Identification and preliminary functional analysis of alternative splicing of Siah1 in *Xenopus laevis*

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ABSTRACT

Siah proteins are vertebrate homologs of the *Drosophila* 'seven in absentia' gene. In this study, we characterized two splicing forms, Siah1a and Siah1b, of the *Xenopus* seven in absentia homolog 1 gene (Siah1). Overexpression of xSiah1a led to severe suppression of embryo cleavage, while that of xSiah1b was not effective even at a high dose. Competition analysis demonstrated that co-expression of xSiah1a and 1b generated the same phenotype as overexpression of xSiah1a alone, suggesting that xSiah1b does not interfere with the function of xSiah1a. Since xSiah1b has an additional 31 amino acids in the N-terminus compared to xSiah1a, progressive truncation of xSiah1b from the N-terminus showed that inability of xSiah1b to affect embryo cleavage was associated with the length of the N-terminal extension of extra amino acids. The possible implication of this finding is discussed.

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1. Introduction

The seven in absentia homolog (Siah) proteins are homologs of the *Drosophila* seven in absentia (sina) gene. This gene codes for a protein downstream from the Sevenless tyrosine kinase receptor that degrades the transcriptional repressor Tramtrack and is required for R7 cell fate specification during *Drosophila* eye development [1–3]. Humans have two unlinked Siah genes, SIAH1 and SIAH2 [4], but mice have two Siah1 (Siah1a and Siah1b) and single Siah2 genes [5]. The Siah1 protein contains an N-terminal RING finger domain, required for interaction with E2 ubiquitin conjugating enzymes [6], as well as a coiled coil domain to form homo- and heterodimers [7]. As an essential component of E3 ubiquitin ligase complexes, Siah1 is responsible for the ubiquitin proteosome degradation that terminates cellular signaling and also for turnover of many key proteins, such as DCC [8], β-catenin [9], Kid [10], c-Myb [11], OBF-1 [12,13], Numb [14], Synaptophysin [15], TIEG-1 [16], Synphilin-1 [17], CtIP [18], T-STAR [19], Polycystin [20], Af4 [21], PHD1 and PHD3 [22], HIPK2 [23], FIH [24,25], PLCepsilon [26] and TRB3 [27].

Siah1a knockout mice exhibit severe growth retardation and male sterility due to a blockade in spermatogenesis [28]. Several splicing variants of SIAH1 appear to play important regulatory roles; for example, SIAH1S, an alternative splicing form of SIAH1,

acts as a dominant negative inhibitor of SIAH1 in the regulation of β-catenin activity [29]. SIAH1L, another splicing variant of SIAH1, is induced in response to p53 and plays a key role in the regulation of β-catenin activity [9,30,31].

In *Xenopus laevis*, overexpression of xSiah2 led to small eyes [32] due to degradation of PHD2 [33], indicating its critical role in retinal development. However, the function of Siah1 in *Xenopus* embryogenesis still remains largely unknown. In the present study, we report for the first time the identification of two splicing forms of Siah1 in *X. laevis* and their distinct functional roles.

2. Materials and methods

2.1. Isolation of xSiah1a and xSiah1b in X. laevis

Two *Xenopus* Siah1protein sequences were found based on a Blastp search using the human Siah1 coding sequence; one was from *X. laevis* (NP_001085438), the other was from the *Xenopus tropicalis* (NP_001015836). The predicted open reading frame (ORF) of the *X. laevis* gene was longer than that of *X. tropicalis*. Comparison of the genomic organization of the two ORFs using Blat revealed that the *X. laevis* Siah1 has an alternative exon in the UTR (Fig. 2A). The full *X. laevis* Siah1a sequence (GU377277) was cloned by RT-PCR using the forward primer 5′-TCCGGTGTCTTTCTATG-GAAGCGGT-3′ and the reverse primer 5′-CATGTACACACCCAG-CTGGGCATCTTTTGTA-3′. The correct clones were verified by sequencing.

