TGF-β Signaling Controls Embryo Development in the Parasitic Flatworm Schistosoma mansoni

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Over 200 million people have, and another 600 million are at risk of contracting, schistosomiasis, one of the major neglected tropical diseases. Transmission of this infection, which is caused by helminth parasites of the genus Schistosoma, depends upon the release of parasite eggs from the human host. However, approximately 50% of eggs produced by schistosomes fail to reach the external environment, but instead become trapped in host tissues where pathological changes caused by the immune responses to secreted egg antigens precipitate disease. Despite the central importance of egg production in transmission and disease, relatively little is understood of the molecular processes underlying the development of this key life stage in schistosomes. Here, we describe a novel parasite-encoded TGF-β superfamily member, Schistosoma mansoni Inhibin/Activin (SmInAct), which is key to this process. In situ hybridization localizes SmInAct expression to the reproductive tissues of the adult female, and real-time RT-PCR analyses indicate that SmInAct is abundantly expressed in ovipositing females and the eggs they produce. Based on real-time RT-PCR analyses, SmInAct transcription continues, albeit at a reduced level, both in adult worms isolated from single-sex infections, where reproduction is absent, and in parasites from IL-7R−/− mice, in which viable egg production is severely compromised. Nevertheless, Western analyses demonstrate that SmInAct protein is undetectable in parasites from single-sex infections and from infections of IL-7R−/− mice, suggesting that SmInAct expression is tightly linked to the reproductive potential of the worms. A crucial role for SmInAct in successful embryogenesis is indicated by the finding that RNA interference–mediated knockdown of SmInAct expression in eggs aborts their development. Our results demonstrate that TGF-β signaling plays a major role in the embryogenesis of a metazoan parasite, and have implications for the development of new strategies for the treatment and prevention of an important and neglected human disease.

Introduction

Amongst the Bilateria, transforming growth factor–β (TGF-β) signaling is recognized as playing an essential role in embryogenesis in deuterostomes and in arthropod protostomes, but its role in lophotrochozoan protostomes is unclear [1]. Schistosomes, the causative agents of schistosomiasis, one of the major neglected tropical diseases [2,3], are metazoan parasites that belong to the lophotrochozoan phylum Platyhelminthes.

Components of TGF-β signaling have been molecularly characterized in metazoans throughout the animal kingdom. Activation of this pathway begins at the cell surface when a dimeric ligand binds a complex consisting of types I and II receptor serine/threonine kinases [4]. Upon ligand binding, the constitutively active type II receptor phosphorylates and activates the type I receptor, which then phosphorylates cytoplasmic Smad proteins that translocate to the nucleus, where they mediate gene expression [4]. Components of a functional TGF-β pathway(s), including one type I receptor [5] (Schistosoma mansoni receptor kinase-1 [SmRK1], S. mansoni transforming growth factor–β type I receptor [SmTβRI]), one type II receptor [6,7] (SmRK2, SmTβRII), and three Smads [8–10], have been identified in S. mansoni, with nearly all components localized to either the surface of the worm or reproductive tissues of the female [5–9,11]. Nevertheless, while nearly the entire transcriptome of S. mansoni has been examined with the identification of 163,000 expressed sequence tags (ESTs) [12], a ligand of parasite origin for the TGF-β pathway(s) has remained elusive. This has led to the hypothesis that the ligands for schistosome TGF-β receptors are of host origin [5,13,14], and a suggestion that host TGF-β signaling through SmRK2, plays a role in the pairing of male and female parasites [7].

Sexually mature S. mansoni live within the mesenteric vasculature, where each female produces approximately 300 eggs each day. Transmission of schistosomiasis depends upon the release of parasite eggs from the human host. Development of an immature egg into a mature egg containing a miracidium, the stage of the parasite that invades the intermediate fresh water snail host, occurs outside of the female worm, and takes approximately 5 d. Many of the eggs produced by schistosomes fail to reach the external environment, but instead become trapped in host tissues, where...
pathological changes caused by the immune responses to secreted egg antigens cause disease [15]. Despite the central importance of egg production in transmission and disease, and recent advances in proteomics and transcriptomics [12,16–18], essentially nothing is known of the molecular pathways involved in embryogenesis in schistosomes.

