

MAPK regulation of maternal and zygotic Notch transcript stability in early development

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Spatiotemporal modulation of the evolutionarily conserved, intercellular Notch signaling pathway is important in the development of many animals. Examples include the regulation of neural-epidermal fate decisions in neurogenic ectoderm of *Drosophila* and somitogenesis in vertebrate presomitic mesoderm. In both these and most other cases, it appears that Notch-class transmembrane receptors are ubiquitously expressed. Modulation of the pathway is achieved primarily by the localized expression of the activating ligand or by alteration of receptor specificity through a glycosyl transferase. In contrast, we present this report of an instance where the abundance of the Notch-class mRNA itself is dynamically regulated. Taking advantage of the long cell cycle of the two-cell-stage embryo of the leech *Helobdella robusta*, we show that this regulation is achieved at the levels of both transcript stability and transcription. Moreover, MAPK signaling plays a significant role in regulating accumulation of the transcript by virtue of its effect on *Hro-notch* mRNA stability. Intracellular injection of heterologous reporter mRNAs shows that the *Hro-notch* 3' UTR, containing seven AU-rich elements, is key to regulating transcript stability. Thus, we show that regulation of the Notch pathway can occur at a previously underappreciated level, namely that of transcript stability. Given that AU-rich elements occur in the 3' UTR of Notch-class genes in *Drosophila*, human, and *Caenorhabditis elegans*, regulation of Notch signaling by modulation of mRNA levels may be operating in other animals as well.

Helobdella | leech | mRNA turnover | 3' UTR

Notch signaling functions in a variety of developmental contexts in different embryonic and tissue systems (1). The main framework of the pathway is evolutionarily conserved; thus, its many roles reflect cooption of an ancestral signaling pathway. Notch signaling regulates diverse functions such as neurogenesis and pattern formation in embryonic development. Regulation of the Notch signaling pathway has been studied extensively, with the conclusion that regulation of Notch itself occurs mainly at the translational or posttranslational levels (2, 3). For example, in the eight-cell *Caenorhabditis elegans* embryo the *notch* homolog *glp-1* is expressed uniformly in all of the cells; restriction of signaling is achieved by regulated translation of the *glp-1* mRNA in a cell-specific manner (3). Posttranslation regulation of Notch is achieved by processes such as glycosylation (4) or by regulated degradation of the signal-transducing Notch intracellular domain (5). Little is known about the regulation of *notch* mRNA itself.

Here, we report that both transcription and mRNA stability are involved in generating a dynamic pattern of *Hro-notch* mRNA expression in the two-cell embryo of the leech *Helobdella robusta*. The cellular simplicity and the long cell cycle of the two-cell leech embryo allowed us to address the temporal dynamics of *Hro-notch* mRNA stability in detail. Our demonstration of 3' UTR-regulated, oscillating expression of Notch-class transcript levels represents a previously uncharacterized mode of regulation of this ancient and ubiquitous signaling pathway. Furthermore, the presence of pentameric AU-rich elements (AREs) in the 3' UTR of *Hro-notch* as well as those from other species, and the correlation of *Hro-notch* expression

with p38MAPK phosphorylation suggest that this mechanism may also be operative in other organisms. Such 3' UTR-mediated transcript stability has been reported for mRNAs encoding cytokines (6), but this work demonstrates that this mechanism can also operate for the Notch receptor. Previous studies have demonstrated a role for the ERK pathway in regulating Notch expression and signaling in *Drosophila* and *C. elegans*, respectively (7, 8). The role of the p38MAPK pathway in regulation of Notch transcript levels, introduces another mode of cross-talk between two distinct signal-transduction pathways.