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2.2. Plasmid construction and in vitro transcription

Total RNA was extracted with Trizol (Invitrogen) according to the manufacturer's instructions, and cDNAs were synthesized using random primers and M-MLV (Promega). PrimerSTAR (Takara) was used to amplify xSiah1a, xSiah1a-n9, xSiah1a-n16, xSiah1a-22 and xSiah1b, which were cloned into the pCS2plus. Primers used are as following: xSiah1a forward primer (5′-CATGAGCCGACAGACTGCTACAGCAATC-3′); xSiah1a-n9 forward primer (5′-TATGAGTGACCCGAAGCGAAGGAAAGAAATG-3′); xSiah1a-n16 forward primer (5′-TATGGTGCTGCTCACGTGCTTGTCCGGTAGTG-3′); xSiah1a-22 forward primer (5′-TATGTTGTACCAGTGGAAAGGGGTGCTGCTC-3′); xSiah1b forward primer (5′-AGCGCGATGAACTGTCTAACTCCTCATGAC-3′) and a shared common reverse primer (5′-CTGCCAATTCAGCACATTGAGATCG-3′). For the xSiah1a-P2A-GFP construct, the reverse primer was (5′-GCACATTGAGATCGTAACGTTTATTC-3′), and PCR products were inserted to pCS2-P2A-GFP. xKid was amplified with the forward primer (5′-GAATGGTTCTTACTGGGCCTCCCCAAAGAG-3′) and the reverse primer (5′-GCTGGAGATGCTGCTCAGGATATTTGCCT-3′). The resulting fragment was cloned into pCR3.1-flag vector. All constructs were verified by sequencing.

To generate mRNA, constructs were linearized by NotI. Capped mRNA in vitro transcription was carried out using an mMESSAGE Machine Sp6 kit (Ambion). Synthesized mRNA was purified using an RNAeasy kit (Qiagen).

RT-PCR was carried out using following primers: xSiah1a forward primer (5′-CAAGGCGAACAGGAAGAGGCA-3′) and reverse primer (5′-GCAGGTTGGGCAGCAAGTGAG-3′), xSiah1b forward primer
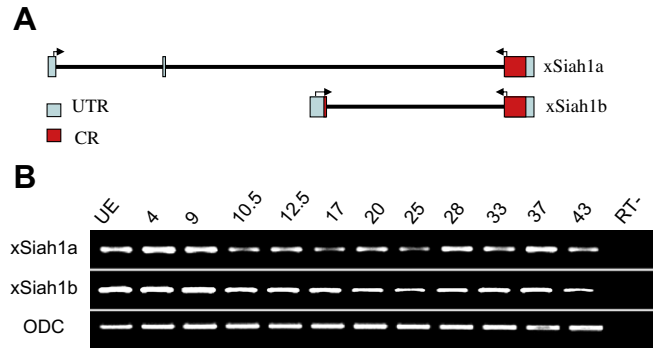
Fig. 2. Temporal expression of xSiah1a and xSiah1b during *Xenopus laevis* embryogenesis. (A) Schematic diagram of xSiah1a and xSiah1b in the genome. Positions of the primers in the Siah1 gene used for RT-PCR are indicated by arrows. UTR, untranslated region; CR, coding region. (B) RT-PCR analysis of xSiah1a and xSiah1b during *Xenopus laevis* embryogenesis. UE, unfertilized eggs, RT−, negative control, ODC, ornithine decarboxylase as a loading control.

(5′-TGACCCGAAGCGAAGGAAAGA-3′) and reverse primer (5′-GCAGGTTGGGCAGCAAGTGAG-3′).

ODC (ornithine decarboxylase) forward primer (5′-TGAATTGATGAAAGTGGCAAGG-3′) and reverse primer (5′-CAGGGCTGGGTTTATCACAGAT-3′).

