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ORIGINAL PAPER

# Role of p97/Valosin-containing protein (VCP) and Jab1/CSN5 in testicular ischaemia–reperfusion injury

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**Abstract** The most significant complication of testicular ischaemia is loss of the testis, which may lead to infertility. Testicular ischaemia damages protein degradation pathways which, together with the overproduction of damaged proteins and consequent upregulation of ubiquitin-conjugated protein aggregates. Despite recent advances, the factors leading to impairment of spermatogenesis owing to testicular ischaemia remain poorly understood. This study was undertaken to gain insight into the cellular and molecular mechanism underlying torsion induced germ cell apoptosis. Male rats were subjected to 2 h torsion, and testes were examined at 2, 4, 12 and 24 h after torsion repair (reperfusion). Ischaemia–reperfusion (IR) of the testes resulted in apoptosis which was revealed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) technique. At 12 h after torsion repair germ cell loss reached peak, then decreased at 24 h repair. Western blotting showed that apoptotic proteins (active caspase 3, caspase 9 and Bax) gradually was upregulated at 12 h reperfusion, however anti-apoptotic protein (Bcl2) was downregulated in the relevant IR treatment. Furthermore, Jab1/CSN5 expression was gradually upregulated and p97/VCP expression was downregulated in IR injury according to western blotting and immunohistochemistry. To test further whether polyubiquitination was also involved in IR injury, the expression of polyubiquitinated proteins was examined, which showed that polyubiquitinated proteins were significantly increased in IR injury. These finding suggest that p97/VCP and Jab1/CSN5 provide a novel signaling pathway for testicular ischaemia and may play an important role in IR injury induced cell death in rat testis.

**Keywords** p97/VCP · Jab1/CSN5 · Cell death · Ischaemia–reperfusion injury · Testis · Rat

## Introduction

Testicular torsion is a urologic emergency that mainly affects newborns, children, adolescents and young adults (Cuckow and Frank 2000; Cuervo et al. 2007). Testicular torsion causes edema, testicular ischaemia, testis dysfunction and permanent loss of spermatogenesis including male infertility (Akgur et al. 1993; Cosentino et al. 1986; Turner and Brown 1993). Torsion reduces the oxygen supply to the testes, and reperfusion leads to the formation of nitrogen and reactive oxygen (Filho et al. 2004; Parlaktas et al. 2014). Highly toxic metabolites of oxygen induce the overproduction of ROS and the activation of oxidizing enzymes, consequently leading to cytoskeletal, cell membrane and mitochondrial damages and germ cell specific apoptosis (Kanter 2010; Lopez-Neblina et al. 2005; Lysiak et al. 2000; Reilly et al. 1991; Turner et al. 1997). Evidences are accumulating to show a pivotal role of

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the ubiquitin–proteasome pathway in ischaemia–reperfusion injury (Meller 2009; Sun et al. 2008).

The ubiquitin–proteasome system (UPS) is a catalytic machinery that targets numerous cellular proteins for degradation, thus being essential to control a wide range of basic cellular processes and cell survival (Ciechanover 1994). Degradation of intracellular proteins via the UPS is a tightly regulated process initiated by tagging a target protein with a specific ubiquitin chain. Alterations in UPS activity may induce pathological responses, ultimately leading to cell death. Testicular ischaemia also damages protein degradation pathways which, together with the overproduction of damaged proteins and consequent upregulation of ubiquitin-conjugated proteins, contribute to the accumulation of ubiquitinated proteins (Sun et al. 2008). Despite recent advances, the factors leading to deposition of such aggregates and triggering apoptotic signaling after ischemic injury remain poorly understood. For this reason, it is a challange to examine whether key molecules of UPS such as Jab1/CSN5 and p97/VCP would be involved in testis ischaemia–reperfusion injury. p97/VCP and Jab1/CSN5 have been identified as interacting partners working in protein degradation pathway and have also an active role in the cell survival (Cayli et al. 2009; Lizano et al. 2013).

p97/VCP is a ubiquitously expressed and highly conserved member of the type II AAA (ATPases associated with various cellular activities) family with an apparent molecular weight of 97 kDa. It contains four main domains: a N-terminal domain binding ubiquitinated substrates, two ATPase domains (D1 and D2), and a C-terminal domain involved in nuclear localization (Dai and Li 2001). p97/VCP participates in various cellular processes requiring ubiquitination and proteasome degradation, including cell cycle control, transcriptional regulation, apoptosis, membrane fusion, and endoplasmic reticulum (ER)-associated degradation (Braun and Zischka 2008; Dai et al. 1998). Increased expression of p97/VCP correlates with cell growth and survival, particularly in cancer cells (Asai et al. 2002; Vandermoere et al. 2006), whereas its depletion, oxidation, or mutation lead to apoptosis triggered by ERstress (Kobayashi et al. 2002; Wojcik et al. 2004). However, it is discrepant whether p97/VCP play a role in testicular IR induced germ cell apoptosis.

Jun activation domain-binding protein-1 (Jab1) was initially identified as a protein that interacts with c-Jun and stimulates the binding of c-Jun and JunD to AP-1 sites, potentiating them as transcription factors (Claret et al. 1996). Jab1 is also known as CSN5, which is the fifth member of the COP9 signalosome (CSN) complex consisting of eight subunits, CSN1–CSN8 (Deng et al. 2000). Jab1/CSN5 exists and functions both as a monomer and as a subunit of the CSN complex. Although Jab1/CSN5 has been shown to be a key player in plant light signaling, development, cell cycle control, and stability of various proteins in a number of systems (Chamovitz and Segal 2001; Wei et al. 2008), the specific role of Jab1/CSN5 and its cellular mechanism in the testicular ischaemia still remain obscure. Thus, it is interesting to examine whether p97/VCP and Jab1/CSN5 can be involved in testis ischaemia–reperfusion injury. In addition polyubiquitination level during IR-induced germ cell apoptosis, would contribute to the understand the molecular aetiology of the testis ischaemia–reperfusion injury.

