Cell Stress and Chaperones (2011) 16:681–687
DOI 10.1007/s12192-011-0277-3

SHORT COMMUNICATION

Stress response gene activation protects sea urchin embryos exposed to X-rays

Rosa Bonaventura · Francesca Zito · Caterina Costa · Salvatore Giarrusso · Filippo Celi · Valeria Matranga

Received: 13 June 2011 / Accepted: 15 June 2011 / Published online: 1 July 2011

Abstract We used *Paracentrotus lividus* sea urchin embryos, a well-established model in developmental biology and ecotoxicology, for investigation on stress/anti-apoptotic protein expression elicited in response to harmful ionizing radiation, such as X-rays. We evaluated the acute effects of a high-dose exposure (5 Gy) on *P. lividus* analyzing by Western blotting the accumulation levels of HSP60, HSP70, BAG3 and a putative p63 at 24 and 48 h after irradiation. We found an increase in the HSP70, BAG3, and p63 protein levels only 48 h after irradiation, whereas no HSP60 increase was detected either at 24 or 48 h. Levels of the mRNA coding for HSP70 and p63 were also investigated by relative RT-PCR and were found to increase 24 h after irradiation, returning to their initial levels at 48 h. Results demonstrate the presence of an adaptive regulatory mechanism operating at the transcriptional level at 24 h, followed by a translational activation at 48 h post-irradiation. In conclusion, our findings confirm the sea urchin embryo as a sensible bioindicator of cell damage and we propose this model for studies on the protective pathways activated in response to X-rays. The novel result of the involvement of BAG3 and p63 in the response to X-rays, never tested so far in any other embryonic system, opens the way for their use as biomarkers of X-ray hazards.

R. Bonaventura · F. Zito · C. Costa · V. Matranga (✉)
Consiglio Nazionale delle Ricerche, Istituto di Biomedicina e Immunologia Molecolare "Alberto Monroy",
Via Ugo La Malfa 153,
90146 Palermo, Italy
e-mail: matranga@ibim.cnr.it

S. Giarrusso · F. Celi
INAF, Istituto di Astrofisica Spaziale e Fisica Cosmica,
Via Ugo La Malfa 153,
90146 Palermo, Italy

Keywords Echinoderms · Development · Ecotoxicology · HSP70 · BAG3 · p63

Introduction

Sea urchins, like many other organisms living in the water column for part of their lives as embryos and larvae, can be exposed to a variety of biotic and abiotic stresses. The sea urchin embryo is a well-recognized developmental biology model the use of which in eco-, embryo- and genotoxicological studies has been recently appreciated (Aluigi et al. 2008; Bonaventura et al. 2005; Bellas et al. 2008; Byrne et al. 2010; Geraci et al. 2004; Kiyomoto et al. 2010; Romano et al. 2010). Many defense strategies and pathways have evolved to protect these embryos and larvae against adverse environmental conditions, permit optimal development, and guarantee species survival (Goldstone et al. 2006; Hamdoun and Epel 2007). A major concern about physical agents impacting on the marine environment comes from the UV radiation reaching the Earth and penetrating waters (Schroder et al. 2005; Shick et al. 1996). In laboratory experiments, sea urchin embryos have been used to study the response to UV-B radiation by evaluating the effects at the morphological, gene, and protein levels (Bonaventura et al. 2005, 2006; Lesser et al. 2003; Nahon et al. 2009; Russo et al. 2010; Schroder et al. 2005). Other natural ionizing radiations are X-rays, components of radionuclide emissions originating from the space which do not penetrate the atmosphere. However, X-rays can be released from radionuclides directly and/or indirectly into the air, soil, and water, as for example in the case of nuclear accidents which regrettably cause radioactive contamination of the nearby areas, including the marine environment (Aumento et al. 2005; Garcia-

