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Dynamic Expression Patterns of *RhoV/Chp* and *RhoU/Wrch* During Chicken Embryonic Development

Cécile Notarnicola,¹ Ludovic Le Guen,¹ Philippe Fort,² Sandrine Faure,^{2†} and Pascal de Santa Barbara^{1*†}

Rho GTPases play central roles in the control of cell adhesion and migration, cell cycle progression, growth, and differentiation. However, although most of our knowledge of Rho GTPase function comes from the study of the three classic Rho GTPases RhoA, Rac1, and Cdc42, recent studies have begun to explore the expression, regulation, and function of some of the lesser-known members of the Rho GTPase family. In the present study, we cloned the avian orthologues of *RhoV* (or *Chp* for Cdc42 homologous protein) and *RhoU* (or *Wrch-1* for Wnt-regulated Cdc42 homolog-1) and examined their expression patterns by in situ hybridization analysis both during early chick embryogenesis and later on, during gastrointestinal tract development. Our data show that both GTPases are detected in the primitive streak, the somites, the neural crest cells, and the gastrointestinal tract with distinct territories and/or temporal expression windows. Although both proteins are 90% identical, our results indicate that *cRhoV* and *cRhoU* are distinctly expressed during chicken embryonic development.

Key words: Rho GTPases; RhoV; RhoU; neural crest; gastrointestinal tract development; chick embryo

INTRODUCTION

Rho family GTPases are crucial regulators of a variety of cellular functions, including actin cytoskeleton dynamics, cell adhesion and motility, vesicular trafficking, cell growth, and survival (Etienne-Manneville and Hall, 2002; Wennerberg and Der, 2004; Hall, 2005). Like most other Ras-like members, Rho GTPases oscillate between inactive GDP-bound and active GTP-bound states, the switch of their activity being tightly regulated by the auxiliary proteins GEFs, GAPs, and

GDIIs (Van Aelst and D'Souza-Schorey, 1997; Rossman et al., 2005). Once activated, Rho GTPases interact with specific downstream effectors and regulate a variety of intracellular processes including the F-actin cytoskeleton dynamics, gene transcription, and activation of kinase cascades, such as the JNK and p38 MAP kinases (Fort, 1999; Ridley, 2001; Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2002). Although the Rho family of GTPases is made of 20 members in vertebrates, little is known on

the cellular and physiological roles of most Rho members, because studies published to date have focused on RhoA, Rac1, and Cdc42, the most conserved and expressed members from lower eukaryotes to mammals (Bou-reux et al., 2007).

Among the Rho members found early in eukaryotes, RhoV (also designated as Chp for Cdc42 homologous protein; Aronheim et al., 1998) and its close relative RhoU (also designated as Wrch-1 for Wnt-1-regulated Cdc42 homolog; Tao et al., 2001) form a sep-

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arate branch of the Rac/Cdc42 subfamily with atypical features (Boureux et al., 2007; Aspenström et al., 2007). Indeed, although RhoV and RhoU share functional similarities with Rac- and Cdc42-related proteins, they both possess additional N- and C-terminal sequences that distinguish them from the classic Rho structure (Shutes et al., 2006). In addition, they both lack the canonical C-terminal CAAX motif required for prenylation and require palmitoylation for membrane association, suggesting that they might exert their activities in distinct subcellular locations compared with other Rho members (Berzat et al., 2005; Chenette et al., 2006; Shutes et al., 2006). The high similarities in protein sequence and biochemical properties between RhoU and RhoV suggest that the two proteins might fulfill redundant biological functions. Along this line, both proteins exhibit intrinsic transforming activities, whereas other Rho members only cooperate in cell transformation (Berzat et al., 2005; Chenette et al., 2005, 2006). We also recently showed that RhoU can rescue the loss of RhoV function during neural crest development in *Xenopus* (Guémar et al., 2007). One could imagine that the specific role of RhoV and RhoU depends on their expression pattern.

In the present study, we examined the expression profiles of *RhoV* and *RhoU* from Hamburger and Hamilton (HH) stages 5 to 12 of chick development, and later on, during gastrointestinal tract development. In the light of their expression patterns, we discuss the relationships between both GTPases in terms of developmental functions.

