ISLET-BRAIN1/JNK INTERACTING PROTEIN-1 IS REQUIRED FOR EARLY EMBRYOGENESIS IN MICE

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SUMMARY

Islet-Brain1/JNK-Interacting Protein-1 (IB1/JIP-1) is a scaffold protein which organizes the c-Jun NH2-terminal kinase (JNK), MKK7 and MLK1 to allow signalling specificity. Targeted disruption of the gene MAPK8IP1 encoding IB1/JIP-1 in mice led to embryonic death prior to blastocyst implantation. In culture, no IB1/JIP-1−/− embryos were identified indicating that accelerated cell death occurred during the first cell cycles. IB1/JIP-1 expression was detected in unfertilized oocytes, in spermatozoa and in different stages of embryo development. Thus, despite the maternal and paternal transmission of the IB1/JIP-1 protein, early transcription of the MAPK8IP1 gene is required for the survival of the fertilized oocytes.

INTRODUCTION

Mitogen-activated protein kinases (MAPKs) are key enzymes involved in diverse cellular processes in response to extracellular stimuli. In mammals, three major groups of MAPK have been identified: ERK, P38 and the c-Jun NH2-terminal kinase (JNK) (1-3). The JNK pathway is activated by many forms of stress including cytokines, heat shock or radiation. In response to these stimuli, specific MAPKKKs are activated which modulate MAPKK such as M KK4 and M KK7 which sequentially phosphorylate the JNK kinase. Once activated, the JNK phosphorylates c-Jun which in turn increases transcription activity of many target genes. The functions of the signal transduction mediated by the JNK group of MAP kinases include the control of cell survival and apoptosis, the regulation of cell proliferation and embryonic development.
morphogenesis (1-3). JNK homologs have been identified in *Drosophila* and this signaling pathway is required for embryonic epithelial cell sheet movement and planar polarity (4). In *Xenopus laevis* eggs and embryos, the JNK pathway is activated during oocyte maturation and stays constitutively activated until the early gastrula stage of embryogenesis suggesting a critical role of the kinase during oocyte maturation and embryogenesis (5).

The specificity of JNK activation and function requires the presence of the scaffold protein “c-Jun N-terminal kinase interacting protein-1” (JIP-1) (6;7). This protein was recently identified as the mammalian homolog of the yeast STE5 that functions as a scaffold protein which organizes the MAPK cascade into a specific module (8). JIP-1 binds to JNK, M KK7 and MLK1 and potentiate the JNK activation (1;6;7). JIP-1 contains a SH3 and PID domain in the carboxy-terminal part of the protein which interact with p190 RhoGEF, the reelin receptor ApoER2, the LDL receptor-related protein, megalin and more recently kinesin (9-12). JIP-1 was initially cloned from a mouse library using JNK as a bait in a two-hybrid system (6). The rat and human homolog of JIP-1 was independently identified and termed Islet-Brain1 (IB1) since it is expressed in the insulin-secreting β-cells of the pancreas and in neurons (13;14). The function of IB1/JIP-1 was partially investigated in insulin secreting cells. In these cells, activation of the JNK cascade by interleukin-1 reduces IB1/JIP-1 content and promotes apoptosis (15). Reduction of the content of IB1/JIP-1 in these cells increased phosphorylation of c-Jun and the apoptotic rate. Conversely, overexpression of IB1/JIP-1 prevented JNK-activation and apoptosis (15). The *MAPK8IP1* gene encoding the human IB1/JIP-1 was mapped to chromosome 11p11.12 (14). *MAPK8IP1* is a candidate-gene for type 2 diabetes since a missense
mutation within the coding region of this gene was linked to human diabetes in a large pedigree (16). The mutation was shown to be associated \textit{in vitro} with an accelerated apoptosis and a decreased insulin transcriptional activity (16).