Orellana et al. 2009; IAEA-TECDOC-1429 2005). Although, only a few old studies addressed the sea urchin embryo as a model system to investigate the effects of X-rays (Kimura 1974; Nakamura 1975), the unfortunate occurrence of the recent nuclear accident at Fukushima in Japan in March 2011 renewed the interest for a sentinel species in order to observe the damage caused by dangerous radiation. In a recent previous report, we demonstrated that sea urchin embryos exposed at the cleavage stage (32 cells) to different single doses of X-rays (from 0.1 to 5 Gy) showed a dose-dependent number of embryonic abnormalities (Matranga et al. 2010). When the highest dose was applied, i. e., 5 Gy corresponding to 5 Sv, nearly no embryos were able to develop normally, even a long time after exposure (48 h), although no lethal effects were detected. In the same study, we correlated for the first time the morphological abnormalities observed to the downregulation of two skeleton terminal differentiation genes, Pl-SM30 and Pl-msp130 (Matranga et al. 2010). As a matter of fact, the occurrence of bone cancer (osteosarcoma) has been correlated to the exposure to ionizing radiation in humans, following radiotherapy which patients suffering from other cancers, underwent (for a review see Ottaviani and Jaffe 2009).

Previous reports have shown that HSP60 protein levels increase after heat shock or cadmium exposure in *Paracentrotus lividus* embryos (Roccheri et al. 2001, 2004). HSP70, generally used for the assessment of vertebrate cellular health state (Gupta et al. 2010) and tumor occurrence (Romanucci et al. 2008), has been recognized as a valid biomarker of exposure to pollutants and UV-B radiation in embryos, as well as in adult immune cells of the sea urchin (Bonaventura et al. 2005; Geraci et al. 2004; Matranga et al. 2000, 2006, 2010; Pinsino et al. 2008; Roccheri et al. 2004). It is known that the activity of HSP70s is regulated by many types of co-chaperones, including BAG3 (Takayama et al. 1999), which is able to interact with it through a conserved domain. BAG3, not yet identified and characterized in the sea urchin, has been shown to be overexpressed in several cell types subjected to stressful conditions, such as high temperature and heavy metals (Pagliuca et al. 2003). However, to the best of our knowledge, BAG3 has never been correlated to X-ray exposure in any other organism, including humans where radiotherapy is generally used for cancer patients.

Proteins of the p53 gene family have been known for their involvement in DNA damage repair following genotoxic stress, including ionizing radiation (Lavin and Gueven 2006). A p63/p73 common ancestor gene has been found in almost all invertebrates, including the sea urchin (Belyi et al. 2010). It's duplication produced the p53 gene in early vertebrates which has been widely studied, due to its regulatory function in apoptosis, inhibition of cell cycle progression, senescence, differentiation, and DNA repair (Oren 2003). A gene homolog of the p53 family has been annotated in the sea urchin genome (Fernandez-Guerra et al. 2006), although its detailed description is still awaited. Nevertheless, as the expression of the p53 family proteins is sensitive in response to genotoxic conditions, their use as potential biomarkers of X-ray radiation in echinoderms is worth investigating.

In this paper, we studied the stress response of sea urchin embryos exposed to a high single dose of X-rays analyzing the protein levels of: (1) HSP60, the heat shock-inducible mitochondrial chaperonin; (2) HSP70, the acknowledged cellular stress and anti-apoptotic protein; (3) BAG3, an anti-apoptotic multifunctional protein interacting with HSP70; and (4) p63, a member of the p53 protein family involved in many cell functions, including stress response. Levels of the mRNA coding for HSP70 and p63 were also investigated.

Materials and methods

Embryo culture and X-rays irradiation

Sea urchin adults of the species *P. lividus* were collected along the northwestern coast of Sicily. After fertilization, embryos were reared at 16°C in Millipore-filtered seawater supplemented with 30 mg/l penicillin and 50 mg/l streptomycin sulfate, at the dilution of 4,000/ml, until they reached the 32-cell stage, after about 3 h. Embryos were then dispensed in 96-well plates, 1,000 per well in 100 µl. Irradiations were performed, as described in (Matranga et al. 2010). Briefly, X-rays were generated by the Ag K-lines radiation produced by a Seifert air-insulated diffraction X-ray tube. Embryos were irradiated once with 5 Gy, in triplicate, and then transferred to 24-well plates, 1 ml per well, and cultured at 16°C. The same procedure was carried out in parallel for unexposed embryos, which served as controls. Morphological analyses were performed 24 and 48 h after irradiation, according to Bonaventura et al. (2005).

