Centro de Investigación en Alimentación y Desarrollo, A.C.

PARTICIPACIÓN DE p53 Y METALOTIONEÍNA EN LA RESPUESTA APOPTÓTICA A HIPOXIA EN EL CAMARÓN BLANCO *Litopenaeus vannamei*, Boone 1931

Por:

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A José Alfredo,
Compañero, yo sé que puedo contar con Usted…
a pesar de la veta o tal vez porque existe…
Hagamos el trato, ahora estoy lista.

A Omar y Monserrat, mis padres. A Carlos Lara, mi choco.
Ustedes son mis primeros formadores y
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¿De quién este logro, si no suyo?

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RESUMEN

El camarón blanco *Litopenaeus vannamei* es un organismo capaz de sobrevivir a periodos de hipoxia a través de estrategias que incluyen cambios en el metabolismo energético. Este trabajo se condujo para investigar si es la evasión apoptótica, mediada por las proteínas p53 y metalotioneína (MT), una estrategia más de *L. vannamei* para tolerar la hipoxia. Se caracterizó el gen MT de *L. vannamei* y se encontró que comparte con otros organismos el arreglo de tres exones/dos intrones. Entre los elementos de respuesta presentes en su región promotora se encuentra uno para p53. En condiciones de normoxia, MT se expresa diferencialmente en hemocitos, intestino, branquias, pleópodos, músculo y -con los niveles más altos- en hepatopáncreas y corazón. Se analizaron los transcritos de MT en hepatopáncreas y branquias después de 3 y 24 h de hipoxia (1.5 mg $OD \cdot L^{-1}$), encontrándose que aumentan a las 3 h, posiblemente para conferir mayor capacidad antioxidante al organismo. Además, el silenciamiento de p53 con RNA de doble cadena (dsRNA) induce un dramático aumento en la expresión de MT tanto en condiciones de hipoxia como de normoxia, sugiriendo que p53 participa en el control de la expresión de MT mediante un mecanismo regulatorio de interferencia transcripcional. La apoptosis evaluada por actividad de caspasa-3 y citometría de flujo, revela una alta actividad apoptótica en hemocitos de camarón aún en normoxia (5 mg $OD \cdot L^{-1}$). Bajo condiciones de hipoxia (1.5 mg $OD \cdot L^{-1}$), la apoptosis en hemocitos aumenta significativamente. Al silenciar p53 para evaluar su participación en el proceso apoptótico, éste se disminuye significativamente después de 24 h de hipoxia, efecto que no se observó a las 48 h. Lo anterior sugiere que en hemocitos de camarón pudieran existir vías de apoptosis tanto dependientes como independientes de p53. El silenciamiento de MT no tuvo efecto significativo sobre la frecuencia apoptótica frente a hipoxia en estos experimentos. El silenciamiento de p53 y MT bajo condiciones de normoxia aumenta significativamente la apoptosis, lo que sugiere que el estrés impuesto por el silenciamiento de estos genes, desencadena vías apotóticas independientes de p53 y MT.

Palabras clave: Hipoxia, crustáceo, apoptosis, metalotioneína, p53, silenciamiento

ABSTRACT

The white shrimp *Litopenaeus vannamei* is an organism capable of surviving hypoxia periods through strategies that include changes in the energetic metabolism. This work was conducted to investigate the apoptotic evasion mediated by the proteins p53 and metallothionein (MT), as an additional strategy of *L. vannamei* to tolerate hypoxia. The MT gene from *L. vannamei* was characterized and it was found that shares the three exons/two introns array with other organisms. Amongst the response elements in its promoter region, one is found for p53. In normoxic conditions, it expresses differentially in hemocytes, intestine, gills, pleopods, muscle and –with the highest levels- in hepatopancreas and heart. The MT transcripts were analyzed after 3 and 24 h of hypoxia (1.5 mg $OD \cdot L^{-1}$), and an increase was found after 3 h, possibly to confer greater antioxidant capacity to the organism. In addition, the silencing of p53 with double-stranded RNA induces a dramatic increase in the MT expression both in hypoxic and normoxic conditions, suggesting that p53 participates in the expression control of MT by a regulatory mechanism of transcriptional interference. The apoptosis evaluated by caspase-3 activity and flow cytometry, reveals a high apoptotic activity in shrimp hemocytes even in normoxia (5 mg $OD \cdot L^{-1}$). Under hypoxia conditions (1.5 mg $OD \cdot L^{-1}$), the apoptotic frequency in hemocytes significantly increases. Upon p53 silencing to evaluate its participation in this apoptotic process, apoptosis decreases significantly after 24 h of hypoxia but not after 48 h. This indicates that in shrimp hemocytes there might be p53-dependent and -independent apoptotic pathways. The MT silencing had no significant effect on the apoptotic frequency upon hypoxia in these experiments. The silencing of p53 and MT under normoxia significantly magnifies apoptosis, suggesting that the stress imposed by the silencing of these genes triggers apoptotic pathways independent of p53 and MT.

Keywords: Hypoxia, crustacean, apoptosis, metallothionein, p53, silencing

INTRODUCCIÓN

Los hábitats de las distintas formas de vida en la Tierra están definidos por la disponibilidad de los recursos necesarios para garantizar su permanencia. Uno de los más importantes recursos para la mayor parte de las formas de vida eucariota es el oxígeno ambiental, necesario para los procesos de respiración celular y producción de ATP. Las fuentes de oxígeno ambiental son el aire y el agua, con concentraciones variables de oxígeno disuelto, pero en agua son siempre menores a las del aire. Todos los seres vivos están expuestos a variaciones discretas en la concentración de oxígeno en sus ambientes. Sin embargo, estas variaciones son más severas y prolongadas en ambientes acuáticos. Mientras que la mayoría de los mamíferos terrestres requieren concentraciones de oxígeno ambiental cercanas a 21%, hay otros organismos cuya permanencia en la Tierra ha dependido de su habilidad para evolucionar y adaptarse a condiciones de escasez de oxígeno. Tal es el caso del topo *Spalax*, en cuyas madrigueras se registran concentraciones de oxígeno de 7% (Ashur-Fabian et al., 2004), del yak *Bos grunniens* que habita tierras a 3,500 m s. n. m. (Lan et al., 2016), la tortuga de orejas rojas *Trachemys scripta elegans* que para soportar el invierno hiberna sumergida en aguas gélidas (Madsen et al., 2015) y de insectos como *Drosophila melanogaster* (Lavista-Llanos et al., 2002), entre otros. Por su parte, los organismos marinos también han desarrollado y fijado estrategias fisiológicas y de comportamiento que le permiten adaptarse a los constantes cambios de temperatura, salinidad o presión de oxígeno que suceden en su hábitat de manera natural o inducida.

Litopenaeus vannamei es una especie originaria del continente americano que actualmente es explotada también en Asia. Sin embargo, a pesar de su peso económico, hay aspectos relevantes de su biología aún no estudiados. Es importante conocer los elementos que actúan para conferir a este organismo la notable resistencia a condiciones de otra forma adversas para su subsistencia. Entre los aspectos de su biología que aún no

están completamente investigados, figura su capacidad de adaptación/tolerancia a condiciones bajas de oxígeno sin que éstas resulten en detrimento de su integridad. En los últimos años, nuestro grupo de investigación ha generado conocimiento concerniente a los mecanismos moleculares en el metabolismo energético y a enzimas antioxidantes que se activan para conferir esta tolerancia en *Litopenaeus vannamei* (Garcia-Triana et al., 2010; Soñanez-Organis et al., 2010; Soñanez-Organis et al., 2012; Trasviña-Arenas et al., 2013; Cota-Ruiz et al., 2015). Sin embargo, un aspecto de la biología del camarón en el que aún existe vacío de información es la manera en que las células sometidas a bajas concentraciones de oxígeno mantienen su integridad y sus funciones, en contraste con animales no tolerantes a hipoxia, cuyas células son blanco inmediato de apoptosis —o muerte celular programada— en respuesta a este estímulo. Consecuentemente, en camarones peneidos el organismo completo es capaz de sobrevivir en condiciones en las que otros animales sucumbirían. Un símil de esta situación se encuentra en la patología del cáncer, en la que las células tumorales en regiones con baja disponibilidad de oxígeno logran sobrevivir y perpetuarse gracias a mecanismos de evasión apoptótica. Dicha evasión está dada por un funcionamiento deficiente de la proteína p53 en aproximadamente la mitad de los tipos de cáncer. Además, se ha encontrado que las metalotioneínas (MTs) contribuyen a esta resistencia a apoptosis, a través de su interacción con p53 (Meplan et al., 2000; Ostrakhovitch et al., 2016).

Con base en lo anterior, el objetivo del presente estudio ha sido evaluar el efecto de la hipoxia sobre apoptosis en *L. vannamei* e investigar un posible mecanismo de regulación mediado por p53 y metalotioneína.

Así, esta investigación se ha guiado bajo la hipótesis de que en *Litopenaeus vannamei* ocurre evasión de apoptosis mediada por p53 y MT en respuesta a hipoxia. Las condiciones *sine qua non* para abordar esta hipótesis fueron: 1) la existencia en *L. vannamei* de los genes p53 y MT, así como su expresión en respuesta a hipoxia; en el **capítulo II** se aborda lo concerniente a MT, y 2) la ocurrencia o evasión de apoptosis ante hipoxia en células del camarón. Posteriormente se evaluó el efecto que el silenciamiento de MT o p53 tiene sobre la apoptosis en respuesta a hipoxia en este organismo, de acuerdo a los resultados reportados en el **capítulo III**. En el **capítulo I** se detalla el marco teórico sobre el cual se formuló este trabajo y en los **capítulos II y III**

se describe la consecución de los objetivos perseguidos en la investigación. Una de las herramientas clave utilizadas fue la manipulación de la expresión de p53 y MT, explotando el mecanismo de RNA de interferencia (RNAi) propio de la célula. El **capítulo IV** está compuesto por una revisión de literatura acerca de los mecanismos moleculares que intervienen en el proceso de silenciamiento génico por RNAi en crustáceos.

CAPÍTULO I

Apoptosis en respuesta a hipoxia:
el marco teórico para su estudio en crustáceos

ANTECEDENTES

Hipoxia

La concentración de oxígeno disuelto (OD) en las masas de agua disminuye como consecuencia de procesos naturales por sí mismos o en combinación con procesos antropogénicos. Los procesos naturales que generan esta hipoxia son principalmente la producción fotosintética de carbono y la respiración microbiana (Helly and Levin, 2009). La hipoxia en los sistemas acuáticos se intensifica por eventos naturales de surgencia y estratificación, en los que ocurre alta movilización de carbono hacia las capas de agua inferiores. Esta condición se exacerba en los sistemas acuáticos con actividades humanas —tanto de producción acuícola como industrial y agrícola con la eliminación de desechos sobre los cuerpos de agua— en los que la carga de nutrientes aumenta la producción y acumulación de carbono orgánico, incrementando la demanda respiratoria de oxígeno (Rabalais et al., 2010).

En términos amplios, un ambiente con 2.8 mg $OD \cdot L^{-1}$ se considera en situación de hipoxia para organismos bentónicos (Díaz and Rosenberg, 1995), mientras que, en general, concentraciones de entre 6 y 4.5 mg $OD \cdot L^{-1}$ afectan los procesos de crecimiento para organismos que habitan la columna de agua (Gray et al., 2002). En el caso de camarones peneidos como *Litopenaeus vannamei*, niveles de 4.5 a 5.8 mg $OD \cdot L^{-1}$ corresponden al nivel crítico de oxígeno (Rosas et al., 1999; Villareal et al., 1994), siendo éste definido como la concentración mínima de oxígeno en la que organismos oxi-reguladores pueden sostener un consumo constante del mismo sin importar su disponibilidad (Hagerman and Weber, 1981). Debajo de este nivel crítico, los peneidos son oxi-conformadores pues su consumo de oxígeno depende de la concentración de éste en el medio (Re and Díaz, 2011; Rosas et al., 1999). Además de esta clasificación para los organismos aerobios, los órganos y tejidos también responden como

oxi-conformadores u oxi-reguladores teniendo diferentes sensibilidades a la hipoxia (Stamati et al., 2011). La disminución en la tensión de oxígeno podría limitar la supervivencia de los organismos en hábitats con condiciones hipóxicas, de no ser porque éstos han evolucionado desarrollando estrategias que les permiten tolerar tales cambios en su ambiente (Gorr et al., 2010).

La tolerancia a hipoxia es una consecuencia importante de la presión de selección en condiciones de baja disponibilidad de oxígeno, la cual es común entre invertebrados, vertebrados menores y tumores. Los organismos acuáticos evolucionaron con una mayor habilidad para tomar oxígeno del ambiente, forzando grandes volúmenes de agua a través de sus branquias. Esto dio lugar a su adaptación a nuevas condiciones de oxígeno, desarrollando sistemas respiratorios más eficientes que les permitieran maximizar su supervivencia (Berner et al., 2007). Así, en condiciones de hipoxia, el langostino *Pacifastacus leniusculus* muestra un comportamiento hipotérmico (Morris, 2004) y la carpa *Carassius carassius* produce etanol en vez de ácido láctico (Nilsson, 1988). Por otro lado, la angiogénesis tumoral, la glucólisis y la evasión apoptótica son consideradas por muchos las principales adaptaciones de los tumores para resistir la hipoxia (Gorr et al., 2010). La respuesta celular a hipoxia está regulada en parte por el factor de transcripción inducido por hipoxia-1 (HIF-1), heterodímero compuesto por las subunidades HIF-1α y HIF-1β que contienen el dominio básico hélice-vuelta-hélice y poseen dominios Per-ARNT-SIM (PAS) que le permiten la unión al DNA y heterodimerización (Wang et al., 1995). HIF-1α tiene además un dominio de degradación dependiente de oxígeno (ODD) (Fig. 1).

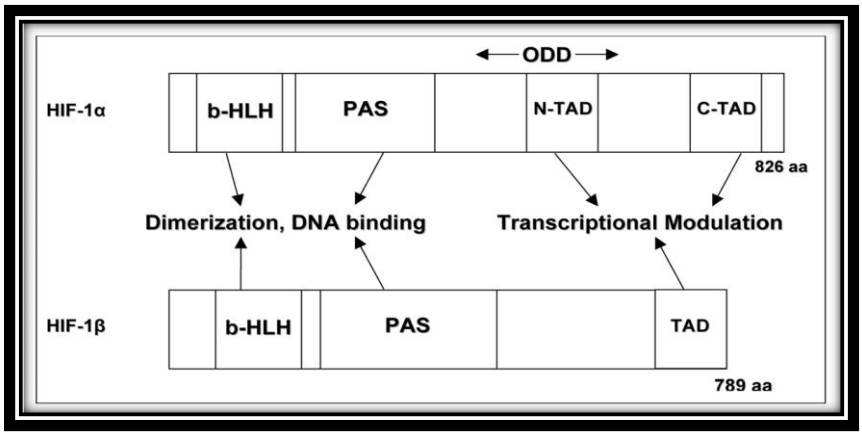

Figura 1. Estructura del complejo HIF-1 tomada de Duffy et al. (2003).

El dominio ODD de HIF-1α funciona por un lado como activador transcripcional que estimula la expresión génica mediante interacciones entre proteína-proteína y por otro lado como sensor de los cambios en niveles de oxígeno que conducen a degradación de HIF-1α (Jeong et al., 2002). Se sabe que bajo condiciones de hipoxia severa o anoxia, la proteína p53 se acumula y se une al dominio ODD de HIF-1α. Esta unión inhibe la activación de genes dependiente de HIF-1 o facilita la degradación de la subunidad HIF-1α (Sánchez-Puig et al., 2005).

El factor de transcripción HIF-1 funciona protegiendo a las células de las consecuencias de la hipoxia moderada promoviendo la activación y aceleración de rutas generadoras del ATP necesario para el funcionamiento y supervivencia celular bajo estas condiciones. En *L. vannamei* bajo condiciones de hipoxia, HIF-1 induce la transcripción de enzimas involucradas en el metabolismo energético glucolítico y gluconeogénico (Soñanez-Organis et al., 2011; Soñanez-Organis et al., 2012; Cota-Ruiz et al., 2016). Por otro lado, en condiciones severas de hipoxia, p53 es activada para promover apoptosis y proteger las funciones fisiológicas normales de tejidos y órganos (Zhao et al., 2009).

Apoptosis

Los metazoarios requieren mecanismos que aseguren el desecho seguro y económico de células superfluas o potencialmente dañinas (Menze et al., 2010). Dichos mecanismos son ejecutados por procesos de muerte celular programada entre los que se incluye la apoptosis. Además de mantener la homeostasis de los tejidos y de ser clave durante el desarrollo, la apoptosis es un mecanismo de protección ante insultos celulares ambientales como radiación, xenobióticos, proteínas virales o bacterianas, especies reactivas de oxígeno (ROS, por sus siglas en inglés), hipoxia, etc. (Ferri and Kroemer, 2001). La apoptosis, o muerte celular programada tipo I (PCD I, por sus siglas en inglés), fue descrita inicialmente por Kerr et al. (1972) como "…un mecanismo de supresión celular controlado… en la regulación de poblaciones celulares animales". Gracias a la gran cantidad de información que se ha generado acerca de este proceso, a esta definición

inicial de apoptosis, se agrega que es un proceso coordinado, dependiente de energía, que involucra una compleja cascada de eventos entre los que se incluye la activación de proteasas de cisteína llamadas “caspasas”, que conectan el estímulo inicial a la muerte final de la célula a través de la proteólisis del citoesqueleto (Elmore, 2007; Elmore et al., 2016). La apoptosis se caracteriza a nivel celular por eventos que incluyen —entre otros— encogimiento celular, fragmentación nuclear, condensación de cromatina (picnosis) y fragmentación del DNA (cariorrexis) (Ferri and Kroemer, 2001; Kerr et al., 1972). Las células apoptóticas no liberan su contenido celular, sino que mantienen la integridad de sus membranas; son rápidamente fagocitadas por células circundantes sin producción de citocinas pro- o anti-inflamatorias y como consecuencia, no hay reacción inflamatoria asociada a este tipo de muerte (Kurosaka et al., 2003; Xu et al., 2009). Una gran variedad de estímulos y condiciones pueden ocasionar apoptosis, aunque no todas las células mueren en respuesta al mismo estímulo inductor (Elmore, 2007).

Existen dos vías apoptóticas principales: la extrínseca o “vía de receptores de muerte celular” y la intrínseca, o “vía mitocondrial” (Fig. 2) (Eum and Lee, 2011). La vía de señalización extrínseca se desencadena por la unión de receptores de muerte de la superfamilia del factor de necrosis tumoral (TNF, por sus siglas en inglés), con sus ligandos extracelulares, formando un complejo de muerte. Ejemplos de estos receptores y sus ligandos son FasR/FasL, TNFR1/TNFα, DR3/Apo3L (Ouyang et al., 2012). Al recibir el estímulo inductor, estos receptores/ligando reclutan proteínas adaptadoras con dominios de muerte como FADD y TRADD (Fas-*associated death domain* y TNF-*associated death domain*, respectivamente) que a su vez se asocian con la procaspasa-8 (Elmore, 2007). De esta manera se forma un complejo de señalización inductor de muerte (DISC), en el que ocurre la activación autocatalítica de la procaspasa-8, seguida de la fase ejecutora de apoptosis (Kischkel et al., 1995). La regulación de esta vía está mediada por proteínas inhibidoras como c-FLIP y Toso que se unen e inactivan a los adaptadores FADD y a la caspasa-8 (Hitoshi et al., 1998; Scaffidi et al., 1999). La vía de señalización intrínseca desencadena apoptosis en respuesta a estímulos que perturban la homeostasis celular (hipoxia, radiación, radicales libres, etc.) y que no son mediados por receptores sino por eventos que están bajo el control de pro-enzimas mitocondriales. Estos estímulos ocasionan la apertura del poro de transición de permeabilidad mitocondrial (MPT, por sus

siglas en inglés) y liberación de proteínas pro-apoptóticas como citocromo *c*, Smac/DIABLO, HtrA2/Omi. El citocromo *c* recluta y activa a las proteínas Apaf-1 y caspasa-9 formando un "apoptosoma", que a su vez activa e inicia la cascada de señalización de la caspasa 3, culminando –como en la vía extrínseca-, en la fase ejecutora de apoptosis (Ouyang et al., 2012). Las vías apoptóticas extrínseca, intrínseca y de perforina/granzimas, convergen en una fase ejecutora común que inicia con la activación de las caspasas efectoras 3, 6 y 7. Estas caspasas hidrolizan proteínas nucleares y del citoesqueleto que finalmente causan los cambios bioquímicos y morfológicos que caracterizan la apoptosis (Elmore, 2007).

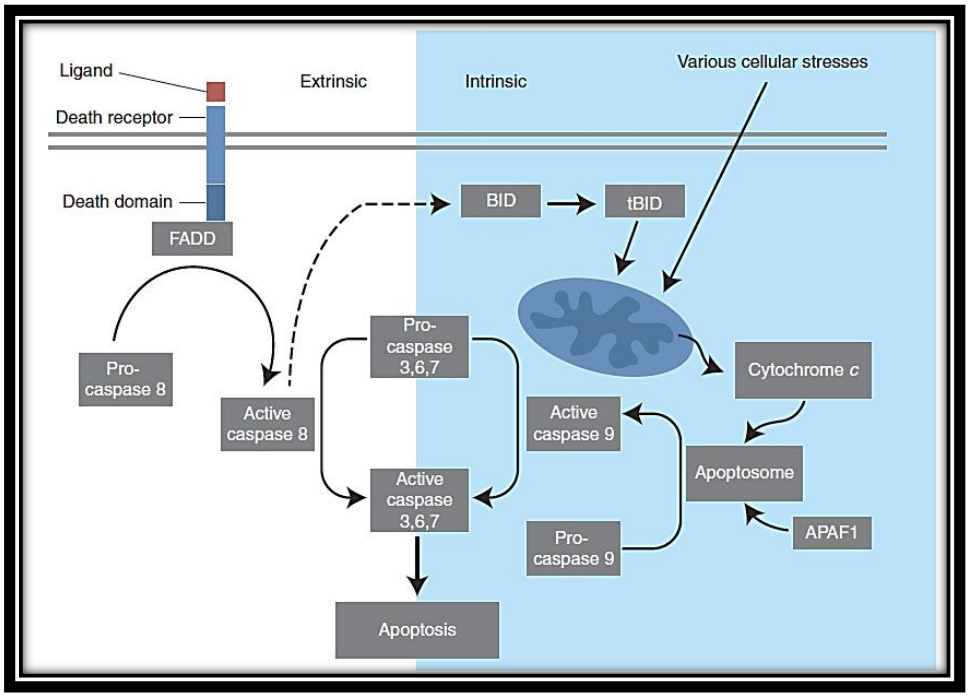

Figura 2. Vías apoptóticas extrínseca e intrínseca. La vía extrínseca se activa a través de la unión de un ligando a su receptor, que a su vez recluta, dimeriza y activa a la caspasa-8, con la ayuda de proteínas adaptadoras (FADD/TRADD). La caspasa-8 activa inicia apoptosis activando a las caspasas efectoras o activando la vía intrínseca por la hidrólisis de BID. La vía intrínseca o mitocondrial puede activarse por diferentes estreses que conducen a la liberación de citocromo *c*, ATP y caspasa-9. La caspasa-9 activada inicia apoptosis mediante la hidrólisis y activación de las caspasas efectoras. Tomado de McIlwain et al. (2013).

La regulación de la vía mitocondrial está dada por proteínas pro- y anti-apoptóticas de la familia Bcl-2, que regulan la liberación de citocromo *c* de la mitocondria alterando la permeabilidad de su membrana (Elmore, 2007).

En crustáceos se han encontrado —como ESTs traducidos— ortólogos de las principales proteínas de las vías apoptóticas presentes en mamíferos, pero la integración de esta información en la fisiología y patofisiología de los crustáceos está lejos de estar completa y debe ser validada por datos experimentales bioquímicos y moleculares. Algunos estímulos pro-apoptóticos descritos en crustáceos son la radiación UV, toxinas ambientales, calor e infecciones virales (Menze et al., 2010; Wu and Muroga, 2004), sin embargo, los mecanismos de ejecución de apoptosis no se han estudiado. No hay evidencia experimental de la existencia de la vía apoptótica extrínseca en crustáceos. La contribución de las caspasas al proceso apoptótico es evolutivamente conservada (Kumar, 2007). Para la vía intrínseca se han clonado y caracterizado 14 diferentes caspasas y una proteína inhibidora de apoptosis (IAP), pero aún no hay datos sobre la función que confirmen la actividad de esta última. La importancia del hallazgo de al menos siete proteínas Bcl-2 sumamente diversas en crustáceos, también merece ser explorado, ya que no hay evidencia de que en estos organismos ocurra activación de caspasas por el citocromo *c* como en mamíferos; por el contrario, datos experimentales demuestran que en *Artemia franciscana* la actividad de caspasa 9 aumenta a altas concentraciones de Ca^{2+} intracelular, pero este aumento es independiente de la liberación de citocromo *c*, lo cual sugiere un mecanismo de activación de caspasas distinto al de vertebrados (Menze and Hand, 2007). Sin embargo, el papel del citocromo *c* podrá ser descartado sólo hasta que la estructura molecular del apoptosoma crustáceo sea resuelta (Menze et al., 2010).

Metalotioneína

Las metalotioneínas (MTs) son proteínas altamente conservadas de 6 a 7 kDa, ricas en cisteína (Ostrakhovitch et al., 2007). Contienen hasta siete iones metálicos bivalentes por molécula (Braun et al., 1992), mismos que puede obtener removiéndolos de metaloenzimas y de factores de transcripción para regular la función de los mismos (Cano-Gauci and Sarkar, 1996; Roesijadi et al., 1998). Los genes que codifican MTs están presentes tanto en eucariotes como en algunos procariotes, usualmente en múltiples copias (Palmiter, 1998). En animales se han encontrado MTs en la mayoría de los tejidos y su

sobre-expresión se ha reportado en varios tipos de tumores humanos (Cherian et al., 2003). En ratón se conocen cuatro isoformas de metalotioneína (MT-1, MTII, MTIII, MTIV), mientras que en humanos hay por lo menos 16 de ellas (Palmiter, 1998). En el **capítulo II** se describen metalotioneínas de crustáceos y se comparan con la encontrada en *L. vannamei* en el marco de este trabajo. Las MTs están involucradas en la homeostasis de metales esenciales en virtud de sus propiedades de unión a los mismos (Ostrakhovitch et al., 2007). Su primera función reconocida fue la detoxificación de metales pesados como cadmio, mercurio, zinc y cobre (Klaassen et al., 1999; Shestivska et al., 2011). Se sabe también, que protegen contra la radiación, el daño oxidativo y contribuyen al control de la proliferación celular y de la apoptosis (Klaassen et al., 1999). Se ha comprobado un incremento en la síntesis de MTs desencadenada por estrés oxidativo (Sato and Bremner, 1993) y se ha demostrado su alta actividad antioxidante contra la peroxidación de membranas lipídicas y del DNA (Fang et al., 1997; Kondoh et al., 2003). Esta cualidad de las MTs está dada por las cisteínas presentes en su estructura, que pueden atrapar oxhidrilos libres en grupos –SH (tiol) y detener el daño por oxidación al DNA y proteínas (Good et al., 1991). Las MTs pueden incluso revertir el daño ocasionado por procesos oxidativos, donando un átomo de hidrógeno al lugar dañado por radicales libres, con lo cual el DNA regresa a su estructura normal (Renan and Dowman, 1989). Las MTs pueden también participar como co-factores en la activación de la síntesis de la glutatión peroxidasa, contribuyendo a los mecanismos antioxidantes también de esta manera (Hayes and McLellan, 1999; Matsubara, 1987).

