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Disruption of Mouse Cenpj, a Regulator of Centriole Biogenesis, Phenocopies Seckel Syndrome

Rebecca E. McIntyre¹, Pavithra Lakshminarasimhan Chavali², Ozama Ismail³⁵, Damian M. Carragher³⁵, Gabriela Sanchez-Andrade⁴*, Josep V. Forment⁵, Beiyuan Fu⁶, Martin Del Castillo Velasco-Herrera¹, Andrew Edwards⁵, Louise van der Weyden¹, Fengtang Yang⁶, Sanger Mouse Genetics Project³, Ramiro Ramirez-Solis⁷, Jeanne Estabel³, Ferdia A. Gallagher⁸, Darren W. Logan⁴, Mark J. Arends⁹, Stephen H. Tsang¹⁰,¹¹, Vinit B. Mahajan¹⁰, Cheryl L. Scudamore¹², Jacqueline K. White³, Stephen P. Jackson⁵, Fanni Gergely², David J. Adams¹*.

¹Experimental Cancer Genetics, Wellcome Trust Sanger Institute, Hinxton, United Kingdom, ²Cancer Research UK Cambridge Research Institute, Li Ka Shing Centre and Department of Oncology, University of Cambridge, Cambridge, United Kingdom, ³Mouse Genetics Project, Wellcome Trust Sanger Institute, Hinxton, United Kingdom, ⁴Genetics of Instinctive Behaviour, Wellcome Trust Sanger Institute, Hinxton, United Kingdom, ⁵The Gudzon Institute and Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom, ⁶Molecular Cytogenetics, Wellcome Trust Sanger Institute, Hinxton, United Kingdom, ⁷Wellcome Trust Center for Human Genetics, Oxford, United Kingdom, ⁸Department of Radiology, Addenbrooke’s Hospital, University of Cambridge, Cambridge, United Kingdom, ⁹Department of Pathology, Addenbrooke’s Hospital, University of Cambridge, Cambridge, United Kingdom, ¹⁰Department of Ophthalmology and Visual Sciences, University of Iowa, Iowa City, Iowa, United States of America, ¹¹Bernard and Shirlee Brown Glaucoma Laboratory, Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, New York, New York, United States of America, ¹²Department of Pathology and Infectious Diseases, Royal Veterinary College, London, United Kingdom.

Abstract

Disruption of the centromere protein J gene, CENPJ (CPAP, MCPH6, SCKL4), which is a highly conserved and ubiquitously expressed centrosomal protein, has been associated with primary microcephaly and the microcephalic primordial dwarfism disorder Seckel syndrome. The mechanism by which disruption of CENPJ causes the proportionate, primordial growth failure that is characteristic of Seckel syndrome is unknown. By generating a hypomorphic allele of Cenpj, we have developed a mouse (Cenpj<sup>em/emb</sup>) that recapitulates many of the clinical features of Seckel syndrome, including intrauterine dwarfism, microcephaly with memory impairment, ossification defects, and ocular and skeletal abnormalities, thus providing clear confirmation that specific mutations of CENPJ can cause Seckel syndrome. Immunohistochemistry revealed increased levels of DNA damage and apoptosis throughout Cenpj<sup>em/emb</sup> embryos and adult mice showed an elevated frequency of micronucleus induction, suggesting that Cenpj-deficiency results in genomic instability. Notably, however, genomic instability was not the result of defective ATR-dependent DNA damage signaling, as is the case for the majority of genes associated with Seckel syndrome. Instead, Cenpj<sup>em/emb</sup> embryonic fibroblasts exhibited irregular centrosome numbers and mono- and multipolar spindles, and many were near-tetraploid with numerical and structural chromosomal abnormalities when compared to passage-matched wild-type cells. Increased cell death due to mitotic failure during embryonic development is likely to contribute to the proportionate dwarfism that is associated with CENPJ-Seckel syndrome.


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* E-mail: da1@sanger.ac.uk

¹ These authors contributed equally to this work.

Introduction

Seckel syndrome is a clinically and genetically heterogeneous primordial dwarfism disorder that is characterised by intrauterine growth retardation, postnatal dwarfism, severe microcephaly, mental retardation, a prominent curved nose and receding chin, together with other clinical abnormalities [1,2,3]. Mutations in five loci have been linked with Seckel syndrome: SCKL1 and SCKL2 are due to mutation of the genes for the DNA damage response proteins ATR and CtIP (RBBP8), respectively; SCKL4 and SCKL5 are due to mutation of the genes for the centrosomal proteins CENPJ (Centromere protein J, or centrosomal P4.1-associated protein, CPAP; Figure 1A) and CEP152; while the gene responsible for SCKL3 is currently unknown [4,5,6,7]. Mutations in PCNT (pericentrin), another centrosomal protein, have been associated with both Seckel syndrome and the overlapping...
dwarfism disorder, microcephalic osteodysplastic primordial dwarfism type II (MOPDII) [8,9,10]. Interestingly, mutations in the centrosomal proteins CEP152 (MCPH4) and CENPJ (MCPH6), which are thought to interact with each other during centriole biogenesis [11,12], have also been associated with primary autosomal recessive microcephaly, a genetically heterogeneous condition caused by mutation of one of eight loci (MCPH1–8; [13,14,15]) result in clinically indistinguishable features that include mental retardation and a severely reduced brain size of greater than two standard deviations below the average. Primary autosomal recessive microcephaly presents at birth or becomes apparent within the first few years of life [16]. Primary microcephaly is thought to be caused by a reduction in neurogenesis while the proportionate dwarfism of Seckel syndrome is thought to be the result of premature death of proliferating cells; it is not clear why different mutations in centrosomal proteins cause Seckel syndrome, MOPDII or primary microcephaly.

CENPJ is a conserved, ubiquitously expressed centrosomal protein with a key role in centriole biogenesis [17,18,19,20,21]. The centrosome is a major microtubule-organizing centre in somatic cells that undergoes a duplication cycle that is tightly coupled with DNA replication (reviewed by [22] and [23]). Briefly, in G1 the centrosome is composed of a pair of loosely connected centrioles that are embedded in a proteinaceous matrix. In concert with DNA replication, a single procentriole forms next to each parental centriole during S-phase. The procentrioles continue to elongate and by the onset of mitosis two centrosomes are present, each comprising an older and younger centriole. The two centrosomes aid the formation of the poles of the bipolar spindle, the molecular machinery responsible for correct segregation of sister chromatids into daughter cells. Centrosome attachment to the poles also ensures that each daughter cell inherits a single centrosome, thus tightly regulating ploidy and centrosome numbers. Impaired centrosome duplication cycles or a failure of centrosome segregation result in abnormal centrosome numbers that in turn perturb bipolar spindle assembly and chromosome segregation [24].

CENPJ contains 17 exons and encodes a 1338 amino acid residue protein with a chromosomal segregate ATPase domain and a T-complex protein 10 (TCP10)-like C-terminal domain. Seckel-syndrome of a consanguineous Saudi Arabian family has been associated with a homozygous splice acceptor mutation in the last nucleotide of CENPJ intron 11 (Figure 1A) that results in the production of three transcripts lacking either exon 12, exons 11 and 12 or exons 11, 12 and 13 [4]. Three CENPJ-microcephaly mutations in three consanguineous Pakistani families have been reported to date and all are predicted to cause a truncating stop codon (Figure 1A; [14,15]).

ATR, RBBP and CEP152 have been shown to play a role in maintaining genomic stability through regulation of the DNA damage response [3,6,25], however such a role has not yet been defined for CENPJ. We set out to develop a mouse model of CENPJ-Seckel syndrome in order to establish the mechanism by which mutation of CENPJ results in this subtype of primordial dwarfism. We show that the Cenpj hypomorphic mouse that we created recapitulates many key features of Seckel syndrome, including microcephaly with memory impairment, dwarfism from birth, and skeletal abnormalities. We further establish that wide-scale genomic instability is the likely cause of cell death within Cenpjmut/mut embryos and suggest that this contributes to the developmental phenotypes observed in CENPJ-Seckel patients.

Results

Generation and phenotyping of a Cenpj hypomorphic mouse

Knockout mice carrying the Cenpjmut/EUCOMM/Wtsi allele (Figure 1A and Figure S1A) were generated on a C57BL/6NTac; C57BL/6-Tyrαβ-1d background by the Sanger Mouse Genetics Project as part of the European Conditional Mouse Mutagenesis Program ([EUCOMM; [26]). Correct gene targeting in founder mice was determined by a combination of standard and quantitative PCR (Figure S1). LacZ staining was detected in the brain and kidneys, while strong staining was present in the testes of mice heterozygous for the Cenpjmut/EUCOMM/Wtsi allele (Figure S2A).

