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Mutations in Pericentrin cause Seckel syndrome with defective ATR-dependent DNA damage signalling.

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Introductory paragraph (150 words.)

Expansion of the brain is one of the defining characteristics of modern humans. Seckel syndrome (MIM210600), a disorder of markedly reduced brain and body size¹, is associated with defective ATR-dependant DNA damage signalling². Previously, only a single hypomorphic mutation of ATR has been identified in this genetically heterogeneous condition³. We now report that mutations in pericentrin (PCNT) also cause Seckel syndrome, resulting in its loss from the centrosome, where it has key functions anchoring both structural and regulatory proteins⁴,⁵. Furthermore, we find that PCNT-Seckel patient cells have defects in ATR-dependent checkpoint signalling, providing the first evidence linking a structural centrosomal protein with DNA damage signalling. These findings also suggest that other known microcephaly genes implicated in either DNA repair responses⁶ or centrosomal function⁷,⁸, may act in common developmental pathways determining human brain and body size.
Seckel syndrome is an autosomal recessive disorder of intra-uterine growth retardation, severe proportionate short stature and marked microcephaly\textsuperscript{1,9}. The ataxia-telangiectasia and Rad3-related (ATR) gene is mutated in Seckel syndrome (ATR-Seckel)\textsuperscript{3}, and encodes an phosphotidylinositol-3-kinase-like kinase which has distinct, but overlapping functions with ATM in co-ordinating the response to DNA damage\textsuperscript{10}. ATR is activated by single stranded DNA whilst ATM responds to DNA double strand breaks. MCPH1, another gene whose mutations cause severe (primary) microcephaly, also acts in the ATR damage response pathway\textsuperscript{6}. Furthermore, other, non-ATR Seckel patient cell lines also exhibit ATR-pathway dysfunction\textsuperscript{2}, suggesting that mutation of further genes encoding proteins involved in this DNA damage response cascade may cause this disorder.

To identify such components, we performed a SNP-microarray genome-wide homozygosity scan on two consanguineous families from the Middle-East with a clinical diagnosis of Seckel syndrome (Supplementary Fig. 1 and 2) and cellular evidence of defective ATR signalling. Multipoint linkage analysis identified a novel locus, Sckl4, with a maximum LOD score of 4.03 at 70.9cM on chromosome 21q22.3 between rs1598206 and rs2330591 (Fig. 1 and Supplementary Fig. 2). 55 Refseq annotated genes lay within this interval, including \textit{pericentrin} (PCNT), which encodes a centrosomal protein. As several centrosomal genes, \textit{CenpJ}, \textit{ASPM} and \textit{CDK5RAP2}, cause the related condition, primary microcephaly\textsuperscript{7,8}, we postulated that \textit{PCNT} might be the causative gene and therefore sequenced its 47 coding exons in affected individuals from both families.

We identified a homozygous nonsense mutation in exon 4 of family 1 (E220X). A homozygous single base-pair deletion in exon 12 in family 2 (S629fs, Fig. 1c,d) produces a frameshift, predicted to result in premature protein truncation after an additional 65 amino acids. Both mutations segregated with the disease in each family, with all parents being heterozygous for the respective mutations. From screening of additional cases we identified a further patient of Moroccan ancestry with a homozygous single base-pair insertion in exon 18, resulting in a frameshift at codon 1190 (C1190fs). Neither this, nor the other two mutations were present in over 200 control alleles screened. Taken together, these mutations disrupt all described
mammalian protein isoforms of PCNT\textsuperscript{11,12}. Therefore we surmised that homozygous loss of function mutations in \textit{PCNT} cause Seckel syndrome.

