Reference: *Biol. Bull.* **211:** 7–17. (August 2006)

Characterization and Role of p53 Family Members in the Symbiont-Induced Morphogenesis of the *Euprymna scolopes* Light Organ

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Abstract. Within hours of hatching, the squid *Euprymna scolopes* forms a specific light organ symbiosis with the marine luminous bacterium *Vibrio fischeri.* Interactions with the symbiont result in the loss of a complex ciliated epithelium dedicated to promoting colonization of host tissue, and some or all of this loss is due to widespread, symbiont-induced apoptosis. Members of the p53 family, including p53, p63, and p73, are conserved across broad phyletic lines and *p63* is thought to be the ancestral gene. These proteins have been shown to induce apoptosis and developmental morphogenesis. In this study, we characterized *p63*-like transcripts from mRNA isolated from the symbiotic tissues of *E. scolopes* and described their role in symbiont-induced morphogenesis. Using degenerate RT-PCR and RACE PCR, we identified two *p63*-like transcripts encoding proteins of 431 and 567 amino acids. These transcripts shared identical nucleotides where they overlapped, suggesting that they are splice variants of the same gene. Immunocytochemistry and Western blots using an antibody specific for *E. scolopes* suggested that the p53 family members are activated in cells of the symbiont-harvesting structures of the symbiotic light organ. We propose that once the symbiosis is initiated, a symbiont-induced signal activates p53 family members, inducing apoptosis and developmental morphogenesis of the light organ.

Received 29 December 2005; accepted 26 May 2006.

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Introduction

The Hawaiian bobtail squid *Euprymna scolopes* Berry, 1913, forms a specific, environmentally transmitted, light organ symbiosis with the marine luminescent bacterium *Vibrio fischeri.* The juvenile squid hatch from egg clutches after about 20 days of embryogenesis. On hatching, the symbiont is harvested from the water column by the activity of two elaborate fields of ciliated epithelia that are specific to the juvenile light organ (reviewed in Nyholm and McFall-Ngai, 2004). The harvested symbionts colonize crypt spaces within the light organ. Once the symbiosis is initiated, the juvenile light organ undergoes a series of symbiont-induced developmental changes. The most conspicuous of these changes is the apoptosis and regression of the cells of the ciliated epithelial field over the first 4 days of the association—a morphogenesis that occurs several cell layers away from the colonizing population of *V. fischeri* (Montgomery and McFall-Ngai, 1994; Doino and McFall-Ngai, 1995; Foster *et al.,* 2000). Derivatives of the microbe-associated molecular patterns (MAMPs) lipopolysaccharide and peptidoglycan, which are shed from the surfaces of colonizing *V. fischeri,* act in synergy to induce this morphogenic change (Koropatnick *et al.,* 2004). Juveniles of *E. scolopes* exposed to other marine environmental bacteria but not to *V. fischeri* retain the complete ciliated epithelial fields, and their light organ remains uncolonized (McFall-Ngai and Ruby, 1991). Thus, the ciliated epithelial fields are critical for the initiation of the symbiosis; the specificity of the response is determined by the select access of symbiont

ligands to host receptors; and the host response involves tissues that are remote from the colonizing symbionts in the crypts of the light organ.

Members of the p53 family of proteins have been implicated in the induction of apoptosis and developmental morphogenesis (White and Prives, 1999; Yang *et al.,* 1999, 2000; Mills *et al.,* 1999; Celli *et al.,* 1999). The p53 family consists of a number of structurally and functionally related, sequence-specific transcription factors. The best studied member of this family is the tumor-suppressor p53, the most frequently mutated gene in many types of tumors (Greenblatt *et al.,* 1994; Hainaut *et al.,* 1998). Native p53 is a homotetramer in solution and is maintained at a low protein level in cells by its negative regulator MDM2 (Kubbutat *et al.,* 1997; Haupt *et al.,* 1997). Both p53 and MDM2 shuttle between the nucleus and the cytoplasm. In the absence of a suitable signal, p53 and MDM2 form a complex in the nucleus that causes the ubiquitination of p53 and its nuclear export to the cytoplasm, where it is degraded by the proteasome. In response to activation factors, such as developmental cues, DNA damage, or hypoxia, p53 is stabilized by phosphorylation. This modification in the protein reduces the interaction between p53 and MDM2, interfering with both the nuclear export and ubiquitination of p53 and resulting in a rapid increase in the level of p53 in the nucleus and its activation as a transcription factor (Ashcroft and Vousden, 1999; Jin *et al.,* 2000; Gottifredi and Prives, 2001).

Two other proteins that are structurally related to p53 have been discovered in vertebrates: p63 and p73 (Schmale and Bamberger, 1997; Kaghad *et al.,* 1997). Although these proteins share similar transcriptional functions and the ability to induce apoptosis, each appears to play a distinct role in development and tumor suppression. Alternate 5′ promoter sites, as well as splicing at the 3′ end of *p63* and *p73*, generate additional complexity and may further modify the actions of these genes (Yang *et al.,* 2002) Homologs of the p53 family have also been identified in *Drosophila melanogaster* (Jin *et al.,* 2000; Ollman *et al.,* 2000) and *Caenorhabditis elegans* (Derry *et al.,* 2001), and in the molluscs *Loligo forbesi* (Ishioka *et al.,* 1995), *Mya arenaria* (Kelley *et al.,* 2001), *Spisula solidissima* (Jessen-Eller *et al.,* 2002), *Mytilus edulis* (Muttray *et al.,* 2005; Ciocan and Rotchell, 2005), and *Mytilus trossulus* (Muttray *et al.,* 2005). Phylogenetic analyses indicate that the *p63* gene is ancestral to the divergence of the Ecdysozoa, Lophotrochozoa, and Deuterostomia (Aguinaldo *et al.,* 1997; Kelley *et al.,* 2001; Yang *et al.,* 2002). The invertebrate homologs of the p53 family appear to have expression and subcellular localization patterns similar to those of the vertebrate p53 family members. However, the cellular effect of the invertebrate p53 homologs, resulting in apoptosis and cell death, has been studied only in *D. melanogaster* (Jin *et al.,* 2000; Ollman *et al.,* 2000) and *C. elegans* (Derry *et al.,* 2001).

In addition to their role in development and tumor suppression, members of the p53 family have been implicated in the responses of host tissues to microbes. Several types of pathogenic bacteria induce apoptosis in the cells of host tissue with which they associate (Norimatsu *et al.,* 1995; Guichon and Zychlinsky, 1996; Zychlinsky *et al.,* 1996), and *Helicobacter pylori* infection has been linked to increased *p53* expression in gastric mucosa (Li *et al.,* 2005; Ozturk *et al.,* 2005). However, unlike the effects that *V. fischeri* has on the cells of the ciliated epithelial field of the *E. scolopes* light organ, the effects in these studies are produced by bacteria that are closely associated with the host tissues undergoing apoptosis.

In this study, we characterize members of the p53 family in the squid *Euprymna scolopes,* and we investigate the presence and role of these proteins in the symbiont-induced developmental morphogenesis of host tissues.