RESULTS AND DISCUSSION

Cloning and Sequence Analysis of cDNAs Encoding Chick *RhoV* and *RhoU*

To study the expression patterns of *RhoV* and its closest relative *RhoU* during chicken embryonic development, we first searched chick cDNA sequences coding for rat RhoV (accession no. AF097887.1) and human RhoU (accession no. AF378087.1) orthologues using the NCBI tblastn program (<http://www.ncbi.nlm.nih.gov/>

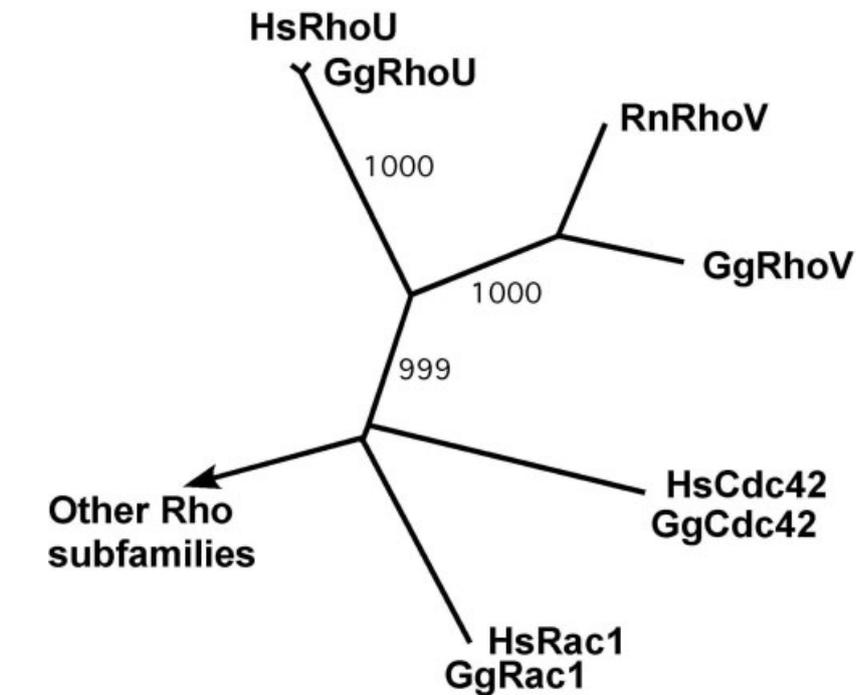


Fig. 1. Similarity tree of human (Hs), rat (Rn), and chicken (Gg) Rho sequences. Cdc42 and Rac1 were used as outgroup.

blast). The search identified several putative chicken mRNA sequences (XM_426425.2 for RhoV, XM_426146 and XM_427270 for RhoU) predicted by automated computational analysis (Gnomon, NCBI). RhoV XM_426425.2 was derived from a three-exon gene structure (ENSGALG00000008476, <http://www.ensembl.org>), in consistency with *RhoV* and *RhoU* gene structures in vertebrates (Boureux et al., 2007), and encodes a full-length protein that shares 82% amino acid (aa) identity with rat RhoV. The two putative *RhoU* transcripts were derived from the partially assembled ENSGALG00000011073 locus: XM_426146, spanning over the putative second and third exons and encoding aa 89-228 (compared with human RhoU) and XM_427270, spanning over the last 108 N-terminal (nt) of the second intron and over the third exon (456 nt coding for aa 107-258, as well as 2.5 kb of 3' noncoding region). Searches in the chick expressed sequence tag (EST) database produced no EST for *RhoV* and 20 for *RhoU*, all located in the 3' noncoding region except BU477343 and BU255458, encoding the 41 and 7 Carboxyterminal amino acids, respectively. Such long putative 3' noncoding region is in agree-

ment with those observed in human (NM_021205, 2.9 kb) or mouse (NM_133955, 2.5 kb). Neither in EST nor in genomic databases could we detect the RhoU N-terminal sequence (i.e., homologous to the 88 amino acids in the human first exon). The partial RhoU protein (aa 89-258) shares 98% identity with human RhoU. Protein sequence alignments and phylogenetic analyses confirmed that the identified chick genes are bona fide *RhoV* and *RhoU* orthologues that we will refer to as *cRhoV* and *cRhoU* (Fig. 1). We next generated in situ hybridization probes by reverse transcriptase-polymerase chain reaction (RT-PCR) and cloning of *RhoV* and *RhoU* open reading frame (ORF) fragments from HH stage 8 embryo mRNA (see the Experimental Procedures section).

