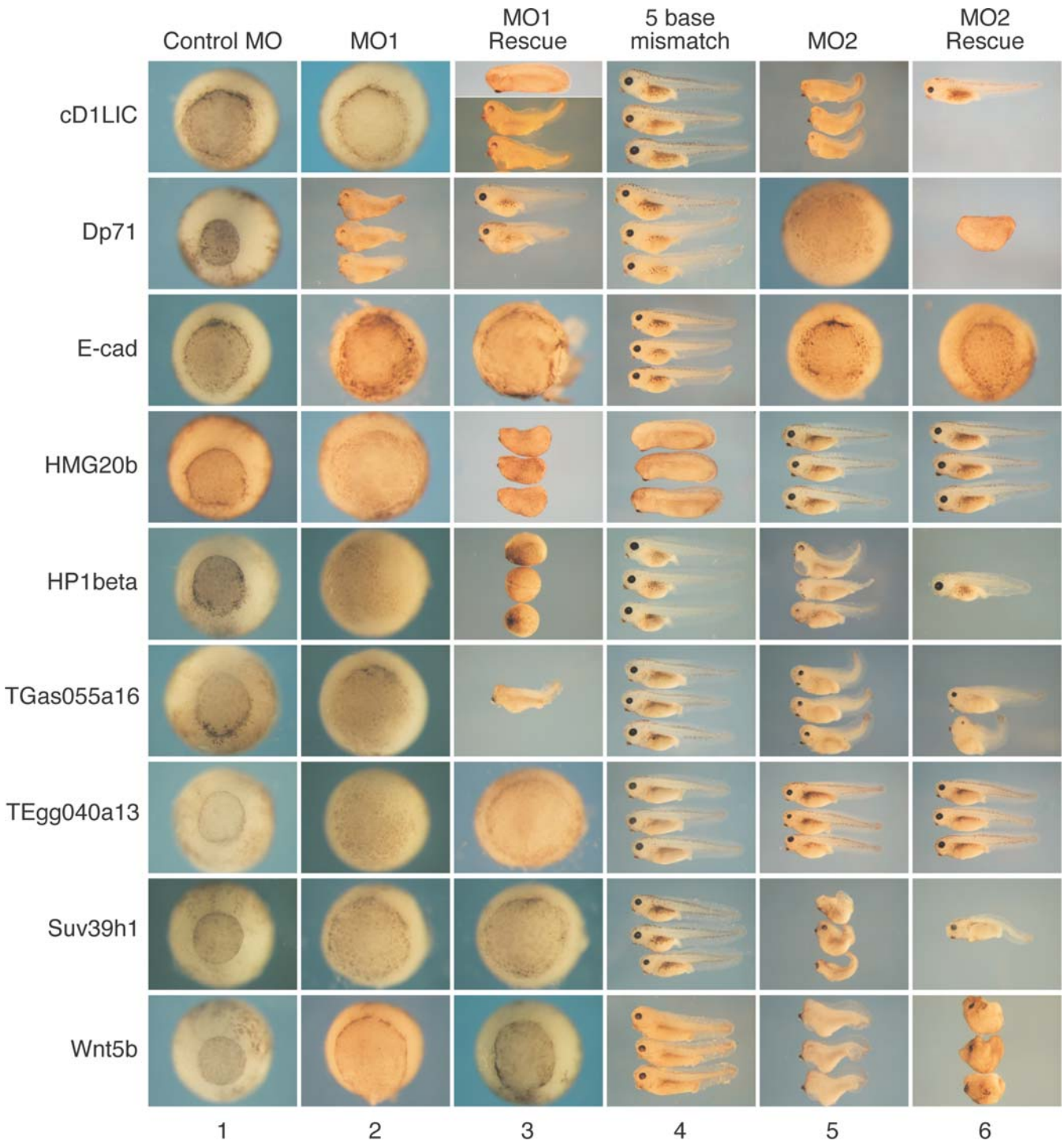

Figure 15. Tests of the Specificities of the Phenotypes Observed in the Gastrula Defects Phenotypic Class

The specificities of the MOs used to define this phenotypic class were investigated by injecting 10–15 ng of the Gene Tools standard control MO (Column 1); the original antisense MO (Column 2); MO1 together with 1 ng of a form of the target RNA that lacks the MO target sequence (Column 3); MO1 (or, in the case of Dp71, MO2) with five mismatched bases (Column 4); MO2 (Column 5); MO2 together with 1 ng of a form of the target RNA that lacks the MO target sequence (Column 6).