SDS–PAGE and western blot

After 24 and 48 h from irradiation, embryos were harvested, centrifuged at 1,200 rpm for 5 min, and the pellets were lysed at 4°C in RIPA buffer, supplemented with: 2 µg/µl antipain and leupeptin, 1 µg/µl aprotinin and pepstatin, 1 mM benzamidine, and 0.1 mM PMSF. Lysates were centrifuged at 12,000 rpm for 10 min, at 4°C, supernatants collected, and total protein concentrations determined by the Bradford method (Bradford 1976). Samples (20-µg proteins) were re-suspended in the Laemmli sodium dodecyl sulfate polyacrylamide gel elec-

trophoresis (SDS–PAGE) buffer (Laemmli 1970), and denatured at 100°C for 5 min. Proteins were separated by 10% SDS–PAGE minigels, transferred to nitrocellulose membranes (Towbin et al. 1979), and blocked by nonfat dried milk dissolved in 20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1% Tween 20 (TBS-T). Primary antibodies, (diluted in 3% BSA–TBS-T) that we used were: anti-bovine brain 70-kDa heat shock protein mouse monoclonal (Sigma H-5147) 1:1,000; anti-heat shock protein 60 mouse monoclonal (Sigma H3524) 1:500; anti-p53 rabbit polyclonal (Cell Signalling, 9282) 1:1,000; anti-BAG3 rabbit polyclonal TOS-2 1:3000, kindly provided by Tosco A, University of Salerno (Fontanella et al. 2010). Secondary antibodies used were: anti-mouse peroxidase-conjugated (Amersham Pharmacia, NA931) and anti-rabbit peroxidase-conjugated (Cell Signalling, 7074), diluted 1:2,000 in TBS-T. Proteins were detected by chemiluminescence (SuperSignal West Pico, Pierce) according to manufacturer's instructions. Films were scanned and band intensities quantified by the ChemiDoc system (Bio-Rad) equipped with the software Quantity One, version 4.2.1.

RNA preparation and relative RT-PCR analysis

Total RNA was isolated according to the manufacturer's instructions using GenElute Mammalian total RNA kit (Sigma) from manually collected controls (25) and 5-Gy irradiated (25) embryos, frozen in liquid nitrogen and stored at −80°C until use. A single-step reverse transcription-polymerase chain reaction (RT-PCR) was carried out using SuperScript One-Step RT-PCR kit (Invitrogen). Three cDNA fragments were amplified from total RNA having the following size: 180nt–hsp70, 327nt–p63, and 248nt–S24, with the latter used as internal reference (Zito et al. 2003). The primers amplifying hsp70 cDNA fragment were previously described (Bonaventura et al. 2006). *P. lividus* p63 cDNA amplicon was amplified utilizing oligonucleotides designed on the predicted p63 sequence generated by automated computational analysis of the *Strongylocentrotus purpuratus* genome (Sea Urchin Genome Sequencing Consortium 2006). The whole RT-PCR reactions were analyzed on 2% agarose gel. Band intensities for each amplification product were previously quantified by densitometric analysis using the GelDoc1000 imaging detection system (Bio-Rad, Hercules, CA, USA) equipped with a Multi-Analyst image analysis software, version 1.1. The relative expression levels of Pl-hsp70 and Pl-p63 were obtained by normalizing measurements of amplicon band intensities to the values of Pl-S24, a transcript encoding a ribosomal protein which has been assumed constant during development (Zito et al. 2003).