Materials and Methods

General procedures

Adult specimens of *E. scolopes* were collected from shallow sand flats of Oahu, Hawaii, and breeding colonies were maintained as described previously (Doino and McFall-Ngai, 1995; Foster *et al.,* 2000). Upon hatching, the juveniles were rinsed and placed individually in 5 ml of either filter-sterilized seawater (FSSW) or FSSW containing 5×10^3 cells ml^{-1} of wild-type *V. fischeri* strain ES114 (Boettcher and Ruby, 1990). Colonization of the light organ was monitored using a photometer (Turner Designs TD-20/20 luminometer, Sunnydale, CA) to measure bacterial luminescence emitted by each animal. All juveniles emitting luminescence above background levels are symbiotic, as confirmed by plating experiments (McFall-Ngai and Ruby, 1991). The light organs of juveniles that had been exposed to *V. fischeri* (symbiotic animals) and juveniles that had not been exposed to *V. fischeri,* and therefore were not colonized (nonsymbiotic animals), were dissected from the animal at specific times after hatching. The light organ was exposed by opening the ventral mantle cavity of the juvenile and then, using fine forceps (Ted Pella, Redding, CA), peeling back the siphon. The exposed light organ was then dissected away from the surrounding tissue. RNA and protein were extracted from whole light organs. Unless otherwise stated, reagents were purchased from Sigma-Aldrich (St. Louis, MO).

RNA extraction

Light organs were collected in RNAlater (10 light organs per 100 μl; Ambion, Austin, TX) and stored at -20 °C until use. For use in cloning and rapid amplification of cDNA ends (RACE) PCR techniques, total RNA was extracted from hatchling and 12-h and 24-h symbiotic and nonsym-

biotic light organs (100 each time/treatment) with the use of TriPure Isolation Reagent (Roche, Mannheim, Germany) following the manufacturer's protocol. Eukaryotic messenger RNA (mRNA) was purified from the total RNA pools using the MPG mRNA purification kit (CPG, Lincoln Park, NJ). Total RNA for use in quantitative real-time PCR applications was extracted using the MasterPure complete DNA and RNA purification kit (Epicentre, Madison, WI) following the manufacturer's protocol. Quality and quantity of RNA was determined by gel electrophoresis and spectrophotometric methods, respectively.

Cloning of the squid p53-like mRNA

Primers p53F1 (5′-CTACAACATGGACTTATTCT-3′) and p53R1 (5′-TGCTTTTCTATCTCTTC-3′) were designed to the conserved p53 DNA-binding region from alignments (Clustal X; Thompson *et al.,* 1997) of DNA sequences of arrow squid (*Loligo forbesi;* GenBank accession number U43596) and clam (*Mya arenaria;* GenBank accession numbers AF253323 and AF253324). These primers were used with 12-h symbiotic light organ RNA template in reverse transcription-PCR (Access RT-PCR System; Promega, Madison, WI) using 1× AMV/*Tfl* buffer, 1.5 m*M* $MgSO_4$, 0.2 m*M* dNTP, 0.2 μM primers, 0.1 U AMV reverse transcriptase, 0.1 U *Tfl* DNA polymerase, and 1 ng mRNA template in a 50-μl reaction volume. Thermal cycling conditions were as follows: 45 min at 48 °C; 2 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 50 °C, 1 min at 68 °C; 5 min at 68 °C. The resulting PCR product was purified using the Geneclean kit (Bio101, Carlsbad, CA) following the manufacturer's instructions, and ligated into pGEM-T Easy Vector (Promega, Madison, WI). Calcium-competent cells of *Escherichia coli* DH5α were transformed with the resultant plasmids. Recombinant colonies were screened for the insert of the predicted length, and plasmids were prepared for sequencing using QIAprep spin miniprep kit columns (Qiagen, Valencia, CA). PCR products were sequenced from the recombinant plasmid using T7 and SP6 primers (Promega, Madison, WI) at the University of Hawaii sequencing facility. The resulting sequence was identified as being similar to the DNA-binding domain of *p53* family genes through BLAST searches of GenBank (Altschul *et al.,* 1990). The *E. scolopes* sequence was compared to the *p53* family DNA-binding domains of other organisms by alignment using Clustal X (Thompson *et al.,* 1997).

5′- and 3′-rapid amplification of cDNA ends (RACE)

The 5′- and 3′-RACE PCR was performed using the SMART RACE cDNA amplification kit including the Advantage II PCR kit (BD Biosciences, Palo Alto, CA). The 5′- and 3′-RACE-ready cDNA was produced using 1 μg of 24-h symbiotic light organ mRNA following the manufacturer's instructions. Primers p53RACEF5 (5′-TGTCCGCTGTGAACACAAATTGGCAA-3′) and p53RACER1 (5′-GGCAAGCACATATTCGCACTCAACAGCT-3′) were constructed internal to the amplification product of p53F1 and p53R1. The 5′- and 3′-RACE reactions were performed following manufacturer's instructions with the following thermal cycling conditions: 5 cycles of 30 s at 94 °C, 3 min at 72 °C; 5 cycles of 30 s at 94 °C, 30 s at 70 °C; 3 min at 72 °C; and 25 cycles of 30 s at 94 °C, 30 s at 68 °C, and 3 min at 72 °C. The resulting amplification products were gel purified using the Geneclean kit (Bio101, Carlsbad, CA) and were cloned and sequenced as above. For each RACE amplification product, a consensus contiguous sequence was generated by aligning all sequenced clones (5′-RACE $n = 4$; 3′-RACE (628 bp) $n = 4$; 3′-RACE (1033 bp) $n = 9$). The *E. scolopes* derived amino acid sequences were compared to the p53 family members of other organisms by alignment and the construction of an unrooted bootstrapped (1000 iterations) neighbor-joining tree based on the Kimura 2-parameter correction using Clustal X (Thompson *et al.,* 1997) with gapped positions excluded.

Quantitative real-time PCR (QRT-PCR)

Fifty nanograms of total RNA from each timepoint/treatment was converted to cDNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA) following the manufacturer's instructions. QRT-PCR was performed using 1 μl of cDNA using the iCycler system and the iQ SYBR green supermix (BioRad, Hercules, CA) following the manufacturer's instructions. Briefly, reactions were carried out using 1 × iQ SYBR green supermix, 0.2 μM forward and 0.2 μM reverse primers, in a 25-μl reaction volume. *E. scolopes p53*-specific primers pairs, p53F1 (above) and p53RTR1 (5′-CTTGTACATGTTCTGGTTTC-3′) yielded a single amplicon of 150 bp within the DNA-binding domain. Reactions were normalized to β-actin, the transcript levels of which do not change at the time points investigated (Kimbell and McFall-Ngai, 2004). β-actin was amplified using primer pair actin6773 (5′-GAGCGTAAATACTCTGTC-3′) and actinRTR1 (5′-GAGAATTTGTAGAGTAGCG-3′), yielding a single amplicon of 148 bp. Thermal cycling conditions for both sets of primers were as follows: 30 min at 60 °C; 50 cycles of 30 s at 95 °C, 30 s at 54 °C, 30 s at 72 °C; and 60 cycles of 10 s at 60 °C with an increasing increment of 0.5 °C every cycle. Fold differences were determined through the efficiency-compensated delta delta Ct method (Pfaffl, 2001). The efficiency (E) of the reaction was determined by constructing standard curves generated using serial dilutions of template, and using the formula $E = (10^{-1/\text{standard curve slope}}) - 1$. A slope of -3.32 generates an efficiency of 100%. Only reactions with greater than 95% efficiency were used in fold-difference determination. The specificity of the reaction was confirmed by observing a single peak on analysis of reaction-product melt curves.

Data were obtained from three separate total RNA extractions at each time point/treatment. Each reaction was carried out in triplicate per QRT-PCR run.

