

Telomerase RNA recruits RNA polymerase II to target gene promoters to enhance myelopoiesis

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Dyskeratosis congenita (DC) is a rare inherited bone marrow failure and cancer predisposition syndrome caused by mutations in telomerase or telomeric proteins. Here, we report that zebrafish telomerase RNA (*terc*) binds to specific DNA sequences of master myeloid genes and controls their expression by recruiting RNA Polymerase II (Pol II). Zebrafish *terc* harboring the CR4-CR5 domain mutation found in DC patients hardly interacted with Pol II and failed to regulate myeloid gene expression *in vivo* and to increase their transcription rates *in vitro*. Similarly, *TERC* regulated myeloid gene expression and Pol II promoter occupancy in human myeloid progenitor cells. Strikingly, induced pluripotent stem cells derived from DC patients with a *TERC* mutation in the CR4-CR5 domain showed impaired myelopoiesis, while those with mutated telomerase catalytic subunit differentiated normally. Our findings show that *TERC* acts as a transcription factor, revealing a target for therapeutic intervention in DC patients.

dyskeratosis congenita | myelopoiesis | telomerase RNA component | zebrafish

Telomerase is an RNA-dependent DNA polymerase that synthesizes telomeric repeats at the end of eukaryotic chromosomes (1). This enzyme complex consists of a catalytic protein with a telomere-specific reverse transcriptase activity, telomerase catalytic subunit (TERT), a long noncoding RNA (lncRNA), telomerase RNA component (*TERC*), that functions as a template for the synthesis of telomeric repeats, and several associated proteins (2). Telomerase is essential for maintaining pools of proliferating cells in adulthood, including hematopoietic stem cells (HSCs) (3). Telomerase complex mutations have been associated with several human diseases, such as cancer and aging, as well as to some rare disorders, such as dyskeratosis congenita (DC) (4, 5) and idiopathic pulmonary fibrosis (6).

DC is a rare inherited bone marrow (BM) failure and cancer predisposition syndrome (7). DC patients have defects in telomere biology, mainly affecting the ability of HSCs to self-renew (8, 9). All mutations identified to date in DC patients are found in the telomerase complex itself or in telomere-stabilizing proteins (10). The BM abnormalities in DC patients also predispose them to aplastic anemia, and these patients have an increased risk of developing myelodysplastic syndromes (11). The incidence of these hematopoietic phenotypes is higher in patients harboring mutations that affect *TERC* compared to patients with other mutations, and this observation cannot be explained only by telomere shortening (7).

In addition to its function in telomere biology, there is an increasing body of evidence showing that the telomerase complex

has noncanonical roles independent of telomere lengthening in both mammals and zebrafish. TERT has been reported to modulate gene transcription and cell proliferation in mammals (12–16), while *TERC* regulates cellular senescence through a short form (*TERC-53*) processed in the mitochondria (17) and apoptosis via a small peptide translated from an open reading frame contained in *TERC* (18). Furthermore, we have previously reported a telomere-independent function of zebrafish *terc* in myelopoiesis by regulating the expression of the gene encoding the cytokine colony stimulation factor 3 (granulocyte) (*csf3a*) and by maintaining an appropriate balance between the myeloid transcription factor Spi-1 proto-oncogene b (*spilb*, also known as *pu.1*) and the erythroid transcription factor GATA binding protein 1a (*gata1a*) (19). Notably, this function of *terc* is dispensable for hematopoietic stem and progenitor cells (HSPC) emergence, erythropoiesis, and lymphopoiesis (19). Although the

Significance

Dyskeratosis congenita (DC) is a rare inherited bone marrow failure and cancer predisposition syndrome caused by mutations in telomerase or telomeric proteins. Although all patients show telomere shortening, the symptoms are highly variable and, therefore, other mechanisms have to be involved. Here, we report that telomerase RNA component (*TERC*) regulates the expression of master myeloid genes in zebrafish and humans by recruiting the transcription machinery. Induced pluripotent stem cells derived from DC patients with a *TERC* mutation in a specific domain, the CR4-CR5, shows impaired myelopoiesis, while those from DC patients with mutation in telomerase catalytic subunit differentiated normally. Our findings reveal a target for personalized and precision medicine approaches for DC patients.

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mechanism of the noncanonical role of *TERC* is unknown, a genome-wide study identified a large amount of *TERC* binding sites in the genome of human cells using Chromatin Isolation by RNA Purification (ChIRP) (20), suggesting that this DNA binding ability could be underpinning some of the noncanonical functions of *TERC*.

In this work, we set out to investigate the molecular mechanism by which *TERC* regulates myelopoiesis using zebrafish and human cells. *terc* levels controlled both myeloid gene expression and neutrophil numbers in zebrafish larvae. Mechanistically, *terc* bound in vivo to *terc* binding sites present in the regulatory regions of myeloid genes as well as to RNA polymerase II (Pol II). Importantly, *terc* harboring mutations in the CR4-CR5 domain that are found in DC patients (21), although able to bind DNA, poorly interacted with Pol II and thus failed to increase the transcription rates of myeloid genes in vitro and to regulate myeloid gene expression and myelopoiesis in vivo. This mechanism is evolutionarily conserved since human neutrophil and monocyte precursor cells with decreased *TERC* levels showed reduced Pol II occupancy at myeloid gene promoters and decreased myeloid gene expression. Finally, induced pluripotent stem (iPS) cells derived from DC patients with a mutation affecting the CR4-CR5 domain of *TERC* showed impaired myelopoiesis while those with TERT mutations differentiated normally. Altogether, our results describe *TERC* as a type of lncRNA that with transcription factor properties controls myeloid gene expression, paving the way for designing new therapies for DC patients.

Results

terc Controls Myeloid Gene Expression and Myelopoiesis in Zebrafish.

We have previously shown that *terc* regulates *csf3a* and *spi1b* messenger RNA (mRNA) expression in zebrafish, in a TERT and telomere length-independent manner (19). We confirmed these results and observed a similar regulation of the transcript levels of *csf3b* and *spi1a*, paralogous genes of *csf3a* and *spi1a*, respectively, shown to have similar functions in zebrafish myelopoiesis (*SI Appendix, Fig. S1 A–E*) (22, 23). *terc* also regulated the *spi1b/gata1a* and *spi1a/gata1a* expression ratios (*SI Appendix, Fig. S1 F and G*). To test whether *terc* causally regulates myelopoiesis, we created a genetic *terc* knockout (*terc* KO) zebrafish model using Transcription Activator-Like Effector Nuclease (TALEN) technology (Fig. 1 *A* and *C*). Importantly, *terc* KO zebrafish larvae showed both reduced myeloid gene expression and reduced number of neutrophils in the caudal hematopoietic tissue (CHT), the main hematopoietic tissue at this developmental stage (Fig. 1 *D–G*). Whole-mount in situ hybridization (WISH) revealed expression of *terc* at the CHT and confirmed the altered expression of *gata1a* and *spi1b* in *terc* KO embryos (*SI Appendix, Fig. S1 H and J*). In addition, lymphopoiesis (*rag1* expression in the thymus at 5 day post fecundation [dpf]), thrombopoiesis (Cd41^{Hi} cells), and HSPCs (Cd41^{Lo}) emergence were unaffected in *terc* KO, confirming previous results in *terc* morphants (*SI Appendix, Fig. S1 I and K*).

Conversely, expression of *terc* RNA in blood cells using the *draculin* regulatory region (Fig. 1 *B* and *C*) (24) led to enhanced myelopoiesis as evidenced by increased myeloid gene transcript levels and number of neutrophils in the CHT (Fig. 1 *D–G*). These results further confirm that *terc* regulates myelopoiesis through increasing myeloid gene expression.

