Journal of Scientific Research & Reports
6(7): 504-513, 2015; Article no.JSRR.2015.175
ISSN: 2320-0227

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Analysis of *Xenopus laevis* RECK and Its Relationship to Other Vertebrate RECK Sequences

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Authors' contributions

This work was carried out in collaboration between all authors. Author JAW designed and carried out experiments, analyzed data, and wrote the initial draft of the manuscript. Author MAN aided with embryo work and in situ hybridization. Author MC aided with RNA extraction and PCR analysis. Author SD provided funding and facilities, as well as writing the final draft of the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JSRR/2015/17044
Editor(s):
(1) Mihaly Mezei, Department of Structural and Chemical Biology, Icahn School of Medicine at Mount Sinai, USA.
Reviewers:
(1) Anonymous, Turkey.
(2) Anonymous, Greece.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=968&id=22&aid=8751

Original Research Article

Received 24th February 2015
Accepted 30th March 2015
Published 10th April 2015

ABSTRACT

Aims: Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) is a membrane-anchored protein that regulates cell migration by both inhibiting matrix metalloproteinases and modulating cellular pathways. Our study is the first to clone and examine the expression pattern of *reck* during early *Xenopus laevis* development.
Study Design: Expression and sequence analysis.
Place and Duration of Study: Department of Biology, University of Western Ontario, 2 years.
Methodology: *Xenopus laevis* RECK cDNA was sequenced and used to make probes to analyze RECK temporal and spatial expression using PCR and *in situ* hybridization respectively. The *X. laevis* sequence was also compared to a breadth of vertebrate and a few invertebrate RECK amino acid sequences to ascertain conserved regions and domains that may be present in all vertebrate and other animal species.
Results: Semi-quantitative PCR, whole mount in situ hybridization, and immunohistochemistry

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were used to examine the expression pattern of *reck* during early *X. laevis* development. *reck* expression is low during gastrulation but increases during neurulation and throughout organogenesis. RECK is also present in neural and vascular structures of the *X. laevis* embryo, consistent with its role in other vertebrates. The predicted full-length *X. laevis* RECK amino acid sequence was found to be highly conserved with other RECK proteins. All RECK proteins are found to have 3 inhibitory Kazal motifs, where the first is the most conserved in all animals. Furthermore, all RECK proteins contain 6 conserved cysteines in each of 5 knot motifs, with the 3rd knot being the most highly conserved. Knot domains were more conserved amongst birds, than amongst herptiles and mammalians.

Conclusion: The conservation of expression and sequence suggest that RECK has a very conserved function in vertebrates and possibly all animals.

Keywords: RECK; development; ECM remodeling; Xenopus laevis; MMPs.

1. INTRODUCTION

During development the extracellular matrix (ECM) undergoes extensive remodeling to allow needed large-scale cell movements to occur. Degradation of the ECM occurs through a large family of extracellular proteases called matrix metalloproteinases (MMPs). Aberrant regulation of MMPs is associated with developmental defects and therefore, a delicate balance between the levels of MMPs and their endogenous inhibitors is crucial for proper development. REversion-inducing Cysteine-rich protein with Kazal motifs (RECK) has recently been discovered to play a role in regulating MMP activity [1].

The *reck* gene encodes a glycosylphosphatidylinositol (GPI)-anchored protein with three serine-protease inhibitor-like domains (Kazal motifs) and five repeats of a putative cysteine knot motif. The N-terminus contains a signal peptide sequence and the C-terminus contains a GPI-anchoring signal sequence [1]. All mammalian *reck* genes that have been sequenced are highly conserved at the amino acid level and share the domains mentioned above. Mammalian RECK proteins are 971 amino acids in length and mass of approximately 110 kDa [1]. *Reck* has also been sequenced from a variety of non-mammalian vertebrate and invertebrate species and has been found to be evolutionarily conserved, however, *Xenopus laevis reck* has yet to be characterized. Indeed while numerous putative *reck* genes can be predicted as many organism genomes are being sequenced, proportionally reptile and amphibian (herptile) *reck* sequences are incomplete, of poor quality, or lacking in various phylogenic databases.