Immunocytochemistry

A 336-bp amplified product of the p53-like DNA-binding domain was cloned and expressed using the IMPACT-CN System (New England Biolabs, Beverly, MA) following the manufacturer's instructions. The expressed protein was intein tagged and purified by binding the tagged protein to a chitin column and then performing an on-column cleavage of the intein tag. The purified expressed protein was concentrated using a Centricon-30 microconcentrator (Millipore, Bedford, MA), and rabbit polyclonal antibodies were made to concentrated protein (Covance, Denver, PA). These antibodies were used in the immunocytochemical staining of nonsymbiotic and symbiotic *E. scolopes* at 4 h, 14 h, and 24 h post-inoculation. These time points were chosen because they coincide with the onset of apoptosis (4 h), the peak of apoptosis (14 h), and the onset of visible regression (24 h) in symbiotic juveniles. Unless indicated, the immunocytochemistry procedures were carried out at 4 °C. Symbiotic and nonsymbiotic juvenile squid were fixed overnight in 4% paraformaldehyde in marine phosphate-buffered saline (mPBS: 50 m*M* sodium phosphate buffer, pH 7.4, with 0.45 *M* NaCl). The animals were rinsed in mPBS, and their mantles were ventrally cut to expose the light organ and gills. The specimens were permeabilized by soaking in 1% Triton-X in mPBS for 2 d, and blocked overnight with 1% Triton-X in mPBS, 1% goat serum. and 0.5% BSA. They were then exposed to the *E. scolopes* p53 antibodies (1:50 dilution), or pre-immune serum as a control, for 4 days. After rinsing with 1% Triton-X in mPBS and an additional overnight of blocking, the specimens were exposed overnight to fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG secondary antibodies and then rinsed with 1% Triton-X in mPBS. Propidium iodide was used to counterstain the samples to allow visualization of cell nuclei. Animals were RNase treated by incubating at 37 °C for 20 min with 100 μg ml^{-1} DNase-free RNase in 2X SSC (0.3 *M* NaCl, 30 m*M* sodium citrate, pH 7.0) and then incubated at room temperature for 5 min with 500 n*M* propidium iodide in 2× SSC. Animal tissues were visualized using a Zeiss LSM510 confocal microscope.

Protein extraction and Western blot

Symbiotic and nonsymbiotic juveniles were collected at 14 h, flash frozen, and stored at −80 °C. Nuclear and cytoplasmic proteins were extracted from the light organs of these animals (500 per treatment) using a modification of the method described by Dignam *et al.* (1983); *i.e.,* "buffer C" was made isosmotic to seawater using NaCl (final concentration of 0.53 *M*) and the final dialysis step was omitted. The time point of 14 h was chosen because it coincides with the peak of apoptosis and with visualization of a large difference in the mean volumes of cross reactivity of the *E. scolopes* p53-like antibody between symbiotic and nonsymbiotic animals. Nuclear and cytoplasmic extracts were concentrated using Centricon-10 microconcentrators (Millipore, Bedford, MA). Protein concentrations were determined using the Bradford method (Bradford, 1976). Samples (30 μg) of the resulting cytoplasmic and nuclear total protein isolations were loaded into each lane and run on a SDS-PAGE gel following standard protocol. The companion SDS-PAGE gel was silver stained using standard procedures (Oakley *et al.,* 1980). Western blots using the *E. scolopes* p53-like DNA-binding domain antibody described above were also performed. Briefly, proteins were electrophoretically transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA) (procedure modified from Towbin *et al.,* 1979). Blots were blocked for 2 h in 50 m*M* Tris, 150 m*M* NaCl, and 0.5% Tween 20, pH 7.5 (TTBS), with the addition of 4% milk. Blots were then incubated overnight in 1:100 dilution of antiserum in 1% milk/TTBS. After three washes in TTBS, blots were incubated for 45 min in a mixture of 1% milk/TTBS, 1:3000 goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA), and 1:3300 avidin-conjugated horseradish peroxidase (to detect biotinylated molecular mass markers) (Bio-Rad, Hercules, CA). Bands of cross-reactivity were detected by chemiluminescence (ECL plus chemiluminescence kit, Amersham Bioscience Corp., Piscataway, NJ). The experiment was performed in duplicate.

Results

Isolation of E. scolopes *p53-like cDNA*

Using primers designed to the conserved *p53* DNA-binding region, a 489-bp product was amplified from mRNA of 12-h symbiotic light organs. The *E. scolopes* sequence was most closely related to the DNA-binding domain of a *p53*-like transcript from the arrow squid, *Loligo forbesi,* with 88% identity at the nucleotide level and 96% identity at the amino acid level. Critical residues of the DNA-binding region that have been identified in human p53 are conserved in the *E. scolopes* sequence (Fig. 1). Multiple 5′-RACE reactions reproducibly identified a single *p53*-like coding sequence of 913 bp, implying a single 5′ promoter site, consistent with studies of other invertebrate *p53* family members (Jin *et al.,* 2000; Ollmann *et al.,* 2000; Kelley *et al.,* 2001; Jessen-Eller *et al.,* 2002). Multiple 3′-RACE reactions reproducibly amplified at least two different coding sequences of lengths 628 bp and 1033 bp. The design of the RACE primers provided 190 bp of overlapping sequence between the primers (Fig: 2) when the 5′- and 3′-RACE sequences were aligned. This overlap, coupled with the identification of multiple identical sequences from

