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Unraveling the Pathogenesis of Hoyeraal-Hreidarsson Syndrome, a Complex Telomere Biology Disorder

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SUMMARY

Hoyeraal-Hreidarsson (HH) syndrome is a multisystem genetic disorder characterized by very short telomeres and considered a clinically severe variant of dyskeratosis congenita (DC). The main cause of mortality, usually in early childhood, is bone marrow failure. Mutations in several telomere biology genes have been reported to cause HH in about 60% of the HH patients, but the genetic defects in the rest of the patients are still unknown. Understanding the aetiology of HH and its diverse manifestations is challenging because of the complexity of telomere biology and the multiple telomeric and non-telomeric functions played by telomere-associated proteins in processes such as telomere replication, telomere protection, DNA damage response and ribosome and spliceosome assembly. Here we review the known clinical complications, molecular defects and germline mutations associated with HH, and elucidate possible mechanistic explanations and remaining questions in our understanding of the disease.

Keywords

Hoyeraal-Hreidarsson syndrome; dyskeratosis congenita; telomere; immunodeficiency; cerebellar hypoplasia

INTRODUCTION

Hoyeraal-Hreidarsson (HH) syndrome is a multisystem genetic disorder typically caused by germline mutations in telomere biology genes. It is considered a clinically severe variant of dyskeratosis congenita (DC) and represents the extreme phenotype caused by aberrant telomere biology. Very short (<1st percentile for age) leucocyte telomere lengths are diagnostic of DC and HH. Patients with HH typically present early in childhood with the

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constellation of cerebellar hypoplasia, immunodeficiency, progressive bone marrow failure, and intrauterine growth retardation (IUGR). The DC-associated mucocutaneous triad of nail dysplasia, lacy skin pigmentation and oral leucoplakia may be present at diagnosis or develop over time in patients with HH. To date, X-linked recessive mutations in *DKC1* (encoding dyskerin), autosomal dominant mutations in *TINF2* (encoding TIN2, also termed TINF2) and autosomal recessive mutations in *TERT*, *ACD* (encoding TPP1, also termed ACD) and *RTEL1*, have been reported to cause HH. All HH-associated genes encode proteins with specialized telomeric functions: TERT and dyskerin are components of the telomerase ribonucleoprotein (RNP) complex, TIN2 and TPP1 are components of the telomeric shelterin complex, and RTEL1 is a helicase important in telomere biology. However, dyskerin, RTEL1 and TERT have also been reported to have non-telomeric functions. Therefore, the question remains whether non-telomeric defects contribute to the pathology of HH, perhaps distinguishing it from DC.

EARLY DESCRIPTIONS OF HOYERAAL-HREIDARSSON SYNDROME

The eponym “Hoyeraal-Hreidarsson syndrome” (HHS or sometimes referred to as HH) was first proposed by a 1995 case report describing a child presenting with progressive pancytopenia, cerebellar hypoplasia, prenatal growth retardation, microcephaly and developmental delay (Aalfs, *et al* 1995). These clinical features were noted as strikingly similar to the clinical description of the patients reported by Hoyeraal *et al* (1970) and Hreidarsson *et al* (1988). Since the initial description by Hoyeraal *et al* (1970), about 50 cases of HH have been reported (Ballew, *et al* 2013a, Berthet, *et al* 1994, Cossu, *et al* 2002, Deng, *et al* 2013, Knight, *et al* 1999a, Kocak, *et al* 2014, Lamm, *et al* 2009, Le Guen, *et al* 2013, Malbora, *et al* 2014, Revy, *et al* 2000, Sznajer, *et al* 2003, Touzot, *et al* 2012, Walne, *et al* 2008, Walne, *et al* 2013b). Progressive bone marrow failure (BMF), cerebellar hypoplasia, immunodeficiency and IUGR appear to comprise a majority of the clinical complications in patients with HH.

CLINICAL MANIFESTATIONS

Clinical Overlap with Dyskeratosis Congenita

In addition to HH-specific symptoms, DC-associated manifestations are also found in HH. DC is classically diagnosed by the presence of the mucocutaneous triad of nail dysplasia, lacy skin pigmentation and oral leucoplakia or by the presence of one feature of the triad in combination with BMF and two other DC-associated findings (Vulliamy, *et al* 2006) (Figure 1). Patients with DC are at very high risk of progressive BMF, pulmonary fibrosis, leukaemia and squamous cell cancer of the head, neck or anogenital regions (Ballew and Savage 2013). Other DC-associated medical problems include non-alcoholic, non-infectious liver fibrosis, stenosis of the oesophagus, lacrimal ducts, and urethra, avascular necrosis of the hips or shoulders and premature greying of the hair (Table I).

Neurological Complications

The original description by Hoyeraal *et al* (1970) reported brothers with the co-occurrence of cerebellar hypoplasia and pancytopenia. The subsequent case by Hreidarsson *et al* (1988) also described a patient with cerebellar hypoplasia and progressive pancytopenia. When

Aalfs *et al* (1995) proposed the term “Hoyeraal-Hreidarsson syndrome,” they also reported the presence of cerebellar hypoplasia in all cases and somewhat more variable presentations of IUGR, microcephaly, developmental delay, immunodeficiency and BMF. Consequently, cerebellar hypoplasia is now considered to be a requirement for the diagnosis of HH (Savage and Alter 2009, Savage and Bertuch 2010) (Figure 1). The underdevelopment of the cerebellum suggests a complex brain developmental abnormality that is probably the underlying cause of HH-associated microcephaly and developmental delay. Additional central nervous system involvement has also been reported in HH. Spastic paresis was reported in three of the four first reported cases (Aalfs, *et al* 1995). Specifically, one patient presented also with peripheral demyelinating neuropathy. Two patients were reported to have seizures (electroencephalogram was normal in one while displaying multifocal epileptiform abnormality in the other) (Ozdemir, *et al* 2004, Pearson, *et al* 2008). A hypoplastic corpus callosum and delayed myelination have also been reported (Kuwashima 2009). Occasionally, patients may also have multiple intracranial calcifications which expands the differential diagnosis to include congenital TORCH infection (Toxoplasmosis, Others Agents, Rubella, Cytomegalovirus and Herpes infection), Aicardi Goutieres syndromes (Knoblauch, *et al* 2003), or even the related telomere biology disorder, Coats plus (Anderson, *et al* 2012, Polvi, *et al* 2012, Savage 2012).

