Targeted inactivation of nuclear interaction partner of ALK disrupts meiotic prophase

Anna Lena Illert1, Hiroyuki Kawaguchi2,*,‡, Cristina Antinozzi3,‡, Florian Bassermann1, Letitia Quintanilla-Martinez6, Christine von Klitzing1, Mitsuteru Hiwatari2, Christian Peschel1, Dirk G. de Rooij5, Stephan W. Morris2, Marco Barchi3 and Justus Duyster1,§

SUMMARY
NIPA (nuclear interaction partner of ALK) is an F-box-like protein that monitors the timing of mitotic entry. Constitutively active NIPA delays mitotic entry by preventing accumulation of nuclear cyclin B1. Here, we have investigated the consequences of Nipa inactivation by using a conditional knockout strategy. Nipa-deficient animals are viable but show a lower birth rate and reduced body weight. Furthermore, Nipa-deficient males are sterile owing to a block of spermatogenesis during meiotic prophase. Whereas Nipa–/– mouse embryonic fibroblasts show no severe phenotype, Nipa–/– spermatocytes arrest during stage IV of the epithelial cycle with subsequent TUNEL-positive apoptosis resulting from improper synopsis, defects in the repair of DNA double-stranded breaks and synaptonemal complex formation. Moreover, we show nuclear accumulation of cyclin B1 with a subsequent premature increase in G2/M kinase activity in Nipa–/– spermatocytes. Together, these results reveal a novel role for NIPA in meiosis.

KEY WORDS: Cell cycle, Knockout mouse, Meiosis, NIPA (ZC3HC1), CCNB1, Checkpoint

INTRODUCTION
The regulated oscillation of protein expression and degradation is an essential mechanism of cell cycle control (Hochstrasser, 1996). F-box proteins (FBPs) play a crucial role in the ubiquitin-mediated degradation of regulatory proteins, as they are substrate recognition subunits of SCF-type E3 ubiquitin ligases. These complexes consist of four subunits: S-phase kinase-associated protein 1 (SKP1), Cullin protein 1 (CUL1), RING-finger protein (ROC1) and the interchangeable FBP (Cardozo and Pagano, 2004). The core components of the SCF (SKP1, CUL1, ROC1; RBX1 – Mouse Genome Informatics) are abundant throughout the cell cycle and interact with the FBPs through their SKP1-binding domain (Cardozo and Pagano, 2004). The substrate specificity of SCF ligases is dictated by different FBPs that act as substrate-targeting subunits: therefore, large families of FBPs are present in all eukaryotes to ensure high specificity. In mammals more than 70 FBPs have been identified, yet only six have been linked to specific substrates (SKP2, β-TRCP (BTRC – Mouse Genome Informatics), NIPA, FBXL3, FBXO4, FBXW7 and FBXO31) (Santra et al., 2007; Skaar et al., 2009a; Skaar et al., 2009b). Gene knockout phenotypes have recently revealed several important functions of selected F-box proteins in vivo. For example, inactivation of β-TRCP leads to defective spermatogenesis, subtle mitotic defects and chromosome overduplication (Guardavaccaro et al., 2003).

Targeting of the Skp2 locus results in hypoplasia of most organs, centrosome overduplication and defects of mitotic entry (Nakayama et al., 2000), whereas the Fbxw7 knockout mouse has an embryonic lethal phenotype, probably owing to morphogenetic cardiovascular defects (Tetzlaff et al., 2004). Thus, with the exception of FBXW7, FBP knockout animals are viable despite the variety of FBP substrate proteins affected. Likewise, knockout studies have identified prominent cell cycle regulatory genes, such as Cdc25c or Cdk2, as non-essential genes (Berthet et al., 2003; Chen et al., 2001; Ortega et al., 2003). These observations highlight the possibility that cell cycle regulation in embryonic stem (ES) cells is different from that in differentiated or tumor cell lines.

We have previously reported the cloning of Nipa (nuclear interaction partner of ALK; Zc3hc1 – Mouse Genome Informatics) in complex with constitutively active oncogenic fusion forms of ALK (anaplastic lymphoma kinase) (Ouyang et al., 2003), which contribute to the development of certain lymphomas, sarcomas and lung carcinomas (Li and Morris, 2008; Morris et al., 1994; Soda et al., 2007). We characterized NIPA as a mammalian F-box-containing protein that defines an SCF-type ubiquitin E3 ligase (SCFNPBA) (Bassermann et al., 2005b). Interestingly, the SCFNPBA complex displays oscillating activity, which is controlled by cell cycle-dependent inhibitory phosphorylation of NIPA in late G2 phase, resulting in NIPA dissociation from the SCF-core (Bassermann et al., 2005b). Cyclin B1 (CCNB1) was identified as a substrate, which is targeted by the SCFNPBA for ubiquitylation in interphase within the nuclear cell compartment (Bassermann et al., 2005b) (supplementary material Fig. S1).

Entry into mitosis is triggered by CCNB1/CDK1 (the M-phase-promoting factor or MPF). Nuclear abundance of CCNB1 is a major determinant of MPF activity, thus requiring regulatory machinery to time subcellular localization of CCNB1. In this regard, phosphorylation of the CRS (cytoplasmic retention signal) has been identified as a means to localize CCNB1 to the nucleus prior to mitosis (Pines and Hunter, 1991; Yang et al., 1998). The SCFNPBA complex degrades nuclear CCNB1 in interphase, thus
providing a further safeguard mechanism to protect the cell from untimely nuclear CCNB1 accumulation (Bassermann et al., 2005b). The phosphorylation of NIPA at G2/M induces its release from the S-CF core complex, leading in turn to an increase of nuclear CCNB1 levels required for the progression into mitosis. Consequently, our last studies demonstrated that expression of phosphorylation-deficient mutants of Nipa (constitutively active) results in a block in mitotic prophase owing to insufficient nuclear CCNB1 accumulation (Bassermann et al., 2005; Bassermann et al., 2005b).

