Germline NPM1 mutations lead to altered rRNA 2′-O-methylation and cause dyskeratosis congenita

Daphna Nachmani¹, Anne H. Bothmer¹, Silvia Grisendi¹, Aldo Mele²,³, Dietmar Bothmer⁴, Jonathan D. Lee⁵, Emanuele Monteleone⁵, Ke Cheng¹, Yang Zhang¹, Assaf C. Bester¹, Alison Guzzetti¹, Caitlin A. Mitchell¹, Lourdes M. Mendez¹, Olga Pozdnyakova⁶, Paolo Sportoletti¹, Maria-Paola Martelli⁷, Tom J. Vulliamy⁸, Modi Safra⁹, Schraga Schwartz⁹, Lucio Luzzatto¹⁰, Olivier Bluteau¹¹, Jean Soulier¹¹, Robert B. Darnell²,³, Brunangelo Falini⁷, Inderjeet Dokal⁸, Keisuke Ito¹², John G. Clohessy¹ and Pier Paolo Pandolfi*¹

RNA modifications are emerging as key determinants of gene expression. However, compelling genetic demonstrations of their relevance to human disease are lacking. Here, we link ribosomal RNA 2′-O-methylation (2′-O-Me) to the etiology of dyskeratosis congenita. We identify nucleophosmin (NPM1) as an essential regulator of 2′-O-Me on rRNA by directly binding C/D box small nucleolar RNAs, thereby modulating translation. We demonstrate the importance of 2′-O-Me-regulated translation for cellular growth, differentiation and hematopoietic stem cell maintenance, and show that Npm1 inactivation in adult hematopoietic stem cells results in bone marrow failure. We identify NPM1 germline mutations in patients with dyskeratosis congenita presenting with bone marrow failure and demonstrate that they are deficient in small nucleolar RNA binding. Mice harboring a dyskeratosis congenita germline Npm1 mutation recapitulate both hematological and nonhematological features of dyskeratosis congenita. Thus, our findings indicate that impaired 2′-O-Me can be etiological to human disease.

Post-transcriptional RNA modifications, also known as the epitranscriptome, are crucial regulators of gene expression. rRNAs are among the most heavily modified molecules in the cell and require post-transcriptional modification to facilitate their processing and secondary-structure formation. Therefore, rRNA modifications are essential for functional fidelity of the ribosome and proper gene expression. 2′-O-Me is the most abundant rRNA modification since each ribosome harbors more than 100 2′-O-Me residues. However, what role 2′-O-Me may play in human disease is unclear.

NPM1 is an abundant and highly conserved phosphoprotein that mainly resides in nucleoli. The NPM1 gene undergoes frequent genetic alterations in hematological diseases and bears the most frequent mutation in acute myeloid leukemia (AML), with approximately 30% of people with AML presenting with a frameshift mutation that results in NPM1’s aberrant cytoplasmic localization (NPMc). This suggests that nucleolar localization of NPM1 is essential for normal cellular function. While NPM1 is a bona fide RNA-binding protein, little is known regarding its RNA-related function(s) in the nucleolus and whether it contributes to pathogenesis.

By studying the RNA-binding function of NPM1 we demonstrated its key role in rRNA 2′-O-Me regulation through the direct binding of C/D box small nucleolar RNAs (snoRNAs). We suggest that 2′-O-Me is crucial for hematopoietic stem cell maintenance and that deficiencies in 2′-O-Me contribute to pathogenesis.

Results

NPM1 regulates 2′-O-Me through snoRNA binding. We initiated our study by determining the repertoire of RNA species bound by NPM1. We performed a high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HiTS-CLIP) analysis using mouse embryonic fibroblasts (MEFs) from our previously established Npm1−/− mouse model. Computational analysis revealed that NPM1 binds to several distinct sequence motifs (Fig. 1a), suggesting that it has a complex RNA-binding capacity.

We found snoRNAs to be the most abundant RNA species bound by NPM1 (Fig. 1b and Supplementary Table 1). The two main classes of snoRNAs are the C/D box and the H/ACA box snoRNAs, which associate with core proteins in small nucleolar ribonucleoprotein (snoRNP) complexes to mediate rRNA modifications. Together with the RNA 2′-O-methyltransferase fibrillarin

¹Cancer Research Institute, Beth Israel Deaconess Cancer Center, Department of Medicine and Pathology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA. ²Laboratory of Molecular Neuro-Oncology and Howard Hughes Medical Institute, The Rockefeller University, New York, NY, USA. ³New York Genome Center, New York, NY, USA. ⁴Hochschule Zittau/Görlitz, Institute of Ecology and Environmental Protection, Zittau, Germany. ⁵Molecular Biotechnology Center and Department of Molecular Biotechnology and Health Sciences, University of Turin, Turin, Italy. ⁶Department of Pathology, Brigham and Women’s Hospital, Boston, MA, USA. ⁷Institute of Hematology-Centro di Ricerche Emato-Oncologiche, University of Perugia, Perugia, Italy. ⁸Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, UK. ⁹Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel. ¹⁰Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania. ¹¹INSERM UMR944 and CNRS UMR7212, Hôpital Saint-Louis, Paris, France. ¹²Ruth L. and David S. Gottesman Institute for Stem Cell and Regenerative Medicine Research, Albert Einstein College of Medicine, New York, NY, USA. *e-mail: ppandolf@bidmc.harvard.edu
NPM1 controls internal ribosome entry site (IRES) translation through 2′-O-Me regulation. The 2′-O-Me sites that are affected by Npm1 deletion are located in the 285 rRNA at nucleotide positions 1327, 3764, 3866, 3904 and 4198 (numbers according to the human nomenclature; Fig. 1c). Notably, these residues are localized in highly structured regions50,51, with positions 3904 and 4198 located within the peptidyl transferase center; therefore, they have the potential to impact ribosome structure and function52. In view of this, we assessed whether global translation is compromised in the absence of Npm1. Both 18S-Met incorporation and 1-homopropargylglycine (HPG) metabolic labeling demonstrated that global translation in Npm1−/− MEFs was not defective (Supplementary Fig. 2a,b, respectively). In addition, Npm1−/− and Npm1+/− MEFs were comparable in their cell size (Supplementary Fig. 2c), RNA content (Supplementary Fig. 2d) and RNA processing (Supplementary Fig. 2e). Immunoblot analysis of the mTOR signaling pathway, a major pathway regulating translation49,50, did not find any significant differences between Npm1−/− and Npm1+/− MEFs at steady state (Supplementary Fig. 2f). We have also previously shown that ribosome composition is not affected in the absence of Npm1 (ref. 52). Taken together, these data support the conclusion that in spite of the compromised 2′-O-Me levels, global translation is unaffected.

However, ribosome alteration can cause gene-specific effects14–26. For example, low accessibility of otherwise functional ribosomes might affect translation of messenger RNAs that have low translation initiation and elongation rates, such as those that have a strong secondary structure of their 5′-UTR17–22. To test in an unbiased manner for gene-specific effects, we performed microarray analysis of polysome-associated transcripts from Npm1−/− and Npm1+/− MEFs. (Npm1−/− provides a physiologically relevant model system to test translational skewing.) Polysome microarray analysis revealed a skew in the distribution of polysome-associated transcripts on loss of even just one NPM1 allele, with the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis identifying the affected cellular pathways (Fig. 1f). Since the 5′-UTR plays an important role in translational regulation, we carried out sequence analysis of enriched/depleted 5′-UTRs. While we did not detect any sequences or motifs that may lead to differential translation, minimum free energy (MFE) analysis found that Npm1−/− polysome-depleted transcripts display a lower MFE value (Fig. 1g), indicating that these transcripts have, on average, a more structured 5′-UTR.

(FBL), C/D box snoRNPs facilitate 2′-O-Me1, whereas H/ACA box snoRNPs associate with the pseudouridines synthase dyskerin (DKC1) to facilitate pseudouridylation14. Thus, our data suggest that NPM1 might have a role in facilitating post-transcriptional modifications of rRNA.

We first examined whether NPM1 deficiency would result in a deficit in pseudouridylation. However, no pseudouridine deficit was detected in Npm1−/− MEFs by pseudouridine site identification sequencing (PSI-seq)14 (Supplementary Fig. 1a). Next, we measured byRTL-P (reverse transcription at low deoxyribonucleoside-5-triphosphate concentrations followed by PCR)14 the levels of individual rRNA 2′-O-Me residues targeted by the NPM1-interacting C/D box snoRNAs. In the absence of Npm1 we observed a significant reduction in the levels of five specific 2′-O-Me residues (Fig. 1c), and confirmed that NPM1 binds to the corresponding snoRNAs (Supplementary Fig. 1b). Notably, we did not observe changes in FBL levels (Supplementary Fig. 1c) or in the abundance of the specific snoRNAs (Supplementary Fig. 1d).

Reexpression of NPM1 in Npm1−/− MEFs restored 2′-O-Me levels (Supplementary Fig. 1e) and led to an increase in other 2′-O-Me modifications (Supplementary Fig. 1e). This suggests that individual 2′-O-Me residues are differentially regulated and is in accordance with recent findings of 2′-O-Me regulation14,26.

To gain additional molecular insight, we focused on the five snoRNAs whose corresponding methylation was reduced in Npm1−/− MEFs (Fig. 1c) and tested whether their loading into snoRNPs was dependent on NPM1. In FBL immunoprecipitation (IP) experiments we observed a significant reduction in FBLs ability to interact with the NPM-bound snoRNAs in Npm1−/− MEFs (Fig. 1d). Given the observed effect on snoRNPs loading and NPM1′s capacity to interact with a variety of proteins1, we hypothesized that NPM1 might interact directly with C/D box snoRNPs to facilitate snoRNA loading. Reciprocal co-immunoprecipitation showed a robust interaction between NPM1 and FBL (Fig. 1e). This interaction is specific since neither nucleolar protein Snord4 (C/D box snoRNP component) nor DKC1 (a member of the H/ACA box snoRNP) was detected in the NPM1 immunoprecipitation (Supplementary Fig. 1f). In addition, we found that the NPM1–FBL interaction is RNA-independent since neither Rnase A nor micrococcal nuclease treatment released FBL to the supernatant (Supplementary Fig. 1g,h). These data demonstrate that NPM1 is a regulator of rRNA 2′-O-Me through interaction with C/D box snoRNAs and FBL.