Immunodeficiency

Immunodeficiency is an under-recognized feature of HH even though it was specified by Berthet in the third case of HH published in 1994 (Berthet, *et al* 1994, Berthet, *et al* 1995, Cossu, *et al* 2002). HH patients are reported to have a progressive immune deficiency manifesting as an increased susceptibility to life-threatening infections. Lymphopenia is the most common immunological abnormality observed in patients with HH (approximately 56 % of patients). A decreased count of B cells and Natural Killer cells is often the most remarkable signature. In particular, a virtual absence of B lymphocytes from birth has been observed in HH (Berthet, *et al* 1994, Cossu, *et al* 2002, Hoyeraal, *et al* 1970, Hreidarsson, *et al* 1988, Jyonouchi, *et al* 2011, Le Guen, *et al* 2013, Revy, *et al* 2000, Touzot, *et al* 2010, Touzot, *et al* 2012). Hence, dysfunction of humoral immunity is the most consistent immunological feature of HH and consists of hypogammaglobulinaemia that can affect all immunoglobulin subtypes (IgG, IgM, or IgA). The antibody response toward specific antigens is sometimes impaired, rendering vaccination ineffective. The marked involvement of the B cell compartment is probably due to the additional cell proliferation that B lymphocytes undergo during their development (thus resulting in accelerated telomere shortening, see below) coupled with a shorter life span as compared with T lymphocytes (Hodes, *et al* 2002). The T cell compartment appears to be less frequently affected, with 16% of reported patients exhibiting a decrease in T cell counts (CD4 and/ or CD8 counts) and an inversion of CD4/CD8 ratio. Abnormalities of T cell proliferation in response to specific antigens (candida and tetanus) and, less frequently, to mitogens have also been observed, indicating reduced T cell function (Ballew, *et al* 2013b, Sznajer, *et al* 2003). Some cases presenting as severe combined immunodeficiency have also been reported (Cossu, *et al* 2002). Thus the diagnosis of HH should be considered in any child presenting with humoral deficiency or combined immunodeficiency associated with neurological features (such as microcephaly and/or cerebellar hypoplasia).

Bone Marrow Failure

In some cases, the development of BMF in a patient with developmental delay may be the first indication of HH. Subsequent clinical evaluation, including brain magnetic resonance imaging scan, can lead to the diagnosis of HH. Most patients with HH appear to develop some degree of BMF in the first decade of life, although the severity may be variable. Haematopoietic stem cell transplantation (HSCT) is the only curative therapy for both BMF and immune deficiency. Unfortunately, the outcome of the procedure is burdened by the occurrence of graft-versus-host disease and active post-transplant infections. Moreover, HSCT does not prevent the evolution of the disease in other organs, as HH patients can present with lethal pulmonary or liver fibrosis post-HSCT (Deng, *et al* 2013, Touzot, *et al* 2012).

Gastrointestinal Complications

Digestive tract anomalies and/or feeding difficulties have been documented in most reported cases and constitute one of the most important challenges in the supportive treatment of HH (Sznajer, *et al* 2003) (Borggraefe, *et al* 2009, Touzot, *et al* 2012). Initially, infants with HH and IUGR may present with feeding difficulties due to delayed development. Oesophageal strictures, sometimes requiring repetitive endoscopic dilations, are present in about 25% of reported cases. Severe enteropathy, manifesting as protracting noninfectious diarrhoea, is also reported in another quarter of cases. Digestives biopsies may show a change in the proliferative compartment of the digestive mucosa, leading to glandular atrophy (Sznajer, *et al* 2003). Colitis has also been described with non-specific lymphoplasmocytic infiltrate and chorionic oedema or more severe lesion consisting of granulomatous lesions associated with mononuclear and eosinophilic infiltrate (Borggraefe, *et al* 2009, Touzot, *et al* 2012).

Other Clinical Complications

There have been reports of skeletal malformations (Malbora, *et al* 2014, Touzot, *et al* 2012), urinary tract abnormalities including vesico-ureteral reflux and kidney duplication (Aalfs, *et al* 1995, Ballew, *et al* 2013b), and ophthalmological signs (enophthalmy, mild optic atrophy, and retinopathy) (Aalfs, *et al* 1995, Touzot, *et al* 2012). It can be challenging to differentiate retinopathy of prematurity in HH patients from those with Revesz syndrome. Revesz syndrome is characterized by the presence of bilateral exudative (“Coats”) retinopathy in the presence of DC-associated features including early-onset BMF, fine, sparse hair, nail dystrophy and intracranial calcifications (Revesz, *et al* 1992, Sasa, *et al* 2012, Walne, *et al* 2008).

Overall, HH is a severe multisystemic disorder with a high rate of early mortality linked to aplastic anaemia and immune deficiency. Notably, conversely to DC, in which very high incidence of cancers is observed (Alter, *et al* 2009), cancer has not been reported as a cause of early death of HH patients. This may be due to the early mortality caused by severe BMF and immunodeficiency.

THE AETIOLOGY OF HOYERAAL-HREIDARSSON SYNDROME

Telomere Biology

The telomeres correspond to the ends of linear chromosomes and are composed, in vertebrates, of hexameric TTAGGG repeats bound by specialized proteins (de Lange, *et al* 1990, Wright, *et al* 1997). A 3′ G-rich overhang is a conserved and essential feature of telomeres, and it was suggested to facilitate the formation of a protective telomere (t)-loop by invasion into the duplex part of the telomere (Griffith, *et al* 1999). During cell division, conventional DNA polymerases are unable to fully replicate the end of chromosomes, leading to the progressive loss of telomeric sequences (Olovnikov 1973, Watson 1972). In germ cells, some stem cells, and activated lymphocytes, telomere shortening is counteracted by telomerase, a reverse transcriptase that adds TTAGGG repeats to the 3′ overhang (Blackburn 2001). The telomerase RNP complex includes the telomerase RNA moiety (hTR; encoded by *TERC*), which contains the template for telomeric repeat synthesis, the telomerase reverse transcriptase protein (TERT) and other factors, such as dyskerin, NOP10, NHP2 and GAR1, which form a complex that binds to box H/ACA snoRNAs (including hTR) (Walne and Dokal 2008), and TCAB1 (also termed WRAP53), which directs the telomerase RNP to the Cajal bodies and facilitates telomere synthesis (Venteicher and Artandi 2009). The t-loop structure, formed and maintained by a specific telomeric protein complex called shelterin, hides the 3′ end of the telomeric overhang and prevents the telomeres from being recognized and processed as DNA-double strand breaks. The core shelterin complex is composed of TRF1 (TERF1), TRF2 (TERF2), RAP1 (TERF2IP), TIN2, TPP1 and POT1 (de Lange 2005). TRF1 and TRF2 bind the double-stranded telomeric DNA and are connected by TIN2 to each other and to the TPP1-POT1 heterodimer, which binds to the telomeric overhang. It protects the chromosome ends from degradation, activation of the DNA damage response (DDR), and fusion to other telomeres. Shelterin also regulates the activity of telomerase (de Lange 2005). Another complex, CST (CTC1-STN1-TEN1), also binds the telomeric overhang and functions in the regulation of telomerase and the coordination of the telomere lagging strand synthesis by Pola/primase (Chen, *et al* 2013). Other proteins, such as helicases, nucleases and DDR factors, play essential roles in telomere maintenance.