Some of the physiological importance of the JNK signaling pathway has been investigated by gene targeting in mice. Mice deficient in either JNK1, 2 or 3 have no obvious phenotype while double mutants (JNK1 \textsuperscript{-/-} and JNK2 \textsuperscript{-/-}) die at mid-gestation with defective neural-tube closure due to an increased apoptosis (17;18). Mice lacking the JNK3 gene are resistant to kanaic-acid induced apoptosis in hippocampal region of the brain (19). Thus, JNK functions are important during development and may play permissive role for cell survival and/or for cell apoptosis. The selective disruption of MKK4 and c-Jun have a much more drastic phenotype than disruption of JNK1,2 or 3. Both MKK4 and c-Jun defective mice have an early embryogical lethality caused by an abnormal liver development (20;21).

Due to the pleiotrophic role of the JNK transduction signal in regulating cellular proliferation, apoptosis and tissue morphogenesis, the physiological function of IB1/JIP-1 \textit{in vivo} is unpredictable. The present study was therefore undertaken to clarify the biological role of IB1/JIP-1 using a gene targeting strategy.
RESULTS

The murine gene encoding IB1/JIP-1 was cloned from a 129/SvJ mouse genomic library. A targeting vector was designed to replace exon 3 through exon 8 of the MAPK8IP1 gene with the neomycin resistance gene (Fig 1). Three embryonic stem cell clones that had undergone homologous recombination were microinjected into C57BL/6 host blastocysts. One clone produced a germline chimeric male resulting in offspring heterozygous for the disrupted allele (Fig 1). The heterozygotes IB1/JIP-1+/- mice were intercrossed in two different genetic background (C57BL/6 and 129 SvJ) and the progeny genotyped. No mutant IB1/JIP-1-/- were found in live-born progenies of IB1/JIP-1+/- intercrosses, indicating that the complete absence of IB1/JIP-1 caused embryonic lethality. Embryos were genotyped at various stages of gestation to determine when embryonic death occurred. As shown in Table 1, a total of 148 pups were analyzed and one single mutant IB1/JIP-1-/- embryo was detected at day 3.5 post coitum. A total of 98 pups (66%) were heterozygous (IB1/JIP-1+/-) whereas the remaining 49 pups (33%) were wild-type animals (IB1/JIP-1+/+), which is in accordance with the expected Mendelian distribution.

To test whether IB1/JIP-1-/- embryos survive to the blastocyst stage, embryos were isolated from IB1/JIP-1+/- intercrosses 24 hours post-coitum and grown in culture up to 5 days. For genotyping the embryos, we used a PCR strategy to identify the wild-type and disrupted allele by amplifying DNA fragments located either in exons 4 and 5 of the murine MAPK8IP1 gene or in exon 3 and in the neomycin gene. As shown in Fig 2, the genomic PCR amplification using the primers located in exons 4 and 5 identified a 406 bp fragment which includes the intronic sequence of 170 bp present between the two exons (lane 1 of Fig 2). Embryos were genotyped at
two-cells, morula and blastocyst stages. A total number of 41 embryos from 3
different litters were genotyped: 21 were heterozygotes (IB1/JIP-1\(^{+/−}\)) and 20 were
wild type (IB1/JIP-1\(^{+/+}\)). The absence of null mutant embryos (IB1/JIP-1\(^{−/−}\)) in these 41
embryos was suggestive of a very early embryological death due to the absence of
the MAPK8IP1 gene.