We have now characterized the consequences of NIPA deficiency in vivo by targeting the Nipa locus by homologous recombination in ES cells and analyzed the effects on somatic cells and testicular germ cells (GCs). We were able to observe evidence for a new unexpected role of the S-CF NIPA complex in the regulation of meiosis.

MATERIALS AND METHODS

Generation of Nipa–/– mice
The conditional knockout vector was generated using ‘recombineering’ strategy (Liu et al., 2003). Briefly, two 500 bp and four 200 bp fragments were amplified, larger fragments were ligated into vector pL253 for retrieval and smaller fragments into pL452 or pL452 for mini targeting. BAC clones were purchased from Invitrogen and the Nipa fragment was retrieved from one BAC clone by co-transfecting pl253/ABXY and BAC-DNA in the recombinogenic cell EL350. Vectors which obtained Nipa fragments were subjected to mini-targeting with pL452/CDEF2 to insert loxP site 5′ of the Nipa-promoter region. Thereafter, another mini-targeting with pL452/GH212J2 integrated a Neo-gene-cassette flanked by two FRT sequences and a 3′ downstream loxP site. After electroporation of the targeting vector into the ES cell line (CMT1; Speciality Media), selected (300 µg/ml G418; 2 mM ganciclovir) and correctly targeted clones were identified by PCR and Southern blotting. Neo-cassette was deleted by transfecting FLP recombinase expression vector pcAGGS-FLPs (Gene Bridges). Two mutant ES cell clones were microinjected (C57BL/6 blastocysts) and resulting male chimeras were mated with female C57BL/6 mice. Heterozygous offspring was intercrossed to (C57BL/6 blastocysts) and resulting male chimeras were mated with (Germany). All experiments were performed in at least eight-generation mouse strain (Rajewsky et al., 1996; Schwenk et al., 1995). All mice were maintained in animal facility at St Jude Children’s Research Hospital (Memphis, TN, USA) and Technical University of Munich (Germany). All experiments were performed in at least eight-generation C57BL/6-baccrossed mice, unless otherwise stated. Mendelian ratio was calculated using mice with mixed background (SV129/C57BL76) and backcrossed C57BL/6 mice.

NIPA is a synonym for zinc-finger C3HC type-containing 1 protein (ZC3HC1) with the NCBI RefSeq accession number NM_016478.3 (mRNA transcript) and NP_057562.3 (protein).

Histology and immunohistochemistry
For histology, tissues were dissected, fixed (4% formalin) and paraffin embedded. Sections (3-5 µm) were cut and stained with Hematoxylin and Eosin. Immunohistochemistry was performed on an automated immunostainer (Ventana Medical Systems, Tucson, AZ, USA), according to the manufacturer’s protocol.

Preparation of MEFs
Primary MEFs were prepared from embryos on embryonic day (E) 13.5 and cultured as described previously (Hartmann et al., 2002).

Plasmids and antibodies
Details of the construction of plasmid pcDNA3.1-Flag-mNipa are available upon request. Mouse-monoclonal antibodies were purchased from Sigma [anti-FLAG (M2), anti-β-actin] and Santa Cruz [anti-CCNB1 (GNS-1), anti-αtubulin]. Polyclonal rabbit antibodies were from Santa Cruz [anti-CCNA2, anti-WEEl, anti-CDC25B, anti-CCNB1 (H-433), anti-Dmc1], Cell Signaling [anti-phospho-CDC2 (Tyr15), anti-CDC2, anti-caspase 3, anti-cleaved-caspase 3, anti-PLK1, anti-MAPKp42/p44], R&D (anti-survivin), Novus (anti-SYCP1) and Upstate (anti-phospho-H3). Polyclonal-rabbit antibody against H1t was a gift from P. Moens (York University, Canada). The mice were maintained in animal facility at St Jude Children’s Research Hospital (Memphis, TN, USA). Antibodies to CCNA2, anti-WEE1, anti-CDC25B, anti-CCNB1 (H-433), anti-Dmc1, anti-phospho-CDC2 (Tyr15), anti-CDC2, anti-caspase 3, anti-cleaved-caspase 3, anti-PLK1, anti-MAPKp42/p44, R&D (anti-survivin), Novus (anti-SYCP1) and Upstate (anti-phospho-H3). Polyclonal-rabbit antibody against H1t was a gift from P. Moens (York University, Canada). A peptide (LDHFADDRTKTSK) was used to raise polyclonal-antibodies against murine NIPA in rabbits. Human NIPA was detected as previously described (Bassermann et al., 2005; Bassermann et al., 2007).

Transient transfection and siRNA
Transient transfections were performed using Lipofectamine 2000 (Invitrogen). siRNAs were used as previously described (Bassermann et al., 2005b).

Isolation, cell culture and treatments of germ cells
Testicular GC isolation was suspended and cultured following the methods described by Jeyaraj et al. (Jeyaraj et al., 2002) and Sette et al. (Sette et al., 1999). Isolated cells were either fixed (70% cold methanol) or cultured overnight (MEM, 0.5% BSA, 1 mL sodium pyruvate and 2 mL sodium lactate; 32°C). After 12 hours, cells were treated with 5 µM OA, and culture was continued up to 5 hours. For time course experiments, aliquots were taken and processed as described below. For cytological analysis and kinase assays, aliquots were taken and processed accordingly.

Flow cytometry analysis of germinal cells
Ethanol-fixed GCs (1×105-2×106) were washed twice (PBS) and incubated in 500 µL of 0.2% pepsin (10 minutes at 36°C). After centrifugation, cells were stained with 25 µg/ml PI, 40 µg/ml RNase and 0.3% Tween-20 in PBS (room temperature for 30 minutes). For TUNEL labeling, 2×105 GCs were washed, treated with pepsin and TdT-mediated dUTP-biotin nick end labeling was performed using the APO-Direct Kit (BD Biosciences).

