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**Title page:**

**The expression of the testis-specific Dyrk4 kinase is highly restricted to step 8 spermatids but is not required for male fertility in mice**

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**Summary:**

Members of the dual specificity tyrosine phosphorylated and regulated kinase family (Dyrk) were shown to have a highly testis-abundant or testis-restricted expression pattern. Moreover, for some members of the family an involvement in gene expression regulation by phosphorylating transcription factors has been shown. Since little is known about the complex regulation of germ cell differentiation in spermatogenesis, we analysed the possible involvement of Dyrk kinases in this process. ISH experiments showed specific distribution of Dyrk kinases mainly in postmeiotic germ cell. We identified Dyrk4 as testis specific kinase with a very restricted expression to stage VIII postmeiotic spermatids. In vitro and in vivo experiments proved the enzymatic activity and suggested the cytoplasmatic localisation of Dyrk4. Finally, analysis of a Dyrk4 deficient mouse line showed that Dyrk4 is dispensable for male fertility hence suggesting a functional redundancy of some Dyrk isoforms during spermiogenesis.

Keywords: Dyrk; Dyrk4; male fertility; testis specific kinase

Dyrks are an emerging family of serine and threonine directed kinases that play key roles in cell proliferation, survival and development. Five mammalian Dyrk genes have been described so far, namely Dyrk1A, Dyrk1B, Dyrk2, Dyrk3, and Dyrk4 (Becker and Joost 1999). The Dyrk kinase family is characterized by activation through autophosphorylation of the second tyrosine in a conserved "YT/QY" motive (Lochhead, Sibbet et al. 2005) and by a unique conserved "Dyrk homology box" (DH-Box) of unknown functionality immediately located 5' of the kinase domain (Becker and Joost 1999). Dyrk1A, the founding member of this kinase family, was first described as the mammalian orthologue of the drosophila gene minibrain (Kentrup, Becker et al. 1996). Together with Dyrk1B, its closest homologue (Leder, Weber et al. 1999), these two kinases define a subgroup within the Dyrk family displaying specific properties. Both kinases do possess a functional, bipartite NLS sequence 5' of their kinase domain resulting in nuclear localisation upon recombinant expression (Becker, Weber et al. 1998; Leder, Weber et al. 1999), and a PEST domain controlling protein degradation in their N-terminus. In contrast Dyrk2, Dyrk3 and Dyrk4, the members of the second subgroup, are devoid of any additional domains and consequently expected to be cytosolic. Interestingly, recombinant Dyrk2 (Becker, Weber et al. 1998) was localized in the nucleus and the cytosol, and Dyrk3 (Lord, Creasy et al. 2000) shows nuclear localisation. The subcellular localisation of

Dyrk4 has not been clarified so far. Upon recombinant expression, all Dyrks except Dyrk4 have been shown to be active kinases phosphorylating histones (Becker, Weber et al. 1998) and specific substrate peptides (Himpel, Tegge et al. 2000; Campbell and Proud 2002).

Most interestingly, except for the ubiquitous expression of Dyrk1A, all other Dyrk kinases show a testis-predominant or testis-specific expression. In a comprehensive Northern Blot experiment, the expression of Dyrk1B appeared to be restricted to skeletal muscle and testis in human and rat (Leder, Weber et al. 1999). Also by Northern Blot, Dyrk2, Dyrk3 and Dyrk4 expression has shown to be restricted to testis in rats and to be up-regulated upon testicular maturation (Becker, Weber et al. 1998). In addition, a more thorough analysis showed Dyrk3 to be expressed in erythroid progenitor cells (Lord, Creasy et al. 2000).

Functional data indicate an involvement of Dyrk1A in Down syndrome (Altafaj, Dierssen et al. 2001) and Dyrk1B in survival of cancer cells (Mercer and Friedman 2006), both by phosphorylation and regulation of transcription factors (Woods, Cohen et al. 2001; von Groote-Bidlingmaier, Schmoll et al. 2003; Sitz, Tigges et al. 2004). Cytosolic Dyrk2 has been implicated in down-regulation of protein translation (Woods, Cohen et al. 2001) and degradation of proteins (Nishi and Lin 2005). Interestingly, Dyrk1A (Yang, Ahn et al. 2001) and Dyrk3 (Li, Zhao et al. 2002) have also been described to directly or indirectly stimulate phosphorylation and transcriptional activity of the cAMP response element binding protein (CREB) supporting speculations for an important function of the Dyrks within the transcriptional control of germ cell development and differentiation.