Since lethality occurred early during embryogenesis, we then evaluated
whether MAPK8IP1 gene expression could be detected in these early developmental
stages. RNA from two-cell embryos and blastocysts were isolated and RT-PCR was
performed using the oligonucleotides located in exons 4 and 5. The RT-PCR strategy
identified a 236 bp amplicon which correspond to the IB1/JIP-1 RNA lacking the
intronic sequence located within the two exons (Fig 2). The IB1/JIP-1 transcript was
detected in the two-cell embryos indicating that either early transcription occurred
during the process of embryonic genome activation and/or that IB1/JIP-1 transcripts
were already present in the unfertilized oocyte (Fig 2). IB1/JIP-1 mRNA was also
found to be present in testis by Northern blot analysis (data not shown) and
immunocytochemistry studies were then performed in unfertilized oocytes, mature
spermatozoa and during various stages of embryonic development using affinity-
purified antibodies raised against IB1/JIP-1 (Fig 3A-J). IB1/JIP-1 was
immunodetected in zygote in pronucleate stage, in blastocysts and in mature
spermatozoa (Fig 3A-J). To explore the possibility that the disrupted allele could be
associated with a loss of IB1/JIP-1 protein in a fraction of the spermatozoa, aliquots
of freshly isolated spermatozoa were smeared on microscope slides. After
immunostaining using the anti-IB1/JIP-1 antibodies, we counted the spermatozoa for
positive and negative staining. Over 600 separate mature spermatozoa isolated
form three heterozygous animals (IB1/JIP1+/−) were evaluated and all expressed IB1/JIP-1. This result indicate that the presence of IB1/JIP-1 in all mature spermatozoa of heterozygous mice (IB1/JIP1+/−) is the consequence of a translated RNA transcribed during a dizygotic stage of spermatogenesis. Similarly, we detected the presence of IB1/JIP-1 in unfertilized oocytes, even when studying heterozygous animals (IB1/JIP1+/−) (data not shown). These results indicated that IB1/JIP-1 may play a role during fertilization, spermatogenesis and oogenesis, possibly linked to the activated JNK pathway which was described in Xenopus laevis to be selectively activated during oocyte maturation (5).

Given the potential role of IB1/JIP-1 during spermatogenesis or oogenesis, we evaluated whether the IB1/JIP-1 haploinsufficiency was associated with an alteration of fertility. In a separate set of experiments, we therefore examined the genotypic ratios of the progeny obtained from male heterozygous mice (IB1/JIP-1+/−) with female heterozygous mice (IB1/JIP-1+/−), from male heterozygous mice (IB1/JIP-1+/−) with wild type female (IB1/JIP-1+/+) and conversely the progeny of female heterozygous mice (IB1/JIP-1+/−) with wild type male (IB1/JIP-1+/+). As shown in Table 2, the genotypic ratios of these intercrosses were in accordance with the Mendelian distribution, thus excluding an impaired fertility linked to the haploinsufficiency of the IB1/JIP-1 protein.

We next examined by Western blotting the IB1/JIP-1 content in wild-type and IB1/JIP-1+/− deficient mice. IB1/JIP-1 content was decreased by approximatively 45% in the brain of heterozygous IB1/JIP-1+/− mice. Figure 3K depicts the IB1/JIP-1 content in brain during various development stages. Once normalized with the tubulin content, the IB1/JIP-1 reduction found in the heterozygous IB1/JIP-1+/− mice
was more apparent prior to day 15 post natal. Glucose homeostasis was also monthly evaluated on regular chow in wild-type and heterozygous mice during 6 months in two different genetic background (C57BL/6 and 129 SvJ). Fasting plasma levels of glucose and insulin were similar between wild-type and heterozygous IB1/JIP-1+/− mice (data not shown). Lastly, pancreas histology was performed and no obvious difference was observed in wild-type and heterozygous IB1/JIP-1+/− animals.
DISCUSSION

We have found that disruption of the murine gene MAPK8IP1 encoding IB1/JIP-1 leads to an early embryological death, prior to the blastocyst implantation. In culture, no complete null mutant IB1/JIP-1−/− was identified as early as two-cell stage suggesting the requirement of IB1/JIP-1 in the first cell cycles. In accordance to the potential importance of the scaffold protein IB1/JIP-1 in early embryogenesis, we detected mRNA encoding the protein from two-cell and blastocyst stage. IB1/JIP-1 RNA and protein were also detected in unfertilized oocytes and in spermatozoa. Despite the expression of IB1/JIP-1 in unfertilized oocytes and testis, we did not find an alteration in fertility in heterozygote IB1/JIP+/- mice. The early death of fertilized null mutant IB1/JIP−/− oocyte indicates therefore that de novo transcription of the MAPK8IP1 gene is required for the survival of the fertilized oocyte.

