A p53-dependent mechanism underlies macrocytic anemia in a mouse model of human 5q− syndrome

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Abstract

The identification of the genes associated with chromosomal translocation breakpoints has fundamentally changed our understanding of the molecular basis of hematological malignancies. By contrast, the study of chromosomal deletions has been hampered by the large number of genes deleted and the complexity of their analysis. We report the generation of a mouse model for the human 5q− syndrome using large-scale chromosomal engineering. Haploinsufficiency of the Cd74 – Nid67 interval (containing the Ribosomal protein S14 gene – Rps14) causes macrocytic anemia, prominent erythroid dysplasia and monolobulated megakaryocytes in the bone marrow. This is associated with defective bone marrow progenitor development, increased apoptosis and the appearance of bone marrow cells expressing high levels of p53. Notably, intercrossing with p53-deficient mice, completely rescues the progenitor cell defect, restoring CMP/MEP, GMP and HSC bone marrow populations. This novel mouse model suggests that a p53-dependent mechanism underlies the pathophysiology of the 5q− syndrome.

5q− syndrome was first described in 1974 by Van den Berghe who reported the consistent association of the deletion of the long arm of chromosome 5 [del(5q)] with hematological abnormalities: macrocytosis, anemia, normal or high platelet count and hypolobulated megakaryocytes in the bone marrow1. The disease has a female preponderance and a low rate of transformation to acute myeloid leukaemia (AML) although most patients become transfusion dependent resulting in significant morbidity2. Recently, the immunomodulatory drug lenalidomide has been shown to induce cytogenetic remission3, though its mode of action remains to be fully determined.

The 5q− syndrome is recognized as a distinct clinical entity according to the WHO classification and is defined by a medullary blast count of < 5% and the presence of the

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AUTHOR CONTRIBUTIONS
A.N.J.M. conceived and designed the project; A.N.J.M., D.R.H., L.R.H., A.L.L., L.F.D., R.P. and H.E.J. designed and generated gene targeted mice; A.N.J.M., J.L.B., L.F.D., S.L.A., A.L.L., A.M., J.B., S.H.W., J.S.W. and A.J.W analysed hematopoiesis; J.S.W. and J.B performed gene expression profiling analysis and provided unpublished information; H.E.J. performed Annexin V analysis. All authors contributed to the writing of the paper.

COMPETING FINANCIAL INTERESTS
The authors declare that they have no competing financial interests.
del(5q) as the sole karyotypic abnormality. The deletion involved in 5q− syndrome is typically a large interstitial deletion and contains a number of genes involved in hematopoiesis. The del(5q) is also commonly found in other myelodysplastic syndromes (MDS) and AML (10-15% of all patients) although it is cytogenetically indistinguishable across these disorders. The del(5q) in the 5q− syndrome is widely believed to mark the location for a tumour suppressor gene(s), the loss of which may affect important processes such as growth control and normal hematopoiesis.

Boultwood et al., first identified the commonly deleted region (CDR) in 5q− syndrome, based on fine mapping of patients with cytogenetically small deletions, and subsequently narrowed the CDR to approximately 1.5 Mb between 5q31 and 5q32, flanked by D5S413 and the GLUTAMATE RECEPTOR ALPHA-1 (GLRA1) gene. The CDR contains 24 known and 16 predicted genes, 33 of which were found to be expressed in CD34+ cells, and represent potential candidate genes. The 5q− syndrome CDR is distinct from a more proximal 5q− CDR which has been associated with more advanced MDS and AML.

To date, studies have failed to identify mutations within the retained alleles, or potential submicroscopic homozygous deletions in the CDR. This suggests that either haploinsufficiency promotes the 5q− syndrome, or that a retained tumour suppressor allele has undergone epigenetic inactivation.

Several potential candidate genes for the 5q− syndrome map within the CDR including: FAT TUMOUR SUPPRESSOR HOMOLOG 2 (FAT2), a homologue of the Drosophila fat tumour suppressor protein; SECRETED PROTEIN ACIDIC AND RICH IN CYSTEINE (SPARC), a matricellular glycoprotein that is expressed primarily in tissues that undergo consistent turnover, or at sites of injury and disease; and RIBOSOMAL PROTEIN 14 (RPS14) a component of the 40S ribosomal subunit. Haploinsufficiency of RPS14 has been described in CD34+ cells of patients with the 5q− syndrome. Indeed, an RNA interference screen in CD34+ human hematopoietic progenitor cells identified RPS14 as a key factor in erythroid development in vitro. These results suggested that 5q− syndrome might have an aetiology related to the RPS19-dependent pathology of Diamond Blackfan anemia (DBA).