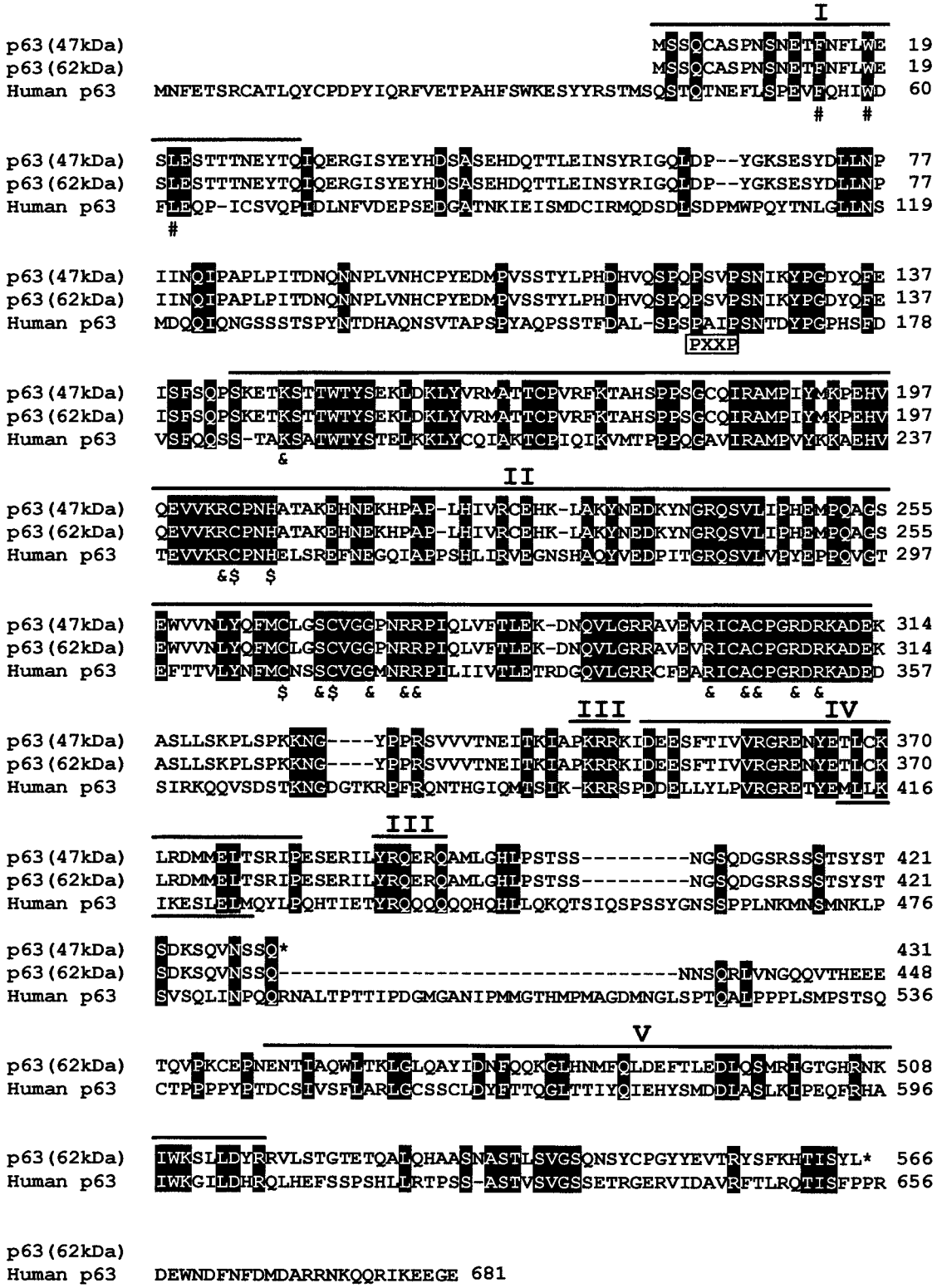

Figure 1. Alignment of the derived amino acid sequences for the *Euprymna scolopes* p63 sequences with human p63. The *E. scolopes* p53-like molecules at molecular masses of 47 kDa and 62 kDa are identical where they overlap. Comparison of the *E. scolopes* sequences with p53 family proteins from other organisms revealed a number of conserved domains: transactivation domain (I), DNA-binding domain (II), nuclear localization signals (III), oligomerization domain (IV), nuclear export signal (underlined within the oligomerization domain), and sterile alpha motif (SAM)-like domain (V). Residues that form the zinc ion coordination site (required for sequence-specific DNA binding) are marked with "$". Residues that have a role in the structural integrity of the domain and DNA contact are marked with "&." Conserved hydrophobic residues that in human p53 interact with MDM2 are indicated with "#." PXXP indicates a proline-rich domain. Identical residues are shaded in black; * indicates potential stop codons. Genbank accession numbers: DQ247973; DQ247974; NM_003722.

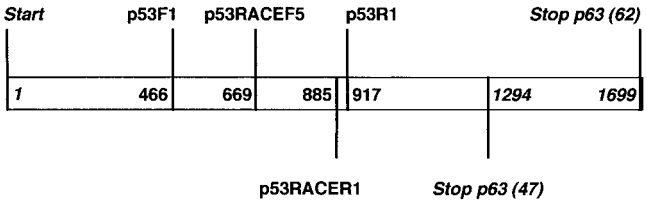

Figure 2. Schematic of the nucleotide sequences of *Euprymna scolopes p63* transcripts showing location of the primer sites. Numbers within the bar indicate the position of the first nucleotides of the primers or the start and stop codons (italics) of each transcript. Genbank accession numbers for *E. scolopes p63* (47) and *E. scolopes p63* (62) are DQ247973 and DQ247974, respectively.

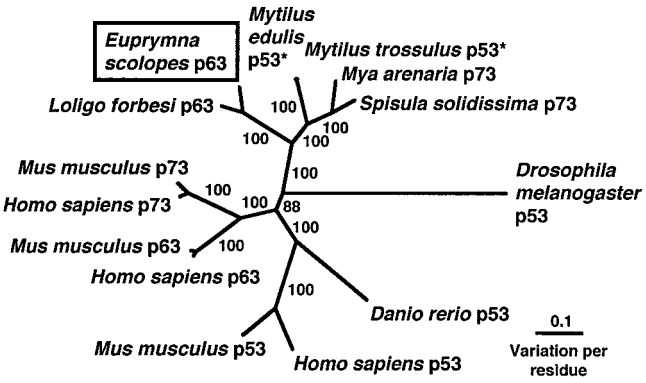

Figure 3. The relationship of p53-like protein sequences from *Euprymna scolopes* and other organisms. An unrooted, neighbor-joining tree with gapped positions excluded. Bootstrap values (%) are indicated. Genbank accession numbers clockwise from *Euprymna scolopes:* DQ247974, AY579472; AY611471; AF253324; AY289767; NM_206545; NM_131327; XM_043215; NM_011640; NM_003722; NM_011641; NM_005427; NM_011642; U43595. Note that only one sequence from *E. scolopes* is represented on the tree because both isoforms of *E. scolopes* p63 are identical where they overlap. Similarly, only one sequence from *M. arenaria* is represented on the tree because *M. arenaria* p53 and p73 are identical where they overlap. * *M. edulis* and *M. trossulus* p53 have only 1 amino acid difference and are therefore barely resolvable on the tree.

different 5′- and 3′-RACE reactions, allowed the construction of two full-length *p53*-like transcripts with open reading frame (ORF) lengths of 1296 bp and 1701 bp (Fig. 2). These lengths correspond to proteins of approximate molecular mass of 47 kDa and 62 kDa. These sequences share identical nucleotides where they overlap, adding to the evidence suggesting alternate splicing of *p53*-like genes in invertebrate systems, as reported in the clam *Mya arenaria* (Kelley *et al.,* 2001). The full-length *E. scolopes p53*-like transcripts are most similar to the *p53*-like transcript from the arrow squid, *Loligo forbesi* (Fig. 3) with identities of 83% and 84% at the nucleotide level, and 80% and 81% at the amino acid level for the 1293-bp and 1701-bp transcripts, respectively. Comparison of the two inferred p53-like proteins from *E. scolopes* with p53 family proteins from other organisms revealed regions of potential protein-protein interaction, including a proline-rich motif and a SAM (sterile alpha motif)-like domain in the 62-kDa *E. scolopes* p53-like protein. Studies of human p53 have shown a PXXP motif within the proline-rich domain to be involved in apoptosis, possibly by binding proteins containing a SH3-domain (Walker and Levine, 1996; Venot *et al.,* 1998). Similarly, residues that have been implicated as essential in binding the p53 negative regulator MDM2 (an E3 ubiquitin ligase) in human p53 (Kussie *et al.,* 1996; Dobbelstein *et al.,* 1999) are conserved in the *E. scolopes* p53-like proteins (amino acid residues F14, W18, and L21). Both *E. scolopes* proteins contained nuclear localization signals (NLS), and a nuclear export signal (NES) within the oligomerization domain (Fig. 1). The mollusc p53-like proteins form a separate well-supported group (Fig. 3). Vertebrate and invertebrate p53-like sequences form separate groupings, with mollusc p53-like proteins being most closely related to vertebrate p63. The fruitfly sequence has least similarity to the other p53-like sequences. Since the *E. scolopes p53*-like transcripts appear to be splice variants of the same gene and since the mollusc p53-like sequences are most similar to vertebrate p63, the *E. scolopes* p53 family members have been designated as p63 accompanied by their predicted subunit molecular weight.

