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SHORT COMMUNICATION

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Expression cloning in ascidians: isolation of a novel member of the asctacin protease family

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Abstract The small genome size and gene number of ascidians makes them an ideal model system in which to screen for conserved genes that regulate the development of chordates. Expression cloning has proven to be an effective strategy for isolating genes that play a role in embryogenesis. We have taken advantage of the large size and ease of manipulation of *Xenopus* embryos for use as an assay system to screen for developmental regulatory genes from the ascidian *Ciona intestinalis*. Many invertebrate genes have been shown to function in vertebrates, providing us with precedent for our cross-species analysis. The first clone isolated from this screen is an astacin class metalloprotease. This ascidian astacin, named *no va*, causes a gastrulation defect in *Xenopus*. In *C. intestinalis*, *no va* is expressed both maternally and zygotically. The zygotic expression is seen in the mesenchyme of gastrula and neurula staged embryos.

Keywords Ascidian · Expression cloning · Astacin metalloprotease · Mesenchyme · *Xenopus*

Introduction

Ascidians are invertebrate members of the chordate phylum. Despite being closely related to the vertebrates, ascidians are estimated to have only around 15,000 protein-encoding genes, which is less than one-quarter of what is expected for vertebrates (Simmen et al. 1998). Additionally, the ascidian genome is estimated to be approximately 5% of the size of the human genome, and is thought to have less functional redundancy (Satoh 1994). Ascidians are also morphologically simple. The free-swimming ascidian larva (tadpole) has approximately 2,800 cells, and is comprised of relatively few cell types (Satoh 1994). These features make ascidians an attractive model organism for both embryology and genetics.

One particularly fruitful approach for isolating developmentally important genes from vertebrates has been expression cloning (Lustig et al. 1997; Smith and Harland 1991). In this strategy in vitro transcribed RNAs are made from pools of clones from a cDNA library and injected into early *Xenopus* embryos. The injected embryos are allowed to develop, and then screened for developmental abnormalities. Pools with activity are subdivided into progressively smaller pools until single clones are isolated. This approach has yielded a number of genes that gave rise to pronounced phenotypes. The expression cloning approach is not biased toward any particular gene type, and, significantly, genes that act both cell autonomously, such as components of signal transduction pathways and putative transcription factors, and genes that encode secreted inducing factors, and thus act non-cell autonomously, have been isolated. Examples include the cell autonomous genes in the MAD family of signal transducing factors (Baker and Harland 1996). Examples of non-cell autonomous factors include the gene *Xwnt-8* (Smith and Harland 1991).

The expression cloning approach is biased toward genes whose products have high specific activity. This problem has lead to the use of the “small pool” modification of the original expression cloning strategy (Lustig et al. 1997). The difficulty with the small pool strategy is the large numbers of pools that need to be screened in order to achieve the same degree of saturation, particularly since normalized cDNA libraries are not typically used. We have reasoned that the reduced genetic complexity of the *Ciona* genome could provide a distinct advantage in expression cloning. Because of the smaller number of genes, we should be able to survey a larger representation of the transcripts by screening the same number of pools. Furthermore, the increased genetic complexity of vertebrates appears to be due to two successive whole-genome duplications that occurred after the split with ascidians (Sidow 1996). Thus, the bulk of

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the increased complexity of vertebrates in comparison to ascidians appears to be an increase in the number of members of gene families, not in the number of gene families themselves. Therefore, expression screens with ascidian cDNAs should allow for a more complete representation of the classes of gene families.

While expression cloning of ascidian libraries by injection into ascidian embryos themselves would appear to be the most direct assay system, and in fact this approach has been used successfully and has led to the isolation of a syndecan from *Ciona savignyi* (Satou et al. 1999), the microinjection of ascidian embryos is laborious and difficult. To circumvent the difficulties of injecting the large number of ascidian embryos that would be needed, we chose instead to use *Xenopus* embryos to assay for ascidian genes by expression cloning. Although this assay requires that the genes work cross-species, there is ample precedent for invertebrate genes functioning in *Xenopus*, including *Drosophila easter*, *spatzle* and *toll* (Armstrong et al. 1998).