To get more insight into the potential function of the Dyrks during the process of spermatogenesis and spermiogenesis, we thoroughly examined the cellular distribution pattern of all Dyrk kinases in murine testis, and showed their stage-

specific distribution in postmeiotic germ cells. Furthermore, we showed Dyrk4 to be an enzymatically active kinase which is located in the cytosol upon recombinant heterologous expression. Based on its very restricted testicular expression pattern, Dyrk4 was considered as a potential target enzyme for male fertility control. We therefore functionally analysed the kinase in a Dyrk4 knock-out mouse. The examination of the mouse phenotype did not reveal any functional defect in spermatogenesis, which might be due to functional redundancy.

## Results

### **Expression analysis of the Dyrk kinases in the rat spermatogenesis via *in situ*-hybridisation**

To narrow down the putative function of distinct members of the Dyrk-kinase family in the process of spermatogenesis a thorough expression analysis was carried out in rat testis via *in situ*-hybridisation (ISH). To identify the different stages of spermatogenesis in which Dyrk kinase mRNAs are expressed, we employed a novel one-step procedure. Development of the ISH signal was followed by a treatment with a PNA-Alexa conjugate and propidium iodine (PI). PNA, a peanut agglutinin, specifically binds to sugar residues exposed in the acrosome and its conjugated fluorophore Alexa thereby allowing an easy visualisation of the acrosome (Aviles, Castells et al. 1997; Maier, Medrano et al. 2003). Additional treatment with PI resulted in a specific staining of the nuclei. We then used two different merged pictures for the expression analysis: ISH/PNA-Alexa provided us with detailed localisation of positive cells within the seminiferous tubules (due to space constraints

this overlay is not presented in Figure 1) and PNA-Alexa/PI allowed for an accurate staging of the respective tubules.

With the exception of Dyrk1A, which does not show detectable expression in the testis by ISH, all other homologues have a highly specific expression pattern in germ cells. Dyrk1B, starting in late pachytene spermatocytes (stage XI - XII) and stopping in step 16 spermatids (stage II-III), shows the longest expression period during spermatogenesis (see Fig. 1 A). Expression of Dyrk2 starts in step 6 spermatids (stage VI) and ends at step 11 (stage XI) (Fig. 1 B). Transcripts of Dyrk3 are first detectable in late pachytene spermatocytes (stage XII), expression abruptly stops in step 7 spermatids (stage VII) (Fig. 1 C). Dyrk4, the Dyrk homologue with the most restricted expression, is only detectable in spermatids at step 8 (stage VIII) (Fig. 1 D). To summarize, four of the five Dyrk homologues are expressed during the process of spermatogenesis. They all have a unique expression pattern, with Dyrk1B being the kinase with the broadest expression and Dyrk4 with highest specificity.

### **Dyrk4 is exclusively expressed in the testis**

The exclusive expression of Dyrk4 in only one stage of spermiogenesis is suggestive of a specific function in this process. Furthermore, studies with a restricted number of tissue samples indicated a predominant expression of Dyrk4 in the testis of rats (Becker, Weber et al. 1998). We therefore focused on Dyrk4 for further functional analysis. To confirm the testis-specific expression pattern, we performed quantitative RT-PCR on a comprehensive collection of murine tissues. Figure 2 illustrates the predominant expression of Dyrk4 in the murine testis confirming the data previously described for the rat.