RECK was first described as a tumor suppressor protein due to its anti-invasive properties. Numerous *in vitro* studies showed that RECK can inhibit the proteolytic activity of three types of MMPs: MMP-2, -9, and MT1-MMP [1,2,3]. Since RECK functions by inhibiting MMPs, its presence during development would suggest that it plays important embryonic roles as well. Vertebrate RECK importance was demonstrated in knockout and knockdown studies in mice and fish respectively. Oh et al. [2] revealed that RECK-deficient mice died halfway through embryogenesis with halted vascular development. In addition, RECK knockdown in zebrafish resulted in impaired vascular integrity and lack of dorsal root ganglia (DRG) formation [4]. RECK has also been shown to be important during mouse secondary palate development [5] and mouse forelimb development [6]. More recently RECK has also been shown to modulate cell-migratory signaling pathways, independent of its MMP inhibitory role [7,8]. As the developmental processes of neural development and vascular branching both involve RECK and tightly controlled cell migration and ECM remodeling events in mice and fish, this implicates the importance of RECK as a key embryonic regulator in other vertebrates. While much *in vitro* analysis of RECK function is currently being carried out, there is limited *in vivo* information regarding this protein, particularly in non-mammalian vertebrates. In this study, we cloned and analyzed the sequence of *X. laevis reck,* and examined its expression pattern during development temporally using semi-quantitative PCR, and spatially using whole mount *in situ* hybridization and immunohistochemistry. *X. laevis reck* expression in frog in consistent with its vascular and nervous system roles seen in mouse and fish. Further, the newly identified *X. laevis reck* sequence was used to analyze the sequence conservation of this protein in numerous animals.

2. MATERIALS AND METHODS

2.1 Animal Care and Rearing

Adult *X. laevis* were purchased from Xenopus 1, Inc (Dexter, MI). Animal rearing and fertilizations were carried out as previously described [9]. Developing embryos were staged according to Nieuwkoop and Faber [10]. Animals were housed and treated in accordance with UWO and CCAC guidelines.

2.2 Cloning *X. laevis* Reck

Since the *X. laevis reck* sequence was not available online at the beginning of this project, the *X. tropicalis* database was used instead as the sequence of this closely related species is better annotated. Based on the putative *X. tropicalis reck* sequence [XM_002938937.2], forward (5'-ATGTGTCGTGATGTATGTGA-3') and reverse (5'-TGGCGACAAAGAATACACCA-3') primers were engineered flanking the predicted full-length coding region of the *reck* gene. Total RNA was isolated from adult *X. laevis* lung tissue using an RNeasy Kit (Qiagen) according to manufacturer's instructions. First-strand cDNA synthesis and PCR were performed on total RNA using qScript Reverse Transcriptase (Quanta Biosciences) and Kapa Hi-Fi Taq PCR Kit (Kapa Biosystems) according to manufacturer's instructions.

The putative *X. tropicalis reck* sequence was only partial, and lacked the signal peptide sequence at the 5' end and the GPI-anchoring signal sequence at the 3' end of the gene. Therefore, the nested gene specific primers for the 5' end (outer: 5'-ATGTGTCGTGATGTATG TGAACAG-3' and inner: 5'-CCTCCCTTAGTTCAGTGTGT-3') and 3' end (outer: 5'-CCTGCAATTCCTGTCACAG-3' and inner: 5'-CAACATGTCTCTGTACCTCAG-3') were also generated to isolate the missing domains of *X. laevis reck* using the FirstChoice RLM-RACE Kit (Life Technologies) according to manufacturer's instructions. The amplicons were cloned into the pCR 4-TOPO vector (Life Technologies) and sequenced at the DNA Sequencing Facility (Robarts Research, London, ON, Canada). The final amplification of the full-length coding region of *X. laevis reck* was done using SuperTaq Plus (Life Technologies).