**Figure 1 | NPM1 regulates 2′-O-Me through snoRNA binding.** a, RNA-binding motifs of NPM1 identified by NPM1 HiTS-CLIP. b, Functional annotation of the RNA species identified in NPM1 HiTS-CLIP. c, Levels of specific 2′-O-Me residues (x axis) mediated by C/D box snoRNAs in HiTS-CLIP. Fold change was calculated for Npm1−/− relative to Npm1+/+ MEFs. Data are presented as the mean ± s.d. of n = 3 independent experiments and compared to the levels of Ctrl modification. U1804 is the Ctrl that is mediated by snoRD20, which was not identified in the HiTS-CLIP analysis. d, FBL immunoprecipitation was performed using Npm1−/− and Npm1+/+ MEFs. Snord enrichment was calculated relative to total input and fold enrichment in Npm1−/− MEFs and compared to the levels of enrichment of the Snord Ctrl, Snord13, which was not identified to interact with NPM1 by HiTS-CLIP. The bars present the mean ± s.d. of n = 4 independent experiments. e, Immunoprecipitation of either NPM1 (upper panel) or FBL (lower panel) was performed using nuclear extracts of Npm1−/− and Npm1+/− MEFs. The representative image out of four independent experiments is shown. IgG, immunoglobulin G. f, KEGG pathway analysis of Npm1−/− polysome microarray analysis relative to Npm1+/+. Statistical significance was determined by GSEA. g, Cumulative distribution of MFE of the 5′-UTRs of polysome-depleted and polysome-enriched transcripts, normalized to 5′-UTR length. h, Diagram of the dual luciferase reporter testing for both cap-dependent and IRES-dependent translation. i, Npm1−/−, Npm1+/− and Npm1+/+ MEFs were transfected with the cap/IRES luciferase reporter (diagram in h). The bars represent the s.d. of n = 6 independent experiments. j, Immunoblot of the IRES-translated genes p27 and XIAP in Npm1−/− and Npm1+/− MEFs. RPL22 served as the loading Ctrl. Representative data out of six independent experiments are shown. k, 5′-UTRs of Cdkn1b and Xiap lead to reduced translation of the luciferase reporter in Npm1−/− compared to Npm1+/+ MEFs. The IRES versus cap translation ratio was calculated relative to the activity in Npm1−/+. Data are presented as the mean ± s.d. of three independent experiments and compared to the levels in Npm1+/+ MEFs. l, Transcript enrichment in Npm1−/− activated ribosomes (prPS6 immunoprecipitation) was calculated relative to the enrichment in Npm1+/+ MEFs. Data are presented as the mean ± s.d. of four independent experiments. m, IRES activity was calculated relative to the activity in Npm1+/+ MEFs. Data are presented as the mean ± s.d. of three independent experiments. n, Analysis of specific 2′-O-Me in clinical remission (n = 14) and patients with NPMc− AML (NPMc−, n = 16). The error bars represent the s.e.m. a.U., arbitrary unit. Statistical significance was calculated using a Mann–Whitney U-test. For all relevant panels, unless otherwise stated, statistical significance was determined by one-tailed Student’s t-test. NS, not significant. Uncropped blot images are presented in Supplementary Fig. 11.
One of the best characterized 5'-UTR structured elements is the IRES element, which enables cap-independent translation initiation\(^2\). To assess whether Npm1 loss impairs IRES function, we first performed gene set enrichment analysis (GSEA) using a recently published dataset of IRES genes\(^3\) and observed a statistically significant negative enrichment of putative IRES genes (Supplementary Fig. 2g, top panel), a pattern that is in line with our findings. We also observed a negative trend with a more stringently filtered gene list, albeit not statistically significant (Supplementary Fig. 2g, bottom panel). Taken together, this strongly suggests that IRES-dependent translation is affected in Npm1\(^{+/–}\) polosomes.

To confirm this experimentally, we used a dual luciferase reporter designed to evaluate cap- and IRES-mediated translation (Fig. 1h). We observed reduced IRES-dependent translation,
in an NPM1 dose-dependent manner, whereas cap-dependent translation was unaffected (Fig. 1i and Supplementary Fig. 2h). As with Npm1+/- MEFs we did not observe any difference in snoRNA abundance in Npm1+/- (Supplementary Fig. 2i); however, methylation of only four methylation residues was affected in Npm1+/- (Supplementary Fig. 2j). This difference might explain the gene dosage-dependent reduction in IRES translation of the dual lucerase reporter (Fig. 1i).

We continued our investigation by looking at well-characterized IRES-containing transcripts, such as X-linked inhibitor of apoptosis (XIAP) and cyclin dependent kinase inhibitor 1B (Cdkn1b/p27)31–33; we used these as ‘endogenous IRES reporters’ in subsequent analyses. Indeed we observed reduced protein levels of XIAP and p27 (Fig. 1j), with no changes in transcript abundance (Supplementary Fig. 2k), which is suggestive of a defective translational mechanism. This was confirmed by replacing the hepatitis C virus IRES element in our reporter with the 5’-UTRs of Cdkn1b or XIap (or of Actb as a non-IRES negative control (Ctrl)), which demonstrated reduced activity with Cdkn1b and XIap, but not with Actb (Fig. 1k). Polysome fractionation of Npm1+/- and Npm1+/- MEFs demonstrated a shift to lighter polysome fraction of Cdkn1b and XIap transcripts as well as of additional 5’-UTR structured transcripts (Supplementary Fig. 2l,m) in Npm1+/- MEFs. To support these findings even further, we performed immunoprecipitation of phosphorylated RPS6 (pRPS6 immunoprecipitation)34,35 and found that IRES-containing transcripts, but not the Ctrl transcript, were depleted from Npm1+/- translating ribosomes (Fig. 1l).

We validated the involvement of snoRNAs in NPM1-mediated translational control by overexpressing and knocking down (via gapmers) the NPM1-associated snoRNAs, Snord47 and Snord52. This led to increased and decreased 2′-O-Me levels respectively, with concomitant alterations to protein levels of XIAP and p27 but no changes in transcript levels (Supplementary Fig. 3a–g).

Intriguingly, expression of the cytoplasm-localized NPMc+ mutant (commonly found in AML) or the NPM1–RARα fusion protein (found in acute promyelocytic leukemia and lacking a number of RNA-binding domains) failed to rescue the IRES-dependent translation deficiency of the lucerase reporter (Fig. 1m and Supplementary Fig. 3h). Consistent with these findings, we observed impaired RNA 2′-O-Me in human NPMc+ AML samples (Fig. 1n), as well as in the human leukemia cell line OCI-AML3 harboring the NPMc+ mutation (Supplementary Fig. 3i), while snoRNA levels were unaffected (Supplementary Fig. 3j). These data, together with previous reports of aberrant 2′-O-Me in leukemia36, strongly suggest that altered 2′-O-Me may contribute to leukemogenesis.

NPM1 regulates cellular growth and differentiation through 2′-O-Me. Since NPM1 mutations are associated with hematological disease, we next investigated what effects perturbations of NPM1, snoRNAs and 2′-O-Me might have on growth and differentiation of hematopoietic cells, using the human erythroleukemia cell line K562.

As expected, efficient NPM1 depletion (Supplementary Fig. 4a) resulted in decreased 2′-O-Me levels (Supplementary Fig. 4b) and reduced levels of IRES-translated proteins, while FBL levels and the abundance of snoRNAs remained intact (Supplementary Fig. 4a and Supplementary Fig. 4c, respectively). NPM1 depletion also led to a substantial reduction in colony formation potential, demonstrating its role in clonogenic growth (Fig. 2a). Moreover, using hemin treatment to induce erythroid differentiation37 (Fig. 2b), we found that NPM1 depletion in K562 cells led to increased differentiation (Fig. 2c).

To understand whether C/D box snoRNAs have a role in these processes, we generated individual snoRNA-inactivated K562 cells by CRISPR–Cas9. Genome editing of snoRNAs was confirmed by Sanger sequencing in the bulk population (Supplementary Fig. 4d). SnoRNA inactivation led to a significant loss in specific snoRNA abundance (Fig. 2d) and to a consequent reduction in rRNA-specific methylation (Fig. 2d), while other unrelated methylations were preserved (Supplementary Fig. 4e). As was the case with NPM1 depletion, inactivation of SNORD15, SNORD47 and SNORD104 led to reduced colony formation (Fig. 2e). Inactivation of SNORD15, SNORD47, SNORD52 and SNORD58 led to increased erythroid differentiation (Fig. 2f). These data demonstrate that individual snoRNAs may have distinct roles in specific biological processes.

To investigate whether the NPM1 depletion phenotypes are mediated by 2′-O-Me, we performed rescue experiments by expressing each snoRNA in NPM1-depleted K562 cells (Fig. 2g). Cells were analyzed for individual snoRNA overexpression and specific 2′-O-Me levels (Fig. 2h). Since SNORD47 failed to overexpress, with no change in the specific 2′-O-Me residue (Fig. 2h), we regarded these cells as an additional negative Ctrl.

In colony assays, only SNORD52 increased colony number (Fig. 2i). On the other hand, all snoRNAs except SNORD47 reduced the rate of differentiation of NPM1-depleted K562 on hemin treatment (Fig. 2j). Whereas individual snoRNAs partially corrected the effects of NPM1 depletion, no individual snoRNA could fully rescue the phenotype of NPM1-depleted K562 cells. This suggests that the combined activity of several snoRNAs is required for a full rescue of NPM1 loss. Since efficient coexpression of all five snoRNAs is technically challenging, we sought to simultaneously affect several methylation residues by overexpressing the snoRNA-dependent
methyltransferase FBL (Fig. 2g). Overexpression of FBL led to increased methylation of most methylation sites (Supplementary Fig. 4f). Most importantly, functionally, overexpression of FBL in NPM1-depleted K562 cells fully rescued both colony formation (Fig. 2k) and differentiation (Fig. 2l). While it may be possible that rescue of such functional assays could be mediated by 2′-O-Me changes to other RNA species, such as mRNAs, 2′-O-Me modifications of mRNA is currently not ascribed to FBL but to other snoRNA-independent methyltransferases\(^40,41\). Hence, these data support the notion that 2′-O-Me is a major mechanism through which NPM1 controls cell growth and differentiation.

