Defective sister-chromatid cohesion, aneuploidy and cancer predisposition in a mouse model of type II Rothmund–Thomson syndrome

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Received December 17, 2004; Revised and Accepted January 25, 2005

Type II Rothmund–Thomson syndrome (Type II RTS) is a rare autosomal recessive genetic disorder characterized by a congenital skin rash, birth defects of the skeleton, genomic instability and cancer predisposition. It is caused by mutations in the *RECQL4* gene and thus represents one of the three cancer-prone genetic diseases that are caused by mutations in a RecQ helicase-encoding gene. Genomic instability has been suspected as a major underlying cause of this disease, and analyses of Type II RTS patient-derived cells demonstrate unusually high frequencies of chromosomal aberrations, suggesting the involvement of chromosomal instability. However, the nature of the instability induced by *RECQL4* mutations has not been clearly defined. We created a viable Recql4 mutant mouse model. These mice exhibit a distinctive skin abnormality, birth defects of the skeletal system, genomic instability and increased cancer susceptibility in a sensitized genetic background. Thus, they provide a useful model for studying Type II RTS. In addition, we demonstrate that cells from these mutant mice have high frequencies of premature centromere separation and aneuploidy. Thus, our observations provide evidence for a previously unsuspected role for Recql4 in sister-chromatid cohesion, and suggest that the chromosomal instability may be the underlying cause of cancer predisposition and birth defects in these mutant mice.

INTRODUCTION

Aneuploidy is a hallmark of most classes of solid tumors (1–4). The fidelity of segregating chromosomes, required for maintenance of euploidy, is facilitated by cell cycle checkpoints, particularly the mitotic spindle checkpoint (5–7). Prior to anaphase initiation, tension produced by the bipolar attachment of microtubules to the centromeres of paired sister-chromatids is required (5,8). Therefore, sister-chromatid cohesion has an essential role in establishing this bipolar attachment (9,10).

RecQ DNA helicases are an evolutionary conserved family of enzymes that share a seven-motif helicase domain (11). In both budding and fission yeast, null mutants of the single RecQ DNA helicase gene, sgs1 and rqh1<sup>+</sup>, respectively, exhibit hyper-recombination and chromosome mis-segregation (12–16). The chromosome mis-segregation phenotype of these mutants provided the first genetic evidence for a link between RecQ DNA helicases and chromosome segregation. Unlike unicellular organisms which have a single RecQ DNA helicase, mammals have five genes that encode different RecQ DNA helicases. For example, humans have *RECQL4*, *RECQL*, *BLM*, *WRN* and *RECQL5* (17,18). Loss-of-function mutations in *WRN*, *BLM* and *RECQL4* cause the autosomal recessive disorders Bloom (BS), Werner (WS) and Type II Rothmund–Thomson (Type II RTS) syndromes, respectively (19–21). Genomic instability and cancer susceptibility are common features of all three disorders, but the nature of the instability and tumor profiles vary among these syndromes. *WRN* deficiency results in large chromosomal deletions (22) and an increased susceptibility to malignancies, particularly sarcomas (23), whereas *BLM* deficiency leads to...
elevated rates of crossovers in mitotic cells and increased susceptibility to all types of cancers observed in the general population (24). However, neither WRN nor BLM deficiency leads to defective mitotic chromosome mis-segregation or aneuploidy.

Type II RTS is a rare autosomal recessive genetic disorder characterized by a congenital skin rash, birth defects of the skeleton, genomic instability and increased susceptibility to malignancy, particularly early-onset osteosarcoma (25–30). Chromosome analyses of Type II RTS patient-derived cell cultures demonstrate unusually high frequencies of chromosomal instability (29,31–34), suggesting that RECQL4 may have a role in chromosome segregation. However, the nature of the instability induced by RECQL4 mutations has not been established.

We report using gene targeting to generate a viable mouse model of Type II RTS. These mutant mice recapitulate the major phenotypes found in Type II RTS patients and hence provide a useful mouse model for delineating the mechanism(s) which contribute to the Type II RTS phenotype. Recql4"/" cells, derived from these mice, are aneuploid. We demonstrate that the aneuploidy phenotype is due to a defect in sister-chromatid cohesion. These findings reveal an important role of Recql4 helicase in sister-chromatid cohesion and mitotic chromosome segregation, and provide new insights into the link between chromosomal instability, birth defects and cancer predisposition.

RESULTS

Generation of a mouse model of Type II RTS by gene targeting

A majority of Type II RTS patients have RECQL4 mutations that are predicted to result in the production of truncated polypeptides lacking an intact helicase domain (28), indicating that the RecQ helicase domain in RECQL4 is essential for its role in maintaining genome stability and suppressing cancer. Thus, we used gene targeting in mouse ES cells to generate a mouse model carrying a mutant Recql4 allele that lacks an intact RecQ helicase domain. We replaced a 1075 bp fragment spanning from within exon 9 through exon 13 of the Recql4 gene and encoding the conserved helicase domain. We replaced a 1075 bp fragment spanning from within exon 9 through exon 13 of the Recql4 gene and encoding the conserved helicase domain (35), with a PGKHprt mini-gene (Fig. 1A).