2.3 Sequence Analysis

The *X. laevis reck* sequence obtained from PCR was translated using the ExPASy Bioinformatics Resource Portal online Translate tool (http://web.expasy.org/translate/). This *Xenopus laevis reck* sequence has been submitted to genbank as [AIZ00509.1]. Other well annotated, full length RECK protein sequences along with the accession numbers used for comparison were from the following species: human *Homo sapiens* [NP_066934.1], chimp *Pan troglodytes* [XP_520575.2], rhesus monkey *Macaca mulatta* [XP_001083599.1], dog *Canis lupus familiaris* [NP_001002985.1], cow *Bos taurus* [NP_001179394.1], mouse *Mus musculus* [NP_057887.2], tiger *Panthera tigris altaica* [XP_007076663.1], alpaca *Vicugna pacos* [XP_006204234.1], little brown bat *Myotis lucifugus* [XP_006102759.1], 13 lined squirrel *Ictidomys tridecemlineatus* [XP_005326326.1], common shrew *Sorex araneus* [XP_004600290.1], fresh water dolphin *Lipotes vexillifer* [XP_007456041.1], platypus *Ornithorhynchus anatinus* [XP_007661391.1], tasmanian devil *Sarcophilus harrisii* [XP_003761399.1], chicken *Gallus gallus* [XP_418897.4], penguin *Pygoscelis adeliae* [XP_009322770.1], bald eagle *Haliaeetus leucocephalus* [XP_010575445.1], ibis *Nipponia nippon* [XP_009473764.1], hummingbird *Calypte anna* [XP_008500730.1], pike *Esox lucius* [XP_010884005.1], croaker *Larimichthys crocea* [XP_010749852.1], zebra fish *Danio rerio* [XP_009295477.1], sole *Cynoglossus semilaevis* [XP_008305649.1], alligator *Alligator sinensis* [XP_006019052.1], soft shell turtle *Pelodiscus sinensis* [XP_006113101.1], frog *Xenopus tropicalis* [XP_002938983.2], ghost shark *Callorhinchus milii* [XP_007896317.1], coelacanth *Latimeria chalumnae* [XP_006005911.1], fruit fly *Drosophila melanogaster* [NP_001261853.1], mosquito *Anopheles gambiae* [XP_311364.4].

Full-length RECK amino acid sequences were aligned using Clustal Omega Multiple Sequence Alignment web software at the European Bioinformatics Institute (EBI) site (http://www.ebi.ac.uk/Tools/msa/clustalo/) and given a score based on similarity. The signal sequence, structural domains of RECK (cysteine knots and Kazal motifs) and GPI anchor were determined using the SMART web software (at http://smart.embl.de/). All analyses were performed using default program settings.

2.4 Expression Analysis

2.4.1 Semi-quantitative PCR

Total RNA was isolated from embryos at the indicated stages using an RNeasy Kit (Qiagen). First-strand cDNA synthesis and PCR were performed on total RNA using qScript Reverse Transcriptase (Quanta Biosciences) and KapaTaq (KAPA Biosystems) according to manufacturer's instructions. The amplification program consisted of 95ºC for 2 minutes, then cycling of 95ºC for 30 seconds, 60ºC for 30 seconds, and 72ºC for 30 seconds for 30 cycles (in the exponential phase). The gene-specific primers used to amplify a fragment of the *reck* gene (147 bp) were: forward (5'-GGATGTTTACAGGTCTACCC-3') and reverse (5'-GGCTCTGTTCCTCCAAAGAT-3'). *Reck* PCR products were quantified against *ef1α* (268 bp). The gene-specific primers used for *ef1α* were: forward (5'-CAGATTGGTGCTGGATATGC-3') and reverse (5'-CTAGGAGTCATCAAGGCAGT-3'). Data represents an average of three biological replicates.

2.4.2 Whole mount in situ hybridization

A fragment of the RECK gene (483 bp) was amplified using the following primers: forward (5'-GGATGTTTACAGGTCTACCC-3') and reverse (5'-ATGGACACTGCCAGGATGTT-3') and cloned into the pGEM-T vector (Promega). Digoxigenin (DIG)-labeled antisense RNA probes were generated from the *reck* cDNA clone using SP6 RNA Polymerase and the DIG RNA Labeling Kit (Roche) according to manufacturer's instructions. DIG-labeled antisense control probes against cardiac troponin 1, a heart-specific marker, and negative no-probe controls were used. Whole mount in situ hybridization was performed as previously described [11]. For imaging, embryos were cleared in a 2:1 mixture of benzyl benzoate:benzyl alcohol to observe internal structures. Embryos were visualized using an Olympus SZX9 microscope system and images were captured using a Nikon Coolpix 990 camera. Images were processed using Adobe Photoshop CS5 Extended, version 12.0.