Recently, Bagchi et al. have demonstrated the use of chromosome engineering to identify the tumour suppressor gene CHROMODOMAIN HELICASE DNA BINDING PROTEIN 5 at human 1p3617, however, the only reported large-scale analysis of a targeted deletion of genes syntenic to human 5q− was a 450 kb region on mouse chromosome 11 (outside of the CDR of the 5q− syndrome), and this showed that the genes between Interferon regulatory factor 1 (Irf1) and Granulocyte-macrophage colony stimulating factor (Csfgm) were not involved in 5q− syndrome. To enable progress towards understanding the 5q− syndrome we applied our determination of the minimal CDR to the generation of a mouse model for human 5q− syndrome. This was achieved by a comprehensive chromosomal engineering approach to explore the region syntenic to the 5q− syndrome CDR for the presence of genes that alter normal hematopoiesis due to haploinsufficiency. Using Cre-loxP recombination to delete the syntenic regions in the mouse equivalent to the 5q− CDR we have generated a mouse model, with a deletion on chromosome 18 flanked by the Cd74 antigen (Cd74) gene and Nerve growth factor-induced differentiation clone 67 (Nid67) gene, that displays a macrocytic anemia, with dysplastic features in the bone marrow. Mechanistically, we found that these changes correlated with an increase in a population of highly p53 positive cells in the bone marrow and elevated apoptosis. Intercrossing of Cd74−/lox Nid67−/lox, LIM domain only 2 (Lmo2)Cre+ mice with Transformation related protein 53 (Trp53)−/− mice resulted in a reversal of the observed deficit in hematopoietic progenitor cell development, thereby demonstrating for the first time the mechanistic involvement of p53 in 5q− syndrome.
RESULTS

Cd74 - Nid67 interval deletion results in a 5q− syndrome-like phenotype

The CDR on the long arm of human chromosome 5 is divided into two regions of synteny on mouse chromosomes 11 and 18, with complete conservation of gene order and orientation (Fig. 1a). To narrow the CDR we generated a series of nested random deletions on chromosome 11, from the Sparc gene, and ranging from a few kilobases to over 5 megabases (Supplementary Fig. 1a-c). Two deletions were selected for further analysis. Clone 91 contained a deletion extending from Sparc to beyond Glutathione peroxidase 3 (Gpx3), a deletion of at least 0.7 Mb, but less than 1.5 Mb (Fig. 1b). Clone 2 contained a deletion extending from Sparc to Neuromedin U 2 receptor (Nmu2r), a deletion of approximately 0.5 Mb (Fig. 1c). In combination these deletions removed all of the genes mapping to the syntenic region on mouse chromosome 11 that are commonly deleted in 5q− syndrome. However, we observed no appreciable changes in the hematopoietic system (Supplementary Fig. 1d,e and data not shown) in heterozygote mice carrying these deletions even at 12 – 14 months of age. We were unable to generate homozygous mice for either deletion (data not shown).

Within this region both Sparc and Fat2 have been proposed as candidate genes for 5q− syndrome8,14. To assess their role in hematopoiensis we generated both Sparc−/− mice (Supplementary Fig. 1a-c) and Fat2−/− mice (Supplementary Fig. 2a-c). We found no evidence for Sparc or Fat2 playing roles in the development of red blood cells (RBC) or platelets (Supplementary Fig. 1f and data not shown).

The syntenic region on mouse chromosome 18 corresponding to the CDR defined in 5q− syndrome8 extends from Nid67 to Adrenergic beta-2-receptor (Adrb2) (Fig. 1a). We subdivided this region for the creation of deletions to avoid the Treacher Collins-Franceschetti syndrome 1 (Tcof1) gene, deletion of which has been reported to result in haplolethality 20. The deletion of the interval extending from Arylsulfatase i (Arsi) to MICER clone MHPP344I1 located in the Colony stimulating factor 1 receptor (Csf1r) gene was achieved by targeting both genes in cis and intercrossing with Lmo2Cre knock-in mice21 (Fig. 1d and Supplementary Fig. 3a-c), but failed to cause substantial changes in RBC numbers and hemoglobin levels (Supplementary Fig. 3d,e). By contrast deletion of the interval extending from Cd74 to Nid67, achieved by targeting loxP sites to both genes in cis (Fig. 1e and Supplementary Fig. 4a-c), and intercrossing with the Lmo2Cre knock-in mice resulted in striking perturbations to the hematopoietic system.