When telomere proteins fail to protect the telomeres, or telomerase fails to maintain their length, telomeres activate DDR and the cells stop proliferating and enter p53 (TP53)-induced replicative senescence or apoptosis (d'Adda di Fagagna, *et al* 2003, Zou, *et al* 2004). p53 activation in turn can induce the degradation of TRF2, exacerbating the telomere defect in a positive feedback loop (Donehower 2009, Fujita, *et al* 2010). Telomere dysfunction occuring on a large scale may cause a telomere biology disease (also termed telomeropathy) with severe manifestations, particularly in tissues with high turn over of cells (e.g., bone marrow).

Telomere Length in Hoyeraal-Hreidarsson Syndrome

Patients with HH have been included in studies of DC since the discovery of their common aetiology. Most of the DC and HH patients exhibit extremely short telomeres in comparison with age-matched controls. Givien that all the HH-causing genes identified so far participate

in length regulation and/or protection of telomeres, HH is now recognized as a telomere biology disorder. Telomere lengths less than the 1st percentile for age, measured by flow cytometry with fluorescent *in situ* hybridization (flow FISH), are greater than 95% sensitive and specific for differentiating DC and HH patients from their healthy relatives or patients with other inherited BMF syndromes (Alter, *et al* 2007, Alter, *et al* 2015, Alter, *et al* 2012). Telomere lengths in HH patients are shorter than those of aged-matched patients with classic DC (Alter, *et al* 2012). This may account, in part, for the clinical severity seen in HH. Although severely short telomeres in the blood is a common feature of HH patients, the direct causality between critically short telomeres and the diverse clinical manifestations in various tissues is not clear. Some DC and HH patients have been reported to have normal or near-normal telomere lengths in tissues with clinical or molecular manifestations of the disease (Lamm, *et al* 2009, Touzot, *et al* 2012, Vulliamy, *et al* 2012). On the other hand, some individuals from HH families exhibit very short telomere lengths (< 1st percentile) without any clinical manifestation (Kocak, *et al* 2014).

Genetic anticipation, the worsening of a disease phenotype in successive generations, has been reported in DC and could conceivably also result in a child with HH (Savage and Bertuch 2010, Vulliamy, *et al* 2004). In addition to inheritance of a germline mutation (*e.g.*, a *TERT* mutation), short telomeres are also inherited from the carrier parent, resulting in a more severe clinical phenotype. However, to our knowledge, only one case was reported where specific HH clinical features (IUGR, microcephaly, cerebellar hypoplasia) were associated with disease anticipation (Vulliamy, *et al* 2005). While exceedingly short telomeres are considered the main cause of HH, the contribution of other telomeric or non-telomeric defects to disease progression, and particularly to the HH-specific manifestations, remains to be understood.

Genetic and Molecular Defects in Hoyeraal-Hreidarsson Syndrome

One of the major breakthroughs in the understanding of HH aetiology came from two articles published in 1999. First, Knight *et al* (1999a) identified mutations in *DKC1* in the patient described by Aalfs *et al* (1995) and in four additional HH patients. As *DKC1* was previously found mutated in X-linked DC (Heiss, *et al* 1998), this suggested that HHS represents a severe variant of DC. Moreover, the same year, Mitchell and colleagues showed that dyskerin was a component of the telomerase RNP complex, which is involved in the maintenance of telomere length (Mitchell, *et al* 1999a), and further showed that DKC1-mutated patient cells exhibited short telomeres. These findings linked the DC and HH diseases to exceedingly short telomeres due to insufficient level of the telomerase RNP complex. Later, mutations in other telomere biology genes were identified, indicating other primary defects causing the disease.

Only a few *in vitro* phenotypic analyses have been reported in cells from HH patients: fibroblasts, lymphoblastoid cell lines (LCLs) and activated T cells. In addition to overall telomere shortening, these analyses have revealed diverse molecular phenotypes, such as shortening of the telomeric overhang, the activation of DDR at telomeric and non-telomeric sites, hypersensitivity to genotoxic stress, loss of hybridization signal from individual telomeres, telomere fragility, telomere-telomere fusions and anaphase bridges and the

formation or the disappearance of extrachromosomal t-circles (Ballew, *et al* 2013b, Lamm, *et al* 2009, Le Guen, *et al* 2013, Touzot, *et al* 2010, Touzot, *et al* 2012). These diverse molecular phenotypes probably reflect diverse molecular functions and pathways implicated in HH (Figure 2). Some telomere biology genes, such as *TERC, NHP2, NOP10, TCAB1* and *CTC1*, were implicated only in DC. Other genes, such as *TERT* and *DKC1*, are mostly implicated in DC, but when implicated in HH are likely to reflect a more severe dysfunction caused by homozygous or compound heterozygous mutations (*TERT*) or a more damaging mutation *(DKC1)*. Mutations in other genes, such as the shelterin genes *TINF2* (*TIN2*) and *ACD* (*TPP1*), tend to cause HH at higher frequency and recessive mutations in the helicase *RTEL1* appear to only cause HH. These observations suggest that there might be a qualitative difference between DC and HH in the nature of the telomere biology dysfunction, and not only in its clinical severity. The genes and mutations implicated in HH are described below, in chronological order of discovery.

Dyskerin (*DKC1*)

DKC1, encoding a protein named dyskerin after the name of the disease dyskeratosis congenita (DC), was the first gene implicated in DC and HH (Heiss, *et al* 1998, Knight, *et al* 1999a, Knight, *et al* 1999b, Knight, *et al* 1998, Vulliamy, *et al* 1999). Dyskerin is an essential nucleolar protein expressed in all tissues and highly conserved among living organisms. It comprises three main domains: a dyskerin-like domain with a yet unknown function at the N-terminus of the protein, TruB pseudouridine synthase catalytic domain, and PUA RNA binding domain. Dyskerin is part of the essential box H/ACA small nucleolar RNP (snoRNP) complex. In these snoRNPs, dyskerin and 3 other proteins, NHP2, NOP10 and GAR1, are associated with small nucleolar RNAs (snoRNA) through a box H and ACA consensus sequences (Angrisani, *et al* 2014). The snoRNAs guide the conversion of specific uridine residues to pseudouridines by base pairing to specific target RNAs, such as ribosomal RNAs, in the nucleolus. This modification is important for the folding and processing of the target RNAs (Kiss, *et al* 2010). A subset of box H/ACA snoRNPs, called small Cajal body RNPs (scaRNPs), are directed to the Cajal bodies by the protein TCAB1, which binds to a sequence called CAB box within the scaRNA (Tycowski, *et al* 2009). scaRNPs modify small nuclear RNAs (snRNAs), which are components of the spliceosome. Vertebrate telomerase RNAs contain H/ACA and CAB boxes, and assemble a typical box H/ACA scaRNP, which is important for the stability and recruitment of telomerase to telomeres (Mitchell, *et al* 1999b, Mitchell, *et al* 1999a, Venteicher and Artandi 2009).