The tm1a/EUCOMM/Wtsi gene-trap cassette that was introduced into the Cenpj locus is designed to truncate mRNA expression and to generate out-of-frame products following the deletion of a critical exon. Previous studies have indicated that mRNAs of certain microcephaly-associated genes are very stable [27] prompting us to perform a detailed analysis of expression and splicing at the Cenpjmut/EUCOMM/Wtsi locus. We generated Cenpjmut/EUCOMM/Wtsi/tna/EUCOMM/Wtsi (Cenpjtna) mouse embryonic fibroblasts (MEFs; 13.5 d.p.c.) and performed SYBR Green qPCR on cDNA using primers spanning the boundaries between different exons (Figure 1A). We observed a low but detectable amount of splicing over the gene-trap cassette in Cenpjtna MEFs (2.1±0.5% of wildtype exon 4–5 levels) and immunoblotting (Figure 1B) confirmed the production of low levels of apparently full-length Cenpj protein [27]. Splicing from exons 3 to 6 and 4 to 6 was detected in both Cenpjtna and wildtype MEFs (Figure S2B). Between exons 3 and 6 the level of splicing detected in Cenpjtna MEFs was increased relative to the levels in control MEFs (444±95%), while decreased levels of splicing were observed between exons 4 and 6 (2.1±0.5%). Using the web-based ExPASy translation tool (http://web.expasy.org/translate/) we predict that mRNAs that are spliced between exons 3–6 and exons 4–6 lead to the production of proteins truncated in exon 6 (Figure S2C). Upstream of the tm1a/EUCOMM/Wtsi cassette (exons 1–2) Cenpj mRNA levels were 68±19% of wildtype levels. Downstream (from exon 6 to 17) of the tm1a/EUCOMM/Wtsi cassette Cenpj mRNA levels were approximately 20% of the levels observed in MEFs from wildtype littermates (mean±SEM, n = 3). In summary, Cenpjtna MEFs are able to produce small amounts of full-length Cenpj protein due to splicing over the tm1a/EUCOMM/Wtsi gene-trap cassette (exons 4–5) and we predict that small amounts of truncated, N-terminal Cenpj protein (corresponding to exons 1 to 3 or 1 to 4) will also be produced.
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Figure 1. Generation of a mouse model of CENPJ-Seckel syndrome. A. The CENPJ gene spans 40 kb and comprises 17 exons. The 3′ and 5′ untranslated regions are depicted in grey. Mutations in CENPJ have been associated with either primary microcephaly (MCPH) or Seckel syndrome (SECKEL). The mutation in intron 11 that has been associated with Seckel syndrome results in the generation of three transcripts: one lacking exon 12, one lacking 11 and 12 and one lacking exons 11, 12 and 13. Disruption of mouse Cenpj by insertion of a cassette (depicted by the blue square) between exons 4 and 5 results in low levels of splicing over the cassette and cryptic splicing between exons 3 and 4 or 5; the latter two transcripts are predicted to result in truncated proteins. The allele was designated Cenpjtm1a(EUCOMM)Wtsi and abbreviated to Cenpjtm. Percentages show mean expression of Cenpj across exon boundaries as determined by quantitative RT-PCR relative to Gapdh for Cenpjtm/tm relative to Cenpj+/+ for RNA extracted from n = 3 murine embryonic fibroblast (MEF) lines. B. Immunoblot to show reduction in Cenpj levels in protein extracted from Cenpjtm/tm (tm/tm), Cenpj+/tm (+/tm), and Cenpj+/+(+/+) MEFs. KAP1 was used as a loading control. C. Table shows frequency of Cenpjtm/tm mice born from heterozygote intercrosses. Cenpjtm/tm showed partial embryonic lethality as shown by their reduced frequency at E18.5 and P14 (25% expected, *P = 0.02, **P = 0.0001, χ² test). D. Representative images of E18.5 skeletal preparations of Cenpj+/+ and Cenpjtm/tm embryos. Staining with alcian blue (cartilage) and alizarin red (bone). Cenpjtm/tm embryo with a sloping forehead and polysyndactylism of digit one of the left hindpaw (inset). E. Bodyweights of male Cenpjtm/tm (n = 8), Cenpj+/tm (n = 7), Cenpj+/+(+/+) and baseline wild-type controls (n = 912) from 3–16 weeks of age. Data show that Cenpjtm/tm are significantly smaller than Cenpj+/+ mice at all ages (P = 2.2 × 10⁻¹⁶, Mann-Whitney-Wilcoxon test). F. Skeletal preparations of E18.5 Cenpjtm/tm embryos showed irregular ossification of the cranium and sternum. G. X-Rays show that adult Cenpjtm/tm mice may present with a flatter, sloping forehead (A), mild elevation of the parietal bone (B), a short humerus with a prominent deltoid tuberosity (C), prominent medial epicondyle (D), an irregular ribcage (E), extra sacrocaudal transitional vertebrae (H), short, abnormally fused caudal vertebrae 2/3 – caudal vertebrae 7/8 (I) and reduced intervertebral joint space (J).

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Phenotyping of mice was performed at the Wellcome Trust Sanger Institute [http://www.sanger.ac.uk/mouseportal/search?query=acenpj]. *Cenpj*<sup>tm1a(EUCOMM)Wtsi/+* mice intercrosses gave close to the expected Mendelian frequency (25%) of homozygote embryos at E14.5 (23.5%); however, by E18.5 this had reduced to 11.6%, suggesting that disruption of *Cenpj* causes partial embryonic lethality between E14.5 and E18.5 (close to the expected Mendelian frequency (25%) of homozygote paw in 2/9 mice, however we noted polysyndactylism of the first digit of the left hind paw in 2/9 embryos (Figure 1D, inset), which has also been reported in mutant *Pnt* (pericentrin) mice [31]. Furthermore, retarded ossification and decreased bone age is reported in the majority of cases of Seckel syndrome, although this clinical abnormality was not specifically addressed for the *CENPJ*-Seckel kindred [1]. A higher proportion of *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* embryos showed incomplete or irregular ossification of the parietal and occipital bones when compared to controls (Figure 1F; 3/5 *Cenpj<sup>tm1a(EUCOMM)Wtsi/+*; 1/30 *Cenpj<sup>+/+*). In addition, a subset of Seckel patients, including a *CENPJ*-Seckel patient, have 11 ribs instead of the usual 12 [4,30,32]. Although all ribs were present in *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* embryos, we noted that the attachment of the ribs to the sternum followed an irregular pattern that closely corresponded to the asymmetrical distribution of ossification centers along the sternum (Figure 1G; 3/5 *Cenpj<sup>tm1a(EUCOMM)Wtsi/+*). Adult *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* mice displayed an irregular ribcage, with crowding of the ribs (Figure 1H; 9/15). Moreover, a subset of Seckel patients have been reported to have bilateral dislocation of the hips and elbows, with a decreased range of motion at the elbows [1]. While we did not find any evidence of dislocation we noted that the humeri of adult *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* mice were anatomically disproportionate when compared with those of wild-type mice; the deltoid tuberosities were closer to the greater tubercle when normalized to humeri length (mean ± SEM: right humeri, *Cenpj<sup>+/+* 49.6 ± 0.28 mm, *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* 47.1 ± 0.93 mm; *P* = 0.02, t-test; Figure 1H), and humeri were sometimes bowed (6/15) with a very prominent medial epicondyle (11/15; Figure 1H). Furthermore, all adult *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* mice (15/15) displayed an abnormal pelvis that was wider at the iliac crest (Figure 1H, iliac crest normalised to ischiac, mean ± SEM: *Cenpj<sup>+/+* 69.7 ± 1.18%, *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* 60.8 ± 1.72%; *P* = 0.0003) and sometimes asymmetrical. Finally, we observed that all *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* mice had a reduced intervertebral joint space in the lumbar and caudal regions (Figure 1H). In general, lumbar and sacral vertebræ were shorter and *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* mice had one to two extra sacrocaudal transitional vertebræ as a result. In 13/15 *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* mice, Caudal 2/3 – Caudal 7/8 were abnormal in morphology and fused (Figure 1H).

### Cenpj-deficiency causes intrauterine and postnatal growth retardation

One of the defining characteristics of primordial dwarfism disorders, such as Seckel syndrome, is a fetus that is small for its gestational age with postnatal growth retardation [8,28,29]; specifically, the Saudi Arabian *CENPJ*-Seckel kindred all have anthropometric values at least seven standard deviations below the mean [4]. *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* mice showed intrauterine growth retardation (Figure 1D; mean ± SEM bodyweight at E18.5, *Cenpj<sup>+/+* 1.12±0.03 g, *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* 0.8±0.04 g; *P* = 0.0001, t-test; crown-rump length at E18.5, *Cenpj<sup>+/+* 23.5±0.26 mm, *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* 20.7±0.54 mm; *P* = 0.0001, t-test). From 3–16 weeks, *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* mice were significantly smaller than wild-type controls (Figure 1E; mean ± SEM bodyweight at E18.5, *Cenpj<sup>+/+* 39.1±1.35 g, *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* 23.4±2.34 g; *P* = 9.3×10⁻¹⁰, t-test; Figure 1E) and length was 76% of controls (mean ± SEM nose-to-tail base length at 14 weeks, *Cenpj<sup>+/+* 10.4±0.05 cm, *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* 7.9±0.07 cm, *P* = 2.2×10⁻¹⁰, t-test; Figure S2D).

### Skeletal abnormalities and abnormal ossification of bone from *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* mice

The skeletal abnormalities of Seckel syndrome associated with an intron 11 mutation in *CENPJ* include a receding chin, high forehead and prominent nasal spine [4]. Although the facial features of the *CENPJ*-Seckel kindred (two siblings and three cousins) were strikingly similar, the skeletal survey of sibling one (a subset of Seckel kindred) were largely normal and that of sibling two revealed 11 ribs instead of the usual 12 [4,30,32]. Although all ribs were present in *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* embryos, we noted that the attachment of the ribs to the sternum followed an irregular pattern that closely corresponded to the asymmetrical distribution of ossification centers along the sternum (Figure 1G; 3/5 *Cenpj<sup>tm1a(EUCOMM)Wtsi/+*). Adult *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* mice displayed an abnormal pelvis that was wider at the iliac crest (Figure 1H, iliac crest normalised to ischiac, mean ± SEM: *Cenpj<sup>+/+* 69.7 ± 1.18%, *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* 60.8 ± 1.72%; *P* = 0.0003) and sometimes asymmetrical. Finally, we observed that all *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* mice had a reduced intervertebral joint space in the lumbar and caudal regions (Figure 1H). In general, lumbar and sacral vertebræ were shorter and *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* mice had one to two extra sacrocaudal transitional vertebræ as a result. In 13/15 *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* mice, Caudal 2/3 – Caudal 7/8 were abnormal in morphology and fused (Figure 1H).