2.5 Immunohistochemistry and Fluorescence Microscopy

Embryos were fixed in 3.7% paraformaldehyde in 1x PBS for 2 hours at room temperature. Fixed embryos were sent to the Molecular Pathology Core Facility at Robarts Research Institute (London, ON) for paraffin embedding and sectioning. 10 µm de-waxed sections were probed with primary antibody (mouse anti-RECK, Santa Cruz) followed by fluorescently-labeled secondary antibody (Alexa Fluor goat anti-mouse IgG, Life Technologies). Slides were mounted using ProLong Gold Antifade Mountant with DAPI (Life Technologies). Slides were visualized using a Leica automated inverted microscope.

3. RESULTS AND DISCUSSION

3.1 Sequence Analysis

Since no record existed to date, we began our study by cloning and sequencing *X. laevis reck*. The full-length coding region of *X. laevis reck* was amplified from adult lung tissue RNA by PCR using primers designed against the *X. tropicalis reck* sequence [XM_002938937.2] and 5' and 3' RACE. Our putative *X. laevis reck* full-length mRNA sequence was submitted to GenBank with accession # [AIZ00509.1]. The derived *X. laevis reck* had a full length coding region of 2,904 bp and a predicted protein length of 967 amino acids (Fig. 1). SMART domain analysis of *X. laevis* RECK was used to identify the hallmark protein domains characteristic of other RECK proteins (Fig. 1a). This included an N-terminal signal sequence and a C-terminal GPI anchor signal – both of which are removed in the mature protein. Also present were 5 characteristic cysteine knot motifs and 3 Kazal inhibitory domains. We then analyzed the predicted *X. laevis* RECK protein sequence along with RECK sequences from both vertebrate and insect species. Clustal Omega was used to align various RECK protein sequences, and conserved amino acids that were identical for all were counted (Fig. 2). Conserved RECK amino acid sequences were examined in all 31 animals selected in this study. RECK proteins demonstrated a 33% and higher amino acid identity when compared across all the vertebrates and insects. Vertebrate RECK amino acids were 58% and higher identical, with fish, herptile and mammalian RECK being 68, 75 and 85% and higher identical within each group. Bird RECK amino acids showed the most identity with a conservation of 93% and higher.

Hallmark domains of RECK were further analyzed to determine conservation within RECK proteins that may reveal important domain functions. A hallmark of all RECK proteins is

A Predicted *X.laevis* RECK amino acid sequence

MKRRSGDVMHAAVRVMSASASCLGLLGIWFFLLIFLLLEAADASCCNQAKDNLMCRDVCEQILSSKSESRIKHLLL
RAPDYCPTSMIDVWTCINSSLPGVSKKSEGWVGLGCCELAIAVECRRACKQASSQNDISKSCRKQYETALISCINR
NEMGSVCCSYAGRHTNCREYCQAIFRTDSSPGPSQIKAVENFCASISPPLVQCVNNYTQSYPMRNPVDSLYCCDRA
EDPQCQSACKRILMSKKTEPEIVDSLSEGCTKPLPQDPLWQCFLESSRTVHSGVNIVPPPSAGLDGAELHCCSKAN
SSNCRDLCTKLYSTSWGNTQVWQEFEFACEYNPLEAPMLTCLADVREPCQLGCRNLTFCTNFNNRPTELFRSCNVQ
SDQGAMNDMKLWEKGSIKMPFMNIPVLDIKKCHPEMWKAIACSLQIKPCHSKSRGSIICKTDCVEILTKCGDHSRF
PESHTAESICELLSPSDENEDCIPLDTYLRSSPLDNAIEEVTHPCNPNPCPANHLCEVNRKGCLPGEPCLPYFCSQ
GCKLGETSDFLVRHGVLIQMPSGSVGCYKICTCGQSGTLENCLDMQCVDLHKSCLVGGQRKNHGESFKVDCNICSC
VAGTLRCSNHQCPHSEEDRRMFTGLPCNCEDQFVPVCGQNGRTYPSACIARCVGLLDHQFEFGLCSSVCNPNPCSR
NGIPKRKVCLTSYEKFGCAQYECIPRHLKCEHSRDPMCDTENVEHINLCTLYQRGRLLSYKGSCQPFCKSAEPVCG
HNGETYPNVCSAYSDRVAVDYYGHCQDVGIFSDQGLHNECLSIQCPAIPVTVCKPIIPPGACCPLCAGVLRILFDK
EKLDTFATATKNTPITVMDILQKIRQHVSVPQCDVFGYLSMESDIIILIVPVDSPPKSIQIDACNKEAEKINSLIN
SDSPTLVSQVPLSALITSEVQVS***TTLNSDCNRICLSIHYIYLYLGIALLYVTLNA***