**Acute deletion of Npm1 in adult mouse hematopoietic stem cells (HSCs) leads to bone marrow failure (BMF).** Previous studies have implicated aberrant ribosome biogenesis and function in the etiology of various hematological diseases and inherited
Npm1 deletion in adult mouse HSCs leads to BMF. a–c. Acute loss of Npm1 in the hematopoietic system leads to the human ribosomopathy phenotype; 7–10 d after plpC injection, dysmegakaryopoiesis (a) and erythroid developmental defects (b) were observed, while 28 d after plpC injection peripheral blood shows dysplastic features (c). a, Representative morphology (n = 20 biologically independent samples) of Npm1-deficient megakaryocytes (panels 2–4) 10 d after plpC injection. b, Staining for TER-119/CD71 positivity (n = 4 biologically independent samples) 10 d after Npm1 deletion. c, Smears of peripheral blood of Npm1loxP/loxPMx1Cre− (panel 1) or Npm1loxP/loxPMx1Cre+ mice (panels 2–9) 4 weeks after plpC administration show images of dysplastic erythroid cells (polychromasia, 2; poikilocytosis, 3 and arrow, 4), dysplastic neutrophils (5 and 6; hypersegmented neutrophils, 7 and 8) and dysplastic platelets (giant platelet, 9). Scale bars, 10 μm. Representative blood smears out of n = 20 biologically independent samples are shown. d, Npm1 deletion in adult HSCs leads to impairment of maintenance of quiescence. Percentage of cells in the G0 phase in Npm1-deleted HSCs (gated on LSK;CD48+;CD150+) 4 d after plpC injection (n = 4 biologically independent samples). e, Relative number (±s.d.) of LSK cells 4 and 21 d after Npm1 deletion, compared with plpC-treated Npm1loxP/loxPMx1Cre− mice (n = 4 biologically independent samples per group). f, Npm1 deletion leads to increased apoptosis of LSK cells 21 d after plpC injection. Data are presented as the mean ± s.d. (n = 4 biologically independent samples per group). g, Results depict mean colony numbers ± s.d. per 500 LSK cells (n = 3 biologically independent samples for each group). h–i, Reconstitution of donor cells in peripheral blood and bone marrow was monitored by staining blood cells with antibodies against CD45.2 (donor) and CD45.1. Data are presented as the mean ± s.d. per 500 LSK cells (3 biologically independent samples for each group). i, Percentage donor-derived cells 21 d after pIpC injection. Data are presented as the mean ± s.d. (3 biologically independent samples for each group).

syndromes, including myelodysplastic syndrome, Diamond–Blackfan anemia and dyskeratosis congenita, hence classifying them as ribosomopathies42–47. These conditions share key features, particularly BMF and cancer susceptibility48,49. In light of the in vitro phenotype observed in NPM1-depleted cells, we aimed to assess the consequences of Npm1 inactivation on adult hematopoiesis in vivo. To this end we generated an Npm1loxP mouse (Supplementary Fig. 5a) and crossed it with Mx1Cre transgenic mice to achieve a hematopoietic-specific conditional knockout of Npm1 (Supplementary Fig. 5b–d).

Seven to ten days after Npm1 deletion, 20 out of 20 Npm1loxP/loxPMx1Cre− mice exhibited dysmegakaryopoiesis (Fig. 3a), defective erythroid maturation (Fig. 3b), dysplastic and low platelet counts (Fig. 3c and Supplementary Fig. 6a) and dysplastic neutrophils (Fig. 3c), which are all features observed in human ribosomopathies50. Flow cytometry analysis of bone marrow cells revealed that acute deletion of Npm1 resulted in exit from quiescence of HSCs/}

hematopoietic progenitor cells (HPCs: Lineage−;Sca1+;c-Kit+, referred to as LSK cells; Supplementary Fig. 6b), as well as of the HSC compartment (CD150−;CD48−; cells; Fig. 3d). Exit from quiescence was cell-autonomous, since it was also observed ex vivo (Supplementary Fig. 6c).

The impact of acute Npm1 loss on proliferation of LSK cells was then determined in vivo by using a competitive bone marrow transplantation assay. When compared to control LSK cells, Npm1-ablated LSK cells showed increased cell cycle entry (Supplementary Fig. 6d). Consistent with this, the number of LSK cells was increased 4 d after Npm1 ablation (Fig. 3e), followed by a significant loss of LSK cells at day 21 (Fig. 3e) due to extensive apoptosis (Fig. 3f).

In vitro Npm1-ablated LSK cells had a decreased capacity to form short-term (Supplementary Fig. 6e) and long-term colonies (Fig. 3g and Supplementary Fig. 6f). To test the repopulating capacity of Npm1-deficient HSCs in vivo we carried out competitive bone marrow transplantation experiments. Within 5 weeks following Npm1
**Fig. 4 | NPM1 germline mutations identified in patients with dyskeratosis congenita.** a. Schematic illustration of NPM1. Presented are the amino acid sequences of the acidic domain (pink) in wild-type NPM1 (upper sequence) and in the two NPM1 mutants, NPM1D178H and NPM1D180del identified in patients with dyskeratosis congenita. b. RNA affinity purification of biotinylated Snord15 in NPM-FLAG-expressing cells. The immunoblot is representative of n = 3 independent experiments. c. FBL immunoprecipitation to evaluate snoRNA enrichment in snoRNPs in CM108 and BV311 cells. Enrichment in CM108 cells was calculated relative to total input and fold enrichment in control BV311 cells. Data are presented as the mean ± s.d. of n = 3 independent experiments. Significance was calculated relative to the enrichment of the non-coding SNORD Ctrl (SNORD13, which was not identified in NPM1 HiTS-CLIP). d. Analysis of specific 2′-O-Me (x axis) in CM108 compared to BV311 cells. Data are presented as the mean ± s.d. of n = 3 independent experiments. 2′-O-Me at 1804 18S rRNA served as a control (Ctrl). e. A representative analysis is shown. Data are presented as the mean ± s.d. of n = 4 independent experiments. FITC, fluorescein isothiocyanate; PI, propidium iodide. f. Reduced IRES activity in CM108 patient cells compared to BV311 Ctrl cells. Data are presented as the mean ± s.d. of n = 3 independent experiments. NS, not significant (one-tailed Student’s t-test). Uncropped immunoblot images are presented in Supplementary Fig. 11.

ablation, Npm1-deficient HSCs were significantly reduced in recipient mice and were eventually outcompeted by wild-type HSCs, confirming BMF and exhaustion (Fig. 3h,i). Thus, we show that Npm1 deletion in adult HSCs leads to BMF. Furthermore, in line with the fact that one of the hallmarks of BMF syndromes is increased susceptibility to cancer51, Npm1 deletion in adult HSCs in conjunction with Trp53 deletion led to the development of an aggressive and fatal form of myelocytic leukemia (Supplementary Fig. 6g–i).
NPM1 germline mutations identified in patients with dyskeratosis congenita. Given the importance of NPM1 for adult murine hematopoiesis, we investigated whether mutations in NPM1 may be associated with other BMF disorders.

Dyskeratosis congenita is an inherited disease characterized by mucocutaneous abnormalities, lung fibrosis, BMF and predisposition to cancer. Dyskeratosis congenita exhibits considerable clinical and genetic heterogeneity. The most common genetic lesion discovered in dyskeratosis congenita is in the X-linked dyskerin pseudouridine synthase 1 (DKC1) gene, the pseudouridine synthase that associates with HACA box snoRNAs. However, in more than 40% of dyskeratosis congenita cases no mutation has been identified.

Mutant DKC1 was shown by others and by us to underlie reduced levels of rRNA pseudouridylation, as well as aberrant translation. Given the shared molecular and cellular features of the NPM1 loss of function described earlier, with those of mutations of DKC1, we wondered whether germline mutations of NPM1 might also cause dyskeratosis congenita. A review of whole-exome sequence datasets of patients with dyskeratosis congenita and dyskeratosis congenita-related conditions (after excluding patients with known dyskeratosis congenita-causing mutations), led us to uncover NPM1 variants in two patients with classic dyskeratosis congenita.

One patient, who presented with severe growth defects at birth, thumb abnormalities and thrombocytopenia, harbored an NPM1 missense mutation (NG_016018.1(NM_001355006.1):c.532G>C) leading to p.D178H substitution. A second patient presented with skin pigmentation abnormalities, nail dystrophy, microcephaly, developmental delay, short stature, skeletal abnormalities in the radius and BMF by the age of 6 and harbored an in-frame deletion (NG_016018.1(NM_001355006.1):c.538_540del (D180del)). Interestingly, both mutations are located in an acidic D/E repeat region that regulates the specificity of NPM1 binding to RNA by its C-terminal basic domain (Fig. 4a).

In view of this, we tested NPM1D180H and NPM1D180del for their capacity to bind snoRNAs and found that it was reduced in both (Fig. 4b and Supplementary Fig. 7a), whereas their ability to interact with FBL was unaltered (Supplementary Fig. 7b). By using skin fibroblasts from the NPM1D180H patient (referred to as CM108) and from an aged-matched healthy control (referred to as BV311), we found that although snoRNAs levels were similar (Supplementary Fig. 7c), binding of FBL to the NPM1-bound snoRNAs was reduced in patient cells (Fig. 4c). Concomitantly, 2'-O-Me levels at the previously identified sites were reduced in dyskeratosis congenita patient cells (Fig. 4d). Since post-transcriptional rRNA modifications affect rRNA secondary structure, they can affect the binding site of ribosome-targeting antibiotics (for example, puromycin and ansomycin), and hence affect sensitivity to antibiotic treatment. Indeed, dyskeratosis congenita patient cells were hyposensitive to antibiotic treatment (Fig. 4e), further indicating that NPM1D178H affects ribosome structure and function. Using the cap/IRES luciferase reporter, we demonstrated a reduction in IRES-dependent activity in patient cells (Fig. 4f), validated by reduced protein levels of XIAP and p27, while NPM1 and FBL levels were unaltered (Supplementary Fig. 7d). Importantly, overexpression of FBL in dyskeratosis congenita patient cells rescued the reduced levels of XIAP and p27 (Fig. 4g). The pS6 immunoprecipitation experiments demonstrated that XIAP and CDKN1B transcripts were indeed depleted from actively translating ribosomes in patient cells (Fig. 4h), while transcript abundance did not change (Supplementary Fig. 7e), which is indicative of translational dysregulation. Global protein synthesis analysis (Supplementary Fig. 7f), mTOR signaling (Supplementary Fig. 7g), rRNA processing analysis (Supplementary Fig. 7h) and polysome fractionation curves (Supplementary Fig. 7i) did not show any significant differences between patient and Ctrl cells, indicating that global translation is not affected. Interestingly, NPM1D178H function was preserved in other respects. NPM1D180H retained nuclear localization (Supplementary Fig. 7j), as well as regulation of p53 (Supplementary Fig. 7k) and of centosome numbers (Supplementary Fig. 7l). Thus, this particular NPM1 mutation specifically compromises its snoRNA binding capacity, leading to altered 2'-O-Me levels and aberrant IRES translation in dyskeratosis congenita patient cells.

