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The nuclear import factor importin α4 can protect against oxidative stress

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ABSTRACT

The importin (IMP) superfamily of nuclear transport proteins is essential to key developmental pathways, including in the murine testis where expression of the 6 distinct IMPα proteins is highly dynamic. Present predominantly from the spermatocyte stage onwards, IMPα4 is unique in showing a striking nuclear localization, a property we previously found to be linked to maintenance of pluripotency in embryonic stem cells and to the cellular stress response in cultured cells. Here we examine the role of IMPα4 in vivo for the first time using a novel transgenic mouse model in which we overexpress an IMPα4–EGFP fusion protein from the protamine 1 promoter to recapitulate endogenous testicular germ cell IMPα4 expression in spermatids. IMPα4 overexpression did not affect overall fertility, testis morphology/weight or spermatogenic progression under normal conditions, but conferred significantly (>30%) increased resistance to oxidative stress specifically in the spermatid subpopulation expressing the transgene. Consistent with a cell-specific role for IMPα4 in protecting against oxidative stress, haploid germ cells from IMPα4 null mice were significantly (c. 30%) less resistant to oxidative stress than wild type controls. These results from two unique and complementary mouse models demonstrate a novel protective role for IMPα4 in stress responses specifically within haploid male germline cells, with implications for male fertility and genetic integrity.

1. Introduction

Regulated nuclear import of proteins through members of the importin (IMP) superfamily of transport proteins is an important mechanism controlling cellular differentiation and development [1–4]. This is particularly evident in the testis, where differential expression and subcellular localization of specific IMPα proteins in flies, mice and even humans are closely aligned with specific stages of spermatogenic maturation that reflect distinct cargo nuclear import requirements for each developmental phase [5–10]. In particular, germ cells undergo mitosis, meiosis, differentiation, and extensive morphological changes, with a significant requirement for IMP function late in spermatogenesis when transcription has ceased and elaborate nuclear remodeling occurs [8,11–13].

IMPα proteins are best understood as adaptors that both recognize nuclear localization signals (NLSs) within specific cargo proteins and bind specifically to the IMPβ1 protein, thereby bringing together a complex that can traverse the nuclear envelope-localized nuclear pore complexes (NPCs) to enter the nucleus [14,15]. The 6 murine (7 in human) IMPα proteins [1,3] recognize both overlapping and discrete sets of cargo proteins [16]. IMPs also have important roles in cellular processes distinct from nuclear transport, including in NPC and mitotic spindle assembly, and serving as adaptors for microtubule motors [17–19].

IMPα4 (kpna4, QIP1) was initially identified as the IMPα preferentially recognizing DNA helicase Q1 [20] and p53 [21]. A "non-transport" role for IMPα4 and other IMPαs during the response of cells to stress conditions has been postulated based on analyses in HeLa cells where IMPα nuclear localization has been linked to a transcriptional function [22], while a role in embryonic stem cell (ESC) pluripotency is suggested by our recent observation that as ESCs transform from the pluripotent to differentiated state, IMPα4 relocalizes from the nucleus to the cytoplasm [23]. During spermatogenesis in adult mice, IMPα4 is robustly detectable from the spermatocyte stage onwards, with predominant nuclear localization in the post-mitotic germ cells, suggestive of a unique non-transport function in these differentiating cells [6,7].

To investigate the role of IMPα4 in the latest stages of spermatogenesis, we developed a novel overexpression transgenic mouse model in which IMPα4 overexpression is specifically restricted to the post-meiotic germ cells (spermatids) via the protamine 1 promoter. IMPα4 overexpression did not appear to disrupt normal spermatogenic progression, but conferred enhanced resistance to in vitro oxidative stress. This correlated with reduced stress resistance in

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spermatids lacking IMPα4. Importantly, this establishes a new role for IMPα4 in testicular germ cells that may be critical to protecting the germline from environmental insults.

2. Materials and methods

2.1. Generation of transgenic mice

All investigations conformed to the NHMRC/CSIRO/AAC Code of Practice for the Care and Use of Animals for Experimental Purposes and were approved by the Animal Welfare Unit of the University of Queensland, under the Queensland Animal Care and Protection Act, and the Monash University Standing Committee on Ethics in Animal Experimentation, as appropriate. The pTransgene–protamine1–EGFP–IMPα4 transgenic construct was generated by cloning a PCR-generated 650 bp fragment of the *protamine 1* promoter into the pTransgene–EGFP vector (Dr. David Sherr, Boston, USA; Accession Number AF515846). The promoter fragment was chosen to confer high-level spermatid-specific expression [24]. The IMPα4 coding sequence (296–1853 bp; accession number NM_008467; lacking the start codon) was also PCR-amplified and subcloned into the pTransgene–protamine 1–EGFP backbone in-frame with the EGFP sequence, to produce an EGFP–IMPα4 fusion protein. Construct integrity was confirmed by DNA sequencing (The Gandel Charitable Trust Sequencing Centre, Monash University). For transgene introduction, the majority of the construct backbone was removed by Sph1/Xho1 digestion, and then injected by standard pronuclear microinjection procedures into CBAxC57BL6 F2 blastocysts. The presence of the transgene was subsequently confirmed by PCR genotyping and Southern hybridization (see below). Hemizygous (Tg/+) transgenic mice were mated to generate the homozygous transgenic (Tg/Tg) lines, with the latter confirmed through genotyping of offspring and throughout ongoing breeding.