cRhoV and *cRhoU* Expression Patterns During Early Development

We next examined the spatiotemporal expression patterns of *cRhoV* and *cRhoU* during early development by whole-mount in situ hybridization of embryos from HH stages 5 to 12 (Hamburger and Hamilton, 1951). At HH stage 5, both *cRhoV* and *cRhoU*

are expressed throughout the primitive streak and in Hensen's node (Figs. 2A, 3A). In addition, *cRhoU* expression, detected in the entire epiblast, is excluded from the most anterior region of the embryo (black arrowhead in Fig. 3A) but is enriched in an elliptical region centered over the Hensen's node corresponding to the prospective anterior neural plate. As development proceeds, expression of *cRhoU* decreases in the primitive streak and in the adjacent posterior open neural plate (Fig. 3B,C), while it is reinforced both in the head fold and in mesodermal cells lateral to the open neural plate as indicated by transverse sections (Fig. 3C,C'). From HH stage 7, expression of *cRhoV* is reinforced posteriorly in the primitive streak as shown by transverse sections (Fig. 2B,B'), while *cRhoU* is no longer detected in this structure from HH stage 7 (Fig. 3C,C'). At HH stage 8, both *cRhoV* and *cRhoU* are expressed in the head neural folds (Figs. 2C,D, 3D,E), although highest levels of *cRhoV* transcripts are detected in the primitive streak (Fig. 2C,C'). At this stage, both GTPases are also expressed in the newly undifferentiated epithelial somites, while undetectable in the unsegmented somite mesoderm (Figs. 2C,D, 3D,E). At later developmental stages, stronger *cRhoV* expression is detected in the anterior neural folds and also at weakest levels from HH stage 9 in the posterior neural folds as well (Fig. 2E-I). Surprisingly, examination of transverse sections reveals disjunctive *cRhoV* and *cRhoU* patterns in the anterior neural folds, *cRhoV* transcripts being predominantly found in the ectoderm adjacent to the neural folds but absent from the neural folds and the neural plate (Fig. 2F,F',G,G'), while *cRhoU* transcripts are restricted to the dorsal-most region of the neural tube and undetectable from the surrounding ectoderm (Fig. 3E,E'). Of interest, *cRhoV* expression profile in the neural folds resembles that of the neural crest inducer *Wnt6* (Garcia-Castro et al., 2002), while the *cRhoU* pattern in the dorsal part of the neural tube is similar to *Bmp4* (Garcia-Castro et al., 2002). These observations in the chick embryo are consistent with our previous study identifying *RhoV* as an essential regulator of neural crest induc-

tion in *Xenopus* (Guémar et al., 2007). At HH stage 9, in addition to the dorsal neural tube, *cRhoU* is expressed in the lateral plate mesoderm and in the somites (Fig. 3F,G,G'). As development proceeds, the broad expression of *cRhoU* in the dorsal side of the neural tube decreases; however, a strong expression was detected in the head, both anteriorly in the olfactory placodes and laterally to the neural tube, in the migrating cranial neural crest cells at HH stage 10 (Fig. 3H,I,I'). In addition, expression of *cRhoU* remains restricted to the most dorsal part of the mature somites from HH stage 12 (Fig. 3L,L'), while it is expressed in the dorsoventral part of the epithelial somites from HH stage 8 (Fig. 3G,G'). At this stage, *cRhoU* is also detected in the optic vesicles and in the splanchnic mesoderm that will contribute to the mesoderm of the gastrointestinal (GI) tract (Fig. 3L,L').

***cRhoV* and *cRhoU* Expression Patterns During GI Tract Development**

It was previously shown that *RhoU* mRNA is highly expressed in the adult stomach (Kirikoshi and Katoh, 2002), and *RhoV* mRNA has been reported to be up-regulated in several primary gastric cancers (Katoh, 2002). *RhoU* expression was also shown to be down-regulated in the jejunum of *W³⁷/W³⁷* mice (Daigo et al., 2004), which show a reduced number of interstitial Cajal cells, which act as a pacemaker for the contraction of the visceral smooth muscles in association with the enteric nervous system (Lecoin et al., 1996). For these reasons, we next addressed the spatiotemporal distribution of *cRhoV* and *cRhoU* during GI tract development. In the primitive uniform GI tract, *cRhoV* expression is first detected at E4 in the foregut and the caudal part of the hindgut (Fig. 4A). From E5, a stronger *cRhoV* signal is detected in the gizzard (muscular stomach) and the cloaca (Fig. 4B,E,F). As demonstrated by transverse sections (Fig. 4C,D), *cRhoV* transcripts are restricted to the endodermal layer of these structures. A fainter *cRhoV* signal was also detected in the endoderm of the proventriculus (glandular stomach) at E6 (Fig. 4E). The expression