2.3. Embryo manipulation

Xenopus laevis embryos were obtained from HCG-induced eggs and in vitro fertilization, and were dejellied in 2% cysteine, then

Fig. 1. Sequence comparison of the alternative splicing variants of Siah1 among human and frog. hSiah1L (EAW82726), hSiah1b (NP_001006611), hSiah1a (NP_003022), xSiah1a and xSiah1b.

cultured in 0.1 MBS. In vitro transcribed mRNA and MG132 (Beyotime) were injected into the embryos at the 1 or 2 cell stage. Embryos were fixed in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EDTA and 3.7% formaldehyde) for 1 h, washed in ethanol three times and stored at −20 °C in ethanol. For β-galactosidase staining, embryos were fixed in MEMFA for 20 min at room temperature, and then washed in PBS twice before staining in a buffer containing 1 mg/ml X-gal, 20 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe(CN)_6$ and 2 mM $MgCl_2$. For DAPI whole mount staining, MEMFA fixed embryos

were washed with PBS five times, stained with DAPI for 30 s, and then washed in PBS five times.

2.4. Cell transfection and Western blotting analysis

293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were homogenized with RIPA lysis buffer
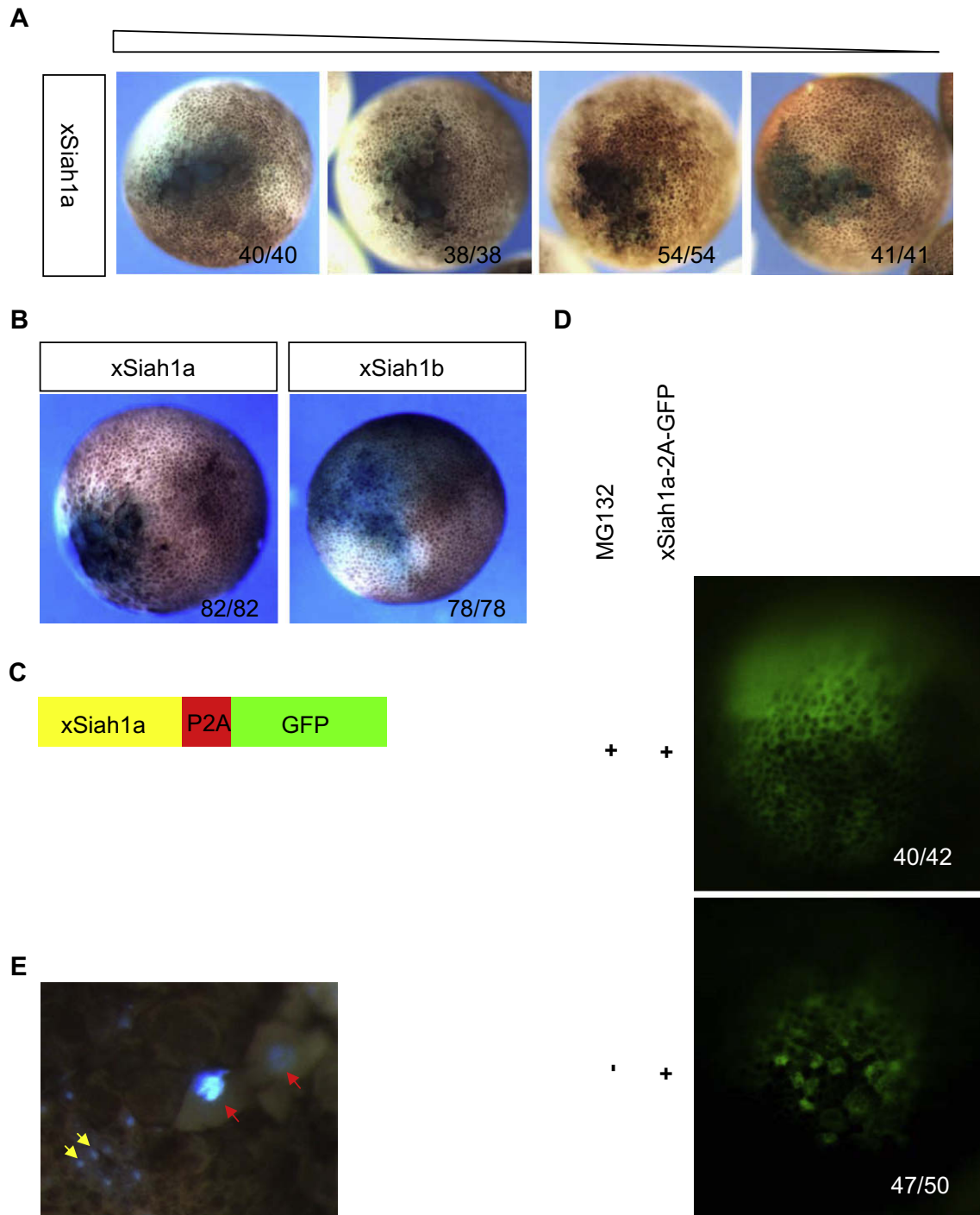
Fig. 3. xSiah1a suppresses *Xenopus* embryo cleavage through a proteosome dependent degradation pathway. (A) Overexpression of xSiah1a suppresses *Xenopus* embryo cleavage in a dose-dependent manner. (B) Two hundred picograms xSiah1a mRNA injection compared to 2 ng xSiah1b mRNA injection. (C) Schematic representation of the construct of xSiah1a-2A-GFP. (D) Expression of XSiah1a was suppressed by MG132, a proteosome inhibitor. MG132 was injected to embryos after injection of 500 pg of xSiah1a-2A-GFP mRNA. (E) Overexpression of xSiah1a formed multinuclear cells. Red arrow shows an enlarged nucleus; yellow arrow indicates a normal nucleus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Beyotime), and protease inhibitor cocktail (Sigma–Aldrich) and 100 mM PMSF were added according to the manufacturer's recommendation. Proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose membrane (Millipore). Anti-flag (Sigma–Aldrich) and anti-GAPDH (Abcam) antibody were diluted as recommended by the suppliers. Detection was by HRP-labeled secondary antibodies and ECL.