B LAD-RSC domain conservation

zebra fish	LADVREPCQLGCKELSYCTNFNNRPTELFRSC
coelacanth	LADVREPCQLGCKDLTFCTNFNNRPTELFRSC
x laevis	LADVREPCQLGCRNLTFCTNFNNRPTELFRSC
chicken	LADVREPCQLGCRNLSYCTNFNNRPTELFRSC
hummingbird	LADVREPCQLGCRNLTYCTNFNNRPTELFRSC
platypus	LADVREPCQLGCRNLTFCTNFNNRPTELFRSC
human	LADVREPCQLGCRNLTYCTNFNNRPTELFRSC
fruit fly	IESVDAPCELGCQGLSFCSNFNNRPTELFRSC
mosquito	IDEVDEPCELGCDGLSFCSNFNNRPTELFRSC
	: .* **:*** * :*:**********

Fig. 1. *Xenopus laevis* RECK amino acid sequence

A) X. laevis RECK is 967 amino acids in length and contains an N-terminus signal sequence and a C-terminus glycosylphosphatidylinositol (GPI)-anchoring sequence (bold and italic). The N-terminal region of RECK contains 5 repeats of a putative cysteine knot motif (underlined). The middle portion of RECK contains a highly conserved 32 amino acid stretch we term a LAD-SCR domain (dashed underline). 3 Kazal motifs serine-protease inhibitor-like domains (wavy underline) are found towards the C-terminus. B) Amino acid sequence of our described X. laevis LAD-SCR domain and its Clustal Omega alignment with other select animal. The star symbols identify the identical amino acids that are found in all sequences, a colon is strong similarity, and a period represents weak similarity

three protease inhibitor-like domains (Kazal motifs). According to Takahashi *et al.* only the first domain in human RECK completely matches a Kazal motif sequence, whereas the second and third domains contain partial Kazal motif sequences [1]. A recently published in silico search of nucleotide databases revealed the presence of members of the Kazal-family in the four major subphyla of the Arthropoda [12]. While most insect Kazal motifs contain the 6 cysteine residues that are characteristic to this domain and are responsible for the formation of 1-5, 2-4, and 3-6 disulfide bridges between the 6 cysteines, only Kazal motif 1 has these 6 cysteines in vertebrates. This conserved presence of Kazal domains in animals that have very diverse developmental histories also suggests that such proteins may also play important roles in general tissue maintenance, as well as in developmental processes. While SMART domain analysis of *X. laevis* RECK identified only two Kazal motif domains (this is the same for *X. tropicalis* and drosophila RECK proteins), our analysis showed that all three Kazal motif domains are highly conserved and contain conserved cysteines. In agreement with Takahashi, who suggested that the first domain best matches a classic Kazal motif sequence [1], the first Kazal motif is best conserved in all animals and within animal groups. Moreover, all vertebrate Kazal motifs contain the same number of amino acids between conserved cysteines among all species examined (data not shown). The conservation of all cysteines in all motifs suggests that the two partial Kazal motifs also contribute an important function to the protein in

vertebrates. As the Kazal motifs are protease inhibitor-like domains and play an important role in inhibiting MMPs [8], the consistent high conservation of Kazal motif 1, and the presence of three such domains in all vertebrates, suggests a conservation of RECK function as an MMP inhibitor in these species.

The N-terminal region of RECK contains five large repeats of a cysteine knot motif. Collectively these five putative cysteine knot motifs share relatively moderate similarity among all animals (17-23%) with higher conservation of identity being seen in vertebrates and the subgroups examined, with the most striking conservation of these domains being in birds (Fig. 2). While in general it was cysteine knot 3 and 4 that were the most conserved, all knot motifs in all animals had six conserved cysteines. This included two cysteines at the beginning of each knot, a gap of 7 or 8 amino acids, followed by the third cysteine with 3 or 4 amino acids before cysteine 4. The conservation of 6 cysteines in 5 knots in all animals, with the spacing of the first 4 cysteines being almost identical in animals ranging from insects to amphibians to mammals is quite remarkable. The conservation in the number of cysteines and their spacing, but the dissimilarity in amino acids between the cysteines, suggests that these motifs play structural roles that are important to the overall functions of folded RECK proteins.