2.2. PCR genotyping

The presence and integrity of the transgene were initially established using 6 primer sets (Supplementary Table 1A), with ongoing genotyping performed with EGFP and Rn18S primer sets (Supplementary Table 1B). For genomic DNA preparation, tail biopsies (2 mm) were collected at weaning and digested in 250 μl lysis buffer (10 mM Tris–HCl (pH 8.5), 50 mM KCl, 0.1 mg/ml gelatin, 0.45% Nonidet P-40, 0.45% Tween 20, and 10 mg/ml of proteinase K) at 55 °C overnight. Lysates were centrifuged, and supernatants precipitated with 250 μl isopropanol. Following centrifugation and washing with 70% ethanol, DNA pellets were resuspended in 100 μl TE buffer, then placed at 90 °C to fully dissolve. 1 μl of DNA was subsequently used as a template for genotyping via PCR. Reactions were performed on the Applied Biosystems GeneAmp PCR system 2700 under the following conditions: 94 °C for 1 min; 35 cycles of 94 °C denaturation for 30 s, 60 °C annealing for 30 s, and 72 °C elongation for 1 min; and a final elongation at 72 °C for 7 min. Reaction products were identified following electrophoresis on 1.5% agarose/TAE gels with Generuler DNA size standards (Fermentas, Canada).

2.3. Southern hybridization

Southern hybridization was performed on DNA extracted from tails of euthanized mice. Approximately 1 cm of tail was digested overnight at 55 °C in tail buffer (1% SDS, 0.1 M NaCl, 0.1 M EDTA, 0.05 M Tris, 0.5 mg/ml proteinase K) followed by phenol and phenol:chloroform:isoamyl alcohol extractions and aqueous phase precipitation using 100% ethanol. Genomic DNA was spooled, rinsed in 70% ethanol, then resuspended in TE buffer. Genomic DNA (10 μg) was digested overnight according to the manufacturer's instructions with either BstXI, XbaI, ApaI or ApaI/BstXI (NEB, Massachusetts, USA). Digested DNA was electrophoresed on 0.8% agarose/TAE gels with Generuler DNA size standards then transferred overnight to Hybond N+ nylon membranes (Amersham/GE Healthcare, Little Chalfont, UK) in 0.4 N NaOH. Membranes were rinsed in 20× SSPE (3 M NaCl, 200 mM sodium phosphate and 20 mM EDTA, pH 7.4) and pre-hybridized in dextran hybridization mix (10% dextran sulfate, 1% SDS, 10 μg/ml denatured herring sperm DNA) for 2 h at 65 °C with rotation. Membranes were hybridized with radiolabeled probes (see below) overnight at 65 °C with rotation, then rinsed in 2× SSPE/0.1% SDS at 65 °C, and washed in 2× SSPE/0.1% SDS at 65 °C for 10 min, followed by 2 washes in 0.1× SSPE/0.1% SDS at 65 °C. Membranes were then dried, sealed in plastic and exposed overnight to X-ray film. Southern hybridization probe consisted of a BpmI (NEB) fragment spanning 240 bp of the EGFP sequence isolated from the pTransgene–PRTM1–EGFP–IMPα4 construct following restriction digestion, agarose gel electrophoresis, and purification using the QIAquick (Qiagen, Hilden, Germany) gel purification kit. Southern probes underwent random-primed labeling with ^{32}P-α[CTP] (dCTP[α-^{32}P] 3000 Ci/mmol, Perkin Elmer, Australia) using the NEBlot random primed labeling kit (NEB), according to the manufacturer's instructions. Labeled probes were purified through a QIAquick spin PCR purification column (Qiagen) and denatured for 2 min at 95 °C prior to addition to membranes in pre-hybridization solution. Following exposure, X-ray films (FujiFilm, Tokyo, Japan) were developed in a Konika Minolta developer bath, scanned, and comparative signal strength quantified using the Java-based public domain ImageJ analysis software (http://rsbweb.nih.gov/ij/).

2.4. Relative copy number estimation by Q-PCR

Genomic DNA extracted as per 2.3 above was used for Q-PCR to determine transgene copy number. Genomic DNA (6.5 ng total) was used in a reaction mix of 10 μl containing 5 μl of SYBR-Green PCR master mix (Roche Diagnostics, Castle Hill, NSW, Australia) and 500 nM each of the forward and reverse primers (Supplementary Table 1B). PCR was performed on a 7900HT real-time system (Applied Biosystems) at 95 °C for 10 min, with 45 cycles of amplification at 95 °C for 15 s, and 62 °C for 30 s. Each reaction was performed in duplicate and averaged. Amplified products were verified by melting curve analysis. Correlation of threshold values (crossing points) with target DNA levels was enabled through a relative standard curve analysis (SDS 2.3 software) generated from genomic DNA, with a ratio of EGFP:Rn18S determined for each sample.

2.5. Analysis of testis and body weights

Wildtype and transgenic mice were assessed over a range of postnatal ages for calculation of the testis:body weight ratios. Mice were killed by carbon dioxide asphyxiation followed by cervical dislocation before tissue removal. Body weights were measured immediately following euthanasia and testis weights measured on trimmed testes immediately following dissections. Data from the different ages was imported and analyzed using Graphpad Prism™ software. A 2-way ANOVA with Tukey post-test was employed to determine statistical significance.

2.6. Immunohistochemistry

Immunohistochemistry was performed as previously described [25]. Briefly, sections from mouse testes fixed in Bouins fixative were dewaxed in histosol, then rehydrated through graded ethanol washes. Sections were treated with glycine (50 mM, pH 3.5) for antigen retrieval, endogenous peroxidases were quenched with 3% H_2O_2, and following blocking, incubated overnight at room temperature without (control) or with primary antibody. Primary antibodies were goat anti-IMPα4 {Kpna4} (Abcam, Cambridge, MA, USA), or mouse anti-GFP (Roche, Quebec, Canada), used at 1:500 diluted in Tris-buffered saline (TBS: 0.05 M Tris HCl, 0.15 M NaCl)/0.1% bovine serum