Next, we functionally analyzed NPM1D180del. To this end, since patient-derived cells were unavailable, we generated NPM1D180del MEFs by CRISPR-Cas9. NPM1D180del MEFs were similar to NPM1D178H patient cells in all respects: ribosomal profiles (Supplementary Fig. 8a); reduced IRES translation (Supplementary Fig. 8b); and reduced XIAP and p27 protein levels. NPM1 levels were not affected (Supplementary Fig. 8c). Similar to the NPM1D178H mutant, the NPM1D180del mutant also showed reduced loading of snoRNAs onto C/D box snoRNPs (Supplementary Fig. 8d) and defective regulation of 2'-O-Me (Supplementary Fig. 8e).

As a further demonstration that these NPM1 mutations entail loss of function, we found that while wild-type NPM1 fully rescued the NPM1 depletion phenotype in K562 cells (Fig. 2), NPM1D180H or NPM1D180del were unable to do so (Fig. 4i) and Supplementary Fig. 8f). Thus, we demonstrated that NPM1D178H and NPM1D180del, two NPM1...
Heterozygous mutants found in the germline of patients with dyskeratosis congenita, have low snoRNA binding capacity and are dysfunctional in regulating cellular functions such as growth and differentiation.

**NPM1<sup>D180del</sup>** mice show multi-organ features of dyskeratosis congenita. Next we sought to establish a causal link between NPM1 germline mutations and dyskeratosis congenita. To this end we generated a knockin NPM1<sup>D180del</sup> allele using CRISPR–Cas9 (ref. S). NPM1<sup>D180del</sup> heterozygous and homozygous mice were born in Mendelian ratios, had no overt developmental or behavioral abnormalities and appeared healthy. However, at 2 months of age both heterozygous and homozygous mice showed features of aberrant hematopoiesis, which developed into BMF by 6 months of age (Supplementary Fig. 9 and Fig. 5, respectively). Since we found no significant difference in the phenotype of NPM1<sup>D180del</sup> heterozygous and homozygous mice, both mouse genotypes were analyzed together as NPM1<sup>D180del</sup> mice.

At 2 months of age NPM1<sup>D180del</sup> mice demonstrated low numbers of long-term HSCs (Supplementary Fig. 9a), as well as increased megakaryocyte erythroid progenitors (Supplementary Fig. 9b).
and increased erythroblasts (pro-erythroblasts and erythroblasts; Supplementary Fig. 9c). Accordingly, red blood counts and platelets were elevated in the peripheral blood (Supplementary Fig. 9d).

At 6 months of age NPM1\textsuperscript{D180del} mice also displayed extramedullary hematopoiesis, probably as a consequence of BMF. Specifically, in the bone marrow, we observed reduced cellularity (Fig. 5a), as well as reduced long-term HSCs, short-term HSCs and multipotent progenitors (Fig. 5b). Progenitor cell analysis revealed a decrease in common myeloid progenitors and megakaryocyte erythroid progenitors, together with a concomitant increase in granulocyte–monocyte progenitors (Fig. 5c). Flow cytometry analysis of bone marrow cells identified an increase in the myeloid Gr1\textsuperscript{+}CD11b\textsuperscript{+} population in the bone marrow of NPM1\textsuperscript{D180del} mice (Fig. 5d).

These data indicate that the HSC compartment of NPM1\textsuperscript{D180del} mice undergoes exhaustion. To further evaluate this, we carried out in vitro colony assays and serial plating of 6-month-old mouse hematopoietic stem and progenitor cells (HSPCs). Initially, we observed no difference in total colony numbers, but a higher percentage of myeloid (CFU-granulocyte–macrophage) colonies was found in NPM1\textsuperscript{D180del} plates (Fig. 5e). However, second replating identified fewer total colonies in NPM1\textsuperscript{D180del} plates and a lower percentage of burst-forming unit-erythroid colonies (Fig. 5e). On third replating, despite normal growth in control plates, no colonies were observed in the NPM1\textsuperscript{D180del} plates (Fig. 5e). Once again, expression of FBL in NPM1\textsuperscript{D180del} HSPCs rescued colony growth (Fig. 5f) and differentiation (Fig. 5g). Taken together these data further substantiate the exhaustion of NPM1\textsuperscript{D180del} HSPCs and suggest defective 2'-O-Me as the underlying mechanism.

Interestingly, NPM1\textsuperscript{D180del} mice were also found to have enlarged spleens (Fig. 5h). Spleen architecture was abnormal with increased red pulp (Fig. 5i,j), as well as increased erythroid blast populations (Fig. 5k), reflecting extramedullary hematopoiesis probably arising in response to HSPC exhaustion.

Peripheral blood analysis identified abnormal red blood cells and dysplastic neutrophils in a number of NPM1\textsuperscript{D180del} mice (Supplementary Fig. 9e), with one of these mice progressing to an overt myeloid proliferation disorder (Supplementary Fig. 9g–i).

In line with our previous data (Supplementary Fig. 8), bone marrow cells from NPM1\textsuperscript{D180del} mice exhibited aberrant tranlastion, whereby XIAP and p27 levels were reduced (Supplementary Fig. 9j) and their transcripts were depleted from actively translating ribosomes (Fig. 5l). Finally, while snoRNAs levels were comparable between NPM1\textsuperscript{D180del} and Ctrl mice (Supplementary Fig. 9k), 2'-O-Me levels were low in NPM1\textsuperscript{D180del} HSPCs (Fig. 5m).

Consistent with dyskeratosis congenita being a multi-organ syndrome, NPM1\textsuperscript{D180del} mice presented with several nonhematopoietic abnormalities. One-third (4 out of 12) displayed acanthosis, a thickening of the skin that is frequently observed in dyskeratosis congenita (Fig. 5n). Additionally, NPM1\textsuperscript{D180del} males demonstrated reduced testicular size (Fig. 5o) with atrophic seminiferous tubules identified fewer total colonies in NPM1\textsuperscript{D180del} mice (Fig. 5p). Flow cytometry analysis of bone marrow cells identified an increase in the myeloid Gr1\textsuperscript{+}CD11b\textsuperscript{+} population in the bone marrow of NPM1\textsuperscript{D180del} mice (Fig. 5d).

Discussion

By identifying NPM1 mutations in the germline of patients with dyskeratosis congenita and modeling these mutations in the mouse, we suggest that aberrant 2'-O-Me is a pathogenic mechanism. Our findings provide a basis for the speculation that additional RNA or protein components of the 2'-O-Me snoRNP complex may also be targeted for dysregulation in dyskeratosis congenita and possibly in other human disorders.

Ribosomopathies are a heterogeneous group of human disorders, sharing characteristics of BMF and cancer susceptibility. With the ribosome being a ubiquitous and essential cellular engine, it is difficult to understand the mechanism driving the tissue-specific features of these diseases. Two main and noncontradictory views try to explain the pathological specificity of ribosomopathies\textsuperscript{27}. The first argues that ribosome concentration and hence availability is crucial for translation and that mRNAs are differentially translated depending on ribosome availability/concentration\textsuperscript{28,30}. The second view, referred to as the ‘specialized’ ribosome hypothesis, argues that ribosome heterogeneity and diversity in either protein and/or RNA composition can be critical in regulating the protein expression of specific mRNAs within a specific cell type\textsuperscript{28,31–35}. Regardless of the underlying mechanism, it is commonly thought that ribosome dysfunction would be most detrimental in certain specific cells or tissues that require either the expression of specific rate-limiting gene(s) or high protein synthesis rates\textsuperscript{36}.

In this study, we find that ribosome function is regulated by a distinct pattern of 2'-O-Me on discrete rRNA residues, and that alteration to snoRNP-complex-associated proteins can result in ribosome dysfunction. We propose a model where 2'-O-Me levels are differentially regulated by NPM1 to maintain an optimal translational program. We hypothesize that the presence of mutant NPM1 in the FBL methylation complex reduces its functionality (but does not abolish it) by limiting snoRNA loading to snoRNPs, leading to aberrant translation and resulting in dysfunctional HSCs and BMF (Supplementary Fig. 10a,b). Nevertheless, it remains possible that mutant NPM1 also affects other proteins in the snoRNP complexes. Interestingly, heterozygosity for specific NPM1 mutations is sufficient to cause dyskeratosis congenita. Since NPM1 is known to pentamerize\textsuperscript{21}, it is probable that one or more abnormal monomers make the entire pentamer dysfunctional, in line with a dominant-negative mechanism of action (Supplementary Fig. 10a,b).

In addition, we have established that NPM1 function as a 2'-O-Me modulator is impaired in several NPM1-mutated malignancies. It is interesting to note that NPM1\textsuperscript{+} mutation and Npm1 deletion knockout share some similarities; most importantly, both lead to increased HSC proliferation (Fig. 3d–e and ref.\textsuperscript{20}). While in the Npm1 conditional knockout model this brisk HSC expansion is followed by their rapid exhaustion, in the NPM1\textsuperscript{+} model this increased proliferation ultimately leads to leukemia development. Interestingly, however, combining Npm1 conditional knockout with additional genetic lesion(s) (that is, Trp53 deletion) also triggers the development of aggressive leukemia (Supplementary Fig. 6g–i). These findings further support a role for aberrant 2'-O-Me in the development of hematological malignancies at large. In agreement with this notion, it was recently demonstrated that induction of C/D box snoRNA/RNP function by AML1-ETO translocation constitutes an important mechanism toward leukemogenesis\textsuperscript{18}.