H1 kinase assay
Cells (2×105) were collected in 10 µL of storage solution (10 mM p-nitrophenyl phosphate, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 5 mM EGTA, 10 µg/ml leupeptin and 10 µg/ml aprotonin), then immediately frozen (−80°C). Cells were thawed, lysed for 10 minutes in 50 µL hypotonic H1 kinase buffer [25 mM MOPS (pH 7.5), 60 mM β-glycerophosphate, 15 mM EGTA, 15 mM MgCl2, 0.1 mM sodium orthovanadate, 1 mM DTT, 10 µg/ml leupeptin and 10 µg/ml aprotonin], centrifuged for 10 minutes (10,000 g at 4°C) and soluble extracts were collected. Kinase reactions using 10-15 µL of extracts were carried out for 60 minutes at 30°C in a total volume of 25 µL in H1 kinase-buffer containing 100 µg/mL H1 (type III-S; Sigma), 1 µg/ml cAMP-dependent protein kinase inhibitor (Sigma), and 1 µCi [γ-32P] ATP (Amersham). Additional aliquots were collected for protein measurement by BIO-RAD DC Protein Assay (Lowry et al., 1951). Input of lysates into kinase assay was thereby calculated.

Chromosome spreads of spermatocytes
Testis cell preparation were prepared for surface spreading as described previously from 16 dpp mice unless otherwise stated (Barchi et al., 2009; Barchi et al., 2005). An indirect immunofluorescence analysis of spread chromosomes was performed using described methods and antibodies (Barchi et al., 2005).

Immunoprecipitation and immunoblot analysis
Immunoprecipitations and immunoblot analysis were performed as described previously (Ouyang et al., 2003).

RESULTS

Phenotypic analysis of NIPA knockout mice
To generate Nipa-deficient mice, we introduced two loxp sites into the Nipa locus by homologous recombination in ES cells (Fig. 1A). Removal of these sequences by CRE recombinase eliminated the
first two exons of Nipa. Correct targeting was confirmed by Southern blot analysis (Fig. 1B) and PCR (supplementary material Fig. S2). Full ORF of the Nipa cDNA could be amplified from wild-type but not from Nipa–/– mouse embryonic fibroblasts (MEFs) (Fig. 1C). Western blot analysis confirmed that NIPA protein was absent in both Nipa–/– MEFs and testicular cells (Fig. 1D). Notably, testicular cells demonstrated elevated expression levels when compared with MEFs. Despite widespread expression in somatic tissues and during embryogenesis (Fig. 1E), Nipa–/– mice were viable and the frequency corresponded to reduced Mendelian ratio (Nipa+/+ 16.36%, Nipa−/− 54.03%, Nipa+/− 29.60%; n=1736) with a normal male: female ratio (51.7% versus 48.3%; n=282). Further analysis of NIPA expression by immunostaining of whole adult organs (left panel) and during embryogenesis (right panel). Murine tissue and embryo mRNA blot were hybridized with a specific Nipa probe and reprobed with β-actin. (E) Nipa expression patterns in various adult organs (left panel) and during embryogenesis (right panel). Data are mean±s.e.m.

### Table 1. Fertility of wild-type and Nipa mutant mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Fraction fertile (fertile/total)</th>
<th>Litters/fertile pair</th>
<th>Mean litter size (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nipa+/+</td>
<td>5/5</td>
<td>4.3</td>
<td>7.9</td>
</tr>
<tr>
<td>Nipa−/−</td>
<td>10/10</td>
<td>4.2</td>
<td>8.1</td>
</tr>
<tr>
<td>Nipa+/−</td>
<td>4/10</td>
<td>2.3</td>
<td>3.3</td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nipa+/+</td>
<td>5/5</td>
<td>4.3</td>
<td>7.9</td>
</tr>
<tr>
<td>Nipa−/−</td>
<td>10/10</td>
<td>4.2</td>
<td>8.1</td>
</tr>
<tr>
<td>Nipa+/−</td>
<td>0/10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Males and females (8-12 weeks of age) of the three different genotypes were tested for fertility for a period of ~3 months with young wild-type males and females. Copulatory behavior was judged to be normal and vaginal plugs were regularly found. Sixty percent of Nipa−/− females were sterile and the remaining 40% had reduced fertility, when judged by the number of litters generated and the mean litter size. All of the tested Nipa−/− males were sterile.

### NIPA is essential for progression through prophase of meiosis I

Nipa−/− males showed progressive atrophy of the gonads (Fig. 1G; supplementary material Fig. S5) starting before sexual maturity. Reduction of testis weight in Nipa−/− animals was already evident in three-week-old pups. In male mice, spermatogonial stem cells are located at the periphery of the seminiferous tubule. These cells undergo several mitotic divisions before giving rise to spermatocytes, where meiosis takes place. In immunohistological analyses, we observed an overall reduction of seminiferous tubule diameter resulting from depletion of all germinal cells, which