We reported here that p97/VCP in testis was downregulated and Jab1/CSN5 is upregulated thus, ubiquitination level was impaired, which may trigger the germ cell apoptosis in testis ischaemia–reperfusion injury.

## Materials and methods

### Animals

Male Wistar albino rats, 5- to 6-months-old and weighing between 230 and 270 g, were obtained from the Gaziosmanpasa University Experimental Animal Research Laboratory. The rats were handled in the laboratory according to institutional guidelines as well as the *Guide for Care and Use of Laboratory Animals* of the National Research Council and the ethic rapor was approved by the local ethics committee. All rats were observed for several days to ascertain the health before sample collection. They were kept in a temperature-controlled room (20–23 °C), on a 12-h light/dark cycle with food (commercial rat chow) and fresh water available ad libitum.

### Testicular torsion

All of the rats were anesthetised with intramuscular ketamine (50 mg/kg) and xylazine (10 mg/kg). The surgical procedures were performed under sterile conditions through standard ilioinguinal incisions. Thirty five male rats were divided randomly into five groups, each containing seven rats. The animals in group I (sham-operated control group, n: 7) underwent a sham operation to determine the basal level of protein expression. In this group, the left testis was exposed through an incision and displaced to the hemiscrotum, and the wound was closed. At the end of the 4 h experimental period, the testes were removed. Group II (I/R injury group, 2 h reperfusion, n: 7) was designed to study the effects of 2 h I/R injury on testicular tissues. The left testis was rotated 720° in a clockwise direction and maintained for 2 h by fixing the left testis to the scrotum with a 4–0 silk suture as described elsewhere (Parlaktas et al. 2014). Detorsion was performed by untwisting the testis and maintaining the position for

2 h. At the end of the experimental period, the testes were removed. In group III (IR injury group, 4 h reperfusion, n: 7), group IV (IR injury group, 12 h reperfusion, n: 7) and group V (IR injury group, 24 h reperfusion, n: 7) the left testicles of the animals were also subjected to the same duration of ischaemia (2 h); however, after 4, 12 and 24 h of detorsion, the testes were removed and harvested for various experiments.

### Apoptotic assay

Apoptosis in the testicular tissue was detected by enzymatic labelling of DNA strand breaks using a TUNEL (Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labelling) kit (TUNEL system, 1684809, Roche, Mannheim, Germany). Paraffin sections were deparaffinised in xylene, rehydrated and treated in a microwave oven in 10 mM citrate buffer, pH 6.0, twice for 5 min and allowed to cool for 20 min. After three washes in phosphate buffered saline (PBS), endogenous peroxidase activity was inhibited with 3 % hydrogen peroxide. The sections were then incubated with equilibration buffer for 10–15 s, and TdT enzymatic labelling of nuclear DNA strand breaks was performed in a humidified atmosphere at 37 °C for 60 min. The typical labels were revealed by adding an alkaline phosphatase-converter with subsequent staining with NBT/BCIP solution as the chromogenic substrate. Each step was separated by careful washings in PBS. Counter staining was performed in Mayer's haematoxylin. The average percentage of apoptotic tubules was estimated by examining 100 cross-sections of seminiferous tubules from each specimen. The seminiferous tubules that contained at least one TUNEL-stained nucleus were considered apoptotic (Yazawa et al. 2001). The percentage of apoptotic cells that stained pink was determined.

### Immunohistochemistry

The testes were fixed in Bouin's fluid for 24 h immediately upon collection, dehydrated, and embedded in paraffin for immunohistochemistry. Immunohistochemistry was performed according to the procedure described previously (Cayli et al. 2011). Briefly, serial sections, 5 μm thick, were collected on poly-L-lysine-coated slides (Sigma-Aldrich, St. Louis, MO, USA) and incubated overnight at 56 °C. The tissue sections were deparaffinised in xylene and rehydrated in a graded series of ethanol. The sections were then treated in a microwave oven in 10 mM citrate buffer, pH 6.0, for 5 min twice and allowed to cool for 20 min. After three washes in PBS, endogenous peroxidase activity was quenched by 3 % hydrogen peroxide in PBS for 20 min and again washed three times in PBS. The sections were then incubated in a blocking serum (ScyTek Laboratories, UT, USA) for 10 min to block non-specific binding. Subsequently, the sections were incubated overnight at 4 °C with mouse monoclonal p97/VCP (ab11433, 1:750, Abcam, UK), rabbit polyclonal Jab1/CSN5 (ab12323, 1:500, Abcam, UK) and rabbit polyclonal ubiquitin (ab 7780, 1:200, Abcam, UK) in a humidified chamber. After incubating with primary antibodies at 4 °C overnight, the sections were washed three times in PBS and incubated biotinylated polyvalent antibodies (BioGenex, Fremont, California, USA) and peroxidase-labeled streptavidin (Biogenex). Immunohistochemistry was performed using a horseradish peroxidase-labeled streptavidin biotin kit (Biogenex) according to the manufacturer's instructions.

After three washes with PBS, bound peroxidase was developed with DAB (Sigma tablets) chromogen, and the sections were counterstained with Mayer's haematoxylin (ScyTek Laboratories, Utah, USA) and mounted with Permount (Fisher Chemicals, Springfield, NJ, USA) on glass slides. For controls, sections were treated with the appropriate isotype mouse IgG or normal rabbit IgG, depending on the primary antibody used, which was diluted to the same final protein concentration as the primary antibody. Photomicrographs were taken with a Leica microscope (Leica DM2500, Nussloch, Germany).