IB1/JIP-1 functions as a scaffold protein which organizes the JNK signal transduction pathway (1;2;7). As mentioned, the JNK pathway plays several roles during embryogenesis (1-3). In Xenopus laevis eggs, the JNK pathway is activated during oogenesis and stays activated in early embryogenesis (5). The selective disruption of genes involved in the control of the JNK activity leads, for several of them, to embryological death (17;18;20;21;22). However, the embryological death observed in IB1/JIP-1−/− embryos is the earliest one observed in the JNK cascade indicating the critical role of early transcription of MAPK8IP1 during the first cell cycles. These early cell stages are characterized by the conversion of the two parental genomes into a single embryonic genome with the initiation of the first divisions(24). Early embryonic cells proliferate rapidly with doubling time as short as
2 hours at day 6.5 p.c (24). The oocyte cytoplasm plays a critical role by controlling and reprogramming nuclear function. The maternal cytoplasmic functions include the repression of genes in the first 10 to 20 hours post fertilization followed by reactivation of appropriate specific genes. Since IB1/JIP-1 is detected in wild type and heterozygote IB1/JIP-1+/− oocyte, it is unlikely that cytoplasmic IB1/JIP-1 contributes to the reprogramming of the nuclear function. On the other hand, it has been shown that too early transcription or an inappropriate temporal transcription could induce embryonic lethality (24). A non-coordinated expression of specific genes during this process of embryonic genome activation may result in loss of normal embryogenesis. The earliest steps of embryonic genome activation involved decondensation of nuclear sperm, formation of the maternal and paternal pronuclei and followed by an alteration of chromatine structure to initiate early gene transcription. The disruption of any of these genes events may lead to inappropriate cell division and differentiation. Our data established that the MAPK8IP1 gene encoding IB1/JIP-1 is one of these critical genes for which early transcription is required to allow early embryo cleavage and survival. IB1/JIP-1 transcription in these stages may be required to allow proper JNK signaling to occur and/or to prevent an inappropriate level of JNK activation. In ex vivo experiments, it was shown that low cellular content of IB1/JIP-1 is associated with an increased apoptosis rate while increasing experimentally the cellular IB1/JIP-1 content could confer protection to stress-induced apoptosis(15).

In conclusion, our data indicates that despite the maternal and paternal transmission of IB1/JIP-1 in heterozygote IB1/JIP-1+/− mice, the level of transmitted
protein is unable to allow proper cell survival. The \textit{MAPK8IP1} gene encoding IB1/JIP-1 needs therefore to be transcriptionally active during the first cell cycles.
MATERIALS AND METHODS

Generation of IB1/JIP-1 mutant mice

A rat IB1 cDNA probe was used to isolate a 13.5 Kb genomic clone from a 129 SvJ mouse library. The murine gene organization was partially characterized and a targeting vector was constructed by replacing a portion of exon 3 through exon 8 of the murine IB1/JIP-1 gene with a neomycin resistance cassette which contained the PGK-1 promoter and the poly(A) site. A thymidine kinase cassette was also inserted in the targeting vector for negative selection. Homologous recombination was obtained in HM-1 embryonic stem cells by transfection of the Xba1 linearized vector. G418 and gancyclovir resistant colonies were screened by Southern blot analysis following a Xba1 and Sal1 digestion, using the indicated probe. The recombined ES cells were detected by the presence of a 7 kb digested DNA fragment together with a 13 kb genomic fragment corresponding to the endogenous IB1/JIP-1 gene. Four clones were injected into C57BL/6 host blastocysts. Embryos were transferred into the uterus of 2.5-day post-coital pseudopregnant CD-1 females. Resulting chimeric mice were bred with C57BL/6 mice and a single clone produced a germline transmission. Heterozygous males of the F1 offspring were repeatedly backcrossed to wild-type dams of the strains C57BL/6 and the 129SvJ for three generations, in order to investigate the effects of the mutation into two different genetic backgrounds. Intercrosses between N3 heterozygous were performed in the C57BL/6 line, as well as in the 129SvJ and between the two strains, in order to test for hybrid vigour.
**Mouse Oocytes and Zygotes**