### **Protein kinase activity and cellular localisation of Dyrk4**



Dyrk4 is the only kinase of the Dyrk kinase family for which activity has not been described yet. In silico analysis validated the presence of three general motifs within the catalytic domain which are thought to be critical for catalytic function of kinases, the VAIK, the HRD and the DFG motif (Hardie and Hanks 1995). Nevertheless, to ensure that Dyrk4 is truly an active kinase the coding sequence of the human Dyrk4 cDNA has been cloned and expressed as a GST-fusion protein in SF9 cells, either in its native sequence or bearing an inactivating point-mutation (K133R). Both GST-fusion proteins were expressed at expected size as shown in Figure 3 A. The myelin basic protein (MBP) is subjected to phosphorylation by the majority of the known non-receptor protein kinases; hence MBP is used as a standard substrate in most kinase assays. The native form of Dyrk4 is catalytically active as demonstrated by phosphorylation of the MBP substrate as well as its autophosphorylation (Fig. 3 A, right panel). As expected, the substitution of lysine at position 133 by an arginine residue abolishes its activity (see Fig. 3 B).

The presence of nuclear localization signals (NLS) in the mRNA sequences of Dyrk1A and 1B predicts these kinases to show a nuclear localisation, while Dyrks 2, 3 and 4 lacking NLS are expected to be cytosolic. Experimental data indeed confirmed the nuclear localisation of Dyrk1A and Dyrk1B and surprisingly also for Dyrk3 (Lord, Creasy et al. 2000), while Dyrk2 was shown to be cytosolic (Becker, Weber et al. 1998; Leder, Weber et al. 1999). The localisation of Dyrk4 has not been described so far. We therefore transfected Dyrk4 into eukaryotic COS7-cells and localised the proteins via immunohistochemistry directed against their C-terminally attached myc-tags. Figure 3 C shows that Dyrk4 is exclusively restricted to the cytosol. This localization is independent of its catalytic activity, as Dyrk4-K133R shows the same subcellular distribution. To summarize, Dyrk4 is a catalytically active, cytosolic kinase.

***Dyrk4 deficient mice***

We obtained a *Dyrk4* deficient mouse line from Deltagen Inc. and confirmed the correct targeting of the *Dyrk4* locus by Southern blot analysis (supplementary figure 1 A and C). To validate the lack of *Dyrk4* mRNA in the homozygous knockout mice we employed a RT-PCR and a Northern blot strategy. The absence of *Dyrk4* transcripts was confirmed as depicted in figure 4 A (Northern blot, supplementary figure 1 B).

Mice deficient for *Dyrk4* are viable and heterozygous matings resulted in almost the expected distribution according to Mendel (1:2:1). In detail, taken 135 newborns into account, we obtained 49% *Dyrk4*<sup>+/+</sup>, 23.5% *Dyrk4*<sup>-/-</sup> and 27.5 *Dyrk4*<sup>+/-</sup> mice.

The *Dyrk4* knockout construct expresses the reporter gene  $\beta$ -galactosidase under the control of the native *Dyrk4* promotor. This enables us to detect the reporter gene protein with immunohistochemistry (IHC) exactly in those germ cell stages where native *Dyrk4* protein would have been originally expressed. Figure 4B-D shows the detection of  $\beta$ -galactosidase in heterozygous *Dyrk4* mice. For better orientation, the acrosome and the nuclei are visualized by PNA-Alexa and PI, respectively. In accordance with our ISH data, the translation product of the reporter gene as an indicator of the of native *Dyrk4* protein occurs only in postmeiotic, elongated spermatids. We also analysed a representative number of mice with respect to normal spermatogenesis and spermiogenesis by classical histopathology. Contrary to our expectation, morphology of testes and epididymides from *Dyrk4*<sup>-/-</sup> mice was normal as compared to wild type littermates (Fig. 4E-J). Progression of spermatogenesis and spermiogenesis, as analysed by comprehensive staging, and the appearance and quantity of epididymal spermatozoa are apparently unaffected.