---

**Figs. 1. Generation of mice lacking NIPA and phenotypic analysis of Nipa−/− mice.** (A) Nipa alleles. The mouse Nipa locus contains 10 exons spanning 25 kb. Coding sequences (boxes), non-targeted coding sequences (white boxes), targeted exons (black boxes), loxP sites (black triangles) and Flp sites (gray triangles) are indicated. neo, PGK-neo cassette; Cre, bacteriophage Cre recombinase; Flp, Saccharomyces cerevisiae Flp recombinase; E, EcoRI; K, Kpn; EV, EcoRV; a, 5’ probe; b, 3’ probe. Wild-type and mutated Nipa alleles are indicated. (B) Southern blot analysis of genomic DNA. After KpnI digestion, hybridization with the 3’ probe b detects a 20 kb wild-type and a 10 kb mutant Nipa DNA fragment. (C,D) Level of Nipa transcripts (C) and NIPA protein (D). Total RNAs were prepared from MEFs and processed for RT-PCR using exon primers for full-length Nipa and β-actin (C). Lysates from testicular cells and MEFs were subjected to immunoblotting (D). β-Actin served as loading control. (E) Nipa expression patterns in various adult organs (left panel) and during embryogenesis (right panel). Murine tissue and embryo mRNA blots were hybridized with a specific Nipa probe and reprobed with β-actin. (F) Testes from wild-type and Nipa−/− 3-month-old mice. (G) Testicular weight at different ages (wt/x, n=40; ko/ko, n=32). Data are mean±s.e.m.
became more profound in older animals. Representative tubules from 21 dpp (days post partum), 60 dpp and 180 dpp Nipa<sup>−/−</sup> and control mice are shown in Fig. 2A-F. At 21 dpp, when many of the spermatocytes are expected to have reached and passed the pachytene stage, abundant numbers of spermatogonial stem cells and spermatocytes were visible in tubules of wild-type mice. At 60 dpp, spermiogenesis is completed and mature sperms can be seen. By contrast, Nipa<sup>−/−</sup> mice showed reduced tubule diameter (Fig. 2B) as result of reduced cellularity. However, although spermatogonia appeared to present in a roughly normal number, spermatocytes content was obviously reduced and neither metaphase I/metaphase II cells nor round spermatids or sperms were seen (Fig. 2B,D). At 180 dpp (Fig. 2E,F), we observed entirely empty tubules in Nipa<sup>−/−</sup> males, without residual spermatogonia, thus suggesting a potential mitotic proliferation defect of Nipa<sup>−/−</sup> GCs.

Detailed analyses revealed that Nipa<sup>−/−</sup> spermatogenesis was mostly arrested at stage IV of the epithelial cell cycle by massive apoptosis of virtually all spermatocytes (Fig. 2G). However, some spermatocytes occasionally escaped this arrest, as judged by the presence of some post-meiotic cells, but these cells gradually disappeared as they entered apoptosis at later stages, implying that cells that escape pachytene arrest could not complete spermatogenesis (Fig. 2H). In histological analyses up until 47 dpp, no spermatocytes reached meiotic divisions and round spermatids were not seen. This observation was confirmed by immunofluorescent staining of chromosome spreads for the mid-pachynema-specific marker histone 1t (H1t). At 16 dpp, H1t-positive cells were seen in control but not in Nipa<sup>−/−</sup> testis (supplementary material Fig. S6). At 21 dpp, Nipa<sup>−/−</sup> testis showed exceptionally H1t-positive cells (arrows in Fig. 2I). Staining of Nipa<sup>−/−</sup> testis with a M-specific marker (phospho-Histone-3) revealed that, at 55 dpp, diplotene MI and MII cells are nearly absent compared with wild-type animals (supplementary material Fig. S7). However, mid-pachynema H1t-positive cells were visible in some Nipa<sup>−/−</sup> testes at 90 dpp, as well as few round and elongated spermatids (Fig. 2J).

Immunostaining with a NIPA-specific antibody revealed highest expression of NIPA in pachytene spermatocytes and weaker staining in the post-meiotic compartment (supplementary material Fig. S8). The developmental arrest of Nipa<sup>−/−</sup> spermatocytes resulted in the complete absence of spermatozoa in the tubule lumens and in the epididymis (supplementary material Fig. S9).
Fertility of females was also affected by deletion of Nipa. Female GCs complete early stages of meiotic prophase I during fetal development and undergo cell cycle arrest around birth. After birth, each surviving oocyte becomes surrounded by somatic cells, forming a primordial follicle that remains arrested until recruited for further development during the reproductive life (van den Hurk and Zhao, 2005). Ovaries of Nipa–/– animals showed mostly complete absence of primordial follicles and corpora lutea. Instead, cysts lined with a layer of epithelial cells replaced the ovarian tissue (supplementary material Fig. S10). The absence of primordial follicle development in Nipa–/– females was a consequence of failure during embryogenesis. Analyses of embryonic ovaries displayed the loss of Nipa–/– oocytes during the course of prophase. Nipa–/– ovaries isolated by the beginning of meiotic prophase (E13.5-E14.5) showed extensive apoptosis in early prophase (Fig. 2K; supplementary material Fig. S11), whereas heterozygous littermates showed normal development of early prophase oocytes. As a consequence of massive oocyte loss, only very few oocytes are left at E18.5 in Nipa–/– ovaries (Fig. 2L). By contrast, wild-type ovaries demonstrated many oocytes in late pachytene as judged from nuclei size and morphology of contracted chromosomes.