Given that dyskerin is also important for ribosome biogenesis and function, *DKC1* mutations were suggested to cause or contribute to X-linked DC/HH by compromising protein synthesis, and particularly internal ribosome entry site (IRES)-dependent translation (Jack, *et al* 2011, Ruggero, *et al* 2003). However, in human cells obtained from X-linked DC patients, ribosomes biogenesis and function were intact or only slightly affected (Carrillo, *et al* 2013, Thumati, *et al* 2013, Wong and Collins 2006) while the levels of hTR were significantly reduced, and some of the mutations were shown to disrupt the physical interaction with hTR (Ashbridge, *et al* 2009). Furthermore, TERC mutations in the box H/ACA motif found in DC patients had a similar effect on hTR levels. These observations support the notion that X-linked DC/HH is mainly caused by reduced levels of the

telomerase RNP (Mitchell, *et al* 1999a, Mochizuki, *et al* 2004, Wong and Collins 2006, Zeng, *et al* 2011). Impaired protein synthesis does not appear to be a major factor causing the disease, but may still contribute to some of the symptoms, such as cancer predisposition (Bellodi, *et al* 2010). It is possible that protein translation is a more robust pathway that can tolerate some perturbations in dyskerin function, while telomere length homeostasis requires a delicate balance between telomere shortening and lengthening factors; even a minor decrease in telomerase activity may cause a disease over time.

DKC1 mutations cause DC and HH in an X-linked recessive manner. To date, over 50 different inherited and *de novo DKC1* mutations have been found in association with DC, 13 of them also cause HH (P10L, I38T, T66A, T67I, H68Q, H68Y, S121G, R158W, K314R, A353V, R378Q, A386T and IVS12+1), and two mutations were only found in HH (T49M and S304N; Figure 3A; (Alder, *et al* 2013, Borggraefe, *et al* 2009, Cossu, *et al* 2002, Du, *et al* 2009, Heiss, *et al* 1998, Knight, *et al* 1999a, Knight, *et al* 1999b, Knight, *et al* 2001, Pearson, *et al* 2008, Sznajer, *et al* 2003, Vulliamy, *et al* 1999, Vulliamy, *et al* 2011, Vulliamy, *et al* 2006, Yaghmai, *et al* 2000). Most *DKC1* mutations cluster into two regions outside the catalytic TruB domain: amino acids 2-72 (N-terminus of the protein) and 314–420 (PUA domain; (Knight, *et al* 1999b, Knight, *et al* 2001, Vulliamy, *et al* 2006). The three-dimensional structure of human dyskerin reveals that these domains are contiguous and probably form a binding site for additional factors (Rashid, *et al* 2006, Walbott, *et al* 2011). Some disease-causing mutations in both regions were shown to affect the binding of the H/ACA RNP assembly factor SHQ1 (Grozdanov, *et al* 2009). SHQ1 is known to stabilize the dyskerin protein and prevent its misfolding and degradation. Grozdanov *et al* (2009) suggested that both increase and decrease of SHQ1 binding reduces the dyskerin availability for RNP assembly. Recently, Brault *et al* (2013) reported that N-terminal *DKC1* mutations fell into a consensus SUMOylation motif and showed that impaired dyskerin SUMOylation leads to reduced dyskerin and telomerase RNP levels, impaired telomerase activity and telomere shortening. Furthermore, forced SUMOylation of mutated dyskerin almost fully restored normal levels of telomerase. The authors suggested that SUMOylation regulates dyskerin stability and provide additional evidence that low dyskerin levels may contribute to DC (Brault, *et al* 2013). Finally, a growing body of evidence demonstrates that reduced expression level of dyskerin in the absence of mutations in the coding sequence may also cause the disease (Knight, *et al* 2001, Parry, *et al* 2011)

There is no clear correlation between the severity of the disease and the location of the mutation; some of the mutations cause only DC while others may cause DC or HH (Vulliamy, *et al* 2006). For example, the A353V mutation is associated with variable severity (from late onset DC to HH), even among siblings (Lai, *et al* 2011, Vulliamy, *et al* 2006). However, two mutations, T49M and S304N, were only found in HH probands (Knight, *et al* 1999a, Sznajer, *et al* 2003, Vulliamy, *et al* 2006). Interestingly, two HH-associated mutations (S121G and R158W) fall into the catalytic TruB domain, suggesting that the catalytic pseudouridinylation activity (and not merely the binding of dyskerin to hTR) is important for telomerase function (Knight, *et al* 1999a, Knight, *et al* 2001). Indeed, it has been shown that the TruB pseudouridine synthase domain of dyskerin alone is essential and sufficient for rescuing both hTR levels and telomerase activity in primary DC-

affected cells (Machado-Pinilla, *et al* 2007). Interestingly, pseudouridylation of an essential stem loop (p6.1) of hTR was shown to affect the structure of p6.1 and the *in vitro* activity of telomerase (Kim, *et al* 2010). The *in vivo* pseudouridylation of uridine 307 within p6.1 was confirmed by transcriptome-wide mapping of pseudouridines, and the levels of pseudouridylated U307 were diminished in DC patient cells with DKC mutations (Schwartz, *et al* 2014). Altogether, these observations indicate that not only hTR is assembled as a snoRNA but it is also the target for pseudouridylation within one of its essential functional domains. Therefore, dyskerin may function not only by physically interacting with and stabilizing the telomerase RNP complex, as suggested above, but also by using its catalytic activity to pseudouridylate p6.1. The detailed molecular mechanism underlying the role of dyskerin in telomerase function and how DKC mutations cause a telomere disease remain to be further investigated.

Telomerase Reverse Transcriptase (*TERT*)

Most DC and HH cases examined to date share the feature of very short telomeres, which is thought to be the major defect causing the disease. More than 50 autosomal dominant (AD) or autosomal recessive (AR) mutations have been described in *TERT*, with diverse clinical presentations from pulmonary fibrosis and aplastic anaemia to DC and HH (Armanios, *et al* 2005, Basel-Vanagaite, *et al* 2008, Marrone, *et al* 2007, Tsakiri, *et al* 2007, Vulliamy, *et al* 2006, Vulliamy, *et al* 2005, Yamaguchi, *et al* 2005). Among them, only five mutations are implicated in HH (P530L, T567M, A880T, R901W, F1127L; Figure 3B), and four of them cause HH if homozygous (T567M and R901W, (Gramatges, *et al* 2013, Marrone, *et al* 2007) or compound heterozygous (P530L and A880T; (Vogiatzi, *et al* 2013). The heterozygous carriers of these mutations have short telomeres with minimal clinical consequences (Gramatges, *et al* 2013, Marrone, *et al* 2007, Vogiatzi, *et al* 2013).