Results and Discussion

Helobdella development is characterized by determinate lineages, beginning with an unequal first cleavage. Prolonged cell cycles and the ability to stage embryos precisely (relative to polar body formation or the initiation of first cleavage) permit us to observe developmental events with good temporal resolution relative to the cell cycles and cell divisions during cleavage. The examination of such carefully timed embryos by *in situ* hybridization under identical reaction conditions reveals a dynamic pattern of *Hro-notch* mRNA levels during the two-cell stage. *Hro-notch* is present as a maternal transcript (Fig. 1A), and *in situ* hybridization shows *Hro-notch* at uniform, basal levels in the zygote and in both blastomeres (AB and CD) at time points immediately after first cleavage (265–280 min after zygote deposition (AZD)) (Fig. 1B). Beginning \approx 15 min after cytokinesis, however, the *in situ* signal starts to fluctuate in a reproducible manner, being stronger first in CD (in 78%; $n = 14$ of 18 stained embryos at 280–295 min AZD; Fig. 1C), and then in AB (in 84%; $n = 67$ of 80 stained embryos at 305–325 min AZD; Fig. 1D), and finally in CD again (in 58%; $n = 18$ of 31 stained embryos at 340–355 min AZD; Fig. 1E). We call these periods of biased expression the CD-1, the AB, and the CD-2 phases, respectively. Embryos fixed at most time points show a mix of staining patterns that we attribute to errors in timing, developmental noise, and the transition from one phase to another [Fig. 1G; and see supporting information (SI) Fig. 6]. After the CD-2 phase, none of the embryos processed for *in situ* hybridization showed staining in either of the blastomeres (Fig. 1F) until the second cleavage. *Hro-notch* expression continues in various cells throughout cleavage (9), but to analyze mechanisms regulating this dynamic expression pattern, we chose to take advantage of the cellular simplicity of the two-cell leech embryo, in which we have documented a similarly dynamic pattern of WNT protein expression and signaling (10).

We hypothesized that this dynamic pattern of *Hro-notch* mRNA expression reflects a well regulated and cell-specific

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Abbreviations: ARE, AU-rich element; AZD, after zygote deposition; PAE, polyadenylation element; sqRT-PCR, semiquantitative RT-PCR.

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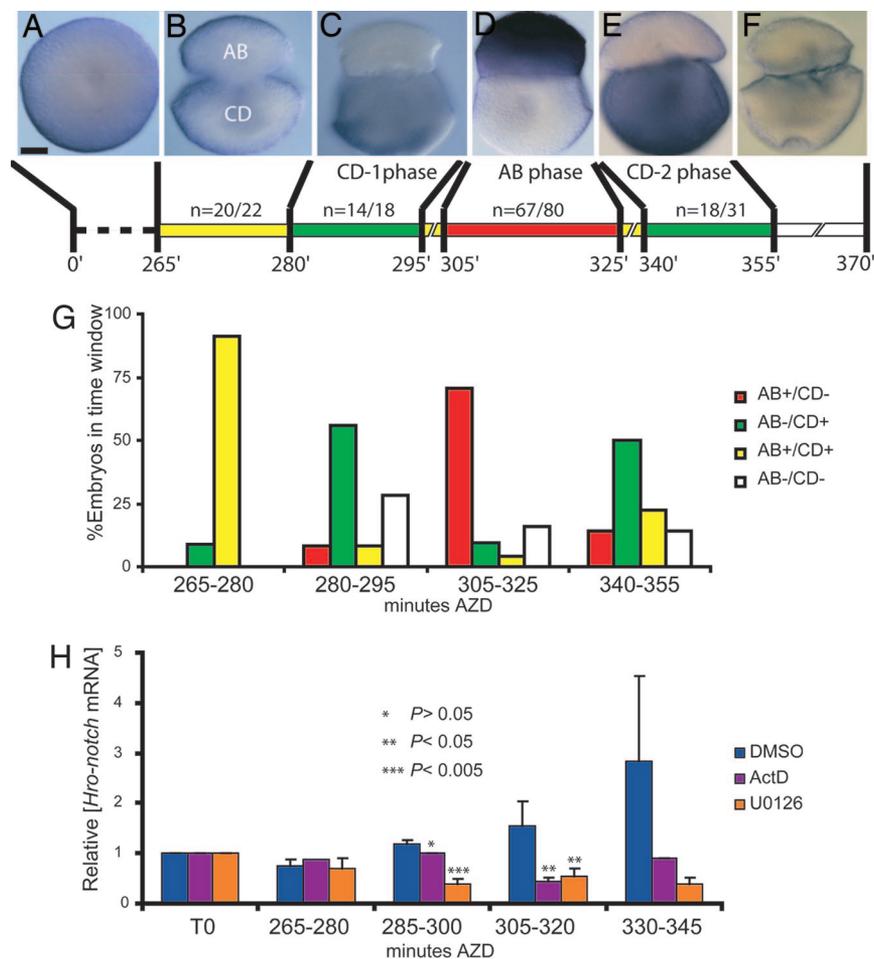