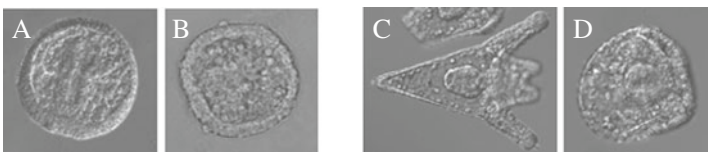
Statistical analysis

Values obtained from the morphological analysis, expressed as percentage of abnormal embryos, were reported as the mean of five independent experiments ± SE. Measurements of band intensities obtained by densitometric analysis for each analyzed protein were reported as the mean of three independent experiments ± SE. Band intensities of amplification products, obtained by densitometric analysis, were

Table 1 Effects of 5-Gy X-rays on embryo development

	24h		48h	
Trial no.	Normal	Abnormal	Normal	Abnormal
1	20.8	79.2	0	100
2	31.9	68.11	7.94	92.06
3	4.44	95.56	5.37	94.62
4	4.63	95.37	2.42	97.58
5	12.26	87.74	9.7	93.3
Mean	14.80	85.19	5.08	95.51
SE	5.22	5.22	1.76	1.44

Percentage of normal and abnormal embryos recorded in 5-Gy irradiated embryos in five independent trials. Representative morphology of normal (**a**, **c**) and abnormal embryos (**b**, **d**) at 24 (**a**, **b**) and 48 h (**c**, **d**) after irradiation



reported as the mean of two independent ± SE. Data was compared using the one-way analysis of variance (ANOVA) test followed by the multiple comparison test of Tukey. The analyses were performed using the Origin 8 (version 8.1) statistical program, and level of significance was set to $P \leq 0.05$; at P value <0.05, the population means were found to be significantly different.

Results and discussion

Sea urchin embryos respond to X-ray exposure by increasing Hsp70, Bag3, and p63 protein levels

It has been reported that the exposure of sea urchin embryos to X-rays at the high dose of 5 Gy greatly affects their development and morphogenesis, as described in our previous study (Matranga et al. 2010). Here, to confirm previous results and validate subsequent molecular analyses, X-ray-exposed embryos were microscopically inspected and scored as reported in Table 1. The percentage of normal and abnormal development observed in 5 Gy irradiated embryos from five different trials is detailed; in the lower panel, photographs of representative morphologies observed at 24 (A, B) and 48 h (C, D) after exposure are shown. In agreement with our previous results, we found that most exposed embryos developed abnormally, with major defects in skeleton elongation and gut differentiation (Matranga et al. 2010). We found 85.19% (SE±5.22) and 95.51% (SE±1.44) abnormal embryos 24 and 48 h after irradiation, respectively. Controls developed correctly with a low percentage of abnormally developed embryos, namely 3% (SE±0.46) and 6% (SE±1.29) at 24 and 48 h, respectively (not reported/shown). In previous studies, we reported the upregulation of HSP70 protein expression in sea urchin embryos exposed to UV-B (Bonaventura et al. 2005, 2006), suggesting that this could be a way to cope with