Here we report the isolation of a novel member of the astacin protease family from the ascidian *C. intestinalis* by expression cloning in *Xenopus* embryos. The new member has been named "*no va*" because of the gastrulation block it causes in *Xenopus* embryos. Members of the astacin family include the vertebrate bone morphogeneic protein 1 (BMP1) and *Drosophila* tolloid. However, the similarity of *no va* to these family members does not extend beyond the metalloprotease domain. Instead *no va* shows greatest similarity to a different class of astacin proteases that have C-terminal "toxin" domains. This class has not been previously identified in chordates, and is typified by the cnidarian astacins PMP1 and HMP1. PMP1 is expressed in the medusa or larval stage of developing jellyfish, and then later in the gut. This expression pattern suggests a role both in development and digestion (Pan et al. 1998). HMP1 is expressed in *Hydra* especially during regeneration of head structures and anti-sense experiments suggest that HMP1 is necessary for proper head regeneration (Yan et al. 2000). In *C. intestinalis*, *no va* is expressed maternally and zygotically. The zygotic expression was observed in the mesenchyme of neurula- and tailbud-stage embryos. The presence of homologues of *no va* in diverse phyla from Cnidaria to Chordates, coupled with its affect on *Xenopus* development, suggests that this subclass of astacins may play important roles in development.

Materials and methods

Library screening

A cDNA library was constructed from gastrula-stage *C. intestinalis*. Sperm and eggs from *C. intestinalis* collected from the Santa Barbara Harbor were obtained by dissection. The eggs were fertilized with dilute sperm solutions in filtered seawater. The embryos (1.5 ml of packed embryos) were raised at room temperature to gastrula stage at which time the embryos were homogenized in a Dounce homogenizer in 5 ml 100 mM NaCl, 20 mM Tris-Cl, pH 7.8, 10 mM EDTA, 1% SDS, and 0.2 mg/ml proteinase K (embryo lysis buffer). After incubation at 42°C for 1 h, the lysates were twice extracted with phenol/chloroform, once with chloroform, and the nucleic acid ethanol precipitated. The nucleic acid was suspended in water and the RNA was selectively precipitated in 2.5 M LiCl. Poly-A^+ RNA was isolated from the total RNA using Oliogtex (Qiagen) and cDNA was synthesized using the Superscript Plasmid System for cDNA synthesis (Gibco BRL). cDNA (10 ng) was then cloned into the pSport expression vector (Gibco BRL).

The library was transfected into Supercompetent XL1-Blue MRF′ *Escherichia coli* (Stratagene). A total of around 13,000 colonies were generated. Pools of around 1,300 colonies were created by combining the bacteria from each colony. Part of each pool was used to make a glycerol stock and the remainder was used to isolate plasmid. Isolated pooled plasmid was linearized with *Not*I and used in a T7 in vitro transcription reaction using mMessage mMachine (Ambion). Ten nl of pooled RNA was injected into one-cell *Xenopus* embryos at 100 pg/nl. When pools of 1,300 were sub-divided, the glycerol stock was used to inoculate 12–20 plates with 150–200 *E. coli* colonies each. Each plate was amplified by pressing the original plate onto a velvet cloth. A replica plate was repeatedly pressed to the velvet more than 20 times. Replica plates were grown overnight. Bacteria from each amplified plate were collected, a glycerol stock made, and plasmid isolated from the remainder. Glycerol stocks of positive pools were used to generate ten plates of 50–100 colonies. These plates were amplified, glycerol stocks made, and plasmid isolated. Once a positive plate was identified, each colony on the original plate of 50–100 was used to grow individual 2-ml liquid cultures. Each liquid culture was used to make a glycerol stock and to isolate plasmid. Pools were created from combining ten individual clones, which were then used in the assay. Positive pools of ten were then separated into their individual clones for analysis.

Sequencing and primary sequence analysis

No va was sequenced using a combination of the dideoxy chain termination method using a combination of Sequenase DNA Sequencing Kit (Amersham Life Science) and the Big Dye Chain Termination Kit (PE Applied Biosystems) on both strands. The isolated cDNA contained a single long open reading frame suggesting that the entire open reading frame was isolated along with its associated untranslated regions.

Northern blot and in situ hybridization

The *no va* northern blot was performed using 10 µg total RNA. The *no va* probe was generated using Prime-A-Gene (Promega) with an *Xba*I fragment of *no va* (nucleotides 199–1987). The same blot was striped and probed with a full-length tubulin probe, which was generated using Prime-A-Gene. The probes were hybridized in Quickhyb (Stratagene) and washed in 2× SSC for 15 min at room.