### Evaluation of the immunohistochemistry

The evaluation of the immunohistochemical labelling was performed using H-SCORE analyses, as previously described (Cayli et al. 2012). The intensities of p97/VCP, Jab1/CSN5 and ubiquitin immunoreactivities were semi-quantitatively evaluated using the following intensity categories: 0 (no staining), 1+ (weak but detectable staining), 2+ (moderate or distinct staining), and 3+ (intense staining). For each tissue, an H-SCORE value was derived by calculating the sum of the percentages of the cells that stained at each intensity category and multiplying that value by the weighted intensity of the staining, using the Formula H-SCORE: $\sum Pi(i + 1)$, where 'i' represents the intensity scores and 'Pi' is the corresponding percentage of the cells. In each slide, five randomly selected areas were evaluated under a light microscope (40× objective), and the percentage of cells for each intensity within these areas was determined at different times by two investigators who were not informed about the type and source of the tissues. The average score of both observers was used.

### Western blotting

Total testicular proteins were extracted using modified radioimmunoprecipitation assay buffer [RIPA; 1 % NP-40; 0.25 % sodium deoxycholate; 150 mmol/L sodium chloride (NaCl); 1 mmol/L EDTA; 1 mmol/L phenylmethylsulfonyl fluoride (PMSF); 1 mg/mL each of aprotinin, leupeptin, and pepstatin; 1 mmol/L sodium vanadate ($Na_3VO_4$); and

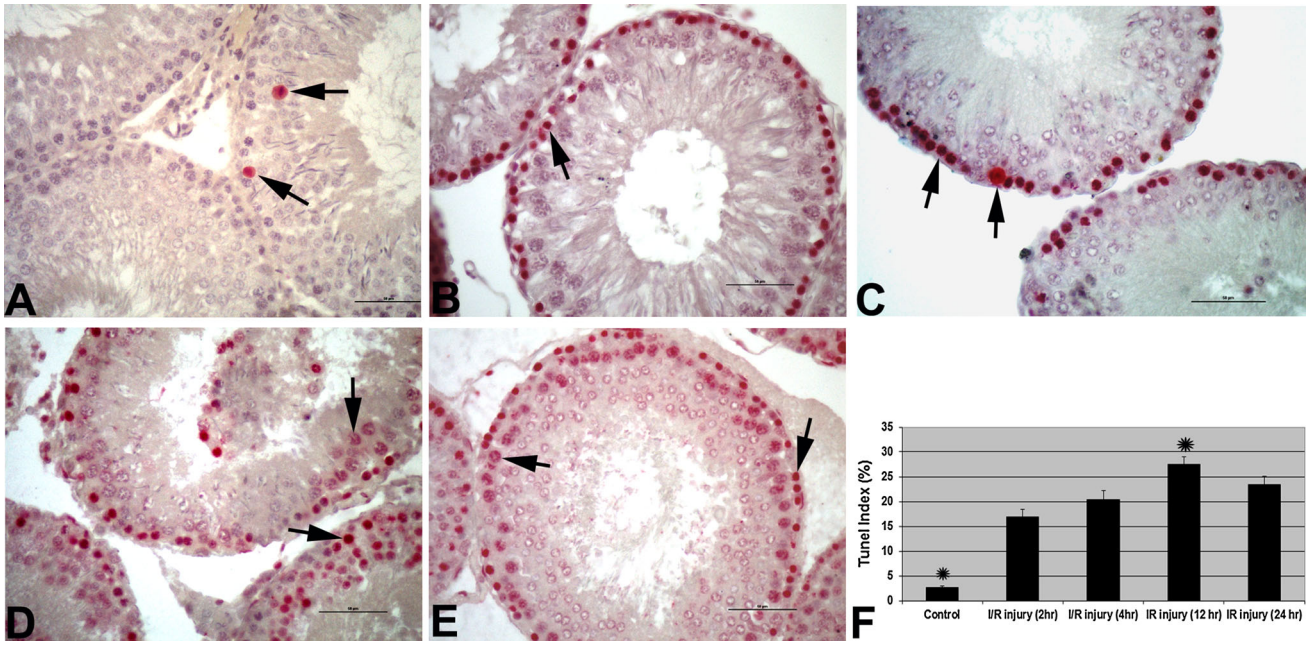



**Fig. 1** TUNEL-positive germinal cells of seminiferous tubules of testes in the control (**a**), group II (I/R injury, 2 h) (**b**), group III (I/R injury, 4 h) (**c**), group IV (I/R injury, 12 h) (**d**) and group V (I/R injury, 24 h) (**e**). The *scale bar* represents 50 µm. The data are represented as the mean ± SEM. *Asterisks*: $p < 0.05$, I/R injury (2 h) versus control, I/R injury (4 h) versus control, I/R injury (12 h) versus control, I/R injury (24 h) versus control, I/R injury (2 h) versus I/R injury (12 h), I/R injury (4 h) versus I/R injury (12 h), I/R injury (12 h) versus I/R injury (24 h)

1 mmol/L sodium fluoride (NaF) in 50 mmol/L Tris–Cl, pH 7.4] and quantitated using the Bradford procedure (Bio-Rad, Hercules, California). Samples (40 µg) were separated on a NuPAGE 4–12 % Novex Bis–Tris gel (Invitrogen) and blotted onto a nitrocellulose membrane through the iBlot transfer system (Invitrogen). The membrane was blocked with 5 % nonfat dry milk in PBS containing 0.1 % Tween 20 (PBS-T) for 1 h. Subsequently, the membrane was incubated overnight with the primary antibody against p97/VCP (ab11433, 1:3000, Abcam, UK), Jab1/CSN5 (ab12323, 1:2000, Abcam, UK), ubiquitin (ab 7780, 1:1000, Abcam, UK), Bax (ab7977, 1:500, in 5 % nonfat dry milk; Abcam), active caspase 3 (ab13847, 1:400, in 5 % nonfat dry milk; Abcam), Bcl2 (ab101568, 1:500, Abcam, UK.), caspase 9 (ab2014, 1:500, in 5 % nonfat dry milk; Abcam) and β-actin (ab8226, 1:3000 in 5 % nonfat dry milk; Abcam, UK). The membrane was washed with PBS-T for 1 h and incubated with horseradish peroxidase–conjugated anti-mouse and anti-rabbit antibodies diluted in 5 % nonfat dry milk in PBS-T. After three washes with PBS, bound secondary antibodies were visualised by enhanced chemiluminescence substrate (ab133406, Abcam, UK). After chemiluminescence detection, the membranes were incubated with stripping solution (100 mM 2-mercaptoethanol, 2 % SDS, 62.5 mM Tris–HCl, pH 6.8) for 15 min at RT and reutilised for further detections. The immunoblot bands for proteins were quantified using an Alpha DigiDoc 1000 gel documentation unit (Alpha Innotech Corporation, California). The optical density (OD) values for active caspase 3, caspase 9, Bax, Bcl2, p97/VCP, Jab1/CSN5 and ubiquitin bands were divided by the OD values of the cognate β-actin bands to normalise the OD values for loading differences.