Heterozygous mice carrying the Recql4 mutant allele (designated Recql4^tm1Glu or Recql4^−) were readily obtained (Fig. 1B) and were indistinguishable from their wild-type siblings (data not shown). Matings between heterozygous mutant mice yielded the expected Mendelian ratios of wild-type, heterozygous and homozygous mutant offspring (data not shown). Southern blotting confirmed that the 1075 bp fragment between exon 9 and 13 was deleted from the genome of homozygous mutant mice (Fig. 1C). Accordingly, RT–PCR analysis also revealed that cells from these homozygous mutant mice did not express any wild-type Recql4 transcript, but did express three major aberrant transcripts (Fig. 1D). Sequence analysis showed that all three contain exon 1 through 8 of the normal Recql4 transcript followed by one of two cryptic exons from within the PGK promoter of the PGKHprt cassette. These cryptic exons contain in-frame stop codons with respect to the normal Recql4 open-reading frame. Therefore, all three aberrant transcripts are expected to produce either one of the two truncated polypeptides consisting of the N-terminal 480 amino acids of Recql4 plus 13 or 15 additional amino acids (either G M F V S S S L L N A N Q or A R V V Q D V T N G S S T S H) instead of the 1216 amino acids wild-type protein (35) or no protein products because of nonsense-mediated mRNA decay. These data confirm that the Recql4^tm1Glu mutant allele is similar to those RECQL4 mutations identified in Type II RTS patients whose transcripts are expected to produce either no proteins or truncated polypeptides lacking the conserved helicase domain (28).

Although all homozygous mutant mice were born alive, 16% (50/314) of them died prior to 24 h of age, a significant increase in perinatal lethality when compared with the 4% (8/189) observed in control (wild-type and heterozygous) littermates (χ² = 16.09, P = 0.003). We have not yet determined the precise cause(s) of death for these mice.

![Image](https://example.com/image.png)
The remaining 84% of homozygous Recql4 knockout mice were able to survive to adulthood and grew at similar rates as compared to wild-type littermates (data not shown). However, they displayed defects of the skin and skeleton, consistent with the organ systems affected in Type II RTS patients. Specifically, by 12 months of age, all homozygous mutant mice spontaneously developed a hypo-/hyper-pigmented skin phenotype on their tails (Fig. 2A and B; unpublished data), reminiscent of the hallmark skin feature of Type II RTS patients (20,26,28,29,32 and 33). In addition, 5.7% (18/314) of the homozygous mutant mice had skeletal defects of the limbs at birth, with most of these defects being preaxial polydactyly of the hind limbs (Fig. 2C and D). The incidence of polydactyly in Recql4−/− mice (15/314) differed significantly from controls (1/189; a single heterozygous mouse had preaxial polydactyly of the left hind limb; \( P = 0.007, \chi^2 = 7.01, \text{Fisher’s exact test} \)). More severe limb defects such as reduction deficiency of the left arm were also observed (Fig. 2F). In addition, we also observed palatal patterning defects in all the mutant mice examined (50/50), but not in the controls (0/30). These defects varied in severity ranging from cleft palate (Fig. 2H) to subtle patterning defects (Fig. 2J). Palatal defects have not been recognized as a common feature for Type II RTS (26).

**Recql4 deficiency modifies the multiplicity and size of intestinal adenomas in Apcmin mice**

In humans, Type II RTS is associated with increased susceptibility to early-onset osteosarcoma (28). In a systematic analysis of 100 Recql4−/− mice and 43 Recql4+/− or Recql4+/+ control mice, we found that five Recql4−/− mice and no Recql4+/− or Recql4+/+ control mice died of cancer prior to 20 months of age. Among these five Recql4−/− mice that died of cancer, two had osteosarcoma and three developed lymphomas. The observed tumor incidence is not sufficient to determine whether there exists a significant difference in tumor susceptibility between Recql4−/− mice and wild-type controls. Thus, we bred the Recql4 mutant mice with Apcmin mice (36). The Apcmin allele creates a sensitized background that is useful to evaluate the effect of specific genetic mutations on gastrointestinal tumor susceptibility in the mouse (37,38). Thus, we examined the effect of the Recql4 deficiency on the susceptibility and progression of intestinal tumors in Apcmin mice either at 120 days, when macroadenomas in these mice can be readily scored, or at the time of their natural death.

At 120 days of age, we observed a two-fold increase in the multiplicity of macroadenomas along the entire GI tract in Recql4−/−, Apcmin/+ when compared with Recql4+/−, Apcmin/+ mice (Fig. 3A; Table 1). In addition to developing more macroadenomas, the average maximal diameter of the macroadenomas was larger in Recql4−/−, Apcmin/+ when compared with Recql4+/−, Apcmin/+ mice (Fig. 3B; Table 1). Finally, the Recql4−/−, Apcmin/+ mice always developed tumors in the large intestine, a site that is inconsistently affected in Recql4+/−, Apcmin/+ mice or in Recql4+/+, Apcmin/+ mice (36).