While the overall RECK protein demonstrates high levels of sequence conservation, analysis revealed a 32 amino acid sequence following the 5th cysteine knot that was the most conserved of any other region of the protein. We have termed this a LAD-RSC domain. This stretch of amino acids shares no homology with any characterized functional domain. This stretch of amino acids is, however, 68% identical between all of the animals we investigated, 88% identical in vertebrates, and over 90% identical within the various vertebrate sub-groups. While domains such as the cysteine knot and Kazal motifs are hallmarks of RECK, these hallmark domains make up only a very small part of its almost 1000 amino acid coding sequence. The high conservation of other regions, such as in this LAD-RSC domain, suggest novel and as yet to be characterized roles for RECK, such as its roles in cell signaling [7] - functions that are MMP-independent and thus may not involve the Kazal motifs.

3.2 Embryonic Analysis

As previous research has demonstrated both a neural and vascular role for RECK during development of fish and mouse [2,4], we sought to investigate if a similar embryonic expression pattern existed for *X. laevis reck*. First, semi-quantitative PCR was performed to examine the

	Full Coding	Kn1 (48aa)	Kn2 (38aa)	Kn3 (47aa)	Kn4 (48aa)	Kn5 (47aa)	LAD-RSC (32aa)	KZ1 (46aa)	KZ2 (43aa)	KZ3 (35aa)
All	33%	(8) 17%	(7) 18%	(8) 17%	(11) 23%	(11) 23%	(21) 67%	(19) 41%	(5) 12%	(7) 20%
Vertebrates	58%	(15) 31%	(17) 45%	(28) 60%	(20) 42%	(19) 40%	(28) 88%	(27) 59%	(11) 26%	(14) 40%
Mammals	85%	(35) 73%	(35) 92%	(42) 89%	(41) 85%	(41) 87%	(29) 91%	(40) 87%	(28) 65%	(21) 60%
Birds	93%	(48) 100%	(35) 92%	(46) 98%	(46) 96%	(45) 96%	(30) 94%	(46) 100%	(36) 84%	(31) 89%
Fish	68%	(29) 60%	(30) 79%	(38) 81%	(34) 71%	(31) 66%	(31) 97%	(31) 67%	(28) 57%	(20) 57%
Herptile	75%	(37) 77%	(30) 79%	(40) 85%	(38) 79%	(30) 64%	(31) 97%	(38) 83%	(26) 60%	(27) 77%

Fig. 2. Percent amino acid identity of select RECK domains

Select full length RECK amino acid coding sequences were aligned with Clustal Omega http://www.ebi.ac.uk/Tools/msa/clustalo/. Cysteine knot (Kn) and Kazal (KZ) motif domains were identified at http://smart.embl.de/. Clustal outputs were used to identify identical amino acids amongst all species within a given group of animals. For the full coding region, the percent identity (%) listed is the minimum amongst the group. For the various domains (whose amino acid size is indicated in brackets) the number of identical amino acids in each aligned group (also in brackets) is represented as a percentage of amino acid identity within that domain

temporal expression pattern of *reck* during early *X. laevis* development. *Reck* mRNA levels were measured relative to *elongation factor 1 alpha* (*ef1α*; a housekeeping gene). *Reck* expression was examined during gastrulation (stage 10), neural tube closure (stage 18), axis elongation (stage 24), and organogenesis (stages 32, 37, and 40). This was done to identify possible correlations between *reck* expression and the timing of developmental events including neurulation, neural crest cell migration and vasculogenesis, as well as the formation of other organs. Low levels of *reck* transcripts were present early in development during gastrulation (stage 10), but were more readily detectable at stage 18, a time during which the neural tube closes, and *reck* levels remained elevated during axis elongation (stage 24) through to organogenesis (stages 32-40) (Fig. 3). Low levels of *reck* expression during gastrulation mirrors low *timp* expression at this time and is consistent as gastrulation is an event that requires extensive ECM remodeling [13], whereas higher *reck* levels during later stages is consistent with processes that require more localized and refined ECM remodeling. Further, the peak of *reck* at stage 18 as well as *timp* [13] is coincident with a decrease in global apoptosis levels in the embryo at this time [14]. As ECM remodelling can facilitate cell death, the high levels of MMP inhibitors (*reck* and *timp*) at this time is consistent with these molecules' contribution to a decrease in apoptotic signals. Roles for reck in neural crest cell migration and vasculogenesis occur at stages 13-21 and 26 onwards respectively, are consistent with RECK's role in these processes both in *X. laevis* and fish and mouse [2,4].