In this study, we describe the cloning and characterization of a *S. mansoni* TGF-β homolog, *S. mansoni Inhbin/Activin (SmInAct)*. Although we found SmInAct to be expressed in adult male and female parasites, and in eggs, the localization of SmInAct expression to the reproductive organs of female parasites focused our attention on the role of this gene in egg production. A role for SmInAct in reproduction was supported by analyses of female parasites recovered from infertile infections, in which we found that SmInAct protein was undetectable. Confirmation of the importance of this TGF-β superfamily member in the reproductive process was obtained from RNA interference (RNAi) studies, in which targeted knockdown of SmInAct in female worms or directly in the eggs that they produce resulted in a marked cessation of embryogenesis.

**Results**

**Cloning and Sequence Analysis of SmInAct**

SmInAct was identified through a *blastn* search of the Wellcome Trust’s Sanger Institute’s *S. mansoni* genome sequence using the C-terminal region of the *Drosophila melanogaster* dActivin sequence. We were unable to identify SmInAct in EST databases regardless of whether we searched using the coding or 3′-untranslated region (UTR) sequences. The 5′ and 3′ ends of SmInAct were amplified via rapid amplification of cDNA ends (RACE) using primers designed from within putative coding sequence and adult *S. mansoni* cDNA as template. The 1.3-kb, full-length SmInAct transcript contains 10 base pairs (bp) of 5′UTR, 808 bp of 3′UTR, and a poly-A tail. The deduced amino acid sequence of SmInAct is 161 residues long and contains many of the molecular hallmarks for a TGF-β, including a putative basic proteolytic cleavage site located at position 32 as RQRR where the bioactive, C-terminal domain (126 amino acids) is enzymatically separated from the N-terminal pro-domain. Nine invariant cysteine moieties, and invariant proline and glycine residues (Figure 1A) essential for the proper dimerization and tertiary structure of a TGF-β homolog, are all predicted in SmInAct. The deduced amino acid sequence of SmInAct contains one putative N-linked glycosylation site at position 110. Within the bioactive domain, SmInAct is 27% identical to both DAF-7 from *Caenorhabditis elegans* and dActivin from *D. melanogaster*, and 29% identical to human TGF-β1 (Figure 1A). Phylogenetic analysis of SmInAct among other TGF-β superfamily members groups this homolog with members of the TGF-β/Activin subfamily (Figure 1B), and further clusters SmInAct phylogenetically with TGF-β homologs from the free-living nematode *C. elegans* (DAF-7) and the parasitic nematodes *Brugia malayi* (Bm-TGH-2) and *Strongyloides stercoralis* (Ss-TGH-1).

**SmInAct Transcript and Protein Expression and Localization**

To determine the expression of SmInAct at the transcript level, real-time reverse transcriptase–polymerase chain reaction (RT-PCR) was performed on cDNA from eggs, adult male parasites, and adult female parasites from mixed-sex infections. As seen in Figure 2A, SmInAct is expressed in all stages tested at relatively similar levels. Western analyses using polyclonal antibodies against recombinant SmInAct were used to determine the protein expression profile of SmInAct. The anti-SmInAct serum recognized a 28-kDa protein in egg antigen extracts and a doublet (32 kDa and 28 kDa) in adult male and female extracts (Figure 2B, lanes 1–3); these bands presumably represent the unprocessed (32 kDa) inactive and processed (28 kDa) active forms of the molecule. The relative molecular weights of the two bands recognized by anti-SmInAct antiserum in parasite extracts are larger than that predicted by the sequence, presumably due to detergent and reducing agent-resistant dimerization, and/or to glycosylation at amino acid 110. Glycosylation plays an important role in the solubility and secretion of other members of the TGF-β superfamily [19,20]. Eggs appear to contain only the lower molecular weight, putatively active form of SmInAct. To localize SmInAct within the parasite, we performed in situ hybridization on sections of adult worms. Anti-sense probes localized SmInAct transcripts to the reproductive tissues of the adult female, with strong signals in the vitellaria and ovary (Figure 2C), whereas in adult males, SmInAct transcripts localized to various subtegumental regions (Figure 2D).

