Dyskeratosis congenita: molecular insights into telomerase function, ageing and cancer

Anna Marrone and Inderjeet Dokal

Dyskeratosis congenita (DC) is a severe, inherited, bone marrow failure syndrome, with associated cutaneous and noncutaneous abnormalities. DC patients also show signs of premature ageing and have an increased occurrence of cancer. DC can originate through: (1) mutations in *DKC1*, which result in X-linked recessive DC; (2) mutations in the RNA component of telomerase (TERC), which result in autosomal dominant DC (AD-DC); and (3) mutations in other, currently uncharacterised, genes, which result in autosomal recessive DC (AR-DC). As *DKC1* encodes dyskerin, a protein component of small nucleolar ribonucleoprotein (snoRNP) particles, which are important in ribosomal RNA processing, DC was initially described as a disorder of defective ribosomal biogenesis. Subsequently, dyskerin and TERC were shown to closely associate with each other in the telomerase complex, and DC has since come to be regarded as a telomerase deficiency disorder characterised by shorter telomeres. These findings demonstrate the importance of telomerase in humans and highlight how its deficiency (through *DKC1* and *TERC* mutations) results in multiple abnormalities including premature ageing, bone marrow failure and cancer. Identification of the gene(s) involved in AR-DC will help to define the pathophysiology of DC further, as well as expand our insights into telomere function, ageing and cancer.

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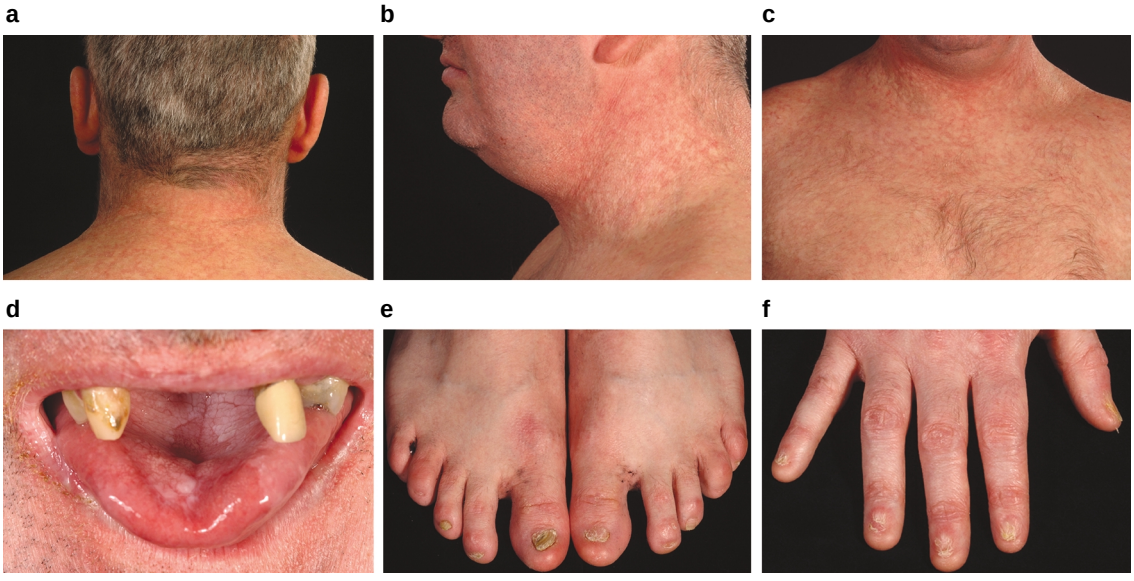
Dyskeratosis congenita (DC; also known as Zinsser–Cole–Engman syndrome) is a severe, inherited, bone marrow (BM) failure syndrome, with an estimated prevalence of approximately 1 per 1000 000 persons (Refs 1, 2, 3). The disease is characterised by a triad of abnormal skin pigmentation, mucosal leukoplakia (thick white patches on the tongue and mucous membranes) and nail dystrophy, as well as various noncutaneous abnormalities (Refs 4, 5, 6, 7) (Fig. 1; Table 1). Mortality in DC patients is usually a result of BM failure but there is also an increased occurrence of fatal pulmonary complications and malignancy (Refs 7, 8) (Table 1). The presence of these features, along with shorter telomeres, premature hair loss or greying, osteoporosis and other signs of ageing, has meant that DC has also been described as a premature ageing syndrome (Refs 9, 10).

In recent years, significant progress has been made in understanding the molecular basis of DC. Key findings include identification of the X-linked recessive gene *DKC1*, identification of the autosomal dominant (AD) gene *TERC*, and strong evidence (shorter telomeres, and *TERC* and *DKC1* mutations) linking DC with several other diseases: Hoyeraal–Hreidarsson (HH) syndrome, which is a multisystem disorder characterised by aplastic anaemia (AA), immunodeficiency, microcephaly, cerebellar hypoplasia and growth retardation; AA, in which the BM ceases to produce sufficient numbers of blood cells; and myelodysplastic syndromes (MDSs), which are a group of BM neoplastic diseases. This review summarises these genetic discoveries and explains how these revelations have given us molecular insight into telomerase function, ageing and cancer, as well as defining the pathophysiology of DC.

Clinical and genetic aspects of DC

DC is a clinically and genetically heterogeneous disease (Refs 7, 11). Three modes of inheritance have been found: X-linked recessive (MIM #305000), AD (MIM #127550) and autosomal recessive (AR) (MIM #224230) (Table 2). Clinically, X-linked recessive DC is more severe than the autosomal forms of DC, of which AD-DC is milder than AR-DC, although exceptions to this rule do exist (Refs 12, 13).

X-linked recessive inheritance of DC, the most common form of DC, is associated with a



Clinical characteristics of dyskeratosis congenita

Figure 1. Clinical characteristics of dyskeratosis congenita. Dyskeratosis congenita (DC) is characterised by a triad of cutaneous features and is also associated with many additional clinical features (Table 1). A selection of these features are shown in this 32-year-old X-linked recessive DC patient: abnormal skin pigmentation (a, b, c), premature greying of hair (a), leukoplakia (d), premature loss of teeth (d), and nail dystrophy (e, f).

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Table 1. Summary of clinical features associated with dyskeratosis congenita

Key clinical features	Percentage of patients affected
Main mucocutaneous triad	
Skin pigmentation	89%
Nail dystrophy	88%
Mucosal leukoplakia	78%
Additional clinical features[a]	
Bone marrow failure	85.5%
Pulmonary disease	20.3%
Premature loss of teeth	16.9%
Premature hair loss/greying	16.1%
Cancer	9.8%

[a] There are also many other somatic abnormalities in any given patient.

mutated *DKC1* allele in these patients (Ref. 14) (Fig. 2; Table 3). *DKC1* encodes dyskerin, which is highly conserved among eukaryotes. On the basis of functional analysis of its homologues, human dyskerin appears to be a multifunctional pseudouridylation protein that catalyses the isomerisation of uridine to pseudouridine in certain RNA molecules and is involved in ribosomal (r)RNA processing, ribosomal subunit assembly and/or centromere or microtubule binding (Refs 15, 16, 17, 18, 19). As such, dyskerin is a component of the small nucleolar ribonucleoprotein particles (snoRNPs), and binds small nucleolar RNA (snoRNA) (Refs 20, 21). SnoRNAs guide the snoRNP complex to the modification site of the target rRNA via sequences in the snoRNA that associate with the target site; the protein then catalyses modification (pseudouridylation in the case of dyskerin) of the rRNA. Characterisation of X-linked recessive DC patients has identified 41 mutations in *DKC1*, some of which overlap regions containing specific domains (Fig. 2; Table 3).

AD-DC is a rare subtype of DC where patients have been found to have a heterozygous mutated *TERC* allele (Ref. 10). *TERC* encodes the RNA component of the telomerase complex and is responsible for maintaining the telomere repeats at the ends of chromosomes (Refs 22, 23, 24). At present, 18 mutations have been identified within *TERC* (discussed in more detail below); most are located within the pseudoknot domain, which appears to be involved in catalytic activity, and many are predicted to reduce telomerase activity when present in vivo. However, owing to the high number of polymorphisms also detected within this relatively small molecule, each mutation requires biological clarification before stating whether these mutations cause DC.