The *no va* in situ hybridization was performed as described previously (Wada et al. 1995) using an anti-sense probe corresponding to the first 1,179 nucleotides of *no va* and a full-length sense *no va* probe. The color reaction was performed at room temperature overnight in BM Purple.

Results and discussion

To screen for gene products with developmental activity from the ascidian *C. intestinalis*, a gastrula-stage cDNA library was first tested in ten pools of 1,300 clones. Capped, poly-A^+ RNA was in vitro transcribed from each pool and injected into one-cell *Xenopus* embryos. The initial assay was to cut ectodermal explants at blastula

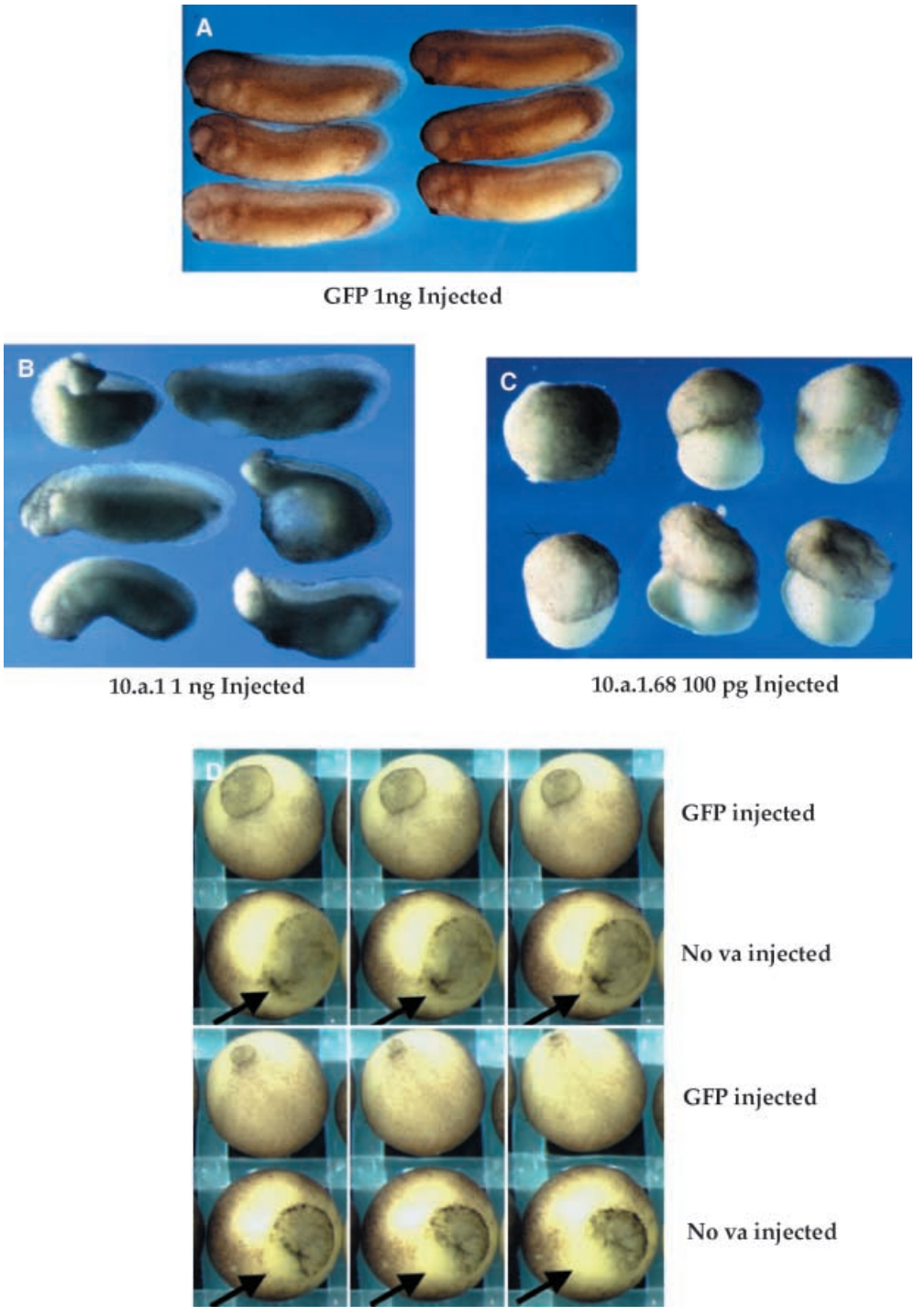

Fig. 1A–C Expression cloning results. **A**. GFP RNA was used as a negative control injection. **B**. Embryos with reduced anterior structures as a result of a pooled RNA injection (10.a.1). **C**. Single clone (10.a.1.68) RNA injection, after sib-selection, responsible for the effect seen in **B**. **D** Gastrulation block viewed by time-lapse photography. A photograph was taken every 5 min throughout gastrulation (not all photographs are included). In each image, the embryos are oriented with the vegetal pole *up*, the *arrow* points to the blastopore lip in the *no va* RNA-injected (100 pg) embryo that appears to "fall out." The control, GFP RNA-injected (1 ng) embryo is at the *top* of each image

stages, grow the explants in isolation until tailbud stages, and then assay for induction of neural or muscle genes by RT-PCR. Pool number 10 in the initial screen was selected as a putative muscle inducer based on this screen. This pool was divided into 12 plates of approximately 140 clones and the assay repeated. Muscle inducing activity was not observed in any pool from the subdivided pool number 10. However, one pool of 150 clones was found to cause anterior deletions in *Xenopus* when whole embryos were screened at tailbud stages. The pool causing the anterior deletions was subdivided and sib-selected until a single clone, 10.a.1.68, was identified that completely radialized *Xenopus* embryos (Fig. 