Deletion of the Cd74 – Nid67 interval causes macrocytic anemia

Blood parameters were analysed from cohorts of the four genotypes (Cd74+/lox Nid67+/lox, Lmo2Cre+ mice; Cd74+/lox Nid67+/lox, Lmo2Cre+ mice; Cd74+/lox Nid67+/lox, Lmo2Cre+ mice; Cd74+/lox Nid67+/lox, Lmo2Cre+ mice). We observed a striking 40 - 50% reduction in the numbers of circulating RBC in the Cd74+/lox Nid67+/lox, Lmo2Cre+ mice when compared to controls (Fig. 2a). Furthermore, hemoglobin levels were also significantly decreased (p ≤ 0.0020) in the Cd74+/lox Nid67+/lox, Lmo2Cre+ mice (Fig. 2b). This correlated with the appearance of pencil-shaped cells in the blood of the anaemic mice (Supplementary Fig. 5a). Mean corpuscular volume (Fig. 2c), mean RBC distribution width (Fig. 2d) and microscopy (Fig. 2e) showed RBC to have an increased size in the Cd74+/lox Nid67+/lox, Lmo2Cre+ mice. Flow cytometric analysis supported the development of macrocytes in the Cd74+/lox Nid67+/lox, Lmo2Cre+ mice, with cells in the TER119+ RBC populations in the bone marrow displaying increased cell size (Fig. 2f). These data indicate that the deletion of the Cd74 – Nid67 interval leads to macrocytic anemia, a key characteristic of 5q− syndrome. In addition we also observed modest thrombocytopenia in the blood of Cd74+/lox Nid67+/lox,
Lmo2Cre<sup>+</sup> mice (Fig. 3a), though a reduction in platelets was also observed in the Cd74<sup>+/lox</sup>Nid67<sup>+/lox</sup>, Lmo2Cre<sup>+</sup> control mice (Fig. 3a) and statistical significance was not always attained (Supplementary Fig. 8c). Although blood lymphocyte and monocyte numbers were not reduced appreciably (Supplementary Fig. 5b,c), total granulocyte numbers were decreased in the blood of the Cd74<sup>+/lox</sup>Nid67<sup>+/lox</sup>, Lmo2Cre<sup>+</sup> mice (Supplementary Fig. 5d), probably due to a deficit in eosinophils and polymorphonuclear leukocytes (Fig. 3b).

Cd74 – Nid67 interval deletion impairs progenitor cell production

We observed a reproducible 50 – 60% reduction in the numbers of cells flushed from the femurs of the Cd74<sup>+/lox</sup>Nid67<sup>+/lox</sup>, Lmo2Cre<sup>+</sup> mice as compared to the controls (Fig. 3c), and the Cd74<sup>+/lox</sup>Nid67<sup>+/lox</sup>, Lmo2Cre<sup>+</sup> bone marrow sections ranged from mildly to highly hypocellular (Fig. 3d). Hypocellularity was due to decreases across the hematopoietic cell lineages, since the relative proportions of these cell types remained relatively similar for all genotypes (Fig. 3e). One notable exception to this was the elevation of mast cells in the bone marrow of Cd74<sup>+/lox</sup>Nid67<sup>+/lox</sup>, Lmo2Cre<sup>+</sup> mice (Fig. 3e and 4f,h). The reason for the increased presence of these cells is unclear. However, the principle abnormalities observed were prominent dyserythropoiesis and monolobulation of the megakaryocytes in cytospins (Fig. 4a - h) and sections (data not shown).

To ascertain if the reduction in peripheral RBC resulted from a defect in the proliferation and differentiation of erythroid and myeloid progenitors we performed assays for colony forming unit-erythroid (CFU-E), CFU-granulocyte/macrophage (GM) and CFU-megakaryocyte (CFU-MK). The numbers of CFU-E (Fig. 5a), CFU-GM (Fig. 5b) and CFU-MK (Fig. 5c) were considerably depressed in the bone marrow of the Cd74<sup>+/lox</sup>Nid67<sup>+/lox</sup>, Lmo2Cre<sup>+</sup> mice. These data indicate that there is a deficit in the hematopoietic progenitor population following the deletion of the Cd74 – Nid67 interval. However, those progenitors that do differentiate, although greatly reduced, remain capable of populating the hematopoietic lineages in proportions similar to those in the control mice (Fig. 3e).

Candidate genes in the Cd74 – Nid67 interval

The deleted Cd74 – Nid67 interval contains eight known genes encoding: CD74 (Cd74) an immunoregulatory protein; Rps14; N-deacetylase/N-sulfotransferase 1 (Ndst1) a heparin sulphate modifying enzyme; synaptopodin (Synpo) an actin-binding protein; myozenin 3 (Myoz3) a cytoskeletal component; RNA binding motif protein 22 (Rbm22) an RNA-binding protein; dynactin 4 (Dctn4) a cytoskeletal component; and Nid67.

We found no evidence for anemia or thrombocytopenia in blood samples from mice with heterozygous or homozygous deficiencies for Ndst1<sup>22</sup> or Cd74<sup>23</sup> (Supplementary Fig. 6a,b). Of the remaining six genes Rps14, encoding a component of the 40S ribosome, has recently been reported to play a key role in 5q<sup>−</sup> syndrome in vitro 15.