Por otro lado, el incremento de la expresión de MT se ha correlacionado con la resistencia a apoptosis en numerosos estudios, independientemente del tipo celular en el que se ha estudiado (Shimoda et al., 2003; Wang et al., 2001). En otros estudios, la sub-expresión de MT inhibe el crecimiento celular y activa apoptosis (Abedel-Mageed and Agrawal, 1997; Deng et al., 1998). La expresión de los genes de MT responde a varios elementos, entre otros, está regulada en *cis* por los elementos de respuesta a metales (MRE, siglas de su nombre en inglés), cuya secuencia es altamente conservada y se encuentra en múltiples copias (Stuart et al., 1984). El factor de transcripción 1 de respuesta a metales (MTF-1, siglas de su nombre en inglés) regula la expresión basal de MT y la expresión estimulada por metales uniéndose a los MREs (Ostrakhovitch et al., 2007). Además del zinc y otros

metales pesados, la actividad transcripcional de MTF-1 es inducida por hipoxia, estrés oxidativo, óxido nítrico y altas temperaturas (Andrews, 2000; Dalton et al., 1994; Murphy, 2004). En respuesta a hipoxia, la inducción de la expresión de MTs depende también de la interacción MTF-1/MRE (Murphy et al., 1999). Sin embargo, en ausencia de HIF-1α, MTF-1 es reclutado al promotor de MT-I pero no es suficiente para activar la expresión del gen en respuesta a hipoxia. Adicionalmente, han demostrado que MTF-1 forma un complejo transcripcional que incluye a HIF-1α y que ambas proteínas son reclutadas al promotor de MT *in vivo* en respuesta a hipoxia (Murphy et al., 1999).

Además de las interacciones mencionadas, diversas investigaciones indican una fuerte relación entre p53 y MT. La alta expresión de MT en tumores de alto grado se asocia consistentemente con la presencia de p53 mutante (Méplan et al., 2000). Elevadas concentraciones de MT en ciertos tumores pueden bloquear apoptosis y relacionarse con la progresión del tumor, en tanto que un papel central de p53 es actuar como supresor de tumores mediante la inducción de apoptosis (Cherian et al., 2003). Esta relación ha sido investigada, encontrándose que la metalotioneína modula la actividad transcripcional de p53 al actuar como un fuerte agente quelante que remueve el zinc de p53 a través de una reacción de transferencia; esto resulta en un plegamiento de p53 que da lugar a una conformación no permisiva para su unión a DNA (Méplan et al., 2000). Sin embargo, cuando MT es expresada experimentalmente a niveles bajos, cataliza las reacciones de transferencia de metales regulando en p53 el plegamiento del dominio de unión a DNA e incrementando así su capacidad de unión al DNA (Méplan et al., 2000).

Estudios realizados en *L. vannamei*, muestran una inducción de proteínas con características de MT desencadenada por altas concentraciones de cadmio y zinc (Wu and Chen, 2005), por lo que se ha utilizado esta proteína como bio-indicador de exposición a metales pesados (Moksnes et al., 1995). La secuencia codificante de *L. vannamei* y su expresión en respuesta a salinidad se reportó en 2014 por (Wang et al., 2014). El **capítulo II** detalla el artículo que nuestro grupo publicó en el mismo año (Felix-Portillo et al., 2014) donde se reporta que la mayor expresión de transcritos de MT bajo condiciones fisiológicas sucede en hepatopáncreas, y que ésta aumenta significativamente después de 3 h de hipoxia, sugiriendo la contribución de MT a la "preparación para estrés oxidativo" ocasionado por este estrés. Se presenta también la secuencia del gen MT, en cuya región

promotora se encuentran elementos de respuesta para HIF-1, MTF-1 y p53. La funcionalidad del elemento de repuesta para p53 se abordó indirectamente en estudios posteriores reportados en el **capítulo III.** El exacerbado aumento de la expresión de MT en hemocitos de animales en los que p53 fue silenciado, sugiere la participación de p53 en el control de la expresión de MT a través de un mecanismo de interferencia transcripcional.

p53, Nodo de Respuesta a Estrés

Numerosos estudios realizados a partir del descubrimiento de p53 en 1979, han evidenciado el prominente papel que esta proteína juega en varias tareas celulares de tal importancia que a menudo se le ha llamado "guardián del genoma" (Lane, 1992). Junto con p63 y p73 forma una familia de proteínas estructural y funcionalmente relacionadas (Bai and Zhu, 2006) (Fig. 3).

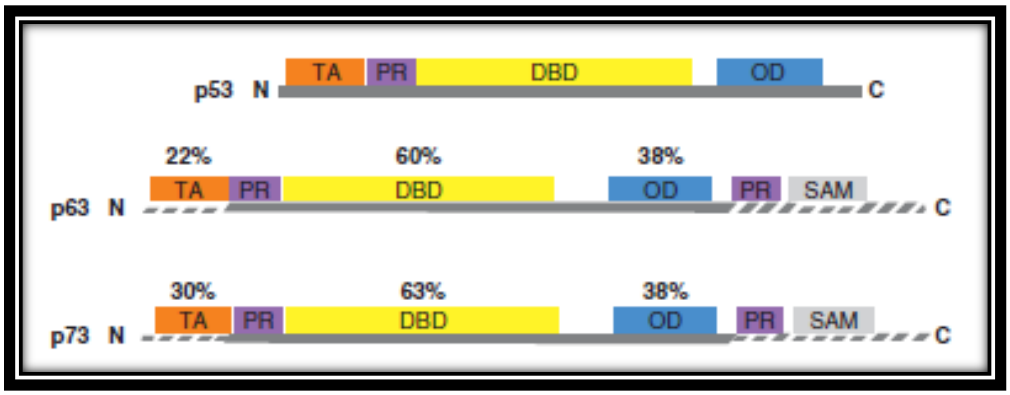

Figura 3. Representación esquemática de la estructura modular de los miembros de la familia p53. La estructura general de p53, p63 y p73 es conservada y consiste en un dominio de transactivación (TA), un dominio central de unión a DNA (DBD) y un dominio de oligomerización carboxi-terminal (OD). Las isoformas más largas de p63 y p73 contienen un motivo alfa estéril (SAM), que es un dominio putativo de interacción proteína-proteína presente en muchas proteínas de señalización y factores de transcripción. En porcentaje se indica la identidad de p63 y p73 con p53. Tomado de Dotsch et al. (2010).

Los análisis filogenéticos de la familia p53 muestran que en invertebrados, los miembros de esta familia parecen tener una estructura más parecida al dominio de p63 (p63-like) de vertebrados que al de p53, lo que indica que p63 podría ser el ancestro evolutivo de estos genes (Rutkowski et al., 2010). La estructura general de estas proteínas y la organización de sus genes parecen estar conservadas desde la mosca *D. melanogaster* hasta mamíferos (Zhou et al., 2000).

En humanos, el gen p53 es de 19,000 pb y contiene 11 exones y tres promotores alternativos (Fig. 4) (Bourdon et al., 2005). Hasta ahora se han identificado por lo menos 13 diferentes isoformas de la proteína que se expresan normalmente de manera tejido-específica (Garcia-Alai et al., 2008; Marcel et al., 2013; Marcel et al., 2011; Murray-Zmijewski et al., 2006) Por su parte, en *D. melanogaster* p53 (Dmp53) codifica tres proteínas de 385, 495 y 110 aminoácidos generadas por splicing alternativo y por el uso diferencial de sus dos promotores (Bourdon et al., 2005).

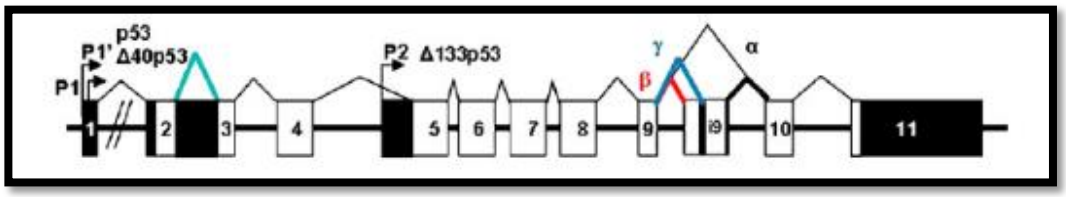

Figura 4. Esquema de la estructura del gen p53 en humanos. Se indican sitios de splicing alternativo (*α, β, γ*) y promotores alternativos (P1, P1´ y P2). Tomado de Bourdon et al. (2005).

La proteína p53 ha sido ampliamente estudiada en diversos modelos animales y en humanos (TP53) debido a las funciones que tiene en procesos oncogénicos. A partir de estos estudios se sabe que p53 es un tetrámero que funciona como factor de transcripción (Brosh and Rotter, 2009) y que el correcto plegamiento de p53 para su conformación de unión a DNA requiere la incorporación de zinc a su estructura (Hainaut and Milner, 1993; Méplan et al., 2000).

Bajo condiciones normales, p53 se expresa a niveles bajos y tiene una vida media corta (Zhao et al., 2009). Su activación es desencadenada por estrés celular de varios tipos como daño al DNA, señalización oncogénica e hipoxia (Liu et al., 2007). Al ser activada, p53 es capaz de promover la transcripción de más de 150 genes involucrados en regulación del ciclo celular, inducción de apoptosis, supresión de tumores, senescencia celular, diferenciación celular, segregación cromosómica, reparación del DNA, regulación de vías metabólicas y de citocinas requeridas para la implantación embriónica (Zhao and Keating, 2007) (Fig. 5).

Los mecanismos que regulan la activación de p53 pueden ser de dos tipos: a) pre-traduccionales, que incluyen la inhibición de la degradación del p53 mRNA por una parte y el incremento de su síntesis por otra (Takagi et al., 2005); y b) post-traduccionales, que involucran la modificación de p53 por reacciones de: i) fosforilación y acetilación que

incrementan tanto su estabilidad como su concentración en respuesta a estrés celular proveniente de diversas fuentes, ii) ubiquitinación, que contribuye a asegurar la latencia y bajo nivel estable de p53 en homesotasis, iii) neddilación, que inhibe la actividad transcripcional de p53, iv) sumoilación, que incrementa la actividad transcripcional de p53, y v) metilación, cuyas consecuencias funcionales pueden ser de represión o de activación de p53, dependiendo del sitio de la modificación y del número de grupos metilo unidos (Ostrakhovitch et al., 2007; Dai and Gu, 2010). Además de estas formas de control, recientemente se han reportado un gran número de microRNAs que regulan y son regulados por p53 en complejas vías de retroalimentación (Takwi and Li, 2009; Wade et al., 2013).

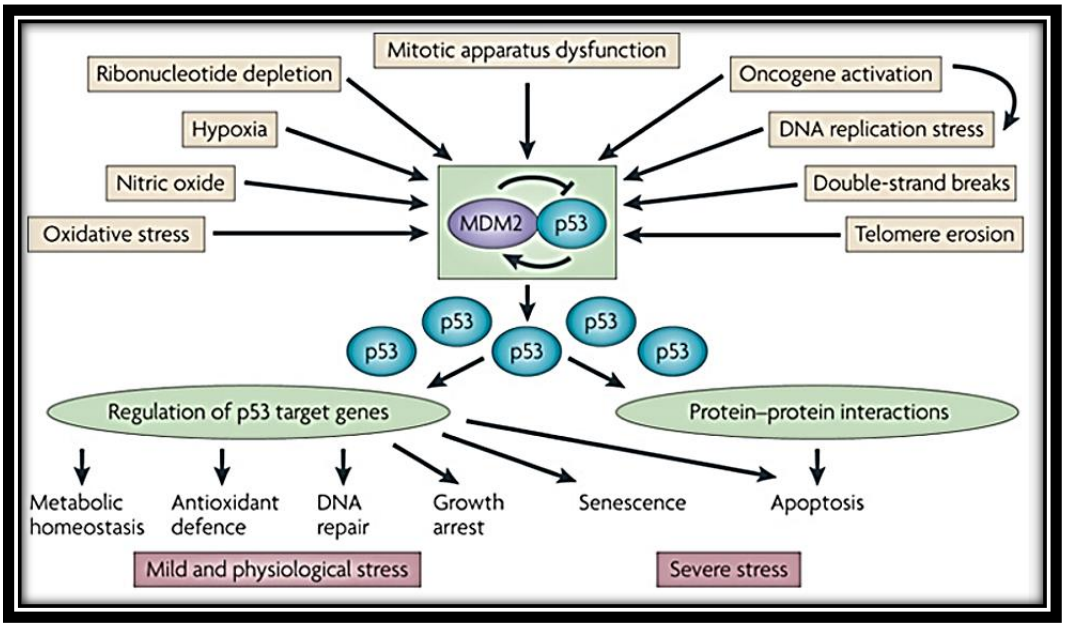

Figura 5. Esquema simplificado de la vía p53. Tomado de Levine and Oren (2009).

Una vez activada, p53 reprime o activa los genes efectores de las distintas funciones que controla. Los criterios exactos a los que p53 obedece para estimular arresto del ciclo celular o apoptosis no se conocen con exactitud, pero incluyen el tipo de estrés que activa a p53, la duración y magnitud del estímulo estresante (Zhao et al., 2009), el tipo celular y el contexto celular durante la exposición a estrés (Balint and Vousden, 2001). Sin embargo, en experimentos en los que la única variable es la abundancia de p53, se ha encontrado que es un “mecanismo de umbral” el que decide el destino celular entre arresto del ciclo celular o apoptosis. Siguiendo este mecanismo, para desencadenar apoptosis las

células deben rebasar un umbral apoptótico cuya altura está determinada por la abundancia de proteína p53, la duración de su expresión y el contexto celular. Debajo de este umbral, p53 y sus proteínas blanco son suficientes para inducir arresto pero no apoptosis. Por arriba de este umbral, p53 y sus genes/proteínas blanco desencadenan apoptosis extensiva (Kracikova et al., 2013).

Aunque hay vías apoptóticas independientes de p53, ésta es una de las proteínas más frecuentemente activadas en este proceso de muerte celular programada. En células de mamífero sometidas a hipoxia, p53 promueve apoptosis pero no arresto del ciclo celular (Koumenis et al., 2001). La hipoxia suprime la asociación de HIF-1α a VHL (*von Hippel-Lindau*) y de p53 a MDM2 (*Murine Double Minute 2*). Siendo VHL y MDM2 componentes de las ubiquitin-ligasas respectivas (Haupt et al., 1997; Yu et al., 2001), HIF-1α y p53 se estabilizan y se acumulan en sus formas transcripcionalmente activas. Así, p53 se acumula en el núcleo de la célula y participa en las vías apoptóticas intrínseca y extrínseca (Pflaum et al., 2014). El control de la vía apoptótica intrínseca (también llamada mitocondrial) ocurre a través de proteínas de la familia Bcl-2, que a su vez, están controladas por p53. Estas proteínas gobiernan la permeabilidad de la membrana mitocondrial y pueden ser pro-apoptóticas (Bcl-10, Bax, Bak, Bid, Bad, Bid, Bik, Blk, entre otras) o anti-apoptóticas (Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG, entre otras) (Elmore, 2007). Además de actuar como represor de la expresión de la β-tubulina y la α-tubulina, p53 inicia la transcripción de genes como PUMA, Bax, Bid en la vía apoptótica intrínseca y de CD95 y TRAIL en la vía apoptótica extrínseca (Beckerman and Prives, 2010; Koumenis et al., 2001).

Además, p53 también tiene funciones apoptóticas que son independientes de su actividad transcripcional. En el citosol, p53 induce muerte celular formando complejos inhibitorios con las proteínas anti-apoptóticas Bcl-XL y Bcl-2, lo cual conduce a la permeabilización de la membrana mitocondrial y a la liberación de citocromo *c* (Pflaum et al., 2014). En su localización mitocondrial, p53 induce oligomerización de Bax y Bak, antagoniza el efecto antiapoptótico de Bcl-2 y Bcl-XL, y forma un complejo con la ciclofilina D en la membrana mitocondrial interna (Fig. 6). Estos cambios ocasionan una acentuada disrupción de las membranas mitocondriales y la subsecuente liberación de factores

apoptogénicos activadores de caspasas o con actividad de endonucleasas como citocromo *c*, Smac/DIABLO o AIF/Endo G (Amaral et al., 2010).

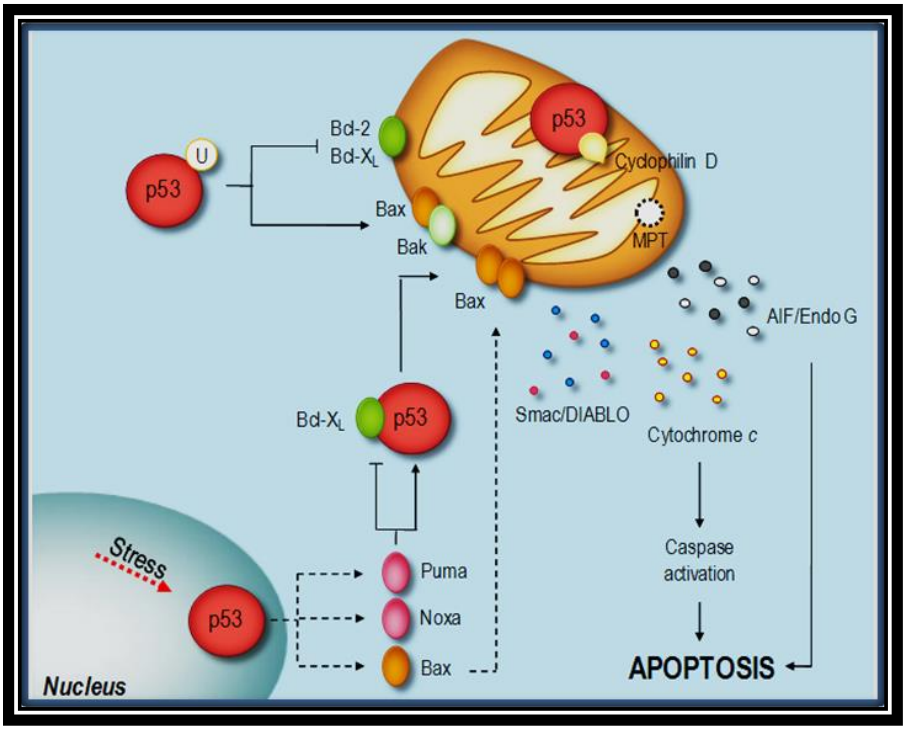

Figura 6. Vías apoptóticas dependientes de p53 en núcleo, citosol y mitocondria.
Tomado de Amaral et al. (2010).

En todos los invertebrados en los que la función de la familia p53 se ha estudiado, se ha encontrado que p53 induce a apoptosis en respuesta a daño al DNA, en tanto que en el caso de vertebrados, incluso habiendo varios miembros de esta familia, un papel común de p53 es la apoptosis en células germinales. Lo anterior indica que es probable que ésta sea una de las funciones ancestralmente comunes de p53 (Rutkowski et al., 2010).

Apoptosis Mediada por p53 y MT en Invertebrados en Respuesta a Hipoxia

El conocimiento existente en cuanto a la regulación de apoptosis en invertebrados se basa principalmente en los hallazgos en *D. melanogaster* que probablemente disten mucho de ser representativos de todos los mecanismos de muerte celular programada que siguen las demás especies del *phylum* Arthropoda. El papel de p53 se ha estudiado en algunos

invertebrados además de *D. melanogaster* y *Caenorhabditis elegans* y en todos ellos se ha encontrado para esta proteína una función común de inducción a apoptosis en respuesta a daño a DNA (Rutkowski et al., 2010). En el estudio de crustáceos específicamente, se han encontrado un gran número de homólogos de proteínas importantes en mecanismos apoptóticos tanto de *D. melanogaster* como de humanos. Sin embargo, son pocos los análisis bioquímicos y genéticos que se han hecho acerca de la función de estos genes en organismos no modelo (Menze et al., 2010). Por ejemplo, en el sistema nervioso central del cangrejo de manglar, *Ucides cordatus*, la radiación UV induce un aumento de proteína p53 y una disminución de la proteína p21 -efectora del arresto del ciclo celular-, con la consecuencia final de apoptosis (Hollmann et al., 2016). Sin embargo, al iniciar la presente investigación se desconocía si en respuesta a hipoxia esta función se mantiene.

En el caso de los camarones peneidos *Penaeus monodon* y *Litopenaeus vannamei* se sabe que ocurre apoptosis en respuesta a infecciones virales, a hipertermia y a hipotermia (Granja et al., 2003; Vousden and Ryan, 2009; Wongprasert et al., 2003). Recientemente se ha reportado apoptosis inducida por cadmio en peneidos e incluso se ha evidenciado la existencia de algunas proteínas involucradas en el proceso apoptótico en este organismo (Yang et al., 2013). Hasta el inicio de este estudio, el papel de p53 como "gen maestro" en dicho proceso, no había sido aún investigado y tampoco su interacción con MT de manera análoga a la encontrada en células de mamífero (Cherian et al., 2003; Méplan et al., 2000).

Mecanismo de Apoptosis Dependiente de p53 Inducida por Hipoxia

En vertebrados superiores, condiciones de hipoxia severa inducen una respuesta de daño al DNA (DDR) única, ya que la hipoxia no genera daño detectable al DNA. Dicha respuesta incluye vías de señalización mediadas por ATR (Ataxia-telangiectasia Rad3-related) y ATM (Ataxia-telangienctasia mutated) (Olcina and Hammond, 2014). Por otro lado, la activación de p53, es una de las principales consecuencias de la DDR inducida por hipoxia (Green and Kroemer, 2009). Subsecuentemente, p53 induce apoptosis y no arresto del ciclo celular en el punto de control G_1/S, como sucede ante el daño al DNA por estrés genotóxico (Olcina and Hammond, 2014). La manera en la que la señal de

hipoxia es transducida a p53 ha sido estudiada en líneas celulares y cultivos primarios embriónicos de mamíferos; así, se ha demostrado que bajo condiciones de hipoxia severa pero no moderada, ocurre una disminución en los niveles de nucleótidos disponibles y arresto de la replicación de manera simultánea. Estos eventos resultan de la actividad disminuida de la ribonucleótido reductasa en estas condiciones (Pires et al., 2010). Esta enzima cataliza la conversión de ribonucleótidos a deoxirribonucleótidos de manera oxígeno-dependiente, pues se requiere oxígeno molecular para regenerar un radical libre en su sitio activo (Probst et al., 1989). Como consecuencia de la disminución de deoxirribonucleótidos ocasionada por la hipoxia severa, disminuye la velocidad de la horquilla de replicación e incluso se detiene, ocasionando estrés replicativo (RS). El RS genera regiones con acumulación de ssDNA a las que se recluta ATR. ATR es una cinasa de serina/treonina que fosforila a MDMX, proteína inhibidora de p53. En consecuencia, p53 se estabiliza y activa para inducir apoptosis. El papel de ATM en hipoxia severa no es claro, sin embargo mutantes nulos de ATM son altamente sensibles a hipoxia/reoxigenación (Freiberg et al., 2006; Lee et al., 2012; Wade et al., 2013), pero es posible que ATR activada por la hipoxia contribuya a la fosforilación y activación de ATM (Olcina and Hammond, 2014).

En animales que viven en condiciones de hipoxia en ambientes acuáticos o lodosos se ha encontrado una mutación R174K en p53 (con respecto a TP53). Estas especies incluyen *Xenopus*, *Xiphophorus*, *Loligo forbesi* y *Mya arenaria* (Zhao et al., 2009). La importancia de esta mutación ha sido investigada en el topo subterráneo israelí *Spalax*, cuyo hábitat se limita a madrigueras subterráneas con bajas tensiones de oxígeno. En la proteína p53 de este organismo, los aminoácidos en los sitios 172 y 207 son lisina (K) en tanto que los aminoácidos en los sitios correspondientes en humanos (174 y 209) son arginina (R) (Ashur-Fabian et al., 2004). La R174, y en menor medida la R209, están altamente conservadas a lo largo de la evolución. La mutación R174K en p53 de *Spalax* reduce su capacidad de unión al DNA (Ashur-Fabian et al., 2004). Una consecuencia de esto es que en este organismo p53 no se une al dominio ODD de HIF-1α en respuesta a hipoxia, hecho que conduce a la transactivación dependiente de HIF-1 y que favorece la transactivación por p53 de efectores de arresto celular pero no de apoptosis evitando así el daño celular

que en otros organismos es inducido por p53 en condiciones de hipoxia (Ashur-Fabian et al., 2004 ; Zhao et al., 2009).

El camarón blanco, *Litopenaeus vannamei*, es un organismo capaz de sobrevivir en condiciones de hipoxia prolongada. Se desconoce si en este animal la hipoxia induce apoptosis mediada por p53, como sucede en las células sanas de otros organismos, o si, por el contrario, la respuesta a hipoxia es reminiscente a aquella de células cancerígenas o de otros organismos tolerantes a hipoxia que aquí se han mencionado. Aunque p53 se encuentra presente en todos los animales en los que se la ha buscado, al iniciar este trabajo de investigación, no había información disponible acerca de la existencia de p53 en el camarón blanco. El primer reporte de este gen en *L. vannamei* se publicó de manera simultánea con nuestras propias investigaciones. Este reporte indica que p53 regula de manera positiva la expresión y actividad de las enzimas antioxidantes superóxido dismutasa manganosa MnSOD y glutatión peroxidasa GPx en camarones sometidos a estrés por metales o pH (Qian et al., 2014); sin embargo, no hace alusión a apoptosis ni al estrés oxidativo ocasionado por la hipoxia. La secuencia encontrada en nuestro laboratorio se reporta en el artículo que compone el **capítulo III** de esta tesis, en el que se describe el estudio de la participación de p53 y MT en la ocurrencia de apoptosis en *L. vannamei* frente a hipoxia. Ambos genes fueron silenciados de manera independiente en camarones que posteriormente se sometieron a hipoxia. Mediante análisis de citometría de flujo y actividad de caspasa-3, se encontró que la apoptosis aumenta en los hemocitos de estos camarones y que este aumento es parcialmente dependiente de la abundancia de transcritos de p53 en tiempos cortos de estrés por hipoxia.

RNA de Interferencia como Herramienta de Estudio de Genes en Crustáceos

Históricamente, el estudio de la función de genes ha sido abordado de manera inicial en organismos modelo, por las ventajas que éstos ofrecen: intervalos generacionales cortos, alta fecundidad, bajos costos de mantenimiento, entre otros. Además, estos organismos modelo poseen rutas bioquímicas o metabólicas conservadas con respecto a otras especies, de manera que pueden establecerse analogías a partir de las cuales se estudien estas otras especies (Ankeny and Leonelli, 2011). A través del tiempo los organismos modelo han

sido modificados, de manera que actualmente es posible manipular la expresión y actividad de genes y proteínas para investigar su función. Otro soporte biológico importante para estudiar funcionalidad de genes y procesos celulares, está dado por las líneas celulares estables que también son manipulables a nivel molecular. Una multitud de líneas transgénicas de organismos modelo y de líneas celulares de diversos orígenes son utilizadas de manera rutinaria en los laboratorios de investigación y sin duda han sido cruciales para el avance del conocimiento de los sistemas biológicos. Sin embargo, a pesar de los esfuerzos de numerosos grupos científicos desde los años noventa y de la importancia económica que los camarones peneidos representan, no ha sido posible establecer aún una línea celular estable proveniente de estos organismos (Ma et al., 2015). El estudio de múltiples patologías de origen viral y bacteriano en peneidos se facilitaría sustancialmente si pudieran analizarse directamente en líneas celulares de la propia especie. También los estudios de ciencia básica de camarón, como el que se presenta en esta tesis, se verán acelerados una vez que estas líneas estén disponibles. Mientras tanto, en los estudios de función génica en organismos no modelo, es necesario hacer uso de estrategias de manipulación de genes que prescindan del uso de líneas celulares para estudiar su función. Para este fin, en este trabajo de investigación se aprovechó el mecanismo de RNA de interferencia (RNAi) propio de las células eucariotas, utilizando RNA de doble cadena (dsRNA) exógeno como desencadenante del mencionado mecanismo. El **capítulo IV** de esta tesis describe el mecanismo de silenciamiento o "*knock-down*" de genes por RNAi y revisa los avances en la descripción de las proteínas clave para este proceso en crustáceos.