The A880T and R901W mutations fall in the catalytic reverse transcriptase domain, and lead to extremely low levels of telomerase activity. The T576M substitution occurs in the T-motif, which lies within the telomerase RNA binding domain, and drastically affects telomerase processivity (Gramatges, *et al* 2013). However, the P530L substitution does not have an apparent effect on telomerase activity *in vitro* although it is associated with short telomeres even in heterozygous carriers, indicating impaired telomerase function *in vivo* (Vogiatzi, *et al* 2013). Heterozygous carriers of the mutations implicated in HH are healthy but have short telomeres, indicating that these heterozygous mutations may be better tolerated than the DC-causing autosomal dominant mutations. The only reported autosomal dominant mutation associated with HH, F1127L, was found also in the asymptomatic mother, indicating disease anticipation or the presence of another paternal mutation, yet to be found. The C-terminal domain of TERT, where this mutation is located, was shown to be involved in its interactions with TPP1, which is known to recruit telomerase to telomeres (Banik, *et al* 2002, Wang, *et al* 2007, Zaug, *et al* 2013, Zhong, *et al* 2012). Most reported DC patients with *TERT* mutations are monoallelic heterozygous, and the activity of telomerase is an average of the homozygous WT and mutant, suggesting that the disease is caused by haploinsufficiency (Armanios, *et al* 2005, Tsakiri, *et al* 2007, Xin, *et al* 2007, Yamaguchi, *et al* 2005, Zaug, *et al* 2013). Severe HH phenotypes associated with biallelic *TERT* mutations are distinguished from DC only by the severity of telomerase deficiency.

TERF1-interacting nuclear factor 2 (*TINF2*)

TINF2, encoding the shelterin component TIN2, was first associated with HH by Savage *et al* (2008). Over twenty *TINF2* mutations have been described for DC, and three of them also for HH (K280X, R282H, P283S; Figure 3C) (Podlevsky, *et al* 2008, Savage, *et al* 2008, Touzot, *et al* 2012, Walne, *et al* 2008). Notably, several of these mutations are causative of Revesz syndrome, a related disorder characterized by the presence of bilateral exudative retinopathy in addition of features of DC (Podlevsky, *et al* 2008, Savage, *et al* 2008, Touzot, *et al* 2012, Walne, *et al* 2008). The reported *TINF2* mutations are often *de novo* but may also be inherited in autosomal-dominant manner (Walne, *et al* 2008). All *TINF2* mutations reported to date cluster in exon 6a (amino acids 269–298) of *TINF2* (Sasa, *et al* 2012, Savage, *et al* 2008, Touzot, *et al* 2012, Vulliamy, *et al* 2012, Walne, *et al* 2008). Interestingly, despite the overall severe disease manifestations a few individuals were found to be clinically unaffected carriers at the time of evaluation with extremely short telomeres (Savage, *et al* 2008).

TIN2 is a shelterin protein, which links the double-stranded DNA binding proteins TRF1 and TRF2 to each other and to the single-stranded DNA binding heterodimer TPP1-POT1. The DC mutations cluster is located 8 amino acids downstream of the TRF1 binding domain (FxLxP) of the protein, but most of the mutations do not affect the ability of TIN2 to bind TRF1 (Sasa, *et al* 2012, Xin and Ly 2012). DC-associated mutations were suggested to compromise the ability of TIN2 to recruit telomerase to telomeres in a TPP1-dependent manner (Yang, *et al* 2011). TIN2 is also important for cohesion of sister telomeres. It interacts with heterochromatin protein 1 gamma (HP1γ) through the canonical binding site PTVML, which is located within the DC-associated mutation cluster (Walne, *et al* 2008). Several patient-derived cell lines displayed reduced associations between TIN2 and HP1γ and reduced sister telomere cohesion (Canudas, *et al* 2011). As the TIN2-associated DC and HH patients have very early onset and severely short telomeres, the authors suggested that severe telomere dysfunction might occur during embryogenesis because non-cohered telomeres were not efficiently elongated by telomerase. However, recent work in a mouse model suggested that telomere shortening in *TINF2* mutation carriers is telomerase independent (Frescas and de Lange 2014).

Another possibility for the effect of *TINF2* mutations is that TIN2-mediated telomere cohesion is important for recombinational telomere elongation during early embryogenesis. It was suggested that mice telomeres lengthen by a recombination-based mechanism in early embryogenesis, while only later in embryogenesis is telomerase activated to maintain telomere length (Liu, *et al* 2007). The ZSCAN4 family of proteins is essential for telomerase-independent telomere elongation in two-cell mouse embryos and ES cells (Falco, *et al* 2007, Zalzman, *et al* 2010). ZSCAN4 activates telomere sister chromatid exchange (T-SCE) and reduces non-telomeric SCE, ensuring the long-term genomic stability in mice ES cells (Zalzman, *et al* 2010). These data suggest that impaired sister telomere cohesion caused by TIN2-DC mutations compromises recombination-dependent telomere elongation in early embryogenesis, thus causing severe DC and HH. The severity of the HH phenotype caused by *TINF2* mutations may be explained by its essential role in shelterin assembly and/or in cohesion-dependent telomere elongation during early embryogenesis.

Regulator of Telomere Elongation Helicase 1 (*RTEL1*)

RTEL1 is an essential DNA helicase that belongs to a small family of iron-sulfur-containing DNA helicases, together with XPD, FANCJ and DDX11/ChlR1, which are implicated in different genomic instability diseases (van der Lelij, *et al* 2010, White 2009, Wu, *et al* 2009). Mouse *Rtel1* was genetically associated with telomere length and its deletion in embryonic stem cells led to the loss of telomeres and to chromosomal abnormalities, suggesting that it is required for both telomere maintenance and genomic stability (Ding, *et al* 2004). Mouse RTEL1 (mRTEL1) was found to associate with telomeres, transiently, during S phase (Uringa, *et al* 2012). Human RTEL1 was reported to interact with TRF1 and TRF2, indicating its recruitment to telomeres (Deng, *et al* 2013, Sarek, *et al* 2015, Sfeir, *et al* 2009). mRTEL1 was reported to have two distinct roles at telomeres: to prevent telomere fragility by resolving G-quadruplexes formed during telomere replication, and to disassemble t-loops to allow telomere replication and prevent t-loop excision (Sfeir, *et al* 2009, Uringa, *et al* 2012, Vannier, *et al* 2012). However, mRTEL1 also has important non-telomeric functions in DNA replication, DNA lesion repair and homologous recombination (Uringa, *et al* 2012, Vannier, *et al* 2013), and human RTEL1 was shown to unwind (CTG-CAG) trinucleotide repeat hairpins to inhibit repeat-mediated chromosome fragility (Frizzell, *et al* 2014) and to play a role in nuclear and cytoplasmic trafficking of pre-U2 RNA (Schertzer, *et al* 2015).