Characterisation of DC and AD-DC has led to significant progress in understanding the molecular basis of DC (summarised in Table 2). The presence of *DKC1* mutations initially suggested DC was a disease of defective rRNA processing (Refs 19, 25, 26) (Fig. 3b). However, as both dyskerin and TERC closely associate together within the telomerase complex (Ref. 27), and thus the X-linked recessive DC and AD-DC mutations converge on this complex (Fig. 3c), DC has recently been reclassified as a defective telomere disorder. Indeed, patients with *DKC1* and *TERC*

Table 2. Summary of dyskeratosis congenita subtypes

DC subtype	Percentage within the DCR[a]	Disease phenotype	Gene characterisation		
			Chromosomal location	RNA/protein product	Mutations identified
X-linked DC	35%	DC, HH	Xq28	Dyskerin	41
AD-DC	5%	DC, AA, MDS	3q26	TERC	18
Uncharacterised	60%	DC, HH	Unknown[b]	Unknown	Unknown

AA, aplastic anaemia; AD, autosomal dominant; AR, autosomal recessive; DC, dyskeratosis congenita; DCR, dyskeratosis congenita registry; HH, Hoyeraal–Hreidarsson syndrome; MDS, myelodysplastic syndrome; TERC, RNA component of telomerase.
[a] Approximate percentage according to the current DCR.
[b] Might represent more than one locus.

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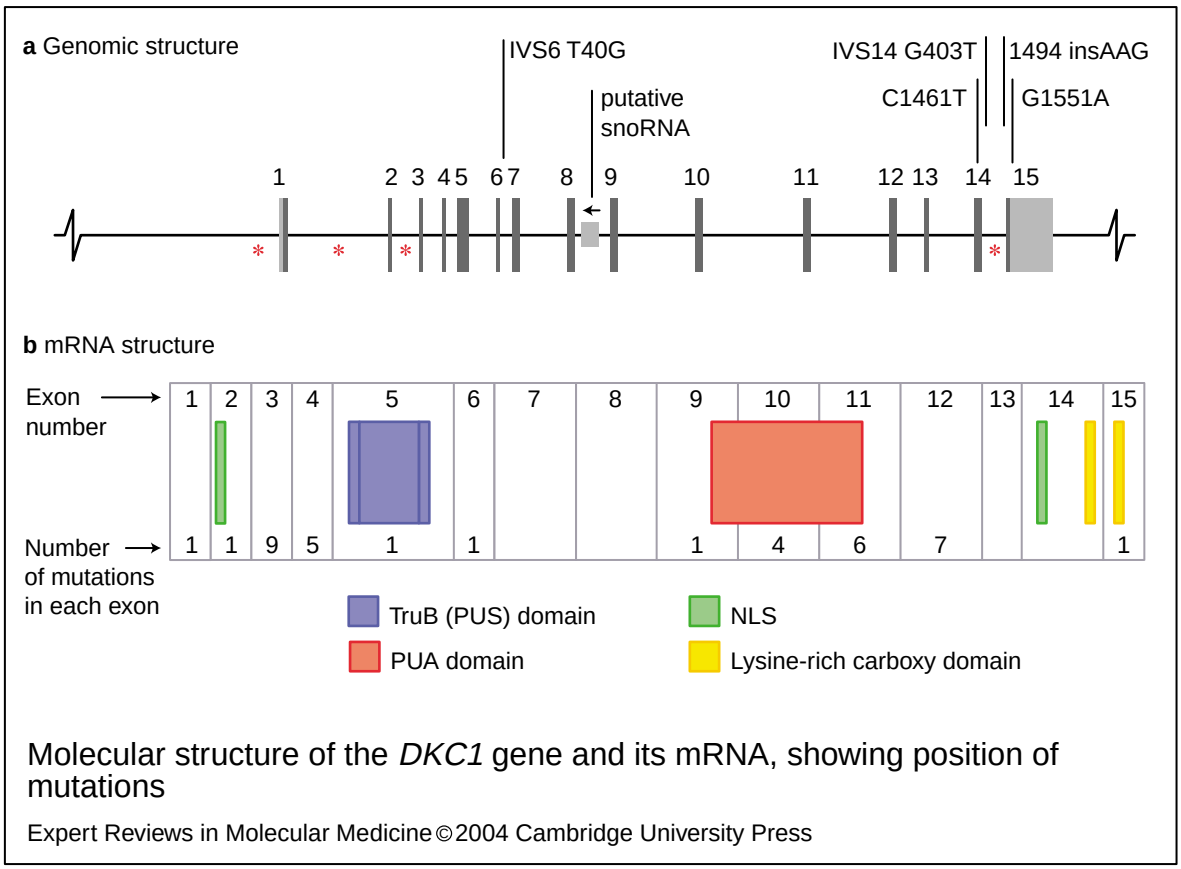

Molecular structure of the *DKC1* gene and its mRNA, showing position of mutations

Figure 2. Molecular structure of the *DKC1* gene and its mRNA, showing position of mutations. Characterisation of X-linked recessive dyskeratosis congenita (DC) patients identified the ~17 kb *DKC1* gene as being responsible for the disease. The gene contains 15 exons that are transcribed into a ~2.5 kb mRNA, which is translated into the 514 amino acid (~57 kDa) dyskerin protein. Forty-one mutations have currently been described for this gene: 37 are in exons and four in noncoding regions (see Table 3). (a) The genomic organisation of the *DKC1* gene is shown, together with the putative small nucleolar RNA (snoRNA) located in intron 8, and the approximate positions of the four mutations in the noncoding regions (asterisks; C–142G in the promoter, C592G in intervening sequence 1 (IVS1), IVS2 C473G and IVS14 A473G), and five polymorphisms (labelled above genomic structure). (b) The crystal structure of dyskerin has yet to be elucidated but several domains have been identified within its mRNA structure: nuclear localisation signals (NLSs; at 11–20 and 446–458 amino acids), a pseudouridine synthase domain [TruB/PUS; at 107–247 amino acids (truB I at 91–104 and TruB II at 121–134 amino acids)], a pseudouridine synthase and archaeosine-specific transglycosylase domain (PUA; at 296–371 amino acids) and lysine-rich carboxy domains (at 474–479 and 498–506 amino acids). Purple numbers on the mRNA structure indicate the number of mutations assigned to each exon; some overlap regions containing a specific domain (i.e. exons 5, 9, 10, 11 and 15), but half are located in exons that have no apparent conserved function.

mutations have been found to have shorter telomeres than age-matched controls (Refs 10, 28), whereas no significant changes were found in their snoRNA accumulation or function (Ref. 27). The clinical features of DC patients also appear to develop within tissues that are predicted to have high cellular renewal or proliferation and therefore high levels of telomerase activity (i.e. skin, oral mucosa and BM). This suggests that the pathophysiology of DC is consistent with the dysregulation of telomerase activity within these tissues (Refs 27, 29).

Telomeres and telomerase

Telomeres are essential for maintaining genome integrity in linear chromosomes (Ref. 30) (Fig. 4). These multifunctional 6 bp repeat structures cap eukaryotic chromosomes to protect them from

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Table 3. Summary of mutations identified in *DKC1*

Mutation	Amino acid substitution	Exon	Disease	Refs
C–142G	–	Promoter	DC	122
C5T	A2V	1	DC	123, 124
IVS1 C592G	–	IVS1	DC	122
C29T	P10L	2	HH	125
IVS2 C473G	–	IVS2	DC	123
C91G	Q31E	3	DC	126
T106G	F36V	3	DC	14
109–111 ΔCTT	ΔL37	3	DC	14
T113C	I38T	3	HH	127
A115G	K39E	3	DC	123
C119G	P40R	3	DC	14
G121A	E41K	3	DC	123
A127G	K43E	3	DC	128
C146T	T49M	3	HH	129, 130
G194C	R65T	4	DC	123
A196G	T66A	4	DC	123
C200T	T67I	4	DC	125
C204A	H68Q	4	DC	125
CT214–215TA	L72Y	4	DC	14
A361G	S121G	5	HH	129
C472T	R158W	6	DC	122
A838C	S280R	9	DC	122
A941G	K314R	10	DC	125
C949T	L317F	10	DC	131
C961G	L321V	10	DC	123
G965A	R322Q	10	DC	131
T1049C	M350T	11	DC	123
G1050A	M350I	11	DC	123
C1058T	A353V	11	DC, HH	123, 128, 132, 133, 134
G1075A	D359N	11	DC	125
C1150T	P384S	11	DC	131
C1151T	P384L	11	DC	122
G1156A	A386T	12	DC	125
T1193C	L398P	12	DC	135
G1204A	G402R	12	DC	123
G1205A	G402E	12	DC	14
C1223T	T408I	12	DC	125
C1226T	P409L	12	DC	136
AG1258–1259TA	S420Y	12	DC	125
IVS14 A473G	–	IVS14	DC	125
Δ Exon 15	Δ493–514	15	DC	6

Abbreviations: DC, dyskeratosis congenita; HH, Hoyeraal–Hreidarsson syndrome; IVS, intervening sequence.

degradation and end-to-end fusions. They also ensure the complete replication of the genome; during normal semi-conservative DNA replication, the lagging strand of each linear chromosome fails to replicate fully at the end of each cell cycle, and the 6 bp telomere sequences can be lost without affecting the rest of the gene repertoire. In addition, telomeres allow cells to distinguish chromosomal ends from double-stranded DNA breaks, and therefore prevent the unnecessary application of DNA-damage checkpoint cascades.