CONCLUSIONES

Ante el vacío de información en cuanto a la regulación de los procesos apoptóticos en el camarón blanco, en este trabajo se propuso explorar la intervención de metalotioneína (MT) y de p53 en la ocurrencia de apoptosis ante el estímulo de hipoxia. A partir de los resultados obtenidos se concluye que en hemocitos de camarón ocurre apoptosis basal independiente de p53 o MT, mientras que en respuesta a hipoxia una vía de apoptosis dependiente de p53 contribuye a la tasa apoptótica total. Los análisis de apoptosis por citometría de flujo y actividad de caspasas realizados, demuestran una respuesta fisiológica que se contrapone a la hipótesis de que en *Litopenaeus vannamei* ocurre evasión de apoptosis mediada por p53 y MT en respuesta a hipoxia. En los dos tiempos ensayados hay apoptosis en hemocitos de camarones sometidos a hipoxia ocasionada por 1.5 mg $OD \cdot L^{-1}$. Aunque la interpretación de estos resultados es directa: *la hipoxia induce apoptosis en hemocitos de camarón blanco*, en la medida en que otros tejidos sean analizados, una respuesta a nivel organismo podrá ser entendida.

PERSPECTIVAS

La respuesta observada en hemocitos solamente explica los efectos de dos concentraciones de oxígeno (~5.0 y ~1.5 mg OD·L^{-1}) y de dos tiempos de exposición a estas concentraciones. Es importante explorar en futuros estudios cuáles son las condiciones de hipoxia que pueden considerarse moderadas o severas en términos de supervivencia celular. Este conocimiento es de interés no sólo en términos de fisiología comparada, sino que podría incorporarse a modelos de simulación de cambio ecológico en las zonas hipóxicas emergentes.

Por otro lado, el grado de estrés que se impuso en el bioensayo es más severo que las condiciones que ocurren en campo, tanto en términos de concentración de oxígeno como de duración de la hipoxia.

Adicionalmente, se desprenden de esta investigación resultados que sugieren que, ante hipoxia MT tiene una función antioxidante protectora, contribuyendo a un mecanismo fisiológico de "preparación para estrés oxidativo" ante las especies reactivas de oxígeno generadas por la hipoxia. La confirmación de esta hipótesis permitiría añadir MT al repertorio de proteínas antioxidantes y de mecanismos importantes para la supervivencia del camarón en ambientes hipóxicos.

Esta investigación de tipo exploratorio exigía como prerrequisito obtener las secuencias codificantes de MT y p53. Durante la estrategia para obtener estas secuencias solamente un gen para cada una de estas proteínas fue encontrado. En otras especies, las metalotioneínas están codificadas por una familia multigénica. Aunque los hallazgos de un solo gen MT aquí descritos fueron consistentes a lo largo de la experimentación, es posible que otros genes codificantes de metalotioneínas estén presentes en *L. vannamei*. De ser así, sería importante investigar la contribución de estas metalotioneínas al proceso apoptótico desencadenado por la hipoxia.

Estudios anteriores en este grupo de investigación, demuestran que en el camarón blanco la aceleración de la ruta glucolítica anaerobia contribuye a sostener las funciones celulares indispensables para resistir la hipoxia. La introducción celular de glucosa -mediada por transportadores - es un punto clave para alimentar dicha ruta metabólica. Es bien conocido que en mamíferos p53 regula la expresión de proteínas transportadoras de glucosa. Hallazgos recientes en nuestro grupo de trabajo revelan la presencia de un elemento de respuesta para p53 en la región promotora del transportador de glucosa GLUT1. El análisis de la expresión de GLUT1 a nivel de transcritos y de proteína en camarones silenciados con dsp53 en hipoxia, daría información acerca del papel regulador de p53, constituyendo —junto con HIF1— un nodo medular de control que integra vías metabólicas, mecanismos antioxidantes y destino celular en crustáceos en ambientes hipóxicos.

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CAPÍTULO II

The metallothionein gene from the white shrimp *Litopenaeus vannamei*: Characterization and expression in response to hypoxia

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RESUMEN

Los animales acuáticos se encuentran con variaciones en la tensión de oxígeno que conducen a la acumulación de especies reactivas de oxígeno (ROS) que pueden dañar a los organismos. Bajo estas circunstancias algunos organismos han evolucionado para tolerar la hipoxia. En mamíferos, las metalotioneínas (MTs) protegen contra ROS generados por la hipoxia. Aquí reportamos el gen MT del camarón blanco *Litopenaeus vannamei* (LvMT). LvMT se expresa diferencialmente en hemocitos, intestino, branquias, pleópodos, corazón, hepatopáncreas y músculo, con los niveles más altos en hepatopáncreas y corazón. El mRNA de LvMT aumenta durante la hipoxia en hepatopáncreas y branquias después de 3 h a 1.5 mg $OD \cdot L^{-1}$. La estructura de este gen se asemeja a sus homólogos en vertebrados e invertebrados, con tres exones, dos intrones y en la región promotora tiene elementos de respuesta para el factor de transcripción inducido por metales (MTF-1), el factor de transcripción inducido por hipoxia (HIF-1). Durante la hipoxia, el complejo HIF-1/MTF-1 podría participar induciendo MT para contribuir a la tolerancia a la toxicidad por ROS. La importancia de las MT en organismos acuáticos podría también incluir procesos detoxificantes de ROS.

Palabras clave: metalotioneína, especies reactivas de oxígeno (ROS), mRNA, hipoxia, camarón, elementos de respuesta, hepatopáncreas, branquias

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ABSTRACT

Aquatic animals encounter variation in oxygen tension that leads to the accumulation of reactive oxygen species (ROS) that can harm the organisms. Under these circumstances some organisms have evolved to tolerate hypoxia. In mammals, metallothioneins (MTs) protect against hypoxia-generated ROS. Here we report the MT gene from the shrimp *Litopenaeus vannamei* (LvMT). LvMT is differentially expressed in hemocytes, intestine, gills, pleopods, heart, hepatopancreas and muscle, with the highest levels in hepatopancreas and heart. LvMT mRNA increases during hypoxia in hepatopancreas and gills after 3 h at 1.5 mg L^{-1} dissolved oxygen (DO). This gene structure resembles the homologs from invertebrates and vertebrates possessing three exons, two introns and response elements for metal response transcription factor 1 (MTF-1), hypoxia-inducible factor 1 (HIF-1) and p53 in the promoter region. During hypoxia, HIF-1/MTF-1 might participate inducing MT to contribute towards the tolerance to ROS toxicity. MT importance in aquatic organisms may include also ROS-detoxifying processes.

1. Introduction

Variations in dissolved-oxygen levels ranging from anoxia to hyperoxia occur in the diversity of environments where shrimp and other crustacean live (Helly and Levin, 2009). Although episodes of hypoxia are common in the aquatic environment, the frequency and spatial extent of these low-oxygen regions have increased considerably due to anthropogenically-driven increases of contaminant and nutrient inputs into estuaries and coastal zones (Rabalais et al., 2010). We are interested in understanding how aquatic organisms cope with these variations in oxygen concentration, and in using the white shrimp *Litopenaeus vannamei*, a native species from the Pacific Ocean as a non-model organism because of the significant and growing importance of this species in aquaculture in America and Asia (Bostock et al., 2010), and also for its potential use in the investigation of protective processes against free radical cellular damage, tissue injury and the concomitant physiological consequences leading to diseases (Lushchak, 2011).

The generation of reactive oxygen species (ROS) in aquatic organisms as a consequence of changing levels of oxygen availability has been extensively reported (Welker et al., 2013). Hypoxia increases ROS levels in several aquatic species (Abele et al., 2007) provoking damage and detrimental effects on proteins, lipids and DNA. If the cellular defense systems -such as the antioxidant repertoire to sustain a redox balance- are exceeded by the ROS levels, the fate of the irreversibly injured cell may be death (Kowaltowski et al., 2009). Cellular protection mechanisms against ROS include several antioxidant enzymes. Some of these enzymes have been described in the white-leg shrimp, including cytosolic manganese superoxide dismutase (cMnSOD) (Garcia-Triana et al., 2010a; Gomez-Anduro et al., 2006), selenoprotein M (SelM) (Clavero-Salas et al., 2007; García-Triana et al., 2010b), catalase (Trasviña-Arenas et al., 2013), glutathione S-transferase (Contreras-Vergara et al., 2008), glutathione peroxidase and total superoxide dismutase (Parrilla-Taylor and Zenteno-Savín, 2011). Additionally, non-enzyme proteins such as metallothioneins participate in this protection (Lazo et al., 1995).

The importance of metallothioneins in metal-detoxifying processes in aquatic invertebrates is well known, but their relevance extends to ROS-detoxifying mechanisms necessary for organisms that are under oxidative stress during their life time. Metallothioneins (MTs) are highly conserved cysteine-rich small proteins ranging from 6 to 7 kDa (Kagi and Kojima, 1987) that are widely

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distributed across kingdoms and phyla and contain up to seven bivalent metallic ions per molecule (Braun et al., 1992). These metals can be obtained from other metalloenzymes and transcription factors, thus regulating the activities of these proteins (Cano-Gauci and Sarkar, 1996; Roesijadi et al., 1998). Due to their metal binding properties, MTs are involved in the homeostasis of essential metals. MTs first recognized function was the detoxification of heavy metals such as cadmium, mercury, zinc and cooper. MTs also protect against radiation and oxidative damage and contribute to the control of cellular proliferation and apoptosis (Ruttkay-Nedecky et al., 2013). As shown in mammals, increased MT synthesis is triggered in response to oxidative damage (Sato and Bremner, 1993) and its high antioxidant activity counteracts peroxidation of lipid membranes and DNA (Fang et al., 1997; Kondoh et al., 2003). In *Caenorhabditis elegans* MTs have an *in vivo* protective role against oxidative stressors and *in vitro* scavenging of hydrogen peroxide (Zeitoun-Ghandour et al., 2011). The regulation of MT by free radicals and its involvement in the protection against H_2O_2 was also demonstrated in cell lines derived from aquatic organisms (Kling and Olsson, 2000). The cysteine residues in MTs can sequester free hydroxyl radicals by their thiol groups and stop oxidative damage to DNA and proteins (Good et al., 1991). MTs can even revert oxidative damage by donating a hydrogen atom to the site damaged by the free radical, thus restoring DNA back to its normal structure (Renan and Dowman, 1989). Additionally, MTs participate as co-factors in the activation of synthesis of glutathione peroxidase (Hayes and McLellan, 1999). Most healthy animal tissues contain MTs, but are highly induced in several types of human tumors (Cherian et al., 2003). There are at least 16 isoforms of MTs in humans and four in mice (Palmiter, 1998). MT genes have been found in eukaryotes as well as in some prokaryotes, usually in multiple copies (Palmiter, 1998) and recently were identified in several crustaceans (Mao et al., 2012) where there are reports of more than one gene present, such as in the water flea *Daphnia pulex* and in the blue crab *Callinectes sapidus* with four and two MT genes identified, respectively (Asselman et al., 2012; Syring et al., 2000). MT expression in crustaceans has been mainly studied in the toxicological context for environmental monitoring (Amiard et al., 2006), revealing that its expression is controlled by various elements, including *cis*-regulated metal response elements (MRE) with a highly conserved core sequence usually present in multiple copies. The metal response transcription factor 1 (MTF-1) regulates the basal expression of MT as well as its expression in response to heavy metals by binding to the MREs (Ostrakhovitch et al., 2007). Besides zinc and other heavy metals, the transcriptional activity of MTF-1 is also induced by oxidative stress (Andrews, 2000) and hypoxia (Murphy et al., 2008).

In response to hypoxia, the induction of MT expression also depends on the MTF-1/MRE binding Murphy et al. (1999). However, (Murphy et al., 2008) demonstrated that in the absence of the hypoxia-inducible factor 1α (HIF-1α), MTF-1 is recruited to the MT-1 promoter but this is not sufficient to drive MT expression in response to hypoxia. Additionally, they also showed that MTF-1 interacts *in vivo* in a transcriptional complex which includes HIF-1α and that both proteins are recruited to the MT promoter in response to hypoxia. In *L. vannamei*, induction of MT-like proteins (MTLP) is triggered by high concentrations of cadmium and zinc, therefore MT has been proposed as a heavy metal exposure biomarker (Moksnes et al., 1995; Wu and Chen, 2005).

The *L. vannamei* MT cDNA sequence was recently reported and its expression studied in response to salinity stress (Wang et al., 2014), however, the MT complete gene sequence was not included nor was its involvement as a hypoxia-generated ROS scavenger. Herein we report the structure and sequence of the MT gene from the shrimp *L. vannamei* (LvMt), its promoter region sequence, and the mRNA tissue-expression under normoxia and in response to hypoxia in gills and hepatopancreas.

2. Materials and methods

2.1. LvMT cDNA cloning

Specific primers were designed based on the sequence of an EST from a muscle cDNA library previously generated in our group and from other *L. vannamei* EST sequences found in GenBank. One of the ESTs corresponded to hepatopancreas (GenBank accession no. BF023848.1) and the other to WSSV infected hemocytes (GenBank accession no. EE572310.1). Total RNA was obtained from hepatopancreas of adult shrimp using TRI REAGENT® (Sigma–Aldrich, St. Louis, MO, USA). Total RNA quantity and quality was assessed by absorbance at 230, 260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Only RNA samples with 260/280 ratio between 1.9 and 2.1 and 260/230 ratio greater than 2.0 were used for the analysis. RNA integrity was analyzed in agarose gels and 1 μg was used for reverse transcription using the QuantiTect® system (Qiagen, Valencia, CA, USA). The MT-cDNA fragment was first obtained by PCR using the primers pair MTF1/MTF2rv (Table 1). To determine the 5′ and 3′ ends of the LvMT transcripts, rapid amplification of cDNA ends (RACE) was performed on a cDNA SMART™ library generated from hepatopancreas of an adult intermolt shrimp (SMART™ cDNA Library Construction Clontech, Palo Alto, CA, USA) using the primer pairs 5′PCR/MTF1rv for the 5′UTR and MTF2/CDSIII3′ (Table 1) for the 3′UTR. All the primers used in this report were synthesized by Integrated DNA Technologies (Coralville, IA, USA) (Table 1). The PCR reactions were carried out in a final volume of 25 μL containing Platinum PCR SuperMix (Invitrogen Inc., Carlsbad, CA, USA), 2.5 μM of each primer and 1 μL of cDNA (synthesized from 50 ng of total RNA). The PCR reactions were carried out in a DNA Dyad Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). An initial denaturing step of 94 °C for 4 min was followed by 35 cycles of 94 °C for 30 s, 58.5 °C for 40 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The amplicons obtained were cloned in a pGEM®-T Easy Vector System (Promega, Madison, WI, USA) using Top 10 *Escherichia coli* cells. Two clones of each amplicon were thoroughly sequenced on both sense and anti-sense strands at the Laboratory of Genomic Analysis and Technology Core of the University of Arizona. Based on the obtained cDNA sequences, the primers MTS1F and MTS1rv as well as the primers mentioned below

Table 1
Primers used to obtain the LvMT nucelotide sequence and to evaluate expression.

Primer name	Sequence (5′–3′)
5′PCR	AAGCAGTGGTATCAACGCAGAGT
MTF1	GGCAGGGTTACTTTCAGAGC
MTF2	CGACTGTCTTCTGCTGCTTG
MTF1rv	GCTCTGAAAGTAACCCTGCC
MTF2rv	CAAGCAGCAGAAGACAGTCG
CDSIII3′	ATTCTAGAGGCCGAGGCGGCCGACATG-$(T)_{30}N_{-1}N$ (N = A, G, C, or T; N_{-1} = A,G, or C)
MTS1F	ATGCCTGATCCATGCTGTAAC
MTS1rv	TGGGCAGCACTTGCATGG
MTSP1	TGCGCAAAGGACTGCAAGTGCGCCAG
MTSP2	GTTGCAAATGCGCCCCTTGTGAGAAG
MTSP2rv	TGGCGCACTTGCAGTCCTTTGCGCAG
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCACGCGTGGT
MTRTF	CTGATCCATGCTGTAACGAG
MTRTRv	CATCTTGTTGCACACTCCTC
L8F2	TAGGGAATGTCATCCCCATT
L8R2	TCCTGAAGGGAGCTTTACACG

(Table 1), were designed using the Primer3web V.4.0.0 suite (Untergasser et al., 2012) to amplify the entire coding sequence from the start to the stop codons. The resultant amplicons were also cloned and sequenced as described previously.

2.2. LvM gene cloning

The pair of primers MTS1F/MTS1rv was used to amplify the sequence corresponding to the coding region using genomic DNA (gDNA) as template to investigate the presence of introns. Genomic DNA was prepared from 300 mg of muscle of an adult shrimp using the DNAzol® Reagent (Life Technologies™, Gaithersburg, MD, UDA) according to the manufacturer's protocol. The full-length LvMT gene sequence was obtained using the Genome Walker™ kit (Clontech, Palo Alto, CA, USA). Briefly, Genome Walker™ libraries (GW DNA libraries) representing the genome of *L. vannamei* digested with *Dra*I, *Eco*RV, *Pvu*II, *Stu*I, *Sma*I, *Ssp*I, *Sac*I were used as templates for the following PCR amplifications: a) to obtain the 5′-flanking region, adaptor primers AP1 and AP2 along with specific primer MTSP2rv were used in seminested PCR reactions (initial denaturation for 5 min at 95 °C followed by 7 cycles of 25 s at 94 °C and 3 min at 72 °C, 35 cycles each of 25 s at 94 °C and 3 min at 67 °C and a final extension of 7 min at 67 °C) and; b) to obtain the 3′-flanking region, specific primer MTSP1 and adaptor primer AP1 were used in a primary PCR reaction with the same conditions as in a) and the resulting product served as template for the nested secondary PCR reaction using the specific primer MTSP2 and adaptor primer AP2 with the same PCR conditions as in a) with only 5 cycles of the first extension round. The PCR reactions were carried out in a final volume of 25 μL containing HotStart Taq 2× master mix (New England BioLabs®Inc, Ipswich, MA, USA), 1 μL of each primer (5 μM), 8 ng of GW library DNA and nuclease-free water (Integrated DNA Technologies, Coralville, IA, USA). All primer sequences are shown in Table 1. The amplicons obtained were cloned and sequenced as previously described. The LvMT gene sequence was deposited in GenBank (Accession no. KJ701600).

2.3. Shrimp maintenance and hypoxia challenge

Juvenile shrimp were obtained from a shrimp farm and raised in a 150 L tank at laboratory conditions until reaching an average weight of 11.4 ± 0.8 g, acclimated to a salinity of 35 ppm, temperature of 28 °C and constant aeration to maintain normoxic conditions (5.0–6.0 mg DO L^{-1}, pH 8.0). Water was exchanged to maintain total ammonia concentrations within the safety levels ≤3.0 mg L^{-1} (Lin and Chen, 2001) throughout the experiment, monitored by flow-injection/gas-diffusion analysis (Hunter and Uglow, 1993). Twenty-eight healthy shrimp at intermolt stage were selected for the bioassay. Seven animals were assigned to the normoxic group and twenty-one to the hypoxic groups. The two groups were kept separately in 80 L tanks and acclimated for 72 h before lowering dissolved oxygen concentrations. Hypoxia was induced by injecting nitrogen gas and air at fixed ratios to maintain 1.45 ± 0.2 mg DO L^{-1}. Five shrimp from the hypoxic tank were collected after 3, 24 and 48 h. The control normoxic group was kept at 5.28 ± 0.3 mg DO L^{-1} and the animals were collected at 48 h.

2.4. LvMT mRNA expression by RT-qPCR

MT mRNA relative expression was evaluated under normoxic and hypoxic conditions using at least four biological replicates with four technical replicates from each treatment group. To study tissue expression, total RNA from samples in normoxic conditions was isolated separately from hemocytes, muscle, hepatopancreas, gills, intestine, pleopods and heart of five juvenile shrimp using TRI REAGENT® (Sigma–Aldrich, St. Louis, MO, USA). To analyze expression under hypoxia regimes, total RNA from gills and hepatopancreas was extracted. Removal of residual genomic DNA was done by treating with DNase I (Roche Diagnostics, Indianapolis, IN, USA) and 1 μg of this RNA was used for reverse transcription using the QuantiTect® Reverse Transcription (Qiagen® Valencia, CA, USA) system. Real-time PCR primers MTRTF and MTRTRv for the MT gene were designed to amplify a fragment of 145 bp (Table 1). The ribosomal protein L8 gene (GenBank accession no. DQ316258.1) was chosen as a reference gene and amplified using the primers L8F2 and L8R2 (Trasviña-Arenas et al., 2013). To construct the calibration curves for MT and L8, serial dilutions from 5×10^1 to 1.28×10^{-4} ng/μl of cDNA (equivalent of total RNA) from hepatopancreas and gills were made, respectively. Two cDNAs were synthesized from each total RNA sample and two qPCR reactions for each cDNA were carried out on a final volume of 20 μL containing 10 μL of 2× iQ SYBR Green Supermix (BioRad Laboratories, Hercules, CA, USA), both L8 primers to a final concentration of 1 μM or both MT primers to a final concentration of 100 nM, 1 μL of template cDNA (equivalent to 25 ng of total RNA) and molecular grade water. In each run, positive and non-template controls were included. All reactions were performed on an iQ5 Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA, USA) after optimizing the parameters to the following program: 94 °C for 3 min followed by 40 cycles of 95 °C for 30 s, 57 °C for 35 s, 72 °C for 55 s taking the fluorescence reading at the extension step. A melting curve was generated from 60 °C to 95 °C by increasing 0.3 °C each 20 s. Efficiency and linearity of the dynamic range for both L8 and MT genes were determined with the software iQ5 (BioRad, USA) from each calibration curve. Relative quantification of the MT mRNA levels was calculated using the comparative Ct method (Schmittgen and Livak, 2008).

2.5. Statistical analysis

For tissue and hypoxia expressions, the data were statistically analyzed by one-way ANOVA and a Tukey–Kramer test was used. Differences were considered when $p < 0.05$. The analysis was performed in NCSS and PASS, 2007.

2.6. Bioinformatic analysis

Database homology searches were done using the National Center for Biotechnology Information BLAST 2.2.28 (Altschul et al., 1997) server. *In silico* translation and search for open reading frames were done using proteomic tools available from ExPASy (Artimo et al., 2012). The multiple sequence alignments were performed using the CLUSTAL Omega program (Sievers et al., 2011). The phylogenetic tree was generated with the MEGA5.05 software using the Maximum Likelihood method supported on the Jones-Taylor-Thornton matrix-based model. Bootstrap analyses were performed using 1000 replicates (Tamura et al., 2011). Deduced protein domains/family/motifs were predicted using the InterProScan platform (Zdobnov and Apweiler, 2001). The presence of consensus sequences of response elements was identified using the NSITE V.2.2004 program (Softberry, Inc) and the Patch program of the TRANSFAC database V.7.0 available at http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi.

3. Results and discussion

3.1. LvMT cDNA and deduced amino acid sequence

The full LvMT cDNA sequence from the 5′UTR to the 3′UTR was obtained and submitted to GenBank with the accession number KJ701600 (Fig. 1A). The 5′UTR is 69 bp long and the 3′UTR is 117 bp

long. The ORF is 177 bp and *in silico* translation generates a protein of 58 amino acid residues. A cDNA of MT from *L. vannamei* (GenBank accession no. JN707684) was recently reported by Wang et al., 2014, and its expression was analyzed in response to stress salinity. Though, there are some differences of that sequence compared to the one herein reported, most of them located in the untranslated regions and only one synonym substitution at position +45 from the start codon.

Clustal Omega analysis revealed the conserved distribution and position of the cysteine residues in the shrimp MT compared to the MTs in *Homo sapiens*, *Drosophila melanogaster*, *D. pulex*, *C. sapidus* MT–Ia and MT-IIa, *Bythograea thermydron*, *Cyanagraea praedator*, *Dromia personata*, *Homarus americanus* and *Pacifastacus leniusculus* (Fig. 1B). A further InterProScan analysis classifies this sequence as a family 3 crustacean metallothionein organized in the characteristic manner P-[GD]-P-C-C-X-(3,4)-C-X-C (where X is an amino acid other than cysteine). The deduced protein contains seven lysine residues in vicinity to cysteine residues. Other invertebrate MTs such as *D. pulex* and *D. melanogaster* contain only five lysines whereas *H. americanus* has nine. These lysine residues are thought to stabilize the interaction between MTs and metal ions due to electrostatic interactions that bridge the protonated basic residues and the negatively charged metal-thiolate complex (Cody and Huang, 1994). When analyzed in PSI Blastp, the deduced amino acid sequence has the highest identity (72%) with the lobster *H. americanus* MT (GenBank accession no. CAC42504.1), and with other MTs from crustaceans with identities ranging from 62 % to 69 % that were then used to construct the phylogenetic tree (Fig. 2), where Decapoda clusters together in a monophyletic group.

3.2. The shrimp MT gene contains introns and putative control elements in the promoter

From the start to the stop codons, the shrimp MT gene was 757 bp long and includes three exons interrupted by two introns, similar to other MT orthologues. The MT gene family appears to conserve exon number and length, where exon I is considerably shorter than exons II and III and have split codons. Additionally, exon location and length are similar amongst the crustacean revised in this study as shown in Fig. 3A.