Pericentrin (also known as kendrin) is a huge 360kDa coiled-coil protein with a C-terminal PACT domain that targets it to the centrosome (Fig. 1d). Two protein isoforms have been described in humans\textsuperscript{11}, the full length protein, pericentrin B, and a C-terminally truncated isoform, pericentrin A. Pericentrin localises to the pericentriolar material (PCM), where it interacts with several structural centrosomal proteins, including γ-tubulin and PCM1\textsuperscript{13,14} and is important for microtubular nucleation and spindle organisation\textsuperscript{15,16}. Anti-pericentrin antibodies disrupt mitosis, suggesting it is essential for mitotic progression\textsuperscript{15}. However, its \textit{Drosophila} homolog, D-PLP, though required for timely recruitment of PCM components, is apparently dispensible for cell division\textsuperscript{17}. Furthermore, as well as its structural roles, pericentrin acts as a scaffold to recruit signalling proteins, such as Protein Kinase A (PKA) and Protein Kinase C\textsubscript{βII}, to the centrosome\textsuperscript{4,18}.

As all mutations identified were homozygous and significantly truncated the protein, this would suggest that they are functionally ‘null’ alleles. To test this prediction, we examined pericentrin localisation in patient lymphoblastoid cell lines (LCLs). Centrosomal pericentrin staining was lost in three independent LCLs, in contrast to control cell lines (Fig. 2a and Supplementary Fig. 3), both in interphase and mitosis. As different isoforms of pericentrin have been described, we also examined protein expression by immunoblotting (Fig. 2b and Supplementary Fig. 3). Two bands were detected in controls (Wild-type (WT), and heterozygous relatives, PCNT\textsuperscript{E220X/+}, PCNT\textsuperscript{S629fs/+}), representing two isoforms of pericentrin, which were not present in mutant LCLs. A smaller ~190kD band was present in PCNT\textsuperscript{E220X} lymphoblastoid cells, (probably representing an aberrant truncated PCNT protein product), while no significant PCNT protein was apparent in the other two mutant cell lines, therefore supporting the molecular genetic findings that protein function is significantly, if not completely, disrupted.

We next examined other components of the centrosome, to establish if they were affected by the absence of pericentrin. ASPM, PCM-1, ninein and centrin were all localised normally (Fig 2a and Supplementary Fig. 4). However, during mitosis, γ-
tubulin was reduced or absent (Fig 2c, d), consistent with previous RNAi experiments\(^5\).

Other patients with Seckel syndrome are defective in the ATR DNA damage response pathway\(^2\). We therefore assessed PCNT-Seckel patient cell lines to see if loss of this key centrosomal protein is associated with the defects in ATR signalling previously found to be associated with this disorder\(^2,3\). Indeed, PCNT-Seckel (PCNT\(^C^{1190fs}\), PCNT\(^E^{220X}\) and PCNT\(^S^{629fs}\)) LCLs exhibited defective UV-induced G2-M checkpoint arrest, similar to that seen in ATR-Seckel patients (Fig. 3a), in contrast to cell lines established from heterozygote relatives (PCNT\(^E^{220X/+}\), PCNT\(^S^{629fs/+}\)) and an unrelated wild type control. This suggested that mutations in PCNT impact on ATR-dependent cell cycle checkpoint activation. Similarly, PCNT-Seckel LCLs also exhibited elevated nuclear fragmentation following replication fork stalling (Fig. 3b) and supernumerary mitotic centrosomes (Fig 3c), two further cellular phenotypes caused by impaired ATR-signalling\(^2\).

Since, these findings suggested a direct role for PCNT in ATR-dependent DNA damage response signalling, we examined further steps in this signalling cascade in PCNT-Seckel cells. We found that ATR-dependent $\gamma$H2AX formation was normal in Seckel-PCNT LCLs, similar to controls (Fig 3d). This was distinct from ATR-Seckel LCLs and suggested that ATR is able to phosphorylate a key substrate in PCNT-Seckel syndrome. Thus, Pericentrin functions downstream of ATR kinase activation in this pathway.

We next examined hydroxyurea-induced 53BP1 foci formation in PCNT-Seckel cell lines. Strikingly, both PCNT-Seckel and ATR-Seckel LCLs failed to form such foci in contrast to control cell lines (Fig 3e). This phenotype is dependent on both ATR and Chk1 kinases, indicating that PCNT has a downstream role in ATR-pathway function. To determine whether PCNT mutations affect other DNA damage responses, we assessed the G2-M checkpoint response to DNA double strand breaks induced by ionising radiation. This response is specifically dependent on ATM and not ATR signalling. PCNT-Seckel LCLs arrested as efficiently as control LCLs under these conditions. ATR-Seckel, unlike ATM mutant LCLs also had a normal response,
confirming the ATR-independence of this assay (Fig 3f). Therefore, we concluded that mutations in PCNT have specific effects on the downstream stage of the ATR-pathway including G2-M checkpoint activation.