The expression pattern in the female suggested a role for SmInAct in egg production. We focused on this possibility, and reasoned that if this were the case, SmInAct expression might be diminished in unfertile females. In vivo, successful oogenesis requires the presence of male schistosomes [21], and, for reasons that have remained unclear, an intact CD4 T lymphocyte compartment within the host [22]. Therefore, we analyzed SmInAct expression in female parasites from mice harboring single-sex infections, and in parasites from severely lymphopenic interleukin-7 receptor knockout (IL-7R−/−) mice carrying mixed-sex infections, which produce a significant number of dead eggs [23,24]. Real-time RT-PCR demonstrated that SmInAct mRNA levels were significantly decreased,
but not absent, in females from these infections (Figure 2E). Of particular interest, SmInAct protein was undetectable by Western analyses in females from single-sex infections as well as from infections of IL-7R/C0/C0 mice (Figure 2B).

While the localization of SmInAct transcripts to the male subtegumental region is not immediately informative in terms of function in the male, we nevertheless noted that male parasites recovered from infertile infections in IL-7R/C0/C0 mice were similar to female parasites in terms of transcriptional and post-transcriptional regulation of SmInAct expression (Figure 2B and 2F). Moreover, this was also the case for male parasites recovered from male single-sex infections (Figure 2B and 2F).

### RNAi-Mediated Knockdown of SmInAct Expression

To gain a better understanding of the function of SmInAct and the signaling pathway it activates, this TGF-β homolog was targeted for knockdown via RNAi [25–27]. Pairs of adult males and females recovered from infected mice were soaked in double-stranded RNA (dsRNA) corresponding to SmInAct (1 μg/ml) or an irrelevant control dsRNA (luciferase) for 1 wk in vitro, followed by RNA extraction and real-time RT-PCR analyses. SmInAct dsRNA-treated worms showed a consistent

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**Figure 1.** SmInAct Is a Member of the TGF-β/Activin Subfamily

(A) ClustalW alignment of the C-terminal domain of the SmInAct protein with three other members of the TGF-β/Activin subfamily. Amino acids identical to the SmInAct sequence are shaded gray. Numbers to the right indicate position of the last amino acid in the row within each respective full-length sequence. Stars indicate invariant amino acid residues in TGF-β homologs.

(B) Phylogenetic dendrogram demonstrating that SmInAct is a member of the TGF-β superfamily. SmInAct (red) is shown clustering among members of the TGF-β/Activin subfamily (solid line), and not with members of the BMP/growth differentiation factor subfamily (dashed line). Conserved residues in the C-terminal region of each homolog (final 94–106 amino acids) were used in the analysis. Percentages at branch points are based on 1,000 bootstrap runs.

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and significant decrease in SmInAct expression of >40% when compared to SmInAct expression in worms soaked in the irrelevant control dsRNA (Figure 3A). No consistently significant difference in the numbers of eggs produced by control versus SmInAct dsRNA-treated worm pairs was observed, suggesting that SmInAct is not important for egg production per se. However, in examining these cultures, we noted that eggs produced by SmInAct dsRNA–treated parasites failed to develop (unpublished data). To specifically address the role of SmInAct in egg development, we treated eggs directly with SmInAct dsRNA. Approximately 20% of eggs laid by adult parasites during the first 2 d of in vitro culture will develop over the ensuing 5 d to contain miracidia [28], with a typical progression of development through six
stages illustrated in Figure 3B. Therefore, eggs produced by worm pairs for the first 2 d ex vivo were collected and soaked in dsRNA (1 μg/ml) corresponding to SmInAct or an irrelevant dsRNA for 5 d, and their development was scored. Relative to eggs soaked in an irrelevant dsRNA, where ~20% of the eggs developed through stage 6, eggs treated with SmInAct dsRNA aborted development at stage 2 (Figure 3C and 3D). An absence of SmInAct transcripts (Figure S1), and a nearly 10-fold decrease in SmInAct protein (Figure 3E), were associated with the failure of SmInAct dsRNA–treated eggs to develop. This phenotype was not observed when eggs were treated with dsRNA corresponding to luciferase, a sequence not encoded in the schistosome genome (Figure 3C and 3D), or to S. mansoni cathepsin B1 (SmCB1), a cathepsin B detectable in eggs (Table 1).