The results of these experiments are summarized in Table 5. In Column 1 (control MO) embryos are shown at the mid-gastrula stage. Embryos in Column 2 (MO1) are at the same stage as those in Column 1, but (with the exception of Dp71) gastrulation is delayed or inhibited. In the case of Dp71, MO1 does not inhibit gastrulation but does cause embryos to develop with a shortened axis. Column 3 indicates that for five of the nine MOs studied, complete or partial rescue of the phenotype was obtained by injection of the cognate RNA. In these experiments, embryos were allowed to develop beyond gastrula stages to tailbud or tadpole stages. In the case of D1LIC, rescue was more complete at tailbud stages (upper panel) than tadpole stages (lower panel). Column 4 shows that for each of the nine MOs, changing five bases caused them to lose the ability to disrupt development.

Use of a second site MO usually causes a milder phenotype than is observed with MO1 (Column 5), but the phenotype is usually specific, in the sense that it can frequently be rescued by injection of the cognate RNA (Column 6).

MO1, original antisense oligonucleotide; MO2, second site MO.

doi:10.1371/journal.pgen.0020193.g015

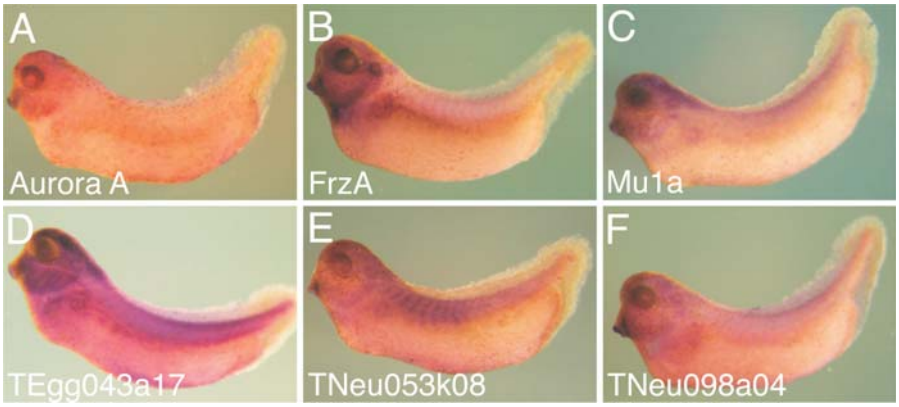

Figure 16. Members of the Same Synphenotype Group Do Not Necessarily Have the Same Expression Patterns

The six examples shown here are all from the motility defects class. (A–C) Expression patterns of the three members of the swimming in circles synphenotype group. (D–F) Expression patterns of three members of the normal appearance but paralyzed synphenotype group. Members of each group are not expressed in the same patterns and so do not belong to the same synexpression group (see text).

doi:10.1371/journal.pgen.0020193.g016

To our knowledge, 57 of the genes analyzed in this paper have been targeted in mouse embryos [49] (Table 6). For 29 of these, the *X. tropicalis* phenotypes resemble those observed in the equivalent mouse homozygous null mutant and in five cases a phenotype is observed in neither species. For example, loss of *Rad51* function in both *X. tropicalis* and mouse causes a disruption of gastrulation and neurulation [50] (Figure 5; Tables 4 and 5), while another gene with a conserved biological role proves to be *Cdx2,* which in *X. tropicalis* is required for proper posterior development and tailbud elongation (Figure 7; Tables 4 and 5). In this case, conventional gene targeting in the mouse embryo does not reveal this function because homozygous null *Cdx2* mutant mice die before implantation [51]. However, tetraploid aggregation experiments show that the gene is also required later in development, during gastrulation and tailbud elongation in a role that resembles its function in frogs [52]. These observations suggest that experiments in *X. tropicalis* can help reveal the post-implantation functions of genes that cause early lethality.