Fig. 1. Dynamic, expression of *Hro-notch* mRNA in the two-cell *Helobdella* embryo (265–370 min AZD; AB is up in this and all subsequent figures). (A–F) Zygote and two-cell-stage embryos processed by *in situ* hybridization for *Hro-notch* under identical conditions; those shown in B–E are from a single experiment. (G) Numerical analysis of two-cell embryos stained for *Hro-notch* shows that predominant patterns follow the progression summarized in the color-coded time line in A–F and in subsequent figures. Data presented are from a total of 178 two-cell embryos. (H) sqRT-PCR from control embryos (blue) reveals a steady increase in *Hro-notch* mRNA during the two-cell stage. This increase is abolished by treatment with actinomycin-D (purple) or U0126 (orange). Actinomycin-D has no effect on mRNA levels until after 300 min AZD ($P > 0.05$). U0126 treatment, however, induces a significant decline in the amount of *Hro-notch* mRNA starting from early stage 2 ($P < 0.005$). sqRT-PCR was done on groups of five embryos in each time window. Data shown are a compilation of a total of five experiments; at each time point, error bars represent standard error (SEM). (Scale bar: 100 μm .)

combination of differential mRNA stability and/or zygotic transcription. Comparing embryos fixed during the different phases and processed in parallel for *in situ* hybridization revealed that staining intensity of blastomere CD during the CD-1 phase is roughly comparable to the basal levels seen in the zygote and in both blastomeres immediately after first cleavage. This finding suggested that the CD-1 phase of cell-specific *Hro-notch* expression resulted from the loss of *Hro-notch* mRNA in cell AB. In contrast, staining in the AB and CD blastomeres during AB-1 and CD-2 phases, respectively, is more intense than the earlier staining. This observation, coupled with the reappearance of *in situ* signal after it has been lost in both AB and CD, suggests that the dynamic *in situ* pattern reflects zygotic transcription of *Hro-notch*, at least during the mid and late two-cell stage (305 min AZD onwards).

To test whether zygotic transcription is associated with the observed *in situ* hybridization pattern, we performed semiquantitative RT-PCR (sqRT-PCR) on precisely timed two-cell embryos, cultured either in medium containing a transcription inhibitor (500 $\mu\text{g}/\text{ml}$ of actinomycin-D in Htr medium containing 0.1% DMSO) or an equivalent concentration of DMSO alone. The DMSO-treated control embryos show a significant increase

in *Hro-notch* mRNA during the AB and CD-2 phases of the two-cell stage relative to earlier time points, and actinomycin-D treatment abolishes this increase (Fig. 1H). Similar results were obtained from embryos injected with another transcription inhibitor (α -amanitin), and inhibitor-treated embryos show a decrease in the *in situ* staining associated with the AB and CD-2 phases (data not shown). These data indicate that the AB and CD-2 phases of *Hro-notch* expression are associated with accumulation of zygotic transcripts in AB and CD blastomeres, respectively, and that zygotic transcription contributes little to the CD-1 phase. Thus, the CD-1 phase is a reflection of selective stabilization of maternal *Hro-notch* mRNA in blastomere CD relative to that in blastomere AB.

Differential mRNA stability is affected by intracellular factors that interact with structural elements commonly located within the 3' UTR of specific mRNAs (11). The p38MAPK pathway has been implicated in regulating the stability of mRNAs bearing the pentameric AREs, AUUUA (6). It has been proposed that p38MAPK influences the interaction between AREs and cytoplasmic factors by phosphorylating such transacting factors (12–15); specifically, activated p38MAPK stabilizes ARE-bearing mRNAs by in-

results do not rule out possible contributions of MAPK pathways other than p38MAPK in this process. In particular, given the U0126 results, it seemed reasonable to consider that ERK signaling also played a role in activating *Hro-notch* transcription during late stage 2, as has been shown for *Drosophila* (7). However, we were unable to detect phosphorylated ERK by using a monoclonal Ab specific for the phosphorylated protein.