To date, 18 mutations in *RTEL1* were described in 17 cases of HH from 14 families (Figure 3D; (Ballew, *et al* 2013b, Ballew, *et al* 2013a, Deng, *et al* 2013, Le Guen, *et al* 2013, Walne, *et al* 2013a): 15 missense mutations (E251K, M492I, E591D, A621T, I699M, L710R, G739V, V745M, K897Q, R957W, F964L, R974X, R986X, C1244R, R1264H), a deletion (del398-422) and two intronic splicing mutations (C102+2T>C, Ivs24+5G>A). Most of the *RTEL1* mutations observed were biallelic, with either homozygous or (mostly) compound heterozygous autosomal recessive inheritance. A few apparently-monoallelic cases may actually have a second *RTEL1* mutation yet to be identified, or may be explained by AD inheritance with genetic anticipation (Ballew, *et al* 2013a). The mutations were located mostly in the helicase domains, or in the harmonin-like or C-terminal RING finger domains, possibly involved in protein-protein interactions or ubiquitin transfer (Ballew, *et al* 2013b, Le Guen, *et al* 2013). The *RTEL1* R1264H mutation was shown to compromise the interaction of RTEL1 with TRF2, causing t-loop excision and telomere length heterogeneity (Sarek, *et al* 2015). This mutation was determined to occur on a common haplotype found in individuals of Ashkenazi Jewish ancestry. Notably, the carrier frequency of this mutation was 1% in a collection of Orthodox Ashkenazi samples and 0.45% in the general Ashkenazi Jewish population (Fedick, *et al* 2014). This led to the clinical recommendation that this mutation be included in carrier screening panels in the Ashkenazi population.

All HH patients with *RTEL1* mutations had similar clinical manifestations including IUGR, cerebellar hypoplasia, BMF and very short telomeres in white blood cells. Normal telomere length in fibroblasts was described for some HH patients, despite poor growth and the formation of DNA damage foci at telomeres (Lamm, *et al* 2009). Most patients developed complications as young children; none of them had significantly abnormal skin pigmentation and only some of them had nail dystrophy and leucoplakia (Ballew, *et al* 2013a, Ballew, *et*

al 2013b, Deng, *et al* 2013, Le Guen, *et al* 2013, Walne, *et al* 2013a). RTEL1 is important for both telomere maintenance and genome stability (Ding, *et al* 2004, Frizzell, *et al* 2014, Uringa, *et al* 2012, Vannier, *et al* 2012). Some studies describe altered DNA replication, elevated levels of total DNA damage and defects in DNA repair in some HH-derived cell lines (Ballew, *et al* 2013b, Le Guen, *et al* 2013). However, most of the cells derived from HH patients harbouring RTEL1 mutations had significant defects only in telomere maintenance (Deng, *et al* 2013, Walne, *et al* 2013a). This suggests that either some of described mutations specifically affect the telomeric function of RTEL1, or that the non-telomeric functions are redundant and/or not essential in humans.

The suggested role of RTEL1 in resolving G-quadruplexes during telomere replication, is consistent with the telomere fragility observed in patients cells, presumably leading to telomere fusion, anaphase bridges and mitotic arrest (Deng, *et al* 2013, Le Guen, *et al* 2013). Another role was suggested for RTEL1 in resolving t-loops during telomere replication (Vannier, *et al* 2012). Defects in t-loop disassembly can induce t-loop excision yielding increased levels of t-circles. However, existing data about t-circle levels in RTEL1 deficient cells are conflicting (Ballew, *et al* 2013b, Deng, *et al* 2013, Uringa, *et al* 2012, Vannier, *et al* 2012, Walne, *et al* 2013a). The discrepancy may be explained by the different mutations having different effects on RTEL1 function or by the different assays used (a rolling circle amplification assay *versus* two-dimensional gel electrophoresis). RTEL1 is the first factor associated predominantly with HH rather than DC. Although it does not function only at telomeres, its implication in HH indicates that it is essential for telomere maintenance. Yet, its precise telomeric functions remain unclear. HH-causing mutations were described in different domains and likely affect different functions of RTEL1 (Figure 3D). Further characterization of the RTEL1 mutants will help distinguishing between different functions of RTEL1 and their implications in HH.

ACD (TPP1)

TPP1 (abbreviated from the former names TINT1, PTOP and PIP1; also termed ACD) is encoded by the *Adrenocortical Dysplasia Homolog (ACD)* gene and is one of the core components of the shelterin telomere protection complex. TPP1 has distinct domains with specific telomeric functions (Figure 3E). The C-terminal domain binds TIN2 to form a bridge between the single-stranded binding POT1 and the double-stranded binding TRF1 and TRF2 (Takai, *et al* 2011). The central domain of TPP1 is required for heterodimer formation with POT1 (Liu, *et al* 2004, Ye, *et al* 2004). In addition, an area on the surface of the N-terminal OB domain of TPP1, termed TEL patch, is required for the interaction of TPP1 with telomerase, telomerase processivity, and for the recruitment of telomerase to telomeres (Nandakumar, *et al* 2012, Zhong, *et al* 2012).

Whole exome sequencing of a patient with HH and his family members discovered a TEL patch mutation (delK170) in the proband and his father, and a missense mutation (P491T) in the proband and his mother (Kocak, *et al* 2014). The TEL patch single amino acid deletion resulted in significant reduction of telomerase processivity and recruitment to telomeres. The P491T missense mutation was less clearly deleterious but may have an effect on TIN2 binding. Similarly, the same TEL patch deletion was identified in a family with aplastic

anaemia and other features consistent with DC (Guo, *et al* 2014). This family showed autosomal dominant inheritance of disease.

Apollo

Some cases of HH were reported in which telomere lengths in both blood cells and fibroblasts were normal (Touzot, *et al* 2010, Touzot, *et al* 2012). The disease causing mutation is unknown, but in one patient, the cells were found to express an aberrant splice variant of Apollo, a nuclease that plays a role in the 3′ overhang formation at leading-strand telomeres (Touzot, *et al* 2010, Wu, *et al* 2012). The authors suggested that this variant, lacking the TRF2-binding domain, has a dominant-negative effect and impairs telomere function. However, the germline mutation(s) that cause the aberrant splicing of *Apollo* is yet to be identified.

Genes implicated in Dyskeratosis Congenita but not in Hoyeraal-Hreidarsson Syndrome

TERC (hTR)

AD mutations in the telomerase RNA component, *TERC*, were identified through linkage analysis of a large family (Vulliamy, *et al* 2001). To date 50 *TERC* mutations are described, resulting in spectrum of diseases, from mild aplastic anaemia to DC (Podlevsky, *et al* 2008). Among 14 DC-associated mutations that include large and small deletions and nucleotide substitutions, 9 fall into the pseudoknot-containing telomerase template domain (Podlevsky, *et al* 2008). Most of *TERC* mutations implicated in DC significantly affect telomerase activity (Vulliamy, *et al* 2011). One HH-associated *TERC* mutation (242C>T) was found in a patient whose mother and sister were both asymptomatic carriers. The telomerase activity of this allele was surprisingly higher than for the rest of the DC-causing alleles (46.8%), indicating that HH in this patient was probably caused by another unidentified mutation, either alone or in combination with the *TERC* mutation described (Vulliamy, *et al* 2011).