Telomerase is a large, mainly uncharacterised, RNA–protein complex that is responsible for the de novo synthesis and maintenance of telomere ends. The basic telomerase core complex consists of the RNA component (TERC), which contains the template region required for addition of

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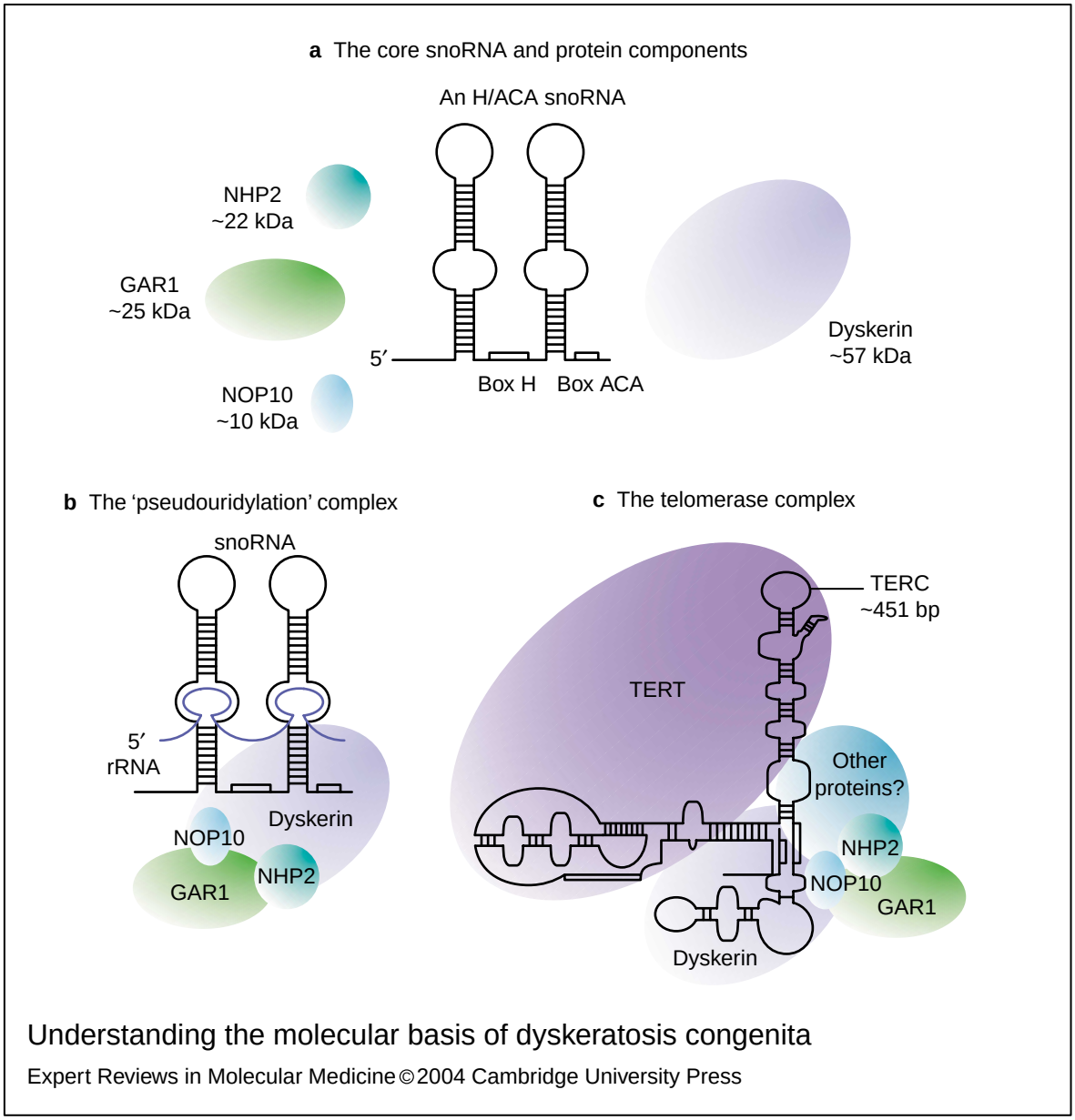

Figure 3. Understanding the molecular basis of dyskeratosis congenita. Three modes of inheritance of dyskeratosis congenita (DC) have been identified: X-linked recessive, autosomal dominant (AD)-DC and autosomal recessive (AR)-DC (Table 2). Initial investigations found mutated *DKC1* genes in patients with X-linked recessive DC. *DKC1* encodes the protein dyskerin, which is a protein component of small nucleolar ribonucleoprotein (snoRNP) particles also comprising GAR1, NHP2, NOP10 and an H/ACA snoRNA (identified by the box H and box ACA motifs) (a). This complex is important in pseudouridylation and ribosomal (r)RNA processing (b), suggesting DC might be a defective ribosomal biogenesis disorder. However, more-recent investigations have found that *TERC* is mutated in patients with AD-DC. TERC (the RNA component of telomerase) is an H/ACA snoRNA that also associates with dyskerin, GAR1, NHP2 and NOP10, along with TERT (telomerase reverse transcriptase) to form the telomerase complex (c). This suggests that DC is also a defective telomerase disorder because all currently characterised DC mutations converge on the telomerase complex. At present, AR-DC remains uncharacterised but by identifying the loci that induce AR-DC, it is hoped that the critical pathways (either pseudouridylation and/or telomere maintenance) in DC will be elucidated. Abbreviations: GAR1, glycine and arginine rich domain 1 [also known as nucleolar protein family A member 1 (NOLA1)]; NHP2, nonhistone protein 2 (also known as NOLA2); NOP10, novel nucleolar protein 10 (also known as NOLA3).

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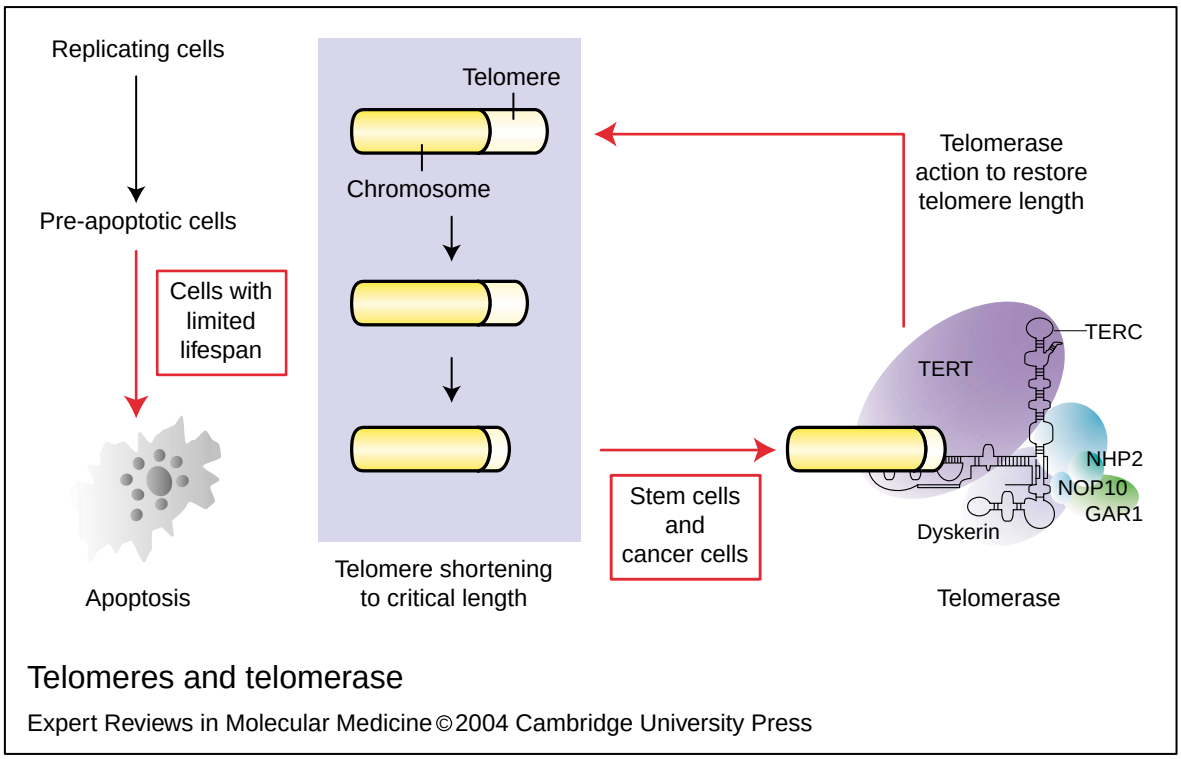