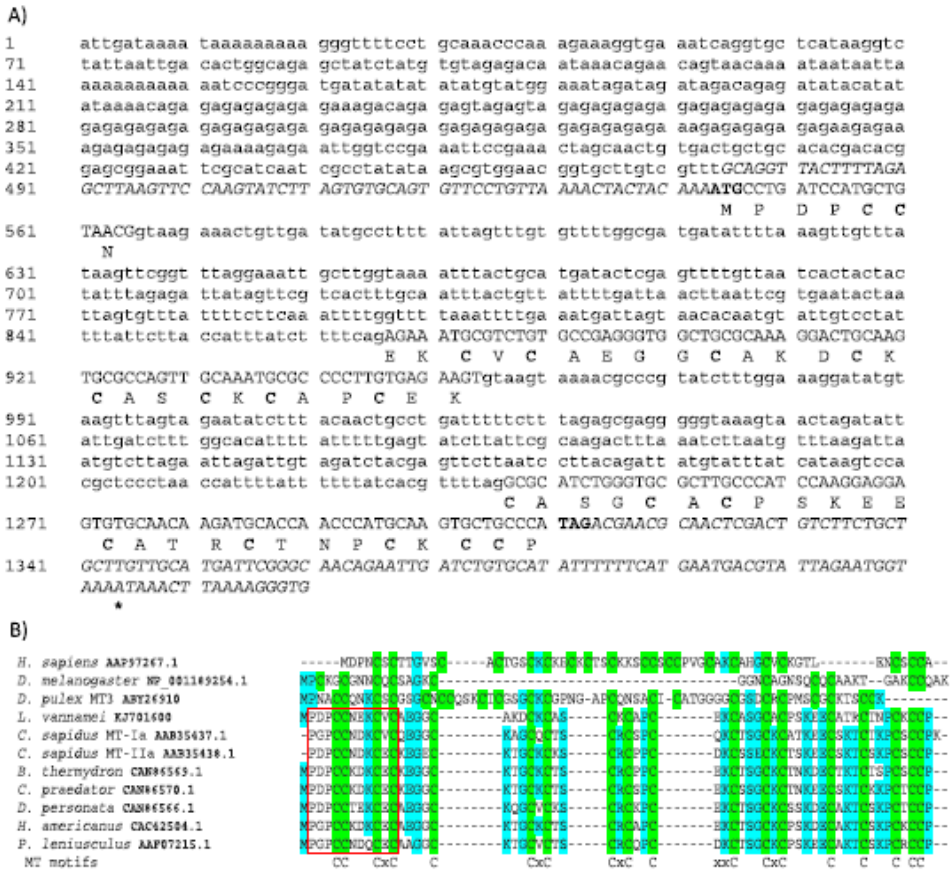

Fig. 1. (A) Nucleotide and deduced amino acid sequences of LvMT gene. The start and stop codons are indicated in bold. Deduced amino acids sequence is indicated as capital letters under each codon of the coding sequence also indicated as capital letters. The 18 cysteine residues characteristic of MTs are shown in bold. The italic letters indicate UTRs present in the mRNA and gDNA sequences obtained. The introns and upstream sequence from the start codon are in lowercase letters. The polyadenylation signal is indicated by the asterisk, and; (B) Clustal alignment of metallothionein amino acid sequences from various organisms. Cysteine residues are shaded in green. Crustacean MT N-terminus motives P-[GD]-P-C-C-X-(3,4)-C-X-C are shown in the red box. Amino acid residues conserved in at least seven of the included species are shaded in blue. Cysteine residues characteristic of metallothioneins are indicated in the last line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

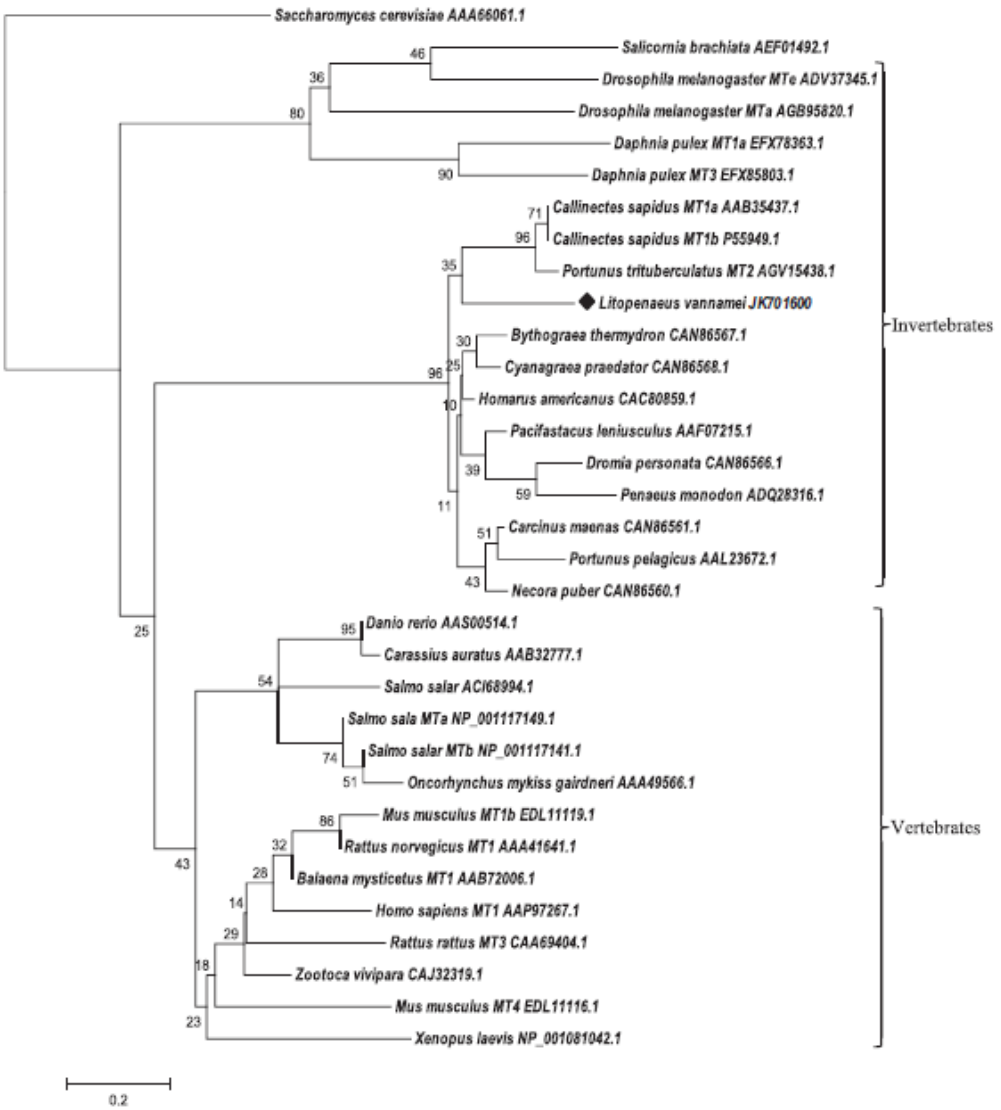

Fig. 2. Molecular phylogenetic analysis by maximum likelihood. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. GenBank accession numbers are provided next to each species name.

The Genome Walker strategy yielded a sequence of 543 bp upstream the translation initiation codon. Analyses of this region by NSITE and TRANSFAC revealed the presence of 198 motifs representing 64 different response elements (Fig. 3B). This proximal promoter region contains more than 50 GAGA response elements that may be bound by GAGA factors (GAF) that regulate up to 28 cellular pathways by activating or pausing transcription (Kwak et al., 2013; van Steensel et al., 2003) during chromatin remodeling (Berger and Dubreucq, 2012), four TATA-binding-protein (TBP) elements and two cAMP-response element binding protein (CREB)-responsive elements. Three different metal response elements (MRE) were found: MREe ctgTGCACACtggcg and MREa on the coding strand, and a metal response element for binding factor-I (MBF-1) on the antisense strand (Dalton et al., 1996; Imbert et al., 1989; Labbe et al., 1991). Remarkably, a HIF-1 response element (HRE) RCGTG was also found on the coding strand. Two p53-response elements were also found in this region (Bourdon et al., 2005; Tamir and Bengal, 1998). Metallothionein promoters in higher eukaryotes contain multiple, yet imperfect, repeats of metal response elements (MRE) (Karin et al., 1987). The differences in MRE sequences also reflect their variable strength to drive transcription (Culotta and Hamer, 1989). The highly conserved core

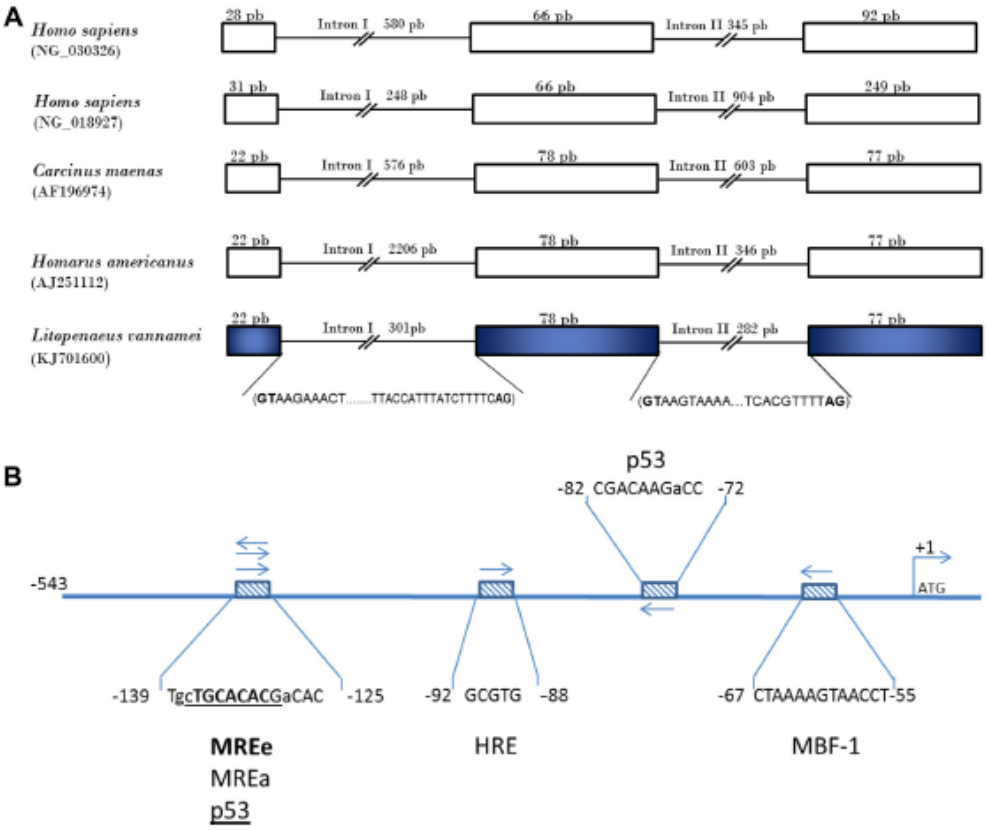

Fig. 3. Panel A) Structural organization of MT genes from various organisms. Boxes and lines represent exons and introns, respectively. Conserved splicing sequences are indicated in parentheses. GenBank accession numbers are given under each species. Taken and modified from (Leignel et al., 2008), and; Panel B) Regulatory elements in the proximal promoter of the LvMT gene. Only the regulatory sequences relevant to this study are shown. The location and orientation of the MREs, HRE and p53-reponse elements are indicated by the arrows.

sequence TGCPuCNC (Culotta and Hamer, 1989) is essential for the induction of MTs by metals. However, the overall expression levels are given by the additive effect of MRE and non-MRE upstream elements (Imbert et al., 1989). Moreover, as shown in mammalian systems, MT expression does not only respond to heavy metals but also to other environmental stresses such as redox fluctuations (Andrews, 2000; Palmiter, 1998) and hypoxia, where the duo hypoxia-inducible transcription factor-1α (HIF-1α)/metal-transcription factor-1 (MTF-1) is essential to induce MT expression by hypoxia (Murphy et al., 2008). The presence of a HRE in the LvMT gene promoter region may reflect its involvement in the response/adaptation to hypoxia in shrimp and the RT-qPCR results herein obtained indicate that there is indeed an effect of hypoxia increasing MT mRNA levels rapidly (Section 3.4). Like in other species, in *L. vannamei* HIF-1α is regulated by hypoxia and its expression levels varies in different tissues in both normoxic and hypoxic conditions (Soñanez-Organis et al., 2009), although the protein has not been quantitated. MT highest mRNA expressions are found in hepatopancreas, where HIF-1α mRNA is lower. Conversely, gills have the highest HIF-1α and the lowest MT expression levels. Whereas the response to hypoxia is the primary role of HIF-1, MT is generally considered to function in metal homeostasis and detoxification processes. Nevertheless, if the MT activation by hypoxia occurs by means of the HIF-1α/MTF-1 duo, it is sensible to think that HIF-1α could be imposing a sort of tissue limitation. If MT constitutive levels are already high in hepatopancreas because of its metal-related processes, then probably low HIF-1α levels will suffice to increase MT expression to the required levels under hypoxic conditions. Conversely, in gills high HIF-1α constitutive levels could quickly drive expression of the scarce MT transcripts. Thus, the differences in HIF-1α tissue expression levels could possibly account for a HIF-1 role not only in glycolysis regulation as reported by Soñanez-Organis et al. (2010) but also in the activation of antioxidant defenses of the sort of MTs. Clearly, the contribution of MTF-1 is also expected to be of paramount importance in this mechanism and needs to be investigated in shrimp. Additionally, whether the mRNA levels of MT and of α and β subunits of HIF-1 are paralleled by the protein levels is yet to be tested.

3.3. *Tissue distribution of LvMT mRNA*

The LvMT mRNA transcripts were quantified by real-time RT-qPCR and detected in all seven tissues studied from normoxic shrimp (Fig. 4). The qPCR reaction efficiencies of the dynamic ranges were 94.2%–100.5% for L8 and 93%–100.3% for MT. In all qPCR runs, R^2 values were ≥0.987. The lowest relative expression

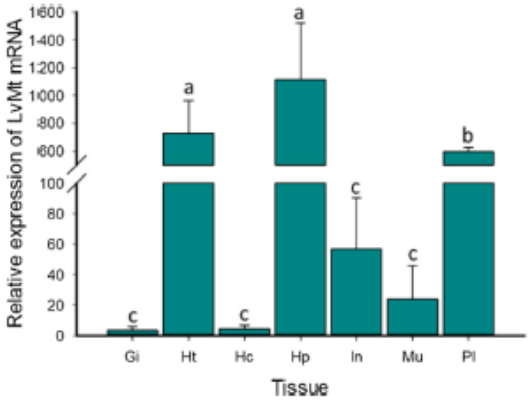

Fig. 4. Relative expression of LvMT mRNA in different tissues. Statistical analysis was based on comparisons of the expression of LvMT relative to L8 ($2^{-\Delta Ct}$ values). Each bar represents means ± SD from data obtained from at least four independent biological samples with four technical replicates ($n \geq 4$). Data were analyzed by one-way ANOVA and multiple Tukey–Kramer tests. Statistical significance was considered at p values <0.05. Different lowercase letters indicate statistically significant differences among different tissues. Abbreviations: Gi, gills; Ht, heart; Hc, hemocytes; Hp, hepatopancreas; In, intestine; Mu, muscle; Pl, pleopods.

was found in gills, followed by hemocytes (1.3 fold-change compared to gills), muscle (6.9 fold-change), intestine (16.4 fold-change) and the highest expressions were in pleopods (172.4 fold-change), heart (211 fold-change) and hepatopancreas (322.2 fold-change) (Fig. 4). Statistically significant differences ($p < 0.05$) were found between tissues.

MT is widely expressed in adults of aquatic invertebrates after metal treatments in various tissues including gills, hepatopancreas, muscle, heart and gonads and the highest expressions were often found in hepatopancreas and gills (Mao et al., 2012). In *L. vannamei*, the highest expression of the MT mRNA was detected in hepatopancreas, which appears to be the major organ that sequesters and detoxifies metals in invertebrates (Ahearn et al., 2004). The ridge-tail white prawn, *Exopalaemon carinicauda* expresses a copper-induced metallothionein only in hepatopancreas even without a copper challenge (Zhang et al., 2014). A similar trend was found in the shrimp *Rimicaris exoculata*, where the higher abundance of MT proteins (determined by differential pulse polarography, DPP) in hepatopancreas rather than in gills and abdomen was explained by the higher ratio of soluble metals in the former tissue (Geret et al., 2002). Nevertheless, studies on inter-organ MT expression differences in several other crustaceans, have shown remarkable higher levels of the protein in hepatopancreas than in gills, even when similar metal concentrations were found in both tissues (Amiard et al., 2006).

Although physiological and cellular studies are needed in *L. vannamei*, the tissue-specific expression of MT in non-stress conditions, could indicate its involvement in normal physiological processes of Cu and Zn regulation at the cellular level that control growth and reproduction (Engel, 1987). Also, it is plausible that different metallothioneins are expressed in different tissues in response to different challenges.

3.4. Expression profile of MT under hypoxia challenge

Despite the tissue expression profile in this study showed relative low levels of MT mRNA in gills, an increased MT transcription in response to hypoxia should not be ruled out in this organ, especially knowing that in aquatic organisms hepatopancreas and gills are the main tissues involved in metal uptake, storage and excretion, having a high capacity to synthesize MTs (Amiard et al., 2006). Moreover, gills are key for oxygen uptake, and hence, are primary tissues to sense hypoxia. Therefore, the LvMT mRNA relative expression was quantified in gills and hepatopancreas under different hypoxia times (Fig. 5). In both tissues tested, there was an increase of MT transcripts after 3 h in hypoxia followed by a decrease after 24 and 48 h in hypoxia. In hepatopancreas there was a 1.5-fold increase of MT levels at 3 h of hypoxia and after 24 and 48 h the levels had decreased approximately 1.5-fold below the normoxic levels. A similar response was found in gills, where a more dramatic 4.0-fold increase in LvMT was found after 3 h of hypoxia and by 24 and 48 h of hypoxia MT transcripts returned to pre-exposure levels. However, it should be noted that LvMT mRNA expressions are much higher in hepatopancreas than in gills and this remains true also for the expression after 24 and 48 h of hypoxia. These results reflect the status of LvMT mRNA at only three time points and one oxygen level (1.45 ± 0.2 mg DO L^{-1}), but the effects of hypoxia might be dose and time dependent. Nevertheless, the changes here detected in MT transcripts are reminiscent to those found in the intertidal snail *Littorina littorea* where MT expression is triggered after only 1 h of anoxia and rises to maximum values after 12 h (English and Storey, 2003). This might mirror the transcriptional response of MT in mammals, where a high rate of transcription occurs after 1–2 h, although the rate and extent to which these transcripts accumulate vary according to the inducing metal (Miles et al., 2000). As for aquatic vertebrates, a significant increase on MT mRNA occurs in the gills of the rainbow trout *Oncorhynchus mykiss* in response to 4 h of copper-induced hypoxia (van Heerden et al., 2004). An early increment of MT protein is also seen in the copepod *Tigriopus brevicornis* after 1 day of cadmium exposure (Barka et al., 2001) and in the marine crab *Charybdis japonica*, where an induction of MT is first observed in gills and hepatopancreas after 3 days of cadmium exposure (Pan and Zhang, 2006). In both cases, no measurements were taken before the stated time points, thus, an even earlier rise in MT concentration could have occurred without being detected. More studies have reported an increase of MT proteins or MTLP in aquatic invertebrates, however, the course of exposure to the triggering agent (mostly metals) is usually in the range of days, weeks or months rather than hours. In *L. vannamei*, the induction of MT proteins (measured by DPP) upon cadmium exposure was tested after 0, 1, 3, 5 and 9 days, detecting elevated levels after three days in hepatopancreas (Moksnes et al., 1995). Similarly, Wu and Chen, (2005) quantified MTs (by silver-saturation method) in response to cadmium and zinc after 4, 7, 14, 21, 28, 56 and 84 days, finding an increase of the protein only after 28 days in both hepatopancreas and gills of the white shrimp.

Given the long half-lives of MTs (reviewed by Amiard et al., 2006), the increase of MT transcripts after 1 h of hypoxia might be sufficient to sustain MT proteins levels necessary under more restrictive oxygen availability or reoxygenation. In the scallop *Chlamys nobilis* upon metal stress there was rapid MTLP synthesis and relatively slow degradation of the newly synthesized proteins (Liu and Wang, 2011). Some hypoxia-tolerant animals resist oxidative stress by increasing the expression and/or activity of antioxidants (Welker et al., 2013). Even more, the effects of metals or hypoxia on metallothioneins and antioxidant enzymes are significantly different to the effect upon exposure to the combined stresses, as Garcia-Sampaio et al. (2008) found in the pacu neotropical fish *Piaractus mesopotamicus*. Antioxidant enzymes studied in *L. vannamei* in response to hypoxia include catalase, that is induced in expression and activity in gills after only 6 h of hypoxia (1.5 mg DO L^{-1}) and these increments are sustained through

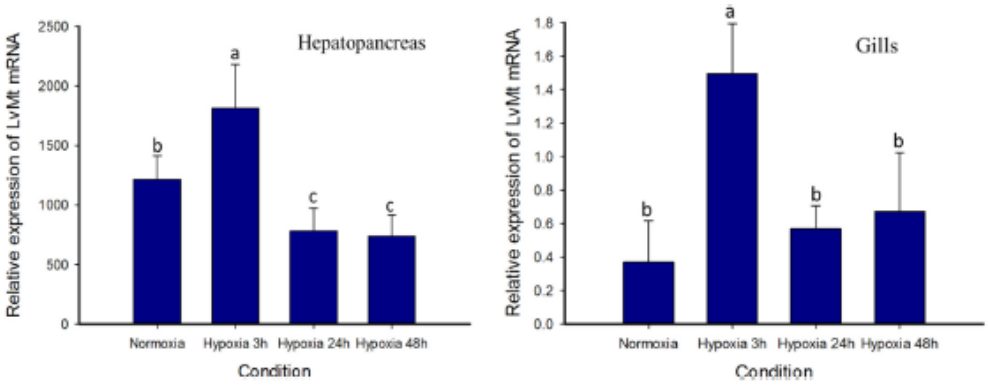

Fig. 5. Relative expression of LvMT mRNA in response to hypoxia. Animals were challenged with hypoxia at 1.5 mg DO L^{-1} for 3, 24 or 48 h. Control animals were maintained at 5 mg DO L^{-1}. Statistical analysis was based on comparisons of the expression of LvMT relative to L8 ($2^{-\Delta Ct}$ values). Each bar represents means ± SD from data obtained from at least four independent biological samples with four technical replicates ($n \geq 4$). Data were analyzed by one-way ANOVA and multiple Tukey–Kramer tests to compare mRNA relative expression levels in different conditions. Statistical significance was considered at p values <0.05. Different lowercase letters indicate statistically significant differences among different treatments.

1 h reoxygenation, 24 h of hypoxia and 1 h reoxygenation, consecutively (Trasviña-Arenas et al., 2013). Similarly, total SOD activity increases activity after 24 h of hypoxia (1.0 mg DO L^{-1}) in hepatopancreas and muscle (Parrilla-Taylor and Zenteno-Savín, 2011). GPx activity is triggered in muscle after 1 h reoxygenation but decreases in hepatopancreas (Parrilla-Taylor and Zenteno-Savín, 2011), and SOD activity is induced upon 6 h reoxygenation (García-Triana et al., 2010). Also, the mRNA expression of cMnSOD, GPx and methionine sulfoxide reductase (MsrB) increased after 4 h hypoxia (4.0 kPa) whereas GST and thioredoxin (TRX-1) transcripts decreased, suggesting a selective down-regulation of these latter transcripts instead of a global decrease of protein synthesis as part of the metabolic depression strategy in response to hypoxia (Kniffin et al., 2014).

The increased LvMT mRNA expression along with the rapid increased expression or activity of catalase (Trasviña-Arenas et al., 2013), total SOD and GPx in response to hypoxia (Parrilla-Taylor and Zenteno-Savín, 2011), and also the increased cMnSOD transcript levels and SOD activity in response to reoxygenation (García-Triana et al., 2010) is in line with the "preparation for oxidative stress" hypothesis proposed by Hermes-Lima et al. (1998), which states that in organisms evolutionary adapted to transitions between normal and extreme oxygen concentrations, an exposure to extreme situations induces the adaptive response that helps them survive at recovery by attenuating the effects of increased ROS formation during hypoxia/reoxygenation. MT antioxidant activity might indeed contribute to protect *L. vannamei* from oxidative damage, since it can remove $O_2^{\cdot-}$ and ·OH simultaneously and can react with OH about 10,000 times faster than SOD (Mao et al., 2012). These results should be complemented with studies on abundance of the LvMT protein since it is well known that MT is regulated both at the transcriptional and at the post-translational levels (McCormick et al., 1991).

In summary, LvMT expression was detected in several tissues under physiological conditions with the highest expression in hepatopancreas. A rapid increase in transcript levels in hepatopancreas and gills is induced in response to hypoxia. This induction could be mediated by HIF-1 and MTF-1 since their response elements are present in the LvMT promoter region. The MT protein possibly has a protective effect against ROS generated during hypoxia stimuli. Although these hypotheses remain to be tested, the findings herein reported point towards the function of MT as part of the strategy displayed by the shrimp to cope with stresses other than metal-exposure, importantly to hypoxia. Hence, care should be taken when considering shrimp MT mRNA only as a heavy metal-pollution biomarker.

Acknowledgments

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CAPÍTULO III

Hypoxia drives apoptosis independently of p53 and metallothionein transcript levels in hemocytes of the whiteleg shrimp *Litopenaeus vannamei*

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RESUMEN

Los mecanismos celulares utilizados por el camarón *Litopenaeus vannamei* para responder a la hipoxia han sido estudiados desde los ángulos del metabolismo energético y de antioxidantes. Aquí investigamos la participación de p53 y metalotioneína (MT) en el proceso apoptótico en respuesta a hipoxia en hemocitos de camarón. Los genes *Lvp53* o *LvMT* fueron silenciados eficientemente por inyección de RNA de doble cadena correspondiente a *p53* o *MT*. El efecto del silenciamiento sobre apoptosis se midió por la actividad de caspasa-3 y por citometría de flujo en hemocitos después de 24 y 48 h de hipoxia (1.5 mg $OD \cdot L^{-1}$). Los hemocitos de los animales sin silenciar tuvieron niveles de apoptosis significativamente más altos en ambos tiempos de hipoxia. Los niveles apoptóticos disminuyeron pero no se suprimieron en hemocitos de camarones silenciados con *dsp53* pero no con *dsMT* luego de 24 h de hipoxia, lo cual indica una contribución de Lvp53 a apoptosis. La apoptosis en normoxia fue significativamente más alta en animales silenciados con dsp53 y dsMT que en los controles sin silenciar, lo cual apunta hacia un papel citoprotector de LvMT y Lvp53 durante el programa apoptótico basal en normoxia. Estos resultados indican que la hipoxia aumenta la apoptosis en hemocitos de camarones y altos niveles de transcritos de *Lvp53* y *LvMT* no son necesarios para esta respuesta.

Palabras clave: Hipoxia, apoptosis, caspasas, silenciamiento con dsRNA, p53, *Litopenaeus vannamei*

1 **Hypoxia drives apoptosis independently of p53 and metallothionein transcript levels**

2 **in hemocytes of the whiteleg shrimp *Litopenaeus vannamei***

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Abstract

The cellular mechanisms used by the shrimp *Litopenaeus vannamei* to respond to hypoxia have been studied from the energetic metabolism and antioxidant angles. We herein investigated the participation of p53 and metallothionein (MT) in the apoptotic process in response to hypoxia in shrimp hemocytes. The *Lvp53* or *LvMT* genes were efficiently silenced by injection of double stranded RNA for *p53* or *MT*. The effects of silencing on apoptosis were measured as caspase-3 activity and flow cytometry in hemocytes after 24 and 48 h of hypoxia (1.5 mg DO L^{-1}). Hemocytes from unsilenced animals had significantly higher apoptosis levels upon both times of hypoxia. The apoptotic levels were diminished but not suppressed in *dsp53*-silenced but not *dsMT*-silenced hemocytes after 24 h of hypoxia, indicating a contribution of Lvp53 to apoptosis. Apoptosis in normoxia was significantly higher in *dsp53*- and *dsMT*-silenced animals compared to the unsilenced controls, pointing to a possible cytoprotective role of LvMT and Lvp53 during the basal apoptotic program in normoxia. Overall, these results indicate that hypoxia augments apoptosis in shrimp hemocytes and high mRNA levels of *Lvp53* and *LvMT* are not necessary for this response.

Keywords: Hypoxia, apoptosis, caspases, dsRNA silencing, p53, *Litopenaeus vannamei*

27 **1. Introduction**

28 In the diversity of environments in which shrimp and other crustacean inhabit, either
29 naturally or in commercial farms, variations in the dissolved oxygen levels occur (Helly
30 and Levin, 2004). Broadly, oxygen concentrations below 3.0 mg DO L^{-1} are considered
31 hypoxia for aquatic organisms (Diaz and Rosenberg, 1995; Gray et al., 2002), but since
32 these animals are continuously exposed to such oxygen drops, some of them have
33 developed strategies to tolerate such environmental changes.

34 The strategies used by invertebrates to handle oxygen changes include
35 hypometabolic/hypothermic behavior, production of ethanol instead of lactic acid
36 during low oxygen episodes, enhancement of the antioxidant system and bradycardia,
37 among others (Gorr et al., 2010; Joyce et al., 2016; Welker et al., 2013). For some other
38 hypoxia-tolerant organisms, like *Artemia franciscana* and *Spalax spp*, apoptosis evasion
39 is a potent strategy to cope with hypoxic challenges (Band et al., 2010; Menze and Hand,
40 2007). In the penaeid shrimp *Penaeus monodon* and *Litopenaeus vannamei* apoptosis is
41 triggered by viral infections, hyperthermia and hypothermia (Chang et al., 2009; Granja
42 et al., 2003; Wongprasert et al., 2003). Whether this apoptotic induction occurs in
43 response to hypoxia is unknown.