PCNT is a key structural centrosomal protein nucleating microtubular assembly during mitosis\textsuperscript{15,16}. It was thus surprising to identify homozygous truncating mutations that appear to cause absence of known forms of the PCNT protein from centrosomes. So remarkably, PCNT would seem to be dispensable for most aspects of human development. \textit{Pericentrin} mutations do however dramatically reduce both body and brain size by \(\sim 8\) standard deviations below the norm. Other centrosomal genes (\textit{ASPM}, \textit{CDK5RAP2}, \textit{CENPJ}) have been implicated in determining human brain size, being mutated in primary microcephaly. They are presumed to act through effects on neurogenic mitosis, perhaps modulating symmetric/asymmetric cell division\textsuperscript{7,8,19}. Given PCNT’s role in mitotic spindle assembly\textsuperscript{15,16} and in co-ordinating mitotic entry\textsuperscript{5}, perturbation of mitosis may similarly result in globally reduced cell number, so additionally causing reduced body size. Alternatively as reduced body size is also present in both ATR-Seckel and MCPH1 primary microcephaly, which correlates with impaired ATR-signalling, reduced cell survival could provide an alternative explanation for reduced brain and body size.

\textit{ASPM} and \textit{MCPH1} primary microcephaly genes have undergone significant adaptive evolution in primates, suggesting that functional alterations in the proteins they encode may have contributed to the evolution of human brain size\textsuperscript{20}. Morphologically, Seckel syndrome also has evolutionary parallels. \textit{Homo Floresiensis}, the recently described Indonesian hominid, has similarly marked reduced brain and body size (cranial volume -6.1 s.d, height -9.5 s.d, relative to modern humans)\textsuperscript{21}. Consequently, changes in this or similar genes could underlie the anatomical differences seen in this hominid\textsuperscript{22}.

Through study of a human disease phenotype, we have for the first time implicated a structural protein of the centrosome in the ATR-dependent DNA damage response. Centrosomes have a central role in regulating cell cycle progression\textsuperscript{23} and pericentrin has an established role in recruiting regulatory proteins to the centrosome\textsuperscript{4,18}.
Therefore a role in checkpoint signalling would be consistent with its known functions.

Components of the ATR-pathway have been observed to localise to the centrosome\(^{24}\), most notably Chk1\(^{25,26}\). Chk1 centrosomal localisation, mediated by activating phosphorylation at Serine317 and 345 residues\(^{27}\), is enhanced by DNA damage. At the centrosome, Chk1 is thought to mediate G2/M arrest through inhibiting activation of Cyclin B-Cdk1 by Cdc25\(^{26}\). Intriguingly, fusion of Chk1 with a PACT domain prevents G2/M mitotic progression\(^{26}\), suggesting that endogenous pericentrin, one of only two PACT domain containing proteins, could provide the means of Chk1 localisation to mediate G2/M checkpoint arrest (model, Fig. 4).

The identification of Pericentrin mutations in Seckel syndrome also provides an interesting convergence between microcephaly genes implicated in ATR signalling (\textit{MCPH1, ATR}) and those involved in centrosomal function (\textit{ASPM, CDK5RAP2, CENPJ}). This suggests that further biochemical and developmental investigation is likely to be fruitful in delineating a common cellular pathway responsible for the brain and body size phenotypes in these syndromes.