***terc* Controls the Activity of Myeloid Gene Promoters in a *terc* Binding Site-Dependent Manner.** It has been shown that *TERC* binds to numerous regions in the human genome through a sequence-specific consensus binding site (CC[A/C]CC[A/C]CCCC) (20). Therefore, we searched for *terc* binding sites in the upstream regulatory regions (arbitrary length of 5 kb upstream of the transcription start site [TSS]) of zebrafish *csf3a* and *csf3b*. We found two potential *terc* binding sites in the *csf3b* promoter region that we named *tercbs1* (-1.5 kb) and *tercbs2* (-1.4 kb) (*SI Appendix, Fig. S2A*) but none in the *csf3a* promoter. Morpholino (Mo)-mediated

knockdown of *terc* decreased the activity of both *csf3a* and *csf3b* promoters (2 kb upstream of the TSS) in luciferase reporter assays in zebrafish larvae (Fig. 2*A* and *SI Appendix, Fig. S2B*), while *terc* RNA overexpression increased the activity of the promoters (Fig. 2*B* and *SI Appendix, Fig. S2C*). Deletion of *tercbs1*, but not *tercbs2*, reduced the activity of the *csf3b* promoter (Fig. 2*C*). Intriguingly, *terc* overexpression was still able to induce *csf3b* promoter activity in the absence of *tercbs1* (*SI Appendix, Fig. S2D*). We speculated that this effect could be due to the higher amounts of Spi1a and/or Spi1b transcription factors observed when *terc* is overexpressed. Deletion of a putative Spi1 binding site in the *csf3b* promoter fully abrogated the *terc*-mediated induction of the promoter activity (*SI Appendix, Fig. S2E*). This result suggests that *terc* could also regulate *spi1a* and/or *spi1b* expression. We searched for *terc* binding sites in the regulatory regions of *spi1* genes and found one site in the 3' region of *spi1a* (around 500 base pair [bp] downstream of the end of the gene) (*SI Appendix, Fig. S2F*) but none in *spi1b*. Deletion of this *tercbs* in the *spi1a* 3' region resulted in decreased activity of the *spi1a* promoter (2 kb upstream of the TSS), and *terc* overexpression was unable to increase *spi1a* promoter activity when *tercbs* was not present (Fig. 2*D*). These results support the hypothesis that *terc* directly controls the expression of myeloid genes.

terc Binding Sites in Regulatory Regions of Myeloid Genes Are Occupied by *terc* and Are Essential for Myelopoiesis In Vivo.

To evaluate whether *terc* was able to bind to these putative *terc* binding sites in vivo, we performed ChIRP experiments (20) in whole kidney marrow (WKM), the main hematopoietic organ in adult zebrafish. ChIRP for WKM cells of wild-type zebrafish showed that *terc* is bound to the *tercbs1* present in the *csf3b* promoter in vivo and, to a lesser extent, to the *tercbs* in the 3' region of *spi1a* (Fig. 2*E*). To confirm that *terc* directly binds to DNA, we performed *terc*-DNA binding assays. Wild-type *terc* was able to directly bind to a *csf3b* promoter double-strand DNA (dsDNA) fragment in vitro, in a *tercbs1*-dependent manner (Fig. 2*F*). Importantly, deletion of the *csf3b*-promoter *tercbs1* in vivo using TALEN technology in *mpx::eGFP* transgenic larvae carrying GFP-labeled neutrophils (25) resulted in neutropenia (Fig. 2*G* and *SI Appendix, Fig. S2G*). We conclude that *terc* binds to regions that regulate myeloid gene expression in vivo and that at least one of those regulatory regions is essential for myelopoiesis.

terc Increases the Efficiency of Zebrafish *csf3b* Promoter to Drive In Vitro Transcription.

Our results show that the RNA component of telomerase, *terc*, might act as a transcription factor. To further confirm this hypothesis, we performed in vitro transcription assays using either nuclear extracts from HL60 cells or recombinant proteins as a source of the transcriptional machinery. While *terc* was able to increase the transcription rates of a luciferase reporter driven by wild-type *csf3b* promoter (Fig. 3 *A–D*), a CR4-CR5 *terc* mutant harboring a point mutation found in DC patients (CR4-CR5^M) (*SI Appendix, Fig. S3A*) failed to do so (Fig. 3 *C* and *D*).

***terc* Interacts with RNA Pol II through Its CR4-CR5 Domain.** The fact that *terc* binds to consensus binding sites present in regulatory regions of myeloid genes led us to hypothesize that *terc* might recruit proteins to regulate expression of these genes. Luciferase reporter experiments in zebrafish larvae showed that deletion of *tercbs1* did not alter the regulation of *csf3b* promoter activity by the transcription factors Spi1b and Gata1a (*SI Appendix, Fig. S4*), known to regulate myeloid versus erythroid fate in zebrafish (26). Next, we tested whether *terc* was able to recruit the transcriptional machinery itself to the DNA. Strikingly, RNA pull-down (using in vitro transcribed and biotinylated *terc*) and in vivo RNA Immunoprecipitation (RIP) experiments demonstrated that *terc* interacted with both total and the active form—phosphorylated at

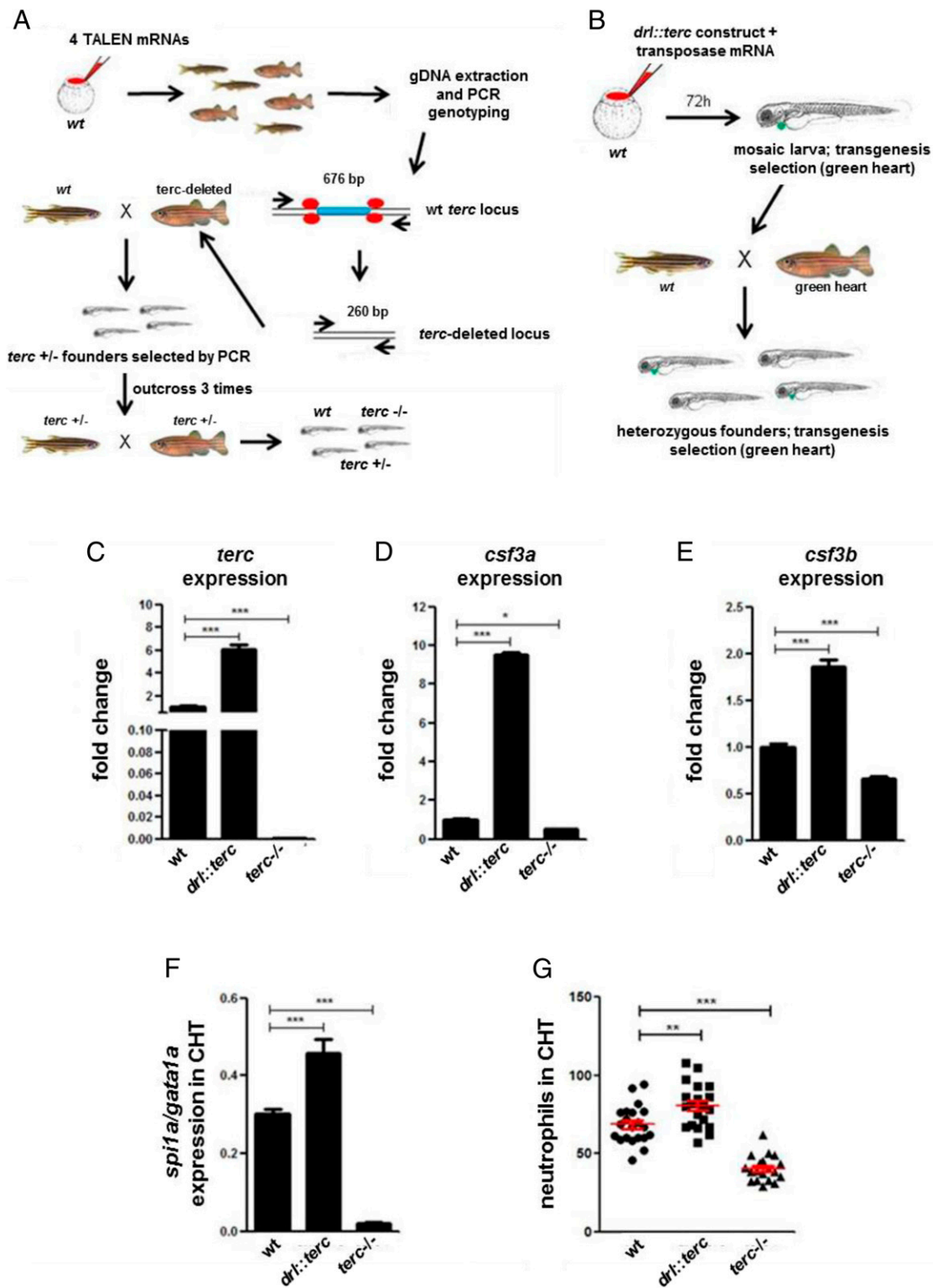


Fig. 1. Zebrafish *terc* controls the expression of promyelopoietic genes in vivo. (A) Workflow of the generation of the *terc*^{-/-} (*terc* KO) zebrafish line. (B) Workflow of the generation of the Tg(*drl::terc*) zebrafish line. (C–F) Expression of the indicated genes in the CHT of wild-type (wt), Tg(*drl::terc*), and *terc* KO fish larvae at 2 dpf. Data are representative results of three independent experiments; *n* = at least 20 larvae per group. (G) Neutrophil counts in the CHT of *mpx::GFP* larvae (neutrophils labeled by GFP) at 3 dpf. Data are the average of two independent experiments; *n* = 21 for wt and Tg(*drl::terc*), and *n* = 19 for *terc* KO. Data values shown are fold change over the value of wt sample in C, D, and E. All data are mean + SEM. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 for one-way ANOVA plus Bonferroni posttest.