Deletion of genes expressed in spermiogenesis might also result in normal testicular and epididymal histology, but showing severe functional defects at the sperm level (Esposito, Jaiswal et al. 2004; Schuh, Cartwright et al. 2004). We therefore analysed a potential involvement of Dyrk4 in sperm mobility by computer assisted sperm analysis (CASA) (Amann and Katz 2004). By use of this technique, we could show that kinematic parameters such as Straight-line Velocity (Dyrk<sup>+/+</sup>:  $70.7 \pm 13.4 \mu\text{m/s}$  versus Dyrk<sup>-/-</sup>:  $62.4 \pm 6.0 \mu\text{m/s}$ , mean  $\pm$  SD, wt: n=3, -/-: n=6) and Straightness (Dyrk<sup>+/+</sup>:  $67 \pm 5 \%$  versus Dyrk<sup>-/-</sup>:  $61 \pm 4 \%$ , mean  $\pm$  SD, wt: n=3, -/-: n=6) from spermatozoa released from the cauda epididymides of wildtype and Dyrk4 deficient mice did not differ. Furthermore, no effect of Dyrk4 deficiency on the percentage of motile spermatozoa (Dyrk<sup>+/+</sup>:  $60 \pm 10 \%$  versus Dyrk<sup>-/-</sup>:  $66.7 \pm 15.1 \%$ , mean  $\pm$  SD, wt: n=3, -/-: n=6) as well as on the overall quantity and quality of intact sperm head morphology (Dyrk<sup>+/+</sup>:  $92.4 \pm 2.9 \%$  intact versus Dyrk<sup>-/-</sup>:  $87.7 \pm 5.2 \%$  intact, mean  $\pm$  SD, wt: n=3, -/-: n=6) released from the cauda epididymides was found. Altogether none of the investigated traits of spermatozoa released from the cauda epididymides differed between wildtype and Dyrk4 deficient mice.

Even though no obvious differences between wildtype and Dyrk4 deficient mice have been observed in means of testicular and epididymal histology and sperm morphology and activity, it could not be excluded that the absence of Dyrk4 may cause infertility or at least a sub-fertility by interfering with later stages of fertilization, e.g. sperm-egg recognition and fusion.

To assess fertility, we performed a standardized mating test pairing five age matched male Dyrk4<sup>+/+</sup> and Dyrk4<sup>-/-</sup> mice with two wildtype females each. Both genotypes performed equally in the test with 8 pregnant female mice (male fertility rate of 1.6) within each group. Also the litter size per pregnant female shows no significant differences, with an average of 8.3 litters for Dyrk4 deficient and 8.6 litters for

wildtype male mice. These results showed that fertility of Dyrk4  $-/-$  mice is not different from wt mice.

## Discussion

Available data on the Dyrk family of kinases suggest their involvement in spermatogenesis and sperm function. This hypothesis is supported by the analysis of the expression pattern revealing a predominant or even exclusive presence of Dyrk-isoforms in testis. We could show here that the mRNA of Dyrk isoforms do have a distinct individual pattern of expression in male germ cells during the later stages of spermatogenesis and spermiogenesis. Our analysis also revealed a partial overlap in expression in postmeiotic spermatids. We were interested if one particular Dyrk may have an essential function during spermiogenesis and aimed to characterize a Dyrk deficient mouse line with respect to male fertility. It has been described that Dyrk1A deficient mice are embryonic lethal and that Dyrk1B is essential for the differentiation of skeletal muscles (Deng, Ewton et al. 2003; Fotaki, Martinez De Lagran et al. 2004; Deng, Ewton et al. 2005). The predominant function of Dyrk2 and Dyrk3 has been assigned to embryogenesis/neurogenesis and erythropoiesis, respectively (Geiger, Knudsen et al. 2001; Lochhead, Sibbet et al. 2003; Zhang, Li et al. 2005).

In contrast to these well studied Dyrk isoforms, barely no data, apart from its raw sequence, were available for Dyrk4. We hypothesized a crucial role of Dyrk4 in spermiogenesis based on its highly restricted testicular expression pattern turning it into a interesting candidate for a knockout analysis. Consequently, we analysed a Dyrk4 deficient mouse line with respect to the potential involvement of the kinase in spermiogenesis and sperm function. However, Dyrk4 deficient mice are fertile without

any obvious dysfunctions in spermatogenesis, sperm motility and fertilization. One explanation might be that another Dyrk isoform having an overlap in the expression pattern functionally compensates the lack of Dyrk4. The analysis of the expression pattern of the other Dyrk isoforms revealed an overlap in mRNA expression at stage eight for Dyrk1B, Dyrk2 and Dyrk4 (Fig. 5, for an overview see Fig. 6). An even broader overlap in expression on protein level, including Dyrk3, is likely but could not be analysed due to the lack of appropriate antibodies. Nevertheless, since Dyrk4 (Fig.2) and Dyrk2 (Becker and Joost 1999) are, in contrast to the nuclear expressed Dyrk1A/B (Becker and Joost 1999) and Dyrk3 (Lord, Creasy et al. 2000), localised to the cytoplasm functional compensation might most likely occur between these two isoforms.