\textbf{Methods}

\textbf{Affected individuals with Seckel syndrome}

All affected individuals included in this study had a diagnosis of Seckel syndrome, having intrauterine growth retardation, severe proportionate short stature, marked microcephaly and developmental delay. Blood samples were obtained with informed consent from affected children, parents and unaffected siblings. Lymphoblastoid cell lines were established by EBV transformation and Genomic DNA extracted using standard methods. The study was approved by the Scottish Multicentre Research Ethics Committee 04:MRE00/19), and University of Sussex School of Life Sciences Research Governance Committee.
Genotyping and linkage analysis
SNP microarray genotyping was performed by AROS Applied Biotechnology (Denmark) using Affymetrix Human Mapping 50k Xba240 Genechips. SNP genotypes were analysed using Alohomba and multipoint linkage analysis performed with GENEHUNTER (version 2.0β) under a model of autosomal recessive inheritance with full penetrance, using a disease allele frequency estimated at 1 in 1,000. Decode genetic map distances and Affymetrix SNP allele frequencies were used.

Mutation detection
Primers were designed using the ExonPrimer tool in UCSC Genome Browser to amplify coding exons of pericentrin (primer sequences listed in Supplementary Table 1). Purified PCR amplification products were sequenced using dye-terminator chemistry and electrophoresed on an ABI 3730 capillary sequencer (Applied Biosystems). Mutation analysis was performed using Mutation Surveyor (Softgenetics). Anonymised control samples were screened by sequencing.

Cell lines and cell culture
LBLs were cultured in RPMI 1640 with 15% fetal calf serum (FCS). Cells used were Wild-type (WT; GM02188), ATR-Seckel (DK0064), Ataxia Telangiectasia-mutated (ATM; GM03189D), all of which are described previously. PCNT\textsuperscript{E220X} (CV1559; affected Seckel, PCNT 658G>T), PCNT\textsuperscript{E220X/+} (CV1584; unaffected brother of CV1559), PCNT\textsuperscript{S629fs} (CV1576; affected Seckel, PCNT 1887delA), PCNT\textsuperscript{S629fs/+} (CV1582; unaffected mother of CV1576) and PCNT\textsuperscript{C1190fs} (12061; affected Seckel, PCNT 3568_3569insT).

Immunofluorescence and Western Blotting
Immunostaining was performed as previously described\textsuperscript{29}. Antibodies were used at the following dilutions: pericentrin (Abcam, ab448-100) 1:1000; pericentrin (N-20) (Santa Cruz Biotechnology) 1:100; γ-tubulin (Sigma) 1:1000; Centrin (A.Merdes) 1:1000; PCM-1 (A.Merdes) 1:1000; ASPM (Bethyl labs, IHC-00058); 1:250; Ninein (A.Merdes) 1:100. Secondary antibodies were FITC or Texas Red conjugated (Jackson Immunoresearch lab), 1:200. Three dimensional data sets were collected with a DeltaVision system (Applied Precision, Issaquah, WA) based on an Olympus
IX-70 with a Chroma Technology Sedat filter set driven by SoftWorx software under standard conditions. All images were archived as raw (r3d) and deconvolved (d3d) files. Images were subjected to standard deconvolution algorithm. Three dimensional data sets were converted to Quick Projections in SoftWorx and then converted to TIFF files and imported into Adobe Photoshop for final presentation. For the quantification of γ-tubulin staining in mitosis, a defined volume containing the centrosome was used to measure the total intensity of the signal. The same volume was used to identify the background noise as intensity outside the cells and the non-centrosomal gamma tubulin. The ratios of centrosome-bound and dispersed gamma tubulin was averaged for each slide after background subtraction and the relative intensity was calculated in comparison to the WT control slide. Immunoblotting was performed using pericentrin (Abcam, ab448-100) antibody at 1:1000, and HRP conjugated anti-rabbit IgG secondary 1:5000 (Cell signalling 7074), detected with ECL-Plus (Amersham).

