Characterization of *hsp* genes in planarian stem cells

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ABSTRACT. Planarians are a model system known for regenerative potential, body plasticity and continuous turnover of all differentiated cell types. These characteristics are based on the presence of pluripotent stem cells, called neoblasts. Damage or reduction in the number of neoblasts deeply affects planarian regeneration and survival. Heat shock proteins (HSPs) are known to perform essential cytoprotective functions in all organisms. To investigate the potential role of *hsp*-related genes on the dynamics of planarian stem cells, representative *hsp*-related genes were identified and characterized in normal conditions and after different stress stimuli. Our work revealed that two different *hsp* genes (*Djhsp60* and *Djmot*) are constitutively expressed in neoblasts, suggesting that their products play important roles in cytoprotection of these cells. RNAi-based functional studies provide evidence of an involvement of *Djhsp60* and *Djmot* in the adaptive response of planarian stem cells to stress and indicate that expression of these genes is critical for planarian survival.

KEY WORDS: *Dugesia japonica*, neoblasts, regeneration, heat shock genes, RNAi

INTRODUCTION

Stem cells are crucial to homeostasis and regeneration of all metazoans (Rando, 2006; Pellettieri & Sánchez Alvarado, 2007). High stress tolerance concomitant to an increased expression of Heat Shock Proteins (HSPs) in stem cells has recently attracted attention, suggesting that HSP members are fundamental to modulate behavior, prevent senescence and prolong proliferative capacity of these cells (reviewed in Prinsloo et al., 2009). HSPs can be detected in all organisms and form the most ancient defence system. The heat shock response was first identified in the salivary glands of *Drosophila melanogaster* upon application of a transient exposure to elevated temperatures (Ritossa, 1962). Later, more functions involving HSPs were uncovered, indicating HSPs as the major regulatory proteins in the cell (Sreedhar & Csermely, 2004). HSPs are functionally related proteins classified into families according to molecular weight. Within each class there are members that are constitutively expressed or finely regulated, and/or specific of different compartments (Prinsloo et al., 2009). Although HSPs are highly expressed in stressed cells and could be considered only as cell stress "buffers", they are also involved in gene expression regulation, DNA replication, signal transduction, differentiation or immortalization (Jolly & Morimoto, 2000). Various studies demonstrate that HSP-induced cytoprotection promotes cell survival by an important contribution to the signals directing the cell to senescence, apoptosis, or necrosis. These proteins can be also involved in immune response stimulation (Sõti et al., 2003; Sreedhar & Csermely, 2004).

Planarian flatworms - well known for regenerative potential and body plasticity - represent a unique model system to study adult stem cells. Planarian stem cells, named neoblasts, are pluripotent cells continuously recruited to replace aged differentiated cells, and allow regeneration in these organisms (recently reviewed by Pellettieri & Sánchez Alvarado, 2007; Handberg-Thorsager et al., 2008; Rossi et al., 2008). The long lifespan of planarians, coupled with the possibility to analyze *in vivo* the behavior of their stem cells during tissue homeostasis and regeneration, provides a unique opportunity for understanding how stem cells respond to stress in more complex metazoans, including humans. Damage or reduction in number of neoblasts deeply affects planarian regeneration and survival. To investigate the potential role of HSP-related genes on the dynamics of neoblasts, we characterized representative HSP-related genes in normal conditions and after different stress stimuli and identified two neoblast-specific HSP members, *Djhsp60* and *Djmot*, belonging to the HSP60 and HSP70 gene families, respectively (Rossi et al., 2007; Conte et al., 2009). Both these genes are involved in the adaptive response of planarian stem cells to stress conditions. *Djhsp60* or *Djmot* RNAi-mediated functional ablation causes growth arrest in neoblasts. The possibility that DjMot plays an essential role in a conserved mechanism of cytoplasmic sequestration of p53, thus antagonizing its nuclear entry, is discussed.