3. Results and discussion

3.1. Isolation of xSiah1a and xSiah1b

Searching the *Xenopus* protein database using the human SIAH1 protein sequence found one *X. tropicalis* Siah1 (xtSiah1) and one *X. laevis* Siah1 (xSiah1). Searching the nucleotide sequences in the *X. tropicalis* genome using Blat tools revealed large differences in the 5′ untranslated region. The xSiah1 has an upstream initiation codon and encoded an additional 31 amino acids compared to human Siah1s (Fig. 1). We then obtained the short xSiah1 through prediction based on bioinformatics using xSiah1 sequences, was and designated this as xSiah1a (GU377277), while the long form was designated xSiah1b.

Searching the *X. tropicalis* genome for xSiah1a and xSiah1b using the Blat program revealed that xSiah1a and xSiah1b shared most sequences except the N-terminus. XSiah1b consists of two exons

and encodes a 313 amino acid protein, while xSiha1a consists of three exons and encodes a 282 amino acid protein (Fig. 2A). The sequences are conserved between humans and *Xenopus* (Fig. 1). These splicing variants have also been found in humans, but their functional significance is not known.

Expression analysis during different stages of embryogenesis revealed that mRNAs for xSiah1a and xSiah1b are both maternal and can be detected at all developmental stages analyzed (Fig. 2B). XSiah1a and xSiah1b are abundantly present before the gastrulation stages (Fig. 2B), indicating their potential roles in *Xenopus* embryogenesis.

3.2. Xenopus cleavage was exclusively suppressed by xSiah1a

Siah1 is involved in the cell cycle, apoptosis and proliferation [10,34,35]. Stable overexpression of SIAH1 in a human lung cancer MCF7 cell line showed cell growth inhibition due to perturbed mitosis through degradation of Kid [10,35], a protein needed for chromosome alignment in metaphase and that should be degraded in anaphase to allow for chromosome separation [36]. Gene ablation of Siah1a in mice demonstrated that Siah1a is also required for traversing metaphase during meiosis I of spermatogenesis [28].