Cd74 – Nid67 interval deletion induces p53 expression and apoptosis

Dysregulation of ribosome biogenesis through insufficiency of ribosomal subunits has been demonstrated to activate a p53 checkpoint and regulate developmental pathways24-26. To determine if the haploinsufficiency of Rps14 (or its flanking neighbours) within the Cd74 – Nid67 interval could lead to the induction of p53 we assessed intracellular p53 levels in bone marrow cells. Significantly, a greater number of bone marrow cells (p ≤ 0.0024) from the Cd74<sup>+/lox</sup>Nid67<sup>+/lox</sup>, Lmo2Cre<sup>+</sup> mice stained for elevated p53, as compared to controls (Fig. 5d,e). In Cd74<sup>+/lox</sup>Nid67<sup>+/lox</sup>, Lmo2Cre<sup>+</sup> samples that demonstrated elevated p53 expression, co-staining for cKit demonstrated that the p53-positive cells were predominantly cKit<sup>int</sup> suggesting that they may represent immature progenitor cells (Fig. 5e).
The stabilisation of p53 leads to cell-cycle arrest and apoptosis 27,28. To assess the levels of apoptosis in the Cd74/off Nid67/off, Lmo2Cre+ mice and their controls, bone marrow cells were stained with Annexin V. Annexin V staining was generally elevated amongst the bone marrow cells isolated from the Cd74/off Nid67/off, Lmo2Cre+ mice as compared to the controls (Fig. 5f), though variation in staining due to the spectrum of disease, and very low frequency of the progenitor cells within the bone marrow, was observed.

These data suggest that elevated p53 in the hematopoietic progenitors of the Cd74/off Nid67/off, Lmo2Cre+ mice induces cell death resulting in a deficit in the erythroid lineage.

**Cd74 – Nid67 interval deletion causes progenitor cell deficiencies**

To assess the effect of deletion of the Cd74 - Nid67 interval on the development of specific hematopoietic progenitor populations we stained bone marrow for markers of common myeloid progenitors (CMP) and megakaryocytic-erythroid progenitors (MEP) (both lin−cKithighSca1−CD16/CD32low), and granulocyte-monocyte progenitors (GMP, lin−cKithighSca1−CD16/CD32high). Bone marrow from the Cd74/off Nid67/off, Lmo2Cre+ mice had a striking deficit in the CMP/MEP and GMP lineages when compared to controls (Fig. 6a, top panels).

Analysis of the IL-7Rα−lin−cKithighSca1+ hematopoietic stem cells (HSC) also showed a notable scarcity of these cells in the bone marrow from the Cd74/off Nid67/off, Lmo2Cre+ mice (Fig. 6b). Similar results were also observed in the spleen, though the proportion of progenitors was considerably lower (Supplementary Fig. 7a). The use of SLAM markers29 confirmed the reduction of HSC (CD150+CD48−CD244−) and more committed CD150−CD48− lineages (data not shown).

**Inhibition of p53 rescues the deletion-induced progenitor cell deficit**

To test whether inhibiting the p53 pathway would ameliorate the anemia and dysplasia observed in the Cd74/off Nid67/off, Lmo2Cre+ mice we intercrossed the Cd74/off Nid67/off, Lmo2Cre+ mice with Trp53−/− mice. Cd74/off Nid67/off, Lmo2Cre+ mice were then crossed with Cd74+/+ Nid67+/+ Trp53+/− mice to generate the twelve genotypes displayed in Figure 6. Absence of p53 from the Cd74/off Nid67+/+ Lmo2Cre+ mice completely reversed the deficit in the CMP/MEP and GMP populations that was observed in the Cd74+/+ Nid67+/+ Lmo2Cre+ mice (Fig. 6a lower panels), with heterozygous expression of p53 having an intermediate phenotype (Fig. 6a middle panels). Similarly, absence of p53 also resulted in complete rescue of HSC development in the Cd74+/+ Nid67+/+ Lmo2Cre+ mice, again with heterozygous expression of p53 having an intermediate phenotype (Fig. 6b). Similar progenitor cell results were also observed in the spleen (Supplementary Fig. 7a).

CFU-E and CFU-GM assays were also undertaken with bone marrow from the twelve genotypes. Deletion of Ttp53 completely rescued the CFU-E (Fig. 6c) and CFU-GM (Fig. 6d) activity in the Cd74/off Nid67+/+ Lmo2Cre+ bone marrow. Similar results were also observed in the spleen (Supplementary Fig. 7b,c).

Due to a combined effect of the Lmo2Cre and Ttp53 genotypes we were unable to determine a positive role for the inhibition of p53 in the reversal of RBC levels in the Cd74/off Nid67+/+ Lmo2Cre+, Ttp53+/− mice (Supplementary Fig 8a). Similar results were also obtained for HGB (data not shown). However, we did find that ablation of p53 reversed the increased MCV observed in the Cd74+/+ Nid67+/+ Lmo2Cre+, Ttp53+/− mice (Supplementary Fig. 8b). Deletion of Ttp53 also resulted in an increase in platelet levels, reversing the thrombocytopenia observed in the Cd74+/+ Nid67+/+ Lmo2Cre+ mice.
(Supplementary Fig. 8c). Moreover, the bone marrow dysplasia observed in the Cd74\(^{+/lox}\)Nid67\(^{+/lox}\), Lmo2Cre\(^{+}\) mice was also reversed by the absence of p53 (data not shown). Thus modulation of p53 plays a critical role in maintaining the hematopoietic progenitor cells following deletion of the Cd74 – Nid67 interval, and that concurrent inhibition of p53 reverses many of the features induced by deletion of the Cd74 – Nid67 interval.