### Statistical analysis

The pairwise multiple comparisons for immunohistochemistry and western blots were analysed with non-parametric ANOVA on ranks (Holm–Sidak Method) followed by the Kruskal–Wallis test. For the TUNEL assay, non-parametric ANOVA on ranks (Tukey test) was performed. The statistical calculations were performed using SigmaStat for Windows, version 3.5 (Jandel Scientific Corp., San Rafael, CA). Statistical significance was defined as $p < 0.05$.

## Results

### Evaluation of germ cell apoptosis in ischaemia–reperfusion injury

The percentage of apoptotic cells in testes was evaluated by the TUNEL technique (Fig. 1). TUNEL positive apoptotic cells were mostly observed in the intratubular elements, where spermatogonia and spermatocyte located (Fig. 1a–e).

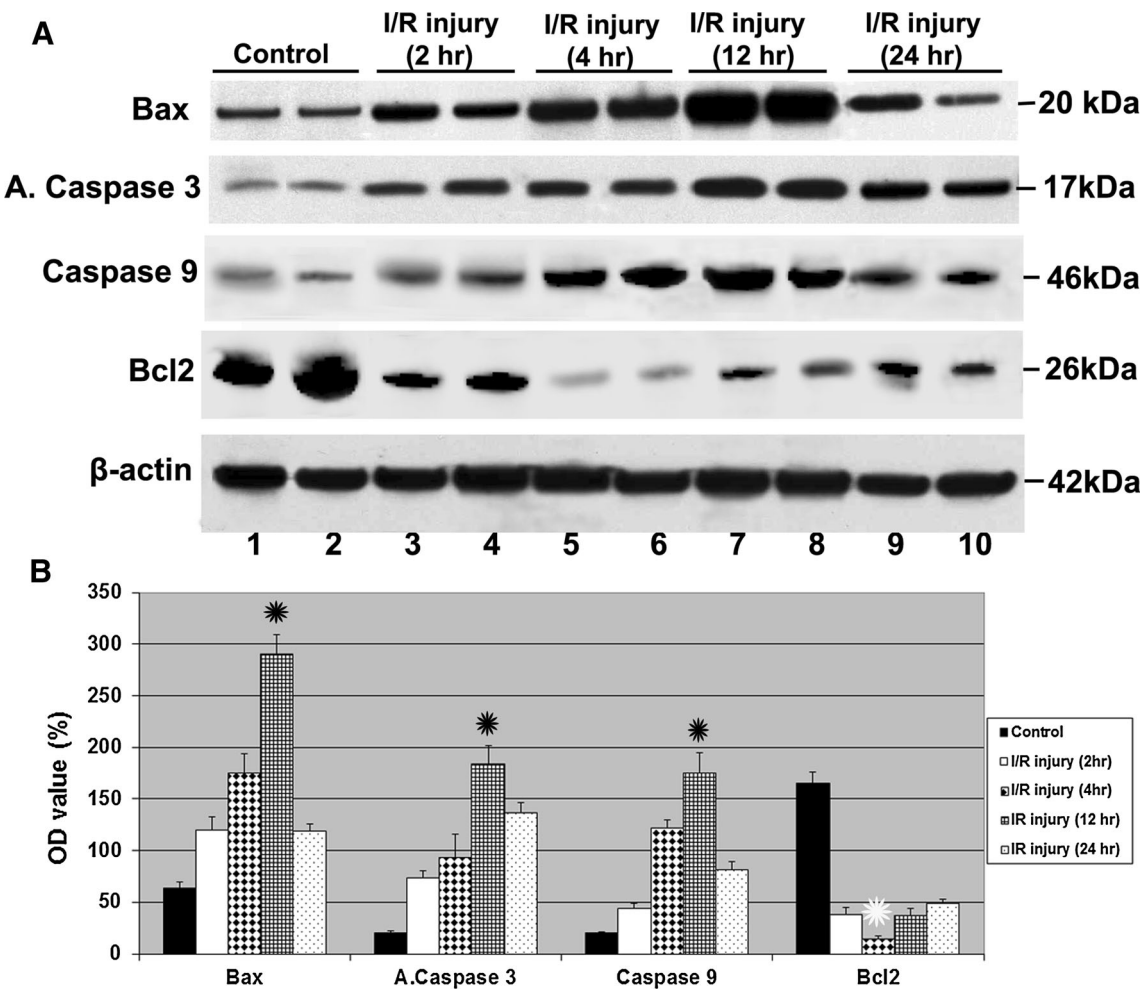



**Fig. 2** **a** Lysates from control (*lanes* 1 and 2), I/R injury, 2 h (*lanes* 3 and 4), I/R injury, 4 h (*lanes* 5 and 6), I/R injury, 12 h (*lanes* 7 and 8) and I/R injury, 24 h (*lanes* 9 and 10) were used for immunoblotting. Bax (20 kDa), active caspase 3 (17 kDa), caspase 9 (46 kDa) and Bcl2 (26 kDa) were detected by immunoblotting and β-Actin (43 kDa) was used as the loading control. **b** Immunoblot bands were quantified by an optical densitometer. The optical density values of active caspase 3, caspase 9, Bax and Bcl2 were normalised to the OD values of the β-actin bands. Bax, active caspase 3 and caspase 9 protein expressions reached maximum level at 12 h of I/R injury (*black asterisks*), however Bcl2 protein expression reached minimum level at 4 h of I/R injury (*white asterisk*). The values are the mean ± SEM. *Asterisks* $p < 0.05$. All comparisons were significantly different except following comparisons: I/R injury (2 h) versus I/R injury (24 h) is not significant (NS) for Bax, a. caspase 3, caspase 9 and Bcl2. I/R injury (2 h) versus I/R injury (4 h) is NS for a. caspase 3, caspase 9 and Bcl2. I/R injury (4 h) versus I/R injury (24 h) is NS for a. caspase 3, caspase 9 and Bcl2. I/R injury (12 h) versus I/R injury (24 h) is NS for Bcl2

Although TUNEL-positive cells were observed in all groups, the percentage of TUNEL positive cell apoptosis showed a increased trend until 12 h of I/R injury (Fig. 1d, f) and reached peak at 12 h of I/R injury compared to control and other stages. The percentage of TUNEL-positive cells was decreased at 24 h of I/R injury but not significantly different compared to 12 h of I/R injury (Fig. 1e, f).