pattern of *cRhoU* during GI tract development appeared much more dynamic. Its expression is first detected at E4 in the anterior foregut that will generate the lung, as well as in the glandular stomach, the caecum bud, and the caudal hindgut (Fig. 4G). A weaker but significant *cRhoU* signal was also detected in the midgut. At E5, expression of *cRhoU* invades the whole GI tract with the exception of the colon (Fig. 4I). It is expressed at high levels in the lung, the glandular stomach, the caecum, and the cloaca and at low levels in the small intestine and the gizzard. In all these structures, *cRhoU* expression is restricted to the mesodermal layer of these tissues, as previously reported for *Bmp4* (Goldstein et al., 2005). It is noteworthy that, in the cloaca, *cRhoU* transcripts are restricted to the mesodermal cell layer (Fig. 4J-M), while *cRhoV* transcripts are localized to the adjacent endodermal layer (Fig. 4C,D). At E5 and E6, *cRhoU* expression is observed in the distal domain of the stomach as a ring corresponding to the pyloric sphincter, a muscular structure separating the stomach from the duodenum (Fig. 4I,N). At E6 and E7, *cRhoU* transcripts accumulate in the lung, the glandular stomach, the caecum, and the cloaca (Fig. 4N,O).

In conclusion, although *cRhoV* and *cRhoU* display a few overlapping domains of expression, they are mostly expressed in distinct territories and/or temporal expression windows. For instance, although both expressed in the primitive streak from HH stage 5, only *cRhoV* remains expressed in this structure by HH stage 7. Similarly, although the two genes are expressed in the newly segmented somites, only *cRhoU* is found in the mature somites. Although *cRhoV* and *cRhoU* are expressed in the forming cranial neural crest cells, only *cRhoU* is found in the migrating ones. Later during development, *cRhoU* is exclusively expressed in the mesoderm of the GI tract, while *cRhoV* is detected in the stomach epithelium. The differential expression of *RhoV* and *RhoU* supports a scenario according to which both proteins fulfill similar functions in specific tissues or at different times. Indeed, duplicated genes have been shown to be retained through evolution by neofunctionaliza-

tion, in which mutations on one gene leads to a protein with new properties, or by subfunctionalization, in which the two genes become expressed in distinct territories through the modi-

fication of their regulatory elements (for a review, see Li et al., 2005). These mechanisms are not mutually exclusive, because once differentially expressed, each paralog has the capac-

ity to evolve independently. The Rho family of GTPases illustrates both types of evolution: on the one hand, RhoA, Rac1, and Cdc42 perfectly match the neofunctionalization crite-

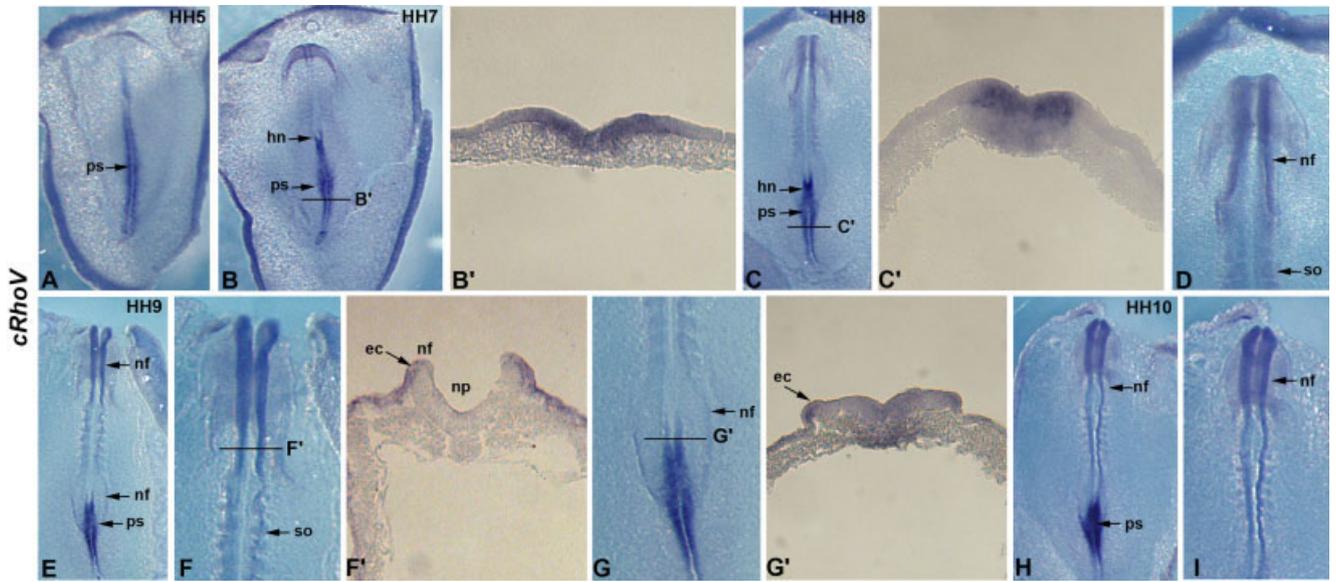


Fig. 2.