1). Analysis of 10.a.1.68-injected embryos throughout early development revealed a gastrulation defect starting at stage 11, when a complete blastopore ring formed but failed to close (Fig. 1D). Because of its gastrulation defect, clone 10.a.1.68 has been named *no va*. No va in Spanish means "doesn't go." The characterization of the gastrulation block in *Xenopus* will be discussed elsewhere.

A.

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  1 MMLHITSYVL LLDLFIGHHL AFAQTEKECN DVVLQQGQNY PLEVINACAQ
 51 RRLRQNSPSG ALNSPCTAEP DCGVETCVFG FRKDASGCRV SCQCSDGEGL
101 YEGDIELTDL TKTFLLKNVD TGVPTPAPTY DPLVQHAAGR SIRLWNNIRE
151 GNNFVVPYAV SRGIGSSGRA AIAAAVRDFD ANTCIRLRPS TRYSGRPYLY
201 MYPGGGCSSP VGRQSSRQQV SLASGCWQKG TVIHEILHSL GFWHEQSRPD
251 RDSHVRINTA NIFRGMAYNF NKMSNRQINS RNSPYDIGSV MHYNSYAFSS
301 NRRPTITDLQ GRPITTQRNG FSRQDLIQLN AMYGCTTTGT GGGGTGTGGG
351 GTGTGGGGTG TGGGGTGTGG GGGGGGGGGS CVDKNSLCSS WAQNGECRNN
401 PRYMLPNCCR SCRGGTTCTD NSVNCPVWAR RGECQNNPRW MTPNCCRSCR
451 ASPTRPPTTT PRPRPTSCSD KSRNCPAWAR ARYCSARLYA SWMSINCQRS
501 CNRCSRG
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B.

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No va       CVDKN--SLCSSWAQNGECRNNPRYMLPNC---CRSC

PMP1        CPNNH--SRCQEWADRGECQKNPRYMLRNCKKSCKQC

HMP1        CEDSH--SNCAAWAKANECNKNPNWMRPNCKKSCGTC

T04G9.2     CRNLR--GDCDDLAKQGWCIRNPGWMRANCPISCGMC

ShK-Toxin CIDTIPKSRCTAFQ----CKHSMKYRLSFCRKTCGTC
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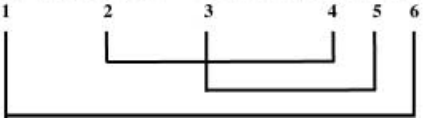

Fig. 2 **A** Predicted amino acid sequence of *no va*. The predicted signal peptide is *boxed*. The conserved metalloprotease domains corresponding to HEXXHXXGFXHEXXRXDR and SXMHY are indicated in *bold*. The glycine rich domain is *italicized* and the three toxin domains are *underlined*. **B** Comparison of the toxin domains of *no va*, the cnidarian astacins *PMP1* and *HMP1*, the predicted *Caenorhabditis elegans* astacin *T04G9.2* and the sea anemone *ShK-toxin*. Identical residues are in *bold*. The six conserved cysteines are as indicated with the predicted intrachain bonding diagrammed *below*