### Neuropathological abnormalities and memory impairment

Microcephaly is one of the defining characteristics of Seckel syndrome [1]. Microcephaly has been clinically defined as a head circumference of at least two standard deviations below the normal range; and in the case of Seckel syndrome associated with mutations in intron 11 of *CENPJ*, head circumference is seven standard deviations below the mean [4,33]. The average *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* mouse brain weight was two standard deviations below that of control mice (Figure 2A; *P* = 0.0002, t-test). Although the two and four-year-old siblings with *CENPJ*-Seckel syndrome described to date had relatively normal magnetic resonance imaging (MRI), cranial MRI of adult patients with Seckel syndrome has revealed several neuroanatomical abnormalities aside from a reduction in brain volume [4,32,34,35]. We therefore assessed the area of brain regions and the thickness of the neuronal layers of the adult mouse brain (16 weeks; Figure S3). Although the patterning of the hippocampal layers appeared normal, the length of the dentate gyrus was significantly reduced in *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* when compared to *Cenpj<sup>+/+* control mice (mean ± SEM: *Cenpj<sup>+/+* 4380±264 μm, *Cenpj<sup>tm1a(EUCOMM)Wtsi/+* 3797±181 μm; *P* = 0.01, t-test; Figure 2B). The average thickness of the cortex, which is often reduced with mutation of microcephaly genes in mice [36,37], and of the molecular, stratum radiatum and oriens layers of the hippocampus were not significantly different to wild-type controls. Similarly, the total areas of the hippocampus, corpus callosum and dorsal third ventricle were unchanged as was the total internal length of the pyramidal cell layer.

History was suggestive of normal cognitive and motor development for four of the five cases within the *CENPJ*-Seckel
kindred while one patient clearly had intellectual impairment (IQ 60; MRI not performed [4]). Since the hippocampus is involved in learning and memory formation and since Seckel patients generally display learning impairments [3,34], we performed a social recognition test with Cenpjtm/tm and control animals [38,39,40]; Figure 2C and 2D). Thus, on day one, mice were tested for habituation-dishabituation: male mice were presented with a novel, anaesthetized stimulus mouse and the time of investigation was recorded. Mice were then given a 10 minute resting period before this was repeated a further three times with the same stimulus mouse. On the fifth trial, mice were presented with an unfamiliar stimulus mouse (Figure 2C). Both Cenpjtm/tm (n = 7) and Cenpj+/+ (n = 7) mice recognized and habituated to the novel stimulus mouse, as there was a decline in investigation time over the first four trials that was recovered on trial five (Figure 2C, trial four vs. trial five, *P = 0.0033 and **P = 0.0014, two-way ANOVA followed by post-hoc t-test). D. A discrimination test was performed 24 h later the habituation-dishabituation test. When given a choice between the familiar (same stimulus animal used for trials one to four) and a new unfamiliar mouse 24 h later, Cenpjtm/tm mice could not discriminate as shown by the similar investigation time for both stimulus animals (Cenpj+/+ P = 0.0326, Cenpjtm/tm P = 0.957, t-test). E. Representative images of immunohistochemical stainings of E14.5 embryo sections. Cenpj was highly expressed in areas of active neurogenesis within the telencephalon. Scale bar 400 μm. There was a generalized increase in cleaved (activated) caspase-3-positive (scale bar 100 μm) and Ser139-phosphorylated H2AX (γH2AX; scale bar 200 μm) cells throughout embryos, images of striatum are shown.. The number of cells positive (as a percentage of total in two different 75 μm² areas) for cleaved (activated) caspase-3 (C3A+) and pan-nuclear Ser139-phosphorylated H2AX (γH2AX) was increased in areas of active neurogenesis within the striatum and cortex. *P<0.05; Mann-Whitney with continuity correction, Cenpjtm/tm n = 3 and Cenpj+/+ n = 3. Data shows mean and SEM. F. Neuron densities were counted in three different areas (75 μm²) of active neurogenesis for each of the striatum (STR), cortex (CTX) and pro-hippocampus (HIP) and three areas of 150 μm² in the mid-striatum (M-STR) of E14.5 embryos, *P = 0.0008, t-test, Cenpjtm/tm n = 3 and Cenpj+/+ n = 3. Data shows mean and SEM. doi:10.1371/journal.pgen.1003022.g002

Figure 2. Neuropathological abnormalities. A. Cenpjtm/tm mouse brain weights were two standard deviations below that of control mice (n = 144 baseline control mice). *P = 0.0002, t-test, Cenpjtm/tm, n = 6 and Cenpj+/+ n = 10. The lower whisker extends to the lowest datum still within 1.5 Inter-quartile range (IQR) of the lower quartile. The upper whisker extends to the highest datum still within 1.5 IQR of the upper quartile B. The dentate gyrus was significantly shorter in Cenpjtm/tm mice (n = 3) when compared to Cenpj+/+ control mice (n = 30), *P = 0.01, t-test. Scale bar 1 mm C. Social recognition test. When tested for habituation-dishabituation, both Cenpjtm/tm (n = 7) and Cenpj+/+ (n = 7) mice recognized a novel stimulus mouse as shown by a decline in investigation time over the first four trials that was recovered on trial five, when they were exposed to a novel mouse (trial four vs. trial five, *P = 0.0033 and **P = 0.0014, two-way ANOVA followed by post-hoc t-test). D. A discrimination test was performed 24 h later the habituation-dishabituation test. When given a choice between the familiar (same stimulus animal used for trials one to four) and a new unfamiliar mouse 24 h later, Cenpjtm/tm mice could not discriminate as shown by the similar investigation time for both stimulus animals (Cenpj+/+ P = 0.0326, Cenpjtm/tm P = 0.957, t-test). E. Representative images of immunohistochemical stainings of E14.5 embryo sections. Cenpj was highly expressed in areas of active neurogenesis within the telencephalon. Scale bar 400 μm. There was a generalized increase in cleaved (activated) caspase-3-positive (scale bar 100 μm) and Ser139-phosphorylated H2AX (γH2AX; scale bar 200 μm) cells throughout embryos, images of striatum are shown.. The number of cells positive (as a percentage of total in two different 75 μm² areas) for cleaved (activated) caspase-3 (C3A+) and pan-nuclear Ser139-phosphorylated H2AX (γH2AX) was increased in areas of active neurogenesis within the striatum and cortex. *P<0.05; Mann-Whitney with continuity correction, Cenpjtm/tm n = 3 and Cenpj+/+ n = 3. Data shows mean and SEM. F. Neuron densities were counted in three different areas (75 μm²) of active neurogenesis for each of the striatum (STR), cortex (CTX) and pro-hippocampus (HIP) and three areas of 150 μm² in the mid-striatum (M-STR) of E14.5 embryos, *P = 0.0008, t-test, Cenpjtm/tm n = 3 and Cenpj+/+ n = 3. Data shows mean and SEM. doi:10.1371/journal.pgen.1003022.g002

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similar investigation time for both stimulus animals (Figure 2D, P = 0.957, t-test; Normalized discrimination \( \text{Cenpj}^{+/+} \) vs \( \text{Cenpj}^{+/-} \), \( P = 0.0417 \)). In summary, short-term memory and olfaction appear to be unaffected in \( \text{Cenpj}^{+/-} \) mice, however long-term memory was significantly impaired. All other tests of neurological function were normal, including open field, grip strength, modified SHIRPA (the SmithKline Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment is a set of behavioural tests designed to test muscle, cerebellar, sensory and neuropsychiatric function), auditory brainstem response and hot plate assessment.

### Increased DNA damage, apoptosis, and reduced neuron density in \( \text{Cenpj}^{tm/tm} \) mice

All of the genes associated with Seckel syndrome have so far been shown to result in defective DNA damage responses and a lowered apoptotic threshold [5,6,7,25,41]. To test whether \( \text{Cenpj} \)-deficiency is associated with elevated levels of DNA strand breaks and/or apoptosis, we performed immunohistochemical staining of E14.5 embryos. Phosphorylation of histone H2AX on serine 139 (\( \gamma \text{H2AX} \)) by the ATR, DNA-PK or ATM kinases occurs at sites flanking DNA strand breaks and enhances the recruitment of DNA repair proteins to sites of damage [42]; if the damage is irreparable then the cell death cascade is normally activated [43]. Compared to controls, there was a general increase in the number of \( \gamma \text{H2AX}- \)positive cells throughout \( \text{Cenpj}^{tm/tm} \) embryos (Figure S4) and this was most pronounced in the developing telencephalon (Figure 2E; mean±SEM. \( \gamma \text{H2AX}- \)positive cells as a percentage of total; striatum \( \text{Cenpj}^{+/+} \) 0.47±0.02%, \( \text{Cenpj}^{tm/tm} \) 2.3±0.3%, \( P = 0.004 \); cortex \( \text{Cenpj}^{+/+} \) 0.0%, \( \text{Cenpj}^{tm/tm} \) 1.3±0.3%, \( P = 0.01 \); pro-hippocampus \( \text{Cenpj}^{+/+} \) 0.0%, \( \text{Cenpj}^{tm/tm} \) 0.9±0.3%, \( P = 0.03 \). Mann-Whitney with continuity correction). Similarly, there was an increase in the number of cleaved caspase-3-positive cells throughout \( \text{Cenpj}^{tm/tm} \) embryos (Figure S4), although this was most pronounced in areas of active neurogenesis (as determined by Ki67 staining; Figure S4) within the telencephalon, where \( \text{Cenpj} \) was most highly expressed (Figure 2E; mean±SEM. cleaved caspase-3-positive cells as a percentage of total cells: striatum \( \text{Cenpj}^{+/+} \) 0.0%, \( \text{Cenpj}^{tm/tm} \) 1.9±0.2%, \( P = 0.001 \); cortex \( \text{Cenpj}^{+/+} \) 0.0%, \( \text{Cenpj}^{tm/tm} \) 0.6±0.2%, \( P = 0.05 \); pro-hippocampus \( \text{Cenpj}^{+/+} \) 0.0%, \( \text{Cenpj}^{tm/tm} \) 0.5±0.3%, \( P = 0.14 \), t-test). There was no detectable difference in the patterns of cellular proliferation between \( \text{Cenpj}^{tm/tm} \) and \( \text{Cenpj}^{+/+} \) embryos when examined using the marker Ki67 (Figure S4).