### Discussion

We report for the first time the generation of a mouse model for a human myelodysplasia involving a large-scale chromosomal deletion: a mouse model for human 5q\(^{-}\) syndrome. We have shown that segmental haploidy (generated using Cre-loxP-mediated chromosomal engineering) of a locus (or loci) residing in the interval between Cd74 and Nid67, syntenic with a region contained within the 5q\(^{-}\) syndrome CDR, results in macrocytic anemia, a key feature of 5q\(^{-}\) syndrome. Bone marrow cell morphology revealed prominent dysplasia within the erythroid lineage and monolobulated megakaryocytes. Bone marrow analysis demonstrated a marked reduction in erythroid and myeloid progenitors that correlated with the appearance of highly p53-positive bone marrow cells and elevated apoptosis, correlating with reduced cellularity. The results from a large study of patients with the 5q\(^{-}\) syndrome by Giagounidis et al has shown that the cellularity of the marrow of patients with the 5q\(^{-}\) syndrome is variable with 40% of patients showing a hypercellular marrow, 40% showing a normocellular marrow and 20% of patients showing a hypocellular marrow. Thus a proportion of patients with 5q\(^{-}\) syndrome have a hypocellular marrow in accord with the hypocellular marrow observed in the Cd74\(^{+/lox}\)Nid67\(^{+/lox}\), Lmo2Cre\(^{+}\) mice2. Thus our mouse model exhibits important features of 5q\(^{-}\) syndrome including macrocytic anemia, prominent dyserythropoiesis and monolobulated megakaryocytes.

To achieve deletion of the Cd74 – Nid67 interval in the hematopoietic lineage we used an Lmo2Cre mouse line as the Cre driver, since this is expressed in HSC (M. Metzler, R. Codrington, K. Akashi and T. H. Rabbitts, personal communication). Although we noted a mild, but reproducible, reduction of myeloid lineages in Lmo2Cre mice, probably due to offsite effects due to persistent high-level expression of Cre recombinase30, the phenotype of the deletion of the Cd74 – Nid67 interval was more profound than that of the Lmo2Cre controls.

Clearly haploinsufficiency of a gene(s) (or non-coding sequences) in this region underlies the macrocytic anemia and megakaryocyte dysplasia characteristic of 5q\(^{-}\) syndrome. The Cd74 – Nid67 interval harbours eight known genes and we have excluded the individual involvement of Nsd1 and Cd74, leaving Rps14, Synpo, Myoz3, Dctn4, Rbm22 and Nid67. Of these, the Rps14 gene is a strong candidate since it has been proposed, following an in vitro RNAi-screening approach, to play a role in 5q\(^{-}\) syndrome15. Moreover, we have shown that haploinsufficiency of RPS14 in the hematopoietic stem cells of patients with the 5q\(^{-}\) syndrome is associated with deregulated expression of multiple ribosomal- and translation-related genes31, further supporting the proposal that the 5q\(^{-}\) syndrome represents a disorder of defective ribosome biogenesis.

The yeast Rps14 homologue is essential for the assembly of 40S ribosomal subunits32,33, although its specific function in the human remains largely unknown. Recent evidence has implicated aberrant ribosomal biogenesis in a number of diseases, including the bone marrow failure syndromes34. Mutation and resultant haploinsufficiency of a number of ribosomal genes, including those encoding structural components of both the large and small ribosomal subunits have been identified to underlie Diamond Blackfan anemia (DBA)16,35, a congenital disease characterised by macrocytic anemia36. Furthermore, it has also been
suggested that defective ribosomal 60S subunit maturation caused by mutations in the Shwachman-Bodian-Diamond syndrome (SBDS) gene may underlie the hematological dysfunction characteristic of this disease37.