To further characterize the failure of meiotic progression in Nipa–/– animals, we isolated and quantified testis cell content. As expected from the histological analyses, we found a dramatic decrease in testicular cell number in Nipa–/– males (to 12% of that in wt/het) (Fig. 3A). Immediately before prophase, GCs duplicate their DNA content from 2C to 4C. Through two meiotic divisions, haploid 1C cells are formed. We compared DNA content of enriched GCs from testis at different ages (Fig. 3B). Nipa–/– mice showed a distinct different DNA distribution compared with wild-type littermates. The first wave of spermatogenesis starts shortly after birth and GCs enter meiotic prophase at about 7 dpp, where the same distribution of testicular cells could be found in knockout and wild-type mice (with slightly increased 4C cell population of Nipa–/– males). Two weeks later, cells that have passed the first and second meiotic division were observed with a haploid chromosome...
set in wild-type animals. In marked contrast, Nipa<sup>−/−</sup> mice displayed a massive reduction of cells after the meiotic divisions, resulting in a lack of haploid 1C cells. Nipa<sup>−/−</sup> GCs arrested in meiosis I, as demonstrated by a relative accumulation of the 4C population at 23 and 40 dpp. Quantification revealed more than 50% of Nipa<sup>−/−</sup> testicular cells with 4C DNA content (Fig. 3C). In agreement with these data, RT-PCR analyses of 60 dpp Nipa<sup>−/−</sup> testis showed a distinct downregulation of markers for meiotic and postmeiotic cells, i.e. transition protein 1 (Tpt1; Tpt1– Mouse Genome Informatics), protamine 1 and 2 (Prm1, Prm2), and cAMP-responsive element modulator (Crem) (Fig. 3D). The expression profile of Nipa<sup>−/−</sup> testicular cells showed high amounts of mitotic/meiotic proteins such as CDK1, CDC25B, phosphorylated WEE1 and CCNB1 (Fig. 3E), which could be due either to the different composition of testicular cells in Nipa<sup>−/−</sup> tests, where relatively more mitotic spermatogonia and early meiotic spermatocytes are present compared to wild type, or to changes in protein abundance in a particular cell type.

The progressive degeneration of Nipa<sup>−/−</sup> testicular GCs prompted us to determine whether the meiotic arrest causes apoptosis. IHC analysis with activated caspase 3 antibody showed apoptosis of Nipa<sup>−/−</sup> spermatocytes starting at 10 dpp (supplementary material Fig. S12). In agreement with this and with the histological examination, TUNEL assays of enriched GCs showed increased apoptosis in 35% of Nipa<sup>−/−</sup> cells (supplementary material Fig. S13), which was restricted to spermatocytes. In addition, combined TUNEL/DNA-content analysis revealed that TUNEL positivity largely occurred in the 4C population of Nipa<sup>−/−</sup> testicular cells (65%), whereas the 2C and 1C populations were affected to a lesser extent (Fig. 3F). Moreover, Nipa<sup>−/−</sup> testicular cells displayed markedly decreased expression of survivin, an IAP-family member whose expression and activity of CCNB1, a previously described substrate of the SCF<sub>NIPA</sub> complex. As expected, cytoplasmic staining of CCNB1 was visible both in wild-type and heterozygous GCs (Fig. 4A). Strikingly, Nipa<sup>−/−</sup> testicular cells showed accumulation of CCNB1 in the nucleus, the subcellular compartment where the SCF<sub>NIPA</sub> complex regulates CCNB1 abundance (Fig. 4A). In accordance with function of NIPA as an ubiquitin-ligase of CCNB1 in murine cells, binding analyses revealed a specific interaction between the two murine proteins (Fig. 4B,C). To rule out the possibility that nuclear CCNB1 accumulation was merely a consequence of a specific cell cycle arrest in meiosis, where high CCNB1 levels are present, we analyzed CCNB1/CDK1 activity in GCs stimulated to enter G2/M in culture. To this end, we stimulated enriched GCs culture, isolated at 10 dpp from Nipa<sup>−/−</sup> and Nipa<sup>−/−</sup> males, with okadaic acid (OA), which has previously been shown to promote chromosome condensation and increased H1-kinase activity in mouse spermatocytes (Sette et al., 1999; Wiltshire et al., 1995), as well as in mitotic cells of the testis (Cobb et al., 1999). Stimulation of Nipa<sup>−/−</sup> cells led to an increase in H1-kinase activity after 1 hour, with a maximum (twofold) after 2.5-5 hours, in accordance with previous reports. By contrast, OA stimulation of Nipa<sup>−/+</sup> cells induced a more rapid, elevated and sustained (2.6-fold after only 30 min) H1-kinase activity when compared with wild-type cells (Fig. 4D; supplementary material Fig. S18). Intriguingly, basal H1-kinase activity in Nipa<sup>−/−</sup> cells was increased. Thus, untimely nuclear CCNB1 accumulation may lead to premature G2/M-kinase activity and one may speculate that this could cause meiotic arrest with subsequent apoptosis of Nipa<sup>−/−</sup> GCs.

We next investigated the impact of NIPA deficiency on CCNB1 levels in somatic tissues. In particular, we focused on non-cycling tissues (liver, brain) to rule out cell cycle-dependent effects on CCNB1 abundance. These experiments revealed that deletion of NIPA leads to a significant accumulation of CCNB1 both in brain (Fig. 4E) and liver tissue (supplementary material Fig. S19) of adult Nipa<sup>−/−</sup> mice. As we did not isolate cell subpopulations of the respective tissues, we cannot exactly determine the specific cell type that contributes to the observed CCNB1 accumulation.

NIPA deficiency leads to an aberrant progression of prophase-I cells

In order to examine further the characteristics of meiotic arrest, we performed immunofluorescence analyses of chromosome spreads of 16 dpp spermatocytes (Fig. 5A–P). During meiotic prophase, homologous chromosome pairing is stabilized by the synaptonemal complex (SC) and synapsis can be monitored by following deposition of the lateral element (anti-SYCP3, Fig. 5A) and the axial element (anti-SYCP1, Fig. 5B). At pachynema, homologous autosomes are fully synapsed along their length, which is marked by completely overlapping SYCP3 and SYCP1 staining (Fig. 5D). By contrast, late zygonema/pachynema Nipa<sup>−/−</sup> cells showed chromosome synapsis defects (Fig. 5E–P). These were indicated by the presence of pachynema-like cells, characterized by several unsynapsed chromosomes in combination with fully synapsed bivalents (Fig. 5H,P), and by interruptions of the SYCP3/SYCP1 staining along the SC formation. In some cases, SYCP3/SYCP1 stretches were dissociated from each other (SC fragments), suggesting that chromosome fragmentation might have occurred (Fig. 5H,L, arrowheads). In other cases, stretches were close to each other (Fig. 5H,L, arrows), suggesting defects in SC deposition or localized SC disassembly (SC gaps). To quantify these observations, we counted the number of late zygonema/pachynema cells with aberrant morphology, including incomplete synapsis, SC fragments and SC gaps. As seen in Fig. 5Q, more than 60% of Nipa<sup>−/−</sup> cells displayed an aberrant morphology, pointing to a severe defect in meiotic progression. Furthermore, quantification of cells with interruptions of SYCP3/SYCP1 staining at pachynema (Fig. 5R) revealed major defects in SC appearance in Nipa<sup>−/−</sup> cells with 4.28±0.05 interruptions/cell (n=123) versus 0.76±0.45 (n=123) in wild-type animals.
Synaptonemal complex structure defects in Nipa<sup>–/–</sup> spermatocytes