Figure 4. Telomeres and telomerase. During normal DNA replication, the lagging strand of each linear chromosome fails to replicate fully at the end of each cell cycle (highlighted in mauve box). To prevent loss of viable coding DNA, 6 bp repeats (telomeres) exist at the ends of chromosomes. These can be safely lost, extending the life cycle of each cell, until a critical length is reached and the cell enters senescence or apoptosis (indicated by the red arrow on the left). In cells that require an extended cell life, such as stem cells, the germline and also the majority of cancerous cells, the systematic loss of telomere repeats can be avoided through the activation of telomerase. This enzyme acts to re-establish the 6 bp repeats on the telomeres to prevent the cell from entering senescence or apoptosis prematurely (indicated by the red arrows on the right).

multiple 6 bp (TTAGGG) telomere repeats, and the enzymatic component (TERT), which acts as the reverse transcriptase for this reaction (Refs 31, 32, 33, 34). The pseudoknot domain of the TERC RNA molecule contains an 11 bp template region (5'-CUAACCC*UAAC*-3') (Fig. 5). It has been suggested that the alignment domain (italics) hybridises to the 3' terminus of the DNA substrate and positions the telomere ready for extension (Refs 35, 36, 37).

Usually, telomerase expression is low or absent in most somatic tissues (Ref. 38), suggesting that these cells undergo slow telomeric DNA loss during each cell cycle (Fig. 4). At present, it is still unknown whether telomerase and telomeres function through the overall length of the telomere repeat or through the secondary structure the multiple 6 bp repeats form along with the telomere-associated proteins (Ref. 39). However, eventually critical telomere length or loss of the telomere secondary structure induces these cells to enter either senescence or apoptosis. This process is thought to act as a checkpoint to guard against genomic instability induced by telomeric loss and to prevent uncontrolled oncogenic cell growth (Refs 40, 41). By contrast, telomerase is believed to be expressed in stem cells and germline cells and is found to be upregulated in the majority of cancer cells, allowing the renewal of telomere length at each replication (Refs 42, 43) (Fig. 4). This ensures that tissues with high cell turnover, such as activated lymphocytes, germline cells and tissue stem cells, remain genetically viable throughout the lifespan of the organ (Refs 43, 44, 45, 46).

TERC mutations in DC

TERC is a highly structured RNA molecule. Although the sequence and length of TERC vary considerably (Ref. 47), vertebrate telomerase

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RNAs share a highly conserved secondary structure that can be divided into four domains: the pseudoknot domain; the H/ACA box and CR7 domains; the CR4–CR5 domain; and the hypervariable region (Ref. 48) (Fig. 5). The secondary structure for this RNA molecule has been determined by phylogenetic covariation analysis, compensatory mutational analysis and nuclear magnetic resonance analysis (e.g. Refs 49, 50, 51, 52).

DC is the first human disease identified to result from mutations within the TERC RNA molecule (Ref. 10). The 18 novel mutations so far identified in *TERC* have been found in DC, AA and MDS patients, as well as some wild-type (WT) controls (Fig. 5; Table 4). The first *TERC* mutation to be identified was the large 3' end deletion (378Δ→3'; O in Fig. 5 and Table 4), which substantiated the claim that mutated *TERC* underlies AD-DC disease (Ref. 10). The pseudoknot mutation GC107-8AG and the CR7 domain mutation C408G were also found to segregate with the disease in two other, unrelated, DC families (Ref. 10). Novel *TERC* mutations were then sequentially found in AA, DC and MDS patients from several different laboratories: G58A, C72G and 110–113ΔGACT (Ref. 53); C116T and C204G (Ref. 54); C–21T, G228A, G305A, G322A, G450A and T467C (Ref. 55); and 96–97ΔCT and G143A (Ref. 56). Although several papers have been published that describe the function of the secondary structure through deletion analysis of *TERC* and *Terc* (the mouse gene) (e.g. Refs 57, 58), at present only eight of these mutations have been shown to have an effect on telomerase activity within AD-DC patients (Refs 50, 51, 58, 59, 60, 61, 62). At present, the clinical data available suggest that *TERC* mutations act through haplo-insufficiency rather than a dominant-negative effect – that is, half the levels of WT telomerase activity are not sufficient for normal telomere maintenance (Refs 59, 62).

Recent investigations using mouse models have also suggested that *Terc* mutations assert their effect through haplo-insufficiency (Refs 63, 64). Knockout *Terc* mice were found to be viable for at least six generations (Ref. 65). It was thought that this lag was due to this laboratory mouse strain having abnormally long telomeres that took several generations to reach their critical length. By the sixth generation, phenotypic abnormalities similar to those seen in DC patients were reported. Features related to ageing appeared to be noticeable from the third generation (Refs 66, 67). Genetic crosses between *Mus musculus domesticus* (long telomeres of around 50 kb) and SPRET/Ei (short telomeres of around 10–15 kb) showed that telomere elongation was possible during normal development (Ref. 68) and the telomere length increases were mediated by telomerase (Ref. 63). Heterozygous *Terc* mice appeared to be unable to maintain their telomere length, suggesting that the limiting telomerase expression resulted in haplo-insufficiency. Additional research has gone on to show that the expression of *Terc*, the RNA component, and not *Tert*, the reverse transcriptase, is the rate-limiting step for murine telomere length maintenance in vivo (Ref. 64).

The promoter region

A novel C–99G mutation was found in a paroxysmal nocturnal haemoglobinuria (PNH) patient, and subsequent investigations found that this base substitution would disrupt one out of the four Sp1-binding sites in the core *TERC* promoter (Refs 69, 70). This result is interesting for two reasons: (1) it is the first mutation to be described within the promoter region of *TERC*; and (2) it is the first mutation to be described in a patient with PNH. PNH is a clonal blood disorder associated with AA and is characterised by the presence of one or more blood cell clones with a somatic *PIGA* mutation that leaves these cells without glycosylphosphatidylinositol (GPI)-anchored proteins (Refs 71, 72). It has been generally accepted that PNH development requires two factors: a *PIGA* mutation and BM failure. The detection of a *TERC* promoter mutation that disrupts *TERC* activity suggests that disrupted telomerase expression can contribute to the pathogenesis of PNH through BM failure.

The pseudoknot domain mutations

The pseudoknot and CR4–CR5 domains bind to TERT (Fig. 3; Fig. 5). Various studies have validated the presence of the pseudoknot region and have found it to be essential for catalytic function (Refs 48, 49, 57, 58, 73, 74, 75, 76), with bases 33–147 (along with 163–330 from the CR4–CR5 domain) being critical TERT-binding sites (Refs 74, 77).

The majority of mutations currently identified in *TERC* are located within the pseudoknot domain (Fig. 5). The mutations either alter the secondary structure of TERC, by disrupting the base pairing between the nucleotides, or prevent