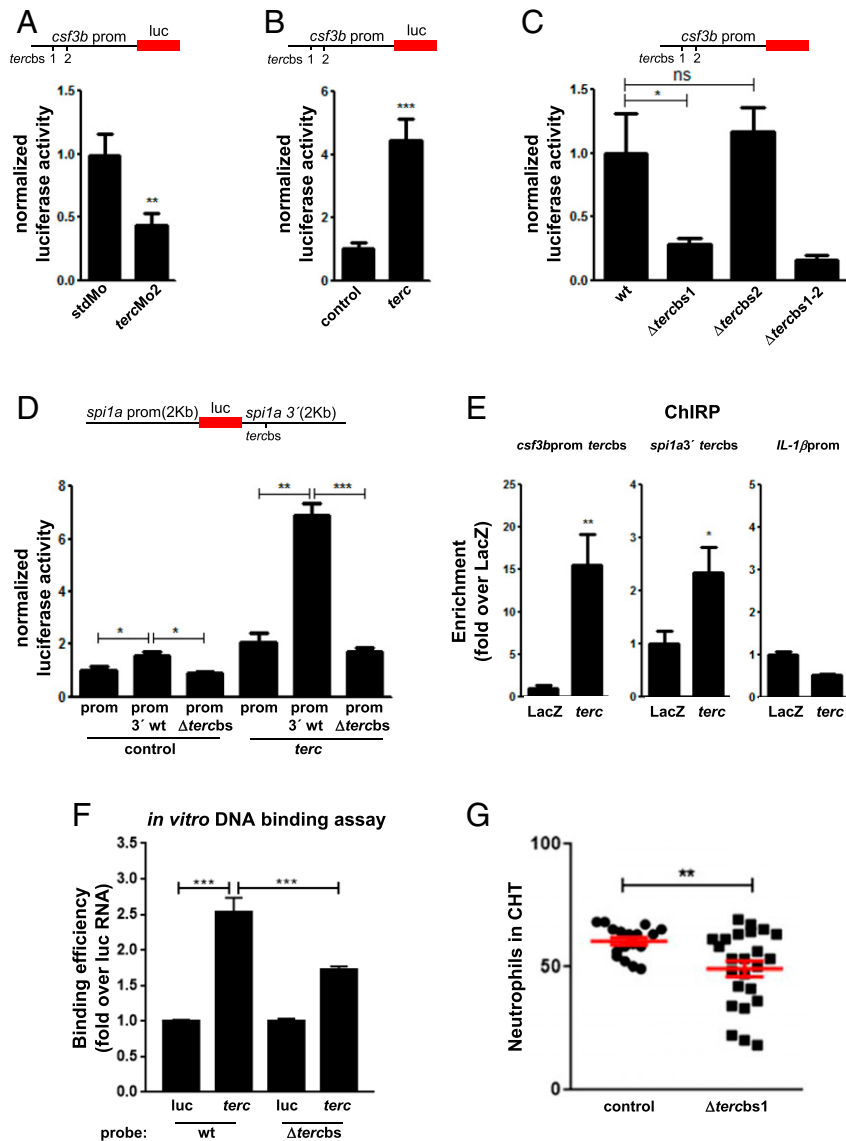


Fig. 2. Telomerase RNA binds to target sequences in regulatory regions of zebrafish promyelopoietic genes and controls the activity of their promoters. (A and B) Activity of *csf3a* and *csf3b* promoters (~2 kb upstream) in the CHT of larvae at 2 dpf after injection of the luciferase reporter constructs combined with *tercMo2* or 200 pg/embryo of *terc* RNA. Data are representative results of three independent experiments. $n =$ at least 30 larvae per group (see *Materials and Methods*); luc, luciferase. (C) Activity of *tercbcs*-deleted *csf3b* promoter in the CHT of larvae at 2 dpf after injection of the luciferase reporter constructs. Data are representative results of three independent experiments. $n =$ at least 30 larvae per group (see *Materials and Methods*). (D) Activity of the *spi1a* promoter (~2 kb upstream) with wild-type (wt) or *tercbcs*-deleted 3' region (~2 kb downstream) in CHT of larvae at 2 dpf after injection of the luciferase reporter constructs in combination with control or *terc* RNA. Data are representative results of three independent experiments. $n =$ at least 30 larvae per group (see *Materials and Methods*). (E) qRT-PCR of ChIP eluates of *tercbcs*-containing DNA fragments encompassing the *csf3b* promoter and *spi1a* 3' region. Data are representative results of two independent experiments; $n = 20$ WKM of adult fish. (F) qRT-PCR of in vitro DNA binding assay eluates. For each probe, data are the average of two independent experiments. luc, luciferase RNA. (G) Neutrophil counts in the CHT of *mpx::GFP* larvae at 3 dpf after injection of TALEN mRNAs to destroy the *csf3b* promoter *tercbcs1* in vivo (see *Materials and Methods*). Data are the average of two independent experiments; $n = 17$ and 24 larvae in control and *tercbcs*-deleted groups, respectively. Data values shown are fold change over the value of the stdMo (standard morpholino) sample in A, the control sample in B, the *csf3b* wt promoter sample in C, the *spi1a* wt promoter + control sample in D, and the luc + wt probe sample in F. All data are mean + SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for Student's *t* test in A, B, E, and G and for one-way ANOVA plus Bonferroni posttest in C, D, and F. ns, not significant.

Serine 5—of Pol II, the core component of the transcriptional machinery (Fig. 4A and B and *SI Appendix, Fig. S3B*).

We next aimed to map the region of *terc* responsible for the interaction with Pol II. The *terc*-Pol II interaction in vitro was blocked by adding a Mo targeting the small Cajal body-specific RNAs (ScaRNAs) domain (*tercMo2*) and one targeting the CR4-CR5 domain (*tercMo3*) but not the one targeting Pseudoknot/Template domain (*tercMo1*) (Fig. 4C and *SI Appendix, Fig. S3A and C*). In those conditions, the amount of biotinylated *terc* bound

to the streptavidin beads was not decreased (*SI Appendix, Fig. S3D*). Strikingly, overexpression of *terc* RNA harboring a point mutation found in the CR4-CR5 domain (CR4-CR5^M) of patients affected by DC (*SI Appendix, Fig. S3A and E*) (21) was unable to increase the activity of the *csf3b* promoter in luciferase reporter experiments (Fig. 4D). In addition, CR4-CR5^M *terc* also failed to increase the number of neutrophils in *mpx::eGFP* transgenic larvae, whereas wild-type *terc* did (Fig. 4E). In contrast, *terc* with mutations found in DC patients affecting the template domain