Consistent with increased levels of apoptosis in the developing telencephalon of \( \text{Cenpj}^{tm/tm} \) embryos, reports of fetal stage Seckel syndrome (loci responsible unknown) have shown reduced neuron density and disorganization of cortical layers at 30 weeks gestation [28,29]. We therefore quantified neuron densities in areas of active neurogenesis within the telencephalon (areas of Ki67-positive staining; Figure S4) and in the mid-striatum of embryos during mid-neurogenesis (E14.5) and found that, in general, the number of neurons were decreased in \( \text{Cenpj}^{tm/tm} \) embryos and the reduction was significant for the striatum (Figure 2F; mean±SEM. for \( n = 3 \) (average of two different 75 \( \mu \text{m} \) areas) in striatum: \( \text{Cenpj}^{+/+} \) 104.9±3.1, \( \text{Cenpj}^{tm/tm} \) 94.7±3.8, \( P = 0.0008 \), t-test).

### Delayed puberty of female \( \text{Cenpj}^{tm/tm} \) mice

Several clinical reports of patients with Seckel syndrome have described precocious puberty or premature thelarche [44,45]. It is not yet known whether the sexual development of patients with Seckel syndrome associated with \( \text{Cenpj} \) mutations is normal since the cases described so far report the phenotype of infants [4]. A thorough histopathological analysis of adult male and female \( \text{Cenpj}^{tm/tm} \) and \( \text{Cenpj}^{+/+} \) mice (\( n = 3 \) of each gender and genotype at 16 wks) revealed several anomalies, including corticomedullary pigmentation in the adrenals of female \( \text{Cenpj}^{tm/tm} \) mice (Figure 3A). Corticomedullary pigmentation is associated with ‘X-zone’ degeneration in female mice, a sex hormone-dependent change that occurs during puberty in virgin females and is often complete by 16 weeks in C57BL/6 mice, or earlier in pregnant females [46]. By using cleaved-caspase 3 as a marker of apoptosis, we found that the adrenals of virgin \( \text{Cenpj}^{tm/tm} \) female mice have pronounced and ongoing X-zone degeneration at 16 weeks when compared to virgin \( \text{Cenpj}^{+/+} \) females (Figure 3A). Wild-type C57BL/6 female mice reach sexual maturity at around 6-7 weeks of age. These findings suggest that, in contrast to Seckel syndrome patients, puberty is delayed in \( \text{Cenpj}^{tm/tm} \) female mice. In support of this, breeding records of females set up with \( \text{Cenpj}^{tm/tm} \) males at 6-7 weeks of age showed that \( \text{Cenpj}^{tm/tm} \) females produce their first litter around four weeks later than \( \text{Cenpj}^{+/+} \) females (\( P = 0.012 \), t-test; Figure 3B). There were no morphological differences in the reproductive tract of male or female \( \text{Cenpj}^{tm/tm} \) animals when examined at 16 weeks of age (data not shown).

### Abnormal development and structural abnormalities of the eye in \( \text{Cenpj}^{tm/tm} \) mice

Although not reported for the \( \text{CENPJ}-\text{Seckel} \) kindred [4], several individuals that have been clinically diagnosed with Seckel syndrome have ocular defects, such as spontaneous lens dislocation, myopia, astigmatism, and retinal degeneration [47] [48]. A higher proportion of \( \text{Cenpj}^{tm/tm} \) embryos had secondary anophthalmia (E18.5, 0/13 \( \text{Cenpj}^{+/+} \), 1/38 \( \text{Cenpj}^{tm/tm} \)) and 1/3 \( \text{Cenpj}^{tm/tm} \) and a higher proportion of \( \text{Cenpj}^{tm/tm} \) pups still had their eyes closed at 15 (5/28 \( \text{Cenpj}^{+/+} \) vs. 1/46 wild-type). At 16 weeks of age, histological analysis of the eyes from \( \text{Cenpj} \) mice showed various structural abnormalities. In the anterior segment, the corneal endothelium and Descemet’s membrane was occasionally broken (Figure 3C). The anterior chamber was of normal depth but the angle was anteriorly displaced in some cases (Figure 3C). Significant cataracts were not observed in \( \text{Cenpj}^{+/+} \) mice but the iris showed adhesions to the lens and its base was anteriorly shifted in relation to the ciliary body (Figure 3C). Also, the ciliary body processes were spaced far apart or blunt in \( \text{Cenpj}^{tm/tm} \) animals, and in some cases, ciliary process morphology was abnormal (Figure 3C). In the retina, the photoreceptor nuclei were variably reduced in number and columns were loosely packed or disorganized in \( \text{Cenpj}^{tm/tm} \) animals (Figure 3C). Other cell layers, including the retinal ganglion, inner nuclear, and retinal pigment epithelium appeared normal, and the optic nerve did not show thinning. At E14.5, \( \text{Cenpj} \) was highly expressed in the retina inner-nuclear layer, where cells are rapidly differentiating and proliferating, but not in the innerretinal ganglion cell progenitor layer (Figure 3D).

### Delayed response to glucose challenge in \( \text{Cenpj}^{tm/tm} \) mice

During routine phenotyping, mice were subject to an intra-peritoneal glucose tolerance test, in which mice were fasted for 16 hours, a bolus of glucose was administered intraperitoneally and blood glucose concentration was monitored for 2 hours. Fifteen minutes after administration of glucose, 4/4 female \( \text{Cenpj}^{tm/tm} \) mice (\( \text{Cenpj}^{+/+} \) 20.5±0.7 mmol/l, \( \text{Cenpj}^{tm/tm} \) 30.7±1.6 mmol/l, \( P = 2.8 \times 10^{-5} \), t-test) and 2/5 male \( \text{Cenpj}^{tm/tm} \) had blood glucose levels greater than or equal to the 97.5th centile of baseline controls (\( n = 670 \) females, \( n = 669 \) males), although this had returned to normal by 30 minutes (Figure 3E).
Cenpj-deficiency is associated with karyomegaly of cardiomyocytes in young mice

Despite being one of the less frequently reported characteristics of Seckel syndrome, there are numerous case-reports of severe cardiac anomalies in Seckel syndrome patients [49, 50, 51, 52, 53]. Strikingly, the majority of 16-week old Cenpj<sup>tm/tm</sup> mice (5/6) and only 1/6 Cenpj<sup>+/+</sup> and 0/4 wildtype mice showed disorganization of cardiomyocytes with an increased incidence of karyomegaly and multinucleate cells, predominantly within the interventricular septum, papillary muscle and inner myocardium (Figure S2E). Cardiomyocyte karyomegaly has previously been observed in wild-type mice [54] where it may be associated with reparative processes [55] and may represent polyploidy [56]. Although the incidence and extent of karyomegaly was noticeably increased in hearts from Cenpj<sup>tm/tm</sup> mice compared to wildtype animals in this study, there was no evidence of fibrosis (consistent with previous cardiac damage) based on trichrome staining or alterations in apoptosis or proliferation (cleaved caspase-3 and Ki67, respectively; data not shown). Interestingly, the preponderance of karyomegaly in cardiomyocytes, hepatocytes and cells of the Figure 3. Delayed onset to puberty and ocular, endocrine, haematological, and plasma abnormalities. A. Periodic acid-Schiff (PAS) staining and cleaved (activated) caspase-3 immunostaining of adrenal sections from 16-week-old virgin female Cenpj<sup>tm/m</sup> mice (n = 3) confirmed corticomedullary pigmentation and ongoing apoptosis in the X-zone, respectively (representative images, scale bars 100 μm). B. Breeding records of Cenpj<sup>tm/m</sup> females set up with Cenpj<sup>tm/m</sup> males at 6-7 weeks of age showed that Cenpj<sup>tm/m</sup> females produce their first litter around four weeks later than Cenpj<sup>+/+</sup> females, *P = 0.012, t-test. C. Top panel shows normal cornea from a Cenpj<sup>+/+</sup> mouse. Cenpj<sup>tm/m</sup> mice had disruption of the Descemet’s membrane and corneal endothelium (arrow). Middle panel shows normal anterior segment from a Cenpj<sup>+/+</sup> mouse. The angle was displaced anteriorly in eyes from Cenpj<sup>tm/m</sup> mice and ciliary process morphology was abnormal. (a, angle; i, iris; cb, ciliary body; l, lens). Bottom panel shows normal retina from a Cenpj<sup>+/+</sup> mouse eye. The retina photoreceptor cells of Cenpj<sup>tm/m</sup> mice were reduced in number and showed columnar disorganized (arrow). (ONL, outer nuclear layer). D. Immunohistochemical staining for Cenpj in Cenpj<sup>+/+</sup> embryo eye (E14.5; RNL retinal neuroblast layer). E. Intra-peritoneal glucose tolerance test to show that female Cenpj<sup>tm/m</sup> mice have a 15 minute delay in response to glucose challenge (n = 4 Cenpj<sup>tm/m</sup> vs. n = 32 Cenpj<sup>+/+</sup>, *P = 2 × 10⁻³, t-test). Graph also shows n = 9 Cenpj<sup>+/+</sup> and n = 670 baseline wildtype controls. F. Plasma albumin levels were decreased in Cenpj<sup>tm/m</sup> males (n = 8 Cenpj<sup>tm/m</sup> vs. n = 35 Cenpj<sup>+/+</sup>, *P = 4.9 × 10⁻³, t-test). Graph also shows n = 7 Cenpj<sup>+/+</sup> and n = 768 baseline wildtype controls. G. Flow cytometric analysis of peripheral blood leukocytes in Cenpj<sup>tm/m</sup> mice revealed an increase in the number of CD8<sup>+</sup> and H. total CD3<sup>+</sup> cells. Data shows total counts per 30 000 propidium-iodide (PI) negative, CD45-positive cells from male mice. For n = 9 Cenpj<sup>tm/m</sup> vs. n = 30 Cenpj<sup>+/+</sup> CD3<sup>+</sup> cells and CD8<sup>+</sup> *P = 0.0002 and CD3<sup>+</sup> *P = 2.9 × 10⁻³, Mann-Whitney-Wilcoxon test. Graphs also show n = 7 Cenpj<sup>+/+</sup> and n = 356 baseline wildtype controls. For all ‘Box and Whisker’ plots, the lower whisker extends to the lowest datum still within 1.5 Inter-quartile range (IQR) of the lower quartile. The upper whisker extends to the highest datum still within 1.5 IQR of the upper quartile.