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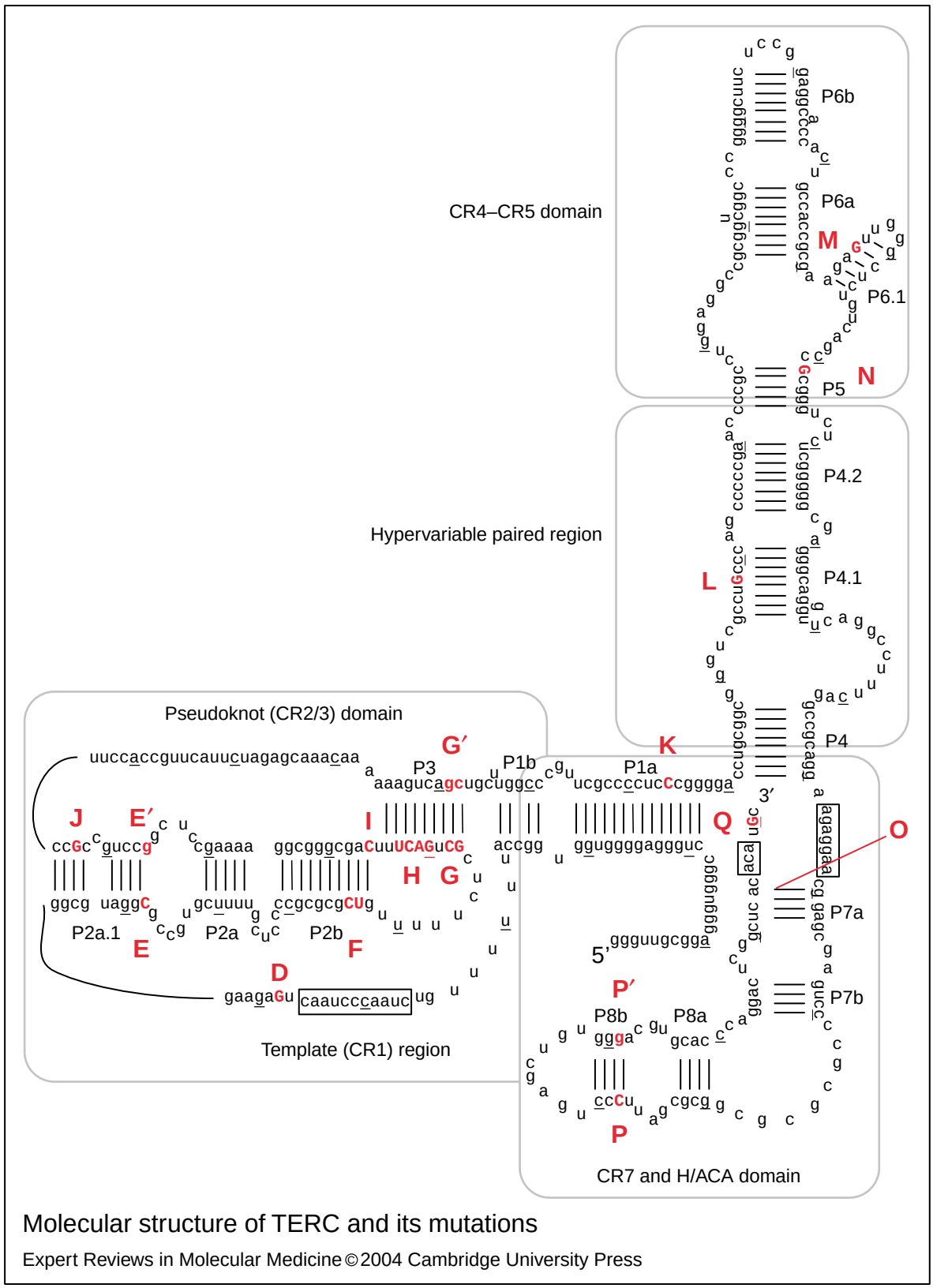

Figure 5. Molecular structure of TERC and its mutations. *(See next page for legend.)*

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Figure 5. Molecular structure of TERC and its mutations. *(Legend; see previous page for figure.)* The 451 bp TERC RNA molecule forms a specific secondary structure with four structural domains (dotted boxes): the pseudoknot, the CR4–CR5 domain, the hypervariable paired region and the CR7 and H/ACA domains. The pseudoknot and CR4–CR5 domains appear to control the catalytic function of TERC, whereas the CR7 and H/ACA domains are required for stability and localisation. At present, 18 mutations (A to Q: see Table 4) have been identified within this RNA molecule, with the majority located within the pseudoknot domain and predicted to reduce telomerase activity when present in vivo. Underlined nucleotides represent every 10th base; bold red uppercase nucleotides represent mutated sites found in DC patients (see Table 4); bold red lowercase nucleotides (E', G' and P') represent mutated sites designed to determine whether the primary sequence or secondary structure was critical for the loss of telomerase activity for the corresponding DC mutation (E, G and P). The artificial changes to TERC showed that the secondary stem structure was critical for in vitro telomerase activity.

Table 4. Summary of mutations identified in *TERC*

Position[a]	Mutation	Disease	Domain	Refs
A	~2 kbΔ to 316 nt	DC	5' UTR and 5' of TERC	56
B	C–99G	AA, PNH	5' UTR	69
C	C–21T	WT	5' UTR	55
D	G58A	DC, AA, IAA, MDS, WT	Near template	53, 55, 92
E	C72G	CAA	Pseudoknot stem P2a.1	53
F	96–97ΔCT	DC	Pseudoknot stem P2b	56
G	GC107–108AG	DC	Pseudoknot stem P3	10
H	110–113ΔGACT	CAA	Pseudoknot stem P3	53
I	C116T	IAA	Pseudoknot stem P2b	54
J	G143A	MDS	Pseudoknot stem P2a.1	56
K	C204G	IAA	Pseudoknot stem P1a	54
L	G228A	ALT, DC, AA, WT	CR4–CR5 domain stem P4.1	55, 88, 92
M	G305A	NSAA	CR4–CR5 domain stem P6.1	55
N	G322A	MDS	CR4–CR5 domain stem P5	55
O	378Δ→3'	DC	H/ACA domain	10
P	C408G	DC	CR7 domain stem P8b	10
Q	G450A	SAA	H/ACA domain	55
R	T467C	AA	3' UTR	55

[a] See Figure 5.
Abbreviations: AA, aplastic anaemia; ALT, alternative lengthening telomerase; CAA, constitutional aplastic anaemia; DC, dyskeratosis congentia; IAA, idiopathic aplastic anaemia; MDS, myelodysplastic syndrome; NSAA, nonsevere aplastic anaemia; nt, nucleotides; PNH, paroxysmal nocturnal haemoglobinuria; SAA, severe aplastic anaemia; UTR, untranslated region; WT, wild type (normal).

binding to TERT. Initial investigations in vitro (rabbit reticulocyte lysate assays) and in vivo (WI-38 VA13 telomerase-negative cell transfection studies) into four of the pseudoknot domain mutations (C72G, 96–97ΔCT, GC107–108AG and 110–113ΔGACT) have shown that reconstituted telomerase activity is reduced by different rates, such that deletion mutations appear to be more severe in their effect than nucleotide substitution mutations (Refs 59, 62). It has been proposed that the drop in telomerase activity with these TERC mutations is due to the mutations compromising the catalytic function of the telomerase complex rather than to the accumulation of the mutated TERC RNA (Refs 51, 58, 59, 62).

Could these changes in structural conformation act as a molecular switch in telomerase function? Investigations into C72G and GC107–108AG (along with C408G) suggested that disruption of the TERC secondary structure, rather than the change to the primary sequence, induced the reduction in telomerase activity (Refs 51, 58, 62). Additional biophysical experiments have shown that the GC107–108AG mutation reduces telomerase activity by hyperstabilising the P2b and P3 pseudoknot stem structure so that it cannot

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shift between its two conformations (Refs 61, 78). Studies have shown that the pseudoknot exists in two states: (1) where the stem forms the P3 and P2b loops (WT conformation 1); or (2) where the P3 stem dissociates and allows the P2b stem to extend further along the TERC RNA molecule (WT conformation 2) (Ref. 61). Normally, the TERC RNA molecule coexists in equilibrium because there is no change in relative energy required to switch between these two different conformation states. In the presence of the GC107–108AG mutation, although the TERC RNA molecule can still form both conformations similar to WT TERC, the energy required to switch between the two states causes the mutated TERC molecules to stabilise within the second conformation state (GC107–108AG conformation 2). It has been proposed that the lack of switching between the two states is the cause for the reduction in telomerase activity seen with this mutation (Refs 50, 51, 58, 59, 62), and therefore explains the shortened telomeres observed within patients with this mutation (Ref. 28).

The CR4–CR5 domain mutations

As with the rest of the TERC molecule, mutation analysis, deletion analysis and structural analysis have validated the presence of the CR4–CR5 domain base-paired structure. Recent studies have found that the junction between base-paired regions P5 and P6 in the CR4–CR5 domain forms an additional stem–loop structure called P6.1 (Refs 58, 79) (Fig. 5). Since nucleotides 163–330 of this domain are required for telomerase activity, it was proposed that this region might also be required for RNA–RNA interactions. Mobility-shift assays have shown that the P6.1 stem–loop structure and sequence are required for in vitro RNA–RNA interaction, which appears to occur at the template region (Ref. 80). This suggests either that the TERC molecular model previously proposed is 'too loose' or that the stem–loop structure is required for TERC dimerisation (Ref. 81) (Fig. 6).

At present, it is believed that the telomerase complex contains at least two TERC RNA molecules and two TERT molecules, but how these interact with each other is not clear. Research currently suggests that telomerase acts as an interdependent multimer containing at least two binding sites (Refs 82, 83, 84). It appears that TERC can dimerise in vitro (Ref. 58) and that the internal J7b–8a loop (the junction between P7b and P8a; see Fig. 5) from the CR7 domain might be involved in RNA–RNA interaction (Ref. 60), along with a region located within the CR4–CR5 domain and pseudoknot (Ref. 58). These data suggest that the current model for the TERC secondary structure and binding to TERT might be incomplete and, therefore, that the telomerase complex could bind in two very different conformations (Refs 60, 80, 81) (Fig. 6).