Nuclear localization of E. scolopes *p53 family proteins in the light organ*

Western blots using an antibody raised against the DNA-binding domain of *E. scolopes* p63 indicate that at least three proteins are more abundant in the nuclei of light organs from 14-h symbiotic animals than in the nuclear protein component of the light organs of 14-h nonsymbiotic animals (Fig. 4). Multiple bands are expected because the antibody was raised against a domain conserved among all p53-like proteins. The three proteins showing the greatest abundance in the nuclei of 14-h symbiotic light organs have molecular masses of approximately 46, 63, and 95.5 kDa. The former two proteins are coincident with the sizes of the derived amino acid sequences of the *E. scolopes p63* transcripts identified by RACE PCR (approximately 47 and 62 kDa). The protein of mass 95.5 kDa is likely to be similar to another, larger p53 homolog that has also been detected in the bivalve molluscs *Mya arenaria* (Stephens *et al.,* 2001) and *Spisula solidissima* (Jessen-Eller *et al.,* 2002). In 14-h nonsymbiotic light organs, the three *E. scolopes* p53 family proteins are more abundant in the cytoplasmic protein extraction than in the nuclear protein extraction (Fig. 4B). Conversely, in 14-h symbiotic light organs, the three *E. scolopes* p53 family proteins exhibit higher abundance in the nuclear protein extraction than in the cytoplasmic protein extraction. These findings suggest that these three proteins have localized to the nucleus from the cytoplasm in the 14-h symbiotic light organs. The same amount of total protein was loaded into each lane of the companion SDS-

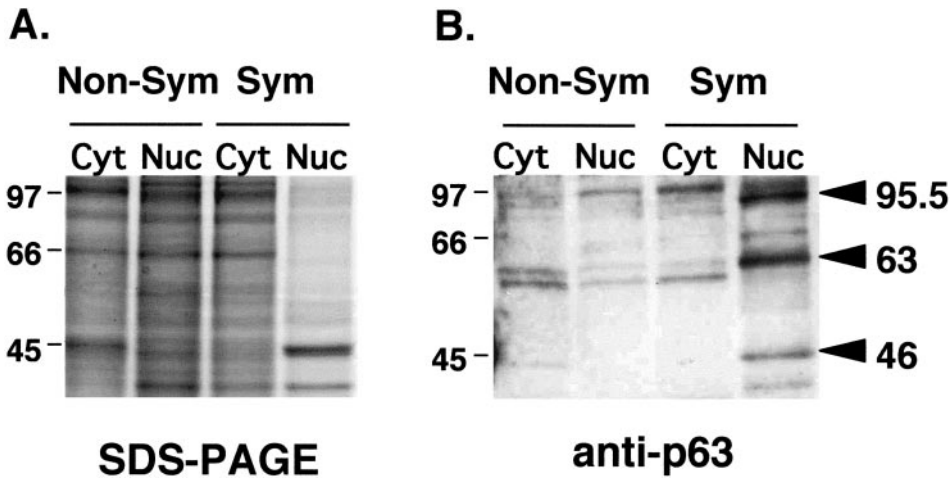

Figure 4. Localization of p63-like proteins in the light organ of *Euprymna scolopes* at 14 h post-inoculation. (A) Silver-stained companion SDS-PAGE gel to the Western blot in (B) showing total protein extracts from the cytoplasm (Cyt) and the nuclei (Nuc) of symbiotic (Sym) and nonsymbiotic (Non-Sym) animals. (B) Western blot showing cross-reactivity of an *E. scolopes* p53-like antibody with proteins from the cytoplasm and the nuclei of symbiotic and nonsymbiotic animals. Numbers represent protein size in kilodaltons. Results are representative of duplicate experiments.

PAGE gel. Therefore, the differences in staining of the total protein components of 14-h symbiotic and nonsymbiotic light organs suggest that there are fewer, more abundant proteins in the nuclei of 14-h symbiotic light organs than in the cytoplasm and nuclei of 14-h nonsymbiotic light organs and in the cytoplasm of 14-h symbiotic light organs (Fig. 4A).

Patterns of E. scolopes *p53 family protein occurrence and gene expression in light organ tissues*

In immunocytochemistry experiments, the p53-like proteins were visualized as small spots of cross-reactivity within the nuclei of the cells of the ciliated epithelial fields of the light organ (Fig. 5A) and within the nuclei of the gill cells. The gill was used as a control tissue because, like the light organ, it is in direct contact with *V. fischeri* in the seawater. Previous immunocytochemical studies of p53 family members in certain invertebrates and vertebrates have shown similar punctate nuclear staining (Liang *et al.,* 1993; Bonsing *et al.,* 1997; Yin *et al.,* 1997; Kelley *et al.,* 2001), which was attributed to the possibility that p53 participates in the modulation of transcription in the nucleolus (Liang *et al.,* 1993). No difference in the number of cross-reactive sites in the nuclei of cells of the superficial ciliated field was detected between each of the treatments/timepoints. Assuming these sites of cross-reactivity to be spherical, we compared the mean volumes of cross-reactive sites. The cells of the superficial ciliated field of symbiotic juveniles had significantly larger cross-reactive sites than those of nonsymbiotic animals at each time point (Fig. 5B). No significant differences were found between the size of cross-reactive sites in the gill cells of symbiotic and nonsymbiotic animals. QRT-PCR revealed no difference in *p53* transcript levels between 12-h symbiotic and nonsymbiotic juvenile light organs, or between 24-h symbiotic and nonsymbiotic juvenile light organs (data not shown).

Discussion

The results of our analyses provide evidence for the activity of p53 family members in the developmental morphogenesis of the juvenile light organ at the initiation of the *Euprymna scolopes-Vibrio fischeri* symbiosis. Two transcripts were identified that have high sequence similarity to other vertebrate and invertebrate p53-family members, as well as conservation of critical domains. Although the *E. scolopes* transcripts were of different lengths, they were identical where they overlapped and thus appear to be splice variants of the same gene. Alternate splicing is well documented in vertebrate *p63* and *p73* gene transcripts, resulting in several functional, differentially expressed isoforms (Yang *et al.,* 2002). Similarly, splice variants of *p53*-like genes have been described in the bivalve molluscs *Mya arenaria* and *Spisula solidissima* (Kelley *et al.,* 2001; Stephens *et al.,* 2001; Jessen-Eller *et al.,* 2002; Cox *et al.,* 2003).

Our data, coupled with other invertebrate p53 family sequence data, suggest that the current nomenclature applied to invertebrate p53 family sequences may be misleading. Assignment of names to invertebrate p53 family members has thus far been based on the vertebrate nomenclature and currently relies more on sequence length and presence or absence of a conserved domain than on sequence differ-