We further investigate the germ cell apoptosis by using Western blot (Fig. 2). Firstly, we showed that apoptotic protein (Bax, a. caspase 3 and caspase 9) expressions were gradually upregulated during I/R injury and reached maximum level at 12 h of I/R injury however, weakened at 24 h of I/R injury. Secondly, antiapoptotic protein (Bcl2) expression was down regulated gradually and reached minimum level at 4 h of I/R injury (Fig. 2a, b).

## p97/VCP was downregulated in testicular I/R injury

We have already shown the expression of p97/VCP and Jab1/CSN5 in the postnatal rat testis (Cayli et al. 2011), but their role in testis is still unknown. p97/VCP immunopositivity was mainly detected in spermatogonia, spermatocyte and Sertoli cells of adult testis. To investigate the effects of I/R injury on the regulation of p97/VCP expression, tissue sections from I/R injuried testes were immunostained. p97/VCP expression was observed in the spermatogonia,

spermatocyte and Sertoli cells in the control groups, however in I/R injuried groups p97/VCP immunostaining was found mostly in Sertoli cells. Immunohistochemistry revealed that the number of immunopositivity and the intensity of p97/VCP immunostaining were significantly reduced in I/R injuried groups compared to control (Figs. 3, 4). p97/VCP expression began to restore at 24 h of I/R injury (Figs. 3e, 4) and the number of the cells and the staining intensity were nearly consistent with those of the control group.

Expression of p97/VCP in control and I/R injuried groups was further examined by Western blot (Fig. 5). Western result confirmed gradually downregulation of p97/VCP in I/R injuried groups. Minimum expression of p97/VCP was observed at 4 h of I/R injury and even up to almost the control level at 24 h of I/R injury (Fig. 5b).

### Jab1/CSN5 was upregulated in testicular I/R injury

In order to clarify whether Jab1/CSN5 involved in I/R injury, we examined the expression of Jab1/CSN5 in different time point of I/R injury by using immunohistochemistry and Western blotting (Figs. 3, 4, 5). In control groups, Jab1/CSN5 distribution was localized predominantly in spermatogonia and spermatocyte (Fig. 3f) and partial reactivity was showed with nuclear staining in spermatogonia and cytoplasmic staining in spermatocytes. Both the nuclear and the cytoplasmic immunoreactivity of Jab1/CSN5 increased in I/R injuried groups (Fig. 3g–j). The changes of Jab1/CSN5 immunoreactivity were consistent with the results of Western blotting (Fig. 5). We observed that Jab1/CSN5 expression level showed a time dependent up regulation and reached a maximum level at 12 h of I/R injury and decreased again at 24 h of I/R injury (Fig. 5a, b).