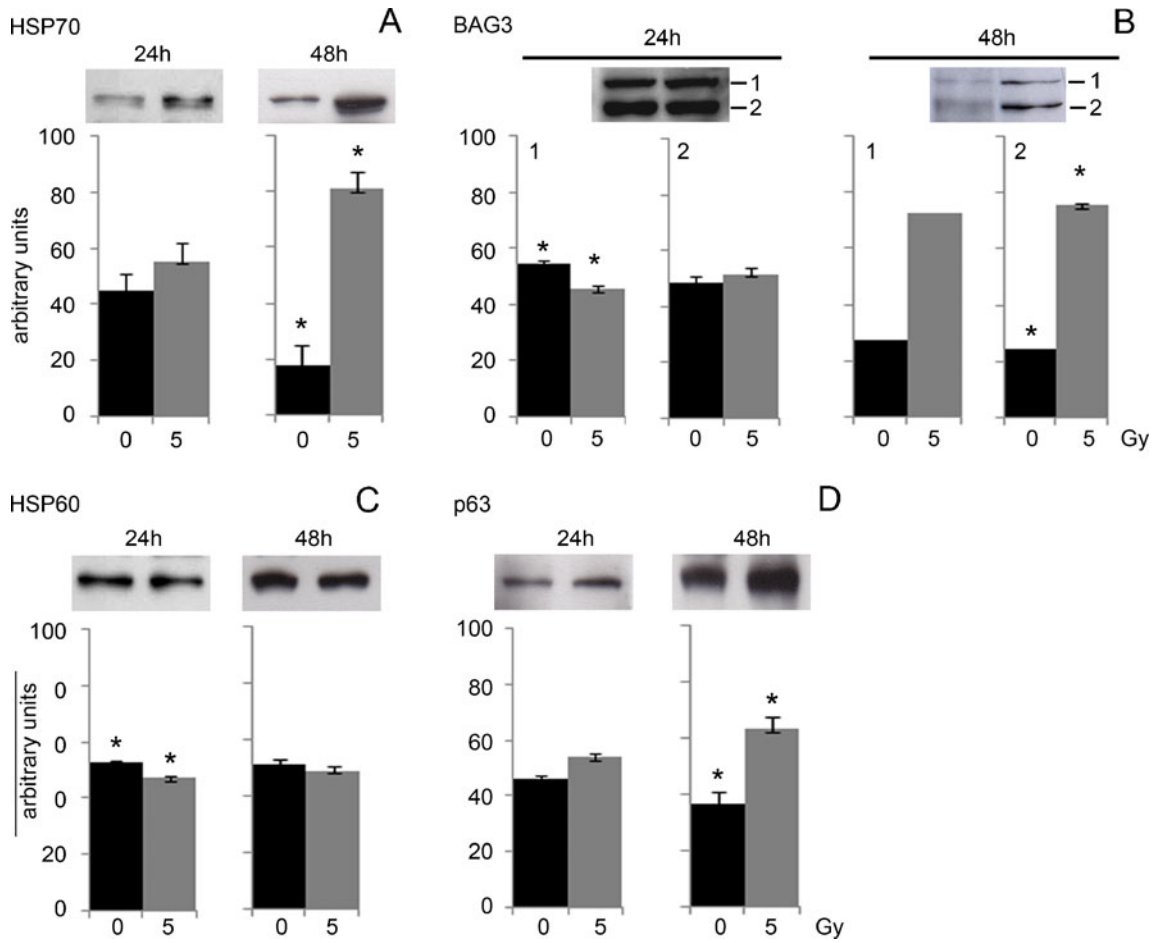


Fig. 1 Effects of X-rays on protein levels 24 and 48 h after irradiation. Western blot tests of total lysates from unexposed (0 Gy) and exposed (5 Gy) embryos were reacted with anti-hsp70, anti-hsp60, anti-Bag-3, anti-p53, respectively, shown in panels **a**, **b**, **c**, **d**. Each histogram reports the volumetric analysis data obtained from Western blot shown on the *upper part* (representative WB images). Each *bar* represents the mean of two/three independent WB experiments ± SE. *Asterisks* (*) indicate significant difference between control and exposed embryos by the ANOVA test ($P<0.05$) followed by the Tukey test