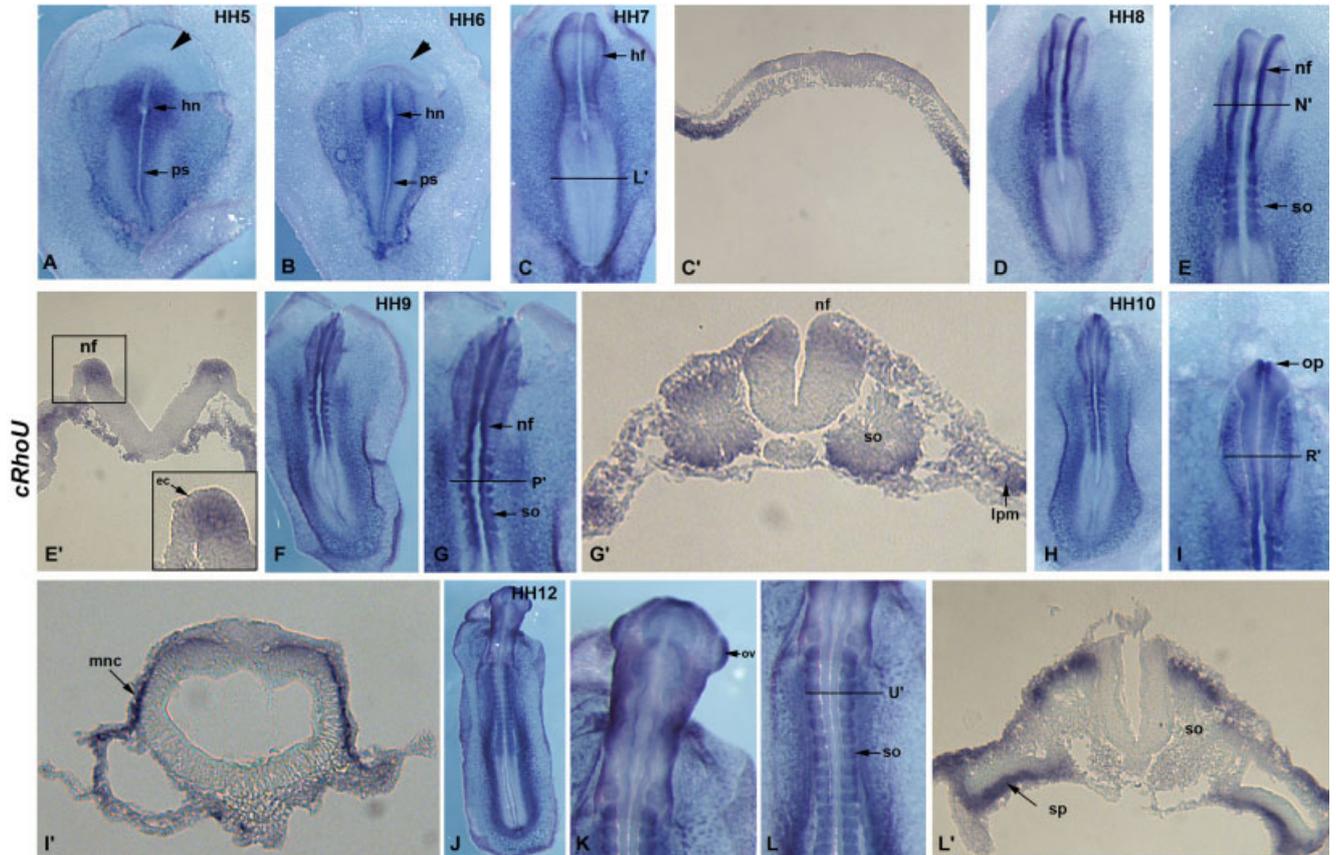


Fig. 3.

tion because they are coexpressed in many cell types from yeast to mammals and have gained specific properties in terms of subcellular distribution and binding partners (Bustelo et al., 2007). On the other hand, more recently duplicated genes such as Rac1-3 or RhoA-C proteins show distinct tissue distribution (Boureux et al., 2007), while exhibiting very similar biochemical properties, which clearly meets the subfunctionalization criterion. The present study strongly suggests that it is also the case for *cRhoV* and *cRhoU*, which predicts that the corresponding proteins still fulfill very similar cellular functions.