DNA damage response assays
For ATR-dependent G2-M arrest cells were untreated (UNT) or irradiated with 5 J/m² UV-C (UV) and immediately seeded into complete medium, incubated for 2 hrs, before being cytopspun onto poly-D-lysine coated slides and stained with DAPI as previously described⁶. For the ATM-dependent G2-M arrest cells were either untreated (UNT) or irradiated with 2Gy ionising radiation (IR) and processed as above. For γH2AX formation and 53BP1 foci formation, cells were either untreated (UNT) or treated with 5 mM hydroxyurea (HU) for 2 hrs and processed for immunofluorescence (IF) staining as previously described². Cells were also pulse labelled (15 mins) with 50μM BrdU to allow quantification of S-phase using anti-BrdU by IF. Supernumerary mitotic centrosomes were determined following treatment with 1.5μM nocodazole for 24 hrs as previously described⁶. Anti-γH2AX was obtained from Upstate Technology (Hampshire, UK), anti-53BP1 (BL181) from Universal Biologicals (Cambridge, UK), anti-BrdU from Autogen Bioclear (Wiltshire, UK) and anti-γ tubulin from Sigma-Aldrich UK Ltd (Dorset, UK).
References


The Dodson paper actually conflicts with our Seckel work! This is not the same process and we have major issues with how these experiments were conducted (very high doses of IR and very long incubation time). We have spoken to C, Morrison about this and have not been able to reconcile.

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**URLs**

UCSC Human Genome Browser
http://genome.ucsc.edu

SMART, (Simple Modular Architecture Research Tool)
http://smart.embl-heidelberg.de/
Figures

Figure 1. Schematic of the Sckl4 critical region and the PCNT gene depicting location of identified mutations.

(a) Genetic map of chromosome 21q22.3 with the refined Sckl4 locus, defined by overlapping homozygous chromosomal segments in two consanguineous families, and extending over a 2.9 Mbp interval between SNP markers rs1598206 and rs2330591 (43,883,204 – 46,751,852, UCSC Browser, March 2006 Assembly). Genetic distances, Decode genetic map. (b) PCNT spans 122kb of genomic sequence in 47 exons and encodes a 3336 amino acid protein. (c) Sequence electropherograms of PCNT mutations. (d) Schematic of the pericentrin protein indicating position of identified mutations and protein structure. Pericentrin contains internal repeats (red), coiled-coil regions (green), and a PACT domain (blue). Protein structural regions as predicted by SMART. Sites of interaction with PKA and calmodulin indicated by asterisks.

Figure 2. Pericentrin localisation and function is disrupted in PCNT-Seckel cell lines.

(a) Pericentrin is not localised to the pericentriolar material in PCNT-Seckel cells. Deconvolved immunofluorescent images from PCNT-Seckel and control lymphoblastoid cell lines. Pericentrin (ab448-100, green) and centrin (red). Scale bar 5μm. Control, heterozygote relative, PCNT\textsuperscript{220X/+}. (b) Immunoblot of LCL cell lysates with Pericentrin ab448-100 antibody, that detects both Pericentrin A and B isoforms. Two pericentrin (arrowheads) isoforms are absent from PCNT-Seckel cells, but present in control lymphoblastoid cells from heterozygous relatives. A smaller protein product of ~170kD is detected in the PCNT\textsuperscript{220X} cell line, possibly produced as the result of aberrant, re-initiated translation. Loading control, alpha-tubulin. (c,d) γ–tubulin localisation is frequently reduced or absent during mitosis in PCNT-Seckel cells. (e) Quantification of γ-tubulin signal in PCNT-Seckel mitotic cells, relative to wild-type. n=20. error bar, s.d. (d) In PCNT-Seckel mitotic cells (astericks), γ–tubulin
centrosomal staining is reduced (arrows) or absent, relative to centrosomal staining in adjacent interphase cells, or in control cells from a heterozygous relative.