Three mutations have been located to the CR4–CR5 domain (Table 4); however, only the G228A mutation has been investigated and it appears to have no specific effect on in vitro or in vivo reconstituted telomerase activity (see below). Further research is still required to establish the effect of these new mutations in the patients. This will aid our understanding of telomerase function and hopefully help to elucidate the final components for the telomerase complex and how they interact together to form this important enzyme.

The H/ACA domain and CR7 domain mutations

The H/ACA box and CR7 domains appear to be required for nucleolar localisation, 3'-end processing and RNA stability (Refs 85, 86, 87). These two domains have been shown to be dispensable for in vitro reconstitution of telomerase activity (Ref. 77). Deletions and base-pair substitutions within the CR7 domain reduce TERC accumulation, which ultimately leads to the observed reduction in telomerase activity (Refs 59, 62, 76). This has also been found to be true for two DC families who have a base-pair substitution (C408G) and a large 3' deletion (378Δ→3'); during in vitro reconstitution, these mutations led to WT levels of activity, but activity was severely reduced after reconstitution in vivo (Refs 59, 62). It appears that the C408G substitution causes a reduction in telomerase activity through unstable TERC RNA that fails to accumulate in the cell and therefore fails to produce functional telomerase complexes (Refs 50, 59). Recent data suggest that this is induced by a change in the secondary structure rather than a change in primary sequence (Ref. 62).

As mentioned above, the CR7 domain might also play a role in TERC RNA–RNA interactions (Ref. 60), implying that mutations in this domain are more likely to alter telomerase activity through destabilisation of the telomerase complex rather than a direct change in telomerase activity levels. This would explain why there is no reduction in

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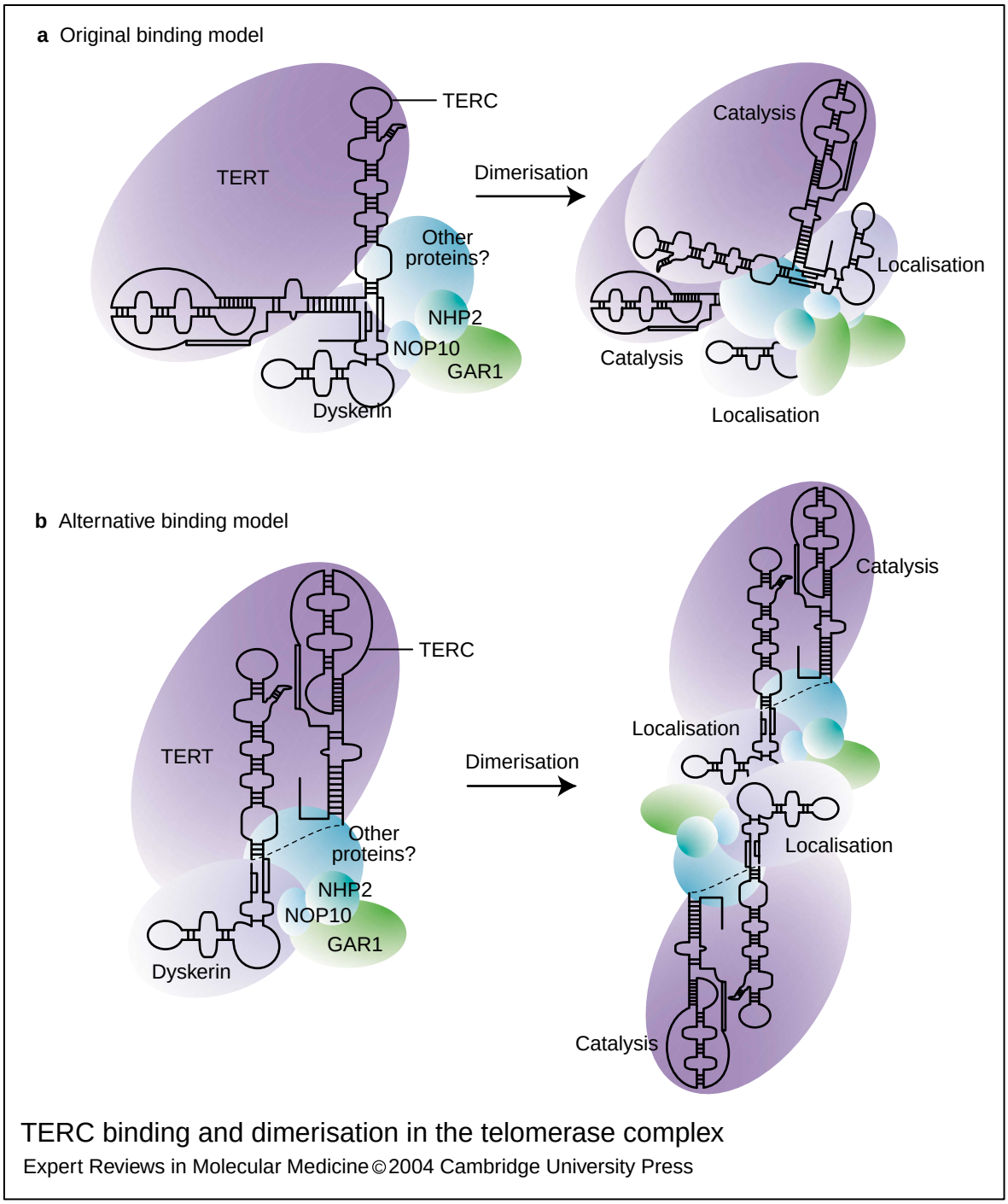

TERC binding and dimerisation in the telomerase complex

Figure 6. TERC binding and dimerisation in the telomerase complex. Traditionally, TERC is thought to have a specific secondary structure (Fig. 5) and has been predicted to form a specific RNA–protein complex (a), but recent research has suggested that the pseudoknot domain binds to the CR4–CR5 domain (b). However, the model shown in part (a) could still hold true if the pseudoknot and CR4–CR5 domain binding occurred between two separate RNA molecules. It has been predicted that the CR7 domain also remains in contact with the CR7 domain of the second TERC molecule during dimerisation, which is also possible within the two proposed binding models. In each case, the pseudoknot and CR4–CR5 domains form the catalytic part of the complex, whereas the CR7 and H/ACA domains form the localisation/stabilisation part of the complex. Additional research is required to determine whether pseudoknot and CR4–CR5 domain binding occurs inter-molecularly (a) or intra-molecularly (b).

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telomerase activity with these mutations within the in vitro assays. In the in vitro system, only TERC and TERT, and not additional telomerase components, are present, and so the 3' end of the TERC RNA is redundant. When assayed in vivo, the additional telomerase components interact with the 3' end of the TERC RNA, and therefore the mutations located in this area can alter telomerase activity through disruption of the telomerase complex itself rather than altering the catalytic function of TERC and TERT (Refs 59, 62).

Polymorphic sites in TERC

Even at this early stage of investigation, it is becoming apparent that TERC could be a highly polymorphic RNA molecule: polymorphisms have been identified at G228A, G58A and C–21T. The G228A mutation was originally found in the human cell line GM847 (Ref. 88), which maintains its telomere length through the alternative telomere maintenance (ALT) pathway rather than through telomerase activity. This pathway was initially recognised in approximately a third of in vitro human cell lines that were immortalised and had abnormally long telomeres, yet had no detectable telomerase activity (Ref. 89). The biological mechanism behind the ALT pathway is still to be fully elucidated but it does not appear to be activated in response to inactivated TERC, TERT or telomerase and might involve a type of sister chromatin recombination mechanism (Refs 90, 91). The G58A mutation lies just 3' of the template region (Ref. 53). This suggested that the G58A and G228A mutations might leave the resulting TERC RNA molecules nonfunctional. However, since the initial identification of mutations G58A and G228A, they have been found at polymorphic frequencies in African-American populations (Refs 55, 92). Subsequent investigations have also shown that both G58A and G228A mutations result in normal telomerase activity in vitro and in vivo (Refs 58, 62), although one study has indicated that the G58A mutation resulted in a fivefold reduction in activity (Ref. 59).

C–21T has been found only in WT controls, so it is very likely that this nucleotide substitution also results in WT telomerase activity. The fact that it currently appears that two TERC mutations result in WT levels of telomerase activity highlights the need to investigate fully any mutations using segregation analysis and biological assays before assigning them as DC-causing mutations.

Does telomerase deficiency predispose to cancerous growth?

It appears that DC-specific *TERC* mutations alter telomerase activity through (1) reducing the catalytic efficiency of the complex, or (2) decreasing TERC stability or (3) disrupting the assembly of the telomerase complex. If DC is a result of defective telomerase activity and is associated with increased occurrences of cancer, this leads to an apparent paradox regarding telomerase activity and cancer. High telomerase expression is usually associated with cancers (~85% of tumours are telomerase-positive; Ref. 42) which has made the development of anticancer drugs through manipulation of telomerase activity appealing. By contrast, DC patients have a high incidence of cancer yet it has been predicted that they would have deficient telomerase activity.