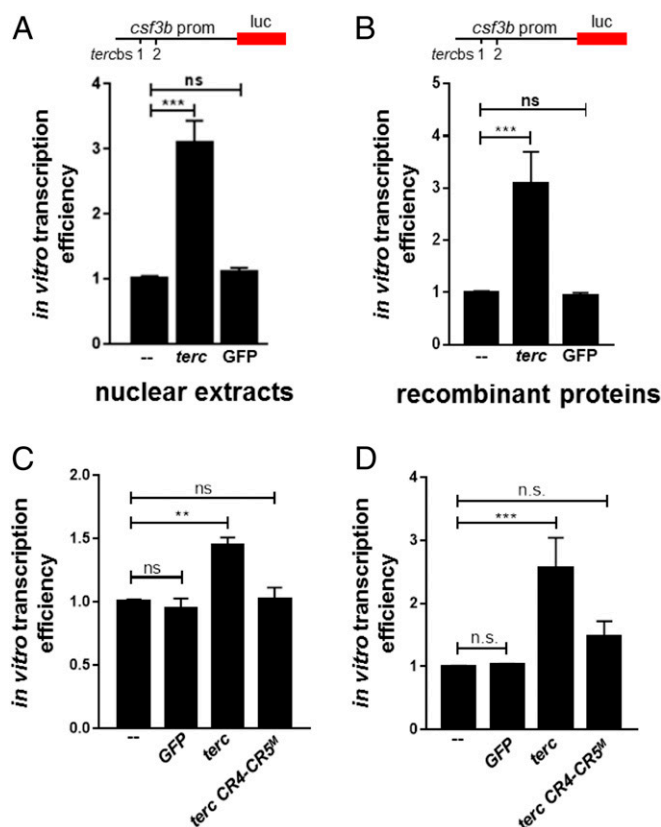


Fig. 3. Telomerase RNA increases the efficiency of in vitro transcription. (A) In vitro transcription efficiency of *csf3b* promoter-driven luciferase reporter in the presence of 25 nM GFP, *terc* RNA, or no RNA as control, using nuclear extracts from HL60 cells as the source of the transcriptional machinery. Data are the average of at least three independent experiments. luc, luciferase. (B) Same as in A, using recombinant proteins as source of the transcriptional machinery. (C) In vitro transcription efficiency of *csf3b* promoter-driven luciferase reporter in the presence of 10 nM GFP, *terc*, CR4-CR5^M mutant *terc* RNA, or no RNA as control, using nuclear extracts from HL60 cells as the source of the transcriptional machinery. Data are the average of at least three independent experiments. (D) Same as in C, using recombinant proteins as source of the transcriptional machinery. Data values shown for all panels are fold change over the no RNA control sample. All data are mean + SEM. ** $P < 0.01$ and *** $P < 0.001$ for one-way ANOVA plus Bonferroni posttest in A, B, C, and D. ns, not significant.

and the ScaRNA domain (*SI Appendix, Fig. S3 A and E*) (21) increased *csf3b* promoter activity and the number of neutrophils at levels similar to wild-type *terc* (Fig. 4D and E). Interestingly, CR4-CR5^M mutant *terc* hardly interacted with active Pol II in RNA pull-down experiments (Fig. 4F), whereas CR4-CR5^M mutant *terc* was still able to interact with the *gcsfb* promoter dsDNA probe (Fig. 4G). Collectively, these data show that *terc* interacts with Pol II and that the interaction is essential for robust myelopoiesis in zebrafish. In addition, this interaction is mainly mediated by the CR4-CR5 domain of the *terc* molecule.

***TERC* Controls Myelopoiesis in Humans.** Next, we examined whether the mechanism of *terc* regulation of myeloid gene expression we discovered in zebrafish is evolutionarily conserved. We used short hairpin RNA (shRNA) constructs and lentiviral transduction to reduce *TERC* levels in the human neutrophil precursor cell line HL60 (HL60sh*TERC*) and in the human monocyte precursor cell line U937 (U937sh*TERC*). HL60sh*TERC* cells barely showed altered transcript levels of *TERT* or altered telomerase activity, while U937sh*TERC* cells had augmented *TERT* expression and telomerase activity compared to the scrambled shRNA controls

(*SI Appendix, Fig. S5 A and B*). However, telomere length was unaffected (*SI Appendix, Fig. S5C*).

We analyzed the expression of myeloid genes in these conditions, that is, in the presence of unaltered telomere length but reduced *TERC* expression. Down-regulation of *TERC* expression by two- or fourfold decreased the expression of *CSF2*—encoding granulocyte-macrophage-colony stimulating factor—in both HL60sh*TERC* and U937sh*TERC* cells (Fig. 5A and B). We also found lower transcript levels of *CSF3*, which encodes granulocyte-colony stimulating factor (G-CSF), and *SPI1* in HL60sh*TERC* cells, whereas U937sh*TERC* cells showed a weak reduction of the mRNA levels of *CSF1*, which encodes macrophage-colony stimulating factor (Fig. 5A and B and *SI Appendix, Fig. S5D*). *CSF1* was not detected in HL60 cells, while *CSF3* was not detected in U937.

As we had demonstrated for zebrafish, we found *TERC* to bind to total and active Pol II in both HL60 and U937 cells by RIP. As expected, known *TERC* binding proteins, such as TERT and dyskerin (DKC), also interacted with *TERC* in these cells, while none of them interacted with control RNAs such as U6 small nuclear RNA (snRNA) and GAPDH mRNA (Fig. 5C and D and *SI Appendix, S6 A and B*). To further show such an interaction, we performed Proximity Ligation Assays (PLA) in HL60 cells. PLA shows up fluorescence only when the tested molecules are in close proximity (nominally 40 nm) (27). We used *TERC* probes and phospho Serine 5 Pol II antibodies to visualize *TERC*–Pol II interaction in the cell nucleus (Fig. 5E, Bottom), and this interaction was not visualized when no antibodies or *TERC* sense probe were used (Fig. 5E, first and third panels). We also detected *TERC*–TERT interaction as positive control (Fig. 5E, second panel). To test whether *TERC* recruits Pol II to enhance myeloid gene expression, we performed Chromatin Immunoprecipitation (ChIP) experiments. Notably, the TSS of *CSF2* and *SPI1* in HL60sh*TERC* cells showed reduced occupancy of both total and active Pol II, and also U937sh*TERC* cells had less Pol II bound around the TSS of *CSF2* (Fig. 5F–H and *SI Appendix, S6 C–E*). These data indicate that *TERC* controls the expression of myeloid master transcription factor and cytokine genes in human myeloid precursor cells by interacting with Pol II and recruiting it to the promoters of target genes to initiate transcription.

CR4-CR5 *TERC* Mutant iPS Cells Show Impaired Myelopoiesis. The incidence of aplastic anemia and cancer is higher in DC patients with *TERC* mutations compared to those affected by mutations in other factors (7), suggesting that *TERC* also plays a noncanonical role in DC. To investigate whether the regulation of myelopoiesis is altered in DC patients with mutations in *TERC*, two iPS cells derived from DC patients and one derived from a healthy donor (HD) were differentiated into hematopoietic cells through embryonic body (EB) formation: three-dimensional cell aggregates that can differentiate into cells of all three germ layers (Fig. 6A). DC patient iPS cells harbored either a heterozygous mutation in the CR4-CR5 domain of *TERC* (nG319A) (28) or a heterozygous pathogenic missense point mutation in TERT (A716V). Consistent with our findings, while both HD and TERT mutant iPS cells were able to generate granulocytic-monocytic colonies (GM) at similar levels in colony-forming unit (CFU) assays, iPS cells harboring the nG319A mutation in the CR4-CR5 domain of *TERC* only produced very few GM colonies. Interestingly, nG319A mutant iPS cells had an increased ability to generate erythroid colonies (Fig. 6C). Both mutant iPS cells generated lower numbers of CFUs than the HD line (Fig. 6D). We then analyzed EBs derived from all three iPS cells by fluorescence-activated cell sorting to study their cell type composition (Fig. 6B). All EBs had a similar percentage of CD31⁺ hemogenic progenitors (HEP), consisting of bipotential precursors (hematopoietic and endothelial cells), and of CD45⁺ CD34⁺ hematopoietic progenitor cells (HPC), at days 15 and 21 of culture (Fig. 6E). However, CR4-CR5 mutant EBs showed slightly increased CD45⁺ mature blood cells at day 21 (Fig. 6E). These