To test the hypothesis that the 3' UTR of *Hro-notch* is sufficient for the observed expression pattern in the two-cell embryo, we injected zygotes with *egfp::N3' UTR-pae*, a reporter mRNA containing eGFP coding sequence, the 3' UTR of *Hro-notch* and an SV40 polyadenylation element (PAE) to prevent degradation of nonpolyadenylated mRNA (25, 26). As a control, we injected mRNA containing eGFP and PAE sequences only (*egfp-pae*). We chose to analyze the *in situ* pattern of the exogenous *egfp* mRNA at 275', 285', 310', and 350' (to represent Pre-CD1, CD-1, AB, and CD-2 phases of *Hro-notch*, respectively), because these time points showed the most robust and least variable staining of *Hro-notch* during the respective phases (SI Fig. 6). Using *in situ* hybridization for *egfp* to follow the injected mRNAs after first cleavage, we found that, in embryos injected with *egfp-pae*, blastomeres AB and CD stain with equal intensities at all time points, and the *in situ* signal showed a gradual decline over time ($n = 49$) (Fig. 3 A–D). In contrast, the staining pattern in embryos injected with *egfp::N3' UTR-pae* recapitulated the early portion of the dynamic *Hro-notch* pattern (Fig. 3 A' and B'). AB and CD stained equally in most of the embryos fixed soon after cleavage. Staining was lost from AB in a significant number ($\approx 30\%$) of embryos by time points corresponding to the CD-1 phase (Fig. 3C') and was lost in both cells in most ($\approx 71\%$) of the embryos by the beginning of CD-2 phase (Fig. 3D'). A significant number of embryos during the CD-1 and AB phases retained staining in both cells; we attribute this to the variability in the amount of mRNA delivered intracellularly, which might be above physiologically relevant concentrations in these embryos. Notwithstanding this observation, we point out that none of the embryos showed detectable staining in blastomere AB after the onset of the CD-1 phase, which reflects the rapid degradation of the mRNA in blastomere AB as compared with that in blastomere CD. This finding suggests that the *Hro-notch* 3' UTR targets the reporter mRNA molecule for degradation and that *egfp::N3' UTR-pae* mRNA is selectively stabilized in the cell with activated p38MAPK. Moreover, the disappearance of *egfp::N3' UTR-pae* reporter mRNA during the CD-2 phase further corroborates the interpretation that the *Hro-notch in situ* signal during this time is indeed due to the accumulation of zygotic transcript.

As a further test of the hypothesis that p38MAPKpp signaling inhibits ARE-mediated degradation of endogenous *Hro-notch* mRNA, we injected zygotes with *in vitro*-transcribed *Hro-notch* 3' UTR. We predicted that the synthetic mRNA would compete with endogenous transcripts for binding the postulated p38MAPKpp-activated stabilization factors, thereby destabilizing the endogenous transcripts. As predicted, the *in situ* signal for endogenous *Hro-notch*, during the AB and CD-2 phases, was reduced in most embryos injected with the 3' UTR (Fig. 4 C and D) compared with those injected with water (Fig. 4 A and B). Using sqRT-PCR to quantify this result, we measured roughly equivalent reductions of endogenous *Hro-notch* mRNA (26% and 32% in the AB and CD-2 phases, respectively) in embryos injected with *Hro-notch* 3' UTR relative to those injected with water (data not shown). The observed level of transcript reduction reflects the balance between enhanced degradation and ongoing transcription. It should be noted that the sqRT-PCR measurements underestimate the extent of the reduction because they were carried out on pools of five embryos each for which the success of the mRNA injections could not be ascertained. These results suggest that even the later (AB and CD-2)