To further analyze the defect in SC formation and fragmentation, we stained chromosome spreads with SYCP3 and the telomere-marker TRF1. As seen in Fig. 6A-D, all wild-type chromosomes have two TRF1 foci at the end of each paired chromosomes, whereas Nipa<sup>–/–</sup> spermatocytes show aberrant chromosome pairing (Fig. 6E-H) with homologous chromosomes showing only one TRF1 focus (Fig. 6I), and paired homologs with two TRF1 foci but a SYCP3 interruption (Fig. 6J), which is most probably not a broken chromosome but a SYCP3 deposition defect.

To characterize SC interruptions as potential chromosome structure defects, we examined staining of STAG3, a meiosis-specific cohesin subunit that is axis associated (Fig. 6K-P). Analysis of 14 dpp spermatocytes revealed SC gaps or fragments of the SYCP3 staining in 23% of Nipa<sup>–/–</sup> chromosomes. Importantly, we found that SYCP3 staining gaps were almost always associated with gaps in the STAG3 staining (132 of 158 SYCP3 anomalies or 83%) (Fig. 6N-P). The remaining fraction was mostly represented by SYCP3 gaps not associated with gaps in the STAG3 staining. In addition, SYCP3 fragments in 14 and 16 dpp Nipa<sup>–/–</sup> mice, which are completely separated from other chromosomes and not included in the ‘gap’-class, always colocazalized with STAG3, suggesting that the integrity of the chromosome axis might be compromised in Nipa<sup>–/–</sup> spermatocytes.

Nipa-deficient mice display normal meiotic sex chromosome inactivation

Sex chromosomes are transcriptionally silenced during prophase by meiotic sex chromosome inactivation (MSCI). One key event in MSCI is H2AX (H2afx – Mouse Genome Informatics) phosphorylation by ATR. Indeed, if H2AX is absent or H2AX phosphorylation (γH2AX) does not occur properly, this results in ectopic expression of XY-linked genes with consequent elimination of spermatocytes at stage IV (Barchi et al., 2005; Fernandez-Capetillo et al., 2003; Ichijima et al., 2011). To examine whether the apoptotic elimination in Nipa-deficient spermatocytes is due to defects in MSCI, we analyzed γH2AX-staining on sex chromatin. Our analysis revealed a normal pattern of γH2AX, suggesting that MSCI occur normally (data not shown). To further challenge our interpretation, we stained chromosome spreads with an antibody that recognize RBMY, a Y-linked gene. The analysis revealed no
differences in pachytene Nipa−/− and Nipa+/+ spermatocytes (Fig. 6Q–S), whereas H2afx−/− pachytene spermatocytes were RBMY positive (Fernandez-Capetillo et al., 2003) and indicate that NIPA is dispensable for correct MSCI.

**Nipa-deficient mice display a delay in the repair of double strand breaks**

The disappearance of double-stranded break (DSB) markers from meiotic chromatin is coincident with synapsis (Mahadevaiah et al., 2001), so the synapsis defect in Nipa−/− mice led us to test whether these cells are defective in the repair of dsDNA breaks. DSB repair can be followed indirectly by staining chromosomes for γH2AX (Mahadevaiah et al., 2001), and for strand-exchange proteins RAD51 and DMC1, which form cytological complexes at sites of ongoing recombination (Moens et al., 1997; Plug et al., 1996). During zygonema, the number of RAD51/DMC1 foci peaks and γH2AX progressively disappears where synapsis has occurred. In early pachynema, most of these early DSB markers have disappeared from autosomes and only few of RAD51/DMC1 foci remain on the unsynapsed regions (Fig. 7A–C) along with the DSB-independent γH2AX in the sex body. In our analysis (Fig. 7J), 16 dpp pachytene-like Nipa−/− spermatocytes displayed appreciably more DMC1 foci with 93.56±9.2 foci versus 50±4.41 foci in wild-type early pachynema cells, where essentially all DMC1 foci disappeared in late pachynema. The increase in number of DMC1 foci might be due to a delay in DSB repair or the consequence of the arrest of Nipa-deficient spermatocytes. To distinguish between these two possibilities, we analyzed an earlier time point during the first wave of spermatogenesis (14 dpp) and compared the number of Nipa−/− DMC1 foci at pachynema with that of early-pachynema (H1t-negative) wild-type cells. The analysis revealed an even more pronounced increase in DMC1 foci number in Nipa−/− 14 dpp (121.55 foci/cell) mice (Fig. 7F, J), where early pachynema cells first appear, indicating a delay in the repair of DSB. In adult Nipa−/− mice, some mid-pachynema H1t-positive cells were present. Importantly, these cells show normal levels of DMC1 foci (Fig. 7G–I), so it is most likely that these cells escape the arrest and – as cause or consequence – successfully repair their DSBs.