At natural death, we observed no difference in the mean or maximal lifespan between Recql4−/−, Apcmin/+ and Recql4+/−, Apcmin/+ cohorts, despite dramatic increases in tumor load in the intestines of the Recql4−/−, Apcmin/+ mice (Table 1). Remarkably, Recql4−/−, Apcmin/+ mice died with numerous tumors occupying the entire interior surface of the distal ileum, compared with fewer tumors in the same area in Recql4+/−, Apcmin/+ sib mice (Fig. 3C and D). Despite the increase in number and size of tumors in Recql4−/−, Apcmin/+ mice, their histological grade did not differ from those of Recql4+/−, Apcmin/+ mice with respect to tumor invasiveness (data not shown). These data demonstrate that Recql4 deficiency significantly modifies tumor susceptibility, but not tumor invasiveness in an Apcmin genetic background.

**Aneuploidy in Recql4−/− cells**

Chromosomal instability has been observed in cell lines derived from Type II RTS patients, suggesting that it may be a cellular feature of this syndrome (29,31–34). Therefore, we utilized cells from our mouse model to interrogate the nature of genomic instability in Recql4−/− cells. Chromosome analysis was carried out using primary mouse embryonic fibroblast (MEF) cultures of wild-type and Recql4−/− cells. At passage 2, wild-type cells displayed a stable, near-diploid mean number of chromosomes (39.5 ± 0.20 chromosomes/cell) with <1% aneuploid cells (Fig. 4A and C). In contrast, at the same passage, 24% of Recql4−/− cells were hyperploid and had an abnormal mean number of chromosomes (45.0 ± 2.25 chromosomes/cell; Fig. 4B and C). We also found that Recql4−/− MEFs had a significantly higher frequency of spontaneous micronuclei than their wild-type counterparts (two-factor ANOVA, \( P < 0.001 \) for all Bonferroni corrected null hypotheses, Fig. 4D). In addition to chromosome counts, each metaphase spread was screened for evidence of other types of chromosomal instability. We did not observe increased rates of sister-chromatid exchange (data not shown), multi-radial chromosomes, chromosome breakages or fusions. However, we noticed that in several spreads from Recql4−/− cells sister-chromatids of metaphase chromosomes were no longer associated with one another at their centromeric regions. These spreads were excluded from chromosome counts and analyzed in more detail in subsequent analysis.

To determine whether the aneuploidy phenotype was unique to MEFs or is a common feature of many Recql4−/− cells, we extended our chromosome analysis to include primary cells isolated from adult mice. We found that all cell types from Recql4−/− mice examined, including adult bone marrow, B- and T-cells, displayed higher rates of aneuploidy than wild-type controls (Fig. 4C). Thus, the aneuploidy phenotype appears to be a common feature of many, if not all Recql4−/− cell types.

**Premature centromere separation in Recql4−/− cells**

In mammalian cells, aneuploidy may arise through several mechanisms, including defective telomere metabolism,
defective spindle checkpoint control and multi-polar spindles due to abnormal numbers of centrosomes (39). Thus, we systematically tested Recql4<sup>2/2</sup> MEFs for defects in each of these pathways. Observations on more than 1000 Recql4<sup>2/2</sup> metaphase spreads revealed no statistically significant increase in end-to-end fusions when compared with wild-type cells (data not shown), indicating that Recql4<sup>2/2</sup> cells have normal telomere metabolism. We then examined the response of cells to the microtubule-spindle poison Colcemid. We found that Recql4<sup>2/2</sup> cells accumulated mitoses and had similar mitotic indices to wild-type cells, indicating normal spindle checkpoint function in Recql4<sup>2/2</sup> MEFs (Fig. 5A). To test for centrosome defects, we performed immunostaining with anti-γ-tubulin antibodies and counterstained with DAPI. We found that the number of metaphase cells with greater than or equal to three centrosomes did not differ between Recql4<sup>2/2</sup> (1.6%) and wild-type (1.9%) cells (Fig. 5B, C and E). In contrast, p53<sup>−/−</sup> cells, which are known to have centrosome defects (40), had a much higher frequency of cells with greater than or equal to three centrosomes per cell (15.6%; Fig. 5D and E). Thus, none of these previously described mechanisms appears responsible for the aneuploid phenotype in Recql4<sup>2/2</sup> cells.