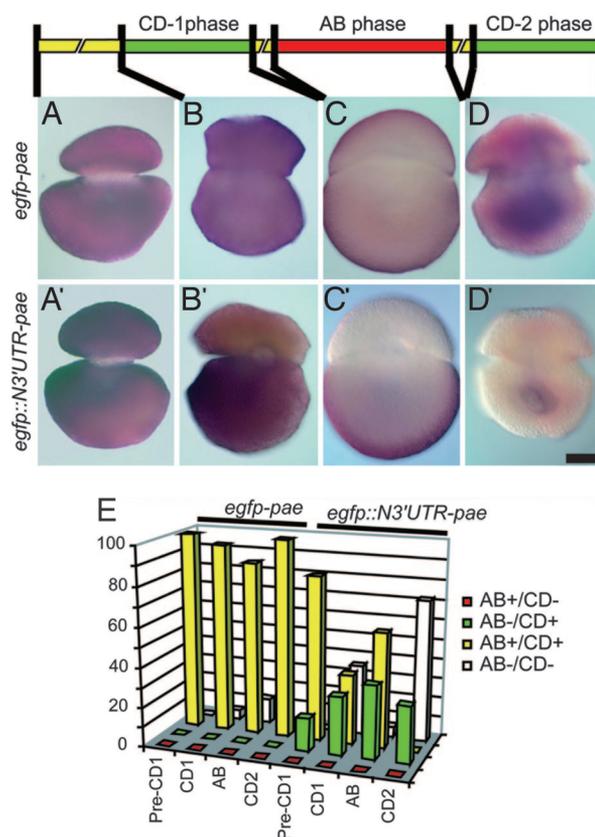


Fig. 3. Destabilization of reporter mRNA by the *Hro-notch* 3' UTR. Zygotes, injected with equal concentrations of *egfp-pae* mRNA (A–D) or *egfp::N3' UTR-pae* mRNA (A'–D'), were fixed at time points corresponding to the color-coded phases of *Hro-notch* expression (see Fig. 1) and then processed by *in situ* hybridization for *egfp*. *egfp-pae* mRNA is inherited uniformly by blastomeres AB, and CD and is largely stable in both the cells through the two-cell stage. *egfp::N3' UTR-pae* mRNA is inherited equally by blastomeres AB and CD at first cleavage (A') but shows more pronounced degradation in blastomere AB during the CD-1 phase (compare B and B'). The *egfp::N3' UTR-pae* mRNA is cleared from blastomere AB by the AB phase (C') and is mostly degraded in blastomere CD by the CD-2 phase (D'). (E) Numerical distribution of *in situ* hybridization patterns for *egfp* in embryos injected with *egfp-pae* or *egfp-N3' UTR-pae*. Note that none of the embryos injected with *egfp::N3' UTR-pae* mRNA showed a stronger signal in AB than in CD. All embryos (except A and A') were processed for *in situ* hybridization simultaneously. Coloration reaction for all embryos was limited to 10 min at room temperature in the dark. (Scale bar: 100 μm .)

phases of *Hro-notch* accumulation reflect the competing dynamics of mRNA transcription, stabilization, and degradation. It appears that when this dynamic is disrupted by the introduction of exogenous *Hro-notch* 3' UTRs that effectively sequester the available stabilization factors, endogenous *Hro-notch* mRNA is targeted for degradation by virtue of the presence of the AREs in its 3' UTR.

In conclusion, we show that transcript levels of a *Notch*-class gene (*Hro-notch*) oscillate antiphasically in the AB and CD blastomeres of the two-cell embryo in the leech *H. robusta*, i.e., high transcript levels in AB are associated with low levels in CD and vice versa (Fig. 5). Moreover, the *Hro-notch* levels are controlled by dynamic activation of one or more of the MAPK (p38MAPK and ERK) signaling pathways. Initially, the *Hro-notch* level in each cell reflects primarily inherited maternal transcripts. Later, the production and turnover of zygotic transcripts becomes important. The 3' UTR of *Hro-notch* mRNA confers a relatively short half-life to the transcripts, apparently because of the presence of multiple AREs. This instability is

under identical conditions of probe concentration (2 ng/ μ l), time and temperature of hybridization, and coloration reactions. Details of the *in situ* hybridization and protocol used are the same as described in ref. 9.

sqRT-PCR. Freshly laid zygotes were synchronized as described above. Batches of five embryos for each time window were collected in 25 μ l of Lysis buffer (cells-to-cDNA kit; Ambion, Austin, TX) on ice. Subsequent steps were as recommended by manufacturer. Nineteen microliters of the lysate from each of the reactions was used to set up the RT reaction with 100 units of SuperScript II RT (Invitrogen, Carlsbad, CA). The resulting cDNA was purified through a Qiaquick PCR purification column (Qiagen, Valencia, CA) and eluted in 30 μ l of distilled water. PCR was set up in the presence of 1 unit of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) and 1.5 mM MgCl₂. A 153-bp fragment was amplified by using the following primer pair: NRTF1, AACAAACCTTCCTACCTGGC and NRTR1, ATCCAGATCGGTGCGAACCTGT.