### Ubiquitination in testicular I/R injury

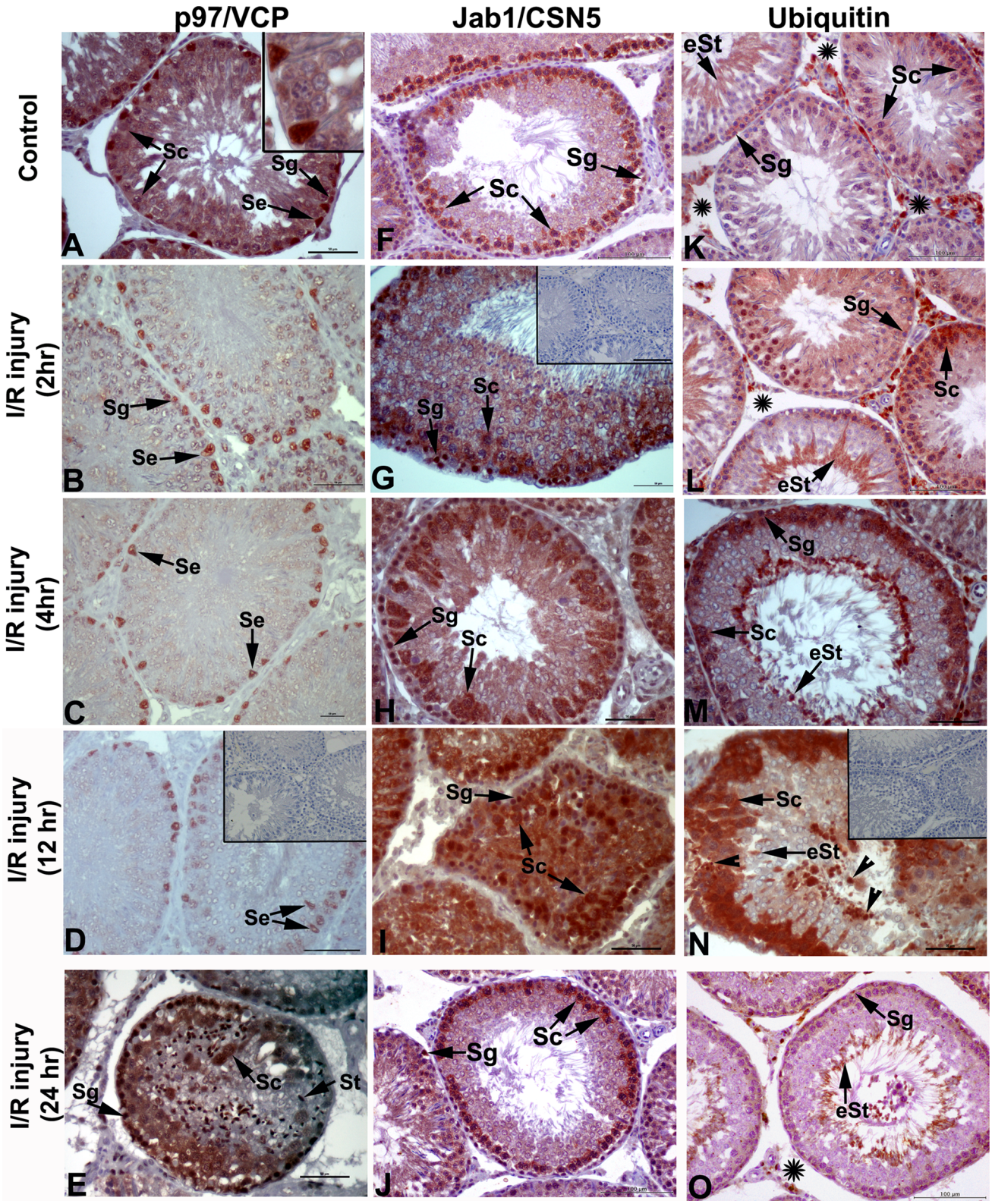
Jab1/CSN5 and p97/VCP are two important proteins playing pivotal role in maintaining the intracellular ubiquitin level. To test whether the polyubiquitinlation was also involved in testicular I/R injury, polyubiquitination in control and I/R injury groups was examined by immunohistochemistry and Western blotting (Figs. 3, 4, 5). In control group, ubiquitin expression was observed in spermatogonia, spermatocytes, elongated spermatids and interstisial cells (Fig. 3k–o). Similar to Jab1/CSN5 expression, ubiquitin expression was gradually increased until 12 h of I/R injury thereafter decreased to almost control level within 24 h of I/R injury. Immunohistochemical expression of ubiquitin was further confirmed by Western blotting (Fig. 5). We found significantly increased expression of polyubiquinlated proteins in I/R injuried testes compared to control (Fig. 5a, b).

**Fig. 3** Immunohistochemical distribution of p97/VCP (**a–e**), Jab1/CSN5 (**f–j**) and Ubiquitin (**k–o**) in the control, I/R injury (2 h), I/R injury (4 h), I/R injury (12 h) and I/R injury (24 h) groups. No significant staining was observed in the negative controls (**d**, **g**, **n**, *inserts*). **a** p97/VCP is moderately expressed in the cytoplasm of spermatocytes (Sc) and spermatagonia (Sg), whereas Sertoli cells (Se) (*insert*) are strong immunopositive for p97/VCP. **b** p97/VCP is moderately expressed in Sg and Se cells. **c**, **d** Weak to moderate expression of p97/VCP is displayed in Se and Sg cells, while no immunolabeling is observed in Sc and Spermatids (St). **e** Sg, Sc, St and Se are moderate to strongly immunpositive for p97/VCP. **f** Jab1/CSN5 is localized weakly in Sg and moderately in Sc. **g–i** Strong expression of Jab1/CSN5 is seen in Sg and Sc. **j** Moderate expression of Jab1/CSN5 is detected in Sg and Sc. **k** Ubiquitin immunoreactivity is observed in Sg, Sc and elongated St (eSt). Interstitial cells of testis (*asterisks*) are also immunpositive for ubiquitin. **l–n** Strong expression of ubiquitin in Sc and eSt is displayed and ubiquitin aggregate is seen in seminiferous tubules (*arrowhead*). **o** Weak to moderate expression of ubiquitin is observed in Sg and eSt. The *scale bar* represents 50 μm for **a–e**, **g–i**, **m**, **n** and 100 μm for **f**, **j**, **k**, **l**, **o**

## Discussion

Testicular torsion is a serious urologic emergency that is mostly encountered in newborn and adolescent males. Previous studies have shown that the ischaemia–reperfusion (I/R) injury due to testicular torsion has been implicated in the pathogenesis of testicular damage (Cosentino et al. 1986). However, molecular mechanism how spermatogenesis impaired owing to the testicular torsion remain obscure. We provided here cellular and molecular event when ischaemia–reperfusion (I/R) injury occured. We found that p97/VCP was downregulated and Jab1/CSN5 was upregulated in I/R injuried testis. Additionally, polyubiquitination was upregulated due to the p97/VCP and Jab1/CSN5 expressions. These results suggest that both p97/VCP and Jab1/CSN5 seem to be an essential factors for spermatogenesis through regulation of polyubiquitin expression levels.