In nine cases, MOs elicited a different phenotype in *X. tropicalis* and mouse, in eight cases a phenotype has been recorded in the mouse, but was not observed in *X. tropicalis* in this study, and for six genes a phenotype was observed in frog but not in mouse. These differences may reflect different developmental strategies in the two species, including the deployment of different gene family members to accomplish a specific developmental task, or, particularly for the last group of six genes, functional redundancy in the mouse embryo. The gene *Fgfr4a* illustrates this point. Two MOs

Table 5. Specificity Controls Applied to the Gastrulation Synphenotype Group

Wellcome Clone Identifier	Gene	First MO			Second MO		
		Synphenotype Group	5-Base Mismatch	Rescue ($28 \leq n \leq 32$)	Synphenotype Group	5-Base Mismatch	Rescue ($28 \leq n \leq 33$)
TGas061b15	*D1LIC*	Gastrula	100% normal	Complete: 90% Partial: 0% None: 10%	Normal body, wavy tail	ND	Complete: 100%, Partial: 0%, None: 0%
TNeu143c03	*Dp71/40*	Short axissurviving to tailbud	ND	Complete: 30% Partial: 70% None: 0	Gastrula	100% normal	Complete: 0%, Partial: 100%, None: 0%
TNeu073g23	*E-cadherin*	Gastrula	100% normal	Complete: 0% Partial: 0% None: 100%	Gastrula	ND	Complete: 0%, Partial: 0%, None: 100%
TGas142e24	*HMG20b*	Gastrula	100% normal	Complete: 50% Partial: 50% None: 0%	No phenotype	ND	Complete: 100%, Partial: 0%, None: 0%
TEgg130i17	*HP1beta*	Gastrula	100% normal	Complete: 20% Partial: 5% None: 75%	Bent-up tail	ND	Complete: 33% Partial: 67% None: 0%
TEgg137c14	*Suv(3–9)1*	Gastrula	100% normal	Complete: 0% Partial: 0% None: 100%	Curved body	ND	Complete: 0% Partial: 100% None: 0%
TEgg040a13	Novel	Gastrula	100% normal	Complete: 0% Partial: 0% None: 100%	No phenotype	ND	N/A
TGas055a16	Novel	Gastrula	100% normal	Complete: 0% Partial: 100% None: 0%	Normal body, wavy tail	ND	Complete: 0% Partial: 75% None: 25%
TGas012b13	*Wnt5b*	Gastrula	100% normal	Complete: 0% Partial: 0% None: 100%	Bent-down tail	ND	Complete: 0% Partial: 0% None: 100%

ND, not determined.

doi:10.1371/journal.pgen.0020193.t005

Table 6. Comparison of *Xenopus tropicalis* and Mouse Phenotypes

Comparison of *X. tropicalis* and Mouse Phenotypes	Cases ($n = 57$)	Genes
Similar phenotypes	29	*14-3-3ε, Blimp1, BMP4, Brachyury, cdx-2, Dp71, E-cadherin, eed, Fgfr3, Hesx-1(Xanf-1), HoxC8, Lefty-b, Lim1, Mu2, nodal, rad51, Rarb, Rb, Rb2, SerpinA1 Smad1, Smad2, Smad4, Smad6, Suv39h1, Suv39h2, Tbx2,Tbx6, vhlh*
Different phenotypes in *X. tropicalis* and mouse	9	*14-3-3zeta, Cdc42, dlgh1, frizzled6, Hox7.1, integrinβ1, p53, Smad3,Tbx3*
Phenotype observed in mouse but not in *X. tropicalis*	8	*Cdx1, D2LIC, Eomes, Fgfr1, Otx2, Rac1, HoxB3, HoxD1*
Phenotype observed in *X. tropicalis* but not in mouse	6	*Fgfr4a, Tieg2, frizzled8, frzA, AHNAK, TGIF1*
No embryonic phenotype in either species	5	*Frizzled10, 53bp1, HBEGF, SerpinE2, OA1*

Mouse phenotype data were retrieved from the Mouse Genome Database, Mouse Genome Informatics Web site, The Jackson Laboratory, Bar Harbor, Maine, United States (http://www.informatics.jax.org).

doi:10.1371/journal.pgen.0020193.t006

directed against this gene product yielded the same phenotype in *X. tropicalis*, in which the body axis appeared normal but the tail of the tadpole was "wavy." No such phenotype has been observed in the mouse embryo [53], although the gene is required for muscle regeneration in this species [54]. However, we note that mutations in the related gene *Fgfr1* cause abnormal mesodermal patterning in the mouse embryo, with loss of somites and expansion of axial mesoderm at the expense of paraxial tissues [55].