We observed that a population of bone marrow cells from the Cd74+/lox Nid67+/lox, Lmo2Cre+ mice expressed higher levels of p53 than controls. The stabilisation of p53 is known to lead to apoptosis27 and we found that increased bone marrow cell apoptosis was also apparent in these mice. Our finding that introduction of the p53-deficient background into the Cd74+/lox Nid67+/lox, Lmo2Cre+ mice completely reverses the observed deficit in bone marrow progenitors and ameliorates the thrombocytopenia in this model is important given the emerging evidence that ribosomal stress leads to activation of the p53 pathway25,26,38. For example, it has been shown that RPS19 deficiency in the zebrafish leads to developmental abnormalities and defective erythropoiesis through activation of the p53 protein family39. Recently, two mouse dark skin (Dsk) loci with mutations in Rps19 and Rps20 have been reported25. The underlying pathophysiology was shown to involve stabilization of p53 leading to cKit ligand expression and epidermal melanocytosis, indicating that p53 regulation may induce discrete phenotypes dependent on the cell type in which expression occurs. Another disorder involving ribosome biogenesis and p53 stabilisation is Treacher Collins syndrome (TCS). Haploinsufficiency of TCOF1 (encoding TREACLE, the protein mutated in TCS)40,41 perturbs mature ribosome biogenesis42, and this has been shown recently to result in the stabilization of p5342. Inhibition of p53 prevented apoptosis of neuroepithelial cells and rescued the cranioskeletal abnormalities associated with mutations in Tcof124.

Our genetic data now implicate p53 in the molecular mechanism underlying 5q− syndrome. Interestingly, we have previously reported the up-regulation of multiple transcriptional targets of p53 in the hematopoietic stem cells of patients with the human 5q− syndrome, including WILD TYPE P53-INDUCED GENE 1 (WIG1), PLECKSTRIN HOMOLOGY-LIKE DOMAIN, FAMILY A, MEMBER 3 (PHLDA3), TNF RECEPTOR SUPERFAMILY, MEMBER 6 (FAS), BCL2-ASSOCIATED X PROTEIN (BAX), B-CELL LYMPHOMA/LEUKAEMIA 11B (BCL11B) and FERREDOXIN REDUCTASE (FDXR)10,31. Moreover, our pathway analysis using the DAVID gene ontology application (http://niaid.abcc.ncifcrf.gov) and these gene expression profiling data sets10,31 have shown that the p53 pathway is significantly up-regulated (p ≤ 0.024) in patients with the 5q− syndrome. As postulated for TCS, the ribosome biogenesis checkpoint that leads to activation of p53 function may also provide a therapeutic avenue for the treatment of the human 5q− syndrome, though this will only be possible if the pathway does not influence the key tumour suppressor role played by p53. It has been reported recently that disruption of 40S ribosome biogenesis leads to p53 induction through a ribosomal protein L11-translatability-dependent mechanism that might be targeted therapeutically43.

Our data clearly identify an important chromosomal region for the development of macrocytic anemia, and would support a role for Rps14. Typically human MDS are associated with thrombocytopenia as in the mouse model presented here, although at diagnosis many patients with the human 5q− syndrome have normal or slightly elevated platelet counts. The explanation for this apparent difference is not known; it may be due to a human/mouse species difference or due to additional genetic abnormalities in the human. However, it is also possible that other genes in the Cd74− Nid67 interval may play an additional role. For example Dcta4 encodes Dynactin 4, a subunit of the Dynactin complex that binds Dynein allowing the motor to migrate on the microtubule lattice. It has been reported that disruption of Dynactin/Dynein function can inhibit proplatelet elongation and this may reduce platelet formation44. However, our CFU-MK data suggest that it is a primary defect in megakaryocyte progenitor production that leads to thrombocytopenia.
Rbm22 is highly conserved from yeast to human and has been proposed to be involved in RNA splicing\textsuperscript{45}; aberrations in which have been linked to cancer\textsuperscript{46}. Moreover, we have recently shown that patients with the 5q\textsuperscript{−} syndrome show a marked reduction in the expression of \textit{RNA BINDING MOTIF PROTEIN 22 (RBM22)}\textsuperscript{10}. By contrast Synaptopodin, Myozenin 3 and Nid67 appear unlikely candidates\textsuperscript{47,48}.

It is possible that additional genes within the CDR could have additive roles to those identified herein. Future studies using competitive cell transfers will help to clarify whether cells carrying the \textit{Cd74 – Nid67} deletion interval have a competitive survival advantage or whether other genes/mutations are responsible for clonal survival and outgrowth in 5q\textsuperscript{−} syndrome.

We report the generation of the first mouse model of the human 5q\textsuperscript{−} syndrome and the demonstration that a p53-dependent mechanism underlies this disorder. This animal model will enable the analysis of both emerging therapeutic compounds and the assessment of the mode of action of existing drugs such as lenalidomide. This study shows the feasibility of generating large-scale chromosomal deletions in mice for the genetic analysis of haploinsufficiency in myelodysplasia.

**METHODS**

**Mouse strains**

We obtained \textit{Cd74}\textsuperscript{−/−} mice\textsuperscript{23} from Jackson Labs. \textit{Trp53}\textsuperscript{−/−} mice were as described\textsuperscript{49}. \textit{Lmo2Cre} knock-in mice were as described previously\textsuperscript{21}. Mice were on a mixed 129 x C57BL/6 background. The methods for the generation of all the other mouse strains used in this study can be found in Supplementary Methods.