Premature centromere separation (PCS) refers to a situation in which sister-chromatids become precociously separated prior to the metaphase/anaphase transition (41). As noted earlier, we observed PCS in standard metaphase spreads from Recql4<sup>2/2</sup> MEF cells. To confirm the PCS phenotype, we first performed fluorescence in situ hybridization (FISH) experiments using two probes specific for chromosome 8 and chromosome 11. On a normal metaphase chromosome spread, a chromosome-specific FISH probe is expected to detect two pairs of signals with each pair representing targets on sister-chromatids. In contrast, on a chromosome after undergoing PCS, a chromosome-specific FISH probe is expected to detect two pairs of signals with each pair representing targets on sister-chromatids. In contrast, on a chromosome undergoing PCS, the probe would detect two separate unpaired signals. We found that this was the case in many

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**Figure 2.** Major morphological phenotypes of Recql4<sup>2/2</sup> mice. Photographs of the tails of a wild-type mouse (A) and a Recql4<sup>2/2</sup> mouse (B) at 12 months of age. Note the striking hypo-/hyper-pigmented areas on the tail of the Recql4<sup>2/2</sup> mouse. (C–F) Radial ray defects in mice. X-ray radiographs of the hind limbs of an adult wild-type (C) and Recql4 mutant (D) mouse. Note that the Recql4<sup>2/2</sup> mouse has an extra digit and a bifurcated digit of the hind limb (white arrow). Photographs of a wild-type mouse (E) and a Recql4<sup>2/2</sup> mouse (F) showing the truncated forelimb in the Recql4<sup>2/2</sup> mouse (white arrowhead). (G–J) Palatal photographs. Photographs of the palates of a wild-type (G) and a sibling Recql4<sup>2/2</sup> mouse (H) at postnatal day 1. Note the severe cleft palate in the mutant mouse (back arrow). Photographs of the palates of wild-type (I) and Recql4<sup>2/2</sup> (J) mice at 12 weeks of age. Note the patterning defect in the palate of the mutant mouse (denoted by a circle).
of the Recql4<sup>−/−</sup> MEF cells (Fig. 6B and C). This observation confirmed our cytological diagnosis of PCS and excluded the possibility that we were counting tetraploid cells.

Subsequently, we systematically analyzed metaphase preparations from primary Recql4<sup>−/−</sup> and wild-type MEFs. The results demonstrated a significant increase in PCS in Recql4<sup>−/−</sup> MEFs when compared with wild-type MEFs (Fig. 6C). As PCS may occur in cultured mammalian cells subjected to prolonged treatment with spindle microtubule inhibitors (42), we confirmed that the increased frequency of PCS is an intrinsic property of Recql4<sup>−/−</sup> MEFs by measuring the incidence of PCS in metaphases from untreated, asynchronous MEF cultures (Fig. 6C). These results demonstrated a nearly 10-fold increase in PCS in Recql4<sup>−/−</sup> MEFs when compared with wild-type controls (7.5% versus 0.8%, respectively; Fig. 6C). Thus, Recql4 deficiency leads to a significant increase in the frequency of PCS in MEFs.

Figure 3. Multiplicity and size of adenomas in the mouse intestine. (A and B) Histograms showing tumor multiplicity and size from mice aged to 120 days. Each dot represents the number of tumors in the intestine of a single mouse and the horizontal line represents the mean of the population. (A) A histogram showing the tumor multiplicity in the intestines of Recql4<sup>−/−</sup>, Apc<sub>Min</sub><sup>+</sup> and Recql4<sup>+/−</sup>, Apc<sub>Min</sub><sup>+</sup> mice. Note the significant 2-fold increase in the mean tumor multiplicity in Recql4<sup>−/−</sup>, Apc<sub>Min</sub><sup>+</sup> mice when compared with Recql4<sup>+/−</sup>, Apc<sub>Min</sub><sup>+</sup> mice. (B) A histogram showing the average maximal diameters of intestinal tumors at 120 days of age. Note the significant difference in the mean tumor size (denoted by horizontal lines) between Recql4<sup>−/−</sup>, Apc<sub>Min</sub><sup>+</sup> and Recql4<sup>+/−</sup>, Apc<sub>Min</sub><sup>+</sup> mice. (C and D) Photographs of tumors from the distal ilea of a Recql4<sup>+/−</sup>, Apc<sub>Min</sub><sup>+</sup> mouse (C) and its Recql4<sup>−/−</sup>, Apc<sub>Min</sub><sup>+</sup> sibling (D) at 6 months of age. Note the numerous tumors covering the interior surface of the intestines in the Recql4<sup>−/−</sup>, Apc<sub>Min</sub><sup>+</sup> mouse when compared with the fewer tumors in the same region in its Recql4<sup>+/−</sup>, Apc<sub>Min</sub><sup>+</sup> sibling.
To determine whether the PCS phenotype is also a property of Recql4-deficient cells in vivo, we analyzed primary cells derived from adult mice. All cell types examined in Recql4<sup>−/−</sup> mice, including adult bone marrow, B-cells and T-cells, displayed a higher frequency of PCS than that observed in their control counterparts (Fig. 6C). Taken together, these data demonstrate that the chromosomal instability and PCS phenotypes are present in all Recql4<sup>−/−</sup> cell types. As sister-chromatid cohesion is essential for normal mitotic chromosome segregation (9,10), our findings indicate that a cohesion defect contributes to the chromosomal instability in Recql4<sup>−/−</sup> cells.