In the ATP-dependent ubiquitin pathway, the attachment of ubiquitin to a target protein, referred to as ubiquitination, is carried out by E1, E2 and E3 (Hochstrasser 1995). The main purpose of ubiquitination is to deliver the ubiquitinated proteins to a cellular trash bin, a lysosome, an autophagosomal vacuole, or a 26S proteasome. Ubiquitinated proteins can either be transferred directly to the proteasome or indirectly transferred via p97/VCP. In addition, during endoplasmic reticulum-associated degradation, p97/VCP dislodges ubiquitinated proteins from the endoplasmic reticulum (ER) and chaperones them to the cytosol for proteasomal degradation (Meusser et al. 2005). Therefore, p97/VCP plays an important role in the maintaince of intracellular ubiquitin level and the regulation of ubiquitin pathway. A number of prior studies have observed an extensive accumulation of ubiquitin conjugates in p97/VCP deficient cells, suggesting impairment of protein degradation pathways (Kirchner et al. 2013; Wojcik et al. 2004). Accordingly, our result showed that p97/VCP


p97/VCP
Jab1/CSN5
Ubiquitin
Control
I/R injury (2hr)
I/R injury (4hr)
I/R injury (12 hr)
I/R injury (24 hr)
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
Sc
Sg
Se
St
eSt

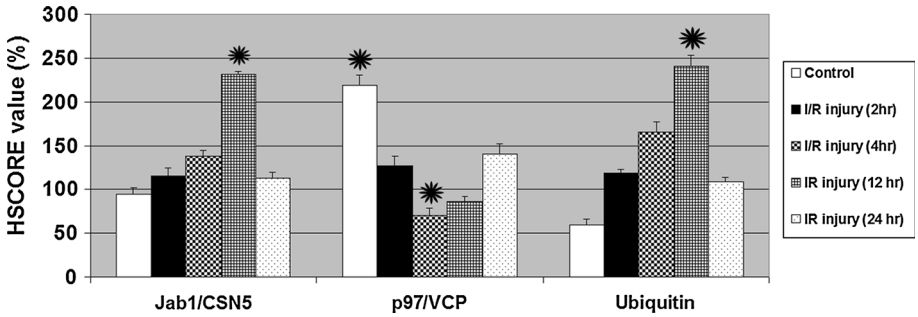



**Fig. 4** Comparison of the HSCORE values of p97/VCP, Jab1/CSN5 and ubiquitin in the control, I/R injury (2 h), I/R injury (4 h), I/R injury (12 h) and I/R injury (24 h). Jab1/CSN5 and ubiquitin expressions are gradually increased and reached the maximum level at 12 h of I/R injury, whereas p97/VCP expression is gradually decreased and reached the minimum level at 4 h of I/R injury. The data are represented as the mean ± SEM. *Asterisk* $p < 0.05$, I/R injury (12 h) versus control, I/R injury (12 h) versus I/R injury (2 h), I/R injury (12 h) versus I/R injury (24 h), I/R injury (4 h) versus control for Jab1/CSN5 control versus I/R injury (4 h), control versus I/R injury (12 h), I/R injury (4 h) versus I/R injury (24 h), I/R injury (12 h) versus I/R injury (24 h) for p97/VCP control versus I/R injury (12 h), I/R injury (12 h) versus I/R injury (2 h), I/R injury (12 h) versus I/R injury (4 h), I/R injury (12 h) versus I/R injury (24 h), I/R injury (4 h) versus control, I/R injury (4 h) versus I/R injury (24 h), I/R injury (4 h) versus I/R injury (2 h), I/R injury (2 h) versus control, I/R injury (24 h) versus control for ubiquitin

expression significantly decreased in I/R injury, and thus polyubiquitination increased.

A number of studies have investigated protein ubiquitination, accumulation and proteasome activity following ischaemia in brain and testis (Caldeira et al. 2014; Huang et al. 2012; Meller 2009). These studies suggest detrimental effects of the ubiquitin proteasome system following ischaemia, resulting in damage to cell components, mediating inflammatory responses, leukocyte infiltration and apoptosis. In line with these studies, we also observed gradually increase in the expression of a.caspase 3, caspase 9 and Bax, whereas Bcl2 expression gradually decreased in I/R injuried testis. In the same I/R injury times, p97/VCP was down regulated and Jab1/CSN5, polyubiquitin protein expressions were upregulated. Our results strongly suggest that testicular I/R injury result in cell death due to the altered expression of p97/VCP, Jab1/CSN5 and polyubiquitin.

Furthermore, it is well known the role of p97/VCP as a survival agent in cancer cells (Asai et al. 2002) and it is also demonstrated its depletion triggers apoptosis by ER stress (Kobayashi et al. 2002). In accordance with these findings, in this study we found minimal expression of p97/VCP in 4 h of I/R injury coincidence with the minimal expression of anti-apoptotic protein, Bcl2. Additionally, our experiments further confirmed the upregulation of ubiquitination when the p97/VCP was downregulated in the relative I/R injury periods. These also supports the conclusion that decreased expression of p97/VCP causes apoptosis and the accumulation of polyubiquitinlated proteins in I/R injuried testis.

In recently published data showed that p97/VCP deficiency caused profound mitochondrial uncoupling leading to decreased mitochondrial membrane potential and increased mitochondrial oxygen consumption (Bartolome et al. 2013). They found that this mitochondrial uncoupling resulted in a significant reduction of cellular ATP production and cell death. The authors also demonstrated that p97/VCP deficient cells were more vulnerable to ischaemia due the less production of ATP. As mentioned in their findings, in our study, the decrease expression of p97/VCP most probably effected the mitochondrial membrane potential and ATP production and thus testicular cell death occuried. As a conclusion our results suggest that p97/VCP is critical molecule regulating multiple steps (apoptosis, ER stress and ubiquitination vs.) in I/R injuried testis.

In addition to p97/VCP, we investigated the expression of Jab1/CSN5 in testicular ischaemia. In contrast to p97/VCP, Jab1/CSN5 is upregulated in I/R injuried testis and seems to protect against cell death in response to ischaemia. Jab1/CSN5 overexpression is reported in many cancer cells (Ahn et al. 2009; Osoegawa et al. 2006), but its physiological significance remains to be investigated. This is the first report to demonstrate that a pathophysiological condition such as ischaemia can increase the expression of Jab1 in the testis. Why do ischaemia increase Jab1 protein levels? We propose that Jab1/CSN5 plays an important role as a cell protector in ischaemia-mediated cell death. Similar results observed in different studies support our data (Fukumoto et al. 2006; Yun et al. 2010). However, there is no previous study that directly reports the role of Jab1/CSN5 as a cell protector in testicular ischaemia.