EXPERIMENTAL PROCEDURES

Chick Embryos

Timed fertilized white Leghorn eggs (Haas Farm, France) were incubated at 38°C in a humidified incubator (Coudelou, France) until used experimentally. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951) for early embryogenesis stages and by embryonic day (E) for gastrointestinal tract analysis. Whole embryos or dis-

sected gastrointestinal tissues were fixed in 4% paraformaldehyde for 2 hr at room temperature, washed in phosphate buffered saline (PBS), gradually dehydrated in methanol to store the sample at -20°C before processing as described below.

Cloning of Chick *RhoV* and *RhoU*

Searches were performed in *Gallus gallus* gene and EST databases with the NCBI tblastn program (<http://www.ncbi.nlm.nih.gov/BLAST>) using the *Rattus norvegicus* RhoV and the *Homo sapiens* RhoU proteins as queries. ORF fragments were obtained by RT-PCR from HH stage 8 chick embryo mRNAs using the following primer sets: RhoV, forward 5'-cc gaa ttc gca gcc atg ccc cc-3', reverse 5'-cc ctc gag cac gtg gta cgc atc c-3'; RhoU, forward 5'-cc gaa ttc gcc gtg gtg tct gt-3', reverse 5'-cc ctc gag ggt tgc tgc tgg gta tc-3'. RT-PCRs were performed with *pfu* polymerase (30 cycles, 94°C 1 min, 57°C 30 sec, 72°C 2 min). PCR products were 474 bp for RhoV and 438 bp for RhoU, containing aa 1-158 and aa 89-234 of the corresponding human sequences, respec-

tively. Fragments were subcloned into the PCR4-TOPO vector (Invitrogen) and checked on an ABI310 automatic sequencer (Perkin-Elmer, Foster city, CA) before subcloning in the pCS2+ vector.

Whole-Mount In Situ Hybridization

Whole-mount in situ hybridization was carried out as described by Faure et al. (2002) and Moniot et al. (2004). In summary, tissues were gradually rehydrated in PBS, washed in PBT (PBS, 0.1% Tween) and incubated for 1 hr in 6% hydrogen peroxide (Sigma, France). Samples were next permeabilized by treatment with proteinase K (10 µg/ml) for 5 min (early stage) and 10 min (gastrointestinal tract), washed with glycine in PBT and fixed in 4% paraformaldehyde/0.2% glutaraldehyde in PBT for 20 min. Tissues were then hybridized with antisense *cRhoV* and *cRhoU* digoxigenin-labeled (Roche) riboprobe overnight at 70°C. After posthybridization washes at 70°C, tissues were incubated in 10% sheep serum for 2.5 hr at room temperature and finally mixed with preabsorbed anti-digoxigenin coupled

Fig. 2. A-I: *cRhoV* expression pattern during early development. Hamburger and Hamilton (HH) stage 5 (A), stage 7 (B), stage 8 (C,D), stage 9 (E-G), and stage 10 (H,I) embryos were analyzed by whole-mount in situ hybridization using digoxigenin (DIG) -labeled RNA probes for *cRhoV*. Transverse sections were performed where indicated on HH stages 7, 8, and 9 embryos labeled (B', C', F', G'). **A:** At HH stage 5, *cRhoV* is expressed in the primitive streak (arrow). **B:** At HH stage 7, *cRhoV* expression is detected in the posterior primitive streak and in the Hensen's node. **B':** Cross-section as indicated in B showing *cRhoV* expression in the primitive streak. **C:** At HH stage 8, *cRhoV* expression is detected in the head neural fold and in the remaining primitive streak. **C':** Cross-section as indicated in C showing *cRhoV* transcripts localization in the primitive streak. **D:** Enlargement of C in the head fold level. **E:** At HH stage 9, *cRhoV* expression is observed in the anterior and posterior neural folds. **F:** Enlargement of E in the head fold level showing that *cRhoV* expression is detected in the head neural fold and in the epithelial somites. **F':** Cross-section as indicated in F showing *cRhoV* expression in the ectoderm adjacent of the anterior neural fold. **G:** Enlargement of E in the posterior neural fold level showing *cRhoV* expression in the remaining primitive streak and in the posterior neural folds. **G':** Cross-section as indicated in G showing *cRhoV* expression in the ectoderm adjacent to the posterior neural fold. **H:** At HH stage 10, *cRhoV* expression is mainly observed in the posterior primitive streak and in the neural folds. **I:** Enlargement of H at the head level showing *cRhoV* expression in the cranial neural folds. Ec, ectoderm; hn, Hensen's node; nf, neural folds; np, neural plate; ps, primitive streak; so, somites.