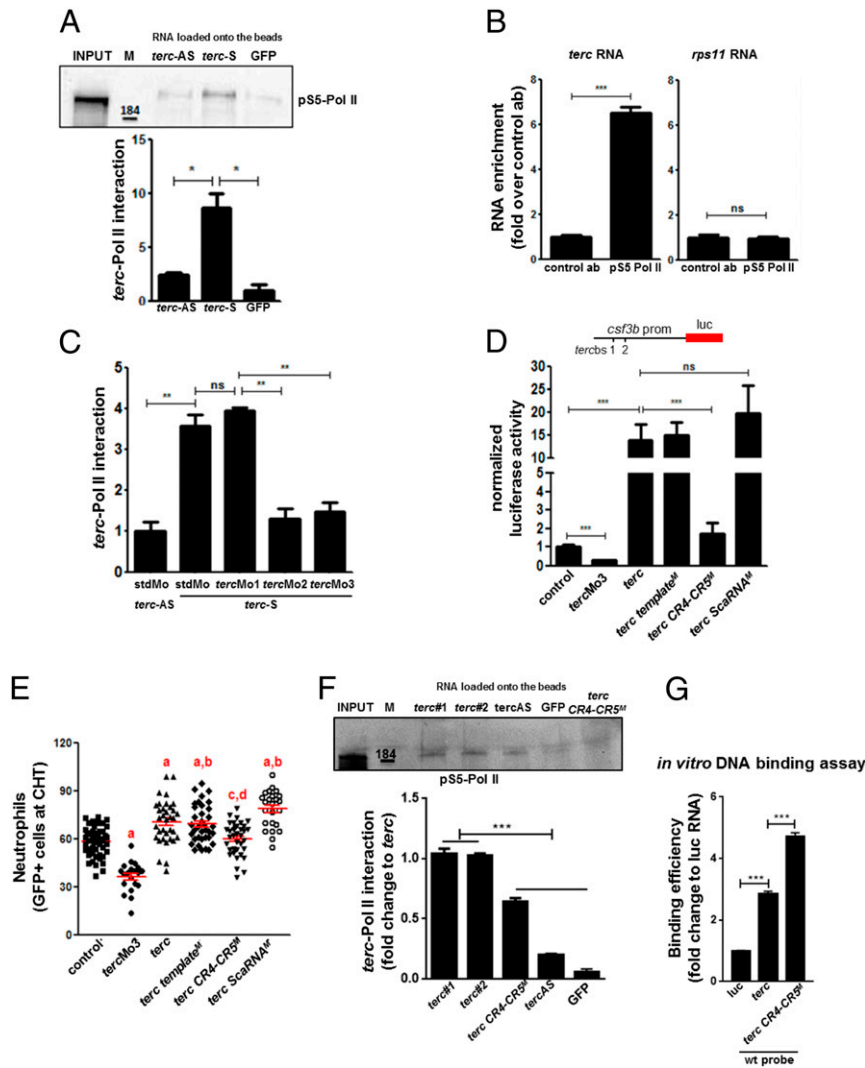


Fig. 4. Telomerase RNA binds to RNA Pol II through its CR4-CR5 domain to promote developmental myelopoiesis in zebrafish. (A) Western blot of RNA pull-down eluates using anti-Phospho S5 RNA Pol II antibodies. For quantification of the interaction, Quantity One software was used; $n = 2$ independent experiments; M, molecular weight marker (kDa); $n =$ at least 500 2-d larvae per group (see *Materials and Methods*). (B) qRT-PCR of RIP experiment using anti-Phospho S5 RNA Pol II assessing enrichment of *terc* RNA. Data are representative results of three independent experiments; $n =$ at least 500 2-d larvae per group (see *Materials and Methods*). (C) Quantification of *terc*-RNA Pol II interaction in the presence of *terc* Mos (reference *SI Appendix, Fig. S3C* for representative Western blot); $n = 2$ independent experiments with at least 500 larvae at 2 dpf per group (see *Materials and Methods*). (D) Activity of the *csf3b* promoter in CHT of larvae at 2 dpf after injection of the luciferase reporter construct in combination with *terc*Mo3 or *terc* RNA at 200 pg/embryo; $n =$ at least 30 larvae per group; luc, luciferase; ^M, mutated (reference *SI Appendix, Fig. S3A*). (E) Neutrophil counts in the CHT of *mpx::GFP* larvae at 3 dpf after injection of *terc*Mo3 or *terc* RNA; $n = 50, 20, 35, 36, 36,$ and 24 larvae at 3 dpf in control, *terc*Mo3, wt *terc*, *terc* template^M, *terc* CR4-CR5^M, and *terc* ScarNA^M, respectively; ^M, mutated (reference *SI Appendix, Fig. S3A*). (F) Western blot of RNA pull-down eluates using anti-Phospho S5 RNA Pol II antibodies and quantification of the interaction. *terc*#1 and *terc*#2 are two independent pull-down samples; $n = 2$ independent experiments; M, molecular weight marker (kDa). (G) qRT-PCR of *in vitro* DNA binding assay eluates. luc, luciferase RNA. Data values shown are fold change over the value of GFP sample in A, the control antibody (ab) sample in B, the stdMo (standard morpholino) + *terc* - AS sample in C, the control sample in D, the *terc*#1 sample in F, and the luciferase sample in G. All data are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for Student's *t* test in B and for one-way ANOVA plus Bonferroni posttest in A, C, D, E, F, and G; a is $P < 0.05$ versus control, b is not significant versus wt *terc* molecule, c is not significant versus control, and d is $P < 0.05$ versus wt *terc* molecule, one-way ANOVA plus Bonferroni posttest; ns, not significant.

data indicate that the emergence of HEP and HPC are not impaired in the three iPS cells. We conclude that a TERC CR4-CR5 domain mutation in iPS cells derived from a DC patient are defective in myelopoiesis, and it is independent of TERT and telomere shortening.

Discussion

DC is an inherited disease provoked by mutations affecting the telomerase complex or telomere-stabilizing components (10) and patients typically die of BM failure due to HSC exhaustion (8, 9). DC patients carrying *TERC* mutations develop aplastic anemia and cancer more frequently than other DC patients (7), suggesting

noncanonical roles for *TERC* in DC pathogenesis. Recent studies have shown that TERT-*TERC* complexes bind to ribosomal DNA promoters and stimulate transcription by Pol I during regeneration and in tumorigenesis (29) and that inhibition of TERT and *TERC* impaired myelopoiesis of human iPS cells independently of telomere length (30). We have found that *terc* plays an essential role in myelopoiesis in zebrafish by regulating myeloid gene expression but in a manner independent of telomere length and also TERT (19). In the present study, we uncover the mechanisms underlying *terc*-mediated regulation of *spi1a* and *csf3b*, the master regulators of zebrafish myelopoiesis (22, 23), and we also extend our observations to human cells. We have found that reduced