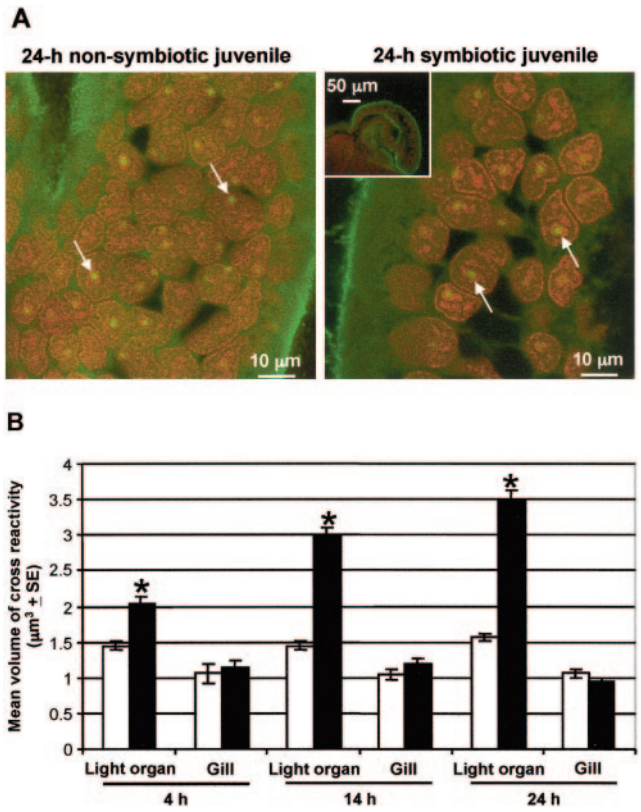

Figure 5. The amount of cross-reactivity of p63 protein in the nucleus of the superficial ciliated field cells. (A) Confocal micrographs showing sections through the anterior appendage of 24-h nonsymbiotic and symbiotic juveniles at the same magnification. Propidium iodide stains cell nuclei red, while the FITC-labeled secondary antibody stains green. Areas of cross-reactivity in the nuclei of cells are visualized as yellow spots. Pre-immune serum controls (inset) do not exhibit spots of cross-reactivity. Apoptosis of cells and regression results in a reduction in the compaction of cells within the superficial ciliated field cells of 24-h symbiotic juveniles. (B) Comparison of the mean volume of cross-reactivity of p53-like proteins in the cells of the superficial ciliated field ($n = 50$ at each timepoint) and gill ($n = 25$ at each timepoint) at 4 h, 14 h, and 24 h post-inoculation in nonsymbiotic (open bars), and symbiotic (filled bars) juveniles. Asterisks indicate means that are significantly different ($P < 0.05$; ANOVA followed by Tukey's pairwise comparison) from that of the nonsymbiotic treatment. Error bars represent standard error of the mean. Volume was calculated by assuming the site of cross-reactivity to be spherical and measuring its diameter.

ences. This protocol wrongly attributes splice variants of the same gene to different groups within the p53 family. For example, human p53 and p73 are different lengths and exhibit different sequences where they overlap when they are aligned, so that human p53 is more similar to mouse p53 than to human p73 (Fig. 3). Conversely, while *Mya arenaria* "p73" contains a sterile alpha motif (SAM) domain and is longer than *M. arenaria* "p53" (Kelley *et al.,* 2001; Stephens *et al.,* 2001), alignment of these sequences shows that they are almost identical where they overlap. Therefore, *M. arenaria* "p53" and "p73" are more similar to each other than they are to other molluscan p53 family members or to their respective vertebrate namesakes, and could be splice variants of the same gene (Kelley *et al.,* 2001; Stephens *et al.,* 2001). Further, current data indicate that the *p63* gene antedated the divergence of the Ecdysozoa, Lophotrochozoa, and Deuterostomia (Aguinaldo *et al.,* 1997; Kelley *et al.,* 2001; Yang *et al.,* 2002). This ancestral position of p63 is supported by the occurrence of SAM-like domains in all three of the major branches of the animal kingdom and by the finding that invertebrate p53 family members are most similar to vertebrate p63. Therefore, we have chosen to designate the p53 family sequences of *E. scolopes* as p63 accompanied by their predicted subunit molecular weight.

Another aim of this study was to determine the activity of *E. scolopes* p53 family members in the cells of the light organ. We chose to detect activation of p53 family members by examining their accumulation in the nuclei of activated cells (Gottfredi and Prives, 2001; Zhang and Xiong, 2001; Inoue *et al.,* 2005). Immunoblotting using an antibody raised against the DNA-binding domain of *E. scolopes* p63 identified at least three proteins that coincide with sizes of p53 family members in the molluscs *Mya arenaria* (Kelley *et al.,* 2001) and *Spisula solidissima* (Jessen-Eller *et al.,* 2002, Cox *et al.,* 2003). We were not able to identify the transcript associated with the 95.5-kDa protein by using the same primers we employed to isolate the transcripts of *E. scolopes* p63 (47) and p63 (62), suggesting that the 95.5-kDa protein has a similar, but not identical, sequence to *E. scolopes* p63. As well as picking up the three strongly cross-reactive bands, the Western blot with the *E. scolopes* p63 antibody also revealed a number of less strongly cross-reactive bands. The amount of squid material (see Materials and Methods) needed for each Western blot precluded the running of multiple gels to perfect blocking and thus minimize nonspecific cross-reactivity. We are confident, however, that at least the three strongly cross-reactive bands described represent *E. scolopes* p53 family members because the antibody was raised against the DNA-binding domain of *E. scolopes* p63. In addition, the cross-reactive bands correspond to the sizes of bands that would be expected from *E. scolopes* p63 proteins and other molluscan p53 family members (Kelley *et al.,* 2001; Jessen-Eller *et al.,* 2002). Further, their patterns of nuclear and cytoplasmic localization within the light organ cells correlate with the onset of symbiosis-induced apoptosis.

Immunocytochemistry indicated that *E. scolopes* p53 family members were present in the nuclei of cells of the ciliated epithelial field in both symbiotic and nonsymbiotic juveniles. This result is not unexpected since p53-like molecules shuttle between the nucleus and the cytoplasm (Ashcroft and Vousden, 1999; Jin *et al.,* 2000; Gottifredi and Prives, 2001). However, more p53-like protein was detected in the nuclei of the ciliated epithelial field of symbiotic juveniles at 4, 14, and 24 h post-inoculation than in similarly aged nonsymbiotic juveniles, as determined by volu-

metric measurement of cross-reactivity and by Western blots of the cytoplasmic and nuclear extracts of 14-h symbiotic and nonsymbiotic light organs. This increase in the amount of protein in the nuclei of cells of the ciliated epithelial field of symbiotic juveniles was observed in all three detectable *E. scolopes* p53-like proteins and was not the result of increased transcript levels, implying that the increase in protein levels was the result of accumulation of existing p53-like protein in the nuclei of ciliated epithelial cells in symbiotic juveniles. Although recent studies suggest that the level of *p53* transcription may be important in some cancers and during the cell cycle (Reisman and Loging, 1998; Raman *et al.,* 2000; Boggs and Reisman, 2006; Niu *et al.,* 2005), the amount of p53 protein in cells is predominantly determined by the rate at which p53 is degraded rather than the rate it is synthesized (Grand *et al.,* 1995; Giaccia and Kastan, 1998; Vogelstein *et al.,* 2000).

The accumulation of p53-like proteins in the nuclei suggests that the proteins were activated in these cells. If this is the case, activation of p53-like proteins occurs as early as 4 h after exposure to *V. fischeri,* a time that coincides with the migration of the symbiont into crypts of the light organ and the onset of apoptosis in the ciliated epithelial fields of symbiotic juveniles. Activation continues through the peak of apoptosis (14 h post-inoculation) and through the onset of visible regression of the ciliated epithelial fields (24 h post-inoculation). Because this developmental morphogenesis does not occur in *E. scolopes* juveniles exposed to other environmental bacteria but not to *V. fischeri* (McFall-Ngai and Ruby, 1991), the signal to initiate the accumulation and activation of p53-like proteins in cells of the ciliated epithelial field is apparently specific to the colonization of the light organ by *V. fischeri.* How the signal reaches the cells of the ciliated epithelial field that are undergoing cell death from the colonizing symbionts in the crypts of the light organ remains to be determined. However, because the interaction of the microbe-associated molecular pattern (MAMP) lipopolysaccharide with the epithelial cells of the light organ crypt also induces apoptosis in cells of the ciliated epithelial field (Foster *et al.,* 2000), the host is likely to detect colonization of the light organ by receptors to MAMPs. A signaling cascade involving MAMP receptors and molecules that function in the innate immune response NF-κB pathway has been identified in transcripts isolated from the light organ (Goodson *et al.,* 2005). Moreover, there is evidence of cross talk between the NF-κB and p53 pathways (Webster and Perkins, 1999; Ryan *et al.,* 2000; Dreyfus *et al.,* 2005). Future studies will be focused upon how detection of the symbiont is translated to a remotely acting signal that initiates apoptosis and regression in the ciliated epithelial fields of the light organ.