Fig. 3. *cRhoU* expression pattern during early development. Hamburger and Hamilton (HH) stage 5 (A), stage 6 (B), stage 7 (C), stage 8 (D,E), stage 9 (F,G), stage 10 (H,I), and stage 12 (J-L) embryos were analyzed by whole-mount in situ hybridization using digoxigenin (DIG) -labeled RNA probes for *cRhoU*. Transverse sections were performed where indicated on HH stages 7, 8, 9, 10, and 12 embryos labeled (C', E', G', I', L'). **A, B:** *cRhoU* is expressed in the primitive streak, in the entire epiblast with the exception of the most rostral part of the embryo (arrowhead) at HH stages 5 (A) and 6 (B). **C:** At stage 7, *cRhoU* expression is reinforced in the head fold and lateral tissues. **C':** Cross-section as indicated in C showing *cRhoU* expression in the lateral mesoderm. **D:** At HH stage 8, *cRhoU* expression is detected in the head neural fold, lateral tissues, and somites. **E:** Enlargement of D showing *cRhoU* expression in the undifferentiated somites. **E':** Cross-section as indicated in E showing *cRhoU* transcripts localized in the dorsal-most region of the neural tube. **F:** At HH stage 9, *cRhoU* expression is observed in the dorsal neural tube and lateral mesoderm. **G:** Enlargement of F showing *cRhoU* expression in the dorsal neural tube and the somites. **G':** Cross-section as indicated in G showing that *cRhoU* is expressed in the dorsoventral domains of the epithelial somites and in the lateral plate mesoderm. **H:** At HH stage 10, *cRhoU* expression is observed in the olfactory placodes, the cranial neural crest, the somite, and in the lateral mesoderm. **I:** Enlargement of H showing *cRhoU* expression in the cranial neural crest and the olfactory placodes. **I':** Cross-section as indicated in I showing *cRhoU* expression in the migrating cranial neural crest. **J:** At HH stage 12, *cRhoU* expression is observed in the somites and lateral mesoderm. **K:** Enlargement of J showing *cRhoU* expression in the optic vesicles. **L:** Enlargement of J showing *cRhoU* expression in the mature somites and lateral mesoderm. **L':** Cross-section as indicated in L showing that *cRhoU* is expressed in the dorsal mature somites and splanchnic lateral mesoderm. Ec, ectoderm; hf, head fold; hn, Hensen's node; lpm, lateral plate mesoderm; mnc, migrating neural crest cells; nf, neural folds; op, olfactory placode; ov, optic vesicle; ps, primitive streak; so, somites; sp, splanchnic mesoderm.