Bessler et al. (Ref. 93) have proposed a model for DC pathogenesis that might explain this inconsistency. For DC patients, critically short telomeres appear to be the principal cause of BM failure. As these telomeres reach a critical length, a checkpoint is triggered that induces irreversible cell-cycle arrest (senescence) and cell death (apoptosis) in the rapidly dividing cells, which then leads to the recruitment of additional quiescent stem cells into the cell cycle. *TERC* mutations affect the maintenance of telomere length whereas *DKC1* mutations destabilise TERC and/or reduce rRNA processing of these recruited stem cells. Additional telomere shortening in these cells then leads to increased genomic instability, cell crisis, senescence and eventually apoptosis, whereas the constant activation of stem cells exhausts this supply of progenitor cells. In some rare cases, the recruited stem cells manage to maintain their telomere integrity long enough for these cells to pass through their cell crises, and these clones survive to act as potential malignant cells (Fig. 7). This model also helps to explain the apparent increased risk of cancer as the normal human population ages: the accumulation of cellular damage, changes in metabolic rates and oxidative stress over time in normal cells increases the risk of malignancy and cellular senescence (Ref. 94).

This suggests that the majority of telomere-dysfunctional cells undergo senescence and/or apoptosis, thereby inhibiting cancer formation by preventing these cells from dividing further (Ref. 95). Yet, for tissues to function normally, it is required that the rate of cellular loss is equal to

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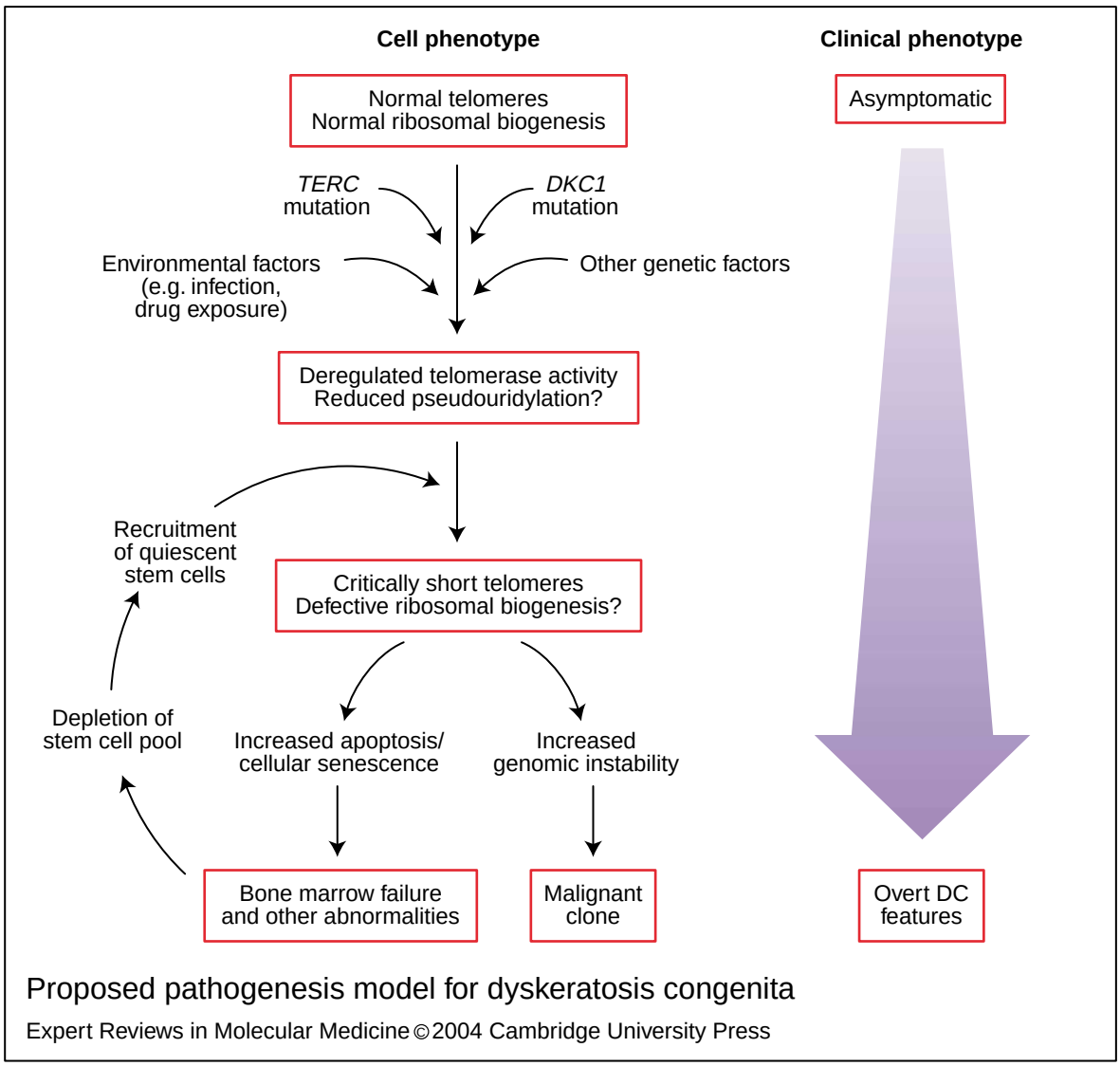

Figure 7. Proposed pathogenesis model for dyskeratosis congenita. Dyskeratosis congenita (DC) mutations converge on the telomerase complex, suggesting that this disease is principally due to defective telomerase activity within rapidly dividing cells, where telomerase function is critical. Yet, one of the somatic features of DC is the increased risk of cancer, which is usually associated with increased telomerase expression. Here we have tried to demonstrate how these two features can occur within the same disease. Following a mutation in *TERC* or *DKC1*, or other uncharacterised genetic or environmental factors, normal cells undergo deregulated telomerase activity and possibly reduced pseudouridylation, which results in shortened telomeres and possibly defective ribosomal biogenesis. The critical telomere shortening leads to increased cell death and chromosomal instability. This progressively leads to depletion of cells (including stem cells) and the development of disease features. The reduction of cells can be expected to lead to recruitment of quiescent stem cells, which contributes to further telomere shortening and genomic instability resulting in yet further loss of cells. Some cells survive the crises and acquire additional genetic changes that lead to the emergence of malignant cells.

the rate of cellular renewal. When either cellular loss is increased or tissue repair is retarded, the clinical outcome is weakness, ageing and eventually death. Therefore, the lack of cellular renewal after stem cell activation in the tissues affected by deficient telomerase activity in DC patients ultimately leads to ageing of these tissues, suggesting that cancer and ageing are linked in vivo (Ref. 96). This puts the therapeutic effects of telomerase-derived therapies into perspective.

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If such a drug is to work, it has to be tightly regulated and highly specific to the cancerous tissue, because the therapy itself might otherwise lead to sterilisation (defective germline development), premature ageing and BM failure.

Does pseudouridylation still have a role to play in DC?

Although dyskerin and TERC closely associate together in the telomerase complex, dyskerin itself is predicted to play an important role in pseudouridylation, a key step in rRNA processing (Fig. 3). The known protein components of telomerase (GAR1, NHP2 and NOP10) are also present as core components of the snoRNP (Ref. 25) (Fig. 3). At present, the precise pathophysiology of X-linked recessive DC remains unclear. Although DC is now regarded as being largely a disease of defective telomere maintenance, the role of defective pseudouridylation cannot be excluded. The latest research in mice seems to suggest that pseudouridylation might still have an important role to play in the pathophysiology of DC.

Targeted mutagenesis of murine *Dkc1* has shown that a lack of dyskerin is embryonically lethal (Ref. 97). As it is already known that *Terc*-knockout mice are viable for at least six generations, these effects of mutated murine dyskerin are unlikely to be due to defective telomerase activity. Mice with a hypomorphic mutation of *Dkc1* had reduced levels of pseudouridine, slower rRNA processing but retained normal telomere lengths (Ref. 98). As laboratory strain mice have exceptionally long telomeres, and overexpression of telomerase in murine haematopoietic stem cells has no effect on cell lifespan in vivo, it has been suggested that telomere length is not a crucial part of murine ageing (Refs 99, 100) and therefore it is possible that other pathways, such as pseudouridylation, are more important. Meanwhile, investigations into EBV-transformed cells from X-linked recessive DC patients have shown no detectable snoRNA accumulation, pseudouridylation or rRNA-processing defects, but did show a reduction in TERC accumulation and telomerase activity (Refs 27, 29).