**DISCUSSION**

**Modeling Type II RTS using Recql4 mutant mice**

There have been prior attempts to model Type II RTS in the mouse by creating mutations in Recql4 (43,44). However, these models have had limited success because of the lethality...
Figure 5. Mitotic indices and centrosome status in Recql4^{-/-} MEFs. (A) Mitotic indices of wild type and Recql4^{-/-} MEFs. Cells were treated with Colcemid, released and then harvested at different time points, stained with DAPI and analyzed by fluorescence microscopy. Note that both genotypes consistently respond to the spindle arrest in a similar manner. (B–D) Centrosome status in MEFs. Metaphase spreads were stained with anti-γ-tubulin (green) and counter-stained with DAPI (blue). (B) A representative metaphase spread of a normal diploid wild-type cell with a pair of centrosomes. (C) A representative metaphase spread of an aneuploid Recql4^{-/-} cell with two centrosomes. (D) A representative metaphase spread of an aneuploid p53^{-/-} cell with four centrosomes. (E) Histogram showing the percentage of cells with no more than two (normal) or more than two (abnormal) centrosomes. Note the similarity between the wild-type and the Recql4^{-/-} cells and the significantly higher frequency of cells with abnormal numbers of centrosomes in p53^{-/-} cells (**P < 0.0001; Fisher’s exact test).
associated with these mutants (43,44). In contrast, the Recql4 mutant mice reported here are viable, fertile and they recapitulate the major features of Type II RTS.

The reason for the discrepancy in phenotypes observed in the previous Recql4 knockout models and in our model is not clear at present. In the first two models generated by Ichikawa et al., (43) the mutant mice were embryonic lethal, whereas in the model generated by Hoki et al., (44) only 5% of the mutant mice survived to 2 weeks of age (44). These observations raise the question of whether Recql4 is essential for viability. Both our Recql4 mutant allele and the one generated by Hoki et al. express aberrant Recql4 transcripts that could potentially be translated into N-terminal truncated Recql4 polypeptide, a situation similar to many mutant alleles identified in Type II RTS patients. Although we have not been able to detect truncated polypeptide using currently available anti-Recql4 antibodies, this may reflect low sensitivity of the antibodies rather than absence of the protein. Therefore, it remains formally possible that our Recql4 mutant allele and many of the RECL4 alleles identified in Type II RTS patients are hypomorphic alleles that retain some functions of RECQL4/Recql4 related to viability. Future experiments are needed to address this issue.

Interestingly, our Recql4 model differs from the viable model generated by Hoki et al. (44) reported that their mutant mice had skin atrophy, colorless hair, hair loss, short stature, bone dysplasia, dystrophic teeth and immunological abnormalities. Importantly, their mice failed to develop poikiloderma and malignancies, both of which are hallmark features observed in Type II RTS patients (25–30). In contrast, the mutant mice reported here developed a pigmented skin feature reminiscent of poikiloderma (Fig. 2; unpublished data) and have increased cancer susceptibility in a sensitized Apcmin genetic background. Furthermore, our mutant mice had an increased incidence of limb defects and a chromosomal instability phenotype, two additional common features of Type II RTS that were not described in the report of Hoki et al. It should be stressed that the phenotypic differences between our Recql4 knockout mice and those generated by Hoki et al. may reflect differences in the mutant Recql4 alleles in these mice. Hoki et al. (44) replaced exon 13 with a PGKneo cassette, whereas we replaced exons 9 through 13 with a PGKhppt cassette. Thus, although both
alleles express aberrant Recql4 transcript, only the allele generated by Hoki et al. expresses an aberrant transcript that could be translated into a polypeptide containing both the N- and C-terminal domains of Recql4.

Our observation of a complete penetrance palatal defect in our Recql4 knockout mouse is intriguing. Although the palatal patterning defects we observed have not previously been reported in Type II RTS (26), abnormal palates are a component feature of RAPADILINO syndrome (OMIM#266280) which is also caused by RECQL4 mutations (45). In contrast to Type II RTS patients, RAPADILINO patients carry at least one copy of a founder hypomorphic RECQL4 mutant allele (45). Future work is required to determine whether palatal patterning defects represent a common feature that is caused by Recql4 or RECQL4 deficiency in mice and humans, or a defect that is associated only with specific types of Recql4 or RECQL4 mutations. Intriguingly, another disorder exhibiting palatal defects may also be linked to Recql4; that is, in Roberts syndrome (RBS; OMIM no. 268300), a disorder characterized by a PCS phenotype in many cell types, patients exhibit congenital skeletal defects, including defects of the palate. The disease causing gene for this autosomal recessive genetic disorder has not been identified. Possibly, RBS is attributable to mutations in a gene that associates with RECQL4 or to an as yet unidentified mutation(s) in RECQL4 itself.