MATERIALS AND METHODS

Asexual specimens of *D. japonica* (GI strain) were maintained at 18°C in autoclaved stream water, fed weekly with chicken liver and used for experiments after 10 days of starvation. Thirty days-starved planarians were used for starvation analysis. Regenerating fragments were produced by transverse amputation. Some intact worms were exposed to a lethal dose (30Gy) of X-rays as described by Conte et al. (2009). For heat shock treatment, intact planarians were

maintained o/n at 28°C before harvesting for RNA extraction. In situ hybridization and RNAi were performed as described by Conte et al. (2009). Total RNA for real time reverse transcription (RT)-PCR experiments was extracted from three planarians, in triplicate, with the NucleoSpin RNAII kit (Macherey and Nagel). Each extraction was tested for the absence of genomic DNA by amplifications performed in the absence of reverse transcriptase. Superscript First Strand Synthesis System kit (Invitrogen) was used for cDNA synthesis. SYBR Green chemistry-based reactions were carried out as described by Conte et al. (2009) with a Rotor-Gene 6000 (Corbett Research). The mRNA levels of specific genes were compared with controls using planarian *elongation factor 2 (Djef2)* as reference gene to normalize RNA input (Rossi et al., 2007). The primer sets used in the experiments, generated using NetPrimer software, were as follows:

Djhsp60 forward primer:
5'TATTGTCGCATCGTTGAAAGC3';
Djhsp60 reverse primer:
5'CCAATTCATCATGTAATGTTTT3';
Djmot forward primer:
5'GCATTCCACCAGCACCTC3';
Djmot reverse primer:
5'CATATTTTCAATTTCATCTTTACTCAA3'.
Djef2 forward primer:
5'CAATCGAAGACGTTCCATGTG3'
Djef2 reverse primer:
5'AACACGAACAACAGGACTAAC3'

RESULTS

Based on the analysis of a *Dugesia japonica* EST collection (Mineta et al., 2003) we identified some cDNAs coding for HSP members with different molecular mass. In particular, several cDNA fragments coding for HSP70-related proteins were found. In order to distinguish more precisely the number of HSP*70*-related genes in *D. japonica*, several couples of primers were designed to amplify cDNA regions included between the different gene fragments (data not shown). RT-PCR analysis demonstrated that at least three HSP*70*-related genes exist in planarians. Two of these genes code for very similar constitutive HSP70 forms (HSC70), while the third gene encodes a peculiar HSP70 member showing high similarity with mammalian Mortalin (*Djmot*: Conte et al., 2009). One of the two HSC70 appeared to be identical at the nucleotide level to *Djhsc70* (accession number ABY83101). In situ hybridization experiments revealed that the HSC70 genes were ubiquitously expressed (not shown), while *Djhsp60* (EST 32903936: Mineta et al., 2003) and *Djmot* had a pattern similar to that shown by other neoblast-specific genes (for example, *Djmcm2*: Salvetti et al., 2000) (Fig.1 A-F). Both *Djhsp60* and *Djmot* parenchymal expression was strongly downregulated in animals exposed to a lethal dose of X-ray (30Gy) (a treatment that destroys neoblasts: Hayashi et al., 2006; Rossi et al., 2007; 2008; Conte et al., 2009) while other *hsp* genes were not affected (not shown). Different stress conditions, such as heat shock (28°C o/n) or a long period of starvation, strongly activated *Djhsp60* transcription (Fig.2 A,B), while *Djmot* upregulation was only observed after prolonged starvation (Conte et al., 2009).

Expression profile of *Djhsp60* and *Djmot* during regeneration was analyzed by in situ hybridization. Regenerating fragments showed strong upregulation of *Djhsp60* transcripts in the blastema, the postmitotic area where neoblast progeny undergo differentiation, and in the stump region beneath the blastema (postblastema), comprised of actively proliferating neoblasts. This accumulation, clearly detected by 2 days after amputation, declined from the regenerating area as regeneration proceeded (Fig.3 A-H).
Djmot transcription appeared upregulated in the postblastema, and a certain level of hybridization signal could be detected also in the blastemal tissue by 2 days after amputation. However, after 4 days, *Djmot* expression was completely absent from the blastema and declined in the postblastema to the normal levels observed in intact animals (Fig. 3 I,J). These results suggest that *Djmot* transcripts are present in proliferating neoblasts, and residual expression may transiently remain in early postmitotic progenitors (Conte et al., 2009).