**Discussion**

Multiple components of a TGF-β signaling pathway have been characterized in S. mansoni, but a ligand of parasite origin for the pathway has remained elusive. Additionally, while functions in host–parasite interactions have been proposed based on the expression of receptors on the parasite surface, and on the responsiveness of the parasite receptors to host TGF-β [5–7,14], the function that TGF-β signaling plays in S. mansoni has remained unclear. In this study, we report the expression of SmInAct, a TGF-β–like
ligand in the parasitic flatworm *S. mansoni*, the production of which is coupled to the reproductive potential of the worms. We provide evidence that SmInAct plays a crucial role in embryogenesis.

Understanding of the developmental processes regulated by TGF-β in invertebrates is based largely on data from the model organisms *D. melanogaster* and *C. elegans*. Decapentaplegic, a bone morphogenetic protein (BMP)-like homolog in *D. melanogaster*, acts as a morphogen by determining cell fate along the dorsal–ventral axis in a gradient-dependent manner [29]. Also in *D. melanogaster*, a type I receptor, *baboon*, stimulates cellular proliferation and is essential for normal embryonic development [30]. Presumably, SmInAct could be fulfilling functions in the schistosome egg analogous to these known roles for decapentaplegic and/or baboon. None of the three characterized TGF-β homologs in *C. elegans* are important for patterning or growth of the embryo [31–33]; however, two TGF-β homologs have yet to be examined (tig-2 and Y46E12BL.1), and, intriguingly, serial analysis of gene expression (SAGE) tags for both homologs have been found in the *C. elegans* embryo [34]. Like the other *C. elegans* TGF-β homologs that are resistant to RNAi affects, tig-2 and Y46E12BL.1 have no phenotype in genome-wide RNAi screens [35,36]; therefore, direct mutagenesis will likely be required to determine the function of these genes.

The identification of SmInAct, a TGF-β superfamily member, as a key component of egg development in *S. mansoni*, a member of the Platyhelminthes, the earliest branch of the Bilateria [37], underscores the central role played by this pathway in embryogenesis. While one type I and one type II TGF-β receptor have been characterized for *S. mansoni*, there appears to be at least three type I receptors and two type II receptors present in the genome based on a preliminary blast search for homologs. It will be important to delineate which of the *S. mansoni* type I and type II TGF-β receptors are involved in SmInAct signaling and to identify the Smads important for transmitting the signal induced by this growth factor. Furthermore, identifying the genes regulated by SmInAct signaling will provide information regarding the precise function that this growth factor serves in egg maturation, as well as the functions the pathway may serve in other life stages of the parasite, including the adult male.

SmInAct protein was not detectable in infertile females recovered from single-sex infections or from IL-7R⁺ mice, despite the fact that these parasites contained SmInAct transcripts (although at lower levels than in fecund parasites). This strongly indicates that SmInAct is both transcriptionally and post-transcriptionally regulated by worms of the opposite sex as well as by signals from the host. It is well established that parasites recovered from hosts lacking CD4⁺ T cells are developmentally stunted and produce significantly fewer fertile eggs than those recovered from mixed-sex infections of immunocompetent hosts. Translation of SmInAct mRNA is the first identified molecular process downstream of the effect of the host immune system on schistosome development [22–24], and as such, could open the way towards an increased understanding of this unusual feature of schistosome biology. The finding that the production of SmInAct in males is under the same constraints as in females is curious and perhaps indicates an additional function(s) for SmInAct in *S. mansoni*. We are unaware of a link between the site of expression of SmInAct in the male schistosome and reproductive events, and further work is required to elucidate the function of SmInAct in male worms.