Moreover, we asked whether the SC fragmentation and possible chromosome axis defects colocalize with sites of unrepaired DNA DSBs. Intriguingly, many SYCP3 abnormalities (interruptions or gaps) were associated with cytological markers for DSB repair. DMC1 foci were found at one or both ends of the SYCP3 staining interruption at an average of 2.06 interruptions per cell or 47.7% (n=105) of the interruptions were associated with DMC1 (Fig. 7K) in Nipa−/− animals, whereas wild-type mice show an average of 0.16 interruptions with DMC1 colocalization (n=105) (Fig. 7L).

To summarize, wild-type pachynema spermatocytes are fully synapsed and SC interruptions occur only occasionally and are not related to DMC1 labeling. By contrast, Nipa−/− animals show a significantly higher rate of SC interruptions and nearly 50% of the SYCP3 abnormalities are associated with DMC1, suggesting a possible mechanistic link between chromosome axis defects and the ongoing process of DNA repair.

**DISCUSSION**

Although previous studies investigated the consequences of constitutively active SCF(NIPA) (Bassermann et al., 2005b), we here studied the effect of NIPA deficiency in vivo and found that ablation of NIPA has profound consequences for the meiotic cell division.

**Nipa deficiency leads to accumulation of CCNB1 in vivo**

Nipa−/− GCs showed high levels of CCNB1 in the nucleus, the subcellular compartment where SCF(NIPA) regulates CCNB1 abundance (Bassermann et al., 2007; Bassermann et al., 2005b; von Klitzing et al., 2011). Other non-cycling tissues such as brain and liver showed high levels of CCNB1, consistent with the previous described function of the SCF(NIPA) complex. However, we found Nipa−/− mice to be viable even though smaller and MEFs displayed no clear cell cycle phenotype. It has previously been shown that NIPA acts as a safeguard mechanism in CCNB1 regulation, preventing untimely nuclear CCNB1 accumulation (Bassermann et al., 2005b; von Klitzing et al., 2011). Primary cells, which have appropriate CCNB1 regulation independent of SCF(NIPA), appear to be able to compensate for the loss of NIPA. Conversely, cells with impaired checkpoints or subtle defects in their CCNB1 regulation machinery might be more dependent on SCF(NIPA) to guarantee correct cell division.
This notion is supported by our experiments in the tumor cell line HeLa, where we show that NIPA knockdown leads to premature mitotic entry and subsequent apoptosis.

Another explanation for the lack of a clear cell cycle phenotype in MEFs can be the relatively low expression of NIPA in MEFs. Interestingly, NIPA is highly expressed in early pachytene spermatocytes and expression data demonstrate that CCNB1 levels are also highest in pachytene spermatocytes (Chapman and Wolgemuth, 1994). Any failure in the CCNB1 regulatory machinery may therefore have substantial consequences at this stage of differentiation and cannot be compensated for by other CCNB1 regulation factors. The reduction of body weight in Nipa–/– deficient animals could either be connected to cell cycle defects or is more likely to be the result of hormone deprivation resulting from testis and ovarial atrophy. Taken together, the NIPA knockout mouse substantiates previously published studies (Bassermann et al., 2007; Bassermann et al., 2005b; von Klitzing et al., 2011) and shows the primary physiological function of NIPA as an important ubiquitylation factor of CCNB1 in vivo.

**NIPA is required for proper meiosis in male and female mice**

The primary finding of this study is the phenotype of male infertility and female subfertility with arrest of the meiotic prophase. Virtually no spermatocytes progress beyond a late zygotene to early pachytene stage and reach an aberrant pachynema stage, where the SC is not assembled properly with incomplete synapsis and SYCP3 polycomplexes. Meiotic asynapsis (as well as unrepaired damage, see below) is not tolerated in mammalian spermatogenesis and all meiotic ‘asynaptic’ mutants that have been examined so far arrest in an epithelial stage IV checkpoint (Barchi et al., 2005; de Rooij and de Boer, 2003). In accordance with this, Nipa–/– spermatocytes become apoptotic in epithelial stage IV, although some rare knockout spermatocytes get past the arrest. Epithelial stage IV apoptosis is also triggered by a failure in MSCI (Royo et al., 2010). However, MSCI seems to occur normally in absence of NIPA, pointing to chromosome synapses defects as one primary cause of spermatocyte elimination. Another important finding in Nipa–/– testis is the empty appearance of the seminiferous tubules in older mice, demonstrating a progressive testicular atrophy with fibrosis and a Sertoli-only syndrome-like appearance. This may argue for an additional mitotic failure of Nipa–/– spermatogonia beyond the role of the protein in meiosis. However, as our knockout strategy does not differentiate between ubiquitously expressed NIPA having a functional role in GCs or in other cell types that regulate gametogenesis (e.g. sertoli cells, Leydig cells, granulosa cells), indirect effects of the GC-surrounding compartment cannot be excluded.

Previous reports on the inactivation of other important mitotic cell cycle regulators (FBPs, CDKs, cyclins and CDK inhibitors) (Cardozo and Pagano, 2004) showed additional spermatogenesis...
defects, reflecting similar mechanisms in mitotic and meiotic regulation. Accordingly, \textit{Btrec}-deficient mice displayed defective spermatogenesis with accumulation of metaphase I spermatocytes (Guardavaccaro et al., 2003), \textit{Cdk2} (cyclin-dependent kinase 2)-deficient spermatocytes proceed to the end of prophase I of the meiotic division (Ortega et al., 2003) and cyclin E2-deficient animals have also extensive apoptosis of spermatocytes, taking place especially in the meiotic divisions (Geng et al., 2003).