44 Knowledge regarding apoptosis regulation in invertebrates is mainly limited to
45 *Drosophila melanogaster* and *Caenorhabditis elegans* and these models might not reflect
46 the apoptotic mechanisms in other species within the Arthropoda *phylum* (Menze et al.,
47 2010). Nevertheless, apoptosis induction in response to DNA damage, occurs in all
48 invertebrates in which the p53 superfamily function has been studied so far (Rutkowski

49 et al., 2010). The p53 protein has been widely investigated in human and in several
50 animal models due to its determinant role in oncogenic processes. Functional p53 is a
51 tetrameric transcription factor that requires the incorporation of zinc into its structure
52 for the proper folding needed for DNA binding (Brosh and Rotter, 2009; Meplan et al.,
53 2000). Activation of p53 is triggered by different cellular stresses such as DNA damage,
54 oncogenic signaling and hypoxia. Once activated, p53 promotes the transcription of over
55 150 genes involved in cell cycle regulation, induction of apoptosis, tumor suppression,
56 regulation of metabolic pathways, cell senescence, and DNA repair, among others
57 (Levine and Oren, 2009). Besides its transcription-dependent apoptotic role, p53 also
58 exerts transcription-independent apoptosis by accumulating in the cytosol or in the
59 mitochondria where it induces oligomerization of pro-apoptotic Bax and Bak proteins
60 and antagonizes Bcl-2 and Bcl-X_L anti-apoptotic effect (Amaral et al., 2010; Speidel,
61 2010). Studies in mammalian cell lines indicate that, specifically in response to hypoxia,
62 p53 induces apoptosis but not cell cycle arrest (Koumenis et al., 2001). Hypoxia
63 suppresses the association of p53 to its ubiquitin-ligase, MDM2 (*Murine Double Minute*
64 *2*), therefore, p53 stabilizes and accumulates in its transcriptionally active form (Haupt
65 et al., 1997). On the other hand, numerous studies have correlated high metallothionein
66 (MT) levels with apoptosis resistance. It appears that independently of the cell type, MT
67 protects cells from apoptosis induced by oxidative stress, metals and anti-cancer drugs
68 and it has been proposed to have a role in regulating the apoptotic process (Shimoda et
69 al., 2003; Wang et al., 2001). MTs are small cysteine-rich proteins that can bind up to
70 seven bivalent metallic ions and are involved in the homeostasis of essential metals

71 (Braun et al., 1992; Ostrakhovitch et al., 2007). The metallic ions can be obtained from
72 metalloenzymes and transcription factors, therefore contributing to the regulation of
73 their activity (Cano-Gauci and Sarkar, 1996; Roesijadi et al., 1998). *In vitro*, MT acts as a
74 strong chelator to remove zinc from p53, whereas in intact mammalian cell lines, it
75 modulates p53 transcriptional activity (Meplan et al., 2000).
76 The white shrimp, *Litopenaeus vannamei* is capable of surviving hypoxic conditions and
77 studies on the molecular mechanisms underlying the adaptation to oxygen deprivation
78 in this species in our group have been mainly focused on antioxidant defense and energy
79 metabolism pathways (Cota-Ruiz et al., 2015; Garcia-Triana et al., 2010b; Martinez-
80 Quintana et al., 2015; Martinez-Quintana et al., 2014; Martinez-Quintana et al., 2016;
81 Soñanez-Organis et al., 2010; Trasviña-Arenas et al., 2013). However, cell fate
82 mechanisms under hypoxia in this organism are still unknown and although there is
83 evidence of some proteins involved in the apoptotic process in crustacean (Leu et al.,
84 2012), studies on their functionality in the physiological context are scarce (Menze et al.,
85 2010). Whether hypoxia triggers p53-dependent apoptosis in penaeid shrimp or if it
86 displays apoptotic resistance as in other hypoxia-tolerant organisms, is yet unknown.
87 In this study, the roles of p53 and MT in response to hypoxia in *L. vannamei* were
88 investigated. We herein report the occurrence of hemocyte apoptosis in response to
89 hypoxia. Furthermore, through dsRNA-driven silencing, we show a p53-dependent
90 caspase activity in these cells. These findings contribute to the understanding of how
91 homeostatic basic processes such apoptosis occur in crustacean.

92 **2. Materials and methods**

93 2.1. *Synthesis and injection of Lvp53 and LvMt dsRNA*

94 A region of 696 bp of *Lvp53* coding sequence (positions 451 to 1145, GenBank accession
95 no. KX179650) was selected to be targeted by the dsRNA. As for the LvMT silencing, a
96 331 bp fragment corresponding to the full coding sequence and UTRs was used (positions
97 489-565, 866-945 and 1227-1402 of the complete gene, GenBank accession no.
98 KJ701600.1) The Lvp53 and LvMT fragments obtained from cDNA by PCR were cloned
99 into pGEM®-T Easy Vector System (Promega, Madison, WI, USA) in both sense and
100 antisense directions. The fragments including the T7 promoter sequences from the
101 plasmid vector were used as templates to produce *in vitro* single stranded RNA (ssRNA)
102 using the T7 RiboMAX™ Large Scale RNA Production System (Promega, Madison, WI,
103 USA). Complementary hybridization to obtain double stranded RNA (dsRNA) for each
104 gene, was done by mixing together equal amounts of each ssRNA, heating the mixture
105 at 80 °C for 10 min and allowing slow cooling down at room temperature until reaching
106 28 °C. The resultant dsRNA were assessed by 2% agarose gel electrophoresis, by verifying
107 changes in migration compared to the ssRNAs (Garcia-Triana et al., 2010a). The dsRNAs
108 were quantified spectrophotometrically at 260 nm.

109 Shrimp were intramuscularly injected 25 µg of *LvMT* dsRNA (ds*MT*) or *Lvp53* dsRNA
110 (*dsp53*) in a final volume of 100 µL of 150 mM NaCl sterile saline solution (SS). Unsilenced
111 control animals were injected 100 µL of saline solution. Shrimp were subjected to
112 hypoxia treatments 24 h after the dsRNA injection for silencing.

113 *2.2. Experimental animals*

114 Healthy juvenile whiteleg shrimp (*L. vannamei*) were obtained from a local shrimp farm
115 and maintained in the Laboratory of Marine Invertebrates Physiology of CIAD (Sonora,
116 Mexico) in 100 L fiberglass tanks with constant aeration through air-diffusing stones, in
117 preconditioned seawater at 28°C and salinity of 35 ppt. The seawater was previously
118 pumped through sand-filters, followed by 5 µM and 10 µM bag-filtration, ozone
119 treatment, ozone destruction and finally passed through 5 µM and 10 µM filters once
120 again. Dissolved oxygen, temperature and salinity were continuously monitored
121 throughout the experiment. Animals were fed a commercial 35% protein feed at a rate
122 of 3% body weight per day. Static-renewal system was used where water quality was
123 monitored daily and water exchange was done to maintain less than 3.0 $mg \cdot L^{-1}$ of total
124 ammonia levels. Groups of 12 intermolt animals weighting 12.5 ± 0.59 g were assigned
125 to each of 12 tanks and were acclimatized for three days before being subjected to the
126 following treatments: 1) normoxia (5.13 ± 0.11 mg of DO L^{-1}) injected with SS; 2)
127 normoxia (5.02 ± 0.11 mg of DO L^{-1}) injected with 25 µg of ds*MT*; 3) normoxia (4.87 ±
128 0.23 mg of DO L^{-1}) injected with 25 µg of ds*p53*; 4) hypoxia (1.54 ± 0.28 mg of DO L^{-1})
129 injected with SS; 5) hypoxia (1.53 ± 0.20 mg of DO L^{-1}) injected with 25 µg of ds*LvMT* and,
130 6) hypoxia (1.64 ± 0.25 mg of DO L^{-1}) injected with 25 µg of ds*p53*. The DO concentration
131 in the hypoxia treatments was induced by mixing nitrogen gas and air. Dissolved oxygen
132 was measured every hour with an oximeter (YSI model 55, Yellow Spring, OH, USA) and
133 adjusted if necessary. The 25 µg doses for injection were determined in preliminary
134 experiments (data not shown). The dsRNA was resuspended in saline solution and the
135 shrimp were injected 24 h before the beginning of the hypoxia challenge. Nine shrimp

136 from each of these groups were collected after 24 h of normoxia or hypoxia induction.
137 Nine animals from six identically treated groups were collected after 48 h of normoxia or
138 hypoxia induction. Animals were fasted for 12 h before the collections.

139 *2.3. Hemolymph collection*

140 Hemolymph (300 µL) from individual animals was extracted from the ventral sinus of the
141 cephalotorax using a 1 cc syringe containing pre-chilled shrimp salt solution (SSS, 450
142 mM NaCl, 10 mM KCl, 10 mM EDTA-Na_2, 10 mM HEPES, pH 7.3). Pools with 100 µL and
143 150 µL of hemolymph from each of three shrimp were made for the caspase assay and
144 RT-qPCR quantification, respectively. Hemolymph from individual shrimp was used for
145 the flow cytometry analyses.

146 *2.4. LvMt and Lvp53 mRNA quantification in hemocytes by RT-qPCR*

147 Total RNA from hemolymph (450 µL) of shrimp in normoxic and hypoxic regimes was
148 isolated using TRI Reagent® (Sigma-Aldrich, St. Louis, MO, USA) and treated with DNase
149 I (Roche Diagnostics, Indianapolis, IN, USA) to remove residual contaminant genomic
150 DNA. The cDNA synthesis was done using the QuantiTect® Reverse Transcription
151 (Qiagen® Valencia, CA, USA) system according to the manufacturer instructions using
152 500 ng of DNase-treated total RNA. Real time PCR primers Lvp53F3 (5´-
153 CCAAGCAGCAATGTGTCAG-3´) and Lvp53F4rv (5´-CTTGTTGCGATCTTT GTTGC-3´) for the
154 Lvp53 gene were designed to amplify a fragment of 177 bp. For *LvMT* RT-qPCR,
155 previously reported primers MTRTF (5´-CTGATCCATGCTGTAACGAG-3´) and MTRTRv (5´-
156 CATCTTGTTGCACACTCCTC-3´) were used to amplify a 145 bp fragment (Felix-Portillo et

157 al., 2014). The gene for the ribosomal protein L8 (GenBank accession no. DQ316258.1)
158 was used as reference and a 166 bp fragment was amplified using the primers L8F2 (5´-
159 TAGGCAATGTCATCCCCATT-3´) and L8R2 (5´-TCCTGAAGGGAGCTTTACACG-3´) (Trasviña-
160 Arenas et al., 2013). Standard quantification curves were made with serial dilutions from
161 25 ng to 6.4 x 10^{-2} pg μL^{-1} of cDNA (equivalent of total RNA) from gill (for *L8*
162 quantification) or hepatopancreas (for *LvMT* and *Lvp53* quantification). For each
163 treatment group, four technical replicates for each of three pools of hemocytes were
164 analyzed by RT-qPCR to assess mRNA expression and the silencing efficiency. The RT-
165 qPCR reactions were carried out in a final volume of 20 µL containing nuclease-free
166 water, cDNA derived from 12.5 ng of total RNA, 2x iQ SYBR Green Supermix (BioRad
167 Laboratories, Hercules, CA, USA), *L8* primers to a final concentration of 1 µM, or *LvMT*
168 primers to a final concentration of 125 nM, or *Lvp53* primers to a final concentration of
169 200 nM. Non-template controls were included in each run. Reactions were done in a
170 CFX96™ Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA).
171 Amplification programs for *L8* and *LvMT* were as previously reported (Felix-Portillo et al.,
172 2014). For the *Lvp53* amplification the following program was used: 95 °C for 3 min
173 followed by 40 cycles of 95 °C for 30 s, 56 °C for 35 s, 72 °C for 55 s, taking the
174 fluorescence reading at the extension step. Efficiency and linearity of the dynamic range
175 for the three genes were determined and relative expression of *Lvp53*, *LvMT* and *L8* were
176 calculated by the $2^{-\Delta Cq}$ method (Schmittgen and Livak, 2008). The silencing efficiency was
177 measured by the percentage of LvMT or Lvp53 transcripts reduction in the dsRNA-
178 injected animals relative to the saline solution injected animals.

179 *2.5. Caspase-3 activity*

180 The activity of caspase-3 was determined using the colorimetric CaspACE™ Assay System
181 (Promega, Madison, WI, USA), in which the caspase substrate Ac-DEVD is labeled with *p*-
182 nitroaniline (pNA). Upon cleavage, free pNA produces a yellow color proportional to
183 DEVDase activity in the sample and is read spectrophotometrically at 405 nm as in
184 (Thornberry, 1994). Diluted hemolymph (300 uL) was centrifuged at 300 x g, 4 °C for 20
185 min and the pellet was resuspended in 50 µL of the provided cell lysis buffer. The cells
186 were lysed by two freeze/thaw cycles at -80 °C for 80 s / 37 °C for 20 s, then incubated
187 on ice for 15 min. The cell lysates were centrifuged at 17,400 x g, at 4 °C for 15 min and
188 the supernatant fractions were collected to measure caspase activity and protein
189 concentration. For the caspase assay, 10 µL of the supernatant were added to 40 µL of
190 the caspase reaction mix containing 16 µL of caspase assay buffer, 1 µL of DMSO, 5 µL of
191 DTT (100 mM), 17 µL of deionized water and 1 µL of the Ac-DEVD-pNA (10 mM)
192 substrate. Duplicate reactions for each sample were incubated at room temperature for
193 20 h. The absorbance at 405 nm was measured using a Multiskan GO microplate reader
194 (Thermo Scientific, USA) at times 0 and 20 h after the incubation with the substrate. A
195 pNA standard curve was prepared according to the manufacturer and was used to
196 calculate caspase-specific activity. Protein concentrations were determined by the
197 Bradford method using a BSA standard curve and used to normalize caspase activity
198 (Bradford, 1976).

199 *2.6. Analysis of apoptosis by flow cytometry*

200 Apoptosis of hemocytes collected at hypoxia 48 h was assessed by flow cytometry using
201 the Alexa Fluor®488 annexin V/Dead Cell Apoptosis kit with Alexa® (Invitrogen, USA)
202 according to the manufacturer´s protocol with slight modifications. Hemolymph was
203 centrifuged at 300 x g for 10 min at 4 °C and hemocytes were washed once in 2X SSS and
204 centrifuged at 300 x g for 10 min at 4 °C. Cell viability was verified by trypan blue
205 exclusion before centrifugation. Hemocytes were resuspended in 1X annexin-binding
206 buffer at 1 x 10^6 cells mL^{-1}, followed by addition of 7.5 µL of Alexa Fluor®488 annexin V
207 and 1 µL of 100 µg $µL^{-1}$ propidium iodide (PI). Hemocytes were incubated 15 min in the
208 dark and were then added 400 µL of 1X annexin-binding buffer. Cells were analyzed by
209 flow cytometry (BD FACSCanto II, San Jose, CA) measuring fluorescence emission at 530
210 nm and 575 nm, using 488 nm excitation wavelength. Results are expressed as mean
211 percentage of apoptotic cells.

212 *2.7. Statistical analyses*

213 Statistical analyses were carried out in NCSS and PASS, 2007, and GraphPad Prism, 2007
214 programs. The effect of hypoxia on the relative expression of *LvMt* and *Lvp53* was
215 analyzed by one way ANOVA and Tukey-Kramer means comparison test. The effect of
216 silencing on relative expression was analyzed with a Student t-test and the caspase-3
217 activity was analyzed by two-way ANOVA (hypoxia x silencing) and Fisher *post hoc* means
218 comparison test. Differences were considered significant when $p<0.05$.

219 **3. Results and Discussion**

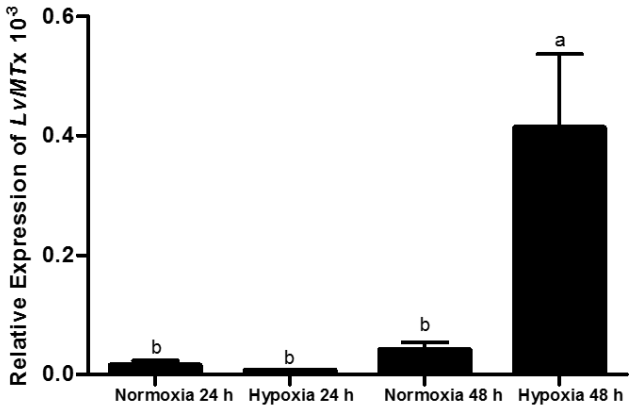
220 *3.1 Expression of LvMt in hemocytes increases after 48 h of hypoxia*

221 Our RT-qPCR results show that metallothionein mRNA transcripts in hemocytes are 9.8
222 fold-change higher after 48 h of hypoxia (1.44 ± 0.26 mg DO L^{-1}) compared to the
223 normoxic control (5.10 ± 0.11 mg DO L^{-1}) (Fig. 1). We previously reported normoxic *LvMt*
224 transcript profiles in different tissues, finding hemocytes among the lowest expressing
225 levels and were therefore not tested in the hypoxia challenge. In that study, expression
226 levels in hepatopancreas and gills increased in response to 3 h of hypoxia (1.45 ± 0.2 mg
227 DO L^{-1}), but not after 24 or 48 h (Felix-Portillo et al., 2014). The 3 h hypoxia challenge was
228 not tested in the present study, so an increase of transcripts at this time point in
229 hemocytes cannot be ruled out. Although MTs are recognized as important metal
230 scavengers, their role as antioxidants against reactive oxygen and nitrogen species has
231 been vastly supported. Reactive oxygen species are generated during hypoxia (Azad et
232 al., 2011; Clanton, 2007; Chandel et al., 2000; Sun et al., 2016) and/or hypoxia-
233 reoxygenation events (Lawniczak et al., 2013; Zenteno-Savin et al., 2006) and must be
234 counteracted by antioxidant enzymatic and non-enzymatic proteins. Thus, the enhanced
235 expression of *LvMt* at 48 h of hypoxia probably reflects the contribution of the MT to the
236 redox cycle (Ruttkay-Nedecky et al., 2013) to maintain oxidative homeostasis in *L.*
237 *vannamei* during oxygen depletion/restitution episodes. The efficiency of ds*Mt* silencing
238 was evaluated in both normoxia and hypoxia. In the case of normoxia 24 h, *LvMt*
239 transcripts in the ds*Mt*-silenced animals were below the detection limit of our dynamic
240 range (25 ng to 6.4 x 10^{-2} pg/µL of cDNA). As for the hypoxia analysis at the same time
241 point, the silencing was 98% efficient compared to hemocytes from animals injected with

242 saline solution only (Fig. 2A). The LvMt knockdown efficiencies at 48 h were 73.5% and

243 93% for normoxia and hypoxia, respectively (Fig. 2B).

244



245

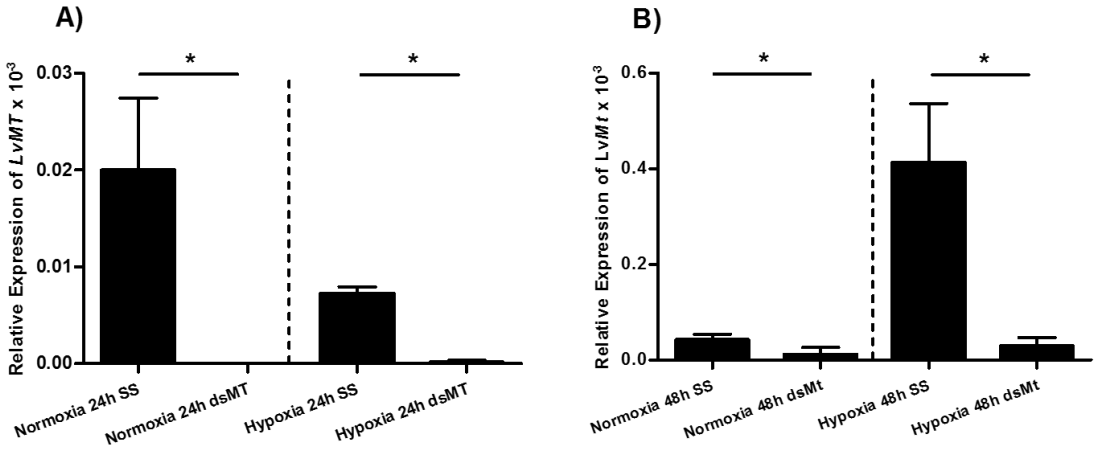
246 **Figure 1. Effect of hypoxia on the *LvMT* expression levels in hemocytes.** Relative

247 expression of *LvMt* in response to 24 h and 48 h of hypoxia and their respective controls

248 in normoxia. Statistical analysis was based on comparison of the expression of *LvMt*

249 relative to *L8* ($2^{-\Delta cq}$). Bars indicate means ± SD. Different lowercase letters indicate

250 significant differences between means ($p<0.05$) to $n \geq 3$.



251

252 **Figure 2. Effect of silencing on the expression level of *LvMt* in hemocytes.** Animals were
253 injected with saline solution (SS) or 25 µg of ds*Mt* and exposed to normoxia (5.0 mg DO
254 L^{-1}) or to hypoxia (1.5 mg DO L^{-1}) for (A) 24 h or (B) 48 h. *LvMt* expression levels relative
255 to *L8* are shown as $2^{-\Delta Cq} \times 10^{-3}$. Bars represent means ± SD from n≥3 with four technical
256 replicates. Data were analyzed by Student t-test. Asterisks indicate significant differences
257 between means ($p<0.05$). Cumulative mortalities were 1.9% and 3.8% for SS and ds*Mt*,
258 respectively.

259 *3.2 Lvp53 silencing results in enhanced expression of LvMt transcripts*

260 The LvMt mRNA levels were also evaluated in hemocytes from the ds*p53*-injected
261 animals. LvMt transcript abundances were higher in the ds*p53*-injected animals than in
262 the SS-injected animals in all treatments (Fig. 3). Statistically significant higher levels
263 were found in normoxia 24 h (1,250 fold-change), normoxia 48 h (53 fold-change),
264 hypoxia 24 h (715 fold-change) and hypoxia 48 h (75 fold-change) compared to the
265 corresponding controls. These results suggest a regulatory activity of *Lvp53* over LvMT
266 expression. The promoter region of the LvMt gene contains at least two p53 response
267 elements on the non-coding strand (Felix-Portillo et al., 2014). This could point towards
268 a mechanism of transcriptional interference (Shearwin et al., 2005), whereby the
269 transcription of LvMt on the coding strand is suppressed *in cis* by a transcriptional
270 process from the p53 response element on the non-coding strand. The presence of
271 overlapping convergent promoters may lead to collision of RNA polymerases on opposite

272 strands, thus causing one of them to stall and backtrack (Palmer et al., 2011). This would
273 provide yet another mode of LvMT regulation at the transcriptional level during
274 physiologically related and opposing processes such as p53-dependent apoptosis and
275 apoptosis resistance aided by the MT protein. In a recent study, (Ostrakhovitch et al.,
276 2016) found that inactivation of the p53 protein in human liver carcinoma cells led to
277 higher expression levels of different MT isoforms, and that p53 might be negatively
278 regulating MT-3 in epithelial cancer cells. It has also been shown that MT is upregulated
279 by hypoxia and mediates stabilization of HIF-1α, therefore constituting a possible
280 mechanism of hypoxia tolerance (Kojima et al., 2009). In line with this, MT modulates
281 p53-mediated transcription possibly by chelating the zinc atom required for the DNA-
282 binding conformation of p53 (Meplan et al., 2000). In *L. vannamei* only one Mt gene
283 generating one transcript has been reported to date, its expression level is tissue-specific
284 and probably regulated in response to metals but also to other environmental cues.
285 Thus, the regulatory interplay of different transcription factors such as MTF-1 (Metal-
286 responsive Transcription Factor 1), p53 and HIF-1 might compensate the lack of several
287 tissue- and stimuli-specific isoforms of the protein in the shrimp.
288

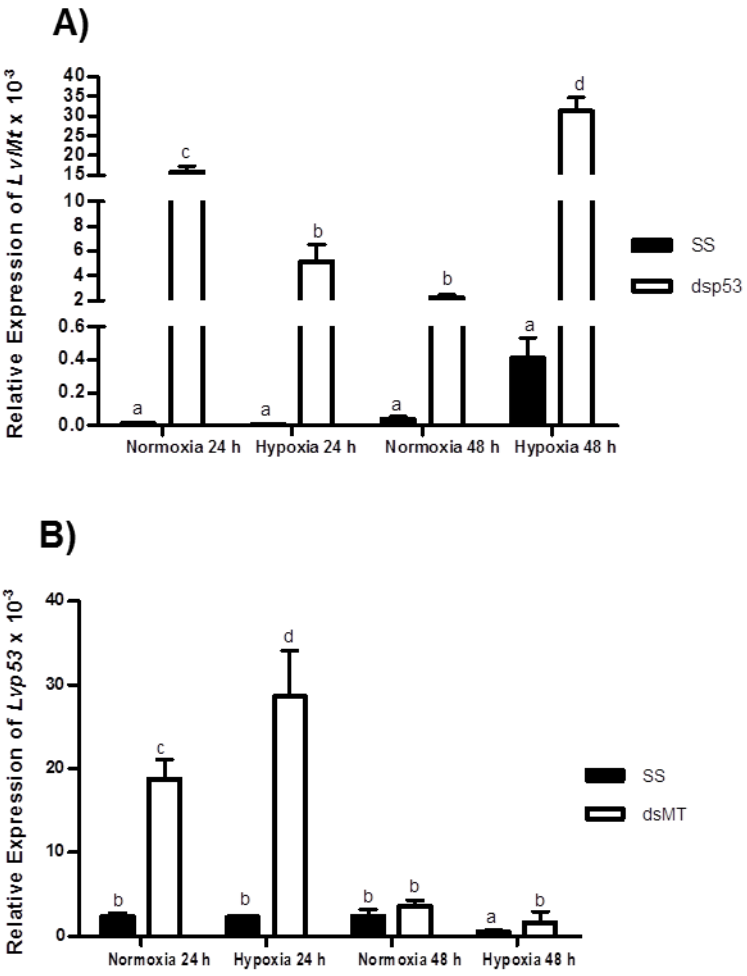
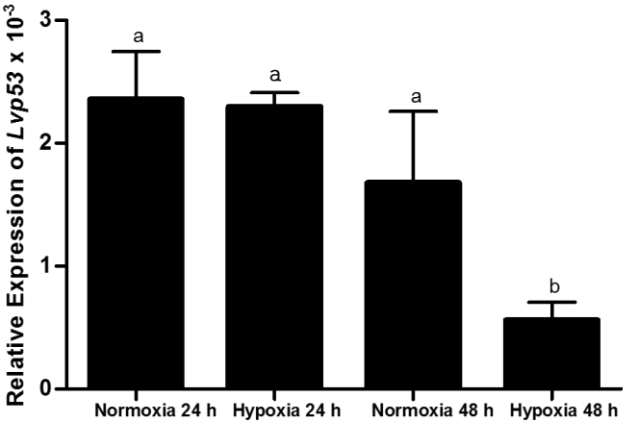


Figure 3. Effect of reciprocal silencing on relative expression of *Lvp53* and *LvMt* in hemocytes. A) Relative *LvMt* expression when silencing *Lvp53* and B) Relative *Lvp53* expression when silencing *LvMt*, in response to 24 h and 48 h of hypoxia and their respective controls in normoxia. Statistical analysis was based on comparison of the expression relative to L8 ($2^{-\Delta cq}$). Bars indicate means ± SD. Different lowercase letters indicate significant differences between means ($p<0.05$) to $n \geq 3$.

296 *3.3 Lvp53 expression drops in response to 48 h hypoxia*

297 The *Lvp53* mRNA expression levels in hemocytes in response to hypoxia were evaluated
298 through RT-qPCR. The *Lvp53* transcripts were equally abundant at normoxia 24 h,
299 hypoxia 24 h and normoxia 48 h, but were significantly lower (3 fold-change) after 48 h
300 of hypoxia (Fig. 4). This change in expression is opposite to the reported in the
301 hepatopancreas of the river prawn *Macrobracchium niponesse*, in which the p53
302 transcripts peak after 48 h of hypoxia (2.0 mg of DO L^{-1}) (Sun et al., 2016). Nevertheless,
303 differences in p53 expression patterns along tissues (hemocyte and hepatopancreas in
304 this case) and species are not surprising. In the Mediterranean mussel *Mytilus*
305 *galloprovincialis*, p53 mRNA levels raise 3 h after UV-irradiation and drop back at 6, 24
306 and 48 h post-irradiation (Estévez-Calvar et al., 2013). In our study, only two time points
307 were tested and it is possible that a rapid response in p53 mRNA levels was missed out
308 before the 24 h of hypoxia. The classical model of p53 regulation is greatly regarded as
309 post-translational, relying on several p53 protein modifications such as methylation,
310 acetylation, phosphorylation, glycosylation, ubiquitination, neddylation and
311 sumoylation. Ultimately, these modifications contribute to p53 stabilization, activation
312 and degradation (Dai and Gu, 2010; Kruse and Gu, 2009). However, recent studies show
313 that p53 regulation also occurs at the mRNA level via its 5´and 3´untranslated regions,
314 microRNAs activity and protein:p53 mRNA interactions (Devany et al., 2013; Jo et al.,
315 2014; Otsuka and Ochiya, 2014; Takwi and Li, 2009). Additionally, autoregulatory loops
316 exist at all levels of p53 regulation (Lu, 2010). The p53 expression and response depend
317 largely on the cellular type, thus, the change in expression levels that we found in

318 hemocytes might not reflect the change in other tissues. It is possible that p53 is
319 responding to the hypoxia stimuli at the protein rather than at the mRNA level.
320 Moreover, in *L. vannamei*, only one p53-family member has been described, thus the
321 compensatory expression and effects of other members cannot be ruled out. Upon
322 dsp53-mediated silencing, *Lvp53* transcripts were significantly less abundant than in the
323 unsilenced controls. In normoxia and hypoxia at 24 h, p53 mRNA levels were efficiently
324 silenced by 97.2% and 96.8%, respectively (Fig. 5). Similarly, at 48 h of normoxia and
325 hypoxia the silencing efficiencies were 91.6% and 99.1%, respectively (Fig. 5B).