The mice analysed within each experiment were of similar age, genetic background and gender where possible. All animal experiments outlined in this report were undertaken with the approval of the UK Home Office.

**Flow cytometry**

We stained bone marrow cell suspensions for cell surface markers. Antibodies to mouse TER119, conjugated to FITC, (clone TER-119) and to mouse CD71, conjugated to PE, (clone C2) were used for the analysis of erythroid cells. For lineage markers antibodies against the following molecules were used all conjugated to FITC: mouse CD4 (clone H129.19), mouse CD3e (clone 145-2C11), mouse Ly-6G and Ly-6C (Gr-1) (clone RB6-8C5), mouse CD11b (Mac-1) (clone M1/70), mouse CD45R/B220 (clone RA3-6B2), mouse FceR1 alpha (clone MAR-1), mouse CD8a (clone 53-6.7), mouse TER119/Erythroid cells (clone Ter119). For HSC markers antibodies to mouse Sca-1, conjugated to PE (clone as above) and to mouse CD117 (cKit), conjugated to APC, (clone 2B8) were used. HSCs were defined as lineage\textsuperscript{−}Sca-1\textsuperscript{+}cKit\textsuperscript{high}. For the CMP population the following antibodies were used: mouse Sca-1 conjugated to PE-Cy7 (clone as above); mouse IL-7Ra conjugated to biotin (clone SB/199, subsequently stained with streptavidin PE Cy7); mouse CD117 (cKit) conjugated to APC (clone as above); mouse CD16/32 PE (clone 93). CMP/MEPs were defined as lineage\textsuperscript{−}Sca-1/IL-7Ra\textsuperscript{−}CD16/32\textsuperscript{low}cKit\textsuperscript{high}. GMPs were defined as lineage\textsuperscript{−}Sca1/IL-7Ra\textsuperscript{−}CD16/32\textsuperscript{high}cKit\textsuperscript{high}.

For intracellular staining the cells were fixed and permeabilised, following manufacturer's instructions (BD Biosciences) and stained for p53 or an IgG\textsubscript{1} isotype control (anti-p53 or IgG\textsubscript{1} isotype conjugated to PE, BD Biosciences). Cells were stained at 1 \times 10^{6} cells per well and intracellular antibodies were added at 20 \mu{l} per well.
For Annexin V staining 1 × 10^6 bone marrow cells were analysed using the Annexin V-FITC apoptosis detection kit 1 according to the manufacturer’s instructions (BD Pharmingen).

**Colony forming cell assays**

We collected femurs and flushed them using IMDM with 2% FBS (StemCell Technologies) to obtain bone marrow cells. Spleens were isolated and disrupted through a cell strainer, before red cells were lysed using ammonium chloride lysis. Bone marrow cells and splenocytes (2 × 10^5 cells per 35 mm dish) were plated in Methocult®-GF M3434 for CFU-GM assays and Methocult® M3334 for CFU-E assays (StemCell Technologies). For CFU-MK, 1 × 10^5 or 5 × 10^5 bone marrow cells were plated in Megacult®-C in the presence of recombinant human (rh) thrombopoietin, rm IL-3, rh IL-6, rh IL-11 following the protocol from StemCell Technologies. After six days in culture the CFU-MK colonies were stained for acetylcholinesterase as described in the instructions from StemCell Technologies.

**Blood cell counts**

We measured blood parameters such as RBC, mean corpuscular volume, red distribution width, hemoglobin concentration, platelet number and WBC using a Sci1 Vet abc Animal Blood Counter.

**Differential cell counts**

We collected blood samples into EDTA coated tubes. In some cases spiculation of the red cells was observed, but this did not correlate with specific genotypes. Blood smears or bone marrow cell (from flushed femurs) cytospin slides were stained with Giemsa (Sigma-Aldrich), following the manufacturer's instructions. Differential cell counts were made blind on 100 cells per slide under oil and at a 63x magnification.

**FISH**

We treated ES cells with 0.1 μg/ml colcemid (Invitrogen, Paisley, UK) for 90 minutes, trypsinised and resuspended in 75 mM KCl for 30 minutes. Cells were then washed four times in ice-cold fixative (3 parts methanol, 1 part acetic acid). Cell suspensions were dropped onto slides then put through an ethanol dilution series. FISH probes were produced from BAC DNA using a Roche DIG or BIOTIN nick translation kit (Roche Diagnostics, East Sussex, UK) as detailed in the manufacturer’s instructions. Slides were denatured in 70% formamide at 65 °C, quenched in ice cold 70% ethanol, then dehydrated through an ethanol dilution series and air dried. The denatured probe mix (biotin-labelled BAC-1 DNA and DIG-labelled BAC-2 DNA, cot1 DNA and hybridisation buffer (Cambio, Cambridge, UK)) was added and hybridised at 42 °C for at least 16 hours. The slides were washed then incubated with either anti-digoxigenin or avidin, conjugated to fluorescein or rhodamine Fab fragments, dictated by the template for detection (Roche Diagnostics, East Sussex, UK). Slides were air dried and analyzed at 1,000X. Image analysis was performed using Smart Capture 2.1 software (Digital Scientific, Cambridge, United Kingdom).