To assess whether *Djhsp60* and *Djmot* play a role in neoblasts, we performed sequence-specific gene silencing by double strand(ds)RNA-mediated interference. Similarly to that observed after *Djmot* RNAi (Conte et al., 2009), functional inhibition of *Djhsp60* caused tissue regression in intact planarians, possibly indicative of homeostatic defects (Fig. 4 A,B). However, only 15% (23/150) among the *Djhsp60* dsRNA-injected specimens showed a morphologically detectable phenotype, in spite of the consistent reduction of the level of *Djhsp60* endogenous transcripts (not shown).

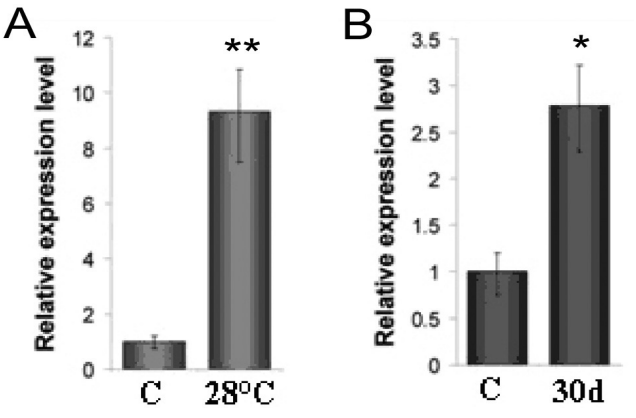

Fig. 2. – ***Djhsp60* expression level in different stress conditions, visualized by real time reverse transcription (RT)-PCR.** (A) heat shock (28°C) (B) 30 days of starvation. d: days of starvation. Expression levels are indicated in relative units, assuming the value of the untreated specimens (C: control) as unitary. Each value is the mean±s.d. of three independent samples, analyzed in triplicate. Samples were compared using the un-paired t-test.$^{**}P<0.001$, $^{*}P<0.05$.

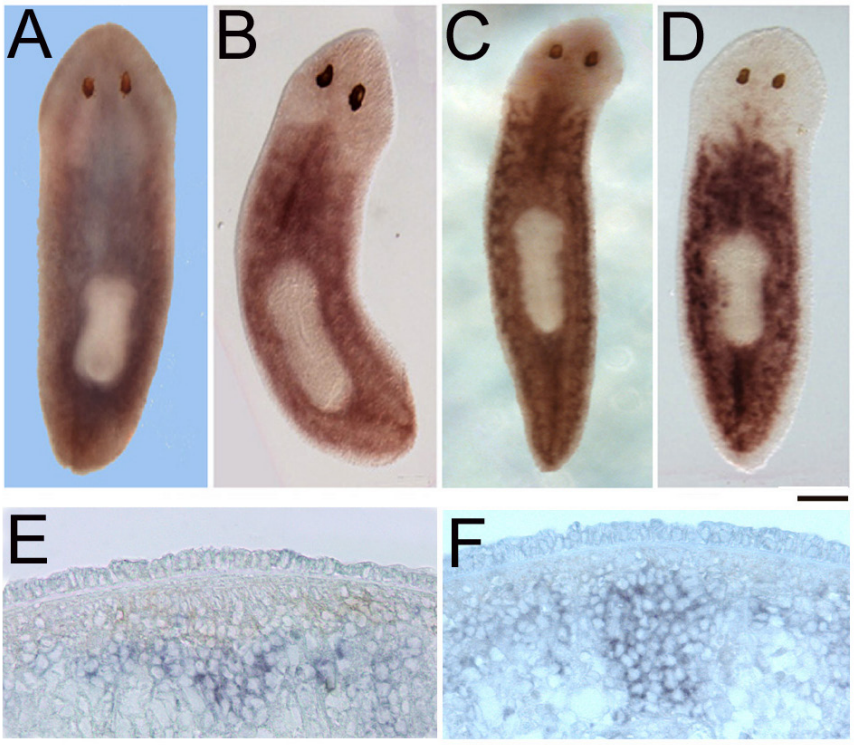

Fig. 1. – **Expression pattern of *hsp* genes in *D. japonica*.** (A-D) Whole mount in situ hybridization on intact planarians. (A) *Djhsc70*; (B) *Djhsp60*; (C) *Djmot*; (D) *Djmcm2* is shown for comparison. (E,F) In situ hybridization on transverse sections, visualized by NBT/BCIP chromogen precipitation in small neoblast-like cells of the dorsal midline region at the prepharyngeal level. (E) *Djhsp60*. (F) *Djmot*. Scale bars: 500 µm in A-D, 100 µm in E,F.