326

327 **Figure 4. Effect of hypoxia on *Lvp53* expression levels in hemocytes.** Relative expression
328 of *Lvp53*, in response to 24 h and 48 h of hypoxia and their respective controls in
329 normoxia. Statistical analysis was based on comparison of the expression of *Lvp53*
330 relative to *L8* ($2^{-\Delta cq}$). Bars indicate means ± SD. Different lowercase letters indicate
331 significant differences between means ($p<0.05$) to $n \geq 3$.

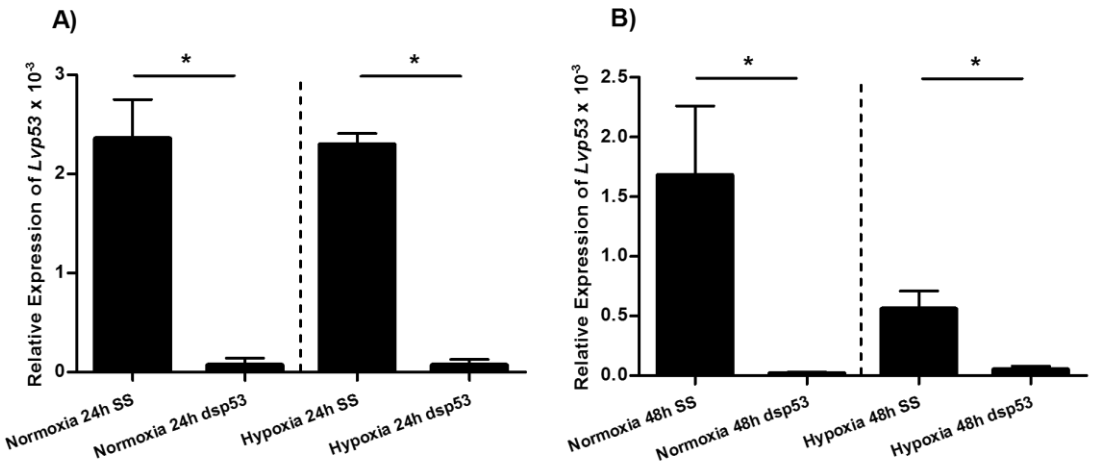
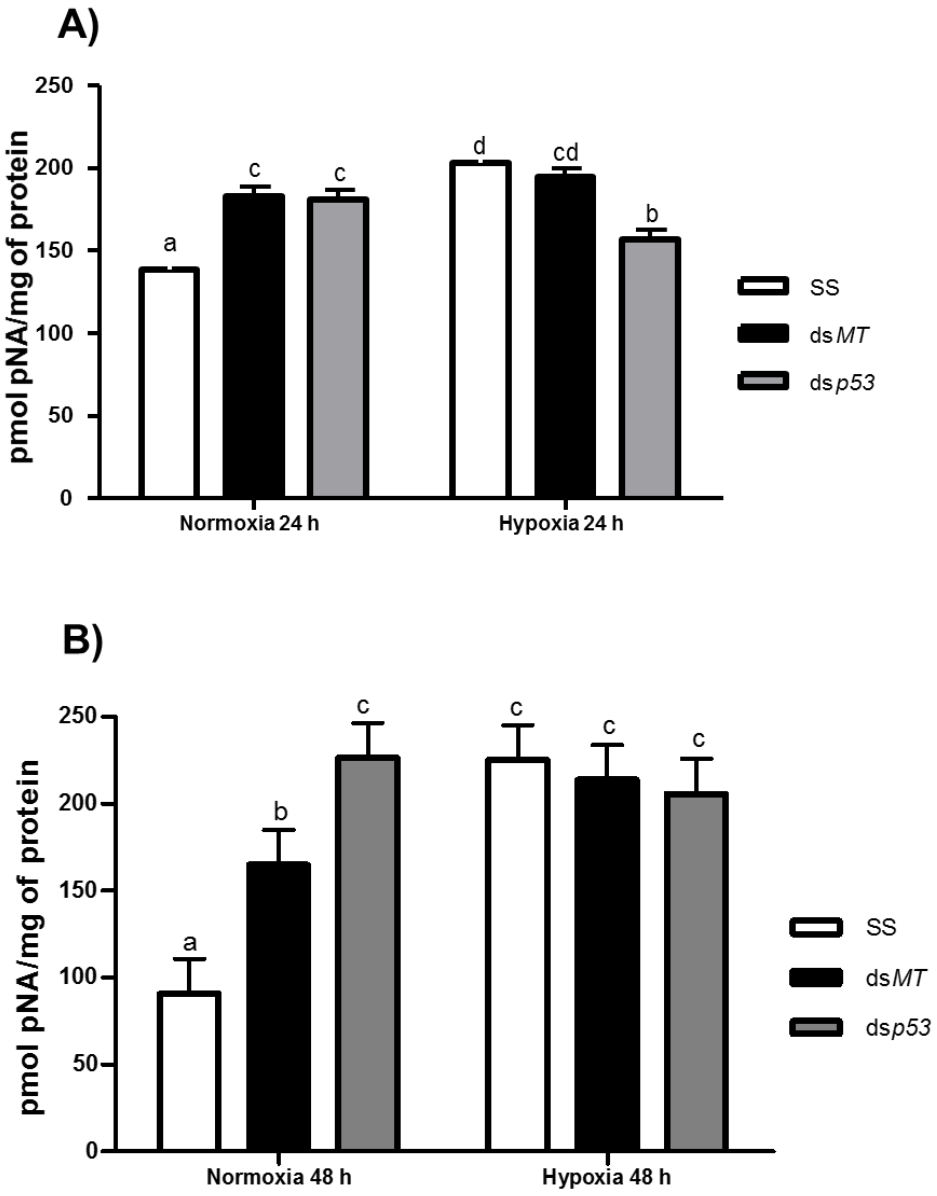


Figure 5. Effect of silencing on the expression level of *Lvp53* in hemocytes. Animals were injected with saline solution (SS) or 25 µg of ds*p53* and exposed to normoxia (5.0 mg DO L^{-1}) or to hypoxia (1.5 mg DO L^{-1}) for (A) 24 h or (B) 48 h. Expression levels relative to *L8* are shown as $2^{-\Delta Cq}$ x 10^{-3}. Bars represent means ± SD from n≥3 with four technical replicates. Data were analyzed by Student t-test. Asterisks indicate significant differences between means ($p<0.05$). Cumulative mortalities were 1.9% and 17.3% for SS and ds*p53*, respectively.

3.4 Apoptosis increases in response to hypoxia

Apoptosis was assessed in terms of caspase-3 activity in hemocytes corresponding to all given treatments (Fig.6) and additionally by flow cytometry for the 48 h treatments (Fig. 7). In hemocyte lysates from shrimp injected with saline solution, apoptosis significantly raises after 24 and 48 h of hypoxia. Silencing with dsMt has no statistically significant effect on apoptosis in response to 24 h of hypoxia in these cells; in contrast, when p53 is silenced, caspase-3 activity decreases at 24 h of hypoxia, pointing towards a p53

347 contribution to the apoptotic pathway triggered by this stress. Cell fate decisions
348 mediated by p53 (cell cycle arrest or apoptosis) rely on an apoptotic threshold
349 determined by p53 and p53-target protein levels and these, in turn, depend on the
350 cellular context (Kracikova et al., 2013). Thus, in p53-depleted cells the apoptotic
351 response is partially abolished. However, after 48 h of hypoxia, apoptotic levels in ds*p53*-
352 silenced hemocytes are comparable to those in the unsilenced control and to normoxic
353 hemocytes. This could indicate that although p53 is the first line of response against
354 hypoxia, the severity imposed by 48 h of low DO might signal alternative apoptotic
355 pathways that come into play because such conditions are not permissive for cell
356 survival. In mammalian cell lines, p53 activation by hypoxia triggers apoptosis primarily
357 through the interaction with corepressors, -proteins that bind transcription factors to
358 prevent transcription- at the promoter regions of genes such as α-tubulin and β-tubulin,
359 thus forming complexes that repress transcription (transrepression), rather than
360 transcriptional activation (transactivation) of pro-apoptotic genes (Koumenis et al.,
361 2001). Indeed, although most classical p53 target genes (p21, MDM2, PUMA, and Bax)
362 are not induced by hypoxia, the death receptor Fas/CD95 is significantly up-regulated in
363 a p53-dependent manner, therefore triggering apoptosis through the extrinsic pathway
364 (Liu et al., 2007). Nevertheless, there is no evidence of the extrinsic apoptosis pathway
365 in crustacean (Menze et al., 2010) and although some of its important players such as
366 the tumor necrosis factor (TNF) and TNF receptor (TNFR) have been described for *L.*
367 *vannamei* (Wang et al., 2012), their involvement in apoptotic processes has not been
368 tested.



369

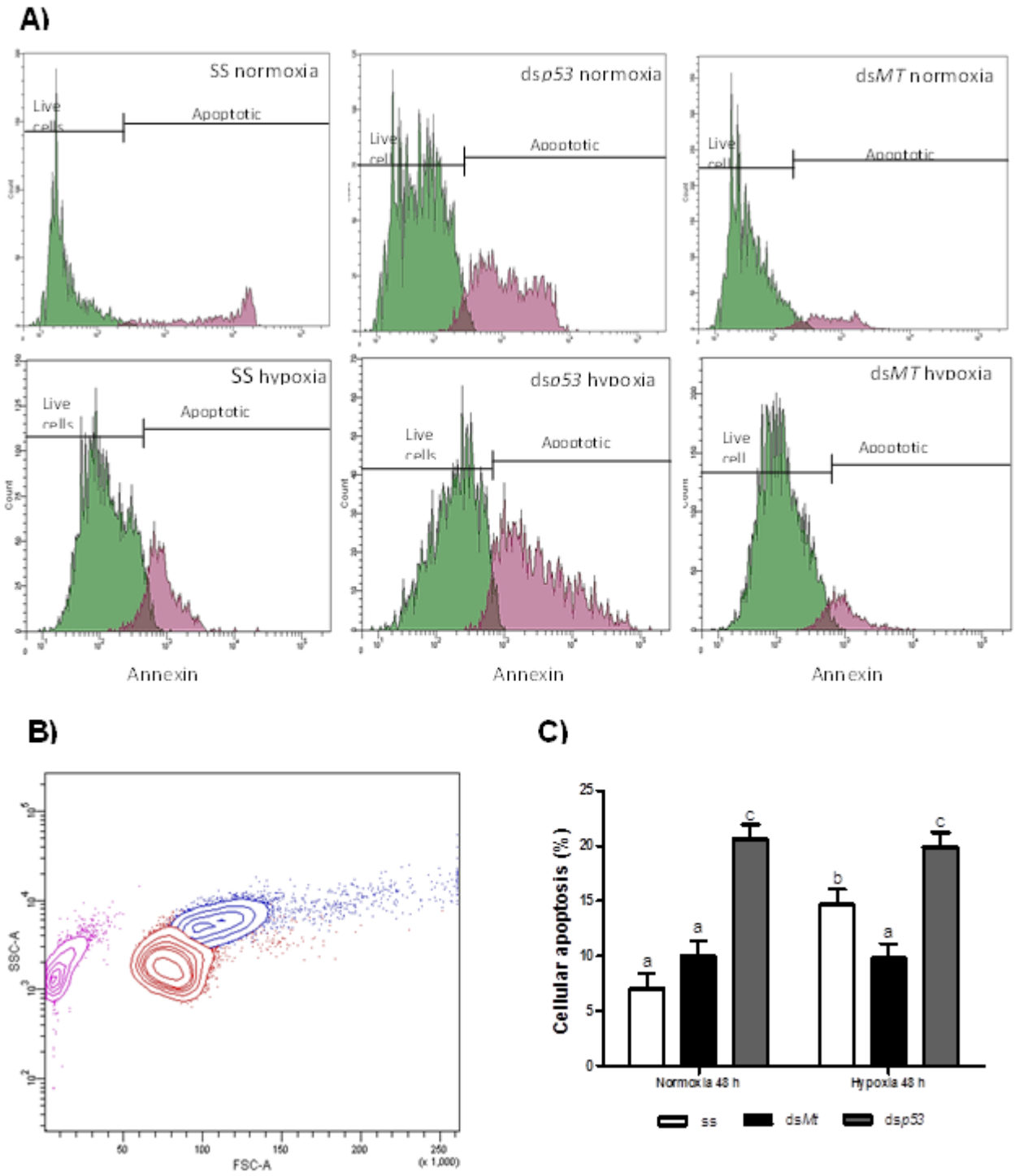
370 **Figure 6. Caspase-3 activity in cell lysates**. Enzymatic caspase-3 activity was measured

371 in hemocyte lysates from shrimp injected with saline solution (SS), ds*MT* or ds*p53* at (A)

372 24 h, and (B) 48 h of normoxia or hypoxia. Specific activities were normalized to total

373 protein content. Results are expressed as means ± SE and statistically significant

374 differences ($p<0.05$) are indicated by the lowercase letters.



375

376 **Figure 7. Flow cytometry of hemocytes from *L. vannamei*.** Panel (A) Distribution

377 histograms of Annexin V with Alexa Fluor® 488-H. Panel (B) Dot plot of FSC and SSC on *L.*

378 *vannamei* hemocytes. Three subpopulations are indicated as hyaline cells, (H); granular

379 cells, (G) and semingranular cells (SG). Panel (C) Apoptotic percentage of hemocytes
380 under normoxia and hypoxia at 48 h. Results are expressed as means ± SE and statistically
381 significant differences ($p<0.05$) are indicated by the lowercase letters.
382
383 It is noteworthy that in our study, even without the low oxygen stress, at normoxic levels
384 (5.02 ± 0.11 and 4.87 ± 0.23 mg of DO L^{-1} for dsMt-treated and dsp53-treated shrimp,
385 respectively) dsp53 and dsMT hemocyte lysates had greater caspase-3 activity than SS
386 hemocyte lysates at both 24 and 48 h, indicating a possible p53-independent and/or Mt-
387 independent caspase activation which is in fact, exacerbated in the absence of these
388 mRNAs. This could point towards alternative p53-independent apoptosis pathways that
389 come into play when the cell is been depleted of LvMT and Lvp53 transcripts.
390 Mechanisms of p53-independent apoptosis include p19 (ARF) and mitogen-activated
391 protein kinases (MAPKs) such as p38, JNK and ERK, and they can also augment the
392 apoptotic activity of p53 (McNamee and Brodsky, 2009; Paliwal et al., 2006; Vousden
393 and Lu, 2002). The involvement of Cdc42 and MAPK in the cadmium-induced apoptotic
394 response has been demonstrated in *L. vannamei* (Peng et al., 2015), thus their role in
395 response to hypoxia deserves to be investigated. Apoptotic cell death via p53-dependent
396 and independent mechanisms has been observed in lymphocytes of an identical subtype
397 ($CD8^{-}CD4^{+}$) but from different organs (thymus and spleen) and it is partially attributed to
398 differences in cell maturation stages (Hotchkiss et al., 2000). Thus, it is possible that
399 granular, semi-granular and hyaline hemocytes, each exerts different responses and
400 mechanisms that could contribute to apoptosis. Indeed, hypercapnic hypoxia appears to

401 alter the normal function of hemocytes in response to pathogens (Holman et al., 2004).
402 Furthermore, events in hemocytes are not necessarily reminiscent of the cellular
403 processes in other cell types. In *Drosophila*, hemocytes –but not other cells -, are
404 protected against ROS generated during hypoxia (Azad et al., 2011). It is then important
405 to study the apoptosis involvement of MT and p53 in tissues where they are more
406 abundantly expressed, e.g. hepatopancreas. It is also important to investigate in *L.*
407 *vannamei*, the existence of other members of the p53 superfamily, namely p63 and p73,
408 which could be partially rescuing the p53-silencing effect, given that in mammals the
409 different p53-superfamily proteins interplay and have overlapping although distinct
410 functions (Belyi and Levine, 2009; Levrero et al., 2000).
411 This study provides an insight into the p53 role in the crustacean cell fate under hypoxia
412 by suggesting the existence of both p53-dependent and p53-independent apoptotic
413 pathways in crustacean.
414

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421

422 **Author contributions**

423 MFP and GYP conceived and designed the experiments, MFP, JAMQ and MAP performed
424 the experiments, MFP, JAMQ and VMH analyzed and interpreted the data, SG
425 coordinated the shrimp hypoxia bioassay, MFP and GYP drafted the MS and all authors
426 critically revised and gave final approval of the MS to be published.
427

428 **Competing interests**

429 Authors declare no competing or financial interests.
430

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CAPÍTULO IV

Molecular players for gene silencing in crustaceans

Review

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RESUMEN

Arthropoda es el más grande filo animal representando el 80% de las especies vivientes. Cómo ocurren las vías de RNA de interferencia (RNAi) en este filo, se basa en gran medida en estudios en la mosca de la fruta *Drosophila melanogaster*. La falta de líneas celulares de crustáceos, disponibles para estudios genéticos ha llevado a los investigadores a la búsqueda de formas alternativas de estudio de la función de genes en estos organismos. Con este fin, el RNA de interferencia ha proporcionado al área una poderosa herramienta que ha permitido el estudio de varios genes de crustáceos *in vivo* que de otra manera hubieran permanecido desconocidos. Durante los últimos diez años, una considerable cantidad de investigación en el subfilo Crustacea ha hecho uso del RNAi para elucidar mecanismos de defensa virales o para combatir patógenos en especies de crustáceos comercialmente relevantes. Se han encontrado ortólogos crustáceos de la mayoría de las proteínas involucradas con los mecanismos de RNAi identificados en mamíferos, *D. melanogaste*r u otros organismos modelo; pero la integración de estas proteínas en los contextos fisiológico, patológico y bioquímico en crustáceos es aún incipiente. La caracterización de los participantes moleculares y de las vías por las que contribuyen a la maquinaria del RNAi en crustáceos proporcionará nuevo conocimiento acerca de la regulación génica en este grupo de organismos.

Palabras clave: crustáceo, dicer, silenciamiento de genes, RISC, RNAi

Molecular players for gene silencing in crustaceans

Review article

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Abstract

Arthropoda is the largest animal phylum representing over 80% of the living species. How the RNA interference (RNAi) pathways occur in this phylum is largely based on studies in the fruit fly *Drosophila melanogaster*. The lack of readily available crustacean cell lines for genetic studies has prompted researchers to the quest of alternative ways to study gene function in these organisms. To this end, RNA interference has provided the field with a powerful tool that has allowed the study of several crustacean genes *in vivo* that would have otherwise remained in the unknown. During the last ten years a considerable amount of research in the Crustacea subphylum has made use of the RNAi work to either elucidate viral defense mechanisms or target pathogens in commercially relevant crustacean species. Crustacean orthologues have been found for most of the proteins involved in the RNAi mechanisms identified in mammals, *D. melanogaster* or other model organisms, but integration of these proteins in the physiological, pathological and biochemical context of crustaceans is still in its infancy. The characterization of the molecular players and pathways and their contribution to the machinery of RNAi in crustaceans will provide new insights about gene regulation in this group of organisms.

Keywords: crustacean, dicer, gene silencing, RISC, RNAi

1. Introduction

The RNA-mediated gene silencing process was serendipitously encountered in petunia flowers upon the introduction of a chimeric petunia chalcone synthase gene in an attempt to create a darker color in the flower. Instead, a white and/or patterned color progeny was obtained and this effect was called "co-suppression" (Napoli et al., 1990). The fungi researchers also provided proof of gene silencing, referred to as "quelling" in *Neurospora crassa*, a model in which they demonstrated the reversal of an albino phenotype after transforming with *al-1* or *al-3* genes, a process shown to be progressively reversible and monodirectional, in the sense that once relieved from inhibition, quelling does not happen again (Romano and Macino, 1992). The term "RNA interference" (RNAi) was first used by Rocheleau et al. (1997) and Fire et al. (1998) to refer to the then-unknown mechanism that could interfere with the function of specific genes in *Caenorhabditis elegans* upon injection of double-stranded RNA (dsRNA). From then on, not only has RNAi been used in many organisms to molecularly explore the function of a great number of genes, but has also had therapeutic applications against viral infections in a variety of species, and as an anti-cancer therapy (Pushparaj and Melendez, 2006).

Non-model organisms portrait a much wider biochemical, ecological and physiological richness than that represented by model organisms. However, the study of the fascinating diversity of non-model organisms has been hindered by the lack of genetic tools that would allow the manipulation of gene expression. In this context, the use of RNAi in non-model species has enable the -otherwise impossible- knock-down of specific genes, leading to a wealth of knowledge that keeps on being generated.

In crustaceans, several research groups have made use of the RNAi-mediated silencing to study development, growth, immune system, metabolism and reproduction. Others have made a biotechnological application of this gene-silencing approach. These aspects have been masterfully addressed by Sagi et al. (2013). We, however, wish to give an overview into the RNAi molecular players that have so far been studied in crustaceans.

2. The mechanism of RNAi

The regulation of gene expression is pivotal for cell functioning and this regulation occurs in several layers of control which include -but are not restricted to- transcription, mRNA degradation/stability and translation. The RNAi regulatory mechanism represents one such layer of post-transcriptional control that encompasses two related but different pathways: 1) micro RNAs (miRNAs), which are small non-coding RNAs mainly involved in endogenous gene regulation, and 2) small- or short-interfering RNAs (siRNAs), mostly implicated in the defense against invasive nucleic acids (Valencia-Sanchez et al., 2006).

The RNAi mechanism involving siRNAs initiates with the presence of long dsRNA in the eukaryotic cell. The dsRNAs are then recognized by a dsRNA-binding protein RDE-4 (Wang and Barr, 2005). This binding enables the activity of the enzyme RNase III called "Dicer", cleaving the dsRNAs into 21-25 bp siRNAs (Bernstein et al., 2001; Ketting et al., 2001). An ATP-dependent helicase unwounds the siRNAs generated by Dicer (Nykanen et al., 2001), providing single-stranded siRNAs that —associated to a protein complex called RNA-Induced silencing complex (RISC)— acts as a guide to base-pair to target homologous transcripts and endonucleolytically cleave them (Hamilton and Baulcombe, 1999; Hammond et al., 2000). This whole process allows for the target mRNA to be degraded before translation into the cognate protein occurs in the cytoplasm.

miRNAs are hairpin-derived small RNAs which are processed in the nucleus by a "Microprocessor" protein complex that includes the RNase III enzyme "Drosha" and a double-stranded RNA binding protein called "Pasha" in *Drosophila* (Denli et al., 2004; Lee et al., 2003). After being processed into 65-75 nt-long precursor miRNAs, they are exported to the cytoplasm where they are further cleaved into ~22 nt miRNAs by the RISC which is composed of Dicer-TRBP-Argonaute 2 protein complex (Chendrimada et al., 2005; Gregory et al., 2005). A single strand of this miRNAs is kept as a guide RNA that has imperfect complementarity to the 3´untranslated regions of mRNA targets but is highly complementary to the 2-8 nt "seed" region. This recognition may result in deadenylation, translational repression and mRNA decay (Chen and Xie, 2012).

The source of dsRNA can be exogenous as it is the case of pathogen nucleic acids and experimentally introduced dsRNA, but it is clear that the entire silencing machinery has evolved and been maintained because it offers an intrinsic benefit to the cell. Indeed,

naturally occurring RNAi is initiated by viral transcripts, or by endogenous miRNAs (Grishok et al., 2000), repeat RNA transcripts originated from satellite repeats (Lippman and Martienssen, 2004) and short-hairpin RNAs (shRNAs) resulting from single-stranded mRNA products. These non-dsRNA silencing triggers are aided by members of the RNA-dependent RNA polymerases (RdRPs) family, which has been extensively characterized and is highly conserved in most eukaryotes (Iyer et al., 2003). RdRPs recognize single-stranded mRNAs (either endogenous or transgenes) as abnormal and then engage in primer-independent synthesis of complementary RNA, thus deriving dsRNA as an intermediary, which is then processed by Dicer and follow the downstream events that are the hallmark of RNAi silencing (Baulcombe, 1996; Makeyev and Bamford, 2002).

3. Molecular players for RNAi in crustaceans

RNAi silencing in crustaceans has been successfully used during the last decade and although the exact silencing mechanism in this subphylum has not been biochemically elucidated just yet, it is thought to be evolutionarily conserved amongst eukaryotes. Regarding the RNAi machinery, several crustacean orthologues have been found and genetically characterized. The functionality of some of them has also been demonstrated (Fig. 1).

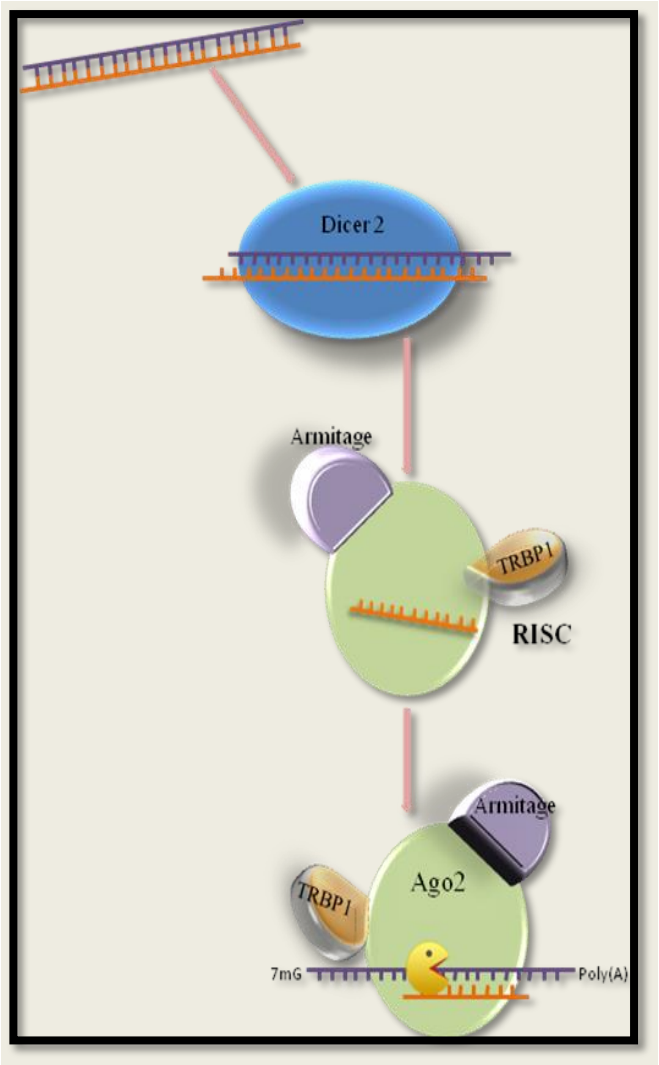

Figure 1. Known crustacean RNAi players in a model of dsRNA-guided post-transcriptional regulation of gene expression. Artificially introduced long dsRNAs or viral RNAs are processed by Dicer into 21-23 nucleotide dsRNA intermediates. The RNA helicase Armitage assists the formation of the single-stranded siRNA-containing RISC. RISC cleaves the target mRNA in the middle of the complementary region.