As temporal expression analysis revealed that *reck* mRNA levels do not peak until neurulation, DIG-labeled anti-sense RNA probes were used to detect *reck* transcript spatial distribution following neural tube closure (stage 24, Fig. 4a) and through organogenesis (stages 28, 34, and 38, Figs. 4b-e). DIG-labeled anti-sense RNA probes for cardiac troponin 1, a heart-specific marker, were used as a positive control (Fig. 4f), and no probes were used as a negative control (data not shown). *Reck* transcripts were present in areas of the developing eye as the primary eye vesicle was folding as at stage 24 [10] and persist in the eye through stage 34 (Figs. 4a, b, d). *Reck* transcripts were also present in dorsal axial structures, including the notochord and the dorsal side of the neural tube at the late tailbud stage (stage 28) (Fig. 4b). During later stages of organogenesis (stage 34), *reck* transcripts were not detectable in the notochord but remained localized in the neural tube (Fig. 4d). The presence of *reck* in the notochord at stage 28 is again consistent with a role for reck in inhibiting apoptosis.

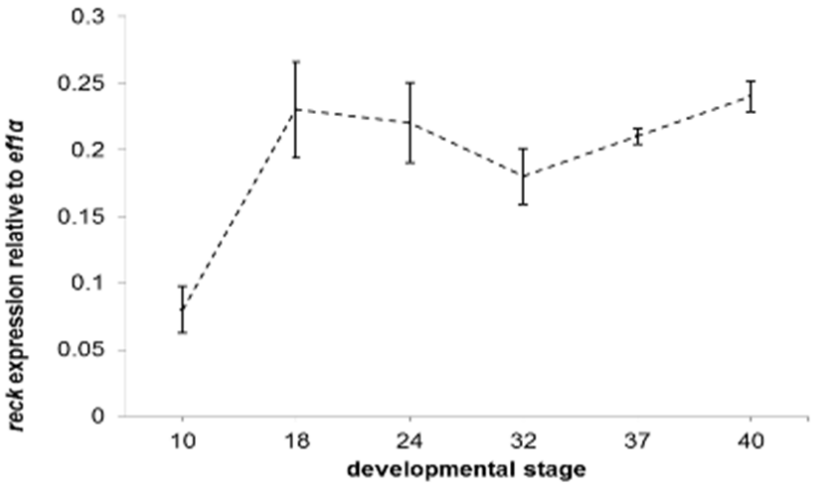

Fig. 3. Temporal expression pattern of *reck* mRNA levels during early *X. laevis* development

Semi-quantitative PCR analysis of reck transcripts measured relative to EF1α was performed on embryos at stages 10 through to 40. reck transcript levels are low early on in development during gastrulation (stage 10) and increase during neurulation (stage 18 to 24) and into organogenesis (stages 32, 37 and 40). Standard error bars are representative of three biological replications

Cells of the notochord undergo increasing levels of cell death up until stage 25 [15]. The absence of *reck* at stage 24 and its presence at stage 28 again coincide with a role in inhibiting apoptosis. Throughout all stages examined, *reck* transcripts were present in most anterior structures including the branchial arches (Figs. 4b, d). Intervening stages were also examined but results were omitted as *reck* expression pattern did not change in these intervening stages (data not shown).

To confirm neural and branchial arch *reck* localization, immunohistochemistry was performed to localize RECK protein on histological sections of late tailbud embryos (Fig. 5). Punctate staining of RECK proteins was present in the dorsal side of the neural tube (Fig. 5b).