Intriguingly, The Cancer Genome Atlas analysis highlights mutations in the acidic repeats of Npm1 as one of the most frequent alterations found in a variety of human cancers (Supplementary Fig. 10c), suggesting that aberrant 2'-O-Me may underlie the development of cancers of various histological origins. Therefore, our findings provide genetic support to the notion that an aberrant epitranscriptome can be pathological and suggest a role for 2'-O-Me in human disease.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41588-019-0502-z.

Received: 23 January 2019; Accepted: 19 August 2019; Published online: 30 September 2019

References

1. Davalos, V., Blanco, S. & Esteller, M. SnapShot: messenger RNA modifications. Cell 174, 498–498.e1 (2018).
32. Holcik, M., Lefebvre, C., Yeh, C., Chow, T. & Korneluk, R. G. A new motif in 3'-UTR RNA that can act as a translational enhancer. Nature 451, 236–239 (2008).
31. Dahiya, S., Marchand, V., Motorin, Y. & Lafontaine, D. L. J. Identification of a new 3'-UTR RNA element that can act as a translational enhancer. Nat. Struct. Mol. Biol. 15, 236–239 (2008).
30. Weingarten-Gabbay, S. et al. Systematic discovery of cap-independent translation enhancers. Nat. Struct. Mol. Biol. 20, 646–650 (2003).
29. Sonenberg, N. & Hinnebusch, A. G. Regulation of translation initiation in eukaryotes. Annu. Rev. Biochem. 86, 1–32 (2017).
28. Mills, E. W. & Green, R. Ribosomopathies: there's strength in numbers. Nat. Rev. Genet. 19, 560–575 (2018).
27. Reschke, M. et al. Characterization and analysis of the composition and dynamics of the mammalian ribosome. RNA 20, 1324–1332 (2014).
26. Ludlow, L. S. et al. Altered translation of GATA1 in Diamond–Blackfan anemia. Nat. Med. 20, 748–753 (2014).
25. Schuler, R. et al. Kinetics of CrPV and HCV IRES-mediated eukaryotic translation using single-molecule fluorescence microscopy. RNA 23, 1626–1635 (2017).
24. Sonenberg, N. & Hinnebusch, A. G. Regulation of translation initiation in eukaryotes: mechanisms and biological targets. Cell 136, 731–745 (2009).
23. Weingarten-Gabbay, S. et al. Systematic discovery of cap-independent translation sequences in human and viral genomes. Science 351, aad4939 (2016).
22. Holcik, M. & Korneluk, R. G. Functional characterization of the X-linked inhibitor of apoptosis (XIAP) internal ribosome entry site element: role of La autoantigen in XIAP translation. Mol. Cell. Biol. 20, 4648–4650 (2000).
21. Holcik, M., Lefebvre, C., Yeh, C., Chow, T. & Korneluk, R. G. A new internal ribosome-entry-site motif potentiates XIAP-mediated cytoprotection. Nat. Cell Biol. 1, 190–192 (1999).
20. Jiang, H., Coleman, J., Miskimins, R., Srivivasan, R. & Miskimins, W. K. Cap-independent translation through the p27 5'UTR. Nucleic Acids Res. 35, 4767–4778 (2007).
19. Natchiar, S. K., Myasnikov, A. G., Kratzat, H., Hazemann, I. & Klaholz, B. P. The epitranscriptome of noncoding RNAs in eukaryotes: mechanisms and biological targets. Nat. Rev. Mol. Cell Biol. 19, 748–753 (2018).
18. Falini, B. et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia and other hematologic malignancies. Blood 115, 312–320 (2010).
17. Krogh, N. et al. Profiling of 2-′O-methyltransferase RNA modifications in the aminoglycoside binding sites of ribosomal RNAs. Nat. Chem. Biol. 9, 227–232 (2013).
16. Grisendi, S., Mecucci, C., Falini, B. & Pandolfi, P. P. Nucleophosmin and its role in leukemia. Cancer Cell 19, 259–262 (2010).
15. Pham, T. K., Bousquet, A., Zhan, J. & Rhoads, S. E. Structural analysis of nucleophosmin/B23–LAD1 interaction and its implications for nuclear transport. J. Biol. Chem. 283, 259–265 (2008).
14. Grisendi, S., Falini, B. & Pandolfi, P. P. The epitranscriptome of noncoding RNAs in eukaryotes: mechanisms and biological targets. Nat. Rev. Mol. Cell Biol. 19, 748–753 (2018).
13. Grisendi, S., Falini, B. & Pandolfi, P. P. The epitranscriptome of noncoding RNAs in eukaryotes: mechanisms and biological targets. Nat. Rev. Mol. Cell Biol. 19, 748–753 (2018).
12. Falini, B. et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia and other hematologic malignancies. Blood 115, 312–320 (2010).
11. Grisendi, S., Mecucci, C., Falini, B. & Pandolfi, P. P. Nucleophosmin and its role in leukemia. Cancer Cell 19, 259–262 (2010).
10. Borer, R. A., Lehner, C. F., Eppenberger, H. M. & Nigg, E. A. Major nucleolar landmarks of genome evolution. Chromosoma 127, 560–575 (2018).
9. Watkins, N. J. & Bohnsack, M. T. The box C/D and H/ACA snoRNPs: key players in the modification, processing and the dynamic folding of ribosomal RNA. Wiley Interdiscip. Rev. RNA 3, 397–414 (2012).
8. Kiss, T. Small nucleolar RNAs: an abundant group of noncoding RNAs with diverse cellular functions. Cell 109, 145–148 (2002).
7. Watkins, N. J. & Bohnsack, M. T. The epitranscriptome of noncoding RNAs in eukaryotes: mechanisms and biological targets. Nat. Rev. Mol. Cell Biol. 19, 748–753 (2018).
6. Kiss, T. Small nucleolar RNAs: an abundant group of noncoding RNAs with diverse cellular functions. Cell 109, 145–148 (2002).
5. Chawla, M., Oliva, R., Bujnicki, J. M. & Cavallo, L. An atlas of RNA base modifications in the human genome. Genome Biol. 13, R76 (2012).
4. Esteller, M. & Pandolfi, P. P. The epitranscriptome of noncoding RNAs in eukaryotes: mechanisms and biological targets. Nat. Rev. Mol. Cell Biol. 19, 748–753 (2018).
3. Frye, M., Harada, B. T., Behm, M. & E. C. RNA modifications modulate gene expression during development. Science 361, 1346–1349 (2018).
2. Safra, M. et al. The mRNA landscape on cytosolic and mitochondrial mRNAs at single-base resolution. Nature 551, 251–255 (2017).
1. Frye, M., Harada, B. T., Behm, M. & E. C. RNA modifications modulate gene expression during development. Science 361, 1346–1349 (2018).
66. Vassiliou, G. S. et al. Mutant nucleophosmin and cooperating pathways drive leukemia initiation and progression in mice. Nat. Genet. 43, 470–475 (2011).

Acknowledgements
D.N. was supported by an EMBO long-term fellowship (no. EMBO-LTF498-2014). K.I. was supported by National Institutes of Health grants (no. R01DK98263, R01DK115577 and R01HL148852), and is a Scholar of The Leukemia and Lymphoma Society. A.H.B. was supported by the Damon Runyon Cancer Research Foundation (no. 2142-12). This work was supported in part by the European Research Council Consolidator (grant no. 311660) and Cancéropole Ile-de-France (no. 2011-1-LABEL-1-AXE2-UP7-3) to J.S.; Medical Research Council (grant no. MR/P018440/1) and Bloodwise (grant no. 14032) to I.D.; the Fondazione AIRC per la Ricerca sul Cancro IG 2016 (grant no. 18568) and the European Research Council Advanced Grant 2016 (no. 740230) to B.F.; Medical Research Council (grant no. MR/PO18440/1) and Bloodwise (grant no. 14032) to I.D.; the Fondazione AIRC per la Ricerca sul Cancro IG 2016 (grant no. 18568) and the European Research Council Advanced Grant 2016 (no. 740230) to B.F.; and by an Outstanding Investigator Award R35 (grant no. CA197529) and the SHINE grant (no. 5R01DK115536) awarded by National Institutes of Health to P.P.

Author contributions
D.N., J.G.C. and P.P. designed the experiments and discussed the data. D.N., J.G.C., L.L. and P.P. wrote the manuscript. D.N. designed and performed the biochemical and translation-related experiments and analyzed the data. D.N. generated the NPM\textsuperscript{D180del} mouse model, and designed and executed the experiments. S.G. generated the Npm1 conditional knockout mouse model. K.I., S.G. and P.S. performed the conditional Npm1 knockout experiments. A.H.B., A.M. and R.B.D. designed and performed the HiTS-CLIP experiments. D.B. and E.M. performed the computational analysis of the HiTS-CLIP data. Y.Z. designed and performed the northern blot experiments. A.C.B. provided critical reagents. A.G. and C.A.M. provided animal technical assistance. M.P.M. and B.F. provided the human AML samples. K.C. and I.D. performed and analyzed the microarray experiments and data. L.L., L.M.M. and O.P. performed the pathology analyses of the hematopoietic features of the NPM1 mutants. T.J.V., I.D., O.B. and J.S. provided and analyzed the dyskeratosis congenita exome dataset. M.S. and S.S. performed and analyzed the PSI-seq experiments and data.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41588-019-0502-z.