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Harderian glands was increased in aged Cenpj<sup>+</sup> mice (13-month old) when compared to age-matched control (Figure S2E).

**Hypoalbuminemia of Cenpj<sup>−/−</sup> mice**

Clinical chemistry was performed on animals at 16 weeks of age. Albumin levels were generally decreased in Cenpj<sup>−/−</sup> mice of both genders compared to controls, and this was statistically significant for males (mean ± S.E.M, Cenpj<sup>−/−</sup> 25.1 ± 0.31, Cenpj<sup>+/+</sup> 21.9 ± 0.32, P = 4.9 × 10<sup>−5</sup>, t-test; Figure 3F).

**Increased levels of CD3<sup>+</sup>CD8<sup>+</sup> T cells in Cenpj<sup>−/−</sup> mice**

Flow cytometric analysis of peripheral blood leukocytes at 16 weeks of age revealed a marked increase in the frequency of the CD8<sup>+</sup> T cell subset (CD3<sup>+</sup>CD8<sup>+</sup>) in both genders of Cenpj<sup>−/−</sup> mice compared to wild-type controls (mean ± S.E.M Males: CD8<sup>+</sup>CD3<sup>+</sup> gated on PI<sup>−</sup> CD45<sup>+</sup>: Cenpj<sup>−/−</sup> 4.3% ± 0.18, Cenpj<sup>+/+</sup> 7.5% ± 0.55, P = 0.0002 Mann-Whitney-Wilcoxon test; Figure 3G). Furthermore, the increase in the proportion of the peripheral blood CD8<sup>+</sup> T cell population was reflected in a concomitant increase in the frequency of total T cells in Cenpj<sup>−/−</sup> mice (mean ± S.E.M, CD3<sup>+</sup> gated on PI<sup>−</sup> CD45<sup>+</sup>: Cenpj<sup>−/−</sup> 9% ± 0.39, Cenpj<sup>+/+</sup> 14% ± 0.41, P = 2.9 × 10<sup>−5</sup>, Mann-Whitney-Wilcoxon test; Figure 3H). The frequency of CD4<sup>+</sup> T cells in the peripheral blood was unaffected (data not shown).

**Increased genomic instability in embryonic fibroblasts from Cenpj-deficient mice**

CENPj depletion in cultured cells has been reported to impair centriole assembly, disrupt centrosome integrity and lead to the formation of monopolar and multipolar spindles instead of bipolar spindles [19,20,21,24,57]. To assess the cellular phenotype of Cenpj<sup>−/−</sup> mice, MEFs were derived from Cenpj<sup>−/−</sup>, Cenpj<sup>−/+</sup> and Cenpj<sup>+/+</sup> littermates. For all experiments, MEFs were of early passage (P<5) and were passage-matched. Cenpj protein levels were reduced in the centrosomes of Cenpj<sup>−/−</sup> MEFs, but residual protein remained detectable in most cells (Figure 4A). Consistent with previous findings, frequencies of both monopolar and multipolar spindles were elevated in two independently derived Cenpj<sup>−/−</sup> MEF lines (Figure 4B; Cenpj<sup>−/−</sup>: 2.1% monopolar and 8% multipolar; Cenpj<sup>−/+</sup> (1): 10.6% monopolar and 20.8% multipolar; Cenpj<sup>−/+</sup> (2): 8.9% monopolar and 18.9% multipolar). Distribution and intensities of the centrosomal proteins ß-tubulin (Figure 4A), CDK5RAP2 (Figure 4B), and pericentrin (data not shown) were unaffected in the mutant. We next asked whether spindle abnormalities are accompanied by aberrant centrosome and centriole numbers in Cenpj<sup>−/−</sup> MEFs. A normal mitotic cell contains two centrosomes, each containing a pair of centrioles. Supernumerary centrioles were visible in mitotic Cenpj<sup>−/−</sup> cells (Figure S5). To facilitate counting of centrioles, cells were arrested in mitosis using monastral, a microtubule motor poison that prevents separation of spindle poles and thereby generates monopoles [58]. Cells with three or four centrioles were considered normal, since it is not always possible to resolve centrioles within a pair. We observed an increase in cells containing both too few (≤2) and too many centrioles (≥5) in the mutant (Figure 4C). To survive, cells with supernumerary centrosomes must either inactivate these or cluster active centrosomes into two poles, a process that ensures bipolar division [25,39]. Clustered centrosomes were indeed observed in Cenpj<sup>−/−</sup> MEFs (Figure 4B). Centrosome clustering however does not result in unequal partitioning of centrosomes into daughter cells (Figure S5), which ultimately causes a disassociation between centrosome numbers and DNA ploidy.

Cell-cycle analysis of Cenpj<sup>−/−</sup> MEFs revealed a significant increase in the number of 4C and elevated levels of >4C cells (Figure 5A), indicative of polyploidy. We examined the ploidy of fifty metaphase spreads of Cenpj<sup>−/−</sup> and Cenpj<sup>+/+</sup> MEFs and found that a remarkably high percentage of Cenpj<sup>−/−</sup> cells were near tetraploid (Cenpj<sup>−/−</sup> 11% vs. Cenpj<sup>+/+</sup> 41%). Twenty metaphase spreads with good fluorescent in situ hybridization (FISH) signals were selected for multiplex-FISH karyotyping which confirmed that many cells were near tetraploid and revealed additional defects such as aneuploidy, centromere loss, centric fusions (Figure 5B) and translocations (Figure 5C; for a breakdown of anomalies see Figure S2F). Consistently, we found evidence of lagging chromosomes in anaphase Cenpj<sup>−/−</sup> MEFs (Figure S5). Furthermore we show that adult Cenpj<sup>−/−</sup> mice have an increased prevalence of micronucleated normochromyatic erythrocytes (P = 0.000004, t-test; Figure 5D), thus confirming that these mutants have spontaneous genomic instability.

**The genomic instability observed in Cenpj<sup>−/−</sup> mice does not reflect defective ATR- or ATM-dependent DNA-damage signaling**

Seckel syndrome belongs to a group of genome instability disorders collectively referred to as DNA-damage response and repair-defective syndromes [60]. So far, all cells derived from Seckel patients have been found to be impaired in signaling mediated by the DNA-damage responsive protein kinase ATR, and therefore display reduced phosphorylation of downstream ATR substrates such as the checkpoint kinase Chk1, and have impaired G2/M cell-cycle checkpoint arrest upon treatment with DNA-damaging agents [60,61]. We therefore treated fibroblasts from Cenpj<sup>−/−</sup> embryos (13.5 d.p.c) with the DNA damaging agent camptothecin, a DNA topoisomerase I inhibitor that causes DNA double-strand breaks specifically in S-phase [62]. Analyses revealed that Cenpj<sup>−/−</sup> fibroblasts were proficient for ATR-dependent and ATM-dependent phosphorylation of Chk1 (pS345) and KAP1 (pS824), respectively, and showed normal activation of γH2AX (Figure 5E). We found no evidence of an impaired G2/M DNA damage checkpoint as determined by the percentage of MPM2-positive cells following irradiation (Figure S2G). Furthermore, CtIP-Seckel cells show defective phosphorylation of replication protein A (RPA) after camptothecin treatment, a phenotype associated with impaired DNA-end resection and homologous recombination [6]. However, we found no evidence of this in Cenpj<sup>−/−</sup> MEFs (Figure 5E).