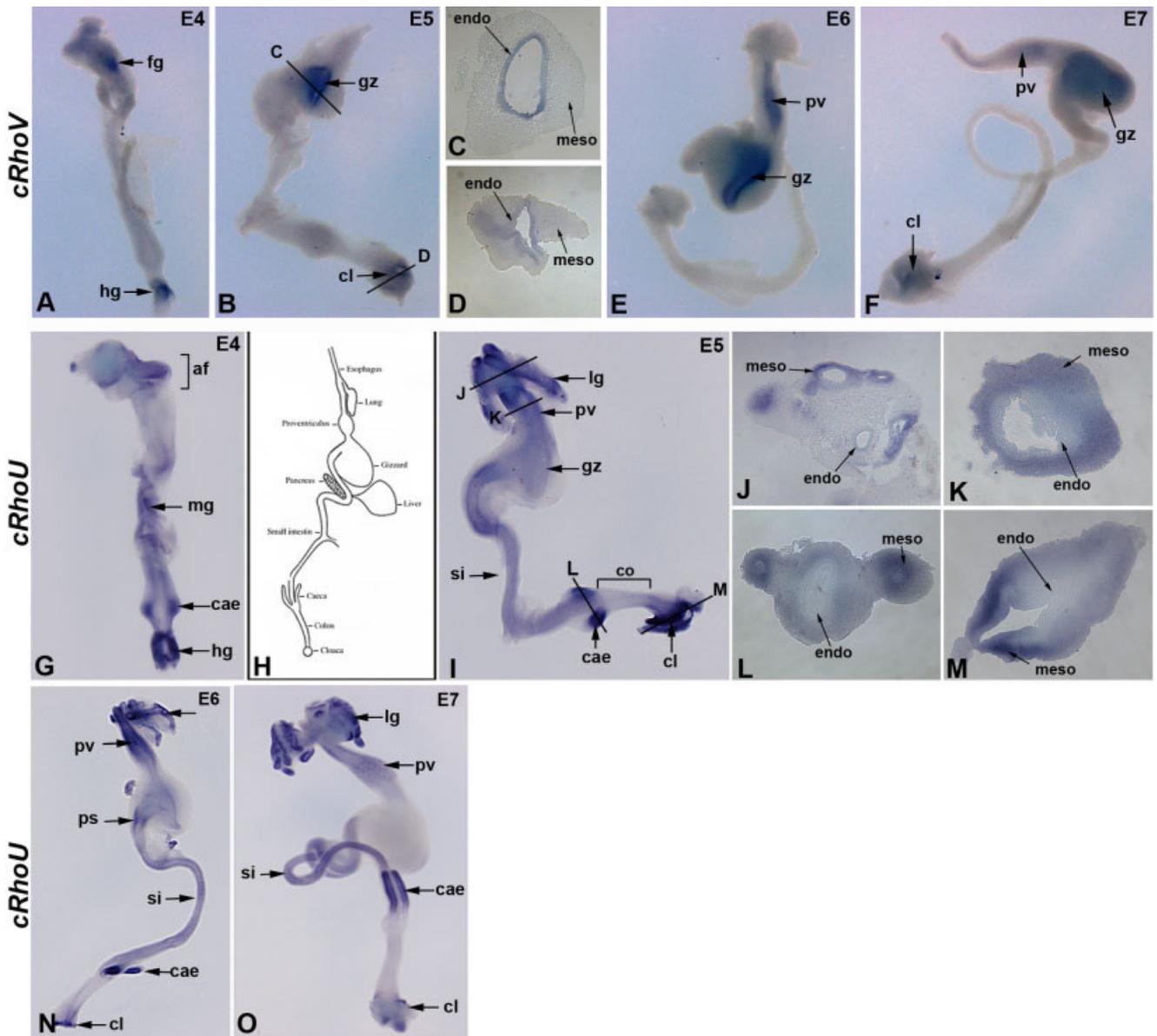


Fig. 4. *cRhoV* and *cRhoU* expression profiles during chick gastrointestinal (GI) tract development. A–O: Whole-mount in situ hybridization of GI tract from embryonic day (E) 4 to E7 showing expressions of *cRhoV* (A–F) and *cRhoU* (G–O). **A:** E4 GI tract showing *cRhoV* expression in the foregut and the hindgut. **B:** E5 GI tract showing *cRhoV* expression in the gizzard and the cloaca. **C:** Transverse section through the gizzard as indicated in B showing *cRhoV* expression in endodermal cell layer. **D:** Transverse section through the cloaca as indicated in B showing *cRhoV* expression in the endodermal cells. **E:** E6 GI tract showing *cRhoV* expression in the stomach (proventriculus and gizzard) and the cloaca. **F:** E7 GI tract showing *cRhoV* expression in the gizzard, proventriculus, and the cloaca. **G:** E4 GI tract showing *cRhoU* expression in the anterior foregut, the caecum bud, and the caudal part of the cloaca. **H:** E6 chick gut cartoon, ventral view. **I:** E5 GI tract showing strong *cRhoU* expression in the lung, proventriculus, caecum, and cloaca. A faint *cRhoU* expression is also detected in the stomach and in the small intestine. **J–M:** Transverse sections of the tissues shown in I at the levels indicated showing *cRhoU* expression in the mesodermal cells of the lung (J), the proventriculus (K), the caecum (L), and the most ventral cloacal structure (M). **N:** E6 GI tract showing strong *cRhoU* expression in the proventriculus, the pyloric sphincter, caecum, and the cloaca. Weak but detectable expression was also observed in the whole mesodermal GI tract with the exception of the colon. **O:** E7 GI tract with *cRhoU* expression in the lung, proventriculus, small intestine, caecum, and the cloaca. af, anterior foregut; cae, caecum; cl, cloaca; co, colon; endo, endoderm; fg, foregut; gz, gizzard; hg, hindgut; lg, lung; meso, mesoderm; mg, midgut; ps, pyloric sphincter; pv, proventriculus; si, small intestine; sto, stomach.

with alkaline phosphatase antibody (Roche) overnight at 4°C. The complexes were detected with BM purple solution (Roche). For sectioning, tissues were equilibrated in 30% sucrose in PBS, then embedded in OCT (Optimal Cutting Temperature Compound,

Tissue-Tek) and sectioned at 20 µm on a cryostat.

Photography

Images were collected in whole-mount under a Nikon SMZ1000 scope and in sec-

tion under a Zeiss Axiophot microscope, both using Nikon DXM1200 camera.

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