NOP10 and NHP2

In just a few cases of AR DC, mutations in two additional components of the box H/ACA snoRNPs, NOP10 and NHP2, have been described. Homozygous R34W mutations in *NOP10* impaired hTR binding and blocked pre-RNP formation (Trahan, *et al* 2009, Walne, *et al* 2007). Homozygous Y139H substitution in NHP2 was described in a patient with severe DC, and compound heterozygous V126M and a stop-loss mutation X154R was found in a patient with classic DC (Vulliamy, *et al* 2008). Patients with mutations in NOP10 or NHP2 had decreased hTR levels and short telomeres, similar to those having mutations in dyskerin (Vulliamy, *et al* 2008, Walne, *et al* 2007). No disease-causing mutations were so far identified in other proteins that are involved in snoRNP assembly and function (Trahan, *et al* 2009).

WRAP53 (TCAB1)

Another telomerase holoenzyme component found mutated in AR DC is TCAB1 (encoded by *WRAP53*; (Zhong, *et al* 2011). TCAB1 binds a consensus sequence termed CAB box in scaRNAs, including hTR, and is required for telomerase trafficking to Cajal bodies and telomere elongation (Stern, *et al* 2012, Venteicher and Artandi 2009). The DC-causing

mutations were compound heterozygous (F164L and R398W; H376Y and G345R) and disrupted telomerase localization to the Cajal bodies, leading to hTR accumulation in the nucleoli and impairing telomere elongation (Zhong, *et al* 2011).

CTC1

Two different groups identified compound heterozygous mutations in *CTC1* (conserved telomere maintenance component 1) and short telomeres in patients with Coats plus syndrome (Anderson, *et al* 2012, Polvi, *et al* 2012). Later, compound heterozygous *CTC1* mutations were found in DC patients (Keller, *et al* 2012, Walne, *et al* 2013b). The connection of CTC1 to telomere biology and the clinical overlap suggests that Coats plus is also a telomere biology disorder. To date more then 20 disease-causing mutations are found in *CTC1*, 10 of them associated with DC (Walne, *et al* 2013b). Although *CTC1* mutations have various effects on telomere length, all of them significantly affect semiconservative replication of telomeric DNA (Chen, *et al* 2013). These findings demonstrated that telomerase deficiency leading to excessive telomere shortening is not the only pathway causing a telomere biology disorder.

CONCLUSIONS

The clinical consequences of abnormal telomere biology are most significant in HH. However, there is substantial clinical overlap between HH, Revesz Syndrome, Coats plus, DC and other apparently isolated illnesses (*e.g.*, pulmonary fibrosis, or aplastic anaemia). These disorders represent different extremes of the illnesses caused by germline mutations in key telomere biology genes. The presence of cerebellar hypoplasia in the setting of immunodeficiency in a young child should prompt a clinical evaluation for HH. The clinical differences caused by germline mutations in genes in the same pathway are probably due to differences in the functional consequence of a specific mutation in a protein, as well as incomplete penetrance and variable expressivity of the mutation in different individuals. The genetic overlap of HH with DC and related telomere biology disorders is well established. *De novo* and inherited (AD, AR and X-linked) mutations have been identified in about 60% of the HH patients. Many HH-associated mutations also cause DC. In some cases (e.g. *ACD*/TPP1) monoallelic mutations caused DC and biallelic mutations caused HH (Guo, *et al* 2014), suggesting that HH is a severe form of DC. On the other hand, mutations in the telomerase core genes *TERC* and *TERT* cause only or mostly DC, while mutations in genes with additional non-telomeric functions, such as *RTEL1* and *DKC1*, cause only or mostly HH, suggesting that non-telomeric defects may contribute to the HH-specific manifestations.

Novel mutations causing HH and other telomere biology disorders are likely to be identified soon with the increasing availability of exome and whole genome sequencing. Some of these mutations may be found in telomere biology genes not yet implicated in DC or HH, or in other genes not known to be involved in telomere maintenance. Since p53 activation can induce degradation of TRF2 (Fujita, *et al* 2010), genetic defects activating p53 may contribute to the progression of telomere biology diseases by combining increased senescence or apoptosis and telomere dysfunction (Donehower 2009). Studying the aetiology of telomere biology diseases is invaluable for understanding the mechanisms

involved in normal telomere maintenance and function. Such understanding will facilitate the design of therapeutic approaches, not only for telomere biology diseases but also for other clinical manifestations of telomere dysfunction, such as cancer and aging-associated pathologies.

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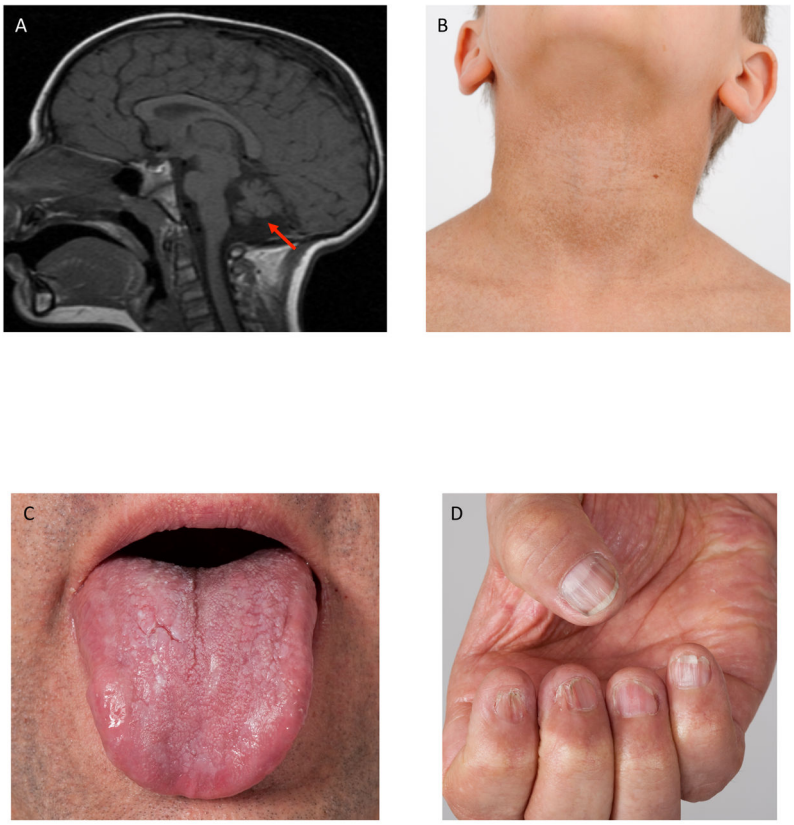

Figure 1. Clinical Features of Patients with Hoyeraal-Hreidarsson Syndrome

A) cerebellar hypoplasia (arrow), B) skin pigmentation abnormalities, C) oral leucoplakia, D) nail dystrophy