Cycling parameters for the amplification were 95°C for 20 sec, 58°C for 30 sec, and 72°C for 30 sec for an optimal number of cycles (23–32 cycles). To control for RNA extraction variability between individual samples, an \approx 450-bp fragment of 18S cDNA was amplified in parallel by using 18S Classic primer pair (Ambion). Conditions for amplifying the 18S fragment were 95°C for 1 min, 60°C for 1 min, and 72°C for 30 sec for five cycles, followed by 95°C for 1 min, 58°C for 1 min, and 72°C for 30 s.

Amplicons were resolved by electrophoresis on a 1.5% agarose gel containing Etbr. Densitometry analysis was carried out on bands by using Alphaimager (Alpha Innotech, San Leandro, CA). After normalizing for background, ratios of band intensity for *Hro-notch* to that of 18S from different samples were plotted against time.

For sqRT-PCR on embryos treated with actinomycin-D or U0126, embryos were cultured in Htr media containing 500 μ g/ml actinomycin-D (catalog no. A5156; Sigma-Aldrich, St. Louis, MO) or 50 μ M U0126 (catalog no. V1121; Promega, Madison, WI) and 0.1% DMSO. Actinomycin-D and U0126 were suspended in water or DMSO to obtain stock concentrations of 20 mg/ml and 50 mM, respectively.

Immunostaining. Zygotes were synchronized as described above; details of subsequent steps were as described in ref. 10. Embryos

were incubated in anti-dpp38MAPK (catalog no. M8177; Sigma-Aldrich, St. Louis, MO) in 1 \times PBS with 1% Tween 20 and 10% normal goat serum (PTN) at 4°C. Subsequently, embryos were rinsed in 1 \times PBS containing 1% Tween 20 (PBTw) and incubated in peroxidase-conjugated goat anti-mouse (catalog no. 115–036-003, 1:1,000; Jackson ImmunoResearch, West Grove, PA) in PTN at 4°C overnight. Embryos were then washed in PBTw at room temperature with frequent changes of the wash buffer. Color reaction was carried out in the presence of 0.5 mg/ml diaminobenzidine in 0.75 \times PBS and 0.003% H₂O₂. After color development, embryos were dehydrated through an ethanol series and mounted in Murray's-Clear for bright field microscopy (E800; Nikon, East Rutherford, NJ).

Histology. Embryos immunostained for p38MAPKpp were dehydrated through an ethanol series and embedded in glycol methacrylate resin (JB4 embedding kit; Polysciences) as per the manufacturer's instructions. Embryos were sectioned by using a glass knife on a microtome (MT-2B; Sorvall, Newtown, CT). Sections were stained with DAPI (1:1,000 dilution in water) in the dark for 30 min, rinsed with water, air dried, and mounted in Fluormount (BDH) for fluorescence and bright-field microscopy (Nikon).

Plasmid Constructs and *in Vitro* Transcription. *Hro-notch* 3' UTR was cloned into XbaI/PstI linearized pCS2-egfp plasmid. *egfp-pae* and *egfp::N3'* UTR-*pae* mRNA was transcribed from linearized plasmids by using the mMessage-machine kit as per the manufacturer's instructions (Ambion). mRNA was injected at the indicated concentrations, and embryos were subsequently processed for *in situ* hybridization. Synthetic *Hro-notch* 3' UTR was prepared from linearized pGEMT-Easy bearing *Hro-notch*-3' UTR by using the T7-megascript kit as per the manufacturer's instructions (Ambion). Purified mRNA was injected into zygotes that were subsequently processed for *in situ* hybridization for *Hro-notch*.

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