Correspondence and requests for materials should be addressed to P.P.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature America, Inc. 2019
Methods

NPMT1-HITS-CLIP. Ultraviolet cross-linking and cell lysis. Npmt1−/−Trp53−/− MEFs or Npmt1−/−Trp53−/− MEFs were grown to 80% confluency in DMEM, 10% FCS, 5% penicillin-streptomycin. Before ultraviolet cross-linking, the medium was aspirated and dishes containing cells were placed on ice. Ultraviolet cross-linking was performed in a hybridization oven at a setting of 400 mJ cm−2, followed by an additional 200 mJ cm−2 after 1 min of recovery. Cross-linked cells were washed once with ice-cold PBS, collected and pelleted for 5 min at 4 °C. PBS was aspirated and cells were snap-frozen on dry ice and stored in −80 °C until further processing. Cross-linked cells were lysed in 1× PXL buffer (1× PXL, 0.1% SDS, 0.5% deoxycholate (DOC), 0.5% NP-40) for 5 min on ice, followed by the addition of RQI DNase, which was incubated at 37 °C for 10 min. To partially digest the cross-linked RNAs, RNase A was added to the lysates at a 1:10,000 dilution and incubated at 37 °C for 5 min. (A titration experiment was performed beforehand to determine the optimal concentration.) Next, the lysates were spun at 14,000 r.p.m. in a benchtop centrifuge for 20 min at 4 °C.

Immunoprecipitation and calf intestinal phosphatase treatment. The NPMT1 antibody (Sigma-Aldrich, clone FCB2291) was coupled to Dynabeads Protein G for Immunoprecipitation (cat. no. 1003) according to the manufacturer’s protocol. After coupling the NPMT1 antibody to the magnetic beads, the supernatant of the spun lysate was added to the beads and incubated at 4 °C for 2 h. After immunoprecipitation, the magnetic beads were washed twice for 5 min each in 5× wash buffer (5× PXL: 5× PBS, 0.1% SDS, 0.5% DOC, 0.5% NP-40; polycationic phosphate (PKN) buffer: 50 mM Tris-Cl, pH 7.4, 10 mM MgCl2, 0.5% NP-40). Next, the RNA pellet was resuspended in 10 μl 0.5% calf intestinal phosphatase phosphate (cat. no. EP0651) according to the manufacturer’s instructions for 20 min at 37 °C, followed by several washing steps: once in PKN buffer; once in PKN buffer + 20 mM EDTA; and twice in PKN buffer.

Linker ligation. The RNA linker (SRA3) was obtained from Dharmacol and ligated using T4 RNA ligase 1 (Fermentas) according to the manufacturer’s protocol at 16 °C overnight, followed by three washes in PKN buffer.

Phosphorylation of RNA tags. Next, RNA tags were radioactively labeled with 32P-γ-ATP (3,000 Ci mmol−1) and T4 PNK (New England Biolabs) according to the manufacturer’s protocol for 20 min at 37 °C, followed by three washes in PKN buffer.

SDS–polyacrylamide gel electrophoresis (PAGE) and transfer to nitrocellulose membrane. Beads with radioactively labeled RNAs were eluted with 60 μl elution buffer (30 μl 1X PKN + 30 μl lithium dodecyl sulfate loading buffer) and eluates were incubated at 70 °C for 10 min, followed by SDS–PAGE on a 10% Bis-Tris Novex gel and transfer to a nitrocellulose membrane at 30 V for 2 h. Next, the membrane was washed in 1X PBS and exposed on film for 15 min.

RNA isolation from nitrocellulose membrane. RNA bands were excised from the nitrocellulose membrane approximately 20 kDa above the expected molecular weight of the protein. The slice was cut into very small pieces and put into Eppendorf tubes. Then, 200 μl of proteinase K solution (4 μg ml−1 protein kinase stock was diluted 1:5 in 1X proteinase K buffer: 100 mM Tris-Cl, pH 7.5, 50 mM NaCl, 10 mM EDTA) was added to the membrane pieces and incubated for 20 min at 37 °C. Next, 200 μl of proteinase K buffer with 7 M urea was added and incubated for 20 min at 37 °C, followed by adding 400 μl RNA phenol + 130 μl chloroform + isomyl alcohol, which was incubated in the thermomixer at 37 °C at 1,000 r.p.m. for 20 min, followed by RNA extraction by ethanol/isopropanol precipitation in the presence of glycogen.

Reverse transcription reaction. Beads for 5-bromo-2′-deoxyuridine (BrdU) immunoprecipitation were prepared as follows. Protein G beads: three washes with antibody-binding buffer (composition as in the manufacturer’s protocol), followed by adding 20 μl of 1X antibody-binding buffer, 5 μl of 50X Denhardt’s solution and 25 μl (5 μg) of anti-BrdU antibody (catalog no. sc-33233; Santa Cruz Biotechnology). Rotation at room temperature for at least 45 min, followed by 3 washes in 1X immunoprecipitation buffer (10X saline–sodium phosphate–EDTA (SSPE), 1 mM EDTA, 0.05% Tween-20). In the meantime, the RNA pellet was resuspended in water, denatured at 65 °C for 5 min (in a microfuge tube) and put on ice. The reverse transcription reaction was performed using Superscript II (Invitrogen) according to the manufacturer’s protocol, in the presence of 1 μl 5-bromo-2′- deoxyuridine 5′-triphosphate sodium salt/reaction (8.2 mM; catalog no. B0631; Sigma-Aldrich) and 1 μl of an RNA linker reverse complement containing the reverse transcription primer (25 μM); Cycling conditions: 45 min at 50 °C, 15 min at 55 °C, 5 min at 85 °C, 4 °C hold. Primers used in this experiment also contained an index for identifying a barcode to identify PCR artifacts. (Please see Supplementary Table 2 for the reverse transcription protocols.) Following the reverse transcription reaction, 1 μl RNase H (2 μl−1; catalog no. 18021-071; Invitrogen) was added and incubated for 20 min at 37 °C and spun through a G-25 column according to the manufacturer’s instructions. Next, the volume of the flowthrough was measured and water up to 40 μl was added, followed by 10 μl 50X Denhardt’s solution (catalog no. D2352; Sigma-Aldrich) and 50 μl 2X immunoprecipitation buffer (0.6X SSPE, 2mM EDTA, 0.1% Tween-20). The mix was incubated for 5 min at 70 °C, and 2 min at 25 °C; it was then added to the prepared tube of BrdU-coated beads. Immunoprecipitation was performed for 30 min at room temperature, followed by the following washes: once with 1X immunoprecipitation buffer with 5X Denhardt’s solution, twice with Nuclear Low Salt Buffer (15 mM Tris, pH 7.5, 5 mM EDTA, 1X Denhardt’s solution), twice with Nuclear Stingent Buffer (15 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2.5 mM EGTA, 1% Triton X-100, 1% sodium DOC, 0.1% SDS, 120 μM NaCl, 25 mM KCl 1X Denhardt’s solution) and twice with 1X immunoprecipitation buffer. To elute the immunoprecipitated complementary DNA, 50 μl of 100 μM BrdU (catalog no. B5002; Sigma-Aldrich) was added in 1X immunoprecipitation buffer and incubated at room temperature for 30 min. The eluate was collected using a magnet and spun through a G-25 column according to the manufacturer’s instructions. Afterwards, the volume was measured and water was added up to 79.5 μl, in addition to 35 μl 37.5 mM 32P-γ-ATP (1.2X SSPE, 4 mM EDTA, 0.2% Tween-20) and 15 μl 50X Denhardt’s solution.

Circularization, relinearization, PCR amplification and HiTS. Next, cDNA was purified using Agencourt AMPure XP beads (Beckman Coulter Life Sciences) according to the manufacturer’s instructions.

HITS-CLIP computational analysis. The CLIP reads had a 4-nucleotide sample multiplexing index and an 8-nucleotide random barcode at the 5′ end. The libraries generated with the BrdU-CLIP adapters were first filtered according to their quality scores. We required a minimum average quality score of 20 for the read. Since Npmt1 is known to bind to tRNA and tRNA would confound the alignment due to its repetitive nature in the genome, we computationally subtracted all sequences that aligned to the SS, 3′SS, 18S and 28S rRNAs. The remaining read sequences (actual RNA tags without index and barcodes) were mapped to the reference genomes (mm9) with the NovoAlign v.3.01.00 program (http://www.novocraft. com/products/novosAlign/) with no more than two mismatches (substitutions, insertions or deletions) allowed per read. PCR duplicates while retaining genuinely unique reads representing independent events. Next, we calculated peaks, which were defined as regions that contain overlapping reads. Peak intensity refers to the number of independent reads that falls within the boundary of the peak. Statistical significance was calculated using the program Piranha v.2.7.0 as described previously.

PSI-seq. To analyze the pseudouridine levels within the rRNA, PSI-seq was performed, essentially as described in Schwarz et al. 3, on total RNA extracted, in triplicates, from either wild-type or Npm1 knockout MEF cells. δ values, capturing the ratio between the number of reads beginning at a position (and indicative of read recruitment) versus the number of reads overlapping that position were calculated for each of the annotated sites in rRNA, obtained from the MODOMICS database. 4 The mean values, for each position, obtained in Npm1−/− versus Npm1+/+ samples are plotted.

RNA motif scanning. We identified all unique peaks among the bona fide peak positions. As part of this peak, we marked only peaks that overlapped by at least 25 base pairs, by using an in-house Perl script. Genomic regions were initially scanned for de novo motif discovery using Homer v.4.10 suite 5 using the hypergeometric distribution, searching for 6–12 nucleotide-long motifs. The resulting significant motifs were further collapsed together by comparing the Euclidean distances of the position weight matrices.

CRISPR-mediated generation of Npmt1−/− mice and MEFs. Mice were generated as described in Quadros et al. 6. Injections to C57BL/6 mice were performed at the Beth Israel Deaconess Transgenic Core Facility. The single-stranded oligodeoxynucleotide for homologous recombination was purchased from...
IDT. For genotyping, a Dral restriction site was inserted into the single-stranded oligo-deoxyribonucleotide by mutating the sequence TTTCAAA to TTATTA. Cas9 for injection, as well as the single guide RNAs, were purchased from PNA Bio. To generate the NPM1*Δ178H/Acry CRISPR MEFs, all of the reagents were transfected to Tpr53−/− MEFs and single colonies were screened with the Dral restriction enzyme for positive clones.

Additional mice. Npm1*Δ178H/Acry mice were crossed with Mx1Cre transgenic mice (purchased from The Jackson Laboratory) and deletion of exon 1–6 of the Iox1 Npm1 allele was detected by PCR. C57BL/6 mice (B6 CD45.2) and C57BL/6 mice congenic for the CD45 locus (B6 CD45.1) were purchased from The Jackson Laboratory. To induce the Mx1 promoter, polyinosine-polycytidylate (pIpC; Sigma-Aldrich) was resuspended in Dulbecco’s PBS at 2 mg ml−1 and passed through a 0.22-μm filter. Mice received 25 μg of pIpC per gram of body mass every other day for 2 weeks.