dangerous radiation, which probably prevents apoptosis. Indeed, in vertebrates, the anti-apoptotic events triggered by stress-inducing agents have been shown to involve an increase in the levels of heat shock proteins (Beere 2005; Jiang et al. 2011). Then, it was of interest to study the involvement of HSP70 in response to X-ray exposure and to evaluate the participation of HSP60, a stress protein we have shown to increase in response to cadmium (Roccheri et al. 2004). Similarly, as it was demonstrated that BAG3 and p63 are implicated in the stress response to different external stimuli (Belyi et al. 2010; Pagliuca et al. 2003), we hypothesized their possible involvement in response to X-rays. In this study, we determined the accumulation levels of the above-mentioned proteins by Western blots on total cell lysates obtained from embryos collected 24 and 48 h after X-ray exposure. We used antibodies to human recombinant BAG3 (Fontanella et al. 2010) and MBP-p53 proteins for the first time on sea urchin lysates. The former of which recognized two bands with an apparent molecular size of 84 and 70 kDa, which we referred to as BAG3-1 and BAG3-2, and the latter a protein band of about 63 kDa, which we referred to as p63 putative protein. As shown in Fig. 1, we did not find remarkable variations in the HSP70, HSP60, BAG3, and p63 protein levels 24 h after irradiation. The modest increase in HSP70 (Fig. 1a) and p63 protein (Fig. 1d) levels was considered not significant by the ANOVA test. The poor decrease in HSP60 levels observed in irradiated embryos (Fig. 1c), although statistically significant, in our opinion is not biologically relevant. These findings did not agree with the expected HSP70 protein upregulation we have previously demonstrated to occur when embryos were exposed to UV-B (Bonaventura et al. 2005). This suggests that, at least at 24 h after irradiation, the response to X-rays is not activated at the translational level, although we cannot exclude the possibility that HSP70 levels increased earlier after irradiation.

A remarkable increase in HSP70, BAG3, and p63 protein levels was observed in irradiated embryos collected after 48 h. In particular, we found a significant increase in the HSP70 levels (Fig. 1a), corresponding to 4.5-fold raise (P=0.0192) as well as 2.6- and 3.1-fold increases in BAG3-1 and BAG3-2 protein levels, respectively (Fig. 1b). The finding of two BAG3 protein bands is in agreement with other studies where BAG-3 is normally found as a 74-kDa cytoplasmatic protein but, depending on the cell type under investigation or following cell exposure to stressors, a doublet can be observed (Rosati et al. 2007). It has been proposed that BAG3 acts as a downregulator of apoptosis, probably exerting this biological function through its interaction with the HSP70 (Rosati et al. 2007). Thus, the finding of an increase of both HSP70 and BAG3 in sea urchin embryos irradiated with X-rays could support the hypothesis that these proteins cooperate to promote cell survival. Indeed, in human cells it has been reported that HSPs, including HSP70, could regulate/inhibit apoptosis at multiple levels (Beere 2005; Jiang et al. 2011). Our findings demonstrated the involvement of BAG3 in the stress response to X-rays for the first time, never tested so far in any other embryonic model. This adds an important contribution to the developmental biology and stress response fields, and suggests BAG3 as a novel biomarker of X-ray hazard.

Similar to HSP70 and BAG3, a significant 1.7-fold increase (P=0.04066) in the p63 protein levels was found, thus calling for a protective role played by the protein, in agreement with reports describing p63 critical roles in DNA repair following ionizing radiation exposure (Lin et al. 2009). However, we cannot exclude the possibility that high levels of p63 found in this study directly correlate to apoptosis, as demonstrated in the case of the high p53 levels detected in *Strongylocentrotus droebachiensis* embryos exposed to UV-B (Lesser et al. 2003). Further studies on *P. lividus* embryos exposed to X-rays are needed to establish a direct correlation between increased p63 protein levels and induced apoptosis, also taking into account a certain degree of physiological apoptosis occurring in some cells at late developmental stages (Roccheri et al. 2002).

To summarize, in embryos irradiated with a single 5-Gy dose of X-ray at the cleavage stage, the protein levels of HSP70, BAG3, and p63 increase 48 h after irradiation,