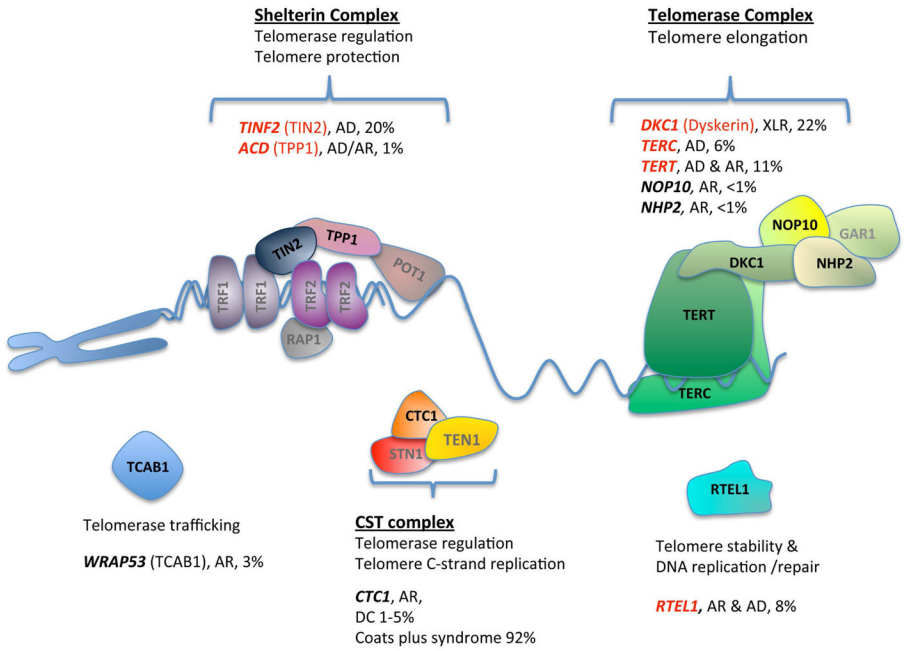

Figure 2. Factors involved in telomere maintenance and associated with telomere biology disorders

Factors implicated in Hoyeraal-Hreidarsson syndrome (HH) are marked with red. The percentages are based on unpublished National Cancer Institute dyskeratosis congenita (DC) cohort data and review of the literature. They represent the combination of DC, HH, and Revesz syndrome. The shelterin complex binds along the telomeric DNA, protects the telomere end by forming a t-loop, suppressing DDR, and regulating telomerase action. TCAB1 regulates telomerase trafficking to the Cajal body. The telomerase ribonucleoprotein complex elongates the 3′ end of the telomere. The CTC1-STN1-TEN1 (CST) complex contributes to telomere replication and telomerase regulation. RTEL1 is an essential DNA helicase involved in telomere maintenance. Abbreviations: AD, autosomal dominant; AR, autosomal recessive; XLR, X-linked recessive.

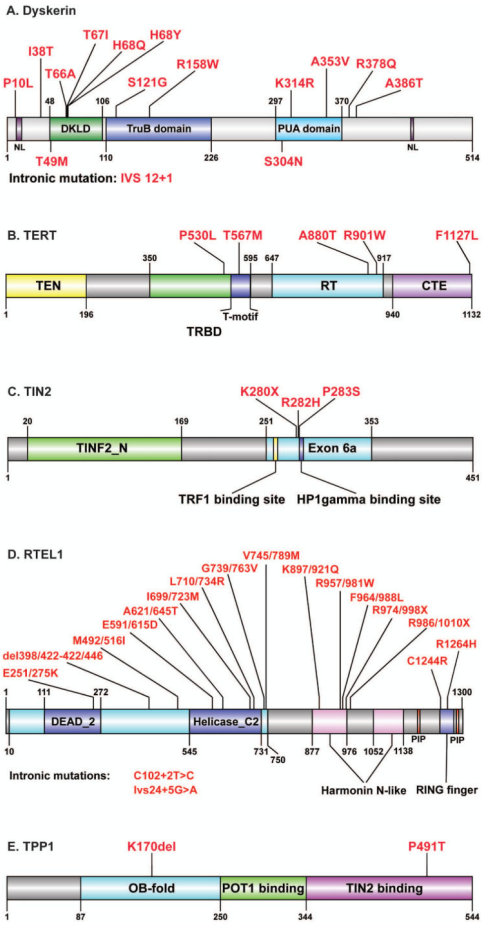

Figure 3. Hoyeraal-Hreidarsson syndrome-associated mutations

A) Dyskerin (DKC1). Indicated are nuclear localization (NL) signals (purple), dyskerin-like domain (DKLD; green), TruB pseudouridine synthase catalytic domain (blue) and PUA RNA binding domain (cyan). **B)** TERT. Indicated are TEN domain (yellow), telomerase RNA binding domain (TRBD; green), T-motif within TRBD (blue), reverse transcriptase (RT) domain (cyan) and C-terminal extension (CTE; magenta). **C)** TIN2 (TINF2). Indicated are TIN2_N domain (green), exon 6a (cyan), TRF1 binding site (yellow) and HP1γ binding site (blue). **D)** RTEL1. Indicated are RAD3-related helicase domain (cyan) containing the helicase type 2 ATP binding and C-terminus domains (blue), PIP boxes (red), Harmonin N-like domains (pink) and RING-finger domain (light violet). Amino acid numbering refers to the main, 1,300aa, splice variant of RTEL1 (NM_001283009; left) and to a 1243aa splice variant, which includes an alternative 24aa exon close to the C-terminus (NM_032957; right). **E)** TPP1 (ADC). Indicated are OB fold (cyan), POT1 binding domain (green) and TIN2 binding domain (magenta). The schemes were illustrated using DOG 1.0 (Ren, *et al* 2009).

Table I

Clinical features associated with classical Hoyeraal Hreidarsson Syndrome

Clinical feature/abnormality	Approximate % of reported patients^
Classical signs	96
Intra-uterine growth retardation	96
Microcephaly	96
Cerebellar hypoplasia	96
Progressive bone marrow failure	100
Developmental delay	92
Associated features	
Mucocutaneous features	71
Immunodeficiency	56
Prematurity	33
Dysmorphology	29
Gastrointestinal features	42
Neurological symptoms	13*

^Percentages based on a compilation of the following references (Ballew, *et al* 2013a, Ballew, *et al* 2013b, Berthet, *et al* 1994, Cossu, *et al* 2002, Deng, *et al* 2013, Knight, *et al* 1999a, Kocak, *et al* 2014, Lamm, *et al* 2009, Le Guen, *et al* 2013, Malbora, *et al* 2014, Revy, *et al* 2000, Sznajer, *et al* 2003, Touzot, *et al* 2012, Walne, *et al* 2008, Walne, *et al* 2013a)

*Represents neurological features not necessarily related to cerebellar hypoplasia