**Figure 3**

**PCNT is required for ATR dependent DNA damage signalling.**

(a) G2-M checkpoint arrest was observed 2 h post UV treatment in control but not in ATR- or PCNT-Seckel LCLs. The mitotic index was examined 2 hours after treatment with 5 J m⁻² UV. Average of 3 experiments, error bars, s.d. Controls:- heterozygote relatives PCNT⁺⁻/⁺ and PCNT⁺⁻/⁺ ; and an unrelated wild type (WT) control. UNT, untreated; UV, UV-C treated. (b) ATR- and PCNT-Seckel LCL cells have increased hydroxyurea-induced nuclear fragmentation. Nuclear fragmentation was examined 24 h after treatment with 5mM hydroxyurea (HU). Percentage of cells displaying nuclear fragmentation (NF) is shown. (c) ATR- and PCNT-Seckel LCL cells exhibit elevated levels of supernumerary centrosomes in mitosis. Percentage of mitotic (phospho-histoneH3 (Ser10) positive cells) with > 2 centrosomes determined following 24 h incubation with nocodazole. (d) γH2AX foci formation is normal in PCNT-Seckel LCLs. Phosphorylation of the histone H2AX (termed γH2AX) by the PI3K-kinases is one of the earliest detectable responses to DNA damage, and is ATR-signalling specific after hydroxyurea treatment. Percentage of γH2AX foci was determined 2 hours after treatment with 5mM hydroxyurea.(e) ATR- and PCNT-Seckel cells show reduced 53BP foci formation after treatment with 5mM hydroxyurea for 2 hrs, reflecting impairment in ATR/Chk1 dependent signalling. Immunofluorescent staining with anti-BrdU confirmed that all LCLs had equivalent S-phase populations (range: 20-23%) prior to exposure to HU. (f) G2-M checkpoint arrest after Ionizing Radiation (IR) is normal in PCNT-Seckel and ATR-Seckel LCLs but not in Ataxia Telangiectasia mutated (ATM) LCLs.

**Figure 4** Model of Pericentrin’s role in ATR-dependent G2/M checkpoint arrest

Low-dose UV and Hydroxyurea activate ATR kinase which then phosphorylates downstream targets including Chk1 kinase. Activating phosphorylation is required for accumulation of Chk1 at the centrosome after DNA damage. Direct or indirect binding of Chk1 by pericentrin could mediate Chk1’s localisation to the centrosome.
Chk1 localisation at the centrosome inhibits Cdc25, preventing Cyclin B/Cdk1 activation\textsuperscript{26} and thus the transition from G2 in to mitosis.

**Supplementary Figure 1**

(a) Clinical details of probands from families 1-3. Growth parameters expressed in standard deviations from the relevant age and sex-related mean. Actual measurements in brackets. Wgt, weight; Hgt, height, OFC, occipitofrontal circumference. Previous reported growth parameters in an unselected cohort of Seckel patients\textsuperscript{1}, were mean birth weight, 1.54kg (range, 1.0-2.06 kg), and height at examination of –7s.d. (± 2.1 s.d.), and a mean OFC of –8.8 s.d (range –5.1 to –13.3 s.d.). (b) A 13 year-old individual with PCNT-Seckel, showing markedly reduced stature and microcephaly, compared with her unaffected xx year-old sister.

**Supplementary Figure 2**

(a) Pedigrees of Families 1 and 2. Asterisk indicates individuals included in genome-wide SNP genotyping and linkage analysis. (b) Total multipoint LODscores for families 1 and 2 for chromosome 21. Distance in centiMorgans, Decode genetic map. Critical region defined by overlapping homozygous segments in the three affected individuals indicated by arrows (rs1598206, 64.25cM; rs2330591, 77.9cM).

**Supplementary Figure 3**

Pericentrin is not detectable at the centrosomes in PCNT-Seckel LCLs using the PCNT N-20 antibody. Control, heterozygote relative, PCNT\textsuperscript{220X/+}.

**Supplementary Figure 4**

ASPM (a), PCM1 (b), and Ninein (c) are normally localised in PCNT-Seckel LCLs. A normal mitotic spindle and midbody is formed in PCNT-Seckel cells (a). Control, heterozygote relative, PCNT\textsuperscript{220X/+}. Scale bar 5µm.

**Supplementary Table 1**

PCNT primer sequences.
selling points for letter to editor

- New human disease gene, in a key gene thought essential for mitosis in vertebrates Cell, Doxsey.
- phenotype, with evolutionary parallels similar to *Homo Floresiensis*
- New component of the ATR signalling pathway, and implicating a structural centrosomal protein in this pathway

Convergence of centrosomes and ATR signalling in the aetiology of microcephaly