3.1 Dicer protein family

Dicer proteins cleave dsRNA into siRNAs or miRNAs, which are involved in viral infections and endogenous gene regulation, respectively (Maroney et al., 2006; Ramaswamy and Slack, 2002). Dicer is a member of the RNase III family of enzymes and has several domains (Fig. 2) that include: ATP-dependent RNA helicase, DUF283 (domain of unknown function), PAZ (Piwi/Argonaute/Zwille), dsRNA binding and RNase III domains (Matsuda et al., 2000). This RNase III cleaves dsRNAs associated to other proteins like transactivating response RNA-binding protein (TRBP), whereas the

PAZ domain binds to single-stranded RNA at the 3´end of the molecule (Hall, 2005). Dicer proteins are a highly conserved family found in plants, fungi and the Metazoa, having up to five representatives in some species (de Jong et al., 2009). As for crustaceans, phylogenetic analysis of Dicer protein sequences available for some crustaceans, revealed that the whiteleg shrimp *Litopenaeus vannamei*, the tiger shrimp *Penaeus monodon*, the shrimp *Marsupenaeus japonicus*, the sea louse *Lepeophtheirus salmonis* and the water flea *Daphnia pulex* have Dicer-1 and Dicer-2 genes, with *D. pulex* possessing three paralogues of Dicer-2, probably derived from Dicer-2 duplications (Lozano et al., 2012). A sequence alignment we made of the Dicer-1 proteins from *M. japonicus*, *L. vannamei* and *P. monodon* reveals identities of 95-97% and the amino acid differences are mainly found on the N-termini half, along the first inter-domain region and within the DUF283 domain (Fig. 3). The DUF283 domain has been suggested to have a functional role in target selection during small RNA processing (Qin et al., 2010). The alignment of the corresponding Dicer-2 proteins shows identities of 91-98% with the differences being distributed along the whole sequence (Fig. 4). Regarding the Dicer-2 orthologue in *L. vannamei* (LvDcr2), not only has it been cloned and characterized, but the heterologous expressed protein has also been shown to interact in a complex with *L. vannamei* Argonaute-2 and TRBP1 recombinant proteins in co-immunoprecipitation assays and pull-down assays (Chen et al., 2011). In the *Drosophila* system, Dicer-2 has been mainly related to siRNA-directed mRNA cleavage, whereas Dicer-1 has been found to be essential for miRISC-directed translational repression (Lee et al., 2004), which suggests that the multi-protein complex detected in the shrimp corresponds to the putative siRNA-induced silencing complex (siRISC). Nevertheless, Dicer-1 in *L. vannamei* also was up-regulated after virus injection in adults, but its differential expression across the larval stages, suggests a role for LvDcr1 in the early innate immunity during larval development (Yao et al., 2010). For *P. monodon*, Dicer-1 (PmDcr1) appears to be indirectly involved in antiviral defense in shrimp, since levels of the Dicer 1 messenger did not correlate with viral loads in naturally infected shrimp, although its knock-down resulted in higher mortalities in virally-infected animals (Su et al., 2008). Dicer-2 (PmDcr2) mRNAs are upregulated in response to viral but not to bacterial infection (Li et al., 2013). In *M. japonicus*, Dicer-1 was an important protein in the biogenesis of miR-7, which had a large

impact on virus infection (Huang and Zhang, 2012b), with the caveat that the Dicer-1 and Dicer-2 dsRNAs used in the study were based on the *L. vannamei* and *P. monodon* sequences.

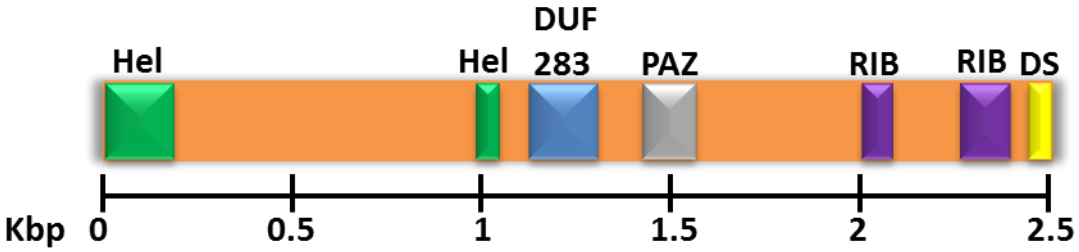

Fig. 2. Graphical representation of the five Dicer domains from shrimp. The boxes represent each domain; the N and C terminal Helicase, the unknown DUF283, the Piwi-Argonaute-Zwille, the bidentate ribonuclease III and the dsRNA binding. The spaces between boxes are the inter-domain regions.

3.2 Argonaute protein family

Argonaute2 was genetically and biochemically identified in *Drosophila* as the effector nuclease component of the RISC complex (Hammond et al., 2001). An RNA-Seq analysis in the sea lice *Caligusrogercres seyi* identified several Argonaute variants which are transcriptionally modulated along different developmental stages and the confirmation of the presence of its functional domains, along with the finding of Pasha in the same study, suggest a mechanism of miRNA processing in this organism during ontogeny (Valenzuela-Miranda et al., 2015). *P. monodon* Argonaute (Pem-AGO) was the first RNAi machinery component identified in crustacean. Its functionality was indirectly tested precisely by dsRNA-mediated silencing in lymphoid organ primary cell culture which showed defective RNAi in response to ds-RNA of *P. monodon* serotonin receptor (Dechklar et al., 2008). Three Argonaute 1 (Ago1) isoforms called Ago1A, Ago1B and Ago1C were found in *M. japonicus.* Ago1A and Ago1B were up-regulated in lymphoid organ and hemolymph in response to viral challenge. Moreover, when Ago1B was knocked-down with dsRNA, Ago1A was able to compensate for the silencing, both at the mRNA level and at the apparent functional level, as the viral loads were maintained as low as in the un-silenced control cells (Huang and Zhang, 2012a). Findings in *L. vannamei*

suggested a possible shared pathway for antiviral immunity and induction of RNAi. These findings included the presence of two Argonaute family members Lv-Ago1 and Lv-Ago2 as well as the orthologue of a membrane channel-forming protein Sid-1, Lv-sid1, involved in the cellular import of dsRNA. Transcript abundance of Lv-Ago2 but not Lv-Ago1, was strongly up-regulated in response to an exogenous-gene dsRNA injection, suggesting a non-redundant activity of these proteins and that Lv-Ago2 is part of the RNAi pathway machinery, although both Lv-Ago1 and Lv-Ago2 possess a conserved essential motif for the catalytic activity of Argonaute proteins. Importantly, this study also shows that >50-bp specific dsRNAs, but not siRNAs, induced silencing of the corresponding mRNA (Labreuche et al., 2010).

In Fig. 5 it can be seen that the proteins argonaute 1 from the three shrimp species are highly identical, indeed *P. monodon* and *M. japonicus* sequences are 100 % identical to each other while *L. vannamei* has 99 % of identity with them. On the other hand argonaute 2 from *L. vannamei* present 76 % and 47 % of identity with *M. japonicus* and *P. monodon* respectively. Even though argonaute 1 and argonaute 2 from *L. vannamei* contain in the primary structure the signature domains PAZ and Piwi of the argonaute subfamily proteins, the identity between them is only of 39 % even in the PAZ domain however the identity between the Piwi domains is 52 % as can be seen in Fig. 6.

3.3 RNA helicases

The RNA helicases are a group of enzymes implicated in several activities of RNA processing in the cell, separating RNA-RNA or RNA-DNA duplexes in an ATP-dependent manner (Fairman-Williams et al., 2010). Within the context of RNAi, the RNA helicase Moloney leukemia virus 10 (mov-10) is an orthologue of the *Drosophila* translational repressor Armitage and one of the RISC proteins essential for miRNA-mediated silencing in humans, with orthologues found also in plants (Zheng et al., 2010). A *M. japonicus* mov-10 (Mj-mov-10) was recently characterized. Its expression was significantly up-regulated after dsRNA injection, and its silencing augmented shrimp mortalities upon viral challenge, thus playing a crucial role in antiviral defense, probably by hindering virally-induced RNAi machinery (Phentrungnapha et al., 2015).

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MjDcr1 MMNRVQPENVHSTIFTPREYQVELVDACLKGNTLSVLASRSTRTFLITMVTREMAHLTRSKEQGGKGQRTLLTGWSGPGL 80
LvDcr1 MMNRVQPENVHSTIFTPREYQVELVDACLKGNTLSVLASRSTRTFLITMVTREMAHLTRSKEQGGKGQRTLLTGWSGPGL 80
PmDcr1 MMNRVQPENVHSTIFTPREYQVELVDACLKGNTLSVLASRSTRTFLITMVTREMAHLTRSKEQGGKGQRTLLTGWSGPGL 80
       ********************************************************************************

MjDcr1 VRAGEAIQQNTNLAVTTYTRLEQVDGWLPSRWSHTFTEAQVIIMTVDVLEKGLETGLLQLDMLNLLVITDAHRVATFPPL 160
LvDcr1 VRAGEAIQQNTNLAVTTYTRLEQVEGWLPSRWSQTFTEAQVIIMTVDVLEKGLETGLLQLDMLNLLVITDAHRVATFPPL 160
PmDcr1 VRAGEAIQQNTNLAVTTYTRLEQVEGWLPSRWSQTFTEAQVIIMTVDVLEKGLETGLLQLDMLNLLVITDAHRVATFPPL 160
       *************************:********:*****************************************

MjDcr1 IKVLNLCRGCRILGMTSPVLSHACSPPQLESFLTHLQDATSCVVDTSSEIVTVLRYVNKPVEKIVMCRDPDPDERSEVEL 240
LvDcr1 IKVLNLCRGCRILGMTSPVLSHACSPPQLESFLTHLQDATSCVVDTSSEIVTVLRYVNKPVEKIVMCRDPDPDERSEVEL 240
PmDcr1 IKVLNLCRGCRILGMTSPVLSHACSPPQLESFLTHLQDATSCVVDTSSEIVTVLRYVNKPVEKIVMCRDPDPDERSEVEL 240
       ********************************************************************************

MjDcr1 EVRRLVGEALSFLDNHRYDLVEVYGPEYQEFCDDLPDPNKEPRQILMDFLDVLNNLGLWCADRAALYALVEVEKLKKKTA 320
LvDcr1 EVRRLVGEALSFLDNHRYDLVEVYGPEYQEFCDDLPDPNKEPRQILMDFLDVLNNLGLWCADRAALYALVEVEKLKKKTA 320
PmDcr1 EVRRLVGEALSFLDNHRYDLVEVYGPEYQEFCDDLPDPNKEPRQILMDFLDVLNNLGLWCADRAALYALVEVEKLKKKTA 320
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MjDcr1 YDRHYLLLCMIFTVMARIRAVVEQAFDKYSELDQILKFSTPKVLRLVEILRQVRPLNFVDPRKRRDRLETLENGKPAAAL 400
LvDcr1 YDRHYLLLCMIFTVMARIRAVVEQAFDKYSELDQILKFSTPKVLRLVEILRQVRPLNFVDPRKRRDRLEVFENGKAAAAL 400
PmDcr1 YDRHYLLLCMIFTVMARIRAVVEQTFDKYSELDQILKFSTPKVLRLVEILRQVRPLNFVDPRKRRDRLEAFENGKSAAAL 400
       *************************:*********************************************.:**** ****

MjDcr1 ECSENTQEDKPLAEDESAQEDPLKLATEVGNTENQQGSASKSKVEMSCEGSAEPSDTTTTLCVQESIYGISEIPLVSSGD 480
LvDcr1 EGSEDTQENKTSSEDKNVEKDSTKPVTDGGNTENLQGSSSKLKIEMSCESSEEPSDT--TLCVQESIYGISEIPLVSSGD 478
PmDcr1 EGSEDTQENKTSAEDKNVHEDPTKLATDGGNTENLQGSASKSETEMSCEGSEEPSDT--TLCVQGSIYGISEIPLVSSGD 478
       * **:***:*  :**:...:*  * .*: ***** ***:** : *****.* *****  ***** ***************

MjDcr1 GAKDPNDECEVNSGNSMPDLEAEEELSEDHSQIHGNSVVLTNSTEPASEDPFVADENSTEDKSELSHGAPSNVFCGSSIE 560
LvDcr1 GAKDPNDECEVNSGNAMPDLKAVEELSEGHSQIHG--------TEPSSEDPFLADENSTEDKSELSHGAPSNMFCGSSIE 550
PmDcr1 GANDPNNECEVNSGNAMPDLKAMEELSEGHSQIHG--------TEPASEDPFLADENSTEDKSELSHGAPSNMFCGSSIE 550
       **:***:********:****:* ***** ******        ***:*****:*******************:*******

MjDcr1 DSSEVVDISTDGGYFTAVSDVSPKCTSKAVSDSFTNDIEDCMMKANCNNHEVNVSRMSEVNTEDFRAEITDRYSCLQDSL 640
LvDcr1 DSSEVVDISTDGGYFTAVSDVSPKCTSKAVSDSYTNDIEDCMMKANCNNYEVNVSRMTEINTENVCAEVTDKYSCLQDSL 630
PmDcr1 DSSEVVDISTDGGYFTAVSDVSPKCTSKAVSDSYTNDIEDCMIKANCNNHEVNVSRMTEVNTENFCAEVTDKHSYLQDSL 630
       **********************************:********:******:*******:*:***:. **:**::* *****

MjDcr1 EDAVEFTEHSMKGRAADDMHTTVQTCPETTQYCSNTCEDVVAKTNNENGSALEDSLCRLNIKESSCTDGCDTESIKSCDL 720
LvDcr1 EDGVDFTKHSMKERAADDMHFTVQTCPETTQYCSNACEDVVAKTNDEDGSALEDSLCRLNIKESSCTDGCDTESIKSCDL 710
PmDcr1 EDGVDFTKHSMKERSADDMHITVQTCPETTQYCSNACEDVAAKTNDEDGSTLEDSLCRLNIKESSCTDGCDTESIKSCDL 710
       **.*:**:**** *:***** ***************:****.****:*:**:******************************

MjDcr1 HNGVLSAKNELLDTAMIQNMKNMTLCNGFEHGSKEDIMDSEKDITSKMNGHVDPYDYDSESEDISDKYKSCESVQSQASV 800
LvDcr1 HNGVLSAKNDLLDTAMIQNVKNMTLCNGFEHGNKEDIMDSEKDITSKMNGHVDPSEYDSESEDISDKYKSCESVQSQASV 790
PmDcr1 HNGVLSAKNDLLDTAMIQNVKNMTLCNGFEHGSKDDIMDSEKDITSKMNGHVDPSEYDSESEDISDRYKSCESVQSQASV 790
       *********:*********:************.*:******************* :**********:**************

MjDcr1 ETSSQEGSISDTFVPPATSDLPCKADATSAVDETATTSDSSPNDQELPAHTDGVEESQDLALVSTSGTTQNTPSCSDSAA 880
LvDcr1 ETSSKEGSISDTYVPPATSDLPCKADATSAVDETATPSDSAPSEQELPTCTNGVENTEDPALVSTSGTTQNTIICSDSAA 870
PmDcr1 ETSSKEGSISDTFVPSATSYLPCKADATSAVDETATPSDSAPNEQELPTCTSGVENTEDPALVSTSGTTQNTTICSDSGA 870
       ****:*******:** *** **************** ***:*.:****: *.***:::* ************  ****.*

MjDcr1 FSNASNGTQPATQAVSSEAAAMADTLAMLLPNSGKGRKRRDDIKEKVKVHNPEDPDSVCGLIFVHHRSMAKIIYRLLKEL 960
LvDcr1 FSNANNGIQPTTQAVSSEAAAMADTLAMLLPNSGKGRKRRDDVKEKVKVHNPEDPDSVCGLIFVHHRSMAKIIYRLLKEL 950
PmDcr1 FSNNSNGTQPTTQTVSSEAAAMADTLAMLLPNSGKGRKRRDDVKEKVKVHNPEDPDSVCGLIFVHHRSMAKIIYRLLKEL 950
       *** .** **:**:****************************:************************************

MjDcr1 SDIGGDFAWIFPQYTVEAKESVKEDPRAAEAEHKKQEEVLRRFRHHECNILVSTRVLEEGIDVPQCNLVLRFDPPTDYRS 1040
LvDcr1 SDIGGDFAWIFPQYTVEAKESVKEDPRAAEAEHKKQEEVLRRFRHHECNILVSTRVLEEGIDVPQCNLVLRFDPPTDYRS 1030
PmDcr1 SDIGGDFAWIFPQYTVEAKESVKEDPRAAEAEHKKQEEVLRRFRHHECNILVSTRVLEEGIDVPQCNLVLRFDPPTDYRS 1030
       ********************************************************************************

MjDcr1 YVHSCGRARGHDTFYFHLITKNQELSFLHDMATYSAFQQVLVSHCGSVEVGTDREVLSSEANAAHAPYITPAAAAVTMAS 1120
LvDcr1 YVHSCGRARGHDTFYFHLITKNQEISFLHDMATYSAFQQVLVSHCGSVEVGTDREVLSSEANAAHAPYITPAEAAVTMAS 1110
PmDcr1 YVHSCGRARGHDTFYFHLITKNQEISFLHDMATYSAFQQVLVSHCGSVEVGTDREVLSSEANAAHAPYLTPAEAAVTMAS 1110
       *************************:*********************************************:*** *******

MjDcr1 AIGLLNKYCAKLPSDTFTRLTAMWDVEEMEETAG-ALKYKCKIMLPINSPLKGTIEGPWQDKVSLAKMAAALECCRRLHQ 1199
LvDcr1 AIGLLNKYCAKLPSDTFTRLTAMWDVEEIKEAEALTPVYKCKIMLPINSPLKGTIEGPWQKKVSLAKMAAALECCRRLHQ 1190
PmDcr1 AIGLLNKYCAKLPSDTFTRLTAMWDVEEIEEAEVEIPKYKCKIMLPINSPLKGTIEGPWQSKVSLAKMAAALECCRRLHQ 1190
       ****************************::*:      **********************.******************

MjDcr1 MGELDDQLQPVGKESMKLDDHLCAPPADDQVPEGMPRPGTTKRRQYYYKKVAVCLTGEQPKQGLDLFVYKLDMVLTCPIP 1279
LvDcr1 MGELDDQLQPVGKESMKLDDHLCAPPADDQVPEGMPRPGTTKRRQYYYKKVAVCLTGEQPKQGLDLFVYKLDMVLTCPIP 1270
PmDcr1 MEELDDQLQPVGKESMKLDDHLCAPPADDQVPEGMPRPGTTKRRQYCYKKVAVCLTGEQPKQGLDLFVYKLDMVLTCPIP 1270
       * ********************************************** ********************************
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198

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MjDcr1 DEQNTRGRKIYRPEQSSRSFGIITTKPISQVSGFPVFTRSGEVVVHVQEIERKVNVTQDQLSALQYFHKFTFTHVLRLEK 1359
LvDcr1 DEQNTRGRKIYRPEQSSRSFGIITTKPISQVSGFPVFTRSGEVVVHVQEIERKVNVTQDQLSALQYFHKFTFTHVLRLEK 1350
PmDcr1 DEQNTRGRKIYRPEQSSRSFGIITTKPISQVSGFPVFTRSGEVVVHVQEIERKVNVTQDQLSALQYFHKFTFTHVLRLEK 1350
       ********************************************************************************

MjDcr1 YPIKFDPTNARTAFYIVPLNKFNGSESIDWEFVKEIQSEGDPRPVPPQDDARKKFQFQHDLYEDAVVMPWYRNQDQPQYF 1439
LvDcr1 YPIKFDPTNARTAFYIVPLNKFNGSEGIDWEFVKEIQSEGDPRPVPPQDDARKKFQFQHDLYEDAVVMPWYRNQDQPQYF 1430
PmDcr1 YPIKFDPTNARTAFYIVPLNKFNGSEGTDWEFVKEIQSEGDPRPVPPQDDARKKFQFQHDLYEDAVVMPWYRNQDQPQYF 1430
       **************************. ************************************************

MjDcr1 YVAEICTHLNPQSDFPDAGFETFEKYYLTKYGLQICNLAQPLLDVDHTSGRLNLLTPRHVNRKGVALPTTSEETKRAKRE 1519
LvDcr1 YVAEICTHLNPQSDFPDAGFETFEKYYLTKYGLQICNLAQPLLDVDHTSGRLNLLTPRHVNRKGVALPTTSEETKRAKRE 1510
PmDcr1 YVAEICTHLNPQSDFPDAGFETFEKYYLTKYGLQICNLAQPLLDVDHTSGRLNLLTPRHVNRKGVALPTTSEETKRAKRE 1510
       ********************************************************************************

MjDcr1 NLQQKQILVPELCTIHPFPASLWRKAVCLPTILYRINALLLADQLRLSVASEVGLGLQNLLPDFSWPPLDFGWSLADVLK 1599
LvDcr1 NLQQKQILVPELCTIHPFPASLWRKAVCLPTILYRINALLLADQLRLSVASEVGLGLQTLLPDFSWPPLDFGWGLADVLR 1590
PmDcr1 NLQQKQILVPELCTIHPFPASLWRKAVCLPTILYRINALLLADQLRLSVASEVGLGLQTLLPDFSWPPLDFGWSLADVLR 1590
       ********************************************************.**************.*****:

MjDcr1 KNQENQEQDDKSNNSSEATNNKQKATKAENPECESPTLTAKKKAPKKTNDMEIGTWSNDMAVDPPTPPFDMDTMNGPDEF 1679
LvDcr1 KNQENQEQDDKSNIATEATNSKQKASKAENPECESPTLAAKKKAPKKGNDMEIGTWSNDMAVDPPTPPFDMDTMNGPDEF 1670
PmDcr1 KNQENQEQDDKSNIASEATNSKQKATKTENSECESPTLAAKKKAPKKGNDMEIGTWSNDMAVDPPTPPFDMDTMNGPDEF 1670
       ************* ::****.****:*:** *******:******** ********************************

MjDcr1 EIDTFDPNVALPDNLTLLNGFSGADDDIEGELGADWGTGITERRTKSSCNKDSKGGMFRVGSPSNFESDGWDMFGSSGYN 1759
LvDcr1 EIDTFDPNVALPDNLTLLNGFSGADDDIEGELGADWGTGITERRTKSSCNKDSKGGMFRVGSPSNFESDGWDMFGSSGYN 1750
PmDcr1 EIDTFDPNVALPDNLTLLNGFSGADDDVEGELGADWGTGITERRTKSSCNKDSKGGMFRVGSPSNFESDGWDMFGSSGYN 1750
       ***************************:****************************************************

MjDcr1 GGYGGYGSYDAYGGYGDFQGLADDLEGCESDVSSDMDDDDKSQTEKLWDEEGTKRRSSMAADEGSSDEEIDLNWPDEDEE 1839
LvDcr1 GGYGGYGSYDAYGGYGDFQGLADDLEGCESDVSPDMDDDDKSQTEKLWDEEGTKRRSSMAADEGSSDEEIDLNWPDEDEE 1830
PmDcr1 GGYGGYGSYDAYSGYGDFQGLADDLEGCESDVSSDMDDDDKSQTEKLWDEEGTKRRSSMAADEGSSDEEIDLNWPDEDEE 1830
       ************.******************** ***********************************************

MjDcr1 VRTEKVEEFQAFLEEKQKIIKESSCYLAENESLLIEKAHRKLQNEGKSSPEKVSHRTESSTTEGCEQLSSKGCDNAQQST 1919
LvDcr1 VRNEKEKEFQPFLEDKQKIIKESSCYLAESESLLIEKAHRKLQNESKSSPEKVSHRTESSTTEGCEQLSSKGCDNAQQST 1910
PmDcr1 VRNEKEKEFQSFLEDKQKIIKESSCYLAESESLLIEKAHRKLQNESKSSPEKVSHRTESSTTEGCKQLSSKGCDNAQQST 1910
       **.** :*** ***:**************.***************.*******************:**************

MjDcr1 SIMIRTVNRDETLALKRPDSEVQCSSWNSICEEMSFSFDFQPDLLNHPGPSPSVILQALTMSNANDGVNLERLETIGDSF 1999
LvDcr1 SIIIRTVNRDETLALKRPDSEVQCSSWNSICEEMSFSFDFQPDLLNHPGPSPSVILQALTMSNANDGVNLERLETIGDSF 1990
PmDcr1 SNIIRTVNRDETLALTRPDSEVQCSSWYSICEEMSFSFDFQPDLLNHPGPSPSVILQALTMSNANDGVNLERLETIGDSF 1990
       * :*************.*********** ****************************************************

MjDcr1 LKYAITTFLYCTYPQRHEGKLSYLRSKQVSNLNLYRLGKRKGLGECMVATKFEPHDNWLPPGYFVPRELEEALIDSGVPA 2079
LvDcr1 LKYAITTFLYCTYPQRHEGKLSYLRSKQVSNLNLYRLGKRKGLGECMVATKFEPHDNWLPPGYFVPRELEEALIDSGVPA 2070
PmDcr1 LKYAITTFLYCTYPQRHEGKLSYLRSKQVSNLNLYRLGKRKGLGECMVATKFEPHDNWLPPGYFVPRELEEALIDSGVPA 2070
       ********************************************************************************

MjDcr1 GHWNMADLPGLHDLASDEIRRLVQERSEQIKRSKNEQATSELMATQNPHDLPIFIPYNLLTQHSIPDKSIADCVEALIGA 2159
LvDcr1 GHWNMADLPGLHDLASDEIRRLVQERSEQIKRSKSEQATSELMATQNPHDPPIFIPYNLLTQHSIPDKSIADCVEALIGA 2150
PmDcr1 GHWNMADLPGLHDLASDEIRRLVQERSEQIKRSKSEQATSELMATQNPHDLPIFIPYNLLTQHSIPDKSIADCVEALIGA 2150
       **********************************.*************** ****************************

MjDcr1 YLTTCGPRGALLFMSWLGIKVLPCTLEDSPETGQLIIYGHLESPQSPLYHCPLTDTKKELDLLLSGYQVFEEKIGYTFQD 2239
LvDcr1 YLTTCGPRGALLFMSWLGIKVLPCTLESSPEACELITYGHLESPQSPPYHCPLTDTRKELDLLLSGYQVFEEKIGYTFRD 2230
PmDcr1 YLTTCGPRGALLFMSWLGIKVLPCTLESSPEASELITYGHLESPQSPLYHCPLTDTRKELDLLLSGYQVFEEKIGYTFRD 2230
       ***************************.***: :** ********** ********:*********************:*

MjDcr1 RSYLLQAFTHASYYKNRLTDCYQRLEFLGDAVLDYLITRHLYEDKRQHSPGALTDLRSALVNNTIFASLAVKYDYHKYFR 2319
LvDcr1 RSYLLQASTHASYYKNRLTDCYQRLEFLGDAVLDYLITRHLYEDKRQHSPGALTDLRSALVNNTIFASLAVKYDYHKYFR 2310
PmDcr1 RSYLLQAFTHASYYKNRLTDCYQRLEFLGDAVLDYLITRHLYEDKRQHSPGALTDLRSALVNNTIFASLAVKYDYHKYFR 2310
       ******* ******************************************************************************

MjDcr1 HFSPGLDRVIRDFVKMQEENGHKINEEYYFMEEDECEAAEDIEVPKALGDVFESVAGAIFLDSGSSLDAVWSVYYTMMCR 2399
LvDcr1 HFSPGPDRVIRDFVKMQEENGHKINEEYYFMEEDEREAAEDIEVPKALGDVFESVAGAIFLDSGSSLDAVWSVYYTMMCP 2390
PmDcr1 HFSPGLDRVIRDFVKMQEENGHKINEEYYFMEEDECEAAEDIEVPKALGDVFESVAGAIFLDSGSSLDAVWSVYYTMMCR 2390
       ***** ***************************** ****************************************** *

MjDcr1 EIEQFSGVVPKSPIRELLEMEPETAKFGKPERLVDGKVRVSVEIFGKGSFSGVGRNYRIAKSTAAKRALRHLKKLQMMAN 2479
LvDcr1 EIERFSGVVPKSPIRELLEMEPETAKFGKPERLVDGKVRVSVEIFGKGSFSGVGRNYRIAKSTAAKRALRHLKKLQMMAN 2470
PmDcr1 EIEQFSGVVPKSPIRELLEMEPETAKFGKPERLVDGKVRVSVEIFGKGSFSGVGRNYRIAKSTAAKRALRHLKKLQMMAN 2470
       ***:****************************************************************************

MjDcr1 QGI 2482
LvDcr1 QGI 2473
PmDcr1 QGI 2473
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HelN (N-terminal helicase domain)
HelC (C-terminal helicase domain)
DUF283 (domain of unknown function 283)
DS (dsRNA-binding motif)
PAZ (Piwi-Argonaute-Zwille domain)
RIBc-a (bidentate ribonuclease III domain)
RIBc-b (bidentate ribonuclease III domain)

Figure 3. Clustal Omega multiple sequence alignments of the Dicer-1 proteins of *M. japonicus* (MjDcr1, ADB44075), *L. vannamei* (LvDcr1, ACF96960) and *P. monodon*

(PmDcr1, ABR14013). MjDcr1 was 95% identical to LvDcr1 and PmDcr1. LvDcr1 and PmDcr1 were 97% identical.