There are also similarities between the phenotypes we observe in *X. tropicalis* and those obtained in the zebrafish (Table 7). Of the 20 genes for which data are available for both species [56], 12 phenotypes appear to be similar and in five cases a phenotype was observed in neither species. The remaining three differences between *X. tropicalis* and the zebrafish may derive from differences in biology, a failure to obtain null mutations in zebrafish, a failure of the *X. tropicalis* MO to inhibit the gene in question, or the whole genome duplication that occurred in zebrafish [9,10].

Together, the similarities between the phenotypes observed in *X. tropicalis* and those obtained in other species argues that work in *X. tropicalis* should shed light on general vertebrate developmental mechanisms.

Synphenotype Groups and Developmental Pathways

Genetic screens in organisms such as *D. melanogaster* and the zebrafish *Danio rerio* allow one to identify genes that function in the same developmental pathway [57], and the same is likely to be true of genes that are members of the same synphenotype groups that are defined in this study. This possibility is illustrated by synphenotype group 6.1 (Short body, upturned tail, dorsalized), which includes the genes *SET8*, *delangin*, *flamingo1.2*, *frizzled2*, *RBBP1*, *TBX6.2*, *Smad6*, and three novel genes. The protein encoded by the first of these genes, SET8, functions as a Histone 4–lysine 20 (H4–K20) methyltransferase. Targeting of this enzyme to heterochromatic regions requires Suv39h1 [58], which itself interacts with pRb [59], which in turn interacts with RBBP, another member of synphenotype group 6.1.

Another more obvious example of genes falling within the same synphenotype group and that function in the same pathway are BMP4, and the molecule that functions downstream of this transforming growth factor type β family member, Smad1 [60]. Significantly, MOs directed against BMP4 and Smad1 can act synergistically, in the sense that simultaneous expression of low doses of BMP4 and Smad1 MOs causes stronger phenotypes than when the oligonucleotides are injected individually (unpublished data).

Although members of a single synphenotype group may indeed be involved in the same developmental or molecular pathway, it is also possible that loss-of-function of different members of a particular molecular pathway may yield distinct phenotypes. Thus, although *RBBP1* is a member of synphenotype group 6.1, its partner *pRb* is required earlier in development and is a member of synphenotype group 2.2 (gastrula or neurula defects). Other members of this group include three novel genes as well as *Anillin*, *Par6B*, *Rad51*,

Table 7. Comparison of *Xenopus tropicalis* and Zebrafish Phenotypes

Comparison of *X. tropicalis* and Zebrafish Phenotypes	Cases ($n = 16$[a])	Genes
Similar phenotypes	12	*Blimp1/Prdm-1/U-boot, Brachyury/Ntl, Chimerin E-cadherin/half-baked, Frizzled7/Frizzled7a,b, Frizzled8/Frizzled8a,b,c, Lefty-b/Lefty-2/Lefty-1, Nodal-related 1/Squint, Tbx2/Tbx2a,b, Tbx6/Tbx16/spadetail, Wnt8/Wnt8a,b, Wnt11/Silberblick*
Different phenotypes in *X. tropicalis* and zebrafish	1	*CPSF4/no arches*
Phenotype observed in zebrafish but not in *X. tropicalis*	1	*Eomes*
Phenotype observed in *X. tropicalis* but not in zebrafish	1	*p53*
No embryonic phenotype in either species	5	*Cdx1/cdx1a, CPSF1, CPSF3, Fgfr1, HoxB3*

[a]Zebrafish phenotype data were retrieved from the Zebrafish Information Network (ZFIN) and the Zebrafish International Resource Center, University of Oregon, Eugene, Oregon, United States (http://zfin.org).

doi:10.1371/journal.pgen.0020193.t007

Smad4, and *Tbx3,* and it may be significant that the Rb can form a complex that includes members of the Smad family [61].