**Discussion**

The Cenpj hypomorphic mouse (Cenpj<sup>−/−</sup>) that we have created displays many of the classical clinical features of Seckel syndrome, including intrauterine and postnatal dwarfism, microcephaly, a sloping forehead, neuropathological abnormalities, memory impairment and genomic instability [1,4,7,15,28,63]. In addition, we have shown that Cenpj<sup>−/−</sup> mice display some of the less frequently reported characteristics of the syndrome, including retarded bone ossification [1,32,64], as well as vertebral abnormalities and several other interesting histopathological and hematological abnormalities that have not previously been reported in patients.

**Microcephaly versus dwarfism of Cenpj<sup>−/−</sup> mice**

Neuroepithelial cells have apical-basal polarity, and the switch from proliferative, symmetric to neurogenic, asymmetric division is controlled by the orientation of the spindle pole during mitotic division [65]. Primary microcephaly is caused by mutations of centrosomal proteins and is thought to arise from an increase in
Figure 4. Centrosome and mitotic spindle abnormalities are elevated in Cenpj-deficient cells. A. Images show examples of Cenpj staining in centrosomes of Cenpj\textsuperscript{+/+} and Cenpj\textsuperscript{tm/tm} mouse embryonic fibroblasts (MEFs). Cells were stained with antibodies against Cenpj (green in merge) and the centrosomal protein γ-tubulin (red in merge). Framed areas are shown at higher magnification. B. Graph shows mitotic spindle phenotypes in Cenpj\textsuperscript{+}/+, Cenpj\textsuperscript{tm/tm} (1), and Cenpj\textsuperscript{tm/tm} (2) cells. C. Total number of centrioles in mitotic cells.
Functionality of Cenpj during neurogenesis is required for normal brain development. Cenpj is evolutionarily conserved from C. elegans to humans, and mutations in CENPJ are associated with a range of neurodevelopmental disorders, including primary microcephaly, Seckel syndrome, and neurocognitive deficits.

Cenpj expression is highest in the developing telencephalon, and it is required for normal neuronal density and long-term memory. Cenpj is involved in the regulation of DNA damage and cell cycle checkpoints, and its loss results in increased levels of DNA damage, apoptosis, and defects in spindle pole orientation.

The microcephaly of Cenpj-/-/-/- mice can be phenocopied by the Cenpj tm/tm mouse line, which shows a reduction in neuron density and long-term memory. These results suggest a role for Cenpj in neurodevelopmental disorders, and further investigation is needed to understand the mechanisms by which Cenpj regulates neurogenesis.

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Figure 5. Genomic instability is associated with abnormal ploidy of Cenpjtmt/tm cells rather than an impaired DNA damage response.

A. Cell cycle analysis of Cenpjtmt/tm mouse embryonic fibroblasts (MEFs) by flow cytometry showed an increase in the percentage of cells in G2 (4C) and cells containing >4C DNA content when compared to Cenpj+/+ cells. Percentages represent means of n = 3 independent MEF lines per genotype (each pair of +/+ and tm/tm cells were passage-matched (passage<5) and derived from littermates), *P<0.05, t-test. PI, propidium iodide. B. Example
mice showed increased genomic instability when compared to karyotype is near tetraploid, with centric fusions (white arrows) and chromosomes that have apparently lost their centromeres (black arrows). C.
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amounts of Cenpj are available in the mutant for cilia formation in or renal cystic disease, suggesting that sufficient
centrioles, Cenpjtm/tm

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ciliogenesis has not been extensively explored in mammals, but
both primary and motile cilia [70]. The role of CENPJ in
esis requires centriole biogenesis and therefore dSas-4 mutants lack
assembly, this effect is relatively mild, and therefore suggests that
Centrioles, mitotic spindles, and ploidy
dSas-4 is the mouse CENPJ. Unlike dSas-4-depleted cells or dSas-4 mutant flies that progressively lose centrioles, Cenpjtm/tm
MEFs contain centrioles even after several passages [70,71]. While the increase in Cenpjtm/tm cells with two or fewer centrioles is consistent with an impairment of centriole assembly, this effect is relatively mild, and therefore suggests that the mutant expresses residual, functional Cenpj protein. Gliogenesis requires centriole biogenesis and therefore dSas-4 mutants lack both primary and motile cilia [70]. The role of CENPJ in gliogenesis has not been extensively explored in mammals, but depletion of CENPJ in cultured cells is reported to impair primary cilium formation [72]. Cenpjtm/tm mice (16 weeks old) did not display phenotypes normally associated with cilopathies such as situs inversus or renal cystic disease, suggesting that sufficient amounts of Cenpj are available in the mutant for cilium formation in the majority of cells. However, the abnormalities in ciliary processes and photoreceptor nuclei within the eye may be attributed to ciliary defects. Moreover, unlike dSas-4 mutant males that display loss of flagella and sperm motility, Cenpjtm/tm male mice are fertile [70], which could again be due to residual expression of Cenpj.

While Cenpjtm/tm MEFs displayed irregular centriole numbers and mono- and multipolar spindles, they also showed extensive polyploidy and aneuploidy. Thus, we cannot conclude whether abnormal centriole and centrosome numbers are the cause or consequence of aberrant ploidy. Figure S6A shows the possible sequence of events that may lead to the abnormal ploidy of CENPJ-Deficient cells. Aberrant centrosome numbers are known to cause mitotic spindle abnormalities, culminating in mitotic delay, chromosome missegregation, cytokinetic failure and polyploidy. Prolonged mitotic delay can cause DNA damage, cell cycle arrest and apoptosis [73,74]. Chromosome missegregation can also damage chromosomes, hence triggering activation of DNA damage checkpoints [68,75]. Chromosome instability could therefore explain the increase in γH2AX levels and potentially, the increase in apoptosis in the mutant embryonic brain. Of all chromosome aberrations detected in the mutant MEFs, tetraploidy was the most prominent. A common cause of tetraploidy is an abortive mitotic cell cycle whereby cells enter but fail to complete mitosis [76]. Mitotic spindle abnormalities in Cenpjtm/tm cells could trigger extended mitotic arrest followed by mitotic slippage producing a tetraploid cell (Figure S6A). Tetraploid Cenpjtm/tm MEFs seem to be able to proliferate, since they represented almost 40% of the metaphase cells obtained for karyotyping. Interestingly, dSas-4 mutant flies show only a small increase in the proportion of aneuploid cells (1% in wild-type vs. 3% in mutants) and no polyploidy [70], whereas the proportion of near tetraploid Cenpjtm/tm embryonic fibroblasts was surprisingly high (~10% in wildtype vs. ~40% in Cenpjtm/tm MEFs). We suspect that Cenpj-deficiency exacerabate tetraploidy in MEFs, which are particularly susceptible to tetraploidy with passaging [77]. Nonetheless, adult Cenpjtm/tm mice show increased micronucleus induction, which is likely the result of lagging chromosomes and chromosome breakage.

Polyploidy as a potential cause of karyomegaly in Cenpjtm/tm tissues
Cenpjtm/tm mice of both genders showed an increased incidence of hypertrophic, disorganized cardiomyocytes with karyomegaly in the endocardium and interventricular septum when compared to wild-type mice. The areas showed no evidence of degeneration or repair, however since a high proportion of Cenpjtm/tm MEFs are polyploid, this is likely to be the cause of the karyomegaly. Although one of the less frequently reported characteristics of Seckel syndrome, there are numerous case-reports of severe cardiac anomalies in Seckel syndrome patients, including atrial and ventricular septal defects, pulmonary atresia, patent ductus arteriosus and congenital heart disease [49,50,51,52,53]. It will be interesting to see whether CENPJ-Seckel patients develop cardiac defects as they age. At 16 weeks of age Cenpjtm/tm mice showed hypoalbuminemia, which is associated with chronic liver and kidney diseases, although histopathological analysis of their livers and kidneys did not reveal any abnormalities. However, the preponderance of karyomegaly in the liver and Harderian glands was increased in aged Cenpjtm/tm mice. Cenpj-deficiency may exacerbate this phenomenon in the cells of both of these tissues, which are prone to karyomegaly [78].
Susceptibility to malignancy

Familial syndromes associated with genomic instability often predispose to cancer formation since DNA damage is the source of mutations that drive malignant transformation. However only a few cancers have been reported for Seckel syndrome patients, possibly due to the shorter life-expectancy of patients with primordial dwarfism. Furthermore, since mutation of each of the five known Seckel genes, ATR, PCNT, CENPJ, CEP152 and RB1BP1 (CIPB), cause genomic instability that is associated with apoptosis, it is possible that Seckel cells may not have the opportunity to accumulate cancer-causing mutations. The chromosomal instability that is associated with Cenpj-deficiency could result in aneuploidy or translocations that cause loss of tumour suppressors or the formation of oncogenic fusion proteins, respectively [29]. We are currently ageing a cohort of Cenpj<sup>+/−</sup> mice (currently 7–14 months old) to determine whether Cenpj-deficiency alters the frequency of malignancy or shortens life-expectancy.