The *No va* cDNA was found to have a single, long open reading frame (Fig. 2A). The predicted amino acid translation of *no va* contained homology to the astacin family of metalloproteases (Bond and Beynon 1995). The genes most closely related to *no va* are the cnidarian astacin metalloproteases PMP-1 (Pan et al. 1998), and HMP-1 (Yan et al. 2000) and a *C. elegans* putative astacin metalloprotease, T04G9.2 (*C. elegans* Sequencing Consortium 1998). These four astacins are characterized by their "toxin" domains. The toxin domain was first identified in a family of sea anemone potassium channel poisons (Tudor et al. 1996). This domain is characterized by six conserved cysteine residues (Fig. 2B). The spacing of these cysteines and their pattern of intrachain bonding distinguish them from other cysteine folds including the EGF domain. PMP1 and HMP1 both have a single toxin domain following the metalloprotease domain, while No va and T04G9.2 both have three repeats of the toxin domain. A search of GenBank reveals the presence of the toxin domain in a wide variety of proteins including vertebrate tyrosinases, and other proteases. However, no vertebrate homologues were found that contain the combination of an astacin protease and a toxin domain. An alignment of the toxin domains from No va, T04G9.2, PMP-1 and HMP-1, as well as a non-astacin toxin domain from sea anemone (ShK-toxin), shows the six conserved cysteine residues (Fig. 2B). However, the four astacins show greater amino acid sequence similarity among themselves than they do to the sea anemone potassium channel toxin domain. Other astacin family members such as BMP-1 and Tolloid are characterized by repeated CUB and EGF domains at the C-terminus (Bond and Beynon 1995). Because No va does not contain the EGF and CUB domains that are found in these astacins, it is unlikely to be a direct orthologue of these genes. In fact, there is little homology between No va and the Tolloid family members outside of the metalloprotease domain. The percent identity of No va to Xolloid is 25%, while the similarity is 31%. In comparison, No va and PMP1 are 42% identical and 50% similar. In addition, No va, unlike all other known astacins, has a glycine-rich domain between the metalloprotease and toxin domains (Fig. 2A).

The expression of *no va* was examined by northern blotting using total RNA from staged *C. intestinalis* (Fig. 3A). The *no va* transcript was detected throughout development, including in the unfertilized egg, indicating that the gene is maternally expressed. We were unable to detect the *no va* transcript by in situ hybridization before the neurula stage, indicating that the early expression of *no va* may be ubiquitous, and thus difficult to detect by in situ hybridization. In the neurula and tailbud embryos *no va* is expressed strongly in the mesenchyme of the trunk. The mesenchyme of ascidians is induced beginning at the 32-cell stage (Kim et al. 2000), suggesting that the peak in expression at the 64-cell stage may be a reflection of this induction and may mark the onset of zygotic transcription of *no va*. In the adult, *no va* is expressed in heart and intestine, but not the ovary.

We have attempted to obtain functional data in ascidians for *no va*, including misexpression by plasmid electroporation, overexpression by RNA injection, and antisense experiments. So far, these experiments have not resulted in a detectable phenotype. However, the phenotype of *no va* in *Xenopus* is very pronounced, and may provide clues to the function of this class of astacin proteases.

While certain structural features of the toxin class of astacins are conserved between chordates, nematodes and cniderians, the developmental mechanisms between these animals have diverged so extensively that it unlikely that the precise function of these metalloproteases is conserved. It is most likely that the toxin domains of these astacin proteases play a role in determining substrate specificity, as C-terminal domains have been shown to function in other astacin proteases (Bond and Benyon 1995). The presence of *no va* in ascidians and additional homologues in cnidarians and nematodes coupled with the gastrulation defect caused by *no va* RNA injection in *Xenopus* indicates that this subclass of astacins plays important roles in development. Analysis of the cnidarian homologues supports this hypothesis. *HMP1* is required for head regeneration in *Hydra* (Yan