The Future

Together, our results suggest that it should be possible to use MOs together with DNA sequence analyses in *X. tropicalis* [24] to perform a large-scale, low-cost, functional screen in a vertebrate embryo. Such a screen should provide valuable information about gene function in development and disease, and by injecting different doses of MOs it should be possible to study gene dosage effects, thereby creating the equivalent of an allelic series for each gene product. Although in this paper we have not been able to address the efficacy of individual MOs with respect to their ability to depress levels of the targeted protein, this may be possible in the future by use of techniques such as iTRAQ [62] or AQUA [63].

Materials and Methods

Embryos and in vitro fertilization. Adult *Xenopus tropicalis* were obtained from NASCO (Fort Atkinson, Wisconsin, United States), and are outbred Nigerian frogs derived from University of Virginia stock. Females were primed with 10 U pregnant mare serum gonadotrophin 1–5 d before use and they then received 100 U Chorulon 3–4 h before egg harvesting. Testes were dissected from sacrificed males and macerated in L15 medium containing 10% sheep serum. The sperm suspension was added to eggs and sperm and was activated 3–4 min later by flooding with 0.1× MMR. Embryos were de-jellied using 2% cysteine hydrochloride (pH 7.9–8.1) 8–9 min after flooding and they were then rinsed in 0.01× MMR prior to injection [64].

MO design and microinjection. MOs were designed to complement sequence between −80 and +25 nucleotides of the translation start site of the target mRNA. We excluded regions containing polymorphisms by referring to a clustered full-length gene sequence database (http://informatics.gurdon.cam.ac.uk/online/xt-fl-db.html) and then by performing EST searches using the *Xenopus tropicalis* EST Blast Server at the Wellcome Trust Sanger Institute, Cambridge, United Kingdom (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/x_tropicalis). Morpholinos were designed either by Gene Tools (Philomath, Oregon, United States) or by following the instructions outlined at the Gene Tools Web site (http://www.gene-tools.com). When there were several choices of optimal MO, the sequence closest to the initiating AUG of the target mRNA was selected. Morpholinos were modified by the addition of either Carboxyfluorescein or Lissamine fluorochromes. This allowed us to ensure that all scored embryos had indeed received injections of MOs and that the MOs were uniformly distributed throughout the embryo. For each MO tested, at least 40 *X. tropicalis* embryos were injected at the one-cell stage with either 10–15 ng or 30 ng of MO at a concentration of 10 ng/nl in H2O. The Gene Tools standard control oligonucleotide 5′-CCTCTTACCTCAGTTACAATTTATA 3′ was used as a control and injected at doses of 10–15 ng and 30 ng.

Samples of the MOs used in this study are available on request while stocks last.

Specificity and rescue experiments. To test the specificity of the phenotypes in the gastrulation synphenotype group, embryos were injected with MOs (10–15 ng) that differed in five bases from the target sequence (details available on request). Rescues were carried out by using the PCR to create expression constructs that retain the Kozak sequence of the endogenous mRNA but lack that part of the 5′ UTR which is recognized by MO2. In addition, six base changes were introduced into the region of the mRNA that is recognized by MO1. Details are available on request.

TUNEL analysis was performed as described previously [65]. Embryos were injected at the one-cell stage with 15 ng specific MO or 30 ng of the Gene Tools standard control MO.

Recording of results. Embryos were examined and photographed at gastrula (stages 10–12), tailbud (stages 24–28), and tadpole (stages 37–41) stages. Samples were fixed in MEMFA (0.1 M MOPS [pH 7.4], 2 mM EGTA, 1 mM $MgSO_4$, and 3.7% formaldehyde) or 4% formaldehyde. For a particular MO to be classified as causing a "phenotype," we required that its injection should cause at least 50% of embryos ($n \geq 40$) to develop in a similar abnormal fashion.

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Author contributions. AAR and JCS conceived and designed the experiments. AAR and CC performed the experiments. AAR and MJG analyzed the data. AAR and JCS wrote the paper.