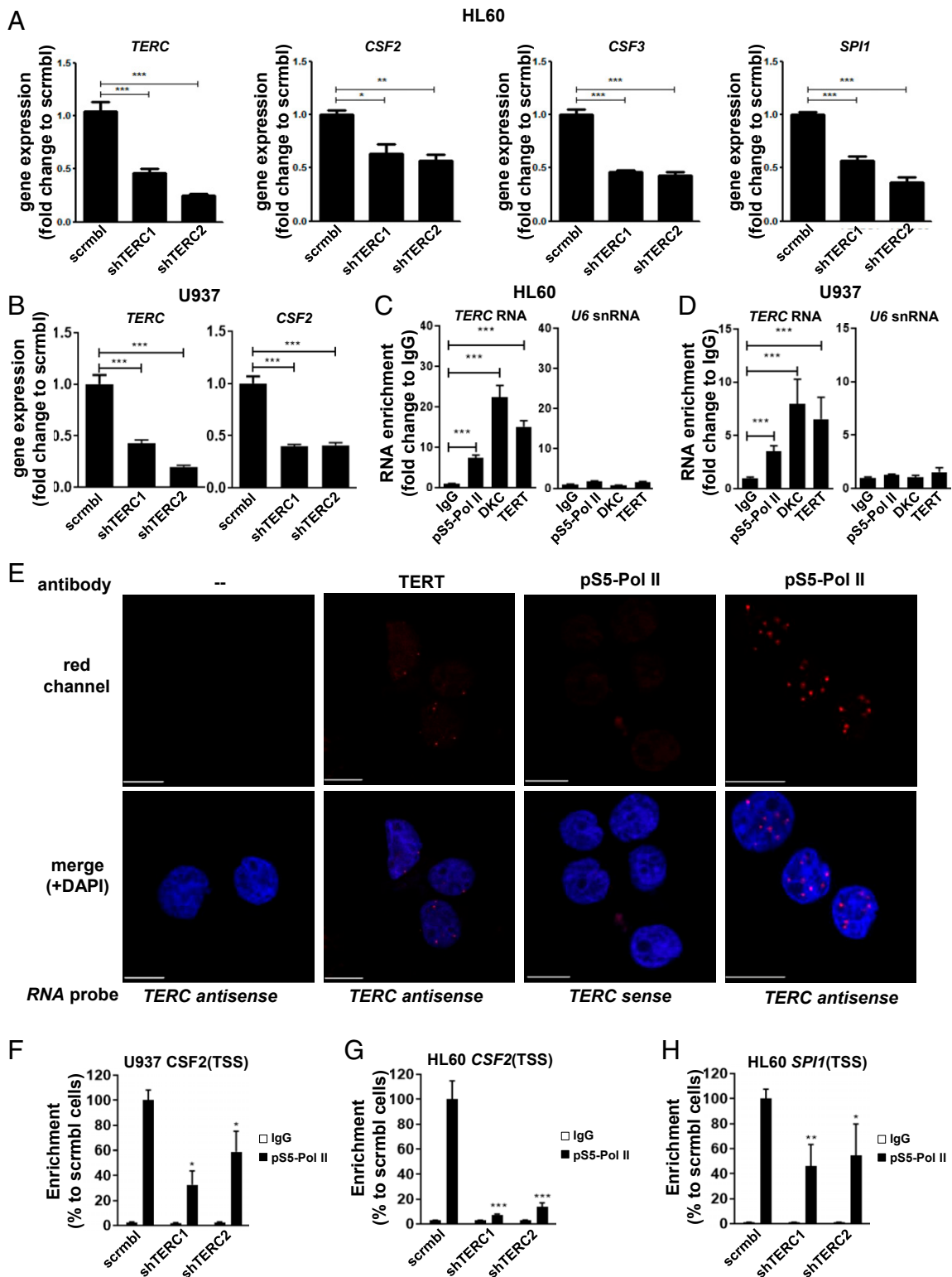


Fig. 5. The regulation of myelopoiesis by the interaction of telomerase RNA with RNA Pol II is evolutionarily conserved. (A) qRT-PCR for indicated pro-myelopoietic genes in neutrophil-like HL60 cells transduced with two different shRNAs against *TERC* (shTERC1 and shTERC2) or a scrambled control shRNA (scrambled). Data are representative results of three independent experiments. (B) Same as in A, using promonocytic U937 cells. (C) qRT-PCR of RIP eluates in neutrophil-like HL60 cells assessing enrichment of indicated RNAs in the presence of anti-Phospho S5 RNA Pol II antibody, Dyskerin (DKC), TERT, or IgG controls. Data are representative results of three independent experiments. (D) Same as in C, using promonocytic U937 cells. (E) PLA cells showing *TERC*-pS5 Pol II interaction in the nucleus of HL60 cells. Note the foci of red fluorescence (indicating interaction) when pS5-Pol II and TERT antibodies were used. (F) qPCR for indicated locus after CHIP with anti-Phospho S5 RNA Pol II antibody or IgG control in promonocytic U937 cells transduced with two different shRNAs against *TERC* (shTERC1 and shTERC2) or a scrambled control shRNA (scrambled). Data are the average of at least two independent experiments. (G) Same as in F, using neutrophil-like HL60 cells. (H) Same as in G but for the indicated locus. Data values shown are fold change over the value of scrambled sample in A and B and the IgG sample in C and D. Data in F through H are % of enrichment over scrambled sample. All data are mean + SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for one-way ANOVA plus Bonferroni posttest in A through D and for Student's *t* test in F and G.

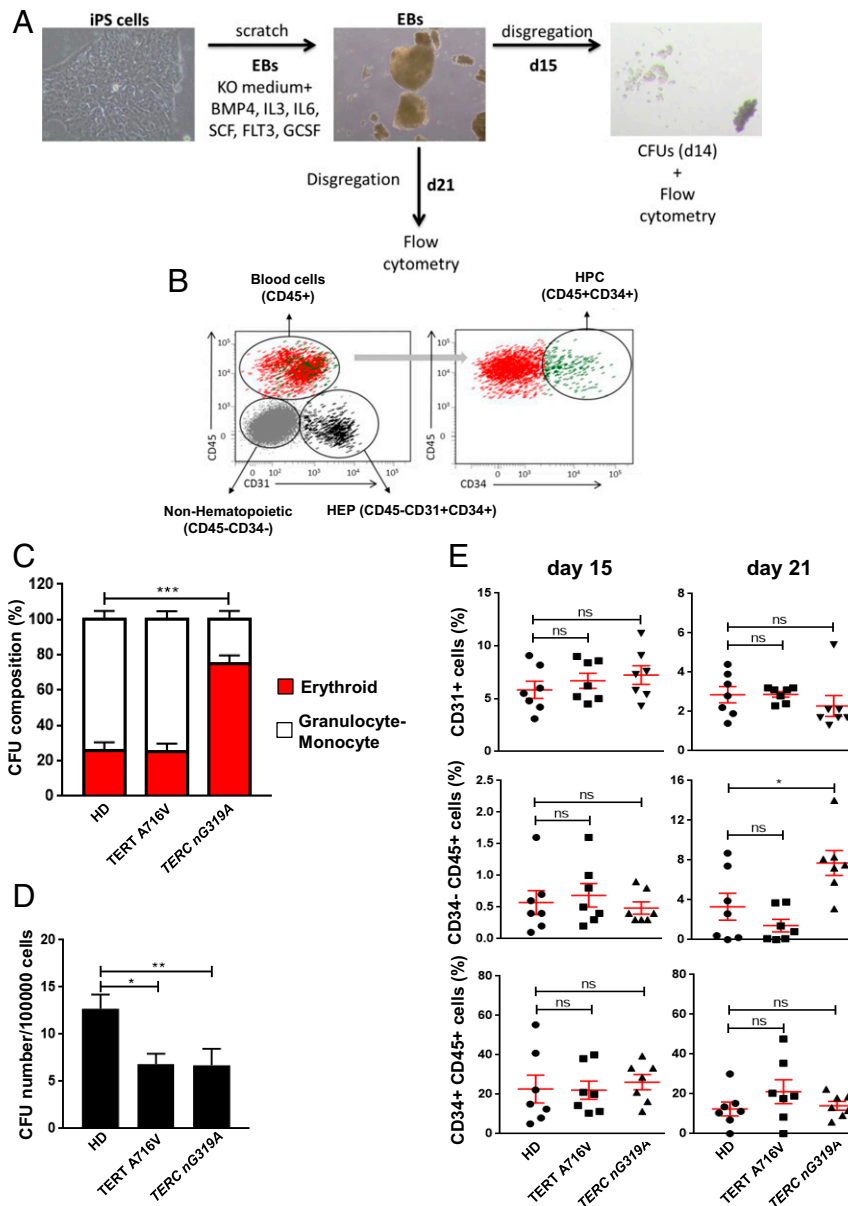


Fig. 6. iPS cells carrying a mutation in the CR4-CR5 domain of *TERC* fail to properly differentiate into myeloid cells. (A) Workflow for differentiation of iPS cells. (B) Dot plot and identification of different cell populations in EBs by flow cytometry. CD31⁺ HEP (bipotential precursors of hematopoietic and endothelial cells), CD45⁺ CD34⁺ HPC, and CD45⁺ mature blood cells. (C) Composition of erythroid and granulocyte/monocyte colonies in CFU assays, using EBs derived from the indicated iPS cell lines. Data are the average of seven independent EB formation experiments. (D) Number of colonies per 100,000 EB cells. Data are the average of seven independent EB formation experiments. (E) Quantification of cell populations in EBs by flow cytometry. CD31⁺ (HEP), CD34⁺CD45⁺ (HPC), and CD45⁺ (mature blood cells) cells in day 15 and 21. Data are the average of seven independent EB formation experiments. All data are mean + SEM. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 for one-way ANOVA plus Dunnett's posttest in all the panels. ns, not significant.

TERC levels resulted in decreased myeloid gene expression in HL60 and U937 cells, human neutrophil and monocyte progenitor cell lines, respectively, without reducing either telomerase activity or telomere length. Importantly, we also show that, while HD and TERT-mutant-derived iPS cells showed a similar ability to differentiate into myeloid cells, myelopoiesis was impaired in CR4-CR5 mutant *TERC* iPS cells. All these observations indicate that *TERC* controls the expression of one of the main gene networks required for robust myelopoiesis in zebrafish and humans, besides its typical function in telomere biology.