Birth defects and genomic instability

The results from our study of Recql4 mutant mice demonstrate a role for Recql4 in development of the palate and the limbs. Although the developmental mechanism(s) for the abnormalities in Recql4−/− mice are not fully understood, we speculate that they are secondary to elevated rates of genomic instability. Such a supposition would be consistent with the extensive documentation in man and in experimental animal models linking genomic instability and the occurrence of birth defects, particularly palatal and radial-limb abnormalities. For example, exposure of fetuses to clastogenic compounds or ionizing radiation can induce genomic instability and result in cleft palate and radial-limb defects (46,47). In addition, similar patterns of birth defects have been observed in patients with other genetic disorders that are associated with chromosomal instability, including RBS, Fanconi anemia (OMIM no.227650), Nijmegen breakage syndrome (OMIM no. 251260) and immunodeficiency-centromeric instability-facial anomalies syndrome (OMIM no. 242860).

The precise mechanism by which genomic instability affects patterning and growth of these systems remains to be elucidated.

Aneuploidy and cancer susceptibility

Our study demonstrates that Recql4 deficiency in mice leads to elevated levels of aneuploidy and increased cancer susceptibility. We believe that aneuploidy is also the main genomic instability of Type II RTS, which is consistent with the increased chromosomal instability in cells derived from Type II RTS patients (29,31–34). An aneuploidy phenotype in Type II RTS is also consistent with a significant increase in susceptibility to early-onset osteosarcoma, which is well documented to involve chromosomal instability (48,49).

Although Recql4 deficiency alone is not sufficient to have a significant effect on the susceptibility to spontaneous osteosarcoma in mice, this may not be surprising. It is well documented that there are numerous intrinsic differences between mice and humans that can contribute to the differences in tumor susceptibilities between the two species. For example, there is a major difference in the lifespan and the total number of mitoses within the lifetime of mice and humans. Thus, it is possible that a specific genomic instability may have a major impact on cancer susceptibility in humans but not in mice. This is illustrated by the fact that specific mutant mouse strains that have genomic instability, but lack a significant increase in spontaneous cancer susceptibility (50,51).

Importantly, we showed that Recql4 deficiency led to a dramatic increase in the multiplicity and size of intestinal tumors in an ApcMin+/− tumor model, most notably in the large intestine, where the macroadenoma penetrance was increased from 50% in Recql4−/−, ApcMin+/− mice to 100% in Recql4−/−, ApcMin+/− mice. The effect of Recql4 deficiency on tumorigenesis in the mouse intestine likely results from the chromosomal instability caused by Recql4 deficiency, which would be consistent with the previous observations that link chromosomal instability to the etiology of gastrointestinal cancer in humans (52).

Given our observations of high rates of aneuploidy and PCS observed in all cell types examined in this study, we believe that these phenotypes represent a general feature of many, if not all, Recql4−/− mice. It is reasonable to assume that the fate of a given aneuploid cell may be different between the in vitro culture conditions and the in vivo micro-environments, and may vary among different cell types, thus accounting for the relatively lower frequency of aneuploidy in cells from in vivo tissues when compared with MEF cells grown in culture. On the basis of this assumption it is interesting to note that cancer susceptibility is limited to distinct tissue types in Type II RTS patients. The reasons for such tissue/organ specificity are not yet clear; however, it is reasonable to suggest that the cellular response to PCS may differ among cell types. Nonetheless, a link between cancer and increased PCS is well established in humans. Patients with other genetic disorders associated with PCS and aneuploidy, such as RBS and mosaic variegated aneuploidy syndrome (OMIM no. 257300), also show an increased cancer susceptibility (41,53,54).

Recql4 deficiency and chromosomal instability

The observation of a chromosome mis-segregation phenotype in budding yeast sgs1Δ mutants (12,13) provides direct genetic evidence for an important role for RecQ DNA helicase in modulating chromosome segregation. However, the mechanism(s) that lead to such a defect in sgs1Δ cells have not been experimentally defined. The common hyper-recombination phenotype observed in many RecQ mutants has led to the speculation that the failure of timely resolution of Holliday junctions may impede the dis-entanglement of sister chromatids and hence contribute to the chromosome segregation defects (55). We report here that Recql4
deficiency results in PCS and aneuploidy in a subset of Recql4−/− cells. We speculate that the chromosome mis-segregation phenotype of sgs1Δ mutants is caused by the same mechanism. Indeed, a recent study has provided genetic evidence for a link between Sgs1 and Ctf4, a component of the cohesion establishment complex, and showed that the sgs1Δ mutants exhibited a PCS phenotype (56). Such conservation between yeast and mice may also suggest that a similar mechanism(s) is responsible for the chromosome instability observed in cells from Type II RTS patients (29,31–34). We have shown that Recql4−/− MEFs have normal frequency of sister-chromatid exchange (unpublished data), demonstrating that unlike its homologs Blm or Wrn, Recql4 does not have a significant role in regulating mitotic recombination. This finding sheds light on the apparent paradoxical notion that despite a high degree of similarity between BLM, WRN and RECLQ4, their deficiency causes three clinically distinct syndromes. Our findings also indicate that in mammals, where five RecQ DNA helicase genes act in concert to maintain genomic stability, individual RecQ homologs have acquired specialized, non-redundant roles. In this context, genetic uncoupling of the functional roles related to recombination and chromosome segregation in mammals exists whereby regulation of the former is carried out by BLM and WRN and regulation of the latter is allocated to RECLQ4.