Furthermore, recent studies have shown that Jab1/CSN5 overexpression induces ubiquitination of some interacting partners (Wan et al. 2002; Zhang et al. 2008). In accordance with these studies, the upregulation of ubiquitinated proteins in I/R injury may occur due to the increase expression of Jab1/CSN5 in our study.

Taken together, the significance of this study is not only providing direct evidence for p97/VCP and Jab1/CSN5

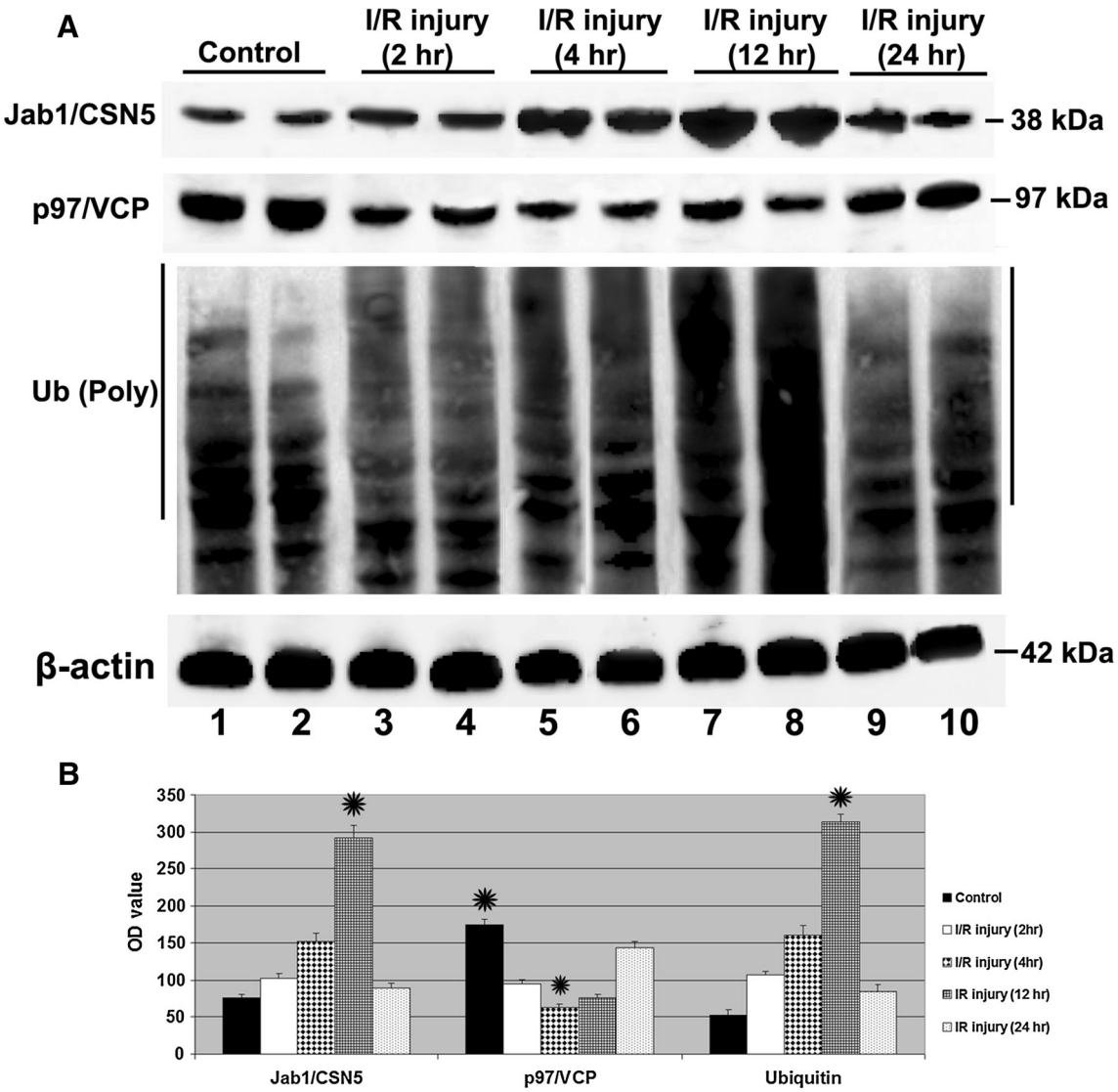



**Fig. 5** **a** Lysates from control (*lanes* 1 and 2), I/R injury, 2 h (*lanes* 3 and 4), I/R injury, 4 h (*lanes* 5 and 6), I/R injury, 12 h (*lanes* 7 and 8) and I/R injury, 24 h (*lanes* 9 and 10) were used for immunoblotting. Jab1/CSN5 (38 kDa), p97/VCP (97 kDa) and polyubiquitin were detected by immunoblotting and β-Actin (43 kDa) was used as the loading control. **b** Immunoblot bands were quantified by an optical densitometer. The optical density values of Jab1/CSN5 (38 kDa), p97/VCP (97 kDa), ubiquitin were normalised to the OD values of the β-Actin bands. The values are the mean ± SEM. *Asterisk*: $p < 0.05$, I/R injury (12 h) versus control, I/R injury (12 h) versus I/R injury (2 h), I/R injury (12 h) versus I/R injury (24 h), I/R injury (4 h) versus control for Jab1/CSN5 control versus I/R injury (4 h), control versus I/R injury (12 h), I/R injury (4 h) versus I/R injury (24 h), I/R injury (12 h) versus I/R injury (24 h) for p97/VCP All group comparisons were significant for ubiquitin except I/R injury (2 h) versus I/R injury (24 h)

participating testiscular I/R injury but also providing vital information towards understanding the mechanism of cell death. Therefore, these data provided insights into biology at the cellular level and the roles of p97/VCP and Jab1/CSN5 in the testicular I/R injury.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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