**Statistics**

Student’s t-test was used to determine statistical significance and two-tail p values are shown. Error bars represent the standard error of the mean.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
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Figure 1.
Representation of human 5q− syndrome CDR in humans and alignment with mouse regions of synteny. (a) The human 5q− syndrome CDR aligned with mouse chromosomes 11 and 18. (b) Confirmation of Clone 91 using FISH. (c) Confirmation of Clone 2 using FISH. (d) Confirmation of the Arsi – micerMHPP344i11 deletion by PCR analysis (see also Supplementary Fig. 3). (e) Confirmation of the Cd74 – Nid67 deletion by Southern analysis (see also Supplementary Fig. 4). S, spleen; L, liver; BM, bone marrow; T, tail. WT, wildtype. Arrows indicate the transcriptional orientation of the genes. The BAC clones and dye colours (red or green) used for FISH are marked in their relative positions.
Figure 2.
Deletion of the \( \text{Cd}74^{+/\text{lox}} \text{Nid}67^{+/\text{lox}} \) interval leads to macrocytic anemia. Blood from the specific genotypes was analysed (\( n = 28 – 52 \)). (a) Red blood cell numbers. (b) Hemoglobin levels. (c) Mean corpuscular volume (MCV). (d) Red cell distribution width (RDW) (\( n = 28 – 52 \)). (e) Live RBC imaged using light microscopy. Scale bar, 20 \( \mu \text{m} \). (f) Bone marrow cells gated on CD71 and TER119 expression and analysed for forward scatter as a measure of cell size. Data are representative of two experiments with five mice per group.
Figure 3.
Blood and bone marrow cell proportions vary following deletion of the \textit{Cd74}^{+/\text{lox}}\textit{Nid67}^{+/\text{lox}} interval. (a) Platelet numbers in blood (n = 28 – 52). (b) Differential white blood cell counts (n = 5 per group). (c) Bone marrow cell numbers (n = 5 per group). Data are representative of at least two experiments. (d) Hematoxylin and eosin stained bone marrow sections. Scale bar, 20 μm. Representative of five mice per group all of similar age. (e) Bone marrow differential cell counts from cytospins (n = 8 - 10 per group).
Figure 4. Giemsa-stained bone marrow cytospins following deletion of the $Cd74^{+/lox}Nid67^{+/lox}$ interval to show prominent dyserythropoiesis, megakaryocytes with monolobulated nuclei and mast cells. Scale bar 40 μm. (a) dysplastic erythroblast (arrow); (b) inter-cytoplasmic bridging (arrow); (c) mitotic figure (arrow); (d) inter-nuclear bridging (arrow); (e) binucleate erythroid precursors (arrow); (f) mast cell (arrow); (g) megakaryocyte with monolobulated nucleus (arrow); (h) megakaryocyte with multiple monolobulated nuclei (star). Also includes a mast cell (arrow).
Figure 5.
Hematopoietic cell progenitors and p53 positive and apoptotic cells in the 
Cd74<sup>+/loxp</sup>Nid67<sup>+/lox</sup>, Lmo2Cre<sup>+</sup> mice. (a) Number of colony forming units – erythroid in bone marrow cultures (n = 5 per group). Data are representative of two experiments. (b) Number of colony forming units – granulocyte/macrophage in bone marrow cultures (n = 7 per group). (c) Number of colony forming units – megakaryocyte in bone marrow cultures (n = 3 per group). (d) Numbers of p53<sup>+</sup> bone marrow cells (n = 5 per group). Representative of two similar experiments. (e) Intracellular staining for p53 in cKit<sup>+</sup> cell surface stained cells. Example is of a highly p53-positive Cd74<sup>+/loxp</sup>Nid67<sup>+/lox</sup>, Lmo2Cre<sup>+</sup>, sample to show cKit<sup>+</sup> cell involvement. (f) Annexin V staining of bone marrow cells (n = 5 per group).
Deletion of p53 reverses the progenitor cell deficits that result from the deletion of the Cd74lox/lox Nid67lox/lox interval. (a) Bone marrow cells stained for CMP, MEP and GMP cell surface markers (representative of five mice per group). (b) Bone marrow cells stained for HSC cell surface markers. Cells initially gated on Lin⁻Sca1⁺ (representative of five mice per group). (c) Number of colony forming units – erythroid in bone marrow cultures (n = 6 per group). (d) Number of colony forming units – granulocyte/macrophage in bone marrow cultures (n = 6 per group).