RNA modification takes up a large part of cellular function. It has been estimated that 3–11% of the coding capacity of eukaryotes, archaea and bacteria genomes is devoted to modifying RNA (Ref. 101), and therefore RNA-modifying enzymes are believed to be one part of the minimal set of molecules required for life (Ref. 102). Pseudouridine synthases modify a specific uridyl residue in RNA by disconnecting the nucleobase and ribose moieties, rotating it and then rejoining it without the aid of cofactors (Ref. 103). Pseudouridine is the most abundant post-transcriptional nucleobase modification in cellular RNAs (Ref. 104). Dyskerin is a member of the TruB enzyme family, such that the RNP it is associated with is responsible for many pseudouridine residues in rRNA (Ref. 105). Given the importance of this function, how could mutations in this enzyme lead only to visible telomerase deficiency in DC patients and not alter pseudouridylation in humans as seen with mutated *Dkc1* mice? Does such an important biological pathway have an alternative route in humans? Is the biological activity of dyskerin so high that any reduction has no significant physiological effect?

Such questions might be answered by the presence of a PUA (for 'pseudouridine synthase and archaeosine-specific transglycosylase') domain as well as a TruB domain (Refs 14, 18, 106, 107) in dyskerin. The PUA domain is required for tRNA-guanine transglycosylase (TGT) enzymes to catalyse the post-transcriptional replacement of a guanine base with a 7-deazaguanine derivative. Sharing of domains between unrelated RNA-modifying enzymes is not uncommon (Ref. 101) but does this mean that other enzymes could biologically 'fill the gap' induced by the loss of dyskerin function in DC patients? Further studies are necessary to determine the significance of the presence of a PUA domain, and to establish the relative contribution of telomere dysfunction and defective pseudouridylation in the pathogenesis of X-linked recessive DC (Ref. 108).

Research in progress and outstanding research questions

Correct diagnosis of DC and other BM failure syndromes

Although relatively few DC patients show the AD mode of inheritance, many related syndromes appear also to have a mutated *TERC* allele. Idiopathic AA patients (IAA) also show shortened telomeres compared with age-matched controls (Refs 109, 110, 111). As the multiple clinical features seen in DC are genetically induced by mutations within a single gene, it is feasible to screen numerous patients with other related BM syndromes. *DKC1* and *TERC* mutations have since

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been found in a selection of different BM failure syndromes. This suggests that the severity of the disease can range from HH disease [very severe (Ref. 112)], through to DC (severe), through to patients with relatively late-onset AA (Refs 53, 54, 111). Additional investigations have begun to link MDS and DC together through a common link of *TERC* mutations (Refs 55, 56).

With such clinical and genetic variation in one disease, correct diagnosis in new DC cases can be very complicated. Further investigations into the DC and AA families characterised with *TERC* mutations have suggested that there is 'disease anticipation', whereby a disease increases in severity in successive generations such that children show an earlier age of onset and experience a more rapid development of the disease. Indeed, there appears to be an earlier onset of disease features in successive generations, with older generations remaining relatively asymptomatic compared with the younger, symptomatic generations, possibly as a result of progressively shortening telomeres in the younger generation (Ref. 56). This is consistent with the lag of phenotype seen in murine heterozygous and null *Terc* models (Refs 63, 65). DC disease anticipation can therefore make the disease appear to be 'nonfamilial' because the parents might be asymptomatic at the time of first presentation. Furthermore, diagnosis of DC based on clinical criteria can be difficult in patients developing haematological features before cutaneous ones. Whereas the recent genetic advances mentioned within this review are facilitating clinical diagnosis, all is still highly dependent on the clinician correctly identifying the presence of a BM failure syndrome in patients and relatives who might show little or no symptoms.

For patients developing severe BM failure, current treatments are limited to the use of BM transplants. As for other diseases, results are better using matched sibling donors, but these are usually not available for most patients. The identification of mutated *DKC1* and *TERC* genes in X-linked and AD-DC cases, respectively, now makes DC an attractive candidate for gene therapy, particularly as there are several lines of evidence that suggest phenotypically corrected cells should possess a growth/survival advantage over affected cells. In this respect, DC shares this characteristic with the inherited immunodeficiency disease X-SCID, in which retroviral-mediated gene therapy is now a clinical reality (Ref. 113).

Genetic characterisation of the AR-DC families

Further clarification of the relative roles of defective telomere maintenance and/or ribosomal biogenesis in the pathogenesis of X-linked recessive DC might come from the genetic characterisation of AR-DC families. Currently, no genetic mutations have been found to cause AR-DC. Screening of obvious candidate genes (e.g. *GAR1*, *NHP2*, *NOP10*) in AR-DC families have failed to yield any coding mutations to date (Refs 12, 13). Several other candidate genes still require investigating but no consistent areas of linkage have been determined in the current set of AR-DC families investigated (A. Walne et al., Dokal laboratory, unpublished). The hope is that, as with the mouse models, genetic characterisation of the AR-DC families will help to determine the critical pathway(s) altered in DC and answer the question of whether DC is a defective telomerase disorder and/or an impaired pseudouridylation syndrome.

Additional telomerase and telomere research

Despite the recent spate of research into telomerase function, and its important role in chromosome maintenance, cellular immortalisation and oncogenesis, still surprisingly little is known about the ribonucleoprotein structure, expression, activation and repression. To understand the biogenesis and function of telomerase, it is desirable to characterise the subcellular localisation and intracellular trafficking of telomerase core components, especially as telomerase is such an atypical polymerase molecule. The latest research has shown that TERT localises throughout the nucleoplasm but is concentrated in the nucleoli (Refs 114, 115, 116), whereas in telomerase-positive cells TERC has been localised to the Cajal bodies, another relatively uncharacterised nuclear compartment, where it is proposed that assembly and/or function of human telomerase might occur (Ref. 117). This suggests that TERC might be a small Cajal body (sca) RNA that guides pseudouridylation of pre-rRNA within the nucleolus or small nuclear RNAs (snRNAs) within the Cajal body itself, and suggests that the Cajal body is a site of telomerase biogenesis and/or function (Refs 117, 118, 119). Additional investigations are now required to determine how TERC and TERT form active telomerase complexes that go on to function

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spatially and temporally within the telomerase biogenesis pathway. Spatially, investigations in yeast are suggesting that telomerase biogenesis requires several different subcellular compartments: the nucleolus, nucleoplasm and cytoplasm (Ref. 120). Temporal regulation appears to be regulated by a complicated set of positive and negative regulators. So far, Sip1, Mad1, Menin, RAK and BRIT1 have been found to repress TERT expression whereas hSIR2 acts to promote TERT expression, and it is likely several other proteins are also involved (Ref. 121).

Concluding remarks

Research into cancer and ageing has suggested that they are linked in vivo, such that treatment of one could lead to detrimental problems associated with activation of the other (Ref. 96). Owing to their relatively long lifespan, stem cells have a greater risk of accumulating genetic mutations that could ultimately result in their cellular progeny transforming into cancerous tissues. Meanwhile, the damage repair checkpoints in these diseased cells induce senescence and apoptosis to remove these cells, and promote activation of stem cells to replace them at the risk of depleting stem cell pools and inducing premature organ/organism ageing in those tissues. Studies described here suggest that pathways connecting ageing and cancer are complex. The recent developments in understanding DC, a disease characterised by both ageing and cancer yet induced by a single genetic mutation, have provided a functional connection between these diseases and defective telomerase/telomeres. It is likely that further studies on this mendelian genetic disease will facilitate a refinement of our understanding of the relevant biological pathways that are involved, with prospects for developing beneficial therapies for ageing-related and cancer-related diseases in the future.

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Further reading, resources and contacts

Detailed information about telomerase, telomeres and the pathways they are involved in can be found at the following websites:

http://genome-www4.stanford.edu/cgi-bin/SGD/GO/go.pl?goid=7004
http://www.biocarta.com/pathfiles/tercpathway.asp
http://www.biocarta.com/pathfiles/tertpathway.asp
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM
http://www.telomere.org
http://www.swmed.edu/home_pages/cellbio/shay-wright/index.html

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Features associated with this article

Figures

Figure 1. Clinical characteristics of dyskeratosis congenita.
Figure 2. Molecular structure of the *DKC1* gene and its mRNA, showing position of mutations.
Figure 3. Understanding the molecular basis of dyskeratosis congenita.
Figure 4. Telomeres and telomerase.
Figure 5. Molecular structure of TERC and its mutations.
Figure 6. TERC binding and dimerisation in the telomerase complex.
Figure 7. Proposed pathogenesis model for dyskeratosis congenita.

Table

Table 1. Summary of clinical features associated with dyskeratosis congenita.
Table 2. Summary of dyskeratosis congenita subtypes.
Table 3. Summary of mutations identified in *DKC1.*
Table 4. Summary of mutations identified in *TERC.*

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