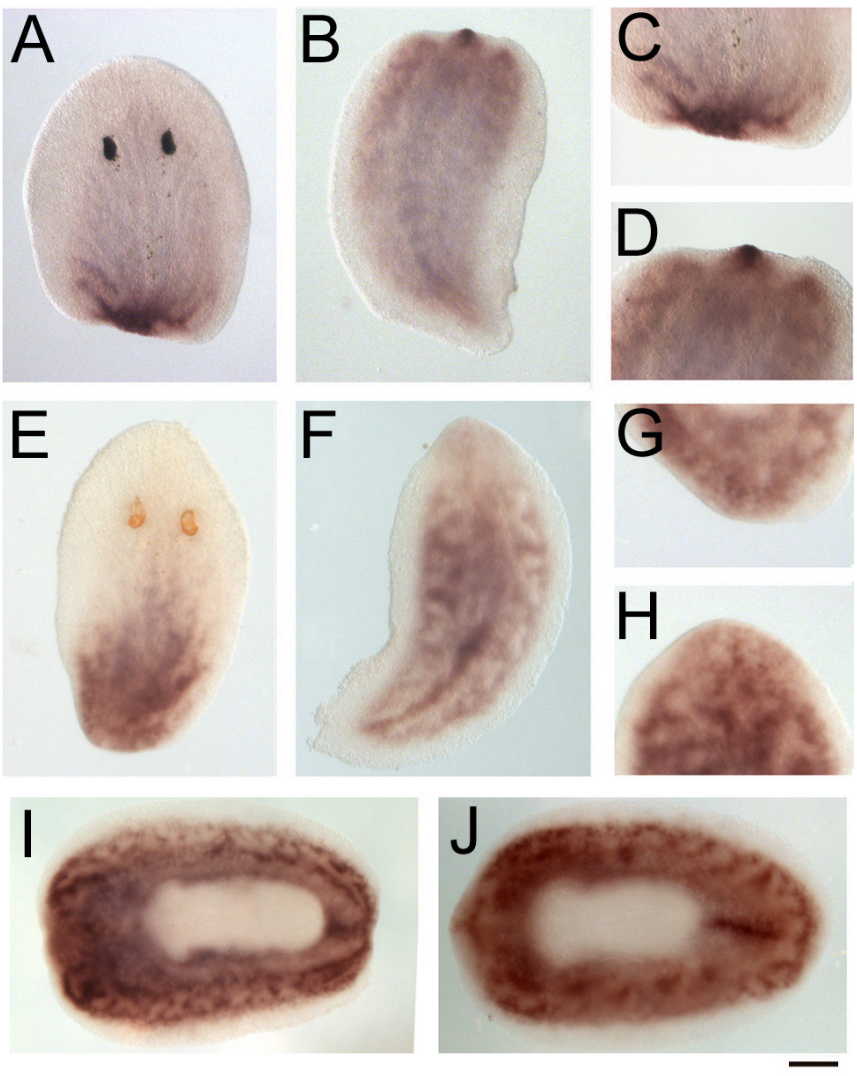

Fig. 3. – **Expression pattern of *Djhsp60* and *Djmot* during regeneration, visualized by whole mount in situ hybridization.** (A-H) *Djhsp60*. (A) posterior and (B) anterior regeneration, 2 days of regeneration. (C) Enlarged view of the regenerating area depicted in A. (D) Enlarged view of the regenerating area depicted in B. (E) posterior and (F) anterior regeneration, 4 days of regeneration. (G) Enlarged view of the regenerating area depicted in E. (H) Enlarged view of the regenerating area depicted in F. Anterior is on the top. (I,J) *Djmot*. (I) A trunk fragment regenerating both a head and a tail, 2 days of regeneration. (J) A trunk fragment regenerating both a head and a tail, 4 days of regeneration. Anterior is on the left. Scale bars: 50 µm in A, B, E, F, I, J; 100 µm in C, D, G, H.