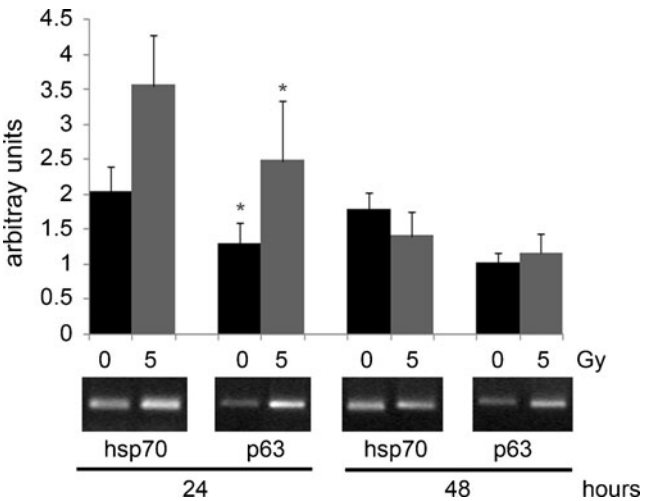


Fig. 2 Semiquantitative analysis of hsp70 and p53-like mRNA levels in embryos exposed to X-rays. Each *bar* represents the mean of the two independent experiments ± SE with six replicates in samples analyzed 24 h after irradiation and three replicates in samples analyzed 48 h after irradiation. Values obtained from measurements of band intensities for each amplification product were normalized to the Pl-S24 values. *Asterisks* (*) indicate significant difference between control and exposed embryos by the ANOVA test (P<0.05) followed by the Tukey test

suggesting that adaptation, cellular repair, and/or recovery are taking place by means of the elevation of stress/anti-apoptotic proteins levels within a period of 48 h after irradiation.

X-ray effects on hsp70 and p63 gene expression

Since we found increased levels of HSP70 and p63 as a consequence of X-ray irradiation, it was of interest to obtain information on the effects at the gene level. The isolation and characterization of the *P. lividus* p63 cDNA which do not fall within the scopes of this report, which have never been described, will be reported in a separate study (Costa et al., in preparation). The expression of Pl-hsp70 and Pl-p63 transcripts was monitored by relative RT-PCR in embryos exposed to X-rays and harvested 24 and 48 h after irradiation (Fig. 2). We observed variations in the levels of Pl-hsp70 and Pl-p63 mRNA (P=0.02693), with a 1.7- and a 1.9-fold increase, respectively, 24 h after irradiation. No changes in the expression of Pl-p63 were detected 48 h after irradiation, while there was a moderate reduction in the expression of Pl-hsp70, though not statistically significant.

We did not take into consideration the Pl-hsp60 gene expression as we found no changes at the protein level, both 24 and 48 h after irradiation. It would also be interesting to know what happens to the BAG3 mRNA expression. The Bag3 sequence has not been characterized so far in the sea urchin genome, although predicted partial sequences similar to proteins of the BAG family have been reported by automatic modeling. Given the detection of two BAG3 cross-reacting bands by Western blotting, a more accurate study at the gene level needs to be performed. Experiments are in progress to isolate the bag3 cDNA from *P. lividus* embryos. In conclusion, we found that embryos elevated p63 and hsp70 mRNA levels at 24 h after X-ray exposure, suggesting that their survival might be mediated by the time-dependent activation of protective anti-apoptotic machinery.

In summary, the sea urchin developmental model, which enables the ethically friendly production of large quantities of embryos to be used for biochemical and biomolecular analyses, has been proven to possess a protective anti-apoptotic machinery operating at the transcriptional level after 24 h, followed by its translational activation at 48 h post-irradiation. The use of the novel biomarkers of X-ray hazards described in this study might be applied to other higher organisms for the estimate of the stress response in development. Lastly, on the basis of the results presented in this report, we confirm the sea urchin embryo as a suitable model for studies on the effects of ionizing radiation and propose its use for the dissection of the stress/protection mechanisms operating in response to X-ray.

Acknowledgments This work has been fully supported by the Italian Space Agency, MoMa Project (contract 1/014/06/0). RB has received a fellowship within the above-mentioned grant. Partial financial support from the EU 7thFP ITN: Biomintec (Contract N° PITN-GA-2008-215507) and CNR Flagship Project FBdQ 2011. We thank the Department of Physics, University of Palermo for space and support in hosting the X-ray facility. We are grateful to all the other members of the group for their helpful contributions and suggestions and the stimulating working environment.

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