The high rates of PCS and aneuploidy observed in all Recql4−/− cells examined suggest that this abnormality pre-disposes cells to aberrant mitosis and chromosome mis-segregation. It remains unclear exactly how Recql4 is involved in sister-chromatid cohesion. RECLQ4 expression has been shown to be cell cycle regulated, with peak expression occurring during the G1- and S-phases (57), coinciding with the timing of cohesion establishment (58). These data may suggest that Recql4/RECLQ4 has a role in the establishment of cohesion during S-phase. Interestingly, it was recently reported that human RECLQ4 physically interacts with UBR1 and UBR2 (59), two key components of the N-end rule ubiquitination pathway (60), which has been shown to have an important role in controlling chromosome segregation in yeast (61). However, it remains unclear how, or whether, UBR1-RECLQ4 or UBR2-RECLQ4 interactions can affect sister-chromatid cohesion in mammalian cells. Finally, it should be noted that the majority of Recql4-deficient cells (75%) appear to establish the appropriate cohesion necessary for normal mitoses, suggesting that Recql4 may function as a regulator rather than as a component of the cohesion complex. Further work is required to experimentally elucidate how Recql4 helicase modulates sister-chromatid cohesion.

MATERIALS AND METHODS

Generation of Recql4 knockout mouse

To construct the replacement gene targeting vector pRecqKO-tk, a 4854 bp EcoRI/SauAI fragment of the Recql4 gene (accession AB042529) was cloned into pBluescript (pBSSK, Stragenig, La Jolla, CA, USA). A 1.075 kb KpnI/SacI fragment (nucleotides 585–1660) was deleted and replaced with a KpnI/SacI digested PGK-HPRT-mini gene cassette (62). The pRecqKO-tk vector was linearized with ScaI, purified and electroporated into AB2.2 (129S7/ SvEvBrd-Hprtm2) mouse ES cells as described (63). Targeted clones were identified by screening HAT resistant colonies by Southern blotting using a 5′ external probe (5P in Fig. 1). This 5′ probe consisted of a 0.4 kb HindIII–EcoRI fragment which detects a 4.3 kb wild-type fragment and a 2.5 kb mutant fragment from HindIII digested genomic DNA. The 3′ probe consisted of a 0.35 kb BglII–HindIII restriction fragment which detects a 12 kb BamHI fragment of the wild-type Recql4 allele and an 8 kb BamHI–Eco RV fragment of the targeted Recql4 allele.

One of these correctly targeted ES cell clones, Recq4KO.1, was expanded, purified and used to generate heterozygous Recql4tm1Glu mutant mice as described (37). Briefly, chimeric males were mated with C57BL/6J (B6) females to obtain heterozygous mutant mice in a (B6×129S7/SvEvBrd-Hprtm2) mixed genetic background. All Recql4 mutant mice used in this study, unless otherwise specified, were in a mixed genetic background consisting of 75% B6 and 25% 129S7 genomes. Homozygous mutants were obtained by intercrossing heterozygous mice. The genotypes of all Recql4 mutant mice were determined by Southern blotting.

RT–PCR analysis of Recql4 expression

A pair of primers, Recql4.5ex8: 5′-AGAGCTACAGGTG CCTCATTG-3′ and Recql4.3ex15: 5′-CATGAAGGCCTG TTGTACTC-3′, corresponding to sequences in exon 8 and 15, respectively, were used to amplify a 981 bp fragment of Recql4 cDNA. Total RNA was isolated from the testis of 6-week-old mice using TRIzol® (Invitrogen, Carlsbad, CA, USA) according to the instruction provided by the manufacturer. All RT–PCR reactions were carried out using Superscript™ II RT/Platinum® Taq mix (Invitrogen).

Mice

B6 and B6.ApcMin/+ (Min) mice (36) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in an American Association of Laboratory Animals (AALAC) accredited barrier-free facility at Case Western Reserve University School of Medicine. Mice were fed (ad libitum) a commercially available rodent breeder diet, 5010 (PMI® LabDiet®, St Louis, MO, USA). All procedures were approved by the Case Western Reserve University Institutional Animal Care and Use Committee (IACUC).