Six to 9-week-old IB1/JIP-1 heterozygous females were stimulated (day 1) with one peritoneal injection (10 IU/0.2mL) of FSH (Folligon; Intervet AG, Pfäffikon, Switzerland) followed on day 3 by a second injection (10 IU/0.2mL) of hCG (Pregnyl; Organon, Zürich, Switzerland) to induce ovulation (26). For the recovery of oocytes, females were killed 13 hours after hCG administration by cervical dislocation. For the recovery of zygotes, females were mated after hCG injection with 8- to 16-week-old heterozygous males and killed 24 hours after hCG injection.

**Immunolabeling of testis, spermatozoa and zygotes**

For light microscopy studies, mouse gonads were rapidly excised and cut in fragments that were quickly frozen in 2-methylbutane precooled in liquid nitrogen. Testis fragments were frozen in OCT medium (Miles Inc., Elkhart, IN, USA) and cryo-sectioned at 5 µm thickness. Aliquot of freshly isolated spermatozoa were smeared on precleaned microscope slides, allowed to air dry 2 h, fixed in methanol for 10 min, and left to air dry before being rinsed in PBS. To improve the staining of zygotes, we drilled the zona pellucida by laser light (27).

**Western blot analysis**

Whole brains were obtained from mice at different developmental stages. A total of 100 µg of the extracts were separated using a 10% SDS-PAGE and the protein were transferred to nitrocellulose. Detection of IB1/JIP-1 was performed using the IB1/JIP-1 antiserum (13;25) in 3/10'000 dilution.
Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification

Mouse zygotes (two-cell or blastocyst stages) were rapidly frozen in liquid nitrogen and homogenized in sterile water. The lysate containing the RNA was reverse-transcribed using oligo dT primers and 0.2 mM of each dNTPs in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl$_2$ and 0.01% gelatin. The reverse-transcribed products was used for PCR reaction in the presence of 20ng sense and antisense primers (Microsynth GmbH, Switzerland). The antisense primer sequence for mouse IB1/JIP-1 located in exon 5 was 5'-CTC GAG CCG CAC ATC TGC C-3'; the sense primer, located in the exon 4, was 5'-AAG CAC AGT TGG CAG GAC CG-3'. These primers generated an amplicon of 275 bp. A second step of amplification was performed using a nested set of primers located within the first amplified fragment of 275 bp. The antisense primer located in exon 5 was 5'-TGA TAG TGG ATT CGA TCT C-3', the sense primer, located in exon 4 was 5'-TGT GTC TCG ATC CTC C-3'. These primers generated a fragment of 236bp. As negative controls, we submitted to PCR amplification samples of total RNA that had not been reverse-transcribed. Another control was obtained by PCR amplification of mice genomic DNA. The primers generated a fragment of 406 bp due to the presence of an intron of 170 bp located between exon 4 and 5.
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LEGENDS TO FIGURES

**Fig 1. Gene targeting of the IB1/JIP-1 locus.**

Wild-type IB1/JIP-1 gene organization and the coding exons (E3 and E8, striped boxes) are schematically depicted in the upper line. A 1 kb cDNA probe (dark box) was designed 5’ of the recombined sequence and used for Southern blot analysis. Middle line: targeting vector including the herpesvirus tk gene (PGK-TK) and the neomycine gene (PGK-NEO). Lower line: disrupted IB1/JIP-1 allele with insertion of the neomycine resistance gene. Xb:XbaI, Xh:XhoI, N:NotI, B: BamHI, P: PstI, S: Sall. *Inset:* Southern blot analysis of genomic DNA obtained from two wild-type (IB1/JIP-1\(^{+/+}\)) and two heterozygous (IB1/JIP-1\(^{1+/-}\)) mice. The disrupted allele gives a 7 kb Xb-S digested fragment while the wild-type allele is 13 kb.