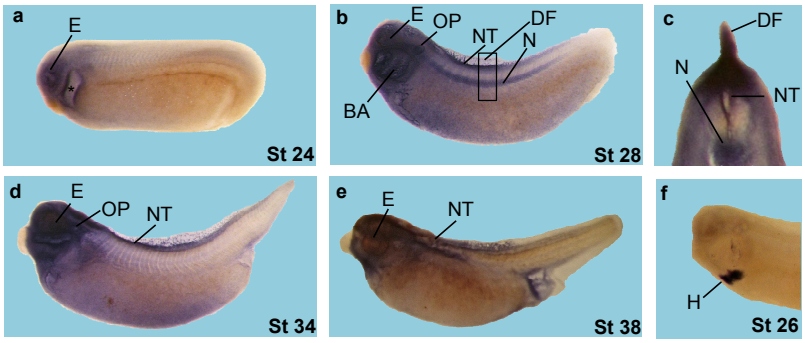

Fig. 4. Spatial expression pattern of *reck* mRNA during early *X. laevis* development. a-e)
*Whole mount in situ hybridization was performed on embryos at stages 24, 28, 34, and 38 using antisense Digoxigenin-labeled reck RNA probes. f) Antisense Digoxigenin-labeled cardiac troponin 1 RNA probes were used to establish specificity in the experiment (stage 26 shown). c) is a magnified view of a cross section indicated in panel b. reck expression is present in anterior structures, including the eye, otic placode, and branchial arches throughout early development (a-e). At the late tailbud stage (b), reck expression is present in dorsal neural structures, including the dorsal side of the neural tube and the notochord. However, by stage 34 (d), reck expression disappears from the notochord and appears on the ventral side of the embryo. Abbreviation: BA=branchial arches, DF=dorsal fin, E=eye, H=heart, N=notochord, NT=neural tube, OP=otic placode. *cavity*

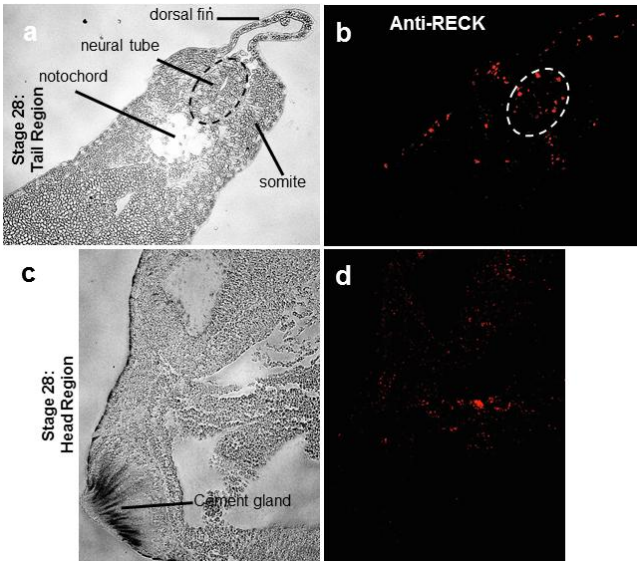

Fig. 5. Localization of RECK proteins in late tailbud embryos
Immunohistochemistry was performed on sections of late tailbud embryos to detect RECK protein (red). a, c) Brightfield DIC images are shown. b) RECK proteins are localized on the dorsal side of the neural tube and dorsal fin. d) RECK proteins are localized in regions consistent with branchial arches

In the head region of late tailbud embryos, RECK proteins were localized in the area of the branchial arches (Fig. 5d). RECK presence in neural structures and branchial arch regions of the developing frog embryo is similar to that observed in zebrafish embryos, and consistent with the finding that RECK plays an important role in the differentiation of neural crest cells into the DRG [4]. Additionally the expression of *reck* in anterior structures coincides with the expression of ECM proteinases (*stromelysisn-3* and *ADAM19*, [16,17]) and *timps* [13], reflecting the complex regulation of the ECM remodelling events occurring in these anterior tissues. Further, RECK presence in the area of the branchial aches, which will contribute to the formation of the gills, is consistent with a role for RECK in vascular development as its localization is consistent with the expression of Xfli1, whose similar expression demarks endothelial cell differentiation [18].

4. CONCLUSION

This study has revealed the important developmental roles of RECK by demonstrating its high level of evolutionary sequence conservation and developmental localization. All examined animal RECK amino acid sequences contain all hallmark domains, and an exceptional number of cysteine residues - both in number and position. Interestingly, even insect RECK contained the same cysteine knot and Kazal hallmark domains. Expression analysis of *reck* during early *X. laevis* development revealed that *reck* is present during given ECM remodeling events associated with neural function and vasculogenesis, as it is in fish and mice. Its downregulation during other important events, such as gastrulation and axis elongation, indicate the importance of regulating RECK during early *X. laevis* development.

ETHICAL APPROVAL

Xenopus laevis frogs were housed and treated in accordance with University of Western Ontario and Canadian Council on Animal Care guidelines.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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