Analysis of ApcMin/+ mice

B6.129S5-Recql4tm1Glu/tm1Glu (F6N2) female mice and ApcMin/+ male mice in a B6 background, both carry the B6 Mom1 susceptibility allele Pla2g2aS/S, were mated to generate Recql4−/+ , ApcMin/+ progeny. These Recql4−/+ , ApcMin/+ mice were then crossed to Recql4−/− females to obtain the experimental and control littermate animals. Genotyping of the Apc+ and ApcMin alleles was carried out by standard PCR methods available from The Jackson Laboratory website. For data at 120 days of age, Recql4−/+ , ApcMin/+
and Recql4−/−, ApcMin/+ mice were euthanized by CO2 asphyxiation for quantitative analysis of gastrointestinal adenomas. For data at natural death, Recql4−/+ , ApcMin/+ and Recql4−/−, ApcMin/+ littermate mice were euthanized when they displayed overt symptoms of intestinal neoplasia and anemia, including rectal bleeding/prolapse, significant weight loss and progressive loss of color (whitening) of the snout and paws. The intestine was resected en bloc, washed in phosphate buffered saline (PBS), opened longitudinally and pinned luminal side up on black dissection wax. Intestinal adenomas (macroadenomas with maximal diameters >0.5 mm) along the entire intestine were counted by microscopic examination at 10× magnification followed by fixation with 10% formalin in PBS (Fisher Scientific, Fairlawn, NJ, USA). Digitized images of polyps and a metric ruler were captured using a SPOT software 3.2.5 for Macintosh™ and RT color SPOT camera mounted on a Leica MZFLIII workstation (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). The images were imported into Adobe Photoshop 6.0 program where the ruler function was used to obtain the length of 1 mm from the ruler in each image. The measure of the largest average diameter per polyp was divided by the length of the 1 mm standard to determine polyp sizes. Statistical analyses were performed with the two-sample Student’s t-test (normality tests for all data sets were \( P > 0.05 \)) using the Prism 3 for Macintosh statistical package (GraphPad Software, Inc., San Diego, CA, USA).

**Histopathologic analysis of tumors**

Tumor tissues were fixed in 10% phosphate buffered formalin. Histological analysis was performed by standard methods. Sections of polyps (5–7 μm) stained with hematoxylin and eosin (H&E) were used to compare histological morphology.

**Cell culture**

Primary MEFs were generated from 13.5 day embryos by standard methods (64). The genotype of each MEF cell line was determined by Southern blotting. Cells were cultured at 37°C (5% CO2) in Iscove’s modified eagle’s medium (IMEM) containing 10% fetal bovine serum (FBS), unless otherwise noted.

**Chromosomal analysis and FISH analysis**

For cytogenetic assays, rapidly growing, passage 2 MEFs were treated with Colcemid (at a working concentration of 0.03–0.05 μg/ml) for 4 h and processed using standard cytogenetic procedures. Briefly, cells were washed with 1× PBS, trypsinized, incubated in 0.075 M KCl hypotonic solution at 37°C for 10 min, followed by fixation in 3:1 methanol–acetic acid. Cell suspensions were dropped onto freshly prepared slides and stained with Vectashield® Hardset™ mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA). Images were captured on a Zeiss Axiohot upright microscope with Photometrics Sensys CCD camera and the Quips Genetic Workstation software.

Bone marrow cells were isolated from the femur, tibia and pelvis of 6-week-old male wild-type and Recql4−/− littermate mice using RPMI-1640 supplemented with 10% FBS and 10 U/ml heparin. B- and T-cells were isolated from spleen and thymus, respectively, of 4–6 week old mice. For B-cells, four to six spleens of the same genotypes were pooled to create a single cell suspension. The cells were then incubated in RPMI-1640 containing 10% FBS, 2 mM l-glutamine, 1 mM sodium pyruvate and 2 × P/S for 48 h followed by a 4 h incubation in the presence of Colcemid. For T-cells, single cell suspensions were incubated in RPMI-1640 containing 10% FBS and Colcemid for 30 min. Spreads were prepared and analyzed as described above.

For FISH experiments, fluorescent DNA probes were prepared using bacterial artificial chromosomes (BACs) specific for chromosomes 8 (Wrg) and 11 (Brcal), respectively, using the SpectrumGreen™ or SpectrumRed™ direct-labeled dUTP by nick translation as per the manufacturer's instructions (VYSIS, Inc., Downers Grove, IL, USA). These probes were then used to hybridize to fixed nuclei as described (65). After the hybridization, nuclear chromatin was counterstained with DAPI.

**ACKNOWLEDGEMENTS**

The authors wish to thank Pat Hunt, Matt Warman and Karen Mann for helpful comments on the manuscript and Josh Friedman, Youngji Park and Cheryl Urban for technical assistance. G.L. also wishes to thank Dr Allan Bradley for his generous support during the course of this study. This work was supported by grants from the National Cancer Institute (CA 89391) and The March of Dimes (5-FY00-570) to G.L. M.B.M. is supported by an NIH predoctoral fellowship to the Department of Genetics (5T32GM008613-08).

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