Cenpj-deficient mouse phenotypes for which there are currently no clinical correlates

We noted a small number of abnormalities in Cenpj<sup>+/−</sup> mice that have not been previously reported for Seckel syndrome patients or mouse models. Seckel syndrome is associated with ocular defects in humans, including spontaneous lens dislocation, myopia, astigmatism, and retinal degeneration. Ocular examination of CENPJ-Seckel patients has not yet been reported [47,48], however Cenpj was highly expressed in the rapidly proliferating retinal neuroblast layer in the 14.5 d.p.c. mouse embryo and Cenpj-deficient mice presented with a number of ocular abnormalities. Furthermore, a small number of reports suggest that Seckel-like syndromes are associated with precocious puberty or premature thelarche [44,45]. In contrast, female Cenpj<sup>+/−</sup> mice showed signs of delayed puberty, although the reproductive tract appeared normal at 16 weeks. We built a protein-protein interaction network using all known Seckel Syndrome associated genes as query (Figure S6B). By using gene ontology enrichment analysis we showed that 265 biological processes (level 3 classification) are significantly over-represented in the Seckel syndrome network (Table S1). As expected, many of the processes were involved in the regulation of cell cycle, cell growth and cell death. Interestingly, the network was also enriched for genes involved in the ‘response to hormone stimulus’ ‘ovulation cycle process’ and ‘sex differentiation’ (Table S1). Transient insulin resistance during puberty is a well documented phenomenon [80]. Cenpj<sup>+/−</sup> mice of both genders had a delayed response to glucose challenge although this was more marked in 16 week-old female mice, which may be explained by delayed puberty in female Cenpj<sup>+/−</sup> mice. While there are no reports of an association between abnormal glucose homeostasis and Seckel syndrome, interestingly, most individuals with MOPDII, including PCNT-MOPDII, develop insulin resistance and diabetes during childhood [81,82]. Aside from centrosome-mediated regulation of the cell-cycle, PCNT is thought to regulate insulin secretory vesicle docking in mouse pancreatic β-cells [83]. Whether CENPJ plays a role in glucose homeostasis remains to be determined. Finally, the proportion of CD8+CD3+ T cells were elevated in Cenpj<sup>+/−</sup> mice, although this was more pronounced in males. The Seckel syndrome protein-protein interaction network that we generated was significantly enriched for genes involved in ‘leukocyte mediated immunity’, ‘leukocyte mediated cytotoxicity’, ‘leukocyte activation’ and ‘interleukin-2 production’ (Table S1). While we are uncertain of the biological basis for these relationships, it will be interesting to see whether there are clinical correlates for these abnormalities and whether other mouse models of Seckel syndrome or primordial dwarfism share these anomalies.

Summary

Mouse models of Seckel syndrome may go some way towards the molecular genetic delineation of this heterogeneous condition. The generalized activation of apoptosis as a result of genomic instability in ATR-Seckel and Cenpj<sup>+/−</sup> mouse embryos provides one explanation for the proportionate dwarfism of Seckel syndrome patients. In agreement with the intron 11 CENPJ-Seckel mutation, which results in the formation of three transcripts, we showed that Cenpj expression is rescued to some extent by cryptic splicing over the cassette to produce a variety of truncated mRNAs, and that there is a moderate degree of individual variation in the ability of an organism to perform this rescue. These data highlight the need for detailed mRNA expression, splicing studies, and protein analysis to establish how individual mutations affect the normal and cryptic splicing of CENPJ mRNAs for each patient directly, and not with prediction analysis tools, so as to understand why some CENPJ mutations cause microcephaly and others Seckel syndrome and how the same CENPJ mutation can cause clinical heterogeneity [4].

Materials and Methods

Confirmation of correct targeting and animal husbandry

Mutant mice carrying the Cenpj<sup>+/−*/EUCOMM(0)Wtsi</sup> allele were generated on a C57BL/6NTac; C57BL/6-Tyr<sup>Brd</sup> background (clone EPD0028_7_G05) by the Sanger Mouse Genetics Project as part of the European Conditional Mouse Mutagenesis Program (EUCOMM; [26]). Correct gene targeting in founder mice was determined by a combination of standard PCR and quantitative PCR (qPCR; see Figure S1 for more details). Following confirmation of correct targeting, mice were genotyped for the Cenpj<sup>+/−*/EUCOMM(0)Wtsi</sup> allele by PCR using primers specific to the wildtype and mutant Cenpj alleles and to LacZ (Figure S1D). In individual experiments, all mice were matched for age and gender. A cumulative baseline was generated from data arising from controls from the same genetic background, age and gender. The care and use of all mice in this study was carried out in accordance with UK Home Office regulations, UK Animals (Scientific Procedures) Act of 1986. Mice were maintained in a specific pathogen free unit on a 12 hr light: 12 hr dark cycle with lights off at 7:30 pm and no twilight period. The ambient temperature was 21±2°C and the humidity was 55±10%. Mice were housed using a stock density of 3–5 mice per cage (overall dimensions of caging: L×W×H 363×207×140 mm, floor area 530 cm<sup>2</sup>) in individually ventilated caging (Tecniplast Seal Safe1284L) receiving 60 air changes per hour. In addition to Aspen bedding substrate, standard environmental enrichment of two nestlets, a cardboard Fun Tunnel and three wooden chew blocks was provided. Mice were given water and diet ad libitum. At 4 weeks of age, mice were transferred from Mouse Breeders Diet (Lab Diets, 5021–3) to a high fat (21.4% fat by crude content) dietary challenge (Special Diet Services, Western RD 829100).

Quantitative real-time PCR

To test for expression of Cenpj and cryptic splicing, total RNA was isolated from mouse embryonic fibroblasts (13.5 days post coitum (d.p.c.), n = 3) using the RNeasy minikit (Qiagen). RT-PCR was performed using the BD Sprint kit containing random hexamers (BD Clontech, CA, USA). Primers were designed to exon boundaries and sequences are available on request. cDNA was quantified using SYBR Green on an ABI7900HT (ABI, CA,
USA). Gene expression was normalised to Gapdh and to wild-type control.

**Immunoblotting**

Protein extracts were prepared from mouse embryonic fibroblasts (13.5 d.p.c.) by directly harvesting cells in Laemmlı buffer. Proteins were separated by SDS-PAGE, and membranes were incubated with primary antibodies to CENPJ [Stratech, Newmarket, UK; Rabbit 1:500], KAP1 (Abcam, Cambridge, UK; Rabbit 1:10 000), KAP1 phospho-Ser-824 (Bethyl, Texas, US; Rabbit 1:1000), Chk1 phospho-Ser-345 (Cell Signaling, Boston, US; Rabbit 1:5000), RPA phospho-Ser-4/8 (Bethyl, Texas, US; Rabbit 1:10 000), H2AX phospho-Ser-139 (γH2AX, Millipore, Billerica, US; Mouse 1:1000). Camptothecin was from Sigma (Poole, UK).

**LacZ staining**

LacZ staining was performed on tissues perfused, removed and fixed (30 min) with 4% paraformaldehyde (pH8). Tissues were then washed three times in PBS. Tissues were then incubated at 4°C for 48 h in staining buffer (5 mM K$_4$Fe(CN)$_6$, 5 mM K$_4$Fe(CN)$_6$, 0.02% IGEPA, CA-630, 0.01% deoxycholate, 2 mM MgCl$_2$, 1 mg/ml bromo-chloro-indolyl-galactopyranoside and data were represented as a percentage. was normalized to the right greater tubercle – trochlea length (mm) and data were represented as a percentage. To show that humeri were anatomically dispropor-

**Histology**

Adult tissue samples were fixed in 10% neutral buffered formalin and E14.5 embryos were fixed in 4% PFA. Samples were dehydrated, paraffin embedded and 4 μm sections were cut before hematoxylin and eosin staining. Neuron densities of E14.5 embryo brains were determined by counting the number of nuclei in three different areas that were 75 μm$^2$ for the densely populated ventricular zones and 150 μm$^2$ for the less densely populated mid-striatum. Adult neuroanatomical measurements (Figure S3) were carried out on 40 μm thick Nissl stained sections using ImageJ freeware (NIH). Heart fibrosis was assessed by Mason’s Trichrome and X-zone pigmentation of the adrenals was confirmed by Periodic Acid-Schiff’s staining using standard methods. Whole mouse eyes were enucleated, fixed, and sectioned for histological studies as previously described [85].

**Immunohistochemistry**

Sections of paraffin embedded tissues were dewaxed and antigen retrieval was performed in boiling 10 mM citrate buffer pH6. Endogenous peroxidases were quenched in 3% hydrogen peroxide (Sigma, UK) before blocking in normal serum (Vector-Labs, UK). Sections were incubated in primary antibodies to Cenpj [Stratech], cleaved caspase-3 (Cell Signaling Technologies, CA, USA), Ki67 (DAKO Ltd, UK) and phospho-Ser 139 H2AX (γH2AX, Cell Signaling Technologies, CA, USA). Vectorstain ABC kit and DAB (VectorLabs, UK) were used according to the manufacturer’s instructions. Sections were counterstained with haematoxylin before clearing and mounting. The number of cells positive for cleaved-caspase 3 or γH2AX were counted in two areas of 75 μm$^2$ the striatum, cortex and pro-hippocampus and data shown are the percentage of total cells in the area. Counts were performed independently by two individuals to confirm reproducibility.

**Neurobehavioral and sensory assessment**

At nine weeks of age, open field, grip strength and modified SHIRPA were performed as described previously [86,87]. Hot plate assessment was performed at 10 weeks of age using standard techniques. Tests for social recognition and olfaction (Cenpj$^{tm/tm}$, n = 9 and Cenpj$^{+/+}$, n = 8) were performed on male mice at 3–6 months of age. Mice were habituated to a test arena identical to their home cage for 10 min. For social recognition, a stimulus mouse was placed into the test arena for 1 min, repeated four times at 10 min intervals. In the fifth trial, a second stimulus mouse was presented. 24 h later the test animals were presented with the familiar animal from trials 1–4 and a new unfamiliar animal, for 2 min. Trials were performed under red light and recorded with an overhead camera and the videos scored blind of genotype. The amount of time the test animal spent investigating, by oronasal contact or approaching within 1–2 cm, was recorded. Stimulus animals, 2–4 months old, were weight-matched to Cenpj$^{tm/tm}$ mice and sedated with ketamine/xylazine (i.p. 1 g/ 0.1 g per kg of body weight). C57BL/6NTac mice were used for trials 1–4 and for the 24 h discrimination test, 129P2/OlaHsd mice were used for trial 3. Three animals (two Cenpj$^{tm/tm}$ and one Cenpj$^{+/+}$) were taken out of the analysis because of their low investigation times (less than 10 s on trial one or during the discrimination test).