Regulation of gene expression is influenced and controlled by multiple factors. Core promoters just upstream of the TSS serve as a binding platform for the transcriptional machinery and are

sufficient to drive transcription with a basal activity, which can be further enhanced by upstream DNA elements called enhancers that bind regulatory proteins including transcription factors (31). lncRNAs are now recognized as major regulators of multiple cellular processes, such as cell differentiation and development, chromosome dosage compensation, regulation of gene expression, and cell cycle control (32–36). In addition, they have been shown to bind the genome and function as scaffolds for the recruitment of chromatin remodelers and transcription factors (37, 38). It has been shown that *TERC* binds numerous regions across the genome through a C-rich consensus binding site (20). These data together with the ability of *TERC* to regulate myeloid gene expression led us to hypothesize that *TERC* was a factor

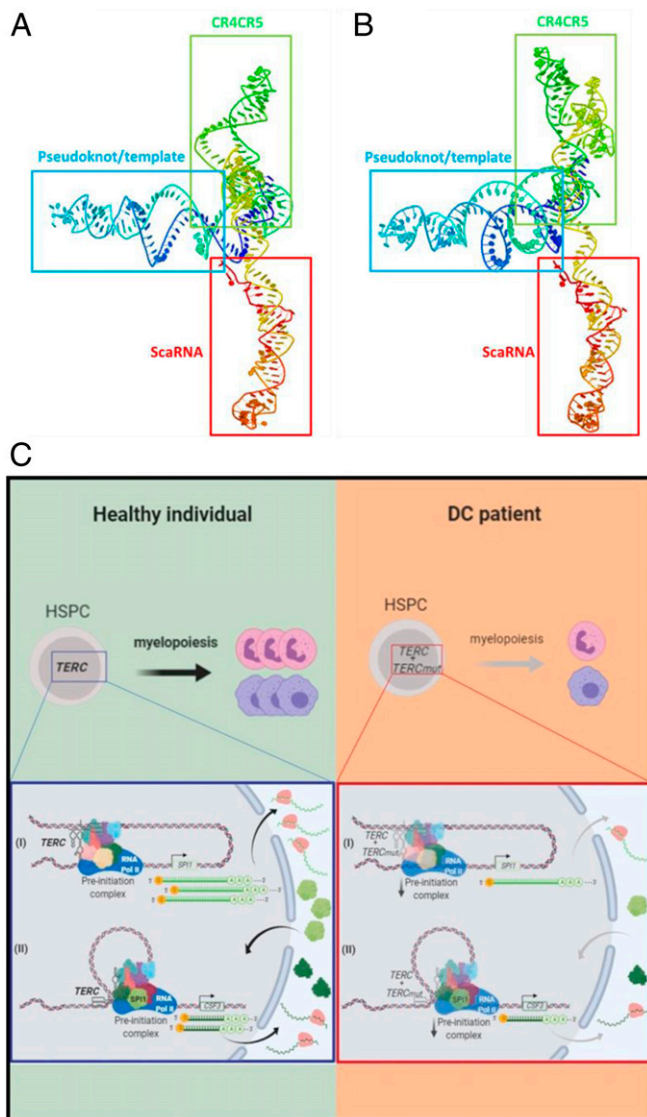


Fig. 7. Predicting structure and proposed model of *TERC* function in myelopoiesis. (A and B) Prediction of *TERC* and *TERC* nG319A tertiary RNA structure. The processing of the structure was done with Jmol application of RNA Composer system (<http://rnacomposer.cs.put.poznan.pl/>), an open-source Java viewer for chemical structures in the third dimension (48). The different domains of the molecule are in different colors, starting 5' in dark blue, followed by green, yellow, orange, and ending 3' in red. (A) *TERC* wild-type structure. (B) *TERC* nG319A structure. (C) *TERC* regulation model of myeloid gene expression. Under normal conditions, *TERC* can regulate the expression of myelopoiesis genes (*SPI1* and *CSF3*) in zebrafish and humans. Telomerase RNA binds DNA through its consensus binding sites and with RNA Pol II, facilitating its recruitment to myelopoietic gene promoters, ensuring robust myelopoiesis. DC patients with a mutation in the *TERC* CR4-CR5 domain, possibly due to a change in RNA structure, do not properly recruit RNA Pol II to promoters and fail to regulate the expression of myeloid genes, resulting in an alteration in myelopoiesis that causes a drop in myeloid cell production. This mechanism could serve as the basis for designing new strategies for therapeutic intervention. CR4-CR5 mutations will anticipate the development of myelodysplastic syndrome and signal the need for precision, personalized treatment. Pink cells are neutrophils and purple cells are macrophages; dark green proteins are G-CSF cytokine and light green protein are master transcriptional factor *SPI1*.

controlling the activity of myeloid gene promoters. We observed that the zebrafish *csf3b* promoter, as well as a regulatory sequence downstream *spi1a*, contained *terc* binding sites that were bound by *terc* and were necessary for robust promoter activity.

Curiously, *terc* also regulated *csf3a* promoter activity that lacks *terc* binding sites. This could be due to the increased transcript levels of *spi1a* and/or *spi1b* caused by *terc* overexpression, since the *csf3a* promoter harbors a putative *spi1* binding site. However, it cannot be ruled out that *terc* also increases promoter activity by another mechanism. Interestingly, there are consensus *TERC* binding sites in the promoters of human *CSF2*, *CSF3*, and in the 3' region of *SPI1* (20), suggesting that *TERC* could also directly regulate these genes. Altogether, these results demonstrate that *terc* binds to *terc* binding sites in vivo and that this interaction is crucial for a stable activity of myeloid gene promoters and thus robust myeloid gene expression driving myelopoiesis.

One of the main findings of this study is that *TERC* behaves as a typical transcription factor, despite being a lncRNA. On one hand, it was able to physically interact with Pol II, the main component of the transcriptional machinery, in vitro and in vivo, in both zebrafish and humans. On the other hand, reduced levels of *TERC* in human myeloid progenitors resulted in a decreased occupancy at the TSS of myelopoietic genes by Pol II, and thus, lower levels of myeloid transcripts. Strikingly, *terc* was able to increase the efficiency of zebrafish *csf3b* promoter to drive in vitro transcription, further suggesting that *terc* is a class of transcription factor. Although it has been shown that several lncRNAs are able to negatively regulate transcription (39), this study shows a lncRNA, the telomerase RNA, is endowed with functions characteristic of positive transcription factors, including binding to upstream regulatory sequences, recruiting Pol II, and stimulating gene expression. Although the DNA binding ability of *terc* is not mediated by the CR4-CR5 domain, we demonstrate that the *terc*-Pol II interaction is dependent on this domain, which is frequently mutated in DC patients. In fact, the *terc* molecule harboring a mutation in the CR4-CR5 domain found in DC patients was unable to regulate myeloid gene promoter activity and only poorly interacted with Pol II, whereas other mutant *terc* molecules did. This suggests that the function of *terc* in myelopoiesis is not only supported by appropriate *terc* levels but also by a fully competent structure of the molecule and that a correct structure of the CR4-CR5 domain is necessary for a productive interaction with Pol II. Therefore, it is tempting to speculate that the mutations in the CR4-CR5 domain found in DC patients, despite conserving DNA binding capacity, might alter the conformational structure of the domain (Fig. 7A and B), preventing a robust interaction with Pol II that results in decreased myeloid gene expression and eventually in a deficient myelopoiesis.

Using iPS cells derived from DC patients, we further confirmed the relevance of the CR4-CR5 domain of *TERC* in myelopoiesis. We showed that a mutation in the CR4-CR5 domain of *TERC* RNA, which is frequently found in DC patients, was responsible for the reduced ability of the iPS cells to differentiate into myeloid cells. In contrast, iPS cells harboring a mutation in *TERT*, the other main component of the telomerase complex, differentiated normally. These data indicate that the noncanonical function of *TERC* in myelopoiesis may be critically impaired in DC patients harboring mutations that affect the CR4-CR5 domain of *TERC*. The total number of colonies in CFU assays was reduced in iPS cells derived from DC patients. This is not surprising since both *TERT* and *TERC* mutations result in compromised telomerase activity and thus proliferation defects [Boyras et al. (28)]. The fact that the composition of the colonies is only altered in iPS cells with mutation in *TERC*, and not in *TERT*, indicates that impaired myelopoiesis is independent of *TERT* and telomere shortening.