In other settings, the uncoupling of transcription and translation is linked to the activation of the integrated stress response [38–41]. This mechanism, conserved in eukaryotes, reprograms cells to conserve energy in response to stress signals such as amino acid deficiency and oxidative stress by restricting the translation of transcripts requiring an active translation initiation complex [38–41]. Limited cellular energy is then used for the expression of genes necessary to maintain cell viability [42]. In this context, parasites in single-sex infections and in mice lacking CD4⁺ T cells may be considered stressed due to the lack of signals received from the opposite sex and immunocompetent host, thereby restricting the translation of non-essential transcripts. SmInAct protein expression may be considered expendable considering the role it plays in embryogenesis rather than in crucial cellular functions linked to the survival of the adult worm. A more thorough investigation of the *S. mansoni* homologs of translation factors involved in the stress response and of the regulation of other transcripts and protein expression will be required to evaluate this possibility.

Post-transcription regulation of eukaryotic transcripts is controlled in part by the 3'UTR [43]. This region can bind elements (including microRNAs and proteins) that inhibit the translation and/or decrease mRNA stability. For example, 3'UTRs of several mammalian cytokines contain adenosine- and uridine-rich elements (AREs) that bind ARE-binding proteins (ARE-BPs) (reviewed in [44]). The binding of ARE-BPs to these transcripts causes either rapid decay or inhibits their translation. While AREs are somewhat divergent in sequence, they often contain the consensus “AUUUA” and

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### Table 1. Comparison of the Development of Eggs Soaked in SmInAct dsRNA versus SmCB1 dsRNA

| dsRNA Treatment | Developed Eggs* (number) | Undeveloped Eggs* (number) | Percent Developed | Significancea |
|-----------------|--------------------------|---------------------------|------------------|--------------|
| Control         | 71                       | 342                       | 17.2             | p ≤ 0.0001   |
| SmInAct         | 49                       | 721                       | 6.4              | p = 0.446    |
| SmCB1           | 124                      | 520                       | 19.3             |              |

*The number of eggs that reached stage 6 of development (Figure 3B).

bThe number of eggs that failed to develop past stage 2 (Figure 3B).

*p-values based on Yates' chi-square between SmInAct or SmCB1 dsRNA-treated eggs and control.

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are found in a uridine-rich environment. Interestingly, the long 3' UTR of SmInAct has two exact repeats of "UUUCAAUUA" that contain the consensus "AUUUA" ARE (underlined). Furthermore, the 3' UTR of SmInAct is U-rich (45% uridines). It will be interesting to determine whether these repeats, or other regions of the long 3' UTR, play a role in the post-transcriptional regulation of SmInAct expression. It is of interest when considering the relationship of schistosomes with their mammalian hosts to note that in other systems, TGF-β superfamily members have been shown to function across phylum boundaries [45,46]. For example, the Drosophila BMP homologs DPP and 60A are able to induce bone development when injected into the skin of rats [45], and mammalian BMP-4 can rescue Drosophila DPP mutants [46]. Consequently, we believe that it is feasible that SmInAct could act as a ligand to initiate signaling in host cells. It is clear that proteins produced by eggs have distinct immunomodulatory functions [47], and SmInAct could conceivably participate in these effects if secreted/lacreted from the schistosome egg. Our identification of SmInAct as a cytokine that is molecularly conserved between host and parasite, coupled with the description of an effective method for altering gene expression in the schistosome egg, allows these and other issues to now be addressed. Despite recent advances in vaccine design [48], a solution for schistosomiasis remains an elusive goal. Current attempts to control schistosomiasis depend on repeated administration of one drug, praziquantel, with no replacements waiting in the wings should resistance develop. Understanding how schistosome eggs develop could provide targets for intervention in the schistosome life cycle and for blocking disease progression.

Materials and Methods

Parasites and animals. The Puerto Rican/NMRI strain of S. mansoni was used in all experiments. Adult schistosomes were recovered by hepatic-portal perfusion from C57BL/6 female mice or B6 IL-7R−/− (The Jackson Laboratory, http://www.jax.org) that had each been percutaneously exposed to ~60 cercariae 8 wk earlier. Adult parasites and eggs laid were maintained in vitro in M199 (Gibco, http://www.invitrogen.com), 10% fetal calf serum, 1% Antibiotic/Antimycotic (Gibco), and 1% HEPES in a 37°C 5% CO2 atmosphere as previously described [11,28].