**NIPA deficiency leads to aberrant SC assembly**
Successful completion of meiosis in mice depends on the assembly of the SC (Jessberger, 2002). Importantly, there is evidence that CCNB1/CDK1 kinase activity is needed for correct SC disassembly (Dix et al., 1997). Premature MPF activity could lead to precocious SC disintegration, followed by an arrest of spermatocytes due to checkpoint activation, and to apoptotic elimination at stage IV. Intriguingly, besides nuclear CCNB1 accumulation, we found evidence of increased basal level of H1-kinase activity in \textit{Nipa}–/– testicular cells. One may speculate that untimely activation of CCNB1/CDK1 have led to premature onset of meiotic cell division. Consequently, \textit{Nipa}–/– zygote and early pachytene spermatocytes fail to complete meiotic prophase by aberrant SC formation and asynapsis, thereafter arrest at the epithelial stage IV checkpoint and undergo apoptosis. Leptotene/zygotene cells are not competent to respond to OA with increase in H1-kinase activity (Cobb et al., 1999). Therefore, increased H1 activity after OA treatment in \textit{Nipa}–/– testicular cell suspension is most likely to be due to mitotic dividing spermatogonia, which could be an explanation for the loss of spermatogonia in older \textit{Nipa}-deficient animals.

**Role of NIPA in DNA repair, SC-fragmentation and chromosome axis defects**
Here, we report that \textit{Nipa}–/– meiocytes exhibit persistent cytological markers for DSB repair proteins in meiotic prophase with more than twice as many DMC1 foci as control animals. This could be either a consequence of stage IV arrest, where \textit{Nipa}–/– spermatocytes do not progress to the point where DSBs are removed from paired chromosomes, or due to delayed repair of DNA DSBs. Kinetic analysis of the first wave of spermatogenesis showed increased DMC1 foci in \textit{Nipa}–/– cells as soon as early pachynema cells appear (13- 14 dpp), thus arguing for an involvement of NIPA in the repair of DSBs. Moreover, we show a subpopulation of rare \textit{Nipa}–/– spermatocytes that passes early pachynema and get to mid-pachynema without any aberrant increase in DMC1/RAD51 foci, implying that some of the knockout spermatocytes can fully repair their DSBs and DMC1 foci persist only in the sex chromosomes. These cells are most likely to be the ones that pass the stage IV arrest and eventually become round spermatids.

\textit{Nipa}-deficient spermatocytes exhibit numerous abnormalities in SYCP3 and STAG3 staining, indicating that possible chromosome axis defects (discontinuities in SYCP3 staining) were often associated with compromised chromosome axis integrity (discontinuities in STAG staining), leading to overt chromosome fragmentation. One possible explanation for these observations could be that ‘weak spots’ on axes are susceptible to disruptive forces from chromosome spreading procedures, resulting in extreme stretching or even complete severing of the DNA from all four chromatids. Alternatively, stretching and/or chromatid severing occur in vivo, perhaps as a consequence of large-scale chromosome movements that have been documented during meiotic prophase in some organisms (Scherthan et al., 2007).

The frequent association of abnormalities in SYCP3 staining and DSB markers (DMC1 foci) suggests a possible mechanistic link between inappropriate SC fragmentation and the ongoing process of DSB repair. This phenotype shares similarities with the \textit{Atm}–/–\textit{Spo11}–/– mice (Barchi et al., 2008) in many aspects, where chromosome fragmentation is coincident with defects in DSB repair. Moreover, we show that \textit{Nipa} deficiency does not lead to a
defect in MSCI, despite epithelial stage IV apoptosis. To our knowledge (besides Trip13 mutant) Nipa–/– knockout mice represent one of the few male mutants in which a defect in MSCI is not involved in the activation of the checkpoint response that leads to stage IV apoptosis. This observation indirectly supports our model, in which elimination of Nipa–/– spermatocytes is due to activation of a checkpoint monitoring synapsis and proper DNA-damage repair.

Nipa–/– females are subfertile with early and severe meiotic defect during embryogenesis. It has been shown that elimination of oocytes during embryogenesis can be due to DNA-damage-independent (synapsis defects in Spo11–/– mice) and -dependent checkpoints (Dmc1–/–, Msh5-knockout mice) (Di Giacomo et al., 2005). As we did not look at SC assembly or DSB repair in mutant females, one can only speculate that elimination of Nipa-deficient oocytes is triggered by one of these two checkpoints or the combination of both.

Taken together, the phenotype of Nipa-knockout mice is a definitive proof of the meiotic significance of NIPA. Arrest and apoptosis from Nipa-deficient spermatocytes is most probably the result of one of two different—or combined—mechanisms: one reason of meiotic failure in Nipa–/– spermatocytes is the upregulation of CCNB1, which leads to SC disassembly, premature CCNB1/CDK1 kinase activity and activation of a checkpoint, which is triggered by defects in homologues chromosome synapsis (Baudat et al., 2000; Odorisio et al., 1998). The other reason could be defective chromosome axis stability of Nipa-mutant spermatocytes, leading to a defect/delay in DSB repair and inappropriate SC fragmentation, thereby activating the DNA-damage-dependent checkpoint with subsequent apoptosis. The latter might be a CCNB1 independent effect and one may speculate the involvement of a new, still unknown, substrate of the SCF-NIPA complex or alternatively a direct function of NIPA as a factor for chromosome stability.

Acknowledgements
We thank Dr Xiaoli Cui and Petra Schenk for excellent technical assistance.

Funding
These studies were supported by a grant from the Deutsche Forschungsgemeinschaft (DFG) and the Wilhelm Sander Stiftung to J.D. (2007.043.1). A.L.I. was supported by a research grant from the Technical University of Munich [KKF-807-08]. J.D. and A.L.I. were supported by DFG [DU2273-1]. M.B. was supported by a grant from Associazione Italiana per la Ricerca sul Cancro (AIRC) [CA69129, CA21765] and by American Lebanese Syrian Associated Charities (ALSAC, St Jude Children’s Research Hospital). F.B. was supported by grants from the German Research Foundation [Emmy Noether Programme, BA 2851/3-1] and German Cancer Aid [109543]. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl?doi:10.1242/dev.073072/-/DC1

References