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References

1. Nusslein-Volhard C, Wieschaus E (1980) Mutations affecting segment number and polarity in *Drosophila*. Nature 287: 795–801.
2. Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, et al. (2003) Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. Nature 421: 231–237.
3. Sonnichsen B, Koski LB, Walsh A, Marschall P, Neumann B, et al. (2005) Full-genome RNAi profiling of early embryogenesis in *Caenorhabditis elegans*. Nature 434: 462–469.
4. Baeg GH, Zhou R, Perrimon N (2005) Genome-wide RNAi analysis of JAK/STAT signaling components in *Drosophila*. Genes Dev 19: 1861–1870.
5. DasGupta R, Kaykas A, Moon RT, Perrimon N (2005) Functional genomic analysis of the Wnt-wingless signaling pathway. Science 308: 826–833.
6. Yamada L, Shoguchi E, Wada S, Kobayashi K, Mochizuki Y, et al. (2003) Morpholino-based gene knockdown screen of novel genes with developmental function in *Ciona intestinalis*. Development 130: 6485–6495.
7. Wianny F, Zernicka-Goetz M (2000) Specific interference with gene function by double-stranded RNA in early mouse development. Nat Cell Biol 2: 70–75.
8. Eckfeldt CE, Mendenhall EM, Flynn CM, Wang TZ, Pickart MA, et al. (2005) Functional analysis of human hematopoietic stem cell gene expression using zebrafish. PLoS Biol 3: e254. doi:10.1371/journal.pbio.0030254
9. Van de Peer Y, Taylor JS, Meyer A (2003) Are all fishes ancient polyploids? J Struct Funct Genomics 3: 65–73.
10. Woods IG, Wilson C, Friedlander B, Chang P, Reyes DK, et al. (2005) The zebrafish gene map defines ancestral vertebrate chromosomes. Genome Res 15: 1307–1314.
11. Blomme T, Vandepoele K, De Bodt S, Simillion C, Maere S, et al. (2006) The gain and loss of genes during 600 million years of vertebrate evolution. Genome Biol 7: R43.
12. Postlethwait JH, Woods IG, Ngo-Hazelett P, Yan YL, Kelly PD, et al. (2000) Zebrafish comparative genomics and the origins of vertebrate chromosomes. Genome Res 10: 1890–1902.
13. Smith JC (2005) *Xenopus* genetics and genomics. Mech Dev 122: 259–262.
14. Amaya E, Offield MF, Grainger RM (1998) Frog genetics: *Xenopus tropicalis* jumps into the future. Trends Genet 14: 253–255.
15. Heasman J, Kofron M, Wylie C (2000) Beta-catenin signaling activity dissected in the early *Xenopus* embryo: A novel antisense approach. Dev Biol 222: 124–134.
16. Draper BW, Morcos PA, Kimmel CB (2001) Inhibition of zebrafish fgf8 pre-mRNA splicing with morpholino oligos: A quantifiable method for gene knockdown. Genesis 30: 154–156.
17. Heasman J (2002) Morpholino oligos: Making sense of antisense? Dev Biol 243: 209–214.
18. Kenwrick S, Amaya E, Papalopulu N (2004) Pilot morpholino screen in *Xenopus tropicalis* identifies a novel gene involved in head development. Dev Dyn 229: 289–299.
19. Baldessari D, Shin Y, Krebs O, Konig R, Koide T, et al. (2005) Global gene expression profiling and cluster analysis in *Xenopus laevis*. Mech Dev 122: 441–475.
20. Niehrs C, Pollet N (1999) Synexpression groups in eukaryotes. Nature 402: 483–487.
21. Chen JA, Voigt J, Gilchrist M, Papalopulu N, Amaya E (2005) Identification of novel genes affecting mesoderm formation and morphogenesis through an enhanced large-scale functional screen in *Xenopus*. Mech Dev 122: 307–331.
22. Driever W, Solnica-Krezel L, Schier AF, Neuhauss SC, Malicki J, et al. (1996) A genetic screen for mutations affecting embryogenesis in zebrafish. Development 123: 37–46.
23. Haffter P, Granato M, Brand M, Mullins MC, Hammerschmidt M, et al. (1996) The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. Development 123: 1–36.