Bone marrow isolation. Long bones from 8-week-old mice were collected and crushed. The isolated cells were treated with ACK lysing buffer for 2 min, washed and then subjected to further analysis. Animal experiments were performed in accordance with the guidelines of the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Flow cytometry and antibodies. For the flow cytometry analyses, we used monoclonal antibodies specific for the following: CD41 (clone eBioMWRag30); CD34 (clone RAM34); c-Kit (clone 2B8); Sca-1 (clone D7); Flt-3 (clone Avas12a1); CD45 (clone RAM11); CD11b (clone M1/70); IgM (clone II/41); CD19 (clone eBioI3D3); and NK-1.1 (clone PK136); all were sourced from eBioscience. Anti-CD130 (clone TC1S12F12.2) and anti-CD48 (HM48-1) antibodies were sourced from BioLegend. We used a mixture of monoclonal antibodies against CD4, CD8, CD3e, B220, CD19, CD45, NK-1.1 as a lineage marker (Lineage). Pyronin Y staining was performed as follows: cells were stained with 10 μg ml−1 of Hoechst 33342 (Sigma-Aldrich) in Hoechst staining buffer (Hank’s Balanced Salt Solution + 3% FCS + 10 mM HEPES) at 37 °C for 45 min. Then pyronin Y (1 μg ml−1; Sigma-Aldrich) was added to the buffer and the cells were further incubated for 45 min. For additional immunolabeling, the antibodies were added to the Hoechst staining buffer supplemented with Hoechst/pyronin Y. Apoptosis was measured by flow cytometry using an APC Annexin V Apoptosis Detection Kit (BioLegend). Cells were gated and only double-positive cells were regarded as apoptotic cells. The anti-NPM1 antibody used in the HiTS-CLIP assay and immunoblotting was purchased from Sigma-Aldrich (clone FC8291). Also, anti-FBL (clone H-140, for immunoblotting), anti-p53 (clone DO-1) and anti-p21 were purchased from Santa Cruz Biotechnology. Anti-γ tubulin (clone GTU-88), anti-FLAG M2 (clone F1804), anti-GAPDH (clone G9545) and anti-RPL22 (clone SC-10696) were purchased from Sigma-Aldrich; anti-p56 and anti-XIAP (clone B9E) were purchased from CST. We also used anti-FBL (catalog no. ab3821, in the immuno precipitation and immunofluorescence experiments; Abcam) and anti-p27 (catalog no. 610242; BD Transduction Laboratories).

Long-term cultures and colony-forming assays. For long-term cultures, we cocultured 105 LSK cells with irradiated OP9 cells in Medium Essential Medium, α modification (Sigma-Aldrich) containing 10% FCS and 1% antibiotic/antimycotic. At 4 weeks, we performed competitive reconstitution assays. LSK cells were sorted, treated with 1 μg ml−1 of Hoechst 33342 (Sigma-Aldrich) in Hoechst staining buffer supplemented with Hoechst/pyronin Y. Apoptosis was measured by flow cytometry using an APC Annexin V Apoptosis Detection Kit (BioLegend). Cells were gated and only double-positive cells were regarded as apoptotic cells.

Competitive reconstitution assay. We sorted 1.5 × 107 LSK cells from Npm1*Δ178H/Acry, Mx1Cre+ or Mx1Cre− mice (CD45.2), and transplanted cells into lethally irradiated CD45.1 congenic mice in competition with bone marrow mononuclear cells from CD45.1 mice. Reconstitution of donor (CD45.2) cells was monitored by staining blood cells with antibodies against CD45.2 and CD45.1. For inducible Npm1 inactivation in the trisomy bone-reconstituting assay, 15 μg ml−1 of engraftment of the donor-derived cells in recipient mice 8 weeks after bone marrow transplantation, we subsequently treated recipient mice with pIpC (25 μg of pIpC per gram of body mass every other day for 2 weeks).

Nuclear extract for immunoprecipitation. Cells were washed with PBS, dislodged and pelleted by centrifugation and resuspended in the cell lysis buffer: 10 mM HEPES, pH 7.5, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5% Nonidet-P40 with protease inhibitor cocktail (Sigma-Aldrich) and allowed to swell on ice for 15–30 min with intermittent mixing. Tubes were vortexed to disrupt cell membranes and then centrifuged at 12,000g at 4 °C for 15 min. The pelleted nuclei were washed three times with the cell lysis buffer and resuspended in the nuclear extraction buffer containing 20 mM HEPES (pH 7.5), 300 mM NaCl, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail, incubated on ice for 30 min. Nuclear extract was collected by centrifugation at 12,000g at 15 min at 4 °C. Protein concentration of the nuclear extract was estimated using the Bradford reagent (Bio-Rad Laboratories).

Protein extraction. Cells were lysed with lysis buffer (50 mM Tris, pH 7.5, 10% glycerol, 5 mM MgCl2, 150 mM NaCl, 0.2% NP-40) supplemented with complete protease inhibition cocktail (Roche) and phosphatase inhibition cocktail (Roche). Following lysis, protein concentrations were measured with BCA protein assay (ThermoFisher). For the expression of mouse snoRNAs, we used the Direct-zol RNA MiniPrep Plus Kit (Zymo Research). On-column Dnase treatment was performed for all samples. To isolate RNA after IP, standard TRIzol (Ambion) isolation was performed according to the manufacturer’s protocol.

Collection of human samples. Written informed consent for the analysis of bone marrow/peripheral blood samples was obtained from each patient. Approval from the local ethics committees was obtained at Perugia Hospital, Institute of Hematology-Centro di Ricerca Emato-Oncologiche, University of Perugia.

Polysome fractionation. For polysome preparation, cells were then incubated with cycloheximide (CHX) at a final concentration of 100 μg ml−1 for 15 min. Plates were then washed with ice-cold PBS containing 100 μg ml−1 CHX, scraped and collected in ice-cold PBS/CHX. Cells were pelleted by centrifugation and subsequently lysed in polysome lysis buffer (20 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 150 mM NaCl, 1% Triton X-100, 1% DOC, 2.5 mM DTT, 0.5 mM EDTA, 100 μg ml−1 RNasin, 100 μg ml−1 CHX, 0.01% protease inhibitor cocktail (Roche), protease inhibitor set without EDTA (G-Biosciences), π1-antitrypsin (EMD Biosciences)) and incubated on ice for 10 min with occasional mixing. Lysates were centrifuged at 7,000,000 r.p.m. for 5 min at 4 °C and the supernatant was carefully removed. Protein concentrations for lysates were measured by Bradford assay and equal amounts of protein were loaded on a 15–50% sucrose gradient containing 100 mM CHX, 0.2 mg ml−1 heparin and 1 mM DTT. Gradients were centrifuged at 36,000 r.p.m. for 3 h at 4°C in a SW 40 rotor (Beckman Coulter Life Sciences) and subsequently fractionated using a Foxy Jr. fraction collector (ISCO). Polysome profiles were reordered using a UA-6 absorbance detector connected to the fraction collector and measuring absorbance at 254 nm. RNA was subsequently extracted from each fraction, by using TRizol and the RNeasy Mini Kit (QIAGEN).

Affymetrix GeneChip and analysis. Polysome and total RNA of Npm1−/− and Npm1+/+ MEFs was extracted as described in the Polysome fractionation section. The extracted RNA was then subjected to analysis via transcriptome analysis, using the Affymetrix Mouse Genome 430 2.0 Array. Double-stranded cDNA was synthesized from total or polysomal RNA (1–10 μg). Linear amplification with T7 RNA Polymerase (Ambion) and biotin labeling (ENZO) were performed by in vitro transcription according to the manufacturer’s instructions. The resulting biotin-labeled complementary RNA was fragmented and hybridized to the Affymetrix Mouse Genome 430 2.0 Array oligonucleotide 2.488 gene microarray chip for 16 h at 45 °C. Following hybridization, the probe array was washed and stained on a Fluidics Station and immediately scanned on a Hewlett Packard GeneArray Scanner. Raw microarray data were imported into R and subjected to robust multi-array analysis using the affy package (v.1.56.0). Using the probe annotation information downloaded from the product website, robust multi-array analysis-transformed probe intensities corresponding to each RefSeq database transcript ID were aggregated by arithmetic mean. The log2(fold changes) in polysome-associated RNA intensity were calculated between the heterozygous and wild-type samples by taking the difference of the robust multi-array analysis-transformed probe intensities and then normalize it by subtracting these values with the
corresponding log₂(fold changes) of total RNA intensities. Pathway analysis was performed using the fgsea package (v.1.4.1) and KEGG pathway annotations 

Dual luciferase assay. Cells were seeded at 80% confluence; after 24 h, 250 ng per well of the cap/IRES reporter were transfected using Lipofectamine 2000 (Thermo Fisher Scientific). The ratio of IRES-dependent translation (Firefly) was calculated relative to cap-dependent translation (Renilla) normalized to control cells. The reporter used contained a Renilla luciferase under the cytomegalovirus promoter, followed by a hepatitis C virus IRES element that drives the expression of the Firefly reporter. To clone the 5′-UTRs of ACTB, CDKN1B and XIAP, hepatitis C virus IRES was excised using the BamHI and EcoRI restriction enzymes and inserts were cloned via the Gibson Assembly Master Mix (NEB), according to the manufacturer’s instructions.

Immunofluorescence. Cells were fixed with 4% paraformaldehyde for 10 min, washed with PBS and permeabilized with PBS and Triton X-100 0.2% for 10 min. Blocking before antibodies was performed in PBS, Triton X-100 0.2% and 10% goat serum for 30 min. Primary antibodies were incubated overnight in blocking buffer and secondary antibodies were diluted 1:500 (Jackson ImmunolResearch) and incubated for 2 h at room temperature. DAPI was used for nuclear staining.