In summary, the findings described in this work depict a scenario where telomerase RNA regulates the expression of *csf3a*, *csf3b*, *spi1a*, and *spi1b* genes in zebrafish and their human counterparts *CSF2*, *CSF3*, and *SPI1*. Telomerase RNA binds to DNA through its consensus binding sites, and to Pol II, recruiting it to the promoters of myeloid genes, assuring an efficient Pol II occupancy around the TSS of these genes, their appropriate expression and, therefore, robust myelopoiesis. This mechanism is impaired in DC

patients (Fig. 7C). Overall, our results provide molecular insights into DC pathogenesis and could serve as a basis for designing new strategies for therapeutic intervention, particularly in DC patients harboring mutations that affect the CR4-CR5 domain of telomerase RNA.

Materials and Methods

Animals. Zebrafish (*Danio rerio*, Cypriniformes, Cyprinidae) were obtained from the Zebrafish International Resource Center and mated, staged, raised, and processed using standard procedures (40). The transgenic line Tg(*mpx::eGFP*) (25) was kindly provided by S. Renshaw, University of Sheffield, Sheffield, UK. The Tg(*drl::terc*) fish line was generated by microinjecting the *drl::terc* [generated by MultiSite Gateway assembly of pCM293 (24)], pME-*terc*, p3E-SV40polyA, and pDestTol2pA2 of the Tol2kit (41) together with transposase mRNA into the cell of one-cell-stage zebrafish eggs. The *terc* KO zebrafish line and the *csf3b* promoter *tercbs1*-deleted mosaic fish were generated by TALEN-mediated deletion of the *terc* genomic locus (see Fig. 1A) (42).

RNA WISH. Wild-type and *terc*^{-/-} embryos were used for RNA WISH. *terc*, *spi1b*, *gata1a*, and *rag1* RNA probes were generated using the DIG RNA Labeling Kit (Merck) from linearized plasmids. Embryos were imaged using a Scope.A1 stereomicroscope equipped with a digital camera (AxioCam IC 3, Zeiss).

Immunofluorescence. Cells from wild-type and *terc*^{-/-} embryos were stained with a monoclonal antibody against zebrafish Cd41 and analyzed by flow cytometry as previously described (43).

Cloning of Zebrafish Myelopoietic Genes Regulatory Regions and *tercbs* Deletions. The regulatory regions cloning of the *csf3a* 2-kb promoter region has been already described (19). The *csf3b* 2-kb, *spi1a* 2-kb, and *spi1a* 2-kb 3' regulatory regions were amplified from zebrafish genomic DNA using Phusion High-Fidelity DNA Polymerase (New England Biolabs). For deleting the *tercbs* present in the *csf3b* promoter and *spi1a* 2-kb 3' regulatory region, a two-step overlapping PCR strategy was used. They were then cloned into pGL3basic vector (containing firefly luciferase, Promega).

Dual Luciferase Assay. Luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) in a Luminometer Optocomp I (MGM Instruments) in dissected tails of at least 30 larvae of 48 hours post fecundation per experimental condition (44).

ChIRP. ChIRP experiments were performed in WKM as described elsewhere (20).

In Vitro *terc*-DNA Binding Assay. A 100-bp 3' biotinylated DNA probe of the zebrafish *csf3b* promoter encompassing the *terc* binding sites as well as 80-bp probes (same sequence but deleting the *terc* binding sites) bound to Dynabeads MyOne streptavidin C1 magnetic beads was incubated with 50 ng luciferase (Promega), wild-type *terc*, and CR4-CR5^M mutant RNAs; beads dsDNA-RNA complexes were washed, RNA eluted, reverse transcribed, and subjected to qPCR.

In Vitro Transcription Assay. In vitro transcription assays were performed using either HL60 nuclear extracts or recombinant proteins as previously described (45), with some modifications.

RNA Pull-Down. RNA pull-down experiments were performed with biotinylated RNA and extracts of 2 dpf larvae as described (32), with some modifications. The eluted proteins were subjected to polyacrylamide gel electrophoresis followed by Western blotting using anti-RNA Polymerase II CTD Repeat YSPTSPS (anti-Phospho S5 RNA Pol II) mouse monoclonal antibodies ([4H8], ab5408, Abcam).

RIP. RIP experiments were performed with 2 dpf larvae or human cells as described (32), with some modifications. The following antibodies were used: anti-p55 RNAPol II, anti-RNA polymerase II CTD repeat YSPTSPS (total RNAPol II, ab817, Abcam), anti-Dyskerin (DKC, sc-373956, Santa Cruz Biotechnologies), anti-TERT (600-401-2525, Rockland), or control antibodies (mouse IgG1, eBioscience or

anti-Histone H3 trimethyl-lysine 4 mouse antibodies ab1012, Abcam). Input and immunoprecipitated RNAs were extracted with phenol:chloroform, reverse transcribed, and subjected to qPCR for detection of expression of *terc* or of control RNAs (*rps11* or *GAPDH*).

PLA. PLA for specific RNA-protein interaction was performed as described (27). Briefly, HL60 cells seeded in poly-L-Lys coverslips were washed with phosphate-buffered saline (PBS) and fixed in 4% formaldehyde, permeabilized, and blocked. Cells were incubated with 100 nM TERC-specific oligonucleotide probes in fresh blocking buffer at 70 °C for 3 min. Subsequently, cells were washed three times with PBS and blocked in PBS-T (PBS 0.1% Tween 20) containing 1% bovine serum albumin and 20 µg/mL salmon sperm sheared (sss) DNA at room temperature (RT) for 1 h.

The samples were washed once with PBS, once with 300 mM NaCl, 30 mM sodium citrate buffer pH 7 (2xSSC) with 0.1% Tween 20, once with PBS, and then incubated with 1:100 anti-p55 RNAPol II, 1:100 of anti-TERT, or no antibody as control, in PBS-T at RT for 1 h. Then, the probe solution was prepared by diluting the corresponding species-specific minus PLA probe (Duolink PLA Fluorescence kit, Merck) 1:5 into PBS-T containing 20 µg/mL sssDNA and allowing the mixture to sit for 20 min at RT. After three washes with PBS, the coverslips were incubated with the probe solution for 1 h at 37 °C.

The following ligation, amplification and labeling steps were performed using the Duolink PLA Fluorescence kit (Merck), following manufacturer's instructions. Samples were washed twice and incubated with fresh ligation mix for 30 min at 37 °C, washed twice, and further treated with fresh amplification mix for 100 min at 37 °C. Finally, cells were washed twice and mounted onto glass slides in Duolink PLA Fluorescence mounting medium with DAPI. Images were captured with a Leica TCS SP8 confocal microscope.

ChIP. ChIP experiments were performed using the MAGnify Chromatin Immunoprecipitation System kit (Thermo Fisher Scientific) using cross-linked HL60 or U937 cells and anti-Phospho S5 RNA Pol II, total RNA Pol II, or mouse IgG antibodies.

Telomerase Activity Assay. To assess telomerase activity, a real-time quantitative telomerase repeated amplification protocol analysis was performed (46, 47).

iPS Cell Culture, Differentiation toward Hematopoietic Lineage, and CFU Assay. The human iPS cell maintained and differentiated as depicted in Fig. 6A. iPS cell lines were maintained in a feeder-free culture system with mTeSR Plus medium (Stem Cell Technologies). For hematopoietic differentiation, undifferentiated iPS cells at 70 to 80% confluence were treated with Matrigel 24 h before starting the differentiation. The medium was changed the next day (day 1) with the same differentiation medium supplemented with hematopoietic cytokines SCF, FLT3LG, IL-3, IL-6, G-CSF (all from R&D), and BMP4 (Miltenyi). EBs were dissociated and single-cell suspensions stained with anti-CD34-fluorescein isothiocyanate, anti-CD31-phycoerythrin, and anti-CD45-allophycocyanin (all from Becton Dickinson) antibodies and 7-actinomycin D and analyzed by flow cytometry. CFU assays were performed by plating 100,000 cells from EBs at day 15 into methylcellulose culture medium H4434 (Stem Cell Technologies).

Statistical Analysis. Data were analyzed by ANOVA and Bonferroni or Dunnett's posttest using GraphPad Prism software.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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