Members of the p53 family are well known for being effectors of development and of response to environmental stresses (White and Prives, 1999; Yang *et al.,* 1999, 2000; Mills *et al.,* 1999; Celli *et al.,* 1999), including responses to microbes (Norimatsu *et al.,* 1995; Guichon and Zychlinsky, 1996; Zychlinsky *et al.,* 1996; Li *et al.,* 2005; Ozturk *et al.,* 2005). Here we propose that the "stress" of detection of the symbiotic bacterium, *V. fischeri,* within the crypt spaces of the light organ of the host, *E. scolopes,* activates p53 family members. The activated p53 family members contribute to the developmental morphogenesis of the light organ by initiating apoptosis within cells of the host that potentiate the initial colonization of the organ by *V. fischeri.*

Acknowledgments

We thank C. Chun, J. Troll, E. Vu, and A. Wier for helpful discussion and comments on the manuscript. We also thank T. Koropatnick for confocal microscopy assistance. This research was supported by NSFIOB 0517007 (to M. M.-N. and E. G. Ruby), NIH AI50611 (to M. M.-N.), NIH grant RR12294 (to E. G. Ruby and M. M.-N.), and the W. M. Keck Foundation (to E. P Greenberg and M. M.-N.). This is HIMB contribution 1232.

Literature Cited

Aguinaldo, A. M., J. M. Turbeville, L. S. Linford, M. C. Rivera, J. R. Garey, R. A. Raff, and J. A. Lake. 1997. Evidence for a clade of nematodes, arthropods and other moulting animals. *Nature* **387:** 489–493.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215:** 403–410.

Ashcroft, M., and K. H. Vousden. 1999. Regulation of p53 stability. *Oncogene* **18:** 7637–7643.

Boettcher, K. J., and E. G. Ruby. 1990. Depressed light emission by symbiotic *Vibrio fischeri* of the sepiolid squid *Euprymna scolopes. J. Bacteriol.* **172:** 3701–3706.

Boggs, K., and D. Reisman. 2006. Increased p53 transcription prior to DNA synthesis is regulated through a novel regulatory element within the p53 promoter. *Oncogene* **25**:555–565. doi: 10.1038/sj.onc.1209076; published online: 12 September 2005.

Bonsing, B. A., W. E. Corver, M. C. Gorsira, M. van Vliet, P. S. Oud, C. J. Cornelisse, and G. J. Fleuren. 1997. Specificity of seven monoclonal antibodies against p53 evaluated with Western blotting, immunohistochemistry, confocal laser scanning microscopy, and flow cytometry. *Cytometry* **28:** 11–24.

Bradford, M. M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.

Celli, J., P. Duijf, B. C. Hamel, M. Bamshad, B. Kramer, A. P. Smits, R. Newbury-Ecob, R. C. Hennekam, G. Van Buggenhout, A. van Haeringen, C. G. Woods, A. J. van Essen, R. de Waal, G. Vriend, D. A. Haber, A. Yang, F. McKeon, H.G. Brunner, and H. van Bokhoven. 1999. Heterozygous germline mutations in the p53 homolog p63 are the cause of EEC syndrome. *Cell* **99:** 143–53.

Ciocan, C. M., and J. M. Rotchell. 2005. Conservation of cancer genes in the marine invertebrate *Mytilus edulis. Environ. Sci. Technol.* **39:** 3029–3033.

Cox, R. L., R. E. Stephens, and C. L. Reinisch. 2003. p63/73 homologues in surf clam: novel signaling motifs and implications for control of expression. *Gene* **320:** 49–58.

Derry, W. B., A. P. Putzke, and J. H. Rothman. 2001. *Caenorhabditis*

elegans p53: role in apoptosis, meiosis, and stress resistance. *Science* **294:** 591–595.

Dignam, J. D., R. M. Lebovitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11:** 1475–1489.

Dobbelstein, M., S. Wienzek, C. Konig, and J. Roth. 1999. Inactivation of the p53-homologue p73 by the mdm2-oncoprotein. *Oncogene* **18:** 2101–2106.

Doino, J. A., and M. J. McFall-Ngai. 1995. A transient exposure to symbiosis-competent bacteria induces light organ morphogenesis in the host squid. *Biol. Bull.* **189:** 347–355.

Dreyfus, D. H., M. Nagasawa, E. W. Gelfand, and L. Y. Ghoda. 2005. Modulation of p53 activity by IκBα: evidence suggesting a common phylogeny between NF-κB and p53 transcription factors. *BMC Immunol.* **6:** 12.

Foster, J. S., M. A. Apicella, and M. J. McFall-Ngai. 2000. *Vibrio fischeri* lipopolysaccharide induces developmental apoptosis, but not complete morphogenesis, of the *Euprymna scolopes* symbiotic light organ. *Dev. Biol.* **226:** 242–254.

Giaccia, A. J., and M. B. Kastan. 1998. The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev.* **12:** 2973–2983.

Goodson, M. S., M. Kojadinovic, J. V. Troll, T. E. Scheetz, T. L. Casavant, M. B. Soares, and M. J. McFall-Ngai. 2005. Identifying components of the NF-κB pathway in the beneficial *Euprymna scolopes-Vibrio fischeri* light organ symbiosis. *Appl. Environ. Microbiol.* **71:** 6934–6946.

Gottifredi, V., and C. Prives. 2001. Getting p53 out of the nucleus. *Science* **292:** 1851–1852.

Grand, R. J., P. S. Lecane, D. Owen, M. L. Grant, S. Roberts, A. J. Levine, and P. H. Gallimore. 1995. The high levels of p53 present in adenovirus early region 1-transformed human cells do not cause up-regulation of MDM2 expression. *Virology* **210:** 323–334.

Greenblatt, M. S., W. P. Bennett, M. Hollstein, and C. C. Harris. 1994. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res.* **54:** 4855–4878.

Guichon, A., and A. Zychlinsky. 1996. Apoptosis as a trigger of inflammation in *Shigella*-induced cell death. *Biochem. Soc. Trans.* **24:** 1051–1054.

Hainaut, P., T. Hernandez, A. Robinson, P. Rodriguez-Tome, T. Flores, M. Hollstein, C. C. Harris, and R. Montesano. 1998. IARC Database of p53 gene mutations in human tumors and cell lines: updated compilation, revised formats and new visualisation tools. *Nucleic Acids Res.* **26:** 205–213.

Haupt, Y., R. Maya, A. Kazaz, and M. Oren. 1997. Mdm2 promotes the rapid degradation of p53. *Nature* **387:** 296–299.

Inoue, T., L. Wu, J. Stuart, and C. G. Maki. 2005. Control of p53 nuclear accumulation in stressed cells. *FEBS Lett.* **579:** 4978–4984.

Ishioka, C., C. Englert, P. Winge, Y. X. Yan, M. Engelstein, and S. H. Friend. 1995. Mutational analysis of the carboxy-terminal portion of p53 using both yeast and mammalian cell assays in vivo. *Oncogene* **10:** 1485–1492.

Jessen-Eller, K., J. A. Kreiling, G. S. Begley, M. E. Steele, C. W. Walker, R. E. Stephens, and C. L. Reinisch. 2002. A new invertebrate member of the p53 gene family is developmentally expressed and responds to polychlorinated biphenyls. *Environ. Health Perspect.* **110:** 377–385.

Jin, S., S. Martinek, W. S. Joo, J. R. Wortman, N. Mirkovic, A. Sali, M. D. Yandell, N. P. Pavletich, M. W. Young, and A. J. Levine. 2000. Identification and characterization of a p53 homologue in *Drosophila melanogaster. Proc. Natl. Acad. Sci. USA* **97:** 7301–7306.