In our experiments, overexpression of xSiah1a through mRNA injection was found to suppress cleavage of *Xenopus* embryos in a dose-dependent manner (Fig. 3A) and eventually led to severe
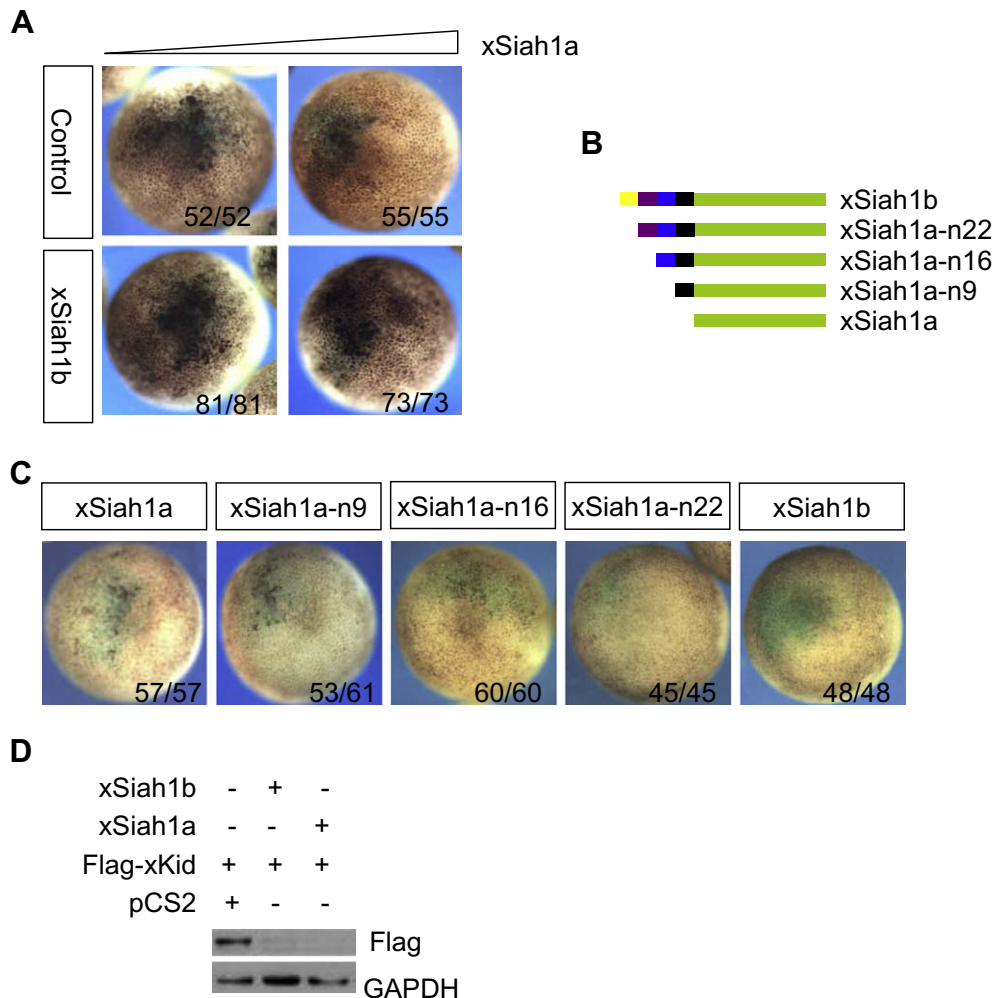
Fig. 4. Analysis of xSiah1b function. (A) Co-injection of xSiah1a and xSiah1b. Two nanograms of xSiah1b mRNA was co-injected with 100 and 200 pg xSiah1a mRNA, respectively. (B) Diagram of the constructs of xSiah1b with different additional N-terminal truncations. (C) Injection of 50 pg of mRNA. (D) Western blot analysis of xKid expression to detect xSiah1a and xSiah1a activity in 293T cells. Equivalent amounts of xSiah1a, xSiah1b and pCS2 (250 ng), were co-transfected with Flag-xKid (1 μg).