Isolation of full-length SmInAct cDNA from S. mansoni. The C-terminal, translated region of the Drosophila activin homolog dActivin (amino acids 565–669) was used to search the Wellcome Trust’s Sanger Institute’s S. mansoni genome assembly using the tblastn algorithm. A contig (0020320) with significant similarity to dActivin was detected using ECL reagents as per manufacturer’s instructions [49]. DIG-labeled sense and anti-sense transcripts were generated using Roche's DIG DNA labeling mix (http://www.roche.com) as per manufacturer’s instructions with T7-tagged amplicons as template (sense: forward 5'-TTGACATCCTTGGGGACAAC-3' and reverse 5'-TTGACATCCTTGGGGACAAC-3'). PCR efficiency (E) was determined for both primer sets by plotting cycle thresholds from a 10-fold serial dilution of cDNA and inputting the slope in the equation E = 10^-1/slope. For expression analyses, quantification of SmInAct transcripts relative to 28S rRNA was calculated using:

\[ \text{Ratio} = \frac{E_{\text{SmInAct}}}{E_{\text{28S}}} \]

where E_{SmInAct} is the PCR efficiency of the reference gene, E_{28S} is the PCR efficiency of target gene, and CT is the cycle threshold. For analysis of RNAi-induced knockdown, quantification of SmInAct transcript relative to paramyosin (paramyosin) primers were: forward 5'-CGGAGGTCCTGCGAGATG-3' and reverse 5'-GCAGTGGCAAATTCGTT-3'. The amplified product was cloned into the expression vector PET28a+ (Novagen, http://www.cellsignal.com) and sequenced.

Western analyses. Drosophila eggs, 72–84 h post oviposition, were used to detect SmInAct expression. Eggs were double stained with rabbit antibodies, while an affinity purified HRP-conjugated horse anti-rabbit antibody (Jackson ImmunoResearch, http://www.jacksonimmuno.com) was used to detect bound antibodies. Affinity purified IgG (Cell Signaling Technology, http://www.cellsignal.com) was used to detect bound rabbit antibodies, while an affinity purified HRP-conjugated horse anti-rabbit IgG (Cell Signaling Technology) was used to detect the anti-paramyosin monoclonal antibody. The secondary antibodies were detected using ECL reagents as per manufacturer’s instructions (GE Healthcare, http://www.gehealthcare.com).

In situ hybridization. Localization of SmInAct in 5-μm sections of adult S. mansoni was as previously described [49]. DIG-labeled sense and anti-sense transcripts were generated using Roche's DIG RNA labeling mix (http://www.roche.com) as per manufacturer’s instructions with T7-tagged amplicons as template (sense: forward 5'-TATACGACCTACATAGGTTGTACTCCAAAAAGGTGTGGATTTGTAACAG-3' and reverse 5'-ATAATAGTGAATATTGTTGTTCCA-3'). The unrooted phylogenetic tree was drawn using amino acids within the conserved C-terminal domain of SmInAct, and known TGF-β superfamily members and distances were drawn using the Dayhoff Pam matrix and neighbor-joining algorithm in the PHYLIP software package developed by J. Felsenstein, University of Washington, Seattle, Washington, United States (http://evolution.genetics.washington.edu/phylip.html). Percentages at branch points are based on 1,000 bootstrap runs.

Real-time RT-PCR. Total RNA was extracted from parasites using Qiagen's RNeasy Mini kit (http://www.qiagen.com), and contaminating genomic DNA was removed by on-column DNAase treatment. Turbo DNA-free endonuclease (Ambion, http://www.ambion.com). First-strand cDNA was synthesized using 500 ng of RNA, SuperScript II reverse transcriptase (Invitrogen), and oligo dT as a primer. RT-PCR controls were performed to confirm the absence of genomic DNA (unpublished data).