Kaghad, M., H. Bonnet, A. Yang, L. Creancier, J. C. Biscan, A. Valent, A. Minty, P. Chalon, J. M. Lelias, X. Dumont, P. Ferrara, F. McKeon, and D. Caput. 1997. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* **90:** 809–819.

Kelley, M. L., P. Winge, J. D. Heaney, R. E. Stephens, J. H. Farell, R. J. Van Beneden, C. L. Reinisch, M. P. Lesser, and C. W. Walker. 2001. Expression of homologues for p53 and p73 in the softshell clam (*Mya arenaria*), a naturally-occurring model for human cancer. *Oncogene* **20:** 748–758.

Kimbell, J. R., and M. J. McFall-Ngai. 2004. Symbiont-induced changes in host actin during the onset of a beneficial animal-bacterial association. *Appl. Environ. Microbiol.* **70:** 1434–1441.

Koropatnick, T. A., J. T. Engle, M. A. Apicella, E. V. Stabb, W. E. Goldman, and M. J. McFall-Ngai. 2004. Microbial factor-mediated development in a host-bacterial mutualism. *Science* **306:** 1186–1188.

Kubbutat, M. H., S. N. Jones, and K. H. Vousden. 1997. Regulation of p53 stability by Mdm2. *Nature* **387:** 299–303.

Kussie, P. H., S. Gorina, V. Marechal, B. Elenbaas, J. Moreau, A. J. Levine, and N. P. Pavletich. 1996. Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science* **274:** 948–953.

Li, J. H., X. Z. Shi, S. Lv, M. Liu, and G. W. Xu. 2005. Effect of *Helicobacter pylori* infection on p53 expression of gastric mucosa and adenocarcinoma with microsatellite instability. *World J. Gastroenterol.* **11:** 4363–4366.

Liang, X. H., M. Volkmann, R. Klein, B. Herman, and S. J. Lockett. 1993. Co-localization of the tumor-suppressor protein p53 and human papillomavirus E6 protein in human cervical carcinoma cell lines. *Oncogene* **8:** 2645–2652.

McFall-Ngai, M. J., and E. G. Ruby. 1991. Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. *Science* **254:** 1491–1494.

Mills, A. A., B. Zheng, X. J. Wang, H. Vogel, D. R. Roop, and A. Bradley. 1999. p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* **398:** 708–713.

Montgomery, M. K., and M. J. McFall-Ngai. 1994. Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Euprymna scolopes. Development* **120:** 1719–1729.

Muttray, A. F., R. L. Cox, S. St-Jean, P. van Poppelen, C. L. Reinisch, and S. A. Baldwin. 2005. Identification and phylogenetic comparison of p53 in two distinct mussel species (*Mytilus*). *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **140:** 237–250.

Niu, G., K. L. Wright, Y. Ma, G. M. Wright, M. Huang, R. Irby, J. Briggs, J. Karras, W. D. Cress, D. Pardoll, R. Jove, J. Chen, and H. Yu. 2005. Role of Stat3 in regulating p53 expression and function. *Mol. Cell Biol.* **25:** 7432–7440.

Norimatsu, M., T. Ono, A. Aoki, K. Ohishi, T. Takahashi, G. Watanabe, K. Taya, S. Sasamoto, and Y. Tamura. 1995. Lipopolysaccharide-induced apoptosis in swine lymphocytes in vivo. *Infect. Immun.* **63:** 1122–1126.

Nyholm, S. V., and M. J. McFall-Ngai. 2004. The winnowing: establishing the squid-vibrio symbiosis. *Nat. Rev. Microbiol.* **2:** 632–642.

Oakley, B. R., D. R. Kirsch, and N. R. Morris. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* **105:** 361–363.

Ollmann, M., L. M. Young, C. J. Di Como, F. Karim, M. Belvin, S. Robertson, K. Whittaker, M. Demsky, W. W. Fisher, A. Buchman, G. Duyk, L. Friedman, C. Prives, and C. Kopczynski. 2000. *Drosophila* p53 is a structural and functional homolog of the tumor suppressor p53. *Cell* **101:** 91–101.

Ozturk, Y., E. Ozer, B. Lebe, O. Bekem, and B. Buyukgebiz. 2005. Immunohistochemical evaluation of p53 expression and proliferative activity in children with *Helicobacter pylori* associated gastritis. *J. Pediatr. Gastroenterol. Nutr.* **40:** 467–470.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29:** e45.

Raman, V., S. A. Martensen, D. Reisman, E. Evron, W. F. Odenwald, E. Jaffee, J. Marks, and S. Sukumar. 2000. Compromised HOXA5 function can limit p53 expression in human breast tumours. *Nature* **405:** 974–978.

Reisman, D., and W. T. Loging. 1998. Transcriptional regulation of the p53 tumor suppressor gene. *Semin. Cancer Biol.* **8:** 317–324.

Ryan, K. M., M. K. Ernst, N. R. Rice, and K. H. Vousden. 2000. Role of NF-κB in p53-mediated programmed cell death. *Nature* **404:** 892–897.

Schmale, H., and C. Bamberger. 1997. A novel protein with strong homology to the tumor suppressor p53. *Oncogene* **15:** 1363–1367.

Stephens, R. E., C. W. Walker, and C. L. Reinisch. 2001. Multiple protein differences distinguish clam leukemia cells from normal hemocytes: evidence for the involvement of p53 homologues. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **129:** 329–38.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25:** 4876–4882.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76:** 4350–4354.

Venot, C., M. Maratrat, C. Dureuil, E. Conseiller, L. Bracco, and L. Debussche. 1998. The requirement for the p53 proline-rich functional domain for mediation of apoptosis is correlated with specific PIG3 gene transactivation and with transcriptional repression. *EMBO J.* **17:** 4668–4679.

Vogelstein, B., D. Lane, and A. J. Levine. 2000. Surfing the p53 network. *Nature* **408:** 307–310.

Walker, K. K., and A. J. Levine. 1996. Identification of a novel p53 functional domain that is necessary for efficient growth suppression. *Proc. Natl. Acad. Sci. USA* **93:** 15335–15340.

Webster, G. A., and N. D. Perkins. 1999. Transcriptional cross talk between NF-κB and p53. *Mol. Cell Biol.* **19:** 3485–3495.

White, E., and C. Prives. 1999. DNA damage enables p53. *Nature* **399:** 734–735, 737.

Yang, A., R. Schweitzer, D. Sun, M. Kaghad, N. Walker, R. T. Bronson, C. Tabin, A. Sharpe, D. Caput, C. Crum, and F. McKeon. 1999. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* **398:** 714–718.

Yang, A., N. Walker, R. Bronson, M. Kaghad, M. Oosterwegel, J. Bonnin, C. Vagner, H. Bonnet, P. Dikkes, A. Sharpe, F. McKeon, and D. Caput. 2000. p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature* **404:** 99–103.

Yang, A., M. Kaghad, D. Caput, and F. McKeon. 2002. On the shoulders of giants: p63, p73 and the rise of p53. *Trends Genet.* **18:** 90–95.

Yin, Y., W. C. DeWolf, and A. Morgentaler. 1997. p53 is associated with the nuclear envelope in mouse testis. *Biochem. Biophys. Res. Commun.* **235:** 689–694.

Zhang, Y., and Y. Xiong. 2001. A p53 amino-terminal nuclear export signal inhibited by DNA damage-induced phosphorylation. *Science* **292:** 1910–1915.

Zychlinsky, A., K. Thirumalai, J. Arondel, J. R. Cantey, A. O. Aliprantis, and P. J. Sansonetti. 1996. *In vivo* apoptosis in *Shigella flexneri* infections. *Infect. Immun.* **64:** 5357–5365.