2′-O-Me detection. Measurement of 2′-O-Me levels was carried out via the RTL-P method as described by Dong et al. with the following modifications. To detect the 2′-O-Me sites in the target RNAs, reverse transcription PCR was performed in a 20-µl reaction mixture containing 25–50 ng of RNA, 50 pmol of reverse transcription primers and a low (0.5µM) or high (1 µM) concentration of deoxyribonucleoside-5-triphosphates. The primer/RNA mixture was denatured at 70 °C for 5 min and then chilled on ice. Following an initial annealing step at 42 °C for 10 min, 200 µl of Moloney Murine Leukemia Virus Reverse Transcriptionase (Invitrogen) and 0.3 U RNaseOUT (Invitrogen) were added. The reaction was incubated at 37 °C for 1 h and then heated at 75 °C for 15 min to deactivate the reverse transcriptase.

Hemin treatment. Cells were cultured with 30 µM Hemin (catalog no. 51280; Sigma-Aldrich) for 72 h. Cell were collected, washed once with PBS and resuspended in a ratio of 1:1 with benzidine solution (1.6 mol acetic acid, 485.4 ml H2O, 1 g benzidine dihydrochloride) and a final concentration of 0.1% of hydrogen peroxide.

Colony formation assay. A total of 1,500 cells were plated in MethoCult H4034 optimum medium in 35 mm Petri dishes (Falcon) in duplicate. Plates were incubated at 37 °C with 5% CO2 and 95% humidity for 7 days. Colonies were scored using an inverted microscope. To visualize the colonies, MTT reagent (Thermo Fisher Scientific) was added and incubated at 37 °C for 1 h and then heated at 75 °C for 15 min to deactivate the reverse transcriptase.

SnoRNA quantitative PCR detection. Total RNA was isolated by TRI reagent (Invitrogen). The poly(A) tail-length reaction (Thermo Fisher Scientific) was performed using 250 ng of total RNA. Reverse transcription (Moloney Murine Leukemia Virus Reverse Transcriptase; Invitrogen) was performed using an adapter primer following the manufacturer’s instructions. SnoRNA detection was performed using a mix of a snoRNA-specific primer and an abridged reverse transcription primer.

IRES GSEA. We determined the set of genes tested by Weingarten-Gabby et al., for enrichment in our polysome enrichment dataset. Genes from this set were selected first based on enhanced GFP levels above background (NaN), resulting in 3,694 putative IRES genes. This gene set included XIAP and CDKN1B, well-annotated IRES genes; more stringent filtering resulted in the loss of these positive-control genes. We used this set of genes for GSEA with the fgsea package in R.

Whole-exome sequencing (WES) analysis. WES, carried out by J.S. (NPM1D178H mutation), was performed on DNA from cultured skin fibroblast cells as described in Bluteau et al. (including an extensive description of the analysis pipeline).

Statistical analysis. Data are presented as the mean ± s.d. unless otherwise specified. At least three independent repetitions were performed for all experiments. A Student’s t-test was used to determine statistical significance unless otherwise specified.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Raw and preprocessed sequencing and microarray data may be accessed from the Gene Expression Omnibus with accession number GSE135726. Since the informed consent obtained from dyskeratosis congenita patients does not allow for public deposition of the data, the WES data from patient CM108 (NPM1D178H) and healthy controls can be communicated upon reasonable request to I.D. and T.J.V.

References

67. Uren, P. J. et al. Site identification in high-throughput RNA-protein interaction data. Bioinformatics 28, 3013–3020 (2012).
68. Machnicka, M. A. et al. MODOMICS: a database of RNA modification pathways—2013 update. Nucleic Acids Res. 41, D262–D267 (2013).
69. Heinz, S. et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol. Cell 38, 576–589 (2010).
70. Arai, F. et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 118, 149–161 (2004).
71. Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y. & Morishima, K. KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res. 45, D353–D361 (2017).
72. Bluteau, O. et al. A landscape of germ line mutations in a cohort of inherited bone marrow failure patients. Blood 131, 717–732 (2018).
73. Pontikos, N. et al. Phenolxen: an open platform for harmonization and analysis of genetic and phenotypic data. Bioinformatics 33, 2421–2423 (2017).
Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

| Item                                                                 | Status |
|----------------------------------------------------------------------|--------|
| The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement | Confirmed |
| A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly | Confirmed |
| The statistical test(s) used AND whether they are one- or two-sided | Confirmed |
| A description of all covariates tested | Confirmed |
| A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons | Confirmed |
| A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) | Confirmed |
| For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted | Confirmed |
| Give P values as exact values whenever suitable. | Confirmed |
| For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings | Confirmed |
| For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes | Confirmed |
| Estimates of effect sizes (e.g. Cohen's d, Pearson’s r), indicating how they were calculated | Confirmed |

Software and code

Policy information about availability of computer code

**Data collection**

- Hewlett Packard GeneArray ScannerR

**Data analysis**

- Homer (version 4.10)
- Prism (GraphPad, version 7.0)
- BD FACSDiva (version 8.0.1)
- FlowJo (version 10)
- Novoalign program (version V3.01.00)
- R (version 3.5.0)
- MODOMICS (2017 update)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Raw and preprocessed sequencing and microarray data may be accessed from the Gene Expression Omnibus with accession number GSE135726. As the informed consent obtained from DC patients does not allow for public deposition of the data, WES data from patient CM108 (NPMD178H) and healthy controls can be communicated upon reasonable request to J.S. and WES data from patient harboring NPM1D180del mutation can be communicated upon reasonable request to
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [x] Life sciences
- [ ] Behavioural & social sciences
- [ ] Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample sizes were chosen to reliably measure experimental parameters while keeping with standards in the relevant fields, and remaining in compliance with ethical guidelines to minimize the number of animals used. |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | Ribosomal RNAs were excluded for some HITS-CLIP analyses.                                                                                                                                  |
| Replication | Sample sizes were chosen to reliably measure experimental parameters while keeping with standards in the relevant fields, and remaining in compliance with ethical guidelines to minimize the number of animals used. The number and type of repeats for each experiment is described in the corresponding figure legend. |
| Randomization | Randomization was used with respect to time point of data collection. Animal subjects were not randomly allocated to experimental groups as all comparisons were between known genotypes. |
| Blinding | Experiments did not involve blinding, but randomization was used with respect to time point of data collection. Animal subjects were not randomly allocated to experimental groups as all comparisons were between known genotypes. |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | n/a | Involved in the study |
|----------------------------------|-----|-----------------------|
| Antibodies                       | [x] |                       |
| Eukaryotic cell lines            | [x] |                       |
| Palaeontology                    | [ ] |                       |
| Animals and other organisms      | [x] |                       |
| Human research participants      | [x] |                       |
| Clinical data                    | [x] |                       |

| Methods                          | n/a | Involved in the study |
|----------------------------------|-----|-----------------------|
| ChIP-seq                         | [x] |                       |
| Flow cytometry                   | [x] |                       |
| MRI-based neuroimaging           | [x] |                       |

Antibodies

| Antibodies used | CD41 (eBioMWRag30, ebioscience), Flt-3 (Avas12a1, ebioscience), CD34 (RAM34, ebioscience), c-Kit (2B8, ebioscience), Sca-1 (E13-161.7, ebioscience), CD3e (145-2C11, ebioscience), CD4 (L3T4, ebioscience), CD8 (53-6.72, ebioscience), B220 (RA3-6B2, ebioscience), TER-119 (TER-119, ebioscience), Gr-1 (RB6-8C5, ebioscience), CD11b (M1/70, ebioscience), IgM (114/41, ebioscience), CD19 (eBio1D3, ebioscience), NK-1.1 (PK136, ebioscience), anti-CD150 (TC15-12F12.2, BioLegend), CD48 (HM48-1, BioLegend), anti-NPM1 (FC82291, Sigma Aldrich for HITS-CLIP and WB), anti-FBL (H-140, SCBT, for WB), anti-p53 (DO-1, SCBT), anti-p21 (SCBT), Anti-gamma tubulin (GTU-88, Sigma-Aldrich), anti-FLAG M2 (F1804, Sigma-Aldrich), anti-GAPDH (G9545, Sigma-Aldrich), anti-RPL22 (SAB2107796, Sigma-Aldrich), anti-pS6 (S2211, CST), anti-XIAP (3B6, CST) anti-FBL (ab5821, Abcam was used in IP and IF experiments), anti-p27 (610242, BD transduction laboratories). In FACS analyses antibodies were diluted 1:100, for WB experiments antibodies were diluted 1:1000. |

Validation

All antibodies are validated for detecting mouse or human proteins by the manufacturer. Antibodies were confirmed, in house, for each specific application using cells of known origin and differentiation state and compared to isotype controls and cells that are known to express or lack the antigen.
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  Cell lines were obtained from the ATCC. The K562, OCI-AML2 and OCI-AML3 cell lines were used

Authentication  none of the cell lines have been authenticated in house

Mycoplasma contamination  All cell lines were checked bi-weekly for mycoplasma by MycoAlert Mycoplasma Detection Kit (Lonza) and were found negative for mycoplasma

Commonly misidentified lines (See ICLAC register)  None of the cell lines belongs to the ICLAC registry

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals  The following murine models were used in this study: transgenic Mx1Cre, conditional Npm1 flox mice generated in house and NPM1D180del generated in house. All murine models are of B6 genetic background. For conditional Npm1 KO mice age 8 weeks were used. In experiments describing NPM1D180del findings mice aged 2 months and 6 months were used.

Wild animals  The study did not involve wild animals

Field-collected samples  The study did not involve field-collected samples

Ethics oversight  Animal experiments were performed in accordance with the guidelines of the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☑ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☑ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☑ All plots are contour plots with outliers or pseudocolor plots.

☑ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation  Long bones from 8 weeks old mice were collected and crashed. The isolated cells were treated with ACK for 2 minutes, washed, and then subjected to further analysis. FACS staining were performed in PBS supplemented with 2%FBS.

Instrument  BD Fortessa HTS

Software  BD FACSDiva (version 8.0.1) and FlowJo (v10)

Cell population abundance  100% of the post-sort fraction were the relevant Lineage-;Sca1+;cKit+ cells. For FACS analyses (not sorting) the abundance is stated in the figure.

Gating strategy  For sorting of LSK cells - Cell were gated on singlets, then viable cells, then Lineage negative population (by an antibody cocktail) and finally the Sca1;cKit double positive population was sorted. For additional gating strategies please see the Methods section.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.