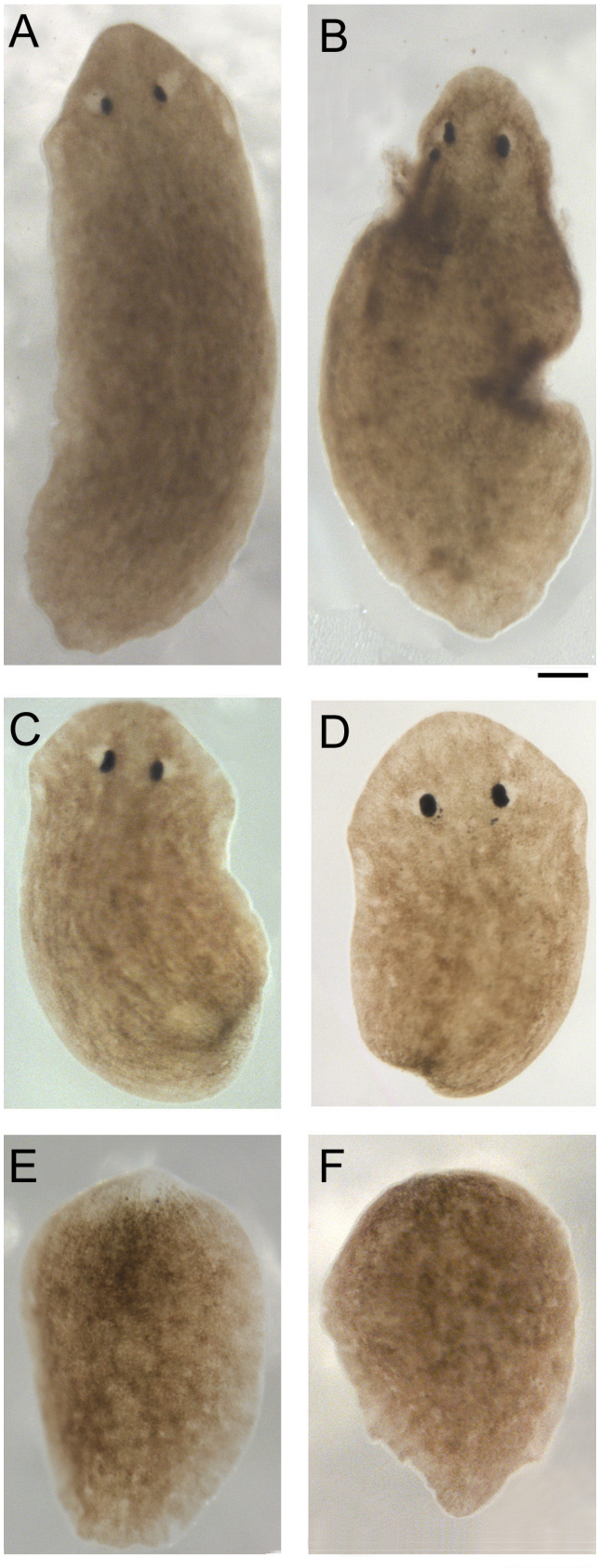

Fig. 4. – **Analysis of *Djhsp60*RNAi phenotypes.** (A,B) Bright-field images of intact planarians, 20 days after the first injection. (A) A water-injected control. (B) A *Djhsp60*RNAi phenotype. (C-F) Bright-field images of regenerating planarians. (C,D) posterior and (E,F) anterior regeneration, 5 days after amputation. (C) A water-injected control. (D) A *Djhsp60*RNAi phenotype unable to regenerate. (E) A water-injected control. (F) A *Djhsp60*RNAi phenotype unable to regenerate. Anterior is on the top. Scale bars: 300 μm.