apoptosis (data not shown). However, xSiah1b was unable to suppress *Xenopus* embryo cleavage, even at a high dose (Fig. 2B). Overexpression of human SIAH1 in cell lines showed cell growth inhibition and more than 50% cells formed multinucleated giant cells [35]. DAPI staining showed that the enlarged cells suppressed by xSiah1a overexpression had giant nuclei (Fig. 3E). Overexpression of xSiah1a has been suggested to disturb mitosis during *Xenopus* early cleavage. To better trace the mRNA distribution in *Xenopus* embryos, we used 2A peptide [37] to link xSiah1a with GFP (Fig. 3D) so that GFP positive cells would express xSiah1a. Co-injection of MG132, a well-known inhibitor of the proteosome with xSiah1a-P2A-GFP diminished the phenotype of cell cycle suppression (Fig. 3C), indicating that suppression of embryo cleavage by xSiah1a is proteosome dependent. In contrast, injection of 2 ng xSiah1b mRNA into embryos had no significant effect, even at a later tadpole stage (data not shown). This result suggests that xSiah1b is inactivated in *Xenopus*.

Siah1 can form homo- or heterodimers with other Siah1proteins through its C-terminal domain [7]. To determine whether xSiah1b can affect xSiah1a activity by formation of a heterodimer with xSiah1a, a low dose of xSiah1a was co-injected with a high dose of xSiah1b. The effect was the same as injection of xSiah1a alone (Fig. 4A), thus eliminating the possibility that xSiah1b serves as a dominant negative form of xSiah1a in *Xenopus*.

3.3. N-terminal 31 amino acids are responsible for xSiah1b activity in the Xenopus embryo

The underlying mechanism for inactivation of xSiah1b during *Xenopus* embryonic development was further explored by examining the functional role of the additional N-terminal sequences. To determine which amino acids at the N-terminal of xSiah1b are required for suppression of xSiah1 activity, we made a series of constructs of Siah1b with different lengths of the N-terminus (Fig. 4B). Overexpression by mRNA injection demonstrated that xSiah1b activity was correlated with the length of the N-terminus (Fig. 4C). On the other hand, xSiah1a and xSiah1b had the same effect with respect to degradation of substrate xKid when transfected into 293T cells (Fig. 4D), indicating that other protein(s) in the *Xenopus* embryo is (are) likely to bind to xSiah1b through the 31 N-terminal amino acids, resulting in inhibition of xSiah1b. Clearly additional efforts are required to determine the detailed mechanism.

In summary, this study reports, for the first time, the presence of alternative splicing variants of Siah1—xSiah1a and 1b—in early embryogenesis of *X. laevis*. xSiah1b seems to be inactivated in *Xenopus*. xSiah1 activity also is apparently regulated at the posttranscriptional level. Transient overexpression of SIAH1L, which has an additional 15 amino acids in the N-terminal than does SIAH1 (Fig. 1), in a 293 cell line resulted in cell growth arrest [31]. Injection of SIAH1L mRNA at 1.5 ng per egg caused hypodorsalization of *Xenopus* embryos due to downregulation of β-catenin signaling but had no effect on early cleavage during *Xenopus* embryogenesis [9]. Overexpression of xSiah2, which is conserved with xSiah1a in its C-terminal, also did not show suppression of embryo cleavage at 2 ng per embryo (data not shown) but resulted in the development of small eyes in the tadpole stage [32,33]. A recent report suggests that suppression of Siah1 activity can reduce tumor growth and prolong survival of the mice with neoangiogenesis [38]. Our present results may prove useful in providing more insight into the regulation of xSiah1 activity in a *Xenopus* model system.

Acknowledgments

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