SmInAct transcript levels in egg and adult stages were quantified relative to 28S rRNA using Applied Biosystems' 7500 real-time PCR system and SYBR green PCR Master Mix (Applied Biosystems, http://www.appliedbiosystems.com). Total reaction volume was 10 μl with 0.8 μl of each primer. 2 μl of first-strand cDNA was used, and 0.5 μl of cDNA as template (or water as a negative control). SmInAct primers were: forward 5'-AACCTGTGTGCTACACCTC-3' and reverse 5'-AATACAAAGCACACCTCCTAAC-3'. 28S rRNA primers were: forward 5'-CCAGCAAATCAGATGGTAGA-3' and reverse 5'-TTGACATCCTTTGGGGACAAC-3'. PCR efficiency (E) was determined for both primer sets by plotting cycle thresholds from a 10-fold serial dilution of cDNA and inputting the slope in the equation E = 10^-1/slope. For expression analyses, quantification of SmInAct transcript relative to 28S rRNA was calculated using:

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where E_{SmInAct} is the PCR efficiency of the reference gene, E_{28S} is the PCR efficiency of target gene, and CT is the cycle threshold. For analysis of RNAi-induced knockdown, quantification of SmInAct transcript relative to paramyosin (paramyosin) primers were: forward 5'-CGGAGGTCCTGCGAGATG-3' and reverse 5'-GCAGTGGCAAATTCGTT-3'. The amplified product was cloned into the expression vector PET28a+ (Novagen, http://www.cellsignal.com) and sequenced. Dissociation curves were generated for each real-time RT-PCR to verify the amplification of only one product.

Recombinant SmInAct expression, antisense production, and Western analyses. EcoRI-digested SmInAct and XhoI (1:1000) were used to amplify the C-terminal bioactive region of SmInAct (forward 5'-GAATTCTGATTTACAAAGGATGTA-3' and reverse 5'-CCGCTGGATCATACAACTACAGCATTACA-3'). The amplified product was cloned into the expression vector PET28a+ (Novagen, http://www.cellsignal.com) and sequenced.

Dissociation curves were generated for each real-time RT-PCR to verify the amplification of only one product.

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conjugated anti-DIG antibody (Roche), and visualized using NBT (337.5 µg/ml) and BCIP (175 µg/ml) in 0.1M Tris-HCl, 0.1M NaCl, 0.05 M MgCl2. Worm sections were photographed using a Leica DMRB microscope and DC500 camera (Leica, http://www.leica.com).

dsRNA synthesis and RNA interference. dsRNA was synthesized using the T7 Megakit script (Ambion) as per manufacturer's instructions. T7-tagged primers were used to generate a 381-mer SmInAct-dsRNA template encompassing the active ligand domain (forward 5'-TAATACGACTCACTATAGGGGCGATCATTAAGG-3', reverse 5'-TAATACGACTCACTATAGGGAACTTACAGCACCTATAAAACAA-3'). Luciferase and SmCB1 dsRNAs (negative controls) were generated as described [25]. For dsRNA treatment of worms, five adult pairs were cultured in the presence of 1 µg/ml dsRNA for 7 d with medium and dsRNA changes occurring every other day. For dsRNA treatment of eggs, five adult pairs were cultured as above (in the absence of dsRNA) for 2 d, worms were removed, and dsRNA was treated at 1 µg/ml. Eggs were photographed using a Leica DMRB microscope and DC500 camera.

Statistical analyses. Student t-test was used for statistical analyses of dsRNA-induced knockdown of SmInAct expression, change in expression of SmInAct in single-sex and IL-7R-/- mice, and egg developmental phenotypes (control versus SmInAct dsRNA). Chi-square analyses were used to test the statistical significance of the egg developmental phenotype. The Yates correction was applied because we specified only two categories: undevolved and developed (Table 1).

Supporting Information

Figure S1. SmInAct Transcript Is Not Detectable in SmInAct dsRNA-Treated Eggs

SmInAct mRNA levels in eggs treated with SmInAct dsRNA or control dsRNA for 5 d were measured using real time RT-PCR using paracymosin as a reference gene for expression. dsRNA treatment is indicated on the x-axis. Data are presented as the mean fold change in SmInAct expression. N/D = not detected. In this experiment, paracymosin mRNA was detectable in the SmInAct dsRNA–treated eggs. However, in most experiments in which eggs were treated with SmInAct dsRNA, it was not possible to recover mRNA from which reference transcripts could be detected by RT-PCR. Eggs treated with control dsRNA always yielded high quality mRNA.

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