Redundancy describes the phenomenon that two or more gene products are performing the same or overlapping function in a given physiological or cellular context. Inactivation of one of these genes is compensated by its counterpart and has little or no effect on the biological phenotype (Nowak, Boerlijst et al. 1997; Krakauer and Nowak 1999). Redundancy seems to be a widespread concept in genomes of higher organisms (Brookfield 1997; Brookfield 1997; Johnson and Brookfield 2003). Examples of functional redundant genes come from numerous studies of developmental biology (Wang, Schnegelsberg et al. 1996), immunology (Schorle, Holtschke et al. 1991), neurobiology (Pekna, Hietala et al. 1998), the cell cycle (Roche, Fumagalli et al. 1995) and reproductive biology (Escalier 1999; Escalier 2001). Analysis of knockout mice revealed unexpected redundancy of some germ cell expressed genes (Escalier 1999; Escalier 2001). For example, it was shown that knockout mice for the germ cell expressed genes *Tnp2* (Adham, Nayernia et al. 2001), *Acr* (Baba, Azuma et al. 1994) and *Smcp* (Nayernia, Adham et al. 2002) on

mixed genetic background are fully fertile. However, double deficient mice for *Tnp2* and *Smcp* or *Smcp* and *Acr* are nearly infertile, illustrating that these gene products do perform overlapping tasks that are essential for the development of functional sperm (Nayernia, Meinhardt et al. 2003).

The homeodomain-interacting protein kinases (HIPKs) belong to the larger family of the Dyrks and consequently are closely related to *Dyrk1-4* (Becker and Joost 1999). Interestingly, it has been shown recently that even though *Hipk1* and *Hipk2* single deficient mice are viable and fertile, *Hipk1* and *Hipk2* double deficiency leads to an embryonic mortality at mid-gestation (Isono, Nemoto et al. 2006). This indicates that members of the *Dyrk* superfamily do act in a functional redundant manner.

Nevertheless, the highly specific expression of *Dyrk4* in the testis combined with its presence only in late stages of spermiogenesis renders the question what its function in this process might be or what signalling cascade it is part of. In general, an essential involvement of kinases in postmeiotic processes has been reported frequently. For example *CaMK4*, a multifunctional serine/threonine protein kinase, is involved in post-meiotic chromatin condensation. Disruption of the *Camk4* gene in mice results in male sterility, impairment of spermatogenesis and abnormal sperm head morphology (Wu, Ribar et al. 2000). Recently, similar observations were made in mice with targeted disruption of the small serine/threonine kinase (*SSTK*) gene (Spiridonov, Wong et al. 2005) which is a member of the testis-specific serine/threonine kinase (*TSSK*) family. Targeted deletion of the *SSTK* gene in mice resulted in male sterility due to profound impairment in motility and morphology of spermatozoa. Interestingly, *Dyrk3* and *Dyrk 1A* (Li, Zhao et al. 2002; Chen, Lin et al. 2005) as well as members of the *TSSK* family have been described to be involved in

gene regulation through direct or indirect phosphorylation of the CREB transcription factor (Yang, Ahn et al. 2001). Variants of CREB and CREM have been shown to be key regulators of a tight and reciprocal interaction between Sertoli and germ cells which is indispensable for the remarkable biochemical and morphological remodelling of the germ cell to form a spermatozoon (Don and Stelzer 2002). While CREM $\tau$ , being the main regulator of transcription in early spermatids, is rather activated by co-expression of ACT (activator of CREM in testis) (Fimia, Morlon et al. 2001), a new member of the CREB protein family present in postmeiotic germ cells, CREB3L4, might also be regulated by phosphorylation (Stelzer and Don 2002; Nagamori, Yabuta et al. 2005; El-Alfy, Azzi et al. 2006). The available data support speculations that Dyrk4 might be involved in such kind of transcriptional regulation.