24. Gilchrist MJ, Zorn AM, Voigt J, Smith JC, Papalopulu N, et al. (2004) Defining a large set of full-length clones from a *Xenopus tropicalis* EST project. Dev Biol 271: 498–516.
25. Takeuchi M, Nakabayashi J, Sakaguchi T, Yamamoto TS, Takahashi H, et al. (2003) The prickle-related gene in vertebrates is essential for gastrulation cell movements. Curr Biol 13: 674–679.
26. Klee EW, Shim KJ, Pickart MA, Ekker SC, Ellis LB (2005) AMOD: A morpholino oligonucleotide selection tool. Nucleic Acids Res 33: W506–W511.
27. Piepenburg O, Grimmer D, Williams PH, Smith JC (2004) Activin redux: Specification of mesodermal pattern in *Xenopus* by graded concentrations of endogenous activin B. Development 131: 4977–4986.
28. Trindade M, Messenger N, Papin C, Grimmer D, Fairclough L, et al. (2003) Regulation of apoptosis in the *Xenopus* embryo by Bix3. Development 130: 4611–4622.
29. Tao Q, Yokota C, Puck H, Kofron M, Birsoy B, et al. (2005) Maternal Wnt11 activates the canonical Wnt signaling pathway required for axis formation in *Xenopus* embryos. Cell 120: 857–871.
30. Gaiano N, Amsterdam A, Kawakami K, Allende M, Becker T, et al. (1996) Insertional mutagenesis and rapid cloning of essential genes in zebrafish. Nature 383: 829–832.
31. Hensey C, Gautier J (1998) Programmed cell death during *Xenopus* development: A spatio-temporal analysis. Dev Biol 203: 36–48.
32. Conlon FL, Smith JC (1999) Interference with brachyury function inhibits convergent extension, causes apoptosis, and reveals separate requirements in the FGF and activin signaling pathways. Dev Biol 213: 85–100.
33. Carlson H, Ota S, Song Y, Chen Y, Hurlin PJ (2002) Tbx3 impinges on the p53 pathway to suppress apoptosis, facilitate cell transformation, and block myogenic differentiation. Oncogene 21: 3827–3835.
34. Ito A, Asamoto M, Hokaiwado N, Takahashi S, Shirai T (2005) Tbx3 expression is related to apoptosis and cell proliferation in rat bladder both hyperplastic epithelial cells and carcinoma cells. Cancer Lett 219: 105–112.
35. Muller P, Kuttenkeuler D, Gesellchen V, Zeidler MP, Boutros M (2005) Identification of JAK/STAT signaling components by genome-wide RNA interference. Nature 436: 871–875.
36. Mitchell KJ, Pinson KI, Kelly OG, Brennan J, Zupicich J, et al. (2001) Functional analysis of secreted and transmembrane proteins critical to mouse development. Nat Genet 28: 241–249.
37. Hansen J, Floss T, Van Sloun P, Fuchtbauer EM, Vauti F, et al. (2003) A large-scale, gene-driven mutagenesis approach for the functional analysis of the mouse genome. Proc Natl Acad Sci U S A 100: 9918–9922.
38. Conlon FL, Sedgwick SG, Weston KM, Smith JC (1996) Inhibition of Xbra transcription activation causes defects in mesodermal patterning and reveals autoregulation of Xbra in dorsal mesoderm. Development 122: 2427–2435.
39. Amaya E, Musci TJ, Kirschner MW (1991) Expression of a dominant-negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. Cell 66: 257–270.
40. Amaya E, Stein PA, Musci TJ, Kirschner MW (1993) FGF signaling in the early specification of mesoderm in *Xenopus*. Development 118: 477–487.
41. Itoh K, Sokol SY (1999) Axis determination by inhibition of Wnt signaling in *Xenopus*. Genes Dev 13: 2328–2336.
42. Hoppler S, Brown JD, Moon RT (1996) Expression of a dominant-negative Wnt blocks induction of MyoD in *Xenopus* embryos. Genes Dev 10: 2805–2817.
43. Graff JM, Thies RS, Song JJ, Celeste AJ, Melton DA (1994) Studies with a *Xenopus* BMP receptor suggest that ventral mesoderm-inducing signals override dorsal signals in vivo. Cell 79: 169–179.
44. Suzuki A, Thies RS, Yamaji N, Song JJ, Wozney JM, et al. (1994) A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early *Xenopus* embryo. Proc Natl Acad Sci U S A 91: 10255–10259.