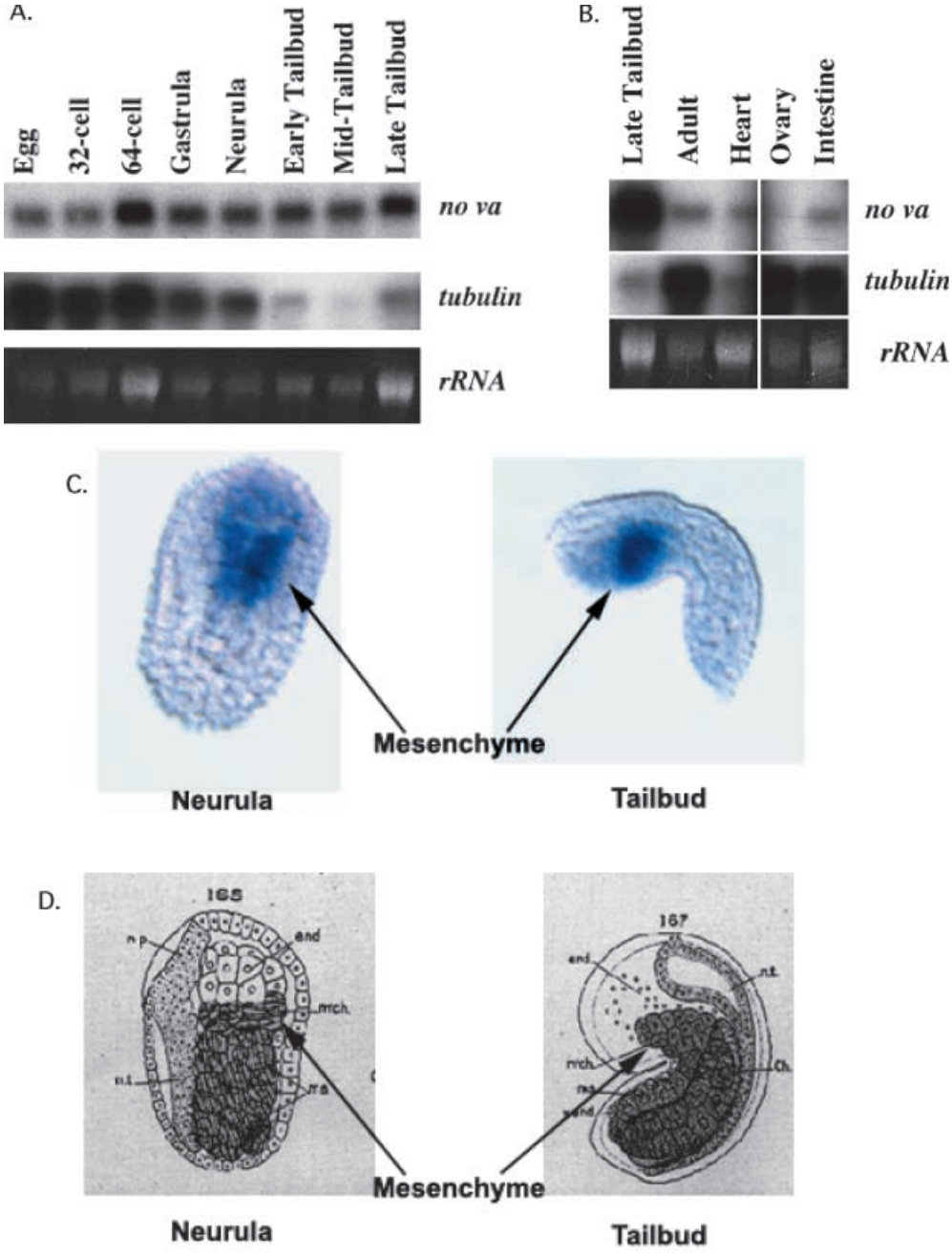

Fig. 3A–D Developmental northern blots and in situ hybridization. **A** *No va* expression is seen throughout early development. *Tubulin* is maternally expressed and its expression level declines throughout development. The ethidium bromide-stained *rRNA* is included as a loading control. **B**. *No va* is expressed in adult tissue, but at lower levels than embryonic stages. The blot was exposed for a longer period of time compared to the embryonic northern. The late tailbud stage is included for comparison of the expression levels between adult tissues and embryonic stages. rRNA is included as a loading control. **C** In situ hybridization for *no va*. *No va* expression is seen in the mesenchyme of neurula (ventral view) and tailbud (lateral view) staged embryos. **D** Conklin's schematic drawings of the lateral view of neurula and tailbud staged embryos (Conklin 1905)

et al. 2000). Based on in situ hybridization analysis, *PMP1* in jellyfish may have two functions. The first would be specification of medusa bud development, and the second would be digestive (Pan et al. 1998).

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