However, the molecular bases are still unclear and future work has to face the open question how the Dyrks are involved in spermiogenesis. In this respect should the future analysis of Dyrk2/4 double deficient mice provide a more detailed view on the synergistic action of the Dyrk kinases and their contribution to spermiogenesis.

Finally, a more detailed analysis of the Dyrk4 dependent signal transduction cascade including a comprehensive screen for putative substrates should give more insight into the molecular function of Dyrks.

## Material and Methods

*In situ hybridization analysis.* In situ hybridization was performed following a standard procedure with digoxigenin-labelled antisense riboprobes as described in Albrecht et al, 1997 (Albrecht 1997) with minor variations. Templates used for riboprobe synthesis resembled positions 1011-1919 of entry XM\_218378 for Dyrk1B, pos. 1167-1750 of entry XM\_235179 for Dyrk2, pos. 965-1766 of entry NM\_001024767 for Dyrk3, and pos. 1622-2115 of entry XM\_232364 for Dyrk4, respectively. Testes were obtained from adult rats after perfusion of the animals with 4%PFA in PBS. Organs were fixed in Bouin-solution overnight and after dehydration transferred to paraffin. Tissue sections of 5  $\mu$ m were cleared in ProTaqClear<sup>TM</sup> 3 x 10 min, rehydrated and then denatured in 2 N HCl in PBS. Thereafter slides were washed 15 min with 2 x SSC at 70°C, subjected to proteinase K treatment (2  $\mu$ g/ml, 10 mM Tris-HCl, pH 8.0, 20min at 37°C), followed by a fixation in 4%PFA-solution at 4°C for 5 min. Sections were washed 5 min in PBS and then acetylated for 10 min in 0.25% acetic anhydride in 0.1 M triethanolamine. After 4 h of prehybridization in 50% formamide containing 5x Denhardt's solution (Sigma), 5x SSC, and 0.2 mg/ml yeast tRNA (Sigma), 0.1 mg/ml hering sperm DNA (Sigma) slices were hybridized in the same buffer containing digoxigenin-labeled probes (2 mg/ml) for 16 h at 72 °C. The slides were washed under high-stringency conditions (4x SSC for 5 min at 72 °C, 5x SSC for 1 min at 72 °C, 40 $\mu$ g/ml RNase in 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM DTT for 1 h at 37°C, 0.2x SSC for 60 min at 68 °C, and 0.2x SSC for 5 min at room temperature). After a 1h incubation in blocking reagent (Roche Molecular Biochemicals), slides were incubated for 1h with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Molecular Biochemicals, 1:5000 in blocking reagent). Color development with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate was carried out for 16 h. Finally, slides were incubated with 10  $\mu$ g/ml propidium iodide in PBS for 1 min, followed by 1h treatment with 50



µg/ml PNA-Alexa conjugate in PBS. After two brief washing steps in PBS the slides were mounted in GVA mounting solution and subjected to stage dependent expression analysis.

*Quality of spermatozoa.* 18 - 32 week old male mice were euthanized by CO<sub>2</sub>. After dissection of the cauda epididymis spermatozoa were released into 500 µl medium (MEM α (Invitrogen) supplemented with pyruvate (100 mM) and BSA (3 mg/ml)) and incubated at 37°C and 5% CO<sub>2</sub>. Spermatozoa were subjected to a computer assisted sperm analysis (CASA) (Hamilton Thorne Research, Beverly, MA, USA), to a motility assessment (%) and to a manual quantification of intact spermatozoa heads. For the analysis by CASA, spermatozoa were diluted 20-times in medium 10 minutes after collection. Subsequently, about 15 µl of the suspension were placed in a 80 µm deep sperm analysis chamber at 37°C (2X-CEL from Hamilton Thorne Research, Beverly, MA, USA). 10 digitised film sequences were recorded by the machine (60 Hz). Later the sperm tracks from the recording were analysed by CASA (Animal Motility, version 12.2 g). The settings used are as follows: frame rate: 60 Hz, number of frames analysed: 100, minimum contrast: 30, minimum size: 4, minimum number of detected data points for a track: 10.