**Fig 2. IB1/JIP-1 mRNA is detected during early embryogenesis.**

Reverse-transcribed RNAs, extracted from oocytes and zygotes at two different developmental stages (two-cell embryos and blastocysts) were PCR amplified using primers specific for the MAPK8IP1 gene. This procedure resulted in the amplification of a single 236 bp product, seen after ethidium bromide staining. PCR amplification of mouse genomic DNA resulted in the amplification of a 406 bp product. Lane 1: PCR from mouse genomic DNA, lane 2: PCR from mouse genomic DNA and RT-PCR from wild-type oocytes RNA to show the size difference between genomic and spliced IB1/JIP-1 RNA.
Figure 3 A-J: Distribution of IB1/JIP-1 in the testis, spermatozoa and zygotes

IB1/JIP-1 is immunodetected in mature spermatozoa with a cellular expression restricted to the tail. (A: low magnification, B: phase-contrast, C: higher magnification, D: phase-contrast, E: positive immunostaining for IB1/JIP-1 is restricted to the tail but not the head and intermediary piece of the mature spermatozoa, F: phase-contrast)

Immunodectection of IB1/JIP-1 in zygote at pronucleate stage (G and H: phase-contrast), and at expanded blastocyst stage (I and J: phase-contrast)

Bar = 55 µm in A and 5.5 µm in C and E and 2.5 µm in G and I.

Figure 3K: IB1/JIP-1 content in brain during development

Western blot analysis of IB1/JIP-1 of brain extracts obtained at day 0 (P=), day 5 post natal (P5), day 15 (P15) and in adult stage from wild-type and heterozygous IB1/JIP-1+/− mice. Tubulin (TUB) was subsequently immunodetected on the same blot for normalization. IB1/JIP-1 content in brain peaked within the first 15 days (25) and its normalized content was reduced by 40 to 50% in the heterozygous IB1/JIP-1+/− mice (+/+ = wild-type animals and +/- = heterozygous IB1/JIP-1+/− ).
**TABLE 1:**

Genotypic ratios of embryos from IB1/JIP-1*+/- heterozygotes intercrosses

<table>
<thead>
<tr>
<th>Age*</th>
<th>No. litters</th>
<th>No. pups</th>
<th>No. of each genotype</th>
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<tr>
<td></td>
<td></td>
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<td>+/+</td>
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<tr>
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</tr>
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<td>E12.5</td>
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</tr>
<tr>
<td>P 28</td>
<td>10</td>
<td>72</td>
<td>23</td>
</tr>
<tr>
<td>Total (%)</td>
<td>22</td>
<td>148</td>
<td>49(33%)</td>
</tr>
</tbody>
</table>

*E3.5, E9.5, and E12.5 represent day 3.5, 9.5, and 12.5 of gestation; P 28 represents 28 days after birth. +/+, Wild-type; +/-, heterozygotes; +/-, homozygous for the IB1/JIP-1 allele.
## TABLE 2:

**Genotypic ratios of the progeny obtained by intercrossing male or female IB1/JIP-1\(^{\text{hr}}\) heterozygotes**

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<thead>
<tr>
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<th>Female</th>
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<th>average of pups/litter</th>
<th>offspring genotype</th>
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<td>+/-</td>
<td>88</td>
<td>6.3 ± 2.1</td>
<td>296 (53%) 261 (47%) 0</td>
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<tr>
<td>+/-</td>
<td>+/-</td>
<td>22</td>
<td>5.6 ± 1.9</td>
<td>56 (46%) 67 (54%) 0</td>
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<td>+/-</td>
<td>21</td>
<td>7.2 ± 2.0</td>
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Fig. 2

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<tr>
<td></td>
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<td></td>
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Fig. 3 k

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<th></th>
<th>P0</th>
<th>P5</th>
<th>P15</th>
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<tr>
<td>+/-</td>
<td>+/-</td>
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IB1/JIP-1
TUB
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