45. Hawley SH, Wunnenberg-Stapleton K, Hashimoto C, Laurent MN, Watabe T, et al. (1995) Disruption of BMP signals in embryonic *Xenopus* ectoderm leads to direct neural induction. Genes Dev 9: 2923–2935.
46. Latinkic BV, Umbhauer M, Neal KA, Lerchner W, Smith JC, et al. (1997) The *Xenopus Brachyury* promoter is activated by FGF and low concentrations of activin and suppressed by high concentrations of activin and by paired-type homeodomain proteins. Genes Dev 11: 3265–3276.
47. Smith JC, Price BM, Green JB, Weigel D, Herrmann BG (1991) Expression of a *Xenopus* homolog of *Brachyury* (T) is an immediate-early response to mesoderm induction. Cell 67: 79–87.
48. Strong CF, Barnett MW, Hartman D, Jones EA, Stott D (2000) Xbra3 induces mesoderm and neural tissue in *Xenopus laevis*. Dev Biol 222: 405–419.
49. Eppig JT, Bult CJ, Kadin JA, Richardson JE, Blake JA, et al. (2005) The Mouse Genome Database (MGD): From genes to mice—a community resource for mouse biology. Nucleic Acids Res 33: D471–475.
50. Shu Z, Smith S, Wang L, Rice MC, Kmiec EB (1999) Disruption of muREC2/RAD51L1 in mice results in early embryonic lethality which can be partially rescued in a p53(-/-) background. Mol Cell Biol 19: 8686–8693.
51. Strumpf D, Mao CA, Yamanaka Y, Ralston A, Chawengsaksophak K, et al. (2005) Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. Development 132: 2093–2102.
52. Chawengsaksophak K, de Graaff W, Rossant J, Deschamps J, Beck F (2004) Cdx2 is essential for axial elongation in mouse development. Proc Natl Acad Sci U S A 101: 7641–7645.
53. Weinstein M, Xu X, Ohyama K, Deng CX (1998) FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung. Development 125: 3615–3623.
54. Zhao P, Caretti G, Mitchell S, McKeehan WL, Boskey AL, et al. (2005) Fgfr4 is required for effective muscle regeneration in vivo: Delineation of a MyoD-Tead2-Fgfr4 transcriptional pathway. J Biol Chem 281: 429–438.
55. Yamaguchi TP, Harpal K, Henkemeyer M, Rossant J (1994) fgfr-1 is required for embryonic growth and mesodermal patterning during mouse gastrulation. Genes Dev 8: 3032–3044.
56. Sprague J, Doerry E, Douglas S, Westerfield M (2001) The Zebrafish Information Network (ZFIN): A resource for genetic, genomic, and developmental research. Nuc Acids Res 29: 87–90.
57. Anderson KV, Ingham PW (2003) The transformation of the model organism: A decade of developmental genetics. Nat Genet 33: 285–293.
58. Schotta G, Lachner M, Sarma K, Ebert A, Sengupta R, et al. (2004) A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. Genes Dev 18: 1251–1262.
59. Nielsen SJ, Schneider R, Bauer UM, Bannister AJ, Morrison A, et al. (2001) Rb targets histone H3 methylation and HP1 to promoters. Nature 412: 561–565.
60. Liu F, Hata A, Baker JC, Doody J, Carcamo J, et al. (1996) A human Mad protein acting as a BMP-regulated transcriptional activator. Nature 381: 620–623.
61. Kowalik TF (2002) Smad about E2F. TGFbeta repressionof c-Myc via a Smad3/E2F/p107 complex. Mol Cell 10: 7–8.
62. Shadforth IP, Dunkley TP, Lilley KS, Bessant C (2005) i-Tracker: For quantitative proteomics using iTRAQ. BMC Genomics 6: 145.
63. Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP (2003) Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. Proc Natl Acad Sci U S A 100: 6940–6945.
64. Khokha MK, Chung C, Bustamante EL, Gaw LW, Trott KA, et al. (2002) Techniques and probes for the study of *Xenopus tropicalis* development. Dev Dyn 225: 499–510.
65. Hensey C, Gautier J (1997) A developmental timer that regulates apoptosis at the onset of gastrulation. Mech Dev 69: 183–195.