For counting the number of spermatozoa with apparently normal sperm heads air dried sperm smears were stained with PNA-Alexa/DAPI (Invitrogen/Sigma Aldrich) diluted in PBS. Nuclear morphology and acrosomal staining were assessed by examining at least 30 (more) spermatozoa per mice.

*Histology and Immunohistochemical Analysis.* Testes and Epididymides were removed from adult mice and decapsulated prior to fixation in Bouin's solution. Fixed tissues were embedded in paraffin, sectioned, and either stained with H&E or with a rabbit anti- $\beta$ -galactosidase antibody (1:500, Invitrogen) according to the manufacturer's instruction.

*RT-PCR Analysis of Dyrk4.* Total RNA was isolated from different tissues with the TRIzol reagent (Invitrogen) and treated with deoxyribonuclease I (Invitrogen) and reverse-transcribed with oligo-(dT)<sub>20</sub> using the Superscript III RT-PCR system (Invitrogen). The cDNA synthesized from 250 ng of RNA was amplified applying the real-time SYBR Green PCR technology with the use of an ABI sequence detection system 7700 (Applied Biosystems). Dyrk4 forward primer 5'-ACCTGCCCACTTCACCCAGA-3' and reverse primer 5'-GCAGAGAAGGCTCCCACACC-3'. EF1 $\alpha$  forward primer 5'-AATTCACCAACACCAGCAGCAA-3' and reverse primer 5'-TGCCCCAGGACACAGAGACTTCA-3'. The PCR-running conditions were as followed: 10 min at 95°C followed by 40 cycles of 15 sec 95°C, 1 min 60°C.

#### *Site-Directed Mutagenesis*

The full-length coding region of the human Dyrk4 (NM\_003845) was subjected to Site-Directed Mutagenesis in order to mutate the VAIK to a VAIR motif to abolish kinase activity. Mutagenesis were done following the user manual of the QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene).

*Protein Expression* . The full-length coding region of the human Dyrk4 (NM\_003845) was amplified from a human testis cDNA pool (BD Clontech) with the forward primer

5'-CACCATGCCGGCCTCAGAGCTCAAGGCT-3' and the reverse primer 5'-TTATCATAACAATAGGGGGTAAAACGTT-3' into the gateway vector system compatible for expression in insect cells (Invitrogen). GST-hDyrk4 were expressed in SF9 cells and purified with Glutathione Sepharose 4B beads (Amersham Bioscience) following the manufacture's instructions. Expression were verified by SDS-Page (10%) followed by Western-blotting and the use of a GST specific antibody (Amersham Bioscience)

*In Vitro Assay of Protein Kinase Activity.* A sample of the recombinant kinase were incubated in phosphorylation buffer (20 mM MOPS pH 7.2, 10 mM MgCl<sub>2</sub>, 5 mM EGTA, 0,01% NP-40, 25 mM glycerophosphate, 1 μM ATP, 1 mM DTT, 1% BSA, 1mM NaF, 1 x protease inhibitor cocktail (Sigma)) for 30 min at 30°C. Substrate (MBP) were added to a final concentration of 0.04 μg/ml. Reaction products were separated by SDS-PAGE (10% gels) and visualized by autoradiography of the dried gels.

*Expression in COS-7 cells.* The full-length coding region of the human Dyrk4 (NM\_003845) was cloned into pcDNA3.1-myc/HIS-B (Invitrogen). For microscopic analysis, COS-7 cells were seeded on coverslips and transiently transfected with FuGENE 6 (Sigma). After 24 h, recombinant protein were detected with anti-myc/anti mouse-Alexa antibodies (BabCo) and the fluorescence monitored with an Olympus BX61 microscope and the software "ANALYSIS".

*Dyrk4 deficient mice.* A Dyrk4 deficient mice strain was licensed from Deltagen Inc. Exons 6 and 7, containing parts of the ATP binding domain, were replaced by a NEO-LacZ cassette. Absence of Dyrk4 transcript in mouse testis was confirmed by

multiplex PCR using the oligonucleotides Dyrk4-For 5'-AAGGGGCCCCATGCAGTATCA-3' Dyrk4-REV 5'-CGGCTTGAGGTCACAGTGGA-3' and EF1 $\alpha$  forward primer 5'-AATTCACCAACACCAGCAGCAA-3' and EF1 $\alpha$  reverse primer 5'-TGCCCCAGGACACAGAGACTTCA-3'. Total RNA was isolated from wildtype and Dyrk4 deficient mice with the TRIzol reagent (Invitrogen) and treated with deoxyribonuclease I (Invitrogen) and reverse-transcribed with oligo-(dT)<sub>20</sub> using the Superscript III RT-PCR system (Invitrogen). The PCR-running conditions were as followed: 15 min at 95°C followed by 30 cycles of 30 sec 95°C, 30 sec 60°C and 20 sec at 72°C. PCR-Result was monitored on a 1% agarose gel.