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PmDcr2 MDASKDEAAADFCPRSYQLELFNCALKKNSILVLGTGSGKTFISILLIKELGHQIRESLKGGSKRTVFVVNTVPLVHQQAAAIQQHTPFEVGKYEGSMGVDYWTDEHWQN 110
LvDcr2 MDASKEEASADFCPRSYQLELFNCALKKNSILVLGTGSGKTFISILLIKELGHQIRESLKGGSKRTVFVVNTVPLVHQQAAAIQQHTPFEVGKYEGSMGVDYWTDEHWQN 110
MjDcr2 MDASKEEASADFCPRSYQLELFNCALKKNSILVLGTGSGKTFISILLIKELGHQIRESLKGGSKRTVFVVNTVPLVHQQAAAIQQHTPFEVGKYEGSMGVDYWTDEHWQN 110
       *****:**:************************************************************************************************

PmDcr2 ELEKHEVLVVVAQIFLDLILHARLPLSSVNLLIMDECHHTTGSHPMREIMRAYETLKQHSPDKLPRIMGLTACVVHRKCKRKDVLKYMESLEAAMDCALVTSIDPVTVLK 220
LvDcr2 ELEKHEVLVVVAQIFLDLILHAKLPLSSVNLLIMDECHHATGSHPMREIMRAYENLKQHSPEKLPRIMGLTACVVHRKCKRREVLKYMESLEAAMDCALVTSVDPETVLK 220
MjDcr2 ELEKHEVLVVVAQIFLDLILHAKLPLSSVNLLIMDECHHATGSHPMREIMRAYENLKQHSPEKLPRIMGLTACVVHRKCKRREVLKYMESLEAAMDCALVTSVDPETVLK 220
       **********************:***************:**************.******:*******************::*******************:** ****

PmDcr2 YTTGPKEKLICYFTENITSDYQETVKAQLAGLECDIITDCFINEKARKQIKKKVKNIEHIMSDLGDWCVARAIKYEIEHLDETEGMEEDPKVREILKLLKERLRDVYIAC 330
LvDcr2 YTTGPKEKVICFYTESITSDYQETVKAQLAGLECDIITDCFINYKARKQIKKKVKNIEHIMSDLGDWCVARAIKYEIEHLDETEGMEEDPKIREILKLLKERLREVYVAC 330
MjDcr2 YTTGPKEKVICFYTESITSDYQETVKAQLAGLECDIITDCFINYKARKQIKKKVKNIEHIMSDLGDWCVARAIKYEIEHLDETEGMEEDPKIREILKLLSEKLREVYVAC 330
       ********:**::**.************************* ********************************************:*******.*:**:**:**

PmDcr2 ELKEKCMSNPLEHITFKVKRLLEIFSKCNKEVFGLIFVERRNTAKILYDLLLEIAQQNPVFSFVRPSYVVGCNSRPGIDIRLAELELRKQKETLEKFRKEETNFLISTSV 440
LvDcr2 ELKEKGMANPVEHITFKVKRLLEIFSKCNKEVFGLIFVERRNTAKILYDLLLEIAKQNPEFSFVKPSYVVGCNSRPGIDIRLAELELRKQKETLENFRKGETNFLISTSV 440
MjDcr2 ELKEKGMANPVEHITFKVKSLLEIFSKCNKEVFGLIFVESRNTAKILYDLLLEIAKQNPEFSFVKPSYVVGCNSRPGIDIRLAELELRKQRETLENFRRGETNFLISTSV 440
       ***** *:**:******** ******************* ***************:*** ****:*************************:****:**: **********

PmDcr2 LEEGVDIRKCNVVVRFDIPQNYRAYVQSRGRARAKPSVYLLMVEEAKVSEMLECIGVYREIEASLQYLCHDRNLPTAQVTRKHFEEDEYIKPYEPYGPVGPKITSNSAIQ 550
LvDcr2 LEEGVDIRKCNVVVRFDMPQNYRAYVQSRGRARAKPSVYLLMVEEAKVSEMLECIGVYREIEDSLQYLCHGRNLPTAQTTRKHFAEDEYIKPYEPYGPVGPKITSNSAIQ 550
MjDcr2 LEEGVDIRKCNVVVRFDMPQNYRAYVQSRGRARAKPSVYLLMVEEAKVSEMLECIGVYKEIEDSLQYLCHGRNLPTAQTTRKHFAEDEYIKPYEPYGPVGPKITSNSAIQ 550
       *****************:****************************************:*** ******* *******.***** *************************

PmDcr2 LVNLYCGKLPSDKFTDNLPEVVEEVKEGIVTVGIRLPIMAPLKDMIYGSGWANKDLAKKSAALQLCIKLHEMGELDSNLRPKESSDDSLVEGLVDLPPEEPVPEGTPESG 660
LvDcr2 LVNLYCGKLPSDKFTDNLPEVVEEVKEGMTTVGIRLPIMAPLKDMIYGSGWANKDLAKKSAALQLCIKLHEMGELDSNLRPKESNDDSLVEGLVDLPPEEPVPEGTSESG 660
MjDcr2 LVNLYCGKFPSDKFTDNLPEVVEEVKEGMTTVGIRLPIMAPLKDMIYGSGWANKDLAKKSAALQLCIKLHEMGELDSNLRPKESNDDSLVEGLVDLPPEEPVPEGTSESG 660
       ********:*******************:.*****************************************************.******************** ***

PmDcr2 TRKRKRIYAKELCYPFSNVHRRSYCLYSINIKPFGQTDQSVTIDSFTCINKIGFICKEGLLCSPFPLFFSKWGDVMVNVKEVGPLTDLDTSLFDKIKKFHRLVFENLIEL 770
LvDcr2 TRKRRRVHAKDLCYPFSNEHGGSYCLYSIDIKPFGQSGESVTIDSSTCISKIGFICKEGLVSSPFPLFFSKWGDVMVNVREVGPLTDLDTSLFDKIKKFHRLVFENLIEL 770
MjDcr2 TRKRRRVHAKDLCYPFSNEHGGSYCLYSIDIKPFGQSGESVTIDSSTCISKIGFICKEGLVSSPFPLFFSKWGDVMVNVREVGPLTDLDTSLFDKIKKFHRLVFENIIEL 770
       ****:*::**:******* *  *******:******: :****** ***.**********:.*****************:*************************:***

PmDcr2 KENLINFDPEESKWDVFVVPLDFSEIIDLDLLNEIYSLDSIKPVMMRSHNQSTKFVRSKFEDGLIYPLYGIPSDQMYYVSQIFDELNPCNTFPEGKKGYESYERYFLERY 880
LvDcr2 NENLMSFDPEKSKWDVFIVPLDFSEIIDLNMLNEIYSFDSIKPVMLKSHNQNTKFVRSKFEDALIYPQYGIPSNQMYYVSHIFDELNPCNTFPEGKKGYESYERYFLERY 880
MjDcr2 NENLMSFDPEKSKWDVFIVPLDFSEIIDLNMLNEIYSFDSIKPVMIKSHNQNTKFVRSKFEDALIYPQYGIPSNQMYYVSHIFDELNPCNTFPEGLKGYESYERYFLERY 880
       :***:.****:******:***********::******:*******::****.**********.**** *****:******:************** **************

PmDcr2 GLEIKDKNQPLLEAKHIPKELNYLKRIPKKAKEKKGNLVPRFVPEFCGIIPIRASLWWQIMCMPSIFHRLNSLNLMHEFIMETKITSPAVKNKVLDFSWTRKFIHELLGS 990
LvDcr2 GLEIKDKNQPLLEAKHIPKELNYLKKIPKKAKEKKGNLVPRFVPEFCGLIPIKASLWWQIMCMPSIFHRLNSFNLMHEFIVKTKITSPAIKNEVLDFSWTKKFIYELLGS 990
MjDcr2 GLELKDKNQPLLEAKHIPKELNYLKKIPKKAREKKGNLVPRFVPEFCGLIPIKASLWWQIMCMPSIFHRLNSFNLMHEFIVKTKITSPAIKNEVLDFSWTKKFIYELLGS 990
       ***:*********************:*****:****************:***:*******************:*******::*******:**:*******:***:*****

PmDcr2 SENAVPIFLDRKKQEYIHPFNMLHAFTLKEASEDKDFNLERLELLGDSFLKFVTGEALFLRDESYHEGRLSQSRGKLVCNKTLFRLACQRGLEGKIAAKVFNPKRNGHIP 1100
LvDcr2 SENTVPIFLDRKKQEYIHPFSMLHAFTLKEASEDKEFNLERLELLGDSFLKFVTGEALFLRDESYHEGRLSQSRGKLVCNKTLFRLACQKGLEGKIAAKVLNPKKNGSIP 1100
MjDcr2 SENTVPRFLDRKKQEYIHPFSMLHAFTLKEASEDKEFNLERLELLGDSFLKFVTGEALFLRDESYHEGRLSQSRGKRVCNKTLFRLACQKGLEGKIAAKVLNPKKNGSIP 1100
       ***:** *************.**************:************************************* ************:**********:***:** **

PmDcr2 GFIVKAEFEESLRSHEVPHDIWHQVPNTVKNVGDLKEAVYDLMSDDASKDDKAEKKGSCYNPWTEHEVSDKSIADCVEAIIGAYLLVCGNEAATSFLDWLGVGVFKDNSL 1210
LvDcr2 GFRIKPEFEESLRSLEVPHDFWHQVPNTVKDVGDLKEAVFALMSDDASKDEKAEKKGSCYNPWTEHEVSDKSIADCVEAIIGAYLLVCGNEAAAAFLHWLGVGVFRDNSL 1210
MjDcr2 GFRIKPEFEESLRSLEVPHDFWHQVPNTVKDVGDLKEAVFALMSDDASKDEKAEKKGSCYNPWTEHEVSDKSIADCVEAIIGAYLRVCGNEAAAAFLHWLGVGVFRDNSL 1210
       ** :* ******** *****:*********:********: *********:********************************** *******::**.*******:****

PmDcr2 LMEPKTIRTAYVCKEHAQDLNIFYKKAVLDRLEKKIGYTFRDRSFIVQAVTHSSYVNNQVTDCYQRLEFLGDAVLDYLVTCHIFCRFTDYSPGQLSDLRSYFVKNDTLGL 1320
LvDcr2 LMEPKTVRTARVRKECAQDLTIFYKKAVLDRLEKKIGYTFRDRSFIVQAVTHSSYVNNQVTDCYQRLEFLGDAVLDYLVTCHIFCRFTDYSPGQLSDLRSYFVKNDTLGR 1320
MjDcr2 LMEPKTVRTARVRKECAQDLTIFYKKAVLDRLEKKIGYTFRDRSFIVQAVTHSSYVNNQVTDCYQRLEFLGDAVLDYLVTCHIFCRFTDYSPGQLSDLRSYFVKNDTLGR 1320
       ******:*** * ** ****.*****************************************************************************************

PmDcr2 AAAKGKLHQHLLHLNPKLEASIDKFLSILSQDPLGEELNTEDDVQDCDDVDIPKALGDMVEAIIGAVYLDSNRSLKVAWQVIERILGDLLNTPIQAIQLNPVRQLLEEVP 1430
LvDcr2 AAAKGKLHQHLLHLNPKLEASIDKFLSILHQDPLGEELNTEDDVQDCEDVEIPKALGDMVEAIIGAVYLDSNRSLKVAWQVIERILGDLLNTSIQTIQLNPIRQLLEEVP 1430
MjDcr2 AAAKGKLHQHLLHLNPKLEASIDKFLSILHQDPLGEELNTEDDVQDCEDVEIPKALGDMVEAIIGAVYLDSNRSLKVAWQVIERILEDLLNTSIQTIQLNPIRQLLEEVP 1430
       *************:*************** *****************:**:********************************** ***** **:*****:********

PmDcr2 GEKQVEFVKGQDDESGKSSYTVTVRGFGTFTTSGKNYRVAKKKVAAIALKEIYKLQAERAISQLPQLPKSPV-- 1502
LvDcr2 GEKQVEFVKGEDDGSEKSTYVVKVKGFGTFTASGKNYRVAKKKVAAIALKEIHNQQ--AAISQLPQLPNPPSSF 1502
MjDcr2 GEKQVEFVKGEDDGSEKSTYVVKVKGFGTFTASGKNYRVAKKKVAAIALKEIHNQQ--AAISQLPQLPNPPSSF 1502
       **********:** * **:*.*.*:******:********************:: *   *********: *
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- DEXDc (N-terminal helicase domain)
- HELICc (C-terminal helicase domain)
- DUF283 (domain of unknown function 283)
- DSRM (dsRNA-binding motif)
- PAZ (Piwi-Argonaute-Zwille domain)
- RIBOc-a (bidentate ribonuclease III domain)
- RIBOc-b (bidentate ribonuclease III domain)

Figure 4. Clustal Omega multiple sequence alignments of the Dicer-2 proteins of *M. japonicus* (MjDcr2, AFB82635), *L. vannamei* (LvDcr2, AEB54796) and *P. monodon* (PmDcr2, AGL08684). PmDcr2 was 91% identical to LvDcr2 and MjDcr2. LvDcr2 and MjDcr2 were 98% identical.

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Lv Argo1   MYPVGQPPGPPGPPGPSGPGGPPGPAGPPVPRPLNLPPGPTPVPGPITTIVPQAPGTPAVATGTGMTALLPPELP---TF   77
Pm Argo1   MYPVGQPPGPPGPPGPSGPGGPPGPAGPPVPRPLTLPPGPTPVPGPITTIVPQAPGTPAVATGTGMTALLPPELPNTPAF   80
Mj Atgo1   MYPVGQPPGPPGPPGPSGPGGPPGPAGPPVPRPLTLPPGPTPVPGPITTIVPQAPGTPAVATGTGMTALLPPELPNTPAF   80
           **********************************.*************************************   :*

Lv Argo1   VAPRRPNLGREGRPITLRANHFQISMPRGYIHHYDISITPDKCPRKVNREIIETMVHAFPRIFGTLKPVFDGRSNLYTRD  157
Pm Argo1   VAPRRPNLGREGRPITLRANHFQISMPRGYIHHYDISITPDKCPRKVNREIIETMVHAFPRIFGTLKPVFDGRSNLYTRD  160
Mj Argo1   VAPRRPNLGREGRPITLRANHFQISMPRGYIHHYDISITPDKCPRKVNREIIETMVHAFPRIFGTLKPVFDGRSNLYTRD  160
           ******************************************************************************

Lv Argo1   PLPIGNEKMELEVTLPGEGRDRVFKVAMKWLAQVNLYTLEEALEGRTRTIPYDAIQALDVVMRHLPSMTYTPVGRSFFSA  237
Pm Argo1   PLPIGNEKMELEVTLPGEGRDRVFKVAMKWLAQVNLYTLEEALEGRTRTIPYDAIQALDVVMRHLPSMTYTPVGRSFFSA  240
Mj Argo1   PLPIGNEKMELEVTLPGEGRDRVFKVAMKWLAQVNLYTLEEALEGRTRTIPYDAIQALDVVMRHLPSMTYTPVGRSFFSA  240
           ******************************************************************************

Lv Argo1   PDGYYHPLGGGREVWFGFHQSVRPSQWKMMLNIDVSATAFYKAQAVIEFMCEVLDIREIGEQRKPLTDSQRVKFTKEIKG  317
Pm Argo1   PDGYYHPLGGGREVWFGFHQSVRPSQWKMMLNIDVSATAFYKAQAVIEFMCEVLDIREIGEQRKPLTDSQRVKFTKEIKG  320
Mj Argo1   PDGYYHPLGGGREVWFGFHQSVRPSQWKMMLNIDVSATAFYKAQAVIEFMCEVLDIREIGEQRKPLTDSQRVKFTKEIKG  320
           ******************************************************************************

Lv Argo1   LKIEITHCGAMRRKYRVCNVTRRPAQMQSFPLQLENGQTVECTVAKYFLDKYKMKLRFPHLPCLQVGQEHKHTYLPLEVC  397
Pm Argo1   LKIEITHCGAMRRKYRVCNVTRRPAQMQSFPLQLENGQTVECTVAKYFLDKYKMKLRFPHLPCLQVGQEHKHTYLPLEVC  400
Mj Argo1   LKIEITHCGAMRRKYRVCNVTRRPAQMQSFPLQLENGQTVECTVAKYFLDKYKMKLRFPHLPCLQVGQEHKHTYLPLEVC  400
           ******************************************************************************

Lv Argo1   NIVPGQRCIKKLTDMQTSTMIKATARSAPDREREINNLVRKADFNNDPYMQEFGLTISTAMMEVRGRVLPPPKLQYGGRT  477
Pm Argo1   NIVPGQRCIKKLTDMQTSTMIKATARSAPDREREINNLVRKADFNNDPYMQEFGLTISTAMMEVRGRVLPPPKLQYGGRT  480
Mj Argo1   NIVPGQRCIKKLTDMQTSTMIKATARSAPDREREINNLVRKADFNNDPYMQEFGLTISTAMMEVRGRVLPPPKLQYGGRT  480
           ******************************************************************************

Lv Argo1   KQQALPNQGVWDMRGKQFFTGVEIRVWAVACFAPQRTVREDALRNFTQQLQKISNDAGMPIIGQPCFCKYANGPDQVEPM  557
Pm Argo1   KQQALPNQGVWDMRGKQFFTGVEIRVWAVACFAPQRTVREDALRNFTQQLQKISNDAGMPIIGQPCFCKYANGPDQVEPM  560
Mj Argo1   KQQALPNQGVWDMRGKQFFTGVEVRVWAVACFAPQRTVREDALRNFTQQLQKISNDAGMPIIGQPCFCKYANGPDQVEPM  560
           ************************:*************************************************

Lv Argo1   FRYLKSTFTGLQLVCVVLPGKTPVYAEVKRVGDTVLGMATQCVQAKNVNKTSPQTLSNLCLKINVKLGGINSILVPGIRP  637
Pm Argo1   FRYLKSTFTGLQLVCVVLPGKTPVYAEVKRVGDTVLGMATQCVQAKNVNKTSPQTLSNLCLKINVKLGGINSILVPGIRP  640
Mj Argo1   FRYLKSTFTGLQLVCVVLPGKTPVYAEVKRVGDTVLGMATQCVQAKNVNKTSPQTLSNLCLKINVKLGGINSILVPGIRP  640
           ******************************************************************************

Lv Argo1   KVFNEPVIFLGADVTHPPAGDNKKPSIAAVVGSMDAHPSRYAATVRVQQHRQNGSTTQGQSASDGSRPRQLTFARTAHDE  717
Pm Argo1   KVFNEPVIFLGADVTHPPAGDNKKPSIAAVVGSMDAHPSRYAATVRVQQHRQNGSTTQGQSASDGSRPRQLTFARTAHDE  720
Mj Argo1   KVFNEPVIFLGADVTHPPAGDNKKPSIAAVVGSMDAHPSRYAATVRVQQHRQNGSTTQGQSASDGSRPRQLTFARTAHDE  720
           ******************************************************************************

Lv Argo1   VIQELSSMVKELLIQFYKSTRFKPNRIILYRDGVSEGQFQTVLQHELTAMREACIKLEADYKPGITYIAVQKRHHTRLFC  797
Pm Argo1   VIQELSSMVKELLIQFYKSTRFKPNRIILYRDGVSEGQFQTVLQHELTAMREACIKLEADYKPGITYIAVQKRHHTRLFC  800
Mj Argo1   VIQELSSMVKELLIQFYKSTRFKPNRIILYRDGVSEGQFQTVLQHELTAMREACIKLEADYKPGITYIAVQKRHHTRLFC  800
           ******************************************************************************

Lv Argo1   SDKKEQSGKSGNIPAGTTVDVGITHPTEFDFYLCSHQGIQGTSRPSHYHVLWDDNHFDSDELQCLTYQLCHTYVRCTRSV  877
Pm Argo1   SDKKEQSGKSGNIPAGTTVDVGITHPTEFDFYLCSHQGIQGTSRPSHYHVLWDDNHFDSDELQCLTYQLCHTYVRCTRSV  880
Mj Argo1   SDKKEQSGKSGNIPAGTTVDVGITHPTEFDFYLCSHQGIQGTSRPSHYHVLWDDNHFDSDELQCLTYQLCHTYVRCTRSV  880
           ******************************************************************************

Lv Argo1   SIPAPAYYAHLVAFRARYHLVEKEHDSGEGSHQSGNSEDRTPSAMARAVTVHVDTNRVMYFA  939
Pm Argo1   SIPAPAYYAHLVAFRARYHLVEKEHDSGEGSHQSGNSEDRTPSAMARAVTVHVDTNRVMYFA  942
Mj Argo1   SIPAPAYYAHLVAFRARYHLVEKEHDSGEGSHQSGNSEDRTPSAMARAVTVHVDTNRVMYFA  942
         ***********************************************************
```

PAZ domain
Piwi domain

Figure 5. Clustal Omega multiple sequence alignments of the Argonaute 1 proteins of *L. vannamei* (Lv Argo, ADK25180.1), *P. monodon* (Pm Argo 1, `ABG66640.1`) and *M. japonicus* (Mj Argo1 ADB44074.1).

A)

```
Lv Argo1 VIEFMCEVLDIREIGEQRKPLTDSQRVKFTKEIKGLKIEITHCGAMRRKYRVCNVTRRPAQMQSFPLQLENGQTVECTVA
Lv Argo2 VLDFMKETLDFREFDF-HDTLEDIVRLKLEKNLKGMKVKVTHSPV-NRTYKIIRVMNAGARDQEFEM--EPG--KFITVE
         *::** *.**:**:   :. * *  *:*: *::**:*:::**. . .*.*:: .* .  *: *.* :  * *     **

Lv Argo1 KYFLDKYK-MKLRFPHLPCLQVGQEHKHTYLPLEVCNIVPGQ
Lv Argo2 KYFKDTYPRTKLQYPYLNVIRAAPETRTIYLPIECCRIQKGQ
         *** *.*   **::*:*  ::.. * :  ***:* *.*  **
```

B)

```
Lv Argo1 VCVVL-PGKTPVYAEVKRVGDTVLGMATQCVQAKNVNKTSPQTLSNLCLKINVKLGGINSILVPGIRPKVFNEPVIFLGA
Lv Argo2 ILVNLPSKKGDKYGRVKKMGDREFSVVTQCILSKTLKNPKPATVNNVLLKINGKMGGVNNTLGRESSTFILTSPVMIMGA
         : * *   *   *..**::**  :.:.***: :*.::: .* *:.*: **** *:**:*. *       ::..**::**

Lv Argo1 DVTHPPAGDNK-KPSIAAVVGSMDAHPSRYAATVRVQQHRQNGSTTQGQSASDGSRPRQLTFARTAHDEVIQELSSMVKE
Lv Argo2 DVNHPPADDRKGTPSLAAVVGSMDCFASNYAAQVRQQI---------------------------SCKEIIQDLKEMTRN
         **.**** *.* .**:********.. *.*** ** *                            : .*:**:*..*.::

Lv Argo1 LLIQFYKSTRFKPNRIILYRDGVSEGQFQTVLQHELTAMREACIKLEADYKPGITYIAVQKRHHTRLFCSDKKEQSGKSG
Lv Argo2 LLIAFFRKTGKKPERLIMFRDGVSESQFYTVLGYELKAMREACKSLQQDYKPGMTFIVVQKRHHTRLFCDDKD-GIGRSK
         *** *::.*  **:*:*::******.** *** :**.****** .*: *****:*:*.***********.**.   *:*

Lv Argo1 NIPAGTTVDVGITHPTEFDFYLCSHQGIQGTSRPSHYHVLWDDNHFDSDELQCLTYQLCHTYVRCTRSVSIPAPAYYAHL
Lv Argo2 NVPPGTIVDQIITHPSEIDFYLCSHQGILGTSKPTHYRVLWDDNDMTMDQLQSMSYAMCHTYSRCTRSVSIPAPAYYAHL
         *:* ** **  ****:*:********** ***:*:**:******.:  *:**.::* :**** *****************

Lv Argo1 VAFRARYHLVEK
Lv Argo2 AAYRAKVH----
         .*:**: *
```

Figure 6. Clustal Omega sequence alignments for PAZ domain (A) and Piwi domain (B) of the Argonaute 1 and Argonaute 2 proteins from *L. vannamei* (Lv Argo 1, ADK25180.1 and Lv Argo 2, ADK25181.1).

3.4 TRBP and eIF6

As mentioned before, transactivating response RNA-binding protein (TRBP) serves as a bridge for the interaction between Dicer and the dsRNA within the RISC complex. TRBPs orthologues have been found in the Chinese white shrimp *Fenneropenaeus chinensis* and in *M. japonicus* and in both cases they were proved to physically interact with the eukaryotic initiation factor 6 (eIF6), also a RISC component, which is a ribosome inhibitory protein that prevents productive assembly of the 80S ribosome, therefore

involved in miRNA translational repression (Chendrimada et al., 2007). When either Mj-TRBP or Mj-eIF6 were knocked down via dsRNA injection, the mRNA levels of the ds-RNA-targeted Mj-prophenoloxidase remained high, indicating that both Mj-TRBP and Mj-eIF6 were essential for eliciting RNA silencing (Wang et al., 2012; Wang et al., 2009). LvTRBP was shown to interact with its RISC partners LVDcr2 and LvAgo2 (Chen et al., 2011). *P. monodon* TRBP (PmTRBP) is significantly regulated by bacterial and viral injected challenges (Yang et al., 2013). Besides the crustacean TRBPs functionally or genetically characterized, a BLAST search also identifies several other crustacean sequences, namely for the sea lice *Caligus rogercresseyi* and *Caligus clemensi* and the salmon louse *Lepeophtheirus salmonis*. All crustacean TRBPs here mentioned possess the three double-stranded motifs that are the signature of the TRBP family.

4. Conclusions

Significant efforts have been directed towards the elucidation of the RNAi machinery in crustaceans, mainly aiming to improve the suggested RNAi-based methods for disease control and to unveil gene function in basic research. Yet, unraveled matters in this vast group of arthropods include the investigation of ds-RNA-mediated gene super-induction of Dicer-2, as it has been demonstrated for the cockroach *Blattella germanica* (Lozano et al., 2012).

It is clear that much work still remains to be done. In addition to the genetic approaches to characterize the components of the RNAi machinery, more biochemical evidences need to be generated to validate the function and contribution of each element to the silencing mechanism. The new focus of the RNA world appears to be the microRNAs biosynthesis, their wide-range of mechanisms to exert silencing and their intricate and overlapping functions. The next years will undoubtedly see a continued use of the progressively improved genetic and therapeutic toolbox of RNAi silencing.

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