**Glucose tolerance**

We assessed glucose tolerance in mice fed on a high-fat diet (Western RD, 829100, Special Diets Services) from 4 weeks of age until 13 weeks of age. At 13 weeks, mice were fasted overnight before a blood sample was taken and glucose was measured using an Accu-Chek Aviva (Roche). To perform an intra-peritoneal glucose tolerance test (IP-GTT), mice were fasted for 16 h, a bolus of glucose was administered intraperitoneally and blood
concentration from the tail vein was measured using Accu-Chek Aviva (Roche) after 15, 30, 60 and 120 min.

Clinical chemistry

We performed clinical chemistry on 16-week-old mice. Mice were terminally anesthetized and blood was collected from the retro-orbital sinus into lithium-heparin tubes. The plasma was immediately analyzed on an Olympus AU400 Analyzer.

Flow cytometry

At 16 weeks of age blood samples were collected into heparin-coated tubes. The main leukocyte populations in peripheral blood were characterized by 8-colour flow cytometry with an LSRII (BD Bioscience, UK Biosciences, UK) and associated software. Briefly, samples were centrifuged at 5000 g for 10 min at 4°C to remove the plasma layer. Red blood cells were lysed (Pharmalyse, BD Bioscience, UK Biosciences, UK) and samples were centrifuged at 400 g for 3 min to pellet the white cells. White cells were resuspended in buffer (PBS pH 7.45 containing 0.5% BSA), transferred to 96-well plate and washed in buffer several times before incubation in 50 µl 10 µg/ml Mouse FcBlock (BD Bioscience, UK Biosciences, UK) for 15 min on ice. Cells were washed and incubated with 50 µl antibody mix from two staining panels (Tables S2 and S3) for 15 min on ice. Propidium iodide (2.5 mg/ml; Sigma, UK) was added to each well and cells were incubated for a further 5 min. Cells were washed in buffer several times before 30 000 propidium iodide-negative, CD45-positive events were collected. Data were interpreted using FlowJo (v7.6, Tree Star, Inc., OR, USA). The frequency of micronucleated normochromatid erythrocytes was determined by flow cytometry (FC500, Beckman Coulter, USA) as described previously [88] and data were interpreted using FlowJo (v9.3.1, Tree Star, Inc., OR, USA). Cell cycle analysis was performed on mouse embryonic fibroblasts (13.5 d.p.c.). Briefly, cells were fixed in 70% ethanol overnight and stained with PI solution before analyzing on a flow cytometer (LSR Fortessa, BD, USA). A total of 10,000 events were acquired per sample. The cells were gated on PI fluorescence area versus PI fluorescence width to discriminate any doublets and clumps. The gated events were displayed on a histogram plot of PI fluorescence area. Data were analyzed using FlowJo (v9.3.1, Tree Star, Inc., OR, USA).

Immunofluorescence

Mouse embryonic fibroblasts (13.5 d.p.c.) were collected and cultured using standard methods. Primary antibodies used in this study were CDK5RAP2 (Bethyl), centrin-3 (Abnova), TPX2 (Abnova), gamma-tubulin (GTU88; Sigma-Aldrich), alpha-tubulin (DM1A; Sigma-Aldrich). Secondary antibodies conjugated to Alexa Fluor 488 and 555 (Invitrogen) were used. DNA was stained with Hoechst (Sigma-Aldrich). To detect centrosomal markers, cells were fixed in −20°C methanol for 5 min. After fixation, cells were processed for immunofluorescence and microscopy as described in [89]. For centriole counts in Figure 4C cells were treated with 100 µM monastrol for 16 h before fixation.

G2 checkpoint assay

Cells were irradiated with 3 Gy ionizing radiation using a Faxitron cabinet X-ray system and left recover for 8 h in the presence of 1 µg/ml of nocodazole (Sigma, Poole, UK) to trap mitotic cells. Cells were trypsinized and fixed with 4% paraformaldehyde, permeabilized with 1× phosphate buffered saline containing 0.2% Triton X-100 for 30 min on ice, and incubated in the presence of MPM2 primary antibodies (Millipore, Billerica, US, Mouse 1:100) and then Alexa-Fluor-488 secondary antibodies (Life Technologies, Paisley, Scotland. Goat anti-mouse 1:200) to detect mitotic cells.

Multiplex-fluorescence in situ hybridization (FISH)

Metaphase spreads from MEFs (Cenpjtm/+ and Cenpjtm/− littermates) were prepared as for metaphase FISH and multiplex-FISH was carried out as previously described [90].

Network generation and analysis

A network of Protein-Protein interactions for Seckel syndrome genes was generated using the Cytoscape 2.8.1 [91] plug-in BisoGenet 1.41.00 [92]. The Entrez Gene symbols of the Seckel syndrome associated genes (CENPJ, PCNT, CEP152, ATIP, RBBP8 (CILP), SCKL3, Entrez Gene IDs = 55835, 5116, 22995, 545, 3992 and 386616 respectively) [4,5,6,41] were used as query to build a network of experimentally validated Protein-Protein interactions, by adding neighbours to the input nodes up to a distance of one. All the interactions are downloadable by BisoGenet from its own database SysBiosimc and have been validated by one or different experimental methodologies such as X-ray crystallography, surface plasmon resonance, two hybrid systems, three hybrid systems and Western blot. The network’s characteristic path length was calculated using Cytoscape’s built-in plug-in NetworkAnalyzer [93].

Gene Ontology (GO) over-representation analysis

The GOs over-representation analysis was performed using the Over-representation analysis tool of the Consensus Path Database website [94]. Statistical significance of the different GOs represented in our network was calculated by the website’s tool using a Hypergeometric test. After calculating the p-value, the tool corrects it for the false discovery rate generating the corrected q-value. A q-value <0.01 was used as threshold for all significant results.

Statistical analyses

A Shapiro-Wilk normality test was performed to assess whether data were normally distributed followed by an F-Test to assess whether equality of variances could be assumed. The significance of the difference between the means of both data sets was tested by applying a two-sided T-Test, assuming variance equality whenever the F-Test was positive (P<0.05). For all cases where the Shapiro-Wilk normality test was negative (P>0.05) a Mann-Whitney non-parametric test was applied to assess the significance. Statistical analyses were performed using R-2.13.0 [95].

Supporting Information

Figure S1 A. Design and validation of the Cenpj allele. The L112 gt1 cassette was inserted at basemap 57174548 of chromosome 14 upstream of a Cenpj critical exon (exon 5, Build 37). The cassette is composed of an FRT-flanked lacZ/neomycin sequence followed by a loxP site. An additional loxP site is inserted downstream of the targeted exon at basemap 57173663. The critical exon is thus flanked by loxP sites. Further information on targeting strategies used for this and other KOMP alleles can be found at http://www.knockoutmouse.org/aboutkompstrategies. B. Correct targeting in founder mice was confirmed by standard PCR using the primers shown in Figure S1D (for more details on cassette quality control see http://www.knockoutmouse.org/kb/ entry/907/). Gels show the presence of LacZ, 5′FRT and loxP sites, generation of a mutant band (MUT) and absence of
backbone (VF4). C. Correct targeting was also confirmed by loss of wildtype allele qPCR. A TaqMan qPCR assay was designed to the wildtype sequence removed during recombineering of the mutant allele. Samples were amplified in a multiplex reaction with a TaqMan qPCR assay designed to the wildtype sequence removed during recombineering of the mutant allele. Samples were amplified in a multiplex reaction with a TaqMan qPCR assay designed to the wildtype sequence removed during recombineering of the mutant allele. Samples were amplified in a multiplex reaction with a TaqMan qPCR assay designed to the wildtype sequence removed during recombineering of the mutant allele. Samples were amplified in a multiplex reaction with a TaqMan qPCR assay designed to the wildtype sequence removed during recombineering of the mutant allele. Samples were amplified in a multiplex reaction with a TaqMan qPCR assay designed to the wildtype sequence removed during recombineering of the mutant allele. Samples were amplified in a multiplex reaction with a TaqMan qPCR assay designed to the wildtype sequence removed during recombineering of the mutant allele. Samples were amplified in a multiplex reaction with a TaqMan qPCR assay designed to the wildtype sequence removed during recombineering of the mutant allele. Samples were amplified in a multiplex reaction with a TaqMan qPCR assay designed to the wildtype sequence removed during recombineering of the mutant allele. Samples were amplified in a multiplex reaction with a TaqMan qPCR assay designed to the wildtype sequence removed during recombineering of the mutant allele. Samples were amplified in a multiplex reaction with a TaqMan qPCR assay designed to the wildtype sequence removed during recombineering of the mutant.
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Author Contributions

Conceived and designed the experiments: D'JA [JKW RR-R REM GS-A OI DMC AE JP]. Performed the experiments: REM OI DMC GS-A A-MDCV-H AE VBM PLCJ GF BF FY JE LevlWJWK MJ A. Analyzed the data: REM OI GS-A A-MDCV-H AE FAG DWI. MJ A SHT SMGP CLS FG. Wrote the paper: REM FG SJJ D'JA.

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