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**Figure 1. A,** expression analysis of Dyrk kinases during the different stages of rat spermatogenesis and spermiogenesis. The top row of panels shows the ISH signal alone whereas the bottom panels display the merged results of the PNA-Alexa staining (green) and the DNA-staining via propidium-iodine (red) of the respective upper panel. The stages are indicated in Roman numbers. Dyrk1B is detectable during spermatogenesis from late pachytene spermatocytes (stag XI-XII) till step 16

spermatids (stage II-III) (A). Dyrk2 is expressed in spermatids between step 6 and step 11 (stage VI till stage XI) (B). Dyrk3 shows expression starting in late pachytene spermatocytes (stage XII) and stopping in step 7 spermatids (stage VII) (C). Dyrk4 transcripts are only detectable in spermatids at step 8 (stage VIII) (D). Exemplary sense controls are presented in (A) and (D), right panels.

**Figure 2. RT-PCR.** Tissue distribution of Dyrk4 in the mouse. Different cDNA samples from a broad variety of tissues were subjected to quantitative RT-PCR with oligonucleotides specific for murine Dyrk4. Expression was normalised to the housekeeping gene EF1 $\alpha$ , expression in the testis was arbitrarily set as 100%. The analysis revealed, that Dyrk4 is almost exclusively expressed in the testis.

**Figure 3.** Functional analysis and subcellular localisation of human Dyrk4. Human Dyrk4-GST and Dyrk4-K133R-GST were expressed as a GST fusionprotein in SF9 cells. Expression of full length Dyrk4-GST fusionproteins (arrowhead) were detected by an anti GST antibody (left panel). Purified Dyrk4-GST fusionprotein was used in a kinase assay with the myelin basic protein (MBP, right panel). Dyrk4 phosphorylates MBP (arrow) and shows characteristic autophosphorylation (arrowhead) (A). Mutation of the VAIK motif in Dyrk4-K133R-GST diminished its kinase activity (B). Dyrk4 is localised to the cytosol, as revealed by transient transfection and subsequent detection via a C-terminal introduced myc-tag (top panels). The kinase activity is not necessary for this restricted localisation (bottom panels) (C).

**Figure 4.** A, RT-PCR with cDNA derived from wildtype or Dyrk4 deficient mice demonstrates the absence of Dyrk4 mRNA in the knockout animals. B-D, immunohistochemistry for reporter  $\beta$ -galactosidase in Dyrk4 $\pm$  testes. The reporter

gene  $\beta$ -galactosidase is expressed exclusively in elongated spermatids (B-C), brownish colour, arrows). PNA-lectin (green in B and D) and propidium-iodide (red in D) for acrosome and nuclei detection, respectively. E-H, histopathological analysis of Bouin's fixative treated and H&E stained 5 $\mu$ m paraffin sections of testes (D and E) and epididymides (F and G) derived from Dyrk4<sup>-/-</sup> and Dyrk4<sup>+/+</sup> littermates. The data suggest that the absence of Dyrk4 does not alter the morphology of the testes and the epididymides.

I and J, morphology of wildtype (I) and Dyrk4 deficient (J) sperm. Spermatozoa were stained with PNA/DAPI. Acrosomes and cytoplasmic droplets were stained green by PNA, and DNA and the tail were stained blue by DAPI.

**Figure 5.** Expression of Dyrk4 isoforms during spermatogenesis and spermiogenesis. Comprehensive expression analysis based on in situ hybridisation revealed that Dyrk isoforms have distinct overlaps in expression during spermiogenesis (modified from (Russell 1990) with permission of Cache River Science).