When amputated, only a limited number of injected fragments did not form a regeneration blastema (20%, 20/100, from three independent experiments) (Fig. 4 C-F). The majority of *Djhsp60* RNAi specimens were able to regenerate, although some of them exhibited a smaller blastema.

Conversely, *Djmot* RNAi strongly inhibited blastema formation and most of the injected fragments completely failed to regenerate. In intact planarians *Djmot* knockdown did not produce any gross morphological defect. All injected specimens (both intact animals and amputated fragments), however, started to die in 4-5 weeks after the first injection, while the survival of controls was not affected (Conte et al., 2009). As it has been demonstrated that HSP60 may be functionally associated with mitochondrial HSP70 (mtHSP70/Mortalin) (Deocaris et al., 2006; Wadhwa et al., 2005) we also coinjected an equimolar mixture of *Djmot* and *Djhsp60* dsRNA. The results did not show any significant variation either in the type or the number of phenotypes (not shown), suggesting that the activity of these two genes in neoblasts involves different mechanisms. In addition, we observed that the levels of *Djhsp60* transcripts were not affected by *Djmot* RNAi (not shown).

DISCUSSION

Djhsp60

Djhsp60 is expressed in the parenchyma in small neoblast-like cells that are specifically eliminated by X-ray irradiation. This finding confirms the data obtained by Rossi and coworkers who found selective *Djhsp60* down-regulation after X-ray treatment (Rossi et al., 2007). However, in this work we have observed that a *DjHSP60*-mediated stress response is specifically activated in postmitotic progenitors during regeneration. *Djhsp60* expression was in fact strongly upregulated in the early blastema, a region devoid of mitotic activity. The loss of body parts probably subjects the remaining cells to a number of stresses, and HSP60 is a chaperone involved in the appropriate folding and assembly of polypeptides into protein complexes. Literature data demonstrate that wounding can induce specific HSP60 response, indicating that appropriate stress response processes may be correlated with regenerative success also in other organisms (Laplante et al., 1998; Makino et al., 2005). Recent studies during limb regeneration in newts reveal that high apoptosis levels are present in the first days after amputation, while a few apoptotic cells are detected one week post-amputation (Vlaskalin et al., 2004). Our data support

the possibility that upregulation of *Djhsp60* transcripts may play a role in preventing programmed cell death in order to protect integrity of the new tissue during the early regeneration events. *Djhsp60* induction after heat shock or starvation further supports the possible cytoprotective role of HSP60 in planarians. *Djhsp60*RNAi experiments also indicated that induction of this gene may be required for appropriate regeneration. However, the overall percentage of abnormal phenotypes observed in *hsp60* RNAi animals was very low, probably due to redundant effects of other *hsp60*-related genes. Although the presence of other *hsp* transcripts may compensate for the loss of *Djhsp60* function, we cannot completely exclude that DjHSP60 plays only an auxiliary role in the regulation mechanisms involved in survival/maintenance of planarian cells, including stem cells (Patterson & Klingenberg, 2007).

Djmot

Djmot is a planarian gene that encodes a protein showing high identity with a heat-uninducible member of the mammalian HSP70 family, identified as Mortalin. In physiological conditions high levels of *Djmot* transcripts are detected in proliferating neoblasts and their descendants, while no detectable expression of this gene can be found in differentiated cells (Conte et al., 2009). Literature data demonstrate that Mortalin-like proteins are essential for cell viability in different organisms, such as yeast, *C. elegans* and mammals (Kimura et al., 2007; Wadhwa et al., 2002; Wadhwa et al., 2005). The phenotypes associated with *Djmot* RNAi also indicate an involvement of this gene in maintaining neoblast viability (Conte et al., 2009). Recent work demonstrates that Mortalin-like proteins may sequester the tumor suppressor protein p53 in the cytoplasm, preventing senescence and apoptosis, and thus promoting lifespan and immortalization of cells in different organisms (Sherman et al., 2007; Wadhwa et al., 2002; Walker et al., 2006). The molecular events that allow neoblasts to escape cellular senescence remain undefined so far. Consistent with the possibility that *Djmot* is necessary to prevent growth arrest in dividing stem cells of planarians, a variable number of flattened, enlarged cells with condensed chromatin were detected in dissociated cells of *Djmot*RNAi planarians (Fig. 5 A,B). This type of cell was never observed in control animals and we suggest that these cells could be senescent cells. Unfortunately, no biomarkers for senescent cells are available in planarians. It is well known that two tumor suppressor proteins, p53 and Rb, play a crucial role in the senescence response (Campisi, 2005). We are tempted to speculate that the relationship between *Djmot* functional ablation and the induction of senescent cells could be related to ability of planarian Mortalin to function as a buffer of a planarian p53-like protein, modulating its activity. Functional inhibition of *Djmot* would allow release of p53 into the nucleus and consequent activation of cell senescence programs (Fig. 5 C,D). Apoptosis is widely cited as the primary mode of stem cell deletion during adult tissue homeostasis. However, it is very difficult to investigate how stem cells die in planarians. Recent investigation of cell death in planarians, including TUNEL assay, yielded ambiguous results (Pellettieri & Sánchez Alvarado, 2007). Our preliminary results, obtained by a Comet Assay protocol adapted for planarians (Prá et al., 2005), provided evidence that, after *Djmot* RNAi, about 10-20% nuclear comets could be detected, a percentage that reflects the presumptive number of neoblast-like cells in planarians (Baguñà & Romero, 1981). Further studies, including characterization of *p53* genes, are needed to further elucidate the molecular pathways implicated in growth control of planarian stem cells.

Conclusions

The molecular characterization of the planarian genes *Djhsp60* and *Djmot* reveals a first insight into the complex scenario of the stress response in planarians and, in particular, in the analysis of genes involved in the protection of the pluripotent stem cell system of these organisms. Certainly, *hsp60* induction seems to be a shared requirement for regeneration of body parts in vertebrate and invertebrate organisms. However it is still unknown whether this gene product plays a general role in cellular stress or, under traumatic circumstances, can be released from cells to regulate immune or inflammatory responses (Calderwood et al., 2007). Conversely, a fundamental and conserved role of Mortalin-like proteins appears to be the modulation of p53 activity in immortalized or stem cells, thus preventing senescence and

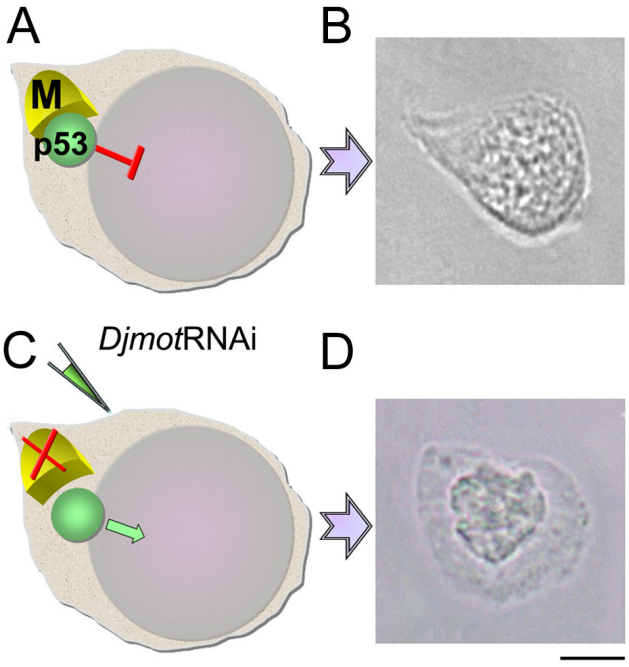

Fig. 5. – **Hypothetical model for DjMot function in neoblasts.** (A) DjMot (M) prevents nuclear translocation of p53–like protein**.** (B) Phase contrast image of a neoblast. (C) *Djmot*RNAi disrupts DjMot-p53 interaction and allows nuclear translocation of p53. (D) Phase contrast image of a senescent cell, as detected after *Dmot*RNAi. Scale bar: 5µm.

prolonging proliferative capacity of these cells (Prinsloo et al., 2009). Further investigation will be essential to identify conserved mechanisms that regulate both stress response in adult stem cells and injury-induced epimorphic regeneration of different organisms.

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